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A glass polyalkenoate cement carrier for bone morphogenetic proteins

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Abstract This work considers a glass polyalkenoate cement (GPC)-based carrier for the effective delivery of bone morphogenetic proteins (BMPs) at an implantation site. A 0.12 CaO–0.04 SrO–0.36 ZnO–0.48 SiO_2 based glass and poly(acrylic acid) (PAA, Mw 213,000) were employed for the fabrication of the GPC. The media used for the water source in the GPC reaction was altered to produce a series of GPCs. The GPC liquid media was either 100 % distilled water with additions of albumin at 0, 2, 5 and 8 wt% of the glass content, 100 % formulation buffer (IFB), and 100 % BMP (150 μg rhBMP-2/ml IFB). Rheological properties, compressive strength, ion release profiles and BMP release were evaluated. Working times (T_w) of the formulated GPCs significantly increased with the addition of 2 % albumin and remained constant with further increases in albumin content or IFB solutions. Setting time (T_s) experienced an increase with 2 and 5 % albumin content, but a decrease with 8 % albumin. Changing the liquid source to IFB containing 5 % albumin had no significant effect on T_s compared to the 8 % albumin-containing BT101. Replacing the albumin with IFB/BMP-2 did not significantly affect T_w. However, T_s increased for the BT101_BMP-2 containing GPCs, compared to all other samples. The compressive strength evaluated 1 day post cement mixing was not affected significantly by the incorporation of BMPs, but the ion release did increase from the cements, particularly for Zn and Sr. The GPCs released BMP after the first day, which decreased in content during the following 6 days. This study has proven that BMPs can be immobilized into GPCs and may result in novel materials for clinical applications.

1 Introduction

Bone morphogenetic proteins (BMPs) are a group of growth factors (GFs) that are members of the transforming growth factor β (TGFβ) superfamily [1]. BMPs can also be defined as cytokines that are significant in bone formation and healing. An example includes the recombinant human BMPs (rhBMPs) which are widely used in tissue engineering for the complete regeneration of cartilage or bone [2, 3]. Several BMPs influence osteoprogenitor cell migration, proliferation and differentiation [1, 4]. Among them, BMP-2 has been identified as the most potent and has been approved for clinical use in spinal fusion, long bone non-union healing and alveolar ridge augmentation [5–10].

Carriers, also known as scaffolds, can be used to deliver BMPs into a targeted area in order to aid bone healing. Seeherman and Wozney [11] define successful carriers in terms of their biocompatibility, resorbability and ability to allow regenerative tissue forming cells into the area to proliferate and differentiate. Ideally when a carrier is implanted, the exogenous BMP concentration needs to be above the critical minimum required for a positive host response for a sufficient time, as to allow rapid cell

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population and to generate a positive tissue response [11, 12]. The exogenous BMP release profiles are dependent on the properties of the carrier, and as numerous release profiles exist; the carrier design is important.

BMPs can be immobilized into different carriers by three main techniques: (1) adsorption, (2) entrapment or (3) covalent bonding. The latter is the most preferred technique since adsorption results in conformational changes associated with less sustained release while entrapment may result in denaturation of the protein (disrupting the structure of the protein and un-coiling it into a random shape) due to pH or temperature changes during material processing [13, 14]. Current carriers can be divided into natural polymers, inorganic materials, synthetic polymers and their co-polymers with some examples of these being calcium phosphates [15, 16], phosphate-based cements [17], poly(lactic-co-glycolic acid) [18], lipid based composites [19], bio gelatin microparticles [20] and silica nanotubes [21]. Additionally, allograft and autograft materials have also been used [22, 23]. The most common carriers are collagen sponges, however they are unable to retain their shape against the compressive forces that they experience after implantation, preventing cell ingrowth and only weakly binding to the BMP which may result in a large bolus release of the BMP from the carrier within hours. This "burst profile" results in the need to apply large amounts of BMP to ensure sufficient concentrations remain during the healing period [10]. These supraphysiological doses have been reported to elevate cancer risk when released in very large amounts [24–26], alongside stimulating an immune response [27, 28]. However initial burst profiles have been shown to aid in the signalling of BMP response cells [29].

