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Review

Bioinorganics and biomaterials: Bone repair

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ABSTRACT

The field of bioinorganics is well established in the development of a variety of therapies. However, their application to bone regeneration, specifically by way of localized delivery from functional implants, is in its infancy and is the topic of this review. The toxicity of inorganics is species, dose and duration specific. Little is known about how inorganic ions are effective therapeutically since their use is often the result of serendipity, observations from nutritional deficiency or excess and genetic disorders. Many researchers point to early work demonstrating a role for their element of interest as a micronutrient critical to or able to alter bone growth, often during skeletal development, as a basis for localized delivery. While one can appreciate how a deficiency can cause disruption of healing, it is difficult to explain how a locally delivered excess in a preclinical model or patient, which is presumably of normal nutritional status, can evoke more bone or faster healing. The review illustrates that inorganics can positively affect bone healing but various factors make literature comparisons difficult. Bioinorganics have the potential to have just as big an impact on bone regeneration as recombinant proteins without some of the safety concerns and high costs.

1. Introduction

The field of bioinorganics is well established in the development of a variety of therapies, such as bipolar disorder (lithium compounds), treatment for testicular and ovarian cancers (cisplatins), and of course fluoride as an anti-cariogenic. Until 20 years ago, gold was one of the most commonly used disease-modifying anti-rheumatic drugs and is still the subject of active research [1]. Other substances under various stages of acceptance and research include strontium as an anti-osteoportic agent, vanadate as an anti-diabetic, bismuth as an anti-ulcer therapy, and daily most people deliberately apply fluoride and more recently fluorophosphates to their teeth in order to help combat caries. The field of bioinorganic therapies is highly active and is reviewed elsewhere, e.g. by Thompson and Orvig [2] and Bakhtiar and Ochiai [3]; however, their application to bone regeneration specifically by way of localized delivery from functional implants is in its infancy and is the topic of this review.

While the therapeutic use of heavy metals for example, may seem counter-intuitive, the words of Paracelsus are pertinent: "Alle Ding' sind Gift, und nichts ohn' Gift; allein die Dosis macht, daß ein Ding kein Gift ist" ("Everything is poisonous and nothing is non-toxic, only the dose makes something not poisonous") [4]. In therapeutic applications, chelation is often critical in controlling the bioavailability and toxicity of bioinorganics, and is key to the safe use of, for example, medical imaging contrasting agents. The toxicity of inorganics is species, dose and duration specific; for example, the toxic properties of silver are well known yet, since mammalian cells are less susceptible to it than bacterial cells, silver-coated endotracheal breathing tubes are approved for clinical use.

Often little is known about how inorganic ions are effective therapeutically since their use is the result of serendipity, observations from nutritional deficiency or excess and genetic disorders. As such, then, there is an unhelpful association between the field and non-scientific and unregulated remedies, and even quackery, as is poignantly evident in tragically uninformed or misguided sufferers of argyria, victims of unsafe nasal sprays and other silver-containing "remedies" (Fig. 1) [5], whose skin is permanently colored grey by the precipitation of silver salts. Exacerbated by the internet, the promise of cheap cures, combined with weak regulation and advice and the wide availability of inorganic dietary supplements, may cause unintended harm. The overzealous self-medication with zinc supplements as a cure for prostate problems and acne has resulted in some cases of copper deficiency (hypocupremia) [6]; indeed, fatal hypocupremia has been inadvertently caused by use of zinc-based denture adhesive [7]. Additionally, chromium used as a muscle building and weight loss supplement is reported as being at best ineffective [8] and at worst possibly genotoxic [9].

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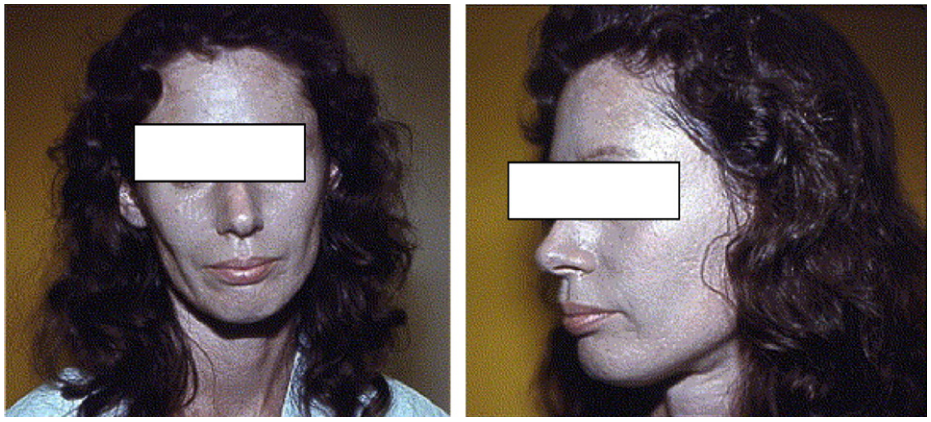

Fig. 1. Argyria sufferer, following childhood exposure to silver containing nasal drops. Improper exposure to silver resulted in permanent grey colored skin due to silver precipitation (reproduced from Ref. [5]). The research was originally published in Ref. [5] © Elsevier.

The effect of inorganic ions on health is largely known through documented effects of deficiency of essential micronutrients, epidemiological studies, nutritional studies of food, animals and tissue health during disease or pharmaceutical treatment. These studies are concerned with systemic levels of ions and have in some cases yielded a wealth of knowledge of the dependency of various systems in which these species play a role, e.g. fluoride [10]; however, in other cases, uncertainty still surrounds the mechanisms by which these substances act. Largely speaking, metal ions can be essential cofactors of enzymes, either as coenzymes or prosthetic groups, and can activate ion channels or secondary signalling by acting directly or as an analog (e.g. strontium for calcium).

In search of new commercial opportunities and purportedly faster healing, there have been a series of "substituted" inorganics evaluated in preclinical studies and even sold as products that release into, or present trace levels of specific ions to, the host tissue. In some cases these bioinorganic ions have a clear role in healing, while in others they are known only to cause mild effects when great lengths are taken to eliminate them from the diet of developing animals. Intentionally or not, the line dividing "structural" biomaterials and controlled released drugs in the form of bioinorganics is blurring and this will impact upon the future of bone regeneration technology and the associated biomaterials disciplines. In order to verse the reader on the general opportunities and risks this change represents, this review attempts to consider the effect ions released from inorganic materials may have on bone tissue. At present, the literature available to the community seems startlingly inadequate to draw firm conclusions. Therefore we have limited our review to the effect of bioinorganics for which there is both a firm basis from which to expect an effect on bone tissue, such as clinical observation, dose response, an identified or heavily implicated biological function, or preclinical studies (including a comparable control) where it is either demonstrated or very probable that a known quantity of an ion of interest is released. Many are essential inorganic micronutrients (Table 1), while some do not have a known essential biological function.

It should be remembered that these quantities listed in Table 1 refer to elements that are ingested and, as such, the amounts entering body fluids are likely to be lower. Additionally, many of these compounds are rapidly excreted from the body even if they pass through the intestine wall. Further, dilution in approximately 40 l of water, immobilization by bone mineral and proteins, and so forth will greatly reduce the ionic concentrations "seen" by a cell within the body, and one can liberally estimate that, from a normal diet, the concentration of elemental zinc (Table 1) might peak in the range of 100s ppm to 100s ppt above physiological norms and at subtoxic levels of 10s of ppm. In contrast, implants can release ions for long periods directly into bone and soft tissues. Here the release rate will affect concentrations of constituent and impurity ions. ASTM standards aim to address this by specifying limits of toxic and heavy metals, as shown in Table 2. By comparison with Table 1, it is apparent that biologically active elements are not all heavy metals, or even metals and so are not restricted.

If we are to learn from prior errors made in patient education and marketing regulation with regard to supplements and medicines containing inorganic compounds, the potential for delivery of non-physiological doses of compounds never previously presented to tissues in this manner should be recognized, especially if there is evidence to suggest a biological response.

