Degree project

UNIVERSITY OF SKÖVDE

Blood interactions with bioactive peptide-functionalized nanocellulose: An evaluation of the activation of the coagulation and complement system.

Master Degree Project in Bioscience

Second Cycle 30 credits

Spring term 2022

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Abstract

A current trend utilizing the biomedical approach in the field of wound care is focused on the increased potential to develop wound healing materials designed to address specific types of wounds or underlying pathologies to achieve improved healing. The work presented in this thesis evaluates the blood response to wood-derived nanocellulose functionalized with a peptide, with the ultimate aim of characterizing the material as a potential wound dressing for chronic wound care. The material was evaluated based on the response toward the innate immune system. These interactions between the material and blood were studied using an *in vitro* whole blood loop model, and then, the coagulation and complement system activation markers were quantified using enzyme-linked immunosorbent assays. The platelet count and the levels of the thrombin-antithrombin complex reported for the material showed no activation of the coagulation cascade whereas there was an activation caused in the complement system showing higher levels of C3a and s-C5b9 components as compared to the controls. The observations obtained from this interdisciplinary project can be considered as a stepping stone toward the need for further analysis of the material in advanced wound care applications. This can be achieved by targeting the specific phases of the wound healing process in order to promote effective wound management.

Popular Scientific Summary

The skin surrounds the complete human body forming the largest organ. This large and exposed area of skin is prone to a constant risk of injury. Any injury caused can affect the traditional mechanism of repair in the body which is necessary to maintain proper health. While acute wounds tend to heal faster and in a specific time period following an orderly fashion, this process can be truly complex in the case of chronic wounds. Chronic wounds are a big concern in the health care sector today with an estimated expenditure of USD 25 billion annually in the US alone. According to the research conducted it has been estimated that about 1-2% of the population experiences a chronic wound once in their lifetime, especially in developing countries. These wounds are a cause of tremendous pain and patient suffering and therefore lead to a major challenge for the healthcare systems worldwide. The wound healing process occurs in a very systemic approach divided into four phases-hemostasis, inflammation, proliferation, and maturation. However, one of the most important transitions in the wound healing process is the one between the hemostasis and the inflammatory phase. Chronic wounds are characterized by a persistent inflammatory phase which could be due to a lot of factors for example bacterial or microbial infection at the wound site. In order to better understand the pathogenesis behind this prolonged inflammation a deeper insight into the phases is required.

Wound dressing materials to aid the healing of wounds have made constant advancements with more than 5,000 different products available in the market today. Progressive research made in the fields of materials science and nanotechnology provides an opportunity to better understand and develop wound healing dressing materials. In the last decade, all the different approaches available in the treatments of impaired wound healing are focused on factors like infection clearance or nutrition or mechanical support and protection. An emerging trend in the wound care industry is the introduction of targeted therapies including the application of biomaterials like wood-derived cellulose nanofibrils (CNF), nanomaterials like nanoparticles (silver, titania), and growth factors like PGDF and EGF being used. But, particularly for the growth factors the clinical efficacy reported has not been very impressive but limited. The incorporation of host defense peptides has been on the rise as well since they are highly preserved and found in all living organisms. This is a very broad and upgraded version of antimicrobial peptides since these can modulate the immune response and also play a key role in processes like re-epithelialization and angiogenesis. To our knowledge, there have been quite a few studies hinting at the role of KR12 in wound care applications as they are still in progress whereas there are quite a lot of studies utilizing CNF as the potential material for the making of the wound dressing.

The studies presented in this thesis revolve around the possibility of using cellulose nanofibers functionalized with host-defense peptides as a new platform for advanced wound healing. This material was evaluated at different concentrations to get a better understanding of its interaction with blood. This novel functionalized material combined the tunable properties of nanocellulose required for the development of wound dressings and the wound healing properties of the host-defense peptide-KR12. The material evaluation is based on the immune response since it plays a very crucial role not only in the inflammatory phase but throughout all the phases of wound healing. The results observed demonstrated no activation of the coagulation system by any of the samples i.e., CNF, CNF-KR12, and KR12. But there was activation of the complement system by the samples wherein CNF showed the highest level of activation of the complement system, KR12 showed no activation at all whereas CNF-KR12 showed an intermediate level of activation of the complement system. This property seems to be important from the perspective of healing chronic wounds since activation of the complement system leads to the recruitment of immune cells which helps in the skin repair mechanism.

Abbreviations

AP- alternative pathway

BSA- bovine serum albumin

CHS- corline heparin surface

CNF- cellulose nanofibrils

CP- classical pathway

EDTA- ethylenediaminetetraacetic acid

ELISA- enzyme-linked immunosorbent assays

HDP- host defense peptides

HRP- horseradish peroxidase

LP- lectin pathway

PBS- phosphate buffer saline

PFP- platelet-free plasma

rpm- rotations per minute

RT- room temperature

SEM- standard error of the mean

TAT- thrombin-antithrombin

TGA- thrombin generation assay

TMB- 3,3′,5,5′-tetramethylbenzidine

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Introduction

Wound healing and chronic wounds

The human skin consists of three tissue layers: an upper layer called the epidermis; a lower layer called the dermis as well as another deeper inner layer called the hypodermis. All these layers together form a protective barrier against the environment (Yousef et al., 2022). A skin wound is the breakdown of this epidermal layer integrity. In order to restore tissue integrity during the events of any cutaneous injury, a systemic process called wound healing is activated (Diegelmann & Evans, 2004). Wound healing is a natural physiological and dynamic phenomenon that involves a complex interplay of different cell types and factors like cytokines, mediators, and the vascular system (Glenn & Goldman, 1976). The process consists of four overlapping, continuous, and precisely programmed phases namely hemostasis, inflammation, proliferation, and remodeling or maturation phase (Guo & Dipietro, 2010). Figure 1 below illustrates these four phases on a time scale progressively, how these overlap with each other and take forward the process of healing (Häggström, 2014). The physiological functions of these phases must occur in a properly organized pattern for a specific duration at an optimal intensity (Mathieu & Wattel, 2006). In case any disruption is caused, it leads to impaired tissue repair or the formation of chronic wounds. Chronic wounds can be classified as wounds that failed to heal in four weeks (Frykberg & Banks, 2015). The healing process in such cases can be affected by many local or intrinsic and extrinsic factors. Local factors include moisture (Metzger, 2004), edema, a faulty technique of wound closure, ischemia, necrosis, and foreign bodies (Iconomou et al., 1993). Extrinsic factors include friction across support surfaces, nutrition, smoking, alcohol consumption, stress, etc amongst other factors (Arnold & Barbul, 2006; Iconomou et al., 1993; Siana et al., 1989; Vileikyte, 2007). Also, both, excessive and impaired inflammation can be detrimental to wound healing (Landén et al., 2016). Hence, for a wound to heal, the primary causative factor for it should be resolved (Glenn & Goldman, 1976). However, a better understanding of the influence caused by these factors on the repair mechanism is required to attain improved therapeutics for wound healing.

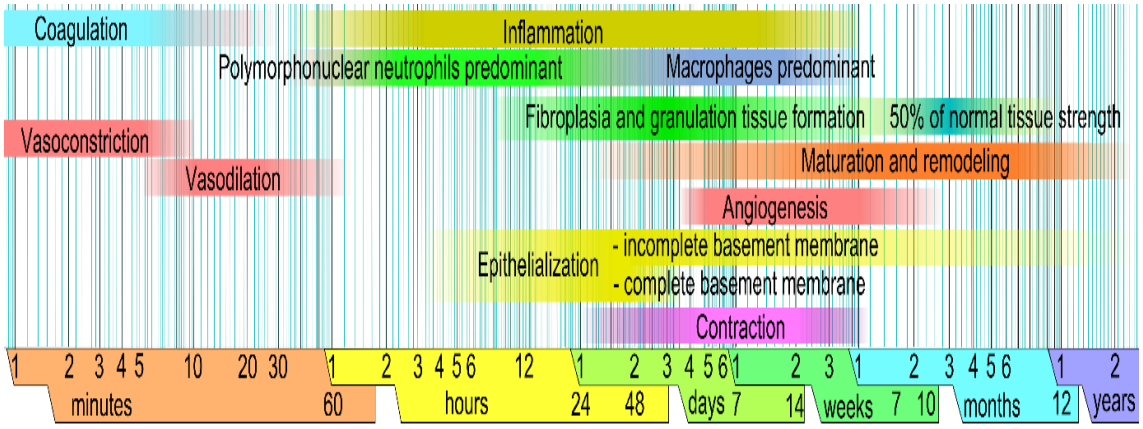

Figure 1. Illustration of different phases of wound healing on a logarithmic scale. The regions with faded intervals mark substantial variation between the phases depending on wound size and healing conditions (Häggström, 2014); used with permission.

Need for novel therapeutic approaches to treat chronic wounds

Skin chronic wounds can be looked upon as a matter of growing health concerns. It has increased manifolds in these years reaching epidemic proportions (Hamdan et al., 2017). This calls for an

immediate need for new and improved therapeutic approaches. Some innovative approaches can be designed to target different phases of wound repair modulating the wound environment and actively promoting the healing of the wounds (Hamdan et al., 2017). Nanotechnology-based approaches are gaining immense popularity as they offer flexibility toward the complex process of normal wound healing. These approaches enable targeting multiple dysfunctional cellular processes and identifying the optimal timing for delivery of the therapeutic agents (Hamdan et al., 2017). Other advantages include cell-type specificity (Hamdan et al., 2017).

There is an increasing inclination toward biologics, gene therapy, and new strategies for the delivery of drug molecules to accelerate the process of wound healing. These emerging approaches are essential from the perspective of clinical and pharmaceutical research (Öhnstedt et al., 2019).

