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Title: A Comparison of the Degradation Behaviour of 3D Printed PDLGA Scaffolds incorporating Bioglass or Biosilica.

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Abstract: (240)

Silica incorporation into biomaterials, such as Bioglass and Si-substituted calcium phosphate ceramics has received significant attention in bone tissue engineering over the last few decades. This study aims to explore the dissolution behaviour of natural biosilica isolated from a freshwater diatom, *Cyclotella meneghiniana*, that has been incorporated into 3D printed poly (DL-lactide -co - glycolide) (PDLGA) scaffolds using extrusion and additive manufacturing. In the study, two different dry weight percentage (1 wt.% & 5 wt.%) of diatom-silica were incorporated into PDLGA scaffolds that were then degraded in phosphate buffered saline (PBS) cell free media. In addition, pure PDLGA scaffolds and 5 wt.% Bioglass scaffolds were used as control groups. The degradation study was performed over 26-weeks. The release rate of Si⁴⁺ ions from diatom-PDLGA scaffolds was found to increase exponentially with respect to time. The compressive strength of scaffolds was also measured with the Diatom-PDLGA scaffolds found to maintain their strength for longer than either pure PDLGA scaffolds or 5 wt.% Bioglass scaffolds. ¹³C NMR data showed that diatom biosilica containing scaffolds had less degradation than pure or bioglass-containing scaffolds at comparable time-points. Overall, the Diatom-PDLGA scaffolds were found to have more desirable physiochemical properties for bone repair compared to Bioglass.

Key words: Diatom-silica, PDLGA, additive manufacture, FDM, bone repair.

1. Introduction:

Interest in three-dimensional (3D) printing technologies has developed at a remarkable pace in bone tissue engineering over the past decade. This is due to their ability to produce bone scaffolds with customised-designed shape and tailored porous structures and properties¹. The need for bone replacement strategies as a result of degenerative bone diseases and fractures has increased significantly due to an increase in ageing population and overall population growth². The advances in 3D printing of bone scaffolds includes fabrication techniques, morphological profile design, material development, and more recently the ability to print composite materials^{3,4}. To produce optimised porous scaffolds with appropriate properties (e.g. biodegradability, biocompatibility, and bioactivity), new composite materials are being developed that are suited to 3D printing with bioactive properties to enhance bone replacement⁵⁻⁷. However, there are still a number of challenges to overcome using these technologies that include improved mechanical properties and a reduction in process induced polymer degradation⁷.

Fused deposition modelling (FDM) is a common 3D printing technology that uses a layer-by-layer technique to build 3D scaffolds^{8,9}. To create the layers, thermoplastic polymers/composites with thermally lowered viscosity are deposited onto a moving platform. In recent years, the ability to print composite materials has made this technology more attractive for bone repair¹⁰. Recent studies have shown the feasibility of printing polymer filaments that contain hydroxyapatite (HA) and beta tricalcium phosphate (β -TCP) that have resulted in improved mechanical and bioactive properties using standard FDM 3D printers^{11,12}. Some new FDM 3D printer contain dual nozzle heads or in-nozzle impregnation

that enable polymers to be blended with fibre or particles during printing⁶. In addition, unlike other solid freeform scaffold fabrication technology, FDM does not require using any solvent, making it a greener approach to scaffold fabrication¹³.

Biodegradable polymers have been used for bone repair since the 1990s¹⁴. They include polymers, such as polylactic acid (PLA), polyglycolic acid (PGA), polyvinyl alcohol (PVA), polycaprolactone (PCL)^{15–21} and more recently poly (DL-lactide-co-glycolide) (PDLGA, or sometimes abbreviated in literature to PLGA)¹⁵. All of these polymers are suitable for 3D printing^{15,16}. PDLGA is an amorphous co-polymer made up of DL-lactic acid and glycolic acid, which is non-toxic and approved by Food and Drug Administration (FDA) as a component of a range of medical devices²². The ability to manipulate the degradation profile of PDLGA through the modification of the copolymerisation ratio of its monomers offers significant promise in biomedical applications. Different ratios of these monomers, lactic and glycolic acid²³ can vary its degradation profile from 1 week (PDLGA 50:50) to 6 weeks (PDLGA 85:15)²⁴, making it suitable for applications ranging from drug delivery system to bone scaffolds^{25–28}. It is also highly versatile in terms of fabrication and has been successfully formed into plates, meshes and screws for medical applications^{24,29}.

In recent years, silica has attracted more attention in terms of its role in bone repair^{30–32}. Over the last 30 years the literature has shown strong evidence that dietary silicon is beneficial to bone health^{33,34}, but the exact biological role(s) of silica in bone repair is still not clear either in terms of dietary or implanted silica³³. Additional evidence to support the therapeutic benefit of silicon in bone is provided by *in vivo* and *in vitro* studies with silicon-containing implants and ceramics, such as Si-substituted hydroxyapatites and Bioglass^{33,35,36}.

