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Review article

Gallium containing calcium phosphates: Potential antibacterial agents or fictitious truth

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ABSTRACT

Amidst an ever-increasing demand for the enhancement of the lifestyle and the modulation of modern diseases, the functionalization of biomaterials is of utmost importance. One of the leading materials for the aforementioned purpose have been calcium phosphates (CaPs). They have been widely used in bone regeneration displaying favourable regenerative potential and biological properties. Many studies have placed their entire focus on facilitating the osteogenic differentiation of stem cells and bone progenitor cells, while the aspect of antibacterial properties has been surmounted. Nevertheless, increasing antibiotic resistance of bacteria requires the development of new materials and the usage of alternative approaches such as ion doping. Gallium (Ga) has been the potential star on the rise among the ions. However, the obstacle that accompanies gallium is the scarcity of research performed and the variety of amalgamations. The question that imposes itself is how a growing field of therapeutics can be further entwined with advances in material science, and how will the incorporation of gallium bring a new outlook. The present study offers a comprehensive overview of state-of-the-art gallium containing calcium phosphates (GaCaPs), their synthesis methods, antibacterial properties, and biocompatibility. Considering their vast potential as antibacterial agents, the need for a methodical perspective is highly necessary to determine if it is a direction on the brink of recognition or a fruitless endeavour.

Statement of significance

Although several studies have been published on various metal ions-containing calcium phosphates, to this date there is no systematic overview pointing out the properties and benefits of gallium containing calcium phosphates. Here we offer a critical overview, including synthesis, structure and biological properties of gallium containing calcium phosphates.

1. Introduction

Bone remodelling encompasses a myriad of interconnected pathways that uninterruptedly unravel during the entire lifetime, while at the same time being subjected to the constantly varying laws of biomechanical forces. The end goal of this process is to have a functional model, which has to possess appropriate mechanical strength. Nonetheless, due to chronic bone diseases such as cancer, hypercalcemia, osteoporosis, or trauma, bone defects are being formed, lacking the ability to be healed or remodelled on their own [1]. In order to overcome this tremendous burden, clinicians and researchers are continuously developing new bone graft materials for bone defect repair, thus creating a high demand for further amelioration of biomaterials [2].

To come as close as possible to the creation of a suitable bone graft model, primarily the composition of bone must be considered. Bone consists of the organic part (30 wt% of mineralized collagen) [3] and inorganic part (60 wt%) - mineral phase, known as hydroxyapatite (HAp) [3,4]. The remaining 10 wt% of the bone composition is water [4]. Considering that HAp is probably the

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most recognized member of the calcium orthophosphate family, the rationale for using calcium phosphates (CaPs) imposed itself quite easily. Furthermore, extensive studies have shown that different CaPs showed promising results as bone graft materials and their outstanding track record has been verified throughout the last few decades. The most commonly used CaPs, to date, are HAp ($Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP; $Ca_3(PO_4)_2$), followed by an abundance of various derivatives [5,6]. In addition to their well-suited composition, they have shown exceptional bioactivity and biocompatibility with cell lines, which are the main participants of the bone microenvironment [7–11]. On the other hand, while the CaPs have been characterized in terms of their osteoconductive properties, the material still is not as competent as the natural apatite [12,13]. A hindrance to the otherwise noteworthy case history of CaPs has been their poor mechanical strength under continuous stress circumstances, most probably connected with challenges associated with the sintering process and their low surface area (typical 2–5 m^2/g) [14].

An additional important aspect of the clinical application of biomaterials is their antibacterial potential. The lack of antibacterial properties of CaPs could cause a risk of infection after the implantation of biomaterial. Infection is related to bacteria adhesion and colonization on implant or material surface. Additionally, the formation of biofilm can be a potential source of systematic infection [15]. Infection is an outcome of bacterial contamination, which can occur in a dry state or under wet conditions. Dry state contamination commonly appears before implantation, and it may be transmitted in the surgical room or due to the unsterile supplies that are being used. By using adequate sterilization this contamination can be controlled [15]. Wet condition contamination is related to bacteria adhesion on the biomaterial surface in an aqueous solution. This type of contamination is affected by different parameters of material, physiological environment and pathogens. In particular, surface morphology, physicochemical properties of material, environment pH, temperature and electrolytes [15,16]. The traditional approach for treating these infections is based on the use of systemic administration of the antibiotics (vancomycin, rifampicin, fluoroquinolone, etc.) for up to six weeks [17]. This approach has several drawbacks, including systemic toxicity, uneven distribution throughout the body and low accumulation in the target site [18,19]. Therefore, methods are being sought to ensure local delivery of antibiotics at the site of implantation. Additionally, the World Health Organisation (WHO) data shows that antibiotic resistance is becoming a global health threat. Successful treatment of infections in modern medicine would be at an even greater risk without developing alternatives. New antibiotic development is a time-consuming procedure, and in the report submitted in 2019, the WHO identified 32 antibiotics in clinical development, of which only six were classified as innovative [20].

Nevertheless, pure antibiotics are not the only answer to modern perils. Different metal ions had shown antibacterial properties and were used a long time before antibiotics were discovered [21–23]. Copper (Cu) and silver (Ag), for example, have been mentioned as far back as ancient Egypt, as components used for water disinfection. Due to their characteristics, non-essential metal ions, Ag or tellurium (Te) being one of them, can be toxic to the biological system, if they are admitted in abundant concentrations [24,25]. However, using metal ions in small concentrations can provide a desired antibacterial effect.

Antibacterial properties of metal ions depend on the charge and electronegativity. In the biological system, metal ion chemistry could enforce different reactions and diverse bindings to the proteins, causing a beneficial after effect [21,26]. The exact mechanism of metal ion interaction with bacteria has not been fully determined and understood. However, several antibacterial activity mechanisms of metal ions or nanoparticles have been proposed and outlined below (Fig. 1):

1) Ion fixation – due to electrostatic forces fixation could occur, leading to the membrane damage. It affects the free transport of protons, and other molecules passing in and out of the bacteria cell, resulting in the loss of membrane potential [21,27].
2) Metal ion has the potential to interact with the bacteria cell membrane and to take action in regulating the production of adenosine triphosphate (ATP), as well as the replication process, which causes the deoxyribonucleic acid (DNA) damage [21,27].
3) Formation of the free radicals (reactive oxygen species: ROS) – in this step, oxygen stress starts to form, resulting in the damage of bacteria membrane, DNA and mitochondria, leading lastly to bacteria death [5,21,28,29].
4) Protein dysfunction and loss of enzyme activity [21].

