2008

A Bacterial Cellulose - Nanosilver System

Andrew Norman

A Bacterial Cellulose - Nanosilver System

(Spine title: A Bacterial Cellulose – Nanosilver System

(Thesis format: Monograph)

By

Andrew Norman

Graduate Program in Biomedical Engineering

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Engineering Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

THE UNIVERSITY OF WESTERN ONTARIO
School of Graduate and Postdoctoral Studies

CERTIFICATE OF EXAMINATION

Supervisor

Dr. Wankei Wan

Examiners

Dr. Andy Sun

Dr. Gordon Campbell

Dr. Argyrios Margaritis

Thesis By:

Andrew Norman

Entitled:

A Bacterial Cellulose – Nanosilver System

Is acceptance in partial fulfillment of the
requirements for the degree of
Master of Engineering Science

Date_____

_____
Chair of the Thesis Examination Board

Abstract

Both nanocrystalline silver and bacterial cellulose have been used as biomedical materials. Silver has been used as an antimicrobial agent, and bacterial cellulose as a wound dressing. The combination of both these technologies has the potential to create a synergistic scenario. A novel method for the attachment of nanocrystalline silver to bacterial cellulose has been developed. The cellulose is oxidized with sodium metaperiodate to dialdehyde cellulose and functionalized with silver using thiosemicarbazide, silver protienate and ammoniacal silver. The samples were prepared using both a commercially available bacteria cellulose wound dressing, Biofill, and lab made, wet pellicle, as the substrate. The antimicrobial efficacy against *E. coli* and *S. aureus* has been determined using a modified disk diffusion test procedure, and the release profiles of silver into deionized water were determined. These tests have shown an antimicrobial efficacy ranging between 1 day for the Biofill prepared samples and 5 days for the pellicle based samples.

Key Words

Bacterial cellulose, nanocrystalline silver, nanoparticles, wound dressing, disk diffusion test, silver, cellulose, nanofibers, nanomaterials.

This work is dedicated to my family.

Acknowledgments

I would like to acknowledge and thank my supervisor Dr. Wankei Wan, who has offered invaluable advice and support.

I'd like to acknowledge Mr. Ganesh Guhados who first introduced me to the project.

Dr. Xinsheng Li, who was always to willing to engage in a conversation about even the tinniest of details of my project.

Mr. Kenneth Wong for spending many hours at the scanning electron microscopy taking many of the pretty pictures contained within this thesis.

Ms. Donna Padavan, who lent her experience and intellect to my aid on countless occasions.

Mr. Mina Mekhail, who was always there to act as a sounding board and offer words of dis/encouragement.

And Dr. Chandra Panchel and Axcellon, who kindly lent me use of their facilities and supplied Biofill for my experiments.

Table of Content

List of Tables

List of Figures

List of Appendices

Nomenclature

AAS – Atomic absorption spectroscopy

Ag-NPs – Silver nanoparticles

ATCC - American type culture collection

BC – Bacterial cellulose

DAC – Dialdehyde cellulose

FT-IR – Fourier transform infrared spectroscopy

HIV – Human immunedeficiency virus

ICP-MS – Inductively couple plasma mass spectrometry

LPS membrane – Lipo-polysaccharide membrane

PA-TSC-SP – Periodate – thiosemicarbazide – silver protienate

SEM – Scanning electron microscopy

SP – Silver proteinate

TSC – Thiosemicarbazide

Chapter 1 – Introduction

"Great things are done by a series of small things brought together." -Vincent Van Gogh

There is no doubt that Van Gogh was considering things more abstract than the field of material science when he uttered the above words; but, they could now easily be applied to nanotechnology. Nanotechnology is the science of bringing together the smallest of things in an attempt to achieve the very great. A nano-object is commonly defined as something having dimensions of less than 100 nm, a nanometer being one billionth of a meter. Nanomaterials are small, so small that it becomes possible and even reasonable to quantify the number of atoms that they contain. It is this realm that many researchers are choosing to explore. Nanotechnology has become an expansive field of research in a short time and has had or will have an impact on many facets of everyday life, including in the electronic, chemical and biomedical fields.

In the field of biomedical engineering, new materials are sought to improve medical devices. The wound dressing, possibly one of the first ever biomedical devices, is now starting to incorporate nanotechnology. Plant derived cellulose, in the form of cotton gauze, is the most common material used in wound dressings, but now the more pure bacteria derived cellulose has found a niche in areas where cotton is less than ideal. *Acteobacter xylium*, a bacteria, produces a ultra-pure form of cellulose in a non-woven mesh of nano-fibers. Bacterial cellulose (BC) has demonstrated several noted advantages over plant derived cellulose, including immediate pain relief, a close adhesion to the wound bed, transparency that allows for easy wound inspection, faster

healing, and improved exudates retention. [1]. In addition to an established role in the field of wound dressings, bacterial cellulose is finding a place in other medical devices, Millon et al. explored the use of bacterial cellulose as a constituent in a poly(vinyl alcohol) aorta prosthesis, and others are exploring it as a possible tissue engineering scaffold [2] [3].

To further improve upon the properties of BC, we have devised a method for building in an antimicrobial efficacy using nanocrystalline silver. Nanocrystalline silver as an antimicrobial agent has garnered a lot of attention in recent times with a proven efficacy against a wide spectrum of infectums, including bacterial, viral, and fungal pathogens. It has been described as having the following advantages over other topical antibiotic treatments: it is effective against antibiotic resistant bacteria; it is difficult for bacteria to develop resistance against it; it has no known major side effects; and it potentially has anti-inflammatory properties [4]. Research has been conducted on incorporating it into a wide variety of medical devices, including orthopedic implants, heart valves, surgical masks, and wound dressings, with varying degrees of success.

This report will examine a novel method for chemically binding nanocrystalline silver to a bacterial cellulose substrate. The method is comprised of three steps:

1. Selection of the BC substrate
2. Oxidation of the BC substrate
3. Attachment of Ag-NPs to the BC substrate

These steps will be explored and the resulting material will be objectively examined for possible use as an antimicrobial biomaterial.

Chapter 2 – Review of Literature

In this chapter a short historical perspective is provided for each of the major components of this project followed by an in depth exploration of on-going research in the respective field.

2.1 – Silver nanoparticles

The spread of antibiotic resistant bacteria has become a major concern for today's health care professionals. Nosocomial infections have been estimated to cause over 8000 deaths yearly in Canada [5], and has been described as the fourth most common cause of death in the United States [6]. These incredible statistics have grown with the increase in antibiotic resistant 'super bugs', and have led to widespread use of alternative forms of infection control. One important alternative to antibiotics is silver.

The use of silver for its antimicrobial properties can be traced through history to superstitious and anecdotal practices, such as the ancient Romans storing drinking water in silver containers to prevent the spread of illness [7], or in more recent times to the coating of washing machines or toilet seats with silver nanoparticles (Ag-NPs) [8]. While the true effectiveness of these practices is questionable, as early as the 17th century silver nitrate had found an important and somewhat verifiable role in medicine; it was frequently included in the medical supplies of a ship's surgeon and used to treat sores. [9] In the 18th century, it took on a larger role in treating dermal ulcers, and the next century saw silver nitrate used to treat large burns. Silver nitrate was adopted for these uses not because of its antimicrobial properties, which were incomprehensible with the level of a medical knowledge of the time, but as a cauterizing agent to remove

granulized tissue and promote scab formation. It seems obvious with today's knowledge that silver nitrate's unknown antimicrobial role aided many unwitting physicians and patients in the treatment of wounds [9]. It was not until 1874 that the antimicrobial properties of silver salts were first investigated, and at this time silver nitrate was used in the treatment ophthalmianeonatorum, the infection of a newborn's eyes with gonorrhea. This treatment is still used today. The use of silver nitrate for the treatment of burns increased until World War II when instances of its use quickly declined with the advent of penicillin and other antibiotics. Silver nitrate returned to the medical fray with a paper published in 1965 by Moyer. [10] At this time, silver sulphadiazine was developed "...to combine the *inhibitory* action of silver with the *antibacterial* effect of sulphadiazine." [11] Whether the effects of silver can be described as inhibitory or antimicrobial will be discussed in the next section.

Accompanying the success of silver salts in fighting infection, there was an increased interest in using metallic silver in a similar fashion. Silver has been incorporated in both urinary and venous catheters, with moderate success [12]. There have also been attempts to liberate silver ions from metallic silver with novel methods such as using an electric current. One such study, conducted by Falcoe and Spadaro show a greater inhibition of bacteria at the anode of a silver coated piece of nylon, compared to the control [13]. Yet, they fail to discuss the independent effects of an electrical current on the growth of the bacteria.

The use of metallic silver has been over shadowed by the success of silver nanoparticles (Ag-NPs). Ag-NPs are generally held to be particles, sometimes crystalline in nature, of less than 100nm in size. The antimicrobial efficacy is inversely

proportional to size, with smaller particles being more effective [14]. One important thing to note is while frequently described as 'nanosilver,' some are composed of a large percentage of silver oxide, this is due to the large ratio of surface to bulk silver molecules [15]. Silver oxide has been used as an antimicrobial coating in the past [16], and the silver oxide component of the nanoparticles could play a significant role in their antimicrobial efficacy. Djokic and Burrell investigated silver oxide's role, concluding, "An essential factor leading to an antimicrobial activity of metallic silver is a presence of Ag oxide(s) at the surface of this material." [17]

There are now efforts to incorporate Ag-NPs into a wide range of medical devices, including but not limited to bone cement [18], surgical instruments [19], surgical masks [20], and wound dressings; the latter is the primary focus of the research presented in this thesis. Ag-NPs are considered a significant advancement in wound care technology, and several large biomedical companies, including Smith and Nephew, Inc.; Johnson & Johnson Medical Ltd.; ConvaTec ER; Squibb and Sons LLC; Argentum Medical LLC; Coloplast Ltd.; Medline Industries Inc.; and others have available products. In a sort of feedback loop, these biomedical companies have sponsored additional research into their products, increasing the available literature several fold [8] [4]. One review article author, after wading through the plethora of research, found it necessary to remind the reader to remain objective in the face of such a "marketing blitz." [21]

2.1.1 – How does silver work?

One important characteristic of the antimicrobial nature of silver is that bacteria have difficulty developing resistance to it. It has been hypothesized that this is the result

of ionic silver having multiple modes of lethality, requiring a high level of adaptation in any given bacteria species [22]. These modes of lethality are still being debated in the literature and are summarized by the following: an uncoupling of the respiratory chain from oxidative phosphorylation [23]; the collapse of the proton-motive forces across mitochondrial membranes [24]; a destructive interaction with thiol- groups of membrane bound enzymes and proteins [25]; and the binding to and seizing-up of DNA replication machinery. Ag-NPs act as reservoirs for ionic silver encompassing its modes of lethality, but additionally it punches holes in the cellular membrane of a bacterium, essentially causing the cell to bleed to death [26].

In addition to its antimicrobial role, there has been some research conducted on a potential role for silver compounds as antiviral agents. Silver sulfadiazine has been investigated as a tool to prevent ocular infection [27] and another silver-sulfur complex has been investigated as an antiviral coating for consumer products [28]. Ionic silver has also been investigated as a method to inactivate viruses in drinking water when combined with UV radiation [29]. More recently, Ag-NPs have been shown to have antiviral properties too. In 2005, two studies were published that described the interaction between Ag-NPs and HIV-1. One study showed that Ag-NPs bind to the surface of the virus where the glycoproteins essential to the infectious properties of the virus are assumed to be; this study went on to show an *in vitro* reduction of infection with silver treated HIV [30]. Another study showed that apoptosis can be delayed in human T-cells infected with HIV, by using Ag-NPs to reduce the viral load. [31] The same study showed Ag-NPs reduced HIV replication.

Ag-NPs have been portrayed as a wonder drug when it comes to the wound healing industry, and in addition to the widely published and accepted antimicrobial ability of Ag-NPs, there has been some published work on other wound healing benefits. One study shows that Ag-NPs decrease inflammation, decrease cell death and reduce the concentration of harmful enzymes in the wound [32]. While inflammation is the body's way of clearing tissue debris, fighting infection and triggering the growth of new tissue; in a properly treated wound, a prolonged inflammation response can be counterproductive. Several papers report that Ag-NPs can either diminish inflammation or the results of it. Wright et al. states that Ag-NPs can "...play a role in altering or compressing the inflammatory events in wounds and facilitating early phases of wound healing." [32] Shin et al. shows that Ag-NPs can alter cytokine production in human cells *in vitro*, and suggests it could be used as a treatment for inflammatory diseases [33]. This is further confirmed in a porcine model by Nadworny et al [34]. It should be noted that most of this research has been conducted by groups with a vested interest in their silver systems.

There are aspects of Ag-NPs that are debated in the literature, such as the effect on the rate of reepithelialisation. One study suggests that Ag-NPs increase the rate of reepithelialisation by up to 70% [35], while another suggests it delays reepithelialisation by over 50% [36]. These conflicting results are not limited to those two studies, Honari et al. also shows improved healing at skin graft donor sites [37], where as Vlachou et al. says delayed healing is experienced [38] when a Ag-NP dressing is used. These counter claims emphasize the on going debate in the literature about the effects of Ag-NPs dressings. One point that is not contended is that Ag-NP dressings can be left on the

wound for a period of up to seven days, whereas silver nitrate and silver sulphadiazine need to be reapplied every 2 and 12 hours respectively. Conventional wisdom is that the less disturbed a wound is during the healing process the better.

2.1.2 – Methods use for creating Ag-NPs

There are many different procedural pathways that can produce Ag-NPs. They can be divided into three broad categories; physical vapor deposition, ion implantation, or wet chemistry.

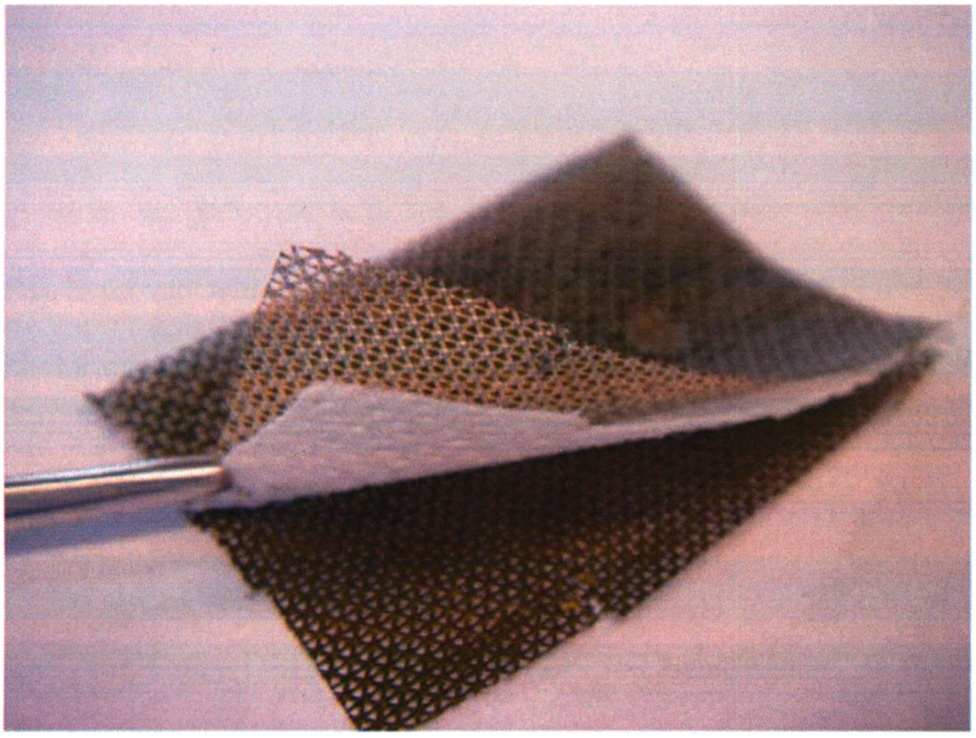

Figure 2.1: Acticoat - three layers exposed.

Acticoat is widely accepted as the gold standard of Ag-NP wound dressings; and is a good example a silver system prepared by a physical vapor deposition process. In the case of Acticoat, the physical vapor deposition process involves placing the substrate (in this case HDPE) in a chamber opposite a silver cathode. The chamber is filled with argon gas; a current is passed through the gas, driving argon atoms into the silver

cathode knocking free some silver atoms. The silver atoms are deposited in nanoparticle form on the substrate [39]. Several other researchers have also used physical vapor deposition to create silver particles [40] [41] [42]. The particles created in this manner generally have been shown to be crystalline with crystallite grain diameters of approximately 15 nm [15]. A photo of a sample of Acticoat can be seen in Figure 2.1, Scanning Electon Micrographs are presented and discussed in the Results and Discussion chapter.

Although it may seem counter-intuitive, ion-implantation has been used to create Ag-NPs [43] [44]. This process has been shown to produce silver particles embedded in polyurethane, silicone, polyethylene, and polymethylmethacrylate [45] [46]. The particles grow in the substrate with the bombardment of ions. The existence of nanoparticles is proven with optical absorbance, and the exact nature of the particles created with this method is not known.

There are several wet chemistry methods for creating silver nanoparticles, including the use of a reducing agent like sodium borohydride ($NaBH_4$). Sodium borohydride has been used with polyvinyl alcohol [47], poly(vinylpyrrolidone), bovine serum albium [30], citrate [48] [49] and cellulose as stabilizing agents. In the case of BSA, the sulfur-, oxygen- and nitrogen-bearing groups mitigate the high surface energy of the nanoparticles during the reduction [30]. Whereas the hydroxyl groups on the cellulose are reported to help stabilize the particles. Citrate and cellulose have been used to create Ag-NPs independent of a reducing agent as well. An additional novel wet chemistry method used to create Ag-NPs took advantage of ß-D-glucose as a reducing sugar and a starch as the stabilizer. [50]

In addition to the methods of nanoparticle formation discussed in this section, it should not be interpreted as an exhaustive list; there are alternative pathways for creating Ag-NPs, but they are relatively uninvestigated [51] [52] [53]. Also, it is important to note, not all nanoparticles are created equal. The size and shape have been shown to have an impact on its efficacy [22]. Additionally, crystal facet size, oxide content and several other factors could also affect the antimicrobial properties [4].

2.1.3 – Potential health concerns with nanosilver

Ionic silver has had a long history of use in medical applications, and it has been shown irrefutably that ionic silver, in the right quantities, has a net positive benefit in treating wounds. Ag-NPs do not have such a long pedigree; and although over the last ten years it seems to evidently be an effective agent, it would be irresponsible to look at any relatively new technology without considering the potential problems.

First, it is best to dispel two common misconceptions about the safety of a topical Ag-NP dressing.

Allergic Reaction: While there is anecdotal evidence suggesting the possibility of a silver allergy, an extensive review of the medical literature does not lend any credence to this possibility [54]. Some silver alloys that include nickel do elicit an allergic reaction, but those alloys are outside the scope of this thesis.

Argyria and Staining: Ingested silver compounds, like colloidal silver, can cause a condition called argyria, which is manifested as a *permanent* bluish-grey

hue. A systemic change in colour is not a possible contradiction with the topical application of silver. The only reference to such an outcome in the literature is a case study of a 17-year-old man, who sustained burns to 30% of his body, and experienced a *temporary* bluish-grey hue after several days of treatment with Acticoat [55]. Argyria is the deposition of silver in deep tissues, a condition that cannot happen on a temporary basis, raising the question of whether the cause of the man's discoloration was argyria or even a result of the silver treatment [56]. Silver dressings are known to cause a "transient discoloration," that dissipates in 2-14 days, but not a permanent discoloration [57].

To properly evaluate the safety of the topical application of silver, three criteria must be examined; how much of the silver is absorbed; what is its effect; and what is its longevity in the body [58]. Normal levels of silver in blood plasma are below .0005 ug/mL, and levels of up to .440 ug/mL have been observed as the result of the application of silver sulfadiazine cream, without any adverse side effects [59]. Although silver levels of .193 ug/ml in blood plasma for a sustained period (three weeks) has been suggested as the cause of renal failure and death in one case study [60]. After discontinuing a silver treatment, silver blood plasma levels remain elevated for approximately three weeks before returning to normal. Silver is cleared through the urine, and some caution is required when treating a patient with poor kidney function, as they are likely to maintain a high blood silver concentration. [61]. A study conducted *in vitro* showed ionic silver levels of 13 to 56 ug/mL were toxic to human fibroblasts, the authors suggests the mechanism for toxicity was the same as heavy metal toxicity [62].

Most papers that discuss the health implications of topical silver application conclude it is a safe and effective treatment [62].

Ag-NPs have different characteristics than ionic silver and are potentially tolerated differently. One point of contention in the literature is how readily Ag-NPs from a wound dressing can penetrate the skin. It has been observed that TiO_2 *micro*particles found in sun-block creams can penetrate to the dermis [63], but there is no direct evidence suggesting that silver levels increase in the blood when Ag-NPs are applied to intact skin []. Rarely however, are these dressings used on intact skin, and it is important to evaluate the dressings in real world scenarios: on compromised skin. An increase in blood silver levels has been observed after the application of a Ag-NPs dressing; in a study with 30 burn patients, a maximum silver blood plasma level of .230 ug/ml was observed, with a mean of .074 ug/ml at the end of treatment [38]. The authors suggest that the higher blood silver levels were related to kidney function - affecting silver clearance. No statistically relevant liver toxicity was observed in this study.

Paknikar et al. have investigated the effect of 7-20 nm Ag-NPs on human fibrosarcoma (HT-1080) and human skin/carcinoma cells (A431) [65]. They found levels of .78 to 6.25 ug/ml for the human fibrosarcoma cells and 1.56 to 6.25 ug/ml in the human skin/carcinoma cells initiated apoptosis; whereas levels of 12.5 ug/ml caused necrosis in both. In an *in vivo* (rats) study conducted to determine the systematic toxicity of Ag-NPs 60nm in size, found induced liver toxicity and coagulation in peripheral blood, at the end a 28 days of oral administration [66]. To summarize, the potential health implications, of using Ag-NPs: blood plasma levels do not reach a level

of anywhere near concern with standard usage of Ag-NP dressings. If they do reach dangerous levels, one would look at the liver and the kidneys to observe the effects. Additionally, with silver being cleared through the kidneys, caution should be taken when considering the use on a patient with impaired kidney function.

No discussion of the health impact of silver would be complete without a review of the St. Jude's Silzone heart valve – a significantly notable event in the medical device industry. The Silzone valve was a standard bileaflet mechanical heart valve that was complemented with a silver coated Dacron sewing cuff [67]. Figure 2.2 shows an example of such a heart valve. While the nature of the silver coating on the cuff was not discussed in the literature, it was applied using a technique of ion beam-assisted deposition [68] suggesting it could be in the form of Ag-NPs. The silver was included in an attempt to decrease instances of endocarditis, an infection of the valve annulus. The valve was approved for sale in Canada, Europe, the United States, and most other markets around the world. In a post-commercialization study, researches showed that the valve prevented tissue ingrowth, created paravalvular leakage, valve loosening, and in the worst cases the valves have to explanted. After 3 years on the market and 36 000 implants, St. Jude discontinued and voluntarily recalled the valve [69]. This detrimental effect of silver has not been observed in the case of wound dressings – with the exceptions of the previously discussed conflicting reports on the rate of reepithelialisation.

