# The phosphoanhydride bond: one cornerstone of life

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Phosphorus, the second element of the fifth group of the periodic table, is heavily embroiled in the energy metabolism of living beings. This element, together with oxygen, forms phosphoanhydride bonds, one of the most energy-rich linkages in biomolecules. The most well-known occurrence of these bonds is within the triphosphate chain of ATP. More recently, besides ATP, increasing attention has been paid to a much more energy-rich molecule, consisting of long chains of phosphate units. This inorganic polyphosphate (polyP) acts as an energy storage and donor in prokaryotes and higher eukaryotes (animals and humans), particularly within the extracellular space. It turns out that this unique biopolymer, prepared in a bioinspired way, has great potential for regenerative medicine applications.

## Phosphorus—an essential element of life

The complex biochemistry of life is based upon only a few elements, i.e., carbon (C), nitrogen (N), oxygen (O), hydrogen (H), sulphur (S) and phosphorus (P). The stability of the covalent bonds, integral to biomolecules, can be attributed to the strength of the binding energy between these elements, as well as the high activation energies required for bond cleavage. Although the bond energy values for C-O and P-O are very similar, there is a big difference between the energies during hydrolytic cleavage of the C-O-P bond (ester: alcohol/acid) and the P-O-P linkage (phosphoanhydride: acid/acid). The free energy of hydrolysis of phosphoanhydride bonds, e.g., within ATP, amounts to -30 kJ/mol, and is considerably higher than for ester linkages, e.g., between adenosine and phosphoric acid, with -15 kJ/mol. Importantly, the high energy packed in the phosphoanhydride bonds in living systems can readily be released *via* the action of hydrolytic enzymes, such as alkaline phosphatase (ALP). During this hydrolytic cleavage, much of the released energy is dissipated in the form of heat. However, a portion of the free energy arising from such catabolic reactions can be stored in a metabolically re-usable form (Figure 1).

## The essential role of polyphosphate for initial forms of life

Living systems need to maintain a highly ordered state which intensifies energetic disequilibrium with its environment. This implies that the initial 'living' system (protocell) needed energy to maintain this state of disequilibrium and to gain enthalpy at the expense of entropy (Figure 2). However, both ATP and GTP, the primary metabolites possessing the phosphoanhydride bond as a major energy store, also feature organic structures (adenosine and guanosine, respectively) connected to the phosphate units. These are more complex than might be plausible as initial biomolecules and lack the

