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Chitosan coatings for surfaces to prevent implant-related infections

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ABSTRACT

Chitosan coatings for surfaces to prevent implant-related infections

Implant-related infections are one of the leading causes of nosocomial biofilm infections. As a result of the increasing number of patients with indwelling implants, public health has concerns about the increase in chronic biofilm-infections. The primary reasons for those concerns are that biofilm infections are resistant to the host´s immune response and have tolerance against high doses of antibiotics. Researchers have concluded that the most efficient way of combating biofilm infections in implants is to prevent them in the first place. Antibiofilm coatings are rapidly becoming a primary component of the global mitigation strategy. Ideal coating agents should possess high antibiofilm efficacy, as well as excellent biocompatibility and nontoxicity. The criteria apply to the semi-natural polysaccharide chitosan, which has been tested as a coating for various applications, including some medical devices.

However, there were limited published studies about chitosan-coated catheters, a common type of medical implants. Catheters mostly made of silicone are an inert substrate that needs to be activated to allow the conjugation of chitosan covalently to the surface. This work reported in the thesis was aimed at developing a chitosan coating strategy for silicone substrates using a silanization method and to investigate the antimicrobial properties of such surfaces.

It was demonstrated that silicone surfaces could be activated by treatment with a piranha solution. Silicone could be further modified by silanization and crosslinking to attach chitosan to the surface. Quantitative ninhydrin assays and qualitative FT-IR analysis were carried out to confirm successful chitosan coating of silicone substrates, and glass which was used as reference material. Both glass and silicone substrates modified with amino silane agents (APTMS), and then the amino groups of chitosan and APTMS were crosslinked together using glutaraldehyde, exhibited superior results relative to a glycidyl (GPTMS) silanization agent. Antimicrobial studies were carried out, but results were inconclusive, showing that further development of the assay procedure is needed.

ÁGRIP

Kítósanhúðanir fyrir yfirborð til að fyrirbyggja sýkingar af völdum ígræðlinga

Sýkingar af völdum ígræðlinga er ein helsta rót spítalasýkinga sem myndast út frá lífhimnum (e. biofilms). Aukinn fjöldi sjúklinga með ígræðlinga hefur leitt til aukinnar hættu á krónískum lífhimnusýkingum. Slíkar sýkingar eru oft ónæmar fyrir ónæmissvörunum líkamans og hafa þol gegn háum skömmtum sýklalyfja. Vísindamenn hafa komist að þeirri niðurstöðu að skilvirkasta leiðin til að hindra slíkar sýkingarnar séu fyrst og fremst að koma í veg fyrir þær. Húðanir sem hindra myndun lífhimna er hratt vaxandi hluti forvarnaraðgerða til að koma í veg fyrir lífhimnusýkingar. Heppilegt húðunarefni fyrir ígræðlinga ætti að búa yfir þeim eiginleikum að geta hindrað myndun lífhimna, góðum lífsamrýmanleika og lítil eitrunaráhrif. Kítósan er að hluta náttúruleg fjölsykra sem hefur þessa eiginleika og hefur því verið rannsökuð sem húðunarefni, þ.á.m. fyrir ígræðlinga.

Hins vegar hefur lítið verið birt um kítósanhúðun leggja (e. catheters) sem eru algeng tegund ígræðlinga í lækningum. Leggir eru að mestu gerðir úr silíkoni. Það hefur óvirkt yfirborð sem þarf að virkja til þess að það sé mögulegt að binda kítósan við það með samgildum tengjum. Markmið verkefnisins var að þróa aðferðir sem byggja á kísileringu (e. silanization) til að húða silíkonyfirborð með kítósan. Einnig var markmið að rannsaka bakteríudrepandi eiginleika húðaðs silíkons.

Sýnt var fram á að hægt er að virkja silíkon yfirborð með piranha lausn. Þessu var fylgt eftir með kísileringu og krossbindingu (e. crosslinking) til þess að festa kítósanið við yfirborðið. Framkvæmdar voru ninhydrin mælingar og eigindlegar FT-IR greiningar til að staðfesta kítósanhúðun við silíkonyfirborðsins. Glerplötur voru húðaðar og rannsakaðar til samanburðar. Aðferð þar sem gleri eða virkjuðu silíkon yfirborð sem var breytt með amínókísilefnum (APTMS) og glútaraldehýði gáfu góða bindingu kítósans. Þessi yfirborð gáfu betri niðurstöðu en þau yfirborð sem breytt voru með glýsidíl kísilefni (GPTMS). Bakteríudrepandi prófanir voru framkvæmdar en niðurstöður reyndust ómarktækar og er því þörf á frekari aðferðaþróun til að ákvarða þennan eiginleika yfirborðanna.

LIST OF ABBREVIATIONS

AMP	Antimicrobial Peptides
APTMS	3-(Aminopropyl)trimethoxysilane
CAUTI	Catheter-associated Urinary Tract Infection
CH_3COOH	Acetic acid
CNS	Central Nervous System
CVC	Central Venous Catheters
DA	Degree of Acetylation
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DS	Degree of Substitution
EPS	Extracellular Polymeric Substances
EtOH	Ethanol
FDA	Food and Drug Administration
FT-IR	Fourier-Transform Infrared Spectrophotometer
GPTMS	3-(Glycidyloxypropyl)trimethoxysilane
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
MeOH	Methanol
MHA	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
MW	Molecular Weight
$NaBH_4$	Sodium Borohydride
NIH	National Institute of Health
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
QAC	Quaternary Ammonium Compounds
SAM	Self Assembled Monolayer
Ti	Titanium
UTI	Urinary Tract Infection
UV-Vis	Ultraviolet-Visible Spectrophotometer
MAC	Mean Area Concentration
AC	Area Concentration
SD	Standard Deviation

E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
K. pneumoniae	*Klebsiella pneumoniae*
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabilis	*Proteus mirabilis*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*

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DECLARATION OF CONTRIBUTION

I hereby declare that all work made in the laboratory and processing of the results in the thesis were performed by me under the supervision of Professor Már Másson for this project. I was also assisted by Ph.D. student Vivien Nagy and M.Sc. student Sigríður Ólafsdóttir. The development of the project was initiated by Professor Már Másson, as he provided me guidance and close supervision through the laboratory work as well as the writing of this thesis. The laboratory work for this project was done in his laboratory at the Faculty of Pharmaceutical Sciences in the Hagi building. Ph.D. student Vivien Nagy provided me guidance in the laboratory with operating instruments, preparing reagents, as well as the identification of the compounds. M.Sc. student Sigríður Ólafsdóttir provided me guidance in preparing and performing the antimicrobial assays in a laboratory in the Stapi building. In consultation with Professor Martha Á. Hjálmarsdóttir, a new method was developed in observing the antimicrobial activity of the samples. Unfortunately, I was not able to test this new method because of the COVID-19 pandemic. All MS students were denied access to the laboratory of Stapi building in mid-March. It affected the project's progress at its final stages, which affected the author's results for that particular chapter in the thesis. The project was in general based on published data discovered by the author as well as data provided by the supervisor. Data was also processed from measurements made from the UV-Vis and FT-IR spectrophotometers in the project.

1. INTRODUCTION

1.1 Medical implants

Medical implants are devices that are inserted into the body to enhance the quality of life. Their history can be traced back to the early Egyptians and South-Central American cultures. Although some of the ancient achievements were impressive, current applications are impacted by the significant development in biological and chemical sciences that have occurred since then (Saini, Singh, Arora, Arora, & Jain, 2015; Teo et al., 2016).

The term implant refers to a medical device placed inside the human body for medicinal purposes, generally for long periods. The main goal of those implant devices is the preservation of human lives and to extend the functionality of vital human body systems ("Medical Implants," 2019; Teo et al., 2016).

An increase in longevity and life expectancy influences the need for implants because the rise in the average age of the world´s population leads to an increase in age-related diseases, e.g., joint diseases (Khan, Muntimadugu, Jaffe, & Domb, 2014; Saini et al., 2015).

Implants can replace various body parts with human-made objects to regulating bodily functions with an implantable device. Human-made implants provide physical support by replacing body parts (e.g., hips or knees), deliver medications through synthetic blood vessels for pain relief and to maintain continuity of lumens of the urethra (e.g., catheters). Monitor implants (e.g., pacemakers) are used to regulate body functions, e.g., abnormal heart rate. Numerous studies have been made on various medical implants with different function in the human body. Recent improvements in biomedical implants make the present and future for this treatment modality very promising ("Medical Implants," 2019; Teo et al., 2016).

1.1.1 Implant biomaterials

Materials being used to create medical devices, prosthesis, and replace natural body tissues are called biomaterials. The definition of the term biomaterial is:

A biomaterial is a nonviable material used in a medical device, intended to interact with biological systems (Williams, 1987).

Contrary to what the word may implicate, a biomaterial is not necessarily biological or based on a bio-related material, though it can be bio-inspired and derived from nature. It is a special class of materials that have been designed to take a form that, alone or as part of a complex system, is used to coexist with a biological system with which it interfaces, i.e. biocompatibility (Detsch, Will, Hum, Roether, & Boccaccini, 2018; Ratner, Hoffman, Schoen, & Lemons, 2013).

The biocompatibility of materials is of considerable interest because implants can corrode in an *in vivo* environment; this can lead to loss of load-bearing strength and consequent degradation into toxic products. For that, a biomaterial should be chemically and biologically inert, as well as nontoxic, to achieve the best performance. In other words, it is reasonable to say that a biomaterial should not give off anything from its mass unless it is specifically engineered to do so. In some cases, the biomaterial is designed to release toxic agents to give an advantage, e.g., a drug delivery system that targets cancer cells and destroys them. Also, biomaterials can promote bioactivity, e.g., bone regeneration, or minimize undesirable bioactivity, such as infection. The selection criteria must be kept in mind when choosing an implant biomaterial because it is the primary criterion for proper functioning (Desai, Bidanda, & Bártolo, 2008; Khan et al., 2014; Raghavendra, Varaprasad, & Jayaramudu, 2015).

Medical implants are made from synthetic biomaterials and broadly divided into three categories: ceramics, metals, and polymers (Khan et al., 2014)

1.1.2 Ceramics

Historically, ceramics are the oldest human-made implant materials. The use of ceramics was motivated by their inertness in the body and excellent biocompatibility, osteoconductivity, corrosion resistance, and better strength for load-bearing applications. However, applications of ceramics in some cases are severely restricted due to brittleness, low elasticity, poor fracture toughness, and extremely high stiffness (Khan et al., 2014; Raghavendra et al., 2015).

The ceramic biomaterial can form ionic bonds after implantation that aids strong bonding to bone tissue. Hence, this can influence bone health (e.g. osteogenesis) and enhance biocompatibility, while strengthening the mechanical properties of the implants. Furthermore, based on their excellent biocompatibility, ceramics are used as implants within bones, joints, and teeth. More specifically, the biomaterial is used as parts of the musculoskeletal system, artificial knees, bone grafts, hip prosthesis,

cardiac valves, dental and orthopedic implants (Desai et al., 2008; Khan et al., 2014; Raghavendra et al., 2015).

The bio-ceramic materials include, among other things: the alumina and zirconia (bioinert ceramics), bioactive glasses and glass-ceramics (bioactive ceramics), and calcium phosphates (bioresorbable ceramics) (Pina, Reis, & Oliveira, 2017).

1.1.3 Metals

Initially, metals were introduced for other uses than implants, such as for parts in the aircraft industry. However, later on, these metals and their alloys have been found to be suitable for use as an implant biomaterial. Good mechanical strength and resistance to fracture are the qualities of metallic biomaterials that give reliable long-term implant performance in major load-bearing applications (Davidson & Kovacs, 1992).

This class of biomaterial has various favorable characteristics, such as high stiffness, ductility, toughness, wear resistance, electrical and thermal conductivity. Metallic implants are used in orthopedics and dentistry primarily, the two areas in which highly loaded devices are the most common. The good electrical conductivity of metals favors their use for neuromuscular stimulation devices, e.g. cardiac pacemakers. All of those properties listed are related to the metallic interatomic bonding that characterizes this class of biomaterial (Desai et al., 2008; Niinomi, 2008).

Metals are susceptible to degradation by corrosion after implantation, a process that can release by-products that elicit toxic or hypersensitivity responses in the body. That makes the corrosion resistance of a surgically implanted alloy an essential characteristic and researchers today give great importance in understanding that problem in order to develop more biocompatible metals (Santos, 2017). Also, metals do not possess bio-functionalities such as bioactivity, bone conduction, and blood compatibility. Hence, surface modifications are required to improve their bone conduction by coating the metals with bio active ceramics (e.g., hydroxyapatite). The blood compatibility is improved by coating the metals with biopolymers (e.g. chitosan) (Hermawan, Ramdan, & Djuansjah, 2011).

The metallic biomaterials include pure metals and metal alloys, but the most successfully used are stainless steels, cobalt alloys, titanium (Ti), and titanium alloys (Khan et al., 2014). Pure titanium and Ti-alloys offer advantages over the stainless steel and cobalt alloys because of its excellent corrosion resistance. Also, superior biocompatibility, high strength, and lower modulus of elasticity (e.g. closer to that of bone) (Davidson & Kovacs, 1992; Khan et al., 2014).

Biomedical application for pure titanium includes dental implants, pacemaker cases, implantable infusion drug pumps, housings for ventricular assist devices, craniofacial implants, screws, and staples for a spinal operation. Biomedical application for Ti-alloys includes fracture fixation plates, femoral hip stems, intramedullary screws and nails (Khan et al., 2014).

1.1.4 Polymers

Polymers are a promising class of materials with a wide range of medical applications and compared to ceramic and metallic biomaterials, they are considered one of the best materials for medical purposes. Polymers are macromolecules composed of many repeating subunits, called monomers, bonded with covalent bonds. Characteristics such as non-cytotoxicity, versatility, biocompatibility, and biodegradation make polymeric biomaterial often an excellent candidate to be used in implants (Devi, 2017; Nag & Banerjee, 2012; Rebelo, Fernandes, & Fangueiro, 2017).

The earlier polymers used in implantable devices were natural in origin, but later on found to have many formulation problems, such as instability, irreproducibility, and uncontrollable formulation characteristics. Consequently, synthetic polymers have been the polymer of choice for implants because they are efficiently produced and available, making it cost-effective for fabrication. Synthetic polymers are either modified from natural polymers or completely synthesized from monomers by using condensation and polymerization techniques to achieve long chains of desired shape and quality. A wide range of chemical and physical attributes can be made based on the monomer subunits. The current trend in the biomedical field is the combination of materials to synthesize composites to provide more mechanical strength, flexibility or functionality (Adikwu & Esimone, 2009; Maitz, 2015; Nag & Banerjee, 2012; Teo et al., 2016).

Polymers are the most widely used implant materials for dental, orthopedic, soft-tissue, and cardiovascular applications, as well as for drug delivery devices. Synthetic polymers have been widely used as implants for treating conditions associated with soft tissues, whereas harder tissues (e.g. bone) are better treated with ceramics and metals. The majority of disease conditions are associated with problems of soft tissues. Applications of synthetic polymers include encapsulants and carriers (e.g. catheters), functional load-carrying and supporting implants (e.g. vascular grafts), prosthetic limbs and medical instrumentation. The range of synthetic polymeric systems include polysiloxanes (silicones), polyethylene, polyesters, polyamides, etc. (Pegoretti &

Dorigato, 2017; Raghavendra et al., 2015). The polysiloxanes will be discussed further in the following chapter 1.1.4.1, considering its surface will be thoroughly researched in this thesis.

The main disadvantage of the utility of synthetic polymers is the general lack of biocompatibility, hence they are often associated with immunological rejection by the body. Furthermore, the biodegradation products from the synthetic polymers may present concerns in the body and lead to an unwanted immunogenic response. The biodegradation of the synthetic polymer occurs by hydrolysis, thereby producing carbon dioxide, which lowers the local pH and could result in tissue necrosis. Hence, returning to the usage of natural polymers has been attracting the researcher´s interest as a possible solution to the problem, as it has more benefits over synthetic polymers. The natural polymers are obtained from natural sources, plant or animal origin, which makes them similar to their biological environment and much better biodegradable qualities. Also, they are less likely to cause toxic and immunological response when located inside the human body. The natural polymers can be divided into three main categories, proteins (e.g. collagen, silk), polysaccharides (e.g. chitin/chitosan, cellulose), and polynucleotides (Khan et al., 2014; Raghavendra et al., 2015; Rebelo et al., 2017).

The main differences between synthetic and natural polymers are shown in table 1, both advantages and disadvantages are taken into account.

Table 1. Advantages and disadvantages of both natural and synthetic polymers as implants.

Natural polymers		**Synthetic polymers**
• Biodegradable • Biocompatibility • Less toxic • Easily available	**Advantages**	• Mechanical and chemical properties readily altered • Reproducible • Reasonable cost
• Structurally more complex • Very complicated extraction process (high cost) • Batch-to-batch variability • Poor mechanical strength	**Disadvantages**	• More toxic • Immunogenic response • Non-biodegradable • Complicated synthetic process

1.1.4.1 Polysiloxanes

Polysiloxanes, or silicones, are an essential class of synthetic polymers that are widely used for medical applications. They exhibit a wide variety of properties that range from being liquids to gels and elastomers. Silicones are the polymer of choice for long-term use in the body where an elastomer is required and demanded for biocompatibility and biodurability. The reason for its high biodurability is a result of other properties of the silicone, such as hydrophobicity, low surface tension, thermal, and chemical stability. Silicones consist of a silicon-oxygen backbone with organic groups attached to the silicon atoms, usually methyl groups. Poly(dimethylsiloxane) (PDMS) is the most common member of this polymeric family and has a long history of use in implantable applications. The lack of any polar groups in its structure, as seen in figure 1, leads to this very hydrophobic polymer (Hacker & Mikos, 2011; Hill, 2005).

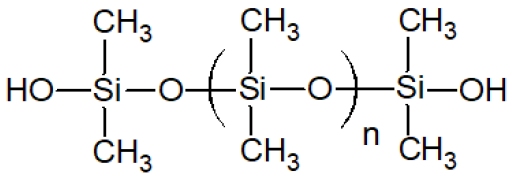


Figure 1. A polymeric structure of PDMS, the most commonly used polysiloxane or silicone (Polymer Properties Database, 2015).

Silicone surfaces are used for the fabrication of various blood-collecting instruments, such as silicone-coated needles and syringes because they have been found to inhibit blood from clogging for many hours. Due to their hemocompatibility, the polymer is also used as heart valves, heart-bypass machines and blood-oxygenator. The most prominent application of silicones is their use as cosmetic implants in aesthetic plastic surgery (e.g. breast, chin, etc.) Silicone elastomers have various medical applications, for example in catheters, drains, shunts, and tubular implants (e.g. artificial urethra) (Hacker & Mikos, 2011).

1.2 Infections and implants

Bacterial contamination is a critical issue that can affect many applications in the medical field, such as implants and medical devices, and subsequent infections can have a significant threat to human health. Implant-related infections remain a challenge and are known as a catastrophic complication, and unfortunately, these are rather common in patients with medical implants. Following the insertion of the implant, they

get coated with plasma and connective tissue, which subsequently may serve as specific receptors for biofilm-forming bacteria. Various factors in the implant's surface play a role in increasing the rate of infection, such as differences in surface charge, hydrophilicity, and the biomaterial itself (Ferraris & Spriano, 2016; Khatoon, McTiernan, Suuronen, Mah, & Alarcon, 2018; Wang & Tang, 2019).

Over half of the nearly two million healthcare-associated infections reported by the Centers for Disease Control can trace back to the indwelling medical implants. The cost associated with implant-related infections can be expensive, not only for the patient but also for the health care system, which includes drug treatments and revision surgeries (VanEpps & Younger, 2016; Wang & Tang, 2019).

Despite more careful and sterile techniques today, it is impossible to create a predictable sterile incision in implant insertion areas, even under laminar flow air conditions. The initial contamination occurs most likely from a small number of microorganisms via the patient´s healthcare worker´s skin, contaminated water, or other external environmental sources. Intact host defense systems usually eliminate transient contamination unless the inocula exceed the threshold levels. Then, the host defense system becomes impaired which could lead to an implant-related infection (Percival, Suleman, Vuotto, & Donelli, 2015; Schierholz & Beuth, 2001).

1.2.1 Biofilms

Humans can be infected by various pathogenic agents, such as bacteria, viruses, and fungi. Bacterial infections are the most common type, both acute and chronic, having two life forms during growth and proliferation. Firstly, planktonic bacteria that are single independent cells and secondly organized sessile aggregates, commonly referred to as biofilm see *figure 2* below (Bjarnsholt, 2013).

Modern antibiotics have been extensively studied for approximately a century and developed mainly to target planktonic bacteria. Generally, they are effective in treating acute infections. Now, however, the research focus has been shifted to the category of chronic infections caused by biofilms. Whenever planktonic bacteria succeed in forming a biofilm (i.e., sessile bacteria) within a human host, it can be challenging to eradicate the infection because biofilms are very resistant to antibiotics. Also, biofilms have a capacity for evading the host defenses developing into a chronic state (Bjarnsholt, 2013; Khatoon et al., 2018).

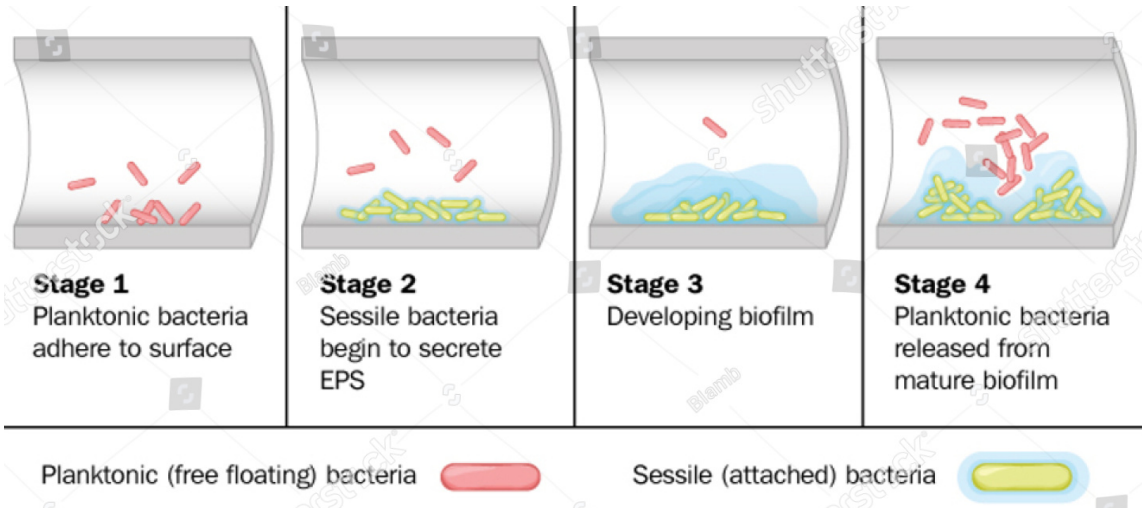


Figure 2. Different stages of planktonic and sessile bacteria in biofilm formation (Simmons, 2019).

The initial steps of chronic infection are exposure to the pathogen and bacterial adhesion, as soon as the bacteria become firmly adhered to a surface, such as an implant surface, they begin to proliferate. Often the increase of surface bacteria is physically and irreversibly aggregated that leads to a self-producing extracellular matrix containing different types of extracellular polymeric substances (EPS). Proteins, polysaccharides, fatty acids, and a variety of nucleic acids is a mixture of EPS that the biofilm secretes. EPS plays a significant role in biofilm formation by protecting the bacteria from the host defenses, such as white blood cells, antibodies, or monocytes. EPS defense mechanism in biofilms is to colonize in a suitable habitat to retain nutrients and to ensure survival. Also, the EPS inside the biofilm's matrix is a primary platform for surface adhesion and attachment. Finally, after the biofilm maturation, planktonic bacteria can disperse from the biofilm and invade surrounding tissues. The dispersed bacterial infection is more difficult to treat because the biofilm source persists (Khatoon et al., 2018; Wang & Tang, 2019).

As a result of the increasing number of patients with indwelling implants, public health concern increases because of biofilm-associated infections. Biofilm infections cannot be treated in the same way as acute infections since antibiotics alone are not capable of eradicating biofilm infections. However, the most efficient way is to mechanically remove the infected area or body part because the biofilm is resistant to the host's immune response and has tolerance to high doses of antibiotics (Bjarnsholt, 2013; Junter, Thebault, & Lebrun, 2016; VanEpps & Younger, 2016). The most efficient way of combating biofilm implant-related infection is to prevent them in the first place, as will be discussed in section 1.2.2.1. It is the most feasible approach to develop a

therapeutic choice that prevents the onset of biofilm in its first steps of colonization on the surface of the implant (Barbosa et al., 2019; Wang & Tang, 2019).

1.2.1.1 Nosocomial infections

In humans, biofilms account for up to 80% of the total number of nosocomial infections, according to the National Institute of Health (NIH). The most common nosocomial infections, considering severity and frequency, are those related to surgeries, such as surgical site infections, implants, and medical devices. Approximately 45% of all nosocomial infections are implantable device-related infections. That percentage includes urinary tract infection (UTI) from catheterization, pneumonia in patients intubated on a ventilator, and sepsis from using an intravascular catheter (Guggenbichler, Assadian, Boeswald, & Kramer, 2011; Percival et al., 2015; Schierholz & Beuth, 2001).

The most significant nosocomial implant-related infections are ventilator-associated pneumonia, which represents 22,8% of cases, catheter-associated UTIs (17,2%), and surgical site infections (15,7%). In each case, it is vital to isolate and identify these infections before giving any advice on prevention and treatment. Hence, antibiotic treatments should restrain before knowing further information about the bacteria causing the infection. Due to increasing antibiotic resistance, the antibiotic should be carefully determined by knowing the antibiotic susceptibility of the bacteria (Bjarnsholt, 2013; Percival et al., 2015).

1.2.1.2 Common bacteria in implant-related infections

Primary bacterial colonizers that are most associated with nosocomial implant-related infections are Gram-positive and Gram-negative bacteria. The Gram-positive bacteria include *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis,* and *Streptococcus viridian*. The Gram-negative bacteria include *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa.* All these pathogens can form biofilms composed of single or multiple species. Initially, the implant can be composed of single species, but the longer in-dwelling time develops a multispecies biofilm (Nandakumar, Chittaranjan, Kurian, & Doble, 2012).

The staphylococcal species are a diverse group of Gram-positive bacteria that mainly colonize the human skin and mucous membranes. *S. aureus* and *S. epidermidis*, are the primary bacteria causing nosocomial infections on the surgical site where the implantation took place. They are responsible for two-thirds of all implant-

related infections, as well as being the primary pathogens involved in orthopedic and cardiovascular infections. *S. aureus* and *S. epidermidis* are the causative agents in about 87% of septicemia, which develops mostly as a result of catheter-related infections and approximately 40-50% of prosthetic heart valve infections. Some tissue damage will unavoidably be caused by the heart valve and bacteria have a higher tendency to colonize locations where tissue damage has occurred. Also, *S. aureus* and *S. epidermidis* cause about 50-70% of catheter biofilm infections, including urinary catheters and central venous catheters (Donlan, 2001; Khatoon et al., 2018; Nandakumar et al., 2012; Stamm, 1978).

Urinary catheters are tubular medical devices mainly made of latex or silicone, and the longer it remains in place, the higher the tendency for bacteria to develop biofilms and subsequently resulting in UTI. The most common bacteria, including the staphylococcus species, to contaminate the urinary catheter and develop biofilms are *K. pneumoniae, E. coli, E. faecalis, P. mirabilis*, and *P. aeruginosa*. Intravenous catheters, such as central venous catheters (CVC), are indwelling medical devices that are particularly necessary for managing critically ill patients to deliver fluids, blood products, and medications. Colonization of CVC can occur rapidly, perhaps within a day, and biofilm formation is dependent on the duration of catheterization. According to Raad et al. (1993), the short-term CVC (<10 days) had more biofilm formation in the external surface but long-term CVC (>30 days) had more on the catheter inner lumen. The most common bacteria, including the staphylococcus species, to develop biofilm on CVC are *K. pneumoniae, E. faecalis,* and *P. aeruginosa* (C. Von Eiff, 2005; Donlan, 2001; Percival et al., 2015).

A common Gram-negative bacterium which is well known to adapt to harsh environments and antibiotics rapidly, thereby making them antibiotic-resistant, is the bacteria *P. aeruginosa* (Khatoon et al., 2018).

1.2.1.3 *Pseudomonas aeruginosa*

The organisms of *Pseudomonas* spp. are mostly free-living bacteria widely distributed in water, marine environments, soil, and on animal and human skin. The *Pseudomonas* spp. are strictly aerobic, although occasionally anaerobic growth is possible if a nitrate source can be utilized. Thus, cultures grow best with aeration, regardless of media (Golemi-Kotra, 2008; LaBauve & Wargo, 2012; Wisplinghoff, 2017).

P. aeruginosa is the most commonly and thoroughly studied bacteria while using surface-based *in vitro* systems to study biofilm developmental processes. *P.*

aeruginosa is a ubiquitous Gram-negative and rod-shaped bacterium that can cause significant disease as an opportunistic pathogen. It has extensive metabolic diversity which allows the bacteria to thrive in a wide variety of environments and nutrient sources. Additionally, this metabolic diversity enables *P. aeruginosa* to succeed as an opportunistic bacterium and causing more serious disease in immunocompromised patients. The bacteria can for instance cause UTI, sepsis, pneumonia, and central nervous system (CNS) infections. *P. aeruginosa* is a common cause of nosocomial infections and has been found responsible for approximately 11% of all the nosocomial infections. Their ability to form biofilms is probably one of its main survival strategies when infecting a host, making *P. aeruginosa* multi-drug resistant bacteria (Bjarnsholt, 2013; LaBauve & Wargo, 2012).

Electrostatic interactions and hydrophobicity mediate bacterial adherence to the implant´s surface. Either, the hydrophobic pathogen adheres well on hydrophobic implant surfaces, or the hydrophilic pathogen adheres well onto hydrophilic implant surfaces. In this case, *P. aeruginosa* adheres well onto hydrophilic, electrically neutral, and smooth polymeric surfaces, e.g., catheters. It shows that not only does hydrophobicity alone determine the bacterial adhesion but also the electrostatic interactions. These electrostatic interactions are essential in determining the attraction or repulsion of the bacterial surface to the implant. But of course, this varies to the type of bacterial strain and the implant-material involved (Nandakumar et al., 2012).

Patients with indwelling implants or medical devices, such as catheters, are at great risk of getting infected by *P. aeruginosa.* Infections caused by *P. aeruginosa* are complicated to treat, causing both more extended hospitalization and increased mortality. When urinary or intravascular catheters are localized into fragile patients, the risk of getting septicemia increases. The mortality in this group is extremely high, i.e. it occurs to almost two-thirds of cases (Olejnickova, Hola, & Ruzicka, 2014).

1.2.2 Treatment for implant-related infection

Post-operative complications, such as implant-related biofilm infections, may have serious consequences leading to necessary curative approaches: prolonged antibiotic therapy, revision surgery, or eventually, removal of a particular implant. Each therapeutic approach must be tailored to the needs of the patient, as well as their medical condition (Ferraris & Spriano, 2016; Moriarty et al., 2017).

