Biological properties of copper-doped biomaterials for orthopedic applications: A review of antibacterial, angiogenic and osteogenic aspects

Aurélie Jacobs, Guillaume Renaudin, Christiane Forestier, J.-M. Nedelec, Stéphane Descamps

Biological properties of copper-doped biomaterials for orthopedic applications: a review of antibacterial, angiogenic and osteogenic aspects

Aurélie JACOBS,[1] Guillaume RENAUDIN,[1]*, Christiane FORESTIER,[2] Jean-Marie NEDELEC,[1] Stéphane DESCAMPS[1]

[1] Université Clermont Auvergne, CNRS, SIGMA Clermont, ICCF, F-63000 Clermont-Ferrand, France.

[2] Université Clermont Auvergne, CNRS, LMGE, F-63000 Clermont-Ferrand, France.

Abstract: Copper is an essential trace element required for human life, and is involved in several physiological mechanisms. Today researchers have found and confirmed that Cu has biological properties which are particularly useful for orthopedic biomaterials applications such as implant coatings or biodegradable filler bone substitutes. Indeed, Cu exhibits antibacterial functions, provides angiogenic ability and favors osteogenesis; these represent major key points for ideal biomaterial integration and the healing process that follows. The antibacterial performances of copper-doped biomaterials present an interesting alternative to the massive use of prophylactic antibiotics and help to limit the development of antibiotic resistance. By stimulating blood vessel growth and new bone formation, copper contributes to the improved bio-integration of biomaterials. This review describes the bio-functional advantages offered by Cu and focuses on the antibacterial, angiogenic and osteogenic properties of Cu-doped biomaterials with potential for orthopedic applications.

Key words: copper, biomaterials, antibacterial, angiogenic, osteogenic

* Contact: Pr Guillaume Renaudin, guillaume.renaudin@sigma-clermont.fr

1. Introduction

Several situations can lead to the use of bone substitutes. It is the case, for example, after a trauma, a bone infection, or more generally after a local bone disease requiring bone tissue removal. In these situations, bone defects may occur, corresponding to a poor filling of the damaged area. With the increase in life expectancy, problems of bone fragility and fractures are also becoming more frequent, resulting in a multiplication of the number of orthopedic surgery procedures. Every year 2.2 million bone graft procedures are carried out worldwide [1].

Conventional solutions present limits in terms of both quality and quantity. Indeed, the autograft remains the ideal transplant, since it has all the desired characteristics: perfect biocompatibility, enabling osteoinduction and osteoconduction with no toxicity. However, it is limited in terms of quantity and usually requires a second sampling site, which increases the risks associated with the surgical procedure. Allografts are possible, but present risks of immune intolerance in the recipient patient, and the osteogenic properties are not always retained, depending on the conservation methods used. This is why the use of synthetic bone substitutes is necessary [2]. These are orthopedic biomaterials whose purpose is to rebuild a deficient bone stock; they must be able to replace the bone and integrate the recipient tissue in a completely safe way. As a basic minimum, the use of synthetic material must have no harmful effect on the biological environment, and ideally the biomaterial must be bioactive and osteoconductive, and present long-term stability or bioresorbability [3]. In this paper, we focus exclusively on biomaterials used for orthopedic applications. These must be bioactive and designed for more interactive purposes than others with benign functions [4].

The risk of infection is an important factor that needs to be controlled during the implantation of a bone substitute. For elective surgeries infection rates vary from 0.7% to 4.2%, and up to 30% in the case of third-degree open fractures [5]. Infections in bone sites are difficult to treat because of their deep localization in the tissue, and depend on the microorganism involved. Bone can be poorly vascularized tissue, and infections or trauma can lead to tissue necrosis. The implant is then recognized as a foreign body; bone sequestration occurs and promotes the persistence and relapse of infection [6]. Consequences for the patient are generally severe: delayed healing, often necessary iterative surgery, longer hospitalization times and therefore increased costs. This is why antibiotics are used in prophylaxis, to avoid the development of infections [7]. However, the massive use of antibiotics has led to the development of resistant bacterial strains [8]. In 1999, Europe set up a supervision system that tracks the increase in antibiotic resistance of 7 pathogenic bacteria [9]. This is especially the case for Methicillin-Resistant *Staphylococcus aureus* (MRSA). During the implantation of a biomaterial, a "race for

surface colonization" takes place between host cells and potentially present bacteria [10]. Indeed, initial colonization by bone cells prevents bacteria adhesion. On the other hand, if the bacteria colonize the surface first, they can form a biofilm; a bacterial environment allowing multiplication and resistance to treatments [11]. In 2002, Karlov et al. demonstrated the strong adsorption of *S. aureus* at the surface of titanium discs coated with calcium phosphate, compared to uncoated discs [12]. Therefore, in recent years, research has focused on the development of synthetic bone substitutes providing a preventive solution to the development of infection. To meet the biological needs, an alternative is to chemically modify these biomaterials during their synthesis. This process is named chemical doping, especially with metals ions (such as Ag^{+} [13], Cu^{2+} [14], Fe^{2+} [15,16], Zn^{2+} [17] and Mn^{2+} [18] among many others). Co-doped materials have also been detailed in recent reviews [19,20]. It is an effective system because ions have an activity directly at the site of implantation, unlike antibiotics, and they have no toxicity at low concentration [21].

Copper (Cu) is an essential trace element, necessary for human development by catalyzing metabolic processes, and is involved in bone formation [22,23]. It also has antibacterial properties and promotes angiogenesis [24,25]. Thus, the Cu^{2+} copper cation appears to be a promising dopant considering its high antibacterial properties and its limited cytotoxicity [26]. This review focuses on the interest of using Cu for its antibacterial properties, mainly when combined with biomaterials. In addition, its angiogenic and osteogenic capacities are discussed. Both metallic (Cu°) and cationic (Cu^{2+}, and also Cu^{+}) forms are concerned here, introduced either in ceramic (bone substitutes, prosthesis coatings), composite or metallic (prostheses) biomaterials.

A bibliographic search via Web of Science ®, using the search string "(copper OR Cu) AND biomaterial*" dates the first paper back in 1965 but shows a real takeoff in the 2000s. Since then, an exponential increase in the literature devoted to the topic can be observed (Figure 1). If we focus more on the biological aspects covered by this review using the search string "(copper OR Cu) AND biomaterial* AND (antibacter* OR osteogen* OR angiogen*), the first search result dates from 1998 and a similar increase is observed, with more than 400 papers published in the last 3 years (Figure 2). This review – involving more than a hundred studies – complements the previous review from Jin et al. on a closely-related topic [27]. This update was motivated by the numerous recently-published related studies based on the biological interest of copper in orthopedic biomaterials; nearly a quarter of the studies described in this work date from the past two years and almost one half are papers published after 2016. These include the effects of Cu ions alone and mainly of Cu-doped biomaterials in antibacterial,

angiogenic and osteogenic properties. First, we will detail the different types of biomaterials that can insert copper atoms. On this basis, the variations in their biological properties, when associated with Cu, will be described. This review also provides a critical analysis of the experimental conditions used to evaluate these biological effects.

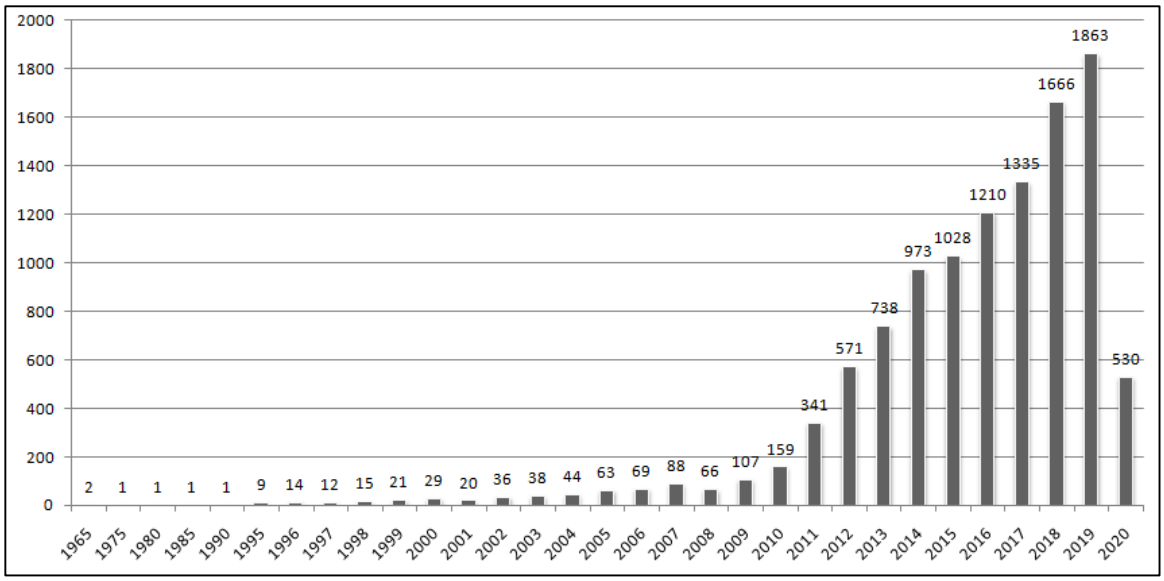

Figure 1. Number of articles on copper-doped biomaterials as a function of published year (for the search "(copper OR Cu) AND biomaterial*" from Web of Science ®).

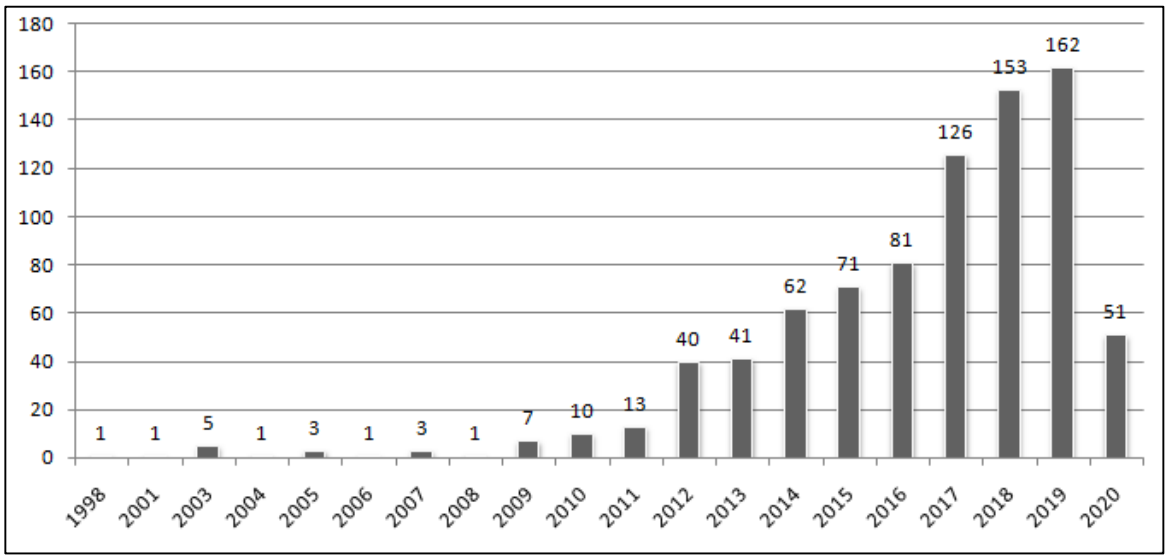

Figure 2. Number of articles on the biological aspects of copper-doped biomaterials as a function of published year (for the search "(copper OR Cu) AND biomaterial* AND (antibacter* OR osteogen* OR angiogen*)" from Web of Science ®).

Despite the promising characteristics for orthopedic applications engendered by doping with copper, the results in the literature show that researchers are still far from commercial success. This is not simply due to the regulatory burden linked to the launch of such metal-doped materials on the health market, but also to the difficulty of proving experimentally the intrinsic effect of the doping element itself. The challenge today in finalizing such a project is to combine in the same study the mastery of all the involved characteristics, namely the material (chemical composition, phase composition, forming and handling), biomechanical aspects (brittleness, mechanical resistance and oxidation resistance) and biological aspects (cytotoxicity, bactericidal, osteogenic and angiogenic properties). The difficulty, or even the impossibility, of extracting all this information from the literature comes from the fact that the materials studied and the experimental protocols used are never, or only rarely, the same.

2. State of the art on the biological interest of copper

2.1. Copper involved in biological processes

Cu is an essential trace element which must be provided by external food sources and water [28]. This metal is required by all living organisms because it is involved in numerous physiological functions like respiration, production of energy, formation of tissues, angiogenesis, neuromodulation and several metabolic processes, where it acts as enzyme cofactors [22]. It is also well established that Cu is involved in the maturation and growth of numerous tissue collagens, and especially in bone collagen [29]. Cu appears essential for bone mineralization and osteoblast functions [23].

According to the U.S. Institute of Medicine (IOM), the suitable dosage of Cu for adults is 0.9 mg/day, and the European Food Safety Authority (EFSA) recommends 1.6 mg/day for men and 1.3 mg/day for women [30,31]. For adults, the tolerable upper intake level is 10 mg/day; above this value, damage can appear [30]. Cu is the third most prevalent mineral present in the body. 100 mg of Cu is present in a 70 kg healthy body, with ⅔ located in the skeleton and muscles and the highest concentrations are found in the liver, brain, kidneys and heart [32].

Cu deficiency leads to several disorders such as anemia, leucopenia, myeloneuropathy and Menke's disease, which causes neuronal degeneration, connective tissue and hair abnormalities and bone fragility [33,34]. Menke's disease is due to mutations of the Cu-ATPase ATP7A that normally facilitates Cu export from enterocytes to the blood, resulting in an intestinal accumulation of Cu [35]. On the contrary, an excess of Cu concentration in the body is toxic and results in Wilson's disease, which is a genetic disease linked to an accumulation of Cu in

the body and causing damage to the liver and nervous system [36]. Another clinical manifestation is the formation of the Kayser Fleischer ring, corresponding to a hallmark brown discoloration of the cornea. This ophthalmologic manifestation appears in nearly 100% of patients with neurological Wilson's disease and about 50% of those with hepatic symptoms [37]. Alzheimer's disease, diabetes and cancers are diseases that can be impacted by excess amounts of Cu due to a failure in Cu regulation and homeostasis mechanisms [32].

2.2. The use of copper as an antimicrobial in water, air and hospital environments

Cu has been used for its anti-microbial properties for centuries; the Egyptians used it for the preservation of water [38]. Nowadays it is still widely used for drinking water distribution, because it resists corrosion and limits bacterial growth, more particularly that of enteric bacteria [39,40]. Other studies have shown that Cu is also effective in aquatic environments and ventilation systems. Swain et al. in 2014, synthesized CuO-NPs (copper oxide nanopowders) and demonstrated their antibacterial properties against 8 bacterial strains and 4 fungal organisms frequently responsible for contamination in aquaculture [41]. In 2012, Schmidt et al. compared the formation of biofilms after 30 weeks on Cu and Al heat exchangers. The results showed that bacterial concentrations in biofilms on Cu were at a much lower level than on the aluminum heat exchangers [42].

