Exogenous polyphosphate is not readily utilized for mineralization in vitro

Marianne Ariganello[1], Sidney Omelon[2], Rima Wazen[1], Fabio Variola[2], Antonio Nanci[1]
[1]Université de Montréal, Montréal, Québec and [2]University of Ottawa, Ottawa, Ontario, Canada

Overview

INTRODUCTION: Polyphosphates (PolyP) naturally occur in many cells types with higher concentrations in osteoblastic cells. More detailed characterization of PolyP function(s) may be pertinent to both physiological and pathological mineralization.

METHODS: We evaluated the capacity of Saos-2 and MC-3T3 bone cell lines to induce mineralization (as measured by von Kossa, Alizarin Red, and EDX) in the presence of exogenous PolyP. We also utilized lentiviral vectors (LV) to overexpress the alkaline phosphatase (ALP) transgene in MC-3T3 cells.

RESULTS: We were unable to demonstrate PolyP-induced mineralization with either cell type, nor in MC-3T3 cells over-expressing alkaline phosphatase. We identified that Alizarin Red interacts uniquely with Ca-PolyP to produce a false positive stain.

CONCLUSION: The straightforward addition of exogenous PolyP is insufficient to induce *in vitro* mineralization. Care must be taken when choosing a method of mineral quantification to accurately detect Ca-P mineral, and Alizarin Red should not be used to measure mineralization in the presence of Ca-PolyP.

Polyphosphates and Alkaline Phosphatase

Polyphosphates (PolyPs) are inorganic chains of phosphates, which could provide a source of phosphate in the body.

PolyP can also be a reservoir for calcium due to its ability to chelate large amounts of calcium.

Figure 1: Schematic of an (A) inorganic polyphosphate polymer and (B) how polyphosphates chelate Ca^{2+}

This chelation could contribute to the initial nucleation event in mineralization: when PolyPs are metabolized, concentrations of total Ca^{2+} and PO_4^{3-} above physiological saturation would be generated locally.

Alkaline Phosphatase (ALP) is an enzyme that plays a role in mineralization *in vivo*, and has been shown to cleave PolyP *in vitro*.

If PolyPs initiate or promote mineralization, then these polymers may also act as novel agents to enhance bone formation when delivered clinically.

PURPOSE: Evaluate the capacity of MC-3T3 and Saos-2 cells to sustain mineralization in the presence of exogenous polyphosphate.

Experimental Design

A) In vitro mineralization assay

- MC-3T3s were treated with 3 mM orthophosphate (Pi); 3 mM β-glycerophosphate (βGP) or 294 µM* polyphosphate (PolyP17, chain length: 17 PO_3^-) for 21 days
- Saos-2 cells (having a significantly higher endogenous ALP expression than MC-3T3s) were treated with 5 mM Pi, 5 mM βGP or 294 uM* PolyP17 for 8 days
- MC-3T3 cells overexpressing the ALP transgene (generated using lentiviral vectors) were treated with 3 mM Pi, 3 mM βGP or 294 µM 17-PolyP for 21 days. ALP overexpression was confirmed visually with Fast Blue staining and spectrophotometrically

** Note: 294 µM PolyP17 contains ~5 mM PO_4^- ions*

B) Mineral Analysis

- Cells were stained with Alizarin Red (identifies Ca^{2+}) or von Kossa (PO_4^{3-})
- Cells were processed for electron microscopy

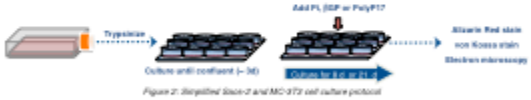


Figure 2: Simplified Saos-2 and MC-3T3 cell culture protocol

Mineralization Analysis: Saos-2

High concentrations of PolyP prevent βGP-mediated mineralization by Saos-2 cells; but not low PolyP concentrations

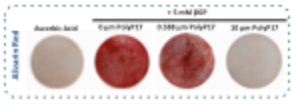


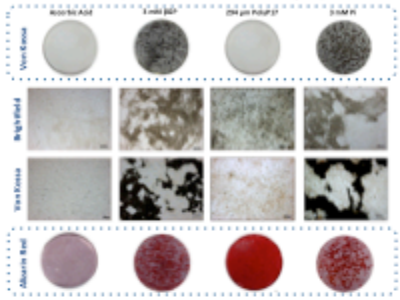
*Figure 3: **Alizarin Red staining of Saos-2 cultures.** Saos-2 cells cultured for 8 days in the presence of βGP were able to form mineral only in presence of low levels of PolyP (less than 6 µM PolyP); in the presence of higher amounts of PolyP, no mineral could be detected. Note: 0.588 µM PolyP17 contains ~ 10 µM Pi ions, and 30 µM PolyP17 contains 176 µM Pi ions.*

Saos-2 cells are unable to utilize PolyP as the sole phosphate source for mineral

*Figure 4: **Alizarin Red and von Kossa staining of Saos-2 cultures.** Saos-2 cells cultured for 8 days in mineralizing conditions (50 µM Ascorbic Acid + a phosphate source) displayed punctate deposits of mineral after βGP or Pi treatment, while no deposits were seen after PolyP treatment. The staining patterns for Alizarin Red and von Kossa were in agreement.*

Mineralization Analysis: MC-3T3

MC-3T3 cells are unable to utilize PolyP as the sole phosphate source for mineral; unique Alizarin Red staining represents a false positive



Proposed Interaction of Ca-PolyP:Alizarin Red

Due to the staining discrepancy between von Kossa and Alizarin Red for PolyP treated MC-3T3s, we propose that Alizarin Red binds to calcium that has been chelated by the PolyP polymer. In this way, Alizarin Red is identifying the presence of residual PolyP polymer, rather than the presence of an apatite mineral. Similarly, von Kossa staining is negative due to the absence of any free PO_4^- ions (all PO_4^- ions are incorporated into the polymer). It is not clear why Saos-2 cells do not stain similarly when treated with PolyP.