While one can appreciate how a deficiency can cause disruption of healing, this cannot explain how a locally delivered excess in a preclinical model or patient who is presumably of normal nutritional status can evoke more bone or faster healing. There are various potential avenues for either promoting bone formation or reducing bone loss. These can broadly be categorized as induction

Table 1
List of essential inorganic elemental micronutrients with values for RDA (recommended dietary allowance) where available, AI (adequate intake), UL (upper limit) and if UL is nonexistent NOAEL (no observed adverse effect level) in the US for adult males [196].

Element	mg day^{-1} RDA/AI	UL/NOAEL
Potassium	4700 AI	31,300 (maximum kidney excretion rate).
Chloride	2300 AI	3600 UL
Sodium	1500 AI	2300 UL
Calcium	1000 AI	4000 NOAEL
Phosphorus	700 RDA	4000 UL
Magnesium	420 RDA	350 UL
Zinc	11 RDA	40 UL
Iron	8 RDA	45 UL
Fluoride	4 AI	10 UL
Manganese	2.3 AI	11 UL
Copper	0.9 RDA	10 UL
Iodine	0.15 RDA	1.1 UL
Selenium	0.055 RDA	0.4 UL
Molybdenum	0.045 RDA	2 UL
Chromium	0.035 AI	NA
Arsenic	None	NA, 1 mg kg^{-1} is toxic
Boron	None	20 mg
Nickel	None	1 mg UL
Silicon	None	NA, 6.5 mg day^{-1} over years as magnesium trisilicate may be associated with urolithiasis
Vanadium	None	1.8 UL
Sulfate	NA	NA, 1500 may cause diarrhoea in infants

Table 2
Maximum impurity limits permitted in resorbable calcium phosphate (ASTM F1088-04) [197].

Element	ppm, max
Pb	30
Hg	5
As	3
Cd	5
Other heavy metals (such as lead)	50

of angiogenesis to accelerate healing, induction of osteogenic differentiation, stimulation of osteoblast proliferation, and control over osteoclast proliferation and resorptive activity, and all can be modulated by inorganic ions.

2. Structural elements of bioceramics

2.1. Calcium phosphate bone graft substitutes

The use of calcium- and/or phosphate-based compounds to replace bone has a long history, and a variety of compounds, spanning the range in extremes of solubility from calcium sulfate (CS) to hydroxyapatite (HA), have been demonstrated as being safe. Following the development of sintered HA in the 1980s, interest grew in resorbable materials that could be replaced by bone and this led to products with Ca:P ratios from 1.7 to 1, and even calcium-free magnesium phosphates [11]. In the vast majority of these studies the graft substitute was considered to be performing a beneficial role only in the solid state. Mainly only bone volume and graft "resorption" (encompassing dissolution, fragmentation, phagocytosis and osteoclast remodeling) were reported. Generally speaking, inorganic bone graft substitutes are considered to have a "structural" function, acting both as a physical barrier to soft tissue invasion and an osteoconductive conduit, and it is thought necessary to ensure that the linear bone growth rate is higher than the "resorption" rate to prevent fibrous tissue invasion. Linear bone growth rates clearly vary, but can be as high as 100 μm day^{-1}. Assuming a bone density of 1.8 g cm^{-3}, a fluid exchange volume of 2 ml/100 g min^{-1} for cortical bone [12] and a spherical implant with a density of 3 g cm^{-3}, this rate of bone growth could be matched by an equivalent dissolution rate achieved by a solubility of 500 mg l^{-1}. While this very approximate calculation is likely to vary by ±1 order of magnitude, it is important to note that such a range encompasses the range of solubility of inorganic bone graft substitutes, from 2 g l^{-1} for CS to 10 mg l^{-1} for HA. One can thus appreciate that the degree of ion release from the graft can vary considerably from material to material. Despite an abundance of literature on the role of calcium ions and, to a lesser extent, phosphate ions on cell signalling and other vital biological pathways and functions, there is a dearth of information on the specific role of ions released from "structural" non-organic resorbable grafts and implants on the subsequent healing of the surrounding tissue. There is a school of thought that suggests that precipitation of apatite *in vivo* on the surface of an implant material, which presumably incorporates proteins, is an important indication of bone bonding ability, often referred to as bioactivity [13]. This has given rise to the use of surface precipitation in simulated body fluid (SBF) as a supposed indicator of bioactivity [14]. However, it has never been demonstrated *in vivo* that the component ions of the precipitated apatite layer originated from the graft, and in the case of "bioactive" polymers such as Polyactive® [15] they clearly cannot; therefore this "predictor" is now being questioned [16].

There have been very few studies that have attempted to isolate effects of ions released from inorganic materials *in vivo*. By radiolabelling calcium in β-tricalcium phosphate (β-TCP) ceramic, it has been shown that dissolution products originating from the implants were partially detected in the surrounding bone tissue [17]. In a number of studies *in vitro*, the presence of calcium phosphate ceramics has been shown to affect the levels of calcium and phosphate in the medium. In a study by Anselme and co-workers [18], HA-coated titanium plates, as received, washed in water, polished and washed with water, or immersed in cell culture medium, were used to culture primary human osteoblasts. The authors observed that cells only grew on medium-preconditioned substrates, and suggested that inhibition of proliferation on non-preconditioned substrates was due to elevation of calcium and phosphate ions in cell culture medium up to threefold of the original concentration. Doi and colleagues [19] compared various calcium phosphate ceramics in an *in vitro* assay using primary rabbit osteoclasts and showed that osteoclasts were unable to proliferate on and resorb highly soluble ceramics such as dicalcium phosphate dihydrate (DCPD), α-TCP and tetracalcium phosphate, possibly due to elevated concentrations of calcium and/or phosphate concentrations of the medium. Knabe and colleagues [20] demonstrated that proliferation and osteogenic differentiation of human bone-derived cells was feasible on resorbable α-TCP ceramic, two resorbable glassy crystalline materials and a carbonated apatite cement, although the amounts of calcium and phosphate ions in the medium during cell culture were not determined. These studies are examples of *in vitro* experiments in which the authors have attempted to correlate viability or bioactivity of cells grown on different bone graft substitutes with their resorbability and consequent calcium and phosphate changes in cell culture medium. It should be noted, however, that in the case of calcium phosphate biomaterials calcium and phosphate ion changes occur simultaneously, making it difficult to isolate their individual effects. Besides, resorption of a biomaterial often leads to material surface changes, which, independent of the calcium and phosphate ion concentration changes in the medium, may have an effect on cell behavior. Finally, all changes occurring in a closed environment of an *in vitro* system may be very different from the *in vivo* environment for which they are intended, suggesting a limited predictive value of the *in vitro* data. This is therefore not only the case for calcium and phosphates, but also for the other inorganics discussed in this review.

In cell culture studies performed in the absence of calcium phosphate bone graft substitutes, the addition of calcium and/or phosphate ions to the cell culture medium showed a dose-dependent effect on viability and osteogenic differentiation of osteoblasts. In the study by Meleti and colleagues [21], primary human osteoblast-like cells were treated with inorganic phosphate in concentrations of 1–7 mM (about 95–665 mg l^{-1}). At 96 h, the percentage of viable osteoblasts treated with 5 mM phosphate was 30%, whereas 7 mM caused complete loss of cell viability. The authors suggested that cell death occurred through apoptosis, which was induced by a mitochondrial membrane permeability transition caused by the anion. In a later study by the same group [22], it was demonstrated that an elevation of calcium ion concentration alone did not have an effect on osteoblast viability, though a modest increase in the medium calcium concentration of 0.1–1 mM (about 4–40 mg l^{-1}) caused a fast and profound increase in phosphate-induced death of cultured osteoblasts. In a study by Dvorak and colleagues [23], fetal calvarial cells were treated with calcium ions in concentrations of 0.5–3 mM (20–120 mg l^{-1}). The results of this study showed that treatment of cells for 7 days or longer with elevated concentrations of calcium (1.8 and 2.5 mM) led to a significant increase in cell proliferation, whereas a decrease in calcium concentration to 0.5 mM significantly decreased cell proliferation, partly due to increased cell death. An increase in the expression of the osteogenic markers core binding factor α1, osteocalcin (OC), osteopontin (OP) and collagen type I (Col-I)

mRNAs was also observed at elevated calcium concentrations, as was mineral nodule formation, suggesting that small deviations of calcium concentrations from physiological values have a considerable effect on bone cell fate *in vitro*, independent of systemic calciotropic peptides. These studies demonstrated a role for calcium and phosphate in controlling osteoblast survival, growth and differentiation, and emphasize the importance of translating these data (e.g. concentrations in relation to cell numbers) to the *in vivo* environment surrounding a calcium phosphate bone graft substitute upon implantation, which is a complex issue.

