Collagen Biomineralization In Vivo by Sustained Release of Inorganic Phosphate Ions

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Synthetic alternatives to replace bone autograft are actively sought to overcome limitations such as extended operative time, the risk of infection, and, most importantly, limited quantities of autologous bone. Bone itself is a biocomposite of aligned (and so high tensile modulus) type I collagen fibers reinforced with ~60 wt % nanocrystals of carbonate substituted hydroxyapatite.[1] Collagen is widely used in wound repair since it promotes healing and angiogenesis.[2,3] Collagen reconstituted from solution lacks the fibrillar alignment required for mechanical reinforcement and is often prepared as very weak, low density gels (~3 wt %). While this technique has recently been improved,[4] it remains mechanically inadequate for hard tissue repair. Various attempts at replicating the biocomposite structure have been reported with techniques ranging from simple mixing[5] and compression[6] to co-precipitation[7] and today there are a number of collagen-apatite bone graft products (e.g., Healos,[8] Collagraft, and NeuGraft[9]). While they combine collagen's excellent bioactivity with the osteoconductive and stiffening properties of calcium phosphates, they fall short of replicating the mechanical properties of bone and use xenogenic (usually bovine) derived collagen.

Mineralization of type I collagen not associated with bone, is thought to be prevented by the inorganic pyrophosphate ion (PPi).[10,11] Hydrolysis of PPi by alkaline phosphatase (ALP), produced by differentiating osteoblasts, locally increases inorganic orthophosphate (Pi) concentration, thus promoting collagen mineralization and simultaneously removing inhibitory PPi ions.[12–14] This process has been mechanistically validated both in vitro and in vivo.[15] This approach has been mimicked by the use of implants with immobilized ALP in a variety of materials combinations;[16–22] however, effective use of recombinant proteins presents a regulatory and potential safety barrier to the realization of this technique as a clinical treatment.

The rate of impaired or delayed fracture healing is 5–10% and global annual fracture rates can be reasonably estimated as being in the order of 10^7.[23] In the event of non-healing, the bone defect becomes filled with soft tissue comprised of type I collagen. The lack of both rigidity and osteoconduction of this tissue allows movement which impedes healing and prevents bone bridging. The ability to mineralize non-callous forming tissues in a localized and controlled way is desirable for increasing their stiffness and promoting osteoconduction. Indeed it potentially offers a route to minimally invasive interventions, without recourse to use supraphysiological doses of recombinant proteins with associated high costs,[24] and potential health risks are reported.[25,26]

In this study, we present a new strategy for mineralized tissue formation in vivo based on localized delivery of Pi sufficient to supersaturate tissue surrounding an implant and induce mineralization of collagen. A polymeric-delivery vehicle not known to elicit a strong tissue reaction within the time frame studied,[27] acted as a diffusion controlling matrix implanted in an intramuscular site. Mildly acidic and alkaline monosodium and disodium phosphates, and a pH neutral combination of these two salts were investigated. After 15 days implantation mineral formation around the implants could be detected by X-ray in all animals irrespective of the phosphate salt used. Histology and electron microscopy indicated two populations of apatite; interfibrillar microcrystals and mineral associated with collagen in what was essentially mineralized tissue. Curiously, within the implant matrices, elemental analysis also revealed the formation of magnesium phosphates, this is thought to be the first in vivo observation of magnesium salt deposition outside the urinary system.

To test the hypothesis that raised Pi concentration could overcome the inhibitory effects of PPi, a series of precipitations were performed in vitro whereby the concentration of PPi was varied and the concentration of Pi required for visible precipitate formation in the presence of physiological concentrations of calcium was measured. We found that if the Pi concentration increased by a factor of 2, then an order of magnitude increase in PPi was required to prevent precipitation (Fig. S1 of the Supporting Information). Given the apparent sensitivity of the system to [Pi], the hypothesis was then tested in vivo. Model depot-delivery implants were constructed by sandwiching sodium phosphate powders in polycaprolactone (PCL) and then cutting to expose the sodium phosphate impregnated core on one side

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(Fig. 1a). Scanning electron microscopy (SEM) revealed that the loaded core area was approximately 4 mm^2 and consisted of microcrystal fragments <38 μm closely integrated within the polymer matrix. A second implant type designed to give a burst release was made by compressing an equivalent mass of pH neutral sodium phosphate mixture into a pellet.

