A bilayered tissue engineered in vitro model simulating the tooth periodontium

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Aim of the study: to develop a bilayered in vitro construct that simulates the tooth periodontal ligament and attached alveolar bone, for the purpose of tissue regeneration and investigation of physiologic and orthodontic loading.
Methods: Two types of materials were used to develop this construct: Sol-Gel 60S10Mg scaffold (University of Erlangen) representing the hard tissue component of the periodontium and commercially available Geistlich Bio-Gide® collagen membrane representing the soft tissue component of the tooth attachment. Each scaffold was dynamically seeded with periodontal ligament cells. Scaffolds were either cultured separately, or combined in a bilayered construct, for 2 weeks.
Characterization of the individual scaffolds and the bilayered constructs included biological characterization: (cell viability, SEM imaging to confirm cell attachment, qRT-PCR expression for periodontium regeneration markers), and mechanical characterisation of scaffolds and constructs.
Results: HPDLCs enjoyed a biocompatible 3D environment within the bilayered construct components. Gene expression showed no drop in expression in the bilayered construct compared to the between individual... |
scaffolds.
Figure (1): Image showing Sol-gel (A), BioGide® (B) individual scaffolds and an assembled biliary construct (C).

263x141mm (150 x 150 DPI)
Figure (2): Viability and growth of HPDLCs after 2w in 3D cultures A: BioGide® membrane porous side, B: BioGide® membrane dense side C: BGC porous side (BioGide® membrane detached from construct) D: BGC dense side (BioGide® membrane detached from construct), E: Sol-gel scaffold & F: SGC (Sol-gel scaffold detached from construct), after 2 weeks in culture.

(G): Bar graph showing LDH assay levels in media of seeded 3D cultures; Sol-gel, BioGide®, membrane and bi-layered 3D constructs as well as mono-layers HPDLCs at different time points of culture (2, 5, 7 & 14 days). Positive controls are included and represented by the red bars. Values are represented as mean ± S.D.
Figure (3): SEM images of individual scaffolds and bilayered constructs including: unseeded Bio-Gide membrane showing; a cross sectional view of the BioGide membrane (A), the difference in the structure is evident between unseeded dense side (B) and the unseeded porous side (C). The unseeded Sol-Gel scaffolds showing oval and inter connected pores (D), and the seeded Sol-Gel scaffolds after 2W in culture (E).

305x187mm (96 x 96 DPI)
Figure (4): SEM images at the interface between the two components of the bilayered construct (BioGide® membrane (A,B,&D) and Sol-Gel scaffold C, E&F)). Images showing HPDLCS spreading and stretching within the bilayered construct components, within the scaffold pores and on the surface. Images showed sheets of HPDLCS growing within the scaffold with typical fibroblast-like appearance. Also showing the remnants of SGC on BGC after their detachment (D).
Figure (5), I: Line graph showing load-extension curves after compression of 3D structures both seeded and unseeded kept for 2W in the same culturing conditions. A: Un-seeded Sol-Gel scaffolds, B: Seeded Sol-Gel scaffolds, C: Un-seeded BioGide®, D: Seeded BioGide®, E: Seeded Bi-layered Construct, F: Un-seeded Bi-layered construct.

Figure (5): (II) (A) Graph showing individual scaffold stiffness (Sol-Gel scaffold and BioGide® membrane), (B) Graph showing comparison between stiffness of seeded and unseeded constructs.

(III) Graph showing Peak failure strength during stage I loading. Note: samples that did not show peak failure at stage I were excluded.

215x185mm (150 x 150 DPI)
Figure (6): Relative change in gene expression in HPDLCs cultured for 2 weeks in monolayers, Sol-Gel, BioGide® and bi-layered construct components after detachment (SGC& BGC). The relative change in expression was normalized to control (zero time point). The data is presented as log 10 of the mean $1\Delta Ct \pm SD$. Results analyzed using Kurskal Wallis multiple comparison test (*$=P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

334x177mm (150 x 150 DPI)
Title: A bilayered tissue engineered *in vitro* model simulating the tooth periodontium

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Running Title: A bilayered tissue engineered periodontium model

Abstract

Due to the complexity of the structure of the tooth periodontium, regeneration of the full tooth attachment is not a trivial task. There is also a gap in models that can represent human tooth attachment *in vitro* and *in vivo*.

Aim of the study: to develop a bilayered *in vitro* construct that simulates the tooth periodontal ligament and attached alveolar bone, for the purpose of tissue regeneration and investigation of physiologic and orthodontic loading.

Methods: Two types of materials were used to develop this construct: Sol-Gel 60S10Mg scaffold (University of Erlangen) representing the hard tissue component of the periodontium and commercially available Geistlich Bio-Gide® collagen membrane representing the soft tissue component of the tooth attachment. Each scaffold was dynamically seeded with periodontal ligament cells. Scaffolds were either cultured separately, or combined in a bilayered construct, for 2 weeks. Characterization of the individual scaffolds and the bilayered constructs included biological characterization: (cell viability, SEM imaging to confirm cell attachment, qRT-PCR expression for periodontium regeneration markers), and mechanical characterisation of scaffolds and constructs.
Results: HPDLCs enjoyed a biocompatible 3D environment within the bilayered construct components. Gene expression showed no drop in expression in the bilayered construct compared to the between individual scaffolds.

Key words: periodontal regeneration, physiologic simulation, in vitro models, bilayered construct, tissue engineering, periodontal ligament cells

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Introduction
Due to the complexity of the tooth periodontium (Jang et al., 2014), and root cementum, regeneration of the full tooth attachment is not a trivial task, especially
when attempting to restore function and simulate physiological dynamics (Sumita et al., 2006; Young et al., 2002).

