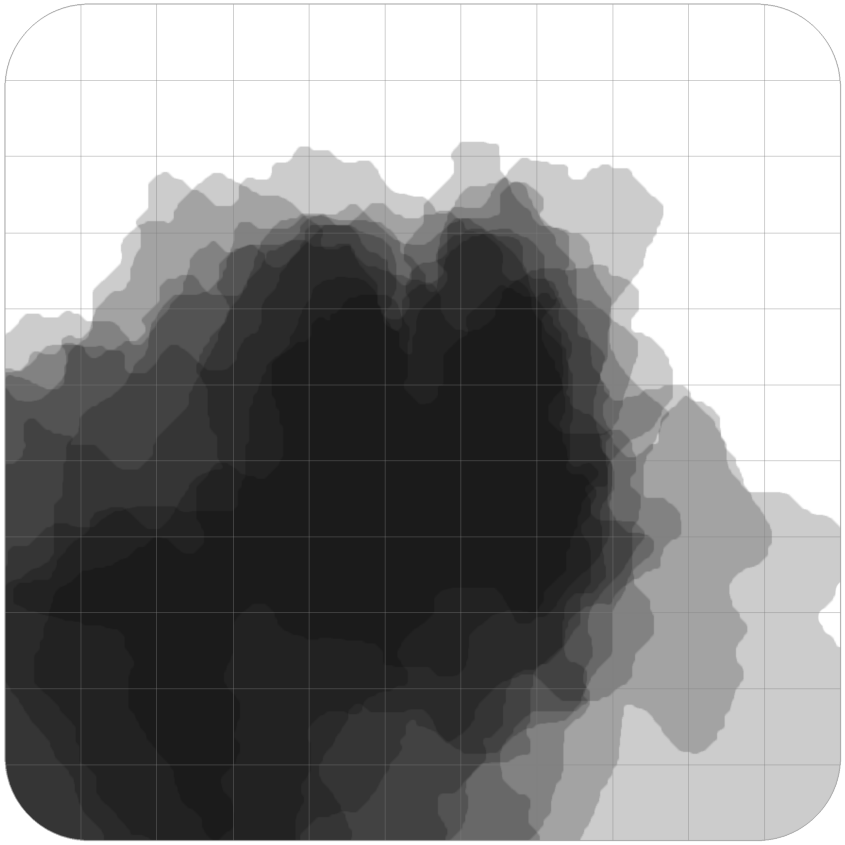

Development of Simulated Wound Fluids

Investigating mass transport and aggregation of proteins in a wound exudate model

Master's thesis in Biotechnology

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Master's thesis 2025

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Cover: Spreading patterns of a serum-containing wound fluid model in a wound dressing.

Abstract

This Master's thesis, carried out in collaboration with Mölnlycke Health Care, investigated the aspect of protein aggregation in simulated wound fluids (SWFs) in comparison to a serum-containing solution (SCS). The primary focus was on a fluid called SWF A, developed by Mölnlycke Health Care as a potential standard test fluid for evaluating wound care products. SWF A is intended to mimic biological wound exudate in terms of wetting, spreading, and drying behavior within a wound dressing, which involves a wide range of physico-chemical properties.

SWF A has demonstrated strong similarities to SCS 1, a fluid known to closely mimic biological exudate, in various tests, especially those where a product is exposed to excessive amounts of fluid. However, in the more complex and clinically relevant method FLUHTE, where the spreading of a fluid within a wound dressing is assessed, SWF A has been observed to differ from SCS 1 under certain conditions, especially in more advanced multilayer foam dressings. To better understand these differences, the aggregation of BSA (Bovine Serum Albumin) in SWF and the diverse mixture of serum proteins in SCS was assessed, primarily using DLS (Dynamic Light Scattering).

The results demonstrated that wound fluids represent a complex system for protein aggregation, where proteins should be regarded as active particles interacting in various ways with their environment. Three main conclusions could be drawn:
(i) Protein aggregation was found to significantly influence the spreading patterns of fluids within a wound dressing. A high aggregation propensity correlated with increased clogging of the dressing and fluid being forced upward within the product, whereas low aggregation propensity resulted in fluid spreading in the direction of gravitational force. (ii) The ionic composition of a fluid had a major impact on protein aggregation, with the formation of insoluble salts likely serving as nucleation points. (iii) Electrolyte composition is not the only factor in serum that affects protein aggregation, as the protein aggregation in SCS 1, despite having the same ionic environment as SWF, still occurred differently. Although no conclusive solution for regulating protein aggregation within SWF was reached, the combined results from this study will hopefully provide a solid foundation for further research within the area of protein aggregation in simulated wound fluids.

Keywords: Wound fluid, exudate, protein aggregation, bovine serum albumin, BSA, serum proteins, wound care products, peptone water, aggregates, mass transport, wound dressings, spreading pattern, serum-containing solution, SCS, DLS, precipitation

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Ida Nilsson

Gothenburg, May 2025

List of Acronyms

Below is the list of acronyms that have been used throughout the project, listed in alphabetical order:

AG	Alkyl Glucoside
BSA	Bovine Serum Albumin
CMC	Critical Micelle Concentration
DLS	Dynamic Light Scattering
ECM	Extracellular Matrix
FHC	Fluid Handling Capacity
FLUHTE	Fluid Handling Test Equipment
HSA	Human Serum Albumin
PDT	Pendant Drop Tensiometry
ROS	Reactive Oxygen Species
SCS	Serum Containing Solution
SWF	Simulated Wound Fluid

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1
Introduction

This project is a collaboration between Chalmers University of Technology and Mölnlycke Health Care. It is also a continuation of a previous master's thesis project, presented in the report *Optimizing the composition of simulated wound fluid to mimic the physico-chemical properties of chronic wound exudate* by Ida Johansson, 2023 [4].

1.1 Background

Chronic wounds are a common condition, affecting roughly 1% of the population in developed countries [5, 6]. Among them are many patients with diabetes suffering from so-called diabetic ulcers, which are a type of chronic wound caused by prolonged high blood sugar levels [7]. The care and management of chronic wounds account for approximately 3-6% of healthcare expenses [8].

Chronic, or non-healing, wounds are wounds that have stagnated in the inflammatory phase of the healing process, leading to slow or no regeneration or healing. The timeframe for what is considered a chronic wound varies depending on the clinical perspective, but typically it is considered chronic if it has not healed after four weeks under normal conditions [8].

For a wound to heal properly, the wound exudate must be managed correctly [8]. The wound should not become too wet and soggy, as this can promote bacterial growth and lead to infection. It can also result in fluid leakage from the product, causing discomfort and inconvenience for the patient. However, the wound should not dry out either, as wounds heal best in a moist environment [2]. Thus, wound care products such as dressings and bandages need to have good exudate management. This is even more important for chronic wounds, as they typically excrete a larger amount of exudate, which also contains more inflammatory components that irritate the wound and counteract healing [2].

When developing wound care products, one needs to make sure that they have adequate exudate absorption, spreading, and evaporation of the fluid. Many companies currently test products using pure saline solution or different kinds of electrolyte solutions [9]. One such fluid frequently used is the European Standard Test solution A, that contains electrolytes in specified amounts (142 mmol NaCl and 2,5 mmol $CaCl_2$) to mimic the ionic concentration of human serum (blood plasma) and wound

exudate [3].
However, real wound fluids differ significantly in composition. Biological wound fluids are fluids leaked from blood capillaries and contain in addition to water and salts also inflammatory components, white blood cells, growth factors, other proteins, nutrients and waste products [8]. All these components interact with each other and with the surrounding environment and can also aggregate. Therefore, test methods using salt solutions lead to inadequate test results and a limited understanding of the products actual performance in wound care.

1.2 Previous research at Mölnlycke Health Care

At Mölnlycke Health Care, fluids like Solution A have, for certain methods, not been considered sufficiently similar to wound exudate. Therefore, for some applications, a serum-containing solution (SCS 1) is used instead, which includes biological horse serum. However, using serum-based solutions presents several challenges, such as potential batch to batch variations and limited knowledge about their exact composition related to the use of animal-derived components.

Mölnlycke Health Care has therefore worked on improving its testing methods by further developing Solution A into a more representative wound exudate fluid. This work resulted in the development of a new simulated wound fluid, SWF A, which was described in a 2024 publication under the title *The importance of the simulated wound fluid composition and properties in the determination of the fluid handling performance of wound dressings* [10]. Their new formulation contained an improved electrolyte composition and included the protein albumin, specifically bovine serum albumin (BSA), at a concentration equivalent to the average total protein level found in serum [10]. SWF A demonstrated significantly improved properties and closely mimicked SCS 1 in tests assessing fluid handling capacity. However, when tested using the FLUHTE method, a technique simulating an exuding leg ulcer, where the spreading of the wound fluid is observed, SWF A performed differently compared to biological wound fluid.

Therefore, a previous masters thesis project was conducted in 2023 by Ida Johansson, with the title *Optimizing the composition of simulated wound fluid to mimic the physico-chemical properties of chronic wound exudate.* This project investigated the physico-chemical properties of the solution, with a primary focus on surface tension, aiming to modify the surface chemistry for improved performance regarding spreading pattern [4]. The project resulted in a new solution containing the same electrolytes as SWF A, with a modified amount of BSA and the further addition of the surfactant alkyl glucoside. This fluid was named SWF B. The fluid performed well under initially used conditions, closely mimicking the spreading patterns of the reference. However, when changes of external conditions like excretion rate, running time and temperature later were made, the spreading patterns differed significantly.

More research is needed on wound exudate and the interactions between its components in order to develop a more robust solution that more accurately mimics natural wound fluid across a broader range of conditions. Many aspects remain unexplored, particularly considering that the inclusion of BSA, a protein, introduces greater complexity. Due to their large size and three-dimensional structure, proteins can interact with their surroundings in numerous ways. Some protein-related aspects that remain unexplored include how BSA might aggregate as the fluid dries, and how it may adhere to the surfaces or pores of a wound dressing.

1.3 Project description

With background in previous research performed at Mölnlycke Health Care, this project was developed to further investigate the area of simulated wound fluids. This section defines the scope of the project, outlines its boundaries, and presents the research objectives.

1.3.1 Purpose and Aims

This project aims to develop a simulated wound fluid, that more accurately mimics the biological characteristics of chronic wound exudate than currently available alternatives. The focus lies specifically on the spreading behavior of the fluid within a wound dressing, with protein aggregation studied as a potential contributing factor. Through this, the research seeks to improve the understanding of how specific behaviors can be replicated in a laboratory context.

A better simulation of wound exudate for product testing would support the development of smarter and more effective wound care products with improved exudate management. This, in turn, could reduce patient discomfort and suffering, as better fluid control may lead to fewer complications and faster healing. More efficient wound dressings would also lower the need for medical interventions and enable more accurate wear-time recommendations, ultimately reducing both waste and healthcare costs. Thus, a more realistic test fluid would contribute to more reliable product performance and better outcomes for both patients and care providers.

Specific aims

To achieve the goal of the project, the following specific aims were established:

1. Evaluate protein aggregation as a factor to consider when formulating synthetic wound fluids for testing of wound dressings.
2. Assess protein aggregation in the already existing synthetic wound fluids in comparison with the reference.
3. Identify and assess relevant wound fluid component candidates for better mimicking the protein aggregation and achieving the behavior of real wound fluid, using additives such as surfactants, proteins, electrolytes, and/or polymers.

1.3.2 Delimitations

To carry out this research, certain limitations and boundaries were defined to clarify the scope of the study and outline what was not included.

1. **Researched aspect:**
   There are many aspects to consider when studying the behavior of wound fluids. This project focuses solely on the aspect of protein aggregation, as it was deemed relevant and feasible within the given time frame. Other potentially relevant aspects are considered outside the scope of this project.

2. **Scope of Wound Fluids:**
   There are significant differences in wound fluid composition between chronic (non-healing) and acute (healing) wounds. This study will focus exclusively on simulating chronic wound fluids, as they represent a larger global health concern. Additionally, only superficial and not internal wounds will be considered.

3. **Reference Solution:**
   The reference solution used in the laboratory research was a serum containing solution (SCS 1) with horse-derived serum in concentrations representative of human wound exudate. This solution has been shown to produce results similar to real wound exudate in terms of spreading patterns in wound dressings and will therefore be considered a good enough reference. No human samples or actual wound exudate was used due to ethical concerns, potential health and safety risks and limited availability.

4. **Environmental Conditions:**
   Analytical conditions such as temperature, pressure, excretion rate, and running time were chosen to reflect values that wound fluid in a dressing would typically be exposed to (e.g., from room temperature to body temperature, and durations of a few days). Given the differences between a synthetic solution with only a few components and real serum, fully replicating its behaviour under all external conditions was not feasible. Therefore, the experimental conditions were limited to selected values considered relevant for the intended application and research context.

5. **Wound Dressing Selection:**
   Spreading pattern tests, using the FLUHTE method, was performed using only one type of wound dressing. The selected product was a commonly used, non-specialized multilayered foam dressing. The specific product was used since the study will focus on parameters relevant to most foam wound dressings. Other properties, such as interactions with specialized coatings or exposure to certain therapies was not considered in this research.

6. **Commercial Availability:**
   Only commercially available additives were considered, as this simplifies long term supply for future testing.

2
Theory

In this chapter, the theoretical background necessary for clarifying the content of the report is presented. The theory covers relevant knowledge within the areas of wounds and wound exudate, the wound dressing development process, and key aspects of wound exudate interaction with dressings with a particular focus on protein aggregation.

2.1 Healing of chronic wounds

There are many causes of wounds, and thereby there is also a wide variety of wound types. In general the healing process of a wound is divided into four phases, hemostasis, inflammation, proliferation and remodeling [8, 11].

1. The first phase, **hemostasis**, is the acute response to injury, during which bleeding occurs [11]. In this phase blood vessels contract to reduce blood loss and clotting factors initiate coagulation. In this process platelets and fibrinogen bind together in the formation of a thrombus, commonly known as a blood clot.

2. The following **inflammatory** phase is when the wound site usually exhibits characteristics like redness, swelling, heat, and pain [11]. In this phase phagocytes (certain types of white blood cells), such as neutrophils and macrophages, are recruited to the wound site to release antimicrobial substances, reactive oxygen species (ROS) and proteases (enzymes) that kills and degrades the pathogens [12]. The cells also engulf and digest the pathogens and dead material from the wound site. The release of certain signaling substances during this phase is often called the signaling cascade, since the inflammation induces more and more inflammatory functions. Eventually, the cascade is however down-regulated due to anti-inflammatory signaling substances that are also part of the inflammatory response.

3. In the third phase, the **proliferation** phase, new tissue is generated [11]. The wound regains blood supply through the formation of new blood vessels (angiogenesis) and new extracellular matrix (ECM) is produced. The wound site contracts, and new epithelial cells are formed over the wound, restoring the skin barrier. This process is primarily initiated by environmental factors such as pH, as well as signaling substances secreted by macrophages.

4. Lastly, the **remodeling** phase is characterized by scar tissue formation [11]. The newly produced ECM components are degraded and reorganized by matrix metalloproteinases (MMPs), and together with the new cells, they are gradually structured into mature tissue.

A chronic wound is a wound stuck in the inflammatory phase of healing. In a normally healing wound, the inflammatory phase typically lasts for up to a week and within two to four weeks the wound is often completely healed [8]. However for a chronic (non-healing) wound, the inflammatory phase persists for much longer, with disruptions in the normal healing process that leads to a wound environment that continually triggers more inflammation, creating a cycle of non-healing. Such a wound can take months to years to heal, and during that time it requires careful, continuous care in order to eventually heal.

The abnormal disruption in the healing and the prolonged inflammatory phase leads to some significant differences in the chemical wound environment in comparison to a healing wound. The chemical environment in a chronic wound is characterized by elevated levels of pro-inflammatory cytokines, free radicals and ROS, as well as certain inflammatory enzymes, all released by inflammatory cells [8]. Chronic wounds also show reduced levels of albumin, the most abundant serum protein, along with an overall lower total serum protein content compared to acute wounds. This is believed to be due to the prolonged inflammatory phase causing reduced availability of proteins as well as nutrients in the wound site [8]. The chemical changes also result in an increase in pH at the chronic wound site, rising from the normal value of 4–6 to a significantly more alkaline level of approximately 7.15–8.9 [13]. The altered wound environment leads to increased production of wound exudate [8]. The significantly elevated pH, along with the increased amount of wound fluid in chronic wounds, actually creates conditions more favorable for bacterial growth, which contradicts the protective function of the inflammatory phase. To reduce the risk of infection, and enable the transition to eventual healing, effective exudate management becomes even more crucial in chronic wound care.

2.2 Wound exudate

Wound exudate is the clear to yellowish fluid released from a wound, primarily during the inflammatory phase. It is derived from blood plasma that leaks out of capillaries into the surrounding tissue in response to injury [14]. Although it originates from blood, it is not blood and generally lacks the components responsible for coagulation, such as platelets, and it contains significantly lower levels of the coagulation protein fibrinogen. Instead, its composition is similar to that of serum (blood plasma without coagulation components), containing water, electrolytes, proteins, and inflammatory mediators.

The primary functions of wound exudate are to maintain a moist environment and to serve as a transport medium for nutrients and waste [15,16]. Moisture is essential

for cellular function and tissue regeneration, and without it, cells in the wound site risk drying out. In general, moist wounds heal faster than dry wounds. Exudate also facilitates the delivery of oxygen and nutrients to the wound site. These substances are originally transported by blood plasma, which, upon leaking through capillaries into the wound bed, becomes exudate [15]. As a result, the exudate acts as a medium for nutrient delivery. Additionally, it helps remove metabolic waste products and cellular debris as it drains from the wound.

Wound exudate is often considered a healing agent. However, this is more accurate for healing than chronic wounds, since the prolonged inflammatory phase in a chronic wound creates a biochemical environment that can sustain inflammation rather than promote healing [15]. Furthermore, some studies suggest that instead of providing antimicrobial effects as exudate in healing wounds does, chronic wound exudate may actually promote bacterial growth in certain species, partly due to its elevated pH [15].

Exudate can be used in wound diagnostics, since the wound fluid represents the ECM of the wound site and thus, parameters such as volume, consistency, odor, and color can provide valuable information about the wounds condition [2, 15]. For example, a wound in the inflammatory phase, like a chronic wound, produces a larger volume of exudate than a wound progressing into healing. Also, exudate with distinct color or odor may indicate bacterial infection [16].