Silicon-containing bone fillers and scaffolds have been reported to bond more effectively to bone compared to non-silicon-containing implants. This has been attributed to the spontaneous formation of a biologically active apatite-like layer on their surface³⁷. Silicon has also been shown to be involved in gene upregulation, osteoblast proliferation and differentiation, type I collagen synthesis and apatite formation. For example, a study from Mieszawska *et al.*, found the presence of silica upregulated the gene expression in osteogenesis³⁸. One recent paper reported more ordered collagen fibrils and mature bone formation with Si-substituted hydroxyapatite granular implants³⁹. The function of silicon in bone regeneration has not been clearly established. The concentration of Si in bone tissue has been reported to be in the range of 100 – 150 ppm in bone and 200 – 600 ppm in cartilage/connective tissues^{36,40}.

Bioglass 45S5, which contains 45 wt.% SiO₂, has shown promise in bone tissue engineering. The major advantages of Bioglass is its bone-bonding capability, therapeutic ion release and enhanced cell growth and differentiation^{41,42}. However, its incorporation into polymer scaffolds, has resulted in lower mechanical strength and poorer fracture toughness^{43,44}. In recent years, diatom frustule (i.e. cell walls) has received some positive interested in bone tissue engineering, mostly because of its chemical composition and unique morphological structure. Thi *et al.*,⁴⁵ demonstrated that diatom biosilica can undergo dissolution in aqueous environments to release silicon ions. They also reported that diatom biosilica particles have little or no cytotoxic effect *in vitro* study using embryonic Swiss mouse fibroblast cells (3T3). This was supported by Walsh *et al.*, who reported that diatom biosilica was non-cytotoxic to J774.2 macrophage cells *in vitro*, and supports cell proliferation and growth of hBMSCs⁴⁶.

This study investigates the effect of natural biosilica (freshwater diatom, *Cyclotella meneghiniana*) compared to synthetic silica (Bioglass® 45S5) in FDM 3D printed PDLGA polymer scaffolds; in terms of their degradation profile and mechanical properties to assess their potential in bone repair applications. The FDM 3D printed scaffolds were fabricated using the method described by Little et al.,⁷ with slight modification to processing conditions to accommodate the addition of the different biocerami filler. This study aimed to understand the degradation rate of the scaffolds in terms of polymer degradation and silica release. In addition, the compressive strength of the scaffolds was measured over the duration of the study. The study was performed over 26-week in PBS. Molecular characterisation of the hydrolytic degradation of PDLGA was studied using ¹³

2. Materials and Methods:

2.1 Materials

2.1.1 Biosilica Bone Filler – Diatoms and Bioglass

C. meneghiniana diatoms were collected from the Mississippi River, USA, isolated and cultured as previously described by Walsh *et al.*,⁴⁶. A nitric acid (HNO₃) digest was used to partially remove the organic fraction by Prof. Julius group. The resultant samples were then freeze dried and sealed in a vacuum-packed bag for transport to the UK. On receipt of the samples the diatoms were washed repeatedly (x10) with 1000mL of deionised (DI) to neutralise the residual acid. Thereafter, the diatom samples were heat in an Elite chamber furnace (BMF 11/7) at 550 °C for 1 hour to remove the remaining organic matter. The resultant diatom sample was assigned the name “heat treated (HT) diatom” in subsequent studies. Bioglass 45S5 (SylcTM, Research Ltd, London, UK) from Denfotex (EJR, D095325/K062502, Reg. No. 8126030) of mean particle size of 5 to 80 µm was also used in this study. Prior to use the purified diatom biosilica and Bioglass 45S5 were vacuum dried at 40°C under 10 mbar of pressure for 2 days before they were blended with PDLGA.

2.1.2 Poly (DL-lactide-co-glycolide) (PDLGA) Polymer

Medical grade PDLGA (85:15) polymer granules (Boehringer Ingelheim Resomer RG 859 SR) were stored in the fridge (-4 °C) in a sealed bag to prevent the degradation of polymer. Prior to use, PDLGA polymer was brought to room temperature overnight. It was then opened and dried under the same conditions as the bioceramic fillers (described above) before being blended together.

2.1.3 Phosphate Buffered Saline (PBS) Buffer

PBS was selected at pH 7.4 to mimic the normal pH environment in human body⁴⁷. The main characteristic from PBS buffer is stability for a long term study⁴⁸, hence being recommended as the *in vitro* soaking solution in BS ISO 13781:2017; Implants for surgery — Homopolymers, copolymers and blends on poly(lactide) — In vitro degradation testing. The PBS was prepared by adding 0.19 g of potassium chloride (KCl) with 0.20 g of monopotassium phosphate (KH_2PO_4), 8.06 g of sodium chloride (NaCl) and 1.14 g disodium phosphate (Na_2HPO_4) to $\approx$ 800mL deionised water. The pH was adjusted to 7.4 using hydrochloric acid (HCl). The solution was mixed then topped up to a final volume of 1000mL in a volumetric flask. The PBS was left overnight to check the stability of the pH before use in degradation study.