Because calcium phosphates are often used in a "structural" capacity, it is extremely difficult to make two identical components that differ only in phase since thermal stability, processing route, strength, crystal size and so forth are all altered by the phase. One of us has previously correlated the thickness of fibrous capsule formation with the solubility of dense sintered calcium phosphates of similar grain size in a subcutaneous model and proposed that the capsule served to modulate the flux of ions [24]. Particulates are theoretically a route to making comparable test materials, but phagocytosis and a difficulty in defining the interfaces in an orthotopic implantation provide a logistical barrier. Further, it must be remembered that bone graft substitutes function in a complex milieu containing ionized, complexed and protein-bound calcium and phosphate ions. Consequently, minerals (e.g. DCPD [25] and calcium carbonate [26]), oxide layers on metals [27] and polymers [15,28] are known to form an apatitic layer in the body and so the surface that is implanted may not remain at the same composition during implantation.

2.1.1. Calcium

Since both calcium and phosphate ions have effects on skeletal cells, the obvious choice of calcium-free bone graft substitutes that could be used to determine the effect of calcium would be the magnesium phosphates. While it is known that the inorganic corrosion products of magnesium are non-toxic and a magnesium phosphate product has been approved by the US Food and Drug Administration (FDA), we were unable to find any report directly comparing a calcium phosphate with a magnesium phosphate of similar solubility to enable the correlation of any biological differences with the released cations.

Serum calcium concentration is normally held within a tight range between 2.25 and 2.75 mM (90–110 mg l^{-1}). This is achieved by a daily balance between intestinal absorption and re-excretion and renal excretion of some 300 mg of calcium. Additionally, bone resorption by osteoclasts can increase serum calcium levels. Systemically, calcium levels are sensed by calcium receptors (CaR) in the thyroid and parathyroid glands. Low calcium levels result in production of parathyroid hormone (PTH), which acts to raise serum calcium by all three mechanisms – increased bone resorption; decreased calcium and increased phosphate renal excretion; and increased intestinal adsorption of calcium and phosphate – by stimulating the kidneys to synthesize calcitriol from vitamin D. Similarly, elevated serum levels of calcium induce the thyroid to release calcitonin, which acts in an opposing manner to PTH to reduce serum calcium [29]. While the role of endocrine calcium receptors is well established, the role of bone tissue CaRs is less clear. They are thought to sense localized changes in calcium [30]. Elevated calcium has been identified as having a number of stimulating effects on osteoblasts *in vitro* [22,23]. As illustrated in Fig. 2, extracellular calcium-induced activation of CaR in bone cells has been shown to induce a number of intracellular signaling pathways, resulting in the regulation of osteoclast and osteoblast activity *in vitro* [30]. Gene knock out studies are more confusing since CaR ablation resulted in hyperparathyroidism, hypercalcemia and hypophosphatemia but the skeletal phenotype was similar to rickets [31]. Double knock outs, which also prevented PTH formation, resulted in skeletally normal mice, and this has led to speculation that other receptors may duplicate the CaR function [32]. Recently, as understanding of the role of CaRs has grown, an appreciation for the potential for a therapeutic effect from calcium released from bioceramics has appeared in the literature [33].

Additionally, calcium receptors are promiscuous in that many cations (both inorganic and organic) may stimulate them to some degree, both as agonists and antagonists, and calcimimetics and calcilytics [34] are an active area of research for pharmaceutical manipulation of PTH. One can appreciate, then, that many biomaterials have the capacity to activate bone tissue CaRs. However, if one considers CS, which is the most soluble inorganic bone graft substitute, while it has been postulated that calcium released from the grafts may accelerate bone healing [35], there is no direct evidence to support this. Indeed, implantation of CS into an osteoporotic spine model, where elevated calcium levels might be expected to inhibit osteoclast resorption, actually resulted in more rapid graft degradation compared with healthy animals [36].

2.1.2. Phosphate

Regarding the role of phosphate on physiological functions, it should first be remembered that the term "phosphate" is imprecise. The orthophosphate anion (PO_4^{3-}) is found in body fluid in the typical range of 73–125 mg l^{-1} (24–41 mg l^{-1} as phosphorus), and much phosphate is associated with proteins through phosphorylation of serine, threonine and tyrosine amino acid residues. "Pi" is used in biology to distinguish inorganic orthophosphate.

The degree of protonation of Pi depends on pH, and at physiological pH Pi is present as 61% HPO_4^{2-} and 39% HPO_4^{-}. Furthermore, Pi is capable of forming polyphosphates; perhaps the most widely known example is the formation and cleavage of the phosphoester bond in adenosine, di- and triphosphates (ADP and ATP). Of crucial importance to bone mineralization is the dimeric ion pyrophosphate (or diphosphate), $P_2O_7^{7-}$, designated PPi. PPi and other polyphosphates are potent inhibitors of mineralization. PPi is formed by the enzymatic degradation of extracellular ATP and is also transported extracellularly by the ANK transporter. Gene knock out studies that reduce PPi below normal ranges (3 μM l^{-1}) have confirmed the role of PPi in inhibiting soft tissue mineralization, and much of what is currently known about the process has been neatly summarized in a recent editorial [37].

However, less is known about the role phosphate plays on physiological functions than the role calcium plays. Pi is known to form part of the negative feedback loop for PTH [38], and there is suggestion that a phosphate receptor may exist, since phosphate regulates expression of the OP [39] and activates the Raf/MEK/ERK and ERK1/2 pathways. Both fibroblast growth factor (FGF) 23 and extracellular phosphate activate Raf/MEK/ERK pathway via FGF receptors in HEK293 cells [40]. Low levels of phosphate have been shown to stimulate osteoclastic resorption, as well as osteoblastic differentiation [41], whereas high levels of phosphate induced apoptosis of osteoblasts *in vitro* [21]. Additionally, phosphate has been suggested to play a role in early chondrogenesis [42].

Hydrolysis of PPi by alkaline phosphatase (ALP), produced by differentiating osteoblasts, locally increases Pi concentration, thus promoting collagen mineralization and simultaneously removing inhibitory PPi ions [43–46]. However, this seems to be a simplification, as there are at least two more phosphatases known to be active at the site of mineralization. PHOSPHO 1 generates Pi from phospholipids [47] and inorganic pyrophosphatase generates Pi from cytosolic PPi in osteoblasts [48]. This latter pyrophosphatase was also found to significantly regulate collagen expression. It should be remembered that osteoclasts are routinely characterized by the expression of acid phosphatase, but its role in bone mineralization is not well understood. Acid phosphatase knockout models showed an abnormal skeletal phenotype with delayed cartilage

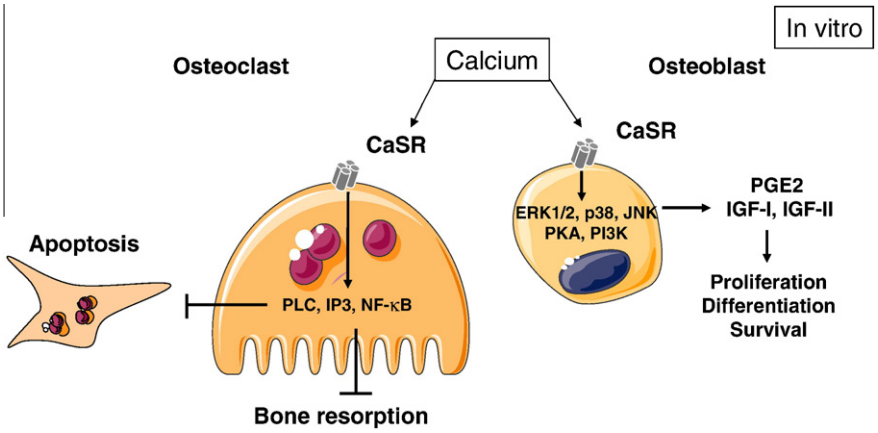

Fig. 2. Role of the calcium sensing receptor in regulation of osteoclast and osteoblast activity *in vitro*. Reproduced from Ref. [30]. The research was originally published in Ref. [30] © Elsevier.

mineralization and reduced bone remodeling and mineralization [49]. Acid phosphatase has also been shown essential for collagen synthesis [50]. Resorption by active osteoclasts is likely to generate locally elevated calcium and phosphate concentrations that may act upon osteoblasts during remodeling.

