Decellularized Tobacco Hairy Roots as Novel Tissue Culture Platform

A thesis submitted to the faculty of Worcester Polytechnic Institute
In partial fulfillment of the requirements of the
Degree of Master of Science in Biomedical Engineering

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May 6th, 2021

Abstract

The use of human mesenchymal stem cells (hMSCs) is gaining increased traction in clinical research and therapies. However, current tissue culture platforms for the large-scale expansion of hMSCs, such as microcarrier beads in bioreactors, result in lowered viability of the cells and affect differentiation potential by reducing surface antigen expression when detached using traditional dissociation agents such as trypsin. Microcarrier beads, commonly made from polystyrene, are well characterized in the expansion of other cell types. It is known that plant-derived scaffolds can be used to culture mammalian cells. Our lab has previously shown that decellularization of bright yellow-2 (BY-2) cell cultures, a tobacco (Nicotiana tabacum) plant cell, can yield matrices for the culture of human fibroblasts (hFFs). Tobacco hairy roots are plant-based tissue cultures from the same transgenic Nicotiana tabacum plant.

In this thesis, we evaluated decellularized tobacco hairy roots as a novel culture platform for hFFs, which served as an analog for other human cell types such as hMSCs. The hairy roots were genetically modified to express enhanced green fluorescent protein (EGFP). The hairy roots were mechanically decellularized via lyophilization followed by chemical treatment with DNase I to remove the DNA. The DNase I treatment resulted in 99% DNA removal, resulting in a DNA content less than 50 ng/mg tissue. This threshold level is considered to be sufficiently decellularized for reseeding with human cells in clinical and tissue engineering applications. Quantification of protein content showed 20% protein retention after decellularization of the hairy roots. Fluorescence microscopy confirmed removal of nuclei and retention of EGFP within the matrices.

To realize this matrix as a mammalian cell expansion platform, we evaluated matrix degradation using cellulase, the in vitro biocompatibility of cellulase, and the ability to detach viable hFFs from the matrix. First, a degradation protocol for the tobacco hairy root matrices was developed using cellulase as the hydrolytic enzyme as an alternative means of cell dissociation. The enzymatic degradation resulted in approximately 30% mass loss of the hairy root matrices in physiological conditions. In vitro biocompatibility experiments showed no adverse effects on hFF viability. Fluorescence imaging confirmed that the hFFs attached to the hairy root matrices. Subsequently, cells could be detached from the matrices using trypsin or the plant degrading enzyme, cellulase. The detached cells remained viable and could continue to be cultured on standard tissue culture plastic. Our studies show that decellularized tobacco hairy root matrices could be used for the support of human cell culture.

Acknowledgements

I would like to thank first Dr. Jeannine M. Coburn for giving me her time and guiding me through not only my graduate studies but also for my undergraduate years. She has given me the opportunity to expand my knowledge in my field and I believe that I have grown as person as a result. Without her, I would not be able to come thus far in my research and would have been lost along the way.

I would then like to thank my committee members, Dr. Marsha Rolle and Dr. Glenn Gaudette, for their invaluable feedback and support throughout this project. They have been truly beneficial in their advice and generous in accommodating additional time and trust in this project.

I would also like to call out the members of the Functional Biomaterials lab, both past and present, and show them my appreciation for encouraging me and providing me helpful critiques on my project and in my time at WPI. I would like to thank the following members:

Alycia Abbott	Katelyn Mistretta	Natalia Vargas Montoya	Kimberly Ornell
Nhi Phan	Elizabeth van Zyl	Carolina Villarreal	Melissa Wojnowski

Next, I would like to thank Dr. Jianfeng Xu, from Arkansas State University, for kindly offering us the tobacco hairy root culture, on which this project is based on. I would also not want to forget in thanking Dr. Sakthikumar Ambady and Jyotsna R. Patel in providing me support in planning out experiments and providing me with reagents necessary for my studies. And I appreciate Dr. Ronald Grimm, Dr. John D. Obayemi and Boquan Li in assisting me with the sputter coater and prepping samples for the SEM. I would like to especially thank Dr. Grimm who was very generous in taking the SEM images during the Covid-19 pandemic when regular students did not have access to many of the facilities and equipment at WPI.

Lastly, I would like to thank my family; Mohammed Mostafizur Rahman, Umme Salma Abdullah and Aniqua Antora, without whose support and encouragement, I would not be able to study abroad at WPI.

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Chapter 1: Introduction

With the advent of patient specific therapies and the ability to repair damaged tissue using the patient's own stem cells, human mesenchymal stem cells (hMSCs) are gaining popularity in research and clinical settings[1]. One of the primary challenges in utilizing stem cells is *in vitro* expansion to obtain sufficient cell numbers for therapeutic relevance[2]. That is especially true for the expansion of hMSCs in common large scale expansion methods[3]. Traditional use of microcarrier beads in bioreactors which increase the surface area to volume ratio for cell expansion is well characterized and helpful in reducing cost and labor[4]. Commercially available products such as the Synthemax II microcarriers® have been developed to improve the large-scale expansion of stem cells such as hMSCs[5-6]. Various studies have been done to understand the microenvironment influence on hMSC potency and microcarrier cultures have the potential to augment hMSCs in therapeutics[7]. However, hMSCs have been found to have reduced viability and do not grow as well on microcarrier beads as they do on traditional planar tissue culture platforms[8]. Also, one of the current challenges of using microcarrier technologies is their reliance on plastics[9]. Plastic produced for biological, medical, and agricultural purposes worldwide has created problems with plastic waste dumping which can have long term environmental impacts[10]. Plastics for cell culturing are primarily single-use items including microcarriers[11]. Alternative tissue culture surfaces for hMSCs or other human cell types that have less environmental impact could prove to be beneficial in cell-mediated therapies.

Plant based scaffolds have gained a lot of attention for growing human cells[12]. These plant-based materials are stripped of their native cells and genetic content, or decellularized, so that they can be reseeded with other cell types. *In vitro* cultured plant materials are another option. Commercially, plant cultures are used to synthesize designer proteins and molecules for the

pharmaceutical industries, cosmetics, food, and so on.[13] Plant-based cultures give flexibility in functionalization by engineering them to produce specific protein or molecules which can benefit human tissue culture. Decellularized BY-2 cells from the plant *Nicotiana tabacum* have been shown to support human fibroblast growth[14]. Hairy roots, derived from the same plant, are another form of plant culture that shows equal promise in the field of decellularized scaffolds. Plant based scaffolds are a renewable resource composed primarily of cellulose. As a result, they are completely degradable via the same routes plants decompose in nature and can disposed of safely unlike plastic-based microcarriers [15]. We propose that tobacco hairy roots could be used as a novel tissue culture surface for the growth of human cells.

Chapter 2: Background

Biomedical research has utilized various avenues for testing and understanding biological systems, disease, and methods of improving patient life. Throughout history, we have used animal models, due to their availability and feasibility, and it has helped us in understanding human health and for the testing and safety of potential medicines. The primary issue with this, apart from ethical concerns, is that human models could yield better results than using other animals as analogs. Problems arising from species extrapolation can be eliminated by using human tissue and cell lines to test diseases and drugs instead of using animal subjects.

Non-animal methods can be employed in testing and research. The use of databases and computational modelling from prior clinical trials can help in reducing future wet lab research. *In vitro* models, utilizing cells and tissues, and human studies have allowed us to study how our bodies are affected by new drugs and to see the effects of diseases on human specific cells[16]. Of the many methods employed, cell cultures are probably the most utilized in biomedical research. Cell cultures provide the advantage of being obtained from different tissue types, to help see organ or tissue specific effects, and many protocols and methods exist that are well characterized. Apart from drug research and disease modelling, cell cultures can provide a therapeutic approach to treat human diseases, whether it be in providing protein production for drug use, or to repopulate damaged tissue or artificial organs.

Relative to human history, cell culture in practice is quite young. The term ‘cell’ was first coined in 1665 in Robert Hooke’s *Micrographia*[17]. But it was not until 1885 when Wilhelm Roux managed to successfully keep embryonic chicken cells alive in a saline solution that helped establish the preliminary principles of tissue culture. Ross Granville Harrison was then credited to successfully grow animal tissue, specifically frog embryo nerve fibers, outside of the body in 1907.

A little over a century later, we are now able to grow 3D cultures and utilize bioprinting techniques to make organs outside of the body. Fundamentals of tissue culture have been developed and criteria governing many types of cell propagation and differentiation have been identified. One of the most important aspects of tissue culture is the ability to propagate cells to a minimum population threshold applicable to the current need. This may mean that a scientist in a research setting may need to expand a cell line from a small inoculum to generate 5 million cells for a small-scale cell study, or that with the same initial seeding conditions, a yield in the scale of trillions may be needed to seed an artificial organ or to manufacture therapeutic molecules in a clinical setting[18].

2.1. Human Mesenchymal Stem Cells in Biomedical Research

As modern medicine begins to observe a shift towards therapies tailored for each patient, human mesenchymal stem cell (hMSC) research is gaining an increased traction[1]. Stem cells, unlike the specialized and differentiated cells that make up most of our tissue and organs, have the potential to develop into various kinds of tissue. As a result, they serve in repairing and replenishing damaged tissue and cells. In the body hMSCs are responsible for making and repairing skeletal tissues and fat. As of March 2021, there are about 110 clinical trials with hMSCs in the United States alone that are active and/or recruiting, of which 85 are within phase I and II (www.clinicaltrials.gov). MSCs exist in almost all tissues, are easily harvested from bone marrow, synovium and fat, and depending on the requirements of a biomedical application, they can be differentiated into several cell types[19-20]. This makes them ideal for tissue regeneration. The surface of hMSCs do not express significant histocompatibility complexes or molecules that can stimulate the immune system. This helps in their potential role in therapies that need to avoid an immune response in the body or prevent tissue rejection[21-22]. MSCs exhibit clonal growth and are characterized by their adherent expansion on plastic culture plates[2]. They exhibit the mesenchymal

surface makers CD29, CD73, CD90 and CD105, while are negative for hematopoietic lineage markers CD34, CD45 and HLA-DR[23]. MSCs are multipotent, and unlike pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs), they can differentiate into limited cell types such as chondrogenic, adipogenic and osteogenic cell types[24].

2.2. Bioprocessing Strategies for Human Mesenchymal Stem Cells

For clinical applications, product manufacturing utilizes primarily MSCs derived from human bone marrow, and then followed by adipose derived stromal cells[1]. For manufacturing hMSC based products, cell yield and production cost need to be considered to maximize efficiency. Typically, manufacturing processes vary in scale based on the therapy approach (allogenic vs autologous). Upstream processing involves in collecting cells from a doner or patient and forming a master cell bank and/or a working cell bank. This is then followed by cell expansion at large scale (**Figure 1**). Downstream processing involves harvesting the cells. The hMSCs need to be detached from their growth surfaces, separated, and then washed. Allogenic therapies, utilizing cells from a donor to treat other patients, require the generation of more doses per batch during the cell expansion step and subsequently will treat more patients than an autologous approach where the sole patient themselves provide the cells for the treatment. Due to the high cost of patient specific therapies, and the need for quality control and security in autologous approaches, allogenic stem cell therapies are the commercially preferred option. Also, various economic studies have shown that upstream processing, particularly cell expansion methods is the primary cost driver[25-26]. Traditional planar cultivation platforms, often used in research settings, are not very cost or space efficient for allogenic therapy approaches to achieve yields of between 10^{12} and 10^{13} cells needed per batch. Typical planar cultivation systems result in 25,000 to 30,000 cells/cm^2. Even

when stacked up to 40 layers, target cell yield cannot be achieved and it is difficult to maintain consistency between batches[27-28].

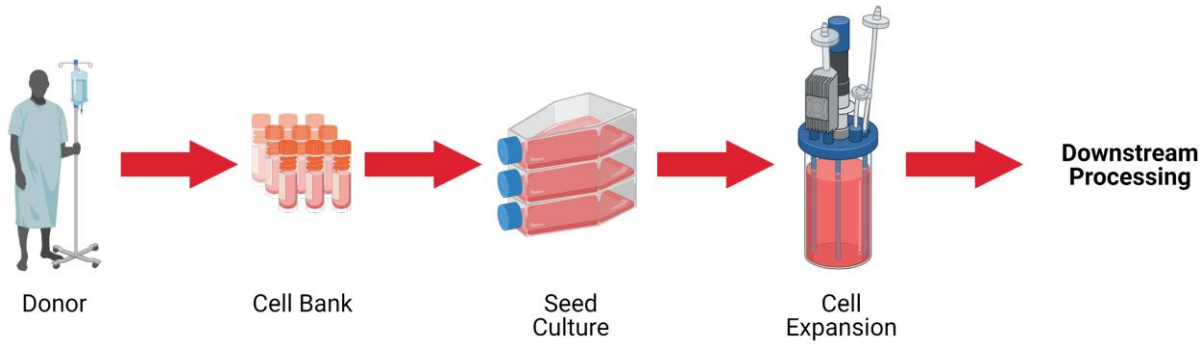

Figure 1: Simplified upstream processing flow diagram for hMSC production
Cells are collected from a donor and stored by freezing to make a master cell bank. Cells are taken from the working cell bank and cultured to make the inoculum or seed culture which is then used to seed a bioreactor for large scale expansion. Cells then need to be dissociated from their culture surfaces, purified, and collected in the downstream processing before they can be ready for clinical or research applications.

2.3. Two-Dimensional (2D) vs Three-Dimensional (3D) Culture Platforms

Expansion on planar platforms is highly inefficient. For large scale attachment and proliferation, several passages are required[8]. Maintaining uniform distribution and growth and proper harvesting methods are crucial to reduce heterogeneity while increasing cell yield[29]. Though studies have shown the capability of hMSCs to differentiate into multiple cell types, often achieved using cell-specific differentiation media, expansion via monolayers usually results in phenotypic changes[2]. This can alter cell fate and their potential for differentiation[30]. As a result, other avenues have been looked at, specifically 3-D culture methods, to better model the native environment of hMSCs (**Figure 2**). These various 3-D culture systems can be broken into either utilizing biomaterials or none thereof, under dynamic or static conditions[2]. A 2-D or planar tissue culture platform consists of a flat surface, usually tissue culture plastic, which serves as the facet

for cell attachment. Cells are seeded at a low density and allowed to grow until they reach a suitable final density, also known as the desired confluency[31]. The cells are then detached using a proteolytic enzyme such as trypsin that breaks down the extra cellular matrix (ECM) and focal adhesions which helps the cells to stay latched on to the surface. A 3-D culture on the other hand does not employ horizontal planar surfaces and instead consists of culturing cells on spherical or irregular shaped biomaterials in suspension or as free-floating spheroid bodies. Examples of 3-D based culture platforms are discussed in the following sub-sections.

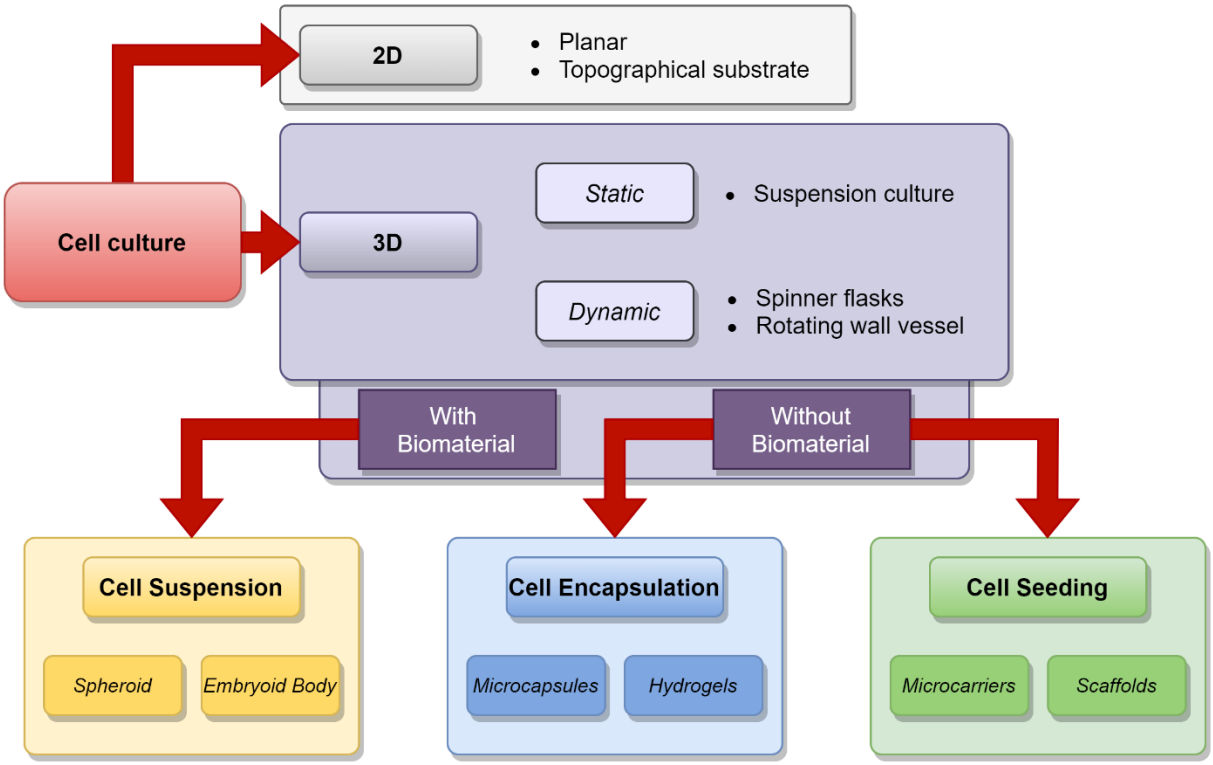

Figure 2: The different types of tissue culture platforms for cell expansion
Tissue culture can be broken up into two primary categories, 2D or 3D culture platforms. 2D culture comprises mainly traditional flat tissue culture plastics utilized in research settings such as flasks or plates. It can also include seeding cells on alternative flat biomaterials or modified surfaces. 3D culture is a broad topic encompassing various aspects of seeding cells on non-planar surfaces to increase cell yield or for encapsulating cells for different seeding strategies in clinical or research settings. Microcarrier beads are an example of 3D cell culture platforms. Other than the cell suspension method for cultivating cells, most platforms for cultivating anchorage dependent cells, other than the cell encapsulation method utilizes trypsin as a dissociating agent in cell culture.

2.3.1. Spheroids

Perhaps the simplest method for a 3-D culture system is via the formation of multicellular aggregates, or spheroids. This allows for the 3-D interactions of cells and the ECM without the need for additional exogeneous substrates that is typical with planar culture platforms[32]. These spheroids can be formed from adherent cells using spontaneous or forced aggregation techniques in suspension culture. Cells can be grown at high densities in tubes or hydrophobic materials, forcing the cells to form aggregate clumps[14]. They are comprised of cells that range from highly proliferative to apoptotic cells. This is due to the oxygen diffusion limit to the center of the spheroids. This hypoxic environment limits the size of the spheroids[33]. Due to the heterogenous nature, PSC spheroids have assisted in modelling complex 3-D cell structures and in cell differentiation and cancer research[34]. But for hMSCs, short term spheroid cultures have been utilized for maintenance and expansion[32]. Spheroid grown hMSCs can exhibit an undifferentiated morphology when subcultured back onto a 2-D culture platform. This is credited to increased ECM deposition compared to adherent grown cells[35]. These cells displayed increased multipotency and clonal growth[36]. Long term culture, however, induces differentiation in hMSCs due to increased heterogeneity and microenvironmental differences[37].