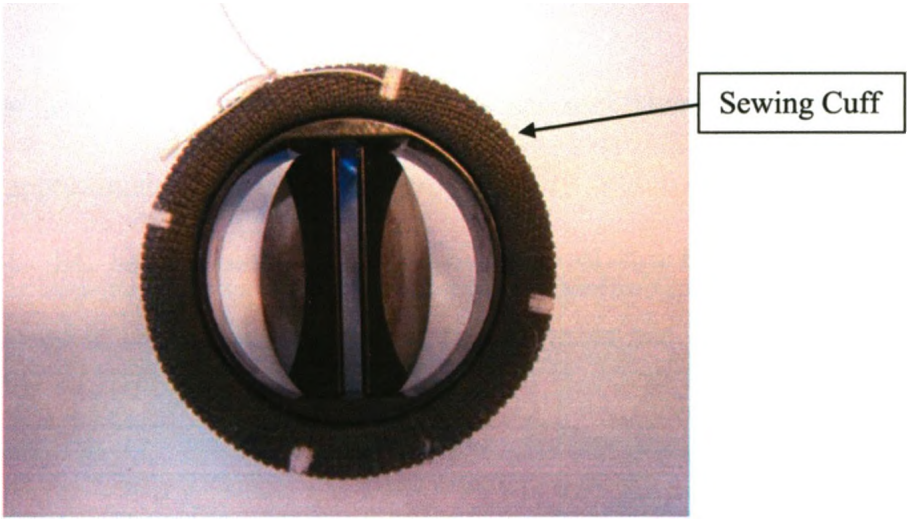

Figure 2.2: St. Jude Heart Valve

2.1.4 – Summary

As discussed in this section, silver is an exceptionally useful antimicrobial agent, with a long history of use in medical applications. Ag-NPs are a more recent development, but share the positive attributes of silver salts, and also have limited drawbacks. The implantation of Ag-NP materials would raise concern in the medical community, given the failure of the Silzone heart valve, but the topical application of Ag-NPs in the form of a wound dressing has been proven safe and effective. The only thing lacking is a controlled method for delivering silver to the wound environment to both create a sustained antimicrobial effect and decrease the potential for discoloration.

2.2 – Bacterial Cellulose

Cellulose is the most prevalent polysaccharide in nature. There is a global annual production of approximately 1.5 trillion tons, primarily found in the woody structure of plants [70]. BC has the same ß-1,4-glucan chemical structure shown in Figure 2.3, but is produced in an ultrapure form without the lignin and hemicellulose found mixed with plant sources of cellulose. It also has a different morphology and different physical properties. Plant cellulose has a degree of polymerization of 13000 – 14000 where as BC has a degree of polymerization of 2000 – 6000. Depending on culture conditions BC can be produced in a less structured non-woven mesh-like form or in a freely suspended fiber form, with fiber bundles having a diameter of approximately 50 nm (although this too can be a function of culture conditions). BC has a great affinity for water, with water content of up to 99% by mass. The loss of this water during drying results in an irreversible structural change due to loss of the hydrogen bonds; this will be discussed further in Chapter 4 [70].

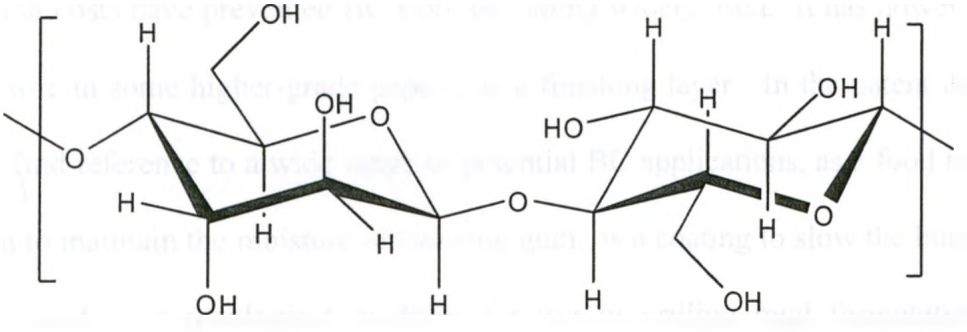

Figure 2.3: Chemical structure of cellulose

Although, there are a few genera of bacteria that produce cellulose including *Acetobacter*, *Rhizobium*, *Agrobacterium* and *Sarcina*, the species that has garnered the most attention is *Acetobacter xylium* (also known as *Gluconacetobacter xyliuns*) [71].

A. xylium is a gram-negative, rod shaped aerobic bacterium with a long history of cultivation. Originally used for vinegar production, it is relatively easy to culture and has a high yield of cellulose; both factors that make it a prime research and commercialization target.

Plant cellulose was first described by Anselm Payen in 1838, who determined its chemical composition by way of elemental analysis. While BC has been used for hundreds of years in the traditional Philippine dessert *nata-de-coco* – a BC pellicle soaked in syrup - it was first confirmed as cellulose in 1886 by AJ Brown [72].

The focus of the original research into *A. xylinum* and BC followed two avenues. First, it was used as a vehicle to examine the biochemical pathways of cellulose production, research that could be used to model cellulose metabolism in higher organisms [72]; and second, for the commercial application of paper production - a primary use of plant cellulose [73]. The original hope was that BC could become a substitute for plant-derived cellulose in the paper making process, but the relatively high production costs have prevented BC from becoming widely used. It has however found a niche role in some higher-grade papers, as a finishing layer. In the patent databases, one can find reference to a wide range of potential BC applications, as a food thickener; as a film to maintain the moisture of chewing gum; as a coating to slow the burning of a cigarette; and as a rheological modifier for use in drilling mud formulations [74]. Despite the ingenuity for finding potential applications, only three have met with success: *nata-de-coco* (the previously mentioned Philippine dessert); as an acoustic membrane in high-end Sony earphones [75], and as a wound dressing [1].

One continuing mystery surrounding BC is its exact biological function. *A. xylium* is a successful and prevalent bacterium in nature, frequently finding a home in rotting fruits and sweetened liquids. The most familiar form of BC is that of a pellicle on the top of a static cultured growth media. It has thus been hypothesized that cellulose acts as a floatation device bringing the bacteria to the oxygen rich air-media interface. This hypothesis has largely been discredited by experiments conducted on submerged oxygen-permeable silicone tubes that show cellulose grows well submerged if enough oxygen is present [76]. Others suspect that cellulose is used to immobilize the bacteria in an attempt to keep it near the food source; or as a form of protection against ultraviolet light [77].

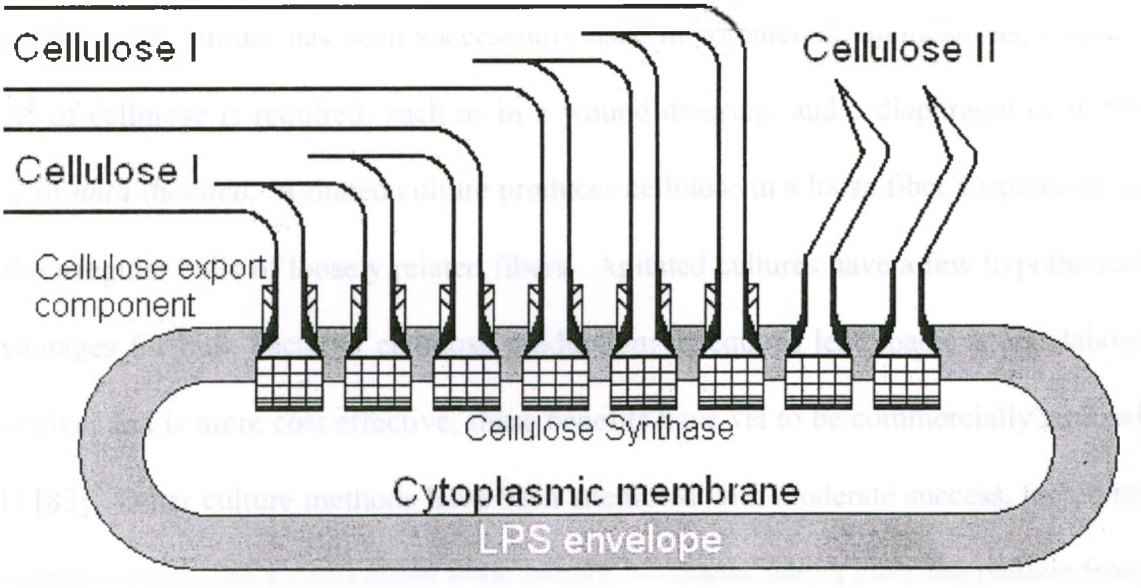

Figure 2.4: Cellulose synthesis redrawn from [78].

BC is assembled extra-cellularly. Cellulose monomers are synthesized by the enzyme, *cellulose synthase,* within the cell and are excreted from two rows of 50 to 80

pores in the cell membrane, where they form protofibrils of between 2 – 4 nm. These in turn are bundled into fibrils of 80 nm by 4 nm, which are assembled in fiber form. Figure 2.4 shows this in detail, with the protofibrils being secreted from the pores in the membrane and being assembled into larger fibers. These fibers are considered cellulose type I, whereas cellulose type II fibers are ß-1,4 glucan chains arranged in disorganized and random manner. The fibers have been described as morphologically falling between algal and plant cellulose [79]. An interesting occurrence with BC is the division of the fiber with cell division - the fiber branches into two as the cell divides and the fiber forming apparatus is divided as well [80].

2.2.1 – Production of cellulose

BC can be produced in either static or agitated cultures. In static culture, the cellulose is produced in a nonwoven nano-mesh that grows on top of the media - a pellicle. Static culture has been successfully used in commercial applications, where a sheet of cellulose is required, such as in a wound dressing, audio diaphragm or in the case of *nata-du-coco*. Agitated culture produces cellulose in a loose fiber suspension, or in the irregular balls of loosely related fibers. Agitated cultures have a few hypothetical advantages for bulk bacterial cellulose production: it requires less space, is less labour intensive, and is more cost effective; these benefits have yet to be commercially realized [81] [82]. Other culture methods have been attempted with moderate success, including an air-lift reactor and a continuous static culture bioreactor which pulls the pellicle from the surface of the media at a rate equal to its growth [83]. Additionally, shake flask cultures are frequently used to produce BC in smaller quantities for research purposes.

There is a wide range of medias used for the production of BC. The traditional *nata-de-coco* cottage industry uses coconut milk mixed with sucrose, with approximately a fifth of the media coming from the left-overs of the last batch. The coconut milk can be substituted for other fruit juices like pineapple juice, to create desserts like *nata-de-pina* [84]. One frequently used research media is Schramm and Hestrin Medium [85]. Developed by Schramm and Hestrin and described in a 1954 paper, it includes (% w/v): glucose, 2.0; peptone, .5; yeast extract, .5; disodium phosphate, .27; citric acid, .115. The pH of the media is adjusted to a pH of 6.0 with diluted HCl and NaOH [86]. There are many variations on this basic media description, but all iterations contain a carbon source whether it is in the form of glucose, fructose, sucrose, monosaccharides, or unrefined sugar sources like fruit juice, or beet molasses [87]. They also must have a nitrogen source, this is the yeast extract in the case of the Schramm and Hestrin media, but could be corn steep liquor, or an synthetic form a nitrogen.

Researchers have used a wide variety of media additives, from antifoam in shaken and agitated cultures, to a wide variety of vitamins including: inositol, nicotinic acid, pyridoxine hydrochloride, thiamnine hydrochloride, D-pantothenic acid calcium, riboflavin, p-aminobenzoic acid, folic acid and d-biotin [88]. Thickeners have been used to allow for higher agitation speed, while lessening the effects of shear. These include xanthan, agar, acetan, polyacrylamide-co-acrylic acid and alginate. In one such case, the addition of 0.4% (w/v) agar to the media was shown to enhanced the production rate of cellulose by over 50% [88]. Polyacrylamide-co-acrylic acid was shown by Joseph et al. to have a similar effect by more than doubling BC production from 2.7±.8 to 6.5±.5 g

dm^{-3} [89] Others have learned that the addition of alginate prevents cellulose clump formation increasing cellulose yield [90]. Ethanol has also been added to culture medium, with mixed results. One group showed that ethanol reduced the production of gluconicacis, lowering pH levels to sub-optimal levels; [91] while others have reported an increased yield in instances where ethanol is added. [92]

In relation to additives there are several other culture conditions that affect BC production and yields. These include: pH, temperature, dissolved oxygen, and shear rate. The optimal pH for BC growth has been described as falling within 5-7 or 4-6, with most research papers reporting using 5. *A. xylium* produced gluconic acid, which lowers the pH requiring a pH control system to maintain the ideal level [93]. Frequently 4N NaOH and 4 N H2SO4 are used to control pH [88]. Dissolved oxygen is usually maintained at 30%. [88]. A temperature range of 25-30 degrees Celsius is described as the optimal temperature range, but the narrower range of 28-30 degrees Celsius is usually used.

One potential method of increasing the cellulose yield is through co-culture. Seto et al. in attempting to isolate a culture of *A. xylium* discovered a colony of *A. xylium* growing in close association with *Lactobacillus mali* [94]. It was found that when cultured together the cellulose yield was tripled. The researchers could not sufficiently explain the increased yield; they eliminated the possibility of a direct role for extracellular polysaccharide produced by *L. mali*, and did not observe any active extracellular enzymatic activity. Regardless, the increased cellulose production is an interesting observation.

2.2.2 – Bacterial cellulose as a wound dressing

It is difficult to know for certain what the first medical interventions were in human history, but to hypothesize that it was the covering of a wound - would be a good guess. The dressing of a wound is essential for the health of a patient; infection can threaten anyone regardless of wound size if left unsterilized and exposed; fluid loss can kill those with extensive burns; and, dermal ulcers can threaten both life and limb. Cosmetically, the covering of a wound can reduce scarring. The characteristics that make an ideal dressing include biocompatibility, sterility, semiocclusiveness, strength, conformability, and moisture retentive properties [95]. While wound dressing technologies based on materials such as alginate or synthetic polymers go part way towards addressing the required characteristics, BC does a better job.

BC has a wound care pedigree, being chemically identical to cotton – which in the form of cotton gauze has been an essential dressing material. BC, like cotton gauze, is highly biocompatible. Research conducted at Chalmers University of Technology in Goteborg Sweden has confirmed this [96]. With the intent of developing a tissue engineering scaffold, the group conducted systematic biocompatibility studies by placing pieces of BC subcutaneously in a rat model. The group observed no signs of inflammation or other symptoms of a negative host response. The group has continued to pursue their tissue engineering scaffold, and have found BC performs favorably with both smooth muscle and endothelia cells [3]. As of late 2006, this group is testing their tissue engineered BC small diameter vessels in a large animal model.

Bacterial cellulose, like Ag-NPs, can be used to treat dermal ulcers and burns. Dermal ulcers are notoriously difficult to cure even after the underlining pathology is

treated. A dermal ulcer is usually defined as a wound which has a loss of integrity, is infected, and has difficulty healing; with treatment they can take weeks or months to heal and in severe cases require radical intervention like amputation. Alvarez et al. tested Xcell, a dressing based on wet BC pellicle, in the treatment of venous leg ulcers, and found considerable improvement over the standard treatment [97]. The two areas of greatest improvement were in: autolyic debridement, with a 40% increase in the removal of nonviable tissue; and pain, with a statistical significant reduction in reported pain. Alvarez et al. stated that the BC dressing "...creates a protective, hypoxic, moist environment similar to an undisturbed wound protected by its own blister roof."

In addition to the treatment of ulcers, studies conducted on using BC in the treatment of burns have been promising. Legeza et al. studied the impact of bacterial cellulose on radiation and thermal burns and showed that a BC dressing accelerated wound healing by between 6 and 17 %, with scab detachment occurring 14-15% sooner [98]. Apart from the commercially available products, Czaja et al. have investigated using a never-dried bacterial cellulose membrane for the treatment of 2^{nd} and 3^{rd} degree burns in animal models [1]. They were found to accelerate the healing process. He also listed the additional benefit of being able to produce sheets of a variety of sizes, therefore eliminating the need to staple together several smaller sheets, as is standard procedure with other wound dressings.

The commercialization of BC wound care products has proceed in an anfractuous manner, with Johnson and Johnson conducting the original research into a bacterial cellulose wound care system in the1980s, but not actually producing a marketable product [99] [100]. Johnson and Johnson later sold their bacterial cellulose technology

to Xylos Corp. Xylos produced a BC dressing under the trade name XCell. The company continued research into the biomedical applications of BC filing patents on a bacterial cellulose wound dressing customized for chronic wounds and for an implantable soft tissue repair graft [101]. But, Xylos Corp sold their cellulose wound care line to Lohmann and Rauscher GmbH, a company much better suited to the marketing and delivery of the product. Bionext Produtos Biotechnologicos Ltda, a Brazilian company, developed their own line of wound care products independent of Johnson and Johnson's original research. Bionext's first generation product was called Biofill, but has since been replaced by a next generational product called Bionext [102]. While the only commercialized medical applications of BC are in the field of wound care there is continued research into other uses. These include skin tissue engineering [103], cartilage tissue engineering [104], small diameter blood vessel replacements [105], and as a brain dura substitute [106].

Chapter 3 – Experimental Methods

3.1 – Bacterial cellulose production

Two types of bacterial cellulose were used to conduct experiments: dried pellicle in the form of the commercial product Biofill and lab-made, wet pellicle. Biofill, obtained via Axcellon Inc. from Bionext Produtos Biotechnologicos Ltda., is a dried and stretched BC pellicle. The lab-made cellulose pellicle was produced using the following procedure and was preserved in its wet form through all chemical reactions, and dried prior to characterization and testing.

The BC prepared in the lab was grown from *Acetobacter xylinum* BPR2001 subsp. *Sucrofermentans*: American Type Culture Collection (ATCC) number 700178. A 500mL shake flask filled with 250mL of the media with composition listed in Table 3.1, was inoculated with three loops of *A. xylinum*. The shake flask was placed in a shaker incubator at 28 degree Celsius and 175 RPM for three days. A 10 mL aliquot of this culture was added to a Pyrex dish measuring 26 by 36 cm and filled to a depth of 8 millimeters, with 750 mL the media listed in Table 3.1. The dish was covered with tinfoil and placed in a static incubator for 7 days.

Table 3.1: Media Composition

	(%w/v)
Yeast Extract	5.3
Fructose	4
Potassium Dihydrogenphosphate	.33
Ammonium Sulphate	.1
Magnesium Sulphate	.025
Trisodium Citrate	.042
Citric Acid	.88

3.1.1 – Cleaning of bacterial cellulose pellicle

The pellicle was removed from the surface of the media as seen in Figure 3.1. It was rinsed thoroughly in deionised water for 5 minutes. It was then placed into a clean Pyrex pan, and autoclaved for 15 minutes. It was rinsed again, and placed in 500mL of 1 N sodium hydroxide (NaOH). The BC and NaOH were maintained at 80 °C for 90 minutes, removed and rinsed thoroughly. It was soaked over night and rinsed repeatedly until all colour was removed from it and its pH was neutral.

Figure 3.1: Pellicle from Static Culture

3.2 – Silver Loading

3.2.1 – Dry bacterial cellulose pellicle

The procedure was adapted from Guhados [107] . A sheet of Biofill with a mass 50 mg was placed in 50 mL of .16 M sodium metaperiodate ($NaIO_4$) solution, to achieve a molar ratio of 1:13, cellulose to $NaIO_4$. The cellulose and solution were left at room temperature for 15, 30 or 60 minutes. The cellulose was then rinsed once in ethylene glycol, to arrest the oxidation, and then rinsed 10 times in deionised water. The cellulose was then placed in a solution of 1 % thiosemicarbazide and 5 % acetic acid, and reacted for 90 minutes at 60 °C. It was then thoroughly rinsed in deionized water was placed in a solution of 1% silver proteinate and 2% sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) for 1 hour in the dark. The silver proteinate solution was prepared by mixing the two constituents and filtering the resulting mixture through a 45 um cellulose-nitrate filter. An ammoniacal silver solution was prepared by adding 1 gram of silver nitrate ($AgNO_3$) to 8 mL of deionised water and then adding ammonia hydroxide drop wise until the brown precipitate dissolved. This solution was diluted to a volume of 75 mLs. The cellulose was placed in the ammoniacal silver solution and was maintained at 60 °C for 15 minutes. The cellulose was then rinsed and dried in a convection oven at 23 °C.

3.2.2 – Lab made, wet pellicle

The dry mass of a lab-made pellicle was determined by cutting a section of approximately 2 cm^2 of wet pellicle, weighing it and drying it at 60° C in a convection oven. This was done in triplicate.

A piece of wet pellicle was cut to a size that corresponded to a dry mass of 50 mg and was placed in 100 mL of sodium metaperiodate ($NaIO_4$) solution, so a molar ratio of .5:1, 1:1, 1.5:1, or 2:1 - $NaIO_4$:cellulose was achieved . The cellulose and solution were left at room temperature for 24 hours. The cellulose was then rinsed once in ethylene glycol, to arrest the oxidation, and then rinsed in deionised water and soaked for 72 hours. The cellulose was then placed in a solution of 1 % thiosemicarbazide and 5 % acetic acid, and reacted for 90 minutes at 60 °C. The cellulose was thoroughly rinsed and soaked for 72 hours. The samples was placed in a solution of 1% silver proteinate and 2% $Na_2B_4O_7 \cdot 10H_2O$. The solution was prepared by mixing the two constituents and filtering the resulting solution through a 45 μm cellulose nitrate filter. An ammoniacal silver solution was prepared by adding 1 gram of $AgNO_3$ to 8 mL of deionised water and then adding ammonia hydroxide drop wise until the brown precipitate dissolved. This solution was diluted to a volume of 75 mLs. The cellulose was placed in the ammoniacal silver solution and was maintained at 60 °C for 15 minutes. The cellulose was then rinsed and dried in a convection oven at 23 °C.

3.2.3 – Determination of aldehyde groups

The aldehyde group analysis was conducted on pieces of BC taken through the oxidation step, but prior to the addition of thiosemicarbazide. The cellulose was dried and weighed. The BC was cut into thin strips, and placed in 10 mL of .05 M sodium hydroxide (NaOH). It was kept at 60°C for 30 minutes, at which point 5 mL of .1 M hydrochloric acid (HCl) was added to neutralize the NaOH. The solution was then

titrated with .01 M NaOH, using phenolphthalein as an indicator [108]. Tests were repeated in triplicate.

3.2.4 – Determination of silver loading

A piece of silver loaded BC with a mass of 20 mg was placed in vials filled 5 mL solution of 50% nitric acid. The solution was left for 24 hours. The silver concentration in the samples was measured by a Varian Spectr AA 55, Atomic Absorption Spectrometer (AAS). Some vials were diluted prior to measuring the concentration, to ensure the concentration of silver fell within the range detectable by the AAS. The AAS was preformed at a wavelength of 338.3 nm, using air and acetylene gas for the flame. The spectrometer was calibrated with .2, .5, 1, 5, 10, 15 ppm of silver standards; the standards were measured before after sampling to ensure measurement stability. Tests were repeated in triplicate.

