Session A3
8009

BIOFUNCTIONALIZING PLANTS: USING NATURE AS TISSUE ENGINEERING SCAFFOLDS

Adam McNeill, adm150@pitt.edu, Balawejder 5:00, Jason Zheng, jlz42@pitt.edu, Balawejder 5:00

Abstract— *Decellularizing plants and using them as tissue scaffolds is a potential solution to issues faced by the field of regenerative medicine. This bioengineering method was implemented in the last few years to overcome the structural complexities of delivering a sustainable amount of nutrients to human tissue. The lack of efficient vascularity is the main problem that arose when developing artificial organs. Since plant and animal tissues share so many structural similarities, engineers are able to extract plant cells from leaves and stems using decellularization techniques and implant human cells back into these plant tissue scaffolds. Simply put, researchers are able to replicate the mechanical properties of human tissues through the usage of various plant geometries since the vascular networks of plants act as efficient passageways for nutrients in human tissue development.*

Another reason for seeking an alternative tissue engineering process is the lack of organ donors. Conventional methods such as decellularizing and repurposing human tissues result in a lack of biocompatibility and efficient vascularity. These processes are also too costly, and varying biological compositions of donor tissues often lead to organ rejection. On the other hand, plants are easily accessible, which makes them cost-effective. Using plant specimens could also potentially minimize biodegradation since plants are found to be more compatible in laboratory environments due to their cellulose composition. With further experimentation, biofunctionalizing plants could be the future of regenerative medicine by increasing access to organ transplants through a more promising and sustainable process.

Key Words— Artificial Organs, Biocompatibility, Decellularization, Extracellular Matrix (ECM), Recellularization, Tissue Scaffolds, Vascularity

DO WE NEED MORE ORGANS?

Effects of the Organ Transplant Crisis

The vast shortage of viable organ replacements is alarming, and current tissue engineering methods have proven to be ineffective in addressing this issue. The biggest concern surrounding current artificial organs is biocompatibility. It is often seen that the newly created organs either degrade over time or lack a functional vascular system. Correlation between experimental setbacks and the availability of organs can also be seen in stats regarding organ transplants. According to a study, "in the U.S. alone, more than a hundred thousand people need heart transplants each year but only about 2,000 receive one" [1]. Scientific research cannot increase the number of organ donors but being able to create organs will drastically increase the number of annual transplants. To achieve this, however, complications of current engineering methods must be overcome.

Because of this organ transplant crisis, scientists decided to build upon current research and develop a new way of engineering artificial organs and tissues that would maximize biocompatibility, while simultaneously be easily accessible to all organ transplant patients [2]. After considering all the factors that made current methods unreliable, researchers turned to bioengineered plant scaffolds. Instead of using traditional methods and human tissue scaffolds to make artificial organs, research on using plant tissues has slowly taken precedence. This new process avoids the need for human tissue donors, potentially eliminating current limitations caused by functionality and vascularity of artificial organs, biocompatibility, affordability, and availability.

Engineering a More Sustainable Pathway

While sustainability has become a buzz word, it has brought on several varying definitions. This new tissue engineering technology embodies many of these definitions as it is sustainable in many respects. The most significant aspect of sustainability in regard to biofunctionalizing plants is its potential to improve a patient's quality of life. Conventional tissue engineering methods have proven to be unreliable in supplying artificial organs due to adverse effects of inconsistencies dealing with biocompatibility and organ rejection. Since plants are easily obtainable, this new direction in using plants as tissue engineering scaffolds will provide an alternative pathway to making artificial organs, one that will produce tissues with greater functional reliability and accessibility.

Using plant scaffolds for tissue engineering purposes is also sustainable in respects to economics and the environment.

Current methods of making artificial organs involve experimentation with human tissues. Often times, the handling of such materials can produce toxic byproducts, which are difficult to properly dispose. On the other hand, using plants could potentially minimize the amount of biodegradability and environmentally harmful substances. Furthermore, human tissues are much less affordable than plants. According to a study, human tissues could cost up to $800 per cubic centimeter [3]. Because plants are cheap and could be obtained directly from the supermarket, the availability of plant scaffold research has the potential to be widespread. All these factors help define the sustainability of this new technology, which means that in the future, more people will have access to artificial organs, and there will finally be a reliable solution to the organ transplant crisis.

THE CLASSIC WAY TO ENGINEER TISSUES

Background of the Process

The conventional method of tissue engineering utilizes human tissue scaffolds. This process involves growing new cells on preexisting human tissues. In order to initiate this process of repurposing (or repopulation with new cells) human tissues, donors must be sought out. In many instances, harvesting new cells and tissues using this method can be quite expensive [3]. The cost and availability of donors, however, aren't the only factors preventing artificial organs from resolving issues faced by the lack of organ donors. Even when organs are created by conventional methods, setbacks stemming from inefficient vascularity, organ rejection, and biodegradability leaves much to be desired. The entire process seems inefficient and unreliable in helping those on the transplant list, but it is currently the best approach to providing help to those in need of organs and tissues.