Biomaterials, containing different metal ions, empower long-term local ion release, providing antibacterial action at the surgical site [30,31]. Such metal ions for doping or substituting have been used: silver (Ag^+), zinc (Zn^{2+}), strontium (Sr^{2+}), sodium (Na^+), copper (Cu^{2+}) and others [2,32]. Additionally, metal ions, for instance calcium (Ca^{2+}), iron (Fe^{2+}), magnesium (Mg^{2+}), manganese (Mn^{2+}) and others are essential elements in our body, found primarily in protein metabolism [33,34].

2. Gallium

Gallium (Ga) is a semi-metallic III group element [35] and a non-essential element in the human body [36]. Its ionic radius of 0.620 Å, rendered as octahedral, is very close to the radius of ferric ion (Fe^{3+}) – 0.645 Å [5]. In addition, the tetrahedral ionic radii of both ions are also very close, 0.47 Å for Ga^{3+} and 0.49 Å Fe^{3+}.

From the 1970s gallium compounds were investigated as therapeutic agents, while simultaneously they were exhibiting different behaviours, including the inhibition of bone resorption [37,38] and promotion of bone growth. Even more, they were involved in the effective treatments of autoimmune diseases – cancer, osteoporosis [39] and copious infectious diseases. Gallium compounds play an important role in the bone-tumour imaging and they have shown antimicrobial and antibacterial properties [35,40,41]. Gallium nitrate is used as a drug to treat hypercalcemia in humans, while gallium maltolate showed toxic influence on lymphoma cells, which accordingly inhibit the growth of the cancer cells [42,43]. Additionally, the aforementioned gallium salts such as gallium maltolate, nitrate and citrate, along with the complex siderophores and hemes are being used as carriers. They deliver Ga^{3+} to a wide range of bacteria, making it a perspective treatment for bacterial infections [6,35,41,44].

When the inhibitory, immunomodulating, anti-hypercalcemic and analgesic operations of Ga^{3+} are in question, the importance falls on the capability to mimic iron species and act as a competitor for tumour cells that require positively charged ion to grow and survive [5]. These actions are supported by the fact that gallium and ferric ions have the same oxidation state (+3) and a similar ionic radius. Gallium starts its therapeutic effect by disrupting the “native” ferric iron’s binding in the enzyme active site. As it is redox inactive it impels the enzyme’s activity in question [45,46]. The metalloprotein that is pivotal for anticancer therapy is ribonucleotide reductase, which is Fe^{3+} dependent and indispensable for the DNA synthesis in living cells. The mechanism transpires through two parallel actions. The first one is the uptake of gallium ion instead of ferric ion, thus inhibiting the proliferation of the cancer cell, which requires a greater amount of ferric ions to function properly. The second process incorporates the redox inactive Ga^{3+} in the active site, stopping the production of DNA and causing the apoptosis of the cancer cell [45].

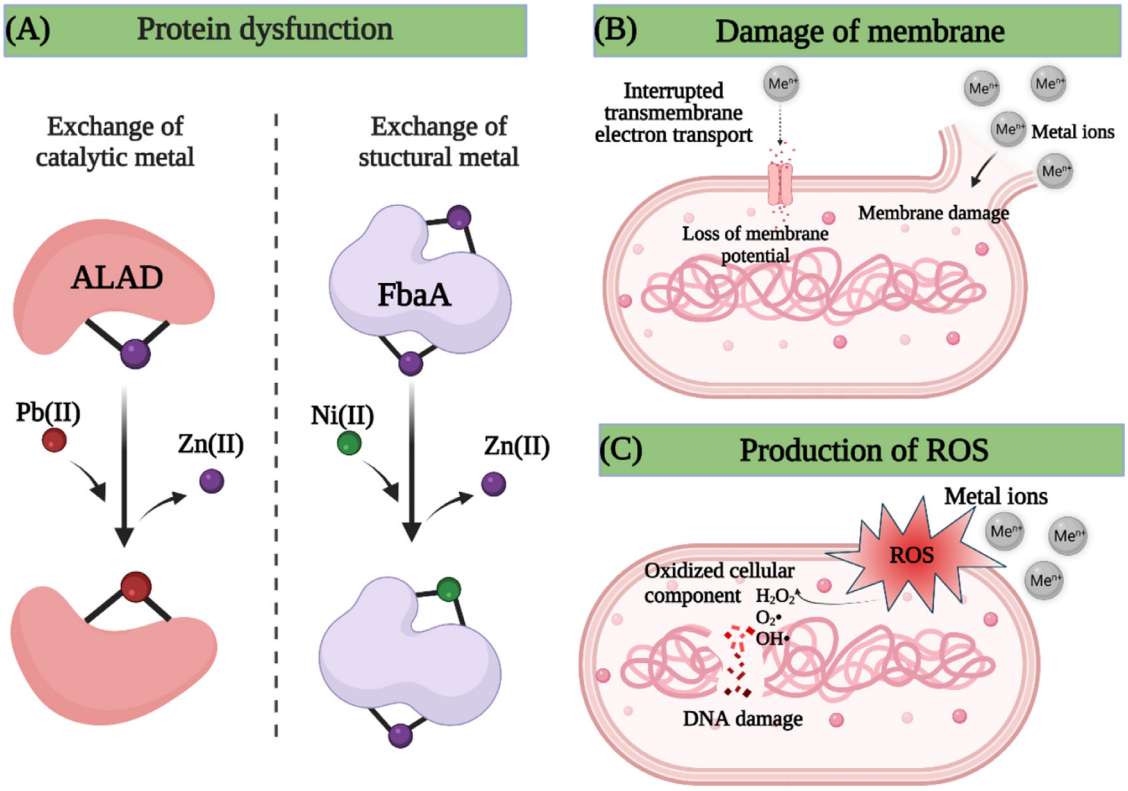

Fig. 1. Mechanism of metal ion interaction with bacteria (A) Metals leading to protein dysfunction by enzyme metal exchange; (B) Metal ion fixation leading to the membrane damage; (C) Production of reactive oxygen species (ROS); ALAD - δ-aminolevulinic acid dehydratase; FbaA - fructose-1,6-bisphosphate aldolase; ROS - reactive oxygen species (created with BioRender.com; adapted from [21,27]).