Silver levels were computed using the following equation (1):

$$Silver\ Fraction = \frac{(5\ ml + dilution\ volume) * mass\ factor * absorbance * calibration\ factor}{mass\ of\ sample} \quad (1)$$

3.3 – Characterization

3.3.1 – X-ray diffraction

AgNP-BC samples were characterized using a Rigaku MiniFlex X-ray diffractometer. Samples were scanned at a 2θ range of 10° to 90° at 0.1° increments. The samples were stored at room temperature in the dark prior to scanning.

3.3.2 – Scanning electron microscopy

The scanning electron microscopy (SEM) was conducted on a Leo-1530 Scanning Electron Microscope with an accelerated voltage of 1 kV. No coatings were used. Particle size was determined using ImageJ software. A sample for 500 particles were measured, 50 per SEM image over 10 SEM images.

3.3.4 – Fourier-transform infrared spectroscopy

The FT-IR spectra of BC and dialdehyde-BC were determined using PerkinElmer FTIR Spectrum BX. A resolution of 2.0 cm^{-1} was used with an interval of 0.5 cm^{-1}, over an interval of 64 scans. The BC sample tested was Biofill, using the preparation conditions as stated early in the chapter for Biofill, with the exception that the Biofill was oxidized for 24 hours, as opposed to 60 minutes.

3.4 – Determination of silver release kinetics

Amber glass vials were filled with 5 mL of deionized water, and BC-silver samples of sizes shown in Table 3.2 were placed in the water and maintained at 37 ° C. The samples were transferred to a new vial at 5 minutes, 20 minutes, 40 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours, 48 hours, and 96 hours. Once the sample had been transferred to a new vial, the old vial containing the water and released silver was stored at 4°C, until the silver concentration was measured using AAS. The fractional release of silver was also determined using equation (1) seen on page 28. Tests were repeated in triplicate.

Table 3.2: Release kinetic sample sizes

Sample Type	Sample mass in mg
Acticoat	80
Silver enhanced Biofill	30
Silver enhanced pellicle	40

3.5 – Antimicrobial tests

The antimicrobial protocol used was a modified Kirby Bauer Method; adapted from Taylor et al. and Clinical and Laboratory Standard Institute's procedures [4] [109]. *Escherichia coli* and *Staphylococcus aureus* Newman were cultured in Trypic Soy Broth for 24 hours. The culture was diluted to an approximate cell density of 1.5 $*10^8$, determined by measuring an optical absorbance of 0.132 at 600 nm [109]. In 100 µL aliquots, the bacteria was then placed on Mueller Hinton agar plates and spread using a cell spreader. Three disks, 1 cm in diameter, of the prepared cellulose film were placed on the agar in an arrangement that would prevent any overlap of potential clear zones. The plates were incubated for 24 hours at 35 °C. The clear zones were measured by

scanning the plates and using the image processing software "Image J." New agar plates were then inoculated using the same procedure and the samples were transferred to the new plates. This process was repeated every 24 hours, until no clear zone remained [4]. The clear zone diameter was measured as seen in Figure 3.2 and the zone of inhibition was determined using equation 2:

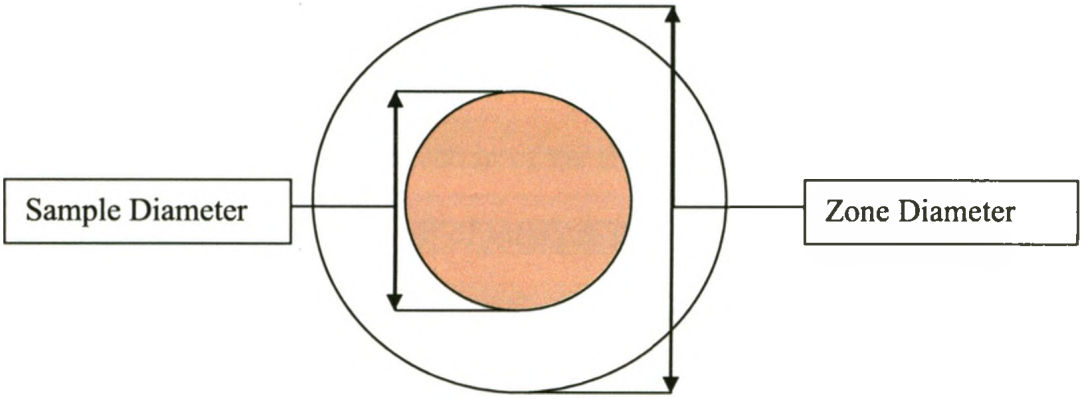

Figure 3.2: How the clear zone size is determined

$$Zone\ of\ Inhibition = \frac{Zone\ Diameter - Sample\ Diameter}{2} \quad (2)$$

$$Sample\ Diameter = 1\ cm$$

An example of a plate is seen in Figure 3.3.

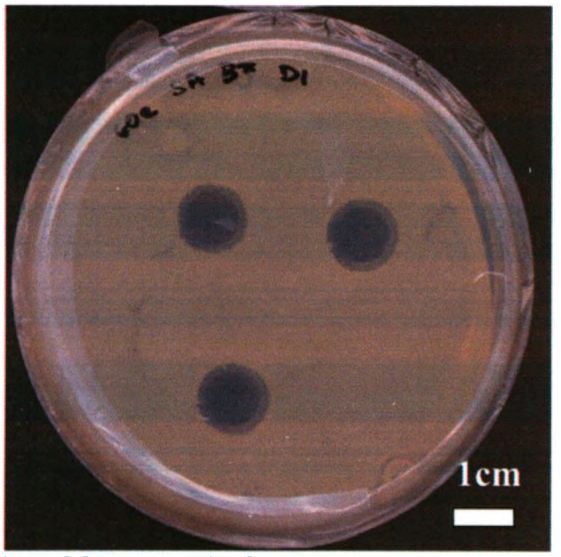

Figure 3.3: An example of a clearzone test on a sample of silver loaded BC. Tested against *S. Aureus*

Chapter 4 – Results and Discussion

The primary purpose of this research project is to explore a novel antimicrobial material for possible use in biomedical applications – with wound dressing being the area of chief consideration. The design requirements considered while evaluating the material are the ability to tune the silver loading, maintain the release of silver overtime, and exhibit antimicrobial properties. The material is a BC substrate with Ag-NPs attached to it. It is created in a three-step process (as detailed in the Methods chapter). The first step is the selection and/or production of the BC substrate. Biofill, a dried and stretch BC pellicle, was one of two sources of cellulose that was used; it has a successful commercial history, and the quickest path to making available a silver loaded BC wound dressing is to predicate it on an already successful product. The second form of cellulose used was produced in the lab; this, as we will see is morphologically different from Biofill, and has correspondingly different characteristics. The second step is the oxidation of the BC substrate; this step prepares the sample for functionalization with thiosemicarbazide and silver. And it follows, that the last step is the functionalization or attachment of Ag-NPs to the BC substrate. Results of these steps, and their corresponding characterization will be detailed and discussed in this chapter.

4.1 – Bacterial cellulose substrate

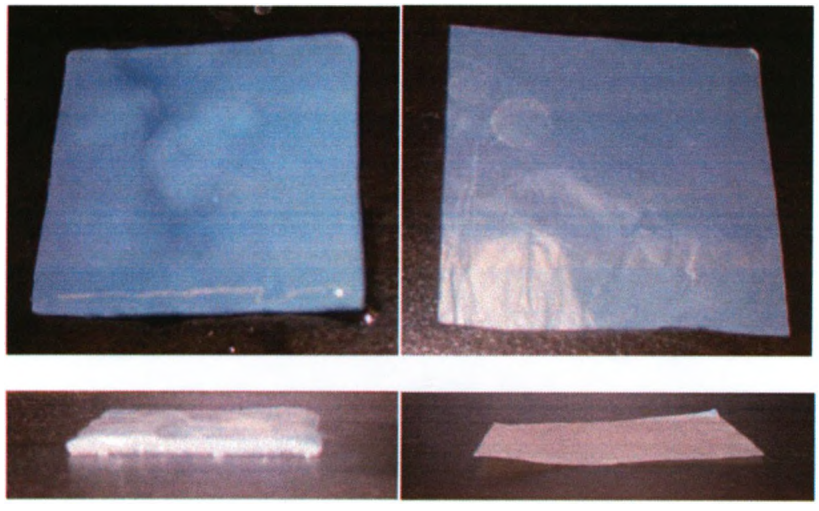

Figure 4.1: Labmade cellulose (left) versus Biofill (right). Top and side views.

A piece of lab made, wet pellicle versus Biofill can be seen in Figure 4.1. The wet pellicle, as produced by the procedure listed in the Methods chapter is comprised of 99% water by mass, with only the remaining 1% being cellulose. When wet it is 2 –3 mm thick, but decreases to a thickness of 60 ± 10 μm upon drying (the other dimensions remain unchanged), and does not reswell upon rewetting; whereas, Biofill is 40 ± 10 μm regardless of whether it is dry or wet. The nanostructure of wet pellicle can be seen in Figure 4.3. This SEM was taken using critical point drying to preserve the nanostructure. The highly porous 3-D structure is irreversibly lost during the normal air drying process; one can note the flattening of the fibers and the collapse of the pores, due to the loss of hydrogen bonds and their role in preserving the nanostructure, as illustrated by the SEM of Biofill [110].

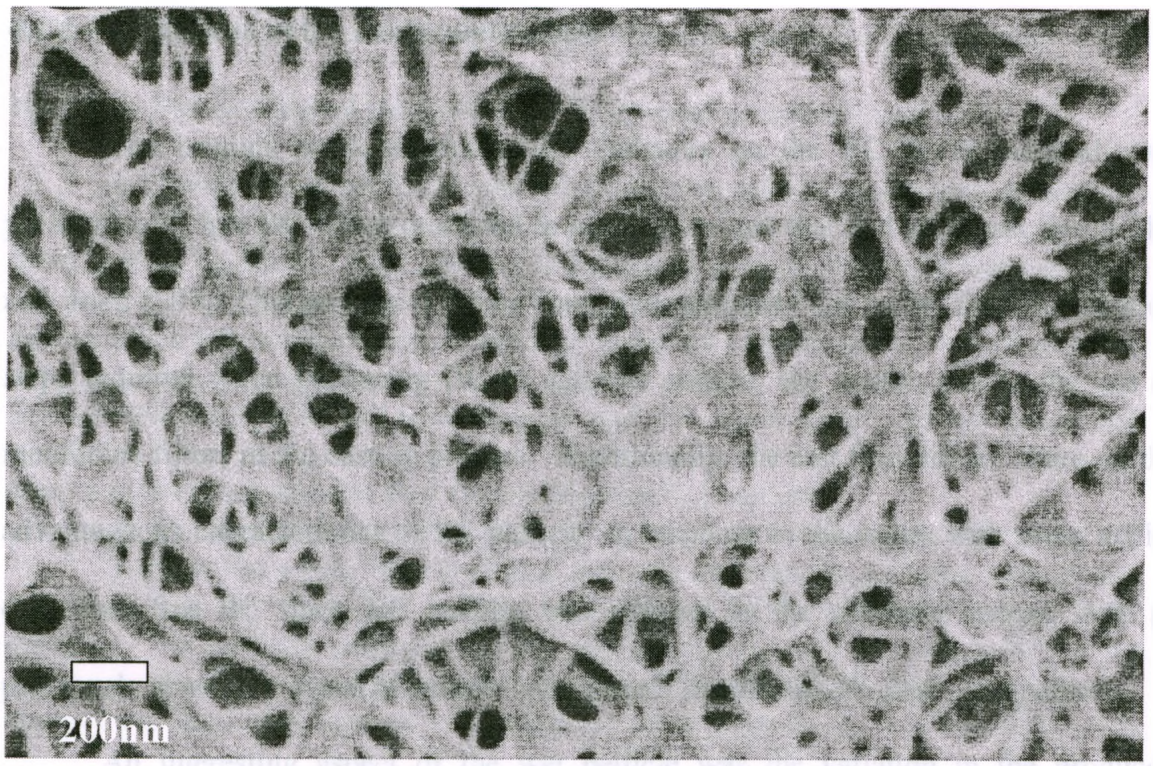

Figure 4.2: SEM of dry pellicle (Biofill)

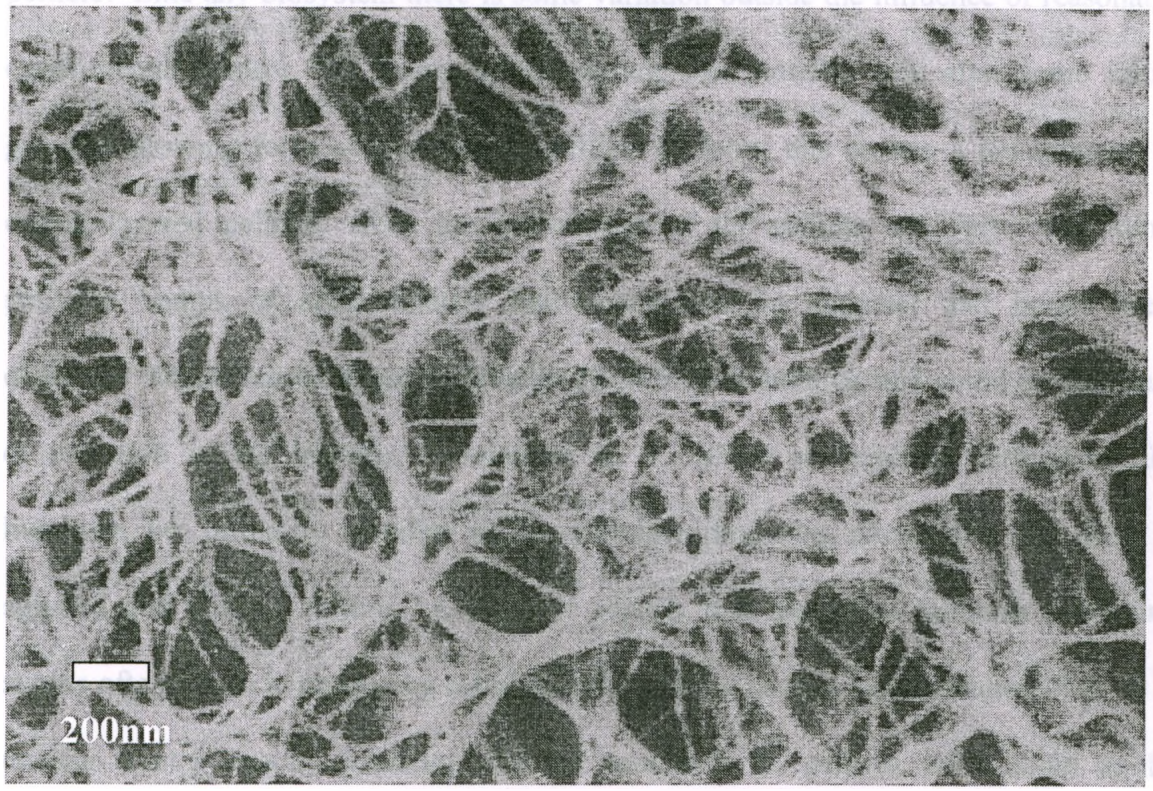

Figure 4.3: Lab made pellicle after critical point drying.

4.1.1 – Production of lab made, wet pellicle

The original work conducted by Guhados on the BC silver material, was preformed on free BC fibers produced in agitated culture that had been cast into a film and dried [107]. This process is labour intensive, primarily due to the fiber purification steps required when producing BC in agitated culture, and costly, due to the use of membrane filters in the casting process. Additionally, the cast film did not share all the advantageous characteristics of BC pellicle. In response to the issues with using a film cast from free BC fiber, BC pellicle was selected as the substrate instead. And as previously stated two types of pellicle were tested: Biofill and lab made, wet pellicle.

An interesting aspect of the wet pellicle was the variation in the produced pellicle. Like any biosystem there is some variation outside the influence of reasonable experimental control. For example, the pellicle can partially sink after some initial growth, allowing for a second thinner pellicle to form; this creates a delaminated or double-layered pellicle. Also, the smoothness of the pellicle can vary; with some pellicles bumps of approximately half-a-centimeter in diameter and height were observed. This occurred in approximately 20 – 25 % of cultures, and could be the result of mutation of the bacteria. Mutations in *A. xylium* are reported throughout the literature, but primarily manifest as non-cellulose producing phenotypes that arise in agitated and shaken culture. One paper reports that these non-cellulose producing strains often return to producing cellulose when placed back in static culture [111]. It is unclear what is exactly occurring with the unsmooth BC pellicle, but it could be the result of mutation in the seed culture - produced in shaken culture - and the return of those bacteria to cellulose producing pheonotypes once placed in static culture. From a

pragmatic point of view, this problem was overcome qualitatively, with some pellicles being rejected on personal judgment, like in any quality control inspection.

A growth period of 7 days was selected as the pellicle had an area density, when dried, similar to Biofill. While Biofill is described as being dried and stretched, the lab made pellicle was not stretched, as the exact procedure for doing this is not known and it is suspected that specialized equipment is used. So, while Biofill and lab made pellicle share a similar area density, they are not necessarily identical.

If grown for a long period of time and with more media, a thicker pellicle can be produced. The pellicles produced for this research project had a thickness of 2 – 3 mm where there have been reports in the literature of pellicles of 2 – 4 cm thick, in cultures maintained over 30 days [112]. As we will see during the rest of this chapter, starting from a wet pellicle will increase the loading of silver – primarily due to an increase in accessibility of the internal structure – and correspondingly the antimicrobial effectiveness of the end material.

4.2 – Oxidation of the bacterial cellulose substrate

The second step, the oxidation of the BC, was the original focus of the research, as it was assumed to provide a facile and effective method for tuning silver loading. While this proved to be somewhat true, as we will see, the source of the cellulose, step one, plays a more important role. The BC was oxidized using sodium metaperiodate ($NaIO_4$) to obtain 2,3-dialdehyde cellulose (DAC). $NaIO_4$ breaks the C-2-C-3 bond of the glucose residues resulting in the structure shown in Figure 4.4 [113].

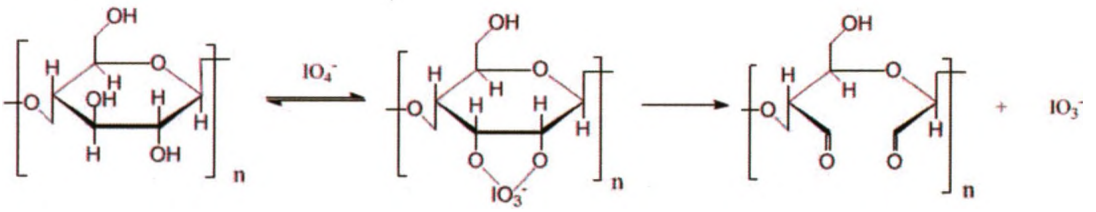

Figure 4.4: Oxidization Reaction

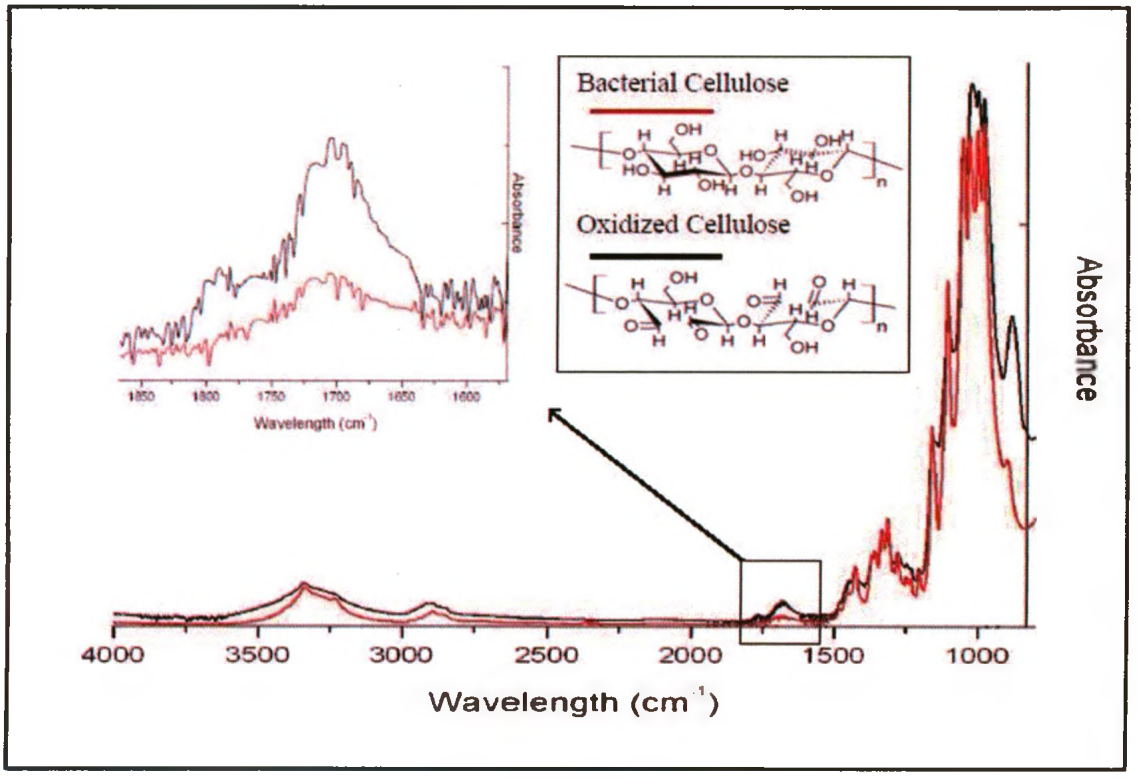

Figure 4.5: FTIR spectrum of bacterial cellulose and oxidized cellulose

The oxidation was confirmed using fourier transform infrared spectroscopy (FT-IR). The low level of oxidation made it difficult to detect a change in the FT-IR spectra, and so a sample of Biofill was oxidized for 20 hours as opposed to the single hour used for sample preparation. The oxidized samples show peaks at 1735 cm^{-1} and 880 cm^{-1} which

correspond to the stretching of the free aldhyde and hemiacetal structure respectively. A flattening of the OH peak is also observed [114].

4.2.1 – Quantify oxidation level: Biofill

The oxidation level was measured using the Cannizzaro reaction as described by Pommerening et al. [108] This procedure provided a simple and repeatable method to determine the number of aldehyde groups, expressed as a percentage of cellulose monomers. An alternative method for determining the oxidation level involves using optical absorbance to measure the initial periodate concentration and remaining concentration after oxidation, to determine the percentage consumed. This method was reported by Maekawa and Koshijima [115]. Both methods were compared by RoyChowdhury and Kumar and showed to yield similar results under ideal conditions [116], making using a redundant method moot. The optical absorbance method was attempted, but ultimately failed to yield usable results, due most likely to the high ratio of $NaIO_4$ to BC, with an expected change in $NaIO_4$ levels on the order of a fraction of a percent. In other words, the sensitivity of that method was not great enough for this application.

Table 4.1: Oxidation Level Biofill.

Sample (oxidation time)	Oxidation Level
15 minute	.52 ± .03%
30 minute	.91 ± .03%
60 minute	1.89 ± .08%

As can be seen by the small standard deviations, in Table 4.1, the oxidation reaction produced highly repeatable results and the Cannizzaro method proved precise. The oxidation times of 15, 30 and 60 minutes were selected as a continuation of Guhados's

work; he had preformed the reaction on microcrystalline cellulose and BC produced in shaken and agitated culture [107].

