A 3D Bioprinted In Situ Conjugated-co-Fabricated Scaffold for Potential Bone Tissue Engineering Applications

Mduduzi N. Sithole, Pradeep Kumar, Lisa C. du Toit, Thashree Marimuthu, Yahya E. Choonara, Viness Pillay*

Wits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 7 York Road, Parktown, 2193, South Africa

*Corresponding Author
Professor Viness Pillay
Tel: +27-11-717-2274
Fax: +27-11-642-4355
Fax2Email: +27-86-553-4733
Email: viness.pillay@wits.ac.za

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Abstract

There is a demand for progressive approaches in bone tissue engineering to repair and regenerate bone defects resulting from trauma or disease. This investigation sought to engineer a single-step in situ conjugated polymeric scaffold employing 3D printing technology as an innovative fabricating tool. A polymeric scaffold was engineered in situ employing sodium alginate as a bio-ink which interacted with a poly(ethyleneimine) solution on bioprinting to form a polyelectrolyte complex through ionic bond formation. Silica gel was included in the bio-ink as temporal inorganic support component and for ultimate enhancement of osteoinduction. Characterization of the biorelevant properties of the scaffold was undertaken via Fourier Transform Infrared Spectroscopy, Differential Scanning Calorimetry and Thermogravimetric Analysis, X-Ray diffraction, Scanning Electron Microscopy, and biomechanical testing. The scaffold maintained its 3D architecture for the duration of the 28 day degradation investigation, while potentially permitting the infiltration of nutrients, growth factor and cells evident by the increased solvent penetration into the scaffold observed via Magnetic Resonance Imaging studies. The scaffold porosity and pore size were found to be 60% and 360µm, respectively. Biomechanical evaluation revealed a Young’s modulus of 18.37MPa highlighting that the scaffold in its current form possesses the mechanical capabilities for certain bone tissue engineering applications. This investigation provided highlighted the applicability of alginate-poly(ethyleneimine)/silica for 3D bioprinting as a scaffold which could possess potential as a bone tissue engineering scaffold.

Keywords: Bone tissue engineering, 3D Printing, scaffold, polyelectrolyte complex
1. Introduction

The traditional repair treatments for critical-sized or non-union bone defects due to trauma or disease have centered on orthopedic implants, allografts, and autografts. However, these techniques suffer from a number of limitations \(^\text{(1-2)}\) such as lack of available donors and inadequate biomechanical properties \(^\text{(3)}\). Hence, bone tissue engineering employing scaffolds has emerged as an innovative and growing field of research in tissue engineering as an alternative technique to overcome the limitations of current approaches \(^\text{(2)}\). Tissue engineering techniques involve the use of an appropriate scaffold biomaterial, relevant growth factor and/or cells \(^\text{(4-5)}\).

Polymers have been investigated as biomaterials for a range of medical applications due to their biological properties \(^\text{(6)}\). Major breakthroughs in tissue engineering is owing to the success in development of novel biomaterial-based approaches that simulate native organ and tissue parts \(^\text{(7)}\). Manipulation of materials and their biorelevant characterization has been the fundamental to their application in tissue engineering \(^\text{(8)}\).

The properties of the scaffold depend mainly on the fabrication methods and the nature of the biomaterial \(^\text{(6)}\). Two-dimensional (2D) patterns of biochemical and mechanical cues have been generated through the development of numerous intricate methods, but for many cell types, the 2D culture condition may not be appropriate for propagating tissue regeneration. Additionally, 2D fabrication methods cannot be easily transformed into 3D methods, hence, there is a need for more progressive methods to precisely capture the intricate 3D cellular environment \(^\text{(7)}\). Scaffolds with controlled surface chemistry, pore size distribution, pore volume, pore interconnectivity and architecture cannot be fabricated with traditional processing.
techniques\textsuperscript{(9-10)} such as solvent casting or particle leaching, freeze-drying and gas foaming\textsuperscript{(7)}. Therefore, additive manufacturing processing technologies such as 3D printing have been explored as an innovative tool since 1988 for the production of 3D structures, such as medical devices and tissue engineering scaffolds\textsuperscript{(11)}. The advantages of 3D printed scaffolds for bone tissue engineering include the fabrication of a well-defined architecture with patient specific geometries as well as enabling the necessary spatial organization (e.g. of bioactives or cells) within the scaffold for enhancing biological functionality\textsuperscript{(1)}. However, appropriate material selection is a major challenge in bioprinting. This could be addressed through conceptualizing tunable bio-inks with a range of material properties; this may be achieved through creation of novel composite mixtures to incorporate desirable features or properties\textsuperscript{(12)}. This approach is enabled via 3D printing which allows multiply biomaterials to be simultaneously plotted\textsuperscript{(12)}. During 3D printing of hydrogel scaffolds, further co-fabrication approaches, such as chemical or physical crosslinking reactions, could be implemented to influence the 3D structural integrity and properties\textsuperscript{(13)}.