2.3.2. Scaffolds

To help mimic the biochemical and biophysical properties that stem cells require to either propagate or differentiate, the use of biomaterials in *in vitro* cultures has become more integral in biomedical research[38]. Biomaterials can be natural or synthetic in origin and provide biocompatibility and often biodegradability to meet some biological function. Biomaterials with tunable mechanical properties allow for the alteration of viability, growth and differentiation of cells[39]. Based on the method or technique used to form the biomaterial, such as electrospinning,

3-D printing, or casting, various scaffolds can be produced with varying tensile strengths, elasticities and pore sizes[40]. From this point on, a 3D biomaterial or scaffold will be used interchangeably to signify a material for cell seeding applications[40-41].

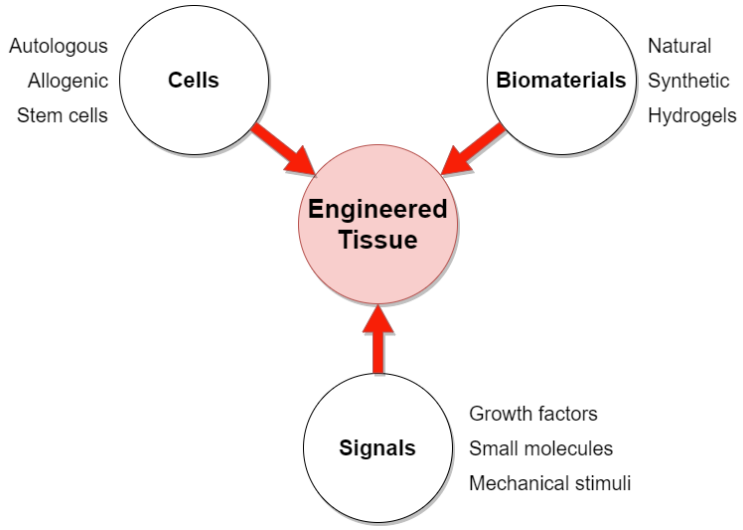

Figure 3: Tissue engineering triad
The three aspects of tissue engineering which includes the biomaterial scaffold to serve as a construct or support; cells to seed the scaffold for research or therapeutic purpose and biochemical or mechanical cues to induce differentiation or biological activity so that the cells can serve their function[41].

Tissue engineering is a field that combines the principles of engineering, cells and materials to mimic and ultimately improve or replace biological tissues[42]. This also takes into consideration chemical cues necessary to fully integrate into the physiological environment. A scaffold is basically an amalgamation of all these factors that then acts as a medium for tissue formation (**Figure 3**)[41]. These scaffolds are usually cell seeded prior to implant along with growth factors. With the use of a device to induce biophysical stimuli such as bioreactor, the scaffold can be artificially subjected to mechanical and chemical cues to bring about the necessary differentiation in the cells and to attain the necessary mechanical properties of the tissue type it was made to replace[43]. Cell seeded scaffolds can be cultured *in vitro* to attain the desired cell environment or implanted directly as an acellular scaffold into the injured site. The latter uses the body's natural

regenerative functions can induce tissue and organ formation *in vivo*[41]. For the culturing of stem cells, scaffolds can be generally thought of two types: 'prefabricated scaffolds' that require post cell seeding or migration of cells, often used for differentiation protocols, and 'self-assembled scaffolds' that encapsulate cells at the time of scaffold formation[2]. Self-assembly is usually performed via different crosslinking methods. Scaffolds that are crosslinked physically, usually by changes in pH and/or temperature, are called reversible gels[44]. These are generally weak mechanically. If the crosslinking utilizes covalent bonds, the result is a mechanically stronger scaffold[45]. Hydrogel scaffolds help mimic ECM of natural soft tissue and as such can be used to grow and differentiate cells *in vitro* and help integrate cells *in vivo*. This is further assisted by the ability to modify hydrogels with drugs or growth factors[46-47]. It has been shown that hMSCs that were encapsulated in nondegradable photopolymerized polyethylene glycol (PEG) hydrogels had improved viability when the scaffolds were coupled with ligands derived from fibronectin and laminin[48]. Proliferation, however, is limited by matrix stiffness and rate of scaffold degradation as observed in a study with hyaluronic acid (HA) based scaffolds[49]. Hydrogel degradation also showed increased hMSC viability even with the absence of cell adhesion ligands.

2.3.3. Microcarriers

Another alternative to planar cultivation systems, bioreactors utilizing microcarriers are a viable option for hMSC expansions[50]. Microcarriers, also called beads, increase the surface area to volume ratio for cells to adhere to and grow on. It enables larger scale expansions of cells that would otherwise be limited by regular planar culture platforms such as flasks or the CellSTACK®. To increase safety in upstream processing, single use bioreactors are preferred over reusable ones[26]. This allows for the production of large scale quantities of homogeneous cells needed for therapeutic or research applications, which is not feasible via 2-D culture systems[8]. For large scale

expansion, regulating pH, temperature and dissolved oxygen concentrations is crucial[51]. Equally as important is making sure to remove waste products and metabolites.

The bioreactor design for cell expansion can be static or dynamic, meaning the media supplementing the cells can either be left standing or kept in motion using a pump or rotor. Microcarriers are usually utilized in dynamic conditions but can also be used in static bioreactors. However, one of the challenges with static cultures is that when cells are seeded at high concentrations, clustering of cells may occur, which can reduce oxygen and nutrient transfer. This can potentially increase cell death in large cell aggregates[52]. Spinner flasks are simple and more common place due to feasibility and ease of use. The mechanism utilizes an internal impeller. Cells are continuously agitated and kept in suspension via stirring[53]. Hydrodynamic shear stress is generated as a result which allows for the mixing of cells and nutrients, but as microcarrier beads are always in close contact with each other and the impeller, excessive agitation can cause cell death[54]. Rotating wall vessel (RWV) bioreactors were designed to reduce shear stress by rotating horizontally[55]. In these systems, culture conditions are controlled via flow perfusion systems. Media has to be changed at intervals to avoid waste accumulation that would otherwise inhibit cell growth[56]. The third type of bioreactor system is the mechanical force bioreactor that is designed to simulate native tissue physiology by using tensile and compressive forces[57]. This has been used primarily to condition scaffolds and for differentiating tissue types. All of the systems stated above for bioreactor technology can be further built upon by the use of appropriate biomaterials to allow for growing cells at high concentrations[52].

In the case of scale up for hMSCs in dynamic bioreactors, a lower cell viability and proliferation is observed compared to static planar culture platforms[58-59]. Studies performed with hMSCs grown on different microcarriers showed that biomaterial signaling plays a crucial role in

cell proliferation[60]. Cell-adhesive coatings such as collagen can promote growth[61]. Use of functionalized biomaterials in microcarrier design can enable either increased proliferation or differentiation based upon media composition and the protein being released from the cell-attachment surface. TGFβ3, when released from microcarriers, helped improve chondrogenic differentiation of hMSCs[62].

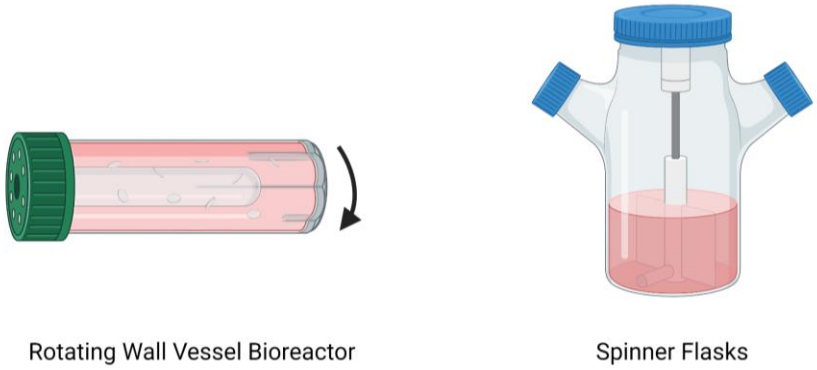

Figure 4 : Two different kinds of bioreactors
(Left) Rotating wall vessel (RWV) bioreactor and (Right) spinner flask for use in tissue cultures.

Microcarriers are primarily used as the cell culture platform in bioreactor systems (namely impeller and RWV based systems). The beads, ranging between 100 and 300 µm, are manufactured from various materials including collagen, gelatin, dextran and polystyrene[60]. They can be designed to have different surface topographies or porosities to promote cell attachment and/or infiltration. These different surface properties increase the surface area to volume ratio for cell attachment and growth allowing to achieve a higher cell density in a given volume of space. After which the cells are detached using enzymatic action[60, 63-64]. Microcarriers have been shown to support human embryonic stem cells (hESCs) proliferate to high densities in spinner flasks[65-66]. The use of coatings, such as ECM proteins, on microcarriers is a common practice to increase PSC attachment[8]. ECM proteins such as vitronectin and laminin can be used to promote stem cell growth[67]. Matrigel, a protein mixture primarily composed of reconstituted basement membrane obtained from the extracts of Engelbreth-Holm-Swarm mouse sarcomas, has been used to aid in

the attachment of hESCs onto cellulose microcarriers for long-term expansion and maintenance[68]. It has been observed that in the absence of animal derived materials, hESCs adhere and grow more readily in perfusion based dynamic culture systems[69]. Corning® Synthemax™ Surface coating is a product available as a microcarrier technology for xeno-free culture conditions for stem cells[6]. It is a proprietary surface coating composed of RGD peptides (Arg-Gly-Asp) immobilized on an acrylate coating to mimic the natural cell environment. Human MSCs were shown to be able to grow on the culture surface and achieve >10,000 fold expansion while maintaining hMSC phenotype and karyotype[5].

2.3.3.a. Plastics in Microcarrier Technology

Another study compared hMSC growth on different commercially available microcarriers such as Cytodex™, SphereCol®, Cytopore™, SoloHill Plastic, and Synthemax II™. SoloHill Plastic microcarriers were determined to be the optimal microcarrier based on the extent of cell proliferation, the flexibility for xeno-free processing, and the effects of cell harvesting on immunophenotype and differentiation capacity[60]. However, since most commercially available microcarriers are plastic in nature, their impact on the environment can be significant[9]. Single-use plastics usually replace more sustainable materials in laboratory and research settings[70]. Their ease of manufacture and ability to be modified to serve various biological purposes makes them desirable. These plastic-based tissue culture platforms such as microcarriers are usually not recycled, leading to an overall increase in plastic waste in the world[11, 71]. Millions of metric tons of plastic waste are generated yearly[72]. In 2014, an estimated 5.5 million metric tons of plastic waste were produced through biological, medical, and agricultural institutions worldwide[10]. Plastic-based microcarrier beads saves time and reduces costs but contributes to microplastic waste

in the world's oceans[71]. As such, for a greener tomorrow, it may be prudent to switch to alternative and sustainable sources of tissue culture biomaterials.

2.3.3.b. Biodegradable Microcarriers

Another avenue for suspension-based culture platforms are the use of microporous and biodegradable microcarriers, and microencapsulated cells[2]. Biodegradable microcarriers such as poly-l-lactic acid (PLLA), poly-co-glycolic acid (PLGA), hydroxyapatite, collagen (Cellagen^{TM}) and gelatin (CultiSpher^{TM}) have recently gained interest, especially in the field of regenerative medicine[73]. By being biodegradable, it may be possible to directly transplant hMSCs *in vivo* without having to go through downstream processing steps, hence increasing total cell recovery. Macroporosity and microencapsulation strategies allow cells to grow internally and reduce the effects of hydrodynamic shear on cells[74]. This can also protect cells from immune reactions post-transplantation[75]. Though not used in practice, development of degradable microcarriers could help in forming new methods for cell detachment and increase recover rate[73].

Cell yields for hMSCs on microcarrier based systems are low even when optimized under xeno-free conditions[76-77]. Cell damage and death from volumetric shear forces while expanding in upstream processing, poor recovery rate of cells from downstream processing and the low affinity of hMSCs for microcarrier based systems provide not many benefits over planar culture methods. Even with the higher operating efficiency of bioreactors for the expansion of cells and the need for fewer man hours, clinical applications may opt for multi-layered flasks for scale up since hMSC markers are not affected by shear stress[78-80]. It is reported that 30% of cells are lost during loading of bioreactors, and another 30% during collection[81]. Cell harvesting efficiency can be as low as 45%[82]. Large-scale expansion of hMSCs needs to be improved for both upstream and downstream processing.

2.3.3.c. Challenges in Cell Detachment

Adherent cells on microcarriers are primarily detached using enzymatic dissociation. The use of digestive enzymes to break down the ECM binding the cells to the culture surface is necessary to make hMSC suspension[83]. Since they are protein digestive enzymes, they can influence cell surface marker expressions which can have detrimental effects on cell function. Trypsin, the current gold standard for cell detachment, can significantly reduce $CD44^+$, $CD55^+$, $CD73^+$, $CD105^+$, $CD140a^+$, $CD140b^+$ and $CD201^+$ numbers within 30 minutes of exposure[83-84]. Trypsin functionality is also regulated by the presence of fetal bovine serum (FBS) in the culture media. Ideally xeno-free media should be standard for hMSCs, however, FBS is still the most common component in growth media for stem cells[85]. Most studies are carried out using FBS at concentrations of between 10% and 20%[86]. FBS contains factors belonging to the family of inter-alpha-trypsin inhibitors[87]. They help in stabilizing cumulus ECM produced by cells, but also, they inactivate trypsin to prevent unnecessary damage to the cell membrane. In large scale bioreactors, one of the challenges is allowing for sufficient dissociation of adherent cells using dissociation enzymes. For maximum efficacy of trypsin, a thorough wash with phosphate buffered saline (PBS) solution without Ca^{2+}/Mg^{2+} ions or PBS (-) is usually employed on planar culture platforms before treatment to remove residual protease inhibitors[88]. The presence of the divalent cations can strengthen focal adhesion of cells to the platforms and as such PBS (+) is only used when it is impertinent to maintain cells on the surface. Due the nature of bioreactors, it is difficult to remove the supernatant, which contains the inhibiting FBS, affecting trypsin activity[89]. Using a higher concentration of trypsin to digest the ECM could also potentially reduce hMSC markers[83-84].

TrypLE™ Express is an alternative to trypsin used when looking for animal-origin free reagents[83]. It is a recombinant enzyme that is gentle on the cells during detachment and results in

reduced effects on surface antigen expression compared to trypsin[83]. Its main drawback, however, is its cost. TrypLE costs almost twice as much as trypsin (www.thermofisher.com).

To add to the challenge of harvesting cells, microcarrier beads necessitate proper separation methods during the downstream processing. Current methods include dead-end sieving, tangential flow filtration (TFF) and counter-flow centrifugation elutriation (CEE)[90]. At small-scale, cells are separated from microcarriers by sieving after trypsinization. For larger scales, the cell-bead suspension is flowed tangentially over a hollow fiber filter to reduce filter fouling. Washing is achieved via diafiltration. The alternative is the CEE where the opposing forces of fluid flow and centrifugation create a fluidized bed, allowing for volume reduction steps and subsequent washing. This helps in creating a lower shear environment for cells. However, clogging from sieving and TFF, and the sheer bulkiness and operating costs for CEE pose disadvantages[91].

2.4. Biomaterial Scaffold Design

One of the challenges in designing an improved tissue culture platform for large-scale expansion is biomaterial selection for the adherent cells. A biomaterial is any such material that has been engineered to meet certain biological requirements, usually for a medical purpose. Selecting an alternative material for tissue culture purposes will have to meet certain conditions. The requirements for an ideal biomaterial that may be used universally are as follows[92]:

- Functionality → the material offers suitable mechanical or chemical properties tuned for the specific implant or purpose.
- Biocompatibility → the material is compatible with native tissue and does not induce an immune response and/or cause unnatural cell death.

- Stability → the material offers sufficient stability against physiological media for a specified duration.
- Biodegradability → the metabolization of the of the material if it were to occur should not cause any adverse conditions.
- Simple Processing → it should be simple to procure said material.
- Shelf-life → it should be able to be stored after processing for a reasonable duration before use.
- Sterilization → the material should be sterilizable without changes in structure and composition.

The ideal biomaterial would satisfy all the above conditions, but in most cases do not have to. Depending on the purpose certain requirements will take precedence over others while others can be foregone (**Table 1**). Cellulose based materials have been used in the past for tissue culture purposes and as microcarriers (Cytopore)[93]. They can be sourced sustainably and are biodegradable which reduces their impact on the environment compared to plastic-based materials. For material selection for our tissue culture platform, we would firstly require it to be biocompatible so that it will be possible to culture cells. It should also be biodegradable so that it can solve the problem that has plagued most plastic based culture platforms. Finally, allowing for functionalization will improve cell attachment and proliferation and the material should be easy to procure and process.

Table 1: Criteria for biomaterial selection

Types			*Attributes*
Synthetic biomaterial	Polymer	PLA, PGA, PMMA	Easy to modify
			Tunable properties
	Ceramic	HA, TCP, glass	Good mechanical strength
			Poor degradability
			Poor tensile strength
	Metal	Titanium, steel	Good mechanical properties
			Non-degradable
			Poor cell adhesion
Natural biomaterial	Collagen, gelatin, fibrin, cellulose		Biocompatibility
			Less likely to trigger an immune response
			Usually has an inherent biosignal
			Shorter degradation time
			Poor mechanical strength

Biomaterials can be natural or synthetic in origin and depending on the type of biomaterial it can have varying mechanical and/or functional properties. Different biomaterials will serve different applications based on what qualities or attributes are desired[94].

2.5. Plants as Scaffolds for Cell Growth

Tissue engineering is a very interdisciplinary field. This domain heavily relies on the use of 3D scaffold materials to provide an apt environment for cell propagation and the subsequent generation of different tissues or organs[41]. As a result, researchers worldwide have experimented with different types of materials to achieve the desired effects. Plants and plant based polymers are seen as a green and sustainable source due to the fact that it is renewable, cheap and very easy to mass produce[95]. They usually require minimal amounts of processing and can provide wider ranges for chemical composition or internal architecture based on what plant or plant-based material it is being derived from.

Plants being used as scaffolds is a more recent concept, however, it has seen use for much longer in the sense of biomedical sciences and medicine. The history of medicinal herbs and therapeutic extracts being used to alleviate ailments and illnesses is as old as mankind itself[96]. In the last 3 decades, genetic engineering has allowed for the generation of a plethora of foreign

proteins in plant-based cultures[97]. This allows for the mass scale production of various hormones and therapeutic proteins which naturally do not form within plant cells.