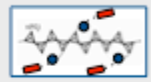


*Figure 5: **Von Kossa and Alizarin Red staining and light microscope analysis of MC-3T3 cultures. A.** MC-3T3s produced mineral after either βGP or Pi treatment but not after PolyP treatment. **B.** At higher magnifications, patches of mineral could be observed in brightfield in either βGP or Pi treated cultures. Cells treated with PolyP showed small clumps under brightfield, but these did not stain with von Kossa, implying that these clumps were not mineral deposits. **C.** When these cultures were stained with Alizarin Red, the typical dark red punctate pattern was observed for both βGP or Pi treated cultures; however uniform bright red staining was seen in PolyP treated cultures, a staining pattern that is not indicative of normal in vitro mineralization. This lack of mineralization with PolyP is consistent with the results of Hasc et al.[7]*

ALP Overexpression in MC-3T3

Overexpression of an ALP transgene by lentiviral vectors

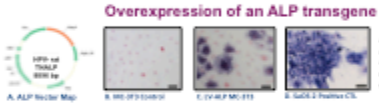


*Figure 7: **LV-ALP Expression. (A)** Tissue non-specific ALP lentiviral vector. **(B)** Non-infected controls minimally stain for Fast Blue, while **(C)** after LV-ALP infection many cells express high levels of ALP. After 5 passages, LV-ALP cells have 40x higher ALP activity over controls.*

High expression of ALP is insufficient to induce PolyP-mediated mineralization

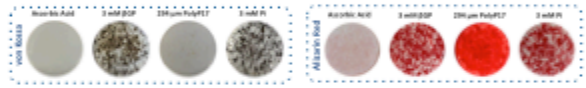
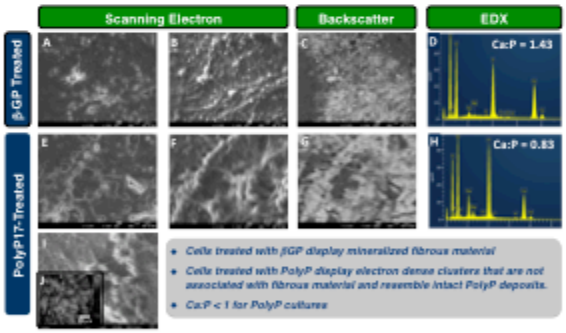


*Figure 8: **Alizarin Red Staining of LV-ALP MC-3T3 Cultures.** Although LV-ALP cells mineralized earlier in the presence of βGP, no mineralization was detected in cultures treated with PolyP, as shown by the negative von Kossa staining. Alizarin Red staining was not altered when LV-ALP MC-3T3 cells were used, demonstrating that even in the presence of high levels of ALP, residual Ca-PolyP remains on the surface of the cultures.*

Results are consistent with the findings of Saos-2 cells, which endogenously express high levels of ALP

Electron Microscopy Analysis

Electron microscopy corroborates Alizarin Red staining and demonstrates the presence of residual PolyP after long-term culture of MC-3T3 cells



- *Cells treated with βGP display mineralized fibrous material*
- *Cells treated with PolyP display electron dense clusters that are not associated with fibrous material and resemble intact PolyP deposits.*
- *Ca:P < 1 for PolyP cultures*

*Figure 9: **Scanning Electron Microscopy.** Secondary electron imaging (A,B,E,F,I) and backscatter electron image (C,G) of MC-3T3 cell cultures after 21 days. EDX analysis (D,H) reveals a Ca:P ratio similar to a substituted apatite mineral for βGP treated cultures, but a very low Ca:P ratio (<1) for PolyP treated cultures, which is indicative of a calcium-polyphosphate complex. (J) Polyphosphate standard.*

Discussion & Conclusions

- Under standard cell culture conditions, neither Saos-2 nor MC-3T3 cells were able to utilize exogenous PolyP as the sole source of phosphate to produce mineral.
  - Is this due to inhibitory mechanisms or incomplete metabolism of PolyP in the *in vitro* setting?
- The ineffectiveness of high levels of ALP (either endogenous or overexpressed) in the metabolism of PolyP leading to mineral deposition indicates that (A) ALP is unlikely the primary enzyme implicated in PolyP metabolism or (B) optimal PolyP processing by ALP requires more conducive conditions.
- PolyP studies reveal conflicting observations: PolyP is reported as stimulating bone mineralization[2-4] as well as inhibiting (pathological) mineralization[1,5,6]. Our results diverge from the former observations and align more with the latter.
- Such discrepancies surrounding the role of PolyP in mineralization warrant more systematic and comprehensive analyses of both *intra-* and *extra*-cellular PolyP; particularly given the complex interactions between PolyP and divalent cations (Ca^{2+}).
- Better understanding of the initial mechanism(s) of mineral nucleation, including the potential role of PolyPs, may lead to novel therapeutic approaches to treat bone afflictions.

[1]Omelon S. Bone 2013. [2]Müller-Ackie Biomaterialia 2011. [3]Wang J Tissue Eng Regen Med 2012. [4]Yaochuu J. Dent Res 2007. [5]Fleisch H. Nature 1966. [6]Schröder O. Experientia 1966.