Three-dimensional Nano-architected Materials as Platforms for Designing Effective Bone Implants

Thesis by

Alessandro Maggi

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

CALIFORNIA INSTITUTE OF TECHNOLOGY

Pasadena, California

2018

(Defended October 25, 2017)

Alessandro Maggi

All Rights Reserved

Acknowledgements

I would like to thank my academic advisor, Professor Julia Greer, for always being encouraging, inspiring and above all for helping me grow both as a scientist and as a person. Julia has been a role model, and I will always aspire to be as brave, undeterred, and understanding as she is. Throughout these last fours years she's been an amazing scientific advisor and irreplaceable personal counselor.

I am immensely grateful to Professors Guruswami Ravichandran, Chiara Daraio, Joel Burdick, and Mikhail Shapiro both for accepting to be on my thesis committee and for being incredibly inspiring scientists. I would also like to thank Professor Sergio Pellegrino and Guruswami Ravichandran for teaching outstanding classes that provided me with solid knowledge for my research work.

I would also like to thank our wonderful collaborators at UCSF, Professor Tejal Desai, Dr. Giada Genchi, Dr. Jessica Allen and Professor Kai Zinn, Dr. Hanqing Li and Michael Abrams at Caltech, for letting us use their facilities, for their work on cell growth and for their deep expertise in biology. A big thank you to Aroutin Khachaturian and Professor Ali Hajimiri for letting us use their facilities.

The work detailed in this thesis would not have been possible without the numerous labs and user facilities around Caltech. I am extremely grateful to Professor George Rossman for educating and training me on FTIR and Raman spectroscopy, which were essential for my research projects.

I am also immensely grateful to the Kavli Nanoscience Institute (KNI) at Caltech for providing great cleanroom facilities and amazing staff. I would like to thank Dr. Matthew Sullivan Hunt for his priceless technical support with SEM, FIB, EDS and for the invaluable conversations we

have had throughout these years. My thanks to Melissa Mendeles for her infinite patience, for always being available to help and for her technical tips. Steven Martinez and Nils Asplund for being amazing engineers and for teaching me everything I know about vacuum systems. Smith "Bud" Johnson for his positive spirit. Guy DeRose for constantly trying to improve the KNI and for his very diplomatic attitude. Alex Wertheim, Bert Mendoza and Nathan Sangkook Lee for giving me great technical insights into deposition and etching technologies. Finally I would like to thank Mary Sikora, Jennifer Palmer and Tiffany Kimoto for always putting a smile on my face. I would like to thank the Greer Group for making graduate school such a memorable adventure. Lucas, Chen, Eric, Carlos, Arturo and Viki, thank you for the wonderful research discussions we've had. Xiaoxing, thank you for being a great source of inspiration, your determination is unparalleled. Thank you to Amanda for being a wonderful research partner and for being incredibly patient with me.

Finally I would like to express my greatest gratitude to my family: Ottman, Tiffany and my parents. Ottman, thank you for being an amazing friend and scientist, you are the reason why I developed some idea of what common sense is.

Tiffany, thank you for being so crazy to decide to be my wife, you are the most patient, uplifting and understanding person I know. Even if I don't' like to voice my feelings, it means a lot to have you along this ride with me, much more than I could ever express in words. Above all, I would like to thank my parents who are the reason why I am writing this thesis today. You have always been and will always be my heroes. Your encouragement, love and endless support motivated me everyday to pull through especially during difficult times.

Lastly, I would like to acknowledge the Amgen graduate fellowship, the Vannevar-Bush fellowship, the Institute for collaborative biotechnologies and the Caltech EAS discovery fund

for the financial support that allowed me to do this work.

Abstract

The growing world population coupled with longer human life expectancy warrants the need for better medical implant development. Recent advances in lithographic techniques have opened the door to a variety of approaches to tackle the aforementioned issue. However, several scientific hurdles must be overcome before patients can use fully synthetic and effective implants.

Identifying the optimal material, porosity, and mechanical properties of the scaffold to induce cell functionality are key obstacles. Limitations in established fabrication techniques have hindered the ability to fully understand cell behavior on 3D substrates. 3D printing is limited to feature sizes that are at least one order of magnitude larger than a single cell (\sim 10µm); electrospinning is able to yield features that are on the same scale as cells, but its stochastic nature leads to scaffolds with poor mechanical properties; salt leeching doesn't allow for control of pore size and distribution which have detrimental effects on nutrient diffusion and cell ingrowth, thereby thwarting the formation of functional tissue.

Much effort has been made to create a suitable platform for regenerating a relatively less complex organ, such as bone, yet the inability to fully understand cell mechanics on 3D scaffolds has curbed the fabrication of effective bone implants.

The first part of this thesis focuses on the suitability of nanoarchitected materials as 3D platforms for bone-tissue growth. We employed two-photon lithography to create polymeric and hydroxyapatite-coated 3D nanolattices to explore scaffold biocompatibility and material effects on osteoblast attachment and growth. Our experiments showed that the unit cell geometry, tetrakaidekahedron, and size, 25µm, were adequate for cell attachment and infiltration, which are

hallmark signs of biocompatibility. Our study also corroborated previous findings that mammalian cells respond differently to different materials that they come in contact with. To isolate structural effects, we fabricated nanolattices coated with a uniform 20nm-thick outermost layer of TiO₂ These nanolattices, which had fixed porosity and unit cell size (25µm) while they varied in structural stiffness (~2-9MPa) were used to explore the influence of scaffold properties on the viability of osteoblasts in a microenvironment similar to that of natural bone. Upon growing osteogenic cells on the nanolattices, significant cell attachment and presence of various calcium phosphate species, which are commonly found in natural bone, were observed. These findings suggest that 3-dimensional nano-architected materials can be used as effective scaffolds for bone cell growth and proliferation.

The second part of the thesis investigates the effects of nanolattice structural stiffness and loading conditions on osteoblast behavior. We fabricated nanolattices with stiffness ranging from ~ 0.7 MPa to 100MPa. Experiments done by seeding osteoblast-like cells on these nanolattices revealed that both stress fiber concentration and bioapatite deposition were higher on the most compliant nanolattice, (0.7 MPa) by $\sim 20\%$ and $\sim 40\%$ respectively. These results provide insights into cell behavior in 3D microenvironments which can lead to a better understanding of stress shielding at the cellular level. Preventing stress shielding by creating scaffolds with structural stiffness and porosity that enhances osteoblasts activity could lead to the creation of effective implants with improved mechanical stability which ultimately improves osteointegration.

In addition to investigating static cell-scaffold interactions we took advantage of the nanolattices tunability to study the effects of dynamic loading on cell behavior. Bone adaptation is driven by dynamic, rather than static loading, however there is still wide controversy on whether stress,

strain or loading frequency plays the most significant role in bone remodeling, which drives bone healing.

In order to understand cell sensitivity to varying loads, displacements and frequencies, we fabricated hollow TiO nanolattices with stiffness ranging from ~0.7-35MPa which were populated with osteoblast-like cells and subjected to cyclic compression to either a constant stress or strain. After seeding SAOS-2 cells on the nanolattices for 12 days different dynamic loading conditions were tested: (1) cyclic uniaxial compressions to strains ranging from ~0.3-2% strain were carried out to investigate the effects of strain magnitude on cell behavior. (2) Cyclic uniaxial compressions to stresses spanning from ~0.02-1MPa were performed to explore the role of stress magnitude on the cells' stress fibers formation. (3) The nanolattices were cyclically loaded at different frequencies, ~0.1-3Hz, while maintaining stress and strain constant, which provided insights into how loading frequency affects osteoblasts behavior.

Cell activity, which was measured by monitoring f-actin and vinculin fluorescence intensity, revealed increased fluorescence in those cells that were mechanically stimulated as opposed to those that were statically grown on the nanolattices regardless of loading condition. Cell response was most drastically affected by varying the loading frequency. A \sim 30% increase in f-actin fluorescence was observed in the cells grown on the nanolattices that were loaded at \sim 3Hz compared to those that were grown on the nanolattices that were cyclically compressed at \sim 0.1Hz.

The last part of this thesis is focused on developing a three-dimensional architected capacitor that could be used as a strain gauge to further our understanding of cell mechanics in 3D. We took advantage of the mechanical tunability of the nanolattices to fabricate a 3D parallel-plate capacitor with a basal capacitance of ~280fF and able to sense forces as low as ~30 μ N. This

work points to nano-architected materials as promising candidates for ideal platforms to investigate more realistic cellular conditions which can immensely benefit the field of tissue engineering.

Published Content

Allen, J., Ryu, J., Maggi, A., Flores, B., Greer, J.R. and Desai, T., 2015. Tunable microfibers suppress fibrotic encapsulation via inhibition of TGFβ signaling. *Tissue Engineering Part A*, 22(1-2),142-150. doi.org/10.1089/ten.tea.2015.0087.

<u>Contributions</u>: extracted samples for mechanical testing, performed bending and uniaxial compression experiments, analyzed data, partially made figures and wrote manuscript.

- (2) Maggi, A., Allen, J., Desai, T. and Greer, J.R., 2017. Osteogenic cell functionality on 3-dimensional nano-scaffolds with varying stiffness. *Extreme Mechanics Letters*, 13,1-9. doi.org/10.1016/j.eml.2017.01.002
 <u>Contributions</u>: designed, fabricated and characterized samples (EDS, Raman), performed uniaxial compression experiments, analyzed data, and wrote manuscript.
- (3) Meza, L.R., Phlipot, G.P., Portela, C.M., Maggi, A., Montemayor, L.C., Comella, A., Kochmann, D.M. and Greer, J.R., 2017. Reexamining the mechanical property space of three-dimensional lattice architectures. *Acta Materialia*, *140*,424-432. doi.org/10.1016/j.actamat.2017.08.052.
 <u>Contributions</u>: partially prepared samples, performed and uniaxial compression experiments, and analyzed data.
- (4) Maggi, A., Li, H. and Greer, J.R., 2017. Three-Dimensional Nano-Architected Scaffolds with Tunable Stiffness for Efficient Bone Tissue Growth. *Acta Biomaterialia*. <u>doi.org/10.1016/j.actbio.2017.09.007</u>.
 <u>Contributions</u>: designed and fabricated samples, performed experiments, characterized samples, derived analytical model, analyzed data and wrote manuscript.
- (5) Maggi, A., Abrahams, M,. Tertuliano, A.O. and Greer, J.R. Probing osteoblasts sensitivity to dynamically loaded 3D nanoarchitected materials. (In preparation) <u>Contributions</u>: designed and fabricated samples, performed experiments, performed compression experiments with living cells grown on the lattices, characterized samples using fluorescence microscopy, analyzed data and wrote manuscript.
- (6) Maggi, A., Bouman R.A., Khachaturian, A., Hajimiri, A., Greer, J.R. Mechanically Tunable 3D strain gauge for cellular biomechanics. (In preparation)
 <u>Contributions</u>: designed and fabricated samples, performed mechanical and elemental characterization, analyzed data and partially wrote manuscript.

Table of Contents

Copyright Page	ii
Acknowledgements	iii
Abstract	v
Published Content	ix
List of Figures	xiii
List of Tables	xiv
Chapter 1: Introduction and Background	1
1.1. Current Bone Implants	1
1.1.1. Commercially available implants	1
1.1.2. Limitations of Current Bone Implants	2
1.1.3 Current Efforts in Bone Scaffolds Development	6
1.2. Architected Cellular Solids	8
1.2.1 What are Cellular Solids?	8
1.2.2 Nature's Architected Materials	10
1.3. Architected Cellular Solids as Platforms for Cell Scaffolding	10
1.4. Thesis Overview	
Chapter 2: Investigating the Biocompatibility of Two Topologically Different 3D	Nano-
Architected Materials: the Kagome and Tetrakaidecahedron Geometries	14
2.1. Chapter Summary	
2.2. Methods	17
2.2.1. 3D Nanolattice Design and Fabrication	17
2.2.2 Beam Stiffness Calculations	
2.2.3. In-Situ Uniaxial Compression Experimental Setup	23
2.2.4. Cell Culture Conditions	
2.2.5. Characterization of Cell-Scaffold Interactions	
2.3. Results	
2.3.1 Porosity and Material Effects on Cell Response	25
2.3.2. Morphological Characterization of Cell-Scaffold Interactions	
2.3.3. Nanomechanical Compression Results	
2.3.4. Spectroscopy for Molecular Cell-Secretions Analysis	
2.4. Discussion	
2.4.1. Pore Size and Material Effects on Cell-Scaffold Activity	
2.4.2. Mechanical Characterization	
2.4.3. Cell- Secretion Characterization	40
2.4.4. Cell-Nanolattice Interactions	43
2.5. Concluding Remarks	45

Chapter 3: Probing Nanolattices' Stiffness Effects on Osteoblasts-like Cells	.46
3.1. Chapter summary	47
3.2. Methods	49
3.2.1. Sample Preparation	49
3.2.2. Quasi-static Uniaxial Compression Experimental Setup	54
3.2.3. Cell Culture Conditions.	55
3.2.4. Cell Imaging and Secretions Characterization via Raman Spectroscopy and EDS	56
3.3. Results	57
3.3.1. Nanomechanical Experiments	.57
3.3.2. Cell Experiments: F-actin & Vinculin Fluorescence Microscopy	59
3.3.3. Cell Experiments: Cellular Secretions Characterization & Quantification	.61
3.4. Phenomenological Model Formulation	.65
3.5. Discussion	71
3.5.1. Mechanical Characterization	.72
3.5.2 Cell Response: F-actin and Vinculin Distribution	74
3.5.3 Cell Response: Mineralization	76
3.6 Concluding Remarks	.79
Chapter 4: Dynamic loading effects on cell attachment and stress fibers formation	80
4.1. Chapter summary	81
4.2. Methods	84
4.2.1. Sample Preparation	84
4.2.2. Monotonic and Cyclic Compression of the Nanolattices	.87
4.2.3. Cell Culture Conditions	.88
4.2.4. Stress Fibers and Focal Adhesion Fluorescence Imaging	.89
4.3. Results	.90
4.3.1. Monotonic Nanomechanical Experiments	.90
4.3.2. Cyclic Nanomechanical Experiments	.93
4.3.3 Cell Response: F-actin and Vinculin Distribution	.98
4.4. Discussion	105
	106
4.4.1. Mechanical Characterization: Monotonic Uniaxial Compression	
4.4.1. Mechanical Characterization: Monotonic Uniaxial Compression	107
 4.4.1. Mechanical Characterization: Monotonic Uniaxial Compression	107 113

Chapter 5: Mechanically Tunable Force Sensor for Measuring Cell Forces in 3D	118
5.1. Chapter Summary	118

5.2 Sensor Design	xii 121
5.3. Methods and Results	
5.3.1 Fabrication Route 1: the Traditional Approach	123
5.3.2 Fabrication Route 2: SiO ₂ Pre-Patterning	126
5.3.3 Fabrication Route 3: Si Pre-Patterning	128
5.3.4 Mechanical Characterization	133
5.3.5 Electrical Characterization	135
5.4 Summary and Outlook	137
References	138

List of Figures

Figure 1. Typical uniaxial stress-strain response for a foam under compression	9
Figure 2: CAD modeling of the kagome and tetrakaidecahedron geometries	17
Figure 3: Nanolattice fabrication process	19
Figure 4: Material systems design and fabrication	20
Figure 5: Post SAOS-2 cell seeding SEM and fluorescence microscopy characterization	26
Figure 6: Nanolattice biocompatibility	27
Figure 7: Post SAOS-2 cell seeding SEM characterization	29
Figure 8: Mechanical tests on the three material systems	30
Figure 9: EDS spectra of features found on the nanolattices	32
Figure 10: Raman analysis of SAOS-2 secretions	34
Figure 11: Design and Fabrication of the nanolattices	50
Figure 12: IP-Dip autofluorescence suppression	52
Figure 13: Shadow mask fabrication	53
Figure 14: Uniaxial compression experiments	57
Figure 15: Fluorescence microscopy images and quantitative analysis	59
Figure 16: SEM images of the SAOS-2 cells' products after 8 and 12 days growth	61
Figure 17: SEM image of the sub-aggregates coating the nanolattices' beams	62
Figure 18: EDS spectra and quantification of Ca and P secreted by the SAOS-2 cells	64
Figure 19: Substrate-dependent f-actin activation model	67
Figure 20: Fluorescence microscopy of f-actin signal on the additional material system	71
Figure 21: Nanolattice height variations	73
Figure 22: Raman spectroscopy analysis of the organic matrix deposited by SAOS-2	77
Figure 23: Sample Design and Fabrication	85
Figure 24: Square trench fabrication	87
Figure 25: Uniaxial Compression Experiments on Hollow Nanolattices	92
Figure 26: Constant load cyclic compressions	94
Figure 27: Constant strain cyclic compressions	96
Figure 28: Pre-post cyclic compression nanolattice morphology9) 7

	xiv
Figure 29: Varying loading frequency cyclic compressions	
Figure 30: Fluorescence microscopy after constant load cyclic compressions	100
Figure 31: Fluorescence microscopy after constant displacement cyclic compressions	.103
Figure 32: Relative fluorescence intensity as a function of loading frequency	.104
Figure 33: Nanolattice modulus as a function of the number of unit cells	.110
Figure 34: controller's response to cyclic loading.	.111
Figure 35: Matlab-generated image of the tetrakaidecahedron unit cell design	.123
Figure 36: SEM images of the steps involved in fabrication route 1	.125
Figure 37: Steps involved in fabrication route 2: SiO ₂ pre-patterning	.127
Figure 38: CAD representation of fabrication route 3: Si pre-patterning	.128
Figure 39: SEM images of the steps involved in fabrication route 3: Si pre-patterning	131
Figure 40: SEM images and corresponding EDS mapping of the fabricated capacitor	133
Figure 41: Mechanical characterization of polymer tetrakadecahedron nanolattices	.134
Figure 42: Electrical characterization of the fabricated tetrakadecahedron capacitor	.136

List of Tables

Table I: Beam stiffness as a function of boundary conditions for each material system	23
Table II: Comparison of the maximum deflection caused by a distributed load (q) using pinn	ned-
pinned as opposed to fixed boundary conditions	23
Table III: Elemental ratios in the Sphere and Spongy phase obtained via EDS	33
Table IV: peak assignment of the organic phase taken from Raman data	35
Table V: peak assignment of the spongy phase taken from Raman data	36
Table VI: Comparison of the elemental ratios predicted by the metaphosphate hypothesis and	l the
ratios found in the Spongy and the Sphere phase via EDS analysis	42
Table VII: Elastic modulus, E*, and compressive strength, σ_i , of each material system measurements of the system measurement of the system measurement of the system measurement of the system of the system measurement of th	ured
via uniaxial quasi-static compression	58
Table VIII: Elastic modulus, E^* , and compressive strength, σ_r , of each material system measurements of the system measurement of the system measurement of the system measurement of the system of the system measurement o	ured
via uniaxial quasi-static compression	.92
Table IX: SiO ₂ etch recipe used for fabrication route 2	6

	xv
Table X: Inductively coupled plasma etching of Si using a Bosch recipe	129
Table XI: Nanolattice geometric features and corresponding structural modulus (E^*)	

Chapter 1. Introduction and Background

1.1. Current Bone Implants

1.1.1. Commercially Available Implants

The skeleton is a remarkably complex organ that provides the necessary structural support for the human body to perform mechanical functions. Upon injury, bone has the ability to self-heal by clearing out necrotic tissues and depositing freshly generated minerals'.

This unique restorative capacity, allows bones to heal and reach strengths comparable to those of pristine bone. However this regenerative ability is slow and can lead to shape and size abnormalities³. Efforts to develop bone grafts that would optimize and accelerate bone healing have been made for more than 350 years; bone implants are designed to facilitate and enhance the bone healing process by providing adequate mechanical and physiological cues to the patient's bone³. The implant's ultimate goal is to provide a platform for the patient's cells to secrete a collagenous extracellular matrix which gets mineralized over time. This newly formed mineralized tissue is then remodeled, resulting in the formation of mature bone around the implant, which secures it to the surrounding bone³.

There are 3 main types of bone implants⁶: (1) autografts, which are osseous matter that is harvested from one anatomic site of the patient and transplanted to the target injured site. This type of implant has the advantage of preventing any immunologic adverse response at the cost of donor site morbidity, increased blood loss and increased operative time.

(2) Allografts are implants whereby bone is harvested from human cadavers, sterilely processed and transplanted to a recipient. Allografts have the advantage of preserving the native mechanical properties and structural features of natural bone but high costs, low availability and risks such as viral transmission make them an imperfect substitute.

(3) Fully synthetic implants represent a good alternative to the aforementioned bone grafts. Given their wide-availability, comparatively low cost and absence of risks such as donor site morbidity and viral transmission, they have become the most commonly adopted bone implants.

Commercially available synthetic orthopedic implants are primarily manufactured out of stainless steel and titanium alloys to achieve the required fatigue strength, high strength-to-weight ratio, flexibility, resistance to corrosion, and biocompatibility⁷. Recently, the focus has shifted from metallic implants toward ceramic ones mainly composed of different ratios of Ca, Na, Mg, P, S, O. This shift was primarily motivated by an attempt to increase biocompatibility, osteogenesis, and osteocoductivity, which is the ability of the implant to provide an adequate platform for new bone growth⁴⁹.

1.1.2. Limitations of Current Bone Implants

Bone is a complex composite material whose mechanical properties arise from its hierarchical structure. The constituent materials of bone, collagen and calcium phosphate, combine to form its lamellar structure[®]. The lamellae are arranged in different ways to compose the two distinct types of bone, cortical and trabecular: (1) cortical bone, which comprises 80% of the total bone mass, is found in the shaft of long bones and in the shell of flat bone. (2) Trabecular bone, which comprises the remaining 20% of bone mass is a highly porous (40–90%) network of beams and plates mainly found at the ends of long bones and within the core of flat bones^{10,10}. These two types of bone display significantly different mechanical properties: cortical bone provides stiffness and a higher strength than trabecular bone with an elastic modulus of ~18 GPa and a

compressive strength of 193MPa. The complex network of trabecular bone, which functionally behaves like stochastic foam, provides excellent energy absorption mechanisms which are reflected in its extraordinary toughness¹³.

Traditionally, bone implants have been made from metallic biomaterials such as stainless steels, Co-Cr alloys, and titanium (Ti) and its alloys. The choice of these materials coupled with their monolithic design led to implants that are stiffer and stronger than cortical bone. The elastic modulus of Co-Cr alloys is ~200 GPa, that of Ti is ~100 GPa and the lowest value of Young's modulus reported for a titanium alloy implant (Ti-35Nb-4Sn) is around 40 GPa. These values are at least 2 times higher than those of cortical bone and one order of magnitude higher than the Young's modulus of trabecular bone (~ 1 GPa)⁴⁴.

This discrepancy in stiffness between bone and the implant results in insufficient mechanical load transfer from the implant to the surrounding tissues, which leads to stress shielding, a phenomenon by which the orthopedic implant absorbs most of the imposed mechanical load and minimizes load transfer to the surrounding cells^{15,16}.

Wolff's law predicts two possible outcomes for bone growth in response to mechanical loading: (1) applying an adequate mechanical load to bone causes the surrounding osteoblasts to respond by initiating a remodeling process that leads to the formation of denser and stronger bone over time or (2) a lack of mechanical load on bone activates osteoclasts, which start breaking down bone tissue which leads to bone resorption¹⁷. As a consequence of the implant being stiffer than the surrounding tissue, the injured bone adapts to these reduced stresses relative to its natural state, decreasing its mass, which prevents the bone from anchoring to the implant and leads to implant loosening and eventual failure^{136,19}. Osseointegration, which is defined as the degree of

consolidation between the implant and the surrounding osseous tissues is compromised by the consequences of stress shielding²⁰⁻²². Hutmacher et al. (2000) postulated that an ideal implant should retain durability in the body and have mechanical properties that match those of the natural bone that is being replaced²³. This remains to be demonstrated experimentally, especially at the cellular level.

In addition to mechanical compatibility, which strongly contributes to osseointegration, an ideal implant should be biocompatible, showing no evidence of adverse cellular response, and osteoconductive, enabling the healing bone cells to grow and deposit new bone tissue. Current implants display other limitations in both of these two areas of research:

(1) limited biocompatibility

For decades, bone implants have been made from metallic materials (Ti, Co, Cr, Fe, Al, V, Mo) which were thought to be necessary to provide structural stability to the regenerating tissue. However, studies have shown that the corrosion byproducts accumulate around the injured area and spread throughout the body over time, which lead to local and systemic toxicity. Significantly elevated metal contents have been measured both in areas adjacent to the injured site and in the blood and urine of patients with metallic implants. Metal levels of up to 21 ppm Ti, 10.5 ppm Al, and 1 ppm V and up to 2 ppm Co, 12.5 ppm Cr, and 1.5 ppm Mo have been observed³⁴²³.

(2) Inadequate porosity

Porosity is defined as the percentage of empty space in a solid and is a fundamental component of cell scaffolds for bone regeneration. It serves as a template for cell infiltration and nutrient diffusion, which are necessary for bone-extracellular matrix deposition to provide structural support to the newly formed tissue. Commercially available implants exhibit lower porosity than cortical bone (~10%), which increases their stiffness and decreases the rate of vascularization. Inadequate vascularity leads to insufficient nutrient delivery to the healing site, which increases the incidence of atrophic bone nonunion, failure of tissue-implant integration^{**}.

The limitations of currently available implants are largely a consequence of the insufficient knowledge we have about cell behavior at the microscale. Until recently, bone implant research has mainly revolved around large-scale integration and mechanics with a specific focus on creating light, stiff, high cycle fatigue implants that were thought to be essential features of successful bone prostheses⁴²⁴.

Recent findings have shown that cell-scaffold interactions at the single cell level have profound effects on tissue growth, morphology and functionality^{27,28}.

Our knowledge about cell mechanics, adhesion, migration, and cytoskeletal function comes primarily from studies on 2D tissue culture²⁹⁻³¹. Recent literature has shown that cell activity on 2D planar substrates is markedly different from cell activity on 3D scaffolds^{32,33}. In-vivo environments are inherently 3D, therefore understanding cell behavior in such a threedimensional space is paramount to designing better implants.

Studies have shown that relative to 2D protein-coated substrates, 3D-matrix interactions led to enhanced cell adhesion by a factor of 6 and enhanced cell infiltration by a factor of 1.5, which caused the cells to assume the spindle-shaped morphology characteristically seen in vivo. At the molecular level, it was observed that adhesive proteins, paxillin and integrins, co-localized in 3D matrices while they separately localized when cells were grown on 2D substrates²². Current

implants are made of features that are several orders of magnitude larger in size than cells and therefore to a single cell, the implant feels like a flat 2D substrate. New technological advancements in micro fabrication techniques and material processing have opened the doors to probing cell activity on 3D scaffolds with features size on the same order as that of single cells.

1.1.3 Current Efforts in Bone Scaffolds Development

The aforementioned limitations associated with the fabrication of currently available bone implants have not been able to meet their large demand. Successful implant integration and long-term healing of bone fractures remain a major surgical challenge and suboptimal outcomes can have significant economic repercussions and negatively affect quality of life^{34,35}.

In order to understand the root causes of the problems plaguing current commercially available bone implants, much research has been devoted to studying native extracellular matrix (ECM). Engler has shown that mesenchymal stem cells can differentiate into neurons, myoblasts, or osteoblasts when they are grown on substrates with stiffness similar to that of the brain (0.1–1 kPa), muscle (8–17 kPa), or cartilage (20-40kpa), respectively^{29,36,37}.

A growing body of evidence has shown that ECM architecture, porosity and stiffness can modulate stem cell adhesion, proliferation, migration and differentiation. Studies using decellularized bone ECM have shown that 3D porous materials have a great potential to unveil the fundamental causes of cell-scaffold load transfer, mass transport at the single cell level and cell-material response^{ss}. However, typical procedures to decellularize native matrix are chemically and physically aggressive and lead to modification in the morphology and mechanical properties of the extracted ECM. Since it has already matured when at the stage of decellularization, the extracted ECM does not represent the true microenvironment that led to its

formation, minimizing its usefulness. Besides, extracted ECM represents the frame of the tissue at a mature level and doesn't necessarily give clues about the micro-environment that led to its formation.

Synthetic cellular solids provide a great platform to make 3D scaffolds modulate porosity, material, size, and stiffness. The modulation of these properties can give important insight into the optimal microenvironments that bone cells build to form mature collagenous ECM, which eventually becomes mineralized into bone.

Recent research that took advantage of the tunability of cellular solids as cell scaffolds has shown that mammalian cell viability, attachment and migration strongly depend on mean pore size and specific surface area of the 3D cellular scaffolds^{36,39,40}. O'Brien et al. (2005) discovered that as the pore size increased from 95µm to 150µm, cell viability decreased by a factor of 2 and cell attachment scaled linearly with increasing specific surface area⁴. Harley et al. (2008) showed that cell migration and cell speed, measured as the distance covered by a cell in a given amount of time increased by a factor of 2 when the pore size was reduced from 151µm to 96µm ⁴². Raimondi et al. (2013) fabricated polymeric scaffolds and observed that a minimum pore size of 10µm was necessary to allow for cell infiltration into their scaffold³. Tayalia et al. (2008) utilized polymeric scaffolds and showed that cells are more uniformly dispersed inside scaffolds with pore sizes of 52µm compared to 12µm²⁷. Despite a growing body of literature on 3D cell-scaffold interaction, the main focus has been on investigating the relationship between porosity and cellular behavior. Limited information about the role of scaffold stiffness on cell behavior is available. The 3D scaffolds that have been investigated either have MPa-level structural stiffness and strut dimensions of hundreds of microns, which is an order of magnitude larger than bone

cell size, or strut dimensions on the same order as cell size (1-10 μ m), but stiffness spanning 10-200 kPa^{33,2,43}.

