Copper-containing bioactive glasses and glass-ceramics: From tissue regeneration to cancer therapeutic strategies

Copper-Containing Bioactive Glasses and Glass-Ceramics: From Tissue Regeneration to Cancer Therapeutic Strategies

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Abstract

Copper is one of the most used therapeutic metallic elements in biomedicine, ranging from antibacterial approaches to cancer theranostics. This element could be easily incorporated into different types of biomaterials; specifically, copper-doped bioactive glasses (BGs) provide great opportunities for biomedical engineers and clinicians as regards their excellent biocompatibility and regenerative potential. Although copper-incorporated BGs are mostly used in bone tissue engineering, accelerated soft tissue healing is achievable, too, with interesting potentials in wound treatment and skin repair. Copper can modulate the physico-chemical properties of BGs (e.g., reactivity with bio-fluids) and improve their therapeutic potential. Improving cell proliferation, promoting angiogenesis, reducing or even prohibiting bacterial growth are counted as prominent biological features of copper-doped BGs. Recent studies have also suggested the suitability of copper-doped BGs in cancer photothermal therapy (PTT). However, more research is needed to determine the extent to which copper-doped BGs are actually applicable for tissue engineering and regenerative medicine strategies in the clinic. Moreover, copper-doped BGs in combination with polymers may be considered in the future to produce relatively soft, pliable composites and printable inks for use in biofabrication.

Keywords: Bioactive glasses (BGs); Copper; Angiogenesis; Antibacterial; Bone tissue engineering; Skin wound healing; Cancer theranostics

1. Introduction

Copper is among the necessary micronutrients for the human body, playing a critical role in the body hemostasis [1]. This transition metal exhibits two different ionic oxidation states (Cu(II) or Cu(I)), affecting the activity of numerous enzymes in the body, including the metabolism of methane, nitrite, and nitrous oxide [2]. Regarding its ability to act as an enzymatic cofactor or structural component, copper directly impacts cellular functions (e.g., respiration and free radical defense) as well as tissue biosynthesis [3]. For instance, it shows a pivotal role in the synthesis and stabilization of the extracellular matrix (ECM) of skin proteins as well as angiogenesis [4]. Experimental studies have shown that the misbalance in the copper amount in the body may result in adverse effects on vital functions such as tissue and organ development [5, 6]. Reduced bone mineral content and bone strength have been mentioned as a result of copper deficiency leading to skeletal abnormalities such as osteoporosis [7-9].

From a regenerative point of view, copper is required for the repair of several tissues and organs such as bone and skin. In this regard, it has been previously verified that the healing process of broken bones occurs faster in the presence of copper [10]. The desirable conditions for the acceleration of tissue healing are provided by some intrinsic properties of copper, including the abilities to improve cell proliferation, stimulating new vessel formation (i.e., angiogenesis), as well as inhibiting bacterial growth [11-14]. In an attempt to take benefits from these properties, a large number of studies focus on copper utilization in the appropriate formulations to enhance the reconstruction process of damaged tissues [15-18]. The incorporation of copper into the structure of bioactive glasses (BGs) is among the most promising approaches developed for tissue engineering strategies.

BGs show excellent physico-chemical and biological features regarding reconstructive procedures of human tissues like the bone and skin [19-21]. Although the initially developed BG, 45S5 Bioglass®, is comprised of four oxides in a simple composition (45 wt% SiO_2, 24.5 wt% CaO, 24.5 wt% Na_2O, and 6.0 wt% P_2O_5), newer formulations have been produced over the years through adding other therapeutic elements to improve the glass biological properties. Copper-containing BGs in different forms (e.g., powder, fibers, and porous scaffolds) have been found as potent materials for enhancing the healing process of both hard and soft tissues. As an illustration, three-dimensional (3D) scaffolds made of copper-containing mesoporous BGs (MBGs) were approved as multifunctional biomaterials for bone regeneration regarding their angiogenic capacity, osteostimulation, and antibacterial activity [22]. Importantly, the incorporation of copper into the BG network causes no adverse effects on glass bioactivity, i.e., the formation of an apatite-like layer on the glass surface is preserved during contact with biological fluids as well as the bonding to the bone *in vivo* [23, 24]. However, a high release of copper (Cu^+ and Cu^{2+} ions) from the construct into the biological environment may be toxic to the living systems [25]. Over time, in addition to bone-repair applications, copper-containing glasses and glass-ceramics were found to be useful in other interesting areas of science and medicine such as cancer therapy, opening up new horizons for biomedical scientists, especially for those focusing on the photothermal enhanced chemotherapy [26]. However, the number of *in vitro* and *in vivo* studies on cancer therapy via copper-doped BGs is fairly restricted, and more research should be conducted in the future to fully disclose the real potential of these constructs in cancer theranostics.

In the present study, we provide a state-of-the-art picture of the importance of copper-containing BGs as versatile materials in medicine, from hard and soft tissue engineering to cancer

photothermal therapy (PPT). For this aim, different types of copper-doped BGs along with their fabrication and characterization approaches are firstly introduced, and then the physico-chemical and biological outcomes will be well discussed. As an important part of the current study, we represent all medical aspects of copper-containing BGs to pave a way ahead of their clinical usage. To the best of the authors' knowledge, this is the first review paper that specifically focuses on the role and potential of Cu-doped biomedical glasses and glass-ceramics.

2. Biological roles of copper

2.1. Copper affects osteogenesis

Copper is recognized as an essential metal for the normal growth and development of the human skeleton; previous studies clarified the ability of this element in inducing osteogenic differentiation of mesenchymal stem cells (MSCs) [27]. In addition, copper is reported as an effector in improving ALP activity, collagen type I secretion, and osteogenesis-related genes (collagen-I and osteocalcin) expression in mouse bone marrow stromal cells (BMSCs) [28]. Oncostatin M (OSM) pathway was found to be one of the signaling pathways activated by copper for advancing osteogenic differentiation of MSCs [29]. However, some reports stated that copper at high concentrations (> 5 μM) may inhibit osteogenesis via the down-regulation of Runx2, i.e., the main regulator of osteoblast phenotype [30]. The effect of copper in collagen deposition was also evaluated, exhibiting a stimulatory impact on the formation of newly organized collagen fibrils and fibers [31]. These biological phenomena could be useful for accelerating the bone healing process. In brief, it seems that more research is required to reveal

other molecular signaling pathways by which copper stimulates osteogenesis in mammalian bone cells and osteogenic differentiation in stem/progenitor cells.

2.2. Copper promotes angiogenesis

Angiogenesis, the sprouting of new blood vessels from pre-existing ones, is among the most vital parameters for having a successful regeneration process of living tissues, as it is responsible for supplying nutrients and oxygen as well as removing the wastes from cells and tissues. Several biomolecules (e.g., vascular endothelial growth factor (VEGF) and angiogenin (ANG)); extracellular signaling pathways (e.g., hypoxia-inducible factor (HIF)-1 and the notch/delta); and intracellular proteins (e.g., hedgehog and sprouty) play critical roles in angiogenesis process [32, 33].

The first evidence on the angiogenic effects of copper was observed by McAuslan and Reilly in 1980 when they found that copper salts could stimulate the migration of endothelial cells (ECs) *in vitro* [34]. Since then, determining molecular events involved in angiogenesis, which was triggered by copper, has been the concept of a large number of studies from those concentrated on cancer treatment to those focused on tissue repair and regeneration. The outputs of previously performed work clarify the regulatory effects of copper on angiogenesis via remodeling of the extracellular matrix (ECM) [31, 35]. In addition, copper could stimulate angiogenesis by implying its influence on various molecules involved in the initiation process (vasodilation and vascular permeabilization) as well as maturation (ECs proliferation, migration, and morphogenesis) (see Figure 1). For example, it was reported that Cu^{2+} ions could increase the binding of angiogenin, a potent angiogenesis stimulator, to calf pulmonary artery ECs by 4.3-fold *in vitro*, thus improving new blood vessel formation [36]. Several experimental studies have

clarified the stimulatory effect of copper on the growth, proliferation, and migration of human ECs in vitro [37]. In addition, copper in the form of $CuSO_4$ at a final concentration of 5 mmol/L could stimulate the growth of human umbilical vein endothelial cells (HUVECs) via the VEGF-independent pathway, which was demonstrated by the increase of the number of cells in the S phase [38]. However, caution should be exercised in the case of copper nanoparticles as they may induce oxidative DNA damage and cell death in vascular ECs [39].

VEGF is a potent mitogen factor for endothelial cells and has a significant impact on angiogenesis through the activation of a couple of cell signaling pathways [40]. In 2014, Li et al. reported that copper could stimulate angiogenesis at the organ system level (in a manner that depends on VEGF) as well as improve the proliferation of vascular endothelial cells in culture systems (without VEGF dependency) [41]. ANG also has a potent effect on angiogenesis (cell migration, invasion, proliferation, and formation of tubular structures) through the interaction with endothelial cells. This molecule elicits its angiogenic effects via well-defined routes, including (1) applying ribonucleolytic activity; (2) inducing the degradation of the basement membrane; (3) stimulating signaling transduction; and (4) facilitating nuclear translocation and subsequent increasing ribosomal RNA transcription [42]. As previously reported, copper could significantly promote the expression and biogenesis of ANG in endothelial cells, thereby triggering angiogenesis [43].

The role of ECM has been well documented in modulating angiogenesis both in physiological and pathological conditions [44, 45]. Fibronectin, as one of the components of ECM, controls endothelial cell (EC) survival and plays a significant role in microvessel elongation, thereby improving angiogenesis [46, 47]. The experimental data have shown that copper could evoke the biosynthesis of fibronectin in bovine ECs and, thus, it may control the stimulation of EC

migration and angiogenesis [48]. Prostaglandin E-1 (PGE-1) has also been found to stimulate angiogenesis by the up-regulation of VEGF expression [49, 50]. The relation of the copper level with PEG-1 function has been shown in an animal study on rabbits, in which lowering of copper resulted in blockage of angiogenesis stimulated by PGE-1 [51]. Ceruloplasmin, as the copper carrier of plasma, is another molecule that could be angiogenic in the presence of copper [52]. Fibroblast growth factors (FGF 1 and 2) are other potent angiogenic factors, which could indirectly control the neovascularization process in concert with other growth factors like VEGF [53]. Another signaling pathway is related to the ability of copper in regulating potent angiogenic factors like FGFs [54, 55].

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor having two different subunits, i.e., HIF-1α and β. It has been shown that the subunit α is oxygen-sensitive and is over-expressed under hypoxic conditions [56]. This factor has a pivotal role in the activation of a couple of angiogenic molecules like VEGF. Therefore, it is potentially used as a therapeutic target to increase (in tissue reconstruction strategies) or decrease (cancer therapy) angiogenesis. Previous studies have shown that copper could affect HIF-1 α, mediated by a copper chaperone, and stabilize it in the nucleus of cells, thereby simulating hypoxia. All the mentioned events result in the activation of VEGF and subsequently enhanced angiogenesis [57].

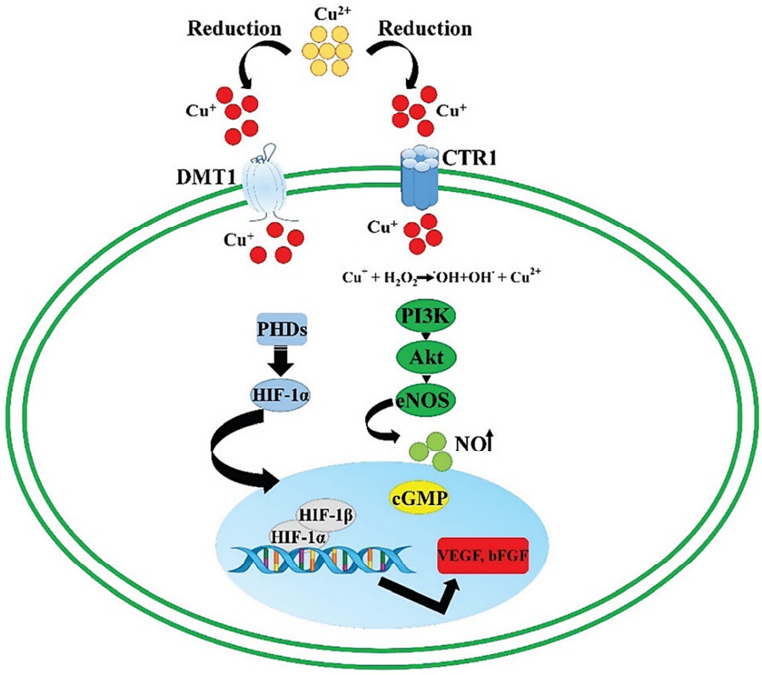

Figure 1. Schematic illustration of the copper roles in angiogenesis regulation. As depicted, the entrance of copper ions into the cells may be mediated by two distinct cell membrane transporters, i.e., the copper transporter Ctr-1 and DMT1 proteins. In the cell, copper transport proteins (chaperones) such as Atox-1 deliver this ion to intracellular proteins. Copper can facilitate the translocation of the factor into the nucleus via inhibiting PHD-mediated hydroxylation of HIF-1 α, which results in its dimerization with HIF-1 β and consequent interactions to hypoxia-responsive elements and VEGF gene over-expression. In addition, copper ions can activate the molecular signaling pathways leading to an increase in the production of nitric oxide (NO) and thereby induce angiogenesis. Reproduced from ref [58].

