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Article

# Developing "Off the Shelf" Pancreases for Diabetic Patients Using Bacterial and Kombucha Tea Waste

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## SUMMARY

**The advent of tissue and organ engineering provides long-term treatment for conditions previously considered chronic and untreatable. Through advances in the field of tissue engineering, patients have the potential to receive synthetic replacements of damaged tissues and cellular grafts. Scaffolds, based in either synthetic or natural polymers, are fundamental to tissue engineering approaches because they provide a structure that mimics the native environment which facilitates cellular differentiation, organization, and functionality. However, current protocols for creating scaffolds remain complex and difficult to translate to the clinic. Our investigation aimed to provide potential solutions to fundamental issues associated with islet cell transplantation, a possible cure for type 1 diabetes. In this study, we used bacterial cellulose as the polymer to create scaffolds. We modulated the structural characteristics of the bacterial cellulose to aid cellular infiltration. After processing, scanning electron micrographs of the scaffolds showed that a favorable blood vessel morphology was maintained and an open pore geometry was obtained. We effectively manipulated the porosity of the scaffold by altering agarose concentrations. The 0.5% agarose-cellulose composite group had an average pore area of 20,000 μm$^2$, which permitted the establishment of regionalized cells islands. Notably, the popular fermented tea drink, kombucha, produces bacterial cellulose as a waste product. Kombucha is made using a symbiotic culture of bacteria and yeast (SCOBY). Normally, SCOBYs are simply discarded after the fermentation process is complete, but could have untapped potential as a platform for tissue engineering and transplantation procedures. To verify that bacterial cellulose can be used as a biomimetic platform, INS-1 cells (an insulin-secreting beta cell-derived line) were seeded in the bacterial cellulose scaffold. We observed morphological changes, such as cytoplasmic extensions and clustering, suggesting reestablishment of functional cell islands. Hence, the results of our experiments suggested that bacterial cellulose is a cost-effective scaffolding platform that can be used to house islet cells as a promising tool in islet cell transplantation, a possible cure for diabetes.**

## INTRODUCTION

Utilizing artificially engineered tissues has the potential to provide holistic and long-term treatment for a wide range of diseases by offering large scale replacement of damaged organs and tissues. However, the clinical success of tissue engineering is currently confined to low-metabolism, acellular, prevascularized, and thin tissues (1). This investigation sought to use techniques from tissue engineering for islet cell transplantation, a promising cure for type 1 diabetes. Type 1 diabetes is a lifelong autoimmune disorder which results in the destruction of islets, the clusters of different cell types found within the pancreas. One of the cell types present, the beta cell, is responsible for insulin production. By receiving islet cell transplants as a replacement, patients can eliminate their dependence on external sources of insulin (2). However, one of the primary factors limiting the progression of tissue engineering and islet cell transplantation is failure to recreate the physical and chemical microenvironment the cells would naturally be found in (3).

Transplantation of islets requires a high cell density equivalent to that of 2-3 donors for each procedure. Within the context of the current organ shortage, where 1.2 million people are in need of an organ for transplantation, it is not likely that islet transplantation can be translated into a cure for type 1 diabetes (3). This high cell density required is partially attributable to the perishing of 60% of newly transplanted islets during the first 48 hours of implantation (4). In the initial steps of transplantation, islets are often irreversibly damaged through standard isolation protocols that involve chemical digestion by proteolytic enzymes, intense mechanical stress, and subsequent purification techniques. Without a support structure, islets are subsequently unable to reestablish their native clustered microenvironment. As a result, functionality is hindered, hypoxic injuries are induced, and effective maintenance of normal levels of blood sugar is prevented (5).

Scaffolds are gaining attention within the scientific community as a 3D framework for cell attachment and proliferation. An ideal scaffolding would be biocompatible, biodegradable, and cost-effective (6). The findings of this study suggest that bacterial cellulose, the most abundant biopolymer, is a cost-effective, sustainable, and easily tunable platform that can fulfill these requirements.

