

Nanomaterial and Biomaterial Approaches for Treating Chronic Wounds

By

Caitlin Lazurko

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Department of Biochemistry, Microbiology, and Immunology, Faculty of Medicine

University of Ottawa

University of Ottawa Heart Institute

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Abstract

Diabetic foot ulcers (DFUs) are a common and severe adverse event associated with diabetes, as 25% of diabetic patients will experience DFUs. The lack of effective DFU therapies results in 20% of diabetic patients requiring amputation. We first developed an algorithm to account for polydispersity when calculating nanoparticle concentration, which will reduce variability between batches and treatments. We also developed a novel 2-layer biomaterial, which combines anti-microbial properties of CLKRS peptide coated silver nanoparticles (CLKRS-AgNPs) with a pro-regenerative collagen matrix embedded with microscopic skin tissue columns (MSTC), to promote DFU wound healing. The collagen hydrogel formulation was optimized, and the physical properties, biocompatibility, and wound healing properties were assessed. Our results indicate that the CLKRS-AgNPs prevent bacterial growth and the collagen matrix provides a regenerative environment. Last, we developed and tested antimicrobial fabrics which can also be applied to chronic wounds, such as DFUs, to prevent and treat infections.

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List of Abbreviations

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid	HEPES
Ammonium Persulfate	APS
Bone Marrow Derived Macrophages	BMDM
Bovine Serum Albumin	BSA
Chondroitin Sulfate	CS
Diabetic Foot Ulcer	DFU
Differential Scanning Calorimetry	DSC
Dulbecco's Modified Eagle Medium	DMEM
Dynamic Light Scattering	DLS
Egtazic Acid	EGTA
Enzyme-Linked Immunosorbent Assay	ELISA
Ethanol	EtOH
Ethylenediaminetetraacetic Acid	EDTA
Fetal Bovine Serum	FBS
Fourier-Transform Infrared Spectroscopy	FTIR
Human Serum Albumin	HSA
Hydrochloric Acid	HCl
Inductively Coupled plasma Mass Spectrometry	ICP-MS
Interleukin	IL
Lithium Chloride	LiCl
Lysogeny Broth	LB
Matrix Metalloproteinase	MMP

Microscopic Skin Tissue Columns	MSTC
Nanoparticle	NP
Paraformaldehyde	PFA
Phenylmethysulfonyl Fluoride	PMST
Phosphate Buffered Saline	PBS
Poly-D-Lysine	PDL
Poly-L-Lysine	PLL
Scanning Electron Cryomicroscopy	Cryo-SEM
Silver Nanoparticle	AgNP
Sodium Chloride	NaCl
Sodium Dodecyl Sulfate	SDS
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis	SDS-PAGE
Tetraacetythylenediamine	TEMED
Tissue Inhibitor of Metalloproteinase	TIMP
Transmission Electron Microscopy	TEM
Tryptic Soy Broth	TSB
Ultraviolet	UV
Vascular Endothelial Growth Factor	VEGF

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1.0 Introduction

1.1 Diabetes and chronic wounds

Diabetes mellitus is one of the most prevalent chronic diseases around the world (1). Globally in 2010, 6.4% of adults were diagnosed as diabetic, and that number is expected to rise to 7.7% by 2030 (1). The prevalence of diabetes around the world is continuously increasing, mostly as a result of aging populations and lifestyle changes, including increasing obesity rates and a lack of physical activity (1, 2). There are several adverse events associated with diabetes, one of the most common and severe being diabetic foot ulcers (DFUs) (2-6). Foot ulcers are often characterized as chronic wounds, with healing times greater than one month (7). Foot ulcers affect both type I and type II diabetic patients and will affect 25% of patients at one point in their lifetime (2, 3, 8, 9). Foot wounds are the biggest cause of hospitalization associated with diabetes, and often progress to a point where the patient requires amputation (2, 10, 11). While up to half of amputations and foot ulcers can be prevented with patient education and identification, 20% of diabetic patients still undergo amputation resulting from DFUs (2, 3, 10, 12). This reflects poor treatment and care options for diabetic patients (2).

1.1.1 Diabetic foot ulcers

DFUs are defined as full-thickness wounds, indicating that the wound protrudes into the subcutaneous tissue in the skin on the foot or ankle (6, 12). DFUs result from trauma, often in combination with neuropathy associated with diabetes and peripheral artery disease (2, 3, 5, 9-11). Infection is a common secondary phenomenon of foot ulcers (3). Neuropathy is characterized by the loss of nerve fibres as a result of decreased blood flow and hyperglycemia, which impacts the duration and severity of the neuropathy (2). Severe neuropathy can cause small-muscle wasting

and loss of sensitivity, which when combined, can increase the pressure applied to the foot and place the patient at greater risk for ulceration (2, 3). Diabetes and neuropathy also increase the patient's risk of peripheral artery disease. Peripheral artery disease results in the occlusion of peripheral blood vessels in the lower limbs. This is often the result of atherosclerosis, the narrowing of the artery as a result of plaque (6, 9, 13, 14). This decrease in blood flow impedes wound healing and can allow a small wound to increase in size and become infected as a result of prolonged healing (13). The presence of atherosclerosis is common in diabetic patients as a result of an abnormal metabolic state, with its prevalence increased two to four-fold in diabetic versus non-diabetic patients (13, 14). Many patients who present with both diabetes and peripheral artery disease also have endothelial cell dysfunction. In a healthy patient, endothelial cells produce nitric oxide, a vasodilator that helps maintain vascular homeostasis. However, it has been shown that endothelial cell dysfunction in diabetic patients impairs nitric oxide-mediated vasodilation, which in turn impairs vascular homeostasis, increases susceptibility to atherosclerosis, and stimulates the body to produce additional vasodilators (14, 15). Overall, neuropathy and peripheral artery disease contribute strongly to the presence of DFUs, and can be detrimental to the patient's health, quality of life, and wound healing ability (5, 6, 8, 9, 14).

DFU classification is important for determining treatment options and patient care plans, as well as for research categorization (16). There are many different research and clinical classification systems used to assess diabetic foot ulcers, such as classification systems released by the Infectious Diseases Society of America, International Working Group on Diabetic Foot, or one developed at the University of Texas (3-5, 16). The PEDIS system, introduced by the International Working Group on Diabetic Foot, classifies foot ulcers based on perfusion, extent or size of the wound, depth, infection, and sensation (5, 16). The University of Texas classification

system has four grades which examine the depth of the wound and four stages which indicate the presence or absence of ischemia and infection (3-5). Many of these classification systems can be used in order to predict foot ulcer development and influence patient education and daily management for patients at risk of foot ulcers (16).

1.1.2 Wound healing

Human skin is the principle exterior defense system. It regulates body temperature and allows information from the external environment to be transmitted to our conscience (17, 18). Human skin is comprised of three layers: the epidermis, dermis, and subcutaneous tissue, and because it acts as a protective barrier against the external environment, breaks in the skin must be protected and healed quickly (19, 20). Wound healing is a dynamic and complicated series of cellular events, orchestrating many different cell and tissue types so that timing of each event allows the skin to return to a healthy state (2, 9, 19). Cutaneous wound healing has four major overlapping phases: hemostasis, inflammation, proliferation, and remodelling, as shown in Figure 1 (2, 9, 20-22). The phases are identified by essential molecular, cellular, and physiological events (20). During hemostasis, the initial reaction is to stop blood loss, so many events are signalled including vasoconstriction and the release of coagulation factors and platelets to close the initial wound and reform the skins natural barrier. The clot acts as a natural matrix for cells, such as fibroblasts and keratinocytes, to migrate through to fill the wound. It is rich in cytokines and growth factors that are released as the platelet degranulates to aid in future wound healing phases (19, 20). Next, inflammatory cells circulating in the blood, specifically neutrophils and macrophages, invade the wound area during the inflammatory phase which occurs within hours of the injury. Neutrophils kill bacteria present in the wound site and assist in wound healing by

removing damaged matrix proteins and releasing pro-inflammatory cytokines. Macrophages also aid in killing bacteria and removing cellular and tissue debris, as well as promoting angiogenesis and tissue granulation. They also remove neutrophils from the wound site (19, 20). Macrophages release cytokines and growth factors to promote cell migration, proliferation, and matrix formation which triggers the proliferation stage of wound healing (20). It has been shown that macrophages are essential for wound healing, as a decrease in macrophage presence results in decreased debridement and fibrosis (23, 24). During the proliferation stage, fibroblasts and keratinocytes migrate to fill the wound. Many of these cells originate from hair follicles and sweat glands within the wound and from the epidermal layer around the edge of the wound (19, 20). While the epidermal repair is occurring, angiogenesis begins with the release of many growth factors, including vascular endothelial growth factor (VEGF), in order to form new blood vessels. At this point in the wound healing process the granulation tissue appears consisting of fibroblasts, macrophages, blood vessels, and proteins. As healing progresses, fibroblasts differentiate into contractile myofibroblasts which help close the wound by pulling at the wound edges (20). It has been shown that various matrix metalloproteinases (MMPs) are needed in the matrix to allow cells, especially keratinocytes, to migrate through the matrix and fill the wound (19). The final stage of the wound healing process is the remodelling phase where collagen type III is converted to collagen type I with the help of MMPs, and the tensile strength of the skin increases (20).

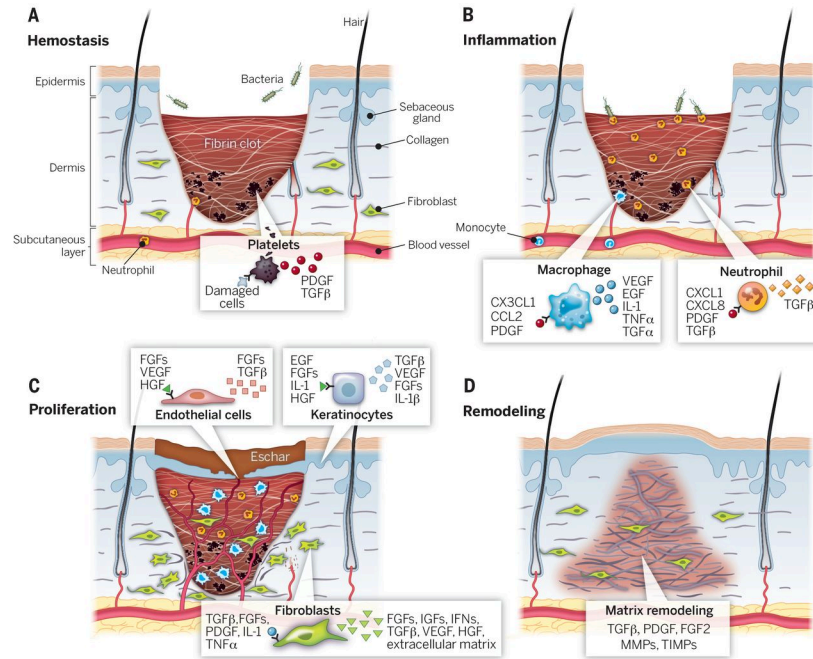


Figure 1. Wound healing phases. The four stages of wound healing include: (A) hemostasis, (B) inflammation, (C) proliferation, and (D) remodeling. These overlapping stages are highly coordinated and identified by specific molecular and cellular occurrences. PDGF, platelet-derived growth factor; TGF, transforming growth factor; FGFs, fibroblast growth factors; IL-1, interleukin-1; TNF, tumor necrosis factor; KGF, keratinocyte growth factor; IGF, insulin-like growth factor; IFN, interferon; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase. From Sun, Siprashvili, and Khavari, 2014 (20). Reprinted with permission from AAAS.

1.1.3 Foot ulcer wounds

Diabetes and diabetic foot ulcers can pose a challenge for proper wound healing. Diabetes, wound dryness, and infection are all associated with pronounced delays in wound healing (6, 11, 18). Moreover, chronic wounds, such as foot ulcers, have a high chance of infection because avascular eschar is a breeding ground for microorganisms (6, 17). In addition, the physical properties of the skin of diabetic patients differs from that of non-diabetic patients, for example it is biomechanically weaker (2). Diabetic patients lack some of the essential cellular and molecular signals necessary for proper wound healing (9, 11, 25-27). There is also a marked difference in the

expression of growth factors, chemokines, and cytokines in the wound that are necessary for wound healing (2, 9, 28, 29). Diabetic patients also have increased inflammation, tissue hypoxia, higher MMP, and lower tissue inhibitor of matrix metalloproteinase (TIMP) concentration which results in higher degradation of the extracellular matrix compared to healthy patients, which impairs the ability to progress through the wound healing stages, thus delaying the overall healing of the wound (9, 30).

As mentioned above, matrix metalloproteinases have an established role in wound healing. Their role is to degrade both matrix and non-matrix proteins to aid in wound healing, repair, and remodelling (28, 31). TIMPs are also essential, as their role is to regulate and inhibit MMPs. The balance between MMPs and TIMPs is necessary for maintaining a healthy environment (29-34). Dysregulation of MMPs can lead to serious diseases including chronic ulcers, cancer, and arthritis (31). MMP activity is low in healthy states, but signals from inflammatory cytokines, hormones, and growth factors can trigger its expression. The human body has 25 different MMPs, each with specific targets. For example, MMP1 cleaves type I collagen to allow keratinocytes to migrate to the open wound during the proliferation stage of healing and MMP9 cleaves many different ECM molecules including elastin and collagen type IV. It has been shown that the MMP9 to TIMP1 ratio is an important indicator of wound healing, as high and prolonged MMP9 concentration and low TIMP1 concentration has been associated with delayed healing. This high ratio of MMP9 to TIMP1 has been demonstrated in DFUs, reflecting poor healing rates (29-34).

DFU wound healing is further impaired in older patients, males, those with larger ulcer size, and patients presenting with comorbidities including heart failure, end-stage renal disease, those who cannot walk or stand unassisted, peripheral artery disease, and peripheral neuropathy (8). Given the severity of DFUs, and the fact that treatment options are limited, successful

treatment of foot ulcers is largely dependent on prevention, as well as type of treatment, including the dressing applied to the wound (2, 28).

1.2 Wound dressings and treatment options

1.2.1 Current treatments for foot ulcers

As mentioned earlier, current treatment options for DFUs are limited and often result in the patient requiring amputation (2). It has been demonstrated that a multidisciplinary approach to treating foot ulcers is beneficial, but implementation of this approach is slow, resulting in limited treatment options with poor therapeutic results (35). Preventative approaches include patient education, as well as prevention and early detection of ulcers (5, 9). Although significant research has focused on understanding the pathophysiology of DFUs, the effectiveness of DFU treatments is still limited (2, 9). Current treatment options for DFUs typically involve wound debridement to remove dead cellular debris, pressure relief by either rest or specialized footwear, infection control, and a wound dressing (5, 9, 11, 12). Other treatment options include surgical intervention for vascular reconstruction, blood glucose control, and hyperbaric oxygen therapy (5, 12). Improper diabetes monitoring and care, as well as infection have been shown to further delay wound healing in diabetic patients (9).

1.2.2 Traditional wound dressings

Understanding the pathophysiology of wounds, specifically the complications associated with DFU wounds has allowed researchers to develop new treatments and improve traditional wound dressings. While there have been advancements in treatment options, traditional and commonly used wound treatments still have very limited healing properties. Dressings such as

gauzes and bandages are frequently used but often result in leakage and dryness and can cause additional damage upon removal, which does not foster a healing environment (22, 36). Moreover, dressings often have a single use such as for debriding, hydrating, or antimicrobial applications, which limits the scope of this treatment, especially in complicated wounds such as DFUs (2). Andrews *et al.* state that dressings should not be the only treatment modality when treating DFUs, thus a multifaceted wound treatment approach is ideal in order to address all aspects of DFU wounds, including ischemia and infection (2).

1.2.3 Skin grafting

Large and extensive skin wounds that are too large to heal normally will often require a skin graft or transplant. Skin grafts have been shown to be an effective treatment for DFU wounds, as they increase the rate of wound healing and decrease the rate of amputation (12). Skin grafts are often characterized into three categories: (i) autografts, (ii) allografts and xenografts, and (iii) bioengineered materials (12). Autografts are skin grafts obtained directly from the patient and the graft is then placed directly on the wound site, ensuring full contact between the graft and the wound. Autografts are typically in the form of full- or split-thickness skin grafts. Full-thickness skin grafts contain both the epidermal and dermal layers of the skin, while split-thickness skin grafts contain the epidermal layer with minimal to no dermal layer (12, 20). Allografts are grafts taken from other patients, while xenografts are grafts obtained from another species that have a similar tissue structure as the recipient. However, both allografts and xenografts are often rejected by the body, so they are considered a more temporary dressing (20).

While autografts are superior to allografts and xenografts since they improve wound healing and are frequently used in wound healing applications, the harvesting of tissue for the graft

is invasive and painful for the patient and puts the patient at risk of additional infection and scarring (37, 38). Moreover, split-thickness skin grafts lack many dermal structures needed for full-thickness tissue regeneration, such as hair follicles and sweat glands. While full-thickness grafts overcome these limitations by containing all dermal structures, they are extremely invasive to the patient, have a high metabolic demand, and require proper blood supply, which limits the size of the graft (37). Tam *et al.* have developed microscopic skin tissue columns (MSTCs) which are microscopic skin fractions collected with a harvesting needle, a double-edged cutting needle, which contain the dermal structures necessary for full-thickness tissue regeneration, while being less invasive than skin grafts. MSTCs have been shown to improve wound healing, with the restored skin resembling healthy skin tissue in terms of epidermal structure, cell populations, and sweat production, with negligible impact on the donor site (37, 38).

Skin grafts are associated with challenges, such as rejection and size of both the donor site and wound area. An ideal graft would incorporate all the therapeutic benefits of skin grafts without the associated risks. Unfortunately, there is yet to be a perfect skin substitute, but this is a gap that the field of bioengineering and biomaterials is trying to fill (20).

1.2.4 Bioengineered materials

Bioengineered materials are skin substitutes engineered in the laboratory from either natural or synthetic material that may or may not contain growth factors or cells which can aid in the wound healing process (12, 36). They are intended to be introduced into the human body to replace damaged organs or tissues and often interact with the cells and tissues in the human body, therefore they must be biocompatible (39). There are three classes by which biomaterials can be categorized (i) synthetic, (ii) natural, or (iii) hybrid materials. Synthetic materials include materials

derived from metals, polymers, and ceramics, while natural biomaterials include materials obtained from plants or animals. Hybrid materials contain a combination of synthetic and natural materials (39).

There has been significant progress made in the development of biomaterials in the last 30 years. First generation biomaterials focused solely on replacing organs or tissues, for example they were often used as medical implants. The goal when designing these implants was to ensure they were not toxic to the human body but maintained the required physical and mechanical properties to be compatible with the body. As the field of biomaterials advanced, second generation biomaterials were developed to be bioactive, indicating the materials had an effect in the body. Today, third generation biomaterials are able to interact with cells in the body and stimulate specific cellular responses (39). These materials are being designed to mimic endogenous tissues in terms of the structure and architecture, with the overarching goal of replacing or regenerating entire organs (20, 39). Bioengineered materials can be designed to repair tissues alone as a matrix or by incorporating cells, growth factors, or pharmaceuticals into the material (39-41). Some of the benefits of tissue engineering include its application in personalized medicine, reduced medical costs, and increased availability (39). Biomaterials are being used for various applications, but there is increasing interest in using biomaterials to repair and replace damaged skin, especially in the case of foot ulcers and other chronic wounds (18, 21, 39, 41-43).

