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# The Influence of Scaffold Designs on Cell Seeding Efficiency in Establishing A Three-Dimensional Culture

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**Abstract**. The concept of Bone Tissue Engineering (BTE) based on cell-seeded scaffold holds great promise for the treatment of bone defects. However, much optimization is required in establishing a three-dimensional (3D) culture before BTE can be successfully applied clinically. In this study, we have assessed the influence of scaffold designs on the initial cell seeding efficiency of anchorage cells. The rapid prototyping designed polylactic acid (PLA) scaffolds fabricated using fused deposition modelling (FDM) designated by 2C and 4C scaffolds were compared to the commercial hydroxyapatite (HA) scaffold. Prior to cell seeding, 2C and 4C scaffolds were deposited with silicon carbonated HA nanopowders/ Hyaluronic acid/ Collagen Type I by Polyelectrolyte Multilayers (PEMs) coating and sterilized under ultra-violet (UV) radiation. Human Bone Marrow derived-Mesenchymal Stem Cells (hMSCs) were then cultured on the scaffolds using different volume of cell suspension, seeding method (one-side and two-sides; coated and non-coated well plates) and culture condition (static and rotary bioreactor). Regardless of scaffold designs, cell-seeded scaffold with small volume of cell suspension and two-sides seeding using coated well plate resulted in better cell attachment and distribution across the surface of the scaffolds than one-sided seeding. In any case, commercial HA exhibited lower percentages of cell attachment. Culturing these scaffolds in different media but culturing these scaffolds in rotary bioreactor has pronounced impact on the cell survival as compared to static condition.

## 1. Introduction

In tissue engineering (TE) particularly scaffold-based TE, two critical challenges remained are the homogenous seeding of cells throughout a porous scaffold and providing sufficient oxygen as well as

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nutrients to the seeded cells throughout the culture[1]. Cell seeding of scaffolds involves the distribution of a cell suspension across the surface of a scaffold and potentially growth within the pores. It is considered as a determining factor for the development of tissue formation as it is the first step involved in establishing a three-dimensional (3D) culture[2]. Therefore, it has been highlighted that homogenous cell seeding can be considered as the building block to uniform tissue formation. However, even for a small scaffold (e.g. 5 mm diameter X 2 mm thick), it can be major challenge to distribute a high density of cells efficiently and homogenously throughout the entire scaffold[2,3].

Several methods have been proposed for the cell seeding into the microporous scaffolds such as static and dynamic seeding. Although static seeding is still the most commonly used, it is characterized by low seeding efficiencies and non-uniform distribution[1]. In order to improve cell distribution as well as providing sufficient oxygen and nutrients for cell growth and survival after seeding, dynamic culture system have been developed, for example, spinner flasks, perfusion, rotating vessel, compression and magnetic force bioreactors[4]. Besides seeding method, the scaffolds architecture (i.e. channel size, porosity, surface roughness and surface area) and properties of the scaffolding material itself are responsible for the cell activities, which subsequently leads to tissue formation[1,5]. Numerous kinds of scaffolds have been fabricated using polymer, ceramic or natural based materials for example poly (lactic acid) (PLA), poly (glycolic acid) (PLGA), hydrogel, chitosan, hydroxyapatite (HA), and so forth by various fabrication techniques[2,6]. Recently, Rapid Prototyping (RP) techniques such as Fused Deposition Modelling (FDM) has become an attractive alternative to fabricate an intricate scaffolds design for bone TE, as it allows customization of scaffold designs to treat the variable needs of patients. For instance, a section of bone defect from the patient can be imaged using Magnetic Resonance Imaging (MRI) or X-Ray computed tomography (CT scan), which can then be converted to the file format for RP manufacturing (usually in stereolithographic or .STL format). The final stage is to print a customized 3D scaffolds for that individual patient[7]. In addition, fabricating scaffolds by FDM also allows the pore/ channel networks of that particular scaffold to be designed such as to reduce resistance of mass transport by shortening diffusion paths[1].

To gain further insight into the influence of cell seeding efficiency on 3D scaffolds, we firstly fabricated two different designs of three-dimensional printed (3DP) hybrid scaffolds via FDM method followed by polyelectrolyte multilayers (PEMs) coating as described in our previous report[5]. We cultured human bone marrow derived mesenchymal stem cells (hMSCs) on the fabricated scaffolds to demonstrate the optimal cell seeding efficiency by investigating the effect of culture method (i.e., one-sided and two-sided seeding, coated and non-coated plate and cell volume suspension) and culture conditions (static and rotary bioreactor) on cell adhesion and viability.

