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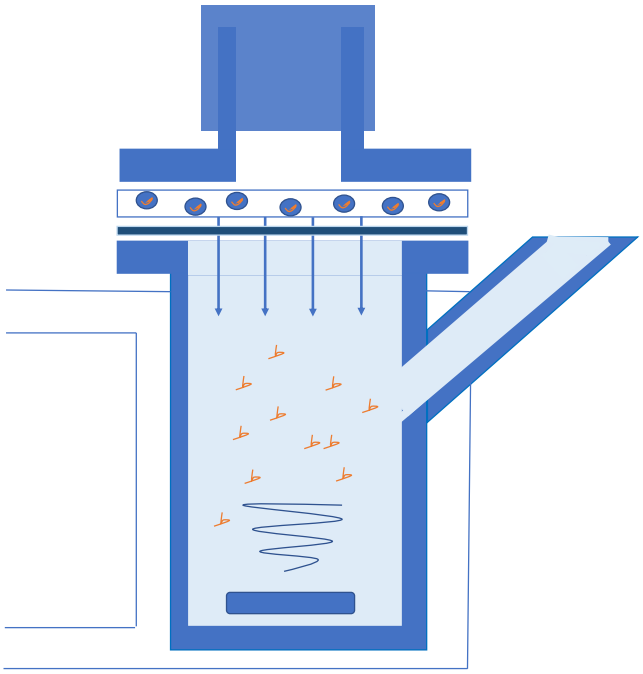

# Method development for measuring the release of antimicrobial substances from wound care dressings

## Utilizing the methodology of the Franz cell diffusion system

Master's thesis in Materials Chemistry

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Department of Chemistry and Chemical Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2019

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Cover: An overview sketch of a typical Franz cell.

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# Abstract

All over the world, there is a rapid increase in chronic diseases, such as diabetes. This is partly a result of an ageing population and a change in lifestyles. One of the most common complications of diabetes is hard-to-heal wounds such as foot ulcers. Hard-to-heal wounds are at high risk of being infected, which has led to the development of wound care dressings containing antimicrobial substances. Controlling the release rate of active substances is an important field within the pharmaceutical industry but have not been as explored in some topically applied medical device products, such as wound care dressings. By controlling the release rate of the antimicrobial substance from the wound care dressing, the wastefulness of the substance is reduced, leading to a reduced risk of bacterial resistance development. The lifetime of the wound care dressing can be prolonged as the antibacterial properties may be prepared in a protective formulation allowing for sustained release. For the development of a material with a controlled release of active substances, a reliable method for measuring the release is required. This Master's thesis project aims to develop a method utilizing the Franz cell diffusion system for measuring the release of antimicrobial substances from wound care dressings.

A method was established for the release of the antimicrobial substance silver sulfate, impregnated in a PVA based gelling fiber material, to both water and simulated wound fluid (SWF). The conclusions were drawn that the volume of water added to the material and temperature of the cells did not affect the release. It was found that the release of silver sulfate was considerably lower into simulated wound fluid (SWF) compared to pure water. The hypothesis is that the proteins in the SWF adsorb to the fabric and the silver binds with the proteins in the material, thereby hinders the diffusion to the receiving SWF.

The method was applied for release measurements of the two organic antimicrobial substances, octenidine dihydrochloride (OCT) and benzalkonium chloride (BAC) in different media. The conclusions were that the release of OCT impregnated on a non-woven cellulose material was detectable in the following receiving media; water, 20% ethanol, and 6wt% Brij. The highest release of OCT was in 20% ethanol. The organic substance BAC impregnated on a non-woven cellulose material was released entirely in water after 72 h, and the release in SWF was low but detectable.

Keywords: Franz Cell diffusion system, Release rate, Antimicrobial, Wound care dressing, Silver sulfate, Octenidine dihydrochloride (OCT), Benzalkonium chloride (BAC), Simulated wound fluid (SWF).

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Alice Flodin, Gothenburg, June 2019

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# 1
# Introduction

The world's population is increasing, living longer and changing lifestyle, which entails in an increased rate of diseases and health care costs. One disease associated with the aging population, the increase in obesity, and a more sedentary lifestyle is diabetes [1],[2]. The risk for developing hard-to-heal wounds is significantly increased for diabetes patients [3]. Unlike the majority of people, where the healing process of a wound is quite simple and fast, patients with hard-to-heal wounds are suffering from a prolonged healing process which is often associated with persistent pain [4]. One of the complications of diabetics is reduced blood flow. The reduction of blood flow increases the risk of foot ulcers, often being developed into hard-to-heal wounds, that often become infected and in the worst cases eventually, lead to amputation or mortality [5]. The increase of hard-to-heal wounds does not only impact the public health but is also a great cost for society. Only in the U.S., there is an estimated US$25 billion spent annually on the treatment of hard-to-heal wounds. Over 6.5 million patients each year are affected, and the number is continuously growing [6].

The largest concern with a hard-to-heal wound is the increased risk of possible infections. To reduce the risk of infection, wound care dressings with topical antimicrobials have been used for a long time in health care. Bacteria are a normal part of the skin flora and thereby in wounds, but there is a critical threshold of the bioburden where the bacteria might impede the wound healing. To keep the bacteria below this critical level, there are different alternatives; one is utilizing silver as an antimicrobial substance in the wound care dressings [7]. Silver has been used to treat and prevent wound infections for over 2000 years.[8].

An alternative to silver as an antimicrobial agent is to incorporate organic antimicrobial substances in the wound dressing. If a layer in the wound dressing is impregnated with an antimicrobial substance, the release rate of the substance to the wound exudate will be high. The rapid release is due to the short diffusion distance, the maximum length being the thickness of the material. The concentration of the impregnated antimicrobial substance needs to be sufficiently high to ensure substantial protection against infection in the wound. By encapsulating the antimicrobial substance in microcapsules, the wastefulness of antimicrobial substance can be reduced [9]. By doing this, the aim is to be able to control the release rate of the active substance and maintain the antimicrobial properties of the wound care dressing over a longer period of time[10]. This would extend the lifetime of the wound care dressing and limit the concentration of the active substance used [9].

## 1.1 Scope and aim

Controlling the release rate of active substances is a vast field within the pharmaceutical industry [10]. However, the area of pharmacokinetics has not been as explored in topically applied medical device products yet. Today, there is no established method for measuring the release rate of antimicrobial substances from wound care dressings. The aim of this Master's thesis project is, therefore, to develop a method for determining the release of antimicrobial substances from wound care dressings.

This project aims to investigate how to develop a reliable method that describes the release of the antimicrobial substances from the wound care dressing. The release rate will be determined by utilizing the "Franz Diffusion Cell System" [11]. To determine the concentration of active substances released into the medium, two different analysis methods will be used, and the release rate profile can be determined. Throughout the project, the most critical method parameters and considerations will be examined during the release measurements. The project will also investigate the relationships between the release profiles and depending on the use of different antimicrobial substances, different receiving media, different material, and preparation techniques.

### 1.1.1 Limitations

The project will mainly focus on method development, using the methodology of Franz diffusion cells, for measuring the release of antimicrobial substances. As one receptor medium, SFW will be used, and not actual wound exudate. This due to the variation between patients and SWF is standardized exudate, further described in section *2.4*.

Two different analysis techniques will be used for analyzing the concentration of the antimicrobial substance. For analyzing the concentration of silver, an inductively coupled plasma-optical emission spectroscopy (ICP-OES) will be used. For the organic antimicrobial substances, a liquid chromatography-mass spectrometry (LS-MS) will be used. The development of the analytic techniques will, however, be excluded from this project. In this project, the investigation of any microbial measurements or the delivery to target, i.e., wound surface will not be investigated.

# 2
# Theory

This chapter will cover the theoretical background of this Master's thesis project. It will start by introducing the theory of wound healing, both acute and hard-to-heal, then address possible treatments of wounds. The later part of the theoretical background concepts the theory regarding the release of antimicrobial substances, antimicrobial concentration levels and microcapsules.

## 2.1 Wound healing

Our skin's primary function is to act as a barrier, protecting our body, against external threats. If the barrier is compromised through an improperly healed wound, external germs such as bacteria and viruses can invade our body. This can potentially lead to critical organ damage. Therefore, making the process of wound healing crucial [12].

The biological mechanism of wound healing is a complex process and achieved through four stages: hemostasis, inflammation, proliferation, and remodeling. The steps are overlapping, but the events of each phase must occur in a precise and regulated manner. Any deviation, interruptions or prolongation may lead to a delay in the healing process and potentially ending up as a hard-to-heal wound [13].

Coagulation is needed for hemostasis and is the process where the bleeding stops, being the first phase that begins immediately after wounding [14]. The process includes vascular constriction and fibrin clot formation. The clot interacts with surrounding wound tissue and releases pro-inflammatory cytokines and different growth factors. Inflammatory cells start to migrate into the wound once the bleeding is controlled and promotes the inflammatory phase. The inflammation process is important for the removal of contaminating micro-organisms and is a normal part of the process of wound healing. However, if the decontamination of micro-organism is ineffective, the inflammation might be prolonged, leading to a more or less permanent state where the wound does not heal [13].

The proliferation phase begins as the inflammatory phase of wound healing fades out, wound contraction begins, and new tissues are rebuilt. All are essential components of the last two phases of the wound healing. The last two phases are similar to the other phases very delicate and dependent on complex biological processes. Several steps are critical, such as the balance between contraction and keratinocyte-

dependent closure; which depends on the depth, location, and inflammation of the wound [14].

During the first three weeks of the healing process, fibrillar collagen accumulates relatively quickly, but the wound has only gained about 20 percent of its ultimate tensile strength. In the last phase, the remodeling phase, the rate of accumulation of collagen is much slower, and the rate at which wounds gain tensile strength is also slow. During the remodeling phase, where the collagen is remodeling and forming bundles, the number of intermolecular cross-links is increased. However, the skin will never recover the same tensile strength as unwounded skin, it will at maximal strength reach 70 percent of its previous tensile strength [15].

### 2.1.1 Hard-to-heal wounds

Chronic wounds, also known as hard-to-heal wounds, do not proceed through the same linear progression of the four phases of wound healing as acute wounds [16]. Hard-to-heal wounds are highly individualized, and caution is needed when extrapolating theories concerning acute wounds to hard-to-heal wounds [17]. All wound types have the potential to become chronic, and a wound is defined as hard-to-heal when it has failed to heal through the normal four-phase process during what is considered to be a normal healing time [18]. For some cases of hard-to-heal wounds, different areas of the wound occur in different phases at the same time, and synchrony of the progression to the next phase is presumably lost. It is not solely the lack of progression that separates an acute from a hard-to-heal wound. A problem for diabetic ulcers seems to be the failure of timely and rapid contraction [16].

A major problem for hard-to-heal wounds is that they have a high incidence of bacterial biofilms, unlike acute wounds, which is often characterized by low bacterial burden [16]. More than two-thirds of all hard-to-heal wounds suffer from biofilm and which may lead to complications such as persistent inflammation, excessive proteolysis, and degradation of critical growth factors such as receptors and extracellular matrix [19],[16]. Biofilms are complex communities of aggregated bacteria, and mature biofilms develop protected microenvironments. Bacterias in a biofilm are more resistant to conventional antibiotic treatments. There are a lot of different types of bacteria in wounds, and for bacterial infections in wounds *Pseudomonas aeruginosa* and *Staphylococcus aureus* seems to be the most commonly occurring species [13].

## 2.2 Antimicrobial treatment of wounds

In the complex process of wound healing, many critical stages can be considered to help the wound to heal correctly, but the most common one to focus on in today's market is the prevention of possible infections. To prevent the infection in wounds, topical antimicrobials have been used empirically for a long time. It is important to note that the skin has a natural microbial flora containing benign bacteria. Although there is a proposed critical threshold of a count of $10^5$ bacteria as the line between colonization and an infection that could impede wound healing [7].

### 2.2.1 Silver as an antimicrobial substance

Silver has been used to treat wounds, skin ulcers, and burns since before medieval times and was the primary drug until the discovery of synthetic antibiotics during the Second World War. Although antibiotics have had great importance in the field of medicine, their overuse has led to a rise of resistant bacteria, making ionic silver once again the primary therapy in wound healing and burns therapy [20]. Silver, in its metal form, does not react with human tissue without being ionized into $Ag^+$. However, silver ionize readily in the presence of moisture, wound fluids and exudates. In its $Ag^+$ form it binds to proteins on cell surfaces, including bacteria and fungi [21]. The dissolution rate of silver ions increases if the silver is in a salt or nanoparticle form compared to metallic silver [22]. In today´s wound care dressings, silver is contained in a variety of forms, ranging from metallic silver in microcrystalline form to inorganic salts such as nitrate, chloride, and many more [21]. Due to the various forms of silver, the same overall concentration of two different wound dressings does not necessarily give the same antimicrobial activity because the difference in free $Ag^+$ ions and release patterns [22].

The primary antimicrobial mechanisms of silver ions are a result of the harmful interaction of the ions with the microbial cell membrane, their interference with the production of energy in the cell, and their prevention of proliferation. The silver ions can also interact with proteins and form insoluble compounds, making the proteins unusable for the bacterial cell. Several factors can reduce the activity of silver ions, such as serum and slightly higher electrolyte concentrations, especially chloride and sulfate. Free silver ions react with proteins and other elements in the wound extrude and create complexes, and the $Ag^+$ antimicrobial effect is thereby reduced in wound exudate [22]. Despite the knowledge of $Ag^+$ interaction with wound exudate most of the systematic studies about silver content and its release from wound dressings are investigated in ultrapure water or a bacterial growth medium. Further investigation of the reduction in the antibacterial effect of the wound exudate needs to be evaluated [20], [23].

### 2.2.2 Benzalkonium chloride

One class of antimicrobial substances are quarternary ammonium compounds (QACs), which are cationic detergents (surfactants or surface-active agents). One such QAC is benzalkonium chloride (BAC), which is today most commonly used as a preservative in nasal sprays, nebulizer, and ophthalmic solutions [24].

The basic structure of a QAC consists of a positively charged nitrogen with four attached groups. The structure of the four attached groups in the QAC can vary, often with some nitrogen atoms, aromatic groups, and branching of the carbon chain. The length of the carbon chains has a significant impact on the antibacterial properties, and the alkyl group lengths of 12 to 14 carbons often show the highest antimicrobial efficiency [25]. Benzalkonium chloride has a surfactant structure with positively charged nitrogen, where one of the attached alkyl groups has various even-numbered alkyl chain lengths. The remaining three groups are two methyl groups

and one aromatic ring, see BACs molecular structure in Figure 2.1 [24]. During this project benzalkonium chloride with a chain length varying between 12 (BAC-12) and 14 (BAC-14) carbons have been used.

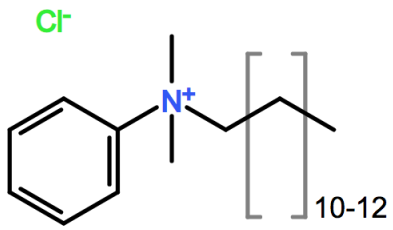


**Figure 2.1:** *Molecular structure of benzalkonium chloride*

For all the QACs there is an anionic counterion. The anion is usually chloride or bromine, and for the case of BAC, it is chloride. Many antimicrobial products contain a mixture of QACs since they can have a different effect on different bacteria. The mechanism behind QACs antimicrobial action originates from their strong interactions with phospholipid membrane. [25].

For BAC it is the cationic part that is responsible for the rapid and prolonged incorporation into cell lipid membranes. The charged part of the molecule has a very specific high-affinity to certain cell membrane proteins; most biological lipid membranes carry a weak negative charge, which influences a variety of cell processes. BAC induces cell death by two different mechanisms, apoptosis and necrosis. Depending on the concentration of BAC; the action is either very rapid at high concentrations or delayed at lower concentrations. In the skin, BAC induces activation of some cells, which in the end may lead to irritation, delayed hypersensibility and allergic reactions [24].

### 2.2.3 Octenidine dihydrochloride

Octenidine is an antiseptic that can be used topically to prevent infection, both prophylactically or therapeutically, on the skin, mucosa, and wounds. It has low toxicity, due to possible metabolites. Another important property of octenidine is that it stays active over a broad pH range (1.6-12.2). In wound care, this is advantageous since, during the healing process of the wound, the pH changes [26].

Octenidine is a surface active agent and has two cationic active centers that are separated by a long aliphatic hydrocarbon chain, see Figure 2.2. The nitrogen in each of the ionic head groups are the ones carrying the positive charge. Octenidine is usually commercially available in its salt form, where there are two negatively charged chlorides creating the dihydrochloride [26].

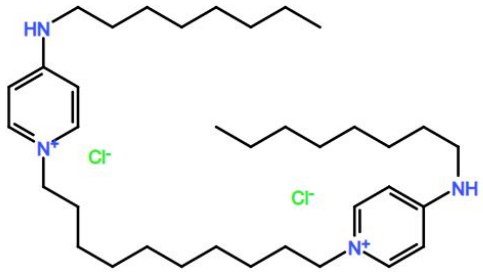


**Figure 2.2:** *Molecular structure of octenidine dihydrochloride*

With the "tail-head-spacer-head-tail" arrangement of octenidine, it resembles a gemini surfactant [27]. Unlike conventional surfactants, like benzalkonium chloride, gemini surfactants consist of amphiphile structure made up of two hydrocarbon tails and two ionic groups linked by a "spacer" [28].

Due to cationic nature of octenidine it binds readily to negatively charged surfaces. Microbial cell membrane and eukaryotic cell membranes are examples of such negatively charged surfaces, and in-vitro studies have shown that octenidine have a strong adherence to bacterial cell membrane components. The unspecific mode of action combined with the strong absorption and interactions to cell walls and cell membrane structures gives octenidine a broad antimicrobial spectrum. It ranges from Gram-positive to Gram-negative bacteria and also includes plaque-forming bacteria and fungi [26]. The antibacterial effect of octenidine does not seem to be comprised of interfering substances like albumin or mucin [29]. Octenidine has the potential to gradually replace conventional older antiseptics like triclosan, PVP-iodine, or chlorhexidine due to its superior safety and biocompatibility properties [30].

### 2.2.4 Surface active antimicrobials

Both benzalkonium chloride (BAC) and octenidine dihydrochloride (OCT) are surfactants, but the structure of OCT most likely gives it more gemini surfactant-like properties while BAC behaves more like a conventional surfactant. A surfactant efficiently lowers the surface tension in the solution until the critical micelle concentration (CMC) is reached. Then the individual surfactants start to self-associate into complex structures, such as micelles, bilayer, and vesicles. The structure of gemini surfactants does, however, give the aggregates some other features compared to conventional surfactants. Conventional surfactants form spherical aggregates (Figure 2.3a), whereas the aggregates of a gemini molecules usually form thread-like (Figure 2.3b) or rod-like structures (Figure 2.3c), see Figure 2.3 for all micelle structures [28].

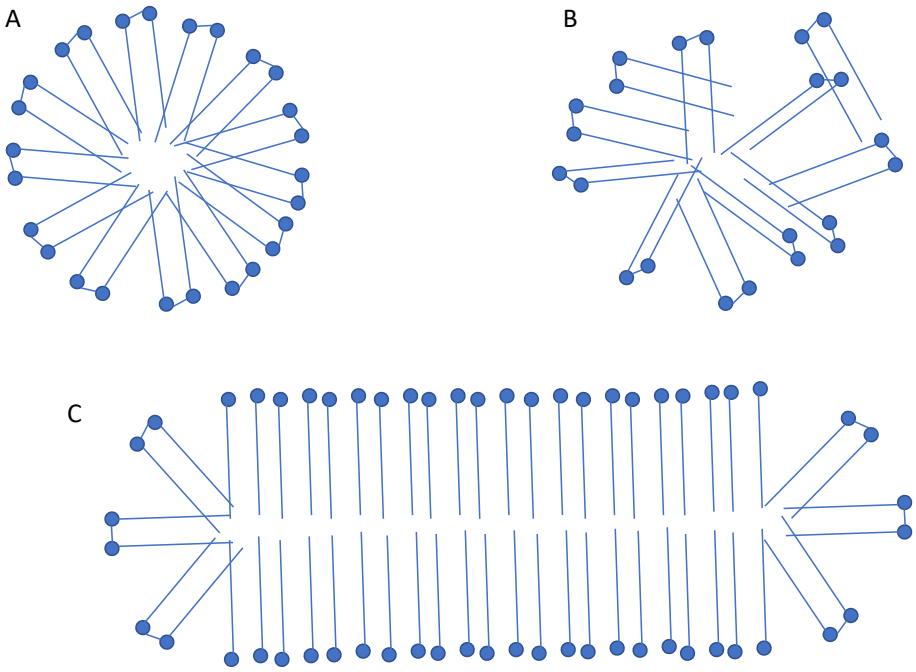


**Figure 2.3:** *Diagram of a) spherical micelle, b) thread-like entangled micelle, c) rod-like micelle*

The gemini surfactants with short hydrophobic spacer usually form the thread-like structure and gemini surfactants with a spacer length comparable to the tail length, usually form rod-like micelles. The long spacer shields the repulsion of the two cationic head groups and favors micelle formation that continues along a single dimension resulting in cylindrical rods. The reduced repulsion, in combination with the increase in the number of hydrocarbons, usually gives the gemini structure a significantly lower CMC compared to conventional surfactants with equivalent chain length [28]. However, the CMC for octenidine has been reported a value of 3.79 mM by monitoring conductivity. Which is not really consider as significantly lower compared to conventional surfactants and the theory regarding the gemini structure giving a low CMC might not apply to octenidine [27]. Due to the double hydrophobic head groups in the gemini structure, they are also more disruptive than individual chains in conventional surfactants. This results in that gemini structures are generally superior in surface activity, and a lower concentration of the gemini surfactants are usually needed to achieve the same surface tension reduction as for conventional surfactants [28].