In considering the biomaterial composition of a bone scaffold it must be noted that bone is a nanostructured inorganic-organic composite (nanocomposite) or multiphase solid material. Alginate has been actively explored in the fabrication of scaffolds due to its capability to enable tissue and organ regeneration\textsuperscript{(14-19)}. For the fabrication of scaffolds using alginate, traditional methods such as electrospinning, crosslinking and lyophilization have been extensively studied. The most commonly employed traditional method for the production of alginate biopolymer scaffolds is via lyophilization\textsuperscript{(20)}. Alginate displays distinct efficacy due its high water absorbing
ability, biodegradability and high biocompatibility. Hence, it has been widely studied for 3D cell culture, tissue regeneration and drug delivery \((21)\). Modification of alginate via diverse approaches is also possible, and a requirement for enhancement of its biomechanical properties for tissue engineering applications. Sun et al. \((22)\) provided an in depth review of alginate hydrogels for regenerative medicine applications through click reaction, free radical polymerization, cell crosslinking, phase transition and ionic crosslinking \((20),(22)\). Polyethyleneimine (PEI) is a branched polycationic polymer that contains a high density of ionizable tertiary, secondary and primary amino groups and has a strong interaction with proteins. It has been employed widely in tissue engineering and promotes cell growth. It has also been utilized to enhance protein loading on solid surfaces and it has been introduced onto the surfaces of organic or inorganic solid spheres using techniques such as covalent grafting, spray drying, electrostatic adsorption and layer-by-layer assembly. Murakami et al. \((23)\) produced PEI-coated hydroxyapatite with various fractions of PEI via a spray drying technique. Further, Xia et al. \((24)\) employed a layer-by-layer technique to produce chemically crosslinked PEI using glutaraldehyde. Therefore, PEI can form a polyelectrolyte (PEC) layer following electrostatic adsorption on a negative substrate \((25)\), such as alginate to create a complex with potentially enhanced capabilities. Silica gel is an exemplary inorganic component commonly included in bone scaffolds. Bioactive materials containing silicon species have demonstrated enhancement of osteogenesis. Silica could thus be included in the bio-ink for improved bone tissue biosimulation.

3D printing technology, as discussed, holds great potential, but there are some important limitations that need to be overcome. There is a great need for an
increased diversity of printable biomaterials for the synthesis of bioarchetypes and biodegradable polymeric biomaterials that will fulfil the requirements of tissue repair, regeneration, restoration and reorganization (26-27). This study proposed to develop a novel bioarchetype employing 3D printing technology as an innovative co-fabrication tool for single-step conjugation of scaffolds. A polymeric scaffold was engineered in situ employing sodium alginate as a bio-ink which interacted with PEI to form a polyelectrolyte complex while the scaffold was simultaneously bioprinted, with inclusion of silica gel as a temporal inorganic bone tissue supporting component for bioarchitectural simulation of bone tissue. Physicochemical and physicomechanical characterizations were undertaken to assess the potential of the scaffold for bone tissue engineering. Ultimately, this research proposes a novel approach for bio-ink design and scaffold bioprinting to address the limited availability of printable biomaterials for bone tissue engineering applications.