Cellulose and nanocellulose

The most abundant polymer on Earth, cellulose is a linear polysaccharide with repeating β (1-4)-bound D- glucopyranose rings. It is biosynthesized in plants, bacteria, tunicates, algae, and fungi (Klemm et al., 2005). The intermolecular forces between the hydrogen bonds in the anhydrous glucose unit give cellulose a hierarchical structure (Khazraji & Robert, 2013). Henceforth, the amorphous and crystalline regions are assembled into elementary fibrils that in turn form cellulose microfibrils (Yusup & Rashidi, 2022). Nanocellulose, the nanoscale form of cellulose, has been the center of increased attention in a broad range of fields namely food coatings, mechanical reinforcement, antimicrobial films, electronic devices, and many more emerging biomedical applications (Trache et al., 2020). Other applications include cement, cosmetics, and paper products as well (Moon et al., 2016; Thomas et al., 2018). It also displays a high specific surface area and good mechanical and viscoelastic properties (Trache et al., 2020). Nanocellulose can be easily functionalized with charged chemical groups. It is categorized into two main types: cellulose nanocrystals and cellulose nanofibrils (CNF) (Lin & Dufresne, 2014). Wood-derived CNF is obtained by the chemical or mechanical treatments of the wood pulp. The mechanical treatment consists of high-pressure homogenization in which the cellulose fibres are forced through a controlled physical geometry under high pressure, causing separation of the microfibrils (Curvello et al., 2019). Individual fibrils in CNF are 2-10 nm in diameter, forming 20-50 nm thick aggregates, and have lengths up to several micrometres (Anžlovar & Žagar, 2022). Promising results have been shown towards the possibility to create CNF-based dressings for wound healing application (Basu et al., 2017).

Wood CNF in wound healing

Progressive research being carried out on the use of wood CNF in wound healing makes it a very interesting topic to explore. The use of wood CNF in the form of sheets, hydrogels (Curvello et al., 2019), and aerogels (Nordli et al., 2019) as wound healing dressings has been supported by various studies. The renewable and easily modifiable nature of wood CNF renders opportunities to combine it with beneficial materials for wound healing resulting in a combination having all the possible properties of a desirable wound dressing. This could be expected to be a good candidate for the development of a platform for advanced wound care applications which serves as the highlight of the work presented in this thesis.

Host Defense Peptide

One of the major hindrances in the process of wound healing is infection. These wound infections have become increasing morbidity and hence, finding a significant treatment is very crucial in this aspect (Mangoni et al., 2016). The multicellular organisms have evolved a plethora of host-defense molecules which includes host defense peptides (HDP) with antimicrobial properties. These molecules are aimed at controlling microbial proliferation and also modulating the host's immune response to injury (Mangoni et al., 2016). The mechanism of their antimicrobial action can be explained via simple electrostatic interaction with the microbes' cell membrane that leads to its disintegration and hence, results in cell death (Epand & Epand, 2009; Wimley, 2010). LL-37 is a human HDP, 37- amino acids long with an alpha-helical structure. This peptide confers properties related to activities ranging from neutralization of inflammatory agents to immune cell chemoattraction and cell proliferation (Mangoni et al., 2016). All these activities make it eligible to be classified as a novel potential anti-infective molecule. Direct use of this peptide for antimicrobial applications due to protease susceptibility is limited. But, as per several studies LL-37 has been shown to modulate the immune response and is under clinical trial phase II for its role in melanoma progression (Dolkar et al., 2018) as well as for the treatment of chronic bacterial middle ear infection (Fjell et al., 2012). Therefore, LL-37 and the peptides derived from it can be engineered to produce biologically stable forms ideal to improve its potential use in clinical aspects (Schmidtchen et al., 2002; Strömstedt et al., 2009).

KR12- functionalized CNF

KR12 is the smallest antimicrobial epitope for the parent peptide human cathelicidin LL-37. Not much is known about this peptide compared to all the properties with supporting evidences present for LL-37. KR12 is known to promote re-epithelialization and angiogenesis beneficial from the aspect of wound care management (Liu et al., 2019). Research is required to exploit its full potential in the era of wound healing. On the other hand, the potential of wood-based nanocellulose for biomedical applications is not only limited to wound healing, but other applications include therapeutic contact lenses (Tummala et al., 2016), drug delivery systems, and tissue engineering and regenerative medicine (Lou et al., 2014; Malinen et al., 2014) among others. Exposed C6 primary alcohol groups present on the cellulose backbone allow easy functionalization of it with chemical groups to obtain novel materials. This offers flexibility to control the bioactivity (Hua et al., 2016), and to produce materials with varying viscosity resulting in materials with tunable mechanical properties that are useful to make wound dressings.

This formed the basis for the hypothesis generated in this research work by combining the CNF with KR12 using material sciences i.e., a modified version of CNF, and evaluating its response in contact with human blood.

Innate Immune system

The innate immune system acts as the first line of defense against foreign surfaces and invading pathogens entering our body. It is an evolutionarily preserved system for host defense encompassing all mammalian tissues (Clark & Kupper, 2005). The intravascular innate immune system is responsible for tissue remodeling and repair (Ekdahl et al., 2015). It functions physiologically, by identifying and removing the foreign substances, including microorganisms, apoptotic and necrotic cell debris, etc, via inflammatory and thrombo-inflammatory responses. Therefore, this system is highly complex and multifaceted.

The complement system

The complement system is a central part of the immune system. Its primary function is the elimination of microorganisms and other foreign particles. For this said purpose, the innate immune cells are recruited to the site and they act upon sensing the 'non-self'. The other biological functions of the complement system include immune recognition and subsequent phagocytosis and cell death (Hajishengallis et al., 2017; Yang et al., 2013). This system can be activated via three different pathways namely- the alternative pathway (AP), the classical pathway (CP), and the lectin pathway (LP). All the three pathways end at a common point which is the assembly of the C3 convertases, which cleaves component C3 into C3a and C3b (Figure 2).

The knowledge regarding the role of the complement system in wound healing is still limited. Some studies show activation of the complement system as a positive effect on wound healing, whereas others show that inhibition of activation of the complement system favors the resolution of chronic wounds (Cazander et al., 2012; Rafail et al., 2015; Sinno & Prakash, 2013). It depends on the wound type and the application of the material. Further, research has suggested that there is an augmenting effect of the complement system on wound healing that relates to its influence on the whole process of it (Cazander et al., 2012).

The terminal complement pathway

The deposition of C3b promotes the generation of C5 convertases that marks the first step of the terminal pathway. C5 convertases then cleave C5 into C5a and C5b (Figure 2). C5b is the first component of the self-assembly of the membrane attack complex (MAC) or its soluble counterpart, the terminal complement complex (sC5b-9) which is formed in the absence of a biological membrane.

Hemostasis and the coagulation cascade

Hemostasis is a physiological phenomenon responsible for the cessation of bleeding at the site of vascular injury while maintaining the blood flow elsewhere in the body. This process is essential to create a balance among the functions of the vessel walls and platelets and the coagulation, which are all necessary for the formation and dissolution of clots (Hemker et al., 1969; Tanaka et al., 2008).

Hemostasis consists of two processes: primary and secondary hemostasis. Primary hemostasis refers to the platelet aggregation and plug formation at the site of injury, whereas secondary hemostasis refers to the interlocking of clot components produced by the deposition of insoluble fibrin. Both of these processes are intertwined and essential to regulating and maintaining hemostasis (Etulain, 2018).

There are two pathways for the coagulation system namely an intrinsic pathway (contact pathway) and the extrinsic pathway (tissue factor pathway). Both the pathways converge at a common point to produce FXa. FXa is an enzyme that is responsible for the cleavage of prothrombin into thrombin. Thrombin is then responsible for the cleavage of fibrinogen into insoluble fibrin leading to the formation of a blood clot (Bäck et al., 2010).

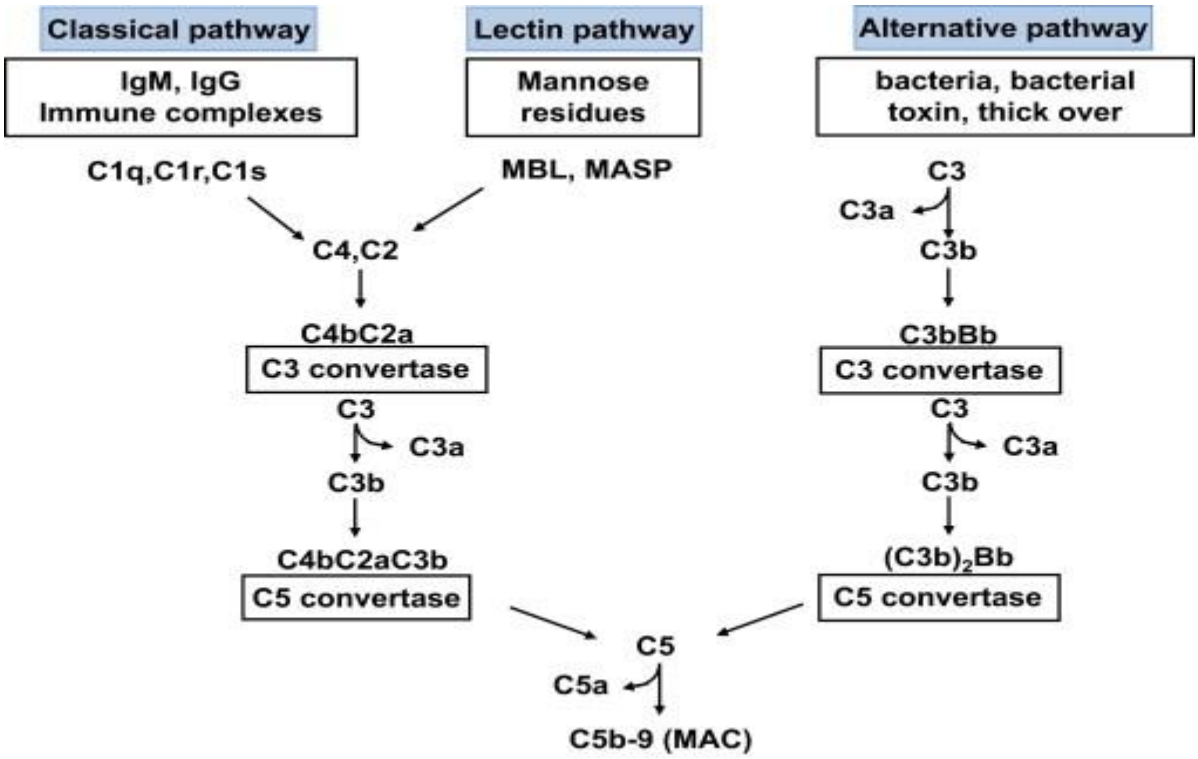

Figure 2: Overview of the complement system. The complement system can be activated by three pathways, the CP, LP, and AP. Recognition molecules from these pathways bind to structures present on non-self-molecules and initiate the assembly of the C3 convertase which cleaves C3 into C3a and C3b. The further downstream reaction leads to the formation of MAC complexes (Noris & Remuzzi, 2013); reproduced with permission from the publisher.

In this thesis, an *in vitro* model utilizing human whole blood was used to study the impact of wood-derived CNF functionalized with the KR12 peptide on the complement system and coagulation cascade.

