Research Article

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Investigating the effect of Copper Addition on $\text{SiO}_2\text{-ZnO-CaO-SrO-P}_2\text{O}_5$ Glass Polyalkenoate Cements: Physical, Mechanical and Biological Behavior

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Abstract: The physical, mechanical, and biological behaviour of copper containing glass polyalkenoate cements were investigated, where copper (Cu^{2+}) was incorporated into a $\text{SiO}_2\text{-ZnO-CaO-SrO-P}_2\text{O}_5$ based glass system. Three GPCs were formulated for this study, a *Control* and two *Cu-GPCs* with 6 (*Cu-1*) and 12 (*Cu-2*) Mol.% of CuO substituted for the SiO_2 in the glass. Rheological evaluation of GPCs determined that the addition of the Cu decreases the working and setting times in the cements. The mechanical properties of the cements were evaluated after 1 - 21 days incubation in DI water. The compressive strength of the cements were found to range between 21-36 MPa, with *Cu-1* having the highest compressive strength. Biaxial flexural strength and Shear Bond Strength of the GPCs were found to increase with respect to time and were higher for the *Cu-GPCs* at 14 MPa and 2.1 MPa respectively. Bioactivity testing was conducted using Simulated Body Fluid (SBF) which revealed CaP precipitants on each of the GPCs surfaces. The effect of Cu addition to the GPCs greatly enhanced the antibacterial inhibition zone (IZ) when tested in *E.coli* (3mm), *S.aureus* (24mm) and *S.epidermidis* (22mm). Cytocompatibility testing revealed more favorable MC3T3 osteoblast cell viability when compared to the *Control* GPC.

Keywords: Glass Polyalkenoate Cement, Copper, Mechanical, Antibacterial, MTT, bone

1 Introduction

Vertebral compression fractures (VCF), predominantly caused by osteoporosis (loss of bone) are very common fractures in the thoracic and lumbar spine. By age of 50, about 20% of men and 50% of women suffer from VCF, and for those patients with severe pain, vertebral augmentation (e.g. vertebroplasty, kyphoplasty) is required [1, 2]. Percutaneous vertebroplasty (PVP) or kyphoplasty (KVP) techniques, are the injections of bone cement in the osteoporotic fractures to stabilize the vertebral body and thereby to relieve pain [3]. An estimated number of 700,000 patients undergo vertebroplasty and kyphoplasty in the United States, each year [4]. The annual incidence of osteoporotic fractures are currently rising and have been predicted to significantly increase in the coming decade [5]. Until now and among the many potential orthopedic cements, Polymethylmethacrylate (PMMA) or its derivatives are the primary cements being used for reconstruction of a damaged vertebral body [6]. These cements have been employed primarily due to their superior mechanical properties, however, shortcomings in terms of their biological performance still exist, which eventually leads to severe complications such as secondary fracture of the vertebral body or loosening of the cement [7]. PMMA cements are not osteoconductive [8], they lack any chemical bonding to host bone tissue, and their adhesion to bone rely primarily on mechanical interlocking [7]. Upon setting reaction, they exhibit an exotherm (up to 91°C) which has been reported to cause thermal necrosis of adjacent bone tissue [9, 10]. Leaching of unreacted monomer of methyl methacrylate (MMA) is also another issue that negatively affects their *in-vivo* biocompatibility [7]. The concerns associated with acrylic cements highlight the significance of developing bioactive skeletal cements.

In an effort to address the issues with the PMMA bone cements, Al-free Glass Polyalkenoate Cements (GPCs) were proposed as potential clinical replacements [11]. GPCs

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Figure 1: Schematic overview of setting reaction in GPCs, and structure after maturation adhesion to bone tissue.

were first developed as restorative dental materials and since then they have found versatile dental applications due to their successful clinical history [12]. Their chemistry consists of a partially degradable glass powder (base), and a water-soluble polymer (acid), which upon mixing, an acid-base reaction takes place resulting in a set cement [13]. The glass component of a conventional GPCs is based on a $\text{CaO-Al}_2\text{O}_3\text{-SiO}_2$ or $\text{CaF}_2\text{-Al}_2\text{O}_3\text{-SiO}_2$ system, and the acid component is typically an aqueous solution of polyalkenoic acid such as polyacrylic acid (PAA) [13]. During the setting reaction, and in the presence of water, the surface of the glass particles are attacked by hydrogen ions from the acid chains. Metal cations, principally Al^{3+} , Sr^{2+} and Ca^{2+} are released from the glass surface into solution, and a gel structure starts to form through ionic crosslinking of leached metallic cations and polyacrylate chains [13, 14]. Degradation occurs on the glass particles surface, while the core remains intact and exists as a filler in the set cement (Figure 1).

GPCs exhibit number of advantages when compared to the PMMA based cements. Good biocompatibility, chemical adhesion to skeletal tissue and surgical metals, minimal toxicity, no exothermic heat generation, and a lack of any significant volumetric shrinkage upon setting make GPCs as promising candidates for skeletal adhesives [14]. The chemical adhesive nature of GPCs is considered to be one of their principal attributes; a key requirement to acquiring a strong interface between the host bone and the implant [15, 16]. The adhesion of GPCs to enamel and dentin is complex, and it is achieved through an ionic exchange at the interface of GPC and hydroxyapatite (HaP)-like mineral of hard tissue. Initially, the mineral phase of tooth will be wetted with GPC, due to hydrophilic nature of both substrates. Following wetting, ionic bonding occurs between the functional groups on the acid (typically carboxylate groups) and Ca^{2+} , and PO_4^{3-} ions on the HaP surface [15, 17]. Ultimately, the diffusion of ions such as Ca^{2+} , Sr^{2+} , and PO_4^{3-} from glass and HaP

substrate within the interfacial zone creates a strongly bonded ion-exchange layer which consists of ions from both sides (Figure 1) [15]. Ionic dissolution of glass particles into the surrounding medium affords GPCs the potential to re-mineralize hard tissue and potentially encourage physiological processes such as bone regeneration [18, 19].

One of the primary concerns with the use of conventional GPCs for skeletal applications, is the presence of Aluminum (Al^{3+}) in the glass composition. Al^{3+} is recognized as being neurotoxic and is implicated in diseases such as Alzheimer's and Parkinson [20, 21]. Moreover, presence of Al^{3+} in skeletal tissue negatively affects bone mineralization [22]. Therefore, to facilitate the transition from conventional dental GPC to skeletal adhesive, Al-free GPC were developed by substitution of Al^{3+} with zinc (Zn^{2+}) [23, 24]. Al^{3+} plays a critical role in the glass structure of GPCs as a network intermediate where it promotes the formation of non-bridging oxygens (NBOs). In the aluminosilicate glass system of conventional GPCs, when the Al/Si ratio is lower than one, Al^{3+} can be either a network modifier or a network former. In the latter case, it replaces the Si^{4+} in the glass network, therefore the network acquires a negative charge and it becomes more susceptible to acid attack. In the glass system of calcium-zinc-silicates, Zn^{2+} provides a similar structural role to Al^{3+} as described in aluminosilicate glasses. Zn^{2+} can act both as a network former and a network modifying, resulting in a negatively charged network of NBOs which is vulnerable to acid attack and GPC formation [25, 26]. Additionally, the introduction of Zn^{2+} improves biocompatibility of cements as it has been cited to improve osteogenesis [27, 28]. Extensive *in vitro* and *in vivo* studies, suggested encouraging results for the biological performance of Al-free GPCs [18, 29]. Studies by Hatton *et al.* showed osteoconduction and new bone formation on the cements (Mp4, Pilkington, UK) surface after six weeks implantation [29, 30]. Despite GPCs presenting good biological performance, their poor mechanical strength and poor fracture toughness limit their application in load-bearing applications [31]. Different compositions of commercially available GPCs exhibit compressive strength ranging between 60 to 300 MPa, elastic modulus of 2 to 10 GPa, and flexural strength up to 50 MPa [14].