The ideal periodontal therapy involves regeneration of the different tissues that comprise the tooth attachment apparatus (Bai et al., 2021), as well as restoration of the physiological function of the periodontium including shock absorbing function under mechanical loading (Cho and Garant, 2000).

Current regenerative periodontal therapies include guided tissue regeneration (GTR) (Araujo et al., 1998), uses a membrane that allows the growth and regeneration of the hard and soft tissues of the periodontium as well as allowing restoration of physiologic architecture of the tooth attachment (Yamada and Cukierman, 2007).

However, current regenerative therapies, although successful in many cases, still have unpredictable outcomes in many others. Furthermore, current GTR treatment is known to be more successful in specific lesions and sites (infra boney defects, molar class II bifurcation) compared to others (Camargo et al., 2005).

There is also a gap in models, that represent the human tooth attachment, and allow investigating regeneration therapies, and the effects of orthodontic tooth movements on the periodontium, in vitro and in vivo (Yang et al., 2015).

In vivo animal models, despite being ethically controversial, can give an idea about the success of periodontal treatment but still lack the exact resemblance to human’s physiology. The results of animal model experiments are often not transferrable to humans, and this is only discovered when clinical trials prove very different results to the animal models (Yijin et al., 2006).

Hence it was important to use the emerging tissue engineering techniques to develop models and constructs that represent the complexity and the diversity of the periodontium for regeneration and in vitro modelling purposes such as studying the effect of orthodontic and physiological mechanical forces on the periodontium. However, many of the available models have limitations in representing tissue diversity or physiological conditions (Kanzaki et al., 2004; Dortgiesen et al., 2012).

In vitro and insitu models that use human cells cultured in monolayers or periodontal multilayered cell sheets lack mechanobiology and simulation of mechanical loading (Arata et al., 2009; Raja et al., 2020; Yang et al., 2015).
In a recent study the development of a whole porcine tooth *in situ* model was attempted. However, the inability to maintain long term sample viability and sterility in culture for more than 8 days, posed a great disadvantage. Furthermore, in spite of this model being the closest to physiological condition, control over tissues and cells and testing individual cell responses have proven to be extremely challenging in this model (El-Gendy *et al.* 2020).

Self-assembling peptide P11-4 a smart scaffolds was successfully used to regenerate PDL critical defects in a rat model (El-Sayed *et al.* 2020). The same scaffold was used by Koch *et al.* 2020 to develop a 3D PDL model. The model tested cell migration and coverage of dentin surface but did not represent the architecture or the tissue hierarchy of the periodontium (Koch *et al.* 2020). Three D printing technologies enabled Lee *et al.* 2013, to create a successful multiphasic periodontal model. However, the complexity involved in developing the model which included printing of 3 different hydroxyapatite phases and seeding 3 cell types on different types of hydrogels, through the printed channels, and a cocktail of growth factors make its cost effectiveness, practicality and versatility questionable (Lee *et al.* 2013).

To create such a complex model, the right choice of stem/progenitor cells as well as materials is required (Ivanovski *et al.* 2006; Yu *et al.* 2014). The stem cells must have the capacity to differentiate into the different tissues that comprise the periodontium (Gay *et al.* 2007). A compartmental multi/biphasic approach must be used to regenerate the periodontium. This approach will require the use of different biomaterials to represent hard tissue and soft tissue components of the periodontium (Chen *et al.* 2008). The diversity of the surface topography of the materials will play a role in directing the differentiation route of the cells (Elisseeff *et al.* 2005; Jäger *et al.*, 2006).

Furthermore, mechanical characterization of multiphasic constructs is essential in future applications of such model (Sabree *et al.*, 2015). However, practicality, cost effectiveness, and versatility of the model are also key characters for the success of the model.

The aim of this study was to develop a biphasic/bilayerd tissue engineered construct with a soft tissue component representing the periodontal ligament (PDL) and a hard tissue component representing the alveolar bone, and to characterize the construct biologically and mechanically.

Future applications of such construct will include periodontium regeneration, skeletal tissue regeneration that requires multiphasic tissue engineering, hard-soft tissue interaction, characterisation of interfaces between tissues, materials, and cells such as:
osteochondral, bone-tendon, bone-ligament interfaces. Investigation the effect of orthodontic loading on periodontium, and biomechanical characterisation of bone and skeletal tissues are overarching translational goals of this construct.

2-Materials and Methods

Cell culture plastics were purchased from Corning. Alpha-modified minimum essential medium, phosphate-buffered saline solution, and fetal bovine serum (FBS) were obtained from Lonza. Antibiotics, growth factors, enzymes, and other reagents were purchased from Sigma, unless stated otherwise.

All scaffolds were sterilized by the Ultraviolet light source (UVP Upland, CA, USA.), for 30 minutes, in the tissue culture hood. Sol-Gel scaffolds were kindly provided by Professor Aldo R. Boccaccini, and Dr Menti Goudouri, University of Erlangen in Germany. The Bio-Gide® Perio was generously supplied by (Geistlich Pharma AG) of Switzerland.

Scaffold fabrication and preparation

Sol-Gel scaffolds

Sol-Gel scaffolds (9X 5X9mm³) produced by the Sol-Gel transformation technique are Gelatin-Genipin coated ceramic scaffolds made of SiO2 [Blehert et al., 2003], CaO (30%) and MgO (Chen et al., 2006). Genipin is a naturally occurring cross linking agent used to cross-link the gelatin as it is considered nontoxic (Tsai et al., 2000). Genipin can spontaneously react with Amino acids or proteins to form dark blue pigments which has been used in the fabrication of food dyes and that what gives the dark blue color of the scaffolds (Fig1A).