Aim

The present master thesis project aims to investigate the effect of HDP- functionalized cellulose nanofibrils (CNF) on the innate immune response, specifically on the activation of the cascade systems of blood. CNF material was functionalized with the HDP KR12 as it is said to be an emerging peptide beneficial for wound care. In order to develop wound dressings or anything which is supposed to be in contact with blood, the evaluation of the response of material concerning the immune system is very important.

Objective

To investigate the effect of CNF-KR12 on the innate immune response. This was carried out in two steps:

a) By carrying out blood experiments using an *in vitro* whole blood loop model. This was employed as a means of interaction between the materials (CNF, CNF-KR12, and KR12) and the blood.

b) By evaluating and quantifying the coagulation system and complement system activation markers using the principle of sandwich ELISA.

Materials and methods

Synthesis of KR12- functionalized CNF (CNF-KR12)

The synthesis of KR12-functionalized CNF was carried out by a researcher at the Division of Nanotechnology and Functional Materials, Uppsala University. It consisted of the covalent incorporation of the KR12 peptide onto carboxymethylated-CNF. Therefore, this synthesis was based on the carbodiimide chemistry reaction where the amine groups in the peptide react with the carboxyl groups onto c-CNF.

Material Preparation for the blood experiment

All the samples used in the study were gel suspensions resuspended in PBS.

CNF-KR12 Material

The starting stock suspensions of CNF-KR12 were prepared in PBS 1x (Thermofisher Scientific) at concentrations of 7.5 mg/mL and 3.75 mg/mL. Subsequently, 100 µl of these suspensions each was added to 1.4 mL of blood in the loops to obtain the final concentrations of 500 µg/mL and 250 µg/mL respectively. Alternatively, stock suspensions of 15 mg/mL and 7.5 mg/mL were prepared and 50 µl of the suspensions was added to 1.45 mL of blood in the loops to obtain the same final concentrations. Note that CNF was used as an internal control at the same concentrations as CNF-KR12.

Soluble antimicrobial peptide KR12

The starting stock solutions of the KR12 peptide (Hangzhou Go Top Peptide Biotech, China) were prepared in PBS 1x (Thermofisher Scientific) at concentrations of 1.5 mM and 0.75 mM, and 0.1 mL of these solutions was added to 1.4 mL of blood in the loops to obtain the final concentrations of 0.1 mM and 0.05 mM each. When 50 µl of the peptide solutions were added to the loops, then the stock solutions prepared were 3 mM and 1.5 mM, keeping the blood volume at 1.45 mL.

Heparinization

The procedure of blood sampling and the experimental model can be the reason for unwanted blood activation which needs to be minimized using heparinization. It is achieved using a Corline heparin surface (CHS) coating, following the manufacturer's protocol (Corline AB, Sweden). The heparin coating is applied in two steps for a double-coated layer to enhance the effect. The first layer is of polymeric amine of 0.25 mg/mL (Corline AB, Sweden) followed by a layer of corline heparin conjugate of 0.05 mg/mL (Corline AB, Sweden). All the lab consumables namely rubber caps used to close the loops, pipette tips (5 mL) used to work with the blood and the falcon tube 50 mL used to collect the drawn blood were heparinized using the above protocol.

Blood Sampling

Fresh human blood was drawn from healthy volunteers using an open system of blood collection. The blood was collected in a 50 mL heparinized falcon tube employing a needle connected to the

heparinized tubing and used within 10 min following the collection. No soluble anticoagulant was added to the collection tube. Ethical approval was obtained from the Regional Ethics Committee reference number- 2008/264 (Uppsala, Sweden). Blood from two different donors was used in the study.

In vitro whole blood loop model

The loop model consisted of pre-heparinized, commercially available tubing with an internal diameter of 6 mm (Cortiva™ bioactive surface from Medtronic, USA). The tubing was cut into small pieces of 8 cm each for the blood experiments. The loops were rinsed using 0.9% NaCl solution prior to the supply of materials into them. Each of them was filled with the respective materials: CNF, CNF- KR12, and KR12 as stated in the "Materials Preparation" section above. Different amounts of each of the materials suspended in PBS were added to the tubing as per the desired final working concentration followed by 1.4 mL and 1.45 mL of freshly drawn blood making up the total sample loop volume of 1.5 mL. Tubing filled with PBS (100 µL or 50 µL) and without PBS i.e., only CHS coated commercial loops along with blood were treated as controls for the experiment. Additional blood was collected that was not exposed to the loop system serving as 0 min initial samples or the reference point for the experiments, supplemented with ethylenediaminetetraacetic acid (EDTA) to a final concentration of 10 mM. The loops were then closed at both ends using heparinized rubber caps. The filled-in loops were then subsequently rotated vertically at a speed of 30 rpm at 37 °C for 1 h in a rotating wheel incubator. Important to note here is that each of the samples at a particular concentration of material and controls was all run in duplicates.

Blood collection, platelet counting, and plasma samples

After incubation at the end of each experiment, 1 mL of blood was collected from each of the loops in Eppendorf tubes supplemented with EDTA to a final concentration of 10 mM and placed on ice. Immediately afterward, all EDTA-treated samples along with the initial blood samples (0 min samples) were analyzed for the measurement of the concentration of the platelets using a XP-300 Hematology Analyzer (Sysmex Corporation, Japan). Then, the blood samples were centrifuged at 2500 x g for 15 min at 4 °C to obtain plasma. These plasma samples were collected and stored at -70 °C until further ELISA analysis.

Enzyme-linked immunosorbent assays (ELISA's) for coagulation and complement markers

For all the ELISA's, PBS (Medicago AB, Sweden) containing 0.05% (v/v) Tween 20 (Sigma Aldrich, USA) was used as the washing buffer, and PBS containing 1% (w/v) bovine serum albumin (BSA, Sigma Aldrich, USA); 0.05% (v/v) Tween 20 and 10 mM EDTA was used as the dilution and blocking buffer. Also, 1M sulphuric acid (Merck KGaA, Germany) solution was used as the stop solution, and 3,3′,5,5′-tetramethylbenzidine (TMB) (Bio-Rad, USA) was used as a color substrate. The endpoint absorbance was measured at 450 nm using a TECAN Sunrise microplate reader (Tecan Group Ltd, Switzerland) for all the samples.

Plasma levels of all the samples from the blood experiments were analyzed by the principle of a sandwich ELISA. A 96-well Maxisorp plate (NUNC, Denmark) was incubated with capture antibody diluted in PBS followed by overnight incubation at 4 °C. Then, the unbound capture antibody was discarded and the ELISA plate is further blocked using the blocking buffer for 60 min at room temperature (RT) and kept on a plate shaker at 700 rpm. The blocking buffer is then

discarded. The samples, standards, and control were all diluted in dilution buffer as stated below. Then, all the samples, standards, and control were added to the plate in duplicates. The plate was incubated for 60 min at RT on a plate shaker at 700 rpm. Next, the plate was washed in the automated plate wash followed by the addition of a detection antibody. The plate was then incubated again at RT for 60 min and kept on the plate shaker at 700 rpm of speed. This was followed by washing the plate again and then horseradish peroxidase (HRP)-conjugated streptavidin was added. It is left for incubation of 15 min at RT on a plate shaker at 700 rpm. Then the plate is washed again and the TMB substrate is added with an incubation of 10 min protected from light at RT. Immediately, after that stop solution is added to the plate in order to stop the reaction. Then, the absorbance in the ELISA plate is measured at 450 nm using the TECAN plate reader.

ELISA's to study the complement activation.

In house C3a ELISA

Mouse monoclonal C3a 4SD17.3, 1 mg/mL (in-house) was used as a capture antibody diluted 1:1000 in PBS to coat the ELISA plate. Biotinylated polyclonal rabbit anti-C3a antibody, 0.823 mg/mL (in-house) was used as the detection antibody diluted 1:500 in dilution buffer to detect the bound C3a in all the plasma samples followed by HRP-conjugated streptavidin (GE Healthcare, Sweden) diluted at 1:500 in dilution buffer. In-house Zymosan activated serum calibrated against a purified solution of C3a was used as standard where the first point is diluted 1:1000 in dilution buffer, followed by 1:2 dilution from the first point until the eleventh point. Hence, a total of 11 standard points. Prediluted zymosan activated serum (in-house) was used as the control. The plasma samples were diluted at 1:300 in the dilution buffer. The results are presented as µg/L.

In house sC5b-9 ELISA

Mouse monoclonal anti-C9 E11 (Diatec Monoclonals AS, Norway) was used as a capture antibody diluted 1:1000 in PBS to coat the ELISA plate. Biotinylated polyclonal anti-C5 (Nordic Bio Site AB, Sweden) was used as the detection antibody diluted 1:2000 in dilution buffer to detect the level of sC5b-9 complexes in the plasma samples followed by HRP- conjugated streptavidin diluted at 1:500 in dilution buffer. In-house Zymosan activated serum was used as standard where the first point is diluted 1:100 in dilution buffer, followed by 1:2 dilution from the first point until the eleventh point. Hence, a total of 11 standard points. Prediluted zymosan activated serum (in-house) was used as the control. The plasma samples were diluted at 1:10 in the dilution buffer. The results are presented as µg/L.

ELISA to study coagulation activation

In house TAT ELISA

Sheep anti-human thrombin antibody (SAHT-AP), (Enzyme Research Labs Inc., USA) was used as a capture antibody diluted 1:500 in PBS to coat the ELISA plate. HRP-conjugated antihuman antithrombin antibody (Enzyme Research Labs Inc., USA) was used as the detection antibody diluted 1:500 in dilution buffer to detect the formed TAT complexes in all the plasma samples. Pooled human serum diluted in normal EDTA plasma (in-house) was used as standard where the first point is diluted 1:100 in dilution buffer followed by 1:3 dilution from the first point until the seventh point. Hence, a total of seven standard points. Prediluted zymosan activated serum (in-house) was used as the control. The results are presented as µg/L.

Statistics

The results of the blood experiments are shown as column graphs. For all the three ELISAs (TAT, C3a, and sC5b-9), the results are plotted as the mean average ± SEM for the two blood experiments with each sample tested in duplicates for each blood experiment, n=2 using GraphPad Prism Version 9.3. Important is to note that for the platelet counting, normalization of all the samples against the initial blood samples was performed.

