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Experimental composite guidance conduits for peripheral nerve repair: An evaluation of ion release

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ABSTRACT

Poly (lactide-co-glycolide) (PLGA) — Pluronic F127 — glass composites have demonstrated excellent potential, from the perspective of controlled mechanical properties and cytocompatibility, for peripheral nerve regeneration. In addition to controlling the mechanical properties and cytotoxicity for such composite devices, the glass component may mediate specific responses upon implantation *via* degradation in the physiological environment and release of constituent elements. However, research focused on quantifying the release levels of such therapeutic ions from these experimental medical devices has been limited. To redress the balance, this paper explores the ion release profiles for Si^{4+}, Ca^{2+}, Na^{+}, Zn^{2+}, and Ce^{4+} from experimental composite nerve guidance conduits (CNGC) comprising PLGA *(at 12.5, and 20 wt.%)*, F127 *(at 0, 2.5 and 5 wt.%)* and various loadings of Si–Ca–Na–Zn–Ce glass *(at 20 and 40 wt.%)* for incubation periods of up to 28 days. The concentration of each ion, at various time points, was determined using Inductively Coupled Plasma–Atomic Emission Spectrometry (Perkin Elmer Optima 3000). It was observed that the Si^{4+}, Na^{+}, Ca^{2+}, Zn^{2+} release from CNGCs in this study ranged from 0.22 to 6.477 ppm, 2.307 to 3.277 ppm, 40 to 119 ppm, and 45 to 51 ppm, respectively. The Ce^{4+} concentrations were under the minimum detection limits for the ICP instrument utilized. The results indicate that the ion release levels may be appropriate to mediate therapeutic effects with respect to peripheral nerve regeneration. The data generated in this paper provides requisite evidence to optimize composition for pre-clinical evaluation of the experimental composite.

1. Introduction

In recent years, significant interest has been placed on the development of bioengineered artificial nerve guidance conduits (NGC) to bridge discontinuities in peripheral nerves which may arise from incidences such as trauma or acute compression. Design specifications for the ideal NGC require that it be: cytocompatible, highly permeable (yet prevents fibrous tissue infiltration and interactions between the myofibroblasts and axon growth), and sufficiently flexible to allow functionality, while possessing suitable degradation rates to provide guidance for regenerating axons [1,2]. The microenvironment within such devices must favor peripheral nerve regeneration; while simultaneously minimizing swelling and inflammatory response, and preferably offering regenerative cues to enhance repair. To date, 11 commercially available NGCs and nerve protectant wraps have been approved by the U.S. Food and Drug Administration (FDA), and a comprehensive review of materials and efficacy for each of these devices is available elsewhere [3]. Present state-of-the-art materials have, in general, enabled good results for patients. However, a device offering an appropriate balance of desirable properties has yet to be realized. As such, much research and development is underway to identify new approaches to realize an ideal NGC device.

Popular devices currently in clinical use for this indication, comprise natural and synthetic biomaterials: Devices fabricated from type I collagen *(e.g. NeuroGen™)* [4–7] are considered to provide biocompatible, bioresorbable *(though challenges of tailored degradation remain)*, non-toxic NGCs, which may provide comparable efficacy to the nerve autograft in discontinuities up to 20 mm [8]. However, such materials may also be associated with significant clinical limitations such as nerve compression, activation of host immune response, and, as necessary, the contiguous use of immunosuppressive drugs [3,9]. Synthetic devices derived from polyesters offer improved mechanical and chemical properties *(e.g. strength, porosity, degradation rate)*, alongside an established history of biocompatibility and safety in other areas of clinical use. Polyesters such as polyglycolic acid (PGA), poly (lactic acid) (PLA), poly-caprolactone (PCL) and their copolymers have been used widely in FDA-approved devices, including

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sutures and drug delivery systems. From the perspective of nerve repair, Poly (DL-lactide-caprolactone) [3,10], has been subjected to extensive examinations by Meek and den Dunnen [10–15], however, swelling and fragmentation *(due to incomplete degradation)* are considered to compromise performance [3,16–19]. As a potential alternative to the above materials, Poly (lactide-co-glycolide) (PLGA) copolymer has also been studied as a potential material for NGC devices: its ability to provide tailored properties (especially in respect of controlled degradation) is deemed a significant advantage in the context of nerve repair [20,21]. In addition, the poloxamer Pluronic F127 (F127) has been added to PLGA NGCs to enhance hydrophilicity and to augment mechanical performance, yielding a PLGA/F127 matrix which shows excellent potential for use in the repair of peripheral nerve discontinuities [22–24]. Nevertheless, the mechanical properties of PLGA-F127 devices require significant enhancement to balance the full suite of properties for an optimum NGC device.

Composite materials, comprising bioresorbable polymer matrices impregnated with bioactive glass fillers, have attracted attention in soft tissue engineering; such composites offer exceptional potential for tailored physical, biological and mechanical properties [25–27]. A primary advantage of the use of bioactive glass in such composites is their ability to trigger specific host responses by virtue of their degradation and contiguous release of therapeutic dissolution by-products [28]. Consequently, the investigation of tailored bioactive glass compositions designed to release specific degradation products to mediate specific host responses in nerve regeneration is a valuable approach to the clinical challenge of peripheral nerve repair, and may yield significant improvements in NGC device design [1,2,9]. The Silicate-Sodium–Calcium–Zinc–Cerium (CNG) glass system has been considered for this application; where such glasses comprise 50% mol. fraction SiO_2 to achieve a Q^2 structure for bonding with soft tissue with additions of NaO to facilitate dissolution [9]. CaO and ZnO are included in such compositions on the basis that integrating Ca^{2+} into the local environment of a regenerating nerve regulates turning (guidance) and extension of the growth cone [29,30]; while the inclusion of zinc (Zn^{2+}) in such biomaterials has been shown to increase antibacterial efficacy [31–34] and it is also an essential component for effective immune system and wound healing mechanisms [34]. In respect of cerium (Ce^{4+}) it has been shown that this oxide is capable of protecting nerve cells from oxidative stress [35] and as such may have neuroprotective capabilities. However of key importance in this regard is the release of Si^{4+}, Na^{+}, Ca^{2+}, Zn^{2+} Ce^{4+} at levels appropriate to mediate such responses that may be beneficial in terms of peripheral NGC clinical performance and, as yet, the levels of release of such elements from CNGC remains to be quantified. This study, a progression from the initial mechanical and biocompatibility experiments (which examined the effects of composition on, the time dependency of tensile strength and modulus, as well as the cytotoxic potential of the composites against L929 Mouse fibroblasts) is designed to evaluate the effect of simulated physiological environments on the dissolution by-products derived from 6 CNGC (identified as the most mechanically stable compositions in previous work) [1,2]. This work quantifies the levels of Si^{4+}, Na^{+}, Ca^{2+}, Zn^{2+} and Ce^{4+} ions released as a function of time and relates the results with the structure of the composite to identify the optimum composite for pre-clinical evaluation as a suitable peripheral NGC.

2. Material and methods

2.1. Glass synthesis and characterization

A glass with composition (mol. fraction) $0.5SiO_2$–$0.2CaO$–$0.13ZnO$–$0.14Na_2O$–$0.03CeO_2$ was synthesized by weighing out appropriate amounts of analytical grade reagents (Sigma Aldrich, Wicklow, Ireland). Pre-fired batches were then thoroughly mixed by shaking (30 min) in a plastic container, prior to firing (1 h, 1520 °C) in platinum crucibles and shock quenching the melts into water. The resulting frit was dried in an oven (24 h, 120 °C), ground and sieved to retrieve a glass powder with a maximum particle size of 45 μm. X-ray diffraction (XRD) was used to validate that the glass was completely amorphous. Diffraction patterns were collected using a Philips XPert MPD Pro 3040/60 X-ray Diffraction Unit (Philips, Netherlands). Disc samples (Φ32 mm × 3 mm) were prepared by pressing selected glass particles (<45 μm) into a backing of ethyl cellulose. Samples were then placed on spring-back stainless steel holders with a 10 mm mask and were analyzed using Cu Kα radiation. A Generator voltage of 40KV and a tube current of 35 mA were used. Diffractograms were collected in the range $10° < 2\theta < 70°$, at scan step size 0.033423° and a step time of 50.16 s.

