Ovalbumin-Based Scaffolds Reinforced with Cellulose Nanocrystals for Bone Tissue Engineering

Benjamin P. Glaesemann

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Abby R. Whittington, Chair
Alex O. Aning
Sean G. Corcoran
Maren Roman

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ABSTRACT

In the field of tissue engineering, a major area of study is developing bone scaffolds that will provide support for osteoblasts. Despite many advances in recent years there is still a significant need for new bio-based 3-D porous scaffolds that possess sufficient initial mechanical properties to prevent immediate failure upon implantation. Ovalbumin (OVA), a glycoprotein from chicken egg whites, has been used to fabricate biodegradable, porous hydrogel bone scaffolds that promote osteoblast attachment and proliferation.

Although ovalbumin scaffolds encourage bioactivity and are naturally resorbed into the body after bone regeneration, they are also very fragile. Extremely stiff cellulose nanocrystals (CNCs), derived from wood pulp, can be utilized to reinforce these scaffolds while improving biocompatibility. When chemically modified to incorporate surface amine groups, cellulose nanocrystals become capable of covalently crosslinking with the OVA matrix for improved mechanical resilience.

Three concentrations (2, 5, 10 wt. %) of CNCs were incorporated and crosslinked to form nanocomposite scaffolds then were compared to pure OVA scaffolds. After fabrication, pore size morphology was compared between each CNC loading using SEM. The images revealed that the 10 wt. % CNC concentration doubled the pore compared to pure OVA scaffolds. Under high magnification, the CNCs were incorporated into the pore walls, providing a contoured surface. AFM was applied to analyze the topography of OVA with CNCs present. The surfaces laden with CNCs had a higher mean surface roughness, but were insufficient to impact cell behavior.

Compression testing was carried out on both Instron and DMA machines to demonstrate any reinforcing effect provided by the CNCs. While the compressive modulus remained constant, the elastic limit and strain increased with CNC loading, indicating a change in the resilience of the reinforced scaffolds. With a MTT Assay, it was shown that MC3T3-E1 preosteoblasts significantly increase in metabolic activity on 2 wt. % films and scaffolds, an indication of proliferation. All scaffolds had a net increase in metabolic activity suggesting overall biocompatibility for OVA scaffolds and those incorporating CNCs. Overall, the 5 wt. % scaffolds had the highest mechanical strength and had a positive cell response.
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List of Abbreviations

ALP Alkaline Phosphatase
AFM Atomic Force Microscopy
BG Bioglass
CNT Carbon Nanotube
DI Deionized
DIOX Dioxane
DMA Dynamic Mechanical Analysis
DMC Dimethylcarbonate
DSC Differential Scanning Calorimetry
DTT Dithiothreitol
ECM Extra cellular matrix
FBS Fetal Bovine Serum
FESEM Field-Emission Scanning Electron Microscope
FTIR Fourier Transform Infrared Spectroscopy
g Gram
GA Glutaraldehyde
GDL Glucono-delta-lactone
GPa Gigapascals
HA Hydroxyapatite
HPMC Hydroxypropyl methyl cellulose
hr Hour
kN Kilonewton
KPa Kilopascals
kV Kilovolt
MCC Microcrystalline Cellulose
MEM Minimum Essential Media
mg Milligram
mm Millimeter
MPa Megapascals
MWCNT Multi-walled Carbon Nanotube
NF Nanofibrous
OCN Osteocalcin
OVA Ovalbumin
PA Polyamide
PBS Phosphate Buffered Saline
PEG Polyethylene Glycol
PCL Polycaprolactone
PLA Polylactic acid
PLLA Poly-L-lactic acid
PLGA Poly(lactic-co-glycolic acid)
PVA Polyvinyl alcohol
SEM Scanning Electron Microscope
SWCNT Single Walled Carbon Nanotube
Tg Glass Transition Temperature
μm Micrometer
wt Weight
1. Introduction and Literature Review

1.1 Material Solutions for Bone Tissue Engineering

In an effort to improve the quality of life by enhancing, restoring, and maintaining tissue and organ function, the field of tissue engineering has been formed in order to study how to combine bioactive materials and tissue cells for a self-healing solution. A scaffold is one of the key components in the tissue engineering paradigm, providing a number of essential functions at once to create a template for new tissue growth [1]. Scaffolds are applied as three dimensional space-fillers within a critical gap in human tissue, thus providing temporary structural support while also serving as a possible delivery vehicle of bioactive molecules. A critical size gap forms when trauma to the bone causes significant tissue loss or when tissue separation is such that the body’s physiological response alone is no longer able to heal the injury site. In these cases, the separation between healthy cells is an obstacle for the deposition of new bone required for healing to occur and function to be restored [2, 3].

The scaffold’s structure organizes the cells and provides the proper environment for the formation of the desired tissue. One of the benefits of these scaffolds is their many variations, where the material can be selected to best accommodate the physical, mass transport, and biological design variables that accompany any tissue regeneration circumstance [4]. Moreover, current medical solutions for the healing of critically-damaged bone involve either an autograft or allograft where the critical gap is filled with bone from donor tissue. These grafting procedures are incredibly painful and have risks associated with them as well, potentially triggering an immune response, inflammation [5], and disease transmission. With polymeric biodegradable bone scaffolds, there is a reduction in cost, pain, and risk of disease from donated tissue, while avoiding a second operation to remove the implant after healing.
Hydrogels are a popular material category for scaffolds, since they are structurally similar to the extracellular matrix and are considered biocompatible. They also can be fabricated with relative ease and in mild conditions to form a three dimensional, swollen environment for cells to encourage new bone or other tissue growth [4, 6]. Although they are often prepared for bone scaffolds, hydrogels can also be utilized as materials for drug and growth factor delivery applications as well as for connective tissue [4, 7].

Furthermore, hydrogels fall under two primary categories which must be considered during material selection processes: synthetic and natural. The advantages for synthetic hydrogels over natural ones range from the ability for photopolymerization, adjustable mechanical properties, and manageable control of scaffold architecture and chemical compositions [6]. Naturally occurring hydrogels are also of interest as they have macromolecular properties more similar to the natural extracellular matrix (ECM) than synthetic hydrogels. For example, collagens are the main protein of mammalian tissue ECM and comprise 25% of the total protein mass of most mammals. Other naturally occurring hydrogels such as chitosan and alginate have also been shown to interact favorably in vivo and thus have been utilized as hydrogel scaffold materials for tissue engineering [4].

Also, most hydrogels occur naturally as degradable materials. Chitosan, for instance, has been found to be structurally similar to naturally-forming glycosaminoglycans, and can be broken down by enzymes in the body [4, 7, 8]. Biodegradability is pivotal for many tissue engineering applications because it allows for complete healthy tissue replacement in the body without a lingering interface between regenerated tissue and the implanted structure. In many current bone scaffold implantation procedures, high-strength materials like titanium are used as permanent fixtures to support the skeletal structure. In these situations, an interface exists...
between the new bone formed and the scaffold with mismatching mechanical properties. Stress shielding can occur as the body relies on the implanted material for support, rather than the native tissue, leading to its deterioration. The interface can become a potential site for recurring failure, regardless of the scaffolds’ ability to rival or exceed the strength of natural bone.

An ideal scaffold for bone tissue regeneration should possess mechanical properties similar to the bone tissue being replaced, good biocompatibility with surrounding tissue, large porosity and pore size, high pore interconnectivity for bone tissue ingrowth, and biodegradability such that it is gradually replaced by new healthy tissue [1]. Despite the success of hydrogels as an osteoconductive material in vivo, they display inadequate mechanical properties necessary as a load bearing, highly porous bone scaffold. Often, their moduli may fall between several hundred KPa to 1-10 MPa, which does not approach the 14.8 and 20.7 GPa moduli of trabecular and cortical bone, respectively [9].

One such modification to improve their fragile condition would be the addition of nano or micro size filler materials, such as a nanofiber, particle, or crystal to reinforce the structure. The strategy in adding a nanofiller is to hinder dislocation or polymer chain movement through interactions that may take place at the atomic level. Nanoparticles have very high surface-to-volume ratios allowing for their high interfacial energy to impact polymer matrix deformation. Classical composite theory predicts that improved bonding between the polymer matrix and the reinforcing phase leads to improved mechanical properties [10]. Moreover, these fillers are typically capable of being functionalized to enhance their reinforcing effect. For instance, carbon nanotubes can be functionalized with a 4-tert-butylphenylene group for better dispersion through steric stabilization [11].
It is widely known that polymeric materials do not offer sufficient mechanical integrity for the stresses and strains of the body’s skeletal system in a load bearing application. There are other applications such as soft tissue engineering or the regeneration of maxillofacial defects that would benefit from polymer implants. While synthetic polymers are easily tuned to exhibit the necessary properties for a given application, natural polymers do provide a more biocompatible template for osteoblast viability, and are less likely to induce inflammation. For this research, the goal was to improve the mechanical properties of natural, biodegradable ovalbumin based bone scaffolds using cellulose nanocrystals.

1.2 Ovalbumin

Many natural polymers are selected with a biomimetic approach. When considering candidate materials for bone tissue regeneration, the chicken egg has great potential. The chicken egg shell’s matrix proteins are similar to bone’s most predominant protein collagen. Albumen serves an analogous purpose to calcium phosphate precipitation on collagen, allowing for eggshell calcium carbonate formation. Albumen, like collagen, also plays significant role in precipitating calcium phosphate for mineralization [12]. This indicates that both proteins provide similar functions in their respective hosts. Moreover, 54% of egg albumen by weight is a globular phosphoglycoprotein from egg white called ovalbumin (OVA) [12]. The amino acid sequence of hen egg white ovalbumin comprises of 385 amino acids, sharing a large portion of them with human serum albumin [12]. Assuming human serum albumin can be isolated from blood, creating a scaffold from ovalbumin provides not just mineralizing capability, but a working model for a scaffold that could be fabricated from an individual’s own proteins.
In addition to ovalbumin’s genetic likeness to human serum albumin, it should possess a structure suitable for the cellular adhesion necessary to seed osteoblasts for tissue regeneration. Half of the ovalbumin amino acids residues are hydrophobic. These residues may be exposed by denaturation of the protein, which may occur by thermal treatment or exposure to an air-water interface [12]. Denaturation of the OVA can occur easily during fabrication due to water interactions, agitation, and chemical crosslinking thus exposing hydrophobic residues. As protein adsorption is critical for a biomaterial to be considered bioactive by mediating cellular interactions, these hydrophobic surfaces have demonstrated a strong tendency to adsorb proteins irreversibly [13]. The driving force for protein adsorption is most likely the unfolding of the protein on the surface accompanied by release of many hydrophobically structured water molecules from the interface, providing a large entropy gain for the system [14]. The interaction of adhesion receptors with adhesion proteins creates a major cellular recognition system on the biomaterial. The target environment for a new biomaterial for tissue engineering is the human body, containing fluids rich in adhesion proteins and growth factors. Such proteins as fibronectin, laminin, and entactin would permit the attachment to and movement of cells on the scaffold [15].

1.3 Nanoparticle Reinforced Synthetic Polymer Scaffolds

Naturally and synthetically derived biodegradable polymers are widely used for fabrication of porous scaffolds in load bearing applications. It has become an abundant dilemma that such porous scaffolds, often hydrogels, do not possess the necessary physical properties as a space-filler in vivo. Polymer matrix reinforcement is an established approach, utilizing carbon nanotubes (CNTs) to enhance the mechanical properties. CNTs high aspect ratio, van der waals
interactions, and ability to mimic the architecture of the ECM make it a viable option for a scaffold nanofiller material [16]. It has been shown in other studies that reinforcement of polymers with CNTs enhances the mechanical properties and leads to improvements in composite strength [17].