To determine release profiles, all implant types were aged in both distilled water and whole serum refreshed daily for 15 days. For PCL implants (~500 mg), the pH of distilled water release media were observed to move from initial values of 8, 7.2, and 4.7 toward the slightly acidic pH of distilled water (Fig. 1b). Cumulative phosphate ion release measurements (Fig. 1c) confirmed that this was due to decreasing amounts of phosphate being released typical of a diffusion controlled release profile as determined by linear fits of data plotted against $t^{1/2}$ (Fig. S2 of the Supporting Information).[28] It appeared a plateau (8 mg) was reached after 7–15 days that did not correlate with the total quantity of loaded Pi (80 mg), this was due to most of the sodium phosphate being located within closed pores. When measurements were repeated in whole serum, its potent buffering effect was very clear with all conditions remaining at pH 8. Furthermore, the quantity and initial release rate of phosphate was decreased by approximately 50% and the variation between samples was notably reduced. The effect of release medium was much greater than the differences in solubility of the salts, the monosodium salt having seven times greater solubility than the disodium salt. In both liquids the compressed sodium phosphate pellet had completely dissolved within 24 h.

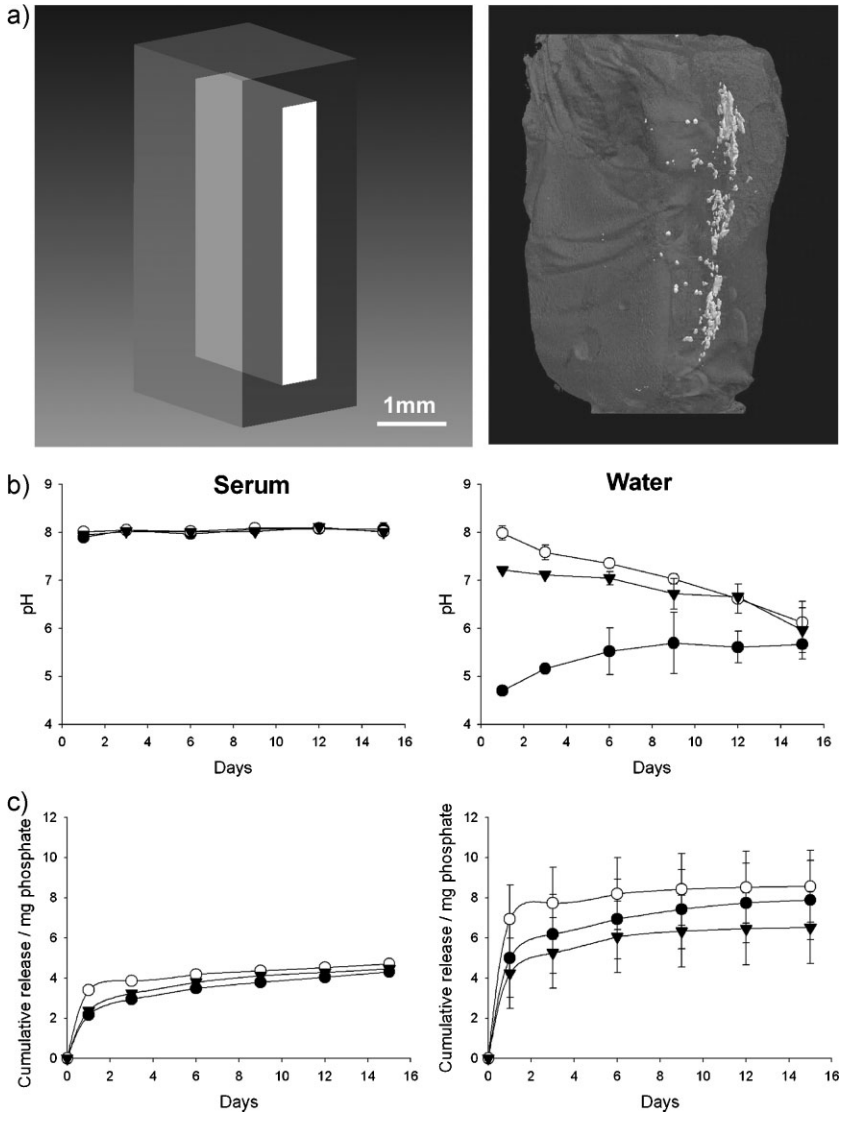

Figure 1. Schematic diagram of implants showing location of polymer-phosphate core (white) in relation to PCL shell (gray) and μCT reconstruction of explanted implant in soft tissue capsule (gray), and location of mineral (light spots) (a). Graphs representing pH change (b) and cumulative phosphate release (c) in serum and in distilled water for monosodium phosphate (•), disodium phosphate (○), and a pH neutral mix of the two (▼).