2.2. PLGA/ F127 solution preparation and CNGC fabrication

PLGA with a lactic to glycolic acid mole ratio, 75:25 (Mw, 113 kDa; IV, 0.74 dL/g; Lot #: LP-443, Lakeshore Biomaterials, Birmingham, AL, USA) was dissolved overnight in tetraglycol T3396, (Sigma Aldrich, Wicklow, Ireland) at 60 °C at an appropriate weight percentage (refer to Table 1 for ranges investigated). F127 was used as a hydrophilic additive (P2443, Sigma Aldrich, Wicklow, Ireland) to the PLGA. The bioactive glass component synthesized in Section 2.1 was added to the solution slowly, using magnetic vibration to ensure homogenous distribution. Six CNGC compositions (Table 1) were prepared, based on a modified immersion precipitation technique described previously [1]. The wt.% of the F127 phase is relative-to the PLGA content and not the overall composite. The wt.% of the glass phase is relative-to the PLGA/F127 composite content. Following fabrication, the CNGCs were subsequently suspended in separate test tubes in the laminar flow hood to dry (48 h) for evaporation of any residual solvent under atmospheric pressure as per methodology outlined by Wen and Tresco [36]. The CNGCs were cut into segments (30 mm length × 1.5 mm inner diameter) using a surgical blade to avoid any compression of the thin walled membrane and stored under moisture free conditions in desiccators (at <10 °C) for subsequent testing. The designations for each composite are based on original designations from the full composite design space and are maintained in this work for ease of reference with other literature.

2.3. Preparation of CNGC extracts

The CNGCs segments (30 mm length × 1.5 mm inner diameter) prepared in Section 2.2 were immersed in 10 mL of tissue culture water (Sigma Aldrich, Ireland) for 1, 3, 7 and 28 day (n = 3) time periods. Each specimen was stored in polypropylene tubes maintained at 37 °C in a shaking waterbath (Stuart Sb40, Reagecon, Shannon, Ireland), agitated at 2 Hz (longitudinal movement) according to ISO10993 part 14 [37]. After each storage period, individual extracts for CNGC specimens were filtered using a sterile 0.2 μm filter (Sarstedt, Ireland). Following that, 3 ml of each filtrate was diluted to 30 ml extract with tissue culture water and stored at 4 °C for future *in vitro* evaluation. For enhanced clarity, the experimental CNGCs may

Table 1
Compositions of composite nerve guidance conduits (values expressed as wt.%).

NGC Designation	PLGA	Pluronic F127	Glass
CNGC-B	20	0	20
CNGC-M	12.5	2.5	20
CNGC-D	20	5	20
CNGC-K	12.5	0	40
CNGC-H	20	2.5	40
CNGC-L	12.5	5	40

be considered as two groups; those comprising 20 wt.% glasses and those comprising 40 wt.% glasses.

2.4. Ionic content analysis

The Si^{4+}, Na^{+}, Ca^{2+}, and Zn^{2+} ionic concentration of each CNGC extract was analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin Elmer Optima 3000, MA, USA). The absorption wavelengths used for the determination of Si^{4+}, Na^{+}, Ca^{2+} and Zn^{2+} are reported in Table 2. Before each cycle of measurement, calibration curves were obtained by preparing standard solutions containing Si^{4+}, Na^{+}, Ca^{2+} and Zn^{2+} (JVA Analytical Ltd, Ireland) at concentrations reported in Table 3. Standard sample concentrations were measured periodically to ensure the accuracy of the calibration curve. Triplicates of each extract (from each incubated CNGC) were measured for each element, with appropriate adjustments in outputs being deployed to balance dilutions of original extracts.

2.5. Statistical analysis

Results are expressed as mean ± standard error of the mean of triplicate determinations. Analysis of the results was carried out using the Student's *t*-test, with a significance level of $P<0.05$. The ion release levels of each element are divided into two groups' relative-to their bioactive glass (20 wt.% group (a) and 40 wt.% group (b)) content.

2.6. Ion release profile modeling

The ion release profiles from the CNGCs are described in terms of the ion release concentration (Y) over incubation time (X). Since the incubation time is not an input of the correlation function, the time dependent functions have been fitted to nonlinear regressive Polynomial, Gaussian, Sine waves and Exponential models using Prism 5.0 software (GraphPad software Inc.). The best fitting model for the four elements with respect to each CNGC is the one phase-decay exponential model:

$$Y = (Y_0 - \text{Plateau}) * \exp(-K * X) + \text{Plateau} \quad (1)$$

where:

'Y' and 'X' are the ion release concentrations in ppm and incubation time in days, respectively;
'Y_0' is the ion release concentration (ppm) at initial ion release; where Y value at $X_0 = 1$;
'Plateau' is the ion release concentration at an infinite time (ppm), where Y value at $X = 28$;
't_{au}' denotes the time necessary for ion release to reach 63% of the estimated 'Plateau' (ppm);
'K' is the rate constant, expressed in reciprocal of the 't_{au}' incubation time and unit is inverse days;
'$t^{1/2}$' denotes the half-life (time) to reach 50% of final 'Plateau' value, '$t^{1/2} = t_{au}$*LN (2)';
'R^2' is the sum of the squares of the distances of the points from the best-fit of the exponential nonlinear regression as determined by Prism 5.0 (GraphPad Inc.) software. The value of R^2 is a fraction between 0.0 and 1.0, with the best-fit line with a R^2 equal to 1.0.

Table 2
Emission lines used for the ICP measurements.

Element	Absorption wavelength	Lower limit	Upper limit	Background correction
Si	288.158	288.073	288.256	±0.026
Na	330.237	330.136	330.348	±0.030
Ca	396.847	396.679	397.039	±0.072
Zn	334.501	334.400	334.614	±0.031
Ce	418.660	418.482	418.864	±0.076

Table 3
Standard concentrations used for the ICP measurements.

Standard	Si^{4+} (mg/L)	Na^{+} (mg/L)	Ca^{2+} (mg/L)	Zn^{2+} (mg/L)
1	2	1	0.5	1
2	4	2	1	2
3	6	3	2	3
4	19	4	3	4
5	15	5	4	5

3. Dissolution results

3.1. Si^{4+} release

The Si^{4+} release levels for each composition of CNGC are illustrated in Fig. 1. Si^{4+} release levels (after incubation at 1, 3, 7 and 28 days) for CNGC comprising 20 wt.% glass (CNGC-B; CNGC-M and CNGC-D) are shown in Fig. 1(a), while Si^{4+} release levels associated with CNGC comprising 40 wt.% glass (CNGC-K; CNGC-H and CNHC-L) are shown in Fig. 1(b). With respect to 20 wt.% glass CNGCs, it was observed that CNGC-B demonstrated Si^{4+} release levels ranging from 1 ppm (±0.028 ppm) at 1 day to 5.11 ppm (±0.07 ppm) at 28 days; the highest mean release of Si^{4+} of the three CNGCs in this series (between the full range of incubation periods examined). CNGC-D exhibited Si^{4+} release levels ranging from 0.76 ppm (±0.04 ppm) at 1 day to 2.92 ppm (±0.137 ppm) at 28 days; while CNGC-M demonstrated Si^{4+} release levels ranging from 1.20 ppm (±0.449 ppm) at 1 day to 2.44 ppm (±0.142 ppm) at 28 days; the lowest mean release of Si^{4+} of the 3 CNGCs tested in this series