As a natural polysaccharide-based polymer, bacterial cellulose shares several chemical similarities to the native extracellular matrix which is rich in glycosaminoglycans, glycoproteins, and glycolipids (7). The ability of cellulose to generate biological cues has been linked to its glycan unit (7). Although bacterial cellulose is chemically similar to plant cellulose, bacterial cellulose does not require intense processing for the removal of intermediates such as lignin and hemicelluloses (8). *Gluconacetobacter xylinus* is a bacterial species known to secrete polymer chains that assemble as a cellulose membrane at the air-liquid interface (8). Amongst easily obtainable sources of *G. xylinus*, the fermented tea

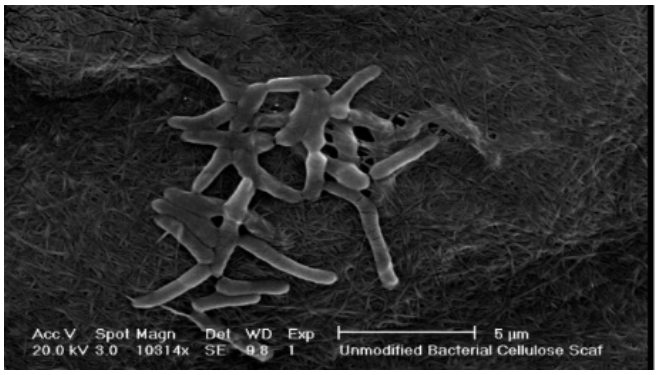



**Figure 1:** Scanning electron micrograph showing the process of bacteria secreting cellulose that forms membranes with interwoven nanofibrous structures.

drink kombucha has symbiotic cultures of acetic acid bacteria (*Gluconoacetobacter* and *Komagataibacter* species), lactic acid bacteria (*Lactobacillus* and *Lactococcus* species), and yeast (*Saccharomyces cerevisiae*). Previous research has demonstrated that the microbial agents within kombucha tea can also create tightly interwoven nanofibrous cellulose structures (9).There has been limited research on the use of bacterial cellulose as a scaffold platform for islet cell transplantation. Therefore, we also sought to study the potential of kombucha cellulose-based scaffolds for islet cell transplantation.

A potential challenge in using unmodified bacterial cellulose membranes as scaffolds is that they lack the macropores necessary for cell attachment, infiltration, and vascularization (10). While techniques ranging from particulate leaching, solvent casting, gas-foaming, microsphere sintering, and electrospinning have been used to create pores, reports of cell toxicity from residues have been noted (11). These techniques also require expensive infrastructure, materials, and equipment (11). Alternatively, agarose is a promising compound capable of creating pores that has been investigated in the context of creating composite scaffolds for bone tissue engineering (12).

In Phase 1 of our investigation, we utilized a range of agarose concentrations to create macroporous scaffolds and manipulate the porosity of the bacterial cellulose scaffolds. We hypothesized that a greater amount of agarose would result in a greater pore size. In Phase 2 of our investigation, we hypothesized that both the bacterial cellulose derived from pristine cultures of *G. xylinus*, and the cellulose derived from symbiotic yeast and bacterial cultures in Kombucha tea would serve as a platform compatible for INS-1 cell attachment.

Bacterial cellulose and agarose composites with macroporous structures were developed as a scaffold platform. Furthermore, preliminary cell seeding studies corroborate the potential applicability of bacterial cellulose for islet cell transplantation scaffolds. The approach we took in this study to provide a platform mimicking the microenvironment is not only cost-effective but also sustainable.