A review of CNTs from the Wake Forest Institute of Regenerative Medicine has discussed the potential for nanotubes in scaffolds, given the weakness of traditionally used materials such as PLGA or PLA, for tissue engineering [18]. One advantage of CNTs is their ability to be functionalized, allowing better dispersion properties in the scaffold. For example, multi-walled carbon nanotubes (MWCNTs) blended with chitosan presented a significant improvement in mechanical properties compared with those of pure chitosan [18]. Nanocomposites composed of 2% MWCNT more than doubled the Young’s modulus and tensile strength compared to neat chitosan [18]. Drawbacks include their non-degradability and a potential to become cytotoxic [18, 19].

Many studies have pointed out inflammatory responses in the lungs and the formation of granuloma which is a small, rounded (from 0.5 mm to 2.0 mm) structure consisting of immune cells such as macrophages. Granulomas indicate tissue injury and are indications of many diseases such as tuberculosis, histoplasmosis, and cryptococcosis. Granulomas in the lungs may also lead to lung cancer [19]. In one such study, the pulmonary toxicity of nanotubes in mice 7 and 90 days after intratracheal instillation was investigated [18]. One tenth mg of material showed no overt clinical signs, though over 50% of the mice died when exposed to 0.5 mg of material. However, over time the mice with intratracheal instillation of CNTs showed granulomas along with other lung lesions [18].
Furthermore, in another study, PVA/CNT hydrogel composites were prepared to evaluate the effect of the nanotubes on the PVA’s mechanical properties [20]. Carbon nanotube weight percents of 0.5, 1.0, and 2.0 were used to test for reinforcing effects. For CNT-0.5 specimen, its tensile modulus, tensile strength and strain at break are increased by 78.2%, 94.3% and 12.7% respectively compared to the control. Contrary to proportionate trends, with the increase in CNTs loading from 0.5 to 2.0%, the hydrogel composites actually saw a decrease in mechanical properties. The reason for this was determined to be related to the CNT’s dispersion and interfacial adhesion with the matrix [20]. It was also discussed PVA has the capability to form acrystalline coating around the nanotubes, maximizing interfacial stress transfer [20]. This may be one of the reasons why the mechanical properties of the hybrid hydrogels were better than the pure PVA hydrogels. With a low CNT weight percent, the interaction between the nanotubes was weaker than that of the PVA molecules and the nanotubes, so homogeneous dispersion in the PVA matrix was favored. With the higher weight percents (1.0 and 2.0 %), the CNTs aggregated, reducing the reinforcing effect [20].

Similarly, hydroxyapatite (HA) has been extensively investigated for years as a biomedical material. It possesses the same chemical composition as bone mineral and features favorable bioactive and biocompatible properties. It is osteoconductive and forms strong bonds to natural bone in vivo. Thus, it has become a common material selected for orthopedic applications [21, 22]. Nano-sized HA in particular may possess special properties given its small size and large specific surface area [23].

In a study by Kathopali et al., scaffolds consisting of PLA and nano HA were prepared using a solvent-casting/salt leeching technique [21]. Using ellipsoidal nanoparticles of HA of a 150 nm length and 25 nm width, loading was varied from 0 to 50 wt. %. The compression
modulus of the scaffolds increased from 4.72 to 9.87 MPa over this range, while the yield strength improved from 0.29 to 0.44 MPa. Also, it was seen that the porosity decreased with an increase in HA content, which may be an effect of the HA occupying the free space available in the pores. Differential scanning calorimetry (DSC) data indicated that the glass transition temperature (Tg) of the scaffolds also increased with HA filler content. This was theorized due to the adsorption of polymer chains onto HA particles, thereby restricting the movement of polymer chains \[21\]. Furthermore, it was hypothesized that there was a favorable interaction between the HA and the matrix due to ion-dipole interactions between the ester groups from the PLA and the calcium from the HA. These types of interactions are likely reasons for the increase of compression modulus with the increasing additions of nano HA. Although, the mechanical properties of the samples for both modulus and strength were still below 10 MPa \[21\], which was much less than the 14.8 GPa modulus of human trabecular bone \[9\].

Similarly, Jie and Yubao discovered similar results in regards to mechanical improvements with increasing nano HA content \[22\]. Biocomposites were fabricated using a co-solution/co-precipitation method to create scaffolds consisting of a polyamide (PA) matrix with needle-like nano HA crystals with a length of 70-90 nm and a diameter of 10-20 nm. At a high weight percent the nano HA/PA composite yielded good homogeneity and high bioactivity. Strong molecule interactions and chemical bindings were present between the nano HA and PA in the composite. From infrared spectroscopy, it could be deduced that nano HA maybe linked with PA66 by hydrogen bonding and by the formation of carboxyl–calcium–carboxyl linkage; supporting the notably high mechanical properties that were observed. Starting at a nano HA wt. % of around 39 and increasing to 65%, the compressive strength increased from 93 to 117 MPa, with similar increases in tensile strength, bending strength, and elastic modulus.
Particularly at higher HA weight percents, the composites showed excellent mechanical properties that almost rival that of natural bone making it a promising candidate for load bearing applications [22]. The primary disadvantage of this scaffold, despite its high strength, is, as a non-hydrogel, it has limited degradability.

In an effort to continue the quest to combine bioactivity with mechanical strength, researchers have taken an interest in bioglass as a nano-sized scaffold material. Compared with micron-sized bioactive ceramic particles, nano-sized glass particles have a large specific surface area and can form a tighter interface with the polymer matrix in composites. Introduction of nano-sized bioglass particles into polymeric materials can not only endow polymer scaffolds with biomineralization capability, but also increase the stiffness of polymer material [24]. Generally, bioactive glasses are able to promote a strong mechanical bond to bone through a bone-like apatite layer on the surface of the glass, enhancing the mechanical properties and bioactivity of the composite [24-27]. Moreover, if their formulation is properly designed, bioglasses can positively interact with soft tissues as well [25].

In a study by Fabbri P et al., highly porous biocompatible composites made of polycaprolactone and 45S5 Bioglass (BG) were prepared by a solid–liquid phase separation method [25]. The composites were obtained with BG weight contents varying from 0–50 wt.%, using either dimethylcarbonate (DMC) or dioxane (DIOX) as solvents. These composites maintained porosity values through the ranges of 88-92%, ideal for cell adhesion and proliferation. Compression tests were executed to compare the moduli and tensile strengths for the various bioglass contents. Over the course of 0-50 wt. % bioglass, the elastic moduli increased from 77 to 156 KPa with DMC as a solvent, and 191-251 KPa with DIOX. At 60%
strain, the compressive stresses increased from 82 to 98 KPa with DMC, and 153 to 214 KPa with DIOX [25].

Furthermore, the PCL–BG composites were immersed in simulated body fluid (SBF) and analyzed at certain time points. The evaluation of the mineralization tests suggested that the introduction of BG particles exerted an integral role in the development of apatite in SBF, since only the samples containing 50 wt. % of BG developed a stable layer of apatite after immersion in SBF for 4 weeks. The authors denoted that the scarce wettability of PCL may have hindered the SBF interaction with the glass particles, making the development of apatite difficult in short periods of immersion. However, high contents of glass and long immersion in SBF may have favored the contact and reaction of the BG particles with the SBF, inducing the mineralization of apatite [25].

Regarding biological analysis, the cell adhesion and proliferation tests performed highlighted a poor cell adhesion and proliferation, attributed to the high hydrophobic nature of the PCL matrix that does not allow for an optimal coating of cell adhesion cues. They suggested this phenomenon should be overcome by copolymerization of PCL with more hydrophilic hydroxyalcanoates or methods of chemical surface modification of the scaffolds [25]. Therefore, it was observed that the administration of bioglass as a nanofiller in polymers could yield not only improved mechanical strength, but also increased mineralization. Unfortunately, the cell studies proved unsuccessful as the osteoblasts did not proliferate or adhere well through the porous composite, although this was hypothesized to be polymer matrix, not the bioglass filler [25].

In a similar study, researchers fabricated PLLA/bioglass ceramic scaffolds with BG contents at 10, 20, and 30 wt. % using a thermally induced phase separation method with
dioxane as the solvent [24]. The composition of the bioglass ceramic in this case was SiO$_2$:CaO:P$_2$O$_5$ at a 55:40:5 molar ratio. The porosities for these composites hovered between 91 and 92% but decreased to 88% at a 30 wt. % BG content. Despite the PLLA based scaffolds having higher mechanical strength than in the previously mentioned study, the trends remained the same. The compressive modulus of the scaffolds increased from 5.5 to 8.0 MPa, while the compressive strength increased from 0.28 to 0.35 MPa as the BG content increased from 0 to 30 wt.% [24]. SEM revealed that the optimal mineralization in SBF was for 20 wt. % BG content, rather than for the higher weight percent. For the 10 wt. % sample, no bioactivity seemed to occur after 21 days, but for 20 wt. % BG content, the biomineralization ability of the scaffold was greatly enhanced. After 1 day of incubation, the apatite clusters covered almost the entire surface of the scaffolds, while for the 30 wt. % the apatite did not nucleate and grow over the entire surface. It was suspected this result was due to an overload of the BG, which may leave too much particle exposure on the surface, thus delaying the apatite growth [24].

Similarly, Barroca et al. used PLLA to create composites using a 3CaO–P$_2$O$_5$–MgO–SiO$_2$ bioglass system using the thermally induced phase separation technique [27]. In this paper, they studied the morphology and reasoned that bioglass acts as a nucleating catalyst agent of the PLLA matrix, promoting polymer crystallization. They also determined the glass solubility controls the pore size. A significant increase in the pore size was observed, up to 150 μm, as the bioglass content increased. This was a significant increase since the pore size of the plain PLLA scaffold after the same quench time was 33 μm. The pore size reflected the decrease in mechanical properties consistent with a more open structure. The elastic moduli decreased from 10.88 MPa at 30 wt. % BG to 6.61 MPa at 50 wt. %. The compressive strength also decreased, from 0.75 MPa at 30 wt. % to 0.62 at 50 wt. % [27].
In accordance with the findings of the prior paper discussed, apatite growth appeared to decrease once the highest BG content was reached. The samples were immersed in SBF for 1 and 14 days and analyzed using SEM images. At the highest tested BG concentration, in this case 50 wt. %, the inorganic matter precipitation on the surface was less evident than on the 30 wt. % sample. For ≥30 wt. % samples, only small agglomerations of inorganic could be seen. Meanwhile, at 30 wt. %, much of the surface was coated with what was determined to be a calcium phosphate formation. In this study, bioglass proved essential in mineralization. Pure PLLA showed a smooth, homogeneous surface even after soaking for 14 days, while for all BG contents the PLLA–BG composites showed deposition of a surface layer after immersion in SBF solution [27].