4.2.2 – Quantify oxidation level: lab made, wet pellicle

The oxidation procedure was adapted when the work shifted to using wet pellicle. Wet pellicle is 99% water, with the remaining being BC. The high percentage of water presented a challenge. For example during the oxidation step of the procedure, a 15 minute oxidation time would not ensure that the $NaIO_4$ had diffused throughout the sample, possibly preventing an even oxidation of the sample. Additionally, it would be difficult to wash all the reagents from the sample afterwards. To overcome these challenges, the procedure was modified. Instead of using a set amount of oxidizing agent and varying the reaction time, the reaction time was kept constant and the oxidizing agent was varied. A time point of 24 hours was selected, and the molar ratio of the *oxidizing agent to BC* was varied between .5:1, 1:1, 1.5:1, and 2:1. As a reminder, a fixed ratio of 13:1 was used for the dry pellicle samples, with the time points varied from 15 minutes, 30 minutes, and 60 minutes. The resulting oxidation levels can be seen in Table 4.2.

The oxidation level was determined using the same quantify procedure as the dry pellicile (Biofill). The samples were dried before determining the oxidation levels.

Table 4.2: Oxidation Level Pellicle

Sample	Oxidation Level
.5 $NaIO_4$: 1 BC	2.6 ± .2 %
1 $NaIO_4$: 1 BC	4.2 ± .3 %
1.5 $NaIO_4$: 1 BC	5.8 ± .3 %
2 $NaIO_4$: 1 BC	6.8 ± .3 %

The overall oxidation level was greater than that for the Biofill samples. This was done intentionally to further increase the silver loading and antimicrobial properties. The upper limit was not arbitrary; 7% was not exceeded because it yielded a material that cracked and flaked easily. The loss of this structural integrity makes it unsuitable consideration as a potential wound dressing.

4.3 – Attachment of silver nanoparticles to the bacterial cellulose substrate

The third step of linking the silver nanoparticles to the BC substrate can be thought of in two discrete sub-steps: first, functionalizing the DAC in preparation for the silver treatment; and second, the silver treatment.

The functionalization of the DAC is accomplished using thiosemicarbazide. Thiosemicarbazide is a ligand, which has historically been used as a chelating agent. It provides an anchor for the silver protienate. The thiolized DAC is seen in Figure 4.6.

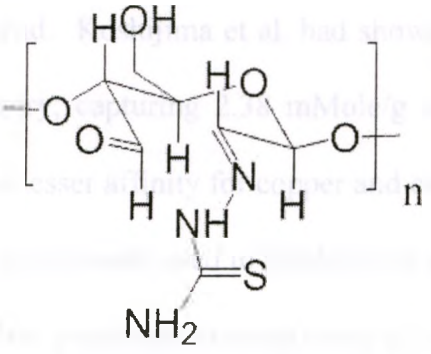

Figure 4.6: Cellulose with thiosemicarbazide

The conformation of the thiosemicarbazide and silver complex was explored by Bonamartini et al. [117] They showed that there are two likely configurations, seen in Figure 4.7 with M representing the metal ion. The first association is the only one that could occur, given the bonding arrangement of the thiosemicarbazide and BC.

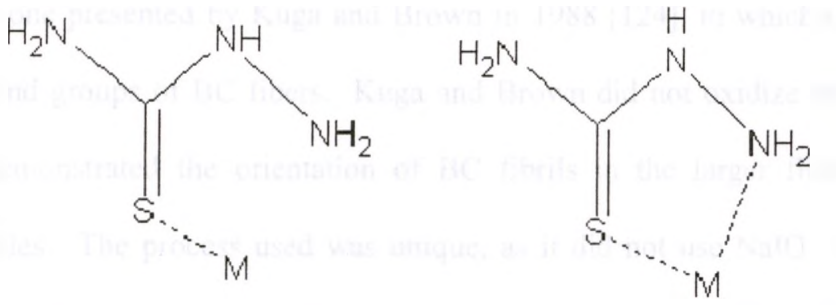

Figure 4.7: Thiosemicarbazide and metal ion bonding arrangement

The material presented in this thesis represents an extension of the work began by Guhados in 2005. He was the first to used thiosemicarbazide with oxidized BC to create a chelating polymer, although previously it has been combined with oxidized plant cellulose to a similar end. Koshijima et al. had shown such a material has a high affinity for silver and mercury, capturing 2.38 mMole/g cellulose and 3.12 mMole/g cellulose respectively, with a lesser affinity for copper and cadmium [118].

There are two silver compounds used to produce the Ag-NPs. The first treatment is with silver proteinate. Silver proteinate is proprietary silver protein complex that was formally sold under the brand names of Argerol or Stillargol, and used to treat certain eye and nose infections; its only modern use is in histological studies. The *second* form of silver used is ammoniacal silver, also known as Tollen's reagent; it is sometimes used in organic chemistry to distinguish between organic functional groups.

If divided differently the entire process can be thought of as a three-step process (described by the main constituents): periodate – thiosemicarbazide – silver protienate (PA-TSC-SP). This is how it is frequently portrayed in histology; where it is used as a method for staining glycogen. The method is described in several textbooks [119] [120] [121], and was widely used in the 1970s and 80s [122] [123]; but, it is difficult to find reference to it in the literature during the 1990s or 2000s. This histological method

resembles the one presented by Kuga and Brown in 1988 [124], in which they stained the reducing end groups of BC fibers. Kuga and Brown did not oxidize the cellulose, but instead demonstrated the orientation of BC fibrils in the larger fibers on non-oxidized samples. The process used was unique, as it did not use $NaIO_4$, but instead relied on the reducing ends of the cellulose fibrils. Guhados also showed that the silver maps to the sulphur locations using energy-dispersive x-ray spectroscopy, when the above procedure was conducted on microcrystalline cellulose [107].

4.3.1 – X-ray diffraction

To properly evaluate the efficacy of the Ag-NP-BC dressing, it is important to compare it to an established material that has a clinical history. In this case Acticoat was selected, as it is widely held as the gold standard of silver dressing. To validate the comparison, the nature of the silver particles must be examined and shown to be akin.

Acticoat is prepared through physical vapour deposition of silver on a polyethylene substrate. As one can see from the diffraction peaks in Figure 4.8 the silver on the BC has the same crystalline structure as those on Acticoat. The XRD fingerprint corresponds to a face-centered cubic-structure [15]. These plots use a 10 point moving average to smooth some of the noise found in the data.

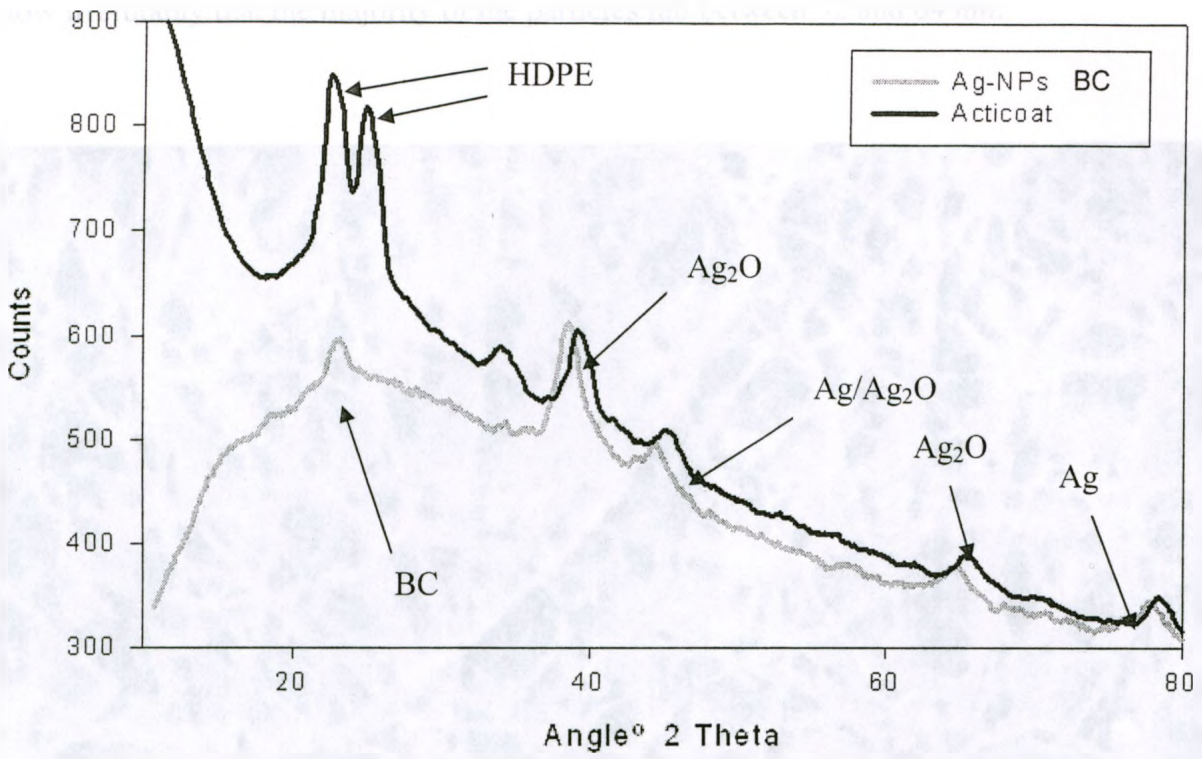

Figure 4.8: XRD specta for and silver-BC and Acticoat samples

4.3.2 – Scanning electron microscopy

The silver particle size was explored using SEM. An image of the silver loaded Biofill (dry pellicle) is seen in Figure 4.9. Multiple micrographs of several independently made samples were taken, showing the repeatability, uniformity, and a particles size that is seen in Figure 4.10. It should be noted that the resolution of the micrographs was 4 nm/1 pixel. The histogram bin size were selected based on 4 pixels, or 16 nanometers, this does not indicate a precision to last digit as the rules of significant figures would normally suggest. It was difficult to obtain a more precise reading on the particle size due to the resolution limitation of the SEM images. Transmission electron

microscopy could not be used due to the sample thickness. Regardless, these results do show irrefutably that the majority of the particles fall between 32 and 64 nm.

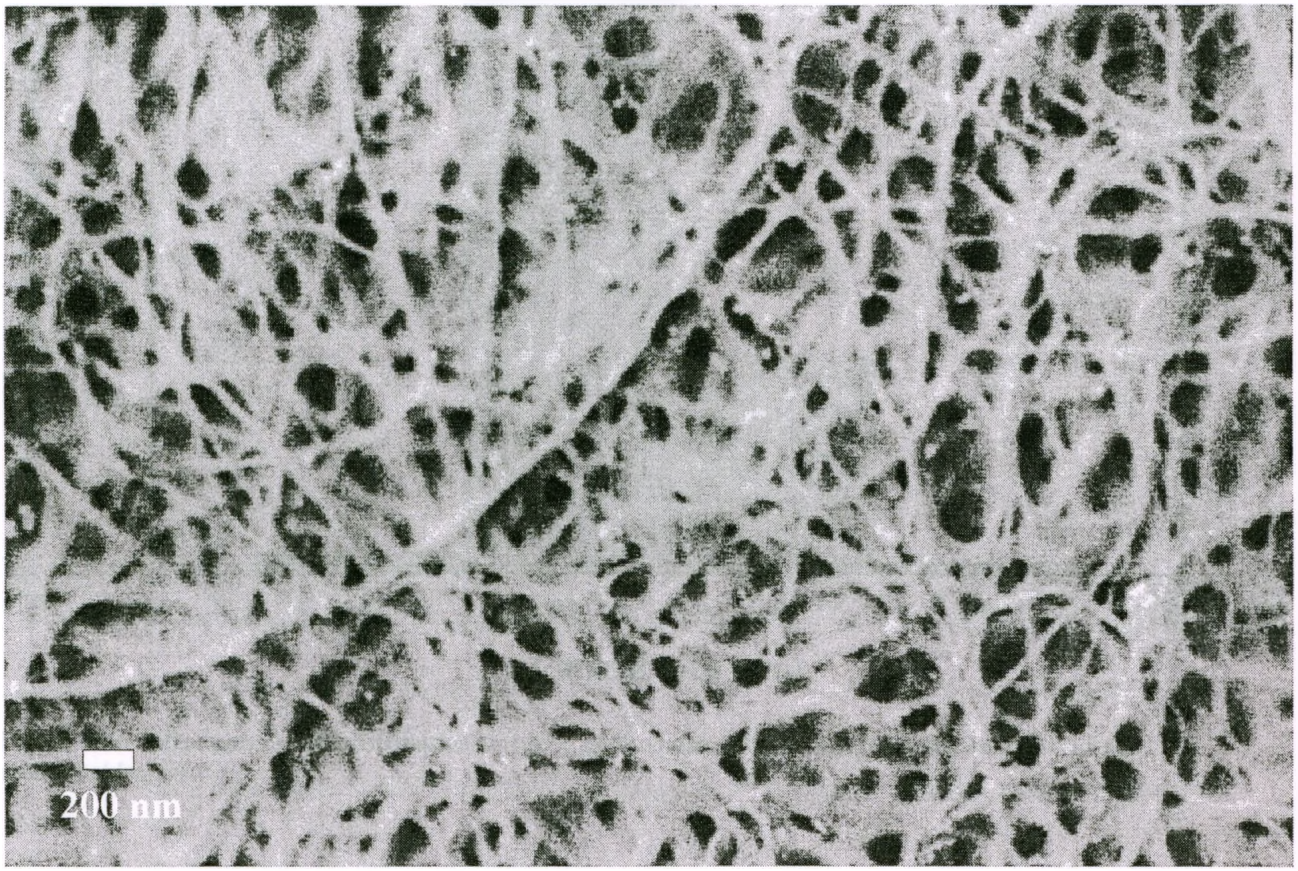

Figure 4.9: SEM of silver loaded Biofill. 1.89 ± .08% oxidized.

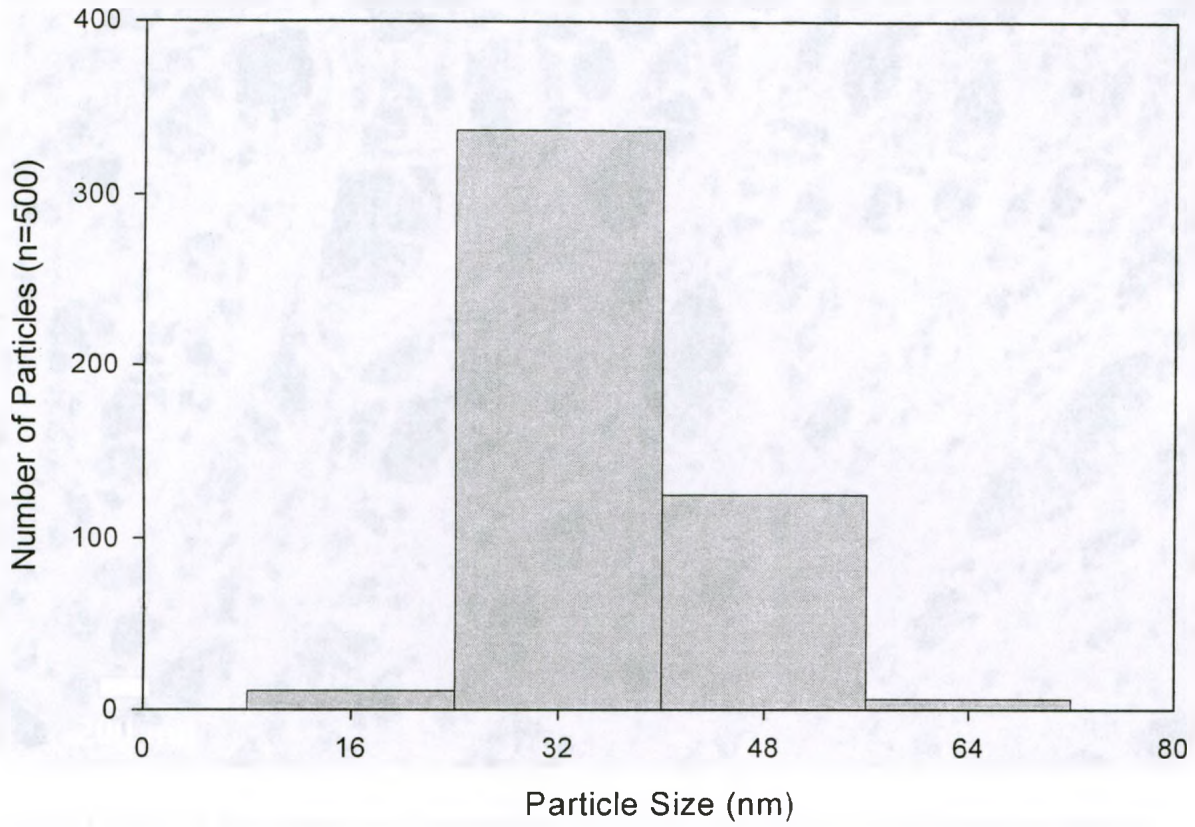

Figure 4.10: Silver particle size distribution on 1.89 ± .08% oxidized Biofill sample. Based on figure 4.5 and additional frames.

The lab made, wet pellicle samples were heavily coated with silver as can be seen in Figure 4.11. While the coating looks particle based, it would be more apt to describe it as BC coated an almost confluent layer of silver. This made particle size determination impossible, hence no particle distribution is presented for the wet pellicle based system.

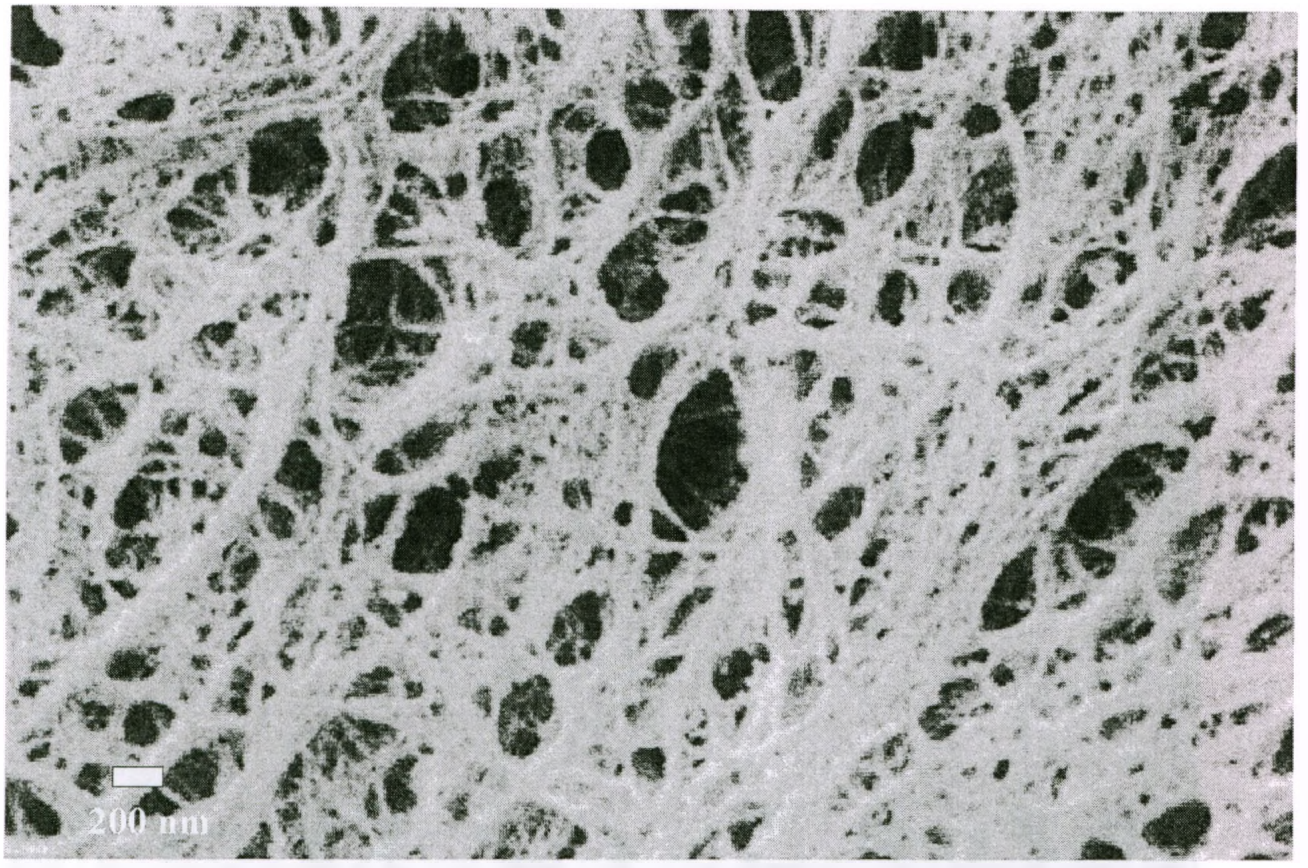

Figure 4.11: SEM of silver loaded on lab-made pellicle (6.8 ± .3 % oxidized) after drying at room temperature.

SEMs of Acticoat yield two different silver morphologies: the discrete particles and grape-bunch types of silver as seen in A and B of Figure 4.12 respectively. The discrete particles have a particles size falling primarily between 32 and 64 nm (Figure 4.13), while the clusters have an estimated size of between 200 and 500 nm, with knobs of between 50 and 150 nm. Both images have been taken from the same piece of Acticoat, calling into question the consistency of the manufacturing process.