2. Material and Methods

2.1. Materials

Sodium alginate (NaAlg, Product number: W201502, sodium alginate), demonstrated to be suitable for biofabrication of 3D hydrogels (28), was obtained from Sigma-Aldrich, Gillingham, UK. Silica gel (Si, $M_w = 60\,000$), poly(ethyleneimine) solution (PEI, $50\%_w/v$ in water, $M_w = 750\,000$), Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal bovin serum (FBS), ethanol, deionized water and additional reagents were purchased from Sigma-Aldrich (Cleveland, OH, USA). Chemicals were utilized without further purification unless stated.
2.2. Fabrication of the 3D bioprinted *in situ* conjugated-co-fabricated scaffold bioarchetypes

The Alg-PEI/Si scaffold was engineered as follows: Sodium alginate (200mg/mL) was prepared as a component of the bio-ink following hydration in ultra-filtrated purified deionized water (Millipore, France). Silica gel (200mg/mL) as an inorganic component was combined with the sodium alginate hydrogel. Separately, PEI solution was prepared by adding 40mL of PEI to 100mL of ethanol. A 3D Bioplotter® (EnvisionTEC, GmBH) was employed to engineer an Alg-PEI/Si 3D porous scaffold.

The 3D printing of these polymeric scaffolds utilized computer aided design (CAD) to create the desired structure to be printed. The importing software of the STL data and the control of the 3D Bioplotter® consisted of two modules: i) the 3D Bioplotter® software for STL data importation and structure (e.g. scaffold) fabrication; and ii) the VisualMachine software for material parameters and machine control. The scaffold was printed at a temperature of 37°C where the Alg/Si Bio-ink was fed into a 30cc cartridge and dispensed through a plastic needle (nozzle diameter 0.41mm), under a pressure of 0.5Bar and deposition speed of 8mm/s into a petri dish containing the solidifying solution of PEI dissolved in ethanol. The layers were deposited at 90 degree angle to the underlying layer in PEI solution. Layers were successively printed with the average solidification time per layer noted. The scaffold was printed using parameters as described in Table 1. The bioprinted scaffold was subsequently washed thrice with double deionized water and dried to constant weight at room temperature (25°C). Table 2 provides visual results of the solidification behavior of the various preliminary solidified scaffolds evaluated, where deionized water and PEI, ethanol and PEI, or ethanol alone were investigated as the solidifying solutions. The preferred scaffold was identified and subjected to further characterizations.
2.3. Determination of the chemical transitions of the 3D bioprinted scaffolds and native biomaterials

The chemical transitions of the scaffold fabricated employing the 3D Bioplotter® (EnvisionTEC) and the native materials used were determined utilizing a Fourier Transform Infrared (FT-IR) spectrometer (PerkinElmer Spectrum 100, FT-IR Spectrometer) fitted with a universal ATR Polarization Accessory (PerkinElmer Ltd., Beconfield UK). The samples were weighed (1-3mg) and analyzed at high resolution with the wavelength ranging from 4000-650cm⁻¹ on a Nicolet Impact 400D FT-IR Spectrophotometer coupled with Omnic FT-IR research grade software (Nicolet Instrument Corp., Madison, WI, USA) and the presence and absence of specific functional groups were evaluated.

2.4. Thermal transition analysis of the 3D bioprinted scaffold

The thermal properties of all the native materials and the bioprinted scaffold were ascertained via differential scanning calorimetry (DSC) (Mettler Toledo, Schwerzenbach, Switzerland). Dried samples of 5-10mg were weighed into aluminium crucible pans (40µL) and a small (0.2mm) hole in aluminium lid was induced to create an open system. The samples were heated under nitrogen atmosphere (Afrox, Germiston, Gauteng, South Africa) with 200mL/min flow rate acting as the purge gas to decrease oxidation. The samples were exposed to a
temperature range between 25°C and 400°C at the rate of 10°C/min and the thermal profiles were analyzed.

