A vascularized bone-on-a-chip model development via exploring mechanical stimulation for evaluation of fracture healing therapeutics

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Abstract

Bone is the major connective tissue maintaining the structural integrity of the human body. However, fracture and many skeletal degenerative diseases can compromise this function. Thus, therapeutics related to bone degeneration are of significant research interest and require good in vitro models for such therapeutic evaluation. Bone is a highly vascularized tissue and incorporation of this feature is significantly important for mimicking the osteogenic microenvironment. In the current study, we developed a vascularized flat bone model via simultaneous mechanical actuation of mechanical strain and fluid shear. The mechanical strain was achieved by static magnetic field actuation of a magnetic nanocomposite scaffold. The fluid shear was generated by developing a micropattern on the magnetic nanocomposite via replica molding and laser-based microfabrication. From the live cell imaging window of the microdevice, both bone and vasculature like cellular morphology was observed. The SEM study showed thick ECM deposition in the dynamic culture. In the PCR study, both osteogenic (Col-1, osteocalcin) and angiogenic phenotypes (PECAM) were observed in the dynamic culture scaffolds while chondrogenic marker (Col-2) was downregulated.

Keywords Bone · Fracture healing · Mechanical strain

Introduction

Bone is the major load-bearing tissue of the human body that has a combination of collagen and calcium-rich extracellular matrix (ECM) and bone cells which also promotes locomotion and joint movement and controls mineral homeostasis. [1] It is also a niche for bone marrow-derived stem cells and major progenitors of blood cells. [2] Bone is majorly

composed of osteoblasts (bone-making cells), osteoclasts (bone-degrading cells), thick calcified ECM, and very rich supply of vasculatures. Bone diseases like osteoporosis, Paget's disease, osteomyelitis, rickets, and many other diseases affect this tissue and often lead a patient's structural and functional deformities. Understanding the effect of drugs on bone diseases is significantly important and often it is difficult to explore animal models.

With significant improvements in microfabrication techniques and biomaterial sciences in the last decade, effective development of in vitro models mimicking different organs has been observed. [3] Top-down approaches such as microfluidics and bottom-up approaches such as 3D bio-printing have developed in vitro models significantly capable of mimicking in vivo systems. There are ample studies available in the literature, which have been objected towards simulating the properties of native bone and its diseases. [4] Coculture-based in vitro bone models already have been explored to show the interplay between osteoblast and osteoclasts for understanding bone remodeling. [5, 6] Bone is an organ with one of the richest blood supply in the body. Hence, a major need for mimicking bone microenvironment in vitro is development of blood vessels. The incorporation

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of microfluidics into the system can mitigate this limitation of currently available bone models. Coculture of mesenchymal stromal cells (MSCs) and endothelial cells into microfluidic platforms helped in forming vascularized bone models in vitro. [7, 8] Bone diseases such as osteosarcoma, cancerous metastasis in bone, and osteomyelitis (bone infection) models have been significantly established for drug screening in vitro platforms. [9–14].

Organ-on-a-chip models require to provide different biophysical stimuli (electric field, mechanical stimulation etc.). While the entire dynamic system mostly works on the functionality of mechanical pumping systems, application of biophysical stimuli is mostly performed via incorporating direct pneumatic system or chemically deposited electrodes. However, these systems are difficult to operate as the device works with cell culture media which is highly conductive. Non-invasive actuation like piezo electric and magnetic actuation is observed to be of recent research interest for microsystem researchers. [15, 16] Magnetic actuators are easy to operate and can be used to generate strain in a controlled manner for mechanical stimulation. Recently, magnetic actuation–based mechanotransduction has been explored in organoid culture, glial cell culture, and cancer-on-a-chip models. [17, 18] We also recently reported iron oxide nanoparticle biopolymer 3D-printed nanocomposites can show osteogenic differentiation of MSCs upon external magnetic actuation in vivo. In our device, also we have explored the possibility of magnetic actuation for osteoblast like cellular phenotype establishment.

