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3D Tissue Scaffold Printing On Custom Artificial Bone Applications

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Abstract: Production of defect-matching scaffolds is the most critical step in custom artificial bone applications. Three dimensional printing (3DP) is one of the best techniques particularly for custom designs on artificial bone applications because of the high controllability and design independency. Our long-term aim is to implant an artificial custom bone that is cultured with patient's own mesenchymal stem cells after determining defect architecture on patient's bone by using CT-scan and printing that defect-matching 3D scaffold with appropriate non-toxic materials. In this study, preliminary results of strength and cytotoxicity measurements of 3D printed scaffolds with modified calcium sulfate composite powder (MCSCP) were presented. CAD designs were created and MCSCP were printed by a 3D printer (3DS, Visijet, PXL Core). Some samples were covered with salt solution in order to harden the samples. MCSCP and salt coated MCSCP were the two experimental groups in this study. Cytotoxicity and mechanical experiments were performed after surface examination withscanning electron microscope (SEM) and light microscope. Tension tests were performed for MCSCP and salt coated MCSCP samples. The 3D scaffolds were sterilized with ethylene oxide gas sterilizer, ventilated and conditioned with DMEM (10% FBS). L929 mouse fibroblast cells were cultured on scaffolds (3 repetitive) and cell viability was determined using MTT analysis. According to the mechanical results, the MCSCP group stands until average 71,305 N, while salt coated MCSCP group stands until 21,328N. Although the strength difference between two groups is statistically significant (p=0.001, Mann-Whitney U), elastic modulus is not (MCSCP=1,186Pa, salt coated MCSCP=1,169Pa, p=0.445). Cell viability (MTT analysis) results on day 1, 3, and 5 demonstrated thatscaffolds hadno toxic effect to the L929 mouse fibroblast cells. Consequently, 3D printed samples with MCSCP could potentially be a strong alternative (biocompatible) for current custom made scaffolds. Desired strength can be acquired with cell inoculation and cultivation of samples in a bioreactor for ossification.

Kişiye Özel Yapay Kemik Uygulamaları için 3B Yazdırma Tekniği Kullanılarak Doku İskelesi Oluşturulması

Anahtar Kelimeler
3B Yazdırma
Yapay Kemik
Doku İskelesi
Biyomekanik

Özet: Kişiye özel yapay kemik uygulamalarında kritik basamak, defekte uygun iskelenin üretilmesidir. Tasarım özgürlüğü ve yüksek kontrol edilebilirlik nedeniyle, 3B yazdırma bilhassa kişiye özel uygulamalar için en uygun yöntemdir. Bu amaçla, 3B yazdırma tekniği kullanılarak mekanik özellikleri kemiğe uygun, toksik olmayan ve kemik doku oluşumunu destekleyecek iskele üretimi oldukça

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Doku Mühendisliği
Biyomedikal Mühendisliği