## 2.3 Antimicrobial substances in wound dressings

The rapid development of antibiotic resistance is one of today's most profound health care issues, and it is the result of overuse and misuse of antibiotics in both human and veterinary medicine as well as agriculture. A critical aspect for the development of new resistant mutants, as well as for the enrichment of pre-existing resistant bacteria, is the use of low antibiotic concentrations. If the concentration of antibiotic is not high enough to kill the bacteria's in the wound, some of the bacteria that survive start to develop a tolerance and may develop resistance [31]. The wastefulness and overuse of antimicrobial substance can be reduced by controlling the release.

The minimal bactericidal concentration (MBC), is the minimum concentration of an antimicrobial substance needed to kill a type kill a particular bacterium and varies for different sorts of bacteria. The minimal inhibitory concentration (MIC) is also essential and defines as the concentration needed of an antimicrobial substance to inhibit the visible growth of a microorganism after overnight incubation [32].

There are standardized methods for determining both MIC and MBC. MIC is used as a facile research tool in the laboratory's to determine the *in-vitro* activity of new antimicrobials, but mainly to confirm the resistance of the antimicrobial substances. MBC is not as frequently used in the field of research but also a valuable tool [32]. In Table 2.1 MIC and MBC values of the antibacterial substances benzalkonium chloride and octenidine against two of the most commonly occurring bacteria's in wounds; *S. aureus* (SA) and *P.aeruginosa* (PA) are presented.

**Table 2.1:** *Table with MIC and MBC values for different antimicrobial substances against S. aureus (SA) and P.aeruginosa (PA)*

| **Substance** | **Benzalkonium (BAC)** | **Octenidine (OCT) [33]** |
|---|---|---|
| **MIC-SA/PA [mg/cm$^2$]** | 2.237/1.409 | 0.021/1.16 |
| **MBC-SA/PA [mg/cm$^2$]** | 7/4.5 | 3.107 - 6.215 |

The values in Table 2.1 was calculated based on the volume 11 ml of the Franz cell and the contact area between the material containing the active substance and the receiving medium. The ratio between MIC:MBC for BAC against SA, may vary between 1:1 and 1:8, but several test procedures and articles support that the ratio is close to 1:3, especially for complex medias [34],[35],[36],[37].

The release rate of the antimicrobial substances is of equal importance as the concentration to achieve an effective antimicrobial response. Figure 2.4 displays three graphs, where graph a) illustrates a rapid release of the antimicrobial substance. The MBC value will be reached if the concentration also is high enough; however, a more sustained release where the antibacterial substance inhibits the growth of bacteria's (MIC) will not last long. Graph b) illustrates a slower release of the active substance, that enables the antimicrobial substance to obtain the properties of the wound care dressing for a longer time.

However, by not reaching MBC the bacteria's might develop resistance towards the antimicrobial substance and thereby change the MIC. The optimal release is, therefore, a combination of both graph a) and b), which are illustrated in graph c). The most common release profile for antibacterial substance is the rapid release in graph a), and can usually be achieved by impregnating the antibacterial substances on the material. The sustained release in graph b) is harder to achieve. For the antibacterial substance to have the release curve in b) the release of the substance from the material needs to be controlled. How to control the release is further explained in the following section, 2.3.1.

The curves in Figure 2.4 is, however, for a dynamic system, with continuous transportation of liquid, like the exudate in a wound. In this project, all the release measurements were performed during static conditions, the release curve presented in this report will therefore mainly resemble curve b) but with different inclinations. The aim with the combination of rapid and slower release is to be able to reach the MBC value fast and decrease the risk of resistance, reduce the waste and also prolong the antimicrobial effect of the wound dressing. By controlling the release rate of the antimicrobial substance, the unnecessary use of antimicrobial substances is reduced, and the wound dressing lasts longer.

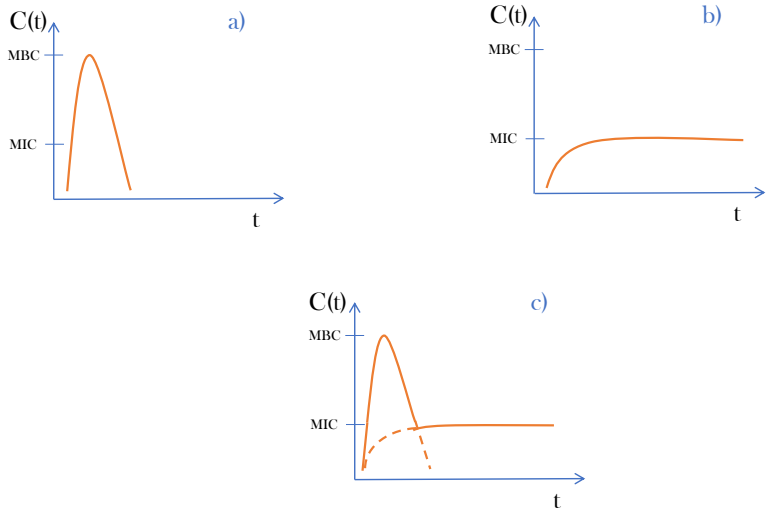


**Figure 2.4:** *Curves of the available antimicrobial substance concentration in the wound as a function of time. Graph a) illustrates a rapid release, graph b) illustrates a slower release. Graph c) is a combination of graph a) and b).*

### 2.3.1 Microcapsules

A common method to obtain a controlled release of an active ingredient over a prolonged period of time is to encapsulate the active ingredient in reservoirs, called microcapsules [9]. There are different types of microcapsules that are classified based on their size and morphology, ranging from 0.1 - 100 µm. The microcapsules consist of a core where the active substances are dissolved, or dispersed, and the core is surrounded by a shell material. It is important that the core material is inert toward the active substances, and that the shell material sustain the core material [38]. Using the microcapsules in wound care dressings, it is also of importance that the material used both for the core and shell is biocompatible.

The morphology of the microcapsules mainly depends on the distribution of the core material. The three basic morphologies of microcapsules are; mononuclear (also known as core-shell) where the shell surrounds the single core. Polynuclear capsules have many cores enclosed within the shell [38]. In a microcapsule of matrix type, the core material is evenly distributed within the shell matrix. Matrix type also include microspheres, which are particles without core material, i.e. only consists of polymer and molecularly dissolved active substance in the polymer shell matrix. [39]. See Figure 2.5 for an overview of the different microcapsules types; core-shell, polynuclear, and matrix form [38].

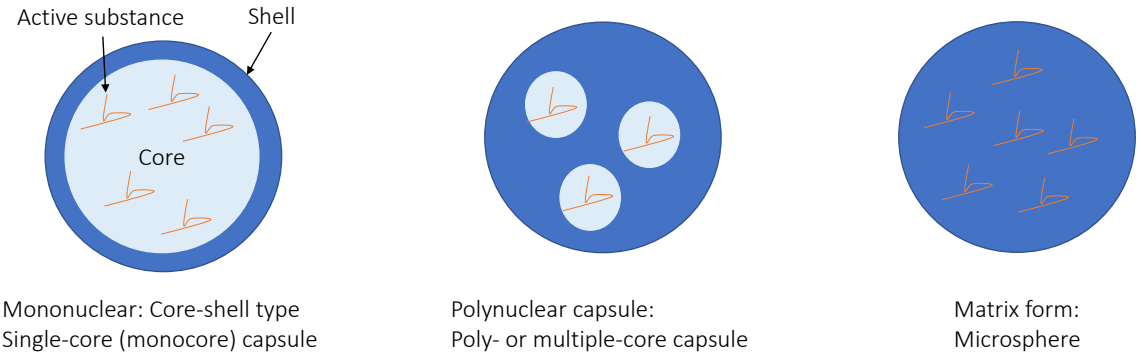


**Figure 2.5:** *Capsules of core-shell type, polynuclear and matrix form*

A variety of release mechanisms from microcapsules have been described in the literature. The microcapsule can be designed to have different release profiles, often either triggered release or sustained release. Triggered release means using a polymer or other material that is destroyed or changed due to external stimuli. The external stimuli can vary of different indicators like; mechanical/shear, temperature, pH, UV-radiation, etc. [40]. For polymers that are inert during the release process, the active substance slowly diffuses through the polymer and then into the surrounding, often aqueous, phase, resulting in a sustained release [9]. However, the size and the wall thickness of the microcapsules often varies, and so does the release rate. The release rate behavior also differs between microcapsules and microspheres, and the classification plays a crucial role [39]. That is why it is essential to examine the release rate from the microcapsules and microspheres on an experimental basis [38].

### 2.3.2 Release of antimicrobial substances

The release antimicrobial substances from both coating films and microspheres to surrounding medium in this project, aims to follow a diffusional transport. If the coating film, or thin material, is considered as plane sheets with homogenously embedded active substance (either molecularly free or in microspheres) and perfect sink conditions assumed, the fractional part of the released amount active substance can be expressed according to Crank as the function 2.1. The fractional part of the released amount active substance is function 2.1 expressed as a function of time t, effective diffusion coefficient, D and the coating thickness L. [41]

$$\frac{m(t)}{m_{tot}} = f(D, L, t) = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} exp\left\{ -\frac{D\pi^2(2n+1)^2}{4L^2} t \right\} \tag{2.1}$$

## 2.4 Simulated Wound Fluid

Wound exudate is defined as the liquid produced from wounds and plays an essential role in the wound healing process. The exudate provides the moist environment required for healing, and it contains vital nutrients for epithelial cells and facilitates the ingress of white blood cells. During the healing process, the most exudate is produced during the inflammatory and proliferative stages. The protein content in exudate is high, but it also contains electrolytes, glucose, cytokines, leukocytes, metalloproteinases, macrophages, and micro-organisms [42].

The composition of the exudate is highly individual, and the difference in both hard-to-heal and acute wounds can vary significantly. It is, however, generally accepted that the protein content of exudates for both hard-to-heal and acute wound is up to 50% of the total protein level of serum, whereas the electrolyte concentration is the same as in serum [43],[44]. Some studies have indicated that proteins in the wound exudate, such as albumin, reduces the antibacterial efficacy of wound antiseptics against certain bacteria [45]. A previously Master thesis project also showed that serum albumin, electrolytes, and glucose, which are all elements in wound fluid, influences the dissolution behavior of silver [23]. Evaluations of silver released from silver-containing wound dressings have also indicated that the complexity of the medium affects the release. Depending on the silver form in the wound dressing, the release might be affected differently [20].

Simulated wound fluid (SWF) was the test medium used in this project to mimic wound exudate. SWF consists of equal proportions of fetal bovine serum (FBS) and Peptone Water (PW); the mixture corresponds to the protein and electrolyte concentrations of wound exudate.

# 3
# Materials and Methods

For this diploma work, the key method is the Franz cell diffusion system, and it is described in detail in section *3.2*. The analytical techniques used for silver was inductively coupled plasma-optical emission spectroscopy (ICP-OES) and for the organic compounds liquid chromatography-mass spectrometry (LC-MS). The materials containing the antimicrobial substance and the release measurements are also described in this section.

## 3.1 Chemicals and Equipment

The following chemicals was used through out the experimental work of the project.

- *Acetone*, $\geq$ 99.8%, Fisher Chemical (CAS: 67-64-1)
- *Benzalkonium chloride (BAC)*, Sigma-Aldrich (CAS: 63449-41-2)
- *Brij L23*, Sigma-Aldrich (CAS: 9002-92-0)
- *Octenidine dihydrochloride (OCT)*, 98%, Alfa Aesar, (CAS: 70775-75-6)
- *Distilled water*, 15 Ωm, Milli-Q®Integral ultrapure water (Type 2), Merck
- *Ethanol*, $\geq$ 99.8%, Fisher Chemical, (CAS: 64-17-5)
- *Ethanol*, 70% denatured with isopropyl alcohol, Solveco (CAS: 64-17-15)
- *Simulated wound fluid (SWF)*, Batch: 614, 989, 1034, Bakteriologiska laboratoriet, Sahlgrenska Hospital, Gothenburg, Sweden.

The simulated wound fluid (SWF) was handled in a aspetic manner, only using sterilized equipment, and the lab bench and gloves were submerged in excess of isopropanol. When handling the 99% ethanol and acetone, which are flammable, this was performed inside the fume hood. Furthermore, the organic compound benzalkonium chloride (BAC) is toxic and was therefore handled inside the fume hood as much as possible. For extra safety precautions, the octenidine dihydrochloride (OCT) was handled in the same manner. The following material and equipment was used during the project:

- *Franz cells*: Hanson Research vertical diffusion cell, 6-cell manual diffusion.
- *Membrane filter*: Supor®-450, 47mm 0.45 $\mu$m. PALL Corporation.
- *Syringes*: Omnifix®-F, 0.01 ml/1.0 ml. B.Braun Melsungen AG.
- *Needles*: 100 Sterican®, $\phi$ 0.8 mm x 120 mm. B.Braun Melsungen AG.
- *Filters*: Minisart®NY25 Syringe Filter 17845, 0.2 $\mu$m, Sartorius.
- *ICP-OES*: iCAP 6000 series, Thermo scientific.
- *LC-MS*: 6100 Series SQ LC/MS, Agilent.

## 3.2 Franz cell diffusion system

Franz cell diffusion system is one of the most common *in-vitro* release testing (IVRT) methods for measuring the drug release of semisolid drug products [46]. Compared to other *in-vitro* release testing methods, the advantage with the Franz cell diffusion system is that the mass transport is purely diffusion-driven, and the volume of the cell is appropriate for analytical measurements. The Franz cell diffusion system employs an open chamber design; a schematic overview can be seen in Figure 3.1.

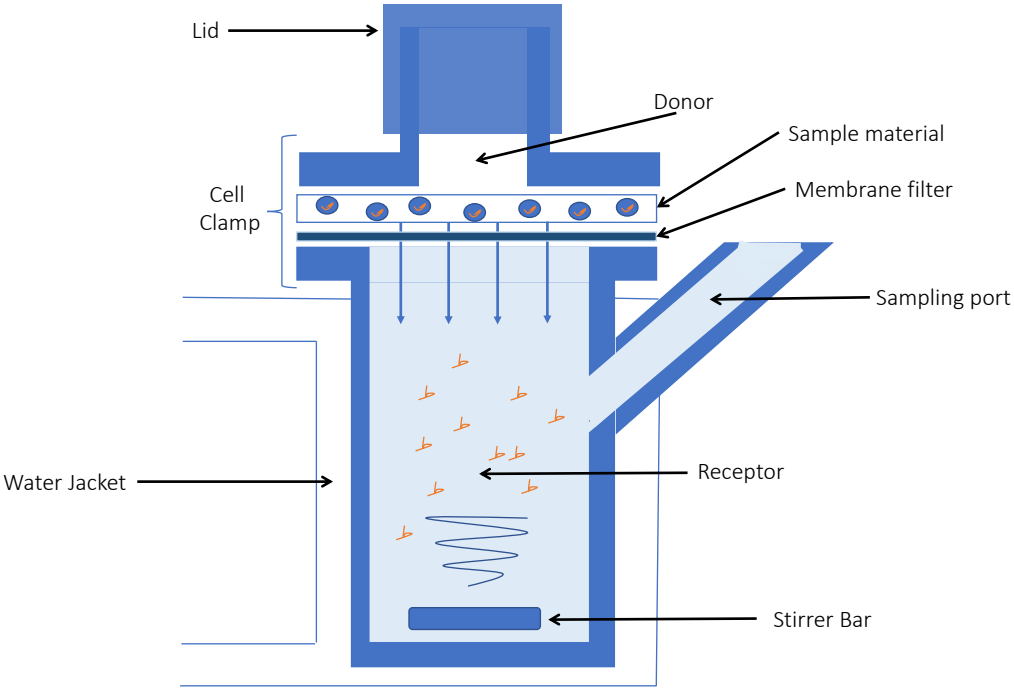


**Figure 3.1:** *An overview sketch of a typical Franz cell*

The Franz cell consists of one donor compartment and one receptor compartment, where the receptor compartment is filled with a receiving medium. These vertical diffusion cells are commonly used for evaluating topical semisolid drug products such as creams, gels, and ointments. The donor department is then filled with the semisolid drug product, and a piece of skin is placed between the two departments to evaluate the penetrating properties and the release rate [46]. However, this project is not about assessing semisolid product like a cream. This project is about measuring the release from solid material containing an antimicrobial substance in a wound care dressing, and therefore will the donor department be empty.

The two compartments are separated by a semipermeable membrane which does not have to be of biological sample origin. It can be a synthetic membrane or a tissue construct. In this project, the separation of the two compartments consisted of the sample material and a membrane filter with a size of $\phi$ 36 mm. The pore size of the membrane filter used allowed for the permeation of both proteins and the active substances. The use of the membrane filter eliminates any influence of

convection. This enables all flow of the active substance to be purely driven by diffusion [11]. A cell clamp ensured that the sample material and membrane filter remains in place during the tests [46]. The contact area between the sample material and membrane filter to the receptor department is the size of $\phi$ 15 mm, and the volume of the receptor department is 11 ml. To assure that the saturation of the receiving medium does not affect the release of the substances, a sink-condition needs to be fulfilled. There is a general agreement for *in-vitro* experiments that to satisfy the sink condition that the maximum concentration of the substance in the Franz cell needs to be less than 10% of the maximum solubility of the substance in the medium [11].

To measure the release of the antibacterial substance in the receptor medium, samples were sequentially collected from the receptor medium, for subsequent analysis. The samples from the receptor department can either be sampled by removing the lid, sample and filter to collect a sample from the receptor compartment. This sampling technique does, however, limit the measurements to one sampling opportunity per sample. The alternative sampling technique is to use a needle and syringe to sample directly in the Franz cell through the sampling port. After each sampling, the receptor compartment was refilled with fresh receptor medium to assure the surface contact between the sample and the medium. This enables several measurements at a different time from the same sample. The concentration from each sampling time needs to be recalculated to compensate for the dilution factor of adding fresh medium, see Appendix A for equations. The temperature of the cells can be kept constant by connecting the water-jacket cells to a thermostatically controlled water bath. Each cell also contains a stirrer bar and an Helix$^{TM}$ stirrer to enable an even concentration distribution; all cells were placed on a magnetic stirrer plate [11]. During every experiment in this project, the magnetic stirrer was set to 550 rpm and there where six identical cells on the magnetic stirrer plate.

## 3.3 Materials

During the development of the method for measuring the release of active substances, different materials containing active substance and receiving media were evaluated. The properties of the materials will be presented in the following sections.

### 3.3.1 PVA based gelling fiber silver containing material

For the measurements of the release of the silver ions, polyvinyl alcohol (PVA) based gelling fiber material, containing silver sulfate, was used. The material was provided by Mölnlycke Health Care and contained approximately 0.2 mgAg/cm$^2$. The material is highly absorbing, and the gelling properties of the material entail in a change in volume and area when in contact with a fluid, see illustrating Figure 3.2. Due to its absorbing properties and to assure contact with the receiving medium, water was added on top of the samples after being placed on the Franz cells.

