Remodeling of Architected Mesenchymal Microtissues Generated on Mechanical Metamaterials

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Running Title

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Abstract

Mechanical metamaterials constitute a nascent category of architected structures composed of arranged periodic components with tailored geometrical features. These materials are now being employed as advanced medical implants due to their extraordinary mechanical properties over traditional devices. Nevertheless, to achieve a desired tissue integration and regeneration, it is critical to study how the microarchitecture affects the interactions between the metamaterial scaffolds and the living biological tissues. Based on human induced pluripotent stem cell technology and multiphoton lithography, we report the establishment of an *in vitro* microtissue model to study the integration and remodeling of human mesenchymal tissues on metamaterial scaffolds with different unit geometries. Microtissues showed distinct tissue morphologies and cellular behaviors between architected octet truss and bowtie structures. Under the active force generated from the mesenchymal tissues, the octet truss and bowtie metamaterial scaffolds demonstrated unique instability phenomena, significantly different from uniform loading from conventional mechanical testing.

Introduction

Mechanical metamaterials are architected structures possessing microscale or nanoscale topological features that are assembled in a specialized order. The material properties of metamaterials depend on the geometry of the unit structure. The rational design of the unit structure and arrangement enables superior material properties at macroscales over natural materials[1,2]. For instance, penta-mode metamaterials, which are made of face-centered-cubic unit cell structures, have high bulk modulus but very low shear modulus[3]. This enables them to function as "liquid" structures and inherit controllable wave propagation. Moreover, novel metamaterial designs encompassing hyper-elastic behavior have been proposed to employ brittle materials for fabricating devices that require large but recoverable deformations. For the applications in biomedical engineering, mechanical metamaterials have been mostly used in design and fabrication of meta-implants[4,5]. Combining metamaterials with different degrees of auxeticity in a hip implant design, implant-bone integration and implant longevity was substantially improved[6]. In order to improve host tissue integration by manipulating the anisotropy of the implant (i.e. the Zener ratio)[7], combination of auxetic and isotropic structures makes it possible to fabricate the meta-implant possessing the failure resistance and adjustable anisotropy that could match with that of specific tissues. Despite these incipient results of meta-implants, the use of metamaterials is still focused on optimization of implant adaptivity to the native conditions. Tissue-scaffold interaction between mechanical metamaterials and biological tissues is still underexplored, while unraveling this response is critical for the purpose of tissue repair and regeneration.

Biomechanical studies are interlaced with the development of *in vitro* models using architected scaffolds[8–10]. With the emergence of multiphoton lithography (MPL), manufacturing complex 3D scaffolds enabled the design of cell niches to control and regulate cell behaviors and functions[11]. The ultra-high printing resolution of MPL makes it possible to recapitulate the nanoscopic topographies of extracellular matrix, which is necessary to create physiologically relevant models. For example, using MPL, the architecture of 3D lattice micro-scaffolds was found to trigger β-catenin activity of breast cancer cells through mechanotransduction pathways, which further promoted their proliferation and invasiveness[12]. In another study, tetrakaidecahedral nanolattices with better compliance were shown to enhance the expression of f-actin and calcium secretion of osteoblasts-like cells[13]. 2D auxetic structures were also found to affect the spreading of mesenchymal stem cells and the division of fibroblasts[14,15]. Though many studies demonstrated the effect of lattice architecture on cellular behaviors and phenotypes, how tissue remodeling process would affect the scaffold architecture via biomechanical interactions has not been elucidated yet.

To quantitatively understand the relationship between tissue remodeling and tissue mechanical conditions, the biomechanical models based on finite element analysis (FEA) elucidated the stress distribution within the 3D microtissues and predicted their remodeling behaviors[23–26]. The accuracy of FEA is dependent on the comprehensive understanding of the mechanical properties for both living tissues and biomaterial scaffolds. For example, the formation of sarcomeres has been linked with the local stress state of cardiac microtissues in a FEA model to computationally simulate the maturation of stem cell derived cardiac microtissues. Furthermore, this computational simulation was used to guide the disease modeling of arrhythmogenic cardiomyopathy by modulating the tissue geometry[27]. More recently, real-time stress distribution within mouse femurs was traced by a micro-FEA model, and then utilized to adapt the mechanical loading and lower the variance across all mice. This helped to prevent the overloading of individual femurs that would create the bone fractures[28].