The most efficient treatment to treat challenging biofilm infections in implantable devices is to mechanically remove the infected area and possibly the infected device

(e.g., implants, catheters). Despite significant developments in surgical and medical practices, it is not always possible procedure without the risk of complications for some patients. If physically removing the implantable device is not an option, there are two main strategies for suppressing and preventing bacterial biofilm infections using antibiotics. Firstly, there is an early aggressive antibiotic treatment preventing the biofilm formation and, secondly, a chronic suppressive antibiotic treatment when the biofilm has formed. The difficulty of antibiotic treatment is greatly increased once mature biofilm is established. However, the problem behind the prolonged antibiotic treatment is the increasing development of antibiotic-resistant bacterial strains (e.g. *P. aeruginosa*) and their narrow spectrum of activity. Therefore, when antibiotics are used to treat difficult bacterial biofilms, there is a demand in using combinations of several different antibiotics to cover different targets. In addition, the antibiotics should be taken in high doses for an extended period of time (Bjarnsholt, 2013; Ferraris & Spriano, 2016; Wang & Tang, 2019).

The development of antibiotic resistant bacterial strains is an ongoing global threat to human health and could become a major challenge for practicing clinicians in the coming decades. Clinically and economically, emphasis should be laid on the prevention of implant-related infections (Ferraris & Spriano, 2016; Moriarty et al., 2017).

1.2.2.1 Prophylactic antibiotic treatments

The prophylactic intervention is the most efficient approach to combat the onset of biofilm in its first steps of bacterial colonization and frequently, it is required to prevent implant-related infections. Pre-operative prophylactic measurements include strict aseptic techniques and also systemic or local antibiotics administration to reduce the infection risk (Moriarty et al., 2017; Wang & Tang, 2019).

Human skin, our natural barrier, is compromised by surgery and during implantation. Accordingly, great attention has to be paid to the prevention of contamination in surgery with using hygienic measures. Thus, surgeons have to exercise extreme caution during implantation while using meticulous antisepsis and strict adherence to hygienic rules. Surgical instruments and garments, operating theatres, air environment and the implant itself must be drastically cleaned to prevent any microbial contamination. These precautions are often combined with the prophylactic administration of antibiotics (Bjarnsholt, 2013; C. Von Eiff, 2005).

Prophylactic systemic administration of antibiotics is routine procedure before implantation. The dose should be sufficient for the drug to reach high bactericidal concentrations in tissues, on the surface of the implant, and in intracellular compartments. The drug should therefore have low toxicity and low tendency to induce bacterial resistance. Bacterial resistance and tolerance is most likely to develop when there is prolonged administration of antibiotics at sub-therapeutic levels (Moriarty et al., 2017; Schmidmaier, Lucke, Wildemann, Haas, & Raschke, 2006).

Prophylactic local administration of antibiotics is an alternative approach to avoid some of the complications and side effects caused by the high-dose systemic administration of antibiotics. Less drug is required to achieve higher local drug levels without risking systemic toxicity. Several strategies for local antibiotic prophylaxis have been attempted using collagen sponges and bone cements loaded with antibiotics, as well as polymethylmethacrylate beads. However, certain aspects of local prophylaxis need to be considered, such as the antibiotic delivery must guarantee a rapid release from the carrier. Also, guarantee that the local drug concentration is well above the minimal inhibitory concentration (MIC) for the particular bacteria (Moriarty et al., 2017; Schmidmaier et al., 2006).

As mentioned above, the increasing number of antibiotic-resistant bacterial strains are an ongoing threat. It has led to research on other types of preventative applications, such as coatings. It is a promising approach to coat implants with antimicrobial or antibiofilm materials which inhibits the initial attachment of bacteria on the implant's surface (Khatoon et al., 2018).

1.3 Antibiofilm coating of implants

Loss of efficacy of conventional antibiotic treatments has led to the development of antimicrobial coatings and surfaces. Multifunctional surfaces and coatings are a promising path that is often used for implantable devices to prevent biofilm formation. Antimicrobial coatings have been an increasingly studied area of research for the biomedical and pharmaceutical science fields. Therefore, tremendous progress has been made in surface modification methods and has prompted the development of surfaces that are able to prevent bacterial adhesion and proliferation. Subsequently, preventing biofilm formation and minimizing the risks of difficult implant-related infections (Narayana & Srihari, 2019; Vaz et al., 2018).

1.3.1 Various coating methods

Various coating methods have been designed using both natural and synthetic biomaterials to produce antibiofilm coatings on implants. These general methods are the following: adhesion resistance (bacteria-repelling), contact-killing and antimicrobial agent release (biocide leaching), see figure 3 (Khatoon et al., 2018; Vaz et al., 2018).

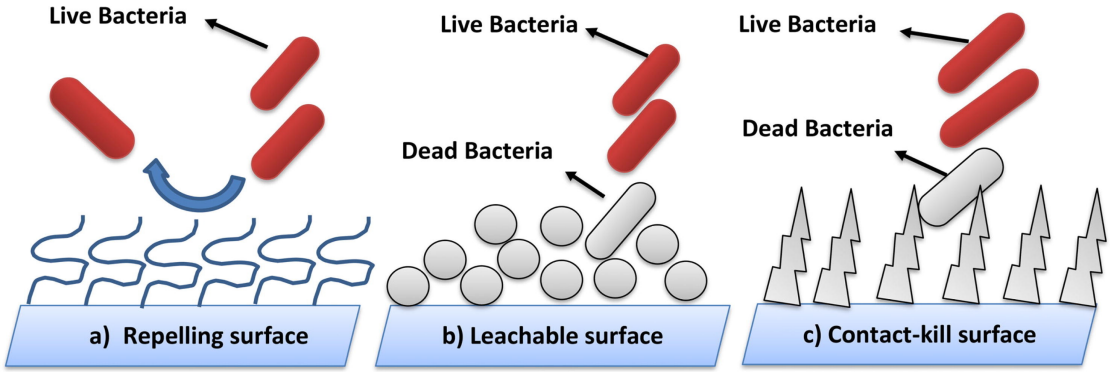


Figure 3. Three main coating methods for designing antibacterial surfaces used for implantable devices (Kaur & Liu, 2016).

1.3.1.1 Leachable surface

The first approach is the oldest method of them all for designing antimicrobial surfaces, in which antimicrobial agents are released. Antimicrobial agents can be incorporated within or on the surface of the biomaterial (or both) to provide antimicrobial characteristics. The various antimicrobial agents used in this method are for example peptides, metal ions and antibiotics. The biomaterial composition should favor the diffusion of the antimicrobial agents in a controlled fashion to induce death for both adherent and adjacent planktonic (non-adherent) bacteria with a long-lasting effect. Biomaterial degradation or hydrolysis of covalent bonds are two approaches to achieve the release of incorporated antimicrobial agents from the implantation site (Lichter, Van Vliet, & Rubner, 2009; Vaz et al., 2018).

1.3.1.2 Repelling surface

The second approach is the anti-adhesive implant surface which reduces the capacity of bacteria to achieve adhesion. As mentioned in chapter 1.2.2, biofilm formation happens in several stages. Bacterial adhesion is the initial crucial stage and interfering with that stage delays or inhibits stable biofilm formation, subsequently reduces the risk of implant-related infection (Vaz et al., 2018).

Implant surface modifications that provide unfavorable conditions for bacterial adhesion can be classified as surface functionalization and surface structuring. The effect of surface functionalization changes the implant´s surface chemistry. While the surface structuring changes the implant´s surface physicomechanical properties using surface topography modifications (Narayana & Srihari, 2019).

The most targeted surface property is the hydrophobicity because the bacterial adhesion depends on it. Generally, metallic implant materials are hydrophilic and negatively charged but polymeric biomaterials depend on their composition. Surface hydrophobicity is characterized by surface wettability, measured by the contact angle. Self-auto assembled monolayers (SAM) are a model of surfaces with controlled chemical properties which are often used for bacterial adhesion studies. SAM can modulate different moieties on the surface and when functionalized with hydrophilic moieties, such as OH and NH_2, they tend to reduce bacterial adhesion. Whereas, hydrophobic surfaces functionalized with methylated groups (e.g., CH_3) do not. Contradictory results do, however, exist. Several candidates with hydrophilic characteristics have been considered to be added to anti-adhesive coatings, such as anionic polysaccharides, hyaluronic acid and heparin (Desrousseaux, Sautou, Descamps, & Traore, 2013; Junter et al., 2016).

Polyethylene glycol (PEG) is used to produce hydrophilic coatings on implant surfaces to reduce bacterial adhesion and the fact that it is only a hydrogen acceptor might be a reason why that coating repels bacteria better than many others. Zwitterionic polymer coatings may also delay or prevent bacterial adhesion effectively, and such surfaces can reduce the adhesion of *P. aeruginosa* by up to 96% (Adlhart et al., 2018; Tiller, 2010).

1.3.1.3 Contact kill surface

The third and last approach is contact killing which aims to eliminate or, at least, disable the proliferation of bacteria adhering to the implant surface. Contact-active surface with antibacterial activity can be created by fixing certain biocides, as discussed in section 1.3.1.1 above, onto the surface with covalent bonding. The previous biocide-leaching method exerts negative impacts on the environment and possibly contribute to the development of bacterial resistance. This disadvantage led to the shift of interest towards a more sustainable, as well as environmentally friendly approach, i.e., contact killing coatings (Kaur & Liu, 2016; Vaz et al., 2018).

Contact killing agents causes them to biochemically inactivate or induce death with cell lysis of the bacteria on contact while being bound to the surface. Biocides are attached irreversibly to the implant surface and therefore do not leach out. The biocides penetrate into the bacteria cell wall which consequently lead to the bacterial death. This approach does not contaminate the environment with their biocides, thus do not contribute to bacterial resistance (Kaur & Liu, 2016; Lichter et al., 2009).

The most effective compounds for contact killing coatings are positively charged chemicals and enzymes (e.g. lysozymes). A significant number of positively charged chemicals have been used in the last decades, that includes antimicrobial cationic peptides, quaternary ammonium compounds (QAC) and chitosan (Vaz et al., 2018).

A simple approach to provide an implantable device surface with contact killing properties involves performing a silanization with QAC-containing silane agents. Previous researchers have described that a concentration of $1.5x10^{-4}$ mol of QAC-containing silane per gram of coating was enough to kill nearly 95% of the viable bacterial colonies after two days of exposure (Ferreira & Zumbuehl, 2009).

1.3.2 Coatings

Antibiofilm coatings are rapidly becoming a primary component of the global mitigation strategy, stimulated by the increasing importance of identifying substitutes for the general administration of antibiotics. The motive of coatings is to prevent bacterial colonization from limiting the spread of complicated biofilm infections. As mentioned in section 1.3.1 above, there are three primary methods in designing those coatings. An ideal coating agent should possess high antibiofilm efficacy, as well as being easily and economically attached to the implant´s surface. Additionally, it needs to be biocompatible and not cause the patient any discomfort (Andersen & Flores-Mireles, 2019; Cloutier, Mantovani, & Rosei, 2015).

1.3.3.1 Implant coatings

The coating of medical devices using antibiofilm agents is effective in inhibiting or killing the planktonic bacteria that does not adhere to the device surface. If the prevention fails and bacteria colonize the implant, biofilm begins to form, and it can cause major complications for the patient being implanted. The complications could involve removing and/or replacing the implant device, as well as strongly treating secondary infection conditions (Cook & Trebella, 2005).

Implants with antibiofilm coatings using the contact-killing method destroys the bacteria upon contact. Metal ions, polycations, antimicrobial peptides (AMP) are an example of antibiofilm agents used for coating implantable devices. Metal ion coatings (e.g. silver, copper, zinc) are used in metallic implants and applies toxic effects on the bacterial membrane, leading to a cell lysis. Polycations are for coating various polymers, ceramic or metallic implants to produce antimicrobial activity by disrupting their cytoplasmic and cell membranes. QAC, chlorhexidine, and chitosan are, for example, polycations used for creating polycationic surface coatings. Glass, titanium and polymers can be coated with AMPs which can inhibit several metabolic mechanisms and kill the bacteria (Narayana & Srihari, 2019).

1.3.3.2 Catheter coatings

Several hydrophobic biomaterials have been used as standard catheters, particularly silicone, they are preferred above other materials because of their superior non-allergenic and flow properties. However, such materials including silicone, are considered as a breeding implant surface for bacteria to colonize and form a biofilm, which leads to infections. Catheter-associated urinary tract infections (CAUTI) are the most common nosocomial infections worldwide and a significant threat to public health. Ongoing efforts to control and prevent CAUTI are present by decreasing bacterial adherence to catheters with antibiofilm coatings (Andersen & Flores-Mireles, 2019; Yassin, Elkhooly, Elsherbiny, Reicha, & Shokeir, 2019).

Clinical trials have validated several antibiofilm agents and many are still under clinical trials and developmental stages. Some of the antibiofilm coatings for catheters under clinical trials include such as silver ions, nitric oxide and AMP. Silver ions are the most popular clinically tested and available coating for catheters but show inconsistent results. Recently, polysaccharides have been showing promising results as a coating for medical applications, such as catheters, and fighting against implant-related infections like CAUTI (Andersen & Flores-Mireles, 2019; Bračič, Strnad, & Fras Zemljič, 2018).

1.3.3.3 Polysaccharide-based coatings

Polysaccharides are increasingly found in various applications in the biotechnology and pharmaceutical fields because of their nontoxicity and antibiofilm properties. Surface treatment by natural or modified polysaccharides is being developed as a repelling surface or contact killing surface for medical devices exposed to biofilm

formation. Some of them have been patented, such as hyaluronic acid, which is a very suitable antibiofilm coating because of its high hydrophilicity. As a result of high-water adsorption capacity, the hyaluronic acid can repel the bacteria, which generally prefers hydrophobic surfaces. Another very common polysaccharide-based coating is heparin, which prevents both thrombosis and colonization of bacteria. Most studies showed anti-adhesive results of heparin coatings though Lange et al. noted no significant difference between the heparin-coated and the non-coated samples. However, polysaccharide-based coatings containing amino groups are extremely promising chemicals with contact killing properties. Chitosan and its derivatives are the most popular amino polysaccharides used as antibiofilm coatings and a number of them have been proposed over the past decade (Bračič et al., 2018; Junter et al., 2016).

1.4 Chitosan

Chitosan is a semi-natural polycationic linear polysaccharide derived from partial deacetylation of chitin, shown in figure 4, and it is the structural element in the exoskeleton of crustaceans (e.g., shrimps, crabs). Chitin is one of the most abundant natural polysaccharides, but the complexity of the chitin structure, insolubility in aqueous solution, and difficulty in its extraction limited the research on this polymer until late 20th century. Recently, chitosan and its derivatives have become the focus of research investigation due to many potential applications in pharmaceutics. They have been considered as a novel carrier material in drug delivery systems, wound healing, hemostatic and antimicrobial agent, among other things (Cheung, Ng, Wong, & Chan, 2015; Ramya, Venkatesan, Kim, & Sudha, 2012).

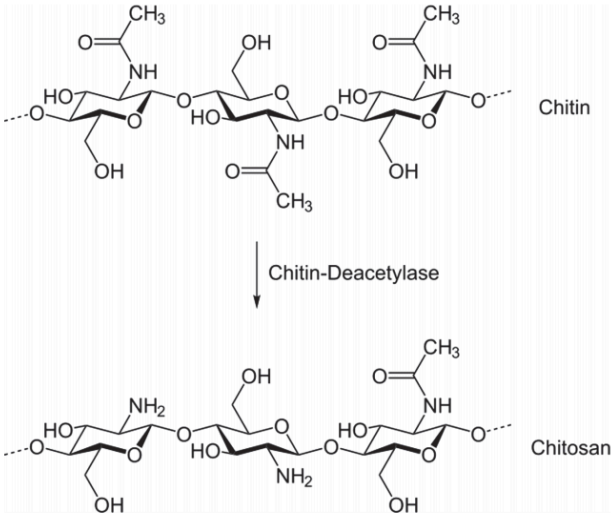


Figure 4. Chitosan is a polysaccharide derived from chitin, the structural element in crustaceans (Ramya et al., 2012).

1.4.1 General characteristics

Chitosan is composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl- D-glucosamine (acetylated unit) monomers within the polymer. For each monomer, the chitosan structure has one primary amine and two free hydroxyl groups, it´s expressed by the formula $C_6H_{11}O_4N$ (Cheung et al., 2015; Goy, Morais, & Assis, 2016).

The benefits and usage of chitosan are limited because of its insolubility in water, high viscosity, and aggregation at higher pH levels. Chitosan becomes soluble in acidic solutions, such as acetic, hydrochloric, or phosphoric acid, with pH below its pKa, lower than 6.5 (Periayah, Halim, & Saad, 2016). The degree of deacetylation, molecular weight (MW), and the acetyl group distribution in the polymers are the main parameters that define solubility and physicochemical characteristics of chitosan. Uneven acetyl group distribution lowers its solubility causing them to aggregate more easily and hinders its applicability. However, molecular modifications on chitosan increase its stability, solubility and their bacteriostatic properties, thus making it more versatile as a biopolymer (Cheung et al., 2015).

A unique feature of chitosan is its natural cationic nature, as the majority of polysaccharides are usually either negatively charged or neutral in an acidic environment. This feature allows chitosan to form multilayer structures or electrostatic complexes with other synthetic or natural negatively charged polymers, transforming it to derivatives or composites (Cheung et al., 2015).

Chitosan and its derivatives have exciting characteristics for use in a wide range of biomedical, pharmaceutical, agricultural and industrial applications. These characteristics include biocompatibility, biodegradability, non-toxicity, and low allergenicity. These advantages in chitosan and its derivatives make them the most significant polysaccharides for the biomedical field, possessing diverse biological activities including antioxidant, antifungal, anticoagulant, anti-inflammatory, anti-tumor, and antimicrobial effects (Cheung et al., 2015; Ngo et al., 2015).

Chitosan derivatization often involves chemically modifying their amino group or hydroxyl group to produce derivatives that contain more cationic or hydrophobic and hydrophilic moieties. The derivatives are particularly aimed at enhancing chitosan´s solubility and antimicrobial properties and their importance as antimicrobial agent are increasing (Sahariah & Masson, 2017).

1.4.2 Antimicrobial properties

Chitosan is well known for its inhibitory effects against bacteria, although the exact mechanism is not yet fully understood. However, several mechanisms may contribute to this. It is believed that the reason for the antimicrobial mechanism of chitosan is considered being a result of its cationic nature, and the target of this antimicrobial action is the negatively charged components of bacteria. When the pH of chitosan is lowered below 6.5 (chitosan pKa), their amino groups will protonate and convert to the positively charged $R\text{-}N(CH_3)_3^+$, making chitosan water soluble. The presence of cationic chitosan is believed to be responsible for binding efficiently to the anionic cell wall of the bacteria. When chitosan binds efficiently to the bacteria´s cell wall, it disrupts their normal functions of the membrane, such as promoting cellular lysis and inhibiting the transport of nutrients into the cells. Therefore, it is expected that polymers with higher charge density are a critical factor for an improved antimicrobial activity (Goy et al., 2016; Ramya et al., 2012; Vinsová & Vavríková, 2011).

The antimicrobial activity of chitosan can only be determined when it has good solubility and that requires the presence of additional number of free amino groups in the polymer. However, chitosan with more N-acetyl groups increases the hydrophobicity. When chitosan has a lesser degree of acetylation (DA), i.e. higher number of free amino groups, the theory is that it enhances the antimicrobial activity towards various strains of Gram-positive and Gram-negative bacteria. According to Sahariah and Masson (2017), the DA appears to have limited effect on antimicrobial

activity when the DA value is less than 50%. Also, the distribution of substituents (DS), i.e. the N-acetyl groups in chitosan, can affect the antimicrobial activity.

The most important factors affecting the antimicrobial activity of chitosan are their structure, chemical properties and DS for the substituents attached to the polymer. Other factors, such as the DA, as mentioned above, as well as the MW of chitosan, can also play a significant factor in antimicrobial activity. However, according to Sahariah et al. (2019) the effect of MW on chitosan´s antimicrobial activity is not considered being as related, as previously thought. Below a certain MW, there is a very marked increase in activity; however, above this value, the MW has a limited or no effect on their antimicrobial activity.

In general, the chitosan´s cationic groups have a positive effect on antimicrobial activity, but it is dependent on factors like DS and the positioning of the cationic charge (Sahariah & Masson, 2017).

1.4.3 Antimicrobial use of chitosan

The versatility and many favorable biological properties of chitosan and its derivatives, especially their antimicrobial activity, has widen the number of their applications. Additionally, the possibility to process chitosan in multiple forms, such as powders, films, gel, solutions, sponges and nanoparticles, has opened the way towards applications in various fields. The main applications of chitosan and its derivatives are summarized in table 2 (Vaz et al., 2018).

Table 2. Different applications of chitosan and its derivatives used in various fields

Field	Applications
Cosmetics	Oral, hair and skin care products, deodorants, lipsticks.
Food industries	Thickener, preservative, moisture loss prevention, coating food to enhance their shelf live.
Agriculture	Biofungicide, antimicrobial prevention from infections.
Textile industries	Antimicrobial coatings, dye absorption, moisture control.
Pharmaceutical	Antibacterial, antioxidant, anticoagulant, antitumor agent. Drug coating and stabilizer, controlled drug release.
Biomedical	Antimicrobial coatings, thromboresistant coatings, wound dressings, gene delivery, cell delivery system, scaffolds for tissue engineering.

Over the past several decades, chitosan has received an increased attention for their antimicrobial properties and the Food and Drug Administration (FDA) approves it as a food ingredient. Another area of growing interest is preparing antimicrobial coatings, where chitosan plays a vital role. One option is the possibility of obtaining thin films and coatings to cover fresh or processed foods to delay the ripening and having an effect on respiration and moisture loss while extending their shelf-life (Bračič et al., 2018; Elsabee & Abdou, 2013).

Surfaces coated with the naturally occurring polysaccharide chitosan resisted biofilm formation by bacteria and is often composed of essential oils, acid compounds, or nanoparticles to increase the antimicrobial activity. Previous researchers suggest that chitosan offers a flexible, biocompatible platform for designing coatings to protect surfaces from infection. The most promising assemblies combine anti-adhesive and bactericidal efficiencies to prevent biofilm formation on implanted devices (Carlson, Taffs, Davison, & Stewart, 2008; Junter et al., 2016; Xing et al., 2016).

1.4.4 Antimicrobial chitosan coatings for implants

The development of antimicrobial coatings in the last decades for biomedical devices has been linked to the increasing awareness and realization of nosocomial infections, particularly implant-related infections. Together with the expanding growth of bacterial resistant strains, it limits the usefulness of the traditional approach in using antibiotics-based treatments. Antimicrobial chitosan coatings can meet those needs with their inherent properties and do not develop resistance like mostly happens with antibiotics (Vaz et al., 2018).

Several techniques can be used to coat chitosan on implant surfaces. The two main techniques commonly used are either noncovalent bonding with the surface (electrostatic, Van der Walls forces or hydrogen bonding) or covalent bonding with the surface. Choosing the method depends on the specific applications. For stable long-term coatings on implantable devices, such as orthopedic prosthesis, dental implants or vascular catheters, the covalent attachment would be preferred. However, if the antimicrobial activity is only needed for short-term coatings (e.g. wound dressing applications) or biodegradation is expected (e.g. drug delivery systems), then the noncovalent attachment is slightly preferred (Vaz et al., 2018).

The unique properties of chitosan, like biocompatibility, osteoconductive properties, and biodegradability, have attracted various researchers around the globe to explore it for coating multiple implants. Preliminary studies have indicated that

chitosan can be a potential for using as a chemically bonded coating for metals used in orthopedic, dental and craniofacial implants (Bumgardner et al., 2003). Chitosan coated dental metallic plates have shown improved clinical longevity in medically compromised patients by affecting their bone-health. Studies have also shown promising results in coating glass substrates with chitosan, however, for other purposes in developing an anticoagulative substrate (Bristow & DeMarco, 2014; Mao et al., 2004). In order for the chitosan coating to covalently bond to the glass and metallic surfaces, their surfaces had to be modified. Surface modifications can offer many different strategies to functionalize, for example, introduce functional groups, change the hydrophilicity, roughness or morphology. Different functionalization strategies have been proceeded with various chemicals, for example silanol, dopamine or phosphates (Bristow & DeMarco, 2014; Vaz et al., 2018).

Metallic and glass substrates have both shown promising results in using the silanization method as a surface preparation (surface modification) step. The silanol functional groups react to the prepared hydroxylated metallic or glass surfaces leading to a stable covalent bonding. Then the terminal group remains available for attaching the chitosan (Vaz et al., 2018). However, glass and metallic substrates have to be prepared with using a harsh acidic solution called piranha to increase the density of hydroxyl groups. Piranha is an oxidation step for substrates that need more density of hydroxyl groups in order to carry out the silanization technique. Acidic-resistant materials like, glass, metallic and silicon substrates (wafers) have used piranha for that purpose (Acres et al., 2012; Vaz et al., 2018).

Surface modification techniques for polymeric substrates are, for example, oxygen plasma treatments that improve surface wettability to enhance the coating. This method has been tried on silicone substrates to coat chitosan with decent results (Chuah, Kuddannaya, Lee, Zhang, & Kang, 2015; Vaz et al., 2018). However, limited studies are about other methods to modify the surface of silicone. No reviews were found about performing the surface modification methods for metals on silicone substrates via silanization. It would be interesting to know if this silanization method would functionalize on silicone surface to covalently bond chitosan, as will be researched in this study.

2. AIM

The primary aim of this thesis is to design chitosan coatings for various surfaces used in preventing implant-related infections. Here, two different substrates were investigated, namely glass and silicone substrates. Silicone is a well-known material used in indwelling catheters, which are considered to be one of the most leading causes of nosocomial biofilm infections. The most efficient way of combating biofilm implant-related infection is to prevent them in the first place by using antibiofilm coatings. Chitosan is believed to be an ideal biocompatible coating material for implants. However, this has not previously been attempted with chemically activated silicone surfaces. No published studies were found about chemically modifying and functionalizing silicone substrates to attach the chitosan. However, numerous studies have been carried out on modifying the surface of glass plates using a silanization method to attach chitosan to the surface with covalent bonds. Glass substrates were therefore also investigated for comparison with the silicone substrate. Additionally, a published article by Acres et al. (2012) has described a silanization method for a pure silicon wafer, which was tried for silicone elastomer, which is though an entirely different polymeric material.
Thus, the specific aims were:

1. To develop procedure for chemical activation of silicone substrates.

2. To use a silanization method to link chitosan to activated silicone and glass substrates.

3. To quantify chitosan that attached to glass and silicone substrates.

4. To compare two different silane agents used for modifying both the glass and silicone substrates when attaching the chitosan.

5. To analyze the chemical structure of the surface coatings on the glass and silicone substrates by FT-IR.

6. To assess activity of chitosan-coated glass and silicone substrates against the bacterium *P. aeruginosa* by contact-killing assay.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Chitosan	Primex ChitoClear TM4030
Lithium acetate dihydrate	Sigma Aldrich
Deionized water (H_2O)	University of Iceland
Glacial acetic acid (puriss. 100%)	Sigma Aldrich
Ninhydrin, A.C.S. reagent	Sigma Aldrich
Hydrindantin dihydrate	Sigma Aldrich
Dimethyl sulfoxide (DMSO) (anhydrous, ≥99.9%)	Sigma Aldrich
Nitrogen (N_2), compressed	ÍSAGA ehf
D-(+)-Glucosamine hydrochloride (≥99%, crystalline)	Sigma
Ethanol (EtOH) 99,9%, contaminated with ethyl acetate	Mjöll Frigg
Toluene (puriss. ≥99.5%)	Sigma Aldrich
(3-Glycidyloxypropyl) trimethoxysilane (GPTMS) (purum. ≥98%)	Aldrich
(3-Aminopropyl) trimethoxysilane (APTMS) (purum. 97%)	Aldrich
Sodium borohydride ($NaBH_4$) (purum. 98%)	Aldrich
Dichloromethane (DCM) (≥99.8%, HPLC)	Honeywell
Acetone (CH_3COCH_3)	Venol
Glutaraldehyde (≈50% in H_2O, technical)	Fluka
Methanol (MeOH) (≥99.9%, HPLC)	Honeywell
Sulfuric acid (puriss. ≥95%)	Sigma Aldrich
Hydrogen peroxide solution (30 wt % in H_2O, A.C.S. reagent)	Sigma Aldrich
Pseudomonas aeruginosa ATCC27853	American Type Culture Collection
Gentamicin 10 µg susceptible discs	OXOID

3.1.2 Instrument/Equipment/Others

Weight scale PB303-S DeltaRange	Mettler Toledo
Magnetic stirrer with heating (MR Hei-Standard)	Heidolph
Magnetic stirrer MS-500D	Witeg
Cover glasses (7/8 inch square, No.1)	N/A
Oven	Whirlpool

Vortex Genie 2	Fischer
GENESYS 150 UV-Visible Spectrophotometer	Thermo Scientific
Orion 3-Star pH meter	Thermo Scientific
NuSil Silicone Elastomer Sheeting MED82-5010-40	Avantor
Branson Ultrasonic 5800 bath	Branson
FTIR-spectrometer Nicolet iZ10	Thermo Scientific
Thermometer	Thermo Scientific
Mediline refrigerator	Liebherr
Pipette (100-1000 µL)	Gilson
Pipette Tips (100-1000 µL)	Exacta Cruz
Mueller Hinton agar plates	N/A
Densichek McFarland reader	BioMérieux
Incubator	Nüve
Parafilm	Bemis
Magnet	N/A

**N/A = manufacturer is not available.*

3.2 Methods

3.2.1 Glass plate surface modification and functionalization

3.2.1.1 Glass plate preparation

Cover glass plates (7/8 inch. square, no.1) were used in the experiment, a square-shaped plate (2.3 cm x 2.3 cm) with the measured surface area of 10.58 cm^2.

3.2.1.2 Silanization procedure

Silanization reagent solutions were prepared as follows: 0.25 ml of (3-aminopropyl)-trimethoxysilane (APTMS) was diluted in anhydrous toluene (25 ml) to obtain 1% APTMS solution. 1% (3-glycidyloxypropyl)-trimethoxysilane (GPTMS) solution was prepared the same way.

Before the coating, the glass plates were rinsed in deionized water and dried in an oven at 80°C overnight. Before the silanization step, the glass plates were cooled at room temperature for about 10 min. They were then transferred to round bottom flasks, containing approximately 10 ml of either the 1% APTMS solution or the 1%

GPTMS solution. The flasks were sealed with aluminum foil and then heated for at least 5 hours in an oil bath (100°C).

After the reaction, the solvent and reagent residues were washed from the glass plates with different solvents of increasing polarity. Thus, the order for the washing was as followed: anhydrous toluene, dichloromethane (DCM) and then finally by acetone which was then removed by drying the glass plate in air. During the washing, each glass plate was washed front and back with each solvent immediately in the correct order using a glass pipette.

3.2.1.3 Glutaraldehyde crosslinking procedure

The glass plates silanized with APTMS were immersed in a 25% glutaraldehyde solution and occasionally shaken for one hour. The glass plates were removed and instantly washed with methanol, then air-dried.

3.2.2 Glass plate coatings

3.2.2.1 Standard solution preparation

The following standard solutions were prepared for coating the glass plate´s surface.

3.2.2.1.1 4% (w/v) chitosan solution

2 ml of acetic acid (CH_3COOH) was diluted with 48 ml of deionized water, giving a 4% (v/v) acetic acid solution. 4 g of chitosan (Batch TM4030) weighed and added to the 4% (v/v) acetic acid solution (≈ 8% (w/v) chitosan solution). The solution became extremely viscous, so it was diluted with 50 ml of deionized water before it was applied on a magnetic stirrer for approximately an hour until dissolution. Then, $NaBH_4$ (0.1 g) was added to the solution until dissolved (final concentration ≈ 4%).

3.2.2.1.2 1% (w/v) glucosamine solution

Glucosamine hydrochloride (2.5 g) was weighed in a volumetric flask and adjusted to 250 ml with a 0.4% (v/v) 1:250 CH_3COOH/H_2O solution.