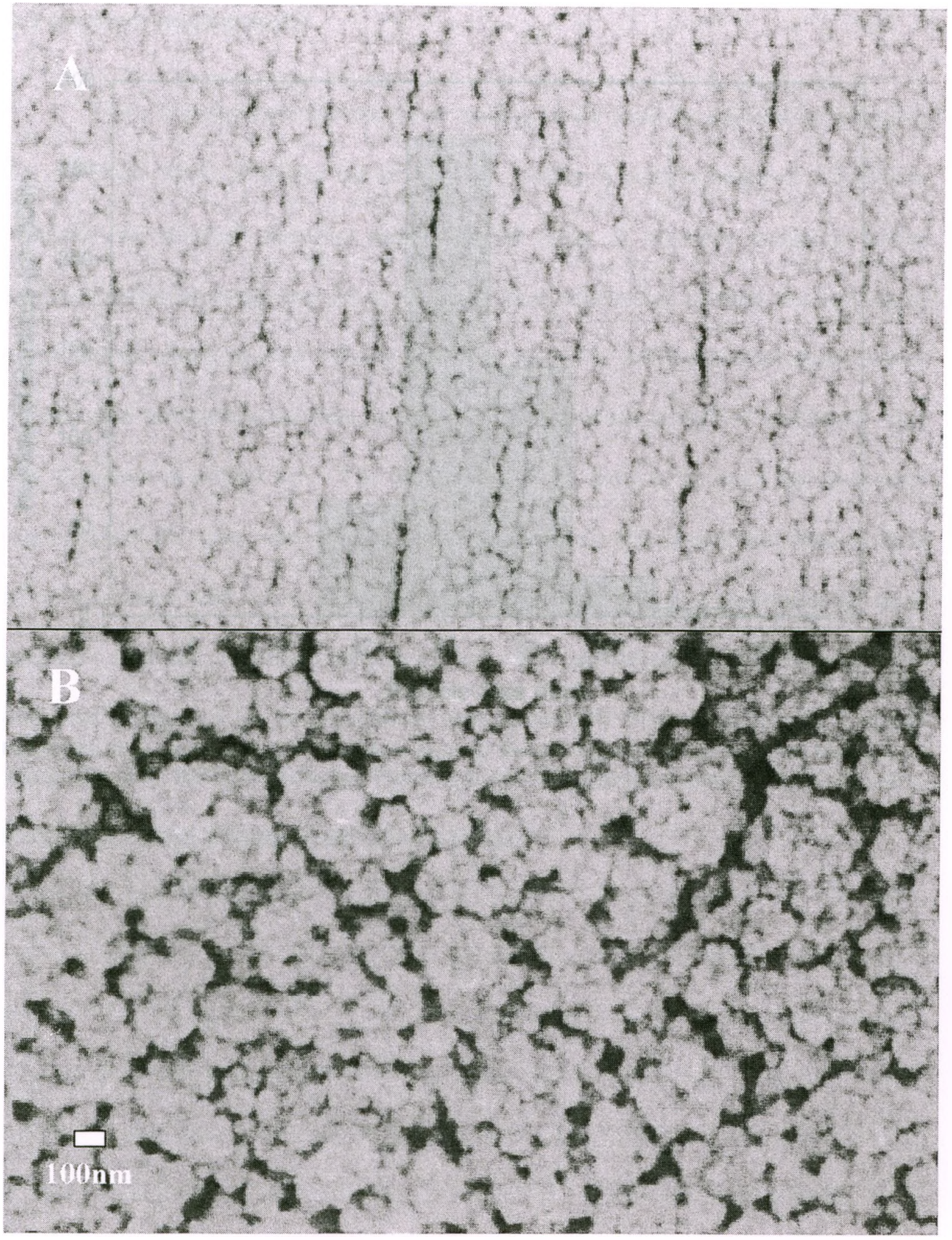

Figure 4.12: SEMs of two different locations on Acticoat, demonstrate two different particles morphologies. Scale bar applies to both A and B.

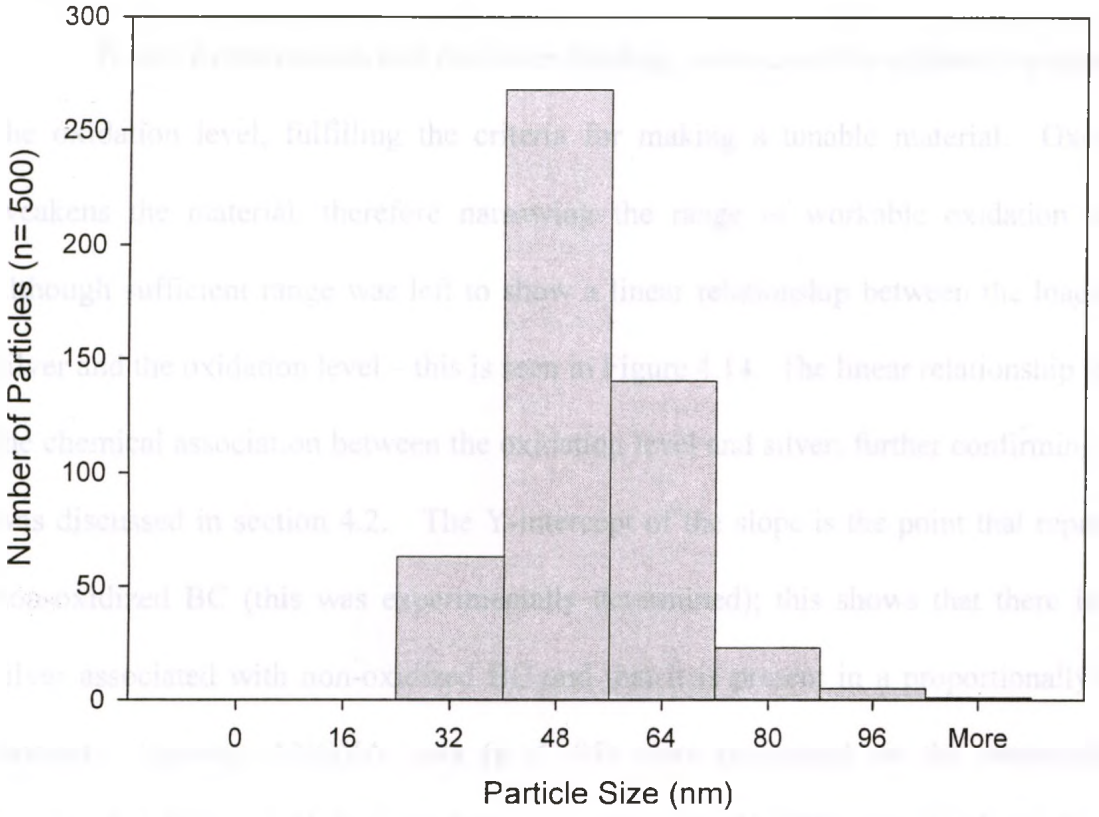

Figure 4.13: Silver particle size distribution from Acticoat. Based on Figure 4.12A and additional frames.

4.3.4 – Silver loading: Biofill

It was hypothesized that the silver loading levels could be adjusted by changing the oxidation level, fulfilling the criteria for making a tunable material. Oxidation weakens the material, therefore narrowing the range of workable oxidation levels; although sufficient range was left to show a linear relationship between the loading of silver and the oxidation level – this is seen in Figure 4.14. The linear relationship proves the chemical association between the oxidation level and silver; further confirming what was discussed in section 4.2. The Y-intercept of the slope is the point that represents non-oxidized BC (this was experimentally determined); this shows that there is both silver associated with non-oxidized BC and that it is present in a proportionally large amount. Oneway ANOVA tests ($p < .05$) were preformed on the measurements presented in Figure 4.14, the populations were statistically different in both the Y and X directions, with the corresponding Tukey test showing that the points in the Y direction are not statistically different from adjacent points.

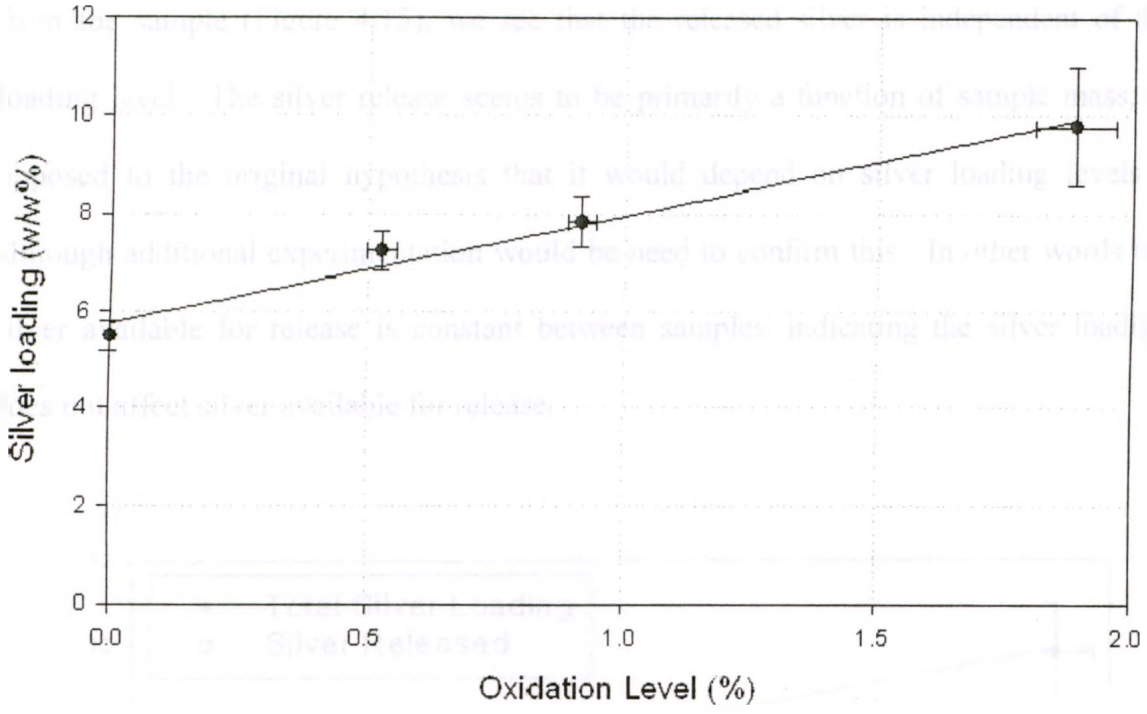

Figure 4.14: Oxidation of Biofill versus silver loading.

Having such a large amount of silver associated with the unoxidized BC, suggests two possible associations. It could be silver bound to the reducing end groups of the cellulose fibrils. As previously mentioned the silver binding process without the oxidation step mimics the procedure that Kuga et al., use for labeling the reducing end groups of BC [124], thus indicating that some of the silver is bound in a similar manner. Or it could be unbound silver that has remained after the rinsing steps; while the samples are rinsed after both the silver loading steps, the rinsing is gentle. It is possible that not all the loose silver is removed during the rinses. In one possible scenario, silver protienate not removed during the rinsing is enhanced with ammoniacal silver in the next step, creating unbound Ag-NPs caught in the Biofill mesh.

If we compare the loaded silver with the silver that is released after 96 hours from the sample (Figure 4.15), we see that the released silver is independent of the loading level. The silver release seems to be primarily a function of sample mass, as opposed to the original hypothesis that it would depend on silver loading levels – although additional experimentation would be need to confirm this. In other words the silver available for release is constant between samples, indicating the silver loading does not affect silver available for release.

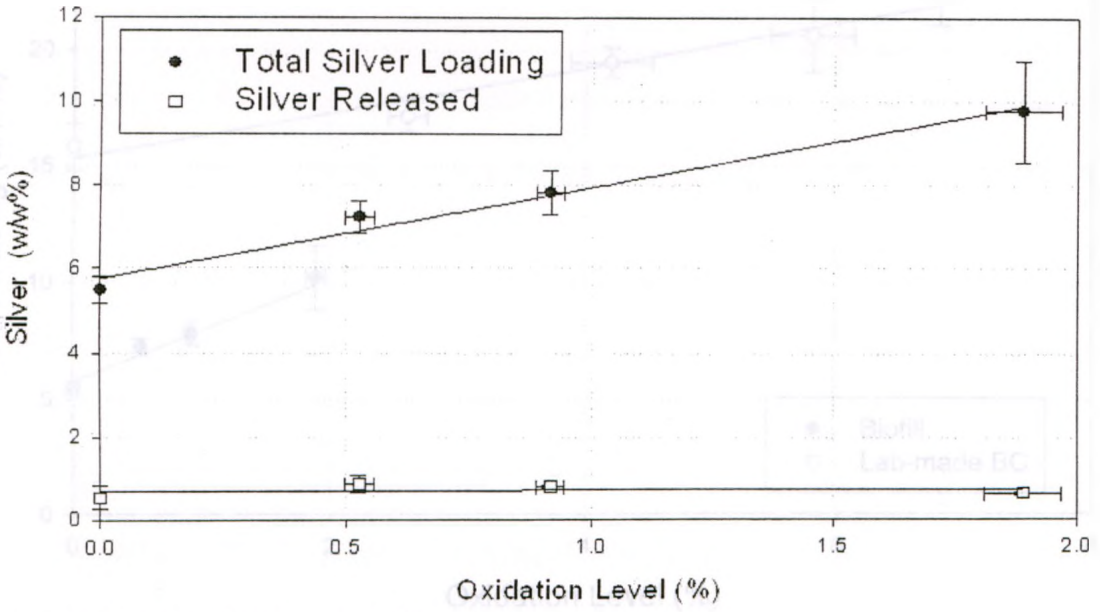

Figure 4.15: Biofill silver loading versus silver release after 96 hours.

4.3.5 – Silver loading: Lab-made, wet pellicle

In Figure 4.15 the silver loading is expressed as a function of oxidation level, for the lab made pellicle and compared to the data from Figure 4.14. It should be noted that there is a much higher loading of silver. This can be explained once again by the 3D structure of the wet pellicle, in which the internal structure is more accessible and

available for silver deposition. When comparing the loading levels in the unoxidized Biofill and wet pellicle samples – this high level of loading in the unoxidation would suggest access to the internal structure makes a large difference. A portion of the silver is bound to the reducing end groups of the cellulose fibrils; in a 3D environment these end groups are more accessible and therefore can bind more silver, compared to the essentially 2D Biofill.

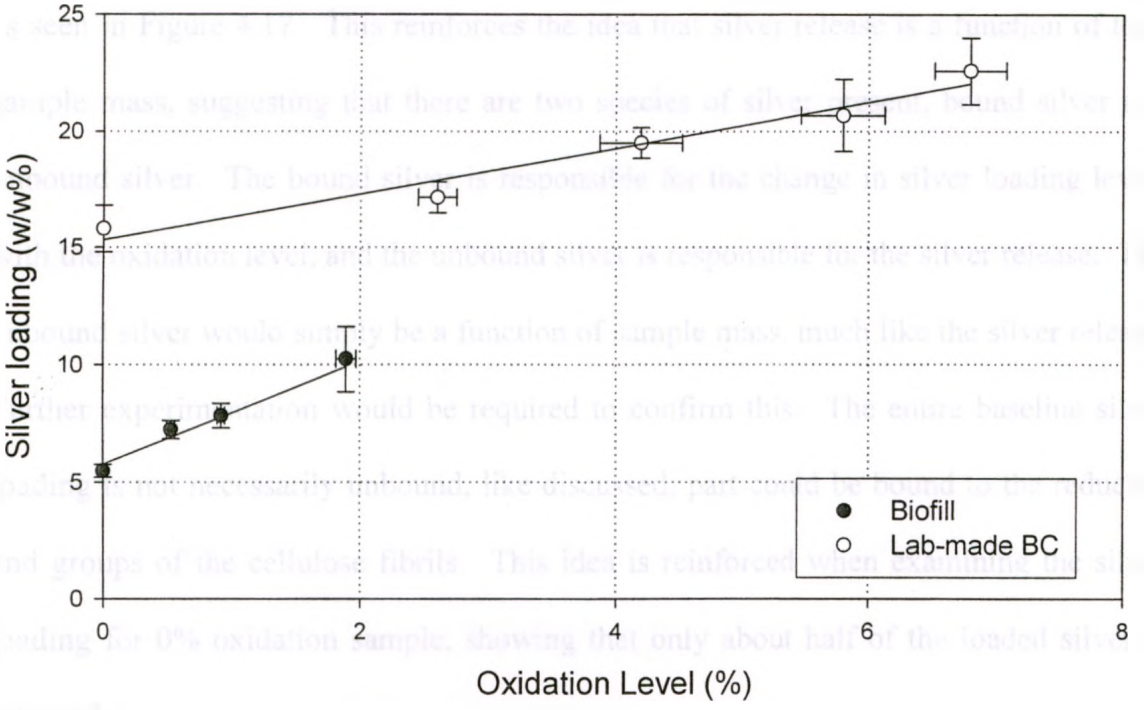

Figure 4.16: Oxidation of lab made, wet pellicle versus silver loading (also including data from Figure 4.14)

Oneway ANOVA tests ($p < .05$) were preformed on the silver loading measurements (Figure 4.16) showing that the population means are statistically different in both the Y and X directions, with a corresponding Tukey test showing the points are not statistically different to adjacent points in the Y direction.

Like previously discussed the linear relationship between the silver loading and the oxidation level is an indication of the association between the aldehyde groups and

the silver. There is a difference in the slopes of the lines, with the lab-made pellicle expressing a less steep slope. The reasons for this are not clear. One possible explanation is that the oxidation loosens the fibers of the Biofill, increasing the surface area for silver binding. This would not have such a large material impact on lab-made cellulose, because so much surface area is already exposed.

Similar to the case of the Biofill (dry pellicle), the amount of silver released over a 96 hour period from the wet pellicle preparation is independent of silver loading levels as seen in Figure 4.17. This reinforces the idea that silver release is a function of total sample mass, suggesting that there are two species of silver present, bound silver and unbound silver. The bound silver is responsible for the change in silver loading levels with the oxidation level, and the unbound silver is responsible for the silver release. The unbound silver would simply be a function of sample mass, much like the silver release. Further experimentation would be required to confirm this. The entire baseline silver loading is not necessarily unbound, like discussed, part could be bound to the reducing end groups of the cellulose fibrils. This idea is reinforced when examining the silver loading for 0% oxidation sample, showing that only about half of the loaded silver is released.

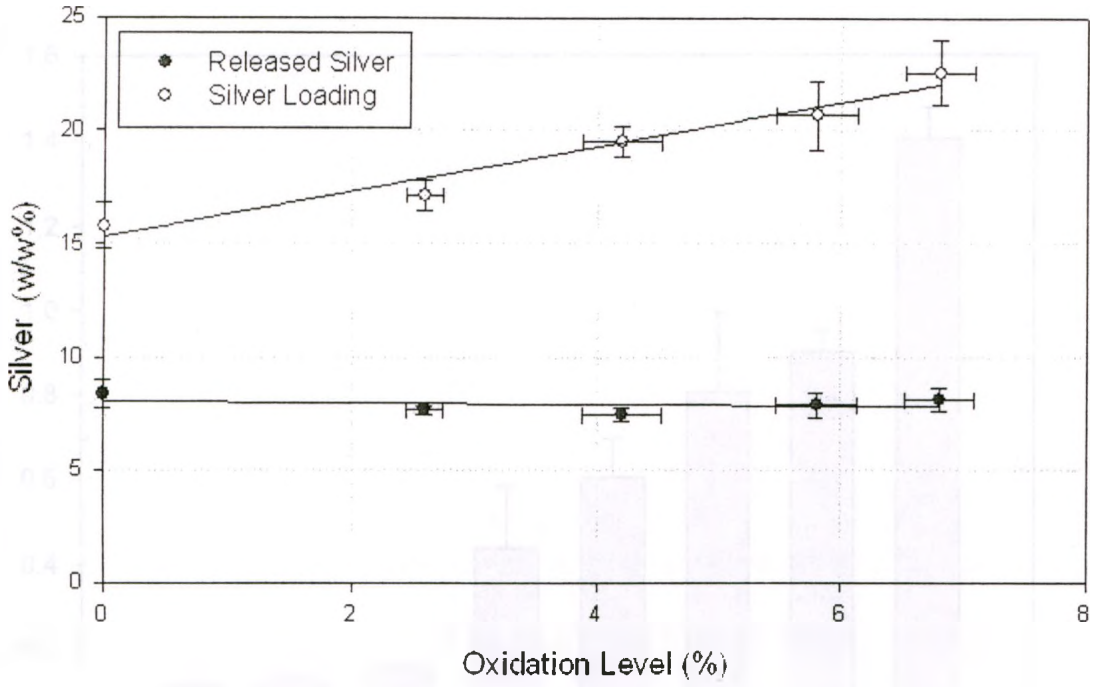

Figure 4.17: Wet pellicle silver loading versus silver release after 96 hours.

4.3.6 – Silver loading by area

An alternative and practical way to look at the prepared samples is silver loading by area. This provides the ability to compare the samples to the loading in Acticoat and gives an idea of the dose that will be delivered to a wound and to the agar in the antimicrobial tests. It should be noted that there is a difference in the thickness of the lab-made cellulose and the Biofill prepared samples. The wet pellicle is 60±10 μm after drying compared to the Biofill of 40±10 μm. This information is presented in Figure 4.18. Acticoat are the heaviest loaded samples.

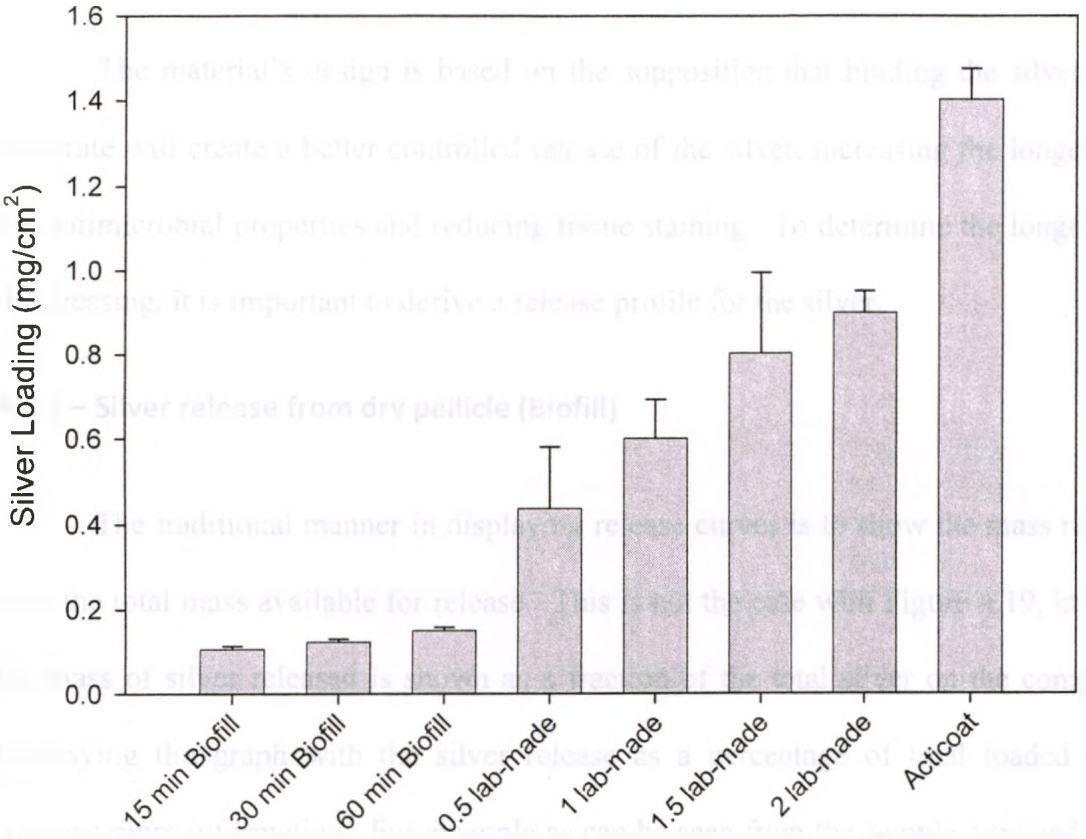

Figure 4.18: Silver loading by area. The biofill samples are labeled according how long they were oxidized for, and the lab-made. wet pellicle are labeled according to the ratio of oxidizing agent to cellulose.

4.4 – Silver release

The material's design is based on the supposition that binding the silver to the substrate will create a better controlled release of the silver, increasing the longevity of the antimicrobial properties and reducing tissue staining. To determine the longevity of the dressing, it is important to derive a release profile for the silver.

4.4.1 – Silver release from dry pellicle (Biofill)

The traditional manner in displaying release curves is to show the mass released over the total mass available for release. This is not the case with Figure 4.19, in which the mass of silver released is shown as a fraction of the total silver on the compound. Displaying the graph with the silver release as a percentage of total loaded silver, conveys more information. For example as can be seen from the sample oxidized for 15 minutes, that silver release seems to level off at a level around 12 %. This suggests a level of about 12 % of the loaded silver is available for release, information that could not have been ascertained otherwise.