Glass polyalkenoate cements (GPCs) were first developed by Wilson and Kent in the 1970s. The original GPCs consisted of a matrix of *fluoro-alumino-silicate* glass, a base, mixed with an aqueous solution of poly(acrylic acid) (PAA), the acid phase. The matrix is obtained by an acid–base reaction between both components in the presence of water, where the protons from the acid rapidly attack the glass network resulting in degradation and release of the glass ionic components. Thereafter, the released cations chelate and crosslink the polymer chains to form a complex composite (cement) that maturates with the presence of water as a medium [30, 31].

It is proposed that an injectable GPC may have the potential to deliver BMPs to a specific surgical site and subsequently release them while the GPC is setting, and throughout the cement's maturation. However only a limited number of studies investigating the effects of incorporating organic molecules on the properties of GPCs have been conducted. Wren et al. [32] investigated the effects of the addition of biological substances including chitin, collagen, cysteine and keratin to an aluminium free Ca–Sr–Zn–SiGPC and reported that these proteins had little influence on the working and setting times of the GPCs; although compressive strength was found to decrease post incorporation. Furlan et al. [33] investigated the effects of grafting PAA carboxylic groups with organic molecules such as chitin on the metal binding ability of calcium (Ca^{2+}) ions. They have recommended the use of chitin-PAA copolymer in the preparation of GPCs due to the improved ability of such polymer to adsorb and bond to Ca^{2+} ions.

The work contained herein investigates the feasibility of a CaO–SrO–ZnO–SiO_2 based GPC as a carrier for BMPs by evaluating the time dependent physical, mechanical and biological properties of composites formulated from this GPC loaded with BMP-2 ligands.

2 Materials and methods

2.1 Glass synthesis

A 0.12 CaO–0.04 SrO–0.36 ZnO–0.48 SiO_2 glass, hereby known as BT101, was formulated by weighing out appropriate amounts of analytical grade reagents (Sigma-Aldrich, Canada) and ball milling (1 h). The mixture was then oven dried (100 °C, 1 h), fired in a platinum crucible (1500 °C, 1 h) and shock quenched in water. The resulting frit was dried, ground and sieved to retrieve a glass powder with a maximum particle size of 45 μm. The glass was then annealed (640 °C, 3 h to relieve internal stresses within the glass network) and used for cement production.

2.2 Cement preparation

PAA (Mw, 213,000) was supplied by Advanced Healthcare Limited (Kent, UK). The cements were formulated in a powder: liquid (P/L) ratio of 1:0.75, i.e. 1 g of glass powder was mixed with 0.37 g PAA and 0.37 ml of liquid. The liquid portion was varied between 100 % distilled water, 100 % formulation buffer (IFB; 2.5 % glycine, 0.37 % glutamic acid, 0.01 % sodium chloride, 0.5 % sucrose and 0.01 % Tween 80, pH 4.5) and 2, 5 and 8 % (of the total glass content) albumin in IFB and 100 % BMP (150 μg rhBMP-2/ml IFB). Thorough mixing of these samples was achieved within 35 s in ambient temperature (23 ± 1 °C). Table 1 outlines the formulations tested.

2.3 Working time

The working time (T_w) of five samples per formulation were measured in ambient air using a technique outlined in ISO 9917-1:2007. The T_w is defined as the time from the

Table 1 Formulation table showing the changing liquid portion of the cement samples

Formulation number	Albumin (%)	Distilled water (%)	IFB (%)	BMP-2 (%)
1 (Control)	0	100	0	0
2	2, 5, 8	100	0	0
3	5	0	100	0
4	0	0	0	100

IFB solution: 5.0 mg sucrose, NF; 25 mg glycine, USP; 3.7 mg L-glutamic acid, FCC; 0.1 mg sodium chloride, USP; 0.1 mg polysorbate 80, NF; 1.0 mL of sterile water (WFI) and a pH of 4.4 ± 0.1

start of mixing, through which the material can be manipulated without having an adverse effect on its properties.