Using Cu is especially interesting in hospital environments where there is a significant risk of nosocomial infections. Indeed, the World Health Organization (WHO) has announced that at any time, 1.4 million people in the world are concerned by nosocomial infections [43]. Considering furthermore the increase in antibiotic resistance, several studies have focused on using Cu on surfaces/items present in hospitals. Prado et al. showed that after 48h, MRSA, *Klebsiella pneumoniae* and *Acinetobacter baumannii* did not adhere to Cu samples, contrary to stainless steel. However, *Pseudomonas aeruginosa* was still able to adhere [44]. In another study, the nosocomial infection rate was studied for one year in an intensive care unit. Some patients were placed in rooms containing Cu alloy surfaces and others in traditional rooms. Then the proportion of patients who developed a nosocomial infection with MRSA or VRE (Vancomycin-Resistant *Enterococcus*) was compared between the two types of rooms. In rooms containing Cu, 7.1 % of the patients developed a nosocomial infection, compared with 12.3% in standard rooms, demonstrating a significant reduction in the number of nosocomial infections thanks to Cu alloy surfaces [45].

It therefore appears that copper is potentially of interest for its antibacterial properties, either in its metallic or cationic form. The following section details the main results of relevant studies carried out during the past decade. The angiogenic and osteogenic properties are then presented. Before studying the three biological functions mentioned above, it is pertinent to approach the subject of cytotoxicity. Indeed, these copper-doped biomaterials should cause no toxicity when implanted; therefore, cytotoxicity is the first aspect to study before evaluating other properties. As with all biological products whose purpose is to be used in the body, the effect is dose-dependent and may vary depending on the living cells/tissues/organisms. In the case of bone biomaterials, the cytotoxic effects can be evaluated using bone cells like Bone Marrow Stem Cells (BMSC), pre-osteoblasts, osteoblasts or osteoclasts. Cells involved in the wound-healing process, like fibroblasts and endothelial cells, may also be considered. In 2016, Wang et al. described the dose-dependent cytotoxicity of Cu^{2+} using fibroblasts with a critical level of 10 mg/mL [46]. In 2019, Li et al. found dose-response relationships between Cu and 3 cell types. The half-maximum inhibitory concentration IC_{50} was 327.9 µM for human endothelial cells, 134.6µM for mouse osteoblastic cells and 0.7µM for rat BMSC [47]. This review focused on the main results concerning the intrinsic biological response provided by copper doping. Cytotoxicity is an experimental step that needs to be evaluated first but is not an intrinsic property of the doping; this feature is therefore not developed further here.

3. Copper as an antibacterial agent in biomaterials and the mechanisms involved

In 2008 Cu was officially recognized as a metallic antimicrobial agent by the Environmental Protection Agency (EPA) [48]. The medical field is highly concerned by infections; in consequence, Cu appears to offer promising perspectives and represents an important research topic. This is even more challenging in the orthopedic field with the development of bacterial resistance to antibiotics.

Studies on different Cu-doped orthopedic biomaterials and the antibacterial experiments performed are listed in **Table 1**. Cu is added to the composition of different types of materials such as calcium phosphate bioceramics, bioactive glasses, biomedical cements, biocomposites and coated/alloyed metals (titanium-based, stainless steel and Co-based alloys). Clinical applications of the different types of biomaterial can be found in the proceeding of Deb and coworkers [49].

3.1. Doped-bioceramics

3.1.1. Calcium phosphate bioceramics

Calcium phosphates are the most widely-used bioceramics due to their chemical and structural similarities with the mineral part of bones [50]. Among them, hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP, $Ca_3(PO_4)_2$) are often studied, alone or mixed together to form Biphasic Calcium Phosphates (BCP) [51–54]. BCP are interesting materials because of the difference in solubility of the two compounds, which enables the regulation of bioresorbability and the kinetic release of the ionic components [55].

Among bioceramics, in 2010 Stanic et al. used Cu-doped hydroxyapatite nanopowders against *Escherichi coli*, *S. aureus* and *Candida albicans* with approximately 95% of microorganism reduction (R%) for the 3 strains. In this study, undoped HAp also showed some bacterial reduction: approximately 60% reduction for *E. coli* and 70% for *S. aureus* and *C. albicans* [56]. Also in 2010, Li et al. evaluated Cu-doped HAp powders against *E. coli* and for all samples the survival rate was less than 1% after 24h. Pure HAp also has an antimicrobial effect with, 26.9% of bacteria surviving [57]. In 2014, Radovanovic et al., used Cu-doped BCP powders (HAp/α-TCP) and obtained a significant R% after 24h of incubation for the 4 tested microorganisms: *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* compared to pure HAp [58]. Shanmugan and Gopal tested fluorapatite (FAp) and HAp doped with Cu against *S. aureus*, *E. coli* and *C. albicans.* For doped HAp, antimicrobial activity was higher against *C. albicans* and *S. aureus* than for *E. coli*. For the doped FAp the antibacterial activity was better against *S. aureus* and *E. coli* than the antifungal activity against *C. albicans*. In this study, pure HAp and FAp also showed some antimicrobial activity [59]. In 2019 Bhattacharjee et al. synthesized Cu-doped hydroxyapatite. All samples tested were effective against *E. coli* and *S. aureus,* with a significant decrease in bacterial viability [60]. It is also important to mention the existence of contradictory data in the literature. In 2017, Marques et al., evaluated Cu-doped BCP (HAp/β-TCP) compacted pellets against *E. coli* and *S. aureus,* and no antimicrobial activity was demonstrated [61]. In this study several cations, including copper, showed no cytotoxicity issues, but only silver exhibited antimicrobial activity against Gram-positive *S. aureus*. Although the biological part of this study was not very detailed, it highlights the importance of the protocol used for the interpretation of bactericidal results, including the ceramic synthesis method, the final shaping of the materials (pellets vs. powders) and the reference used.

3.1.2. Bioactive glasses

Bioactive glasses are amorphous silica-based materials, initially developed by L.L. Hench and coworkers [62], and are generally composed of SiO_2, Na_2O, CaO and P_2O_5. These biomaterials are known to exhibit high bioactivity; these glasses react with body fluids and form HAp at the interface between the material and the bone, which promotes new bone formation [63].

In the field of bioactive glasses, Wu et al. in 2013 worked with Cu-loaded mesoporous-bioactive glass with molar ratio Cu/Ca/P/Si = 5/10/5/80 and evaluated their materials against *E. coli* over 1, 3 and 7 days. Already from day 1 there was a significant decrease in the number of survival bacteria with the doped bioactive glass. Even with the undoped material, from day 3 there was a significant reduction in bacteria survival [64]. In this study, Cu enabled a faster antibacterial effect (day 1 compared to day 3) and, as mentioned previously, it is important to have a short-term effect to prevent bacterial adhesion to the surface of biomaterials, leading to nosocomial infections [10]. The same year, Palza et al. synthesized bioactive glass with Cu ions. The copper content of their samples (in weight percent) was 3.7 wt% and 8.4 wt%. The authors demonstrated antimicrobial properties and found that the bactericidal effect was lower toward ampicillin-resistant *E. coli* DH5α than *Streptococcus mutans*. In this study, they also demonstrated that antimicrobial activity is enhanced when bioactive glass samples are immersed for 24h before the addition of bacteria, in other words when there are more metal ions in the culture media [65]. In 2005 Abou Neel et al. developed phosphate-based glass fibers containing Cu. The tested strain was *Staphylococcus epidermidis* and the results indicated a significant reduction in the number of CFU (Colony Forming Units) with the doped material compared to the control [66]. In 2017, bioactive glass-ceramics containing Cu were evaluated against *S. aureus* and *P. aeruginosa*. The authors found that the inhibitory and bactericidal effects were more significant on *P. aeruginosa* than on *S. aureus*. Furthermore, for *P. aeruginosa* the doped materials showed greater efficiency compared to gentamicin (an antibiotic used in the medical field to treat severe infections) [67]. In another study, Pouroutzidou et al. synthesized Cu-doped bioactive glass nanopowders and tested their materials against 7 bacterial strains: *Listeria monocytogenes*, *Bacillus cereus*, *S. aureus*, *E. coli*, *Salmonella* Typhimurium, *Salmonella* Enteriditis and *P. aeruginosa*. The authors found a significant reduction in the bacterial growth rate for 3 strains: *B. cereus*, *S. aureus* and *P. aeruginosa* [68]. Gupta et al. tested Cu-doped bioactive glasses against *E. coli* and *S. aureus*. A growth inhibition zone (about 2 cm) was observed for both bacterial strains, and this inhibition zone was larger than the inhibition zone observed with the other dopants studied (silver and iron) and similar to the effect obtained with the positive control carried out with the antibiotic gentamicin [69]. Bari et al. studied firstly the antibacterial activity of Cu-containing

mesoporous bioactive glass nanoparticles and secondly the effect of their ionic dissolution extracts. For all tested bacteria, growth inhibition was observed with all tested samples and the study demonstrated a positive correlation between the extract concentrations and bacterial growth inhibition [70]. Still in the same year, Foroutan et al. worked with Cu-doped calcium phosphate glasses. All tested samples showed antibacterial activity against *S. aureus,* and this effect intensifies when the Cu content increases [71].

These five studies published in 2017 [68-71] showed the importance of the ionic dissolution products of bioactive glasses, including dopant dissolution (concentration and kinetic) in the context of bone tissue engineering, which was already reviewed about ten years ago by Hoppe et al. [72] but which remains rarely studied in the literature (**Table 1**).

3.2. Bone cements

Biomedical cements consist of self-hardening mixtures of a solid and a liquid phase. In 2017 Rau et al. synthesized calcium phosphate cement composed of Tricalcium Phosphate doped with Cu (Cu-TCP). The self-hardening process involves the addition of monocalcium phosphate monohydrate ($Ca(H_2PO_4)_2{\cdot}H_2O$) with a solution of citric acid and leads to the precipitation of a brushite phase (Cu-doped $CaHPO_4{\cdot}2H_2O$). The hardened cement showed antibacterial activity exclusively against the Gram-negative strains (*E. coli*, *P. aeruginosa* and *S.* Enteriditis), whereas the growth of the Gram-positive *S. aureus* was not impaired [73]. The antibacterial activity of the copper-containing cement was significantly higher than that of its Cu-doped TCP precursor powder. Recently, Zhang et al. used a Cu-substituted Ca-silicate self-curing cement (composed of Ca_2SiO_4) against 4 strains: *P. aeruginosa*, *S. aureus*, *E. faecalis* and *E. coli*. The authors found that pure cement showed a significant decrease in viable bacteria compared to the control within a few hours. Adding Cu enabled them to prolong the antibacterial effect for several more hours [74].

3.3. Biocomposites

Biocomposite materials consist of an assembly of at least 2 components: an inorganic filler dispersed in a polymer matrix. Boccaccini and coworkers' review of polymer/bioglass nanocomposites exposed the different preparation methods for biocompatible composites [75]. In 2016 Bejarano et al. worked with a biodegradable poly (D, L-lactide) (PDLLA) mixed with a sol-gel bioactive glass doped with 1 mol % of CuO; the final composition of the material was PLA/10-CuBG (10wt % of Cu-doped glass particles). The authors tested their composite against a MRSA. After 1 day and 3 days, antibacterial activity was around 40%, and after 7

days reached 98% compared to the PDLLA scaffold. In this study, samples without Cu, PLA/10-BG also exhibited an antibacterial effect. In fact the bacterial viability rate was 80% after 3 days, and 40% after 7 days [76]. The same year Wang et al., used a biocomposite aerogel composed of Cu-containing mesoporous bioactive glasses and nanofibrillated cellulose (potentially used as wound dressing materials). Their results indicated that the composite aerogels containing Cu significantly inhibit the growth of Gram-negative *E. coli*. Samples composed of composite aerogel without Cu and nanofibrillated cellulose alone did not show any antibacterial effect [46]. In 2010 Sahithi et al. developed a composite based on nano-hydroxyapatite powders soaked with Cu and combined with polyethylene glycol (PEG 400). The combination of these 2 compounds exhibited significant antimicrobial activity against *S. aureus* and *E. coli*, and a greater effect on Gram-positive *S. aureus* [77]. In 2014 in the study of Li et al., a water-soluble Cu°/polyacrylic acid (Cu/PAA) composite was used against 4 bacterial species: *S. aureus*, *B. subtilis*, *E. coli* and *P. aeruginosa*. Results indicated that Cu°/PAA could inhibit bacterial growth of the 4 strains and showed bactericidal activity against three of the tested strains, except for *B. subtilis* [78]. This composite illustrates the fact that copper presents effective bactericidal activity in both its cationic and metallic forms. Finally, in 2020, Narayanan et al. synthesized a tri-component composite containing Chitosan/Polyvinyl pyrrolidone with Cu-Hap, and evaluated the antibacterial properties of these composites against 3 bacterial strains (*S. aureus*, *B. subtilis* and *E. coli*) and 3 fungi (*C. albicans*, *Penicillium notatum* and *Rhizopus stolonifer*). The results indicated that the addition of Cu-HAp increases growth inhibition for all the microorganisms tested [79].

3.4. Coating/alloying of metallic biomaterials

Metallic biomaterials are widely used in orthopedics thanks to their mechanical properties and their corrosion and wear resistance. They are usually composed of stainless steel, or titanium, cobalt and tantalum alloys. However, these materials have no biological interactions with tissues. Coating/alloying is an interesting approach with a view to establishing and enhancing biological properties at the implant-tissue interface [80].

3.4.1. Coatings on Titanium alloys

The interest of choosing titanium alloys for orthopedic implants was detailed in the review of Geehta and coworkers [81]. Concerning coatings on titanium, in 2015 Huang et al. coated titanium with Cu-doped HAp and obtained an antimicrobial ratio >75% against *E. coli* [82]. Kalaivani et al. worked with a Cu-doped $CaSiO_3$ coating and evaluated its antibacterial

properties on *E. coli* and *S. aureus*. Results indicated that pure powder did not exhibit antibacterial properties, whereas there was a gradual increase of the antibacterial activity with the amount of Cu present in the doped coatings [83]. Recently, in 2019, Huang et al. coated titanium with a Cu-containing ceramic. The authors did not observe antibacterial activity against *S. aureus* but demonstrated that their Cu-doped material promoted the bactericidal effect of macrophages [84]. The same year, Ghosh et al. coated Cu-doped hydroxyapatite onto titanium with varying Cu contents and tested these samples against *E. coli* and *S. aureus*. Their results indicated that the number of viable bacteria decreased as the Cu content in the coatings increased, whereas hydroxyapatite without Cu gave results similar to those observed with the control. The antibacterial rate was 78% after 8h of culture for *E. coli*, and 83% for *S. aureus*. In parallel with this experiment, the Cu ions release during antibacterial tests were analyzed by ICP-MS. After 8h, for the control (hydroxyapatite without Cu) the concentration of Cu ions was 0.5 ppm, compared to 31 ppm for the sample with the lowest Cu content and 68 ppm for the sample having the highest Cu content [85]. It is important to mention that this is the only study referenced in which the quantities of copper released were measured during biological tests (**Table 1**). Burghardt et al. prepared titanium plates coated with galvanically deposited copper. The results indicated that after 6h no viable *S. aureus* bacteria was found in a medium containing Cu ions released by the coated material. After 24h of growth on the surface of the materials, a complete removal of adherent bacteria was observed with Cu-coated titanium plates [86]. In 2019, Wolf-Brandstetter et al. coated titanium implants with Cu-doped calcium phosphate. After 2h of culture with *E. coli*, coatings containing the highest amount of Cu showed a significantly lower number of viable bacteria, and this effect was prolonged to 12h. Results also indicated a reduction in adherent bacteria after 12h on the surfaces of implants [87].