After 15 days intramuscular implantation, X-ray photographs revealed ectopic mineral formation in soft tissue adjacent to implants containing sodium phosphates irrespective of the degree of protonation of the sodium salt (Fig. S3 of Supporting Information). In contrast, negative controls made from unloaded PCL did not induce any mineralization of soft tissue in any of the animals tested. Microcomputed X-ray tomography (μCT) revealed that the mineral formation spatially correlated to the tissue adjacent to the exposed sodium phosphate loaded porous PCL core (Fig. 1a).

Back-scattered SEM (BSEM) analysis of the resin embedded soft tissue confirmed mineral formation for all experimental groups (Fig. 2). Although precise quantification was not possible, energy dispersive X-ray analysis (EDX) indicated this mineral had the same calcium phosphate ratio in all samples and was 1.5 ± 0.1, typical of a calcium-deficient apatite.[29] Histological sections showed that tissue around the implants was loose connective tissue and von Kossa staining confirmed mineral formation in all experimental groups (Fig. 2). Both the total amount of mineral formed varied among animals. Although the degree of protonation of the Pi ion did not affect the presence of mineral, it did affect the size and distribution of mineral. Most strikingly, the release of acidic $H_2PO_4^-$ induced the formation of large (50–100 μm) mineral deposits that in some cases were phagocytosed by giant multinucleated cells. However, basic HPO_4^{2-} ion release formed banded deposits of fine (1 μm) mineral arranged parallel to the implant surface. The pH neutral mixture induced mineral similar in morphology to the acidic ion but the mineral size was reduced by up to one order of magnitude (Fig. 2).

Transmission electron microscopy (TEM) indicated that the deposition of microscale

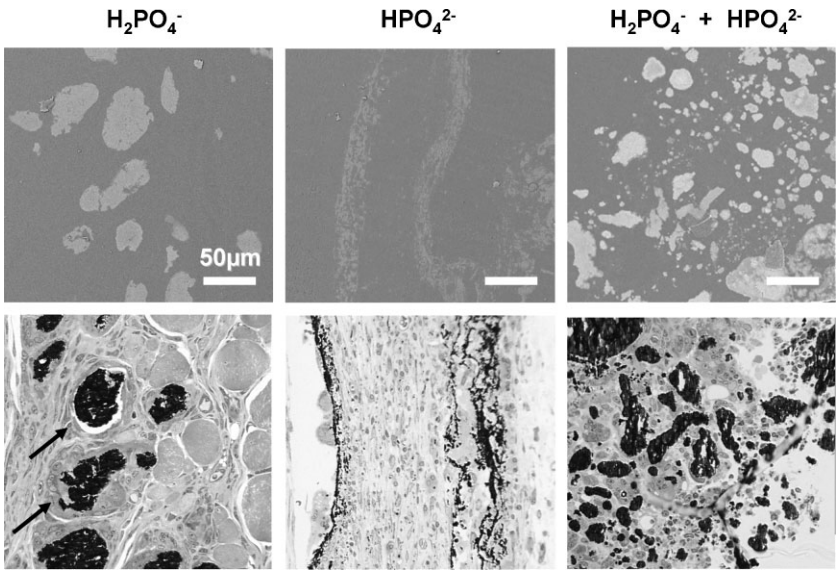

Figure 2. Effect of the degree of protonation of released Pi on the size and morphology of calcium phosphate mineral deposits formed in soft tissue in the proximity of PCL implants and revealed by BSEM (top) and light microscopy (bottom row). Tissue explants were fixed and resin embedded prior to sectioning in an ultamicrotome, mineral was revealed by von Kossa staining. Phagocytosis of large particles was evident (arrows) in tissues exposed to monosodium phosphate.

mineral (as shown in Fig. 2) appeared to be random and not associated with any tissue type or ultrastructural features. However, around the edges of the microscale mineralized deposits, electron dense regions of mineral were observed associated with collagen fibrils (Fig. 3), indicative of mineralized collagen.[15] SAED confirmed this mineral phase to be apatitic.[30] The mineral formed in soft tissue was a mixture of microscale deposits and mineralized collagen in which sodium was not detected using EDX.