2.3. Copper exhibits antibacterial activity

Copper is known to act as both an antibacterial particle (e.g., nanoparticles) and a surface agent against various bacterial strains [59]. This element was reported as the first metallic antimicrobial agent by the United States Environmental Protection Agency (EPA) in 2008 [60] thanks to its low price, ease of mixing with various polymers, and relative stability of its

chemical and physical properties [61]. A few mechanisms have been well-defined by which copper could kill bacteria, like damage to the outer plasma membrane and subsequent disruption of membrane integrity, transport protein activity, and ion permeability [59, 62, 63]. Oxidative stress (i.e., production of reactive oxygen species (ROS)) and DNA degradation are two other actions of copper for killing bacteria via damage to lipids, proteins and nucleic acids [64]. Several parameters could affect the antibacterial property of copper, including size, shape, concentration, and type of microorganisms [65]. For instance, Palza et al. synthesized a series of copper-doped sol-gel silicate BGs and compared their antibacterial effects with the silver-doped counterparts [66]. The basic composition of the glasses was 62.3 SiO_2, 28.9 CaO, and 8.6 P_2O_5 (wt.%), in which small amounts of silver and copper were incorporated. The data obtained from minimal bactericidal concentration (MBC) assay revealed the antibacterial activity of the glass particles against *Escherichia coli* (*E. Coli*) DH5 α ampicillin-resistant (Gram-negative) and *Streptococcus mutans* (*S. Aureus*) (Gram-positive). The authors stated that the antibacterial behavior of the glasses depends on the bacteria strain and the biocide ions in the composition.

Mishra et al. in 2017 assessed the thermal, structural, and antibacterial effects of copper- and iron-doped phosphate glasses with a formulation of [xCuO or Fe_2O_3 + (100 - x) (0.2 CaO + 0.2 SrO + 0.1 Na_2O + 0.5 P_2O_5)] (mol%), in which x ranges from 0 up to 5 [67]. The authors found that the incorporation of copper into the glass increases the sintering window and the dissolution rate of the samples. The results obtained from the antibacterial test confirmed that 5% copper-containing glass particles (< 125 µm) at a concentration of 1200 mg/mL had a significant inhibitory effect on *Staphylococcus epidermidis* in comparison to the commercial S53P4 glass. The addition of copper to mesoporous BGs (MBGs) was also carried out to prepare antibacterial materials. As an illustration, Koohkan et al. could synthesize copper-doped MBGs (68 SiO_2–23

CaO–4 P_2O_5–5 CuO (mol%)) in two distinct settings by using Pluronic P123 (named as Cu-PBG) and without the surfactant (named as Cu-BG) [68]. The copper-doped BGs were incubated with *E. Coli* at a concentration of 100, 50, 25, and 12.5 mg/mL, and the minimum inhibitory concentration (MIC) and MBC assays were performed to show the antibacterial activity of the samples. Their results clarified that the Cu-PBGs had a better antibacterial effect during the first 24 h, probably because of a greater release of Cu^{2+} and Ca^{2+} ions into the environment in comparison to the Cu-BGs. The authors stated that this result could be related to the higher specific surface area of the surfactant-templated mesoporous material than the ‘‘conventional” sol-gel BG, yielding improved ion exchange phenomena in the testing medium. It is worth noting that copper exhibits other antimicrobial properties, including antifungal and antiviral activities, which may be interesting for designing and developing a new class of glasses in the future.

2.4. Copper cytotoxicity

Although copper at low concentrations plays a pivotal role in the regulation of some critical functions of the human body (e.g., hemostasis and bone formation), an excess amount of this element could produce free radicals and results in toxicity or inflammation [69]. It was reported that nano-sized particles (23.5 nm) of copper are more cytotoxic substances than microparticle counterparts (17 μm) [70]. The copper nanoparticles (□100 nm) at different concentrations (5, 10, 20, and 40 μg/ml) showed the ability to inhibit the expression of a series of important genes of the ERK signaling pathway, including BRAF, ERK, and MITF [71]. In another study, Feng et al. reported that copper sulfide nanoplates, a common substance used for PTT, cause a significant decrease in the viability of bone marrow-derived MSCs (RAW 264.7 cell line) and

ECs at a concentration higher than 100 μg/mL. Moreover, the results obtained from *in vivo* study revealed that the maximum tolerated dose of copper is 8.66 mg/kg, and its lethal dose 50 is 54.5 mg/kg. In 2018, Kaur et al. evaluated *in vitro* toxicity of copper-containing MBGs having a formulation of $(25 - x)CaO - xCuO - 10P_2O_5 - 5B_2O_3 - 60SiO_2$ (x= 2.5, 5, 7.5, 10 mol%) [72]. All the samples were toxic against the J774A.1 murine cell macrophages at concentrations 1.95, 3.91, 7.8125, 15.625, 31.25, 62.5 μg/mL. The 2.5% Cu- MBGs was the only sample with no significant toxicity at the concentration of 0.98 μg/mL. The authors reported that all the samples cause higher cell viability in comparison to the controls at concentrations of 0.12, 0.06, and 0.03 at 0.24 μg/mL.

3. Synthesis and physico-chemical properties of copper-doped bioactive glasses and glass-ceramics

3.1. Synthesis methods for the production of copper-doped BGs

Since the invention of BGs, it was clear that one of the most appealing properties of these materials was the possibility to tune their biological response by varying their composition. It was demonstrated that cellular pathways beneficial to bone regeneration could be triggered by the interaction between ion dissolution products and the biological environment [73].

As discussed in the section 2, with specific reference to the role of copper, doping BGs with therapeutic metallic ions could be an effective way to promote osteogenic, angiogenic, and antibacterial response both *in vitro* and *in vivo*. Moreover, this approach could represent a valuable alternative to the use of drugs/antibiotics and expensive growth factors, which are currently behind the clinical treatment of most bone diseases [74, 75].

The design of the glass composition and the synthesis method selected for its production are both key aspects in determining the physico-chemical properties of the final material and, thus, they should be carefully considered in order to avoid undesired effects on the living tissue, i.e., acute cytotoxicity and inhibition of osteogenic activity [76].

Copper can be introduced in the network of BGs at different oxidation states (Cu^{2+} and Cu^{+} ions or metallic Cu^{0}) either in the form of network modifier or metallic nanoparticles [77-79], and several synthesis methods, including the melt-quenching route [80], the sol-gel process [68] and the ion exchange [23] have been already reported to be effective in the production of silicate, borate, and phosphate Cu-doped BGs showing enhanced antibacterial and angiogenetic properties. In general, according to the oxidation state, specific biological effects could be induced. As reported by Meghana et al. [81], Cu_2O nanoparticles exhibited high affinity with bacteria by binding proteins, while the bactericidal effect of CuO nanoparticles was mainly based on the production of ROS.

According to the final purpose, it is possible to modify the Cu oxidation state by means of specific physical and chemical treatments applied to the material. In a recent study, Miola et al. [82] induced the reduction of Cu^{2+} ions directly on the surface of a silicate BG performing a combination of physical and/or chemical treatments, thus increasing the surface-to-volume ratio, the antibacterial action, and the surface functionalization potential.

Among the physical treatments, UV-light irradiation and thermal treatments both in air and in argon were performed, while chemical treatments included the exposure to tannic acid, sodium L-ascorbate, and sodium hydroxide. In this regard, the most effective reducing agents from Cu^{2+} to metallic copper (Cu^{0}) were proved to be tannic acid and sodium L-ascorbate, while thermal treatments in reducing atmosphere (argon flowing) led to the formation of Cu_2O. [82].

The Melt-quenching route is maybe the most common technique used for glass production, not only in the biomedical field [83]. As regards Cu-doped BGs, the melting process was extensively used by several research groups to produce silicate, borate, and phosphate systems for different clinical applications [80, 84-86]. Cu-doped melt-derived BGs can be obtained by following two different strategies. In the first case, Cu is introduced in the glass network by adding a proper precursor to the initial batch of reagents that will be melted at high temperatures. Some examples of reagents used for Cu-doping of melt-derived BGs include $CuNO_3$ [84], CuO [80], and $Cu(NO_3)_2 \cdot 2.5(H_2O)$, all in the form of powders [85]. In the second case, the melt-quenching route is used to produce the basic copper-free glass that will be then doped by ion-exchange procedures, which are typically carried out by immersing glass powders into a copper-containing aqueous solution for a certain period of time. In this regard, Miola et al. [23, 82] performed Cu-doping by immersing melt-derived BG powders inside a copper acetate aqueous solution at 37°C for 1 h in order to introduce copper as a glass modifier in the glass network at the expense of Na^+ and Ca^{2+}.

Cu-doped glasses can also be produced by the sol-gel process, which is a chemical-based synthesis method characterized by remarkably lower processing temperatures and higher versatility – including wider bioactive compositional ranges - as compared to melt-quenching routes. Sol-gel glasses can be obtained in different forms, all characterized by a very high specific surface area, thus determining appealing properties in terms of bioactivity, texture, and ion release [87]. In particular, the possibility of obtaining MBGs, characterized by a well-ordered porosity at the mesoscale, makes the sol-gel process combined with supramolecular chemistry a very effective strategy in the realization of bioactive systems for the controlled release of antibacterial copper ions [88, 89]. This option discloses highly promising perspectives in the

production of antibiotic-free antibacterial – and in general therapeutic – biomedical glasses, as comprehensively discussed by Kaya et al. [90]. In a recent study, Bari and coworkers [17] obtained Sr-Cu co-doped MBG particles with good co-release properties and controlled textural features for drug and biomolecule delivery. This study compared MBGs obtained by ultrasound-assisted sol-gel technique [17, 91] with those obtained by a new aerosol-assisted spray-drying method by considering changes in textural properties, ion release kinetics, and geometrical particle features. The use of different mesostructure directing agents, i.e., CTAB and Pluronic P123 for ultrasound-assisted sol-gel technique and aerosol-assisted spray-drying method, respectively, led to producing MBGs with different mesopore size. In particular, CTAB yielded smaller pores (2.5 nm) compared to P123. As a result, Sr-Cu MBGs produced by the spray-drying method exhibited lower specific surface area (172 m^2/g) as compared to the material obtained by ultrasound-assisted sol-gel synthesis (470 m^2/g), determining different ion release kinetics.

The higher versatility of the sol-gel method is mainly due to the possibility to get different products by properly acting on the high number of process parameters involved in the synthesis process, i.e., pH, temperature, solvent type, oxides precursors, and catalysis conditions (acidic or basic) [92]. As an example, higher ethanol/TEOS ratios and higher amounts of ammonia were demonstrated to improve antibacterial properties and inhibit the hemolytic effect of Cu-doped BG nanoparticles in the quaternary SiO_2–CaO–MgO–CuO system [93].

In 2017, Gupta and coworkers [94] first described a novel approach for the introduction of Cu in the BG network, called a bio-inspired route. The method is based on the self-organization and direct assembly of biological macromolecules, which allow the formation of a material with a well-ordered hierarchical structure, similar to some nanostructured materials available in Nature.