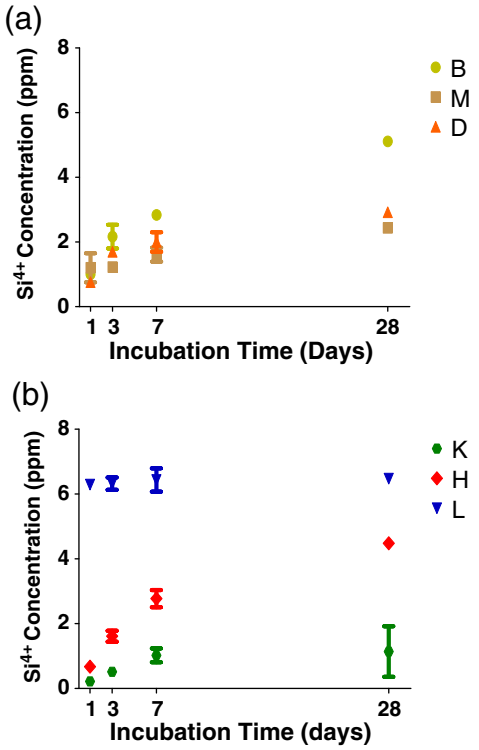

Fig. 1. The Si^{4+} ion release levels of 6 CNGCs with time dependency (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods.

(between 3 and 28 day incubation periods only). In general, CNGC of the series comprising 20 wt.% glass were observed to have significant increases in Si^{4+} release between each incubation period, with the exception of CNGC-M (no significant differences were observed between 1 and 3 days, and 3 and 7 days incubation periods).

With respect to 40 wt.% glass CNGCs, it was observed that CNGC-L demonstrated Si^{4+} release levels ranging from 6.30 ppm (±0.161 ppm) at 1 day to 6.48 ppm (±0.047 ppm) at 28 days; the highest mean release of Si^{4+} of the 3 CNGCs in this series (over the full range of incubation periods studied; reaching a peak after 24 h). CNGC-H exhibited Si^{4+} release levels ranging from 0.67 ppm (±0.055 ppm) at 1 day to 4.48 ppm (±0.096 ppm) at 28 days; while CNGC-K demonstrated Si^{4+} release levels ranging from 0.22 ppm (±0.065 ppm) at 1 day to 1.14 ppm (±0.779 ppm) at 28 days; the lowest mean release of Si^{4+} of the 3 CNGCs tested in this series (over the full range of incubation periods examined). Indeed, with respect to incubation time, the Si^{4+} release levels of CNGC-K significantly increased from 1 day to 3 days, and 3 days to 7 days before finally plateauing. In general, significant increases in Si^{4+} release were observed for CNGC-H between each incubation period; while no significant increase was observed for CNGC-L between all incubation periods examined.

The Student's *t*-test was used to examine the levels of Si^{4+} release for 20 wt.% and 40 wt.% glass containing CNGCs at equivalent incubation periods (represented in Fig. 2(a) and (b), respectively). For 20 wt.% glass CNGCs, it was observed that CNGC-B generally releases significantly more Si^{4+} than either CNGC-M or CNGC-D for each incubation period (the exceptions being: (i) CNGC-B and CNGC-M releasing similar levels of Si^{4+} after 1 days incubation and (ii) CNGC-B and CNGC-D releasing similar levels of Si^{4+} after 3 days incubation). In addition, CNGC-D was observed to release significantly higher amounts of Si^{4+} than CNGC-M at 3 and 28 days incubation.

For 40 wt.% glass CNGCs, it is evident that CNGC-L releases significantly more Si^{4+} than either CNGC-H or CNGC-K for all incubation periods. However, it is also noted that the Si^{4+} release from CNGC-H is significantly higher than the Si^{4+} release from CNGC-K at equivalent incubation periods.

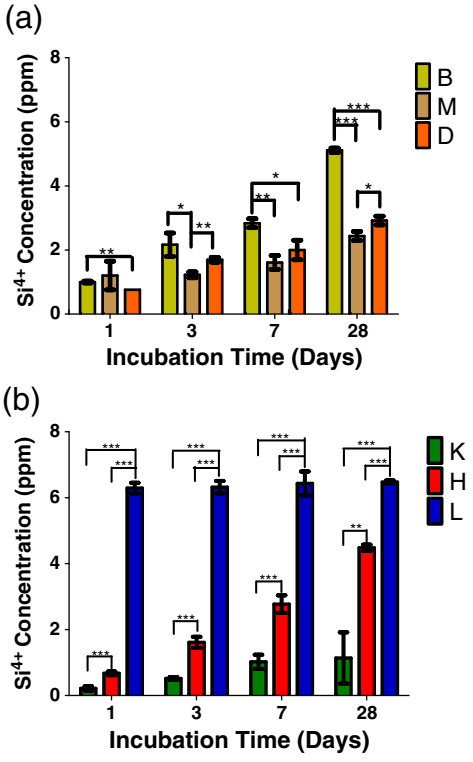

Fig. 2. Student's *t*-test results for the Si^{4+} ion release of the 6 CNGCs (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods (* P<0.05, ** P<0.005, ***p<0.0005).

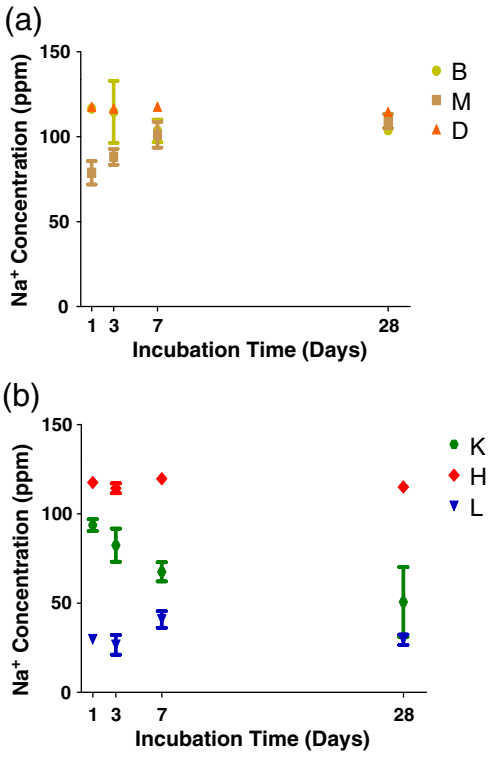

Fig. 3. The Na^+ ion release levels of 6 CNGCs with time dependency (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7, and 28 days incubation periods.

3.2. Na^+ release

The Na^+ release levels for each composition of CNGC are illustrated in Fig. 3. Na^+ release levels (after incubation at 1, 3, 7 and 28 days) for CNGC comprising 20 wt.% glass (CNGC-B; CNGC-M and CNGC-D) are shown in Fig. 3(a); while Na^+ release levels associated with CNGC comprising 40 wt.% glass (CNGC-K; CNGC-H and CNHC-L) are shown in Fig. 3(b). With respect to 20 wt.% glass CNGCs, it was observed that CNGC-D demonstrated Na^+ release levels ranging from 117.6 ppm (±0.854 ppm) at 1 day to 114.6 ppm (±0.945 ppm) at 28 days; the highest mean release of Na^+ of the 3 CNGCs in this series. CNGC-B exhibited Na^+ release levels ranging from 116.53 ppm (±2.248 ppm) at 1 day to 103.77 ppm (±0.907 ppm) at 28 days; while CNGC-M demonstrated Na^+ release levels ranging from 78.76 ppm (±6.9 ppm) at 1 day to 109.2 ppm (±4.157 ppm) at 28 days; the lowest mean release of Na^+ of the 3 CNGCs tested in this series (at 1, 3 and 7 days incubation periods only). The only significant increase in the Na^+ release for CNGCs comprising 20 wt.% glass was observed to occur for CNGC-M between 1 day to 7 days, and 3 days to 28 days.