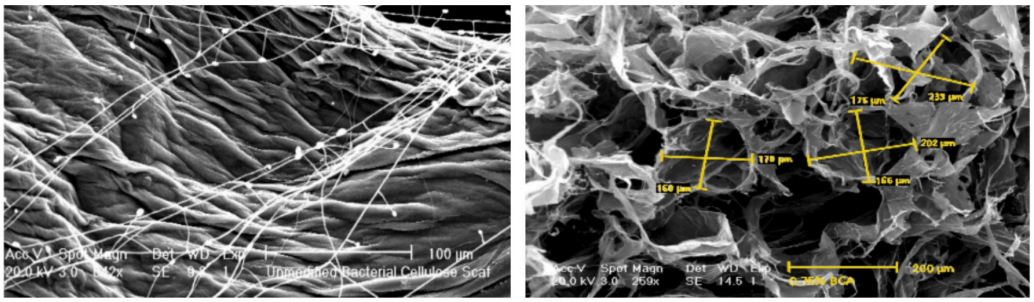
## RESULTS

### Agarose Allows for the Creation of Macroporous Bacterial Cellulose Scaffolds

In Phase 1 of our investigation, we hypothesized that altering agarose concentrations would enable creation of *G. xylinus* (pristine, single culture) cellulose-based scaffolds with different pore morphologies. We ranged the concentrations from 0.5% to 1%. Specifically, we hypothesized that 1% agarose-bacterial cellulose (BCA) would have the greatest average pore area. To characterize the morphology of the scaffolds' fibers and pores, we used scanning electron microscopy to measure the area of each pore.

The fiber morphology naturally produced by *G. xylinus* is analogous to the pancreas' blood vessel morphology and fiber arrangements achieved by the process of electrospinning (**Figure 1**). Electrospinning is a synthetic fabrication technique which creates organized nanofiber structures in polymers. We processed the cellulose membranes by homogenizing with an immersion blender and adding agarose increased the surface area of the 1.5 cm diameter scaffold while maintaining the intrinsic nanofibrous structures unique to cellulose **(Figure 2)**. Specifically, physical interconnections between the fibers were intact, and pores, defined as circular cross sections of 2D spaces, were present in processed agarose-cellulose composites.

The cellulose-agarose composite scaffolds displayed an open pore geometry while the control scaffolds, unmodified bacterial cellulose, displayed no pores on their surfaces (**Figure 2**). The average pore areas were 20,000 µm, 6,000 µm, and 2,000 µm for the 0.5%, 0.75%, and 1% bacterial cellulose agarose composites, respectively **(Figure 3,** $p < 0.05$**).** The statistical significance of these results was analyzed by using a one-tail t-test and comparing the pore areas of the bacterial-cellulose agarose composite groups to those of the unprocessed bacterial cellulose scaffolds with a




A: Unmodified Bacterial Cellulose Scaffold B: Composite Bacterial Cellulose-Agarose Scaffold

**Figure 2:** Scanning electron microscopy of composite scaffolds showed macropores formed through the addition of agarose. A) Scanning electron micrograph of an unmodified bacterial cellulose scaffold containing no pores. B) Scanning electron micrograph of a 0.75% bacterial cellulose-agarose scaffold with pores indicated by yellow outline.

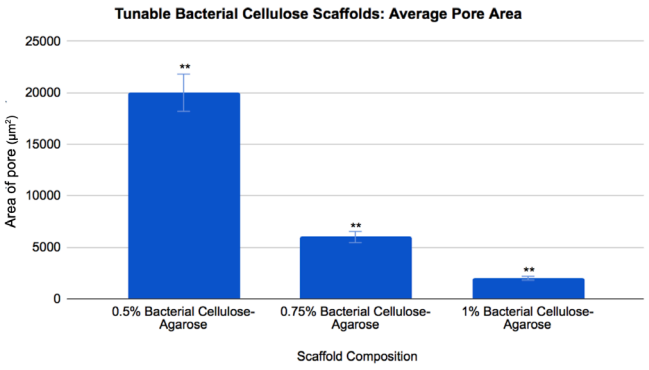



**Figure 3:** The average pore size areas (μm²) were calculated for each scaffold group through scanning electron microscopy. Three samples from each scaffold group were characterized and four randomized images of each scaffold were taken (total of 12 images for each group). The error bars indicate the standard deviation from the average pore size of each respective scaffold group. Asterisks are included on groups with $p < 0.05$ from a one-tailed t-test suggesting statistical significance.

threshold of significance of $p < 0.05$. Our initial hypothesis was not supported as the opposite effect was noted; processing with greater amounts of agarose led to a significantly smaller pore area (up to three times less with $p < 0.05$). Although changes in overall porosity were not predicted, processing with larger quantities of agarose decreased the number of pores.

