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Polyphosphate Nanoparticles: Balancing Energy Requirements in Tissue Regeneration Processes

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Nanoparticles of a particular, evolutionarily old inorganic polymer found across the biological kingdoms have attracted increasing interest in recent years not only because of their crucial role in metabolism but also their potential medical applicability: it is inorganic polyphosphate (polyP). This ubiquitous linear polymer is composed of 10–1000 phosphate residues linked by high-energy anhydride bonds. PolyP causes induction of gene activity, provides phosphate for bone mineralization, and serves as an energy supplier through enzymatic cleavage of its acid anhydride bonds and subsequent ATP formation. The biomedical breakthrough of polyP came with the development of a successful fabrication process, in depot form, as Ca- or Mg-polyP nanoparticles, or as the directly effective polymer, as soluble Na-polyP, for regenerative repair and healing processes, especially in tissue areas with insufficient blood supply. Physiologically, the platelets are the main vehicles for polyP nanoparticles in the circulating blood. To be biomedically active, these particles undergo coacervation. This review provides an overview of the properties of polyP and polyP nanoparticles for applications in the regeneration and repair of bone, cartilage, and skin. In addition to studies on animal models, the first successful proof-of-concept studies on humans for the healing of chronic wounds are outlined.

1. Introduction

Polyphosphate (polyP) is an ancient polymer that is found from prokaryotes to eukaryotes to metazoan animals.[1–3] This physiological linear polymer is composed of hundreds of phosphate residues linked together by high-energy anhydride linkages (reviewed in: refs. [4–7]). Soon after the discovery of ATP[8] and pyrophosphate,[9] polyP was identified first as RNA[10] and later as polyP[11] with its energy-rich phosphate units.[12] The initial studies on polyP were carried out in yeast.[13] Three properties qualify polyP as an essential component of living cells; first, it is the element phosphorus that is indispensable for life, and second, this polymer allows self-replicating and enzyme-containing primordial living units to be compartmentalized as a coacervate,[3,14,15] and third, it is an energy generator both in bacterial cells[16] and the extracellular space of metazoans.[17]

The interest in polyP, especially with regard to its application in human therapy, was pushed by the discovery that this polymer has an anabolic effect on biomineralization in bone and teeth.[18–20] The importance of polyP for biomineral formation is understandable since ≈15% by weight of the human body consists of bone, a calcium phosphate (Ca-phosphate) mineral. Almost simultaneously it was shown, initially using spicules from sponges as an animal model, that not only organic materials but also inorganic materials (biominerals) in living organisms are formed enzymatically, in sponges with the enzyme silicatein.[21–23] Later, with the alkaline phosphatase (ALP) in mammalian bone,[20,24] a further push toward the application of polyP for potential treatment in humans occurred. ALP is a polyP hydrolyzing enzyme.[19] Since any kind of anabolic regeneration requires metabolic energy,[25] with ATP being the main energy-carrying metabolite,[26] it was reasonable to elucidate whether cleavage of the energy-rich bonds in polyP could be exploited for the generation of ATP. In fact, as described in this review, the transfer of energy from polyP to ATP was subsequently demonstrated experimentally.[17] Thereafter, it was possible to successfully conduct proof-of-concept studies with polyP for the treatment of epithelia[27] and chronic wounds.[28]

2. Physiological Synthesis of polyP

In humans and other mammalian organisms, polyP is encapsulated in the dense granules of the blood platelets, which contain large amounts of ADP and ATP together with pyrophosphate and polyP.[29,30] Platelets are abundant cell fragments in the blood that arise from megakaryocytes. When they are activated, polyP

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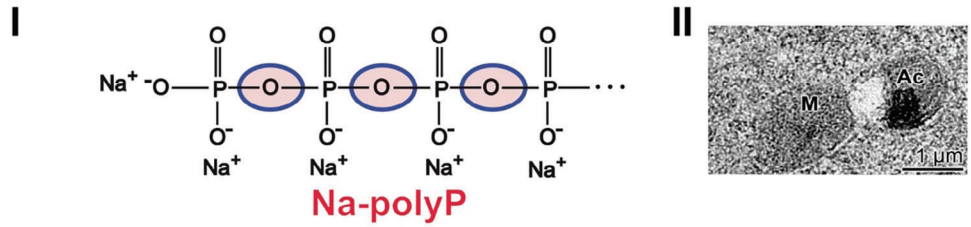

Figure 1. Polyphosphate and its physiological deposition. I) Linear polyP structure as it occurs in cells. The high-energy bonds are circled. II) The polymer is deposited in cells in acidic electron-dense organelles, the acidocalcisomes (Ac), or dense granules. Acidocalcisomes are in close association with mitochondria (M); TEM. Reproduced with permission.[17] Copyright 2019, American Chemical Society.

molecules with a chain length of 60–100 phosphate (P_i) units are secreted into the surrounding fluid.[31–34]

PolyP is a polyanionic polymer that exists in the salt form together with cations, e.g. Na^+ (**Figure 1**-I). For classification, polyP molecules with 60–100 P_i units have been termed medium-chain polyP, while the long-chain polyP fraction includes polymers with several hundreds to thousands of P_i units.[33] In the mitochondria, the polyP chains are comparatively short; a length of 15 P_i units has been determined.[35] In contrast to eukaryotes, bacteria mainly synthesize very long polyP molecules with a size between 100 and 1000 P_i units, catalyzed by a polyP kinase using ATP as a substrate.[36,37]

2.1. Synthesis of polyP

PolyP is synthesized via two routes: first, purely chemically, by polycondensation of monomeric phosphate at high temperatures, while the physiological polyP is formed at ambient temperature using enzymes, most likely intracellularly.

2.1.1. Chemical Synthesis of polyP

PolyP can be prepared by a purely chemical process through temperature-dependent condensation of Na-orthophosphate.[38] Pyrophosphate is formed at a temperature below 240 °C, while longer-chain polyP is obtained at higher temperatures.[39] The products are converted at temperatures above 600 °C into the soluble Graham salt of $NaPO_3$ with chain lengths of 40–50 P_i units or 10–20 P_i units. This product has a glass-like appearance and is amorphous.[40]

2.1.2. Biosynthesis of polyP

In living organisms, polyP synthesis is obviously an enzymatic process. In bacteria, polyP is synthesized by two enzymes, polyP kinase 1 (PPK1) and polyP kinase 2 (PPK2), using ATP as a substrate. Both enzymes catalyze polyP formation in a reversible manner by transferring the γ-phosphate of ATP to the growing polyP chain (reviewed in ref. [6]). PPK1 favors the anabolic synthesis of polyP, while PPK2 mediates the reverse reaction, the phosphorylation of nucleoside mono- or diphosphates, leading to the degradation of the polymer. In eukaryotes, the mitochondria are the main source and intracellular generator of ATP. In mammalian systems, a PPK has not been found. The intracellular storage of polyP in eukaryotes occurs mainly in acidic vacuoles termed acidocalcisomes (lower eukaryotes) or dense granules (mammalian organisms), organelles usually located adjacent to the mitochondria;[41,42] Figure 1-II). In yeast, it has been proposed that polyP is synthesized from ATP during transport into the ≈100 nm large acidocalcisomes.[43] In these organisms, the transmembrane Vtc (vacuolar transporter chaperone) complex has a biocatalytic function and could act as a polyP polymerase.[44,45] In mammalian systems, a genuine polyP-synthesizing enzyme has not yet been identified. However, it is likely that in mammals the synthesis of the high-energy storing polyP is also driven enzymatically since the energy-rich phosphoanhydride bonds in this molecule are characterized by high activation energies and are therefore very stable at room temperature.

It is surprising and perhaps indicative that the ATP level in the platelet-dense granules and acidocalcisomes is high with ≈400 mM[46] compared to the one in mitochondria with ≈1 mM (reviewed in ref. [17]). In extreme contrast to these concentrations, the polyP concentration in the mitochondria is likewise low at ≈1 mM compared to the vacuolar polyP with 150 mM (**Figure 2**). Even more, the ADP concentration in the dense granules/acidocalcisomes (with 600 mM) and the pyrophosphate level (300 mM) are likewise high.[46] In the platelets, harboring the majority of the polyP in their dense granules, kinases are found, such as pyruvate kinase, phosphatidylinositol 3-kinase, and the threonine-specific protein kinase, which are involved in intracellular signaling.[47] The intracellular ATP pool is as high as 3–10 mM, while the ATP level in the extracellular space is only ≈10 nM (see ref. [17]). Therefore, it seems likely that in platelets with their dense granules, which are rich in different kinases and the substrate ATP, polyP is formed perhaps through a (reversible) kinase reaction.

The equilibrium constant of the putative (reversible) kinase reaction that mediates polyP formation is probably close to one (inclusive heat, released during enzymatic decomposition) because in this enzyme reaction, one energy-rich bond (in the reactant ATP) is cleaved on one side and another energy-rich bond (in the polyP product) is formed on the other side. However, since in the acidocalcisomes and platelet dense granules polyP is most likely present predominantly in an insoluble form, complexed with the positive cations Ca^{2+} and/or Mg^{2+}, it is expected that the concentration of the soluble polyP product in these compartments is low compared to the concentration of (soluble) ATP. Since enzyme reactions usually proceed in an aqueous environment, the polyP product in the proposed kinase reaction is therefore immediately

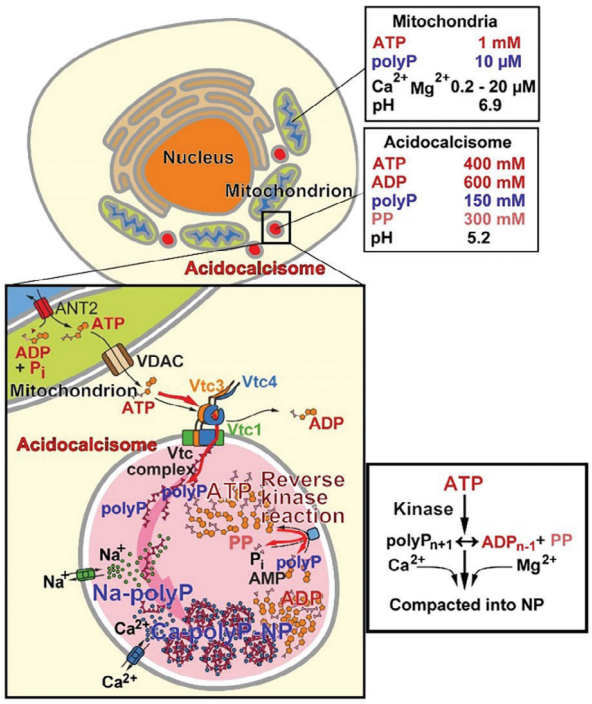

Figure 2. Proposed route of mammalian polyP synthesis. In mitochondria, the reduced coenzymes undergo oxidation in the respiratory chain, resulting in ATP synthesis. ATP is released from the matrix into the intermembrane space via the adenine nucleotide translocase 2 (ANT2) transporter and from there into the extra-mitochondrial cytosol via the voltage-dependent anion channel (VDAC). ATP accumulates in the adjacent dense granules, which correspond to the yeast acidocalcisomes with their Vtc complex, and might drive the synthesis of polyP by a postulated kinase, through a reversible reaction. Through dynamic exchange processes, the polymer forms either the soluble Na^+ salt or Ca-polyP nanoparticles (Ca-polyP-NP). Subsequently, Ca-polyP-NP and Na-polyP are released into the extracellular space upon platelet activation.

removed from the reaction as it precipitates in the presence of divalent cations. Consequently, the overall reaction/equilibrium is shifted toward the polyP product (Figure 2).