önemlidir. Uzun vadede hedefimiz CT taramayla hedef dokuda belirlenen defekte uygun geometride, uygun malzemeyle 3B iskele oluşturulması ve üzerine kişiden alınan mezankimal kök hücre ekilmesi ile oluşturulan nihai kemik dokunun hastaya aktarılmasıdır. Çalışmamızda, modifiye toz kompozit kullanılarak 3B yazdırılmış iskelelerin mukavemet-sitotoksisite ölçümlerinin sonuçları sunulmuştur. Bilgisayarda oluşturduğumuz 3B tasarımlar, modifiye bir kompozit toz kullanılarak 3B yazıcı ile yazdırılmıştır. Örneklerin yarısı sertleştirmek için tuz çözeltisi ile kaplanıp kurutularak iki deney grubu oluşturulmuş, SEM ve ışık mikroskobu altında yüzey özellikleri incelendikten sonra sitotoksisite ve mekanik testleri yapılmıştır. Çekme testleri kontrol grubunda 6, tuzlu grupta 7 tekrarlı yapılmış, elastik modül hesaplanmıştır. Sitotoksisite için 3B iskeleler etilen oksit gaz sterilizatörüyle sterilizayona tabi tutulduktan sonra havalandırılmış ve DMEM (%10 FBS) ile şartlandırılmıştır. L929 fare fibroblast hücre hattı kullanılarak, iskelelere üç tekrarlı ekimler yapılmış ve MTT ile hücre canlılığı belirlenmiştir. Mekanik test sonuçları incelendiğinde kontrol grubunun ortalama 71,305N'a (n=6) dayanabilmekte olduğu, tuzlu grubun ortalama 21,328N'a (n=7) dayanabilmekte olduğu gözlemlenmiştir. Her ne kadar dayanıklılık açısından istatistiksel olarak anlamlı bir fark bulunmuş (p=0,001, Mann-Whitney U) olsada, elastik modülleri arasındaki fark istatiksel olarak anlamlı bulunmamıştır (kontrol=1,186Pa, tuzlu=1,169Pa, p=0,445). MTT sonuçları incelendiğinde de her iki deney grubundaki iskele malzemelerinin toksik olmadığı, 1. 3. ve 5. gün analizlerine göre hücre canlılığının olumsuz etkilemediği görülmüştür. Dolayısıyla, modifiye toz ile 3B yazdırılmış numunelerin sitotoksik açıdan uygun olduğu (biyouyumlu) gözlemlenmiştir. Hedeflenen mukavemete kemik hücreleri ekimi ve numunenin biyoreaktörde kemikleştirilmesiyle ulaşılabileceği düşünülmektedir.

1. Introduction

Many different bone diseases such as bone infections, fractures and osteoporosis are more frequently seen due to the rise in the average age of population or traumatic reasons (Rauh et al., 2011; Bose et al., 2012). Bone can not manage to heal itself when a critical size defect occurs (Lichte et al., 2011). Therefore, in order to treat bone defects, 4million bone grafting or substitutes are performed in the world annually (Brydone et. al, 2010). Autografts and allografts are used for treating critical size defects, however they have some limitations and risks such as donor site morbidity, high infection risk and immune response (Inzana et al., 2014). At this point, tissue engineering (TE) offers different strategies for new bone formation. TE basically involves three main components in convenient environment; scaffold, cells and growth factors (Eslaminejad and Faghihi, 2011).

Scaffolds should have similar mechanical properties with bone (tension, compression, modulus) (Table 1), appropriate physical properties (pore size; macro pore size >100 μm, microporosity pore size < 20 μm (Bose et al., 2012), pore density), compatible biological properties for cells (non-toxic, biodegradable), moderate manufacturing conditions and low cost (Rauh et al., 2011).

Bone structure mainly composes of 10% water, 60% inorganic (Keaveny et al., 2004) and nearly most of the rest (organic) is collagen (95% of it is type I collagen (Carrin et al., 2006)).

Table 1. Anisotropic and Asymmetrical Ultimate Stresses and Elastic properties of Human Femoral Cortical Bone (Keaveny et al., 2004; Reilly et al., 1975)

Longitudinal (MPa)	
Tension	135 (15.6)*
Compression	205 (17.3)
Modulus	17900 (3900)
Transverse (MPa)	
Tension	53 (10.7)
Compression	131 (20.7)
Modulus	10100 (2400)
Shear (MPa)	65 (4.0)

*Standard deviations are given in parentheses

As collagen has high cellular activity, inorganic compounds can be used to enhance mechanical properties of the scaffolds (Ahn et al., 2012). Inorganic materials such as metals, ceramics, bioglasses and organic materials such as polymers are used as scaffold materials (Lichte et al., 2011). Since natural bone has 85-90% calcium phosphate (CaP) of dry inorganic part, ceramics have high potential to be used as inorganic compoundsto develop bone scaffolds (Lichte et al., 2011). Osteoconduction and osteoinduction are required for bone regeneration. While osteoconductivity is a property that allows bone cells to adhere and proliferate, osteoinductivity is the ability of materials

to induce bone formation with molecular signaling (Tangri et al., 2004; Boseet al., 2012). Calcium sulfate (CS), β-tricalcium phosphate (β-TCP) and hydroxyapetite (HA) are three typical ceramic bone engineering materials which are osteogenic, biocompatible, bioresorbable and bioactive. These materials also provide good structural support and sufficient porosity (Bulut and Karakurt, 2011; Lichte et al., 2011; Tangri et al., 2004; Wu et al., 2012).