2.5. Analysis of the thermal decomposition of the 3D bioprinted scaffold

A thermogravimetric analyzer (TGA 400, PerkinElmer Inc., MA, USA) was used to analyze the thermal decomposition of the newly formed scaffolds and the native materials. Samples (10-20mg) were placed in a ceramic pan under nitrogen gas atmosphere. Thermograms were recorded in a range of 30°C to 900°C and revealed the thermal decomposition of the materials.

2.6. Characterization of the 3D bioprinted scaffold swelling and structural integrity transitions

The swelling and structural integrity behavior of the 3D bioprinted scaffold matrices was imaged using a 0.5T open configuration Magnetic Resonance Imaging (MRI) system (Signa SP, General Electric Medical Systems, Milwaukee, WI, USA). The dried scaffold was placed in a prepared phosphate buffered saline (pH 7.4) and positioned within the flow equipped with 0.5T permanent magnet system, stabilized at 37°C. After duly configuring, optimizing the shims and probe tuning, the cone-like lower part of the cell was filled with glass beads to provide laminar flow at 16mL/min of the solvents employed. The scaffold was placed in position and magnetic resonance images were taken at set time intervals with MARAN-I version 1.0 software. The image was acquired after setting the frequency offset and testing gain employing RINMR version 5.7 under continuous solvent conditions. MARAN-I software comprises image acquisition software and image analysis software. MR images were acquired over 24 hours.
2.7. Surface morphology analysis of the 3D bioprinted scaffold

Surface morphology of the fabricated scaffold was examined utilizing scanning electron microscopy (SEM) FEI Nova NanoLab SEM (FEI Company, Hillsboro, Oregon, USA). Gold isotope was used to sputter coat the samples while the sample was mounted on the aluminium spud with an EPI coater (SPI Model sputter-coater and control unit, Hester, PA, USA). After coating the samples for 60s, under constant nitrogen gas conditions, the samples were analysed using a FEI Nova NanoLab SEM (FEI Company, Hillsboro, Oregon, USA).

2.8. Porositometric determination of the 3D bioprinted scaffolds

The 3D plotted scaffold porosity was calculated from the microscopic images of the scaffolds using (Equation 1), in accordance with the theoretical approach of Landers et al. (29):

\[
P = 1 - \frac{V_{\text{scaffolds}}}{V_{\text{cube}}} = 1 - \frac{\pi}{4} \cdot \frac{(d)^2}{d_1 \cdot d_2}
\]

(Equation 1)

Where:

- \( P \) = scaffold porosity,
- \( d \) = scaffold fiber diameter,
- \( d_1 \) = scaffold fiber spacing and \( d_2 \) = layer thickness within each different structure (30).

2.9. Comparative degradation rate analysis of the 3D bioprinted scaffolds

The degradation rates of the bioprinted Alg/Si and Alg-PEI/Si scaffolds were compared. UV-light was used to sterilize the pre-weighed dry scaffolds, which were
subsequently soaked in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovin serum (FBS), and incubated in an atmosphere of 5% CO$_2$ at 37°C (pH 7.4) to simulate the environment in a living organism. Scaffolds were removed at various time intervals and weighed after being dried. The medium was replaced every second day since the degraded residues may contaminate the scaffold because in a living organism degraded residues are eliminated through different processes such as urinating amongst others and the study was performed for a period of 28 days $^{(30)}$. The degradation rate was calculated according to Equation 2 as follow:

$$\text{Degradation rate} \% = \frac{W_0 - W'}{W_0} \times 100$$  \hspace{0.2cm} \text{(Equation 2)}$$

Where:

$W'$ = final weight of the scaffold

$W_0$ = initial weight of the scaffold

### 2.10. Determination of the biomechanical properties of the 3D bioprinted scaffold

The scaffold mechanical properties were measured utilizing a BioTester 5000. This enabled Biaxial testing of the scaffold which is critical for investigating the mechanical properties of biomaterials due to their directionally oriented microstructures. The BioRankes system permits the mounting of samples tissue into the BioTester. The fabricated scaffold was equilibrated by first soaking it in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovin serum (FBS) and incubated at an atmosphere of 5% CO$_2$ at 37°C (pH 7.4) for 24 hours. The sharp BioRankes pierces the most delicate and tough samples as to
afford a constant dispersing site of attachment across the shape of the samples. Following positioning of the samples, a manually-functioning crown press exerted pressure onto the sample.