## 2. Experimental

In this study, two different scaffold designs were fabricated by FDM technique followed by PEMs in order to produce 3DP hybrid scaffolds. Commercially available scaffolds of pure sintered HA scaffolds were used as the experimental control. The basic properties of commercial HA, two channels (designated as 2C) and four channels (designated as 4C) hybrid scaffolds are summarized in Table 1.

**Table 1.** Summary of basic properties of commercial HA, 2C and 4C hybrid scaffolds

| Scaffolds/ Properties | Commercial HA | 4C | 2C |
|---|---|---|---|
| Diameter (mm) | 10 | 10 | 10 |
| Thickness (mm) | 2 | 2 | 2 |
| Pores/ Channels size (μm) | 200-300 | 1500 | 1500 |
| Porosity (%) | 60 | 40 | 20 |
| Nature of materials | 100% HA | SiCHA/ Collagen type I/ Hyaluronic acid coated on PLA | SiCHA/ Collagen type I/ Hyaluronic acid coated on PLA |

### 2.1 Scaffolds fabrication
The 3DP scaffolds were initially fabricated by 3DP technique via FDM method using a synthetic polymer, Poly (lactic acid) (PLA) resin (Product code: 4032 D) purchased from NatureWorks® LLC (United States). Scaffolds were printed using Ultimaker 2 from Ultimaker (United Kingdom). Two different structural designs were fabricated i.e., the two- and four channel scaffolds and the basic properties of the fabricated scaffolds are shown in Table 1. The fabricated scaffolds were coated using Polyelectrolyte Multilayers (PEMs) technique using silicon carbonated hydroxyapatite nanopowders (SiCHA), collagen type I and hyaluronic acid as described in our previous report[5]. SiCHA nanopowders were firstly synthesized based on nanoemulsion method followed by calcination at 500°C, which we have developed earlier[8].

### 2.2 Cell culture and seeding
Human bone marrow derived-mesenchymal stem cells (hMSCs) obtained from a 24-year old male (Lonza, United States) were expanded until passage two when the required cell number was obtained. Cells were cultured and incubated at humidified environment at 37°C with 5% $CO_2$ in proliferation media (PM) consists of 4.5 g/L Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, Penicilin-Streptomycin and Fetal Bovive Serum (FBS; Biosera labtech, United Kingdom). All other reagents were purchased from Lonza, United Kingdom.

Prior to cell seeding, the fabricated 3DP hybrid scaffolds were initially sterilized for three times in the UV Chamber for 90 seconds each cycle followed by pre-wetting in PM for three hours before cell seeding. Commercial hydroxyapatite (HA) scaffolds were used as control samples and these scaffolds required longer soaking in PM (72 hours) as recommended by the manufacturer (Ceramisys, Sheffield, United Kingdom). Pre-wetting is a crucial step to improve the seeding efficiency by promoting a formation of thin layer of proteins which are required for cell adhesion to the scaffolds. Scaffold seeding was carried out in 24 well of coated and uncoated cell culture well plates. The coated well plate used was a 24 well plate coated with 1% w/v Pluronic F-127 (Sigma-Aldrich, United States), to prevent the cell from attached to the well plate. As control, the uncoated well plate (ordinary 24 well culture plate) was used. After cell counting, 100 000 cells were seeded onto each scaffold in the wells. To choose the right seeding method, cells were seeded by two ways i.e. (1) seeded the total amount of cells only one side; (2) seeded half of the amount on one side, incubate for 3 hours and repeat these steps on the other side before toping up with proliferation media. The cell volume suspensions were also optimized using two different volume suspensions namely, 20 and 40 µL. The cellular scaffolds were then cultured for 24 hours at 37°C and 5% $CO_2$ in humidified atmosphere.

### 2.3 Cell seeding efficiency
Cell seeding efficiency of hMSCs on three different designs of the scaffolds was determined through the observation of their adhesion and viability capability.

*2.3.1. Cell adhesion.* Seeding efficiency was calculated using the following formulation:

$$Cell\ seeding\ efficiency\ (\%) = \frac{n_i - n_f}{n_i} * 100$$

where, $n_i$ is the cell number seeded and $n_f$ is the cell number adhered to the scaffolds after 24 hours incubation at 37°C and 5% $CO_2$ in humidified atmosphere, respectively.

