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INSTITUTE OF PHYSICAL AND APPLIED CHEMISTRY
ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

THE EFFECT OF SALTS ON THE HYDROLYTIC STABILITY OF BIOPOLYMER CARRIERS WITH ANTIBACTERIAL SUBSTANCES

VLIV SOLÍ NA HYDROLYTICKOU STABILITU BIOPOLYMERNÍCH NOSIČŮ S ANTIBAKTERIÁLNÍMI LÁTKAMI

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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3) Release of antibacterial substances and measurement of their effectiveness
4) Evaluation and interpretation of results
5) Conclusion

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ABSTRACT

This diploma thesis deals with observing the effects of salt on the stability of biopolymer carriers made of protein and polysaccharide. Theoretical part is concerned with biomaterials and materials for sample preparation and also deals with the current situation of treatment of infected wounds with various materials. Within the experimental part, the carriers were prepared in two different buffers with different concentrations of sodium chloride in order to stabilize bioactive proteins, peptides or enzymes generally used for carrier entrapment. In every series of samples there were prepared both crosslinked and non-crosslinked samples. Properties like hydrolytic stability and swelling ratio were tested and the effect of salt concentration on sample stability was observed. Since the optimal salt concentration has been established, antibacterial enzyme was entrapped into all carriers and its release was monitored. Whereas at non-crosslinked samples significant differences between salt and salt-free samples both stability and enzyme release have been monitored, at crosslinked samples the differences were neglectable. However, in all cases the enzyme released from foamed samples according to first order kinetics, which is concentration dependent. Based on the results from disk diffusion test provided on different strains of *S. aureus*, the concentration of 325 mg/ml, have been chosen for my further work.

Prepared carriers with antibacterial enzyme could be used as both- fast or prolonged-releasing wound dressings for *Staphylococcus aureus* or methicillin-resistant *Staphylococcus aureus* (MRSA) infected tissue.

KEY WORDS

Scaffold, collagen, carboxymethyl cellulose, antimicrobial enzyme, Staphylococcus aureus, MRSA

ABSTRAKT

Táto diplomová práca sa zaoberá vplyvom soli na stabilitu biopolymérnych nosičov z proteínu a polysacharidu. Teoretická časť sa zaoberá biomateriálmi a materiálmi na prípravu vzoriek a taktiež sa zaoberá momentálnou situáciou liečby infikovaných rán rôznymi materiálmi. V experimentálnej časti boli pripravené nosiče v dvoch rôznych pufroch, s rôznymi koncentráciami chloridu sodného v snahe stabilizovať bioaktívne proteíny, peptidy alebo enzýmy, všeobecne používané a vkladané do nosičov. Boli sledované vlastnosti ako hydrolytická stabilita, botnanie a vplyv rôznych koncentrácii soli na stabilitu vzorku. Keď bola stanovená vhodná koncentrácia soli, do vzoriek bol pridaný antibakteriálny enzým a bolo sledované jeho uvoľňovanie. Zatiaľ čo pri nesieťovaných vzorkách boli sledované významné rozdiely v hydrolytickej stabilite a uvoľňovaní pre vzorky so soľou a bez nej, pri sieťovaných vzorkách boli tieto rozdiely zanedbateľné. Vo všetkých prípadoch sa však enzým uvoľnený zo vzoriek uvoľňoval podľa kinetiky prvého rádu, ktorá závisí na koncentrácii. Na základe výsledkov z difúznych testov robených na rôznych kmeňoch *Zlatého stafylokoka* bola pre túto prácu vybraná koncentrácia lyzostafínu 325 mg/ml.

Pripravené nosiče s antibakteriálnymi enzýmami by mohli byť používané ako kryty rán s krátkodobým alebo dlhodobým uvoľňovaním na rany infikované *Zlatým stafylokokom* alebo methycilín rezistentným *Zlatým stafylokokom* (MRSA).

KĽÚČOVÉ SLOVÁ

Scaffold, kolagén, karboxymethyl celulóza, antimikrobiálny enzým, Zlatý stafylokok, MRSA

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DECLARATION

I declare that my diploma thesis was worked out independently and that the used references are quoted correctly and fully. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, VUT.

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student's signature

PREHLÁSENIE

Prehlasujem, že som diplomovú prácu vypracovala samostatne a že všetky použité literárne zdroje som správne a úplne citovala. Táto práca je z hľadiska obsahu majetkom Fakulty chemickej VUT v Brne a môže byť použitá ku komerčným účelom len so súhlasom vedúceho diplomovej práce a dekana FCH VUT.

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LIST OF CONTENT

1 INTRODUCTION

One of the most pressing issues today are infectious complications in patients with acute or chronic wounds arising during either hospitalization or outpatient care. The prevalence of such cases is increasing worldwide, including countries with advanced health care systems. Patients with acute injuries (burns) or with early chronic wounds (venous ulcers, diabetic foot, pressure sores) have completely compromised all barrier functions of the skin. This is the reason why are skin and soft-tissues seriously open to many infective complications. Staphylococci, especially *Staphylococcus aureus*, are the most common cause of skin and soft-tissue infections. The increasing resistance of microorganisms to many antimicrobial preparations has been known for several decades. However, unfavourable acceleration in this field has been recorded particularly over the past ten years which have seen a dramatic increase in the number of resistant PPM (potentially pathogenic microorganisms). This development often leads to a situation where available antimicrobial therapies are not a treatment option.

Current healing of infected wounds uses mainly antibiotics, silver, zinc and other antimicrobial agents based on antiseptics. Biomaterials in combination with other natural materials and bioactive molecules have also been used as wound dressings to accelerate healing and reduce scar formation. Biopolymers, in addition to their function as a physical barrier and absorptive material, stimulate the healing process by promoting the formation of granulation tissue and epithelisation due to direct effect on wound biochemistry. Among other biopolymers there are dextran, alginate, hyaluronic acid, derivatives of cellulose and collagen, which are generally applied.

2 CURRENT STATE OF ART

2.1 Structure and properties of skin

Skin is one of the most important and the largest organ in the human body. The skin covers an area from about 1.5 m^2 to 2 m^2 depending on the weight and height of person. Function of the skin is diverse. The main functions of skin are:

- Protection: against mechanical, thermal and chemical impacts, UV radiation, microorganisms etc.
- Repair and adaptation: self-healing and change in composition and structure when injured or exposed to stress.
- Sensation: through its mechanoreceptors and thermoreceptors.
- Temperature regulation: control of the sweating.

Skin is also responsible for the synthesis of vitamin D and the excretion of water, urea, ammonia, and uric acid. Besides these functions, the appearance of the skin has important implications on social interaction providing information about health, age, gender, etc. Human skin structure is organized in three main layers: epidermis, dermis, and hypodermis. (Figure 1)

The epidermis is thin, with a typical thickness of about 20-150 nm, depending mostly on the body site except for the palms and soles that are thicker. It mainly consists of keratinocytes (about 90 %), which differentiate in the stratum basale and then migrate outwards changing their shape, physiology, and functions. Dead keratinocytes, the corneocytes, are stored in the outermost layer forming the stratum corneum, which has a thickness of about 14 nm. The epidermis acts as a waterproof barrier and contains melanin, which is responsible for skin colour.

The thickness of the dermis varies between 1 and 4 mm. The dermis is built up of collagen and keratin fibers, which provide structural strength to the skin. The dermis contains blood vessels, lymph vessels, hair follicles, and glands that produce sweat, which helps regulate body temperature, and sebum, an oily substance that helps keep the skin from drying out.

The hypodermis or the fat layer lies below the dermis and helps protect the body from heat, cold, and from mechanical shocks. [1, 2]

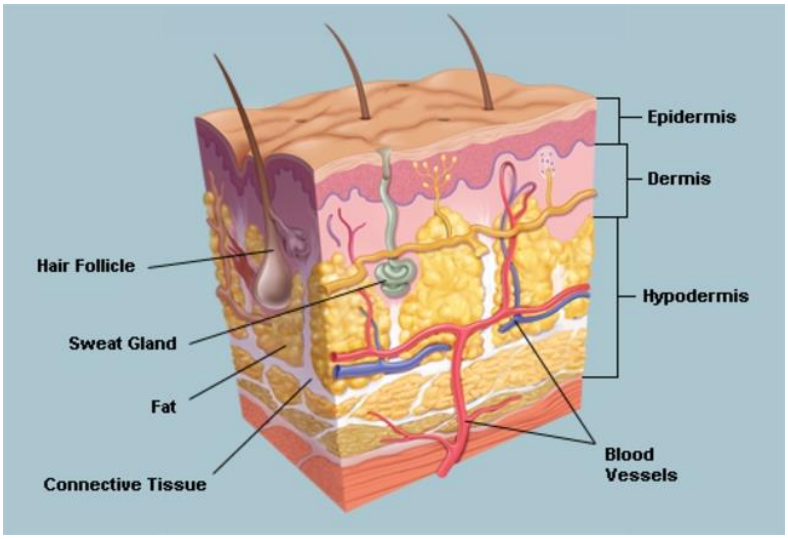

Figure 1: Structural depiction of layers of human skin [3]

2.2 Tissue engineering and scaffolds

Tissue engineering aims to regenerate damaged tissues instead of replacing them by developing biological substitutes that restore maintain or improve tissue function. The main goal of tissue engineering is to synthesise substitutes that mimic the natural extracellular matrix to help guide the growth of new functional tissue. Biological tissues consist of cells, signalling mechanisms, and extracellular matrix. Tissue engineering technologies are based on biological triad and involve the successful interaction between three components:

- The scaffold that holds the cells together to create the tissue physical form.
- The cells that create the tissue.
- The biological signalling molecules that direct the cells to express the desired tissue phenotype.

Scaffold is the central component that is used in tissue engineering to deliver cells, drugs and genes into the body. They are commonly recognized as defined structure biomaterials potentially able to perform some useful functions. The scaffolds provide 3D structure for cell adhesion, proliferation, differentiation and secretion of extracellular matrices to guide new tissue formation and regeneration. The most important properties for scaffolds are biocompatibility, biodegradability, non-toxicity, non-antigenicity, cost-effectiveness and long shell life. They are usually made by processes as lyophilisation, fibre bonding, foaming or 3D printing. [4]

The major applications of tissue engineering skin substitutes are in promoting the healing of acute and chronic wounds. The three-dimensional scaffolds can not only cover wound and give a physical barrier against external infection as wound dressing, but also can provide support both for dermal fibroblasts and the overlying keratinocytes for skin tissue engineering.

An ideal scaffold in skin tissue engineering is the one that mimics the natural environment for skin growth through appropriate cell infiltration, proliferation, and differentiation. It should

be biodegradable, permeable to oxygen, water and nutrient exchange and should be protective against infection and mechanical damage. [5, 6]

Different forms of wound dressings have been developed. Materials used to cover wounds and burns are also called artificial skin, as they fulfil the functions of normal skin within areas with wounds and partly destroyed skin. Wound and burn covering material are classified as traditional dressing, biomaterial-based dressings and artificial dressings.

Traditional dressings are the most commonly used materials for wound and burn dressings. The best sample of these group is gauze and gauze-cotton composites which have very high absorption. As they cause rapid dehydration whereas they are being removed from the wound surface, they can cause bleeding and damage of newly formed skin.

Biomaterial-based dressings are natural dressings with collagen type structures, including elastin and lipid. These dressings can be categorized under following groups: allografts, tissue derivate and xenografts. Allografts used as a most common source fresh or freeze-dried skin fragments taken from patient or patients relatives. The disadvantages of these dressings are risk of the body's rejection to a transplanted tissue and also a difficulty of preparation these dressings. Xenografts are commercially available materials contrary to autographs and allografts. The most common of xenografts is the one derived from pig because it has a long shelf-life, can be sterilized easily and the pig skin is the most similar to the human. Artificial dressings are fabricated from synthetic materials such, as non-biological material and polymers which are not found in skin. Many natural (collagen, chitosan...) and synthetic (teflon, polyurethanes and their derivates, silicon) polymers are being used in the preparation of artificial dressing materials. [7]

There is an urgent clinical need to develop novel antibacterial therapies to destroy biofilms which will henceforth, reduce healthcare infections. Nanostructured materials are attractive because of their capability and selectivity, particularly in pharmaceutical and biological applications. The antimicrobial activities of metal oxide nanoparticles and their selective toxicity to biological systems suggest their potential applications as diagnostic, therapeutics, and nanomedicine-based antibacterial agents. The compensation of using these metal oxides nanoparticles as antimicrobial agents are their better efficiency on resistant bacteria, less toxicity, and heat resistance.

Zinc oxide nanoparticles (ZnO) have many significant features such as physical and chemical stability, high catalysis activity, and effective antibacterial activity.

Titanium dioxide nanoparticles (TiO_2) decompose organic compounds by the formation and constant release of hydroxyl radicals and superoxide ions when exposed to non-lethal UV light, which is highly efficient in inhibiting the growth of MRSA.

Zinc oxide and titanium dioxide nanoparticles are well known for their inhibitory and bactericidal effects. There were studies designed to determine the efficacy of zinc and titanium dioxide nanoparticles against biofilm-producing methicillin-resistant Staphylococcus aureus. Biofilm production was detected by the tissue culture plate method. Biofilms were subjected to antimicrobial activity using commercially available zinc and titanium dioxide nanoparticles.

The nanoparticles showed considerably good activity against the isolates, and it can be concluded that they are promising as antibacterial agents. [8]

Golden nanoparticles are well known to be biocompatible and are extensively used in biomedical domains. The nanoforms of Au alone do not possess antimicrobial activity and must be joined with another chemical species to be effectively applied in antimicrobial applications. For example, Au nanoparticles can be linked with collagen or with gelatin or chitosan to be used in wound healing. In a study, Au nanoparticles were combined with cryopreserved human fibroblasts and topically applied to burn wounds. Treated wounds exhibited a greater healing rate, reduces inflammatory stage, and amplified collagen installation. In another research, Au nanoparticles were loaded into N,N,N-trimethyl chitosan/alginate complex. This nanocomposite presented good biocompatibility and a high wound dressing perspective. [9, 10, 11]

2.3 Biopolymers for skin tissue engineering

Biopolymers and various natural polymers have been used in many biomedical applications such as pharmaceuticals, tissue engineering, tissue regeneration scaffolds, drug delivery agents, etc. In wound care, they are used as dressings for acute or chronic wounds and as regeneration templates. Biopolymers can be derived from a variety of sources (animals, plants, and microorganisms). Due to their similarity with extracellular matrix (ECM), high biocompatibility, and high-water holding capacity, these polymers-based scaffolds are appealing for skin repair and regeneration purposes.

Biopolymers with attractive properties for wound healing applications include alginate, proteins, collagens, gelatin, elastin, fibroin, hyaluronic acid, cellulose, chitosan, etc. [12, 13, 14]

For example, alginate dressing. Water-insoluble calcium alginate is transformed into a hydrophilic gel by exchanging the calcium for sodium ions. The wound exudates are the source of the sodium ions. The hydrophilic gel clears the ulcer and keeps the wound in a moist state, and facilitates granulation and epithelization. Also, it helps wound homeostasis through the release of calcium. This kind of dressing activates fibroblast growth and cell-mediated responses in a wound, and accelerate clotting processes. These types of dressings are used for diabetic foot, inflamed wounds with bacterial contamination, and thanks to the hemostatic features, they can be also used for bleeding wounds. [15, 16]

Another example of using biopolymers as a wound dressing is hydrocolloid dressing. Hydrocolloid dressings are produced in two forms (a plate and self-adhesive hydrocolloid gel), both are made of carboxymethylcellulose, gelatin, and pectins. In the presence of wound exudates, hydrocolloids absorb liquid and form a soft gel, the properties of which are determined by the nature of the formulation. These types of dressings are used for wounds with minimum or medium amounts of exudates in every stage of healing of the wound. These types of dressings should not be used for infected or necrotic wounds. [17]

2.3.1 Collagen

Collagen is a natural protein, a part of ECM contained in various tissues. Several distinct types of collagen had been identified which collectively, represent about one-third of the total protein of vertebrate animals. As a function of structure and supramolecular organization, they were grouped as fibril-forming (types I, II, III, V, XI), fibril-associated (types IX, XII, XIV), membranes (types IV, VII, VIII, X) or other specific function. Fibrils, which are forming collagens are the major structural element of connective tissue, forming a scaffold that gives stability and integrity to tissue and organs. Collagen is a biocompatible and biodegradable material and an essential wound dressing component stimulating wound healing. [18, 19]

2.3.1.1 Structure of collagen

The collagen molecule consists of three polypeptide subunits known as alfa-chains (Figure 2). Each of these chains contains characteristic L-handed amino acid sequence of polyproline, often termed as polyproline type 2 helix. The proper folding of each of these chains requires a glycine residue to be present in every third position in the polypeptide chain. For example, each alfa-chain is composed of multiple triplet sequences of Gly-Y-Z in which Y and Z can be any amino acid. Y is commonly found as proline and Z is usually presented as hydroxyproline. The presence of hydroxyproline in the Y position is also thought to contribute to the stability of the helical form.

Those three alfa-chains are twisted around one another in a rope-like manner to produce the overall tightly packed triple-helical form of the molecule. [20, 21, 22]

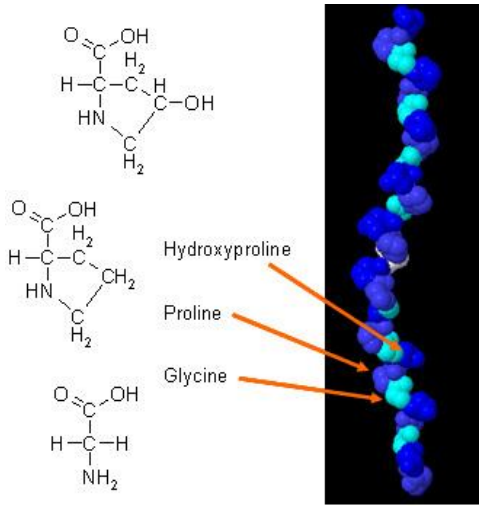

Figure 2: Molecular structure of collagen with glycine, proline and hydroxyproline amino acids residues [22]

There are close to 30 different types of collagen that are found in various types in the human body. The most abundant type of collagen presents in the human body is type I. It can be found in bones, tendons or organs, collagen II based mainly in cartilage, collagen III can be found in reticular fibres, collagen IV is found in the basement membrane of cell membranes and collagen V has its place in hair or nails. [23]

2.3.1.2 Collagen-based scaffolds

Collagen-containing dressings are biologically active, in addition to the absorption effect, stimulate the natural activity of fibroblasts, keratinocytes and macrophages, activate fibronectin, and stimulate angiogenesis. Collagen, therefore, participates in all phases of healing frames due to the specific arginine-glycine-aspartate groups, which induces cellular adhesion. This interaction makes collagen being a natural substrate for cell growth, proliferation, differentiation, and function regulation. There are many already available gels, sheets, lattices, or scaffolds based on collagen, which is used as a temporary skin burns covering reducing scar formation. However, collagen is hardly ever used in its pure unmodified form. Imperfections of collagen (bioactivity, morphology, mechanical properties, etc.) can be eliminated by combination with other biomaterials and bioactive molecules (e.g. hyaluronic acid, chitosan, hydroxyapatite, platelet-rich plasma, etc.)