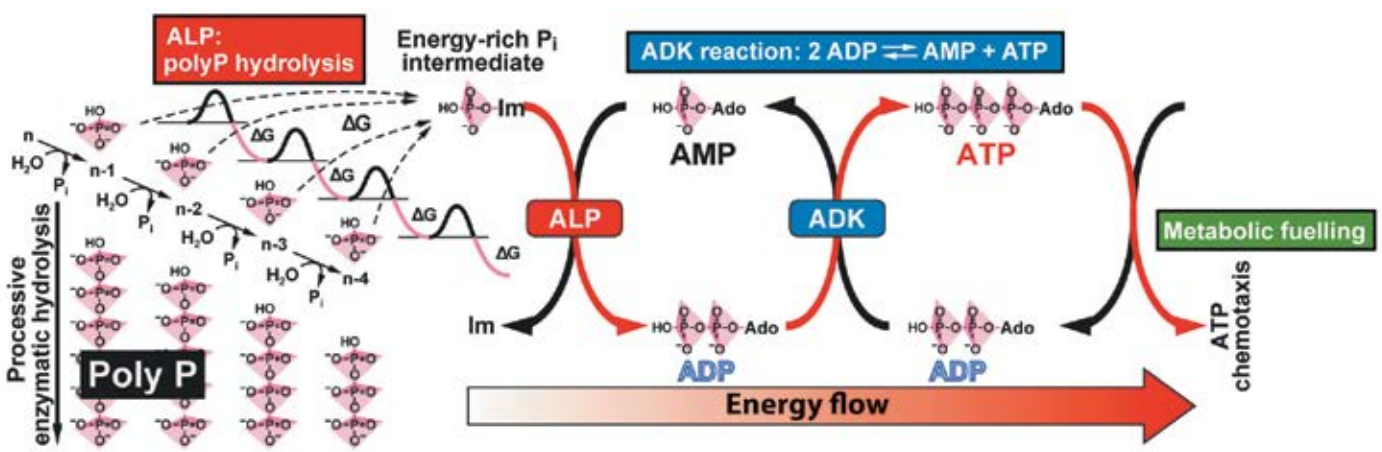


**Figure 1.** The biopolymer, inorganic polyphosphate (polyP), as an energy generator in the extracellular space. The sequential degradation/hydrolysis reactions of polyP, catalysed by the enzyme ALP (alkaline phosphatase), are highly exergonic. The free energy of the released phosphoanhydride bond can either be dissipated in the form of heat or used for the synthesis of ADP from AMP via (most likely) an energy-rich phospho-intermediate ($I_m$). AMP is subsequently converted into ATP, mediated by ADK (adenylate kinase), which then becomes available for energy-consuming processes.



 

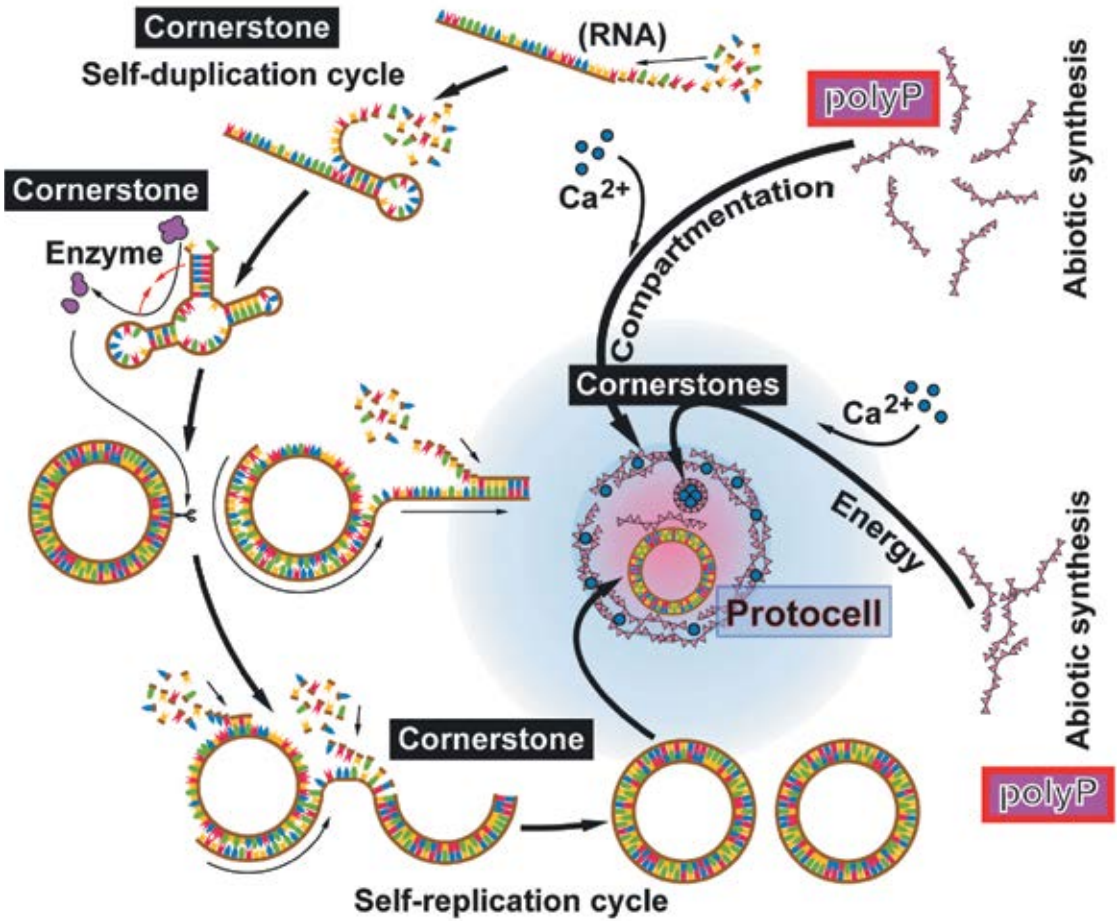


**Figure 2.** The proposed structural and functional prerequisites for the formation of a protocell. Cornerstone 1: most likely an RNA is initially formed that allows the formation of self-duplication/replication cycles. Cornerstone 2: complex secondary structures of RNA allow the emergence of the first enzymes. Cornerstone 3: polyphosphate (polyP), which can be abiotically formed, serves in the presence of $Ca^{2+}$ as the molecule that undergoes coacervation and, in turn, the assembly of a membrane shell around the self-duplicating/replicating system and the enzyme(s). Finally, polyP, in the form of Ca-polyP nanoparticles, can be taken up by the protocell and serves as an energy source for the energetically closed system.

