Bacterial Cellulose as a Potential Bone Graft

Kennedi K. Lowman[1,2,3]

[1]Albany State University, [2]Virginia Polytechnic Institute and State University, [3]Bioengineering/ Bioinformatics Summer Institute- Virginia Polytechnic Institute and State University School of Biomedical Engineering and Sciences ICTAS Stanger St Blacksburg, VA 24061

Abstract

Each year tissue engineering costs the United States $2 million dollars. Bacterial Cellulose (BC), a hydrogel with a fine fiber network, is produced by the bacterium *Acetobacter xylinum* that can be used as a protective coating. In contrast to other polymers, BC possesses high tensile strength, high water holding capabilities, and high mechanical properties. The purpose of the current study is to determine if individual fibers of BC can be functionalized with calcium by applying an electric field. BC was grown and calcium was deposited simultaneously using Corn Steep Liquor (CSL) media, with the addition of fructose, in channels 4 cm long x 5 mm wide x 2.5 mm deep. The channels contained platinum electrodes supplying an electric field of 3 to 7.5 volts for 72 hours in the presence of $CaCl_2$. BC pellicles formed and were then examined using the Environmental Scanning Electron Microscope (ESEM). Energy-Dispersive X-ray Spectroscopy (EDS) was also used to determine the composition in each sample. Calcium was found deposited on the BC fibers at 5.5 volts. Lower voltages, such as 4.0 volts, resulted in no calcium deposition on the fibers. The presence of Carboxymethyl Cellulose (CMC) is critical for the calcium deposition. Calcium deposition will occur at 5.5 volts suggesting there may be a specific electric field requirement for calcium deposition on BC.

Keywords: hydroxyapatite, bacterial cellulose, tissue engineering, bone grafts

1. Introduction

Tissue engineering research is a growing field where researchers experiment with more efficient and inexpensive ways to create scaffolds. These scaffolds will repair or replace whole tissues quicker than previous methods. Researchers are trying to create tissue scaffolds where cell proliferation occurs in the microstructure like it would in the human body.[1] Manufacturing challenges inhibit clinical trials from taking place on existing scaffold fabrication techniques.[2]

Worldwide there are over 2 million bone grafts performed every year. These procedures cost the US billions of dollars every year. There are three types of bone grafts: autografts, allografts, and synthetic grafts;[3] autografts and allografts are most common. Autografts harvest autologous bone from the iliac crest where a long surgical procedure is usually accompanied by residual pain. Typical complications include blood loss, nerve injury, infection, hernia fracture, cosmetic defects, and occasional chronic pain at the donor site.[4] Between autografts and allografts, allografts are the most frequently chosen bone substitutes because of the availability of tissue and its ability to be customized through manufacturing. These synthetic bone grafts should be biocompatible, show minimal fibrotic reaction, undergo remodeling, and support new bone formation. Additionally, mechanically synthetic bone substitutes should have similar strength to the bone it is replacing.[4] In our experiment, we are creating the initial steps for fabricating a synthetic graft by using BC and calcium. The presence of calcium is vital to repair fractures in the bone and initiate bone growth.[3] This type of research is being conducted to create safer, more efficient bone substitutes. By creating a more efficient product to replace bone grafts millions of dollars would be saved worldwide.[3, 5]

BC is a primary metabolite that is mainly used as a protective coating. Cellulose is a biopolymer that is represented as a microbial extracellular polymer. Bacteria forms subfibrils, which are the thinnest naturally-occurring fibers.[6] The subfibrils are crystallized into microfibrils, which form bundles that turn into ribbons. The ribbons accumulate into a fine fiber network forming a mat called a pellicle during static conditions (S-BC). Static conditions refer to the cells at the oxygen-rich liquid/air interface.[6,7] The pellicles form parallel but disorganized planes. S-BC fibrils are extended

and piled on top of one another in a more criss-cross pattern.[6]

The strain of bacteria cellulose used most commonly is *Acetobacter xylinum*. *A. xylinum* is a model microorganism for cellulose studies. It is a gram-negative, acetic acid bacterium.[6] *A. xylinum* grows rapidly and can be maintained under controlled environments in a laboratory setting.

Bone is a composite of collagen and hydroxyapatite. Hydroxyapatite is a chemical composite of natural bone with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$; this ideally mimics synthetic bone structures.[3] Previous experiments created calcium-deficient hydroxyapatite by immersing the BC pellicle into a calcium solution followed by a phosphate solution. Our approach uses electric fields to control the fiber orientation and to deposit calcium throughout the forming structure. We are studying the effects of electric fields on BC growth and calcium deposition using CSL media with the addition of $CaCl_2$.