The Fundamentals of the Process: Decellularization

After the tissues have been obtained from donors, engineers must prepare the sample(s) for decellularization. Decellularization is the process in which existing cells in a sample are destroyed and removed, leaving behind a "ghost tissue" [3]. A "ghost tissue" is a tissue sample that contains the extracellular matrix of cells (ECM) but lacks the cells themselves. According to the US National Library of Medicine, "The ECM represents the secreted products of resident cells of each tissue and organ, is in a state of dynamic reciprocity with these cells in response to changes in the microenvironment, and has been shown to provide cues that affect cell migration, proliferation, and differentiation" [4]. In summary, the ECM offers two main benefits for cells. The first benefit is that the ECM provides biochemical support, where it assists the cells in carrying out chemical processes such as glycolysis and cell-to-cell communication. Additionally, the ECM provides structural support (contains many structural proteins i.e. collagen), which allows for organized compositions of cells and vascular networks that make up tissues and organs. As seen here, it is crucial that the decellularization process leaves the ECM unharmed, while simultaneously removes existing cells.

Selection of Decellularizing Agents

Decellularizing agents must be chosen carefully in order to preserve the ECM. According to researchers, "The most effective agents for decellularization of each tissue and organ will depend upon many factors, including the tissue's cellularity (e.g. liver vs. tendon), density (e.g. dermis vs. adipose tissue), lipid content (e.g. brain vs. urinary bladder), and thickness (e.g. dermis vs. pericardium)" [4]. Damaging the ECM is inevitable during decellularization, but the extent of ECM damage can be greatly reduced when the above characteristics are taken into account. Since multiple factors are considered with a given tissue sample, every piece of sample will require its own unique decellularization method.

With the vast diversity of tissue samples, decellularizing agents can range from chemical substances such as acids, bases and detergents to biological substances such as enzymes and chelates. Many of these agents, however, have drawbacks associated with them. Some could damage the structural integrity of the ECM while others may leave behind cellular residue, which again factors into the discussion on sustainability in regard to waste production. Because of all the risks involved in making the tissue scaffolds, the utmost attention must be placed on this early stage of tissue engineering.

Whole Organ Perfusion Techniques

Whole organ perfusion is an effective and common method to deliver decellularizing agents to tissue and organ samples. This technique takes advantage of the vascular networks already present in the tissues. For example, a study conducted on a rat heart showed that decellularizing agents can perfuse or flow through the circulatory system of the heart to destroy unwanted elements such as cells and residues [4]. The resulting heart retained the original geometry but had a translucent white appearance since all cells were stripped from the organ ("ghost tissue") [4]. Subsequent tests were done on the same rat heart, and the results showed that the vascular network maintained its integrity and was not damaged by the decellularizing agents [4]. The rat experiment not only demonstrated success in organ decellularization but also presented proficiency in the organ's vascularity. Another important takeaway was that the perfusion technique is safe and reliable since the organ itself was minimally damaged. This technique was also adopted by researchers conducting experiments on plant decellularization due to its reliability.

Evaluation of Tissue Scaffolds

After a given sample has been decellularized, a few criteria must be met for it to be tested in vivo. The criteria set forth by previous in vivo testing include but are not limited to the following: the sample must contain <50 ng dsDNA per mg ECM dry weight, <200 base pair DNA fragment length, and a lack of visible nuclear material in stained tissue sections [4]. After testing the decellularized sample for its biological composition, the mechanical and structural integrity of the ECM must also be evaluated. This is necessary because of the potency of some decellularizing agents such as detergent.

In order to move on to the recellularization process, a decellularized specimen must pass all the testing. Even if a specimen passes the examination, there are still risks in using the decellularized tissue sample(s). This is where issues involving sustainability may arise. A donor's tissue composition could potentially interfere with newly introduced cells in the subsequent recellularization phase. This could then result in organ rejection, which is a big concern for many engineers when it comes to the sustainability and biocompatibility of the process.

The Fundamentals of the Process: Cell Seeding and Recellularization

Once the evaluation process is complete, engineers must decide what is the best way to repopulate or recellularize the "ghost tissues" with new cells (most likely an autograft). The most common method of recellularization is cell seeding. There are multiple ways to "seed" or introduce new cells to a tissue scaffold (decellularized tissue with ECM remaining). Again, the ECM will act as a foundation for the new cells to accumulate, grow, and communicate. Passive seeding is the simplest and least efficient form of cell delivery where cell samples are simply pipetted into the scaffold [5]. More efficient methods include dynamic seeding, which makes use of rotational systems [5].

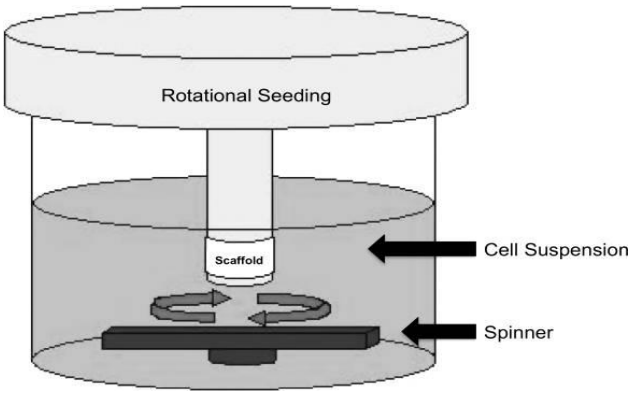

FIGURE 1 [5]
Basic depiction of rotational seeding

As presented in figure 1, scaffolds are placed in a spinner flask with a cell suspension in which the rotation of the system causes a driving force that "pushes" the cells into the scaffolds [5]. Ultimately, the type of seeding depends on the tissue sample and the availability of lab resources.