The roles of ALP, PHOSPHO1 and PPi in mineralization have been mechanistically validated *in vivo* (e.g. [51]). The use of implants with immobilized ALP in a variety of materials combinations [45,52–57] has been shown to induce mineralization *in vivo*, but no distinction between the removal of PPi and the localized increase in Pi was made. Recently we have shown that only sustained localized release of Pi from an implant can mineralize collagen *in vivo* [58]. This occurred by overriding the inhibitory effect of PPi by elevating Pi, and one might envisage that intracellular inorganic pyrophosphatase has a similar effect.

Finally, it should be recalled that pioneering work by Fleisch [59] on pyrophosphate inhibition of ectopic mineralization led to the discovery of the most effective and widely used osteoporosis treatment available today, the bisphosphonates. These are essentially non-hydrolysable analogs of the pyrophosphates, wherein P–O–P is replaced by P–C–P. Bisphosphonates are mineral-seeking ions that bind to bone following administration. Their mode of action is that they interfere with prenylation following internalization by osteoclasts, thereby deactivating them and limiting bone destruction [60] – hence their widespread use as a treatment for osteoporosis [61]. They are also used in the management of bone metastases [62]. Recently bisphosphonates have been shown to enhance initial bone formation when released from implants [63] and could provide a cheap and safe way to enhance bone–implant integration.

2.2. Silicate

Regarding the roles of silicates in physiological processes, it should first be mentioned that the terms "silicon", "silica" and "silicate" are often interchangeably and therefore inaccurately used. While silicon is the second most abundant element in the earth's crust, pure silicon is rarely found naturally. A silicate is a silicon-containing anion, often SiO_4^{4-}, but, as with phosphates, many other configurations are possible, such as $Si_2O_7^{6}$ – and $Si_nO_{3n}^{2n-}$. Silica is a silicate with the formula SiO_2, and is usually made from polymerized SiO_4^{4-} with shared vertices in either a crystalline (e.g. quartz) or amorphous form (e.g. glass). Silicates are poorly soluble but may form silicic acid or uncharged molecules in order to dissolve. Since foods and water contain silicate, deficiency in humans is unknown.

Silicon is mainly found in connective tissues, because it is an integral component of the glycosaminoglycan and their protein complexes [64]. Since the early work of Carlisle and co-workers [64–67], there have been contradictory results with regard to the effect of dietary supplements of silicon on bone formation and bone quality in animals and humans. By an electron probe microanalysis of tibial bones of young mice and rats, Carlisle claimed to localize silicon in active areas of early calcification. Silicon and calcium amounts increased in parallel at relatively low calcium concentrations; however, when the mineral composition approached HA, silicon concentrations fell below the detection limit. These data suggested that silicon is associated with calcium in an early stage of calcification [68]. It should be noted, however, that the closeness in position of the K_α peaks for silicon and phosphorus would certainly reduce the detection limit that the electron probe microanalysis could have provided [69].

In addition to calcium phosphates, silicate-based materials are also used in bone regeneration and therefore are of interest for this review. Silica-based bioactive glasses mainly consist of SiO_2, CaO, Na_2O and P_2O_5 in defined proportions, with SiO_2 being the former of the two-dimensional glass network and alkali metal (sodium, potassium) and alkali earth metal (calcium, magnesium) being network modifiers. The ratio between the network former and the network modifier in a glass determines its solubility in physiological solutions and hence its bioactivity and resorbability [70]. It is generally accepted that silica-based glasses can have bone-bonding capacity. The processes occurring on the surface of bioglass upon immersion in physiological solutions such as SBF can be divided into five stages: sodium leaching and formation of silanols; loss of soluble silica and formation of silanols; polycondensation of silanols to form hydrated silica gel; formation of an amorphous calcium phosphate layer; and crystallization of a carbonated apatite layer [70,71]. Although the exact processes are dependent on the nature of the solution, it is accepted that the ability of these glasses to form a biological apatite layer on their surface is the origin of their bioactivity. Although Gatti and Zaffe [72] observed the presence of silicon ions in the surrounding tissue upon implantation of silica bioglass particles in bone defects in the jaws of sheep, it remains questionable whether there is an active role that silicon ions play in the processes leading to new bone formation.

In a recent leading opinion paper by Bohner [73], the question was raised whether silicon substitution in the crystal structure of calcium phosphate ceramics, such as HA and β-TCP, indeed resulted in biological performance superior to that of their stoichiometric counterparts, as is claimed in a number of research

papers. This review stated that, although there exist publications showing more pronounced bone formation in silicon-substituted HA compared to the phase pure ceramic [74,75], there is no direct evidence that this is attributable to silicate release from the ceramics. By substituting an ion into a ceramic lattice, not only the chemical composition, but also many other physico-chemical properties may change, making it difficult to isolate the effect of a single component.

Apart from silica-based glasses and silicon-substituted calcium phosphate ceramics, efforts have been put into developing collagen–silica hybrids. Coradin and colleagues [76] followed the procedure of co-gellation of Col-I and silicate solutions, followed by lyophilization. The procedure was shown to involve the conformation of an inorganic amorphous network of silica from the sodium silicate by the condensation reaction of silicic acid and self-assembly of collagen helices in the aqueous solution. The same group later showed that sodium silicate interferes with *in vitro* collagen fibrillogenesis in a dose-dependent manner: whereas a high concentration of sodium silicate of 10 mM (about 1.2 g l^{-1}) inhibited fibrillogenesis, a lower concentration of about 800 μM (about 98 mg l^{-1}) resulted in the formation of longer and wider fibrils compared to sodium chloride at the same concentration [77]. By using a paste of fibrillar bovine collagen as an additional organic template for silicification *in vitro*, Heinemann and colleagues [78] prepared monolithic hybrid xerogels that supported fibrillogenesis attachment, proliferation and osteogenic differentiation of human mesenchymal stem cells (hMSCs). The authors also reported that these gels supported the formation of osteoclasts from monocytes and showed that a homogeneous mixture of gels with calcium phosphate cements was feasible [79]. Although silicated collagen mineralizes profusely *in vitro*, there are no reports that we are aware of that reproduce this effect *in vivo*.

2.3. Magnesium

Magnesium biomaterials include the fascinating degradable metals, their inorganic degradation products and magnesium-based cements. Magnesium-based cements have been FDA approved (Osteocrete, Bone Solutions Inc.), while magnesium-based degradable stents are in advanced clinical trials (Biotronik AMS stent) and are being actively researched in orthopaedics. The reader is directed to one of several reviews on degradable magnesium alloys [80], and a recent report by Tamimi and colleagues [81] on cell response to different magnesium phosphates. Despite its long, if not widespread, use, which has demonstrated good tissue tolerance during degradation, there have been few studies that have pointed to a biological role for the release of magnesium ions in bone repair. Otsuka et al. [82] reported a positive effect on bone growth with combined zinc and magnesium substitution in calcium phosphate ceramics, though a clear dose response was not observed and the effects of magnesium were not distinguished from those of zinc. Landi et al. [83] observed enhanced resorption and osteoconduction in magnesium-containing HA compared with controls.

Magnesium is an essential element and the vast majority of studies concerning bone and this element focus on dietary deficiency, which is found to affect bone healing only in adult animals with a severe deficiency [84].