2.2 Methods

2.2.1 Blend Preparation and Filament Fabrication

PDLGA polymer and biosilica bone filler (diatom biosilica and bioglass 45S5) were mixed homogeneously by hand after vacuum drying for individual components for two days. After blending, each prepared blend was dried for a further 1 day in vacuum oven under the same conditions before the filaments were extruded. The four filament groups extruded included: Pure PDLGA filament (Control), 1 wt.% Diatom-PDLGA filament, 5 wt.% Diatom-PDLGA filament and 5 wt.% Bioglass-PDLGA filament. This is similar concentrations used in previous studies that was reported to show better mechanical properties when compared to higher concentrations of 10 and 20%^{49,50}. In addition, small concentrations (typically up to 1.5%) of silica have been reported to induce a therapeutic response in response in bone⁵¹. A Haake

twin-screw extruder (Thermo Fisher Scientific, Germany) was used to produce filaments. The extrusion temperature varied between 165 °C to 175 °C at different stages of the extrusion process. All filaments were fabricated to a diameter of 1.75 mm which was required for the FDM 3D printer.

2.2.2 3D Printing Bone Scaffolds via Fused Deposition Modelling (FDM)

A RepRap Mendel (RepRapPro, UK) 3D printer was used to fabricate the bone scaffolds with a method similar to the one described by Little et al.,⁷. A standard architecture of scaffold was selected and designed using SolidWorks 2010, and then converted from STL to G-Code using opensource Slic3r 1.2.9. Printron (or Printerface) software was connected to the 3D printer to control the movement and amount of polymer filament extruded from the nozzle head ($\varnothing$ 0.4 mm) using G-Code. The printing temperatures and parameters used are summarised in Table 1. The pure PDLGA scaffolds were printed at 190°C, which was comparable with the previous study by Little et al.⁷. After the incorporation of diatom or bioglass 45S5, a trial and error approach was taken between 160 to 245°C. The optimum print temperature was determined according to scaffold size, shape and porous structure after printing, and ease of extrusion from print nozzle. In addition, the platform temperature on 3D printer was maintained at 60 °C throughout the print process. The scaffold was built up by 24 layers switching between printing in horizontal and vertical direction. For example, the polymer strips in the first bottom layer were printed along the X axis, and the second layer from bottom was printed along the Y axis. This cycle was repeated for 12 times to build up the scaffold. There was approximately 150 mm of filament used for each scaffold.

Table 1: FDM Parameters

Parameters	Values
Print Temperatures:	
Pure PDLGA Scaffold	190 °C
1 wt.% Diatom-PDLGA Scaffold	220 °C
5 wt.% Diatom-PDLGA Scaffold	225 °C
5 wt.% Bioglass-PDLGA Scaffold	165 °C
Print Details:	
Fan Speed	100 rpm
Print Speed	22 mm/s
Travel Speed	130 mm/s
Layer height	0.2 mm

2.2.3 Morphology Study on diatoms

To investigate the morphology of the *C. Mercehiniana* diatoms, scanning electron microscopy (SEM), atomic force microscopy (AFM), and transmission electron microscope (TEM) were used on HT diatoms. A FEI Quanta FEG – Environmental SEM from Oxford EX-ACT was used to image the diatoms. For imaging, a few frustules were spread on the carbon tab, which was attached on the SEM specimen stub. The SEM sample was then coated by AGAR Gold Sputter Coater for 40 seconds under an argon environment prior to imaging in the SEM. For the AFM, an Oxford Instrument Cypher ES Environment AFM was used. The sample was prepared by suspending a small number of diatom frustules in acetone in a microcentrifuge tube. Afterwards, the mixture was shaken by Grant Instruments™ PV-1 personal vortex mixer for a few minutes to disperse the diatom frustules into single particles. A dropper was then used to transfer the diatom suspension directly on to the AFM specimen disc. The acetone was left to evaporate off prior to imaging. The preparation of TEM sample was similar to the AFM. HT diatoms were spread on a TEM copper grid instead

of AFM specimen disc. The TEM used was a TF20D Tecnai transmission electron microscope manufactured by Philips.

2.2.4 Micro-Computed Tomography on scaffolds

Micro-CT was used to image the 3D structure of the scaffolds. The micro-CT scanner used in this study was a μ CT 40 from Scanco Medical with a sealed X-ray source (30kVp - 70kVp). The printed scaffold was placed in the sample holder and loaded in the machine to start the scanning process which took approximately one hour under 70kVp.

2.2.5 The Degradation Study on Scaffolds

For the long-term degradation study *in vitro*, each scaffold was immersed in 15 mL of PBS (method see 2.1.3) in a 15 mL metal free Falcon tube for 26 weeks. The timeframe of this study was selected based on previous literature on P(CL-co-LA)-PEG-P(CL-co-LA) scaffolds⁵². A PBS control without any scaffolds was included to assess the stability of the buffer. In this study one scaffold per group (x3 additional replicates) was added to each tube. At set timepoints, the PBS solution was removed and refreshed with new PBS. In addition, during the study, the PBS solution was refreshed every two weeks for all samples. The spent PBS solution was tested using ICP-OES and the cumulative Si ion release was calculated for each timepoint. There were four replicated samples at each time point to calculate the average change in pH and released silica with respect to time. The samples were incubated in a

Stuart shaking incubator SI500 oven at 37°C and were agitated at 70 RPM throughout the degradation study.