Most of the scaffolds that were used in these studies were made out of FDA-approved polycaprolactone, polyglycolic acid, polyglycolide, polylactide, collagen and hydroxyapative-polymer composites which are inherently compliant, which are unable to reach a larger stiffness range without increasing the strut size.

1.2. Architected Cellular Solids

1.2.1. What are Cellular Solids?

Cellular solids are assemblies of geometrical unit cells made up of an interconnected network of solid struts or plates that pack together to fill space in either a stochastic fashion such as in stochastic foams or in an ordered fashion such as in architected materials. By combining constitutive material properties and architectural elements, properties such as stiffness, strength, toughness, thermal conductivity, diffusivity and so forth, can be tailored to meet requirements that were traditionally thought to be impossible. The mechanical properties of cellular solids are generally characterized by their constituent material properties, relative density, which is defined as the volume fraction of the solid material (*V*_i) divided by the representative volume of the unit cell (*V_n*), $\bar{\rho} = V/V_n$ and structural topology, which is defined by the connectivity of the struts or faces comprising the unit cell of the cellular solid⁴.

Cellular solids can be topologically defined as bending or stretching-dominated. This distinction comes from the unit cell's nodal connectivity (Z), which represents the number of struts that meet at an intersection point. A three-dimensional structure with Z < 12 is bending dominated and its main deformation mechanism is via bending of its struts while a structure with $Z \ge 12$ is stretching dominated and resists mechanical deformation via compression and tension of its struts^{44,47}. The structural deformation mechanism directly impacts the modulus (E) and yield strength $(\bar{\sigma}_y)$ of the overall structure⁴⁸. Bending-dominated are structures that allow for bending of their beams, and their strength and modulus scale as $\sigma_y \propto \bar{\rho}^{1.5}$ and $E \propto \bar{\rho}^2$, while stretchingdominated structures have no intrinsic mechanisms that allow for bending deformation, and as a result, their strength and stiffness scale linearly with relative density $\sigma_y \propto \bar{\rho}$ and $E \propto \bar{\rho}^*$.

A cellular material under compression undergoes three regimes of behavior in its stress-strain response, as shown in Figure 1. Initially, the bending of cell edges corresponds to the linear elastic behavior. This linear elastic region is followed by a stress plateau involving cell collapse and specific behavior, such as plastic yielding or brittle failure, dependent on the nature of the solid material from which the cellular solid is composed. The final regime begins when opposite cell walls come into contact, with further deformation compressing the cell wall material itself. This results in the steeply rising regime of the stress-strain response, known as densification^{se}.



Figure 1: Typical uniaxial stress-strain response for an elastic-plastic foam under compression. The three regimes of the mechanical behavior (linear elasticity, plateau, and densification) are labeled. Based on [50].

1.2.2. Nature's Architected Materials

Over millions of years nature developed cellular structures that display complex architected designs spanning nine orders of magnitude from nanometers all the way to meters.

Nature's use of such a large range of length scales may have evolved as a consequence of the mechanical requirements imposed onto biological systems, which involve minimizing weight while keeping high stiffness, strength and toughness.

By reducing density and taking advantage of structural mechanics, nature's architected materials, which are often composed of both ordered and disordered phases across different levels of hierarchy, are prominent examples of the ability to conserve materials while maintaining excellent mechanical properties. For instance hard biological materials such as bone, antler, shell, nacre, and wood are known to have exceptional hardness and toughness and have been reported to have higher fracture toughness than man-made composites of the same composition and density⁴⁴⁵. Hard porous biological materials such as sea sponges, diatoms, and radiolarians are simultaneously stiff, tough, and lightweight, properties that have been shown to contribute to their effective defense against predators. Plant stems and narrow plant leaves have developed tubular structures that exhibit a honeycomb-like or foam-like core supporting a denser outer cylindrical shell, which increase the resistance of the shell to kinking or local buckling failure⁵³⁴. Characterizing and understanding these natural structures has the potential of providing humans with precious guidelines for the design of new advanced materials.

1.3. Architected Cellular Solids as Platforms for Cell Scaffolding

A major goal of bone tissue engineering is to synthesize or regenerate the injured tissue. Architected 3D cellular solids offer a useful platform to investigate key parameters of bone remodeling for the eventual design of more effective implantable orthopedic devices⁵⁵. The tunability of cellular solids has already been successfully exploited in several fields: by increasing porosity while utilizing a rigid geometry, researchers could fabricate an architected cellular solid with low thermal conductivity and high stiffness which is highly desirable for space application⁴⁶. The ability to modulate pore size and their geometrical distribution allowed for the fabrication of an ultralight cellular structure that exhibited excellent energy absorption properties, which is ideal for military application^{57,88}.

Recent advances in 3D manufacturing technologies has enabled the creation of arbitrary geometries with feature sizes spanning from a few hundred nanometers to a few hundred microns, which make it very attractive for biomedical application. Self-propagating photopolymer waveguides, microstereolithography, two-photon lithography, and holographic lithography are techniques that allow for the fabrication of such 3D multi-scale structures³⁹⁻⁶¹. In addition to controlling porosity, unit cell geometry and periodicity, these techniques are well integrated with established semiconductor fabrication techniques (etching, metallization, surface functionalization), which increase the parameter space that is available for cell interrogation. Ultimately, cellular solids could be fabricated with optimal properties for synthetic bone growth. The patient's target cells would be harvested and seeded onto the multi-scale 3D architected scaffold that would serve as an artificial extra cellular matrix for cell growth and tissue formation. The diseased or damaged tissue from the injured site would be removed and the scaffold with attached cells would then be implanted in the patient. Over time, the synthetic matrix would integrate into the patient's body with full tissue regeneration with properties identical to native tissue.

1.4. Thesis Overview

The aim of this thesis is to develop a better understanding of cell behavior, specifically bone cells, on periodic 3D nano-architectures. Chapter 2 describes the fabrication of nanoarchitected materials with different geometries, kagome and tetrakaidecahedron, which were used to identify a suitable unit cell size that allowed for cell infiltration and proliferation. Electron microscopy was used to confirm that a pore size of 25µm appears to be an adequate size for cell infiltration into the fabricated structures.

Material effects were probed by locking unit cell geometry and pore size. Polymeric and hydroxyl-apatite-coated nanolattices with a tetrakaidecahedral geometry were fabricated and osteoblast-like cells were grown on them. SEM analysis revealed that morphological differences in the deposits secreted by the cells were evident, but quantitative analysis could not be performed due to scarcity of deposited material available. These experiments revealed that both pore size and scaffold material affect cell infiltration, proliferation and function.

Based on these findings, we fabricated larger nanolattices of constant porosity and material composition that were used to probe osteoblast functionality, which was measured as a function of collagen and mineral deposition. SEM, energy dispersive spectroscopy (EDS) and Raman spectroscopy were utilized to evaluate the biocompatibility of the fabricated nanolattices. Cell adhesion, proliferation and mineral deposition were observed to be qualitatively indistinguishable across nanolattices with elastic moduli spanning from ~2-9MPa. EDS and Raman spectroscopy analysis revealed the presence of hydroxylapatite (Ca_i(PO_i)_a(OH)_a), tricalcium phosphate (Ca_i(PO_i)_a) and metaphosphates ([Ca_i(P₂O_i)]_a), chemical species normally found in natural bone. Such osteogenic functionality suggests that 3-dimensional nanoarchitected

materials can be used as effective scaffolds for cell growth and proliferation, which could eventually lead to the generation of better bone implants.

Chapter 3 explores the role of scaffold structural stiffness on osteoblast activity. The mechanosensitive response of osteoblast-like cells was explored by tracking mineral secretions and intracellular f-actin and vinculin concentrations after 2, 8 and 12 days of cell culture in mineralization media on nanonattices with tetrakaidecahedral periodic geometry and structural stiffness ranging from 0.7-100 MPa.

Experiments revealed that the most compliant nanolattices had $\sim 20\%$ more intracellular f-actin and $\sim 40\%$ more calcium and phosphate secreted onto them than the stiffer nanolattices, where such cellular response was virtually indistinguishable.

Chapter 4 focuses on investigating the role of different dynamic loading conditions on bone cell activity, which was measured by tracking the concentration of intracellular stress fibers and focal adhesions. By compressing osteoblasts, which had been grown on nanolattices of different structural stiffnesses to varying strains, stresses and loading frequencies we attempted to elucidate which mechanical cues bone cells are more sensitive to. These results can be used to develop medical therapies which have the potential of slowing down, if not preventing bone density loss in patients prone to osteoporosis.

Chapter 5 investigates the potential of combining two-photon lithography with standard semiconductor fabrication techniques to develop 3D architected force sensors that can probe cellular forces in microenvironments which mimic more realistic physiological conditions. Overall this thesis points to 3D nanoarchitected materials as a promising platform for studying complex cellular behaviors and for creating efficient implantable medical devices.

Chapter 2. Investigating the Biocompatibility of Two Topologically Different Three-Dimensional Nano-Architected Materials: the Kagome and Tetrakaidecahedron Geometries.

In this chapter we report on the fabrication of periodic 3D nano-architectures with ~99% porosity, ~2 μ m strut diameters, and ~2-9MPa structural stiffness that were used to explore the influence of scaffold properties on the viability of osteoblasts in a microenvironment similar to that of natural bone.

First we explored the role that geometry and pore size had on cell infiltration and found that a pore size of 25μ m in diameter was sufficient for cell adhesion and proliferation. We also confirmed that nanolattice material affected cell response, which was measured as a function of cell secretions. In order to eliminate such variables we fabricated nanolattices that had unit cells with tetrakaidecahedral geometry and a pore size of 25μ m. The unit cells were tessellated in space to form a lattice with lateral dimensions of 200μ m x 200μ m and a height of 50μ m. Some of the polymer nanolattices were coated with a conformal 120nm-thick layer of SiO₂, others were coated with 120nm of Ti. All nanolattices had a ~20nm-thick outermost layer of TiO₂.

Osteogenic cells were grown on the nanolattices for 28 days, and the resulting cell morphology and depositions were characterized via scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), and Raman spectroscopy. These analyses revealed significant cell attachment and the presence of hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$), tricalcium phosphate ($Ca_5(PO_4)_2$) and metaphosphates ([$Ca_2(P_2O_7)]_8$), chemical species normally found in natural bone. Such osteogenic functionality suggests that 3-dimensional nano-architected materials can be used as effective scaffolds for cell growth and proliferation, which could eventually lead to the generation of better bone implants.

2.1. Chapter Summary

Bone grafting is among the most common surgeries in the US with approximately 400,000 cases per year^{sz}. As detailed in section 1.1.1. and 1.1.2. commercially available implants are made of metallic alloys that exhibit high stiffness, strength and fracture toughness. As a consequence of these properties they cause stress shielding, cytotoxicity, which due to byproducts leeching and their low porosity leads to poor re-vascularization of the injured site.

Clinical data confirms the inadequacy of current bone implants and highlights the importance of understanding the fundamental causes of implant failure at the cellular level. Several questions remain unanswered: (1) what is an adequate pore size for successful cell infiltration and nutrient diffusion? (2) Are cells affected by the scaffold material? (3) Are cells highly sensitive to changes in scaffold stiffness?

When bone fracture occurs, the complex architecture that osteocytes have developed over years ceases to provide the necessary conditions for bone homeostasis. An ideal orthopedic implant needs to provide an ideal microenvironment that induces cells to re-build the native architecture, which can ultimately facilitate osteointegration and osteogenesis⁶³.

The microenvironment in which cells reside *in vivo* exhibits a complex milieu of signals which play an essential role in a diverse set of cellular processes. Cells are capable of sensing and responding to a plethora of signals, consisting of biochemical and biophysical cues, over a wide range of length scales⁴⁴⁵⁵. Cell signaling, which drives all cell behaviors occurs at length scales that range from a few nm to a few hundred microns⁴⁶⁵. Current implants are either monolithic or exhibit features that are on a millimeter length scale which is 2 orders of magnitude larger than

the size of a single cell. As a consequence of this length scale mismatch even the smallest feature of current implants is virtually indistinguishable from a flat 2D substrate to a single cell. Cell response to 2D substrates is remarkably different from 3D substrates (see section 1.1.2. for details) therefore a need to fabricate scaffolds with features size on the sample scale as single cells is evident if we aim at understanding cellular processes that occur in-vivo microenvironments.

The parameter space in 3-D is more complex than 2D and involves investigating the effects of relative density, effective surface area, defined as the total scaffold's surface area available to cells, and scaffold's structural stiffness on cell function. The latter is usually correlated with the deposition of collagen and calcium phosphate. To the best of our knowledge, all existing studies have either adopted scaffolds with unit cell strut/wall size larger than 100 μ m, porosity of 40-60% and high compressive moduli in the hundreds of MPa range OR scaffolds with the beam diameters on the order of single digit μ m, porosity of 80–90% and low compressive moduli ranging from a few tens of kPa to hundreds of kPa¹¹⁹²⁶⁶²⁶⁶⁷.

In most of these studies, the scaffolds were made of various compositions of calcium phosphate mixed with polymeric matrices. They also had either close-cell topologies, which retards nutrient diffusion and hence requires using large pore sizes of ~100 to 700 μ m, or open-cell stochastic foams, where beam and pore sizes vary randomly throughout the scaffold. Because of the nature of stochastic foams, no regularities in strut size, pore geometry or unit cell stiffness is available, which makes such structures not ideal to understand cell behaviors in 3D environments.

In this chapter we describe the fabrication and testing of 3D scaffolds with MPa-level structural stiffness and strut dimensions on the same order as cell size $(1-10\mu m)$. We initially varied unit cell geometry and pore size to evaluate cell adhesion and infiltration. Secondly we kept pore size

and unit cell topology constant to assess cell response as a function of scaffold material composition.

2.2. Methods

2.2.1. 3D Nanolattice Design and Fabrication

Two different geometries were employed in this chapter: kagome and tetrakaidecahedron. Kagome is a stretching periodic-bending dominated structure with a connectivity Z = 6 and the tetrakaidekahedron is a bending periodic bending dominated structure with a connectivity $Z = 4^{\text{mas}}$. The fabricated Kagome unit cell exhibited a relative density of 4.05% and the tetrakaidecahedron unit cell has a relative density of 1.23%, which is close to that of trabecular bone, 5%⁴⁴. We chose the kagome and tetrakaidecahedral geometries to mimic the porous nature and mechanical response of trabecular bone.

The kagome unit cell had a pore size, $U_x = 13.5\mu m$, measured as the linear distance from one triangular face to the opposite one, the beam length (l_x) was 4.25 μm and the beams had an elliptical cross section with a major axis (2*a*) of 1.43 μm and a minor axis (2*b*) of 0.6 μm (Figure 2(a)).



Figure 2: CAD modeling of the kagome and tetrakaidecahedron geometries. (a) 3dimensional CAD representation of the kagome unit cell highlighting the pore size (U_{κ}) , the beam length (l_{κ}) and the beam elliptical cross section with minor axis (2b) and major axis (2a).

(b) CAD model of how kagome unit cells were patterned in space to create a nanolattice. (c) CAD model of a tetrakaidecahedron unit cell highlighting the unit cell pore size (U_{τ}) and beam length (l_{τ}) . (d) CAD model showing the tessellation of the tetrakaidecahedron geometry in space.

The tetrakaidecahedron unit cell had a pore size, $U_r = 25\mu m$, measured as the linear distance from one square face to the opposite one, the beam length (l_r) was 8.33 μm and the beams had an elliptical cross section with a major axis (2*a*) of 2.24 μm and a minor axis (2*b*) of 1.3 μm (Figure 2(c)). Each nanolattice that was used to evaluate pore size as a function of cell infiltration into the scaffold was composed of 4x4x4 unit cells while the nanolattices that were used to test for biocompatibility were composed of 8x8x2 unit cells and were arranged in a square. Figure 2 (b) and (d) show representative images of how the kagome unit cell (b) and the tetrakaidecahedron unit cell (d) were tessellated in space to create a nanolattice.

All scaffolds were fabricated via two-photon lithography (TPL) direct laser writing (DWL), which employs a femtosecond-pulsed laser (780nm) that is rastered in space to selectively crosslink a negative tone photoresist, IP-Dip (Nanoscribe GmbH), into a designed structure. Some of the resulting polymer nanolattices discussed in this thesis were subsequently coated with different materials using either sputtering or atomic layer deposition (ALD) and some were hollowed out via the the focus ion beam (FIB) and oxygen plasma etching. Figure 3 shows a CAD representation of the nanolattice fabrication process.



Figure 3: Nanolattice fabrication process. (a) a CAD model of the final nanolattice is prepared via MATLAB (Mathworks) or Solidworks (Dessault systems). (b) The CAD model is processed by a two-photon lithography instrument (Nanoscribe) which employs an infrared laser (780nm) to cross-link a negative tone resist. (c) Polymer nanolattice skeleton after wet development. (d) Materials can be coated on the polymeric nanolattice scaffold via sputtering, ALD or CVD deposition techniques. (e) The FIB can be used to slice open the edges of the nanolattice to expose to core polymer skeleton, which can be removed by immersing the nanolattice in an O_2 plasma etcher. The inset shows the resulting hollow shell.

To investigate the role of porosity we fabricated nanolattices which were made of polymer, IP-Dip (Nanoscribe GmbH). To investigate nanolattice biocompatibility the nanolattices were made of three material systems: (i) Polymer nanolattice IP-Dip (Nanoscribe GmbH) coated with a 20 nm thick layer of TiO₂ deposited via Atomic Layer Deposition (ALD); (ii) polymer nanolattice coated with 120 nm of sputtered SiO₂ and the same outermost ALD coating of 20nm TiO₂ as (i); and (iii) polymer nanolattice coated with 120nm of sputtered Ti and the same outermost ALD coating of 20nm TiO₂ as (i) and (ii). The three material systems and their arrangement on the chip are shown in figure 4.



Figure 4: Material systems design and fabrication. (a) SolidWorks model of the tetrakaidecahral unit cell and cross sectional view of the three material systems used in the experiments. (B) SEM image of the trekaidecahedral unit cell. (C) SEM image of the nanolattice formed by 8x8x2 unit cells. (D) SEM image of a single chip containing 4 nanolattices.

Sputter deposition was carried out using a TES magnetron sputterer (Temescal BJD-1800). Titanium was sputtered using RF power at 125W, a working pressure of 10 mtorr, Ar pressure of 100 sccm and table rotation set at 100%. An average Ti thickness of 120nm was obtained after depositing for 70 minutes. SiO₂ was deposited using RF power of 125W, a working pressure of 10 mtorr, Ar and O₂ as sputtering gases with a relative concentration of 80% to 20% and table rotation set at 100%. The deposition occurred over 180 minutes to obtain an average coating of 120nm. All 3 material systems were coated with a 20nm-thick outermost layer of TiO₂ that was

deposited using a Cambridge Nanotech S200 atomic layer deposition (ALD) system with H₂O and Titanium Tetrachloride (TiCl₂) as precursors. The relative density of the nanolattices was calculated using Solidworks software by evaluating the volume fraction of the solid material and dividing it by the representative volume of the unit cell.

2.2.2 Beam Stiffness Calculations

We performed analytical calculations to estimate the stiffness of individual struts that comprise the nanolattice. Strut stiffnesses were used to evaluate whether the seeded cells would be able to cause any significant bending of the struts. The slenderness ratio (L/a) of the beams is ~10, which is in the regime of applicability of the Euler-Bernoulli beam bending theory. It is reasonable to assume that the seeded cells exert a distributed load, q, of 100nN/µm, that the beams have fixed boundary conditions at the nodes, and that the bending stiffnesses of the coatings can be linearly superposed.

Assuming pure bending loading conditions, the maximum deflection of a beam, w_{max} , caused by the cells is

$$w_{\rm max} = \frac{qL^3}{384(E_P I_P + E_C I_C + E_{TiO_2} I_{_{TiO_2}})}$$
(1)

and the beam stiffness is:

$$k_{eff} = k_{P} + k_{C} + k_{TiO_{2}} = \frac{384(E_{P}I_{P} + E_{C}I_{C} + E_{TiO_{2}}I_{TiO_{2}})}{L^{3}}$$
(2)

where k_P is the bending stiffness of the polymer beam, k_C is the bending stiffness of the intermediate beam coating material (SiO₂, Ti) and k_{TiO_2} is the bending stiffness of the outermost
TiO₂ coating. E_P , E_C and E_{TiO_2} are the elastic moduli of the polymer, the intermediate coating (SiO₂, Ti) and the outermost TiO₂ coating, respectively. The moment of inertia of the elliptical monolithic beam is $I_P = \pi a^3 b/4$, that of the intermediate coatings of thickness t_e is

 $I_{C} = \frac{\pi}{4} \{ [a + t_{C}]^{2} [(a + t_{C})(b + t_{C}) - ab], \text{ and that of the outermost titania coating of thickness}$ $t_{TiO_{2}} \text{is } I_{TiO_{2}} = \frac{\pi}{4} \{ [a + t_{C} + t_{TiO_{2}}]^{2} [(a + t_{C} + t_{TiO_{2}})(b + t_{C} + t_{TiO_{2}}) - (a + t_{C})(b + t_{C})].$

Determining the proper boundary conditions for the nanolattice beams is not trivial. The main difficulty in choosing the beam boundary conditions comes from the contributions of the nodes to the overall stiffness of the beams. Literature on cellular solids often assumes pin-jointed beams for lattices, which serves as the foundation for the classical theories on their mechanical deformation^{46,30}. Compression experiments performed in our group on several different kinds of unit cell geometries suggests that the nodes in a nanolattice exert a moment on the beams, so perhaps a more accurate boundary condition would be a torsional spring with a prescribed stiffness. To the best of our knowledge, no conclusive data on the rotational stiffness of the nodes has been reported because of the challenges associated with the experiments to obtain such data.

In order to show the importance of the boundary conditions in accurately predicting the stiffness of the nanolattice beams we recalculated the bending stiffness and the maximum deflection of the beams using fixed-fixed and pin-pin boundary conditions and observed a nearly order of magnitude difference in beam stiffness and maximum displacement. Table I summarizes the original and updated calculations for stiffness and Table II summarizes the results for maximum deflection. **Table I**: Beam stiffness as a function of boundary conditions for each material system. All stiffnesses are expressed in kN/m.

B.C.	Polymer-TiO ₂	Poymer-SiO ₂ -TiO ₂	Polymer-Ti-TiO ₂
Pinned-Pinned	0.8	1.8	2.3
Fixed	7.6	17	24

Table II: Comparison of the maximum deflection caused by a distributed load (q) using pinnedpinned as opposed to fixed boundary conditions. All values are expressed in picometers (pm).

B.C.	Polymer-TiO ₂	Poymer-SiO ₂ -TiO ₂	Polymer-Ti-TiO ₂
Fixed	13	6	4
Pin-Pin	121	58	42

This clearly highlights the significant role of the boundary conditions in determining the beam stiffness and its maximum deflection. The SAOS-2 cells used in this work utilize protein complexes that have sizes on the order of tens of nanometers to sense mechanical cues, and the resultant beam deflections are in the subnanometer range regardless of boundary conditions. The difference in maximum beam deflection obtained using either boundary condition should not cause a significant change in cell response.

2.2.3. In-Situ Uniaxial Compression Experimental Setup

The structural stiffness of the nanolattices was obtained by performing uniaxial compression experiments in an in-situ nanomechanical instrument (InSEM, Nanomechanics, Inc., Oak Ridge, TN). Samples were compressed to failure under quasistatic conditions at a strain rate of 10³s⁴ simultaneously collecting load vs. displacement data and observing the deformation process. A stainless steel flat punch with a diameter of 600µm was used to compress the nanolattices. The

load-displacement data was converted into engineering stress vs. strain using $\sigma = F/A$ where *F* is the recorded load and *A* is the footprint area of the nanolattice. Global axial strain ε was calculated as $\varepsilon = (H_i - H_i)/H_i$ where H_i is the initial height of the nanolattice measured from SEM images and $(H_i - H_i)$ is the displacement measured by the nanoindenter. The modulus was calculated form the elastic loading portion of the data as:

$$E^* = \frac{d\sigma}{d\varepsilon} \tag{3}$$

2.2.4. Cell Culture Conditions

SAOS-2 osteosarcoma cell line was used for all in vitro studies. Growth media for SAOS-2 cells consisted of 50/50 DMEM:F12 with 5% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Mineralization media consisted of growth media supplemented with 50 μ g/ml ascorbic acid and 50 μ g/ml β -glycerol-phosphate. Experiments were performed over 14 day to study the effects of pore size on cell infiltration into the nanolattice and 28 days to investigate the biocompatibility of the nanolattices. Cells cultured in growth media for the first 14 days, and mineralization media for the final 14 days. Media was changed every 2 days.

2.2.5. Characterization of Cell-Scaffold Interactions

SAOS-2 cells cultured on the nanolattices were fixed with 4% paraformaldehyde for 15 minutes. For the analysis of cellular infiltration into the kagome nanolattice, in addition to using SEM, confocal microscopy was also performed. Immunocytochemistry staining was performed by using vinculin, which is a marker for focal adhesions that was identified by using a primary mouse IgG anti-vinculin antibody (diluted 1:50 in 10% goat serum, Millipore) and a secondary goat FITC–IgG anti-mouse antibody (diluted 1:50 in 10% goat serum, Millipore). Cytoskeletal factin and cell nucleus staining were carried out by incubating cells with TRITC-conjugated phalloidin (Sigma) and DAPI (Millipore). For SEM analysis, fixed samples were rinsed in phosphate buffered saline and serial dilutions of ethanol in phosphate buffered saline until 100% ethanol was attained. The nanolattices were then dried in a critical point dryer; cell morphology and mineralization were investigated using a FEI Nova 200 Nanolab scanning electron microscope (SEM) equipped with an energy dispersive spectroscopy module (EDAX Genesis 7000). The accelerating voltage that was necessary to survey the energy levels of all the expected elements was found via software simulation (Casino MonteCarlo electron beam simulator) to be 8KeV, which results in an interaction volume of ~500nm. Dead time, which is the time that the detector takes to analyze each incoming x-ray and assign the photon energy to a given element, of 18-20% was kept constant throughout all scans by adjusting the amplification time on the EDAX system. Renishaw M1000 MicroRaman Spectrometer system with a laser wavelength of 514.5 nm and a power density 130W/cm⁻ was used for Raman analysis.

2.3. Results

2.3.1 Porosity and Material Effects on Cell Response

SAOS-2 cells were cultured for 14 days on the kagome and the tetrakaidecahedron nanolattices, both made out of IP-Dip polymer. We observed widespread cell adhesion on both geometries, as shown in figure 5, and no adverse cell response, measured as the number of floating cells in the culture dish was evident.



Figure 5: Post SAOS-2 cell seeding SEM and fluorescence microscopy characterization. (ac) SEM images of the kagome nanolattice showing SAOS-2 cells adhering to the top (a) and sides (b, c) of the polymeric nanolattice. (c) reveals that no apparent cell infiltration inside the kagome nanolattice took place. (d) Confocal fluorescence microscopy confirming that cells grew on the outermost unit cells of the nanolattice. The side view represents a cross sectional slice through the nanolattice revealing no cell presence inside the kagome nanolattice.

(e-h) SEM images showing SAOS-2 cells adhering to the top (e, f) and infiltrating (g, h) the tetrakaidecahedron polymeric nanolattice.

Cell infiltration into the nanolattice with a kagome unit cell with a pore size, $U_x = 13.5 \mu m$, was not observed neither via SEM nor via confocal microscopy (figure 5(c, d)). When SAOS-2 cells were seeded on tetrakaidekahedral nanolattices with a unit cell of pore size, $U_x = 25 \mu m$, both cell adhesion and infiltration were observed as shown in figure 5(e-h). As a result, the tetrakaidekahedron geometry with a pore size of 25 μm was chosen as a suitable scaffold for further cell response investigation.

In order to probe the effects that nanolattice material composition had on cell response we grew SAOS-2 cells on tetrakaidecahedron nanolattices made of either IP-Dip or IP-Dip coated with a

20nm thick layer of ALD-deposited TiO₂, which was chosen because of its widespread use in implantable medical devices. Upon growing SAOS-2 cells on the nanolattice for 14 days we observed morphological differences in cell response. The polymer nanolattice showed the presence of cells adhering to the structure (figure 6 (a)) and exerting forces on the beams of the nanolattice as evident from figure 6 (b).



Figure 6: Nanolattice biocompatibility. (a) SEM image of a polymeric tetrakaidecahedron nanolattice showing cell attachment after growing SAOS-2 cells for 14 days. (b) zoomed-in view of the cells pulling on the nanolattice beams. Cell attachment appeared to be mainly localized to the bottom unit cells. (c) SEM image of a TiO_3 -coated tetrakaidecahedron nanolattice showing the presence of both cells and cellular secretions. (d) zoomed-in view of the cellular secretions that were found throughout the TiO_3 -coated nanolattice.

In addition to cells attaching and exerting forces on the nanolattice (figure 6 (c)), the TiO₂-coated scaffold also displayed the presence of cellular secretions which were found throughout the structure (figure 6 (d)). These secretions appeared to be composed of nano-sized granules interspersed in a filamentous matrix. The size of the nanolattice, 100x100x100um coupled with the cell culture time, 14 days, didn't allow the SAOS-2 cells to deposit enough material to be analyzed via spectroscopic techniques.