Unlike conventional sol-gel synthesis, this method does not require any calcination process to remove organic residues and consolidate the BG structure as the synthesis is thoroughly carried out under ambient conditions and in the aqueous phase, without the need for any organic solvent. The potential of this approach was recognized to be enormous from many viewpoints (easy technology, eco-friendly, relatively inexpensive); moreover, avoiding the calcination step could be beneficial to the textural properties of the material as heating treatments could determine the collapse of the nanostructure.

Table 1 provides an overview of literature results concerning Cu-doped BGs for tissue engineering applications along with the production methods used.

Table 1. A comprehensive overview of the latest advances in the production of Cu-doped BGs for tissue engineering applications.

Material	**Composition**	**Method of production**	**Oxidation state**	**Application**	**Relevant notes**	**Ref**
Cu- Sr mesoporous bioactive glass NPs	SiO_2-CaO-CuO-SrO	US-assisted sol-gel method/ aerosol-assisted spray-drying method	Cu^{2+}	Bone regeneration and prevention of infection	Co-release of antibacterial ions	[17]
Cu-Silicate BGs	SiO_2-P_2O_5-CaO-CuO (0,1,2 wt.%)	SG	Cu^{2+}	BGs with antibacterial properties	Comparison with Ag-doped bioactive glasses	[66]
Cu-Mesoporous silicate bioactive glasses	SiO_2-CaO-P_2O_5-CuO	SG with/without mesostructure directing agent (P123)	Cu^{2+}	Multifunctional scaffolds for BTE applications (drug/biomolecules delivery systems)	P123 improved biocompatibility of the material	[68]
Cu-Silicate BGs	MgO-ZnO-CaO-SrO-P_2O_5-SiO_2-CaF_2-CuO (0,1, 3, 5 mol.%)	MQ	Cu^{2+}	Bone tissue engineering	Improved ion dissolution and hindering of biomineralization mechanism	[80]
Cu- bioactive glass	SiO_2 - Na_2O – CaO - P_2O_5 - B_2O_3 - Al_2O_3	MQ/ion Exchange	Cu^0, Cu^+, Cu^{2+}	BGs with antibacterial properties	In situ reduction of copper by chemical/physical treatments	[82]
Cu-Borate and borosilicate BGs	Na_2O-K_2O-MgO-CaO-B_2O_3-P_2O_5-SiO_2-ZnO-CuO	MQ	Cu^{2+}	Infection treatment/prevention	Dissolution and biomineralization study in different environmental conditions	[85]

Cu mesoporous bioactive glasses NPs	SiO_2-CaO-CuO (2-5 mol.%)	One-pot US-assisted sol-gel method	Cu^{2+}	Bone regeneration and prevention of infection	Bactericidal effect against three different bacterial strains	[91]
Cu-silicate bioactive glasses	SiO_2-P_2O_5-Na_2O-CaO	Bio-inspired synthesis	Cu^{2+}	Bone regeneration	Higher network connectivity in comparison to Cu-free control.	[94]
Cu-Borosilicate BGs	Na_2O-K_2O-MgO-CaO-B_2O_3-P_2O_5-SiO_2- CuO (0,1,3 wt.%)	MQ	Cu^{2+}	BG-scaffolds with angiogenetic properties	Enhanced angiogenetic properties *in vivo*	[95]
Cu-Silicate BGs	SiO_2-P_2O_5-CaO-CuO (1, 5, 10 mol.%)	SG	Cu^{2+}	BGs with antibacterial properties	Better in providing long-term antibacterial protection, compared to Ag-doped glasses	[96]
Cu-BG nanoparticles	SiO_2-CaO-CuO (0,5,10,15 mol.%)	SG/Modified Stober method	Cu^{2+}	Bone regeneration and wound healing products	Good apatite forming ability, sustained release of Cu ions , no cytotoxicity	[97]

* MQ: melt-quenching route; SG: sol-gel route.

3.2. Thermal, microstructural and textural properties of Cu-doped BGs and glass-ceramics

There is convincing evidence of how the incorporation of metallic elements in the glass network may result in specific variations in the thermal response, microstructure, morphology, textural properties, and reactivity of the final material [75].

The effect of copper inclusion on glass particles size and geometry is controversial: despite Goh et al. [96] reported no significant variation for increasing Cu amounts, a very recent study by Chitra and coworkers [98] showed that the incorporation of copper in sol-gel BGs led to a deep modification in the morphology and the structure of particles, which evolved from a spherical shape to cluster-like cubes, as depicted in Figure 2.

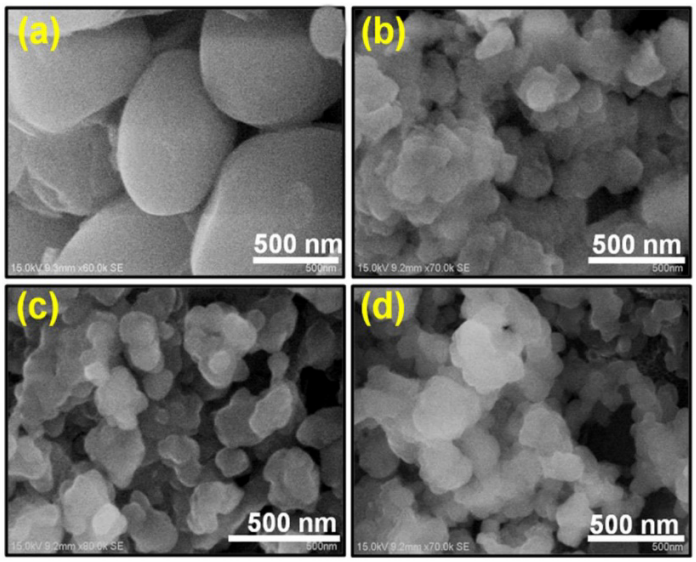

Figure 2. FE-SEM images showing particle morphology for increasing amounts of copper - (a) Cu-free BG, (b) 0.5Cu-BG, (c) 1.5Cu-BG and (d) 2.5Cu-BG. Reproduced from ref [98].

Moreover, XRD assessments showed an increment in cell volume, while FT-IR and Raman spectroscopy revealed dominant phosphate bands due to copper deposited on the phosphate sites and latent silica bands due to the disruption of Si-O-Si bonds. Copper, in fact, is an intermediate element that is able to enter the glass network as a modifier, thus creating two oxygen bridges and causing the weakening of the glass network. In general, when a metallic oxide is introduced in the glass network, Si-O-Si covalent bonds are broken, and metal-oxygen bonds form accordingly. Such bonds, due to their ionic nature, are weaker than the covalent ones, and this may result in a different thermal response of the material, depending on the copper content.

Wers et al. [99] investigated the effect of Cu doping in different amounts (0, 0.1, 1, 5, and 10 wt.%) on the thermal behavior of melt-derived 46S6 bioactive glasses. Consistently to what previously observed, the introduction of Cu in the glass network resulted in significant changes in the thermal response of the material. The introduction of the dopant at the expense of CaO

remarkably increased the degree of internal strain relaxation. As a result, both glass transition temperature (T_g) and crystallization temperature (T_x) decreased with the increase of Cu content (Figure 3a). However, the most important effect was observed on the melting temperature, where the increase of the Cu content led to a linear decrease in the melting point of the material, as shown in Figure 3b.

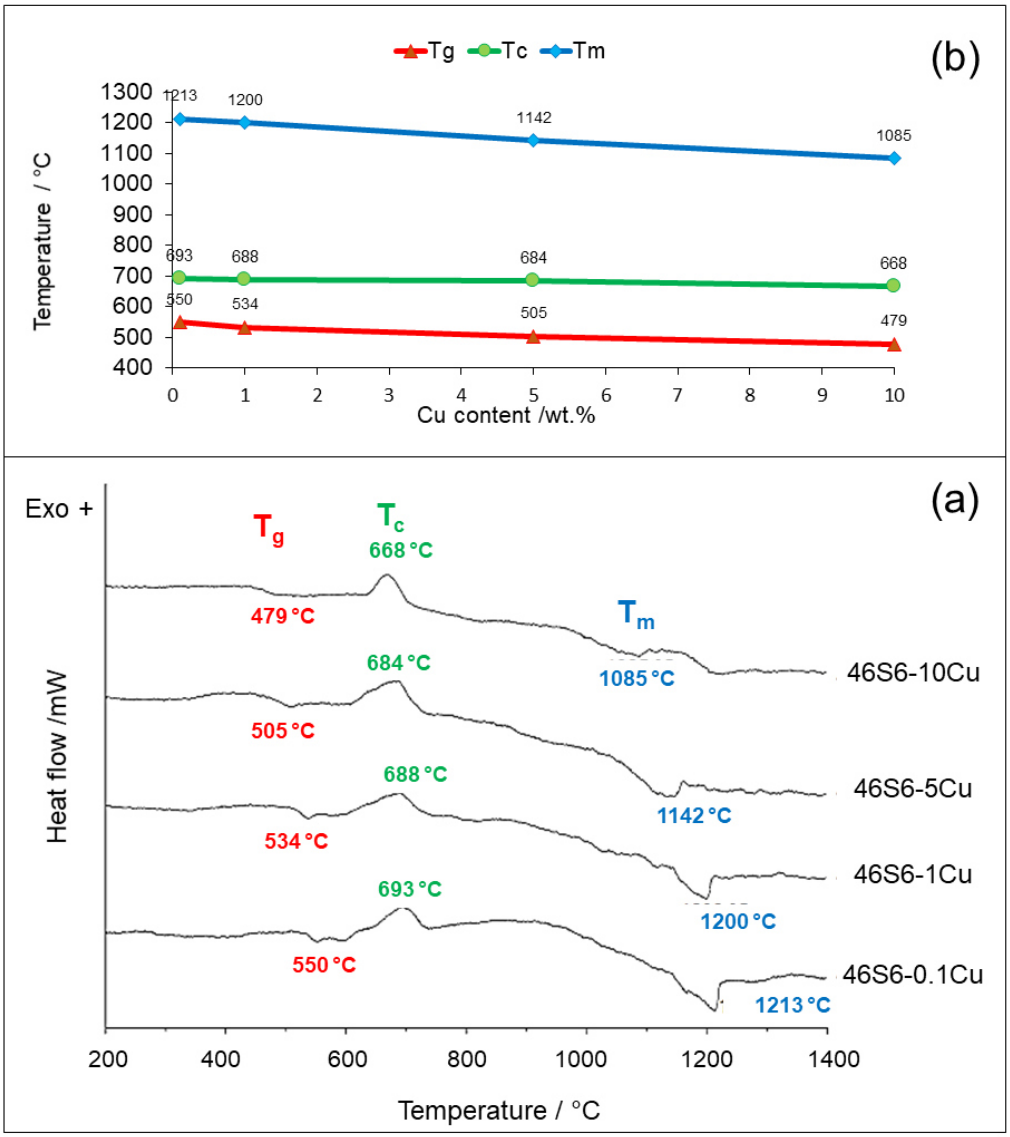

Figure 3. DTA thermograms of Cu-doped 46S6 bioactive glasses (a) and Tg, Tc, and Tm trends as a function of the Cu amount (b). Graphic representation of experimental data reported by Wers et al. [99].

By direct comparison with analogous Ti-doped glasses, the authors attributed the modification in the thermal behavior of the glasses to the chemical characteristic of the dopant, too, considering the influence of the melting point and the electronegativity of copper. A brief overview of the chemical properties of copper compared to those of the most common bivalent ions is provided in Table 2.

Table 2. Chemical features of copper as compared to those of the most common bivalent ions acting as network modifiers in the BG network [100-102].

Chemical element	Ionic radius (A)	Electronegativity	Melting point (°C)
Cu	0.73	1.90	1084.6
Ca	0.99	1	839
Mg	0.72	1.31	649
Sr	1.12	0.95	769

The same effect of Cu-doping on characteristic temperatures was also observed by Miola et al. in a silica-based melt-derived glass in the system 48% SiO_2 - 26% Na_2O - 22% CaO - 3% P_2O_5 - 0.43% B_2O_3 - 0.57% Al_2O_3 [82].

Interestingly, it was found that introducing Cu in the glass network could have a beneficial effect in improving the thermal stability of the glass, broadening the so-called workability window, i.e., the temperature range in which it is possible to thermally treat the material avoiding its crystallization. This could be a remarkable advantage, especially in scaffold manufacturing, where glass sintering is of fundamental importance to obtain mechanically resistant grafts while preserving their bioactivity in contact with body fluids [99]. It is known, in fact, that crystallization upon sintering may result in a dramatic decrease of the apatite-forming ability of

BGs due to the reduction of the amorphous phase, which is more reactive compared to crystalline one(s) and involved in the ion-exchange phenomena behind the bioactive process [103].