Modification in chemical composition of the glass, the acid component, and variations in powder to liquid ratio of GPCs can alter the physical, biological, and mechanical properties, and therefore extend their applications to orthopaedics [32]. Divalent cations in the glass have the potential to link two polyanionic chains, where the ionic strength of the bond proceeds as $\text{M}=\text{Al}^{3+}>\text{Cu}^{2+}>\text{Zn}^{2+}>\text{Ca}^{2+}>\text{Mg}^{2+}$ [33]. This suggests that

Cu^{2+} is a mechanically relevant ion when formulating GPCs, because it can act as a strong ionic crosslink to two carboxylic acid (COO^-) side groups, resulting in hardened set materials [34]. Additionally, Cu^{2+} has been extensively cited in literature for being an antibacterial agent [35] and has been shown to enhance and stimulate the proliferation of osteoblast cells and to promote the bone regeneration [35–37]. Ewald *et al.* showed enhanced osteoblastic cell activity and proliferation on the calcium phosphate cements mixed with Cu^{2+} solution [38]. In a study by Wang *et al.* Cu-doped bioactive borate glass scaffolds showed a significantly higher capacity to proliferate MC3T3-E1 cells compared to the control scaffolds [39]. Several recent studies have demonstrated that Cu^{2+} stimulates the proliferation of endothelial cells and promotes angiogenesis, suggesting Cu^{2+} acts as one of the primary agents for wound healing applications [37, 40–43]. Cu^{2+} can up-regulate the hypoxia-inducible factor 1 (HIF-1) transcription activity; by stabilizing the expression of hypoxia-inducible factor 1 (HIF-1 α), and the secretion of vascular endothelial growth factor (VEGF) [43, 44]. Artificially mimicked Hypoxia, has been established as a major pathway to angiogenesis and vascularization [44].

The primary objective of this research will be to investigate the effect that the Copper (Cu^{2+}) addition has on GPCs formed from a $\text{SiO}_2\text{-CaO-SrO-ZnO-P}_2\text{O}_5$ glass system, and the potential as a GPC based bone cement for PVP or KVP. More specifically the objective of this study is to analyze the effect of Cu^{2+} on the handling and mechanical properties, the adhesive bond strength, and *in-vitro* biological potential of Cu-GPCs.

2 Materials & methods

2.1 Cu-GPC Synthesis

2.1.1 Glass Synthesis

Three glasses were formulated for this study, two copper containing glasses (*Cu-1*, *Cu-2*) in addition to a copper free $\text{SiO}_2\text{-CaO-SrO-ZnO-P}_2\text{O}_5$ glass (*Control*). The copper containing glasses contain incremental concentrations of CuO at the expense of Silica (SiO_2) (Table 1). The powdered mixes of analytical grade reagents (Fisher Scientific, PA, USA) were oven dried (100°C , 1 h) mixed and fired (1400°C , 1 hr) in platinum crucibles and shock quenched into water. The resulting frits were dried, ground and sieved to retrieve glass powders with a maximum particle size of $45\mu\text{m}$.

Table 1: Glass compositions (Mol. Fr) where Si is substituted with Cu.

	SiO ₂	CuO	ZnO	CaO	SrO	P ₂ O ₅
Control	0.48	0.00	0.36	0.06	0.08	0.02
Cu-1	0.42	0.06	0.36	0.06	0.08	0.02
Cu-2	0.36	0.12	0.36	0.06	0.08	0.02

2.1.2 Cement Formulation

Cements were prepared by thoroughly mixing the glass powders (<45µm) with polyacrylic acid (E11 PAA—Mw, 210,000, <90µm, Advanced Healthcare Limited, Kent, UK) and de-ionized (DI) water on a clean glass plate. The cements were formulated with variable powder to liquid (P:L) ratios from 2:1.5 to 2:3.0 with 40wt% additions of PAA (Table 2). Complete mixing was undertaken within 20s.

Table 2: Cement formulation for variable P/L ratios and with 40 wt.% concentration of PAA.

	2:3.0	2:2.5	2:2.0	2:1.5
Glass (g)	1	1	1	1
PAA (g)	0.6	0.5	0.4	0.3
Water (mL)	0.9	0.75	0.6	0.45

2.1.3 Rheological Evaluation

The working time (W_t) of the cements was measured under standard laboratory conditions (Ambient Temp, 25°C), and was defined as the period of time from the start of mixing during which it was possible to manipulate the material without having an adverse effect on its properties. Each sample (where $n = 5$), was measured using a stopwatch on a clean glass plate with a sterile spatula. The setting time (S_t) of the cement series was tested in accordance with ISO9917 [45] which specifies the standard for dental water based cements. Cements were stored at 37°C during setting period, and S_t was measured by lowering a 400 g mass attached to a Gilmore needle into a cement filled mold with 8×9×10 mm internal diameter. S_t was taken as the time the needle failed to make a complete indent in the cement surface. Each measurement was conducted under the same mixing conditions to ensure reproducibility.

2.2 Mechanical Properties

2.2.1 Compressive Strength

The compressive strength (CS) of the cements (6×40mm, where $n = 5$) were evaluated in accordance with ISO9917 [45]. Cylindrical samples were tested after 1, 7, 14, and 21 days incubation in DI water. Samples were stored in sterile DI water in an incubator at 37°C, after the incubation time has expired the cements were removed and tested while wet on an Instron 4082 Universal Testing Machine using a 5 kN load cell at a crosshead speed of 1 mm/min. The CS (MPa) was calculated using Eq. 1. Where ρ is the maximum applied load (N) and d is the diameter of sample (mm).

$$CS = \frac{4\rho}{\pi d^2} \quad (1)$$

2.2.2 Biaxial Flexural Strength

The biaxial flexural strength (BFS) of the cements (where $n = 5$) were evaluated by a method described by Williams *et al.* [46]. Cement discs measuring (2×120mm) were tested after 1, 7, 14, and 21 days. Samples were stored in sterile DI water in an incubator at 37°C. At each time period, the cements discs were removed and tested while wet on an Instron 4082 Universal Testing Machine using a 1 kN load cell at a crosshead speed of 1 mm/min. The BFS (MPa) was calculated using Eq. 2. Where ρ is the fracture load (N), t is the sample thickness (mm) and r is the radius of the support bearing (mm).

$$BFS = \frac{\rho}{t^2} \{0.63 \ln(r/t) + 1.156\} \quad (2)$$

2.2.3 Shear Bond Strength

Shear bond strength (SBS) testing was conducted according to ISO 29022 [47]. Briefly, cylindrical cement samples (6×40mm, where $n = 15$) were mounted on hydroxyapatite (HaP) plates which were pre-mounted in resin and ground using 125µm grit size. Samples were bonded to the HaP surface and left for 1 hour to set. The split ring molds were then removed, and the bonded samples were incubated in DI water for 1, 7, 14, and 21 days at 37°C before testing. The test was conducted at a crosshead speed of 1.0 mm/min until failure. The SBS (MPa) was calculated using Eq. 3. Where ρ is the fracture load (N) and A_b is the bonding area expressed in mm².

$$SBS = \frac{\rho}{A_b} \quad (3)$$

2.2.4 Laser Profilometry

Laser Profilometry was performed on a Solarius Laserscan Profilometer (Solarius, Ca, USA), with a vertical sensor resolution of 10µm, and spot size of 2µm. Analysis of data was performed using Solar Map Version 5.0.4.5261 software.