While a lot of studies focusing on in vitro models of bone cancer, bone marrow, and long bone have been carried out, flat and irregular bones were relatively neglected for in vitro model establishments. [19–22] However, trabecular flat bones take significant roles in the mechanostability of the skeleton. While they are irregular in shape and mechanically anisotropic, they take away the load from the joints and distribute them among the entire skeletal structure. [23] Diseases like osteomyelitis and Paget's disease can affect their mechanical properties and mineralization leading to mechanical failure and skeletal deformities. [24].

Nature has its method of cellular differentiation and multicellular tissue formation via biophysical and biochemical actuation. It has been observed that fluid shear stress upon MSCs can lead them towards endothelial lineage. [25] Mechanical loading and strain have been observed to be effective for the differentiation of MSCs into the osteogenic lineage. [26, 27] In the current study, we have constructed a polyacrylic-based laser-engraved microfluidic device. A micropatterned magnetic scaffold has been explored for simultaneous fluid shear and magnetic actuation–induced mechanical strain to obtain both osteogenic and endothelial phenotypes in the same platform. The shear stress is generated via gradient development in media flow and mechanical

strain is achieved by external magnetic actuation on a magnetic nanocomposite (ultrasmall super paramagnetic iron oxide nanoparticle/USPION and gelatin blend).

Materials and methods

All the materials are used as procured and not purified until mentioned. De-ionized (DI) water was used for all the work. NaOH, sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), HCl, FeCl_3 , FeCl_2 , gelatin (porcine skin), glutaraldehyde, and poly-L-lysine were purchased from Merck, India. Commercially available polyacrylic sheets of thickness 1.5 mm and 3 mm and 0.2 mm were used. 2.5-mm-inner-diameter silicone rubber tubes and 1-mm-thick silicone rubber sheets were procured from the market. 316 stainless steel hex bolts (approximately 8 cm in length and 2 cm in diameter) and matching nuts were also purchased commercially.

Synthesis and characterization of USPIONS

The citrate-capped USPION (ultrasmall super paramagnetic iron oxide nanoparticles) synthesis protocol was inspired by the method described elsewhere with certain modifications. [28] FeCl_2 powder was added to 10 mM sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$) solution (pH raised by NaOH solution to 11 and heated to the temperature of 70 °C) so that the final concentration of FeCl_2 remains 1 mM. In that warm alkaline solution, equal volume 2 mM FeCl_3 solution was added dropwise. The solution turns brownish-black. The solution was cooled to room temperature and neutralized by 1 M HCl. The nanoparticles were collected by centrifugation (1000 RCF) and dried in a vacuum oven. The synthesized USPIONS were characterized with HRTEM (JEOL, JEM-2100), XRD Bruker (Cu-K α), and SQUID (Quantum Design, USA).

Fabrication of USPION/gelatin nanocomposite micropatterned scaffold

The scaffold fabrication slurry was prepared by mixing porcine skin gelatin (20%W/V) in warm DI water. It was mixed with a mechanical stirrer continuously to prepare a homogenous slurry having a USPION concentration (10 mg/mL). The slurry was allowed to mix overnight, and bubbles were diminished by adding n-octanol before casting. The polyacrylic molds (3-mm plates) were prepared by a tabletop computer-controlled laser machine (ULS 2.30, Universal Laser System, UK). The mold had semicircular channels engraved to have features of 200 μm (final diameter) and approximate surface roughness is 20–30 μm ; both are important for creating fluid shear gradient (the entry and exit channel in the bioreactor are approximately 1 mm in diameter

and a scaffold is mostly comprising of 6 microchannels). The slurry was poured on the mold (50-mL slurry for a composite comprising of 20 scaffolds having a dimension of length 1 cm, width 1 cm, and thickness 0.5 cm) slowly and allowed to solidify in vacuum at 4 °C for 2 h. After that, the composites were peeled out of the mold carefully. The composite scaffolds were cross-linked using cold 5% glutaraldehyde (V/V) in DI H₂O in the dark environment for 6 h. The scaffolds were further treated with 10% glycine solution for neutralizing the unreacted aldehyde groups which could be potentially toxic for the biological system. The scaffolds were washed thoroughly with DI H₂O thrice and dried in a vacuum oven putting some weight on the sample to prevent the samples from buckling while drying (Fig. 1).