Polymeric additive containing HA granulates were used in a bone tissue engineering study (Leukers et al., 2005). In this study, CAD data were used to print the scaffold, air was blowed slightly to remove unbound powder, and the scaffold was sintered 2h at 1300^{o} C as a post process. 500 μm diameter pore size was obtained. Bone formation requires minimum pore size between 100-150 μm, however it was reported that pore sizes larger than 300μm enhanced bone formation and vascularization (Karageorgiou and Kaplan, 2005). Leukers et al. (2005) observed that 3D printed HA scaffolds were appropriate for bone replacement. Becker et al. showed that 3D printed HA blocks supported capillary-vessel formation and osteoconduction (Becker et al., 2012).

HA and β-TCP were used as CaP powder by Zhou et al. CaP powders were blended with $CaSO_4$ (25:75 wt.% and 50:50 wt.%). They used water-based binder not to reduce working life of the print head, although they mentioned the reinforcement impact of acidic binders. Better compressive strength wasachieved in the samples with higher CaP:$CaSO_4$ ratio, and HA:$CaSO_4$ powders showed better results than β-TCP:$CaSO_4$ powders. Neither of the CaP powders showed any cytotoxicity. This study showed that the 3D printability of CaP and $CaSO_4$ powders and their combinations with a water based binder (Zhou et al., 2014).

Inzana et al. (2014) studied with 3D printed calcium phosphate (CaP) powder with 1-2% collagen for bone regeneration. CaP was used because of its good osteoinductivity and sufficient mechanical strength, and collagen was used for maximum flexual strength and cell viability. They had four groups; 3D printed CaPs (solely), with 1 wt.% collagen printed (as a binder), with 2 wt.% printed (as a binder), and 0.5 wt.% collagen coated. It was shown that adding collagen to the binder solution enhances the strength of CaP. Highest mechanical strength was seen in CaP printed with 2 wt.% collagen. However, collagen coating produced best results in relative cell viability (viable/dead signal) although 3D printed CaP with 1 wt.% collagen had higher viability than sole CaP (Inzana et al., 2014).

Another ceramic; calcium sulphate hemihydrate (CSH) was studied by Sidqui et al. (1995). It was reported that osteoblastic cells attach on CSH and osteoclasts resorb this material (Sidqui et al.,1995). Wu et al. (2012) demonstrated that addition of amorphous calcium phosphate (ACaP) to calcium sulphate improved osteoconductivity because of the reduction in the resorption rate. They produced synthetic bone graft substitutes (SBGSs) and demonstrated that degradation period of this composite matched with natural bone regeneration rate (Wu et al., 2012).

All these studies support the convenience of ceramic usage as scaffold material in bone tissue engineering. Different ceramics can be combined in different ratios to get better mechanical and biological results. Electrospinning, molecular self-assembly, salt leaching, gas foaming, freeze drying are typical scaffold manufacturing techniques for variable applications (Andrews et al., 2011; Chung and Park, 2007; Subia, et al., 2010). However, these techniques would not let well-defined custom design scaffold fabrication, and manufacturing scaffolds instantly is not possible. SFF offers more geometrical flexibility (free-form) and has broad materials choice. 3D objects can be produced rapidly and with high reproducibility by SFF (Subia et al., 2010). Solid free form is basically manufacturing by additive layers, so it can be called additive manufacturing (AM) at the same time. Many techniques including selective laser sintering (SLS), stereolithography (SLA), fused deposition modelling (FDM), direct metal laser sintering (DMLS), electron beam melting (EBM), 3DP are available for AM (Wong and Hernandez, 2012; Li et al., 2014). These processes can be categorized according to the phases of the raw materials used. If raw material is in liqid form FDM or SLA can be used (Kruth, 1991). If it is possible to have desired material in powder form of suitable size, 3 dimensional printing (3DP) can be used as a scaffold manufacturing method (Butscher et al., 2013). 3D printing is one of the best techniques particularly for custom designs on artificial bone applications because of the high controllability and design independency. 3D images of desired structures are needed in 3D printing process. These images can be designed in CAD software (as Solidworks or Inventor); also they can be captured from 3D scanning like computed tomography (CT-scan) or magnetic resonance imaging (MRI) (Becker et al., 2012). We aim to detect bone defect with CT-scan and get its 3D images to produce defect-matching scaffolds so that 3DP application can support our custom artificial bone study.