With respect to 40 wt.% glass CNGCs, it was observed that CNGC-H demonstrated Na^+ release levels ranging from 117.57 ppm (±0.208 ppm) at 1 day to 115.03 ppm (±0.351 ppm) at 28 days; the highest mean release of Na^+ of the 3 CNGCs in this series (over the full range incubation periods studied; reaching a peak after a 24 h incubation period). CNGC-K exhibited Na^+ release levels ranging from 93.6 ppm (±3.33 ppm) at 1 day to 50.67 ppm (±19.54 ppm) at 28 days; while CNGC-L demonstrated Na^+ release levels ranging from 29.76 ppm (±1.316 ppm) at 1 day to (29.46 ppm±2.93 ppm) at 28 days; the lowest mean release of Na^+ of the 3 CNGCs tested in

this series (over the full range of incubation periods examined). However, with respect to incubation time, the Na^+ release levels of CNGC-L significantly decreases from 1 day to 3 days, with a subsequent increase from 3 days to 7 days, and no significant difference thereafter. Significant decreases in Na^+ release were observed for CNGC-K from 1 day to 7 days, and from 1 day to 28 days.

The Student's *t*-test was used to examine the levels of Na^+ release for 20 wt.% and 40 wt.% glass containing CNGCs at equivalent incubation periods (represented in Fig. 4(a) and (b), respectively). For 20 wt.% glass CNGCs, it was generally observed that Na^+ release from CNGC-D was significantly higher than that released from CNGC-M for each incubation period (the exception being after 28 days; for which similar levels of Na^+ were released by both compositions). Additionally, the mean Na^+ release from CNGC-D is significantly higher than release levels observed for CNGC-B after 7 days incubation; with a subsequently more pronounced increase in significant difference occurring after a 28 day incubation period. The only significant difference in Na^+ release between CNGC-B and CNGC-M was evident after 1 day incubation; with no subsequent significant difference observed thereafter.

For 40 wt.% glass CNGCs, it was observed that CNGC-H releases significantly higher levels of Na^+ than either CNGC-K or CNGC-L for each incubation period. It is also noted that the Na^+ release from CNGC-K is significantly higher than that released from CNGC-L after 1, 3 and 7 days incubation; with no subsequent significant difference observed thereafter.

3.3. Ca^{2+} release

The Ca^{2+} release levels for each composition of CNGC are illustrated in Fig. 5. Ca^{2+} release levels (after incubation at 1, 3, 7 and 28 days) for CNGC comprising 20 wt.% glass (CNGC-B; CNGC-M and CNGC-D) are shown in Fig. 5(a); while Ca^{2+} release levels associated with CNGC comprising 40 wt.% glass (CNGC-K; CNGC-H and CNHC-L) are shown in Fig. 5(b). With respect to 20 wt.% glass CNGCs, it was observed that CNGC-B demonstrated Ca^{2+} release levels ranging from 3.12 ppm (±0.046 ppm) at 1 day to 3.28 ppm (±0.023 ppm) at 28 days; the highest mean release of Ca^{2+} of the three CNGCs in this series (over the full range of incubation periods examined). CNGC-D exhibited Ca^{2+} release levels ranging from 2.86 ppm (±0.025 ppm) at 1 day to 3.15 ppm (±0.035 ppm) at 28 days; while CNGC-M demonstrated Ca^{2+} release levels ranging from 1.59 ppm (±0.038 ppm) at 1 day to 2.31 ppm (±0.059 ppm) at 28 days; the lowest mean release of Ca^{2+} of the 3 CNGCs tested in this series (over the full range of incubation periods examined). In general, CNGC-M and CNGC-D were observed to have significant increases in Ca^{2+} release levels between each incubation period, with CNGC-D demonstrating no significant difference between 3 and 7 day incubation periods.

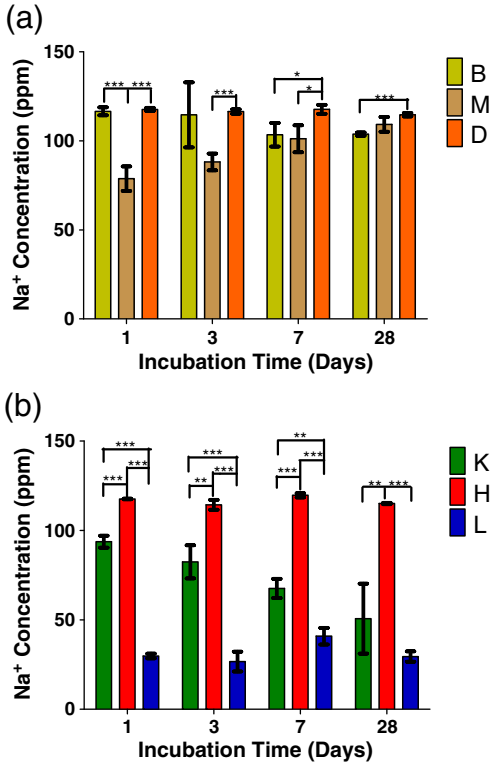

Fig. 4. Student's *t*-test results for the Na^+ ion release of 6 CNGCs (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7, and 28 days incubation periods (* P<0.05, ** P<0.005, ***p<0.0005).

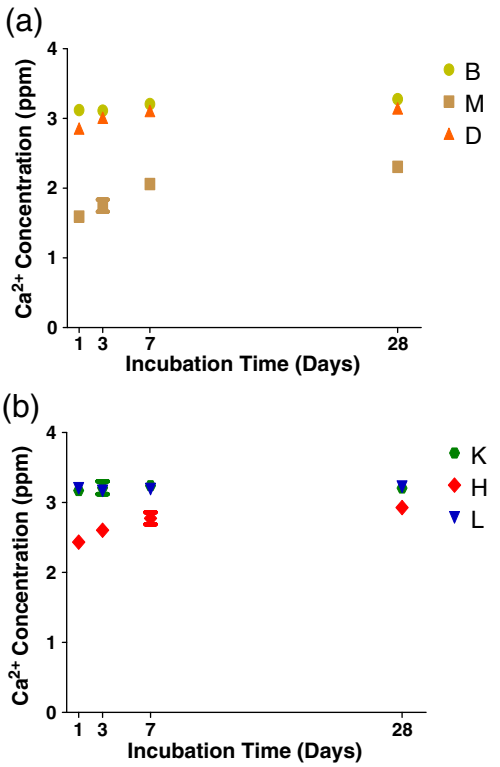

Fig. 5. The Ca^{2+} ion release levels of 6 CNGCs with time dependency (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7, 28 days incubation periods.

With respect to 40 wt.% glass CNGC, it was observed that CNGC-K demonstrated Ca^{2+} release levels ranging from 3.17 ppm (±0.056 ppm) at 1 day to 3.21 ppm (±0.055 ppm) at 28 days; the highest mean release of Ca^{2+} of the 3 CNGCs in this series (reaching a peak after an initial incubation period of 1 day). CNGC-H exhibited Ca^{2+} release levels ranging from 2.43 ppm (±0.072 ppm) at 1 day to 2.93 ppm (±0.023 ppm) at 28 days; while CNGC-L demonstrated Na^+ release levels ranging from 3.20 ppm (±0.046 ppm) at 1 day to 2.23 ppm (±0.029 ppm) at 28 days; the lowest mean release of Ca^{2+} of the 3 CNGCs tested in this series (over the full range of incubation periods examined). However, with respect to incubation time, the Ca^{2+} release levels of CNGC-H significantly increased over each subsequent incubation period, while the Ca^{2+} release levels of CNGC-K and CNGC-L demonstrated no significant difference over each of the incubation periods.