It is assumed that a combination of cellulose fibres with collagen will allow the preparation of materials with improved properties under favourable manufacturing costs. Among the cellulose derivatives, carboxymethylcellulose (CMC) is appearing as a proper option to interference with collagen fibers and possessing good water capacity. The carboxymethylated cellulose fibres themselves produce, in contact with the wound, a cohesive gel absorbent to the fibre structure to maintain optimal moisture and wound environment. As collagenous cover acts as a substrate providing an alternative source of collagen into the wound (which can be degraded by a high concentration of proteolytic enzymes in chronic wounds), we assumed that the combination of collagen with CMC and enzybiotics will offer the ideal product for healing difficult to treat septic chronic wounds. [24, 25]

2.3.1.3 Crosslinking of collagen-based scaffold

Crosslinking of the collagen-based scaffolds is an effective method to modify the biodegrading rate and to optimize the mechanical property for implantation purposes. The principle of a crosslinking reaction relies on the modification of amine and carboxyl groups within the collagen molecules, to allow the formation of covalent bonds.

EDC (1-Ethyl-3-[3-dimethylami-nopropyl]-carbodiimide hydrochloride) is a zero-length crosslinker for biochemical conjugations. It can efficiently form conjugates between two protein molecules, between a protein and a peptide, or between proteins and oligonucleotides, and with small molecules. For higher coupling efficiency and more stable

amine-reactive intermediates, EDC crosslinking protocols often include NHS (N-hydroxysuccinimide) or its water-soluble analog Sulfo-NHS. EDC in conjunction with NHS allows for 2 step coupling of two proteins without affecting the carboxyl's of the second protein.

At first, EDC activates carboxyl groups and forms an amine reactive O-acetylsourea intermediate that naturally reacts with primary amines to form an amine bond and an osourea by product. The O-acylisourea intermediate is unstable in aqueous solutions and failure to react with an amine will cause hydrolysis of the intermediate, regeneration of the carboxyls, and the release of N-substituted urea. Therefore, it is necessary to quench the EDC activation reaction with a thiol-containing compound. EDC couples NHS to carboxyls, which form an NHS ester that is considerably more stable than the O-acylisourea intermediate and allows for efficient conjugation to primary amines at physiologic pH. [26, 27, 28]

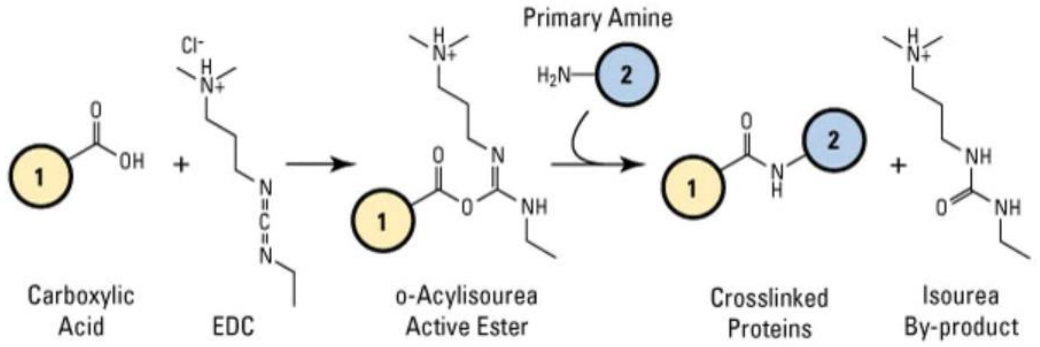

Figure 3: EDC crosslinking reaction scheme [29]

2.3.2 Carboxymethylcellulose (CMC)

CMC is a natural, biocompatible, and biodegradable polysaccharide (Figure 4) used as a viscosity modifier or thickener to stabilize emulsions in various products. It has excellent water retaining capacity and it makes it an attractive candidate for applications such as wound dressing, drug delivery, and skin engineering.

The polar carboxyl or hydroxyl groups make cellulose to be water-soluble and chemically reactive and serve as active sites for preparing CMC gels. CMC can create a stable 3D network by chemical crosslinking. [30, 31]

CMC is widely used in drug delivery systems. There is also an application of carboxymethylcellulose films for bacterial infection wound healing. Furthermore, they are used in hemostatic dressings. CMC is also used in the hydrocolloid dressings which are meant to interact with exudate or wound fluid to form a gel, which leads to a moist environment and speeding the healing process. [32, 33]

Its sodium salt, known as sodium carboxymethyl cellulose (NaCMC) is often used form, synthesized by the alkali-catalysed reaction of cellulose with chloroacetic acid. CH_2COOH groups are substituted on the glucose units of the cellulose chain through an ether

linkage. Sodium carboxymethyl cellulose is a pale yellow, odourless, nontoxic, water-soluble powder, stable in the pH range between 2 and 10, and insoluble in organic liquids. (Equation (*E1*), (*E2*))

$$2\,ClCH_2COOH + Na_2CO_3 \rightarrow 2\,ClCH_2COONa + CO_2 + H_2O \qquad (E1)$$

$$C_6H_7O_2(OH)_3 \cdot NaOH + Cl \cdot CH_2COONa \rightarrow C_6H_7O_2(OH)_2 \cdot O \cdot CH_2COONa + NaCl + H_2O \qquad (E2)$$

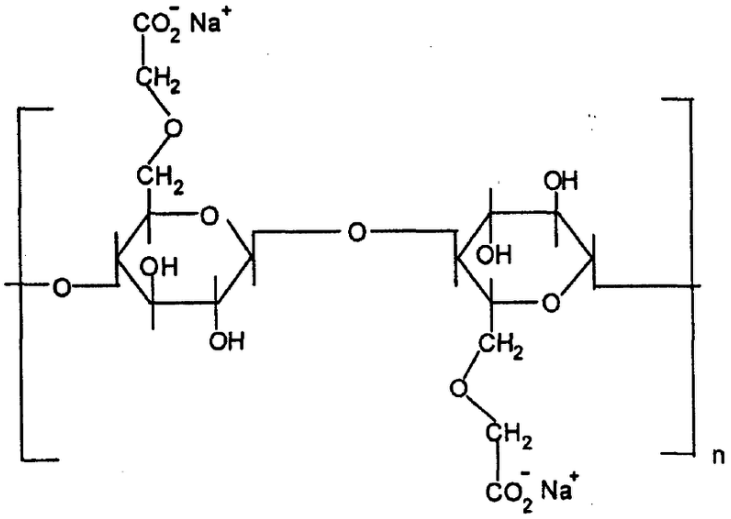

Figure 4: Chemical structure of NaCMC [34]

2.3.3 Polysaccharide-protein interactions

Polysaccharides and proteins are natural polymers that are widely used as functional ingredients for various food colloids or emulsion formulations. Proteins and polysaccharides consist of long linear chains of covalently linked subunits. These two classes may look similar but they differ, in the kind of subunits and consequently, in the structure, they adapt in aqueous solution. The flexibility of the polysaccharide chain depends on the linkage type, through which carbon atoms and the sugar ring are connected (1.4 or 1.6) into the anomeric form of the linkage (alfa or beta). Depending on the flexibility and polysaccharide may adopt a stiff linear, helical or random coil conformation in solution. Polysaccharides commonly show only limited or even no differences between subunits but on the other hand, there are also protein chains that are built up from many different amino acids for every protein in a unique sequence as encoded by genetic material. Those amino acids can be acidic, basic, or neutral and they differ in their polarity (and also a hydrophobicity). Besides by their primary structure, proteins are characterized by their conformation at a secondary, tertiary, and sometimes quaternary level. Proteins can also be subdivided in globular and random-coil like on the basis of their

conformation. Proteins are generally surface-active, polysaccharides, besides some exceptions, are not. In principle, all protein molecules of one type are identical, as opposed to polysaccharides which are heterodisperse in molecular weight and distribution of subunits. The molecular weight of polysaccharides (100 – 1000 kDa) is often much larger than the molecular weight of proteins (10 – 60 kDa). [35, 36]

2.4 Infection and antibiotic resistance

Wound and skin infections are the growth and spread of microbes, usually bacteria, within the skin or a break or wound in the skin. These infections trigger the body's immune system and cause inflammation and tissue damage within the skin, or wound and slow the healing process. The wound healing, in any tissue, is a normal biological process and it involves four complex steps: hemostasis, inflammation, proliferation, and tissue remodelling. [37, 38]

A wound can be classified as chronic and acute. Acute wounds represent the injured skin that heals through the regular phases of wound repair and chronic wounds need longer healing time. The longer healing time is usually attributed to many factors, including amplified levels of inflammatory mediators, wound infection, or poor nutrition. The skin microbiota diversity and the cutaneous microenvironment such as dry, moist, and sebaceous can influence the wound repair process and the occurrence of skin infections. As soon as the skin is impaired, typical microorganisms of the normal skin flora and exogenous bacteria and fungi can soon gain access to underlying tissue, which offers a warm, humid, and nutrient-rich environment for their development. But when the healing is delayed, the normal microbiota of the wound changes and more aggressive microbial types are hosted. Cocci types are present in about 50 % of chronic wound and from that *Staphylococcus aureus* appears the most. An infection at a wound site starts with contamination and continue with colonization and wound infection (Figure 5). [39, 40, 41, 42]

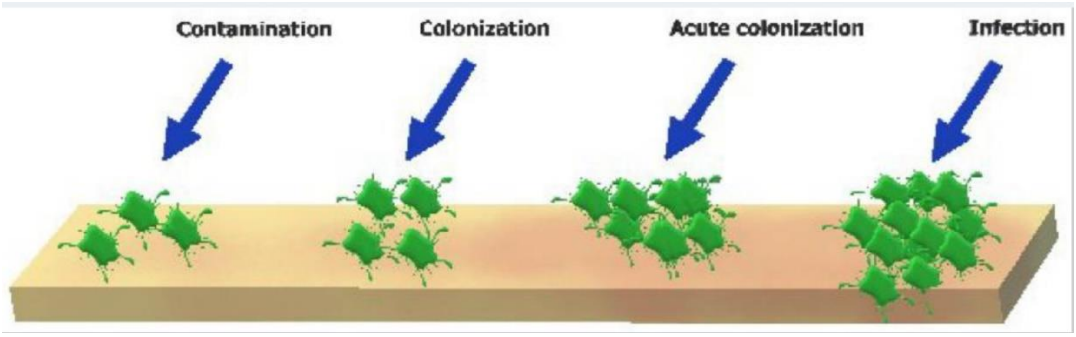

Figure 5: Illustration of the continuum from contamination to wound infection [43]

Infection in chronic wounds is frequently polymicrobial, boosting synergistic effects. This collective effect can be usually supported by oxygen consumption: aerobic bacteria can encourage tissue hypoxia, building advantageous conditions for anaerobic multiplication. Once

anaerobic bacteria species are established, they can obstruct phagocytosis of other microorganisms by producing a short chain of fatty acids. Besides, the nutrient flux from one bacteria can sustain the evolution and proliferation of another. In many types of chronic wounds, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are usually encountered growing simultaneously in co-cultures. Many pathogens can stick together and form biofilms, which are microbial masses surrounded by a polymeric environment, consequently evading the killing activity of antibiotics and host effectors. Biofilms can be considered as a physical obstruction to wound healing where the usual process of the inflammatory phase can be extended. It has been proved that bacterial by-products in the form of fatty acids can impede chemotaxis of neutrophils and phagocytosis of *Staphylococcus aureus* bacterial cells. MRSA infections make the body susceptible to other forms of fungal and bacterial infections which are problematic to eliminate. For example, MRSA represents about 40 % of wound isolates and infect 14-17 % of patients experiencing burn wounds. [44, 45, 46]

In an immunocompromised individual, the bacteria can enter the deeper tissues. With the purpose of accelerating wound healing, topical antimicrobials can promote the treatment of severely infected wounds. Tests and data collected from patients indicate that antimicrobial wound dressing can be helpful in wounds that may be affected by biofilms. [47, 48]

Although antibiotics have significantly improved the health of human beings by treating infections, many of the infectious strain still cause a substantial problem worldwide, by means of antibiotic resistance. [49]

Antibiotics work by inhibiting or killing susceptible microorganisms. Antibiotic resistance evolves naturally via natural selection through random mutation. Infections caused by antibiotic-resistant germs are difficult, and sometimes impossible to treat. In most cases, antibiotic-resistant infections require extended hospital stays. Each year in the U.S. is at least 2,8 million people infected with antibiotic-resistant bacteria or fungi, and more than 35 000 people die as a result. [50]

The most widely used group of antimicrobial agents are the β-lactam drugs. All of the member of these group shares a specific core structure which consists of a four-sided β-lactam ring. Resistance to the β-lactam drugs occurs through three general mechanisms:

1. Preventing the interaction between the target PBP (penicillin-binding proteins) and the drug, usually by modifying the ability of the drug to bind to the PBP.
2. The presence of efflux pumps that can extrude β-lactam drugs.
3. Hydrolysis of the drug by β-lactamase enzymes.

The β-lactamases (originally called cephalosporinases and penicillinases) inactivate β-lactam drugs by hydrolysing a specific site in the β-lactam ring structure, causing the ring to open. The open ring drugs are not able to bind to their target PBP proteins. The known β-lactamases are wide-spread, and the group contains enzymes that are able to inactive any of the current β-lactam drugs. The production of β-lactamases is the most common resistance mechanism used by gram-negative bacteria against β-lactam drugs, and the most important resistance mechanism against cephalosporin and penicillin drugs. [51, 52, 53, 54, 55]

2.4.1 Staphylococcus aureus

Staphylococci are widespread microorganisms including both primarily pathogenic, so commensal species. These are commonly found on the skin and mucosal surfaces of birds and mammals such as part of normal microflora. Staphylococcus aureus does not normally cause infection on healthy skin, however, if it is allowed to enter the bloodstream or internal tissues, these bacteria may cause a variety of potentially serious infections.

Staphylococci were first observed by French microbiologist Louis Paster and Scottish physician and amateur microbiologist Alexander Ogston in 1880.

Staphylococcus aureus is the most dangerous of all of the many common staphylococcal bacteria. These gram-positive, sphere-shaped (coccal) bacteria often cause skin infections (often causing abscesses) but can also cause pneumonia, bone infections, or heart valve infections. The bacteria are spread by having direct contact with an infected person, by using a contaminated object, or by inhaling infected droplets by sneezing or coughing.

A lot of strains have developed resistance to the antibiotics and their effects. If a person takes antibiotics, the antibiotics kill the strains that are not resistant, leaving mainly the resistant strains. The bacteria than may multiply, and if they cause infection, the infection will be more difficult to treat. The resistance of the bacteria often depends on where person got the infection, in hospital or other health care facility or outside. [56, 57, 58]

2.4.2 MRSA

Methicillin-resistant Staphylococcus aureus, commonly known as MRSA, is a form of contagious bacterial infection. It is specific “staph” bacteria that is often resistant to certain antibiotics called beta-lactams. These antibiotics include methicillin and other more common antibiotics such as oxacillin, penicillin, and amoxicillin. MRSA is also called “superbug” an informal term used to describe a strain of bacteria that has become resistant to the antibiotics usually used to treat it.

Most MRSA infections are skin infections that often appear as a bump, a boil or the area is red, tender, and swollen. Sometimes it can be confused with a spider bite. These symptoms can quickly turn into deep, painful abscesses that require surgical draining (Figure 6). [59, 60, 61, 62]

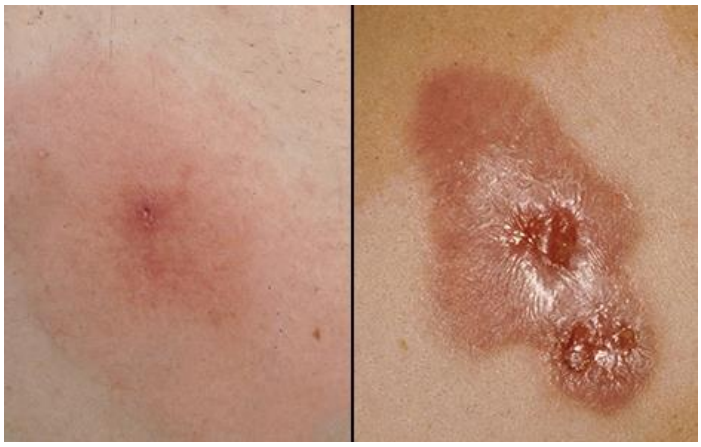

Figure 6: Development of MRSA wound [63]

2.5 Current state of treating infected wounds with dressings

Fibrous scaffolds can be also categorized as porous scaffolds and are made up of nanofibers and have a great potential to mimic the natural environment for human tissue. Nanofibers are synthesized using techniques such as phase separation, drawing, self-assembly and the more widely used electrospinning technique. Nanofibrous scaffolds are used for soft tissue engineering applications and they also act as vehicles for the controlled delivery of drugs and various biological molecules in the form of proteins and DNA. Some synthetic and natural polymers have been utilized for nanofiber fabrications to produce fibrous scaffolds for biomedical applications. These nanofibers can be specially functionalized by a blending or coating technique, or by surface grafting polymerization for attaching ligand molecules and adhesive proteins on the nanofiber surface. Blending of growth factors, genes and drugs directly into the polymer solution during electrospinning is practiced for controlled release properties. There are plenty of natural polymers being used for nanofibrous scaffold fabrication with application as wound dressing and skin substitutes. For example, chitosan nanofibrous scaffolds have been observed to work better than 3D sponges of chitosan in terms of growth, adhesion and differentiation of keratinocytes, fibroblasts and endothelial cells. [64, 65, 66]

2.5.1 Healing infected wounds with silver

The silver ion has been known to be effective against a broad range of microorganisms for a very long time. Today, silver ions are used to control bacterial growth in a variety of medicinal applications, including the healing of infected or burn wounds.

The mechanism of the antimicrobial action of silver ion is closely related to their interaction with thiol groups, but other target sites are a possibility too. Amino acids, such as cysteine, and other compounds containing thiol groups neutralized the activity of silver against bacteria. On the other hand, disulfide bond-containing amino acids, non-sulfur-containing amino acids, and sulfur-containing compounds, such as cystathione, cysteic acid, taurine, and sodium bisulfate,

were all unable to neutralize the activity of silver ions. These and other findings imply that the interaction of silver ions with groups in enzymes and proteins play an essential role in its antimicrobial action, although other cellular components, like hydrogen bonding, may also be involved.

Silver was also proposed to act by binding to key functional groups of enzymes. Silver ions cause the release of K+ ions from bacteria; the bacterial plasma or cytoplasmatic membrane, which is associated with many important enzymes, is an important target site for silver ions. [67, 68]

2.5.2 Zinc oxide and titanium dioxide nanoparticles

There is an urgent clinical need to develop novel antibacterial therapies to destroy biofilms which will henceforth, reduce healthcare infections. Nanostructured materials are attractive because of their capability and selectivity, particularly in pharmaceutical and biological applications. The antimicrobial activities of metal oxide nanoparticles and their selective toxicity to biological systems suggest their potential applications as diagnostic, therapeutics, and nanomedicine-based antibacterial agents. The compensation of using these metal oxides nanoparticles as antimicrobial agents are their better efficiency on resistant bacteria, less toxicity, and heat resistance.