2.3 Characterisation

2.3.1 pH Change in Dissolution and Degradation Study

A Jenway 3510 pH metre was calibrated by reference buffers (pH 4, pH 7 & pH 10) before recording the pH change from PBS during the degradation study. The pH value from the degradation study was recorded after the samples were removed from oven and then cooled down to 20 °C to ensure the accuracy of the measurement.

2.3.2 Inductively Coupled Plasma -Optical Emission Spectrometry (ICP-OES) Analysis

To measure the released of Si^{4+} ion from the scaffolds, an Agilent 5100 ICP-OES instrument was used. Prior to analysis, the instrument was flushed with a 2% nitric acid (HNO_3) rinse solution and then calibrated with a bulk mixed element solution that was applied with different concentration (0.1ppm, 1ppm & 10ppm). Each sample was tested in triplet. The 2% HNO_3 rinse solution was also used at periodic intervals during analysis to ensure the accuracy. Additional information on the ICP-OES method are given in the supplementary information.

2.3.3 The Compressive Strength of Scaffolds

A Zwick/Roell Z100 instrument was used to test the compressive strength of scaffolds in the degradation study at the different timepoints. Samples were tested at weeks 1, 4 and 8. Beyond week 8 the samples became too fragile to handle. Prior to compression testing, the scaffolds were collected from the PBS solution using tweezers and wiped with a dry tissue to remove excess moisture. In addition, the original scaffolds (week 0), before the degradation study, were also tested as the control sample. All scaffolds were compressed to 0.5 strain with a strain rate of 2 mm/min, which means the scaffolds were forced to the half original height at the end of test. The preload was set to 150 N on the scaffolds with the exception of the last time point (8-week) which was set to 5 N due to their reduction in strength.

2.3.4 Degradation Analysis on Scaffolds via ^{13}C NMR Spectroscopy

^{13}C NMR was performed on the crushed scaffolds at the following timepoints: 0, 1, 4, and 8 weeks. Samples were also taken at weeks 12 and 16 which were too fragile to mechanically test but were suitable for NMR analysis. For each NMR spectra 40 mg of the scaffold powder was dissolved in 0.7 mL of deuterated chloroform (CDCl_3) for 2 hours at room temperature ($\approx 20^\circ\text{C}$) using the method described by Petisco-Ferrero *et al.*⁵³. The mixed solution was then analysed on a Bruker DPX400 NMR spectrometer.

2.3.5 Statistics Analysis

IBM SPSS version 25.0 was applied for the statistical analysis of variance (ANOVA) between different groups and factors in this study. Two-way ANOVA was performed on the silicon release and compressive strength data for the scaffolds. Tukey post-doc test was used for all statistical comparisons.

3 Results and Discussion:

3.1 Diatoms and scaffolds

The morphology of HT diatoms was studied with AFM, SEM and TEM. Fig. 1a shows the AFM images of a unicell diatom, which is approximately 8 to 10 μm in diameter. This result is in agreement with the average size of *C. Meneghiniana* diatom (6 – 18 μm) from previous studies^{54–56}. Both the AFM (Fig 1a) and SEM (Fig 1b) images show the elaborate patterns of perforations corrugate, and uniform waves are visible on the surface of diatom. A side view of a single diatom is shown in Fig. 1b from SEM. It is easy to observe the girdle band, which holds the two overlapping diatom valves together. Some tiny pores are also visible from the top valve with an undulating radial pattern. In addition, marginal spines are found at the edge of underside valve on the left side of the SEM image. Fig. 1c displays a fragment from diatom under TEM. Nano-holes ($\sim 50\text{ nm}$) are visible in a uniform pattern along the surface of the frustule. These nano-holes can control the diatom access to the external environment for processes such as waste removal and mucilage secretion⁴⁵. The darker region on the TEM (Fig 1c) indicate a denser material, which could be a thicker section of biosilica. Fig. 2 presents the 3D image of a template PDLGA scaffold after printing from micro-CT. The scaffold is an upright cylinder built up layer by layer with some regular porous structure inside. The average scaffold diameter was $12.5 (\pm 0.2)\text{ mm}$, with a height of $6.0 (\pm 0.1)\text{ mm}$. Each scaffold was found to be approximately $0.44 (\pm 0.02)\text{ g}$, and the width of the strut in the ranged of 0.34 to 0.42 mm. In terms of the porous structure, the ratio of the relative solid volume was found to be 0.35, which means the scaffold has more than half of its total volume taken up by porous space. Supplementary data (Fig. 1) shows the microstructure of

each scaffold type and indicative example of strut width. The distribution of the bioceramic filler within the composites materials is also shown in (Supplementary data, Fig. 2).