2.2.1 Electrolytes

The base of wound exudate is a salt solution composed of the specific selection of ions naturally present in the blood. These electrolytes are shown in table 2.1, which compares their concentrations in serum and wound exudate, based on data from Trengove *et al.* [2].

Electrolytes	**Serum (mM)**	**Exudate (mM)**
Sodium Na^+	142	141
Potassium K^+	4.9	4.4
Chloride Cl^-	101	104
Calcium Ca^{2+}	2.29	2.23
Magnesium Mg^{2+}	0.89	0.94
Phosphate PO_4^{3-}	1.16	1.27
Bicarbonate HCO_3^-	26	19

Table 2.1: Electrolyte levels (median value) in wound fluid compared to serum, from a study by Trengove *et al.* [2]. The highlighted rows are the ones considered statistically significantly different.

The most prevalent ions in exudate are Na^+ and Cl^-. However, there is also the bicarbonate ion HCO_3^-, and Potassium K^+, Calcium Ca^{2+}, Magnesium Mg^{2+} and PO_4^{3-} in considerably lower concentrations.

The bicarbonate ion HCO_3^- functions as a pH buffer in both blood and wound exudate. In blood, this mechanism is referred to as the bicarbonate buffer system [17]. This regulation happens through the reaction in equation 2.1, with a pKa of 6.1. The phosphate ion PO_4^{3-} can also function buffering.

$$HCO_3 + H^+ \rightleftharpoons H_2CO_3 \tag{2.1}$$

The other electrolytes have various functions in the body as well as in the site of a wound, acting as signaling substances in various systems, regulating the water balance in cells (eg. Na^+) and participating in metabolism (eg. Mg^{2+}) [18]. The calcium ion Ca^{2+} functions as a signaling substance in nerve transmission and various intercellular processes.

In the study by Trengove, the electrolytes highlighted in yellow were identified as having statistically significant differences between serum and wound exudate [2]. However, as also noted in the report, the differences are minor, and the electrolyte levels in serum and wound fluid can be considered similar, with the electroneutrality maintained.

2.2.2 Serum proteins

Serum proteins are part of the serum derivative of blood, excluding red blood cells and coagulation components such as platelets. They are broadly categorized into two groups: albumin and globulins, where albumin is the most abundant protein in the blood, and globulins is a collective term for various proteins, including those involved in the immune system.

In the study by Trengove *et al.*, the content of certain plasma proteins in serum and wound exudate was examined [2]. They are shown in table 2.2, and since all proteins were showing a statistically significant difference in concentration between serum and wound fluid, they are all marked yellow.

Plasma proteins	**Serum (g/L)**	**Exudate (g/L)**
Total protein	73	39
Albumin	40	22
α_1-Globulin	3.0	1.4
α_2-Globulin	9.2	4.4
β-Globulin	11	5.8
γ-Globulin	9.8	5.7

Table 2.2: Levels of major plasma proteins in serum and wound fluid, based on data from Trengove *et al.* [2]. All proteins are highlighted since they all are considered significantly different with $p < 0.001$.

In general, as also concluded in the study by Trengove *et al.*, the amount of protein in wound exudate is approximately half of the content in serum [2]. Trengove *et*

al. also compared the concentrations for some of the proteins between healing and chronic wounds. The total protein content differed from 41 g/L in healing wounds to 34 g/L in chronic wounds. The same trend has been seen for albumin and γ-globulin as well, indicating that exudate in chronic wounds have a slightly lower protein content than that of healing wounds. Again, this aligns with the idea that a chronic wound site in inflammation has a less sufficient supply of nutrients, which negatively impacts the protein synthesis for many proteins [2]. Another study by James *et al.* suggests that the total protein content in chronic wounds is even lower, at approximately 30 g/L [19]. Both studies support the statement that protein levels are lower in chronic wounds compared to healing wounds.

Albumin

Albumin, called Human Serum Albumin (HSA) to distinguish it from the albumin of other species, is the most abundant serum protein [20]. In blood it is present at levels of 35-50 g/L, which is equivalent to about half the total protein content. For wound exudate, as earlier described, the level is instead around 20 g/L [2, 19]. It is naturally synthesized in the liver at a rate of about 10–15 g per day, but is not stored there, as it is generally released directly into the bloodstream [20].

HSA is a relatively small serum protein consisting of 585 amino acids arranged in a helical structure, with a weight of 66.5 kDa [20, 21]. It is a globular protein, resembling the shape of a heart, with dimensions of 80 x 80 x 30 Å [21]. It has three main domains, each consisting of two subdomains. The domains are all similar in shape, but are not identical and in each of these domains the protein has sites where it can bind ligands.

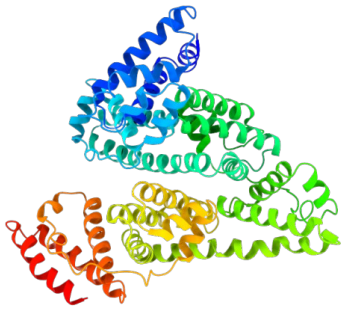


Figure 2.1: Structure of human serum albumin (HSA) [1].

In the body, HSA has two main functions, transport ligands and moderate the oncotic pressure, which is the colloidal osmotic pressure in the vascular system regulated by proteins [20]. Albumin regulates the pressure due to its negative charge (at normal blood pH), enabling it to attract water molecules and hence regulate the pressure. This negative charge, along with the binding sites also makes albumin able to transport both endogenous ligands, like ions or fatty acids, as well as exogenous ligands (drugs) through the blood stream. In the clinical use albumin is also a marker of both nutritional status as well as liver function, since malnutrition leads to disruption in the synthesis of albumin [20].

Globulins

The other fraction of serum proteins, besides albumin, is globulins, which make up about 35–38% of serum proteins. Globulins are a diverse group of proteins, with hundreds of different types including carrier proteins, complement components, immunoglobulins, and various enzymes [22]. They are generally divided into four categories based on their behavior in electrophoresis: α_1-, α_2-, β-, and γ-globulins. As globulins comprise over a hundred types, they vary widely in size. In general, α_1-globulins are the smallest, with a mean size of 93 kDa, while γ-globulins are considered the largest, with a size of approximately 150–160 kDa [23]. However, due to the wide range of proteins, sizes span from 44 to 2400 kDa (lipoproteins among the largest) across the different groups.

In the blood a normal level is 1-3 g/L of α_1-globulins, 6-10 g/L of α_2-globulins, 7-12 g/L of β-globulins and 7-16 g/L of γ-globulins [24]. Similar to albumin, a low level of globulins is seen as a marker for malnutrition, due to disruption of protein synthesis in general [22].

2.2.3 Other components

There are also several other components found in both serum and wound exudate, including metabolic waste products, enzymes and enzyme-related markers, lipids (such as signaling molecules and hormones), and inflammatory mediators. Table 2.3 presents components that, according to Trengove *et al.*, are present in concentrations greater than 1 mM.

Metabolic markers	Serum (mM)	Exudate (mM)
Glucose	6.4	1.8
Urea	8.7	8.9
Lactate	2.7	10.9
Cholesterol	4.4	1.7
Triglycerides	1.9	0.6

Table 2.3: Levels of metabolic markers of concentrations > 1mM in serum and wound exudate, based on data from Trengove *et al.* [2]. The highlighted rows are the ones considered statistically significantly different.

Glucose levels in wound exudate is significantly lower than in serum. This is presumably because the nutrient supply to a wound site is limited, since blood vessels are cut off and the glucose that is delivered to the site is used up before the exudate leaves the wound site [2,15]. In further experiments conducted by Trengove *et al.*, glucose levels in exudate from healing versus chronic wounds were compared. The median glucose concentration in exudate from healing wounds was found to be 2 mM, while exudate from chronic wounds had an even lower level of 1.2 mM (p=0.02). These findings indicate an even more nutrient-deprived environment in chronic wounds.
Lactate, on the other hand, is significantly elevated in wound exudate. As a marker of anaerobic metabolism, this elevation suggests that anaerobic conditions are more

prevalent in the wound environment compared to circulating blood [25]. This is consistent with the suggestion from Trengove and Cutting that nutrient supply to a wound site is challenged, since one could assume that also oxygen supply could be challenged [2, 15]. Other components are also present in wound exudate, like proteinases (protein degrading enzymes) and signaling substances associated with the inflammation and growth factors, however, in very low concentrations [26]. The wound exudate also carries waste products from the wound site, such as dead cells.

2.3 Testing of wound care products

Testing of wound care products is crucial in the development of dressings that provide optimal healing conditions. The wound should remain moist, while excess exudate must be absorbed and transported into the dressing to prevent skin damage and maintain a clean wound surface [16]. The dressing should also be breathable, allowing moisture vapor to escape, while still protecting the wound from bacteria and the external environment. To develop such products, materials must be evaluated and tested for various properties, including absorption capacity, fluid handling, and vapor transmission.

2.3.1 Standard test methods

The fluid handling performances of wound dressings can not be measured clinically since there is a vast variability among patients, wounds, method of practice and wound care protocols. This means that clinicians, product engineers, health care administrators, regulatory and reimbursement bodies critically depend on the outcomes from laboratory testing methods in their decision making processes [27].

The European standard EN 13726:2023, *Test methods for wound dressings – Aspects of absorption, moisture vapour transmission, waterproofness and extensibility*, defines a variety of test methods for wound dressings that are widely used by the industry and in the procurement processes of wound dressings, to evaluate the fluid handling performances in controlled setups and under pre-set test conditions [3]. Among the methods in the standard, fluid handling capacity will be described in greater detail below, as it is one of the more frequently used, in demonstration of the effectiveness of products [10].

Fluid Handling Capacity (FHC)

This method is a widely used test method for evaluating the full fluid absorption of waterproof wound dressings [3]. Fluid handling capacity refers to both the amount of fluid absorbed by the dressing and the moisture vapor lost through it, which corresponds to the total amount of fluid that a product can handle. In the test, a piece of the dressing is sealed over one end of a cylinder, with the absorbent side facing inward. A test solution is then poured into the cylinder, creating a column of liquid in contact with the dressing. Under controlled temperature and humidity

conditions, the dressing's FHC is assessed by measuring the amount of fluid retained and evaporated over time.

2.3.2 The FLUHTE method

The FLUHTE method, short for Fluid Handling Test Equipment, is a method developed by Mölnlycke Health Care, due to the lack of testing approaches that accurately mimic the clinical real-life use of wound dressings [28]. The equipment consists of a metal cylinder, representing a lower leg, with a simulated wound site to which a flow of test fluid can be connected. It assesses the total fluid management of a product, including absorption, retention and evaporation in a clinically relevant setup, and evaluates the vertical spreading pattern of exudate in a wound dressing, where gravitational forces influence fluid distribution [29]. Parameters such as temperature, flow rate, and compression bandaging can be adjusted to produce clinically relevant results under various conditions.

2.3.3 Wound fluids in *in vitro* testing

In the wound care industry, a variety of fluids are used to test products, and many companies have developed their own formulations. This has resulted in diverse testing practices. Some use only water or saline solutions, even though those lack key characteristics of real wound exudate, in terms of physicochemical properties such as pH, surface tension, viscosity and stickiness [30]. However, one test fluid that is widely accepted is the European standard Solution A, an electrolyte solution [3]. Other fluids used in many variations are serum-containing solutions (SCS), where biological serum derived from animal blood is used to mimic the protein content of wound exudate. There are also a limited number of other published wound fluid formulations, where a notable example is SWF A, developed by Mölnlycke Health Care [10].

Solution A

In the European standard EN 13726:2023 the test fluid Solution A is described. The content of the fluid is presented in table 2.4.

Component	**Concentration [mM]**
NaCl	142
$CaCl_2 \cdot 2H_2O$	2.5

Table 2.4: Concentrations of components in Solution A. [3].

According to the standard, these concentrations are considered comparable to the ionic strength of human serum and wound exudate.

SWF A

At Mölnlycke Health Care, Solution A was found to have several weaknesses as a test fluid. Through internal studies conducted by the company, it was shown that when compared to a serum-based solution closely mimicking exudate behavior, Solution A differed significantly in pH, contact angle and surface tension, conductivity, and FHC [10]. As a result, Mölnlycke Health Care developed an artificial wound fluid named SWF A, based on the study of wound exudate composition from Trengove *et al.* This fluid more accurately reflected both the electrolyte composition of wound exudate (including buffers) and its protein content (with BSA used as a representative of the total protein content), as shown in table 2.5, rather than including only a few electrolytes like Solution A [2, 10].

Component	**Concentration [mM]**
NaCl	110
$NaHCO_3$	20.0
KCl	2.67
KH_2PO_4	1.30
$MgCl_2 \cdot 6\ H_2O$	0.500
$CaCl_2 \cdot 2\ H_2O$	2.20
Bovine Serum Albumin	0.512

Table 2.5: Concentrations of components in SWF A.

SWF A was found to perform better than solution A in all the evaluated aspects, closely mimicking the reference solution [10]. It had a pH of 7.5, similar to SCS, compared to the pH of solution A at 5.6. It also demonstrated results equivalent to SCS in fluid handling capacity tests for wound dressings.

SWF B

Further studies showed, however, that the SWF A did not mimic wound exudate well in terms of spreading behavior in wound dressings using the FLUHTE method. In the study by Johansson, an improved fluid was developed and named SWF B [4].

Component	**Concentration [mM]**
NaCl	110
$NaHCO_3$	20.0
KCl	2.67
KH_2PO_4	1.30
$MgCl_2 \cdot 6\ H_2O$	0.500
$CaCl_2 \cdot 2\ H_2O$	2.20
Bovine Serum Albumin	0.301
Alkyl Glucoside	0.695

Table 2.6: Concentrations of components in SWF B.

It was found that the surface tension differed between the SWF A and the reference SCS, and that the higher surface tension in SWF A resulted in stronger capillary action, which to a greater extent counteracted gravitational forces for SWF A in a wound dressing compared to SCS [4]. This led to differences in spreading patterns between the fluids. The study also concluded the amount of BSA representing the total protein content was too high, since it caused severe protein aggregation that resulted in a solid barrier stopping the fluid from spreading downward in dressings. To improve the solution, the addition of the surfactant alkyl glucoside was added, and the protein content was altered. The composition of the resulting SWF B is presented in table 2.6. The resulting mimicked exudate better in spreading patterns under evaluated conditions.

2.4 Wound exudate in the wound care product

Wound dressings can be divided into two major groups; traditional dressings and modern dressings [31]. While the traditional dressings, like gauze and cotton, primarily act as a barrier between the wound and the environment for practical reasons while the wound heals, like trauma and dirt protection and keeping the exudate from staining clothes, modern dressings are meant to actually facilitate the healing of the wound. The proper management of exudate, as described in section 2.2, is an important part in facilitating healing of a wound, and in that includes not only the proper absorption of the exudate but also moisture retention and evaporation of the fluid from the dressing [31]. There are several different types of modern wound dressing materials available on the market, with different material properties tailored to support wound healing and manage exudate in specific ways.

2.4.1 The materials and chemistry of a wound care product

There are generally five types of modern wound dressing materials: films, hydrogels, alginates, hydrocolloids and foams [31,32]. These materials can be used individually, layered, or combined into integrated products depending on the intended application. There are also certain types of wound dressings with antimicrobial properties, using silver ions or iodine to produce a hostile environment for bacteria.

Films are thin, transparent, and flexible membranes that allow gas exchange while retaining moisture at the wound site [32]. **Hydrogels**, a broad class of water-absorbing polymers, typically made of hydrophilic starch polymers, have moderately absorbing and cooling effects. Alginates and hydrocolloids can be considered under-groups of hydrogels from a chemical standpoint, although they are often treated as separate categories in wound care. **Alginates**, derived from seaweed, are highly porous and form a gel upon contact with wound exudate through ion exchange. **Hydrocolloids**, like hydrogels, are composed of hydrophilic polymers, generally cellulose or gelatin, but are cross-linked. They are available in sheet, paste or powder form and progressively absorb fluid, as well as lower the pH on the wound surface, which inhibits bacterial growth.

Foams are the most absorbing material (together with hydrocolloids), and their thickness and elasticity also provide the wound with protection from external trauma [32]. Foams are in general one layer in multi-layered products, together with a contact layer (closest to the skin) and sometimes also additional outer layers. Such products are frequently used for chronic wounds, as those are known to excrete large volumes of exudate [32]. The following layers are frequently found in typical multilayered foam dressings [4]:

1. A silicon **wound contact layer** is often found closest to the skin and the wound site. This layer should be permeable and is often perforated. Due to its hydrophobic properties, silicone adheres to the skin without sticking to the wound itself.

2. The next layer is typically the absorbing **foam layer**. It has a porous structure and is made of a slightly hydrophilic material, designed to transport fluid directly through the layer without causing it to spread laterally. The highly porous structure and hydrophilic behavior give rise to capillary action, which draws the fluid into the dressing.

3. Next is a **spreading layer**, which is a nonwoven material that due to its structure helps the fluid spread evenly in the product. The spreading of the fluid is important, since the evaporation of the fluid is facilitated when spread over a larger area.

4. After the spreading layer comes a **retention layer**, often consisting of super-absorbent fibers, cellulose fibers, and binding polymers, which provides a high fluid retention capacity.

5. Last there is some kind of **backing film** that covers the backside of the dressing, and thereby is the barrier between the wound fluid and the outside environment. This is a thin semi-permeable membrane that allows evaporation of the fluid, while protecting the inside from bacteria and viruses from the outside. The evaporation of fluid from the dressing is of great importance, as greater fluid transport out of the product allows the dressing to be worn longer before becoming saturated and thus failing in fluid management.

2.4.2 Wetting of the wound dressing

When a droplet of wound exudate first reaches the wound dressing, it wets the wound contact layer. As it continues its journey into the wound product, each subsequent layer, with specific material properties influencing the interactions, becomes wetted in turn. The wetting is the ability of a liquid to interact with and form contact with a surface of a solid material [33]. The level of interaction, and thus the wettability, is a balance between the adhesive and cohesive intermolecular forces between the liquid and the solid.