Zinc oxide nanoparticles (ZnO) have many significant features such as physical and chemical stability, high catalysis activity, and effective antibacterial activity.

Titanium dioxide nanoparticles (TiO_2) decompose organic compounds by the formation and constant release of hydroxyl radicals and superoxide ions when exposed to non-lethal UV light, which is highly efficient in inhibiting the growth of MRSA.

Zinc oxide and titanium dioxide nanoparticles are well known for their inhibitory and bactericidal effects. There were studies designed to determine the efficacy of zinc and titanium dioxide nanoparticles against biofilm-producing methicillin-resistant Staphylococcus aureus. Biofilm production was detected by the tissue culture plate method. Biofilms were subjected to antimicrobial activity using commercially available zinc and titanium dioxide nanoparticles. The nanoparticles showed considerably good activity against the isolates, and it can be concluded that they are promising as antibacterial agents. [69]

2.5.3 Enzybiotics

One of the greatest successes of medicine in the 20^{th} century was the discovery and development of antibiotics that allowed to control many diseases caused by microorganisms. But is necessary to search constantly for new therapeutic tools and keep fighting against disease causing microorganisms and that leads us to the concept of enzybiotics. Although microorganisms degrading enzymes have been known since the beginning of the last century, their use was soon forgotten because of the widespread use of antibiotics. The term

enzybiotic is a hybrid word from “enzyme” and “antibiotic” and refers to phages: that is, viruses that attack and lyse bacteria and that can potentially help to fight bacterial diseases.

The most accurate definition of enzybiotics refers to a group of phage associated enzymes that are produced actively during the phage lytic cycle. Phages use them for detaching from the bacterial hosts trough a specific lytic process at the level of the peptidoglycan layer. Also “fungal endoglucanases”, another group of enzymes can be included in this definition. The term “enzybiotics” is wider than was first thought, and it should include all enzymes, regardless of their origin, that are able to act on microbial cells to cause their death.

A bacteriophage is a virus able to infect and kill bacteria and that, in the case of lytic phages, interferes with normal bacterial metabolism, causing the bacterium to lyse. Phages were discovered in 1915 and 1917 by Twort and D’Herelle. Twort was the first author to introduce the term “virus” whereas the name bacteriophage was proposed by D’Herelle.

Biochemically, bacteriophage endolysins (Ply) are peptidoglycan hydrolases enzymes encoded by double-stranded DNA bacteriophages, produced in phage-infected bacterial cells toward the end of their replicative lytic cycle in order to degrade the peptidoglycan of the host cell from inside. This leads to bacterial lysis and subsequent release of progeny phages.

Two proteins, an endolysin and a holin, are crucial for bacteriophage induces bacterial lysis. Holins create membrane pores so that endolysins reach and cleave the peptidoglycan, thus inducing lysis and death of the bacterial cell. As no outer membrane is present in the gram-positive bacteria, peptidoglycan become more susceptible to the action of endolysins due to unrestricted access when applied externally and destroy such microorganisms rapidly. This renders endolysins as interesting antimicrobial candidates, particularly in current scenario of rising bacterial drug resistance. Endolysins being specific peptidoglycan hydrolases, reduce the incidences of antibiotic resistant pathogens rather than merely acting as broad-range antimicrobial. The feature of endolysins targeting unique and highly conserved bonds of peptidoglycan retards the probability of developing resistance against the activity of bacteriophage endolysins. The inimitable capability of endolysins to quickly cleave peptidoglycan in host species specific manner makes them promising potential antibacterial agents. [70]

2.5.3.1 Mechanism of action

Bacteriophages, depending upon structure follow two methods to release their progeny virions from host bacterial cells:

- Filamentous phage is released through bacterial cell walls without killing bacterial cell.
- Non-filamentous phages make use of specific lysine enzymes to either inhibit the synthesis of peptidoglycan in the cell wall of bacteria or hydrolyse the built peptidoglycan using a holin-endolysin system.

Endolysins need a second protein holin to find their substrate molecule in the cell wall. Lysin remains in the cytosol till the late stage of the lytic cycle and hydrolyse the peptidoglycan of

the bacterial cell wall when holins forms pores in the inner membrane of the infected host cell. This results in access of lysin to the peptidoglycan causing rapid cell lysis thus releasing mature phage progeny.

In holin-endolysin system, phage requires both the holin and lysin for host cell lysis. Nevertheless, when lysins are employed as recombinant enzymes and applies exogenously to gram positive bacteria they are well capable of causing rapid lysis as no outer membrane is present to inhibit their access to the cell wall. In gram negative bacteria, the use of endolysin as antibacterial is limited as outer membrane hinders the access of exogenous lysins towards the cell wall peptidoglycan. Phage lysins selectively target specific pathogenic bacteria without affecting surrounding commensal microflora due to narrow host range.

Bacteriophage murein hydrolase enzymes display high specificity towards the cell wall of host bacteria due to presence of well-defined cell wall binding domain that affix the endolysin to its substrate. Bacteriophage induces host lysis with the help of two proteins, endolysin and holin. Endolysin, a kind of muralytic enzyme accumulate in the cytosol during the vegetative cycle and degrade the bacterial cell wall with the help of holin proteins which are accrued inside the cytoplasmatic membrane. Holins as membrane proteins remain in the membrane until a specific programmed time when the membrane becomes abruptly permeable to the endolysins. Destruction of the murein of cell wall and cellular bursting are immediate consequences of lytic action of endolysins. As holin genes direct the length of the infective cycle of lytic phages using holin proteins hence they are subject of deep evolutionary interest. Though action of holins is regulated by a number of diverse proteins, they represent one of the most sundry functional groups, with more than 100 known or putative holin sequences. [71]

2.5.3.2 Major classes of enzybiotics

Bacteriocins

Bacteriocins (narrow spectrum antibiotics) are proteinaceous extracellular substances that are produced by both gram-negative and gram-positive species. They are produced spontaneously or induced by a certain chemical. They inhibit the growth of similar or closely related bacterial strains. Bacteriocins are produced by non-pathogenic bacteria that normally reside in the human body. The loss of these harmless bacteria following antibiotic use may allow opportunistic pathogenic bacteria to invade the human body.

Lysins

Lysins, or also known as endolysins or murein hydrolases are peptidoglycan degrading enzymes released by bacteriophages that help in lysis of bacterial cell wall at the end of the lytic cycle to release the progeny phage particles. They have characteristic lysis or cell-wall binding domain and degrade peptidoglycan with glycosidase, endopeptidase, amidase. They are highly effective against gram-positive bacteria as the outer membrane is absent. The main mode

of antibacterial action of lysins is the enzymatic cleavage of the covalent bonds in peptidoglycan. Depending on their enzymatic specificities, lysin falls into 5 major classes:

- N-acetylmuramoyl-L-alanine amidases
- Endopeptidases
- N-acetylmuramidases (lysozymes)
- Endo-β-N-acetylglucosaminidases
- Lytic transglycosylases

Lysozymes

Lysozymes also known as muramidase or N-acetylmuramide glycanohydrolase, globular protein of 129 amino acid residues. They belong to the class glycoside hydrolase that catalyse the hydrolysis of 1,4-β-linkages. They cleave the 1,4-β-linkages between N-acetyl muramic acid and N-acetyl-D-glucosamine in peptidoglycan structure. Lysozyme is abundant in a number of secretions such as saliva, tears, or human milk. Albumin in egg white is also an abundant source of lysozyme. In human body, lysozyme enzyme is encoded by LYZ gene. Lysozyme is regraded to be a natural antibiotic, it is a significant factor of innate immunity and a unique enzybiotic which has not only antibacterial activity but also antiviral, anti-inflammatory, anti-cancer, and immunomodulatory activities.

Lysozyme is capable of killing only gram-positive bacteria, while gram-negative bacteria are resistant due to the presence of the outer membrane.

Autolysins

An autolysin is an enzyme that hydrolyses the components of biological cell or tissue. It exists in all bacteria containing peptidoglycan. The enzyme functions similar to lysozyme. It cleaves the 1,4-β-linkage between N-acetyl glucosamine and N-acetyl muramic acid. The matrix of peptidoglycan is very rigid, so these enzymes break down the matrix into the small sections so that growth and division of cells can occur. Atl is the major lysin of Staphylococcus epidermidis and Staphylococcus aureus playing an important role in the separation of cells and in virulence, their virulence is also attenuated. For the development of new types of antibiotics, autolysins represent a promising target. [72, 73]

2.5.3.3 Bacteriophages vs Antibiotics

Because bacteriophages are viruses it would be reasonable to speculate that their use in therapy might have undesirable side effects. However, at the same time, it should be recalled that phages are the most abundant organisms in the biosphere, and for example, one liter of seawater contains up to 10000 million phages. So, the human organism is adjusted to living in the presence of these entities.

A phage is unable to replicate if the host is not present. There are data concerning the persistence in the human body of phages administered over several days, so far, there was no evidence of their replication. The rationale suggests that phages, as active therapeutic agents, should increase exponentially inside the organisms and it has been found that a single dose of phage can be more effective than the repeated administration of antibiotics. Also, it has to be considered if the bacteria are able to develop phage resistance mechanisms during treatment. Phage resistant mutants are always generated randomly, but the mechanisms of antibiotic-resistance and phage resistance are quite different. Phages and bacteria have been co-evolving for millions of years, and hence it is more difficult to obtain new, stable phage resistant bacterial strains than antibiotic resistant strains. However, phage therapies are not effective when dormant spores are considered, because spore peptidoglycan is protected from lysozymes and amidases by the external structures of the spore and variations in the chemical nature of the peptidoglycan. These barriers disappear as soon as about 10 minutes after spore germination.

An undesirable side effect when antibiotic therapy is considered is the endotoxin-released when cell lysis occurs. This can be prevented with phage therapy. [74]

The advantages and disadvantages of the use of bacteriophages and antibiotics are summarized in Table 1.

Table 1: Basic properties of bacteriophages and antibiotics for their use as therapeutic agents [74]

Character	Bacteriophages	Antibiotics
Specificity	High	Middle multiple
Side effects	No	Yes
Resistance	No/low	High
Production costs	Low	High
Replication at infection site	Yes	Middle multiple
The disease-causing bacteria must be identified before starting the therapy	Yes	No
Attack to normal microflora	No	Yes
Located in infection site	Yes	No

Biotechnological and medical applications of endolysins

Since the discovery of phages, they have been used in a variety of basic and applied research fields. A classical application of phages has been in diagnostic to identify and classify bacterial species and strains. As knowledge of phage structure increased, more applications of phages have been developed for the detection of pathogens.

Most phage endolysins act only against the bacterial species from which the phage was originally isolated. Endolysins from gram-positive bacteria can also act as exolysins because the peptidoglycan is, accessible from outside.

Lysostaphin and its use in biomedical application

Lysostaphin is an antimicrobial agent belonging to a major class of antimicrobial peptides and proteins known as bacteriocins. It exhibits a high degree of antistaphylococcal bacteriolytic activity, being inactive against bacteria of all other genera. Infections caused by staphylococci continue to be a worldwide problem. Since lysostaphin kills the animal and human staphylococcal pathogens, it has the potential biotechnological applications in the treatment of staphylococcal infections. In vivo and in vitro studies using lysostaphin have shown that it has potential to be used, in combination or solely with other antibacterial agents to treat and prevent bacterial staphylococcal infections. [75]

The concept of using purified murein hydrolases to kill bacteria by breaking bonds of specific bacterial pathogens has proved to be valid with bacterial enzymes. A successful example of this approach is lysostaphin, an endopeptidase produced naturally by *Staphylococcus simulans*, which efficiently cleaves glycyl-glycine bonds in the pentaglycine inter-peptide links of the staphylococcal peptidoglycan. A single dose of lysostaphin, formulated as a cream, was more effective than a single dose of mupirocin ointment to eliminate *Staphylococcus aureus* nasal colonization in a cotton rat model. [76, 77]

Dixon and co-workers tested the effectiveness of lysostaphin followed by treatment with methicillin against established renal abscess lesions in mice. A single dose of lysostaphin, followed by four daily doses of methicillin, produced striking reductions (more than 99,99 %) of the staphylococcal populations. Such reductions were significantly higher than that achieved by either lysostaphin or methicillin alone. Similar results were also reported by Harrison and Zygmunt. [78, 79]

Quickel Jr. and co-workers tested the efficacy of lysostaphin to alter natural Staphylococcus aureus carriage in 95 persistent carriers. In this work, the efficiency of lysostaphin intranasal spray was compared to Neosporin®. The treatment was carried out for five days with either agent significantly reducing carriage rates. Despite, this effect persisted through the fifth day only after therapy with lysostaphin. [80]

Another study studied lysostaphin functionalized cellulose fibers for wound healing. The cellulose-based and lysostaphin functionalized fibers were tested for their antimicrobial activity against *Staphylococcus aureus* to confirm contact-mediated killing. Fiber based mats were washed with water and the resultant wash solutions were tested against *Staphylococcus aureus* suspension. In the case of cellulose-chitosan-lysostaphin fibers, where the enzyme was covalently bound to fibers through glutaraldehyde crosslinking, the bioconjugation was stable as reflected by the insignificant antimicrobial activity in the wash solution. The control cellulose chitosan fibers showed about 20 % bacterial killing after 3 hours of incubation. This result is not unexpected, as chitosan is known to be antimicrobial due to its polycationic nature. However, the functionalized samples with lysostaphin resulted in more than 95 % bacterial killing. [81]

Recently were engineered a lysostaphin-delivering injectable PEG hydrogel to treat *Staphylococcus aureus* infections in orthopaedic implant infections and support fracture repair. In these studies, were characterized the activity, stability, and release of hydrogel encapsulated lysostaphin, as well as antimicrobial and antibiofilm performance. The efficacy of lysostaphin delivering hydrogels were tested in vivo using a murine femur fracture infection model. The synthetic hydrogels were engineered to deliver active lysostaphin to infected bone fractures to clear the infection and promote fracture healing. The synthetic hydrogel delivery vehicle maintained lysostaphin activity over 14 days and controlled the release of active enzyme through passive and protease triggered mechanisms. The hydrogels displayed high activity against various strains of Staphylococcus aureus, as well as a methicillin-resistant Staphylococcus epidermis clinical isolate from prosthetic joint infection in vitro. In a murine model of fracture infection, lysostaphin delivering hydrogels cleared the infection and supported fracture repair, lysostaphin delivering hydrogels cleared the infection and supported fracture repair, with bone formation and mechanical properties equivalent to those of uninfected fractures. Lysostaphin delivering hydrogels restored the local inflammatory environment to that sterile fractures at 7 days. On the other hand, infected fractures treated with either prophylactic antibiotics or soluble lysostaphin showed no differences in bacterial levels and impaired healing compared with the infection controls. Notably, the delivery of lysostaphin with this hydrogel carrier significantly reduced MRSA infections in fracture model. The results of this study show that hydrogel-mediated delivery of lysostaphin eliminates fracture infections, including antibiotic-resistant strains, allowing for the endogenous fracture repair mechanisms to progress and healing occur. [82, 83, 84]

2.6 Methods used in this work

2.6.1 Lyophilization

Lyophilization or freeze-drying is a process in which water is removed from the product after it is frozen and placed under a vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase. The process consists of three separate processes: freezing to convert most of the water into ice, primary drying to sublime the ice, and secondary drying to remove unfrozen water by desorption.

Freezing is a critical step since the microstructure established by the freezing process represents the microstructure of the dried product. The product must be frozen to a very low temperature to be completely solidified. Because freeze-drying is a change of state from solid to gaseous phase, the material must be first pre-frozen. The method of pre-freezing and the final temperature of the frozen product can affect the ability to successfully freeze-dry material.

- Rapid cooling results in small ice crystals, useful in preserving structures to be examined microscopically, but resulting in a product that is more difficult to freeze dry.
- Slower cooling results in large ice crystals and less restrictive channel in the matrix during the drying process.

Most samples that are to be freeze-dried are eutectics, which are mixtures of substances that freeze at a lower temperature than the surrounding water. That's called the eutectic temperature. Eutectic point is the point where all three phases (solid, liquid, gas) coexist.

Lyophilization's second phase is primary drying. After pre-freezing the product, conditions must be established in which ice can be removed from the frozen product through sublimation, resulting in a dry, structurally intact product. This requires carefully control of the two parameters: temperature and pressure involved in freeze-drying system. The rate of sublimation of ice from a frozen product depends on the difference between vapor pressure of the product and the vapor pressure of the ice collector. Molecules migrate from the high-pressure sample to a lower pressure area. Because vapor pressure is relating to temperature, it is necessary that the product temperature is warmer than the ice collector temperature. It is extremely important that the temperature at which a product is freeze-dried is balanced between the temperature that maintains the frozen integrity of the product and the temperature that maximizes the vapor pressure of the product. This is the balance that is key to optimum drying.

Lyophilization's final phase is secondary drying, during which the ionically-bound water molecular are removed. By raising the temperature higher than in the primary drying phase, the bonds are broken between the material and the water molecules. Freeze-dried material retains a porous structure. After the lyophilization process is complete, the vacuum can be broken with an inert gas before the material is sealed. [85, 86, 87, 88]

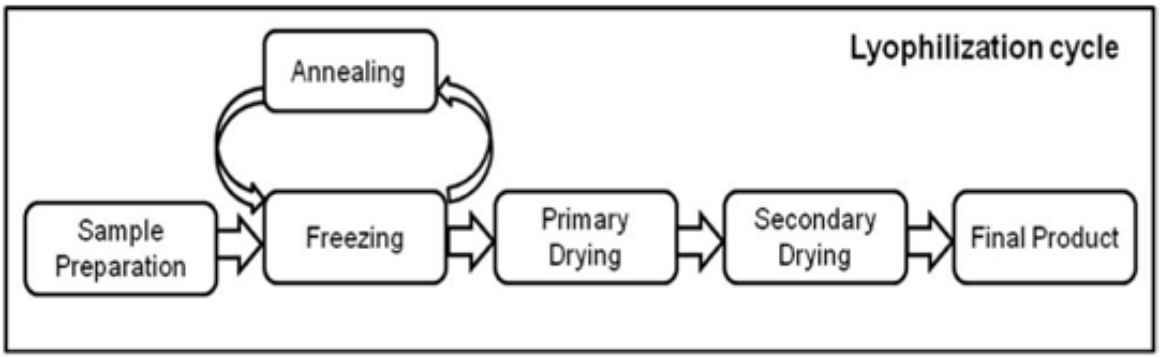

Figure 7: Steps involved in lyophilization process from sample preparation to final product. Annealing is an optional step, that is occasionally used to crystalline substance [85]

2.6.2 Bradford protein assay

The Bradford protein assay is used to measure the concentration of total protein in a sample. The principle of this assay is that the binding of protein molecules to Coomassie Brilliant Blue (G-250) dye under acidic conditions results in a color change from red to blue.

