J Mater Sci: Mater Med (2011) 22:945–959
DOI 10.1007/s10856-011-4263-1

Composition–property relationships for an experimental composite nerve guidance conduit: evaluating cytotoxicity and initial tensile strength

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Received: 26 October 2010 / Accepted: 18 February 2011 / Published online: 3 March 2011

Abstract The objective of this work was to examine the main (individual), combined (interaction) and second-order (quadratic) effects of: (i) poly(D,L-lactide-*co*-glycolide) (PLGA), (ii) F127, and (iii) a zinc-silicate based bioactive glass, on the cytotoxicity and ultimate tensile strength of an experimental nerve guidance conduit (NGC). The experimental plan was carried out according to a Box–Behnken design matrix. The effects of each compositional factor were quantified using response surface methodology (RSM) techniques. Linear and quadratic polynomial equations were developed to examine cytotoxicity (after incubation at 3, 7 and 28 days) and initial ultimate tensile strength (UTS_0). Multiple regression analyses showed that the developed models yielded a good prediction for each response examined. It was observed that the beneficial effects of PLGA and bioactive glass on controlling cytotoxicity appeared greater than that of F127. Furthermore, the experimental conduits (with the exception of CNGC-I and CNGC-K) generally showed superior cytocompatibility when compared with the comparable literature for the clinically used nerve guidance conduit Neurolac®. In this investigation, optimal compositions for cell viability were obtained for the following composition: PLGA = 18.89 wt%/F127 = 0.52 wt%/glass = 12.71 wt%. The optimization of composition with respect to ultimate tensile strength was also established (desired UTS_0 being based on the properties of the control device Neurolac® whose UTS is c.20 MPa). The desired UTS_0 of $\leq$20 MPa was found for the composition: PLGA = 18.63 wt%/F127 = 0.77 wt%/glass = 5.54 wt%. A UTS_0 $\leq$30 MPa was recorded for the composition: PLGA = 18.34 wt%/F127 = 0.62 wt%/glass = 9.83 wt%, such tensile strengths are comparable to, reported values for Neurolac®. Examination of the composition–property relationships with respect to combining cell viability and UTS_0 indicated preferred compositions in the range 17.97–19.90 wt% PLGA, 0.16–1.13 wt% F127 and between 5.54 and $\leq$20 wt% glass. This research demonstrates the value of a design of experiments approach for the design of novel nerve guidance conduits, and shows that the materials examined may have potential for the repair of peripheral nerve discontinuities.

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1 Introduction

Recently, much attention has been directed to the development of synthetic scaffolds for nerve tissue repair and regeneration [1–10]. These efforts have become increasingly important, due in part, to the shortcomings associated with autografts (limited supply, second surgical procedure [11], donor site morbidity [12], mismatch of donor nerve size with recipient site [13], occurrences of neuroma formation [13] and increased recovery time for patients) and allografts (immune rejection, increased risk of cross-contamination, possible transmission of pathogens and secondary infection [14–17]). Clinically, the most popular processed biomaterials used in such indications, include type I collagen conduits [18–20] and polyphosphoesters

such as poly (DL-lactide–caprolactone) [21, 22]. However, there is significant scope for improvement with these materials. Specifically, the requirement to balance suitable mechanical properties, biocompatibility, and semi-permeability, alongside controlled degradability [1, 11, 13, 22] is preferred in addition to materials which can interact with the body to unlock mechanisms of self-repair and regeneration [23]. It is in this regard that composite materials offer significant opportunities for investigation. Composites that have combined biodegradable polymers and bioactive inorganic phases, such as hydroxyapatite (HA) or bioactive glass, have resulted in resorbable scaffolds with tailored biocompatibility and improved physical and mechanical properties, especially in hard tissue regeneration [24, 25]. Additionally, such an approach can be manipulated to tailor in vitro and in vivo degradation behaviour, allowing for controlled release of degradation by-products, some of which can be clinically beneficial [26]. As such, if properly designed and optimized, composite materials may allow the development of scaffolds with tailored physical, biological and mechanical properties to suitably match the application of peripheral nerve repair. Bioresorbable poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and PLGA copolymers are attractive as matrix materials for scaffolds in tissue engineering [27, 28]. These polymers have demonstrated efficacy in clinical use as resorbable sutures, meshes, and in drug delivery systems [29], and are of interest in nerve repair [3, 4, 6, 8]. In addition to this, Pluronic F127 (F127) is a poly(ethylene oxide)–poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymer that can undergo sol–gel transition, depending on its concentration and ambient temperature. It has water-solubility and carries a low toxicity. Approved by the FDA for use in the human body, F127 has been found to significantly enhance the rate of wound and burn healing with several Pluronic-based formulations shown to effectively prevent postoperative adhesions or reduce adhesion area after surgery [30]. Recently, Oh et al. [31] have developed PLGA/F127 composites for peripheral nerve repair, with the view-point of increased F127 content reducing the hydrophobic nature of PLGA; thus enhancing its biocompatibility in vivo.

Guidance in regenerating axons involves turning of the motile tip (the growth cone) [32–37] and is dependant on extra-cellular cues, including ionic messengers. Primary amongst these messengers, is the divalent calcium ion (Ca^{2+}), which regulates turning (guidance) and extension of the growth cone [32–37]. In addition, recent research has identified cerium (Ce^{4+}) as a biocompatible anti-oxidant, [38] associated with beneficial effects in terms of axonal elongation, and prevention of oxidative injury to axons [39, 40]. Additional elements are also of interest in this regard. Zinc (Zn^{2+}) is the second most prevalent trace element in the human body and is required for correct immune function and growth [41–43], it is also a potent antibacterial agent [43, 44], and is of critical importance for effective wound healing [45]. Recently, it has also been suggested that Zn^{2+} may be a valuable specific therapy for uremic polyneuropathy, and nerve development [42]. From a materials standpoint, release of these ionic components in controlled levels is possible via the synthesis of degradable glasses comprising these elements [46]. Combining such glasses with polymers like PLGA, would offer superior mechanical properties, enhanced permeability (if synthesized correctly [47–49]). In addition, the degradation of such composites may be enhanced; both in terms of (i) providing control over the period in which the material resorbs, (ii) and in releasing therapeutic agents which may positively interact with the host to facilitate enhanced outcomes.

However, the effect of such a bioglass (comprising Ca, Zn and Ce), dispersed within a polyphosphoester/poloxamer based NGC has yet to be evaluated. The objective of this research paper is twofold. Firstly, a preliminary evaluation of the composition–property relationships for a new composition of composite nerve guidance conduit (CNGC) will be presented. In this preliminary study, the cytotoxicity and tensile strength of unique CNGCs ($n = 13$) is presented and analysed using response surface methodology (RSM) alongside a desirability approach to identify the most promising CNGC. Secondly, as a result of a design of experiments (DOE) approach to materials design the impact of individual components will be uncoupled and evaluated with respect to key responses. This approach is valuable since there is a significant lack of DOE investigations, which study the effects of composition on glass–polymer composites for soft-tissue repair (replacement), specifically with respect to optimising such materials in terms of cytocompatibility and mechanical strength for nerve guidance conduits.

2 Materials and methods

2.1 Design of experiment

A Box–Behnken design (BBD) with three independent variables (A: % PLGA (w/w); B: % F127 (w/w); C: % glass (w/w)) at three levels was performed, according to Table 1. Thirteen CNGC compositions (Table 2) were derived from this approach and synthesized for sixteen experimental runs using Design-Expert 8.0.2 software (Stat-Ease).