The contact angle from a liquid droplet on the solid surface is a way of measuring the wetting at an interface. An angle of $< 90°$ represents high level of interaction

in the interface, and is considered wetting, while an angle of $> 90°$ is considered non-wetting [33]. Hydrophilicity is the solid materials property, as a result of water wetting the surface. If water wets the surface, the material is considered hydrophilic, while if non-wetting occurs it is hydrophobic [33]. A hydrophobic material often tends to prevent bacterial growth, since the bacteria have difficulty attaching to it, while hydrophilic materials in many applications are considered biocompatible [33]. In wound dressings both types of materials have their advantages and disadvantages. Hydrophobic materials, even though they protect against bacterial growth on the other hand have a poor liquid uptake, while hydrophilic materials instead often have a good liquid uptake, but can stick too much to the wound site and are more prone to bacterial growth [33].

Wound exudate consists primarily of water, but also contains other components such as ions and proteins. These additional constituents influence the interaction at the interface between the exudate and the wound dressing material, and thus the simplified classification of hydrophilicity does not fully capture the behavior. There are two main ways in which the composition affects wetting: through changes in surface tension and through protein adhesion.

Surface tension

One closely related aspect of wetting is the surface tension of the fluid. Surface tension is a property of liquids and describes the cohesive energy present between liquid molecules at the interface with air [34]. Young's equation 2.2 describes the relationship between interfacial free energies and the contact angle θ in the case of a liquid droplet on a solid surface [35]. The indices S (solid), V (vapor), and L (liquid) are used to specify the different interfaces.

$$\gamma_{SV} = \gamma_{SL} + \gamma_{LV} cos\theta \tag{2.2}$$

Under the assumption that the surrounding vapor is air, one can simplify the equation:

$$\gamma_{SL} = \gamma_{S} - \gamma_{L} cos\theta \tag{2.3}$$

As shown in equation 2.3, the interfacial free energy γ_{SL} between the solid material in a wound dressing and the fluid is negatively dependent on the surface tension of the liquid, γ_L. Thus, the higher the surface tension of the fluid, the lower the interaction at the interface. This is because the cohesive forces within the liquid are strong, making the liquid less likely to interact with the solid surface.

Water has a surface tension of 72 mN/m, a relatively high surface tension due to the strong hydrogen bonds between the water molecules [36]. Wound fluid, however, contains not only water but also various other dissolved components. These additional molecules affect the cohesive forces within the fluid and in most cases additives decrease the surface tension, since the inhomogeneous mixture of molecules will give rise to weaker cohesive sites at the surface.

Proteins tend to decrease the surface tension significantly, since proteins due to their hydrophobic sites tends to locate at surfaces and interfaces when in solution [37]. However, at already high protein concentrations an addition of proteins will not have such a pronounced effect, as the protein already occupies the surface area. A water solution containing albumin have a surface tension of about 52 mN/m (a little depending on concentration) [37]. However, electrolytes as an additive often increase the surface tension, due to the formation of ion-dipole bonds with the water molecules instead of ion-ion bonds [36]. Serum that has a quite complex mixture of proteins and electrolytes, has a surface tension of 56 mN /m.

Capillary action is caused by a mismatch between the free energy of a material surface in capillaries and the surface tension of a liquid [38]. If a liquid is wetting a material well, meaning that the material has a higher surface tension than that of the fluid, it will also be prone to have a large capillary action with capillaries of the same material. Thus, a material that is more hydrophilic, will likely do a better job of drawing the fluid into its capillaries.

Protein adsorption

Protein adsorption is an important mechanism with various functions within our body. For example, when we are injured, the blood that is normally only in contact with hydrophilic endothelial cells within the vascular system, is suddenly exposed to hydrophobic surfaces of the damaged tissue. In this situation the adsorption of certain serum proteins such as fibrinogen onto these hydrophobic surfaces of the wound site initiates coagulation and wound healing [39].

Protein adsorption is also a relevant aspect in many engineering application [40]. When designing biomaterials, such as implants that goes into the vascular system, the aim is often to prevent and decrease protein adsorption, since the initiation of coagulation within the circulating blood can cause severe problems [40]. As hydrophobic surfaces in general give rise to more protein aggregation, one therefore often use hydrophilic surface modifications to decrease the adsorption.

Due to their large size, almost all proteins have both hydrophilic and hydrophobic sites and regions [41]. Protein adsorption onto surfaces happens when these regions interact with a surface, making the protein adhere to the material. Due to the specific structures of the protein, as well as the material properties, the nature and strength of this adhesion vary [42].

A protein in aqueous solution, such as serum or exudate, is in its native structure directing its hydrophilic regions outwards. When exposed to a hydrophilic surface it will naturally interact with it, however often relatively weak, as the surround water molecules also exert hydrophilic forces on both the surface and protein [42]. However when exposed to a hydrophobic surface, such a protein can undergo interfacial relaxation, meaning it attaches to the surface using its previously hidden hydrophobic sites and, in the process, spreads out on the surface [43]. This is a form of denaturation of the protein, and the process takes time. After fully adsorbed to a hydrophobic

surface the protein fibrinogen has an increased footprint area (the surface area occupied by the protein) by 3 times in comparison with its native state [43]. Albumin (BSA), in comparison, spread by 5 times its native size.

In the presence of ions, the process of surface adsorption may be enhanced, due to dehydration of the proteins [42]. The ions may occupy more water molecules, removing them from binding to the protein or/and the surface (referred to as the salting-out effect in section 2.5), and instead drive the interaction between the surface and protein.

In a wound care product protein adsorption can be of importance in different ways. In a product used on fresh wounds, where bleeding still occurs, the adsorption of proteins such as fibrinogen onto hydrophobic materials in the dressing can, and probably will cause coagulation [39]. In wound dressing for chronic wounds, however, that is of minor relevance, since wound exudate typically does not contain coagulation components in any substantial amount. However there will be protein adhesion in the pores and capillaries of the dressing materials, and since electrolytes are present, perhaps there may be even more pronounced adhesion, which could be a possible starting point to buildup. One could also imagine that due to adhesion of proteins onto the hydrophobic materials of a dressing, the surfaces will slowly become hydrophilic as the proteins expose their hydrophilic regions out towards the liquid [43]. Thereby, the materials that were meant to exert hydrophobic forces on the water molecules in the fluid, now tend to lose that function.

2.4.3 Drying of the wound dressing

Along with the wetting of the wound care product by exudate from the wound, there is a simultaneous drying process. As the fluid is distributed and managed, water is allowed to evaporate and the vapor can pass through the semi-permeable outer membrane of the product.

Evaporation

The mass balance over the system of a wound dressing has three parts; the incoming wound fluid through absorption from the wound site, the outgoing vapor from simultaneous evaporation of water in the dressing, and the accumulated fluid within the dressing. This mass balance is shown in equation 2.4.

$$\dot{m}_{\text{in}} = \frac{dm}{dt} + \dot{m}_{\text{out}} \tag{2.4}$$

However, the material that leaves the product is only water, and not all of the components in wound exudate. The electrolytes and proteins remain in the wound dressing. Therefore, the mass balance could be further specified into equation 2.5 to 2.7:

$$\dot{m}_{\text{in}}(\text{H}_2\text{O}) = \frac{dm(\text{H}_2\text{O})}{dt} + \dot{m}_{\text{out}}(\text{H}_2\text{O}) \tag{2.5}$$

$$\dot{m}_{\text{in}}(\text{electrolytes}) = \frac{dm(\text{electrolytes})}{dt} \tag{2.6}$$

$$\dot{m}_{\text{in}}(\text{proteins}) = \frac{dm(\text{proteins})}{dt} \tag{2.7}$$

For components other than water, the incoming mass equals the accumulated mass, meaning there will be buildup within the dressing. However, regarding the higher the evaporation and thereby outflow from the product, the lower the accumulation. Lower accumulation means it takes longer time before the dressing becomes saturated (fails in fluid handling), allowing the product to be worn for a longer time before needing replacement. Instead, the product only needs to be changed when necessary due to buildup of solids or the risk of bacterial growth. This reduces the handling of products as well as the waste. Therefore one wants a dressing that promotes good, yet controlled, evaporation.

The evaporation of water, from liquid to gas, involves breaking intermolecular bonds within the water. In pure water these intermolecular forces are hydrogen bonds, and breaking one such bond to evaporate a single water molecule (at room temperature) requires an energy of about 10-12 k_BT (corresponding to 25-30 kJ/mol) [44]. In wound fluid, however, there are not only water molecules, but also other components that give rise to ionic bonds, which slightly alter the required energy.

The evaporation is driven by heat transfer, since without the addition of energy (in this case thermal energy) the intermolecular bonds will remain, as this is the most favorable (lowest energy) state for the molecules. Therefore the rate of evaporation is affected by the thermal conductivity for both the fluid and the wound dressing materials [45]. It is also dependent on the materials capacity to spread of the fluid to the outer layer of the product and over a large area, as this allows for more evaporation.

Protein aggregation

As more and more water evaporates in a wound dressing, the concentration of proteins as well as electrolytes increases, and thereby the likelihood of protein aggregation is increased [46]. Protein aggregation refers to the process in which proteins begin to clump together into larger particles, aggregates, eventually leading to phase separation and precipitation [47, 48]. In the wound dressing, formation of solid particles inside the pores and capillaries may lead to clogging of the material, which in turn can hinder effective distribution and management of wound exudate. Mechanisms of protein aggregation are described further in section 2.5.

2.5 Protein aggregation

In this section, the composition of proteins and the mechanisms and principles of protein aggregation are described. The section also covers strategies for stabilization of protein solutions to prevent aggregation, and highlights some inconsistencies and controversies in the field.

2.5.1 The principle of aggregation

Proteins are composed of one or more polymer chains of amino acids, also known as polypeptides. An amino acid consists of a central carbon atom (α carbon) bonded to a carboxyl group, an amino group, a hydrogen atom and a side group, often denoted R [41]. During formation of a polypeptide, the amino and carboxyl groups form peptide bonds, linking the amino acids together into a long chain. This chain of peptide bonds is referred to as the backbone of the protein

There are 20 different types of amino acids, each with a specific side group [41]. The majority of them have non-polar side groups, while some have polar properties and others are charged (ionic). The sequence of amino acids in the polypeptide is called the primary structure of a protein [41]. However, this sequence also lays the foundation for the secondary and tertiary (three-dimensional) structure of the protein. The different properties of the side groups enables interactions with other side groups as well as the surrounding environment. The non-polar chains will interact through hydrophobic forces, and the polar and charged ones will form hydrogen and ionic bonds, and these bonds will form twists and turns in the polypeptide and fold the protein into its three dimensional shape. Environmental conditions also affect the shape of the protein [41]. In aqueous solutions, such as serum, proteins tend to turn their hydrophilic regions outward, while hiding hydrophobic regions in the interior of the three-dimensional structure. The shape of the protein when found in its native environment, stabilized with numerous side group bonds, is called the native state of the protein [49].

A protein in synthetic solution is typically only marginally stable, as these solutions rarely mimic the complex chemical properties of the native biological environment. Consequently, surrounding molecules in the solution may interact with and destabilize the protein [50]. If the native state of a protein is altered, such as through unfolding or misfolding due to interactions with its environment, it is referred to as denaturation. Unfolded or denatured proteins tend to aggregate. The denaturation and aggregation lead to loss of protein function, and moreover, the process is difficult to reverse. Thus, protein aggregation poses a challenge for protein storage in research applications, and clinical use [50].

Proteins can undergo denaturation through various mechanisms. For example, elevated temperatures can induce denaturation, as the input of thermal energy can disrupt intramolecular bonds [50]. Surface adsorption and foaming can lead to denaturation as the interfaces with other materials provide a different chemical

environment. Shifts in pH can lead to acidification or alkalization of the side groups of the protein, which can alter its structure. High protein concentrations can also cause denaturation as the proteins are more prone to interact with each other instead of the environment.

It is important to distinguish between protein **aggregation** and **precipitation**, although these terms are often used interchangeably. By definition, protein aggregation refers to the formation of protein aggregates as a result of denaturation, while precipitation describes the phase separation of protein particles from a solution, while remaining in their native state [48]. However, the terminology is not always used consistently. Aggregation is sometimes used broadly to describe various forms of protein particle formation. Likewise, the term precipitation is occasionally used to describe the separation of already aggregated proteins from solution.

2.5.2 The role of electrolytes in protein aggregation

There are many ways to stabilize a protein-containing solution, using salts, amino acids and polyols for example [50]. Additives often used can be of three main types; structure stabilizers, denaturants or aggregation suppressors, shown in figure 2.2 [47]. Structure stabilizers, as the name suggests, stabilize and compact the native structure of the protein through various mechanisms. Denaturants, as the name tells, denature proteins at high concentrations, leading to aggregation. However, at lower concentrations, they can instead assist in the refolding of misfolded or partially folded (intermediate) proteins. There are also some additives that do not affect the structure of the proteins but instead disrupt protein–protein interactions, and thereby prevent the initiation of aggregation.

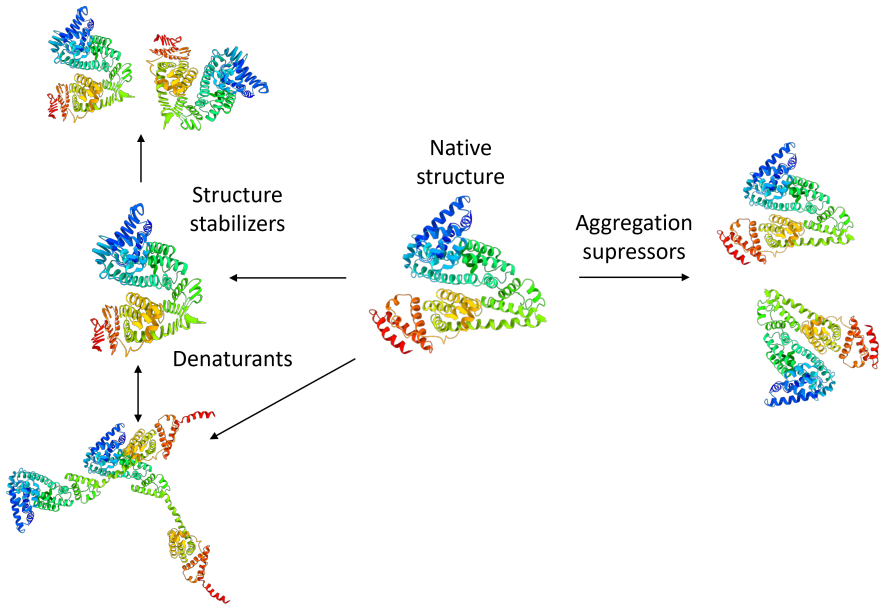


Figure 2.2: Chart of possible actions of additives on protein aggregation.

Electrolytes (salts) are at low concentrations generally stabilizing to protein solutions, due to electrostatic interactions, which mainly depends on the ionic strength of the solution [47]. However, at higher concentrations, specific ions begin to exert distinct effects on proteins, leading to either stabilization or denaturation. The effects of specific ions are described by the Hofmeister series, first introduced by Franz Hofmeister in 1888 [46, 47]. However, since then modifications of the series have been proposed and there are controversies to the mechanisms underlying certain effects [46, 47]. Recent studies suggest that other factors, such as type of protein and environmental factors also can play a role in the outcome [46].

The terms **salting in** and **salting out** regarding the effect of a salt on proteins, have a double meaning. As mentioned, all inorganic salts (generally) have stabilizing properties, meaning the salt increases the solubility of the protein at relatively low salt concentrations. This range of salt concentration is seen as stabilizing due to the neutralization of electrostatic energy between the proteins (that are generally negatively charged) in the solution. This is in some reports referred to as a salting-in effect [4,10,51]. At high salt concentrations the ions and the proteins instead become competitors for binding with water, which reduces the solubility of the proteins and is called salting out.

However, according to the principle of the Hofmeister series, specific ions have either a salting-in or salting-out effect on proteins [10, 46]. These specific interactions are possible due to the negative (anionic) charge of the protein backbone, which cations are prone to interact with [46]. The more **chaotropic** the ion, the stronger its interaction with the protein, potentially leading to the ion and following water molecules "salting in" into the protein and possibly denaturing it. On the other hand, strongly **kosmotropic** anions will instead compete with the anionic protein for water, pulling water molecules away from the protein and forcing it into a more compact native structure. This is called "salting out", and may lead to precipitation.

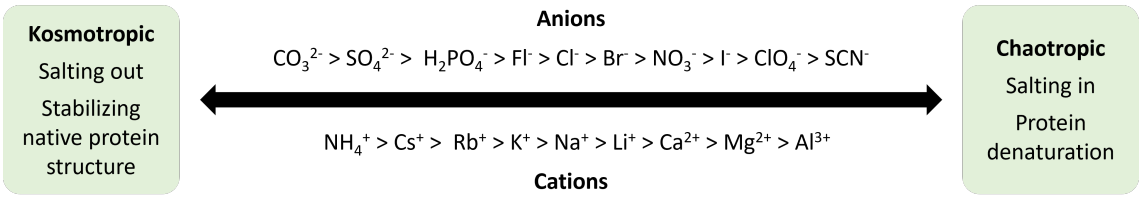


Figure 2.3: The Hofmeister series.

In a recent study by Madeira *et al.* (from 2022) the aggregation of BSA upon exposure to different ionic environments was investigated using dynamic light scattering (DLS) and high performance liquid chromatography (HPLC) [46]. The authors described that the effects of salts on proteins, apart from their kosmotropic/chaotropic behavior, also depend on environmental factors such as pH, as well as the ions tendency to interact with specific regions and functional groups in the protein, not just the backbone. Thus, the specific structure of the protein also influences the outcome.

They further referred to a previous study in which albumin was exposed to various electrolytes at pH 3. In such an acidic environment, cations have no precipitation effect and in that specific situation the anions followed a trend based on electronegativity, where divalent anions induced stronger precipitation than monovalent halogens. However, the same pattern was not found in an environment where the anions exerted an effect.

In their own experiments, Madeira *et al.* used NaCl, whose constituent ions are located near the middle of the Hofmeister series, implying a relatively neutral effect in terms of kosmotropicity or chaotropicity. They also examined the effect of other salts, in comparison to NaCl in relation to the concentration of BSA, observing the following:

- The higher the BSA concentration, the lower the NaCl concentration required to induce aggregation.
- The behavior of BSA when exposed to ions of higher valence was similar to that of NaCl. However, the salt concentration needed to trigger aggregation decreased proportionally to x, x^2, and x^3 for monovalent, divalent, and trivalent ions, respectively.