1.4 Nanoparticle Reinforced Natural Polymer Scaffolds

So far, the polymer matrices discussed have been synthetic. However, many synthetic polymers demonstrate insufficient cell adhesion, often due to a hydrophobic surface that could hinder cell proliferation throughout a three-dimensional architecture. They also require functional groups for further surface modifications to improve biocompatibility [28]. Naturally occurring polymers have also been researched thoroughly, since they are already tailored to interact in a biological fashion. Natural polymers often possess highly organized structures and may contain extracellular ligands which are necessary to bind with cell receptors and are able to guide cells to grow at various stages of development [29]. Still, they tend to be even more fragile than their synthetic counterparts, requiring significant mechanical enhancement before their biocompatibility advantages are of use in a load bearing scaffold application.
Polysaccharides and proteins are typically the natural polymers used for bone tissue engineering. For polysaccharides, alginate and chitosan are popular selections, or some combination of the two. They are often reinforced with hydroxyapatite in the same fashion as a synthetic polymer based scaffold [30]. Alginate, a polysaccharide from seaweed, is widely known for its abundance, low price, low toxicity and biocompatibility [30]. It is often used in drug delivery systems, and while it undergoes gelation in simple conditions, the gelation kinetics, crosslinking, and structural properties are difficult to precisely control [4, 30]. Therefore, uniformity is a difficult quality to achieve when fabricating an alginate hydrogel [30].

Still, progress has been made fabricating uniform alginate hydrogels. Control over the material properties of a hydrogel is crucial for many biomedical applications. Structural uniformity of tissue engineering scaffolds is necessary not only for uniform cell distribution, but also for consistently well-controlled material properties. In an alginate gelation study by Kuo and Ma, the hydrogels were formulated with controlled structure, gelation rate, and mechanical properties for tissue engineering applications [30]. The gelation was initiated by introducing calcium ions and glucono-delta-lactone (GDL) or water with the alginate. They found that for high calcium contents, the gelation rate was faster, yielding heterogeneous gels. For high gelation rates, with CaCO$_4$-2H$_2$O, there was less time to form a uniform gel than using a slower process with CaCO$_3$-GDL. For higher polymer contents, the viscosity increased which hindered polymer chain movement and its ability to crosslink. The slower gelation rate of the CaCO$_3$-GDL system provided time for CaCO$_3$ particles to evenly disperse throughout the suspension during mixing before complete gelation occurred. These uniform gels were produced with leveled surfaces and superior mechanical properties. Further characterization of mechanical properties of the alginate hydrogels made with CaCO$_3$-GDL showed that the compressive
modulus and strength increased with calcium content, presumably due to increased crosslinking density [30].

Although these alginate scaffolds can be tailored to have a level, uniform structure with enhanced mechanical properties, they are still too fragile to be used as a load-bearing bone scaffold. Frequently, researchers will fabricate more intricate composites containing multiple natural polymers with a nanoparticle filler. Chitosan and alginate are often used in tandem to form hybrid scaffolds for bone tissue engineering. Since chitosan is cationic, opposed to the negatively charged alginate, an attraction formed between the two species promoting mechanical stability in the scaffold. Both pure materials are weak and unstable, but have hydrophilic surfaces, are very biocompatible, non-toxic, and are biodegradable under normal body conditions. In a study by Lia Z. et al., SEM showed that alginate-chitosan also managed to reproduce the highly interconnected porous network typical of a plain chitosan scaffold [31]. Both plain and composite scaffolds were shown to be highly porous with a pore size around 100–300 μm, a structure favorable for cell attachment and new bone tissue ingrowth. The porosities of pure chitosan and chitosan–alginate scaffolds in this study were determined to be 84.86% and 91.94%, respectively [31].

Compression tests of both chitosan and chitosan–alginate composite scaffolds were carried out to obtain the mechanical strength data. The compressive yield strength and Young’s modulus were determined to be 0.125 MPa and 2.56 MPa, respectively, for pure chitosan, and 0.467 MPa and 8.16 MPa, respectively, for chitosan–alginate. The threefold increase in Young’s modulus and yield strength for the chitosan–alginate scaffold relative to the chitosan scaffold was attributed to the strong ionic interactions between chitosan and alginate to form a chitosan–alginate complex [31]. Fourier Transform Infrared Spectroscopy (FTIR) revealed shifts of the
amide I and II bands to be replaced by one new band as a result of the interaction between residual carboxylic groups. The shifts in the amide groups suggested the formation of the chitosan–alginate complex as a result of the ionic interaction between the negatively charged carbonyl group (–COOH) of alginate and the positively charged amino group (–NH$_2$) of chitosan [31].

An in vivo cell study on the chitosan-alginate scaffold was conducted with histological evaluation [31]. Taken at 4 and 12 weeks, the histology showed that the scaffolds facilitated rapid vascularization and deposited connective tissue and calcified matrix within the entire scaffold structure [31]. These in vivo results demonstrate a strong aptitude for this scaffold among its natural polymer counterparts for new tissue growth, and while the mechanical properties are much improved from a single component natural polymer scaffold, the matrix requires further reinforcement to become a viable template for bone tissue healing.

As found in synthetic polymer scaffold systems, natural polymers are also reinforced with nanoparticles such as nano HA. Given polysaccharides’ (such as chitosan) notoriously poor mechanical strength, it is crucial to improve its structural properties in order to be able to fully utilize its superior bioactivity. The ability of chitosan to support cell attachment and proliferation is attributed to its chemical properties. As mentioned prior, the backbone of chitosan is structurally similar to glycosaminoglycans, the major component of the extracellular matrix of bone and cartilage [4, 8, 32]. Other advantages of nano HA chitosan composite scaffolds for bone tissue engineering include the formation of highly porous scaffolds with interconnected pores as well as MC3T3-E1 preosteoblast attachment and spreading [8].

In a study by Thein-Hein W. et al., high and medium molecular weight chitosan scaffolds with 0.5, 1 and 2 wt. % fraction of nano HA were fabricated by freezing and lyophilization [8].
Despite the changes in nano HA content, the pore size was not affected and remained in the 50-120 μm range. After performing compression tests, they confirmed the expected increase in compressive modulus with the addition of nano HA. The compression modulus of hydrated chitosan scaffolds was increased on the addition of 1 wt. % nano HA from 6.0 to 9.2 KPa in the high molecular weight scaffold [8]. They also found higher modulus values for the high molecular weight samples compared to the medium weight samples, likely due to an increase in polymer chain entanglement. After verifying improved mechanical properties, the authors checked for improved cell attachment and proliferation. The cell attachment percentage increased from 75% for chitosan scaffolds to 85% for the chitosan nano HA scaffolds after 4 hours. Also, they concluded from fluorescence microscopy that the proliferation of pre-osteoblasts on the nanocomposite scaffolds was significantly greater in relation to the chitosan scaffolds due a noticeably higher cell density after day 7. Cell growth increased by a factor of 10 within 2 weeks on nano HA/chitosan, and after 28 days there were only a few dead cells observed. The authors determined the addition of nano HA successfully improved both the structural integrity of chitosan and its pre-osteoblast biocompatibility [8].

The other segment of natural biopolymers used for hydrogel bone scaffolds are proteins, such as collagen or gelatin. Collagen is the most widely-used tissue-derived natural polymer, and it is a main component of ECM and the most abundant protein in bone [7, 33]. Gelatin is a natural material derived from collagen by hydrolysis and has almost identical composition as that of collagen. Since gelatin is a denatured biopolymer, the selection of gelatin as a scaffolding material can circumvent the concerns of immunogenicity and pathogen transmission associated with collagen [33]. Both collagen and gelatin have limited mechanical strength; however, they can be crosslinked with glutaraldehyde to improve their physical properties. Collagen meets
many of the biological design parameters for tissue regeneration, as it is composed of specific combinations of amino acid sequences that are recognized by cells and degraded by enzymes excreted from the cells [7, 34].

In one study, researchers were able to overcome collagen’s weak mechanical properties and create a porous biodegradable nanocomposite bone scaffold that chemically, structurally and mechanically mimics natural trabecular bone [35]. The authors of the study extracted collagen fibers from rat skin and added synthetically produced apatite nanocrystals to form a foam-like scaffold by freeze drying the frozen slurry. SEM confirmed the microstructure matches that of an actual trabecular bone complete with both nanopores and macropores. Compression tests were performed on the crosslinked scaffolds over a range of a 0 to 77 wt. % nanocrystalline apatite. The maximum performance of the composites was found at 67.5 wt. % nano apatite and 32.5 wt. % collagen with a compressive modulus at 37.3 MPa and a yield strength of 2.7 MPa. The maximum scaffold performance at this particular composition was found to best match with natural trabecular bone in terms of molecular structure, crystalline phase and crystallite size [35].

This synthetic imitation of the trabecular bone also proved to work well when the scaffolds were used to heal critical sized gaps in vivo [35]. The research team was able to perfectly heal a 5 mm gap in the femur of Wistar rats after 5 months. Without a scaffold in place, a non-union fracture in the femur was unable to heal. Furthermore, the scaffold effectively healed a 1x2 cm segmental defect in the tibia of Yorkshire-Landrace pigs. CT scans of the pig tibia showed bone densification at the defect site 3 months post implantation [35].

Researchers have also experimented with gelatin for a variety of tissue engineering applications, as it forms a gel readily with temperature, and as a derivative of collagen, maintains much of its bioactivity [33, 36]. Although the natural polymers discussed thus far were
fabricated with nano-sized apatite particles during the scaffold formation, apatite mineralizing on
the scaffold surface can also play a role in promoting mechanical strength and improved cell
differentiation. At Michigan University, researchers compared a commercial gelatin, a
nanofibrous (NF) gelatin, and a mineralized NF gelatin [33]. The NF gelatin was prepared using
a thermally induced phase separation technique, producing a matrix with fiber diameters
hovering around 150 nm and a porosity unchanged from normal gelatin. The concept was to
better represent the extracellular matrix while producing a higher surface area and subsequently
better mechanical strength. Compression tests proved this hypothesis, showing a ten-fold
increase in the compressive modulus from 80 KPa to 801 KPa for Gelfoam® and NF-gelatin
respectively. As anticipated, once the scaffolds were incubated in SBF, the modulus showed
significant improvement, jumping from 801 KPa to around 1.4 MPa after 7 days. The apatite
formation not only strengthened the scaffold but enhanced osteogenic differentiation. The
expression of bone sialoprotein (BSP) and osteocalcin (OCN) in the osteoblast constructs for the
gelatin-apatite composite was about 5 times and 2 times higher than in the NF-gelatin constructs
4 weeks after cell culture [33]. These biomimetic scaffolds showed encouraging traits for the
mineralization of scaffolds and providing a higher surface area polymer matrix.

In some cases, researchers combine polysaccharides and proteins to develop hydrogel
bone scaffolds. Blending the advantages of polymers like chitosan’s biocompatibility with
gelatin’s cell attachment capabilities has had success [32, 37]. These blended structures may
also be reinforced with nanoparticles to promote mechanical strength and enhance the already
biologically advantageous properties. For instance, the addition of nano-sized bioglass not only
bolsters the compressive modulus but aids in protein adsorption, while nano HA encouraged cell
attachment for chitosan-gelatin systems [32, 37].
1.5 Cellulose Nanocrystals in Tissue Engineering

Currently, numerous efforts are focused on the use of materials from renewable resources as reinforcement agents in nanocomposites. Cellulose nanocrystals have attracted a great deal of interest in the nanocomposites field due to their appealing intrinsic properties such as high surface area, unique morphology, low density, and high mechanical strength. In addition, they are easily chemically modified, readily available, renewable, and in some cases biodegradable. They are formed by acid hydrolysis, where the crystalline region resistant to the acid remains and the disordered portion of the cellulose is hydrolyzed [38, 39]. The resulting dimensions of the crystals varied between 3-5 nm in diameter and 100-300 nm in length, if the cellulose is from wood [40]. Cellulose nanocrystals, given their nanoscale dimensions and ability to interact with the continuous phase, make them ideal candidates to improve the mechanical properties of a scaffold material [38, 39]. The theoretical value of the Young’s modulus for a perfect cellulose crystal has been estimated to be 167.5 GPa, theoretically stronger than steel and similar to Kevlar [38].