ability to form a necessary compartment around the self-replicating and enzyme-holding initial living unit (a coacervate). In contrast, the bioinorganic polymer polyP is a suitable candidate. This simple molecule is comprised of only orthophosphate residues (Pi) that are linked together by phosphoanhydride bonds. polyP can be formed in the abiotic world by condensation of Pi at elevated temperatures (above ≈600°C) and enzymatically at ambient conditions in biotic systems, demonstrating the power of enzymes. Cells accomplish this synthesis by using enzymes that lower the activation energy to trigger the synthesis of the polymer. In turn, this polymer 'absorbs' metabolic energy from the environment in its phosphoanhydride bonds forming phosphate chains of up to 1000 Pi residues.

It is worth reiterating that polyP is built entirely from up to 1000 phosphate residues linked via high-energy phosphoanhydride bonds (Figure 1). Combined with an ability to undergo phase separation (coacervation), this brings together two important cornerstones of protocell formation, and helps to establish conditions for two key characteristics of a prototypical protocell; replication and enzyme-driven metabolic reactions (Figure 2).

## Occurrence of polyphosphate in bacteria and eukaryotes

Initial studies by Liebermann and Ascoli provided the first evidence for the existence of polyP. Shortly after the discovery of ATP, polymeric phosphagen-phosphate was first identified in bacteria and yeast, and finally in all living systems, including plants and animals. The polymer is stored within volutin granules in bacteria and in acidocalcisomes in eukaryotes. Within the cell, polyP is present as salts with inorganic cations, such as $Ca^{2+}$ or $Mg^{2+}$.

The metabolism of polyP remains best characterized in bacteria, with the identification of polyP-anabolic enzymes, most notably polyphosphate kinase. In the eukaryotic yeast *Saccharomyces cerevisiae* polyP is formed by the vacuolar transporter chaperone 4; however, exo- and endo-polyphosphatases have been identified that degrade polyP. ALP is the major polyP catabolic enzyme in animals, which acts as an exo-polyphosphatase, starting cleavage from the terminal phosphate of the polyP chain under the release of Pi. Evidence shows that polyP in human blood or plasma is also hydrolytically cleaved by

an endo-polyphosphatase(s). Even though the polymer is exposed to these phosphatases in the circulation system the half-life of polyP is approximately two hours.

## Polyphosphate the condensed energy storage for and generator of ATP in Metazoa

In living systems, ATP is the universal energy carrier linking cell catabolism and anabolism. ATP contains two energy-rich phosphoanhydride bonds that can be hydrolytically cleaved either to form ADP and Pi or AMP and pyrophosphate (PPi), which is then further hydrolysed into its two phosphate units. Inorganic polyP, however, is composed of multiple (up to hundreds and more) phosphate units linked by anhydride bonds and contains many times the energy that is stored in one ATP molecule.

So, what is the ongoing function of polyP in animals/humans? Has it lost its role in energy metabolism to ATP, or is it still relevant in these organisms? It has been shown that polyP acts as an energy store and supplier. All cells and tissues studied so far, especially platelets, as well as bone-forming osteoblasts or brain tissue, contain significant amounts of polyP (up to 1 mM or more). Intracellularly, polyP is stored in acidocalcisomes and in conjunction with mitochondria, these acidic organelles serve as production sites of polyP. Platelets can transport polyP, and thus energy, over long distances, e.g., to damaged tissue where they release their polyP cargo to support energy-consuming repair processes.

There is no evidence, in animals, that polyP can directly transfer its energy-rich phosphate to a substrate, as seen with ATP. However, both within the cells and exposed to the extracellular space at the cell surfaces, ALP cleaves polyP in a processive manner – the enzyme remains bound to the substrate until the complete polymer is degraded to orthophosphate, releasing huge amounts of free energy (a multifold of –30 kJ/mol); Figure 1. As noted previously, much of the energy is released not in the form of heat. It has been proposed that, in the presence of polyP in the extracellular space, ATP levels increase via a phosphotransfer from polyP to AMP/ADP. In bacteria, polyP is formed from ATP during polyP kinase reactions, which are reversible; ATP can easily be formed by the reverse reaction (transfer of phosphate from polyP to ADP). However, the polyP kinase enzymes necessary to perform this reaction do not exist in animals. Experiments have revealed that ALP acts in concert with a membrane-bound adenylate kinase (ADK); Figure 1. The enzyme activities of ALP and ADK are linked via an energy-rich intermediate, allowing phosphotransfer to AMP in the formation of ADP, which is subsequently converted by ADK into ATP and AMP. In this way, ATP is generated in the extracellular space, using the high-energy acid anhydride bonds in polyP.