3.2.2.2 Chitosan attachment on a glass surface

The glass plates made in chapter 3.2.3 were immersed in either chitosan (glass plates B and D) or glucosamine solution (glass plates A and C). At room temperature, the

glass plates were allowed to soak for one hour. Then removed from the solution, washed with deionized water, and finally air-dried. These glass plates are analyzed using the ninhydrin assay.

3.2.3 Silicone sheet surface modification and functionalization

3.2.3.1 Silicone sheet preparation

Silicone elastomer sheets (MED82-5010-40) from NuSil Technology were used in the experiment. The silicone sheets were cut into squares (2.3 cm x 2.3 cm), the same size as the glass plates (10.58 cm^2).

3.2.3.2 Silicone sheet oxidation procedure

The procedure used was similar to the procedure reported by Acres et al. (2012) for oxidation of silicon surfaces. Any surface contaminants were removed from the silicone substrates with immersing in acetone and ultrasonicated for 30 min. Then rinsed with deionized water and let dry in a heating oven (80°C). While the silicone sheets were drying, the piranha solution was prepared using sulfuric acid and hydrogen peroxide. Sulfuric acid (15 ml) was stirred on a magnetic stirrer first, followed by a hydrogen peroxide (6 ml), which was poured slowly into the solution. Mixing the solution is exothermic, and the resultant heat can bring the solution temperature up to 120°C. (Caution! Piranha solution is extremely corrosive and protective measures should always be taken). The piranha solution was therefore allowed to cool reasonably for about 15 min before the silicone sheets were immersed into the piranha solution (conc. H_2SO_4/H_2O_2 (2.5:1 v/v)) at room temperature. After about 30 min, the piranha solution and the silicone sheets were poured to a ca. 400 ml of deionized water for dilution. The silicone samples were removed from the solution, washed with deionized water, and finally dried in an oven (80°C).

3.2.3.3 Silanization procedure

The same silane coupling agents were used, APTMS and GPTMS, for the silanization procedure. However, the standard solutions for silanizing the silicone sheets were in a higher concentration than the glass plates. APTMS (1.0 ml) was diluted in anhydrous toluene (50 ml) to obtain 2% APTMS solution. The 2% GPTMS solution was prepared the same way.

The dried silicone sheets were taken out of the oven and cooled down, then immersed in the 2% standard solutions (APTMS or GPTMS) for 20 min at room temperature. Subsequently, the substrates were thoroughly rinsed with anhydrous toluene to remove any excess reagent residues. Again, the silicone sheets were heated in an oven (80°C) to dry them.

3.2.3.4 Glutaraldehyde crosslinking procedure

The silicone sheets silanized with APTMS were immersed in a 25% glutaraldehyde solution and occasionally stirred for one hour. The silicone sheets were removed and instantly washed with methanol, then air-dried. This particular step activates the cross-linkage on the silicone sheet´s surface for binding the chitosan.

3.2.4 Silicone sheet coatings

The standard solution preparation for the silicone sheets was the same as for the glass plates, seen in section 3.2.2.1.

3.2.4.1 Chitosan attachment on a silicone sheet

After drying, the silicone sheets were either immersed in chitosan (silicone sheets B and D) or glucosamine solution (silicone sheet A and C). At room temperature, the silicone sheets were allowed to soak for one hour. Then removed from the solution, washed with deionized water, and finally dried in a heating oven (80°C). These silicone sheets are analyzed using the ninhydrin assay.

3.2.5 Ninhydrin reagent preparation

The ninhydrin reagent was prepared in two steps according to Leane et al. (2004).

3.2.5.1 Lithium acetate buffer (4 M)

Lithium acetate dihydrate (10.2 g) was weighed and dissolved in deionized water (approx.15 ml). The following solution was adjusted to pH 5.2 using glacial acetic acid and the volume adjusted to the final volume (25 ml) with deionized water.

3.2.5.2 Ninhydrin reagent

Ninhydrin (1 g) and hydrindantin (0.15 g) were weighed and dissolved in DMSO (37.5 ml). The 4 M lithium acetate buffer (12.5 ml) was then added to the solution while

bubbling the solution with a stream of nitrogen (this is done in order to remove oxygen from the solution). The dark red reagent was then stored in a dark stoppered bottle and refrigerated.

3.2.6 Ninhydrin assay

Two different stock solutions were prepared to make the calibration curves for the ninhydrin assay, an analyte (chitosan) in comparison with a reference (glucosamine).

3.2.6.1 Stock solutions

0.1% (w/v) of stock solutions were prepared for both chitosan and glucosamine.

3.2.6.1.1 0.1% (w/v) chitosan solution

Chitosan (0.25 g) was weighed in a volumetric flask and adjusted to 250 ml with a 0.4% (v/v) 1:250 CH_3COOH/H_2O solution.

3.2.6.1.2 0.1% (w/v) glucosamine solution

Glucosamine hydrochloride (0.25 g) was weighed in a volumetric flask and adjusted to 250 ml with a 0.4% (v/v) 1:250 CH_3COOH/H_2O solution.

3.2.6.2 Calibration curve

The standard solutions were prepared by dilution of the stock solutions (0.1%) as described in the following table 3.

Table 3. Prepared standard solutions in different concentrations.

Standard solutions:	**0.1% chitosan = 1 mg/ml**	**0.1% glucosamine = 1 mg/ml**
Dilution 1	0.1 mg/ml	0.1 mg/ml
Dilution 2	0.05 mg/ml	0.05 mg/ml
Dilution 3	0.02 mg/ml	0.02 mg/ml
Dilution 4	0.005 mg/ml	0.005 mg/ml

The standards were assayed by mixing 1 ml standard solution with 1 ml of ninhydrin reagent in a test tube (final volume = 2 ml) followed by short shaking by hand. The test tubes were protected from UV light and covered with aluminum foil when

needed. A blank was also prepared by mixing 1 ml deionized water with 1 ml of ninhydrin reagent.

The test tubes were heated in a water bath (100°C) for 30 min to allow the reaction to proceed. Following this they were shortly cooled down in a cold water bath and diluted with 5 ml of 50% (v/v) EtOH/H_2O mixture to a final volume of 7 ml. The tubes were then vortexed for 15 sec to oxidize the excess of hydrindantin. The absorbance of each solution was measured at 570 nm on a UV/Vis spectrophotometer (GENESYS 150).

3.2.6.3 Ninhydrin assay of coated glass plates and silicone sheets

The glass plates made in section 3.2.2.2 and the silicone sheets made in section 3.2.4.1 were transferred into 50 ml Erlenmeyer flasks in which they would fit. Sufficient volume was added in the flasks to immerse both sides of the plate/sheet, which is 2 ml of deionized water and 2 ml of ninhydrin reagent to each Erlenmeyer flask (final volume = 4 ml). The flasks were capped, shortly shaken by hand and heated in a water bath (100°C) for 30 minutes to allow the reaction to proceed with the chitosan coatings on the glass plates/silicone sheets. After that, the flasks were shortly cooled down in a cold water bath and each solution poured over into glass tubes while removing the plate/sheet. Then diluted with 5 ml of 50% (v/v) EtOH/H_2O mixture (final volume = 9 ml). The solutions in the glass tubes were then vortexed for 15 sec to oxidize the excess of hydrindantin. The absorbance of each solution was measured at 570 nm on a UV-Vis spectrophotometer (GENESYS 150) and the surface coating identified using FT-IR spectrophotometer (Nicolet iZ10).

3.2.6.4 Analysis of results from the ninhydrin assay

The measured absorbance for the ninhydrin solutions were used to calculate the concentration by using the standard calibration curve where linear equation for chitosan and glucosamine were created. X-axis represents the concentration (mg/ml) and the y-axis the absorbance (nm). The following calculations shown below are listed in table 4 in the results for glass plate D. The calibration curve equation for chitosan (shown in figure 12 in the results) is used for calculating the chitosan concentration. The absorbance of glass plate D was at 0.587 nm after being diluted by 10 to fit within the calibration curve:

$$y = 6.4292x + 0.0055.$$

$$x = \frac{0.587 - 0.0055}{6.4292} = 0.091 * 10(dilution) = 0.91\ mg/ml$$

The ninhydrin reagent volume used for each ninhydrin assay was 2 ml. Thus, the concentration had to be multiplied with the volume of ninhydrin reagent to calculate the mass (mg) of chitosan:

$$0.91 \frac{mg}{ml} * 2\ ml = 1.81\ mg$$

Finally, the concentration on the 10.58 cm^2 glass plate D coated with chitosan was calculated, area concentration (AC), by dividing the mass of chitosan with the area of the glass plate:

$$AC = \frac{1.81\ mg}{10.58\ cm2} = 0.171\ mg/cm2$$

The rest of the calculated results from this particular ninhydrin assay is listed in table 4 in the results, showing the results of the other glass plates. All absorbance measurements from the ninhydrin assays performed in this study on glass and silicone substrates were calculated the same way as was described above.

3.2.6.5 FT-IR analysis

The surface coatings were identified with the Nicolet iZ10 FT-IR spectrophotometer (Thermo Scientific, Madison, USA) in reflection mode. The collected spectra were analyzed with *OMNIC* software. First, the background was collected before loading small amount of chitosan (Primex ChitoClear TM4030) or glucosamine hydrochloride (Sigma) on the sampling accessory to be collected. Thereafter, the coated or uncoated glass and silicone substrates were placed the same way on the sampling accessory for the samples to be collected. The obtained peaks were identified for the coated substrates to compare with the authentic material of chitosan and glucosamine for confirmation.

3.2.7 Antibacterial activity

Four Mueller Hinton agar plates (MHA) were prepared for the agar test, two MHA for each class of samples, glass plates and silicone sheets. The agar test was performed with the bacteria *P. aeruginosa* ATCC27853 and the bacterial inoculum prepared at 0.5 McFarland, measured in a McFarland reader and the correct concentration

adjusted using saline. The MHA were all covered with the bacterial inoculum using cotton swabs before the samples were placed on the MHA. Maximum three samples were placed on each MHA plate including a 6 mm Gentamicin antimicrobial susceptibility disc (OXOID) as a performance control. The four MHA Petri dishes were placed in an incubator at 36°C overnight. The day after, the Petri dishes were inspected to analyze if the samples had contact killing effects.

4. RESULTS

4.1 Surface modification and functionalization

The surfaces of glass plates and silicone sheets need to be modified in order to successfully bind the primary amino groups of chitosan or glucosamine to their substrates. Glass substrates have shown promising results in coating with chitosan, using silanization. However, limited information can be found on coating silicone substrates with chitosan. Here, glass and silicone will be tested the same way using the silanization method, the preparation is described in section 3.2.1 for glass plates and section 3.2.3 for silicone sheets.

4.1.1 Glass plates

The procedure was carried out following the thesis of Milewska and Ostrowska (2016) about engineered chitosan coatings for implants. It contained a method, silanization, carried out on glass samples before coating with chitosan, showing promising results. In this study, that procedure was repeated with a few modifications. Glucosamine hydrochloride was used in this study instead of 1,3-diaminopropane as a standard solution, whereas it is more equivalent to chitosan. Glucosamine contains one amino group in its structure, while 1,3-diaminopropane includes two. Chitosan monomers contain one amino group as well, and the polymer is composed of glucosamine units.

Firstly, the glass plates (7/8 inch. square, no.1) were modified in either of the two chemical ways with the silane coupling agents (GPTMS and APTMS) to activate the surface using the silanization method. Subsequently, the chitosan and glucosamine (positive control) coatings are attached to the activated surface. However, the glass plates silanized with APTMS have an additional step with a glutaraldehyde crosslinker. The overall procedure for the coated glass plates is briefly described in figure 5.

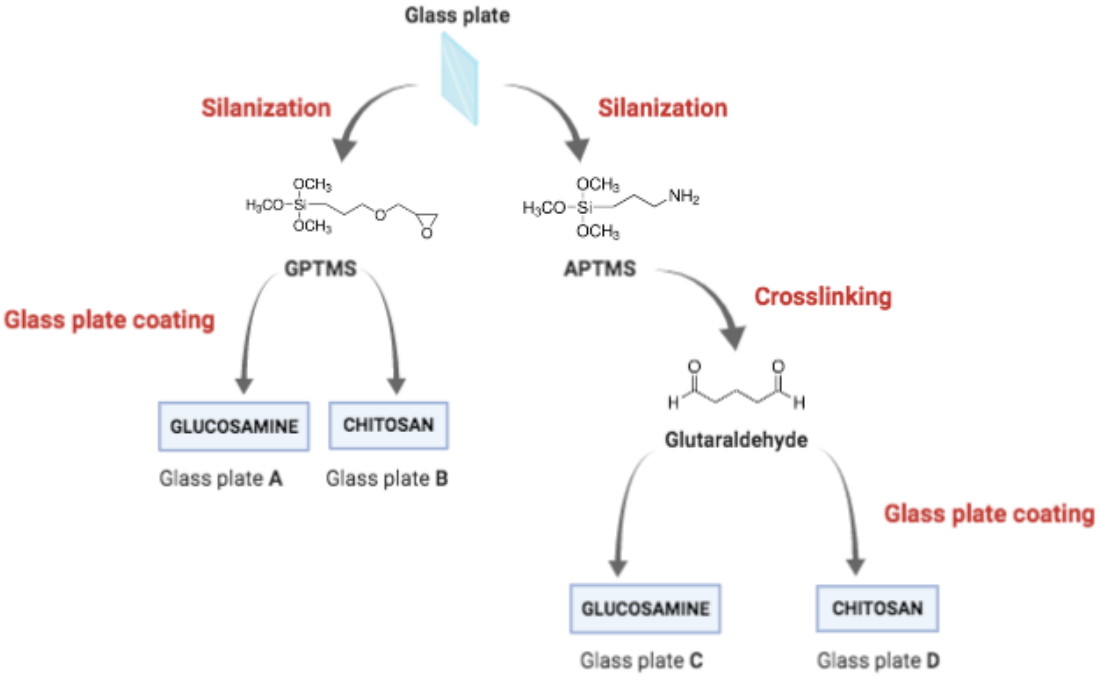


Figure 5. A scheme of the overall procedure of coating the glass plates with chitosan or glucosamine.

4.1.2 Silicone sheets

The procedure used in this study to coat the silicone sheets were not based on any particular research. However, an activation method used for this study was found in an article by Acres et al. (2012). The article focuses on activating silicon substrates, which are a completely different material than the silicone polymer used for this study, so the methods (piranha and silanization) tested on silicone sheets, were uncertain.

The silicone sheets (MED82-5010-40) used in the procedure are identical to various medical catheters used *in vivo* in patients. They contain the same properties, e.g., low surface tension, excellent biocompatibility, thermal stability, as well as high hydrophobicity (Takeichi, 2011). The silicone sheets were first activated with oxidation (piranha solution) before linking either of the two silane coupling agents (GPTMS and APTMS) to the surface, in the same way as the procedure for the glass plates. The overall procedure for the silicone sheets is briefly described in figure 6.

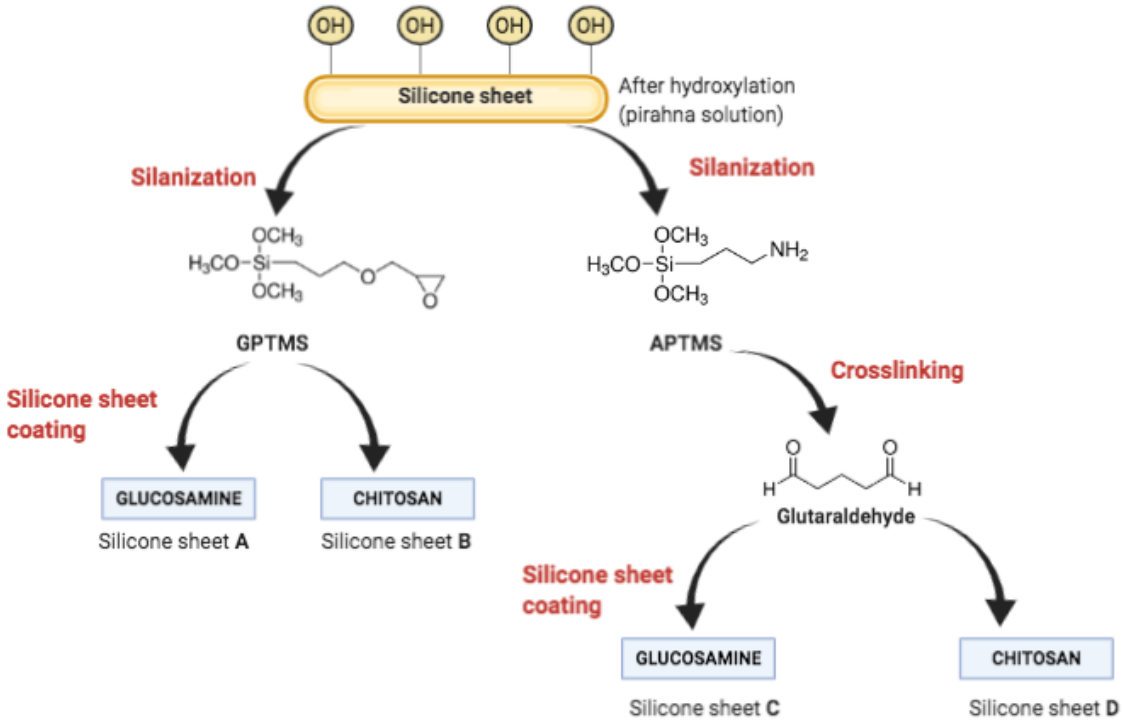


Figure 6. A scheme of the overall procedure of coating silicone sheets with chitosan or glucosamine.

Unlike the glass plates, there is a need for activating the silicone sheet surface before the silanization step. The hydrophobic silicone sheets are immersed in a piranha solution, a strong oxidizer, that removes most organic residues and hydroxylates the surface (adds OH groups). According to Koh et al. (2012), the piranha solution can change the surface properties of the surface and substitutes the methyl groups with silanol groups (Si-OH), shown in figure 7. The increase of silanol groups on the surface increases the polarity, resulting in a surface layer which is highly hydrophilic and subsequently leads to the formation of stronger intermolecular bonds.

Figure 7. Piranha solution oxidizes the surface of the silicone sheet, making it more hydrophilic.

4.1.3 Silanization and crosslinking

Silane coupling agents contain functional groups that act as a sort of intermediary which bonds organic material (chitosan) to inorganic materials (glass, titanium or silicon). The silane coupling agents used to modify the surface, APTMS and GPTMS, are shown in figure 8. Their structures are very similar besides one functional group that separates them, and the discussed groups are circled in red below.

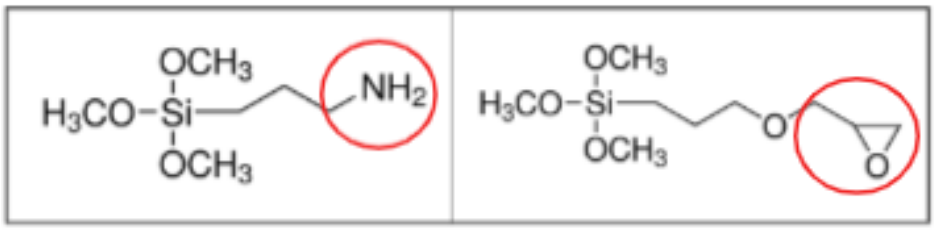
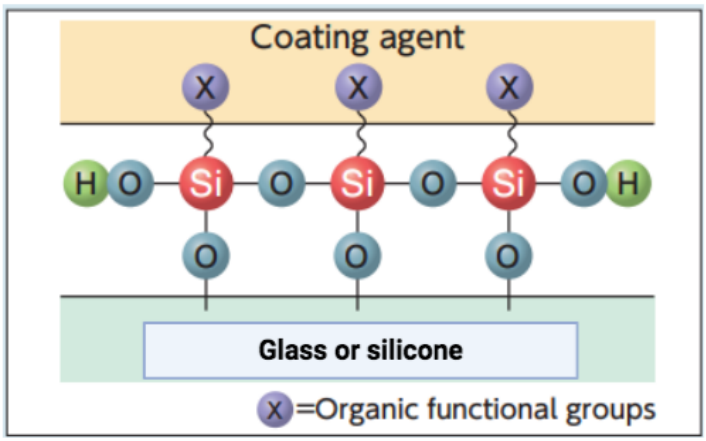


Figure 8. The silane coupling agents APTMS (right) and GPTMS (left) modify the glass plate´s surface.

The silanization method should leave behind an organic functional group (X) on the glass or silicone substrates, like shown in figure 9. The APTMS solution covers the surface with an amino group and the GPTMS with an epoxy group (Shin-Etsu, 2017).



X =	Organic functional groups
APTMS	Amino group
GPTMS	Epoxy group

Figure 9. The glass or silicone surface (inorganic material) after APTMS or GPTMS silanization, giving different functional groups as mentioned in the table (right), either amino group or epoxy group (Shin-Etsu, 2017).

Glutaraldehyde is a dialdehyde chemical and has a role as a crosslinking reagent through amino groups. It is one of the most widely used crosslinkers since it´s relatively inexpensive and reacts rapidly with amino groups (Beppu, Vieira, Aimoli, & Santana, 2007; Imani, Rafienia, & Emami, 2013).

The glutaraldehyde step activates the crosslinkage on the glass or silicone surfaces C and D, thus binding the chitosan to the substrate. Note that the

glutaraldehyde crosslinker was only used to link the APTMS and chitosan´s amino groups together. The free epoxy group from GPTMS bonds to the chitosan´s amino group without using any crosslinker. Detailed description of each step in the coating for the glass plates are shown in figure 10 and silicone sheets in figure 11.

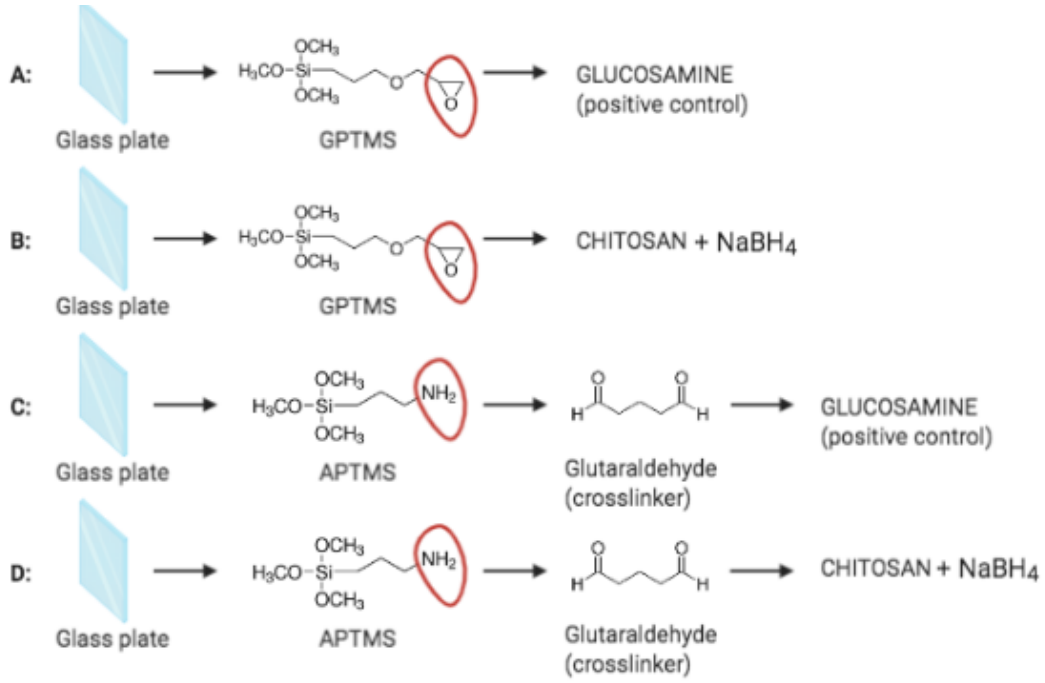


Figure 10. Scheme of each step in the preparation of the coated glass plates A-D (without control samples) in an attempt to attach chitosan to the glass plate´s surface.

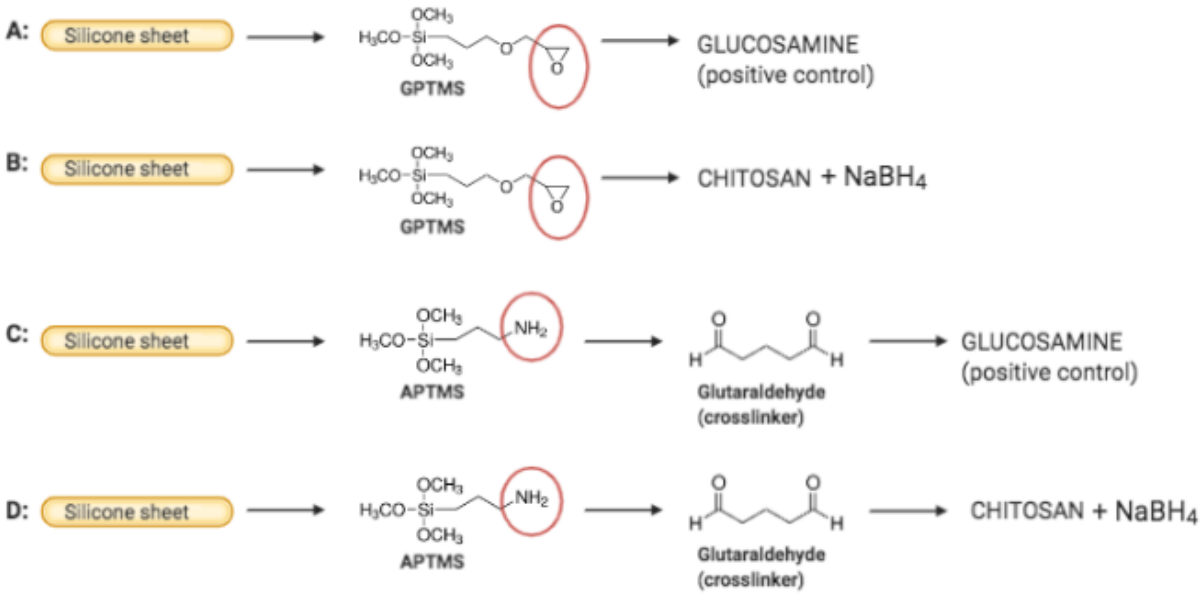


Figure 11. Scheme of each step in the preparation of the coated silicone sheets A-D (without control samples) in an attempt to attach chitosan to the silicone sheet´s surface.

4.2 Ninhydrin quantification assay

Ninhydrin assay is a colorimetric method using ninhydrin reagent to quantify the free primary amino groups in chitosan attached to the glass and silicone surfaces. At 570 nm, the absorbance was measured on a GENESYS 150 UV-Vis-spectrophotometer. The Rheumann´s purple product from the ninhydrin reaction can give a broad estimation of how many free NH_2 groups are bonded to the surface of the glass or silicone substrates, more about the Rheumann´s purple is discussed in section 5.1.

4.2.1 Ninhydrin assay on glass plates

Before performing the ninhydrin assay, a standard calibration curve was prepared on the same day for both chitosan and glucosamine. The calibration curve is used to verify the ninhydrin reagent´s response before performing the ninhydrin assay and the concentration attached can be estimated. The procedure is precisely described in section 3.2.6.2. Figure 12 demonstrates a calibration curve for one of the ninhydrin assays performed, i.e. measurement number two (note that calibration curves 1, 3, 4 are shown in the appendix). The absorbance of glucosamine (orange) and chitosan (blue) were both measured in the UV-Vis spectrophotometer at 570 nm, showing a clear linear response to the reagent.

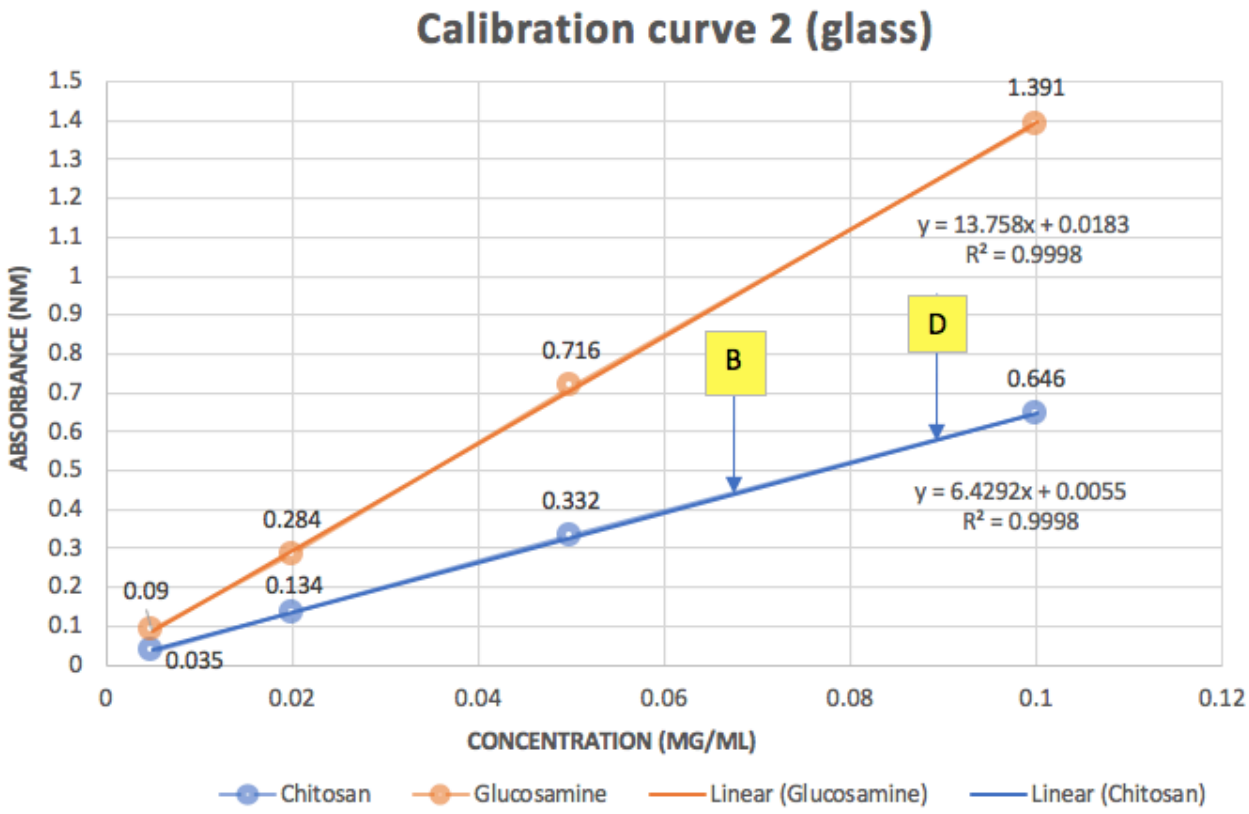


Figure 12. Calibration curve for glass with known concentrations of glucosamine (orange) and chitosan (blue) solutions were measured in an UV-Vis spectrophotometer (GENESYS150). The absorbance at 570 nm shows a linear response to the ninhydrin reagent.

A ninhydrin assay was performed on ninhydrin samples A-H after reacting to the glass plates, the results are listed in table 4 below (results for other assays are in the appendix). The ninhydrin procedure for the glass plates is described in section 3.2.6.3.

Chitosan is a polysaccharide containing numerous NH_2 it should give good ninhydrin response (sample B and D) and therefore a dark purple color. Due to the dark color of samples B and D (containing chitosan), it needed ten-fold dilution to get significant measurements on the UV-Vis spectrophotometer, see figure 13 (left). After dilution (right) the solutions were measured and subsequently could fit within the standard calibration curve. The calculated concentration for glass plate D was greater or 0.91 mg/ml of chitosan while glass plate B measured with 0.72 mg/ml of chitosan (both samples are marked in the calibration curve in figure 12).