The effects of ions on proteins are of complex character, where research in the field sometimes contradicts. The Hofmeister series, along with the theory of concentration-dependent salting-in and salting-out effects, can serve as useful rules of thumb. However, when the electrolyte composition becomes more diverse, with multiple ions present simultaneously, and additional environmental and protein factors come into play, the outcomes are not always predictable.

3
Methodology

In this chapter the procedures of the conducted experiments as well as the principles behind the used methods are described. The experimental setups are also motivated and linked to their underlying hypotheses.

Some assumptions were made that apply to most of the experiments:

1. As the fluids used were aqueous solutions with densities close to that of water, it was assumed that volume and weight proportions were equivalent and interchangeable. Between mass measurements (scales) and volume measurements (pipettes, etc.), the most suitable and practical method for each specific application was used.

2. In many experiments substances were gradually added to a sample. A dilution of the total volume up to 5% due to additives was considered negligible.

3. In motivations and hypotheses, as well as in the interpretation of results, the word *aggregation* is used to describe the process of protein-containing particle formation. Depending on the definition of aggregation, as described in section 2.5.1, the term is sometimes defines as particle formation due to certain specific mechanisms. However, even though the exact mechanisms driving the particle formation discussed in this report are unknown, the term aggregation is still used.

3.1 Sample preparation

The samples examined in this project consisted of wound fluid or, in some cases, water with various additives. The wound fluids were in most cases made as stock solutions of 1L at a time, to reduce the risk of batch variations between experiments. In all stock solutions and prepared samples deionized water or mQ water was used. All salt additives were weighed using a scale with 0.0001 g precision, and all biological dry components (proteins and peptides) were weighed with a precision of 0.01 g.

All stock solutions containing biological material were sterile-filtered as the final step of preparation and subsequently handled under aseptic conditions. Surfaces were disinfected, and exposure to air and the external environment was minimized during use. The stock solutions were stored in the fridge to prevent growth, should they unexpectedly be contaminated. Prepared samples were kept for a maximum of

7 days, and were stored in the fridge if containing biological material. This helped prevent any possible growth and limited air exposure, as small sample volumes are more affected by exchange with air and can change in physical properties.

3.1.1 Preparation of wound fluids

Throughout the experiments, six types of wound fluids were used, either on their own as pure samples or as a base in combination with various additives. In this section the compositions and preparation of the fluids are presented.

SCS - Serum Containing Solutions

Two wound fluids used contained serum, blood plasma, from animal sources. They are referred to as SCS 1 and SCS 2, and their content is presented in table 3.1.

SCS 1		**SCS 2**	
Component	**Amount [g]**	**Component**	**Amount [g]**
Horse serum	500.0	Fetal calf serum	500.0
Solution A	500.0	Peptone water	500.0

Table 3.1: Formulation of SCS 1 and SCS 2 (per 1000g).

SCS 1 is a solution containing horse serum and solution A in a 1:1 ratio. The horse serum was bought sterile from Håtunalab (art. no 153), and stored in the fridge, while the solution A was premade at Mölnlycke Health Care according to the standard EN 13726:2023 [3].

When preparing SCS 1, the horse serum bottles were handled aseptic. The quantities of horse serum, and then solution A were weighed with a precision of two decimal places (0.01 grams). The SCS 1 solution was not a stock solution. Since it is composed of only two components, and the horse serum already were sterile, it was instead always prepared within hours before use to prevent any growth in the final solution.

The horse serum was diluted to half the concentration, as the concentration of serum proteins in wound exudate is approximately half the amount of the protein concentration in serum [2]. However the salt concentration should not be diluted, and therefore solution A was used as the dilution agent. The SCS 1 solution has in tests at Mölnlycke Health care shown great similarities with real wound exudate regarding the spreading patterns in wound care products and was therefore chosen as the positive control solution for this project.

The **SCS 2** solution has been used as a reference solution at another compartment at Mölnlycke Health care. Instead of horse serum it contains fetal calf serum, and instead of solution A it contains peptone water, which is a solution prepared at Sahlgrenska University Hospital containing 1g/L of Peptone Bacteriological Biolife and 8.5 g/L of NaCl within 1 liter of deionized water.

This solution was brought into the project because it was an available liquid at Mölnlycke Health Care. It was initially interesting to investigate since it could provide new relevant knowledge to their daily laboratory work, but also since it could perhaps offer valuable insights for the project when comparing it to SCS 1 and other liquids. The liquid was found to contain a very interesting solution, namely the peptone water, which was further explored in the study.

SWF - Simulated Wound Fluids

Three of the fluids used were variations of SWF A, developed by Mölnlycke Health Care. They consist mostly the same components and followed therefore similar preparations. The compositions of the fluids are presented in table 3.2

Component	**SWF A [g]**	**SWF 20 [g]**	**SWF B [g]**
NaCl	6.428	6.428	6.428
$NaHCO_3$	1.680	1.680	1.680
KCl	0.199	0.199	0.199
KH_2PO_4	0.177	0.177	0.177
$MgCl_2 \cdot 6\ H_2O$	0.102	0.102	0.102
$CaCl_2 \cdot 2\ H_2O$	0.323	0.323	0.323
Bovine Serum Albumin	34.00	20.00	20.00
Alkyl Glucoside 65%	–	–	0.40
Deionized water	1000	1000	1000

Table 3.2: Formulation of SWF A, SWF 20, and SWF B (per 1L).

SWF A is a solution containing salts and buffers with added albumin. All salts except $CaCl_2$, were dissolved in water under stirring. $CaCl_2$ was added later to avoid formation of insoluble $CaCO_3$ when reacting with $NaHCO_3$, as shown in equation 3.1 However when adding proteins before the addition of $CaCl_2$ the ions had already engaged with the proteins, which prevented precipitate formation.

$$CaCl_2 + 2NaHCO_3 \rightarrow CaCO_3 + 2NaCl + CO_2 + H_2O \tag{3.1}$$

The BSA was weighed separately and added to the flask using a funnel. The flask was then closed with a stopper and left for stirring until the protein was completely dissolved. Then the $CaCl_2$ was finally added and stirred until complete dissolution. The surface, gloves and other equipment were then sterilized using ethanol 70% before setting up the equipment for sterile filtration. The filter and flask were connected, and a trap to prevent any contamination were used between the filter and the tap creating the suction. After sterile filtration the bottle were stored in the fridge and handled aseptic.

Since SWF A is Mölnlycke Health Cares newly established standard for certain methods and is well known within the organization, it was also used as a reference.

Although it generally performs well and mimics SCS 1 in FLUHTE for some products and under some conditions, it has been found to differ significantly in certain cases, particularly with more advanced multilayer foam dressings such as the one used in this study. Therefore, it served as a negative control.

SWF 20 was prepared in the same way as SWF A, with the exception that SWF 20 contained 20 g/L of BSA instead of 34 g/L. The BSA amount in this solution was based on the albumin content of biological wound exudate neglecting the remaining proteins, rather than representing the total protein content. SWF 20 was used in multiple tests, primarily as a base solution to test additives with. By using SWF 20 instead of SWF A as a sample base less BSA was used, which was beneficial both economically and practically.

SWF B was also prepared in the same way as the other SWF solutions, with the addition of the surfactant last before the final sterile filtration. The fluid, developed by Johansson during the previous master thesis, served as a starting point of knowledge for this research. However, it was not of particular interest in the subsequent investigation of protein aggregation. As SWF B was only used in a few initial experiments, only one batch of it was prepared during the entire project.

Peptone 20

The last fluid used in the project was referred to as Peptone 20. This fluid did not exist prior to the project but was developed as a result of interesting findings early in the experimental process. It was thereafter frequently analyzed.

Component	**Amount [g]**
Bovine Serum Albumin	20.00
Peptone water	1000

Table 3.3: Formulation of Peptone 20 (per 1L).

Peptone 20, as shown in table 3.3, contained only two components, making the preparation simple. A flask was prepared with a magnetic stirring bar in the bottom and a small volume of the peptone water. The BSA was weighed in a beaker and then transferred into the flask using a funnel. The flask was gently swirled to wet the protein, and the remaining peptone water was used to rinse residual protein down the neck of the flask. The solution was stirred until dissolution, and sterile filtered.

The composition of Peptone 20 can be seen as a combination of SWF 20 and SCS 2. Its protein source is BSA, similar to the SWF solutions. However, instead of using the electrolyte base found in SWF A, peptone water was used, which has a different ionic composition and also contains peptides, that were meant to mimic small biomolecules found in the body.

3.1.2 Additives

The following additives, including surfactants, protein stabilizers and biomolecules, were used in one or several experiments throughout the project.

Alkyl glucoside

Alkyl glucoside (AG) is a low foaming, mild surfactant commonly used in rinsing fluid for washing machines. It is a non-ionic surfactant, with a molar mass of 373.94 g/mol [52]. The molecule consists of a quite small head group composed of a glucose molecule, and a branched fatty alcohol tail. The surfactant, called AG 6202 when purchased from Nouryon, was provided as a 65% stock solution in water [52].

Triton X-100

Triton X-100 is, like AG, a non-ionic surfactant. However, it is not as mild as AG in terms of surface tension reduction. It is widely used in biochemical applications to solubilize proteins and is considered both mild and non-denaturing, despite its stronger surface tension lowering effects compared to AG [53]. Structurally, Triton X-100 differs from AG because of its larger hydrophilic head group as well as a straight (non-branched) polyether tail, with the general formula t-Oct-C_6H_4-$(OCH_2CH_2)_xOH$, where $x \approx 9$–10 [53]. The surfactant is obtained from Sigma-Aldrich in high purity (nearly 100%). Its critical micelle concentration (CMC) is in the range of 0.2–0.8 mM at room temperature according to the supplier.

Peptone Bacteriological

Peptone Bacteriological, purchased from Biolife Italiana, is a powder derived from enzymatic digestion of bovine meat [54]. It is generally used in microbial culture media, as the digested polypeptides provide amino acids to the growing cells. In solution, it provides a neutral pH. This additive was used both in its pure state and in the use of peptone water purchased from Sahlgrenska University Hospital. The peptone water is a aqueous solution, containing 1 g/L of Peptone bacteriological, and 8,5 g/L of NaCl. The use of peptides as an additive was based on the idea that they would mimic the function of small biomolecules in wound fluid, such as biolipids, metabolites and actual amino acids. However, the additive have the downside of providing an excellent environment for bacterial growth if bacteria would be accidentally introduced to it.

Bovine Serum Albumin

BSA, albumin derived from bovine blood, was for this project purchased from Roche in the form of dry crystallized protein flakes, with a purity of $> 98.5\%$ [55]. BSA is a highly available protein, with strong similarities to HSA (human serum albumin), although protein structures differ slightly between species [56]. Therefore it is frequently used as a model protein in research, as well as a component in culture media. It has a neutral pH when in aqueous solution [55].

In the experiments within the project, BSA was not strictly used as an additive, as it was included as part of the different SWF and Peptone 20 fluids. However, its amount varied, either representing the total protein content of exudate or just the albumin fraction.

Bovine γ-globulin

Bovine γ-globulin, purchased from Sigma-Aldrish in a purity of >99%, had the form of dry crystallized flakes, similar to BSA [57]. Gamma globulins, the family of globulins with the overall largest size (150-160 kDa), mostly consist of immune proteins [23]. These globulins, derived from bovine blood, are therefore frequently used in immunology research [57]. However, in this project it was used as a model of larger proteins, in comparison to BSA, to evaluate effects of protein sizes in a fluid.

Urea

According to Jahan *et al.* urea is known to have a non-monotonic effect on proteins and aggregation behavior [58]. On one hand it is known to be a strong denaturant, as proteins unfold in urea, and since denaturated proteins are prone to aggregate, urea thus can promote aggregation. On the other hand, urea can act as an aggregation suppressor, as urea enables stronger hydrogen bonds to the protein than those of water molecule, and thereby they can outcompete protein-protein interactions (that can lead to aggregation) better than a pure aquatic environment. According to the study, the effects seems to be concentration dependent.

Glycerol

Glycerol is an frequently used polyol for protein refolding, as it stabilizes and compacts the native structure of proteins [50]. According to Vagenende *et al.* glycerol reorientates the protein back to its native state by directing hydrophilic regions outwards, as the glycerol bonds to them. Thereby it inhibits denaturation, and prevents protein aggregation. For specific protein complexes, however, it can have the effect of protein association (clustering of native state proteins), which can be a precursor to aggregation.

3.2 Analythical methods

The experiments conducted during the project involved three analythical methods; pendant drop tensiometry, to measure surface tension, DLS to asses protein aggregation and FLUHTE to analyze spreading patterns of fluids. The DLS method in particular, were used throughout multiple experiments. The methods and the principles of the instruments are further described below.

3.2.1 Pendant drop tensiometry - PDT

Pendant drop tensiometry is an optical method for assessment of surface tension of liquids [59, 60]. The components of the experimental setup, as shown in figure 3.1, consists of a light source, a camera and a syringe with the fluid placed in between [59].

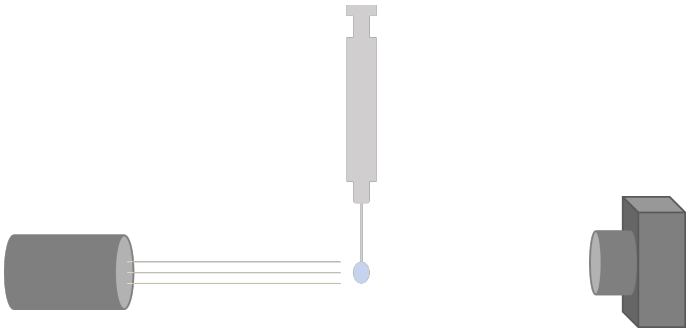


Figure 3.1: Experimental setup for pendant drop tensiometry.

The method is based on the evaluation of the deformation of a liquid droplet hanging from the tip of a syringe needle [60]. The forces acting on the hanging droplet are the gravitational force, pulling the droplet down, and the surface tension counteracting the gravitation [59]. Thus, if high surface tension, the droplet will have a close to spherical shape, while if lower the droplet will be more stretched out.

$$\gamma = \frac{\Delta\rho g R_0}{\beta} \tag{3.2}$$

Based on the shape, the surface tension of the liquid is given from the equation 3.2, where γ is the surface tension of the liquid, $\Delta\rho$ is the density difference between the liquid and the surrounding medium, g is the gravitational constant, R_0 is the radius of curvature of the droplet and β is the shape factor, also referrer to as the Bond number [59, 60]. The Bond number is derived from the Young-Laplace equation, handling the three dimensions of the shape. A $\beta = 0$ would indicate a completely spherical droplet (though physically impossible), and the higher the number, the more the droplet is stretched out [59].

Within this project an Optical Goniometer was used. The fluids were assumed to have negligible density difference from water, therefore $\Delta\rho$ was set to air–water. The syringe had a needle diameter of 0.718 mm, to which the program was calibrated.

All experiments were performed at room temperature, and no consideration was given to tempering the samples, as such a small sample volume as a drop quickly adjusts to room temperature. A few drops of the fluid were discarded before the measurement started. Thereafter, measurements was performed on 10 droplets of each fluid, and a mean value of the surface tension was calculated.

In surface tension curves, the critical micellar concentration (CMC) can be identified as the concentration at which the lowest surface tension is observed. The CMC represents the point at which surfactants no longer exist solely at fluid interfaces but begin to form micelles. This value was, when found, noted.

3.2.2 Dynamic Light Scattering - DLS

Dynamic Light Scattering is one of the most common techniques for measuring the size of nanometer-scale particles in colloidal liquids [61]. The instrument used in this project was a Litesizer 500. Its key components, shown in figure 3.2, include a laser, a cuvette chamber, and detectors at different angles. In the Litesizer 500, there are three detectors, at the angles 15°, 90° (side scattering) and 175° (back scattering).

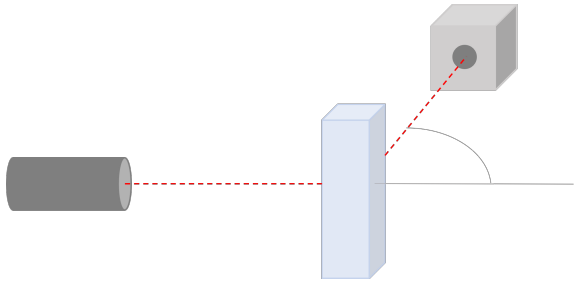


Figure 3.2: Experimental setup for DLS.

The technique is based on the measurement of the Brownian motion. In a liquid unaffected by external forces, particles move due to collisions with other particles in the liquid [61]. From those collisions small particles will gain more speed than large particles. By measuring the intensity of scattered light over time, information about the motion of the particles can be obtained. This due to slower particles scattering light in the same position for longer durations than fast particles.

$$D = \frac{k_B T}{6\pi\eta R_H} \tag{3.3}$$

Using the Stokes-Einstein equation (3.3), the hydrodynamic radius can be calculated [61]. In the equation D is the diffusion coefficient obtained from the movement of particles, k_B is the Boltzmann constant, T is temperature, η is the dynamic viscosity and R_H is the hydrodynamic radius. From the measurement an intensity distribution over sizes is given, as well as the hydrodynamic diameter (D_H, calculated from R_H). The **hydrodynamic diameter** refers to the diameter of a smooth spherical particle with the obtained speed [61]. This means that the calculations assume that the particles are smooth and spherical, and uniform in size. If the actual shape of the

particles differ significantly from this description, the size estimation may be less accurate.

The intensity distribution, which tends to place emphasis on larger particles, since they scatter more light, can be converted into a distribution based on volume or numbers [61]. These often instead place emphasis on smaller particles. However, the distribution used in this project has been the one based on intensity, since it displayed the differences between fluids most clearly. The intensity was obtained from side scattered light (90°).

Each fluid, already at room temperature, was gently mixed before 1 mL of the liquid was transferred into the cuvette for measurements. The temperature was set to 25 °C and the equilibration time was set to 1 min and 30 s each time a new fluid was measured, to ensure proper tempering. For each addition to the fluid in the cuvette, the sample was mixed gently and the equilibration time was set to 30 s to allow potential air bubbles to disappear before starting a new measurement. The only exception, where the equilibration time was set to zero, was during the investigation of the time dependence of aggregation.