The scaffold fabricated was characterized using SEM (Carl Zeiss, EVO 60), Auger electron spectroscopy (ULVAC, PHI), nanoindentation (TI 950 TriboIndenter, Hysitron Inc., USA), and 3D optical surface profilometry (Bruker). The swelling ratio of the samples was measured by finding out the weight of the dry sample, putting the samples in DI H₂O, and measuring weight over a different period of wet samples. The swelling ratio is measured by the following formula:

$$\text{SwellingRatio} = (W - W_0)/W_0 * 100 [W = \text{wet weight}; W_0 = \text{dry weight}]$$

Fabrication of microfluidic device

The microfluidic device was fabricated according to the scheme presented in Fig. 2. Polyacrylic sheets (3-mm thickness) were procured from the market. These were machined using a CO₂ laser machine (ULS 2.30, Universal Laser System, UK) into different layers. 2D CAD models

were created and exported to fabricate microchannel, scaffold attachment spots, and glass windows for live-cell imaging. The sealing of the device was done by silicone rubber membranes (1 mm thick) and the entire device was firmly attached by 360 SS stainless steel screws. Silicone rubber tubing with gas permeability was utilized for media exchange and micro-infusion pumps were used flowing of the media. Plates were fabricated using laser machining including two cover plates, three structural plates (one top, one middle, and one bottom, refer to Fig. 2). The top and bottom plates have scaffold support areas and media entry and exit points. They are attached to a glass window for microscopy. The middle plate has the microfluidic channel for media transport and scaffold chambers. For sealing, silicone rubber membranes (1-mm thickness) are cut into shapes. The entire device is strongly tightened by stainless steel screws and bolts. The top and bottom chambers are covered with glass coverslips which were attached by polyacrylic dissolved with chloroform. A perfusion pump (Masterflex, Cole Parmer India) is used for media persusion.

The entire setup is kept in the CO₂ incubator (37 °C, 5% CO₂) for the complete study (2–3 weeks) except time-to-time checking under the microscope (please find the video submitted with the manuscript).

Cell culture and imaging study

The cell culture study was conducted by culturing mesenchymal stromal cells (MSCs from Wharton's Jelly, 50,000 cells/sample) on the magnetic composite scaffolds at 37 °C and 5% CO₂ using DMEM high glucose,

Fig. 1 The fabrication strategies for microchanneled magneto-active USPIO-gelatin nanocomposite by laser micromachining, replica molding, and cross-linking to prepare magneto-active 3D scaffold with microchannels

Fig. 2 The in-house fabricated bioreactor with in situ bio-imaging setup (for operation, please find the video file submitted). Polyacrylic sheets were micromachined by a laser engraving machine and they were joined by stainless screws and sealed by silicone rubber sheets cut into shapes. Microscopy windows were prepared by attaching glass coverslips

10% FBS, and 2.5% antimicrobial and antifungal solution (HiMedia). A higher concentration of antimicrobial agent application was used to minimize possibilities of infection in the bioreactor setup. 10^5 cells per scaffold were cultured. After 72 h, the scaffolds were transferred into the bioreactor, and media was flown through the samples with a flow rate of 1 mL/h using a medical-grade infusion pump. Two 0.05-T magnetic strength rare earth metal bar magnets (1 cm² area and 2-mm thickness) were attached to both sides of the reactor for magnetic actuation. The magnets were attached to the sides of the reactor chamber so that the center of the magnets remains at an angle of approximately 38° with the center of the scaffold (Fig. 3). The control experiment for cell culture was conducted by culturing the cells on scaffolds in 12-well cell culture plates (Nunc). The microscopic analysis was conducted in both SEM (Carl Zeiss, EVO 60) and fluorescence microscopy (Carl Zeiss). The long-term cell tracking study was conducted using an in-house prepared cell tracking dye (carbon nano dots, detailed protocol mentioned in supplementary section). [29, 30].