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. Second order polynomials were fitted to the experimental

Table 1 Independent variables and their levels used in the response surface design

Independent variable		Factor levels		
No.	Variable	−1	0	+1
A	% PLGA (w/w)	5	12.5	20
B	% Pluronic F127 (w/w)	0	2.5	5
C	% Glass (w/w)	0	20	40

data to obtain the regression coefficients of individual linear, interaction and quadratic terms using; sequential *F* test, lack of fit test in addition to other adequacy measures performed to select the best fit as per the literature [50–53]. In order to visualize and optimize the relationship between responses and each factor, regression coefficients were used to generate 3-D surface plots and contour plots from the fitted polynomial equations [52]. The desirability approach was adopted based on the literature [54] and in terms of the desired responses. All data were expressed as mean ± standard deviation (SD).

2.2 Materials

2.2.1 Polymer solution preparation

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 in tetraglycol T3396 (Sigma-Aldrich, Wicklow, Ireland) using an airtight container with a volume of 100 ml at 60°C (at 5, 12.5 and 20% w/w) in a waterbath to ensure that full dissolution had occurred. Dissolution occurred overnight.

2.2.2 Glass synthesis

Zinc-silicate glass with composition (mol. fraction) $0.5SiO_2$–0.2CaO–0.13ZnO–$0.14Na_2O$–$0.03CeO_2$ was synthesized for this work, as described and evaluated elsewhere [46]. In brief, appropriate amounts of analytical grade reagents; silicon dioxide, calcium carbonate, zinc oxide, sodium carbonate and cerium oxide (Sigma-Aldrich, Wicklow, Ireland) were weighed out as appropriate and thoroughly mixed by shaking (30 min) in a plastic

Table 2 Box–Behnken design matrix of design variables, in coded (and real values: in italics) and responses for cell viability (3, 7 and 28 days) and initial ultimate tensile strength

Sample	Std. no.	Run no.	Factors			Responses			
			A/PLGA (% w/w)	B/Pluronic F12 (% w/w)	C/Glass (% w/w)	Cell viability, 3 days (%)	Cell viability, 7 days (%)	Cell viability, 28 days (%)	Initial UTS_0, 1 day (MPa)
CNGC-A	1	1	*−1 (5)*	*−1 (0)*	*0 (20)*	–	–	–	–
CNGC-B	2	8	*+1 (20)*	*1 (0)*	*0 (20)*	137 (*25.8*)	143 (*21*)	127 (*6.8*)	38 (*3*)
CNGC-C	3	7	*−1 (5)*	*+1 (5)*	*0 (20)*	–	–	–	–
CNGC-D	4	2	*+1 (20)*	*+1 (5)*	*0 (20)*	107 (*3.3*)	120 (*21.1*)	104 (*1.7*)	77 (*13*)
CNGC-E	5	12	*−1 (5)*	*0 (2.5)*	*1 (0)*	104 (*3.3*)	112 (*19.9*)	108 (*3.1*)	–
CNGC-F	6	6	*+1 (20)*	*0 (2.5)*	*1 (0)*	103 (*6.9*)	146 (*32.5*)	113 (*3.1*)	13 (*4*)
CNGC-G	7	5	*−1 (5)*	*0 (2.5)*	*+1 (40)*	–	–	–	–
CNGC-H	8	9	*+1 (20)*	*0 (2.5)*	*+1 (40)*	113 (*8.4*)	167 (*50.1*)	157 (*28.1*)	–
CNGC-I	9	10	*0 (12.5)*	*1 (0)*	*1 (0)*	97 (*3.9*)	94 (*9.7*)	118 (*5.5*)	1 (*0.28*)
CNGC-J	10	13	*0 (12.5)*	*+1 (5)*	*1 (0)*	104 (*14.7*)	156 (*14.1*)	119 (*2.4*)	6 (*1*)
CNGC-K	11	4	*0 (12.5)*	*1 (0)*	*+1 (40)*	91 (*2.5*)	94 (*8.7*)	94 (*7.2*)	–
CNGC-L	12	3	*0 (12.5)*	*+1 (5)*	*+1 (40)*	–	–	–	–
CNGC-M-1	13	11	*0 (12.5)*	*0 (2.5)*	*0 (20)*	111 (*6.9*)	127 (*8.9*)	133 (*9.8*)	28 (*12*)
CNGC-M-2	14	11	*0 (12.5)*	*0 (2.5)*	*0 (20)*	78 (*6.4*)	109 (*8.2*)	119 (*9.5*)	/
CNGC-M-3	15	11	*0 (12.5)*	*0 (2.5)*	*0 (20)*	83 (*5.9*)	124 (*8.8*)	110 (*9.5*)	/
CNGC-M-4	16	11	*0 (12.5)*	*0 (2.5)*	*0 (20)*	85 (*6.4*)	119 (*8.2*)	127 (*9.5*)	/

Standard deviations for responses (cell viabilities and UTS_0) in parenthesis (in italics)

Where '–'denotes insufficient mechanical strength of the prepared CNGC to undergo subsequent tensile testing or MTT assaying. '/' denotes selectively untested

container. Each batch of powder was then fired (1520°C, 1 h) in a platinum crucible and shock quenched into water. The resulting glass frit was dried in an oven (120°C, 24 h), ground and sieved (<45 μm aperture) to retrieve glass powders of an appropriate particulate size.

2.3 Fabrication of composite nerve guidance conduits (CNGCs)

13 variations of CNGCs were synthesized based on the compositions shown in Table 2. Briefly, asymmetrically porous PLGA/F127/glass tubular scaffolds were prepared in the form of CNGCs by a modified immersion precipitation method.

Variable concentrations of F127 (0–5% w/w, PLGA base: P2443: Sigma-Aldrich, Wicklow, Ireland) gel were incorporated into prepared (5–20% w/w) PLGA solutions at 60°C and allowed to dissolve. F127 was used as a hydrophilic additive to PLGA, with tetraglycol (glycofurol) used as a nontoxic co-solvent for PLGA and F127. A given amount of glass (0–40% w/w) powder was subsequently dispersed into the polymer solution using ultrasonification (Model UCB-30, Spectrolab Instrument Pvt. Ltd) for 60 min. Calcium alginate hydrogels (rod-shaped) were fabricated by the injection of 4% (w/w) sodium alginate, $NaC_6H_7O_6$ (medium viscosity: W201502: Sigma-Aldrich, Wicklow, Ireland) into 2% (w/w) calcium chloride, $CaCl_2$, solution (C4901: Sigma-Aldrich, Wicklow, Ireland) using a stainless steel 316 syringe needle, pipetting blunt 90° tip™ (Sigma-Aldrich, Wicklow, Ireland) of gauge size, 14. The diameter obtained for the prepared calcium alginate hydrogel rods can be controlled via the injection process: using different needle gauge sizes in order to obtain the desired inner diameter of the resulting NGC. The prepared calcium alginate hydrogel rod (diameter, ~1.5 mm) with water saturation was immersed into the PLGA/F127/glass mixture solution (10 ml) for a period of 3 min at room temperature. The coating thickness (that is, the CNGC wall thickness) can be increased with a subsequent increase in the calcium alginate hydrogel immersion time in the PLGA/F127/glass solution. PLGA/F127/glass solution was precipitated onto the surface of the calcium alginate hydrogel rod by the diffusion of water molecules from the hydrogel rod into the PLGA/F127/glass solution (in tetraglycol co-solvent). After washing the PLGA/F127/glass coated calcium alginate hydrogel rod in excess water to remove any residual tetraglycol, the calcium alginate hydrogel was removed (slid out) from the outer PLGA/F127/glass thin walled tube to form the final PLGA/F127/glass CNGC. Following fabrication, the CNGC was submersed in deionized water and 25% (w/v) glycerine for 24 h in order to protect the tube walls during the post drying process as per methodology outlined by Wen et al. [49]. 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. The CNGCs were cut into segments (Length: 30 mm; inner diameter 1.5 mm) 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.