Selection of Cells for Implantation

In addition to considering the multiple seeding and recellularizing methods, engineers must also decide which cell types to use. Some of the common cell types used for seeding include fetal and stem cells. In a study done on rats, fetal lung cells were implanted into an approved tissue scaffold, which resulted in successful gas exchange after implantation [6]. This showed that fetal cells associated to the organ of interest are highly effective in the seeding process, since the functionality of the organ will be retained.

Stem cells have also shown success in experiments. After implanting (or seeding) stem cells into a scaffold, the cells are given the proper environment (ECM) to differentiate into specialized cell types. For example, when stem cells are seeded into the coronary arteries of a heart scaffold, differentiation will result in the formation of cardiomyocytes (heart cells) [6]. In either case, the seeded cells could grow on their respective tissue scaffolds. This is essentially the stage of the process where the newly seeded cells are allowed to develop into artificial organs by making use of the structure and nutrients provided by the ECM of the original tissue/organ. All in all, the types of cells used for seeding, the methods of seeding, and the scaffolds of interest are all chosen carefully to ensure that the repurposed tissue and/or organ is fully functional.

Experimental Analysis: Biodegradability and Cross-Linking Agents

The main goal of the decellularization process is to create a scaffold by wiping out preexisting cells and maintaining the structural and functional integrity of the ECM. However, the newly created organ or tissue is at risk of losing their functionality and/or structural composition. According to an article published by the International Journal of Artificial Organs, "Decellularized tissues are exposed to a breakdown process known as biodegradation upon recellularization or after in vivo transplantation, which affects the mechanical strength and durability of the scaffold" [7]. Due to this concern, researchers have developed a way of preventing biodegradation by introducing chemical and biological agents to combat the breakdown process.

The method of introducing foreign agents to the scaffold to prevent biodegradation is known as cross-linking. As stated by Dr. Heung-Myong Woo, "Cross-linking is a process during which cross-linking agents make pairs with two or more molecules, forming intermolecular and intramolecular bonds in the collagen fibers, increasing the mechanical strength, enzymatic degradation resistance, and structural stability of the

biomaterial" [7]. The functionality of these foreign agents is especially important because degradation rates increase while the scaffold is in vivo (or in the body). Countering the natural breakdown process, however, may result in even further damages to the ECM, the scaffold, and most importantly, the vascularity. Cross-linking can help make the process less biodegradable while simultaneously carry the risk of interfering with the new organ's vascular system, which makes it a very ineffective process.

Selection and Analysis of Cross-Linking Agents

To determine which cross-linker is the safest to use, researchers have developed a system of classification. These agents are broken down to 3 types: chemical, biological, and physical. Chemical cross-linkers are most frequently used for direct defense against chemical and enzymatic degradation [7]. As a result, this reduces the degradation rates. Some of these cross-linkers such as the synthetic Glutaraldehyde and naturally derived Genipin are potential toxins [7]. Despite the cross-linkers' effectiveness in combating the breakdown process, they potentially could damage the scaffold even further.

Physical cross-linking methods include the usage of ion beams, gamma rays, and ultraviolet irradiation [7]. These methods can increase the strength of scaffolds by providing thermal stability while simultaneously preventing residue build-up in the biomaterials (scaffold) [7]. Although the physical cross-linking processes sound more promising, the expense of this process is too great for it to be the primary method. In addition, biological cross-linking agents such as Transglutaminase and hydrogen peroxide are still being tested, and many have shown unfavorable results, like toxicity, similar to those of chemical cross-linkers [7]. Because of all the inconsistency and unreliability of cross-linking agents, researchers are continuously looking for ways to balance out the biodegradation process.

Limitations of the Current Approach

As previously outlined, many complications are faced when developing artificial organs using human tissue scaffolds. Many levels of sustainability are impacted during this process. In terms of economics, human tissue donors are very expensive, which makes it hard to obtain resources and conduct consistent experiments. Additionally, reliable cross-linking methods are way too costly. Biocompatibility is also affected as seen in the need of cross-linking agents. Decellularized human tissue scaffolds undergo natural biodegradation processes, which drastically affects the functionality of the final organ that's created. This natural breakdown of the artificial organ further disrupts vasculature and may even produce harmful wastes. Lastly, because tissue samples are donated, organ rejection could possibly occur when the artificial organ undergoes in vivo testing. All these shortcomings of human tissue scaffolds gave researchers the incentive to seek out methods that would improve all the facets of sustainability.

A NEW "ORGAN"-IC PROCESS

Crossing Kingdoms: Plant and Animal Biology

The new proposed method of bioengineering artificial organs involves manipulating the mimicry of plant and animal biology and was first experimented within the last few years to overcome the structural complexities of delivering a sustainable amount of nutrients to human tissue [2]. According to researchers, "plant vasculature follows Murray's Law, which is the physiological law describing the tapered, branching network design of the human cardiovascular system" [2]. Because plant and animal tissues share so many structural similarities, engineers can extract plant cells from leaves and stems using decellularization techniques and implant human cells back into the "plant skeletons."