The Student's *t*-test was used to examine the levels of Ca^{2+} release for 20 wt.% and 40 wt.% glass containing CNGCs at equivalent incubation periods (represented in Fig. 6(a) and (b), respectively). For

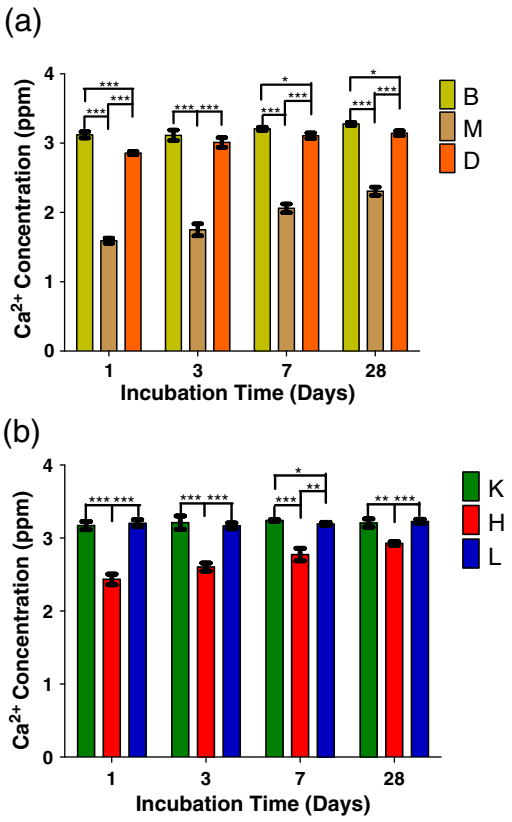

Fig. 6. Student's *t*-test results for the Ca^{2+} ion release of 6 CNGCs (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods (* $P<0.05$, ** $P<0.005$, ***$p<0.0005$).

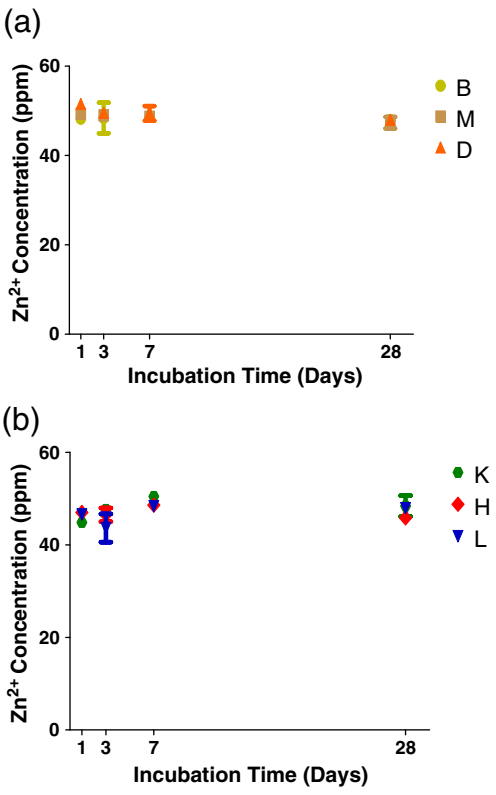

Fig. 7. The Zn^{2+} ion release levels of 6 CNGCs with time dependency (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods.

20 wt.% glass CNGCs: (i) CNGC-D exhibited significantly higher levels of Ca^{2+} release compared to CNGC-B for each incubation period; (ii) CNGC-B demonstrated significantly higher levels of Ca^{2+} release compared to CNGC-M for each incubation period and (iii) CNGC-B demonstrated significantly higher levels of Ca^{2+} release compared to CNGC-D for each incubation period, with the exception of 3 days incubation (where no significant differences were observed between either composition).

For 40 wt.% glass CNGCs: (i) CNGC-K demonstrated significantly higher levels of Ca^{2+} release compared to CNGC-H for each incubation period and (ii) CNGC-L also demonstrated significantly higher levels of Ca^{2+} release compared to CNGC-H for each incubation period and (iii) CNGC-K demonstrated significantly higher levels of Ca^{2+} release compared to CNGC-L after a 7 day incubation period, whereby no significant difference was apparent after a longer incubation period of 28 days.

3.4. Zn^{2+} release

The Zn^{2+} release levels for each composition of CNGC are illustrated in Fig. 7. Zn^{2+} release levels (after incubation at 1, 3, 7 and 28 days) for CNGC of the series comprising 20 wt.% glass (CNGC-B; CNGC-M and CNGC-D) are shown in Fig. 7(a); while Zn^{2+} release levels associated with CNGC comprising 40 wt.% glass (CNGC-K; CNGC-H and CNHC-L) are shown in Fig. 7(b). With respect to 20 wt.% glass CNGCs, it was observed that CNGC-D demonstrated Zn^{2+} release levels ranging from 51.54 ppm (±0.584 ppm) at 1 day to 47.77 ppm (±0.645 ppm) at 28 days; the highest mean release of Zn^{2+} of the 3 CNGCs in this series (after an initial incubation period of 1 day). CNGC-B exhibited Zn^{2+} release levels ranging from 48.21 ppm (±0.67 ppm) at 1 day to 47.94 ppm (±0.29 ppm) at 28 days; while CNGC-M demonstrated Zn^{2+} release levels ranging

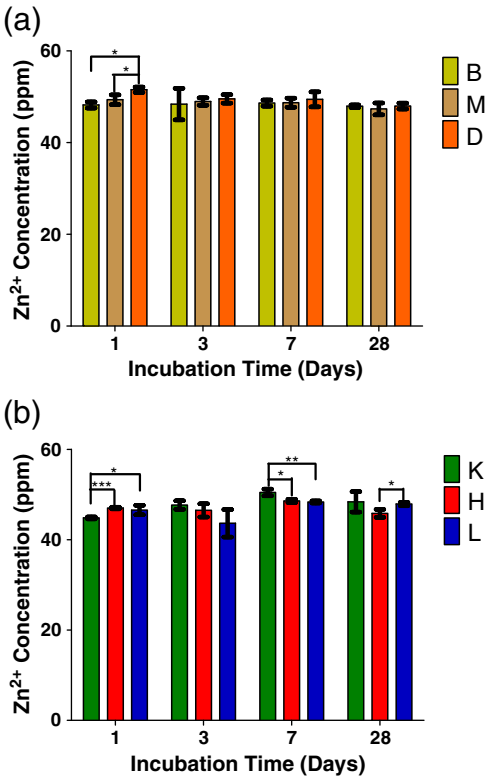

Fig. 8. Student's *t*-test results for the Zn^{2+} ion release of 6 CNGCs (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods (* $P<0.05$, ** $P<0.005$, ***$p<0.0005$).

from 49.35 ppm (±0.1.07 ppm) at 1 day to 47.34 ppm (±1.281 ppm) at 28 days; the lowest mean release of Zn^{2+} of the 3 CNGCs tested in this series (at the first incubation period at 1 day). In general, 20 wt.% glass CNGCs were observed to have no significant difference in Zn^{2+} release at each incubation period, with the exception of CNGC-D (decreasing significantly from 1 day to 3 days and 1 day to 28 days incubation periods).