2.4 Preparation of CNGC extracts

The CNGCs segments (Length: 30 mm; inner diameter 1.5 mm) prepared in Sect. 2.3 were submersed in 10 ml of sterile tissue culture water (Sigma-Aldrich, Ireland) for 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). After each time-period, the CNGC specimens were removed and its filtrate (filtered through Grade 5 Whatman filter paper) retained and stored at 4°C; prior to in vitro evaluation.

2.5 Cell viability test

2.5.1 Cell culture of mouse fibroblast cell line L929

The established mouse fibroblast cell line L929 (European Collection of Cell Cultures (ECACC), NCTC clone 929) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Wicklow, Ireland) supplemented with 10% foetal calf serum (FCS, Sigma-Aldrich, Wicklow, Ireland), 1% (2 mM) L-glutamine (Sigma-Aldrich, Wicklow, Ireland), and 1% Tryptose Phosphate Broth TPB (Sigma-Aldrich, Wicklow, Ireland). Cells were incubated in T-75 flasks (Sarstedt, Ireland) at 37°C in a 5% CO_2 incubator, cell culture media was changed every 2–3 days. When the cells reached confluency (~70%), they were detached using 0.25% trypsin EDTA Solution (Sigma-Aldrich, Wicklow, Ireland), centrifuged and re-suspended in fresh culture media with an appropriate cell concentration (0.1 ml) of cells to new 75 ml flasks to create a new single cell suspension until desired passage was reached.

2.5.2 MTT assay

L929 cells that were grown to the desire passage (passage 7) were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 1 ml of L929 cells were seeded at a density of 1×10^4/ml in 24 well plates (Sarstedt, Ireland). Culture media was used as a negative control in row 1 and culture media plus cells used as a positive control in row 2. Plates were then incubated for 24 h in a cell culture incubator at 37°C (5% CO_2, 37°C, >90% humidity) [55].

24 h later, 100 µl of sterile tissue culture water was added to negative and positive control wells. 100 µl of experimental samples were added to appropriate wells for testing. Analysis of each extract (prepared as per Sect. 2.4) was performed in triplicate (n = 3 (extracts per condition), with 3 cell viability analyses performed on each extract). The plate was then incubated for another 24 h in a cell culture incubator at 37°C (5% CO_2, 37°C, >90% humidity) [55] as per [56]. 24 h later, 3 ml PBS was added in each MTT vial and an amount equal to 10% of culture medium (100 µl) of MTT was added to each well. Plates were then returned to the incubator for 4 h. 4 h later, an amount of MTT solubilisation solution equal to original culture medium volume (1 ml) was added in each well of the plates to dissolve the resulting formazan crystal. Each well was mixed using a pipette in order to enhance dissolution of the crystals, 2 sample extracts (100 µl) from each well were taken and aliquot in 96 well plate. The spectrophotometric absorption was measured (TriStar LB 941, Berthold Technologies, US) at a wavelength of 570 nm and the background absorption of the 96-well plates was recorded at a wavelength of 650 nm [55], then subtracted from the 570 nm measurement to get the accurate spectrophotometric absorption of the testing samples [55]. Cell viability was calculated by comparison with the positive control (100%) using the following equation [56]:

$$\text{Cell viability} = 100 * \text{OD}_{570e}/\text{OD}_{570c} \quad (1)$$

where OD_{570e} is the mean value of the measured optical density of the extracts of the experiment sample and OD_{570c} is the mean value of the measured optical density of the positive control [55].

2.6 Mechanical properties

CNGC specimens (30 mm × 1.5 mm (ID)) were prepared as per Sect. 2.3 and tested in triplicate using an Instron 3345 test machine (crosshead speed: 10 mm min^{-1}) at ambient temperature. Test data was recorded using Bluehill2 software. All sample dimensions were accurately measured using vernier-callipers and the measured average dimensions were 25 mm (±5 mm) length by 1.8 (±0.2 mm) diameter. Compositions containing 12.5 and 20 wt% PLGA were examined (see Table 2 for compositions) as those based on 5 wt% additions PLGA were of insufficient integrity for testing.

3 Results and discussion

3.1 Analysis of variance (ANOVA)

The test for significance of the regression models, test for significance on each model coefficients, and the lack of fit test were carried out. Backward regression methods were selected to determine the significant model terms automatically. The reduced quadratic and linear models present the ANOVA for each response and show the significant model terms. Abstracted ANOVA results are provided in Table 3.

The same tables show additional adequacy measures R^2, adjusted R^2 and the adequate precision. All R^2 and adjusted R^2 adequacy measures are high (and in reasonable proximity to 1), while all values of adequate precision for each response exceed the desired value of >4, which is also in agreement and indicates significant regression models (relationships).

3.1.1 *Development of mathematical models*

Tables 2 and 4 illustrates the cell viability response based on the compositional variables (A, B and C as outlined in Sect. 2.1). In a limited number of cases, large deviations about the mean are recorded, and acknowledged.

The final mathematical models in terms of coded factors as determined by Design-Expert software are shown below in Eqs. 2–4. Based on Eq. 2 the level of significance of positive quadratic effects (based on compositional variables) follows the order $(A^2) > (B^2)$. A significant negative interaction was observed to be (AB). Equation 3 demonstrates that the level of significance for the positive main and interaction effects on cell viability after 7 days was (A) > (AB) while the significance of the negative main, interaction and quadratic effects follows the order $(A^2) > (AB) > (B^2) > (C)$. With respect to Eq. 4 the significant positive main and interaction effects on cell viability after 28 days are (A) > (AB) while the level of significance for the negative main, interaction and quadratic effects follows the order: $(C) > (BC) > (B^2) > (A^2) > (AB)$.

$$\%\ \text{Cell viability (3 days)} = +85.06 - 32.06\,\text{AB} + 27.20\,\text{A}^2 + 17.99\,\text{B}^2 \quad (2)$$

$$\%\ \text{Cell viability (7 days)} = +119.58 + 58.70\,\text{A} - 30.93\,\text{C} - 11.86\,\text{AB} + 41.74\,\text{AC} - 30.79\,\text{BC} - 21.49\,\text{A}^2 - 25.50\,\text{B}^2 \quad (3)$$

$$\%\ \text{Cell viability (28 days)} = +122.25 + 36.79\,\text{A} - 11.96\,\text{C} - 15.99\,\text{AB} + 33.71\,\text{AC} - 23.53\,\text{A}^2 - 15.36\,\text{B}^2 \quad (4)$$

Table 3 Abstracted ANOVA table for the cell viability reduced quadratic polynomial models (3, 7 and 28 days) and UTS_0 reduced linear model

Response	SS_{model}	DF	Mean square	Lack of fit	F value	Prob. > F model	R^2	Adj-R^2	CV (%)	Adq-prec.
Cell viability (3 days)	5076.27	3	1692.009	Not sig.	11.56	0.0028	0.8126	0.7423	11.64	11.061
Cell viability (7 days)	5750.61	7	821.52	Not sig.	18.13	0.0070	0.9694	0.9160	5.35	13.399
Cell viability (28 days)	2744.21	6	457.37	Not sig.	7.93	0.0191	0.9049	0.7907	6.33	10.745
UTS_0	3100.87	1	3100.87	–	8.35	0.0446	0.6761	0.5951	–	4.086