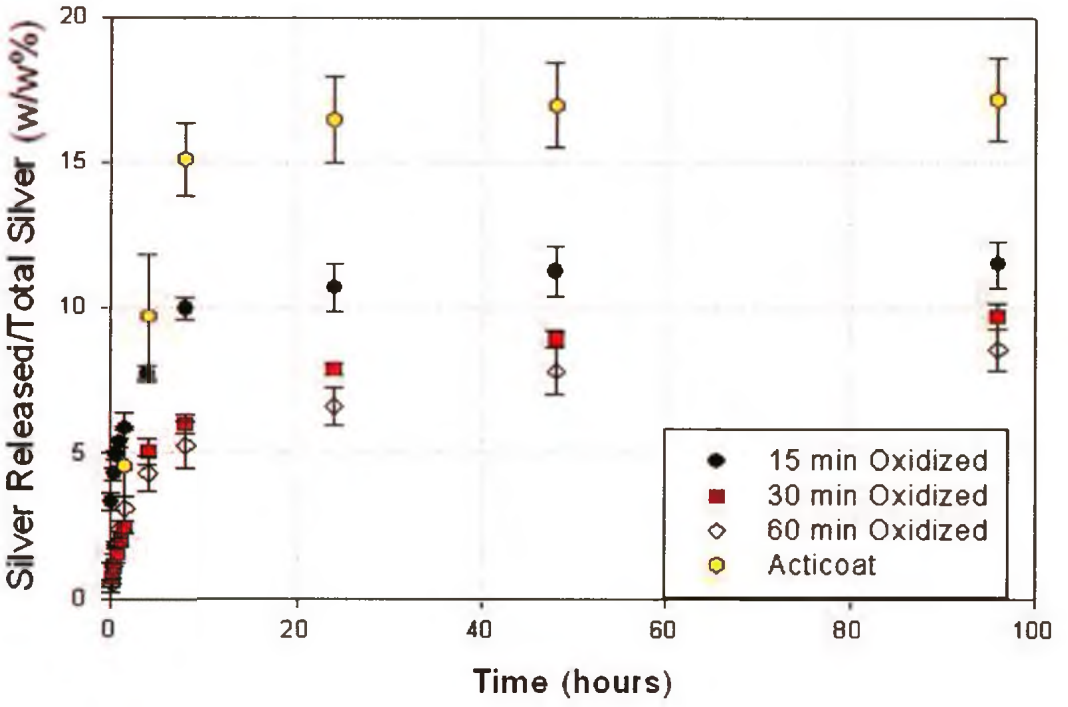

Figure 4.19: Biofill release profile

The data presented in Figure 4.19 might suggest that the 15 minute oxidized sample release more silver that the 60 minute oxidized sample, which is only partially true. The release presented in the graph is a function of a fraction of the total silver, and the 15 minute oxidized sample has less silver loaded than the 60 minute sample, therefore the fractional released is larger.

4.4.2- Lab-made, wet pellicle release

The curves for the samples prepared from the lab made, wet pellicle are similar to the curves seen with samples prepared from Biofill. Figure 4.20 shows the release of silver as a portion of total silver loading, and it is apparent that the samples with lower levels of silver loading release a proportionally larger amount of silver. The results for

Acticoat are shown for comparison. It should be noted that Acticoat is 13 ± .4 % silver by mass, and is tripled layered.

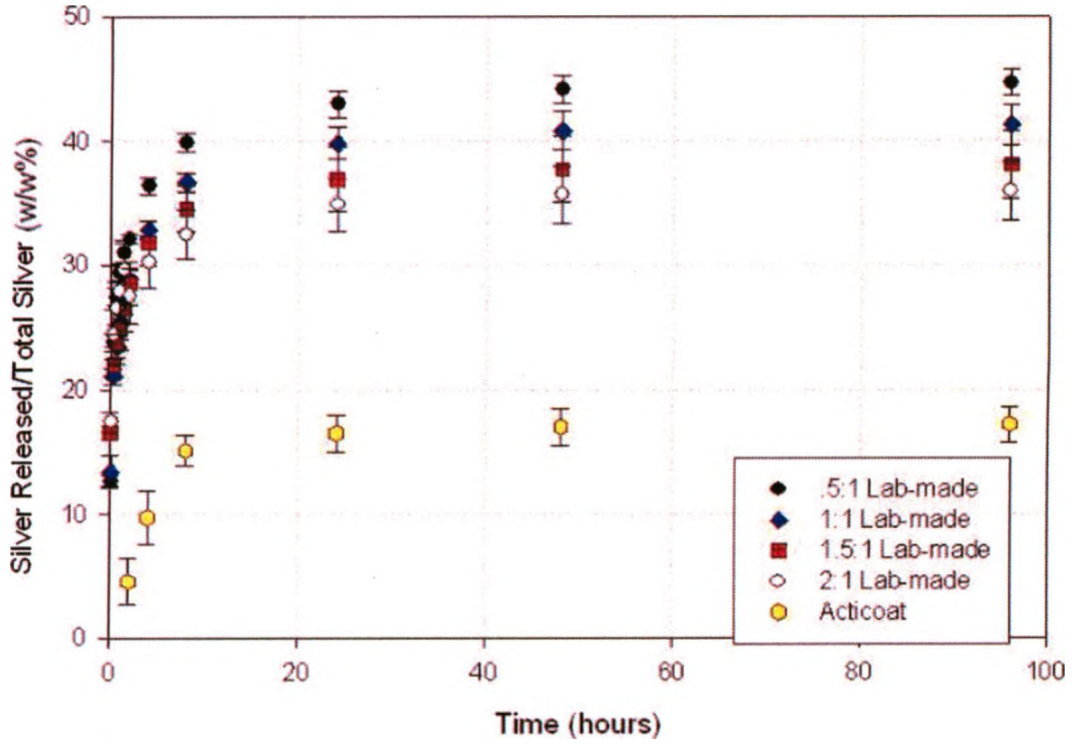

Figure 4.20: Release profile lab-made, wet pellicle

4.4.3 – Silver release mechanism

There are several possible modes of release. For example, when Acticoat is placed in water the silver can be seen simply falling off the polyethylene substrate as a fine black powder – this type of release may also be present in the BC based dressing although does not occur in observable levels. As previously discussed there is the potential for unbound silver to be present in the material, and could influence the silver release profile.

Additionally, it should be noted that the total silver released is a percentage of the total loaded silver. This is best explained by suggesting the largest component of the released silver is unbound silver – which would be independent of the loading level, and instead be a function of substrate mass. But, the simple letting go of the unbound silver is not necessarily the only mode of silver release. The original design was based on the premise that nanosilver dissolves into Ag^+ and Ag^0 clusters (defined as a grouping of 8 or more atoms) as described by Fan and Bard. [125]. Hoskin et al. postulate that it is the oxidation of the silver in an aqueous environment that contributes to this break down with dissolved oxygen playing a role [126], as seen in the following mechanism:

$$O_2(aq) + 4H+(aq) + 4Ag^0(s) \rightarrow 4Ag^+(aq)+2H_2O(l)$$

Fan and Bard explore the release of silver from an Acticoat like material, as it relates to silver clusters and silver ions in a 1 M $NaClO_4$ solution. A limitation of the study conducted for this project is that only total silver release is studied, without making the distinction between Ag-NPs, Ag^0 clusters and Ag^+ ions. But, it is safe to assume, silver release can be summarized as having three distinct types of silver – Ag-NPs, ionic silver and silver clusters.

Silver levels were measured using Atomic Absorption Spectrophotometry, which had the advantages of simplicity and availability, allowing for the testing of a multitude of samples; but had the disadvantage of poor sensitivity, measuring only to.5 ppm. In Gray's PhD thesis, conducted on the nanosilver linked polyurethane substrate, she attempted to use AAS, but was ultimately unsuccessful due to its lack of sensitivity

[127]. She was only able to evince through indirect methods (antimicrobial results) that silver was released.

The other disadvantage of AAS is its inability to handle highly ionic solutions – the silver signal was lost when testing the release in phosphate buffered solution. In the past other groups have investigated the release of silver into several ionic solutions including: alpha-medium, PBS(-), calf serum, .9% NaCl, artificial saliva, 1.2 mass% l-cysteine, 1 mass% lactic acid, .01 mass% HCl, and simulated body fluid [128] [129] – using inductively couple plasma mass spectrometry (ICP-MS). The release of silver into an ionic solution or the wound environment raises interesting questions. The silver release, if in ionic form would quickly bind to negative ions in solution, for example chlorine, forming biologically inactive silver-chloride. In the case of Acticoat, the recommended application procedure in clinical settings requires soaking the dressing in distilled water – as oppose to the standard saline. Whether this step is superfluous or not, as the dressing is then applied to the normally strongly ionic concentration of a wound's exudate, is an excellent question. The answer, unfortunately, is still elusive. Despite this, the release of silver into de-ionised water has been investigated in the literature with proven silver systems [4].

4.5 – Antimicrobial properties

The microbial test preformed on the samples was an *adapted* disk diffusion test. The *genuine* disk diffusion test is a qualitative clinical procedure used to determine the susceptibility of bacteria to a specific antibiotic. The genuine test is preformed by inoculating a Mueller Hinton agar Petri dish with an aliquot of bacteria and placing a standardized sized disk soaked in a specific antibiotic on the agar. The antibiotic diffuses out of the disk preventing the bacteria from growing in a halo like area around the disk. The size of this halo is dependent on several factors; including how susceptible the bacteria is to the antibiotic, how well the antibiotic diffuses through the agar, whether the antibiotic reacts with the agar, and how well the bacteria grows on the agar. In a clinical setting the test results are compared to a set of standards either developed in lab or provided by the Clinical and Laboratory Standards Institute (CLSI) – and a dichotomous result is given: susceptible or resistant.

While CLSI publishes the standards for a wide range of antibiotics, they have yet (as of 2008) to published results for any test involving silver compounds. But, the test remains widely used in the literature to examine the efficacy of silver compounds, although frequently interpreted to offer more qualitative results. Several researchers have raised concerns, with Falcone and Spadaro stating that the inhibition zone from silver materials "...are complex and appear to be self-limited in size due to diffusion and competing binding process." [13] Silver ions react readily with the agar, so much so that in the case of silver nitrate loaded BC, the agar beneath the cellulose sample turns white with what is assumed to be silver chloride. With other silver species no noticeable change in the colour of the agar is observed, but it is highly likely these competing

binding processes are still at work. Additionally, the clear zone is established (although not observable) within the first 3 to 6 hours after placing the sample on the dish [109]. In this short time, silver nanoparticles would diffuse to a lesser extent than smaller antibiotics, creating a clearzone that is comparatively smaller.

Modifying the test has mitigated some of these problems. First the inclusion of an Ag-NPs 'standard' dressing, in the form of Acticoat, was used. This reduces the requirement for a comparison to the CLSI standards, as Acticoat becomes the measuring stick, having a history as an effective silver dressing. As discussed earlier, an examination of Acticoat indicates that the Ag-NPs are of approximately the same nature as those present in the BC prepared sample. And while there is little to be done about the competing binding processes that Falcone and Spadaro alluded to, these will at least be equal in both cases. Additionally, to offset the limitations of the test, it is expanded to a multiday test by following the procedure established by White et al., and instead of drawing conclusions only on the comparative efficacy of the material, information on the longevity of the dressing can be discerned. In a 2005 paper by Callant-Behm et al., published explicitly as an indepth analysis of the disk diffusion test versus a kill-kinetic test for silver dressings, concludes that the White et al. method provides "…useful data as to dressing longevity…" [130]

4.5.1 – Disk diffusion test - Biofill

Table 4.3: Clear zone size - Biofill prepared samples.

Sample	Clear Zone: E. coli	Clear Zone: S. Aureus
15 minute oxidized	0 mm	.5 – 1.5 mm
30 minute oxidized	0 mm	.5 – 1.5 mm
60 minute oxidized	0 mm	.5 – 1.5 mm
Acticoat	1 – 2 mm	1.5 – 2.5 mm

The clearzone sizes are listed in Table 4.3. One should refer to the Methods section to view how these were determined; the procedure is comparable to those published in the scientific literature, but differs from that established by the Clinical and Laboratory Standards Institute (CLSI). The numbers are presented as a range in part to indicate the qualitative nature of the test, as opposed to a more quantitative test. Additionally, it should be noted that while the *E. coli* and *S. aureus* results are displayed in the same table they should not be compared, only numbers within a column should be compared. This is due to the inclination of *E. coli* to Mueller Hington agar (the standard agar used for this test). In other words, *E. coli* grows better that *S. aureus* on Mueller Hington agar, making the clear zones smaller, or in this case non-existent.

Table 4.4: Days of observable clear zone - Biofill

Sample	Days of observable clear zone (*S. aureus*)
15 minute oxidized	1 day
30 minute oxidized	1 day
60 minute oxidized	1 day
Acticoat	6 days

Table 4.4 shows the longevity of the samples. Both tables show that there is little difference between the efficacies and the longevities of the different oxidation levels.

This leads to the conclusion that longevity is based on silver release and is independent of silver loading. The longevity was also much less than Acticoat was the watershed result that eventually led to the revaluation of initial substrate and following that, the use of wet pellicle.

4.5.2 - Disk diffusion test – wet pellicle

The antimicrobial tests conducted on the lab-made cellulose are modified clear-zone tests, the same as those conducted on the Biofill prepared samples. The results were significantly better than the Biofill. One can see in Table 4.5 that the clear zones are significantly larger than those seen with the Biofill samples. The *S. aureus* clear zones are larger than those of the *E. coli*, due to the fact that *S. aureus* does not grow as well as the *E. coli* on the agar used. As discussed in the last section, more silver is present and more silver is released in lab-made, wet pellicle samples than the Biofill prepared samples, this directly results in the larger clear zone.

Like before, the results again are given as a range, indicating the uncertainty in measuring the clearzone size. While some slight variation in the *S. aureus* measurements, the conclusion is that all sample preparations have essentially similar efficacy. This corresponded to the release kinetics, which show antimicrobial efficacy is dependant on silver release, and not silver loading – this is not a totally unexpected result.

Table 4.5: Clear zone sizes – lab-made pellicle samples.

Sample	Clear Zone: *E. coli*	Clear Zone: *S. aureus*
.5 $NaIO_4$: 1 BC	1 – 2 mm	1.5 – 2.5 mm
1 $NaIO_4$: 1 BC	1 – 2 mm	1.5 – 2.5 mm
1.5 $NaIO_4$: 1 BC	1 – 2 mm	1 – 2.5 mm
2 $NaIO_4$: 1 BC	1 – 2 mm	1 – 2 mm
Acticoat	1 – 2 mm	1.5 – 2.5 mm

The longevity of the sample is displayed in Table 4.6. But, once again there was little difference between oxidation levels, suggesting that like antimicrobial efficacy and longevity is dependant on silver release.

The results do show that samples remain active for five days having a similar longevity as Acticoat. If we compare these results to the area density of silver (Figure 4.18) we see that these results are accomplished about one third of silver per square centimeter as Acticoat, as the case with wet pellicle cellulose oxidized with a ratio .5:1 (oxidizer to cellulose). This initial loading difference in silver loading does not necessarily translate into a difference in dose, with the total dose per cm^2 of Acticoat falling within the range of total doses for the wet pellicle samples. The pellicle sample has the added advantage of a closer adhesion of the material to the agar, which would translate into the real world application with a closer adhesion to the wound bed.

Table 4.6: Days of observable clear zone – lab-made pellicle samples

Sample	Days of observable of clearzone: *E. coli*	Days of observable clearzone: *S. aureus*
.5 $NaIO_4$: 1 BC	5 days	5 days
1 $NaIO_4$: 1 BC	5 days	5 days
1.5 $NaIO_4$: 1 BC	5 days	5 days
2 $NaIO_4$: 1 BC	5 days	5 days
Acticoat	5 days	6 days

4.6 – Alternatives

The results lead to the conclusion that the initial premise of binding silver to the BC substrate maybe unnecessary, and should be reevaluated. An alternative is the creation Ag-NPs in the interior mesh like structure of the wet pellicle. There are several manners in which to create silver nanoparticles and several of these were explored. These included using silver nitrate and an autoclave or the reducing agent sodium borohydride. More information can be found on these in Appendix A. While there is substantially more work to be done on these systems to appropriately evaluate their efficacy, the groundwork has been laid.

Chapter 5 – Conclusion and Future Design Considerations

The research outlined in this thesis explores variations on one approach of combining silver and bacterial cellulose, for the production of an antimicrobial material that can be used as a biomedical biomaterial. The process yields a novel material, with nanocrystalline silver bound to the surface. It was expected that the bounded Ag-NPs would release silver in a bioactive form and provide an antimicrobial effect, while this may have occurred it did not do so in distinguishable levels. Instead unbound silver caught in the BC mesh was released and created the antimicrobial effect. This leads to two possible thought pathways: one redesign the material to focus on unbound Ag-NPs, several methods are described in Appendix A for doing this; or examine the effectiveness of the material in such settings where the release of silver is not required. Surgical gloves would be a good example of a device where bound silver could potentially prevent bacteria from sitting on the surface of the glove, decreasing the chance of spreading infection. The effectiveness of non-released or non-releasable silver preventing the buildup of bacterial is not established in the literature, and is suggested here merely as a hypothesis.

One consideration in the development of this biomaterial is the type of BC used. Two types were examined in this thesis, Biofill and lab-made wet cellulose (reacted wet, used dry); a third exists. Guhados originally developed the silver loading process on loose BC fibers. The fibers were produced in shaken and agitated cultures, methods that

have several distinct advantages and disadvantages. One advantage is the potential for scale up, but as stated in the Literature Review this promise has yet to be realized. Another advantage is the direct use of loose fibers in nanocomposites, with the corresponding disadvantage being to use the loose fibers for a wound dressing they would need to be cast in a film. The main disadvantage is the added complexity in production – there is difficulty with pellicle to ensure all reagents are rinsed from the cellulose after each step – this difficulty is multiplied with the use of loose fibers. Loose fibers must be washed in a centrifuge; it would be a time consuming and difficult task to ensure all the reagents were rinsed from the BC. Not all of the color is removed from the cellulose after even a dozen washes, suggesting at least some contaminants remain; purity is an important characteristic for biomedical materials. This leads to the conclusion BC should be produced in pellicle form, except when used in a composite. The data presented in the thesis also leads to the conclusion that the silver loading should be conducted on wet 3D pellicle instead of dry pellicle.

The commercial future of a material based on the three step process explored in this thesis is questionable. Several criteria must be examined before proceeding towards a path of commercialization. First, the cost of silver proteinate is prohibitive. Second, designing a scaled production line would be difficult, especially with rinsing between steps. Third, biocompatibility test need to be conducted; the outcome of which are not assured. While BC is biocompatible, the biocompatibility of the other constituents are either not known or not good. Silver proteinate has had a medical role in the past, and some grades have been sold under the brand Argerol for medical use.

Thiosemicarbazide is both allergenic and toxic, with an oral dosage for a rat of 9 mg/kg, with a specific warning against irritation to skin; whether binding it to BC mitigates this toxicity is unknown. Also, if there is any remaining ammoniacal silver, this too could cause biocompatibility issues.

Some aspects of the design still need to be examined. As mentioned during the discussion, the oxidation process weakens the material; the ideal strength and flexibility of the material depends on the application, but should be qualitatively studied. Additionally, one of the important benefits of BC for a wound dressing application is its transparency; this is compromised by silver loading. Where the ideal balance between silver loading and transparency lies needs to be determined and the oxidation level adjusted to optimize for that criteria. Another feature not tested for was the potential for staining of the skin and wound tissue, any material that leave silver behind will create localized discoloration. In other words, staining should be expected.

Works Cited

[1] W Czaja, A Krystynowica, S Bielecki, and R M Brown, "Microbial cellulose - the natural power to heal wounds," *Biomaterials*, vol. 27, no. 2, pp. 145-51, Jan 2006.

[2] L E Millon, G Guhados, and W Wan, "Anisotropic polyvinyl alcohol-Bacterial cellulose nanocomposite for biomedical applications," *J Biomed Mater Res B Appl Biomater*, vol. 86B, no. 2, pp. 444-52, August 2008.

[3] A Bodin, H Backdahl, L Gustafsson, B Risberg, and P Gatenholm, "Influence of cultivation conitions on mechanical and morphological properties of bacterial cellulose tubes," *Biotechnol bioeng*, vol. 97, no. 2, pp. 425-434, June 2007.

[4] P L Taylor, A L Ussher, and R E Burrell, "Impact of heat on nanocrystalline silver dressings. Part I: Chemical and biological properties," *Biomaterials*, vol. 26, no. 35, pp. 7221-9, Dec 2005.

[5] D E Zoutman et al., "The state of infection surveillance and control in Canadian acute care hospitals," *American Journal of Infection Control*, vol. 31, no. 5, pp. 266-72, Aug 2003.

[6] W R Jarvis, "Selected aspects of the socieconomic impact of nosocomial infections: morbidity, mortality, cost, and prevention.," *Infection Conrol Hosp Epidemiology*, vol. 17, no. 8, pp. 552-7, Aug 1996.

[7] X Chen and H J Schluesener, "Nanosilver: a nanoproduct in medical application," *Toxicol Lett.* , vol. 176, no. 1, pp. 1-12, Jan 2008.

[8] Simon Silver, Le T Phung, and Gregg Silver, "Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds," *J Ind Microbial biotechnol*, vol. 33, pp. 627-624, May 2006.

[9] H Klasen, "Historical review of the use of silver in the treatment of burns. I. Early uses/," *Burns.*, vol. 26, no. 2, pp. 117-130, March 2000.

[10] C A Moyer, L Brentano, D L Gravens, H W Margraf, and W W Monafo, "Treatment of large burns with 0.5 percent silver nitrate solution," *Arch Surg*, vol. 90, pp. 812-67, June 1965.

[11] H J Klasen, "A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver," *Burns*, vol. 26, no. 2, pp. 131-138, June 2000.

[12] Kwan Kew Lai and Sally A Fontecchio, "Use of silver-hydrogel urinary catheters on the incidence of catheter-associated urinary tract infection in hospitalized patients," *American Journal of Infection Control*, vol. 30, no. 4, pp. 221-225, June 2002.

[13] Alfred E Falconoe and Joseph A Spadaro, "Inhibitory Effects of Electrically Activated Silver Material on Cunaneous Wound Bacteria," *Plastic and Reconstructive Surgery*, vol. 77, no. 3, p. 455, March 1986.

[14] J R Morones et al., "The bactericidal effect of silver nanoparticles.," *Nanotechnolgy*, vol. 16, pp. 2346-2353, 2005.

[15] P L Taylor, O Omotoso, J B Wiskel, D Mitlin, and R E Burrell, "Impact of heat on nanocrystalline silver dressings. Part II: Physical properties," *Biomaterials*, vol. 26, no. 35, pp. 7230-40, Dec 2005.

[16] A J Schaeffer, K O Story, and S M Johnson, "Effect of silver oxide/trichloroisocyanuric acid antimicrobial urinary drainage system on catheter-associated bacteriuria," *Journal of Urology*, vol. 129, no. 1, pp. 69-73, Jan 1988.