The bacterial cellulose scaffolds show a remarkable ability to absorb fluids and maintain structural integrity. This feature is important to characterize in scaffolds because it determines the structure's ability to retain cell and fluid without dissolving. The lyophilized bacterial cellulose scaffolds could hold 400 μL of phosphate buffered saline while the lyophilized bacterial cellulose-agarose could hold 800 μL of phosphate buffered saline prior to losing structural integrity and dissolving **(Figure 4).** When normalized to weight, this meant that the lyophilized bacterial cellulose scaffolds had an average swelling ratio of 36 times their initial mass while lyophilized bacterial cellulose-agarose scaffolds had an average swelling ratio of 38 times their initial mass **(Figure 4).** The difference in swelling ratios is likely due to differences in porosity as detailed above.

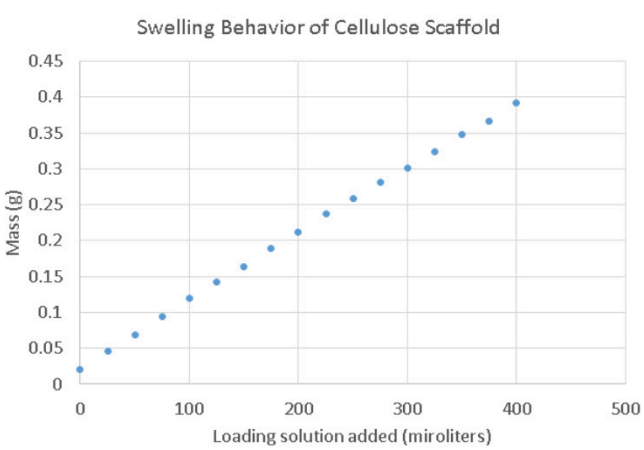
### INS-1 cells are compatible with Pristine Bacterial Cellulose and Kombucha-Based Bacterial Cellulose Scaffolds are Compatible with INS-1 Cells

In Phase 2 of this investigation, we aimed to assess the feasibility of using bacteria-derived cellulose as a platform for cell attachment. We hypothesized that bacterial cellulose-based scaffolds derived from *G. xylinus* and symbiotic cultures would aid in restoring native islet morphology. Kombucha-derived cellulose was utilized to analyze the potential tissue engineering applications of a widespread alternative to single culture-derived bacterial cellulose.

Pictures taken after seeding INS-1 cells in the well plates (coated with kombucha derived cellulose or containing no cellulose coating) and overnight incubation displayed morphological changes that indicate cell attachment onto the scaffolds. Cells seeded in the well plate coated with kombucha-derived cellulose had visible cytoplasmic extensions and congregated in native island-like clusters **(Figure 5D, Figure 5F)**. The INS-1 cells seeded in wells coated with kombucha derived cellulose displayed similar morphologies to INS-1 cells seeded in tissue culture coated wells, the positive control **(Figure 5D, Figure 5B)**. Due to material and time limitations, further quantification of the cells' viability could not be performed.