3.2.3 FLUHTE

The principle of FLUHTE, as described in section 2.3.2, is to measure the management of test fluid in a wound dressing using an instrument setup that mimics a wound on a lower leg. The specific FLUHTE setup used was FLUHTE Basic, seen in figure 3.3, that contains the following components:

1. A pump, to which the syringes with fluid are loaded. The pump regulates the flow rate of fluid out of the wound site.

2. The syringes with wound fluid, connected via tubes to the wound site.

3. The leg model, consisting of two half cylinders, with one wound on each. The wound is simulated by five holes in the metal leg, to which the tubes can be connected. The leg also has a hole for a temperature probe that helps in controlling the temperature.

4. The hot plate that sets the temperature of the leg.

5. The wound dressing, covered with compression bandage or tube socks, which depending on the size of the tube or how tightly the compression bandage is wrapped, generates different pressures.

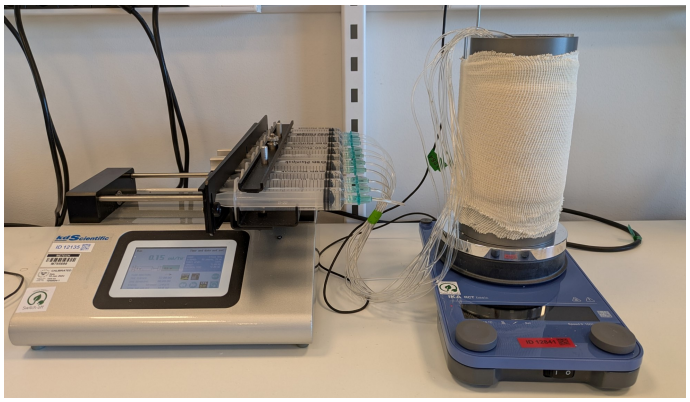


Figure 3.3: Experimental setup of FLUHTE.

In this project, the wound dressing used was a five-layered foam dressing with a size of 15 × 15 cm, of which the absorption area was 11 × 11 cm. The setups used were a flow rate of 0.75 mL/h per each wound site, a temperature of 30 °C, and an application pressure of 40 mmHg achieved by putting three layers of a small-sized tube sock around the leg. The runtime was 72 hours. These specific settings were chosen as they previously had been found to be challenging for the system, including SWF A and advanced foam dressings, resulting in inadequate spreading patterns. For each fluid at least 5 spreading patterns were produced.

Before each experiment, all surfaces and equipment, including pumps, metal legs, and hot plates, were disinfected. The system was then set up with syringes, tubing and compression socks, and waste wound dressings were placed over the wound sites. Syringes were filled with fluid using the pump. After filling, the tube ends were connected to the wound site, and a small amount of fluid was discarded to remove air from the tubes and channels in the wound site. Prior to application of the actual wound dressings, their dry weight was measured the waste dressings were then replaced with the real dressings, covered with three layers of compression socks. The hot plate was switched on and when the target temperature was reached, the pump was started.

After the 72 hours, the equipment was turned off and the wound dressings were carefully removed one by one. Their weights were recorded, and the dressings were then adhered to an overhead film and put on a light board. The edges of the spreading patterns were traced onto another overhead film. The dressings were then cut open, to observe the spreading between the foam and the spreading/retention layers. From the overhead films, the traced patterns were scanned and subsequently layered and formatted for visual presentation using Photoshop. The recorded weights were used to calculate the amount of evaporated fluid, and the results were plotted using Python.

4
Results and Analysis

In this chapter, the findings and results from the experimental analyses conducted within the framework of this project are presented. Additionally, some findings from literature and previous experiments conducted by Mölnlycke Health Care are also included when relevant.

4.1 General evaluation of fluids

This section presents the results from the first stage of experiments, conducted with the intention of characterizing the behavior of six already existing and to varying extent already used fluids at Mölnlycke Health Care. The purpose was to find differences worth further exploration. Some analyses also were carried out in order to assess and evaluate methods for further testing during the project.

4.1.1 Surface tension measurement of the fluids (PDT)

In this experiment the surface tension of five of the six wound fluids, described in section 3.1.1 were analyzed. As the previous master thesis project had focused mainly on surface tension, a first idea was to assess that parameter, to either confirm that there was still significant differences in surface tension between different fluids, or to reject the idea of further work in the area. The question addressed was:

Is surface tension the most relevant parameter?

The results are presented in figure 4.1. The surface tension for all fluids were within the range of 56.0 to 59.3 mN/m. Closest to the SCS 1 value of 58.2 mN/m were SCS 2, while Peptone 20 and SWF 20 had slight higher values, but all of them within the precision of the instrument at about 1 mN/m. The mean value for SWF B ended up slightly lower than the rest of the fluids, but not with a very large difference.

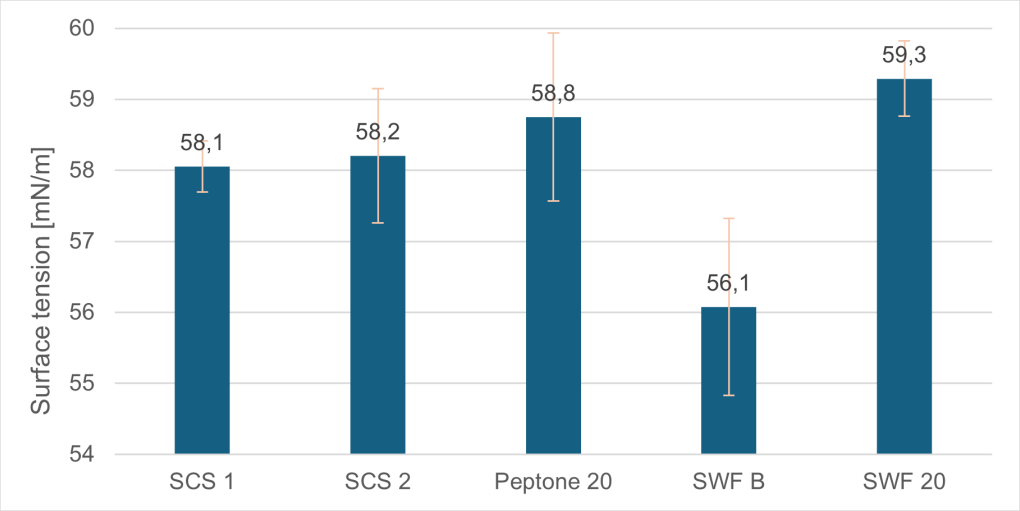


Figure 4.1: Mean surface tension of different fluids.(n=10 per fluid; SWF B: n=20).

The results from this experiment, conducted using pendant drop tensiometry, shows less of a difference between different fluids regarding surface tension, in comparison to what was obtained in the previous master thesis, were instead the Du Noüy ring method was used. In those results, all fluids had a slightly lower value, and the SCS 1, which was referred to as the fluid SCS in the previous report had a remarkably lower value.

However, the outcome of the current experiment shows that there is no significant difference between the fluids regarding surface tension. Instead, the differences are negligible in most cases, as they fall within the margin of error of the instrument's precision and the variance between replicates within each sample.

4.1.2 Particle size distribution of the fluids (DLS)

The idea of this experiment was to assess the differences in particle sizes within all the six fluids in 3.1.1 by DLS measurements. Based of reports like Trengove *et al.* there should be significant differences between serum and the simplified wound fluids. The research question was:

Is the particle size distribution different between the fluids?

In 4.2 the particle size distribution, based on relative intensity of side scattered light (90°), is shown for the fluids along with water as an extra control.

The SWF fluids (SWF A, SWF B and SWF 20) and Peptone 20 had a large peak at about 7 nm. According to different literature the nominal size of BSA is about 7.1 nm, and the dimensions of BSA are 4 x 14 x 14 nm [62, 63].

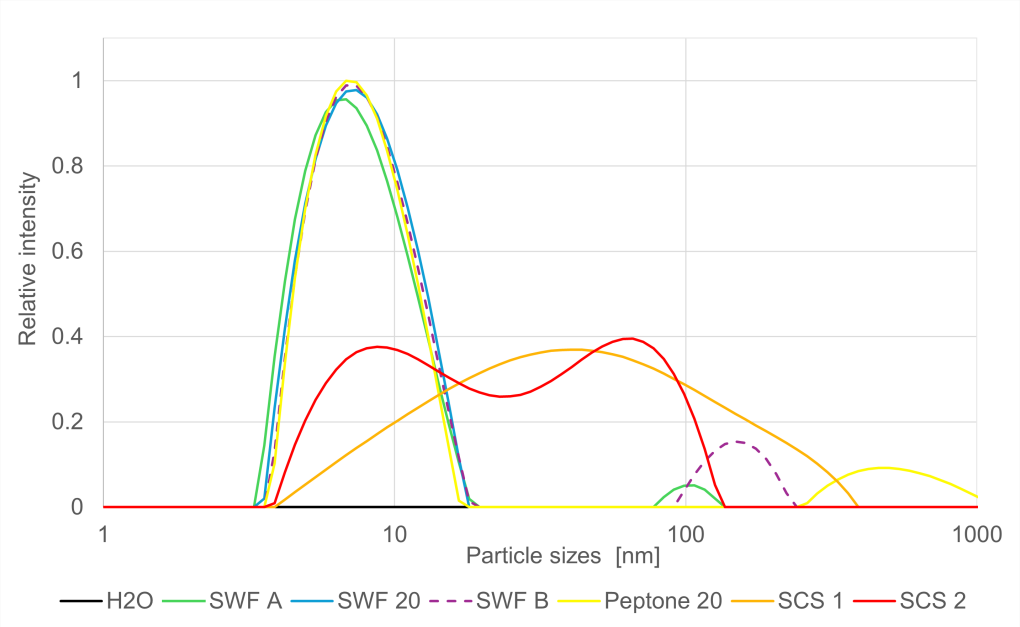


Figure 4.2: Particle size distributions for different test fluids.

This corresponds well to the large peak for these fluids, that contain added BSA. The SCS 1 and 2 had peaks covering approximately the same range of sizes. However, the SCS 1 showed one broad peak with an intensity maximum at about 40-50 nm, while the SCS 2 showed a bimodal distribution, with one maximum at about 8 nm while the other intensity maximum had a significantly larger size of about 60 nm. For comparison the size of gamma globulins, which is the largest plasma protein type that should be present in the serums, are 10-20 nm [64].

4.1.3 Calcium-induced aggregation (DLS)

The same fluids were further examined in the DLS when triggered with $CaCl_2$, to induce protein aggregation. As protein aggregation is a behavior within wound fluids that could significantly affect the spreading pattern in wound dressings, it was of priority to assess. As water evaporates from a fluid in a wound dressing, the concentration of proteins and electrolytes increase. The idea was to investigate how the increase of one salt could affect the protein behavior. The specific use of $CaCl_2$ had three reasons; (i) the fact that it was an already existing salt in the assessed fluids, (ii) the moderately protein denaturating nature of the Ca^{2+} ion, according to the Hofmeister series, and (iii) findings about $CaCl_2$ being utilized in other studies, for example a study by Ju *et al.* where it aggregated whey proteins [65]. The research question became:

Is it possible to induce aggregation of BSA in the fluids using $CaCl_2$?

$CaCl_2$ was gradually added, 10 μL at the time of a 0.25 M solution, to the cuvette containing 1 mL of fluid. The results are presented in 4.3, where the average particle size represented by the hydrodynamic diameter is plotted against the concentration

of $CaCl_2$ caused by gradual addition of highly concentrated $CaCl_2$ solution into the vials. The initial $CaCl_2$ in the fluids are not included since the exact values for the serum based solutions are unknown.

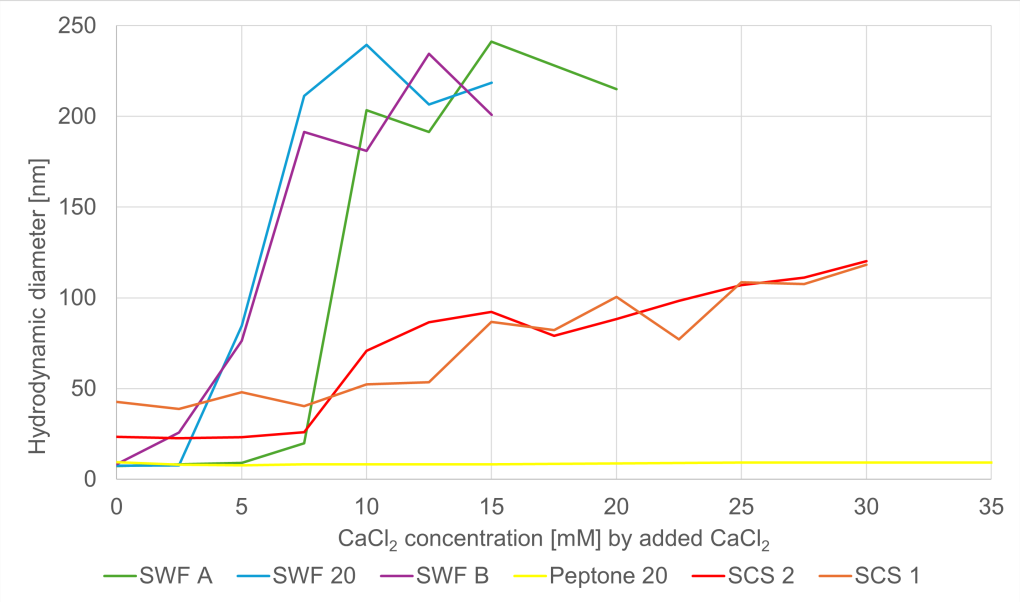


Figure 4.3: Increase of average particle size (hydrodynamic diameter) induced by $CaCl_2$ for six different fluids.

In all fluids except Peptone 20 there was an increase in particle size when $CaCl_2$ was added, indicating that addition of $CaCl_2$ does trigger aggregation in the fluids. For both serum-containing solutions (SCS 1 and SCS 2) there was an increase in particle size as a result of added Ca. However, the increase was gradual, and every small addition of $CaCl_2$ lead only to a slight increase. The SWF fluids were remarkably more affected by the added Ca, forming much larger particles at lower concentrations of Ca. The SWF B and SWF 20 samples showed almost identical patterns, and thus it can be concluded that the surfactant in SWF B had no major effect on the formation of aggregates. However, there was a noticeable difference between those two samples and SWF A, where SWF A showed a shift towards higher $CaCl_2$ concentrations for the start of aggregate formation. Since the only difference between the samples was that SWF A contains 70% more BSA, this may suggest that the delay is related to the BSA concentration.

Additionally, it should be noted that Peptone 20 was not triggered by the added $CaCl_2$ at all. It showed no sign of aggregation regardless of the amount of $CaCl_2$ added (in the measured range of 0-45 mM).

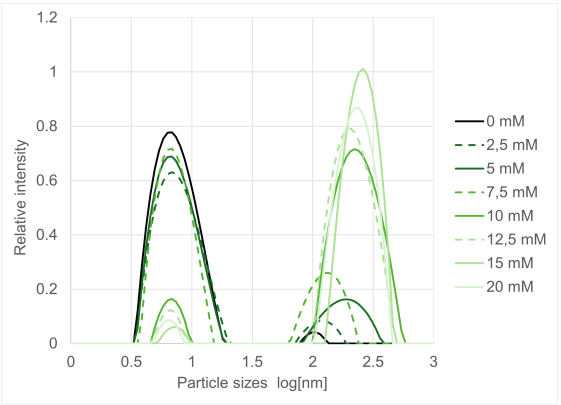
All fluids, except Peptone 20, exhibited a tendency to form sigmoidal patterns. Therefore, sigmoidal regressions were performed on the data series using Python (Spyder). It is important to note that these sigmoidal curves are not based on any theoretical models. However, several different regression functions were tested for comparison, and the sigmoidal curves consistently showed the best fit to the datasets. In table 4.1 the constants from the regressions are presented, where L represents the maximal asymptotic value, x_0 is the x-value at the point of maximum slope and k is the slope parameter.

Sample	**L**	**x_0**	**k**	**R^2**
SWF A	220	10.84	2010	0.98
SWF 20	220	7.54	1380	0.99
SWF B	210	7.67	850	0.97
SCS 1	170	17.92	82	0.89
SCS 2	110	9.53	200	0.91

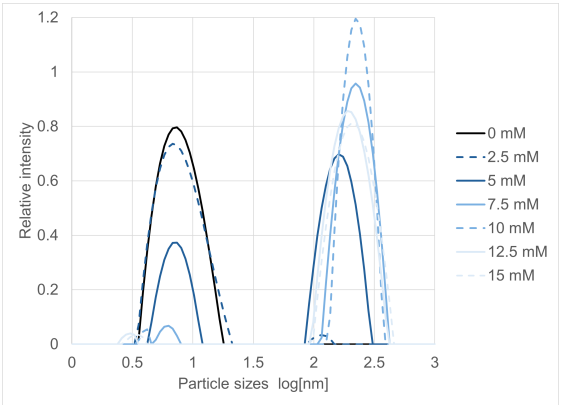
Table 4.1: Parameters for sigmoidal curve regressions of the measured data series.

SWF A showed the steepest slope with k=2010, while SCS 1 showed the flattest, k=82. There was also a clear difference in the asymptotic values, where all the SWF fluids had an L in the range of approximately 210-220 whereas the SCS values were much lower.

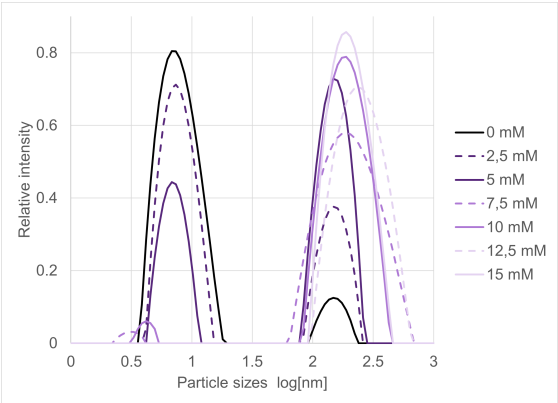
The change in particle size within each fluid sample, as a result of the added $CaCl_2$ is shown in figure 4.4. For the SWF fluids one can see a gradual change from the BSA peak to another distinct peak at about 220 nm. This indicates the formation not of various aggregated forms of albumin, but rather of a specific aggregate, since the same peak occurs in every SWF sample. For Peptone 20 the BSA peak remains unaffected by the Ca ions, as corresponds well to the constant value of the hydrodynamic diameter. The various small peaks are likely caused by air bubbles or contaminants and can be considered negligible, as their size does not exceed that observed in the H_2O sample, which should not exhibit any true peaks. Regarding the two SCS samples they both change into a more distinct bimodal shape with a very heavy peak at the larger sizes.