In the dense granules and acidocalcisomes, which are rich in calcium, polyP is deposited as an electron-dense material. In this state, with Ca^{2+}, the insoluble polyP most likely accumulates in the form of dynamic assemblies.[48] The Ca-polyP deposits are assumed to undergo dynamic exchange with free polyP and most likely with other cations such as Na^+. This dynamic deposition of polyP would also explain the finding that the polyP is released from these organelles as both insoluble Ca-polyphosphate nanoparticles (Ca-polyP-NP) and soluble Na-polyP.[46]

2.2. The Two Phases of polyP: Soluble (Coacervate) and Particle-Associated Forms (Nanoparticles)

In the circulating human blood or in the blood plasma, polyP exists as soluble Na-polyP at a concentration of ≈1 µg mL^{-1} [33] or in a particulate/nanoparticle (NP) form.[46] The polyP chains extruded from the platelets have a length of 60 -100 P_i residues, while the NP-associated polyP is composed of longer polymers.[46]

2.2.1. PolyP Chains in the polyP Coacervate

In the physiological environment, the polyanion polyP remains soluble only with Na^+ as the counterion. The concentration of this cation in serum is ≈100 mM (2.3 g L^{-1}), in contrast to Ca^{2+} at ≈2.5 mM (90 mg L^{-1}). Under these concentration conditions, polyP remains in the soluble form but retains its chelating properties. It should be noted that Na^+ is a harder cation and binds more strongly to hard counter-anions compared to Ca^{2+}.[49] At a concentration of >5 mM polyP (based on P_i), the polymer undergoes coacervation or NP formation, pH-dependently.[50]

PolyP exhibits a biologically distinctive property, the formation of a coacervate. Coacervation is a process in which phase separation occurs, in the case of polyP during salt bridge formation between the polymer and smaller divalent metal cations such as Ca^{2+} or Mg^{2+}. During the coacervation of polyP at pH 7, a Ca^{2+} shell is formed around the condensed polyP polymer phase.[51] Based on modeling/simulation studies, binding of the monovalent cation Na^+ to the polyP polyanion occurs in alternating order, while divalent cations (Ca^{2+} and Mg^{2+}) bind to the polyP strand along an ordered peripheral line;[52,53] **Figure 3**-I. Molecular dynamics simulations of the interaction of polyP and Ca^{2+} ions revealed that, at pH 7, both components first organize rather randomly, while in the following both partners sort themselves apart (Figure 3-IIA,B) and finally form two "layers" with polyP on one side and Ca^{2+} on the other side (Figure 3-IIC,D). These aggregates form liquid droplets, which further associated to organized, two-liquid-phase droplets in the aqueous environment. At pH 10, the Ca^{2+} and polyP components form concentric rings that condense to concrete particles, which further grow to NP that are (almost) devoid of water.[52]

Coacervate assemblies are formed from a colloidal system via separation into two liquid phases. The material formed from polyP and Ca^{2+} ions is highly viscous (**Figure 4**-IA–C). At low magnification, the coacervate blocks appear with a compact morphology traversed by open cavities (Figure 4–ID). At higher magnification, it becomes overt that the coacervate blocks are interspersed with ≈30 nm large granules embedded in the coacervate (Figure 4-IE). These granules are rarely suspended but predominantly associated with clusters via the aqueous gel (Figure 4-IF).

Compared to Ca-polyP-NP formation, the process of coacervation (Ca-polyP-Coa formation) at pH 7 is a slower process;[52] Figure 4-IIA,B. Starting with a solution of 1.2 M $CaCl_2$ added dropwise to a 0.48 M Na-polyP solution with stirring (Figure 4-IIA), the reaction mixture becomes turbid and at a molar ratio of 1:2 (Na-polyP:$CaCl_2$) a viscous gel with a consistency of 10–15 cP[53] is formed (Figure 4-IB,C).

FTIR spectra showed distinct differences between the Ca-polyP-Coa and Ca-polyP-NP as well as to Na-polyP and Ca-dihydrogen phosphate (Ca-P_1). According to published data,[54–56] all polyP samples show the characteristic signals for ν_{as}(P—O—P) at wavenumbers ≈863 cm^{-1} and for ν_s(P-O-P) at ≈733 cm^{-1}.[52] In contrast, Ca-dihydrogen phosphate is distinguished by the presence of the signal $\nu_3(PO_3)^{2-}$ at 1021 cm^{-1} and for $\nu_4(PO_4)$ at 580 cm^{-1}. The Ca-polyP-NP comprises a distinct signal at a wavenumber of ≈1084 cm^{-1} reflecting $\nu_{as}(PO_3)^{2-}$, a band that is only minor in the coacervate or the Na-polyP sample.

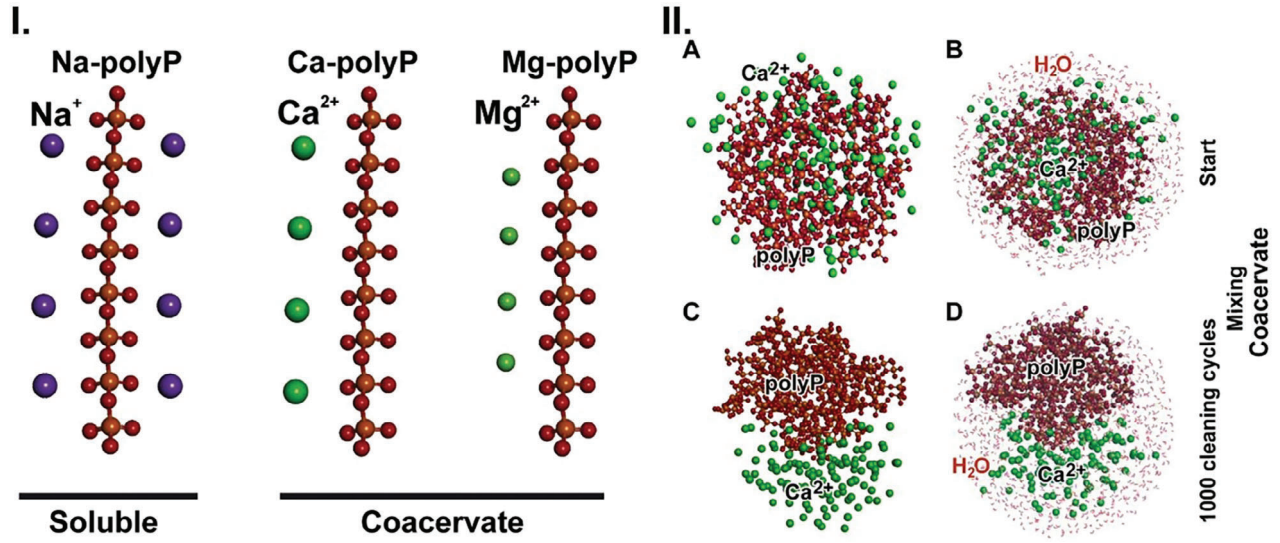

Figure 3. Coacervate formation at pH 7 and NP formation at pH 10. Molecular dynamics simulations. I) Structure of the polyP salts with monovalent and divalent cations. The simulation studies showed that the monovalent ions (Na^+) are arranged alternately, while the divalent cations (Ca^{2+} and Mg^{2+}) are located only on one side of the polyP strand. II) (A,B) At pH 7, the two components, polyP, and Ca^{2+}, arrange stochastically and, after 1000 cleaning cycles, separate into a polyP cluster and a Ca^{2+} assembly. Then, in the aqueous phase, a liquid–liquid phase separation takes place under the formation of the coacervate. Reproduced with permission.[53] Copyright 2023, Springer Nature Switzerland AG.

2.2.2. PolyP Transition from Coacervate to NP

Scanning electron microscopy (SEM) analyses of the samples from the turbidity studies (Figure 4-II) revealed that at pH 7 the coacervate phase is formed from Na-polyP and $CaCl_2$, while at pH 10 NP are assembled (**Figure 5**). The coacervate shows a block-like morphology at low SEM magnification (Figure 5A–C), while at higher magnification grain-like particles become visible (Figure 5D). At the beginning of the pH 10 titration phase, the collected particles, the grains, show an already rounded, NP-like morphology and are often decorated with particles, resulting in wrinkled surfaces. When the concentration ratio in the reaction fluid reaches 480 mM $CaCl_2$ to 400 mM polyP, distinct rounded particles, termed NP, are formed (Figure 5E,F). The ultimately formed NP have a size of ≈100 nm (Figure 5G,H); they have a porous internal morphology (Figure 5H). From these data, it can be concluded that those NP are formed by assembly from the ≈30 nm-sized granules in the coacervate phase.

2.2.3. Growth of polyP Nanoparticles

In the presence of Ca^{2+}, the phosphate anions are less soluble.[57] The degree of solubility is dependent on the pH of the reaction

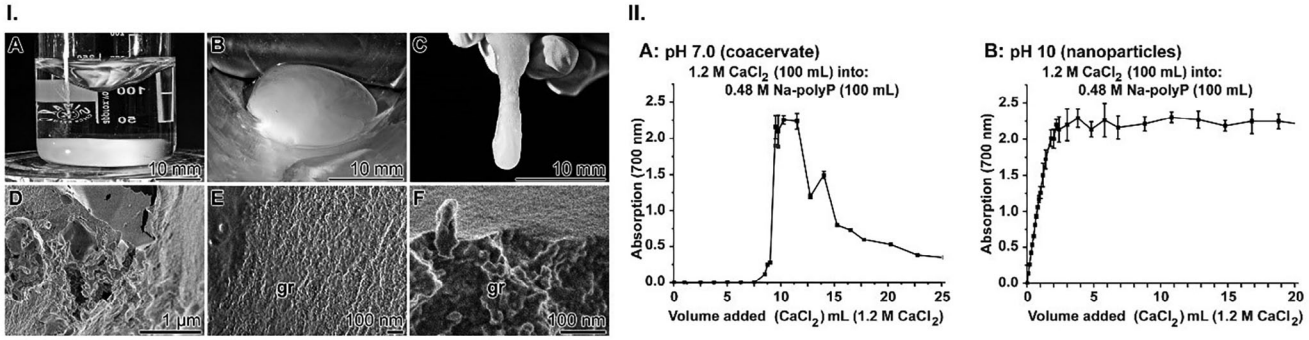

Figure 4. Coacervate formation and characterization. I) Coacervate formation from $CaCl_2$ and Na-polyP. Optically visible aggregate formation at pH 7.0. A) A $CaCl_2$ solution (14 g of $CaCl_2 \bullet 2H_2O$ in 100 mL) is added to a Na-polyP solution (5 g in 100 mL) at room temperature under vigorous stirring. After completion of the process, the B) coacervate with a C) viscous consistency is obtained. At low SEM magnification, D) the coacervate shows a solid morphology interrupted by open cavities. E,F) At higher magnifications, small (≈30 nm) granules (gr) embedded in the viscous coacervate appear. Reproduced with permission (Creative Commons Attribution License).[14] Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. II) Optically visible aggregate formation from $CaCl_2$ and Na-polyP. In a volume-controlled setup, the appearance of aggregates either at (A) pH 7.0 or (B) pH 10 is monitored. At a pH of 7, the first aggregates appear later (after 6 mL) compared to the assay run at pH 10 (0.5–1.5 mL). Reproduced under the terms of the CC-BY-NC license.[52] Copyright 2022, Elsevier Ltd.

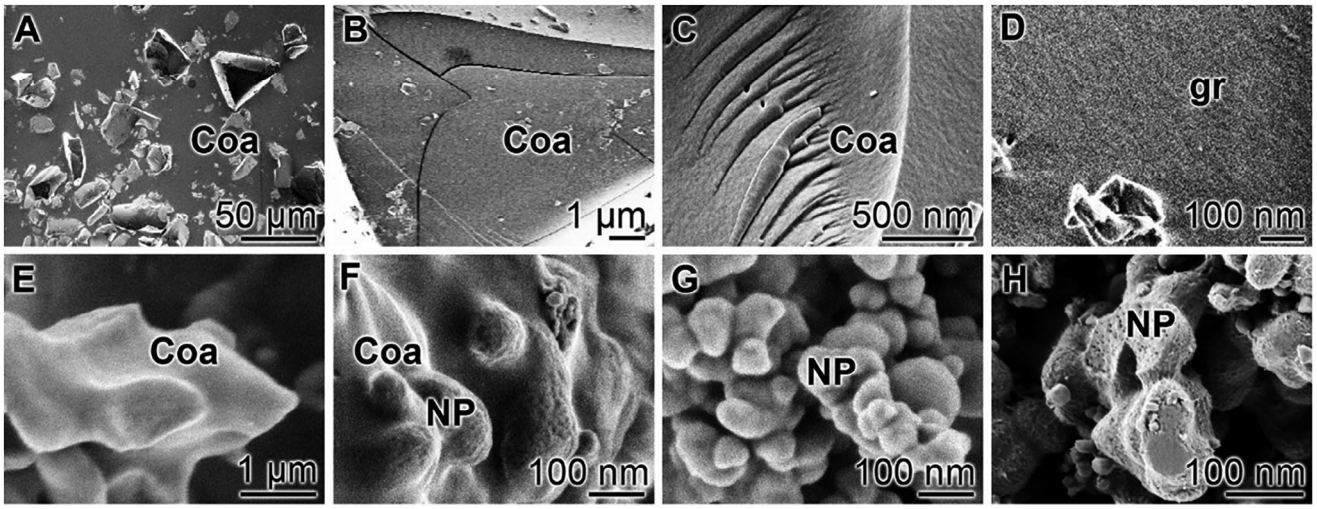

Figure 5. The transition from coacervate to NP. **A–D**) The coacervate (Coa) phase is formed at pH 7. A–C) At lower SEM magnification, the coacervate aggregates appear as blocks. D) At a higher magnification ≈30 nm grains (gr) appear within the liquid phase. **E**,**F**) After raising the pH to 10 the aqueous polymer coacervate droplets develop to NP. **G**,**H**) The NP has a porous internal morphology. A–D) Reproduced with permission (Creative Commons Attribution License).[14] Copyright 2018, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. E–H) Reproduced under the terms of the CC-BY-NC license.[52] Copyright 2022, Elsevier Ltd.

mixture. Phosphoric acid is a weak triprotic Brønsted–Lowry base. Therefore, the concentration of its different dissociated forms, and hence the concentration of the soluble phosphorus, depends on the pH of the solution. The reactivity of PO_4^{3-} to free HPO_4^{2-} and $H_2PO_4^{-}$ increases with decreasing pH. This means that with increasing pH (increase in PO_4^{3-}) the amount of insoluble Ca-polyP increases. The dissociation process is reversible, the equilibrium between the soluble metal cations and phosphate anions will be almost entirely on the side of the (almost) insoluble Ca-phosphate at pH 10.[58] This chemistry could explain the growth of the particles/NP. Furthermore, the reversible conversion of NP to coacervate could also be formulated in this sense, which is needed for understanding the transformation of NP to the biomedically active coacervate form.[14,56]

In the cells, polyP is stored in the dense granules or acidocalcisomes, organelles with a size ≈100 nm.[42] Due to the Ca^{2+} environment, it can be assumed that polyP exists there as NP. It was a challenge to fabricate chemically Ca-polyP-NP of this size (≈100 nm) in order to assess their biomedical potential. Applying a superstoichiometric concentration ratio between soluble $CaCl_2$ and Na-polyP of 2:1, NP could be reliably prepared in an amorphous state at pH 10.[59] Only in the amorphous form can these particles elicit biological activity.[60] The biological activity of the NP is achieved via transformation into the coacervate phase of polyP.