RT-PCR study

The MSCs were cultured on the scaffolds (10^5 cells/scaffolds) and four different groups were prepared (FMg, fluid shear + magnetic actuation; F, only fluid shear; Mg, only magnetic actuation; and control). After 15 days, the experiments are completed and cells were isolated from scaffolds. Total RNA was isolated from the samples after 15 days of culture using an RNA extraction kit (Genetix, India) following the manufacturer's protocol. RNA concentration was balanced via measuring in "Nanodrop" (Bio-Rad). RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was utilized for cDNA synthesis following the manufacturer's protocol.

PCR amplification was done for 35 cycles using the following conditions: 94 °C (denaturation) for 30 s, variable 1 (annealing) for 30 s, and 72 °C extensions for 30 s and a final extension at 72 °C for 10 min in the thermal cycler (Eppendorf, Germany). PCR products were then analyzed by 1% agarose gel electrophoresis. It was watched via adding ethidium bromide solution into the gel using Gel-doc (Bio-Rad) machine. The bands were analyzed using ImageJ software and relative band intensities were plotted. GAPDH was utilized as a housekeeping gene and its expression was checked. The other gene expression, which were considered into account, are collagen-I, collagen-II, BMP-2, PECAM, and osteocalcin (primer sequences and melting temperature are provided in Table 1).

Fig. 3 Static bar magnet-based scaffold actuation system (2 rare earth bar magnets of magnetic strength 0.05 T was used)

Statistical analysis

Statistical analysis was conducted by taking the mean and standard deviation of samples run in triplicates ($n = 3$). The Student's t -test is conducted with $p \leq 0.05$ to be statistically significant concerning control wherever possible.

Results

Physicochemical characterization of the nanoparticles

The synthesized nanoparticles were characterized using TEM, XRD, and SQUID analysis (Fig. 4). The nanoparticles were observed to have mostly spherical in shape and approximately 5.2 ± 1.2 nm. The inter crystal plane distance

Table 1 List of primer sequences and respective melting temperature

Gene name	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Melting temperature (°C)
Collagen-I	GCGCCAGAAGAACTGGTACATCAGCAA	GCGCGCCATACTCGAACTGGAATC	60
Osteocalcin	ATGAGAGCCCTCACACTCCTC	GCCGTAGAAGCGCCGATAGGC	58.5
GAPDH	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	54
Collagen -II	GGCAATAGCAGGTTACGTACA	CGATAACAGTCTTGCCCCACTT	53.9
PECAM/CD-31	TCAAATGATCCTGCG	CCACCACCTTACTTG	58.9
BMP-2	ACCCGCTGTCTTCTAGCGT	CTCAGGACCTCGTCAGAGGG	57

Fig. 4 Physicochemical characterization of the USPIONs: (a) TEM brightfield image, (b) HRTEM images showing inter crystalline planes of the nanostructures, (c) XRD pattern of the USPIONs to confirm its phases, (d) SQUID analysis of the USPIONs for finding the magnetic properties

from the high resolution was observed to be 1.67 Å. The XRD analysis suggested presence of mixed phases in the nanoparticles.

The peaks around 26° and 22° can be attributed to Y-Fe₂O₃. The peaks around 31°, 35°, and 39° could be attributed to Fe₃O₄. The peaks around 41°, 45°, and 51° can be attributed to α-Fe₂O₃. [31] The SQUID analysis suggested the material has significant magnetic properties as per our previous report of carbon dot-assisted SPION synthesis. [32] Thus, the magnetic nanocomposite developed using these particles can be used for mechanical actuation using external magnetic field. The gelatin-USPION composite was prepared by pouring the slurry on the micropatterned mold using replica molding. The surface roughness (Fig. 5a and b) in two orthogonal axes (*X* and *Y*) is plotted. The average roughness (ΔZ) was observed to be in the range of 220 nm which is helpful in cellular attachment. [33] The swelling study was conducted to understand the final dimensions of the microchannels prepared in the scaffolds (Fig. 5c). Considering, there is approximately 55% of swelling, the final channel diameters at steady state should be in the range of 300–350 μm. The SEM analysis shows that the scaffolds have microchannels in the range of 220 ± 30-μm diameter (Fig. 5d).