[17] S S Djokic and R E Burrell, "Behavior of Silver in Physiological Solutions," *J. Electrochem. Soc.*, vol. 145, no. 5, p. 1426, May 1998.

[18] V Alt et al., "An in vitro assessment of the antibacterial properties and cytotoxicity of nanoparticulare silver bone cement.," *Biomaterials*, vol. 25, no. 18, pp. 4383-91, Aug 2004.

[19] Pensel Juergen and Ulrich Sander, "Surgical Microscope," *United States Patent 20060034423*, 2006.

[20] Y Li, P Leung, L Yao, Q Song, and E Newton, "Antimicrobial effect of surgical masks coated with nanoparticles," *Journal of Hospital Infection*, vol. 62, pp. 58-62, 2006.

[21] Bishara S Atiyeh, Michel Costagliola, Shady N Hayek, and Saad A Dido, "Effect of silver on burn wound infection control and healing: Review of the literature," *Burns*, vol. 33, no. 2, pp. 139-148, March 2007.

[22] Sukdeb Pal, Yu Tak, and Joon Song, "Does the antibacterial activity of silver annoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Esherichia coli," *Applied and Environemtal Microbiology*, vol. 73, no. 6, pp. 1712-1720, March 2007.

[23] W J Schreurs and H Rosenberg, "The effect of silver ions on transport and retention of phyosphate by Esherichia coli," *Journal of Bacteriology*, vol. 152, pp. 7-13, 1982.

[24] P Dibrov, J Dzioba, K K Gosink, and C C Hase, "Chemiosmotic mechanism of antimicrobial activity of Ag+ in Vibrio cholerae," *Antimcirob Agents Chemother*, vol. 46, no. 8, pp. 2668-2670, August 2002.

[25] S Y Liau and D C Read, "Interaction of silver nitrate with readily identifiable groups: Relationship to the antibacterial action of silver ions," *Lett Appl Microbiol*, vol. 25, pp. 279-283, 1997.

[26] Ivan Sondi and Branka Salopek-sondi, "Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria," *Journal of Colloid and Interface Science*, vol. 275, pp. 177-182, 2004.

[27] T Tokumaru, Y Shimizu, and C L Fox, "Antiviral activites of silver sulfadiazine in ocular infection," *Res Commun Chem Pathol Pharmacol*, vol. 8, no. 1, pp. 151-8, May 1974.

[28] Hiroaki Oka, Toshikazu Tomioka, Katsumi Tomita, Atsushi Nishino, and Shigeharu Ueda, "Inactivation of enveloped viruses by silver thiosulfate complex," in *Metal based drugs.*, 1994, ch. 5-6, p. 511.

[29] M A Butkus, M Talbot, and M P Labare, "Feasibility of the silver-UV process for drinking water disinfection.," *Water Res.*, vol. 39, no. 20, pp. 4925-32, December 2005.

[30] J L Elechiguerra et al., "Interaction of silver nanoparticles with HIV-1," *Journal of Nanotechnology*, vol. 3, no. 6, pp. 1-10, June 2005.

[31] Raymond Wai-Yin et al., "Silver nanoparticles fabricated in Hepes buffer exhibit cytoprotective activites toward HIV-1 infected cells," *ChemComm*, pp. 5059-5061, 2005.

[32] J Barry Wright, Kan Lam, Andre G Buret, Merle E Olson, and Robert E Burrell, "Early healing events in a porcine model of contaminated wounds: effects of nanocrystalline silver on matrix metalloproeinases, call apoptosis, and healing," *Wound Repair and Regeneration*, vol. 10, no. 3, p. 141, May-June 2002.

[33] Seung-Heon Shin, Mi-Kyung Ye, Hae-Sic Kim, and Hyung-Suk Kang, "The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells," *Intarional Immunopharmacology*, vol. 7, pp. 1813-1818, August 2007.

[34] P L Nadworny, J Wang, E E Tredget, and R E Burrell, "Anti-inflammatory activity of nanocrystalline silver in a porcine contact dermatitis model," *Nanomedicine*, p. epub, June 2008.

[35] M E Olson, J B Wright, K Lam, and R E Burrell, "Healing of porcine donor sites covered with silver-coated dressings.," *Eur J Surg*, vol. 166, no. 6, pp. 486-9, Jun 2000.

[36] M E Innes, N Umraw, J S Fish, M Gomez, and R C Carotto, "The use of silver coated dressings on donor site wounds: a prospective controlled matched pair study," *Burns*, vol. 27, no. 6, pp. 621-627, September 2001.

[37] S Honari, M S Gibran, L H Engrav, A R Carlson, and D M Heibach, "Clinical benefits and cost effectiveness of Acticoat as a dressing for donor sites," *Journal of Burn Care Rehabitation*, vol. 22, p. S74, 2001.

[38] Evangelia Vlachou et al., "The sfaety of nanocrystalline silver dressing on burns: a study of systemic silver absorption," *Burns*, vol. 33, pp. 979-985, 2007.

[39] Ken Dunn and Val Edwards-Jones, "The role of Acticoat with nanocrystaline silver in the management of burns.," *Burns*, pp. S1-S9, 2004.

[40] Kensuke Akamatsu and Shigehito Deki, "NAnoscale metal particles dispersed in polymer matrix," *Nanostructured Materials*, vol. 8, no. 8, pp. 1121-1129, December 1997.

[41] Del Nobile et al., "Effect of ag-containing Nano-composite Active packaging system on surivial of Alicylobacillus acidoterrestris," *Food Engineering and Physical Properties*, vol. 69, no. 8, pp. E379-383, 2004.

[42] S B Sant, K S Gill, and R E Burrell, "Nanostructure, dissolution and morphology characteristics of micocidal silver films depostied by magnetron sputtering," *Actabiomaterialia*, 2007.

[43] A L Stepanov and V N Popok, "Effect of the ion beam current density on the formation of implanted metal nanoparticles in a dielectric matrix," *Technical Physicis Letters*, vol. 29, no. 12, pp. 20-25, 2003.

[44] A L Stepanov, V N Popok, and D E Hole, "Formation of Metallic Nanoparticles in Silivate Glass," *Glass Physics and Chemistry*, vol. 28, no. 2, pp. 90-95, March 2002.

[45] A L Stepanov, V N Popok, I B Khaibullin, and U Kreibig, "Optical properties of

polymethylmethacrilate with implanted silver nanoparticles," *Nuclear Instruments and methods in physics research*, vol. 191, no. b, pp. 473-477, 2002.

[46] J Davenas, P Thevenard, F Philippe, and M N Arnaud, "Surface implantation treatments to prevent infection complications in short term devices," *Biomolecular Engineering*, vol. 19, no. 2, pp. 263-268, August 2002.

[47] George Lisensky. (2008, July) Synthesis of Colloidal Silver. [Online]. http://mrsec.wisc.edu/Edetc/nanolab/silver/index.html

[48] Pal Sukdeb, Yu Kyung Tak, and Joon Song, "Does tha antibacterial activity of silver nanoparticles depend on the shape of the nanoparticles? A study of the gram-negative bacterium Echerichia coli," *Applied and Environemtnal Microbiology*, vol. 73, no. 6, pp. 1712-1720, March 2007.

[49] Chun-Nam Lok et al., "Silver nanoparticles: partical oxidation and antibacterial activities," *J Biol Inorg Chem*, vol. 534, p. 527, 2007.

[50] Poovathinthodiyil Raveendran, Jie Fu, and Scott L Wallen, "Completely "Green" Synthesis and Stabilization of Metal Nanoparticles," *Journal of the American Chemical Society*, vol. 125, no. 46, pp. 13940-13941, October 2003.

[51] S Clemenson, D Leonard, D Sage, L David, and E Espuche, "Metal nanocompostie films prepared in situ from PVA and silver nitrate. Study of the nanostructuration process and morphology as a function of the in situ routes," *Journal of Polymer Science Part A: Polymer Chemistry*, vol. 46, no. 6, pp. 2062-2071, 2008.

[52] R Naik, S Stringer, G Agarwal, S Jones, and M Stone, "Biomimetic synthesis and patterning of silver nanoparticles," *Nature Materials*, vol. 1, no. 3, p. 169, 2002.

[53] J Zhu, S Liu, O Palchik, Y Koltypin, and A Gedanken, "Shape-controlled synthesis of silver nanoparticles by pulse sonoelectrochemical methods," *Langmuir*, vol. 16, no. 16, pp. 6396-9399, 2000.

[54] Palle Homstrup, "Reactions of the oral mucosa related to silver amalgam: a review," *Journal of Oral Pathology & Medicine*, vol. 20, no. 1, pp. 1-7, January 1991.

[55] Marji Trop et al., "Silver-coated dressing acticaot caused raised liver enzymes and argyris-like symptoms in burn patient," *The Journal of Trauma, Injury, Infection and Critical Care*, pp. 648-652, 2006.

[56] A Parkes, "Silver-coated dressing Acticoat," *J. Trauma*, vol. 61, no. 1, pp. 239-40, July 2006.

[57] Smith & Nephew. (2007, October) Acticoat FAQ. [Online]. http://global.smith-nephew.com/us/ACTICOAT_FAQS_8928.htm#17432

[58] Richard White and Keith Cutting, "Exploring the effects of silver in wound management - what is optimal?," *Wounds*, p. http://www.medscape.com/viewarticle/549223, March 2007.

[59] S Maitre, K Jaber, J L Perrot, C Guy, and F Cambazard, "Increased serum and urinary levels of silver during treatment with topical silver sulfadiazine," *Ann Dermatol Venereol*, vol. 129, no. 2, pp. 217-219, 2002.

[60] G Chaby et al., "Topical silver sulfadiazine0induced acute renal failure," *Ann*

Dermatol Venereol, vol. 11, no. 1, pp. 891-3, November 2005.

[61] H M Harrison, "Pharmacology of sulfadiazine silver. Its attachment to burned human and rat skin and studies of gastronintestinal absorption and extension," *Archive of surgery* , vol. 114, no. 3, March 1979.

[62] R L McCavley and H A Linares, "In vitro toxicity of topical antimcirbial agents to human fibroblasts," *Journal of Surgical Res.*, vol. 46, pp. 267-74, 1989.

[63] J Lademann et al., "Penetration of titanium dioxide microparticles in a sunscreen formulation into the horny layer and the follicular orifice," *Skin Pharmacol Appl Skin Physiol*, vol. 12, no. 5, pp. 247-56, September 1999.

[65] S Arora, J Jain, J Rajwade, and K Paknikar, "Cellular respnoses indeuced by silver nanoparticles: in vitro studies," *Toxicology Letters*, vol. 179, pp. 93-100, 2008.

[66] Yong Soon Kim et al., "Twenty-Eight-Day ORal Toxicity, Genotoxicity, and Gender-Related Tissue Distibution of Silver Nanoparticles in Sprague-Dawley Rats," *Inhalation Toxicology*, vol. 20, no. 6, pp. 575-583, April 2008.

[67] A Ionescu et al., "Incidence of embolism and paravalvar leak after St Jude Silzone valve implatation: experience from the Cardiff Embolic Risk Factor Study.," *Heart*, vol. 89, pp. 1055-61, 2003.

[68] Dieter Horstkotte, "Thrombogenicity of the St. Jude Medical Prosthesis with and without silzone-Coated Sewing cuffs," *Ann Thorac Surg*, vol. 71, pp. 1065-73, 2001.

[69] Victor Davila-Roman et al., "Prevalence and Severity of paravalular regurgitation in the artifical valve endocarditis reduction trial (AVERT) echocardiography study," *Infectios Endocarditis*, vol. 44, no. 7, pp. 1467-72, 2004.

[70] Dieter Klemm, Brigitte Heublein, Hans-Peter Fink, and Andreas Bohn, "Cellulose: Fascinating biopolymer and sustainable raw material," vol. 44, no. 22, pp. 3358-3393, 2005.

[71] P Ross, R Mayer, and M Benziman, "Cellulose biosynthesis and function in bacteria," *Microbiol Mol Biol Rev*, vol. 55, no. 1, pp. 35-58, March 1991.

[72] A J Brown, "On an acetic ferment which forms cellulose," *Journal of the Chemical Society*, vol. 41, 1886.

[73] Donald C Johnson, Amar N Neogi, and Henry A Leblanc, "Bacterial cellulose as surface treatment for fibrous," United States Patent 4861427, March 10, 1988.

[74] Glenn Penny and Deborah Lenk, "Drilling Mud Compositions," European Patent EP0588879, June 05, 1992.

[75] Y Nishi et al., "The structure and mechanical properties of sheets prepard from bacterial cellulose," *Journal of Material Science*, vol. 25, no. 6, pp. 2997-3001, 1990.

[76] Tomoyuki Yoshino, Tomoko Asakura, and Kiyoshi Toda, "Cellulose production by Acetobacter pasteurianus on silicone membrane," *Journal of Fermentation and Bioengineering*, vol. 81, no. 1, pp. 32-36, 1996.

[77] Scott Williams and Robert Cannon, "Alternative Environmental Roles for Cellulose Produced by Acetobacter xylinum," *Applied Environmental Microbiology*, vol. 55, pp. 2448-2452, October 1989.

[78] R M Brown,.: Ellis Horwood, 1989, pp. 145-252.

[79] S Kuga and R M Brown, "Silver labeling of the reducing ends of bacterial cellulose," *Carbohydrate Research*, vol. 180, no. 2, pp. 345-350, 1988.

[80] S Yamanaka et al., "The strucutre and mechanical properties of sheets prepared from bacterial cellulose," *journal of Materials Science*, vol. 24, no. 9, pp. 3141-3145, September 1989.

[81] Kouda Tohru, Takaaki Naritomi, Hisato Yano, and Fumihiro Yoshinaga, "Inhibitory effect of carbon dioxide on bacterial cellulose producion by acetobacter in agitated cultue," *Journal of Fermentation and Bioengineering*, vol. 85, no. 3, pp. 318-321, 1998.

[82] Kouda Tohru, Hisato Yano, and Fumihiro Yoshinaga, "Effect of agitator configuration on bacterial cellulose producivity in aerated and agitated culture," *Journal of Fermentation and Bioengineering*, vol. 83, no. 4, pp. 371-376, 1997.

[83] N Sakairi, H Asano, M Ogawa, N Nishi, and S Tokura, "A method for direct harvest of bacterial cellulose filaments during continuous cultivation of Acetobacter xylinum," *Carbohydrate Polymers*, vol. 35, no. 3-4, pp. 233-237, 1998.

[84] m Lapuz, E Gallardo, and M Palo, "The Nata Organism - Cultural Requirements, Characteristics, and Identity," *The Philippine Journal of Science*, vol. 96, pp. 91-109, 1967.

[85] A Krystynowicz et al., "Factors affecting the yield and properties of bacterial cellulose," *Journal of Industrial Microbiology & Biotechnology*, vol. 29, pp. 189-195, 2002.

[86] S Hestrin and M Schramm, "Synthesis of cellulose by Acetobacter xylinum. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose," *Biochem J*, vol. 58, no. 2, pp. 345-352, October 1954.

[87] Sherif Keshk, Taha Rasek, and Kazuhiko Sameshima, "Bacterial Cellulose Production from Beet Molasses," *African Journal of Biotechnology*, vol. 5, no. 17, pp. 1519-1523, September 2006.

[88] S Bae, Y Sugano, and M Shoda, "Improvement of bacterial cellulose production by addition of agar in a jar fermentor," *J Biosci Bioeng*, vol. 97, no. 1, pp. 33-38, 2004.

[89] Gerard Joseph, Gerald Rowe, Argyrios Margaraitis, and Wankei Wan, "Effects of polyacrylamide-co-acrylic acid on cellulose production by Acetobacter xylinum," *Journal of Chemical Technology and Biotechnology*, vol. 78, no. 9, pp. 964-970, September 2003.

[90] L L Zhou, D P Sun, L Y Hu, Y W Li, and J Z Yang, "Effect of addition of sodium alginate on bacterial cellulose production by Acetobacter xylium," *Journal of Industrial Microbiology and Biotechnology*, vol. 34, no. 7, pp. 483-489, April 2007.

[91] S Yunoki, Y Osada, H Kono, and M Takai, *Food Science and Technology*, vol. 10, no. 3, pp. 307-313, 2005.

[92] H Son, M Heo, Y Kim, and S Lee, *Biotechnology Applied Biochemistry*, vol. 33,

pp. 1-5, 2001.

[93] Jung Hwang, Young Yang, Jae Hwang, Yu Pyun, and Yu Kim, "Effects of pH and dissolved oxygen on cellulose production by Acetobacter xylinum BRC5 in agitated culture," *Journal of bioscience and bioengineering*, vol. 88, no. 2, pp. 183-188, 1999.

[94] A Set et al., "Effective cellulose production by a coculture of Gluconacetibacter xylinus and lactobacillus mali," *Applied Microbial and cell physiology*, vol. 73, pp. 915-921, 2006.

[95] S Westaby, *Wound care*, Westaby, Ed. St. Louis: Mosby, 1986.

[96] Gisela Helenius et al., "In vivo biocompatibility of bacterial cellulose," *Journal of Biomedical Material Research: Part A*, vol. 76A, no. 2, pp. 431-438, 2005.

[97] Oscar M Alevarez, Maynak Patel, Juanita Booker, and Lee Markowitz, "Dressing for the treatment of chronic venous leg ulcers: Results of a single center randomized study involving 24 patients," *Wound*, vol. 16, no. 7, pp. 224-223, September 2004.

[98] V I Legeza et al., "Effects of new wound dressings on healing of thermal burns of the skin in acute radiation disease," *Bull Exp Biol Med*, vol. 138, no. 3, pp. 311-5, Sep 2004.

[99] David Ring, Wilson Nashed, and Thurman Dow, "Microbial polysaccharide articles and methods of production," *United States Patent 4655758*, 1986.

[100] David Ring, Wilson Nashed, and Thurman Dow, "Liquid loaded pad for medical applications," *United States Patent 4588400*, 1986.

[101] G Serafica, C Damien, F Wright, and H Beam, "Implantable microbial cellulose materials for various medical applications," Us2007/0128243 A1, June 7, 2007.

[102] L Farah, "Process for the preparation of cellulose film, cellulose film produced thereby, artifical skin graft and its use," 4912049, March 27, 1990.

[103] N Sachavanakit et al., "Growth of human keratinocytes and fibroblasts on bacterial cellulose film," *Biotechnology Prog.*, vol. 22, no. 4, p. 1194, Jul-Aug 2006.

[104] A Svensson et al., "Bacterial cellulose as a potential scaffold for tissue engineering of cartilage," *Biomaterials*, vol. 26, no. 4, pp. 419-31, Feb 2005.

[105] L E Millon and W K Wan, "The polyvinyl alcohol-bacterial cellulose system as a new nanocomposite for biomedical application," *J Biomed Mater Res B App Biomate*, vol. 79, no. 2, pp. 245-53, November 2006.

[106] L R Mello, L T Feltrin, P T Fonter, and F A Ferraz, "Duraplasty with biosynethsis cellulose: an experimental study.," *J Neruosurg*, vol. 86, pp. 143-50, 1997.

[107] Ganesh Guhados, *Studies of nanomaterials - bacterial cellulose and multiwalled carbon nanotubes*. London, Ontario: Faculty of Graduate Studies, 2005.

[108] Klaus Pommerening, Horst Rein, Dieter Bertram, and Reinhard Muller, "Estimation of dialdehyde groups in 2,3-dialdehyde bead-cellulose," *Carbohydrate Research*, vol. 233, pp. 219-223, 1992.

[109] Audrey Wanger, "Disk Diffusion Test and Gradient Methodologies," in *Antimicrobial Susceptibility Testing Protocols*, Richard Schalbe, Lynn Steele-Moore, and Avery C Goodwin, Eds. Boca Raton: CRC Press, 2007, p. 56.

[110] Stanislaw Bielecki, Alina Krystynowica, Marianna Turkiewicz, and Halina Kalinowska, "Bacterial Cellulose," in *Biopolymers*. New York: Weinheim, 2001, ch. 3, pp. 37-46.

[111] R E Cannon and S M Anderson, "Biogenesis of Bacterial Cellulose," *Critical Reviews in Microbiology*, vol. 17, no. 6, pp. 435-447, 1991.

[112] M Hornung, M Ludwig, A M Gerrard, and H P Schmauder, "Optimizing the Production of Bacterial Cellulose in Surface Culture: Evaluation of Substrate Mass Transfer Influences on the Bioreaction (Part 1)," *Eng. Life. Sci.*, vol. 6, no. 6, pp. 537-545, May 2006.

[113] S Vicini et al., "Thermal analysis and characterisation of cellulose oxidized with sodium methaperiodate," *Thermochimica Acta*, vol. 418, no. 1-2, pp. 123-130, March 2004.

[114] U J Kim, M Wada, and S Kuga, "Solubilization of dialdehyde cellulose by hot water," *Carbohydrate Polymers*, vol. 56, pp. 7-10, October 2004.

[115] E Maekawa and T Koshijima, "Properties of 2,3-dicarboxy cellulose combined with various metallic ions," *Journal of applied polymer sicence*, vol. 29, no. 7, p. 2289, 1984.

[116] P RoyChowdhury and V Kumar, "Fabrication and evaluation of porous 2,3-dialdehydecellulose membrane as a portential biodegradable tissue-engineering scaffold," *Journal of biomedical materials research part a*, vol. 76A, no. 2, pp. 300-309, 2005.

[117] Corradi Bonamartini, Fava Gasparri, Ferrari Belicchi, and M Nardelli, "Ligand Configuration and Coordination Geometry in Silver(I)-Thiosemicarbazide Complexes," *Acta. Cryst.*, vol. C43, p. 407, 1987.

[118] Tetsuo Koshijima, Ryutaro Tanaka, Einosuke Muraki, Akibumi Yamada, and Fumiko Yaku, "Chelating polymers derived from cellulose and chitin," *Cellulose chemistry and technology*, vol. 7, pp. 197-208, August 1972.

[119] Hayat MA, *Stains and Cytochemical Methods*, 348th ed.: Springer, 1993.

[120] A M Glauert, B E Beeston, and W C Bigelow, *Practical Methods in Electron Microscopy*. Princeton, NJ: Princeton University Press, 1972.

[121] E Gantt, *Handbook of Phycological Methods.*: Cambridge University Press of America, 1980.

[122] W A Anderson and P Personne, "The localization of glycogen in the spermatozoa of verious invertebrate and vertebrate species," *The journal of Cell Biology*, vol. 44, no. 1, p. 29, 1970.

[123] J Friden, J Seger, and B Ekblom, "Implementation of periodic acid theiocemicarbazide-silver proteinate staining for the ultrastrutural," *Cell and Tissue Research*, vol. 242, no. 1, p. 229, 1985.

[124] Shigenori Kuga and Malcolm Brown, "Silver labeling of the reducing ends of bacterial cellulose," *Carbohydrate Research*, vol. 180, pp. 345-350, 1988.

[125] F F Fan and A J Bard, "Chemical, eletrochemical, gravimetric, and microscopic studies on antimicrobial silver films," *J Phys Chem B*, vol. 106, pp. 279-287, 2002.

[126] Jay S Hoskins, Tanju Karanfil, and Steven M Serkiz, "Removal and Sequestration of Iodide Using Silver-Impregneated Activated Carbon," *Environ. Sci. Technol.*, vol. 36, no. 4, pp. 784-789, Janurary 2002.