3. Results and Discussion

3.1. Preliminary evaluation of the 3D bioprinted scaffold

As depicted in Figure 1, a novel polyelectrolyte complex (PEC) scaffold was designed and printed using a 3D Bioplotter® (EnvisionTEC). PEI was the cationic polymer of choice in this investigation, owing to its potential to interact with anionic functional groups to form complexes. Sodium alginate was a favorable anionic polymer selection, since it contains very reactive anionic functional groups (COO\(^-\)), rendering ionic bonding through electrostatic interaction between the positively charged amino groups of PEI with the negatively charged COO\(^-\) of sodium alginate possible.

3D bioprinting highlights a novel application for the Alg-PEI PEC incorporating silica. The challenge was to investigate a biologically nontoxic solvent that would dissolve PEI, which upon contact with NaAlg/Si bio-ink, would cause it to undergo solidification while enabling the interaction of NaAlg with PEI. Stable solidification is required to enable the next layer to be printed on top of the previous layer, implicit to this layer-by-layer fabrication approach. Ethanol was found to be the solvent of choice because it is nontoxic as well as enabling in situ co-fabrication of the PEC with solidification of the biopotted strands. When water, however, was employed as solvent it did not effectively form or maintain a solid structure for the subsequent layer to be printed upon.
3.2. Chemical transition evaluation of the bioprinted scaffold

The 3D bioprinted scaffolds composed of sodium alginate; PEI and silica gel were successfully fabricated using 3D Bioplotter® (EnvisionTEC). Its fabrication was validated through various techniques as discussed below.

The FTIR spectrum in Figure 2a depicted a significant band at $1054\text{cm}^{-1}$ which is indicative of Si-O-Si in the spectrum of pristine silica gel. This band is overlapped by other vibrational modes in the FTIR spectrum of the bioprinted scaffold (Figure 2d). Figure 2b represents the spectrum of pristine sodium alginate, which displayed a broad stretch at $3500\text{cm}^{-1}$ to $3000\text{cm}^{-1}$ associated with O-H stretching and two distinct peaks at $1595\text{cm}^{-1}$ and $1397\text{cm}^{-1}$ which are attributed to the asymmetrical and symmetrical vibration of COO$^-$ functional groups, respectively. The vibration stretch peak at $1021\text{cm}^{-1}$ is associated with C-O-C functional groups $^{(31)}$.

Figure 2c represents the pristine PEI FTIR spectrum, which possessed the following significant bands: the two peaks at $3345$ and $3287\text{cm}^{-1}$ arise from NH$_2$ stretching vibration; the strong peaks at $2945$ and $2844\text{cm}^{-1}$ are associated with asymmetrical and symmetrical vibrations of CH$_2$ groups, respectively; and the peaks at $1599\text{cm}^{-1}$ and $1645\text{cm}^{-1}$ are associated with N-H bending. The peak at $1465\text{cm}^{-1}$ is associated with CH$_2$ bending and the peak from $1311\text{cm}^{-1}$ to $1000\text{cm}^{-1}$ is associated with C-N stretching $^{(32, 33)}$. 
Figure 2d depicts the spectrum of the bioprinted polymeric scaffold (Alg-PEI/Si). There are still two bands at 2948 cm\(^{-1}\) and 2811 cm\(^{-1}\) that are associated with CH\(_2\) stretching vibrations, originating from the presence of PEI; in addition there is a peak at 1465 cm\(^{-1}\) associated with the CH\(_2\) bending vibration of PEI. Further interpretation highlighted bands at 3262 cm\(^{-1}\) to 3350 cm\(^{-1}\) associated with an NH\(_2\) stretching vibration, as well as a new broad peak at 1562 cm\(^{-1}\) which is due to the newly formed ionic bond between COO\(^-\) and NH\(_3^+\), of NaAlg and PEI, respectively, which resulted in the absence of the peak at 1645 cm\(^{-1}\) observed in Figure 2c. This partially confirms the formation of the polyelectrolyte complex (PEC), however further physicochemical analyses were undertaken to support this finding.