2.4 Net setting time

The net setting time (T_s) of five samples per formulation were tested in ambient air according to ISO 9917-1:2007.

2.5 Compressive strength

The compressive strength (σ_c) of five samples per formulation, one day post mixing, was evaluated in ambient air according to ISO 9917-1:2007. Five cylindrical samples (6 mm height, 4 mm diameter) were tested after being incubated in distilled water (37 °C, 1 day). Testing was undertaken on a United Universal Tester (STM-50KN, United Testing Systems, Inc., Huntington Beach, CA, USA) using a ±2 kN load cell at a crosshead speed of 1 $mm \cdot min^{-1}$.

2.6 Statistical methods

One-way analysis of variance (ANOVA) was used to analyze the data. *Post-hoc* Bonferroni test was used to compare the relative means and to report the statistically significant differences when $P < 0.05$. Statistical analysis was performed using statistical package for the social sciences (SPSS) software (IBM SPSS statistics 21, IBM Corp., Armonk, NY, USA).

2.7 Ion release

Ion release studies were performed according to a method described by Wren et al. [34]. The ion release profiles (n = 5 samples) were measured after 1-day maturation using an Agilent 4100 (Agilent Technologies, Inc., Santa Clara, CA, USA) microwave plasma–atomic emission spectrometer (MP–AES). MP–AES calibration standards for Si, Sr, Ca, and Zn were prepared from a stock solution on a gravimetric basis. Three target calibration standards were prepared for each ion with 0.3, 0.5 and 1.0 parts per million (ppm) concentrations while distilled water was used as a *blank*. Samples for Ca, Sr, and Zn analysis were diluted in a ratio of 1:10 while samples for Si analysis were diluted in a ratio of 1:30. A pilot study was conducted to determine the appropriate ratio for dilution of all elements.

2.8 BMP release studies

Samples of the GPCs loaded with BMP were placed into an Eppendorf tube (n = 5). 1 mL of phosphate buffered saline +0.1 % bovine serum albumin (PBS + BSA) was added to each tube and the tubes were closed and held at 38 °C. After 1 day the PBS + BSA was removed and replaced with a further 1 mL of fresh PBS + BSA. This was incubated for a further 6 days and then removed. The PBS + BSA samples were stored at −20 °C until ready for analysis.

BMP-2 concentrations were determined using an enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Quantikine ELISA Kit; R&D Systems Inc. Minneapolis, MN). The amount of BMP released was normalized to the weight of GPC in each tube.

3 Results

GPCs were formulated in line with the methods section and evaluated physically, mechanically and biologically.

3.1 Working and setting times

The T_w and T_s for BT101 cement formulations with PAA and distilled water (i.e. 0 % albumin (Alb.)), distilled water with 2, 5 and 8 % Alb., IFB with 5 % Alb. and BMP-2 solution are presented in Figs. 1 and 2, respectively.

T_w shows a statistical increase in the BT101_2 % Alb samples, with an increase from 77 to 102 s. Increasing the albumin content to 5 and 8 %, and subsequently changing the water component to IFB with albumin and then IFB with BMP-2 resulted in no statistical difference ($P < 0.05$) in T_w. However all samples are statistically different compared to the *Control* sample, BT101 0 % Alb.