2.3. Biological Importance of Cations in polyP Nanoparticles

The biomedical application of polyP is decisively dependent on the choice of the respective cation that forms the salt with polyP.[61] This circumstance is closely related with the ion environment at the site where the polymer is formed and applied. As an example, the levels of Ca^{2+} (extracellularly normally ≈2.3 mM) and Mg^{2+} (≈0.9 mM) show considerable fluctuations during regeneration, e.g., during wound healing. The Ca^{2+} level drops by 40% immediately after wounding, while conversely the Mg^{2+} concentration increases by 45%.[62] This change is physiologically significant because Mg^{2+} stimulates cell migration into the injured region. In the later phase of cell proliferation, the concentration ratio between the two cations returns to the plasma levels. Ca^{2+} stimulates cell proliferation.[63] This observation indicates that, in addition to the regeneratively acting polymer polyP, the respective cation in the wound bed is critical for the effectivity of the given polyP salt. Consequently, the choice of the counter-cation of polyP allows also a (possible) substitution of the ion milieu in the regeneration area.

PolyP is also suitable for application as Na-polyP, but with the potential adverse side effect that the polymer chelates divalent cations out and reduces thereby their bioavailability. As a smart biomaterial, polyP can guide divalent, substituting ions to the target site along with their own beneficial properties. Monocalcium phosphate (Ca-P_1) and Ca^{2+} tripolyphosphate (Ca-$polyP_3$) have crystal structures and morphologies in the anhydrous phase (**Figure 6**A,B,E,F).[64–66] In solution, Na-polyP can immediately elicit its pharmacological activities, such as the generation of metabolic energy. In the presence of divalent cations, polyP forms NP at a superstoichiometric ratio and under alkaline conditions.[59] Mg^{2+} accelerates the adhesion of mesenchymal stem cells to the fibrillar structures in the cartilage through conformational changes of the integrins and thereby promotes cartilage synthesis.[67] The NP formed with this ion has a size of ≈500 nm (Figure 6C,D). Phosphate (PO_4^{3-}) is a hard base and tends to bind to hard acids such as Mg^{2+}. Ca^{2+} is less hard, larger than Mg^{2+} and has a lower charge state (the charge criterion applies mainly to acids, to a lesser extent to bases), and is weakly polarizable. With this cation, polyP of a chain length of 40 P_i units forms a smaller NP with an average diameter of ≈150 nm (Figure 6I,J). Down the hardness scale, Sr^{2+}-polyP particles are

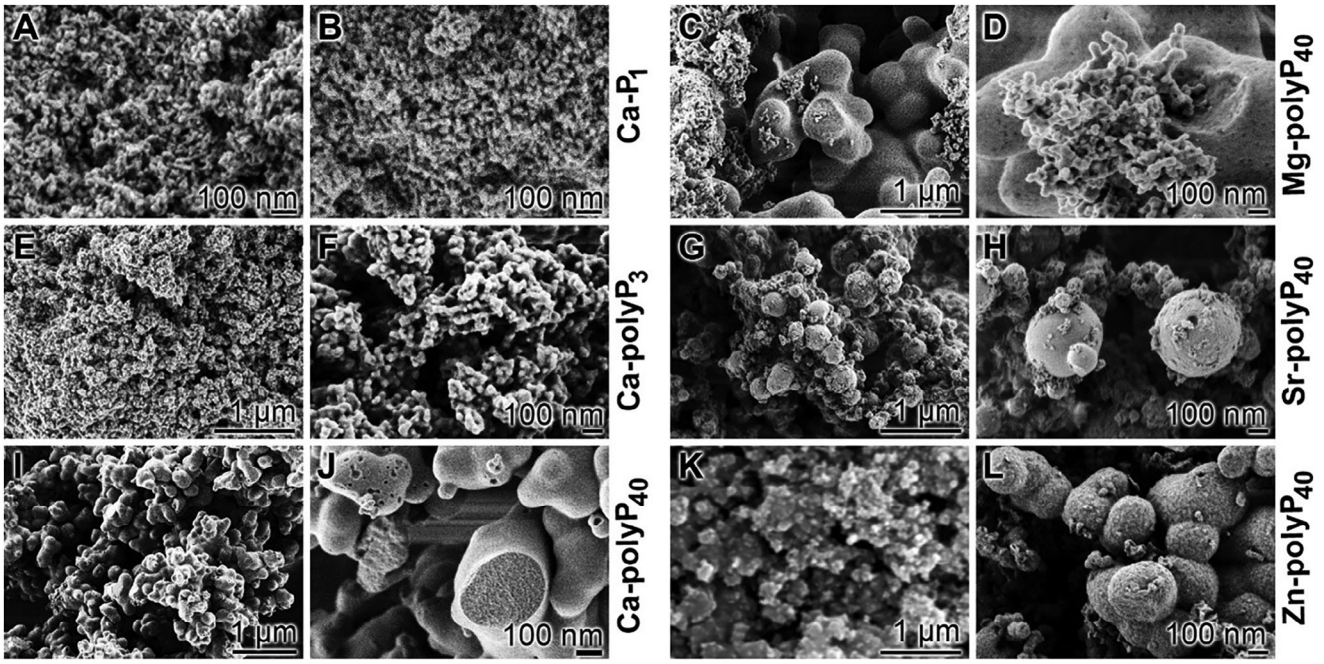

Figure 6. Morphology of therapeutically important phosphate/polyP particles formed with divalent ions; SEM analyses. A,B) Monocalcium phosphate (Ca-P_1); E,F) Ca^{2+} tripolyphosphate (Ca-$polyP_3$), and I,J) Ca^{2+} polyP with a chain length of 40 P_i units (Ca-$polyP_{40}$). Then, C,D) NP are shown that are prepared from Na-polyP with 40 P_i units and Mg^{2+} as the cation (Mg-$polyP_{40}$), or G,H) Sr^{2+} (Sr-$polyP_{40}$), as well as of K,L) Zn^{2+} (Zn-$polyP_{40}$). A–H) Reproduced with permission.[61] Copyright 2018, The Royal Society of Chemistry. K,L) Reproduced with permission.[64] Copyright 2020, The Royal Society of Chemistry.

similarly sized (Figure 6G,H) and for Zn^{2+} NP an average size of ≈150 nm was determined (Figure 6K,L).

Sr^{2+}, as an alkali-earth metal cation, was found to increase the synthesis activity of osteoblasts and is present in higher amounts in the metabolically active trabecular bone compared to cortical bone.[68] In addition, polyP in the form of Zn-polyP NP supports the growth and migration of epidermal keratinocytes, which also makes this formulation a candidate for wound regeneration.[64]

2.4. Interaction with Proteins

Recent studies have shown that polyP may have an additional role in regulating protein activity: PolyP exhibits a chaperone-like activity.[69] The polymer is able to protect cells from stress-induced protein aggregation.[69,70] PolyP binds and stabilizes misfolded proteins in a soluble β-sheet-rich conformation, thereby preventing their aggregation.[70] This mechanism (based on the stabilization of β-sheet-rich unfolding intermediates) is thought to be involved in the protective effect of polyP on neuronal cells against the toxicity of amyloidogenic proteins such as Alzheimer's Aβ protein and may therefore be of interest in diseases that are associated with the deposition of amyloid fibrils.[69,71] In addition to its effect on amyloid aggregation in neuronal tissue, the chaperoning activity of polyP also appears to be involved in the antiviral, anti-SARS-CoV-2 activity of the polymer.[27,72] PolyP has been reported to interact specifically with the receptor binding domain of the viral spike protein,[27] preventing receptor binding and cell infection, but also intracellularly with the host receptor protein angiotensin-converting enzyme 2 (ACE2) as well as the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp).[72] The binding of polyP to positively charged amino acids of the latter proteins leads to their enhanced proteasomal degradation, resulting in inhibition of SARS-CoV-2 replication, as both demonstrated in various cell lines infected with the virus and in experiments with primary human nasal epithelial cells exposed to nebulized SARS-CoV-2.[72] This effect could be suppressed by adding the proteasome inhibitor MG-132.

2.5. Distribution of polyP in Eukaryotic Cells and Tissue

In the precursor cells of the platelets, the megakaryocytes, ATP is generated in the mitochondria during the enzyme/coenzyme-driven respiratory chain. Glucose is metabolized to lactate/pyruvate and transported into the mitochondrial matrix where it is oxidatively decarboxylated to acetyl-CoA. In the citric acid cycle, the reduced coenzymes NADH and $FADH_2$ are formed. These coenzymes then undergo reoxidation to NAD^+ and FAD, which is coupled with ATP production via mitochondrial ATP synthase. ATP crosses the inner mitochondrial membrane through the adenine nucleotide translocase (ANT2) and from there the outer mitochondrial membrane through the voltage-dependent ion channel (VDAC) and reaches the cytoplasm; Figure 2. We propose, as mentioned above, that within the dense granules/acidocalcisomes, Ca^{2+}-bound polyP is synthesized from the abundantly present ATP via the postulated (reversible) kinase(s) reaction(s); Figure 2.

It is essential that any nutrient, energy-delivering system, cytokine or growth factor required for regeneration reach the

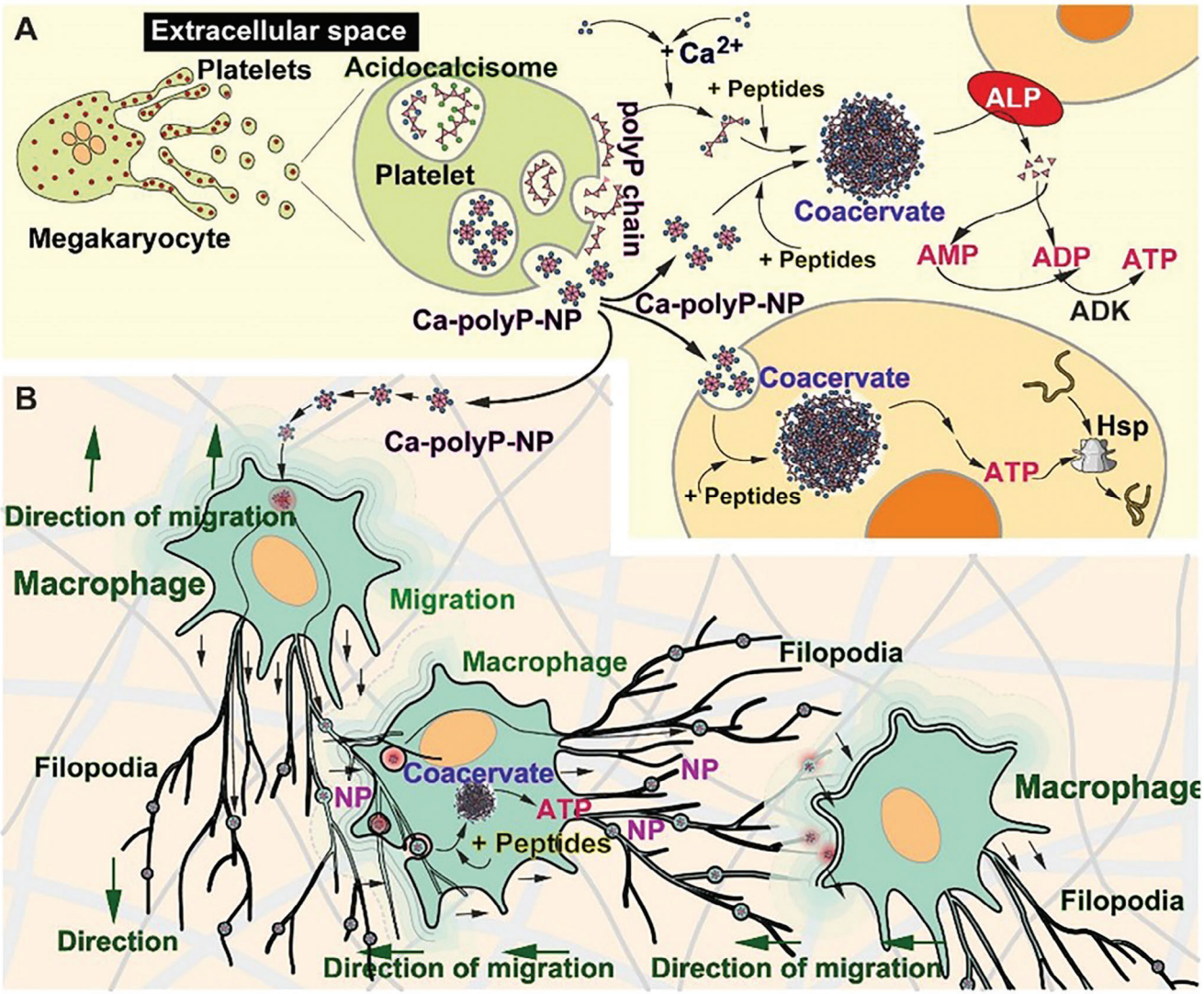

Figure 7. Proposed distribution pathways for Ca-polyP-NP. A) In the extracellular space, the platelets produced by megakaryocytes harbor the bulk of the polyP and primarily distribute polyP in the circulating blood. They release the two forms of polyP (Na-polyP and Ca-polyP-NP), which are subsequently enzymatically cleaved by ALP. The liberated free energy is stored in ADP, which is further processed to ATP in the presence of ADK (adenylate kinase). B) In addition, it is proposed that the macrophages, which readily engulf NP, are involved in supplying injured regions with polyP. These cells comprise migrasomes, organelles produced by migrating cells; they can be filled with NP. The migrasomes migrate along long, retracting fibers.