Geistlich Bio-Gide® Perio membrane

Geistlich Bio-Gide® Perio membrane is a commercially available collagen membrane, made of natural purified porcine collagen without further cross linking or chemical additives. This membrane is used clinically to treat advanced periodontal defects in GTR surgeries (Araujo et al., 1998; Camelo et al., 2001; Ramseier et al., 2006; Sculean et al., 2004). Geistlich’s Bio-Gide® membrane is a double sided, the porous side allows the penetration and growth of osteoblasts hence it is placed facing the alveolar bone during GTR whilst the dense face is placed adjacent to the soft tissue to prevent epithelial growth into the bony defect during GTR (Pietruska, 2001). Each Geistlich Bio-Gide® membrane (30mmX40mm) was cut into 9 circular scaffolds of equal diameters (9mm) using a sterilized circular sharp cork porer (Fig.1B).

Isolation and culture of human periodontal ligament cells (HPDLCs):
Cell isolation

Freshly extracted wisdom teeth were provided through Leeds school of Dentistry skeletal and Dental tissue bank after patients’ informed consent. Human Periodontal ligament cells (HPDLCs) were isolated from middle third of extracted healthy wisdom teeth. The cells were maintained in basal culture medium (α-MEM supplemented with 20% FBS, 200mM of l-glutamine, and 100 units / mL penicillin/streptomycin) at 37°C and 5% CO₂ until 80% confluent.

HPDLCs culture in monolayers

Passage (4) HPDLCs were seeded in 6 well plates at a density of 2.5X10⁵ cells per well (n=3). HDPLCs were cultured under basal conditions (the same conditions as the scaffolds) for 2 weeks. Monolayers were used as controls for qRT-PCR and LDH assay experiments. Media collected for LDH assays up to 14 days. HPDLCs at zero time point (not previously expanded in culture), were used as negative controls for monolayer HPDLCs and 3D structures that were cultured for 2 weeks.

Cell seeding & bi-layered construct assembly.

Individual scaffold seeding

2.5 x 10⁵ cells were dynamically seeded on each scaffold type, n=3/group, for both Sol-Gel scaffolds and Bio-Gide® membrane separately. Dynamic seeding was performed using Macs Mix® (Miltenyi Biotech, Germany) tube rotator machine. After 48hrs of rotation the scaffolds were transferred to 12 well plates and were then cultured for two weeks, the media was changed twice a week.

Bi-layered Construct assembly

After 48 h of dynamic seeding, the Sol-Gel scaffolds were placed on top of the Bio-Gide® membrane in a 12 well plate so that the porous surface of the Bio-Gide® membrane is in contact with the Sol-Gel giving a bi-layered construct (Fig1C). The constructs were then cultured in this arrangement for 2 weeks and media was changed twice weekly.

Scanning Electron Microscopic (SEM) imaging

Unseeded, individually seeded scaffolds (Sol-Gel scaffolds and Bio-Gide® membrane) and seeded bilayered constructs were examined and imaged using SEM (JEOL JSM...
Bio-Gide® membranes were imaged on both porous and dense sides. Seeded scaffolds were also imaged using the SEM to determine the cell attachment, proliferation, and formation of extracellular matrix. Furthermore, bi-layered constructs were examined at the interface between the two scaffolds and each scaffold was imaged after being detached from the bi-layered construct (Sol gel component of the construct (SGC) & Bio-Gide® component of the construct (BGC)). Imaging of seeded scaffolds and constructs was carried out after 2 weeks of culture. All samples were fixed in 4% formaldehyde, dehydrated and gold spattered before being imaged by SEM.

**Cell viability assays**

**Live/dead staining**

After 2 weeks of culture, the Seeded Sol-Gel scaffolds, Bio-Gide® membranes and the bi-layered constructs were fixed in 4% neutral buffered formalin, after being stained with Viability/Cytotoxicity kit (Invitrogen Life Technologies, USA) for mammalian cells, according to manufacturer’s instructions. Briefly, 10 µl Ethidium homodimer-1 (EthD-1) and 5 µl Calcein AM were added to 5ml of plain media. The staining solution was used to stain constructs as follows, 200 µl of the staining solution was added to each scaffold / construct and were incubated in the dark at 37°C and 5% CO₂ for 30 minutes. After staining, the scaffold components (SGC and BGC) were detached from the bilayered constructs. Whereas control individual scaffolds were left as they were and imaged using inverted fluorescent microscopes (LEICA DMI 6000B, Japan). The Bio-Gide® membranes were imaged on both the dense and porous sides.

**Quantification of cell death percentage: Lactate Dehydrogenase assay**

LDH is a cytosolic enzyme present in many different cell types. When the plasma membrane is damaged, LDH is released into the cell culture media. LDH that is released on cell lysis is a stable and cytosolic enzyme and converts a tetrazolium salt in the medium to a red formazan product and its increase in culture medium is proportional to the number of dead cells with a compromised cell membrane.

The LDH CytoTox 96® colorimetric LDH assay kit was used to detect LDH released due to cell death or cell lysis in the media of the monolayers, individual scaffolds, or the bilayered constructs.

Cell death rates were determined by calculating percentage of cell death under each culture condition compared / normalized to a 100% cell kill control.