2.3 Solubility Analysis

2.3.1 Ion Release Profile

Each of the cement discs (2×12ømm, where $n = 5$) were incubated in 10 mL of sterile DI water and rotated on an oscillating platform at 37°C for 1, 7, 14 and 21 days. After each time period, the extracts were prepared by removing the cement disc and filtering the remaining liquid. The ion release profiles of the liquid extracts were measured using Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP–OES) on a Perkin- Elmer Optima 3000DV (Perkin-Elmer, MA, USA). ICP–OES calibration standards for Ca²⁺, Si⁴⁺, Zn²⁺, PO₄³⁻, Sr²⁺ and Cu²⁺ were prepared from a stock solution on a gravimetric basis. Three target calibration standards were prepared for each ion and DI water was used as a control.

2.3.2 Simulated Body Fluid Trial

Simulated body fluid (SBF) was prepared with the ionic concentration similar to that of human blood plasma, and in accordance with the procedure outlined by Kokubo *et al.* [48]. Each cement disc (2×12ømm, where $n = 3$) was subsequently immersed in a fixed volume of SBF according to Eq. 4. Where V_s is the volume of SBF used (mL) and S_a is the exposed surface area of the cement disc (mm²). Specimens were stored in plastic containers for 1, 7, 14 and 21 days. After the specified incubation time, the specimens were removed from the SBF, gently rinsed in DI water and oven dried (37°C, 24 h). Changes in the surface morphology of the incubated samples after soaking in SBF were characterized by scanning electron microscopy (SEM).

$$V_s = \frac{S_a}{10} \quad (4)$$

2.3.3 Scanning Electron Microscopy (SEM)

A FEI Co. Quanta 200F Environmental Scanning Electron Microscope (SEM) equipped with an EDAX Genesis Energy-Dispersive Spectrometer was used to obtain secondary

electron images and carry out chemical analysis of the surface of the discs. All EDX spectra were collected at 15 kV, using a beam current of 26 nA. Quantitative EDX converted the collected spectra into concentration data by using standard reference spectra obtained from pure elements under similar operating parameters.

2.4 Biological Behavior

2.4.1 Antibacterial Analysis - Agar Diffusion

The cement discs (2 × 6ømm, where $n = 3$) were initially sterilized by placing discs under UV light for 3 hours prior to testing. The antibacterial activity of the cements was evaluated using *E. coli* strain ATCC 8739 (LB agar and broth), *S. epidermidis* strain ATCC 14990 (BHI agar and broth), *S. aureus* (TSB agar and broth) using the agar diffusion method. Each microbe was initially grown aerobically in liquid broth at 37°C for 24 hours. The agar diffusion test was performed under standard laboratory sterile conditions in a fumigation hood using sterile swabs for inoculation of bacteria. Each cement disc was placed within an inoculated plate and cultured for 24 hours at 37°C. Preparation of the agar disc diffusion plates involved seeding agar plates with a sterile swab dipped in a 1/50 dilution of the appropriate 24-hour culture of bacteria. Symbiosis protocol 3 colony counter was used for imaging of the bacterial plates. Calipers were used to measure zones of inhibition where each sample was analyzed in triplicate and mean zone sizes ± standard deviations were calculated. Inhibition zone sizes were calculated using Eq. 5.

$$\text{Inhibition Zone (mm)} = \frac{\text{Halo}\varnothing - \text{Disc}\varnothing}{2} \quad (5)$$

2.4.2 Antibacterial Analysis - Bacterial Broth

The antibacterial activity of the cements was evaluated against three strains of bacteria, using the bacterial broth method. Luria agar and broth were used for the culture of *E. coli*, which was grown aerobically at 37 °C. Similarly, *S. epidermidis* strain ATCC 14990, and *S. aureus* were also used and were cultured in BHI, and TSB agar and broth, respectively. Each cement sample (2×6ømm, where $n = 5$) was exposed to 1 mL of sterile DI water and rotated on an oscillating platform at 37°C for 1, 7, 14 and 21 days. After each time period expired, liquid extracts were removed. Bacterial solutions were prepared by growing microbes aerobically in liquid broth at 37°C for 24 hours. The antibacterial efficacy was tested in 98 well plates in 9 repli-

cates, where aliquots (20 μ L) of undiluted cement extracts were added into wells containing (80 μ L) bacterial solution and were inoculated over a time period of 24 hours in a sterile incubator at 37°C. The experimental extracts were compared against a healthy growing population of *E. coli*, *S. epidermidis* and *S. aureus* at each time period. A sterile control (sterile DI water and broth) was also analyzed as the liquid medium background. At each time period, the broth was examined by UV-Visible light spectroscopy (Pharmacia biotech ultrospec 3000 UV-Visible light spectrophotometer) at 600 nm for the transmission (%T) through the liquid medium.

2.4.3 Cytotoxicity - MTT assay

The established cell line MCT3T osteoblasts was used in this study as required by ISO10993 part 5 [49]. Cells were maintained on a regular feeding regime in a cell culture incubator at 37°C/5% CO₂/95% air atmosphere. The culture media used was HyClone MEM Alpha Modification (1X) media (Thermo Scientific, MA, USA), which included L-glutamine, ribonucleosides, and deoxyribonucleosides, and was supplemented with 10 vol% fetal bovine serum (Thermo Scientific, MA, USA). Cement extracts were collected by immersing each cement sample (2×60mm, where $n = 5$) in 1 mL of sterile DI water (≈ 0.5 g/ml) and rotated on an oscillating platform at 37°C for 1, 7, 14 and 21 days. Cytotoxicity of cement extracts was evaluated using the methyl tetrazolium (MTT) assay in 96 well plates in 9 replicates. Aliquots (10 μ L) of undiluted cement extracts over 1,7,14, and 21 days were added into wells containing MCT3T cells in (100 μ L) culture medium. Cells were seeded at a density of 10,000 cells per well and incubated for 24 hours. The MTT assay was then added in an amount equal to 10% of the culture medium volume/well. The cultures were then re-incubated for a further 4 h (37°C/5% CO₂). Next, the cultures were removed from the incubator and the resultant formazan crystals were dissolved by adding an amount of MTT Solubilization solution (10% Triton X-100 in acidic isopropanol (0.1 NHCl)) equal to the original culture medium volume. Once the crystals were fully dissolved, the absorbance was measured at a wavelength of 570 nm. Aliquots (100 μ L) of tissue culture water were used as controls, and cells were assumed to have metabolic activities of 100%.

2.5 Statistical Analysis

Statistical analysis was conducted to compare mechanical properties, antibacterial efficacy, and cytocompatibility of cements in relation to 1.) maturation time in DI water over 1, 7, 14 and 21 days, and 2) differences between the cement compositions (*Control*, *Cu-1* and *Cu-2*) at each individual time period. SPSS 19.0 Statistics Software was used to conduct one-way analysis of variance (ANOVA). Comparison of relevant means was performed using the post hoc Bonferroni test. Differences between groups were deemed significant when $p < 0.05$.