Results

General

The aim of this project was to investigate the effect of KR12- functionalized CNF on the innate immune system. For this, human whole blood along with the material was incubated using an *in vitro* loop model and then changes in the number of platelets along with the levels of blood activation markers were determined. Different activation markers like TAT, C3a, and sC5b-9 were quantified using ELISA. The results for each one of them are discussed separately below.

Synthesized KR12-functionalised CNF (CNF-KR12)

The resultant CNF-KR12 (final product of synthesis) had a peptide substitution corresponding to 0.2 mmol of KR12 peptide per g of CNF. This was obtained by the elemental analysis of the material.

Heparinization

The heparinization of the lab consumables used in the blood experiments was tested against toluidine blue as per the protocol. The purple/ violet pigmentation after incubating the equipment for 2-3 min and then rinsing it with water was observed which confirms heparinization. Hence, all the consumables were heparinized properly.

In vitro whole blood loop model

The response of the materials after getting in contact with the blood was studied and analyzed by means of an *in vitro* whole blood loop model. Here, the experimental setup had two controls (external) i.e., a control with PBS (diluted the blood equivalent to the dilutions present with tested materials) and a control without PBS (blood with no additive). These were used as controls in context with the experimental model to study the reactions and effects caused by the loop model and the PBS dilutions in the blood. However, the loops having CNF material were the internal control as it was used as the same final concentration as the CNF-KR12. The KR12 soluble peptide also acted as an internal control having concentrations equivalent to the amount of peptide present in CNF-KR12. This helped to study and draw a conclusion about how effectively or non-effectively the KR12 peptide influenced the innate immune response in combination with CNF, which is the testing material. Following the setup, all the diluted materials were filled inside the loops along with the blood, and results obtained from ELISAs thereafter, are stated separately below.

Platelet Counting using Hematology Analyzer

Platelet counts for the initial blood samples serving as 0 min samples at the time of the beginning of the experiment and for the samples after 1 h incubation in the loops at 37 °C were all analyzed to see the response of the material in contact with the blood. The number of remaining platelets (%) for the samples and controls was calculated with respect to the initial blood samples. There is a variation in the platelet count between different blood donors and so, normalization was carried out for all the samples against the initial blood samples and then plotted as mean ± SEM (Figure 3). In the case of the activation of the coagulation cascade, a significant reduction in the platelet count is expected in the samples followed by the formation of a clot.

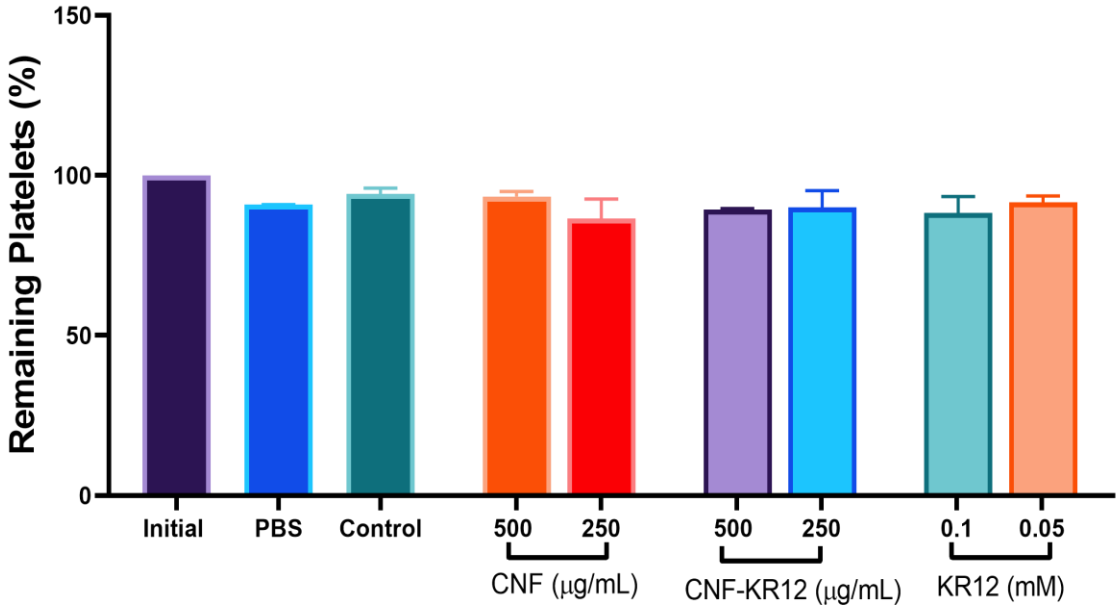

Figure 3: Coagulation cascade activation measured as the remaining platelets (%) by the materials in contact with blood compared to the initial samples which is followed by PBS control, Control, CNF (500 µg/mL and 250 µg/mL), CNF-KR12 (500 µg/mL and 250 µg/mL), and KR12 (0.1 mM and 0.05 mM). The platelet count was measured after 1 h incubation at 37 °C and samples were mixed with EDTA to a final concentration of 10 mM. Data represent mean ± SEM, n=2.

After 1 h incubation, all the materials CNF, CNF-KR12, and KR12 showed a very small reduction in the platelet count (around 10%) (Figure 3). There was no clot formation observed for any of the samples. Similarly, the reduction is very small for the controls (PBS and without PBS) (Figure 3) stating that there was no activation induced by the loop model itself. While some differences in the reduction count can be observed between the two different working concentrations of CNF i.e., between 500 µg/mL and 250 µg/mL, it is still within the range of 10-12% reduction. Similar is the pattern observed between the different concentrations of KR12 peptide i.e., 0.1 mM and 0.05 mM respectively. This shows there was no apparent activation of the coagulation system.

Enzyme-linked immunosorbent assays (ELISAs) for coagulation and complement markers

C3a and sC5b-9 were quantified using sandwich ELISA for the complement system activation whereas TAT levels were quantified for the coagulation cascade activation.

In-house TAT ELISA

The levels of TAT after whole blood incubation in the loop model were measured using TAT ELISA to investigate the pattern of activation of the coagulation cascade by the materials. These levels for TAT were measured in the obtained plasma from the blood samples after the 1 h incubation at 37 °C (Figure 4). Thrombin is an enzyme responsible for the cleavage of fibrinogen to fibrin that functions to bind platelets into a clot. Hence, high concentrations or increased levels of TAT indicate activation of coagulation and are used as a coagulation activation marker.

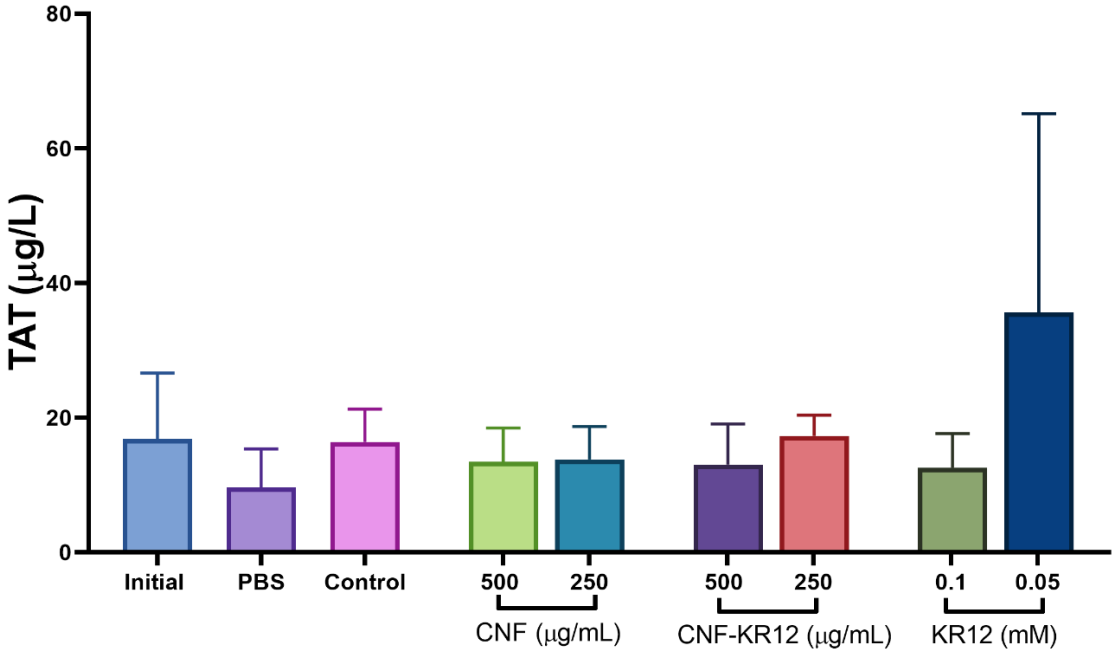

Figure 4: Coagulation cascade activation measured by the formation of TAT after blood contact with the materials: Initial blood sample, PBS control, Control, CNF (500 µg/mL and 250 µg/mL), CNF-KR12 (500 µg/mL and 250 µg/mL), and KR12 (0.1 mM and 0.05 mM) using TAT ELISA. This graph shows the corresponding levels of TAT after the incubation. Data represent mean ± SEM, n=2.

The resulting TAT levels as shown in figure 4 depict that the samples with CNF and CNF-KR12 showed no increase in the level of TAT formation comparable to the initial blood sample as well as both the controls. Also, there was rather a slight decrease in the TAT concentration with respect to the addition of PBS in the control as compared to the control without PBS. As a whole, both the samples with CNF and CNF-KR12 were still within the range of 0-20 (µg/L) as compared to the initial blood samples as well as both the controls and, no clots were observed. This shows there was no activation of coagulation in these samples. There was a small increase in the sample with KR12 peptide for 0.05 mM concentration unlike the sample having a concentration of 0.1 mM. This increase is within the range of 20-40 (µg/L) which is still not sufficient to represent considerable activation of the coagulation. This shows there was no activation of coagulation in the samples with KR12 peptide.

Hence, neither there was a considerable amount of reduction in the platelet count nor were the levels of TAT increased to an extent to support coagulation system activation. It can be stated that there was no apparent activation of the coagulation cascade by the materials.

ELISA's to study the complement activation.

In house C3a ELISA

The levels of the C3a component after whole blood incubation in the loop model were measured using C3a ELISA to investigate the pattern of activation of the complement system by the materials. These levels for C3a were measured in the obtained plasma from the blood samples after the 1 h incubation at 37 °C (Figure 5).