1.2.5 Hydrogels

Hydrogels are a type of biomaterial that are frequently used in tissue engineering that show promise in wound healing applications. Hydrogels are synthesized by crosslinking natural or synthetic polymers (18, 39, 44). The crosslinking can be completed using chemical crosslinkers,

such as organic solvents and chemical reagents, physical crosslinking, such as the formation of hydrogen or Van der Waals bonds, or radiation crosslinking, using radiation (*i.e.* UV-irradiation) to form free radicals that can allow the material to crosslink (18, 44).

Hydrogels are often used in tissue engineering because their 3D structure closely mimics the extracellular matrix, especially when compared to cell monolayers (18, 43, 44). Moreover, collagen hydrogels show additional benefits for wound healing because collagen is the most abundant protein in the extracellular matrix (18, 43). In addition to being an abundant protein in the extracellular matrix, collagen promotes cell migration, attachment, and proliferation as well as protein binding (45-47). Proteins bind to collagen by receptors that recognize the Proline-Hydroxyproline-Glycine sequence of collagen, integrin receptors which bind the Phenylalanine-Hydroxyproline-Glycine sequence of collagen or recognize other motifs, or receptors for non-collagenous regions (46). Many of the proteins that bind to collagen promote cell proliferation and adhesion (46). Collagen also contains chemoattractant properties for fibroblasts which aid in wound healing (45). Moreover, collagen can decrease the elastase levels in chronic wounds, which promotes the healing of these wounds (45). Overall, collagen and its interactions in the wound environment make it an optimal choice as a wound healing dressing.

Traditional bandages and early biomaterials were designed to cover the wound and act as an artificial barrier with little impact on wound healing (18, 21). Today, hydrogels are designed to promote wound healing by increasing epithelial cell proliferation, eliminating scab formation, keeping the wound environment moist, allowing oxygen circulation and gas exchange, and acting as a barrier against bacteria and microorganisms (18, 22, 36). There are many different hydrogels available for treatment of chronic wounds and DFUs. For clinical use, biomaterials should be safe and facile to produce and administer (20). Dermagraft® and Apligraf® are two skin substitutes

that are approved for use in clinic (39, 41, 42). Dermagraft® is a bioengineered hydrogel used in clinic to stimulate angiogenesis and improve wound healing of DFUs. It is a collagen gel reinforced with bioresorbable polyglactin mesh scaffold that contains dermal fibroblasts (39, 42). Apligraf® is a 2-layer skin substitute that uses a collagen hydrogel embedded with fibroblasts as the dermal layer and keratinocytes as the epidermal layer; it is currently an FDA approved DFU treatment (39, 41). Some limitations of Apligraf® is that it has high rates of degradation and poor retention of fibroblasts at the wound site (41). It has been suggested that all skin substitutes be used in conjunction with standard wound healing care procedures in order to provide the most effective healing environment (42).

There is significant research being done in designing hydrogels with various materials for DFU application. For example, Zhang *et al.* demonstrated that pectin and gum arabic hydrogels with basic fibroblast growth factor applied to the wound resulted in increased cell proliferation, wound re-epithelialization, and decreased wound size, indicating improved wound healing (21). Chondroitin sulfate hydrogels have shown promise for drug delivery as well as tissue engineering applications (40, 48, 49). There are many different collagen hydrogel dressings being applied to DFU wound treatment, especially since collagen can easily be modified by incorporating additives to obtain a hydrogel with specific properties and characteristics (29, 33, 34, 41, 50, 51). Helary *et al.* designed a concentrated collagen hydrogel for chronic wound treatment. This hydrogel has shown to improve cell viability, proliferation, and increase gene expression of genes associated with re-epithelialization (41). Ulrich and colleagues demonstrated that a cellulose/collagen matrix successfully decreased protease levels in the wound exudate and decreased wound size (33). Gottrup *et al.* used collagen/oxidized regenerated cellulose (ORC) along with a silver dressing to

improve wound healing by normalizing protease activity in the wound environment and preventing infection (34).

Recent evidence has shown that bioengineered materials alone are able to repair the skin so that it can act as a protective barrier, however, they are not able to restore the full functions of the skin, including touch and temperature sensation, perspiration, and hair growth (29, 39). A combinatory approach to biomaterials which includes skin substitutes, such as MSTCs, will promote skin tissue regeneration.

1.2.6 Antimicrobial dressings

Other materials such as antibacterial functionalized fabrics have been developed for wound healing applications. Cotton fabric is very versatile and can be functionalized with different antimicrobial agents including antibiotics, silver nanoparticles, N-halamine derivatives, and quaternary ammonium salts (52-58). Natural fibers are highly susceptible to bacterial infection, which can play a role in cross-contamination and infection (53, 55, 58). The chemical composition of cotton, which is primarily composed of cellulose, contains hydroxyl groups that make it easily modifiable. These fabrics can be easily modified to help decrease this bacterial susceptibility (53, 55). Functionalization of fabrics limits possible contamination associated with weaving together antibacterial fibers, and can be applied for many different applications, including wound dressing (52, 53, 55). Sadanand *et al.* functionalized cotton fabrics with silver nanoparticles and demonstrated that the fabrics maintained antibacterial properties (52). Functionalized cotton fabrics show diverse potential in terms of application, with the major focus being a facile synthetic route that allows the fabrics to maintain their antimicrobial properties (52-56).

1.3 Nanotechnology

Nanomaterials have been used for centuries with early applications seen in the Lycurgus Cup, and are gaining increasing popularity due to their unique magnetic, optical, and electric properties that can be exploited for many different applications including medicine and nanodevices (59, 60). Many of the unique physical and chemical properties of nanoparticles (NPs) are associated with the shape and size of the particle (59-61). NPs have physical and chemical properties that differ from their bulk material, which are often exploited for various applications (59, 62, 63). Nanoparticles can be synthesized in various methods by either the bottom up method, where atoms or molecules are used to synthesize the nanoparticles, or the top down method, where bulk materials are broken down into nanoparticles (64). An example of bottom up synthesis would be the reduction of metal salt, as shown in Figure 2, where metal salt is used along with a reducing agent and a stabilizer. Citrate can be used as both a reducing agent and a stabilizer. The reducing agent reduces the metal ion, for example silver ion (Ag^+) to Ag^0 , and the stabilizer controls the nanoparticle size and prevents further growth and aggregation (60).

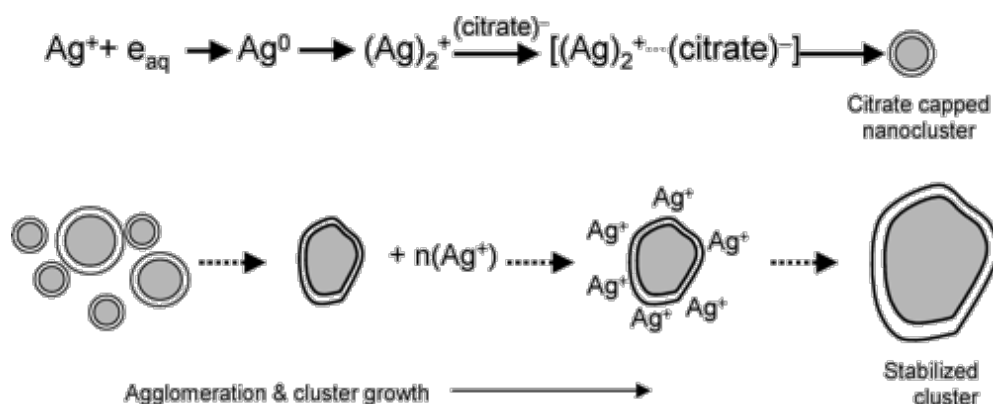


Figure 2. Scheme depicting formation of citrate-capped AgNPs. Reprinted with permission from Pillai and Kamat, 2004 (60). Copyright 2019 American Chemical Society.

1.3.1 Silver nanoparticles

The antimicrobial properties of silver have been applied since early Roman times when drinking water was stored in silver containers to prevent contamination. Hippocrates first described the medical applications of silver for treating ulcers and improving wound healing (59). Silver, as well as silver nanoparticles (AgNPs), have a wide antimicrobial spectrum and its application as an antibacterial agent has been widely studied (36, 63, 65-67). The antibacterial properties of AgNPs have shown to be effective against both anaerobic and aerobic bacteria, gram-negative and gram-positive bacteria, fungi, and viruses (36, 62, 67). AgNPs have demonstrated a greater effect on gram-negative than gram-positive bacteria, which can be explained by the difference in cell wall thickness, with gram-positive cells having a cell wall thickness around 30 nm and gram-negative around 4 nm (62). There is still significant debate over the toxicity of AgNPs, however it has been shown that adding protecting agents to the AgNPs allow them to remain non-toxic and biocompatible (62, 66). The rise in antibiotic resistant bacteria has led researchers to find new ways of inhibiting bacterial growth, and AgNPs have widely been explored for this application (34, 62, 63, 65, 67). It has also been shown that bacteria are less likely to develop resistance against AgNPs compared to antibiotics and that AgNPs have greater antimicrobial efficiency versus other metal NPs (67).

The exact mechanism of antibacterial and antibiofilm activity of AgNPs is still not fully understood; however, two methods, contact killing and ion killing, are currently the most accepted (34, 62). The antibacterial properties of AgNPs are largely dependent on their high surface area to volume ratio (62, 63). The theory surrounding contact killing is that AgNPs attach onto the surface of bacteria and infiltrate the bacterial cell wall. It is believed that the positive charge of the AgNPs and the negative charge of the cell wall aid in this electrostatic interaction. This interaction then

damages the membrane and causes the content of the cell to leak out, resulting in cell death (62, 63, 65, 67). This theory supports the observations that AgNPs are more effective against gram-negative bacteria whose cell wall is thinner than the gram-positive bacteria (62).

Once the AgNPs adhere to the cell wall they can also infiltrate the cell and cause additional damage. AgNPs can interact with and damage proteins, DNA, lipids, and ribosomes. When interacting with ribosomes, AgNPs can impede the synthesis of proteins (34, 62, 63). Nanoparticles have been shown to have size-dependent antibacterial effects, possibly because smaller NPs can invade deeper into the cell and penetrate the cytoplasm (62). Last, AgNPs produce high levels of reactive oxygen species (ROS) and free radicals (34, 62, 67). These products can normally be eliminated by the cell, but AgNPs inactivate the respiratory chain dehydrogenases and can downregulate the expression of antioxidant enzymes, which allows excess ROS to accumulate in the cell (34, 62, 63, 67). High levels of ROS result in oxidative stress, which causes an apoptotic-like response in the cell along with cellular and DNA damage (34, 62).

Silver ions released from AgNPs are also associated with the antibacterial activity of AgNPs. Their release is greatly dependent on surface area, as nanoparticles with the highest surface area release the highest concentration of silver ions (62). This antimicrobial activity relies on Ag^+ forming stable bonds to the sulfhydryl groups in proteins and enzymes, thus deactivating them. Many proteins that become deactivated by the silver ions are involved in cellular respiration and the silver ions prevent ion transport and the transmembrane generation of ATP, therefore inhibiting the electron transport chain from completing oxidative phosphorylation (62, 67). Moreover, Ag^+ can bind nucleic acids and prevent cell division (62). Silver ions also have the ability to increase oxidative stress. Bacteria commonly use the thioredoxin (Trx) system to decrease oxidative stress, which is composed of nicotinamide adenine dinucleotide phosphate (NADP^+), thioredoxin

reductase (TrxR). Silver ions have been shown to bind to the active sites of *Staphylococcus aureus* Trx and TrxR, which disrupts the system and decreases its ability to control oxidative stress levels, resulting in cell damage (62, 67). It is likely that both contact and ion killing play a role in the antibacterial properties of AgNPs (62, 67).

1.3.2 Nanomaterials in a biological environment

When nanomaterials are placed in a biological environment, the surface of the nanoparticle will be modified by the adsorption of proteins from the environment onto the surface of the nanoparticle. This results in the formation of the protein corona, the layer of proteins around the nanoparticle (68-73). These proteins compete with molecules that have been previously adsorbed onto the surface of the NPs and can act as stabilizing agents for the NPs. The formation of the protein corona is dynamic as molecules are constantly adsorbing and desorbing based on the affinity of these molecules for the surface (74). The protein corona then gives the nanoparticle a new biological identity and often new physical and chemical characteristics, such as size, shape, and surface charge, which can allow it to interact in the environment in different manners and result in changes in biodistribution, toxicity, or interactions with the environment (68, 69, 75, 76). It is important to understand these interactions when choosing a capping agent because changes to the protein corona can affect the overall outcome and targets of the NP treatment (66, 68, 69, 75).

1.3.3 Antimicrobial peptide capped AgNPs

Modifying the surface of AgNPs allows one to modify the properties of the AgNPs, including the antibacterial effects (62, 66, 77). Our lab has previously shown that by capping AgNPs with peptides, we are able to produce stable antimicrobial and biocompatible AgNPs (66,

77). In this project we are using the CLKRS peptide as a protecting agent for AgNPs. It is a pentapeptide with the amino acid sequence cysteine-leucine-lysine-arginine-serine which was developed as an anchoring moiety for a collagen mimetic peptide and has been shown to bind with higher affinity to the surface of AgNPs than other similar pentapeptides, such as CLFRS (cysteine-leucine-phenylalanine-arginine-serine) (78, 79). CLKRS can also be used as a capping agent when synthesizing the AgNPs and the concentration of CLKRS will dictate the size and shape of the AgNP, with the average diameter of the nanoparticles decreasing with increasing CLKRS concentration (78). This short chain peptide sequence is of low cost, facile to produce, biocompatible, and our lab has demonstrated that CLKRS-AgNPs have antibacterial and antibiofilm properties (77-79).

1.4 Research Plan

1.4.1 Rationale

Diabetes and related illnesses are on the rise in Canada and around the world. Diabetic foot ulcers (DFUs) are one of the most common and debilitating adverse events associated with diabetes (2-5). One quarter of diabetic patients will experience foot ulcers in their lifetime, and 20% of these individuals will undergo amputation as a result of the ulcer, reflecting a lack of effective medical treatment options that are clinically available (2, 3, 8-12). Wound healing is a complicated series of events, and even more so in diabetic patients due to the lack of essential signals necessary for proper wound healing as well as a change in the expression of factors associated with healing (2, 9, 11, 25-29, 34). Treatment options today are limited (2). Infected DFU wounds are often treated by debridement and antibiotics (5, 9, 11, 12). However, with antibiotic resistant bacteria becoming an increasing concern, researchers are looking for other

options (34, 62, 63, 65, 67). Skin grafts, today's gold standard treatment, are limited by the size and shape of the wound, rejection, and invasiveness of the procedure (20, 37, 38). Bioengineered materials readily repair the skin but lack the ability to restore the functions of the skin (29, 39). By using a combinatory approach which incorporates antimicrobial silver nanoparticles, non-invasive skin columns, and the benefits of biomaterials, we hope to provide an environment which will promote skin tissue regeneration.

1.4.2 Aims and Objectives

1. Develop and test an algorithm that corrects for nanoparticle size polydispersity when calculating nanoparticle concentration.
2. Develop and test a biomaterial that promotes healing of chronic wounds alone and in combination with the microscopic skin tissue columns.
3. Test the biocompatibility and antibacterial effects of antibiotic-grafted fabrics.

1.4.3 Hypothesis

It is hypothesized that by deriving an algorithm that corrects for nanoparticle polydispersity in nanoparticle concentration calculations, we will be able to more accurately determine the true nanoparticle concentration in solution, which will decrease experimental variability between samples that result from non-homogenous sample size distribution. It is also hypothesized that by combining our sprayable formulation of surface-grafted CLKRS-AgNPs with the regenerative properties of MSTCs embedded in a type I collagen matrix we will provide a regenerative environment for wound healing of DFUs. Last, it is hypothesized that the antibiotic-grafted textiles will decrease bacteria in both planktonic and biofilm form while maintaining its biocompatibility.

2.0 Materials and Methods

Chemicals and Reagents

Silver nitrate (AgNO_3), 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone (I-2959), glycine, 25% glutaraldehyde solution, sodium hydroxide (NaOH), chondroitin sulphate (CS), phosphate buffered saline (PBS), trisodium 2-hydroxypropane-1,2,3-tricarboxylate (sodium citrate), sodium chloride (NaCl), Tris base, hydrochloric acid (HCl), calcium chloride (CaCl_2), egtazic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMST), albumin from human serum (HSA), and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich and used without further purification. All solutions were prepared using Milli-Q water, unless otherwise stated. All cell culture media and reagents were purchased from Thermo Fisher Scientific, Gibco brand unless otherwise specified.

Animals

All animal studies were conducted with ethical approval from the University of Ottawa Animal Care Committee (protocol number HI-2527) and in compliance with the National Institutes of Health Guide for the Use of Laboratory Animals. Female C57Bl/6 mice purchased from Charles River Laboratories or CX3CR1-GFP (B6.129P-Cx3cr1^{tm1Litt}/J) mice purchased from The Jackson Laboratory were used for all experiments.

Cell lines and culture conditions

A human dermal fibroblast cell line (ATCC® PCS-201-012™) transfected with green fluorescence protein (GFP) was cultured in supplemented high glucose DMEM media (10% FBS and 1% penicillin-streptomycin) at 37°C with 5% CO_2 . The media was changed every 3 days.

Mouse dermal fibroblasts were isolated from C57Bl/6 mice aged 8-12 weeks as previously described (80). Briefly, mice were euthanized by CO₂ inhalation and cervical dislocation, dorsal hair was shaved, and a skin sample of approximately 1 cm² was collected. The tissue sample was then minced into ~1 mm² pieces and incubated in 700 µL of HBSS and 300 µL digestion buffer (collagenase (Gibco-17101-015) and dispase (Gibco)) for 30-60 mins at 37°C. The sample was rinsed three times by centrifuging at 300g for 5 mins and resuspending in supplemented high glucose DMEM (10% FBS and 1% penicillin-streptomycin) and cells were cultured for 1-2 weeks before use. Murine bone marrow-derived macrophages (BMDMs) were isolated from the femur and tibia of mice, as described in the literature with minor modifications (81). Briefly, the femur and tibia of euthanized mice were obtained. Muscle and tendons were removed from the bone and the bones were flushed with media (high glucose DMEM, 10% FBS, 15% L929 cell media, 1% penicillin-streptomycin). The cells were cultured for 7 days before use, with the media changed every second day. All cells were passaged until reaching 90% confluency by lifting cells with 0.5% trypsin-EDTA solution for 5 mins at 37°C with 5% CO₂, followed by centrifugation at 1000g for 5 mins and counting of cells then seeding cell in a new cell culture plate.

2.1 Silver nanoparticle synthesis and characterization

2.1.1 Synthesis of citrate capped AgNP

Citrate capped AgNPs were prepared as described in the literature with minor modifications (82-84). Briefly a deoxygenated (30 mins N₂) aqueous solution containing 0.2 mM AgNO₃, 0.2 mM I-2959, and either 1.0 mM or 0.2 mM sodium citrate was irradiated with UVA light (8 lamps, in a Luzchem LZC-4 photoreactor at 25.0 ± 0.5°C) for 30 mins. Yellow translucent solutions were obtained in all cases and the solutions were kept at room temperature and protected from light.