2.3.2. Morphological Characterization of Cell-Scaffold Interactions

Our results showed that unit cell geometry, pore size and scaffold outer coating material affected cell response therefore in order to investigate the nanolattice biocompatibility as a function of nanolattice structural stiffness we fixed such variables. We fabricated a tetrakaidecahedron nanolattice, 200x200x50µm, with unit cell size of 25µm and outer coating of TiO₂. Three material systems were created to probe scaffold biocompatibility as a function of structural stufness: (i) Polymer nanolattice IP-Dip (Nanoscribe GmbH) coated with a 20nm-thick layer of TiO₂ deposited via Atomic Layer Deposition (ALD); (ii) polymer nanolattice coated with 120 nm of sputtered SiO₂ and the same outermost ALD coating of 20nm TiO₂ as (i); and (iii) polymer TiO₂ as (i) and (ii) (figure 4 (a)).

We observed that SAOS-2 cells cultured on the nanolattices for 28 days adhered to the scaffolds for all material systems and produced organic and mineral phase deposits shown in figure 7. These deposits had two different morphologies: (1) spherical mineral aggregates, whose diameter ranges from $\sim 2\mu m$ to $\sim 10\mu m$ (figure 7 (e)) and (2) sponge-like mineralized phase, whose size varied from $\sim 1\mu m$ to $\sim 100\mu m$ (figure 7 (f)). The other two commonly observed features were inclusions embedded in the organic matrix phase that were ~150nm-diameter needle-like protrusions, whose lengths were on the order of a few microns (figure 7 (g)), and a network of ~50nm-diameter intertwined filaments (figure 7 (c), (d)). In further discussion, we refer to these four features as sphere (figure 7 (e)), spongy (figure 7 (f)), needle (figure 7 (g)) and filaments (figure 7 (c), (d)).



Figure 7: Post SAOS-2 cell seeding SEM characterization. The cross-sectional schematic view on the top left corner provides a legend for which system a specific phase was found. The white arrow in (b-g) indicates the presence of a specific phase. (a) SEM image showing no evident difference in cell colony formation and cell secretions on our three material systems after growing SAOS-2 cells for 28 days. (b) SAOS-2 cell attached to a unit cell. (c) Image of the filament phase that appears to serve as an anchor between cells and the nanolattice. (d) Zoomed-in view of the filament phase showing filament diameter varying from about 40 to 100nm. (e)

Image of the Sphere phase with a diameter of 9μ m. (f) Image of the Spongy phase. (g) Image showing the presence of needle-like features that grew in close proximity of the Spongy phase.

2.3.3. Nanomechanical Compression Results

We performed quasi-static uniaxial compression experiments to determine the effective structural stiffness of nanolattices. Figure 8 shows representative stress-strain data for nanolattices made from each material system.



Figure 8: Mechanical tests on the three material systems. (a-c) SEM images of the three material systems. From the top: material system (i) composed of solid core polymer coated with 20nm-thick layer of TiO₂. System (ii) comprised of solid core polymer coated with 120nm-thick layer of SiO₂ and a 20nm-thick layer of TiO₂. System (iii) comprised of solid core polymer coated with 120nm-thick layer of TiO₄.

(d-f)stress-strain plots of the three material systems that were tested. The red dashed triangle indicates the region from which the structural elastic modulus was calculated. (g-i) post-compression SEM images for material system (i), (ii),(iii) respectively.

A total of 4 nanolattices were compressed for each material system. The data reveals that all material systems exhibited an extensive so-called toe region, represented by an initial non-linear stress-strain behavior up to $\sim 10\%$ strain followed by a linear region up to 14\% strain at ~ 0.22 MPa for system (i) (polymer nanolattice coated with 20nm TiO_i), after which localized instabilities caused deviations from linearity until the final brittle failure at 24% strain and 0.41 MPa (figure 8 (d)). We observed similar compressive behavior in system (ii) (polymer nanolattice coated with 120nm of SiO₂ and an outermost layer of 20nm TiO₂), which exhibited a large toe region up to $\sim 9\%$ strain followed by a linear elastic region up to 13% strain and 0.24 MPa followed by non-linear deformation and final brittle failure at a strain of 21.5% and a stress of 0.42 MPa (figure 2 (e)). System (iii) (polymer coated with 120nm of Ti and an outermost layer of 20nm TiO₂) exhibited a toe region up to $\sim 7\%$ strain followed by a linear region up to the onset of plasticity at a strain of 12.7% and a stress of 0.56 MPa followed by brittle failure at 14.5% strain and 0.63 MPa (figure 2 (f)). The linear elastic regime from which the stiffness was extracted likely represents a reasonable approximation for the structural modulus of the nanolattices^{49,71}. Using Eq. (3), we found that nanolattices coated with TiO₂ only (system (i)) had a modulus of 1.98 ± 0.28 MPa, nanolattices coated with SiO₂ and TiO₂ (system (ii)) had a modulus of 3.71 ± 0.52 MPa, and nanolattices coated with Ti and TiO₂ (system (iii)) were the stiffest, with a modulus of 8.82 ± 0.22 MPa.

2.3.4. Spectroscopy for Molecular Cell-Secretions Analysis

Figure 9 (a-c) shows the presence of cell secretions at multiple length-scales: at the largest length-scale we observed large aggregates that occupied portions of the nanolattice which extended several microns (figure 9 (a)). At smaller length-scales we noticed the Sphere (figure 9 (b)) and the Spongy phase (figure 9 (c)) within the nanolattice unit cells suggesting a hierarchical assembly of these phases.

We used EDS to identify the chemical composition of the cell products that were deposited on the nanolattices. Figure 9 (d) shows these EDS spectra that reveal the presence of C, O, Ca, P, Mg and Na on our nanolattices.



Figure 9: EDS spectra of features found on the nanolattices. (a-c) SEM images showing the areas that were scanned for EDS analysis. (d-f) EDS spectra of the nanolattice, Sphere phase and Spongy phase respectively. The spectrum taken of the nanolattice (d) shows a large amount of carbon due to the extensive presence of cells and extracellular matrix on the nanolattice; The

EDS spectra (e) and (f) show that the ratio of magnesium to calcium was larger by a factor of 2.4 in the Sphere phase as compared to the Spongy phase.

Table III shows the relative atomic concentrations of the elements typically found in bone (Ca,

Mg, P, O) in the in the individual identifiable phases (sphere and spongy).

Table III: Elemental ratios in the Sphere and Spongy phase obtained via EDS showing that the amount of magnesium compared to calcium in the Sphere phase was larger by a factor of 2.43 compared to the Spongy phase.



We were not able to obtain similar scans for the embedded needle-like features and the filamentous phase because of the low signal to noise ratio. In order to obtain a more detailed picture of the secretions that were observed on the nanolattices we used Raman spectroscopy. Figure 10 shows the Raman spectra collected from the organic phase and the mineral deposits.



Figure 10: Raman analysis of SAOS-2 secretions. (A) Raman spectrum of Spongy phase showing a broad peak in the 930-990 cm⁴ consistent with the presence of several calcium-phosphate compounds; the insert shows a zoomed-in view of the peaks corresponding to Amorphous Calcium Phosphate (ACP) at 946 cm⁴, Hydroxyapatite (HAP) at 962 cm⁴ and Dicalcium-Phosphate-Dihydrate (DCDP) at 985 cm⁴. (B) Raman spectrum of the organic phase showing the presence of nucleic acids in the region spanning from 645 to 731 cm⁴ which indicates the presence of cellular material and the characteristic three peaks at 1248 cm⁴,1452 cm⁴ and 1660 cm⁴ that are typical of collagen molecules.

The spectra collected from the organic phase contained peaks consistent with the presence of mammalian cells, i.e. nucleic, amino, and fatty acids. We observed the most prominent peaks at 1660cm⁴, 1256cm⁴ and at 1450cm⁴, which have consistently been associated with the presence of collagen molecules^{72,73}. Table IV provides a detailed peak assignment for this phase.

Table	IV:	peak	assignment	of the	organic	phase	taken	from	Raman	data.
			0							

Wave number	Assignment
cm ⁻¹	
645	
669	Nucleic Acids
729	
852	C-C proline, hydroxyproline
880	C-C hydroxyproline
1005	Phenylalanine ring breathing
1256	Amide III
1452	CH ₂ wag
1660	Amide I

The spectra collected from the spongy phase revealed the presence of several species of calcium phosphate-based compounds, with a peak at 962 cm⁴, which represents the v_i phosphate stretch, and two broad peaks at 946 cm⁴ and at 970 cm⁴, which are indicative of amorphous calcium phosphate (ACP) and tricalcium phosphate (TCP). The asymmetric phosphate stretch (v_i) at 1030 cm⁴ and a symmetric carbonate stretch (v_i) at 1070 cm⁴ were also identified. The peak at 1090 cm⁴ revealed the presence of MgCO₃^{74/3}. Table V summarizes the peak assignment for the spongy phase. Limitations in optical resolution of the Raman instrument precluded us from investigating additional features found on the nanolattices.

Wave number cm ⁻¹	Assignment
879	Octacalcium-Phosphate (OCP)
946	Amorphous Calcium Phosphate (ACP)
962	Hydroxyapatite (HAP)
970	Tricalcium-Phosphate (TCP)
985	Dicalcium-Phosphate-Dihydrate (DCDP)
1030	Phosphate Asymmetric stretch

Table V: peak assignment of the spongy phase taken from Raman data.

2.4. Discussion

2.4.1. Pore Size and Material Effects on Cell-Scaffold Activity

Existing literature has been largely focused on studying cell behavior on 2D surfaces, the effects of 3-dimensional scaffolds on cell function have not yet b*een fully mapped out****.

We fabricated 3-dimensional nanolattices with different unit cell geometries and pore size. Previous literature showed conflicting results with respect to the minimum adequate pore size for cell growth and infiltration. O'Brien et al. showed that a minimum pore size of 20µm was necessary for osteogenic cell viability^a; Tayalia et al. have shown that the probability of a cell to migrate increased by a factor of 1.5 when the pore size increased from 20µm to 100µm³⁷. Traditionally the smallest pore size for nutrient diffusion and bone formation had been quoted by many as 100µm^{35.41}. However, more recently researchers have shown bone formation in interconnected micropores less than 10µm in size³²⁵.

In order to investigate osteoblast response to scaffold porosity, material composition and stiffness, we took inspiration from trabecular bone, which has been reported to have more osteocytes per unit of volume and higher rate of turnover compared to cortical bone, which is ideal for faster healing¹⁴. The first topology we employed to mimic trabecular bone was the kagome geometry which, like trabecular bone, is bending dominated and has exceptional fracture toughness¹⁴. Two competing factors were taken into consideration when choosing the unit cell pore size: the diameter of osteoblasts in suspension, 10µm, and the maximum beam length to prevent unit cell collapse under its own weight. Based on these constraints we fabricated a kagome nanolattice with pore size of 13.5µm.

SEM images of the polymer kagome nanolattices after growing SAOS-2 cells for 14 days revealed significant cellular attachment on the outermost unit cells of the nanolattice but no cell appeared to penetrate the nanolattice (figure 5(a-d)). Figure 5 (c) suggests that a single osteoblast is able to engage the full unit cell, however the interconnectivity of kagome unit cells, which is made of smaller triangulated structures, as shown in the figure 2, might have drastically hindered cell motion and prevented successful cell migration into the nanolattice. Confocal microscopy (figure 5 (d)) was also employed to check for cell infiltration into the nanolattice. Confocal microscopy confirmed the presence of cells on the outermost unit cells only. The lower arrow in figure 5(d) points to a cross sectional slice of the kagome nanolattice taken via confocal microscopy through the middle of the nanolattice which further proved the absence of cells within the nanolattice. Cell attachment occurred on the flat substrate around the nanolattice and on the outermost unit cells of the nanolattice.

To allow for the fabrication of a nanolattice with larger pore size we introduced a different unit cell geometry, the tetrakaidecahedron, which is also a bending-dominated structure and was

identified as the optimal shape for filling space with minimal surface area^{**}. We were able to fabricate a tetrkadekahedron unit cell with a pore size of 25μ m, ~2x larger than that of the kagome unit cell. Upon growing SAOS-2 cells on polymeric nanolattices with a tetrakaidekahedral geometry we observed cell attachment and cell migration toward the inner unit cells of the scaffold. The SEM images in Figure 5(e), (g) and (h) show that most cells infiltrated the nanolattice and the yellow arrow point to a cell that was able to penetrate the centermost unit cell, which suggests that a pore size of 25μ m was large enough for cell infiltration into the nanolattice. Based on these findings we deemed the tetrakaidecahedron unit cell with a pore size of 25μ m a suitable structure to investigate cell behavior on the nanolattices.

Besides pore size, cell response is affected by the scaffold's surface material. As a consequence of fabrication limitations, most studies involving micro-scale scaffolds have focused on testing structures that were made out of different polymers, PEEK, PLGA, SU-8, PCL, collagen, with varying degree or biodegradability and stiffness. These studies reported differences in cell behavior as a consequence of the scaffold's material³⁷. Recently ceramic and polymer-ceramic composite scaffolds have been fabricated and tested. These materials have shown higher cell viability and mineral deposition than those scaffolds made of polymer only³⁴³⁵⁻³⁷.

In our study we decided to compare polymeric nanolattices (IP-Dip) and TiO₂-coated nanolattices. TiO₂ was our material of choice because of its proven biocompatibility, ease of deposition (ALD) and bioinertness⁵⁸. In order to probe material effects the nanolattices that were tested had the same unit cell geometry, tetrakaidecahedron and same pore size, 25μ m.

SAOS-2 cells, which were grown on the nanolattices for 14 days responded differently to the two nanolattices. Figure 6 shows that cell attachment was present on both nanolattices. In addition to growing on the bottom-most row of unit cells, osteoblasts seeded on the TiO₂-coated nanolattices

also adhered on upper rows of unit cells and secreted material on the nanolattice. The limited size of the nanolattice, 100x100x100µm coupled with a short growth period, 14 days, didn't allow SAOS-2 cells to deposit enough material to be detected via EDS.

In order to characterize these cellular secretions and confirm nanolattice biocompatibility over a range of stiffness (~1-10MPa) that has not been explored before, we fabricated 3 material systems (detailed in section 2.2.1) and grew cells on them for 28 days. We will discuss these material systems' mechanical properties first and then look at their effect on cellular behavior.

2.4.2. Mechanical Characterization

The relative density of the 3 material systems, $\bar{\rho}$ was kept relatively constant at 1.23% and pore size was fixed at 25µm which appear to sufficiently large to ensure nutrient diffusion, cell-to-cell and cell-to-matrix communication. Structural stiffness was determined experimentally through uniaxial compression tests which demonstrated a span of approximately a decade in structural stiffness (figure 8). Material system (i) (polymer nanolattice coated with 20nm of TiO₃) had a modulus of 1.98 ± 0.28 MPa and a stress-strain data with several structural instabilities caused by fracture of individual beams driven by the brittle TiO₃layer. System (ii) (polymer nanolattice coated with 120nm of SiO₃ and 20nm of TiO₃) had a modulus of 3.71 ± 0.52 MPa and displayed similar mechanical behavior as system (i). Both system (i) and (ii) exhibited localized bending and fracture of individual beams up to a stress of ~0.4 MPa, at which inelastic buckling propagated through the unit cells of the top layer similarly to the observations by Oliveira et al. for ceramic foams [41]. System (iii) (polymer nanolattice coated with 120nm of Ti and 20nm of TiO₃) had a modulus of 8.82 ± 0.22 MPa and a ductile stress-strain signature, driven by the deformation of the Ti coating, whose volume fraction was ~22%. This metallic coating led to localized plastic hinge formation in the beams, a behavior previously seen in literature⁵⁵⁰. The onset of global plasticity during deformation of these nanolattices occurred at a stress of 0.56 MPa and a strain of ~13%; the nanolattices ultimately failed via brittle failure at 0.62 MPa, most likely caused by the stress transfer between the Ti and the TiO₂. The substantial toe region present in all nanolattices up to ~10% strain, was caused by the slight initial misalignment and contact between the indenter tip and the initially concave top surface of the nanolattice.

2.4.3. Cell- Secretion Characterization

Figure 7 shows the SEM images of the nanolattices after growing SAOS-2 cells for 28 days, which revealed the presence of extensive cell colonies and cell secretions. We did not observe any geometrical or dimensional changes in the struts, which suggests that the cells did not exert forces that stressed the nanolattices beyond yield. We discovered several common features on the surfaces of the nanolattices made from all material systems: organic proteinaceous phase matrix, as well as spongy, sphere, filaments and needle phases, which suggests that osteogenic functionality was fully induced on each nanolattice. All three material systems elicited a virtually identical response in the seeded cells. Aside from the ubiquitous presence of SAOS-2 on all nanolattices (Figure 7 (a)), filaments on the order of 40-70nm in diameter were found between the cells (figure 7 (c), (d)). These filaments had the dimensions and morphology that is consistent with collagen deposition by osteoblasts on 2D and 3D scaffolds^m. Raman spectra of the regions that contained mostly organic phase had substantial large peaks at 1248 cm⁴, 1452 cm⁴ and 1660 cm⁴, which is consistent with collagen molecules^m.

Needle-like features appeared at random intervals in the vicinity of the spongy phase, and the intensity contrast in the SEM images appeared to be similar for the needle-like features and the spongy phase, which suggests a similar elemental composition of these features. We hypothesize that when the needle phase was nucleated within the organic phase, its growth was directional driven by the mechanical constraints of the surrounding organic phase. When the nucleation of this secondary phase occurred outside of the organic phase it created a more disordered phase, whose morphology is sponge-like (figure 7 (f)).

Resolution limitations of the EDS and Raman instruments prevented us from conclusively demonstrating that the spongy phase and the needle-like deposits (\approx 150nm) were composed of the same molecules. The two deposits, sphere and spongy (figure 7 (e), (f)), had dimensions of several microns that were amenable to a more detailed elemental analysis, which revealed that they were mainly composed of Ca, P, O and C, which represent the main elements in the EDS spectra of bone ⁹²³⁹.

The EDS analysis of the spongy and sphere phases also revealed the presence of Na and Mg, which is also characteristic of bone based on the reports that demonstrated that bone formation and stabilization occurs via inclusions of several positively-charged ions. The presence of Ca, Mg and Na deposits on the nanolattices may have contributed to changing the structural configuration of the phosphorous-containing molecules from long chains to shorter ones, with 2 non-bridging oxygen atoms per phosphate as previously reported^{44,55}.

Several studies on calcium phosphate compounds show that long polymeric chains of oligophosphates (P_2O_3) naturally form in environments with low ion presence, and as the abundance of positive ions increases, the long metaphosphate chains break down into smaller chains^{36,37}. According to these studies, the expected ratio of O:P is 2.5 and of P:X is 2, where X is

a cation of valency of 2° . We used EDS data to calculate the elemental ratios present in the sphere and spongy phases, and we found that in each phase the O:P ratio was 2.5 and that for P:X was 2, which are the theoretical ratios proposed by the metaphosphate stoichiometry. Table VI summarizes the elemental composition of each studied phase.

Table VI: Comparison of the elemental ratios predicted by the metaphosphate hypothesis and the ratios found in the Spongy and the Sphere phase via EDS analysis. The label cation includes the amount of calcium summed to the amount of magnesium present.



We used Raman spectroscopy to confirm the presence of several different phosphate species, which are listed in Table IV.

The Raman spectra collected from the spongy phase revealed the presence of several mineral species that are commonly found in mineralized bone, hydroxyapatite $(Ca_{10}(PO_4)_6(OH)_2)$, tricalcium-phosphate $(Ca_3(PO_4)_2)$ and octacalcium-phosphate $(Ca_8H_2(PO_4)_6)$ [51]. The presence of amorphous calcium phosphate (ACP) and octa-calcium phosphate (OCP) may provide insights

into a possible mechanism of cell-mediated bone mineral deposition, which begins with the deposition of amorphous calcium phosphate that grows to a mature ordered phosphate and finally becomes hydroxyapatite through energetic transformations^{36,38}. We were not able to perform Raman spectroscopy on the sphere phase because they were too small ($\approx 10 \mu m$) to withstand the intensity of the laser without damage.

2.4.4. Cell-Nanolattice Interactions

Based on unit cell size and geometry, we hypothesize that the mechanism for SAOS-2 populating the nanolattices involves the individual osteoblast cells initially exerting traction forces on the individual struts within the nanolattice and subsequently invading an entire unit cell, transferring the mechanical forces onto it. In this mechanism, the entire nanolattice becomes mechanically engaged when a sufficiently large number of cells form a functional unit equivalent to the bone multicellular unit (BMU). A functional unit is formed through cell-to-cell communication and is responsible for matrix-mineral deposition³⁷. Our observations of ubiquitous depositions in the form of extracellular matrix and minerals suggest that the range of stiffnesses in these scaffolds, ~2 to 9MPa, was adequate to enable osteogenic functionality in the seeded cells.

A possible reason for the observed similarity in cell behavior across all three material systems is that the very low forces of ~80-100nN exerted by osteoblasts, apply negligible displacements on the struts. The strut displacements were calculated using Eq. (1) to be 13pm for system (i), 6pm for system (ii) and 4pm for system (iii). These displacements are 3 orders of magnitude smaller than the size of the integrins, ~ 15nm in diameter, which suggests that a cell senses the same stiffness from the nanolattices made from all three material systems.

Deshpande et al. proposed a biomechanical model, which predicts that mammalian cells respond to different substrate stiffnesses by modifying the degree of actin polymerization and concentration of focal adhesion¹⁶⁰. The former is proportional to the number of formed stress fibers, which - along with focal adhesions - provide the mechanism for the cells to exert traction forces on the substrate. We hypothesize that a strut stiffness that ranges from 7.6 kN/m (system (i)) to 24 kN/m (system(iii)), calculated using Euler-Bernoulli beam theory with fixed boundary conditions and a scaffold stiffness of ~2-9 MPa measured experimentally, caused the SAOS-2 cells to maintain a maximum level of polymerized actin, which leads to a virtually identical cell behavior on nanolattices made from all three material systems.

Mineral deposition has been shown to be proportional to the forces that osteoblasts exert on the substrates^m, which may explain why an identical cellular response, measured as a function of mineral deposition, was triggered for nanolattices made out of all three material systems. These results show that osteogenic functionality in osteoblast-like cells could be elicited on three-dimensional scaffolds with stiffnesses spanning from ~2 to ~9MPa, which extends the stiffness boundaries beyond kPa-level for a unit cell size on the same order as osteoblasts dimensions, reported in literature and may have significant implications for understanding cell mechanics in 3D environments.

The versatility of two-photon lithography enabled us to create three-dimensional scaffolds that closely mimic physiological conditions, and allowed for isolating a single parameter at a time in determining its effect on osteoblast activity. In this work, we locked the pore size to be constant and systematically increased the stiffness to monitor osteogenic cell activity solely as a function of increasing scaffold stiffness. Quantifying the upper and lower substrate stiffness boundaries on osteoblast bioactivity will shed light on understanding stress shielding, which has the potential to significantly improve future orthopedic implants.

2.5. Concluding Remarks

We demonstrated the feasibility of using a 3-D architected nanolattices as scaffolds for growing artificial bone tissue and to investigate cell behavior in microenvironments similar to those that are native to the human body. Using different unit cell geometries, the kagome and the tetrakaidecahedron, we observed that a minimum pore size of 25µm was necessary for cell attachment and migration into the nanolattice Further experiments probing the effects of scaffold's material on cell response showed that TiO₂-coated nanolattices induced SAOS-2 cells to secrete extracellular matrix. These findings led us to choose the TiO₂-coated tetrakaidecahdron nanolattice with a pore size of 25µm as the optimal scaffold for investigating cellular behavior.

By using different intermediate coatings and locking the beam size, pore size and outer-most scaffold coating we were able to create nanolattices with different stiffness, 2~9MPa. This enabled us to isolate and to systematically investigate biocompatibility, osteointegration and proliferation as a function of 3D scaffold stiffness. SEM analysis showed that SAOS-2 adhered, migrated and successfully deposited extracellular matrix on the nanolattices. EDS and Raman spectroscopy showed that the cellular deposits were composed of several calcium phosphate species, which are present in freshly deposited bone in vivo. To the best of our knowledge, this is the first work that reports spectroscopic analysis of the bone cell deposits on 3D scaffolds. Based on these arguments, the scientific contributions of this chapter are: (1) fabricating 3D scaffolds with ~2-9 MPa stiffnesses, which have not been explored previously in terms of osteoblast growth/proliferation, (2) investigating cell behavior on a scaffold whose unit cell size is similar

to the size of the cell, and (3) offering precise chemical analysis of the secreted species. Future work will involve the fabrication of scaffolds with a much larger range of structural stiffness, which will be used to investigate how bone cell functionality, measured as a function of mineral deposition, is affected by substrate stiffness. Stiffness-cell interaction has the potential to provide insights into the root causes of stress shielding which is a major issue affecting current bone implants.

Chapter 3: Probing Nanolattices' Stiffness Effects on Osteoblasts-like Cells.

In this chapter we seek to understand how the 3D mechanical environment of an implant affects bone formation during early osteointegration. We employed two-photon lithography (TPL) direct laser writing to fabricate 3-dimensional rigid polymer scaffolds with tetrakaidecahedral periodic geometry, herewith referred to as nanolattices, whose strut dimensions were on the same order as osteoblasts' focal adhesions (~2µm) and pore sizes on the order of cell size, ~10µm. Some of these nanolattices were subsequently coated with thin conformal layers of Ti or W, and a final outer layer of 18nm-thick TiO, was deposited on all samples to ensure biocompatibility. Nanomechanical experiments on each type of nanolattice revealed the range of stiffnesses of 0.7-100 MPa. Osteoblast-like cells (SAOS-2) were seeded on each nanolattice, and their mechanosensitve response was explored by tracking mineral secretions and intracellular f-actin and vinculin concentrations after 2, 8 and 12 days of cell culture in mineralization media. Experiments revealed that the most compliant nanolattices had $\sim 20\%$ more intracellular f-actin and $\sim 40\%$ more Ca and P secreted onto them than the stiffer nanolattices, where such cellular response was virtually indistinguishable.

We constructed a simple phenomenological model that appears to capture the observed relation between scaffold stiffness and f-actin concentration. This model predicts a range of optimal scaffold stiffnesses for maximum f-actin concentration, which appears to be directly correlated with osteoblast-driven mineral deposition. This work suggests that three-dimensional scaffolds with titania-coated surfaces may provide an optimal microenvironment for cell growth when their stiffness is similar to that of cartilage (~0.5-3MPa). These findings help provide a greater understanding of osteoblast mechanosensitivity and may have profound implications in developing more effective and safer bone prostheses.

3.1. Chapter summary

The precise mechanisms that lead to orthopedic implant failure are not well understood; it is believed that the micromechanical environment at the bone-implant interface regulates structural stability of an implant. The number of expected osteoporosis-related fractures is predicted to grow by a factor of 7 in the next twenty-five years because of a substantial increase in the ageing population. By 2030, the demand for hip and knee replacements is predicted to increase by 174% and 673%, respectively^{102,003}. This tremendous need for bone prostheses has motivated significant research efforts to develop a more thorough understanding of properties of bone at each level of its hierarchy, with a focus on scaffold-osteoblast interactions at the cellular level^{6,004}. As

mentioned in the introduction of this thesis, several types of bone grafting scaffolds exist, however, despite the significant efforts that have been made to develop fully synthetic implants for more than five decades⁶ no optimal implant exists yet. Commercially available, fully synthetic orthopedic implants cause stress shielding due to the discrepancy in stiffness between bone and the implant (see section 1.1.2. for more details). As a consequence of the inadequate load transfer from the implant to the cells, the healing bone decreases its mass, which prevents the bone from anchoring to the implant and leads to implant loosening and eventual failure^{214,123}. Hutmacher et al. (2000) postulated that an ideal implant should retain durability in the body and have mechanical properties that match those of the natural bone that is being replaced³⁶. This remains to be demonstrated experimentally, especially at the cellular level.

To date, research on mammalian cells' ability to exert forces onto a 2-dimensional substrate via stress fibers, which are bundles of polymerized actin, has shown that cells exhibit a bell-shaped sensitivity to changes in substrate stiffness^{29,38}. We hypothesize that adhesion and mineralization behavior of bone cells may also exhibit a sensitivity dependence on the stiffness of 3-dimensional (3D) scaffolds^{10,11,2}. Identifying an optimal stiffness range for mineralization on 3D scaffolds has the potential to offer quantitative guidelines for the fabrication of bone implants that minimize stress-shielding while maximizing bone growth.

Challenges associated with fabricating complex three-dimensional scaffolds with strut dimensions on the same order as osteoblasts (~10 μ m) has rendered existing studies to be limited to a stiffness window ranging from ~10-200 kPa^{18,19,26,106}. Most literature has been focused on studying cell behavior on either 2D substrates or on scaffolds with a narrow range of structural stiffness and strut size of at least one order of magnitude larger than the cell's size which has made the cell-scaffold interaction virtually the same as that on a 2D substrate^{26,39,106,107}.

We focused on exploring the dependence of osteoblast-like cells (SAOS-2) on the structural stiffness of porous substrates with a constant pore size. We utilized two-photon lithography, sputtering and atomic layer deposition (ALD) to fabricate periodic, 3-dimensional cellular solids, referred to as nanolattices, with tetrakaidecahedral geometry, measured their structural stiffness, and populated osteoblast-like SAOS-2 cells onto them to study their behavior.

The relative density of the nanolattices in this work, calculated using Solidworks software (Dassault Systems), ranged from 0.14% to 12.2%. The pore size, U, was maintained constant at 25µm for all nanolattices in this work to isolate the effects of the scaffolds' structural stiffness, which was varied by depositing different material coatings onto the original polymer nanolattices (Figure 11). We were able to achieve a range of structural stiffnesses that spans over two orders of magnitude, from ~0.7 MPa to 100 MPa, which covers a region that had not been previously explored: existing literature on scaffolds with similar sizes explored the stiffness range of ~10-200 kPa. SAOS-2 cells were seeded on the nanolattices, and the cells' f-actin concentration was measured after a 48-hour growth period in mineralization media. Longer periods of growth, up to 12 days, were conducted to characterize the relationship between scaffold stiffness and cells' mineralization ability.