Fig. 1. (a) AFM images of diatoms, (b) SEM images of diatom biosilica frustules and valves, and (c) The fragment of diatom from TEM. Abbreviations of diatom features: (po) pore openings, (sp) spines, (gb) gridle band, (v) valve, (f) intact diatom frustule

Figure 2: Typical 3D printed scaffold from micro-CT

3.2 Degradation Study on Scaffolds

After 3D printing, a 26-week degradation study was performed on scaffolds. At each timepoint images were taken to visibly map their changes, shown in Table 2. Both 1 wt.% and 5 wt.% diatom-PDLGA scaffolds maintained their shapes and porous structure within the first 8 weeks. However, the shape of the pure PDLGA scaffolds and 5 wt.% Bioglass-PDLGA scaffolds became slightly distorted after 2-weeks and were tacky to the touch. After 10 weeks, the structure of pure PDLGA scaffold broke down completely, turning it into a viscous substance at the bottom of the tube. The 5 wt.% Bioglass-PDLGA scaffold kept its general shape but became very swollen and distorted before also breaking down at week 10. However, the diatom scaffold groups (1 wt.% and 5 wt.%) showed no obvious swelling and maintained their shape up to week 12. After two more weeks, all scaffold groups degraded into a viscous substance. At the end of the experiment in week 26, there was no polymer left in the pure PDLGA scaffold and 5 wt.% Bioglass-PDLGA scaffold groups. However, both diatom-PDLGA scaffold groups had some polymer residue.

Previous studies of PDLGA with a ratio of 85:15 lactic acid to glycolic acid reported complete degradation occurred between 12 to 16 weeks. Agrawal et al.,⁵⁷ reported a similar degradation period of 12 weeks *in vivo* that implanted PDLGA cylinder subcutaneously into the back of the male adult Wistar rats⁵⁸. Wu et al., reported a slightly longer degradation period of 16 weeks for pure PDLGA (85:15) scaffolds that were fabricated using a compression moulded/particulate leach technique in an cell free *in vitro* degradation study that was performed in PBS (pH 7.4) buffer solution⁵⁹. A detailed study on the hydrolytic degradation of PDLGA with different molecular weights and ratios of lactic to glycolic acid by

Robles-Bykbaev et al., found that degradation was strongly linked to carboxylic group formation, although the 85:15 ratio was not included in their study²². Other factors also influencing the degradation processes included the increase in chemical interactions between the hydrolytic groups, the scaffolds specific surface area²⁶ and the molecular weight of PDLGA (85:15)⁶⁰. The addition of ceramic material has also been found to influence the degradation of PDLGA. A study by Ara et al, found the addition of calcium carbonate and calcium phosphate to PDLGA decreased the rate of degradation of PDLGA⁶¹. A similar finding was observed in our study whereby both diatom-PDLGA and Bioglass-PDLGA scaffolds took longer to degrade (Table 2).

Table 2: Images of the scaffolds in PBS buffer solution during the 26-degradation study.

Duration	Pure PDLGA Scaffold	1 wt.% Diatom-PDLGA Scaffold	5 wt.% Diatom-PDLGA Scaffold	5 wt.% Bioglass - PDLGA Scaffold
2 Weeks
8 Weeks
10 Weeks
12 Weeks
14 Weeks
16 Weeks
20 Weeks
26 Weeks

Fig. 3 shows the pH change during the degradation study. The results found that the control sample (PBS buffer without scaffold) was found to stay stable at pH 7.4 throughout the experiment with little fluctuation. The results from the pure PDLGA scaffold group displayed a significant pH drop at week 8 and 14 to $7.09 (\pm 0.12)$ and $2.35 (\pm 0.12)$ respectively (Fig. 3a), and then recovered to 7.4 in week 24 due to the refreshment of PBS solution every two weeks. The acidic lowering of pH is a result of the degradation of the by-products of lactic acid and glycolide acid. This drop in pH value is comparable to those reported in other studies during PDLGA degradation^{62,63}. Farahani et al., reported that the pH drop was partly related to the release of the lactic acid groups into the local environment⁶⁴. Both diatom-PDLGA scaffold groups displayed a similar pH change with respect to time (Fig. 3b & 3c). The significant pH change started at week 10, as opposed to week 8 like pure PDLGA scaffolds. After week 10, the pH level of PBS solution dropped to $2.32 (\pm 0.02)$ and $2.36 (\pm 0.01)$ from 1 wt.% and 5 wt.% diatom-PDLGA scaffold respectively, until week 18, before recovering to pH 7 in week 24. The lowest pH recorded from diatom-PDLGA scaffolds was similar with pure PDLGA scaffold, which means diatom biosilica did not affect pH environment itself. The acidic condition was caused by the degradation of PDLGA polymer to lactic acid and glycolic acid. However, the addition of diatoms to PDLGA appears to delay the acid burst release by two weeks, suggesting that the addition of the ceramic played a role in inhibiting degradation. This supports findings by Ara et al, as discussed earlier⁶¹. 5 wt.% Bioglass-PDLGA scaffolds, however, turned slightly alkaline increasing in pH to $8.84 (\pm 0.69)$ in the first two weeks. Previous dissolution studies of 45S5 bioglass have shown similar trends, and have been reported to increase to a pH between 8.7 or 9.7 in the aqueous solutions⁶⁵. It is most likely that a reaction is started due to the ionic exchange between Ca^{2+} & Na^{+} ions from Bioglass with the H^{+} and H_3O^{+} ions from the PBS solution, which causes an increase in

pH. Fiume *et al*, study also reported the release of soluble silica from 45S5 Bioglass to form of silanol bonds (Si-OH) that increase the pH of solution.⁶⁶