Glucosamine, however, should give a weak to no response (less purple color), as it is a monosaccharide containing only one NH2 group. Also, the NH2 group of glucosamine should be bonded to either silane agent (APTMS or GPTMS) and therefore does not provide any free NH2 groups for the ninhydrin reagent (sample A and C). The concentration for glass plates A and C were close to zero, as expected.

Control samples E-H are listed as well in table 4. As expected, sample F (APTMS) had a high response in the ninhydrin assay because of the free NH_2 groups on the glass surface. Samples E and H show minor response as expected, close to zero, as they do not contain any amino groups. It should have been a zero response, but the samples could possibly have gotten slightly contaminated during the procedure. Sample G was measured as a negative value, it can be interpreted as zero, as expected. The glutaraldehyde crosslinker is supposed to block the free amino groups of APTMS with their aldehydes, and they do not respond to the ninhydrin assay.

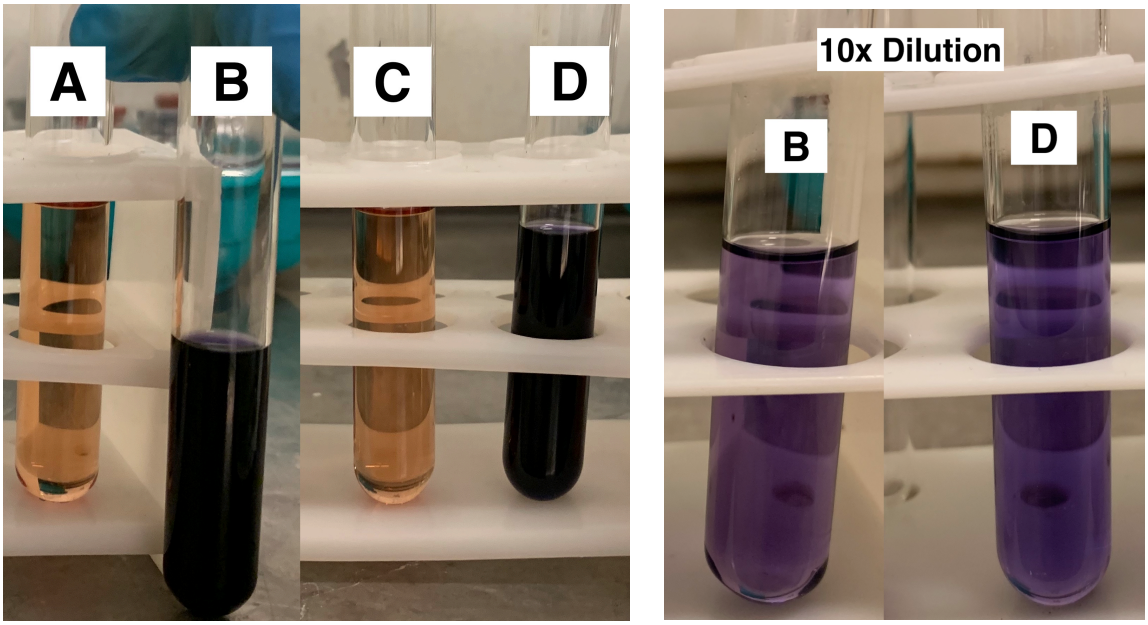


Figure 13. The ninhydrin samples A-D measured in UV-Vis spectrophotometer (left). Sample B and D had to be diluted 10 times to get significant measurements, both of them include chitosan (right).

Table 4. Ninhydrin samples from one assay for the glass plates measured at 570 nm with UV/Vis spectrophotometer (GENESYS 150) and various calculations made from the calibration curve.

Samples	Coatings	Absorbance (nm)	Concentration (mg/ml)	Mass (mg)	Area concentration (mg/cm^2)
Blank	-	-	-	-	-
A	GPTMS Glucosamine	0.017	0	0	0
B	GPTMS Chitosan	0.468 x 10*	0.719	1.438	0.136

C	APTMS Glutaraldehyde Glucosamine	0.032	0.001	0.002	0
D	APTMS Glutaraldehyde Chitosan	0.587 x 10*	0.905	1.810	**0.171**
Controls					
E	GPTMS	0.024			
F	APTMS	1.280			
G	APTMS Glutaraldehyde	-0.019			
H	Toluene	0.038			

**Solutions were measured as inconclusive (>1.5 nm) and were diluted 10 times for absorbance that fitted within the calibration curve, seen in figure 12. The dilutions were considered in the calculations.*

Four ninhydrin assays in total were performed on coated glass plates in this study (see other results in appendix). Only the significant ninhydrin assays were taken into consideration (n=4). The calculated AC for every glass plate in these four assays were compiled to calculate the mean area concentration (MAC). Figure 14 exhibits the MAC results for every glass plate method tested, and it exhibits which method had the highest average response to the ninhydrin assay. Glass plate method D has superior response in comparison with the others. However, the standard deviation (SD) was higher than the average in all cases, which makes the results statistically insignificant. The most probable cause for this high SD is the small number of measurements, and the fact each glass plate method A-D were not measured at the same time. Section 5.2.3 summarizes every possible bias that could have resulted in this high SD in the ninhydrin assays.

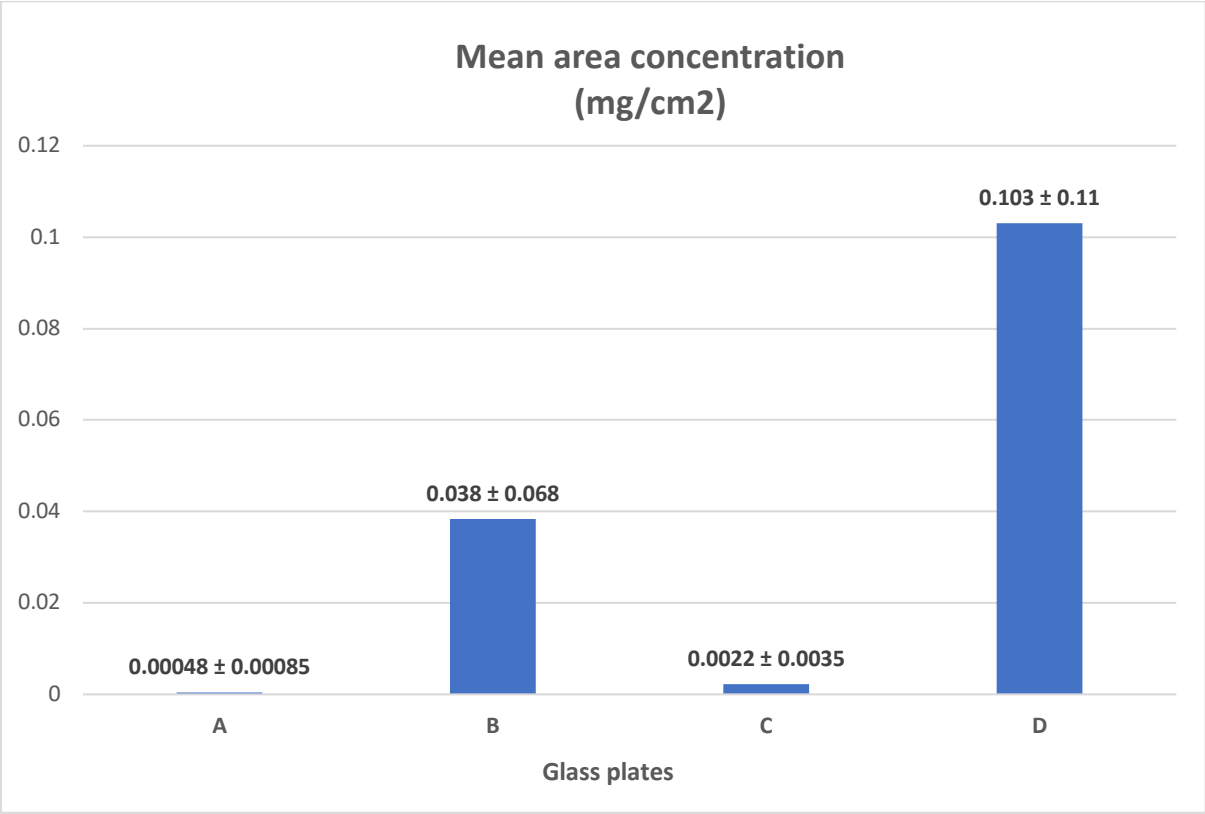


Figure 14. The mean area concentration for glass plates A-D (n=4).

As a result of the insignificant numbers of MAC in figure 14, the AC results in table 4 were substituted to AC ratio wherein glass plate D (light blue) is given the effect of 100% (SD: 0) considering it had, in general, the superior results.

AC ratio for glass plates A-C are then individually examined and compared to the optimum value of D in figure 15 below. Those measurements were performed at the same time and should give more significant results.

Figure 15 exhibits that chitosan-coated glass plate B (82.4%) has 17.6% less activity then glass plate D, indicating that GPTMS did not attach the chitosan as well as the APTMS. However, it does not exclude that the glucosamine did not, in fact, coat the glass plates successfully but it shows that glucosamine does not contain any free NH_2 groups on top the glass plates to react to the ninhydrin reagent. The FT-IR spectrophotometer, discussed later, will analyze that particular factor.

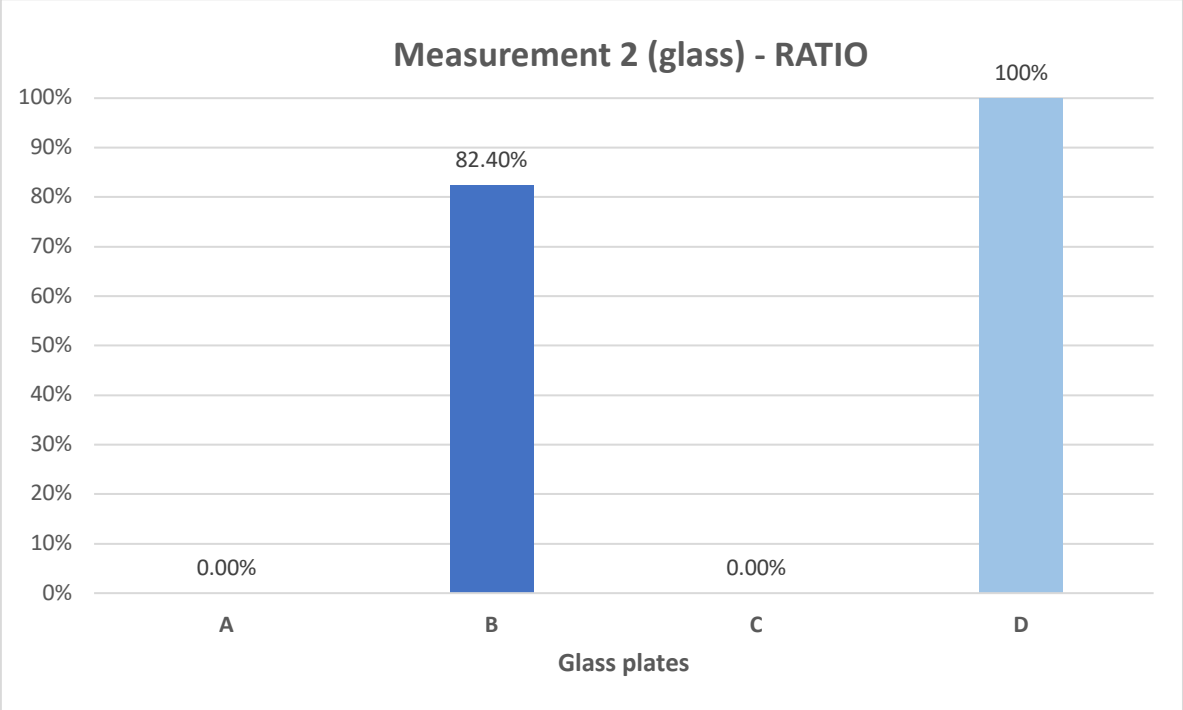


Figure 15. Glass plate D (light blue) shows the most promising results in general and is given the effect of 100% for method comparisons. Glass plate B has decent results, it has about 82.4% effect in comparison with sample D.

4.2.2 Ninhydrin assay on silicone sheets

The same calibration curve procedure was used for the silicone sheets as the previous section described. The absorbance results for ninhydrin samples A-H are listed in the table 5, they represent results for one assay made on the silicone sheets (results from other assays are shown in the appendix).

Same results were expected for the silicone sheets as the glass plates. Sample B and D, containing chitosan, had the highest response, like expected. However, sample A and C, containing glucosamine, had slightly lower response but higher than expected. Sample A measured at 0.201 nm which is most likely caused by contamination of some sort. Sample C measured at 0.343, which is a higher response than expected, the sample could most likely have been either contaminated or the coating procedure for glucosamine unsuccessful. Chances are that this response is caused by the silane agent beneath, APTMS, containing NH_2 group.

Control samle H, shown in table 5, was prepared to assess the importance of oxidizing the silicone substrate by immersing them in a piranha solution before performing the silanization method. For comparison, the absorbance for silicone sheet D measured at 0.464 nm, but when skipping the oxidizing step (the piranha solution) in sample H, it only measured at 0.074 nm. Figure 16 below exhibits the distinct

difference between silicone sheets D and H when observing the Rheumann's purple color after the ninhydrin reaction. Silicone sheet D has a dark purple color while silicone sheet H contains very light transparent purple color.

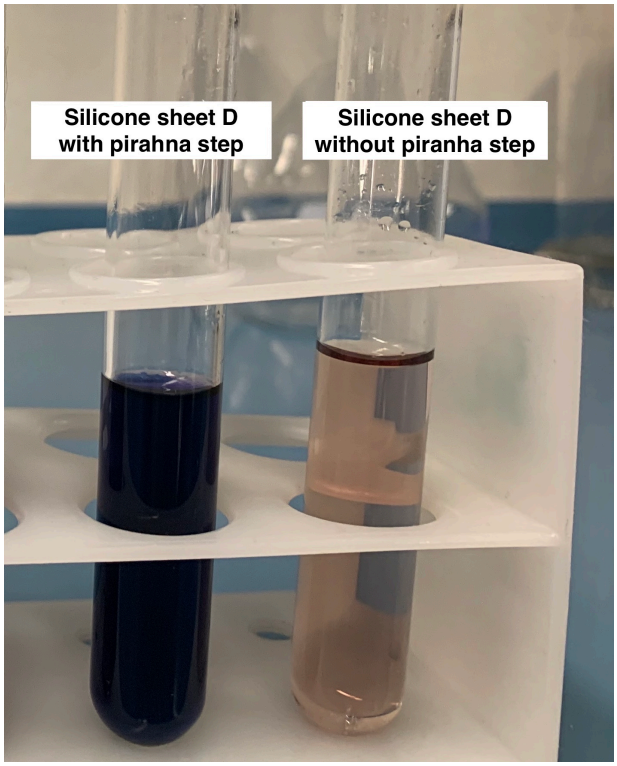


Figure 16. Silicone sheet D (left) and H (right) solutions after the ninhydrin reaction showing a distinct difference in color. The oxidizing step is essential in attaching the chitosan to the silicone sheets.

The AC for each silicone sheet A-D were calculated from a standard calibration equation, like the glass plates (calculations are shown in detail in the previous section). All results from the calculations are listed in table 5. These calculations can indicate which method using either APTMS or GPTMS activation method attaches the chitosan and glucosamine better to the silicone sheet surfaces.

Table 5. Ninhydrin samples from one assay for the silicone sheets measured at 570 nm with UV/Vis spectrophotometer (GENESYS 150) including calculations made from calibration curve.

Samples	Coatings	Absorbance (nm)	Concentration (mg/ml)	Mass (mg)	Area concentration (mg/cm^2)
Blank	-	-	-	-	-
A	GPTMS Glucosamine	0.201	0.013	0.026	0.003

B	GPTMS Chitosan	0.385 x 10*	0.590	1.180	0.112
C	APTMS Glutaraldehyde Glucosamine	0.343	0.024	0.048	0.005
D	APTMS Glutaraldehyde Chitosan	0.464 x 10*	0.713	1.426	**0.135**
Controls					
E	GPTMS	0.011			
F	APTMS	0.790			
G	APTMS glutaraldehyde	0.284			
H	D without the piranha step	0.074			

**Solutions were measured as inconclusive (>1.5 nm) and were diluted 10 times for absorbance that fitted within the calibration curve. The dilutions were considered in the calculations.*

Three ninhydrin assays in total were performed on coated silicone sheets in this study (see results in appendix). The calculated AC for every silicone sheet A-D for these three assays were compiled to calculate the mean area concentration (MAC). Figure 17 exhibits the MAC results for every silicone sheet method A-D, the same way as the glass plates (shown in the previous section). The MAC for silicone sheet D contains approximately 0.083 mg of chitosan per cm^2 of the silicone sheet and silicone sheet B contained 0.037 mg of chitosan per cm^2.

Only the significant ninhydrin assays were taken in consideration (n=3) for the silicone sheets, i.e., only the absorbance measurements that fitted within their calibration curve. Samples were often diluted to fit the measurements within the calibration curve, especially the samples containing chitosan (B and D). In one occasion when the samples were diluted 10-fold, the absorbance measurements subsequently fell below the standard calibration curve. As the glass plates, the MAC´s for the silicone sheets were statistically insignificant because the SD was high and, in some cases, above the average. The most probable cause for this high SD is the small number of measurements and the fact that each silicone sheet method A-D

were not measured at the same time. Section 5.2.3 summarizes every possible error that could have resulted in a high SD in the ninhydrin assays.

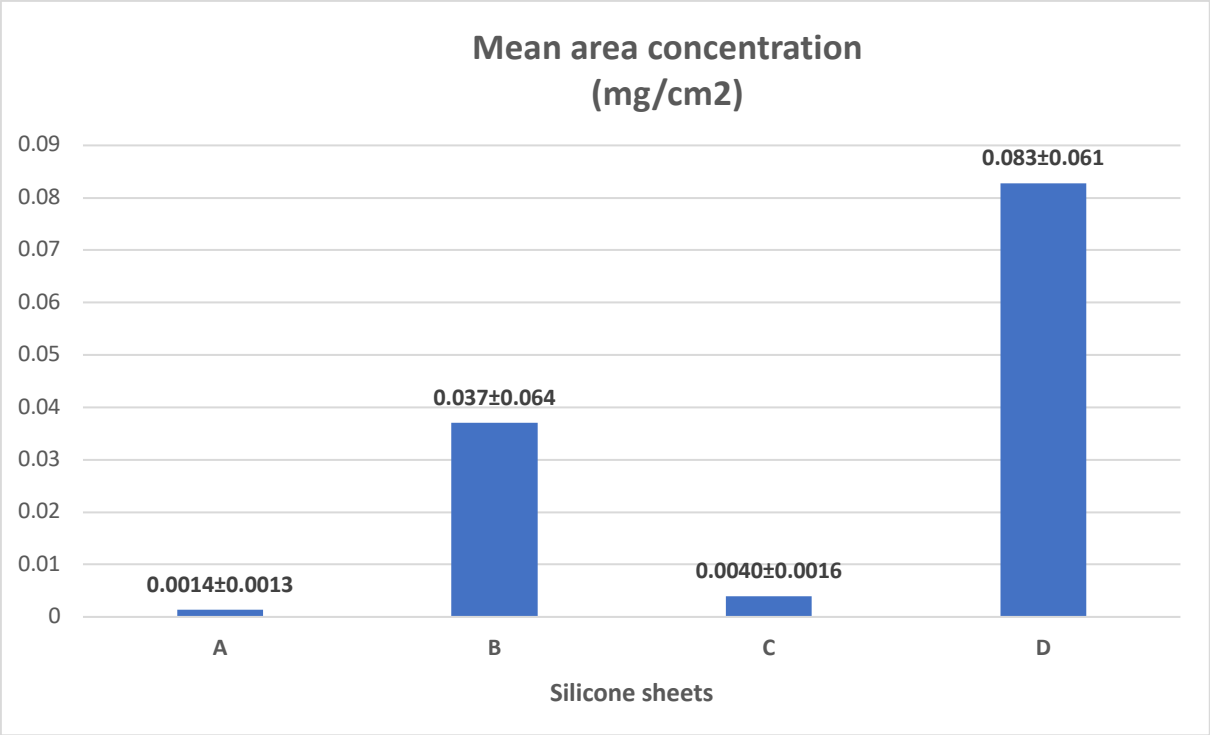


Figure 17. The mean area concentration for each silicone sheet method A-D (n=3).

As a result of the insignificant numbers of MAC in figure 17, the AC results in table 5 were substituted to AC ratio wherein silicone sheet D (light blue) is given the effect of 100% (SD: 0) considering it had, in general, the superior results.

AC ratio for silicone sheets A-C are then individually examined and compared to the optimum value of D in figure 18 below. Those measurements were performed at the same time and should give more significant results.

Figure 18 exhibits that chitosan-coated silicone sheet B (83.0%) has 17.0% less activity then glass plate D, indicating that GPTMS did not attach the chitosan quite as well as the APTMS. The glucosamine-coated silicone sheets A and C had very limited response, 1.86% and 3.30% respectively.

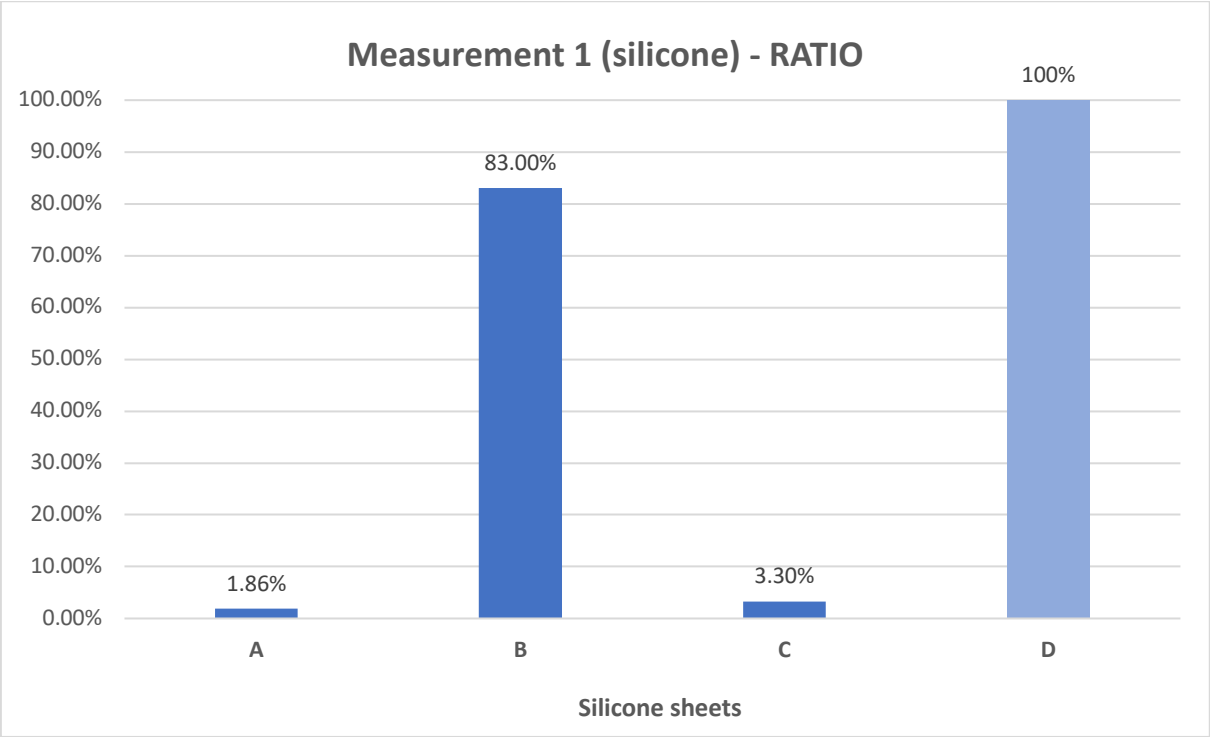


Figure 18. Silicone sheet D (light blue) shows the most promising results in general and is given the effect of 100% for method comparisons. Silicone sheet B has decent results, it has about 83.0% effect in comparison with sample D.

4.3 FT-IR spectra analysis

The coated glass plates and silicone sheets were placed in the FT-IR spectrophotometer (Nicolet iZ10) for analysis in reflection mode. The instrument can identify the surface material and if the surface coating was successful. Thus, its purpose is to indicate if chitosan or glucosamine did attach to the glass or silicone substrates using either of the surface activation method (APTMS and GPTMS). Figure 19 exhibits characterized peaks for the spectra of the authentic form of chitosan (left) and glucosamine (right).

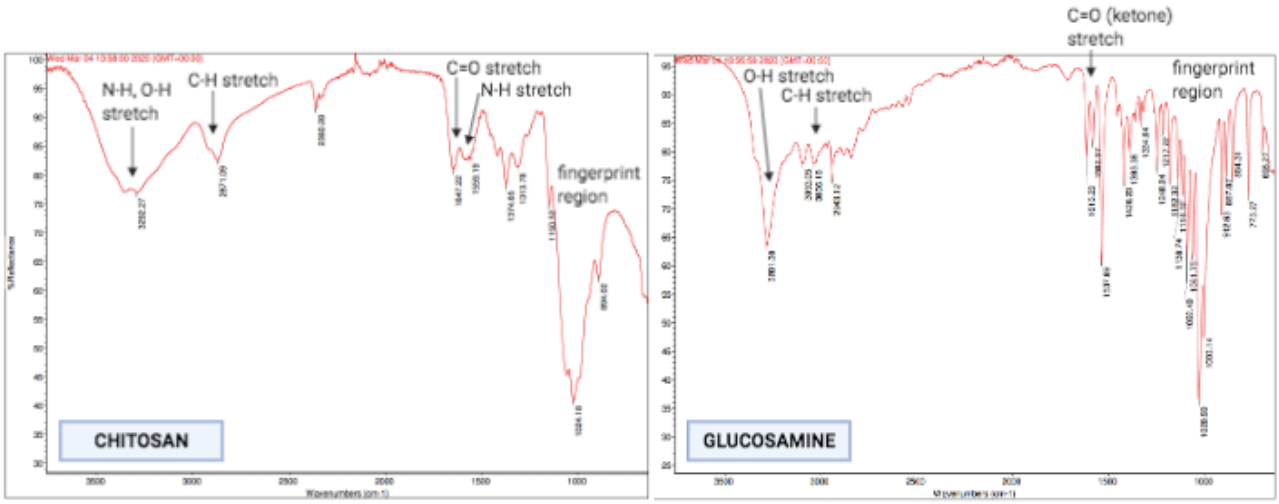


Figure 19. The authentic IR-spectra of chitosan (left) and glucosamine (right) used for comparison in the following analyzation of the coated glass and silicone spectra´s.

4.3.1 Surface coating analysis for glass plates

An untreated glass plate substrate (blank) was measured to know their characteristic peaks and to compare if the chitosan or glucosamine coatings were successful or not, see figure 20. The fingerprint regions were not compared.

Figure 20. FT-IR spectra of untreated glass plate without any coatings for comparison (blank).

Each IR-figure shown below will contain two different spectra. The lower one is always the pure form of the chemical being analyzed, i.e., either chitosan or

glucosamine. Upper spectra exhibit the compound identified on the glass plates surface. Comparing the upper spectrum to the authentic sample (lower spectra) will provide a reasonable confirmation if the coating process was successful.

Figure 21, exhibits the spectra for glass plate D and it is highly equivalent to the spectra of the authentic chitosan below, containing similar characteristic peaks. However, glass plate B did not respond as well as glass plate D and had a more similar spectrum to the untreated glass plate (blank) in figure 20 rather than the chitosan spectrum but weak peaks characteristics for chitosan could be observed. Figure 22 exhibits the spectra for glass plate B as well as the authentic chitosan spectra below.

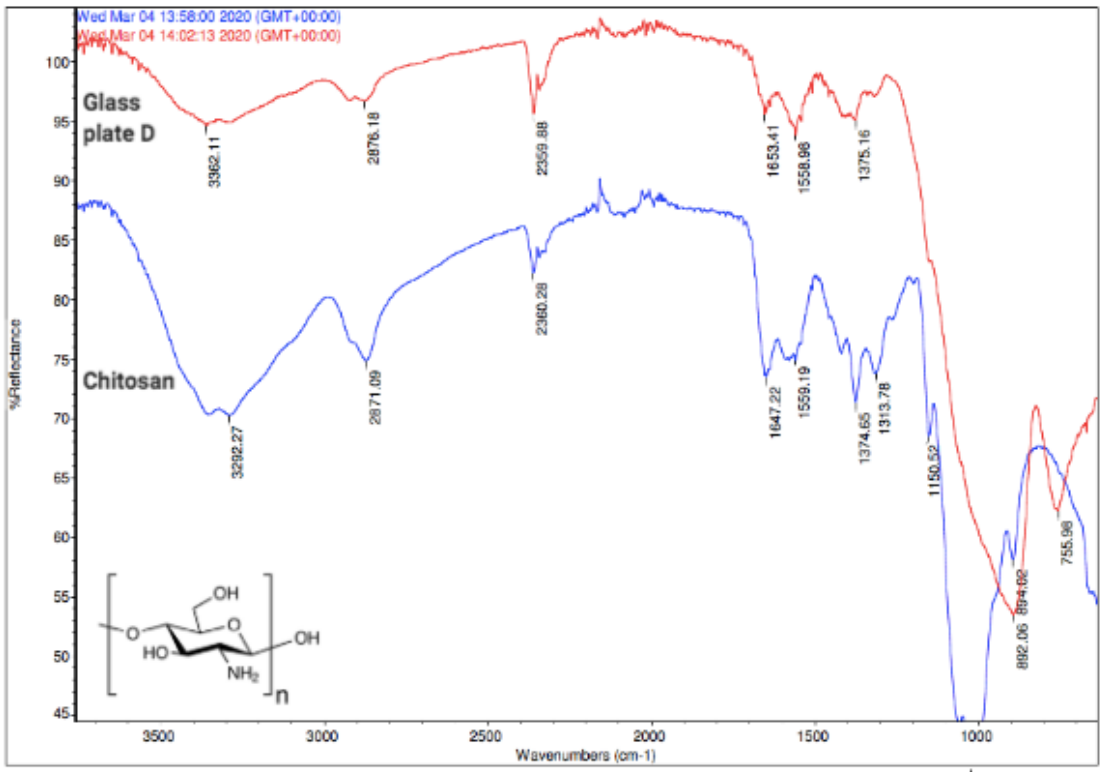


Figure 21. FT-IR spectra of glass plate D coated with chitosan (upper spectra).