3 Results

Three different glasses were synthesized as; *Control*, *Cu-1* and *Cu-2* with *Cu-1* and *Cu-2* containing 6 and 12 Mol.% of copper at the expense of silica (Table 1). *Control*, *Cu-1*, and *Cu-2* glasses were used to form GPCs where the cement formulations (Table 2) were prepared by varying the P/L ratio at a PAA concentration of 40wt%. The GPCs working (W_t) and setting times (S_t) were analyzed as a function of powder to liquid (P/L) ratio, ranging from 2:1.5 to 2:3.0. The results are presented in Table 3. For *Control* and *Cu-GPCs* (*Cu-1*, and *Cu-2*), W_t was decreased by decreasing the P/L ratio from 2:3.0 to 2:1.5, where W_t at P/L of 2:3.0 was found to be 1m, 58s for *Control*, 1m, 38s for *Cu-1*, and 1m, 10s for *Cu-2*. At P/L of 2:1.5, the W_t values were found to be 41s, 46s, 29s, for *Control*, *Cu-1* and *Cu-2*, respectively. Similar trend was observed for the S_t of the cements. The longest S_t was observed at P/L of 2:3.0 with 10m, 16s for *Control*, 7m, 45s for *Cu-1*, and 6m, 04s for *Cu-2* and while the shortest S_t occurred at P/L of 2:1.5, with 130s, 55s, and 18s, respectively. Within each P/L ratio formulation, the *Control* cement presented the longest W_t and S_t , and *Cu-2* showed the shortest W_t and S_t .

To describe the mechanical integrity of the cements, compressive strength, biaxial flexural strength and shear bond strength testing were performed. Initially, cements with differing P/L ratios were incubated in DI water for 14 days. Their compressive strength (CS) were calculated and the results are presented in Figure 2a. The highest CS for *Control* and *Cu-1* were attained at P/L of 2:2.5, with 28 MPa and 30 MPa, respectively. The highest CS for *Cu-2* was found to be 25 MPa at P/L ratio of 2:3.0. Based on the cements handling properties (W_t and S_t) and preliminary CS results, P/L ratio of 2:2.5 was chosen for formulating the cement series and to further analyze mechanical properties and biological behavior. To determine the effects of mat-

Table 3: Working (W_t) and Setting (S_t) times of *Control*, *Cu-1* and *Cu-2* formulated with 40wt% PAA, and variable P/L ratios.

<i>P/L Ratio</i>	<i>Working Times (min/sec)</i>			<i>Setting Times (min/sec)</i>		
	<i>Control</i>	<i>Cu-1</i>	<i>Cu-2</i>	<i>Control</i>	<i>Cu-1</i>	<i>Cu-2</i>
2:3.0	1m, 58s	1m, 38s	1m, 10s	10m, 16s	7m, 45s	6m, 04s
2:2.5	1m, 32s	1m, 37s	57s	4m, 32s	5m, 31s	3m, 45s
2:2.0	1m, 06s	1m, 04s	46s	3m, 16s	2m, 56s	1m, 11s
2:1.5	41s	46s	29s	2m, 10s	55s	18s

Figure 2: a) Compressive strength of *Control* and *Cu-GPCs* using variable P/L ratios after 14 days incubation in DI water, and b) Compressive strength of *Control* and *Cu-GPCs* with P/L ratio of 2:2.5 after 1, 7, 14, and 21 days incubation in DI water.

uration time on mechanical properties, each cement was tested for CS after 1, 7, 14 and 21 days incubation in DI water at 37°C and the results are presented in Figure 2b. For each of the cement composition, CS remained relatively unchanged over the 1 – 21 day incubation period, however, *Cu-1* exhibited higher CS compared to *Control* and *Cu-2*. The CS of *Control*, *Cu-1* and *Cu-2* after 21 days incubation in DI water was 28, 33, and 25MPa respectively. Biaxial flexural strength (BFS) was also conducted over the same time frames and the data is presented in Figure 3a. Compared to the compression testing, BFS was found to be more influenced by maturation time. The lowest BFS was observed after 1 day, and highest was after 21 days incubation in water. The BFS values for *Control*, *Cu-1*, and *Cu-2* were 8, 8, and 10 MPa after 1day maturation in DI water, and 12, 14, and 14MPa, after 21 days, respectively. The next step for studying the mechanical behavior of the cements was to examine the shear bond strength at the bone-cement interface. To further perform Shear Bond Strength (SBS) analysis, *Cu-1* was chosen due to its superior CS and BFS compared to *Cu-2*. A test method was developed ac-

cording to ISO29022 where the cement bars were mounted on HaP discs, and the shear force was analyzed to determine the bone-cement bond strength. SBS values for *Control* and *Cu-1* cements incubated in DI water from 1 - 21 days is presented in Figure 3b. At each time period, the bond strength values of *Control* and *Cu-1* were found to be relatively similar, where increased incubation time leads to increased bond strength in the cements. Over 1 to 21 days of incubation, SBS of *Control* ranged between 1.4 to 1.9 MPa, and for *Cu-1* it ranged from 1.3 to 2.1 MPa. A summary of the mechanical properties of the *Control* and *Cu-GPCs* after different incubation time periods is presented in Table 4. To provide further insight into the mechanism of interfacial failure, laser profilometry was performed on the surface of the tested shear bond strength samples. 3D microtopography of the fracture surface of *Control* and *Cu-1* after 1 and 21 days incubation in DI water is presented in Figure 4, where the smooth blue background represents the HaP surface, and the circular mark illustrates the fractured surface of the cement cylinders. The scale bars in Figure 4 represents the vertical height (Z), which is demonstrated

Figure 3: a) Biaxial flexural strength of *Control* and *Cu-GPCs* and b) Shear Bond strength of *Control* and *Cu-1* after 1, 7, 14, and 21 days incubation in DI water.

Table 4: Summary of mechanical properties of *Control*, *Cu-1*, and *Cu-2* after incubation in DI water for 1, 7, 14, and 21 days.

	Compressive Strength (MPa)			Biaxial Flexural Strength (MPa)			Shear Bond Strength (MPa)	
	<i>Control</i>	<i>Cu-1</i>	<i>Cu-2</i>	<i>Control</i>	<i>Cu-1</i>	<i>Cu-2</i>	<i>Control</i>	<i>Cu-1</i>
1 Day	24.14	27.06	20.55	8.55	8.48	9.86	1.42	1.32
7 Days	27.66	36.21	24.24	8.20	9.03	9.93	1.51	1.81
14 Days	28.28	30.26	21.64	10.97	12.33	10.43	1.82	1.95
21 Days	28.85	33.47	25.24	12.12	14.48	14.37	1.91	2.1

at the same range (0 to 1.5mm) for all the scans ($X = 7$ mm, $Y = 7$ mm, $Z = 1.5$ mm). The surface topography scans presented in Figure 4, highlight the distribution of the residual cements after failure compared to the smooth HaP surface. Projected scan area in Figure 5, shows the vertical height analysis of the scanned area presented in Figure 4, displaying the change in vertical height (Z) of the surface extending from the residual cement disc to the edges of the HaP background. For each scan, three ranges of the surface height values (Z) with the corresponding percentage, are presented. The smoothest surface was observed after 1day incubation, with approximately 63% and 76% of total area of the sample ≤ 0.25 mm in height, for *Control* and *Cu-1*, respectively. Further analysis of the samples surface after 21 days, shows a significant change in the topography for *Cu-1*, displaying distinct circular mark of residual cement. For *Cu-1*, approximately 25% of total area of the sample was determined to be higher than 0.85 mm in height, to maximum of 1.27 mm. After 21 days, *Control* showed lower height parameters compared to *Cu-1*, with maximum height occurring between 0.55 – 0.83 mm cov-

ering approximately 25% of total area. Optical microscopy images of the fracture surface of *Cu-1* cements after 1 and 21 days incubation in DI water are presented in Figure 6a and 6b. In Figure 6, the white background is the HaP surface, and *Cu-1* cement appeared as darker green.