[127] Joy Elizabeth Gray, *The electroless deposition of silver on a biomedical polyurehtane: interfacial interactions, adhesion enhancement, and biological efficacy*. London, Canada: University of Western Ontario, 2000.

[128] Y Okazaki and E Gotoh, "Comparison of metal release from various metallic biomaterial in vitro," *Biomaterials*, vol. 26, no. 1, p. 11, 2005.

[129] J Jones, L Ehrenfried, P Saravanapavan, and L Hench, "Controlling ion release from bioactive glass foam scaffolds with antibacterial properties," *journal of Materials Science: Materials in Medicine*, vol. 17, no. 11, p. 989, November 2006.

[130] Corrie L Callant-Behm et al., "Comparison of in vitro disk diffusion and time kill-kinetics assays for the evaluation of antimicrobial wound dressing efficacy," *Wound Repair and Regeneration*, vol. 13, no. 4, pp. 412-421, 2005.

[131] N Vigneshwaran, A A Kathe, P V Varadarajan, R P Nachane, and R H Balasubramanya, "Functional Finishing of Cotton Fabrics Using Silver Nanoparticles," *Journal of Nanoscience and Nanotechnolgy*, vol. 7, no. 6, pp. 1893-1897, 2007.

[132] V N Popok, A L Stephanov, and V B Odzhaev, "Synthesis of Silver Nanoparticles by the Ion Implantation Method and Investigation of thei Optical Properties," *Journal of Applied Spectroscopy*, vol. 72, no. 2, pp. 229-234, March 2005.

[133] T Maneerung, S Tokura, and R Rujiravanit, "Impregnation of silver nanoparticles into bacterial cellulose for antimicrobial wound dressing," *Carbohydrate polymers*, vol. 72, no. 1, pp. 43-51, April 2008.

[134] W R Jarvis, "Selected aspects of the socioeconomic impact of nosocomial infections: morbidity, cost, and prevention.," *Infect Control Hosp Epidemiol*, vol. 17, no. 8, pp. 552-7, Aug 1996.

[135] W Standford and R W Rappole, "Clincial experience with silversulphadiazine," *J Trauma*, vol. 96, pp. 184-188, 1968.

[136] Shigenori Kuga and Malcolm Brown, "Silver labeling of the reducing ends of bacterial cellulose," *Carbohydrate Research*, vol. 180, pp. 345-350, 1988.

[137] M Iguchi, S Yamanaka, and A Budhiono, "Bacterial cellulose - a masterpiece of nature's arts," *Journal of Materials Science*, vol. 35, pp. 261-270, January 2000.

[138] Smith & Nephew, "Acticoat FAQ," http://global.smith-nephew.com/us/ACTICOAT_FAQS_8928.htm#17432,.

[139] "http://mrsec.wisc.edu/Edetc/nanolab/silver/index.html".

Appendix A – Alternative Methods

There is compelling evidence presented in the body of this thesis indicating that the antimicrobial efficacy of the discussed material is due to unbound Ag-NPs. There are several possible procedures for developing simpler systems, they rely on the idea of creating silver particle *in situ* – in the unwoven fiber mat of either Biofill or lab made, wet pellicle – instead of binding the Ag-NPs to the BC fibers. A few potential methods were explored, they have been evaluated using some of the techniques already discussed in the body of this thesis, but none has been subjectted to the full battery of tests.

A.1 Autoclaved silver

One of the simplest methods for creating a nanosilver dressing is using the steam and pressure of an autoclave. This procedure has been explored using cellulose cotton by Vigneshwaran et al. [131]. That research group created an Ag-NP cotton material by placing a cotton sample in a solution of silver nitrate and autoclaving it. This method was adapted for use with BC, and provides a promising method for creating the novel nanomaterial. The exact mechanism that creates the Ag-NPs is unclear, Vigneshwaran hypothesize that it was starches in the cotton that reduced the silver, these would not be present in the BC, therefore eliminating it as a possibility.

The procedure has one distinct advantage – it uses only compounds that are already used in the treatment of wounds, silver nitrate and BC; no other chemical treatments are required. Additionally, the final product remains flexible and strong, without the degradation of strength that accompanies oxidation. Any unreduced silver

will remain as silver nitrate, which already has antimicrobial properties. During manufacturing, the combination of the autoclaving step for production could be combined with the sterilization step reducing overall costs.

A.1.1 – Autoclaved silver: Methods

Biofill was placed in a solution of $AgNO_3$ of concentration of .1 M, and left for 15 minutes. The Biofill in solution was placed in the autoclave, and run on a 15 minute wet cycle. The cycle reached a temperature of 121° C and a pressure of 15 psi. The samples were removed from the autoclave and solution, and dried in a convection oven at room temperature.

Optical absorbance for the silver loaded BC samples was determined using a Beckman Coulter DU 520 UV-Vis Spectrophotometer. They were backgrounded against Biofill.

For additional characterisation techinques please see Chapter 3: Methods.

A.1.2 – Autoclaved silver: results and discussion

No scanning electron microscopy was performed on the samples. Instead optical absorbance was used to confirm the presence of Ag-NPs. Optical absorbance is frequently used for this purpose with a peak at 450 nm indicating the presence [132] of Ag-NPs. This peak is seen in Figure A.1 and therefore confirms the presence of Ag-NPs. XRD was conducted on the samples, but no peaks corresponding to

nanocrystalline silver were observed (Figure A.2); this could be because there is no crystalline silver, or could be a result of a level that is below the detectible range. Table A.1 shows that the amount of Ag-NPs makes up a smaller proportion of the total sample weight than the materials discussed in the body of the thesis; the silver loading for a 100 mM autoclaved sample is 5.1% silver compared to 5.5% silver for the unoxidized Biofill sample. It should be noted that the method described in the body of this thesis places the samples in two solutions of silver; the first having a significant silver concentration and second has a silver nitrate concentration of about 70 mM prior to adding the ammonia hydroxide. Summing these two treatments would put the amount of silver to prepare those samples at a higher level than the level of silver used to prepare the autoclaved samples.

The results of the antimicrobial tests can be seen in Table A.2. The antimicrobial test showed that there was an antimicrobial effect for the heavier loaded sample.

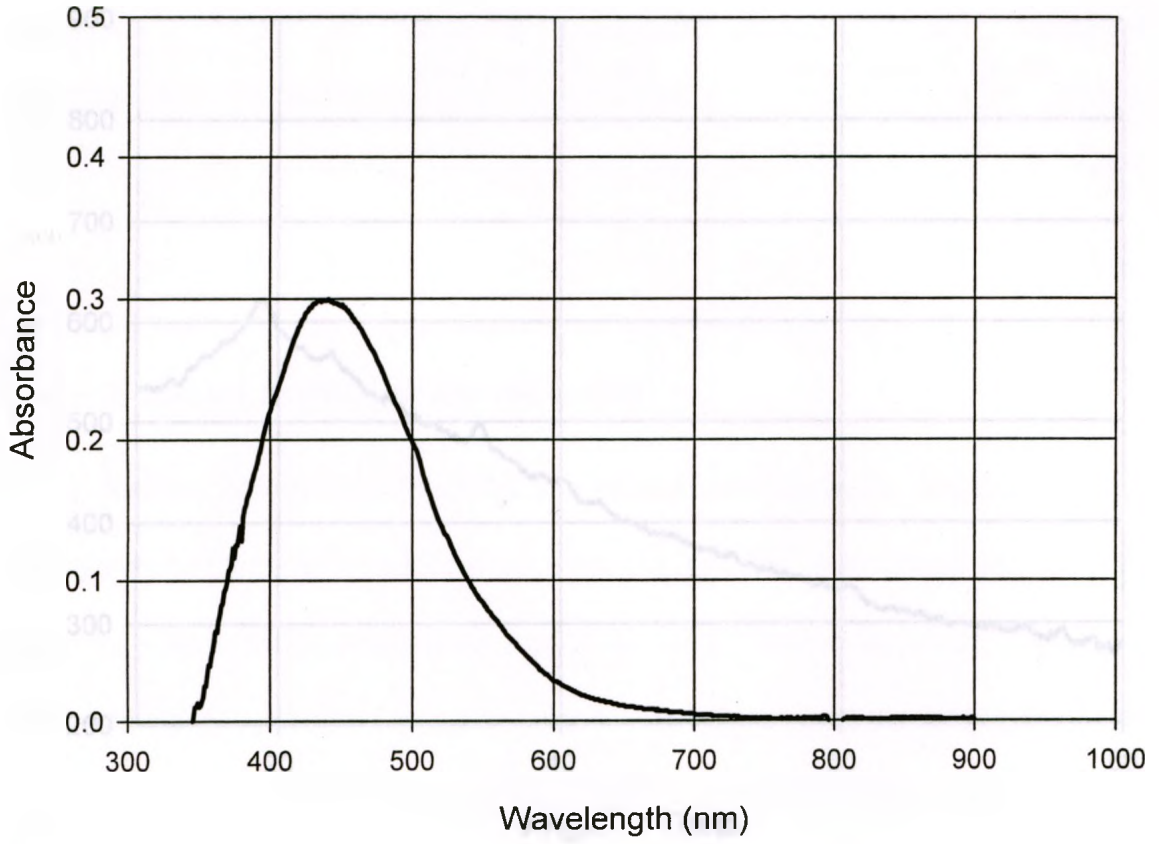

Figure A.1: Autoclaved samples – optical absorbance. Background plain BC.

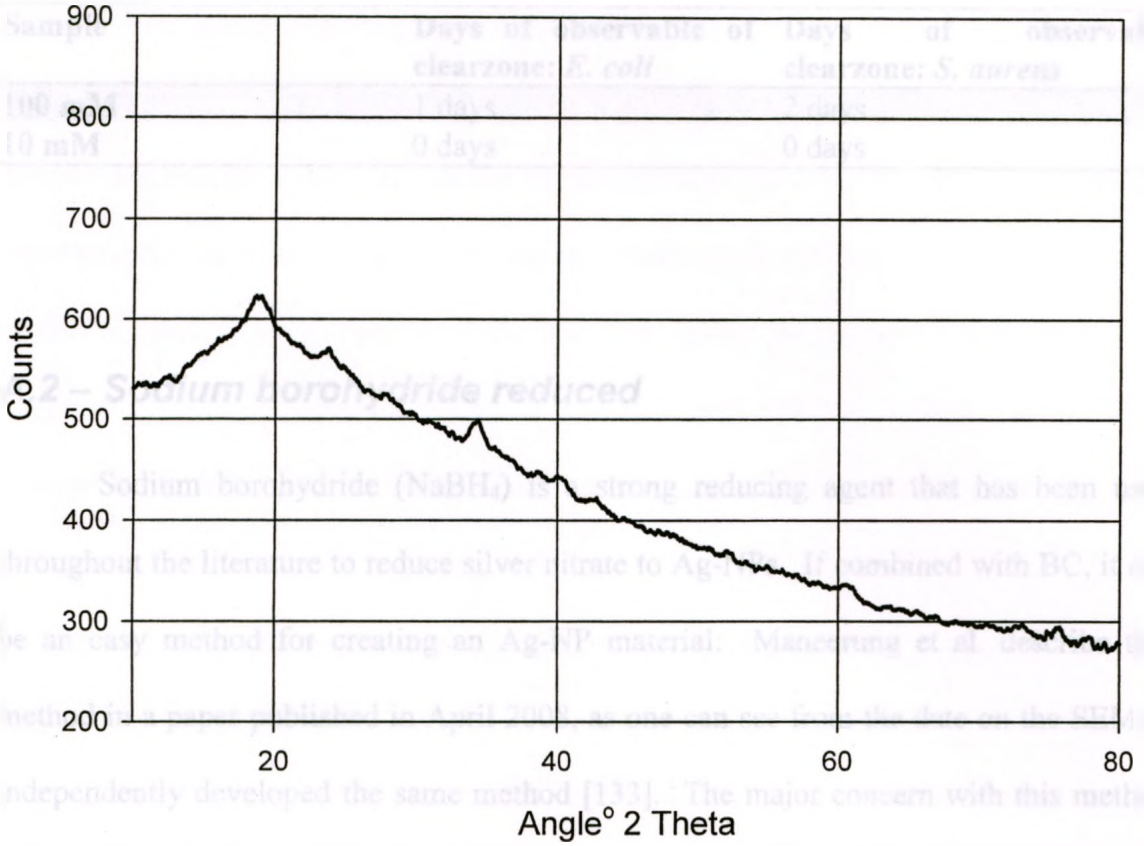

Figure A.2: Autoclaved samples – XRD

Table A.1: Autoclaved samples – silver density

Sample	Silver Density (w/w%)
100 mM	5.1 ± .15
10 mM	1.8 ± .37

Table A.2: Autoclaved samples – antimicrobial size of clearzone

Sample	Clear Zone: *E. coli*	Clear Zone: *S. Aureus*
100 mM	0 - .5 mm	.5 – 1.5 mm
10 mM	0 mm	0 mm

Table A.3: Autoclaved samples – antimicrobial longevity

Sample	Days of observable of clearzone: *E. coli*	Days of observable clearzone: *S. aureus*
100 mM	1 days	2 days
10 mM	0 days	0 days

A.2 – Sodium borohydride reduced

Sodium borohydride ($NaBH_4$) is a strong reducing agent that has been used throughout the literature to reduce silver nitrate to Ag-NPs. If combined with BC, it can be an easy method for creating an Ag-NP material. Maneerung et al. describe this method in a paper published in April 2008, as one can see from the date on the SEMs I independently developed the same method [133]. The major concern with this method is removing the remaining sodium borohydride and/or products of the reduction from the BC. Maneering et al. washes the BC thoroughly to mitigate this concern, but this is not a perfect solution. Washing probably removes some of the silver, creating inefficiency in the process and does not ensure all the sodium borohydride is removed.

A.2.1 – Sodium borohydride reduced: Method

Biofill samples were soaked in silver nitrate solutions of 100 mM and 10 mM of solution for 15 minutes. The samples were removed from the silver solution and placed in a solution of 100 mM of sodium borohydride. They were left for 5 minutes, and then rinsed. The were dried in a convection oven at room temperature.

For characterisation techinque please see Chapter 3: Methods.

A.2.2 – Sodium borohydride reduced: Results and discussion

These samples have the same efficacy as the autoclaved perpared samples, but with less silver. The lower silver level could be the result of a few factors, the autoclaved samples were kept in the silver solutions for a longer period of time (15 minutes, plus the time in the autoclave), this could of allowed for a more silver to soak in and/or more silver to form Ag-NPs; but, more likely the reduced silver content is a result of rinsing the samples. This is a simple and effective system, but with the drawback of dealing with the products of the silver nitrate – sodium borohydride reduction.

The optical absorbance was taken for the 100 mM sample, and a peak as 450 nm is observed (Figure A.3). Like with the autoclaved sample this shows the presence of Ag-NPs. This is further confirmed with SEMs. Figure A.4 shows Ag-NPs on the BC substrate. The Ag-NPs are small on the order of 20 – 50 nm.

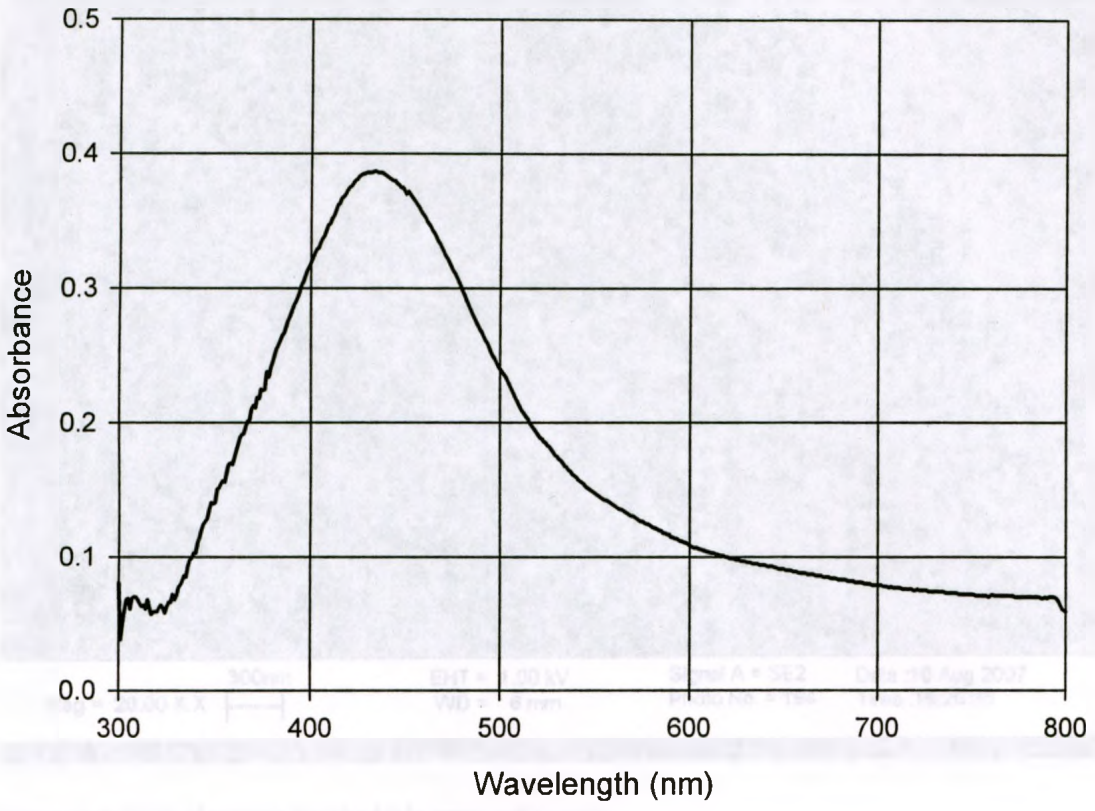

Figure A.3: Optical spectra for sodium borohydride prepared samples. Background plain BC.

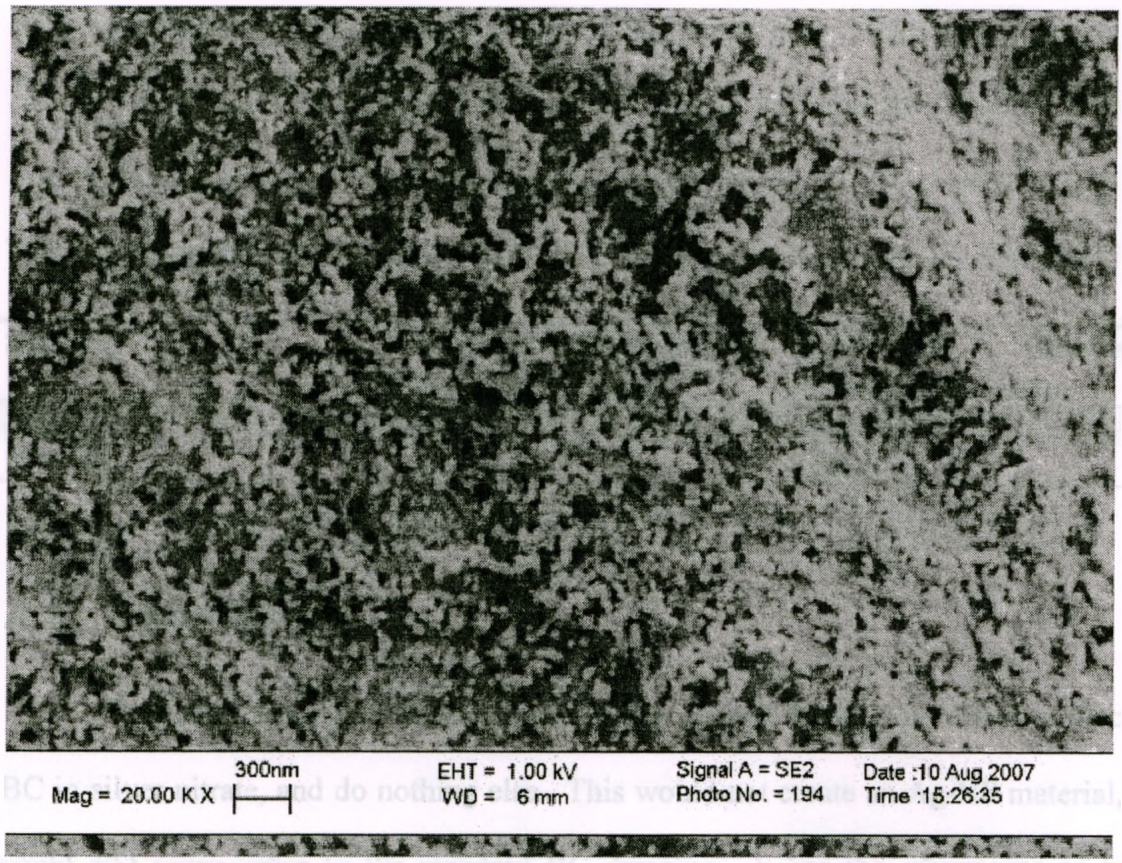

Figure A.4: SEM of sodium borohydride prepared samples

Table A.4: Silver Density on Sodium Borohyride prepared samples

Sample	Silver Density (w/w%)
100 mM	2.4 ± .5
10 mM	1.1 ± .5

Table A.5: Silver nitrate – antimicrobial size of clearzone

Sample	Clear Zone: E. coli	Clear Zone: S. Aureus
100 mM	0 - .5 mm	.5 – 1.5 mm
10 mM	0 mm	0 mm

Table A.6: Silver nitrate - antimicrobial longevity

Sample	Days of observable of clearzone: *E. coli*	Days of observable clearzone: *S. aureus*
100 mM	1 days	2 days
10 mM	0 days	0 days

A.3 – Silver Nitrate

The simplest method to create a BC silver loaded dressing is to soak a piece of BC in silver nitrate, and do nothing else. This would not create an Ag-NP material, but could add some value to the standard BC dressing. It has the advantages of being simple, and having both constituents already used in wound care would allow for an expedited approval process. A secondary objective of testing these samples was to establish controls for the above methods. The silver loading was not measured.

A.3.1 – Silver nitrate: Method

Biofill samples were placed in 100 and 10 mM $AgNO_3$ solutions, and left for 15 minutes. The samples were removed and air-dried. Antimicrobial tests were conducted in triplicate.

A.2.2 – Silver nitrate: Results and discussion

The results show that just adding silver nitrate to BC creates an antimicrobial material; this is an obvious result. The results also show that both the sodium borohydride and autoclaved samples are slightly better when it comes to antimicrobial

efficacy. Further testing will be required to fully explore the difference in activity for the autoclaved and silver nitrate samples. The results are about the same as the results seen in the body of the thesis for Biofill prepared samples – but as previously stated these samples are easier to prepare, and probably more likely to pass regulatory hurdles.

Table A.7: Silver nitrate – antimicrobial size of clearzone.

Sample	Clear Zone: *E. coli*	Clear Zone: *S. aureus*
100 mM	0 – .5mm	0 – 1 mm
10 mM	0 mm	0 mm

Table A.8: Silver nitrate – antimicrobial longevity tests.

Sample	Days of observable of clearzone: *E. coli*	Days of observable clearzone: *S. aureus*
100 mM	1 day	1 day
10 mM	0 days	0 days