Focusing, then, on possible modes of action for localized release from an implant, magnesium, present at physiological levels of 17–25 mg l^{-1}, has been implicated as an essential ion for maintaining the PPi–Pi balance, in which we conjecture it may have two roles. First, it could change the substrate specificity of ALP by altering pyrophosphatase activity of ALP, depending upon the Mg/PPi ratio (Fig. 3) [85]. Secondly, it has been proposed that it is not the PPi ion that is the preferred substrate for ALP but the $Mg_2P_2O_7$ complex, thus explaining the widely observed effect that Mg increases ALP activity above that expected by supplying a sufficient cofactor [86–88]. The activity of the enzyme could therefore be cellularly controlled by a feedback mechanism rather than by specialized regulatory binding sites on the enzyme [85]. Indeed, this is borne out in clinical practice since PPi levels could be controlled by oral administration of magnesium [89], whereas a lack of magnesium is associated with the pathological deposition of calcium pyrophosphate [89]. Finally, it was shown over a decade ago in a clinical trial that short-term oral supplementation with magnesium reduced bone turnover markers in young adult males [90]. Supplementation with 365 mg of magnesium significantly reduced the serum intact parathyroid hormone (iPTH) level, and reduced levels of both serum bone formation and resorption biochemical markers after 1–5 days. Serum bone formation markers (type I procollagen peptide and OC) correlated negatively with ionized Mg^{2+} but not with iPTH or ionized Ca^{2+}. Interestingly, the quantity of magnesium excreted through urine increased over the 30 day trial and did not plateau, suggesting the initial retention of magnesium. More recently, a very similar study in post-menopausal osteoporotic women with a daily dose of 288 mg magnesium showed similar findings [91].

3. Bioactive non-structural ions

3.1. Strontium

Strontium is, like calcium, a group IIa element and, from a chemical point of view, they behave similarly. Although strontium is not an essential trace element, a substantial amount of research has been performed on its properties and effects due to its chemical analogy to calcium. Initial interest in strontium metabolism stems from the fact that 90Sr is an abundant and potentially hazardous by-product of nuclear fission [92]. The fact that strontium behaves metabolically largely like calcium has helped in maintaining interest in this element. An early study has shown that, although the metabolic pathways followed by strontium and calcium are largely similar, there exist quantitative differences in how the body handles these elements: in general, more strontium than calcium is excreted in urine and feces [93]. When fed in large amounts, strontium has been shown to cause rickets in

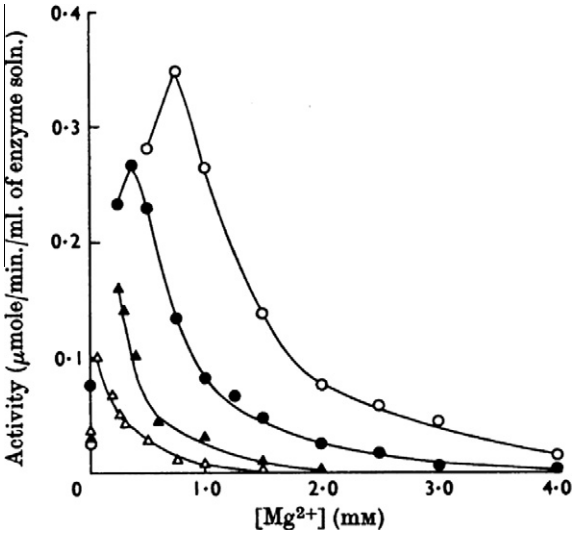

Fig. 3. Inhibition of pyrophosphatase activity by Mg^{2+} at four fixed PPi concentrations: ○ 1.0 mM, ● 0.5 mM, ▲ 0.25 mM, 0.1 mM (reproduced from Ref. [85]). The research was originally published in Ref. [85] © the Biochemical Society.

experimental animals by disrupting the intestinal calcium absorption, synthesis of vitamin D and mineralization [94].

Strontium is a bone-seeking element, of which 98% in the human body can be found in the skeleton [95]. It is therefore not surprising that, among the trace metals present in human bone, strontium was the only one that was correlated with bone compressive strength [96]. Strontium was shown to have dose-dependent effects on bone formation, which was further affected by presence or absence of renal failure. While low doses (0.19–0.34%) of orally supplemented strontium chloride were reported to improve the vertebral bone density and stimulate bone formation in rats with normal renal function, doses of 0.4% and higher were shown to induce defective bone mineralization [97,98]. However, in animals with chronic renal failure, 0.34% of strontium as chloride compound in drinking water induced a bone lesion histologically characterized as osteomalacia [99]. This and further studies on a similar model showed the effect of strontium to be complex and dose dependent.

Strontium is used within certain commercially available dentifrices for treatment of dentin hypersensitivity. The main approach to dentin desensitization is the closure of opened dentinal tubules by applying "active" ingredients in dentifrice such as fluoride, potassium and strontium salts [100,101] or nanosized carbonated apatite [102]. Such topical application of strontium appears to satisfy the criteria of local delivery. It has indeed been shown that brushing teeth with strontium-supplemented toothpastes increased the strontium content in the exposed enamel, as early as 3 weeks into treatment. This increase in strontium content is suggested to be advantageous in the treatment of cariogenesis [103].

Dose-dependent effects of strontium have been observed in cell culture experiments: studies with primary osteoblasts isolated from fetal rat calvaria showed that, at a low dose (1 mg l^{-1} strontium in the culture medium), nodule formation was reduced, while mineralization was not impaired; at an intermediate concentration (5 mg l^{-1}), no effect on either nodule formation or mineralization was observed; while at high concentrations (20–100 mg l^{-1}), mineralization was reduced, while there was no effect on nodule formulation [104]. Strontium was also shown to reduce excessive bone resorption in rats with osteopenia, which was associated with a decrease in the number of osteoclasts [105].

Based on these findings, a distrontium salt, strontium renalate, is used clinically in the treatment of osteoporotic patients in Europe. The dual anabolic and anti-resorptive role of strontium ranelate has been described *in vitro* [106]. Bone biopsies from patients treated with strontium ranelate indeed showed a reduction in bone resorption; however, no evidence of increased bone formation was found. Long-term treatment with a daily dose of strontium ranelate was associated with a 40% reduction in new vertebral fractures in postmenopausal women with osteoporosis [107]. In a different study, a significant reduction in nonvertebral fractures, in particular hip fractures, was observed in a subset of patients that, based on the age and Bone Mineral Density (BMD) score were at higher risk of hip fracture [108].

Although a number of *in vitro* studies have shown both positive effects on ostegenic differentiation and mineral formation and inhibitory effects on osteoclastic differentiation and resorption [109–112], the exact molecular mechanism behind this dual role of strontium is still incompletely understood. In a study by Bonnelye and co-workers [113], it was suggested that the resorptive activity of osteoclasts by strontium ranelate was inhibited by the disruption of actin cytoskeleton organization. In a recent study by Caudrillier and co-workers [114], it was suggested that the inhibition of osteoclast differentiation by strontium ranelate is mediated by the stimulation of CaRs, whereas formation of mature osteoclast is mediated by inhibition of the receptor activator of nuclear factor kappa-B ligand (RANKL)-induced nuclear translocation of nuclear factor kappa-B (NFkB) and activator protein-1 (AP-1) in the early stages of differentiation.

Several attempts have been made to incorporate strontium into calcium phosphate bioceramics, with the goal of locally delivering strontium ions to the area of bone where repair is required. In a study by Kannan and colleagues [115], wherein strontium-doped β-TCP was produced from calcium-deficient apatite through aqueous precipitation and subsequent heat treatment, the substitution of calcium by strontium was shown to have an effect on the lattice constant of the resulting ceramic. Such substitution to a level of 26 wt.% led to a significant expansion in both the *a*- and *c*-axes in a dose-dependent manner, since the ionic radius of strontium (1.13 Å) is larger than that of calcium (0.96 Å). These data were consistent with earlier work by Bigi and colleagues [116,117], who showed that β-TCP was able to host up to 80 at.% (20 wt.%) of strontium without provoking any remarkable rearrangement of the unit cell. Similarly, strontium for calcium substitution was demonstrated in α-TCP ceramic [118]. In the HA ceramic, substitution was proven possible up to 12 wt.%. The solubility of the HA increased upon strontium substitution as a consequence of the increase in the crystal lattice [119–122].