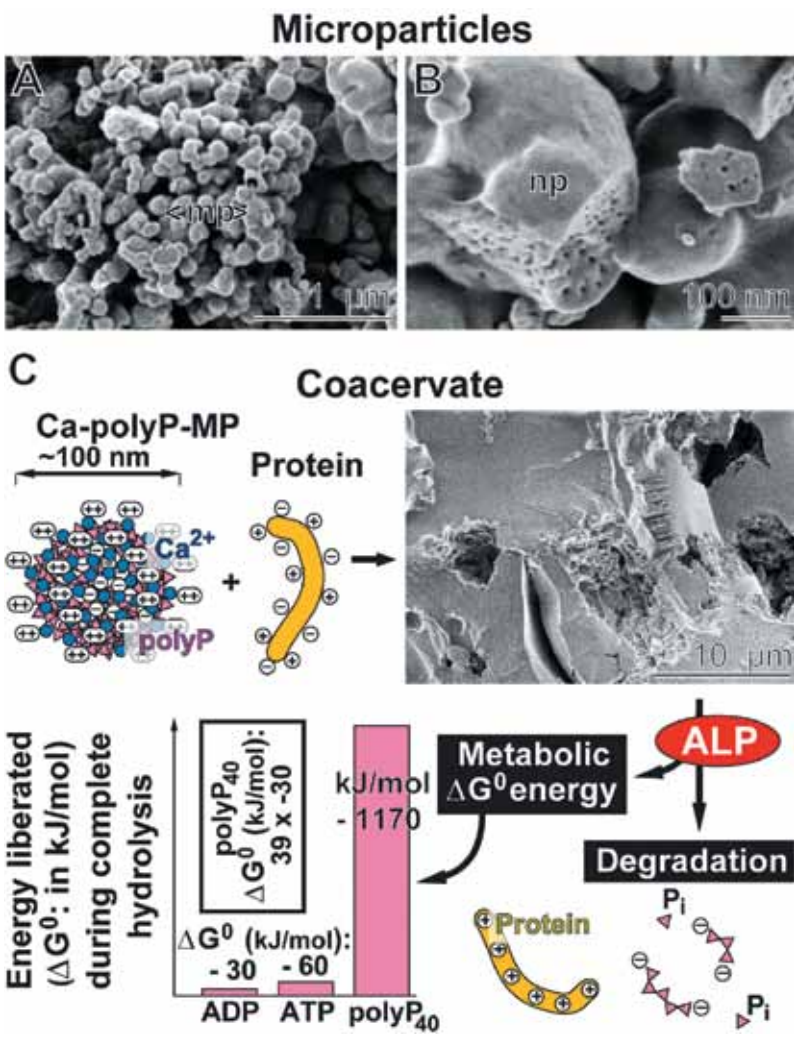


**Figure 3.** Transformation of stable Ca-polyP microparticles (Ca-polyP-MPs) into a biologically active and energy-providing coacervate in the presence of protein containing body fluids. **(A)** Ca-polyP-MPs (diameter about 150 nm); scanning electron microscopy. **(B)** At higher magnification, the porous structure of the particles becomes visible. **(C)** Scheme: the Ca-polyP-MPs are stable over long periods. Administered to tissue, the positively charged calcium counter-ions ($Ca^{2+}$) bound to the negatively charged polyP of these energy storage particles are released due to the formation of a coacervate between the polyP and the positively charged amino acids containing proteins. As a result, the energy-rich polyP is degraded by ALP with the release of large amounts of metabolic energy – a multitude of the energy present in the energy-rich bonds of the universal energy carrier molecule ATP; e.g., polyP40 (39 phosphoanhydride linkages), about –1170 kJ/mol, compared with –60 kJ/mol (ATP: two energy-rich bonds).