site of need in a fast equally safe, and economically potent manner. In this regard, the vascular system is most effective. This is particularly important for processes involved in repair of osteochondral defects affecting cartilage, cartilage-bone interface, and subchondral bone. Importantly, the vascularized periosteum contributes essentially to a successful blood supply.[73] Even in the case of extensive wound regeneration, an operant restoration of the blood circulation must first take place.[74] After reaching the damaged site to be repaired, the blood, which contains the required physical and chemical factors, such as oxygen, nutrients, and regulatory factors, can initiate cell proliferation and remodeling. Growth factors such as bone morphogenetic proteins[75] are also formed together with the (compartmentalized) polyP in megakaryocytes, from which the platelets (thrombocytes) originate.[76] These small cell fragments play a key role in every type of tissue repair in mammals.

Upon platelet activation in response to tissue injury,[77,78] polyP is released from the platelets in two forms, either in the soluble form, as polyP complexed with Na^+ cations, or as insoluble NP with Ca^{2+}, as Ca-polyP-NP with a size of ≈300 nm;[46,79] **Figure 7A**. The polyP chains in the soluble polyP fraction released from the platelets are short with a polymer length of 60–100 P_i residues. In the acidocalcisomes and dense granules, both the pH milieu (5.4) and Ca^{2+} concentration (70 mM) are suitable for NP formation.[80] The release of the two forms of polyP, soluble and nanoparticulate, from the platelets occurs in a controlled manner[81,82] as proposed.[46] In the circulating blood after full platelet activation, the concentration of polyP is relatively high with 0.5–3 μg mL^{-1}.[46,83] The level of Ca-polyP-NP has not yet

been determined due to analytical difficulties. PolyP in the circulating blood or plasma released from activated platelets has only a short half-life with ≈1.5–2 h,[84] compared to quiescent platelets with a life-span of 7–10 d; their density is high with 150–400 • 10^3 platelets per mL. The polyP molecules are of short to largely short chain lengths with ≈50 P_i units for Na-polyP and ≈150 P_i units for the particle-associated form when released from the platelets into the bloodstream. In the circulating blood, polyP, which is incorporated into NP, remains attached to the platelet surface while the soluble polymer chains are set free.[79] When the platelets are exposed to the extracellular matrix (ECM), they are activated by interaction with collagen and laminin, and also with fibrinogen during vascular events.[85] The activation of the platelets upon contact with ECM proteins occurs via their adhesion receptors, especially if the endothelial barrier is disrupted.[86,87]

The concentration of Ca^{2+} in the serum is low at 80–100 µg mL^{-1} (≈2.3 mM),[83] in contrast to Na^+ at 5.7 mg mL^{-1} (142 mM). In turn, the released Na-polyP undergoes at least partial coacervation. The second form of polyP released from platelets is Ca-polyP-NP. This formulation is particulate and, in turn, insoluble. The platelets are the principal disseminating cells in the circulation system. There, via the platelets, polyP is transferred to other body cells where the polyP undergoes enzymatic hydrolysis and processing, generating metabolic energy (ATP);[17] Figure 7A.

Since polyP has a strong potency to regulate the metabolism and defense state in macrophages,[88–90] one might assume that these cells, in addition to the platelets, act as vehicles for NP to be distributed in the body (Figure 7B). These migration-competent cells, which are important for the inflammatory status, produce migrasomes, which are loaded onto retraction fibers that trail behind these migrating cells, the podocytes.[91] The migrasomes are vesicular structures filled with cellular contents and small vesicles. Platelets also produce migrasomes, which they release after activation.[92] It is very likely that Ca-polyP-NP is also involved in this recently published cell migration pathway. The macrophages migrate both in the plasma and in the intercellular matrix as well as in the basement membrane along the fibronectin network.[93] In this cell vehicle the components, polyP, are distributed throughout the body.

3. Distinguished Property of polyP: Generation of Metabolic Activity

Exposure of SaOS-2 cells to Ca-polyP-NP revealed a significant increase in the ATP level in the extracellular space (culture medium).[94,95] The first distinct hint that polyP anabolically upregulates the energy production of the cells came from TEM (transmission electron microscopy) analyses, which showed that cells grown in the presence of polyP have a high accumulation of mitochondria adjacent to the cell nucleus (**Figure** 8-ID–F), while normal numbers of these organelles are seen in controls (Figure 8IA,B).

The generation of ATP by polyP involves two enzymes, ALP and adenylate kinase (ADK).[17] The ALP is a ubiquitous, often membrane-bound ecto-enzyme[96] that hydrolyzes polyP from the ends of the polymer. This enzyme degrades polyP in a processive manner.[97] The mechanism has been described in detail.[17] In response to polyP, cells, mostly SaOS-2 cells and mesenchymal stem cells (MSC) have been used, migrate directionally, and begin to organize into microvessels. This process can be abolished or significantly prevented by the addition of levamisole, an established inhibitor of ALP,[98] as well as by the natural ADK inhibitor, P^1,P^5-di(adenosine-5´)pentaphosphate.[99] The inhibitor studies supported the conclusion that during the release of P_i from polyP, the generated metabolic energy is used for the synthesis of ADP from AMP. ADP is then up-phosphorylated by ADK to ATP.[17,100] In turn, polyP acts as a reservoir for metabolic energy in eukaryotes and especially in the extracellular space of tissues in animals since the beginning of their development.[16,101] The generation of ATP by SaOS-2 cells depends on the concentration of polyP, as shown here by exposing these cells to Ca-polyP-NP in the range between 3 and 30 µg mL^{-1} (Figure 8-II). Overall metabolic activation of the cells with the mineralization activation cocktail[102] composed of β-glycerophosphate, ascorbic acid, and dexamethasone boosts ATP production considerably.

4. Current Areas of Application of the polyP NP

Basically, any area of the body that is injured is subject to polyP substitution and regeneration therapy with polyP.[17] The delivery of polyP focuses primarily on those areas that are not sufficiently supplied with blood and thus with polyP via the blood platelets. At risk are patients suffering from thrombocytopenia, for example during sepsis,[103] chronic wounds[104] bone fractures,[105] and bone marrow disorders.[106] The economic burden on society is high; just for example, the expenditures for chronic wounds in the US have been estimated at $28.1–$96.8 billion.[107]

The fields of application of polyP-NP in human therapy have been elucidated in the last years, and even proof-of-concept studies have confirmed the substantial beneficial effect of polyP and its NPs. Here we focus on the effects of the polymer in the field of hard tissues (bone) and soft tissues (cartilage and wound healing); **Figure** 9.

4.1. Bone Fracture Healing

Bone is built of organic fibers (such as collagen), 60–70% minerals Ca-phosphate components, and only ≈15% water. The blood circulation is limited and bone is hypoxic in some regions, such as the diaphysis, which is devoid of any arterial blood supply. In contrast to this region, which is the midsection (shaft) of a long bone, the apical metaphysis portion with its cartilaginous component (epiphyseal plate) together with its fibrous component, the growth plate of the bone, is heavily vascularized and supplied with blood.

4.1.1. PolyP a Physiological Regulator of Bone Mineral Crystallization

It is established that the mineral deposits in the human body are first deposited as amorphous material that is biologically active and then processed to crystalline minerals.[56] This holds true

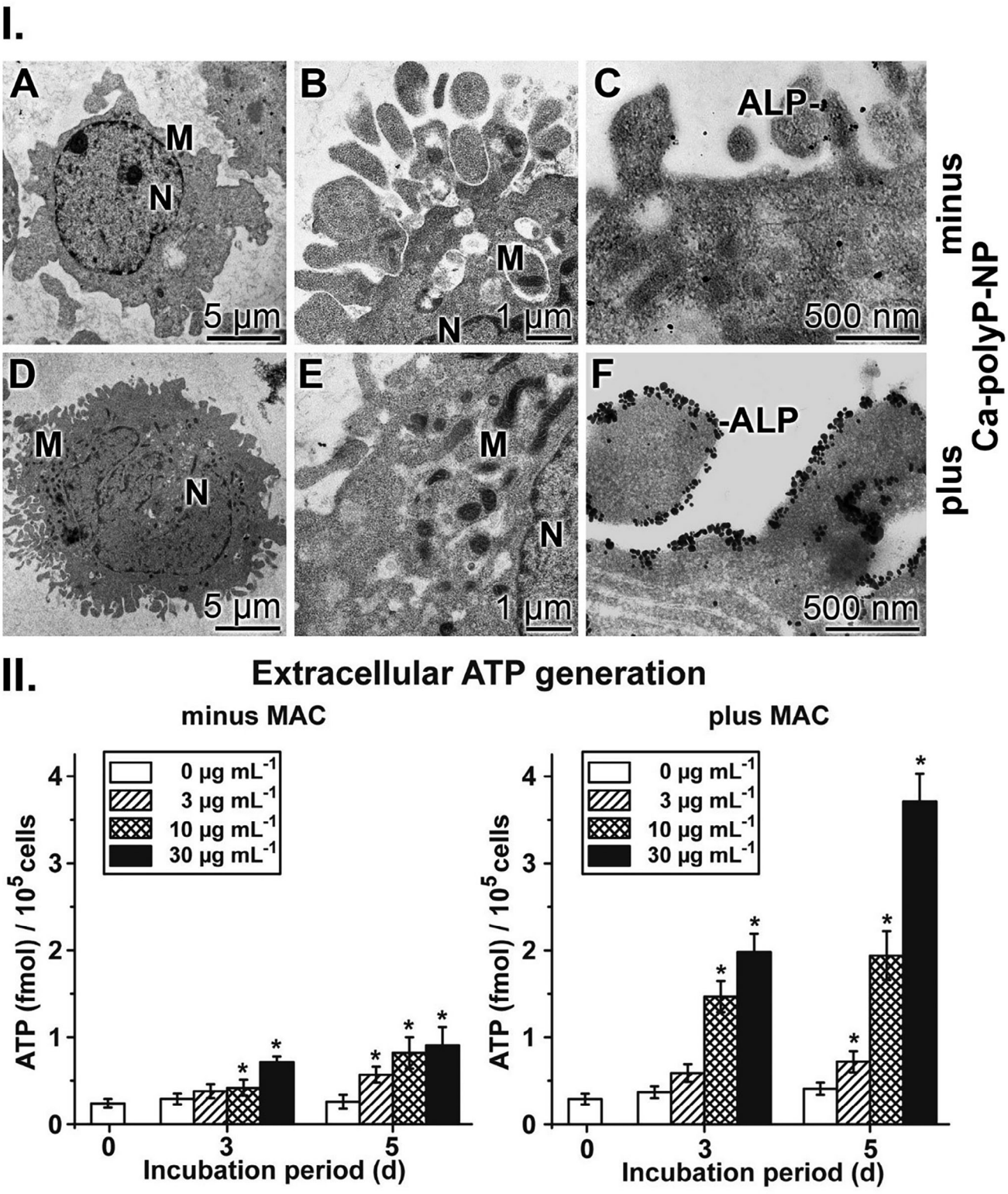

Figure 8. Generation of ATP in response to Ca-polyP-NP incubation in SaOS-2 cells. I) The cells were incubated either in the A–C) absence polyP or were D–F) exposed to 30 µg mL^{-1} of Ca-polyP-NP; TEM. In the absence of the polymer, the abundance of mitochondria (M) in the cells is low, while in the assays with the polymer, their density is high. The cell nucleus is also marked (N). By applying the immunogold labeling technique, the localization of the alkaline phosphatase (ALP) becomes visible; compared to the controls, the density of the ALP immune complexes on polyP-treated cells is high. II) The level of ATP in the extracellular space of SaOS-2 cells increases after exposure of the cells to 3, 10, and 30 µg mL^{-1} of Ca-polyP-NP significantly (*). After activation of the cell metabolism with the mineralization-activation cocktail (MAC) the level of ATP increases drastically. Reproduced under the terms of the CC-BY license.[94] Copyright 2015, The Company of Biologists Ltd.