The basis for the Bradford assay is that in order for the Coomassie dye to bind stably to protein, it needs to be doubly protonated. When the dye comes in contact with protein, the first electron is donated to charged groups on the protein. This disrupts the structure of the protein, resulting in exposure of hydrophobic pockets. The dye binds to these pockets, with the

sulfonic acid groups binding to positive amines. In addition, there is an attraction due to Van der Waals forces. The stably bound Coomassie G-250 is the blue, unprotonated form.

The change in the color from red to blue upon binding protein is measured spectroscopically. In the absence of protein, when the dye is red, Bradford reagent has an absorbance maximum of 470 nm. In the presence of protein, the change to the anionic blue form of the dye shifts the absorbance maximum to 596 nm.

The standards used most commonly for the Bradford assay are bovine serum albumin (BSA) and bovine γ-globulin (BGG). Ideally, the standard should be the same in the same ratios as are found in the sample. However, for most samples, this is not practical or even possible. [89, 90, 91]

2.6.3 Mechanism of drug release from polymer matrix

The mechanism for releasing a drug from a polymer matrix can be described in two slightly different ways. The first method describes the transport of a drug molecule through a polymer matrix. The second way describes the already direct release of the drug into the surrounding environment. Both the transport of the drug molecule through the polymer matrix and its release from the matrix alone determine the rate of release. If a system with the gradual release of a drug is created, it is important to know the release mechanism, along with the physicochemical processes that influence the release. The drug molecule can be released from the system in several ways. Added pharmaceuticals and other additives (salt, surfactants, etc.) may also affect the processes underway in the matrix of the polymer.). The drug's properties, particularly its polarity, can alter the properties of the original polymer. Furthermore, acidic drugs may also increase acid hydrolysis, and conversely, the addition of an alkaline-like drug may slow down acid hydrolysis to possibly neutralize acidic hydrolysis products. The release of the drug from the polymer matrix, therefore, depends on the properties of the polymer, the drug, and their interactions with each other. [92]

The medicinal release profile is sometimes used as a basis to evaluate the mechanism of action of the entire system. Fredenberg et al. [93] described drug release profiles that are composed of several phases. Single-phase release is very rare. Two- or three-phase profiles are much more common. Large systems often exhibit a three-phase release system, due to heterogeneous degradation. In contrast, a two-phase release is very often observed in small systems.

Phase I in the classic tri-phase release profile is usually described as a burst release and has been attributed to non-encapsulated drug particles on the surface or drug molecules close to the surface easily accessible by hydration. Other reasons for burst release may be the formation of cracks and the disintegration of particles. Phase II is often a slow release phase, during which the drug diffuses slowly, either through the few existing pores, while polymer degradation and hydration proceed. Phase III is usually a period of faster release, often attributed to the onset of erosion. This phase is sometimes called the second burst. If the second phase is rapid, there may be a slower phase at the end of the release period. The release profile may not exhibit any burst

release. Some examples of different release profiles are given in Figure 8. The term degradation is used with slightly different meanings in different studies: i.e. both hydrolysis and erosion of the polymer, or the combination of the two processes. [93]

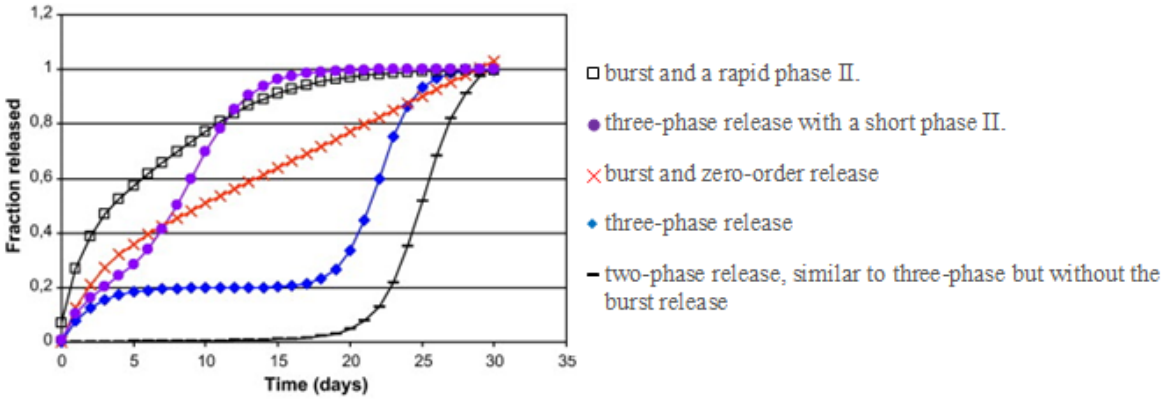

Figure 8: Release profiles consisting of different phases [93]

3 GOAL OF THE WORK

Salts stabilize antibacterial proteins but destabilize their biopolymer carriers. The main aim of this work is to establish the effect of different salts and their concentrations on the stability of biopolymer carriers with following steps:

- Preparation of samples with different buffers and salt concentrations.
- Swelling tests of prepared samples.
- Hydrolytic stability of prepared samples.
- Choosing one suitable buffer and salt concentration to load a lysostaphin into it.
- SEM morphology of prepared sample.
- Release of lysostaphin from samples via UV-VIS.
- Data evaluation ad discussion.

4 EXPERIMENTAL PART

4.1 Materials and methods

4.1.1 Chemicals

- Ultrapure water type II according to ISO 3696
- Woven carboxyl methyl cellulose (P56/16 NAT 60gsm, DS- 0,237, pH- 7,23), Holzbechcer, spol. s.r.o barevna a bělidlo Zličín, Czech Republic
- Bovine Collagen type I., 8 % (Výzkumný ústav pletařský a.s., Czech Republic)
- Ethanol 96% p.a. (PENTA s.r.o., the Czech Republic)
- *N*-(3-Dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 98% (Sigma-Aldrich, Germany)
- Sodium phosphate dibasic for molecular biology (≥98,5 %) (Sigma-Aldrich, Germany)
- Bradford reagent for 0.1 – 1.4 $mg.ml^{-1}$, (Sigma-Aldrich, Germany)
- Lysostaphin (lyophilized powder, Veterinary research institute, v. v. i. Czech Republic)
- Saline solution (Braun, Germany)
- Phosphate Buffered saline, sodium chloride and potassium dihydrogen phosphate (Lach-ner, Czech Republic), potassium chloride, sodium phosphate dibasic and TrisHCl buffer (Sigma-Aldrich, Germany)

4.1.2 Equipment

- Analytic balance SI-234A, DENVER INSTRUMENT, United States of America
- Disintegrator – IKA Ultra Turrax ® T18 basic
- Freeze-dryer Epsilon 2-10D LSCplus, Christ, Germany
- Incubator BPN-80CRH(UV) Series Co2, Hinotek, China
- Milwaukee pH55 Martini, pH meter, United States of America
- UV/VIS Spectrophotometer, Biochrom Libra S22
- Vortex- IKA Vortex 3
- High-resolution Scanning Electron Microscope FEI VERIOS 460L
- Coater Leica EM ACE600

4.2 Preparation of Collagen/CMC Samples

Samples were prepared in four series, in order to choose the right buffer/salt solution and to compare the effect of crosslinking on the properties of the sample. The first two series were prepared in PBS/NaCl or TrisHCl/NaCl buffer solution with four different concentrations. (25mM/125mM, 10mM/50mM, 5mM/25mM and 1mM/5mM). These series of samples were

just freeze-dried. The second two series were prepared in water, then freeze-dried after that crosslinked with EDC/NHS (described below) and then poured with PBS/NaCl or TrisHCl/NaCl buffer solution with different concentrations (25mM/125mM, 10mM/50mM, 5mM/25mM, 1mM/5mM) and freeze-dried again.

4.2.1 Preparation of non-crosslinked Collagen/CMC Samples

Bovine collagen and woven carboxymethylcellulose in a concentration of 0,5 % were prepared according to the next steps. In the first step of the production, the cellulose and collagen were sheared into smaller pieces. Then the preparation of samples continued with the swelling of cellulose in PBS/NaCl buffer for 30 minutes at reduced temperature. The collagen was also retained in PBS/NaCl buffer for 30 minutes at reduces temperature. The final step of preparation was mixing cellulose and collagen, blending them, and converting into homogenous gels. The cellulose solution was stirred for 10 minutes at 6000 revolutions per minute (RPM). The collagen solution was stirred for 5 minutes while cooling and after this, the collagen mixture was added in small portions while cooling the beaker with cellulose and stirred at 6000 RPM. The resulting mixture was placed into prepared plates in a volume of 1 ml and freeze-dried for two days.

The preparation of the samples in TrisHCl/NaCl was the same as PBS/NaCl.

List of non-crosslinked coll/CMC samples in different concentrations are in the table below. (Table 2)

Table 2: List of non-crosslinked Coll/CMC Samples

buffer	concentration	$w_{collagen}$ [%]	$w_{cellulose}$ [%]
PBS/NaCl	25mM/125mM	0.5	0.5
	10mM/50mM		
	5mM/25mM		
	1mM/5mM		
TrisHCl/NaCl	25mM/125mM		
	10mM/50mM		
	5mM/25mM		
	1mM/5mM		

4.2.2 Preparation of crosslinked Collagen/CMC Samples

Preparation of crosslinked scaffolds was almost the same as the previous, only buffer/salt solution was added in different preparation step; collagen and cellulose were sheared into smaller pieces and swelled-up in UPW for 30 minutes. Then homogenized with disintegrator and freeze-dried for two days. After lyophilization, the samples were cross-linked with EDC/NHS in ethanol solution according to Sloviková et al. [94] The agent was washed out

from the samples after two hours of cross-linking. Washing out was carried out two times with 0.1 M Na_2HPO_4 and three times with distilled water. After the last wash, the samples were poured with 1 ml of PBS/NaCl or TrisHCl/NaCl of the required concentration and then freeze-dried again for 2 days. List of crosslinked coll/CMC samples in different concentrations are in the table below. (Table 3)

Table 3: List of crosslinked Coll/CMC Samples

buffer	concentration	$w_{collagen}$ [%]	$w_{cellulose}$ [%]	EDC/NHS
PBS/NaCl	25mM/125mM	0.5	0.5	yes
	10mM/50mM			
	5mM/25mM			
	1mM/5mM			
TrisHCl/NaCl	25mM/125mM			yes
	10mM/50mM			
	5mM/25mM			
	1mM/5mM			

4.3 Loading of lysostaphin into collagen-based samples

Based on evaluation (chapter 5.5), one effective concentration of protein and one concentration of salt have been chosen for following sample preparation.

4.3.1 Preparation of non-crosslinked Collagen/CMC Samples with Lysostaphin

Mixture of collagen and CMC was prepared according to the section 4.2.1. Lyophilized lysostaphin was then dissolved in 1 ml of buffer/salt solution and vortex for 3 minutes. The solution of lysostaphin with effective concentration was poured into coll/CMC mixture. The resulting mixture was pipetted into prepared plates in a volume of 1 ml and freeze-dried for two days.

4.3.2 Preparation of crosslinked Collagen/CMC Samples with Lysostaphin

Mixture of coll/CMC was prepared according to the section 4.2.2. After the last wash, the samples were poured with 1 ml of buffer/salt solution or water with dissolved lysostaphin of the required concentration and then put into the fridge for 24 hours and freeze-dried again for 2 days.

4.4 Characterization of Samples

4.4.1 Swelling ratio and Water Content

Each sample was weighted at its dry state before the swelling test and then placed into ultrapure water at room temperature. Samples were taken out of the ultrapure water in the following intervals 1, 3, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, and 180 minutes and gently placed on filtrate paper for a couple of seconds and weighted again. The swelling ratio was calculated to define the exact amount of swelling caused by water absorption and the swelling curve was obtained. The swelling ratio was calculated as follows:

$$Swelling\ Ratio = \frac{W_s}{W_i}, [-] \qquad (E3)$$

Where W_s is the weight of sample in swollen state and W_i is the initial weight of dry scaffold sample. The ratio of weight increase to the initial weight was characterized as water content:

$$Water\ Content = \frac{W_s - W_i}{W_i}.100, [\%] \qquad (E4)$$

4.4.2 Hydrolytic stability

The samples were weighed at dry state and then placed into vials containing saline solution. The hydrolytic stability was simulated in the incubator at 37 °C. After the regular time, the samples were removed, get rid of surplus saline solution, and re-weighted every day for one week and then after a larger interval of time. The hydrolytic stability was calculated according to the equation where the W_D is the weight of swelled sample in the appropriate day and the W is the stabilized weight at the appropriate time during swelling.

$$Hydrolytic\ stability = 100 - \left(\frac{W_D.100}{W}\right), [\%] \qquad (E5)$$

4.4.3 Morphology and Porosity

The morphology and microstructure of lyophilized samples were studied using scanning electron microscopy (SEM). Samples were sliced into small and thin slices using a razor blade. Slices were then placed on a carbon tape located on the metallic target. Before the measurement started, samples have been coated with a 20 nm gold layer on Coatera Leica EM ACE600 to

receive a better resolution as it is improving secondary electron signal. The gold layer as a conductive layer, inhibits the charging of samples and decreases the damage of samples. The measurement was performed on High-resolution Scanning Electron Microscope FEI VERIOS 460L device at 5 kV acceleration voltage and 25 pA obtaining SEM images.

The average pore size of samples was characterized by the SEM visualization using image analyses program ImageJ.

4.4.4 Release of Lysostaphin from Collagen/CMC Samples

Samples prepared with lysostaphin were placed into large vials with saline solution and kept in the incubator at 37 °C. After regular times, the whole volume was taken out from vial and vortexing. A certain amount of this solution was pipetted into the cuvette with Bradford reagent and it was let to react in a dark place for 15 minutes and then the absorbance at 595 nm was measured.

5 RESULTS AND DISCUSSION

5.1 Prepared Samples

The first series of samples were prepared in PBS/NaCl solution in four different concentrations. The salt was added into all buffers to see the effect of salt on coll/CMC scaffolds and to try to concentrate/stabilize the lysostaphin. Concentration of collagen and CMC was 0.5 %. This concentration was chosen according to previous study [94].

Table 4: Table of all prepared samples with corresponding pH of suspensions

Salts	Salt concentration	Suspension pH	Collagen pH	Cellulose pH	EDC/NHS	
PBS/NaCl	25mM/125mM	3.7	3.1	6.3	No	Yes
	10mM/50mM	3.9	3.3	6.5		
	5mM/25mM	4.3	3.6	6.8		
	1mM/5mM	4.6	3.8	7.4		
TrisHCl/NaCl	25mM/125mM	8.7	8.5	9.3	No	Yes
	10mM/50mM	8.2	8.4	9.2		
	5mM/25mM	7.1	6.5	9.1		
	1mM/5mM	4.7	3.2	8.4		
reference sample	0mM (water)	5.0	2.8	7.2	No	Yes

The pH values of collagen solutions ranged from 3.1-3.8 depending on the PBS/NaCl concentrations. The pH of CMC solutions ranged from 6.3-7.4 depending on the PBS/NaCl concentrations. After mixing the solutions, the pH of the final solutions ranged from 3.7, (for the highest concentration) to 4.6 (for the lowest concentration).

As we can see in the figure below (Figure 9), samples prepared in PBS/NaCl solution after freeze-dried were much smaller and harder than the reference sample prepared in ultrapure water without any salt. The higher the concentration was, the smaller and harder the samples were. The major shrinkage of the samples was observed with highest salt concentration, while minor shrinkage was observed with lower salts concentration.

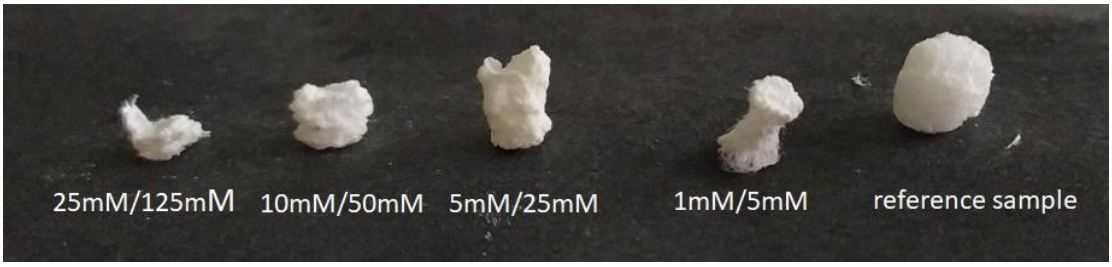

Figure 9: Non-crosslinked samples prepared in PBS/NaCl solutions

The second series of non-crosslinked samples were prepared in TrisHCl/NaCl solution with the same concentrations of collagen and CMC and also with the same concentrations of salt to compare it with PBS/NaCl solution and choose the best for work with lysostaphin. The pH values of collagen solutions ranged from 8.5-3.2 depending on the TrisHCl/NaCl concentrations. The pH of CMC solutions ranged from 9.3-8.4 depending on the TrisHCl/NaCl concentrations. After mixing the solutions, the pH of the final solutions ranged from 8.7, (for the highest concentration) to 4.7 (for the lowest concentration).

As we can see, this series of samples (Figure 10) were also smaller and harder than a reference sample. The pH of the reference sample after mixing solutions of collagen and CMC was 5.

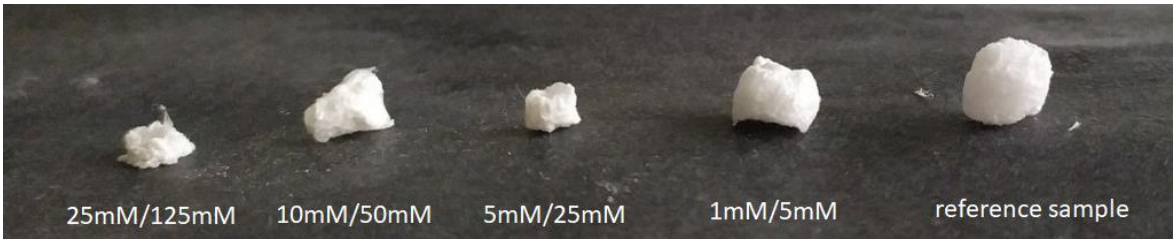

Figure 10: Non-crosslinked samples prepared in TrisHCl/NaCl solutions

The crosslinked series of samples look much better and consistent then non-crosslinked samples (Figure 11, Figure 12). This series of samples were less shrinked because there was a strong hydrogen bond formed during the first lyophilization before the salt was added.