To quantify the ion release from each cement, ICP-OES was performed on the cements discs incubated in DI water over 1, 7, 14, and 21 days. Ion release profiles were determined for each cement composition including Si^{4+} , Zn^{2+} , Ca^{2+} , PO_4^{3-} , Cu^{2+} , and Sr^{2+} , and the corresponding results are illustrated in Figure 7. Si^{4+} release was found to increase with respect to incubation time for all three GPCs. *Control* exhibited higher release of Si^{4+} compared to *Cu-GPCs*, with a high burst of ions over the first 7 days and then a steady release up to 21 days. Si^{4+} release for *Control* ranged from 25 to 104 mg/L. However, Si^{4+} release for *Cu-CPCs* showed a relatively linear trend with consistent release over the 1 - 21 day incubation period. Si^{4+} release for *Cu-1* and *Cu-2* ranged from 49 to 82 mg/L and 43 to 79 mg/L, respectively. Zn^{2+} release was relatively low, with no release in the first 24 hours for all GPCs. *Control* had

Figure 4: Laser profilometry images of the fracture surface of *Control* and *Cu-1* after adhesive shear bond analysis, and 1 and 21 days incubation in DI water.

the lowest Zn^{2+} release with 0 to 0.1 mg/L. *Cu-GPCs* exhibited very similar levels of Zn^{2+} release, but higher than the *Control* cement. *Cu-1* and *Cu-2* released 1.9 and 1.6 mg/L Zn^{2+} after 21 days. Ca^{2+} release profiles of *Control* and *Cu-GPCs* were similar with none of the cements having significantly higher release than others. All the cements showed a gradual increase in their Ca^{2+} release over 1 to 21 days. The same trend was also observed for Sr^{2+} release. Over the experimented time period of 1 to 21 days, Ca^{2+} release was found to range from 0 to 1.3, 1.7 and 1.4 mg/L, and Sr^{2+} release ranged from 1.5 to 5.7, 1.9 to 5.9 and 1.7 to 5.3 mg/L for *Control*, *Cu-1* and *Cu-2*, respectively. On the other hand, the results of PO_4^{3-} release from the cements showed higher ion release for the *Control* cements compared to *Cu-GPCs*, ranging from 3.7 to 8.3 mg/L. However, PO_4^{3-} release of Cu^{2+} series remained constant over the course of 21 days. PO_4^{3-} release from *Cu-1* was found to range from 4.4 to 5.2 mg/L, and for *Cu-2* this was found to range from 3.4 to 4.9 mg/L. *Cu-GPCs* were also examined for the Cu^{2+} release. The results from Figure 7, indicates steady release of Cu^{2+} from GPCs over 1 to 21 days. Cu^{2+} release reached 2.1 mg/L at day 1 for *Cu-1*, and remained relatively constant for the next 21 days, with 2.1 mg/L at day 21. For *Cu-2*, highest

amount of Cu^{2+} release was at day one with 2.5 mg/L. After 21 days incubation, Cu release levels was found to be 2.4 mg/L for *Cu-2*.

Simulated Body Fluid (SBF) trials were conducted for each GPC to evaluate their preliminary *in-vitro* bioactivity. SBF trials were performed on the cement discs over 1 to 21 days of immersion, and SEM images of the depositions on the surface of *Control*, *Cu-1*, and *Cu-2* GPCs after 21 days incubation in SBF, along with their corresponding EDX spectra, are presented in Figure 8. As shown in Figure 8, all three GPCs presented a similar morphology of spherical precipitants on the surface after 21 days incubation in SBF. The clusters of spherical shaped particles are attributed to CaP formation on the surface [50, 51]. EDX spectra presents (Figure 8) the semi-quantitative elemental composition (wt%) of the CaP depositions which showed the presence of Si^{4+} , Sr^{2+} , PO_4^{3-} , Ca^{2+} , Zn^{2+} , and Cu^{2+} (only in *Cu-GPCs*). EDX elemental composition of the spherical surface precipitants after immersion in SBF reveal relatively low amount concentrations of Si^{4+} on the surface with 2, 1, and 1 wt% for *Control*, *Cu-1*, and *Cu-2* respectively. However, considerably higher concentrations of Ca and PO_4^{3-} , and Zn^{2+} are present when compared to

Figure 5: Projected scan areas of laser profilometry images (Figure 3) of *Control* and *Cu-1* fracture surface after 1 and 21 days incubation in DI water, analyzing vertical height (Z) range and corresponding % of total area.

initial glass composition, with 12, 17, and 16 wt% of Ca^{2+} , and 18, 26 and 26 wt% of PO_4^{3-} , and 58, 45, and 44 wt% of Zn^{2+} for *Control*, *Cu-1*, and *Cu-2* respectively. Moreover, EDX results confirmed the presence of 2, and 3 wt% of Cu^{2+} on the surface depositions of *Cu-1* and *Cu-2*, respectively.

To assess the antibacterial efficacy of the cements and to determine the potential of Cu^{2+} ions on the cements antibacterial properties, two different test methods were employed. First, agar diffusion testing was conducted on the

cement discs with a range of bacteria. Result of agar diffusion testing is presented in Figure 9. It is evident from Figure 9 that *Control* cement showed the smallest inhibition zone (IZ) of any bacteria tested compared to *Cu-GPCs*. Measurements of IZ of agar diffusion test are presented in Fig. 10 (a, b, c). No inhibition was observed for *Control* testing in *E. coli*, and *S. aureus*. The only IZ observed for *Control* was 1.7 mm testing in *S. epidermidis*. In contrast to *Control* cement, *Cu-GPCs* demonstrated significantly higher

Figure 6: a) Shear bond strength testing setup and b) optical microscopy images of fracture surface of Cu-1 after 1, and 21 days.

Figure 7: Ion release profiles of Control and Cu-GPCs, investigating Si, Zn, P, Sr, Ca and Cu release after 1, 7, 14 and 21 days incubation in DI water.

Figure 8: SEM micrographs of surface of Control and Cu-GPCs and corresponding EDX after 21 days incubation in SBF.

Figure 9: Agar diffusion antibacterial testing of Control and Cu-GPCs in *E. coli*, *S. epidermidis*, and *S. aureus*.

antibacterial activity, particularly with *S. epidermidis* and *S. aureus* (Figure 9). IZ calculated for Cu-1 cement was 1.9 mm, 21.3 mm, and 20.5 mm when tested in *E. coli*, *S. epidermidis* and *S. aureus*, respectively (Figure 10a, 10b, 10c). Cu-2 exhibited 3.0 mm, 21.6 mm, and 23.9 mm IZ when tested in *E. coli*, *S. epidermidis* and *S. aureus*, respectively. Further, to quantify the antibacterial properties of the cements as a function of time, bacterial-broth test was performed on the liquid extracts incubated in DI water over 1 - 21 days. The same three bacterium were tested and the results are presented in Figure 10(d, e, f). Bacterial viabilities for Cu-GPCs were reduced as low as 5% for *E. coli*, 3% for *S. epidermidis*, and 2% for *S. aureus*. For Control cement, the viability was found to be lower when compared to healthy

growing *E. coli*, and *S. aureus* with 83%, and 95% viability, respectively. However, the bacterial viability of the Control cement in *S. epidermidis*, was increased from 96 to 113, 122, and 123% after 1, 7, 14, and 21 days. Apart from the Control cement in *S. epidermidis*, all the other cements exhibit relatively stable viability with respect to time.