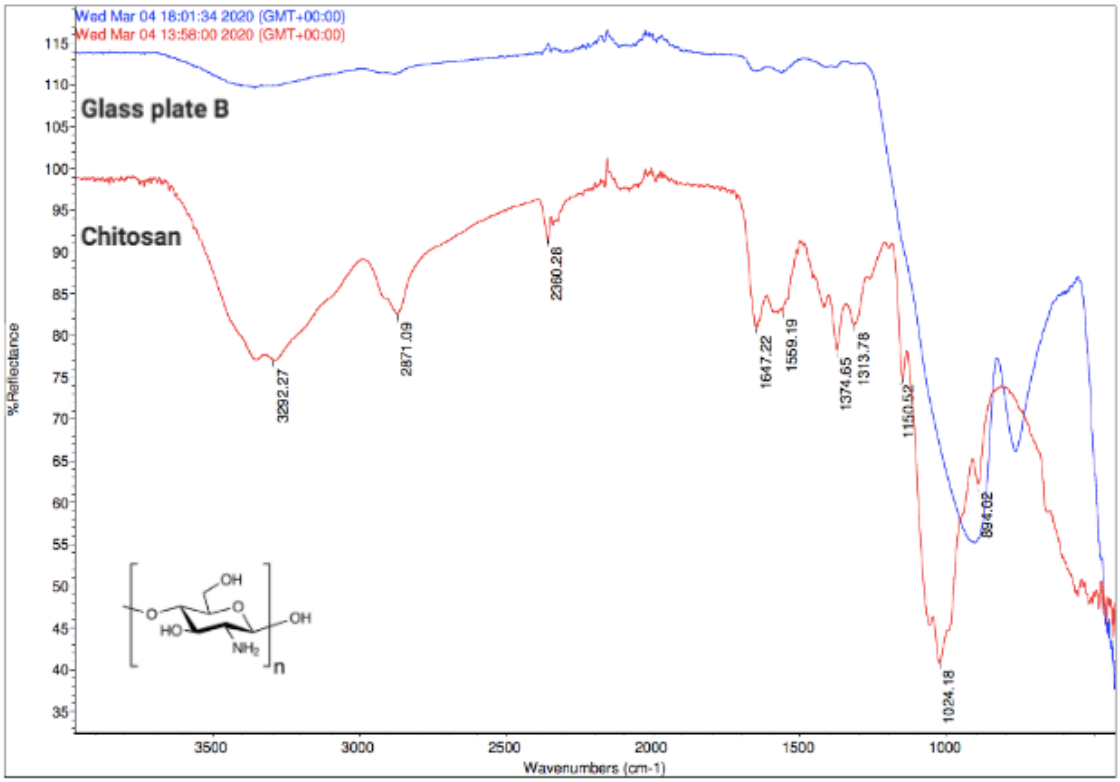


Figure 22. FT-IR spectra of glass plate B coated with chitosan (upper spectra).

Figure 23 and 24 shown below are glass plates C and A coated with glucosamine. The peaks shown in both figures are unknown, they are neither similar the spectra for glucosamine nor the glass plate (figure 20). On the basis of this data, it is not possible to identify if the glucosamine attached to the glass plate surface using either method. These results exhibit chemical modification, whereas the chemical bonds that were supposed to be created are possibly other than initially thought.

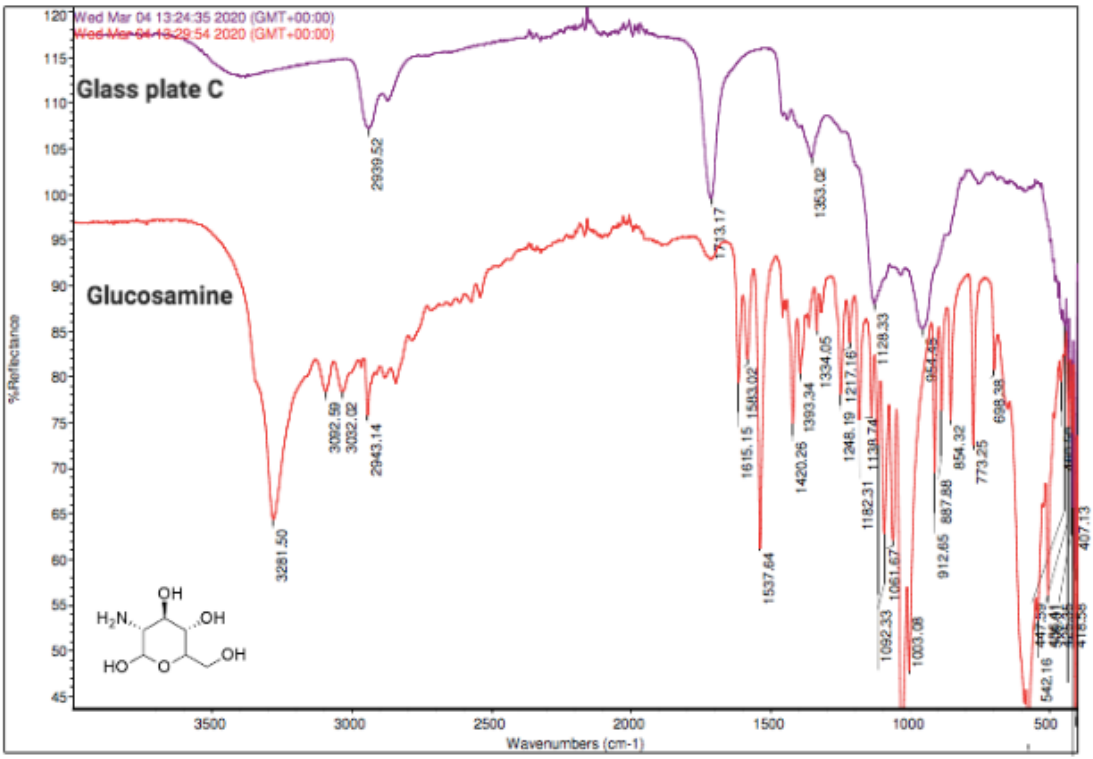


Figure 23. FT-IR spectrum of glass plate C coated with glucosamine (upper spectra).

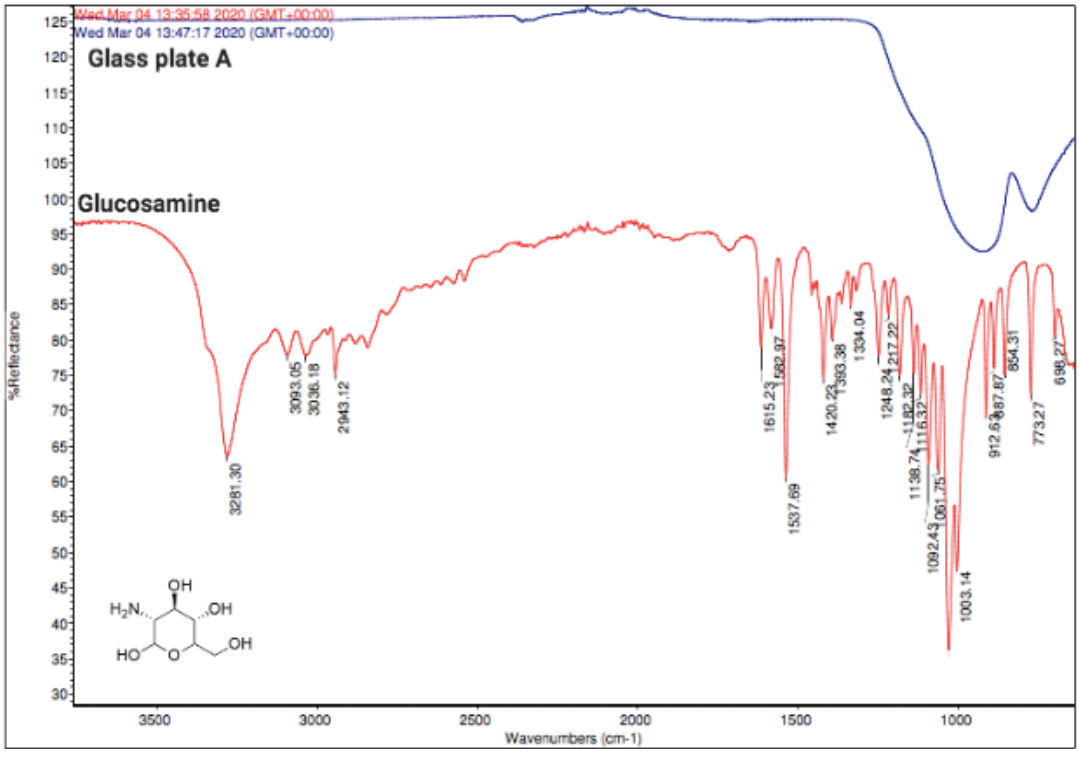


Figure 24. FT-IR spectrum of glass plate A coated with glucosamine (upper spectra).

As mentioned previously, glass plates D exhibited the most promising results when the surface coating was characterized, it had the surface modification method using APTMS for silanization and glutaraldehyde for crosslinking.

Glass plate D in figure 25 (left) exhibits similar spectra to chitosan, as well as characteristic peaks, such as the O-H stretch at 3362.11 cm^{-1}, C-H stretch at 2876.18 cm^{-1}, C=O and N-H (amide) stretch at 1653.41 cm^{-1} and 1558.98 cm^{-1}, respectively.

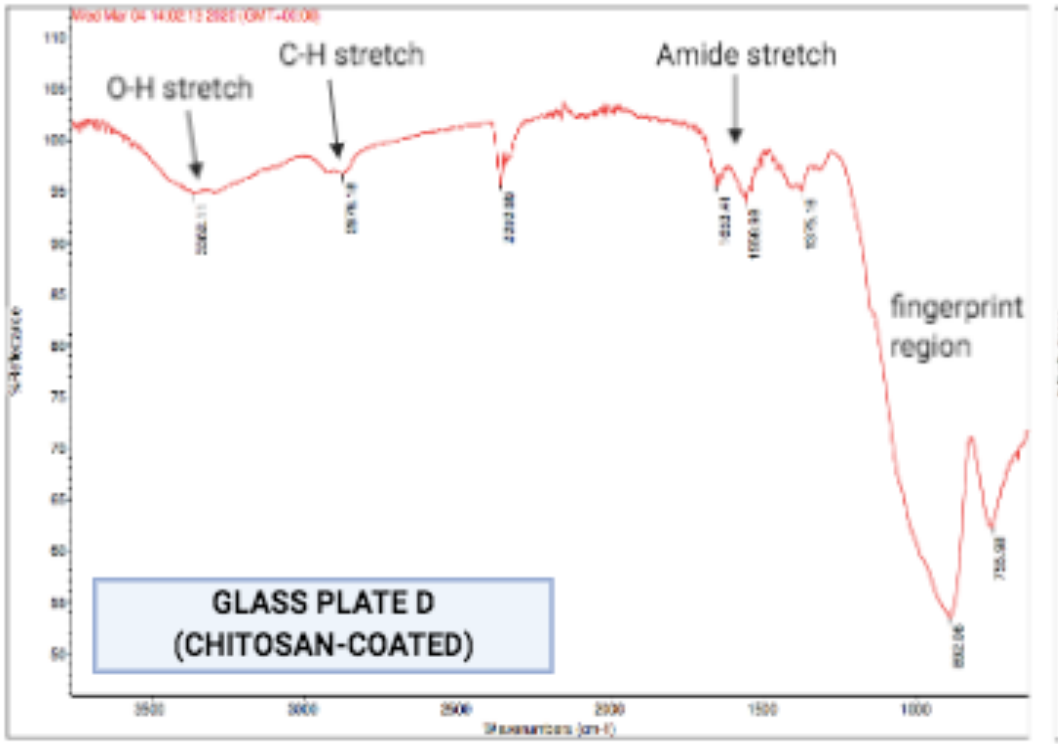


Figure 25. Glass plate D (left) has similar peaks as the pure chemicals for chitosan, indicating that the coating process was successful.

4.3.2 Surface coating analysis for silicone sheets

An untreated silicone sheet substrate (blank) was measured to know their characteristic peaks and to compare if the chitosan or glucosamine coatings were successful or not, see figure 26.

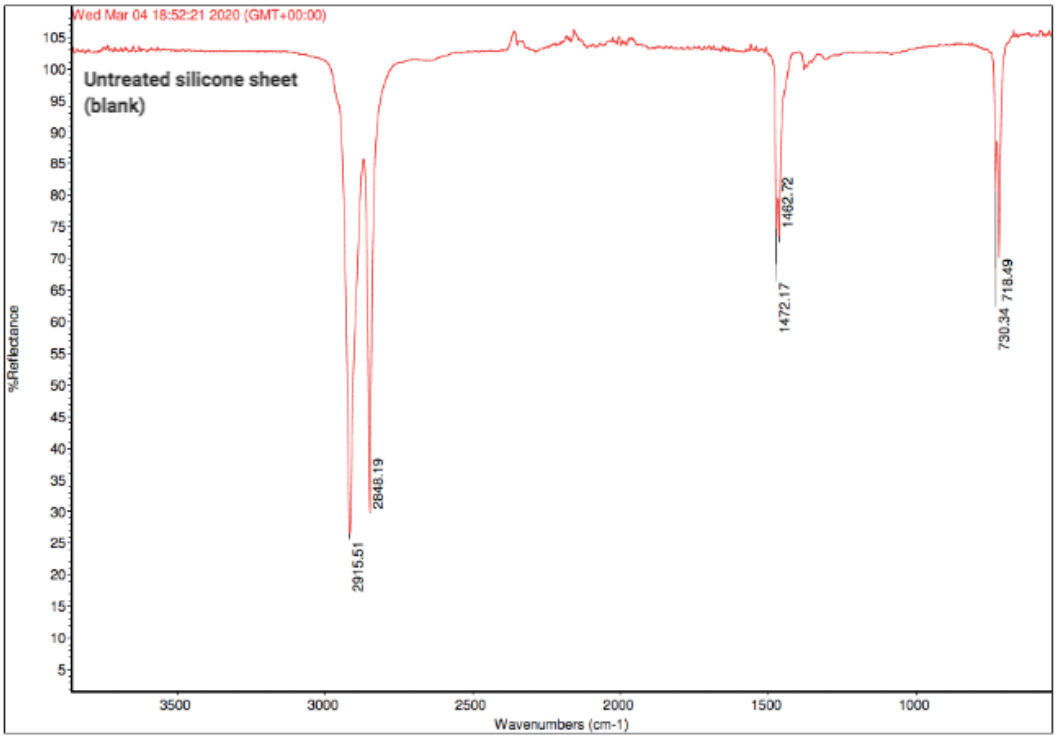


Figure 26. FT-IR spectrum of untreated silicone sheet without any coatings for comparison (blank).

Like the glass plates, each figure will contain two different spectra. Lower spectra are the pure form of either chitosan or glucosamine and upper spectra exhibit the compound identified on the silicone sheet surface. Comparing the upper spectrum to the authentic sample (lower spectrum) will provide a reasonable confirmation if the coating process was successful.

Figure 27, exhibits the spectra for silicone sheet D and it is highly equivalent to the spectra of the authentic chitosan below, containing similar characteristic peaks. However, silicone sheet B did not respond as well as silicone sheet D and had a more similar spectrum to the untreated silicone sheet in figure 26. The spectrum for silicone sheet B as well as the authentic chitosan spectra below is shown in figure 28.

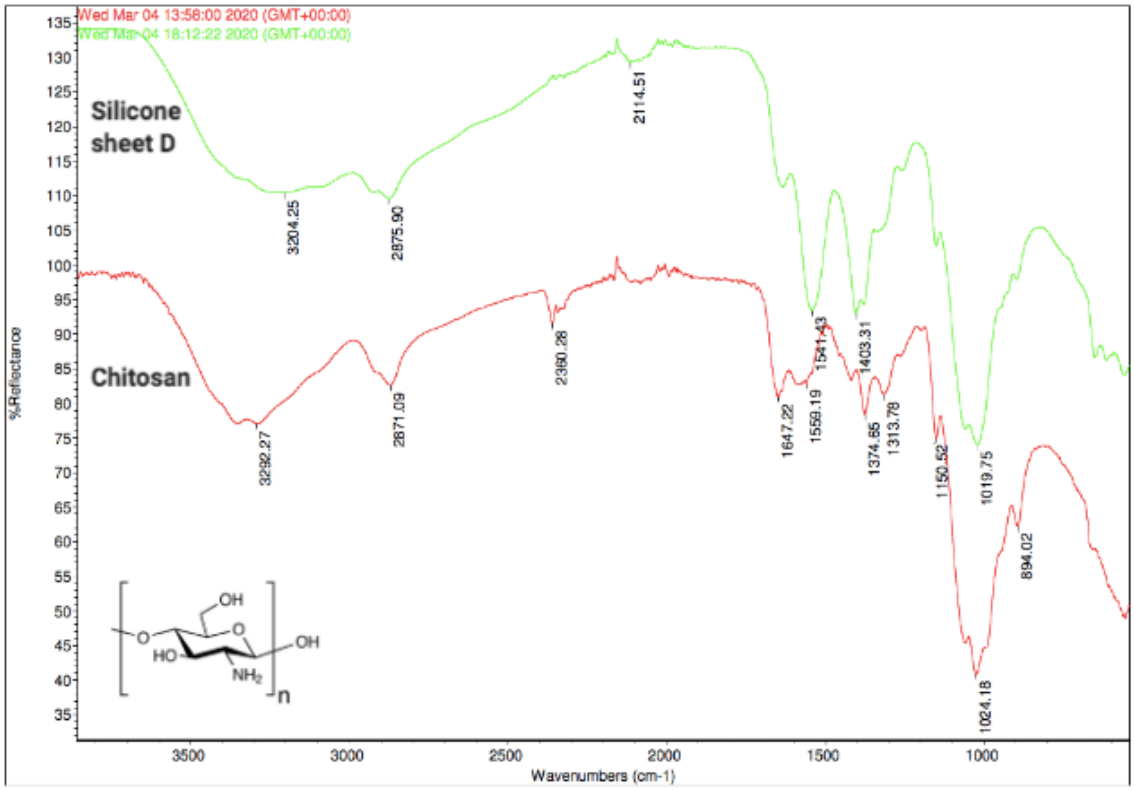


Figure 27. FT-IR spectrum of silicone sheet D coated with chitosan (upper spectra).

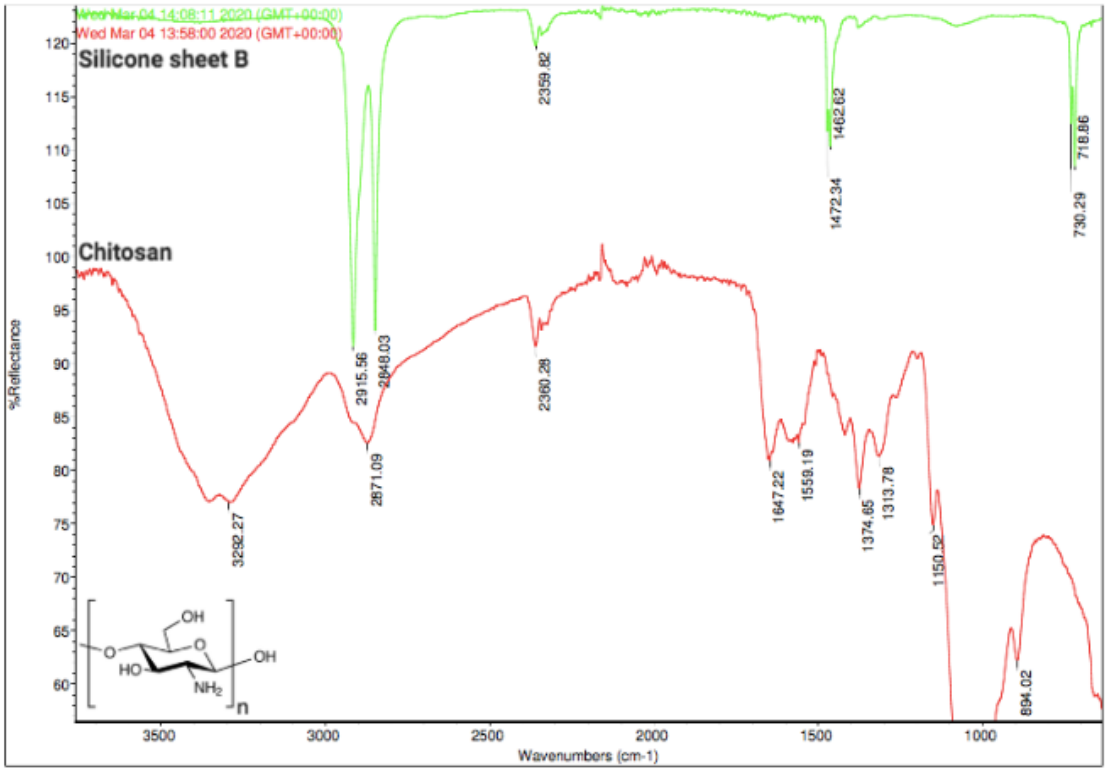


Figure 28. FT-IR spectrum of silicone sheet B coated with chitosan (upper spectra).

Figure 29 and 30 shown below are silicone sheets C and A coated with glucosamine. Silicone sheet C indicates more successful coating than silicone sheet

A in comparison with the spectrum of the authentic glucosamine shown below (lower spectrum). The silicone sheet C exhibits similar but much weaker peaks for glucosamine. However, silicone sheet A responded poorly and had a spectrum more similar to the untreated silicone sheet, shown in figure 26.

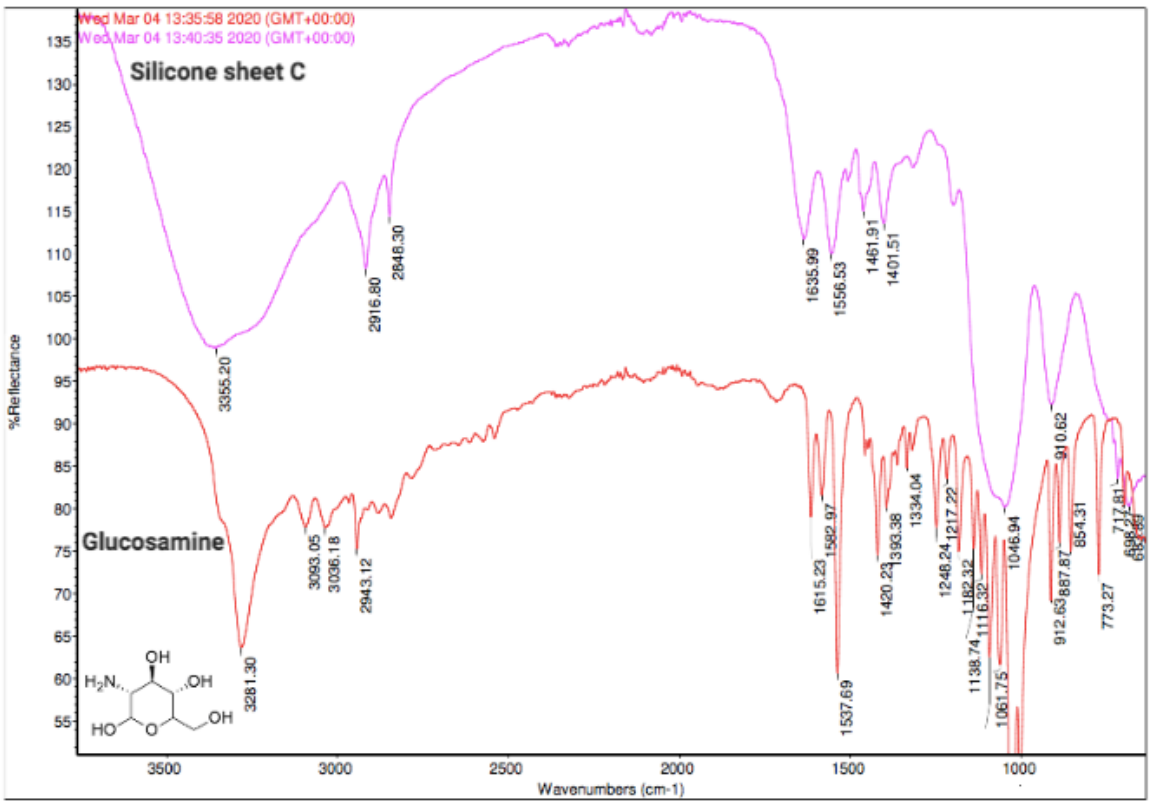


Figure 29. FT-IR spectrum of silicone sheet C coated with glucosamine (upper spectra).

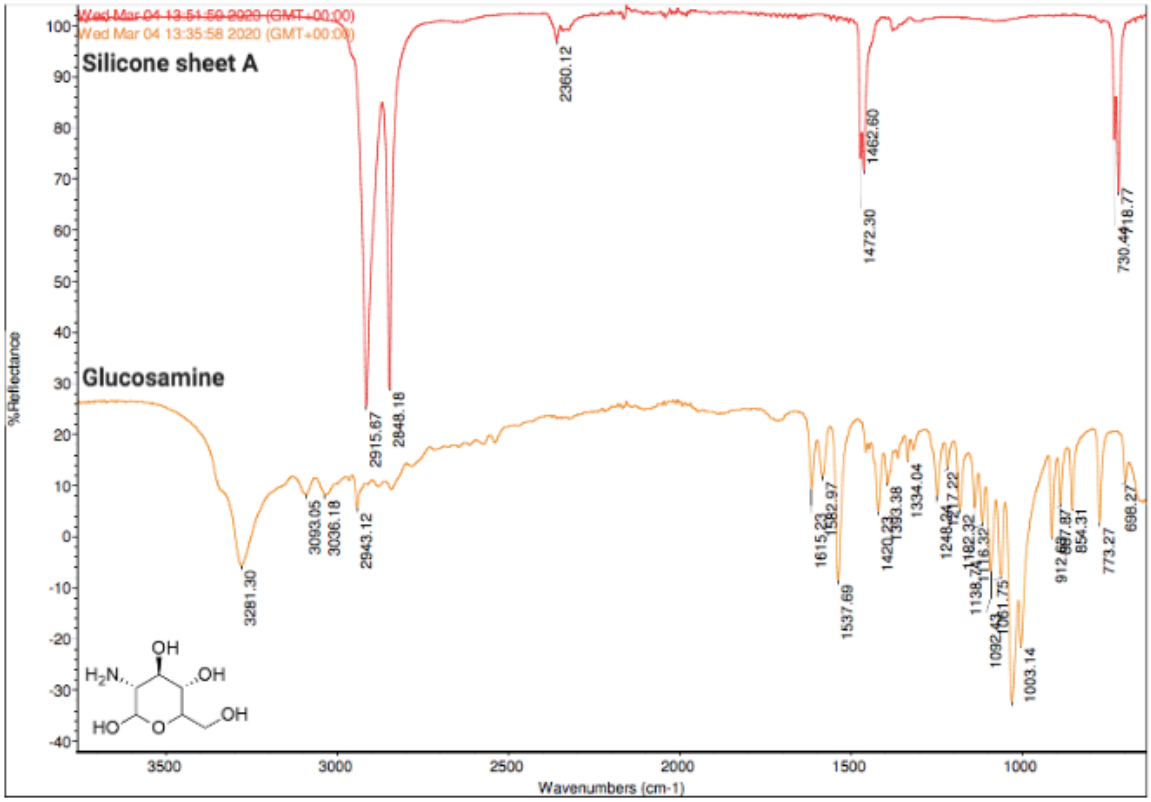


Figure 30. FT-IR spectrum of silicone sheet A coated with glucosamine (upper spectra).

As mentioned previously, silicone sheets D and C exhibited the most promising results when the surface coating was characterized, they had the same surface modification method.

Silicone sheet D in figure 31 (left) exhibits similar spectra to chitosan, as well as characteristic peaks, such as the O-H stretch at 3204.25 cm^{-1}, C-H stretch at 2875.90 cm^{-1}, C=O and N-H (amide) stretch at 1541.43 cm^{-1} and 1403.31 cm^{-1}, respectively.

Silicone sheet C in figure 31 (right) exhibits similar spectra to glucosamine, as well as characteristic peaks, such as O-H stretch at 3355.20 cm^{-1}, C-H stretch at 2916.80 cm^{-1} and possibly C=O (ketone) stretch at 1635.99 cm^{-1}.

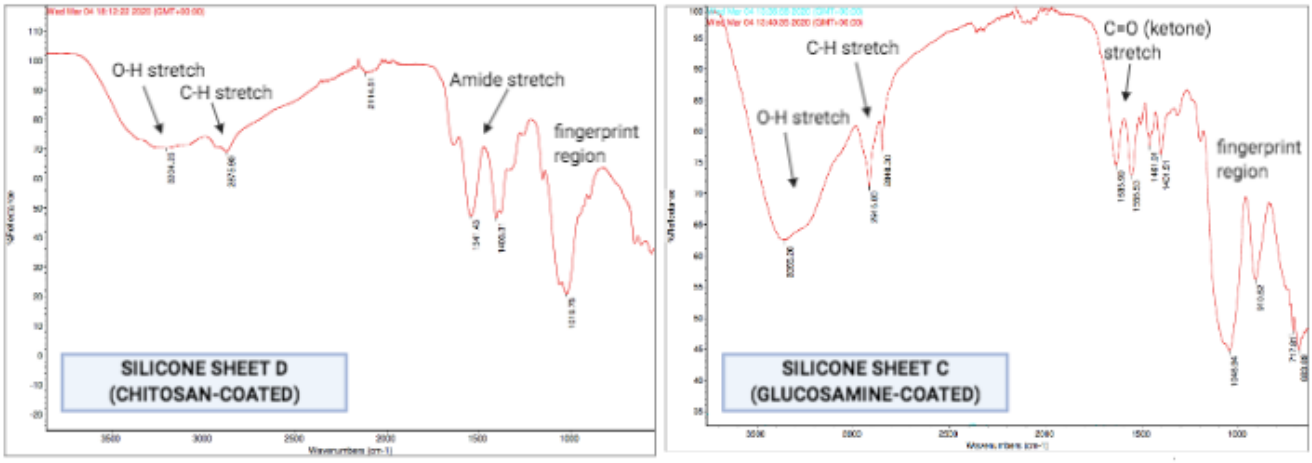


Figure 31. Silicone sheets D (left) and C (right) have similar peaks as the pure chemicals for chitosan and glucosamine, indicating that the coating process was successful.

4.4 Antibacterial activity assay

According to the Clinical and Laboratory Standards Institute (2018), agar disk-diffusion testing is the official method in many clinical microbiology laboratories for routine antibacterial susceptibility testing. The method used in the thesis was similar, but with some modifications. Generally, the purpose of the agar disk-diffusion test is to observe the antibacterial agent´s diffusion into the agar, and then the inhibition growth zone measured. However, the thesis purpose is only to observe the contact killing effects, i.e., if the growth of the testing bacteria is inhibited beneath the antibacterial samples. Also, instead of using 6 mm filter paper discs like described, there were 23 mm glass plates and silicone sheets tested. The antimicrobial activity of ten samples was tested against the Gram-negative bacteria *P. aeruginosa*, grown on blood agar.

The antibacterial activity assay was performed on 5 glass plates and 5 silicone sheets coated with chitosan or glucosamine for comparison, see table 6. The assay procedure is described in section 3.2.7.

Table 6. The glass plate and silicone samples tested for the antibacterial activity against *P. aeruginosa.*

	GLASS PLATES	SILICONE SHEETS
1	Blank	Blank
2	APTMS + glutaraldehyde + glucosamine	APTMS + glutaraldehyde + glucosamine
3	APTMS + glutaraldehyde + chitosan	APTMS + glutaraldehyde + chitosan
4	GPTMS + glucosamine	GPTMS + glucosamine
5	GPTMS + chitosan	GPTMS + chitosan

4.4.1 Antibacterial activity assay for glass plates

After incubation overnight, the agar plates were analyzed. The chitosan-coated glass plates were specifically inspected because of their known contact killing capability. However, the results of the assay indicated a limitation in the procedure rather than antimicrobial activity. All of the glass plate samples inhibited bacterial growth whether or not they contained chitosan, including the blank, as seen in figure 32.