The purpose of this study is to coat individual BC fibers with calcium by applying an electric field to the BC pellicle. The difference between this study and previous studies is that we are trying to apply calcium to each layer of BC as it grows whereas previous studies dipped the pellicle in a calcium solution after it had already formed. Electrolysis is used as a result to coat the BC fibers with calcium. With the right parameters, calcium can be deposited on every fiber in the BC pellicle.

2. Materials & Methods

2.1 Preparation of Media

Corn steep liquor (CSL) media was used during this experiment with the addition of fructose as a culture media. *A. xylinum* bacteria were grown for this experiment in a flask in the incubator for 3 days at 27 °C in the culture media.

2.2 Fabrication of Micro-growth Channels

Polydimethylsiloxane (PDMS) liquid was poured over the stamp of a 4 cm long x 5 mm wide x 2.5 mm deep channel. The stamp and PDMS were set on a hot plate allowing the PDMS to harden for 30 minutes. After taking the mold off the stamp, two holes were punched into each channel, one at each end. The mold was washed with soap, ethanol, and DI water and dried with pressurized air. It was then placed in plasma cleaner with two glass slides for a total of four minutes. The glass slides and mold were bonded together with channels facing down towards the slide.

2.3 Cellulose Production and Deposition

A 1000uL pipette tip was inserted into one side of the channel and a pipette tip with a mixture of 20 mL media, 2 mL of $CaCl_2$, and 1 mL of bacteria was inserted in the other end to fill the channel up. Platinum wire was fed through each tip and connected to a DC supply with a voltage of 3.5, 5.5, 6.0, or 7.0 to apply electric fields to the channels. The experiments were left to run for 72 hours at the set voltage.

2.4 Sample Preparation for ESEM

The PDMS was peeled off the glass slides and the pellicles were pulled out of the channels with tweezers and placed in test tubes of DI water. The test tubes were placed in the sonicator for an hour at 60 °C. Afterwards, new DI water replaced the old and the test tubes were immersed into liquid nitrogen. Immediately after immersing in liquid nitrogen, the test tubes were placed in a jar and connected to a freeze dryer for two days. After the pellicles were freeze dried, they were cut and mounted onto SEM specimen holders with the surface and cross-sectional pieces facing upwards. They were placed in a sputterer and coated with palladium for 45 seconds.

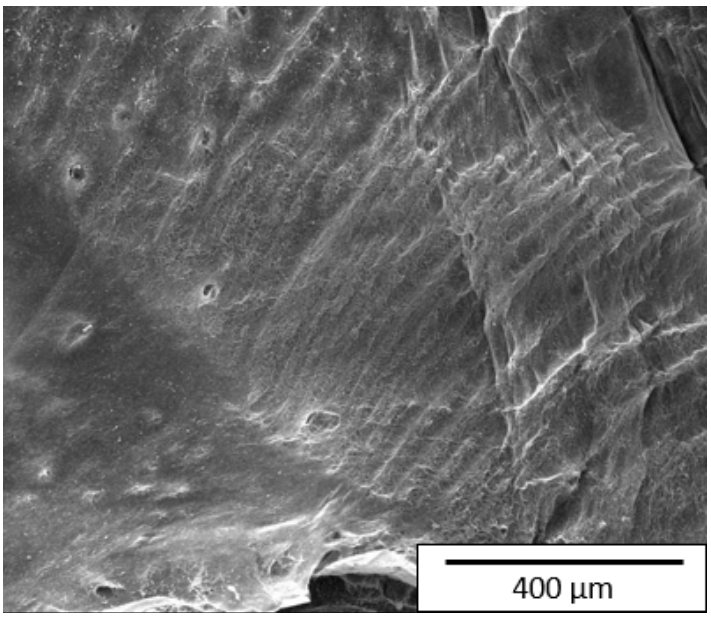

Figure 1: ESEM images of the surface of a pellicle at a voltage of 5.5.