Plant tissues are composite materials organized into complex hierarchical structures(**Figure 5**)[98]. Plant cell walls contain cellulose fibers which help to reinforcing a flexible and hydrophilic matrix of hemicelluloses and other polymers such as pectin and lignin[99]. The cellulose fibers are arranged geometrically and give rise to the sturdiness and strength of plant tissue. The composition of this plant matrix varies from one plant species to another, such as a woody oak tree compared to the stem of a dandelion, or within the same plant species; leaves are softer and more pliable than roots for instance. Depending on the plant tissue the mechanical strengths can vary between as low as 0.3 MPa to as high as 30 GPa[99]. This means that different types of plant tissue could be used for different mammalian tissue types.

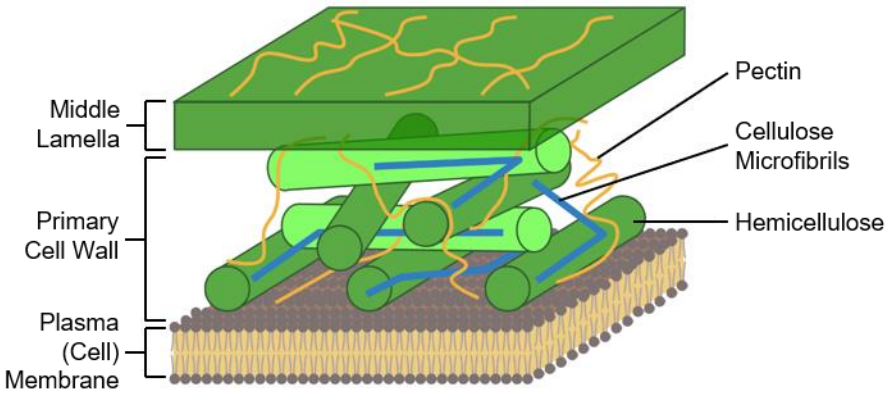

Figure 5: Plant cell wall composition
Plant cell wall matrix composition consisting primarily of cellulose fibers with interconnecting hemicellulose and pectin molecules. Lignin is usually present in woody plants. The cell wall structure provides rigidity to plant cells and provides structural support for the plant itself.

Plant tissue are also known for their well-defined vasculature. In vascular plants, water and nutrients are absorbed through the roots and make its way to the leaves via specialized tissue, the xylem. The phloem assists in transporting organic nutrients from areas of photosynthesis to the growing regions of the plant. These two tissue types usually run parallel to each other, typically

with the phloem surrounding the xylem in a concentric ring, which is then called a vascular bundle, and are easiest to observe with the naked eye in leaves[100]. This interconnected network of pores and vessels allow for the continual motion of water and sap throughout the plant and is akin to vascular networks in mammalian tissue[101].

2.5.1. Decellularized Plant Tissue as Scaffolds

When working with materials of plant origin it is pertinent to remove the native genetic material as foreign DNA can have adverse effects on mammalian cells and can trigger an immune response in the body. Different techniques have been developed to properly remove the genetic content, or decellularize the material to make it suitable for cell seeding applications. As standard practice, a combination of mechanical, surfactant and enzymatic methods have been used to decellularize mammalian organs and tissues[102-103]. There are concerns regarding decellularized mammalian tissues as scaffolds for tissue engineering due to risk of pathogen transmission and contamination. Much research has been undergone into utilizing plant-based tissues as scaffold material. Plant-based scaffolds have been studied as 3D cell culture models, such as apple derived cellulose scaffolds[104]. The Pelling lab has seeded mouse NIH3T3 and C2C12 cells and human HeLA cells on decellularized apple tissue to demonstrate the ability to grow mammalian cells on inexpensive and easily produced tissue[104-105].

The hydrophilic nature of plant-base scaffolds as well as their innate water transport capabilities derived from plant framework enable plant tissues to expand mammalian cell cultures over prolonged periods[106]. The existing microstructure in the plant-derived matrices assist in cell alignment. At the University of Wisconsin in the Murphy lab, bamboo tissue was decellularized using sodium dodecyl sulfate (SDS), a surfactant, in water and then lyophilized (freeze-dried)[106]. The tissue was selected due their high porosity and growth rate, was then biofunctionalized using

dopamine conjugated RGD peptides (Arg-Gly-Asp). Dopamine is an adhesive molecule capable of improving mammalian cell attachment and proliferation. Cell seeded bamboo scaffolds can assist in bone tissue engineering applications.

Similarly, spinach leaves were decellularized and seeded with human cells for cardiac tissue engineering in the Gaudette lab at WPI. Human endothelial cells successfully seeded and colonized the inner surfaces of the plant vasculature. hMSCs and hPSC derived cardiomyocytes were adhered to the outer surfaces and were able to show contractile function[107]. Other plants such as the leek, *Allium porrum*, have been utilized as scaffolds by seeding with SH-SY5Y human neuroblastoma cells for mammalian cell culture studies[108].

2.5.2. *In Vitro* Plant Cultures as Scaffold Material

An alternative option to standard grown plant tissues is *in vitro* plant cell and tissue cultures. *In vitro* plant cell and tissue cultures can be grown in aggregates and are composed of matrices comprising primarily cellulose, hemicellulose and pectin *In vitro* plant cultures can also be used as scaffold systems. They can be grown in aggregates and are composed of matrices comprising primarily cellulose, hemicellulose and pectin[109]. They can be rapidly grown in culture and also offer the ability to be genetically transformed. This allows for the synthesis of designer proteins and therapeutics which is beneficial for vaccine production and pharmaceuticals[110-111]. *Nicotiana tabacum*, is one such transgenic plant that has been used in generating plant cultures for many areas of research. Our lab has shown that tobacco bright yellow-2 (BY-2) cell cultures can be used as plant-based aggregate culture models after decellularization[14]. Human foreskin fibroblasts (hFFs) were shown to grow readily on BY-2 aggregates. The BY-2 cell cultures are known for their high proliferation rate (80-100-fold per week) and have been transformed to express a reporter protein, EGFP (enhanced green fluorescent protein), fused to a target protein for

the visual detection of the synthesized protein. The target and reporter proteins have been shown to facilitate extracellular secretion[112]. Our lab has showed that after decellularizing the BY-2 via lyophilization and deoxyribonuclease I (DNase I) treatment, the EGFP is retained with the matrix by fluorescence imaging and Western blot analysis. The retention of designer proteins may be beneficial as it provides the possibility to biofunctionalize the decellularized plant culture matrices. Other methods of decellularization, such as by using surfactants like 0.25% SDS or 0.1% Triton X-100 in PBS can result in poorer protein retention[14, 101-102, 107].

Tobacco hairy roots derived from the same transgenic plant species are readily grown in fermenters and can also be transformed to express desired target proteins[14]. Unlike the BY-2 cells which form as aggregates of undifferentiated cells, the tobacco hairy root is seen as highly differentiated plant tissue similar in appearance to adventitious roots of natural plants. By infecting the wounded young leaves from *Nicotiana tabacum*, it leads to a neoplastic growth which is the source of the hairy roots[113]. The roots are continuous structures which when intertwined can form a solid structure for scaffolds.

Chapter 3: Specific Aims

The focus of this project is to characterize tobacco hairy roots and evaluate their potential use as a novel platform for tissue culture.

Aim 1: Characterize Biochemical and Physical Properties of Decellularized Tobacco Hairy Roots

Presence of plant or foreign genetic material may have adverse effects on mammalian cell cultures. For this aim, the degree of decellularization will be evaluated based on the presence of double stranded DNA. Decellularization is defined as the removal of genetic material from a material that was originally occupied with cells. Reduction in in genetic content to less than 50 ng of double stranded DNA per mg of dry weight of the material is defined as being successfully decellularized[14]. The retention of double stranded DNA will be quantified and used to evaluate the decellularization of the plant material. DNA retention will also be visually observed by staining the plant samples for nuclei identification. A BCA assay will be used to quantify overall protein retention. Further characterization will be conducted by imaging the surfaces of the hairy roots to better understand the surface topography that will be utilized for mammalian cell adherence.

Aim 2: Develop Degradation Protocol for Tobacco Hairy Root Derived Matrix at Physiological Conditions

The intension of the project is to grow human cells on tobacco hairy root matrices and be able to detach them from the culture surface using alternative methods to proteolytic enzymes such as trypsin. For that aim we hypothesize that plant degrading enzymes, namely cellulase and pectinase, can be utilized in the degradation of the hairy root matrices at physiological pH and temperature.

If the enzymes cannot function properly within those parameters, it will not be possible to degrade the roots to detach cells. However, the optimal conditions for these enzymes are not compatible with human cells and can cause cell death. A degradation study will be carried out with enzyme in various buffer conditions to be able to optimize the procedure and maintain mammalian cell viability. We also hypothesize that the plant degrading enzymes themselves will not negatively affect cell viability and allow for normal proliferation. The human cells may have to be exposed to the plant degrading enzymes for a significant amount of time to ensure proper degradation for cellular dissociation. As such, it needs to be ascertained if cell viability is not affected by exposure to enzymes using by quantifying DNA at different time points post treatment.

Aim 3: Characterize Cell Seeding on Tobacco Hairy Roots and Subsequent Detachment

For this aim, we hypothesize that cells will be able to successfully adhere to the plant derived matrices and be able to proliferate and subsequently dissociate for the purposes of cellular expansion. Surface modification of the matrices should be able to improve cell adhesion and might prove necessary due to cellulose having a negative charge similar to that of cells. Fluorescence imaging will be used to determine if cells adhere to the surface and then are properly removed after treating with a dissociative enzyme. Trypsin will be utilized as a control enzyme as it is the standard method for cell detachment. Quantitative analysis can be performed by measuring DNA content in the mammalian cells.

Chapter 4: Methods

This chapter will detail the procedures followed to meet the specific aims of this project. The methods for collection, transformation and decellularization of the plant derived material and the step taken to characterize them along with mammalian cell seeding protocols are discussed in detail.

4.1. Plant Cell Culture

The *Nicotiana tabacum* plant modified to express $(SP)_{32}$-EGFP by using *Agrobacterium rhizogenes* ATCC 15834 to infect young leaves of the transgenic plant was provided by Jianfeng Xu (Arkansas State University). The $(SP)_{32}$ tag is comprised of 32 repeats of the "Serine-Proline" motif. This protocol is derived from Tobacco BY-2 transformation for the expression of EGFP with $(SP)_{32}$ tag that was used for the detection and quantification of a target protein being produced by the BY-2 cells. Tobacco hairy roots were cultured in Murashige and Skoog basal media for 12 days at 23°C in shaker flasks at 90 rpm. Murashige and Skoog medium is a plant growth medium used in plant cell culture. It is a nutrient blend of inorganic salts, vitamins and amino acids[114]. The hairy roots were filtered out and washed 3 times with sterile distilled water and then stored at -80°C until further processing.

4.2. Decellularization of Plant Material

Obtained plant material was frozen at -80°C until ready for usage for at least 12 hours. Once ready, samples were then lyophilized (Labconco, Kansas City, MO) for a minimum of 24 hours. This step mechanically permeabilizes the plant matrix and preps it for the next steps of decellularization. After lyophilization, samples were stored at room temperature.

The lyophilized tobacco hairy roots were chemically decellularized using 0.5 mg/mL DNase I (Sigma-Aldrich, St. Louis, MO) in a phosphate buffered saline (PBS) containing 0.492 mM Mg^{2+} and 0.9 mM Ca^{2+}. For 2 mg of plant material, 1 mL of the DNase I solution was used. This protocol is based on an optimization study which was previously done in the lab[14]. Though it was designed originally for BY-2 work only, it was adapted for the Hairy Roots also. DNase I powder was dissolved in the PBS+ (the "+" sign indicates presence of divalent cations) to create a 1 mg/mL solution. For every 2 mg of plant matter, 0.5 mL of DNase I solution was used. Samples were degassed while in solution to draw out air bubbles trapped within the matrix for 5 minutes with intermittent re-entry of air. They were incubated at 37°C for either 30 minutes (for the "30 min" samples) or for 12 hours (for the overnight or the "O/N" samples). The samples were then washed 4 times using PBS only with centrifugation at 14,000 rpm for 5 minutes between each wash step (Eppendorf Centrifuge 5415 C).

For the characterization studies of the decellularized hairy roots, control samples were developed. The untreated "Control" consisted of dry plant material that received no additional treatment after lyophilization. The "-DNase" contained plant material that had been immersed in PBS containing 0.492 mM Mg^{2+} and 0.9 mM Ca^{2+} without DNase I for both 30 minutes and overnight. The -DNase samples were also washed and spun down 4 times using PBS similar to the DNase treated samples above.

For the characterization study on the hairy roots, a set of samples were crushed using a micro-pestle to create Pulverized Hairy Roots. This was done to check if the decellularization protocol could be improved for the plant material.

4.3. Characterization of Plant Derived Matrices

This section will cover the different assays and protocols used to characterize the decellularized plant material. It will cover the methodology for the DNA and protein quantification and qualitative analysis using histology and scanning electron microscopy (SEM).

4.3.1. DNA Quantification

A Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA) was used to measure DNA content in the plant material. Two mg of hairy roots were approximated into microcentrifuge tubes. Samples were treated with 0.5 mL of 125 µg/mL papain (Worthington Biochemical Corp., Lakewood, NJ) dissolved in 100 mM sodium phosphate buffer with 10 mM EDTA-Na_2 (PBE) and 11.1 mM L-cysteine and then incubated at 60°C for 16 hours. The samples were centrifuged at 14,000 rpm for 5 minutes and 10 µL of the supernatant was diluted 1:10 with 90 µL of 1× Tris-EDTA (TE) Buffer. The diluted supernatant was then incubated with a 200-fold dilution of PicoGreen dye in TE buffer for 5 minutes at room temperature in the dark using a 96-well black plate as per manufacturer's protocol. Florescence was read at an excitation wavelength of 480 nm and an emission wavelength of 520 nm (Victor Multilabel Plate Reader, Perkin Elmer, Waltham, MA) and compared against a lambda DNA standard curve.

4.3.2. Total Protein Quantification

A Pierce Bicinchoninic Acid (BCA) assay kit (Thermo Scientific, Rockford, IL) was used to quantify total protein retention after all the processing steps. Approximately 2 mg of plant material (exact amounts recorded) was treated with 0.5 mL of 3 M guanidine hydrochloride for 16 hours at room temperature. For the dry "No Treatment" control. This solubilizes any protein retained in the cell walls. This was then centrifuged for 5 minutes at 14,000 rpm. The supernatant

was removed and mixed with an equal volume of the BCA working reagent in a 96-well plate as per manufacturer's protocol. The plate was incubated at 37°C for 30 minutes. Absorbance values were measured at 562 nm (SpectraMax 250, Molecular Devices, San Jose, CA) and compared against a protein standard of bovine serum albumin (BSA).

4.3.3. Fluorescence Imaging

To visually evaluate nuclei and EGFP, tobacco hairy root samples were fixed in 10% formalin for 30 minutes and then stained with Hoechst nuclei stain (ThermoFisher Scientific, Waltham, MA). Images were taken with a digital camera (SPOT Insight CMOS 5.1) on an upright microscope (Nikon Eclipse E600). The nuclei fluorescence was measured using a 352 nm DAPI filter cube. EGFP fluorescence was detected using 485 nm FITC filter cube.

4.3.4. Scanning Electron Microscopy

Scanning Electron Microscope (SEM) images of tobacco hairy roots were taken using the desktop Phenom G1 SEM (ThermoFisher Scientific, Waltham, MA). Samples were first made to undergo different treatments (-DNase or +DNase treatments as mentioned in Section 4.2) and then lyophilized. After freeze drying, samples were sterilized via autoclave or ethylene oxide (EtO) treatment method. Samples were then mounted on SEM pin stub specimen mounts using conductive copper tape and allowed to air dry in the fume hood. Samples were the coated with a 3:2 ratio of gold and palladium particles on the sputter coater (Quorum 150R ES, Electron Microscopy Sciences, Hatfield, PA) to a thickness of 50 nm.

4.4. Degradation Study

To validate alternative detachment protocols for cells seeded on hairy root matrices, a degradation protocol is required at physiological conditions for cultured cells (**Figure 6**).

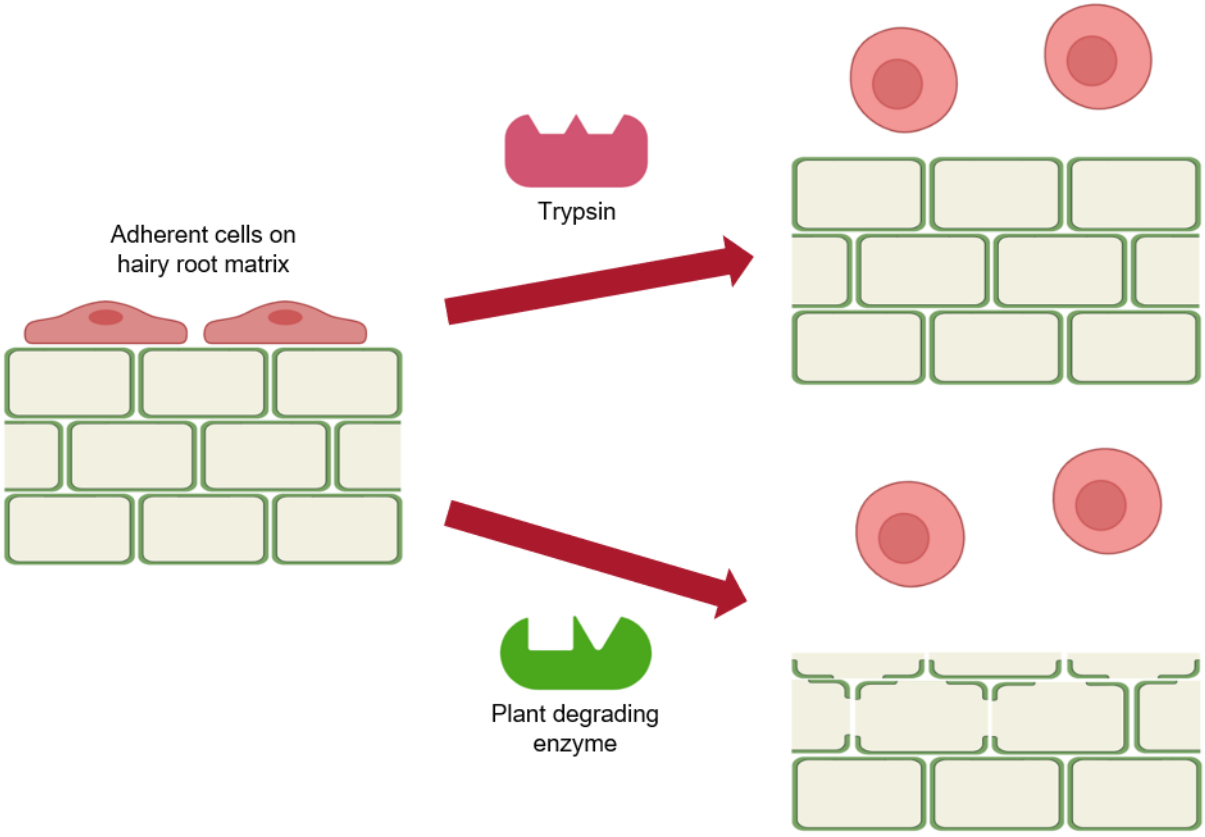

Figure 6: Cell detachment concept
Adherent cells such as hFFs are normally detached from the culture surface using proteolytic enzymes such as trypsin. Trypsin can be harmful to cells if exposed for an extended amount of time and can cause a reduction in surface antigen on hMSCs. If adherent cells can be grown on a degradable substrate such as plant-based materials, it may be possible to detach cells by breaking down the substrate using plant degrading enzymes. Degradation of the underlying plant-based matrix would release the cultured cells into the media without harming them.