Figure 3 represents the XRD diffractograms of pristine Si, pristine NaAlg and the Alg-PEI/Si scaffold for ascertaining the scaffold physicochemical state. The observed XRD patterns of pristine Si (Figure 3a) displayed a broad band centred at 22° as an indication of its amorphous nature\(^{(34)}\). Figure 3b represents pristine NaAlg displaying characteristic peaks at 14°, 22° and 38° confirming its semi-crystalline nature\(^{(35)}\). Figure 3c depicts the XRD for the bioplootted scaffold which had bands at 14°, 23° and at 38°, reminiscent of pristine NaAlg, but with a more amorphous nature.

3.3. Assessment of thermal transitions and stability of the bioprinted scaffold
The thermal stability of the pristine Si, pristine NaAlg and Alg-PEI/Si scaffold was assessed using TGA. Figure 4a depicts the thermal degradation of pristine Si; there is a gradual loss of mass toward 100°C due to the evaporation of water and it proved to be stable up to 400°C. With regard to the TGA of pristine PEI, Wang et al.\(^{(25)}\) highlighted that the weight loss is first observed between 106°C and 162°C corresponding to the evaporation of water from PEI, and between 253°C and 298°C corresponding to the endothermic peak ascribed to the decomposition of PEI\(^{(25)}\). Figure 4b shows the TGA curve for pristine NaAlg where the loss of moisture was distinctly observed at temperatures below 100°C. The major degradation of NaAlg occurred at 242°C. Figure 4c represents the TGA curve of the Alg-PEI/Si scaffold, where the evaporation of water was observed at temperatures below 100°C. There are two major degradations which occurred at 242°C corresponding to Alg and the other at 298°C which may correspond to the ionically cross-linked PEI confirming the presence of both polymers in the product.

The DSC curves of pristine NaAlg, pristine Si and the Alg-PEI/Si scaffold under N\(_2\) are shown in Figure 5. The dehydration was evidenced by an endothermic peak at ~100°C in all the thermal curves and the pristine Si is stable up to 400°C as evidence in Figure 5a. The decomposition of pristine NaAlg in Figure 5b is represented by an exothermic peak at 240–250°C. Finally, the decomposition of the carbonaceous material occurred at 375°C\(^{(36)}\). Figure 5c represents the thermogram of the bioprinted scaffold, which also exhibits an exothermic decomposition at 240–250°C corresponding to initial NaAlg decomposition and a small endothermic peak close to 400°C, but lack of a second decomposition peak. There is delay in eventual decomposition of the scaffold compared to the individual components due to the
ionic interaction \cite{33}. These results are consistent with the thermal stability findings above.

3.4. Evaluation of the morphology porosity, pore size, swelling and structural integrity of the bioprinted scaffold

Figure 6 depicted the scaffold dimensions and morphology for printing. Figure 6a highlighted the thickness of each printed bio-ink strand, which was observed to be 0.4mm, and the length of the space between the strands was visualized to be 0.2mm. The final dried Alg-PEI/Si bioprinted scaffold possessed dimensions of 3.6mmx4.5mmx1mm. Figure 6c provides the SEM of the dried printed scaffold which highlighted the roughness and porous nature of the bioprinted scaffold with consistent channelling of pores which are favorable for nutrient flow and material transfer, as well as providing a large surface area for attachment and proliferation of cells. The porosity of the scaffold was calculated to be 60\% while the pore size was found to be 360±20µm, highlighting shrinkage of the scaffold strands on complex formation and drying. In general the acceptable pore size is in a range of 50 to 1000µm \cite{37} for bone tissue engineering applications.