2.1.2 Surface plasmon band spectra

The surface plasmon absorption band (SPB) was measured with a Libra S50 UV–Vis spectrophotometer (Biochrom, Cambridge, UK) at room temperature, using 1.0 cm path length cuvettes. Sodium citrate was added to AgNP solutions with less than 1.0 mM of sodium citrate so the concentration of sodium citrate among all batches remained consistent. For citrate capped AgNPs, the maximum wavelength absorbance was found at 398 nm for 1.0 mM sodium citrate solutions and 402 nm for 0.2 mM sodium citrate solutions. 1.0 μ M Human Serum Albumin (HSA) was added to the AgNP solutions and the plasmon shift was monitored and recorded.

2.1.3 Transmission Electron Microscopy (TEM)

Samples were prepared by applying $\sim$ 5.0 μ L of fresh AgNP solution to carbon-coated copper grids (400 mesh) and dried in a vacuum system for at least three days. High-resolution electron microscopy images were obtained using a FEI Tecnai G2 F20 TEM operating at 75 kV. Samples were measured at least in triplicate and a minimum of 100 nanoparticles per image were measured to determine nanoparticle diameter.

2.1.4 Dynamic Light Scattering (DLS) measurements

Hydrodynamic size of the citrate@AgNP solutions were measured using a Malvern Zetasizer Nano ZS at 20°C in 1.0 cm path-length disposable plastic cuvettes. Reported values correspond to the average of nine independent batches, each measured in triplicate.

2.1.5 Tryptophan fluorescence of HSA upon adding AgNPs

Tryptophan 214 (Trp-214) fluorescence was performed in a Perkin Elmer LS55 instrument (Massachusetts, USA). Spectra were obtained by exciting samples at 295 nm and monitoring emission at 340 nm at room temperature. Samples were diluted less than 5.0% to minimize changes in fluorescence as a result of dilution. Fluorescence emission intensity was corrected by inner filter effects derived from AgNP absorption using:

$$F_{\text{corr}} = F_{\text{obs}} \times 10^{[(A_{295} + A_{340})/2]}$$

where F_{corr} is the corrected and F_{obs} is the uncorrected fluorescence, respectively, and A_{295} and A_{340} are the AgNP absorbances at the respective wavelengths (85).

2.1.6 Preparation of spray-on AgNP formulation

The spray-on AgNP formulation was adapted from another formulation synthesized by our lab (66). Briefly, the solution was prepared on ice by mixing 60 μL of 20 μM CLKRS solution with 6 mL of 0.83 nM citrate@AgNPs. Next, 192 μL of 0.1 mg/mL solution of rat tail collagen Type I (Corning) was added and vigorously mixed. The pH of the solution was adjusted to 7.4 using 5X PBS and a colour change from yellow to brown was observed. Next, 174 μL of 1.5% glutaraldehyde solution was added to the NPs and the solution was left for 30 mins in the dark. Excess glutaraldehyde was quenched with a 20% glycine solution and incubated again for another 30 min. The final solution was then diluted to a final total silver concentration of 100 μM in 1 $\times$ PBS. A sterile solution of 1 $\times$ PBS was used as control.

2.2 Collagen hydrogel

2.2.1 Collagen hydrogel synthesis

Collagen hydrogels were prepared on ice in a sterile environment by mixing 6 mL of Type I porcine collagen Theracol (1%) (Sewon cel Contech) with 1 mL of 10 $\times$ collagen media (9 mL 10 $\times$

DMEM medium (pH 7.2), 10 mL 1× PBS, 9 mL 10× HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) buffer). Next 600 µL of 1× PBS was added to the solution. The pH was then adjusted to 7.0-7.4 with 1.0 M NaOH, which resulted in a colour change from yellow to pink. Next, 200 µL of 1.5% glutaraldehyde was added to the solution 100 µL at a time, mixing in between additions, and the solution was thoroughly mixed, then left on ice in the dark for 15 mins. 500 µL of 20% glycine was mixed into the solution to quench the unreacted glutaraldehyde and left for 15 mins prior to use. For the PDL hydrogel, 500 µL of 5% PDL solution in PBS was added following addition of the glycine. For the CS hydrogel, 500 µL of 25% chondroitin sulfate solution in PBS was added following addition of the glycine. The CS-PDL hydrogel contained 500 µL of 25% CS solution in PBS and 500 µL of 5% PDL in PBS added following addition of the glycine. Physical properties of the hydrogels were then analyzed to determine viscosity, denaturation temperature, FTIR, water content, collagenase degradation rate, and pore size (Cryo-SEM).

2.2.2 Viscosity

The viscosity of the hydrogels was measured using a Brookfield RS-CPS+ Rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA). Measurements were carried out at 37°C in triplicate for each hydrogel, and the average values are reported.

2.2.3 Differential Scanning Calorimetry (DSC)

First, the hydrogels were washed with 1× PBS and stored in PBS at 4°C until further use. The denaturation temperature of each collagen hydrogel was measured using a Q2000 differential scanning calorimeter (TA Instruments, USA). Heating scans were recorded at temperatures

ranging from 8 to 80°C at a scan rate of 5°C/min. Denaturing temperature was calculated from the curve of heat flow versus temperature increase.

2.2.4 Fourier-transform infrared spectroscopy (FTIR)

The hydrogels were first synthesized and dried in the desiccator. The FTIR spectra were measured on a Nicolet iS5 Spectrometer equipped with an iD7 ATR accessory with diamond crystal (Thermo Scientific, USA) and 300 scans were averaged.

2.2.5 Water content

Water content of the hydrogel was determined by taking the initial mass of the hydrogels then placing them in a desiccator at room temperature. The masses of the hydrogels were measured every 24h for up to 120h. The reported values correspond to the average of three independent samples.

2.2.6 Collagenase degradation *in vitro*

The hydrogels were washed with PBS, cut into pieces of similar weight and shape and incubated in Tris-HCl buffer (pH = 7.4) for 1h at 37°C. The hydrogels were removed from the solution and weighed after carefully blotting away excess liquid. The hydrogels were then incubated in a preheated collagenase solution (5 U/mL in 0.1 M Tris-HCl buffer containing 5 mM CaCl₂) at 37°C for up to 3h, as previously described (86). At each time point the gels were removed from the solution, carefully blotted, weighed, and placed back in a fresh preheated collagenase solution at 37°C to determine the relative degradation *in vitro*.

2.2.7 Scanning Electron Cryomicroscopy (Cryo-SEM)

Microstructure analysis of the collagen hydrogels was assessed using low temperature Cryo-SEM images. Cross-sections of the samples were coated with a 5.0 nm thick carbon film prior to SEM imaging using a low vacuum coater Leica EMACE200 (Wetzlar, Germany), and imaged using the secondary emission detector in a JSM-7500F FESEM from JEOL Inc. (Peabody, MA). ImageJ (National Institute of Health, Bethesda, MD) software was used to measure the pore sizes. Over 100 individual pores were measured from randomly selected areas of each sample, and samples were tested in triplicate.

2.2.8 Hydrogel crosslinking time

Hydrogel crosslinking time was determined by solidifying 250 μ L of hydrogel on a 12-well Costar® well plate at 37°C. Solidification was determined by monitoring the change in opacity of the hydrogels. Each hydrogel formulation was tested independently in triplicate and crosslinking time was monitored for up to 80 minutes.

2.3 *In vitro* hydrogel testing

2.3.1 Cell number

Human dermal GFP fibroblasts (50,000 cells/well) were cultured for 3 days in a 12-well plate in contact with the collagen hydrogel. The cells were quantified at 24, 48, and 72h using a JuliFL Microscope (NanoEntek) and 4 random microscopic images were obtained from each well. The number of fibroblasts were quantified using ImageJ software. Each hydrogel treatment was tested in triplicate.

2.3.2 Macrophage polarization assay

Murine bone marrow-derived macrophages (10,000 cells/well) were seeded on coverslips coated with hydrogel in a 12-well plate and cultured for four days, with the media (DMEM, 10% FBS, 15% L929 cell media, 1% penicillin-streptomycin) changed every second day. After 4 days in culture, the media was removed, and the cells were washed two times with Hank's Balanced Salt Solution (Sigma). The cells were then treated with a solution of 4% paraformaldehyde (PFA) (Sigma) in 1× PBS for 15 min in the dark at 4°C. The PFA was then removed and the cells were washed twice with 50 mM ammonium chloride (NH₄Cl) in PBS, waiting 7 mins between washes. Next, the samples were washed three times with 1× PBS and following the final wash, a 0.2% NaN₃ solution was added to the well and the samples. Next, samples were washed with 1× PBS then incubated in the blocking solution (2% v/v BSA/PBS + 0.5% Triton X-100) at room temperature for 1.5h. Blocking solution was removed and samples were incubated with primary antibodies CD206 (1/1000, Ab64693, abcam) and CD86 (1/500, Ab119857, abcam) diluted in fresh blocking solution (2% bovine serum albumin (BSA) and 0.5% Triton X-100 in 1× PBS) to identify M2 and M1 macrophages, respectively. The following day the samples were washed three times with 1× PBS. Secondary antibodies Alexa Fluor 488 donkey anti-rat (1/2000, Invitrogen A21208) and Alexa Fluor 568 goat anti-rabbit (1/2000 Invitrogen A11036) were diluted in the blocking solution and incubated with the sample at room temperature for 1h. Samples were washed three times with 1× PBS and samples were incubated at 37°C for 15 mins with DAPI. The cells were then washed 1× with PBS, mounted on a slide with Pro-Long™ Gold antifade reagent with DAPI (Invitrogen) and imaged on a Zeiss Axio Imager.Z1 fluorescent microscope. Analysis was completed with ZenBlue (2011-2012) digital imaging software. For quantification, 4 random microscopic images were obtained from each slide and the number of M2 and M1 macrophages

were quantified using ImageJ software. Hydrogels were tested in triplicate with a blank coverslip used as a control.

2.3.3 Microscopic skin tissue column isolation

Standard hypodermic needles were used for harvesting skin tissue columns as previously described (37). The needles were honed to have 2 cutting edges, so that a column of tissue is extracted by inserting and withdrawing the needle through the full-thickness of the skin. Microscopic-skin tissue columns were extracted from dorsal mouse skin for the *in vitro* tests and for *in vivo* tests it was extracted from the skin removed during the creation of the wound.

2.3.4 Microscopic skin tissue column viability

Microscopic skin tissue column (MSTC) compatibility and viability in hydrogels were assessed by plating approximately 30 columns in each well of a 12-well plate in 250 μ L of hydrogel. Samples were also tested with the CLKRS-AgNP spray-on formulation by applying four 5 sec sprays of AgNPs, incubating at 37°C for 10 mins in between each spray and 60 mins after the final spray before applying the MSTCs and hydrogel. Columns were cultured in high glucose DMEM media (10% FBS and 1% penicillin-streptomycin) at 37°C with 5% CO₂. The media was changed every 3 days and cultures were kept for up to 21 days. Samples were imaged with a JuliFL microscope (NanoEntek) to monitor cell migration at various time-points. Viability of the skin columns and migrating cells were assessed with a LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific). Briefly, the samples were washed 3 times with 1× PBS. Next the cells were incubated with calcein-AM (1/2000) and ethidium homodimer-1 (1/500) diluted in 1× PBS at 37°C in the dark for 30 mins. The samples were then rinsed again 3 times

with 1× PBS before imaging with a Zeiss Axio Observer.A1 fluorescence microscope and analyzed with ZenBlue (2011-2012) digital image software. For quantification, 4 random microscopic images were obtained from each well and the number of live and dead cells were quantified.

2.3.5 RNA isolation

RNA was isolated from tissue harvested from the wound that had been treated with the hydrogel by adding 1 mL of TRIzol/TRI-Reagent (Zymo Research) to approximately 25 mg of tissue. Tissues were then homogenized with 0.5 mm zirconium silicate beads (Next Advance) for 10 mins in a Bullet blender tissue homogenizer (Next Advance). Next, 200 µL of chloroform was added, shaken vigorously for 30 sec, and then left at room temperature for 10 mins to allow separation. The samples were centrifuged at 12,000g for 15 mins and the clear top phase containing RNA was collected. Next, 300 µL of isopropanol was added to the RNA phase, the sample was shaken and then kept at -20°C overnight. The sample was centrifuged at 12,000g for 10 mins and the supernatant was aspirated, leaving the RNA pellet. Next, 0.5 mL of ice-cold 75% ethanol was added to the pellet and the sample was centrifuged at 7500g for 5 mins. The liquid was removed, and the pellet was air dried for 10 mins at room temperature then 20 µL of RNase free water (Invitrogen) was added to the tube and heated at 40°C for 10 mins to dissolve the RNA.

2.3.5 RNA Purity and Quantification

Isolated RNA was analyzed with the NanoDrop-1000 Spectrophotometer with V3.3 Software (Thermo Scientific). The machine was blanked with 1 µL of RNase free water (Invitrogen) and 1 µL of purified RNA sample was analyzed for RNA concentration (ng/µL) and purity (260/280

ratio). Samples used had a 260/280 ratio greater than 1.9. If RNA sample purity was low, they were further purified with a DNase purification (0.5 μ L DNase, 41 μ L RNase free H₂O, 9 μ L DNase buffer (Invitrogen)) that was heated for 10 mins at 37°C. Then 0.2 M EDTA (pH 8.0) was added to the solution and heated to 75°C for 10 mins. Next, 2.5 μ L of 4 M LiCl and 75 μ L 99% EtOH was added to precipitate the RNA and the samples were kept at -20°C overnight. The samples were centrifuged at 13,200g for 15 mins, the supernatant was removed, and the pellet was dissolved in 20 μ L RNase free water (Invitrogen).

2.3.6 Reverse Transcription and qPCR

The isolated RNA was transcribed to cDNA using the GoScript™ Reverse Transcriptase kit (Promega). Briefly, 1-4 μ L of RNA was added (1 μ g total RNA) and combined with 1 μ L of Random Hexamer (Invitrogen) and RNase free water (Invitrogen) to 5 μ L. The sample was loaded into the MYCycler (BioRad) and ran with the program: 70°C for 5 mins, 4°C for 2 mins. Then the reverse transcriptase reaction mix containing 4 μ L GoScript™ 5 $\times$ Reaction Buffer (Promega), 1.5 μ L 25 mM MgCl₂, 1 μ L of 10 mM dNTP Mix (GeneDirex), 0.5 μ L GoScript™ Reverse Transcriptase and RNase free water (Invitrogen) to a total volume of 15 μ L was added to the RNA solution in the MYCycler (BioRad). The sample was then run with the program: 25°C for 10 mins, 42°C for 60 mins, 70°C for 15 mins, finishing at 4°C and then the samples were stored at -20°C until use.

Primers used were previously designed in the lab and spanned at least two exons in length.

Table 1. List of primers used for RT-qPCR analysis.

Common Name	Gene Abbreviation	Primer Direction	Sequence
	<i>GAPDH</i>	Forward	TGAAGGGGTCGTTGATGG

Glyceraldehyde 3-phosphate dehydrogenase (Housekeeping)		Reverse	AAAATGGTGAAGGTCGGTGT
Tissue inhibitor matrix metalloproteinase 1	<i>TIMP1</i>	Forward	GTAAGGCCTGTAGCTGTGCC
		Reverse	AGGTGGTCTCGTTGATTCGT
Tissue inhibitor matrix metalloproteinase 2	<i>TIMP2</i>	Forward	GGATTCGAATGACATCTAT
		Reverse	GAATCCTCTTGATGGGGTTG
Matrix metalloproteinase 9	<i>MMP9</i>	Forward	AAGGACGGCCTTCTGGCACACGCCTTT
		Reverse	GTGGTATAGTGGGACACATAGTGG
Cluster of Differentiation 86	<i>CD86</i>	Forward	TCAGTGATCGCCAACTTCAG
		Reverse	TTAGGTTTCGGGTGACCTTG
Cluster of Differentiation 38	<i>CD38</i>	Forward	TGATCGCCTTGGTAGTAGGG
		Reverse	AGCCTCAGAAGCCAACTCAC
Cluster of Differentiation 206	<i>CD206</i>	Forward	CAAGGAAGGTTGGCATTGT
		Reverse	CCTTTCAGTCCTTTGCAAGC
Collagen type III alpha 1 chain	<i>Coll3A1</i>	Forward	TGGTCCTCAGGGTGTAAGG
		Reverse	GTCCAGCATCACCTTTTGGT

Samples for RT-qPCR were prepared by combining 10 μ L SYBR® Select Master Mix (Thermo Fisher), 0.8 μ L of 10 μ M forward and reverse primers combined (Table 1), 7.7 μ L ddH₂O, 0.5 μ L iCycler iQ External Well Factor Solution (BioRad), and 1 μ L of cDNA. Samples were then loaded into the iCycler (BioRad) and the thermocycler program used was as follows: 50°C for 2 mins, 95°C for 2 mins for activation, 45 cycles of denaturation at 95°C for 15 sec followed by annealing/extending at 58°C for 1 min, 55°C for 30 sec, 95°C for 30 sec followed by 60°C for 1 min, and a final melting curve at 60°C for 10 sec for 70 cycles. Data was analyzed using the $\Delta\Delta C_t$

method, where each experimental sample was normalized to the GAPDH housekeeping gene and the PBS control samples.

2.3.7 Protein isolation and Western blot analysis

Protein was isolated from flash frozen tissue samples. Tissue was harvested from freshly euthanized animals, which were sacrificed 7 days after hydrogel treatment, and the wound area was harvested and homogenized in 1 mL of RIPA buffer (1 M Tris HCl, pH 6.8, 5 M NaCl, 1% SDS, 97% sodium deoxycholate, Triton-X 100, ddH₂O and 1× Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific)) per 25 mg of tissue using 0.5 mm zirconium silicate beads (Next Advance) for 10 mins in a Bullet blender tissue homogenizer (Next Advance). The samples were agitated for 2h at 4°C followed by centrifugation at 12,000g for 20 mins. The protein concentration was determined by a bicinchoninic acid assay (BCA, Thermo Fisher Scientific), and 20 µg of protein was added per well in the Western gel. The supernatant was frozen at -80°C until use.