3.4.2. Coating on stainless steel

In 2020 Akhtar et al. coated stainless steel 316L with Cu-chitosan complexes and evaluated their antibacterial effect against *E. coli* and *S. aureus*. Results indicated a strong effect against both strains after 3h with Cu-chitosan complexes, with no bacterial growth, and this effect was still observed after 24h. Coating with chitosan alone did not evince antibacterial activity [88].

3.4.3. Copper-bearing metals

Cu can also be added to the composition of stainless steel, titanium and cobalt alloys; i.e. Cu-alloying. In 2012 Ren et al. used 317L-Cu stainless steel against *E. coli* and *S. aureus*, their

material shows some antibacterial effect after 2h: 20% growth inhibition for *S. aureus* and 40% for *E. coli* and after 12h this rate increased to 80% and 90%, respectively. Finally, after 24h the material killed 99% of the bacteria. In this study, the bactericidal effect observed with *S. aureus* was lower than that observed with *E. coli* in the first hours of the test, but finally after 24h the antibacterial activity was the same for the 2 strains [89]. Two years later, the same authors tested a Cu-bearing titanium alloy on the same 2 strains. After 24h of co-culturing, they observed a significant reduction in the number of viable bacteria, and this effect was accentuated when the Cu content was increased [90]. In 2011 Chai et al. used austenitic stainless steel 317L containing Cu. Quantitative bacterial analyses continuously showed (6, 12, 24 and 48h) that the Cu-doped material inhibited bacterial growth compared to undoped 317L for both *S. aureus* and *E. coli*. After 48h of incubation, almost no viable bacteria were found [91]. 304 Cu-bearing stainless steel was tested against the anaerobe *Porphyromonas gingivalis* in 2013 by Zhang et al. The authors observed the morphologies of the bacteria at the surface of doped and undoped stainless steel. Irregular shapes and sizes were found after 2h, 4h and 6h for the doped material. After 8h the bacterial cells appeared fragmented. The number of viable bacteria slowly decreased after 2h, almost no viable bacteria were found after 8h and an antibacterial rate of 100% was achieved after 10h [92]. The same year, Zhang et al. evaluated an antibacterial titanium-Cu alloy with *E. coli* and *S. aureus* using agar diffusion assay and plate-count method. With agar diffusion assay, no inhibition zone was observed with the Cu-doped material compared to the positive control sample, consisting of antibiotic tablets with erythromycin, penicillin, kanamycin and gentamicin. Then authors evaluated the colonization on the material surfaces by the 2 bacterial strains with plate-count method and after polishing the samples to investigate the corrosion properties. Results indicated that no viable bacteria were found even after polishing, attesting a strong antibacterial effect on the surface and also inside the material [93]. This study shows that the bactericidal activity can come either from direct contact with the biomaterial or from the release into the biological medium of the doping element. In 2015 Ma et al. synthesized Cu-bearing titanium alloy and studied the adhesive capacity of *S. aureus* on this material. All samples showed significant bacterial adhesion reduction (between 62.5% and 98.6%) compared to the control. Moreover, there were only a few randomly-distributed bacteria on the Cu-doped alloy and no formation of a complete biofilm like that observed on the control [94]. In 2012, Nan et al. compared the antibacterial rate of 3 stainless steels: a commercial type 200 steel (Cu wt% = 1.45), a custom-designed one (Cu wt%= 2.77) and a control without Cu. For *E. coli*, antibacterial rates were 0%, 76.70% and 99.99% for the control, the commercial type and the custom type, respectively. For *S. aureus,* the results were 0%,

99.99% and 99.99%. These results confirm the antibacterial efficiency of Cu [95]. Cu-bearing austenitic antibacterial stainless steel was already used against *E. coli* by the same authors in 2008. After 9h of culture on the surface of the doped material the outer membrane collapsed, and after 24h all the bacteria were thin and shriveled. In this study the authors measured the amount of Cu released in the supernatant during bacterial tests, and results showed approximately 0.35 ppm at 1h, 0.55 ppm at 3h, 1.1 ppm at 5h and 1.4 ppm at 7h. They concluded that more cell damage is caused when the quantity of Cu released is increased [96].

Co-Cr-Mo alloys have been also widely used in total hip or knee replacements due to their corrosion resistance and mechanical properties. In 2016 Zhang et al. used Co-Cr-Mo and Co-Cr-Mo-Cu alloys against *S. aureus* over a period of 24h. The alloy without Cu did not demonstrate any antibacterial activity, whereas Cu-added alloys exhibited antibacterial rates >94% [97].

3.5. Assessment of the effect of copper introduced into biomaterials

3.5.1. Comparison of bacteriological results

All these studies clearly demonstrated that the Cu used in these biomaterials has antibacterial activity. However, it is essential to take into account many parameters that can influence the results. Among these factors: the type of bacteria (Gram positive or negative), the type of tests used to evaluate the antibacterial effect, the experimental conditions and the variation in Cu content between studies. Another key factor to consider is the release rate of Cu ions (kinetic and amount) in the biological media.

Bacterial strains, experimental tests and results are presented in **Table 2** (bacterial activity is simply noted as positive (+) or ineffective (-) because it was not possible to classify quantitatively the bactericidal action between studies due to different protocols used). Regarding the strains used in the experiments, results differ according to the Gram of the bacteria. Gram-positive bacteria have negatively charged cell surface and a very thick peptidoglycan membrane (20-80 nm). Cu ions are positively charged, and thus attracted to the negatively-charged surface, resulting in cell damage and the apoptosis of Gram-positive. By contrast, Gram-negative bacteria have a very thin layer of peptidoglycan (6-15 nm) and an outer membrane which can represent a barrier and prevent the diffusion of Cu [70]. It was clearly demonstrated that Cu has an effect on bacterial membranes in the studies of Li et al. and Nan et al., in which morphological modifications of bacterial cells were observed [78,96].

Another key point regarding bacterial strains is their origin. American Type Culture Collection (ATCC) strains were used in all the studies previously cited; enabling comparison of the results

between studies [58]. It is apparent that the use of clinical strains recently isolated from patients with bone infections would provide a better overview of situations encountered in current medical practice.

A significant factor which needs to be considered for the interpretation of the results is the nature of the test used and all its parameters. Among the different experiments used to evaluate antibacterial properties, quantitative determination of the colony forming units (*i.e*. viable bacteria) by plating on agar plates is the method most often applied in the studies. An agar diffusion test and the measurement of optical density are also implemented. Some other methods are also cited, such as MTT assay and the use of resazurin. Thus, it is difficult to correctly compare and interpret results from different experiments. Experiment settings also vary. This is particularly the case for the length of incubation (ranging from a few hours to a few days), for the definition of the reference (as well as positive and negative controls) and for the quantity of materials tested (concentrations vary from µg/mL to g/mL). In some studies, the undoped material has an antibacterial property but the reasons for this effect are not investigated.

Finally, another aspect which is important to underline concerns the measurement of the amount of Cu released during experiments, and therefore the concentration of Cu involved in the antibacterial process. The Cu doping rates of materials are different depending on the studies; thus, it appears essential to know the amount of Cu responsible for the effects observed during bacterial tests. However, among the 37 studies cited in Table 1, only 2 measured the Cu released during antibacterial assays. Nan et al. used Cu-bearing austenitic antibacterial stainless steel with 3.8 wt% of Cu. They measured, using ICP-MS, the concentration of Cu released; results indicate approximately 0.35 ppm at 1h, 0.55 ppm at 3h, 1.1ppm at 5h and 1.4 ppm at 7h [96]. Ghosh et al. coated titanium with Cu-doped hydroxyapatite with a Cu content (atom %) of HA=0, B1= 2.4 et B4 = 6.6. They also measured Cu by ICP-MS after 8h with the same conditions used for the bacterial tests. The results show 0.5 ppm, 31 ppm and 68 ppm for HA, B1 and B4, respectively [85]. It is surprising to find Cu in the undoped sample (HA, perhaps corresponding to the detection limit) and in this study Cu concentrations are much higher than those cited previously (ceramic versus metal doping). These values confirm that it is necessary to measure Cu concentrations, since they can vary considerably between materials and therefore strongly influence the antibacterial results observed. As reported in Table 1, 22 studies did measure the Cu released but not in the exact same conditions as those used for antimicrobial tests (i.e. same culture medium, same concentration of material in the culture medium), so it is impossible to know the amount of Cu responsible for the results observed; and 13 did not

measure Cu concentrations at all, notwithstanding the fact that a significant copper release rate could cause cytotoxicity problems.

To conclude, it appears very important to consider all the parameters mentioned above to correctly interpret and compare results obtained from different studies. It is, however, almost impossible to achieve this via the literature with the aim of deducing simple guidelines concerning the chemical compositions to be favored.

3.5.2. Mechanisms involved in the antibacterial activity of copper

Concerning the modes of action of Cu, several mechanisms have been described in the literature. The most important process is based on the redox properties of Cu; in fact, it can produce reactive oxygen species (ROS) in a Fenton-type reaction and ROS cause damage to lipids, proteins, membrane and DNA. Significant oxidative stress is caused by an increase in the intrinsic amount of Cu and leads to redox cycling between the different forms of Cu: Cu°, Cu^{+} and Cu^{2+} [98,99]. The DNA double helix contains binding sites for copper, which leads, after Cu^{2+} bonding, to disorders in helical structures and the denaturation of DNA [100]. Damage to the membrane is related to the fact that Cu^{2+} can interact with SH-groups and lead to their inactivation [100]. The interaction of copper with proteins was clearly demonstrated by Nandakumar et al. by quantitative proteomic profiling on *E. coli* after exposure to metallic copper surfaces. Results indicated that of 509 proteins identified, 209 were differently expressed after contact with copper. Proteins involved in efflux pumps were up-regulated and proteins related to biogenesis functions were down-regulated [101]. The bacterial killing process can be separated into 4 steps (Figure 3). First, the Cu is released from the Cu-doped surface/material. Secondly, cell damage begins with membrane ruptures leading to a loss of cytoplasmic content. Next, the production of ROS causes further cell damage by interacting with proteins and lipids, and finally DNA is fragmented, leading to cell death [38].

Some mechanisms can protect bacteria from the toxic effects of Cu ions: (i) active export of Cu from the cell with Cu-transporting ATPase pumps, (ii) sequestration of Cu by Cu-binding metal chaperones or metallothionein, (iii) relative impermeability of bacterial membranes and (iv) transformation of Cu^{+} into the less toxic Cu^{2+} form by multi-Cu oxidases [24,100]. These mechanisms only enable tolerance to Cu ions, delaying the deadly effects, and they are not considered as real resistance processes [102].

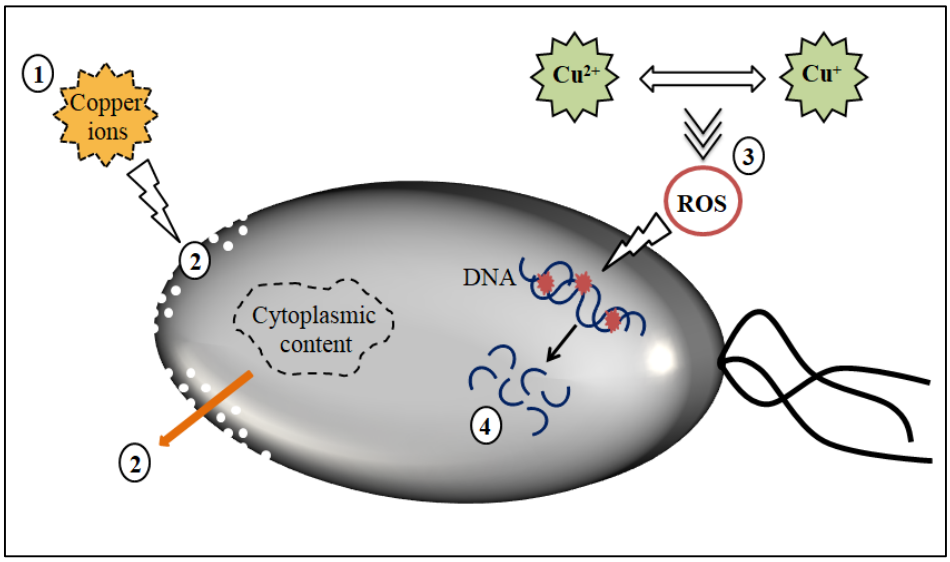

Figure 3. The killing process involved in the antibacterial properties of copper. Copper ions are released from the doped biomaterial (1) and cause membrane damage leading to a loss of cytoplasmic content (2). Then the production of ROS (3) causes DNA fragmentation (4) and cell death.

4. Angiogenic properties of Copper-doped biomaterials

4.1. Effects of copper on angiogenesis

After surgery or injury, the wound healing process takes place in order to repair damaged tissues [103]. In this mechanism, five phases occur: coagulation and hemostasis, inflammation, detersion and proliferation, which encompasses the major healing process and remodeling. It is a complex process in which various cell types and chemical mediators need to be activated and coordinated to enhance healing quality. Angiogenesis is the process of formation and growth of new blood vessels from pre-existing vessels. This physiological mechanism is critical during the complete wound-healing process and plays a vital role during the proliferative phase [104]. Recently it was shown that Cu ions bind to and interact with several growth factors involved in blood vessel formation [105]. Vascular Endothelial Growth Factor (VEGF) is a crucial mediator in the angiogenic process during the proliferative phase. Sen et al. showed that Cu sulfate significantly increased VEGF expression. It accelerated and improved the closure of excisional murine dermal wounds [106]. Angiogenin (ANG) is a ribonuclease and a strong stimulator of angiogenesis that interacts with endothelial cells [107]. Cu can modulate angiogenin transcription and affect the localization of ANG to enhance its function; moreover, it can negatively affect endothelial cell migration [108]. Matrix metalloproteinases (MMPs) are involved in the wound-healing process, in particular by regulating the activity of growth factors

such as VEGF and promoting cell proliferation in angiogenesis. It has been found that Cu in low concentrations stimulates the activity of MMPs, and high concentrations increase the expression of MMPs in fibroblasts [109].

In 2012, Pickart et al. showed that tripeptide glycyl-L-histidyl-L-lysine (GHK) has a strong affinity for Cu. The authors demonstrated that the complex of GHK with Cu ions (GHK-Cu) increases the expression of proteins such as MMPs, VEGF, collagen, elastin, fibroblast growth factor and nerve growth factor. In addition, this complex has an anti-inflammatory action with the suppression of free radicals in order to prevent oxidative stress [110]. Recently, the angiogenic effects of Cu nanoparticles were investigated with human fibroblast, endothelial and keratinocyte cells. The authors observed that Cu nanoparticles can increase cell migration in the 3 cell types, stimulate the proliferation of endothelial and fibroblast cells, enhance collagen deposition and promote skin wound healing *in vivo,* and do not accumulate in the liver [111]. Cu has beneficial effects on healing mechanisms and particularly on angiogenesis. When a biomaterial is implanted, the wound-healing process takes place; it is therefore rational to investigate if these biological properties are enhanced with Cu-doped biomaterials.