**CytoTox 96® Reagent preparation**


Assay buffer was thawed in a 37°C in a water bath, then, 12ml of the buffer solution was added to the bottle of Substrate mix to form **CytoTox 96®** Reagent. This reagent can be stored for 6-8 weeks at -20°C in the dark.

Conditioned media was collected from the different sample groups monolayers, scaffolds, and bi-layered constructs and from the positive control (maximum LDH release or 100% kill), which was prepared by incubating each group in media containing 1% triton x for 2-3 h prior to media collection, the experiment also include a blank (plain media without FBS), negative control (basal media with FBS) in triplicates. Collected samples were centrifuged for 3 min at 10000 rpm. Then triplicates of 50 µl of conditioned media from each sample was transferred to a 96 well plate. Fifty µl of LDH **CytoTox 96®** reaction mix was added to each well and was incubated for 30 minutes at 37°C. During this period, the plate was covered with a foil protecting it from light. After 30 minutes, stop solution (50µl) was added to all wells and then the absorbance was read and recorded by a plate reading spectrometer (Varioskan Flash reader) at 490nm. This data was analysed and presented as absorbance data in comparison to the maximum LDH release.

**Mechanical testing**

The unseeded Sol-Gel scaffold, Bio-Gide membrane and bi-layer construct were tested under compressive mechanical loading using a universal testing machine (LLOYD LR10K, UK) with a 20 N load cell. Constructs and scaffolds seeded with HPDLCs were tested after 2 weeks of culture (n=3). An 8 mm diameter pin was used to compress the samples in 48 well plates submerged in media under semi-constrained compression.

The crosshead displacement was set to zero at the bottom of the plate before each test. The crosshead was then raised to the height of the samples (height in dry state) and the samples were compressed at a rate of 0.5 mm/min. Loading continued until complete failure was observed or the sample reached a compressive load of 18 N.

Mechanical properties were evaluated by calculating the stress, strain, stiffness, and average compressive strength required to induce failure in the Sol-Gel scaffold, Bio-Gide® membrane and the bi-layered construct.

**Quantitative Real Time (qRT-PCR) Gene Expression Assays**

**RNA extraction**
mRNA was extracted using the RNeasy mini kit (Qaigen, UK), according to manufacturer’s instruction. Briefly the RLT+ 10% β-mercapto-ethanol were added directly to HPDLCs monolayers.

Whereas individual scaffolds (Sol-Gel and Bio-Gide®) and scaffolds separated from the construct (SGC & BGC), were minced before adding the RLT buffer. Then, the cell lysates were mixed with 70% Ethanol, homogenised using QI shredder (Qaigen, UK) and centrifuged for 15 sec at 10.000 rpm.

After this step mRNA was then extracted from monolayers and scaffolds similarly as indicated by the manufacturer. Briefly the mRNA was caught in the silicate membrane within the extraction columns, then they undergo a series of washes using RW1 and RPE buffers and finally the mRNA is collected in a 30-50 µl of RNAse - DNAse free water. mRNA was quantified using a Nano drop (Nanodrop2000, Thermo scientific).

**DNAse purification**

After quantification, mRNA from each sample was purified from any remnants of genomic DNA using DNase I kit (Invitrogen, UK) according to manufacturer’s instructions. Briefly in a 20 µl reaction using, 9µl of sample (up to 1 µg of mRNA) was added to 1 µl of DNase enzyme and 10 µl of buffer and incubated at room temperature for 15 minutes followed by adding 1 µl of EDTA and incubating at 65°C in a thermal cycler for 10 minutes.

**Reverse transcription (cDNA synthesis)**

One microgram of mRNA from each sample was used for reverse transcription with the ABI High-Capacity RNA-to-cDNA kit (ABI, UK) in a 20µL reaction volume according to the supplier’s instructions, briefly 1µl of enzyme and 10 µl of buffer added to 9µl of purified mRNA sample. The mixture was incubated at 37°C for 1h followed by 95°C for 5 min in a thermal cycler.

**qRT-PCR gene expression**

The relative changes in osteogenic (COL1A1, and OC), angiogenic (CD31/PECAM1, and VEGFR2), and cementum (CEMP1) marker genes’ expression were compared in HPDLCS cultured under the previously designated conditions after 2 weeks of culture. Furthermore, relative expression of RANK ligand (RANKL)/ osteoprotegerin (OPG) expression were also investigated under the previously mentioned conditions.
Relative change in gene expression for all markers was analyzed using the \( \Delta\Delta Ct \) method according to the following equation:

\[
\frac{2^{-(\text{gene of interest} - \text{housekeeping gene}) \text{ at 2 weeks}}}{2^{-(\text{gene of interest} - \text{housekeeping gene}) \text{ of the 0 time point control}}}
\]

Where housekeeping gene used in this study was GAPDH and the control group was the zero-time point HPDLCs, which have not been expanded in culture.

qRT-PCR was carried out using Roach LC480 light cycler to amplify and detect the following Taqman® probes:

- \( \text{GAPDH} \): Hs99999905-m1
- \( \text{COL1A1} \): Hs00164004-m1
- \( \text{CEMP1} \): HS04185363-s1
- \( \text{PECAM1} \): Hs01065290-m1
- \( \text{VEGFR2} \): Hs00911700-m1
- \( \text{RANKL} \): HS00765721-m1
- \( \text{OPG} \): HS00900360-m1

Statistical analysis

LDH and qRT-PCR results were statistically analysed using the Kruskal-Wallis multiple comparison test with 95% confidence interval. The statistical analysis was carried out using Graph pad prism version 7. All RT-PCR and LDH experiments were repeated 3 times \((n=3)\) and had 3 biological replicates \((n=3)\), total \(n=9\) data was represented in the form of mean±SD.