The separating gel was prepared using 6.9 mL ddH₂O, 4.8 mL 40% acrylamide, 4 mL 1.5 M Tris HCl (pH 8.8), 160 µL 10% SDS, 160 µL 10% ammonium persulfate (APS), and 16 µL tetraacetythylenediamine (TEMED), and the gel plate was filled to 1 cm from the top. Isopropanol was added to the top and left to solidify. The stacking gel was prepared using 5.8 mL ddH₂O, 1.5 mL 40% acrylamide, 2.5 mL 0.5 M Tris HCl (pH 6.8), 100 µL 10% SDS, 100 µL 10% APS, and 10 µL TEMED, and added on top of the separating gel with a 15-well comb inserted. The solidified gels were wrapped in moist paper towel and placed at 4°C overnight. Samples were prepared by adding 5× Loading buffer (Laemmli buffer: 3.55 mL ddH₂O, 1.25 mL 0.5 M Tris

HCl, pH 6.8, 2.5 mL glycerol, 2 mL 10% SDS, 0.2 mL 0.5% Bromophenol blue) and boiled at 95°C for 10 mins, then loaded into the 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with one well containing 5 μ L EZ-RUN Pre-Stained Rec Protein Ladder (Fisher BioReagents). Once loaded, the chamber was filled with Running buffer (25 mM Tris Base, 192 mM glycine, and 3.5 mM SDS with ddH₂O) and run at 120 V for approximately 90 mins. Once the dye-front reached the bottom of the gel, the run was stopped, and the proteins from the gel were transferred to a nitrocellulose membrane for 90 mins at 100 V, in a chilled chamber containing 100 mL of 10 $\times$ transfer buffer (25 mM Tris Base and 192 mM glycine) with 200 mL methanol and 700 mL ddH₂O. Next, the membranes were cut and stained with Ponceau S to ensure bands were present prior to blocking in 5% w/v dry milk in Tris-Buffered Saline with 0.1% Tween-20 (TBST) (100 mL of 10 $\times$ TBS (50 mM Tris Base, 150 mM NaCl with H₂O, pH 7.4 (adjusted with HCl)), 900 mL ddH₂O and 1 mL Tween-20 (Sigma)) for 1h at room temperature on a plate shaker (Scilogex). Primary antibodies were added to the blots, diluted in blocking solution: TIMP1 (1:5000; abcam; ab38978), MMP9 (1:1000; abcam; ab38898), and loading control α -tubulin (1:5000; cell signalling) and incubated overnight at 4°C. The blots were then washed 3 times with TBST for 10 mins before the secondary antibodies were added, 1:10,000 dilution of anti-Rabbit IgG Secondary HRP-linked (Novus) and 1:1000 anti-Mouse IgG, HRP-Linked (Cell Signaling Technologies). After 1h of shaking at room temperature, the blots were washed 3 times with TBST for 10 mins before incubating the blots with equal volumes of Novex ECL HRP Chemiluminescent Substrate Reagent Kit (AP Chemiluminescent Substrate and AP Chemiluminescent Substrate Enhancer (20 $\times$)) for 5 mins in the dark. The blots were then developed using CL-XPosure Film (Thermo Scientific), scanned and analyzed on ImageJ using pixel quantification of protein bands, and normalized to the β -actin loading control.

2.4 *In vivo* hydrogel testing

2.4.1 Animal surgery

Eight week old female C57Bl/6 mice were anesthetized with 2.5% isoflurane through a nose cone inhaler. Their backs were shaved and washed with 70% ethanol and two full thickness dorsal skin wounds were made with a circular punch, 6 mm in diameter. The left wound was used as a control while the right wound received the treatment. The skin surrounding the wound was covered with sterile filter paper and the mice received four 5 sec AgNP spray treatments with 2 mins in between each spray treatment. The hydrogel treatment with and without MSTCs was then applied to the right wound. MSTCs were collected from the skin excised from the wound. Mice were randomly assigned to control or treatment groups. The wounds were kept open with a silicone disk covered with Tegaderm™ and secured with 6 sutures. All animals were monitored for signs of inflammation, and pain was managed by buprenorphine administered pre-surgery. Wound size was monitored by imaging the wounds at the time of the incision and at day 7. The mice were sacrificed seven days following the treatments and organs were freshly harvested for ICP-MS (see below) or tissue samples were isolated for histological analysis (see below).

2.4.2 Silver content quantification

Silver infiltration into the organs was measured following a protocol previous described by our lab (66, 87, 88). Briefly, organs were harvested from freshly euthanized animals, frozen, and freeze-dried for four days. The resulting dry specimens were digested in a *DigiPREP* MS system (SCP Science). Total silver concentration was determined by inductively coupled plasma-mass spectroscopy (ICP-MS; Agilent 7700x) and silver content determined monitoring the 107 m/z signal (100 ms integration), using Argon as a carrier gas (0.85 ml/min, Ar plasma gas flow: 15

L/min). A sample size of $n=6$ was used for the hydrogel treatment with the CLKRS-AgNP and $n=3$ for the PBS treatment.

2.4.3 Histology

Mouse skin samples were harvested, fixed with 4% PFA for 10 mins, rinsed in $1\times$ PBS, then placed in 15% sucrose solution for 4h at 4°C , and 30% sucrose solution overnight at 4°C . The samples were then frozen in OCT (Fisher). Slides with $10\text{ }\mu\text{m}$ tissue sections were prepared using a cryostat device (Microm HM550 Cryostat, Thermo Scientific). The sections were stained with Masson's Trichrome or hematoxylin and eosin (H&E) staining. Images of the stained sections were taken using a Zeiss Axio Imager.A2 microscope and analyzed with ZenBlue (2011-2012) digital image software. Epithelial thickness and collagen content were quantified using ImageJ software.

2.5 Anti-microbial fabrics

2.5.1 General fabric synthesis protocol

Fabrics were synthesized by A. M. Montagut at the Department of Chemistry and Centro de Innovación en Química Avanzada (ORFEO-CINQA), Barcelona. Materials were received and used without modification. Briefly, a piece of cotton fabric ($3\times 3\text{ mm}$) was washed in a solution of soap and K_2CO_3 under reflux for 3h. The cotton fabric was then dried and placed in a 1 M NaOH solution for 1h. Next, the fabric was washed with anhydrous acetone and dried. The fabric was then placed in a multi-reactor tube with a 0.23% W/M solution of the antibiotic molecule that was to be anchored to the fabric in 10 mL of anhydrous tetrahydrofuran (THF). The set up was left under reflux for 3 days.

2.5.2 Antimicrobial assays

The antimicrobial properties of the fabrics were tested against *Staphylococcus aureus* (ATTC 25293) and *Pseudomonas aeruginosa* (PAO1). Overnight cultures were prepared by resuspending one colony in 2.0 mL of LB broth and culturing in an orbital shaker at 37°C for 18h. The overnight culture was diluted to 10⁵ CFU/mL in 10% LB broth and a 3x3 mm piece of fabric was incubated in the bacterial solution for 18h. The number of surviving colonies were quantified by diluting the sample and plating 10 µL of the diluted solution on an agar plate. The agar plates were then incubated at 37°C for 16h and the number of colonies were counted. Fabrics were tested with an n=4.

2.5.3 Anti-biofilm assay

Pseudomonas aeruginosa (PAO1) and *Staphylococcus aureus* (ATTC 25293) biofilms were grown by incubating a bacterial suspension (10⁷ CFU/ml) in M63 medium broth (3g KH₂PO₄, 7g K₂HPO₄, 2g (NH₄)₂PO₄, 0.4% arginine, 1 mM MgSO₄, 1 L ddH₂O) or tryptic soy broth (TSB) broth (Sigma) on glass surfaces arranged into the wells of a 12-well plate for 6 or 24h at 37°C, respectively. Biofilm prevention was tested by incubating the 3x3 mm piece of fabric with the bacteria as the biofilm was growing. The fabrics were also tested on preformed biofilms by removing the bacterial solution and replacing it with sterile saline, applying the 3x3 mm piece of fabric to the formed biofilm and incubating at 37°C for 12h. After incubation, the fabrics were removed, and the surviving biofilm was quantified by scraping the glass surface and plating the bacteria. Triplicate experiments were completed for each treatment.

2.5.4 Antibiotic leaching assay

Antibiotic leaching was testing by incubating a 3x3 mm square piece of fabric with 1 mL of 10% LB broth overnight. The fabric was the removed and 10^5 CFU/mL of *P. aeruginosa* (PAO1) or *S. aureus* (ATTC 25293) was added to the solution and incubated at 37°C for 18h. Bacteria were plated on agar plates and colonies counted as previously described. Triplicate experiments were completed for each treatment.

2.5.5 Cell number

Human dermal GFP fibroblasts (50,000 cells/well in a 12 well plate (Costar)) were cultured for 3 days in contact with a 3x3 mm piece of fabric. The cells were counted at 24, 48, and 72h. For quantification, 4 random microscopic images were obtained using a JuliFL microscope (NanoEntek) from each well and the number of fibroblasts was quantified. Fabrics were tested in triplicate with a blank fabric used as a control.

2.5.6 Cell viability assay

Viability was assessed using the LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Thermo Fisher Scientific). Human dermal fibroblasts were cultured for 3 days in contact with a 3x3 mm piece of fabric. The treatment was then removed, and the cells were washed 3 times with 1× PBS. Next, the cells were incubated with calcein-AM (1/2000) and ethidium homodimer-1 (1/500) diluted in 1× PBS at 37°C in the dark for 30 mins. The samples were then rinsed again 3 times with 1× PBS before imaging with a Zeiss Axio Observer.A1 fluorescence microscope and analyzed with ZenBlue (2011-2012) digital image software. For quantification, 4 random microscopic images were obtained from each well and the number of live and dead cells were quantified. Fabrics were tested in triplicate.

2.5.7 IL-6 Enzyme Linked Immunosorbent Assay

Eight-week-old female C57Bl/6 mice were anesthetized with 2.5% isoflurane through a nose cone inhaler. The dorsal skin was shaved and washed with 70% ethanol then two dorsal full thickness skin wounds were made with a 6 mm circular punch. The left wound was used as a control and treatment was applied to the right wound. A 5x5 mm piece of fabric was placed in the wound bed. The wounds were kept open with a silicone disk covered with Tegaderm™ and secured with 5 sutures. All animals were monitored for signs of inflammation, and pain was managed by Buprenorphine administered post-surgery. The animals were sacrificed 24h after treatment and the wound area was harvested and then homogenized in homogenization buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton-X, 0.5% sodium deoxycholate, 1 mM PMSF, 1× Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific)) using 0.5 mm zirconium silicate beads (Next Advance) for 15 mins in a Bullet blender tissue homogenizer (Next Advance) followed by centrifugation at 10,000g for 10 mins. The supernatant was frozen at -80°C until use. All samples were normalized to a starting concentration of 1 mg/mL of total protein, determined by bicinchoninic acid assay (BCA, Thermo Fisher Scientific). The ELISA assay (Thermo Fisher Scientific) was completed as per the manufacturer's instructions. Briefly, freshly prepared IL-6 standard (10,000 pg/mL) was serially diluted to a working range of 0 – 500 pg/mL and 100 µL of samples and standards were added to wells pre-layered with designated 1° antibodies and incubated for 2h at room temperature. The plate was washed 4 times with wash buffer, followed by addition of 100 µL of mouse IL-6 biotin conjugate solution, which was incubated for 30 min at room temperature. The plate was then washed 4 times with wash buffer. Then 100 µL of streptavidin-HRP (Thermo Fisher) was added to the wells and incubated for 30 min at room temperature. The plate was then washed 4 times with wash buffer and 100 µL

of chromogen was added to the wells and incubated for 30 min at room temperature in the dark. The reaction was halted by the addition of 100 μ L of stop solution and the absorbance was read at 450 nm. Controls were prepared with PBS without biotin conjugate and streptavidin-HRP. The concentration of IL-6 in samples was determined using a standard curve generated from the standard IL-6 solutions.

2.6 Statistical analysis

Linear regression analysis and statistics for nanoparticle sizes (mean, average, standard deviation, and standard error) were carried out in Kaleida Graph 4.5®. One-way ANOVA, Holm-Sidak analysis, and Student's t-test (unpaired data with unequal variance) using a confidence interval of $p < 0.05$ were used to determine statistically significant differences. An outlier test with a confidence interval of $p < 0.05$ was completed for the PCR and Western Blot analysis results. Analyses were carried out in Kaleida Graph 4.5®.

3.0 NANOpoLC Algorithm Correcting Nanoparticle Concentration with Polydispersity

3.1 Introduction

The study of nanoparticles and their interactions in several environments, particularly biological environments, is steadily increasing (68, 75, 89). For example, incorporating nanoparticles into biomaterials in order to modify the physical and therapeutic properties of the material is becoming increasingly popular, and nanoparticles are being used for the development of new technologies in fields such as drug delivery, imaging, sensing, and tissue engineering (75, 89, 90). Unfortunately, determining the precise concentration of nanoparticles in solution remains a difficult task (61, 75, 89, 90). Today, nanoparticle concentration is typically estimated by determining the volume of material used to synthesize the nanoparticles with the mass and the density of the material used. Next, electron microscopy is used to determine the average size of the particles, which is converted into volume, and from these values the concentration of the nanoparticles in solution can be calculated. This calculation relies heavily on estimates and assumes a monodisperse solution. Other methods used to determine nanoparticle concentration, such as using sensors, microscopy, or gravimetric analysis to determine concentration, are often limited by sample dispersity and result in an estimated value (90).

Due to a lack of nanoparticle homogeneity and high batch-to-batch variability, even under consistent synthesis conditions, calculating the concentration of nanoparticles in solution remains difficult (61, 75, 90). This variability is largely a result of low concentration of nanoparticles in solution and the polydispersity of the sample. Polydispersity of nanoparticle samples can occur during chemical synthesis or with the addition of capping agents (75). The discrepancy in sample concentration therefore affects the reliability of the data collected. For example, results can show

that binding affinities for the same system differ by several orders of magnitude (75, 91). Sample polydispersity can be corrected for by considering the distribution of the nanoparticle size in the sample. Based on our review of the literature, there is no algorithm that accounts for sample size distribution when calculating nanoparticle concentration. Therefore, we developed and tested the Nanoparticle Polydispersity Corrector (NANoPoLC) algorithm to correct for sample polydispersity (92).

3.2 Results

Sample polydispersity for NPs in solution is often quite high and overlooked with traditional NP concentration calculations. This can result in significant discrepancies in results obtained when testing NPs in various environments, for example when determining the minimal inhibitory concentration (MIC) of NPs as antibacterial agents (75, 90). We developed the Nanoparticle Polydispersity Corrector (NANoPoLC) algorithm which considers polydispersity when calculating NP concentration and allows one to determine NP concentrations for size distributions that differ from a traditional Gaussian distribution (92). The NANoPoLC algorithm is shown in Equation 1, and a detailed derivation of the equation can be found in the published article (92).

$$[NP] = \frac{2[S]}{\sum [F(d_i)g(d_i) + F(d_{i+1})g(d_{i+1})]h}$$

Equation 1. NANoPoLC algorithm to correct for sample dispersity when calculating NP concentration. [S] represents the initial concentration of the nanoparticle precursor, [NP] denotes the nanoparticle concentration, F(d) corresponds to the number of atoms for a nanoparticle precursor with the diameter “d”, and g(d)h is the ratio of the number of nanoparticles with diameter d to the total number of nanoparticles with the size distribution described by the function “g”(92).

In order to test the algorithm in the present study, we synthesized two different sizes of AgNPs which was completed using two different amounts of sodium citrate, which acts as both the reducing and capping agent. As shown in Figure 3 A, the two batches of AgNPs were synthesized by combining silver nitrate and I-2959, the photo-initiator, along with either 0.2 or 1.0 mM of citrate. The samples were irradiated under UV light for 30 mins. The sample size was then assessed using two methods: dynamic light scattering (DLS) and transmission electron microscopy (TEM). The particle size distribution from each sample and measurement type was plotted (Figure 3 B, C). The results indicate that the 0.2 mM AgNPs are larger than the 1.0 mM AgNPs, which is expected because less capping agent allows the NPs to grow larger in size, and also results in increased sample polydispersity. There is also a noticeable difference in size distribution determined for a representative sample from the DLS and TEM measurements. DLS results indicate that the mean diameter of the 0.2 mM AgNPs is 10 nm and the 1.0 mM AgNPs is 5.2 nm, whereas TEM results indicate the 0.2 mM AgNPs have a mean diameter of 19 nm and the 1.0 mM AgNPs have a mean diameter of 7.0 nm. This variation in size measurements could be the result of the large sample size for the DLS compared to TEM, where only 1100 NPs were measured, or the fact that TEM samples are dried prior to imaging, which could affect aggregation. This could also account for the increase in sample polydispersity seen in the TEM samples compared to the DLS results.

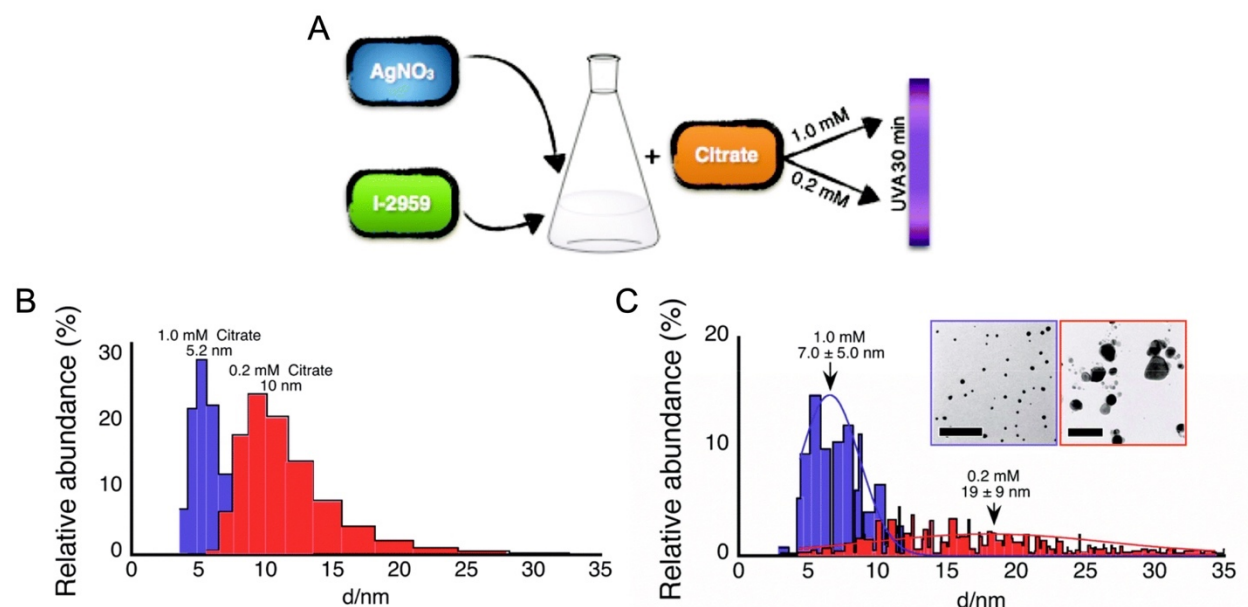


Figure 3. AgNP synthesis and size distribution. (A) Schematic of the preparation of AgNP with two size distributions as a result of different citrate concentrations (0.2 and 1.0 mM). Silver nitrate and I-2959, the photo-initiator, were combined in aqueous solution and then the sodium citrate was added at a concentration of 0.2 or 1.0 mM. Samples were degassed for 30 min and irradiated for another 30 min under UVA. (B) Size distribution of AgNPs synthesized with two different sodium citrate concentrations: 1.0 (purple) and 0.2 (red) mM, determined by dynamic light scattering. Size distribution represents the average mean diameter determined from nine independent batches of nanoparticles. (C) Size distribution of 1.0 mM (purple) and 0.2 mM (red) AgNPs determined by transmission electron microscopy (TEM). Values determined by measuring 1100 individual nanoparticles from TEM images. Inset: representative TEM images for the samples (scale bars are 100 nm). Adapted from Lazurko *et al.*, 2018 (92). Reproduced with permission of The Royal Society of Chemistry.