The following methods were followed to determine degradation profile of tobacco hairy using plant degrading enzymes in various buffer conditions. Decellularized plant material was transferred into microcentrifuge tubes and the dry weight measured (~5 mg). Triplicate samples were treated with enzymes in different buffer conditions (**Table 2**).

Table 2: Degradation study with enzyme cocktail of cellulase and pectinase

Treatment	**Citric Buffer 10%**	**Citric Buffer 1%**	**DMEM (acidic) 1%**	**DMEM 1%**	**CM 1%**	**Control**
Buffer	Citric Acid Buffer	Citric Acid Buffer	DMEM	DMEM	Complete Media	Milli-Q H_2O
pH	6.01	6.01	6.03	8.29	7.4	6.51
Temperature (°C)	60	60	37	37	37	25
Enzyme Conc. (% Stock, v/v)[a]	10	1	1	1	1	0

[a] 1:1 ratio of cellulase and pectinase at stated percentage of each enzyme stock

For the control, samples were treated in water without enzymes. Citric acid buffer was adjusted to a pH of 6 to create the optimal acidic environment for the enzymes[115]. Dulbecco's Modified Eagle Media (DMEM) was prepared for both acidic and physiological conditions. Complete media was prepared by supplementing DMEM with 1% Penicillin/Streptomycin and L-Glutamine, and 10% Fetal Bovine Serum (FBS). The plant degrading enzymes used in this study are cellulase (from *Trichoderma reesei*, 700 U/g) (Sigma-Aldrich, St. Louis, MO) and pectinase (from *Aspergillus aculeatus*, 3800 U/mL) (Sigma-Aldrich, St. Louis, MO). These enzymes are known to breakdown cellulose and pectin, respectively, via hydrolysis which are common components of plant-based materials[99]. The enzymes were diluted to the working concentrations and used in combination in each respective buffer solution. For each sample, 1 mL of the enzyme solution was added. Samples were agitated by rotating at 180 rpm on plate shakers at 37°C (Multi-Platform Shaker, ThermoFisher Scientific) or at 60°C (Multi-Purpose Rotator Model: 2314, ThermoFisher Scientific). The control was left to agitate at room temperature. After 16 hours, the samples were centrifuged at 14,000 rpm for 5 minutes (Eppendorf Centrifuge 5415 C). The supernatant was removed, and the samples were washed once with PBS and twice with Milli-Q H_2O. Samples were allowed to dry at 60°C for 36-48 hours before measuring the sample dry weight.

Weight was measured several times on a scale until constant weight was observed. Weight remaining after degradation was calculated as a percentage using the equation below:

$$Weight\ Remaining\ (\%) = 100 \times \frac{W_t}{W_0}$$

where W_o is the initial dry weight of the sample, and W_t is the dry weight of the sample after treatment.

A second degradation experiment was carried out based on results from the enzyme biocompatibility study. Instead of using a cocktail of cellulase and pectinase, cellulase alone was used at concentrations of 1% and 10% of stock (**Table 3**). All samples were agitated by rotating at 180 rpm on the Multi-Platform Shaker at 37°C. Samples were then collected and washed as mentioned previously. Dry weight was then measured and analyzed.

Table 3: Degradation study with cellulase enzyme

Treatment	**Citric Buffer 10%**	**Citric Buffer 1%**	**DMEM 10%**	**DMEM 1%**	**CM 10%**	**CM 1%**	**Control**
Buffer	Citric Acid Buffer	Citric Acid Buffer	DMEM	DMEM	Complete Media	Complete Media	Milli-Q H_2O
pH	6.01	6.01	8.29	8.29	7.4	7.4	6.51
Temperature (°C)	37	37	37	37	37	37	25
Cellulase (% stock, v/v)	10	1	10	1	10	1	0

4.5. Plant Degrading Enzyme Biocompatibility Study

To check for biocompatibility of the plant degrading enzymes, hFF cells were seeded into 48-well plates for the acute and 5-day toxicity studies, and into a 6-well plate for the replating toxicity study. Cells were exposed to various concentrations of the enzyme stocks. Three primary experiments were performed to check for acute toxicity on the cells, a 5-day study after exposure

to the enzymes, and a replating study where cells were detached after exposure and then replated onto fresh tissue culture plastic.

4.5.1. Acute Toxicity Study

For the acute toxicity study, hFFs were seeded at 5,500 cells per well into a 48-well plate in 300 µL of hFF complete media. Once cells reached confluency, wells were treated with different concentrations of enzyme (**Figure 7**). Cells were incubated at 37°C for an hour. After treatment, cells were rinsed once with complete media and then treated with 300 µL of 0.1% Triton X-100 in TE buffer for 15 minutes to lyse the cells and expose their genetic materials. The cells were vigorously pipetted and then transferred to 1.5 mL microcentrifuge tubes and frozen at -20°C for at least 24 hours. Frozen samples were then thawed and assayed for dsDNA content using a PicoGreen assay as per the DNA quantification protocol (4.3.1.).

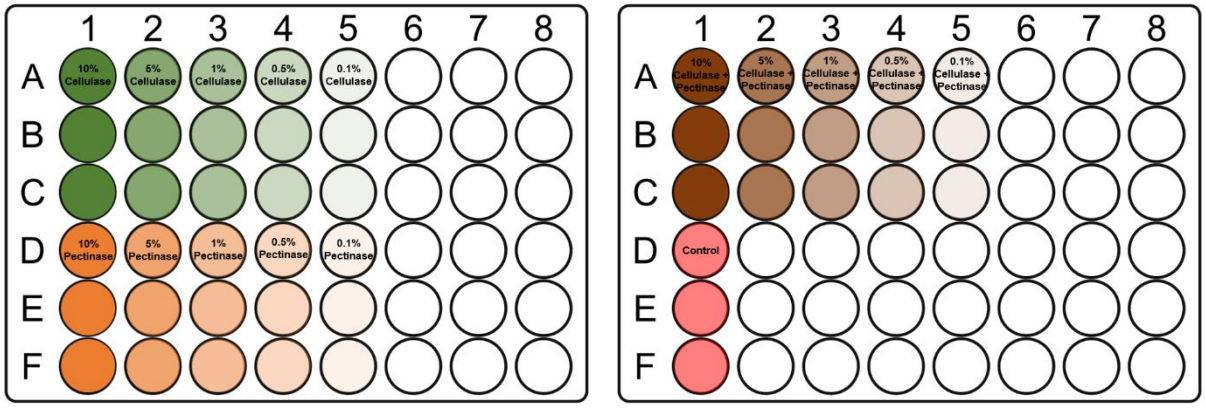

Figure 7: Experimental plan for different enzyme treatments for toxicity studies
48-well plate layouts for the biocompatibility assays for the acute and 5-day toxicity studies are outlined. Triplicates of each enzyme concentration were utilized in testing cell viability after treatment.

4.5.2. Five-Day Toxicity Study

For the 5-day toxicity study, hFF cells were seeded at 5,500 cells per well in a 48-well plate similar to the acute toxicity study (**Figure 7**) and treated with different enzyme concentrations for an hour at 37°C. After enzyme treatment, cells were rinsed once with complete media and then the

cells were allowed to grow in culture in fresh media. After 5 days, the experiment was concluded by treating cells with 300 µL of 0.1% Triton X-100 in TE buffer for 15 minutes and then transferring and freezing the solutions in tubes at -20°C. DNA content was measured using a PicoGreen assay.

4.5.3. Replating Toxicity Study

For the replating study, hFF cells were seeded into a 6-well plate at 48,000 cells in 2 mL of media. Once cells reached confluency, they were treated for one hour with varying concentrations of cellulase at 37°C (**Figure 8**). After treatment, cells were rinsed once with hFF media and then treated with 0.25% trypsin-EDTA to detach the cells. The cells were resuspended in fresh media and then replated into a 48-well plate using a 1:4 split with triplicates per treatment condition and allowed to grow. After 5 days, cells were treated with 300 µL of 0.1% Triton X-100 in TE buffer for 15 minutes and then frozen at -20°C for at least a day before quantifying the DNA content using a PicoGreen assay.

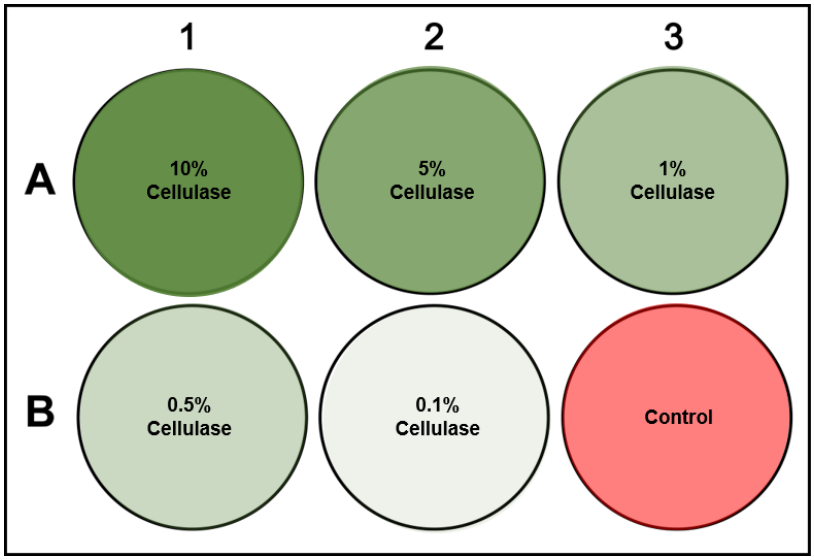

Figure 8: Experimental plan for enzyme replating toxicity studies
6-well plate layout for hFF seeding. Seeded cells were exposed to various concentration of cellulase for an hour and were split and replated using trypsin into a 48-well plate with a layout similar to that for cellulase in **Figure 7**.

4.6. Human Cell Seeding on Tobacco Hairy Roots

4.6.1. Surface Coating and Initial Seeding Strategies

Hairy roots were decellularized in bulk (~100 mg), as per the protocol in section 4.2 with DNase I, in 50 mL conical tubes. Samples were lyophilized and then sterilized using EtO gas. Samples were placed in the fume hood over 2 days to off-gas residual EtO. Five mg of the sterilized plant matrices were loaded into sterile 1.5 mL microcentrifuge tubes for further processing. Hairy roots are primarily composed of cellulose which has net charge which does not promote cell attachment (**Figure 9**). We theorized that the addition of a poly-L-lysine coating would induce a positive charge that would benefit cell attachment. To test the theory, samples were coated with either 0.01% w/v cell culture grade poly-L-lysine solution (P4832, Sigma-Aldrich, St. Louis, MO) or 2% w/v type A gelatin from porcine skin (G1890, Sigma-Aldrich, St. Louis, MO). For both treatment condition, 0.5 mL of the coating solution was added to each sample tube. The tubes were loaded onto a Mini Tube Rotator (ThermoFisher Scientific, Waltham, MA) and rotated for 30 minutes at 10 rpm to ensure sufficient coating. The hairy root samples were transferred to separate wells in a 24-well plate and allowed to dry for at least 2 hours in the biosafety cabinet. Dried samples were then transferred to new sterile 1.5 mL microcentrifuge tubes. Control samples were left uncoated.

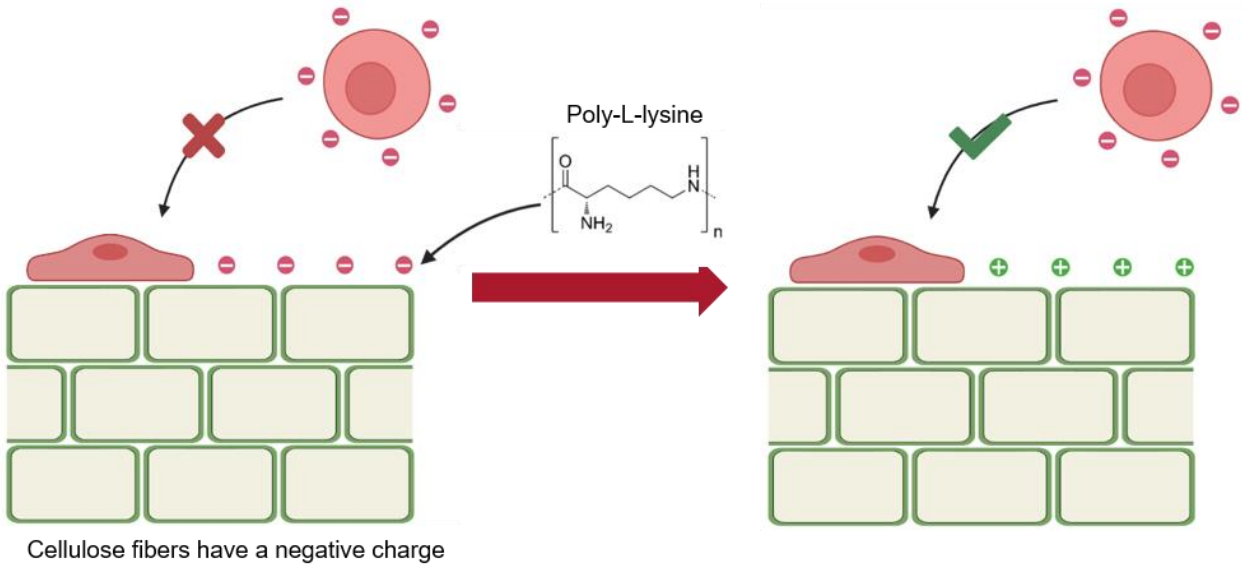

Figure 9: Surface charge affecting cell attachment
Cellulose fibers have a negative surface charge which does not facilitate cell adhesion. The membranes of most adherent cell types have a net negative charge. As like charges repel, modifying the surface of the cellulose via poly-L-lysine adsorption resulting in a positive charge could improve cell adhesion.

For the surface coating study hFFs were seeded at 500,000 cells per microcentrifuge with ~5 mg decellularized hairy roots in 500 µL of hFF media. Cells were allowed to grow at 37°C for 5 days. After the treatment durations, cells on the matrices were stained using Hoechst and imaged using fluorescence microscopy. The cells attached to the matrices were then treated with either 1% cellulase or 0.25% trypsin-EDTA for 5 minutes. The matrices were reimaged to verify detachment of cells.

4.6.2. Five- and Ten-Day Seeding Study

Decellularized tobacco hairy root matrices were sterilized using EtO and then coated using 0.01% w/v cell culture grade poly-L-lysine solution. Approximately 5 mg of the coated hairy roots was aliquoted into microcentrifuge tubes and seeded with 500,000 hFFs. The cells were allowed to grow for 5 and 10 days at 37°C. After the experimental durations for each time point, a set of samples were rinsed and fixed in 10% formalin. Cells were permeabilized using 0.1% Triton X-100 in PBS (+). Nuclei were stained using Hoechst and the actin cytoskeleton was stained using a

1:100 dilution of Phalloidin 594 (Biolegend). The samples were imaged using fluorescence microscopy. Another set of samples were lysed using 0.1% Triton X-100 in TE buffer for 15 minutes and then frozen for PicoGreen assay to quantify DNA content.

For the 5-day and 10-day studies, samples were treated with either 10% cellulase in DMEM or 0.25% trypsin-EDTA to detach the cells. Samples treated with trypsin were incubated for 5 minutes at 37°C. Proteolytic enzyme activity was inhibited by adding hFF complete media to the samples. Cellulase treated samples were agitated at 75 rpm on the Multi-Platform Shaker for 1 hour at 37°C. The detached cells were reseeded into 6-well plates and continued to grow in culture. Detachment from the matrices was verified by Hoechst staining and fluorescence microscopy. The reseeded cells were kept in in culture at 37°C for 5 days and the lysed using 0.1% Triton X-100 in TE buffer for 15 minutes. Samples were frozen at -20°C for at least a day before being assayed using PicoGreen.

4.7. Statistical Analysis

For all experiments, data are presented as mean ± standard deviation from three biological replicates unless stated otherwise. For the PicoGreen and BCA assays an additional technical replicate for each biological replicate was also performed. Statistical significance was determined using a one-way ANOVA followed by a Tukey honest significant difference test using GraphPad Prism (version 9.1.0). Unpaired t-tests were carried out for the 5- and 10-day seeding studies when applicable. Significance was accepted at $p < 0.05$.

Chapter 5: Results

5.1. DNA Quantification

To evaluate the effectiveness of DNase treatment on removing DNA, matrices were quantitatively evaluated for DNA content. The groups evaluated were hairy roots treated with 1 mg/mL DNase I for 30 min (+DNase 30 min), buffer only for 30 min (-DNase 30 min), 1 mg/mL DNase I overnight (+DNase O/N), or buffer only overnight (-DNase O/N). DNA content of the treated hairy roots were compared to an untreated control. It can be seen that DNA content decreases after any of the treatments (**Figure 11**). Samples that were washed with the PBS buffer (-DNase) retained 51.5% of genetic material after 30 minutes of treatment but only 20.9% after treating overnight. PBS is not known to degrade dsDNA, so loss in genetic material is accounted to leaching and endogenous DNase activity. The -DNase treatment also shows that even after leaching some of the DNA, nuclei are still present.

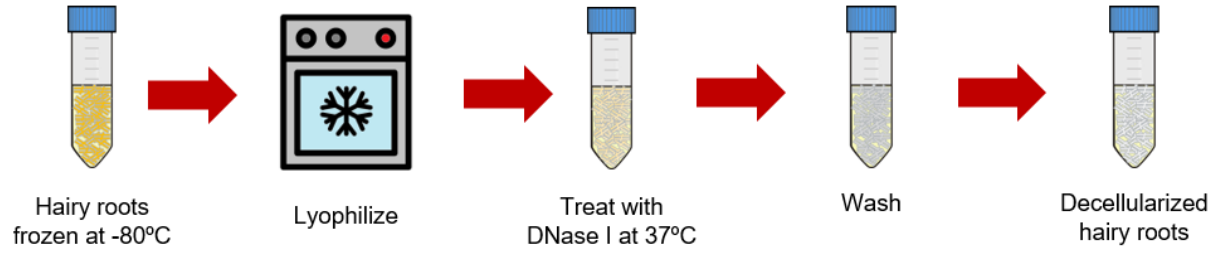

Figure 10: Flow diagram for tobacco hairy root decellularization
Tobacco hairy roots were cultured and then frozen at -80°C. The roots were decellularized mechanically via lyophilization and then chemically using DNase I at 37°C. The processed roots were then washed several times using PBS before being ready for seeding.

DNase I treatment of the hairy roots removes greater than 98% of dsDNA content for both the 30 minutes and overnight treatments. For the +DNase samples, there was no significant difference between sample treatment time indicating that prolonged treatment with the enzyme is not necessary. The level of DNA retained after treatment with DNase I is within the benchmarks of decellularization which is 50 ng of DNA content per mg of decellularized tissue[14]. Fluorescence

imaging shows no apparent nuclei presence within the DNase treated samples. The blue fluorescence observed in images for +DNase is likely due to autofluorescence of the plant-derived material.