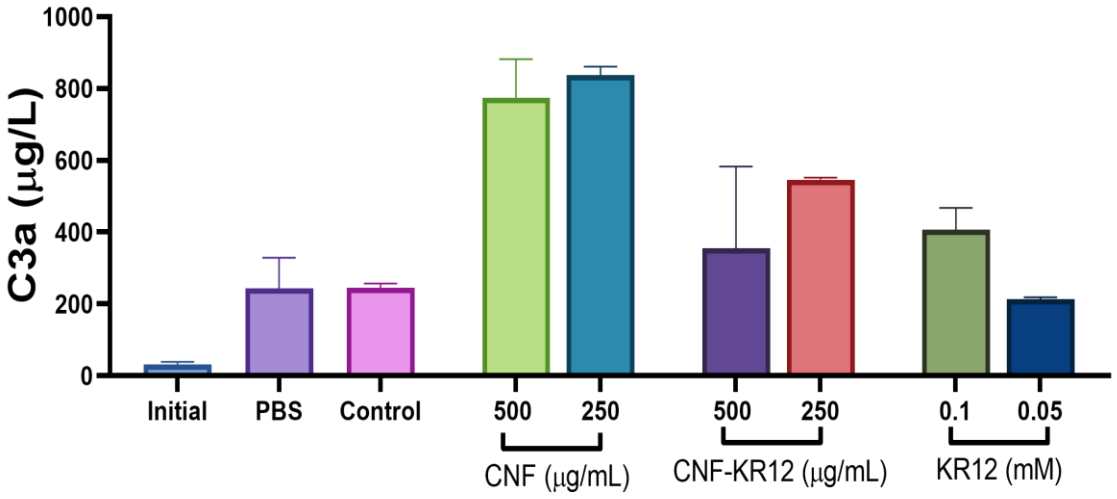

Figure 5: Complement system activation measured by the formation of C3a fragment after blood contact with the materials: Initial blood sample, PBS control, Control, CNF (500 µg/mL and 250 µg/mL), CNF-KR12 (500 µg/mL and 250 µg/mL), and KR12 (0.1 mM and 0.05 mM) using C3a ELISA. This graph shows the corresponding levels of C3a after the incubation. Data represent mean ± SEM, n=2.

C3a is an activation product of the complement system. The increase in the levels of C3a is known to depict activation of the complement system. There is no activation observed in the initial blood samples as expected. The controls (with and without PBS) show slightly higher C3a levels compared to the initial sample but are still very low. This slight activation might be the result of the activation caused by the loops in general. Among the samples, CNF showed a very high activation with a large increase in the levels of C3a as compared to the PBS control, Control, and the initial blood samples (Figure 5). The lowest levels of C3a were present in the KR12 peptide samples, almost at the same levels as the controls. However, the KR12 sample with 0.1 mM showed an increase in C3a. Interesting is to note that the plasma samples in contact with the CNF-KR12 material showed an intermediate increase in the levels of C3a as compared to KR12 and CNF samples i.e., somewhere in between the range of CNF and KR12 samples alone. This suggests that there was a strong promotion of complement activation by CNF and comparatively lower activation by CNF-KR12 whereas KR12 did not trigger any complement activation at all.

In house sC5b-9 ELISA

Another marker of complement activation is sC5b-9 which marks the end product component of the complement cascade. Just like C3a, the levels of sC5b-9 were also quantified in the plasma using sC5b-9 ELISA after incubation of the materials in whole blood incubation for 1 h at 37 °C in the loop model (Figure 6). The controls (with and without PBS) show slightly higher C3a levels compared to the initial sample but are still very low. An increase in the levels of sC5b-9 indicates complement activation. The sC5b-9 levels for all the samples (Figure 6) showed a similar pattern

as the C3a levels (Figure 5). Important to note is the same low degree of activation seen in KR12 samples for both the concentrations tested i.e., 0.1 mM and 0.05 mM. CNF samples showed the highest activation levels of sC5b-9 as compared to both the controls and the initial blood samples (Figure 6). However, the plasma samples in contact with the CNF-KR12 material showed an intermediate level of sC5b-9 as compared to KR12 and CNF samples separately. This suggests that there was a strong promotion of complement activation by CNF and comparatively lower activation in CNF-KR12 whereas KR12 did not promote the activation of the complement system.

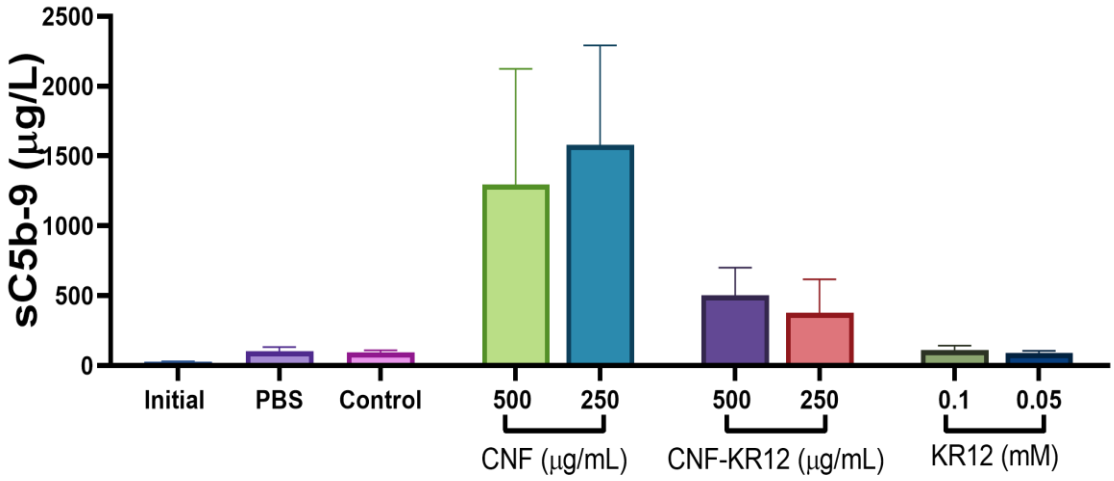

Figure 6: Complement system activation measured by the formation of sC5b-9 component after blood contact with the materials: Initial blood sample, PBS control, Control, CNF (500 µg/mL and 250 µg/mL), CNF-KR12 (500 µg/mL and 250 µg/mL), and KR12 (0.1 mM and 0.05 mM) using sC5b-9 ELISA. Data represent mean ± SEM, n=2.

The CNF-KR12 material showed an interesting pattern of intermediate activation levels for both C3a as well as sC5b-9 components of the complement cascade. However, since all the results are presented as a mean of only two blood experiments, no conclusions can be drawn about the statistical significance of the results.

Discussion

Overview

In the work presented in this thesis, a novel material CNF-KR12 was investigated. This material combines the wood-derived CNF with the HDP KR12. CNF has shown remarkably promising results in wound care healing applications (Basti et al., 2022; Basu et al., 2017). Hence, CNF served as the starting material. On the other hand, KR12 is classified as an HDP conferring several properties supporting wound care (Gomes et al., 2017; Liu et al., 2019; Miao et al., 2021). The hybrid material was assessed in terms of the response generated by the innate immune system by studying the material-induced activation of the coagulation system and the complement system.

Response of the coagulation system – Platelet count and in-house TAT ELISA

In the events of cutaneous injury marked by the damage of blood vessels leads to the exposure of the endothelium. The platelets are activated and adhere to the endothelium and to fibrin, in the formed blood clot. This clotting is initiated by the extrinsic pathway of coagulation consisting of tissue factor and plasma factor VII/VIIa (Mackman et al., 2007). Similarly, the platelets are also activated when the blood comes in contact with a foreign surface via the intrinsic pathway of coagulation initiated by the factor XII by negatively charged surfaces (Renné et al., 2012). The remaining platelets (%) were calculated after normalization of the platelet counts against the initial blood samples (Figure 3). This is done to avoid the variation of the platelet counts between the different donors (here, two different donors). On the other hand, thrombin is responsible for the generation of a clot. The antithrombin acts as a regulator of coagulation and inactivates thrombin by binding to it, forming a TAT complex (Svensson et al., 2021). Measuring the levels of thrombin is difficult since, it is inactivated by the serine protease inhibitor (SERPIN) AT, resulting in the formation of these TAT complexes (Svensson et al., 2021). An advantage of these complexes is that they are very stable and can be easily measured in the plasma making them a reliable method to monitor the coagulation activation (Hong et al., 2005). There was no apparent activation of the coagulation system by any of the materials (CNF, CNF-KR12, and KR12) as reported by the remaining platelet counts (%) as well as the TAT levels (Figures 3 and 4). The 10% (approximate) reduction of platelet count in almost all the samples could be a consequence of background activation. The levels showed variation between the different donors as can be reflected by the large error bars (SEM) for the mean which is the possible reason for the increase in the level of TAT in the KR12 sample alone having 0.05 mM concentration. In a study, CNF was hybridized with different types of nano titania which showed activation of the coagulation system, except one of them which was ammonium oxo-lactato titanate instead inhibited the platelet consumption (Svensson et al., 2021). The coagulation activation induced by CNF hybridized with TiO2 and CNF with captigel showed TAT levels up to 30,000- 35,000 (µg/L) whereas KR12 sample (Figure 4) shows TAT levels up to 20 -40 (µg/L) only which is very small as compared to the samples where activation has been observed. Similarly, CNF crosslinked with calcium sample (Basu et al., 2017) showed TAT levels up to 800 (ng/mL) whereas CNF here, showed TAT levels much lower than that up to 20 (µg/L). The calcium ion crosslinking with CNF were shown to influence the biological response to the material (Basu et al., 2017). In the case of activation of the coagulation system (Basu et al., 2017; Svensson et al., 2021), the platelet reduction was also around 50%. One of the reasons, the coagulation system was activated were the different types of materials and different experimental setup used which suggests that CNF crosslinked with calcium and some forms of nano titania induce the coagulation system efficiently.

Also, platelet activation via a fibrin clot serves as a pool for the cytokines and the growth factors to initiate the wound closure process (Opneja et al., 2019). The activation of coagulation system is the first reaction to stop the bleeding of the wound and maybe an important criterion for the making of hemostatic wound dressings in particular. In the previous research (Basu et al., 2017), a more basic form of CNF linked with calcium was used to evaluate its potential for treating the wounds particularly acute wounds where there are no prolonged inflammatory phases caused by the infection (Frykberg & Banks, 2015) and the primary function is to stop the bleeding. Similarly, TAT levels were also increased in the studies mentioned above. In case of hemophilic conditions also, the material to be hemostatic is the most important factor of healing (Monroe & Hoffman, 2012). Also, the generation of the right amount of thrombin is also necessary for effective hemostasis and wound healing during the coagulation process (Hoffman & Monroe, 2007). With respect to the aforementioned studies, this thesis aimed at investigating the material in the context of chronic wound care in particular, so no activation of coagulation system is not a failure to the testing of the material. In fact, one of the other possible reasons for no activation of coagulation system could be that the material was a low activator at the concentrations of 500 µg/mL and 250 µg/mL which means the increase in concentrations could lead to activation, but to work with higher concentrations of the cellulosic material can be another challenge (e.g., very dense suspensions can be difficult to deal with).