In powder based 3DP, one thin layer of powder material is dispersed on a platform, following that the binder is sprayed onto the powder. The binder ties up the powders to form a layer. This process is repeated many times to produce layer by layer structures (Lichte et al., 2011). 3DP is composed of four main components; material, binder, cells and printer. Four types of liquids can be used as binder; water solutions suspended with polyvinyl materials, chloro

form solvent, acidic solution, and water-based liquids (Zhou et al., 2014). Appropriate binder is chosen according to the scaffold material.

At the end of the printing process, it is possible to apply some post processes to increase the quality of the scaffolds. Post-printing manipulation, depowdering, coating, sintering and infiltration are some of the post process applications. Depowdering is the removal of loose powder with brushing, blowing air, vacuuming, vibration, or wet depowdering (ultrasonicating, microwave-induced boiling and CO_2 bubble generation in soda water). Coating is usually done with a polymer-particle paste or slip casting to improve surface properties. Sintering and infiltration is applied to increase the strength of structures. In sintering, scaffolds are exposed to high temperature and they shrink to consolidate. Dipping part, aerosolizing infiltrant, spraying the part are infiltration techniques to achieve high density structures without large shrinkage (Utela et al., 2008). Inzana et al. (2014) used 8.75% acid solution to maximize cytocompatibility and mechanical strength.

After printing of defect matching scaffolds (captured with CT scan) and post-processes (if needed), the scaffolds need to be seeded with osteoblastic cells or mesenchymal stem cells (MSC). MSCs have high osteogenic differentiation potential and it is easy to access autologous MSCs from patients' own tissues; also MSCs don't cause any immune responses or rejection (Eslaminejad and Faghihi, 2011). So using patient's own MSCs has many advantages.

If we consider that human body have 206 bones in specific sizes, shapes, compositions and roles (Brydone et. al, 2010), we should choose a moderate start point for our study. This study focuses mainly on the evaluation of MCSCP for on custom artificial bone applications. First, scaffolds were designed with CAD software to be printed for mechanical, cytotoxicity tests and SEM examinations. MCSCP scaffolds were manufactured with 3DP technique. Some samples were covered with salt solution in order to harden the scaffolds. Salt coated and regular MCSCP were the two experimental groups in this study. Cytotoxicity and mechanical experiments were performed after surface examination with scanning electron microscope (SEM). Cytotoxicity evaluation was carried out at day 1, 3 and 5. Additionally, scaffold microstructure, pore size and cell attachment success was determined by SEM examination. Tensile stress and Young's modulus were measured for mechanical assessment of MCSCP.

2. Materials and Methods

2.1. Design and fabrication

One more CAD software (Solidworks, Inventor) was used to design scaffolds. Samples were produced as 5x5x5mm bars for scanning electron microscopy (SEM), as 10x10x5 mm bars for cell viability testing and in special dogbone shape for mechanical testing as shown in the Figure 1.

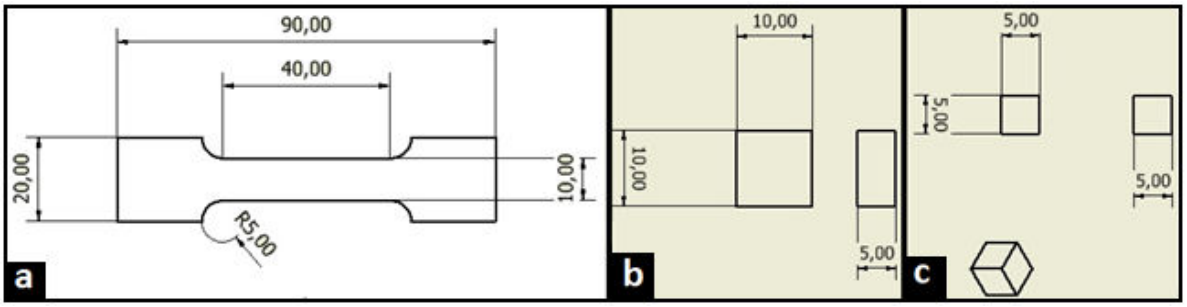

Figure 1. Dimensions of the (a) tensile test specimen, (b) cell viability test specimen, (c) SEM specimen