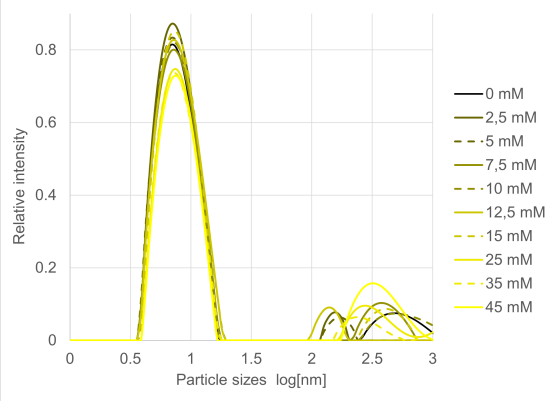
SWF A



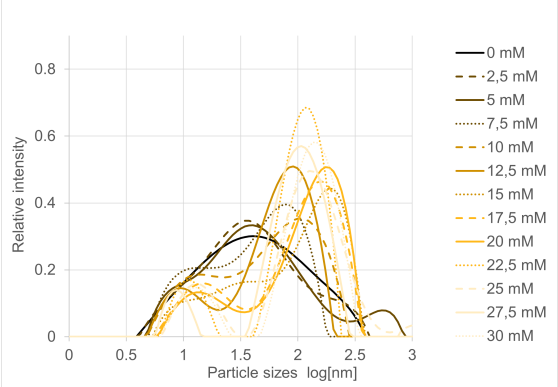
SWF 20



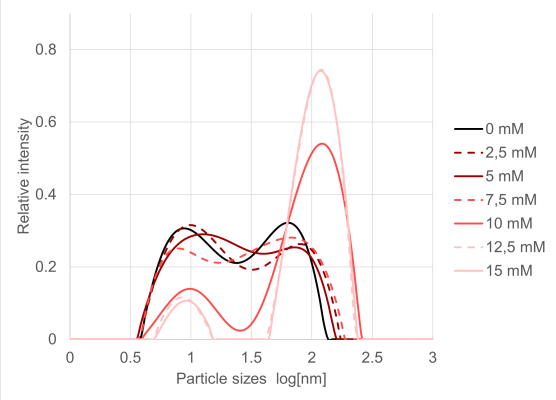
SWF B



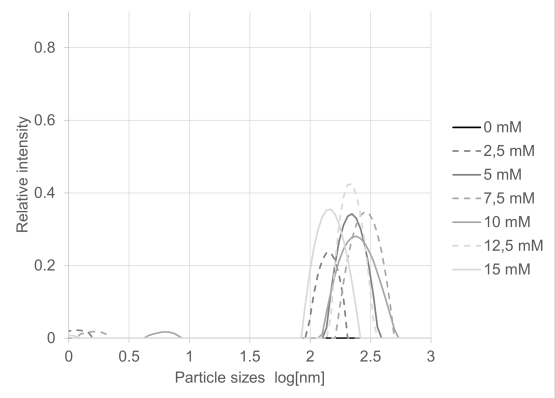
Peptone 20



SCS 1



SCS 2



H_2O

Figure 4.4: Particle size distribution with increasing $CaCl_2$ concentration for the fluids. The curves reflect added $CaCl_2$ only, excluding initial concentrations in the fluids.

4.1.4 Kinetics of aggregation (DLS)

Since the induction of protein aggregation could be measured by DLS, the time dependency and kinetics of the aggregate formation were of further interest. SWF 20 and SCS 1 were investigated to answer the question:

How does the aggregation process evolve over time?

To induce aggregation 30 μL of 0.25 M $CaCl_2$ solution was added to 1 mL of fluid (corresponds to 7.5 mM of $CaCl_2$ in the fluid). The equilibration time of the measurements was set to 0, to allow analysis immediately after the addition. The results are shown in figure 4.5. There was a substantial difference in the aggregation kinetics between the two fluids. SWF 20 had a much faster initial aggregation, which gradually slowed down over time, while SCS 1, in contrast, showed a consistently slow aggregation throughout the entire period. The shape of the curves suggests that SWF 20 could be following a logarithmic aggregation pattern, while SCS 1 shows a trend that appears approximately linear over time.

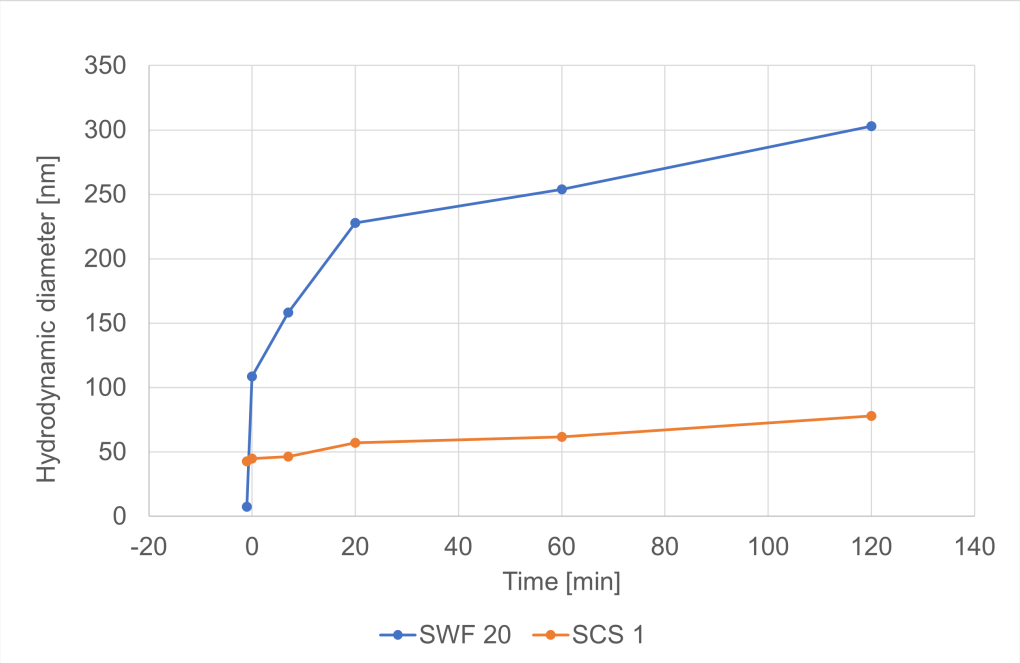


Figure 4.5: Time dependence of aggregate formation.

Table 4.2 shows the inherent growth of particle size in relation to the non-aggregated state for the two fluids. Within the equilibration and measurement time, the SWF 20 fluid had already grown particles on average 14 times (1367% larger than) the size of the non-aggregated fluid. During the same time, the particle size in SCS 1 has only increased by 5.5 %. After two hours the difference is instead a total growth of 3985% in the SWF 20 sample, while in the SCS 1 sample it had only enlarged by 83%.

Time (min)	SWF 20 (%)	SCS 1 (%)
Before addition	100.00	100.00
0	1466.56	105.49
7	2134.66	108.86
20	3074.56	133.76
60	3423.18	145.19
120	4085.08	183.33

Table 4.2: Relative size increase within each sample, calculated by setting the non-aggregated state (prior to $CaCl_2$ addition) as 100%.

4.1.5 Fluids with different aggregation behavior (FLUHTE)

The results from section 4.1.3 showed interesting differences in aggregation between SCS 1 and SWF A, which lead to the suggestion that this difference could be a possible cause to different spreading patterns in the two fluids. To evaluate this hypothesis, the spreading patterns of SWF A, that was highly prone to aggregate when triggered by the presence of Ca^{2+} ions, and SCS 1, which showed a less pronounced aggregation response, were analyzed in FLUHTE. Peptone 20 was also evaluated, as it did not aggregate at all during the DLS experiment. To account for the difference in protein content between SWF A and Peptone 20, and simultaneously investigate the influence of protein concentration on spreading patterns, the fluid SWF 20 was also included in the analysis. The question to be answered was:

Does the propensity of aggregation and/or the amount of albumin in the fluids affect the spreading patterns within a wound dressing?

The results from the FLUHTE analysis are shown in figure 4.6 and 4.7, where the images of the spreading patterns are layered on top of each other in a heat map. Darker colors indicate areas that are more frequently wet. The SCS 1 spread downwards in all replicates. It also showed a tendency to spread towards the sides, most prominently to the left. The SWF A replicates, by contrast, spreads upwards from the center. These results aligns well with what has previously been observed at Mölnlycke Health Care for the specific system.

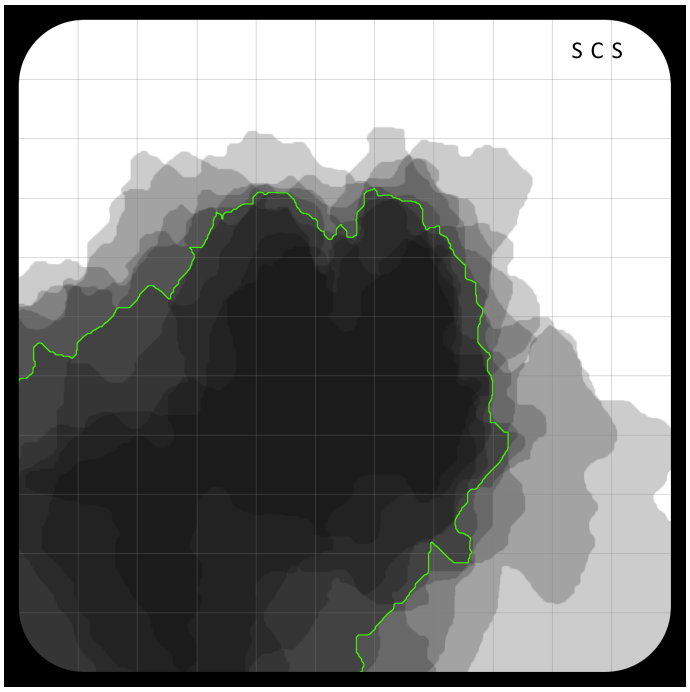
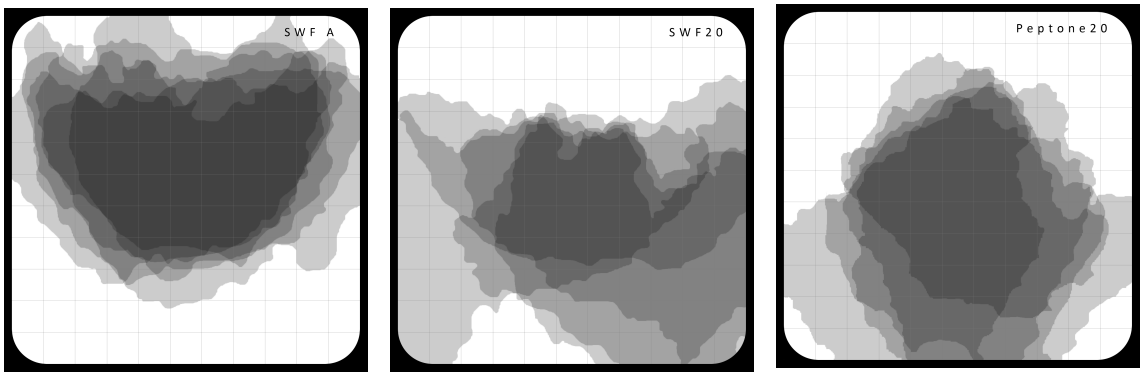


Figure 4.6: Layered spreading patterns of SCS 1 using FLUHTE for 72h at 0.75 mL/h. The green outline indicates the area where at least 50% of the layers overlap. (n=10).



SWF A SWF 20 Peptone 20

Figure 4.7: Layered spreading patterns for three test fluids, using FLUHTE for 72h at 0.75 mL/h. (SWF A: n=6; SWF 20 and Peptone 20: n=5).

The spreading pattern in SWF 20, which contains the same components as SWF A but with a lower protein concentration, did not spread upward or downward, but rather to the sides. This resulted in slightly less homogeneous spreading, as the fluid tended to move laterally, either to the left or right, leading to less overlapping spreading patterns. However the results were somewhere in between those for SCS 1 and SWF A. This indicates that the amount of protein in the liquid plays a role in the spreading patterns, with fluids containing less proteins spreading less upwards.

Lastly, when observing Peptone 20, which was known not to aggregate by $CaCl_2$ in the DLS, the spreading of the fluid looked similar to SCS 1, with the most prominent spreading downwards, and not especially much at all upwards in the wound dressing. Overall this suggests that a fluid with less or no aggregation spreads more downwards in the wound dressing, while fluids more prone to aggregation, like the SWF A and SWF 20, are prevented from spreading down and instead are forced to the sides and upwards. In connection with the DLS findings, the desired fluid behavior may therefore be one with less pronounced aggregation in response to $CaCl_2$, more similar to the response of SCS or even the absence of aggregation observed in Peptone 20.

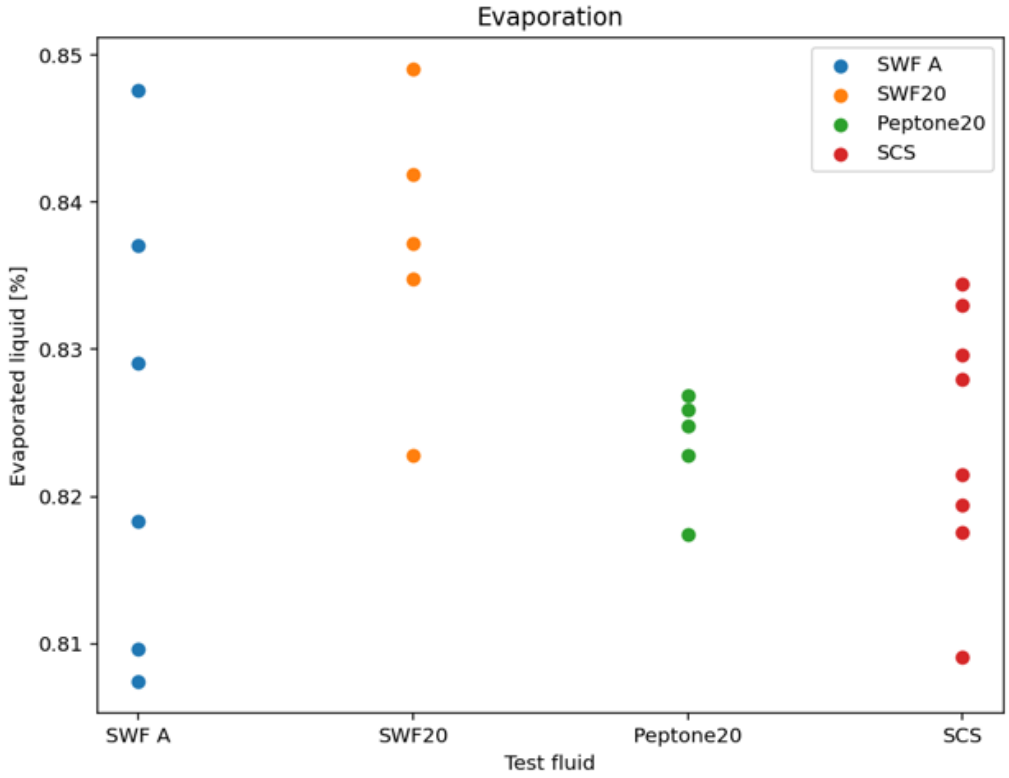


Figure 4.8: Evaporation for the samples in the FLUHTE analysis.

In figure 4.8 the amount of evaporated liquid in all the samples are presented. The percentage of liquid that have evaporated during the 72 h runtime is for all fluids within the range of 80-85%, with SWF A having the most diverse evaporation between replicates, while Peptone 20 on the other hand had less variance. Overall, however, all the fluids had similar degree of evaporation, and since there is some variance between replicates within samples, there are no significant difference between the different types of fluids.

4.2 Effect of biomolecular size polydispersity

In this section the results from a series of experiments done to investigate both some small and large particles as additives are presented. They were investigated since biological wound fluid contains both many different kinds of large proteins, as well as other smaller biomolecules such as peptides, that are not present in the simulated wound fluids. One additive specifically investigated was the peptides found in the Peptone 20 fluid, since it showed many interesting results in the general testing experiments.

4.2.1 Peptone in presence of SWF salts (DLS)

Since Peptone 20, containing peptides from Peptone Bacteriological, exhibited a non-aggregating behavior upon exposure to $CaCl_2$ and also showed a similar spreading pattern to that of SCS 1, its peptides were further examined in solutions with a different electrolyte composition than that of peptone water. The research question became:

Are peptides along with the SWF salts behaving the same as Peptone 20?

To assess this the protein aggregation in samples containing SWF 20 with addition of different amounts of Peptone bacteriological were analyzed by DLS, when exposed to $CaCl_2$. Peptone 20, in comparison to these samples, contains 1.0 g/L of Peptone bacteriological and 20 g/L of BSA in NaCl solution. In figure 4.9 the results from the DLS measurements are shown.

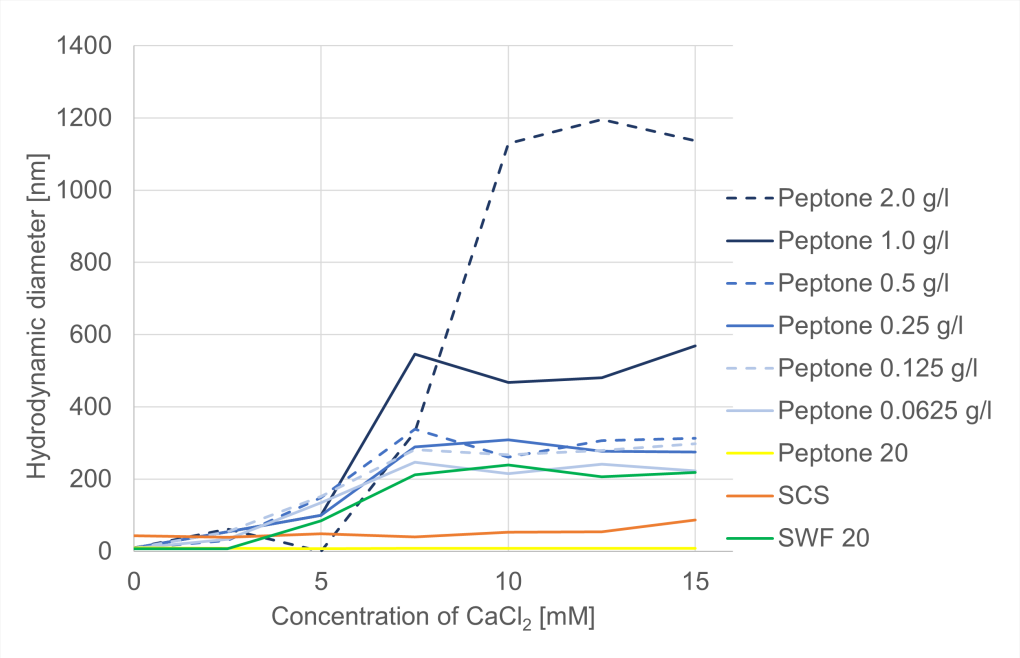


Figure 4.9: $CaCl_2$-induced aggregation in SWF 20 with different peptide concentrations.