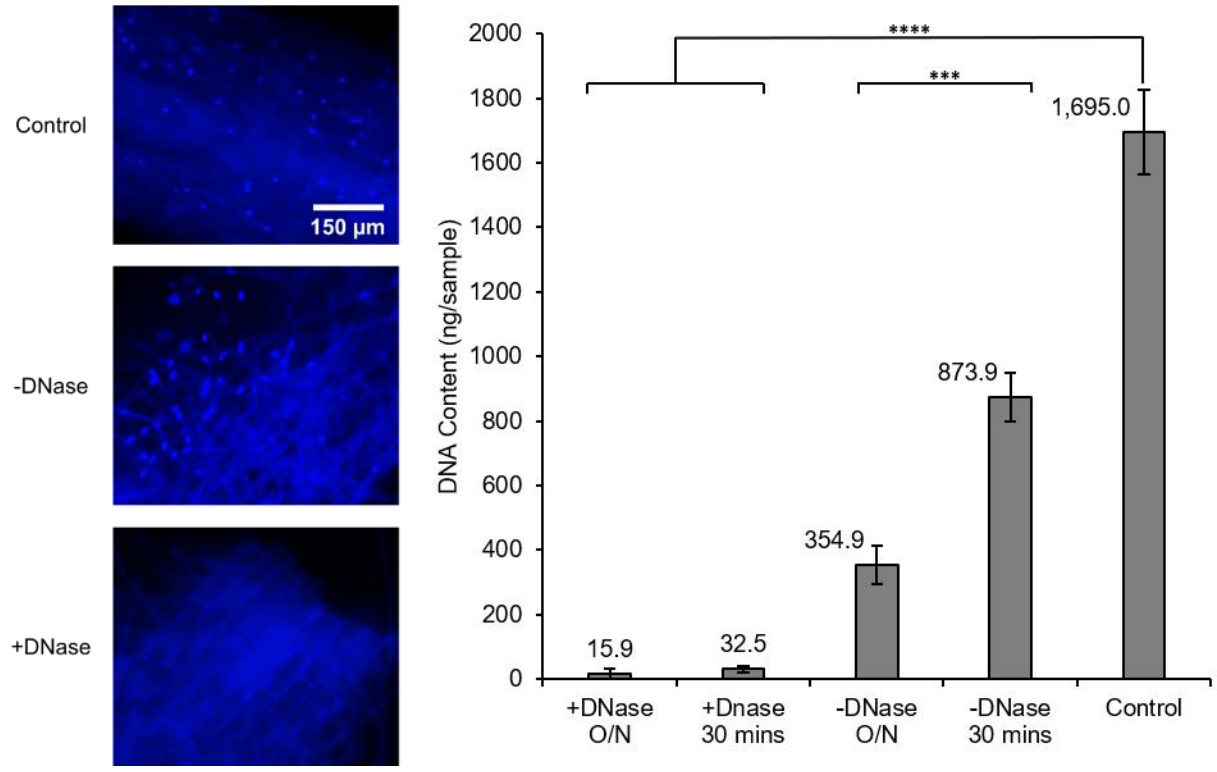

Figure 11: DNA quantification for decellularized tobacco hairy roots
DNA content was evaluated after decellularization. +DNase treatments, both 30 mins and O/N were not significantly different. +DNase treatment removed majority of DNA retained within matrices and can be visually verified by the absence of intact nuclei from fluorescence imaging. -DNase treatment had removed DNA, but not enough to meet decellularization standards. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (******* $p<0.001$, ******** $p<0.0001$).

Given the potential diffusion limitations that the bulk hairy roots may incur on the DNase I, we next evaluated the impact of DNase I treatment on pulverized hair roots. Pulverization results in fine powder formation. The pulverized samples were decellularized and their DNA content was compared to non-pulverized samples (**Figure 12**). This experiment was carried out to test if pulverizing samples could improve the decellularization protocol. Pulverized and uncrushed controls significantly different DNA retentions. The Control had 1,695 ± 130 ng DNA/sample

while the Pulverized Control indicated retention as 2,247 ± 434 ng DNA/sample. This suggests that pulverizing the samples exposed more of the DNA trapped within the tobacco hairy roots that was detected by the PicoGreen assay. For the +DNase treated groups, pulverizing the hairy roots did not show any significant difference to the uncrushed samples. For both the 30 minutes treated and overnight samples, DNA levels are comparable. For all +DNase treated groups, DNA levels are within benchmark levels, and pulverizing the roots does not improve the decellularization protocol.

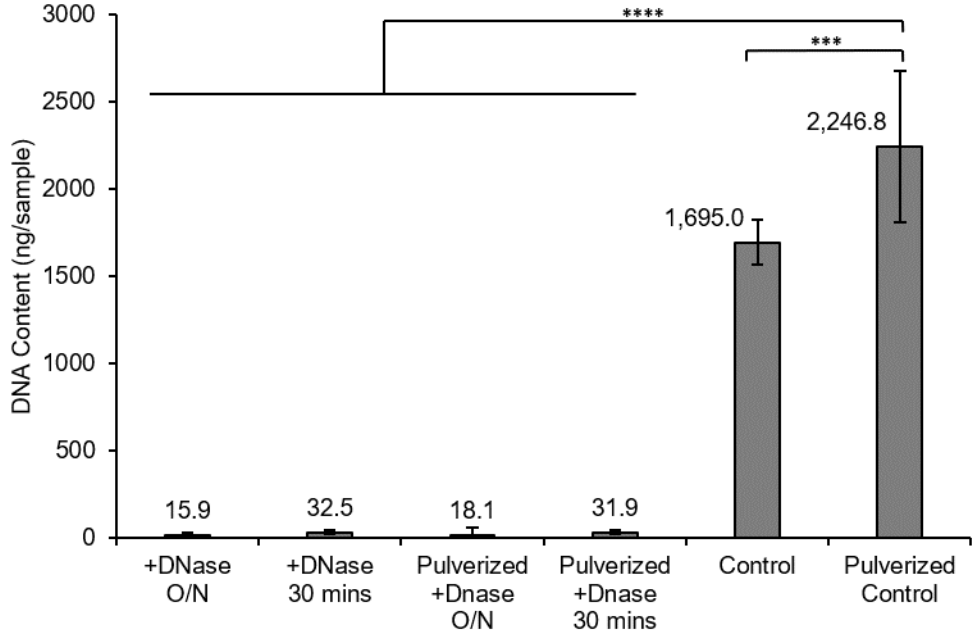

Figure 12: DNA content quantification after pulverizing tobacco hairy roots
Decellularized pulverized tobacco hairy roots were evaluated for DNA content and compared to unpulverized samples. The action of pulverizing did not significantly improve the decellularization protocol. The pulverized control showed significantly higher DNA content compared to the unpulverized control. DNA content was trapped within matrices. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (******* $p<0.001$, ******** $p<0.0001$).

5.2. Protein Quantification

To evaluate the impact of DNase treatment on protein retention, matrices were quantitatively evaluated for protein content. The same non-pulverized groups were evaluated as in the DNA Quantification section. Compared to the untreated control, there is an overall decrease in

retained protein for all other test groups. Overnight treatment for both the +DNase and -DNase groups have lower levels of protein content than their 30-minute counterparts (**Figure 13**). For the +DNase, protein content decreases from 85.2 ± 10.0 μg per sample to 60.6 ± 13.7 μg per sample. Similarly, for the -DNase, protein content decreases from 60.5 ± 10 to 30.2 ± 11.9 μg per sample.

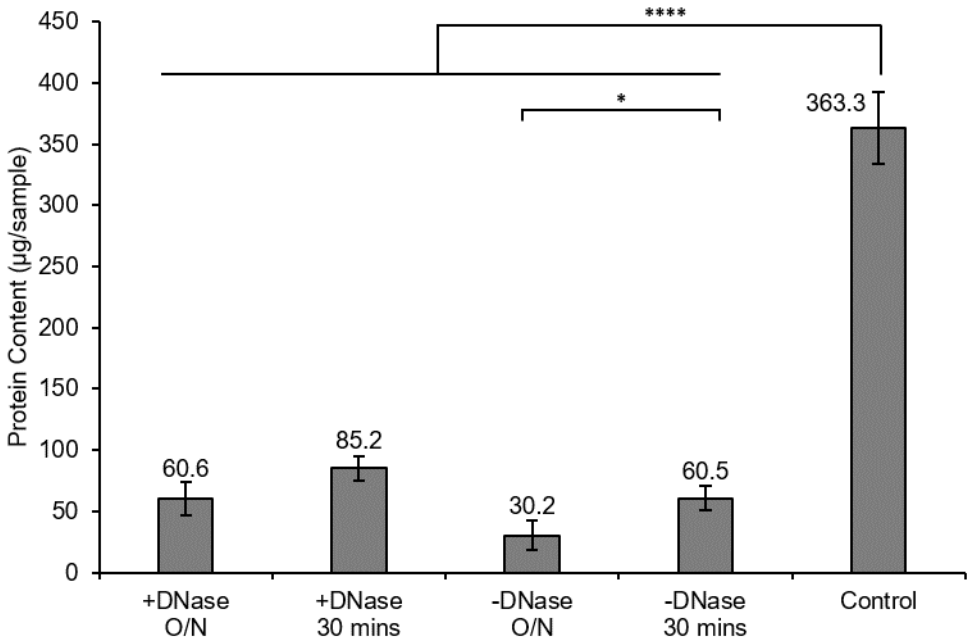

Figure 13: Protein retention for decellularized tobacco hairy roots
Protein retention was evaluated for the different treatments on the tobacco hairy roots. Significant protein loss was observed in the matrices. Protein loss is comparable for the +DNase and -DNase treatments. The -DNase O/N treatment suggests that longer immersion in solution would result in further leaching of proteins. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (***** $p<0.05$, ******** $p<0.0001$).

Longer treatment for the -DNase samples resulted in further removal of protein. Protein loss may be attributed to leaching from the matrices while submerged in solution. It can be concluded that +DNase treatment does not further leach protein and in the context of attempting to keep desirable proteins within the plant-derived matrix, it does not worsen general protein retention (**Figure 13**).

5.3. Fluorescence Imaging

To visually identify nuclei and EGFP within the tobacco hairy roots, fluorescence imaging was performed. Nuclei were stained using Hoechst while EGFP can fluoresce on its own when excited with wavelengths between 460-500 nm. The samples were imaged using a 10x objective and features such as the individual cell compartments and surrounding cell walls can be identified (**Figure 14**). From the brightfield images, the main tubular structure of the roots has hair-like projections protruding from the external surface. This is similar to the fibrous root systems of natural plants. A central channel can also be observed in the brightfield images but is more apparent in the green fluorescence images. The function of these channels is most likely to transport water and other soluble nutrients throughout the hairy root system.

Hoechst dye binds strongly to the minor groove of dsDNA and fluoresces when excited by ultraviolet light. Images for blue fluorescence show solid circular-like bodies in the Control and -DNase samples. These circular bodies are absent in the +DNase samples and indicate the removal of solid nuclei bodies due to the decellularization protocol with DNase I. This information corroborates with DNA quantification data (**Figure 11**). Any blue fluorescence observed not as solid circular bodies but from the matrix may be accounted to unbound genetic material in the samples or autofluorescence of the plant-derived material. The green fluorescence images for EGFP strongly highlight the cell wall structure. The EGFP is escorted out of the cells of the tobacco hairy roots during the plant culture with the help of the $(SP)_{32}$ export protein and was observed to have remained trapped within the matrices after decellularization. Since all the hairy root samples provided express EGFP and there is no control that does not express the reporter protein, it cannot be verified if the green fluorescence is due to the EGFP or autofluorescence of the matrix. It should, however, be noted that the green fluorescence is weaker in the -DNase and +DNase samples than

the untreated Control. If the fluorescence is due to EGFP, it may be reduced due the leaching of the protein when treated in either test group.

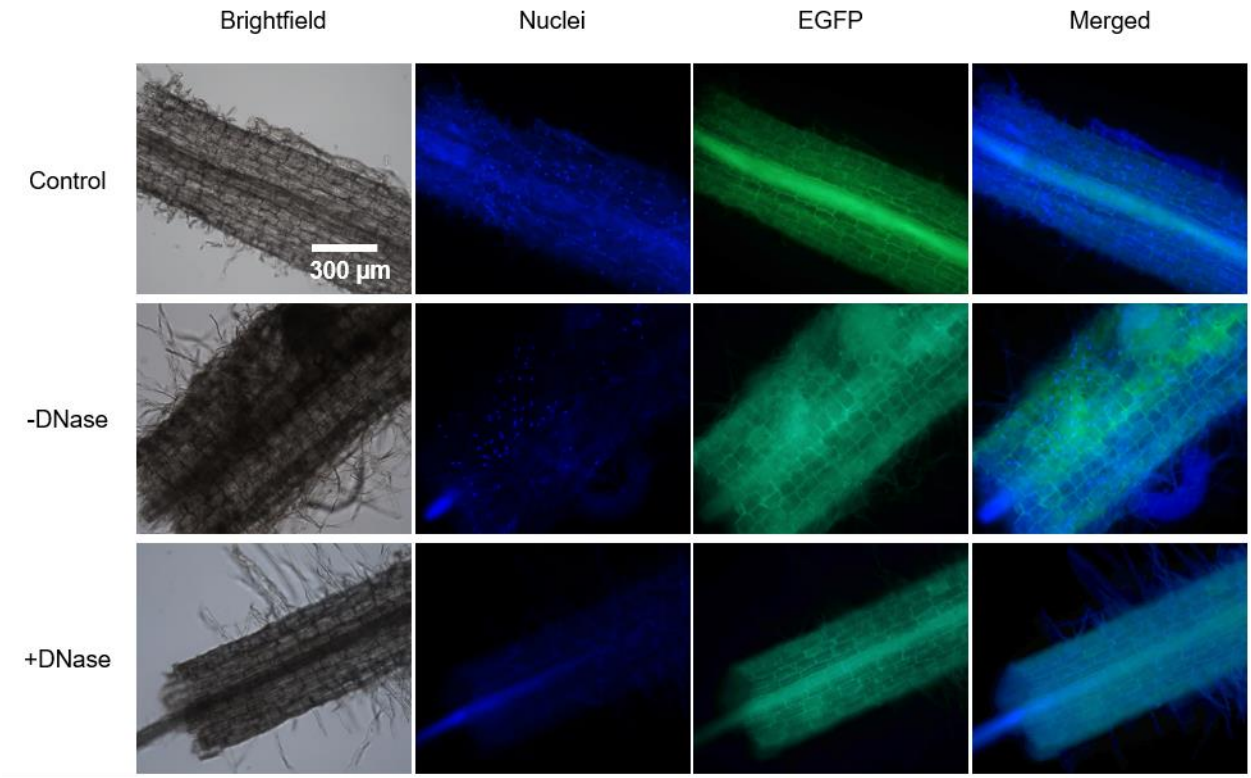

Figure 14: Fluorescence images for decellularized tobacco hairy roots
The effects of the various treatments on the tobacco hairy roots can be visually observed. The control and -DNase samples have intact nuclei present within the matrices. The nuclei are absent in the +DNase samples. EGFP fluorescence is noted to be weaker in the -DNase and +DNase groups.

5.4. Scanning Electron Microscopy

Surface topography of the decellularized tobacco hairy roots after different sterilization treatments was studied with SEM (**Figure 15**). The outer surface of the roots appears to have been damaged from lyophilization and is flaking off. The next layer visible has a smooth characteristic on the Control samples. Autoclaving the roots appeared to cause the smooth layer to cave in on itself. This is apparent as the otherwise smooth surface is replaced with lines and grooves from the internal structure of the cell walls of the hairy roots. The grooves may help in retaining fibroblasts

after seeding but could also trap them and prevent them from spreading out due to contact inhibition of the cells. Precipitates were observed on the surfaces of the -DNase and +DNase treated samples. This is attributed to phosphate salt deposition from the PBS wash steps. Rinsing with water should remove the salt precipitation. This is also noted with EtO treated samples. With the EtO treated samples, the flaky exterior appears to be mostly lost after treatment exposing more of the inner layer.

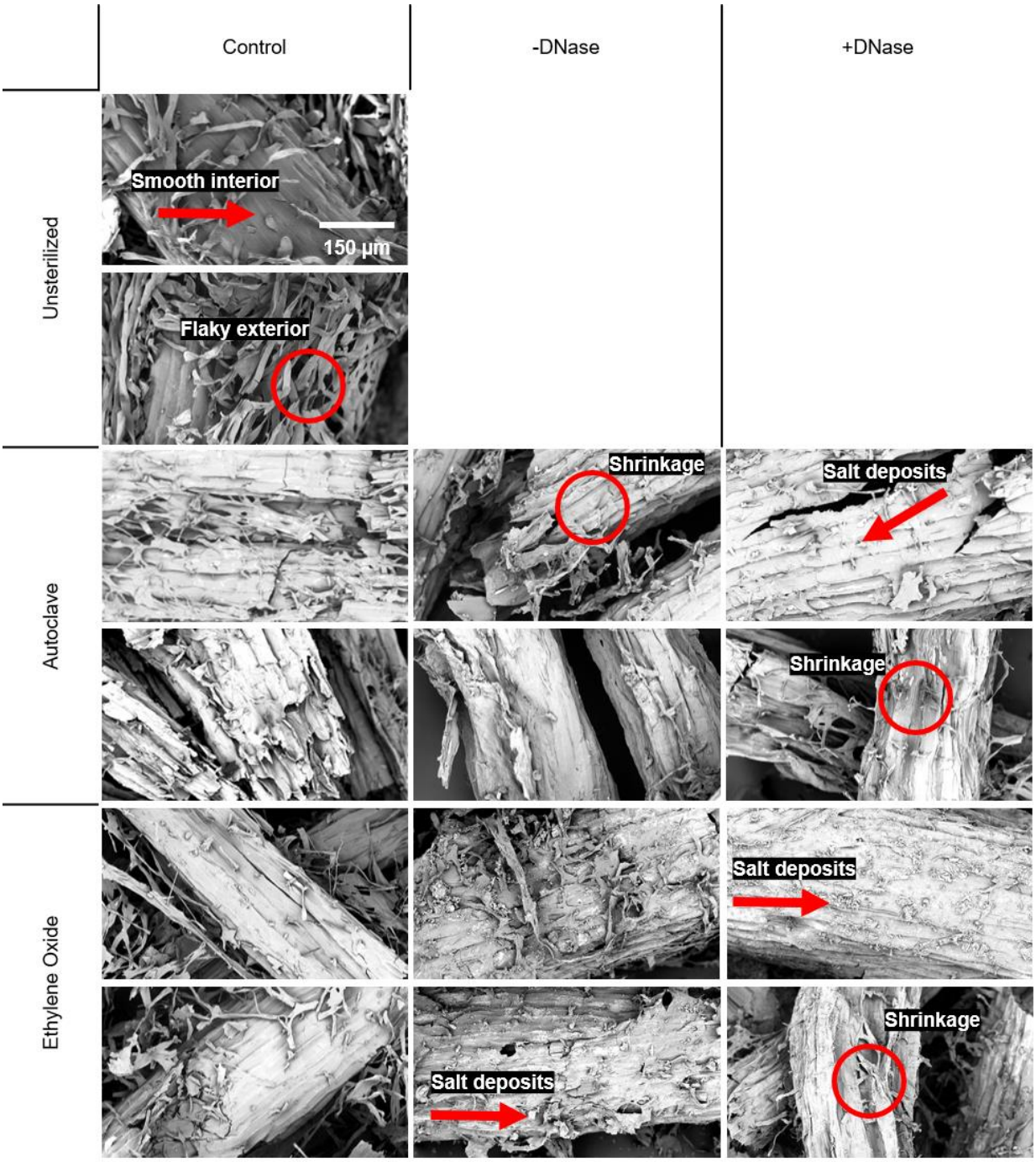

Figure 15: SEM images of tobacco hairy roots after different treatments and sterilization methods
Scanning electron microscopy images were taken for the decellularized tobacco hairy roots and controls after two different sterilization methods. Surface topography of the hairy roots can be observed for the different treated samples. The Controls had more intact root structures with a flaky exterior. Effects of either the +/-DNase treatments resulted in alteration of surface morphology resulting in drier and shriveled up matrices. External damage is pronounced, but general structure of the roots is retained. The +/-DNase treatments resulted in small deposits on the surface of the matrices.