The cell adhesion throughout the scaffolds was observed using MTT, (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. The reagent is reduced to a crystalline, purple salt by viable cells. After 24 hours of incubation, media was removed from samples and they were washed in PBS. Samples were then stained with 500 µL of MTT reagents and incubated for 1.5hours, washed with 1.0 mL of PBS once and immediately imaged using a bright field microscope AMG-EVOS X1 CORE at magnification of 10X.

*2.3.2. Cell viability.* The cell viability was observed using Confocal Laser Scanning Microscope (CLSM) Olympus Fluoview FV 1200 with Fluoview Version 4.1 software (Olympus, UK). The viability of the cells was assessed after 24 hours using the Live/Dead Assay Kit (Invitrogen, United Kingdom) according to the manufacturer's instructions. Calcein-AM ester was used to fluorescently label viable cells (green); the nucleus of dead cells is labelled with Propidium Iodide (red). Briefly, cell culture media was removed from samples. They were washed with PBS then immersed in a PBS staining solution containing 10 µM Calcein-AM and 1 µM Propidium Iodide and incubated at 37°C for 20 minutes in the dark. The samples were then washed once with 1.0 mL of PBS and immediately imaged using CLSM.

## 3. Results and discussion

The concept of Bone tissue engineering (BTE) holds great promise for the treatment of clinical defects. However, much optimization is required before BTE can be broadly applied clinically. This study evaluated various cell seeding methods on the different designs of 3D scaffolds i.e. the HA, 2C, and 4C seeded with different volume of cell suspensions (20 and 40 µL) in coated and non-coated well plates (Table 2).

**Table 2.** Percentages of cell attachment onto the scaffolds with different cell volume suspensions on different scaffold designs

| Scaffolds | Coated well plates | | Non-coated well plates | |
|---|---|---|---|---|
| | 20 µL | 40 µL | 20 µL | 40 µL |
| HA | 55% | 47% | 46% | 45% |
| 2C | 87% | 80% | 56% | 48% |
| 4C | 85% | 77% | 60% | 52% |

The results suggested that scaffolds seeded with small cell volume suspension of 20 µL have higher percentages of cell attachment onto the scaffolds as compared to 40 µL cell volume suspension. Regardless of the scaffold designs, lower cell attachment was found on scaffolds seeded in the non-coated well plates. In order to further optimize the seeding efficiency, scaffolds were then seeded in two different methods using 20 µL volume suspension and coated well plates. Using small volume cell suspension allows the cell to retain on the surface of the 3DP hybrid scaffolds and in the porous structure of the HA scaffolds. While, larger volume of cell suspension could easily flow through the channels/pores of the scaffolds and cells would then rather adhere to the well plate than to the scaffolds. Thus, smaller volume suspension was found to be better on a small scaffold in order to avoid overflowing of the cell suspension around the scaffold, which could lead to the loss of cells that should attached to the scaffolds.

The percentages of cell attachment was found to be higher on 3DP hybrid scaffolds as compared to the HA scaffolds. This showed that the coating materials used to fabricate the 3DP hybrid scaffolds were more suitable for cell attachment than pure HA scaffolds. This proved the role of collagen as one of the elements in the coating materials as demonstrated in our previous study[5]. The presence of collagen on the 3DP hybrid scaffolds has effectively contributed towards better support for cell attachment[9]. While, pure HA scaffolds needed longer pre-wetting time with higher concentration of serum (20% FBS) before seeding compared to the 3DP hybrid scaffolds. This was to create a thin layer of protein on the surface as to provide better cell attachment.

To further improve the seeding efficiency, scaffolds were seeded with hMSCs on different culture substrates. Pluronic F-127 has been used as biological coating in particular to prevent cells adherent to the culture well plate[10]. Pluronic F-127 is a polypropylene oxide-polyethylene oxide (PPO-PEO) tri-block copolymer with two hydrophilic ethylene oxide chains and a hydrophobic propylene oxide chain in the middle, which provides Pluronic F-127 with very flexible molecular chains and high capacity to hydration[11]. Studies have shown that coating substrates with Pluronic F-127 reduces protein and hence cell adhesion, which prevents seeded cells from adhering to the substrate. The results obtained shows that hMSCs seeded on all scaffold designs in the coated well plates resulted in higher percentages of cell attachment compared to the non-coated well plates. This finding highlighted the beneficial use of Pluronic F-127 in improving seeding efficiency.