Despite it being known for decades, it is still unclear what effect gallium has on the bone and its related processes [47]. Data from several studies detected deposition of Ga^{3+} in the bone tissue, presumably related to its linkage with bone remodelling on active metaphyseal growth plates and healing fractures [31,40]. Additionally, Bernstein et al., have introduced Ga^{3+} ion as the inhibitor for the SARS-CoV-2 virus [48].

3. Antibacterial activity of gallium

Due to the above-mentioned similarity between Ga and Fe ions, it is possible to replace Fe^{3+} with Ga^{3+} in the metabolism of the protein, thus interfering with the functioning of bacterial cells [49]. As a result, Ga^{3+} exhibited a less toxic outcome, compared to silver ions [5,35,49]. Extensive research has shown that Ga^{3+} has antibacterial activity against iron-dependent bacteria, such as *P. aeruginosa* [50,51]. Approaches of bacterial iron uptake and metabolism have been shown in the Fig. 2. Fe^{3+} has low bioavailability, so its uptake and transport relays on the iron-binding proteins, transferrin or lactoferrin, heme or heme-containing proteins and siderophores [21,50,51]. Bacteria synthesize siderophores (Greek for "iron" and "bearer"), which are low-molecular-weight compounds that scavenge iron (in the Fe^{3+} oxidation state) from the environment, hence making it important for bacterial growth and survival [52].

During the infection, the place of siderophore-mediated iron uptake plays an important role in the colonization, growth and survival of the bacteria [21,50–52]. The properties that Ga^{3+} shares with Fe^{3+} permit it to bind with high avidity to these iron-binding proteins. Ga^{3+} antibacterial activity is based on "*Trojan horse*" strategy (Fig. 2) [21,53]. This mechanism pathway rests on "tricking" the bacteria to take up the toxic Ga^{3+} that mimics Fe^{3+} ions. Gallium interrelates with the siderophores and lowers

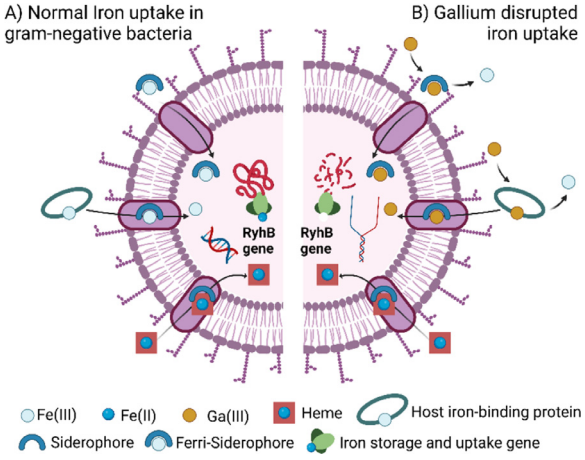

Fig. 2. Iron uptake metabolism approach of the iron-dependent bacteria A) in normal conditions; B) in presence of gallium (adapted from [50], created with Biorender.com).

the number of chelators in the environment designated for providing the ferric ion needed for bacteria. The host cell takes in the newly created gallium-siderophore complex, supplying itself with the redox-inactive cation, ultimately hampering the bacterial metabolism [45,54].

By disrupting the bacteria metabolism, the essential actions needed for them to survive are being destroyed: DNA synthesis and repair, respiration and oxidative stress response. The entire process, combined with several subsequent actions that have been described in the literature, finally results in the bacteria death [50,55]. Several studies have shown that Ga^{3+} has an antibacterial

Table 1
Synthesis routes of CaPs.

Product	Methods	Ref.
Ga-doped HAp	Ultrasound-assisted ion exchange	[63]
HAp modified by gallium	Wet chemical precipitation	[47,49]
Ga-containing HAp	Hydrolysis of urea	[41]
HAp modified by gallium	Solid-state reaction	[49]
Ga-containing HAp	Hydrothermal synthesis	[64]
Ga-containing β-TCP	Solid-state reaction	[40,65–67]
Ga-doped ACP	Wet chemical precipitation	[68]

influence on Gram-negative and Gram-positive bacteria (e.g. *M. tuberculosis* and *M. avium, S. aureus, E. coli* [56], as well as *P. aeruginosa*, which is multidrug-resistant bacteria) [21,57].

As shown, the different activity mechanisms of Ga^{3+} have the ability to separate it from the other metal ions, especially when being used for therapeutic and antibacterial purposes. Furthermore, gallium has the possibility to be incorporated in different CaPs, giving an extended possibility for a controlled and local ion release. Given the growing field of therapeutics, in the next section localized spatiotemporal release effects will be described in more detail.

4. Gallium containing calcium phosphates (GaCaPs)

Being recognized as the most suitable substitute for biological apatite, HAp has undergone the research on different ion incorporations, stemming from monovalent to divalent ions [58–60]. However, even with the wide array of tested ions, the lack of research on using Ga^{3+} for HAp, but also for the other members of the orthophosphate family is evident in the literature [2,61,62]. In order to understand properly the mechanism of Ga^{3+} incorporation and the outcome it elucidates, the synthesis route and the structure of the CaPs have to be taken into account. Furthermore, the range of concentrations of the Ga^{3+} ion must be carefully monitored. In the following subsections synthesis methods of GaCaPs, more specifically amorphous calcium phosphate (ACP), HAp and β-tricalcium phosphate (β-TCP) will be conferred in more detail.

The process of doping encompasses interstitial incorporation of the ion or its placing between atom layers. However, substituted refers to the atom (element) exchange in a certain atomic position – crystallographic term. The impact of whether Ga^{3+} ions are doped or substituted in conventional CaPs is yet to be understood and current state of the art will be presented in the following sections.