In this work, we developed an *in vitro* tissue model based on different designs of mechanical metamaterials to study how the metamaterial architecture would affect the tissue formation. Two types of thoroughly investigated metamaterials (octet truss and auxetic bowtie structures) were fabricated by MPL using SZ2080TM, which is an inorganic-organic hybrid photoresist used for high resolution laser fabrication. Comparing to other commonly used photoresist materials, it shows negligible shrinkage during solvent washing and supports the growth of various cell types[29–31].The microtissues were generated by growing mesenchymal stromal cells derived from human induced pluripotent stem cells (hiPSC-MSCs) on the metamaterial scaffolds. Mesenchymal stromal cells (MSCs) are widely used for general mechanobiological studies due to their presence in many tissue types and differentiation capability in response to different mechanical environments[16–19]. However, primary MSCs obtained from human donors have limited proliferation potency and high variability in cell quality[20]. To overcome these restrictions, human pluripotent stem cells (hiPSCs) have become a promising resource for MSCs, because they can be expanded to a large quantity while maintaining the differentiation potential. There are already many protocols available to robustly generate MSCs from hiPSCs using small molecules and defined media[21,22]. Consistent production of MSCs from the same hiPSC source ensures the model development with high robustness, consistency, and reproducibility. We found that both overall tissue morphology and local cell behaviors were highly dependent on the microarchitecture of metamaterials. Furthermore, mechanical force generated from the microtissues induced unique deformation patterns on the metamaterial scaffolds, leading to structural instability due to buckling. This *in vitro* model provides the avenue to obtain a fundamental understanding of biomechanical interactions between living microtissues and metamaterial scaffolds, which will potentially be utilized to formulate new design principles to guide the generation of artificial tissues for various applications.

Methods and Materials

Multiphoton Lithography Fabrication of Metamaterial Scaffolds

All of the metamaterial scaffolds were fabricated by the MPL process with an organic-inorganic hybrid resin, SZ2080TM, as the basal material[32]. The experimental apparatus and fabrication conditions have been reported previously[33]. Specifically, the MPL apparatus is equipped with a NIR laser (FemtoFiber pro, Toptica) which has 780 nm wavelength, 100 fs pulse width and 80 MHz repetition rate. The laser beam was focused using a 100x microscope objective lens (Plan-ApoChromat 100 x/1.40 Oil M27, Zeiss). The stage was translated such that the laser beam could polymerize inside the material and fabricate the geometry. Each structure was designed in a CAD file using SolidWorks 2019x64. Through an *.STL* file, it was converted into a g-code, so that the stages can be translated accordingly.

hiPSC-MSC Differentiation

The hiPSC line was obtained from Dr. Conklin's lab at the University of California San Francisco (UCSF). The committee on Human Research at UCSF approved the hiPSC research protocol (#10-02521). hiPSCs were maintained on 6-well plates coated with growth factor reduced Geltrex (Life Technologies, Ca# A1413302) in Essential 8 (E8) media (Life Technologies, Ca# A1517001). The protocol to differentiate hiPSC-MSCs has been published previously[23]. Briefly, hiPSCs were treated with Essential 6 (E6) media (Life Technologies, A1516401) supplemented with 10 ng/mL bFGF (R&D Systems Ca# 233-FB), 4 μM SB431542 (Stemgent, Ca# 04–0010–10) and 4 μM CHIR99021 (Stemgent, Ca# 04-2004) for five days. Next, differentiated cells were dissociated and replated in a serum-free MSC culture media (CTS StemPro MSC SFM), (Life Technologies, A1033201) for 4 more passages to obtain differentiated hiPSC-MSCs.

Generation of Mesenchymal Microtissues

To generate the mesenchymal microtissues, one mechanical metamaterial scaffold was placed into one well of a 6-well plate and sterilized with 70% ethanol for 1 hour. After rinsed with Dulbecco's phosphate buffered saline (DPBS, Gibco) three times, the scaffolds were then coated with diluted Matrigel for 1 hour. To seed the hiPSC-MSCs to the metamaterial scaffolds, the cells were dissociated, suspended, concentrated with a density of 1.5×10^7 cells/mL and seeded with a total volume of 20 μL. After cell seeding, the scaffolds were incubated at 37 ˚C for 1.5 hours to promote the initial cell attachment before more cell culture media was added.