Considering sol-gel glasses, the most critical step to be opportunely designed in order to prevent crystallization is the calcination treatment of dried gels, which is required for allowing the thermal elimination of residual organic compounds and the glass consolidation. In order to do this, the thermal characterization of gels could be useful to identify crystallization peaks upon heating.

However, sometimes it could be useful to induce glass devitrification as a potential strategy to enhance the mechanical and biological properties of the material by the production of glass-ceramic systems. As an example, Cu-doped sol-gel quaternary glass-ceramic materials in the SiO_2-CaO-MgO-CuO system with modulable crystallinity and degradation rate were obtained by inducing crystallization at different temperatures. The nucleation of wollastonite multilateral crystalline phases observed above 830 °C was intentionally induced for its capability to independently establish a chemical bond with host bone by the formation of hydroxyapatite on the surface of the material. Moreover, the nucleation of this crystalline phase was reported to favor a controlled and sustained ion release from the material to the biological environment, as well as to improve the mechanical properties [104].

Goh et al. investigated the effect of Cu incorporation on the microstructural properties of sol-gel ternary glasses in the system SiO_2-CaO-P_2O_5 doped with 1, 5, 10 mol.% of CuO. XRD patterns revealed no differences between the undoped system and the doped ones, regardless of the amount of Cu included in the material. As no Cu-containing phases were identified, copper was supposed to be incorporated in the glass network only in the form of the bivalent cation (Cu^{2+}),

and its reduction to metallic copper was avoided by performing the heating treatment in oxidizing conditions [96].

In another study, Bejarano et al. [105] demonstrated that the crystallization tendency of Cu-doped glasses is strongly dependent on the composition of the system analyzed. This study analyzed the crystallization behavior of ternary (58S) and quaternary (Na-doped) glasses (NaBG) produced by the sol-gel method. CuO nucleation was observed both in 58S- and NaBG-based systems, where the intensity of the diffraction peaks assessed by XRD investigations was directly related to the amount of Cu introduced into the composition. In addition, the presence of Cu in NaBG determined the formation of an apatite-like phase together with Ca_2SiO_4. Coesite, cristobalite, and other calcium silicates were also detected for higher amounts of dopant.

The formation of cristobalite and $CaSiO_3$ was also observed in ternary sol-gel systems doped with 0.2-5 mol.% Cu [106], where their formation was attributed to Coulomb repulsion generated by the high ionic potential of Cu ions and the octahedral coordination (CN=6), resulting in immiscibility and phase separation. However, the crystallization tendency of Cu-doped BG was found to be remarkably lower compared to that of Ag-doped BG, as demonstrated by the broad and low-intensity diffraction peaks observed by Palza et al. in SiO_2-P_2O_5-CaO glasses doped with 1 and 2 wt.% CuO upon XRD analysis [66].

Textural properties of Cu-doped gel-derived BGs were also investigated by several research groups in order to elucidate if copper content may play a role in somehow modulating them. Overall, most results suggest that the higher the copper content, the lower the structural arrangement of mesopores and the specific surface area of Cu-doped MBGs. In a study by Wu et al. [22], it was first reported that the inclusion of Cu^{2+} in the glass network led to a decrease of specific surface area, pore volume, and mesopore size as compared to the undoped system. This

tendency was confirmed by Bari et al. [91] and Palza et al. [66], who observed a decrease of specific surface area by half as a result of the introduction of 5 mol.% and 2 wt.% of Cu, respectively, in the glass formulation. These findings are also in line with those reported by Baino [89], showing that the pore volume and specific surface area of Cu-doped MBGs significantly decreased as the copper content increased from 1 to 5 mol.%, while the mean mesopore size (5.1 nm) was not apparently affected by the amount of copper. A similar copper-depending trend of pore volume and surface area was also observed by Luo et al. [107] for Cu-doped nanofibrous mesoporous glass scaffolds. These results suggest that the incorporation of Cu^{2+} ions may have a negative effect on the precursor condensation, disrupting the ordered orientation of $(SiO_4)^{4-}$ units during the self-assembling reaction of the glass. The mechanism behind this effect in MBGs, although being still unclear, might be related to the difference of ionic radii: copper typically replaces calcium (both are bivalent modifiers) but the difference between the ionic radius of copper and the ionic radius of silicon (which is the forming element of glass network) is larger than the difference between the ionic radius of calcium and the ionic radius of silicon (ionic radii: 0.210 nm for Si^{4+}, 0.231 nm for Ca^{2+}, 0.140 nm for Cu^{2+}).

In apparent contradiction with the above-mentioned studies, Bejarano and coworkers [105] reported an opposite trend for the textural properties of Cu-doped 58S-based BGs. Compared to the parent 58S system, Cu-doped quaternary glasses exhibited a reduction of the specific surface area, which was shown to increase for increasing contents of the dopant [105]. This different result compared to the majority of the literature could be attributable to the different processes used to produce the glasses, as the 58S system was synthesized by a conventional sol-gel route without the incorporation of any surface-directing agent, as done during the synthesis of MBGs.

3.3. Bioactive properties of Cu-doped BGs and ion release

The effect of copper inclusion on the apatite-forming ability of BGs and interfacial reactions with biological fluids is still quite controversial. To date, in fact, the available literature does not allow identifying a unique trend related to Cu-doping on the bioactive properties of glasses for tissue engineering applications. Some experimental studies showed a clear improvement of the hydroxyapatite-forming ability on the BG surface with faster formation rates and reaction kinetics [97], while others revealed a sort of inhibitory effect of Cu-doping on the formation of the reaction layer at the glass/tissue interface [95].

For example, Zheng et al. reported that the incorporation of copper into BG nanoparticles led to positive effects on the apatite formation, which was observed after just 3-day immersion in simulated body fluid (SBF). Moreover, a sustained release of both Cu and Si ions was observed up to 14 days, thus proving the suitability of the material to be used as a therapeutically active system for copper delivery [97].

On the contrary, according to other studies, copper was found to have a stabilizing effect on the glass network, thus slowing down the reactivity rate in contact with body fluids, analogously to what was observed in Zn-doped BGs [105].

The second scenario was also supported by a recent work by Kapoor et al. [80], who investigated the effect of transition metal on the properties of alkali-free BGs: bioactivity tests in SBF revealed that Cu-doping increased the dissolution of Si, Sr, Ca, Mg and Zn ions due to the higher covalent character of Cu-O bonds compared to that of Ca-O bonds, but inhibited biomineralization mechanism due to the competition between Ca^{2+} and Cu^{2+} ions in forming phosphate species on the surface of the material [80]. Incorporation of copper within the newly-formed calcium-phosphate layer was also reported [18].

However, despite these apparently controversial results, most of the experimental works agree that Cu-doping does not provoke any really remarkable changes in the bioactive behavior of biomedical silicate glasses and does not hinder the ion release mechanisms through the newly-formed apatite-like layer [66, 82, 91, 96, 108].

In a study by Schuhladen et al. [85], the dissolution and bioactive behavior of Cu-doped borate and borosilicate glasses were tested in different media under both static and dynamic conditions in order to mimic as closely as possible the real biological environment. Higher dissolution rates were reported for borate glasses compared to silica-based ones, while borosilicate systems exhibited an intermediate behavior. Despite continuous flowing conditions favored the overall glass dissolution mechanism, therapeutically relevant amounts of copper were released in all the experiments, while boron release was inhibited in SBF due to the structural differences existing between borate and silicate glasses.

Apart from modulating the reaction kinetics, the introduction of copper was proved to affect the morphology of the apatite layer formed on BGs and apatite crystal size: according to Wers and coworkers, the layer of hydroxyapatite formed on the surface of Cu-doped BGs upon soaking in SBF for 30 days appeared to be constituted by more heterogeneous apatite crystals compared to the Cu-free system [109].

4. Biomedical applications of copper-doped bioactive glasses and glass-ceramics

In order to take advantage of copper-modulated properties, several tissue-engineered (TE) constructs have been developed and applied to mainly improve bone regeneration and wound healing process via an improved cell proliferation, angiogenesis, and prohibition of the bacterial growth in the damaged sites [110, 111]. Regarding the literature, copper-containing glasses,

either alone or in combination with polymers, are being utilized in reconstructive strategies of both hard and soft tissues.

4.1. Bone tissue engineering

Historically, BGs in different forms (powders, particles, and scaffolds) have been considered for the repair and reconstruction of hard tissues like the bone and tooth [112-114]. Several studies have well-documented the effectiveness of BGs towards osteogenic differentiation either in static or dynamic conditions. For example, bioactive phosphate glass microspheres doped with 5 and 7 mol% of titanium dioxide were proposed as suitable platforms for the effective culture of MG63 osteoblastic cells without eliciting any cytotoxic effect [115].

The evaluation of copper-doped glasses for *in vitro* and *in vivo* osteogenesis has been a highly interesting research topic in biomaterials science and regenerative medicine; therefore, plenty of experiments tried to reveal cellular and molecular responses post-exposing to them [116, 117]. There are a couple of studies mentioning that copper has an inhibitory effect on osteogenesis along with down-regulation of osteogenic genes, thus hindering the formation of new bone tissues [118, 119]. For instance, Lin et al. showed that 0.4 and 0.8 wt.% copper-doped silicate 13–93 BG-based scaffolds had no significant effect on the number and alkaline phosphatase activity of pre-osteoblastic MC3T3-E1 cells *in vitro*, while the 2.0 wt.% copper-doped glasses significantly decrease the number and ALP activity of the cells [119]. In contrast, other studies emphasized the substantial role of copper in improving the migration and osteogenic differentiation of MSCs [120, 121]. For example, copper showed to play an effective role in the migration of bone marrow mesenchymal stem cells (MSCs) through Rnd3-dependent cytoskeleton remodeling modulated by the HIF-1α pathway [122]. In another study, Cu-doping

of 45S5 Bioglass® composition resulted in promoting early differentiation of MSCs to osteoblast phenotype, over-expressing of anti-inflammatory interleukins as well as reducing pro-inflammatory interleukins [123]. In general, it should be noted that the biological effects of copper are dose-dependent, highlighting the key importance of having control over copper delivery rates while designing and developing copper-doped biomaterials to be used in bone growth and remodeling [124].

Since improving angiogenesis directly accelerates the bone healing process, the synthesis and application of BGs doped with copper is of great importance in tissue engineering concepts [125]. In this regard, the potential suitability of a 5% (mol.) copper-containing MBG was assessed in a SiO_2-CaO-P_2O_5 composition, where calcium was replaced with copper [126]. The angiogenic effects of copper-containing glasses on the bovine aorta endothelial cells (BAEC) and zebrafish (*Danio rerio*) embryo model confirmed that the ionic dissolution products released from the samples could promote angiogenesis without eliciting any toxicity. Binary doping of BG fibers with strontium and copper was performed to simultaneously improve osteogenesis and angiogenesis properties [127]. The effective concentrations of released ions Sr^{2+} and Cu^{2+} were achieved within 40 h and maintained for 4 weeks. The fibers could successfully promote osteogenic and angiogenic differentiation of stem cells derived from adipose and bone marrow tissues as well as vascularization of progenitor HUVECs while inhibiting osteoclasts activities (mainly due to the action of strontium).

Interesting results were also obtained with non-silicate-based BGs by Wang et al., who prepared Cu-doped borate BG-based scaffolds to improve bone healing in critical-sized rat calvarial defects [120]. The scaffolds, doped with 0–3.0 wt.% copper, exhibited pore sizes in the range of 200–400 μm and were prepared by a polymer foam replication technique. The 3 wt.% copper-

containing samples did not result in any cytotoxicity to human bone marrow-derived stem cells (hBMSCs), while they increased the alkaline phosphatase activity. The *in vivo* results showed induced angiogenesis and subsequently promoted bone regeneration in animals implanted with copper-containing scaffolds as compared to the Cu-free scaffolds (see Figure 4).