4.1. Synthesis routes

The two main principles being used in obtaining GaCaPs are synthesis in aqueous media and solid-state reaction. The most popular method, however, is the wet precipitation route, while sol-gel and hydrolysis methods are not being used as often (Table 1).

4.1.1. Hydrolysis method

Hydrolysis is a water ionization process that results in the diffusion of hydrogen and hydroxide ions. According to Mohd Pu'ad et al. it is the least used wet synthesis method [69]. In the hydrolysis process it is possible to transform the calcium and phosphate precursors to HAp [69]. For example, α-TCP can be transformed to HAp by mixing it with different solvents, succeeded by hydrolysis for 2–120 h at 70 °C. The time needed for completing hydrolysation is dependent on chosen solvent. Hence, using 1-octanol as a solvent would take more than 24 h to completely hydrolyse α-TCP to HAp [69,70]. As one of the most common sources of calcium and phosphorous ions, calcium nitrate tetrahydrate ($Ca(NO_3)_2{*}4H_2O$) and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) are being used. Once urea has been added into the synthesis media, the suspension is heated in the temperature range of 80-90°C [41,71]. Kurtjak et al. used the same approach with an additional step of sonication of the mixture, to obtain Ga-containing HAp [41]. Nonetheless, the hydrolysis synthesis of CaP requires temperatures higher than the room temperature, which is the main reason why this method is unlikely to be used.

4.1.2. Sol-gel method

During the sol-gel method, calcium and phosphorus precursors are mixed in the presence of a solvent thereby forming "sol". Subsequently, the solvent is removed and "gel" is formed by heating. In this method organic solvents, such as ethanol, butanol and others are being used [72,73]. Alkoxides are frequently used as calcium and phosphorus precursors, for instance, calcium acetate, calcium diethoxide, triethyl phosphate or phosphite are some of the representatives [72–74]. This method is suitable for the production of thin-film coatings [72,73]. Additionally, when CaP sol-gel is formed a porous scaffold can be produced by using the foam replica technique [75]. However, so far this method has not been used for obtaining Ga-doped CaPs.

4.1.3. Wet chemical precipitation method

One of the most extensively used synthesis routes, in aqueous media, is wet chemical precipitation. HAp or ACP are commonly obtained by this method. The wet chemical precipitation method is based on the mixing of two salts, where one serves as the source of Ca^{2+} ions and the other as PO_4^{3-} ion precursor. Salts which are diversely used for the precipitation are calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$) and diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) [49,68]. The wet precipitation method consists of two synthesis steps: precipitation of two different ion precursors and ageing of obtained suspension, during which the HAp phase is being formed [69].

Kurjak et al. studied the influence of Ga^{3+} addition in different steps of the synthesis (Fig. 3). Introducing Ga^{3+} precursor at the beginning of the synthesis, together with initial raw materials, is known as co-precipitation of GaHAp(CP) (Fig. 3A). When Ga^{3+} precursor is introduced before the ageing, GaHAp(TR) is obtained by transformation in the wet chemical precipitation method (Fig. 3B). Eventually, if Ga^{3+} is added at the end of the synthesis, together with the final HAp crystals, the ion-exchange approach follows and GaHAp (IE) is obtained (Fig. 3C) [41]. It was found that the various approaches affect the capability of ion release from the material, resulting in diverse antibacterial properties. As there are multiple ways of Ga^{3+} incorporation in CaPs, it can be found in the structure or adsorbed on the surface [41].

Throughout the literature, various combinations of wet chemical precipitation and other methods (i.e., ultrasound-assisted ion exchange, hydrolysis, hydrothermal synthesis) can be found. For instance, Kurtjak et al. combined the wet precipitation method with sonication and hydrolysis [41]. Additionally, the wet chemical precipitation method has different synthesis variables – Ca/P molar ratio, pH, temperature and ageing time, where all of the aforementioned variables can influence the final properties of HAp [69,76]. As gallium sources $Ga(NO_3)_3{*}xH_2O$ [41,49,64,65,77], gallium trichloride ($GaCl_3$) [47] and gallium(III) trioxide (Ga_2O_3) [40,66,67] were used.

For obtaining pure HAp, without the presence of other CaP phases, synthesis pH was maintained from 9 to 10 [49,68]. Once Ga^{3+} ions were present in the equation, the hard acidic nature of Ga^{3+} itself in the aqueous media should be taken into account. If the synthesis conditions are maintained at a wide pH range, hydrolysation can transpire, leading to the formation of hydroxylate species, predominantly gallium gallate $[Ga(OH)_4]^-$. The resulting formation of the hydronium ion (H_3O^+) in aqueous media leaves

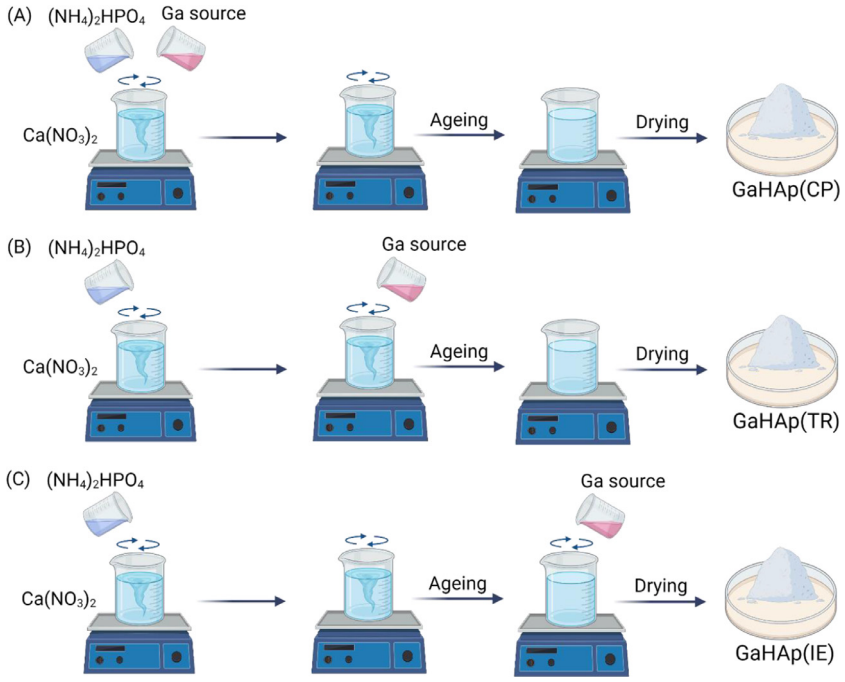

Fig. 3. Wet precipitation method of GaHAp (A) co-precipitation; (B) transformation and (C) ion-exchange (created with BioRender, inspired from [41]).

the synthesis solution highly acidic [45,78,79], leading to a difference in the final synthesis pH, which ultimately affects obtaining of the pure HAp in the presence of Ga^{3+}.