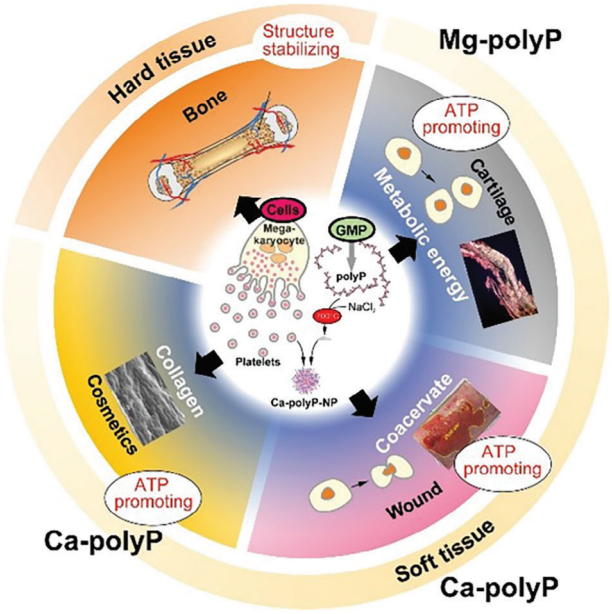

Figure 9. The major application routes of polyP in regenerative medicine, for the repair of hard tissue (bone) and soft tissue such as cartilage and in wound healing or cosmetics.

also for the mineral phase of bone, hydroxyapatite (HA). The non-crystalline precursor of HA is amorphous Ca-phosphate (ACP). This phase can be stabilized by polyP at a percentage of >15% by weight.[108,109]

Central to HA formation is the enzyme ALP, more precisely a specific isoform, the tissue-nonspecific alkaline phosphatase (TNAP), which is strongly expressed in bone, liver, and kidney and plays a key role in the calcification process of bone.[110] This enzyme not only hydrolyzes pyrophosphate, an inhibitor of biomineralization at least in vitro systems.[111] It also provides inorganic phosphate (P_i), which is needed as a source for Ca-phosphate deposition in bone. Pyridoxal phosphate and β-glycerophosphate are considered organic sources of P_i.[102] However, these precursors are quantitatively not sufficient to provide phosphate for HA formation in the body.[112] Since the sites for initial bone formation are highly vascularized[113] and the number of platelets increases during reshaping and reorganization during embryonic development,[114] it is most likely that it is polyP that is the major P_i supplier for bone mineralization.[112] This polymer, polyP, stabilizes the amorphous precursor of HA and it is the ALP that controls the kinetics of transformation of the precursor into the crystalline phase.[109] This transition process of ACP to crystalline Ca-phosphate (CCP) and then to HA can be traced by FTIR (**Figure 10A**) and also by SEM (Figure 10B). The amorphous ACP state is characterized by a non-split $\nu_4(PO_4)$ vibration at a wavenumber of 580 cm^{-1}. Incubation of ACP with ALP (bovine intestinal mucosa) for 60 min, especially for 180 min, leads to a gradual splitting of this vibration signal[109] into two distinct peaks at 599 and 557 cm^{-1}. This shift reflects the transformation from ACP to CCP.

In parallel, X-ray diffraction (XRD) analyses were performed (Figure 10C). The diffractograms show that ACP shows no signs of crystallinity at the beginning of the incubation with ALP, while after 60 and 180 min incubation with the enzyme distinct and characteristic peaks at 30.2°, 46.6° and 54.8° are recorded that are characteristic for HA.[115] SEM analyses confirmed that ACP has a globular shape, while CCP particles are angular to cubic (Figure 10B).

4.1.2. PolyP the Physiological Driving Force of Bone Formation

Two major components drive bone (HA) synthesis, first, phosphate (P_i), which processes fibrous tissue to HA, a crystalline Ca-phosphate ($Ca_{10}[PO_4]_6[OH]_2$), and, second, metabolic energy. The cells involved in bone formation, osteoblasts, and osteoclasts, are provided with sensitive energy-monitoring mechanisms such as the AMP-activated protein kinase and the mechanistic target of rapamycin (mTOR) pathway to control bioenergetics and ATP synthesis in cells of the osteogenic lineage.[116] Furthermore, extracellular ATP is a decisive autocrine and paracrine signaling molecule for bone and muscle cells.[117] Not only bone synthesis but also bone resorption is an energy-intensive metabolic process. An imbalance of both processes contributes to the development and progression of osteoporosis.[118]

Both cornerstones required for bone formation, phosphate, and metabolic energy, are provided by polyP. First, phosphate: as mentioned, polyP is transported to the tissues, including bone, via the blood platelets. There, Na-polyP and also Ca-polyP-NP are subject to enzymatic hydrolysis. In the case of the Ca-polyP-NP, the particles must be enzymatically disintegrated. During this physical/chemical process, the particulate polyP becomes soluble in the coacervate phase. In this state, the polyP can be enzymatically hydrolyzed to P_i, which is then fed to the ACP precursor, most likely amorphous calcium carbonate (ACC), which is subsequently transformed to ACP in the presence of P_i (**Figure 11**).[119]

Second, metabolic energy: during cleavage of the energy-rich phosphoanhydride bonds by ALP, the released Gibbs free energy is stored in AMP to ADP;[17] Figure 7A and Figure 11. The generated ADP undergoes up-phosphorylation to ATP via ADK under the formation of ATP and AMP. ATP is required for phosphorylation of a central protein, the dentin matrix protein 1 (MP-1), present in the ECM of teeth and bone.[120] Impaired expression of MP-1 leads to osteomalacia with hypo-mineralized tissues due to abnormal phosphate supply to the bone-forming centers.[121] MP-1 modulates the transcription factor sore-binding factor a1 (Cbfa1), which is a runt domain family member, and through this interlink, MP-1 is a key molecule in cartilage and bone development.[122] The functional activity is controlled by the Fam20C kinase.[123]

These two prerequisites for a metabolic synthesis and regeneration of bone are met by Ca-polyP-NP with the supply of P_i and metabolic energy; even Ca^{2+} is delivered via this route.

In its capability to provide metabolic energy (ATP) required for energy-dependent tissue regeneration, polyP fundamentally differs from other materials used for bone regeneration that are based on calcium phosphate. These materials such as ß-tricalcium phosphate (ß-TCP) or HA are widely used as bone substitute materials or bone fillers, and their osteoconductive and sometimes osteoinductive properties have been extensively studied.[124] The regeneration activity of the latter materials is largely based on the release of calcium and phosphate ions,

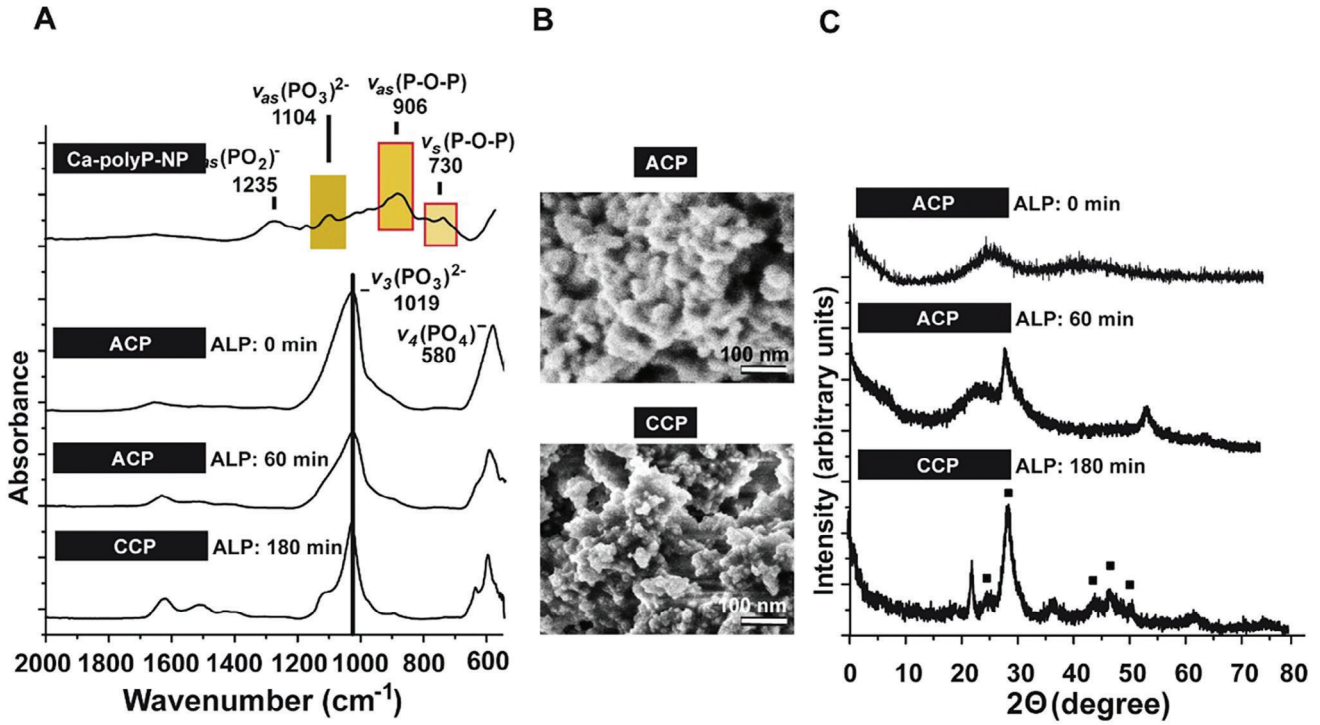

Figure 10. Effect of incubation with the enzyme ALP on the transition kinetics of ACP (amorphous Ca-phosphate) stabilized by 15% (by weight) of polyP to CCP (crystalline Ca-phosphate). A) The FTIR spectrum of ACP shows no splitting at the ν_4PO_4 band, while a split pattern appears during incubation with ALP for 60–180 min. B) Morphology of ACP (globular shape; top) versus CCP (angular to cubic morphology; bottom). C) XRD spectrum of CCP with distinct crystalline signals (cube symbols); the diffractogram for the initial ACP state does not show a sharp peak. After 60 min of ALP treatment, the first sharper peaks appear. A) Reproduced with permission (Creative Commons Attribution).[61] Copyright 2018, The Royal Society of Chemistry. B,C) Reproduced with permission.[109] Copyright 2022 The Academy of Dental Materials. Published by Elsevier Inc.

which are involved in the regulation of maturation and function of osteoblasts and osteoclasts.[125] They induce the expression of osteoblast differentiation marker proteins such as ALP, collagen type I, and bone morphogenetic proteins, are involved in signaling pathways (calcium), and provide the materials for bone mineral deposition.[124,126] However, these materials lack the metabolic energy (ATP) providing function shown by polyP.

There are only a few other strategies to stimulate tissue regeneration by enhancing energy production. However, in contrast to polyP, these strategies rely on the delivery of

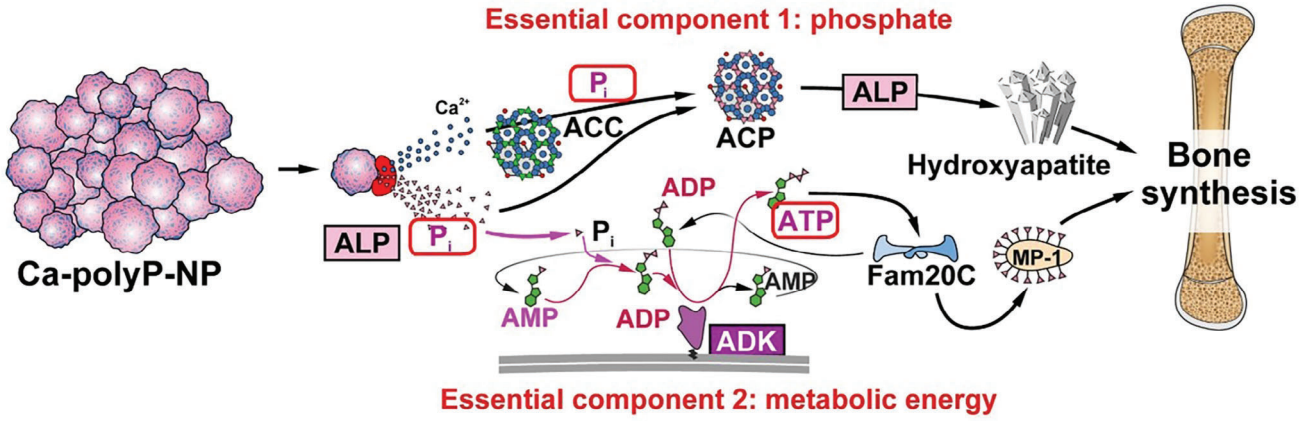

Figure 11. The two prerequisites for controlled synthesis and regeneration of bone; P_i and metabolic energy. Ca-polyP-NP is processed by ALP under the release of P_i. Phosphate is needed for the conversion of ACC to ACP, which proceeds in the absence of enzymes. From the amorphous state, Ca-phosphate (ACP) maturates to hydroxyapatite after enzymatic hydrolysis of the polyP constituent. The release of Gibbs free energy that accumulates during hydrolysis of the energy-rich bonds in polyP is (partially) stored in ADP, which is subsequently converted to ATP. ATP, in turn, feeds the Fam20C kinase while phosphorylating the bone/dentin matrix protein 1 (MP-1). MP-1 is a major controlling factor in bone formation.