In a paper by Peresin M. et al., cellulose nanocrystals (CNCs) were used to reinforce nanofibers in composite mats produced via electrospinning of poly vinyl alcohol with two different concentrations of acetyl groups [39]. The nanocrystals had a maximum diameter of 290 nm for each load investigated, 0-15 wt. % in this experiment. Although the electrospinning increased the crystallinity of the scaffolds fibers, the cellulose nanocrystal additions led to a reduction in crystallinity. Still, the PVA fibers were mechanically weak without some particle reinforcement, and cellulose nanocrystals demonstrated strong interactions with the PVA matrix via hydrogen bonding. Support for this explanation was provided using FTIR, by the band
observed between 3550 and 3200 cm\(^{-1}\). This is characteristic of the stretching O-H from the intermolecular and intramolecular hydrogen bonds. The shape of this band was substantially different when comparing the neat and the CNC filled PVA [39]. For mechanical analysis, to substantiate the effect of the hydrogen bonding, DMA was used in tensile mode. The storage modulus values were charted for increasing CNC content, showing a steady increase in the modulus from 0 to 15% loading. At 0%, the storage modulus was 15.45 MPa and rose steadily in even increments for each measure value to 57.30 MPa at 15% CNC loading. A decrease in the crystallinity would be expected to induce a reduction in the storage modulus. It was concluded that the observed strength enhancement in CNC-loaded PVA mats can only be related to the reinforcing effect of the dispersed phase, via the percolation network held by hydrogen bonds [39].

In a Journal of Agricultural and Food Chemistry article, three sizes of microcrystalline cellulose (MCC) nano-particles were incorporated into hydroxypropyl methyl cellulose (HPMC) thin films at different concentrations [41]. The diameters of the nanoparticles ranged from 32 to 48 nm, while the concentrations were analyzed for tensile strength at 3:0.08, 3:0.4, and 3:0.8 HPMC:MCC ratios. At 3:0.8 the tensile strength increased by 53% from the control samples at 35.6 MPa to 54.5 MPa [41]. Although tensile properties are not as relevant for bone scaffolds, the increase in tensile strength does demonstrate a reinforced structure due to the addition of the cellulose nanoparticles, regardless of particle size in this case. With SEM, it was seen that the nanoparticles were able to generate a rougher surface on the film [41]. While the objective of this study was not to analyze its potential for cell attachment, the rougher surface does provide insight as to the surface characteristics on a given polymer for tissue engineering applications.
On the other hand, the hydrophobicity of the HPMC/MCC nanocomposite may suggest the potential for cell adhesion difficulties on polymers reinforced with MCC.

Much remains to be characterized for any composite consisting of CNCs but it has strong potential for a number of systems given its non-toxicity, slow but eventual degradation, and its ability to be functionalized. Under Dr. Maren Roman at Virginia Tech, generic cellulose nanocrystals were labeled with FITC for bioimaging applications. In this reaction scheme, the surface of the nanocrystals was decorated with epoxy functional groups via reaction with epichlorohydrin in sodium hydroxide. Following dialysis, the epoxy ring was opened with ammonium hydroxide to introduce primary amino groups [42]. The next step would yield the final FITC label, but stopping the process short provides a functionalized nanocrystal surface with amine groups. In theory, this allows for the nanocrystals to not only hinder dislocation movement via van der waals interactions but also crosslink to the polymer matrix, further improving mechanical properties of the scaffold.

1.6 Ovalbumin/Cellulose Nanocrystal Composites

For the bone scaffold system described in this research, the aforementioned aminated cellulose nanocrystals are being used to reinforce a biodegradable ovalbumin scaffold, as they can chemically bind to the ovalbumin matrix for a greater mechanical strength in compression. Ovalbumin is a glycoprotein found in chicken egg whites where of its 385 amino acids, 10% of the amino acid sequence matches that of human serum albumin [43]. Compared with other natural polymers, it is cheaper and more abundant, while mechanically, ovalbumin scaffolds are extremely fragile. They have shown an average yield strength of 0.025 MPa and an elastic modulus of 0.06 MPa [43].
Still, early cell studies showed a substantial increase in cell number after 4 and 96 hours and from alkaline phosphatase (ALP) and osteocalcin (OCN) data it was concluded that cells positively respond to ovalbumin scaffolds resulting in pre-osteoblast differentiation into mature osteoblasts with the beginning of bone matrix formation [43]. Ovalbumin may yet prove to be a great material option for tissue engineering due to its biocompatibility, but its poor mechanical strength remains a critical issue. Creating a nanocomposite with the high strength cellulose nanocrystals may provide the needed structural effects to allow ovalbumin scaffolds to be resilient enough to be handled by surgeons. If the mechanical properties increase substantially, this nano-composite may become capable of being sutured in place for bone regeneration.

Ovalbumin (OVA) offers a strong potential for biocompatibility and approval for medical use by the FDA, as it is derived from a food item. So far, OVA has not been sufficiently explored as a scaffold material when compared to other natural polymers such as collagen, chitosan, or alginate. Reinforcement with cellulose nanocrystals offers an opportunity to blend the biocompatibility of a naturally occurring material, ovalbumin, with the high modulus and stiffness of nontoxic, nanosized cellulose crystals. Although many researchers have been thus far hard-pressed to reach mechanical properties to rival that of bone, it is still advantageous to optimize the mechanical properties of polymer scaffolds. Scaffolds create a hospitable environment for cell growth such that they are resilient enough to be handled and sutured by surgeons. A successful project would involve improving the mechanical strength drastically, discovering the optimal cellulose nanocrystal loading, maintaining scaffold biocompatibility, and ideally improving cell adhesion and proliferation by creating a more contoured scaffold surface.
2. Materials and Methods

2.1 List of Materials

ATCC - MC3T3-E1 sub-clone 4 presosteoblasts
Biomedical Technologies Inc. - Mouse osteocalcin kit
Biotron - Alkaline phosphatase
Benchmark - Fetal bovine serum - triple 0.1 µm sterile filtered
Dubeccos - Phosphate buffered saline 1X
Fisher Chemical - Sodium hydroxide
Gibco - Antibiotic-Antimycotic (100X)
Gibco - PBS pH 7.4
Gibco - MEM α-medium
Gibco - 0.5% trypsin - EDTA
Ricca Chemical Company - Borate buffer, pH 9.5
Riedel-de haen - L(+) ascorbic acid
Sigma Aldrich - DL-dithiothreitol, minimum 99% titration
Sigma Aldrich - Glutaraldehyde solution, grade II, 25%
Sigma Aldrich - Glycine non animal source
Sigma Aldrich - Triton X-100
Sigma Aldrich - Trizma hydrochloride reagent grade
Sigma Aldrich - β-glycerophosphate disodium salt hydrate
Sigma Aldrich - In vitro toxicology assay kit, MTT based
Sigma Life Science - Albumin from chicken egg white, grade II

2.2 OVA solution

To start, 0.01 g of DL-Dithiothreitol (DTT) and 30 mL of borate buffer (pH 9.5) was mixed with 50 mL of deionized (DI) water. Five grams of grade II albumin from chicken egg white (ovalbumin, OVA) was gradually added to the mixture over a period of 30 minutes, while being stirred at 400 rpm. The solution was stirred for 4-5 hr, yielding a 6.25 wt. % solution. The solution was funneled into snake skin dialysis tubing for 3 days, with the water changed twice daily to complete the dialysis procedure. A pH of 8.9 was measured for the final dialyzed solution. The solution was stored in a refrigerator before use. The ovalbumin has an isoelectric point of 4.6 [12].
2.3 Aminated Cellulose Nanocrystal Suspension

Cellulose nanocrystals (CNCs) were prepared from milled (60-mesh) dissolving grade softwood sulfite pulp (Temalfa 93A-A from Tembec, Inc.) as described by Beck-Candanedo et al. using 64 wt% sulfuric acid (10 mL/g cellulose), a temperature of 45 °C, and a hydrolysis time of 60 min [42].

The surface of the nanocrystals was decorated with epoxy functional groups via reaction with epichlorohydrin (6 mmol/g cellulose) in 1 M sodium hydroxide (132 mL/g cellulose) according to the method by Porath and Fornstedt [44]. After 2 hr at 60 °C, the reaction mixture was dialyzed (Spectra/Por 4 dialysis tubing) against DI water until the pH was below 12. Next, the epoxy ring was opened to introduce primary amino groups by adjusting the pH to 12 with 50% w/v sodium hydroxide, then adding ammonium hydroxide (29.4%, 5 mL/g cellulose) followed by heating to 60 °C for 2 hr. The reaction mixture was dialyzed until the pH was 7 [42]. The final concentration of the CNC-NH2 suspension was 2.18% with 1 FITC moiety per 27 nm.$^2$. Figure 1 demonstrates the progression of treatments with resultant functional groups.

![FITC tagging process for CNCs with the 3rd step representing the aminated form used in this study [42].](image)

2.4 Scaffold Fabrication

Using a salt leaching and freeze drying technique [45], an interconnected pore structure was created to provide a suitable habitat for cellular attachment, proliferation, and differentiation.
In a 12 well plate, 1 g of >150 μm NaCl was placed in each well. Two and a half mL of ovalbumin solution was pipetted into the desired number of wells. The 2.18% CNC-NH₂ suspension was weighed and 0.46, 1.03, and 1.77 g to generate 2, 5, and 10 weight % scaffold compositions respectively. The CNC suspension was combined with the NaCl and OVA solution and then sonicated for 5-10 seconds for homogeneity. The OVA/CNC crosslinking scheme is shown in Figure 2. From previous work [43], 250 μL of 25% grade II glutaraldehyde solution (GA) was added for a 10% GA/OVA ratio. The well plate was placed on a shaker at room temperature for approximately 20 hr. The scaffolds were then cut using a 6 mm punch and rinsed in a glycine solution (3.75g/500 mL water) to remove excess GA for 1 hr at 37 °C. Afterwards, the scaffolds were added to DI water for at least 2 days with the water changed twice a day at room temperature to allow for salt leaching. After rinsing, the scaffolds are frozen at -80 °C and then lyophilized for 2 days resulting in a dry, porous, 3-D scaffold.

![Figure 2: Proposed GA crosslinking mechanism between CNC’s amine groups and OVA’s lysine groups.](image-url)
2.5 Thin Film Fabrication:

2.5.1 Films for Cell Studies, SEM, AFM

For pure OVA films, the OVA solution was pipetted into a well plate without salt, along with 10% v/v GA and then stirred. The films (n=3) were then placed on a shaker for 24 hours to crosslink. One mL of the glycine solution heated to 37 °C was poured into each well to remove the excess GA for duration of 1 hr. The films were then left to air dry overnight.

2.5.2 Films for Contact Angle Measurements

Batches were prepared utilizing 500 μL of OVA solution, 50 μL of GA, with additions of 16.8, 43.4, and 91.7 μL for each CNC loading. Fifty μL of each batch was pipetted carefully onto small microscope slides and spread with the pipette tip to cover the slide surface. The films were left to dry under a chemical fume hood overnight.