Table 4 Summary of the significant (positive and negative), main, interaction and quadratic effects associated with compositional factors (order of significance for positive significant effects: highest to lowest and negative significant effects: lowest to highest)

Ranking of effects	Effect of increasing factor on response	Responses			
		Cell viability (%), 3 days	Cell viability (%), 7 days	Cell viability (%), 28 days	UTS_0
Positive effect					
1	↑	A^2	A	A	C
2	↑	B^2	AC[a]	AC[a]	–
Negative effect					
1	↓	AB[a]	C	A^2	–
2	↓	–	BC[a]	AB[a]	–
3	↓	–	B^2	B^2	–
4	↓	–	A^2	C	–
5	↓	–	AB[a]	–	–

[a] Effects of these interactions are examined in Figs. 2, 3, and 4

3.2 Composition–property relationships with respect to cytotoxicity

The perturbation plot (Fig. 1) helps to compare the effect of all the factors from a particular point (centre point: 12.5 wt% PLGA, 2.5 wt% F127 and 20 wt% glass) in the design space. The response is plotted by changing only one factor over its range, while holding the other factors constant. This represents in a decoupled manner the effect of each compositional factor on each response for cell viability. A steep slope or curvature in a factor shows that the response is sensitive to that factor. This data is the basis for the desirability approach used later in Sect. 4.

3.2.1 Factor A (PLGA content: range 5–20 wt%)

With respect to independently evaluating the impact of Factor A on cytocompatibility, variable responses were observed as a function of time. At 3 days (Fig. 1a), varying the content of PLGA between 5 and 12.5 wt% decreases mean cell viability from 113 to 85% as compared with the control (tissue-culture water). Interestingly, an increase in PLGA content from 12.5 to 20 wt% results in mean cell viabilities recovering to 113%, as compared with the control. At 7 days (Fig. 1b), increasing the PLGA content of the CNGC approximates linear behaviour with cell viability in the range 50–150%. By 28 days (Fig. 1c), Factor A shows a linear effect on cell viability between 5 and 12.5 wt%, thereafter its impact is observed to approach a quadratic relationship. The observed dependency of increased cytotoxicity with decreasing PLGA content is likely attributable to reductions in pH associated with the faster degradation of conduits comprising <12.5 wt% PLGA [57]. Even though cytotoxicity is observed for certain additions of PLGA, it must be noted that such observations are limited in terms of severity [31, 57]. More significantly, the results point to optimum contents of PLGA additions in respect of the overall composite; a feature further elucidated in Sect. 4. PLGA is the only factor, in terms of the proposed models, to have a positive main effect on cell viability (Table 4).

3.2.2 Factor B (F127 content: range 0–5 wt%)

With respect to Factor B, cell viability follows the same behaviour patterns as noted for Factor A (Fig. 1). Succinctly, a parabolic effect is noted at 3 days between a maximum of 103% cell viability, to 85% cell viability, recovering to 103% cell viability for loadings of F127 of 0, 2.5 and 5 wt%. However, for 7 and 28-day cell viabilities, a reverse quadratic effect is observed (Fig. 1b, c). Whilst other studies have shown that low concentrations of F127

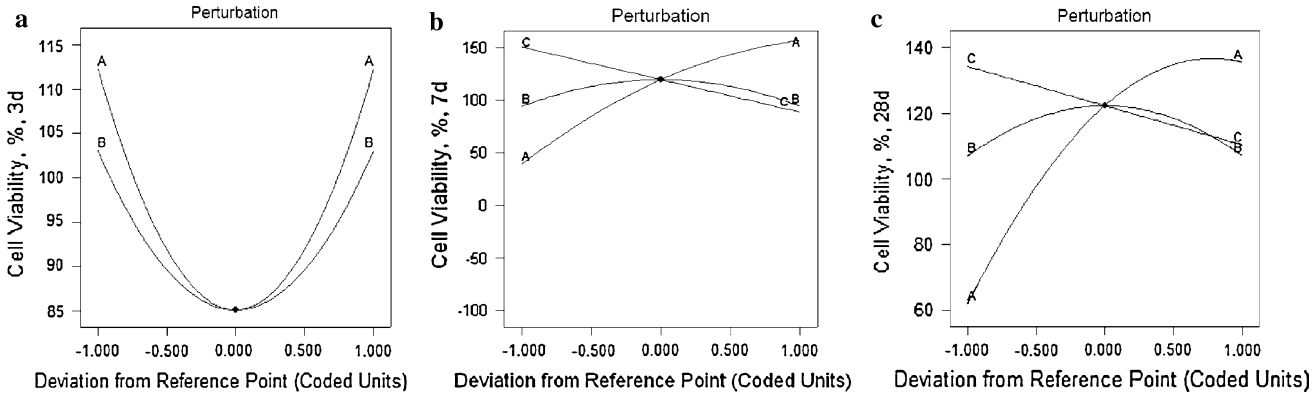

Fig. 1 Perturbation plots showing the effect of each factor on the cell viability at **a** 3 days, **b** 7 days and **c** 28 days from the centre point of the BBD space (0, 0, 0: 12.5, 2.5, 20 wt%)

may alter transport of compounds across cell membranes (normally impermeable), in general F127 has been shown to have good cytocompatibility similar to this ranges reported here [58]. Nevertheless, whilst all cell viabilities recorded represent reasonable levels of cytocompatibility, the reason for the reverse quadratic effect is difficult to explain based on the present data. Based on the models developed and when examined as an isolated factor, F127 does not significantly effect cell viability.

3.2.3 Factor C (glass component: range 0–40 wt%)

For Factor C there is no impact on 3-day cell viability as a result of the glass content (Fig. 1a). This observation is likely due to the fact that there is a limited surface area of glass particles exposed to the incubation media up to this time point. However, the glass component exerts a marked influence on cell viability at 7 and 28 days (Fig. 1b, c). By 7 days, 0 wt% glass provides for cell viabilities in excess of 150% as compared with the control (tissue culture water), which decreases in a linear fashion to c.100% cell viability with the addition of 40 wt% of glass. Similar reductions are noted for the 28-day data. As incubation time increases, and polymeric degradation ensues, increased glass particle exposure to the media (which increases with glass content) occurs and results in the accumulation of metal ions in solution; such accumulations being associated with increased cytotoxicity [59]. However, an interesting point of note is that whilst cell viabilities decrease, the reduction is towards a viability of 100% as compared with the control. Whilst cell viabilities in excess of 100% have been previously reported for zinc-silicate glasses [26] the authors have pointed out that enhanced cell viability (evident by induced cell proliferation) may not correspond with beneficial effects. Rather, genotoxic effects can cause cell DNA damage resulting in malfunctions within the regulation of the cell cycle and subsequent uncontrolled proliferation of mutated cells. As such, relevant genotoxicity testing is warranted for these glasses and their composite derivatives. Based on the models developed, and when examined as an isolated factor, the glass component exerts a significant negative effect on cell viabilities at 7 and 28 days.

3.3 Examination of significant interactions between compositional factors on cell viability

Figures 2, 3, and 4 represents contour plots, which illustrate the significant interaction between compositional factors on cell viability (highlighted in Table 4). The significant interactions are: AB, AC and BD. With respect to the interaction AB (Fig. 2), this interaction has negative significance at each time point (Table 4). At 3 days (Fig. 2a–c) lowest cell viabilities (87%) are obtained for median concentration of PLGA and F127. Enhanced cell viabilities (up to 150%) appear to be dependant on increasing PLGA and decreasing F127, from c.11 and c.1% respectively.