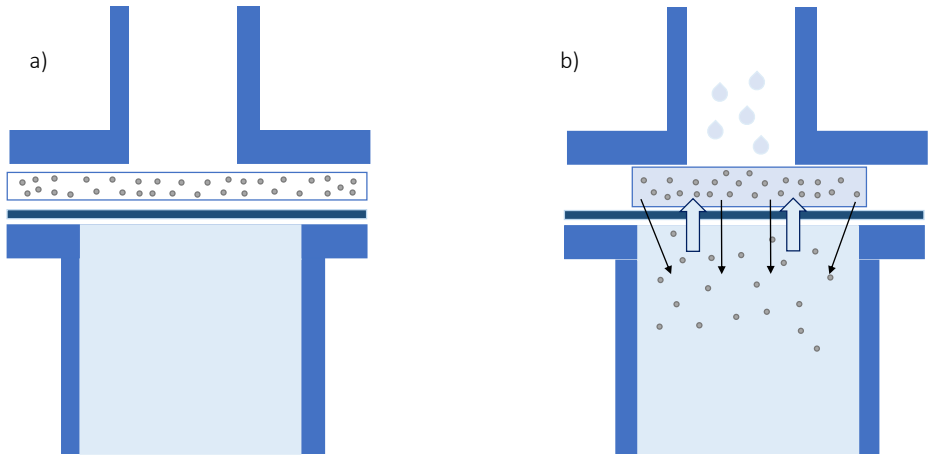


**Figure 3.2:** *Gelling properties of the PVA based gelling fiber silver containing material. Figure a), to the left, illustrates the material in its dry state. The right figure, b), illustrates the material after absorbing fluid. For this state the volume, and thereby, area of the material have changed.*

During all experiments, the size of each sample of the PVA-based fiber material was punched to an exact size of $\phi$ 32 mm in the dry state. The samples where punched randomly from all over the bigger sheet of the material. Due to the absorbing properties of the material, all the silver in the material can diffuse through the material to the receiving media. Therefore, the calculation of the total amount of silver available was based on the total dry area of the sample, and not the contact area between the material and receptor chamber. The maximum concentration of silver in the Franz cells was, therefore, assumed to be 0.146 mg/ml. The solubility of silver in water is 8.3 mg/ml (25 °C) [47], which means the sink conditions of less than 10% of the maximum solubility are to release in the Franz cells are fulfilled.

### 3.3.2 Cellulose fiber material

In addition to the PVA-based hydrogels containing silver sulfate, antiseptic-functionalized cellulose non-wovens were also evaluated. The cellulose non-wovens were functionalized with the antiseptics using either impregnation or by incorporating antiseptic-containing microspheres as described below.

The cellulose fiber material used during the experiments was manufactured by dissolving the cellulose in the ionic liquid: 1-ethyl-3-methylimidazolium acetate. The solution, called dope solution, was then by air force pressured through a nozzle with nine small pipes onto a rotating cylinder. The cylinder was placed in a water bath,

and when the dope solution reached the water surface, the ion solution dissolves in the water. The cellulose then precipitated on the cylinder, leaving a non-woven fiber on the cylinder. The cellulose fiber is then removed, washed in a water bath and then dried.

The cellulose samples used in this project were punched to a size of $\phi$ 26 mm and was weighed before any experiment, due to the inherent inhomogeneity of the cellulose material. For the cellulose sample that was impregnated with the organic substances, an impregnation solution with the substance dissolved in ethanol was prepared and then added to the cellulose. For the samples where the antimicrobial substances were added in the manufacturing process of the cellulose fiber, two different approaches were used. Samples containing OCT in microspheres were prepared by mixing a suspension with the octenidine encapsulated in microspheres with the dope solution. The microspheres consisted of PLLA (poly-L-lactide) and PLGA (poly(lactic-co-glycolic acid)). When producing the cellulose fiber material containing BAC, the antimicrobial substance was not encapsulated in microspheres, and therefore directly incorporated in the fibers. Although the cellulose fiber does not seem to have the same volume and area changing properties as the PVA based gelling fiber silver containing material, the calculation of the total amount of organic substance was based on the whole area of the sample due to the absorbing capacity of the cellulose material. Hence, contributing to the dissolution of the active substance in the entire sample piece.

## 3.4 Silver release measurements

For the development of a robust method utilizing the methodology of Franz cells, it is advantageous to start with a few sources of uncertainty as possible. By evaluate one new parameter at a time, the effect of that parameter can be examine without interference of other parameters. The starting point in this project was, therefore, to measure the release of active substances from a material with a known active substance content, and high solubility in the receiving medium. As described in section *3.3.1*, silver release in water fulfilled the sink conditions in the Franz cell, and the total available amount of silver in the PVA based gelling fiber silver-containing material was known. The release measurement of active substances from different materials utilizing the methodology of Franz cells was therefore developed based on the release of silver from the PVA based gelling fiber silver-contain material in water.

The first parameter evaluated in the method development was how the absorbing properties of the material affected the release. Comparing the difference in adding water of the approximately max absorption volume of the punched out circular pieces of the material (1 ml), and half of the max absorption volume (0.5 ml), this was made to assure contact between the water surface and the sample. The membrane filter placed between was also soaked in water before placing in the Franz cell to ensure the contact. This was made for all measurements. The release of silver in water was measured by removing the Franz cell from the magnetic stirrer plate and sampling 100 $\mu$l from the receptor medium by removing the lid, sample,

and membrane filter. The 100 $\mu$l of the sample was placed in 15 ml polypropylene tube. With this sampling technique, the silver concentrations measured at different sampling times were all from individual PVA based gelling fiber silver containing material samples. The sampling times for the release of silver in water was 0.5 h, 1h, 6h, and 24 h, and the samples were then stored in the fridge before analyzed with ICP-OES. For the measurements of released silver in water from the silver-containing material wet with 1 ml of water, an analysis at 48 h was also done with the same sampling technique.

The Franz cells can be heated by water to retain a constant temperature, connecting the water jacks of each cell in series and then to a water pump with heating. The temperature when evaluating the antimicrobial properties of the substances was executed at approximately 35 °C, to mimic the temperature the skin surface where the temperature usually varies between 30.7 °C and 38.6 °C [48]. The thermometer of the water bath was set to 35 °C, but due to heat losses, the temperature in the Franz cells was about 32 °C. The release of silver to water in room temperature was compared to the release of silver in the water at skin surface temperature.

Simulated wound fluid is a more complex media than water, but more accurately mimics the real environment of the wound dressing. The measurement was made with the same parameters as for the release measurements of silver in water. Heating of the cells with the water bath temperature at 35 °C and the maximum absorption volume of 1 ml water was added on top of the PVA based gelling fiber silver-containing material. The difference in the release of silver in the two mediums was compared. Water being the more ideal media for the release of silver ions due to the high solubility of silver sulfate, making it a suitable media for reference measurements when performing SWF measurements.

In order to optimize the method of measuring the release of active substances with the Franz cell diffusion system, the individual cells were sampled multiple times. This allowed for measurements from a single cell during the whole measurement time and reduced the workload. The first sampling technique used in this project were sampled by removing the entire cell and sampling 100 $\mu$l from the receptor chamber. Thereby is the concentration from each sampling time from an individual sample, since the material with the active substance had to be removed in order to collect a sample. By instead sampling through the sampling port each sampling time, the measurements were from the same sample at all sampling times. At each time sampling of 200 $\mu$l was made from the sampling port, a replenishment of 200 $\mu$l fresh medium was added to the Franz cell. The replacement is necessary since it was crucial for the surface of the receiving media in the receptor chamber to be in contact with the sample. This was repeated five times during the whole measurement time. During the measurements of sampling directly in the cells, three of the cells contained the receiving media water, and the three other cells contained SWF. The samplings were made at 0.5 h, 1h, 6h, 24h, and 48h after adding the first sample on the Franz cells. The PVA based gelling fiber silver-contain material samples were all wet with 1 ml of water. The cells were heated with the water bath temperature

set to 35 °C, however, the heating system for the Franz cells, unfortunately, broke after 30 h of heating making the last sampling at 48 h at room temperature.

To assure the accuracy of the method utilizing the Franz cell diffusion system for measuring the release, the results from the experimental measurements was adapted to the mathematical model described in section *2.3.2*. The results from the release of silver in both water and SWF was adapted. During this project the thickness of the material, L, was consider to be equal for all the measurements of the silver release.

## 3.5 Organic antimicrobial release measurements

The same method used for measuring the release of silver was evaluated for measuring the release of the antimicrobial organic substances; benzalkonium chloride (BAC) and octenidine dihydrochloride (OCT) in different media. To have control over the exact amount of substance available for release into the medium, the first step was the impregnate the cellulose material with a known concentration of BAC and OCT. For this, the solubility of OCT and BAC in different solvents was evaluated. The solubility tests also included the investigation of the sink conditions. To fulfill the condition, the maximum concentration in the cell needs to be less than one-tenth of the saturation concentration. The solubility test was performed by adding the amount of substance in an Eppendorf tube and adding 1 ml of the solvent, then mixing with a vortex overnight and determine by eye if the substance is solved. For the solubility test in SWF for both of the organic substances, was evaluated both in directly in SWF and by filtering the SWF with a Minisart®Filter 0.2 $\mu$m filter to remove any preexisting small white particles in the SWF. The filtering of the SWF was made due to the small white particles included in the SWF matrix that made it hard to determine, after adding the organic substance, if the small particles was a preexisting part of the SWF matrix, or insoluble organic substances.

The cellulose material was impregnated by first preparing an impregnation solution with the organic substance dissolved in ethanol. The concentrations that were infused were intended to be as close to MBC as possible. After weighing the $\phi$ 26 mm sized cellulose material, the samples were placed in individual dishes and the ethanol solution was with an automatic pipette then carefully added in the middle of the piece. Depending on the weight of the sample, the volume and the concentration of the ethanol solution was adjusted, to achieve as homogeneous samples as possible. The amounts ranged from 0.1 to 0.4 ml. The samples were then dried overnight in a container with solvent-absorbing pellets.

Suitable receiving media for the two organic substances were evaluated by placing the impregnated cellulose sample on the Franz cells containing different media. The receiving medias evaluated were water and SWF for BAC and for OCT the evaluation also included 20% ethanol in water and 6wt% Brij in water. The membrane filter was soaked in water before placing the impregnated samples, with the side where the impregnation solution had been added, down towards the receiving

medium. The second sampling technique used was the 200 $\mu$l sampling was made through the sampling port. For SWF as receiving medium, the samples were placed in Eppendorf tubes, and 100 $\mu$l of the sample was with the help of an automatic pipette placed in a new Eppendorf tube in order to be analyzed. For the samples where the receiving medium was another solvent then SWF the 200 $\mu$l of the sample was also placed in Eppendorf tubes, but 100 $\mu$l of those was with the help of an automatic pipette placed in an LC-MS vial with an insert. The samples were stored in the fridge before analyzing with LC-MS.

A comparison of the release of the organic substances for the materials where the active substance had been added during the manufacturing, was performed. The receiving medium for these samples was chosen based on the results from the measurements of the impregnated BAC and OCT cellulose. For both substances SWF was chosen as one of the media due to the high resembles the real wound environment. The other receiving medium for the BAC measurements was water, due to the excellent solubility of BAC in water. For the OCT measurements, the other receiving medium chosen was 20% ethanol, since it was the media where the samples with impregnated OCT had the highest release fraction. The membrane filter was for these measurements also soaked in water before placing on the Franz cells. All the samples were weighed before placed in the Franz cells, and the sampling technique with the sampling port was used. The samples were prepared in Eppendorf tubes and LC-MS vials in the same manner as for the impregnated cellulose samples.

## 3.6 Analytical techniques

For the analysis of the concentration of the antibacterial substance released to the receptor media, two different analytic technique was used. For analyzing the concentration of silver, the analytical technique Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) was utilized. ICP-OES measures silver in all forms in the sample matrix, and the method used is validated for the measurement of silver in both water and SWF.

When measuring the concentration of the antimicrobial organic substances, the analytic technique of Liquid Chromatography-Mass Spectrometry (LC-MS) was utilized. LC-MS is not an established analytical tool for measuring organic antimicrobials and have to be validated for each substance in each matrix. Therefore, the experimental design for this measurement technique had to be developed within the frames of this Master thesis. The whole process from preparation of the samples to the evaluation of the results will, therefore, be described in a more detail manner compared to the ICP-OES technique.

### 3.6.1 Inductively Coupled Plasma-Optical Emission Spectroscopy

To determine the concentration of silver in a solution, the ICP utilizes inductively coupled plasma to ionize the sample [49]. The plasma is an ionized gas that contains several ions, then the inorganic analysts in the form of an aerosol are introduced into the plasma. Some of the atoms in the plasma will become ionized. The plasma can then excite an electron in the inorganic atoms or ions to a higher energy level. These excited electrons then relax back to their initial "ground" state, and the corresponding energy is emitted. This energy has a specific wavelength that is characteristic for each substance and can be detected with optical emission spectroscopy (OES). Thereby quantifying the concentration of the inorganic substance [50].

A preparation procedure of the samples was performed to digest the sample, and ensure that the silver is in solution. This was done by adding 9 ml of a dilution solution to the 100 $\mu$l of the sample in the 15 ml polypropylene tube. The dilution solution was prepared by adding approximately 1000 ml water in a 2000 ml volumetric flask and then adding 600 ml hydrochloric acid (32%) and 200 ml nitric acid (65%). 200 $\mu$l of yttrium standard (1000 mg/l) was added, and the flask was filled with water to the volume of 2000 ml.

### 3.6.2 Liquid Chromatography–Mass Spectrometry

The liquid chromatography is a technique for separation of components based on their physical properties. It is possible to identify and quantify different elements from a mixture by coupling the chromatography column to a mass spectrometer [51]. The physical separation of the components in a liquid mixture is based on that the mixture is distributed between two immiscible phases, called the stationary and the mobile phase. There are many variations in the procedures in the LC, but the most common one today is reversed-phase chromatography and accounts for about 80% of all LC modes and is also used in this project [52]. In the reverse phase mode, the stationary phase is non-polar (hydrophobic), and the mobile phase is polar [53]. In this project, the mobile phase was a gradient of two eluents. For the analysis of OCT, the elutants was A and B, and the eluents gradient can be view in Table 3.1.

A = $H_2O$ + 1 M ammonium formate + formic acid
B = MeOH + 1 M ammonium formate + formic acid

**Table 3.1:** *Eluents gradient for analyzing OCT with LC-MS*

| **Time [min]** | **A [%]** | **B [%]** |
|---|---|---|
| 0 | 75 | 25 |
| 2.5 | 20 | 80 |
| 7 | 0 | 100 |
| 8 | 0 | 100 |
| 8.10 | 75 | 25 |
| 10 | 75 | 25 |

For the analysis of BAC with LC-MS, the eluents are A and C, and the gradient of the eluents can be view in Table 3.2.

A = $H_2O$ + 1 M ammonium formate + formic acid
C = ACN

**Table 3.2:** *Eluents gradient when analyzing BAC with LC-MS*

| **Time [min]** | **A [%]** | **C [%]** |
|---|---|---|
| 0 | 95 | 5 |
| 1 | 10 | 90 |
| 7 | 0 | 100 |
| 8 | 0 | 100 |
| 8.20 | 20 | 80 |
| 9 | 95 | 5 |
| 10 | 95 | 5 |

The stationary matrix is long-chained alkyl groups that are attached to the inside surface of the column. The column used in this project was a *Poroshell 120EC-C18 (4.6x100mm, 2.7 $\mu$m)* and the guard column is a *InfinityLab Poroshell 120 EC-C18 (4.6x5mm, 2.7 $\mu$m).* The temperature of the column was 50°C for the OCT and 40°C for the BAC. The injection volume for analyzing OCT was 3 $\mu$l, and for BAC, it was 5 $\mu$l. The sample is injected into the mobile phase stream; the high-pressure pump produces the stream, and the flow for analyzing both OCT and BAC is 0.8 ml/min.

The mobile phase containing the sample is pumped through the column, and depending on the hydrophobicity of the molecule, the chemical affinity to the stationary phase will be different. The analytes with the highest affinity to the mobile phase will elute out of the column first. The molecules with higher affinity to the stationary phase will have a longer retention time and therefore elute after the more hydrophilic molecule. The analytes are thereby separated based on their hydrophobicity. By placing a detector after the column, the analytes can be identified by the retention time and then quantified [54]. For this project, the detector was a mass

spectrometer. By converting individual compounds and atoms to charged ions, the mass spectrometer measures the molecular masses of substances. The mass spectrometry can, therefore, provide quantitative information of analytes at a level of structure specificity [53]. The fragmentor voltage for quantifying the BAC samples in this project was 180V, and for detecting the OCT samples, the fragmentor voltage was 100V.

#### 3.6.2.1 Calibration curves

To quantify the concentration of the sample, a calibration curve needs to be established. The calibration curve is a linear curve with known concentrations of the sample in the same solvent mixture, or matrix, as the sample with an unknown concentration; by using the same matrix, errors caused by the matrix can be avoided. The information received from the detector is the integrated area from the mass spectrometer signal and retention time of the different analytes. With the calibration curve, the unknown concentrations of the samples can be calculated from the integrated area received from the detector. The calibration curve concentrations were prepared slightly different for the OCT and the BAC samples.

The OCT calibration curves were made in the following matrices; 20% ethanol in water, water:methanol (1:1) and SWF. A first stock solution was prepared with a concentration of 1 mg/ml by adding 10 ml of the matrix to 10 mg OCT and mixed with a vortex. This first stock solution was stored in the fridge at +4°C for upcoming analysis. For the calibration curve in SWF, the first stock solution was the same used for the water:methanol matrix, i.e., the OCT was dissolved in water:methanol instead of SWF for the first stock solution. This was done due to the insolubility of OCT in SWF.

A second stock solution for each matrix was prepared by adding 10 $\mu$l of the first OCT stock solution to 990 $\mu$l of the matrix to reach a final concentration of 10 $\mu$g/ml. The rest of the calibration concentration levels were then prepared by mixing the second stock solution with the matrix, according to Table 3.3.

**Table 3.3:** *Calibration levels preparations for OCT analysis*

| **Calibration point** | **Second stock solution [$\mu$l]** | **Matrix [$\mu$l]** | **Concentration [$\mu$l/ml]** |
|---|---|---|---|
| 1 | 10 | 990 | 0.100 |
| 2 | 25 | 975 | 0.250 |
| 3 | 60 | 940 | 0.600 |
| 4 | 150 | 850 | 1.500 |
| 5 | 300 | 700 | 3.000 |

The calibration curves for BAC was prepared in two matrices; water and SWF. A first stock solution with a concentration of 1 mg/ml was made by dissolving 10 mg BAC in 10 ml of water. This first stock solution with BAC dissolved in water was used to prepare the second stock solution for both water and SWF. The first stock

solution was used for the calibration curves in both matrices due to the insolubility of BAC in SWF. The stock solution was stored in a fridge at +4°C.

The second stock solution, with a concentration of 10 $\mu$g/ml, was prepared by adding 10 $\mu$l of the first BAC stock solution to 990 $\mu$l of the matrix. The calibration levels were prepared by mixing the second standard solution with the matrix according to Table 3.4, and the final concentrations of BAC-12 and BAC-14 were calculated based on the ratio of the areas received from the LC-MS analysis. BAC-12, BAC with a chain length of twelve carbons, and BAC-14, BAC with a chain length of fourteen carbons, will have different retention times due to the difference in hydrophobicity and will, therefore, appear as two separate peaks on the chromatography.

**Table 3.4:** *Calibration levels preparations for BAC analysis*

| **Calibration point** | **Second stock solution [$\mu$l]** | **Matrix [$\mu$l]** | **Concentration BAC-12 [$\mu$g/ml]** | **Concentration BAC-14 [$\mu$g/ml]** |
|---|---|---|---|---|
| 1 | 10 | 990 | 0.0013 | 0.0987 |
| 2 | 25 | 975 | 0.0033 | 0.24675 |
| 3 | 75 | 925 | 0.0098 | 0.74025 |
| 4 | 200 | 800 | 0.026 | 1.974 |
| 5 | 500 | 500 | 0.0065 | 4.935 |

The calibration range for OCT is therefore between 0.1 and 3.00 $\mu$l/ml. For BAC-12 the range is between 0.0013 - 0.0065 $\mu$l/ml and for BAC-14 between 0.0987 - 4.935 $\mu$l/ml. The calibration levels are analyzed on the LC-MS, and a calibration curve is constructed by plotting the concentration of each sample against the integrated area received from the LC-MS instrument. The accuracy of the linearity of the calibration curve is used to decide the limit of detection (LOD) and limit of quantification (LOQ).

For values above the limit of detection, it is with the certainty of the calibration curve that the values detected are from the substance in interest, but the concentration detected is uncertain. For values above the limit of quantification, the concentration of the substance detected is also within the certainty of the calibration curve. If the values are below the LOD or outside the calibration range, there is no certainty that the correct sample or concentration has been detected. Therefore, no values below the LOD or outside the calibration range was included in this project.

However, values that are above LOD but below LOQ have been included, but any conclusion drawn from these samples have been made with caution and are clearly stated. For each analysis, there is a new calibration curve made, two different measurements of the same substance in the same medium might therefore have different LOQ and LOD, depending on the linearity of the calibration curve.

#### 3.6.2.2 Sample preparation

Due to the high sensibility of the LC-MS instrument, some preparation steps of the samples are usually required before analyzing. If the concentrations of the samples of interest are expected to be above the calibration range, the sample should be diluted before the LC-MS analysis. In Table 3.5 below the dilutions of the samples for the different batches of the organic substance samples can be viewed.