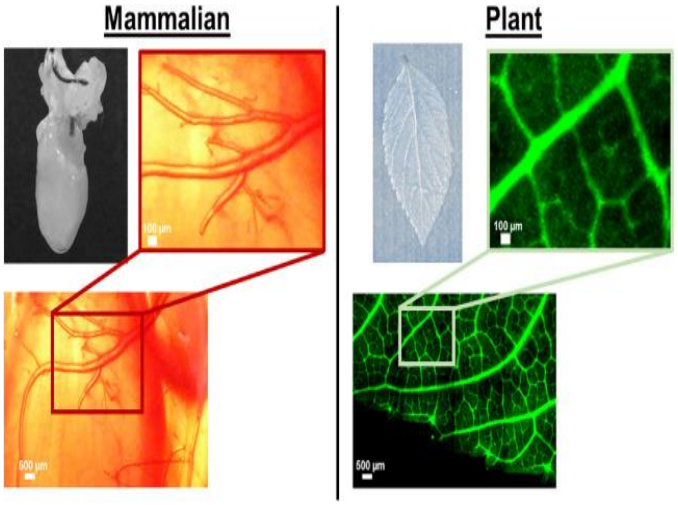

FIGURE 2 [2]
Comparison of the branching networks in mammals and plants

Figure 2 is adapted from experimentation done on rat hearts. The rat testing was carried out to display the similarities between animal and plant vasculature. After isolating and decellularizing the rat heart, red dye was perfused throughout its vasculature. This was then compared with a leaf from the *Buddleja davidii* plant. As seen by the results in the picture, venous structures in the plant could be utilized as a scaffold for human tissue because of the mimicry between the plant and animal tissue. This new direction in regenerative medicine is still in the research and development phase, but shows immense promise of being a solution to the organ transplant crisis.

An Alternative Method

Researchers at the Worchester Polytechnic Institute (WPI) have found promising experimental results in an entirely unique process of repurposing spinach leaves as human heart tissue scaffolds. The importance of these results was emphasized by Glenn Gaudette, PhD, a professor of biomedical engineering at WPI when he stated, "Adapting abundant plants that farmers have been cultivating for thousands of years for use in tissue engineering could solve a host of problems limiting the field" [8]. By evaluating the mimicry of vascular networks between plants and animals, Dr. Gaudette is suggesting a more nature-oriented approach to replace and repair tissues and organs through the use of plant life.

Even though plants and animals exhibit fundamentally different approaches to transporting their essential nutrients, they share astonishing similarities in the structures of their vascular networks. What enables a plant's efficient vascularity is the branching network of thin veins that deliver nutrients to the plant's cells [9]. On the other hand, humans employ a similar vascular geometry as seen in the arteries of the heart. Moreover, the cardiovascular and lymphatic systems of the human body work together to carry nutrients and filter wastes much like the veins of the spinach leaves. Figure 3 depicts the use of red dye in decellularized plant tissue to simulate the transport of blood, oxygen, and wastes.

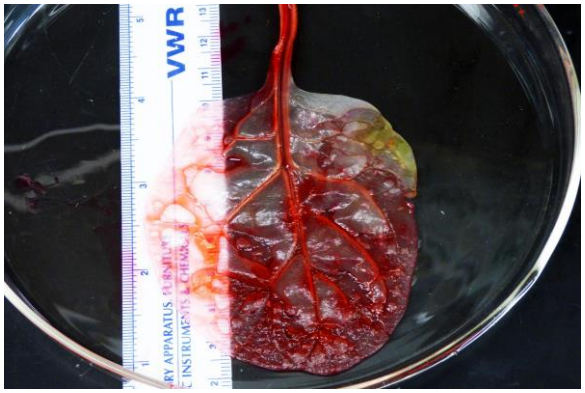

FIGURE 3 [9]
Red dye perfused through "ghost plant tissue"

The basis of this finding propels tissue engineering research into utilizing various plants to mimic the vascular networks of other organs.

Overview of Current Research

Despite the uniqueness of using plant scaffolds, the experiments done on repurposing the spinach leaves involved many similar steps to those of traditional tissue scaffolding methods. For example, the research conducted by the WPI team involved stripping plant cells from the spinach leaves (decellularization), and culturing the leaves with beating human heart cells [8]. This success in replicating human heart tissue opens the door for scientists and engineers to find a way to grow layers of these tissues to form healthy heart muscle that could one day be used to treat the damages suffered by heart attack patients.

Decellularization of the spinach leaf ECM also led researchers to find that what remained was a frame made of cellulose, which was surprisingly similar to the vascular geometry of human heart tissue [9]. According to WPI researchers, cellulose is a biocompatible substance that has already been proven useful in several applications of regenerative medicine (e.g. cartilage tissue engineering, and wound healing) [9]. In the case of plant tissue scaffolds, scientists can mimic the vascularity of human heart tissue by using the compositional cellulose of the ECM as structural support. The cellulose from the spinach "ghost tissue" also serves to reduce structural breakdown of the tissue scaffolds since cellulose molecules are strongly bound together, which makes it more viable than other biomaterials. This remarkable biocompatibility of cellulose will serve as the main motivation for further experiments on plant tissue scaffolds. Results from experimentation done by Dr. Gaudette and a collaborative group of universities are detailed in the subsequent sections.