Calculating the concentrations of the AgNP solutions using the TEM data and the NANoPoLC algorithm resulted in calculated concentrations being significantly smaller than values calculated using the NANoPoLC algorithm with the DLS data or assuming a monodispersed solution (Table 2). For the remainder of this experiment we focused on the DLS data due to the higher sample size obtained, and because DLS is less costly, more accessible, facile, and rapid than TEM. However, there may be some cases where TEM will be superior to using DLS, for example when using a large protecting agent that can interfere with DLS; under these

circumstances, it is important to consider a large sample size (>1000 NPs) from several batches to determine the sample size distribution.

Table 2. Comparison of nanoparticle concentrations calculated assuming monodispersity and polydispersity. Uncorrected calculation refers to the concentration calculation which assumes a monodisperse NP population. This calculation uses the concentration of metal divided by the total number of atoms. 1:1 mix refers to a sample containing a 1:1 mixture of 0.2:1.0 mM AgNPs. *These values show the mean size calculated from 9 sample replicates and the standard deviation from the mean (SD) which are assumed to be proportional to the concentration determined for each sample (4.3 ± 1.2 nm and 10 ± 1.2 nm for 1.0 and 0.2 mM, respectively). **Values calculated using NANoPoLC from data obtained from 9 independent samples by DLS, $\pm$ value is standard error. ***Values calculated using NANoPoLC from data acquired from 9 independent samples by TEM. $^{\pm}$ Value calculated from error propagation from 0.2- and 1.0-mM citrate concentration values. $^{++}$ A sample size of $n=3$ was used for this data set. Adapted from Lazurko *et al.*, 2018 (92). Reproduced by permission of The Royal Society of Chemistry.

Calculation Method	AgNP Concentration (nM)		
	0.2 mM citrate	1.0 mM citrate	1:1 mix
Uncorrected*	3.5 ± 1.1	44 ± 12	$24 \pm 4.8^+$
NANoPoLC – DLS**	0.017 ± 0.003	0.83 ± 0.30	$0.39 \pm 0.20^{++}$
NANoPoLC – TEM***	0.007	0.15	-

To further illustrate the importance of a more reliable NP concentration calculation, we measured the association of human serum albumin (HSA) to AgNPs of different sizes by monitoring tryptophan fluorescence (Figure 4). Fluorescence intensity of tryptophan 214 was monitored, since it is the only tryptophan residue in the HSA sequence (92). Upon addition of HSA to the AgNP solution, we notice a visible red shift in the surface plasmon band of the NPs, likely a result of dielectric constant changes (Figure 4 B, C) (76, 85). We also used fluorescence intensity of HSA to determine the association of the HSA to AgNPs (Figure 4 D). Microliter volumes of HSA was added to the AgNP solutions and the change in tryptophan fluorescence was monitored at 340 nm, representing the emission wavelength of tryptophan. Fluorescence intensities were plotted in Stern-Volmer plot (Figure 4 D), where the slope corresponds to K_{SV} , which is the Stern-Volmer constant that relates the quenching of the fluorescence to the AgNP presence in solution. This slope gives information regarding the

association of HSA to the AgNPs. The results obtained indicate that 0.2 mM citrate AgNPs have a K_{SV} value of $176 \pm 11 \times 10^9 \text{ M}^{-1}$ calculated with NANoPoLC, and $0.86 \pm 0.05 \times 10^9 \text{ M}^{-1}$ calculated with the uncorrected concentration calculation. Similar trends are observed for the 1.0 mM citrate AgNPs which have K_{SV} values of $4.3 \pm 0.21 \times 10^9 \text{ M}^{-1}$ calculated with NANoPoLC and $0.083 \pm 0.004 \times 10^9 \text{ M}^{-1}$ calculated with the uncorrected method, and the 1:1 mixture of 0.2:1.0 mM AgNPs have values of $8.4 \pm 0.5 \times 10^9 \text{ M}^{-1}$ calculated with NANoPoLC and $0.070 \pm 0.004 \times 10^9 \text{ M}^{-1}$ for uncorrected concentration calculations. By correcting for sample polydispersity when calculating K_{SV} values, there is a 200-fold change for 0.2 mM citrate AgNPs and a 52-fold change for the 1.0 mM AgNPs. The 1:1 mix, which represents a polydisperse sample indicated a large proportion of smaller NPs, as the K_{SV} value calculated by NANoPoLC was smaller than the 0.2 mM sample and more similar to the 1.0 mM sample.

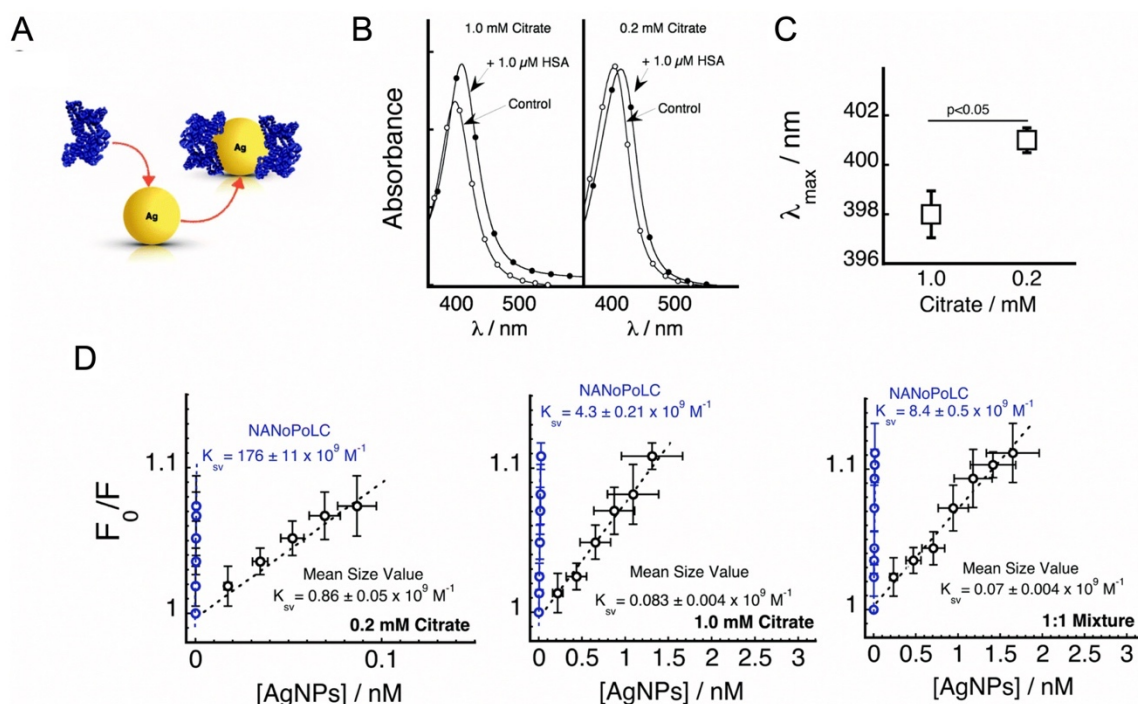


Figure 4. Association of human serum albumin (HSA) with AgNPs. (A) Diagram of the association of HSA (15 μM) to AgNPs. (B) Plasmonic absorption spectrum of AgNPs synthesized with 1.0 and 0.2 mM citrate and the associated plasmon shift associated with the addition of HSA. (C) Maximum absorption wavelength of the two sizes of AgNPs. p value calculated from t-test for unpaired data. (D) Normalized fluorescence intensity of HSA as a function of AgNP concentration determined by DLS for 0.2 mM AgNPs (left), 1.0 mM AgNP (middle), and a 1:1 mixture of 0.2 mM:1.0mM

AgNPs. From Lazurko *et al.*, 2018 (92). Reproduced with permission of The Royal Society of Chemistry.

3.3 Discussion

Nanomaterials are increasingly being incorporated into many different fields, making it important to know the true concentration of NPs in solution to ensure reliability between tests (59, 60, 89, 92). The NANoPoLC algorithm allows a more accurate NP concentration calculation by correcting for the sample polydispersity. This is especially useful for nanoparticle solutions that do not follow a traditional Gaussian size distribution, as well as those that have been shown to have high size variability, even when synthesized under the same conditions (61, 75, 90). It will also allow individuals to use more homogeneous samples and gain a greater understanding of the relation of nanoparticle size to their potency or effect as a treatment.

3.3.1 Polydispersity measurements

The results gathered from testing the algorithm highlight the importance of large sample sizes when calculating NP size distribution and indicated that DLS measurements may be superior to TEM measurements due to the large sample size tested with DLS versus TEM, and due to the fact that NPs remain in aqueous solution with DLS measurements (61, 89). However, DLS measures hydrodynamic radius, while TEM measures size, so if there is a large capping or protecting agent being used, TEM will be superior because it will allow one to measure the NP size without considering the capping agent (61, 89). It is extremely important to understand both the size of the NPs in solution and the concentration, as these can both impact the behaviour of the NPs as well as their physical and chemical properties (93).

The results demonstrate the importance of correcting for NP polydispersity, as there was a large difference in NP concentration determined using the uncorrected calculation vs. the NANoPoLC algorithm. It has been shown that DLS data can be skewed when NP samples are not homogeneous, which likely accounts for the large difference in concentrations determined with the uncorrected calculation and NANoPoLC (89). Due to the many magnitudes of difference in concentration, these results can largely impact the outcomes observed, for example when applying these NPs as a treatment.

3.3.2 Effect of polydispersity on association constants

The association of HSA to the AgNPs and the change in fluorescence intensity also indicate significant differences in the extent of protein association to NP solutions of different sizes. Results suggested that smaller NPs in solution determined the extent of protein association, which was observed with the 1:1 mix sample having K_{SV} values more similar to the smaller sized 1.0 mM citrate AgNP solution than the larger 0.2 mM AgNPs. It is not possible to directly compare the association constants to the literature due to the lack of corrections for sample polydispersity. However, Gebauer *et al.* determined an association constant of $0.014 \times 10^9 \text{ M}^{-1}$ for HSA to AgNPs with a diameter of 42 nm (94), and Roy and Das showed an association constant of $0.912 \times 10^9 \text{ M}^{-1}$ for the small molecule phenylalanine to AgNPs ranging from 20 to 40 nm in diameter (95). These values obtained from the literature are both in the same order of magnitude as the uncorrected values shown here, which shows the impact of not correcting for polydispersity. Overall, these results indicate that the NANoPoLC algorithm corrects for sample polydispersity, and these tests highlight the impact of uncorrected size values on concentration and association constants, which can affect results obtained when using NPs for various applications.

3.3.3 Future directions

One limitation of this study is that the algorithm assumes that all of the ionic silver is reduced into the nanoparticles, therefore it may overestimate the actual silver concentration available for nanoparticles synthesis. The AgNPs were synthesized using I-2959, a strong, effective reducing agent, therefore assuming complete reduction of the silver is reasonable given the protocol.

Additional studies must be completed in order to fully understand how the extent of association varies with nanoparticle size, as was observed. Moreover, additional studies should refine the algorithm to be applicable to samples with mixed NP geometries, elaborate on the catalytic activity of the NPs, quantify binding, and examine how available surface area impacts protein binding.

4.0 Multilayered hybrid biomaterials for treatment of diabetic foot ulcer wounds

4.1 Introduction

Diabetes is one of the most prevalent chronic illnesses with many associated adverse events, such as DFUs (1-5). DFUs are difficult to treat as a result of infection, inflammation, and poor circulation (11, 18). Unfortunately, current treatments for DFUs are severely lacking, resulting in many patients requiring amputation (2). Hydrogels are being applied as a treatment for DFUs because their 3D structure mimics the extracellular matrix, in addition to their ability to keep the wound environment moist, increase cell proliferation and eliminate scab formation (18, 43, 44). Moreover, collagen hydrogels are useful for DFU wound healing and tissue engineering because collagen is the most abundant protein in the ECM, so it creates a matrix that is similar to the endogenous environment (29, 33, 34, 41, 50, 51).

In this project we hypothesize that by combining the antimicrobial properties of CLKRS-AgNPs in conjunction with a novel collagen hydrogel embedded with microscopic skin tissue columns, we will provide a regenerative, wound healing environment. The CLKRS-AgNPs was the first layer of this 2-layered approach, which was applied as a strategy to prevent and treat bacterial infection. This was followed by the application of the collagen hydrogel with MSTCs, which is intended to fill the wound and provide dermal structures necessary for wound healing. This is the first 2-layered approach that we are aware of for treating DFUs. It will allow individualized wound treatment because we are able to control the application of both the antimicrobial and hydrogel treatments, in terms of quantity, duration of treatment, and number of applications.

We focused on a collagen hydrogel due to the abundance of collagen in the ECM (22, 36, 43, 49, 51, 96), and tested the incorporation of both chondroitin sulfate and poly-D-lysine (PDL) in the hydrogel. Chondroitin sulfate is a disaccharide that forms glycosaminoglycan chains that attach to proteins (6). Normally these chains are found in the basement membrane of the skin, between the epidermal and dermal layers, but during wound healing they are found in the granulation tissue. It is also found in the body to help repair tendons and bones, and may have implications in wound healing (6). Chondroitin sulfate has also been shown to decrease fasting glucose levels in healthy and diabetic patients (97). Chondroitin sulfate hydrogels have shown promise for tissue engineering applications, including cell therapy and drug delivery (40, 48, 49, 98). Poly-D-lysine was also added to the hydrogels. Poly-L-lysine (PLL) hydrogels have been shown to promote wound healing and prevent infection (99). Our lab had tested PLL hydrogels, but the hydrogels were rapidly degraded, therefore we decided to test the D enantiomer of the amino acid, which is not found endogenously, to reinforce the hydrogel structure and reduce degradation.

4.2 Results

In order to characterize the effectiveness of the hydrogel as a treatment for DFUs, we first optimized the hydrogel synthesis and characterized the physical properties of the hydrogels before completing any *in vitro* or *in vivo* testing of the gels. The hydrogels were developed based on a parental collagen hydrogel formulation that have previously been described by our lab (100-102). Several different hydrogel formulations were tested until we had optimized its properties. Gel 1 is the optimized collagen hydrogel formulation (Figure 5). PDL contains a 5% solution of Poly-D-lysine (PDL) added to the Gel 1 formulation, CS contains a 25% solution of chondroitin sulfate

(CS) solution added to the Gel 1 formulation, and the CS/PDL gel contains both the 5% PDL solution and the 25% CS solution. So, in total, 4 gel formulations were tested.

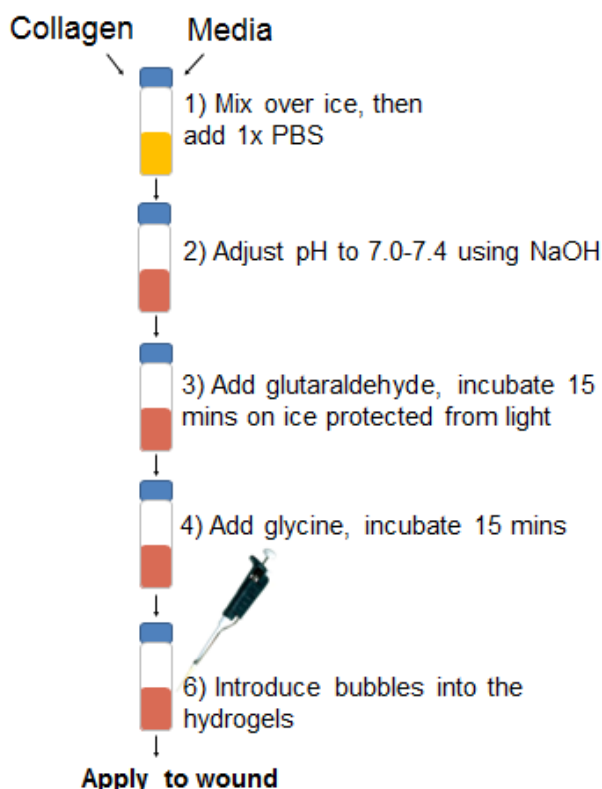


Figure 5. Depiction of collagen hydrogel synthesis. Schematic of hydrogel formulation. For the PDL and CS hydrogels, the PDL and CS solutions are added immediately after addition of the glycine.

4.2.1 Physical characteristics of hydrogels

The physical characteristics of the different hydrogel formulations were determined. Differential scanning calorimetry (DSC) was used to determine the denaturation temperature of the collagen in the hydrogels, which show that the collagen degrades around 50°C, well above physiological temperatures (Figure 6 A). Furthermore, the viscosity of the hydrogel formulations was similar between the gels, at around 200 Pa•s, the Fourier-transform infrared spectroscopy (FTIR) results show that the hydrogels all maintain the bonds indicative of collagen structure, indicating that the collagen is not denatured, and the water content of the hydrogels was above

90% (Figure 6 B-D). Microbubbles were added into the hydrogels in order to increase the pore size to allow cells to migrate through the gels. The pore size of the hydrogels varies significantly with the addition of the microbubbles, from $16\pm4\ \mu\text{m}$ without bubbles to $39\pm21\ \mu\text{m}$ with bubbles for Gel 1, and $10\pm2\ \mu\text{m}$ without bubbles to $31\pm24\ \mu\text{m}$ with bubbles for the PDL gel (Figure 6 E). The crosslinking time also varied significantly, with the Gel 1 and PDL hydrogels solidifying in under 5 minutes, and the CS and CS/PDL hydrogels solidifying in over 60 minutes (Figure 6 F). Last, the collagenase degradation assay indicates that all hydrogels degrade significantly more slowly than the parental formulation ($p<0.05$). Moreover, the PDL hydrogel degrades the slowest and the CS hydrogel has the fastest degradation rate of the modified hydrogels (Figure 6 G). Because all the hydrogels showed similar physical properties, with the crosslinking time being the major difference, we decided to use Gel 1 and PDL for future experiments due to their fast solidification times that make testing and application more feasible. Only Gel 1 was used when there was limited tissue sample for testing MSTCs and for RT-qPCR and Western blot to limit the number of animals used.