Results

Verification of HPDLCs viability

**Live/ dead assay of HPDLCs cultured on 3-D constructs**

HPDLCs seeded Bio-Gide® membrane were imaged on both the porous \((\text{Fig.2A})\) and dense \((\text{Fig.2B})\) sides after 2 weeks of culture. All attached cells displayed viable fibroblast like appearance indicative of cell spreading on both membrane sides with minimal cell death. Seeded Sol-Gel scaffolds after 2 weeks of culture showed a viable layer of HPDLCs growing on all sides of the scaffold as well as penetrating the scaffold pores. These results confirmed biocompatibility of both scaffolds to HPDLCs \((\text{Fig.2E})\).

The **bi-layered constructs** were tested after 2 weeks of culture. The Sol-Gel scaffold detached from the bi-layered construct \((\text{SGC})\) showed confluent, viable layer of HPDLCs covering the scaffold \((\text{Fig.2F})\). The Bio-Gide® membrane detached from the bi-layered construct \((\text{BGC})\) showed viable HPDLCs on both the porous \((\text{Fig.2C})\) and the dense \((\text{Fig.2D})\) sides. However, the porous side showed parts of the remnants of
the Sol-Gel scaffolds attached denoting good attachment between the bilayered components at the interface (Fig. 2C).

Most cells acquired a green stain denoting viability with minimal red stain denoting minimal cell death and maintained a fibroblast like morphology indicating cells were healthy (Fig. 2: A-F).

**Quantification of percentage of cell death using LDH**

Significant increase in LDH levels (P<0.001) were observed at day 7 and at day 14 in the Sol-Gel scaffolds compared to monolayers spontaneous release. The Bio-Gide® membrane showed significant increase in LDH levels (P<0.05) at day 14 only with non-significant increases at days 2, 5& 7 when compared to monolayer LDH levels. However, when scaffolds were combined in a bi-layered construct significant increases in LDH levels (P<0.001) were observed initially at day 2 but then reduced to non-significant levels at days 5, 7 & 14 (Fig.2G).

**SEM imaging of seeded and unseeded scaffolds**

Seeded and unseeded individual scaffolds and constructs were imaged using SEM. Sol-Gel pores had consistent oval shape (Fig.3D) with evident interconnectivity. Pores size ranged between (686.25 ±67.54 X 832.25±106.34µm). Unseeded Bio-Gide® membrane structure was confirmed to be porous on one side and dense fibrous on the other side. The collagen fibres were wavy and arranged longitudinally in a parallel manner. The porous side had more interconnectivity with irregular fibres arrangement compared to the dense side (Fig. 3B&C).

Images of seeded scaffolds (Sol-Gel scaffolds and Bio-Gide® membrane) after 2 weeks in culture showed HPDLCS spreading and stretching on the surfaces and showed sheets of HPDLCS growing within the scaffolds with typical fibroblast like appearance (Fig.3E).

Similar images were observed in the bi-layered constructs after 2 weeks of culture. Furthermore, when examining the interface of the bi-layered constructs, HPDLCS were seen spanning the attachment site after 2 weeks of culture. Seeded scaffolds detached from bi-layered construct (SGC &BGC) showed HPDLCS extension and growth in sheets within the Sol-Gel scaffold and on both sides of the Bio-Gide® membrane (Fig 4E&F).

**Mechanical properties of individual scaffolds and bi-layered construct**
The compressive load/extension behaviour in the Sol-Gel scaffold typically showed an initially high stiffness toe region (stage I), a yield region with micro-brittle fractures (stage II) followed by a high stiffness region of material compaction (stage III) (Fig. 5-I: A & B). The stiffness for stage I and II were calculated as $E_1$ and $E_2$ respectively. On average the unseeded Sol-Gel samples after 2 weeks ($n=4$) had a stiffness ($\pm$ standard deviation) of $511.20 \pm 382.99$ kPa and $219.15 \pm 113.44$ kPa respectively (Fig. 5-I: A). In comparison, the seeded Sol-Gel samples with HPDLCs ($n=4$) showed a considerable decrease in $E_1$ stiffness at $95.70 \pm 30.66$ kPa and an increase in $E_2$ stiffness at $494.39 \pm 71.47$ kPa (Fig. 5-I: B).

The Bio-Gide membrane typically showed a low stiffness toe region followed by a linear increase in stiffness (Fig.5-I: C & D) The stiffness for the toe and linear stiffness regions were calculated as $E_1$ and $E_2$ respectively. The unseeded membrane ($n=2$) after 2 weeks showed $E_1$ and $E_2$ stiffness of $4.39 \pm 0.86$ kPa and $92.68 \pm 17.48$ kPa respectively (Fig.5-I:C). Bio-Gide samples seeded with HPDLCs ($n=3$) showed a decrease in $E_1$ stiffness of $1.61 \pm 0.28$ kPa and a similar $E_2$ stiffness of $100.47 \pm 15.22$ kPa (Fig.5-I: D).

The bi-layer construct showed a non-linear toe region (stage I), with a yield region (stage II) followed by a high stiffness region (stage III), showing similar behaviour to the Sol-Gel samples (Fig. 5-I: E & F). The stiffness for stage I and II were calculated as $E_1$ and $E_2$ respectively. The unseeded construct at 2 weeks ($n=3$) showed $E_1$ and $E_2$ values of $278.73 \pm 224.24$ kPa and $460.14 \pm 374.01$ kPa respectively (Fig.5-I: E). In comparison the seeded construct with HPDLCs at 2 weeks ($n=3$) showed a decrease in $E_1$ at $103.20 \pm 62.01$ kPa and a considerable increase in $E_2$ stiffness at $676.42 \pm 144.77$ kPa.