Fig. 1. The pH changes from 3D printed scaffolds in the degradation study (a) Pure PDLGA Scaffolds, (b) 1 wt.% Diatom-PDLGA Scaffolds, (c) 5 wt.% Diatom-PDLGA Scaffolds, (d) 5 wt.% Bioglass-PDLGA Scaffolds (n=4)

The cumulative ICP data for Si ions over the 26-week degradation study is shown in Fig. 4. The PDLGA scaffolds with 5 wt.% Bioglass exhibited the fastest initial release of Si ions in comparison to both diatom-PDLGA scaffolds. After one week, approximately 28.88 (± 17.36) ppm Si ions were released into the PBS solution from 5 wt.% Bioglass-PDLGA scaffolds. At week 2 the burst release continues, releasing approximately 100.30 (± 35.87) ppm of Si ions into the PBS solution. Thereafter, the release rate of silica was found to slowly increase

linearly up to $169.43 (\pm 14.73)$ ppm in week 20 and $225.94 (\pm 5.70)$ ppm in week 26, where $p > 0.5$. This is accounted for by the likelihood of some bioglass particles being exposed at the surface of the scaffold, so dissolving in the first two weeks, as a burst release, with later release being from bioglass particles that are entrapped within the polymer.

The 5 wt.% diatom-PDLGA scaffold group displayed an increasing rate of Si ion release during the degradation study with no initial burst release, suggesting that the majority of the diatom particles were contained within the polymer rather than exposed at the surface. The concentration released was found to be significantly lower compared to the 5 wt.% Bioglass-PDLGA scaffolds, until week 24 when a significant ($p = 0.19$) increase in Si ions release occurred from $94.19 (\pm 9.73)$ ppm in week 22 to $199.84 (\pm 8.77)$ ppm. In the first week, only $0.58 (\pm 0.02)$ ppm Si ions were released into the PBS solution. However, week 26 recorded a $272.17 (\pm 10.94)$ ppm level of Si ions, which was the highest concentration of ions released between the silica scaffold groups. The 1 wt.% diatom-PDLGA scaffolds displayed a similar increasing rate of Si ions release to that of 5 wt.% diatom-PDLGA scaffold groups, however, at a lower concentration. The maximum release of Si ions in this group was found to be $69.88 (\pm 2.22)$ ppm at the end of degradation study. Both diatom-PDLGA scaffold groups have an initial slower steady release rate of Si ions compared to the Bioglass-PDLGA scaffold groups, with a later near-exponential increase. These differing temporal characteristics in release rate may relate to the differing solubility of bioglass when compared to diatoms (e.g. pH) and/or different physical characteristics, such as specific surface area (SSA), crystallinity and particle size. Previous studies have reported on the significant pH increase in bioglass after initial immersion into PBS solution due to the ion exchange between Na^+ and Ca^{2+} ions in the glass and the H^+ protons in the PBS, which accordingly increases the ionic concentration of the solution^{67,68}. It is likely that this spike in

pH, which is also evident in our results (Fig 3d) contributed to accelerating the degradation/hydrolysis of PDLGA as the buffer solution turned alkaline at a pH just below 9. Polymer degradation is known to accelerate in both alkaline and strong acids conditions⁶⁹. In terms of the physical properties of the fillers, Walsh et al.,⁴¹ reported the SSA of *C. meneghiniana* to be $\sim 33.67 \text{ m}^2 \text{ g}^{-1}$ whereas Bioglass 45S5 has been reported to be significantly lower in the range of 0.15 to $2.7 \text{ m}^2 \text{ g}^{-1}$ ⁷⁰. In theory the higher SSA should dissolve faster, however, the fillers are not directly exposed to the buffer solution, they have been blended with polymers, then melted and formed into polymer filaments. Therefore, the higher SSA may have resulted in a better bond between the polymer and ceramic phase between the diatoms and PDLGA resulting in a slower release rate in the diatom filaments. The desired silica release profile to induce a therapeutic effect in bone still largely unknown in the literature. A review study by Khandmaa *et al.* concluded that the release of 16 – 20 ppm Si ions from biomaterials could stimulate bone repair⁷¹. This was supported by a study by Ruangsuriya *et al.* who found that a concentration of 500 μM (≈ 14 ppm) of Si ions could increase the gene expression associated with osteocalcin activity⁷². Pingping *et al.*⁷³ study also showed that low concentrations of Si ions of 0.625 mM (≈ 17.5 ppm) could significantly enhance the proliferation, mineralisation, bone-related gene expression and bone matrix proteins of bone marrow stromal cells (BMSCs). Silicon in bone has been reported in the range of 100 to 150 ppm and 200 – 550 ppm in extracellular bone^{36,74,75}.