## DISCUSSION

This study aimed to address primary challenges in islet cell transplantation by investigating agarose-bacterial cellulose as a macroporous scaffold platform on which insulin secreting islet cells could be seeded and transplanted with. The findings of this investigation can also be applied to the larger fields of tissue and organ engineering because of the simplicity of the technique utilized. For example a challenge in cardiac tissue engineering is that myocytes require recreation of unique intracellular junctions to produce mechanical contractions that pump blood forward (13). The scaffold described in this study supports the cell-to-cell contact necessary for optimal functionality of islet cells. Other fields such as neural tissue and kidney tissue engineering which culture cells with highly specialized functions could also benefit. The electric



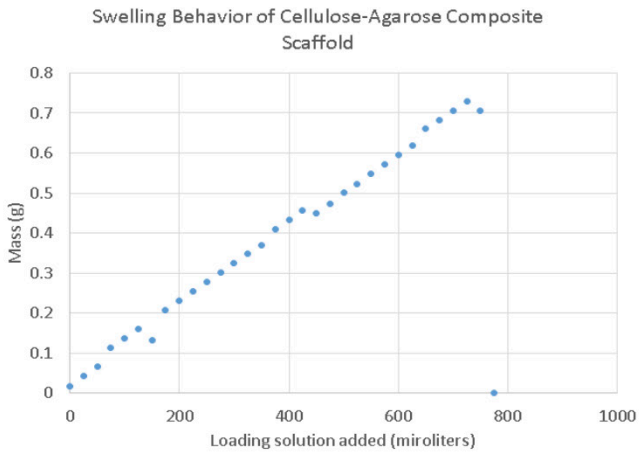




**Figure 4:** Swelling capacities of the lyophilized bacterial cellulose and bacterial cellulose- agarose composite scaffolds were assessed by determining the amount of phosphate buffered saline which the samples could retain. The endpoint was determined once structural integrity was lost. The unmodified scaffold had an average swelling capacity of 36 times original weight while the composite scaffolds had a swelling capacity of 38 times original weight.

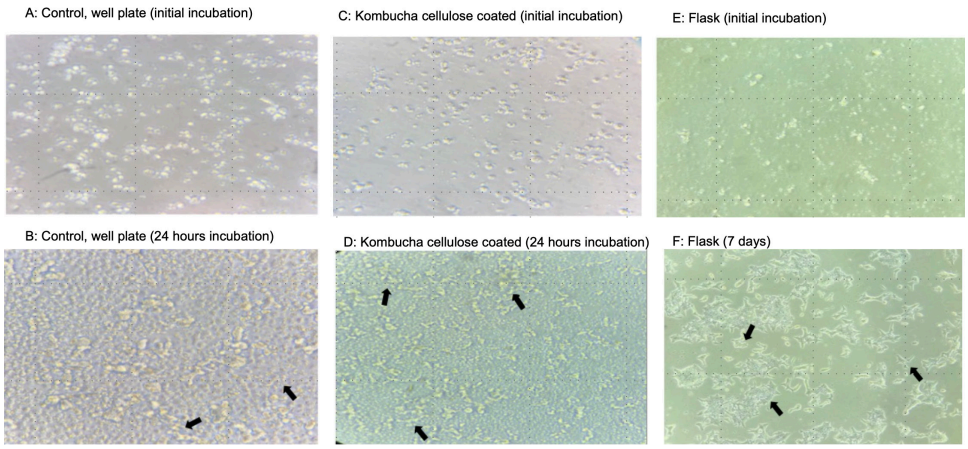



**Figure 5:** Images showing morphologies of the INS-1 cells seeded on control (A) and kombucha-derived cellulose (C). The first row shows INS-1 morphology after the cells were initially seeded (A, C). The second row depicts INS-1 cell morphological changes following 24 hours of incubation (B, D). Native islet cell morphology after 2 hours of seeding (E) and culturing for a week in a flask is (F). The arrows point to the clustering and island-like morphology and cytoplasmic extensions which are characteristic of INS-1 cells.

stimulation of neurons and filtration performed by nephrons are dependent upon cell-to-cell contact and the physical properties of their natural environments (14,15).