The surface chemical characterization of the samples was conducted by AES which reaches until first few nanometers depth of the sample. While gelatin being a denatured protein (collagen) having a random distribution of carbon and oxygen (Fig. 6a and b), the iron nanoparticles (Fig. 6c) were observed to be homogeneously distributed. The samples' micromechanical property was observed to be in the range of human trabecular bone (Figure S3, modulus 9.88 ± 2.06 GPa and hardness 0.365 GPa). [34] The live cell microscopy results showed spindle-like structures in the presence of mechanical stress (Fig. 7a) and formation of bone-like nodular structures (Fig. 7b). We also observe thick ECM deposition (Fig. 7c). However, endothelial cell-like tubular structural features also (Fig. 7d–f) have been observed which suggests presence of both phenotypes (bone cells and endothelial cells). This observation was further verified using PCR analysis (Fig. 8). In PCR, 4 different types of samples were denoted (F is only fluid shear, Mg is only magnetic field, FMg is both fluid shear and magnetic field, and CN is static culture with no magnetic field). It is observed that PECAM (endothelial marker) and BMP-2 were highest in F, whereas OCN (osteocalcin, bone tissue marker) was observed to be highest in Mg. A housekeeping gene (GAPDH) and positive control Coll-2 (chondrocyte marker) were shown to have similar levels of expression suggesting effect of physical forces only modulates bone and endothelial phenotype. Interestingly, the PCR result of FMg in the major ECM protein Col-1 is highest suggesting highest ECM synthesis. This result is as per the SEM result observed in cell culture studies, as just like upregulation of Col-1, very high amounts of ECM deposition

are observed (Fig. 7c and supplementary results: Figure S1). But gene expression of OCN, BMP-2, and PECAM in FMg is next to highest. This result suggests the combination of fluid shear (F) and magnetic field (Mg) is capable of creating both osteogenic and endothelial phenotypes in a single model. Similar results were obtained from H&E histological imaging (supplementary section figure S2).

Discussion

Organ-on-a-chip is a concept mostly explored to mimic in vivo tissue models in vitro. Bone is the major load-bearing connective tissue of the mammalian body as well as it is working as the niche for many hematopoietic cells. Therefore, a bio-mimetic in vitro model for osteogenic tissue is of significant research. Specifically, the pharmaceutical industries working in the area of osteogenic diseases such as rickets, osteoporosis, osteoarthritis, and orthopedic trauma are highly interested in the development of in vitro bone models. [35] While there are certain in vitro bone models that exist in literature, most of them fail to mimic multiple features. Bone is a mechano-responsive tissue. Therefore, the mechanical stimulus must have a specific effect on the system. Secondly, bone is significantly rich in vasculature which helps in nutrient and gaseous transport. Most of the in vitro models available in literature depend upon diffusion for this transport phenomenon. From Fick's law of diffusion, it can be denoted that

$$J = DA(C_1 - C_2)/L$$

(*J* is the flux density (diffusion rate; mol/m²/s); *D* is the diffusion coefficient (function of molecule and medium); *A* is the cross-sectional area; *C*₁ and *C*₂ are the concentrations at source and sink; *L* is the path length, and *D* depends inversely upon density of the medium) [36].

As the flux density depends inversely upon the path length of diffusion, for a thick matrix such as bone, the maximum limit of *L* for effective diffusion is 150–200 μm.

Therefore, lack of vasculature beyond this distance can be considered as a major limitation to the mass transport phenomena of an in vitro bone model. From the data provided in the figures, it is clear that the channels are around 250 μm in dimensions with the distance between channels to be around 50 μm (Fig. 5d). From 1 mm at the entry to the division of the flow to 200 μm generates a shear gradient.