Fig. 2. The accumulated silica release from 3D printed scaffolds during the degradation study (n=4)

3.3 Compression Test on Scaffolds

The mechanical properties were studied under compression testing that was performed on the scaffolds at the earlier time points. Typical stress-strain curves from week 0, 1, 4 and 8 are shown in Fig. 5. Note scaffolds could not be tested for the duration of the study, as they became too difficult to handle. Fig. 5a shows a typical stress-strain curves of the original scaffolds prior to immersion into PBS buffer solution. The pure PDLGA scaffold (blue line) exhibited the largest yield stress, just over 20 MPa, with a Young's modulus of 470 MPa. 5 wt.% Bioglass-PDLGA scaffold (red line) had slightly lower mechanical properties with a yield stress of 18 MPa, compressive stress of 43 MPa and a Young's modulus of 435 MPa. Both diatom-PDLGA scaffolds (1 wt.% - purple line and 5 wt.% - green line) exhibited a similar yield stress of 12 MPa and compressive stress of between 30 – 33 MPa. The Young's

modulus was found to be significantly higher at 260 MPa in 5 wt.% diatom-PDLGA, compared to 1 wt.% diatom-PDLGA scaffold was found to be 190 MPa. Compared with the reference value of natural cancellous bone (2 to 45 MPa)^{76,77}, the compressive strength from the original scaffolds could meet the requirement for cancellous bone.

Fig. 3. Typical compression curves from 3D printed scaffolds after (a) 0 week, (b) 1 week, (c) 4 weeks, (d) 8 weeks. Blue line (pure PDLGA scaffold), red line (5 wt.% Bioglass-PDLGA scaffold), purple line (1 wt.% diatom-PDLGA scaffold) and green line (5 wt.% diatom-PDLGA scaffold).

After one week's immersion in PBS solution, all the scaffolds showed a significant reduction in compressive strength but followed the same general trend as week 0 (Fig. 5b). After week 4 (Fig. 5c), pure PDLGA scaffold and 5 wt.% Bioglass-PDLGA scaffolds showed a significant drop in stress at 0.06 and 0.11 strain respectively, whereas both diatom-scaffold concentrations followed the same trend but with a drop in overall compressive strength. At

week 8 (Fig 5d.) the compressive strength of all scaffold groups was significantly compromised, however, both diatom groups maintained measurable compressive strength (1.5 MPa) up to approximately 0.1% strain.

Fig. 4. The results from Compression test (a) Average Young's modulus, (b) Average yield stress

A summary of the compressive data for each scaffold group is shown in Fig 6. The results showed that 1 wt.% diatom-PDLGA scaffold and 5 wt.% diatom-PDLGA scaffold maintained their Young's moduli and yield strength up to 8 weeks immersion in PBS solution. Whereas, both pure PDLGA scaffold and 5 wt.% Bioglass-PDLGA scaffold groups were very fragile to test beyond week 4. Prior to immersion of the scaffolds into PBS solution (0-week), pure PDLGA scaffold exhibited the largest average Young's modulus at 466 (± 22) MPa, followed by 5 wt.% Bioglass-PDLGA scaffold with a Young's modulus of 434 (± 51) MPa. The addition of diatoms however, both 1 wt.% and 5 wt.% was found to significantly reduce the Young's modulus to 186 (± 40) MPa and 264 (± 34) MPa respectively compared to the pure-PDLGA scaffold group. However, after week 4 the 5 wt.% Bioglass-PDLGA scaffold groups became

the weakest scaffold group with the Young's modulus dropping to $34 (\pm 13)$ MPa. The 5 wt.% Bioglass scaffold groups demonstrated the fastest reduction to 90% of the Young's modulus in all four groups tested in just 4 weeks. This results are also comparable with a previous study by Liu et al., who reported the mechanical response of bioglass scaffolds rapidly changed within 4 weeks⁷⁸.

Fig. 6b, the average yield stress data from the compression test, showed a similar trend to the average Young's modulus. Diatom-PDLGA scaffolds maintained some yield stress up to week 8, whereas, the pure PDLGA scaffold and 5 wt.% Bioglass-PDLGA scaffold only maintained its yield stress up to week 4. This was found to be a statistically significant change between week 4 and 8 ($P = 0.78$). Both diatom-PDLGA scaffold groups kept their shapes during the compression test up to week 4 whereas, the pure PDLGA scaffold and 5 wt.% Bioglass-PDLGA scaffold had no meaningful data after week 1. The addition of diatom biosilica at concentrations of 1 wt.% & 5 wt.% did not initially enhance the compressive strength of PDLGA when compared to the pure PDLGA control. However, both diatom-PDLGA scaffolds maintained their mechanical strength for a long period of time, which is advantageous in bone repair, as bone tissue requires 12 weeks to heal^{79–81}. This is likely to be a result of the diatom biosilica filler which in a previous study by Han *et al.*, were found to inhibit thermal degradation of the PDLGA.