4.1.4. Solid-state reaction

The solid-state reaction is known as a dry method and it requires the use of high-energy processing or high temperatures. This method has been primarily used to obtain Ga-β-TCP ceramics or cements [40,65–67]. The crucial point of the solid-state reaction is the heat treatment temperature, and since TCP has two polymorph forms, α and β (which have different thermal stability), it requires a careful consideration [80,81]. Mellier et al. sintered anhydrous calcium phosphate, calcium carbonate, and gallium oxide mixture at 1000 °C for 24 h and obtained Ga-β-TCP [31,66,82]. Qiu et al. sintered Ga-TCP at 1000 °C for two hours and in order to obtain bioceramics, the attained Ga-TCP was mixed with paraffin, then moulded and sintered at 1250 °C for an additional 2 h. This resulted in TCP bioceramics with different compositions and diverse Ga^{3+} rates in the final product. Products synthesized without Ga^{3+} exhibited the α-TCP structure, whereas the products with up to 1.25 wt% of Ga^{3+} revealed the biphasic TCP, comprised of α-and-β-TCP. When Ga^{3+} was used in the range from 2.5 wt% to 7.5 wt%, pure β-TCP was formed [40]. By applying high temperatures, transformations between TCP polymorph forms occurs. Nevertheless, the reconversion of phases depends on the cooling rate [80].

4.2. Incorporation of the gallium ion in calcium phosphates

Antibacterial properties of various CaPs have been summarized in Table 2, while the biocompatibility has been described in the Table 3.

4.2.1. Gallium doped amorphous calcium phosphate (GaACP)

Yang et al. doped ACP with gallium ions, with initial (Ca+Ga)/P molar ratio being in the range from 1.8 to 3.0. XRD pattern revealed the characteristic wide bump around 30° 2Theta, indicating the formation of CaP with an amorphous structure. From ^{31}P and ^{71}Ga solid-state nuclear magnetic resonance (ssNMR) analysis, it was concluded that formed ACP, consisted of calcium phosphate clusters and gallium hydroxide oxide $Ga_x(OH)_yO_z$ clusters. Rapid Ga^{3+} ion release was observed in 24 h, while the further ion release stayed constant up to 96 h. Inhibition zone against *P.aeruginosa*, when in presence of GaACP, increased with increasing (Ca+Ga)/P molar ratio [68].

4.2.2. Gallium containing hydroxyapatite (GaHAp)

Another intrinsic approach has been the modification of HAp structure with Ga^{3+}. Possible interchange in the HAp structure, secures it from the resorption process and enhances the overall biomechanical properties of the skeletal system [64,77].

Being a member of CaPs that has the most resemblance to the apatite phase of human bone, HAp has been heavily utilized in multiple research areas, stemming from bone tissue engineering to the cell-targeting [83]. HAp ($Ca_{10}(PO_4)_6(OH)_2$) displays hexagonal crystal structure (Fig. 4.), with $P6_3/m$ space group symmetry and lattice parameters of γ=120 °, a=b=9.432 Å and c=6.881 Å [83–85]. However, HAp can be found also in the monoclinic form encompassing the $P2_1/b$ space group, with unit cell parameters of a=9.421 Å, b=2a and c=6.881 Å, while γ is 120° [85]. The Ca/P molar ratio of stoichiometric HAp is 1.67, which differs from the biological apatite, due to the diversion substitutions [84]. Depending on the type of the ion (electric charge, size etc.), different segments (PO_4^{3-}, Ca^{2+} or OH^-) of the crystal lattice have been substituted. An additional influence on the place of substitution in HAp was the net charge of crystal planes. In this instance, the a and b planes are positively charged hence they adsorb negatively-charged molecules, while the c plane is vice versa [74].

As a trivalent ion, Ga^{3+} is incorporated in the crystalline lattice either through heterovalent substitutions or through intercalations [64]. Depending on whether Ga^{3+} is introduced through gallium nitrate [41,87], gallium chloride [88] or sodium gallate [64], the total sum of the ionic radii can steer the mechanism of incorporation. When gallium nitrate is used, ion radii is larger (2.69 Å) than that of the Ca^{2+} (1.98 Å) and gallium forms a solid solution of intercalation. If it is in the form of gallate, the ionic radius is

Table 2
Antibacterial properties of GaCaPs.

Material	The obtained concentration of Ga^{3+}	Experimental material	Method	Result	Ref
GaHAp	13wt%	*E. coli, P. aeruginosa, S. epidermis*	Disk diffusion	*E. coli, S. epidermis* exhibited no inhibition zone; *P.aeruginosa* expressed the inhibition zone	[63]
		P. aeruginosa,	Microdilution antibiogram	Complete inhibition of bacteria growth for concentrations ranging from 0.1 g/L^{-1} g/L	
	3 wt% Co-precipitation 4 wt% Transformation 8 wt%; 15 wt% Ion Exchange	*P. aeruginosa*	Disk diffusion (24 h)	Inhibition zone increase in the order HAp(Ga)Co-Precicpitation < HAp(Ga) IonExchange < HAp(Ga)Transformation	[41]
			Microdilution antibiogram (1 day)	Inhibition of bacterial growth. The minimal inhibitory concentration (MIC): HAp(Ga)CP 0.9 g/L, HAp(Ga)TR 0.3 g/L, HAp(Ga)IE (8 wt% Ga) 0.1 g/L and HAp(Ga)IE (16 wt% Ga) 0.075 g/L	
	0.32 wt%	*P. fluorescen*	Disk diffusion (16–18 h)	Inhibition zone 17 mm, for Ga-HAp obtained with wet precipitation method, and 27 mm for Ga-HAp obtained with solid-state reaction. (HAp inhibition zone 13 mm)	[49]
GaACP	-	*P. aeruginosa*	Disk diffusion	An inhibition zone from 14 to 19 mm was observed. Increased with increasing (Ca+Ga)/P molar ratio.	[68]

Table 3
Biocompatibility of GaCaPs.