Immunostaining and Confocal Microscopy

Architected microtissues were fixed with 4% (vol/vol) paraformaldehyde, permeabilized with 0.2% (vol/vol) Triton X-100 and blocked with 2% (wt/vol) bovine serum albumin (BSA). Next, microtissues were incubated with Phalloidin for 1 hour to stain F-actin and then incubate with DAPI for 10 minutes to stain nuclei. Confocal microscopy (Zeiss U880) was used to capture z-stacks of the microtissues architected by the metamaterial scaffolds, which can be observed simultaneously due to autofluorescence.

Scanning Electron Microscopy (SEM)

The microtissues were fixed in 4% paraformaldehyde overnight with 0.1M PBS at room temperature. After washing three times with PBS, samples were then dehydrated in a series of concentrations of ethanol (15%, 30%, 50%, 70%, 95% and 100%) at room temperature for 15 minutes at each concentration, plus two more 100% ethanol dehydration at last. After dehydration, samples were dried in the vacuum oven for one day. Prepared samples were placed on stubs, and sputter-coated a layer of gold thin film with a thickness of 10 nm. At last, scanning electron microscope (SEM, JSM-IT100LA, JEOL USA Inc.) was used to image the microtissues architected by metamaterial scaffolds.

Helium Ion Microscopy (HIM)

After sample preparation for SEM, the samples were imaged using helium ion microscopy (Zeiss ORION NanoFab). To avoid charging effects, the imaging was conducted using the electron flood gun to negate the accumulating positive charge from a scanning ion beam, permitting the imaging of the samples without additional sputtering, allowing extremely high-resolution imaging that is required to observe the nanofeatures of the cell membranes.

Finite Element Analysis (FEA)

To investigate the effect of the cell attachment to the scaffold, FEA simulations were performed using the Multiphysics Software ANSYS R18.1. Both bowtie and octet truss structures were discretized by 3D-10-node tetrahedral solid elements. Specifically, octet truss structure was discretized with 173018 nodes and 84558 elements, while the bowtie structure was discretized with 32301 nodes and 16648 elements. The mechanical properties were set using the same values that have been reported in the previous work[31]. Based on SEM images, the cells within bowtie structure intended to distribute along the edges of the beam members, while the cells within octet truss structure occupied the sides of the lattice. For this reason, a quasi-static displacement field was applied at these specific members of both lattices for large nonlinear deformations. For the bowtie structure, distributed displacement was set at the nodes of the beam members at the unit edges to apply compression, while the beam members at the top were subject to a distributed displacement that would cause them to bend inwards, which was represented by the HIM images. For the octet truss structure, the side beam members were subject to a distributed displacement that would cause the compression to the array. Since the structures were fixed on the glass substrate, the bottom beam members were defined as fixed supports. After the calculation of the nonzero components of the displacement vector at each node, the force vector was obtained by the following equation:

$$\{\mathrm{K_o} + \mathrm{K_u}(\mathbf{u}) + \mathrm{K_\sigma}(\boldsymbol{\sigma})\}\mathbf{u} = \boldsymbol{f}$$

Where K_o is the linear stiffness matrix, $K_u(u)$ is the initial displacement stiffness matrix and $K_\sigma(\sigma)$ is the initial stress stiffness matrix, u is the displacement vector of all the nodes and *f* is the force vector of all the force components at the respective nodes. The force-displacement curves were obtained by the reaction force at the fixed supports, which is the summation of all the force vectors.

Results and Discussion

We employed two types of unit structure designs, namely the octet truss and the auxetic bowtie structures, to create metamaterial scaffolds. The octet truss, furnishing ultra-stiff and ultra-light material properties, has been used as the building block of load-bearing biomaterials for hard tissue implants[34,35]. The bowtie structure, on the other hand, is heralded macroscopically negative Poisson's ratios, fracture resistance and high energy absorption performance[36,37] (Figure 1A). Previously reported dimensions of structural units were used in our designs (Octet truss: beam thickness: 1.5 μm, base length: 50 um; Bowtie structures: beam thickness: 8 μm, base length: 50 μm)[31,38], which ensure that the scaffolds possess the minimum volume possible, while simultaneously have the structural integrity to hinder collapse. The bowtie structures had beam members of larger diameter to obstruct collapse during the fabrication process. The 3D metamaterial scaffolds was fabricated using a Ti: sapphire femtosecond laser scanning system (power: 1 mW, scanning speed: 10 μm/s) to achieve sub-micron resolution on scaffold printing (Figure 1B)[31]. An organic-inorganic hybrid resin, SZ2080TM, was used as the basal material for fabricating all scaffolds[32].