INSERT FIGURE 4 HERE

INSERT FIGURE 5 HERE

INSERT FIGURE 6 HERE

INSERT FIGURE 7 HERE
This study utilized magnetic resonance imaging (MRI) to observe the swelling and structural integrity behavior of the bioprinted polymeric scaffold in phosphate buffered saline (pH 7.4). The observed images were captured at various time intervals. The light grey area that surrounds the scaffold matrix is the dissolution medium (buffer), while the contrasting black area within the scaffold matrix is the non-gelled non-hydrated region of the polymeric scaffold matrix. It is observed from Figure 7 that, as the scaffold matrix hydrated, it progressively gelled and swelled represented by the white region of the scaffold matrix with each printed strand becoming thicker. Initially there was increased erosion, with loss of some of the incorporated silica gel; thereafter that the scaffold maintained its 3D network (shape) over the 24 hour period of investigation.

### 3.5. Degradation evaluation of the various bioprinted scaffolds

The degradation behavior of Alg/Si and Alg-PEI/Si scaffolds are depicted in Figure 8. The NaAlg/Si scaffold displayed complete erosion in the medium (pH 7.4) within a day (24 hours) as shown in the Figure 8 and this can be attributed to the solubility of NaAlg in water and the fact that the pristine silica gel is not chemically bonded to the pristine NaAlg; the silica gel primarily acts as a bioactive and support structure. The Alg-PEI/Si scaffold demonstrated an initial mass loss of 50% within a day due to the high silica gel content incorporated within the scaffold, but subsequently the scaffold maintained a near constant mass up to day 28. The fact that scaffold maintained its 3D network structure for the 28 day period of investigation highlights that the degradation of the scaffold has the potential to occur over time at a controlled resorption rate correlating with the bone tissue reformation rate (~3-6 months).
3.6. Evaluation of the physicomechanical strength of the bioprinted scaffold

Matrix hardness is the force required to deform scaffold matrices in the presence of an external pressure, while deformation energy is the energy required to withstand the forces within the matrices. Analysis of the physicomechanical strength is employed to indicate the stability of the matrices and their ability to withstand applied pressure, which a bone scaffold would ultimately encounter in vivo. A harder and more resilient matrix indicates a more compact matrix which is likely to have higher Young’s modulus. The hardness of the scaffold is also influenced by the inherent properties of the polymers employed in the formulation of the scaffold.

Alginate was investigated as a preferred material for bone tissue engineering compared to other materials because it can be easily modified, it can introduced into the body in a less invasive manner due to its biocompatibility, and it enables controlled release of tissue induction factors (e.g. MBP). However, due to their less sufficient mechanical properties they cannot bear loads at the initial stages of regeneration without fixation (38). Thus formation of a PEC with 3D printing was applied. In this study, a BioTester was employed to for biaxial determination of the stiffness or the ability of the printed scaffolds to resist deformation under an applied tensile load. Figure 9a depicts the scaffold while it was at rest (before force was applied, indicated by the green dot on the force-displacement curve). Figure 9b
shows the maximum stretching of the scaffold with biaxial testing before breakage (indicated by the green dot on the force-displacement curve). The stress-strain curve generated highlighted the elastic behavior of the scaffold. The Young’s modulus (representative of material stiffness) of the bioprinted scaffold was calculated from the linear elastic portion of the stress-strain curve generated from the average forces and displacements in both x and y-directions, and was determined to be 18.37 MPa (Figure 10). The bioprinted scaffold exhibited the linear elastic behavior recommended for bone tissue engineering (39), and the Young’s modulus attained is within values reported for hybrid and nanobiocomposite alginate scaffolds for bone tissue engineering (~1-30 MPa) (40,41) highlighting that the scaffold in its current form possesses the mechanical capabilities for certain bone tissue engineering applications (e.g. cancellous bone repair). Further strengthening of the bioprinted scaffold by enhanced incorporation of silica or addition of alternative inorganic or biomineral components may be considered for enhancement of the scaffold’ biomechanical properties.