Seeding method is another crucial key towards efficient seeding. The distribution of cells on each scaffold seeded by one-sided and two-sided method was analysed using MTT stains (Figure 1). It was found that cells were more homogenously spread over the entire scaffolds when hMSCs were seeded on the scaffolds by the two ways method. The use of two ways seeding method is indirectly helping the cells to migrate faster throughout the entire scaffolds. While, the one way seeding method requires longer time for the cells to migrate and infiltrate from one side of the scaffold to the other[12].

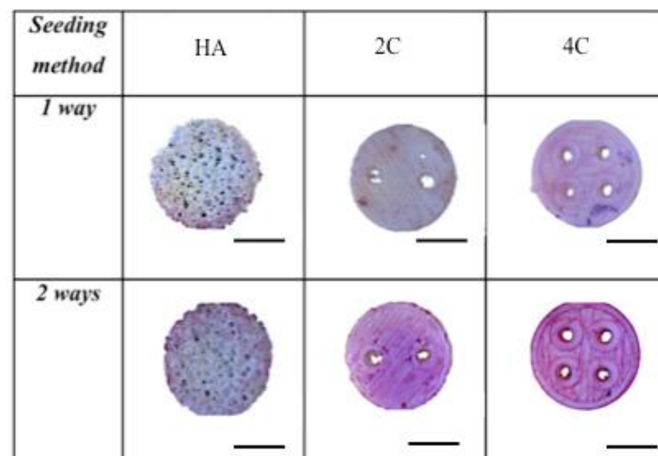


**Figure 1.** Cell distribution on different scaffold designs seeded with one-sided and two-sided seeding method. Scale bar = 1 mm.

The final part of our study investigated on the effect of culture condition on cell seeding efficiency. Rotary bioreactor was used in this study to investigate the impact of a microgravity environment on cells fate upon cultured on different scaffold designs. The aims of using this type of bioreactor were to minimize shear force and maximize the fluid flow throughout the scaffolds to provide enhanced mass transfer across large scaffolds for BTE[4]. Live/dead staining observation revealed that higher proportions of viable cells were found when scaffolds were cultured at 20 rpm compared to 40 rpm. This indicates that the lower rotational speed allows for a better cell attachment across the scaffolds whilst the cells tend to detach from the scaffolds when they were rotated at higher speed. At a higher speed of 40 rpm, scaffolds started to collide with each other and the wall of the chamber, resulted in some loss of cells.

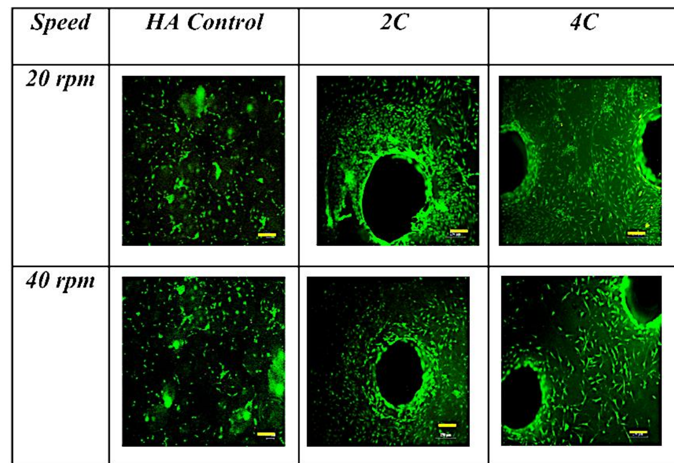


**Figure 2.** Cell viability on different structural designs of 3DP hybrid scaffolds at 20 and 40 rpm, respectively. Results suggested more viable cells were found as cells were cultured at 20 rpm where the scaffolds were free floating in the rotary bioreactor. Scale bar= 500 µm.

## 4. Conclusion

Five main parameters improving cell seeding efficiency were established in this study: (1) suitable composition of the tested scaffolds mimicking bone mineral content, (2) small volume of cell suspension, (3) using coated well plates during cell seeding, (4) seed cells by the two-sided method and (5) controlled speed of rotary bioreactor.

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