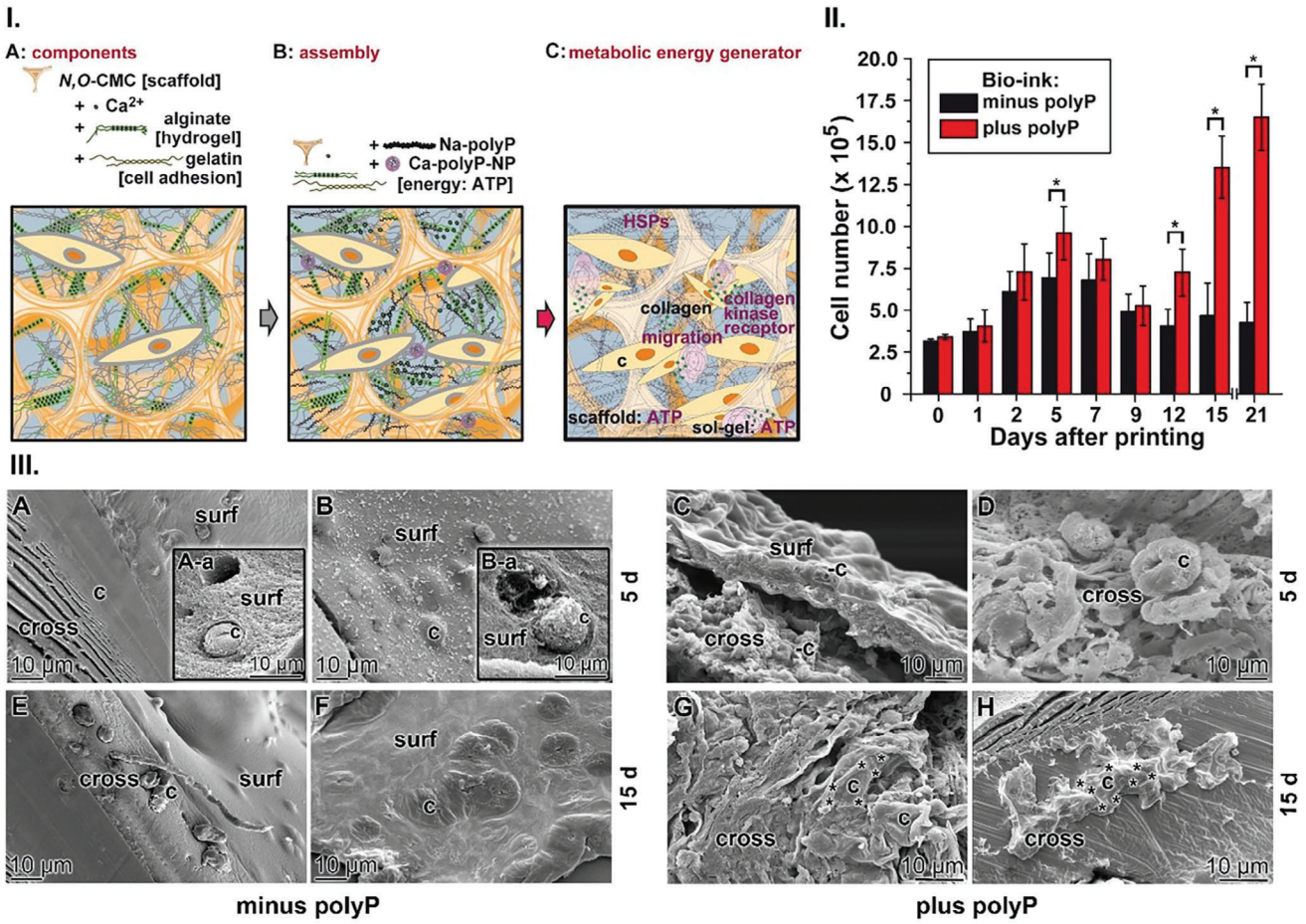

Figure 12. The 3D printing of a hydrogel supplemented with polyP. **I**) Hierarchical composition and organization of the innovative hydrogel. **A**) The components: *N,O*-CMC used for stabilizing the scaffold, alginate for the formation of the Ca^{2+} cross-linked hydrogel, gelatin for the provision of the adhesion substrate (R-G-D sequence) to the cells, and Ca-polyP-NP as a supply of metabolic energy. **II**) Kinetics of MSC growth in the bioink in the absence (minus) or presence (plus) of polyP. After the indicated incubation periods, the cell densities were determined colorimetrically. **III**) Growth of the MSC in the hydrogels in the (**left panel** [A, B, E, F]) absence (minus) or (**right panel** [C, D, G, H]) presence (plus) of polyP. The major differences in the growth pattern between the two hydrogels are the higher cell (c) density of the polyP hydrogel, the distribution pattern of the cells on the hydrogel surfaces (surf), and the arrangement of the cells. In the stratified cross fractures, it can be detected that in the presence of polyP the lobate cells (some boundaries are marked [*]) aggregate to clusters; SEM images. Reproduced under the terms of the CC BY 4.0 DEED license.[129] Copyright 2021, The Author(s). Published by IOP Publishing Ltd.

intracellular (not extracellular) ATP, either through the application of ATP-containing vesicles[127] or through a bioenergetically active, poly(glycerol succinate)/ethanediol-based material that increases mitochondrial energy production. This material is gradually degraded into fragments that are internalized to increase the mitochondrial membrane potential and accelerate bone regeneration.[128]

4.1.3. 3D Bioprinting of Tissue Units

PolyP has also been successfully used for the formulation of a bioink suitable for 3D bioprinting with cells.[129] The bioink was composed of *N,O*-carboxymethyl chitosan (*N,O*-CMC; as a structural component), alginate (the hydrogel) and gelatin (cell adhesion matrix), and polyP as the metabolic energy-providing component (**Figure** 12-IA–C). The components were hierarchically composed and organized in the bioink. As a basic scaffold *N,O*-CMC was chosen to provide stability (Figure 12-IA). Then, alginate was added, which was thereupon bathed in Ca^{2+} solution to form a hydrogel. In addition, the material was supplemented with gelatin to provide the adhesion substrate to the cells (Figure 12-IA). Finally, Ca-polyP-NP was supplemented to enrich the hydrogel with the source of metabolic energy (Figure 12-IB).[129] ATP, generated, served as fuel for heat shock proteins or kinases such as extracellular protein tyrosine kinases.[130] It was found that, in this hydrogel, the cells (MSC) readily proliferate especially within their cavities.[129] A biphasic growth kinetics was determined with a first peak after 5 days (increase of the cell density from 2.5×10^5 to 9.7×10^5 cells mL^{-1}) and a second one after 15 days (to 17.1×10^5 cells mL^{-1}) (Figure 12-II). It is very remarkable that the proliferation potency continued further to 21 days. This strong proliferation was found only in the hydrogel enriched with polyP; in the hydrogel without

polyP, the growth/proliferation capacity of the cells was significantly lower.

The distribution and density pattern of the MSC within the bioink depends on the polyP polymer (Figure 12-III). In the absence of polyP, the cells remain on the surface of the hydrogel (Figure 12-IIIA,B). There, the cells are bulging the surface of the printed hydrogel. Importantly, the cells remain roundish and isolated. Cross fractures showed that the scattered cells have a rounded morphology; they remain isolated (Figure 12-IIIE,F). In contrast, in the hydrogel matrix with polyP, the cells form clusters at the surface of the prints (Figure 12-IIIC,D), and the lobulated MSC form aggregates, as shown in cross fractures (Figure 12-IIIG,H). This series of experiments showed that the growth/aggregate pattern of MSC changes from isolated cells to globular to lobular spheres when embedded in the polyP bioink, which is indicative of cell differentiation.[131]

The master genes that control the switch in MSC toward cartilage and bone differentiation are the two transcription factors RUNX2 (runt-related transcription factor 2) and Sox9 (SRY-box transcription factor 9).[132,133] Gene expression studies revealed a distinct increase in the expression ratio between RUNX2 and Sox9 in cells growing in polyP-supplemented hydrogel, reflecting an increased propensity for differentiation. In addition, the morphogenetic activity of the MSC in the polyP-enriched matrix is underpinned by the finding that the MSC in this polyP hydrogel show a strong induction of the expression of the ALP gene.[129]

4.1.4. In Vivo Studies

The proof-of-concept implying that the precursor for crystalline bone (HA) formation originates from an amorphous precursor,[60] an initial amorphous calcium phosphate phase (ACP), was tested in in vivo studies. ACP transforms into the crystalline apatite.[134] Earlier it was established that ACP is stabilized by polyP (≈15–20% by weight).[135] ACP was found to be superior to crystalline ß-TCP in the calvarial defect regeneration model in rats.[135] Building on the strong evidence for a significant ACP content in crystalline mature bone by the Posner group,[136] a modeling approach revealed that polyP chains can protrude between the Posner molecules and thereby stabilize the ACP phase. Therefore, it was advisable to determine the regenerative effect of amorphous versus crystalline calcium phosphate precursor in vivo.[137] Again, the calvarial defect model (New Zealand White rabbits) was applied. The Ca-phosphate minerals (see Section 4.1.1.), crystalline β-TCP, crystalline Ca-phosphate (CCP [with 5% (w/w) polyP]) and amorphous Ca-phosphate (ACP) [stabilized with 15% (w/w) polyP] were encapsulated into microspheres with poly(D,L-lactide-*co*-glycolide) (PLGA) and applied into the defect. After a healing period of 6 weeks, the animals were sacrificed and assessed. A comparison of the histology of the regenerating areas revealed that in the β-TCP series, all microspheres had dropped out of the tissue slices and no signs of regeneration could be observed. The subsequent SEM analyses confirmed that the microspheres project out of the defect in the calvarial bone. In contrast, strong signs of regeneration were already evident in the CCP series (supplemented with low amounts of polyP); even new blood vessels could be identified. These results from SEM analyses supported the regeneration effect and were underpinned by histological analyses, which showed infiltration of cells into the microspheres and also the presence of regenerative tissue in their surroundings. In conclusion, the ACP-supplemented microspheres showed the strongest regeneration potency.[137]

Bone regeneration, like regeneration of skin tissue (see Section 4.3.), is a highly energy-dependent process. This is reflected in a sharp increase in the ATP content in the callus during fracture healing, indicating intensive energy metabolism in the newly forming bone tissue.[138] Exploiting the energy-supplying function of polyP as well as its ability to convert into a regeneratively active coacervate upon contact with body fluids, a number of further animal studies have been conducted that confirmed the benefits of polyP and its amorphous polyP nanoparticles in bone healing. It was found that polyP encapsulated in PLGA microspheres (polyP content, 7.26 ± 0.92 wt.%) significantly enhanced bone regeneration in critical-size calvarial defects (rat) compared to β-TCP used as a reference material.[139] Bone mineralization was accompanied by strongly upregulated expression of collagen type I and the marker protein ALP in the polyP implant area. Nanoindentation revealed a hardness/Young's modulus of the newly formed tissue around the polyP implant close to that of calvarial bone within a 56-day healing period.[139] In another study, polyP integrated in a *N,O*-CMC matrix was studied, again using the rat calvarial defect model.[140] Both polymers were linked together via Ca^{2+}. The microspheres composed of polyP (20 mg mL^{-1}), *N,O*-CMC (60 mg mL^{-1}), and alginate (60 mg mL^{-1}) were found to be superior in their regeneration-inducing activity to microspheres containing β-TCP or β-TCP together with silica. With a hardness/Young's modulus of 1175 kPa, the mechanical properties of the regenerating areas exceeded those around the β-TCP and β-TCP/silica reference implants by a factor of 2–3 and almost reached those of the pre-existing bone (1840 kPa).[140] Nanoparticles prepared from the strontium salt of polyP showed even higher mineralization-inducing activity than the Ca-polyP particles.[141] The stronger osteogenetic effect of the amorphous Sr-polyP particles was explained by the finding that these particles only slightly upregulated the expression of sclerostin compared to ALP in contrast to the Ca-polyP particles.[141] This antagonist of Wnt/β-catenin signaling suppresses the differentiation and mineralization of bone cells.[142] In the rat calvaria, the bone defect was almost completely restored after an implantation period of 12 weeks.[141]

Recently, in a first-in-human clinical trial the safety, feasibility, and osteoinductivity of Ca-polyP-NP have been evaluated.[143] In this single-blinded, parallel, prospective pilot study, Ca-polyP-NP has been applied either alone (1 g in 1.5 mL saline) or in combination with biphasic calcium phosphate (BCP; composed of 60% HA and 40% β-TCP) (1 g Ca-polyP-NP and 2 g BCP in 3–5 mL saline) for alveolar cleft repair. Based on the results, it was concluded that Ca-polyP-NP is a safe material. None of the patients developed allergic reactions or showed other local or systemic adverse effects.