The Fundamentals of the Process: Decellularization

As previously discussed, the difficulty of maintaining functional vascular networks is the main obstacle when using human tissue scaffolds. According to the team of researchers, viable vascular networks must be able to overcome the oxygen diffusion limit of 100-200 μm within tissues [2]. Because there is so much variation among human tissues, decellularization techniques must be varied, which often times result in scaffolds with inconsistent functionalities. Even with the use of different decellularizing agents, the structural and mechanical integrity of the tissue scaffolds cannot be fully preserved. As a result, scientists have turned to plant-based decellularization in search for a more biocompatible solution of protecting the ECM and vascular efficiency.

When decellularizing human tissues, the remaining ECM was found to contain structural biomolecules such as polysaccharides and proteins. Upon decellularization, the "ghost tissues" of the plants were also found to contain many similar structural compounds. Plant cell walls, for instance, are made up of polysaccharides such as pectin and cellulose, which is the most abundant component of plant cell walls [2]. As discussed earlier, cellulose is a well-studied macromolecule that has been used in many applications of tissue engineering. Furthermore, experiments done on cellulose showed that it was biocompatible and less biodegradable than other ECM components such as collagen even after in vivo transplantation [2]. The success of cellulose research alone was enough for moving towards the study of biofunctionalizing plants. Moreover, the striking compositional similarities between plant and animal ECMs provided even more motivation for

moving away from conventional tissue scaffolding approaches.

Preparations Before Decellularization

In contrast to human tissue decellularization, plant decellularization is much more simplified. Preparation for plant decellularization is also much easier because plants are conveniently obtained unlike human tissues. After gathering plants from local markets, researchers at the University of Wisconsin and WPI, decided on using perfusion techniques similar to the whole organ perfusion techniques done on human tissues [2]. This makes sense because perfusion techniques are effective in specimens that have efficient vascular networks. Likewise, a plant's vascular system closely mimics that of a human's, which also encouraged the use of the same perfusion methods.

The first stage of the preparation process involves cannulating the individual plant specimens [2]. In surgical terms, cannulation refers to the process of introducing a thin tube into a vein or body cavity. Even though each plant had to be cannulated differently, the process still remained relatively straightforward when compared to the preparation of human tissues. Another advantage of using plants is that mistakes can be afforded. Unlike human tissues, plant life is readily available, which allows for sustained experimentation. After this "surgical" stage of decellularization, perfusion techniques were applied to introduce decellularizing agents into the plant specimens.

Adaptations from Whole Organ Perfusion Techniques

Plant structures aren't as varied as human tissue structures and therefore, don't require notably different decellularizing agents. However, the perfusion process involves many intricate steps, which must be closely monitored to minimalize structural damage to the plants' ECM and vascular networks. Before the perfusion process began, the specimens were treated with hexanes and phosphate-buffered saline in order to remove the cuticle layer of the plants (protective film covering the epidermis of leaves) [2]. The unwanted cuticle layer was removed without the use of perfusion techniques because perfusion techniques are most effective when used to alter the internal composition of tissues.

The perfusion process was initiated by the team after removing the cuticle layer. Various solutions were perfused through the plants' cannulas for 5 days [2]. After this initial treatment, additional perfusions were carried out to ensure complete decellularization [2]. As seen by the research done at WPI and other renown universities, the decellularization process is complicated in terms of execution but simplistic in terms of resource acquirement. According to the researchers, decellularized specimens were stored in sterile deionized water at 4 °C until they are needed for experimentation [2]. This process of preservation is not entirely faultless since the plant scaffolds could only be stored for up to two weeks [2]. However, this is typically not a major concern because scientists are quickly making strides in biofunctionalizing plants research, which in turn means that decellularized samples are always in high demand.

Evaluation of Tissue Scaffolds: DNA Analysis

The criteria set forth for evaluating decellularized tissue samples are listed in a previous section above. In summary, the criteria focused on three key requirements, all of which centers around DNA. Those three key requirements established appropriate standards for DNA length, DNA amount, and DNA visibility within decellularized tissue samples [4]. In order to measure these properties of DNA, researchers carried out a series of procedures to quantify DNA. This DNA quantification process made use of conventional sample analysis techniques and technologies. such as Direct Cell Proliferation Assaying and Coomassie Protein Assaying [2].

The assaying of the plant fragments led to major findings in regard to DNA composition. The decellularized plant tissues were found to contain significantly less DNA (<50 ng DNA per mg tissue) and proteins when compared to native plant fragments [2]. This meant that the levels of remaining DNA were able to comply with the "DNA amount" criterion for evaluating decellularized tissues. In the days that followed, the amount of DNA continued to decrease in the plant scaffolds, which further satisfied the requirements set forth by the evaluation test [2]. These results further supported the sustainability of plant scaffolds by showing that minimal plant DNA remained, which could potentially lead to increased biocompatibility following recellularization. However, much like human tissue scaffolds, complications could still arise even if the scaffolds pass the evaluation.

Evaluation of Tissue Scaffolds: Spinach Analysis

Additional findings were also noted by the research team in regard to the spinach leaves, which were used as the "model species" during the experiments. According to the researchers, the decellularized spinach leaves started to lose their green coloration a day after the initiation of the perfusion (decellularization) processes [2]. This result indicated that the plant samples were slowly becoming translucent because chloroplasts and chlorophylls were being destroyed alongside plant cells, leaving behind the structural ECM.