5.5. Degradation Study with Cellulase and Pectinase

The tobacco hairy root matrices were exposed to two types of plant degrading enzymes to be able to break down the cell wall. Cellulase and pectinase were chosen since they target the common components of cell wall material. Samples were treated with the enzymes in different buffer conditions and percentage weight remaining after enzymatic was calculated (**Figure 16**). Weight remaining after enzyme treatments were similar among the different buffers groups and had significant loss in mass compared to the Control. The Control was used to take into account loss in plant material between wash steps due to frequent pipetting. Loss in plant material is comparable for each of the treatment groups. The effects of pH, temperature or concentration do not alter degradation based on the experimental design. This may be due to the enzyme having completed its activity within the treatment time for all buffer conditions, or there may be another variable affecting enzymatic degradation. The 1% w/v enzyme cocktail appears to be able to degrade approximately 30% of the tobacco hairy root matrices at physiological conditions in DMEM basal media. Weight loss is similar to having a 10-fold higher concentration of the enzymes in low pH (6.0) and high temperature (60°C).

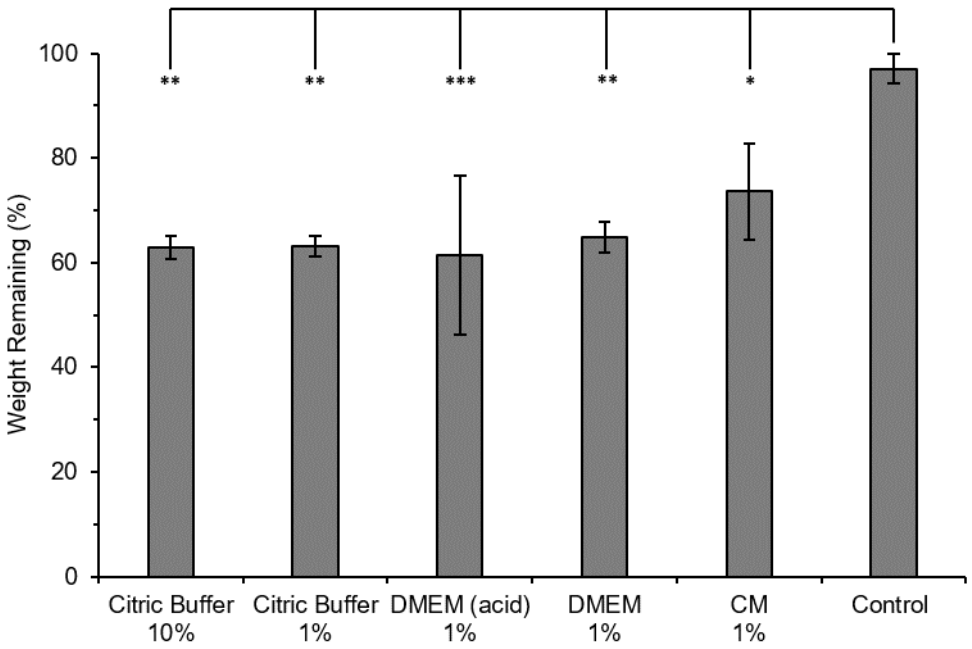

Figure 16: Degradation study of tobacco hairy roots with plant degrading enzymes
A 1:1 cellulase to pectinase was utilized for this degradation study. The enzyme cocktail showed significant loss in plant matrices for all samples compared to the control. No significant difference is observed within sample groups. Changes in pH, temperature or concentration did not vary weight loss in the matrices. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (***** $p<0.05$, ****** $p<0.01$, ******* $p<0.001$).

5.6. Biocompatibility Studies with Plant Degrading Enzymes

For plant degrading enzymes to be used as an alternate cell detachment method by breaking down plant matrices, mammalian cell viability throughout the treatment process and after needs to be maintained. The effect of enzyme on hFF viability was measured using a PicoGreen assay after one hour of treatment and allowing the cells to grow for different durations. For acute toxicity, samples were immediately evaluated after treatment with the enzymes. The control, which is treatment with DMEM basal media without enzymes, is represented as 0% enzyme concentration for each of the different enzyme groups (**Figure 17**).

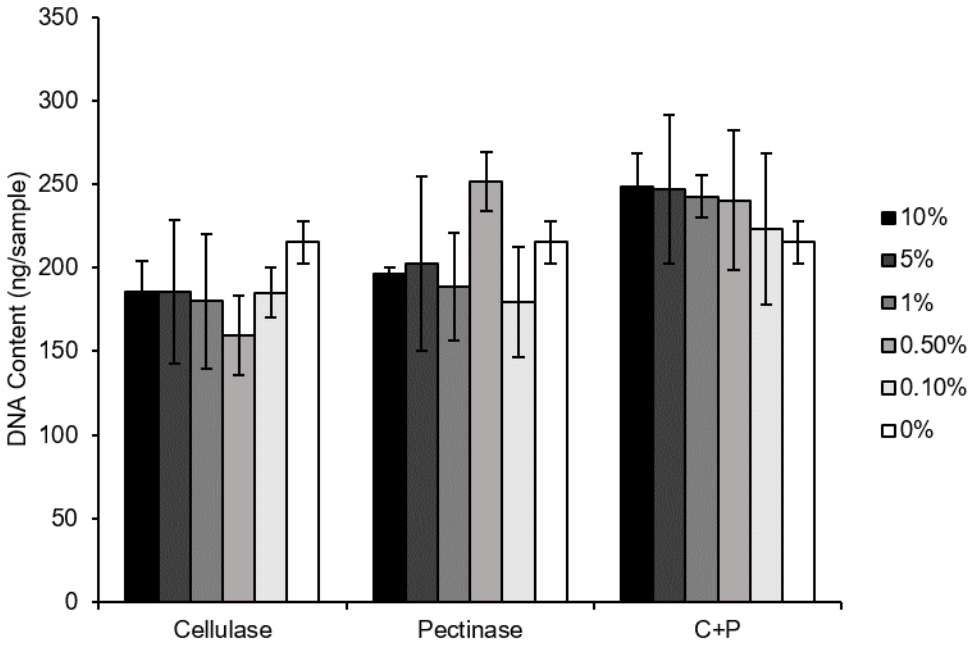

Figure 17: Plant degrading enzyme biocompatibility study (acute)
Acute toxicity was measured by quantify DNA in hFFs immediately after treatment with different concentrations of plant degrading enzymes for 1 hour. Cell viability for cellulase, pectinase and C+P (1:1 ratio of cellulase and pectinase) did not significantly differ from the Control which was represented by the 0% bar in the graph. Data are presented as the mean ± standard deviation of three separate samples.

For the 5-day study, when the hFFs were cultured post-treatment, pectinase exposure appeared to negatively impact the cells (**Figure 18**). Wells treated with pectinase had exhibited reduced cell viability. DNA content fell below around 50% of the control for the pectinase only treated samples. Cellulase alone at all concentrations evaluated did not significantly affect hFF viability. Therefore, subsequent experiments were performed with cellulase and not pectinase.

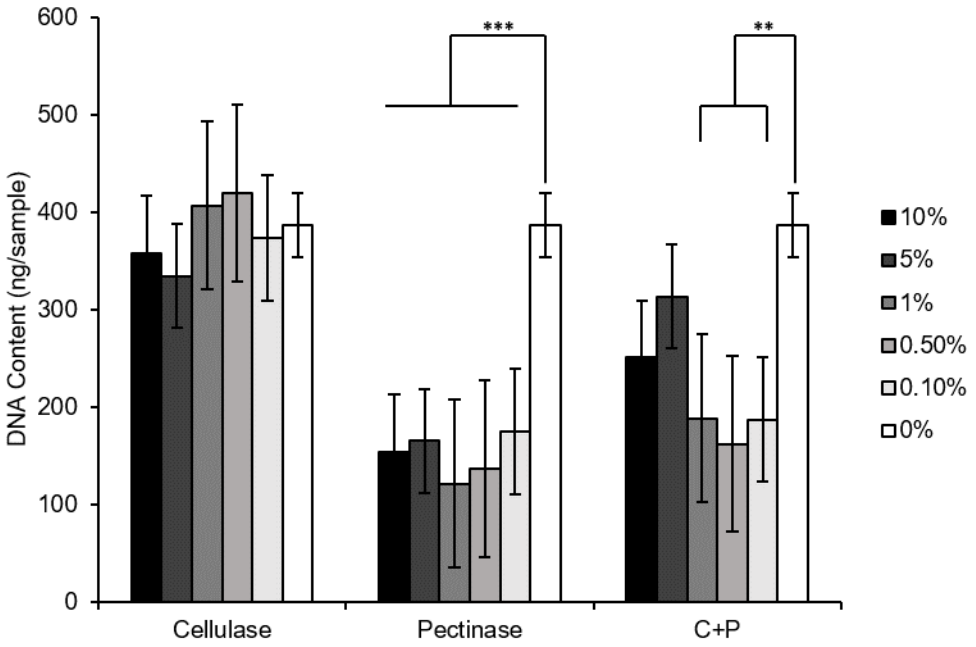

Figure 18: Plant degrading enzyme biocompatibility study (5-day)
Plant degrading enzyme toxicity was evaluated after growing treated cells for 5 days. Cellulase treatment did not cause any adverse effects on the hFFs and was similar to the Control (0%). Pectinase caused significant decrease in DNA content and also resulted in lower hFF viability in the C+P group. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (****** $p<0.01$, ******* $p<0.001$).

To further verify that the hFFs were not negatively impacted by cellulase exposure, a replating study was performed by treating cells with cellulase at various concentration, detached with trypsin, and replated for continued cell growth. DNA content was measured and compared with the 0% control (**Figure 19**). Higher concentrations of cellulase did not significantly affect hFF viability. After allowing the cells to grow for 5 days from replating, cell viability is similar for all test concentrations without any significant difference from the control.

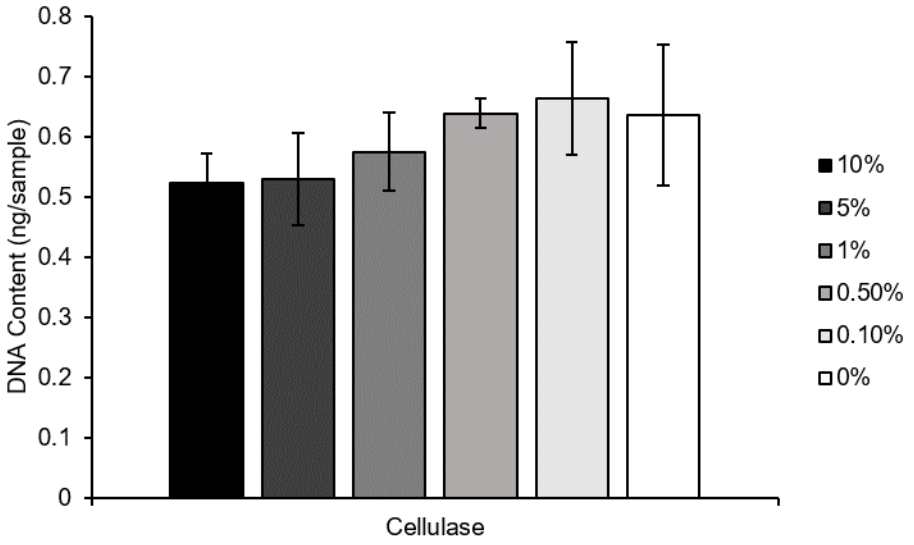

Figure 19: Cellulase biocompatibility study (replating)
hFFs exposed to cellulase for an hour were replated onto new tissue culture plastic using trypsin. After 5 days of culture, cell viability was measured using a DNA assay. Changing the concentration of cellulase did significantly affect hFF compared to the Control (0%). Data are presented as the mean ± standard deviation of three separate samples.

A follow up degradation study was carried by only using cellulase as the sole plant degrading enzyme. Moving forward all studies relating to plant degrading enzymes were carried out using cellulase due to the adverse effects of pectinase.

5.7. Degradation Study with Cellulase Only

As temperature was noted as not being a primary factor in altering enzymatic degradation (**Figure 16**), all samples were incubated at 37°C. Two different concentrations of cellulase (1% and 10%) were tested in three different buffers: Citric buffer (pH 6) and DMEM basal media and hFF complete media (CM) both of which were in the physiological pH range (**Figure 20**). Within each buffer conditions, the effect of concentration does not have any significant effect. Compared to the control, approximately 35% of matrices were reduced through degradation in the Citric Buffer test group. For the DMEM and CM test groups, around 30% of mass was reduced through degradation.

It was shown that it possible to degrade some of the plant matrices using only cellulase at a concentration of 1% w/v to stock in DMEM and complete media at physiological conditions.

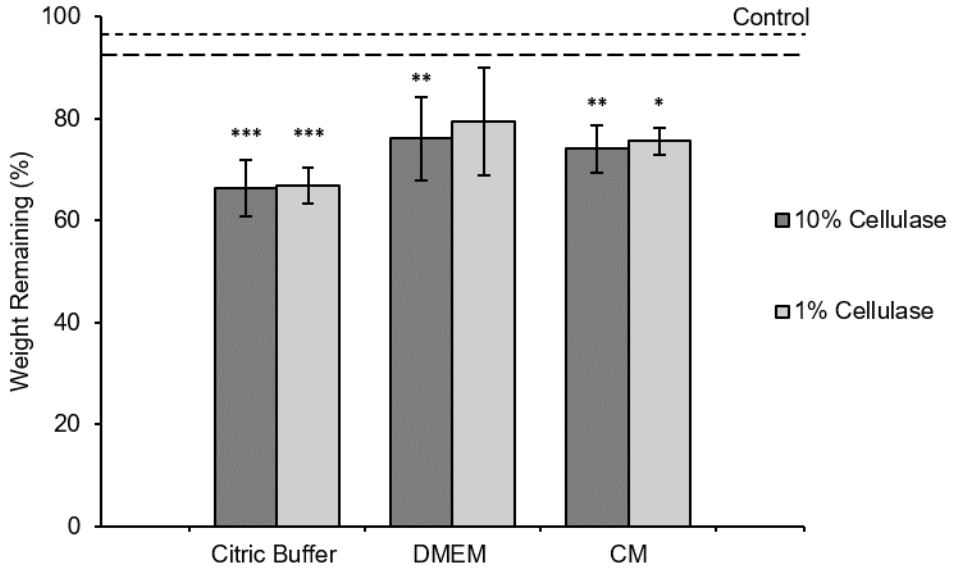

Figure 20: Degradation study of tobacco hairy roots with cellulase
Degradation study with cellulase as the sole enzyme was carried with the tobacco hairy root plant matrices in different buffers and concentrations. No significant differences in weight for the two enzyme concentrations for the different buffers was observed. Significant loss in material occurred for all samples except for DMEM 1% sample. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (***** $p<0.05$, ****** $p<0.01$, ******* $p<0.001$).

5.8. Human Cell Seeding Studies

hFFs were seeded onto tobacco hairy roots with different coatings to determine if it is necessary to achieve cell adhesion. Plant-based cellulose tends to have a negative surface charge which can affect cell adhesion since mammalian cells also have a net negative charge. Tissue culture plastics and microcarrier beads are usually designed with a coating that promotes cell adhesion. One such method is to give the tissue culture surface positive charge. Poly-L-lysine was chosen since it is a protein that is primarily used to provide a net positive charge to any surface. Gelatin was used as it is a known biocompatible material on which mammalian cells readily adhere and proliferate.

Uncoated hairy roots did not promote cell adhesion (**Figure 21**), as few cells, indicated by the stained nuclei, are observed to have settled on its surface. The 2% gelatin coating and 0.01% ploy-L-lysine coating achieved better results in terms of cell adhesion as more nuclei and blue fluorescence is observed. With the samples that were left untouched after seeding (still), concentrated clumps of cells are noted due to the higher concentrations of blue fluorescence making it difficult to single out individual nuclei. Samples that were rotated for 30 minutes (roll) had more even cell spacing and attachment.

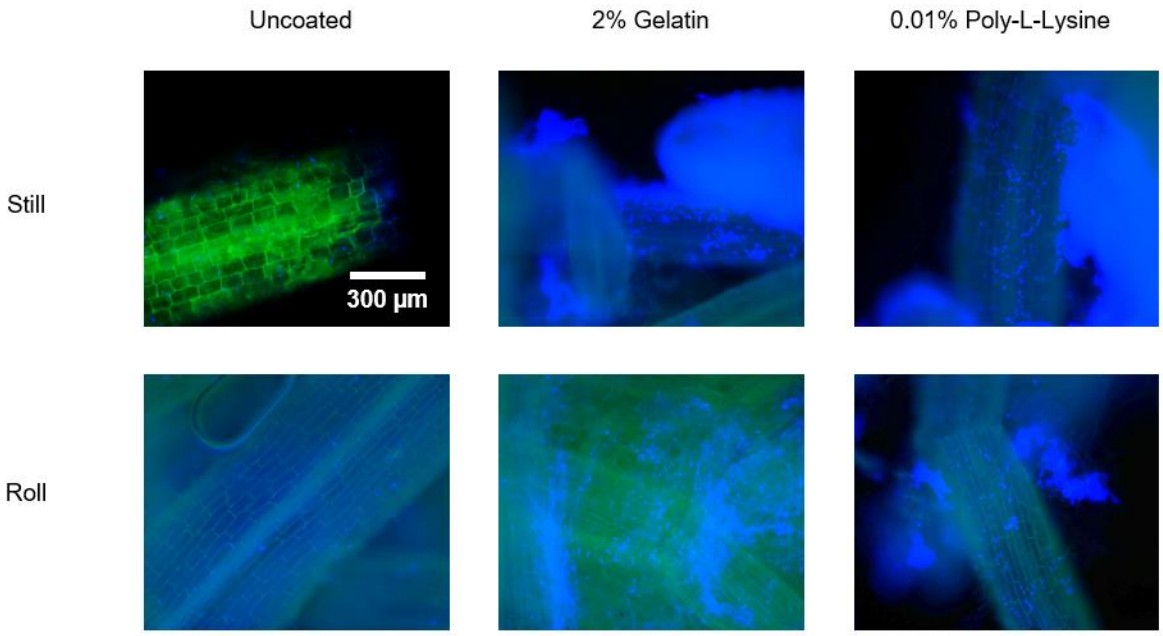

Figure 21: hFFs seeded onto tobacco hairy root matrices using different coatings
Preliminary cell seeding study to determine coating and attachment method onto matrices. (From left) Uncoated, gelatin coated, and poly-L-lysine coated samples were seeded with hFF cells and were either placed still for the entire culture duration or rotated around for 30 minutes initially to achieve even coating of the matrices.