In vitro bioactivity studies in SBF showed that strontium–HA, with a strontium content below 10 mol.% (8 wt.%), showed a more pronounced apatite layer formation on the surface than pure HA, which could be attributed to a higher dissolution rate of strontium-containing ceramic [122,123]. A dose-dependent decrease in reactivity in terms of retarded and slower hydrolysis was observed in strontium-substituted α-TCP cements [118]. Experiments with osteoprecursor cells (OPC1) showed that both the attachment and proliferation were increased in HA containing 20 mol.% (16 wt.%) strontium compared to the control without strontium. An increase in ALP and OP were also observed, suggesting that strontium stimulated osteogenic differentiation of OPC1 cells [123]. Similar findings were also obtained in a study where osteoblast-like cells were cultured on strontium-substituted biphasic calcium phosphate ceramic, consisting of HA and TCP [124]. Pulsed-laser deposition was successfully used to apply a coating of strontium-doped HA, prepared by an aqueous precipitation method, on metallic substrates. Osteogenic differentiation of human osteoblasts MG-63 was stimulated by 0.3–0.6 wt.% strontium, whereas proliferation of osteoclasts was negatively affected [125]. Release of strontium in time was not determined in any of these *in vitro* studies, making it difficult to draw strong conclusions as to whether the effect observed was due to released strontium ions.

In vivo, injection of strontium-containing calcium phosphate cement into rabbit iliac crest cancellous bone revealed that Sr–HA stimulated the formation of new bone formation [126]. Semi-quantitative energy-dispersive X-ray (EDX) microanalysis showed that, upon explantation, strontium was still present on the surface of cement, as well as on the interface between cement and the newly formed bone, though no strontium was detected in the surrounding bone [126].

In addition to calcium phosphate bioceramics, strontium-doped silicate- and phosphate-based glasses have also been developed. The effect of strontium doping on these glasses *in vitro* and *in vivo* has been shown to be highly dependent on the level of strontium substitution, as this affects not only the amount of released strontium ions but also the structure of the glass network, and therewith the associated degradation and bioactivity properties [127–131]. In the *in vivo* study by Gorustovich et al. [128], where no effect of strontium substitution (6 wt.% SrO for CaO) was observed, EDX analyses revealed the presence of strontium in the cement/bone interface. In the *in vitro* study by Gentleman and colleagues [127], the strontium released from bioactive glasses, which enhanced the metabolic activity of osteoblasts and

inhibited the resorptive activity of osteoclasts, showed a burst release profile in cell culture medium, although it was unclear what the total percentage of strontium released from the glasses was. This once again emphasizes how difficult it is to isolate the effect of a single element when used in a "structural" composition such as a ceramic.

In summary, then, while normal supplementation with strontium appears to do no harm, evidence to support its prophylactic use in at-risk patients seems sparse. That said, while bisphosphonates have been shown to reduce bone loss, sometimes with an associated reduction in fracture rate, there is evidence that severe prevention of remodeling reduces bone strength [132] and that, in some patient groups, alendronate seems to be associated with low-impact fractures [133]. However, it is hard to distinguish between bisphosphonate types, dosing regimes, severity and types of osteoporosis. There is a growing consensus that an as yet undefined "bone quality" is perhaps more important for patients than an easily quantified bone mineral density, and work is ongoing to try to define this. It can thus be said that the lack of clarity surrounding strontium supplementation is not surprising, given the complex nature of defining bone "health", especially in older patients, and hopefully patient studies with more statistical power will help patients and clinicians to make better informed choices in the future.

3.2. Lithium

Lithium was widely marketed and became one of a number of medicinal products popular in the late-nineteenth and early-twentieth centuries. It was the active ingredient of a refreshment beverage that was originally named "Bib-Label Lithiated Lemon-Lime Soda" and was marketed specifically as a hangover cure with the slogan "takes the ouch out of grouch". Its name was later changed to 7 Up [134]. Lithium was one of the first drugs to be approved by the US FDA (in the early 1970s) for the treatment of manic depression [135]. The use of lithium is recommended for the treatment of both unipolar and bipolar depressive disorders. Lithium therapy has been shown to be associated with mild, reversible hyperparathyroidism, which is a risk factor for osteoporosis; however, there exist conflicting results with regard to the effect of lithium treatment on bone loss, depending on the duration and treatment intervals. Zamani and co-workers [136] compared bone mineral density of 75 lithium-treated patients to that of matched normal subjects and concluded that the mean bone density in the treated patients was significantly higher at the spine, femoral neck and trochanter, a finding that was possibly due to a lower bone turnover in those patients. In a case control study by Wilting et al. [137] in which never, ever, current, recent and past lithium use were compared to matched controls in 231,778 fracture cases, it was found that the current use of lithium was associated with a decreased risk of fracture, which did not vary with cumulative duration of use. However, among past users, an increased risk of fracture was observed, which increased with time since discontinuation of lithium use. It has been observed that lithium causes granulocytosis and lymphopenia, while it enhances the immunological activity of monocytes and lymphocytes.

The mechanism behind these diverse roles of lithium involves a number of pathways by which glycogen synthetase kinase-3beta (GSK3 beta) is inhibited. This enzyme phosphorylates and inhibits nuclear factors that turn on cell growth and protection programs, including the nuclear factor of activated T cells (NFAT) and Wnt/beta-catenin [138]. The Wnt signal transduction pathway has been associated with cartilage and bone formation. Kugimiya and co-workers [139] showed that GSK-3b controls osteogenesis through the regulation of Runt-related transcription factor-2 (Runx-2) activity. Lithium has been shown to activate Wnt-responsive genes by selectively inhibiting GSK3 activity. Indeed, in the study by Kugimiya et al. [139], the cleidocranial dysplasia in heterozygous Runx2-deficient mice was significantly rescued by the genetic insufficiency of GSK-3b or the oral administration of lithium chloride.

Patients suffering from osteoporosis–pseudoglioma syndrome have an inactivating mutation in the Wnt co-receptor low-density lipoprotein receptor-related protein-5 (LRP5) [140], whereas an activating LRP5 mutation is associated with high bone mass [141,142]. Analysis of LRP5-deficient mice revealed a decreased number of osteoblasts, suggesting that Wnt signaling stimulates bone formation at the level of osteoprogenitor proliferation [143]. De Boer and co-workers [144] showed that overactivation of Wnt by low concentrations (4 mM) of Wnt-mimic-lithium stimulated hMSC proliferation, and that hMSCs extensively expanded in the presence of lithium chloride retained the potential to differentiate into both the osteogenic and adipogenic lineages. They also showed that Wnt signaling induced by low concentrations of lithium chloride inhibited dexamethasone-induced osteogenic differentiation of hMSCs and mineralization of osteogenic hMSCs [145]. In the study by Bain and colleagues [146], lithium chloride concentrations of 10 and 25 mM (42–1050 mg l^{-1}) induced ALP activity in C3H10T1/2 murine multipotent cells, whereas concentrations below 10 mM had no effect on the expression of this early marker of osteogenesis. Recently, it has been shown that lithium inhibits BMP-2 signaling to affect osteogenic differentiation in both osteoblastic cell line MC3T3-E1 and murine myoblastic cell line C2C12 [147].

In the study by Wang and colleagues [148], lithium was deposited with calcium phosphate on titanium substrata using electrolytic deposition (up to 0.5 wt.%). It was shown that the deposition of the calcium phosphate coating was inhibited by the presence of lithium chloride in the electrolytes, and this inhibition was concentration dependent. With a burst release of about 90% of lithium from the deposited calcium phosphate layers, as determined in simulated physiological solution, the attachment and initial proliferation of MG-63 osteoblasts were stimulated [148]. It would be interesting to further investigate the possible effects of lithium on proliferative capacity of osteoprogenitor cells by a controlled release from bioceramics.

In conclusion, in contrast to the proven stimulatory effects of genetic Wnt pathway activation in human and mouse bone tissue formation at the level of osteoprogenitor proliferation, the effect of lithium ions on osteogenic differentiation *in vitro* is dose dependent and possibly related to the level of commitment of a cell to the osteogenic lineage.