Fabricated scaffolds were imaged using scanning electron microscopy (SEM), showing precise microstructures and great mechanical integrity (Figure 1C). The length scale of metamaterial structural units was similar to the size of individual biological cells, which would provide direct associations between biomechanical responses of cells and structural geometry of metamaterial unit. The hiPSC-MSCs showed a spindle-like shape and expression of standard MSC markers including CD73, CD90 and CD105 (Figure S1). hiPSC-MSCs were then seeded onto Geltrex-coated metamaterial scaffolds with a density of $1.5*10^7$/ml. The high cell-seeding density resulted in the formation of a 3D thick tissue on the metamaterial scaffolds. We estimated around four hiPSC-MSCs surrounding each structural unit of metamaterials.

On the octet truss metamaterial scaffolds, we observed efficient penetration of hiPSC-MSCs throughout the 3D scaffolds based on the immunostaining of actin filaments and cell nuclei (Figure 2A). Though many structural branches were present in the octet truss structure, cells were able to traverse the struts, invaded into the pores within the scaffolds and exhibited an elongated morphology. Compared to standard SEM imaging, helium ion microscopy (HIM) enhances the contrast, resolution and depth of field, which enable the visualization of tissue-material interactions under microscopic levels[39]. In order to maximize the attachment to the metamaterials with a low material density, hiPSC-MSCs grew and elongated following the longitudinal orientation of the beams, which is a common phenomenon for cells on most architected scaffolds (Figure S2A)[10,40,41]. To further facilitate the cell adhesion, thin branches of plasma membranes were extended from cell bodies to attach to the adjacent beams (Figure S2B). In addition, micropores were present on cell bodies, which might be created under high mechanical tension when hiPSC-MSCs spanned over different beams (Figure S2C).

The mechanical load generated by the microtissues led to a large deformation of entire octet truss metamaterial scaffold, manifested as the decrease of its height and the inward buckling of struts at the corners (Figure 2A). The stability of center posts maintained an isotropic stress distribution across octet truss structure, in order to keep the overall integrity of metamaterial scaffold. To define the deformation modes of metamaterials, FEA simulations were performed using the Multiphysics Software ANSYS R18.1. The mechanical properties were set with the same values that have been reported in previous work[33]. We first simulated the mechanical behaviors of octet truss structure under uniform mechanical load. The octet truss structure can sustain high mechanical force without fracture with a linear relationship between force and displacement (Figure S4), which confirmed the ultra-stiff property of this structure.

To recapitulate the deformation characteristics of octet truss metamaterial scaffolds under the mechanical loading from microtissues, the beam members at the top part of octet truss structure were subjected to a distributed displacement, which caused a compression of the array (Figure 2B). The bottom part attached on the coverslip, which limited the spatial moving capacity of beam members, were simulated as fixed supports. The force-displacement relationship showed that the octet truss structure experienced a bifurcation buckling mode evidenced by the positive-negative-positive transition of the slope[42]. This distinct deformation mode indicated a unique way of mechanical loading from living biological tissues on metamaterial scaffolds. The mesenchymal tissues growing on the scaffolds formed tight connections with monolayer tissues grown on the surface of the coverslip, which applied a dominant compressive force transmitted through the main axis of beam members of the metamaterial scaffolds. Since octet truss is a stretch-dominated meta-structure, the compression-induced displacement was limited by the interior hardness of materials[43]. However, the top part of octet truss had a better compliance, showing that most of beams buckled under the biological force from mesenchymal microtissues. The buckling of individual beams disrupted the integrity of unit structure, and then modulated the loading bearing mode of the octet truss metamaterial scaffolds. Once the force was applied in perpendicular to the beam axis, it was possible to deform the scaffold to a large extent with less mechanical loading.