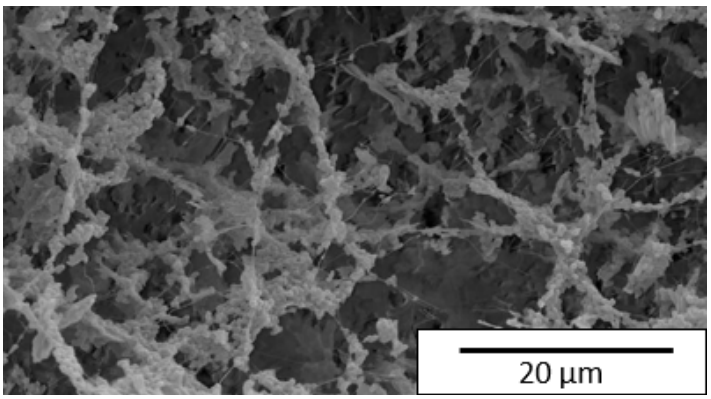

Figure 2: Wire deposition near the 5.5V electrode hole.

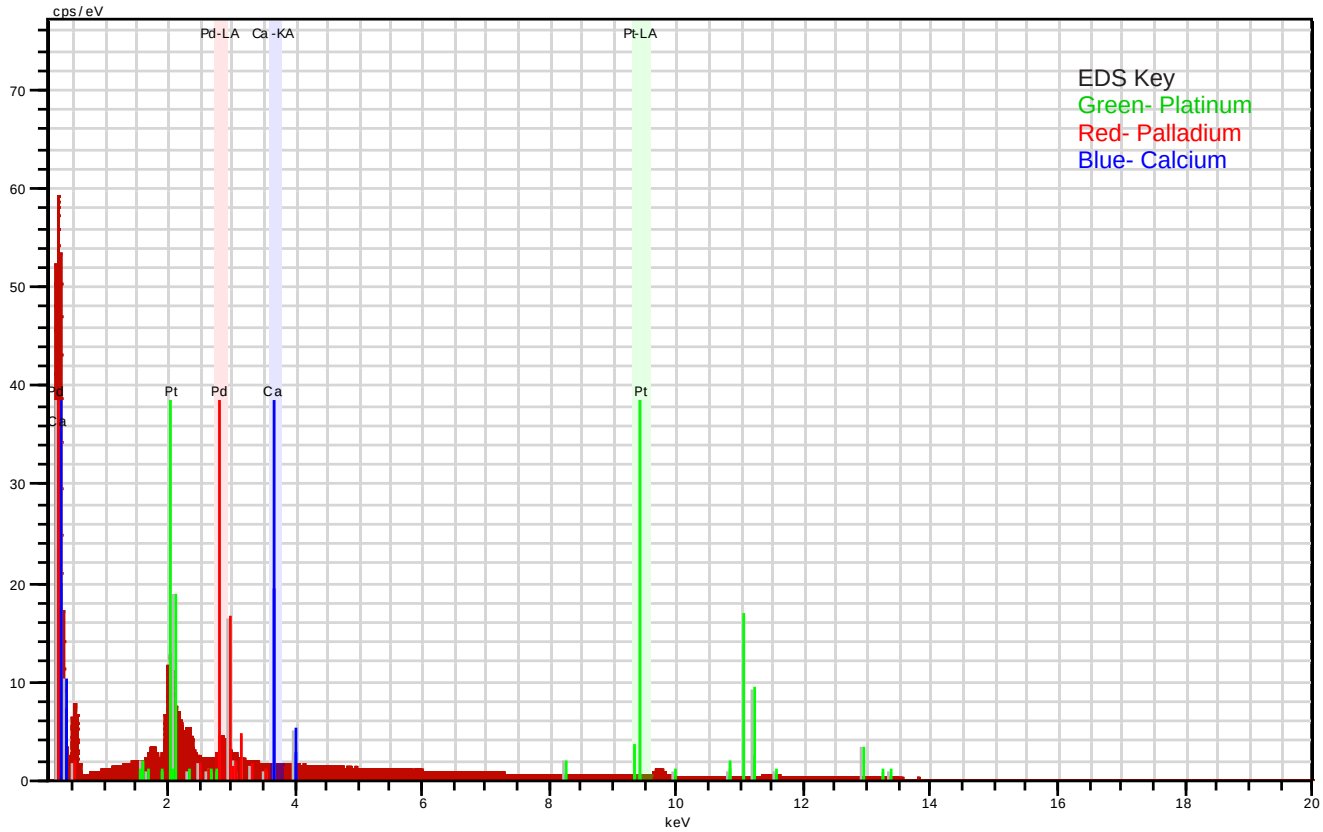

Figure 3: Materials deposited in the 5.5V sample.