Response of the complement system- C3a and sC5b-9 ELISA

Formation of C3a is an important part of the complement activation process as it is said to induce inflammatory responses and activate the T-cells, both of which are very essential for the initiation of the wound healing process whereas sC5b-9 functions to destroy the cell membranes of the pathogens, ultimately facilitating their death. Hence, it is called a membrane attack complex. There was a moderate level activation in the sample CNF-KR12 for both the complement markers C3a and sC5b-9 (Figures 5 and 6). The CNF showed a high activation of the complement system for both C3a and sC5b-9 markers (Figures 5 and 6) whereas KR12 samples did not trigger complement activation at all for both the markers (Figures 5 and 6). There was an induction of the complement system activation in all the samples except the KR12 peptide but an intermediate level of it in the hybrid sample CNF-KR12 best justifies the combination of a highly activating material CNF and the non-activating material KR12. This suggests the peptide KR12 when combined with CNF moderates a highly activating response of the CNF, alone which speaks for the modulation of immune response and the wound healing environment caused by host-defense peptides (here, KR12).

The complement system is a very significant part of the innate immune system and several studies suggests that the C3a component is antibacterial demonstrating an antimicrobial effect of complement activation that nullifies causing excessive inflammation and works more in the killing of bacteria and microbes (Nordahl et al., 2004). Therefore, an activation of the complement system is beneficial for its antibacterial activity. High level of the complement markers, however, suggests a high and immediate inflammatory response which can cause inflammation in the wound area. As a consequence of this, a high activation observed in CNF alone (Figure 5 and 6) might not be the ideal choice for the healing of the chronic wounds as shown by an increased level of it, but its tunability and good mechanical strength best supports the criteria for a potential wound dressing material. But intermediate inflammation or moderate levels of complement activation markers could potentially be beneficial for wound healing. Since, KR12 peptide (smallest epitope of the parent peptide LL-37) is contributing towards the moderation of the highly activating CNF material, several studies support the pro-inflammatory and anti-inflammatory nature of the LL-37 (parent peptide) which suggests that its presence in a particular amount can act to heal wounds

by recruiting immune cells for e.g., keratinocytes that are essential for skin repair and re-epithelialization process while excessive inflammation may lead to tissue damage (Kahlenberg & Kaplan, 2013; Xhindoli et al., 2016; Yang et al., 2020). Hence, it plays an important role in regulation of inflammatory responses (Nijnik & Hancock, 2009). In the studies (Basu et al., 2017; Svensson et al., 2021), the activation of complement system was much lower up to 200-250 (ng/mL) for all the CNF hybrid samples as compared to the work presented here (Figures 5 and 6) that shows levels within the range of 500-600 (µg/L) for both C3a and sC5b-9 markers. These studies also support a normal pattern showing that if the coagulation system is highly activated then the complement system is not activated and vice-versa (Basu et al., 2017; Svensson et al., 2021). However, the main function lies in the application of the material according to the type of wounds. For chronic wounds, a better attention to the modulation of the prolonged inflammatory phase is more essential among other factors. Many studies have previously discussed the complexity of the healing of the chronic wounds (Frykberg & Banks, 2015; Guo & Dipietro, 2010).

LL-37 peptide is shown to support re-epithelialization as well as angiogenesis from the perspective of wound healing (Mangoni et al., 2016). Several other studies (Gomes et al., 2017; Liu et al., 2019) support the human based defense peptide KR12 for the healing of chronic wounds, where they used the peptide under different modifications. For example, in this work (Liu et al., 2019), KR12 is modified by the hyaluronic acid and immobilized on fibrous egg shell membrane. On the other hand, CNF has plethora of evidences supporting its use in the wound care industry. For example one of the recent work included the use of CNF incorporated with silver nanoparticles and chitosan film that showed results successfully inhibiting the bacterial growth (Zaitun Hasibuan et al., 2021).

In vitro whole blood loop model was utilized to study the impact of this material on interaction with the blood. Ekdahl and co-workers developed a holistic approach to studying the interaction of biomaterials with the blood at a molecular level (Ekdahl et al., 2013). This model utilizes commercially heparinized plastic tubing which can be filled with fresh blood or plasma samples to evaluate the biomaterial's interaction with the blood. However, there is room for an air bubble left inside the tubing which ensures enough space for the rotation of the whole volume of blood and prevents clotting due to inadequate flow (Ekdahl et al., 2013). The air bubble can also cause slight activation of the complement system as seen in the controls (Figures 5 and 6) and also could be the reason of the C3a levels in the KR12 samples (Figure 5). Some of the advantages of using loop model over chamber model (discussed below) include the need for less material (CNF and KR12) per experiment which cuts down the expenses and also makes it easier to compare and perform evaluation with soluble peptides (here, KR12). But it also has the limitation of the material being present in the form of gel suspensions which requires testing the same material in different concentrations because the material in the loop needs to be properly suspended in the blood to attain the best results. For that, different concentrations are required to study the response.

Previous research carried out proved the potency of CNF as a hydrogel eligible for the production of wound dressing supported by promising results in *in vitro* and *in vivo* experiments (Basu et al., 2018). Polymers like cellulose have the ability to absorb large amounts of water due to the presence of hydrophilic functional groups attached to the polymer backbone. Wood CNF in the form of films/sheets, hydrogels (Basti et al., 2022; Basu et al., 2017; Curvello et al., 2019), or aerogels (Nordli et al., 2019) is already supported through several pieces of evidence ideal for wound healing dressings due to greater moisture adsorption capacity suitable for exuding wounds like burns (Basti et al., 2022). As a hydrogel it renders moisture donating properties beneficial to maintain the moist wound bed required for re-epithelialization and advanced wound healing applications (Metzger, 2004) unlike loop model where the material is present as a gel

suspension. The material in the form of films and hydrogels can be tested by using an *in vitro* chamber model (Basu et al., 2017; Svensson et al., 2021). This model has the advantage of analysis of the material surface interactions with the blood by using high resolution microscopy methods. However, there is a need for a larger amount of material to make films and hydrogels unlike loop model.

Important factors that can act as sources of error while performing the experiments are the dilutions of the samples while performing the ELISAs, weak or uneven heparinization of materials that will lead to background activation showing non-significant results, or while collecting the blood samples from the healthy volunteers for example the blood needs to be collected from volunteers who have not taken any medications that may affect the blood experiments like paracetamol and ibuprofen can affect the platelet count but not for a very long time, they have reversible effects. Another one includes acetylsalicylic acid (Ekdahl et al., 2013). However, these are all the potential errors that can happen while performing the experiments and will lead to non-significant results.

Conclusion

The combined studies with CNF-KR12 showed that the material does not induce the activation of the coagulation system but it promoted the activation of the complement system. It is difficult to draw a significant conclusion from the above discussion, as the results are not statistically tested. Two blood experiments are not enough to say anything significant about the nature of the material in question. Therefore, the experiments described in the thesis should be repeated to attain enough data for statistical analysis. However, this might be taken as a stepping stone into future research where the material can be tested further either by exploring other *in vitro* models or expanding the range of concentrations tested to get a better insight into its application for wound healing.

Ethical aspects and impact on the society

The collection of blood samples from volunteers presents the same potential risks to the health and safety of the person drawing the blood as it does to the person using it for research or experiments. Therefore, all blood samples must be treated as potentially infectious. All the blood samples were assessed and worked upon in a special lab called “blood lab” with considerable precautions. Ethical approval for the blood sampling was obtained from the Regional Ethics Committee, reference number- 2008/264 (Uppsala, Sweden).

This thesis aimed to address the issue of chronic wound healing with the idea of generating dressings combined with the CNF and HDP-KR12 which might regulate faster healing. Chronic wounds are the cause of ultimate physical pain and trauma. They are often regarded as socially and economically a burden in the life of a patient suffering from them. With research focused on improving the lifestyle of such people- improved and upgraded versions of wound dressings are creating havoc as they are said to be better in terms of accelerating the process of healing. Along with the need for new therapeutic approaches, there is an increasing quest for the replacement of synthetic materials with greener materials, eco-friendly and sustainable for our society. CNF is one of those materials creating a breakthrough in the science of wound healing and has so far shown promising results. It is biodegradable, renewable, and a naturally occurring biopolymer in nature. It is present in abundance and thus, easily available cutting down the expenses and making it an economical choice. Other than this, the simplified manufacturing processes curated after ongoing research for almost 20 years make it an even more interesting research area to further contribute to improving people’s quality of life.

Future perspectives

CNF has attracted the interest of a great number of researchers as an alternative to traditional wound dressings. Traditional wound dressings were said to be limited at the disposal of healing of wounds because they were susceptible to infection. The current and updated versions of the dressings include materials in the form of hydrogels, films, etc, and are designed to treat a specific type of wound. CNF has an easily modifiable nature which exploits its application to be combined with different biomaterials that can facilitate the healing of wounds. Here, the work presented in this thesis shows the CNF functionalized with KR12; a host-defense peptide. The idea behind this functionalization was to introduce antibacterial properties into the material that might help in accelerating the healing process. Successful research based on CNF material already led to some products available in the market encouraging its potential characteristics as a wound care application. Modifications might improve and confer higher and targeted amount of wound healing.

The work presented in this thesis evaluated the response of CNF-KR12 to the innate immune system. CNF-KR12 showed no activation of the coagulation system but activated the complement system. This result shows that the material can be a good candidate for the healing of chronic wounds but further research is required. The best way would be to first, repeat the experiments using the same approach as mentioned in the thesis and statistically interpret the results. The other aspect before moving forward with the material would be to test its interaction with blood in an *in vivo* model as discussed by Basu and co-workers (Basu et al., 2018).