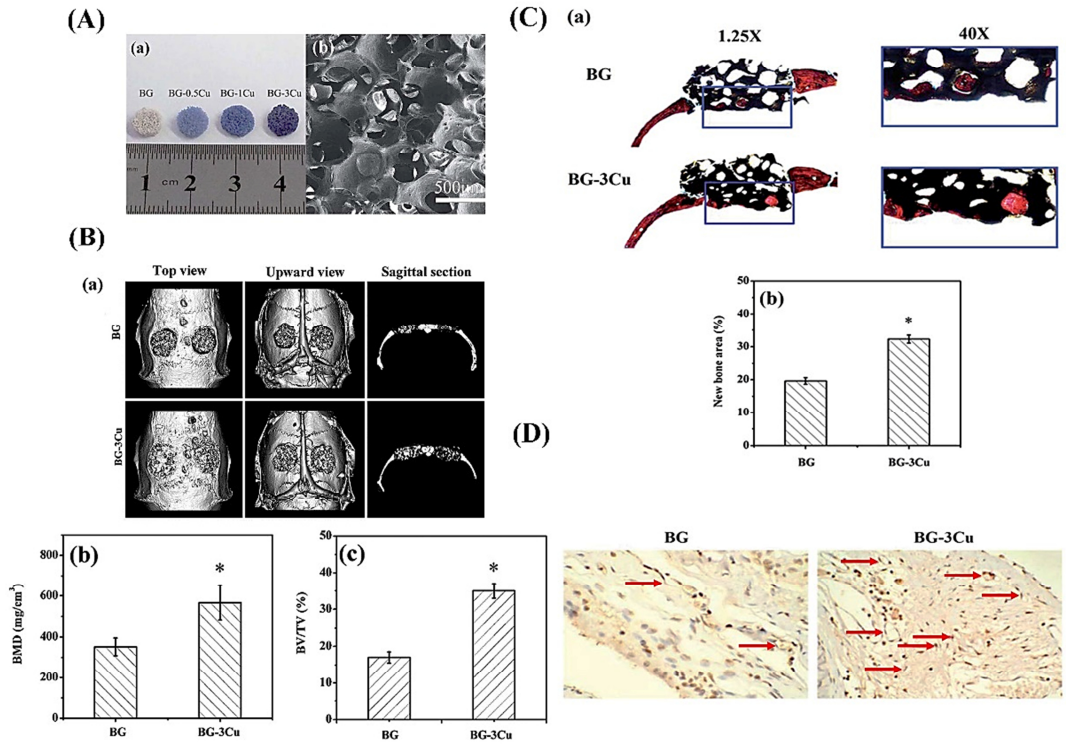

Figure 4. (A) Macroscopic images of different groups of copper-containing scaffolds including undoped (BG) and doped with 0.5 wt% CuO (BG–0.5Cu), 1.0 wt% CuO (BG–1Cu) and 3.0 wt% CuO (BG–3Cu) (a) and SEM micrographs exhibiting the microstructure of the BG–3Cu samples. (B) Micro-CT assessment of bone regeneration process after the implantation of the BG and BG–3Cu scaffolds into the rat calvarial defects at 8 weeks of post-surgery (a) and quantified results showing bone mineral density (BMD) and bone volume/total volume (BV/TV) (b and c, respectively). (C) Transmitted light micrographs of van Gieson picrofuchsin stained sections of the samples received the BG and BG–3Cu scaffolds after 8 weeks of implantation (a) and the graph showing the percentage of the new bone area in the defects (b). (D) microscopic images of the immunohistochemically stained samples for CD31 marker in rat calvarial defects treated with the BG and BG–3Cu scaffolds after 8 weeks of implantation (red arrows indicating newly formed blood vessels). Mean ± SD; n = 3. *Significant difference between groups (*$p < 0.05$). Reproduced from ref [120].

Copper was proven useful to tailor the physicochemical properties and increase the biodegradation time of 3D borosilicate BG scaffolds [95]. The outcomes clarified that the agglutination effect of Cu^{2+} ions and charge balance effect could yield more stable glasses. Hence, copper doping led to lower degradability and lower ion release in the scaffolds, along with a significant improvement in angiogenic properties due to the delivery of Cu^{2+} ions.

The therapeutic potential of composites made of copper-doped BGs and polymers was also examined for the replacement of injured bones [128-131]. Based on the literature, it can be stated that the mixing of copper-doped BGs with polymeric matrices may improve both the mechanical and biological properties of the final products compared to the polymer alone [132]. Ryan et al. prepared collagen scaffolds embedding 2 mol% copper-doped BGs to reduce infection and enhance osteogenesis and angiogenesis [133]. They could fabricate scaffolds with satisfactory micro-architectural and mechanical properties. The biological characteristics of the composite scaffolds revealed an improvement in osteogenesis (up to a 3.6-fold increase in calcium deposition), angiogenesis (in a chick CAM membrane, and tubule formation in Matrigel® assay); and antibacterial activity (up to 66% inhibition against *S. aureus*). Based on the results, the authors claimed that the scaffolds have the potential to be used as one-step therapies for osteomyelitis, decreasing infection, and accelerating bone healing. In another study, 0, 0.5, and 1.5 mol% copper-doped glasses were added to alginate-pullulan polymers to make composites appropriate for bone tissue regeneration [134]. The results obtained from the cell viability assay (Alamar blue®) confirmed that the samples had no adverse effects on fibroblasts and osteoblasts. Furthermore, *in vivo* data documented that the composites implanted in rat subcutaneous pockets did not elicit any immunological responses at 5 weeks post-surgery.

The synthesis and characterization of copper-doped MBGs with or without a surfactant is also under investigation for possible applications in hard tissue engineering [135]. Luo et al. recently reported the successful production of 3D MBG nanofibrous scaffolds having good biocompatibility and antibacterial activities in favor of bone tissue engineering [107]. According to the intrinsic ability of MBGs to load and deliver various drugs, 1.5% and 2.5% copper-doped MBGs were successfully developed to release the anti-inflammatory drugs acetaminophen (ACE) and ibuprofen (IBU) [98]. The obtained data indicated the improved antimicrobial activity in the Cu-doped samples along with the more prolonged drug release action.

Antimicrobial properties of Cu-containing BGs can also be designed by applying proper treatments of the surface of BGs, which is known to play a pivotal role in determining the functional properties [136]. In this regard, Miola et al. tried to do in situ chemical and physical reduction of copper on a silica-based BG surface [82]. The authors first doped the glasses with Cu^{2+} ions by using an ion-exchange process and then exposed them to chemicals (tannic acid, ascorbic acid, and NaOH) as well as physical processes (UV irradiation, thermal treatment in air or argon atmosphere) to enhance the in situ reduction of Cu^{2+} ions to Cu^{+} or Cu^{0} (see Figure 5). The results revealed that the chemical treatments support the formation of Cu^{0}, while thermal treatment promotes the nucleation of CuO (in the air) or Cu_2O (in argon). Importantly, all the treated samples maintained a bioactive behavior, and the samples containing Cu^{2+} ions or Cu^{0} nanoparticles had the best antibacterial activity.

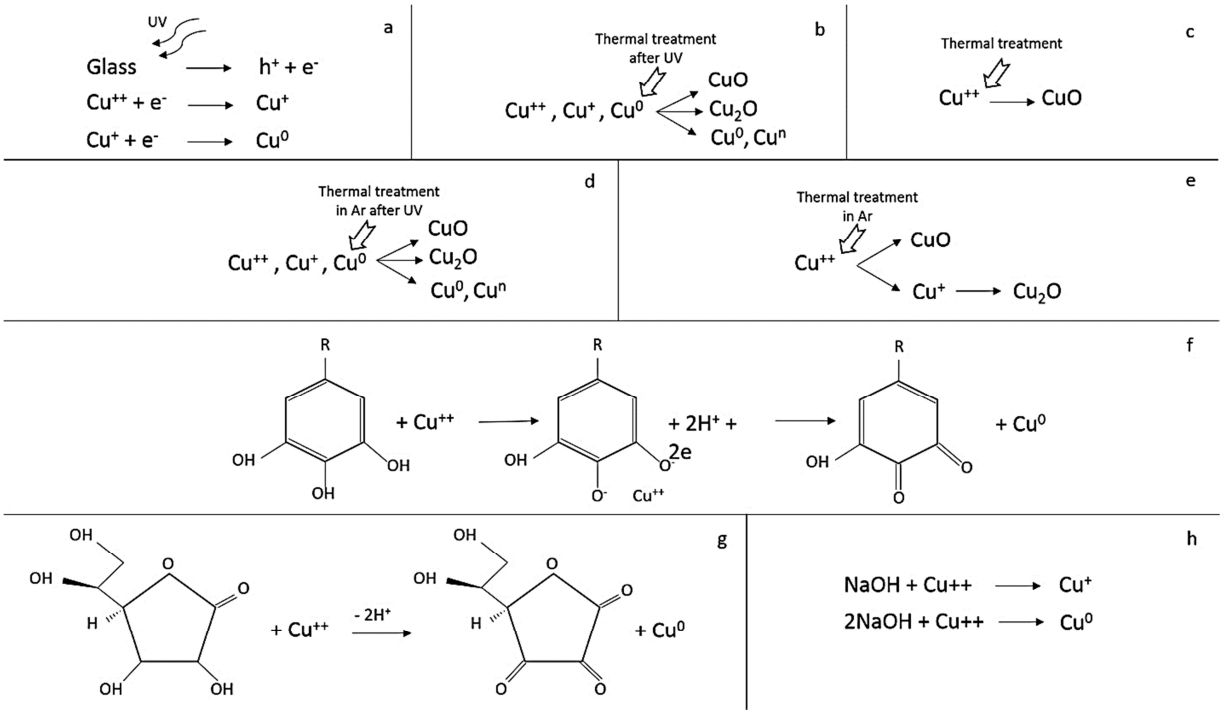

Figure 5. Suggested mechanisms of in situ reduction of copper on BG surface after a) UV irradiation (h^+ and e– are a hole center and an electron, respectively), b) thermal treatment post UV irradiation, c) thermal treatment, d) thermal treatment in Argon (Ar) post UV irradiation, e) thermal treatment in Ar, f) tannic acid treatment, g) sodium L-ascorbate treatment, h) NaOH treatment. Reproduced from ref [82].

4.2. Dental applications

The promising antibacterial properties of copper easily find applications in dentistry, where the incidence of bacterial infections is very high, and the conventional treatments are gradually becoming inefficient due to an excess or improper use of antibiotics, resulting in the development of resistant microorganisms [137]. Among the dental diseases afflicting the global population, periodontitis and peri-implantitis can be considered the most invalidating ones, both from a functional and a cosmetic point of view, as they can shortly lead to the destruction of both hard and soft tissues, followed by loss of teeth or early failure of the implant [138, 139]. Copper

has been widely used in dental applications in different forms, including Cu nanoparticles for the prevention and treatment of resistant bacteria [138], dopant element for the development of antimicrobial metallic surfaces for dental implants [140], and additive to mouth rinse and toothpastes for eliciting an inhibitory effect on caries activity [141].

The use of BGs in dentistry includes the implantation of restorative materials, mineralizing agents, production of bioactive coatings on dental implants, pulp capping, root canal treatment, and air abrasion [142]. However, very few specific studies are available about the use of Cu-doped glass and glass-ceramic materials in dentistry. El-Fiqi et al. recently reported the successful synthesis of amine (NH_2)-functionalized Cu-doped BG nanospheres (Cu-BGn) for managing tooth defects through increasing angiogenesis and reducing bacterial infections at the damaged site [143]. The glass samples were mesoporous, positively-charged, and bone-bioactive with a size ranging between 50–60 nm. The chemical formulation of the amine (NH_2) surface-functionalized Cu-BGn and Cu-free (BGn) were $85SiO_2$–10CaO–5CuO and $85SiO_2$–15CaO (wt%), respectively. The release of Cu^{2+} from the glasses was observed at therapeutically-effective doses, 79 and 152 ppm after 24 and 168 h, respectively. *In vitro* cell assays confirmed the cellular uptake of Cu-BGn via ATP-dependent endocytosis and micropinocytosis. In vitro osteogenesis and odontogenesis of human dental pulp multipotent stem cells (hMSCs) was assessed by using differentiation cell culture media containing 2.5, 5, or 10 μg/mL of BGn or Cu-BGn; the results revealed a significant up-regulation in the early (collagen I alpha) and the late osteo/odontogenic gene markers (dentin sialophosphoprotein, dentin matrix protein 1, and osteocalcin) after 1 and 3 days post-incubation. Cu-BGn at concentrations of 2.5–10 μg/mL induced the migration of HUVECs, whereas Cu-free samples showed a similar migration rate compared to the control. The antibacterial effects of the glasses against one of the most

frequently found pathogens of the pulp tissue infection, i.e., *Enterococcus faecalis* (*E. faecalis*), clarified the critical impact of Cu^{2+} ions on suppressing bacterial growth over 5 h co-culture, while Cu-free samples showed no antibacterial activity. The implantation of BGn (1.5 mg), Cu-BGn (1.5 mg), and EGF-loaded Cu-BGn (EGF@Cu-BGn) (2.25 mg) into rat molar teeth infected with *E. feacalis* proved the dentin regenerative potential of copper-containing glass samples.