As seen in figure 4, by day 5, samples became translucent with a light green hue. To ensure the removal of all residual chlorophylls, sodium chlorite was used to sterilize the samples, which led to completely colorless and translucent spinach leaves by day 7 [2]. However, this physical observation of the spinach leaves alone did not conclude the chloroplast investigation. As a result, histological analysis tests were carried out by the team. These tests were designed for comparative analysis between decellularized spinach leaves and their native counterparts.

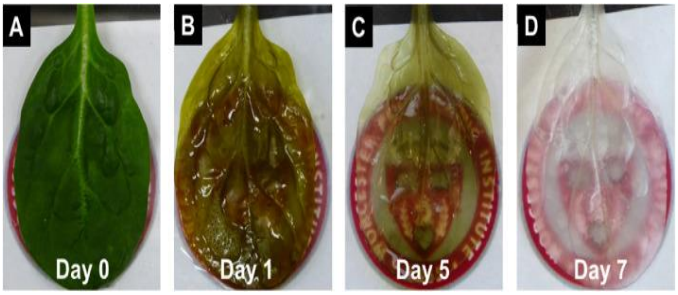

FIGURE 4 [2]
Timeline of a decellularized spinach leaf

The results from the histological analysis showed that native spinach leaves contained plant cells with nuclei and chloroplasts, whereas neither were present in the decellularized samples [2]. This finding allowed the team to further consolidate the effectiveness of the decellularization process by making two strong conclusions. The lack of nuclei indicated that genetic material from the plants was wiped out (as shown by DNA analysis), and the lack of chloroplasts indicated that other organelles and cellular residue may also be destroyed during the perfusion process.

More importantly, the biggest revelation from the histological analysis centered around lignin, a complex organic polymer that acts as a major component of the spinach leaf vasculature [2]. The analysis showed that lignin was present in both the native and decellularized spinach leaves [2]. This indicated that one of the major components of the spinach leaf vasculature was "protected" during decellularization. Furthermore, images of the spinach scaffold produced by scanning electron microscopy (SEM) revealed that pattern and density of vascular networks resembled those of the original leaves [2]. These discoveries continued to reinforce the sustainability of biofunctionalizing plants by showing that plant tissues are more reliable than human tissues during the decellularization process since vascularity was preserved and the ECM remained intact.

Evaluation of Tissue Scaffolds: Testing Vascularity

The main struggle faced by human tissue scaffolds is the lack of a sustainable and functional vascular system, which is why so much focus was placed on vascularity during the plant research. Despite the lignin and SEM findings of the spinach leaves, the team of researchers conducted "perfusion studies" in order to definitively test the vasculature of the newly developed spinach scaffolds. The perfusion studies involved using Ponceau Red dye, which was injected via the cannula of decellularized spinach leaves [2]. As the dye was allowed to perfuse, initial observations led to two important findings. In terms of a major success, the dye was found to flow into smaller branches of the spinach veins, which meant that microvasculature was minimally damaged following decellularization [2]. Even though the dye was able to perfuse through the entire leaf, minor leakage also occurred [2]. Because the results weren't completely successful, more experimentation was done in regard to the vascularity of the spinach leaf scaffolds.

In order to fully understand the capabilities of the scaffold's vascular networks, perfusion tests involving larger substances were carried out. According to Dr. Gaudette from WPI, "Human capillaries have vessel diameters between 5 and 10 μm that support the flow of the 6-8 μm diameter red blood cells" [2]. This scientific fact is the motivation for using larger substances to simulate blood flow through the spinach veins. The large substances chosen for perfusion were different sized fluorescent microspheres (synthetic polymers) [2]. The results indicated that the transport of these microspheres became increasingly restricted (but still possible) as the diameters of the spheres increased. Most notably, all the microspheres within the size range of red blood cells were able to perfuse freely throughout the scaffold [2].

Two implications were drawn from the microsphere perfusion tests. Not only was the spinach scaffold's ECM and vascular network able to transport microspheres in the size range of red blood cells, but its structural integrity was also maintained when transporting microspheres 10 times the size of red blood cells (100 μm microspheres vs. 6-8 μm). These findings again coincided with the initial hypothesis that plant vasculature is able to mimic human vasculature. Despite the minor leakage through the plant leaves, a tremendous amount of progress has already been made in terms of the sustainability and functionality of plant tissue scaffolds.

The Fundamentals of the Process: Cell Seeding and Recellularization

Once the plant scaffolds were thoroughly evaluated by Gaudette and his team, the engineering process moved onto the recellularization phase. For the purposes of simplifying their experiments, the researchers decided to focus in on two major cell lines. These cell lines were the human umbilical vein endothelial cells (HUVEC) and the human pluripotent stem cell-derived cardiomyocytes (hPS-CM) [2]. The HUVEC cell line most likely appealed to the researchers because of their availability and affordability, whereas the hPS-CMs were chosen to test the recellularization capabilities of heart cells. Additionally, the seeding techniques employed during this recellularization phase were similar to those used for the recellularization of human tissue scaffolds because plant and human tissues share similar structural geometries.

Preparation of Scaffolds Before Cell Seeding

Prior to the recellularization process, plant scaffolds had to be properly prepared to ensure successful seeding. All the plant scaffolds were treated with fibronectin (an ECM protein that aids in the binding of ECM components) [2]. The coated specimens were then monitored in a standard incubator for 24

hours and perfused with phosphate-buffered saline (PBS) to wash off residual fibronectin much like in the decellularization phase [2]. The main reason for utilizing fibronectin was to ensure successful cell adhesion during the seeding procedures. Furthermore, the ECM protein also aids in cell growth, differentiation, and migration. It is important to note that although fibronectin isn't found in plants, its ability to assist the functionalities of human cells is advantageous since the plant scaffolds were to be seeded with human cell lines.