T_s experiences a statistical increase from 160 to 188 s with 2 % albumin additions to the water source, which

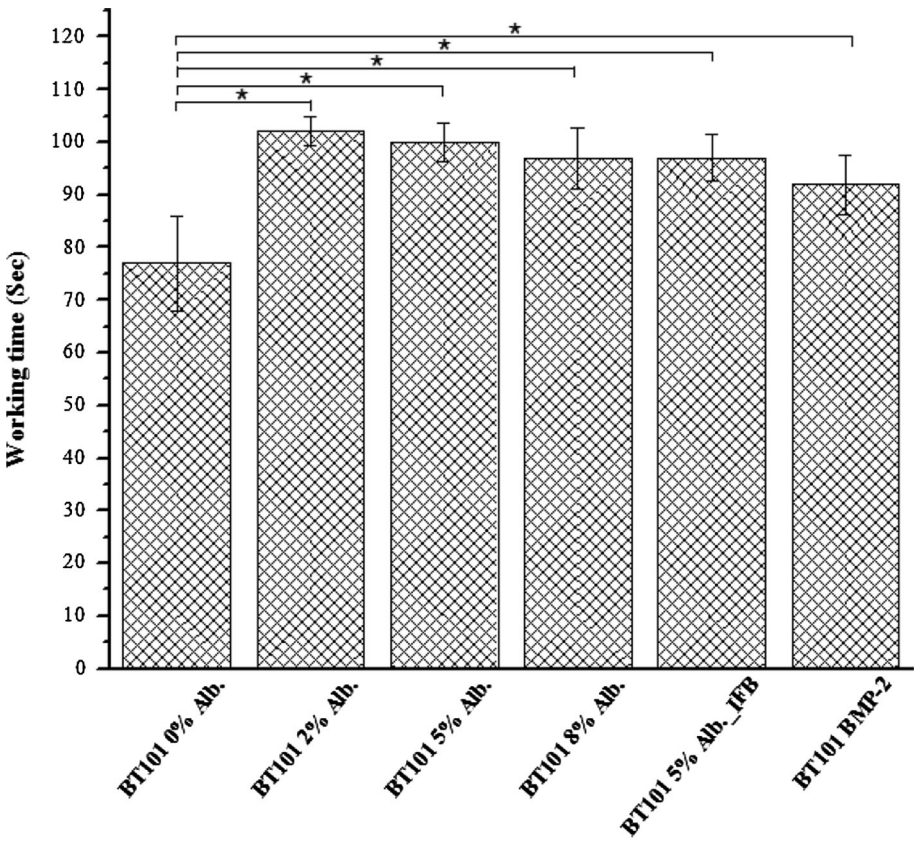

Fig. 1 Working times for BT101 glasses mixed with different albumin (Alb.) loadings (0, 2, 5, 8 wt%), IFB with 5 % Alb. and BMP-2 solution. *Stars* and *bars* show statistical significance ($P < 0.05$)