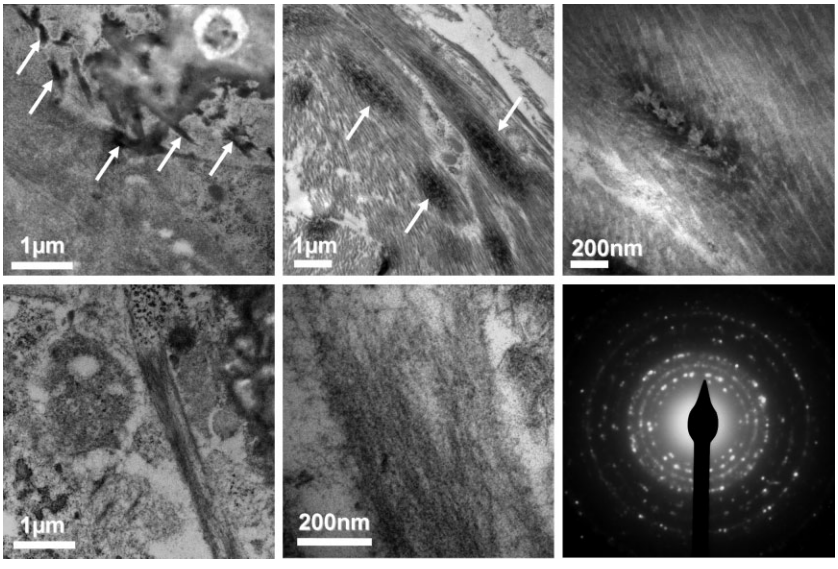

Figure 3. TEM images showing regions of mineralized collagen (arrows) in soft tissue found in the proximity of phosphate loaded implants. SAED confirmed the presence of an apatitic phase.

Within the PCL implants a thin layer of calcium phosphate mineral was formed near the depot/tissue interface and occasionally a few remaining sodium phosphate crystals were observed (Fig. S4a of the Supporting Information). EDX analysis revealed the presence of mainly calcium and phosphorus, and lesser amounts of magnesium and sodium (Fig. S4 of the Supporting Information). Deeper within the implant, >2 mm distal to the depot/tissue interface, crystals of unreacted sodium phosphate and also magnesium phosphate were found. The Mg/P ratio was ~1, indicating a newberyite type deposit (Fig. S4b of the Supporting Information). This may have occurred as a result of two possible mechanisms. Firstly, apatite may be partitioned outside the implant by virtue of its low solubility[29] making the liquid within the phosphate loaded implant essentially calcium free. The serum concentrations of calcium and magnesium are in the order of 2.3 and 1 mM,[31] leaving magnesium in solution within the implant to be salted out by sodium phosphate.[32] Secondly, recent work concluded that polymers can have an ability to template mineral nucleation.[33] It seems feasible that a similar effect could be operative in our system; however, our control material showed no signs of mineral formation, indicating that high phosphate concentration is also a requirement.

Recent work suggested that pyrophosphates prevent ectopic mineralization in all extracellular matrices and Pi cannot induce ectopic mineralization unless PPi is removed from the extracellular milieu.[15] However, this study shows that when Pi is delivered locally rather than systemically, localized mineralization of soft tissue is possible. In this ectopic implantation site the tissue that formed around the implant was a loose connective tissue containing little collagen. Nonetheless proof of the concept that Pi is able to induce mineralization was attained in this model system, supporting our hypothesis developed from in vitro crystallization studies. One can envisage several refinements to improve the quantity of mineralized tissue formed such as delaying the release of Pi until the fibrous capsule had formed, implantation into a more collagen-rich site, using a degradable matrix, and so forth. It is interesting to note that early work on inorganic bone grafts was driven by the idea that a deficiency in serum calcium and phosphate ions was responsible for poor bone healing and that the supply of these ions could rectify this. Indeed in one of the earlier reports on material mediated bone repair, the sodium phosphates were implanted into fracture sites without success, but also without complication.[34] After over 85 years as the role