2.6 Scaffold Characterization

2.6.1 Scanning Electron Microscopy (SEM)

Scaffold cross-sectional area morphologies were viewed using a Leo (Zeiss) 1550 field emission scanning electron microscope (FESEM). Samples were sliced with a razor blade to reveal an internal cross-section. The scaffolds were sputter-coated with a 15 μm thick conductive gold-palladium coating under vacuum in an argon atmosphere. Pure OVA, 2, 5, and 10 wt. % 3-D samples were evaluated for pore size and any apparent interconnectivity at 5 kV.
2.6.2 Compression Testing

The 3-D scaffolds were studied for mechanical behavior via compression testing performed with an Instron 5869 machine using a 10 kN maximum load cell. The tests were conducted on samples with approximately 6 mm diameters and 6 mm heights at a speed of 0.6 mm/min (10% strain/min). Five samples were tested per CNC loading (0, 2, 5, 10 wt. %) in both the dry and wet state. Wet scaffolds were pre-soaked for 2 hr in phosphate buffered saline (PBS) to simulate in vivo conditions. Furthermore, the actual compression tests for the wet scaffolds took place in a cylindrical apparatus filled with PBS (pH 7.4), maintained at 37 °C to replicate mechanical behavior in the human body.

Stress-Strain curves were generated with Blue Hill™ software after each test, whereupon the slope of the linear portion of each curve was used to calculate the Young’s Modulus values. Elastic limit and strain values were also extrapolated from each graph at the onset of plastic deformation. To better understand the composites yield behavior, elastic limit ($\sigma_y$) and strain ($\varepsilon_y$) values were tasked to find resilience ($U$).

$$U=1/2*\varepsilon_y*\sigma_y$$  \[1\]

For an enhanced understanding of scaffolds under compressive forces, a Q800 Dynamic Mechanical Analysis Instrument by TA tested both wet and dry scaffolds using a force ramp, rather than the strain ramp as seen in Instron compression testing. Dry samples (n=3) endured a 0.7 N/min force ramp up with a 15 N threshold and wet samples (n=3) endured a 0.3 N/min force ramp to a 10 N threshold. Compressive moduli, elastic limits, and yield strains were calculated from the stress-strain curves generated.
2.6.3 Swelling Behavior

3-D scaffolds 6 mm in diameter and height were weighed and then submerged in PBS. At time points of 1, 2, 4, and 24 hr, the 3 scaffolds of each composition were quickly rolled on a paper towel to remove unabsorbed PBS and weighed on a scale. Comparing wet weight to dry weight, percent absorbance could be calculated and plotted for each time point to compare swelling for each CNC loading.

\[
\% \text{ Absorption} = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}} \times 100
\]  

[2]

2.7 Thin Film Characterization

2.7.1 Contact Angle

Due to the hydrophilic character of CNCs [38], additions of nanocrystalline cellulose may alter the water contact angle of an OVA hydrogel. The advancing contact angle was measured by dripping DI water onto the film surface. Meanwhile, images were captured frame by frame using an APDR B/W camera. Images were analyzed with FTA Video 2.0 where the droplet contact angle was measured 3 frames before each droplet edge expansion. The capturing speed was 10 frames per second. Three droplets were measured for contact angle per film (n=3) per composition.

2.7.2 Atomic Force Microscopy (AFM)

Living cells can change their morphology, such as spreading, in response to surfaces and other stimuli [15]. Cellular investigations have shown generating rougher surfaces in films generates better cell adhesion, growth and viability [56]. Atomic force microscopy was used to provide information on ovalbumin surfaces and reveal any changes in topography coincident
with CNC additions. Small fragments of dried thin films were adhered to double stick tape and placed in the atomic force microscope. Images were captured over a 1 µm scan size with the AFM in tapping mode in order to avoid disruption from surface artifacts. Mean surface roughness values were obtained using Nanoscope™ 6.1 software to investigate the effects of CNC loading on surface roughness. Three images were captured and processed for surface roughness per thin film composition.

2.8 In Vitro Cell Studies

In order to determine the effect of CNCs on cell response, two in vitro cell studies were performed. In each study, MC3T3-E1 pre-osteoblast cells were seeded and cultured in expansion medium containing α-minimum essential medium (MEM) plus 10% v/v fetal bovine serum (FBS) and 1% v/v antibiotic/antiomycotic. Media was changed and cells were split every 72 hr.

2.8.1 Cell Viability – MTT Assay

For a scaffold to be considered biocompatible, evidence for cell viability, rather than cytotoxicity must be established. MTT Assays are performed to measure a change in the metabolic activity of viable cells between two time points, and therefore indicate any toxic effect provided by the substrate. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide or MTT can be dissolved in a balanced salt solution such as PBS. The MTT in solution will be broken down into water insoluble purple formazan crystals in the presence of the mitochondrial dehydrogenases produced by viable cells [47]. The formazan crystals are then dissolved by acidified anhydrous isopropanol and spectrophotometrically measured. The cell number can be related to the amount of formazan formed based on a standard curve, and thus indicates the degree of cytotoxicity of a scaffold material.
Scaffolds and thin films were placed or formed in a 48 well plate in triplicate. The plate was then sterilized using an ethylene oxide (EtOH) plasma treatment. The scaffolds were then presoaked in α-MEM for 30 minutes. MC3T3-E1 pre-osteoblasts were seeded onto the surface of each scaffold composition (10,000 cells/scaffold) in triplicate and cultured in 700 µL α-MEM for 4 and 96 hr. The samples were incubated for 2 hr with 10% media volume of the MTT reconstituted in PBS. The media and MTT was removed and replaced with 700 µL of 0.1 N HCl in anhydrous isopropanol. Well plates were placed on a shaker for 30 min to allow the formazan crystals time and agitation to dissolve. The absorbance of this solution was spectrophotometrically measured at 570 nm, with the background absorbance subtracted at 690 nm. To correlate the absorbance with cell number, a calibration curve was generated by seeding $10^3$, $10^4$, $10^5$ cells in duplicate in well plates, then measuring the absorbance once the MTT assay procedure was completed as before. The cell count for each sample could then be calculated from the measured absorbance values.

2.8.2 Cell Differentiation – OCN Assay

Cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. Osteoblasts express various phenotypic markers and synthesize collagenous and noncollagenous bone matrix proteins such as osteocalcin (OCN) [48]. OCN is a protein secreted by osteoblasts and is known as a late marker of osteoblastic differentiation and mineralization in the bone formation process [49]. Pre-osteoblast MC3T3-E1 cells can be seeded onto scaffolds, incubated for set time intervals and analyzed for OCN by an enzyme-linked immunosorbant assay. If the presence of OCN is detected, there is some level of osteoblast differentiation occurring.
To induce osteoblastic differentiation, cells were seeded and cultured on three scaffolds for 1, 2, and 3 week periods. MC3T3-E1 pre-osteoblast cells were cultured on scaffolds containing varying CNC amounts in a 48-well plate containing 0.5 mL expansion medium supplemented with ascorbic acid (50 mg/mL) and β-glycerol phosphate (10 mmol/L) to enhance osteoblast differentiation. At the 1 week time period, cells were seeded on scaffold and incubated in non-osteogenic media as a control. OCN levels were measured each week using an enzyme-linked immunosorbant assay (ELISA) kit (Biotechnologies, Inc.) according to the manufacturer’s directions.

2.9 Statistical Analysis

Data collected for each experiment was analyzed and evaluated using One Way ANOVA for determining statistical significance between scaffold compositions. Tukey’s multiple comparison statistics were also generated simultaneously for circumstances involving more complex trends. Statistically significant determinations required p values of < 0.05 to fall within a 95% confidence interval.

3. Results

3.1 Scaffold Design and Fabrication

Three-dimensional porous scaffolds initially were designed to incorporate unmodified cellulose nanocrystals. By a simple stirring and sonicating method, it was difficult to maintain homogeneity during the crosslinking phase. A CNC rich layer was observed on the top of the scaffold, evident by a change in color and texture following lyophilization. Given the swelling nature of hydrogel, unmodified CNCs may filter out of a scaffold in small portions during DI
water rinsing. Modifying the CNCs bound them to the matrix and prevented CNC loss during the rinsing phase in substantial amounts.

Once amine functionalized CNCs were chosen, scaffolds were cut out of each well of a 12 well plate using a cork borer. Figure 3 shows a scaffold as produced from a single well and a 6 mm x 6 mm cylinder as used for testing purposes. The newly developed scaffolds displayed a homogenous light tan coloration throughout the bulk of the sample and a more consistent structure. Also, for any scaffold composition, a darker orange crust would form from the interaction with air during crosslinking.

![Figure 3: Lyophilized 3-D composite scaffolds of OVA and 10 wt. % CNC at diameters of 2 cm and 6 mm.](image)

The thin films fabricated for contact angle measurement, by spreading the OVA solution on a microscope coverslip produced noticeable differences between surfaces with and without CNCs. Films without CNCs had clumps and aggregates of OVA and regions of different thickness. As the wt. % of nanocrystals increased, the films became homogeneous, with no visible artifacts and even coloration on the surface.

### 3.2 Scanning Electron Microscopy

Following fabrication, scaffolds were inspected with SEM to identify pore size, interconnectivity, and any trends created by CNC loading. SEM images, shown in Figure 4,
confirmed a porous morphology with pores ranging from 150 µm to 410 µm for pure OVA (A) scaffolds with a mean pore size of 229.85 µm after 6 measurements, sampling small, medium, and large size pores. 2 wt. % (B) scaffolds had a range of 180 to 460 µm with a mean pore size of 327.8 µm. 5 wt. % (C) scaffolds had a range of 165 to 420 µm with a mean pore size of 273 µm. 10 wt. % (D) scaffolds had a range of 290 to 690 µm with a mean pore size of 459.65 µm. The 10 wt. % scaffolds had approximately twice the average pore size of unmodified OVA scaffolds. Mean pore diameters are compared in Figure 5.

Figure 4: SEM micrographs of A) 0 wt. % (pure OVA), B) 2 wt. %, C) 5 wt. %, and C) 10 wt. % CNC loadings.
Figure 5: Mean pore diameters where N=6, * p < 0.05

Figure 6: SEM micrographs of a A) material surface with no nanocrystals present magnification and B) a surface containing nanocrystals with two exposed CNC portions measuring 146 and 186 nm
SEM images of OVA thin films with A) 10 wt. % CNC and B) 30 wt. % CNC

SEM of thin films confirmed the presence of CNCs in the crosslinked ovalbumin. Figure 6 reveals the clear difference in the surfaces with and without CNCs. Exposed portions of CNCs were measured to lengths of 146 and 186 nm. Figure 7 shows a visible change in the topography of the film, with a noticeable increase in the number CNCs scattered about the surface with increasing CNC concentration. The CNCs are randomly distributed and oriented, with portions embedded in the OVA.

3.3 Scaffold Swell Test

To evaluate how the scaffolds behave in liquid medium, they were soaked in PBS and measured for % absorption. This measurement demonstrates the hydrophilicity of each scaffold by how much PBS imbibed. There was a direct relationship found between the wt. % of CNCs added and the % absorption, with 10 wt. % having the highest absorption (Figure 8). Strangely, the 2 wt. % scaffold had a higher absorption than the 5 wt. % scaffold, but within the standard error. Regardless of any odd values, all scaffolds containing nanocrystals had higher % absorption than the pure OVA.
Figure 8: Percent adsorption of PBS for 0 ( ), 2 ( ), 5 ( ), and 10 ( ) wt. % scaffolds at time points of 1, 2, 4, and 24 hr. The * indicates the data is statistically different than the 0 and 5 wt. % scaffolds (p < 0.05).