**Table 3.5:** *Sample dilution for the LC-MS analysis*

| **Samples** | **Dilution** | **Dilution factor** |
|---|---|---|
| Impregnated BAC in $H_2O$ | 1 $\mu$l sample + 1000 $\mu$l $H_2O$ | 1001 |
| Impregnated BAC in SWF | 5 $\mu$l supernatant + 1000 $\mu$l MeOH | 201 |
| Impregnated OCT in 20% EtOH | 5 $\mu$l sample + 995 $\mu$l 20% EtOH | 200 |
| Impregnated OCT in 6wt% Brij | 2 $\mu$l sample + 1000 $\mu$l 20% EtOH | 501 |
| Impregnated OCT in $H_2O$ | 5 $\mu$l sample + 995 $\mu$l MeOH:$H_2O$ (1:1) | 200 |
| Impregnated OCT in SWF | 3 $\mu$l supernatant + 1000 $\mu$l MeOH:$H_2O$ (1:1) | 334.33 |
| OCT in PLLA spheres in 20% EtOH | No dilution | |
| OCT in PLLA spheres in SWF | No dilution | |
| OCT in PLGA spheres in 20% EtOH | No dilution | |
| OCT in PLGA spheres in SWF | No dilution | |
| BAC incorporated in fibers in $H_2O$ | 20 $\mu$l sample + 980 $\mu$l $H_2O$ | 50 |
| BAC incorporated in fibers in SWF | No dilution | |

For the samples in SWF, there was an additional preparation step before any dilution; this step was made for each calibration standard, blanks, and samples. From each calibration standard, blank and samples in SWF 200 $\mu$l were transferred with an automatic pipette to Eppendorf tubes, and 800 $\mu$l of methanol was added over each one. The Eppendorf tubes were mixed on vortex and then centrifuged at 13 500 rpm for 15 min at 15 °C.

The centrifuging step was done to separate the proteins in SWF from the supernatant methanol phase, because the proteins can not be analyzed on the LC-MS. The supernatants in the methanol phase were then transferred to LC-MS vials. Some of the samples supernatants were also diluted, see Table 3.5, before analyzed.

# 4

# Results

In this chapter, the main results from the different experiments performed during this Master thesis project will be presented. The release methodology for different materials was developed by first analyzing the release and characterization of silver. The key parameters for the method were evaluated before analyzing the organic compounds. The method of measuring the release is, however, material dependent and different parameters need to be considered for different types of materials, active substances, and receiving media.

## 4.1 Release studies of silver

Firstly, the absorbing properties of the PVA based gelling fiber silver-containing material effect on the release of silver was evaluated. Also, the difference in the release of silver in the heated cells was compared to room temperature. The release of silver was then measured in the more complex medium SWF, mimicking the release of silver in an environment closer to the real context of wound care dressings.

### 4.1.1 Release of silver in water

The comparison of adding the maximum absorption volume (1 ml) or half (0.5 ml) that to wet the PVA based gelling fiber silver-containing material is presented in Figure 4.1. The red dots in the graph represent the release of silver ions in water, as the silver-containing material was wetted with the maximum absorption volume of 1 ml water. The yellow dots illustrate the release of the silver ions when the silver-containing material was soaked with a volume of 0.5 ml water.

Since the fraction of released silver do not differ, it should not be any difference in the release depending on the volume of water added to the PVA based gelling fiber silver-containing material. The red dots shows that almost all silver available in the material was released to the water phase within 48 h.

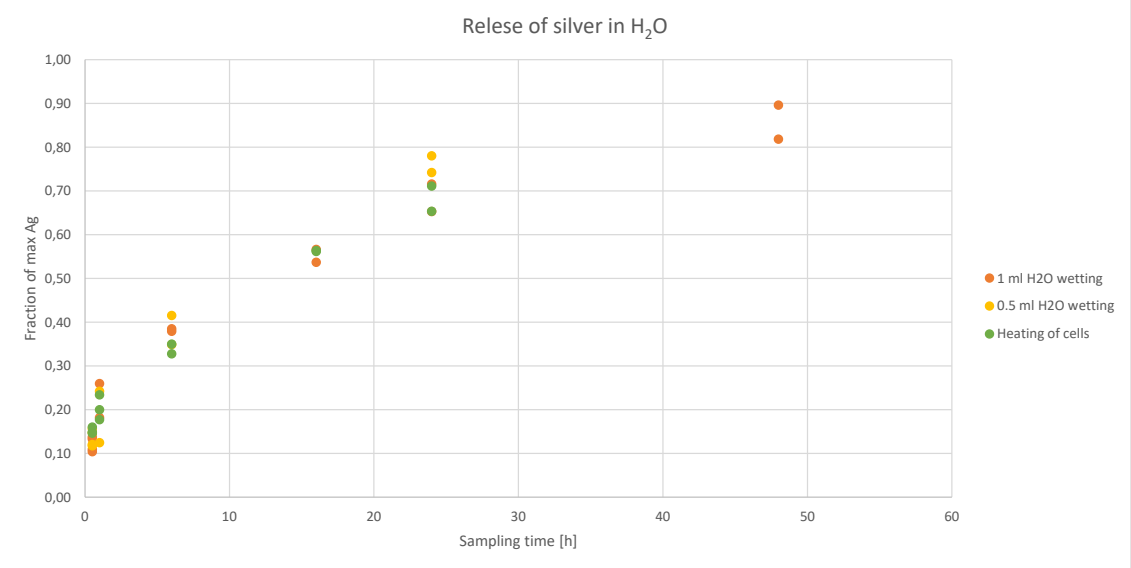


**Figure 4.1:** *Graph over the release of silver in $H_2O$ with different water volumes absorbed, and heating of the Franz cells.*

In Figure 4.1 the green dots represent the release of silver to water when the Franz cell had a temperature of approximately 32 °C. The fractions of silver released are not significantly different from the red and yellow lines, where the measurements were conducted in room temperature.

### 4.1.2 Release of silver in simulated wound fluid

The release of silver ions in water was compared to the release of silver ions in simulated wound fluid. In Figure 4.2, the blue dots represent the fraction of the total available amount of silver in the material released in water over time. The orange dots are the equivalent for of silver in SWF. A graph displaying the release of silver in SWF can be viewed in Figure B.1 in Appendix B.

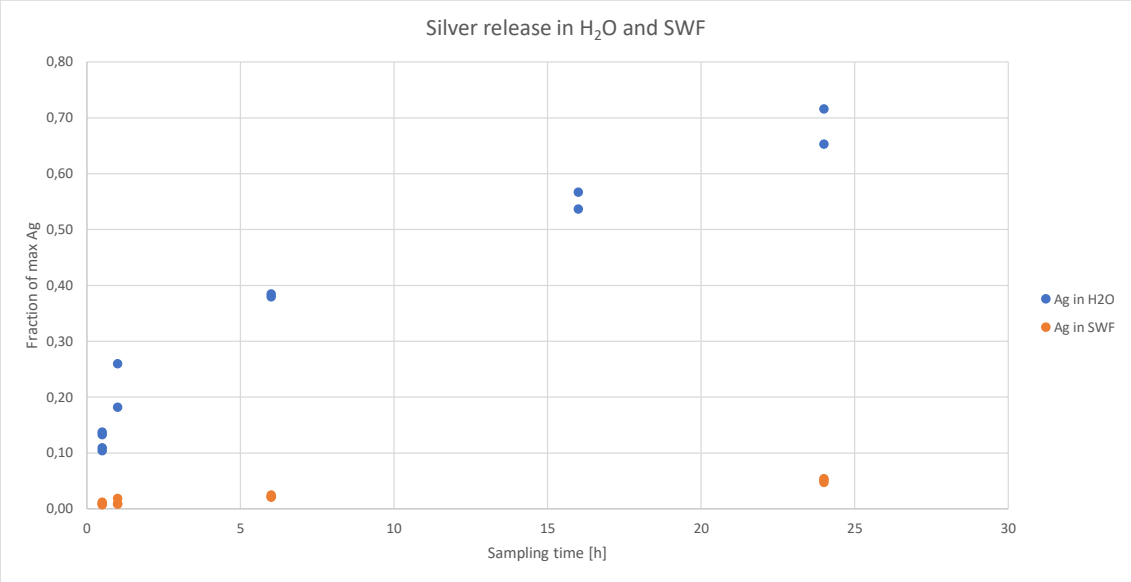


**Figure 4.2:** *Graph that illustrates the release of silver in $H_2O$ and SWF*

The plotted values of both media follow the expected release trend; however, the fractions of the silver released in SWF is significantly lower.

### 4.1.3 Sampling techniques

In Figure 4.3, the fraction of silver released in water over time, where the concentrations had been with the first sampling technique sampled from one cell at a time with individual samples is plotted as green dots. The blue dots represent a fraction of silver released in the water when the sampling have been made with the second sampling technique from the same cell with one sample for all the sampling times. Both of the sampling techniques display the same expected release trend, and the results are comparable.

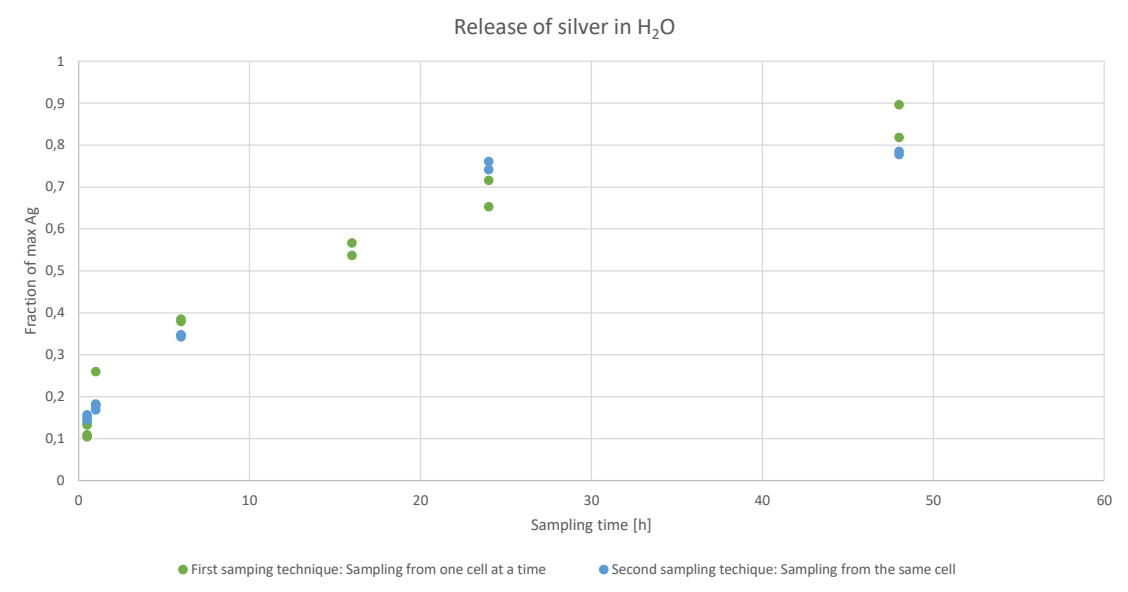


**Figure 4.3:** *Graph of the release of silver in $H_2O$ when using two different sampling techniques; sampling from one cell at a time, and from the same cell each time*

The two different sampling techniques were performed in the same way for evaluating the release of silver in SWF. In Figure 4.4, the orange dots represent the first sampling technique where the sampling was done from one cell at a time. The yellow dots in the figure illustrates the fraction of silver released in SWF when using the second sampling technique sampling was done from a single cell and sample each time. The fractions of released silver at each sampling time are considered equivalent.

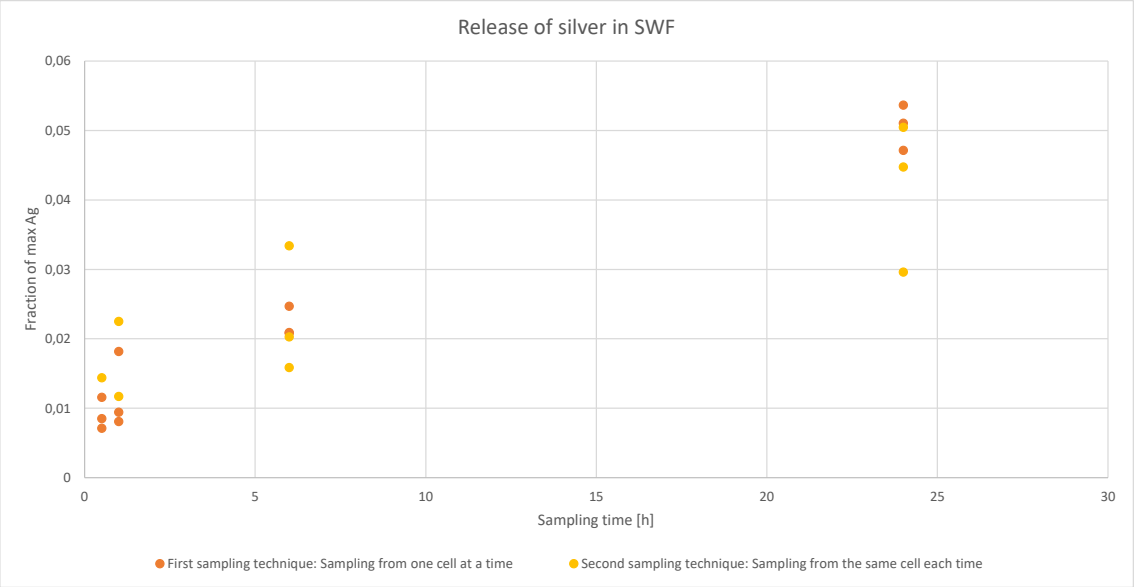


**Figure 4.4:** *Graph of the release of silver in SWF when sampling from one cell at a time and, from the same cell each time*

When using the second sampling technique of sampling from the same cell, three cells were filled with water and three cells were filled with SWF. The fractions from each individual cell at the different sampling times is illustrated in two graphs in Appendix B. For the release in water, see Figure B.2 and for the release in SWF see Figure B.3. The fraction of silver in water are identical for cell 1 and 2. For cell 3, the fraction is lower at the 24 h sampling time. This is considered to be an outlier since the other two cells have similar fractions at all sampling times. For the comparison between the three different cells in the results are not identical; this is probably due to the heterogeneity of SWF. The fractions of silver at different sampling times follow the release trend for all the cells; however, cell 1 has the most distinct release trend shape.

### 4.1.4 Adapting release measurements to physical model

The results from the measurements described in the previous section, *4.1.3*, where adjusted to the function *2.1* in section *2.3.2*. The function describes the physical model of diffusional transport of active substance from thin material containing homogeneously distributed to the surrounding medium. In Figure 4.5 the fractions from the measurements in previous section, *4.1.3* is plotted as coloured dots. The red dots are the fractions from the measurements utilizing the first sampling technique of one cell at a time. The orange dots represent the fractions of silver released in cell number 1 while sampling from the same cell for all the sampling times. The green dots represent cell number 2, and the blue represents cell number 3. The data from all of the measurements were adapted to the function of the physical model

and are plotted as lines in the respective color of the measurements. The lines are very related to both the potted fractions and each other, which indicates that the measuring method agrees well with the physical model of diffusional transport.

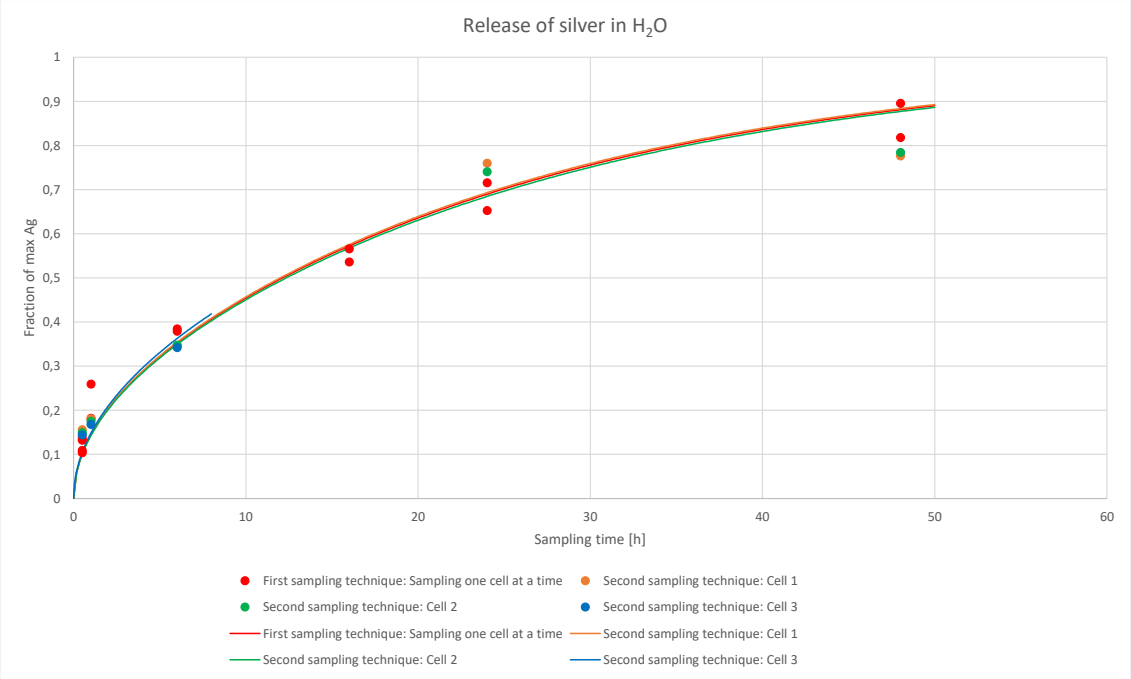


**Figure 4.5:** *Graph that illustrates the release of silver from PVA based gelling fiber silver-containing material in water adapted to the physical model*

From the physical model, the diffusion coefficients for silver in the PVA based material to water based on the measurements from the previous section, *4.1.3*, could be calculated. In Table 4.1 the diffusion coefficients for the different samplings is shown, where L is the thickness of the material. L is assumed to be equal for all samples, and the diffusion coefficients are therefore comparable. In the column to the right in Table 4.1 is the error of the diffusion coefficients for the different samplings.

**Table 4.1:** *Diffusion coefficients for silver release from PVA based gelling fiber silver-containing material in water*

| | $(D/L^2)[h^{-1}]$ | Error of $(D/L^2)$ |
|---|---|---|
| Sampling one cell at a time | 0.016212 | 0.0010196 |
| Cell 1 | 0.016402 | 0.0027601 |
| Cell 2 | 0.015935 | 0.0023155 |
| Cell 3 | 0.017195 | 0.0029265 |

The results from the measurements of silver release from the PVA based gelling silver-containing material in SWF was adapted to the physical model in the same manners as for the silver release in water. In Figure 4.6 are the fractions of released silver in SWF, presented in section *4.1.3*, plotted as dots. The red dots are the fraction of silver released measured while utilizing the first sampling technique with sampling from one cell at a time. The remaining dots represent the different cells while utilizing the second sampling technique with sampling directly in the cell, where the cell number 1 is represented as orange dots. Cell number 2 are represented with green dots and cell number three with blue dots. The data from all of the measurements were adapted to the physical model and are plotted as lines in the respective color of the measurements. The lines are not as alined with the plotted fractions or each other as for the release of silver in water, but over all agree fairly well.

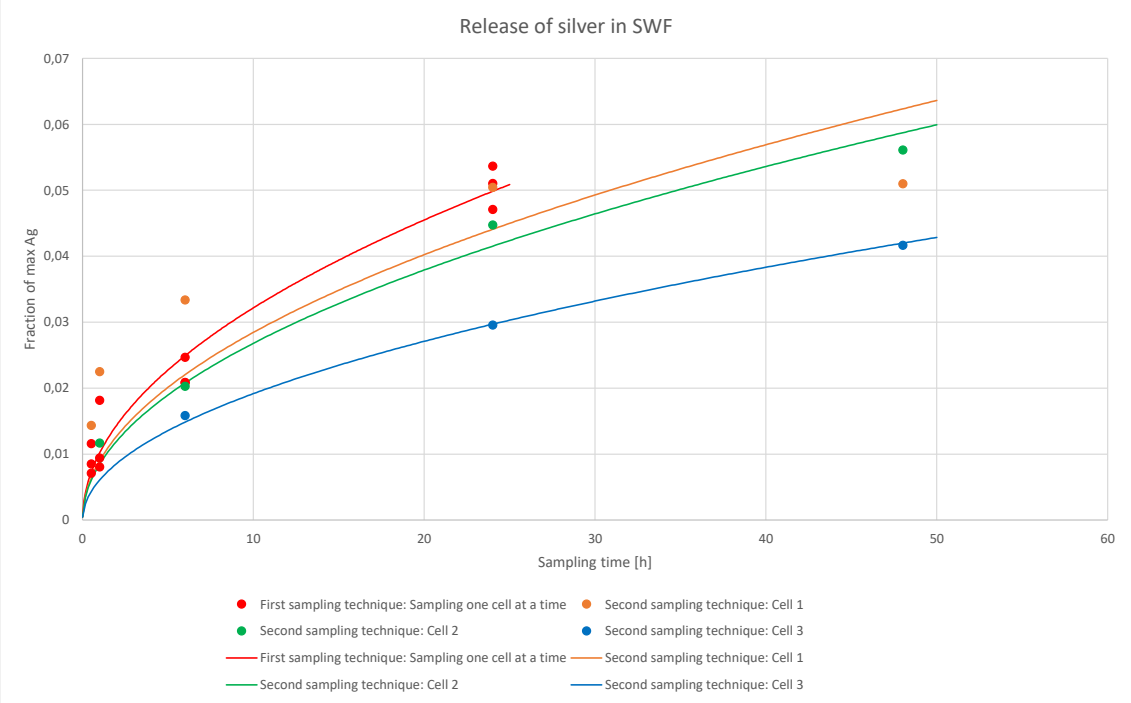


**Figure 4.6:** *Graph that illustrates the release of silver from PVA based gelling fiber silver-containing material in SWF adapted to the physical model*

The diffusion coefficients based on the measurements from the previous section, *4.1.3*, was calculated for the release of silver in the PVA based material to SWF in the same manner as for the measures in water. In Table 4.2 the diffusion coefficients for the different samplings are displayed, where L is the thickness of the material. L is assumed to be equal for all samples, and the diffusion coefficients are therefore comparable. The column to the right in Table 4.2 is the error of the diffusion coefficients for the different samplings.