**4. Conclusion**

The applications of autografts or allografts have shown limitations in repair or replacement of bone injuries. 3D bioprinting technology was introduced as a promising approach for scaffold fabrication; however, limitations exist in terms of available printable biomaterials. Hence, a novel alternative method of scaffold fabrication via *in situ* conjugation-co-fabrication for formation of a PEC-based 3D bioprinted scaffold for bone tissue engineering was employed. This single-step
fabrication approach resulted in a scaffold based on an innovative Alg-PEI/Si biomaterial with appropriate pore size and porosity and maintenance of its 3D architecture over the 28 day period of investigation. The biomechanical properties of the 3D bioprinted scaffold highlighted the linear elastic behavior and a Young's modulus which was favorable for certain bone tissue engineering applications. The application of the 3D printing enabled construction of the scaffold layer-by-layer via low temperature processing for enhanced control over the pore orientation, pore size distribution, pore volume and interconnectivity of the scaffold. This technique thus promotes customization of the spatial organization of the scaffold via variation of printing parameters for optimization of scaffold strength, nutrient transfer and cellular infiltration for the required bone tissue engineering application. Further studies will focus on enhancement of the mechanical properties of the scaffold with evaluation of osteoblast attachment-, proliferation- and differentiation-promoting capabilities of the scaffold.

5. Acknowledgements

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6. Conflict of interest

The Authors report no conflict of interest.

7. References


**Figure Legends**

Figure 1: Schematic and photographic depiction of formation of the 3D bioprinted and *in situ* conjugated-co-fabricated scaffold

Figure 2: FTIR spectra of a) Pristine Si, b) Pristine NaAlg, c) Pristine PEI and d) Alg-PEI/Si scaffold

Figure 3: XRD spectra of a) Pristine Si, b) Pristine NaAlg and c) Alg-PEI/Si Scaffold

Figure 4: The TGA spectra for a) Pristine Si, b) Pristine NaAlg and c) Alg-PEI/Si Scaffold

Figure 5: DSC thermograms of a) Pristine Si, b) Pristine NaAlg and c) Alg-PEI/Si Scaffold

Figure 6: A. Surface schematic of the proposed design of the bioprinted scaffold. B. Cross-sectional schematic of the proposed design of the bioprinted scaffold. C. SEM image of the surface section of the bioprinted Alg-PEI/Si scaffold

Figure 7: Magnetic Resonance Imaging of the bioprinted scaffold

Figure 8: *In vitro* degradation (mass loss, %) of the bioprinted scaffolds

Figure 9: BioTester images and corresponding real-time image analysis and force-displacement graphs representing scaffold strength a) before application of force and b) during maximum stretch

Figure 10: Stress-strain curve depicting Young’s modulus of the bioprinted scaffold
Table 1: Parameters for 3D printing of the bioarchetype scaffold

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Figure 1: Schematic and photographic depiction of formation of the 3D bioprinted and *in situ* conjugated-co-fabricated scaffold

254x190mm (300 x 300 DPI)
Figure 2: FTIR spectra of a) Pristine Si, b) Pristine NaAlg, c) Pristine PEI and d) Alg-PEI/Si scaffold
Figure 3: XRD spectra of a) Pristine Si, b) Pristine NaAlg and c) Alg-PEI/Si Scaffold
Figure 4: The TGA spectra for a) Pristine Si, b) Pristine NaAlg and c) Alg-PEI/Si Scaffold
Figure 5: DSC thermograms of a) Pristine Si, b) Pristine NaAlg and c) Alg-PEI/Si Scaffold

103x190mm (300 x 300 DPI)
Figure 6: A. Surface schematic of the proposed design of the bioprinted scaffold. B. Cross-sectional schematic of the proposed design of the bioprinted scaffold. C. SEM image of the surface section of the bioprinted Alg-PEI/Si scaffold

254x162mm (300 x 300 DPI)
Figure 7: Magnetic Resonance Imaging of the bioprinted scaffold

250x125mm (300 x 300 DPI)
Figure 8: *In vitro* degradation (mass loss, %) of the bioprinted scaffolds

237x176mm (300 x 300 DPI)
Figure 9: BioTester images and corresponding real-time image analysis and force-displacement graphs representing scaffold strength a) before application of force and b) during maximum stretch.
Figure 10: Stress-strain curve depicting Young’s modulus of the bioprinted scaffold

Young’s Modulus = 18.37MPa