3.4 Mechanical Testing

3.4.1 Compression (Instron)

Compression testing was carried out at strain rate of 10%/min until failure at room temperature. Pure OVA scaffolds were compared to OVA/CNC composite scaffolds with 2, 5, and 10 wt. % CNC loadings in terms of the compressive modulus and yield behavior. This data was gathered from force/displacement curves that were converted to stress/strain curves. The modulus was calculated from the linear portion of each curve, while the yield point was chosen at the first significant sign of pore collapse, represented by a stair-step drop in stress, or by a clear change in the slope. Figure 9 presents both pore collapse and a change in slope signifying the material has yielded during dry testing.
Table 1: Numerical dry compression testing results with calculated mean and standard error mean values for each scaffold composition.

<table>
<thead>
<tr>
<th>Variable</th>
<th>0 wt. %</th>
<th>2 wt. %</th>
<th>5 wt. %</th>
<th>10 wt. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain (mm/mm)</td>
<td>0.040</td>
<td>0.028</td>
<td>0.150</td>
<td>0.130</td>
</tr>
<tr>
<td>Stress (kPa)</td>
<td>79.7</td>
<td>25.4</td>
<td>82.8</td>
<td>97.3</td>
</tr>
<tr>
<td>Modulus (kPa)</td>
<td>915</td>
<td>1172</td>
<td>1439</td>
<td>1058</td>
</tr>
</tbody>
</table>

Table 1 lists the dry compression data averaged for five scaffolds per CNC concentration. The 5 wt. % scaffolds had the highest compressive moduli and yield strain values. The 10 wt. % scaffolds generated the highest elastic limits, and overall provided the most repeatable results, as displayed by lower standard error mean numbers. Pure OVA and 2 wt. % scaffolds demonstrated significantly weaker mechanical responses compared with 5 and 10 wt. % scaffolds. Table 2 lists the wet compression data and gathered and averaged for five scaffolds.
per CNC concentration. There were no significant differences in the majority of the wet data, as PBS absorption played an increased role as observed in the swell test (Figure 8).

Table 2: Numerical wet compression testing results with calculated mean and standard error mean values for each scaffold composition.

<table>
<thead>
<tr>
<th>Variable</th>
<th>0 wt. %</th>
<th>2 wt. %</th>
<th>5 wt. %</th>
<th>10 wt. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain (mm/mm)</td>
<td>Mean</td>
<td>SE Mean</td>
<td>Mean</td>
<td>SE Mean</td>
</tr>
<tr>
<td></td>
<td>0.091</td>
<td>0.013</td>
<td>0.095</td>
<td>0.011</td>
</tr>
<tr>
<td>Stress (kPa)</td>
<td>6.07</td>
<td>0.79</td>
<td>7.15</td>
<td>0.91</td>
</tr>
<tr>
<td>Modulus (kPa)</td>
<td>65.8</td>
<td>12.0</td>
<td>71.8</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Once a 10 wt. % CNC concentration was reached, the compressive modulus decreased. Also, the elastic limit did increase substantially between 0 and 5 wt. % in the wet state. Figure 10 clearly presents the as described relationships between 0, 2, 5, and 10 wt. % scaffolds for both dry and wet Instron compression testing.

Furthermore, CNC loading generated smoother stress-strain curves when tested dry. Comparing 0 wt. % scaffolds with 10 wt. % scaffolds, Figure 11, there is an evident decrease in pore collapse events typical in a brittle porous material. As shown in Table 3, the dry 5 and 10 wt. % scaffolds demonstrated improved resilience over unmodified scaffolds while the 5 wt. % scaffolds performed the best in the wet state. Improved resilience indicates better material performance in terms of elastic limit and yield strain.

Table 3: Resilience values calculated from scaffold yield behavior

<table>
<thead>
<tr>
<th>Dry Scaffold</th>
<th>Resilience (kPa)</th>
<th>Wet Scaffold</th>
<th>Resilience (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 wt. %</td>
<td>0.36</td>
<td>0 wt. %</td>
<td>0.28</td>
</tr>
<tr>
<td>2 wt. %</td>
<td>1.59</td>
<td>2 wt. %</td>
<td>0.34</td>
</tr>
<tr>
<td>5 wt. %</td>
<td>6.21</td>
<td>5 wt. %</td>
<td>0.57</td>
</tr>
<tr>
<td>10 wt. %</td>
<td>6.33</td>
<td>10 wt. %</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Figure 10: Instron compression testing results of pure OVA ( ), 2 ( ), 5 ( ), and 10 wt. % ( ) 3-D scaffolds in the dry (A-C) and wet (D-F) state. The charts compare compressive Young’s modulus, elastic limit, and yield strain values for a constant strain ramp. B) ## indicates that the 2 wt. % is significantly different (p < 0.05) than the rest. C) * indicates the 5 wt. % data is significantly different than 2 wt. % data, and ** represents a significant difference between the 10 wt. % data and pure OVA and 2 wt. % data. D) # indicates a significant difference between 5 and 10 wt. %. E) $ indicates a significant difference between the pure OVA and 5 wt. % data.
3.4.2 Dynamic Mechanical Analysis

To further characterize mechanical performance of OVA/CNC scaffolds, DMA was utilized to more easily define when the scaffolds fail. DMA used a similar set up as the Instron compression testing, although instead of a strain ramp, a force ramp was applied. This generated more sudden failure, with large changes in strain for a given force. The plots did not resemble curves, but rather a stair-step progression as shown in Figure 12. Yield points in each

Figure 11: Stress-Strain curves of the dry material reveal the effect of CNC loading on the brittleness of the material. The 10 wt. % scaffold plot is smooth compared to 0 wt. % scaffold plot.

Figure 12: Stress-Strain curve plotted during dry DMA testing. The plot displays a stair-step loading pattern.
scaffold were easy to identify with a force ramp method. Still, the data was largely similar to Instron compression, with large discrepancies within a sample population. From DMA, it became more apparent that the 5 wt. % scaffolds were the stiffest and strongest, demonstrating noticeably improved Young’s moduli and elastic limits. All scaffolds crosslinked with CNCs proved more resilient in terms of stress and strain than 0 wt. % unmodified scaffolds.

Figure 13: Dynamic Mechanical Analysis results of 0 (●), 2 (■), 5 (●), and 10 wt. % (▲) scaffolds. Scaffolds were compressed with a constant force ramp until failure. Compressive modulus, elastic limit, and yield strain values were determined for dry (A-C) samples along with the compressive modulus of wet (D) samples. For dry elastic limit and strain charts, * indicates 0 wt. % scaffolds are significantly different (p < 0.05) than the 2, 5 and 10 wt. % scaffolds.
Testing in the wet state did not show any trends. With the large amount of water/PBS absorbed in each scaffold, modulus values tend to coalesce. DMA results are shown in Figure 13 for visual comparison.

### 3.5 Contact Angle Measurements

The effect of CNCs on the hydrophilicity of the surface was determined by contact angle measurements of DI water droplets on thin films of the composite. The results, Figure 14, suggest that the CNCs form a slightly more hydrophobic surface when crosslinked with ovalbumin, despite being hydrophilic in nature. There was a statistically significant difference between the 5 wt. % scaffolds and the scaffolds without CNCs. Overall, CNCs did not appear to have a strong effect on the contact angles found on the film surfaces.

![Figure 14: Contact angle measurements for 0 (■), 2 (■), 5 (■), and 10 (■) wt. % scaffolds. The * indicates the 0 and 5 wt. % data is statistically different from one another (p < 0.05).](image)

### 3.6 Atomic Force Microscopy

AFM was utilized to provide information on the roughness of OVA/CNC thin film
Figure 15: A) mean roughness values gathered by AFM for 0 ( ), 2 ( ), 5 ( ), and 10 wt. % ( ) OVA/CNC thin films. B-E) each thin film surface (B: 0 wt. %, C: 2 wt. %, D: 5 wt. %, E: 10 wt. %) generated by AFM in tapping mode.

surfaces. It was hypothesized that the addition of CNCs would increase the surface roughness. A slight increase in mean roughness was seen with 2 and 5 wt. % films, with the highest Ra values for 10 wt. % films as seen in Figure 15. This likely indicates a higher surface concentration of nanocrystals.

3.7 In Vitro Cell Studies

3.7.1 Cell Viability - MTT Assay

Figure 16A shows an 11-fold increase in cell number on the 2 wt. % samples. Figure 16B shows a 6-fold increase for the 2 wt. % loading. For both the 2 wt. % films and scaffolds, the material killed some of the original cells seeded, indicated by the decrease in cell number before showing remarkable growth to day 4. All films and scaffolds were biocompatible and increased the cell count from the initial 10,000 cells seeded. The pure OVA without nanocrystals were the least viable.
Figure 16: MTT Assay for cell proliferation and viability assessed after 1 and 4 day incubation periods. A) symbol “a” indicates that the 0 and 2 wt. % films from day 1 are significantly different (p < 0.05). Symbol “b” indicates that the 2 and 5 wt. % films are significantly different. B) # indicates that the 5 wt. % scaffold from Day 1 is significantly different than the other Day 1 scaffolds. The ## indicates that the 10 wt. % scaffold from Day 1 is significantly different than the 2 wt. % scaffold. The * indicates that the 5 and 10 wt. % scaffolds from Day 4 are significantly different (p < 0.05).
3.7.2 Cell Differentiation – OCN Assay

The OCN Assay was performed to measure the amount of osteocalcin, a protein produced by bone-forming cells and a marker for differentiation. The density of osteocalcin for each sample was related to the absorbance values measured using a spectrophotometer. The absorbance values for all the samples matched the absorbance values of the blanks that contain no substance capable of OCN secretion. So, there was no osteocalcin produced by the MC3T3-E1 pre-osteoblasts and therefore no differentiation.

4. Discussion

4.1 Scaffold Design and Fabrication

With the addition of CNCs to OVA, visibly homogeneous scaffolds were fabricated once the glutaraldehyde amount was fixed at a 10% by volume ratio and the CNCs were amine-functionalized. Normally, a low density of CNCs in water produces a suspension of a perfectly uniform dispersion of the whiskers via electrostatic repulsions [38]. In various media, the surface charges of CNCs can be screened allowing for chiral interactions to take over leading to nematic alignment and close packing [38]. Thus, CNCs modified with an amine functional group were sought out to chemically crosslink with the ovalbumin matrix. The goal was to evenly distribute the CNCs throughout the entire fabrication process to avoid the nematic phase formation. Visual observations and general handling of scaffolds indicated that a more homogenous sample was produced once CNCs were crosslinked with the OVA.

Furthermore, given the charges present, CNCs may be capable of interrupting protein aggregates. The steric repulsion forces and other charge effects may disrupt the normal aggregation when CNCs are added to the OVA solution during fabrication. When the
OVA/CNC solution was crosslinked on microscope slides for the contact angle experiment, there was no visible inhomogeneity for the reinforced films, as opposed to a rough topography observed with the control films.

4.2 Scanning Electron Microscopy

Collecting images of the porous microstructure of OVA/CNC scaffolds revealed two important points. First, slight differences were observed between the pore size and CNC loading. There appeared to be a slight increase in the pore size for the 2 and 5 wt.% scaffolds, while the 10 wt.% loading doubled the mean pore size of the scaffolds.