In another study, Theodorou et al. proposed the use of gel-derived glass-ceramic scaffolds doped with zinc or copper to protect the dental tissue regenerative process against bacterial penetration [144]. Specifically, trabecular-like glass-ceramic scaffolds with 74 vol.% of porosity were obtained by traditional foam replication technique. Compared to the un-doped scaffolds, the Cu-doped ones revealed a delay in the process of deposition of the reaction hydroxyapatite layer, attributed to the nucleation of wollastonite upon thermal treatment, while Zn-doped scaffolds showed an inhibited bioactivity mechanism. Despite that, *in vitro* cellular studies performed to evaluate the capability of these materials to support the attachment and the proliferation of Dental Pulp Stem Cells revealed a better biological behavior of Zn-doped materials compared to Cu-containing scaffold due to the very high release of cytotoxic Cu amounts within the physiological environment. In fact, cells grown onto the scaffold surface exhibited a rounded morphology, indicating poor attachment and a potential cytotoxic effect [144].

Nevertheless, the possibility to incorporate therapeutic ions within BGs and glass-ceramics still remains one of the most appealing possibilities in dental applications, which is potentially achievable by an accurate compositional and microstructural design of the material, thus allowing the production of multifunctional systems able to provide mechanical support to dental restoration while providing an effective action against resistant microorganisms [145].

4.3. Skin wound healing

Compared to hard tissue engineering, the use of BGs in the management of soft tissue injuries is a newer concept in biomaterials science and bioengineering [146]. *In vitro* experiments revealed that BGs could induce the growth and proliferation of fibroblasts, promote the production of growth factors (e.g., FGF2), and the formation of granulation tissue [147]. It was reported that the composition of the glasses determines their efficacy in terms of wound healing [148]. Meanwhile, copper has been used as a therapeutic agent in the treatment of various skin wounds like chronic diabetic foot ulcers [149]. This metallic element could induce the production of VEGF, increase blood vessel formation (angiogenesis), inhibit infections, and stimulate the expression and stabilization of extracellular skin proteins [150-152]. Hence, the application of copper-containing BGs alone or in combination with polymers in various forms (fibers, films, and hydrogels) has been an interesting approach for the management of acute and chronic wound defects [153]. Generally, skin wound healing comprises four well-organized and overlapping stages, including (I) hemostasis, (II) inflammation, (III) proliferation, and (IV) remodeling [154]. Copper is confirmed to play positive roles in the mentioned stages, thus contributing in the acceleration of skin wound healing. Copper, with its potent biocidal properties, may prevent wounds from becoming infected; therefore, it directly plays a role in the hemostasis stage. Moreover, copper is identified as an effector in the biosynthesis and stabilization of ECM skin proteins, including collagen types I, II, and V, as well as fiber components (elastin, fibrillins). Stimulation of dermal fibroblast proliferation and neo-vessel formation was also documented as a positive consequence of using copper for treating wounds. All these biological events are

categorized in the proliferation phase of skin wound healing involved in the proliferation phase (see Figure 6) [152, 155].

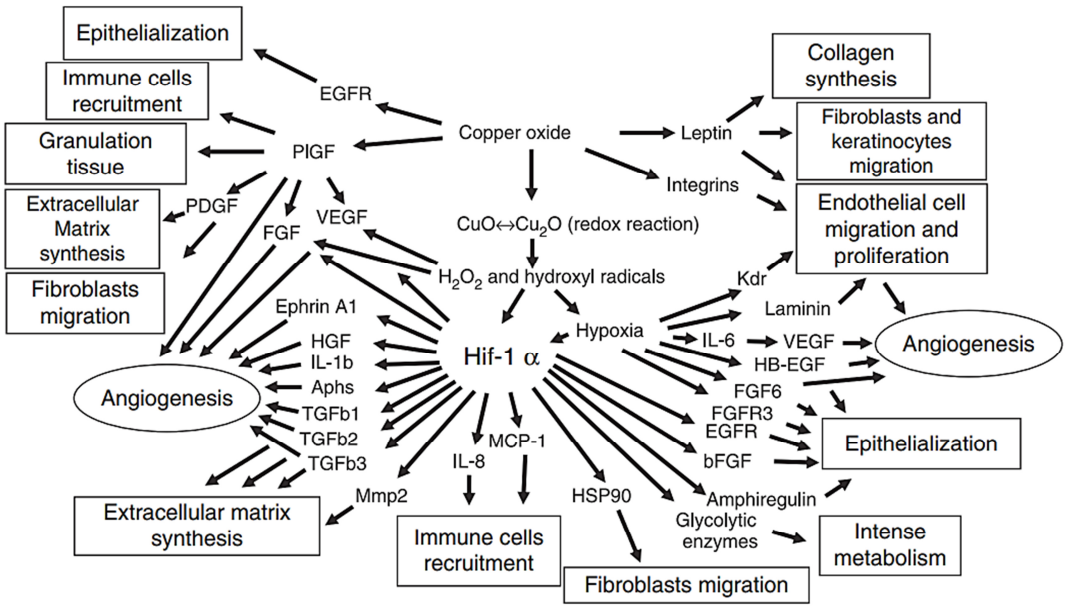

Figure 6. Schematic illustration of the proposed mechanisms by which copper oxide affects skin wound healing. Reproduced from ref.[152] with permission.

In 2015, Stähli et al. successfully prepared copper-doped (2.5 wt.%) 45S5 Bioglass® and evaluated its superior angiogenic effects as compared to Cu-free 45S5 glass powder through the formation of capillary-like networks by endothelial cells cultured onto a 3D matrix of type I collagen [156]. In another study, it was reported that copper-doped BG-based scaffolds could induce the proliferation and growth of human dermal microvascular endothelial cells co-cultured with BMSCs [117]. These results also showed that copper-doped samples promoted the

production of vascular endothelial growth factor (VEGF) without eliciting any adverse effects on the cells.

The use of cotton-like melt-derived glass microfibers is described as one of the most promising forms of wound dressings as they can be produced by a relatively easy method and on a large scale [157]. In this regard, Zhao et al. prepared wound dressings made of copper-doped borate BG microfibers to manage full-thickness skin injuries in a rodent model [111]. They synthesized the glass microfibers (diameter of 0.4-1.2 μm) in a composition of $6Na_2O$-$8K_2O$-8MgO-22CaO-$54B_2O_3$-$2P_2O_5$ (mol%) doped with 0-3.0 wt.% copper. The results obtained from *in vitro* assays clarified that ionic dissolution products of the samples caused no toxicity to endothelial cells and fibroblasts, while they supported cell migration, tubule formation, and expression of angiogenesis-related genes (VEGF, bFGF, and PDGF). The histopathological analyses revealed that 3.0 wt.% copper-doped glass microfibers could significantly induce angiogenesis in treated animals after 4 and 14 days of implantation in comparison to controls (untreated animals). Moreover, collagen deposition, maturity, and orientation were improved in the copper-BG experimental groups (Figure 7). The authors concluded that copper-containing glass fibers could be considered a promising substitutes for healing full-thickness skin injuries.

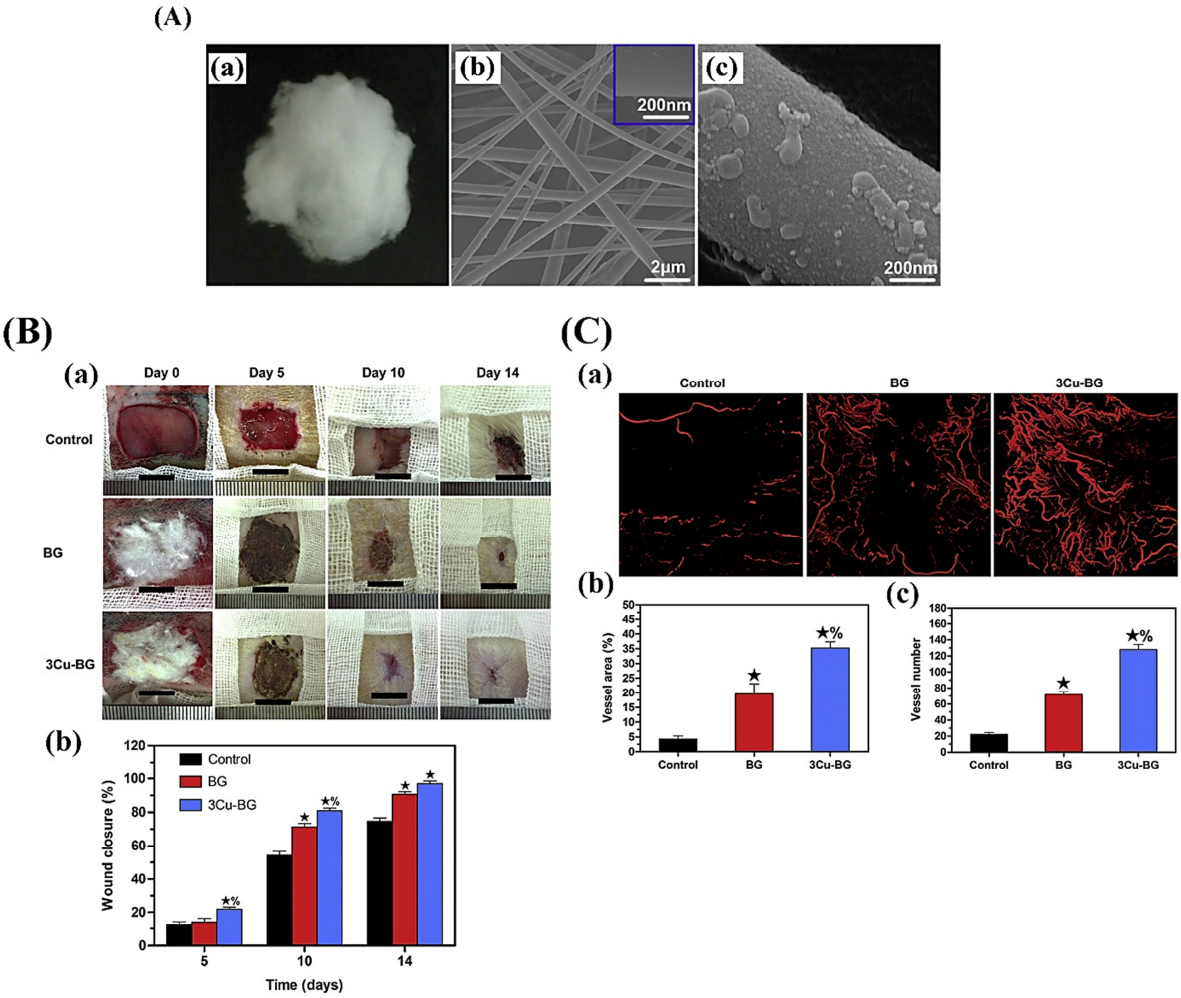

Figure 7. (A) Macroscopic observation of the cotton wool-like appearance of borate-based BG microfibers (3%Cu-BG) (a); SEM micrographs of the 3%Cu-BG microfibers before (b) and after (c) 7 days of the incubation in SBF. (B) Macroscopic images of full-thickness skin injuries in rats left untreated (control) or treated with the BG or 3%Cu-BG microfibers after 0, 5,10, and 14 days of surgery. (Scale bar ¼ 10 mm) (a) and the graph showing the wound closure (%) after 5, 10, and 14 days of surgery (b). (C) the results of micro-CT analysis (3D reconstructed images) of showing newly formed blood vessels in full-thickness skin defects of the different groups at 14 days post-surgery (a), showing the new blood vessels and morphometric analysis of the new blood vessel area and the number of blood vessels. Reproduced from ref [111] with permission.