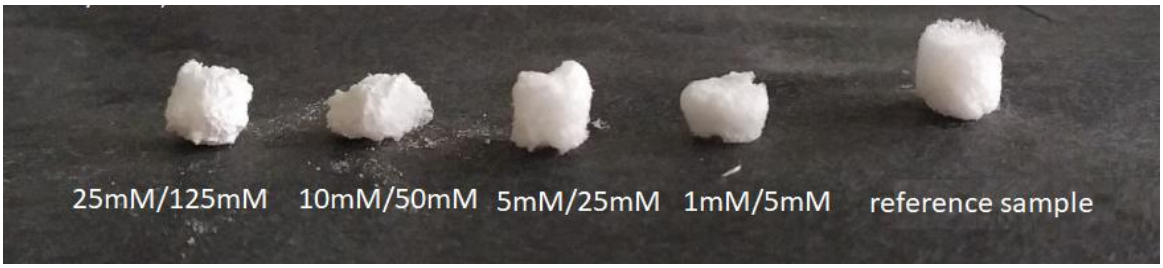

Figure 11: Crosslinked samples prepared in PBS/NaCl solutions

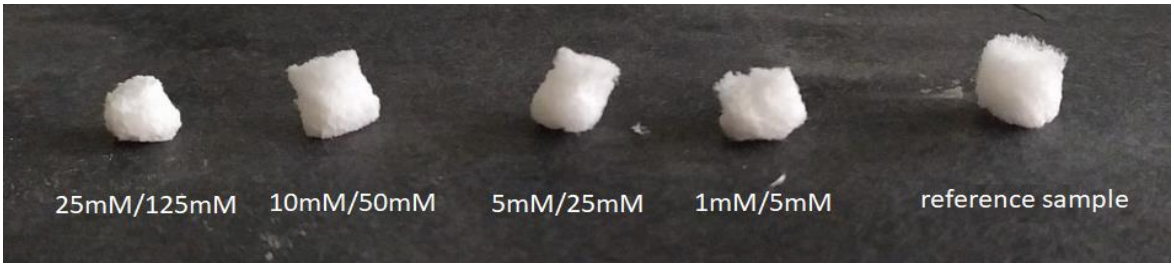

Figure 12: Crosslinked samples prepared in TrisHCl/NaCl solutions

5.2 Swelling ratio

Swelling properties of non-crosslinked coll/CMC scaffolds were studied at ultrapure water. In a few minutes, scaffolds quickly absorbed the maximum of water.

Swelling of fibrous proteins in acid and alkaline solutions is considered to be the result of the excess osmotic pressure (swelling pressure) in the protein phase arising from the difference in concentration of the diffusible ions in the protein and the external phase, equilibrium being reached when the osmotic pressure is balanced by the cohesive forces of the protein. In Figure ***13*** we can see that the reference sample has the biggest swelling ratio of about 15 what is caused by highly hydrophilic carboxyl groups of CMC so it can absorb a lot of water, but it has not survived as long as samples prepared in PBS/NaCl solution. According to the study [95] in acidic solutions, swelling ratio is decreasing as the concentration of sodium chloride is increasing. The swelling ratio in these samples ranges between 5.8 – 10. The lowest value of swelling ratio (5.8) has the sample with the highest concentration of salt. The highest value of SR has the sample with 5mM/25mM salt concentration. The results of the sample with the lowest salt concentration were burdened with a greater error (±83.25) than the samples with a concentration of 5mM/25mM (±33.55) so we can assume that the claim that with increasing concentration of salt, swelling ratio of samples decreases is right because these results indicated that the charge screening effect caused by cations (Na^+) could induce a decline of anion-anion electrostatic repulsions, leading to a decrease in the osmotic pressure between the sample and the external solution. [96]

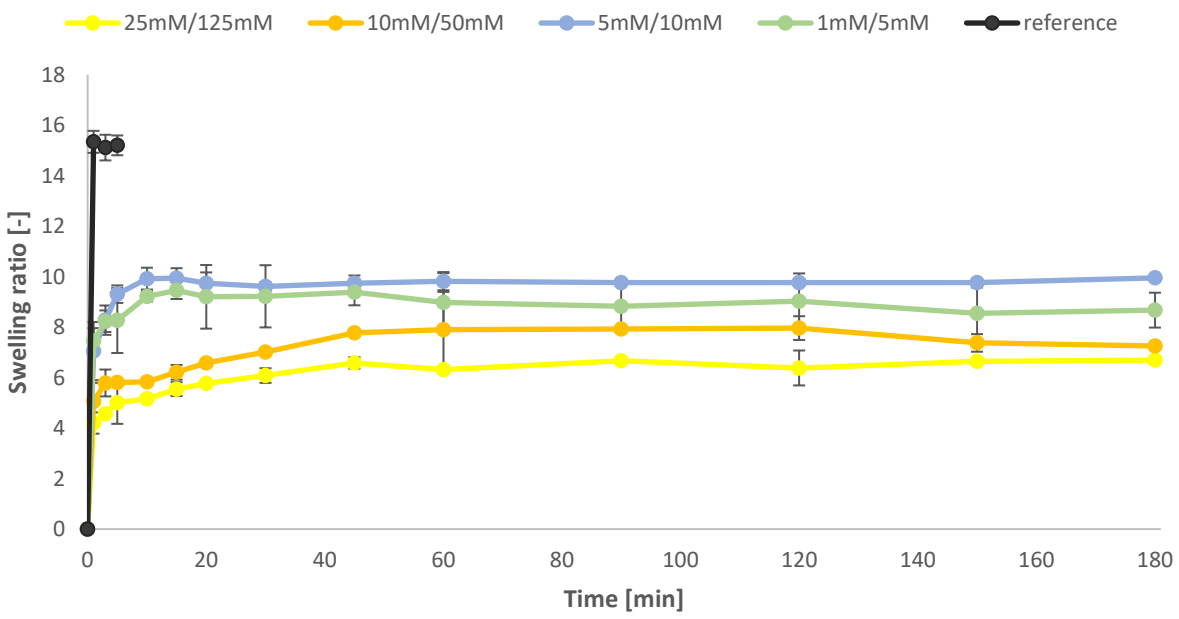

Figure 13: Swelling ratio of non-crosslinked coll x CMC samples in PBS/NaCl solution

The water content was studied as amounts of water absorbed by swelling. Because the referent samples were not stable for 45 minutes these samples were not included in the water

content graph. The highest amount of water absorbed sample with concentration 5mM/25mM and the second-highest amount of water absorbed the sample with the lowest salt concentration (1mM/5mM), but this sample was burdened with a greater error (error for 1mM/5mM was ±51.74, and for 5mM/25mM ±6.72). In this case, we can also say that the water content of the samples is decreasing with the salt concentration increasing (Figure 14). The time dependence of water content was very similar to the curves of swelling ratio and thus they are shown in Appendix 1.

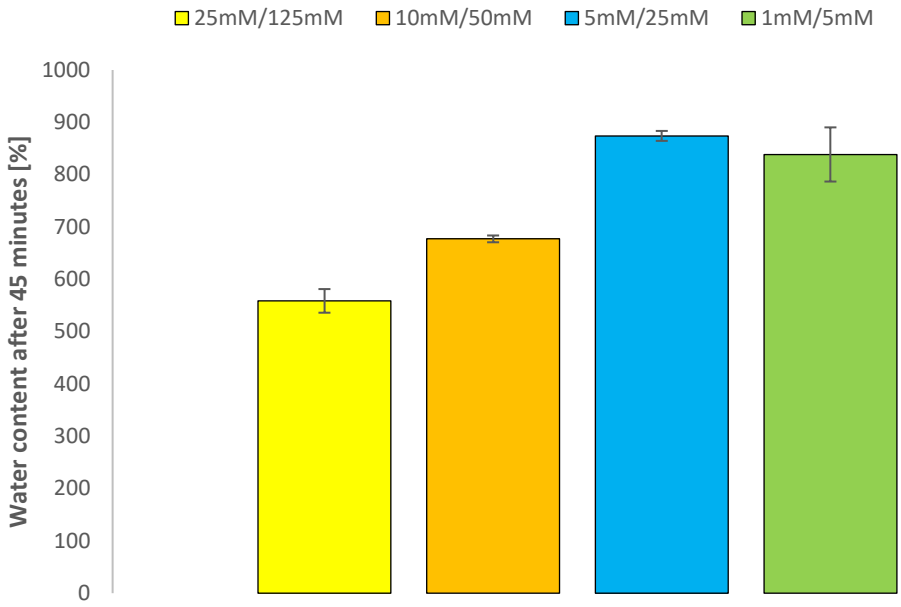

Figure 14: Water content of non-crosslinked samples prepared in PBS/NaCl solution

In the second series of non-crosslinked samples, prepared in TrisHCl/NaCl solution, it can be seen that samples were not stable and almost all of them disintegrated after 10 minutes (Figure 15). This was caused by the pH value of the prepared samples. Values of the pH were 8.7 for 25mM/125mM, 8.2 for 10mM/50mM, 7.2 for 5mM/25mM and 4.6 for 1Mm/5mM concentration. Because the CMC has a negative charge and collagen has also a negative charge at the pH higher than an isoelectric point, so the charges repel and it makes the samples unstable in water solution. The sample with the lowest concentration of salt has pH (4.6), here the collagen has a positive charge, thus it had shown the best stability in this sample.

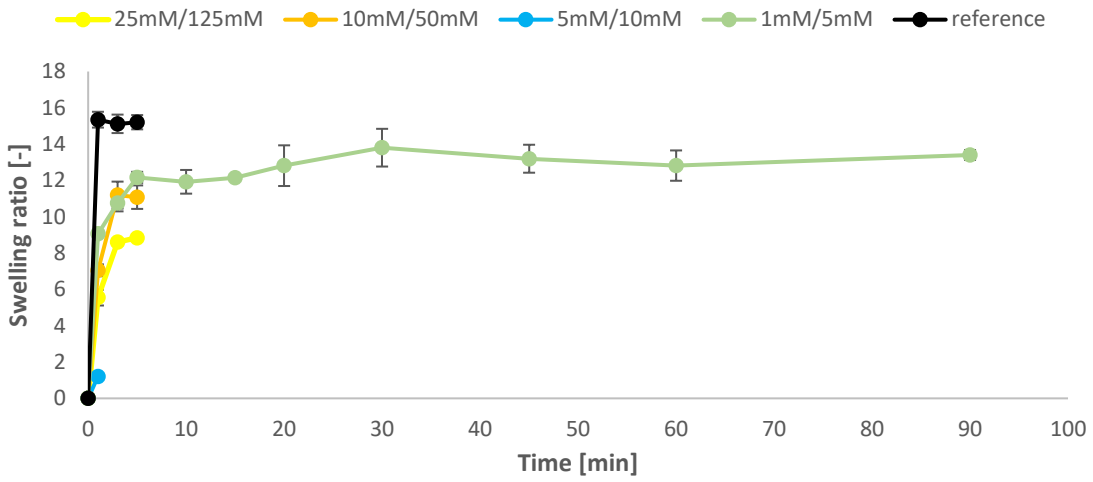

Figure 15: Swelling ratio of non-crosslinked coll x CMC samples in TrisHCl/NaCl solution

The value of SR in crosslinked reference samples rapidly decreased compared to the non-crosslinked reference sample. However, the non-crosslinked reference sample was saturated after 1 minute in water and completely collapsed after 5 minutes. So, it can be said that the production of stable ester due to reaction of EDC/NHS with collagen, as well as hydrogen bond formation due to internal interaction of collagen into matrices during crosslinking brought collagen a more stable structure. [97]

In the crosslinked samples prepared in a salt solution, the higher values of swelling ratio can be seen (Figure 16, Figure 17). With increasing salt concentration, the swelling capacity of samples decreased. This is attributed to a charge screening effect of the additional cations causing a non-perfect anion-anion electrostatic repulsion, leading to a decreased osmotic (ionic) pressure difference between the samples and the external solution. [98]

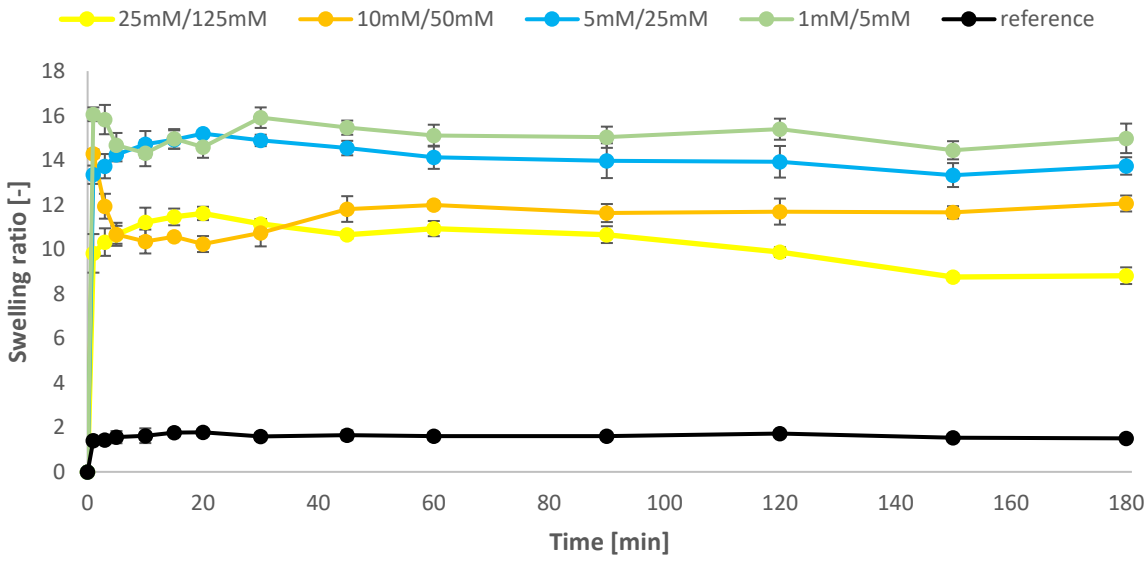

Figure 16: Swelling ratio of crosslinked coll x CMC samples in PBS/NaCl solution

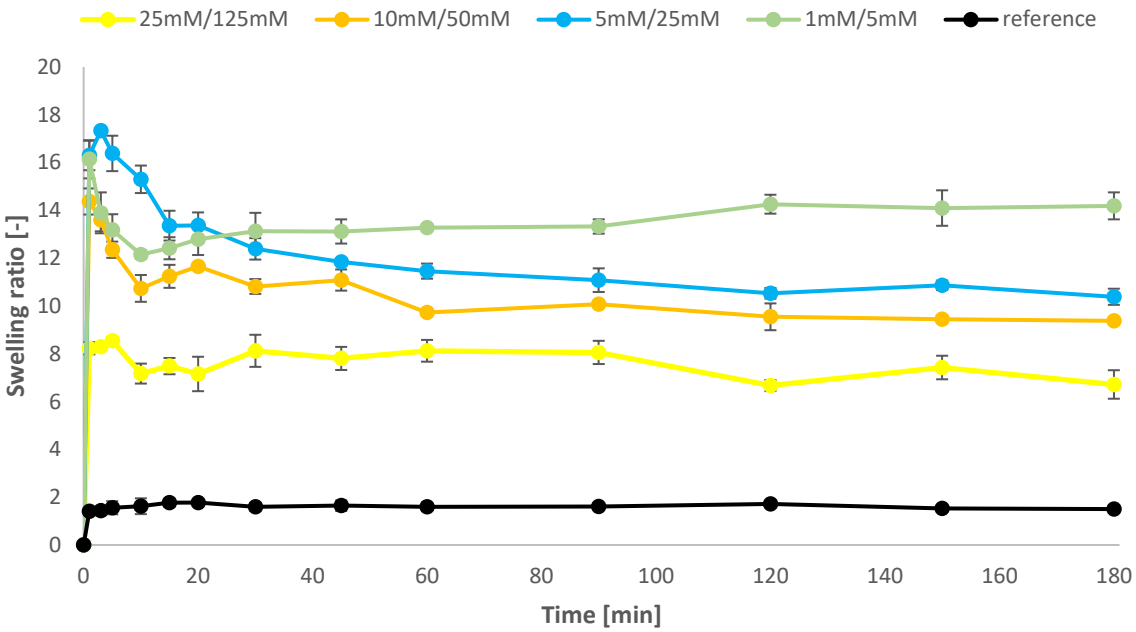

Figure 17: Swelling ratio of crosslinked coll x CMC samples in TrisHCl/NaCl solution

The water content of crosslinked samples is shown in Figure 18 and Figure 19. From these photos, we can also say that the water content of the samples is decreasing with the salt concentration increasing. The time dependence of the water content was very similar to these curves of swelling ratio and thus they are shown in Appendix 2 and Appendix 3.

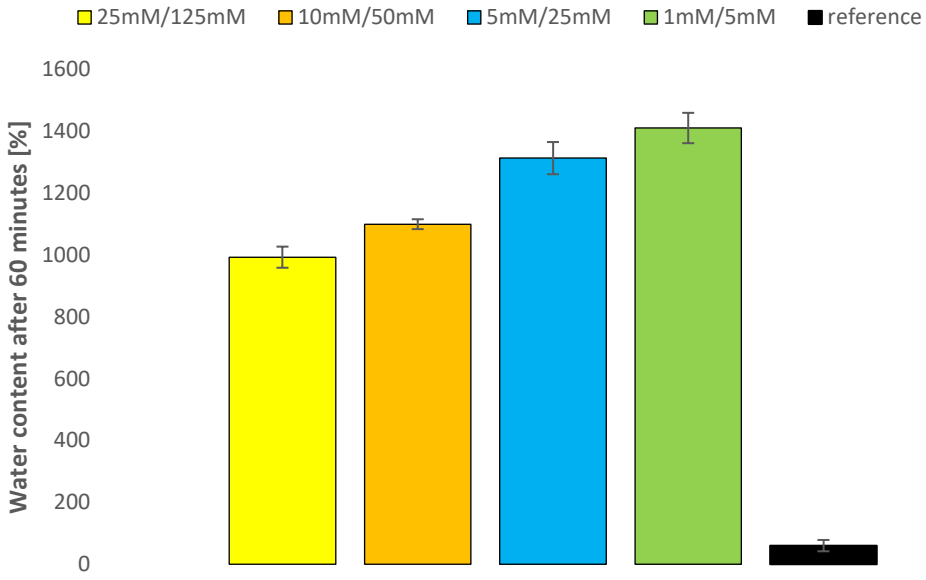

Figure 18: Water content of crosslinked samples prepared in PBS/NaCl solution

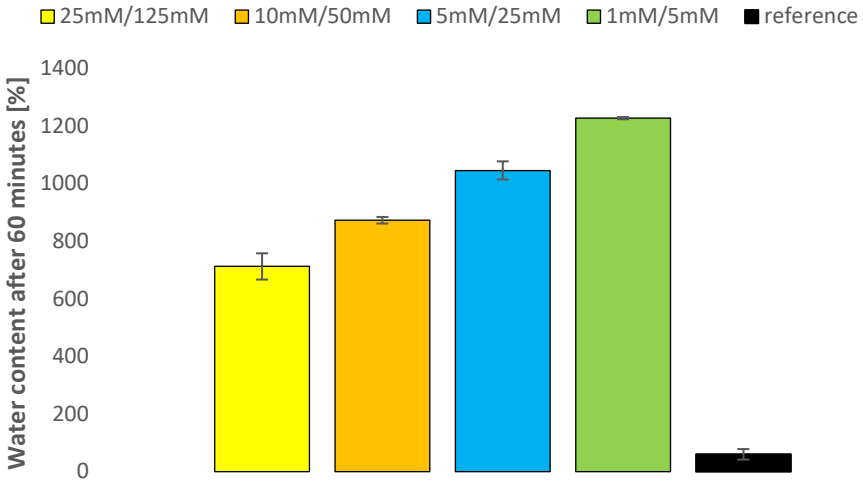

Figure 19: Water content of crosslinked samples prepared in TrisHCl/NaCl solution

5.3 Hydrolytic stability

Hydrolytic stability or degradation is an important property of scaffolds to determine how a sample behaves in an aqueous environment. One of the most common processes that occur in the aqueous environment is hydrolysis.