**Table 4.2:** *Diffusion coefficients for silver release from PVA based gelling fiber silver-containing material in SWF*

| | **(D/L$^2$) [h$^{-1}$]** | **Error of (D/L$^2$)** |
|---|---|---|
| Sampling one cell at a time | 0.000081301 | 0.0000060043 |
| Cell 1 | 0.000063584 | 0.000018501 |
| Cell 2 | 0.000056423 | 0.0000045478 |
| Cell 3 | 0.000028844 | 0.00000080367 |

## 4.2 Release studies of organic antimicrobial substances impregnated on cellulose material

Developing a method for measuring the release of the organic substances, the first obstacle to overcome was the impregnation of the organic substance onto the cellulose material. The first section presents the solubility of OCT and BAC in different solvents. During these solubility test, the requirement of the sink condition was also evaluated. Both of the organic substances were found to have low solubility in SWF and was examined more elaborate. The release of both of the organic agents was then evaluated in different receiving media to find the most ideal receiving media.

### 4.2.1 Solubility of organic substances

The solubility test results for the antimicrobial substances OCT and BAC in the following solvents; ethanol, 20% ethanol in water, acetone, water, and 6wt% Brij in water are presented in the Table 4.3. The concentrations in the Table 4.3 are marked as dissolved if the substance was consider dissolved, not tested if the concentration only was tested for one of the substances. For some concentrations, the substance was insoluble and thereby marked insoluble.

**Table 4.3:** *Solubility of OCT and BAC in different solvents*

| **Substance [mg]** | **Solvent [1 ml]** | **OCT** | **BAC** |
|---|---|---|---|
| 400 | EtOH | Dissolved | Dissolved |
| 1250 | EtOH | Was not tested | Dissolved |
| 100 | 20% EtOH | Insoluble | Dissolved |
| 30 | 20% EtOH | Dissolved | Was not tested |
| 20 | Acetone | Insoluble | Dissolved |
| 6.67 | Acetone | Insoluble | Was not tested |
| 100 | $H_2O$ | Was not tested | Dissolved |
| 30 | $H_2O$ | Dissolved | Was not tested |
| 30 | 6 wt% Brij | Dissolved | Was not tested |

When dissolving OCT in ethanol, the total volume of the solution was about 20% more than the volume of pure ethanol when adding at least 200 mg in 1 ml ethanol. The volume change is not negligible for the mass of OCT dissolved in ethanol and was taken into consideration when calculating the concentrations for the solutions.

The solubility in simulated wound fluid was investigated for OCT and BAC. Even a small concentration of the organic substance creates precipitation or aggregation. The SWF was filtered with Minisart®filters with the pore sized of 2 $\mu$m, to remove any small white particles. In Figure 4.7, test tube number 2 contains filtered SWF where there are no visible particles. To the left in Figure 4.7, test tube number 1, contains 1 mg OCT in 40 ml of the filtered SWF, giving the concentration of 0.025 mg/ml. The arrows in Figure 4.7 points towards precipitates in the SWF containing OCT, indicating that some elements in the SWF interact with the OCT and aggregates.

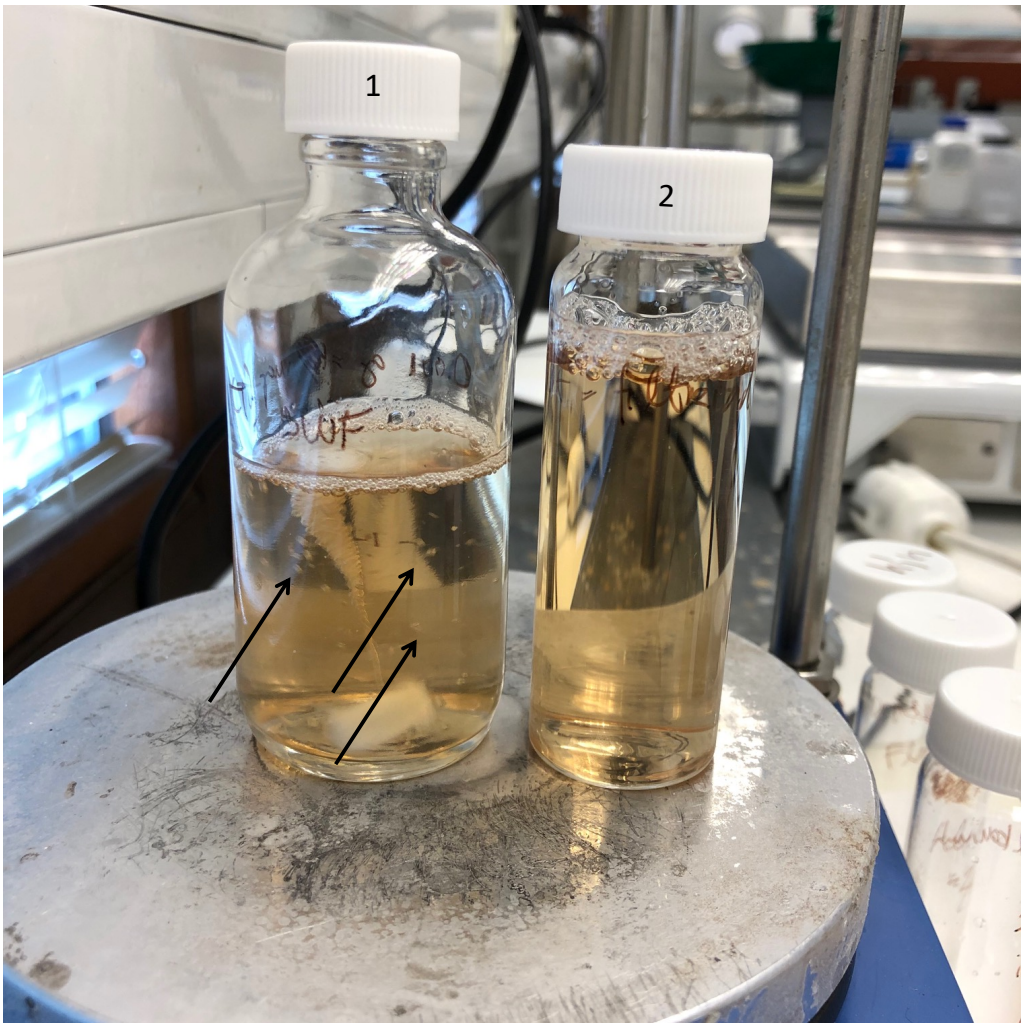


**Figure 4.7:** *Test tube 1 contains 0.025 mg/ml OCT in filtered SWF and test tube 2 contains filtered SWF*

The same solubility test was performed for BAC in SWF. In Figure 4.8, the test tube labeled number 2 contains filtered SWF. Test tube number 3, to the right in Figure 4.8, contains 5 mg BAC solved in 14 ml filtered SWF, giving the concentration 0.36 mg/ml. To the left in Figure 4.8, labeled number 1, contains the solutions from test tube number 3 filtered through the same filter as the filtered SWF. Test tube number 3, with BAC, is opaque compared to test tube number 2 without BAC, stating that the BAC interacts with elements in the SWF. Leaving test tube number 3 overnight, white particles had sedimented to the bottom of the tube.

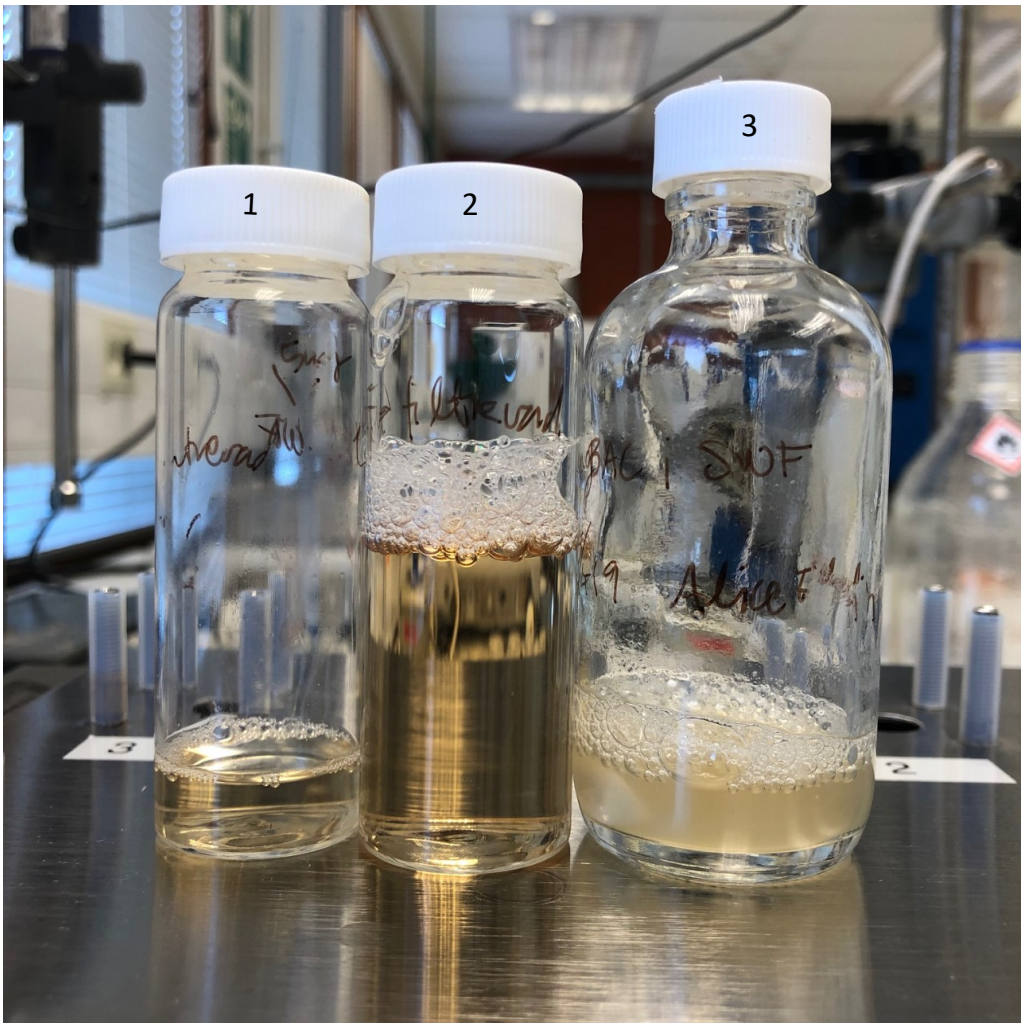


**Figure 4.8:** *Test tube 1 contains filtered content from test tube 3, test tube 2 contains filtered SWF and test tube 3 contains 0.36 mg/ml BAC in filtered SWF.*

### 4.2.2 Release of organic substances impregnated on cellulose to different media

Benzalkonium chloride (BAC) was dissolved in ethanol and impregnated onto the cellulose material with a concentration in the range of the values of MBC, see Table 2.1. Each cellulose sample was weighed before and after impregnation, to calculate the amount of BAC on each sample. The concentration is plotted as a fraction of the total amount of BAC on each sample in Figure 4.9. The blue dots in Figure 4.9 represent the fraction of the total amount BAC released in the water at the different sampling times. Within 24 h all of the BAC is released in water. In Figure 4.9, the release of BAC in SWF is plotted as orange dots. The release of BAC in SWF can be seen in Figure B.4, the three individual cell in water in Figure B.5, and the three individual cells in SWF in Figure B.6, all in Appendix B.

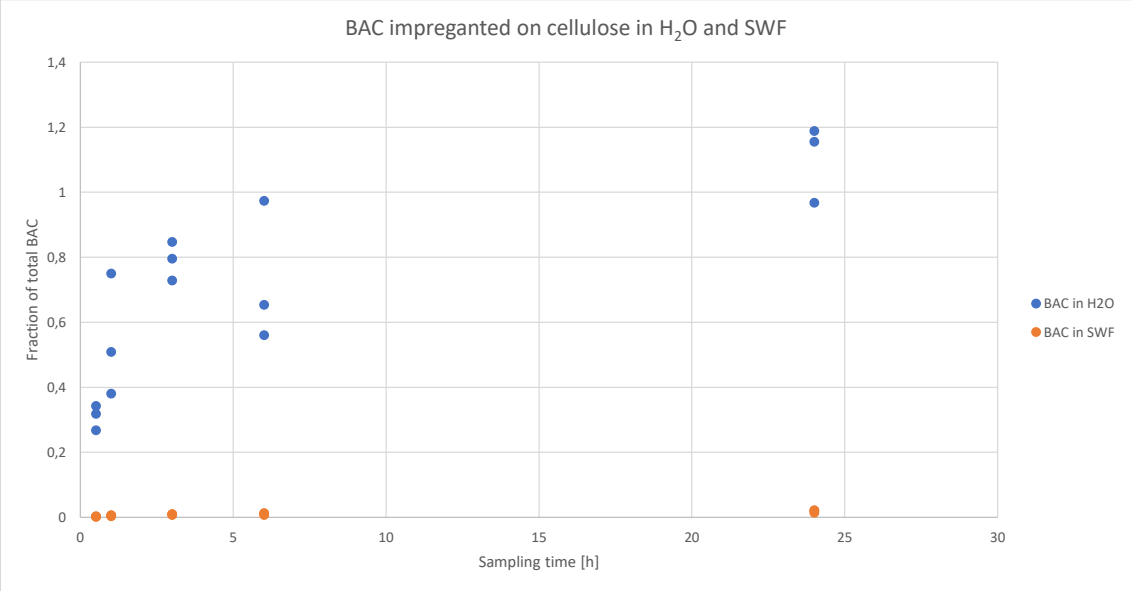


**Figure 4.9:** *BAC impregnated on cellulose release in $H_2O$ and SWF*

Octenidine was impregnated on cellulose in the same manner as BAC with regards to the MBC values in Table 2.1, and the release in different mediums were evaluated. The evaluation covered more medias compared to the release of BAC, due to the presumed lower solubility of OCT in water.

The blue dots in Figure 4.10 represent the release of OCT in 20% ethanol, the green dots represent OCT release in water and the yellow dots represent OCT release in 6wt%wt Brij solution. Some of the concentrations analyzed with LC-MS are below the limit of quantification; these values are presented as hollow rings instead of dots.

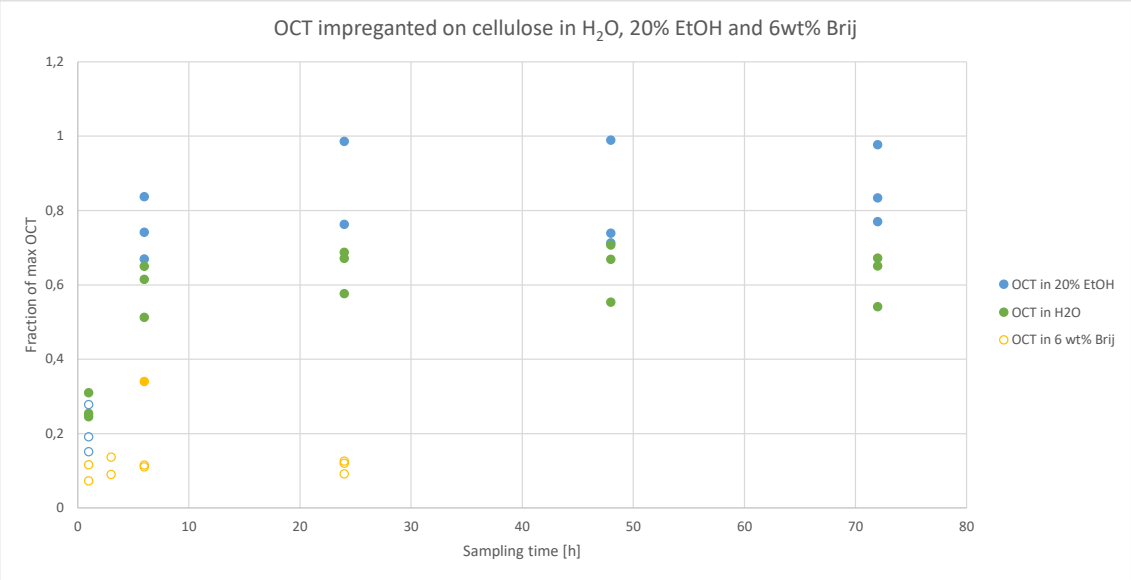


**Figure 4.10:** *Release of OCT impregnated on cellulose in $H_2O$, 20% ethanol and 6wt% Brij. All of the results for OCT in SWF was below the limit of detection and is therefore omitted from the graph.*

See Figure B.7 for a graph of the three individual cells with OCT in 20% ethanol plotted as individuals in Appendix B. The releases of the individual OCT samples in water are presented in Figure B.8 and in the 6wt% Brij solution is shown in Figure B.9, both are presented in Appendix B. The release rate of OCT impregnated on cellulose material in SWF was also evaluated; however, all the concentrations were below the limit of detection in the LC-MS analysis, and no conclusions with certainty could be drawn.

## 4.3 Release studies of the organic substances incorporated during manufacturing

Three cellulose materials were evaluated where the organic substance had been incorporated in the cellulose fiber in the manufacturing of the cellulose material. In Figure 4.11, the release of OCT that had been encapsulated in microspheres of PLLA in SWF is presented. The dots with no fill are concentrations that are below the limit of quantification.

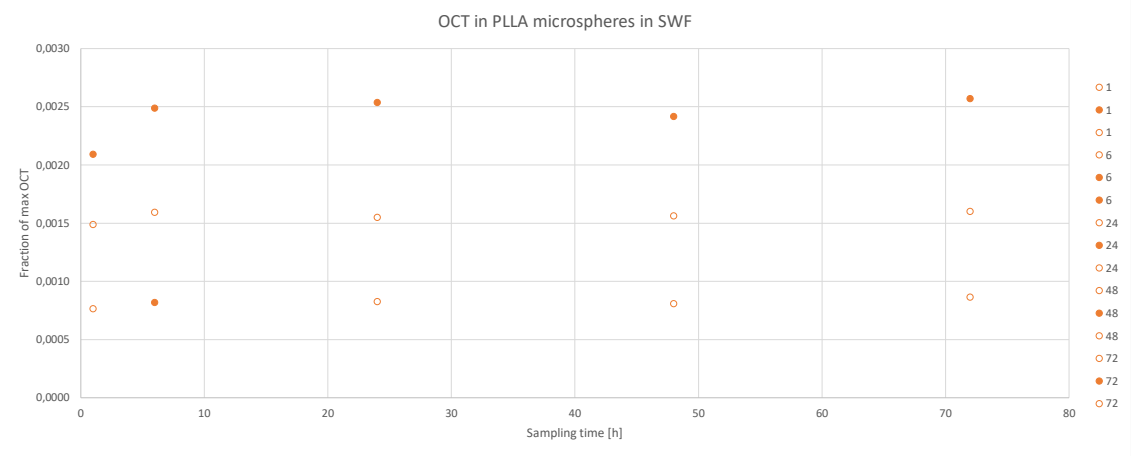


**Figure 4.11:** *Graph of the release of OCT encapsulated in PLLA spheres into SWF*

The concentrations from the individual cells can be view in Figure B.10 in Appendix B. The release of OCT encapsulated in microspheres of PLLA were also evaluated in 20% ethanol but all the values were below the limit of detection.