The designs were exported in rapid prototyping format (.stl) and imported to 3D printing software. Then 3D designs were printed layer by layer with a 3D printer (3DS, Visijet, PXL Core). One thin layer of powder material was dispersed on a platform, following that, the binder was sprayed onto the powder. The binder ties up the powders together to form a stable layer. This process is repeated many times to produce layer by layer structures. Modified Calcium Sulfate Composite Powder (MCSCP) was used as the powder material, and 2-pyrrolidone was used as the binder.

After printing process, samples were depowdered by blowing air to get rid of loose powders as a post process. All surfaces of one half of the test samples were sprayed with 44% Epsom salt (magnesium sulfate) solution and dried in air for 2 days for hardening, so that we have two test groups: MCSCP and salt coated MCSCP for mechanical and toxicity tests.

2.2. Analysis and testing

2.2.1. Mechanical tests

Mechanical properties of MCSCP such as tensile strength and Young's modulus were examined using a computer-controlled Shimadzu Autograph AG-IC Series universal testing machine (Shimadzu Corp., Japan) equipped with a 500N load cell. Trapezium X software was used for machine control and data acquisition.

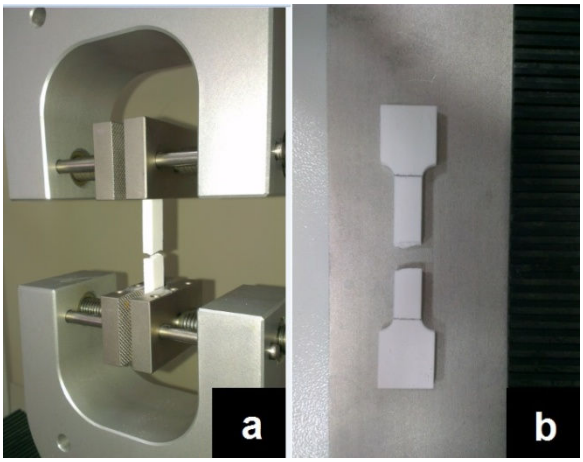

Figure 2. Illustration of tensile testing; breaking off view.

Tensile tests were carried out on the test machine at cross-head speed of 2mm/min at room temperature. The specimens had a length of 90 mm and width of 10 mm (Figure 1a). The average values of MCSCP and salt coated MCSCP samples were reported in the study (n=6 for both).

2.2.2. Scanning electron microscopy (SEM)

Special SEM specimens (5mm length, 5mm width and 5mm height) were printed for cell seeding and imaging process with the SEM that is used for particle size, microstructure characterization of the material and the scaffold. The cell morphology of the L929 cells on scaffold surfaces after cell culture for 5 days were observed using scanning electron microscopy (SEM, JSM-6060 JEOL). The media were aspirated from culture plate wells; the scaffolds were washed with 0.1 M sodiumcacodylate (Sigma Chemical Company, St. Louis, USA) buffer and fixed in 25% glutaraldehyde (Merck, Germany) in 0.1 M sodiumcacodylate at 4 °C for 30 minutes, and post fixed in 1% osmium tetroxide in 0.1 M sodiumcacodylate at 4 °C for 30 min in dark. Following a buffer rinse, the samples underwent gradual dehydration in an ethanol series. Finally, samples were placed in hexamethyldisilazane (HMDS, Sigma Chemical Company, St. Louis, USA) for 30 min, allowed to dry overnight, and stored in a desiccator. Samples were sputter-coated with gold–palladium prior to imaging.