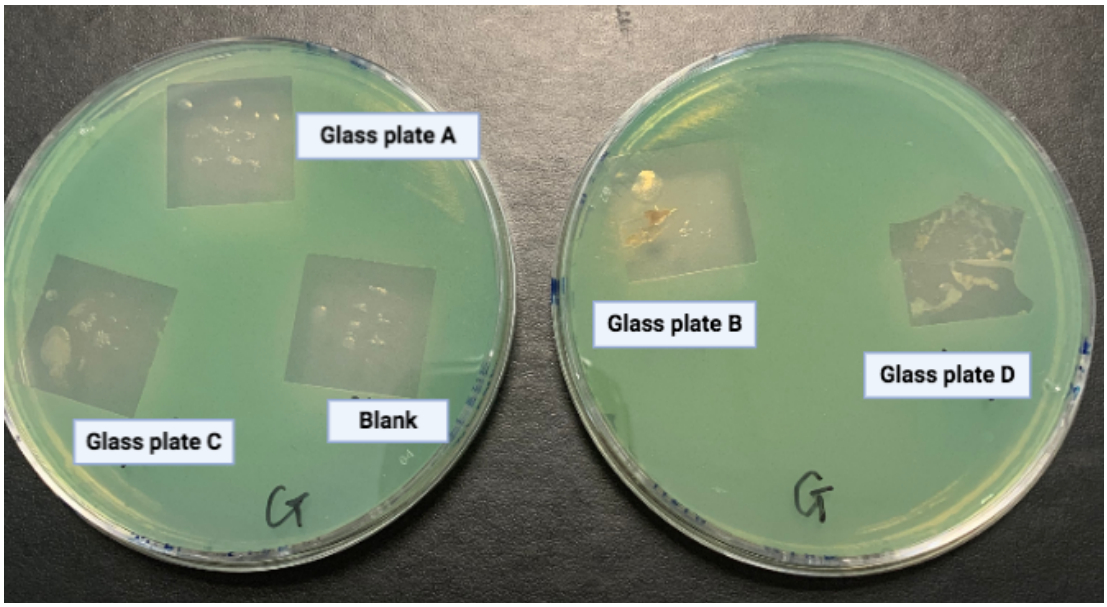


Figure 32. Antimicrobial activity assay on surface-coated glass plates A-D and a blank.

4.4.2 Antibacterial activity assay for silicone sheets

The same results were seen for the silicone sheets as for the glass plates above, the antimicrobial results were the same. The bacteria were killed under the control sheets as well as the samples in figure 33.

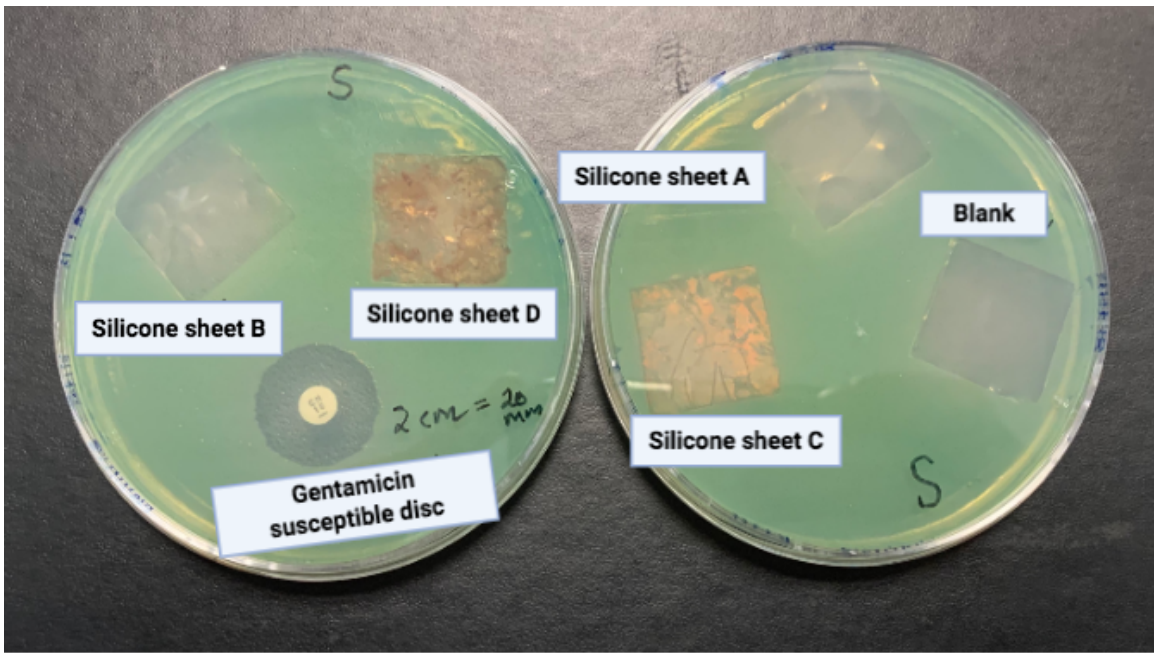


Figure 33. Antimicrobial activity assay on surface-coated silicone sheets A-D, blank and Gentamicin performance control.

The results for the glass plates and silicone sheets in the antimicrobial assay are ineffective. As shown in both figure 32 for glass plates and figure 33 for silicone sheets, it seems like all samples had contact killing effects whether or not their surfaces were coated with chitosan. Thus, the first result of this assay is that this specific method is not suitable for testing the sample´s antimicrobial activity. Whereas, the bacteria (*P. aeruginosa*) seems to be not growing beneath the samples under these conditions. Thus, a more appropriate method is needed to perform the antibacterial activity for the glass plates and silicone sheets.

5. DISCUSSION

The synthetic silicone polymers have been used as implantable catheters for years and have a high risk of developing biofilm infections, such as CAUTI. Preventable actions have been taken by coating catheters with antimicrobial agents and the idea of using chitosan, a natural and biodegradable polymer, to coat silicone substrate is a development at an early stage. There are limited studies found about chitosan-based coatings on silicone catheters. However, chitosan-based coatings on a glass substrate has shown promising results using the silanization method. However, there are no hydroxyl groups on the surface of silicone, so some type of activation procedure is needed. Silicone surfaces have been activated by ozone treatment (Özçam, Efimenko, & Genzer, 2014) but the required equipment was not available in the lab of Hagi. We were looking for a more convenient method and found a study by Acres et al. (2012) which describes the use of piranha solution for activation of silicon wafers, followed by silanization. However, silicone is a completely different substrate, so the outcome was uncertain. The hydrophobic property of silicone needs to be modified by oxidation to introduce OH groups to the surface, in order to increase the binding of the silane agents APTMS or GPTMS to the surface. The study mentioned above contained the same surface modification method (silanization) as the procedure for glass plates. Thus, the same silanization method was used in this study, both for glass plates and silicone sheets to chemically attach chitosan to the surface. Various methods were used to quantify, analyze, and confirm the chitosan coatings, using ninhydrin assay, FT-IR spectrophotometer, and antibacterial assay. The results were favorable for both the glass plates and silicone sheets, whereas they showed promising response using the same surface activation method using APTMS silane agent and glutaraldehyde crosslinker. Samples prepared with that surface activation method seemed to attach chitosan more successfully than the other method tested, as mentioned in section 4.2 in the results.

5.1 Surface modifications and functionalization

Figure 34 describes in detail the chemical modifications made on the glass and silicone substrates and how they attach the coating substance, i.e., chitosan or glucosamine, with or without using a crosslinker. The substrates activated by using the silane agent GPTMS (samples A and B) with a free epoxy group attaching to one free amino group

of glucosamine and chitosan, creating a bond through a secondary amine (-NH group). However, the substrates activated by using the silane agent APTMS (samples C and D) with a free amino group attaching to the glutaraldehyde crosslinker. The crosslinker´s purpose is to link the APTMS´s and chitosan´s amino groups together.

Sample C coated with glucosamine creates a tertiary amine bonding to the crosslinker but sample D coated with chitosan creates a more stable secondary amine bonding. The reason for that is the $NaBH_4$ mixed with the chitosan standard solution preparation before performing the coating, as described in section 3.2.2.1.1. The imine group that would have been produced like in sample C is usually very unstable and that is the reason for adding the reducing agent, $NaBH_4$. It has an impact on the glutaraldehyde´s bonding to chitosan and reduces the imine bond creating a secondary amine which is more stable and attaches the chitosan more successfully. Here, we possibly could have had got better results by mixing NaBH4 with the glucosamine standard solution in section 3.2.2.1.2. Then the reducing agent would have reduced the imine bond and created 2° amine bond and there would have been the same amine bonding in all samples A-D. Thus, sample C could be showing less response than the chitosan sample due to the instability of imine.

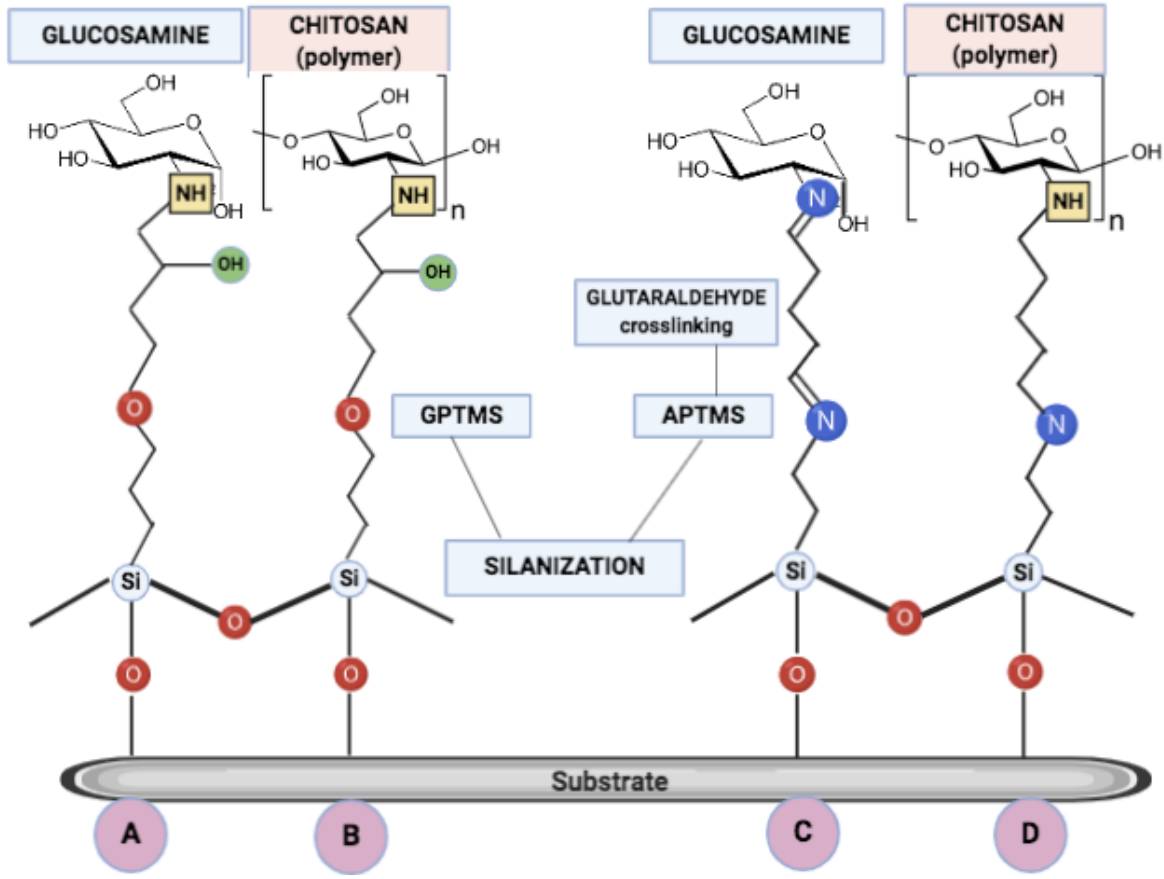


Figure 34. Chemical substrate modifications with APTMS or GPTMS to attach chitosan/glucosamine. The substrate applies to both the glass plates and silicone sheets and exhibits samples A-D.

5.2 Ninhydrin quantification assay

Ninhydrin assay is a general method for quantitative analysis using an anionic dye that quantifies the bound or adsorbed chitosan to the surfaces. The procedure is rapid, inexpensive and suitable for routine analysis of many samples. The reaction of ninhydrin reagent with a primary (1°) amino group will form a colored reaction product, called Ruhemann´s purple, see the following reaction in figure 35 below.

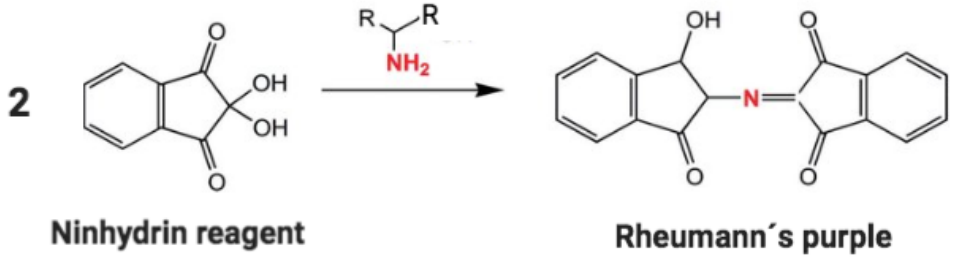


Figure 35. The ninhydrin reacts with a 1° amino group and produces Rheumann´s purple.

The Rheumann's purple is a deep blue or purple-colored substance formed by the reaction of ninhydrin with a 1° amine group and the ninhydrin's reduction product hydrindantin. Both chitosan and glucosamine contain a 1° amine group, and previous studies has been showing good response to the ninhydrin method. It can give a clear indication if the coating procedure was effective or not by viewing the color of both the substrate being tested, as well as the solution that reacted to the substrate.

Ninhydrin's reaction to the glass and silicone substrate gives a quantitative estimation where an increasing purple Rheumann's color represents the number of free 1° amine groups on the surface coating. The ninhydrin reagent reacts to those free amine groups on the substrate, creating purple color and their absorbance measured on the UV-Vis spectrophotometer.

Like mentioned before in figure 34 above, the substrate was activated with using two different silane agents, GPTMS and APTMS and then coated with two different coating materials, glucosamine (samples A and C) and chitosan (samples (B and D). The general conclusion from the ninhydrin assay's results was that chitosan gave a much higher response than glucosamine when attached to the surface. There is an explanation for that, chitosan is a polysaccharide material, but glucosamine is a monosaccharide material. That is to say, chitosan has numerous free 1° amine groups that can react to the ninhydrin reagent, but glucosamine has none. The only available amino group in glucosamine is linked to the substrate's silane agent or crosslinker and

creates a secondary and tertiary amine groups (sample A and C, respectively) that do not respond as well to the ninhydrin assay. However, the glucosamine's results gave the solution an orange-like color which provided minor response in the ninhydrin assay, as mentioned in the results (section 4.2).

5.2.1 Ninhydrin assay on glass plates and silicone sheets

The results from the ninhydrin assays concluded that the process of activating the glass substrates with APTMS and crosslinking with glutaraldehyde (sample D) did attach the chitosan more successfully than the process of using the GPTMS (sample B). The results for the silicone sheets were the same as for the glass plates. The silicone sheet oxidized with piranha solution, silanized with APTMS, crosslinked with glutaraldehyde to attach the chitosan (sample D) showed the best response to the ninhydrin assay. Thus, the method D for both glass and silicone substrates exhibited the most promising results for further developments. It seems that using the crosslinker glutaraldehyde creates stronger bonding to chitosan rather than only using GPTMS.

As for the results for silicone sheets, the importance of the oxidizing step with piranha is clearly noticeable and plays a major role in the coating procedure for silicone. If the silicone sheets are remained nonpolar (no oxidation step), then it seems that the silane agents APTMS and GPTMS will less likely create a bonding with the silicone sheet surfaces.

5.2.2 Standard deviation and variability of the measurements

The calculated standard deviation (SD) for the measurements (shown in the appendix) made for each method A-D was higher than the average, which makes the results less reliable. The number of measures on samples were only 3-4, and that factor immediately makes the SD increase. The SD results make the ninhydrin assay in this study statistically insignificant, where the variability between the quantitative measurements is spread too widely. Various factors could have affected this high variability, e.g., the limited number of measures (like mentioned above), the coating drying time, and varying degrees of activity of the ninhydrin reagent.

The insignificant calibration curves (shown in the appendix) were not included in the measurements, but they could indicate the reason for the variability. Ninhydrin's reagent sensitivity decreases with time due to its unstable nature and that factor could have affected the results, making internal measurement variable. That is to say, the

ninhydrin reagent had different quality dependent on when the measurements were measured in the study. Another factor that possibly could have increased the quantitative variability is the coating drying time before performing the ninhydrin assay. It seemed as the glass or silicone samples that had been let dry overnight showed greater results in the ninhydrin assay than those samples measured the same day. This particular factor is wise to look better at in the future, i.e. the drying time impact on the coatings.

Even though the ninhydrin assay results are quantitatively and statistically insignificant, it still has relatively promising qualitative results making them significant. It is quite obvious by simply watching the purple solutions after reacting to the sample's surface, as well as observing the surface of the glass plates or silicone sheets after the ninhydrin reaction, shown in figure 36 below. The chitosan-coated samples D always showed superior promising results.

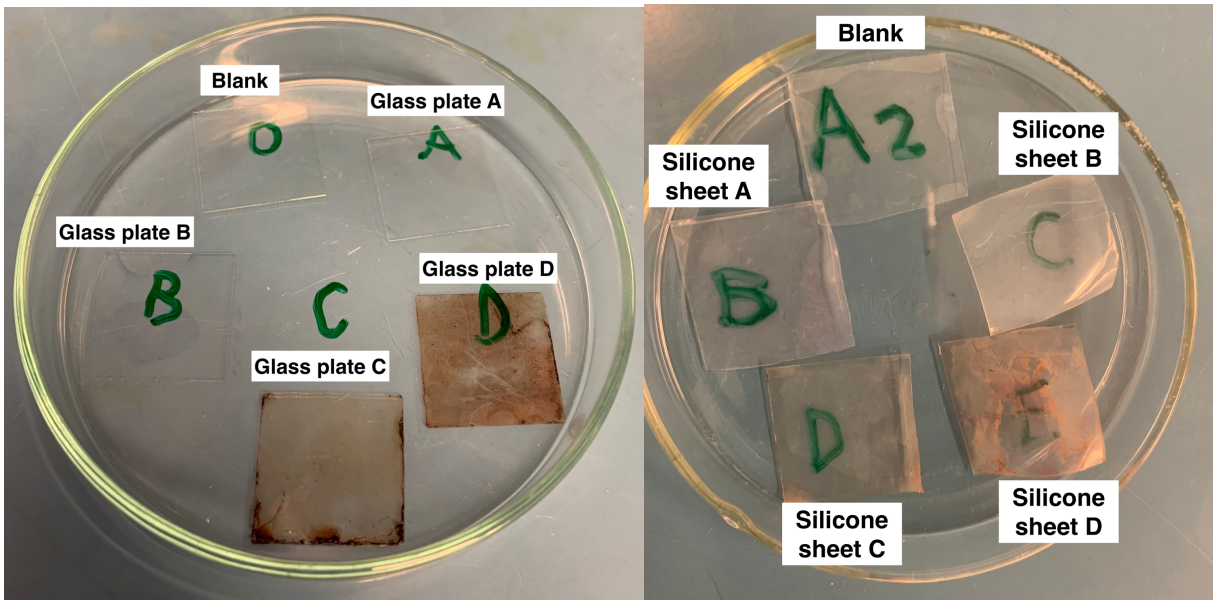


Figure 36. Glass plates (left) and silicone sheets (right) after the ninhydrin reaction. The glass plates exhibit very clear purple color attached on the surface; however, it is not as clear for the silicone sheet.

5.3 FT-IR spectra analysis

The original intention for this study was to use the Nicolet iN10 MX FT-IR microscope to analyze the distribution of chitosan on the surface of coated glass plates and silicone sheets. The procedure did not occur due to unforeseen circumstances, i.e. the FT-IR microscope had some malfunctions which could not be repaired in time. Thereupon, it

was decided to use the procedure with the FT-IR spectrophotometer to identify the actual coatings of the glass plates and silicone sheets.

The ninhydrin assay results indicate that samples silanized with APTMS and crosslinked with glutaraldehyde attaches the chitosan better. The IR spectra indicate the same results. Samples that are silanized with an amino group (APTMS) and crosslinked (glutaraldehyde) to another amino group belonging to chitosan has the best coating results. However, samples silanized with an epoxy group (GPTMS) and react to an amino group belonging to chitosan or glucosamine did not exhibit hoped for results. The IR-spectra tend to be more similar the substrate´s surface, such as the glass plate or the silicone sheet. Some error might be in those results whereas the infrared light is placed on only a small spot of the sample. Thus, it could mean that the surface coating is not as equal for those sample silanized with GPTMS as in for samples silanized with APTMS.

5.3.1 Surface coating analysis for glass plates and silicone sheets

The IR-results for the glass plates indicate that the glutaraldehyde crosslinker in APTMS attaches the chitosan amino groups better than the GPTMS. These results confirm that the surface coating for sample D (chitosan) that responded the best in the ninhydrin assays previously is, in fact, chitosan. However, the surface coating for sample C (glucosamine) using the same method exhibited unknown peaks, and it was impossible to identify if the glucosamine did attach to the glass plate. These results could be presenting a chemical modification, whereas the chemical bonds that were supposed to be created are possibly other than initially thought. Based on the FT-IR and ninhydrin results, it is not possible to know if coating sample C was successful.

The IR-results for the silicone sheets, also indicate that the glutaraldehyde crosslinker in APTMS attaches the chitosan amino groups better than the GPTMS. These results for the silicone sheets confirm two things. Firstly, the surface coating for sample D (chitosan) that responded the best in the ninhydrin assays previously is, in fact, chitosan. Secondly, it seems that the surface coating for sample C (glucosamine) is successful, unlike sample C for the glass plate. Wherein the ninhydrin assay could not give those pieces of information, the FT-IR spectra for sample C looks identical to the authentic sample of glucosamine but showing weaker peaks.

5.4 Antibacterial activity assay

The primary purpose of work was to evaluate the antimicrobial properties of substrates coated with chitosan and the potential to use it as a coating for medical implants to prevent biofilm formation. This test was primarily intended for silicone catheters, where implant-related infections are often caused by the bacterium *P. aeruginosa*. The agar test used in this study has previously worked in the lab for hydrogel membranes and was therefore also tried for the coated glass plates and silicone sheets to evaluate the antibacterial activity.

This test was unsuccessful because the bacteria grown under the area covered by the glass plates or silicone sheets were killed, also in case of controls without coating. The explanation for this may be that this test is not suitable for this particular bacterium. *P. aeruginosa,* which is strictly aerobic bacteria that needs aeration to grow. The bacteria most likely suffocated below the glass plates and silicone sheets. The inhibition zone diameter around the gentamicin measured 20 mm. According to the European Committee on Antimicrobial Susceptibility Testing (2020), the diameter target is the same and within range (17-23 mm). Thus, the antimicrobial assay itself worked as expected. Still, the result is that it was not suitable for analyzing the antimicrobial activity of the samples prepared in the current study.

Thus, another procedure was considered in consultation with Martha Á. Hjálmarsdóttir, a professor in Biomedical Sciences at the University of Iceland. There was a need for changing the process that suited better for the strict aerobic bacteria and its environment. The method involves inoculating a thin layer of bacterial suspension to the surfaces of the silicone samples coated with chitosan. Also, an untreated sample of silicone sheet would be inoculated for comparison. The samples are immersed in the bacterial suspension for a period of time and then dried. Bacterial colony counts should be conducted on all the silicone samples. The chitosan treated samples are analyzed by counting if there have been a reduced number of bacteria compared to the untreated sample.

Unfortunately, it was not possible to test this procedure because of the COVID-19 pandemic, as MS students were not allowed to work in the lab after March 20th but some samples were prepared and we hope to test them in the near future.

6. CONCLUSIONS

The primary aim of this thesis was to focus on designing chitosan coatings for silicone substrates that are often used *in vivo* as catheter-based applications. Catheters are a class of implants that are thought to contribute to chronic antibiotic-resistant biofilm infections. One of the most significant implant-related nosocomial infections. Today, various antimicrobial coated catheters have been clinically tested, but then new problems arise, such as the increasing bacterial resistance and poor biocompatibility. Therefore, the semi-natural polysaccharide chitosan has been suggested and subsequently tested as an antimicrobial coating agent for implants. However, no published studies on chitosan-coated silicone catheters were found. The project is based on promising research for chitosan-coated glass substrates by using a surface activation method called silanization and as well an article describing activation and silanization of silicon wafers (pure Si). The main findings of the study were:

1. Silicone substrate could be activated by treatment with a piranha solution.

2. The chitosan-coated silicone sheets showed promising results when the surface was modified with the amino silane agent (APTMS) and crosslinked with glutaraldehyde. The glycidyl silane agent (GPTMS) method could be used but less chitosan-coated on the substrates than with the APTMS method.

3-4. The ninhydrin assay was useful for the quantitative assessment of the surface activation methods.

5. The FT-IR spectrophotometer could be used to identify the material coated glass and silicone substrates modified with silane agent APTMS and glutaraldehyde crosslinker. However, this method did not work as well with coated substrates modified with the silane agent GPTMS exhibited with only weak or absent peaks for the coating material.

6. The contact-killing activity of the coated substrates against the bacterium *P. aeruginosa*, assessed by agar test was inconclusive. An alternative antimicrobial assay method was proposed but could not be performed due to COVID-19.

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I would like to acknowledge and express my sincerest gratitude to the following people who were involved in this project and gave me support throughout the study.

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Last but not least, I would like to express my gratitude to my mom and dad, as well as my brothers for their support and encouragement during the study.

Thank you all very much,

Una Guðmundsdóttir

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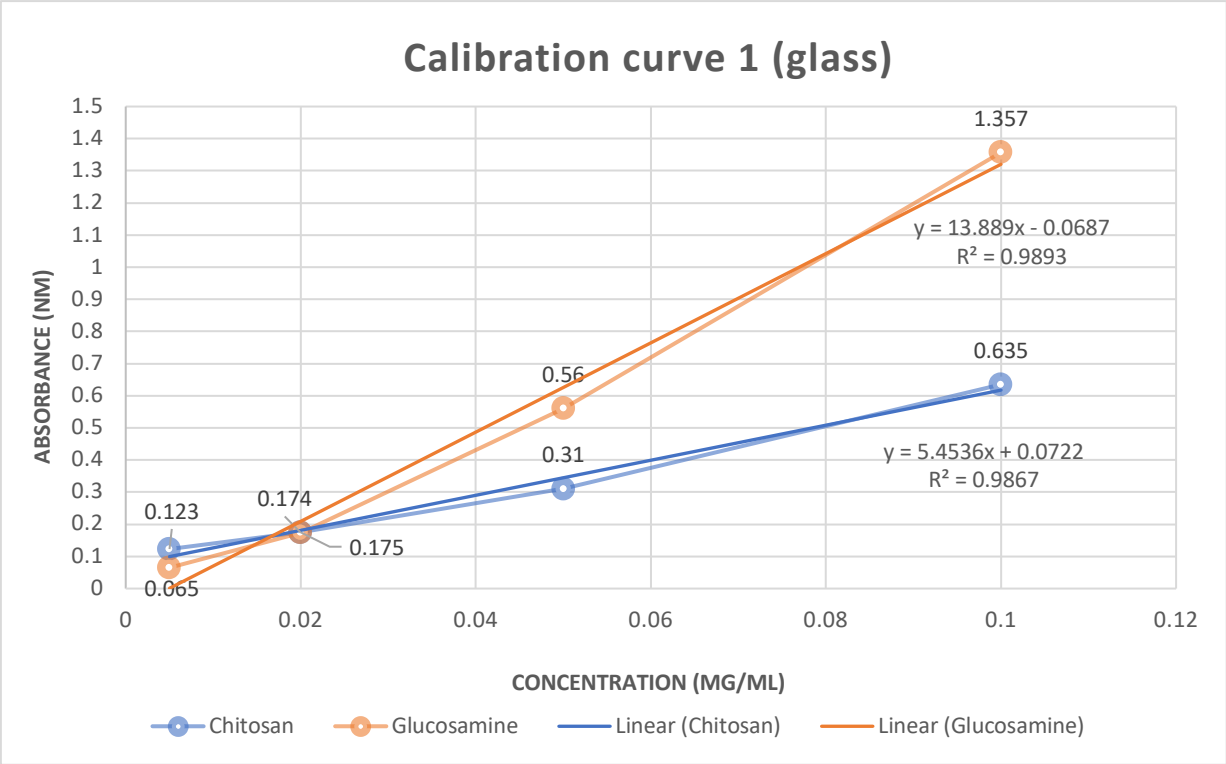
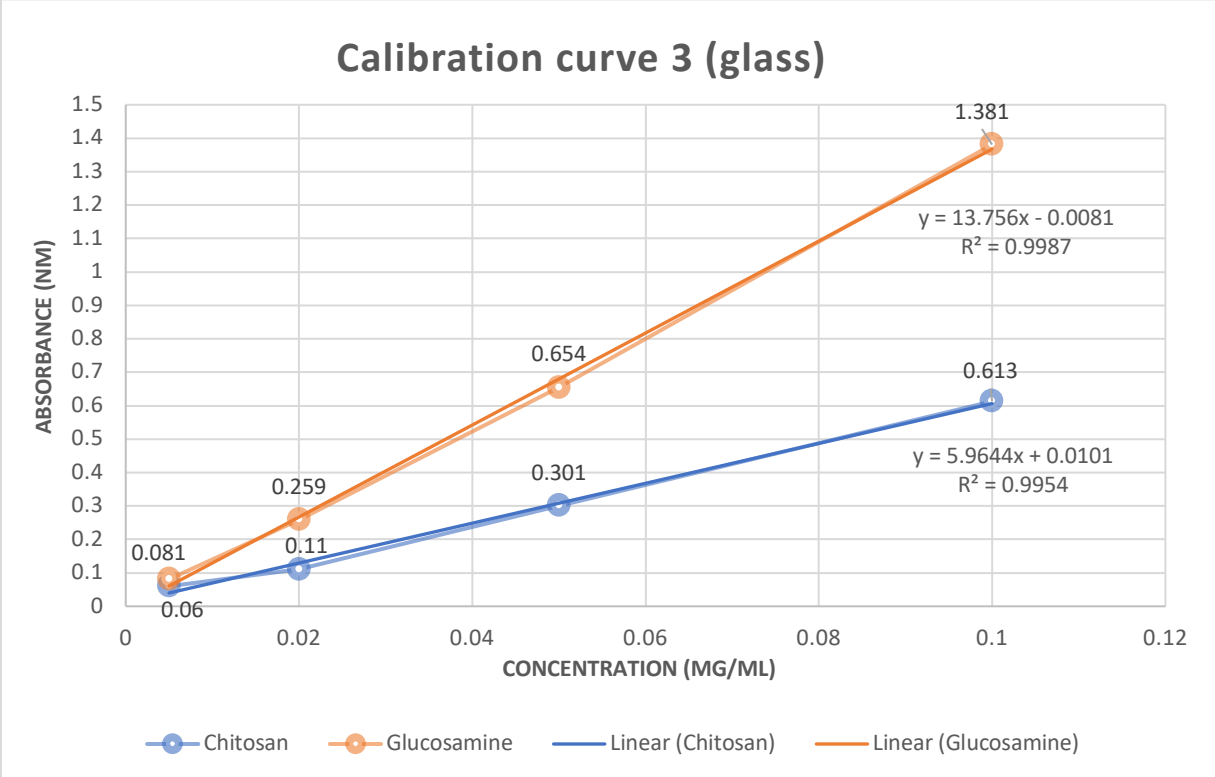
9. APPENDIX