As we can see in Figure 20 the non-crosslinked samples prepared in PBS/NaCl solution were more stable than samples prepared in TrisHCl/NaCl solution and the samples with the highest hydrolytic stability contain lower salt concentrations (5Mm/25mM- lasts 4 hours and lost 37 % of its weight, 1mM/5mM- lasts 4 hours and lost 24 % of its weight). The reference sample lasts 30 minutes longer (4.5 hours) than samples prepared in PBS/NaCl and lost over 60 % of its weight until the point, when the structure was disrupted and impossible to manipulate with it. The reason why the reference sample was more stable in this test than in the swelling test is because here the samples were kept in saline solution, which contains a low concentration of salt and it stabilizes the sample to lasts longer than in water.

In Figure 21 we can see that samples prepared in TrisHCl/NaCl solutions were not stable and dissolved after a very short time.

Provided tests are showing that adding salt and increasing the temperature, decreasing the stability of non-crosslinked samples.

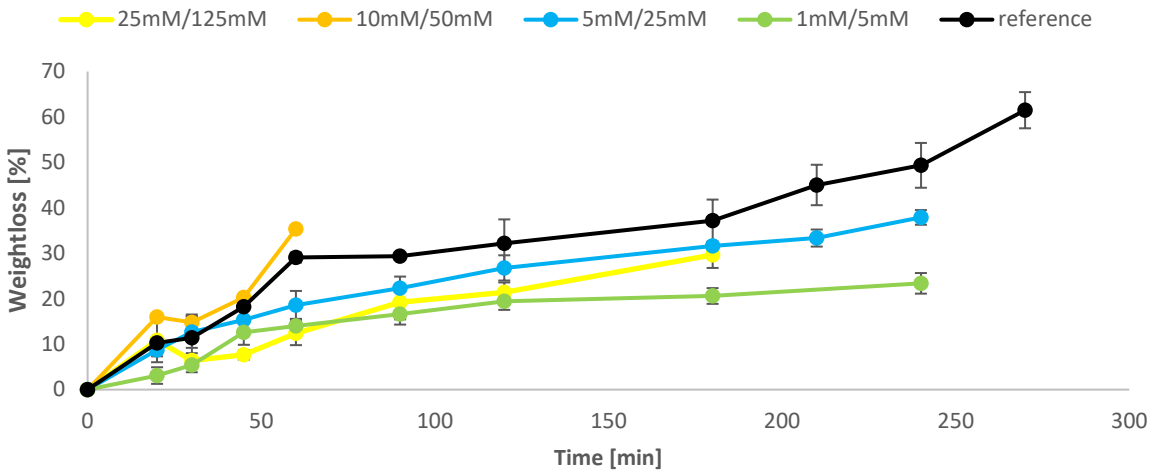

Figure 20: The effect of salt on hydrolytic stability of non-crosslinked PBS/NaCl samples in time

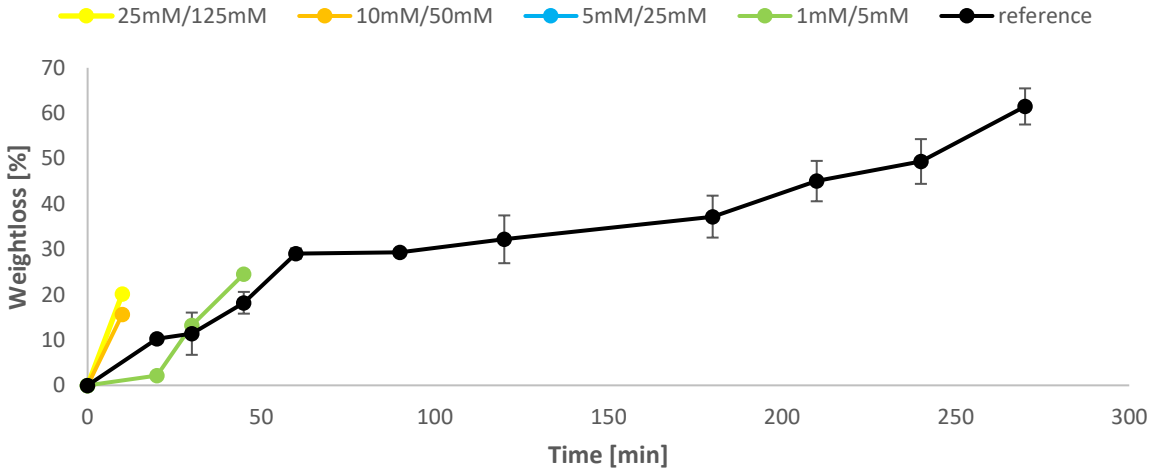

Figure 21: The effect of the salt on hydrolytic stability of non-crosslinked TrisHCl/NaCl samples in time

The hydrolytic stability of crosslinked samples showed that is no big difference in results between samples prepared in PBS/NaCl (Figure 22) or in TrisHCl/NaCl (Figure 23) because of a strong hydrogen bond made during the crosslinking process. The result of the reference sample prepared in UPW showed that its weightloss was lower and last between 8-15 days in compared to buffer/salt samples. However, at the end of the testing (after 40 days), the weightloss of the reference sample was 80 %. This result is, comparing with the samples in buffer/salt 75-85 % almost negligible.

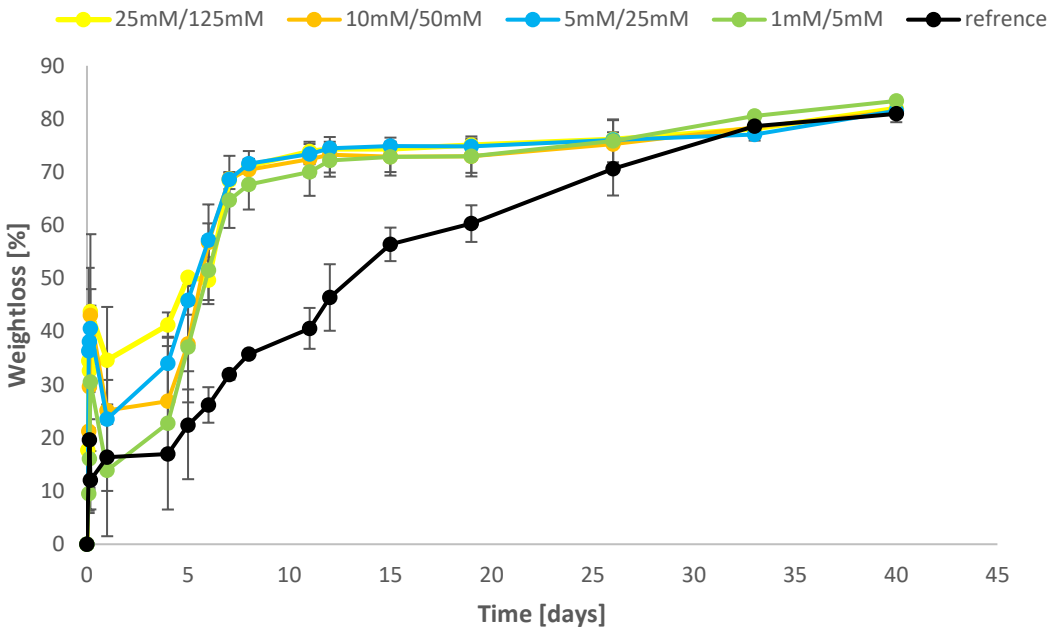

Figure 22: The effect of salt on hydrolytic stability of crosslinked PBS/NaCl samples in time

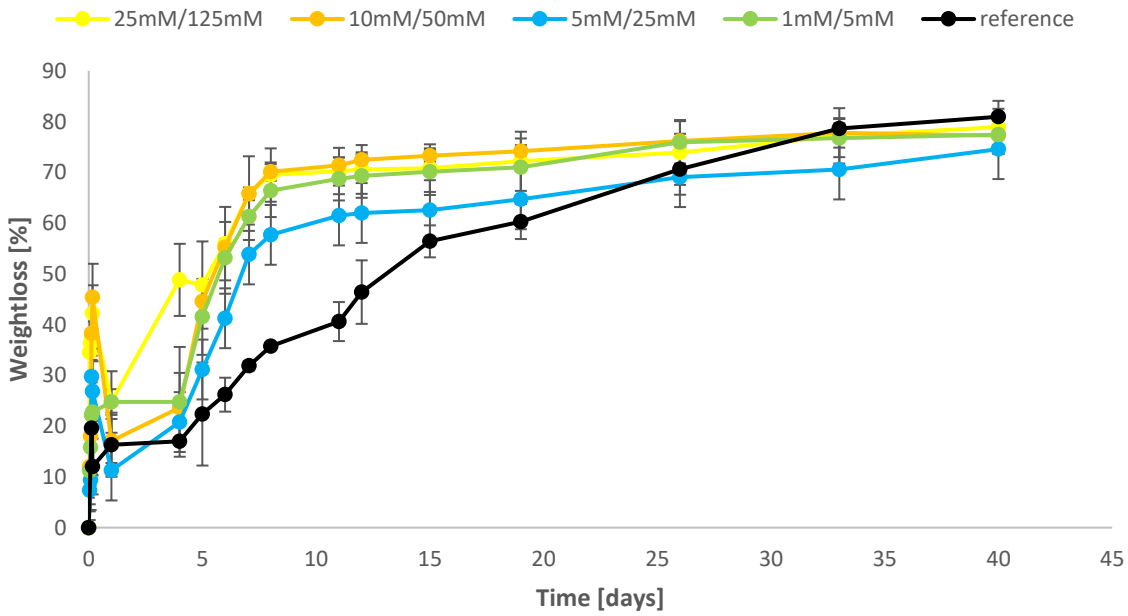

Figure 23: The effect of salt on hydrolytic stability of crosslinked TrisHCl/NaCl samples in time

By evaluating swelling and hydrolytic stability of prepared samples, for further work we decided for next tests to work just with PBS/NaCl solution, with 5mM/25mM concentration.

5.4 Morphology and Porosity

Porous structures allow for optimal interactions with cells. Specifically, pore size determines the efficiency at which cells seed into scaffolds: small pores prevent cells from penetrating the scaffold, whilst large pores prevent cell attachment due to a reduced area and, therefore, available ligand density. A scaffold with an open and interconnected pore network and a high degree of porosity is ideal for the scaffold to interact and integrate with the host tissue.

SEM observation of prepared samples was performed and the following figures (Figure ***25***, Figure 26, Figure 27) are representing porous structures. On the pictures where salt was added, it can be seen a good adhesion of salt to the surface of samples.

From pore sizes summarized in Figure 24, the addition of sodium chloride (5mM/25mM) slightly increases the size of pores in both, crosslinked and non-crosslinked samples. In non-crosslinked samples without salt was pore size 54.06 ± 47.30 and in samples with salt, only slight increase occurred 61.32 ± 53.72, same happened with crosslinked samples (pore size without salt 69.53 ± 56.01, and with salt 93.33 ± 63.95). This claim was also confirmed in the article [99], where sodium chloride was added to the collagen solution, and pore sizes in this case also slightly increase. Because pore size increases the sample can absorb more water as we saw in the swelling tests. Our SEM pictures were made with relatively low salt concentration so there was no unwanted shrinkage of pores as can be assumed for samples with a higher salt concentration as we saw in the pictures of prepared samples (Figure 9, Figure 10, Figure 11, Figure 12), that samples with high salt concentrations were much smaller.

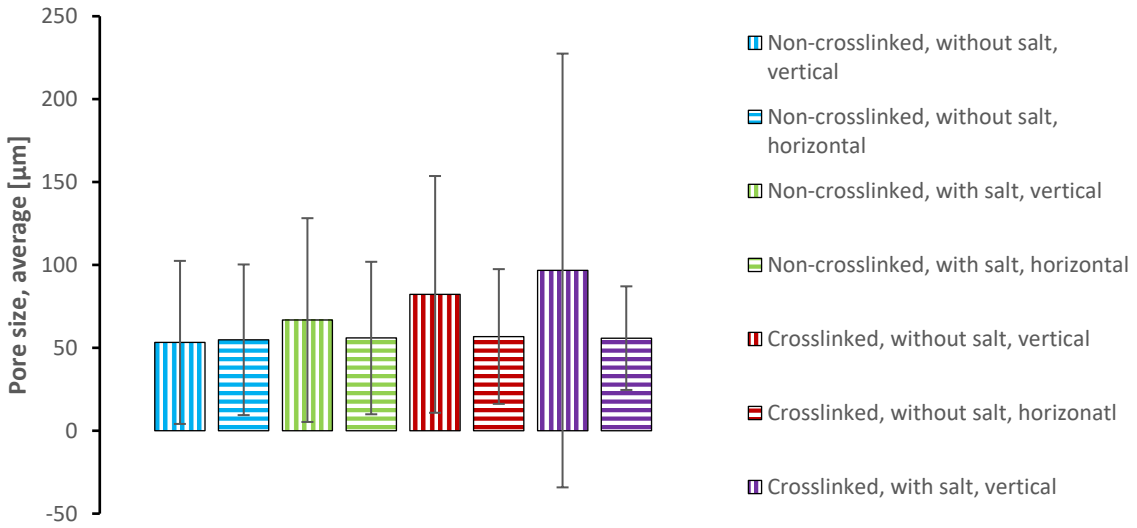

Figure 24: Average pore sizes of crosslinked and non-crosslinked samples, with and without salt

Table 5: Total pore size average

Sample	Pore size average [µm]
non-crosslinked, without salt	54.06 ± 47.30
non-crosslinked, salt	61.32 ± 53.72
crosslinked, without salt	69.53 ± 56.01
crosslinked, with salt	93.33 ± 63.95

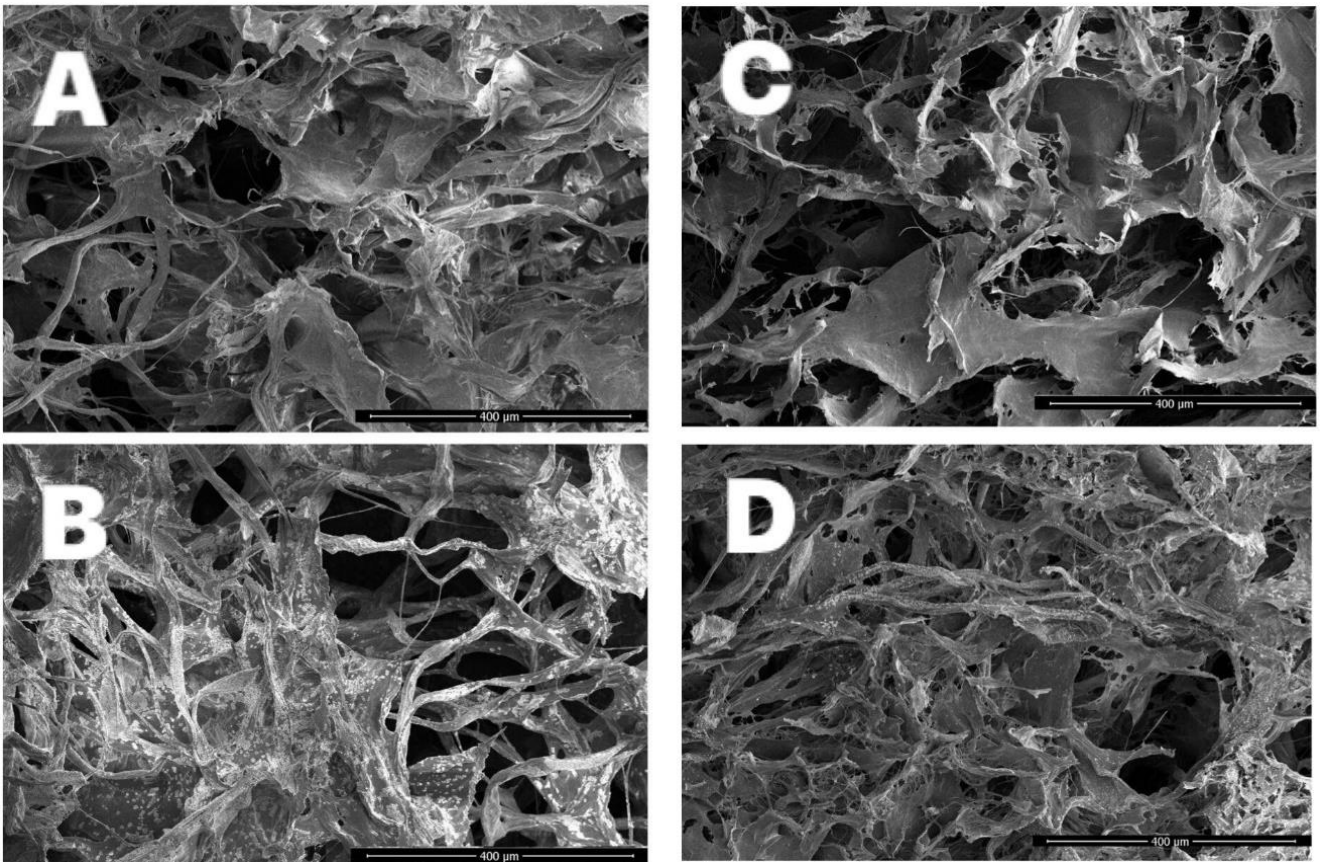

Figure 25: Comparison of SEM analysis images: A) non-crosslinked samples without salt; B) non-crosslinked samples with salt; C) crosslinked samples without salt; D) crosslinked samples with salt

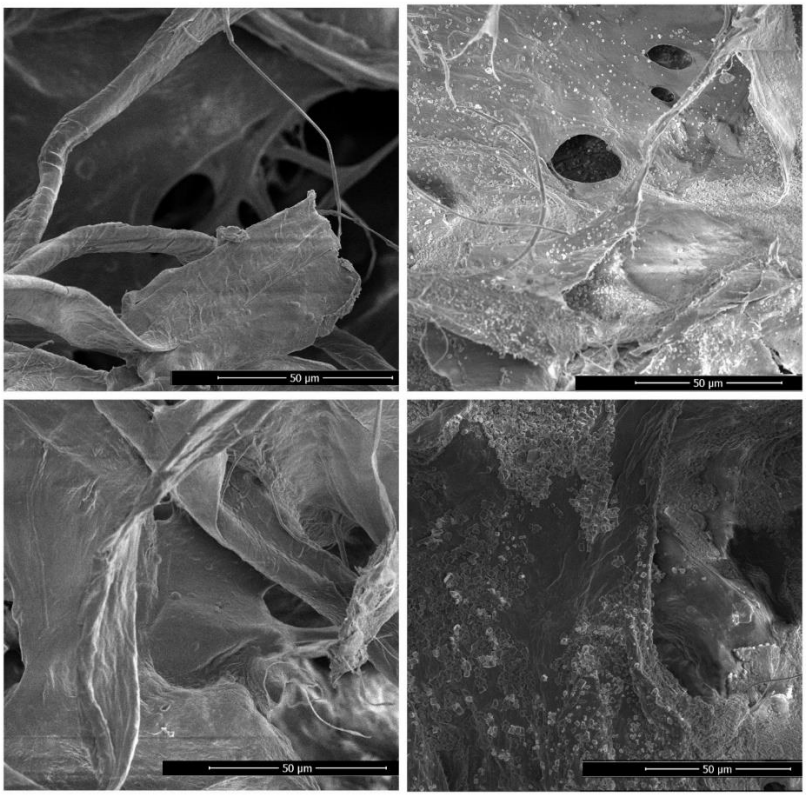

Figure 26: Zoomed pictures of non-crosslinked samples: left side without salt, right side with salt