Comparing unseeded and seeded bi-layered construct after 2 weeks in culture, there was no statistically significant difference between seeded and unseeded constructs at stage I (Fig. 5-II: A). At stage III, significantly higher stiffness was noticed in the seeded constructs. The peak strength of the Solgel and construct samples were calculated as the maximum stress reached during stage I of loading where the foam bears most of the loading before micro-fractures occur in stage II and increasing stress due to foam densification in stage III. The unseeded and seeded Solgel constructs had higher compressive peak strengths at $28.67 \pm 3.46$ kPa and $22.24 \pm 16.98$ kPa compared to the unseeded and seeded construct at $18.57 \pm 5.76$ kPa and $9.16 \pm 0.97$ kPa (Fig. 5-II: B).

**Relative change of gene expression in HPDLCs cultures**
**COL1A1** showed a higher expression in Sol gel (SGC) that was separated from the bi-layered construct after 2 weeks of culture, in comparison to monolayer cultures, sol-Gel and Bio-Gide© cultured individually, at 2 weeks. These results were only significant when comparing the SGC and monolayers (p≤0.05). BGC also showed a significantly higher expression of **COL1A1** compared to monolayers (p≤0.01) and Biogide© cultured individually (p≤0.001) ([Fig 6]).

**OC** showed a significantly higher expression in individual Bio-gide©(p≤0.001) and in monolayer(p≤0.001) cultures compared to expression in individual Sol-Gel, after 2 weeks of culture. SGC of the bi-layered construct showed significantly higher **OC** expression compared to individual Sol-Gel (p≤0.01). The expression of **OC** in BGC showed no significant difference to the expression in all other groups ([Fig 6]).

**CEMP1** showed no significant difference in gene expression between all groups including scaffolds cultured within the bi-layered constructs after 2 weeks in culture ([Fig 6]).

**OPG** expression in individually cultured Sol-Gel, showed significantly higher baseline levels compared to monolayer cultures (p≤0.05), individually cultured Bio-Gide© (P≤0.01) and SGC (p≤0.05). Whereas **OPG** expression in BGC was only significantly higher than that of monolayer cultures (p≤0.05) ([Fig 6]).

**RANKL** expression has been significantly higher in BGC compared to individually cultured BioGide© (p≤0.01) and was also higher compared to other groups except monolayers, however, this was statistically non-significant. Monolayer cultures also showed a significantly higher **RANKL** expression, at 2 weeks, compared to individually cultured Bio-Gide© (P≤0.01) ([Fig 6]).

**VEGFR2** expression in the bi-layered construct: SGC and BGC, was significantly lower than monolayers and individually cultured constructs after 2 weeks of culture ([Fig 6]).

**PECAM1** expression at 2 weeks did not show a significant difference between the bi-layered construct components and the rest of the groups. However, the **PECAM1** expression was significantly higher in individual Bio-Gide© scaffolds compared to individual Sol-Gel constructs ([Fig 6]).

**Discussion**
This study involved designing a bi-layered construct that allows culturing cells on 2 different substrate scaffolds representing soft and hard tissues. To be able to establish this bi-layered construct, diverse surface texture was required in scaffolds and a cell population that was multipotent and able to create a range of diverse tissues had to be used in the study.

HPDLCs proved to be our best choice for this study, since they are mixed stromal cells that included a subpopulation of stem cells, which maintained stem cell phenotype and characters: self-renewal, generating large numbers of progeny and differentiate into multiple mature cell types (Fortier, 2005; Porter and von Fraunhofer, 2005). Furthermore, they are the most appropriate cell source for periodontal tissue engineering (Ivanovski et al., 2006). Moreover, reports showed that cells isolated from PDL of healthy and diseased teeth were identified to have adult stem cells among the mixed population of PDL cells (Nagatomo et al., 2006; Ramseier et al., 2006). An important feature of HPDLCs is having a subpopulation of cells capable of undergoing differentiation into two types of mineralised tissues, bone and cementum (Inanc et al., 2007). Furthermore, their capability to regenerata the soft tissue PDL component of the periodontium made them a suitable candidate for this study (Inanc et al., 2007).

Cells normal growth environment within the periodontium is a three-dimensional environment. Cells grown on flat 2D tissue culture substrates differ considerably in their morphology, cell-cell and cell-matrix interactions, as well as differentiation from those growing in more physiological environments (Yamada and Cukierman, 2007). Furthermore, 3D culture enhance osteogenic differentiation compared to 2D culture (Inanc et al., 2006), (El-Gendy et al., 2013).

Hence, the selection of suitable scaffolds and their surface topography was of an utmost importance to this study.

The Sol-Gel scaffold used in this study is a synthetic Gelatin Genipin coated bioactive glass that fulfilled the essential requirements in scaffolds designed for bone regeneration (Oreffo et al., 1999) (Hutmacher, 2000; Oreffo and Triffitt, 1999; Rose and Oreffo, 2002) (Hutmacher and Dietmar, 2000). It has shown biocompatibility to PDLCS; as scaffolds should be able to support cell viability and growth without initiating an immune response or any toxic reactions.