It should be noted that polyP has also been recognized as safe for use as a food additive by the US Food and Drug Administration (FDA) and the European Union (EU); it is listed under the E-numbers E 452(i) (Na-polyP), E 452(iii) (Na/Ca-polyP) and E 452(iv) (Ca-polyP).[144] Furthermore, this polymer shows no carcinogenicity, no reproductive or developmental toxicity, and no

genotoxicity.[138] The LD_{50} for polyP in rats and mice is very low at >2000 mg kg^{-1}.[145]

4.2. Enhanced Growth Of Human Chondrocytes Onto Cartilage

One reason for the limited regeneration capacity of cartilage is the low proportion of chondrocytes/MSC in this tissue. Only a proportion of 1–5% chondrocytes has been estimated.[146] In addition, the blood supply is very low. It was therefore conceivable to test if polyP, which is normally supplied by the platelets, could substitute for this polymer in ameliorating cartilage damage.

Already in 2012[147] it was reported that polyP (the exact formulation was not given) with the physiological chain length of 45 P_i units stimulates the synthesis of collagens and glycosaminoglycans in chondrocytes; in comparison, phosphate or pyrophosphate had no beneficial effect. These positive effects could be confirmed with cartilage explants cultivated in vitro. Even in vivo (guinea pigs) it could be demonstrated that intra-articular injections of polyP significantly improved the osteoarthritis-outcome score.

In a subsequent study, polyP was investigated in vitro in the formulation of amorphous magnesium (Mg) salts, Mg-polyP-NP.[148] This decision was based on the findings that magnesium is an essential cofactor for a number of enzymes and other functional proteins in the human body.[149] A deficiency in this element leads to neuromuscular, cardiac, or nervous disorders. Mg-polyP-NP were encapsulated into *N,O*-CMC, and alginate and fabricated into tissue units with Young's modulus/stiffness comparable to those of in vivo samples.[148] Gene expression studies revealed that chondrocytes seeded on these specimens strongly upregulate the expression of both collagen type II and aggrecan, the major proteoglycan present in the articular cartilage.

A Ca-polyP-NP-containing porous cryogel with cartilage-like viscoelastic properties, prepared from polyP, karaya gum, and polyvinyl alcohol by Ca^{2+}-mediated ionic gelation and intermolecular cross-linking, has been evaluated in an animal study.[150] Implants in rat muscle with microspheres containing this material were found to be replaced by granulation tissue after just 2–4 weeks of implantation.[142] In vitro, it was shown that the Ca-polyP-NP, generated in situ within this cryogel are converted into the biologically active coacervate upon contact with body fluid, allowing human MSC to invade and proliferate.

4.3. Wound Healing, an Energy-Consuming Repair Process

The skin, as the body's largest organ, protects the individual from bacteria, regulates body temperature, and also controls sensations such as touch and pain. Since the skin is the effective barrier between the environment and the internal milieu, it determines and maintains the body's homeostasis and thus the survival of the organism.[151] It is therefore essential that the repair and regeneration capacity of the skin is maintained. Wound healing is a physiological reaction of the body to tissue injury and is complex. It involves an interplay between different cell types, cytokines, mediators, and the vascular system. It is important that both initial vasoconstriction of blood vessels and platelet aggregation progress and lead to cessation of bleeding. In general, wound healing is subdivided into four phases: Hemostasis, inflammation, proliferation (granulation), and remodeling (**Figure 13-I**).[152]

4.3.1. Hemostasis

Hemostasis is a process by which bleeding is stopped and clotting occurs, causing blood to form from a liquid into a gel. The blood vessels constrict and reduce bleeding. Subsequently, the platelets stick together to form a plug and (temporarily) seal the vessel wall. Finally, coagulation (blood clotting) occurs, a process in which a series of enzyme-driven reactions is activated, finally leading to a fibrin mesh produced around the platelet plug.

Ca^{2+} is a central ion controlling hemostasis during vascular injury.[153] The von Willebrand factor interacts with the platelets leading to an increase in the free Ca^{2+} concentration. Ca^{2+} causes the activation of several coagulation factors, such as Factor XIII. This ion is responsible for cross-linking fibrin clots and maintaining the clot architecture. Factor XIII is associated with a protransglutaminase complex, which, together with thrombin and Ca^{2+}, activates the final stage of the clotting cascade.[154]

It has been proposed that polyP modulates blood clotting,[32] a view that has been disputed.[155] The problem is that the degree of binding (chelate formation) of the respective cations to the polyP anion has often not been taken into account. Therefore, the cation exchange reactions of Na^+, Mg^{2+}, and Ca^{2+} have to be considered in detail. Also, chelating Ca^{2+} from the system effectively prevents blood clotting.[156] In addition, the chain length of the polyP used for the particular study is an important issue.[90] These authors stress that the potency of polyP depends on the chain length of the polymer and highlight the distinctive physiological roles of short-chain polyP released from the platelet-dense granules and long-chain polyP produced in bacteria.

4.3.2. Inflammation

During the inflammation phase, immune cells clean the injured site by phagocytosis, thereby removing damaged cells, pathogens, and bacteria, and neutrophils, macrophages, and monocytes migrate into the wound area. It has been shown that polyP (of the physiological chain length 60–100 P_i units) promotes the differentiation of macrophages and fibrocytes and mediates wound healing anabolically.[89] In addition, monocytes are stimulated by polyP to differentiate into fibrocytes and macrophages. Furthermore, polyP supports antibacterial processes during the inflammation phase by directly affecting both some Gram-positive bacteria and some Gram-negative bacteria.[157]

4.3.3. Proliferation (Granulation)

This phase in particular is very metabolic energy consuming.[158] During this period, angiogenesis, the growth of blood vessels from the existing and accumulating cells, must have to develop. Especially, fibroblasts have to migrate to the injured site, even more fibroblasts have to differentiate to myofibroblasts,

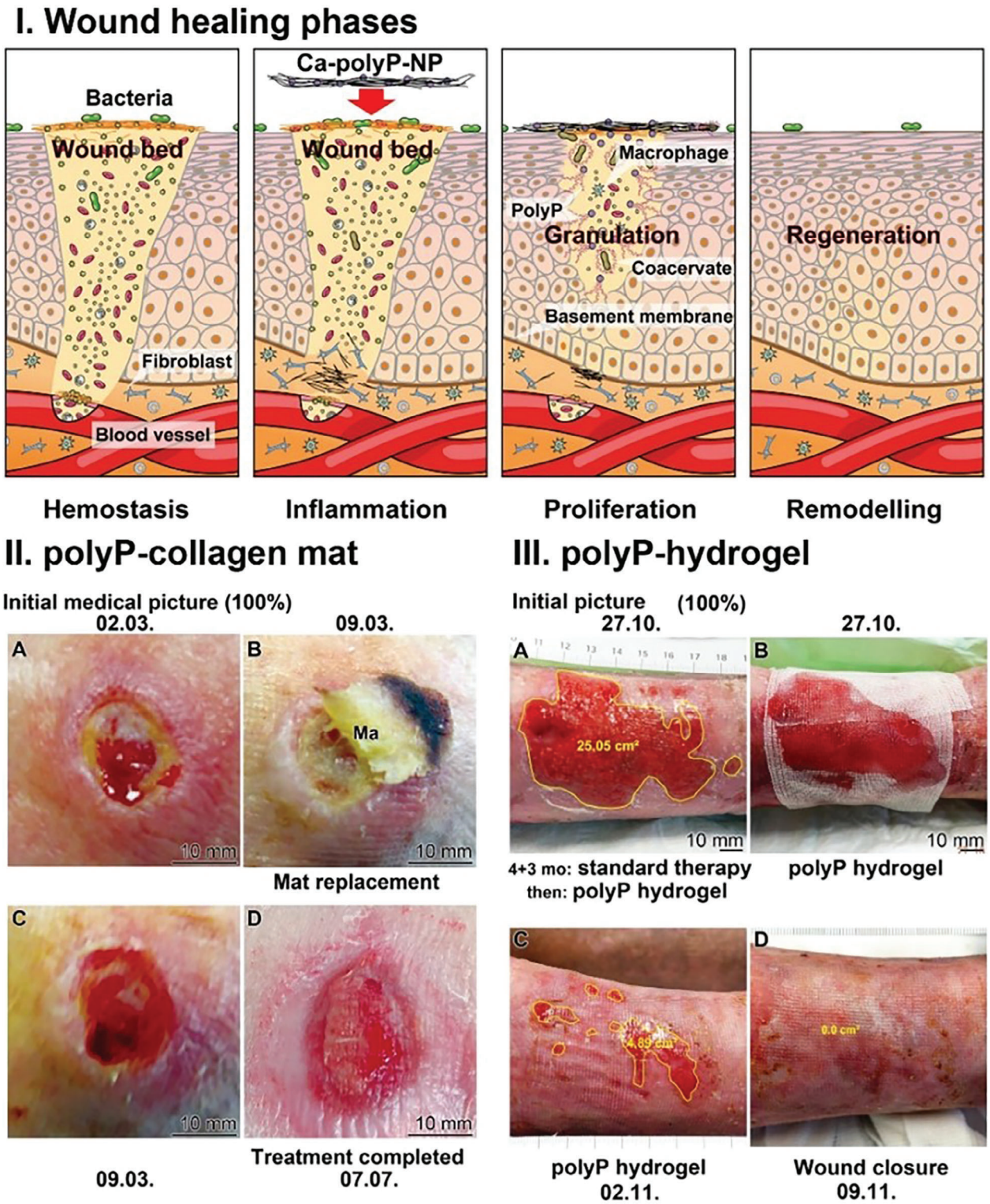

Figure 13. Successful proof-of-concept trials with polyP. I) The four phases of wound healing. II) Application of polyP embedded in a collagen mat (the dates when the respective treatments were performed are also given). A) Initial clinical picture. The treatment started with the collagen-polyP mat. B,C) Then, the bulged mat (Ma) was replaced; a marginal epithelial rim developed around the wound. (D) Termination of the polyP treatment. III) Accelerated treatment of manifested superficial erosions on a prurigo eczema using the polyP (600 µg mL^{-1} Na-polyP and 60 µg mL^{-1} Ca-polyP-NP) loaded hydrogel. A) Initial clinical picture. B) Covering of the wound with the polyP hydrogel and overlaying with a neutral gauze. C,D) During the treatment, the wound area was reduced to 19.5% of the initial size and finally even completely. II) Reproduced under the terms of the CC BY 4.0 DEED license.[28] Copyright 2022, Ivyspring International Publisher. III) Reproduced with permission.[152] Copyright 2022, Elsevier Ltd.

which are smooth muscle-like cells. These myofibroblasts contain the intracellular filament protein vimentin, which assembles in an ATP-dependent way.[159] In addition, also α-smooth muscle actin contracts while consuming ATP.[160] These cells respond to ATP/purinergic $P2Y_1$ receptor signaling circuits in which, e.g., cyclooxygenase-2 and intracellular signaling pathways via p38 mitogen-activated protein kinases and protein kinases C are stimulated.[161] Both kinase systems are essential for completing the inflammatory and proliferative phases of wound healing[162] particularly also during the formation of the granulation tissue matrix that fills wounds at the healing injured site.

4.3.4. Remodeling

The maturation/remodeling phase is completed under physiological conditions three weeks after injury, at a time when the anabolic pathway of collagen synthesis is terminated. Then, a functional organization of the cells in the initially injured wound can take place. Fibrocytes in particular secrete an array of chemokines, cytokines, and growth factors that are required for directional growth and migration of fibroblasts in the physiologically composed wound healing milieu.[163] The ATP-consuming migration of fibroblasts, utilizing the collagen and also the fibrous fibronectin for guided migration, is crucial in wound healing.[164]

4.3.5. In Vivo Studies

Based on the in vitro studies mentioned and in particular the knowledge that polyP supplies cells with metabolic energy, with ATP, it was justified to conduct in vivo animal studies.[165] Tissue defects of 8-mm in diameter were set in the interscapular region of mice. Then, Ca-polyP-NP was applied to the wounds. Already after a healing period of 7 days, the Ca-polyP-NP showed a significant re-epithelialization by 72% compared to controls. Surprisingly, in diabetic mice, a ≈40% re-epithelialization was even measured. In the regenerating wound area, a strong upregulation of collagen gene expression (collagen types I and III) as well as α-smooth muscle actin and plasminogen activator inhibitor-1 was determined using the qRT-PCR method.