To analyze the cytotoxicity of the cement series, MTT assay was performed on the GPC liquid extracts post incubation in DI water after 1, 7, 14, and 21 days. Cell viability was conducted using MC3T3-E1 osteoblast precursor cell line and the results are presented in Figure 11. All the experimental cements tested, had lower viability compared to healthy growing osteoblast cells with a metabolic rate of 100%. Control cement expressed cell viability of 90, 77,

Figure 10: (a, b, c) Inhibition Zone (IZ) of of Control and Cu-GPCs employing the antibacterial agar diffusion method, and (d,e,f) bacterial viability of Control and Cu-GPCs extracts after 1, 7, 14 and 21 days incubation in DI water employing the bacterial broth method. Each test analyzed the GPCs efficacy in *E. coli*, *S. epidermidis* and *S. aureus*.

Figure 11: MC3T3-E1 cell viability of Control and Cu-GPCs extracts after 1, 7, 14 and 21 days incubation in DI water.

78, and 58% at 1, 7, 14, and 21 days incubation time, respectively. Cu-GPCs induced lower cell viability compared

to the Control cement at day 1, however their viability was higher than Control cement after 21 days. Cu-1 exhibited cell viability of 81, 85, 86 and 71%, and Cu-2 expressed viability of 72, 73, 71, and 69% after 1, 7, 14, and 21 days respectively. None of the extracts caused a reduction in cell viability lower than 58%, which was presented by the Control GPC after 21 days incubation.

4 Discussion

The glass formulation used for this study was based on a novel 0.48SiO₂-0.36ZnO-0.06CaO-0.08SrO-0.02P₂O₅ system. The composition was selected such that the resultant Al-free glass is acid degradable and can be formulated to form GPCs with suitable rheological behavior outlined by relevant ISO standards[34]. Characteristic of this novel control glass system includes low SiO₂ to ensure a high degree of solubility and bioactivity. ZnO provides the alternative to Al₂O₃ with similar structural effects on the

formation of chain crosslinks within the GPC [24]. In addition, ions with positive effects on bone metabolism (Ca^{2+} , Sr^{2+} , and PO_4^{3-}) have been included to encourage integration between the GPC and host tissue [58]. Further modification of this glass aims to substitute SiO_2 with 6 and 12 mol% of CuO to tailor the reactivity of the glass. However, higher substitution of the CuO with SiO_2 would adversely affect the glass solubility. CuO is a network modifier oxide and its incorporation into glass may result in the formation of NBOs, which further disrupts the glass network and increases the susceptibility of glass towards acid attack. The structure of the glasses were characterized in an earlier study by the authors [34]. *Control* and *Cu-GPCs* were formulated with a range of different P/L ratios (2:3.0 to 2:1.5) and their W_t and S_t is presented in Table 3. Two independent trends were observed for the rheology as a function of 1) glass chemistry and 2) different P/L ratios. The first observation was that, by incorporating CuO in the glass, the W_t and S_t decreases. For all different P/L ratios, *Control* had the longest and *Cu-2* had the shortest W_t and S_t , respectively. This may be attributed to number of different physiochemical processes. The susceptibility of the glass to acid attack is modified by the composition of the glass and liberation of different metal cations in the aqueous solution. Ions released by the glass particles interact with charged acid chains, resulting in gelation and further hardening of the cement. The reduction in setting times as observed in *Cu-GPCs* is likely due to presence of divalent cations of Cu^{2+} which have the potential to crosslink two polyanionic chains. Release of Cu^{2+} ions from *Cu-1*, and *Cu-2* glasses increases the concentration of metal cations in the solution, which further accelerates the chelation and shortens the setting times. Accordingly, *Cu-2* with a higher concentration of CuO , had shorter W_t and S_t compared to *Cu-1*. An additional observation noted was that by increasing the P/L ratio, the W_t and S_t increases for all the cements. The liquid component consists of the acid (PAA) and water. Typically a higher concentration of PAA results in increasing the number of COO^- groups in the liquid, thus accelerating the setting reaction. However, an opposite trend was observed here. The W_t and S_t were delayed by increasing the liquid in formulation. One possible explanation is that by increasing the liquid content, system becomes more fluid. This makes the cement mix more dilute, decreases the initial viscosity, and this decreases the availability of binding sites for chelation thereby delaying the crosslinking process. An ideal bone cement should have an extended W_t to allow sufficient manipulating, and a rapid S_t to reduce the risk of infection [33]. The experimental GPCs presented here exhibit typical W_t and S_t observed for dental GPCs. For commercially

available dental GPCs the W_t is quoted to range from 1.3 - 3.8 minutes and the S_t is reported to range between 2.7 - 6.2 minutes [33]. Yet, the majority of commercially available acrylic based cements for skeletal applications have longer W_t ranging between 3-8 minutes and S_t ranging between 6-14 minutes [52]. The S_t of the GPCs formulated in this study is comparable with commercially available bone cements, however, their W_t falls shorter than timeframes proposed for Vertebroplasty or Kyphoplasty with a recommended range between 5-8 minutes [52].

The mechanical properties of the cements were evaluated with compression, flexural, and shear bond strength testing outlined by ISO standards where applicable. Figure 2 illustrates the effect of differing P/L ratio and differing maturation time on the GPCs CS. The results from Figure 2a, indicates that the CS was not significantly affected by neither P/L ratio nor maturation time ($p > 0.05$), however, it was influenced by chemistry of the glasses. Highest strength was obtained by *Cu-1* with significantly higher CS values compared to *Control* and *Cu-2* ($p=0.000$) during 1 to 21 days of incubation period. *Cu-2* identified as the weakest cement with significantly lower CS compared to *Control* ($p=0.003$), and *Cu-1* ($p=0.000$). Figure 2b suggests that cements attain most of their CS in their first 24 hours and then retain their strength with respect to incubation time. Compressive strengthening in GPCs can be explained by analyzing the microstructure of cements. As described earlier, after initial stages of setting reaction, the set cement consists of intact glass particles which are surrounded by a crosslinked polysalt matrix [13]. The mechanical strength can be attributed to the magnitude of complexation within the polysalt matrix. The extent, and the strength of the complexation, depends on the glass solubility and release of specific cations involved in GPC maturation, the degree of naturalization, polyacrylic molecular weight and concentration [53]. With regard to *Cu-GPCs*, increased release of Cu^{2+} , facilitates a higher degree of the interaction between the cations and polyanion chains which increases the extent of crosslinking in the cement structure which contributes to increased CS as presented for *Cu-1* (Figure 2b). Unreacted glass particles act as reinforcing agent within the cement matrix. The experimental GPCs examined here, exhibit lower range of CS (20-35 MPa) compared to commercially available bone cements which range between 80-100 MPa for Vertebroplasty or Kyphoplasty [52], and conventional dental GPCs which ranging between 60-300 MPa [14]. CS of Fuji IX and Ketac Molar, one hour after mixing, has been quoted to be of 84 and 86 MPa, respectively [54].