However, counter-intuitively, increasing F127 from c.1% and decreasing PLGA below 11% also provides for increased cell viability (again up to 150%). Based on the data, the glass component exerts no influence on this interaction with respect to cell viability at 3 days. However, by 7 and 28 days the glass exerts influence over the interaction AB (for reasons discussed in Sect. 3.2.3). At 7 and 28 days, it is clear that increased cell viabilities can be controlled based on composition. Specifically, 0 wt% glass will allow for cell viabilities of c.150%. However, the same ratio of A:B with 20 and 40 wt% additions of glass reduces cell viability to c.125 and c.100% respectively. Interestingly, the effect of increased glass content on cell viability may by controlled (increased) by increasing the PLGA content from 14 to 20 wt%, and decreasing F127 to <3 wt% (Fig. 2d–i).

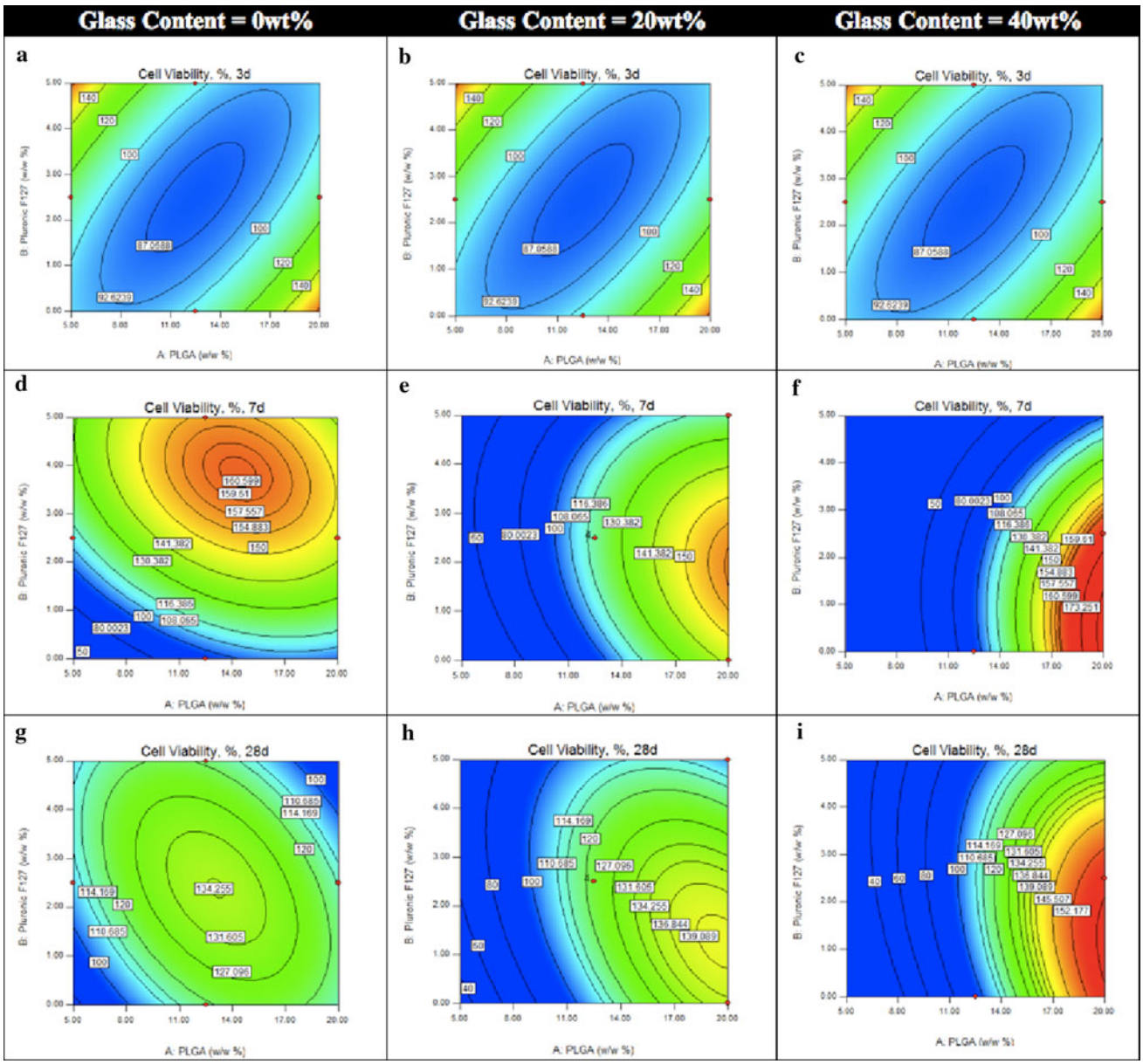

Fig. 2 Contour plots showing the significant interaction effects of AB on the cell viability (**a–c** 3 days, **d–f** 7 days, **g–i** 28 days) for three loadings of glass (0, 20, and 40 wt%)

With respect to the interaction AC (Fig. 3), which has a positive significant effect at 7 and 28 days (Table 4), the following observations are noted; lowest recorded levels of cell viability (<50%) are typically associated with reduced PLGA content (for all additions of F127), indicating that F127 exerts little influence on the AC interaction with respect to low levels of cell viability.

Furthermore, as noted for the AB interaction, increased glass content and increased PLGA content offer highest levels of cell viability (Fig. 3a–f). Again, the contour plots provide an exacting mechanism for selecting preferred viability (between <50% up to in excess of 150%) based on composition (previously noted for the interaction AB). For example, based on the interaction AB, increased cell viabilities at 7 and 28 days (>140%) may be obtained where the following conditions are met:

(i) 0 and 2.5 wt% F127 is used in the CNGC.
(ii) PLGA is kept in the range 17–20 wt%.
(iii) Glass content is in excess of 25 wt%.

For the interaction AC, it is noted that incorporation of 5 wt% F127 requires reduced levels of PLGA at 7 days; for