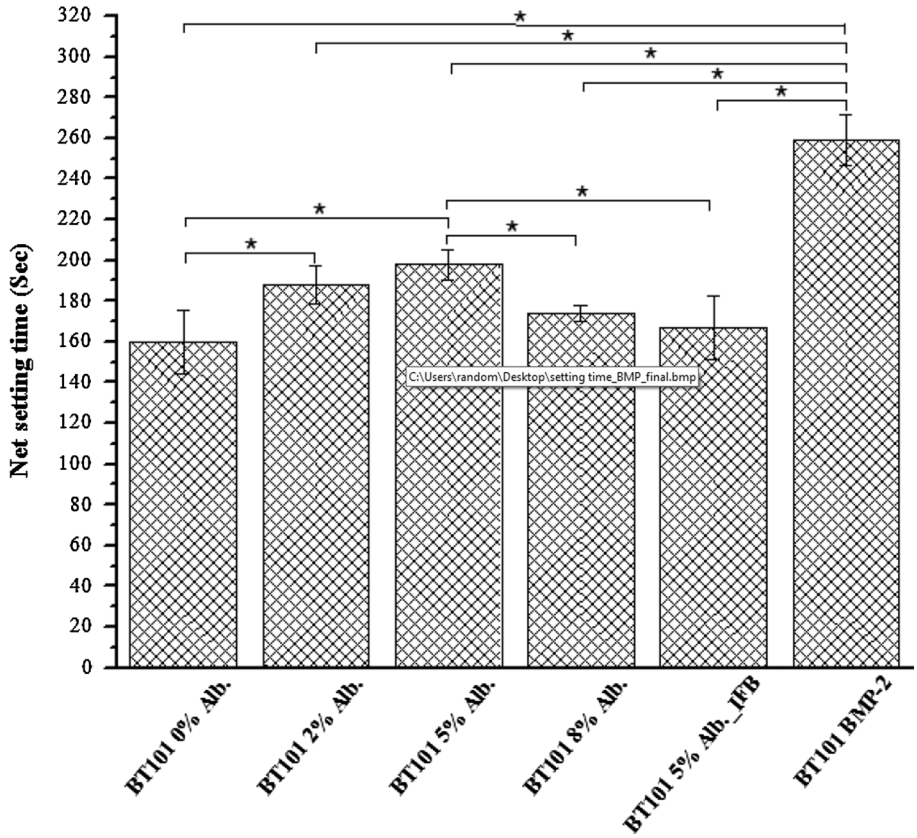

Fig. 2 Setting times for BT101 glasses mixed with different albumin (Alb.) loadings (0, 2, 5, 8 wt%), IFB with 5 % Alb. and BMP solution. *Stars* and *bars* show statistical significance ($P < 0.05$)

increases to 198 s for the BT101 5 % Alb samples, although there is no statistical difference between the 2 and 5 % Alb samples. Introducing 8 % albumin results in a decrease in T_s down to 174 s which is significantly different to the previous BT101 5 % Alb samples, but not compared to the *Control* BT101 0 % Alb. This decrease in T_s continues when the water source is replaced with 5 % Alb in 100 % IFB solution, giving a T_s of 167 s. Removing the 5 % albumin from the IFB and replacing with BMP-2 proteins results in a statistically significant jump in T_s to 259 s.

3.2 Compressive strengths

The compressive strength data for BT101 cements mixed with 0 % Alb., 5 % Alb., IFB with 5 % Alb., and BMP-2 are presented in Fig. 3. Testing was conducted 1 day post cement preparation.

BT101 samples displays a mean strength of ~24 MPa, which decreases down to ~15 MPa with the addition of 5 % Albumin. Replacing the water source for the cement reaction with IFB solution increases the mean strength to ~22 MPa. This mean strength increases to ~30 MPa with the replacement of the 5 % albumin with BMP-2 in the IFB solution. One-way ANOVA shows that there is only a significant difference between the BT101 5 % Alb samples and the BT101_BMP-2 samples.

3.3 Ion release profiles

The ion release profiles for the BMP-2-containing BT101 GPCs were tested, 1d post cement preparation and compared to BT101 GPCs and *blank* water samples. Figure 4 presents the results obtained. Figure 4 shows that the incorporation of BMP-2 into BT101 GPCs resulted in increased dissolution of all cations when compared to the BT101 or water *blank* samples. Si levels were substantially higher when released from the BMP-2 containing samples (19.6 ppm) compared to the *blank* (1.9 ppm). Sr was released at a rate five times higher in the BMP containing samples (3.9 ppm) than the *blank* (0.1 ppm). Ca also released at slightly higher rates from the BMP-2 containing samples (3.6 ppm) than the *blank* (0.54 ppm). Similarly, Zn released at slightly higher rates from the BMP-2 containing samples (3.85 ppm) when compared to the *blank* (0.19 ppm).

3.4 BMP release

Table 2 displays the amounts of BMP released at 1 and 7 days, post cement preparation and incubation with PBS + BSA at 37 °C. After 1*d* the GPCs released a mean amount of 1.01 ± 0.36 ng BMP-2/g GPC. Over the following 6 days a further 1.67 ng/g were released.