In studying the mechanical properties of GPCs, CS is not particularly reliable, since the fracture mode of GPCs

at the atomic level is accompanied by tensile or shear failure rather than compression [47, 55]. Tensile measurement of brittle materials such as GPCs comes with its own limitations, therefore, BFS is considered as the more relevant analysis to assess tensile strength of the GPCs. BFS of *Control* and *Cu-GPCs* as a function of incubation in DI water is illustrated in Figure 3a. *Control* and *Cu-GPCs* show similar values ($p > 0.05$) at each time period: 12, 14 and 14 MPa for *Control*, *Cu-1*, and *Cu-2* after 21 days incubation. The BFS for each GPC was observed to slightly increase with respect to maturation time. This effect may be attributed to the ongoing dissolution of the glass particles resulting in increased crosslinking. The setting reaction of these cements takes place in three stages; dissolution, gelation and maturation [13]. The first two stages are typically completed within the first 24 hours, however maturation accompanied with additional crosslinking can take place over extended time periods [14]. Increased concentration of polysalt crosslinked COO^- groups increases the BFS of cements which range from 8-14 MPa. BFS values attained here can be considered for non-load bearing skeletal applications, and is comparable with the values for commercially available CPCs such as Cementek (Teknimed, Toulouse, France) and α -BSM (ETEX Corporation, MA, USA) that have flexural strengths of 9 MPa and 4 MPa respectively [49].

To further evaluate the mechanical and adhesive nature of these *Cu-GPCs*, shear bond strength (SBS) testing was performed on the *Control* and *Cu-1* cements which exhibited higher CS and BFS compared to *Cu-2*. The SBS results are presented in Figure 3b. SBS testing can provide an insight into the extent of chemical bonding at the cement-bone tissue interface. Studies on the adhesion mechanism of GPCs to dentin and enamel suggest that chemical bonding is developed with the HaP mineral phase, rather than the collagen component [15]. Initially when freshly mixed cement paste is placed on surface, it will be wetted due to the hydrophilic nature of HaP [15]. Gradually, ionic bonds will develop between free COO^- groups from the PAA and calcium and phosphate ions from HaP. As time proceeds a diffusion process occurs where ions from the cement and the HaP diffuse to an interfacial zone. Eventually, the ion exchanged layer consists of calcium and phosphate ions from HaP in addition to ions liberated from the glass phase of the GPC [15, 17]. This may explain the increase in SBS of the GPC with respect to time. Glass dissolution and diffusion of ions over time will contribute to development of a strongly ion exchanged interface. With respect to this study, surface topography was analyzed with laser profilometry of cement-HaP interface post fracture, and the effect of incubation time on the bond strength. The re-

sults from Figure 4 and accompanying surface characteristics (Figure 5) present visible differences in the topography of the fractured samples after 1 and 21 days incubation. Comparison of each GPC surface after 1 and 21 days presents two different failure mechanisms. Smooth fracture surface (Figure 5) of the GPCs after 1 day suggests little to no chemical bonding is developed at the interface and thus cements fail adhesively at the interface. Materials that experience adhesive failure typically exhibit low bond strength [15, 56]. However, after 21 days a considerable amount of the GPC was adhered HaP, post fracture. This suggests an alternative mechanism of failure. Rough fracture surface of the *Control* and *Cu-1* after 21 days in Figure 4 and 5, suggests that cohesive failure occurs within the cement rather than at the interface. This observation is more clear for *Cu-1* cements after 21 days incubation period, (Figure 4 and 5). A considerable amount of residual cement at the interface of *Cu-1* after 21 days suggests that extensive chemical bonding has occurred. This can be further confirmed with optical microscopy images taken from the *Cu-1* fracture surface after 1 and 21 days incubation (Figure 6). It is evident the incubation time leads to increased crosslinking and maturation within the GPC, and additionally the addition of Cu to the glass phase of the GPC greatly improves the adhesive nature of the material.

In order to determine the solubility, and to further analyze potential therapeutic effects of the cements, ion release profiles were analyzed with respect to incubation time. Levels of Si^{4+} , Zn^{2+} , Ca^{2+} , Sr^{2+} , PO_4^{3-} , and Cu^{2+} were released from cements after 1, 7, 14 and 21 days (Figure 7). Overall, *Cu-GPCs* exhibited similar ion release profiles, however, the *Control* and *Cu-GPCs* presented different behavior for Si^{4+} , Zn^{2+} , and PO_4^{3-} release. Gradual and steady Si^{4+} release plays a key role in the process of *in vivo* mineral formation. Release of Si^{4+} ions from the glass into physiological solution results in formation of Si-OH (Silanols) on the surface, which is known as the first initial step for calcium phosphate deposition [57]. *Control* and *Cu-GPCs* exhibited Si^{4+} release in the range of 24 to 104 mg/L. Si^{4+} release, <100 mg/L, is considered favorable to enhance bone formation through inciting stimulatory effects on osteoblast cell proliferation [58]. Zn^{2+} ions have also proven to positively affect bone metabolism by promoting its formation and mineralization [59]. Zn^{2+} can favorably enhance alkaline phosphatase activity in bone tissue, however, it has been reported that Zn^{2+} concentration higher than 16 mg/L could be cytotoxic for surrounding bone tissue [58]. *Control* cement exhibited low Zn^{2+} release, ranging from 0 to 0.1 mg/L over 21 days. *Cu-GPCs* showed general increase in Zn^{2+} release which ranged from 0 to 1.9 mg/L over 21 days of incubation. With respect to Ca^{2+} re-

lease, cements showed a similar profile. However, for PO₄³⁻ ion release, *Control* exhibited a higher rate of P release compared to the *Cu-GPCs*. Overall, release of Ca²⁺ and PO₄³⁻ from the cements showed a gradual increase with respect to time. Ca²⁺ and PO₄³⁻ are the main inorganic constituents in skeletal tissue and it is widely established that the release of these ions triggers new bone formation along the bone-implant interface [60]. *Control* and *Cu-GPCs* exhibited similar Sr release profiles, with a gradual increase, reaching to 6mg/L after 21 days. *In-vivo* studies on the therapeutic effects of Sr²⁺ revealed enhanced osteoblast cell activity, differentiation, and proliferation [61, 62]. In general, lower levels of Si⁴⁺ and PO₄³⁻ release from *Cu-GPCs* may be attributed to structural effects of Cu²⁺ addition in the glass structure. In a previous study, MAS-NMR results of these glasses established that the addition of CuO increased the percentage of Q³ and Q⁴ species within the glass, indicating an increase in the number of bridging-oxygens (BOs) in the glass. This effect results in increasing the network connectivity of the glass structure, [34] and makes the glass less soluble, and will likely decrease the release of network forming ions such as Si⁴⁺ and P. However, network modifier cations such as Sr²⁺ and Ca²⁺, which form crosslinks with PAA groups, are less restricted within the glass structure and more are available to release upon incubation in an aqueous environment [33]. This could explain the similar levels of release attributed to Ca²⁺ and Sr²⁺ observed for each GPC. The *Cu-GPCs* exhibited a relatively constant Cu²⁺ release rate over 21 days which may provide a positive therapeutic benefit as Cu²⁺ ions possess are known to induce antimicrobial effects, and are also cited to improve angiogenesis through stimulation of endothelial cells and new bone formation at implant sites [35, 37].