3. Results

The ESEM was used to observe the surface and cross section of the pellicle pieces for traces of calcium on the BC fibers. Observing the overall surface of the pellicles revealed no calcium on the fibers. However, the fibers near the holes, where the electrodes were placed, calcium was observed at 5.5V.

EDS analysis was carried out for the 5.5V sample and small amounts of calcium along with palladium from the coating, and platinum were detected in the sample.

After observing the electrode hole during our first visit to the ESEM, we took a closer look at the electrode holes during the next set of trials to observe any material deposition.

We experimented with some of our trials and added Carboxymethyl Cellulose (CMC) to the solution. Under the ESEM, the BC pellicles showed crystal formation on the fibers

Through EDS, we found a greater amount of materials deposited in our sample such as calcium, palladium, oxygen, carbon and potassium. Potassium was a material in our media. Carbon and oxygen are BC pellicle growth components.

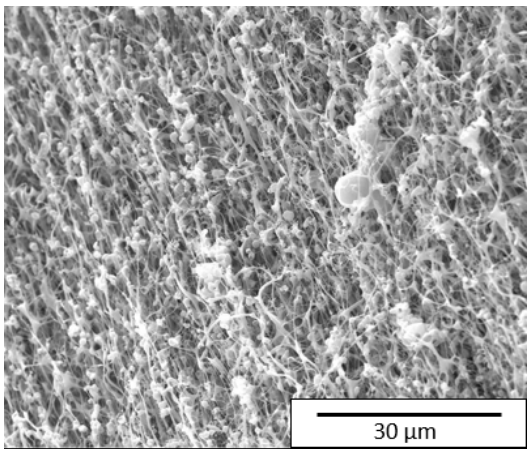

Figure 4: Materials deposited on the BC fibers at 5.5 V.

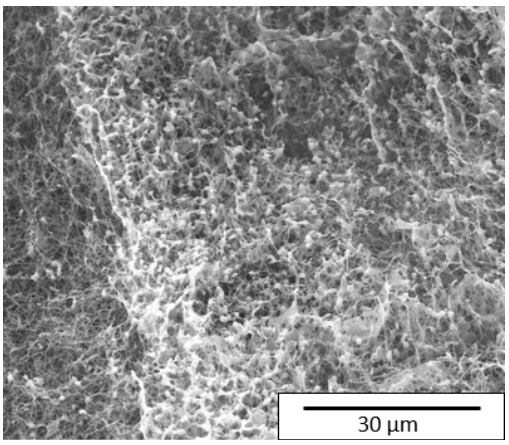

Figure 5: At closer observation EDS reveals calcium on the BC fibers.

4. Discussion

At low voltages, fiber alignment occurs. BC grows at 3.5V to 5.5V but would not grow at 6.0V or 7.0V. When exposed to higher electric fields, the bacteria halts its production of cellulose and it's possible that at these electric fields it may even kill the bacteria. The first couple of trials did not show calcium deposited on the BC fibers but the EDS analysis picked up palladium and platinum. Palladium was used to coat our samples while platinum electrodes were used to apply an electric field. Since BC is made up of carbon and oxygen the EDS showed high values of these materials.

We experimented with some of our trials and added CMC to the solution and received positive results. Previously, CMC was used to initiate a calcium-deficient hydroxyapatite.[3] The CMC physical absorption onto cellulose is thought to occur due to a co-crystallization process but the mechanism is still not well understood. Observing the BC pellicles under the ESEM showed particles or crystals on our fibers as seen in Figure 3. In the experiments without CMC, we found 0.70 atomic percent of calcium in our pellicle, whereas when we added CMC, our atomic percent of calcium increased to 1.23. Many trials were not conducted using CMC until we realized that CMC was helpful for another group's experiments.[3] The experiments without CMC did not show calcium on the fibers when viewed under the ESEM. Also, increasing the amount of calcium and molarity in some later trials the fibers remained to show no calcium deposition.

Although we did not get a high quantity of calcium onto each fiber we were able to deposit calcium by applying a low voltage electric field to each channel.

5. Conclusion

Calcium was successfully deposited onto BC fibers at 5.5 V. However, deposition was only seen locally on the BC and not throughout the entire pellicle. When CMC, a negative polymer, was added to the media, more deposition was seen.

In the future, we could try and find some calcium-coated electrodes. Also, mixing CMC and a phosphorus solution, such as phosphate buffered saline (PBS), into the media may help deposit phosphorus and calcium on each fiber of the pellicle to form hydroxyapatite. More experiments on finding the right channel size to grow the BC in is an important factor for future research since a lot of our trials' solution did not form pellicles even after 72 hours.