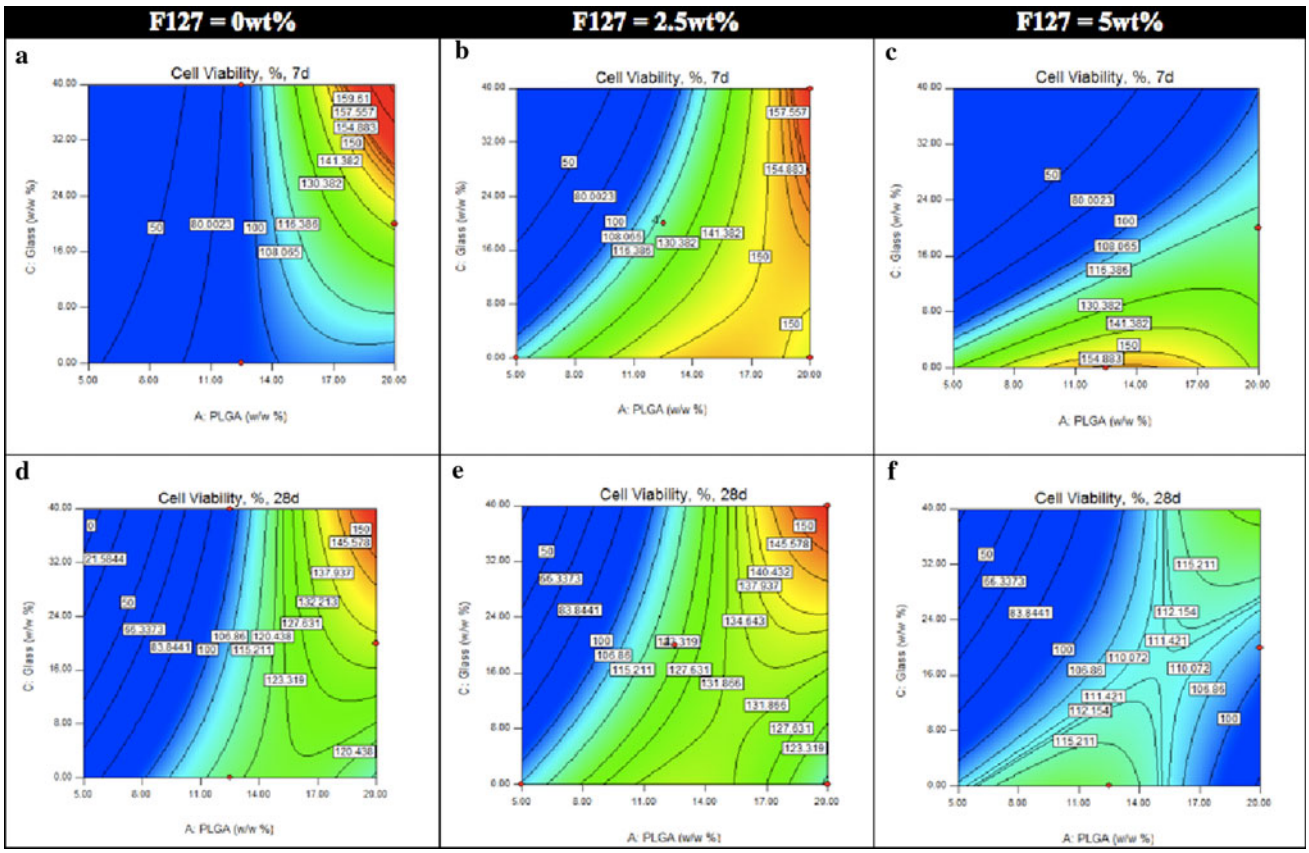

Fig. 3 Contour plots showing the significant interaction effects of AC on the cell viability (**a–c** 7 days, **d–f** 28 days) for three loadings of F127 (0, 2.5, and 5 wt%)

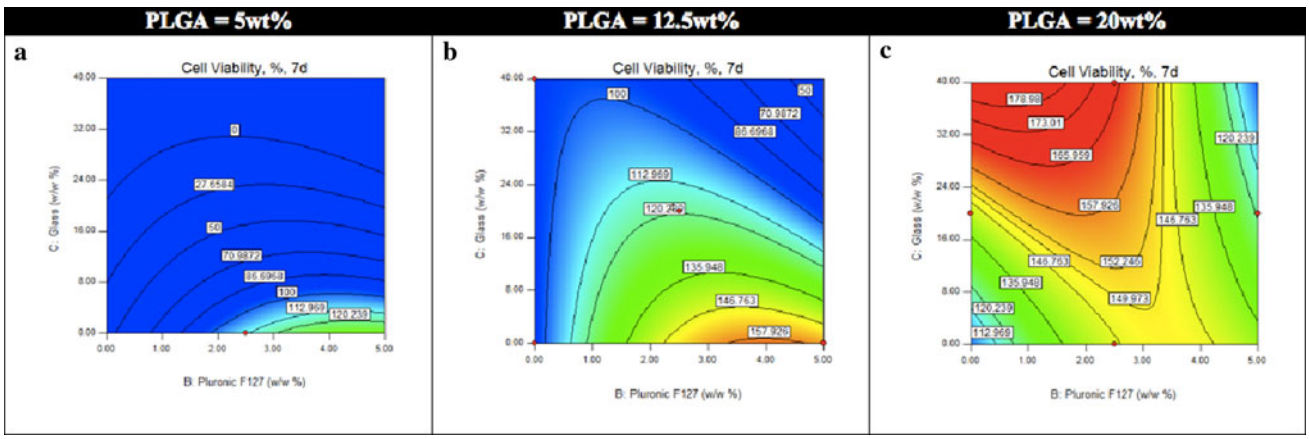

Fig. 4 Contour plots showing the significant interaction of BC on cell viability (7 days only) for three loadings of PLGA (0, 12.5, and 20 wt%)

example PLGA in the range 7–19 wt% and glass loading <3 wt% confer largest cell viabilities at this time point. The final significant interaction reported (Table 4) is BC and demonstrates negative significance at 7 days (Table 4). More particularly, at low concentrations of PLGA (5 wt%), it is required that additions on the order of at least 2 wt% F127 and 2.5 wt% glass are required to obtain cell viabilities exceeding those of the control. It is also evident that increased additions of factors B and C is permitted where the PLGA content is in the range 12.5–20 wt%. Enhanced evaluation of composition–property relationships with respect to cell culture was possible at the main level for the CNGC. The main, combined and quadratic effects of composition on the experimental CNGC has been established in this section, and will allow for enhanced material design and development of these materials.

3.4 Composition–property relationships with respect to UTS_0

CNGC synthesized with 0 and 5 wt% additions of PLGA were unsuitable for mechanical testing due to lack of structural integrity (irrespective of glass content). However, for CNGC synthesized based on 12.5 and 20 wt% additions of PLGA (with a glass content ≤20 wt% glass), UTS_0 values in the range 2.6–1.4 MPa were recorded (Table 2), with highest values being observed for those with 20 wt% additions of PLGA. As expected (Eq. 5), the glass composition was the main factor, which allowed for a significant effect in achieving the UTS_0 within a desirable range (Eq. 5, developed for this response will be used in Sect. 4 to optimize the composite).

$$UTS_0 = +52.88 + 45.47\,C \quad (5)$$

It was noted that glass loadings over 20 wt% compromised the processing of conduits; specifically, high loadings of glass appeared to inhibit precipitation of composite (in solution form) onto the alginate rods. It appeared that the F127 also exerts control on the UTS_0, which is counter to recent literature [31]. Specifically, CNGC-B and CNGC-D differ only in F127 content, 0 and 5 wt% respectively (Table 2) yet observed UTS_0 of 38 and 77 MPa was observed respectively. The reason for this observation, which is counter to published literature is unclear at present, however interaction effects between the glass and the F127 may be the determining factor here. Further analysis, over extended periods of time with additional experimental runs may clarify the root cause of this observation.

4 Optimization based on cytotoxicity and UTS_0

To ensure that CNGCs developed are of adequate mechanical strength and cytocompatibility, it is recommended that the optimal compositions be used. The models established in this work have been developed and checked for their adequacy [52]; consequently, optimization criteria can be set to find the optimum compositions based on the composition–property responses examined. Three optimization criteria (Table 5) were used.

Weight is given for all the factors in the range 1,0. The goal of the first criteria is to identify the optimal composite compositions to maximise the cell viability with no limitation set on either the compositional factors or the mechanical strength. On the other hand, the second criterion is investigated to identify the optimal composite compositions, with no limitation set on the compositional factors, but would maximise the cell viability while achieving an UTS_0 in the range: 5–30 MPa: to provide CNGCs of optimal cell viability with greater tensile strength than the Neurolac® device [22]. In addition to this, for the third criterion, the goal was to identify the optimal compositions with no limitation set on the compositional factors: to maximise the cell viability while achieving an UTS_0 in the range: 5–20 MPa: to provide CNGCs of optimal cell viability of a comparable tensile strength to the Neurolac® device [22]. The top five optimal solutions that fulfil these three sets of criteria are presented in Table 6.