Analyzing the surface depositions of materials incubated in SBF is conducted as a screening test to study the ability of a materials potential for bone bonding in physiological fluids [48]. Precipitation of calcium phosphate on the surface is regarded as a pre-requisite for the material to bond to living bone and provides the driving force for formation of biologically active bone-like apatite [48]. Analysis of SEM images along with their corresponding EDX spectra (Figure 8) of the *Control* and *Cu-GPCs* after 21 days incubation in SBF identifies the presence of Ca²⁺ and PO₄³⁻ containing precipitates on the materials surface. Similar morphologies of the precipitants suggest that the *Control* and *Cu-GPCs* exhibit similar behavior when incubated in the SBF. The initial glass compositions contain 6 and 2 Mol% of Ca²⁺ and PO₄³⁻, respectively (Table 1). Despite the low amount of Ca and P in the starting glass composition, EDX results confirms considerably higher amounts

of Ca²⁺ and PO₄³⁻ are deposited on the surface after SBF incubation, acquiring up to 17 and 26 wt% of Ca²⁺ and PO₄³⁻ for *Cu-GPCs*, respectively. Comparing these values with the initial glass compositions, suggests high degree of reactions occurred between the GPCs and the SBF solution resulting in formation of calcium phosphate. Dissolution of the glass particles from the cements and the release of Si ions leads to the formation of silanol groups (Si-OH) on the surface. Silanol groups are scattered across the surface and eventually form a silica-rich layer. Si-OH groups from the glass, and COOH groups from PAA covers the surface as favored sites for continuous absorption of calcium and phosphate ions from the solution and from the glass [63, 64]. This leads to the formation and growth of amorphous calcium phosphate on top of the silica-rich layer [65]. Low concentrations of Si⁴⁺, and high concentrations of Ca²⁺ and PO₄³⁻ from EDX (Figure 8) suggest the formation of calcium phosphate rich depositions that cover the silica-rich layer on the surface of the incubated GPCs. Further insight into the surface depositions reveal high levels of Zn²⁺ are present. This might be due to high content of Zn²⁺ in the initial glass composition. Zn²⁺ are released into solution and supersaturate the SBF-cement interface, resulting in formation of zinc rich calcium phosphate depositions on the surface of cements [66]. Subsequently, release of Sr²⁺ and Cu²⁺ ions (only in *Cu-GPCs*) into the solution, results in the precipitation and formation of Sr²⁺ and Cu²⁺ containing zinc-calcium-phosphate depositions, as the EDX elemental composition of the precipitates, confirms presence of Sr²⁺ and Cu²⁺ on the surface of cement discs. The presence of therapeutic ions such as Cu²⁺ and Sr²⁺ on the surface depositions and further releasing to surrounding biological environment plays a critical role to enhance bioactivity and antibacterial efficacy [58].

Next, the cements were analyzed for their antibacterial efficacy. Bacterial infection remains a major concern in the field of orthopaedics, and negatively impacts the clinical outcome of the orthopedic devices causing complication such as osteomyelitis (bone infection) [67]. The four most common bacterial species causing osteomyelitis are *S. aureus*, *P. aeruginosa*, *S. epidermidis* and *E. coli*, while *S. aureus* remains as the dominant species accounting for over 45% of all the skeleton infections [68]. In this study, the antibacterial efficacy of the cements was tested against three prevalent infectious bacteria was conducted and the results are presented in Figure 9 and 10. The images of bacterial cultures in Figure 9, clearly shows enhanced inhibition zone (IZ) for *Cu-GPCs*, specifically against *S. epidermidis* and *S. aureus* bacteria. The growth of all three species of bacteria is significantly ($p=0.000$) reduced for

Cu-1 and *Cu-2*, when compared to *Control* cement. *Cu-2* shows significantly greater IZ than *Cu-1* when tested in *E. coli* ($p=0.016$), and *S. aureus* ($p=0.000$). However, no significant change was observed in IZ of *Cu-1* and *Cu-2* when tested in *S. epidermidis* ($p=0.836$). The agar-broth test method results (Figure 10d, 10e, 10f) give a clear insight to the cements antibacterial properties over extended time periods, and when compared to healthy growing bacteria. For all three-bacterium tested, the bacterial viability of *Cu-GPCs* was found to be 1) significantly lower ($p=0.000$) than healthy growing bacteria, and 2) significantly lower ($p=0.000$) than the *Control* cement. However, the *Control* cement showed different behavior when compared against the reference bacteria. For the *Control* cement, the viability was found to be significantly lower ($p=0.000$) than 100% reference bacteria only when tested in *E. coli*, but significantly higher ($p=0.043$) when tested in *S. epidermidis*. No statistically significant change in viability was observed for *Control* cement when tested in *S. aureus* ($p=0.552$). For all three species of bacteria, *Cu-GPCs* exhibit a consistent bacterial viability, as their viabilities do not significantly vary ($p < 0.005$) at each time period and over 1 to 21 days of incubation. Wren *et al.* studied the antibacterial properties of the commercially available bone cements including Simplex P+Tobramycin, Spineplex and Novabone against *E. coli*, *S. epidermidis* and *S. aureus* [69]. No inhibition zone was reported for the bone cements without antibiotics against three different species of bacteria. Maximum IZ of 13mm was reported for Simplex P + antibiotic when tested in *S. epidermidis* [69]. However, experimental *Cu-GPCs* exhibited greater IZs even when compared to antibiotic containing bone cements such as Simplex P. The antibacterial efficacy of the *Cu-GPCs* is greatly enhanced by incorporation of the Cu^{2+} within the glass phase. These can be one alternative to antibiotic-loading of the cements, which has developed serious antibiotic-resistant bacterial pathogens [70]. The exact molecular mechanism in which Cu^{2+} ions act as antibacterial agents is complex and are not understood completely, however, several pathways are suggested. High concentration of Cu^{2+} inside a bacteria cell, induces Cu^{2+} redox cycling, this leads to generation of hydrogen peroxide which results in oxidative damage to bacteria. Formation of reactive oxygen species (ROS) can cause oxidative stress and disrupts the cell hemostasis [71]. Cu^{2+} also obstruct the cell metabolism by disrupting proteins and enzymes in bacteria, resulting in uncontrolled activation of hydrolytic enzymes and causing membrane damage to the bacteria [72].

The MC3T3-E1 cell viability of the untreated GPC extracts are presented in Fig. 11. The MTT assay determines the number of viable cells based on cell metabolism [73].

The results from Figure 11 illustrates that the cell viabilities of *Control* and *Cu-GPCs* were significantly reduced when compared to healthy growing cell population, which was assumed to have a metabolic rate of 100%, with ($p=0.000$) for *Control*, ($p=0.000$) for *Cu-2*, and ($p=0.001$) for *Cu-1*. Additionally, analyzing the behavior of the cements over differing time periods demonstrates that the cell viability of the *Control* cement significantly ($p=0.000$) decreases over the 1 to 21 days incubation period. However, the *Cu-GPCs* exhibited a consistent cell viability profile, with insignificant influence on cell's metabolism over 1 to 21 days, with ($p=0.063$) for *Cu-1*, and ($p=0.870$) for *Cu-2* GPCs. One possible explanation is higher degree of solubility for *Control* cements compared to *Cu-GPCs*. Dissolution products of the glass and acid component of the GPCs can significantly alter the pH of the surrounding solution and consequently affect the number of viable cells. Generally, GPCs consist of highly soluble glass particles, and aqueous solutions of polymeric acids such as PAA, where release of its unreacted monomer is considered as the main reason for their mild cytotoxicity [74].

To conclude, the incorporation of Cu within GPCs can impart beneficial properties for materials applied for vertebroplasty and kyphoplasty. This series of *Cu-GPCs* presents solubility profiles that encourage bioactivity, antibacterial efficacy and retains cytocompatibility. An area that will need addressing in the future are the mechanical properties, in particular the biaxial flexural strength and shear bond strength. Improving the *Cu-GPCs* flexural strength through glass fiber reinforcement and testing their mechanical properties post-incubation in Simulated Body Fluid over extended time periods will be a primary focus. Additionally, cell adhesion studies will be conducted to determine MC 3T3 Osteoblast and L929 Fibroblasts ability to colonize and proliferate on the surfaces.

Conflict of Interests: The authors declare no competing financial interest.

Ethical approval: The research related to animals' use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

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