As a scaffolding platform, bacterial cellulose is different from synthetic and oil-based polymers as it is easier to obtain, environmentally friendly, and can be cultivated from a renewable source. The intrinsic nanofibrillar structure of bacterial cellulose is similar to the pancreatic blood vessel morphology previously well characterized in the literature (16). In Phase 1 of experimentation in which we aimed to create porous scaffolds with dimensions suitable for islet cell culture, the data supported our hypothesis that processing *G. xylinus*-based bacterial cellulose with agarose could create pores with dimensions suitable for islet cell culture. However, the second portion of our hypothesis was not supported. A greater concentration of porogen did not correspond to larger average pore areas. We postulate that this trend was observed because the greater concentrations of agarose had a "clogging" effect between the fibers. This addition of agarose as a one-step modulation to alter the entire structure of the scaffold offers an efficient methodology which enhances porosity. Hence, the need for additional procedures and equipment such as electrospinning, particulate leaching, gas infusion, and phase separation can be eliminated.

In the Phase 2 of experimentation, we performed cell-seeding studies to observe the morphological changes in islet cells when cultured on substrates coated with bacterial cellulose derived from pristine cultures and symbiotic cultures of kombucha. We demonstrated that bacterial cellulose facilitates favorable islet cell morphology changes such as cytoplasmic extensions and cell clustering. The two component system of the bacterial cellulose, derived from pristine bacterial culture and kombucha cellulose scaffold, and INS-1 cells demonstrated the first step towards using this biomaterial as a platform to support INS-1 cells and regulate cell morphology without added adhesion promoters. Although additional viability and metabolic assays could not be performed (such as the MTT assay), the results of this preliminary cell study suggest that bacterial cellulose based scaffolds have the potential to serve as a platform for INS-1 cell attachment and proliferation.

Future research should be done to better elucidate chemical and physical differences between bacterial cellulose produced by the symbiotic colonies of kombucha and the single strain of *G. xylinus*. Although kombucha waste-based bacterial cellulose could be an untapped high-value biomaterial, batch to batch variability should be considered and further studied. The variations in strains of symbiotic bacteria and yeast cultures, supplements added to recipes while brewing, carbohydrate source, pH, and temperature between each factory and distributor can potentially result in alteration to fiber architecture and orientation.

Prior to the clinical translation of the bacterial cellulose-agarose scaffolds proposed in this investigation, several additional material characterizations and cell studies are necessary to verify the biocompatibility of the scaffold. Immune responses to scaffold implants are the leading cause of failure of tissue engineering products (17). Cytokine and chemokine release levels can be used as indicators for inflammatory responses.The chemical properties and constituents of kombucha and pristine bacterial cellulose scaffolds can be profiled for factors known to contribute to inflammatory and immune rejection responses (17). Such chemical analysis can be useful in the process of optimizing alternate purification techniques (e.g. alternatives to the sodium hydroxide incubation) as kombucha tea SCOBYs typically have higher sugar residues on the cellulose membrane when compared to cellulose membranes synthesized by a monoculture of *G. xylinus*.

Furthermore, studies to quantify INS-1 cell proliferation and insulin release while seeded on the bacterial cellulose scaffolds should be performed. Since nuclear factor-κB and poly(ADP-ribose) polymerase (PARP) are proteins involved in major pathways associated with necrosis of islet cells, assays can be performed to characterize up/down regulation of these pathways over an extended period of time in cells seeded onto the bacterial cellulose scaffolds for viability (16). We also recommend that when the aforementioned studies are performed, cell culture conditions should mimic stress induced by islet isolation (18).

Existing literature suggests that the reduced bioactivity of synthetic scaffolds results in lower rates of cell adhesion

and implant fibrosis; however, comparison of the bacterial cellulose scaffolds proposed in this study to existing synthetic and natural polymer-based scaffolds will be better informed by the aforementioned studies. Additionally, there are numerous avenues to tailor the bacterial cellulose scaffold designed in this study. Testing different compositions of the cellulose-agarose composites can allow for the creation of macroporous and microporous bacterial cellulose scaffolds to suit other cell types. Modifications to the nanofibrous structure such as alignment, interconnectivity, width and the role of all these factors in vascularization and insulin release could also be studied.