The second member of the bi-layered construct is the Bio-Gide© membrane. An evident advantage of this membrane is the porous side allowing HPDLCs more surface area to grow within it. In addition, the membrane made up of pure collagen that is the basic structure of PDL (Berkovitz, 2004).
HPDLCS maintained viability and growth after 2 weeks of culture on both types of individual scaffolds as well as within the bi-layered construct. This was confirmed by the cell death assay, which showed no significant difference in LDH levels between the different culture conditions.

The balance between the constructs porosity and the maintenance of its mechanical properties is a requirement determined by the nature of loading that the construct is supposed to bear invivo (O’Brien, 2011). In our case it was of utmost importance to accommodate the balance between the constructs porosity to allow cell growth and the maintenance of its mechanical properties to withstand loading invivo is essential (O’Brien, 2011). The compressive stress-strain behaviour of the Sol-Gel scaffolds were reflective of typical ceramic-based bone supporting scaffolds showing compressive brittle failure (Chen et al., 2006). The bilayer construct showed compressive behaviour of the BioGide under low loading followed by a predominantly brittle loading behaviour as the higher stiffness Sol-Gel scaffold bears the load. The scaffold material tended to crack first at stress concentration sites (stage I) causing the apparent strength to drop temporarily but the specimen as a whole still has the strength to bear more loads causing the stress to rise again and the repetition of this cause the jagged curve in the stage II yield region. The construct material builds up more strength at the beginning of (stage III) due to material compaction.

The unseeded Sol-Gel and construct compressive strength range was a magnitude lower at approximately 0.02-0.03 MPa In comparison to the compressive strength of the bioglass from (Chen et al., 2006) of 0.3-0.4 MPa. However these values are in line with other data published by the group who developed the Sol-Gel scaffolds stating compressive strengths of approximately 0.03 ± 0.005 MPa (Goudouri et al., 2016).

The compressive properties for the Sol-Gel and construct are relatively low in comparison to other ceramic and polymer bone scaffolds. However other bone scaffolds are fabricated for use in high load-bearing environments such as bone defects in the lower and upper limbs, requiring high initial stiffnesses that are comparative to cancellous bone (Lichte et al., 2011). In comparison, the Sol-Gel scaffold is designed for alveolar bone reconstruction, a relatively lower load bearing environment.

The seeded Sol-Gel and construct samples demonstrated smoother stress/strain load behaviour after 2 weeks. This can be attributed to the cell growth within the scaffold filling the micropores and voids, leading to less brittle behaviour as shown in this study. This change also saw a drop in stiffness, this may be attributed to the stiffness contrast between the high stiffness Sol-Gel struts and low stiffness cellular components. With initial compressive loading the cellular components of the scaffold yield first, producing a significant toe region. However, an increase in stage III stiffness
was observed for the seeded Sol-Gel and construct samples, indicating a decrease in porosity and the load contribution from the cellular components during densification. The other factor why the initial compressive properties (stage I) did not increase after 2 weeks incubation was that the seeded samples were not loaded. However the aim of this paper was to characterise the construct and investigations into loading effects were carried out in a study that will be published subsequently in the future.

In this study, the excellent pore interconnectivity and porosity of the system especially within the hard tissue component represented by the Sol-Gel Scaffolds, helped maintain a good tissue perfusion and maintenance of nutrients and cell growth and viability within the bi-layered construct, inspite of doubling the thickness. Furthermore it enhanced attachment and integration between the 2 components of the bi-layered construct (Sabree et al., 2015). It is well known that variation in surface topography can change the cues that cells receive and influence cell differentiation and gene expression (Metavarayuth et al., 2016).

A few studies in the literature have investigated the changes in gene expression in multiphasic models of the periodontium. However, many studies investigated differential gene expression in monolayers (Choi et al., 2011; Lee et al., 2007) or investigated the effect of mechanical loading on the HPDLcs (Berendsen et al., 2009; Shi et al., 2019).

In terms of the length of the culture required for osteogenic and angiogenic differentiation, it was reported in previous studies that at least 2 time points (between 2 and 4 weeks of culture), might be recommended to capture the changes in osteoblastic and angiogenic gene expression profile of stem cells isolated from dental tissues, both in monolayer cultures (Alkharobi et al., 2016; Alkharobi et al., 2018), and on 3D scaffolds (El-Gendy et al., 2015; El-Gendy et al., 2013).

particularly for PDLSCs, Açıl et al., 2016, have compared the osteogenic gene expression under osteogenic induction conditions. Although they have mentioned that they have investigated gene expression after 2 and 3 weeks of culture, they only presented data at 2 weeks only. They have reported no changes to RUNX-2 expression but an increased expression of osteopontin and osteonectin at 2 weeks (Açıl et al., 2016).

Here, we would like to highlight that due to logistical reason we were not able to show data at more than one time point or after 2 weeks of culture. Hence we acknowledge that inability to properly capture the gene expression profile at different timepoints within our model has been a limitation of this study.

We have observed gene expression differences in HPDLCs cultured on solgel and those cultured on Bio-Gide©.
Angiogenic markers such as VEGFR-2 and PECAM1 were both expressed at a higher level in Bio-Gide compared to Sol-Gel scaffolds which were cultured individually. To our surprise the expression of genes associated with mineralisations such as OC had higher expression in individual Bio-Gide compared to Sol-Gel scaffolds. This result was in agreement with Alves et al., 2015, who have found that 2D collagen type I scaffolds showed higher OC expression by PDLSCs compared to those cultured in 3D collagen type I scaffolds (Alves et al., 2015). In our case the Bio-Gide membrane although having rough surface topography on one of its sides was more or less a 2D layer of collagen type I.