With 40 wt.% glass CNGCs, it was observed that CNGC-K demonstrated Zn^{2+} release levels ranging from 44.82 ppm (±0.233 ppm) at 1 day to 48.37 ppm (±2.263 ppm) at 28 days; the highest mean release of Zn^{2+} of the three CNGCs in this series (after a final incubation period of 28 days). CNGC-L exhibited Zn^{2+} release levels ranging from 46.57 ppm (±1.043 ppm) at 1 day to 47.89 ppm (±0.369 ppm) at 28 days; while CNGC-H demonstrated Zn^{2+} release levels ranging from 47 ppm (±0.177 ppm) at 1 day to 45.83 ppm (±0.868 ppm) at 28 days; the lowest mean release of Zn^{2+} of the three CNGCs in this series (after a final incubation period of 28 days). Indeed, with respect to incubation time, the Zn^{2+} release levels of CNGC-K significantly increase over each incubation periods before plateauing after 7 days incubation. In general, 40 wt.% glass CNGCs were observed to have significant increases in Zn^{2+} release from 1 day to 7 days incubation periods, and then subsequently plateau.

The Student's *t*-test was used to examine the levels of Zn^{2+} release for 20 wt.% and 40 wt.% glass containing CNGCs at equivalent incubation periods (represented in Fig. 8(a) and (b), respectively). For 20 wt.% glass CNGCs, it was generally observed that there was no significant difference in the level of Zn^{2+} release from each CNGC over all the incubation periods, (the exception of CNGC-D being significantly different (higher) to both CNGC-B and CNGC-M after 1 day incubation).

For 40 wt.% glass CNGCs, it was observed that CNGC-K released significantly less Zn^{2+} than either CNGC-H or CNGC-L after 1 day incubation. However, after 7 days incubation, CNGC-K subsequently released significantly higher levels of Zn^{2+} than both CNGC-H and CNGC-L. In addition, it was also observed that the Zn^{2+} release from CNGC-L is significantly higher than CNGC-H after a 28 days incubation period.

3.5. Si^{4+} and Ca^{2+} ion release profile modeling

The release profiles for each element were modelled using nonlinear regression curve fitting methodologies; for which Na^{+} and Zn^{2+} did not converge to form the desired nonlinear exponential function. The associated half-life to acquire the highest Si^{4+} release levels was identified for each CNGC using Prism 5.0 software (GraphPad Inc.) as presented in Table 4. With respect to CNGC of the series comprising 20 wt.% glass it was observed that the associated half-life for each CNGC follows the order of CNGC-B > CNGC-D > CNGC-M (Table 4(a)), such that CNGC-B demonstrated the longest half-time of 4.821 days; CNGC-D exhibited a half-time of 2.689 days; while CNGC-M demonstrated the lowest half-time of 2.02 days. In addition, the R^2 values obtained were 0.9745, 0.9273, and 0.8352 for CNGC-B, CNGC-D, and CNGC-M, respectively. With respect to CNGC of the series comprising 40 wt.% glass it was observed that the associated half-life of each CNGC follows the order of CNGC-H > CNGC-K > CNGC-L (Table 4(b)), such that CNGC-H demonstrated the longest half-time of 4.961 days; CNGC-B exhibited a half-time of 3.021 days; while CNGC-M demonstrated the lowest half-time of 0.1731 days. In addition, the R^2 values obtained were 0.9906, 0.5514, and 0.1456 for CNGC-H, CNGC-K, and CNGC-L, respectively.

Table 4
The best fit parameters for non linear one phase association model formed from Si^{4+} release over 4 time points (1, 3, 7, and 28 days).

Conduits	$t^{1/2}$ (days)	t_{au} (days)	y_{max} (ppm)	R^2
(a). Nonlinear fit of Si^{4+} release from CNGCs content 20% glass				
CNGC-B	4.821	6.956	5.117	0.9745
CNGC-M	2.02	2.915	2.168	0.8352
CNGC-D	2.689	3.879	2.785	0.9273
(b). Nonlinear fit of Si^{4+} release from CNGCs content 40% glass				
CNGC-K	3.021	4.359	1.173	0.5514
CNGC-H	4.961	7.158	4.556	0.9906
CNGC-L	0.1731	0.2497	6.41	0.1456

Table 5
The best fit parameters for non linear one phase association model formed from Ca^{2+} release over 4 time points (1, 3, 7, and 28 days).

Conduits	$t^{1/2}$ (days)	t_{au} (days)	y_{max} (ppm)	R^2
(a). Nonlinear fit of Ca^{2+} release from CNGCs content 20% glass				
CNGC-B	0.1874	0.2703	3.199	0.7062
CNGC-M	0.5076	0.7323	2.066	0.9599
CNGC-D	0.2683	0.3871	3.089	0.9058
(b). Nonlinear fit of Ca^{2+} release from CNGCs content 40% glass				
CNGC-K	0.1655	0.2388	3.219	0.1547
CNGC-H	0.3332	0.4807	2.772	0.9267
CNGC-L	NA	NA	NA	NA

The associated half-life to acquire the highest Ca^{2+} release levels was identified for each CNGC using Prism 5.0 software (GraphPad Inc.) as presented in Table 5. With respect to CNGC of the series comprising 20 wt.% glass it was observed that the associated half-life of Ca^{2+} release for such CNGC follows the order of CNGC-M > CNGC-D > CNGC-B; the inverse order that observed for Si^{4+} release in the same group (Table 5(a)). CNGC-M demonstrated the longest half-time of 0.5076 days; CNGC-B exhibited a half time of 0.2683 days; while CNGC-D demonstrated the lowest half-time of 0.1874 days. In addition, the R^2 values obtained were 0.9599, 0.9058, and 0.7062 for CNGC-M, CNGC-D, and CNGC-B respectively. With respect to CNGC of the series comprising 40 wt.% glass it was observed that the associated half-life of CNGC-H and CNGC-K are 0.3332 and 0.1655 respectively (Table 5(b)). The Ca^{2+} release of CNGC-L however did not converge to form a nonlinear exponential function. In addition, the R^2 values obtained were 0.9906, 0.5514, and 0.1456 for CNGC-H, CNGC-K, and CNGC-L, respectively.

4. Discussion

A requisite component for the biological evaluation of medical devices is to quantify degradation by-products under simulated physiological conditions. The importance of such analysis increases significantly when the degradation by-products may establish certain interactions with the local tissue to support effective regeneration. Consequently, and in the context of the devices examined in this paper, the aim of this work was to evaluate the Si^{4+}, Na^{+}, Ca^{2+}, Zn^{2+}, and Ce^{4+} release levels (and profiles where possible) for a group of experimental composites, which have already demonstrated potential as NGCs.

Table 6 summarizes the maximum mean ion release concentration for 20 wt.% and 40 wt.% glass containing CNGCs over all the incubation periods examined. In general it is observed that maximum ion release levels for detectable elements are in the order *(increasing)* of $Si^{4+} < Ca^{2+} < Zn^{2+} < Na^{+}$.

The Si^{4+} release data was suitable for investigation *(given the increasing levels of release over the time period examined)* using a one-phase exponential model. It was observed that CNGC-B had the longest half-life followed by CNGC-D; then CNGC-M in the 20 wt.% glass group (Table 4(a)). With respect to the 40 wt.% glass group it is noted from the R^2 data that the validity of the model becomes compromised; Si^{4+} degradation from CNGC-H had a half-life of 3.021 days ($R^2 = 0.99$, Table 4(b)), however the same model yields R^2 values of 0.55 and 0.14 for CNGC-K and CNGC-L respectively; a

Table 6
The maximum mean Si^{4+}, Na^{+}, Ca^{2+}, and Zn^{2+} release concentration expressed as Mean ± SD, and group by the glass content of (20 wt.% and 40 wt.%).