On the auxetic bowtie metamaterial scaffolds, a thicker tissue formed with a bulky morphology that had a clear tissue boundary following the contour of the scaffolds (Figure 3A). The cells tended to aggregate around the bowtie structures with dense tissue formation, instead of extending cell bodies with thin plasma membrane branches that were observed on the octet truss metamaterial scaffolds. When imaging with HIM, we observed that cell wrapped around the beams and micropores formed on the cell membrane (Figure S3A, B). The flat edges of bowtie structures were significantly deformed inwards by the microtissues, leading to the shrinkage of their diagonal ribs and the closing of the pores (Figure 3A). In addition, the vertical parts flipped 90 degrees and met their neighbors due to the high compression force.

To simulate the deformation characteristics of bowtie metamaterial scaffolds under the mechanical loading from microtissues, the distributed displacement was set at the nodes of beam members at the flat edges, while the top beams were subjected to a displacement that would cause them to bend inwards (Figure 3B). The mechanical force of the microtissue caused a snap through buckling mode on the bowtie structure demonstrated as a plateau on the force-displacement curve[44]. Compared to the octet truss, composed of multidirectional beam members that provide a homogeneous mechanical behavior, high anisotropy degree of the bowtie structure based on its beam arrangement assured sufficient area for cell attachment without stretching cell membrane to form thin branches. Due to the disruption of structural units, the metamaterial scaffold became more compliant. However, the bowtie structure had less stress relaxation comparing to the octet truss, since bowtie structures contained less buckled beams under the mechanical loading. More importantly, the negative Poisson's ratio of bowtie structure allowed the tissues to aggregate in a natural way, which brought the cells in close contact with strong intercellular connections. Hence, the entire scaffold was dramatically compressed, shown as inward buckling of individual beams at scaffold edges, flipping of vertical flat beams and closing up the unit pores.

Results described above evidenced the fact that mechanical load from biological tissues was prone to cause the buckling of individual fibers and trigger the instability of metamaterials. However, the metamaterial scaffolds could sustain a significant distortion without fracture, which was possibly resulted from efficient tissue-scaffold integration as a single "*living composite*". The active remodeling of mesenchymal microtissues made it possible to establish an equilibrium state of the composite under minimal mechanical force and energy potential. In comparison, uniform mechanical loading was passive and externally exerted to the metamaterial scaffolds, thus it was not able to create the buckling forms that generated internally from the living composite. The concept of living tissue-scaffold composite should be taken into consideration for future mechanistic analysis on tissue-material mechanical interaction and evolution of tissue instability. Meanwhile, the optimization of computational models is dependent on the accurate experimental measurement of spatial stiffness of the living tissue constructs. However, conventional atomic force microscopy (AFM) or nanoindentation are limited for this measurement due to the highly hierarchical textures of 3D tissue constructs. Bio-indenters are featured by their large displacement range, high resolution force control and capability of testing samples in a liquid condition, which are essential for measuring the mechanical properties of soft living tissues[45–47]. In future, our microtissue model can be used to establish a correlation between metamaterial geometry with soft tissue mechanics based on bio-indentation measurement.

Conclusion

In summary, we have generated the first 3D human microtissue model on metamaterial scaffolds fabricated using MPL technology. hiPSC-MSCs could assemble into 3D microtissues on different metamaterial scaffolds, but tissue morphology and scaffold deformation mode were significantly different between octet truss and bowtie structures. On microscopic level, we observed the microtissue remodeling adapting to the complex scaffold geometry. FEA simulations unraveled that mesenchymal microtissues induced different deformation modes on the metamaterial scaffolds and caused a large transformation of their geometries. Further quantification of cell surface tension, immunostaining of other cell markers and simulation of force distribution within the meta-structures would benefit a deeper understanding of how unit geometries of metamaterials affect tissue phenotypes.

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Data Availability

The authors declare that all data supporting the findings of this study are available. Source data for the figures are available from the corresponding author on reasonable request.

Author Contribution

Z.M., C.W., Z. V., and C.P.G. conceived and designed the experiments. C.W. conducted the biological experiments and confocal microscopy. Z.V. performed the fabrication of metamaterial scaffolds. T.W. differentiated and provided the hiPSC-MSCs. S.S. performed the SEM imaging. C.W. and Z.V. analyzed and interpreted the data. C.W., Z.V., C.P.G., and Z.M. wrote the manuscript with discussions and improvements from all authors. Z.M. and C.P.G. supervised the project development and funded the study.

Author Disclosure Statement

All authors declare no conflict of interest.

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