The release of OCT encapsulated in microspheres of PLGA in SWF is presented in Figure 4.12. The sampling times were 1 h, 6 h, 24 h, 48 h and 72h for this experiment as well; however, all the values from 48 h and 72 h were below the limit of detection. From sample times 1 h, 6h, and 24 h, only cell number 2 had concentrations over LOD. The release of the OCT was also measured in 20% ethanol, but all of the results from the measurement was below the limit of detection.

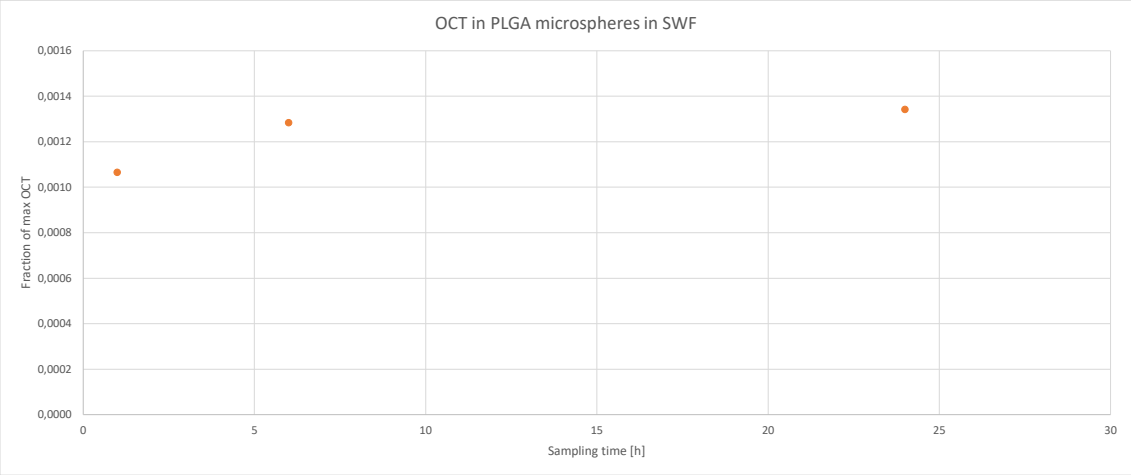


**Figure 4.12:** *Graph of the release of OCT encapsulated in PLGA spheres into SWF*

A cellulose material with BAC incorporated in the fibers during the manufacturing of the material was evaluated. The release in both water and SWF was assessed, however, the concentrations in SWF was below the limit of detection. In Figure 4.13, a graph of the fraction BAC in the water at the different sampling times can be viewed. For this experiment, there was no sampling done at 48 h. All the samples are represented as blue dots with no fill because all the values are below the limit of quantification. The samplings made after one hour was under the limit of detection and therefore not included in the graph. A graph where the three cells are plotted individually and can be seen in Figure B.11 in Appendix B.

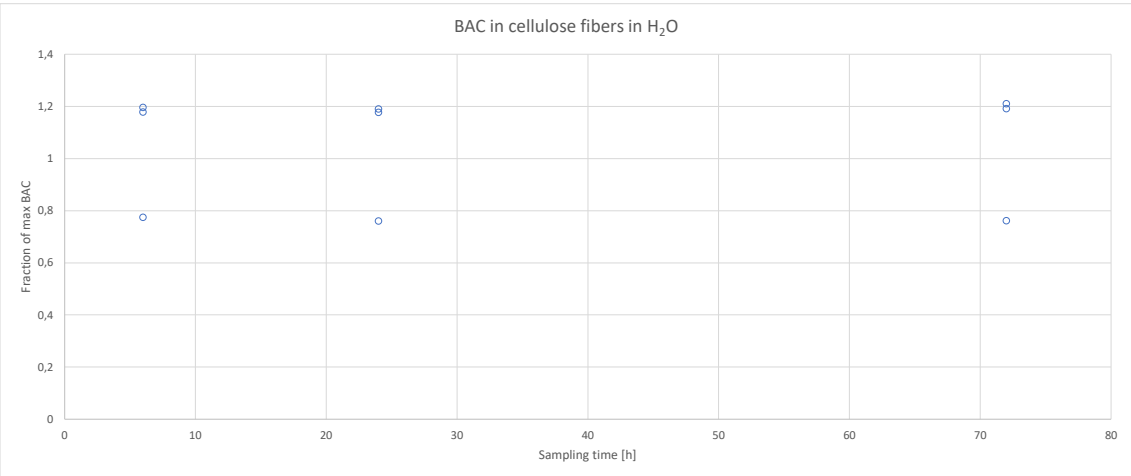


**Figure 4.13:** *Graph of the release of BAC incorporated in the cellulose fibers spheres into water*

# 5

# Discussion

This Master's thesis project aimed to develop an *in-vitro* method utilizing the Franz cell diffusion system for studying parts of the phamacokinetics of active substances in wound care dressings. To achieve a robust method, the parameters of the method were first evaluated for PVA based gelling fiber silver-containing material. The material had a known total amount of silver and the media first evaluated was water, due to silver sulfates high solubility and the simplicity of water as a media. This chapter will discuss the results both of the release of silver in water as well as when the method was applied to more complex mediums and materials.

## 5.1 Method development using silver release

Since the PVA based, gelling fiber silver-containing material was highly absorbing and had dynamic physical properties when in contact with liquid, the first parameter to be evaluated was the absorption. By adding water, trough the donor chamber onto the material, with a volume near the maximum absorption of the material and comparing it to volume half the size effect of how the diffusion of water into the material affected the release of silver could be evaluated. Comparing the two curves with different volumes of water added in Figure 4.1, the difference between the curves is relatively small. The release of silver is thus assumed not to be affected by the material's intrinsic absorption of the receiving medium. However, it was evaluated how the release is effected by not adding water. The water was mainly added to assure contact between the material and the receiving medium, and if there were no contact, the release of silver would decrease. Therefore the PVA based gelling fiber silver-containing material samples were for the following experiments was consistently wetted with 1 ml of water. It is also noteworthy that after 48 h almost all of the silver, approximately 90%, was released in the water. It is confirming that the maximum sampling time required for measuring the release of silver in water is about 48 h. The Franz cell was heated to skin temperature, to evaluate if the release was diverging from the measurements at room temperature, since the increase in enthalpy may lead to an increase in entropy. However, the results from measuring the release of silver in the water at elevated temperature compared to room temperature do not indicate any change in release. The unaffected release due to a slight temperature increase is however only valid for the release of the antimicrobial substance silver.

### 5.1.1 Effect of receiving media

In Figure 4.2, it is clear that the curves of the release of silver in water compared to SWF differ considerably. This difference, however, was not unexpected. With the increasing complexity of the media, the variation and number of interactions between the active substance and the media also increase, as stated in previous studies mentioned in section *2.4*. The composition in wound fluid is highly complex, and more in-depth investigations are required than are possible to investigate within this Master's thesis project to understand all of the interactions between silver and wound fluid. One hypothesis of the low concentrations of silver in SWF is that the proteins that are absorbed in the PVA based gelling fiber silver-containing material binds with the silver in the material and is thereby not released into the receiving SWF. The silver sulfate might also continue to crystallize on the silver binding to the proteins in the material. The high concentration of chloride ions in the SWF is also most likely forming insoluble silver chloride, reducing the concentration of free silver ions available to release to the SWF. By analyzing the amount of silver left in the PVA based gelling fiber silver-containing material after the release measurements on the Franz cells, may be able to prove some justification of the hypothesis. The total amount of silver in the material can be analyzed with ICP-OES. However, the analyzing the silver content of solid materials requires more rigorous preparations and was therefore not included in this project. The potential determination of the total amount of silver in the material is not, however, enough to determine exactly the type of interaction between the silver-containing material and SWF and even further investigations are required. In Figure B.1 in Appendix B, where the release of silver in SWF is plotted, the curve in the graph exhibits the desired release behavior, confirming that silver is released in small concentrations in the SWF.

### 5.1.2 Sampling techniques

For the development of the method, it was of importance to be able to sample directly in the cells. This sampling technique enables the ability to have several data from the same sample at different times. The advantages of this sampling technique were also a reduced practical workload when sampling. It is expected that the measurements are more homogeneous since they are all conducted from the same sample, but it also requires less material for the equal amount of data points. Since there where six Franz cells in total, it was an advantage in running the experiment in two different media at the same time with three replicates in each medium. The opportunity to run two media at the same time enables the analyses to always have an ideal control medium as reference for complex media. As seen in Figure 4.3 there is no difference in the two different sampling techniques, confirming that technique of sampling directly from the cells are equally precise as the first sampling technique, the one where the whole cell is removed. The same comparison was made for the release of silver in SWF, seen in Figure 4.4 where the curve differs slightly more, but that is most probably due to the inhomogeneity in the SWF medium or the sample since there are quite small that are used here, so certain inhomogeneity in the samples might be expected. Comparing the graphs in Figure 5.1 and 5.2, seen below, where the three replicates from the individual cells

are presented, the three replicates for the release of silver in water are very similar, indicating good robustness of the method. The two concentrations measured at 24 h and 48 h in cell 3, displayed as a dotted line, was considered to be deviating and was therefore not included in any conclusions drawn. The three replicates for the release of silver in SWF are not as homogeneous, but that is most likely also due to the complexity of the receiving medium.

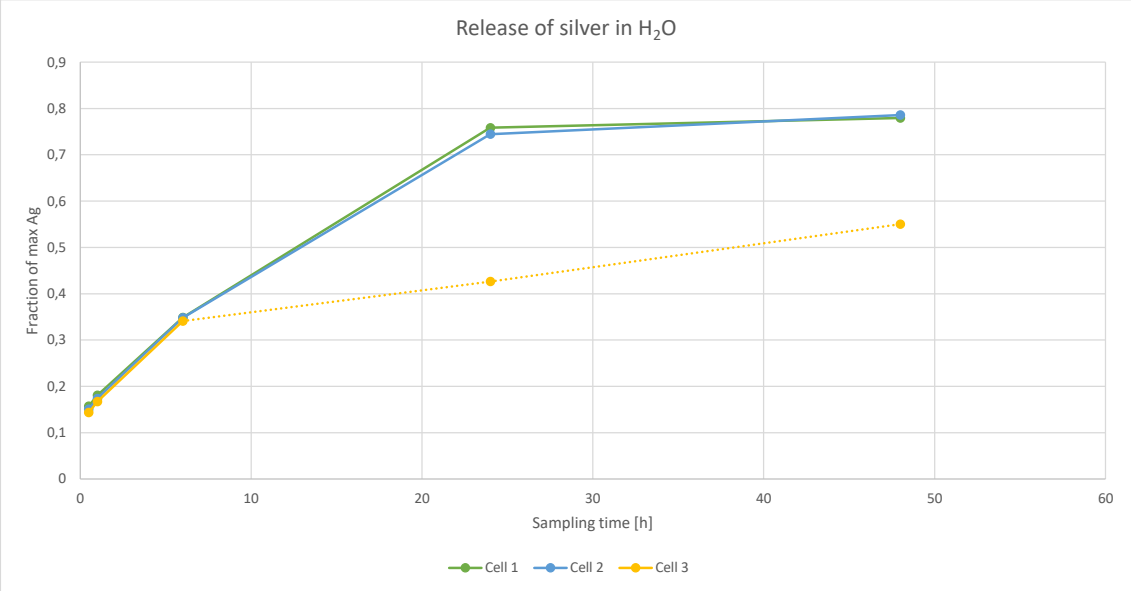


**Figure 5.1:** *Graph of the release of silver in $H_2O$ in the three different cells*

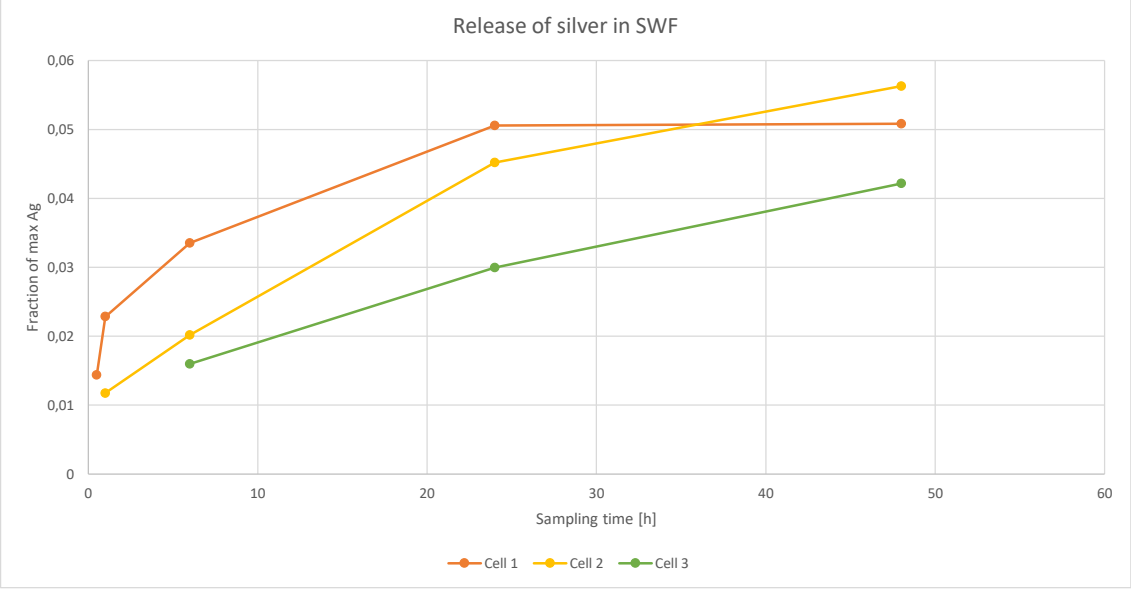


**Figure 5.2:** *Graph of the release of silver in SWF in the three different cells*

### 5.1.3 Adapting release measurements to physical model

The method for measuring the release rate of silver in a PVA based gelling fiber silver-containing material in water agree very well with the physical model of diffusional transport, demonstrating that the method is robust. For the equivalent measurements in SWF was the result was not as consistent but agreed reasonably well with the physical model. The more scattered result is probably due to the lower concentrations and the more complex media, both are factors that increases the variance. This indicates that the Franz cells diffusion system is an good method for measuring mass transport by diffusion for active substances from solid material to a suitable medium.

## 5.2 Method development for the organic substances impregnated on non-woven cellulose

The method was confirmed to be robust, as established via the silver release measurements, described above. The same method was to be applied and evaluated on the release of the organic antimicrobial substances; octenidine dihydrochloride (OCT) and benzalkonium chloride (BAC). First, the OCT and BAC needed to be applied to the cellulose fibers, and the amount used had to be known to be able to calculate the released fraction. As explained in the section *2.3* the MBC is the minimal bactericidal concentration, and in an optimal wound care dressing there would be an antimicrobial substance that reaches MBC to kill the bacteria's to prevent infection. The concentrations impregnated onto the wound care aimed to be as close to the MBC concentrations in the Franz cells as possible.

### 5.2.1 Solubility of the organic substances and impregnation of the non-woven cellulose material

The impregnation technique used required the organic substances to be dissolved in a volatile solvent, and the solubility of the substance was evaluated in Table 4.3. Both of the substance has high solubility in ethanol, and ethanol also has high volatility and was thus a suitable solvent for the impregnation of the cellulose. A quick test of how the ethanol compared to the 20% ethanol in water affected the cellulose material was made, and the high water content in the 20% ethanol solution made the edges of the $\phi$ 26 mm sized cellulose material curl. The change in the shape of the cellulose can make it challenging to place them correctly in the Franz cells, and thereby, one more reason for choosing ethanol as solvent. The sinking conditions were consider fulfilled for OCT release in water. The highest concentration of the OCT impregnated samples was 3.27 mg/ml in the Franz cells, which is slightly above the 10% of the maximum concentration dissolved, 30 mg/ml, but since the sinking condition is just an experimental suggested value, it was considered to be within acceptable limits. For OCT in the 6wt% Brij solution, the same applies as for OCT in water. The maximum concentration of OCT released in 6wt% Brij was 3.45 mg/ml in the Franz cells and was considered to be within reasonable

limits to fulfill the sink condition. For the OCT impregnated samples in the 20% EtOH the highest concentration was 2.45 mg/ml in the Franz cells, and according to the solubility test, the concentration of 30 mg/ml OCT in 20% EtOH and the sink condition is fulfilled. For the measurements of the release of BAC in water, the sink conditions were also achieved. The condition was fulfilled since the maximum concentration of BAC dissolved in water during the solubility tests was 100 mg/ml, and for the samples, where BAC had been impregnated on the cellulose, the maximum concentration is the cells was 8.91 mg/ml.

The solubility of the organic substances in SWF was hard to evaluate due to both substances either precipitates or contribute to aggregation in the SWF. The SWF is, as previously mentioned, a complex medium and contains a lot of different elements, and some of them exist as small white particles in the SWF. When adding the organic substances to the SWF, it was hard to judge only by eye if the tiny white particles were unsolved organic substance or a preexisting element in the SWF matrix. Due to this, the SWF was filtered to remove all the preexisting small white particles, and the organic substances were added to the filtered SWF. When adding the OCT and BAC small white particles were formed even in the filtered SWF, verifying that elements in the SWF precipitate or that the organic substances are not properly dissolved. The solubility of OCT and BAC in SWF is hard to determine with just the eye. Analyzing different concentrations of BAC and OCT in SWF with UV-Vis, could give more answers but is not part of this project.

### 5.2.2 Receiving media

Although the solubility of the organic substances in SWF was not determined, the release measurements in SWF was still of great interest since the SWF is the medium most similar to the real environment of the wound care dressing. For the release measurements of the organic substances, an ideal reference medium was desirable to the SWF. For BAC, water was chosen due to its high solubility, and as Figure 4.9 displays, all of the BAC impregnated on the cellulose are released in water within 24 h. Release measurements for BAC impregnated on cellulose to SWF was also performed, and even though the concentrations released is significantly smaller compared to water the measurements display a desirable release curve, shown in Figure 5.3. Comparing the two Figures 5.4 and 5.5 below, displaying the individual cells with BAC in SWF and water, SWF displays a more continuous result than the individual cells with BAC in water.