Secondly, the pore sizes could be estimated and compared with literature findings in order to establish relevance as a scaffold to promote bone cell activity. It is well known that pore size does have an effect on the attachment, proliferation, and migration of cells. One group performed a study to accurately chart this effect using naturally derived collagen-glycosaminoglycan scaffolds for bone tissue regeneration. The basis was to unravel the claim of another group that hypothesized the critical range of pore size for optimal cellular activity and viability is 20-120 µm [50].

The other group eventually demonstrated that cell attachment decreased with increasing pore size [51]. Therefore, an experiment was devised using a pore size range of 85-325 µm. They were able to show that specific surface area plays a large role in the initial attachment of osteoblasts, so smaller pores around 120 µm had an initial peak in cell count. Once the cell proliferation phase commenced, the early peak disappeared. The scaffolds with the larger pores above 300 µm were able to overcome the initial cell adhesion disadvantage and ended with the highest cell count due to a much higher level of osteoblast infiltration. The scaffolds with a
mean pore size of 325 µm were deemed optimal for bone tissue engineering [51], although they did not experiment with higher pore sizes.

In another study, PLGA-CaP scaffolds with very large pores were assessed for cell activity and viability [52]. Scaffolds were prepared with pore diameters in the ranges 470-590, 590-850 and 850-1200 µm. Pore size did not impact cell growth or ALP activity for these ranges, but there was optimal bone formation and an increased number of blood vessels observed in scaffolds with pores of 470-590 µm after 4 and 8 weeks [52]. These two studies combined indicate that aiming for a 300-600 µm pore diameter range could be a good strategy when designing a material for bone tissue regeneration. The primary concern with higher pore sizes has been weakening and decreased mechanical performance.

After the OVA/CNC scaffolds were imaged with SEM, the pore diameters were measured in the 150 to 460 µm range for the 0-5 wt. % loadings. Therefore, the scaffolds for this project should be well suited for both the attachment and proliferation of osteoblasts infiltrating the scaffold. The 10 wt. % scaffolds had several pores that were almost 700 µm and a mean diameter double that of pure OVA scaffolds (Figure 5). This may be due to the hydrophilic effect of CNCs, allowing larger ice crystals to form during freezing. A majority still fell in the 300-600 µm, but the open, porous structure certainly had a negative effect on the mechanical properties. The 2 wt. % scaffolds had the second largest mean pore size, which may suggest they were better suited for cell proliferation than the 0 and 5 wt. % scaffold, as validated by the MTT assay. Moreover, SEM images all showed interconnected pores. Interconnected pore architectures play a major role in cell seeding efficiency [53] as cells can infiltrate through the porous network. Figures 4B and 4D have the most visible interconnected pores.
Viewing the image of the 2 wt. % scaffold in Figure 4, it was apparent that there was a larger deviation in the size of pores. The pore network was messier, without the clean, rounded pores of the other scaffold compositions. It appeared that the 2 wt. % concentration was inadequate dispersed, and therefore unevenly absorbed water, forming ice crystals of different dimensions depending on the scaffold region.

4.3 Scaffold Swell Test

The % absorption results gathered from the swell test (Figure 8) accurately reflected the assumption that higher amounts of CNCs incorporated in the OVA scaffolds would increase the swelling. As stated before, CNCs are very hydrophilic, so when combined with a hydrogel, a high level of swelling was anticipated. The 10 wt. % scaffolds took on 17 times its original weight in PBS as opposed to the pure OVA scaffolds, which absorbed 9 times its original weight. This enhanced absorption for CNC additions did impact testing, as any scaffolds that were presoaked, or immersed for compressive loading showed more compliance. Testing in the wet state reduced the Young’s modulus significantly, but it also may alter the material’s properties to a more pronounced degree as an absorption threshold is reached. The 10 wt. % scaffolds compression tested wet had the lowest modulus, which could be a result of the extreme degree of swelling observed with a high CNC content.

4.4 Mechanical Testing

4.4.1 Compression (Instron)

Based on the dry compression results, several effects from the addition of CNCs became evident. As stated before, the CNCs are highly charged particles and may break up protein aggregates, forming a more homogeneous material with fewer defects due to clumping. The
CNCs in proper quantity may also crosslink together to form a separate gel-like material distributed throughout the scaffold, plasticizing the material. Additionally, the compression testing of dry samples (Figure 10) has shown an improvement in the elastic limit and strain of the scaffold with increasing CNC additions, where the yield strain increases were the most significant. At 5 and 10 wt. % CNC compositions, the yield strain was much higher than it was for 0 and 2 wt. %.

Also, it was demonstrated that the Young’s compressive modulus did not change significantly between the various CNC loadings. Since CNC additions did not coincide with any change in modulus, the yield point data became the most relevant. With the elastic limit and yield strain, the resilience of the material could be calculated according to Equation 1. In general, resilience describes a material’s ability to perform elastically, absorbing energy and recovering after deformation occurs [54]. According to Table 3, both the dry and wet scaffolds had a marked increase in resilience at CNC loadings of 5 wt. %. Although the 10 wt. % scaffolds performed well in the dry state, the large swelling of the scaffolds during PBS immersion reduced its strength and resilience. More resilient materials could show more promise in a surgical environment where implantable materials need to be handled. General handling would likely deform the material, so the ability of the scaffold to maintain its initial shape and volume would be highly beneficial.

Similar to the irregular force-deformation curves involving micro-collapse of the porous structure, are the patterns displayed by crunchy foods, such as cereal and cheeseballs. In one study, two crunchy cereals were compressed and shown to display extremely jagged force-deformation relationships, comparable to the 0 and 2 wt. % scaffolds in this study (Figure 17). They were also tested in environments up to 85% humidity, which smoothed out the curves to
such an extent that they bore no resemblance to the dry tests [55]. These results reflected similar effects that PBS had on the scaffolds when compressed in the Instron machine.

For the wet compression testing, the numbers were greatly reduced from the dry scaffold results in terms of compressive modulus, elastic limit, and yield strain. The stress-strain curves also differed, showing no presence of brittle fracture with completely smooth plots. Due to the hydrophilic nature of the scaffolds, a large amount of PBS was absorbed by the scaffolds, as demonstrated in the scaffolds swell test.

![Figure 17: Comparison of experimental force deformation curves of cheeseballs stored at 23% relative humidity with a sample 2 wt. % CNC loaded OVA scaffold force-deformation curve.](image)

This water absorption played a role in the modulus and elastic limit values for the 10 wt. % scaffolds, Figure 10, which decreased substantially compared to the 5 wt. % scaffolds. This result differed greatly from the dry 10 wt. % scaffolds that performed second best compared to the 5 wt. % scaffolds during compression. Since 10 wt. % scaffolds had the highest amount of adsorption, as shown in the Swell Test, it became clear that fluid uptake strongly governs the mechanical properties of the scaffolds.

Wet compression test results supported this notion in terms of elastic limit and strain as well. The only significant difference during testing was the elastic limit of the 5 wt. % scaffolds.
The rest of the scaffolds had nearly identical elastic limit values, and all scaffolds showed no noticeable difference in yield strain. Likely, the PBS absorbed reached some critical value that nullified much of the mechanical advantage provided by the CNCs. Regardless, the calculated resilience of each wet scaffolds showed that the 5 wt. % scaffold had a clear mechanical advantage over the others. Combined with the top performance during dry testing, the results suggest that the 5 wt. % scaffold is structurally the most capable as a material to be handled by surgeons.

Much in the way that the PBS or high humidity directly plasticize materials, the hydrophilic effect of CNCs themselves may have had an indirect impact on the mechanical behavior. Figure 11 displays the change in behavior of the dry scaffold during deformation. The plot on the left (Figure 11A) shows a very brittle material, with pore collapse and other fissuring occurring throughout the length of the test. On the right (Figure 11B), the plot shows a smoothed stress-strain curve with a highly reduced amount of cracking and abrupt pore collapse. The only difference between the two materials is a 10 wt. % loading of CNCs. The discrepancies suggest the CNCs indirectly plasticize the scaffold to some extent via water absorption, and may reduce the overall brittleness of the material on some micro or nano-scale level. The overall Young’s modulus did not change between compositions, so there were competing factors that formed the properties calculated during testing. For instance, while the stiffness of the OVA matrix may be lowered with CNC additions, nano-particle reinforcement could compensate for this. The aforementioned increase in pore size due to CNC loading would also be detrimental to the Young’s modulus, but may be counteracted by nano-particle strengthening mechanisms.
Another suspected contributing factor for an unchanging Young’s modulus was the presence of remaining crosslinks. Given the proposed crosslinking scheme that includes OVA binding to both OVA and the CNCs (Figure 2), along with the potential for CNC crosslinking to other CNCs, there were many ways the reaction could progress. It was indeed possible the various conformational possibilities did not always coincide, or the CNCs crosslinked and aggregated apart from the OVA matrix. Either way, the glutaraldehyde may have been depleted to create a material that did not have optimal crosslinking. If increasing amounts of CNCs did produce a higher amount of remaining crosslinks, nanoparticle surface interactions may have counteracted the effect. It has been shown in prior research that if the crosslinking density was decreased, there would be a subsequent reduction in the compressive modulus [42].

4.4.2 Dynamic Mechanical Analysis

To better complement the mechanical data gathered by Instron compression testing, DMA was utilized to support any formed assumptions or tentative conclusions. Instead of using a strain ramp commonly applied for compressive tests, a force ramp was initiated. This set up allowed for more noticeable failure events in each scaffold, where a fissure in the scaffold would create an instant deformation step that was easily detected on the resulting plot as shown in Figure 12.

Compared to the values produced by Instron compression testing, the DMA produced values were consistently lower. This may be due to the faster application of force or sensitivity differences in the load cells. More importantly, the conclusions that were drawn from Instron testing were confirmed with the DMA. The 5 wt. % scaffolds had the best performance in the dry state in all categories, and thus had the highest resilience as well. Also, the scaffolds were tested wet, but the constant application of force yielded no identifiable point of failure, and only
the compressive modulus could be calculated. As observed before, there were no significant
differences found between the scaffold compositions tested wet. The scaffolds prepared for
DMA were only soaked, and not submerged during testing, but the effect of PBS absorption
produced similar results.

4.5 Contact Angle Measurements

Overall, there was a high level of consistency between all the films with CNCs with
reduced error compared to the unmodified films, although the higher contact angles suggest a
more hydrophobic surface. Visual inspection of each scaffold led to the assumption that the
films became more homogeneous with CNC loading. The unmodified films were uneven, with
noticeable aggregation of OVA protein. Any surface roughness imparted by the CNCs or
surface morphology would impact the contact angle measurements. Given the topographical
changes, the high amount of error seen for contact angle measurements on pure OVA seemed to
correlate with the absence of CNCs.

If the lower contact angle for the 0 wt. % films was a legitimate finding, it indicated that
the CNCs increased the hydrophobicity of the film. This was unusual since both CNCs and
OVA are hydrophilic. A potential cause for larger contact angles on CNC/OVA surfaces could
be from the CNCs interfering with OVA aggregates. If CNCs surface charge disrupts the OVA
confirmation, it is possible the hydrophobic residues could be exposed, creating a more
hydrophobic surface as well. As mentioned in Chapter 1, half of ovalbumin’s residues are
hydrophobic [12].
4.6 Atomic Force Microscopy

Sources have shown that treatments of surfaces that generate rougher surfaces can improve cell attachment, growth, and viability [56]. Some experiments have indicated roughness provides marginal improvement in attachment, but is a crucial component in proliferation [57], while others have shown that rougher surfaces only aids in initial cell attachment [58]. In general, optimal roughness values for cell attachment and spreading are seen in the range of hundreds of nanometers [57, 58, 59]. This is still debated as some authors demonstrated that silica-nanoparticles hindered proliferation at surface roughness values of 300 nm, and had optimal attachment and spreading at 50 nm [59].