The results clearly show that when peptides were added to an environment containing the SWF salts, the fluid formed aggregates similar to the ones in SWF 20. The results also show that the more peptides, the larger aggregates were formed. The peptides thus appears to facilitate the formation of larger aggregates. The results also suggest that it is not the peptides in Peptone 20 that prevented the aggregation, but instead the ionic composition of the fluid or possibly the combination of peptides and the different ionic environment.

4.2.2 Significance of the order of exposure to peptides (DLS)

Since Peptone 20 did not aggregate, while SWF 20 with added peptone bacteriological did show aggregation, a hypothesis related to the order of exposure playing a role was formed. In Peptone 20, the proteins were exposed to the peptides in the absence of Ca^{2+}, whereas in SWF 20 Ca^{2+} ions were already present when peptides were added. Therefore the question became:

Does the order of exposure to peptides vs $CaCl_2$ matter?

To assess this, three new solutions were made; (i) one fluid with only 20 g/L of BSA and 8,5 g/L of NaCl, to compare with the Peptone 20, (ii) one fluid called SWF 20 buffer, that was the SWF 20 but without $CaCl_2$ and $MgCl_2$, (iii) and one version where 1 g/L of peptone bacteriological was added to the SWF 20 buffer solution. The solutions aggregation behaviors were assessed with $CaCl_2$ induced aggregation in DLS.

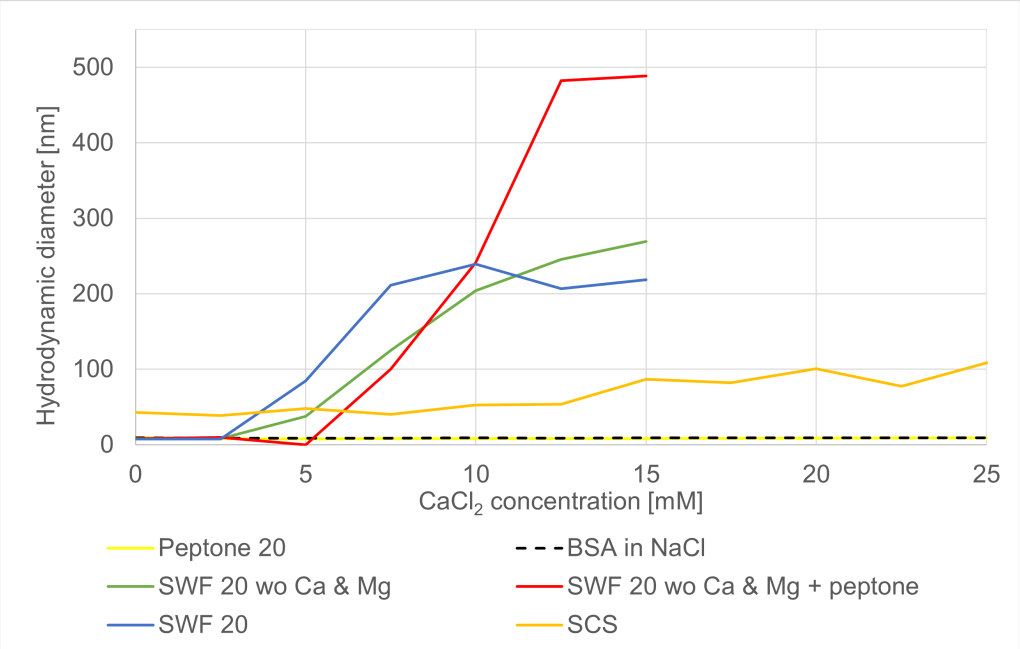


Figure 4.10: $CaCl_2$-induced aggregation in fluids with peptides and different salt environments.

From figure 4.10 some different comparisons can be made. When comparing Peptone 20 and BSA in NaCl without the peptides, the peptone did not have any effect, since

no aggregation was detected in any of the fluids upon exposure to Ca^{2+} ions. It seems like when BSA is in a solution with NaCl as only salt it does not aggregate in the way earlier detected.

When comparing SWF 20 and SWF 20 buffer, which is the SWF 20 solution without $MgCl_2$ and $CaCl_2$, no significant difference was observed. Once $CaCl_2$ is added, the ionic composition of the two solutions becomes comparable, which explains their similar behavior. However, when comparing their behaviour to SWF 20 buffer + peptone, there is a significant difference in the size of the formed aggregates. Again, the peptides appear to facilitate larger particles forming in the solution, similar to the results from section 4.2.1. Thus, even though the peptides this time were added before the first exposure to Ca, they did not prevent aggregation. Instead they facilitated the formation of larger aggregates.

4.2.3 Effect of the addition of γ-globulins (DLS)

Since BSA only contributes with one size of protein, while exudate and serum contain a wide range of sizes, the effect of larger proteins was of interest, forming the following question:

What effect does the larger serum proteins have?

The chosen category of proteins was γ-globulins, as they are the largest-sized category of globulins and they were therefore hypothesized to produce the greatest effects. 14 g/L of these globulins was added to SWF 20 to mimic the 20 g/L of albumin and 14 g/L of globulins found in biological exudate. The solution was analyzed, again using $CaCl_2$-induced aggregation in DLS.

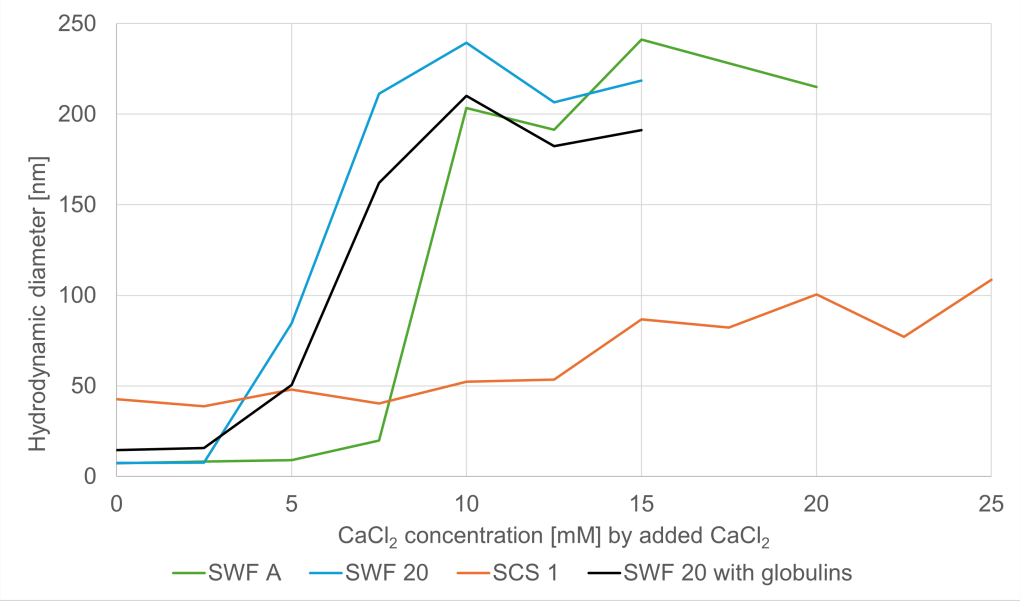


Figure 4.11: $CaCl_2$-induced aggregation in SWF 20 with addition of γ-globulins.

In figure 4.11 the results are presented. When comparing the curve for the γ-globulin-containing sample to those of SWF 20 and SWF A, no significant effect could be seen on the steepness of the slope. Thus, other than a slightly larger initial particle size, there seems to be no substantial effect on the aggregation behavior.

4.3 The role of ions in formation of aggregates

This section presents the results from investigations into how the ionic environment influences aggregate formation. As observed from the results in section 4.2, the presence or absence of specific ions in a sample has a major impact on the extent of aggregation upon exposure to $CaCl_2$. Therefore the properties and effects of the ionic environment were further researched.

4.3.1 The role of buffer content on the aggregation (DLS)

The results in section 4.2.2 demonstrated that BSA did not aggregate in the presence of only NaCl. However, intense aggregation was observed when buffer salts ($NaHCO_3$, KH_2PO_4) and KCl were present. Given that the presence or absence of buffer ions appears to significantly influence protein aggregation, their effect was further investigated, to address the question:

What is the effect of varying the amount of buffer in SWF 20?

In figure 4.12 the aggregation by $CaCl_2$-induction is presented for SWF 20 with different amounts of buffer content. The SWF 20 100% buffer is the original SWF 20 fluid, which had already been tested. Then altered SWF 20 samples were examined, starting with SWF 20 with no buffer. This sample showed no sign of aggregation when being triggered by $CaCl_2$ in the range of 0-15 mM. SWF 20 with 50% and 40% buffer were also examined, showing a shift in the initial aggregate formation. However, from the point when aggregation starts (10 resp. 12.5 mM $CaCl_2$), both slopes have about the same steepness as SWF 20 with its original buffer content.

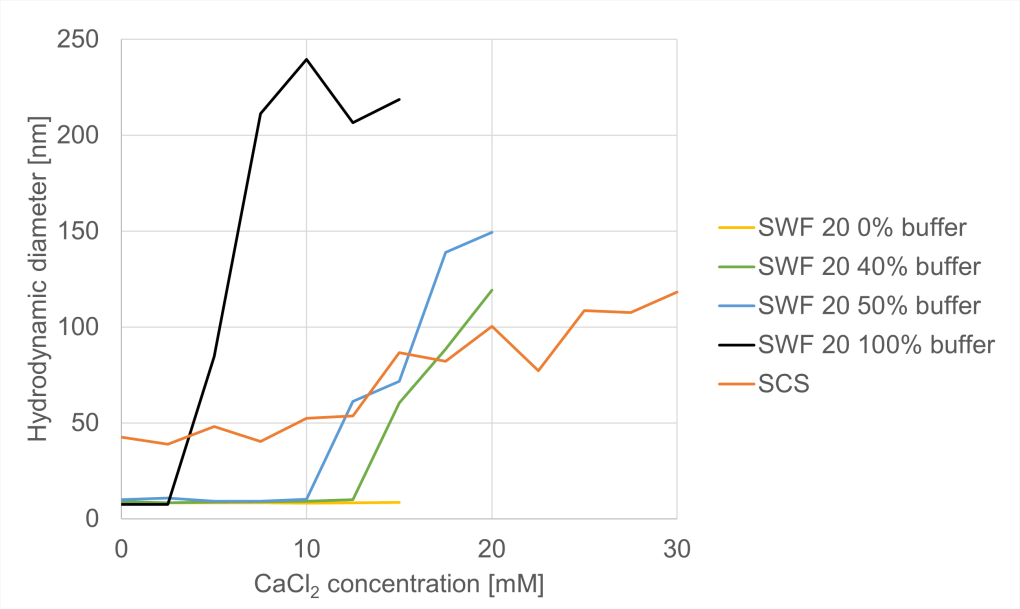


Figure 4.12: $CaCl_2$-induced aggregation in SWF 20 with different buffer concentrations.

Lowering the buffer content in SWF 20 did not result in the desired fluid behavior that was reducing its reactivity to $CaCl_2$ and producing a flatter slope. Instead, the decreased buffer concentration merely shifted the onset of aggregation and increased the $CaCl_2$ concentration required to trigger the start of the aggregate formation, while then having the same steep aggregation as SWF 20. Thus, it was concluded that modifying the buffer content is not a good means to regulate the aggregation.

4.3.2 Aggregation induced by buffer compared to Ca and Mg salts (DLS)

When a fluid evaporates in a wound dressing, the electrolyte and protein content increases. Since both chaotropic and kosmotropic ions could have the potential to aggregate proteins through different mechanisms, the effect of the kosmotropic buffer ions as inducers of aggregation was tested in comparison with the chaotropic Ca^{2+} and Mg^{2+}. To properly investigate drying, all ion concentrations should be increased. However, since Ca^{2+} together with HCO_3^- could form the insoluble $CaCO_3$, both ions could not be added together. For SWF 20, one test was also conducted while completely eliminating one of the two components for precipitate formation, to investigate if there was precipitate formation in the earlier experiments and if this played a role in the discovered aggregation. The researched questions were:

Is it possible to induce aggregation using selected buffer salts instead of $CaCl_2$?
Does aggregate formation still occur when salt precipitation is not possible?

The questions were experimentally assessed using DLS. In figure 4.13 the aggregate formation in SCS 1 induced by buffer vs Ca^{2+} and Mg^{2+} are shown in yellow and orange. The curves show a similar pattern between the both series, which indicates that no matter the ion, aggregation is mildly triggered by it. Thus, the buffer ions does also trigger aggregate formation.

For the SWF 20 curves (blue) the formation of remarkably large particles when exposed to the buffer ions can be seen. The hydrodynamic diameter in earlier aggregation in SWF 20 triggered by $CaCl_2$ have reached a size of max 250 nm, while this curve reaches values of 1600 nm.

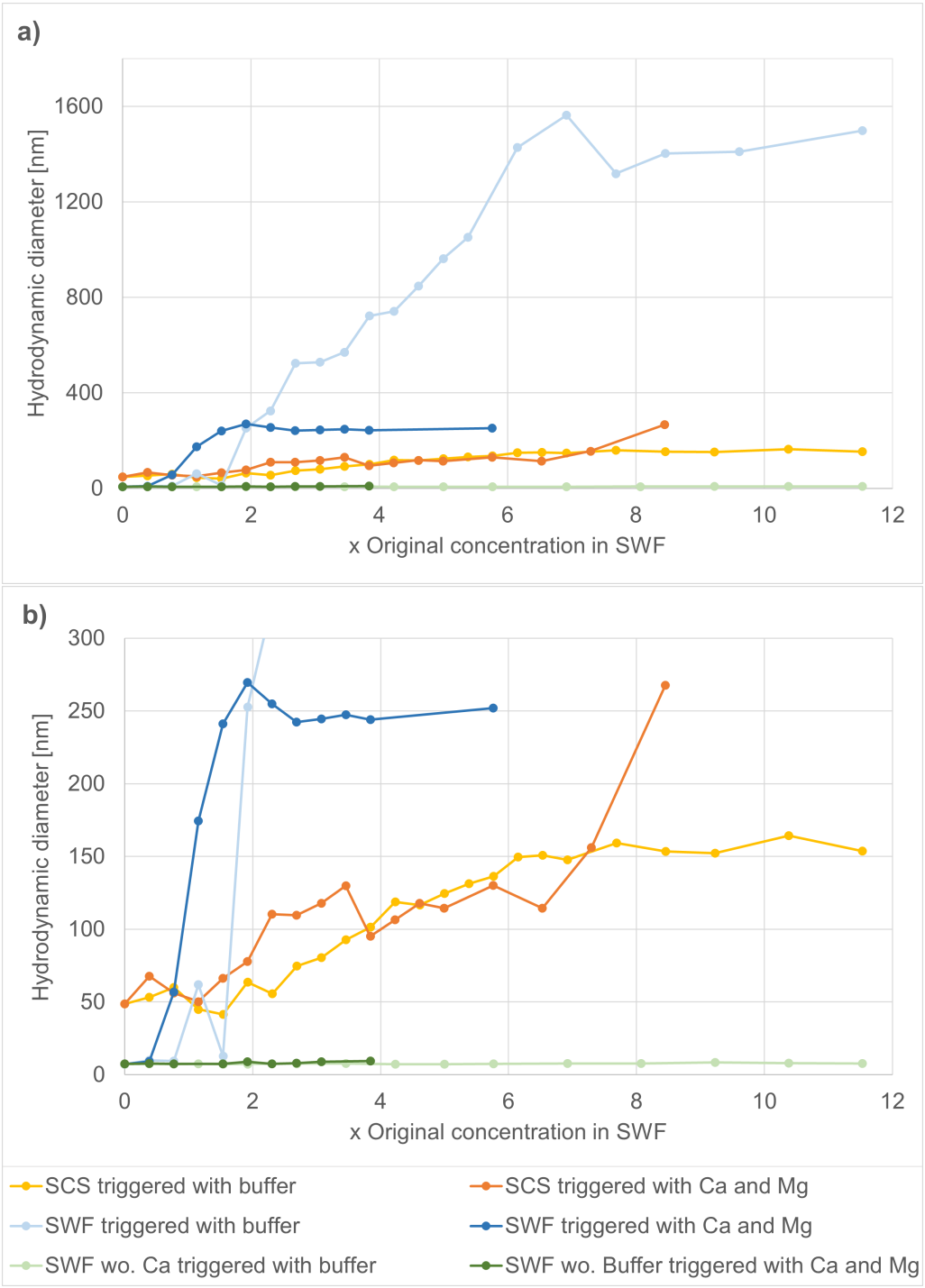


Figure 4.13: $CaCl_2$- and $MgCl_2$-induced vs. buffer-induced aggregation in modified SWF 20. X-axis values indicate multiples of the ionic concentrations in SWF 20.
a) Full measured range.
b) Zoomed-in view of the smaller particle sizes in a.

In the experiment where one of the required components for $CaCO_3$ precipitation was omitted (green), no aggregation was observed. For the SWF 20 without buffer ions (dark green) the lack of aggregation was somewhat expected since BSA with 0% buffer in 4.3.1 did not aggregate either, and the only difference here was the

triggering additive being both $CaCl_2$ and $MgCl_2$ instead of just $CaCl_2$. The more interesting result was seen in the other green curve, SWF 20 without $CaCl_2$ being triggered by buffer, that also showed no sign of aggregation, even though its counterpart containing $CaCl_2$ did form large aggregates. Despite the presence of a chemically similar ion, Mg^{2+}, the lack of specifically the Ca^{2+} ion, leads to no aggregate formation. These findings strongly indicates that when we have aggregation we probably also have precipitate formation, and the precipitate itself may serve as the trigger of aggregation.