3.3. Zinc

Calhoun and colleagues reported that human body contains between 1.4 and 2.3 g of zinc on the biochemical level [149]. The zinc content of bone ash lies between 150 and 250 μg g^{-1} (0.015–0.025 wt.%), which is relatively high compared to other tissues. Zinc has long been known to play an important role in various physiological processes. Zinc is involved in the synthesis of a large number of proteins and is required for their stability; it is one of the constituents of the antioxidant system, and plays a role in cytoskeleton maintenance, immune function and cellular signalling [150]. There exist about 1400 zinc-finger proteins, which account for a large part of the transcription regulatory proteins [151], including co-activators, chromatin-modifying and -remodeling enzymes, DNA-binding transcription factors, members of the general transcription machinery and multisubunit RNA polymerases [152].

Zinc deficiency is associated with a number of skeletal anomalies in fetal and postnatal development, such as decreased bone age, which can be treated with zinc supplementation. Nutritional zinc deficiency has been shown to result in a decrease in zinc

concentrations in bone [149]. Recent studies, however, have shown that high zinc levels, associated with oral supplementation or extended use of denture cream, may lead to hypocupremia, which may cause anemia, leucopenia and neutropenia [153,154]. Zinc retards intestinal absorption of copper by two mechanisms. First, they both share the same carrier in enterocytes and so, with pre-exposure to zinc, copper transport is blocked for some hours. Secondly, zinc induces metallothionein in enterocytes, which binds metals that are subsequently excreted in the feces [154].

An early study indicated an increase in uptake of zinc during bone formation, in particular in the areas of calcification, the development of osteons of compact bone of a young adult dog and newly deposited endochondral bone in the metaphysis of a young rat [155]. In bone, zinc has been shown to play a structural role within the bone matrix. This consists of HA crystals, which contain zinc complexed with fluoride. In addition, a stimulatory role on bone apposition by osteoblasts and an inhibitory role on osteoclastic resorption of bone have also been attributed to zinc [156]. Yamaguchi and co-workers [157], for example, showed an increase in Runx-2, osteoprotegerin and regucalcin mRNA expression by MC3T3-E1 osteoblasts in the presence of zinc in concentrations of 10^{-6}–10^{-4} M (65 μg l^{-1}–6.5 mg l^{-1}). More recently, Kwun and colleagues [158] observed a negative effect of zinc deficiency on the osteogenic activity of MC3T3-E1 cells *in vitro*: bone marker gene transcription was reduced through inhibited and delayed Runx-2 expression and the mineralization of extracellular matrix was reduced through a decrease in ALP activity. The effect of zinc on the human osteoblast-like cell line SaOS-2 was shown to be concentration dependent: ALP expression and mineral nodules formation were stimulated in presence of zinc at concentrations of 1 and 10 μM (65 to −650 μg l^{-1}) but inhibited at concentrations higher than 25 μM (1.6 mg l^{-1}) [159]. When primary murine bone marrow stromal cells and osteoblasts were treated with zinc at concentrations of 10^{-9} M (65 ng l^{-1}) and lower, no effect was observed on proliferation, while an inhibitory effect was proven on both osteogenic and adipogenic differentiation [160]. Similarly, no effect of zinc supplementation to the osteogenic medium was observed on cell number, ALP activity, collagen synthesis, total protein content or matrix mineralization of rat bone marrow-derived stromal cells [161]. As recently reviewed by Yamaguchi [162], zinc has been shown to have an inhibitory effect on bone resorption in tissue culture systems *in vitro* and to suppress osteoclastogenesis of osteoclastic cells derived from bone marrow.

Since zinc has been shown to stabilize the crystal lattice of β-TCP, preventing its conversion to α-TCP [163], a range of experiments were directed towards zinc-containing β-TCP and biphasic calcium phosphate ceramics consisting of HA and β-TCP. Ito and colleagues [164], for example, developed biphasic calcium phosphate ceramics containing up to 7 wt.% zinc. Incorporation of zinc into HA results in instability of the crystal lattice, caused by the lack of adequate substitution sites. Zinc exhibits an inhibitory effect on crystallization of HA, and heat treatment of HA in the presence of zinc resulted in conversion into β-TCP, with a reduced lattice constant in comparison to pure β-TCP [165].

A number of studies have been performed *in vitro* to study the effect of incorporation of zinc into calcium phosphate bioceramics on biological processes related to bone formation and turnover. In the study by Ikeuchi and colleagues [166], rat and human bone marrow-derived stromal cells were cultured in HA/TCP ceramics containing zinc in amounts varying between 0 and 1.3 wt.%. Both human and rat bone marrow cells, cultured in osteogenic medium, showed an increase in ALP expression with increasing zinc content in the HA/TCP ceramic. The authors analyzed the release profile of the zinc-containing ceramics into cell culture medium and showed that the release was relatively high during the first 2 days, then decreased slowly with time. The addition to the cell culture medium of soluble zinc in concentrations equal to those in the ceramics containing 1.3 wt.% zinc showed a similar effect [166]. In another study, a positive effect on the proliferation of the MC3T3 osteblastic cell line was observed in HA/TCP ceramics containing up to 1.3 wt.% zinc, whereas higher concentrations caused cytotoxicity [164]. A mildly positive effect of TCP ceramic doped with 0.3 wt.% zinc was also observed on the proliferation of human fetal bone tissue-derived osteoprecursor cell line [167]. The addition of zinc to brushite-forming β-TCP cement showed a positive effect on adhesion, proliferation, ALP activity and Col-I secretion of the MC3T3-E1 osteblastic cell line, an effect which was even more pronounced when both strontium and zinc were present in the cement [168]. The presence of zinc in β-TCP (up to 0.6 wt.%) has been shown to have a dose-dependent negative effect on the resorptive activity of primary mature rabbit osteoclasts, as indicated by an increase in the number of apopoptic osteoclasts and a decrease in actin ring formation [169,170]. Incorporation of zinc into thin apatite films also led to the decreased resorption by primary osteoclasts, whereas no effect was found on the initial attachment of cells [171].

In an *in vivo* study by Kawamura and co-workers [172], zinc-containing HA and HA/TCP ceramics were used in a rabbit femoral defect to assess new bone formation. HA/TCP ceramic with low concentrations of zinc (about 0.3 wt.%) showed significantly more bone apposition than the control, whereas higher concentrations (about 0.6 wt.%) of zinc in both TCP and HA/TCP ceramic led to the increased resorption of host bone, as indicated by an increase in the size of the medullar cavity. Long-term implantation studies with a ceramic containing about 0.3 wt.% zinc confirmed a positive effect of the zinc in the ceramic with regard to bone apposition, but also demonstrated an increase in bone resorption at 60 weeks of implantation compared to the control [173]. It is remarkable that in *in vitro* studies by the same group, positive effects on osteogenic differentiation by both an osteoblastic cell line and primary bone marrow-derived stromal cells were observed on ceramics containing higher amounts of zinc, of about 1.2 wt.% [164,166]. Also *in vitro*, it was observed that ceramics containing about 0.6 wt.% zinc inhibited the resorptive activity of mature osteoclasts [170], whereas *in vivo*, the same concentration led to increased bone resorption [172]. Recently, Pina et al. [168] qualitatively showed a more pronounced bone formation in zinc- and zinc-and-strontium-containing β-TCP cements than in Norian SRS® cement, in a tarsal bone model in a pilot study in two pigs.

It should be noted that, apart from the studies for which the release analysis of the substituted element from a bone graft substitute was specifically mentioned, in the majority of the *in vitro* and *in vivo* studies described here the release was not determined. Furthermore, the fact that the presence of an additive changes intrinsic properties, e.g. crystallinity and hence solubility of the bone graft substitute, to a different level, depending on the content, makes it very difficult to directly attribute the observed effect to the inorganic additive. It is therefore imperative to always determine release profiles, in cell culture medium, for example, preferably in the presence of cells, to have an idea of the concentrations the cells sense during the culture. Similarly, understanding the release profile *in vivo*, by measurements or modeling, is of great importance in determining whether local concentrations correlate with the biological response observed.