In this study, AFM revealed that the mean surface roughness increased when a 10 wt. % concentration of CNCs was added (Figure 15). Although this seemed, and may be favorable, the highest roughness values were no greater than 1 nm, and therefore were unlikely to play a strong role in cell behavior. The AFM results indicated that all the films had very smooth surfaces. Given the smoothness at the highest CNC loading tested, it was also safe to conclude it did not significantly impact the contact angles measured prior.

4.7 In Vitro Cell Studies

4.7.1 Cell Viability - MTT Assay

The MTT Assay accounted for the number of cells present at time points of 1 day and 4 days. This provided insight, through metabolic activity, into the cell viability, cell proliferation, and cytotoxicity of the scaffold. The results from Figure 16 showed that each scaffold composition tested was not cytotoxic, as more cells were present at each time point than were seeded. It was clear from the results that the cell survival rate was much higher than the cell death rate, since the cells multiplied over time. Therefore, both the films and the scaffolds could
be considered biocompatible. The materials that showed the best cell proliferation from initial seeding to 24 hr were the 0 wt. % films and the 5 wt. % scaffolds. For both the films and the scaffolds, the 2 wt. % CNC composites clearly had the best proliferation between days 1 and 4. While the other film and scaffold compositions displayed small gains in cell count, although they were statistically not significant.

It was clear that the 2 wt. % scaffolds were able to proliferate sufficiently to make up for any lack of initial cell attachment. The reason for this advantage was not clear. The factors that may have played a role include pore size, surface roughness, unbound CNCs, uncrosslinked OVA, and experimental error. The surface roughness, as discussed prior, likely did not play a large role for the films, although the actual roughness of the porous material was never determined. It has been shown that rougher surfaces are sometimes generated with larger pores. For this experiment, the 10 wt. % scaffold exhibited the largest pores. Furthermore, unbound CNCs and uncrosslinked lysine groups may be present in each sample. Lysine groups in the OVA carry a positive charge at a pH of 8.9, which would provide better attraction for negatively charged cells. Unbound CNC sulfate groups carry a negative charge in the OVA solution, potentially repelling cell attachment since the pH is below the CNC isoelectric point. The charge of the CNC would depend on the charge balance between negative sulfate groups and positive amino groups. The MTT assay data could reflect differences in the concentration of positively or negatively charged functional groups.

4.7.2 Cell Differentiation – OCN Assay

The differentiation study detected no increase in OCN levels. Absorbance readings matched that of the buffer solution, thus negating the presence of OCN. In an earlier study, OCN levels did increase significantly compared to the control after a period of 3 weeks for the
unmodified scaffolds [43]. Therefore, pre-osteoblasts have been shown to differentiate on ovalbumin scaffolds, indicating there was a problem with the assay for the current study. One reason the cells did not differentiate could be a result of the change in MC3T3-E1 pre-osteoblast sub-clone. Another reason could be related to the differentiation media. An osteogenic medium containing ascorbic acid and β-glycerol phosphate was used to culture the cells on day 1 instead of day 5 when the proliferation phase ended. Potentially, the cells were not able to proliferate enough to produce detectible levels of OCN.

5. Conclusions

Ongoing research in bone tissue regeneration has moved towards degradable materials that encourage only native tissue growth with no scaffold remaining after complete healing. Naturally derived polymers are often engaged in this area as an alternative to synthetic materials, lending their biocompatibility to the biomimetic strategies employed. Forming a natural, degradable 3-D porous scaffold from ovalbumin hydrogels has had promising results in the attachment, proliferation, and differentiation of osteoblasts [43, 60]. Despite the advantages of this bio-active material, mechanical reinforcement is needed to provide a scaffold that is sturdy enough to be handled by surgeons.

Nanoparticles are often added to strengthen scaffold materials by disrupting polymer chain and dislocation movement. Cellulose nanocrystals were chosen to reinforce the ovalbumin based scaffolds due to their high stiffness and organic, non-toxic composition. The CNCs were functionalized with amine groups to covalently crosslink with the ovalbumin matrix. The CNCs without amine groups hindered crosslinking and did not distribute themselves evenly throughout the scaffold volume. Scaffolds were fabricated with CNC loadings of 2, 5, and 10 wt. % to compare with 0 wt. %, or pure OVA scaffolds. SEM was used to image the porous structure of
each scaffold. The pore sizes increased with CNC loading, with pores on the 10 wt. % scaffolds doubling the diameters found on pure OVA scaffolds.

The scaffolds were submerged in PBS and weighed at several time points to evaluate the amount of swelling generated by absorption. Scaffolds containing CNCs swelled more than the unmodified scaffolds due to the hydrophilic nature of cellulose. The CNCs had a direct effect on swelling since the 10 wt. % scaffolds showed higher % absorption compared to the 2 and 5 wt. % scaffolds. Following compression testing with Instron and DMA instruments, it was determined that the 5 and 10 wt. % scaffolds were the most resilient in the dry state and the 5 wt. % was the most resilient in the wet state. The wet scaffolds were significantly weaker than the dry scaffolds due to the higher amount of PBS absorbed when pre-soaked and /or submerged. Overall, the 5 wt. % scaffolds were deemed the most capable for bone tissue replacement surgeries.

To characterize the effect of CNCs on the scaffolds’ surface properties, thin films were generated to gather contact angle measurements and investigate surface roughness. The contact angles were all the same for films containing CNCs, with slightly higher contact angles than were measured for the unmodified film. The discrepancy between the two was attributed to an irregular surface created without the plasticizing effects of the CNCs, resulting in higher error. The film surfaces were visibly more homogeneous with CNC loading. Next, AFM was used to examine the surface topography. Mean surface roughness was determined for each CNC loading, indicating a significant increase between the 0 and 10 wt. % films. As expected, the CNCs roughened the surface of the hydrogel. All roughness values were below 1 nm, so any differences were thought to be negligible for promoting cell attachment.
Cell studies included an MTT Assay for cytotoxicity and cell proliferation, and an assay designed to identify OCN: an indicator of cell differentiation. The MTT Assay showed that all the scaffolds were non-cytotoxic. A high level of proliferation was seen for 2 wt. % films and scaffolds. The scaffolds with the highest cell count on day 4 were the 2 and 5 wt. % scaffolds. The OCN assay did not show any signs of differentiation after one attempt, although OCN had been present for the unmodified scaffolds in earlier studies [43, 60].

From this study, it can be concluded that the addition of a 5 wt. % aminated CNCs improves the resilience of the OVA scaffold in both the wet and dry state, improves homogeneity, and had the second highest cell count incubated on the scaffolds. Given the mechanical improvements and upgraded cell proliferation, the scaffolds were shown to benefit from all CNC additions, but properties were optimal with a 5 wt. % loading.

6. Future Work

Future directions for the reinforcement of ovalbumin-based scaffolds should include the repetition of several experiments and only after promising results found in vitro should this system be translated to in vivo. There is evidence supporting the differentiation of cells, so OCN and ALP assays need to be repeated for further clarity. Also, all testing could have benefited from doubling the original sample size in each group to identify outliers, reduce error, and reinforce conclusions.

To truly understand the functionality of these scaffolds as a bioactive, osteoconductive material, further cell work is needed. Other cell studies should include a PicoGreen assay to accurately count the number of cells present. The MTT assay only measured metabolic activity, which may reflect the cell number, but does not exclude other determinants. Staining for actin
and integrins would also be beneficial in the examination of osteoblast attachment and spreading on the OVA/CNC surface.

Given the high sources of error in some of the tests and the inconsistencies present, the entire fabrication process should call for the revision of the OVA solution fabrication. The solution would be better described as a colloidal suspension, with an undesirable amount of OVA powder forming insoluble, non-reactive aggregates. By increasing the pH of the solution, the aggregates may be dissolved, forming a more homogenous solution to prepare scaffolds. To investigate, there was a trial experiment adjusting the pH to 9.5. This solution was not able to crosslink due to the high level of separation between particles created by the caustic pH. There may be a lower pH that could prevent large scale aggregation without completely disassociating the OVA agglomerations. The ability of the solution to crosslink is pivotal to forming a cohesive scaffold. If this technique proves unsuccessful, it would still be advantageous to identify a more consistent method for preparing the OVA solution.

Furthermore, the deaggregation of the protein requires investigation. During thin film fabrication there was a noticeable change in the crosslinked material topography. The films became more homogeneous as CNCs were added. The effect of CNCs, given their electrostatic charge should be investigated to find signs of conformational changes in the ovalbumin. CNC charge is also pertinent, as their incorporation would certainly adjust the mineralization capabilities of OVA. The deposition of calcium phosphate on OVA is charge driven [60], so mineralization should be quantified for OVA/CNC composites.

Finally, unmodified CNCs could be added to the scaffolds, relying solely on nanoparticle strengthening mechanisms, rather than the combined effect with chemical crosslinking. The scaffolds could then be characterized by the methods described in this study. Unmodified
scaffolds were initially studied; however there were some issues with keeping the nanocrystals from segregating to the surface of each scaffold. Further experimentation may solve this issue, allowing for the creation of a more traditional, and potentially stronger reinforced biomaterial. To better characterize the surface effects of CNCs on the composite, contact profilometry should be performed to get a larger field of testing for surface roughness measurements.
7. References


[52] Sicchieri LG. Pore size regulates cell and tissue interactions with PLGA-CaP scaffolds used for bone engineering. Journal of tissue engineering and regenerative medicine. 2011:n-a-n/a.


APPENDIX A: Compression Testing for 10 – 30 wt. % CNC Scaffolds

A.1 Introduction

Considering the size, aspect ratio, attractive mechanical properties, and high interfacial energy of cellulose nanocrystals, it is no wonder they have jumped to the forefront of nanocomposite research for the reinforcement of polymeric materials. They have been shown to provide significant increases in the tensile storage modulus of polymers [38] but their effect on the compression behavior of materials has not been well established. Several authors have tested 3-D porous materials over the range of 0 to 10, or at most 15 wt. % concentrations [61-63]. At a certain concentration, the reinforcing effect of these nanoparticles will impart a negative effect on the mechanical properties, as only low amounts are needed for percolation [38]. To find this upper boundary, OVA/CNC composite scaffolds were investigated at loadings of 10, 20 and 30 wt. % CNC.

A.2 Materials and Methods

See Chapter 2.4

A.3 Results and Discussion

The compression testing results indicated that once CNC loadings surpassed a 10 wt. % concentration, the resilience drastically decreased due to a sharp drop in the elastic limit and yield strains. The compressive modulus remained roughly the same for a 20 wt. % loading, but then decreased significantly for the 30 wt. % loading. Given this information, a maximum loading of 10 wt. % was chosen for scaffold characterization. Due to the high aspect ratio of the CNCs, low rather than high concentrations of CNCs are not advisable for strong interactions.
with the polymer matrix. It appeared for loadings of 10 wt. %, the CNCs were overly abundant and did not percolate evenly. As discussed earlier, the hydrophilicity of the CNCs in such high concentrations attracted additional moisture which may have also played a role in diminished mechanical performance.

![Figure 18](image)

Figure 18: A) Dry compression testing results for 10, 20, and 30 wt. % scaffolds. B) Resilience values calculated for 10, 20, and 30 wt. % scaffolds according to equation 1.