Hairy roots without any coating were not suitable for cell attachment. Moving forward, the 0.01% poly-L-lysine treatment was selected as the coating of choice. The gelatin is from a porcine source and as the poly-L-lysine is a synthetic molecule which should not have any adverse immunological effects for human studies, it was preferred over the latter. To ensure even coating

of the hairy root matrices, a 30-minute rotation was employed to agitate the samples during seeding from now on.

Decellularized tobacco hairy roots coated with poly-L-lysine were seeded with hFF cells and kept in culture for 5 and 10 days. For both time points, fluorescence images were taken to visualize cell seeding (**Figure 22**). Solid nuclei were observed on days 5 and 10 after seeding. This was verified as hFF cells attached to the matrices by counterstaining with phalloidin 594 to visualize the actin cytoskeleton. On day 5, cells were observed as clumps and appeared to be fewer in number. By day 10, more cells were seen to have spread over the surface since individual nuclei can be singled out and the actin filaments are more spread out.

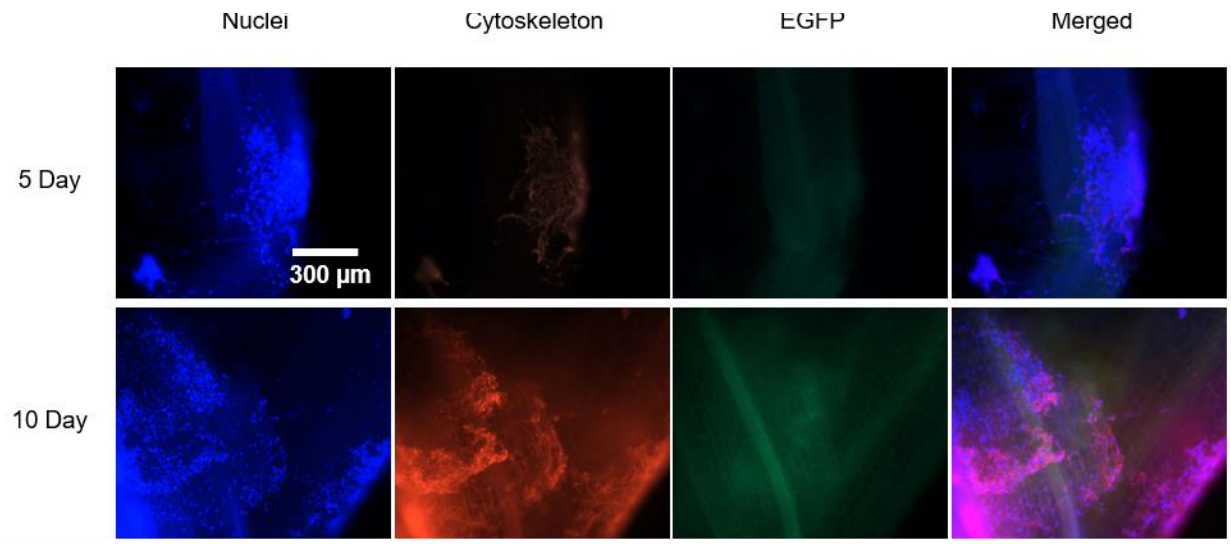

Figure 22: Fluorescence images of hFFs seeded onto tobacco hairy root matrices for 5 and 10 days
Tobacco hairy root matrices were seeded with hFF cells and then imaged after 5 and 10 days of culture. hFFs can be identified by their solid blue nuclei and red cytoskeleton on top of the matrices. Visually more cells can be observed on the surface of the matrices after 10 days and appear to be more spread apart.

The appearance of clumps and fewer number of cells may be attributed to insufficient seeding distribution. Quantitative analysis did not show significant difference between the Day 5 and Day 10 samples (**Figure 23**). The bars indicating samples before treatment with any detachment enzyme did not show proliferation of the cells. This may suggest that even though

cells are able to attach to the tobacco hairy root matrices, they are not able to proliferate, or that the growth of the hFFs are limited due to exposed surface area of the roots. After seeding the matrices, the decellularized plant material tends to settle to the bottom of the tubes and clump up reducing surface area for cells to potentially expand.

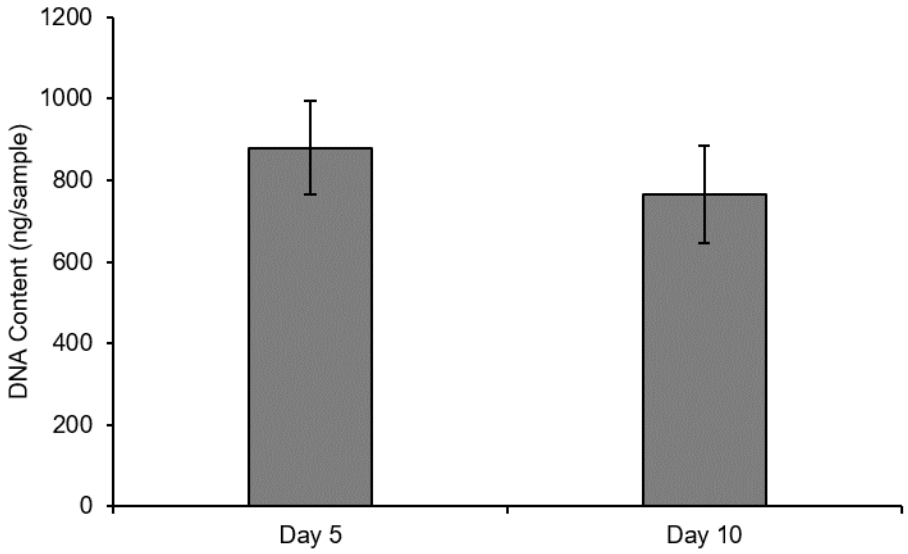

Figure 23: DNA quantification for hFF seeded matrices
DNA content was measured for the seeded tobacco hairy root matrices for the 5- and 10-day studies. Increase in DNA quantity was not observed after 5 days of additional culture time. No significant difference was found within the samples after n unpaired t-test. Data are presented as the mean ± standard deviation of three separate samples separate samples.

The cells are still viable as they retain their doubling capabilities after detachment with proteolytic enzymes. Cells that were cultured on the hairy root matrices for Days 5 and 10 were treated with treated with either 10% cellulase or 0.25% trypsin-EDTA depending on the experimental group. Cell detachment was verified by staining the matrices with Hoechst. Presence of intact nuclei may suggest incomplete detachment of the hFFs.

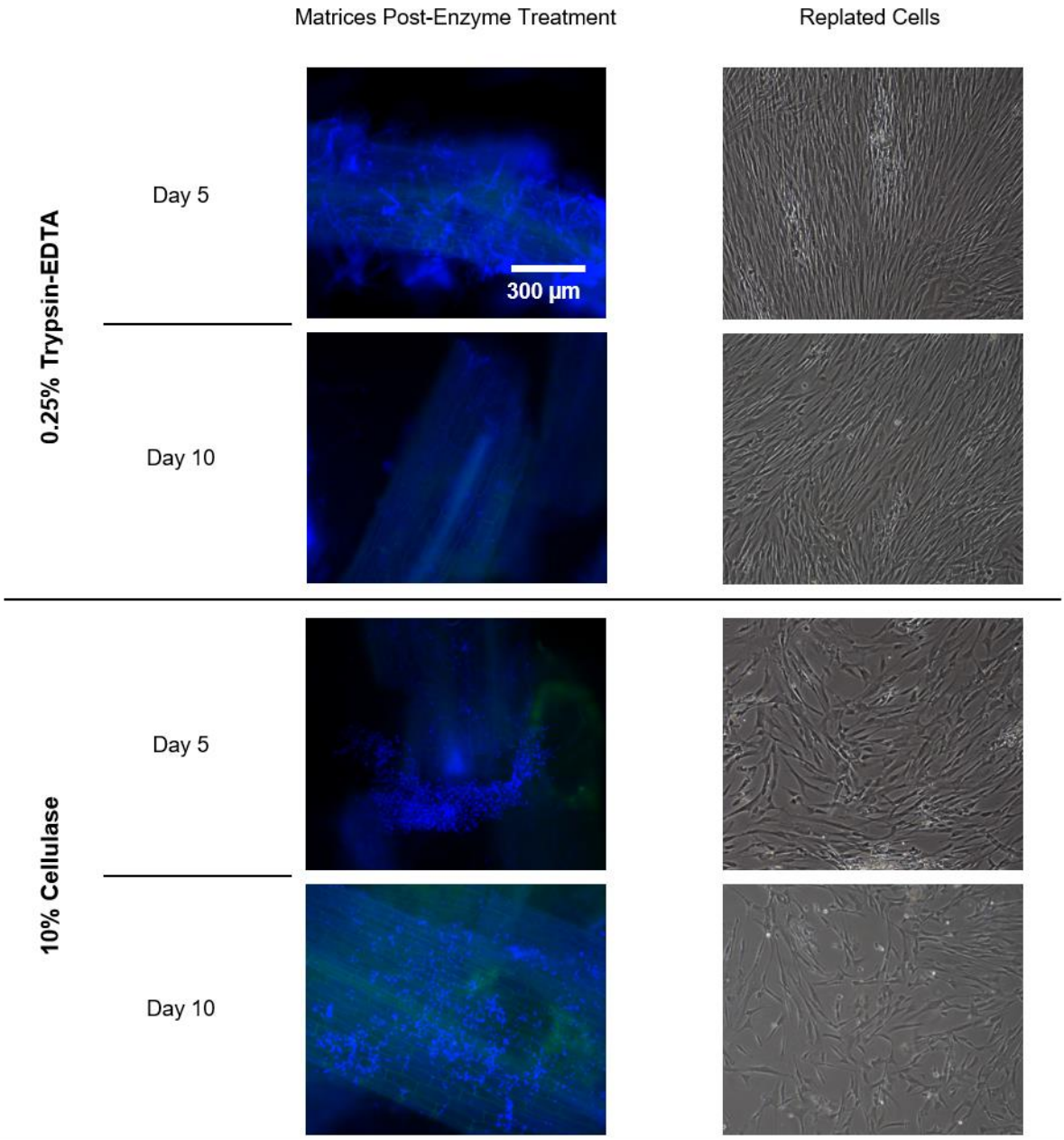

Figure 24: Enzyme detachment and replating study (Day-5 and Day-10 studies)
After 5 and 10 days of culture on the matrices, the hairy roots were imaged using fluorescence microscopy to identify any remaining hFFs on the surface. After the cells were reseeded onto standard tissue culture plastic, it was followed up with brightfield imaging to verify that the cells were growing after being detached from the tobacco hairy roots. After cellulase treatment, nuclei are still observed on the matrices, however, cell detachment was still possible as cells were observed to be growing on the tissue culture plastic. Solid nuclei are not observed for the trypsin treated samples, and more cells are observed after reseeding for those samples.

Trypsin, a commonly used enzyme for the detachment of cells from culture surfaces showed almost complete removal of all hFFs from the hairy root matrices (**Figure 24**). Cellulase

treatment did not achieve the same effect as nuclei are still observed using fluorescence microscopy. However, this did not indicate that the enzyme failed in removing cells as after replating cells are noted to grow for both the Day 5 and Day 10 studies. This indicates that the cellulase detachment protocol is inefficient in removing the cells from the culture surface. That is corroborated by the lower confluency for the cellulase detached cells for both time points.

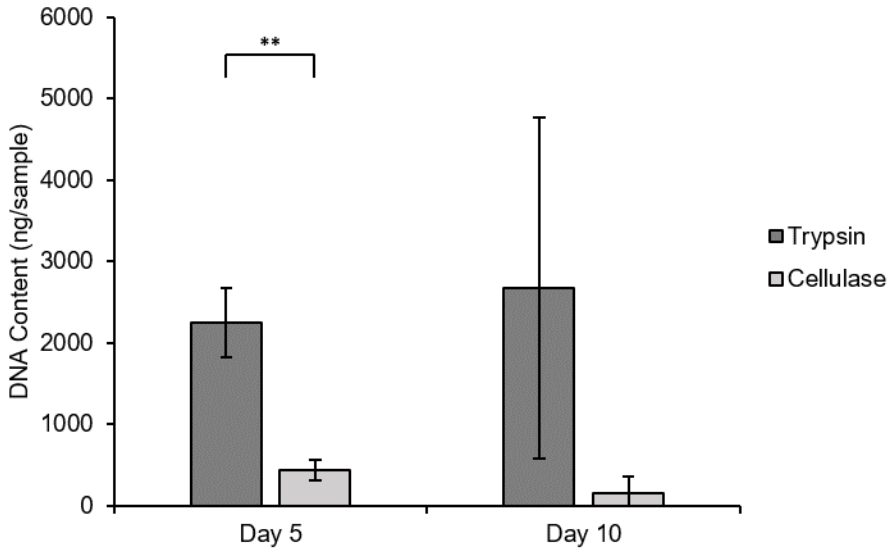

Figure 25: DNA quantification for hFF seeded hairy root replating study
DNA content after replating was measure for the Day-5 and Day-10 samples. Trypsin detached hFFs had a higher DNA content than cellulase treated samples. Post hoc tukey test did not show significant differences between samples. For the Day-5 sample, an unpaired t-test showed that difference between the trypsin and cellulase treated samples was significant. Data are presented as the mean ± standard deviation of three separate samples. Asterisk denotes statistical difference (****** $p<0.01$).

hFF seeded hairy roots were cultured for 5 and 10 days before being treated a cell dissociation protocol (trypsin or cellulase treatment) and subsequently replated into new tissue culture plastic and left in culture for 5 more days. dsDNA content was measured and compared between the replating studies (**Figure 25**). hFF dissociation with trypsin yielded higher genetic content after allowing it grow on fresh tissue culture plastic. The day 10 samples had a high standard deviation but that is to be expected with such a study since we cannot control how much surface area that the hFFs had access to while in culture on the matrices. This can inadvertently

cause large variations in reseeded cell numbers. Cellulase treated hairy roots yielded a smaller DNA content after replating. A large amount hFFs still remained on the matrices after enzyme treatment and as such had a lower reseeding count.

Chapter 6: Discussion and Future Directions

Using plant-based scaffolds for tissue engineering is a rapidly expanding area for biomedical engineers. It is a sustainable source of biomaterials and provides us with alternatives for tissue engineering design considerations. *In vitro* plant cultures are easy to produce in large quantities and is possible functionalize them for tissue culture applications.

Decellularization of the tobacco hairy roots using DNase I resulted in DNA levels well below the benchmark standard (**Figure 11**). DNA levels from 30 minutes of treatment with DNase I resulted in 16.25 ± 4.98 ng of DNA content per mg of tobacco hairy root matrices. The decellularization is 50 ng DNA/mg of tissue[14]. Further modifications to the decellularization by pulverizing the roots did not significantly reduce DNA content further (**Figure 12**). For the rationale of decellularizing the matrices for reseeding purposes, lyophilization of the plant-based tissue followed by a 30-minute treatment with DNase I was shown to be highly efficient. Visual evidence was also provided by fluorescence imaging (**Figures 11 and 14**) which showed reduction of intact solid nuclei within the matrices.

The tobacco hairy roots are genetically engineered to express EGFP along with a $(SP)_{32}$ motif. EGFP is used as a visual reporter protein for the detection of designer proteins which are being produced. One such designer protein is FGF2 which is a fibroblast growth factor, which aids in mammalian cell proliferation[116-118]. The $(SP)_{32}$ protein chain was added to facilitate secretion of attached proteins from within the cytoplasm outside of the cell membrane[119]. For the commercial production of designer proteins, this process is beneficial as it allows for the product to be shuttled out of the cells and be available for collection and purification. Our lab found that some of the protein is still retained within the matrices of decellularized BY-2 cells even after decellularization.

FGF2 or other therapeutic proteins retaining within plant matrices could be beneficial for tissue culture studies. That is why BY-2 cells were characterized and evaluated for human cell seeding[14].

Hairy root cultures from *Nicotiana tabacum* can be engineered to express proteins which can improve cell adhesion and proliferation. Compared to BY-2 cells, EGFP secretion in hairy roots is weaker[119]. For a comparison study, it would be ideal to compare fluorescence between hairy root-EGFP and roots that do not express the protein. Since we were unable to obtain tobacco hairy roots that do not express EGFP, it was not possible to gauge how much green fluorescence was due the root matrices autofluorescing and how much was it due to the EGFP. +DNase and -DNase treatments showed reduction in fluorescence (**Figure 14**). This corroborated with decrease in overall protein content after the different treatments (**Figure 13**). This suggests that the hairy roots, even though they do not secrete EGFP as well as BY-2, retained a limited quantity of the protein. Leaching of the protein from the plant matric is not desirable, and a BCA assay is not sufficient to quantify the target protein. Leached protein content was assumed to be normal as it was observed in the -DNase samples. +DNase treatment did not result in further loss of protein. To better retain desirable proteins such as FGF2, it may be prudent in adding binding modules to the target protein so that it may adhere better to the cellulose or hairy root matrices and still retain within the structure even after post-treatments. This will require genetically engineering the plant cultures to express the desirable protein chains and motifs.

Our collaborator Jianfeng Xu at Arkansas State University has been conducting trials with glycosylphosphatidylinositol (GPI) membrane protein anchors that would bind to our target protein sequence, much like the $(SP)_{32}$ motif. However, unlike the $(SP)_{32}$ protein chain which helps shuttle attached molecules out of the cell membrane and helps in secretion, GPI anchors assist in binding to the cellular membrane itself[120]. GPI, which is a phosphoglyceride, attaches to the C-

terminus of the amino acid chain. This post translational modification takes place in the endoplasmic reticulum and then the protein is transferred to the Golgi apparatus and then finally to the plasma membrane where it remains attached[121]. The theory is that after culturing the plant material *in vitro*, the target protein, aka FGF2, would be retained within the cells and not secreted out. During the decellularization process, the GPI anchor should improve protein retention and may allow for a sustained and controlled release of the growth factors into the surrounding media resulting in uptake by the adherent cells in culture.