4.2. Effects of copper-doped biomaterials on angiogenesis

Studies on Cu-doped biomaterials and their angiogenic properties are listed in **Table 3.** Barralet et al. worked with a degradable osteoconductive copper-adsorbed macroporous scaffold. Results indicate that low doses of Cu enable the formation of micro-vessels and improve the wound-healing process in mice [112]. Wu et al. synthesized Cu-containing mesoporous bioactive glass and found a significant increase in VEGF secreted by human Bone Marrow Stromal Cells (hBMSC) and in alkaline phosphatase (ALP) activity – with copper concentrations between 14.2 and 152.7 ppm – indicating an improvement of the angiogenesis capacity compared to undoped materials [64]. Kong et al. worked with a Cu-doped calcium silicate bioceramic. The authors found that the Cu ions extracted from their material exhibited a significant angiogenic effect, with an increase in the vascularization of Human Umbilical Vein Endothelial Cells (HUVEC) and Human Dermal Fibroblasts (HDF) in co-culture and VEGF expression – with an optimal Cu^{2+} concentration of 0.7 ppm only [113]. A borate-based bioactive glass scaffold doped with Cu was fabricated by Wang et al. in 2014. Their results indicated enhanced blood vessel formation and an increase in new bone formed; in other words better bone regeneration in rat calvarial defects when compared to undoped material [114]. In 2016 the same authors evaluated the angiogenesis potential of their Cu-doped borosilicate bioactive glass in a rat calvarial defect model after 8 weeks of implantation. Results showed a

much higher number of blood vessels with Cu-doped material than with undoped bioactive glass [115]. The same year, a biocomposite aerogel of mesoporous bioactive glass containing Cu and nanofibrillated cellulose was evaluated on HUVECs and 3T3 fibroblasts. The authors demonstrated a major increase in newly-formed vessels and a significantly higher gene expression of angiogenic genes in 3T3 fibroblasts [46]. In 2016, Bejarano et al. synthesized a composite material with PDLLA polymer mixed with Cu-doped bioactive glass. Results indicated that the doped composite promoted the angiogenesis marker VEGF expression of ST-2 mice bone marrow stromal cells [76]. Recently, Romero-Sanchez et al. evaluated the biological effects of ionic products of Cu-containing mesoporous bioactive glass on bovine aorta endothelial cells (BAEC) and *in vivo* with *zebrafish* embryos. The results showed higher endothelial cell migration and an increase of sub-intestinal venous plexus, indicating that those materials promote angiogenesis [116]. In 2016, Lin et al., synthesized Cu-doped silicate bioactive glass scaffolds. An *in vitro* study was performed with mouse pre-osteoblastic cells to evaluate the ability of the scaffolds to support the growth and differentiation of an osteogenic cell line. *In vivo* experiments were carried out in rat calvarial defects with an implantation time of 6 weeks. Samples showed good cell viability, proliferation and attachment to the surface, comparable to the control except for the sample with the highest Cu content (2 wt% CuO). *In vivo* results showed an increase in blood vessel area with increasing Cu content. A significant result was obtained with the scaffolds containing the highest Cu amount compared to undoped material [117]. Rath et al. fabricated scaffolds of bioactive Cu-doped glass and evaluated their angiogenic potential with BMSC and human dermal microvascular endothelial cells (HDMEC). Results indicated that scaffolds in combination with BMSC enhanced angiogenic potential with an increase in VEGF secretion and allow the formation of endothelial tubes with HDMEC [118]. In 2018, Elrayah et al. worked with Cu-substituted hydroxyapatite scaffolds with several micro- and nano-topographic structures. Their results showed that human Endothelial Cell (EC) viability is dependent on the surface morphology of the scaffold and the culture time. A flower-like shape was the most favorable structure for angiogenic proliferation in EC, and Cu-doped scaffolds enabled an increase in blood vessel formation after 8 weeks in a rabbit model [119]. Recently, Mou et al. fabricated a Cu-doped composite with a nano-calcium-deficient hydroxyapatite and a multi (amino acid) copolymer. Results showed that the sample doped with 1% Cu exhibited better cell adhesion with pseudopods with rat BMSC and superior proliferation compared to an undoped composite. *In vivo* studies indicated greater blood vessel formation and bone regeneration with 1% doped material [120]. Finally, Zhang et al. used calcium phosphate cement doped with Cu and evaluated its angiogenic properties with rat BMSC and

HUVEC in co-culture. The results showed good activity and proliferation of both cell types, and an increase in VEGF expression [121].

These studies clearly demonstrate that Cu ions added to biomaterial compositions have beneficial effects on healing mechanisms, and particularly on angiogenesis. Effective concentrations of copper for angiogenesis are heterogeneous; Wu *et al.* indicated efficient Cu^{2+} concentrations ranging from 14.2 to 152.7 ppm [64] and Kong *et al.* demonstrated beneficial results with 0.7 ppm [113]. However, as previously explained, it is essential to take into account several parameters that can influence the results: the type of endothelial cells used, the *in vivo* models, the type of tests used to evaluate the pro-angiogenic effect and the different Cu contents between the biomaterials.

Various endothelial cells were used to investigate angiogenic properties: HUVECS, BAEC, mouse pre-osteoblastic cells, HDMEC. The *in vivo* models used differ between studies: rat, mouse, *zebrafish*. To evaluate the angiogenic effects several parameters were investigated: blood vessel formation, endothelial cell proliferation, level of expression of angiogenic factors like VEGF and of genes characteristic of angiogenesis. As explained before, the wound-healing process and therefore angiogenesis is a complex process involving various cell types and chemical mediators that need to be activated and coordinated to be efficient. Wolf-Brandstetter et al. explained that it appears difficult to evaluate the pro-angiogenic effect of Cu ions using only *in vitro* endothelial cell monoculture models. The authors suggest more complex *in vitro* models like co-cultures with fibroblasts or human mesenchymal stem cells and *ex vivo* models like *zebrafish* embryos, or chick embryo chorioallantoic membranes, in order to show more clearly the impact of Cu ions on angiogenesis [87].

Finally, as previously described, the amount of Cu added to materials varied depending on the studies; thus, it is apparent that this parameter clearly modifies the concentration of Cu release and so the effect on *in vitro* and *in vivo* tests. Even more than the doping rate, it is the dopant dissolution (concentration and kinetics) which will have an impact on angiogenesis, as established in the review of Hoppe et al. [72].

5. Osteogenic properties of Copper-doped biomaterials

5.1. Effects of copper on osteogenesis

Osteogenesis is the process by which new bone tissue is formed, and ideally a bone substitute should allow this mechanism. Osteoblasts are cells derived from mesenchymal stem cells.

These are bone-forming cells and act by controlling mineralization by the deposition of hydroxyapatite crystals, producing collagen which is at the origin of the organic matrix [122]. The relation between angiogenesis and osteogenesis is known. Angiogenesis brings all the necessary elements (vascular growth, oxygen, nutrients, soluble factors and several types of cells) needed for osteoblasts to form new bone [123].

Generally synthetic biomaterials have poor osteogenic properties and in practice the addition of an osteogenic growth factor is often needed to achieve correct bone reconstruction. For example, Bone Morphogenetic Proteins (BMP) have been used successfully in bone repair, although some adverse biological effects were found *in vivo* [117]. The diamond concept described by Giannoudis et al. in 2007 clearly demonstrated the complex interactions needed for bone healing. These include 4 elements: osteogenic cells, osteoconductive scaffolds, growth factors and the mechanical environment [124]. It is known that Cu plays a role in the bone metabolism and severe Cu deficiency leads to bone abnormalities [125]. It has been shown that a Cu deficiency results in a decrease in bone strength in rats [126]. Additionally it has been found that Cu significantly increases the deposition of collagen fibers [25]. Cu has beneficial effects on the healing mechanism and can enhance bone formation. Cleary this is a crucial property when a biomaterial is implanted; it is therefore rational to investigate whether Cu-doped biomaterials can help with bone regeneration.

5.2. Effects of copper-doped biomaterials on osteogenesis

Studies on Cu-doped biomaterials and their osteogenic properties are listed in **Table 3**. In 2016, Lin et al., synthesized Cu-doped silicate bioactive glass scaffolds. An *in vitro* study was performed with mouse pre-osteoblastic cells to evaluate the ability of the scaffolds to support the growth and differentiation of an osteogenic cell line. Samples showed good cell viability, proliferation and attachment to the surface, comparable to the control, except for the sample with the highest Cu content (2 wt% CuO). However, the Cu-doped materials had no significant effect compared to undoped samples *in vitro*. The effects were not significant either on *in vivo* bone formation with rat calvarial defects 6 weeks after implantation [117]. Bi et al., investigated the effects of a bioactive borate glass microstructure and Cu doping on bone regeneration. Their results indicated that the trabecular microstructure showed greater new bone formation than oriented or fibrous microstructures after implantation in rat calvarial defects after 12 weeks. Cu doping (0.4 wt.%) increased osteogenesis only for the fibrous microstructure [127]. In 2014 Wang et al. synthesized borate bioactive glass with 3.0 wt.% CuO. With hBMSCs, the Cu-doped biomaterials increased the ALP activity of the cells and thus had the best capacity to

support their osteogenic differentiation. Furthermore, results showed an increase in bone regeneration and blood vessel formation in rat calvarial defects at 8 weeks post-implantation [114]. Ewald et al., worked with brushite scaffolds loaded with Cu ions and evaluated their effect on the growth and activity of osteoblastic cells seeded on the scaffolds. Results indicated that cell activity and proliferation were increased by Cu ions, and the expression of bone specific proteins was enhanced. The authors demonstrated that the activity of osteoblastic cells also increased on brushite alone, compared to polystyrene, indicating an effect of the material structure also [128]. In 2015 Huang et al. worked with a Cu-substituted hydroxyapatite coating on titanium. Their results showed good cell proliferation with osteoblast-like cells from mouse skulls compared to titanium alone; however there was no significant difference between pure and Cu-doped hydroxyapatite [82]. Ren et al. demonstrated that after 3 days of culture, the ALP activity of osteoblasts and the expression of osteogenic genes such as collagen type I (ColI), osteopontin (OPN) and runt-related transcription factor 2 (Runx2) significantly increased for 317L-Cu steel in comparison to undoped 317L steel [129]. In 2015, Ren et al. worked with Cu-bearing stainless steel and demonstrated *in vivo* that more new bone tissue was formed around the Cu-doped implant [130]. Recently, in 2020, Zhang et al. worked with Cu-substituted dicalcium silicate cement, and the results showed that the quantitative new bone formation was significantly higher with Cu cement than for undoped cement [74]. Burghardt et al. prepared a composite with a Cu deposit on titanium alloy. With 0.1 mM of Cu^{2+} there was a stimulation of proliferation of MSC, an increase in ALP activity, a higher expression of ColI, osteoprotegerin (OPG), OPN and mineralization of the cells [86]. In 2010 Yang et al. worked on inorganic additives to calcium phosphate films. Cu deposition showed an inhibitory effect on osteoblast proliferation and differentiation [131]. Wu et al. prepared Cu-containing mesoporous bioactive glass scaffolds and tested these materials and their extracts on hBMSC. The authors demonstrated a significant increase in the expression of bone-related gene ALP, OPN and osteocalcin (OCN) in the cells [64]. D'Mello et al. evaluated the effect of Cu incorporation into chitosan scaffolds *in vivo* in rat calvarial defects. The amount of bone tissue regenerated was twice as high with Cu compared to the scaffold alone, and eleven times higher compared to empty defects [132]. Huang et al. evaluated the effects of a titanium surface coated with Cu on the regulation of macrophages which could interact with the osteogenic properties of the biomaterial. Their results indicated that macrophages grown with Cu^{2+} or on a Cu-doped substitute surface induce a favorable inflammatory environment for osteoblast cell proliferation and differentiation. *In vivo* results with rat models showed an increase in the expression of osteogenic markers like OCN and Runx-2 with the doped material [84]. Mou et al. synthesized

a Cu-doped composite with a nano calcium-deficient hydroxyapatite and a multi (amino acid) copolymer. The authors found an increase in the ALP activity of rat BMSCs and a higher mineralization level, which demonstrated a stimulation of the osteoblastic differentiation of the cells [120]. Finally, Zhang et al. found an increase in the osteogenic gene expression of Col I, ALP, OPN and Runx2 from rat BMSC cultured with cu-containing calcium phosphate cement [121].

These studies demonstrated that Cu ions added to the composition of biomaterials can have beneficial effects on the osteogenesis process. However, in some studies, no beneficial effect was found compared to undoped materials and results indicated that the positive effect comes from the topographic structuring of the biomaterial. Surface morphometry is also a strategy studied for the antibacterial improvement of biomaterials [133]. Huang et al. explained that material structuring plays an important role in the adhesion step of cells on the surface of the biomaterial, which corresponds to the first step of bone formation, whereas chemical composition contributes to the proliferation and differentiation steps [82]. The relationship between macrophage and osteogenesis is developed in the study of Huang et al. Macrophages are first recruited in response to physiological signals and create an inflammatory microenvironment. Then bone repair cells react to this inflammatory site created by the macrophages and migrate to the material surface to start their osteogenic role. This indicates that the action of macrophages is needed to promote the proliferation and differentiation of osteoblastic cells [84]. Rodriguez et al. found that Cu reduced the proliferation rate of MSC and increased their differentiation rate, indicating that Cu regulates both processes in an opposing manner [125].

Concerning the role of Cu in osteogenesis, it appears difficult to conclude concerning a specific effect on a specific target. Beneficial effects have been demonstrated, but the precise mechanisms of action are not clearly understood. As osteogenesis is a very complex mechanism, related to angiogenesis and including various factors, cell types and biological processes, it is difficult to correctly investigate, understand and describe the effects of Cu-doped biomaterials on this physiological mechanism.

In addition to the chemical composition of biomaterials, such as copper doping covered in this review, surface properties are known to directly influence cell behavior and therefore new bone formation. Feng *et al.* demonstrated that the architecture of β-TCP scaffolds influence *in vivo* defect healing performance, with higher bone formation and vascular ingrowth [134]. A review concerning the impact of the porosity of scaffolds on osteogenesis indicated that high porosity favors bone ingrowth and osteogenesis by allowing vascularization and high oxygenation. The

authors also pointed out that the mechanical properties of the scaffolds can be affected, and are reduced with too high a porosity and too large a pore size [135]. A recent review of Xiao *et al.* reported the effects of surface structure parameters, including structure size, morphology and roughness, on cell behavior. The shape and size of the surface microstructure impact cell morphology, orientation, proliferation and adhesion force, depending on cell type. A combination of micro and nano-structures induce synergistic effects on the proliferation and osteogenic differentiation of bone marrow stem cells. Surface roughness is a key factor which needs to be adjusted. A rough surface can promote cell adhesion and differentiation; however, increasing roughness can lead to cell death. In this review the authors also reported that surface structure impacts osteogenesis by interacting with protein adsorption, cilia modulation and the immune response [136].

It appears clearly that the surface properties of materials strongly influence processes involved in osteogenesis and therefore the osteointegration of orthopedic biomaterials. In a more general context, the biological response depends on a complex interaction between patient-related aspects (age, gender, metabolism, …), material properties (architecture, biodegradation, composition, porosity, …) and induced biological responses (cell adhesion and proliferation, protein adhesion, …) [137].

6. Conclusion

This review focuses on the biological properties provided by Cu-doped biomaterials intended to be implanted in a bone site during orthopedic surgery. Three major properties were discussed: antibacterial, angiogenic and osteogenic, as they represent key steps for ideal bone repair. Concerning antibacterial effects, the vast majority of studies have demonstrated a positive role of copper. Cu ions can produce reactive oxygen species (ROS) and cause damage to lipids, proteins and DNA, leading to the death of the bacteria. Angiogenesis and osteogenesis are related wound-healing processes. Concerning angiogenesis, Cu ions have been found to bind and interact with several growth factors involved in blood vessel formation. For osteogenesis, the specific role of Cu ions is unclear, but some studies have found beneficial effects on osteoblastic cells.