3.2. Methods

3.2.1. Sample Preparation

All scaffolds were fabricated via TPL direct laser writing (DWL), which employs a femtosecond-pulsed laser that is rastered in space to selectively cross-link a negative tone photoresist, IP-Dip (Nanoscribe GmbH), into a designed structure. The resulting polymer

nanolattices were subsequently coated with different materials to create scaffolds that are comprised of 4 different material systems shown in Figure 11 (i).



Figure 11: Design and Fabrication of the Nanolattices. (i) Computer-aided design of the sample showing the four types of nanolattices that were tested. All nanolattices had tetrakaidecahedral unit cells of length (U) = 25μ m and a beam radius (R), which varied from 1 to 1.5µm. The insets show a zoomed-in view of the unit cells that comprise each type of nanolattice: (A) hollow with an 18nm-thick TiO₂ wall. (B) IP-Dip-core coated with 18nm-thick layer of TiO₂. (C) IP-Dip-core coated with ~250nm-thick layer of Ti and 18nm-thick layer of TiO₂. (D) IP-Dip-core coated with ~250nm-thick layer of W and 18nm-thick layer of TiO₂. (ii) Top SEM view of the fabricated samples. (iii) EDS map and spectrum that shows the

composition of the W and Ti nanolattices (material systems C and D). (iv) A zoomed-in side SEM view of the hollow TiO2 nanolattice (material system (A)).

Material system (**A**) was fabricated by first coating the polymer scaffold with an 18nm-thick layer of TiO₂ deposited via ALD and then slicing off the sample edges along each face using a focused ion beam (FIB) (FEI Nova 200 Nanolab) at 30KeV and 5nA. The samples were then placed into an O₂ plasma etcher at 0.6 mbarr and 100W (Diener GmbH) for 24 hours to etch away the original scaffold and to produce a hollow TiO2 nanolattice (Figure 11 (ii), 1(iv)).

Material system (B) was fabricated using the same process as Material system (A) without etching away the polymer scaffold. **Material system (C)** was fabricated by sputtering a ~250nmthick layer of Ti onto the original polymer scaffold and subsequently coating it with an 18nmthick layer of TiO₂ deposited via ALD. **Material system (D)** was fabricated by sputtering a ~250nm-thick layer of W onto the original polymer scaffold and subsequently coating it with an 18nm-thick layer of TiO₂ deposited via ALD.

Some of the original polymer nanolattices (IP-Dip) were used for fluorescence studies, which revealed the need to treat the polymer nanolattices with Sudan Black to suppress autofluorescence at the wavelengths that were used for f-actin and vinculin staining (516nm and 633nm respectively). We incubated the polymeric nanolattices in Sudan Black solution for 2 hours following the protocol developed by Jaafar et al. (Figure 12)¹⁰⁸.



Figure 12: IP-Dip autofluorescence suppression. (i) Fluorescence microscopy images of the polymer nanolattices taken at $\lambda = 561$ nm and $\lambda = 633$ nm showing strong autofluorescence coming from IP-Dip. (ii) Fluorescence microscopy images of the polymer nanolattices after being treated with Sudan Black for 2 hours showing autofluorescence suppression at $\lambda = 561$ nm and $\lambda = 633$ nm.

Sputter deposition was carried out using a magnetron sputterer (Temescal BJD-1800). Titanium was sputtered using RF power at 125W, a working pressure of 6mtorr, Ar pressure of 60sccm and table rotation set at 100%. An average Ti thickness of ~250nm was obtained after depositing for 140 minutes. W was deposited using RF power of 125W, a working pressure of 5mtorr, Ar pressure of 50sccm and table rotation set at 100%. An average W thickness of ~250nm was obtained after depositing for 140 minutes. The outermost 18nm-thick TiO₂ coating was deposited using ALD (Cambridge Nanotech S200) with H₂O and Titanium Tetrachloride (TiCl₂) precursors. A shadow mask, which was fabricated via photolithography and deep reactive ion etching (DRIE), was used to selectively coat Ti on system (C) and W on system (D) that are adjacent to each other on the SiO₂ substrate (Figure 13).



Figure 13: Shadow mask fabrication. (i) Solidworks model of the shadow mask. (ii) SEM side view of the nanolattices housing created via DRIE. (iii) Zoomed-in view of the housing side wall showing a height of 90 μ m, necessary to prevent nanolattice collision with the mask floor during the alignment step. (iv) SEM Top and side view of the shadow mask deposition window confirming successful wafer etch-through.

A 500µm-thick Si wafer was first uniformly thinned to a thickness of ~200µm in an inductively coupled reactive ion etcher (Oxford DRIE System 100 ICP/RIE) using SF, as reactive gas. Subsequently photoresist AZ5214-e was used to pattern a circle of 4mm diameter in the center of the Si chip. 200nm of Al₂O, was then deposited in an e-beam evaporator (Temescal FC-1800). Lift-off of the photoresist (AZ5214-e) revealed a 4mm-diameter circle surrounded by Al₂O, (Figure 12 (ii)), which provided an adequate protective layer against etching. To create a housing for the nanolattices so that they would not be crushed during the alignment procedure, DRIE was employed to etch ~90µm into the Si pattern using a Bosch recipe (Figure 12 (iii)). The final deposition window was made by first using two-photon lithography (Nanoscribe GmbH) to

pattern a polymeric circle of 120µm diameter in the middle of the fabricated trench. Evaporation of 200nm of Al₂O₃ followed by lift-off of the polymeric circular pattern was completed and a final wafer etch-through step using DRIE was performed to expose the deposition window (Figure 12 (iv)).

Figure 11 (iii) provides a map generated by EDS that shows the distribution of Ti and W in material systems (C) and (D). The spraying effect inherent to sputtering deposition was minimized to ~15µm by reducing the size of the shadow mask's deposition window to 120µm x 120µm. To mimic the porous structure of cancellous bone we chose a tessellated tetrakaidecahedral unit cell geometry (Figure 11 (i)) which had circular beams of length L = 8.33µm and a radius R = 1µm for material system (A) and (B) or R = 1.5µm for material systems (C) and (D), and a unit cell size U = 25µm for all material systems (Figure 11 (i)). Each nanolattice contained 8 (length) x 8 (width) x 2 (height) unit cells, and each sample contained 4 nanolattices arranged in a linear sequence from material system (A) to (D) to establish a stiffness gradient (Figure 11 (ii)). The nanolattices were separated by 10µm to allow for precise and selective sputter coating (Figure 11 (iii)).

3.2.2. Quasi-static Uniaxial Compression Experimental Setup

All nanolattices were uniaxially compressed to a maximum strain of 50% at a strain rate of 10^{3} s⁴ in a nanoindenter (G200, Agilent Technologies). The load vs. displacement data collected by the nanoindenter was converted into engineering stress vs. strain. Engineering stress was calculated using $\sigma = F/A$, where F is the applied load and A is the footprint area of the nanolattice, and global compressive strain, ε , was calculated as $\varepsilon = (H_{1}-H_{1})/H_{1}$ where H_{1} is the initial height of the nanolattice measured from SEM images and $(H_{i}-H_{i})$ is the displacement recordered by the nanoindenter. The structural stiffness of the nanolattice, E^{\cdot} , was calculated as the slope of the elastic loading portion of the data, which is indicated by the dashed black line in Figure 14 (ii):

3.2.3. Cell Culture Conditions

All in vitro experiments were performed using the SAOS-2 cell line from ATCC. Cells were cultured in 100 mm dishes. DMEM, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin, were used as the culture media. Media was replaced every 2 days, and cells were split every 4-5 days using Accutase Cell Detachment Solution. Differentiation media consisted of DMEM low glucose, with 10% FBS, 2mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin, 10 mM β -glycerophosphate, 100 nM dexamethasone, and 50 μ M ascorbic acid.

For immunostaining experiments (subset 1), cells were seeded onto the nanolattices from each material system at a density of 12,000 cells/cm³ and grown for 7 days, after which they were transferred into mineralization media and cultured for additional 2 days. Samples were then washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes. Samples were washed again with PBS and blocked with 1% BSA in PBS for 30 minutes. Anti-vinculin diluted in blocking buffer was then added to the cells and incubated overnight at 4°C, and samples were washed three times again with PBST incubated with phalloidin-555 and a 647-conjugated secondary antibody at room temperature for three hours. After the final wash with PBST, the cells were imaged with a confocal microscope (Zeiss LSM 710).

For mineralization experiments (subset 2), cells were seeded onto different nanolattices from each material system at a density of 12,000 cells/cm² and allowed to proliferate for 14 days. Cells were then transferred into mineralization media and cultured for additional 8 or 12 days. These samples were also washed three times in PBS and fixed in 4% paraformaldehyde for 15 min. After fixation, and one more wash with PBS, the cells were incubated in serial dilutions of ethanol for 10 minutes each.

3.2.4. Cell Imaging and Secretions Characterization via Raman Spectroscopy and EDS

After the cells from subset 1 were grown on the nanolattices for 2 days in mineralization media, they were imaged to quantify the amount of fluorescence from f-actin and vinculin staining. Samples were imaged in a Zeiss LSM 710 confocal microscope using a 20x, NA 0.8 lens, which offered the highest magnification to image the entire nanolattice. Z-stack images were captured at a constant spacing of 1µm and a total height of 55µm and were used to calculate the maximum projected intensity using software ImageJ. To quantify the relative amount of fluorescence from every material system, fluorescence data from each individual chip was normalized by the fluorescence intensity of material system (A). A total of 5 chips were used to determine error in fluorescence experiments.

To quantify their propensity for mineralization, SAOS-2 cells from subset 2 were subjected to serial dilutions of ethanol in phosphate buffered saline until 100% ethanol was attained and then processed in a critical point dryer (Tousimis 915B). Cell secretions were morphologically and spectroscopically analyzed using a scanning electron microscope (SEM, FEI Nova 200 Nanolab) equipped with an EDS module (EDAX Genesis 7000). EDS parameters were adopted from Maggi et al. (2017)^{*}, and 3 scans per nanolattice were taken to ensure current stability. Raman

analysis of cell secretions deposited onto the nanolattices was carried out using a micro Raman spectrometer (Renishaw M1000) with a laser wavelength of 514.5 nm and a power density of 130W/cm².

3.3. Results

3.3.1. Nanomechanical Experiments

We performed quasi-static uniaxial compression experiments to ~50% global uniaxial strain to determine the effective structural stiffness and deformation characteristics of each nanolattice. Figure 14 shows SEM images of nanolattices from each material system before and after the compression, as well as the corresponding stress vs. strain data.



Figure 14: Uniaxial compression experiments. (i) SEM images of representative as-fabricated samples from each material system. The circles in the top left corner of each image represent a schematic of the beam cross section for each material system (not to scale). (ii) Representative stress-strain response to quasi-static uniaxial compression of each material system. The inset shows a zoomed-in view of the compression of the hollow nanolattice (wall thickness = 18nm TiO₂. (iii) SEM images of the same samples after compression. All samples from material systems B, C, and D underwent brittle failure, the hollow nanolattice (A) (bottom image)
experienced localized Euler beam buckling and some residual recovery. Scale bars in each SEM image represent 50µm.

The stress-strain data for all samples contains a short initial non-linearity, or toe region, which is primarily caused by a small misalignment between the compression tip and the top surface of the nanolattice. The stiffer material systems (C) (polymer/Ti/TiO₂) and (D) (polymer/W/TiO₂) exhibited a toe region up to 1% strain; the toe region in more compliant systems (B) (polymer/TiO₂) and (A) (hollow/TiO₂) extended to 3% strain. A linear elastic region, indicated by the dashed slopes in Figure 12 (ii), followed the toe region, and was used to calculate the effective structural stiffness, $E^{\text{-grin}}$. The post-elastic behavior varied depending on the constituent material of the nanolattice. Fig. 2(iii), which shows post-compression SEM images of a representative nanolattice from each material system, reveals that all the composite systems (B, C, and D) experienced catastrophic brittle failure at a strain of ~9%, ~13% and ~18% respectively; the hollow material system (A) deformed in a ductile-like fashion with discrete serrations that correspond to individual layer buckling events (Figure 12 (ii)-inset). Table VII summarizes the moduli, E, and compressive strengths, σ_i , for all material systems, which span more than two orders of magnitude.

Table VII: Elastic modulus, E*, and compressive strength, σ_r , of each material system measured via uniaxial quasi-static compression. Error was calculated by taking the standard deviation from 4 data points gathered per material system.

System	E* (Mpa)	σ _r (Mpa)
Α	0.69 ± 0.2	0.019 ± 0.003
В	16.8 ± 0.9	0.45 ± 0.01
С	60.2 ± 7.4	1.78 ± 0.28
D	96.7 ± 6.9	4.53 ± 0.7

3.3.2. Cell Experiments: F-actin & Vinculin Fluorescence Microscopy

SAOS-2 cells were cultured on the nanolattices to determine the effect of substrate stiffness on the production of stress fibers and focal adhesions by the cells. After 2 days of growth in mineralization media, the actin fibers (f-actin) were stained with phalloidin (red) and the focal adhesions were stained with anti-vinculin antibodies (green) to quantify their amounts via fluorescent experiments (Figure 15).



Figure 15: Fluorescence microscopy images and quantitative analysis. (i) Z-stack projections of confocal images of SAOS-2 cells grown on the nanolattices for 2 days that show actin filaments stained with Phalloidin and (ii) focal adhesions stained with anti-vinculin antibodies; the material system is represented by the schematic circle on top of the corresponding nanolattice. Relative amounts of f-actin (iii) and focal adhesions (iv) as a function of nanolattice

stiffness. Fluorescence data was normalized by the intensity of the most compliant material system (A). Horizontal error bars represent standard deviation in nanolattice elastic moduli and vertical error bars represent standard error in fluorescence measurements. (v) Merging of the red and green channels shows higher levels of co-localization (yellow) on the nanolattices compared to the surrounding flat substrate.

Figure 15 shows the results of the fluorescence experiments. A schematic representation of each individual material system is placed directly above the image that was generated via fluorescence microscopy for that specific material system (Figure 15 (i-ii)). Figure 15 (iii) reveals the presence of ~20% more f-actin on the most compliant nanolattice (A) compared to that on the other material systems (B-D), all of which displayed similar levels of relative maximum intensity of f-actin. Figure 15 (iv) shows vinculin staining, which revealed no significant differences in focal adhesion concentration across the four material systems.

Merging the signal from phalloidin (Figure 15 (i)) and vinculin staining (Figure 15 (ii)) produced the images in Figure 15 (v), which show the amount of co-localization (yellow color) between factin and focal adhesions in the nanolattices. These images reveal uniform distribution of colocalized f-actin and focal adhesions along the z-axis with no apparent location preference within the nanolattice. A qualitative analysis also revealed higher levels of co-localization on the nanolattices compared to the flat substrate (Figure 15 (v)). The footprint area of the nanolattices occupied ~0.2% of the total sample area which made it impossible to physically separate the cells attached to the nanolattices from those on the neighboring flat substrate and to perform more quantitative biological assays.

Sudan Black was not able to suppress the inherent autofluorescence of the nanolattice polymer at wavelengths shorter than ~400nm, which rendered nuclear staining, such as DAPI, ineffective in revealing meaningful information about the number of cells on each nanolattice.

3.3.3. Cell Experiments: Cellular Secretions Characterization & Quantification.

Figure 16 shows SEM images of SAOS-2 secretions on the nanolattices after a growth period of 8 and 12 days in mineralization media.



Figure 16: SEM images of the SAOS-2 cells' products after growing for 8 and 12 days in mineralization media. (**i**,**ii**) Top-down SEM images of the samples after 8 days (**i**) and 12 days (**ii**) of growth. Circles above the images represent a schematic of the individual beam cross-sections for each material system. (**iii**,**iv**) Zoomed-in SEM images that reveal large amounts of organic material (white arrows) grown on the nanolattice after 8 days. These deposits were found across all material systems. (**v**,**vi**) Zoomed-in SEM images showing large amounts of mineral formations (orange arrows) on the nanolattices after 12 days. These aggregates were found across all material systems. (**vii**) Raman spectroscopy analysis of SAOS-2 products after 12 days of growth. Spectra collected from all material systems revealed the presence of bioapatite (962 cm⁴) and collagen molecules (854 cm⁴,879 cm⁴). (**viii**) SEM image of the organic phase that

shows the presence of filamentous features with diameters of 75 ± 32 nm, consistent with the size of collagen fibrils. (ix) SEM image of a mineral aggregate that most probably corresponds to bioapatite.

These experiments reveal that SAOS-2 cells deposited organic and mineral compounds on all nanolattices after growing in mineralization media for 8 and 12 days. SEM images in Figure 16 (i-vi) demonstrate the presence of a continuous matrix interspersed with ~50-100nm-diameter filaments that are indicated by arrows in Figure 16 (iii,iv,viii). The mineral deposits, indicated by arrows in Figure 16 (v,vi,ix), appear to have two dominant morphologies: (1) spherical clusters with diameters of ~2-15 μ m (Figure 16 (v,vi)) that are composed of (2) smaller aggregates ranging from ~300nm-1 μ m (Figure 16 (ix)). These smaller aggregates, ~300nm-1 μ m, were also present as a continuous coating on the nanolattice beams and in the larger spherical secretions as shown in Figure 17.



Figure 17: SEM image of the irregularly-shaped sub-aggregates that uniformly coated the nanolattices' beams and that were found to be making up larger spherical nodules. (i) SEM side-image of a nanolattice showing mineral aggregates uniformely coating the nanolattice struts (purple inset) and making up larger spherical nodules (cyan inset). (ii) Zoomed-in view of the mineral aggregates that coat the nanolattice beams. These aggregates were irregularly-shaped and varied in width from ~300nm-1 μ m. (iii) Zoomed-in view of the mineral aggregates making up larger spherical nodules. Similarly to the deposits shown in (ii) these aggregates were also irregularly-shaped and varied in width from ~300nm-1 μ m.

Figure 17 (i) shows the presence of several spherical mineral nodules and a continuous coating on the nanolattice struts. Figure 17 (ii) and (iii) offer a more detailed view of the aggregates making up the continuous coating (ii) and the spherical aggregates (iii). These aggregates share both shape and size (\sim 300nm-1µm) and were hypothesized to be the initial SAOS-2 mineral secretions.

Raman spectroscopy performed on the organic phase revealed peaks at 854 cm⁻¹ and 879 cm⁻¹, which most probably correspond to proline and hydroxyproline, respectively, and suggest the presence of collagen molecules. The spectra taken from the mineral phase exhibited a peak at 962cm⁻¹ (Figure 16 (vii)), which is likely representative of some form of bioapatite.

Figure 18 shows the results of the EDS analysis performed on the scaffolds after 8 and 12 days of cell growth in mineralization media. Nanolattices made from all material systems revealed the presence of C, O, Na, Mg, Ca and P.



Figure 18: EDS spectra and quantification of Ca and P secreted by the SAOS-2 cells. (i,ii) Representative EDS spectra after growing SAOS-2 cells for 8 days (i) and 12 days (ii) in mineralization media. (iii-vi) Relative intensity of Ca (iii,iv) and P (v,vi) after 8 days (iii,v) and 12 days (iv,vi). (v,vi) Horizontal error bars represent the standard deviation in elastic moduli measured over 4 samples and vertical error bars represent the standard error in the intensity of Ca and P obtained from EDS spectra of 3 chips per time point. In each plot, Ca and P concentrations were normalized by their relative amounts on the most compliant material system (A).

EDS spectra of all samples after 8 days of growth reveal the relative intensity of C to be a factor of ~3 higher than those of P and Ca (Figure 18 (i)). EDS spectra after 12 days of growth reveal the amount of C to be ~6% lower than that of P and ~29% higher than that of Ca in all samples (Figure 18 (ii)). Figure 18 (iii-vi) displays the relative intensity of Ca and P after 8 and 12 days of cell growth as a function of nanolattice stiffness. The data from each sample was normalized to the corresponding element intensity on the most compliant material system (A). The intrinsic inability of EDS detectors to reliably capture light elements (Z < 11) limits the accuracy of quantifying the concentration of C. This analysis reveals that after 8 days, the hollow, most compliant material system (A) had ~40% more Ca and P compared with those on stiffer material systems (B-D), all of which displayed similar levels of Ca and P (Figure 18 (iii, v)). After 12 days, a less drastic difference in Ca and P concentration across the material systems was observed. Material system (A) displayed $\sim 15\%$ more Ca and P compared with material system (B), and material system (B) displayed ~10% more Ca and P than material systems (C) and (D) (Figure 18 (iv, vi)). These results show that material system (A) with the lowest structural modulus of 700kPa, had the highest amounts of f-actin and mineral deposits (Ca, P).

3.4. Phenomenological Model Formulation

To explain the observed higher cellular activity on the most compliant 3D substrates, we propose a simple qualitative phenomenological model that is aimed to relate f-actin concentration to substrate stiffness. The ability of a cell to respond to external mechanical stimuli depends on highly interconnected and coordinated networks of signaling events that regulate cell adhesion. Mammalian cells attach to a substrate by reorganizing their cytoskeleton, which is a complex, highly heterogeneous and dynamic system that undergoes constant rearrangement. Multiple components play important roles in cystoskeleton rearrangement. Following the approach of Ingber (1997), in this model we treat the cytoskeleton as a network of microfilaments and microtubules that distribute forces within the cell through a balance of compression and tension without taking into account more detailed structures¹⁰⁷. We setup the model by employing the following elements involved in cell adhesion: (1) a "sticky" element, focal adhesions, (2) an active force-generating element, f-actin, and (3) a compression element, microtubules.

Focal adhesions, which induce monomeric actin (g-actin) to polymerize into f-actin that can autonomously contract, are the anchor points of the cell to the substrate. F-actin pulls on the substrate by using integrins, or transmembrane proteins that serve as adhesive elements between the substrate and the cell. Rod-like protein complexes, or microtubules, resist this actin-driven cell and prevent cell collapse^{100,100}.

Existing models treat actin filaments and microtubules as linear-elastic solids, which predict a linear relationship between f-actin concentration and substrate stiffness^{mine}. This linear relationship saturates when the maximum biologically-allowed concentration of filamentous actin in the cell is reached (~60uM)⁶⁶. These models accurately describe the interactions between f-actin and microtubules and do not account for the integrins, which play an important role in cell attachment and migration¹⁶⁹.

Following the approach of De Santis et al. $(2011)^{m}$, who treated the cellular mechanical elements, f-actin and microtubules, as linear elastic springs, we developed a model that accounts for the f-actin-integrin-substrate interaction. In this model, the cells are in quasi-static equilibrium with the substrate, and the force generated by the filamentous actin (F_{FA}), which is a function of the force developed in the integrins (F_{TT}), is balanced by the compression of the

microtubules (F_{MT}) , and the traction at the cell-substrate interface (F_s) (Figure 19 (i)).



Figure 19: Substrate-dependent f-actin activation model. (i) Model phenomenology: F_{MT} represents the force exerted by microtubules (green), F_{AF} represents the force exerted by f-actin filaments (red) and F_{TT} represents the force exerted by integrins (black). F_s represents the resistive force of the substrate. Microtubules, f-actin and the substrate were modeled as elastic solid springs; the integrins were modeled as sliders. (ii) Cumulative distribution function (CDF(Pi_R)) of a cluster of integrins per micron squared as a function of substrate elasticity that shows more integrin-substrate bonds breaking as stiffness increases. (iii) Model predictions of f-actin concentration change (η_{FA}) as a function of substrate stiffness. (iv) F-actin activation factor, η_{FA} , as a function of the structural stiffness of the substrate. Solid line represents theoretical predictions, open diamond symbols represent experimental data.

To satisfy static equilibrium, the following relation must be true:

$$F_{MT} + F_{FA} + F_S = 0 \tag{4}$$

Each force can be expressed in terms of spring constants and dimensions as:

$$F_{FA} = K_{FA}(L - L_{FAR})/L_{FAR}$$

68

$$F_{MT} = K_{MT} (L - L_0) / L_{FAR}$$

$$F_S = K_S (L - L_0) / L_0$$
(5)

where L_{a} is the rest length of an element, L is the final elongation of an element, and K = EA is the effective spring constant of the element where E is the Young's modulus of the element, and A is the cross sectional of the element. K_{FA} is the effective stiffness of f-actin, K_{ar} is the effective stiffness of microtubules and K_{a} is the effective stiffness of the substrate.

The rest lengths of the microtubule and of the substrate are independent of a cell's pre-stress¹¹³, the f-actin rest length (L_{FAR}) is a function of the pre-stress developed by a cell upon its adhesion to a substrate¹¹⁴:

$$L_{FAR} = (1+P)L_0 (6)$$

where P is a unitless pre-stress coefficient which we estimated using Engler et al.²⁹ Solving equations (4) and (5) gives an expression for the force that f-actin exerts onto the substrate as a function of its stiffness:

$$F_{FA} = \frac{K_{FA}P(K_{MT} + K_S)}{K_{FA} + (K_{MT} + K_S)(1+P)}$$
(7)

Eq. (7) doesn't take into account the integrins, which play a crucial role in cell mechanics. Li et al. (2003) showed that a single integrin-substrate bond has a strength of ~100pN. Once the force exerted by the contracting f-actin exceeds this strength, the integrins dissociate from the substrate¹¹⁵. Following this approach and that of He et al. (2014)¹¹², we modeled the probability of

an integrin-substrate bond rupture (Pi_R) as a function of actin-generated tension. We then calculated the cumulative distribution function (*CDF*) for 4000 integrins (Figure 19 (ii)), which literature has shown to be a reasonable average number of integrins per $\mu m^{2.16}$.

$$CDF(Pi_R) = \frac{1}{2} \int (Pi_R) dF_{FA}$$
(8)

We incorporated the effects of integrin-substrate bonds rupturing on the effective force exerted by f-actin by modeling integrins as sliders that work in series with the actin filaments, as shown in Figure 19 (i). Multiplying Eq. (7), which represents the linear relationship between actin force and substrate stiffness, by the probability of finding an intact integrin-substrate bond (Eq. (8)) gives the f-actin activation factor, η_{r_A} :

$$\eta_{FA} = \frac{\frac{K_{FA}P(K_{MT} + K_S)}{K_{FA} + (K_{MT} + K_S)(1+P)} (1 - CDF(Pi_R))}{F_{FA(\max)}}$$
(9)

 $\eta_{\scriptscriptstyle FA}$ describes the change in f-actin concentration relative to the baseline level of 0, which corresponds to the minimum amount of polymerized actin necessary for the cell to remain attached to a substrate, to a maximum level of 1, which corresponds to the highest possible effective concentration of f-actin in the cell.

Eq. (9) demonstrates that η_{FA} is related to the probability of integrins dissociating from the substrate, which is a function of the force that f-actin exerts (Eq. 8) that is related to the substrate stiffness, K_{A} , as shown in Eq. (7). η_{FA} was normalized by the maximum force that f-actin can exert, which is dictated by the maximum concentration of actin allowed by the cell.

Fig. 19 (iii) shows a plot of η_{rs} as a function of the substrate modulus ($E_s = K_s/A$) predicted by the model, which reveals a linear increase in actin activation with substrate stiffness up to ~2MPa where the role of integrin dissociation becomes dominant. The maximum f-actin activation occurs at the substrate stiffness of 2.3 MPa where about 20% of the integrin-substrate bonds have broken (Figure 19 (ii)). As more integrin-substrate bonds dissociate, η_{rs} rapidly decreases back to the baseline level of 0 at the substrate stiffness of 5.2 MPa, where virtually 100% of the integrin-substrate bonds have broken and only the baseline integrin-substrate bonds, essential for the cell-substrate attachment, remain.

The model predicts a specific range of substrate stiffnesses, 0-2.5MPa, where the f-actin activation factor rises from 0 to 1 and then rapidly decays back to the baseline level for all higher stiffnesses. In reality, the rise and decay of η_{FA} would probably be more gradual because of the dynamic nature of integrin-substrate bond kinetics. This simple model is not able to capture the kinetics of the integrin-substrate bonds and is formulated based on the steady state approximation.

To evaluate the credibility of the proposed model, we fabricated an additional material system, a polymer skeleton with a tetrakaidecahedral unit cell, pore size $U = 25 \mu m$, beam radius of 0.5 μm coated with an 18-nm-thick TiO₂ layer, whose structural stiffness was measured to be ~3MPa, i.e. within the range of non-zero η_{ra} .

We conducted the same fluorescence experiments by growing SAOS-2 cells on the nanolattices for 2 days in mineralization media, staining for actin fibers and measuring the f-actin fluorescence intensity which represents the degree of f-actin activation. The relative fluorescence intensity was calculated as explained in section 3.3.2 and the results were plotted together with the model that we developed as shown in figure 19 (iv).



Figure 20: Fluorescence microscopy images showing f-actin signal on the additional material system. (a) Z-stack projections of confocal images of SAOS-2 cells grown on the nanolattices for 2 days showing actin filaments stained with Phalloidin. The schematics placed on top of each nanolattice reveal which material system the f-actin signal was generated from. In order from the left: material system (A), Additional material system (indicated by the red star), material system (C), material system (D). The white dashed vertical line was used to mark the borders between nanolattices.

Figure 19 (iv) shows the experimentally obtained f-actin fluorescence data plotted together with the model predictions. It appears that the proposed phenomenological framework that is based on coupling the probability of integrins dissociating from the substrate to the existing linear elastic models for cell mechanics accurately captures the experimental observations in the range of stiffnesses studied, 0.7 to 100MPa.