 

## Formation of bioinspired amorphous nanoparticles and the beauty of coacervation

Many diseases in animals are associated with a lack of energy, which affects the extracellular space containing only low levels of ATP. It is evident that the formation of the bulky extracellular matrix (ECM) during tissue regeneration is a very energy-consuming process, especially in cell-poor tissues such as cartilage or bone. In tissues with impaired or not yet fully developed vascularization, e.g., during tissue regeneration, this energy requirement cannot be met, due to inadequate nutrient and oxygen supply; however, the exogenous addition of polyP can deliver the necessary metabolic energy. In addition, polyP triggers signals that stimulate cell attachment, ingrowth and stem cell differentiation into mature, functionally active cells, especially during wound healing and the repair/regeneration of bone and cartilage.

In order to exploit the unique capabilities of polyP (i) to be morphogenetically active (promoting cell differentiation via gene induction) and (ii) to provide the energy required for tissue regeneration/repair, we developed a procedure to prepare bioinspired amorphous polyP particles (diameter of ≈150 nm), by using the particles in nature (in the acidocalcisomes) as a model. By applying a superstoichiometric ratio of calcium (Ca) to P, nanoparticles or microparticles could be prepared from the calcium salt of polyP (Ca-polyP-MPs) that mimic the natural particles (Figure 3 A,B). These particles are amorphous (only the amorphous particles are able to develop biological activity) and stable over long periods (due to their high zeta potential, i.e., the potential difference across phase boundaries between solids and liquids).

However, these polyP particles become biologically active after contact with protein present in body fluids. The particles undergo a transformation into a 'coacervate' phase exhibiting morphogenetic/regenerative activity,