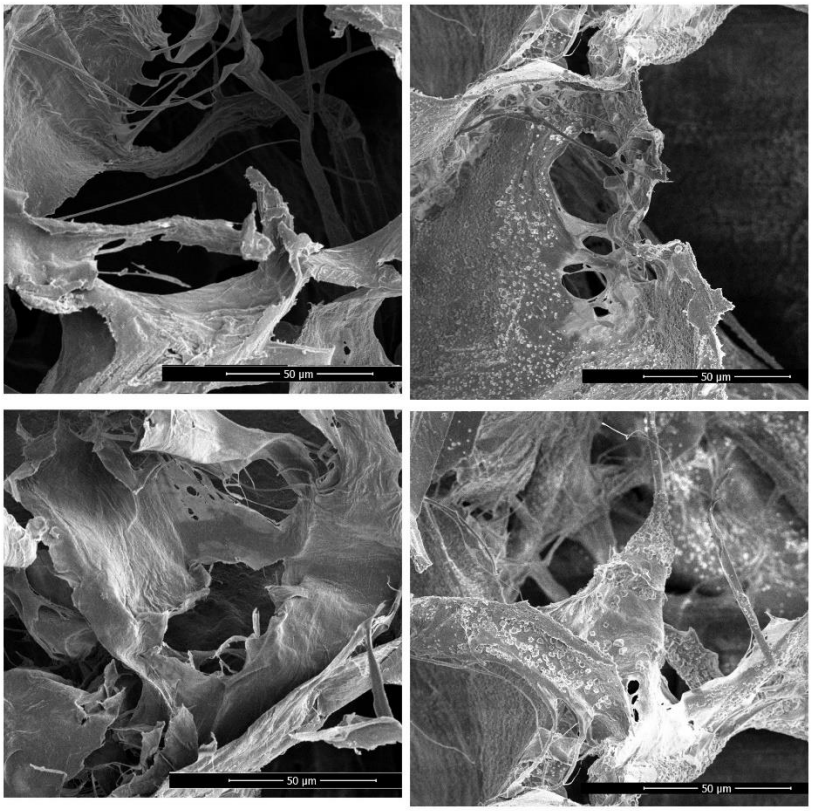

Figure 27: Zoomed pictures of crosslinked samples: left side without salt, right side with salt

5.5 Evaluation of Collagen/CMC Samples with Lysostaphin

By evaluating swelling and hydrolytic stability of prepared samples, one of the most suitable salt concentration was chosen for further work (5Mm/25mM). All samples prepared with lysostaphin are in Table 7, and photos of prepared samples are in Figure 31.

The concentration of lysostaphin used in samples was determined by antibacterial activity. Crosslinked, non-crosslinked samples with 6 different concentrations of lysostaphin (Table 6) were prepared by Ing. Katarína Kacvinská and sent for antibacterial activity tests to Veterinary Research Institute Brno. The samples were tested on two strains of Staphylococcus aureus (strain 244-ST8 and strain 5921-ST22). The bacterial culture was dissolved in PBS solution and applied in Petri dishes with blood agar, then the samples with lysostaphin were applied. The results of the antibacterial activity of samples are in Figure 28 and Figure 29. According to these tests, the first and the highest concentration effective against strains 244 and 5921 of Staphylococcus aureus, have been chosen for our further work.

Table 6: Concentration of lysostaphin loaded into the samples

	Concentration of protein
1	325 ug/ml
2	216.67 ug/ml
3	130 ug/ml
4	52 ug/ml
5	26 ug/ml
6	13 ug/ml

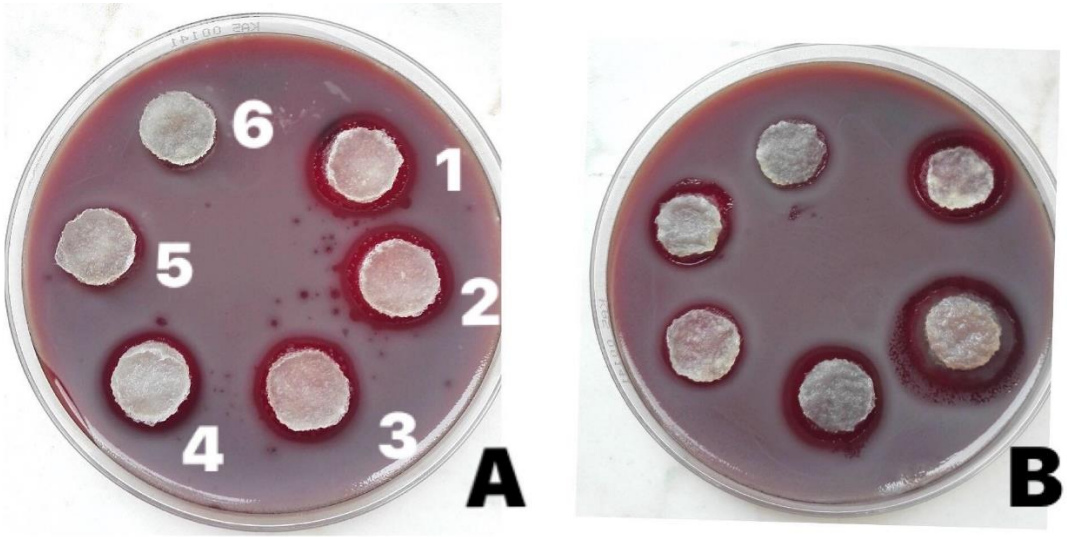

Figure 28: Antibacterial activity of lysostaphin to strain 244- ST8 in: A) non-crosslinked samples; B) crosslinked samples

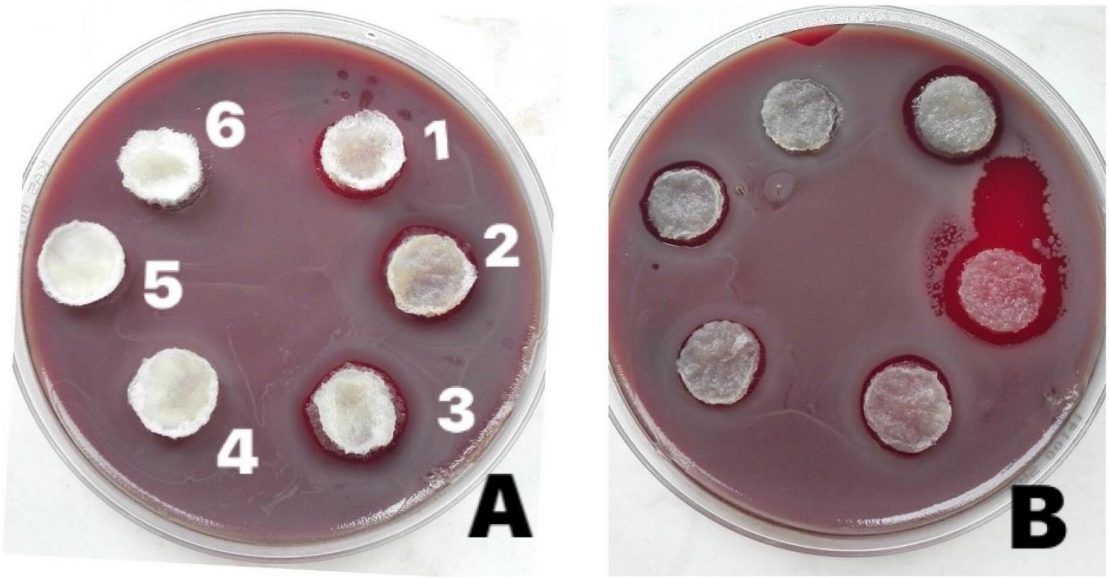

Figure 29: Antibacterial activity of lysostaphin to strain 5921- ST22 in: A) non-crosslinked samples; B) crosslinked samples

Table 7: List of coll/CMC samples prepared with lysostaphin for further tests

	$w_{collagen}$, [%]	$w_{cellulose}$, [%]	EDC/NHS	Lysostaphin
PBS/NaCl (5mM/25mM)	0.5	0.5	x	x
			x	yes
			yes	x
			yes	yes
Ultrapure Water	0.5	0.5	x	x
			x	yes
			yes	x
			yes	yes

Prepared non-crosslinked samples with lysostaphin differed in salt addition, whereas no problematic was the addition of lysostaphin into the saline biopolymer solution, the non-salt presence was accompanied by precipitation. When lysostaphin dissolved in the mixture of collagen and CMC, the two-phase separation occurred. While in up part of the Eppendorf precipitation of proteins occurred, in the down part, a clear UPW solution stayed separated (Figure 30- B, C). This was caused because lysostaphin is a large molecule of protein and it may react with its own groups and make a conglomerate. Therefore, the salt added into protein stabilize lysostaphin and prevent forming these conglomerates. We tried to resolve this problem with the dosing solution of lysostaphin by drops into the coll/CMC mixture and gently mixed with a glass rod. The resulting mixture was placed into prepared plates in a volume of 1 ml and freeze-dried for two days.

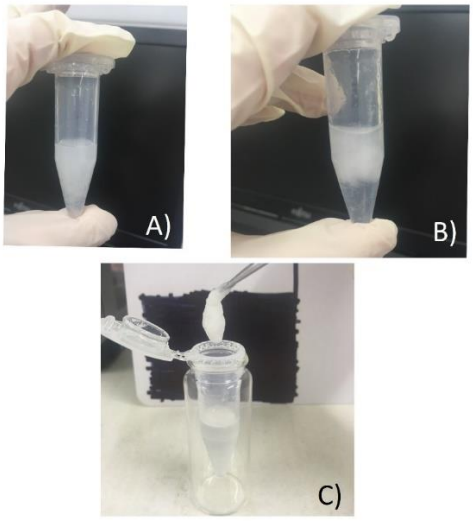

Figure 30: A) coll/CMC mixture in UPW; B) coll/CMC mixture with lysostaphin in UPW; C) conglomerate after adding a lysostaphin into coll/CMC mixture prepared in UPW

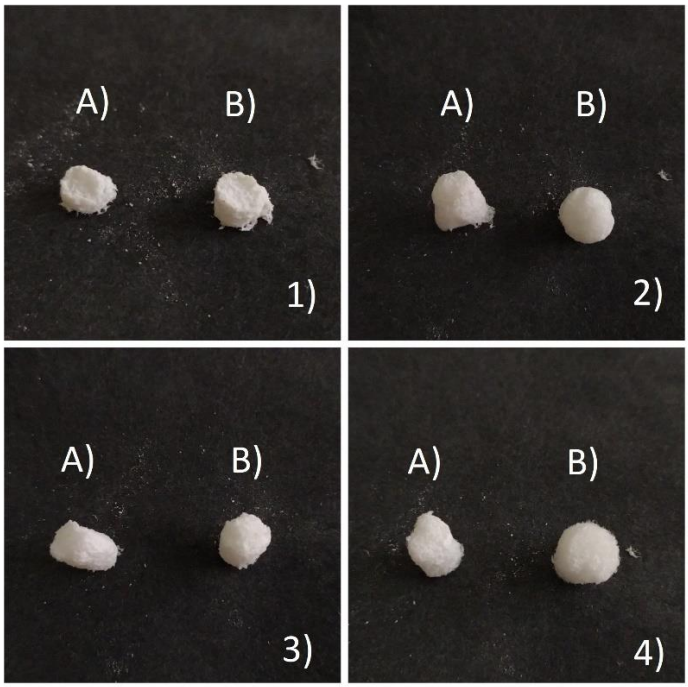

Figure 31: 1) non-crosslinked sample prepared in PBS/NaCl: A) with lysostaphin; B) without lysostaphin;

2) non-crosslinked sample prepared in UPW: A) with lysostaphin; B) without lysostaphin;

3) crosslinked sample prepared in PBS/NaCl: A) with lysostaphin; B) without lysostaphin;

4) crosslinked sample prepared in UPW: A) with lysostaphin; B) without lysostaphin

5.6 Release of Lysostaphin from Collagen/CMC Samples

Calibration curves for three different concentrations of lysostaphin have been made (for high, medium, and low concentration of lysostaphin). For our samples, we decided to use the calibration curve for the low concentration of lysostaphin (Figure 32).

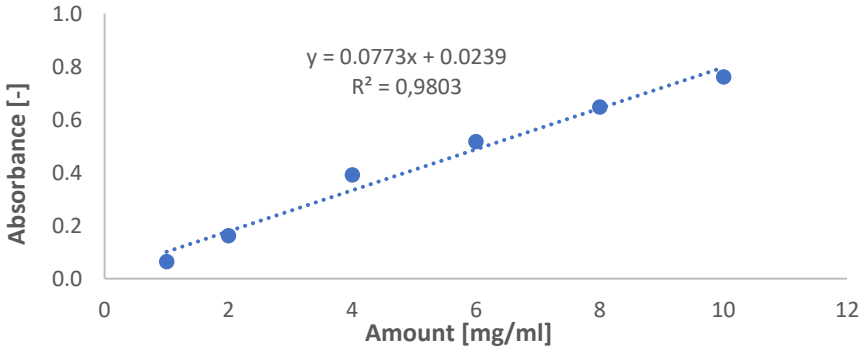

Figure 32: Calibration curve for low concentration of lysostaphin

Because collagen in our samples is also a protein that can react with Bradford reagent, we prepared the same samples with and without lysostaphin to compare values of absorbance to get just the amount of released lysostaphin. A Matlab in-house script was used to determine the fitting curve and parameters. Every sample released lysostaphin according to 1st order kinetics.

In Figure 33 we can see that the non-crosslinked sample prepared in salt lasted just 2 hours and during this period, it released 51.23 mg/ml (15.8 %) of lysostaphin. This amount was released in just one phase (Equitation 6, 7) and eroded quickly.

$$c_1 = c_0 - c_0.\exp(-k.t) \tag{E6}$$

$$k = \frac{ln2}{t_{1/2}} \tag{E7}$$

Non-crosslinked samples prepared without salt (Figure 34) last for 22 days and during this time they released 71.11 mg/ml (21.8 %) of lysostaphin. This amount was released in two phases of release according to Equitation 8. In first phase there was released the greatest amount of lysostaphin and in second phase, slower release of lysostaphin has been observed.

$$c = c_1 - c_1.\exp(-k_1 t) + c_2 - c_2.\exp(-k_2 t) \tag{E8}$$

Both of these releases were according to 1st order kinetics and the greatest amount of lysostaphin has been released in first 30 minutes. In both cases, we not all lysostaphin was released from samples. This can be caused by unwanted interaction of lysostaphin -NH_2 groups with collagen or maybe degradation products may have occurred that we were unable to detect.

Table 8: The comparison of fitting parameters in non-crosslinked samples

	samples without salt	samples with salt
c_1 (mg/ml)	64.76	49.52
$t_1$1/2 (hours)	452.50	0.21
c_2 (mg/ml)	37.52	-
$t_2$1/2 (hours)	5.20	-
k_1	0.002	0.055
k_2	0.133	-

Where c_1 is limit concentration, k_1 is rate constant, and t_1½ is reaction half time for the first phase of release and c_2 is limit concentration, k_2 is rate constant, and t_2½ is reaction half time for the second phase of release.

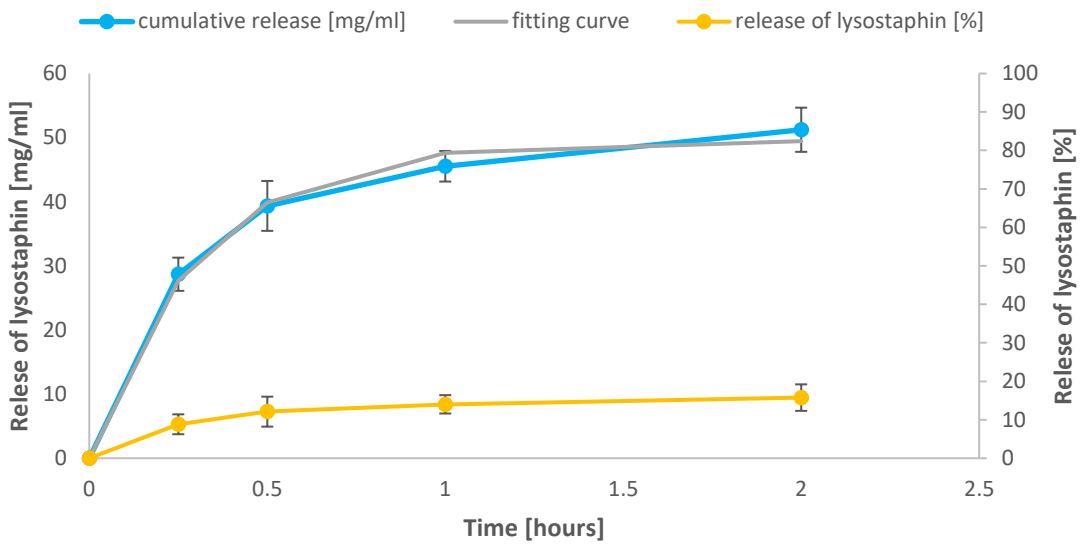

Figure 33: Release of lysostaphin from non-crosslinked samples with salt

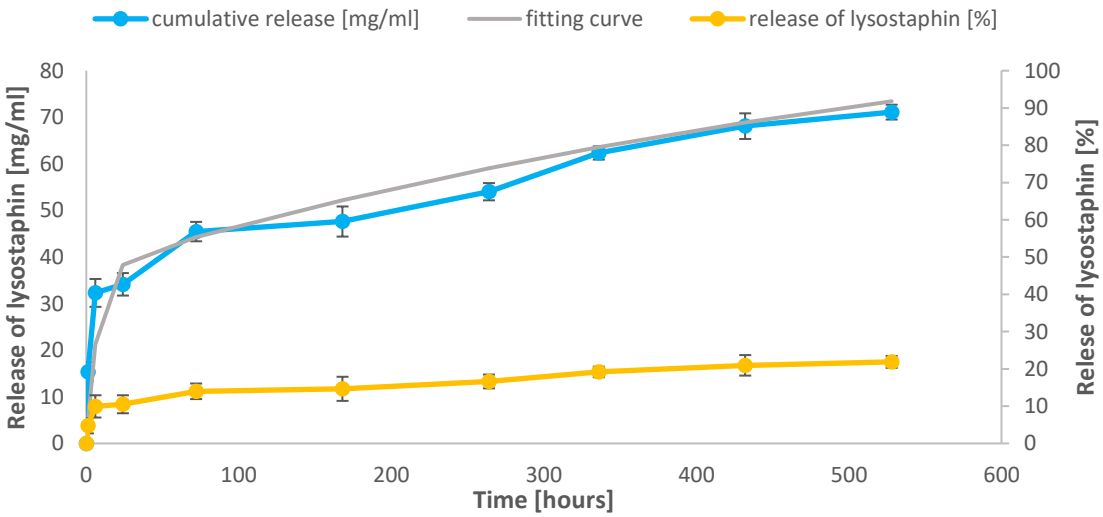

Figure 34: Release of lysostaphin from non-crosslinked samples without salt

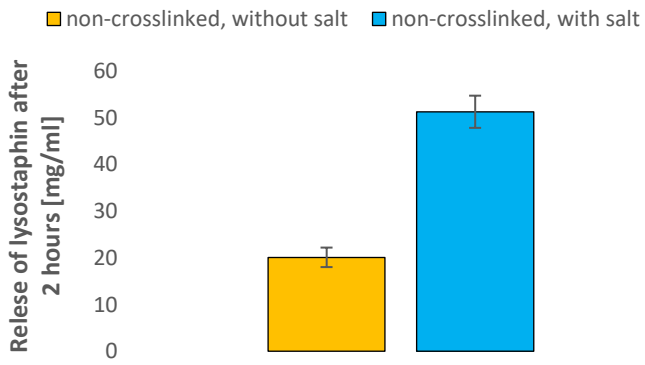

Figure 35: The comparison of non-crosslinked samples with and without salt after 2 hours

In crosslinked samples, amount of released lysostaphin was just slightly different. Crosslinked samples with salt released 34.78 mg/ml (10.7 %) during 18 days (Figure 36) and crosslinked samples without salt released 37.51 mg/ml (11.54 %) (Figure 37). Both of these releases were two phased (E8) and according to 1st order kinetics. In first phase the greater amount of lysostaphin has been released and this may have been caused by some parts of samples not crosslinked and thus it degraded quickly with a greater amount of lysostaphin in it.