Element[a]	CNGCs (20 wt.% glass)			CNGCs (40 wt.% glass)		
	Maximum mean ± SD (ppm)			Maximum mean ± SD (ppm)		
	CNGC-B	CNGC-M	CNGC-D	CNGC-K	CNGC-H	CNGC-L
Na^{+}	116.53 ± 2.248	109.2 ± 4.157	117.67 ± 2.511	93.66 ± 3.325	119.63 ± 1.159	40.84 ± 4.633
Zn^{2+}	48.63 ± 0.673	49.35 ± 1.070	51.54 ± 0.584	50.45 ± 0.743	48s.57 ± 0.868	48.35 ± 0.236
Ca^{2+}	3.28 ± 0.023	2.31 ± 0.059	3.15 ± 0.035	3.24 ± 0.010	2.93 ± 0.023	3.23 ± 0.029
Si^{4+}	5.11 ± 0.070	2.44 ± 0.142	2.92 ± 0.137	1.14 ± 0.779	4.48 ± 0.096	6.477 ± 0.047

[a] Order of element ion release arranged from highest to lowest.

feature attributable to the enhanced dissolution rates associated with each composite (Figs. 1 and 9; Table 4(b)). SiO_2 is incorporated into the compositions to act as the glass network former, and its potential therapeutic value in peripheral nerve regeneration warrants investigation. The Si^{4+} release levels observed for the composites varied between 0.22 ppm and 6.477 ppm; whereby at such levels (from a systemic perspective) Si^{4+} release is unlikely to have adverse effects, since Si^{4+} is frequently absorbed from the diet as orthosilicic acid at levels beyond this threshold. More interestingly the therapeutic efficacy of Si^{4+} release from the conduits is worthy of additional investigation in respect of soft tissue regeneration. Reffitt et al. [38] have shown that collagen Type I synthesis may be increased by orthosilicic acid concentrations between 0.288 and 0.567 ppm (10 and 20 μM), while Hench [28] recently demonstrated that Si^{4+} release may upregulate gene expression for enhanced tissue repair.

Where controlled release of constituent glass components under physiological condition is preferred, additions of Na_2O are beneficial. Na_2O acts as a flux during synthesis and adopts a modifying role in the glass network [39] concurrent to facilitating dissolution [39–41]. In respect of Na^{+} release, it was observed that peak release is generally achieved within an initial 24 h period, with the exception of CNGC-M. Consequently modeling a release profile is not possible (the Na^{+} release at the 4 time points does not supply appropriate information to form nonlinear exponential regression model). An interesting observation in the Na^{+} data is that significant decreases in Na^{+} release were observed for CNGC-K from 1 day to 7 days, and from 1 day to 28 days. While one may intuitively be drawn to conclude interactions between degradation by-products of the polymer and the glass are a possible mechanism for the observed reduction, it is necessary to point out that such trends are not observed for CNGC-H and CNGC-L (which releases a relatively higher and lower concentration of Na^{+} respectively, when compared with CNGC-K (see Figs. 3 and 4). Based on the current data, this observation is difficult to explain. It has been previously reported that Na_2O content in silicate glasses has a positive effect on cell viability by facilitating enhanced specific surface area [9]. The effect of Na^{+} release from glass–ceramics has been published by Li et al. [42]; showing that increased roughness resulting in degradation may be correlated with improved interfacial bonding and cell adhesion. Na^{+} release from CNGCs in this study ranged from 40 to 119 ppm; comparative to human plasma with Na^{+} levels of approximately 3200 ppm (142 mM) [43]. It can be anticipated from a systemic standpoint that the Na^{+} release levels observed are unlikely to lead to any systemic complications, though localized interactions with living tissue warrant investigation given the critical role of Na^{+} in respect of nerve function [44,45].

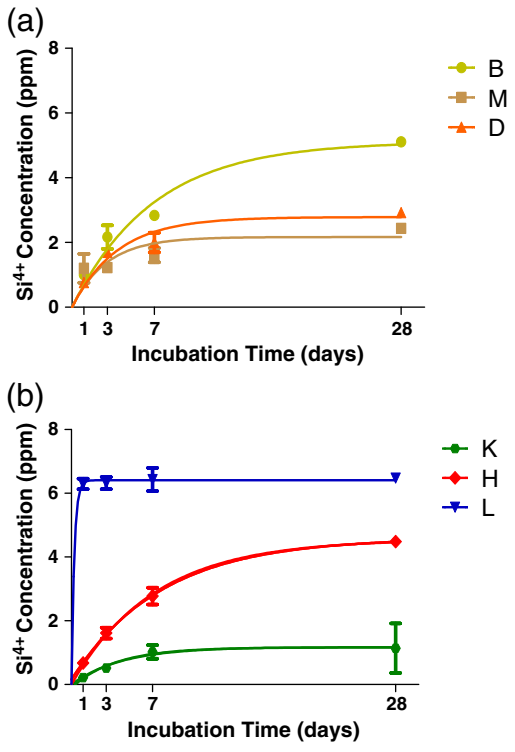

Fig. 9. The Si^{4+} ion release profiles of 6 CNGCs with time dependency (a) CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods.

The Ca^{2+} release rate was also investigated through the one-phase exponential model (Fig. 10 and Table 5). CNGC-M had the longest half-life (about a half day), followed by CNGC-D, then CNGC-B (for the 20 wt.% glass group). An interesting observation in respect of the Ca^{2+} release profiles arise when compared against previous literature [9]; specifically the half-life of Ca^{2+} release for the glass (in solution at pH 7.4) is 2.07 days versus a maximum half-life of 0.5 days when the same glass is used as a filler in the composites devices. It is reasonable to conclude that such behavior is due to a combination of features; acidic degradation by-products from the polymer (which include lactic acid) may act locally to facilitate an acid–base reaction with the glass (comparable to those reactions in dental restorative materials). Such reactions are associated with rapid Ca^{2+} release (on the order of minutes) for glass polyalkenoate cements [46]. In addition, given that the glass is homogenously distributed in the polymer structure the effective surface area is maximized for reaction. In synergy, these factors are likely to contribute to the reduced half-lives of ion release observed, not only for Ca^{2+} but also for the other elements investigated. The maximum Ca^{2+} release is in the range 2.31 ppm to 3.28 ppm (Fig. 5 and Table 6) for the CNGCs examined. Ca^{2+} regulates motility of the axonal growth cone and the guidance of growth cone extension [47–49]. It has been reported that there are grades affecting Ca^{2+} ion concentration on the growth cone behavior, with maximal activity occurring at an optimal level within a permissive range, whereby many calcium-dependent processes also display a similar dependence on optimal Ca^{2+} [50]. For example, intracellular Ca^{2+} levels may regulate outgrowth by affecting the stability of the peripheral cytoskeleton; such that it has previously been claimed that the extension of growth cones appear to be greatest at an optimal Ca^{2+} level of about 0.008 ppm [51]. In addition,