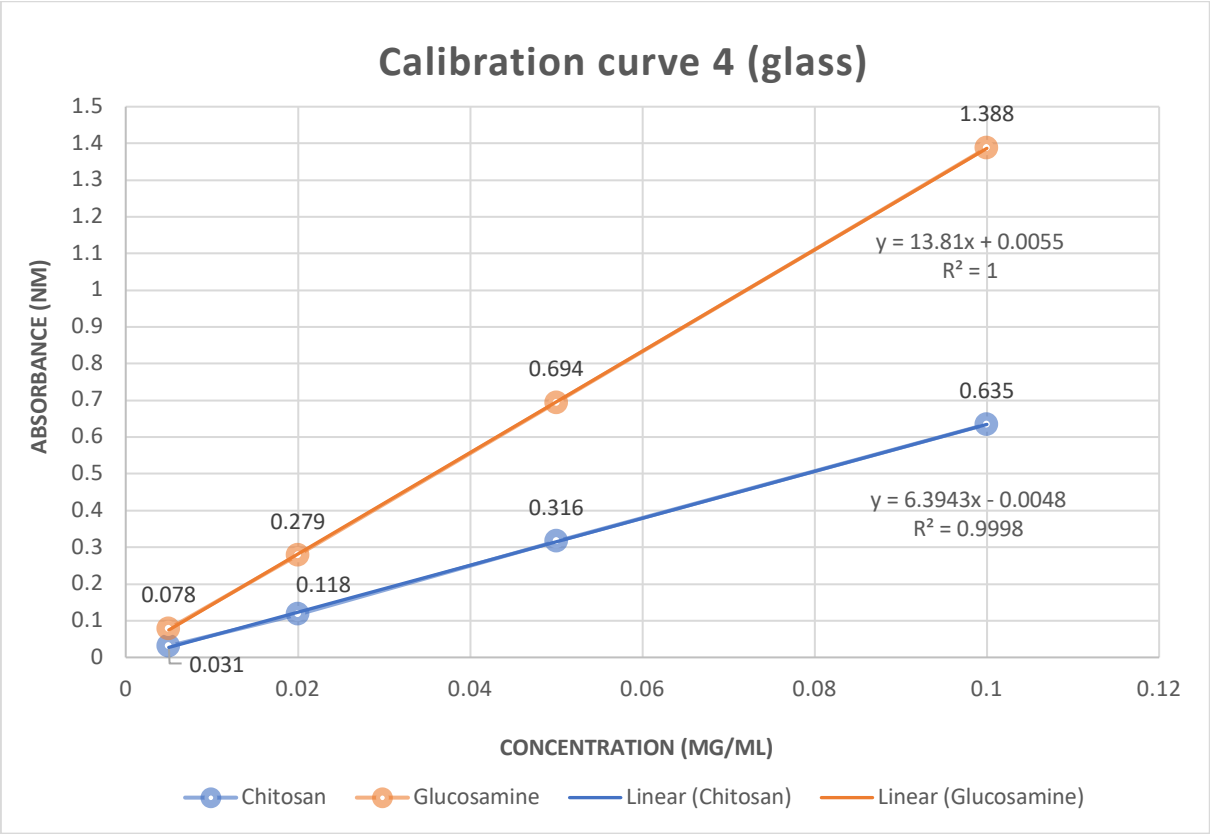
Glass plates



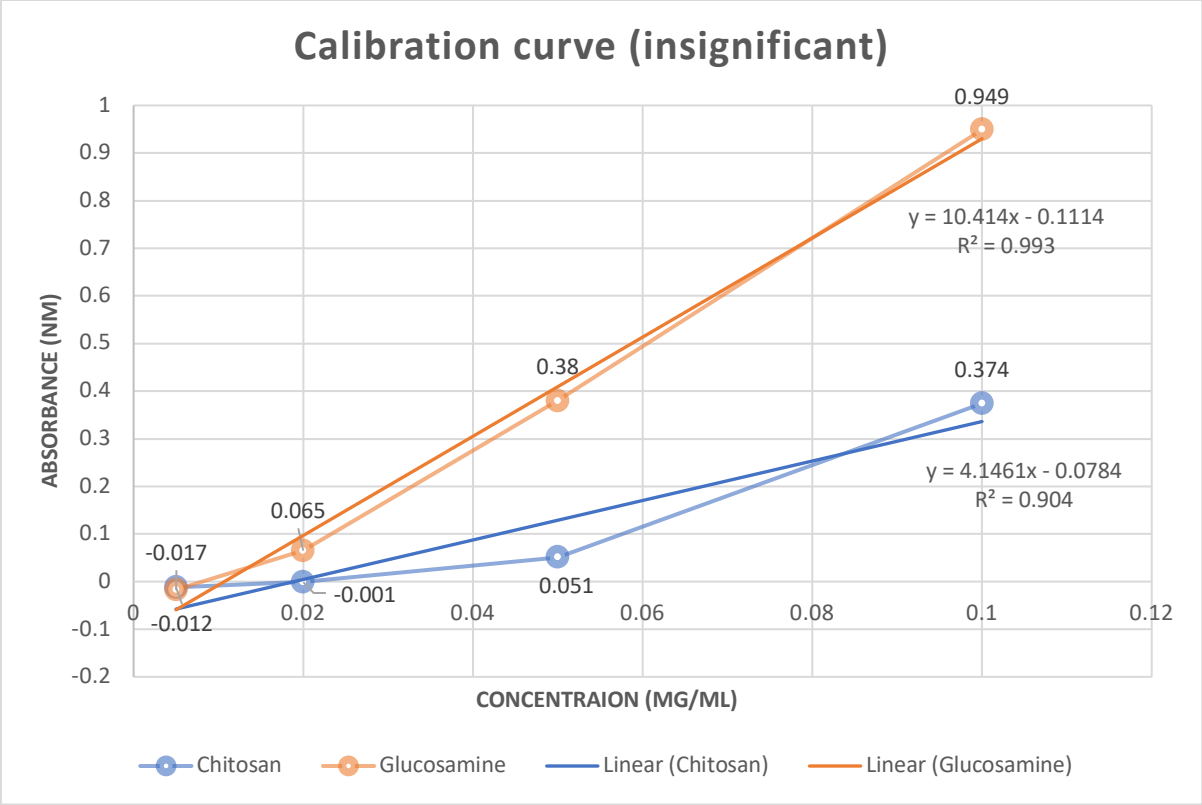
Calibration curve 1.



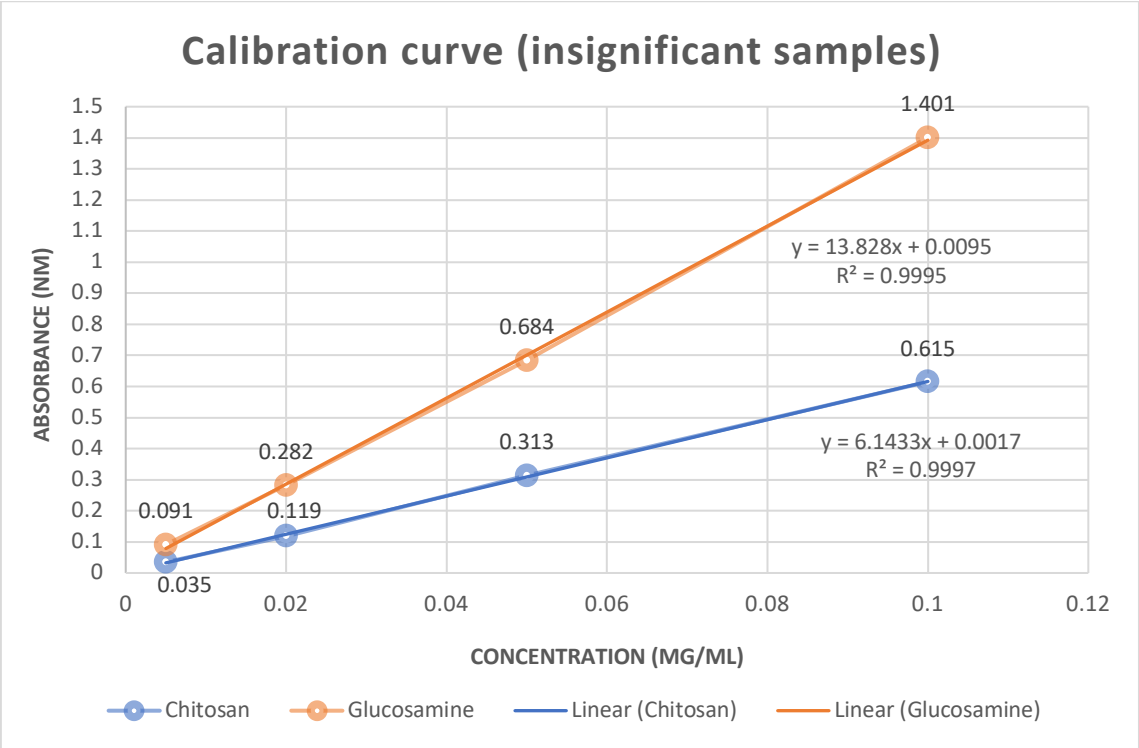
Calibration curve 3.



Calibration curve 4.



Insignificant calibration curve prepared for a ninhydrin assay on glass plates. The ninhydrin reagent responded poorly which led to a poor calibration curve.



Calibration curve prepared for a ninhydrin assay on glass plates. The calibration curve responded well, but the absorbance measurements for the glass plates seemed to be inaccurate. The different drying time for the samples was most likely the explanation.

Table A: All measurements and calculations from the ninhydrin assays for the glass plates.

Measurements	Absorbance (nm)	Concentration (mg/ml)	Mass (mg)	Area concentration (mg/cm^2)
Number 1				
A	0.059	0.0092	0.018	0.00174
B	0.196	0.022	0.044	0.00418
C	0.47	0.039	0.078	0.00734
D	0.711*	1.19	2.38	0.225
Number 3				
A	0.004	0.00088	0.0018	0.000166
B	0.037*	0.045	0.09	0.00851

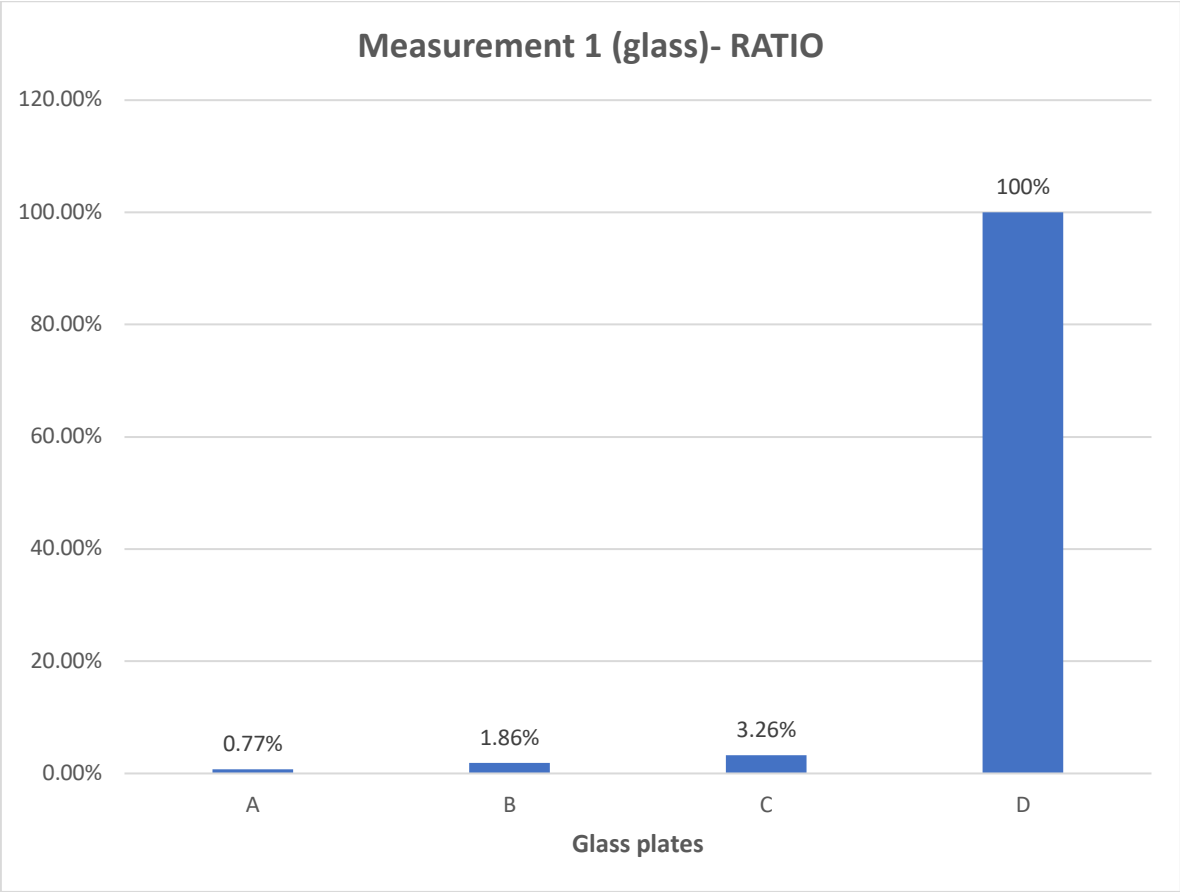
C	-0.016	0	0	0
D	0.022*	0.02	0.04	0.00378
Number 4				
A	-0.009	0	0	0
B	0.016	0.0033	0.0066	0.000624
C	0.111	0.0076	0.0152	0.00144
D	0.04*	0.07	0.14	0.0132
Number E (insignificant)				
A	0.044	0.0025	0.005	0.00047
B	0.165	0.0266	0.053	0.0050
C	0.720	0.0514	0.103	0.0097
D**	0.162	0.0261	0.052	0.0082

** Solutions were measured as inconclusive (>1.5 nm) and were diluted 10 times for absorbance that fitted within the calibration curve. The dilutions were considered in the calculations.*

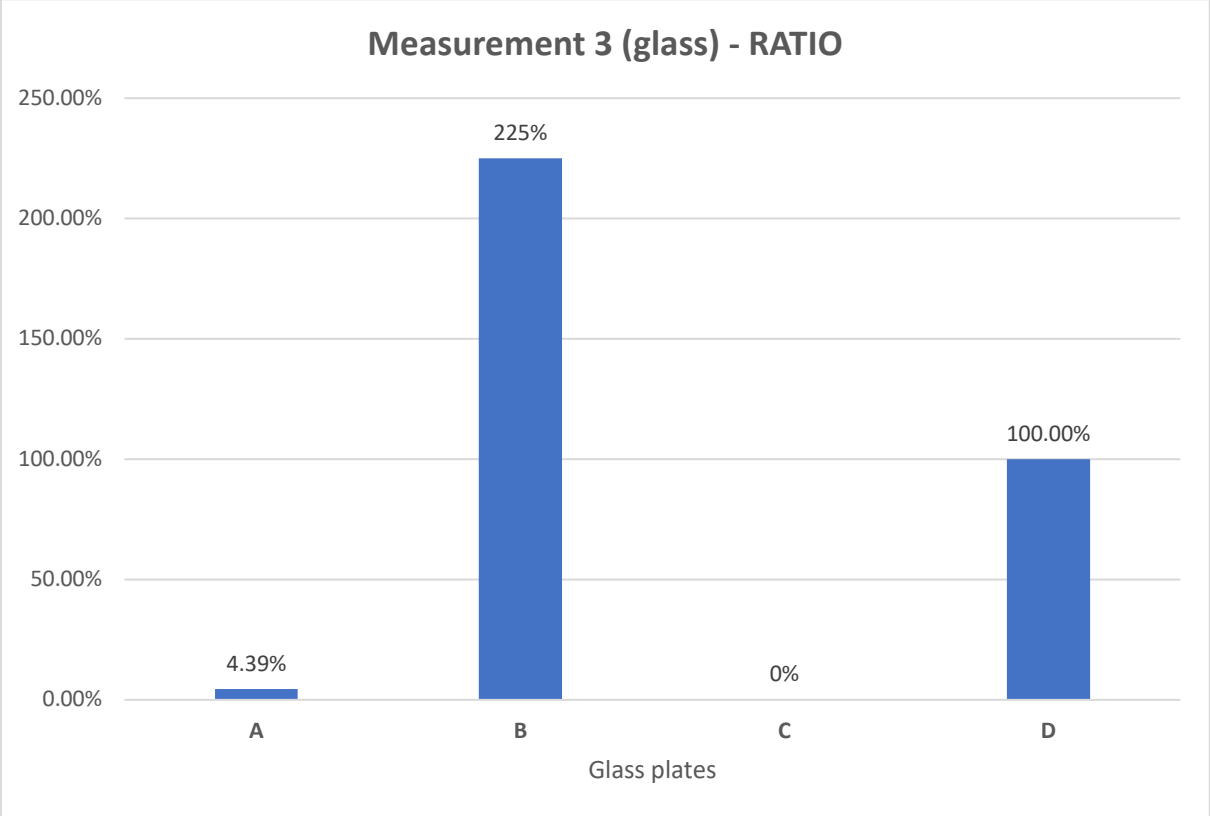
*** Shorter drying time because the coating step had to be repeated. Chitosan significantly adheres to the surface, which led the glass to break when handling the sample.*

Table B: The calculated mean AC and SD for each glass plate method A-D.

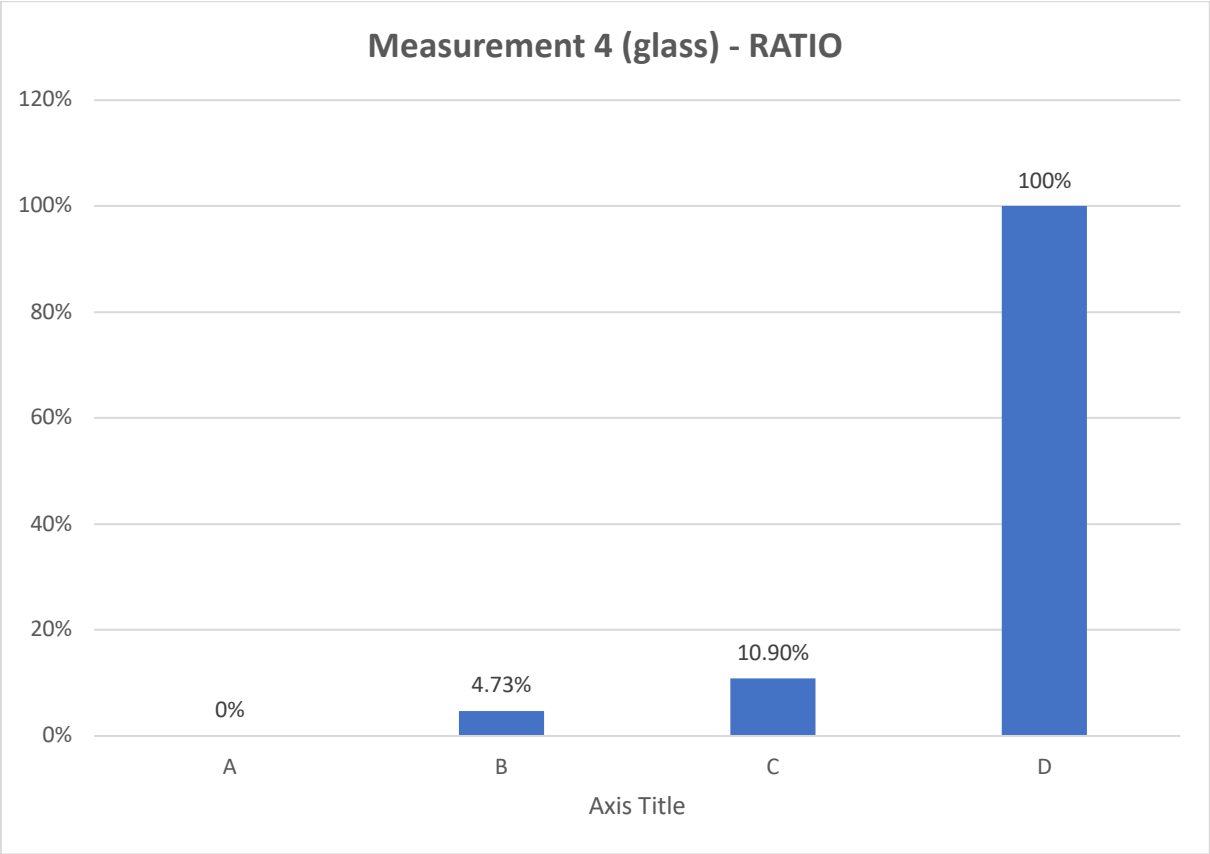
Measurements	Glass A	Glass B	Glass C	Glass D
1	0.00174	0.00418	0.00734	0.225
2	0	0.140	0.000190	0.17
3	0.000166	0.00851	0	0.00378
4	0	0.000624	0.00144	0.0132
Mean AC	**0.000477**	**0.0383**	**0.00224**	**0.103**
Standard Deviation	**0.000846**	**0.0679**	**0.00346**	**0.111**



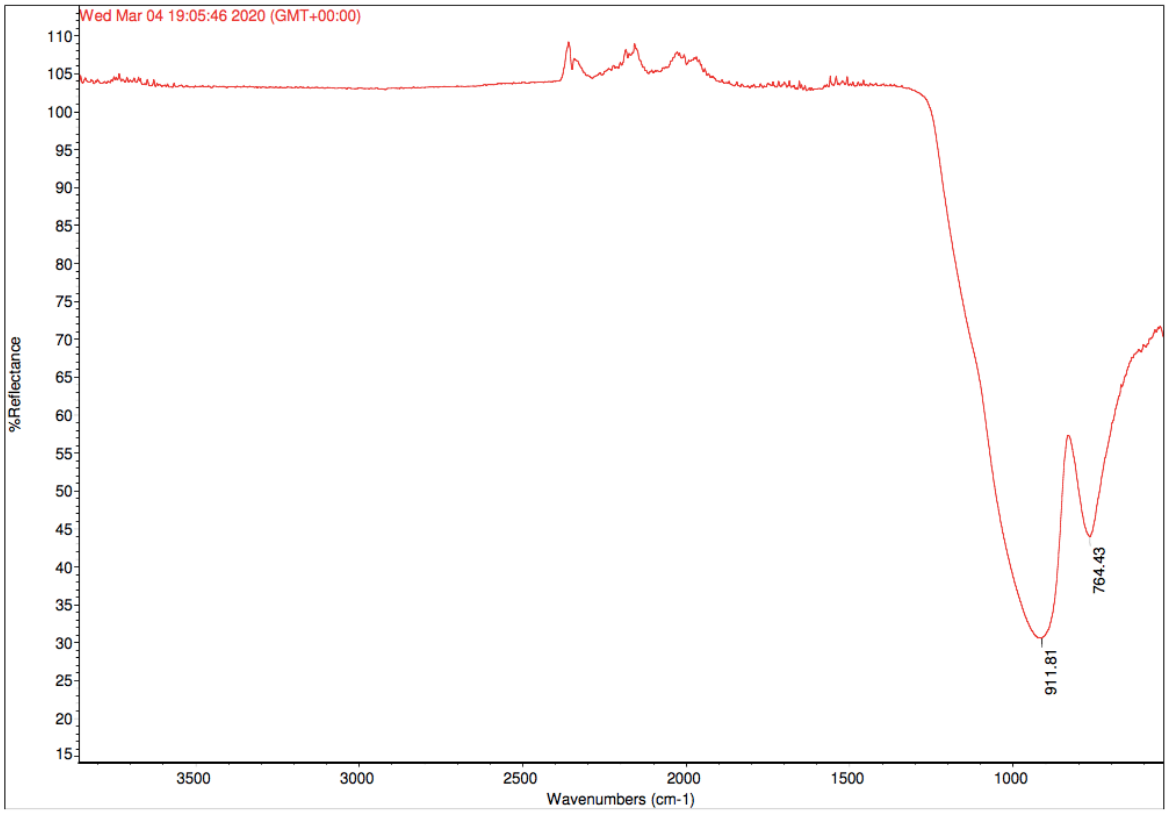
AC RATIO for ninhydrin assay 1 (glass). Sample D = 100% (SD=0).



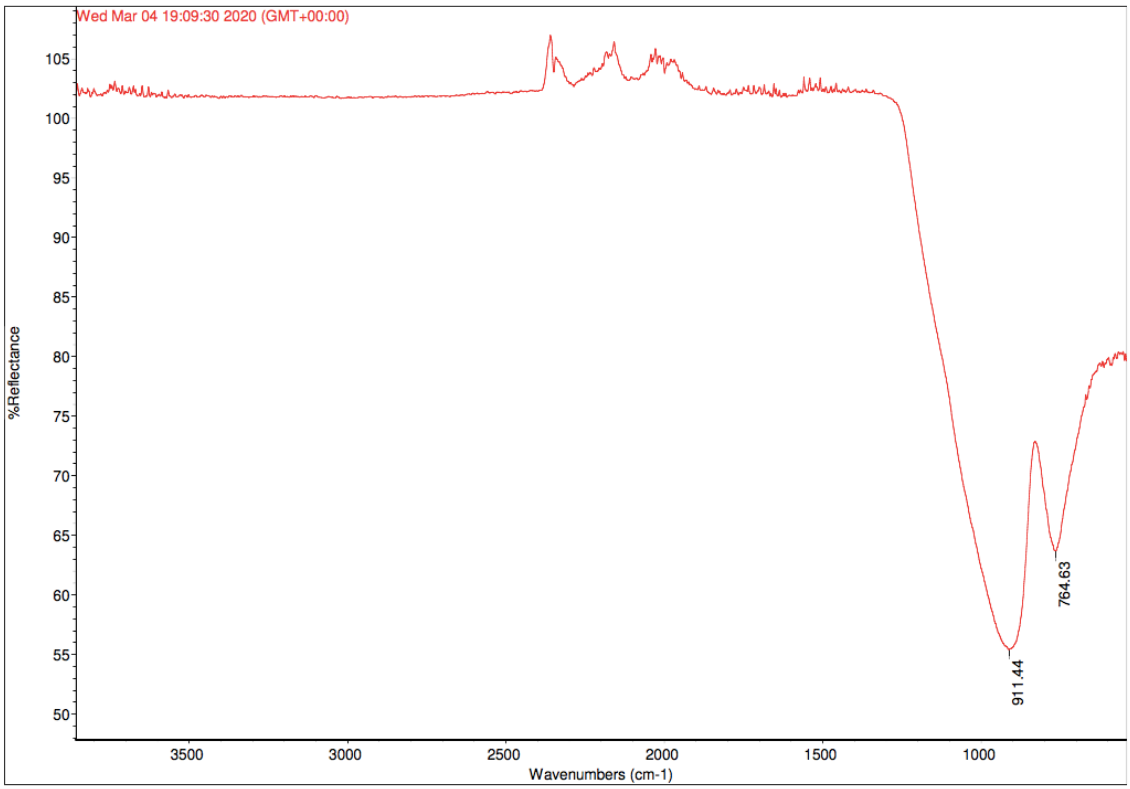
AC RATIO for ninhydrin assay 3 (glass). Sample D = 100% (SD=0).



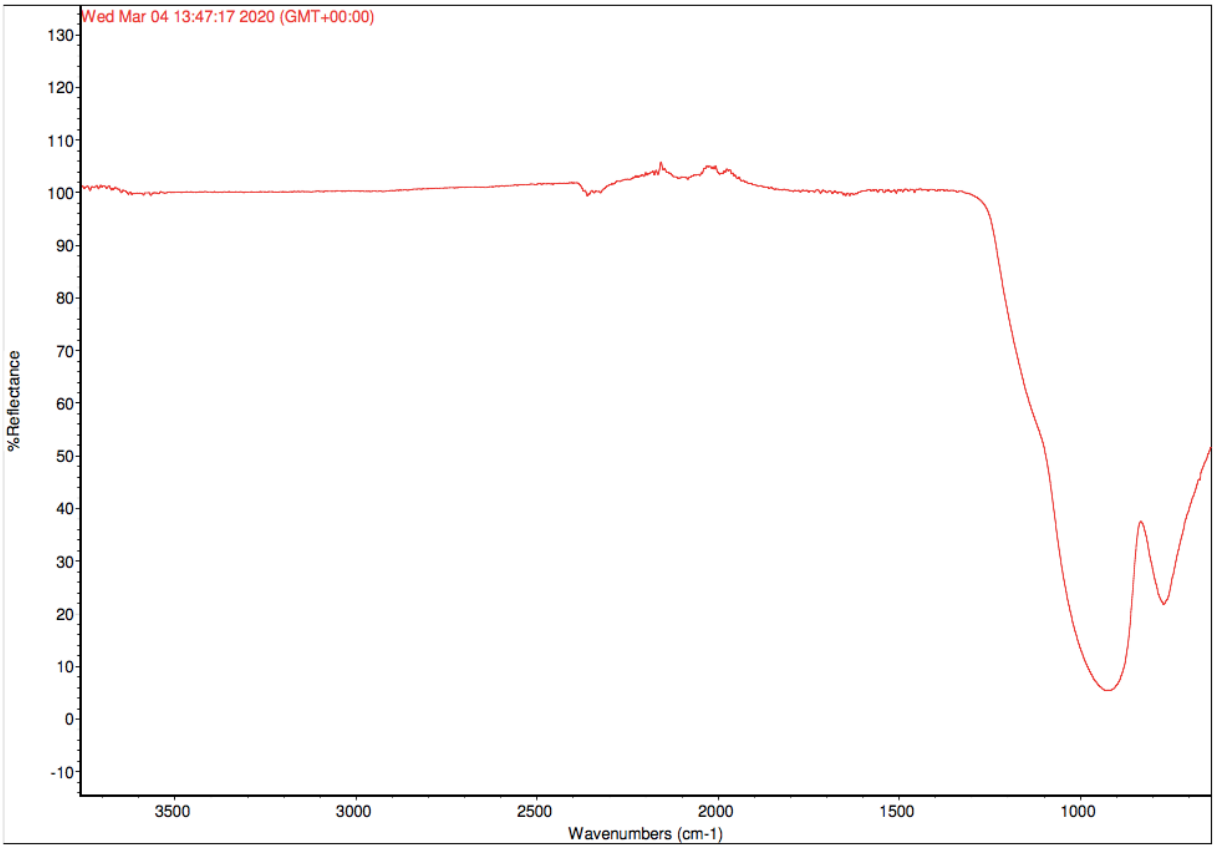
AC RATIO for ninhydrin assay 4 (glass). Sample D = 100% (SD=0).



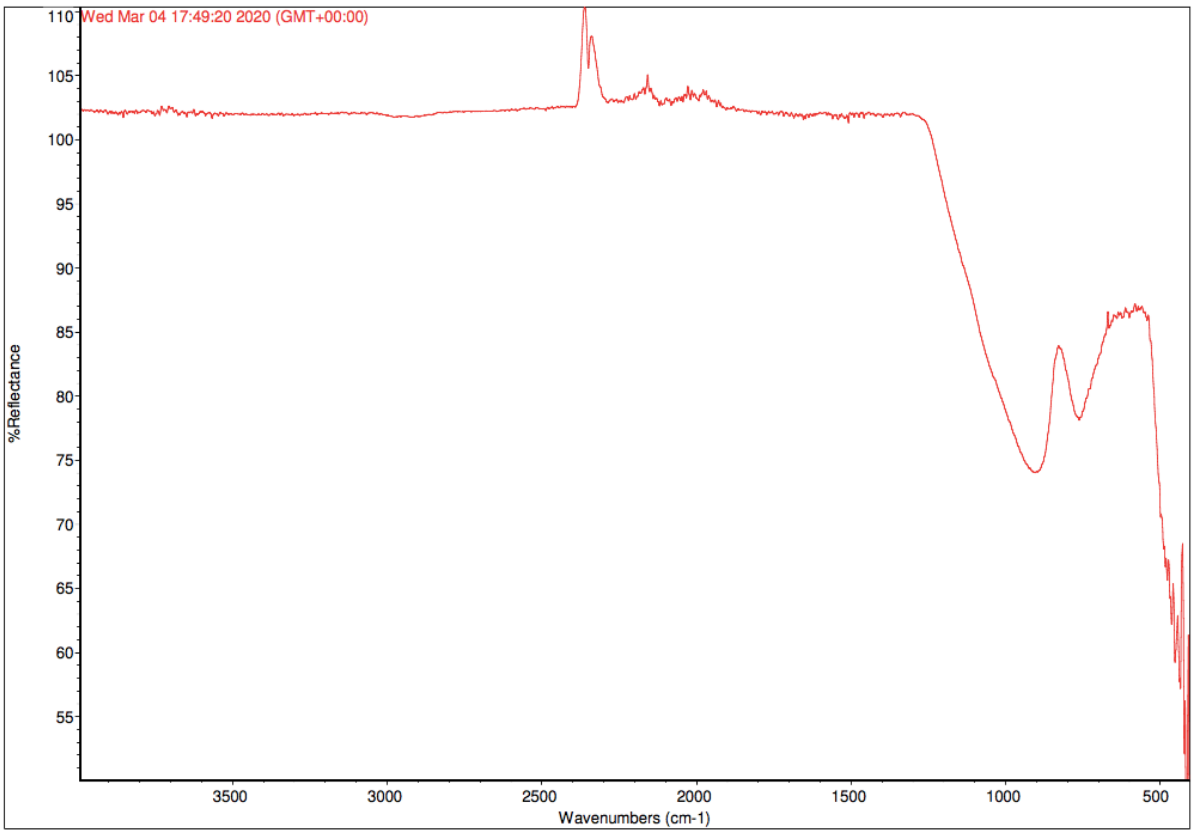
IR-spectra of APTMS treated glass plate.