Overall, the results of this study are promising, as scaffolds can be developed sustainably and processed cost-effectively by using bacterial cellulose and agarose. The kombucha and *G. xylinus*-derived bacterial cellulose macroporous scaffolds have the potential to be used as a platform technology in tissue engineering and can be functionalized (the addition of therapeutics/growth factor like compounds) in the future.

## MATERIALS AND METHODS

### Cultivating and Harvesting Cellulose from *G. xylinus*

We prepared media for bacterial growth using the following reagents purchased from Millipore Sigma: 5.0 grams of yeast extract, 3.0 g of peptone, and 25.0 g of mannitol dissolved in 1 L of distilled water and autoclaved overnight. In Phase 1, bacterial cellulose-based scaffolds were prepared by culturing *G. xylinus* bacteria (ATCC) in mannitol-based broth for two weeks. The cellulose membranes were aseptically removed and washed with distilled water to achieve a neutral pH of 7. The membranes were then sterilized by incubating in a 0.25 M sodium hydroxide solution for seven days.

### Preparing Composite Cellulose- Agarose Scaffolds

To prepare cellulose-agarose composites, cellulose membranes were homogenized using an immersion blender. We added 0.5%, 0.75%, and 1% weight by weight of agarose to the cellulose homogenate. The composite solutions were autoclaved overnight and transferred into 24-well plates (2 g per well). The composites were frozen at -86 °C and then lyophilized.

### Characterizing Physical Properties of Bacterial Cellulose Scaffolds

Scanning electron microscopy was performed on three samples for each composite group after sputter coating with palladium. Four pictures of randomly selected areas were taken for each scaffold sample. ImageJ›s near-distance plug-in was used to standardize pore selection and to measure the area of the 2D circular cross section of the pores in the scaffold samples. Averages and distribution of pore areas were analyzed for the three composite groups followed by statistical analysis using a one-tailed t-test (Excel).

To measure the swelling ratios and rates of the unmodified and cellulose-agarose composite scaffolds, the initial masses of eight scaffolds (four cellulose-agarose composites and four unmodified scaffolds) were noted. Scaffolds were loaded with 15 µL increments of phosphate buffered saline. The masses of the scaffolds were recorded until scaffold integrity was lost. The swelling ratios of scaffolds were calculated by comparing the averages of final swollen mass and initial dry mass of the four unmodified and four composite scaffold samples.

### Cell Seeding Studies

Preliminary cell studies were conducted to validate bacterial cellulose as an islet cell compatible platform. A rat insulinoma-derived islet cell line, INS-1, was provided by AddexBio and used as the model. Kombucha-derived bacterial cellulose was obtained by cultivating a SCOBY (symbotic yeast and bacterial culture) from Kombucha Kamp in a tea medium containing Lipton's "America's Favorite" tea bag, one cup of sugar, and the starter culture liquid provided. The cellulose membranes were sterilized by using the same method as described for sterilizing cellulose membranes synthesized by *G. xylinus*. Slurries of pristine bacterial cellulose and kombucha-derived cellulose were created by adding a 1:1 weight/weight ratio of cellulose and sterile, deionized water. The bottoms of sterile well plates were coated with 100 µL of the suspension and further sterilized using ultraviolet radiation in the cell culture hood for 16 hours. INS-1 cell suspensions were stained with Trypan Blue to count cell viability. The INS-1 cells were then seeded at a density of 300,000 cells per well. Images were taken after 2 hours of incubation and at the endpoint of 24 hours following incubation by randomly selecting 4 areas in each well. Morphological changes such as cytoplasmic extensions and settlement in colonies were documented and compared to morphologies of cells seeded on standard, tissue culture coated wells.

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