2.2.3. Viability of cells cultured on 3D printed materials

Cell Culture

Mouse fibroblast (L929) cells were obtained from the stocks of Animal Cell Culture and Tissue Engineering (EGEREACT) Laboratories, Bioengineering Department, Ege University, Turkey. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biochrome, Merck Millipore, Germany), 10 mg/mL gentamicin (Gibco, BRL) and 2 mM L-glutamine (Gibco, BRL) at 37 °C in a 5% CO_2 humidified environment. After a subconfluent monolayer was achieved, cells were detached by gentle digestion with 0.25% trypsin/EDTA (Sigma Chemical Company, St. Louis, USA), counted with a hemocytometer, and suspended in fresh media before seeding onto scaffolds.

Cell Culture on 3D Printed Scaffolds

Prior to cell seeding, scaffolds were immersed in non-sterile phospahte buffered saline (PBS, Sigma Chemical Company, St. Louis, USA) overnight. After washing with PBS three times, they were treated with UV for 4 hwith UV sterilizer (Goldterm, Turkey). Then, they were submerged in PBS again for overnight and dried. At the end of this, scaffolds were sterilized with ethylene oxide gas sterilizer for 3 hat 54°C (AXIS, AX-60 & AX 135) and ventilated for 72h. Scaffolds were placed in 24-well plates (Costar, Corning, USA) that were coated with 1% agarose (w/v, Sigma Chemical Company, St. Louis, USA) and were conditioned with complete culture medium for 2 hat 37 °C. Next, cells were harvested from cell culture plate and counted with a hemocytometer. 50 µL cell suspension was dropped onto each scaffold that have 1 cm^2 surface area (10mm length, 10mm width and 5mm height) with $5x10^4$ cells/cm^2density and to achieve attachment on the scaffold, the cells were cultured at 37 °C in a 5% CO_2 humidified environment for 30 minutes. After 30 minutes, 1 mL complete medium was added on each well.

MTT Assay for Cell Viability

L929 cell proliferation and viability on the scaffolds were quantitatively evaluated *via* MTT assay (Jin et al., 2008). The assay is dependent on the cellular reduction of MTT [3-(4,5-dimethylthialzol-2-yl)-2,5-diphenyltetrazolium bromide] by the mitochondrial dehydrogenase enzymes of viable cells, to a blue formazan product, which can be measured by spectrophotometer (Suslu et al., 2014). The quantity of purple formazan crystal formation is proportional to the amount of viable cells. 1, 3, and 5 days after cell seeding, culture medium from each well was aspirated and replaced by 1 mL per well of MTT solution (5 mg/mL in DMEM without serum) for each scaffold in 24-well culture plates (Costar, Corning, USA). The plates were incubated for 3.5 h at 37 °C. The media were aspirated and the cells were then

lysed using 600 µL per well of dimethylsulfoxide (DMSO) to release and solubilize formazan crystals. After 15 min. of rotary agitation, 100µL DMSO solution from each sample, which contained dissolved formazan crystals, was transferred into 96-well-plates to read absorbance at 570 nm (690 nm ref.). UV/visible spectrophotometer (Molecular Devices-Versa Max) was used for measurement of absorbance. Amount of viable cells that correlated with the absorbance was determined *via* calibration curve prepared as a cell number/mL unit.

3. Results

3.1. Mechanical tests

Table 2 shows all mechanical test results data that were captured from measurements on MCSCP and salt coated MCSCP samples. According to results, it is seen that MCSCP's mechanical properties are better than salt coated MCSCP. Elastic modulus (E) and max force (F) values were obtained from stress/strain curve that was recorded at the time of measurements. MCSCP group stayed intact until average maximum force reached 71,305N (n=6) and salt coated MCSCP group stayed intact until average maximum force reached 21,328N force (n=7). On the other hand, the difference in elastic moduli (E) was not significant like the difference in maximum force. E values of MCSCP and salt coated MCSCP samples were 1,186Pa and 1,169Pa, respectively.

Table 2. Max. force and elastic modulus values of samples

MCSCP		SALT COATEDMCSCP	
MaxForce(N)	**E(Pa)**	**MaxForce (N)**	**E (Pa)**
58,08	1,380	20,03	2,195
51,42	0,525	8,63	0,972
64,41	0,993	14,90	2,080
78,20	0,708	30,48	1,556
103,00	1,392	23,08	1,169

Although differences in maximum forces between two main groups were statistically significant according to Mann-Whitney U test (p=0.001), differences in elastic modulus (E) between two main groups were not statistically significant (p=0.045).