Now, from Newton's law of viscosity, we get

$$\tau = -\mu \Delta / \Delta z$$

(τ is the shear stress, μ is the coefficient of viscosity (0.78×10^{-3} N.s/m²) for DMEM, *u* is the velocity, and *z* is the distance traversed)

$$\Delta u = -0.2231 m/s, / \Delta z = 0.005 m$$

Fig. 5 Physicochemical characterization of the scaffolds with microfluidic channels. **(a)** 3D surface profilometry images showing micro- and nanoroughness. **(b)** 3D surface roughness profile along the X and Y axes. **(c)** Swelling study until the scaffold reaches to equilibrium.

(d) SEM image to show casted microchannels (scale 250 μm). **(e)** Magnified SEM image to show the microroughness on the surface (scale 100 μm)

$\tau = 0.35 \text{ N/m}^2$ which is at the similar level as observed with previous literature where people observed the effect of shear stress upon endothelial differentiation to enhance endothelial-like differentiation up to 11 to 214 fold. [37] At the same time, magnetic field working upon the scaffold (0.05-T two bar magnets) allows the scaffold to experience

mechanical stretch. The mechanical stretch was generated by magnetic actuation where the magneto-active scaffold (the scaffold's SQUID result is provided suggesting the magnetic susceptibility of the biopolymer-magnetic nanoparticle composite) was kept between two equally strong rare earth metal permanent bar magnets (0.05 T). The scaffold was stuck to its position in the reactor with sterile

Fig. 6 Surface chemical characterization of the scaffold using AES: (a) C1, (b) O1, (c) Fe1, (d) combined showing the distribution of different atoms on the scaffold surface

Fig. 7 Live cell microscopic imaging (7 days). (a) Cellular stretching. (b) Bone-like nodule formation. (c) Thick ECM deposition. (d) Endothelial cell-like tube formation. (d and e) Conduit formation (a, b, d scale, 50 μ m; c, e, f scale, 20 μ m)

Fig. 8 PCR analysis of gene expression after 15 days of cell culture showing high osteocalcin and PECAM expression suggesting both osteogenic and endothelial gene differentiation pathway activation of the MSCs ($n = 3$, $p \leq 0.05$)

double-sided adhesive tape. Hence, the mechanical stretch in the scaffold generated micro-tension to the adherent MSCs in the scaffolds. This micro-tension is significantly effective in osteogenic differentiation of MSCs (bone-like phenotype development). Literature suggests the mechanical stress in the order of 0.3–0.8 MPa is significantly effective for bone-like differentiation. [38] The current model has mechanical stiffness in the range of ECM of trabecular bone. But, at steady state, the shear gradient on the cells is in the range of endothelial differentiation. With almost control equivalent collagen-2 expression, it is also evident that the bone development phenomenon is similar to intramembranous ossification. It is already evident in previous biomechanical studies that combination of tensile strain and fluid shear gradient can lead to intramembranous osteogenesis. [39, 40] Intramembranous ossification is the natural fracture healing mechanism in the human body. [41] To create a similar system in vitro requires activation of an inflammatory microenvironment via cytokines like IL-1 β , TNF- α , and IFN- γ (NF- κ B activation). [42] The only other way to develop such models is via development of a coculture of bone cells and pro-inflammatory (M1) macrophages. [43] Thus, modeling this system is significantly important and economically viable especially for testing therapeutics related to flat bone healing like the skull or mandible [44].

Conclusion

The current study shows that exploring biophysical stimuli can be an alternative to growth factors for modeling complex organ systems. In the developed model, we not only mimic vascularization and osteogenesis in the same experimental chip via competition of two orthogonal mechanical forces, but also find mechanistic similarities between nature's natural fracture healing mechanism (intramembranous ossification). In this model, no growth factors are utilized and the

physical property can be controlled easily (modulation of nanoparticle content, changing magnet strength, changing the channel dimensions, and changing the fluid input velocity). Thus, this experimental model could be significantly useful in exploring therapeutics involved in fracture healing. However, a major limitation of this system is creating its mathematical model so that it can be used to deal with complex bioengineering problems. Here, two different forces are interacting and one of them cannot be calculated directly (magnetic actuation). So, multiplexing the system and creating better mathematical model could enhance the scope of the device.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s44164-021-00004-7>.

Data availability The data is available in electronic supplementary document.

Declarations

Conflict of interest The authors declare no competing interests.

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