Conversely, Lee et al., showed that higher dentin and cemetum gene expressions on phases representing the hard tissue components of the periodontium in their model (Lee et al., 2013). We haven’t seen much significant differences in gene expression between the individual constructs and those detached from the bi-layered construct after 2 weeks of culture. This was a reassuring finding as the gene expression remained similar even after increasing the thickness of the construct by assembling the two layers. This finding was to some extent similar to Zhang et al., 2019, findings as they didn’t find a significant change in the expression of osteogenic genes between their untreated controls and their test group of PDLSCs (grown in monolayers) treated with a combination of A83-01 and FGF-2 after 14 days of culture. However, they have seen a drop in gene expression at 7 days in the test group. There were some exceptions such as HPDLCs seeded on the SGC showing a significant increase in OC compared to its individual counter part denoting a more advanced osteoblastic differentiation, this has been confirmed previously by our group for bioglass scaffolds (El-Gendy et al., 2013). The other couple of exceptions were the significant increase in the expression of RANKL. However, this was not associated with a drop in the OPG, which blocks osteoclastogenesis by RANKL. This increase in RANKL expression is hence not an indication of osteoclastic activity within the constructs. Further investigation in the cell-cell interactions within the construct is required to explain the OPG/RANKL expression observed in this study (Kanzaki et al., 2001).

Furthermore, there was a significant drop in VEGFR2 expression in BGC compared to individual Bio-Gide® which was an indication of proper oxygenation of the construct as hypoxia is a major stimulus to VEGFR2 increased expression (Riddle et al., 2009).

A group of scientists in Cardiff have established what is now known as a gold standard ex vivo model in dentistry and dental regeneration, known as the rat mandibular slice model (Smith et al., 2010). This model has shown great success and versatility in
investigating the destruction and regeneration of bone and dental tissues under physiological and pathological conditions. This model was also successfully loaded to investigate the effect of orthodontic forces on bone and PDL (Colombo et al., 2015; El-Bialy et al., 2011; Sloan et al., 2013; Smith et al., 2010). However, our current bi-layered model in addition to its comparability to the mandibular slice model, it has the added values and versatility of being able to investigate different cell interactions as well as the endless scaffold materials options that can be used in such a model, lending itself not only to dental physiologic simulation and regeneration but also expanding its use to other skeletal tissues that require soft- hard tissue interactions.

**Conclusion:**

A bilayered multiphasic *invitro* model was developed for the purpose of periodontium simulation and regeneration. The model was successfully cultured for 2 weeks maintaining cell viability and attachment as well as gene expression. The model shows versatility that makes it suitable for investigation of cell-cell interactions, cell-materials interaction and the effect or mechanical and orthodontic loading on cell behaviour and tissue regeneration.

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**Conflict of Interest statement:**

The authors would like to declare no conflict of interest for any of them with the conduct of this study.
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Figure legends

Figure (1): Image showing Sol-gel (A), BioGide® (B) individual scaffolds and an assembled bilyaerd construct (C).

Figure (2): Viability and growth of HPDLCs after 2w in 3D cultures A: BioGide® membrane porous side, B: BioGide® membrane dense side C: BGC porous side (BioGide® membrane detached from construct) D: BGC dense side (BioGide® membrane detached from construct), E: Sol-gel scaffold & F: SGC (Sol-gel scaffold detached from construct), after 2 weeks in culture.

(G): Bar graph showing LDH assay levels in media of seeded 3D cultures; Sol-gel, BioGide® membrane and bi-layered 3D constructs as well as mono-layers HPDLCs
at different time points of culture (2, 5, 7 & 14 days). Positive controls are included and represented by the red bars. Values are represented as mean ± S.D.

Figure (3): SEM images of individual scaffolds and bilayered constructs including: unseeded Bio-Gide membrane showing: a cross sectional view of the BioGide membrane (A), the difference in the structure is evident between unseeded dense side (B) and the unseeded porous side (C). The unseeded Sol-Gel scaffolds showing oval and interconnected pores (D), and the seeded Sol-Gel scaffolds after 2W in culture (E).

Figure (4): SEM images at the interface between the two components of the bilayered construct (BioGide® membrane (A, B, & D) and Sol-Gel scaffold C, E & F). Images showing HPDLCS spreading and stretching within the bilayered construct components, within the scaffold pores and on the surface. Images showed sheets of HPDLCS growing within the scaffold with typical fibroblast like appearance. Also showing the remnants of SGC on BGC after their detachment (D).

Figure (5), I: Line graph showing load-extension curves after compression of 3D structures both seeded and unseeded kept for 2W in the same culturing conditions. A: Un-seeded Sol-Gel scaffolds, B: Seeded Sol-Gel scaffolds, C: Un-seeded BioGide®, D: Seeded BioGide®, E: Seeded Bi-layered Construct, F: Un-seeded Bi-layered construct.
Figure (5): (II) (A) Graph showing individual scaffolds stiffness (Sol-Gel scaffold and BioGide® membrane), (B) Graph showing comparison between stiffness of seeded and unseeded constructs.

(III) Graph showing Peak failure strength during stage I loading. Note: samples that did not show peak failure at stage I were excluded.

Figure (6): Relative change in gene expression in HPDLCs cultured for 2 weeks in monolayers, Sol-Gel, BioGide® and bi-layered construct components after detachment (SGC& BGC). The relative change in expression was normalized to control (zero time point). The data is presented as log 10 of the mean $1\Delta ct \pm SD$. Results analyzed using Kurskal Wallis multiple comparison test (*=P≤0.05, **P≤0.01, ***P≤0.001).