3.2. Cell Viability Assay

L929 mouse fibroblastic cells were seeded on to MCSCP and salt coated MCSCP scaffolds and cell viability were measured by MTT assay. MTT assay results are shown on Figure 3. These results demonstrated that cell number on MCSCP scaffolds significantly increased from day 1 to day 3 (p = 0.01<0.05).

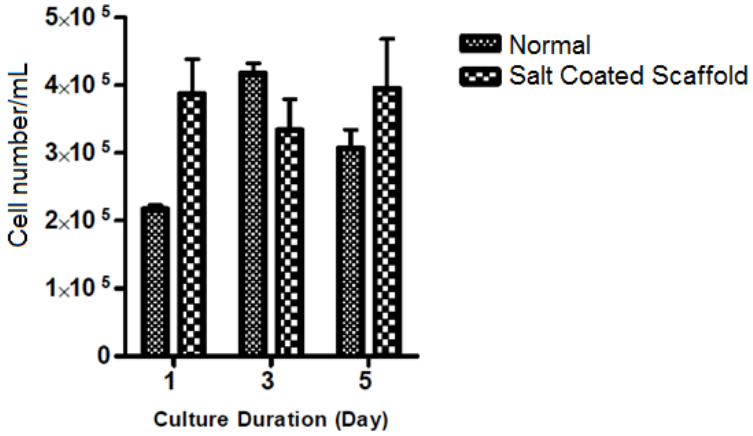

Figure 3. Cell viability on MCSCP scaffolds after cultivation for 1, 3 and 5 days. The experiment was repeated in triplicate (n = 3) for each sample. Data are shown as averages with the error bars indicating standard deviation, * p<0.05.

These results confirm that L929 cells were attached and proliferated on MCSCP scaffolds without facing any toxic effect. However, there were no significant difference observed on salt coated MCSCP compared to MCSCP scaffolds (p = 0.25>0.05). Cell numbers significantly decreased from day 3 to day 5 on MCSCP scaffolds (p = 0.25>0.05); however no significant decrease was observed on salt coated MCSCP scaffolds (p= 0.3>0.05). The comparison of cell numbers on MCSCP and salt coated MCSCP scaffolds on day 3 and day 5 did not show any significant difference. However on day 1, significant difference was observed between the two groups; but the difference might be caused by experimental error because the day 1 result on salt coated scaffold were almost twice as much as the cell number compared to initial cell number seeded on the scaffolds. These results proves that MCSCP scaffolds were biocompatible, however salt coating did not show any further benefit in terms of cell viability on the scaffolds.

3.3. Scanning electron microscopy (SEM)

Non-coated & cell free scaffolds (Figure 4) and non-coated scaffolds with cells (Figure 5) were examined with SEM to evaluate particle size, microstructure characterization of the material and the scaffold.

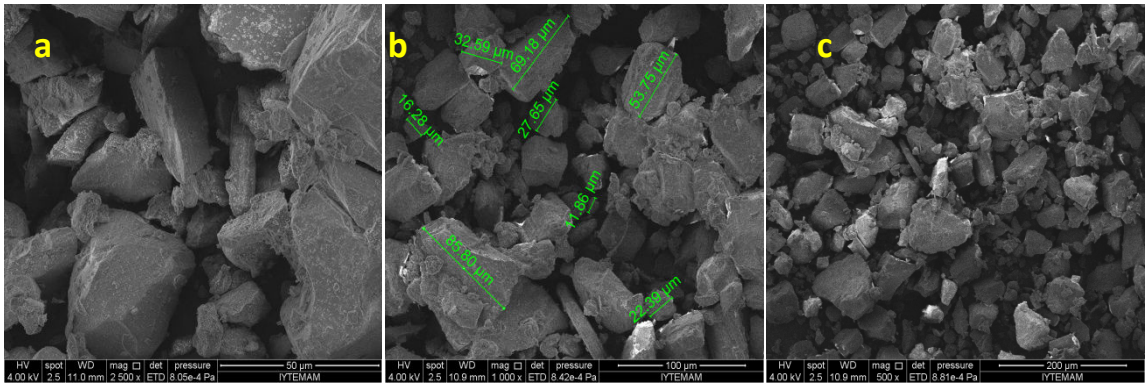

Figure 4. SEM images of non-coated, cell free scaffolds a. 2500x b. 1000x c. 500 x

Scaffold surface is shown in 2500x, 1000x, 500x magnifications in Figure 4. Average particle size was measured as 39,94 µm. Particle size larger than 20µm facilitate fluid migration (Butscher et al., 2011).