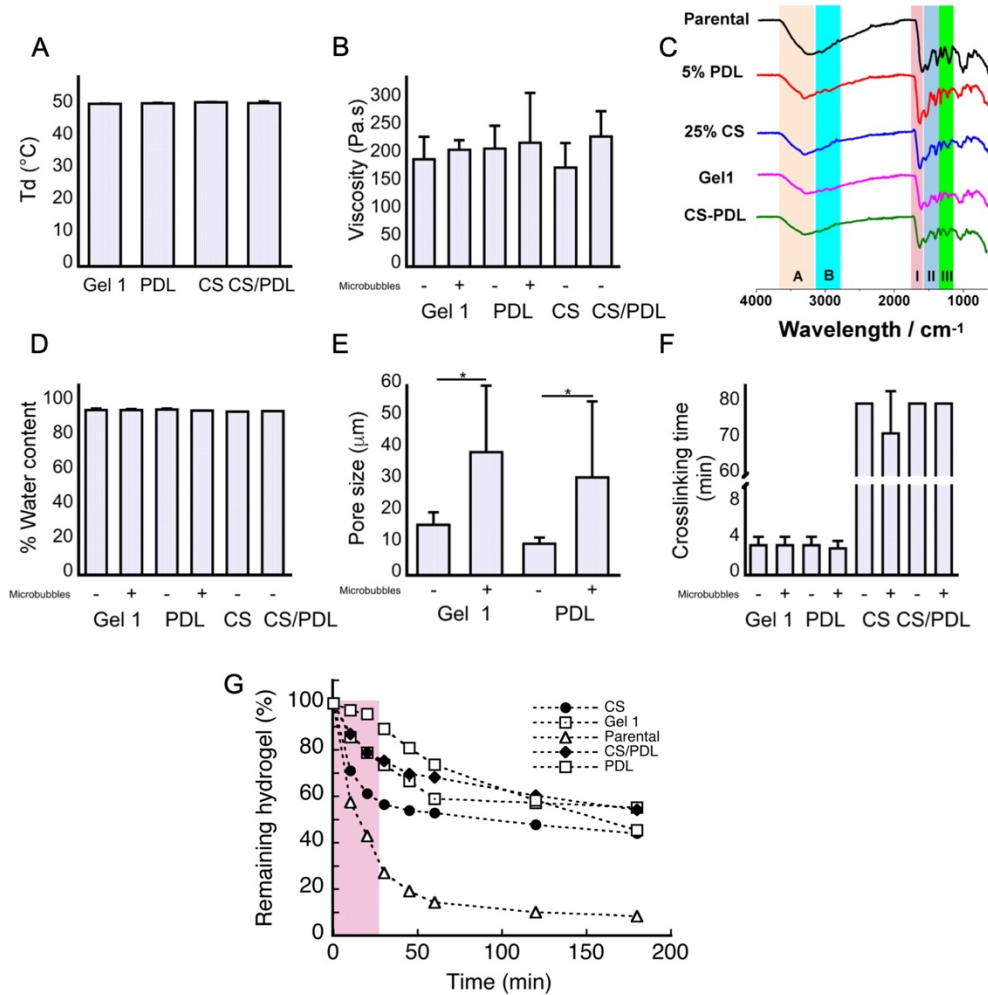


Figure 6. Physical characteristics of the hydrogel formulations. (A) Collagen denaturation temperature determined by differential scanning calorimetry (DSC). (B) Hydrogel viscosity determined with a rheometer. (C) FTIR results indicate that the gels retain collagen structure. (D) Water content was determined by dehydrating and weighing gels determine water mass lost after 120h. (E) Pore size determined by imaging the hydrogels by cryo-SEM results indicate differences in pore size with the addition of microbubbles (*p<0.05), p values determined by Student t-test. (F) Hydrogel crosslinking time was determined by monitoring the time for each hydrogel formulation to crosslink and become a gel. Hydrogels were crosslinked at 37°C and monitored up to 80 minutes. (G) Collagenase degradation was determined by submerging hydrogels in a 5 units/mL collagenase solution and weighing hydrogels at various time points to determine a decrease in hydrogel weight. P values were calculated with one-way ANOVA followed by Holm-Sidak analysis.

4.2.2 Hydrogel biocompatibility

Once the physical characteristics of the hydrogels were determined, we tested the biocompatibility of the hydrogels by monitoring fibroblast proliferation, macrophage polarization, and completing cell viability assays. The fibroblast count, shown in Figure 7, indicates that the fibroblasts proliferate on the hydrogel. This indicates that the hydrogel is capable of supporting cell culture and growth. The results show similar proliferation trends for both the Gel 1 and PDL hydrogels with and without microbubbles.

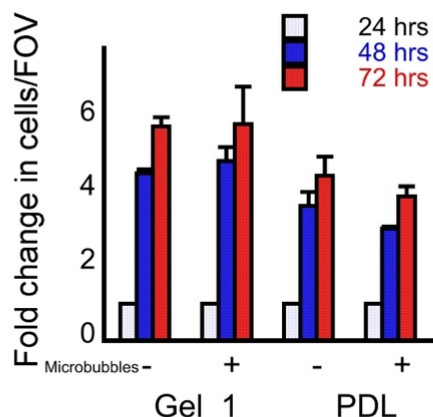


Figure 7. Human dermal fibroblast count. Increase in the number of human dermal fibroblasts per field of view (FOV) measured after seeding 50,000 cells/well on 500 μ L of hydrogel in a 12-well plate. Cells were imaged and counted every 24h for 72h. Cells were kept at 37°C with 5% CO₂. These values correspond to the average of 3 independent samples and the error bars are the standard deviation of the mean.

We then completed a macrophage polarization assay to determine how monocytes polarize when in contact with the hydrogel. Macrophage polarization was monitored by seeding cells on the hydrogels for four days, then staining the macrophages with CD206 for identifying the M2, pro-regenerative macrophages, and with CD86 for the M1, pro-inflammatory macrophages. The results indicate that there are significantly fewer M1 macrophages in both the hydrogel treatments compared to the control (Figure 8).

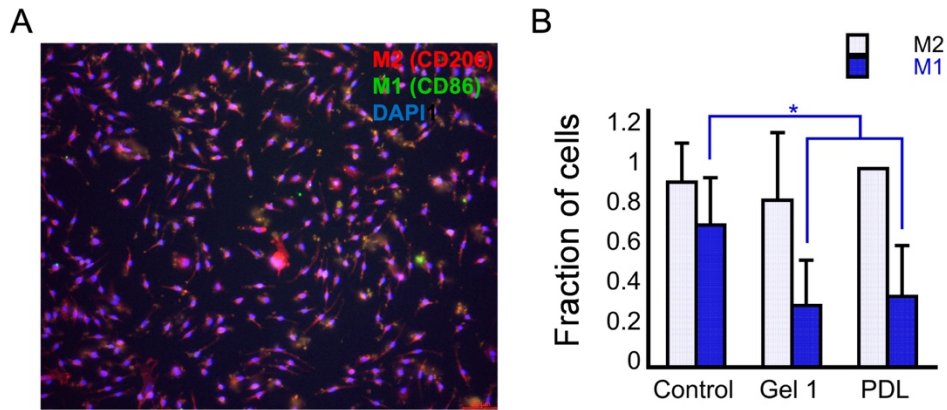


Figure 8. Macrophage polarization upon hydrogel treatment. (A) Representative image of macrophages seeded on hydrogel Gel 1 treatment. (B) Macrophage polarization was determined by plating cells on hydrogels solidified on a coverslip. The cells were left in contact with the hydrogel for 4 days with the media being changed every second day. Cells were then fixed with 4% PFA and stained with CD206 (M2) and CD86 (M1). For quantification, 4 random microscopic images were obtained from each slide and the number of M2 and M1 macrophages were quantified using ImageJ software. Hydrogels were tested in triplicate with a blank coverslip used as a control. These values correspond to the average of 3 independent samples and the error bars are the standard deviation of the mean, p values were calculated with one-way ANOVA followed by Holm-Sidak analysis.

Next, the spreading and viability of the cells from the microscopic skin tissue columns in the hydrogels was tested. This test was to ensure that cells are able to migrate out of the MSTCs and be supported by the hydrogel in order to fill the wound. The results indicate that cells readily migrate out of the MSTCs, and after 14 days are fully confluent in the well plate (Figure 9). The viability of the cells was assessed, and the results show that 96% of the cells remain viable. These tests were completed on MSTCs isolated from murine, porcine, and human skin, and all showed similar results (Figure 10). These results indicate that the hydrogel is suitable matrix for supporting MSTCs.

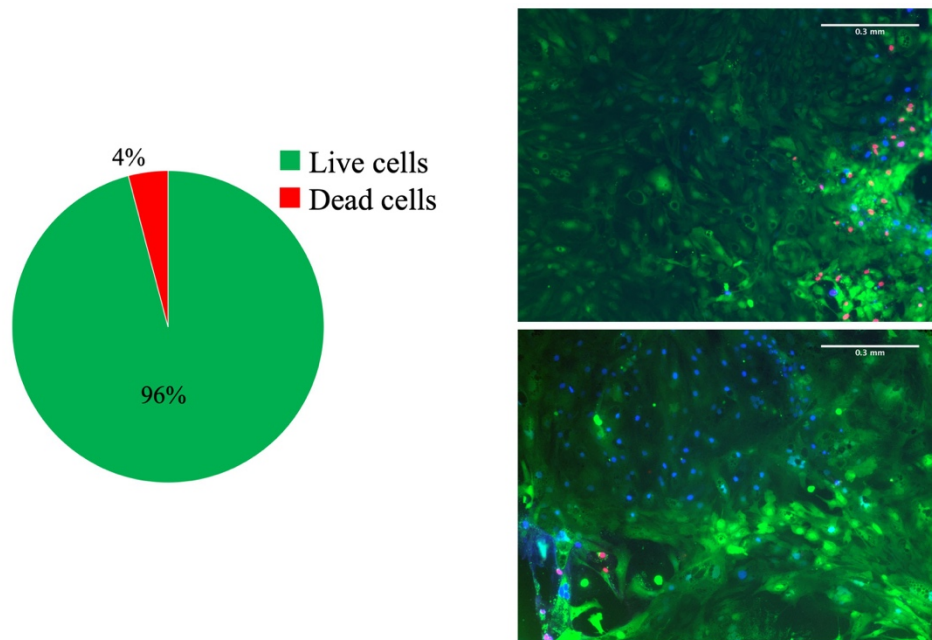


Figure 9. Porcine microscopic skin tissue column viability. MSTCs cultured in Gel 1 hydrogel for 14 days. Microscopic skin tissue column compatibility and viability in hydrogels were assessed by plating approximately 30 columns in each well of a 12-well plate in 250 μ L of hydrogel. Columns were cultured in high glucose DMEM media (10% FBS and 1% penicillin-streptomycin) at 37°C with 5% CO₂. The media was changed every 3 days. Viability of the skin columns and migrating cells was assessed by staining with calcein-AM (green), ethidium homodimer-1 (red), and DAPI (blue). Scale bars represent 0.3 mm. Samples were tested in triplicate.

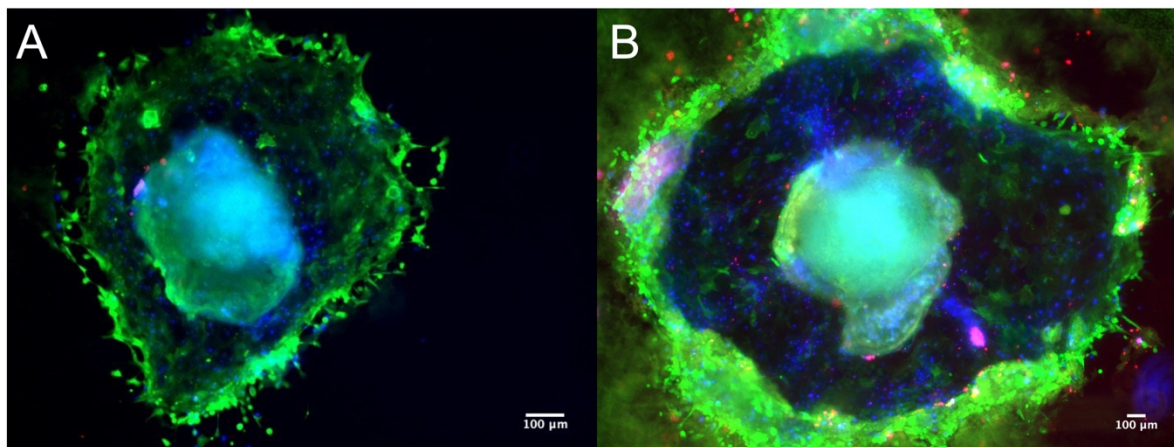


Figure 10. Human microscopic skin tissue columns. Human derived MSTCs cultured in Gel 1 hydrogel for (A) 14 and (B) 21 days. Microscopic skin tissue column compatibility and viability in hydrogels were assessed by plating approximately 30 columns in each well of a 12-well plate in 250 μ L of hydrogel. Columns were cultured in high glucose DMEM media (10% FBS and 1% penicillin-streptomycin) at 37°C with 5% CO₂. The media was changed every 3 days and cultures were kept for up to 21 days. Viability of the skin columns and migrating cells was assessed by

staining with calcein-AM (green), ethidium homodimer-1 (red), and DAPI (blue). Samples were tested in triplicate.

4.2.3 Effect of CLKRS-AgNP and hydrogel treatment on wound healing

Hydrogels were then tested *in vivo* in a murine model. Two 6 mm wounds were created on the dorsal skin of mice (Figure 18, Appendix A). The left wound was used as the internal control, to account for potentially different rates of healing between mice, and the right wound was treated with the hydrogel or PBS control treatment for 7 days. First, silver content in mouse organs was determined by inductively coupled plasma mass spectrometry (ICP-MS). As part of the 2-layered approach, the spray-on CLKRS-AgNPs was first applied to the wound, followed by the application of the hydrogels. The treatment was left for 7 days then the organs were freshly harvested and the silver ion content in each organ was determined by ICP-MS. The results indicate that the majority of the silver ions remain at the wound site, with minute amounts in the other organs. There is some silver ion content present on the skin which could be a result of overspray.

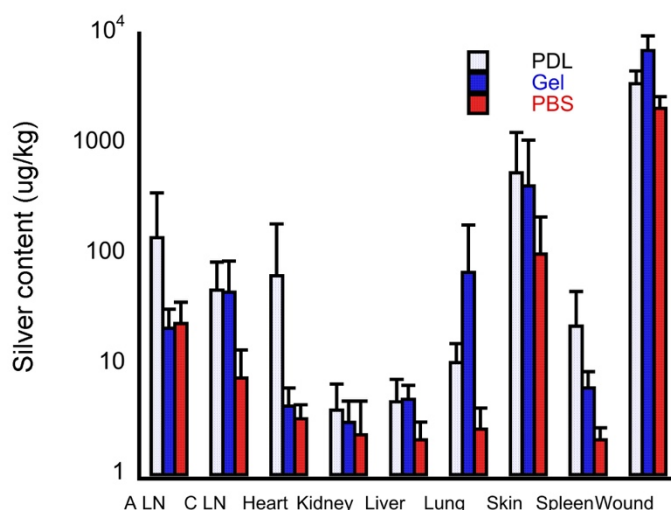


Figure 11. Silver content in mouse organs. Silver ion content measured by ICP-MS in freshly harvested organs 7 days after initial CLKRS-AgNP treatment. Organs were harvested from freshly euthanized animals, frozen, and freeze-dried for four days before ICP-MS analysis. Sample sizes

of 6 mice for the hydrogel treatments and 3 mice for the PBS control and the error bars are the standard deviation of the mean. A LN – axillary lymph nodes, C LN – cervical lymph nodes.

Next, RT-qPCR was completed on freshly harvested skin tissue samples of mice treated with the Gel 1 hydrogel to determine the change in mRNA expression. Several genes of interest were identified, primarily those involved in wound healing such as collagen type III alpha 1 chain, TIMP1, TIMP2, and MMP9, as well as macrophage polarization markers to identify M2 (CD206) and M1 macrophage phenotypes (CD86 and CD38) (103, 104). The results indicate that there is no significant difference in the mRNA expression for the genes studied (Figure 12). There are trends which indicate increasing collagen type III alpha 1 chain expression with Gel 1 and Gel 1 with MSTC treatment, as well as a trend for increased MMP9. Macrophage characterization showed a trend for greater CD206 expression, and a trend for decreased CD38 expression and increased CD86 expression (Figure 12 E-G).

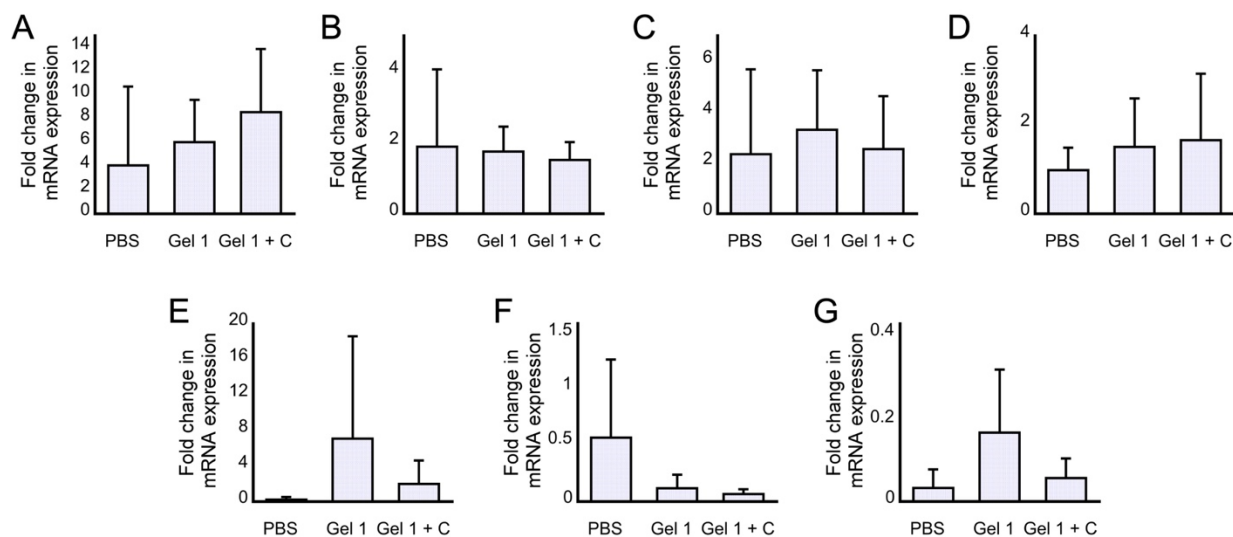


Figure 12. Analysis of change in mRNA expression upon hydrogel treatment in tissue samples. RT-qPCR was performed on mice treated with PBS (n=3), Gel 1 hydrogel (n=3), and Gel 1 hydrogel with MSTCs (n=3) (Gel 1 + C). The change in mRNA expression was tested for the following genes: (A) Collagen type III alpha 1 chain, (B) TIMP1, (C) TIMP2, (D) MMP9, (E) CD206, (F) CD38, (G) CD86. These values correspond to the average of 3 mice per sample group and 3 replicate PCR experiments and the error bars are the standard deviation of the mean.

Western blot analysis was completed to monitor change in protein expression following hydrogel treatment. MMP9 and TIMP1 protein levels were analyzed from tissue samples. The results indicate that there is no significant difference in the level of protein expression, but there is a trend showing a decrease in MMP9 levels with Gel 1 with MSTC treatment and an increase in TIMP1 levels with Gel 1 treatment (Figure 13).