4.3.3 Effect of SWF salts on the aggregation in SCS 1 (DLS)

Since the ionic environment appeared to play a significant role for the aggregation process, the possibility that the difference in aggregation behavior between SCS 1 and SWF A was due to variations in ionic composition could not be ruled out. Although some of the salts examined separately did not show signs of causing that exact effect, the overall combined effect had not yet been examined. Therefore the following question was addressed:

How would SCS behave when exposed to the same ionic environment as SWF A?

In figure 4.14 the $CaCl_2$-induced aggregation of this SCS with SWF salts is compared with SWF A and SCS 1. Even though it produces slightly larger particle sizes for the same $CaCl_2$ additions, it does follow the SCS 1 curve very well and does not at all have a similar curve to SWF A. This suggests that the difference between the solutions is not, or at least not solely, caused by differences in the ionic environment, but also to a high extent by other properties of the solutions.

Figure 4.14: $CaCl_2$-induced aggregation in SCS with SWF salts.

This result corresponds well to the FLUHTE experiments previously conducted by Anna Svensby at Mölnlycke Health Care, where the same SCS with SWF salts solution, along with original SCS 1 and SWF A, was tested under the following conditions: 1 mL/h, 24 hours, 40 mmHg, and 32°C. The results for SCS 1 and SWF A respectively observed in that experiment, showed great resemblance with spreading patterns received within this projects (figure 4.6 and 4.7). The spreading patterns from SCS with SWF salts appeared nearly identical to those of SCS 1.

Although there were some differences in the experimental conditions in this experiment in comparison with the ones conducted within the frame of this project, the results clearly demonstrate that SCS with SWF salts performed similar to original SCS 1 fluid. The findings in 4.14 align well with the FLUHTE results, which reinforces the hypothesis that the measured aggregation by DLS correlates with the resulting spreading patterns for the same fluid.

4.4 Addition of synthetic molecules

Lastly, synthetic additives were tested in various experimental setups, both to characterize the fluids and to evaluate their potential as functional additives. The experiments included two types of surfactants, alkyl glucoside (AG) and Triton X-100, as well as glycerol and urea. The outcomes of these tests are presented in the following section.

4.4.1 Interactions between surfactants and BSA (PDT)

Surfactants are generally known to increase solubility and prevent aggregation. For this experiment two nonionic surfactants were used. As the ions from salts investigated mostly had shown to increase or induce aggregation, the interest now laid in surfactants of non-ionic nature. The two assessed surfactants were AG and Triton X-100, which despite their shared nonionic character, differ significantly in molecular structure. Their effect on surface tension in water, and in SWF 20 was analyzed, to answer the question:

How does the different surfactants interact with BSA in wound fluid?

It was also necessary to understand what effect the surfactants would have on the surface tension, since that is also an important aspect of a wound fluid. In figure 4.15 the four surface tension series (AG in H_2O, AG in SWF 20, Triton X-100 in H_2O and Triton X-100 in SWF 20) are presented. Marked in the AG in SWF 20 series is the value where the AG concentration is equivalent to SWF B. An unexpected dip was also observed in this series, which was initially assumed to be a potential experimental error. To verify this, the experiment was repeated, resulting in the same outcome.

As expected, Triton X-100 was found to reduce the surface tension of the liquids significantly more than AG at the same concentrations. At 0.05 mM, which was the lowest concentration tested for Triton X-100, the surface tension still was lower than that for SWF B, and as shown in figure 4.1 also lower than SCS 1 (58 mN/m). Therefore, the Triton X-100 cannot be seen as a potential future additive to a simulated wound fluid. The CMC of Triton X-100 in water was observed to be 0.4 mM. For Triton X-100 in SWF 20 the CMC was instead somewhere in the range of approximately 3-7 mM. This demonstrates a strong affinity of Triton X-100 to BSA.

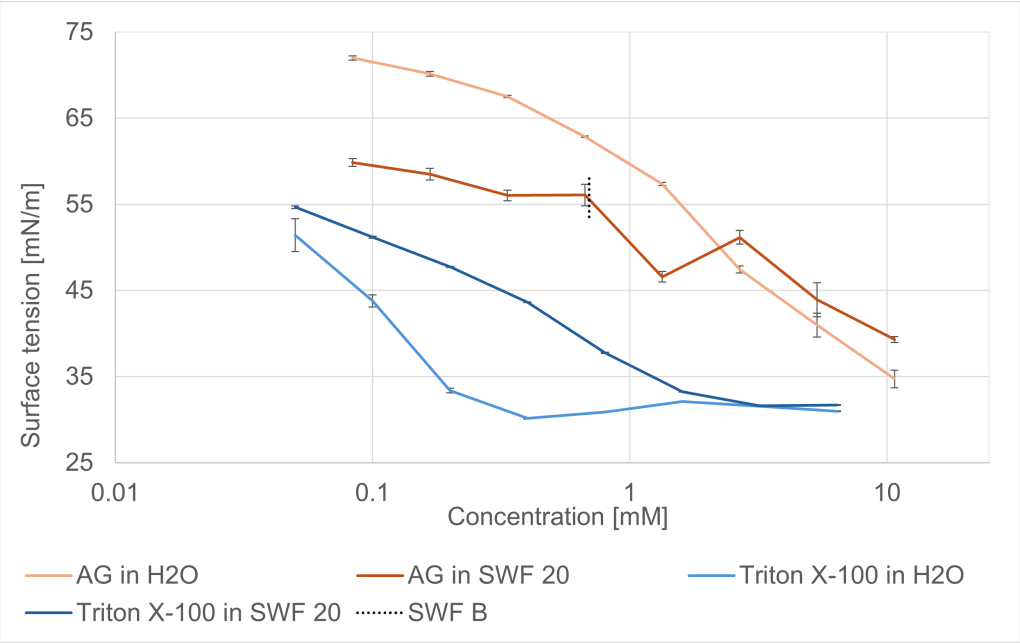


Figure 4.15: Mean surface tension of H_2O and SWF 20 with addition of surfactants. Standard deviation is marked in the error bars.

The AG in H_2O generally produced a slightly higher surface tension than the same surfactant concentration in SWF 20. This indicates that when both AG and BSA is present the both components contribute to decrease the surface tension more than any of the components does on their own. However, regarding Triton X-100 the effect seemed to be the opposite, since the Triton X-100 within SWF 20 solution had a higher surface tension than the surfactant on its own in water. This indicates that the interaction between Triton X-100 and BSA differs markedly from that of AG, with the protein and the surfactant seemingly occupying each other, preventing the surface tension decreasing effect.

4.4.2 Effect of surfactants on already formed aggregates (DLS)

The surfactants were used with the aim of characterizing the aggregates that was formed in the fluids due to $CaCl_2$ exposure. The idea was that surfactants could have the effect of dissolving aggregates, depending on how strongly the aggregates were bound. The assessed question became:

Are the aggregates possible to dissolve using surfactants or are they tightly bound?

After addition of 7.5 mM of $CaCl_2$ to SCS 1 and SWF A respectively, surfactants was introduced to investigate whether they could reduce the particle size again. In SCS 1, a concentration range of 0-0.55 M AG was added, but no effect on the aggregated particle size was observed. It was therefore decided not to continue testing AG, and instead proceed with Triton X-100, based on the assumption that a surfactant with a stronger interaction with BSA might have an effect.

SCS 1 and SWF A with added $CaCl_2$ were tested, along with SCS 1 without any $CaCl_2$, based on the hypothesis that some particles in SCS 1 might be aggregates even in the initial state of the fluid. However, no effects was observed upon addition of Triton X-100 in the range of 0-0.3 M to the aggregated SCS 1, nor in the range of 0-0.2 M to either aggregated SWF A or the initial SCS 1. The aggregates in the fluids appeared to be tightly bound and not affected by the surfactants.

4.4.3 Effect of surfactants on aggregate formation (DLS)

Even though the surfactants had no effects when the aggregates were already formed, the idea of using surfactants to prevent aggregate formation was not rejected. This experiment was therefore conducted, to answer the question:

How is formation of aggregates affected by surfactants?

In figure 4.16 the curves for SWF 20 with addition of 0.5% (weight) AG and Triton X-100, respectively, are shown. Even though according to section 4.4.1 the addition of 0.5% surfactant to SWF would lead to an unacceptably low surface tension, their high amount was used to better visualize any potential effects.

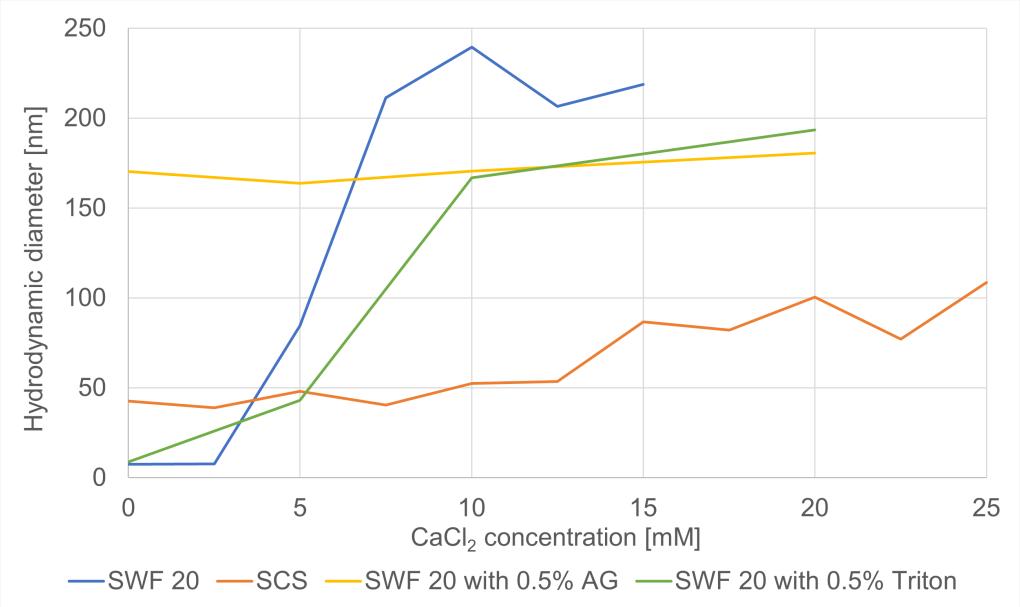


Figure 4.16: $CaCl_2$-induced aggregation in SWF 20 with addition of surfactants.

The sample containing AG had become aggregated already from the beginning, before addition of any Ca. It seems that AG, instead of having the expected effect of preventing aggregation, may actually induce it. The Triton X-100-containing sample shows a slight tendency towards a less steep aggregation curve, marginally more similar to that of SCS 1 than SWF 20 is, although it still remains very similar to SWF 20 overall. However, this observation cannot be confirmed with confidence, as the number of data points is too limited. Moreover, since the effect is minimal despite the high surfactant concentration, Triton X-100 does not appear to be an effective additive for the purpose.

4.4.4 Effect of urea and glycerol on the aggregation (DLS)

As urea and glycerol are known to influence the aggregation of proteins in solution, they were evaluated as additives, with the question:

How is formation of aggregates affected by urea and glycerol?

Due to their reported non-monotonic effects, a suitable concentration was difficult to decide. In the study about urea as stabilizer, the concentration 3, 5 and 8 M was assessed [58]. However, urea is also naturally present in blood and wound fluid, though in low concentrations. According to Trengove *et al.* the concentration, both in serum and exudate is close to 9 mM [2]. In other studies concentrations of glycerol were 17%, 30% etc [50]. However, if using such high amount, the viscosity would be severely affected.

The finally chosen concentrations were 50 mM of urea, and 2% of glycerol, in SWF 20. The additives were expected to affect the sample in some way, and based on the effect, the concentrations could be reevaluated. In figure 4.17 the aggregation upon induction by $CaCl_2$, of SWF 20 with addition of glycerol and urea respectively, is presented.

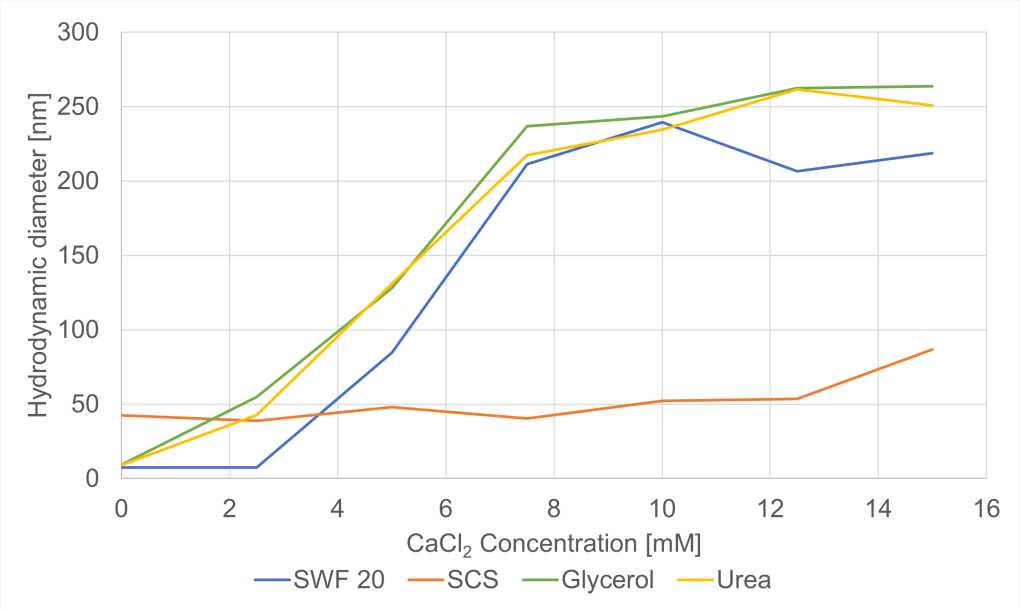


Figure 4.17: $CaCl_2$-induced aggregation in SWF 20 with addition of glycerol and urea.

The additives appeared to have no visible effect, as the SWF 20 samples containing urea respectively glycerol showed aggregation curves very similar to that of pure SWF 20. They began to aggregate at slightly lower $CaCl_2$ concentrations. Due to the lack of effect, glycerol and urea does not seem to be suitable additives, and were therefore not tested further.

5

Conclusion

Wound fluids are complex systems, where interactions between proteins and electrolytes have a particularly important role. Proteins cannot be seen as inert particles affecting viscosity or surface tension alone, but instead as complex molecules that can interact in various ways with their environment potentially changing it.

As shown from the experiments presented in Section 4.1, the formation of aggregates upon the addition of $CaCl_2$ varied between the test fluids. The SWF fluids exhibited more pronounced aggregation and formed larger particles compared to SCS 1, which showed moderate aggregation, and Peptone 20 that did not aggregate at all. This difference was linked to the spreading behavior of the fluids, where the absence of aggregation in response to $CaCl_2$ correlated with a spreading behavior more similar to that of SCS 1, and thus, to exudate. Therefore, it can be concluded that, to develop a fluid more closely mimicking the spreading behavior of exudate, it is necessary to find a way to control and limit protein aggregation within the fluid to a level comparable to the moderate aggregation seen in SCS 1.

It was further found that the electrolytes present in a fluid strongly influence the formation of aggregates. This conclusion was based on results from experiments presented in section 4.2, where salts rather than peptides were shown to be responsible for the aggregation-suppressing effect, and from section 4.3, where the roles of specific ions was further explored.

The absence of aggregate formation when one of the ions required for $CaCO_3$ precipitation was excluded, suggests that the observed aggregation was not pure BSA aggregation due to protein denaturation, but rather it could be induced by the formation of insoluble $CaCO_3$ in the solution. The $CaCO_3$ particles likely serve as nucleation points for proteins to aggregate together with the salt. However it is certain that protein is included in the particles, since the peak of non-aggregated BSA disappears upon particle formation.

In the experiment assessing different buffer concentrations within SWF, a higher concentration of $CaCl_2$ was required to initiate the aggregation when the buffer content was lower. This may indicate the existence of a threshold concentration of Ca^{2+} ions at which HCO_3^- ions begin to be released from proteins and starts to exist free in the solution. When less bicarbonate was available, the threshold increased; whereas at higher bicarbonate concentrations, the release began at lower $CaCl_2$ levels, suggesting that the equilibrium between protein-bound and free bicarbonate

in the solution was shifted.

However, certain results point towards the aggregation behavior being even more complex than this, since SCS 1 also contains the salts required for $CaCO_3$ formation, and in the experiment where SCS was diluted with SWF salts, the ion concentrations were nearly identical. Despite this, the fluid exhibited a different aggregation behavior compared to SWF. This indicates that other unknown components in the serum may interfere with or alter the effects of the salts in SCS 1.

The project has significantly deepened the understanding of the complex nature of proteins within wound fluids and identified an important and essential area for further research and development, namely protein aggregation. By managing protein aggregation within a fluid, the fluid will most likely be able to mimic exudate better, regarding its spreading patterns within a wound dressing.

6
Considerations for Future Work

Even though the results of this project have highlighted several interesting findings regarding BSA and its interactions with electrolytes in simulated wound fluids, future research is needed to further understand this complex system. As certain electrolytes, with potential to cause salt precipitation, has been detected as a key to protein aggregation, it would be of great interest to evaluate chelating agents as potential additives. Chelating agents might be able to offer a controlled gradual release of the key ions, which in turn could provide a way to regulate aggregation.

To better understand the mechanisms behind the observed aggregation, wether it is actual aggregation upon denaturation, or particle formation including salt and native-structured BSA, it could be valuable to complement the results with Circular Dichroism (CD) analysis. This technique provides detailed information about the proteins secondary structure, such as α-helices and β-sheets, and could reveal whether denaturation has occurred. This information could be valuable in the continued search for additives to better mimic the properties of exudate, as it would provide more insight into the functions that such additives should possess.

In the bigger perspective it would also be of relevance to evaluate the protein aggregation upon drying, in fluids within a real wound dressing, to ensure if additional factors present in the realistic setting further influence or alter the aggregation process. The clinically relevant FLUHTE setup offers the realistic setting for such experiments. Combined with analytical techniques, such as various microscopy methods or DLS analysis of extracted fluids, this approach could enable a more comprehensive analysis of the protein aggregation within a wound dressing.

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