In a study conducted under the supervision of Prof. Wu [106], copper-doped BG/natural eggshell membrane nanocomposites were prepared for improving wound healing through inducing angiogenesis and antibacterial activity. The research team could fabricate copper-containing glass nanocoating (40–50 nm) on the eggshell membrane via the pulsed laser deposition (PLD) technique. After being incubated with endothelial cells, 5 mol% copper-doped nanocomposites could enhance the secretion of hypoxia-inducible factor (HIF)-1α protein and a series of genes involved in angiogenesis (VEGF, VEGF receptor 2 (KDR) and endothelial nitric oxide (eNos)), as well as decrease the viability of Gram-negative bacteria (E. coli). *In vivo* angiogenic activity of the copper-doped samples was verified via overexpression of CD31 protein in treated mice. In addition, the formation of a continuous and uniform epidermis layer was observed in the experimental groups *in vivo*.

More recently, the synergic effects of copper-doped borate BG/poly(lactic-co-glycolic acid) wound dressing with vitamin E (0-3.0 wt.%) were investigated as regards the efficiency in stimulating angiogenesis in cells and full-thickness skin wounds healing in Sprague-Dawley (S-D) rats [158]. Similar to the studies mentioned above, the *in vitro* results showed improved angiogenesis (better migration, tubule formation, and VEGF secretion) in endothelial cells treated with copper-doped and vitamin E-loaded samples. Furthermore, substantial improvement was observed in the epithelialization of wound closure and collagen remodeling *in vivo* (see Figure 8).

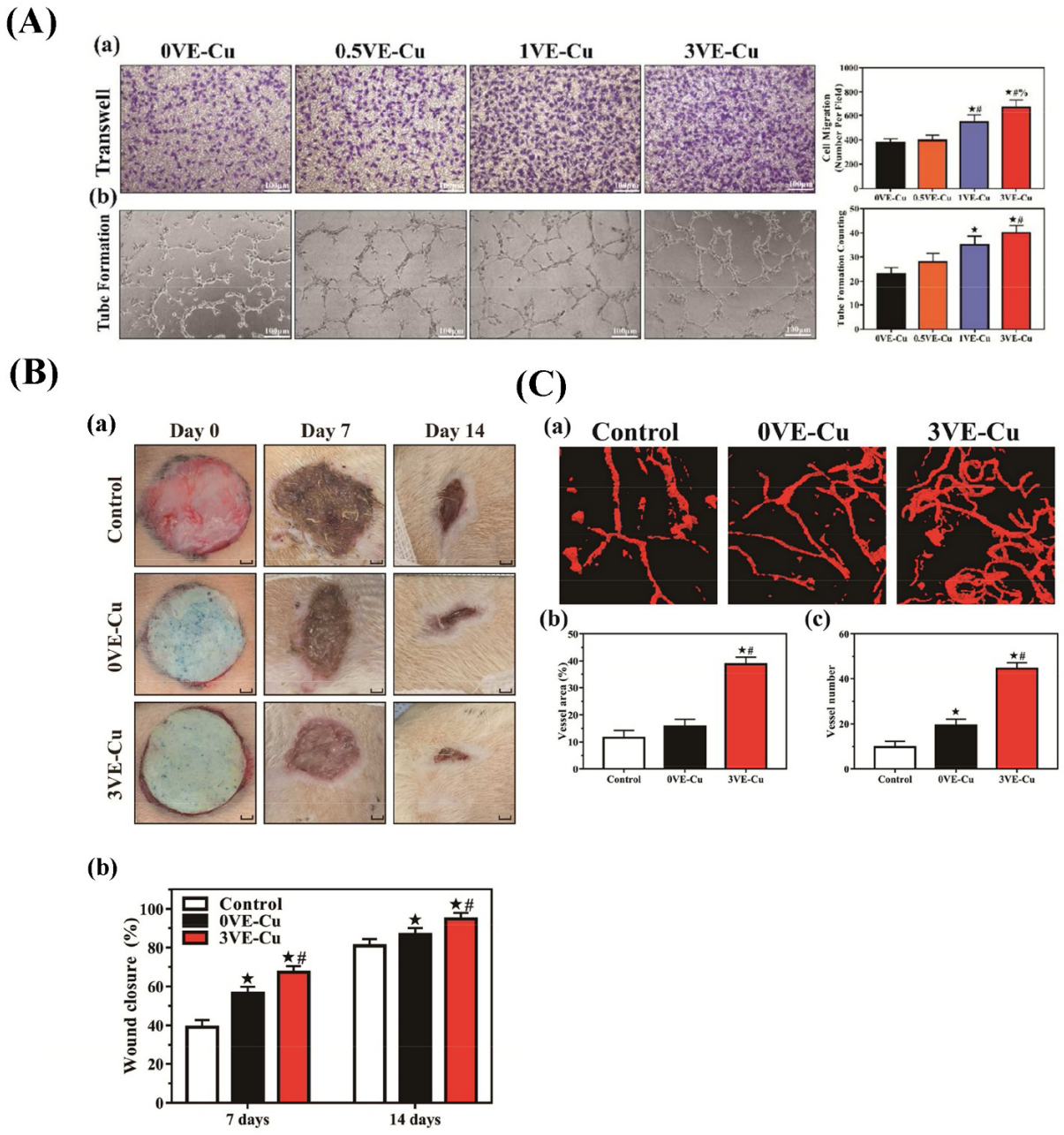

Figure 8. (A) Evaluating the migration and tube formation of human umbilical vein endothelial cells (HUVECs) treated with ionic dissolution product of copper-doped borate BG/poly(lactic-co-glycolic acid) (PLGA) dressings loaded with vitamin E (0, 0.5, 1, and 3.0 wt.% vitamin E) by the transwell method (a) and Matrigel^{TM} (b) after 12 h incubation. (B) Optical images of full-thickness skin defects including untreated (control), treated with the 0VE-Cu, and 3VE-Cu dressings after 0, 7, and 14 days post-surgery (a) (scale bar = 2 mm) and the graph exhibiting the wound closure percentages at 7 and 14 days of post-implantation (b). (C) Micro-CT analysis (3D reconstruction images) of the defects to evaluate blood vessel formation in the untreated (control) or treated animals with the 0VE-Cu or 3VE-Cu dressings on day 14 (a) and the graphs

showing the results of morphometric analysis of the new vessel area and the number of blood vessels (b and c, respectively). Reproduced with permission from ref [158].

4.4. Ocular applications

Some special silicate BG compositions were found suitable to improve the long-term performance of orbital implants, which are implanted in the ocular cavity following surgical procedures of evisceration or enucleation [159]. Cu-doped BGs have recently shown great promise in this regard, as suggested by early experimental evidence.

Orbital implant infections, which usually happen due to implant exposure and subsequent colonization by bacteria, can be effectively treated by antibiotic eye drops or systemic therapy, provided that the implant is porous and vascularized [160]. Implant removal is the most drastic remedy to be carried out if the infection does not resolve pharmacologically, thus involving additional cost and stress to the patient [161]. Porous orbital implants are typically made of polyethylene, hydroxyapatite, or alumina, which are inert with no inherent antimicrobial properties. In the attempt to tackle this issue, Ye et al. [162] coated porous hydroxyapatite orbital implants with a thin layer of Cu-doped MBG, thereby synergistically combining the antibacterial effect of copper ions released from the glass with that of ofloxacin, an antibiotic hosted inside the glass mesopores. MBG coatings doped with 2 or 5 mol.% of CuO were deposited by immersion of the hydroxyapatite implant in the sol and then consolidated via calcination. Cu-doped implants could inhibit the viability of *Staphylococcus aureu*s and *Escherichia coli in vitro*; specifically, the antibacterial halo increased from about 12 to 15 mm as the copper content increased from 2 to 5 mol.%, although the drug loading and release capacity were less efficient in the samples with higher copper concentration. This latter observation not only suggests a

predominant antimicrobial effect associated with the release of copper ions but is also consistent with the "disturbing" effect of copper ions on the formation of the mesoporous structure assessed in other studies (in other words, less ordered the pores, lower the amount of drug that can be hosted inside).

It is worth highlighting that this coating-based approach can potentially succeed in fulfilling two apparently irreconcilable requirements of orbital implants, i.e., the need for a permanent material (the skeleton lying underneath the coating) combined with the release of antibacterial ions (copper) that occurs as the glass layer dissolves over time.

Apart from being antibacterial, Cu-doped orbital implants carry the potential of stimulating fibrovascularization as well, as first hypothesized by Baino in 2015 [163]. This property was confirmed *in vivo* in 2018 by Wang et al. [164], who performed primary angiogenic tests in a panniculus carnosus muscle model in rabbits and reported that the Cu-doped glass coating significantly accelerated the vascularization of porous hydroxyapatite orbital implants compared to Cu-free materials.

Incorporation of copper in nearly-inert alumino-silicate orbital implants was also reported following different strategies [165]. In a first approach, melt-derived Cu-doped strong macro-porous scaffolds (compressive strength about 20 MPa) were produced by sponge replication, but the release of copper ions was inadequate to elicit a therapeutic effect. On the contrary, the deposition of a thin Cu-doped MBG layer on the walls of the previously-prepared porous glass-ceramic foam allowed achieving a more sustained release of copper ions.

The research about the use of ion-releasing BGs for ophthalmic applications is in its very beginning, and several peculiar parameters related to the "working conditions" of orbital implants should be taken into account for developing safe and effective biomaterials for this

purpose. For example, the interaction of copper ions with ocular secretions, the fate of released ions, and the associated risk of local storage and, perhaps, tissue necrosis are all issues deserving careful consideration in future research.

4.5. Cancer treatment

Metal-based strategies play a pivotal role in the detection and treatment of various types of cancers [166, 167]. For example, cisplatin is one of the most famous metal (platinum)-based chemotherapeutics, which is currently being used for treating a wide series of cancers in hard and soft tissues [168]. Over time, more metals have been examined and applied for use in anti-cancer approaches like photothermal therapy (PTT) [169]. Copper, either in pristine or modified form, has been utilized for cancer PTT [170-172]. In addition, copper was successfully incorporated into hollow silicate microspheres to prepare multifunctional bioactive scaffolds with the ability to load and deliver an anti-cancer drug (trametinib) for subsequent use in chemo-photothermal therapy of skin cancers and regeneration of skin tissue [173]. In 2017, Wang et al. also showed the applicability of electrospun nanocomposites containing Cu_2S nanoflowers for simultaneously skin tumor therapy and wound healing [174].

On the other hand, both melt-derived and sol-gel glasses have a successful history in cancer theranostics [175-178]. Accordingly, the possible usability of copper-doped BGs for cancer therapy seems reasonable and feasible. In this regard, copper-doped MBGs were developed to combine photothermal therapy and chemotherapy [26]. The synthesized glasses showed good bioactivity, excellent drug loading capacity, and photothermal property, making them promising substitutes for bone tumor therapy.

Hyperthermia induced by magnetic glasses is another anti-cancer approach applied for the treatment of solid tumors like bone malignant tumors [20]. More recently, copper/iron-codoped magnetic BGs have been produced to act as multifunctional materials able to kill tumors cell, prevent bacterial infections, and improve bone regeneration [68]. The authors showed that the incorporation of copper into the basic glass composition (SiO_2-CaO-Fe_2O_3-P_2O_5) led to an improvement in the magnetic saturation of the samples and elicited no significant cytotoxicity to healthy cells. In another study, Liu et al. fabricated Cu/Fe/Mn/Co-multi doped bioactive glass-ceramic (BGC) scaffolds with photothermal effect and osteogenic differentiation ability by using the 3D-printing method [179]. The results published by the authors show that these metal-multi doped scaffolds, containing up to 5% dopants, have excellent photothermal activity, and their performance could be listed as 5Cu-BGC > 5Fe-BGC > 5Mn-BGC > 5Co-BGC. Moreover, the hyperthermia produced by 5Cu-, 5Fe- and 5Mn-BGC samples could effectively kill tumor cells *in vitro* and inhibit tumor growth *in vivo* (see Figure 9). More specifically, 5Fe- and 5Mn-BGC samples were suggested as promising candidates for PPT of bone tumor and bone regeneration since they showed a better substrate for adhesion of MSCs.