Non-coated scaffold with cells is shown 5000x magnitude in Figure 5. L929 mouse fibroblastic cells were attached to the MCSCP scaffold properly.

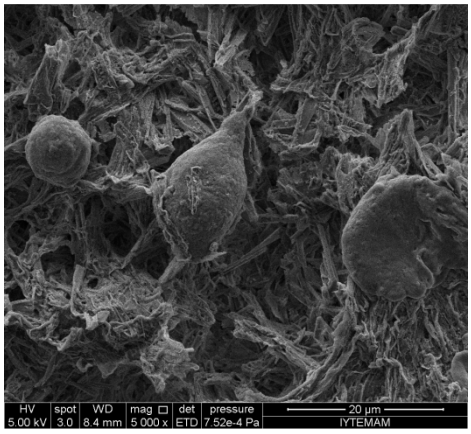

Figure 5. SEM images of non-coated scaffolds with cells, 5000x

4. Discussion and Conclusion

In this study, MCSCP was evaluated as a bone scaffold material. Salt coated MCSCP and MCSCP were investigated in this study. Mechanical test results showed that salt coating did not have any positive effect on strength of scaffolds. Moreover, salt application reduced the tensile strength of samples. This result supported the importance of convenient post-processing. Chosen technique can either increase or reduce the mechanical strength of the scaffolds. In other words, salt spraying was not an advantageous post process for this study. In future experiments, other techniques like dipping in acid solution can be applied for better results. Also, mechanical test results of MCSCP samples were not similar to mechanical properties of bone (Table 1). Therefore, some improvements such as trace element addition or additional post processing would be required to increase the strength and elastic moduli of the scaffolds. We think that, culturing cell inoculated scaffolds in custom designed perfusion bioreactors for ossification would enable bone tissue formation within the scaffolds, and make significant contribution on their mechanical properties.

The mechanical properties of 3D printed samples were aggravated by coating them with salt, because the salt hardened the samples and that made them more brittle. According to mechanical properties of the printed scaffolds, they are not as strong as bone materials. Both scaffolds were so weak in mechanical features, so the scaffolds should be reinforced by modifying the printing process to improve mechanical features of the materials. Scaffolds can be also improved by cultivating osteoblast cells on them. So, ossified scaffolds may be obtained which really improves mechanical properties.

According to biocompatibility results, scaffolds did not show any toxic effect from day 1 to day 3. The reason of the decrease on cell numbers from day 3 to day 5 on MCSCP scaffolds could be the inadequate surface area for further cell proliferation. The experiments might be repeated with less initial cell number. When we compare salt coated and normal samples, it was observed that salt coating did not show any positive effect on scaffolds in terms of cell proliferation.

The SEM image of MCSCP (without cell) demonstrated that samples have suitable particle size for fluid migration, printing resolution (average particle size ~39.94 µm). SEM images of L929 mouse fibroblast cells seeded on MCSP scaffolds (non-coated) showed complete attachment and spreadingof cells on the scaffold surface. That means particle characteristicsare suitable for tissue engineering applications. In further studies,

examination of wettening behavior, particle uniformity, and surface properties can help us to understand the material better. Also, binder characteristics will be studied.

As a result, mechanical and cytotoxicity properties of MCSCP were analyzed. It was shown that 3D printed MCSCP scaffolds were biocompatible, non-cytotoxic, and convenient for cell attachment but need some developments to enhance mechanical properties. In addition, salt coating didn't have any positive effect on our scaffolds. Therefore, other post processing options will be investigated for better results. MCSCP is a promising material for bone tissue engineering on custom artificial bone applications because of its biocompatibility, convenient microstructure and printability. However, number of measurements was not enough. Measurements should be repeated with more samples for better interpretation.

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