3.4. Discussion

The global need for more effective osteogenic scaffolds has motivated a debate on the optimal scaffold specifications, especially about the mechanical properties like scaffold stiffness and

strength¹⁹. At the macroscale, it has been shown that implants with elastic moduli on the order of hundreds of GPa cause stress shielding, which hinders long-term bone healing¹⁵. The fundamental causes of stress shielding likely originate at the microscale and remain largely unknown. This work aims to quantify the effects of structural stiffness of 3-dimensional nano-architected scaffolds on the stress distribution and mineralization capability of osteoblast-like cells (SAOS-2).

3.5.1. Mechanical Characterization:

A relatively large span of relative densities, 0.14%-12.2%, coupled with a tetrakaidecahedral open cellular architecture and different thin film coatings enabled us to fabricate 3-dimensional scaffolds that spanned more than two orders of magnitude in structural stiffness, ~0.7-100 MPa. The mechanical behavior of the nanolattices was analyzed via quasi-static uniaxial compression experiments, which revealed two distinct deformation behaviors: global brittle failure exhibited by composite material systems (B), (C) and (D), and layer-by-layer collapse exhibited by hollow material system (A). A toe region was present in all compressions up to ~3% strain and was likely caused by: (1) a slight initial misalignment between the 600µm–diameter compression tip and the 200µm–wide nanolattice and (2) the incomplete initial contact caused by the fabrication-induced concavity of the top nanolattice surface as shown in figure 21.



Figure 21: Nanolattice height variations. SEM side-view of the nanolattice showing a height difference between the center (purple line) and the side of the nanolattice (cyan line). The orange lines show the presence of a slope between the bottom and top unit cell which suggests shrinking on the upper-most layer.

Figure 21 shows that the height of the nanolattice edge (cyan line) is \sim 51µm while the height of the central unit cells (purple line) is \sim 49µm which causes a height difference between edge and center of the lattice of \sim 2µm. The concavity induced by IP-Dip shrinkage during fabrication is clearly visible by looking at the slope between the top and bottom unit cells shown by orange dashed lines. This concavity coupled with slight nanolattice-compression tip misalignment gave rise to the initial non-linearity that we defined as "toe region".

Following the toe region, nanolattices made from material system (A) (hollow TiO₂nanolattice with 18nm wall thickness) underwent linear elastic loading up to 5% strain and a stress of 12 kPa, followed by a series of discrete strain bursts that correspond to the individual beam buckling events, which ultimately led to brittle fracture of the TiO₂ beam wall¹¹⁷. The initial strain burst was always the most extensive, ~10%, all subsequent strain bursts were $\leq 5\%$. This is likely a result of the substantial accumulation of strain energy in the fully intact sample during loading until its release in the first instability/buckling event, after which the weakened nanolattice is not capable of sustaining as much strain energy between each layer collapse.

Material system (B) (polymer scaffold coated with 18nm of TiO₂) displayed linear-elastic behavior up to 3% strain and ~0.3MPa stress. Inelastic deformation commenced at stresses higher than ~0.3 MPa, which generated high tensile, so-called "hoop", stresses in the outer TiO₂ shell at the nodal connections of the nanolattice and caused brittle fracture of the entire beams and nodes and led to catastrophic collapse of the entire nanolattice^(matrix). Material system (C) (polymer-Ti-TiO₂) and (D) (polymer-W-TiO₂), each containing 26% metal by volume, exhibited similar mechanical behavior characterized by an initial linear elastic response up to ~5% strain followed by yielding and limited plasticity of the composite beams. Global brittle failure occurred at a compressive stress of ~3 MPa for material system (C) and at ~8 MPa for material system (D) because the latter is ~1.5 times stiffer. The ensuing structural collapse occurred because of inefficient load re-distribution within the nanolattice after fracture of the individual nodes and beams, which disabled the nanolattice to be capable of carrying the applied compressive load.

3.5.2 Cell Response: F-actin and Vinculin Distribution

Physical cues, such as substrate stiffness, are known to affect cellular stress states, which activate pathways that control cell behavior³⁰. Studies have shown that stem cell differentiation fate has a bell-shaped dependency on substrate stiffness³⁰. For example, stem cells grown on compliant 2D substrates (0.1~1kPa) had a higher probability of developing into neurons while those grown on stiffer substrates (20~80kPa) had a higher probability of becoming bone cells^{11,154,152}. The large stiffness range of 0.7-100 MPa exhibited by the 4 fabricated material systems in this work allowed us to determine the role of the 3D scaffold stiffness on osteoblast behavior with regards to stress fibers concentration, cell adhesion, and mineral deposition. Fluorescence microscopy

data revealed the presence of stress fibers (f-actin) and focal adhesions in SAOS-2 cells grown in mineralization media for 2 days on all 4 material systems. By measuring relative fluorescence intensity we observed that f-actin expression peaked on the most compliant nanolattices made from the hollow TiO₂(material system (A)) and dropped by ~20% with increasing nanolattice stiffness (Figure 15 (iii)). This suggests that osteoblasts may be highly sensitive to substrate elasticity within a narrow substrate stiffness range of ~0.1-10MPa and virtually insensitive to it at higher stiffnesses. We postulate that when cells grow on a nanolattice with an elastic modulus larger than ~5MPa (Figure 19 (ii,iii)) the f-actin exerts forces that are larger than the tensile strength of the integrin-substrate bond, on the order of 100pN which causes its rupture. When this bond dissociates, the stiffness felt by the contracting actin filaments rapidly decreases and leads to f-actin depolymerization, which manifests itself as a decrease in fluorescence intensity. Fluorescence results also revealed that the spatial distribution of the actin filaments appears to be a function of substrate stiffness. Figure 15 (i) shows that the f-actin was uniformly distributed on the nanolattices of material system (A) and more confined to the nanolattice beams on nanolattices made from material systems (B), (C) and (D). The excessive number of cells present on all nanolattices and the limitations in optical resolution of the instrument prevented us from drawing more quantitative conclusions about the spatial distribution of f-actin on the nanolattices. The relative fluorescence intensity of focal adhesion staining was within the error of the measurement for all material systems, which suggests their relative equivalence. Vinculin was observed along the nanolattice beams, which appear to provide anchor points for cell adhesion (Figure 15 (ii)). These observations may be explained by the functional differences between f-actin and focal adhesions. F-actin serves as an active mechanical element that constantly pulls on the substrate, its function has been reported to be strongly sensitive to

substrate stiffness. Focal adhesions are passive mechanical elements that function as bridges for cell adhesion to the substrate regardless of its stiffness¹²¹. This functional difference may explain why the vinculin appears to be more sensitive to the availability of free surface area than to the substrate stiffness. All nanolattices in this work had a similar surface area available for cell attachment, which could explain the similarity in focal adhesion concentrations across material systems. Overlaying f-actin and vinculin fluorescence images allowed us to qualitatively observe a high degree of co-localization across all material systems (Figure 15 (v)); a signature that was previously observed when cells were grown in natural 3D environments derived from living tissues¹²¹. This finding suggests that the nanolattices used in this study may provide 3D platforms that adequately mimic natural microenvironments and elicit a cellular response comparable to that seen in vivo.

3.5.3 Cell Response: Mineralization

After growing SAOS-2 cells on the nanolattices for 8 and 12 days in mineralization media, we observed that the scaffolds were fully coated with deposits of minerals and of organic matrix. SEM images shown in figure 16 reveal the presence of such deposits on all nanolattices that had two main morphologies: (1) organic cellular/proteinaceous matrix interspersed with ~50-100nm-wide filaments, which are consistent with collagen deposited by osteoblasts on 2D and 3D scaffolds⁴⁴ (figure 16 (iii,iv,viii)), and (2) irregularly-shaped ~300-900nm-sized mineral aggregates which appear to be evenly distributed among the lattice beams (figure 16 (v,vi); (figure 17). These smaller formations appear to coalesce into larger, cauliflower-shaped aggregates, with dimensions of ~2-15µm. Similar deposits have been observed and identified as

calcium phosphate species in our earlier work⁴⁰ (Figure 16 (v,vi,ix)).

Raman spectroscopy of the organic phase reveals the presence of several nucleic acids, fats and amino acids specifically proline and hydroxyproline, which are indicative of collagen (figure 22).



Figure 22: Raman spectroscopy analysis of the organic matrix deposited by SAOS-2 cells after a growth period of 12 days in mineralization media. Spectra collected from all material systems revealed the presence of cellular material (nucleic acids, fats (CH₂ wag), proteins (phring breathing, amide I, II)) and collagen molecules (Proline (854 cm²),Hydroxyproline (879 cm²)).

Analysis of the larger, cauliflower-shaped deposits, indicates the presence of some form of bioapatite, which is the main mineral found in mature bone (figure 16 (vii)). These findings suggest that SAOS-2 cells functionality was induced on the nanolattices. EDS analysis showed that the SAOS-2 cells which resided on the most compliant nanolattice (material system (A)) exhibited ~40% higher levels of Ca and P compared with those on all other scaffolds after growing in mineralization media for 8 days and ~10% higher after a growth period of 12 days.

The relative amounts of Ca and P across material systems (B-D) after 8 and 12 days of cell growth remained relatively constant. After a cell growth period of 12 days, the difference in Ca and P between material system (A) and the other material systems (B-D) was much smaller (~10%) than that observed after 8 days (~40%). These results suggest that: (1) more minerals are secreted onto the most compliant substrates and (2) deposition saturates after a certain amount of cell growth. The large reduction in the difference between mineral amounts between the most compliant system (A) and the other material systems also implies that rate of secretion is non-linear.

SEM images and EDS data also convey that the relative amounts of organic matrix quantified as the relative intensity of the C signal with respect to Ca and P decreased with time.

The intensity of carbon changed from being ~3 times greater than that of Ca and P on day 8 to approximately the same for all three elements on day 12 across all material systems. These results are consistent with the existing in-vivo models that postulate that the osteoblasts initially secrete an organic extracellular matrix, predominantly composed of collagen, which gets mineralized over time and forms several calcium-phosphate compounds^{124,125}. This finding further suggests that the nanolattices may be able to evoke a cellular response similar to that observed in in-vivo studies, which render them a promising framework for future implants.

Elastic moduli of ~0.45 to 1MPa are typical of articular cartilage, which is the natural precursor of bone in mammals¹²⁶. The results of this work suggest that utilizing 3D scaffolds with elastic moduli in that range may be promising in stimulating more efficient bone formation by mimicking embryonic development.

3.6 Concluding Remarks

We used TPL to fabricate three-dimensional rigid polymer nanolattices whose strut dimensions were on the same order as osteoblasts' focal adhesions ($\sim 2\mu m$) and pore sizes of 25µm. Some of these nanolattices were subsequently coated with thin conformal layers of Ti or W, and a final outer layer of 18nm-thick TiO₂ was deposited on all samples to ensure biocompatibility. Nanomechanical experiments on each type of nanolattice revealed their stiffnesses to range from ~ 0.7 MPa to 100MPa. Osteoblast-like SAOS-2 cells were seeded on each type of nanolattice, and their mechanosensitive response was explored by tracking the intracellular f-actin and vinculin concentration after 2 days of cell culture.

Bone-like material that was deposited on the nanolattices by SAOS-2 cells was used as a cell functionality marker. Quantification of such deposits was performed via EDS after 8 and 12 days of cell growth in mineralization media.

These experiments revealed that the most compliant nanolattices, with the stiffness of 0.7 MPa, had a $\sim 20\%$ higher concentration of intracellular f-actin and $\sim 40\%$ more secreted Ca and P compared with all other nanolattices, where such cellular response was virtually indistinguishable.

We developed a simple phenomenological model that appears to capture the experimental observations. The underlying physical foundation of this model comes from incorporating the crucial role that integrins have in cell adhesion into well-established cell mechanics models.

The combination of the experiments and proposed theory suggest that the cell mineralizationinducing ability of 3D substrates is very sensitive to their structural stiffness and that optimal osteoblast functionality is attained on 3D substrates whose stiffness ranges from 0.7 to 3 MPa, similar to that of cartilage. These findings have significant implications for understanding the role that 3D scaffold stiffness plays in inducing mineralization and for introducing the nanolattices as promising platforms for new synthetic bone graft materials.

Chapter 4: Dynamic loading effects on cell attachment and stress fibers formation

In this chapter we explore the role that different dynamic loading conditions have on osteoblasts stress fibers formation, which has been associated with their mineralization capabilities¹⁰¹.

By mechanically compressing the nanolattices with cells grown on them we sought to understand whether stress, strain or loading frequency causes a larger cellular response, which was measured as a function of stress fibers formation.

We employed two-photon lithography (TPL) to fabricate nanolattices with tetrakaidecahedral periodic geometry, with pore size, (U) of 25 μ m, beam dimensions of ~2 μ m in diameter and ~8 μ m in length. Four material systems comprised of hollow-tube nanolattices with different wall thicknesses were created to achieve a wide structural stiffness range, ~0.7-35MPa. All nanolattices had an outer-most layer of TiO₂ which ensured biocompatibility.

Osteoblast-like cells (SAOS-2) were seeded on each nanolattice and compression to different stresses, strains and loading frequencies was carried out. The mechanosensitive response of SAOS-2 cells was measured by monitoring the intracellular f-actin and vinculin concentrations after 3 days of cell culture in mineralization media.

4.1. Chapter summary

Pathologies related to decreased bone mass, such as osteoporosis, disuse-induced bone loss and insufficient bone regrowth upon fracture affect millions of people annually¹²⁷. Osteoporosis alone causes more than 8.9 million fractures annually, resulting in an osteoporotic fracture every 3 seconds¹²⁸.

Decreased bone mass, also known as osteopenia, is a condition whereby osteoclasts, which are cells that break down bone tissue, are more active than osteoblasts, which are cells that synthesize bone. Over time, osteopenia causes a drastic reduction in bone density, which is exponentially related to the incidence of bone fracture¹²⁹.

The populations that are most afflicted by osteopenia-related diseases are astronauts and people over 50 years of age. Today, 1 in 3 women and 1 in 5 men over 50 will experience osteoporotic fractures and by 2050, the population aged 50 and over is projected to increase by ~30%, raising the number of fractures due to bone mass loss¹³⁰⁻¹³². In addition to an increasing aging population, NASA and several private companies have pushed for establishing permanent human habitats on the Moon and on Mars, effectively increasing the number of people venturing into outer space in the near future^{131,134}.

Microgravity, which is any environment where the effective gravity is close to zero and is generally found in interplanetary space, induces bone density loss similarly to bone disuse on Earth, leading to severe osteopenia in astronauts. Traveling one-way to Mars using currently available spacecrafts would cause astronauts to lose about 6% of their bone density, therefore a round-trip to Mars would cause a bone density reduction of 12%, dramatically increasing the risks of bone fracture in astronauts¹²⁷.

Physical activity, which mechanically stimulates bone, has been implicated in bone density regulation. Several studies have shown that decreased physical activity reduces bone mass. Besides disuse, localized bone density loss also occurs as a consequence of fracture immobilization and improper load transfer from the artificial implant to the native bone^{135,136}.

This tremendous need to pin down the root causes of osteopenia has motivated significant research efforts to develop a more thorough understanding of how bone responds to different loading regimes. For instance, Shackelford et al. (2004) showed that an experimental group of human subjects all within the ages 22-56 had greater bone mineral density after 17 weeks of resistive training compared to a control group of subjects of the same age that was kept at bed rest¹⁷⁷. In order to gain better insight into the mechanisms underlying bone remodeling as a function of loading conditions, Turner et al. (1994) applied bending loads ranging from 0-54N to rat tibiae which caused bone to be strained from 0.1-0.4%. A constant load was applied as a sine wave with a constant frequency of 2Hz for 36 cycles (18 seconds) a day. After repeating these cyclical loads for 12 days they observed a 300% increase in bone formation in those samples that were strained to 0.4% compared to those that were strained to 0.1%, concluding that osteocytes are sensitive to deformation¹⁸⁷.

Rubin and Lanyon (1985) performed similar experiments on avian bone and found that the number of cycles per day ceased to have a beneficial effect above a threshold of ~100 cycles a day and the most staggering increase in bone mass were observed up to 40 cycles a day. They concluded that the duration of the mechanical stimulus did not yield proportional increases in bone mass once a certain threshold was exceeded. They also showed that bone is sensitive to both loading frequency and strain magnitude: at a physiologically-relevant loading frequency of 1Hz, which corresponds to slow walking, they observed an increase in bone mass when the

applied load caused a strain of 0.2% (i.e. $2000\mu\epsilon$)¹³¹.

As a consequence of the large length-scale at which these experiments were performed, no data concerning the relationship between cells and mechanical loading was obtained. More recently, studies done by periodically stretching 2D matrices with cells seeded on them have shown that bone-derived cells are responsive to mechanical strain with their response saturating at 1% strain¹⁹. Given the inability to measure bone deposition over short periods of time, studies performed at the cell level have relied on markers such as actin, aggrecan and alkaline phosphatase (ALP) to correlate cellular response to bone remodeling.

While the above studies represent tremendous progress in understanding osteoblast behavior as a function of loading conditions, an important question remains unanswered: Do bone cells sense mechanical deformation, force or loading frequencies?

We employed TPL, sputtering and atomic layer deposition (ALD) to fabricate nanolattices with a constant unit cell geometry, tetrakaidecahedron, porosity, $U = 25 \mu m$ and beam radius size and length of $R = 0.85 \mu m$ and $L = 8.33 \mu m$ respectively [figure 23].

After measuring the structural stiffness of the nanolattices, we seeded osteoblast-like SAOS-2 cells onto them and mechanically loaded the populated nanolattices using different loading conditions. The fabricated hollow nanolattices were coated with different amounts of TiO₂ to reach a structural stiffness spanning from ~0.7-35MPa while maintaining a fairly constant relative density at 0.17%. Three loading conditions were tested:

(1) we investigated the effects of strain magnitude on osteoblasts by compressing the nanolattices to a constant maximum load, 0.65mN corresponding to a stress of ~16kPa, which caused the most compliant nanolattice to experience an average global strain of 0.7% while the stiffest nanolattice experienced an average global strain of 0.35%.

(2) In order to probe the role of stress magnitude on bone cells, we compressed the nanolattices to a constant maximum displacement of ~1 μ m, corresponding to a global strain of ~2%, which caused the most compliant material system to experience an average stress of ~16kPa (0.65mN) and the stiffness system to experience an average stress of ~600KPa (~22mN).

(3) In order to isolate the effects of loading frequency on cell response, nanolattices from a single material system were compressed to a constant stress of 130kPa (4.5mN) and the loading frequency was varied from \sim 0.1-3Hz.

Cellular response was tracked by measuring the f-actin and vinculin concentration of the cells after 320 cycles of mechanical stimulation done over 48 hours.

4.2. Methods

4.2.1. Sample Preparation

Hollow nanolattices were fabricated via TPL (Nanoscribe GmbH) from solid polymer scaffolds and subsequently coated with TiO₂ and some with Ti before being exposed to the focused ion beam and O₂ plasma that was used to remove the polymeric material which yielded a hollow nano-architected shell.

Each sample was composed of 4 nanolattices. The first solid polymeric scaffold was coated with \sim 160nm of sputtered Ti. A second polymeric scaffold was written at a distance of 300µm from the first one and ALD was used to coat 50nm of TiO₂ on the first two polymeric scaffolds. A third solid polymer scaffold was written 300µm away from the second scaffold and an additional 32nm-thick layer of ALD TiO₂ was deposited on the nanolattices. The last polymeric scaffold was written at a distance of 300µm from the third nanolattice and 15nm of ALD TiO₂ was coated

on all four nanolattices. Hollow structures were made by removing the edges of the coated nanolattices using a focused ion beam (FIB) (FEI Nova 200 Nanolab) at 30KeV and 5nA. The internal polymer was etched out by placing the samples into a Zepto Plasma Etcher (Diener GmbH) and by employing O₂ plasma at 0.6 mbarr and 100W for 72 hours. To ensure that no other material but TiO₂ would come in contact with SAOS-2 cells a final 3nm-thick layer of TiO₂ was ALD-deposited on all nanolattices after polymer removal. In summary, 4 material systems were fabricated:

Material system (A): Hollow nanolattice comprised of an 18nm-thick layer of TiO₂.
Material system (B): Hollow nanolattice comprised of a 50nm-thick layer of TiO₂.
Material system (C): Hollow nanolattice comprised of a 100nm-thick layer of TiO₂.
Material system (D): Hollow nanolattice comprised of a 160nm-thick layer of Ti and 100nm-thick layer of TiO₂.



Figure 23: Sample Design and Fabrication. Computer-aided design of the fabricated Si well with 4 nanolattices written in it. The insets show SEM images of each material system and

provide a zoomed-in view of a single unit cell. (A) Hollow with an 18nm-thick TiO_2 wall. (B) Hollow with a 50nm-thick TiO_2 wall. (C) Hollow with a 100nm-thick TiO_2 wall. (D) Hollow with an 160nm-thick Ti and 100nm-thick TiO_2 wall.

Sputter deposition was carried out using a magnetron sputterer (Temescal BJD-1800). Titanium was sputtered using RF power at 125W, a working pressure of 30mtorr, Ar pressure of 200sccm and table rotation set at 100%. An average Ti thickness of ~160nm was obtained after depositing for 140 minutes. TiO₂deposition was achieved using ALD (Cambridge Nanotech S200) with H₂O and Titanium Tetrachloride (TiCl.) precursors.

In order to allow the cells to survive on the nanolattices while mechanical loading was being performed we fabricated a square trench, which was 7x7mm in lateral dimensions and $\sim300\mu m$ in depth, as shown in figure 24. A 500 μm -thick Si wafer was spin-coated with a negative tone photoresist (AZ nLOF-2020) and exposed using a Karl-Suss Microtech MA6 Aligner (i-line) for 12 seconds. Following development for 1 minute in MF-319 we deposited 200nm of Al₂O₂ in an e-beam evaporator (Temescal FC-1800). Lift-off of the photoresist revealed a 7x7mm-square pattern on the Si substrate (figure 24 (b)). DRIE was employed to etch $\sim300\mu m$ into the Si pattern using a Bosch process (figure 24 (c)).



Figure 24: Square trench fabrication: (a) Solidworks model of the square trench etched into a Si substrate. (b) SEM side view of the etched pattern spanning 7x7mm in lateral dimensions. (iii) Zoomed-in view of the trench side wall measuring $\sim 300 \mu m$ in depth.

4.2.2. Monotonic and Cyclic Compression of the Nanolattices

Monotonic and cyclic uniaxial compression experiments were performed on the nanolattices in a G200 XP Nanoindenter (Agilent Technologies). In order to identify each material system's structural stiffness (E^*) all nanolattices were uniaxially compressed to 50% strain at a strain rate of 10^3 s⁴. The load displacement data collected by the nanoindenter was converted into engineering stress strain according to the formulas detailed in section 2.2.3.

The structural stiffness was estimated based on the loading slope of the stress strain curve in the linear regime, which is indicated by the black dashed-line in figure 25 (b).

In order to determine the effects of varying loading conditions on the cells that were seeded on

the nanolattices, cyclic compression experiments were performed.

The first loading condition, i.e. constant maximum load and loading frequency was tested by cyclically loading and unloading the nanolattices from all material systems with cells grown on them for 160 cycles a day for 2 days at a frequency of ~1Hz and a maximum load of 0.6mN which corresponded to a nominal stress 16.6kPa.

The second loading condition, i.e. constant maximum displacement and loading frequency, was tested by cyclically loading and unloading the nanolattices from all material systems with cells grown on them for 160 cycles a day for 2 days at a frequency of 1Hz and a maximum displacement of $\sim 1\mu m$, which corresponds to a strain magnitude of $\sim 0.2\%$.

The third loading condition, i.e. constant stress and strain, different loading frequencies, was tested by cyclically loading and unloading the nanolattices from material system (B) with cells grown on them for 160 cycles a day for 2 days at loading frequencies of 0.1, 1 and ~3Hz.

4.2.3. Cell Culture Conditions

SAOS-2 cell line from ATCC was used for all experiments. The culture media was composed of DMEM, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin. Media was replaced every 2 days, and cells were split every 4-5 days using Accutase Cell Detachment Solution. Differentiation media consisted of DMEM low glucose, with 10% FBS, 2mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin, 10 mM β -glycerophosphate, 100 nM dexamethasone, and 5 μ M ascorbic acid. Cells were seeded on the nanolattices at a density of 12,000 cells/cm⁻² and grown for 12 days in growth media after which they were transferred into mineralization media and

cultured for additional 3 days. On the 2^{ω} and 3^{ω} day in mineralization media, the nanolattices with cells grown on them were taken out of the incubator and mechanically stimulated for ~2 hours per day. Control samples were also taken out of the incubator but no mechanical stimulation was performed on them.

The mineralization media was changed each day after mechanical stimulation. 12 hours after the last mechanical stimulation samples were then washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes. Samples were washed again with PBS and blocked with 1% BSA in PBS for 30 minutes. Anti-vinculin diluted in blocking buffer was then added to the cells and incubated overnight at 4°C, and samples were washed three times again with PBST incubated with phalloidin-555 and a 647-conjugated secondary antibody at room temperature for three hours. After the final wash with PBST, the cells were imaged with a confocal microscope (Zeiss LSM 710).

4.2.4. Stress Fibers and Focal Adhesion Fluorescence Imaging

After mechanically stimulating the cells under different loading conditions for 2 consecutive days, the cells were imaged to quantify the amount of fluorescence from f-actin and vinculin staining. Samples were imaged in a Zeiss LSM 710 scanning confocal microscope using a 10x, NA 0.3 lens, which offered the highest magnification to image the entire nanolattice. Z-stack images were captured at a constant spacing of 1µm and a total height of 50µm and were used to calculate the maximum projected intensity using software ImageJ.

To quantify the relative amount of stress fibers and focal adhesions developed in the cells as a function of loading conditions, fluorescence data from each individual chip was normalized by the fluorescence intensity of material system (A). The amount of fluorescence in the control

samples was also normalized by material system (A). Material system (A) was chosen to be the normalization factor because it was the only system that experienced full contact with the indentor tip for all loading conditions. To determine error in our experiments a total of 4 chips were mechanically tested and 4 chips were used as control.

SEM analysis was performed to confirm that the nanolattices did not experience structural failure during cycling compression experiments. After confocal imaging, SAOS-2 cells were subjected to serial dilutions of ethanol in phosphate buffered saline until 100% ethanol was attained and then processed in a critical point dryer (Tousimis 915B). Nanolattice integrity was investigated using a scanning electron microscope (SEM, FEI Quanta 200).

4.3. Results

4.3.1. Monotonic Nanomechanical Experiments

Figure 26 shows SEM images of each material system before and after compression as well as the stress-strain data that was obtained by performing quasi-static uniaxial compression experiments to 50% strain on the nanolattices. These experiments were done to determine the structural stiffness, deformation characteristics and compression strength of each material system. All nanolattices exhibited an initial non-linearity, also called toe region, which is due to: imperfections during scaffold fabrication. Uneven polymer cross-linking distribution caused structural shrinkage which led to a height difference between the edge unit cells and central unit cells of ~1um (figure 21). Minimum misalignment between the compression tip and the top surface of the nanolattice due to a possible small tilt of the substrate also contributed to the presence the initial non-linearity.

Following the toe-region, the stress-strain data (figure 25 (b)) shows that linear-elastic deformation, which is indicated by the black dashed lines in figure 26 (b), was present and was employed to calculate the nanolattice structural stiffness, E^{*}.

Two distinct deformation signatures were observed during nanolattice compressions. The figure of merit that best describes these two behaviors lies in the thickness-to-radius of the hollow beams, t/R where t is the thickness of the coating material and R is the radius of the nanolattice beams. In accord with the theory developed my Meza et al. (2014)¹⁰⁷ material systems (B), (C) and (D), with $t/R \ge 0.03$ revealed linear elastic loading followed by catastrophic brittle failure at a strain of ~9.7%, ~9.5% and ~7%, respectively, while material system (A), which exhibited 0.02 $\le t/R \le 0.03$, revealed a combination of brittle and ductile-like deformation which led to the presence of strain bursts. Material system (A) exhibited failure at ~5% strain and marginal recovery after compression to ~50% strain. Table VIII summarizes the moduli, E, and compressive strengths, σ_{i} , for all material systems, which span more than one and a half orders of magnitude.



Figure 25: Uniaxial Compression Experiments on Hollow Nanolattices. (a) SEM images of representative as-fabricated samples from each material system. The circles in the top left corner of each image represent a schematic of the beam cross section for each material system (not to scale). (b) Representative stress-strain response to quasi-static uniaxial compression of each material system. The inset shows a zoomed-in view of the compression of material system (A). (c) SEM images of the same samples after compression showing global brittle failure for material system (B), (C) and (D). Localized brittle failure led to the partial recovery observed in material system (A).

Table VIII: Elastic modulus, E^* , and compressive strength, σ_r , of each material system measured via uniaxial quasi-static compression. Error was calculated by taking the standard deviation from 4 data points gathered per material system.

System	E* (Mpa)	σ _, (Mpa)
Α	0.7 ± 0.3	0.02 ± 0.003
В	5.03 ± 0.2	0.19 ± 0.03
С	19.7 ± 0.5	0.69 ± 0.07
D	35.2 ± 1.2	1.1 ± 0.19

4.3.2. Cyclic Nanomechanical Experiments

After growing SAOS-2 cells on the nanolattices for 12 days in growth media and 1 day in mineralization media different cyclic loading conditions were tested. All nanolattices regardless of material system and loading condition were cyclically compressed for 2 days for 4 consecutive sets of 40 load-unload cycles per day.