3.4 Degradation Study via NMR

^{13}C NMR analysis was carried out on scaffold solutions to investigate the change in the polymer at a molecular level during degradation. Fig. 7 shows the ^{13}C NMR spectra of the different scaffold groups with respect to time.

In the monomeric form the three carbon atoms in the structure give characteristic chemical shifts at 20 ppm, 66 ppm and 179 ppm displayed in Table 3. The chemical shifts observed herein correspond well to those observed previously in the literature⁸¹. There is a wide variation in electronegativity across the molecule which gives rise to very distinct chemical shifts. When in the polymeric form there is a slight change in the electron densities across the molecule and therefore a shift in ppm can be observed upon polymerisation or breakdown. This phenomenon makes ^{13}C NMR an ideal tool for observing polymeric break down at the molecular level.

Table 3. Structure and respective chemical shift measured in ppm of key lactic acid monomeric and polymeric ^{13}C peaks.

Structure	Chemical shift (ppm)
20
66
179
16
69
169

Resonances at 168 ppm and 60 ppm are attributed to the carbonyl and β -C peak in the glycolic acid components of the polymer chain, which has been shown previously by Essen *et al.*,⁸². The peaks corresponding to the lactic acid monomer started to become apparent in week 8 for the pure PDLGA scaffolds. This corresponds well with the drop in pH observed after 6 weeks shown in Figure 3. In contrast, the analysis of diatom-PDLGA and Bioglass-PDLGA did not show peaks for the monomeric units until week 16. This would suggest the monomer lactic acid was released from the PDLGA polymer chain after 8 weeks in PBS solution for pure PDLGA scaffold groups, whereas the addition of diatom and Bioglass groups the silica appears to inhibit the release lactic acid until week 16. This supports earlier

data Table 2 and Fig. 3 on pH/silica ion release that the addition of the diatoms delayed the onset of degradation compared to pure PDLGA. Both bioglass and diatom silica have distinctly different material attributes, e.g. surface area, pore size, yet both silica groups have been shown to inhibit the release of lactic acid from PDLGA. The most plausible explanation is hydrogen bonding between functional group/polar atoms in the PDLGA and the surface of the silica. Zhou et al.,⁸³ reported similar bonding interactions between dry composites of PDLLA and HA that were moulded together. They observed hydrogen bonding between C=O groups in the PDLLA with the surface P- OH groups in the HA. However, to the best of our knowledge there is little literature on the bonding mechanism between PDLGA and Silica. To better understand the chemical interactions occurring, further research is required, however, these results clearly show that the addition of silica helps to slow the degradation rate of PDLGA, which is useful on bone tissue applications that requires up to 12 weeks to heal⁷⁹⁻⁸¹.

Fig. 5. ^{13}C NMR spectroscopy during the degradation study on (a) the skeletal formula of PDLGA polymer chain & monomer lactic acid (b) Pure PDLGA Scaffolds, (c) 1 wt.% diatom-PDLGA Scaffolds, (d) 5 wt.% diatom-PDLGA Scaffolds, (e) 5 wt.% Bioglass-PDLGA Scaffolds

4 Conclusion

Diatom biosilica and Bioglass 45S5 PDLGA scaffolds were studied in PBS buffer to assess their Si release and compressive strength during in vitro degradation. The results suggested that diatom addition to PDLGA scaffolds reduces scaffold degradation whereby structural decomposition occurs up to 4 weeks later than pure PDLGA scaffolds. In term of the release rate of Si^{4+} ions, the concentration of Si^{4+} ions released from 5 wt.% Bioglass-PDLGA scaffold was significantly higher than both diatom-PDLGA scaffolds (1 wt.% & 5 wt.%), at comparable time points. For the bioglass composite the early burst release at week 2 may be a risk factor for bone regeneration. The incorporation of diatom biosilica exhibited a slower release rate on Si^{4+} ions than 45S5 Bioglass from scaffolds. In addition, compression tests demonstrated that the compressive strength from diatom-PDLGA scaffolds could be maintained longer than pure PDLGA scaffold and 5 wt.% Bioglass-PDLGA scaffold, which is advantageous in bone repair. This prolonged compressive strength is mostly a result of the silica ions inhibiting the degradation of the PDLGA. For scaffolds it is critical that strength is maintained until tissue has generated and developed structure to the point where the scaffold support is no longer required.

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Author Statement

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RH, FB, PJW - Conceptualization; RH - Data curation; RH, PJW - Formal analysis; MJ - Statistical analysis, RH (Self-funded PhD with 1-year Beaufort Marine Biodiscovery Programme - FB) Funding acquisition; RH, FB, PJW - Methodology; RH Writing - original draft; All authors - Writing - review & editing.

Highlights

1. The compressive strength of Diatom–PDLGA scaffolds were found to maintain their strength for longer than either pure PDLGA scaffolds or 5 wt.% Bioglass scaffolds.
2. Chemical analysis using ^{13}C NMR showed that scaffolds with diatom silica had less degradation than pure or bioglass-containing scaffolds at comparable time-points.
3. The release rate of Si^{4+} ions from diatom-PDLGA scaffolds was found to increase exponentially with respect to time