In another way, a change from *in vitro* loop model to a whole blood chamber model (Basu et al., 2017; Svensson et al., 2021) would allow the material surface analysis and also provides the scope to test the material in the form of hydrogel or films (Hua et al., 2016). The material can then be analyzed using Scanning Electron Microscopy to study the nano and micro-structure of the CNF after coming in contact with the blood. *In vitro* assays like the thrombin generation assay (TGA) using pooled platelet-free plasma (PFP) can be used to further study the hemostatic ability of CNF which would help to assess the real-time formation of thrombin.

Upcoming futuristic approaches in the world of wound care talk about loading the material with stem cells as a potential therapeutic solution but this also requires large clinical trials (Moradpoor et al., 2022). The 3D and 4D printing of biomaterials capable to mimic the extracellular matrix are also looked upon as an alternative towards a promising solution (Moradpoor et al., 2022). To facilitate better healing of the wounds and pertain to higher healing rates, a better understanding of the different types of wounds is required both at the molecular and cellular levels (Frykberg & Banks, 2015).

Acknowledgements

I would like to thank everyone who helped and advised me throughout the journey of my thesis. To begin with, I would like to express my gratitude to my supervisors at Uppsala University- Asso. Prof. Natalia Ferraz and Asso. Prof. Karin Fromell for making the resources available to me. I owe a debt of gratitude for your valuable feedback and insights into making this project possible and paving my way to research. I would also like to thank my examiner Prof. Maria Algerin for her constant support during the finalization of this project.

Lastly, I would thank all the people at Rudbeck Laboratory and Angstrom Laboratory (Uppsala University) for their constant support and cooperation while I was working in the lab.

References

Anžlovar, A., & Žagar, E. (2022). Cellulose Structures as a Support or Template for Inorganic Nanostructures and Their Assemblies. *Nanomaterials*, *12*(11), 1837. https://doi.org/10.3390/nano12111837

Arnold, M., & Barbul, A. (2006). Nutrition and Wound Healing: *Plastic and Reconstructive Surgery*, *117*(SUPPLEMENT), 42S-58S. https://doi.org/10.1097/01.prs.0000225432.17501.6c

Bäck, J., Sanchez, J., Elgue, G., Ekdahl, K. N., & Nilsson, B. (2010). Activated human platelets induce factor XIIa-mediated contact activation. *Biochemical and Biophysical Research Communications*, *391*(1), 11–17. https://doi.org/10.1016/j.bbrc.2009.10.123

Basti, A. T. K., Jonoobi, M., Sepahvand, S., Ashori, A., Siracusa, V., Rabie, D., Mekonnen, T. H., & Naeijian, F. (2022). Employing Cellulose Nanofiber-Based Hydrogels for Burn Dressing. *Polymers*, *14*(6), 1207. https://doi.org/10.3390/polym14061207

Basu, A., Hong, J., & Ferraz, N. (2017). Hemocompatibility of Ca^{2+} -Crosslinked Nanocellulose Hydrogels: Toward Efficient Management of Hemostasis. *Macromolecular Bioscience*, *17*(11), 1700236. https://doi.org/10.1002/mabi.201700236

Basu, A., Celma, G., Strømme, M., & Ferraz, N. (2018). In Vitro and in Vivo Evaluation of the Wound Healing Properties of Nanofibrillated Cellulose Hydrogels. *ACS Applied Bio Materials*, *1*(6), 1853–1863. https://doi.org/10.1021/acsabm.8b00370

Cazander, G., Jukema, G. N., & Nibbering, P. H. (2012). Complement activation and inhibition in wound healing. *Clinical & Developmental Immunology*, *2012*, 534291. https://doi.org/10.1155/2012/534291

Chami Khazraji, A., & Robert, S. (2013). Self-Assembly and Intermolecular Forces When Cellulose and Water Interact Using Molecular Modeling. *Journal of Nanomaterials*, *2013*, 1–12. https://doi.org/10.1155/2013/745979

Clark, R., & Kupper, T. (2005). Old Meets New: The Interaction Between Innate and Adaptive Immunity. *Journal of Investigative Dermatology*, *125*(4), 629–637. https://doi.org/10.1111/j.0022-202X.2005.23856.x

Curvello, R., Raghuwanshi, V. S., & Garnier, G. (2019). Engineering nanocellulose hydrogels for biomedical applications. *Advances in Colloid and Interface Science*, *267*, 47–61. https://doi.org/10.1016/j.cis.2019.03.002

Diegelmann, R. F., & Evans, M. C. (2004). Wound healing: An overview of acute, fibrotic and delayed healing. *Frontiers in Bioscience: A Journal and Virtual Library*, *9*, 283–289. https://doi.org/10.2741/1184

Dolkar, T., Trinidad, C. M., Nelson, K. C., Amaria, R. N., Nagarajan, P., Torres-Cabala, C. A., Ivan, D., Prieto, V. G., Tetzlaff, M. T., Curry, J. L., & Aung, P. P. (2018). Dermatologic toxicity from novel therapy using antimicrobial peptide LL-37 in melanoma: A detailed examination of the clinicopathologic features. *Journal of Cutaneous Pathology*, *45*(7), 539–544. https://doi.org/10.1111/cup.13262

Ekdahl, K. N., Hong, J., Hamad, O. A., Larsson, R., & Nilsson, B. (2013). Evaluation of the blood compatibility of materials, cells, and tissues: Basic concepts, test models, and practical guidelines.

Advances in Experimental Medicine and Biology, *735*, 257–270. https://doi.org/10.1007/978-1-4614-4118-2_18

Ekdahl, K. N., Teramura, Y., Asif, S., Jonsson, N., Magnusson, P. U., & Nilsson, B. (2015). Thromboinflammation in Therapeutic Medicine. In J. D. Lambris, K. N. Ekdahl, D. Ricklin, & B. Nilsson (Eds.), *Immune Responses to Biosurfaces* (Vol. 865, pp. 3–17). Springer International Publishing. https://doi.org/10.1007/978-3-319-18603-0_1

Epand, R. M., & Epand, R. F. (2009). Lipid domains in bacterial membranes and the action of antimicrobial agents. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1788*(1), 289–294. https://doi.org/10.1016/j.bbamem.2008.08.023

Etulain, J. (2018). Platelets in wound healing and regenerative medicine. *Platelets*, *29*(6), 556–568. https://doi.org/10.1080/09537104.2018.1430357

Fjell, C. D., Hiss, J. A., Hancock, R. E. W., & Schneider, G. (2012). Designing antimicrobial peptides: Form follows function. *Nature Reviews Drug Discovery*, *11*(1), 37–51. https://doi.org/10.1038/nrd3591

Frykberg, R. G., & Banks, J. (2015). Challenges in the Treatment of Chronic Wounds. *Advances in Wound Care*, *4*(9), 560–582. https://doi.org/10.1089/wound.2015.0635

Glenn, J. K., & Goldman, J. (1976). Task delegation to physician extenders—Some comparisons. *American Journal of Public Health*, *66*(1), 64–66. https://doi.org/10.2105/ajph.66.1.64

Gomes, A., Teixeira, C., Ferraz, R., Prudêncio, C., & Gomes, P. (2017). Wound-Healing Peptides for Treatment of Chronic Diabetic Foot Ulcers and Other Infected Skin Injuries. *Molecules*, *22*(10), 1743. https://doi.org/10.3390/molecules22101743

Guo, S., & Dipietro, L. A. (2010). Factors affecting wound healing. *Journal of Dental Research*, *89*(3), 219–229. https://doi.org/10.1177/0022034509359125

Häggström, M. (2014). Medical gallery of Mikael Häggström 2014. *WikiJournal of Medicine*, *1*(2). https://doi.org/10.15347/wjm/2014.008

Hajishengallis, G., Reis, E. S., Mastellos, D. C., Ricklin, D., & Lambris, J. D. (2017). Novel mechanisms and functions of complement. *Nature Immunology*, *18*(12), 1288–1298. https://doi.org/10.1038/ni.3858

Hamdan, S., Pastar, I., Drakulich, S., Dikici, E., Tomic-Canic, M., Deo, S., & Daunert, S. (2017). Nanotechnology-Driven Therapeutic Interventions in Wound Healing: Potential Uses and Applications. *ACS Central Science*, *3*(3), 163–175. https://doi.org/10.1021/acscentsci.6b00371

Hemker, H. C., Loeliger, E. A., & Veltkamp, J. J. (1969). *Human Blood Coagulation: Biochemistry, Clinical Investigation and Therapy*. Springer Netherlands. https://doi.org/10.1007/978-94-010-3423-4

Hoffman, M., & Monroe, D. M. (2007). Coagulation 2006: A Modern View of Hemostasis. *Hematology/Oncology Clinics of North America*, *21*(1), 1–11. https://doi.org/10.1016/j.hoc.2006.11.004

Hong, J., Azens, A., Ekdahl, K. N., Granqvist, C. G., & Nilsson, B. (2005). Material-specific thrombin generation following contact between metal surfaces and whole blood. *Biomaterials*, *26*(12), 1397–1403. https://doi.org/10.1016/j.biomaterials.2004.05.036

Hua, K., Rocha, I., Zhang, P., Gustafsson, S., Ning, Y., Strømme, M., Mihranyan, A., & Ferraz, N. (2016). Transition from Bioinert to Bioactive Material by Tailoring the Biological Cell Response to Carboxylated Nanocellulose. *Biomacromolecules*, *17*(3), 1224–1233. https://doi.org/10.1021/acs.biomac.6b00053

Iconomou, T. G., Zuker, R. M., & Michelow, B. J. (1993). Management of major penetrating glass injuries to the upper extremities in children and adolescents. *Microsurgery*, *14*(2), 91–96. https://doi.org/10.1002/micr.1920140202

Kahlenberg, J. M., & Kaplan, M. J. (2013). Little peptide, big effects: The role of LL-37 in inflammation and autoimmune disease. *Journal of Immunology (Baltimore, Md.: 1950)*, *191*(10), 4895–4901. https://doi.org/10.4049/jimmunol.1302005

Klemm, D., Heublein, B., Fink, H.-P., & Bohn, A. (2005). Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angewandte Chemie International Edition*, *44*(22), 3358–3393. https://doi.org/10.1002/anie.200460587

Landén, N. X., Li, D., & Ståhle, M. (2016). Transition from inflammation to proliferation: A critical step during wound healing. *Cellular and Molecular Life Sciences*, *73*(20), 3861–3885. https://doi.org/10.1007/s00018-016-2268-0

Lin, N., & Dufresne, A. (2014). Nanocellulose in biomedicine: Current status and future prospect. *European Polymer Journal*, *59*, 302–325. https://doi.org/10.1016/j.eurpolymj.2014.07.025