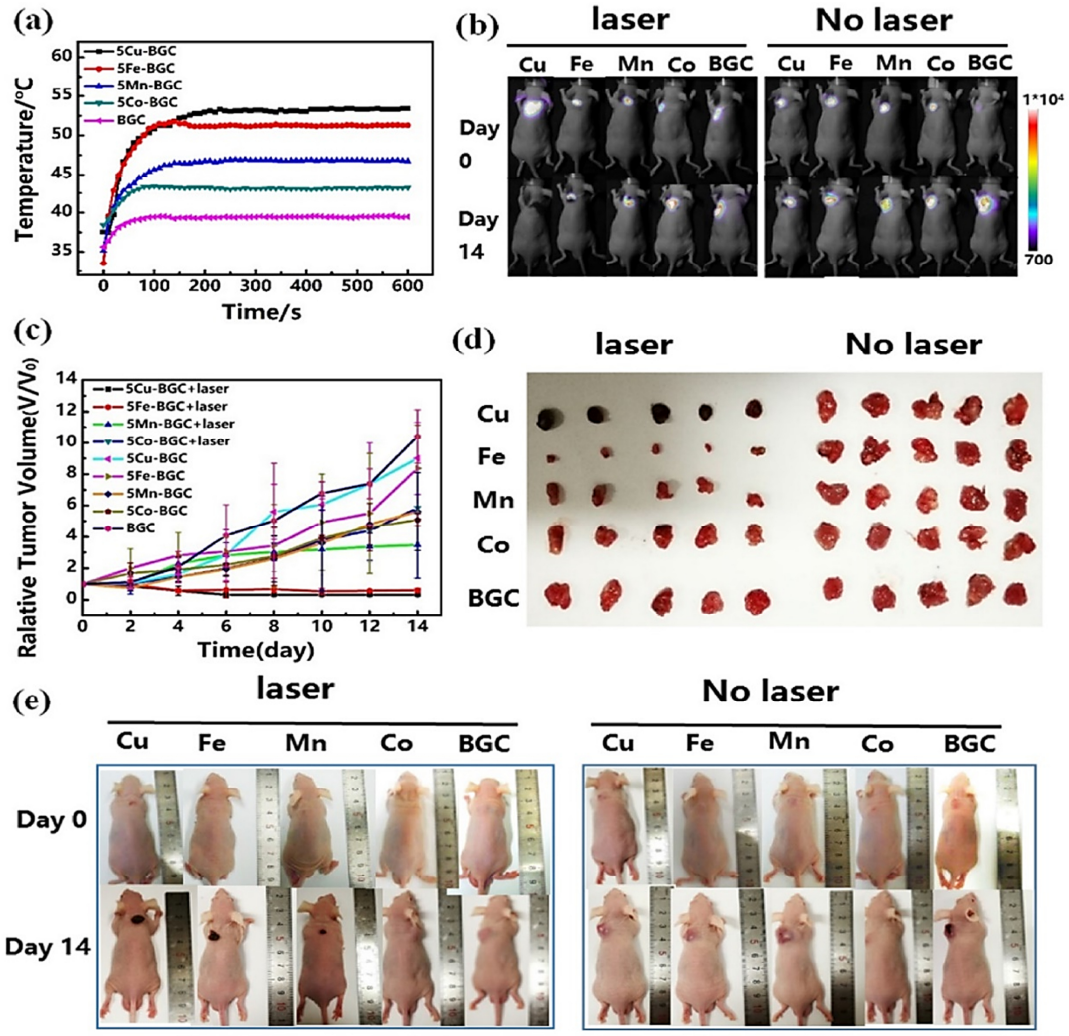

Figure 9. The plot showing heating curves of human osteosarcoma cells (Saos-2 cell line) tumor-bearing mice after the implantation of copper-, iron-, magnesium-, and cobalt-doped bioactive glass-ceramic (BGC) scaffolds (5%Cu-BGC, 5%Fe-BGC, 5%Mn-BGC, 5%Co-BGC) and BGC scaffolds under 808 nm laser irradiation with a power density of 0.75 W/cm^2 for 15 min (a); The fluorescence imaging of animals showing tumors at day 0 and 14 (b); the graph showing tumor volume growth after the treatments (c); macroscopic observation of tumor tissues obtained from the animals at day 14 (d); Optical observation of the animals received treatments at days 0 and 14 (e). The *in vivo* tumor growth was significantly inhibited as a result of the photothermal effect of 5%Cu-BGC and 5%Fe-BGC scaffolds. Reproduced from ref [179].

5. Polymer/Cu-BG composite materials: a step towards biofabrication strategies

Due to the composite nature of biological bone, the production of multicomponent biomimetic scaffolds based on the combination of a polymeric, biodegradable component, and an inorganic bioactive phase (i.e., BGs and glass-ceramics), have recently gained increasing scientific interest [180]. Apart from conferring bioactive properties to the inert, organic matrix, it was demonstrated that the incorporation of BG and glass-ceramic materials within polymers carries several benefits, including enhanced osteoblast adhesion, differentiation, and proliferation, as well as improved angiogenic and antibacterial properties, which are provided by introducing proper therapeutic ions [74, 181].

As discussed in the previous sections, the well-known antibacterial and angiogenic potential of Cu-doped BGs make them materials of choice for the production of composite scaffolds for both hard and soft tissue engineering applications [22, 182, 183]. Despite the enormous potentialities, very few studies describing the preparation of Cu-BG/polymer composite biomaterials are currently available in the literature. As an example, Miola et al. [129] described the production of a PMMA/Cu-BG composite cement with different viscosities obtained by incorporating a melt-derived Cu-doped BG into a commercial cement matrix (Cemex®) used for orthopedic applications. The composite material exhibited a clear antibacterial effect against *S. epidermidis* biofilm and good bioactive properties. The latter advantage was associated with the successful exposure of glass particles on the cement surface, thus representing a promising result for the development of antibiotic-free cements with osteoinductive properties to be used in orthopedic prostheses fixation, temporary prostheses, and spinal surgery [129].

In another study, Sharifi et al. [184] reported the production of biomimetic composite scaffolds resembling the natural composition of bone by incorporating sub-micrometric glass fibers into an alginate/gelatin hydrogel. These Cu-containing composite scaffolds, produced by freeze-drying and electrospinning, revealed improved cell growth and viability and exhibited better biocompatibility compared to both the pure hydrogel and the composite scaffold containing undoped Bioglass® fibers. PDLLA/Cu-BGs composite multifunctional scaffolds for bone regeneration have also been recently produced by lyophilization and salt leaching methods by Bejarano et al. [130, 185]. This study showed that the incorporation of Cu-doped glasses with antibacterial properties into PDLLA conferred to the scaffold improved angiogenetic properties, resulting in improved VEGF secretion, but also improved bioactive potential. However, high levels of metal ion released were associated with cytotoxic effect, thus stressing the importance of the design phase in scaffold manufacturing.

In recent years, many efforts have been addressed to the development of new materials suitable to be used in biofabrication, a novel technological field aimed at the production of 3D structures mimicking biological tissues and organs [186]. These strategies are based on the intimate combination of cells, polymers (typically hydrogels), and other biologically active substances that are hierarchically distributed within the matrix according to their specific function [186]. Compared to conventional tissue engineering strategies, the main advantages related to the use of hydrogel-based “bioinks” include a higher cell loading efficiency and a more homogeneous and tailored cell distribution, which is difficult to be achieved by traditional culture techniques [187]. However, pure polymeric hydrogels are sometimes affected by poor stability and poor printing fidelity, thus making it necessary to put in place new strategies for improving pre- and post-printing features, as well as post-printing cellular viability [188]. In this regard, novel composite

bioinks have been developed by including different inorganic fillers within the polymeric matrix, such as silica nanoparticles, micro- and nano-sized BGs, hydroxyapatite, and β-tricalcium phosphate (β-TCP).

In 2016, alginate dialdehyde-gelatin (ADA-GEL) constructs incorporating Sr-doped BG nanoparticles (BGNPs) were produced by biofabrication in order to obtain a grid-like composite for future application in bone tissue engineering [189]. However, to the best of the authors' knowledge, no studies about bioinks using Cu-doped glasses as inorganic fillers are currently available in the literature. Given the positive effects of Cu-doping in conferring antibacterial and angiogenetic properties to Cu-containing polymer-matrix composites while stimulating osteogenetic pathways, the development of Cu-BG/hydrogel composite bioinks suitable for the biofabrication of 3D cellularized structures would represent an interesting and valuable step towards the design of novel multifunctional implants mimicking biological bone.

6. Concluding remarks and future prospects

The available literature provides convincing evidence of the potential suitability of Cu-doped BGs to be used, alone or in combination with polymeric matrices, for making high-added-value implantable materials for different biomedical purposes. As a major step forward in the field, additive manufacturing has brought great opportunities for patient-specific tissue substitutes, while there are limited studies in which Cu-doped BGs were utilized for fabricating the scaffold to mimic the native structure of tissues [190]. Therefore, more research is required to determine appropriate design and production strategies for generating constructs made of Cu-doped glass

and glass ceramics and evaluating their therapeutic potential for either hard or soft tissue replacement.

Copper ions released from BGs are able to stimulate angiogenesis, which plays a pivotal role in the regenerative processes of both hard and soft tissues. Vascularization is key to allow osteogenesis, which can be further promoted by other ion dissolution products (e.g., silicate and Ca^{2+} ions) that are typically released by BGs and are known to stimulate bone cells towards a path of regeneration and self-repair. Angiogenesis is also highly desirable when the BG-based implant is addressed to wound healing and skin repair or needs to be rapidly vascularized, such as porous orbital implants.

In spite of the promising results coming from quite abundant amounts of *in vitro* and *in vivo* studies, at present, no Cu-doped BG system has been cleared by FDA or has somehow received any form of approval for clinical applications. The reason behind that is due to the various barriers existing between research and market; the following example is particularly instructive in this regard. An academic and industrial US joint team led by Mohamad Rahaman (Missouri University) and Ted Day (ETS Wound Care) conducted many studies to assess the suitability of the Cu-free and Cu-doped 13-93B3 borate BG compositions for wound healing applications. The basic 13-93B3 glass has eventually been commercialized in the form of cotton-like mats for veterinarian applications (FDA approval in 2011) and has also been proved effective in inducing the healing of chronic wounds in diabetic patients who were irresponsive to conventional treatments (ReadiHeal and Mirragen® products). Although the Cu-doped glass was shown to further improve angiogenesis as compared to the basic 13-93B3 material, only the latter composition is currently commercialized. This confirms once more that the market usually prefers relatively simple solutions (i.e., Cu-free vs. Cu-doped glass) if the benefits of the more

complex product are not so outstanding. On the basis of the early available trials in humans, the presence of copper seems even unnecessary to achieve the desired goal (wound healing); further studies should be conducted to understand if copper is really essential in specific pathological cases where angiogenesis would be otherwise impossible or insufficient to allow tissue healing. An interesting field of application for Cu-doped BGs could concern orbital implants, where a quite fast vascularization rate is desirable inside the implant.

Apart from being useful in stimulating tissue regeneration, Cu-doped BGs can also elicit antimicrobial properties. If, on the one hand, copper is a less potent antibacterial agent than silver, on the other hand, Cu^{2+} ions are apparently less prone to induce unwanted side effects like tissue necrosis or late toxicity. Using antimicrobial metallic ions as an alternative to antibiotics is today one of the most challenging topics of research due to the well-known problem of antibiotic abuse in many countries of the world and, hence, the development of resistant bacterial strain. Future studies should clearly elucidate if copper-based therapies might effectively replace fully or at least partly antibiotics. Furthermore, it should be taken into account that antimicrobial properties of copper ions are co-adjuvant to allow tissue regeneration as well: in this regard, Cu-doped BGs are actually multifunctional biomaterials promoting tissue regeneration/angiogenesis while discouraging bacterial growth. Indeed, control of dosage plays a key role as these different therapeutic actions require different levels of copper ions.

Lastly, Cu-doped BGs show promise for cancer treatment via photothermal therapy, alone or in combination with other ions eliciting additional effects like hyperthermia. This field of research is in its beginning and indeed deserves further investigation in the future to assess the suitability and feasibility of such a strategy as an alternative to more stressful and invasive chemotherapy.

Conflict of interest

The authors declare no conflicts in this publication.

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