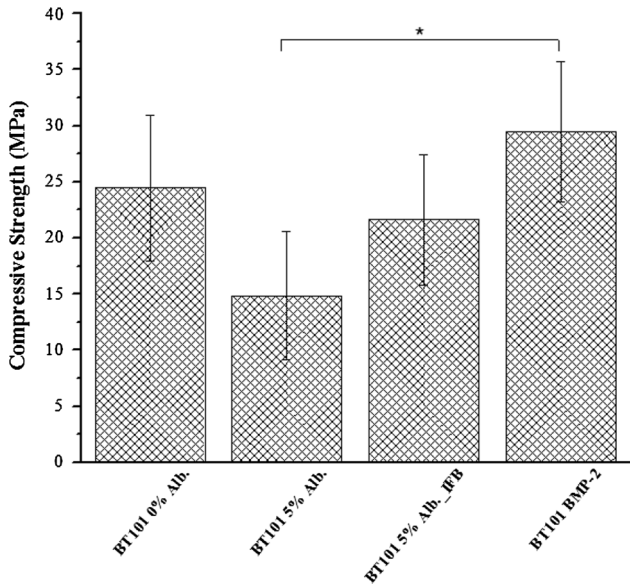

Fig. 3 One day compressive strengths of BT101 glasses mixed with different albumin (Alb.) loadings (0, and 5 wt%), IFB with 5 % Alb. and BMP solution. *Stars* and *bars* show statistical significance ($P < 0.05$)

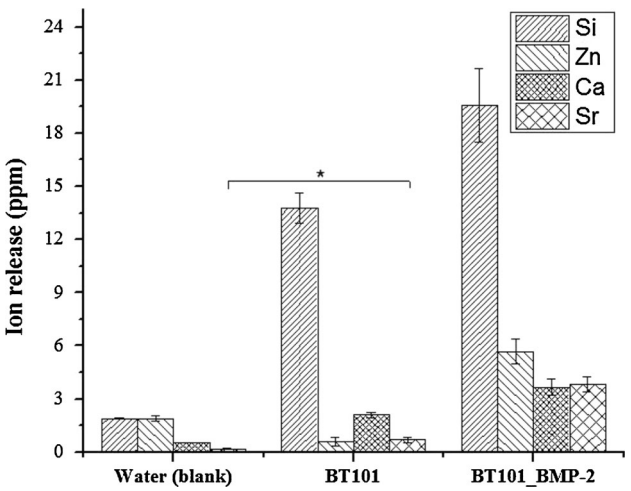

Fig. 4 One day ion release profiles for water (blank sample), Control BT101 and BMP-2 loaded BT-101 cements (ppm). *Stars* and *bars* show statistical in-significance ($P < 0.05$). All P values were <0.05 except for Sr tested for water (blank) and BT101 cement samples

Table 2 ELISA BMP-2 release profiles based on weight, displayed in parts per billion (ng/g)

Samples	Day 1 (ng/g)	Day 7 (ng/g)
Mean	1.01	1.67
St dev.	0.36	1.89

4 Discussion

The aim of this work was to evaluate the effect of incorporating albumin and BMP-2 into a bioactive glass cement, specifically one based on 0.12 CaO–0.04 SrO–0.36 ZnO–0.48 SiO_2 glass. This cement has been the subject of earlier characterisation studies [32, 35, 36].

T_w of the *Control* GPC (BT101 0 % Alb) was found to significantly increase with the addition of 2 % albumin. This value remains relatively constant with no statistical change (Fig. 1) with further increases in albumin content or IFB solutions. The T_s experiences an increase with 2 and 5 % albumin, but a decrease with 8 % albumin and 5 % Alb. in 100 % IFB solution. The incorporation of BMP-2 was found however to increase the T_s significantly to ~259 s (4.3 min).