Table 9: Comparison of fitting parameters in crosslinked samples

	samples without salt	samples with salt
c_1 (mg/ml)	27.36	19.00
$t_1$1/2 (hours)	95.29	113.30
c_2 (mg/ml)	10.94	16.70
$t_2$1/2 (hours)	0.31	0.33
k_1	0.007	0.006
k_2	2.269	2.100

Again, in these samples not the whole amount of lysostaphin was released from samples and in this case, it may have been caused by unwanted interaction of lysostaphin -NH_2 groups with collagen or possibly degradation products may have occurred that we were unable to detect. These samples were not measured until total erosion of samples so with increasing time, the amount of released lysostaphin may also increase.

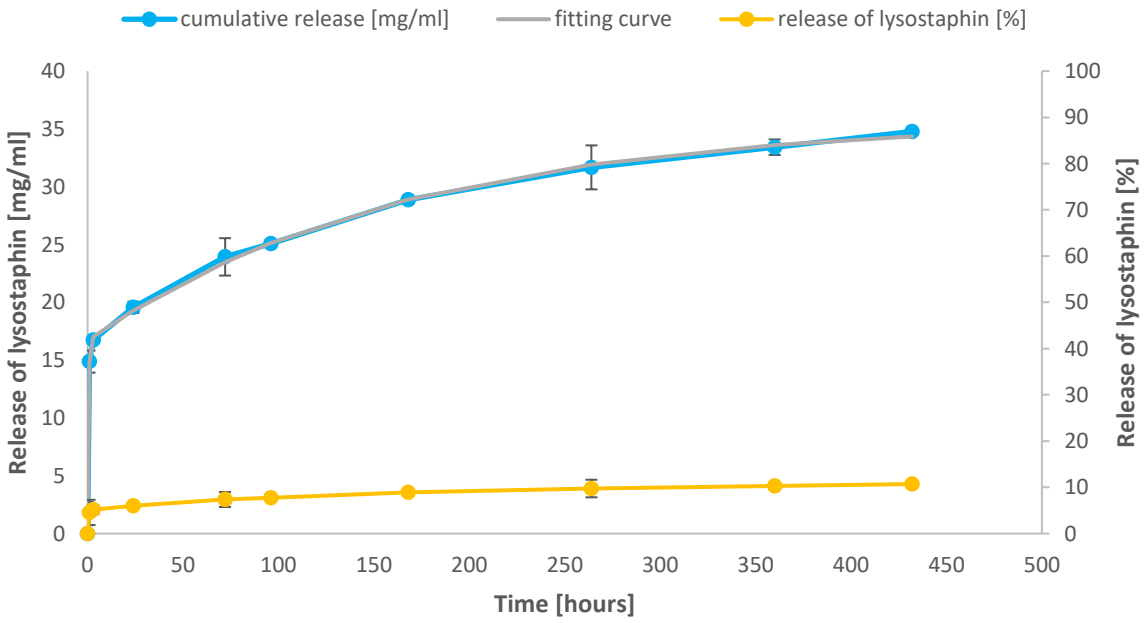

Figure 36: Release of lysostaphin from crosslinked samples with salt

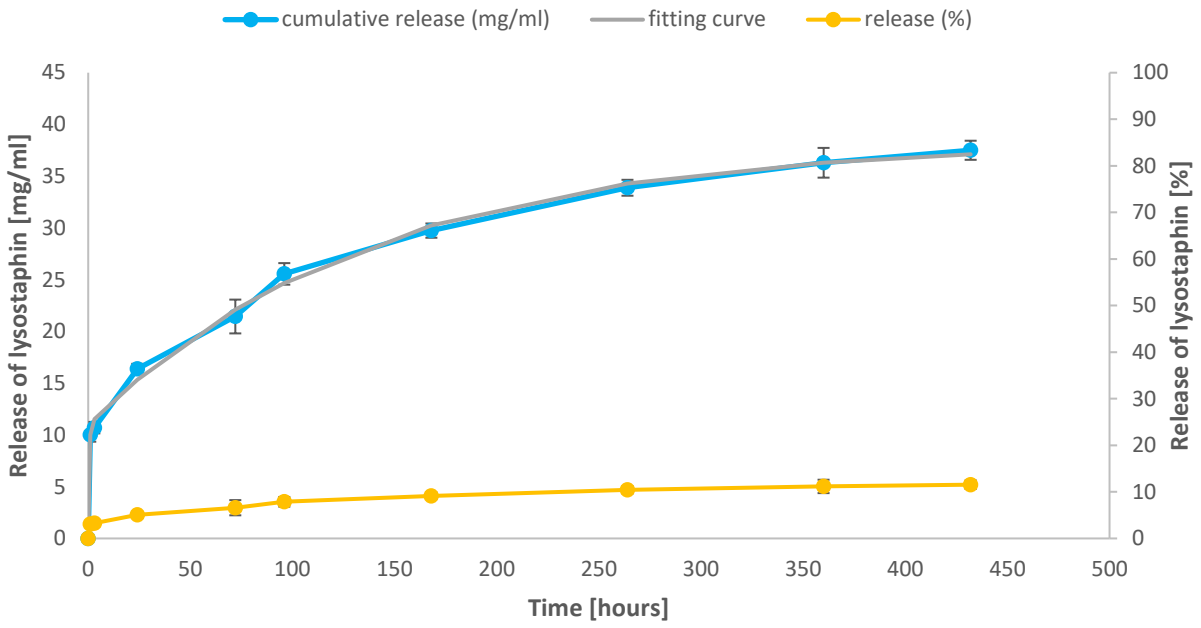

Figure 37: Release of lysostaphin from crosslinked samples without salt

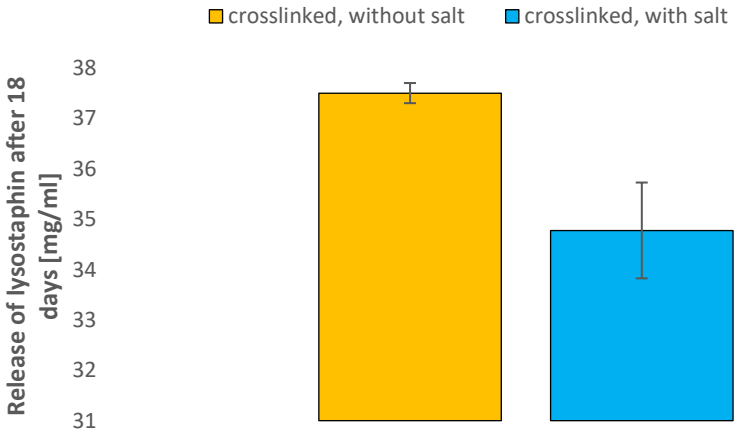

Figure 38: The comparison of crosslinked samples with and without salt after 18 days

From results, it can be said that lysostaphin is released from samples by eroding the matrices. The more the matric eroded, the more lysostaphin was released.

6 CONCLUSIONS

This diploma thesis deals with observing the effects of salt on the stability of biopolymer carriers. Two series of samples (crosslinked and non-crosslinked) in two different buffers/salt solutions (PBS/NaCl and TrisHCl/NaCl) in four concentrations (25mM/125mM, 10mM/50mM, 5mM/25mM, 1mM/5mM) have been prepared. The effect of salt on samples was visible immediately after the preparation of the samples. The samples were getting smaller inversely proportional with the rising salt concentration.

Further on, swelling tests have been conducted, which showed that TrisHCl/NaCl solution is not suited for non-crosslinked samples due to the rapid erosion of all the samples caused by the high pH of the buffer making the samples non-stable. Non-crosslinked samples prepared in PBS/NaCl solution were more stable, even more stable than the reference sample. Also, it has been confirmed that salt increases the swelling ratio, but with increasing concentration of salt the swelling ratio decreases. Based on this test, it can be said that it is better to use a buffer with lower pH and as small concentration of salt as possible.

The addition of salt into crosslinked samples significantly increased the swelling ratio compared to the reference sample. As with non-crosslinked samples, the swelling ratio was decreasing with an increasing concentration of salt. The suitability of samples, according to the swelling ratio was the best with the lowest concentration of salt.

Then the hydrolytic stability of prepared samples has been observed in saline solution at a temperature of 37 °C. Even in this case, the non-crosslinked samples prepared in TrisHCl/NaCl solution eroded in a short time and it confirmed that this buffer is not suitable for sample preparation. The most stable non-crosslinked samples made in PBS/NaCl solution were the ones with low salt concentration. The difference was marginal in crosslinked samples and both series lasted for forty days losing approximately 75-85 % of their mass before eroding.

Based on the swelling test and hydrolytic stability test, it was decided to use only PBS/NaCl solution with a concentration of 5mM/25mM for another sample preparation. Series of crosslinked and non-crosslinked samples with the aforementioned buffer/salt concentration were prepared. The samples were subjected to SEM analysis and results were compared to reference crosslinked and non-crosslinked samples made without salt. I could see slightly enlarged pores in SEM images of samples prepared with salt (5mM/25mM) which could explain better results of the swelling tests. Because during this measurement I have worked with relatively low salt concentration, there was no unwanted shrinking of the pores as we would expect it with higher salt concentrations.

After choosing the right buffer/salt solution and concentration, lysostaphin in a concentration of 325 mg/ml have been added into the samples. Once again, there were prepared two series of samples (crosslinked and non-crosslinked) with lysostaphin, and with or without salt to compare releasing of lysostaphin with Bradford reagent and UV-VIS spectrometer at a wavelength of 595 nm.

It was found that not the whole amount of lysostaphin released from any of the samples which could be caused by interactions between groups of lysostaphin and collagen or carboxymethylcellulose. Another explanation could root down to some degradation products that we were not able to detect. This is the reason why this method is not applicable for measuring the exact amount of released lysostaphin and it would be more suiting to use some other method like HPLC chromatography or measuring release using ELISA.

Based on the results, it can be said that non-crosslinked samples with salt would be more suitable for treating acute wounds because these samples showed releasing a greater amount of lysostaphin (15.8 %) before the erosion of the sample. Non-crosslinked samples without salt lasted longer (22 days) and therefore would be better for treating chronic wounds with a gradual release of smaller amounts of lysostaphin. The main difference between crosslinked samples with or without salt in terms of lysostaphin release was negligible (1 %). Hence it would be beneficial to subject the samples to antibacterial tests to tell whether the released lysostaphin in both samples is still active against strains of *Staphylococcus aureus.*

One of the key points of this diploma thesis was to determine whether the addition of salt is necessary for lysostaphin stabilization because salt generally destabilizes biopolymer carriers. However, data shows that lysostaphin is stable even without the addition of salt mainly for crosslinked matrices and thus its addition is not necessary. However, it seemed that interactions between lysostaphin and both collagen and carboxymethylcellulose should be further studied more in deep to better understand the protein release and its stability.

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8 LIST OF ABREVIATIONS

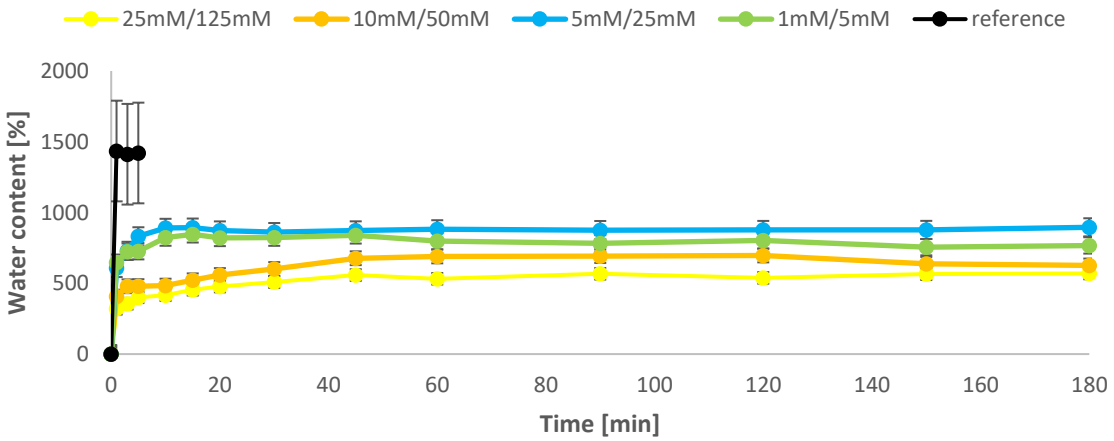
3D	three-dimensional
BSA	bovine serum albumin
BGG	bovine γ-globulin
CMC	carboxymethylcellulose

Coll	collagen
ECM	extracellular matrix
EDC	*N*-(3-Dimethylaminopropyl)-*N´*-ethylcarbodiimide hydrochloride
G-250	Coomassie Brilliant Blue
Gly	glycerine
kDa	kilodalten
MRSA	methicillin resistant Staphylococcus aureus
NHS	*N*-hydroxysuccinimide
pA	picoAmper
PBS	Phosphate Buffered saline
PBP	penicillin binding proteins
PEG	polyethylene glycol
RPM	revolutions per minute
SEM	scanning electron microscope
SR	swelling ratio
TrisHCl	Tris(hydroxymethyl)amino-methane
UPW	ultrapure water
UV	ultraviolet

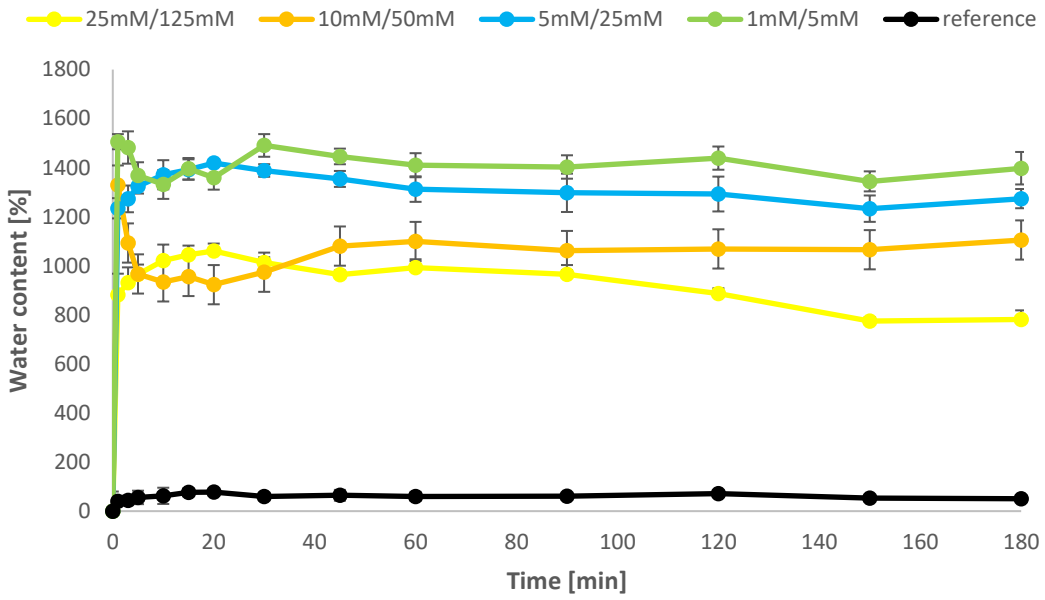
9 LIST OF TABLES

10 LIST OF FIGURES

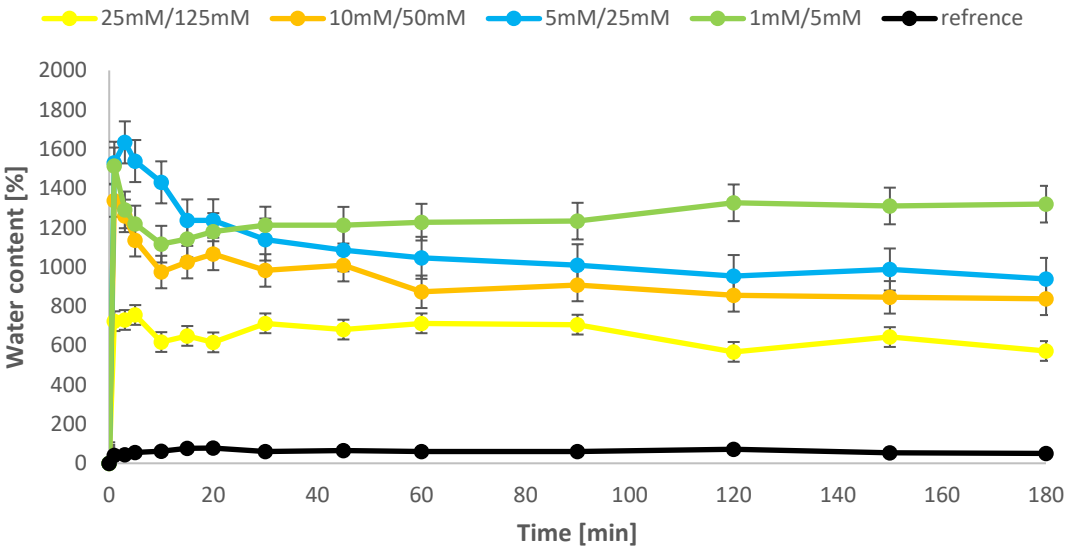
11 LIST OF APPENDIXES



Appendix 1: The dependence of water content on the time for non-crosslinked samples prepared in PBS/NaCl solution



Appendix 2: The dependence of water content on the time for crosslinked samples prepared in PBS/NaCl solution



Appendix 3: The dependence of water content on the time for crosslinked samples prepared in TrisHCl/NaCl solution