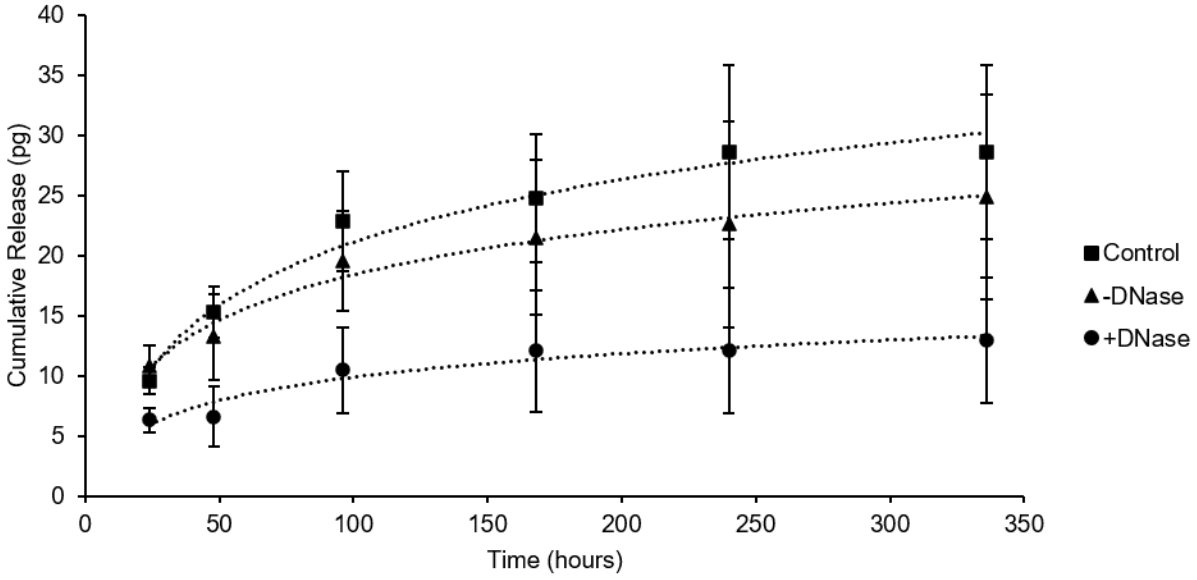

Figure 26: FGF2 release profile for BY-2 cells
BY-2 cells engineered to express FGF2 showed a sustained release of the growth factor over a period of 2 weeks. +DNase treatment on the BY-2 yielded a shallower release profile indicating further leaching of FGF2 during the decellularization process compared the Control and -DNase samples. Data are presented as the mean ± standard deviation of three separate samples.

It would be beneficial to carry out a release study of FGF2 to evaluate the biofunctionalization property of the hairy roots. Our collaborator had provided us with BY-2 cells that expressed FGF2 and was thus used as an analog for the hairy roots in a protein release study. A release profile of FGF2 from BY-2 which had not been sequenced with GPI was quantified by using an ELISA and showed a sustained release of the protein over a course of 2 weeks (**Figure**

26). With the help of binding modules, such as GPI, it may be possible to prolong the release of growth factors as well as increase the amount available for the cultured cells. Plant-based cultures may serve as an easy way of obtaining pre-functionalized scaffold material for human tissue culture. As such, it is important to be able to characterize the decellularization of other plant cultures with the goal of utilizing them as tissue culture platforms.

When using reagents or materials for mammalian cultures, it is important that all processes are carried out aseptically and that anything coming in contact with the cells have been properly sterilized. Unsterilized material may introduce fungal and/or bacterial contaminations to the culture media and that would a serious problem in clinical settings. The ability to be able to sterilize tissue culture surfaces is thus important. The four main methods of sterilizing are steam (autoclave), filtration, radiation (gamma) and gas (EtO)[122]. The method used ultimately depends on the material that needs to be sterilized. Filter sterilization is used for solutions and cannot be used for the hairy roots. Radiation and high heat from the autoclave, though used significantly in clinical practice, are undesirable for the hairy root matrices. To be able to functionalize the matrices with secreted proteins, it is important to retain the protein function. Ionizing radiation and the high heat from autoclaving can significantly damage and denature proteins[123-124]. EtO helps in sterilization through a process known as alkylation[125]. This is a chemical reaction in which a hydrogen atom is replaced with a hydroxyethyl radical in amino acids causing proteins to change their shape. These proteins then penetrate the cell membranes, typically that of microorganisms, and creates a channel leading to the outside. Due to the puncturing of the plasma membrane these cells ultimately undergo lysis and die. Regardless of the chemical changes produced, ethylene oxide does not permanently alter the essential qualities of proteins such as growth factors[126]. For

the purposes of sterilizing the tobacco hairy root matrices without permanently damaging any beneficial proteins, EtO treatment was utilized for all the cell seeding experiments.

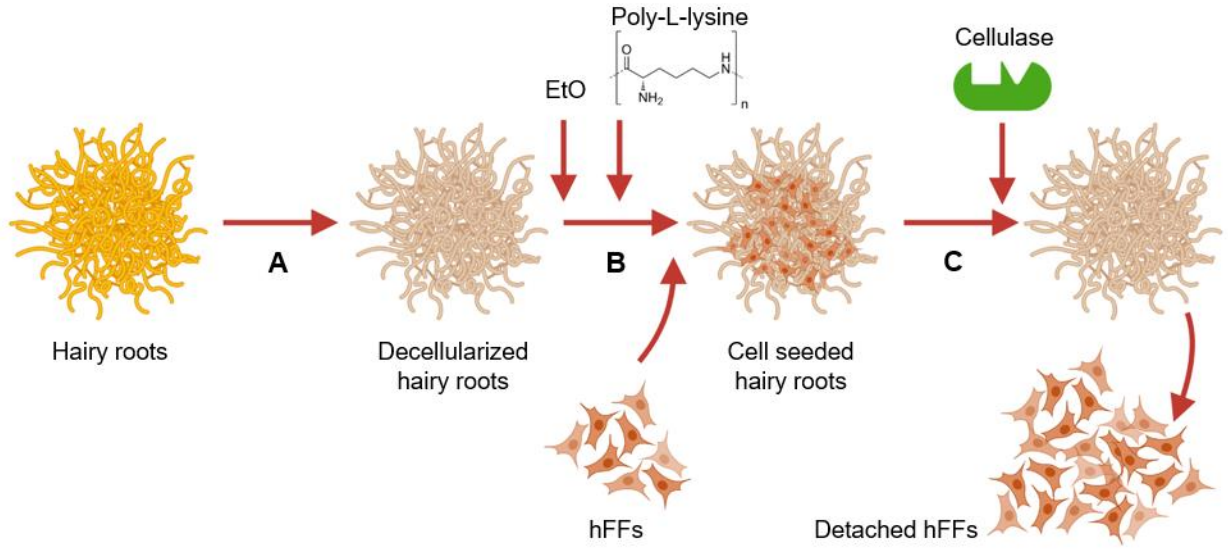

Figure 27: Concept schematic for using decellularized tobacco hairy roots as a tissue culture platform Tobacco hairy roots are cultured to express recombinant proteins that promote cell adhesion and proliferation and frozen prior to further processing. **(A)** The roots are lyophilized and treated with DNase I at 37°C to decellularize them. **(B)** The decellularized roots are sterilized using EtO gas and coated with poly-L-lysine. hFFs are then seeded onto the roots. **(C)** Cells are cultured on the roots for expansion. The matrix is then degraded using cellulase to dissociate the cells which can then be collected for upstream processing.

Hairy root matrices are made up of cell wall material which primarily consists of cellulose and other compounds such as hemicelluloses and pectin for example. Cellulose is very stable under physiological conditions and are only broken down through enzymatic hydrolysis such as when it is decomposed in the soil by bacteria. As such, it makes it an ideal scaffold material in tissue engineering as the only byproduct of degradation, which will have to be forced through additional means, is water and sugar. In tissue culture of adherent cells, the primary method of detachment is via proteolytic enzymes such as trypsin[127]. It is a well characterized enzyme that digests proteins such as those found in the ECM and focal adhesions of the cells to the culture surface. This also helps in breaking up clumps of cells in solution which is useful for counting and seeding close estimates of cells in studies and clinical practice. Cell dissociation from the hairy root matrices

using trypsin was largely successful and it was possible to reseed viable cells onto a new culture plastic (**Figure 24**). However, trypsin is a digestive enzyme secreted by the pancreas into the small intestines of animals like pigs. The enzyme can adversely affect cellular proteins and lyse them if left in culture for too long. FBS is thus necessary to help in inhibiting trypsin activity and is used once cells have been successfully detached from a culture surface. Other than lysis of cells, exposure to trypsin can alter surface antigen expression in hMSCs[83]. This can affect the differentiation potential of these stem cells which can limit their use in clinical practice.

Based on the results from the cell detachment experiments, trypsin was observed to be more effective at dissociating cells compared to cellulase. Alternative detachment solutions, which are also animal free, are available such as TrypLE but are not as commonly utilized as trypsin. TrypLE would be considered ideal as studies showed that it has a lesser adverse effect on hMSCs during dissociation[83]. Other products used as a trypsin substitutes that are also well characterized for use by the FDA and adhere to good manufacturing practices include non-enzymatic cell dissociation solutions, Accutase and TrypZean[128]. Non-enzymatic cell dissociation solutions consist of a proprietary mixture of chelators that assist in disassociating cells and is gentle on the cells. Its main drawback is that it does work well for highly adherent or adhesive cell lines which require a stronger dissociation agent. Accutase and TrypZean are both animal free enzyme-based dissociation agents. TrypZean, similar to TrypLE, is a recombinant version of trypsin but expressed in corn. Accutase is a mixture of proteolytic and collagenolytic enzymes. Both these dissociation agents are gentle on cells and help protect with retention of surface antigens[128]. However, due to the high cost of these alternative enzyme options, trypsin is still the most prevalently used dissociation agent in research and clinical settings (www.sigmaaldrich.com).

Degradable scaffolds for tissue culture are not a novel concept[129]. The ability to grow cells on a scaffold material that can be degraded via alternate pathways that does not affect the quality of the tissue culture would be valuable for the tissue culture industry. It could potentially reduce the cost of manufacturing plastic-based culture platforms and reduce waste as they can be broken down into environmentally friendly materials. Cellulose degradation could be used as cue to dissociate cells without the need for trypsin[130]. Biocompatibility assays for plant degrading enzymes (**Figure 18**) showed that pectinase had adverse effects on the health of hFFs, however, cellulase did not alter cell viability even after exposing them to it for an hour. Cellulase functions optimally in low pH and high temperature conditions which are not suitable for cell culture, but degradation studies with the enzyme (**Figure 20**) showed that it was possible to break down the matrices in the non-optimized physiological conditions. These tests to identify cellulase as a dissociative enzyme in cell culture are not perfect by any means. Cellulase could potentially be affecting antigen expression on the cells, and that will not be readily observed from DNA quantification studies. Studies to identify cell surface markers for hFFs did not lie within the scope of our study and to verify that claim further research will be required.

Cellulase and pectinase are enzymes sourced from the fungi *Trichoderma reesei* and *Aspergillus aculeatus* respectively. After synthesis of the enzymes, they require purification steps to help in concentrating the desired enzyme and removing other contaminants and proteins. Enzyme purity can vary between batches and as such, enzymes used in clinical research have to meet several benchmark standards so that they can pass FDA regulations (www.fda.gov). Plant degrading enzymes are primarily used in plant chemistry or related fields and not in clinical research. For use in clinical studies, proper protocols will need to be taken in enzyme preparation and to ensure proper purity. For instance, the pectinase solution from Sigma-Aldrich contains other

enzymes of undetermined concentrations such as pectintranseliminase, polygalacturonase and pectinesterase, along with small amounts of hemicellulases and cellulases. The presence of these extra enzymes as well as other additives/contaminants unknown to us could be the reason that had negatively affected hFF viability for pectinase (**Figure 18**).

For the cell dissociation studies, cellulase was used in verifying if the hFFs could be detached by breaking down their underlying culture surface. Post-seeding images after replating showed that cells could be detached and grown in standard tissue culture plastics (**Figure 24**). The confluency observed was lower than cells that were dissociated using trypsin. This is corroborated with Hoechst-stained hairy root matrices after cellulase treatment that showed that significant portion of the hFFs was still held back on the roots and were not detached. One of the primary reasons as to why this may have occurred is due to media conditions for the cellulase. The optimal conditions for cellulase are a pH of 6.0 and a temperature of 52°C. The roots were exposed to a 10% w/v stock concentration of cellulase in DMEM basal media for an hour at 37°C. The degradation studies were carried out overnight and similarities between Citric Buffer (optimal) and DMEM and CM (physiological) may be attributed to the enzyme working time provided. Some amount of degradation had occurred which resulted in the dissociation of cells and successful seeding. However, the protocol is not optimized, and time is not sufficient for the complete dissociation of the hFFs. Further studies in varying temperature, pH, concentration, buffer, and time for enzymatic action need to be determined if cellulase is a viable option for the alternate dissociation method for tissue cultures. Alternative strategies can involve obtaining cellulase from other sources such as bacteria that have optimal working conditions close to that of physiological and that are properly prepared and purified.

Traditionally, trypsin is used in conjunction with disodium ethylenediaminetetraacetic acid (EDTA). EDTA is a chelating agent, meaning that it binds to and stabilizes metal ions. Divalent cations (Mg^{2+} and Ca^{2+}) help strengthen cell-cell and cell-matrix interactions[131]. EDTA chelates the divalent cations weakening cellular adhesion and assists in the detachment of cells in culture. Conventionally for mammalian tissue culture 0.25% or 0.05% trypsin in a 0.53 mM EDTA solution. A future study with cellulase in EDTA should be carried out to see if a chelating agent is necessary for complete dissociation of cells. Other than optimizing pH and temperature, enzymes require other variables such as cofactors for them to function properly. Cofactors can be divalent cations. It is known that PBS for instance deactivates cellulase and was avoided in enzyme preparation[132]. However, if Ca^{2+} and Mg^{2+} ions are needed for cellulase activity, the addition of EDTA could potentially inhibit the enzyme. As such, further testing is required.

Various surface characteristics can affect cell adhesion and proliferation such as porosity, hydrophilicity, and surface charges. Cellulose as a material is biocompatible and does not adversely affect cell viability. However, cells do not readily attach to cellulose. Mammalian cell membranes have a net negative charge or are stated to have a polyanionic surface[133]. This is due to the movement of different ion species through the membrane. Cellulose fibers also have a slight negative charge[134]. Due to like charges, the cells tend to not adhere to the surface of plant-based materials. Modifying the surface to better improve cell adhesion is a common approach in tissue engineering and has been applied to cellulose based scaffolds in the past[135]. To induce a polycationic surface for the tobacco hairy root matrices, poly-L-lysine was employed as a surface coating[136]. Compared to the uncoated control which showed very limited cell attachment (**Figure 21**), poly-L-lysine coating substantially increased cell adhesion. Gelatin was also utilized to coat the hairy roots. Gelatin forms a proteinous ECM that greatly improves mammalian cell adhesion

and proliferation[137]. Hoechst staining showed similar results to that of the poly-L-lysine treatment (**Figure 21**). Gelatin used in research is primarily porcine in origin. To establish a protocol for the human cell expansion with as much animal-free reagents, poly-L-lysine was selected to coat the matrices. With that study we also concluded that it was best to seed cells on the hairy roots utilizing a tube rotator to achieve even coating and distribution of the cells as it would reduce clumping and allow more area for the cells to grow on.

A seeding study was carried out over 5 and 10 days to evaluate hFF seeding on the coated hairy root matrices. The hFFs, which were used as an analog for hMSCs, could be seeded and cultured on the hairy roots and a study over the course of 10 days showed that hFF viability did not decrease. We expected to see a significant increase in DNA content for the day 10 study, however, the DNA contents were similar (**Figure 23**). A significant increase in hFF DNA content was not observed in our studies and may have been due to the limitations set by the scope of our research.

To ensure that cells would adhere to the matrices, half a million cells were seeded per samples. This is considered to be overseeding, as standard lab protocol for seeding hFFs on traditional plastic is 5,000 cells/cm^2. The hairy root matrices in theory should be to remain in suspension if proper stirring or agitation is employed. However, after rotating the matrices in cell suspension for 30 minutes, they were placed back in their tubes still for the duration of the culture. The roots settled down over time covering up areas that could be exposed to the cells. It is anticipated that this may have trapped cells at the center of the root clumps creating regions of hypoxia or low oxygen concentration, limiting cell proliferation. Clumping of the hairy roots at the bottom of the tubes resulted in limited surface area for the hFFs to grow on. The resulting presumable hypoxic core within the center of the hairy root clumps would limit proliferation. It is

possible that cells were only able to grow on the outside of these root clumps. Equal weight of root matrices was used for the 2 different time points and should technically have the similar surface areas exposed for cell culture. As we had overseeded the samples, this created a maximum confluency of hFFs on the matrices and hence similar DNA content. Replating studies with the 5- and 10-day studies showed similar results between treatments (**Figure 25**). Trypsin as mentioned before was successful is dissociating most of the cells enabling more cells to be seeded onto tissue culture plastic than the cellulase treated samples. This at the least shows that is possible to grow hFFs on hairy roots and we can detach those cells for further expansion. The cellulase degradation protocol for detachment was not as successful as trypsin.

Attempts at developing an alternative detachment protocol for hFFs showed that there is room for improvement. Plant degrading enzymes such as cellulase were not used before for tissue culture. Further optimization of the enzyme degradation at physiological conditions will be required for it to be a viable option. Alternate buffers or enzyme cofactors would need to be researched to improve the hydrolytic degradation of the matrices.

In this thesis, we evaluated the decellularization of tobacco hairy roots and quantified protein retention within the matrices. The decellularization protocol using DNase I showed satisfactory reduction in xenogeneic DNA content and showed retention of proteins within the matrices. From the standpoint of retaining proteins such as growth factors, this is desirable as it allows for biofunctionalization in the future. Improvements in protein retention will be required and will have to be achieved through further genetical modification of the hairy roots. Further studies with the GPI anchor protein to improve retention of proteins like EGFP and FGF2 will improve the utility of this approach for cell seeding and therapeutic purposes. Further

modifications to the buffer parameters will be needed to better optimize the procedure and the addition of a chelating agent such as EDTA could help with detachment.

Chapter 7: Conclusion

Through the course of this thesis, we have met the criteria to complete the three specific aims that had been proposed. Through DNA and protein quantification we were able to characterize the biochemical properties of the decellularized tobacco hairy roots for Aim 1. That information concluded that decellularization was sufficient to meet the requirements for cell seeding and other tissue engineering applications. Fluorescence microscopy and SEM imaging enabled the visualization of EGFP retention and surface topography. For Aim 2, we were able to develop a degradation protocol for the hairy roots at physiological condition. Cellulase was chosen as the plant degrading enzyme as it did not impair hFF viability in the toxicity tests. Cells replated after 1 hour exposure to various concentrations of cellulase showed similar growth to untreated samples. In Aim 3, we were able to successfully seed hFFs onto the hairy roots and culture them before detaching them and reseeding the cells to regrow on a new culture surface. Trypsin detachment was highly effective in dissociating the hFFs, however cellulase treatment will need to be improved upon.

In conclusion, decellularized tobacco hairy roots can be used as a tissue culture platform for hFFs and potentially other human cell types such as hMSCs but to utilize them for the large-scale expansion of cells, further research will need to take place. This thesis on tobacco hairy roots shows that plant-derived tissue cultures have potential in tissue engineering and mammalian cell culture.

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