Loading Condition 1: constant load cyclic experiments

Figure 26 shows a representative load-displacement curve from material system (C) that exemplifies the first loading condition in which the load across material systems was kept constant at 0.6mN, which was chosen based on the maximum load at failure (~0.7mN) of the most compliant material system, i.e. material system (A). The load-displacement curve shows the presence of linear loading-unloading segments that were accompanied by a small amount of hysterisis, which was likely caused by the friction between the top surface of the structure and the indenter tip. The loading frequency was also kept constant at ~1Hz across material systems, as shown in figure 26 (b), by adjusting the strain rate according to eq. 10.

$$\frac{dF}{dt} = \dot{\varepsilon} \, K_{MS} H \tag{10}$$

where $\dot{\varepsilon}$ is the strain rate, K_{ss} is the stiffness of a given material system, H indicates the height of the nanolattice, dF is the load differential, i.e. the maximum prescribed load (0.6mN) minus the minimum prescribed load (0.1mN) and dt is the time differential which was set at 1 second.


Figure 26: Constant load cyclic compressions. (a) Representative load displacement curve taken from material system (C) undergoing 50 rounds of cyclic loading to a maximum load of ~60mN. (b) Load-time curves showing that all material systems, (A) in purple, (B) in cyan, (C) in fuscia and (D) in green experienced the same leading frequency at ~1Hz. The time interval was chosen arbitrarily. (c) Displacement-time curves from all material systems showing that the most compliant nanolattice (A) was strained significantly more than the other material systems.

Keeping the maximum load constant caused the material systems to be strained to different amounts, as shown in figure 26 (c). The average effective strain was calculated by first measuring the nanolattice global displacement per loading-unloading cycle as a function of time (Figure 26(c)), which was obtained by subtracting the minimum displacement from the maximum displacement per cycle. The net displacement per cycle was then converted into net strain per cycle by employing the formulas detailed in section 2.2.3. The net strain per cycle was then used to find the average effective strain that each material system experienced by applying eq. (11).

$$\bar{\varepsilon}_{effective} = \frac{1}{N} \sum_{j=2}^{N} \left(\varepsilon_{max_j} - \varepsilon_{min_j} \right) \tag{11}$$

where N is the number of cycles used to calculate the average effective strain which was

arbitrarily chosen to be 20, ε_{max} was the maximum strain per cycle and ε_{min} is the minimum strain per cycle. Only the net strain caused by cyclic loading was used for the calculation of the average effective strain hence the strain caused by the first loading segment was discarded, which is reflected by *j* starting at 2.

Material system (A) showed the largest average strain, $0.81\% \pm 0.1\%$; material system (B) exhibited an average strain of $0.57\% \pm 0.05\%$; material system (C) exhibited an average strain of $0.46\% \pm 0.03\%$ and material system (D) exhibited an average strain of $0.44\% \pm 0.03\%$.

Due to the presence of a toe region, which was more prominent for the stiffer material systems, i.e. (C) and (D), the net average strain for these material systems was larger by more than 100% than the one calculated from Hooke's law (eq. (3)).

Based on previous experiments performed in our group, we expect the nanolattice to be in full contact with the indentor tip only after the toe-region has been cleared. Therefore, only material system (A), whose toe region extends to ~0.2mN, was in full contact during the constant-load cyclic compression experiments. The displacement that was recorded for material systems (B), (C) and (D) was effectively that of a number of unit cells, but not that of the full nanolattice.

Loading condition 2: constant maximum displacement cyclic experiments

The second loading condition that was tested involved keeping the nanolattices' global strain and loading frequency constant at ~2% and ~1Hz respectively. Figure 27 (a) shows a representative load-displacement curve from material system (C) which reveals that the loading data in each cycle is characterized by elastic loading followed by elastic unloading. The slope of the loading and unloading regions of the load-displacement curve were virtually identical, confirming the elastic nature of the cyclic compression experiments. Figure 27 (c) shows that the net

displacement across all material systems was kept constant at ~1 μ m, which corresponds to a global nanolattice strain of ~2%. Figure 27 (b) reveals that the loading frequency was kept constant for all material systems at ~1Hz. As a result of keeping the strain magnitude constant, the peak load across material systems varied by more than one order of magnitude as shown in figure 27 (b), which reveals that material system (A) reached a maximum load of ~0.65mN, material system (B) reached a maximum load of ~5mN, material system (C) reached a maximum load of ~16mN and material system (D) reached a maximum load of ~22mN.



Figure 27: Constant strain cyclic compressions. (a) Representative load displacement curve taken from material system (C) undergoing 50 rounds of cyclic loading to a maximum load of ~16mN. (b) Load-time curves showing that by fixing the maximum displacement to ~1mN the 4 material systems experienced different maximum loads: ~0.65mN for material system (A) (purple), ~5mN for material system (B) (cyan), ~16mN for material system (C) (fuscia) and ~22mN for material system (D) (green). (c) Displacement-time curves showing that all material systems experienced approximately the same amount of net displacement calculated as the maximum displacement minus the minimum displacement per cycle.

In order to ensure that cyclic compression testing didn't introduce any mechanical flaws into the nanolattices, SEM images of the nanolattices before and after cyclic compressions were taken. Figure 28 shows representative images of a nanolattice from material system (A) pre-and-post cyclic compression, which confirmed that no permanent deformation or cracking was introduced as a consequence of cyclic compressions. No cells were present on this nanolattice to facilitate the visualization of possible flaws introduced by mechanical experiments.



Figure 28: Pre-post cyclic compression nanolattice morphology. (a) SEM image of a nanolattice from material system (A) before cyclic compression. (b) Zoomed-in view of a node, which is the area that experiences the most stress during compression, before cyclic compression. (c) SEM image of the nanolattice shown in (a) after experiencing 50 rounds of cyclic loading. (d) Zoomed-in view of a node post cyclic compression showing no signs of mechanical failure.

Loading condition 3: varying loading frequency cyclic experiments

In order to probe the effects of loading frequency on cell response we cyclically loaded the nanolattices at frequencies which approximately corresponds to standing (~0.1Hz), slow walking (~1Hz) and running (~3Hz). For this loading condition we arbitrarily selected one material system (B), which allowed us to load the nanolattices to a constant maximum load of 4.5mN and minimum load of 0.5mN while varying loading frequency.

Figure 29 shows a few loading-unloading cycles for all loading frequencies that were tested. At lower loading frequencies, 0.1Hz and 1Hz, a high degree of repeatability with respect to

maximum/minimum load and loading frequency across cycles was observed (Figure 29 (a), (b)). A lower degree of repeatability across cycles was observed at a frequency of \sim 3Hz which caused deviations from the prescribed maximum/minimum load of ±0.5mN. This deviation was likely caused by the instrument's inertial forces that could not be properly controlled at such a loading frequency (figure 29 (c)).



Figure 29: Varying loading frequency cyclic compressions. Only material system (B) was utilized for this loading condition. A maximum load of 4.5mN and minimum load of 0.5mN were kept constant across the three different loading frequencies that were tested. Figure 29 shows representative load time curves taken from cyclically compressing the nanolattices at a loading frequency of ~0.1Hz (a), ~1Hz (b) and ~3Hz (c).

4.3.3. Cell Response: F-actin and Vinculin Distribution

SAOS-2 cells were cultured on the nanolattices and subjected to mechanical stimulation to determine the effects of the three different loading conditions that were tested on the formation of stress fibers and focal adhesions by the cells. The cell culture conditions were kept identical for all loading conditions: SAOS-2 cells were seeded on the nanolattices and they were allowed to proliferate in growth media for 12 days after which they were switched to mineralization media for 3 days. After 1 day in mineralization media, mechanical stimulation was performed for

A few hours (~6hrs) after the last set of mechanical compressions, the cells were fixed and actin fibers (f-actin) were stained with phalloidin (red) while focal adhesions were stained with anti-vinculin antibodies (green).

Loading condition 1: constant load, constant loading frequency, different strain

Figure 30 shows the results of the fluorescence experiments for loading condition (1). A schematic representation of each individual material system is placed directly above the image that was generated via fluorescence microscopy for that specific material system (figure 30 (a), (b)). F-actin and vinculin fluorescence intensity was normalized by the most compliant material system (A). Fig. 30 (c) reveals the presence of 2 clear trends in stress fibers formation. The cells that were grown on the control samples, which were comprised of nanolattices that were not mechanically compressed, showed the presence of \sim 30% more f-actin on the most compliant nanolattice (A) compared to the other material systems (B-D). Nanolattice (B) had \sim 10% more f-actin compared to the other material systems ((C), (D)) which displayed similar levels of relative maximum intensity of f-actin. These results showed agreement with those predicted by the model presented in section 3.4.

On the other hand, the cells that were grown on the nanolattices which were cyclically compressed showed a different trend: material systems (B), (C) and (D) displayed an increase in f-actin fluorescence intensity compared to the control sample by \sim 45%, \sim 50% and 40% respectively, which led to an apparent homogenization of the actin fluorescence intensity signal across material systems.

Figure 30 (b) shows representative fluorescence images of focal adhesions which were formed by cells that were cyclically compressed. No distinct trend was evident from plotting vinculin expression as a function of mechanical compression conditions as shown in figure 30 (d).



Figure 30: Fluorescence microscopy after constant load cyclic compressions. (a) Z-stack projections of confocal images of SAOS-2 which were grown on the nanolattices and cyclically compressed 320 times over 48 hours to the same load (0.6mN) showing actin filaments stained with Phalloidin. (b) Focal adhesions stained with anti-vinculin antibodies; the material system is represented by the schematic circle on top of the corresponding nanolattice. Relative amounts of f-actin (c) and focal adhesions (d) as a function of nanolattice stiffness. The data points in red (c) and green (d) represent the results from the nanolattices that were cyclically compressed. The data points in blue represent the control samples that were not compressed. Fluorescence data was normalized by the intensity of the most compliant material system (A). Horizontal error bars represent standard deviation in nanolattice elastic moduli and vertical error bars represent standard error in fluorescence measurements.

The high density of cells and other materials on the nanoscaffolds rendered it difficult to quantify the number of cells on the scaffolds using such methods as cell membrane staining. Fluorescent staining of genomic DNA, which was done using DAPI, wasn't successful as a result of nonspecific staining.

In order to gather more detailed data on cellular behavior, we considered traditional biological assays, such as immunoblotting to extract quantitative information about up/down-regulation of osteoblasts' specific genes as a function of loading conditions. The number of cells that grew on a single nanolattice was low, on the order of ~130 (this number is based on an approximation given by the number of unit cells present in the nanolattice), which is three orders of magnitude lower than what is necessary for such analysis. In addition to the few cells available for analysis, the very small footprint area of the nanolattices, which was ~0.16% of the total area of the chip precluded selective extraction of cells grown on the nanolattices vs. those grown on the surrounding 2D substrate.

Loading condition 2: constant load, constant loading frequency, different strain

We isolated the role that loading magnitude plays in stress fibers and focal adhesions formation by loading the nanolattices to a constant maximum strain at a fixed frequency, ~1Hz, which led the most compliant material system (A) to experience a maximum load of ~0.6mN while the stiffest material system (D) experienced a maximum load of ~21mN. We employed fluorescence microscopy to monitor the concentration of stress fibers and focal adhesions. SAOS-2 cells were cultured on the nanolattices and four samples were subjected to mechanical stimulation while 4 samples, which were not cyclically compressed, served as control.

Figure 31 (a) shows representative images of actin fluorescence intensity which was recovered from each material system after cyclic compression. F-actin distribution appeared to be uniform across each nanolattice along all planar directions which suggests isotropic attachment of cells

onto the nanolattices. Quantification of the actin intensity signal revealed a similar trend for the cyclically compressed and the uncompressed, i.e. the control samples (figure 31(b)). The control samples revealed that SAOS-2 cells that were grown on the most compliant nanolattice (A) displayed ~20% more fluorescence signal than those that were grown on material system (B), which exhibited ~20% more f-actin than material system (C). Material system (C) revealed ~20% more actin signal than material system (D). The nanolattices that were cyclically compressed revealed an identical trend. Material system (A) displayed the largest fluorescence intensity signal, which was ~10% larger than that of material system (B). Material system (B) revealed ~15% higher fluorescent signal than material system (C), which displayed ~15% more actin fluorescence than material system (D).

Focal adhesions, which were stained green, didn't display an evident trend. Figure 31 (C) shows representative fluorescence images from all nanolattices. Quantification of the fluorescent signal revealed a quite constant amount of the focal adhesions across material systems regardless of presence or absence of mechanical stimulation (Figure 31 (d)).



Figure 31: Fluorescence microscopy after constant displacement cyclic compressions. (a) Zstack projections of confocal images of SAOS-2 which were grown on the nanolattices and cyclically compressed 320 times over 48 hours to the same load (0.6mN) showing actin filaments stained with Phalloidin. (b) Focal adhesions stained with anti-vinculin antibodies; the material system is represented by the schematic circle on top of the corresponding nanolattice. Relative amounts of f-actin (c) and focal adhesions (d) as a function of nanolattice stiffness. The data points in red (c) and green (d) represent the results from the nanolattices that were cyclically compressed. The data points in blue represent the control samples that were not compressed. Fluorescence data was normalized by the intensity of the most compliant material system (A). Horizontal error bars represent standard deviation in nanolattice elastic moduli and vertical error bars represent standard error in fluorescence measurements.

Loading condition 3: single material system, constant load, varying loading frequencies

Fluorescence microscopy revealed a dependency of f-actin and vinculin concentration on loading frequency. The amount of stress fibers (figure 31 (a), (c)) increased as a function of loading frequency. Cells that were grown on the nanolattices that were not compressed revealed the least amount of fluorescence intensity. Those cells that were grown on nanolattices that were grown on the g

compared to the control, i.e. the nanolattice that was not compressed. Higher loading frequencies led to a further increase in f-actin fluorescence. Those cells that were grown on the nanolattices that were compressed at a loading frequency of 1Hz exhibited an increase in f-actin fluorescence of \sim 30% compared to the control and the cells that were compressed at a loading frequency of \sim 3Hz revealed the largest increased in f-actin, \sim 50%, compared to the control (figure 31 (c)).

Vinculin fluorescence revealed a similar trend: increasing loading frequency generally caused an increase in vinculin fluorescence intensity (figure 31 (b), (d)). Loading the nanolattices at the frequency of 0.1Hz, 1Hz and 3Hz led to an increase in vinculin signal by 20%, 50% and 60% respectively compared to the control as shown in figure 31 (d). To quantify the relative amount of fluorescence as a function of loading frequency, fluorescence data from each individual chip was normalized by the fluorescence intensity of the control. A total of 4 chips were used to determine error for loading condition 3.



Figure 32: Relative actin and vinculin fluorescence intensity as a function of loading frequency. All nanolattices that were used for this loading condition were taken from material

system (B). (a) Z-stack projections of confocal images of SAOS-2 which were grown on the nanolattices and stained for actin filaments with Phalloidin. One nanolattice per sample was not compressed and functioned as control. The other nanolattices were cyclically compressed 320 times over 48 hours at the loading frequencies shown on top of the confocal image. (b) Z-stack projections of focal adhesions stained with anti-vinculin antibodies. (c) Relative fluorescence intensity of stress fibers as a function of loading frequency. The points in red represent the results from the nanolattices that were cyclically compressed at different loading frequencies. The point in blue represents the control (not compressed). (d) Relative fluorescence intensity of focal adhesions of cells grown on the nanolattices which were compressed at different loading frequencies. The green data points were obtained from the nanolattices that were cyclically compressed at different loading frequencies. The blue point represents the control. Fluorescence data was normalized by the nanolattice that was not compressed (control). Horizontal error bars represent standard deviation in nanolattice elastic moduli and vertical error bars represent standard error in fluorescence measurements.

4.4. Discussion

Osteopenia is a disease characterized by a decreased amount of bone density and by abnormalities in the architectural organization of bone structures that lead to bone tissue weakening and increased susceptibility to fractures¹⁰. Osteopenia mainly occurs in the ageing population, in astronauts, and in patients who have received bone implants. As a result of the large number of people affected by osteopenia and the lack of a permanent cure, osteopenia is widely viewed as a major public health concern and has enormous costs associated with it. Currently, a variety of drugs, such as bisphosphonates, teriparatides, and monoclonal antibodies, are being used to slow down the progress of osteopenia, however, these drugs often have side effects and are not efficient in inducing the native bone cells to deposit more mineral material¹⁰¹⁰⁴. Research into the root physical causes of osteopenia has been done for over 6 decades and it has shown that at the macroscale, mechanical stimulation of bone leads to tissue remodeling and increased mineral deposition, yet the source of such disease is still unknown¹⁰¹²⁰¹²¹³. By using 3D

106

nano-architected scaffolds with features size on the same order of cellular components, a few microns, we aimed to clarify which mechanical stimuli osteoblasts are most sensitive to.

4.4.1. Mechanical Characterization: Monotonic Uniaxial Compression

We employed nanolattices with a tetrakaidecahedral geometry, a fairly constant low relative density, ~0.14-0.36%, and a structural stiffness spanning from ~0.7-35MPa, which was achieved by fabricating hollow Ti/TiO₂ nanolattices. The mechanical behavior of the nanolattices was analyzed via quasi-static uniaxial compression experiments, which revealed two distinct deformation behaviors: catastrophic brittle failure exhibited by the stiffer material systems (B), (C) and (D), and a combination of localized brittle fracture and ductile-like layer-by-layer collapse exhibited by the most compliant material system, (A) (figure 25). A toe region caused by imperfections in the experimental setup was present in all compression experiments as detailed.

Following the toe region, nanolattices made from material system (A) (hollow TiO, with 18nm wall thickness) exhibited linear elastic loading up to ~4% strain and a stress of ~12kPa followed by discrete strain bursts that corresponded to localized brittle failure events, which led to unrecoverable structural damage. The first strain burst exhibited the largest magnitude, ~15% strain, which was caused by greater elastic strain energy stored in the tube walls upon initial loading. The subsequent strain bursts, which occurred at lower stresses exhibited a smaller magnitude, on the order of ~5% strain which follows from less strain energy being accumulated in the structure. We postulate that the reason behind this layer-by-layer collapse behavior lies in the shell-thickness-to-beam-radius (t/R) ratio for material system (A), which was 0.02. Literature⁸⁴⁰⁷¹⁰⁹ has shown that when (t/R) < 0.03, the global deformation of the nanolattice is

Material system (B), (C) and (D) displayed (t/R) > 0.03, which was shown to cause global brittle fracture at stresses lower than those required for the onset of shell buckling mechanisms. Material system (B) (hollow TiO, with 50nm wall thickness, t/R = 0.055), material system (C) (hollow TiO, with 100nm wall thickness, t/R = 0.11) and material system (D) (hollow TiO, with 160nm Ti - 100nm TiO, wall thickness, t/R = 0.28) displayed a similar deformation behavior: following the initial toe region, the nanolattices experienced linear-elastic loading up to a strain equal to ~7% for material system (B), ~6% for material system (C) and ~5% for material system (D). We suspect that the large tensile stresses generated in the structure due to beam bending gave rise to high hoop stresses in the outer TiO, shell at the nodal connections of the nanolattice, which led to brittle fracture^{mone}. Global brittle failure occurred at a compressive stress of ~0.19 MPa for material system (B), at ~0.69 MPa for material system (C) and at ~1.1 MPa for material system (D). The large amount of strain energy stored in the nanolattice right before global failure appeared to be larger than the amount of energy that the units cells, which remained intact, could absorb leading to a complete structural collapse.

4.4.2. Mechanical Characterization: Cyclic Uniaxial Compression Experiments

Loading condition 1: constant load, constant loading frequency, different strain

After growing SAOS-2 cells on the nanolattices for 12 days in growth media and 1 day in mineralization media, cyclic compressions were performed for 2 days before cell fixation and staining were carried out. To ensure cell survival during the mechanical stimulation, which was

done outside an incubator, a square trench into was etched into the Si substrate. The trench contained the nanolattices with the cells grown on them and served as a reservoir of nutrients during cyclic compressions (figure 24).

Each material system was subjected to 160 loading-unloading cycles per day and the cyclic experiments were repeated for 2 days. It is evident from figure 26 (a) that the first cycle differed slightly from all other loading-unloading cycles. This deviation was likely caused by the indenter tip flattening the unit cells positioned along the edges of the nanolattice which tend to be initially bowed upwards. All other cycles revealed no virtual differences in the loading-unloading path, which suggests full nanolattice recoverability within the stress window that was tested. The small hysteresis shown in figure 26 (a) was likely caused by: (1) frictional forces that developed between the indenter tip and the top surface of the nanolattice; (2) thermal drift, which could not be lowered below ~10nm/s due to experimental constraints.

We measured the structural stiffness of the nanolattices from 20 loading and unloading slopes of the cyclic experiments and observed a discrepancy between the structural modulus measured from the monotonic experiments to failure and that from cyclic loading to 0.6mN for all material systems but the most compliant one (A). The apparent reduction in E^* that we observed for loading condition 1 was calculated to be ~50% for material system (B) and ~65% for material system (C) and (D). This phenomenon was caused by the extent of the toe region which appeared to be dependent of the nanolattice structural stiffness.

The stiffer material systems (B), (C) and (D) exhibited a toe region that extended above the maximum load (0.6mN), leading to incomplete contact between the indenter tip and the nanolattice. Separate experiments that were carried out in our group, which showed that

nanolattice structural stiffness remains relatively constant with respect to the number of unit cells it is comprised of (figure 33) allowed us to estimate the effective contact area between the indenter tip and the nanolattices according to eq. 11:

$$A_{eff} = \frac{H}{E^*} \left(\frac{dF}{dH} \right) \tag{11}$$

where A_{α} is the effective contact area, H is the nanolattice initial height, E is the nanolattice structural stiffness measured from monotonic uniaxial compression to failure and dF/dH is the load displacement data collected by the nanoindenter. The estimated effective area for material system (B) was about 50% of the total footprint area and A_{α} for material system (C) and (D) was ~35% of the total nanolattice area. As a consequence of the inability to fully engage the stiffer nanolattices, the strain magnitudes that were reported for material system (B), (C) and (D) are an overestimation of the actual strains experienced by these material systems. Consequently, we argue that the cells grown on such material systems experienced smaller strains than the ones expected from the data that we collected. Since the effective contact area was larger for material system (B) compared to that of material system (C) and (D), we expect the strain data to be more accurate for material system (B) compared to that of material system (C) and (D). In light of this analysis, we think that the trend observed in figure 26, which shows that $\varepsilon_a > \varepsilon_c > \varepsilon_a$ was preserved and was likely larger than the one measured experimentally.



Figure 33: Nanolattice structural modulus as a function of the number of unit cells. Experiments and simulations performed in our group show that the modulus of the tetrakaidecahedron nanolattice remains relatively constant regardless the number of unit cells and layers that the nanolattice is composed of. (Figure courtesy of Carlos G. Portela)

Figure 26 (c) shows the presence of a non-linear region in the displacement-time curves of all material systems. This non-linearity resulted from intrinsic limitations of the nanoindenter's control system: at a loading frequency of \sim 1Hz the system's inertial forces caused a delay between the end of the loading and unloading segments which manifested itself in the residual displacement shown in figure 26 (c).

Figure 32 shows a zoomed-in view of the delay between the end of the loading segment and the end of the loading displacement segment. The loading rate (cyan) was overlapped to the displacement rate (orange) in order to show that the loading rate remained constant up to the loading peak (0.6mN) after which we observed a non-linearity in the form of a shallow decay

lasting ~200ms, the necessary time for the instrument to reverse the loading direction. This decay caused the non-linear behavior observed in the displacement-time curve (figure 26 (c)), which also lasted for ~200ms. We hypothesize that this non-linearity is a consequence of the inertial forces of the system.



Figure 34: controller's response to cyclic loading. The Load-Time data (cyan) was overlapped to the Displacement-Time data (orange) for a given cyclic compression which revealed a delay of ~200ms between loading and unloading segments.

Loading condition 2: constant load, constant loading frequency, different strain

In order to probe whether osteoblasts are sensitive to different peak loads, we seeded SAOS-2 cells on the nanolattices for 12 days in growth media and 1 day in mineralization media before performing cyclic compressions for 2 days. By fixing the peak strain at $\sim 2\%$, we were able to load all nanolattices past their toe region, which ensured full contact between the nanolattice and the indenter tip. Structural stiffness values measured from cyclic experiments were virtually identical to those measured from quasi-static experiments, which further confirmed full contact

between the nanolattices and the indenter tip.

Figure 27 (a) shows that loading/unloading cycles were nearly self-similar, showing evidence of full recoverability of the structure within the prescribed strain. The small hysteresis loop shown in Figure 27 (a) had a magnitude of ~100nm and was most likely caused by thermal drift and by friction between the lattice and the indenter tip.

Figure 27 (b) reveals that a broad range of peak loads spanning more than one order of magnitude were reached during these experiments, while the net displacement per cycle across material systems was kept relatively constant as shown in figure 27 (c). The most compliant nanolattices (A), were cyclically loaded to a maximum load of 0.65mN, which represented ~80% of the failure load for this material system. In order to reach a maximum strain of ~2%, material system (B) was loaded up to 5mN, which is 70% of its failure load; material system (C) was loaded up to 16mN, which is 66% of its failure load and material system (D) was loaded up to 21mN, which is 54% of its failure load.

Figure 27 (c) shows that a similar net displacement of $\sim 1 \mu m$ was experienced by all material systems and that the non-linear region caused by the system's inertial forces was also present and virtually identical across material systems, which were all loaded at $\sim 1 Hz$.

Loading condition 3: single material system, constant load, varying loading frequencies

The effects of varying the loading frequency on stress fibers and focal adhesion formation were isolated by selecting a single material system (B), which allowed for locking the maximum load (4.5mN) and displacement (~1µm) across the frequency range to be studied. Material system (B) was chosen as the best candidate for this loading condition because full contact could be

established at a relatively low maximum load, which is less likely to cause damage to the cells that were grown on the nanolattices. Three loading frequencies were deemed adequate for this study: 0.1Hz which qualitatively resembles the loading frequency of passive standing; 1Hz which was used to simulate a slow walking condition and finally 3Hz, which reproduced a loading frequency similar to that caused by a slow running condition.

The linearity shown in the load vs. time curves shown in figure 29 reveals that the loading rate could be maintained constant for all loading frequencies which also suggests that the nanolattices were loaded within the linear elastic region. Cyclic repeatability was remarkable for slower loading frequencies (0.1Hz, 1Hz) while instrument instabilities caused slight variations for the faster cyclic compressions, which were performed at ~3Hz.

4.4.3. Cell Response: F-actin and Vinculin Distribution

Mechanical stimuli, which are ever-present between cells and their surroundings, have become increasingly recognized as major regulators of cell behavior and tissue structural organization ^{120,145-} ¹⁴⁷. Mechanotransduction is the process by which cells are able to sense and translate these forces into cellular signaling, which ultimately guides cell migration, matrix assembly, and tissue organization³¹. In addition to sensing passive mechanical cues, such as the stiffness of the surrounding matrix, tissues commonly experience a variety of active loads, such as in the case of bone, which has been shown to initiate tissue remodeling¹⁴⁸.

The first link between bone adaptation and mechanical stimuli comes from a theory developed over 100 years ago by Wolff and Roux who proposed a mathematical model that linked the thickness and number of trabeculae to the quantitative distribution of mechanical stresses in bone¹⁶¹³⁵. A large number of experiments performed over the last 30 years demonstrated that bone mass and architecture continuously adjust to the changing mechanical environment, thus confirming Wolff's law of bone remodeling^{17,36,36}. These experiments also revealed that bone adaptation is driven by dynamic, rather than static, loading and that a short duration of mechanical loading is sufficient to initiate an adaptive response^{17,36,36,47,187}. More recently, experiments have confirmed that the number of loading cycles and loading frequency are important determinants of bone adaptation, however whether osteoblasts are more sensitive to changes in load or displacements is still debated¹⁶³. Attempts to answer this open question were made by stretching osteoblasts that were cultured on 2D polymeric matrices. Literature has shown that there are clear differences in how forces are experienced by cells in 2D versus 3D environments. Strain fields in 2D contexts are smooth and homogeneous, and cell deformation occurs in a predictable manner. In-vivo environments, on the other hand, are 3D and structurally heterogeneous which lead to a much more complex and less predictable strain states^{17,36,36,4}.

To mimic more realistic in-vivo microenvironments, we fabricated 3D nanoarchitected materials with different structural stiffness that allowed us to isolate the role that stress, strain and loading frequency play on the cell's stress fibers (f-actin) and focal adhesions (vinculin) formation, which represents a major indicator of the events involved in mechanotrasduction³¹.

Those samples that were not mechanically stimulated revealed that substrate stiffness contributed to modulating stress fibers concentration in SAOS-2 cells. The most compliant nanolattice (A) exhibited ~20% more f-actin fluorescence intensity than material system (B), ~35% more than material system (C) and ~50% more than the stiffest nanolattice (D). These results support the findings that were detailed in chapter 3 therefore confirming the critical role that nanolattice

structural stiffness plays on stress fibers formation in osteoblasts-like cells.

The application of cyclic loading to the nanolattices led to a cellular response that was different from that of the uncompressed samples, confirming that mechanical stimulation affects bone cell behavior. By fixing the loading frequency (1Hz) and maximum load (0.65mN) the nanolattices experienced different amounts of maximum strains ranging from 0.44% (material system D) to 0.81% (material system A). This loading condition didn't produce an evident trend in cellular response. All cells regardless of the material system where they were grown on exhibited an increase in f-actin fluorescence signal of ~45% suggesting that net strain magnitude might not directly cause an increase in f-actin formation.