Seeding Analysis: HUVEC Cell Line

Another reason for choosing the HUVEC cell line was because endothelial cells form the inner linings of various cavities in the body, which include blood vessels [2]. Consequently, the plant scaffolds were treated with fibronectin right before the seeding phase to ensure maximum efficiency of blood flow in post-recellularization testing. A sample size of roughly 375,000 HUVECs was injected into the plant scaffolds, specifically spinach leaves (the "model species"), via the cannulated stems [2]. This meant that the endothelial cells will slowly develop into the walls of blood vessels with fibronectin assistance by using the cellulose composition of the ECM as a "structural guide."

The researchers then proceeded onto the testing phase once endothelialization of the spinach leaf vasculature using HUVECs was complete. In order to analyze the success of recellularization, comparison tests (similar to those done for DNA analysis) were carried out. Results from LDL assaying showed that all the human cells were able to properly grow inside the spinach leaf scaffolds [2]. As a result of these findings, the researchers concluded that the HUVECs remained viable even after being introduced into a foreign plant environment.

Seeding Analysis: hPS-CM Cell Line

Recellularization using the hPS-CMs differed in that they were implanted on the surfaces of the scaffolds, as opposed to being perfused throughout the leaf structure. Through contractile analysis, the cell clusters were found to reach the highest contractile rates 10 days after seeding [2]. Additionally, contractile rates of the heart cells started to decrease from day 17 to 21. This decrease in contractile frequencies was alarming since it could mean that the cells were losing functionality as time progressed. To investigate the issue, the team carried out a series of calcium-based signal analyses because calcium is essential for heart contractions.

Based on the results of the calcium tests, the researchers concluded that hPS-CMs did not lose functionality after recellularization because they were able to produce strong calcium signals. Furthermore, the researchers came up with an explanation for the inconsistent frequency changes in the previous contractile analyses. According to their report, "this difference in frequency is probably due to the difference in the size of different clusters analyzed" [2]. As seen in figure 5, the heart cell derivatives were able to contract and form differently sized clusters on the surfaces of the leaves. The calcium testing helped to not only provide a reasoning behind contractile inconsistencies, but also demonstrated the hPS-CM's ability to retain functionality.

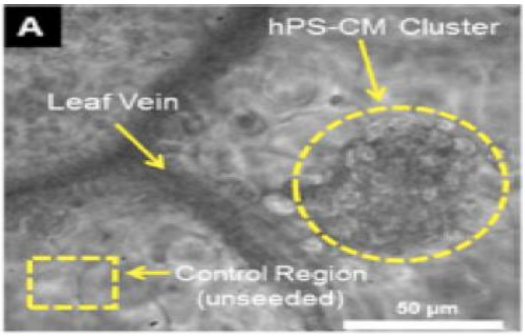

FIGURE 5 [2]
Clustering hPS-CMs on surface of spinach scaffold

STRIVING FOR A GREENER FUTURE

Current research has shown that human tissue scaffolds are not completely biocompatible when tested in vivo. This same concern applies to plant tissue scaffolds despite the versatility and rigidity of cellulose in the plant ECM. According to the researchers, "decellularized cellulose is biocompatible and biodegradable, but it is unclear the in vivo response to whole decellularized plant tissue" [2]. What this implies is that although plant tissue scaffolds have shown to have less drawbacks than human tissue scaffolds, the future of biofunctionalizing plants is strongly dependent upon strategies that will preserve plant ECM and cellulose compositions by preventing and/or limiting biodegradability.

There are also definitive advantages to using plant tissue scaffolds. Human tissues are extremely difficult to obtain and the cost of purchasing them makes human tissue research very scarce. The use of bioengineered plants, however, is much more economically sustainable due to the availability of plants and their low costs. Additionally, recellularization of plant scaffolds provided different insights into the future of biofunctionalizing plants. In terms of HUVEC seeding, the research team was able to conclude that epithelial cells have the potential to grow and develop into blood vessels that mimic the human vascular system within plant scaffolds. Moreover, implantation of the stem cell-derived cardiomyocytes showed that heart cells are able to retain functionality, while simultaneously grow and divide on the surfaces that they were seeded to. The biggest takeaway from the seeding process was that plant scaffolds are able to sustain human cell growth and stable vasculature.

Dr. Gaudette's research proved that many of the limitations faced by human tissue scaffolds could be overcome by utilizing plant tissue scaffolds instead. This new engineering route also has the capability of increasing all facets of sustainability by providing a more reliable and

efficient pathway to artificial organs. The results from his experiments weren't faultless, but the successes were enough to motivate additional research on biofunctionalizing plants. Many questions are left unanswered but with further research and experimentation, a potentially greener and more biocompatible method could very well be the solution to the organ transplant crisis.