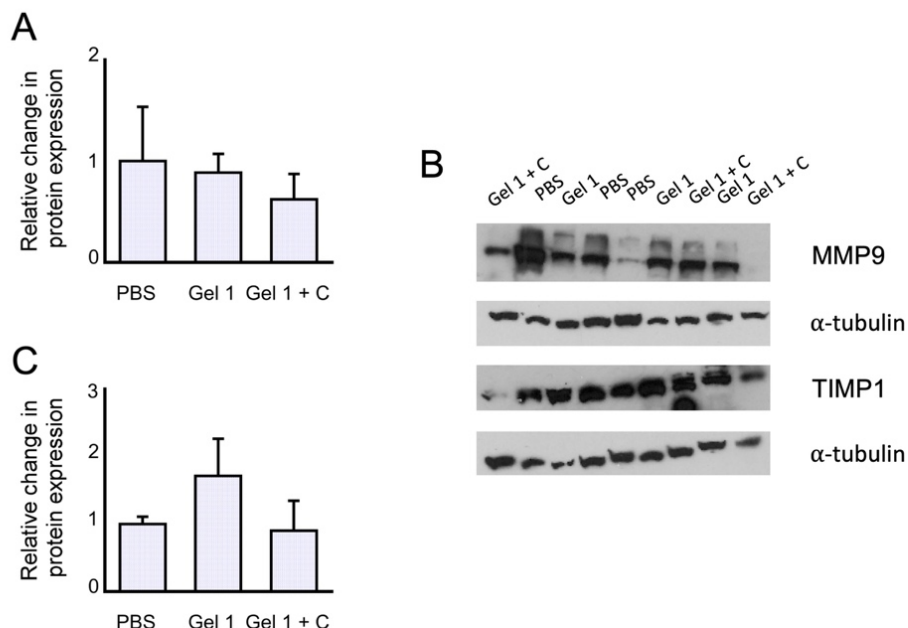


Figure 13. Analysis of MMP9 and TIMP1 protein levels in tissue. Western blot analysis was performed on mice treated with PBS (n=3), Gel 1 hydrogel (n=3), and Gel 1 hydrogel with MSTCs (n=3) (Gel 1 + C). Results were normalized to α -tubulin and compared to the PBS control. **(A)** Relative MMP9 protein levels. **(B)** Western blot results for both MMP9 and TIMP1 as well as the α -tubulin loading control. **(C)** Relative TIMP1 protein levels. These values correspond to the average of 3 mice per sample group and the error bars are the standard error of the mean.

The *in vivo* results of hydrogel treatment in the murine wound model, shown in Figure 14, demonstrate that MSTC and hydrogel treatment results in significantly increased epithelial thickness. Furthermore, hydrogel treatments showed a significant decrease in wound size. Lastly, collagen content at the wound site increased with Gel 1 treatment with MSTCs and PDL with and without MSTCs.

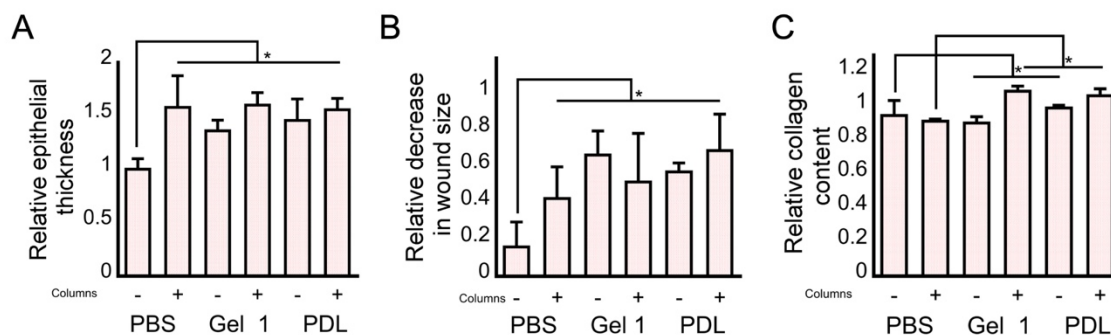


Figure 14. Effect of hydrogel treatment on wound healing. (A) Wound size was determined by imaging the wound at the time of incision and 7 days after treatment. Decrease in wound size was normalized to the internal control (left wound). (B) Epithelial thickness was determined by imaging histological sections stained with Masson's trichrome stain. Thickness was measured on Image J and 3 measurements were taken for each wound. (C) Collagen content at the wound site was determined by imaging histological sections stained with Masson's trichrome stain. The presence of blue dye indicating collagen was quantified in Image J. These values correspond to the average of at least 3 independent samples and the error bars are the standard deviation of the mean. P values were calculated with one-way ANOVA followed by Holm-Sidak analysis.

4.3 Discussion

4.3.1 Physical properties of the hydrogels

First, the hydrogel formulation was optimized based on a parental formulation that had been previously been described by our lab for applications in the heart (100-102). Many different concentrations of PDL and CS were incorporated into the collagen hydrogels, and the physical properties of the optimized formulations were then tested. The thermal stability of the collagen was determined to ensure that the collagen maintained its structure in the hydrogel and upon application to the skin (105). The results indicate that the denaturation temperature of the collagen hydrogels is approximately 50°C, well above human skin temperature. Next, viscosity of the hydrogels was all similar at approximately 200 Pa•s, which is within the same order of magnitude as reported in the literature (106). FTIR was completed to analyze the chemical bonds and molecular composition of the hydrogels (105). Collagen maintains distinct peaks that represent the

amide bonds in the triple helix. Peaks at (A) 3300 and (B) 3087 cm^{-1} are associated with the stretching vibration of the N-H groups, while amide bands at (I) 1650 and (II) 1550 cm^{-1} are from carbonyl group and C-N stretching vibrations and N-H bending, and the band at (III) 1240 cm^{-1} is the result of vibrations from C-N stretching, N-H bending, and CH_2 wagging from the collagen glycine backbone and proline side chains. These results indicate that the type I collagen used to synthesize the hydrogels maintains its triple helix structure (105). The water content of the hydrogels was determined to be approximately 90%, which will allow the hydrogels to maintain a moist wound environment (18, 43, 44). The pore size of a material is important for cell migration (107). It has been demonstrated that a pore size ranging from 20-125 μm is optimal for dermal repair(108). Since the pore sizes of our materials were relatively small, we incorporated microbubbles into the hydrogels in order to increase pore size to facilitate greater cell migration through the gel. By including microbubbles into the hydrogel, we significantly increased the pore sizes to allow cells such as fibroblasts which are approximately 10-15 μm in diameter, macrophages which range from 20-80 μm , and neutrophils which range from 8-10 μm in diameter, to migrate more easily through the hydrogels (109). We expect by increasing the pore size, cells will be able to migrate more easily through the hydrogels and use it more readily as a matrix to heal the wound. The crosslinking time of the hydrogels was very important, as it is not feasible for application in clinic if the hydrogels take more than 15 minutes to crosslink. Hydrogel solidification was monitored by observing a change in the opacity of the hydrogels. Therefore, the solidification time of hydrogels largely influenced the hydrogels chosen for further testing. Our results indicate that the Gel 1 and PDL hydrogels, which take less than 5 minutes to crosslink, are superior to the CS hydrogels which take over an hour to crosslink; therefore, we continued our experiments with the Gel 1 and PDL hydrogels. Last, collagenase degradation indicates the

longevity of the collagen hydrogel. Degradation is important to help rid the hydrogel from the body once the wound has healed, so it is important that degradation occurs. Lack of degradation can result in a foreign-body response by the body (110). The collagenase concentrations used in this assay are higher than endogenous collagenase concentrations, which for DFUs wounds is approximately 2.5×10^{-15} U/mL, indicating that *in situ* the hydrogel may degrade more slowly than indicated by the assay (111).

4.3.2 Hydrogel biocompatibility

The biocompatibility of the hydrogels was tested by monitoring fibroblast proliferation, macrophage polarization, and cell viability. Biocompatibility of biomaterials is essential, but for use in treating DFUs, a material that also possesses wound healing properties would be advantageous (112, 113). Proliferation of cells on a material can provide information regarding the toxicity of the material, shown by a decrease in proliferation and viable cells (112). Our results indicate that the number of fibroblasts seeded on the hydrogel increase over a period of 72h, indicating that the hydrogels are biocompatible and non-toxic. Next, we completed a macrophage polarization assay to assess the inflammatory response to the hydrogels. Monocytes can polarize into M1 or M2 macrophages in response to their environment, with M1 macrophages being a pro-inflammatory response, while M2 macrophages are classified as an anti-inflammatory and reparative response (104); although macrophage polarization is not a distinct end-point, but rather a spectrum of continuous shift and polarization. Macrophages can be identified as M1 or M2 by staining for different protein markers that are more abundant in M1 or M2 populations such as CD86 and CD206, respectively (111). The results from the polarization assay indicate that the macrophages are predominantly M2, with a decrease in M1 macrophages, indicating a pro-

regenerative environment. Last, biocompatibility of the hydrogels with MSTCs was determined by culturing the MSTCs in the hydrogel, similar to how they would be applied *in vivo* as a DFU treatment. *In vitro* results indicate that the cells migrate out of the MSTCs onto the hydrogel to the point of confluency. These cells remain viable for several weeks, indicating that the hydrogel is biocompatible and a suitable matrix to support MSTC culture.

4.3.3 *In vivo* response to hydrogel treatment

The hydrogels were tested in an *in vivo* murine model (Appendix A, Figure 18). First, we tested the organ infiltration of silver ions from the CLKRS-AgNPs spray-on treatment to ensure the silver remained at the wound site, limiting any potential toxic effects of silver in the organs. The results indicate that the silver remained at the wound site, which will allow the treatment and prevention of bacterial infection at the wound site, while reducing any risk of organ toxicity (66, 77).

Next, RT-qPCR was completed on tissue samples isolated from mice treated with hydrogels and MSTCs to analyze mRNA expression of genes associated with wound healing. Collagen type III alpha 1 chain has been shown to be downregulated in diabetics (114, 115). TIMPs and MMPs have an established role in wound healing, repair, and remodelling, and the balance between them is essential for a healthy environment (28-34). Macrophage markers (CD 206, 86, 38) were also analyzed to compare tissue samples to the *in vitro* assay completed to monitor the inflammatory response to the hydrogels. Results also show trends of increased CD206 and CD86 expression with hydrogel treatment, and a decrease in CD38. It is interesting that CD86 and CD38 are both M1 markers and show different trends, but studies have shown that CD38 is a more specific M1 marker for murine macrophages (104). Considering the specificity of the markers, the

results are consistent with the *in vitro* macrophage polarization results and indicate a reparative environment.

Western blot analysis was completed to determine change in TIMP1 and MMP9 protein expression following hydrogel treatment. Similar to the RT-qPCR results, no significance was obtained but trends indicate a decrease in MMP9 protein expression and an increase in TIMP1 expression, especially with the Gel 1 hydrogel treatment. It is possible that because these tests were completed on a small (n=3) sample of healthy mice, we do not observe significant changes in mRNA and protein expression that may be observed in a diabetic mouse model. Therefore, additional experiments should be completed on a larger sample size and further experiments should be completed on diabetic mice with ischemic wounds in order to verify the expression changes in an environment that is more representative of the DFU wound environment.

Last, the wound healing properties of the hydrogel treatment was assessed. After applying the hydrogel dressing to the wound for 7 days, we observed a significant increase in epithelial thickness with hydrogel treatment compared to the PBS control. Similar results were seen with a decrease in wound size and an increase in collagen content with hydrogel treatment. It was observed that alone, the hydrogel and the MSTC treatments have similar wound healing effects, and when combined, we do not observe a synergistic effect, as expected. One explanation is that since these tests were completed on young, healthy mice, the healing potential was maximized with the individual treatments. Therefore, it is important to test these hydrogels in a diabetic mouse with ischemic wounds in order to determine the extent of wound healing of the hydrogel, MSTC, and combined treatment in a non-ideal wound environment that has delayed healing.

4.3.4 Future directions

One limitation to this study was the small number of animals used for *in vivo* testing. These animals were used for preliminary tests of the treatment. This number should be increased in order to gain a comprehensive understanding of the impact of the hydrogel treatment on wound healing. Future studies should be completed on diabetic mice with ischemic wounds in order to analyze change in mRNA and protein expression as well as wound healing. Moreover, additional studies must be completed to test the CLKRS-AgNPs in combination with the hydrogel treatment in a murine model, since the majority of the experiments in the present study tested these treatments separately. Lastly, an *in vivo* infection model should be completed to test the antibacterial properties of the AgNP-CLKRS hydrogel treatment on a pre-existing bacterial infection.

5.0 Antibacterial grafted fabrics as an antimicrobial barrier

5.1 Introduction

Cotton fabrics are frequently used for wound dressing applications, but due to their natural fibres, they are highly susceptible to bacterial infection (53, 55, 57, 58). There have been numerous studies functionalizing cotton fabrics with different antimicrobial agents that can be used for many different applications (52-58). The major focus is to functionalize these materials using a facile synthesis that maintains the antimicrobial properties of the agent used to functionalize the fabric (52-56).

In this study, three types of cotton fabrics were synthesized to incorporate fluoroquinolone acid (7-chloro-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), fluoroquinolone ester (7-chloro-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic ethyl ester), or eugenol (2-methoxy-4-(prop-2-en-1-yl)phenol). Fluoroquinolone derivatives are known antibacterial agents, used to treat a range of bacterial infections (116, 117). Fluoroquinolones are in the same family of quinolone antibiotics, which have been used since the 1960s to treat many different infections, including urinary tract infection, respiratory tract infection, and skin infections (117). Eugenol is derived from cloves and has been shown to possess antifungal, antimicrobial, antibacterial, antiparasitic, and anticarcinogenic properties (118-121). These antimicrobial agents were covalently tethered to the cotton fabric with a long hydrocarbon chain linker between the antimicrobial agent and the cotton. This is expected to improve the hydrophobicity, which has been shown to increase antibacterial activity (122, 123). The hydrocarbon chain also decreases steric hindrance for additional chemical modifications. This is the first step in producing antibiotic-bound cotton fabrics that are facile to synthesize, maintain their antimicrobial properties, and have potential versatility for use with various antibiotics that are specific to their application.

The antimicrobial fabrics were synthesized by our collaborator A. M. Montagut in the Vallribera lab at the Department of Chemistry and Centro de Innovación en Química Avanzada (ORFEO-CINQA), Campus UAB, Barcelona, and in this report we focus on testing the antimicrobial properties along with the biocompatibility of the fabrics. Early tests showed fluoroquinolone acid had no antibacterial effects, so we used the fluoroquinolone ester and eugenol fabrics for the remainder of the experiments.

We hypothesize that these fabrics will maintain the antimicrobial properties of the agents covalently linked to the cotton, allowing them to be applicable to a variety of bacteria, while maintaining their biocompatibility.

5.2 Results

5.2.1 Biocompatibility of fabrics

The biocompatibility of the fabrics was first determined to ensure cells remain viable when in contact with the antimicrobial fabrics. Cell count results indicate that the cells continue to proliferate when in contact with all fabrics and the blank cotton fabric control (Figure 15 A). Next, a cell viability assay was completed after cells had been in contact with the fabrics for three days. The results show that over 94% of the cells remain viable, indicating that they are compatible with the fabrics (Figure 15 B). Lastly, an enzyme-linked immunosorbent assay (ELISA) assay was completed to determine the skin tissue interleukin-6 (IL-6) concentration of mice 24 hours after application of the fabrics. The results demonstrate that there is no significant difference, but there is a trend for a decreased inflammatory response with the fluoroquinolone fabric (Figure 15 C).

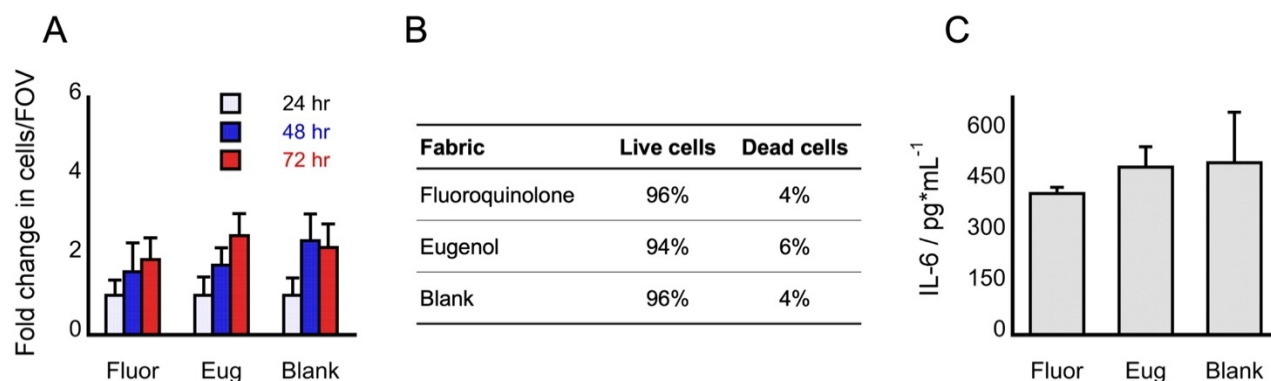


Figure 15. Biocompatibility of antimicrobial fabrics. (A) Number of human dermal fibroblasts per field of view (FOV) measured at different time points after seeding 50,000 cells/well in a 12-well plate. A 3x3 mm piece of fabric was placed in contact with the cells during cell culture and cells were imaged and counted every 24h for 72h. These values correspond to the average of 3 independent samples and the error bars are the standard deviation of the mean. (B) Cell viability of the human dermal fibroblasts was assessed using a live/dead assay completed fibroblasts incubated with fabrics for 72h. Cells were quantified, and viability was assessed. These values correspond to the average number of cells quantified from 4 random images of 3 independent samples. (C) Interleukin-6 (IL-6) levels for murine skin tissue wound beds homogenized after implantation of the fabric for 24h. IL-6 levels were quantified by ELISA on freshly harvested wound tissue. Results are obtained from n=4 per experimental group. Error bars correspond to the standard deviation from the mean.

5.2.2 Antibacterial properties

The antibacterial properties of the fabrics were tested via a planktonic assay and a biofilm assay. The results of the planktonic assay are shown in Figure 16 A, and they indicate a significant decrease in *S. aureus* survival following fluoroquinolone fabric treatment. Moreover, Figure 16 B demonstrates antibiotic leaching from the fabrics. These results indicate that there is no significant decrease in bacteria when grown in broth that had previously been in contact with the antimicrobial fabric, indicating no significant leaching had occurred.

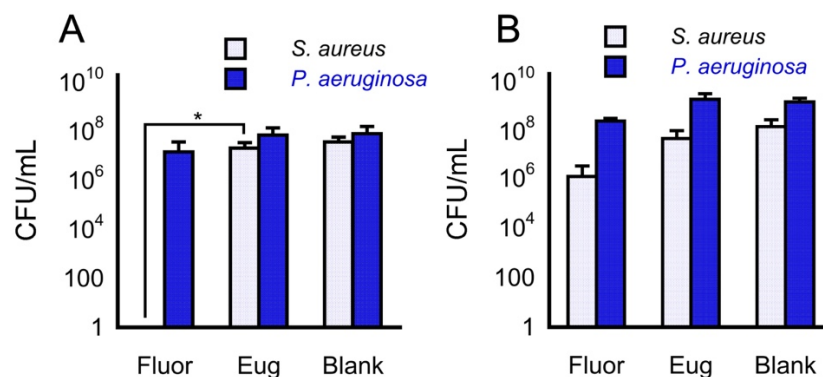


Figure 16. Antibacterial properties and antibiotic leaching assay. (A) Surviving bacterial colonies of *P. aeruginosa* and *S. aureus* following planktonic assay with fabric treatment with fluoroquinolone (Fluor) and eugenol (Eug) and without antibiotics (Blank). The values correspond to the average of 3 independent samples and the error bars are the standard deviation of the mean. **(B)** Leaching assay results. Antibiotic leaching was assessed by incubating the fabrics in LB broth overnight then removing the fabrics and incubating a fresh overnight bacterial culture in the presoaked broth to see if the antibacterial agents leached from the fabric and remained in solution. The values correspond to the average of 3 independent samples and the error bars are the standard deviation of the mean. P values in the plots were calculated from Student t-test (two-tailed).