Similarly, keeping the loading frequency (1Hz) and maximum nanolattice strain (~2%) constant caused the material systems to experience different amounts of load ranging from 0.6mN (material system A) to 22mN (material system D). This loading condition triggered a homogeneous cellular response which led to a similar increase in f-actin fluorescence (~20%) signal across material systems. Varying maximum loading also didn't appear to cause an evident trend in f-actin formation.

Loading condition 3 where the loading frequency was allowed to change while the maximum load and displacement were kept constant at ~4.5mN and ~1 μ m respectively, exhibited a clear trend: increasing loading frequency led to an increase in f-actin fluorescence signal. In order to fix load and displacement, one material system, which was arbitrarily chosen, was used for this loading condition. The control sample, which was not compressed functioned as control and exhibited the least amount of fluorescence signal. The cells grown on the nanolattices that were compressed at a loading frequency of 0.1Hz revealed ~20% more fluorescence intensity than the control sample. When the loading frequency was increased to 1Hz, which mimics the loading

frequency experienced by natural bone when walking, the f-actin intensity increased by $\sim 30\%$ compared to the control. The highest amount of f-actin fluorescence intensity was observed when the nanolattices were compressed at a loading frequency of ~ 3 Hz, which is approximately the same loading frequency experienced by bone while running.

The vinculin staining revealed a similar behavior: focal adhesion fluorescence exhibited an evident trend only when loading frequency was changed. Vinculin fluorescence intensity appeared to be quite constant across material systems for the control samples and for the constant load/displacement loading conditions. Conversely isolating the loading frequency effects by fixing maximum load and displacement revealed a gradual increase in focal adhesion fluorescence signal as the loading frequency was increased from 0.1Hz to 3Hz.

This study confirms that f-actin remodeling is affected by both the structural stiffness of the nanolattice and by the mechanical stimulation that the scaffold is subjected to. Competing factors that affect stress fibers formation appear to be scaffold's structural modulus, load magnitude, strain magnitude and loading frequency.

For all loading conditions the nanolattices that were mechanically stimulated induced an increased cellular response compared to the nanolattices that were not cyclically compressed suggesting that mechanical stimulation might play a more prominent role in stress fibers remodeling than that played by the scaffold's structural stiffness alone.

After mechanical stimulation the difference in fluorescence intensity across material systems with different moduli decreased by more than 20% compared to the control samples, supporting the hypothesis that cyclic compression may be a stronger determinant than structural stiffness with regards to f-actin formation.

Among the different mechanical stimuli loading frequency appeared to have the largest effect as indicated by figure 32. While varying maximum load or displacement didn't produce a detectable trend in f-actin fluorescence signal, increasing loading frequency from 0 to 3Hz caused a 50% increase in stress fibers formation, suggesting that osteoblasts might be most sensitive to loading frequency changes.

4.5. Concluding Remarks

We used TPL to fabricate three-dimensional hollow TiO₃nanolattices whose strut dimensions were on the same order as osteoblasts' focal adhesions (~2µm) and pore sizes of 25µm. We varied the nanolattices structural stiffness by changing the shell thickness from 18nm to 100nm. Nanomechanical experiments on each type of nanolattice revealed their stiffnesses to range from ~0.7MPa to 35MPa. Osteoblast-like SAOS-2 cells were seeded on each type of nanolattice, and their mechanosensitive response was explored by cyclically compressing the nanolattices under 3 different loading conditions: constant load, constant strain, varying loading frequency.

The cellular response was tracked via fluorescence microscopy by staining for intracellular factin and vinculin concentration after 2 days of mechanical stimulation.

These experiments revealed that stress fibers formation was enhanced after cyclically compressing the nanolattices across material systems and that increasing loading frequency provoked the most drastic changes in f-actin and vinculin fluorescence intensity.

These findings have significant implications for understanding the role that external mechanical cues play in cytoskeletal reorganization which could lead to a deeper understanding of the mechanisms underlying osteopenia.

Chapter 5. Mechanically Tunable Force Sensors for Measuring Cell Forces in Three-Dimensions

This chapter details the design, fabrication challenges and characterization of a threedimensional capacitor with tunable electro-mechanical properties. Rather than integrating the capacitive plates with a traditional flexible diaphragm, we combined well-established semiconductor fabrication techniques with two-photon lithography to sandwich an insulating nanolattice in between two conductive parallel plates. Our novel design allowed us to fabricate a tunable 3D parallel-plate capacitor with a basal capacitance (C_s) of 0.3pF which was able to sense forces down to ~30 μ N. The nanolattice was made from polymeric tetrakaidecahedral unit cells with a pore size (*U*) of 30 μ m, which were tessellated in space to form a lattice with lateral dimensions of 900 x 900 μ m and a basal height of 23 μ m. By taking advantage of the structural flexibility of the nanolattices with regards to porosity, stiffness and strength such capacitors can be tailored to sense forces at the cellular level to offer insights into cell mechanics during early tissue formation.

5.1 Chapter Summary

The advent of tissue engineering ~ 30 years ago revolutionized the study of cell mechanics³⁹. Understanding the underlying principles of cell assembly is paramount to create artificial organs. Cell mechanics plays a crucial role in determining how cells arrange themselves in space. Literature has shown that forces exerted by cells on their environments are key in maintaining the normal architecture and function of tissues; disruption of these mechanical cues leads to disease¹⁰. In order to characterize the mechanical properties of cells and the magnitude of the forces that they sense and exert on their surroundings, several techniques have been developed. Two types of probing tools are currently available¹⁰:

(1) Devices that apply a force to the cell and track the cell's response to this stimulus. Examples of tools in this category are: atomic force microscopy (AFM) which uses a cantilever beam with an atomically sharp tip at its free end to probe cellular structures; optical tweezers, which use a highly focused infrared laser to exert pico-Newton forces to the cell by taking advantage of the refractive index mismatch between the media and the cell; magnetic tweezers which adopt ferromagnetic particles to transmit forces to the cell.

(2) Devices that can only sense the forces developed by the cell via structures that undergo measureable deformations. Examples of tools belonging to this category include traction force microscopy, which employs fluorescent beads that are incorporated in a matrix onto which cells exert traction forces. By tracking the beads' displacement field, cellular forces can be calculated. Micropillar arrays employ an array of vertically aligned cantilevers that deflect as a function of the force magnitude that the cells exert on them. By measuring the pillars' deflection, cellular forces can be extracted. Microelectromechanical systems (MEMS) devices take advantage of capacitive sensing by using thin membranes that undergo very small deflections caused by the applied cellular forces. These forces are then transduced into detectable capacitance changes.

These instruments have played a crucial role in advancing the field of cell mechanics, however they suffer from several limitations. Most of these currently available tools require an active probing device and a separate sensing device, which increase the complexity and costs of the apparatus. Moreover, the number of cells that can be studied simultaneously using these devices is limited to only a few, hindering the investigation of the mechanical interaction of large groups of cells, which are commonly found in early tissue formation. To the best of our knowledge, all current devices measure cellular activity in 2D, however native organs are inherently three-dimensional with cell mechanics shown to be remarkably different from 2D, which calls for the development of force sensors that are able to interrogate cell interactions in 3D^{29,121,155}.

Studies have shown that 3D architected materials offer useful platforms to investigate cell behavior. In chapters 3 and 4, we have shown that osteoblast activity, specifically their stress fibers concentration, which is directly related to cellular forces, is affected by the stiffness and loading conditions of the scaffolds. However, several questions remain unanswered:

(1) Do osteoblasts feel the mechanical properties of the individual beams, the unit cell or of the full nanolattice?

(2) How does the magnitude of cellular forces on 3D substrate compare to that on 2D substrates?

(3) Does the magnitude of these forces change as a function of externally applied stresses?

In order to answer these questions, we developed a 3D tunable micro-capacitor that is able to actively engage multiple cells by applying and sensing forces spanning three orders of magnitude, \sim 30µN-10mN. We took inspiration from 2D capacitive sensing devices and substituted the classic dielectric thin film with an insulating nanolattice, which provides increased sensing flexibility and an adequate environment for cells to grow and mechanically interact. We fabricated a device that consists of one electrode placed on a fixed substrate and the other placed on top of a nanolattice. We combined traditional semiconductor fabrication techniques, such as photolithography, thin film deposition and deep reactive ion etching (DRIE)

with two-photon lithography (TPL), which enabled us to incorporate arbitrary 3D geometries on a pre-patterned substrate. The forces exerted by the cells grown on the 3D architected dielectric cause a change in the distance between the top and bottom plates giving rise to a capacitance change, which can be used to resolve cellular forces in 3D.

5.2. Sensor Design

Capacitance is the ability to store electrical charge, upon the application of a voltage V, on two parallel metal plates separated by a distance L. The resulting electric charge remains on the plates even when the voltage is removed. Traditionally an insulating solid material with a dielectric constant K higher than of air is inserted in between the metal plates to increase the overall capacitance of the device, which can be calculated via the following equation:

$$C_0 = K\varepsilon \frac{A_0}{L_0} \tag{12}$$

where C_{0} is the basal capacitance, K is the dielectric constant of the material inserted between the metal plates, ε is the permittivity of free space that is equal to $8.8542 \times 10^{-12} \text{ C}^{2}\text{N}^{-1}\text{m}^{-1}$, A_{0} is the initial overlapping area between the top and bottom plates and L_{0} is the initial distance between the top and bottom plates. By using two-photon lithography we were able to substitute the solid dielectric material, separating the two plates, with an architected material with tunable mechanical properties.

As a consequence of the porous nature of architected materials, the two plates of the capacitor are in contact with both air and the polymeric material (Ip-Dip Nanoscribe GmbH) from which the nanolattice is made, effectively creating a situation equivalent to two capacitors connected in parallel. The net basal capacitance of the fabricated capacitor can then be estimated using the following equation:

$$C_{T_0} = C_{lat} + C_{air} = \frac{\varepsilon}{L_0} (K_{lat} A_{lat} + K_{air} A_{air})$$
(13)

where $C_{r_{i}}$ is the total basal capacitance of the fabricated 3D capacitor, C_{uu} is the capacitance due to the lattice being in contact with the metal plates, C_{uv} is the capacitance due to the plate area being in contact with air, K_{uv} is the dielectric constant of IP-Dip which is ~3, K_{uv} is the dielectric constant of air which is 1, A_{uv} is the footprint area of the lattice on the metal plate and A_{uv} is the contact area between the metal plate and air. For the final device L_v was fixed at 23µm and A_v , which is the sum of A_{uv} and A_{uv} was kept constant at 0.57mm². According to eq. (13) the total basal capacitance of the fabricated nanolattice was calculated to be 286fF.

By introducing a nanolattice in place of a thin film solid dielectric, we were able to fabricate a variable distance capacitive displacement sensor whose sensitivity is given by the following equation:

$$\frac{dC}{dL} = -\frac{\varepsilon}{L^2} (K_{lat} A_{lat} + K_{air} A_{air})$$
(14)

The effective distance between the plates *L* changes as a function of the forces that the cells apply, giving rise to a capacitance change which can be used to evaluate cellular forces. In order to tune the device initial capacitance C_r and its sensitivity to forces exerted by cells, we varied the unit cell elevation angle θ (figure 32) from 30° to 45° and the beam radius *R* from 0.7µm to 1µm. By varying θ and *R* we were able to fabricate porous dielectrics with structural stiffness *E** values spanning from ~0.2-2MPa. The final device that was electrically tested displayed a structural stiffness of 0.57MPa.



Figure 35: Matlab-generated image of the tetrakaidecahedron unit cell design. (a) unit cell with an elevation angle (θ) of 30°. (b) Unit cell with an elevation angle (θ) of 37.5°. (c) Unit cell with an elevation angle (θ) of 45°. Varying the elevation angle allowed us to vary the mechanical properties and basal capacitance of the fabricated capacitor.

5.3. Methods and Results

Several challenges associated with integrating a 3D architected material in a parallel-plate capacitor led to the exploration of three different fabrication routes:

5.3.1. Fabrication Route 1: the Traditional Approach

A 300µm-thick SiO₂ slide that served as the insulating substrate, was dehydrated at 115C° for 5 minutes and subsequently spin-coated with a negative photoresist AZ nLOF2020 which was exposed for 12 seconds in soft contact mode using a Karl-Suss Microtech MA6 aligner with a power density of 15mW cm². After a post-exposure bake (PEB) of 45 seconds at 110C° the sample was developed in MF319 for 2.5 minutes at room temperature. 20nm of Ti and 80nm of Au were evaporated in a Kurt J. Lesker Lab Line at a rate of 1Å/s and a first lift-off procedure of the photoresist was carried out in PG remover at 60C°, revealing the bottom fixed plate of the capacitor and the electrical contacts (figure 33 (a)).

TPL direct laser writing (Nanoscribe GmbH) was used to fabricate a nanolattice which included

the top plate of the capacitor and a slanted bridge that was necessary to ensure electrical continuity between the top plate of the capacitor and the bottom electrical contact as shown in figure 33 (b).

Photoresist nLOF2020 was used a second time to mask the area surrounding the nanolattice. For this step, a maximum spinning speed of 1000rpm could be achieved in order to avoid nanolattice/bridge collapse. Karl-Suss Microtech MA6 was used in proximity mode with a gap distance of 50µm and exposure time was set for 10 seconds to induce photoresist crosslinking. Following PEB at 105C° for 45 seconds we developed the pattern in MF319 for 5 minutes to ensure complete removal of the photoresist that resided within the nanolattice (figure 33 (c)). A 50nm-thick Au film was deposited at 1Å/s using a Kurt J. Lesker Lab Line e-beam evaporator, which effectively metallized the top plate and the bridge of the capacitor. In order to release our device, a second lift-off process was carried out in PG remover at 60C°. This last step led to failure of fabrication route 1. Multiple iterations of the lift-off conditions were carried out with no success. Figure 33 (d-g) shows that the failure was caused by multiple problems: figure 33 (d) shows warping of the top plate, which contributed to the detachment of the nanolattice from the substrate. Figure 33 (e) reveals substantial Au delamination from the polymeric nanolattice, which also contributed to device failure by breaking electrical continuity. Figure 33 (f) shows the presence of large amounts of photoresist left within the nanolattice, which were caused by the inability to precisely pattern the photoresist due to the large gap distance required by proximity exposure modality (figure 33 (g)).



Figure 36: SEM images of the steps involved in fabrication route 1 (a) SiO2 substrate (dark gray) with bottom Au-coated electrical contacts patterned on it. (b) Nanolattice written on the bottom Au-coated capacitor plate. (c) Second photoresist spinning to reveal the nanolattice upper plate. (d) Nanolattice warping upon second lift off of the photoresist shown in (c). (e) Au delamination from the polymeric substrate following the second lift-off. (f) Presence of photoresist within the nanolattice after post lift-off. (g) Photoresist positive profile caused by proximity mode expose.

5.3.2. Fabrication Route 2: SiO₂Pre-Patterning

Photoresist AZ nLOF2020 was spun on a SiO₂ substrate and exposed following the same conditions detailed in section 5.3.1. A protective etch mask composed of a 300nm-thick layer of Cr was deposited in an e-beam evaporator (Temescal FC-1800) at a rate of 1.5Å/s. Removal of the photoresist upon evaporation was carried out by dipping the sample in warm (60C°) PG remover for 10 minutes revealing the electrical contacts and the capacitor's bottom plate (figure 34 (a)). Substrate pre-patterning was achieved via SiO₂ etching which was performed using an Oxford Systems Plasma Lab 100 ICPRIE. The etching conditions that were used are given in table IX.

Table IX: SiO₂ etch recipe used for fabrication route 2.

02	10 sccm	ICP Power	2100 W
C4F8	40 sccm	Forward Power	200 W
Temperature	20 C	Pressure	10 mt

The etch rate was determined experimentally by etching multiple substrates for multiple times and was determined to be 85nm per minute. The substrate was etched for 25 minutes to a depth of $\sim 2\mu m$ (figure 34 (b)). The Cr mask revealed an erosion of $\sim 4nm/min$, this gives a selectivity of 20:1. Sidewalls undercut, which was essential for the success of this fabrication route, could not be achieved via dry plasma etching therefore the sample was dipped in buffered hydrofluoric acid (HF) for 10 minutes and agitation was applied. Figure 34 (c-d) reveal that HF wet etch was successful in yielding a small undercut which was measured to be $\sim 200nm$. Following the etching process, 80nm of Au were evaporated following the same conditions given in section 5.3.1. Figure 34 (e) shows one of the pre-patterned (raised) electrical contacts after Au deposition; the high contrast of the SEM image between the Au (clear) vs SiO2 (dark) and the EDS map provided in (figure 34 (F)) reveal that the etching depth and the amount of undercut weren't adequate to maintain electrical separation between the bottom substrate and the raised pattern leading to a short-circuited sample. The difficulties in achieving a depth larger than a few microns and the inability to control the etching profile precisely led to the failure of fabrication route 2.



Figure 37: Steps involved in fabrication route 2: SiO, **pre-patterning.** (a) Au-coated electrical contacts (orange arrows) and capacitor's bottom plate (cyan arrow) are revealed following Au

deposition and photoresist lift-off. (b) SiO₂ plasma etching following the recipe detailed in table IX reveals a positive profile incompatible for further processing. (c) SiO₂ wet etch in buffered HF for ~10 minutes to introduce undercutting necessary for further processing. (d) Zoomed-in view of the undercut profile created via HF wet etching. (e) Post Au deposition image showing a small separation between the Au on the bottom substrate and that present on the raised contact. The light gray signal comes from Au. The dark gray signal comes from SiO₂, which is highlighted by the black arrow. (F) EDS map of the undercut section of the sample showing possible sources of electrical continuity between the bottom substrate and the raised electrical contacts. Au is shown in yellow. SiO₂ is shown in blue.

5.3.3. Fabrication Route 3: Si Pre-Patterning

This approach, which resulted in the successful fabrication of a 3D micro-capacitor was based on Si dry etching, which is very well-characterized and controllable. Figure 35 shows a CAD rendering of the fabrication steps.



Figure 38: CAD representation of fabrication route 3: Si pre-patterning. (a) A Si wafer was spin-coated with a positive photoresist, Microposit Shipley 1813, which was exposed for 15 seconds using a Karl-Suss Microtech MA6 mask aligner. (b) Dry Si etching was performed to create the electrical contacts and the bottom plate of the capacitor. (c) Wet oxidation was performed to introduce an insulating layer. (d) Au deposition was carried out to metalize the electrical contacts and bottom plate. (e) The nanolattice along with the top plate of the capacitor were written using TPL. (f) Au deposition was carried out to metallize the top plate of the capacitor and the connecting bridge.

Deep reactive ion etching (DRIE) was performed in an Oxford Systems Plasma Lab 100 ICPRIE using a Bosch process and photoresist (Microposit Shipley 1813,) as etch mask. The Bosch recipe consists of 2 steps: an etch step that was set for 18 seconds and a deposition step that was set for 13 seconds. The details of the recipe are given in table X. After etching for 27 cycles which corresponds to ~15 minutes a depth of ~50 μ m was obtained (figure 36 (a)). Figure 36 (b) shows an SEM image of the pattern after Si dry etching which reveals a ~5 μ m undercut. Following DRIE, thermal oxide was grown on the etched Si substrate to create an insulating substrate. Wet thermal oxidation at 1100C° was performed for 15 hours yielding an oxide thickness of ~1.5 μ m (figure 36 (c)). SEM images that were taken after thermal oxidation revealed smoothening of the surface as shown in figure 36 (d).

Table X: Inductively coupled plasma etching of Si using a Bosch recipe.

Etching step:

SF ₆	160 sccm	ICP Power	2000 W
C_4F_8	0 sccm	Forward Power	30 W
Temperature	15 C	Pressure	35 mt

Deposition step:

SF ₆	0 sccm	ICP Power	1750 W
C_4F_8	140 sccm	Forward Power	10 W
Temperature	15 C	Pressure	10 mt

Evaporation of 50nm of Ti and 80nm of Au was carried out in a Kurt J. Lesker Lab Line e-beam evaporator as schematically shown in fig xxx. A Basal pressure lower than 10⁻⁷ torr and a rate of
1Å/s were observed to be necessary for an adequate directional deposition, which ensured electrical separation between the lower substrate and upper patterns. The large difference in atomic number between Au and SiO₂ gave rise to a stark contrast in the SEM as evidenced in figure 36 (e, f), which revealed a clear separation between the bottom and top Au film. This first Au deposition formed the bottom capacitor plate and the electrical contacts. A nanolattice comprising a top solid plate and a slanted bridge was written on top of the bottom plate as shown in figure 36 (g). The last step consisted of evaporating 80nm of Au on the sample, which effectively gave rise to the top plate of the capacitor (figure 36 (h)). Electrical continuity between the top plate and one of the bottom electrical contacts was established via the connecting bridge as shown in figure 36 (k).



Figure 39: SEM images of the steps involved in fabrication route 3: Si pre-patterning. (a) Si substrate etched using a Bosch recipe (table X). The purple square indicates the section of the sample that is detailed in (b). (b) Zoomed-in image of the etch profile. A significant undercut (\sim 5µm) was created via DRIE. (c) Post oxidation of the Si substrate showing substrate charging which is indicative of the insulating nature of the SiO₂ that formed following oxidation. (d) zoomed-in view of the area outlined in the cyan box in (c) revealing surface smoothening after oxidation at 1100 C°. (e) First deposition of Ti (50nm) and Au (80nm) used to metallize the electrical contacts and the bottom plate of the capacitor. The light gray signal in the image comes from Au. The dark gray signal comes from SiO₂. Zoomed-in view of the area outlined in the green area. The dotted black lines were introduced on top of the border between the Au-coated area and the SiO2 area to highlight the separation between these two elements. (g) TPL was used to write a nanolattice on top of the bottom capacitor place, indicated by the cyan arrow. The orange arrows point to the electrical contacts. (h) Image showing the nanolattice after the second Au deposition used to metallize the top plate of the

capacitor, indicated by the purple arrow. (k) Zoomed-in view of the bridge that connects the top plate of the capacitor and one of the electrical contacts.

EDS mapping shown in figure 37 confirmed that no electrical continuity existed between the bottom and the top plate of the 3D capacitor confirming the success of this fabrication approach. The low resolution of the EDS map was due to multiple factors: (1) the EDS detector in the SEM chamber that was used (FEI Nova 200) is positioned so that maximum signal is achieved when the sample is not tilted. Our sample needed to be tilted to an angle of 52° in order to probe for the presence of Au on the sides of the nanolattice which would cause a short circuit. (2) As a consequence of the weak signal introduced by tilting the sample a higher voltage (15keV) had to be used to collect enough x-ray. The high voltage caused charging of the nanolattice, which was made from insulating polymer, leading to charging-induced signal "vibrations" that effectively cause slight wobbling of the image. Despite this less-than-ideal situation figure 37 shows that a clear separation between the bottom and top Au-coated plated existed.



Figure 40: SEM images and corresponding EDS mapping of the fabricated capacitor.

(a) SEM image (top) and EDS map (bottom) of the nanolattice written on the bottom plate of the capacitor. The EDS map shows that Au (in yellow) was present on the top and bottom plates of the capacitor and a separation (in black) existed between the two. (b) SEM Zoomed-in view of a unit cell and part of the capacitor's top plate. EDS map (bottom) of the nanolattice unit cell revealing that no Au was present on the side of the nanolattice. (c) SEM image of the area outlined by the cyan in figure 36 (c) showing that no Au was present on the sidewalls of the fabricated sample. The absence of the polymer making up the nanolattice decreased surface charging leading to a higher resolution EDS map.

5.3.4. Mechanical Characterization

Uniaxial monotonic compression experiments on the nanolattices were carried out at a constant strain rate of 10^{4} s⁴ to ~25% strain using a nanoindenter (G200, Agilent Technologies).

Upon compression, the load-displacement data was collected and converted into stress-strain data. The effective structural stiffness of the nanolattices was calculated as detailed in section 3.2.2. All nanolattices that were fabricated were made from IP-Dip. Figure 38 (a) shows SEM images of some of the nanolattices that were tested; from the left: $R = 0.7 \mu m/\theta = 37.5^{\circ}$; $R = 1 \mu m/\theta = 30^{\circ}$ and $R = 1 \mu m/\theta = 45^{\circ}$. All fabricated nanolattices reveal bowing of the top plate, which was likely caused by polymer shrinking during development.

The corresponding stress-strain curve for each nanolattice system is shown in figure 38 (b). An initial toe-region was present for all nanolattices with $R = 1\mu m$ while it was absent for nanolattices with $R = 0.7\mu m$, which was likely due to limitations in instrument resolution; a probable scenario is that for very compliant nanolattices ($E^* \le 0.5$ MPa) the nanoindentor might have started recording the "load on sample" only after a noise threshold level, which appeared to be ~10 μ N had been reached. The toe-region was likely caused by the bowing of the upper plate and slight misalignment between the compression tip and the nanolattice.

A linear elastic region, indicated by the red slopes in figure 38 (b), followed the toe region and

was used to calculate the effective structural stiffness, E^{*4971} .



Figure 41: Mechanical characterization of polymer tetrakadecahedron nanolattices. (a) SEM images of some of the nanolattices that were fabricated. From the left: $R = 0.7 \mu m/\theta = 37.5^{\circ}$; $R = 1\mu m/\theta = 30^{\circ}$ and $R = 1\mu m/\theta = 45^{\circ}$. (b) Representative stress-strain response to quasi-static uniaxial compression of the nanolattices shown in (a). The red line represents the slope taken from the linear region of the stress-strain curve which was used to calculate the E^* of the nanolattice.

Table XI summarizes the structural stiffness results we recovered from the compression experiments. We observed that for a fixed unit cell size U_r and elevation angle θ , changing Rfrom 0.7µm to 1µm caused an increase in E^* by more than 100%. On the other hand, changing θ from 30° to 45° while keeping R and U_r fixed led to a decrease in E* by ~50% for $U_r = 25$ µm and ~200% for $U_r = 30$ µm. Changing beam radius thickness had more drastic effects on the smaller unit cell size, $U_r = 25$ µm, while changing elevation angle had larger effects on the larger unit cell size, $U_r = 30$ µm.

Radius (µm)	Width (µm)	θ (°)	H ₀ (μm)	Modulus (kPa)
0.7	25.0	37.5	19.2	587.9 ± 11.4
0.7	25.0	45.0	25.0	678.3 ± 9.2
0.7	30.0	37.5	23.0	282.3 ± 13.7
0.7	30.0	45.0	30.0	340.6 ± 10.2
1.0	25.0	30.0	14.4	1244.5 ± 17.2
1.0	25.0	37.5	19.2	1781.7 ± 21.7
1.0	25.0	45.0	25.0	1925.9 ± 18.1
1.0	30.0	30.0	17.3	317.2 ± 11.3
1.0	30.0	37.5	23.0	570.6 ± 17.4
1.0	30.0	45.0	30.0	901 8 + 14 2

Table XI: Nanolattice geometric features and corresponding structural modulus (E^*) .

The post-elastic behavior was characterized by a plateau region, which is a typical behavior observed in cellular solids and likely caused by the formation of plastic hinges at the nodes of the unit cell. The polymeric nature of the nanolattices that were tested led to structural recovery without visible mechanical damage.

5.3.5. Electrical Characterization

The electrical performance of the fabricated 3D capacitor was monitored by using a network analyzer which measured reflected coefficient (S_n) in comparison to the characteristic impedance (Z_n) of 50 Ω across a range of frequencies ($\omega = 0.2$ -4GHz). For small capacitance values, the capacitance values can be extracted from S_n according to the following equation:

$$C = \frac{\frac{1}{S_{11}} - 1}{j2Z_0\omega}$$
(15)

Advanced design system (ADS) simulations were also carried out to include the effects of the instrument's parasitic capacitance. Figure 39 shows a plot of the capacitance as a function of frequency and includes the experimental data (orange), the values obtained from ADS simulations (blue) and theoretical capacitance calculated from equation (13). The theoretical capacitance was expected to be 286fF for the fabricated capacitor which was comprised of 540 unit cells. Each unit cell had lateral dimensions of 30µm x 30µm and a height of 21µm.



Figure 42: Electrical characterization of the fabricated tetrakadecahedron capacitor. Simulated (light blue line), theoretical (dashed black line) and measured (orange line) capacitance was plotted as a function of frequency.

Figure 39 reveals that the measured data varied within ~17% of the theoretical capacitance for frequencies lower than 3GHz. The instrument's noise due to higher impedance at frequencies higher than 3GHz led to the measulinered capacitance to reach values close to ~360fF which deviated from the theoretical capacitance by ~25%. The simulated data incorporated the

instrument's parasitic capacitance and accurately predicted the experimentally measured capacitance for frequencies close to 3GHz where the instrument's parasitic capacitance plays a bigger role.

5.4 Summary and outlook

In this chapter we detailed the fabrication and testing of a mechanically tunable 3D capacitive sensor with a basal capacitance of ~280fF that could be used to monitor cell forces in 3D microenvironments. Currently it is unknown at what level of hierarchy cells engage the nanolattices. The fabricated device has the capability to indicate whether cells mechanically engage the nanolattice at the single unit cell level or at a more global level. This information could shed light on cell mechanics in 3D microenvironments, which represent more physiologically realistic conditions. Future work will be focused on determining the sensitivity of the fabricated sensor by continuously recording capacitance changes during dynamic uniaxial compression experiments.