SOURCES

[1] M. Greshko. "Sheep-Human Hybrids Made in Lab—Get the Facts". National Geographic. 2.18.2018. Accessed 2.21.2018. https://news.nationalgeographic.com/2018/02/sheep-human-hybrids-chimeras-crispr-organ-transplant-health-science
[2] J. Gershlak et al. "Crossing kingdoms: Using decellularized plants as perusable tissue engineering scaffolds." Science Direct. 2.11.2017. Accessed 1.12.2018. https://www.sciencedirect.com/science/article/pii/S0142961217300856
[3] J. Gamble. "Growing Organs on Apples." The Atlantic. 7.27.2016. Accessed 1.12.2018. https://www.theatlantic.com/science/archive/2016/07/growing-organs-on-apples/493265/
[4] P. M. Crapo et al. "An overview of tissue and whole organ decellularization processes." US National Library of Medicine. 4.2012. Accessed 2.18.2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3084613/#__ref-listidm139628941024608title
[5] G. A. Villalona et al. "Cell-Seeding Techniques in Vascular Tissue Engineering." US National Library of Medicine. 2.22.2010. Accessed 2.18.2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2946885/
[6] M. E. Scarritt et al. "A Review of Cellularization Strategies for Tissue Engineering of Whole Organs." US National Library of Medicine. 3.2015. Accessed 2.18.2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378188/
[7] K. H. Hussein et al. "New insights into the pros and cons of cross-linking decellularized bioartificial organs." The International Journal of Artificial Organs (IJAO). 1.25.2017. Accessed 1.24.2018. https://www.researchgate.net/publication/313025587_New_insights_into_the_pros_and_cons_of_cross-linking_decellularized_bioartificial_organs
[8] M. Cohen. "WPI Team Grows Heart Tissue on Spinach Leaves." Worchester Polytechnic Institute. 3.22.2017. Accessed 1.13.2018. https://www.wpi.edu/news/wpi-team-grows-heart-tissue-spinach-leaves
[9] D. Chambers. "Spinach Leaf Transformed Into Beating Human Heart Tissue." National Geographic. 3.25.2017. Accessed 1.13.2018. https://news.nationalgeographic.com/2017/03/human-heart-spinach-leaf-medicine-science/

ADDITIONAL SOURCES

"A new spin for spinach: A scaffold for working heart cells". Harvard Health Publishing. 6.2017. Accessed 1.27.2018. https://www.health.harvard.edu/heart-health/a-new-spin-for-spinach-a-scaffold-for-working-heart-cells
D. Chambers. "Spinach Leaf Transformed Into Beating Human Heart Tissue." National Geographic. 3.25.2017. Accessed 1.13.2018. https://news.nationalgeographic.com/2017/03/human-heart-spinach-leaf-medicine-science/
M. Cohen. "WPI Team Grows Heart Tissue on Spinach Leaves." Worchester Polytechnic Institute. 3.22.2017. Accessed 1.13.2018. https://www.wpi.edu/news/wpi-team-grows-heart-tissue-spinach-leaves
P. M. Crapo et al. "An overview of tissue and whole organ decellularization processes." US National Library of Medicine. 4.2012. Accessed 2.18.2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3084613/#__ref-listidm139628941024608title
G. dePillis. "Scaffold To Grow Hearts in Laboratory." Linkedin. 5.5.2017. Accessed 1.25.2018. https://www.linkedin.com/pulse/scaffold-grow-hearts-laboratory-g-j-depillis
K. F. Eng. "A promising way to grow body parts … using an apple." TED. 2.16.2016. Accessed 1.24.2018. https://ideas.ted.com/a-promising-way-to-grow-body-parts-using-an-apple/
J. Gamble. "Growing Organs on Apples." The Atlantic. 7.27.2016. Accessed 1.12.2018. https://www.theatlantic.com/science/archive/2016/07/growing-organs-on-apples/493265/
J. Gershlak et al. "Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds." Science Direct. 2.11.2017. Accessed 1.12.2018. https://www.sciencedirect.com/science/article/pii/S0142961217300856
M. Greshko. "Sheep-Human Hybrids Made in Lab—Get the Facts". National Geographic. 2.18.2018. Accessed 2.21.2018. https://news.nationalgeographic.com/2018/02/sheep-human-hybrids-chimeras-crispr-organ-transplant-health-science/
K. H. Hussein et al. "New insights into the pros and cons of cross-linking decellularized bioartificial organs." The International Journal of Artificial Organs (IJAO). 1.25.2017. Accessed 1.24.2018. https://www.researchgate.net/publication/313025587_New_insights_into_the_pros_and_cons_of_cross-linking_decellularized_bioartificial_organs
M. E. Scarritt et al. "A Review of Cellularization Strategies for Tissue Engineering of Whole Organs." US National Library of Medicine. 3.2015. Accessed 2.18.2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4378188/
"Using Plants as 3-D Scaffolds for Tissue Engineering." University of Wisconsin-Madison. 3.22.2017. Accessed 1.14.2018. https://www.technologynetworks.com/cell-

science/news/using-plants-as-3-d-scaffolds-for-tissue-engineering-286511
G. A. Villalona et al. “Cell-Seeding Techniques in Vascular Tissue Engineering.” US National Library of Medicine. 2.22.2010. Accessed 2.18.2018. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2946885/

ACKNOWLEDGEMENTS

We would like to thank Beth Newborg for coming into our engineering class and going over the details of every step in the process. We would also like to thank our writing instructor, Rachel McTernan, our co-chair, Conrad Li, and our two chairs, Alexander Sundermann and Suyesh Acharya, for answering all our emails and providing feedback on our submissions.