Material	Type of study	Experimental material	Result	Ref
GaHAp	*In vitro*	Mouse fibroblasts L929	Good cell survivability of HAp(Ga) materials: 80% for HAp(Ga)TR, 70% for HAp(Ga)IE (8 wt% Ga). Cytotoxic effect for HAp(Ga)IE (16 wt% Ga), cell viability less than 50%.	[41]
		Cercopithecus aetiops kidney cells VERO (ATCC)	No significant difference in cell growth between control and GaHap samples. Conclusion: inert, biologically compatible material.	[77]
		Mammalian cell line BALB/c 3T3 clone A31	Nontoxic effect of GaHap obtained with wet precipitation method. Toxic effect of GaHap obtained with solid-state reaction.	[49]
Ga-β-TCP ceramics	*In vitro*	Mouse bone mesenchymal stem cells	The proliferation of cells was not observed in the presence of β-TCP bioceramics containing Ga^{3+}ions.	[40]
		RAW264.7 murine monocyte cell line	Inhibition of osteoclastic-activity-related genes in presence of 2.5 wt% Ga-β-TCP ceramics.	
Ga-CaP cement (GaCPC)	*In vitro*	RAW264.7 murine monocyte cell line	Gallium ion release increased in presence of RAW263.7 cells.	[66]
	In vivo	Filing of cylinder defect in knee joint of New Zealand White rabbit	New bone formation was observed in close contact with cement surface after four weeks.	
	In vitro	Human osteoblasts and monocytes	Differentiation of human primary monocytes into osteoclasts when seeded alone on CPC or GaCPC was not observed.	
	In vivo	Lewis rats, Bilateral femoral	Higher amounts of fibrillar collagen were synthesized when the reconstruction occurred with Ga containing cements. Presences of Ga^{3+}induced a significant 23% increase in defect filling	[31]

1.64 Å, and it is possible to replace Ca^{2+} in the lattice, with no changes in crystallographic parameters [64]. As an alternative approach to doping, HAp nanorods can be coated with metallic Ga nanoparticles. Material obtained in this manner showed a characteristic crystalline structure of HAp and a non-crystalline layer of Ga particles with an oxide/hydroxide layer [89].

GaHAp revealed the characteristic hexagonal structure of HAp. Modification of Ga^{3+} did not cause a shift in the reflections of representative maxima of HAp. XRD pattern did not reveal additional reflection of new phases or changes in unit cell parameters [41,49,63,64,77,89]. However, different studies observed and described a decrease in crystallinity of GaHAp (Fig. 5), revealing the inhibition effect of Ga^{3+} on the formation of HAp crystals [41,47,49]. With the addition of Ga^{3+}, the amorphous surface layer of HAp in the form of gallium derivatives (e.g., gallium phosphates ($GaPO_4$)) can be observed [41,49,64,77]. The 3 wt% of water in GaHAp can be attributed to the mentioned disordered surface layer [64]. The amorphous structure of GaHAp surface layer was analysed with NMR and revealed that it has broader OH^- bands, compared to the pure HAp [41]. Theories assume that the surface layer, in the form of $GaPO_4$ is more toxic compared to $Ga(NO_3)_3$ [41]. The biological properties of Ga^{3+} are affected by its complexation with ligands in solution. For example, in water with neutral pH, precipitation of gallium hydroxide occurs. However, gallium ion remains in solution in presence of citrate. Additionally, its uptake by bacteria or cells is influenced by chemical speciation (the distribution of an element amongst chemical species in a system) of Ga^{3+} that occurs by ligand exchange [54].

GaHAp showed antibacterial properties against bacteria, e.g., *P.aeruginosa* and *P.fluorescen.* With the disc diffusion method, the inhibition zone was observed after 24 h against *P.aeruginosa* [41,63] and after 16-18 h against *P.fluorescen* [49]. HAp nanorods coated with Ga particles showed antibacterial activity against *P.aeruginosa* [89]. Inhibition concertation against *P.aeruginosa* for GaHAp was determined to be from 0.1 to 1 g/L of GaHAp suspension in Mueller Hinton broth [41,63]. Kurjak et al. observed inhibition for the gallium concentration even below 0.1 g/L [41,89]. The results were compared by the various incorporations of Ga^{3+} into the structure by using different synthesis methods. GaHAp produced by ion exchange (GaHAp(IE)) has sufficient antibacterial properties compared to the GaHAp obtained through transformation and co-precipitation method (GaHAp(TR)) mostly due to the fast ion release [41]. GaHAp(TR) and GaHAp(IE) showed superior antibacterial activity against *P.aeruginosa*, whereas GaHAp(IE) exhibited minimal inhibition concentration (MIC) of 0.075 g/L [41]. When GaHAp was functionalized with gold (Au) particles it showed antibacterial activity against *E.Coli, S.aureus* and *S.epidermidis,* whereas GaHAp alone did not have an antibacterial effect [63].

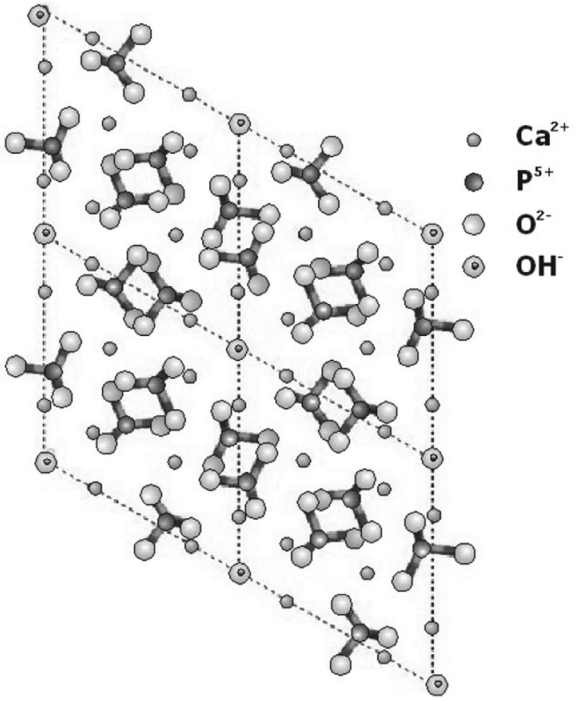

Fig. 4. Structure of hydroxyapatite (reprinted from [86] with permission).