Proteins have been reported to possess the ability to bind cations [37], there by causing conformational changes within the cement matrix and the subsequent delay in the initial handling properties. This is in good agreement with the results reported in this study. Following the argument that proteins can bind cations, it is safe to postulate that albumin and BMPs also have the ability to bind cations and change the handling properties when they are incorporated into GPCs. The incorporation of different percentages of albumin resulted in a random increase and decrease in the working and setting times, as discussed earlier in Sect. 3.1. It was however shown that albumin or BMP-containing samples have resulted in longer or relatively equivalent setting and working times when compared to those values obtained for the *Control* samples. The albumin used was in freeze-dried form when added directly to the distilled water source for cement preparation. As such the number of water molecules available for the reaction is likely reduced with increasing albumin content, thus reducing T_s over critical amounts (>5 %) of added albumin. Changing the liquid source to IFB containing 5 % albumin (BT101 5 % Alb_IFB) had no significant effect on the T_s compared to the BT101 8 % Alb. The decrease in the T_s for BT101 8 % Alb and BT101 5 % Alb with 100 % IFB might also be attributed to the change in the electrostatic field of the cement paste. High values of Alb. (>5 %) or the replacement of water with IFB may change the charge balance and pH of the paste (during charge compensation), thus changing the electrophoretic mobility in such a way that the liberated cations migrate within the paste at a quicker rate resulting in quicker chelation of PAA chains, and obviously quicker setting properties. This was found to be in good agreement with the literature [37], which interrelated the protein mobility to the material's overall charge and stated that the interaction of metal ions with proteins would normally result in shorter protein migration time due to a less negative surface of the protein through interacting with a positively charged ions or ligands. The incorporation of BMP-2 ligands (Fig. 5a) on the other hand, is expected to interrupt the glass-polymer network during the setting process, so that the formation of the polymeric-silicic acid (which later condenses to form a silica gel) is delayed. Thereafter, BMP would be absorbed (Fig. 5b) and covalently bind the released glass cations (Fig. 5c). Conformational changes or denaturation of BMP-2 stem from complex formation. On mixing, the pH is high causing the PAA chains to ionize and create an electrostatic field which allows for the release of cations from the glass network into the aqueous cement paste. Glass cations may bind to BMP-2 ligands forming additional complexes with anions from the surrounding solution, which may explain the ability of the cement matrix to set while aging. The retardation in the setting reactions of GPCs as a result of incorporating BMPs can also be explained by assuming that BMP-2 ligands enter into the aqueous phase of the cement paste, causing the PAA chains to coil up and reducing their capacity to ionize, and hence retarding the setting chemistry of the cement matrix [30, 37–41].

The increase in T_s (up to 4.3 min) as a result of incorporating BMP-2 is interesting when compared to that of the *Control* BT101 (2.6 min). The latter was reported as too short for surgical deployment in any application [42]. The end of the T_w and the beginning of the T_s is defined where

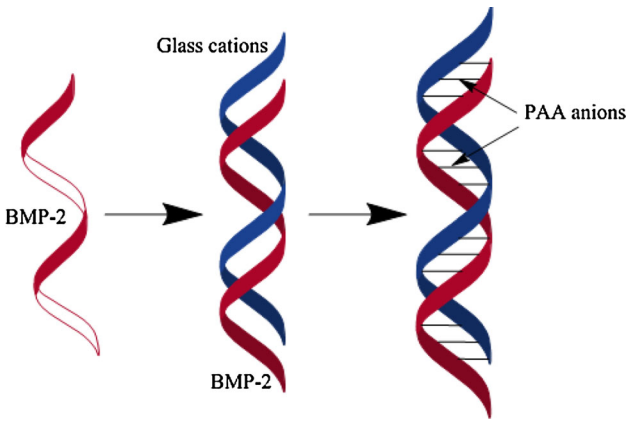

Fig. 5 **a** BMP-2 ligands, **b** BMP-2 ligands bonding to the released cations and **c** bonded BMP-cations chelate PAA chains during cement maturation

the material turns from a plastic to an elastic material and is related to a critical amount of crosslinking occurring in the cement structure between the glass ions and un-bonded carboxylic acid groups (COO^-) [38]. This process continues until the cement has fully set.