Regarding Cu-doped biomaterials, antibacterial properties are very often found, sometimes with variations in efficacy between studies, depending on various factors such as the Gram of the bacteria, the experimental conditions used and the Cu content of the material. Cu-doped biomaterials exhibited clear angiogenic abilities with the formation of new blood vessels. Osteogenic properties are found in some papers, with new bone formation, but are not described

in all studies, which indicates the presence of complex mechanisms, including the undeniable role of the structure of the material, and supports the fact that more studies are necessary to understand clearly how Cu ions interact with the physiological processes of osteogenesis.

The lack of homogeneity concerning chemical and biological characterizations prevents the specification of an ideal model for orthopedic biomaterial. A harmonization between studies would strengthen the bio-adaptation of materials to biological requirements.

Although it appears difficult to concluded on definite guidelines concerning ideal characteristics for these copper-doped materials, it is apparent that copper provides beneficial effects on biological properties. The very numerous recent studies devoted to the role of copper in biomaterials show the interest of the scientific community, and demonstrate the real potential of copper doping for biological/medical applications. Today we are clearly far from past fears linked to the alleged toxicity of this element [138] but, to our knowledge, we are also far from a commercial reality for orthopedic applications.

References

[1] J.T.B. Ratnayake, M. Mucalo, G.J. Dias, Substituted hydroxyapatites for bone regeneration: A review of current trends, J. Biomed. Mater. Res. - Part B Appl. Biomater. 105 (2017) 1285–1299. https://doi.org/10.1002/jbm.b.33651.

[2] S. V. Dorozhkin, Biocomposites and hybrid biomaterials based on calcium orthophosphates., Biomatter. 1 (2011) 3–56. https://doi.org/10.4161/biom.1.1.16782.

[3] S. V Dorozhkin, Bioceramics of calcium orthophosphates, Biomaterials. 31 (2010) 1465–1485. https://doi.org/10.1016/j.biomaterials.2009.11.050.

[4] A. Tathe, M. Ghodke, A.P. Nikalje, A brief review: Biomaterials and their apllication, Int. J. Pharm. Pharm. Sci. 2 (2010) 19–23.

[5] M. Clauss, A. Trampuz, O. Borens, M. Bohner, T. Ilchmann, Biofilm formation on bone grafts and bone graft substitutes: Comparison of different materials by a standard in vitro test and microcalorimetry, Acta Biomater. 6 (2010) 3791–3797. https://doi.org/10.1016/j.actbio.2010.03.011.

[6] J. Calhoun, M.M. Manring, M. Shirtliff, Osteomyelitis of the Long Bones, Semin. Plast. Surg. 23 (2009) 059–072. https://doi.org/10.1055/s-0029-1214158.

[7] G.J.A. ter Boo, D.W. Grijpma, T.F. Moriarty, R.G. Richards, D. Eglin, Antimicrobial

delivery systems for local infection prophylaxis in orthopedic- and trauma surgery, Biomaterials. 52 (2015) 113–125. https://doi.org/10.1016/j.biomaterials.2015.02.020.

[8] F.C. Tenover, Mechanisms of antimicrobial resistance in bacteria, Am. J. Infect. Control. 34 (2006) S3–S10. https://doi.org/10.1016/j.ajic.2006.05.219.

[9] EARSS, On-going surveillance of Pneumoniae, S Aureus, S Coli, E Faecium, E Faecalis, E Pneumoniae, K Aeruginosa, P. aeruginosa, 2008. www.rivm.nl/earss,.

[10] A. Gristina, Biomaterial-centered infection: microbial adhesion versus tissue integration, Science (80-.). 237 (1987) 1588–1595. https://doi.org/10.1126/science.3629258.

[11] R.M. Donlan, Biofilms and Device-Associated Infections, Emerg. Infect. Dis. 7 (2001) 277–281. https://doi.org/10.3201/eid0702.010226.

[12] A. V. Karlov, I.A. Khlusov, V.A. Pontak, V.P. Ignatov, M.A. Ivin, S.Y. Zinatulina, Adhesion of Staphylococcus aureus to implants with different physicochemical characteristics, Bull. Exp. Biol. Med. 134 (2002) 277–280. https://doi.org/10.1023/A:1021567804286.

[13] Jacobs, Gaulier, Duval, Renaudin, Silver Doping Mechanism in Bioceramics—From Ag+: Doped HAp to Ag°/BCP Nanocomposite, Crystals. 9 (2019) 326. https://doi.org/10.3390/cryst9070326.

[14] S. Gomes, C. Vichery, S. Descamps, H. Martinez, A. Kaur, A. Jacobs, J.-M. Nedelec, G. Renaudin, Cu-doping of calcium phosphate bioceramics: From mechanism to the control of cytotoxicity, Acta Biomater. 65 (2018). https://doi.org/10.1016/j.actbio.2017.10.028.

[15] S. Gomes, A. Kaur, J.M. Grenèche, J.M. Nedelec, G. Renaudin, Atomic scale modeling of iron-doped biphasic calcium phosphate bioceramics, Acta Biomater. 50 (2017) 78–88. https://doi.org/10.1016/j.actbio.2016.12.011.

[16] G. Renaudin, S. Gomes, J.M. Nedelec, First-row transition metal doping in calcium phosphate bioceramics: A detailed crystallographic study, Materials (Basel). 10 (2017). https://doi.org/10.3390/ma10010092.

[17] S. Gomes, A. Kaur, J.M. Nedelec, G. Renaudin, X-ray absorption spectroscopy shining (synchrotron) light onto the insertion of Zn2+ in calcium phosphate ceramics and its influence on their behaviour under biological conditions, J. Mater. Chem. B. 2 (2014) 536–545. https://doi.org/10.1039/c3tb21397h.

[18] F. Westhauser, S. Wilkesmann, Q. Nawaz, S.I. Schmitz, A. Moghaddam, S.C. Injury, K. Aschaffenburg-alzenau, Osteogenic properties of manganese-doped mesoporous bioactive glass nanoparticles, J. Biomed. Mater. Res. (2020) 1–10. https://doi.org/10.1002/jbm.a.36945.

[19] B. Yilmaz, A.Z. Alshemary, Z. Evis, Co-doped hydroxyapatites as potential materials for biomedical applications, Microchem. J. 144 (2019) 443–453. https://doi.org/10.1016/j.microc.2018.10.007.

[20] I. Cacciotti, Multisubstituted hydroxyapatite powders and coatings: The influence of the codoping on the hydroxyapatite performances, Int. J. Appl. Ceram. Technol. 16 (2019) 1864–1884. https://doi.org/10.1111/ijac.13229.

[21] F. Paladini, M. Pollini, A. Sannino, L. Ambrosio, Metal-based antibacterial substrates for biomedical applications, Biomacromolecules. 16 (2015) 1873–1885. https://doi.org/10.1021/acs.biomac.5b00773.

[22] P. Chellan, P.J. Sadler, The elements of life and medicines, Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. 373 (2015). https://doi.org/10.1098/rsta.2014.0182.

[23] N.M. Lowe, W.D. Fraser, M.J. Jackson, Is there a potential therapeutic value of copper and zinc for osteoporosis?, Proc. Nutr. Soc. 61 (2002) 181–185. https://doi.org/10.1079/pns2002154.

[24] M. Vincent, R.E. Duval, P. Hartemann, M. Engels-Deutsch, Contact killing and antimicrobial properties of copper, J. Appl. Microbiol. 124 (2018) 1032–1046. https://doi.org/10.1111/jam.13681.

[25] C. Gérard, L.J. Bordeleau, J. Barralet, C.J. Doillon, The stimulation of angiogenesis and collagen deposition by copper, Biomaterials. 31 (2010) 824–831. https://doi.org/10.1016/j.biomaterials.2009.10.009.

[26] F. Heidenau, W. Mittelmeier, R. Detsch, M. Haenle, F. Stenzel, G. Ziegler, H. Gollwitzer, A novel antibacterial titania coating: Metal ion toxicity and in vitro surface colonization, J. Mater. Sci. Mater. Med. 16 (2005) 883–888. https://doi.org/10.1007/s10856-005-4422-3.

[27] S. Jin, L. Ren, K. Yang, Bio-Functional Cu Containing Biomaterials: a New Way to Enhance Bio-Adaption of Biomaterials, J. Mater. Sci. Technol. 32 (2016) 835–839. https://doi.org/10.1016/j.jmst.2016.06.022.

[28] C. and H.-A.M. Linder, Copper biochemistry and molecular biology, AM J Clin Nutr. 63 (1996) 797S-811S. https://doi.org/10.1093/ajcn/63.5.797.

[29] W. Opsahl, H. Zeronian, M. Ellison, D. Lewis, R.B. Rucker, R.S. Riggins, Role of copper in collagen cross-linking and its influence on selected mechanical properties of chick bone and tendon, J. Nutr. 112 (1982) 708–716. https://doi.org/10.1093/jn/112.4.708.

[30] Institute of Medecine, Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and

zinc, 2001. https://doi.org/https://doi.org/10.17226/10026.

[31] EFSA, Scientific Opinion on Dietary Reference Values for copper, EFSA J. 13 (2015) 1–51. https://doi.org/10.2903/j.efsa.2015.4253.

[32] A.P. Ingle, P. Paralikar, S. Shende, I. Gupta, J.K. Biswas, L.H. Da Silva Martins, M. Rai, Copper in medicine: Perspectives and toxicity, Biomed. Appl. Met. (2018) 95–112. https://doi.org/10.1007/978-3-319-74814-6_4.

[33] J.Y. Uriu-Adams, C.L. Keen, Copper, oxidative stress, and human health, Mol. Aspects Med. 26 (2005) 268–298. https://doi.org/10.1016/j.mam.2005.07.015.

[34] S.M. Wazir, I. Ghobrial, Copper deficiency, a new triad: anemia, leucopenia, and myeloneuropathy, J. Community Hosp. Intern. Med. Perspect. 7 (2017) 265–268. https://doi.org/10.1080/20009666.2017.1351289.

[35] J.H. Kaplan, S. Lutsenko, Copper transport in mammalian cells: Special care for a metal with special needs, J. Biol. Chem. 284 (2009) 25461–25465. https://doi.org/10.1074/jbc.R109.031286.

[36] A. Hordyjewska, Ł. Popiołek, J. Kocot, The many "faces" of copper in medicine and treatment, BioMetals. 27 (2014) 611–621. https://doi.org/10.1007/s10534-014-9736-5.

[37] M.T. Lorincz, Neurologic Wilson's disease, Ann. N. Y. Acad. Sci. 1184 (2010) 173–187. https://doi.org/10.1111/j.1749-6632.2009.05109.x.

[38] G. Grass, C. Rensing, M. Solioz, Metallic copper as an antimicrobial surface, Appl. Environ. Microbiol. 77 (2011) 1541–1547. https://doi.org/10.1128/AEM.02766-10.

[39] V.B.P. Sudha, K.O. Singh, S.R. Prasad, P. Venkatasubramanian, Killing of enteric bacteria in drinking water by a copper device for use in the home: laboratory evidence, Trans. R. Soc. Trop. Med. Hyg. 103 (2009) 819–822. https://doi.org/10.1016/j.trstmh.2009.01.019.

[40] T.A. Dankovich, J.A. Smith, Incorporation of copper nanoparticles into paper for point-of-use water purification, Water Purif. (2014) 245–251. https://doi.org/10.4103/0974-8520.105261.

[41] G. Swain, N. Shinjo, Comparing biofouling control treatments for use on aquaculture nets, Int. J. Mol. Sci. 15 (2014) 22142–22154. https://doi.org/10.3390/ijms151222142.

[42] M.G. Schmidt, H.H. Attaway, P.A. Sharpe, J. John, K.A. Sepkowitz, A. Morgan, S.E. Fairey, S. Singh, L.L. Steed, J.R. Cantey, K.D. Freeman, H.T. Michels, C.D. Salgado, Sustained reduction of microbial burden on common hospital surfaces through introduction of copper, J. Clin. Microbiol. 50 (2012) 2217–2223. https://doi.org/10.1128/JCM.01032-12.

[43] WHO, Improved Hand Hygiene to Prevent Health Care-Associated Infections, Patient Saf. Solut. 1 (2007). https://doi.org/10.1016/s1553-7250(07)33134-6.

[44] V.J. Prado, M.M. Esparza, R.A. Vidal, C.T. Durán, Actividad bactericida de superficies de cobre frente a bacterias asociadas a infecciones nosocomiales, en un modelo in vitro de adherencia y sobrevivencia, Rev. Med. Chil. 141 (2013) 291–297. https://doi.org/10.4067/S0034-98872013000300002.

[45] C.D. Salgado, K.A. Sepkowitz, J.F. John, J.R. Cantey, H.H. Attaway, K.D. Freeman, P.A. Sharpe, H.T. Michels, M.G. Schmidt, Copper Surfaces Reduce the Rate of Healthcare-Acquired Infections in the Intensive Care Unit, Infect. Control Hosp. Epidemiol. 34 (2013) 479–486. https://doi.org/10.1086/670207.

[46] X. Wang, F. Cheng, J. Liu, J.H. Smått, D. Gepperth, M. Lastusaari, C. Xu, L. Hupa, Biocomposites of copper-containing mesoporous bioactive glass and nanofibrillated cellulose: Biocompatibility and angiogenic promotion in chronic wound healing application, Acta Biomater. 46 (2016) 286–298. https://doi.org/10.1016/j.actbio.2016.09.021.

[47] K. Li, C. Xia, Y. Qiao, X. Liu, Dose-response relationships between copper and its biocompatibility/antibacterial activities, J. Trace Elem. Med. Biol. 55 (2019) 127–135. https://doi.org/10.1016/j.jtemb.2019.06.015.

[48] U.S. EPA Approves Registration of Antimicrobial Copper Alloys, Copp. Dev. Assoc. Inc. (2008). https://www.copper.org/about/pressreleases/2008/pr2008_Mar_25.html (accessed March 12, 2020).

[49] P. Deb, A.B. Deoghare, A. Borah, E. Barua, S. Das Lala, Scaffold Development Using Biomaterials: A Review, Mater. Today Proc. 5 (2018) 12909–12919. https://doi.org/10.1016/j.matpr.2018.02.276.

[50] S. V. Dorozhkin, Calcium orthophosphates, J. Mater. Sci. 42 (2007) 1061–1095. https://doi.org/10.1007/s10853-006-1467-8.

[51] G. Daculsi, R.Z. Legeros, E. Nery, K. Lynch, B. Kerebel, Transformation of biphasic calcium phosphate ceramics in vivo: Ultrastructural and physicochemical characterization, J. Biomed. Mater. Res. 23 (1989) 883–894. https://doi.org/10.1002/jbm.820230806.

[52] W. Suchanek, M. Yoshimura, Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants, J. Mater. Res. 13 (1998) 94–117. https://doi.org/10.1557/JMR.1998.0015.

[53] S. V. Dorozhkin, Bioceramics of calcium orthophosphates, Biomaterials. 31 (2010)

1465–1485. https://doi.org/10.1016/j.biomaterials.2009.11.050.