3.4. Copper

In plasma, 90% of copper is present in ceruloplasmin and most of the remainder is bound to albumin. Ceruloplasmin is an oxidase enzyme and low levels (caused by copper deficiency) result in iron overload in tissues such as the brain and liver. Indeed, one of copper's first biological functions to be identified was its role in iron

metabolism [174]. For a review of the history of the study of the iron–copper relationship, including colorful accounts of early case studies, e.g. on the lack of anemia amongst female factory workers exposed to copper, the reader is directed to Fox [175]. Copper is a cofactor for several other enzymes in the body, such as superoxide dismutase, which protects the body against harmful effects of superoxide (O_2^{2-}) by decomposing it into hydrogen peroxide and oxygen. An enzyme relevant to musculoskeletal repair is lysyl oxidase, which catalyzes the formation of aldehyde-based crosslinks from lysine residues in collagen and elastin precursors. Excess copper can occur in some genetic disorders, notably Wilson's disease, which results in liver disease and neurological symptoms [176]. Copper deficiency is potentially life threatening, causing cardiovascular deformities culminating in rupture. Copper deficiency is intentionally introduced by selective complexing agents in anti-angiogenic strategies for tumor treatment trials since copper is essential for blood vessel formation [177]. Copper reduction in cell culture is also known to stimulate haematopoietic stem cell (HSC) proliferation and is used to improve HSC expansion clinically [178]. In the event of either dietary or genetic copper deficiency in humans and animals, osteogenesis is diminished, with thinning of the cortex and trabeculae of long bones. Mechanically, bones of copper-deficient animals are brittle. This has been attributed to a 300% higher solubility (less cross-linked) collagen [179]. However, while one can appreciate that copper is essential for a number of enzyme-based processes for bone formation – and, for that matter, survival – it does not explain why localized or systemic delivery of copper should impart any benefit for healing other than for correction of deficiency. Having said that, there is no evidence with which to reject this hypothesis either. While plasma albumin binds between 5 and 180 ng ml^{-1} of Cu^{2+} under physiological conditions [180], binding of up to 4 μg ml^{-1} of Cu^{2+} to its copper-binding glycylhistidyllysine (GHK) tripeptide residue can occur [181]. The free tripeptide GHK is found in human plasma, saliva and urine, and during wound healing proteolysis may liberate this tripeptide.

3.4.1. GHK–Cu

GHK–Cu has been observed to have a number of biologically stimulatory effects. It has been widely studied for wound repair and is marketed as a cosmeceutical for scar reduction, as recently reviewed by Pickart [182]. GHK–Cu has been shown to increase messenger RNA production for various extracellular matrices (collagen, elastin, proteoglycans, and glycosaminoglycans in fibroblasts [183,184]), as well as stimulating angiogenesis *in vivo* at levels of 10^{-12} M (1 ng l^{-1}) [182]. *In vitro*, it also increases the growth of human marrow stromal cells and promotes the attachment of human osteoblastic cells, while *in vivo* it promotes the filling of bone defects in femurs and bone attachment prostheses [185].

3.4.2. Inorganic copper

Much less has been published on the potentially therapeutic effects of copper ions than on the GHK–Cu complex. Like many other micronutrients, it has an essential role in bone formation and healing, as reviewed previously [186], yet this alone does not support any basis for supplying ions locally at a site of healing. Nonetheless, inorganic copper has been shown to exert angiogenic and wound-healing responses *in vivo* and *in vitro* [187–189]. With regard to bone, Giavaresi et al. [190] reported a higher vascular density in and around subcutaneously implanted allografts and hyaluronan-based hydrogel loaded with 2 dry wt.% copper as copper sulfate compared with copper-free controls. Barralet et al. reported a number of studies whereby inorganic copper was implanted and released from resorbable scaffolds [191–193]. A comparison between copper sulfate-loaded bioceramics with negative (unloaded) and positive (vascular endothelial growth factor (VEGF)) controls was performed. Some 56 ng of copper sulfate per implant was found to be optimal for blood vessel ingrowth, and the design of the implants was such that much of the copper should be released into the closed end of a 1 mm diameter pore of volume ~8 μl, giving a maximal average concentration in the order of 100 μM. This dose was found to give similar vascularized tissue ingrowth to an optimal dose of 200 ng VEGF and microscopy revealed organized connective tissue in the vascularized pores [191,192]. In an attempt to find *in vitro–in vivo* correlation, copper sulfate, VEGF and FGF were added to an *in vitro* endothelial cell angiogenesis assay. About 300 μM (48 mg l^{-1}) copper sulfate induced the formation of cord-like and tubular structures, and was found to have an additive effect, especially with FGF. The implantation of dead-ended collagen tubes with 560 ng of copper sulfate demonstrated microvessel infiltration and collagen deposition *in vivo*, and again the effect was additive with FGF [193]; this points to another possible mode of action: namely, potentiating the effect of endogenous growth factors. Recent work has shown that copper seems to be externalized by endothelial cells during tubulogenesis [194]. It is our view that collagen formation in turn physically supports blood vessel formation *in vivo* and that this is likely a main or the main contributing factor to the rapid vascularization observed in copper-loaded porous scaffolds. The stimulation of extracellular matrix (ECM) formation as a means of accelerating angiogenesis and healing represents a new approach to bone regeneration, distinct from previous approaches. It is potentially highly relevant to filling pores in defect repair materials to accelerate bone formation throughout the defect rather than by accelerating bone ingrowth from the periphery. It is feasible that the GHK tripeptide, which may be formed through proteolysis during healing and subsequent tissue remodeling, is therefore required to biologically "activate" the inorganic copper. Nonetheless, there are several *in vitro* works demonstrating a direct effect on ECM production by inorganic copper (e.g. [195]), where presumably the GHK supply is limited. Further, inorganic copper is extremely stable and lends itself to incorporation of implants that may have room temperature shelf lives of years.

4. Discussion and summary

In summary, bioinorganics can positively affect bone healing, though until recently their use has been based on serendipitous observation, often with a lack of clear rationale or hypothesis. While serendipity has a vital role to play in discovery, it is of limited use in exploitation. Apatites, as every bioceramics scholar knows, are capable of a huge variety of anionic and cationic substitutions; however, this does not render them ideal delivery matrices for these ions. The field of pharmaceutical bioinorganics has a lot to teach us and it is time for the bioceramics community to change its approach. The growing number of reports of apparent benefits of various ions in many combinations is doing little to clarify what species is effective at what dose over which duration. More fundamental studies are vital in order to address this, and the effect of a cation, for example, needs to be separated from its counter anion. This may require the use of experimental pumps or reservoirs to precisely deliver ions locally, in order for their effect to be better understood. The use of substituted ceramics or surface adsorption is very limited as an approach and we are near its limit. Once the localized effect of a species on bone growth is understood, we can begin to design release profiles with a specific goal in mind, rather than using a range based on substitution or adsorption limits and relying on dissolution or desorption to hopefully provide a therapeutic effect. Just as Paracelcus remarked some 500 years ago, dose is critical. Complex formation can be used to limit toxicity, optimize biological activity and more precisely maintain a therapeutic window, and has not been employed in biomaterials applications, in contrast with bioinorganics in general.

This review has illustrated that potentially several compounds act similarly while others may have very specific actions. Further, some ions, such as inorganic phosphate and copper, have a well-demonstrated essential role in bone repair and/or wound healing, while the role of others, such as silicate, is ambiguous. We have also touched upon the issue that the use of ions as drugs is poorly regulated since industry standards previously only anticipated systemic hazards from known toxic impurities, rather than from a localized tissue-stimulating effect. By analogy with prior errors in rash advocacy of a particular metal and the poor outcomes of several patients, the lack of apparent regulatory awareness is potentially a concern. That said, perhaps bioceramics have always been acting as drugs, and effects such as osteoconduction are merely a result of ion release or localized ion concentration modification. Several decades of bioceramics research have sadly made little difference to the patient requiring a bone graft, though the variety of essentially similar synthetic bone graft substitutes has surely grown. It is perhaps pertinent to recall that, to date, there is no satisfactory explanation for the mechanism of tissue bonding observed in many ceramics with bone and soft tissue, a property we take as read and rarely question. Here we suggest that, by considering some inorganics as pharmaceutical agents, at least our understanding of these biomaterials will improve and, at best, their biological performance can be improved to the point that synthetics begin to challenge autograft's dominance as the bone replacement of choice.

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Appendix A

Figures with essential color discrimination. Certain figures in this article, particularly Fig. 1, are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.actbio.2011.03.027.

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