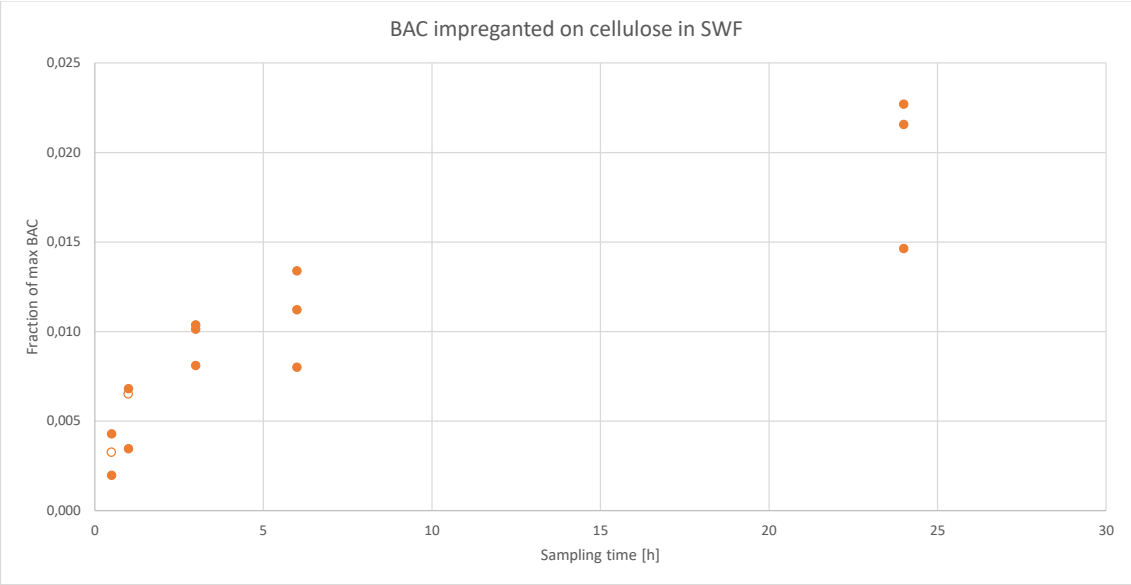


**Figure 5.3:** *Graph of the release of BAC impregnated on cellulose in SWF*

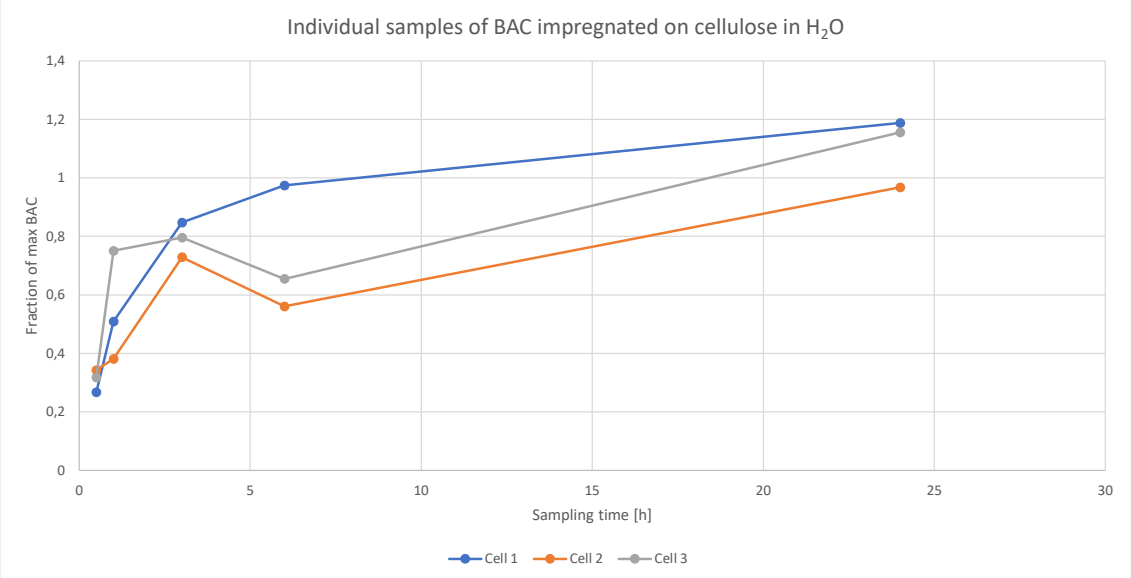


**Figure 5.4:** *Graph over the release of BAC impregnated on cellulose in $H_2O$ displayed as the three individual cells*

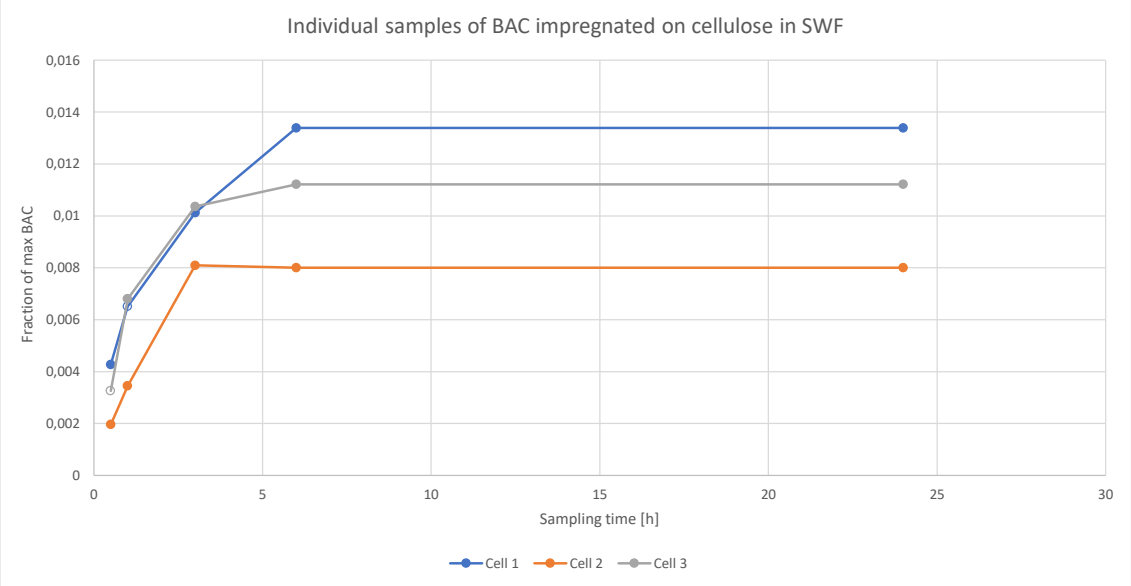


**Figure 5.5:** *Graph over the release of BAC impregnated on cellulose in SWF displayed as the three individual cells*

There is a deviation in the measurements, where cell 2 and 3 have had a higher concentration at one hour than after six hours, this might be due to mishandling while sampling, variations in the measurement technique or deviations in preparing the samples for the LC-MS measuring. The most likely reason for the unexpected lower concentration after 6 hours, is the insecurity in the sample preparation for the LC-MS. The samples were diluted with 1 $\mu$l sample + 1000 $\mu$l $H_2O$ using an automatic pipette, 1 $\mu$l is probably not enough sample to represent the concentration in Franz cell.

For OCT, several media were tested as the receiving medium. In Figure 5.6 below, the releases of OCT impregnated on cellulose into 20% ethanol, water, and 6wt% Brij solution is presented. Almost all the OCT concentrations measured in the 6wt% Brij solution was below the limit of quantification except one. However, all of the samples where diluted 2 $\mu$l sample + 1000 $\mu$l 20% ethanol, if the samples where not to be diluted the concentrations might have been above the limit of quantification. The samples were diluted with 20% ethanol instead of 6wt% Brij, which might have an impact on the results in 6wt% Brij. The samples probably have to be diluted to avoid high concentrations of Brij in the instrument, but this needs to be further investigated. More importantly, the release of OCT is much higher in both 20% ethanol and water. For the release of OCT in the water, there are indications that a plateau has been reached when about 60% of the total amount of impregnated OCT has been released. In the 20% ethanol solution, on the other hand, almost all of the OCT infused on the cellulose seems to be released within 72 h. Making the 20% ethanol to a suitable receiving medium for comparison studies of OCT. Since all the results were below the limit of detection for the impregnated OCT in SWF, there is probably no need to dilute these samples, or dilute them less.

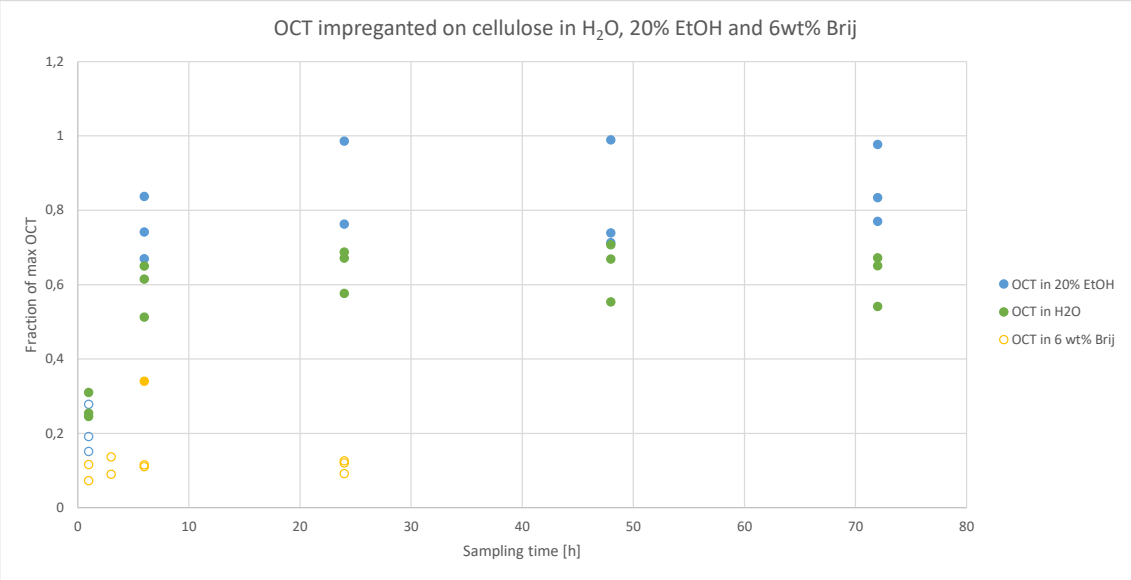


**Figure 5.6:** *The release of OCT impregnated on cellulose in $H_2O$, 20% ethanol and 6wt% Brij. All of the results for OCT in SWF was below the limit of detection and is therefore omitted from the graph.*

## 5.3 Organic substances incorporated in non-woven cellulose during the manufacturing

To control the release of the organic substance, they were incorporated in microcapsules, or in this case microspheres, the theory behind this is explained in section *2.3.1*. The cellulose material was manufactured containing microspheres with OCT encapsulated. The capsules were based on either PLLA or PLGA. For the release measurements of OCT in PLLA microspheres, the receiving medium was 20% ethanol and SWF, however, the OCT concentrations released was only above the limit of detection in SWF. Most of the OCT concentrations in SWF was also below the limit of quantification and thereby increasing the uncertainty of the results. The release measurement of the OCT in PLGA microspheres was conducted in the same way with even fewer concentrations above the limit of detection. The release measurements with the concentrations that were above the limit of detection in SWF can be seen in Figure 4.11 for the PLLA microspheres and in Figure 4.12 for the PLGA microspheres. The behavior was unexpected since the OCT had a higher release to the 20% ethanol for the cellulose samples with impregnated OCT. Neither of the samples where diluted, so nothing could be done differently in the preparation step of the samples to receive detectable concentrations, see Table 3.5. However, the linearity of the calibration curve was slightly worse in 20% ethanol, and thus LOD was slightly higher compared to LOD in SWF. This may be an explanation for the fact that the OCT concentrations were detectable in SWF but not in 20% ethanol. The ethanol in the solution is probably also affecting the polymer in microspheres and needs to be further investigated.

The samples with OCT in PLGA microspheres was after the 72 h on the Franz cells placed in individual test tubes filled with 11 ml of 99% ethanol to dissolve all the remaining OCT in the samples in the ethanol. The results from the LC-MS were all slightly above the calibration curve range, and therefore not included in the results of this Master's thesis project, but all where approximately 10% of the expected maximum value. The predicted maximum value was calculated on the assumption that OCT was nearly insoluble in water; however, these results indicate that up 90 % of the OCT can be lost in the current manufacturing process.

The samples were the BAC was incorporated in the cellulose fibers during the manufacturing, the concentrations released in SWF were all below the limit of quantification. The BAC concentrations released in water were all below the limit of quantification. The samples in SWF was not diluted before analyzing with LC-MS, meaning there is no improvement to make in the preparation step to achieve measurable concentrations. However, the samples for BAC incorporated in the fibers released in water the samples where diluted 20 $\mu$l sample + 980 $\mu$l $H_2O$. If no dilution were to be made on those samples, the concentrations might have been above the limit of quantification.

## 5.4 Analytical techniques

For the analysis of silver in water and SWF, the ICP-OES analytical technique used was validated. During this master's thesis project, there is nothing that indicates that the results from the measuring method of ICP-OES are not reliable.

The analytical technique used for measuring the organic substances was, however, not validated, and continuously developed during the project. There are some aspects in the measurement of the concentrations of the organic substances that need to be further evaluated and are currently considered to be error sources. The dilution of the samples is one critical parameter since it is hard to know the concentration of the sample in advance, and the dilution is done in regards to the highest possible concentration. If the concentrations are high, the samples need to be diluted due to the sensitivity of the instrument and to fit in the calibration curve range. For the samples that were diluted during this project, the volumes taken from the samples was 1 - 5 $\mu$l. For these small volumes, it is usually hard to get a representative concentration for the whole sample. Especially in samples in which there has been aggregation and precipitation, as for the organic substances in SWF. More investigations regarding the storage of the samples are required as they may aggregation and precipitation in more solvents the SWF overtime and fridge conditions.

For the substances, OCT and BAC that are also known surface active agents, some of the substance do most likely attach to the surface of the pipette tip during the dilution. The attachment of the surface active agents to any surface is, however valid for all the steps when handling the samples. Including when sampling with the needle in the cell and adding the 100 $\mu$l to the LC-MS vial. The smaller volumes should, however, be more affected. For the calibration curves produced for

each measurement, the lowest volume pipetted was 10 $\mu$l. The concentrations used in the calibration curves are linear and repeatable, so assumable volumes of 10 $\mu$l or larger are valid, but volumes below 10 $\mu$l might lead to uncertainties.

By comparing the calibration curves in suitable solvents for the organic substances, such as water for BAC, and water:methanol (1:1) for OCT, with the calibration curves in SWF the amount of organic substance loss during the preparation step, can be calculated. However, since the calibration curve for the samples in SWF also is composed in SWF, any matrix effects should been accounted for. There is one difference between the SWF samples in the calibration curve and the SWF sample from the Franz cells, though. For the calibration curve samples, the organic substance is first dissolved in a suitable solvent, water for BAC and water:methanol (1:1) for OCT, before adding a concentration of 1 mg/ml of those stock solutions to the SWF samples. For the SWF samples on the Franz cells, the organic substance is dissolved directly in the SWF from the cellulose samples containing the organic substances. More investigations are required on the effect of the organic substances being in a dissolved state before added to the SWF, compared to adding them in their natural state.

## 5.5 Outlooks

To further develop the method for measuring the release of organic substances in different media, the analytical technique for the measurements needs to be validated. During this Master's thesis project, the analytical method with LC-MS for both OCT and BAC have been continuously developed, but still, more knowledge is needed in terms of preparation of the samples.

The unexpected low concentrations of OCT in the polymeric microspheres in the 20% ethanol solution needs to be further investigated. Further investigation regarding the most ideal medium for measuring the release of OCT in the polymeric microspheres may be evaluated if moving forward with the project. Water might be a more appropriate medium if the microspheres are affected by the ethanol.

Adding excipients, either in the SWF or in the material containing the active substance might lead to more robust measurements. Since the heating of the Franz cells broke before any evaluation of how temperature affects the release of the organic substances, would this aspect also be an interesting parameter to evaluate.

More detailed investigation regarding the behavior of the antimicrobial substances in different matrices, especially OCT, would be interesting. With UV-Vis or light scattering, it might be possible to study the aggregation and surface interactions of the surface active agents and then maybe connect it to different behaviors exhibited during the release measurements.

# 6
# Conclusion

Currently, there are no standardized methods for measuring the release rate of antimicrobial substances that are incorporated in wound care dressings. In this project, the methodology of the Franz cell diffusion system was utilized for the development of a method for measuring the release rate of active substances. For the method to be reliable, the method was evaluated for each specific material, active substance, and receiving medium.

Firstly, the different parameters that might affect the release of silver sulfate from a PVA based gelling fiber silver-containing material were evaluated. The conclusion could be drawn that the release curves were similar, whether the maximum absorption volume of water of half of the maximum absorption volume was added to gelling the silver-containing material. The temperature had neither effect on the release of silver in water, comparing the release at room temperature and approximate skin temperature. Two different sampling techniques were used and the concentrations measured using them both was equal, concluding that both sampling techniques are valid. However, the sampling technique using a needle and syringe through the sampling port is more practical, time-saving, and enables several measurements on the same specimen, reducing errors caused by variation between replicates. Therefore, the sampling technique through the sampling port was considered to be the superior one. The adaptations of the results to the physical model showed that the method was robust for the measurements of the release of silver from a solid by diffusion into water. The adaptions also indicated that the method was suitable for the same measurement in SWF.

From the measurements of the organic substances, the conclusion that the release of octenidine impregnated on cellulose in water, 20% ethanol and 6wt% Brij in water is detectable. However, the OCT concentrations in 6wt% Brij is very low, and any release profiles are hard to distinguish. For OCT impregnated on cellulose, almost all the OCT released in 20% ethanol within 72 h. The organic substance BAC impregnated on cellulose is also completely released in water after 72 h, and the release in SWF is low but detectable.

For measuring the release of the organic substances encapsulated in polymeric microspheres, where the aim is to achieve a controlled release rate, more studies are required. Further investigations of the analytical technique LC-MS, more specifically in the preparation step of the samples, are needed for the continued development of the method for measuring the release of the active substance.

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# A

# Appendix: Calculations

When samples are removed from the receiver chamber and replaced with fresh receiving medium, a correction must be made to account for the dilute factor. This correction is additive and can be calculated as follows:

- c = concentration measured with LC-MS at the sampling [mg/ml]
- $V_{exchange}$ = volume sampled from the cell [ml]
- $V_{cell}$ = total volume in the cell [ml]
- $m_{out}$ = mass substance sampled out from the cell [mg]
- $m_{cell}$ = mass substance in the cell [mg]
- $m_{totalreleased}$ = corrected mass at the sampling (no dilution) [mg/ml]
- $m_{max}$ = Total mass of substance on sample [mg]

First sampling

$$m_{out1} = c_1 * V_{exchange} \tag{A.1}$$
$$m_{cell1} = c_1 * V_{cell} \tag{A.2}$$
$$m_{totalreleased1} = m_{cell1} \tag{A.3}$$
$$Fraction = m_{totalreleased1}/m_{max} \tag{A.4}$$

Second sampling

$$m_{out2} = c_2 * V_{exchange} \tag{A.5}$$
$$m_{cell2} = c_2 * V_{cell} \tag{A.6}$$
$$m_{totalreleased2} = m_{cell2} + m_{out1} \tag{A.7}$$
$$Fraction = m_{totalreleased2}/m_{max} \tag{A.8}$$

Third sampling

$$m_{out3} = c_3 * V_{exchange} \tag{A.9}$$
$$m_{cell3} = c_3 * V_{cell} \tag{A.10}$$
$$m_{totalreleased3} = m_{cell3} + m_{out1} + m_{out2} \tag{A.11}$$
$$Fraction = m_{totalreleased3}/m_{max} \tag{A.12}$$

Fourth sampling

$$m_{out4} = c_4 * V_{exchange} \tag{A.13}$$
$$m_{cell4} = c_4 * V_{cell} \tag{A.14}$$
$$m_{totalreleased4} = m_{cell4} + m_{out1} + m_{out2} + m_{out3} \tag{A.15}$$
$$Fraction = m_{totalreleased4}/m_{max} \tag{A.16}$$

Fifth sampling

$$m_{cell5} = c_5 * V_{cell} \tag{A.17}$$
$$m_{totalreleased5} = m_{cell5} + m_{out1} + m_{out2} + m_{out3} + m_{out4} \tag{A.18}$$
$$Fraction = m_{totalreleased5}/m_{max} \tag{A.19}$$