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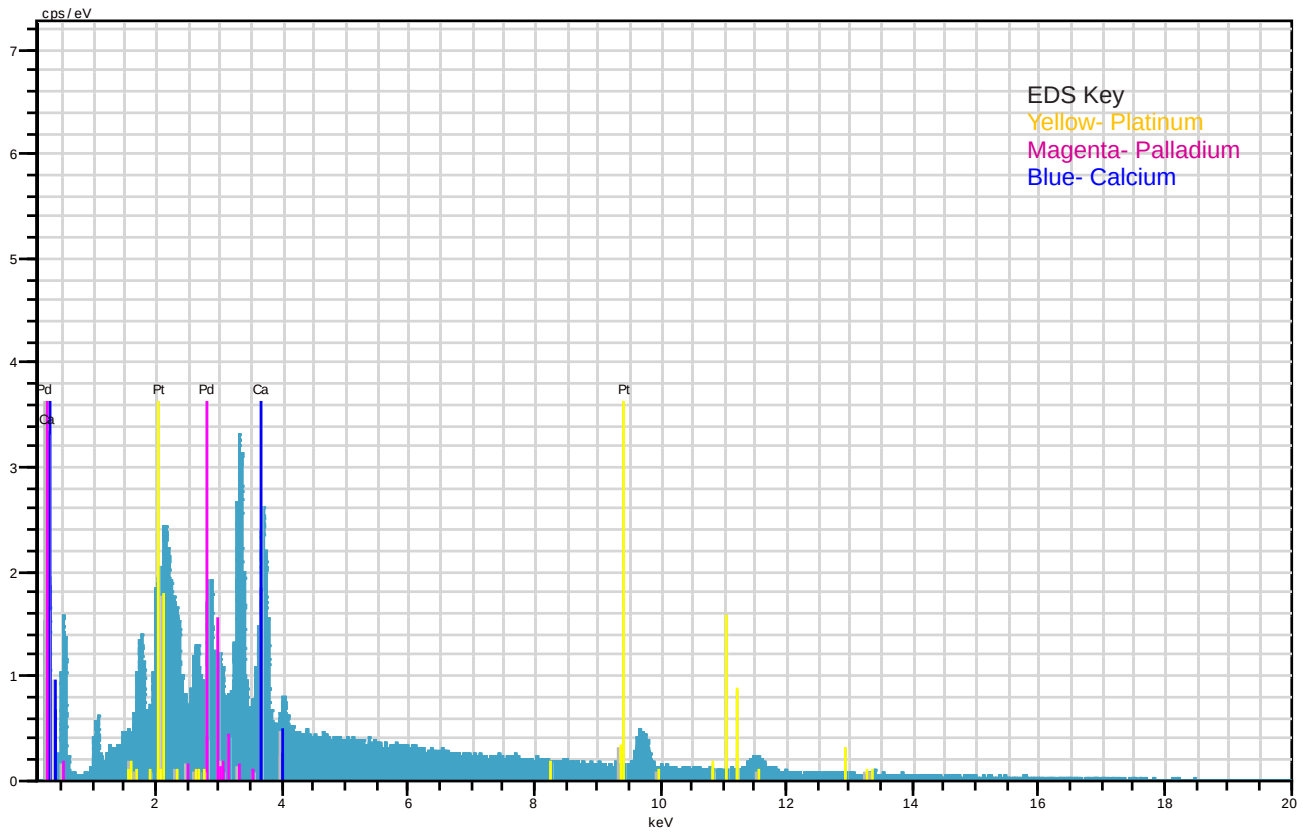

Figure 6. EDS results show calcium deposition at 5.5 V.

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About the Author

Kennedi Lowman is a native of Columbus, GA. She loves reading, writing poems, hanging with family & friends, spending time with her nieces & nephews, cooking, & playing recreational sports. Kennedi would love to travel outside of the continental US more. Fall 2010, Kennedi graduated with a Bachelor of Science degree in Biology with a concentration in Biotechnology from Albany State University in Albany, GA. In the Spring of 2015, she graduated Cum laude with a Bachelor of Science degree in Clinical Laboratory Science from Tuskegee University in Tuskegee, AL. She currently works at Northside Medical Center in Columbus, GA as a Medical Technologist. Her ultimate career goal is to become an R&D scientist. Kennedi is undecided about what she would like to further her research in right now, but cancer is at the top of her list, specifically breast cancer. Contact at: klowman87@gmail.com