Liu, M., Liu, T., Zhang, X., Jian, Z., Xia, H., Yang, J., Hu, X., Xing, M., Luo, G., & Wu, J. (2019). Fabrication of KR-12 peptide-containing hyaluronic acid immobilized fibrous eggshell membrane effectively kills multi-drug-resistant bacteria, promotes angiogenesis and accelerates re-epithelialization. *International Journal of Nanomedicine*, *14*, 3345–3360. https://doi.org/10.2147/IJN.S199618

Lou, Y.-R., Kanninen, L., Kuisma, T., Niklander, J., Noon, L. A., Burks, D., Urtti, A., & Yliperttula, M. (2014). The use of nanofibrillar cellulose hydrogel as a flexible three-dimensional model to culture human pluripotent stem cells. *Stem Cells and Development*, *23*(4), 380–392. https://doi.org/10.1089/scd.2013.0314

Mackman, N., Tilley, R. E., & Key, N. S. (2007). Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *27*(8), 1687–1693. https://doi.org/10.1161/ATVBAHA.107.141911

Malinen, M. M., Kanninen, L. K., Corlu, A., Isoniemi, H. M., Lou, Y.-R., Yliperttula, M. L., & Urtti, A. O. (2014). Differentiation of liver progenitor cell line to functional organotypic cultures in 3D nanofibrillar cellulose and hyaluronan-gelatin hydrogels. *Biomaterials*, *35*(19), 5110–5121. https://doi.org/10.1016/j.biomaterials.2014.03.020

Mangoni, M. L., McDermott, A. M., & Zasloff, M. (2016). Antimicrobial peptides and wound healing: Biological and therapeutic considerations. *Experimental Dermatology*, *25*(3), 167–173. https://doi.org/10.1111/exd.12929

Masson-Meyers, D. S., Andrade, T. A. M., Caetano, G. F., Guimaraes, F. R., Leite, M. N., Leite, S. N., & Frade, M. A. C. (2020). Experimental models and methods for cutaneous wound healing assessment. *International Journal of Experimental Pathology*, *101*(1–2), 21–37. https://doi.org/10.1111/iep.12346

Mathieu, D., & Wattel, F. (2006). Physiologic Effects of Hyperbaric Oxygen on Microorganisms and Host Defences Against Infection. In D. Mathieu (Ed.), *Handbook on Hyperbaric Medicine* (pp. 103–119). Springer-Verlag. https://doi.org/10.1007/1-4020-4448-8_7

Metzger, S. (2004). Clinical and Financial Advantages of Moist Wound Management: *Home Healthcare Nurse: The Journal for the Home Care and Hospice Professional*, *22*(9), 586–590. https://doi.org/10.1097/00004045-200409000-00003

Miao, F., Li, Y., Tai, Z., Zhang, Y., Gao, Y., Hu, M., & Zhu, Q. (2021). Antimicrobial Peptides: The Promising Therapeutics for Cutaneous Wound Healing. *Macromolecular Bioscience*, *21*(10), 2100103. https://doi.org/10.1002/mabi.202100103

Monroe, D. M., & Hoffman, M. (2012). The clotting system - a major player in wound healing: THE CLOTTING SYSTEM IN WOUND HEALING. *Haemophilia*, *18*, 11–16. https://doi.org/10.1111/j.1365-2516.2012.02889.x

Moon, R. J., Schueneman, G. T., & Simonsen, J. (2016). Overview of Cellulose Nanomaterials, Their Capabilities and Applications. *JOM*, *68*(9), 2383–2394. https://doi.org/10.1007/s11837-016-2018-7

Moradpoor, H., Mohammadi, H., Safaei, M., Mozaffari, H. R., Sharifi, R., Gorji, P., Sulong, A. B., Muhamad, N., & Ebadi, M. (2022). Recent Advances on Bacterial Cellulose-Based Wound Management: Promises and Challenges. *International Journal of Polymer Science*, *2022*, 1–24. https://doi.org/10.1155/2022/1214734

Nijnik, A., & Hancock, R. E. (2009). The roles of cathelicidin LL-37 in immune defences and novel clinical applications: *Current Opinion in Hematology*, *16*(1), 41–47. https://doi.org/10.1097/MOH.0b013e32831ac517

Nordahl, E. A., Rydengård, V., Nyberg, P., Nitsche, D. P., Mörgelin, M., Malmsten, M., Björck, L., & Schmidtchen, A. (2004). Activation of the complement system generates antibacterial peptides. *Proceedings of the National Academy of Sciences*, *101*(48), 16879–16884. https://doi.org/10.1073/pnas.0406678101

Nordli, H. R., Pukstad, B., Chinga-Carrasco, G., & Rokstad, A. M. (2019). Ultrapure Wood Nanocellulose—Assessments of Coagulation and Initial Inflammation Potential. *ACS Applied Bio Materials*, *2*(3), 1107–1118. https://doi.org/10.1021/acsabm.8b00711

Noris, M., & Remuzzi, G. (2013). Overview of Complement Activation and Regulation. *Seminars in Nephrology*, *33*(6), 479–492. https://doi.org/10.1016/j.semnephrol.2013.08.001

Öhnstedt, E., Lofton Tomenius, H., Vågesjö, E., & Phillipson, M. (2019). The discovery and development of topical medicines for wound healing. *Expert Opinion on Drug Discovery*, *14*(5), 485–497. https://doi.org/10.1080/17460441.2019.1588879

Opneja, A., Kapoor, S., & Stavrou, E. X. (2019). Contribution of platelets, the coagulation and fibrinolytic systems to cutaneous wound healing. *Thrombosis Research*, *179*, 56–63. https://doi.org/10.1016/j.thromres.2019.05.001

Rafail, S., Kourtzelis, I., Foukas, P. G., Markiewski, M. M., DeAngelis, R. A., Guariento, M., Ricklin, D., Grice, E. A., & Lambris, J. D. (2015). Complement deficiency promotes cutaneous wound healing in mice. *Journal of Immunology (Baltimore, Md.: 1950)*, *194*(3), 1285–1291. https://doi.org/10.4049/jimmunol.1402354

Renné, T., Schmaier, A. H., Nickel, K. F., Blombäck, M., & Maas, C. (2012). In vivo roles of factor XII. *Blood*, *120*(22), 4296–4303. https://doi.org/10.1182/blood-2012-07-292094

Schmidtchen, A., Frick, I.-M., Andersson, E., Tapper, H., & Björck, L. (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37: Bacterial proteinases

degrade and inactivate LL-37. *Molecular Microbiology*, *46*(1), 157–168. https://doi.org/10.1046/j.1365-2958.2002.03146.x

Siana, J. E., Rex, S., & Gottrup, F. (1989). The effect of cigarette smoking on wound healing. *Scandinavian Journal of Plastic and Reconstructive Surgery*, *23*(3), 207–209. https://doi.org/10.3109/02844318909075119

Sinno, H., & Prakash, S. (2013). Complements and the wound healing cascade: An updated review. *Plastic Surgery International*, *2013*, 146764. https://doi.org/10.1155/2013/146764

Strömstedt, A. A., Pasupuleti, M., Schmidtchen, A., & Malmsten, M. (2009). Evaluation of Strategies for Improving Proteolytic Resistance of Antimicrobial Peptides by Using Variants of EFK17, an Internal Segment of LL-37. *Antimicrobial Agents and Chemotherapy*, *53*(2), 593–602. https://doi.org/10.1128/AAC.00477-08

Suzana Yusup & Nor Adilla Rashidi. (2022). *Value-chain of biofuels: Fundamentals, technology, and standardization*. https://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=2929470

Svensson, F. G., Manivel, V. A., Seisenbaeva, G. A., Kessler, V. G., Nilsson, B., Ekdahl, K. N., & Fromell, K. (2021). Hemocompatibility of Nanotitania-Nanocellulose Hybrid Materials. *Nanomaterials*, *11*(5), 1100. https://doi.org/10.3390/nano11051100

Tanaka, K., Davie, E. W., & Ikeda, Y. (Eds.). (2008). *Recent advances in thrombosis and hemostasis 2008*. Springer.

Thomas, B., Raj, M. C., B, A. K., H, R. M., Joy, J., Moores, A., Drisko, G. L., & Sanchez, C. (2018). Nanocellulose, a Versatile Green Platform: From Biosources to Materials and Their Applications. *Chemical Reviews*, *118*(24), 11575–11625. https://doi.org/10.1021/acs.chemrev.7b00627

Trache, D., Tarchoun, A. F., Derradji, M., Hamidon, T. S., Masruchin, N., Brosse, N., & Hussin, M. H. (2020). Nanocellulose: From Fundamentals to Advanced Applications. *Frontiers in Chemistry*, *8*, 392. https://doi.org/10.3389/fchem.2020.00392

Tummala, G. K., Rojas, R., & Mihranyan, A. (2016). Poly(vinyl alcohol) Hydrogels Reinforced with Nanocellulose for Ophthalmic Applications: General Characteristics and Optical Properties. *The Journal of Physical Chemistry B*, *120*(51), 13094–13101. https://doi.org/10.1021/acs.jpcb.6b10650

Vileikyte, L. (2007). Stress and wound healing. *Clinics in Dermatology*, *25*(1), 49–55. https://doi.org/10.1016/j.clindermatol.2006.09.005

Wimley, W. C. (2010). Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model. *ACS Chemical Biology*, *5*(10), 905–917. https://doi.org/10.1021/cb1001558

Xhindoli, D., Pacor, S., Benincasa, M., Scocchi, M., Gennaro, R., & Tossi, A. (2016). The human cathelicidin LL-37—A pore-forming antibacterial peptide and host-cell modulator. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1858*(3), 546–566. https://doi.org/10.1016/j.bbamem.2015.11.003

Yang, B., Good, D., Mosaiab, T., Liu, W., Ni, G., Kaur, J., Liu, X., Jessop, C., Yang, L., Fadhil, R., Yi, Z., & Wei, M. Q. (2020). Significance of LL-37 on Immunomodulation and Disease Outcome. *BioMed Research International*, *2020*, 8349712. https://doi.org/10.1155/2020/8349712

Yang, K., DeAngelis, R. A., Reed, J. E., Ricklin, D., & Lambris, J. D. (2013). Complement in action: An analysis of patent trends from 1976 through 2011. *Advances in Experimental Medicine and Biology*, *735*, 301–313.

Yousef, H., Alhajj, M., & Sharma, S. (2022). Anatomy, Skin (Integument), Epidermis. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK470464/