References

- 1. Dimitriou, R., Jones, E., McGonagle, D. & Giannoudis, P. V. Bone regeneration: current concepts and future directions. *BMC Med*. **9**, 1–10 (2011).
- 2. Pilia, M., Guda, T. & Appleford, M. Development of composite scaffolds for load-bearing segmental bone defects. *BioMed Research International* **2013**, (2013).
- 3. Obwegeser, J. A. Bioconvertible screws made of allogenic cortical bone for osteosynthesis following sagittal split ramus osteotomy without postoperative immobilisation. *J. Cranio-Maxillofacial Surg.* **22**, 63–75 (1994).
- 4. Holy, C. E., Fialkov, J. a, Davies, J. E. & Shoichet, M. S. Use of a biomimetic strategy to engineer bone. *J. Biomed. Mater. Res. A* **65**, 447–453 (2003).
- 5. Cooper, L. F. *et al.* Fluoride modification effects on osteoblast behavior and bone formation at TiO2 grit-blasted c.p. titanium endosseous implants. *Biomaterials* **27**, 926–936 (2006).
- 6. Roberts, T. T. & Rosenbaum, A. J. Bone grafts, bone substitutes and orthobiologics: the bridge between basic science and clinical advancements in fracture healing. *Organogenesis* **8**, 114–24 (2012).
- 7. Nagels, J., Stokdijk, M. & Rozing, P. M. Stress shielding and bone resorption in shoulder arthroplasty. *J. Shoulder Elb. Surg.* **12**, 35–39 (2003).
- 8. Andreiotelli, M., Wenz, H. J. & Kohal, R. J. Are ceramic implants a viable alternative to titanium implants? A systematic literature review. *Clinical Oral Implants Research* **20**, 32–47 (2009).
- 9. Lind, M., Overgaard, S., Bünger, C. & Søballe, K. Improved bone anchorage of hydroxyapatite coated implants compared with tricalcium-phosphate coated implants in trabecular bone in dogs. *Biomaterials* **20**, 803–808 (1999).
- 10. Tertuliano, O. A. & Greer, J. R. The nanocomposite nature of bone drives its strength and damage resistance. *Nat. Mater.* **15**, 1195–1202 (2016).
- 11. Weiner, S., Traub, W. & Wagner, H. D. Lamellar Bone: Structure–Function Relations. J. Struct. Biol. **126**, 241–255 (1999).
- 12. Chen, P.-Y. *et al.* Structure and mechanical properties of selected biological materials. *J. Mech. Behav. Biomed. Mater.* **1**, 208–26 (2008).
- Kopperdahl, D. L. & Keaveny, T. M. Yield strain behavior of trabecular bone. J. Biomech. 31, 601–608 (1998).
- 14. Niinomi, M. & Nakai, M. Titanium-based biomaterials for preventing stress shielding between implant devices and bone. *Int. J. Biomater.* **2011**, (2011).
- 15. Huiskes, R. Stress shielding and bone resorption in THA: clinical versus computersimulation studies. *Acta Orthop. Belg.* **59 Suppl 1,** 118–129 (1993).
- 16. Ridzwan, M. I. Z., Shuib, S., Hassan, A. Y., Shokri, A. A. & Mohammad Ibrahim, M. N. Problem of stress shielding and improvement to the hip implant designs: A review. *Journal of Medical Sciences* **7**, 460–467 (2007).
- 17. Frost, H. M. Wolff's Law and bone's structural adaptations to mechanical usage: an overview for clinicians. *Angle Orthodontist* **64**, 175–188 (1994).
- 18. Fu, Q., Saiz, E. & Tomsia, A. P. Bioinspired strong and highly porous glass scaffolds. *Adv. Funct. Mater.* **21**, 1058–1063 (2011).

- 19. Wagoner Johnson, A. J. & Herschler, B. A. A review of the mechanical behavior of CaP and CaP/polymer composites for applications in bone replacement and repair. *Acta Biomater*. **7**, 16–30 (2011).
- 20. Ellingsen, J. E., Thomsen, P. & Lyngstadaas, S. P. Advances in dental implant materials and tissue regeneration. *Periodontology 2000* **41**, 136–156 (2006).
- 21. Le Guehennec, L. *et al.* Histomorphometric evaluation of bone response to different titanium implant surfaces. *20th International Symposium on Ceramics in Medicine* 613–616 (2007). at <<Go to ISI>://000253480200152>
- 22. Puleo, D. A. & Thomas, M. V. Implant Surfaces. *Dental Clinics of North America* **50**, 323–338 (2006).
- 23. Hutmacher, D. W. Scaffolds in tissue engineering bone and cartilage. *Biomaterials* **21**, 2529–2543 (2000).
- 24. Puleo, D. A. & Nanci, A. Understanding and controlling the bone-implant interface. *Biomaterials* **20**, 2311–2321 (1999).
- 25. F.F., H. *et al.* Nickel-, chrom- and cobalt-concentrations in human tissue and body fluids of hip prosthesis patients. *J. Trace Elem. Electrolytes Health Dis.* **6**, 239–43 (1992).
- 26. Karageorgiou, V. & Kaplan, D. Porosity of 3D biomaterial scaffolds and osteogenesis. *Biomaterials* **26**, 5474–5491 (2005).
- Tayalia, P., Mendonca, C. R., Baldacchini, T., Mooney, D. J. & Mazur, E. 3D cellmigration studies using two-photon engineered polymer scaffolds. *Adv. Mater.* 20, 4494– 4498 (2008).
- 28. Marino, A. *et al.* The Osteoprint: A bioinspired two-photon polymerized 3-D structure for the enhancement of bone-like cell differentiation. *Acta Biomater.* **10**, 4304–4313 (2014).
- 29. Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **126**, 677–689 (2006).
- 30. Discher, D. E., Janmey, P. & Wang, Y.-L. Tissue cells feel and respond to the stiffness of their substrate. *Science* **310**, 1139–43 (2005).
- 31. Janmey, P. A. & McCulloch, C. A. Cell mechanics: integrating cell responses to mechanical stimuli. *Annu. Rev. Biomed. Eng.* **9**, 1–34 (2007).
- 32. Cukierman, E. Taking Cell-Matrix Adhesions to the Third Dimension. *Science* (80-.). **294,** 1708–1712 (2001).
- 33. Chitcholtan, K., Asselin, E., Parent, S., Sykes, P. H. & Evans, J. J. Differences in growth properties of endometrial cancer in three dimensional (3D) culture and 2D cell monolayer. *Exp. Cell Res.* **319**, 75–87 (2013).
- 34. Roohani-Esfahani, S.-I., Newman, P. & Zreiqat, H. Design and Fabrication of 3D printed Scaffolds with a Mechanical Strength Comparable to Cortical Bone to Repair Large Bone Defects. *Sci. Rep.* **6**, 19468 (2016).
- 35. Buma, P., Schreurs, W. & Verdonschot, N. Skeletal tissue engineering-from in vitro studies to large animal models. *Biomaterials* **25**, 1487–1495 (2004).
- 36. Murphy, W. L., McDevitt, T. C. & Engler, A. J. Materials as stem cell regulators. *Nat. Mater.* **13**, 547–557 (2014).
- 37. Wu, S., Liu, X., Yeung, K. W. K., Liu, C. & Yang, X. Biomimetic porous scaffolds for bone tissue engineering. *Mater. Sci. Eng. R Reports* **80**, 1–36 (2014).
- 38. Chen, G., Dong, C., Yang, L. & Lv, Y. 3D Sca ff olds with Di ff erent Sti ff ness but the Same Microstructure for Bone Tissue Engineering. (2015). doi:10.1021/acsami.5b02662

- 39. Raimondi, M. T. *et al.* Three-dimensional structural niches engineered via two-photon laser polymerization promote stem cell homing. *Acta Biomater*. **9**, 4579–4584 (2013).
- 40. Maggi, A., Allen, J., Desai, T. & Greer, J. R. Osteogenic cell functionality on 3dimensional nano-scaffolds with varying stiffness.
- 41. O'Brien, F. J., Harley, B. A., Yannas, I. V. & Gibson, L. J. The effect of pore size on cell adhesion in collagen-GAG scaffolds. *Biomaterials* **26**, 433–441 (2005).
- 42. Harley, B. A. C. *et al.* Microarchitecture of three-dimensional scaffolds influences cell migration behavior via junction interactions. *Biophys. J.* **95**, 4013–4024 (2008).
- 43. Freyman, T. M., Yannas, I. V. & Gibson, L. J. Cellular materials as porous scaffolds for tissue engineering. *Prog. Mater. Sci.* **46**, 273–282 (2001).
- 44. Gibson, L. J. & Ashby, M. F. Cellular solids: Structure and properties. Materials Science and Engineering: A 123, (1990).
- 45. Deshpande, V. S., Ashby, M. F. & Fleck, N. A. Foam topology: Bending versus stretching dominated architectures. *Acta Mater*. **49**, 1035–1040 (2001).
- 46. Montemayor, L. C. & Greer, J. R. Mechanical Response of Hollow Metallic Nanolattices: Combining Structural and Material Size Effects. *J. Appl. Mech.* **82**, 1–10 (2015).
- 47. Meza, L. R. *et al.* Reexamining the Mechanical Property Space of Three-Dimensional Lattice Architectures. 1–20
- 48. Deshpande, V. S., Fleck, N. A. & Ashby, M. F. Effective properties of the octet-truss lattice material. *J. Mech. Phys. Solids* **49**, 1747–1769 (2001).
- 49. Meza, L. R. & Greer, J. R. Mechanical characterization of hollow ceramic nanolattices. *J. Mater. Sci.* **49**, 2496–2508 (2014).
- 50. Gibson, L. J. Biomechanics of cellular solids. J. Biomech. 38, 377–399 (2005).
- 51. Fratzl, P. & Weinkamer, R. Nature's hierarchical materials. *Progress in Materials Science* **52**, 1263–1334 (2007).
- 52. Meyers, M. A., McKittrick, J. & Chen, P.-Y. Structural Biological Materials: Critical Mechanics-Materials Connections. *Science* (80-.). **339**, 773–779 (2013).
- 53. Aitken, Z. H., Luo, S., Reynolds, S. N., Thaulow, C. & Greer, J. R. Microstructure provides insights into evolutionary design and resilience of Coscinodiscus sp. frustule. *Proc. Natl. Acad. Sci.* **113**, 2017–2022 (2016).
- 54. Weaver, J. C. *et al.* Hierarchical assembly of the siliceous skeletal lattice of the hexactinellid sponge Euplectella aspergillum. *J. Struct. Biol.* **158**, 93–106 (2007).
- 55. Harvey, E. J., Henderson, J. E. & Vengallatore, S. T. Nanotechnology and bone healing. *J. Orthop. Trauma* **24 Suppl 1,** S25–30 (2010).
- 56. Dou, N. G. & Minnich, A. J. Heat conduction in multifunctional nanotrusses studied using Boltzmann transport equation. *Appl. Phys. Lett.* **108**, (2016).
- 57. Ajdari, A., Nayeb-Hashemi, H. & Vaziri, A. Dynamic crushing and energy absorption of regular, irregular and functionally graded cellular structures. *Int. J. Solids Struct.* **48**, 506–516 (2011).
- 58. Gong, L., Kyriakides, S. & Jang, W. Y. Compressive response of open-cell foams. Part I: Morphology and elastic properties. *Int. J. Solids Struct.* **42**, 1355–1379 (2005).
- 59. Jacobsen, A. J., Barvosa-Carter, W. & Nutt, S. Micro-scale truss structures formed from self-propagating photopolymer waveguides. *Adv. Mater.* **19**, 3892–3896 (2007).
- 60. Bertsch, A., Lorenz, H. & Renaud, P. 3D microfabrication by combining microstereolithography and thick resist UV lithography. *Sensors Actuators, A Phys.* 73,

14-23 (1999).

- 61. Sharp, D. N. *et al.* Photonic crystals for the visible spectrum by holographic lithography. *Opt. Quantum Electron.* **34**, 3–12 (2002).
- 62. Hing, K. a. Bone repair in the twenty-first century: biology, chemistry or engineering? *Philos. Trans. A. Math. Phys. Eng. Sci.* **362**, 2821–50 (2004).
- 63. Nag, S. & Banerjee, R. Fundamentals of Medical Implant Materials. *ASM Handbook, Mater. Med. Devices* 23, 6–17 (2012).
- 64. Lund, A. W., Yener, B., Stegemann, J. P. & Plopper, G. E. The natural and engineered 3D microenvironment as a regulatory cue during stem cell fate determination. *Tissue Eng. Part B. Rev.* **15**, 371–380 (2009).
- Crotti, S. *et al.* Extracellular Matrix and Colorectal Cancer: How Surrounding Microenvironment Affects Cancer Cell Behavior? *Journal of Cellular Physiology* 232, 967–975 (2017).
- 66. Milo, R. & Phillips, R. Cell Biology By the Numbers. Current opinion in plant biology (2014). doi:10.1016/j.pbi.2009.10.009
- 67. Rho, J. Y., Tsui, T. Y. & Pharr, G. M. Elastic properties of human cortical and trabecular lamellar bone measured by nanoindentation. *Biomaterials* **18**, 1325–1330 (1997).
- 68. Sullivan, R. M., Ghosn, L. J. & Lerch, B. A. A general tetrakaidecahedron model for open-celled foams. *Int. J. Solids Struct.* **45**, 1754–1765 (2008).
- 69. Pellegrino, S. & Calladine, C. R. Matrix analysis of statically and kinematically indeterminate frameworks. *Int. J. Solids Struct.* **22**, 409–428 (1986).
- Ashby, M. F. The properties of foams and lattices. *Philos. Trans. A. Math. Phys. Eng. Sci.* 364, 15–30 (2006).
- 71. Papka, S. D. & Kyriakides, S. In-plane compressive response and crushing of honeycomb. *J. Mech. Phys. Solids* **42**, 1499–1532 (1994).
- 72. Manoharan, R., Wang, Y. & Feld, M. S. Histochemical analysis of biological tissues using Raman spectroscopy. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **52**, 215–249 (1996).
- 73. Draper, E. R. *et al.* Novel assessment of bone using time-resolved transcutaneous Raman spectroscopy. *J.Bone Miner.Res.* **20**, 1968–1972 (2005).
- 74. Carden, A. & Morris, M. D. Application of vibrational spectroscopy to the study of mineralized tissues (review). *J. Biomed. Opt.* **5**, 259–268 (2000).
- 75. Stewart, S. *et al.* Trends in early mineralization of murine calvarial osteoblastic cultures: A Raman microscopic study. *J. Raman Spectrosc.* **33**, 536–543 (2002).
- 76. Zhu, H. X., Knott, J. F. & Mills, N. J. Analysis of the elastic properties of open-cell foams with tetrakaidecahedral cells. *J. Mech. Phys. Solids* **45**, 319–343 (1997).
- 77. Marquez, J. P., Elson, E. L. & Genin, G. M. Whole cell mechanics of contractile fibroblasts: relations between effective cellular and extracellular matrix moduli. *Philos. Trans. R. Soc. A Math. Phys. Eng. Sci.* **368**, 635–654 (2010).
- 78. Chen, C. S. et al. Geometric control of cell life and death. Science 276, 1425–8 (1997).
- 79. He, L. H., Standard, O. C., Huang, T. T. Y., Latella, B. A. & Swain, M. V. Mechanical behaviour of porous hydroxyapatite. *Acta Biomater*. **4**, 577–586 (2008).
- 80. Suchanek, W. & Yoshimura, M. Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants. *J. Mater. Res.* **13**, 94–117 (1998).

- 81. Hing, K. A. Bioceramic bone graft substitutes: Influence of porosity and chemistry. *International Journal of Applied Ceramic Technology* **2**, 184–199 (2005).
- 82. Lan Levengood, S. K. *et al.* The effect of BMP-2 on micro- and macroscale osteointegration of biphasic calcium phosphate scaffolds with multiscale porosity. *Acta Biomater.* **6**, 3283–3291 (2010).
- 83. Lan Levengood, S. K. *et al.* Multiscale osteointegration as a new paradigm for the design of calcium phosphate scaffolds for bone regeneration. *Biomaterials* **31**, 3552–3563 (2010).
- 84. Montemayor, L. C., Meza, L. R. & Greer, J. R. Design and fabrication of hollow rigid nanolattices via two-photon lithography. *Adv. Eng. Mater.* **16**, 184–189 (2014).
- 85. Zhang, X. *et al.* Functionalized mesoporous bioactive glass scaffolds for enhanced bone tissue regeneration. *Sci. Rep.* **6**, 19361 (2016).
- 86. Owen, S. C. & Shoichet, M. S. Review Design of three-dimensional biomimetic scaffolds. 1321–1331 (2010). doi:10.1002/jbm.a.32834
- 87. Wang, H. *et al.* Biocompatibility and osteogenesis of biomimetic nanohydroxyapatite/polyamide composite scaffolds for bone tissue engineering. *Biomaterials* 28, 3338–3348 (2007).
- Li, B., Liu, X., Cao, C. & Ding, C. Biocompatibility and antibacterial activity of plasma sprayed titania coating grafting collagen and gentamicin. *J. Biomed. Mater. Res. Part A* 83, 923–930 (2007).
- 89. Banhart, J. & Baumeister, J. Deformation characteristics of metal foams. *J. Mater. Sci.* **33**, 1431–1440 (1998).
- 90. Ramamurty, U. & Paul, A. Variability in mechanical properties of a metal foam. *Acta Mater*. **52**, 869–876 (2004).
- 91. Verma, D., Katti, K. S. & Katti, D. R. Osteoblast adhesion, proliferation and growth on polyelectrolyte complex-hydroxyapatite nanocomposites. *Philos. Trans. A. Math. Phys. Eng. Sci.* **368**, 2083–97 (2010).
- 92. Jensen, S. S., Pinholt, E. M., Melsen, F. & Ruyter, E. Tissue Reaction and Material Characteristics of Four Bone Substitutes. *Int. J. Oral Maxillofac. Implants* **11**, 55–66 (1996).
- 93. Kourkoumelis, N., Balatsoukas, I. & Tzaphlidou, M. Ca/P concentration ratio at different sites of normal and osteoporotic rabbit bones evaluated by Auger and energy dispersive X-ray spectroscopy. J. Biol. Phys. **38**, 279–291 (2012).
- 94. Posner, A. S. The mineral of bone. *Clin Orthop* 87–99 (1985). at http://www.biomednet.com/db/medline/86054209>
- 95. Van Wazer, J. R. & Callis, C. F. Metal Complexing by Phosphates. *Chem. Rev.* 58, 1011–1046 (1958).
- Pemberton, J. E., Latifzadeh, L., Fletcher, J. P. & Risbud, S. H. Raman Spectroscopy of Calcium Phosphate Glasses with Varying CaO Modifier Concentrations. *Chem. Mater.* 3, 195–200 (1991).
- 97. Bertoluzza. This work concerns the structural characterization of biomaterials, their prosthetic use and the correlation with their biocompatibility. The structural method used is Raman spectroscopy which, being a non destructive and non invasive technique, is capabl. **1403**, 40–49 (1990).
- 98. Crane, N. J., Popescu, V., Morris, M. D., Steenhuis, P. & Ignelzi, M. A. Raman

spectroscopic evidence for octacalcium phosphate and other transient mineral species deposited during intramembranous mineralization. *Bone* **39**, 434–442 (2006).

- 99. Vacanti, C. A. & Vacanti, J. P. Bone and cartilage reconstruction with tissue engineering approaches. *Otolaryngol. Clin. North Am.* 27, 263–276 (1994).
- 100. Deshpande, V. S., McMeeking, R. M. & Evans, A. G. A model for the contractility of the cytoskeleton including the effects of stress-fibre formation and dissociation. *Proc. R. Soc. A Math. Phys. Eng. Sci.* **463**, 787–815 (2007).
- Khang, D., Sato, M., Price, R. L., Ribbe, A. E. & Webster, T. J. Selective adhesion and mineral deposition by osteoblasts on carbon nanofiber patterns. *Int. J. Nanomedicine* 1, 65–72 (2006).
- 102. Garellick, G. *et al.* Arthroplasty Future Clinical and Economic Impact of Revision Total Hip and Knee Future Clinical and Economic Impact of Revision Total Hip and Knee Arthroplasty. *J Bone Jt. Surg Am* **89**, 144–151 (2007).
- 103. Kurtz, S. M. *et al.* Future Clinical and Economic Impact of Revision Total Hip and Knee Arthroplasty. *J Bone Jt. Surg Am* **89**, 144–151 (2007).
- Huiskes, R., Ruimerman, R., van Lenthe, G. H. & Janssen, J. D. Effects of mechanical forces on maintenance and adaptation of form in trabecular bone. *Nature* 405, 704–706 (2000).
- 105. Pedersen, J. A. & Swartz, M. A. Mechanobiology in the third dimension. *Ann. Biomed. Eng.* **33**, 1469–1490 (2005).
- 106. Cui, Y. *et al.* The nanocomposite scaffold of poly(lactide-co-glycolide) and hydroxyapatite surface-grafted with l-lactic acid oligomer for bone repair. *Acta Biomater*. 5, 2680–2692 (2009).
- Fu, Q., Saiz, E., Rahaman, M. N. & Tomsia, A. P. Bioactive glass scaffolds for bone tissue engineering: State of the art and future perspectives. *Mater. Sci. Eng. C* 31, 1245– 1256 (2011).
- 108. Jaafar, I. H. *et al.* Improving fluorescence imaging of biological cells on biomedical polymers. *Acta Biomater*. **7**, 1588–1598 (2011).
- 109. Wang, N., Butler, J. P. P. & Ingber, D. E. E. Mechanotransduction across the cell surface and through the cytoskeleton. *Science* **260**, 1124–1127 (1993).
- 110. Rodriguez, M. L., McGarry, P. J. & Sniadecki, N. J. Review on cell mechanics: Experimental and modeling approaches. *Appl. Mech. Rev.* **65**, 060801 (2013).
- 111. McGarry, J. G. & Prendergast, P. J. A three-dimensional finite element model of an adherent eukaryotic cell. *Eur. Cell. Mater.* **7**, 27–33; discussion 33–34 (2004).
- 112. He, S., Su, Y., Ji, B. & Gao, H. Some basic questions on mechanosensing in cell-substrate interaction. *J. Mech. Phys. Solids* **70**, 116–135 (2014).
- 113. De Santis, G. *et al.* How can cells sense the elasticity of a substrate? an analysis using a cell tensegrity model. *Eur. Cells Mater.* **22**, 202–213 (2011).
- 114. Gefen, A. Cellular and biomolecular mechanics and mechanobiology. Computer Methods in Biomechanics and Biomedical Engineering (2012). doi:10.1080/10255842.2012.705081
- 115. Li, F., Redick, S. D., Erickson, H. P. & Moy, V. T. Force measurements of the alpha5beta1 integrin-fibronectin interaction. *Biophys. J.* 84, 1252–1262 (2003).
- 116. Cavalcanti-Adam, E. A. *et al.* Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. *Biophys. J.* **92**, 2964–74 (2007).

- 117. Meza, L. R., Das, S. & Greer, J. R. Strong, lightweight, and recoverable three-dimensional ceramic nanolattices. (2014). doi:DOI 10.1126/science.1255908
- 118. Bauer, J., Hengsbach, S., Tesari, I., Schwaiger, R. & Kraft, O. High-strength cellular ceramic composites with 3D microarchitecture. *Proc. Natl. Acad. Sci. U. S. A.* 111, 2453– 8 (2014).
- 119. Meza, L. R. *et al.* Resilient 3D hierarchical architected metamaterials. *Proc. Natl. Acad. Sci. U. S. A.* **112,** 11502–7 (2015).
- 120. Ozdemir, T., Xu, L.-C., Siedlecki, C. & Brown, J. L. Substrate curvature sensing through Myosin IIa upregulates early osteogenesis. *Integr. Biol. (Camb).* **5**, 1407–16 (2013).
- 121. Mrksich, M., Huang, S., Whitesides, G. M. & Ingber, D. E. Geometric control of cell life and death. **276**, (1997).
- 122. Yeung, T. *et al.* Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskeleton* **60**, 24–34 (2005).
- Cukierman, E. Taking Cell-Matrix Adhesions to the Third Dimension. *Science* (80-.).
 294, 1708–1712 (2001).
- 124. Owen, T. A. *et al.* Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.* 143, 420–430 (1990).
- 125. Gilbert SF. Developmental Biology. Osteogenesis: The Development of Bones. *Dev. Biol.* 6th Ed. http://www.ncbi.nlm.nih.gov/books/NBK10056/ (2000). doi:10: 0-87893-243-7
- 126. Mansour, J. M. Biomechanics of Cartilage. *Kinesiol. Mech. pathomechanics Hum. Mov.* 66–79 (2009). doi:10.1002/art.23548
- 127. Nagaraja, M. & Jo, H. The Role of Mechanical Stimulation in Recovery of Bone Loss High versus Low Magnitude and Frequency of Force. *Life* **4**, 117–130 (2014).
- 128. Johnell, O. & Kanis, J. A. An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. *Osteoporos. Int.* **17**, 1726–1733 (2006).
- 129. Helgason, B. *et al.* Mathematical relationships between bone density and mechanical properties: A literature review. *Clin. Biomech.* **23**, 135–146 (2008).
- 130. Melton, L. J., Atkinson, E. J., O'Connor, M. K., O'Fallon, W. M. & Riggs, B. L. Bone density and fracture risk in men. *J. Bone Miner. Res.* **13**, 1915–1923 (1998).
- 131. Rubin, C. T. & Lanyon, L. E. Regulation of bone mass by mechanical strain magnitude. *Calcif. Tissue Int.* **37**, 411–417 (1985).
- Rubin, C., Xu, G. & Judex, S. The anabolic activity of bone tissue, suppressed by disuse, is normalized by brief exposure to extremely low-magnitude mechanical stimuli. *FASEB J.* 15, 2225–9 (2001).
- 133. NASA. NASA's Journey to Mars, Pioneering Next Steps in Space Exploration. NASA's journey to Mars 1–36 (2015). at https://www.nasa.gov/sites/default/files/atoms/files/journey-to-mars-next-steps-20151008_508.pdf>
- Crawford, I. A. Dispelling the myth of robotic efficiency : why human space exploration will tell us more about the Solar System than will robotic exploration alone . *Astron. Geophys.* 53, 1–12 (2012).
- 135. Krølner, B. & Toft, B. Vertebral bone loss: an unheeded side effect of therapeutic bed rest. *Clin. Sci. (Lond).* **64,** 537–40 (1983).

- 136. Nagaraja, M. & Jo, H. The Role of Mechanical Stimulation in Recovery of Bone Loss High versus Low Magnitude and Frequency of Force. *Life* **4**, 117–130 (2014).
- 137. Shackelford, L. C. Resistance exercise as a countermeasure to disuse-induced bone loss. *J. Appl. Physiol.* **97**, 119–129 (2004).
- 138. Turner, C. H., Forwood, M. R., Rho, J.???Y & Yoshikawa, T. Mechanical loading thresholds for lamellar and woven bone formation. *J. Bone Miner*. *Res.* **9**, 87–97 (1994).
- 139. Dahl, A. C. E. & Thompson, M. S. in *Comprehensive Biotechnology, Second Edition* 5, 217–236 (2011).
- 140. Kanis, J. A. *et al.* A new approach to the development of assessment guidelines for osteoporosis. *Osteoporosis International* **13**, 527–536 (2002).
- 141. Ebeling, P. R. New drugs for osteoporosis. Aust. Prescr. 34, 176–181 (2011).
- 142. Sibonga, J. D. Spaceflight-induced bone loss: Is there an Osteoporosis Risk? *Curr. Osteoporos. Rep.* **11**, 92–98 (2013).
- 143. Turner, C. H. Three rules for bone adaptation to mechanical stimuli. *Bone* **23**, 399–407 (1998).
- 144. Rubin, C. T. & Lanyon, L. E. Dynamic strain similarity in vertebrates; an alternative to allometric limb bone scaling. *J. Theor. Biol.* **107**, 321–327 (1984).
- 145. Cheng, B. *et al.* Energetics: An emerging frontier in cellular mechanosensing. *Phys. Life Rev.* **1**, 6–11 (2017).
- 146. Cheng, B. *et al.* Cellular mechanosensing of the biophysical microenvironment: A review of mathematical models of biophysical regulation of cell responses. *Physics of Life Reviews* (2017). doi:10.1016/j.plrev.2017.06.016
- 147. Baker, B. M. & Chen, C. S. Deconstructing the third dimension how 3D culture microenvironments alter cellular cues. *J. Cell Sci.* **125**, 3015–3024 (2012).
- 148. Toben, D. *et al.* Fracture healing is accelerated in the absence of the adaptive immune system. *J. Bone Miner. Res.* **26**, 113–124 (2011).
- Wolff, J. Das Gesetz der Transformation der Knochen. Dtsch. Medizinische Wochenschrift 19, 1222–1224 (1893).
- 150. Carter, D. R. Mechanical loading histories and cortical bone remodeling. *Calcif. Tissue Int.* **36 Suppl 1,** S19–S24 (1984).
- Du, P., Cheng, C., Lu, H. & Zhang, X. Investigation of cellular contraction forces in the frequency domain using a PDMS micropillar-based force transducer. J. *Microelectromechanical Syst.* 22, 44–53 (2013).
- 152. Nowatzki, P. J., Franck, C., Maskarinec, S. A., Ravichandran, G. & Tirrell, D. A. Mechanically tunable thin films of photosensitive artificial proteins: Preparation and characterization by nanoindentation. *Macromolecules* **41**, 1839–1845 (2008).
- Pathak, A., Deshpande, V. S., McMeeking, R. M. & Evans, A. G. The simulation of stress fibre and focal adhesion development in cells on patterned substrates. *J. R. Soc. Interface* 5, 507–524 (2008).
- 154. Kumar, S. *et al.* Viscoelastic retraction of single living stress fibers and its impact on cell shape, cytoskeletal organization, and extracellular matrix mechanics. *Biophys. J.* **90**, 3762–73 (2006).
- 155. Maggi, A., Allen, J., Desai, T. & Greer, J. R. Osteogenic cell functionality on 3dimensional nano-scaffolds with varying stiffness. *Extrem. Mech. Lett.* **13**, 1–9 (2017).