of pyrophosphate and orthophosphate ions in controlling biomineralization becomes apparent, we show that this long neglected initial approach was in principle correct, albeit because of different mechanisms. We have shown that only sustained localized release of orthophosphate can mineralize collagen. This occurs not by correcting a deficiency in phosphate as was thought to occur in the early part of last century, but by overriding the inhibitory effect of PPi. This simple and inexpensive method of inducing in vivo mineralization may find a role in a number of orthopedic applications such as the treatment of fracture non-union, delayed callous formation, and acceleration of osteodistraction (lengthening of bones by applying tensile force on a mineralizing callus), whereby soft tissue formed can be stiffened and more importantly, rendered osteoconductive. Indeed the ability to selectively deposit osteoconductive calcium phosphates simply by locally overriding the body's natural ''anti-fouling'' system by supplying Pi can in some indications offer significant advantages over operative placement of grafts.

Experimental

Material Preparation: Polycaprolactone (PCL) (360 mg, $M_w = 80\,000$, Sigma–Aldrich) beads were placed in a mold and melted at 70 °C until a homogenous layer was formed. After drying at 75 °C, monosodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4) (Fisher Scientific), or a mix of the two in a ratio that gave an aqueous solution of pH 7.4, was crushed in a mortar and sieved through a 38 μm sieve. These powders (160 mg) were then distributed over the layer of PCL, on top of which another PCL layer was melted. The set block was then cut in half along the plane perpendicular to the polymer and salt layers resulting in the sodium phosphate layer exposed on one side sandwiched between PCL. Sodium phosphate pellets were made by uniaxially compressing the powder (80 mg) in a 5 mm diameter die at 100 MPa.

In Vitro Phosphate Release: Samples ($n = 3$) of PCL containing either mono- or disodium phosphate or a pH neutral mixture were immersed in distilled water or heat-inactivated adult bovine calf serum (BCS) (20 mL) and incubated at 37 °C for 15 days with daily solution changes. pH was measured daily. At the end of the experiment, ion chromatography (IC) analysis (Dionex, Sunnyvale, CA) was performed to measure Pi release with time.

In Vivo Study: All animal experiments were performed according to the Guidelines of the Canadian Council for Animal Care and approved by the Animal Care Committee, Laval University. Prior to implantation, all implants were sterilized with 70% alcohol. Under anesthesia, PCL blocks ($n = 4$) containing the different sodium phosphates were implanted in thoracic muscles (*Erector spinae* muscle) of CD1 (20–25 g) mice. Implants were positioned such that the sodium phosphate exposed side was inside the muscle. Empty blocks and mixed sodium phosphate pellets without PCL served as controls for the effect of sodium phosphate and the effect of sustained release, respectively. Each animal received two implants according to a randomized scheme. The thoracic muscle and skin were sutured separately. Animals were sacrificed 15 days after implantation, and implants with some surrounding tissue were excised and stored in sodium cacodylate buffer for further processing.

X-ray images of all samples were made upon explantation using a Faxitron MX20 (Wheeling, USA). In addition, μCT images were made of selected implants using a SkyScan 1072 (Aartselaar, Belgium).

Mineralized tissue was then dissected, fixed in 0.1 M sodium cacodylate (pH 7.3), 1% glutaraldehyde, and 4% paraformaldehyde, dehydrated in a series of ethanol, infiltrated with increasing concentrations of LR White acrylic resin (London Resin Company), and transferred to gelatin capsules for polymerization. Von Kossa staining was performed on trimmed sections. Ultrathin sections were made for observation with a TEM (Philips Technai 12, Eindhoven, The Netherlands) at 120 kV. Tissue sections were stained with tannic acid and uranyl acetate for increased contrast. Selected area electron diffraction was performed on unstained sections using a Philips CM200 TEM operating at 200 kV. The remaining embedded samples were sputter coated with Au/Pd and examined using SEM (VP-SEM, Hitachi S-3000 N) under back-scattered electron mode at an accelerating voltage of 15 kV. EDX analysis was performed using an Oxford detector and INCA software. Excised PCL blocks after removal of tissue were dried at 37 °C, prior to EDX and high resolution SEM analysis at 2 kV (FE-SEM, Hitachi S-4700).

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