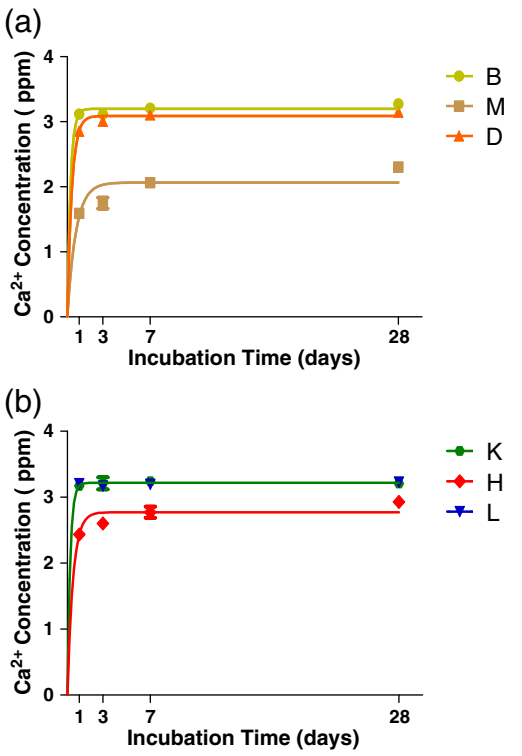

Fig. 10. The Ca^{2+} ion release profiles of 6 CNGCs with time dependency (a)CNGCs with 20 wt.% bioglass, (b) CNGCs with 40 wt.% bioglass over 1, 3, 7 and 28 days incubation periods.

pre-synaptic action potentials produce approximately 0.4008 ppm (10 μM) free Ca^{2+}at the calyx of held synapse to give a fast speed of Ca^{2+} signaling [52]. Moreover, the mean resting Ca^{2+} of olfactory ensheathing cell cultured alone or with neurons is 1.202 ppm in an external calcium solution for a rat [53]. Tucker et al. [54] have studied the spatial Ca^{2+} distribution in hair cells at a concentration of 3.407 ppm, which could promote extrusion turtle hair cells in the micro-domain of synaptic release sites. The appropriate Ca^{2+} concentration for the above application therefore ranges between 0.008 ppm to 3.407 ppm. Hence, it can be perceived that Ca^{2+} levels detected within these upper and lower ranges will have similar beneficial effects. The Ca^{2+} release concentrations for CNGC reported herein range from 2.3 ppm to 3.32 ppm in tissue culture water over all the incubation periods examined (Figs. 5 and 6). Hence, Ca^{2+} release concentrations from these CNGCs appear to be in a range, which may offer beneficial efficacy in respect of peripheral nerve regeneration.

The Zn^{2+} release was observed to occur quickly, with levels peaking within an initial 24 h period (consequently, the Zn^{2+} release levels reported over the 4 incubation periods could not give sufficient information to form a nonlinear model). Correlating the Zn^{2+} release profiles of the composites versus the glass particulates in isolated form (from previous literature [9,55]; it is noted that the half-life for Zn^{2+} from the glass is 1.4 days at pH = 7.4. However, as one would anticipate; Fig. 7 and Table 6 indicate the half-life for Zn^{2+} release profiles from the CNGCs is less than 1 day; a feature, as previously mentioned, may be associated with to localized acid attack of the glass from early degradation by-products of the polymer. Zinc is present in all body tissues and fluids. The concentration of Zn^{2+} in grey matter is about 9.75–13 ppm (0.15–0.2 mM), and 28 ppm in spinal cord. Moreover, human Zn^{2+} blood plasma levels have been shown to be approximately 6.4 ppm [56]. Zn^{2+} levels previously reported to have clinically beneficial effects on nerve growth were reported in the range of 0.065 ppm to 3.27 ppm in toad olfactory epithelia, with higher levels appearing to inhibit neural firing rates; indicating the important nature of quantification and control of degradation by-product release profiles and levels [56]. Similarly, research has also demonstrated that Zn^{2+} may be required for neuroprotection following sub-lethal ischemia in both *in vivo* and *in vitro* models [57]. More recently research has suggested that about 19.5 ppm (300 μM) concentration of Zn^{2+} is released during neurotransmission, and the optimal Zn^{2+} release for muscle cells is about 40–50 μM (2.615–3.27 ppm) [9]. In addition, the antibacterial effects of Zn^{2+} have been evaluated by broth dilution methods [58], in which bacterial growth was inhibited in the most concentrated Zn^{2+} oxide (0.719 ppm and 1.046 ppm) suspension. The Zn^{2+} concentration between 0.065 ppm and 19.5 ppm in neuronal cell may involve some aspects of peripheral nerve regeneration (PNR) function. However, Zn^{2+} release from the CNGC exceeded the reported optimal ranges, which may produce undesirable cytotoxic effects [59,60]. On the other hand, limited literature exists in respect of such ion release local to regenerating peripheral nerves and requires further examination. In respect of Ce^{4+}, its concentration in the tissue culture water was under the minimum detection limits of the ICP standard solutions and instrument utilized for all the CNGCs; a feature attributable to its low concentration and structural role in the glass [61].

The present investigation is not orientated around examining the effects of composition on property directly; rather its focus is on evaluating levels of dissolution by-products in order to identify the optimum device compositions. With respect to ion release data from the CNGCs prepared in this it is also important to note that variations in the internal composite structure, such as inhomogeneities in glass dispersion and variable porosity (decreases in the number and size of macrovoids with increased PLGA content) due to processing may also impact ion release levels. F127, is considered to impart a significant effect on the degradation rates of such composite devices, since its inclusion in similar compositions (without glass additions) for PNR has been reported to control properties pertaining to hydrophilicity, permeability, porosity and mechanical strength [1,2,23].

This paper explores the levels and profiles (where possible) of ionic degradation by-products from novel composites designed for PNR. However, it is critically important to clarify that the peripheral nervous system (PNS) is very sensitive to ionic imbalances and such imbalances may lead to dysfunction (e.g. the functional disturbances of voltage gated calcium channels). On the other hand, Ca^{2+} is directly involved in secretion of neurotransmitters and hormones, and plays an essential role in the development of motor co-ordination and sensory processing [62]. In particular, Ca^{2+} influx has a significant effect on the Na^{+} voltage gate, thus may affect the voltage potential on the membrane, and hence the ionic concentration in the neuron is very sensitive to pharmacological augmentation of an endogenous voltage and Ca^{2+} regulator. PNR is a complex phenomenon involving interactions between neurons and Schwann cells (SC), where SC proliferation and myelination processes have been critically linked to appropriate levels of Zn^{2+} ions [31,63]. There may be greater benefit of zinc supplementation for infections in implantation sites specific to nerve (motor and sensory) recovery. This paper has shown that it is possible to synthesize novel NGCs based on unique composites that are designed to release potentially therapeutic elements (and/or regenerative cues) local to the implant site. Such an approach is a fresh departure from contemporary materials approaches to a significant clinical problem. Further studies with specific cell lines (*e.g.* Schwann cells), and polymeric degradation profiling will comprise the next steps of analysis prior to considering pre-clinical evaluations (*e.g.* intracutaneous reactivity) of these novel devices in PNR indications.

5. Limitations of the study

As with any scientific study, intrinsic limitations exist which must be acknowledged such that the impact of the discussed data is as

relevant as possible. We would like to address the limitations; which may arise as a function of our methodology:

1. The composites evaluated in this work were selected on the basis of mechanical property evaluation and cell culture data provided for in a previous publication (*i.e.* the optimum compositions in the selected design space as per a design of experiments methodology; based on cytotoxicity, tensile strength and modulus, where selected for this study) a consequence of this approach meant that limited data on the influence of F127 and PLGA on the degradation behavior of the materials exists.
2. Future work will be required to evaluate and profile the release rates inside a 24 h period to generate earlier profile and degradation data, which was not possible in the present study.

6. Conclusions

The use of composite materials is a fresh approach to the challenge of PNR. In line with the emerging philosophy of having degradation by-products establish key interactions with living tissues for the purposes of regeneration, we have constructed a composite glass filler which permits release of ionic components which may facilitate regeneration and protection in the peripheral nervous system. These devices have previously demonstrated controlled mechanical properties, and cytocompatibility and the present paper has now quantified and profiled (where possible) the release of each of its inherent ionic components. Such data will now be leveraged to select the optimum compositions for specific cell line testing prior to further biological evaluation.

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