From the optimal solutions outlined (Table 6) it is evident that for the three sets of criteria (as per the top 5 ranked solutions for each criterion), the working ranges for both PLGA and F127 compositions are similar. RSM graphical optimization (Fig. 5) allows for visual selection of the optimum compositions for each of the components in order to form an idealised CNGC of tailored mechanical strength and biocompatibility.

In fact, to reach any of the three criteria, the PLGA and F127 are required to range between 17.97 to 19.90 wt% and 0.16 to 1.13 wt%, respectively, while the working range for glass addition varies between 5.54 to 34.80 wt%. With respect to the glass content, the prediction of up to 34.80 wt% is based on responses examined and does not recognise difficulties in synthesizing CNGC with >20 wt% glass. As such a glass loading of <20 wt%, based on the results is preferred. If the compositions of the three components in the CNGC are free to change in order to

Table 5 Design optimization parameters

Factor/response	First criterion		Second criterion		Third criterion	
	Goal	Importance	Goal	Importance	Goal	Importance
PLGA % (w/w)	In range	+++	In range	+++	In range	+++
Pluronic F127 % (w/w)	In range	+++	In range	+++	In range	+++
Glass % (w/w)	In range	+++	In range	+++	In range	+++
Cell viability, 3 days (%)	Maximise	+++	Maximise	+++	Maximise	+++
Cell viability, 7 days (%)	Maximise	+++	Maximise	+++	Maximise	+++
Cell viability, 28 days (%)	Maximise	+++	Maximise	+++	Maximise	+++
Initial ultimate tensile strength, UTS_0 (MPa)	None	N/A	In range (5–30 MPa)	+++++	In range (5–20 MPa)	+++++

Table 6 Optimal solution as obtained using Design-Expert (*using the parameters in* Table 4)

Solution number	A	B	C	Cell viability (%), 3 days	Cell viability (%), 7 days	Cell viability (%), 28 days	Initial UTS (MPa)	Desirability
First criterion								
1	18.89	0.52	12.71	137.740	135.403	131.563	–	1.000
2	19.90	0.16	26.78	156.938	158.377	144.158	–	1.000
3	18.31	0.48	17.18	133.254	139.194	134.611	–	1.000
4	19.56	1.03	13.91	133.157	145.450	133.558	–	1.000
5	17.97	0.25	34.80	134.995	158.216	143.975	–	1.000
Second criterion								
1	18.34	0.62	9.83	130.445	132.242	130.059	29.7658	1.000
2	18.49	0.70	9.50	130.109	133.525	130.017	28.9988	1.000
3	18.27	0.60	9.92	130.186	131.896	130.084	29.9524	1.000
4	19.08	0.95	9.74	130.319	138.513	130.181	29.5567	1.000
5	18.86	0.84	9.53	130.706	136.244	130.047	29.0779	1.000
Third criterion								
1	18.63	0.77	5.54	130.000	130.000	127.014	19.9999	0.971
2	19.02	0.94	5.54	130.000	133.152	126.600	19.9997	0.971
3	19.10	0.98	5.54	130.000	133.746	126.470	19.9998	0.970
4	19.45	1.13	5.54	128.596	130.001	127.321	19.9997	0.969
5	19.45	1.13	5.54	130.108	136.093	125.700	19.9999	0.958

maximise the cell viability (3 days $\approx$ 138%; 7 days $\approx$ 135%; 28 days $\approx$ 132%) without any limitations on the mechanical strength, the PLGA composition needs to be between 17.97 to 19.90 wt%, with a low level of F127 composition between 0.16 to 1.03 wt% and glass composition ranging between 13.91 to 34.80 wt%. On the other hand, if an UTS_0 of $\sim$30 MPa is required using the same conditions as set for the first criteria, it is verified that, the PLGA composition has to range between 18.27 to 19.08 wt% and the F127 composition between 0.60 to 0.95 wt%.

The results for the graphical optimization are also displayed as overlay plots (for enhanced detail), as shown in Fig. 6, which satisfy the third and first solutions in both the first (Fig. 6a) and second criteria (Fig. 6b), respectively. The third solution (18.27 wt% PLGA, 0.60 wt% F127 and 9.92 wt% glass) has been selected for the second criteria since its combination contains a desirability factor of 1, and moreover the inclusion of about 10 wt% glass may also aid with ease of process-ability while not compromising on levels of cell viability. To reduce the UTS to <20 MPa however, 5.54 wt% glass is required (Fig. 6b) in combination with 18.63 wt% PLGA and 0.77 wt% F127.

These types of plots may be used as a manufacturer's reference guide to prepare the proposed CNGCs in choosing the values of the compositions for the composite material comprising of PLGA, F127 and glass, which would achieve the appropriate levels of mechanical strength tailored to suit the anatomical site at a maximum level of biocompatibility. The yellow/shaded areas on the overlay plots are the regions that meet the proposed criteria. The mechanical stability of the conduit is an additional requirement for the success of tissue regeneration. For the case of the CNGCs developed in the present study, the conduit requires a high degree of porosity to allow rapid tissue infiltration, while having enough composite matrix material to retain its structural integrity for an appropriate duration to enable the degradation process match the rate of peripheral nerve regeneration. The addition of rigid fillers to polymer matrices is common practice in composite technology to improve the stiffness, mechanical strength and structural integrity of components. Boccaccini et al. [48], applying a model developed based on an equation by Ishai and Cohen [60], estimated that the addition of 1 wt% Bioglass® particles in their composites lead to a 5% increase of elastic modulus of neat PLGA materials of equal porosity. The approach in this study, and the RSM outputs allow enhanced evaluation of composition–property relationships with respect to such composites.

5 Limitations of the study

As a preliminary study, limitations exist which must be noted. Primarily, rather than evaluate specific cell responses (e.g. biosynthetic activity) this study emphasized phase 1 cytotoxicity evaluation using L929 mouse fibroblasts. Whilst, this is a robust and accepted model for preliminary