These data underscored that polyP in the form of NP has significant beneficial anabolic effects on cells involved in wound regeneration. The conclusion that this effect is due the enzymatic conversion of polyP to ATP[166] was supported by the equally impressive finding that complete wound healing in animals is also achieved after direct application of ATP via an intracellular ATP delivery system.[167]

The regenerative effect of the direct application of ATP in the healing of skin wounds has been demonstrated in (diabetic) rabbits.[127] However, the mechanism of accelerated wound healing through intracellular ATP delivery appears to be different from that of extracellular ATP generated from polyP and involves the proliferation and M2 polarization of macrophages, leading to increased release of proinflammatory cytokines, stem cell proliferation, and collagen formation.[168,169]

Release of extracellular ATP can also be induced by mechanical stress, e.g., by the application of low-intensity pulsed ultrasound,[170] which is used in the treatment of chronic skin wounds or to improve osteogenesis in the healing of bone defects.[171] The underlying mechanism has been attributed to the binding and activation of extracellular ATP to purinergic P2 receptors (ionotropic, ligand-gated P2X or metabotropic, G-protein-coupled P2Y receptors)[172] on the cell surface. Such an autocrine signaling mechanism, induced by the release of endogenous ATP by mechanical stimuli and activation of P2X or P2Y receptors, leading to wound healing, has been reported for a number of cell types and tissues such as bone, cartilage, skin, and connective tissue.[173–176]

Some effects of polyP could also be caused by the direct binding of polyP to P2 purinoreceptors. The subtype of the receptor as well as the effect and mode of action depend on the cell type and have still been relatively little investigated. For example, exposure of primary thymocytes to polyP has been shown to result in an increase in intracellular calcium level ($[Ca^{2+}]_i$), an effect that is primarily mediated by P2X receptors as shown by inhibitor studies and molecular docking experiments, which revealed binding energies similar to ATP.[177] In astrocytes, polyP interacts with $P2Y_1$ receptors, leading to an increase in $[Ca^{2+}]_i$ via the phospholipase C/inositol 1,4,5-trisphosphate signaling pathway.[178] Activation of $P2Y_1$ receptors of neurons by polyP protects cells from glutamate excitotoxicity.[179] The increase in $[Ca^{2+}]_i$ in chondrocytes by extracellular polyP can be blocked by calcium channel inhibitors such as gadolinium and nifedipine.[180] Using human umbilical vein endothelial cells (HUVEC), extracellularly applied Ca-polyP-NP was found to promote angiogenic tube formation, as demonstrated in the in vitro tube formation assay.[100] Evidence for the involvement of extracellular ATP generated from polyP in tube formation was provided by inhibitor experiments with apyrase (depletion of extracellular ATP), levamisole (inhibitor of ALP) and P^1,P^5-di(adenosine-5')pentaphosphate (Ap_5A; ADK inhibitor). A possible involvement of P2Y receptors was indicated by the suppression of the stimulatory effect of Ca-polyP-MP by MRS2211, a $P2Y_{13}$ antagonist.[100]

4.4. Chronic Wounds and Chronic Wound Healing

Undersupply of energy-delivering metabolites can cause retarded wound healing or even the occurrence of chronic wounds that are difficult to treat.[181] The underlying causes range from malnutrition to stress, diabetes, and metabolic syndrome to a predisposition of a patient to develop chronic, nonhealing wounds.[182] Pressure ulcers or decubitus are examples of chronic wounds and have an incidence rate of 3–5% in hospitalized patients.[183] Chronic wounds are defined as those that fail to proceed through the controlled regenerative wound healing phases and do not develop anatomic and functional integrity within a period of 3 months.[183] Healing of chronic wounds can take several years, a period during which patients experience severe pain as well as emotional distress. Medical wound care imposes a heavy financial burden on society and the annual expenses are expected to reach $15–22 billion by 2024.[184]

The treatment of chronic wounds is a complex task and is difficult or, at present, even impossible to pinpoint to a single root. Currently, the healing concept relies on the improvement of the most appropriate physical and chemical environment by

removing waste and necrotic tissues from the wound. This is to prevent reinfection, in parallel with adequate moisturizing of the wound.[185] In addition, some successes could be achieved by promoting restoration of the local disruption of vascularization combined with a balanced supply of lacking oxygen or nutrients. Having this in mind, it appears to be justified to subdivide chronic wounds into a general metabolic and tissue-localized syndrome based on venous insufficiency, diabetes, and neuropathies, among others.

4.5. PolyP and Chronic Wound Healing (Proof-of-Concept Application)

For the successful biomedical treatment of patients with chronic wounds (after standardized therapeutic attempts in clinical practice according to §37 of the Declaration of Helsinki), three formulations have been developed: first, the application of polyP in collagen scaffolds (mats) for chronic deep skin wounds, second, formulation of a functional hydrogel, and third, a wetting solution applied for both procedures to keep the regeneration site at physiological humidity.[186]

4.5.1. Healing with polyP in Collagen Scaffold (Mats)

The healing process of chronic wounds is often arrested already in its first inflammation phase, which leads to increased exudate production. While the increased blood platelet concentration supplies enough growth factors for acute wound healing, these components are reduced in chronic wounds.[187] In principle, increased exudate promotes healing, but in chronic wounds with the stalled inflammation phase, the healing progress is retarded due to adverse bacteria growth and toxin production. Skin grafts or collagenous skin substitutes are often recommended to provide an ECM surrogate and cellular stimuli to aid in the healing process of chronic wounds. In our biomedical approach, compressed collagen mats enriched with polyP were fabricated to provide a nascent wound bed and enhance its stability.[188]

During the wound repair phases, keratinocytes are in active cross-talk interactions with immune cells.[189] These cells heavily proliferate and differentiate in the polyP-enriched collagen mats.[28] In Figure 13-II, the healing progress of deep chronic wounds is illustrated for an 82-year-old patient. In the initial state, in March 2021, the wound was in a sloughy necrotic state with a crater-like depression. The patient suffered from intense pain and showed a severe inflammation zone in the surrounding tissue (Figure 13-IIA). After 4 days treatment, the polyP-supplemented mat (partially) slipped out (Figure 13-IIB) and was replaced by a new polyP-enriched mat. During this short time, the peripheral redness strongly decreased and a marginal epithelial rim already developed around the wound (Figure 13-IIC). Regularly, the wound was treated with the wetting solution in the wound area. After four months, the wound area substantially decreased and was stabilized by granulation tissue (Figure 13-IID). The treatment could be terminated and the wound area covered by adhesive plaster.

Based on these results, it was concluded that polyP is a novel, highly beneficial component in mats for comprehensive healing of chronic wounds. This conclusion was supported by biopsy studies, which showed that an intensive differentiation process of fibroblasts to myofibroblasts takes place in the regenerating wound area.

4.5.2. Healing with a polyP Formulation Based on a Functional Hydrogel

Hydrogels are hydrophilic cross-linked polymers (either through electrostatic forces or covalent bonds) that act as superabsorbent polymers that vigorously swell in water. In turn, they have the potency to encapsulate bacteria that are present in the wound exudate, thus aiding in wound debridement care. Wound hydrogels are particularly suitable for the treatment of more superficial chronic wounds, which not only extend beyond the epidermis but also disintegrate the tissue down to the adventitial dermis with its loosely woven meshwork and the periadnexal-reticular dermis, which is characterized by coarse collagen bundles.

For the proof-of-concept application, a hydroxyethyl cellulose-based hydrogel with a low to average dynamic viscosity of 25 Pa s was applied in a phosphate buffer (pH ≈6.5) to which Na-polyP and Ca-polyP-NP were added.[152] The healing success of a patient is documented in Figure 13-III. The 90-year-old male patient had manifested superficial erosions on the right lower leg together with prurigo eczema for 4 months. Initially, the patient was treated with standardized and modern wound dressings. After another 3 months, only a slight improvement could be observed (Figure 13-IIIA). Then the wound was treated with the polyP hydrogel enriched with 600 µg mL^{-1} Na-polyP and 60 µg mL^{-1} Ca-polyP-NP (one substitution every 3 d); Figure 13-IIIB). Even after a period of one week, a significant reduction in the wound area was recorded (Figure 13-IIIC). Complete regeneration was achieved after a further 7 days (Figure 13-IIID).[152]

In addition to this successful "bench to bedside" translation, the results with this polyP hydrogel proved that bacteria can be entrapped in the hydrogel and subsequently eliminated. With the hydrogel formulation, a further new therapy option for chronic wounds could be documented, which is also based on the generation of metabolic energy after enzymatic cleavage of the polymer.

5. Conclusion: PolyP a Physiological Polymer That Balances Energy in Tissue Repair

Cells are biologically open systems and constantly exchange mass and energy with their environment – an equilibrium is never reached. In this context, with the elucidation of the functional significance of polyP, especially with regard to bioenergetics, a paradigm shift in regenerative medicine has been initiated.

Bioenergetic processes during tissue regeneration, like normal cellular bioenergetics, must also follow the laws of thermodynamics. In biological systems, biochemical reactions only proceed spontaneously when they release energy and/or increase the degree of disorder. The increase in order within a given system is in turn dependent on an increase in disorder outside that system.[190] For biological syntheses, including regeneration, it is imperative to couple the anabolic, energy-consuming reactions with preceding or subsequent energy-releasing and/or entropy-increasing reactions. Such biological processes are always driven by enzymes. Hence, anabolic enzymes can only accomplish the reactions they catalyze together with coenzymes

(such as ATP or coenzyme A), which supply energy via their energy-rich bonds.[191] As shown here, polyP also meets these requirements as it delivers the metabolic energy (ATP) needed for kinases, as well as for heat shock proteins and the extracellular matrix structural organization.

It has been a long way to uncover the biological and biochemical basis of regeneration. Already in the Second Book of Aristotle entitled "History of Animals" (cited from ref. [192]) he wrote in 350 B.C. "...The tails of saurian and of serpents, if they are cut off, will grow again." Revolutionary was the discovery that the cnidarian Hydra has the potency to regenerate missing parts after the individuals had been dissected.[193] With the inclusion of genetics,[194] the causal analytical line of thinking was implemented to understand regeneration. In the 1985s, stem cell biology was flourishing with the discovery that stem cells reside in adult tissues, where they have a robust regenerative capacity in vivo while being rapidly lost in culture.[195] Growth factors have been found to regulate cell functions and stimulate their proliferation and differentiation, often in combination with other growth factors such as cytokines. In turn, the concept of harnessing stem cells for biomedical purposes became more realistic.[196]

There is increasing evidence that bioenergetics, i.e., the adjustment of energy metabolism, is crucial for the progression of stem cell differentiation.[197] Of particular importance is the Sirtuin 1 (SIRT1) cycle, which directly links the cellular metabolic status to gene expression and regulates glucose and lipid metabolism in the liver and other organs, promotes fat mobilization and stimulates remodeling of brown fat.[198] In addition, the nutrient sensor SIRT1 promotes bioenergetic pathways in stem cell activation and regulates the autophagic flux in response AMP-activated protein kinase.[199] Also worth mentioning is that the reduction of ATP supply reduces pluripotency.[200]

With the application for wound healing in patients, polyP has successfully mastered the proof-of-concept phase. It was also a significant advancement to show that polyP accelerates the regeneration of bone and cartilage defects in vitro and in animals. For all these healing applications, there is a common denominator: the lack of metabolic energy during the costly regeneration processes.

Often the ECM has been explained in terms of supramolecular assemblies organized from small, modular subunits forming homopolymers and heteropolymers.[201,202] The energetics of supramolecular fabricating can only partially be explained in terms of free energy minimization.[202] Only after feeding the system with ATP, the dynamics of the migration of polymers can be kept running and self-assembly interactions of macromolecules can continue.[202]

In conclusion, this review outlines the biomedical potential of polyP, especially for regenerative processes aimed at repairing organs that are poor in cells. The formulation of polyP, in the NP form, turned out to be advantageous because the application can be carefully targeted and the high surface-to-volume ratio of the particles allows a shaping of the pharmacokinetic profile. For polyP, the breakthrough came after the successful elaboration of the method to prepare amorphous polyP-NP in a bioinspired way as synthesized in the acidocalcisomes or dense granules.[59] As with any functionally active bio-inorganic material, polyP must be present in an amorphous form in the NP.[59,203] To become biomedically active, the NP fabricated must have a ξ potential that allows adsorption to the cell surfaces in the target organ and also endocytotic uptake, as elaborated with Ca-polyP-NP.[204] Here, polyP has the distinguished property of changing from the NP state to the coacervate phase,[14] whereby the "biologically inert" NP are converted into a functionally active form. In the second step, the polyP coacervate undergoes enzymatic hydrolysis or phosphotransfer mediated by ALP and ADK to generate extracellular metabolic energy that speeds up regeneration processes, as highlighted for bone and wounds.

It is hoped that this review will contribute to a further understanding of the secrets of physiological regeneration induced by nanoparticles formed by polyP.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.N. and S.W. performed the experiments, analyzed the results, and revised the original draft of the manuscript. W.E.G.M., H.C.S., and X.H.W. wrote the manuscript and supervised the whole project. W.E.G.M. and X.H.W. provided the resources. All authors discussed mutually and intensely the results and commented on the manuscript.

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biomaterials, coacervate, energy storage, inorganic polyphosphate, metabolic energy, nanoparticles, regenerative activity

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