Next, the anti-biofilm properties of the fabrics were tested to determine their ability to prevent biofilm formation (Figure 17 A, C) and kill pre-existing biofilm (Figure 17 B, D). The results indicate that the eugenol fabrics significantly prevented *S. aureus* biofilm formation, whereas the fluoroquinolone fabrics significantly decreased a pre-existing *S. aureus* biofilm. Moreover, the fabrics showed no significant difference in their ability to prevent *P. aeruginosa* biofilm formation, but the eugenol fabric significantly decreased pre-existing *P. aeruginosa* biofilms.

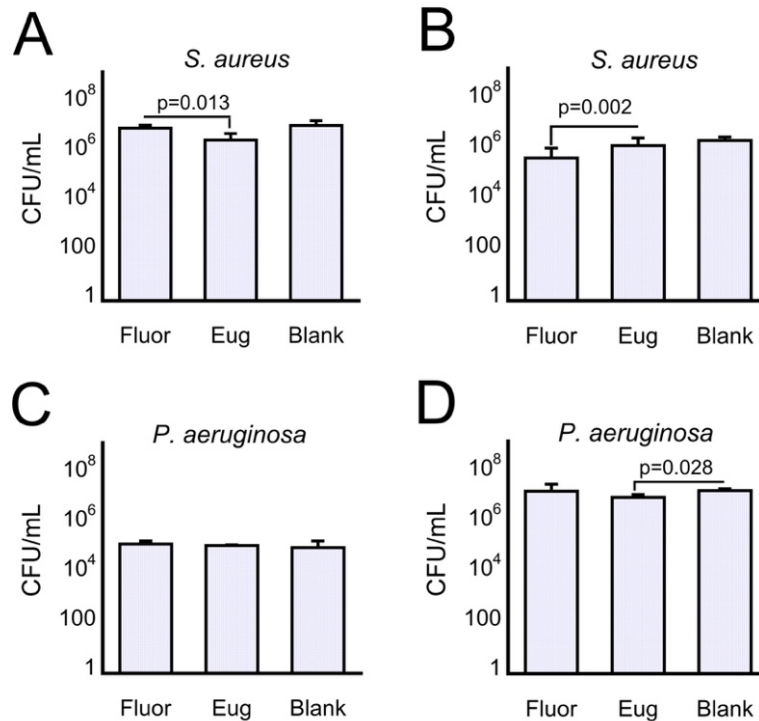


Figure 17. Effect of antibiotic fabric on bacterial colony survival. Biofilm bacterial survival for *S. aureus* (A & B) and *P. aeruginosa* (C & D). The assays measured the ability of the fabrics to prevent biofilm formation (A & C) as well as kill bacteria in a preformed biofilm (B & D). Values reported correspond to the average of 3 independent measurements, and error bars are the standard deviation from the mean (y-axis is in log-scale). P values in the plots were calculated from Student t-test (two tails).

5.3 Discussion

5.3.1 Antimicrobial fabric biocompatibility

Biocompatibility is an essential requirement for any materials in contact with the human body (112, 124). Biocompatibility of the antimicrobial fabrics was assessed using a cell count assay, cell viability assay, as well as by analyzing the inflammatory response associated with *in vivo* fabric application. Cell proliferation and viability assays provide important information on the growth behaviour of cells when they are in contact with the material of interest (112, 125, 126). Proliferation assays indicate toxicity of materials by means of loss of proliferation and the

reduction of living cells, which can allow one to determine the cytotoxicity of the material as well (112). Viability assays allow the direct quantification of viable and dead cells in the sample (112). In addition, certain materials in contact with the body can cause an inflammatory reaction, which can be determined by monitoring inflammatory markers, such as interleukin-6 (IL-6) (113, 125). IL-6 was chosen because it is synthesized rapidly at the wound site during the initial stage of inflammation (113). The results obtained from these assays indicate that the fabrics are biocompatible, as they allow cells to proliferate, show limited cell death, and elicit no increase in immune response.

5.3.2 Antibacterial properties

Planktonic organisms are those that are not attached to a surface, whereas biofilms are the result of bacterial cells attaching to a surface (127). Planktonic assays are used to determine the minimum bactericidal concentration for specific antibacterial agents against planktonic bacteria (128). Since the concentration of antibacterial agents attached to the fabrics is fixed, we monitored the extent of bacterial growth with and without antimicrobial fabric treatment. The results indicate that the fluoroquinolone fabrics significantly decreased *S. aureus* growth, with little impact on *P. aeruginosa*. This is likely due to the fact that *S. aureus* is a gram-positive bacterium, whereas *P. aeruginosa* is a gram-negative bacterium; while studies have shown that fluoroquinolone antibiotics are effective against both types of bacteria, it is known that different fluoroquinolone derivatives are more suited to either gram-negative or gram-positive bacteria (129, 130).

5.3.3 Antibiotic leaching

An antibiotic leaching assay was completed to determine if the antibiotic agents leached off of the fabric and remained in the solution. Briefly, the fabrics were soaked in broth overnight then removed from the broth, bacteria cultures were added to the solution, and bacterial growth was monitored. If a decrease in viable bacteria was observed it can be concluded that the antibiotics leached off the fabric, and if there was no decrease in viable bacteria, then no leaching occurred. The results indicate that no significant leaching was observed, but there was a trend showing fluoroquinolone leaching as there was slight decrease in *S. aureus* and *P. aeruginosa* bacteria, though it was not significant. Leaching has been associated with loss of antimicrobial activity, antibiotic resistance, as well as release of bioactive substances into the environment which has potential health risks, including sensitizing patients to anaphylaxis and skin irritation (57, 58, 131-133). Yu *et al.* demonstrated that by covalently bonding an antimicrobial agent to cotton fabric, the fabric maintains antibacterial properties with no toxic or irritating effects to the skin (57). Overall, decreasing antibiotic leaching, as seen with the antimicrobial fabrics tested, prolongs the lifetime of the antimicrobial fabric and reduces the risk of potential adverse events associated with the antimicrobial agent, such as irritation or antibiotic resistance (57, 58, 131-133). However, in order to enhance the antimicrobial properties of the textiles it would be beneficial to tether additional antimicrobial molecules to the fabrics that are able to exert their antibacterial properties through contact with the cell rather than having to infiltrate the nucleus, such is the case with fluoroquinolone. Otherwise, using a timed-release method for tethering fluoroquinolone may further enhance the antibacterial properties.

5.3.4 Antibiofilm properties

As mentioned above, biofilms are formed when bacterial cells attach to a surface (127, 134). Biofilms are much more difficult to treat as opposed to planktonic cells because mature biofilms are coupled by a network of extracellular polymeric substances that protect the bacteria and make infiltrating the biofilm challenging (127). The results indicate that the eugenol fabric prevented *S. aureus* biofilm formation and the fluoroquinolone fabric eliminated pre-existing *S. aureus*, while the eugenol fabric eliminated preformed *P. aeruginosa* biofilm. It has been shown that *P. aeruginosa* is resistant to fluoroquinolone treatment, which is seen in both the planktonic and biofilm results obtained (135). However, fluoroquinolone resistance has also been associated with *S. aureus*, but resistance to the antibacterial fabrics was not observed (136). These results are consistent with findings that have shown that eugenol and fluoroquinolone are effective at both preventing biofilm formation and eliminating pre-existing biofilm, and that modified cotton fabrics maintain the antibiofilm properties of the agents they are modified with (58, 121, 135, 137, 138).

In conclusion, the results obtained for the antimicrobial fabrics indicate that all fabrics are biocompatible, as they allow cell proliferation and maintain high cell viability, with no increase in inflammation or significant leaching. The fluoroquinolone fabric demonstrated a reduction in *S. aureus* bacteria present in planktonic and biofilm form, and the eugenol fabric prevented *S. aureus* biofilm formation and eliminated preformed *P. aeruginosa* biofilm.

5.3.5 Future directions

These antimicrobial textiles can be used for a variety of applications, including clothing, hospital linen, surgical masks and gowns, as well as for wound healing dressings. The next steps for this material are to attempt to tether additional antibiotics and antimicrobial agents to the fabric

with this facile synthesis in order to create a diverse range of antimicrobial textiles that can be tailored to specific applications.

6.0 Conclusion

In conclusion, these studies highlight the importance of proper NP concentration calculations and indicate the wound healing properties associated with MSTCs embedded in the collagen hydrogel. It also demonstrates the antibacterial and antibiofilm properties of the biocompatible antibiotic grafted fabrics. These three projects can be combined for optimal DFU wound healing by using AgNPs with known concentrations to prevent and eliminate bacterial growth combined with the regenerative properties of collagen hydrogels embedded with MSTCs followed by the application of antimicrobial fabrics to cover and protect the wound from microbial infection (Figure 18). While this project focused on treating diabetic foot ulcers, this project has potential applications in other non-healing chronic wounds and burn wounds.

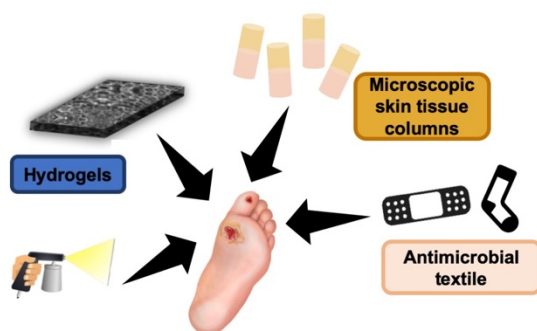


Figure 18. Schematic representation of the three projects as a combinatory treatment for DFUs.

7.0 References

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List of Contributions

Training and technical support was provided by various members of the Alarcon and Suuronen labs. Funding of this project was made possible because of Dr. Emilio Alarcon, Dr. Marc Ruel and Dr. Suuronen. Drs. Emilio Alarcon and Erik Suuronen were responsible for the overall design and organization of this project. Animal care was responsible for the maintenance of all animals used throughout the study. NANoPoLC algorithm was derived by Francisco Valenzuela-Henríquez, testing of the algorithm was completed by Caitlin Lazurko and Manuel Ahumada (Alarcon lab). Bacterial testing for the hydrogel was completed by Zohra Khatoon (Alarcon lab), Li Zhang (Mah lab), and supervised by Dr. Thien-Fah Mah. Danielle Giroux (Suuronen lab) performed the Western Blot experiments. The antimicrobial fabrics were synthesized by A. M. Montagut (Vallribera lab).

Appendix A – Schematic representation of murine model.

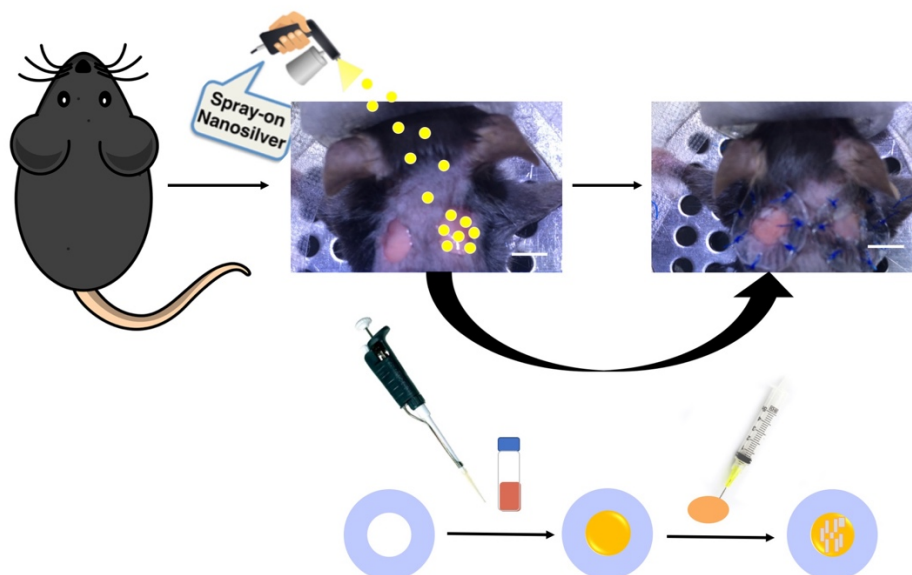


Figure 19. Schematic representation of murine model for hydrogel testing. Two 6 mm wounds are created on the dorsal skin of mice. The left wound was used as the internal control, to account for different rates of healing between mice, and the right wound was treated for 7 days. The CLKRS-AgNP spray-on treatment is applied directly to the wound before the hydrogel treatment. The hydrogel is left to crosslink in silicone disks to keep the wound open during healing and prevent skin contraction. The MSTCs are harvested from the skin excised when creating the wounds and are placed directly in the hydrogel. About 100 μ L of hydrogel is placed directly into the wound bed before applying the disk with crosslinked hydrogel and MSTCs. The mouse is treated for 7 days then sacrificed to quantify wound healing. Scale bars represent 0.5 cm.

Appendix B – Permissions and Authorizations

Section 1.3 Nanomaterials, Figure 2.



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Curriculum Vitae

EDUCATION

10/2017-2019	M.Sc., Biochemistry, University of Ottawa
10/2013-04/2017	B.Sc, Honors, specialization in Biochemistry, University of Ottawa

AWARDS AND SCHOLARSHIPS

2019	Second place, University of Ottawa BMI Seminar Day
2018	Award of Excellence in Graduate Studies – MSc in Biochemistry, University of Ottawa
2018	Researcher in a Supporting Role Research Award, University of Ottawa Heart Institute
2018	Queen Elizabeth II Graduate Scholarship in Science and Technology
2018	Coralie Lalonde Innovation Award, Ottawa Cardiovascular Research Day
2017-2019	University of Ottawa Admissions Scholarship
2017	First Place, Poliquin Day Research Award, Department of Otolaryngology, University of Ottawa
2016	Women in Science Research Award, University of Ottawa
2015	NSERC Undergraduate Student Research Award, University of Ottawa
2015	University of Ottawa Undergraduate Research Opportunity Program Award
2013-2017	University of Ottawa Dean's Honor List, B.Sc. summa cum laude
2013-2017	University of Ottawa Entrance Scholarship

RESEARCH EXPERIENCE

07/2017 - present	Biomaterials and Tissue Engineering Research BioEngineering and Therapeutic Solutions Lab, University of Ottawa Heart Institute, Ottawa, ON <i>Visiting academic at Harvard Medical School, Boston, MA, USA</i> • Engineering new hydrogel matrices to promote wound healing and testing in vitro/in vivo applications • Developing in vivo models for testing hydrogel matrices
05/2015-07/2017	Chemistry Research Assistant Scaiano Laboratory, University of Ottawa, Ottawa, ON • Synthesize silver nanoparticles as a treatment for chronic rhinosinusitis

- Test the bacterial inhibition of the silver nanoparticles against laboratory bacteria strains and bacteria samples from patients with chronic rhinosinusitis
- Complete cell viability and nanoparticle stability tests as well as planktonic and biofilm assessments to determine bacterial inhibition

10/2016-04/2017

Molecular Medicine Laboratory Honor Student

Molecular Medicine Lab, University of Ottawa, Ottawa, ON

- Synthesize a novel fluorogenic probe to detect specific ketones and aldehydes *in vitro*
- Use laboratory equipment such as NMR, mass spectrometry, and HPLC to confirm the synthesis of specific compounds
- Test efficacy of the probe using Chemical Exchange Saturation Transfer MR techniques and fluorescence imaging
- Supervise undergraduate students and teach them proper laboratory procedures and techniques, chemical syntheses, and equipment protocols

03/2014-01/2017

Thoracic Surgery Data Collection and Analysis

The Ottawa Hospital, Thoracic Division, Ottawa, ON

- Collect thoracic surgery historical data pertaining to pre-operative variables, operative information, and post-operative complications
- Re-classify the data according to standardized procedure categories, and complication grades, and affected organ system
- Analyze summary statistics of surgical volume (i.e. number of cases performed) and quality reporting (i.e. post-operative complication rates)

RESEARCH CONTRIBUTIONS

Publications

Lazurko C., Harden S., Suuronen EJ, Alarcon EI. Biomaterials for Organ and Tissue Repair. *Front. Young Minds.* 7:8. doi:10.3389/frym.2019.00008.

Suchy M., **Lazurko C.**, Kirby A., Dang T., Liu G., Shuhendler A. Methyl 5-MeO-N-aminoanthranilate, a minimalist fluorogenic probe for sensing cellular aldehydic load. *Org Biomol Chem.* **2018**. doi:10.1039/C8OB02255K.

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Conferences

**presenting author*

C Lazurko* and Dr. E Alarcon. “Infection-fighting gel for treatment of diabetic foot ulcers”. Ottawa Cardiovascular Research Day, April 2018.

T Dang, **C Lazurko**, M Suchy*, W Oakden, Y Truong, G Facey, G Stanisiz, and A Shuhendler. “Hydrazo-CEST: A carbonyl-activatable CEST-MRI contrast agents towards aldehyde imaging in vivo”. World Molecular Imaging Congress, Philadelphia, PA, USA, September 2017.

C Lazurko*, J.C. Scaiano, and S Kilty. “Silver Nanostructures as a Treatment for the Bacteria of Chronic Rhinosinusitis”. Department of Otolaryngology-Head and Neck Surgery Poliquin (Research) Day, Ottawa, ON, Canada, May 2017.

Poster Presentations

**presenting author*

C Lazurko*, K Goel, Dr. B McNeill, Dr. E Suuronen, and Dr. E Alarcon. “Collagen hydrogels for the treatment of diabetic foot ulcers”. Ottawa Cardiovascular Research Day, April 2018.

C Lazurko, S Foster, M Suchy*, and A Shuhendler. “Fluorescence Fingerprinting of Aldehydes for Blood-based Detection of Disease & Injury”. World Molecular Imaging Congress, Philadelphia, PA, USA, September 2017.

T Dang, **C Lazurko**, M Suchy*, W Oakden, Y Truong, W Lam, G Facey, G Stanisiz, and A Shuhendler. “Hydrazo-CEST Probes Towards Imaging Aldehydes In Living Subjects”. Music City CEST, Nashville, TN, USA, August 2017.

C Lazurko*, T Dang, M Suchy, and A Shuhendler. “Molecular imaging probes for the bioorthogonal detection of endogenous aldehydes and ketones”. University of Ottawa Honour’s program poster presentation, Ottawa, ON, Canada, April 2017.

C Lazurko* and A Seely. “What is the true incidence of adverse events following surgical resection for esophageal and gastric cancer?”. Undergraduate Research Opportunity Program (UROP) poster presentation, April 2015.

Book Chapters

M Ahumada, **C Lazurko**, E.I. Alarcon, Chapter 1 - Fundamental concepts on surface chemistry of nanomaterials. In Photoactive Inorganic Nanoparticles, Prieto, J. P.; Béjar, M. G., Eds. Elsevier: 2019; pp 1-19.