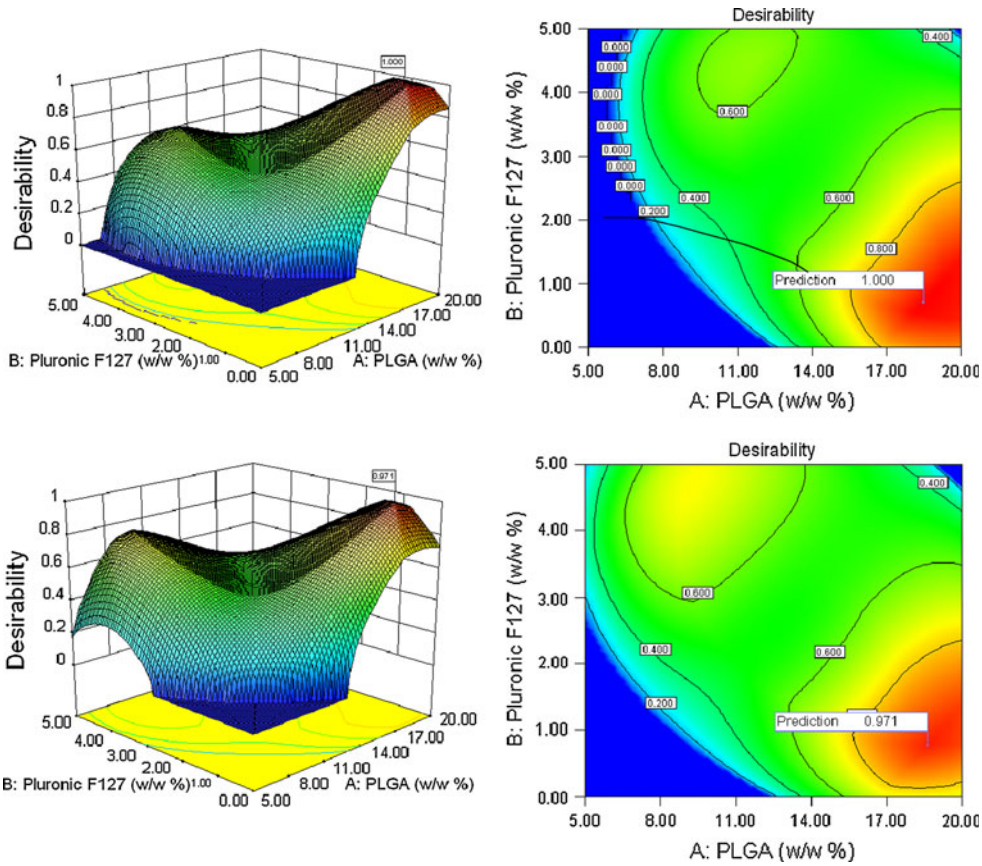

Fig. 5 RSM plots (in 3D and 2D) show the desirable region for optimal compositions of PLGA, Pluronic F127 and glass in the final CNGC based on the second and third criterion

screening, additional experimentation using more representative cells (e.g. glial cells) is now warranted. Secondly, and again in respect of the cell culture experimentation; whilst the study did capture the influence of individual components (main effects) and the interaction of components on cytocompatibility, the study did not capture the root-cause of enhanced cell proliferation associated with some CNGC; a feature which may or may not be positive. As pointed out in the discussion, full genotoxicity screening of the materials is warranted based on these results.

The design space was reduced from an original set of 13 compositions to 9 based on difficulty synthesizing mechanically stable CNGCs. However, the models developed on these 9 compositions are statistically robust, based on:

- The normal probability plot of the studentized residuals, that is, the number of standard deviations of the actual values from their respective predicted values were also found to follow a normal distribution for each response.
- The probability P value is used to quantify this probability and is a very good indicator of significance. All developed regression models had high R^2 values and are comparable to numerous other studies in the literature; moreover no significant lack of fit was interpreted within any of the models.
- The CV as the ratio of the standard error of estimate to the mean value of the observed response (as a percentage) is a measure of reproducibility of the model and as a general-rule a model can be considered reasonably reproducible if its CV is not greater than 10% [61]. The model fitted for cell viability at 3 days is greater than 10% of CV. By applying diagnostic plots including normal probability plot of residual, plot of residuals versus predicted for cell viability at 3 days, the assumptions of normality, independence and randomness of the residuals were satisfied. The fitted model for cell viability at 3 days was thus accepted.
- The adequate precision value is a measure of the "signal-to-noise ratio" for the responses. A ratio >4 is considered to be adequate model discrimination. The adequate precisions for the responses (cell viabilities (%) at 3, 7 and 28 days are 11.061, 13.399 and 10.745 respectively, and UTS_0 is 4.086) are all above four.

6 Conclusions

This paper has successfully synthesized a unique set of CNGC based on (i) a unique, phosphate free ion leachable glass comprising elements associated with enhanced

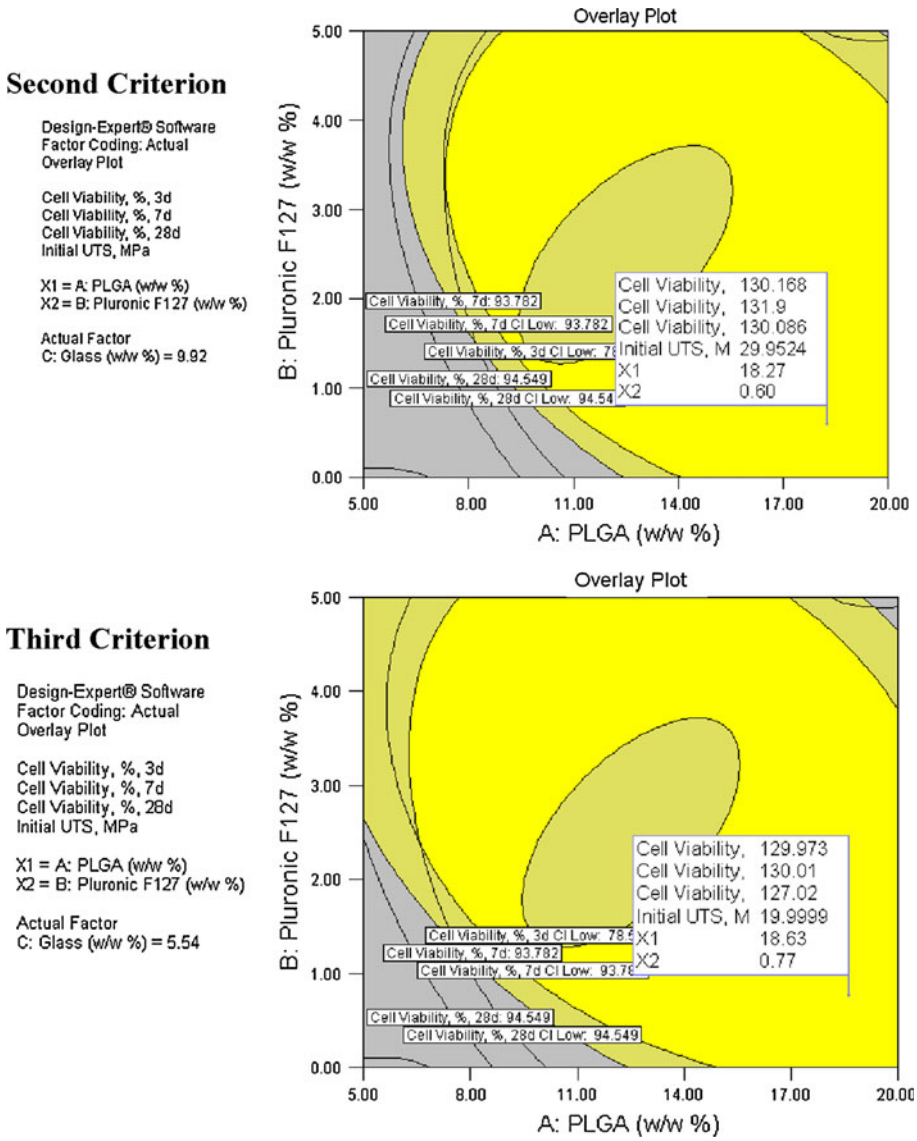

Fig. 6 Overlay plots show the region of the optimal compositions for PLGA, Pluronic F127 and glass in the final CNGC based on the second (**a** solution number 3) and third criterion (**b** solution number 1)

peripheral nerve regeneration and protection, alongside (ii) a PLGA/F127 polymeric matrix, synthesized as tubular constructs. The main (individual), interaction and quadratic effects of composition of experimental CNGC has been rigorously examined with respect to cytotoxicity and mechanical properties using a design of experiments approach. The recommended ranges reported correlate with the design space investigated herein: which were deemed as appropriate for navigation as per Design-Expert software (as a result of the statistical adequacy measures performed). The desirability/optimization methodology employed was used as a basis to select preferred compositions (based on cytotoxicity and mechanical properties). The preferred compositions are in the range 17.97–19.90 wt% PLGA, 0.16–1.13 wt% F127 and between 5.54 and ≤20 wt%. The experimental material warrants further examination as a CNGC.

Acknowledgments This work was supported by Enterprise Ireland as part of a proof of concept (PC/2008/315) project under the National Development Plan.

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