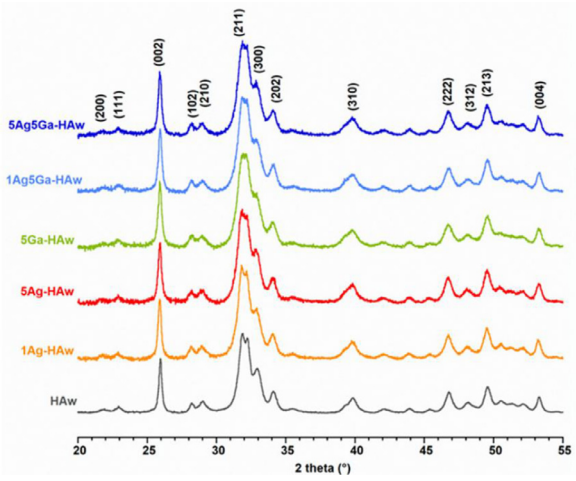

Fig. 5. XRD pattern of GaHAp samples synthesized by the wet chemical precipitation method (reprinted from the reference [49]).

Cytotoxicity of GaHAp *in vitro* was investigated with mammalian cells [49], human or animal fibroblasts [41,89] and monkey kidney cells VERO [77]. Furthermore, human osteoblasts and osteocytes were tested in the presence of gallium substituted calcium phosphate cement (CPC) [31]. At GaHAp concentration below and at minimal inhibition concentration (MIC) both human and mouse fibroblast cell viability showed good results of 80% [41,89]. However, in the values above MIC, human fibroblast viability decreased down to 50%. In contrast, mouse fibroblast viability in presence of 0.1 g/L GaHAp was 70% [89]. It should also be mentioned that GaHAp obtained with the ion-exchange method showed a toxic effect on mouse fibroblasts above MIC and cell viability was less than 50% [41,89]. A significant difference in the cell viability of African green monkey *Cercopithecus aetiops* kidney cells (Vero) was not observed both in the presence of HAp and GaHAp. Melnikov et al. showed in their study that GaHAp is a promising biocompatible material without toxic effect on monkey kidney cells VERO [77].

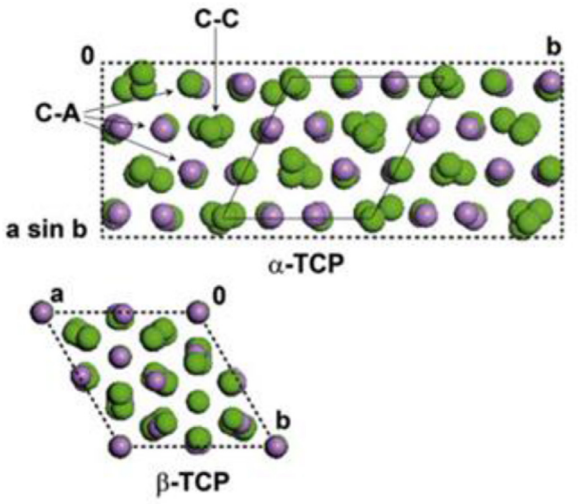

Fig. 6. Schematic structure of α-TCP and β-TCP unit cells along the [0 0 1] direction; C-C, cation–cation column; C-A, cation-anion column (reprinted from [91] Copyright (2011), with permission from Elsevier).

Furthermore, Pajor et al. observed the toxic effect of GaHAp obtained by the solid-state reaction at a concentration smaller than 12.5 mg/L. However, the toxic effect of GaHAp with a nominal Ga amount of 0.35 mass% obtained by the wet precipitation method was not observed [49]. The authors had no explanation for this observation; however, it can be related to the difference in solubility of the samples obtained by different synthesis methods.

4.2.3. Gallium containing β-tricalcium phosphate (Ga-β-TCP)

β-Tricalcium phosphate (β-TCP) is biocompatibile and has showed osteoconductive properties [67]. TCP exists in two polymorphs forms – α – and β – TCP, with a Ca/P molar ratio of 1.5 [40]. β-TCP displays rhombohedral structure (Fig. 6), with space group R3c symmetry and cell parameters of $a = b = 10.4$ Å, $c = 37.4$ and $\gamma = 120°$. The structure itself consists of 5 different Ca sites in which Ca atoms are distributed [90,91]. However, TCP can be found in α-TCP polymorph form as well, where it represents the monoclinic space group P21/a, with lattice constants being $a = 12.8$ Å, $b = 27.3$ Å, $c = 15.21$ Å, and $\gamma = 90°$ [91,92]. β-TCP has been found to exist in temperatures lower than 1125°C, whereas at a higher temperature it transformed into α-TCP [93].

Gallium incorporation affects β-TCP structure mainly because of the difference in ion radius of Ca^{2+} (0.99 Å) and Ga^{3+} (0.62 Å). Moreover, the electrovalence difference between Ga^{3+} and Ca^{2+} ions could lead to the formation of vacancy in the structure [40,67]. The theory says that gallium ions only occupy the octahedral M5 calcium site, while calcium occupation of the M4 site decreases in inverse proportion to the gallium content in the structure [66,82]. Possibly due to the different electrostatic repulsion between cation and phosphorous in M4 and M5 environments [94]. The XRD pattern displays the decrease in the unit cell parameters when in presence of Ga^{3+}. The shift of the characteristic peaks to the higher angles reveals the contraction of the cell unit. Ga-β-TCP dense ceramics have higher compressive strength compared to the pure β-TCP and it increases with increasing the Ga^{3+} content [66,82].