# B

# Appendix: Graphs

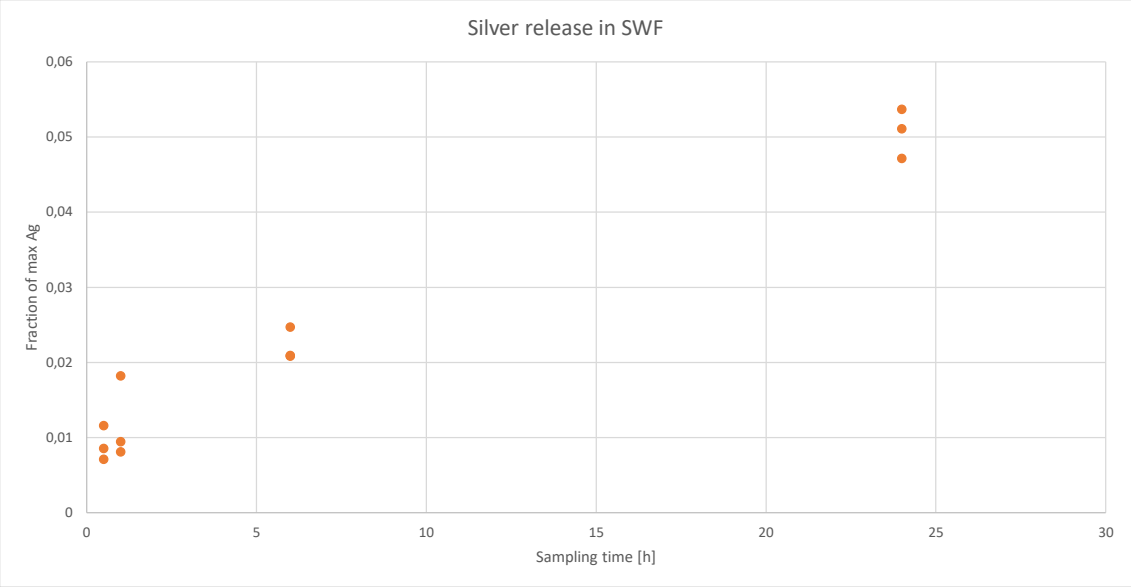


**Figure B.1:** *Graph of the release of silver in SWF*

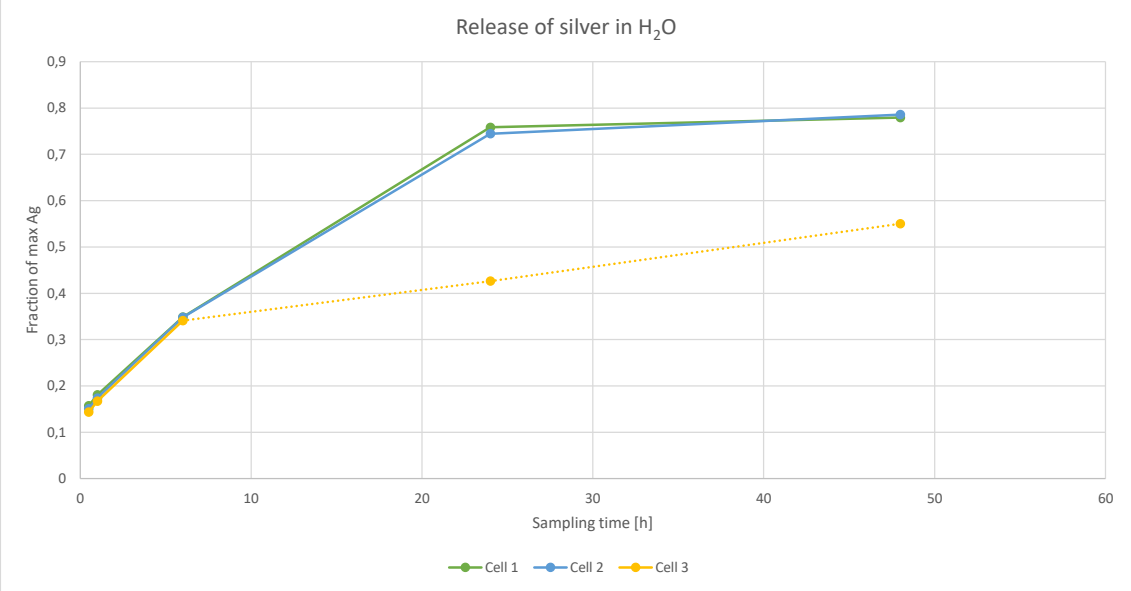


**Figure B.2:** *Graph of the release of silver in $H_2O$ in the three individual cells*

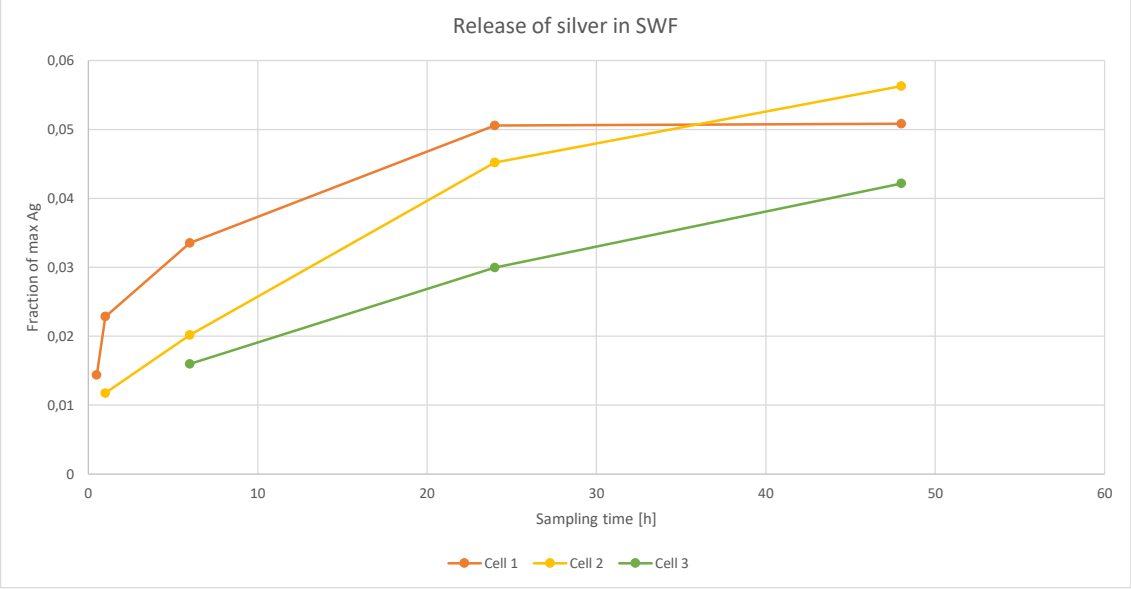


**Figure B.3:** *Graph of the release of silver in SWF in the three individual cells*

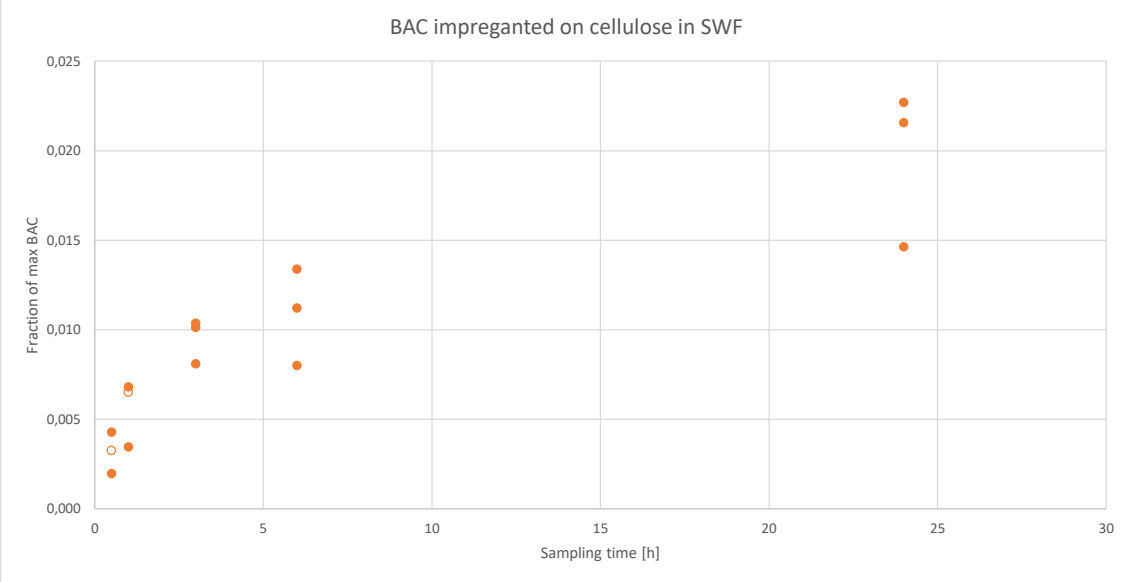


**Figure B.4:** *Graph of the release of BAC impregnated on cellulose in SWF*

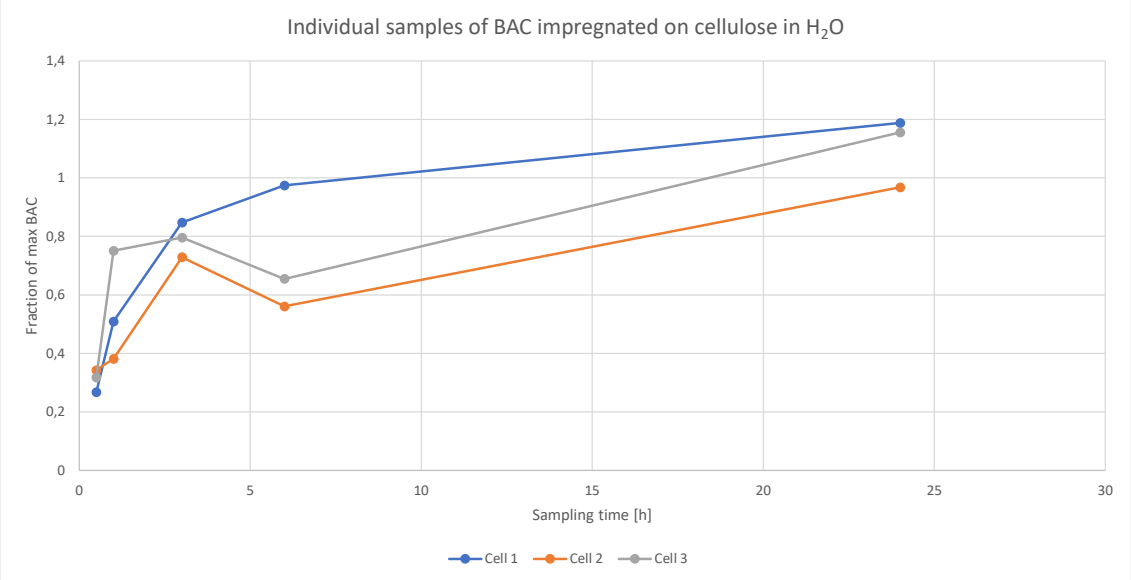


**Figure B.5:** *Graph of the release of BAC impregnated on cellulose in $H_2O$ displayed as the three individual cells*

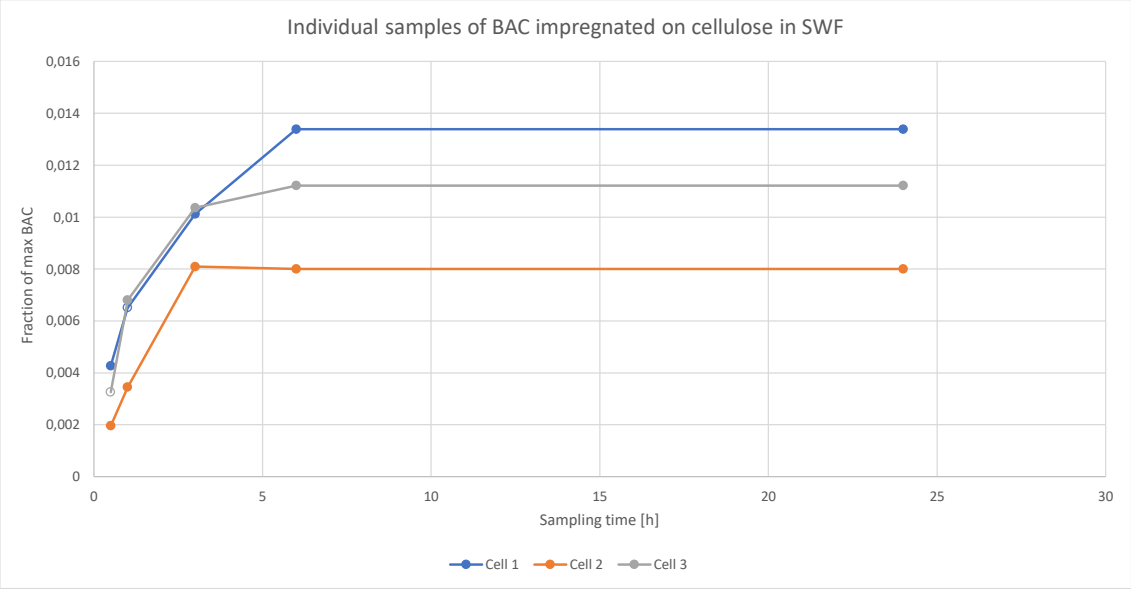


**Figure B.6:** *Graph of the release of BAC impregnated on cellulose in SWF displayed as the three individual cells*

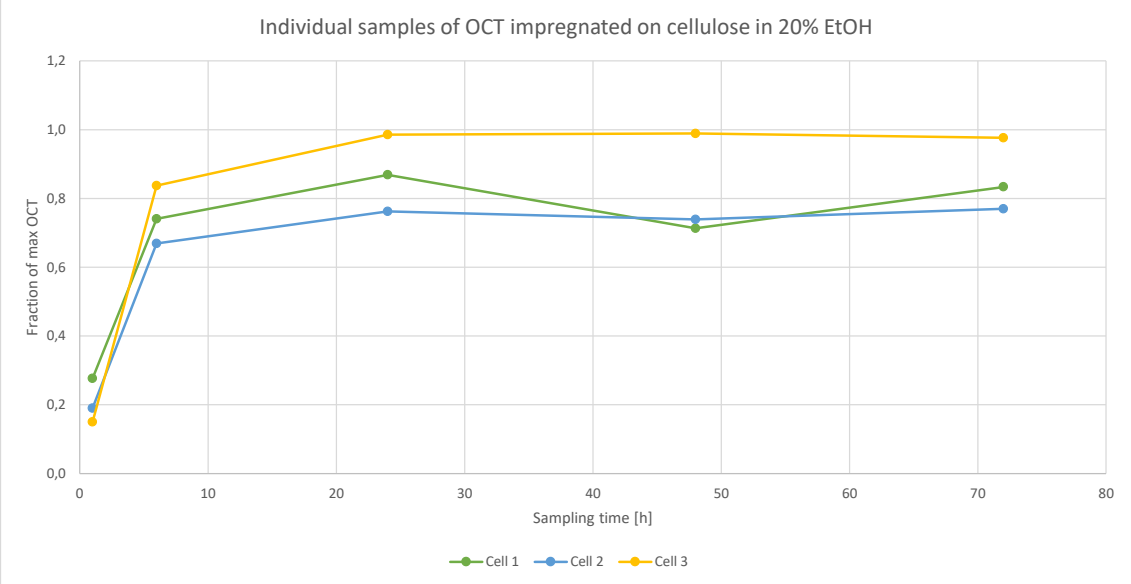


**Figure B.7:** *Graph of the release of OCT impregnated on cellulose in 20% ethanol displayed as the three individual cells*

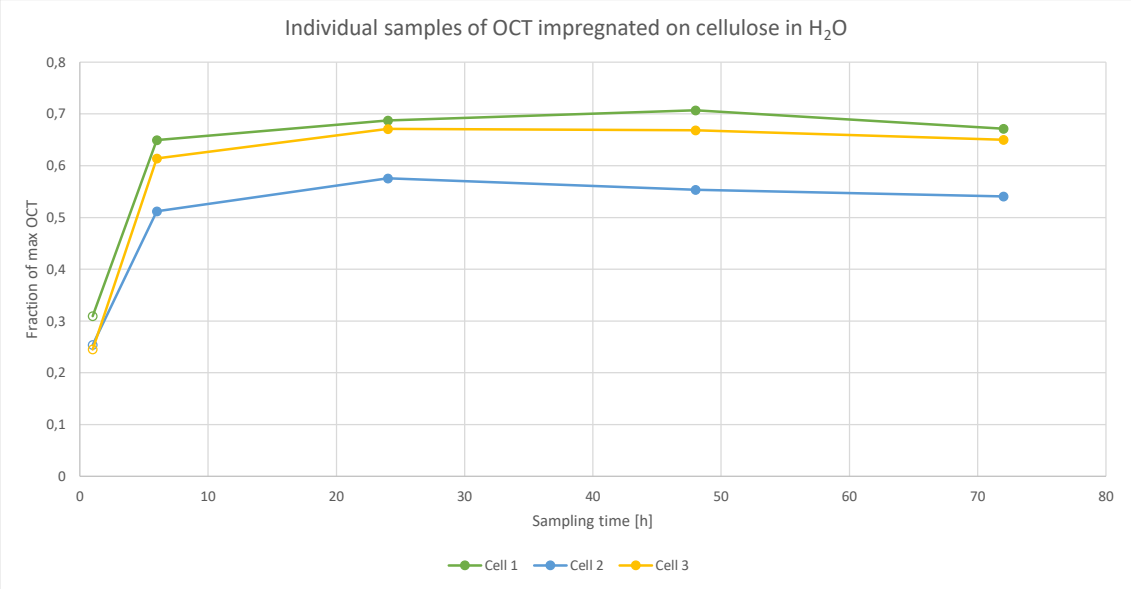


**Figure B.8:** *Graph of the release of OCT impregnated on cellulose in $H_2O$ displayed as the three individual cells*

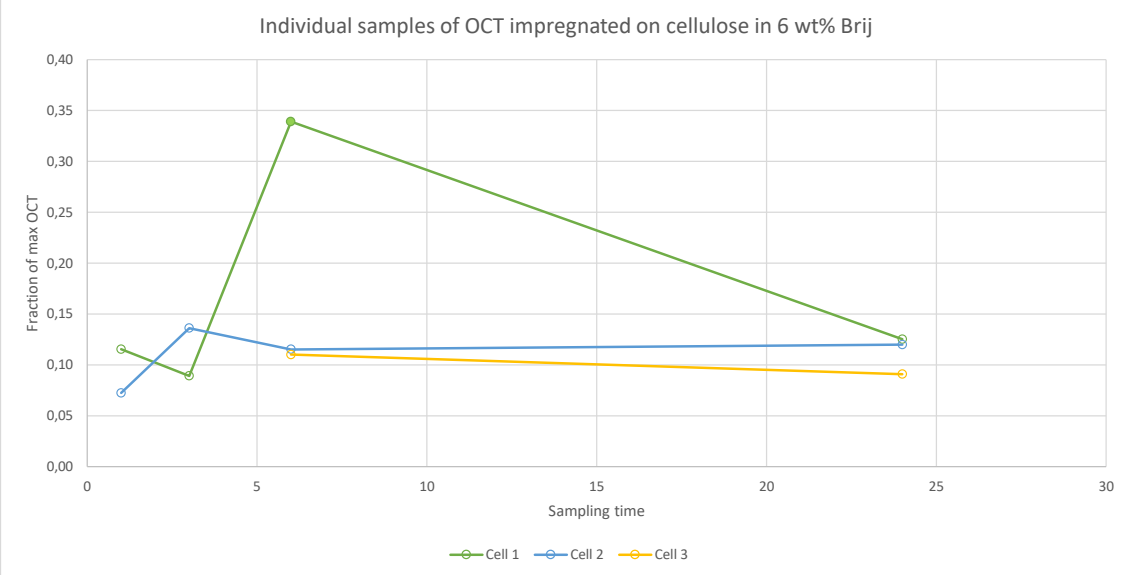


**Figure B.9:** *Graph of the release of OCT impregnated on cellulose in 6wt% Brij displayed as the three individual cells*

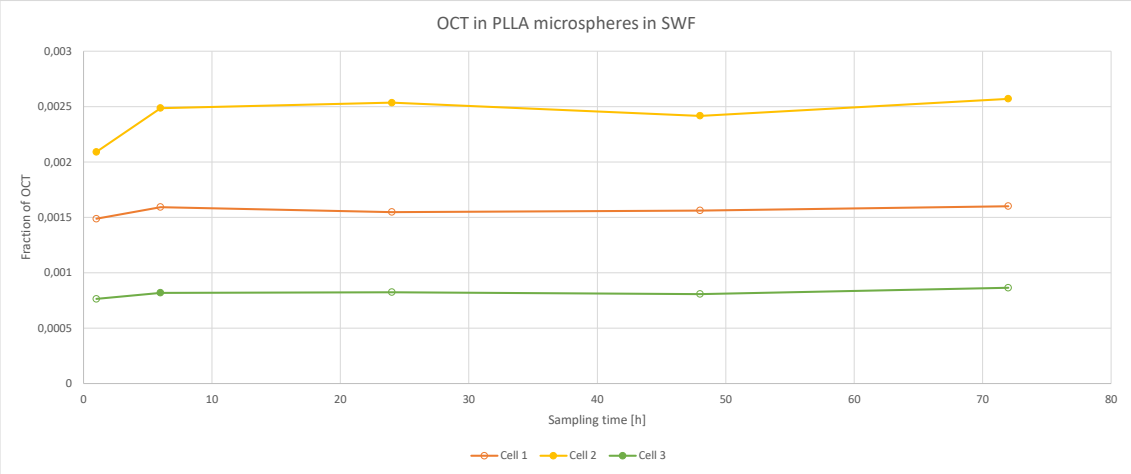


**Figure B.10:** *Graph of the three individual samples of release of OCT in PLLA microspheres in SWF*

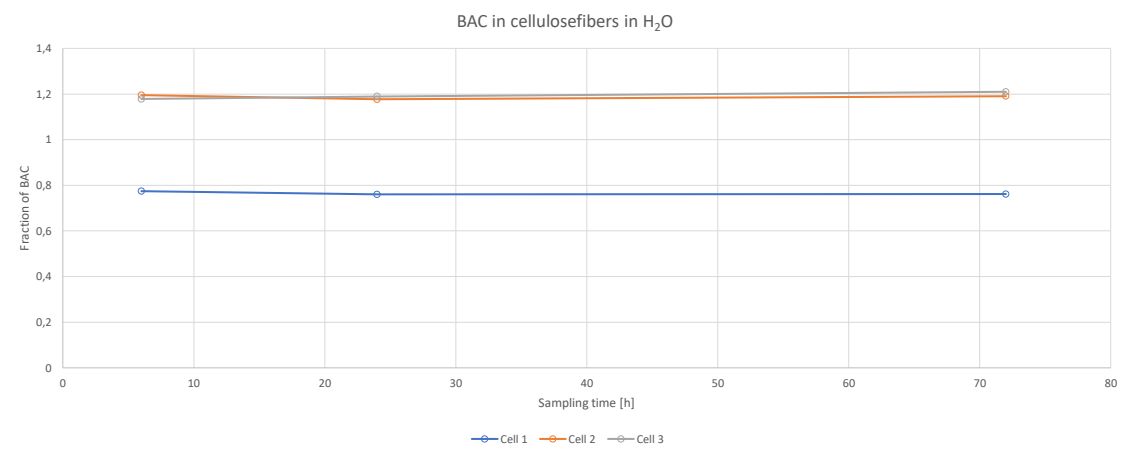


**Figure B.11:** *Graph of the three individual samples over the release of BAC incorporated in the fibers of the cellulose material*