The compressive strength of the BMP-2 containing cements, one day post cement mixing, were found to be not significantly different ($P < 0.05$) to those of the *Control* BT101 GPCs. It was explained earlier that this slight increase in strength could result from additional complexes between cations and BMP-2 ligands (bonded together) and the polymer chains, as the cement ages. The compressive strength of human trabecular bone is heavily dependent upon storage and testing conditions but has been reported as being between 2 and 10 MPa. The compressive strength of the GPC, with or without BMP, is approximately 24 MPa, a strength comparable to that of the trabecular bone it is likely to be implanted within [43]. On the other hand, the obtained strength values are considerably low, when compared to the strength of similar materials reported in the literature for dental applications. For example, a compressive strength of 171 and 225 MPa, 24 h post cement preparation, were reported for *Ketac Fil* and *Opusfil* commercial GPCs, respectively [30].

Ion release studies were performed in order to evaluate the solubility of the cement series in relation to incorporation of Alb and BMP. The incorporation of albumin and the type of liquid source for the cement reaction does affect the ion release profiles from these cements (Fig. 4) as cation bonding to the COO^- reduces in the presence of albumin (as implied by T_s data), thus the availability of unbonded cations in the cement increases. The substitution of distilled water with BMP loaded IFB results in increased ion release from the cements over 1 day, particularly for Zn^{2+} and Sr^{2+} ions. This increased ion release supports the hypothesis that IFB is retarding the setting reaction.

ELISA was conducted to evaluate the solubility of BMPs from the cement matrix as the cement ages in distilled water. These results have shown that the GPC was able to release BMP after just 1 day. This release of BMP-2 is most likely due to diffusion from the unset surface of the GPC due to the elongation of T_s, in a similar fashion to the ion release profiles. If the BMP loading was increased, the amount of BMP released would be expected to increase. The amount released over the first 24 h was similar to the amount released over the following 6 days, suggesting that the BMP release rate was reduced as the GPC aged. This could be due to the post-setting (cross-linking) reactions in the GPC that contributes to its maturation, thus making it harder for the BMP to diffuse into medium solution, or it could be a case that the GPC was not loaded with enough BMP to sustain the release. Thus this GPC has an initial "burst" profile that reduces as the cement ages. Additionally, it is worth mentioning that the maximum amount of recorded BMP-2 released after 1 day is very low when compared with concentrations (230 ng/g) previously shown to induce bone anabolism and increased bone remodeling in mice [44]. Other studies in the field [45, 46] have indicated that the threshold dose of BMP for in vivo bone induction is several orders of magnitude (milligram) greater than that (nanogram) of cell responses in vitro. The recorded release of BMP would, therefore, have no effects, beneficial or deleterious, on bone growth and modelling. However this preliminary study has proven the novelty of GPCs as carriers for BMP proteins, which can behave as an in situ tissue-engineered composite that adapts to the physiological environment and provides long-lasting repair. The study has also shown improved strength and longer handling properties of GPCs as a result of incorporating small amounts of BMP-2. Increasing and monitoring the amount of released BMP, up to levels that would induce bone remodeling, would be a suitable subject for future research.

5 Conclusions

- Adding IFB/BMP2 to the control GPC carrier has no significant effect on working time but does extend setting time, increasing the opportunity for the carrier to release BMP at a specific site.
- The increased setting time of the BMP-2 containing GPC, measured one day post mixing, does not affect compressive strength, when compared to the control samples. The compressive strength of the cements are similar to human trabecular bone and should not result in either BMP being squeezed out of the carrier early nor should they cause stress shielding; consequences of a carrier too weak or too strong in compression, respectively.
- Using BMP loaded IFB solution results in an increased ion release profile of the cements compared to the unfilled GPC. This is likely related to the IFB solution extending setting times.
- Increased setting time facilitated the release of BMP-2 from the carriers in amounts that were recordable by the ELISA test.

It has been shown that BMP can be incorporated into, and released from, GPCs without having a deleterious effect on the compressive strength of the GPC itself. The GPC released BMP-2 in the largest amounts in the first day, which was followed by reduced BMP-2 release as the GPC aged.

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