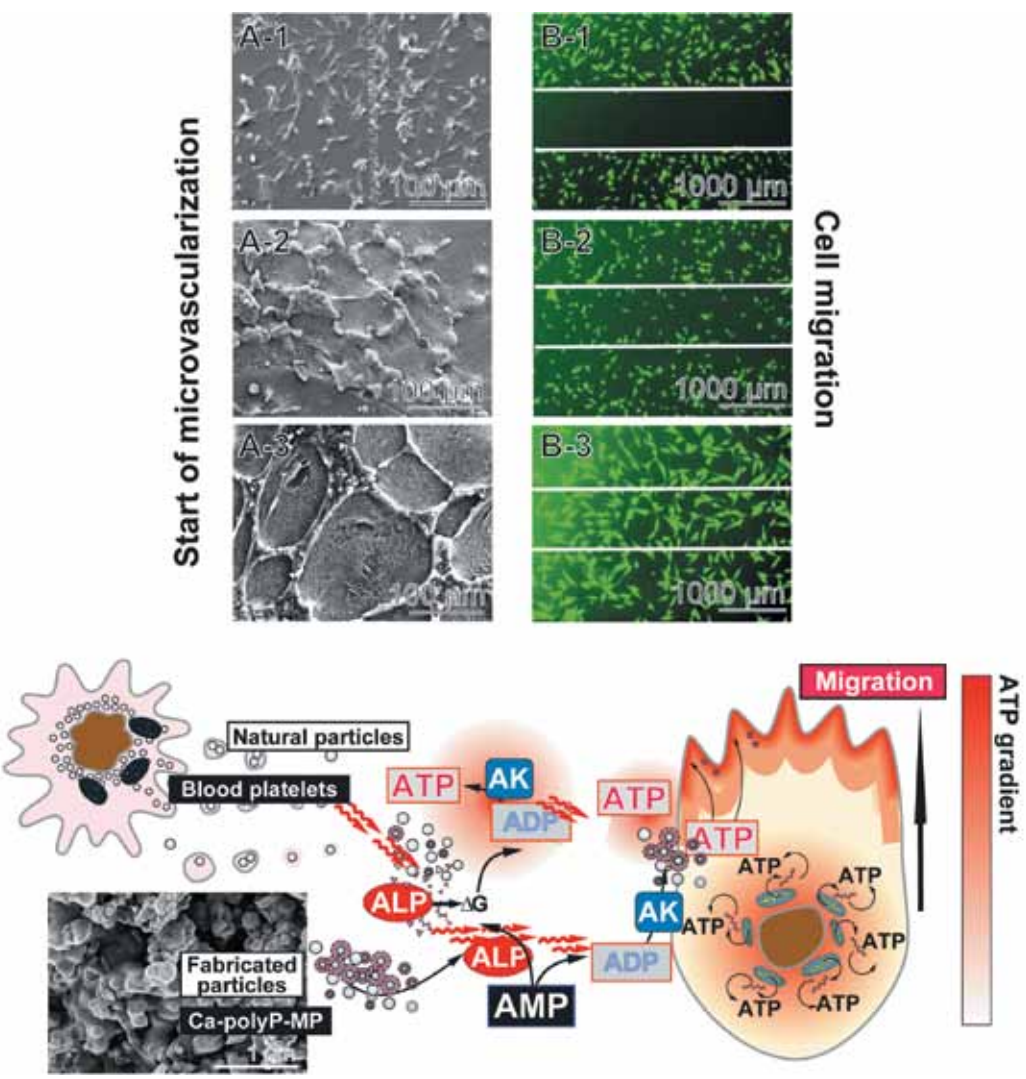


**Figure 4. Top:** The polyP-induced micro-vessel formation visualized electron microscopically (A-1: minus polyP; A-2 and A3: plus polyP; scanning electron micrographs) and endothelial cell migration in the scratch assay (scratch between the 2 white lines; B-1: minus polyP; B-2 and B-3: plus polyP; cells are stained with Calcein AM for fluorescence microscopy). **Bottom:** building an ATP gradient for cell migration/ microvascularization from natural or synthetic polyP microparticles. Through the combined action of ALP and ADK (isoform AK1ß), the energy stored in polyP, either released from platelets (natural mechanism) or administered as biomimetic polyP particles (therapeutic application), is used for the formation of an ATP gradient both extracellularly and intracellularly. The polyP-induced endothelial cell migration proceeds along the formed ATP gradients.

biodegradability and providing energy/ATP; Figure 3C. Only in the coacervate form (Figure 3C) do the particles stimulate energy-consuming processes such as skin repair or bone hydroxyapatite synthesis. The coacervate attracts cells, e.g., mesenchymal stem cells that begin to infiltrate and become embedded in the coacervate, which provides a 'niche' to the cells by delivering growth signals and providing metabolic energy.

## Application in biomaterial science for wound healing

Despite advances in medicine, wound therapy, especially the therapy of chronic, non-healing wounds, remains a medical challenge. In particular, patients with diabetes and immobilized patients often suffer from open wounds due to insufficient perfusion and oxygenation. Here, the energy-delivering polyP opens a new therapeutic approach, not feasible with other materials. By using this polymer, it becomes possible to provide the metabolic energy necessary for wound healing. Animal experiments have demonstrated that Ca-polyP-MPs significantly accelerate wound healing in healthy and diabetic animals. This effect is based on the property of polyP to induce sprouting of new blood vessels into the wound area. Using the *in vitro* tube formation assay of endothelial cells (Figure 4 top, A-1 to A-3), it was shown that the Ca-polyP particles accelerate microvascularization (the initial stage of angiogenesis) by forming a chemotactic ATP gradient, both intracellularly and extracellularly (Figure 4 bottom) that promotes cell migration (Figure 4 top, B-1 to B-3).

The application of polyP for wound healing appears closest to the market but there are many other potential applications of this unique biopolymer. For example, polyP also induces the tissue regeneration of bone and cartilage defects. Further possible applications are in 3D printing, including 3D cell printing (bio-printing) in combination with hydrogel-forming polymers and perhaps, in future, 4D printing utilizing $Ca^{2+}$-inducible *in situ* polyP nanoparticle formation.

## Polyphosphate: an evolutionary driving force

Our group has proposed that all metazoans originated from one common ancestor, closely related to the sponges (Porifera), about 800 million years ago. These earliest metazoan animals consist of, with a few exceptional marker molecules that function as host-defence systems, all the characteristic structural and functional proteins of 'crown' taxa, such as mammals. It was surprising that in these basal animals, the cells are embedded in a bulky ECM allowing them to functionally interact and establish an individual organism with tissue-like units. In spite of this already complex organization of the cells, the ECM of these animals is simple in comparison to mammalians, with a network of lectins and a collagen. In sponges, like in any other metazoan taxa, the cells migrate in the ECM without destroying its organization. Unsurprisingly, these animals are filled with polyP that changes concentration during different morphogenetic events; its level decreases in parallel with an increasing metabolic activity and *vice versa*.

This observation suggests that polyP is used as an energy reservoir during a progressive upregulation of energy metabolism. We suppose that parallel with the emergence of the adhesion molecules (such as integrins) and signal transduction mechanisms (such as the transmembranous receptor tyrosine kinases) the extracellular ATP-generating system evolved, with polyP as the source.

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