[54] M. Bohner, B.L.G. Santoni, N. Döbelin, β-tricalcium phosphate for bone substitution: Synthesis and properties, Acta Biomater. 113 (2020) 23–41. https://doi.org/10.1016/j.actbio.2020.06.022.

[55] M. Ebrahimi, M.G. Botelho, S. V. Dorozhkin, Biphasic calcium phosphates bioceramics (HA/TCP): Concept, physicochemical properties and the impact of standardization of study protocols in biomaterials research, Mater. Sci. Eng. C. 71 (2017) 1293–1312. https://doi.org/10.1016/j.msec.2016.11.039.

[56] V. Stanić, S. Dimitrijević, J. Antić-Stanković, M. Mitrić, B. Jokić, I.B. Plećaš, S. Raičević, Synthesis, characterization and antimicrobial activity of copper and zinc-doped hydroxyapatite nanopowders, Appl. Surf. Sci. 256 (2010) 6083–6089. https://doi.org/10.1016/j.apsusc.2010.03.124.

[57] Y. Li, J. Ho, C.P. Ooi, Antibacterial efficacy and cytotoxicity studies of copper (II) and titanium (IV) substituted hydroxyapatite nanoparticles, Mater. Sci. Eng. C. 30 (2010) 1137–1144. https://doi.org/10.1016/j.msec.2010.06.011.

[58] Ž. Radovanović, B. Jokić, D. Veljović, S. Dimitrijević, V. Kojić, R. Petrović, D. Janaćković, Antimicrobial activity and biocompatibility of Ag + - and Cu 2+ -doped biphasic hydroxyapatite/α-tricalcium phosphate obtained from hydrothermally synthesized Ag + - and Cu 2+ -doped hydroxyapatite, Appl. Surf. Sci. 307 (2014) 513–519. https://doi.org/10.1016/j.apsusc.2014.04.066.

[59] S. Shanmugam, B. Gopal, Copper substituted hydroxyapatite and fluorapatite: Synthesis, characterization and antimicrobial properties, Ceram. Int. 40 (2014) 15655–15662. https://doi.org/10.1016/j.ceramint.2014.07.086.

[60] A. Bhattacharjee, Y. Fang, T.J.N. Hooper, N.L. Kelly, D. Gupta, K. Balani, I. Manna, T. Baikie, P.T. Bishop, T.J. White, J. V. Hanna, Crystal chemistry and antibacterial properties of cupriferous hydroxyapatite, Materials (Basel). 12 (2019). https://doi.org/10.3390/ma12111814.

[61] C.F. Marques, S. Olhero, J.C.C. Abrantes, A. Marote, S. Ferreira, S.I. Vieira, J.M.F. Ferreira, Biocompatibility and antimicrobial activity of biphasic calcium phosphate powders doped with metal ions for regenerative medicine, Ceram. Int. 43 (2017) 15719–15728. https://doi.org/10.1016/j.ceramint.2017.08.133.

[62] L.L. Hench, R.J. Splinter, W.C. Allen, T.K. Greenlee, Bonding mechanisms at the interface of ceramic prosthetic materials, J. Biomed. Mater. Res. 5 (1971) 117–141. https://doi.org/10.1002/jbm.820050611.

[63] M. Vallet-Regí, A.J. Salinas, Ceramics as bone repair materials, in: Bone Repair Biomater., Elsevier Inc., 2009: pp. 194–230. https://doi.org/10.1533/9781845696610.2.194.

[64] C. Wu, Y. Zhou, M. Xu, P. Han, L. Chen, J. Chang, Y. Xiao, Copper-containing mesoporous bioactive glass scaffolds with multifunctional properties of angiogenesis capacity, osteostimulation and antibacterial activity, Biomaterials. 34 (2013) 422–433. https://doi.org/10.1016/j.biomaterials.2012.09.066.

[65] H. Palza, B. Escobar, J. Bejarano, D. Bravo, M. Diaz-Dosque, J. Perez, Designing antimicrobial bioactive glass materials with embedded metal ions synthesized by the sol-gel method, Mater. Sci. Eng. C. 33 (2013) 3795–3801. https://doi.org/10.1016/j.msec.2013.05.012.

[66] E.A. Abou Neel, I. Ahmed, J. Pratten, S.N. Nazhat, J.C. Knowles, Characterisation of antibacterial copper releasing degradable phosphate glass fibres, Biomaterials. 26 (2005) 2247–2254. https://doi.org/10.1016/j.biomaterials.2004.07.024.

[67] R.A. Popescu, K. Magyari, A. Vulpoi, D.L. Trandafir, E. Licarete, M. Todea, R. Ştefan, C. Voica, D.C. Vodnar, S. Simon, I. Papuc, L. Baia, Bioactive and biocompatible copper containing glass-ceramics with remarkable antibacterial properties and high cell viability designed for future: in vivo trials, Biomater. Sci. 4 (2016) 1252–1265. https://doi.org/10.1039/c6bm00270f.

[68] G.K. Pouroutzidou, G.S. Theodorou, E. Kontonasaki, I. Tsamesidis, A. Pantaleo, D. Patsiaoura, L. Papadopoulou, J. Rhoades, E. Likotrafiti, C.B. Lioutas, K. Chrissafis, K.M. Paraskevopoulos, Effect of ethanol/TEOS ratios and amount of ammonia on the properties of copper-doped calcium silicate nanoceramics, J. Mater. Sci. Mater. Med. 30 (2019) 1–13. https://doi.org/10.1007/s10856-019-6297-8.

[69] N. Gupta, D. Santhiya, S. Murugavel, A. Kumar, A. Aditya, M. Ganguli, S. Gupta, Effects of transition metal ion dopants (Ag, Cu and Fe) on the structural, mechanical and antibacterial properties of bioactive glass, Colloids Surfaces A Physicochem. Eng. Asp. 538 (2018) 393–403. https://doi.org/10.1016/j.colsurfa.2017.11.023.

[70] A. Bari, N. Bloise, S. Fiorilli, G. Novajra, M. Vallet-Regí, G. Bruni, A. Torres-Pardo, J.M. González-Calbet, L. Visai, C. Vitale-Brovarone, Copper-containing mesoporous bioactive glass nanoparticles as multifunctional agent for bone regeneration, Acta Biomater. 55 (2017) 493–504. https://doi.org/10.1016/j.actbio.2017.04.012.

[71] F. Foroutan, J. McGuire, P. Gupta, A. Nikolaou, B.A. Kyffin, N.L. Kelly, J. V. Hanna, J. Gutierrez-Merino, J.C. Knowles, S.Y. Baek, E. Velliou, D. Carta, Antibacterial

Copper-Doped Calcium Phosphate Glasses for Bone Tissue Regeneration, ACS Biomater. Sci. Eng. 5 (2019) 6054–6062. https://doi.org/10.1021/acsbiomaterials.9b01291.

[72] A. Hoppe, N.S. Güldal, A.R. Boccaccini, A review of the biological response to ionic dissolution products from bioactive glasses and glass-ceramics, Biomaterials. 32 (2011) 2757–2774. https://doi.org/10.1016/j.biomaterials.2011.01.004.

[73] J. V. Rau, V.M. Wu, V. Graziani, I. V. Fadeeva, A.S. Fomin, M. Fosca, V. Uskoković, The Bone Building Blues: Self-hardening copper-doped calcium phosphate cement and its in vitro assessment against mammalian cells and bacteria, Mater. Sci. Eng. C. 79 (2017) 270–279. https://doi.org/10.1016/j.msec.2017.05.052.

[74] F. Zhang, M. Zhou, W. Gu, Z. Shen, X. Ma, F. Lu, X. Yang, Y. Zheng, Z. Gou, Zinc-/Copper-Substituted Dicalcium Silicate Cement: Advanced Biomaterials with Enhanced Osteogenesis and Long-term Antibacterial Properties, J. Mater. Chem. B. (2020) 1–21. https://doi.org/10.1039/c9tb02691f.

[75] A.R. Boccaccini, M. Erol, W.J. Stark, D. Mohn, Z. Hong, J.F. Mano, Polymer/bioactive glass nanocomposites for biomedical applications: A review, Compos. Sci. Technol. 70 (2010) 1764–1776. https://doi.org/10.1016/j.compscitech.2010.06.002.

[76] J. Bejarano, R. Detsch, A.R. Boccaccini, H. Palza, PDLLA scaffolds with Cu- and Zn-doped bioactive glasses having multifunctional properties for bone regeneration, J. Biomed. Mater. Res. - Part A. 105 (2016) 746–756. https://doi.org/10.1002/jbm.a.35952.

[77] K. Sahithi, M. Swetha, M. Prabaharan, A. Moorthi, N. Saranya, K. Ramasamy, N. Srinivasan, N.C. Partridge, N. Selvamurugan, Synthesis and characterization of nanoscale-hydroxyapatite-copper for antimicrobial activity towards bone tissue engineering applications, J. Biomed. Nanotechnol. 6 (2010) 333–339. https://doi.org/10.1166/jbn.2010.1138.

[78] B. Li, Y. Li, Y. Wu, Y. Zhao, Synthesis of water-soluble Cu/PAA composite flowers and their antibacterial activities, Mater. Sci. Eng. C. 35 (2014) 205–211. https://doi.org/10.1016/j.msec.2013.11.006.

[79] V. Narayanan, S. Sumathi, N. Arunai Nambi Raj, Tri-component composite containing Copper-hydroxyapatite/chitosan/ polyvinyl pyrrolidone for bone tissue engineering, J. Biomed. Mater. Res. (2020). https://doi.org/10.1002/jbm.a.36950.

[80] J. Wilson, Metallic biomaterials : State of the art and new challenges, Elsevier Ltd, 2018. https://doi.org/10.1016/B978-0-08-102205-4.00001-5.

[81] M. Geetha, A.K. Singh, R. Asokamani, A.K. Gogia, Ti based biomaterials, the ultimate

choice for orthopaedic implants - A review, Prog. Mater. Sci. 54 (2009) 397–425. https://doi.org/10.1016/j.pmatsci.2008.06.004.

[82] Y. Huang, X. Zhang, R. Zhao, H. Mao, Y. Yan, X. Pang, Antibacterial efficacy, corrosion resistance, and cytotoxicity studies of copper-substituted carbonated hydroxyapatite coating on titanium substrate, J. Mater. Sci. 50 (2015) 1688–1700. https://doi.org/10.1007/s10853-014-8730-1.

[83] S. Kalaivani, R.K. Singh, V. Ganesan, S. Kannan, Effect of copper (Cu2+) inclusion on the bioactivity and antibacterial behavior of calcium silicate coatings on titanium metal, J. Mater. Chem. B. 2 (2014) 846–858. https://doi.org/10.1039/c3tb21522a.

[84] Q. Huang, Z. Ouyang, Y. Tan, H. Wu, Y. Liu, Activating macrophages for enhanced osteogenic and bactericidal performance by Cu ion release from micro/nano-topographical coating on a titanium substrate, Acta Biomater. 100 (2019) 415–426. https://doi.org/10.1016/j.actbio.2019.09.030.

[85] R. Ghosh, O. Swart, S. Westgate, B.L. Miller, M.Z. Yates, Antibacterial Copper-Hydroxyapatite Composite Coatings via Electrochemical Synthesis, Langmuir. 35 (2019) 5957–5966. https://doi.org/10.1021/acs.langmuir.9b00919.

[86] I. Burghardt, F. Lüthen, C. Prinz, B. Kreikemeyer, C. Zietz, H.G. Neumann, J. Rychly, A dual function of copper in designing regenerative implants, Biomaterials. 44 (2015). https://doi.org/10.1016/j.biomaterials.2014.12.022.

[87] C. Wolf-Brandstetter, R. Beutner, R. Hess, S. Bierbaum, K. Wagner, D. Scharnweber, U. Gbureck, C. Moseke, Multifunctional calcium phosphate based coatings on titanium implants with integrated trace elements, Biomed. Mater. 15 (2020) 1–18. https://doi.org/10.1088/1748-605X/ab5d7b.

[88] M.A. Akhtar, K. Ilyas, I. Dlouh, F. Siska, A.R. Boccaccini, Electrophoretic Deposition of Copper (II)– Chitosan Complexes for Antibacterial Coatings, Int. J. Mol. Sci. 21 (2020) 1–19. https://doi.org/10.3390/ijms21072637.

[89] L. Ren, K. Yang, L. Guo, H.W. Chai, Preliminary study of anti-infective function of a copper-bearing stainless steel, Mater. Sci. Eng. C. 32 (2012) 1204–1209. https://doi.org/10.1016/j.msec.2012.03.009.

[90] L. Ren, Z. Ma, M. Li, Y. Zhang, W. Liu, Z. Liao, K. Yang, Antibacterial properties of Ti-6Al-4V-xCu alloys, J. Mater. Sci. Technol. 30 (2014) 699–705. https://doi.org/10.1016/j.jmst.2013.12.014.

[91] H. Chai, L. Guo, X. Wang, Y. Fu, J. Guan, L. Tan, L. Ren, K. Yang, Antibacterial effect of 317L stainless steel contained copper in prevention of implant-related infection in

vitro and in vivo, J. Mater. Sci. Mater. Med. 22 (2011) 2525–2535. https://doi.org/10.1007/s10856-011-4427-z.

[92] D. Zhang, L. Ren, Y. Zhang, N. Xue, K. Yang, M. Zhong, Antibacterial activity against Porphyromonas gingivalis and biological characteristics of antibacterial stainless steel, Colloids Surfaces B Biointerfaces. 105 (2013) 51–57. https://doi.org/10.1016/j.colsurfb.2012.12.025.

[93] E. Zhang, F. Li, H. Wang, J. Liu, C. Wang, M. Li, K. Yang, A new antibacterial titanium-copper sintered alloy: Preparation and antibacterial property, Mater. Sci. Eng. C. 33 (2013) 4280–4287. https://doi.org/10.1016/j.msec.2013.06.016.

[94] Z. Ma, L. Ren, R. Liu, K. Yang, Y. Zhang, Z. Liao, W. Liu, M. Qi, R.D.K. Misra, Effect of Heat Treatment on Cu Distribution, Antibacterial Performance and Cytotoxicity of Ti-6Al-4V-5Cu Alloy, J. Mater. Sci. Technol. 31 (2015) 723–732. https://doi.org/10.1016/j.jmst.2015.04.002.

[95] L. Nan, J. Cheng, K. Yang, Antibacterial Behavior of a Cu-bearing Type 200 Stainless Steel, J. Mater. Sci. Technol. 28 (2012) 1067–1070. https://doi.org/10.1016/S1005-0302(12)60174-1.

[96] L. Nan, Y. Liu, M. Lü, K. Yang, Study on antibacterial mechanism of copper-bearing austenitic antibacterial stainless steel by atomic force microscopy, J. Mater. Sci. Mater. Med. 19 (2008) 3057–3062. https://doi.org/10.1007/s10856-008-3444-z.

[97] E. Zhang, C. Liu, A new antibacterial Co-Cr-Mo-Cu alloy: Preparation, biocorrosion, mechanical and antibacterial property, Mater. Sci. Eng. C. 69 (2016) 134–143. https://doi.org/10.1016/j.msec.2016.05.028.

[98] C. Espírito Santo, N. German, J. Elguindi, G. Grass, C. Rensing, Biocidal mechanisms of metallic copper surfaces, in: Use Biocidal Surfaces Reduct. Healthc. Acquir. Infect., Springer International Publishing, 2014: pp. 103–136. https://doi.org/10.1007/978-3-319-08057-4_6.