As in the case of GaHAp, the amorphous phase was also detected in Ga-β-TCP. Ga^{3+} prevented the transformation from β phase to α phase at Ga concentrations up to 2.5mol% [40]. Phase composition influences Ga^{3+} ion release in cell culture media, and since α-TCP is more soluble than β-TCP, it released higher concentrations of Ga^{3+}. Furthermore, bioceramics doped with Ga^{3+} at higher amounts of α-TCP phase have less porosity and higher com-

pressive strength [40]. Composite scaffolds with sintering agent phosphate-based glasses (PBG), with the composition of Ga-PBG and β-TCP, had increased compressive strength due to smaller macropore size. TCP-Ga-PBG scaffolds showed noticeably higher shrinkage, lower total porosity, smaller macropore size and denser microstructure [67]. *In vitro* studies reveal Ga-β-TCP did not affect the stem cells, however, inhibition of osteoclastic activity related genes was observed in the presence of Ga-β-TCP [40].

4.2.4. Gallium containing calcium phosphate cements (GaCPC)

Cement is ascribed with properties such as moldability and injectability, which after the injection into the bone, self-sets and hardens [31]. Calcium phosphate cement (CPC) is being used with different compositions that contain HAp, α-TCP and β-TCP, in different phase ratios. Ga^{3+} containing cement has been obtained by adding raw material, doped with Ga^{3+}. Cements with Ga-β-TCP showed higher setting time and cements containing gallium loaded calcium-deficient apatite (Ga-CDA) had higher compressive strength than cements without Ga^{3+} ion presence [65].

In vitro cell viability of human osteoblast decreased after 3 and 6 days in the presence of GaCPC, when compared to the pure CPC. However, in *in vivo* studies on Lewis, both CPC and Ga-CPC exhibited attributes of bone regeneration in the central area of the used implants, while the cortical regeneration transpired without the signs of inflammation or fibrous encapsulation [31].

Ga- cements in in vivo studies on New Zealand White rabbit femoral defects exhibited new bone formation on the surface of the implanted material after four weeks, compared to cements not containing gallium. [69]. Interestingly, gallium ion release increased in the presence of animal macrophages RAW264.7 cells [65], indicating phagocytic break down of particles.

5. Future directions and conclusions

In recent years, the possibility of gallium ion incorporation in multiple biomaterial strategies, by using calcium phosphates, has upheld the interest of the scientific community. Ga^{3+}-functionalized CaPs have proven to be effective antibacterial agents, while simultaneously promoting bone regeneration without inducing any immediate toxic effect. Even though gallium's similarity to iron exhibited an uncial antibacterial mechanism against bacterial infection and cancerous sites, it is still unclear how the differentiation, without a toxic effect on mammalian cells, occurs in biological processes (cell metabolism). It should also be taken into consideration that the biological properties of GaCaPs depend on the synthesis route of the material and the phase composition of CaPs. These variables have been found to influence the release of Ga^{3+} ions from the material, as well as ion incorporation in their structure. Furthermore, what was interesting to observe is that the addition of Ga^{3+} decreased crystallinity of HAp, but induced changes in unit cell parameters and shrinkage of the crystalline lattice of TCP.

From presented studies, GaCaP's showed promising inhibition of bacteria growth without noticeable toxic effect on human and animal cells. However, to date there is still a handful of reports on GaCaPs in the context of interfering with regenerative pathways or tissue engineering, particularly when compared to other well-examined ions in this field (e.g., Fe^{3+}). With an array of preliminary results indicating that the incorporation of gallium has promise in substituting Fe^{3+}, more materials favouring its properties should be considered as a step forward. For example, studies on Ga-β-TCP discuss material biocompatibility without data on antibacterial properties.

Additional hindrance, when it comes to the antibacterial properties of GaCaP, is related to the limited amount of data obtained from the investigations on a small number of bacteria strains. To fully cover the area of GaCaP antibacterial activity, a more comprehensive range of bacteria needs to be tested, for example, on anaerobic bacteria that is primarily present in the oral cavity. Nevertheless, the scarcity of research done on GaCaACP is evident. To the best of our knowledge, only one study was published so far. Considering that the results obtained were promising, further studies should be conducted to explore the potential of the GaACP combination. An added benefit to exploring the range and direction of the aforementioned combination is that ACP serves as a precursor for the formation of HAp [95–97]. Significant input can be done by investigating how Ga^{3+} ions influence the stability of ACP.

The so far presented results on the *in vitro* and *in vivo* cytotoxicity of GaCaP raise the question of what effect material has on bone remodelling related processes, especially on osteogenesis, osteoclast and osteoblast activity. Nevertheless, gallium, as the representative of the new, highly perspective ion is starting to be in the forefront of the research in the field of bone regeneration. The spectra of benefits, at this stage, can be easily challenged by the opulence of uncertainties. Given the many reported, but still understudied effects, we expect gallium to push its way to the forefront of research, as a part of materials tackling antimicrobial resistance and aiding in regeneration of tissues (Fig. 7).

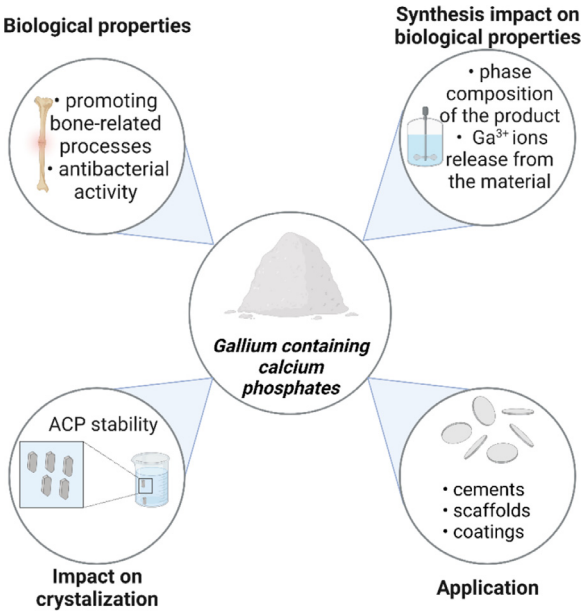

Fig. 7. Future perspectives and conclusions about gallium containing calcium phosphates (Created created with BioRender).

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Declaration of Competing Interest

Authors do not have any conflicts of interest to declare.

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