[99] M. Hans, S. Mathews, F. Mücklich, M. Solioz, Physicochemical properties of copper important for its antibacterial activity and development of a unified model, Biointerphases. 11 (2016) 018902. https://doi.org/10.1116/1.4935853.

[100] G. Borkow, J. Gabbay, Copper as a Biocidal Tool, Curr. Med. Chem. 12 (2005) 2163–2175. https://doi.org/10.2174/0929867054637617.

[101] R. Nandakumar, C.E. Santo, N. Madayiputhiya, G. Grass, Quantitative proteomic profiling of the Escherichia coli response to metallic copper surfaces, BioMetals. 24 (2011) 429–444. https://doi.org/10.1007/s10534-011-9434-5.

[102] E. Ladomersky, M.J. Petris, Copper tolerance and virulence in bacteria, Metallomics. 7 (2015) 957–964. https://doi.org/10.1039/c4mt00327f.

[103] R.S. Kirsner, W.H. Eaglstein, The wound healing process, Dermatol. Clin. 11 (1993) 629–640. https://doi.org/10.1016/s0733-8635(18)30216-x.

[104] T. Velnar, T. Bailey, V. Smrkolj, The wound healing process: An overview of the cellular and molecular mechanisms, J. Int. Med. Res. 37 (2009) 1528–1542. https://doi.org/10.1177/147323000903700531.

[105] A.P. Kornblatt, V.G. Nicoletti, A. Travaglia, The neglected role of copper ions in wound healing, J. Inorg. Biochem. 161 (2016) 1–8. https://doi.org/10.1016/j.jinorgbio.2016.02.012.

[106] C.K. Sen, S. Khanna, M. Venojarvi, P. Trikha, E. Christopher Ellison, T.K. Hunt, S. Roy, Copper-induced vascular endothelial growth factor expression and wound healing, Am. J. Physiol. - Hear. Circ. Physiol. 282 (2002) 1821–1827. https://doi.org/10.1152/ajpheart.01015.2001.

[107] X. Gao, Z. Xu, Mechanisms of action of angiogenin, Acta Biochim Biophys Sin. 40 (2008) 663–669. https://doi.org/10.1111/j.1745-7270.2008.

[108] C. Giacomelli, M.L. Trincavelli, C. Satriano, Ö. Hansson, D. La Mendola, E. Rizzarelli, C. Martini, Copper (II) ions modulate Angiogenin activity in human endothelial cells, Int. J. Biochem. Cell Biol. 60 (2015) 185–196. https://doi.org/10.1016/j.biocel.2015.01.005.

[109] N. Philips, H. Hwang, S. Chauhan, D. Leonardi, S. Gonzalez, Stimulation of cell proliferation and expression of matrixmetalloproteinase-1 and interluekin-8 genes in dermal fibroblasts by copper, Connect. Tissue Res. 51 (2010) 224–229. https://doi.org/10.3109/03008200903288431.

[110] L. Pickart, J.M. Vasquez-Soltero, A. Margolina, The human tripeptide GHK-Cu in prevention of oxidative stress and degenerative conditions of aging: Implications for cognitive health, Oxid. Med. Cell. Longev. 2012 (2012) 1–8. https://doi.org/10.1155/2012/324832.

[111] S. Alizadeh, B. Seyedalipour, S. Shafieyan, A. Kheime, P. Mohammadi, N. Aghdami, Copper nanoparticles promote rapid wound healing in acute full thickness defect via acceleration of skin cell migration, proliferation, and neovascularization, Biochem. Biophys. Res. Commun. 517 (2019) 684–690. https://doi.org/10.1016/j.bbrc.2019.07.110.

[112] J. Barralet, U. Gbureck, P. Habibovic, E. Vorndran, C. Gerard, C.J. Doillon,

Angiogenesis in Calcium Phosphate Scaffolds by Inorganic Copper Ion Release, Tissue Eng. Part A. 15 (2009) 1601–1609. https://doi.org/10.1089/ten.tea.2007.0370.

[113] N. Kong, K. Lin, H. Li, J. Chang, Synergy effects of copper and silicon ions on stimulation of vascularization by copper-doped calcium silicate, J. Mater. Chem. B. 2 (2014) 1100–1110. https://doi.org/10.1039/c3tb21529f.

[114] H. Wang, S. Zhao, J. Zhou, Y. Shen, W. Huang, C. Zhang, M.N. Rahaman, D. Wang, Evaluation of borate bioactive glass scaffolds as a controlled delivery system for copper ions in stimulating osteogenesis and angiogenesis in bone healing, J. Mater. Chem. B. 2 (2014) 8547–8557. https://doi.org/10.1039/c4tb01355g.

[115] H. Wang, S. Zhao, W. Xiao, J. Xue, Y. Shen, J. Zhou, W. Huang, M.N. Rahaman, C. Zhang, D. Wang, Influence of Cu doping in borosilicate bioactive glass and the properties of its derived scaffolds, Mater. Sci. Eng. C. 58 (2016) 194–203.

[116] L.B. Romero-Sánchez, M. Marí-Beffa, P. Carrillo, M.Á. Medina, A. Díaz-Cuenca, Copper-containing mesoporous bioactive glass promotes angiogenesis in an in vivo zebrafish model, Acta Biomater. 68 (2018) 272–285. https://doi.org/10.1016/j.actbio.2017.12.032.

[117] Y. Lin, W. Xiao, B.S. Bal, M.N. Rahaman, Effect of copper-doped silicate 13-93 bioactive glass scaffolds on the response of MC3T3-E1 cells in vitro and on bone regeneration and angiogenesis in rat calvarial defects in vivo, Mater. Sci. Eng. C. 67 (2016) 440–452. https://doi.org/10.1016/j.msec.2016.05.073.

[118] S.N. Rath, A. Brandl, D. Hiller, A. Hoppe, U. Gbureck, R.E. Horch, A.R. Boccaccini, U. Kneser, Bioactive copper-doped glass scaffolds can stimulate endothelial cells in co-culture in combination with mesenchymal stem cells, PLoS One. 9 (2014) 1–24. https://doi.org/10.1371/journal.pone.0113319.

[119] A. Elrayah, W. Zhi, S. Feng, S. Al-Ezzi, H. Lei, J. Weng, Preparation of micro/nano-structure copper-substituted hydroxyapatite scaffolds with improved angiogenesis capacity for bone regeneration, Materials (Basel). 11 (2018). https://doi.org/10.3390/ma11091516.

[120] P. Mou, H. Peng, L. Zhou, L. Li, H. Li, Q. Huang, A novel composite scaffold of Cu-doped nano calcium-deficient hydroxyapatite/multi-(Amino acid) copolymer for bone tissue regeneration, Int. J. Nanomedicine. 14 (2019) 3331–3343. https://doi.org/10.2147/IJN.S195316.

[121] J. Zhang, H. Wu, F. He, T. Wu, L. Zhou, J. Ye, Concentration-dependent osteogenic and angiogenic biological performances of calcium phosphate cement modified with copper

ions, Mater. Sci. Eng. C. 99 (2019) 1199–1212. https://doi.org/10.1016/j.msec.2019.02.042.

[122] I. Couret, Biologie du remodelage osseux, 28 (2004) 57–65.

[123] J.M. Kanczler, R.O.C. Oreffo, Osteogenesis and angiogenesis: The potential for engineering bone, Eur. Cells Mater. 15 (2008) 100–114. https://doi.org/10.22203/eCM.v015a08.

[124] P. V. Giannoudis, T.A. Einhorn, D. Marsh, Fracture healing: The diamond concept, Inj. Int. J. Caree Inj. 38 (2007) S3-6. https://doi.org/10.1016/s0020-1383(08)70003-2.

[125] J. Pablo Rodriguez, S. Rios, M. Gonzalez, Modulation of the proliferation and differentiation of human mesenchymal stem cells by copper, J. Cell. Biochem. 85 (2002) 92–100. https://doi.org/10.1002/jcb.10111.

[126] J. Jonas, J. Burns, E.. Abel, M.. Cresswell, J.. Strain, C.. Paterson, Impaired mechanical strength of bone in experimental copper deficiency, Ann Nutr Metab. 37 (1993) 245–252. https://doi.org/10.1159/000177774.

[127] L. Bi, M.N. Rahaman, D.E. Day, Z. Brown, C. Samujh, X. Liu, A. Mohammadkhah, V. Dusevich, J.D. Eick, L.F. Bonewald, Effect of bioactive borate glass microstructure on bone regeneration, angiogenesis, and hydroxyapatite conversion in a rat calvarial defect model, Acta Biomater. 9 (2013) 8015–8026. https://doi.org/10.1016/j.actbio.2013.04.043.

[128] A. Ewald, C. Käppel, E. Vorndran, C. Moseke, M. Gelinsky, U. Gbureck, The effect of Cu(II)-loaded brushite scaffolds on growth and activity of osteoblastic cells, J. Biomed. Mater. Res. - Part A. 100 A (2012) 2392–2400. https://doi.org/10.1002/jbm.a.34184.

[129] L. Ren, K. Yang, Bio-functional design for metal implants, a new concept for development of metallic biomaterials, J. Mater. Sci. Technol. 29 (2013) 1005–1010. https://doi.org/10.1016/j.jmst.2013.09.008.

[130] L. Ren, H.M. Wong, C.H. Yan, K.W.K. Yeung, K. Yang, Osteogenic ability of Cu-bearing stainless steel, J. Biomed. Mater. Res. - Part B Appl. Biomater. 103 (2015) 1433–1444. https://doi.org/10.1002/jbm.b.33318.

[131] L. Yang, S. Perez-Amodio, F.Y.F. Barrère-de Groot, V. Everts, C.A. van Blitterswijk, P. Habibovic, The effects of inorganic additives to calcium phosphate on in vitro behavior of osteoblasts and osteoclasts, Biomaterials. 31 (2010) 2976–2989. https://doi.org/10.1016/j.biomaterials.2010.01.002.

[132] S. D'Mello, S. Elangovan, L. Hong, R.D. Ross, D.R. Sumner, A.K. Salem, Incorporation of copper into chitosan scaffolds promotes bone regeneration in rat calvarial defects, J.

Biomed. Mater. Res. - Part B Appl. Biomater. 103 (2015) 1044–1049. https://doi.org/10.1002/jbm.b.33290.

[133] D. Campoccia, L. Montanaro, C.R. Arciola, A review of the biomaterials technologies for infection-resistant surfaces, Biomaterials. 34 (2013) 8533–8554. https://doi.org/10.1016/j.biomaterials.2013.07.089.

[134] Y.F. Feng, L. Wang, X. Li, Z.S. Ma, Y. Zhang, Z.Y. Zhang, W. Lei, Influence of Architecture of β-Tricalcium Phosphate Scaffolds on Biological Performance in Repairing Segmental Bone Defects, PLoS One. 7 (2012) 1–12. https://doi.org/10.1371/journal.pone.0049955.

[135] V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomaterials. 26 (2005) 5474–5491. https://doi.org/10.1016/j.biomaterials.2005.02.002.

[136] D. Xiao, J. Zhang, C. Zhang, D. Barbieri, H. Yuan, L. Moroni, G. Feng, The role of calcium phosphate surface structure in osteogenesis and the mechanisms involved, Acta Biomater. 106 (2020) 22–33. https://doi.org/10.1016/j.actbio.2019.12.034.

[137] M. Bohner, Y. Loosli, G. Baroud, D. Lacroix, Deciphering the link between architecture and biological response of a bone graft substitute, Acta Biomater. 7 (2011) 478–484. https://doi.org/10.1016/j.actbio.2010.08.008.

[138] M.E. Letelier, S. Sánchez-Jofré, L. Peredo-Silva, J. Cortés-Troncoso, P. Aracena-Parks, Mechanisms underlying iron and copper ions toxicity in biological systems: Pro-oxidant activity and protein-binding effects, Chem. Biol. Interact. 188 (2010) 220–227. https://doi.org/10.1016/j.cbi.2010.06.013.

Table 1. Studies that evaluated the antibacterial effect of copper-doped biomaterials.

Biomaterial	Chemical composition	Copper concentration released	Bacterial strains tested	References
Calcium phosphates bioceramics	FAp ($Ca_{10-x}Cu_x(PO_4)_6F_2$) and HAp ($Ca_{10-x}Cu_x(PO_4)_6(OH)_2$ powders with $x = 0.05$ to $x = 0.5$	n.s.	*E. coli* *S. aureus* *C. albicans*	[59]
	Biphasic HAp($Ca_{10-x}Cu_x(PO_4)_6(OH)_2$)/α-TCP powders with $x = 0.02$ or 0.04	n.s.	*E. coli* *S. aureus* *C. albicans* *P. aeruginosa*	[58]
	HAp nanopowders (CuHAP1 and CuHAP2 with Cu/(Ca+Cu) = 0.0004 and 0.004 respectively	n.s.	*E. coli* *S. aureus* *C. albicans*	[56]
	HAp pellets and powders (Cu/Ca = 0.001, 0.05, 0.1, 0.15)	n.s.	*E. coli*	[57]
	BCP (HAp 50% and β-TCP 50%) powders compacted into pellets with Cu = 2.6mol%	n.s.	*E. coli* *S. aureus*	[61]
	Cu-doped HAp$Ca_{10}(PO_4)_6[Cu_x(OH)_{2-2x}O_x]$ with $x = 0.2$, 0.4, 0.6 and 0.8, synthesized by Wet Chemical Method and Solid State Method	n.m.	*E. coli* *S. aureus*	[60]

Bioactive glasses	Cu-loaded mesoporous-bioactive glasses (CU-MBG): 0Cu-MBG (molar ratio Cu/Ca/P/Si = 0/15/5/80) and 5Cu-MBG (molar ratio Cu/Ca/P/Si = 5/10/5/80)	n.s.	*E. coli*	[64]
	Bioactive glass (BG) and BG with copper ions (CuBG1 and CuBG2 with CuO = 3.7 wt.% and 8.4 wt.% respectively)	n.s.	*E. coli (ampicillin-resistant)* *S. mutans*	[65]
	Bioactive glass-ceramics: $60SiO_2 \cdot (32\text{-}x)CaO \cdot 8P_2O_5 \cdot xCuO$ with x = 0; 0.5; 1.5; 2.5 and 4(mol%)	n.s.	*S. aureus* *P. aeruginosa*	[67]
	Bioactive glass nanopowders with composition in mol%: 60 SiO_2, 30 CaO, 7.5 MgO and 2.5 CuO	n.m.	*L. monocytogenes* *B. cereus* *S. aureus* *E. coli* *S.* Typhimurium *S.* Enteriditis *P. aeruginosa*	[68]
	Copper doped bioactive glass (Cu = 2wt%)	n.m.	*E. coli* *S. aureus*	[69]
	Copper-containingmesoporous bioactive glass nanoparticles: Cu-MBG 2% (molar ratio Cu/Ca/Si = 2/13/85)	n.s.	*E. coli* *S. aureus* *S. epidermidis*	[70]

	Phosphate-based glass fibers (PGF) containing CuO with CuO mol% = 0, 1, 5 and 10	n.s.	*S. epidermidis*	[66]
	Cu-doped calcium phosphate glasses (CPG, CPG-Cu2, CPG-Cu4 and CPG-Cu6 with 0, 2.2, 4.5 and 6.3 mol % of Cu respectively	n.s.	*S. aureus*	[71]
Bone cements	Cu-TCP cement: Cu-substituted TCP (Cu = 0.30 wt %) powder with Monocalcium Phosphate Monohydrate (MCPM) and Carbonated Hydroxyapatite (CHA) (mass proportion 1/0.764/0.091) mixed with citric acid (0.45M) to form Dicalcium Phosphate Dihydrate (DCPD, brushite)	n.s.	*E. coli* *S. aureus* *S.* Enteriditis *P. aeruginosa*	[73]
	Cu-substituted dicalcium silicate cement: - C2S-5Cu = 0.06 Cu^{2+} (molar ratio) - C2S-10Cu = 0.11 Cu^{2+} (molar ratio)	n.s.	*P. aeruginosa* *S. aureus* *E. faecalis* *E. coli*	[74]
Biocomposites	PDLLA (poly(D, L-lactide)) scaffolds with Copper doped BG particles (CuBG = 60SiO_2-25CaO-1Na_2O-4P_2O_5-1CuO in mol %): composite with 10 wt % of glass particles	n.s.	*Methicillin-resistant* *S. aureus*	[76]
	Biocomposite aerogel composed of Cu-doped mesoporous BG (Cu-MBGs with molar ratio Si/Cu/Ca/P=75/5/15/5)) and nanofibrillatedcelluloseNFC: MBGSi75Cu5 (5:2) and NFC:MBGSi75Cu5 (10:1)	n.s.	*E. coli*	[46]

	Water-soluble Cu/polyacrylicacid (Cu/PAA) composites	n.m.	*S. aureus* *B. subtilis* *E. coli* *P. aeruginosa*	[78]
	Nano HAp powders soaked with copper (nHAp-Cu), and combined with PEG 400 (nHAP-Cu/PEG 400)	n.m.	*E. coli* *S. aureus*	[77]
	Tri-component composite: Chitosan/Polyvinyl pyrrolidone (CS/PVP) with Cu-HAp weight ratios (0, 20, 40, 60, and 80 wt%)	n.m	*S. aureus* *B. subtilis* *E. coli* *C. albicans* *P. notatum* *R. stolonifer*	[79]
Coated/alloyed metals (Titanium, stainless steel and Co-based alloys)	CuHAP coating on titanium ($Ca_{10-x}Cu_x(PO_4)_6(OH)_2$ with x = 0.025)	n.m.	*E. coli*	[82]
	Copper doped $CaSiO_3$ coated on Ti: 1x-CS, 2x-CS, 3x-CS, 4x-CS and 5x-CS with Cu (wt%) = 1.654, 2.939, 4.399, 5.768, 7.268 respectively	n.s.	*E. coli* *S. aureus*	[83]
	Micro-Ti surface: TiO_2 matrix with amorphous CaO, $CaSiO_3$ and SiO_2. Cu-Hier-Ti surface: $CaO{\cdot}3CuO{\cdot}4TiO_2$ and $CaO{\cdot}TiO_2{\cdot}SiO_2$	n.m.	*S. aureus*	[84]

	Cu-HAp coated titanium (Cu-HA-coated Ti) with Cu content (atom %) HA=0, B1 = 2.4, B2 = 2.7, B3 = 4.2, B4 = 6.6	HA = 0.5 ppm * B1 = 31 ppm * B4 = 68 ppm *	*E. coli* *S. aureus*	[85]
	Titanium plates Ti6Al4V with Cu layer at 1μg/mm^2	n.s.	*S. aureus*	[86]
	Cu-doped calcium phosphate based coating on titanium implants with Cu01 = 4.1 μg of coating and Cu03 = 25.1 μg	n.s.	*E. coli*	[87]
	Cu-chitosan complexes coated on stainless steel 316L with Cu (%) = 2.93, 5.82, 11.45 and 16.50	n.m	*E. coli* *S. aureus*	[88]
	317L-Cu stainless steel (317L-Cu SS) with (wt.%): Cr 19, Ni 13, Mo 3.5, Cu 4.5 and Fe in balance	n.m.	*E. coli* *S. aureus*	[89]
	Ti-6Al-4V-*x*Cu (*x* = 1, 3, 5 wt%)	n.m.	*E. coli* *S. aureus*	[90]
	317L = 00Cr19Ni13Mo3 and 317L-Cu = 00Cr19Ni13Mo3-4.5 with Cu wt% = 4.5	n.s.	*E. coli* *S. aureus*	[91]
	304 Cu-bearing stainless steel with (wt.%): C 0.016, Cr 18.52, Ni 8.36, Mn 0.43, Si 0.61, Cu 3.90, and Fe in balance	n.m.	*P. gingivalis*	[92]
	Ti-Cu alloys	n.s.	*E. coli* *S. aureus*	[93]
	Cu-bearing titanium alloy Ti-6Al-4V-5Cu with (wt%) Al/V/Cu/Fe/C/N/O/H/Ti = 6.06/3.75/4.85/0.06/0.01/0.002/0.05/0.001/Balance	n.s.	*S. aureus*	[94]

	Commercial stainless steels type 200 (200-C) with 1.45 wt % of Cu and stainless steels enriched with 2.77 wt % of Cu	n.m.	*E. coli* *S. aureus*	[95]
	Austenitic stainless steel (0Cr18Ni9) and Cu bearing austenitic antibacterial stainless steel (0Cr18Ni9- 3.8 wt % Cu)	0.35 ppm, 1h ** 0.55 ppm, 3h ** 1.1 ppm, 5h ** 1.4 ppm, 7h **	*E. coli*	[96]
	Co-Cr-Mo-Cu alloys (Co-*x*Cu) with 1 wt % Cu, 2 wt % and 4 wt %.	n.s.	*S. aureus*	[97]

n.s.: not significative (measured but not related to antimicrobial activity)

n.m.: not measured

* Measured by ICP-MS after 8h with the same conditions used for bacteria growth

** Measured by ICP-MS

Table 2. Antibacterial test parameters in studies with Cu-doped biomaterials.

Bacterial type	Bacterial strain	Biomaterial	Type of experiment	Antibacterial effect*	References
Gram -	*E. coli*	Calcium phosphates bioceramics	Quantitative method and Agar diffusion test	+	[56]
			Quantitative method and Agar diffusion test	+	[57]
			Quantitative method	-	[59]
			Quantitative method	+	[58]
			Agar diffusion test	-	[61]
			Quantitative method, MTT assay and Optical density	+	[60]
		Bioglasses	Quantitative method	+	[64]
			MTT assay	+	[70]
			Agar diffusion test	+	[69]
			Quantitative method and Optical density	-	[68]
		Biomedical cements	Quantitative method and Agar diffusion test	+	[73]
			Quantitative method	+	[74]

		Biocomposites	Optical density	+	[77]
			Optical density	+	[78]
			Optical density	+	[46]
			Agar diffusion test	+	[79]
		Coated/alloyed metals	AFM analysis	+	[96]
			Quantitative method	+	[91]
			Quantitative method	+	[95]
			Quantitative method	+	[89]
			Quantitative method and Agar diffusion test	+	[93]
			Quantitative method	+	[90]
			Quantitative method	+	[83]
			Quantitative method	+	[82]
			Quantitative method and Optical density	+	[85]
			Optical density and LIVE/DEAD staining	+	[87]
			Quantitative method	+	[88]
	P. aeruginosa	Calcium phosphates bioceramics	Quantitative method	+	[58]
		Bioglasses	Resazurin test	+	[67]

			Quantitative method and Optical density	+	[68]
		Biomedical cements	Quantitative method and Agar diffusion test	+	[73]
			Quantitative method	+	[74]
		Biocomposites	Optical density	+	[78]
	S. Typhimurium	Bioglasses	Quantitative method and Optical density	-	[68]
	S. Enteriditis	Bioglasses	Quantitative method and Optical density	-	[68]
		Biomedical cements	Quantitative method and Agar diffusion test	+	[73]
	P. gingivalis	Coated/alloyed metals	Quantitative method and SEM	+	[92]
Gram +	*S. aureus*	Calcium phosphates bioceramics	Quantitative method and Agar diffusion test	+	[56]
			Quantitative method	+	[58]
			Quantitative method	+	[59]
			Agar diffusion test	-	[61]
			Quantitative method, MTT assay and Optical density	+	[60]
		Bioglasses	Resazurin test	+	[67]
			MTT assay	+	[70]

			Agar diffusion test	+	[69]
			Quantitative method and Optical density	+	[68]
			Quantitative method	+	[71]
		Biomedical cements	Quantitative method and Agar diffusion test	-	[73]
			Quantitative method	+	[74]
		Biocomposites	Optical density	+	[77]
			Optical density	+	[78]
			Agar diffusion test	+	[79]
		Coated/alloyed metals	Quantitative method	+	[91]
			Quantitative method	+	[89]
			Quantitative method	+	[95]
			Quantitative method and Agar diffusion test	+	[93]
			Quantitative method	+	[90]
			Quantitative method	+	[83]
			Quantitative method	+	[86]
			Quantitative method and SEM	+	[94]
			Quantitative method	+	[97]
			Quantitative method	-	[84]
			Quantitative method and Optical density	+	[85]

			Quantitative method	+	[88]
	L. monocytogenes	Bioglasses	Quantitative method and Optical density	-	[68]
	S. mutans	Bioglasses	Quantitative method	+	[65]
	B. subtilis	Biocomposites	Optical density	+	[78]
			Agar diffusion test	+	[79]
	B. cereus	Bioglasses	Quantitative method and Optical density	+	[68]
	S. epidermidis	Bioglasses	Quantitative method	+	[66]
			MTT assay	+	[70]
	E. faecalis	Biomedical cements	Quantitative method	+	[74]
Yeast	*C. albicans*	Calcium phosphates bioceramics	Quantitative method and Agar diffusion test	+	[56]
			Quantitative method	+	[58]
			Quantitative method	+	[59]
		Biocomposites	Agar diffusion test	+	[79]
	P. notatum	Biocomposites	Agar diffusion test	+	[79]
	R. stolonifer	Biocomposites	Agar diffusion test	+	[79]
Antibiotic resistance	*E. coli* (ampicillin resistant)	Bioglasses	Quantitative method	+	[65]
	S. aureus (methicillin-resistant)	Biocomposites	Quantitative method	+	[76]

*"+"= antibacterial effect and "-" = no antibacterial effect

Table 3. Studies of angiogenic and osteogenic properties of Cu-doped biomaterials

Biomaterial		Angiogenic properties		Osteogenic properties		Ref.
Family	Description	Experimental method	Results*	Experimental method	Results*	
Bioceramics	Macroporous bioceramics scaffolds loaded with Cu	Intraperitoneal implantation during 15 days in mice	↗ micro-vessels formation			[112]
	Cu addition to calcium phosphate films			MC3T3-E1 culture (mouse pre-osteoblastic cells) Primary osteoclasts isolated from rabbit long bones	↘ MC3T3-E1 proliferation → MC3T3-E1 differentiation ↘ Primary osteoclasts resorptive activity	[131]
	Brushite scaffolds loaded with Cu			MG 63 osteoblastic cell culture	↗ activity and proliferation of osteoblastic cells ↗ expression of bone specific proteins (OPN, integrine β-1)	[128]

	Cu-doped calcium silicate bioceramics	HUVEC- HDF co-culture	↗ angiogenic patterns (nodes, circles and tubes) ↗ VEGF secretion			[113]
	Cu-substituted hydroxyapatite coating on titanium			MC3T3-E1 culture (mouse pre-osteoblastic cells)	→ cells proliferation	[82]
	Cu-substituted hydroxyapatite scaffold	Subcutaneously implantation in rabbit models for 8 weeks	↗ blood vessel formation			[119]
Bioglasses	Cu-doped bioactive borate glass			Implantation in rat calvarial defects for 12 weeks	↗ bone regeneration	[127]
	Cu-doped mesoporous bioactive glass scaffolds	hBMSC culture	↗ VEGF expression	hBMSC culture	↗ ALP activity from hBMSCs ↗ gene expression of ALP, OPN and OCN	[64]

	Bioactive Cu-doped glass scaffold	hBMSC culture hDMEC culture	↗ VEGF secretion from hBMSCs ↗formation of endothelial tube from HDMEC			[118]
	Borate-based bioactive glass scaffold doped with Cu	hBMSC culture Implantation in rate calvarial defect for 8 weeks	↗ ALP activity ↗ blood vessels formation	Implantation in rat calvarial defects for 8 weeks	↗ bone mineral density	[114]
	Cu-doped silicate bioactive glass scaffolds	MC3T3-E1 culture (mouse pre-osteoblastic cells) Implantation in rat calvarial defects for 6 weeks	→ cell viability, proliferation and attachment to the surface ↗ blood vessel formation	Implantation in rat calvarial defects for 6 weeks	→ bone formation	[117]
	Cu-doped borosilicate bioactive glass scaffold	Implantation in rat calvarial defects for 8 weeks	↗ blood vessels formation			[115]

	Cu-containing mesoporous bioactive glass	BAEC culture *Zebrafish* (Danio rerio) embryo assay	↗ cells migration ↗ number and thickness of subintestinal venous plexus			[116]
Biocomposite	Cu incorporation into chitosan scaffolds			Implantation in rat calvarial defects for 4 weeks	↗ bone formation	[132]
	Composite material with PDLLA polymer mixed with Cu doped bioactive glass	ST-2 mice bone marrow stromal cells culture	↗ VEGF secretion			[76]
	Biocomposite aerogel of mesoporous bioactive glass containing Cu and nanofibrillated cellulose	HUVEC culture 3T3 fibroblasts culture	↗ angiogenic patterns (branching point, tube length) ↗ blood vessel formation ↗ gene expression of Vegfa, Vegfc, Pdgf and Fgf2 for 3T3 fibroblast			[46]

	Cu-doped composite with a nano calcium-deficient hydroxyapatite and a multi-(amino acid) copolymer	rBMSC culture Implantation in rabbit femoral defect for 12 weeks	↗ adhesion and proliferation ↗ ALP activity ↗ blood vessel formation	rBMSC culture Implantation in rabbit femoral defect for 12 weeks	↗ ALP activity ↗ bone formation	[120]
Bone cements	Cu-containing calcium phosphate cement	mBMSC and HUVEC co-culture	↗mBMSC and HUVEC proliferation ↗VEGF	mBMSC and HUVEC co-culture	↗ gene expression of Col I, ALP, OPN and Runx2	[121]
	Cu-substituted dicalcium silicate cement			Implantation in a mandibular bone defect of rabbit for 16 weeks	↗ bone formation	[74]
Coated/alloyed metals	317L-Cu steel			MC3T3-E1 culture (mouse pre-osteoblastic cells)	↗ ALP expression ↗ expression of osteogenic genes (Col I, OPN, Runx2)	[129]

	Galvanic Cu deposit on titanium alloy			hBMSC culture	↗ ALP expression ↗ expression of osteogenic genes (Col I, OPG, OPN) ↗ mineralization of the cells	[86]
	Cu-bearing stainless steel			MC3T3-E1 culture (mouse pre-osteoblastic cells) Implantation in the lateral epicondyle of rats for 15 days	↗ ALP expression ↗ expression of osteogenic genes (Col I, OPN, Runx2) ↗ bone mineral density	[130]
	Titanium surface coated with Cu			SaOS-2 culture Implantation in rat femoral defect for 8 weeks	↗ gene expression of OCN and Runx2	[84]

*↗ : improvement, →: constant, ↘ : decrease