IR-spectra of GPTMS treated glass plate.

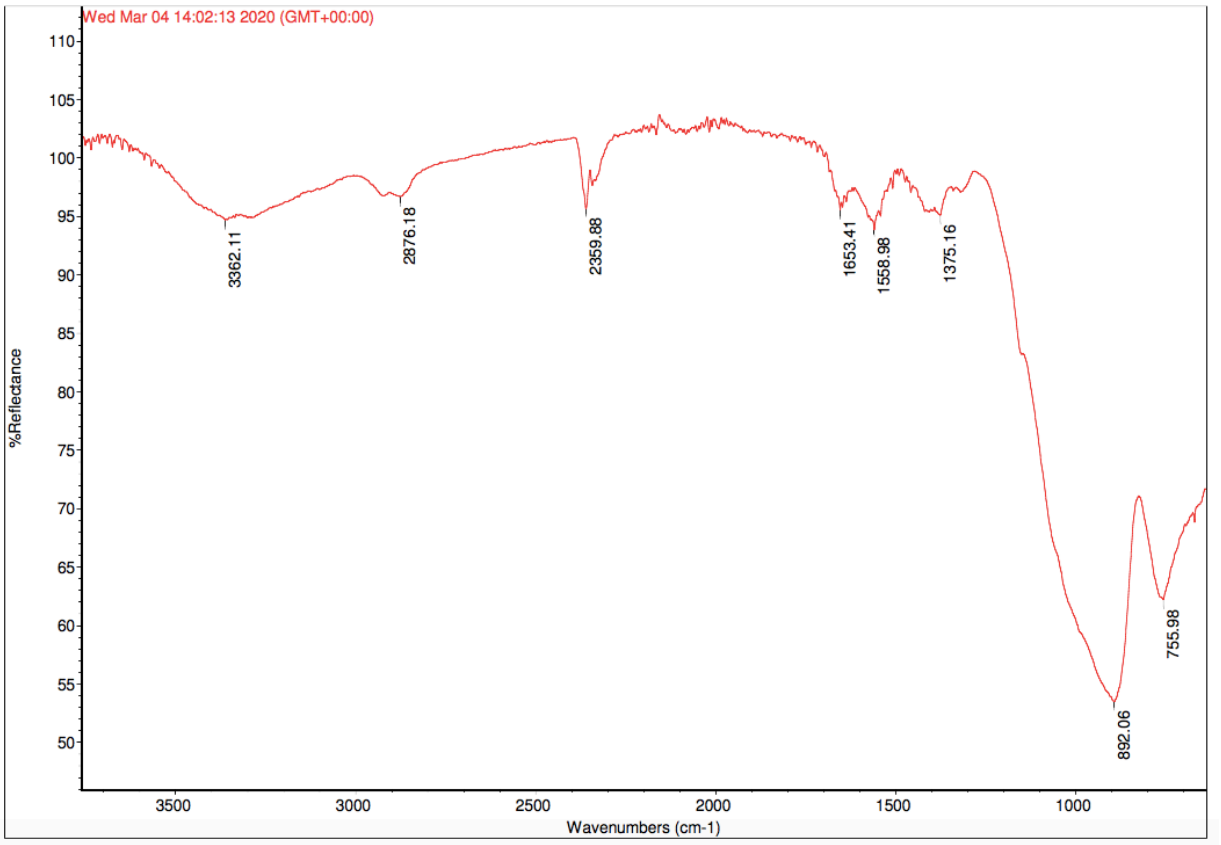


IR-spectra of glass plate A (GPTMS + glucosamine).



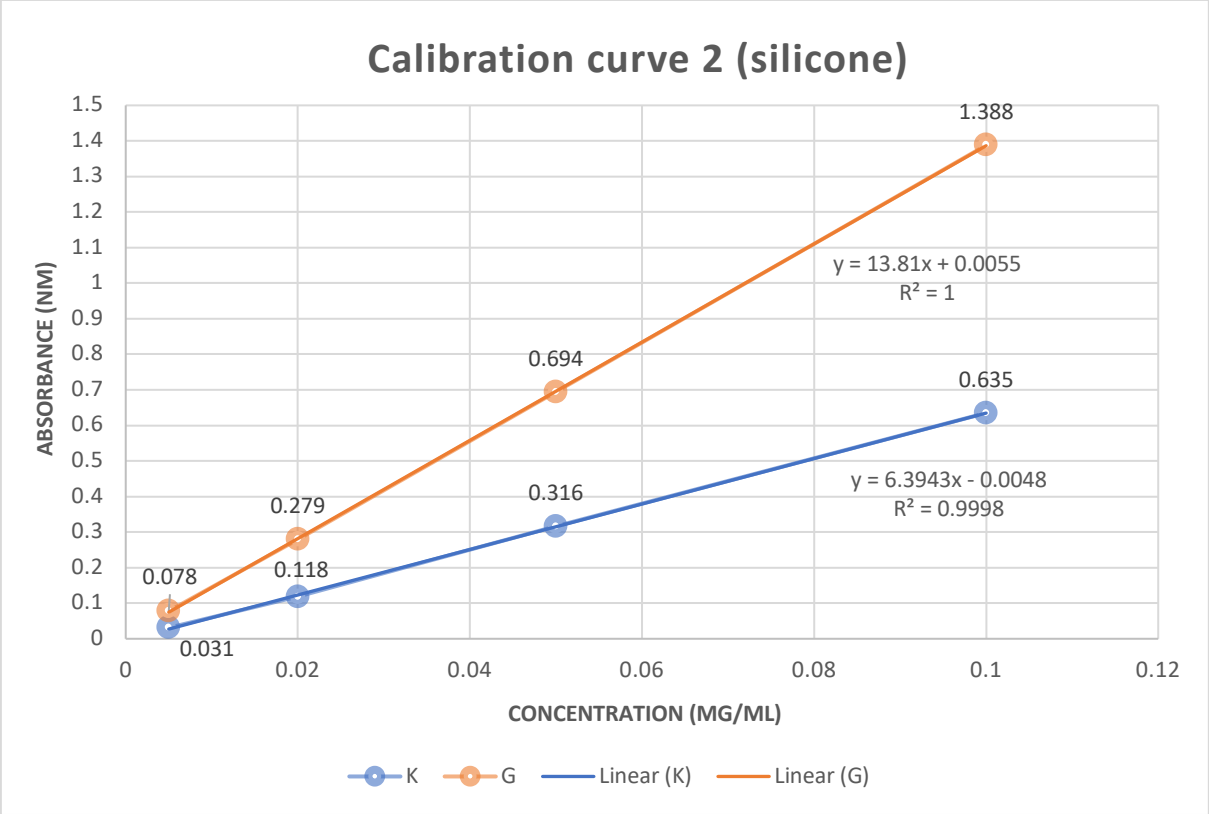
IR-spectra of glass plate B (GPTMS + chitosan).

IR-spectra of glass plate C (APTMS + glutaraldehyde + glucosamine).

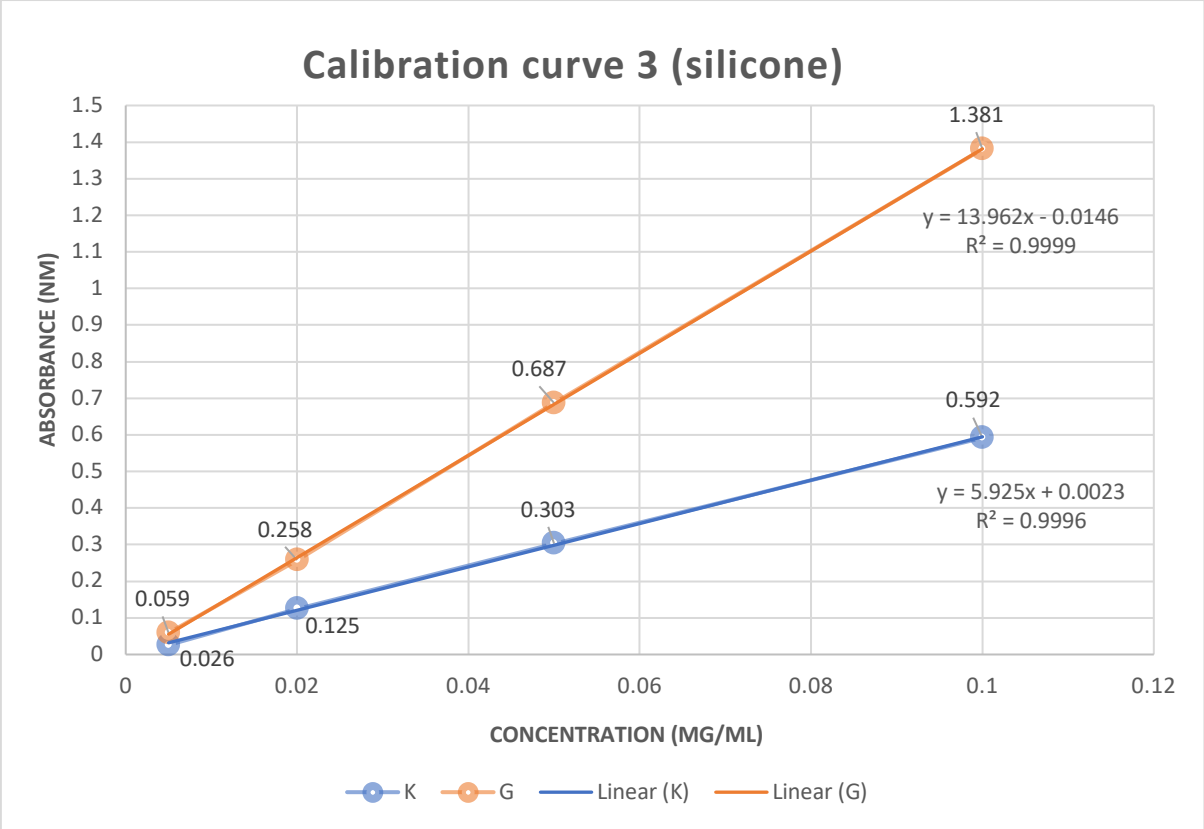


IR-spectra of glass plate D (APTMS + glutaraldehyde + chitosan).

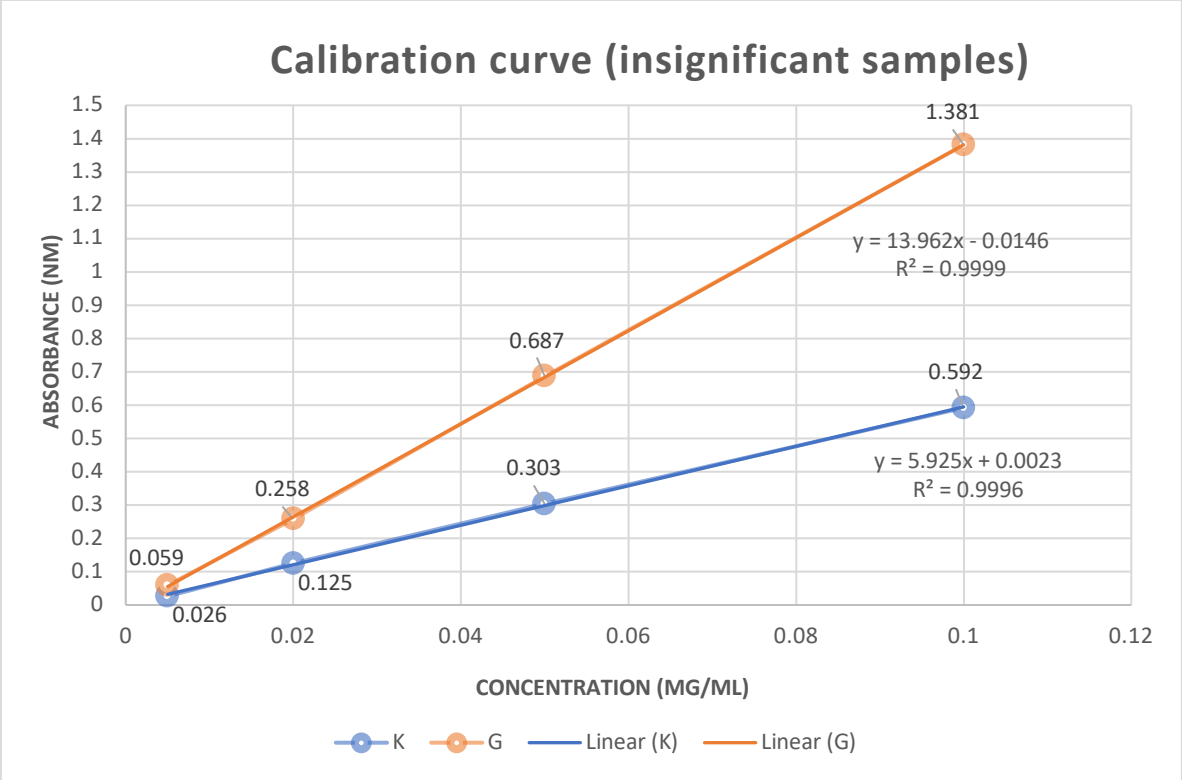
Silicone sheets



Calibration curve 2.



Calibration curve 3.



Calibration curve prepared for a ninhydrin assay on silicone sheets. The calibration curve responded well, but the absorbance measurements for silicone sheets A-D were insignificant before and after the dilution for unknown reasons.

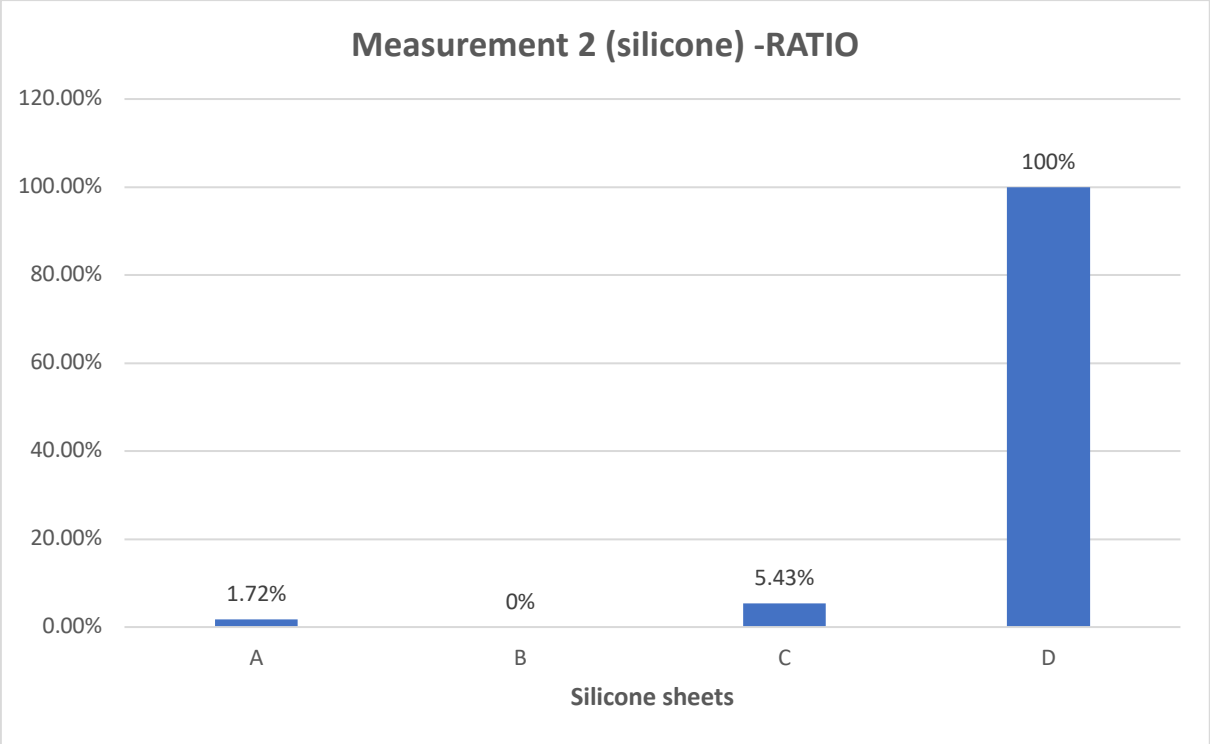
Table C: All measurements and calculations from the ninhydrin assays for the silicone sheets.

Measurements	Absorbance (nm)	Concentration (mg/ml)	Mass (mg)	Area concentration (mg/cm^2)
Number 2				
A	0.128	0.009	0.018	0.0026
B	0	0	0	0
C	0.393	0.028	0.056	0.0053
D	0.325 x 10*	0.520	1.03	0.0975
Number 3				
A	-0.022	0	0	0
B	-0.024	0	0	0
C	0.150	0.012	0.024	0.0022
D	0.495	0.083	0.166	0.0157
Number ? (insignificant)				
A	0.080	0.007	0.014	0.0013
B	0.628	1.060	2.120	0.2003
C	-0.012 x 10*	0	0	0
D	0.177 x 10*	0.030	0.059	0.0056

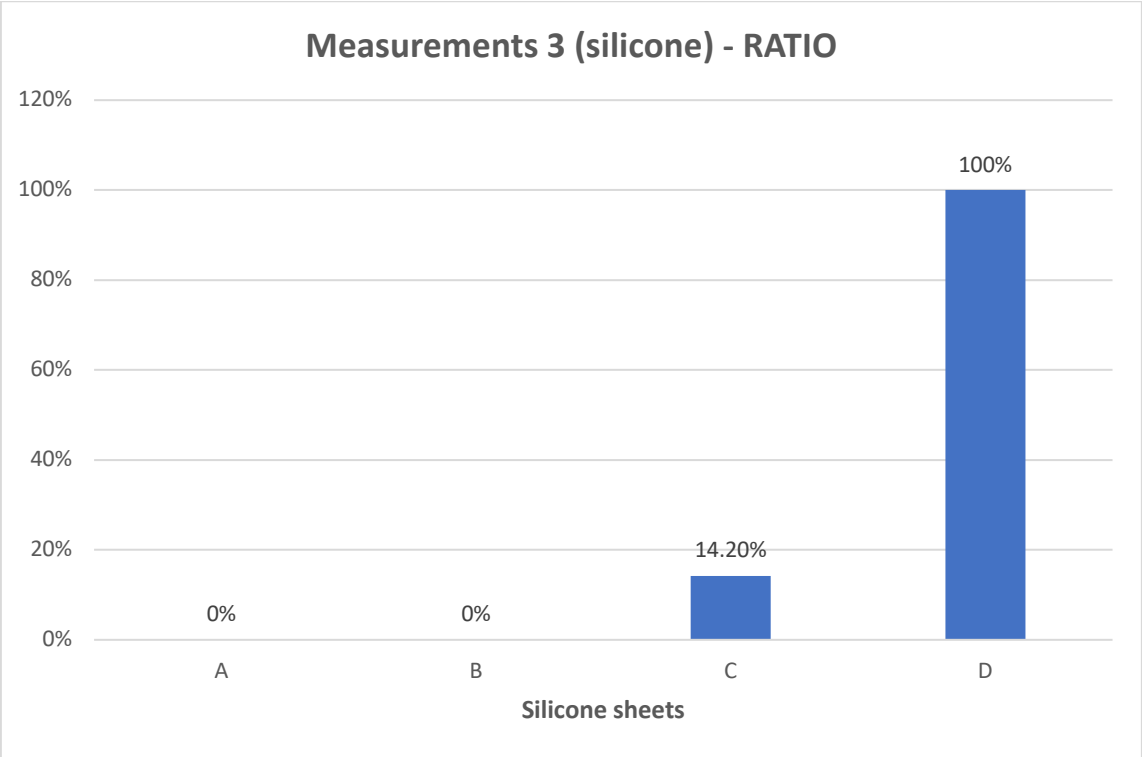
** Solutions were measured as inconclusive (>1.5 nm) and were diluted 10 times for absorbance that fitted within the calibration curve. The dilutions were considered in the calculations.*

Table D: The calculated mean AC and SD for each silicone sheet method A-D.

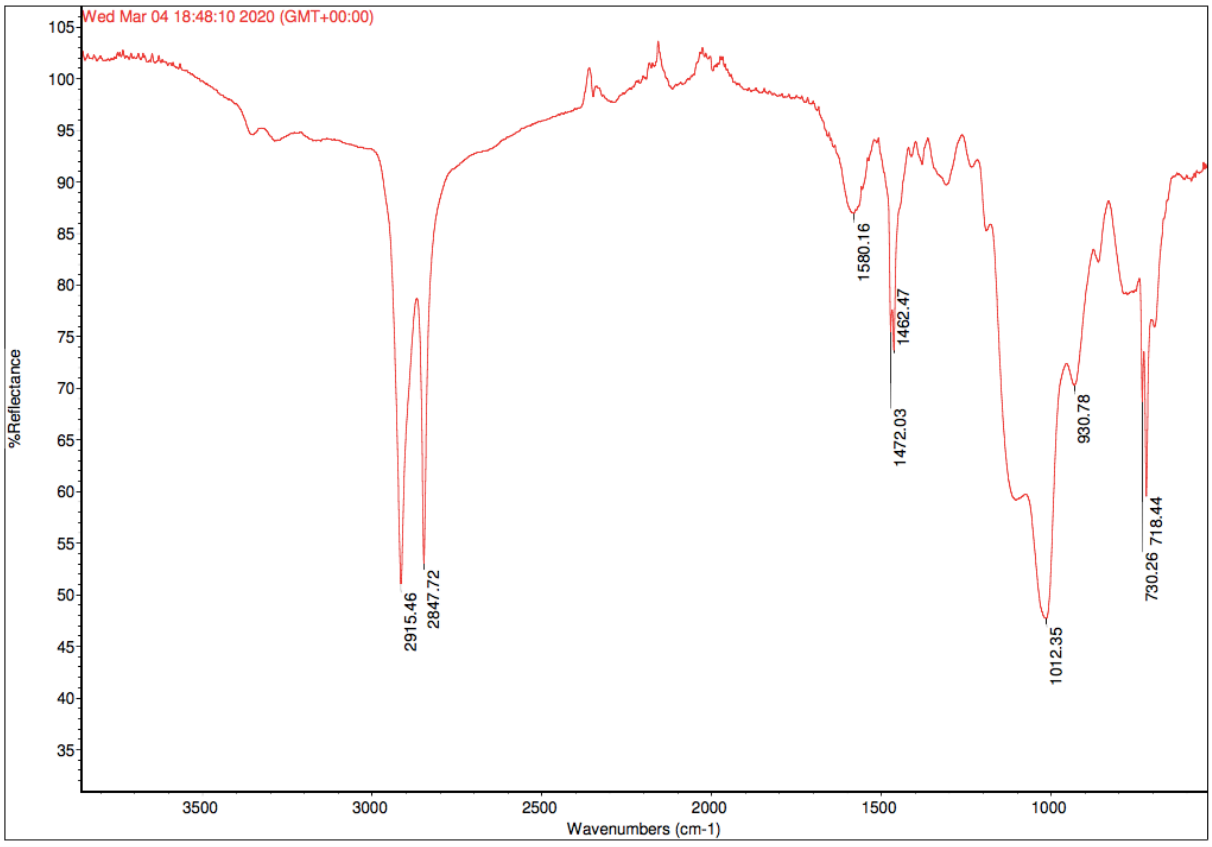
Measurements	A	B	C	D
1	0.00251	0.112	0.00446	0.135
2	0.00168	0	0.00529	0.0975
3	0	0	0.00223	0.0157
Average	**0.00136**	**0.0371**	**0.00399**	**0.0827**
Standard Deviation	**0.00128**	**0.0647**	**0.00158**	**0.0610**



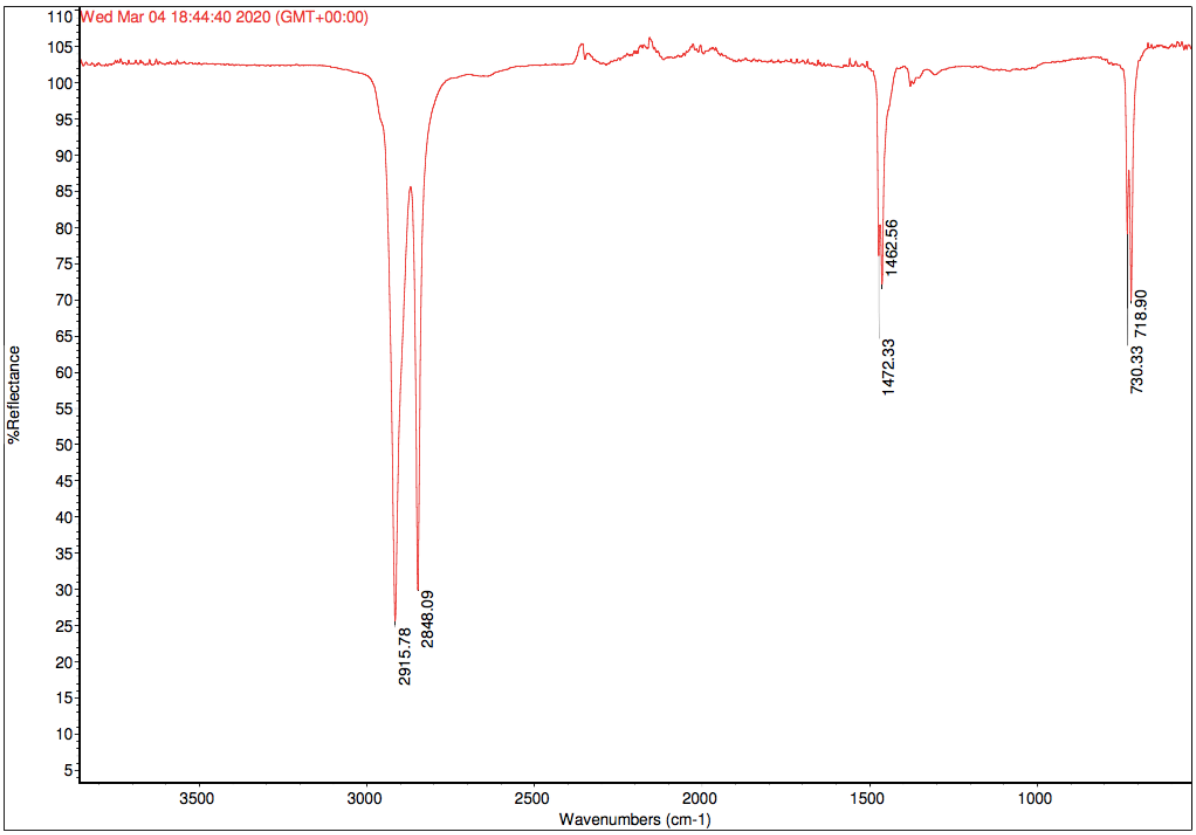
AC RATIO for ninhydrin assay 2 (silicone). Sample D = 100% (SD=0).



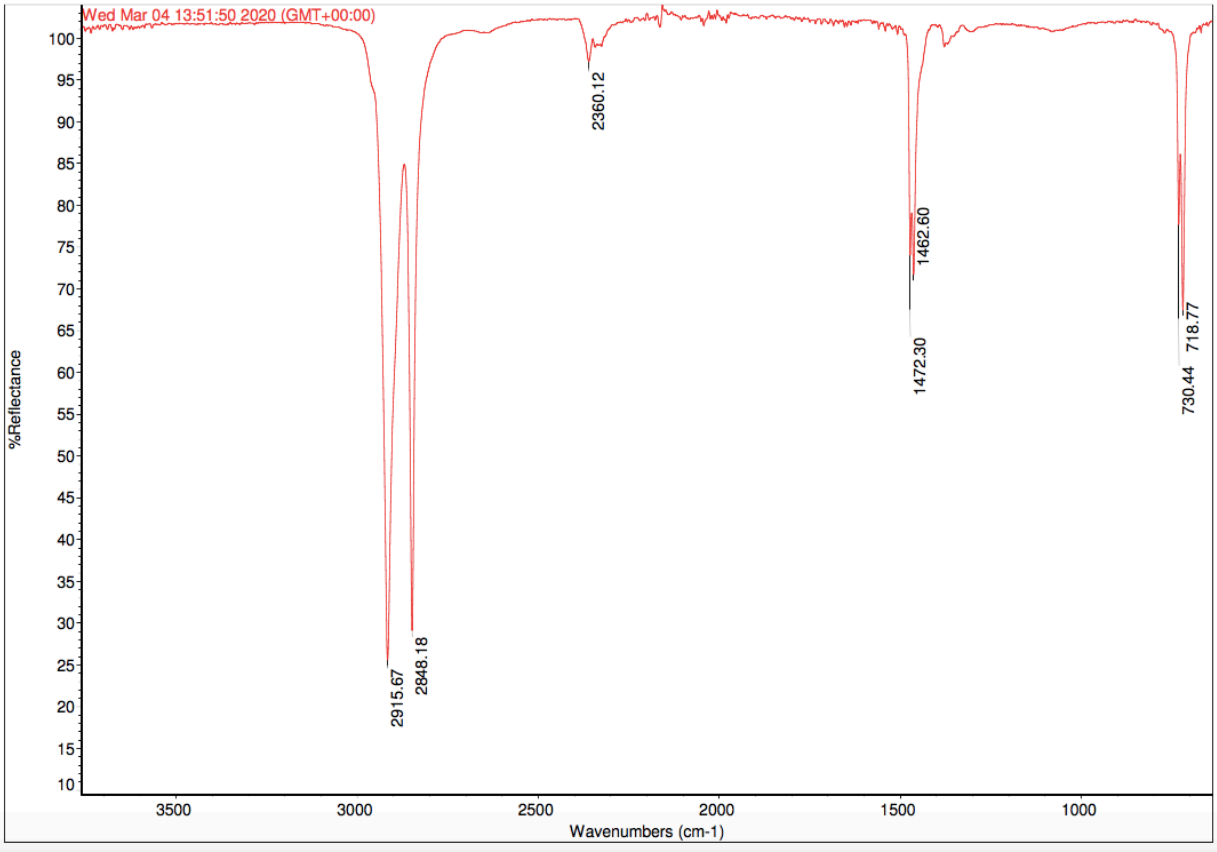
AC RATIO for ninhydrin assay 3 (silicone). Sample D = 100% (SD=0).



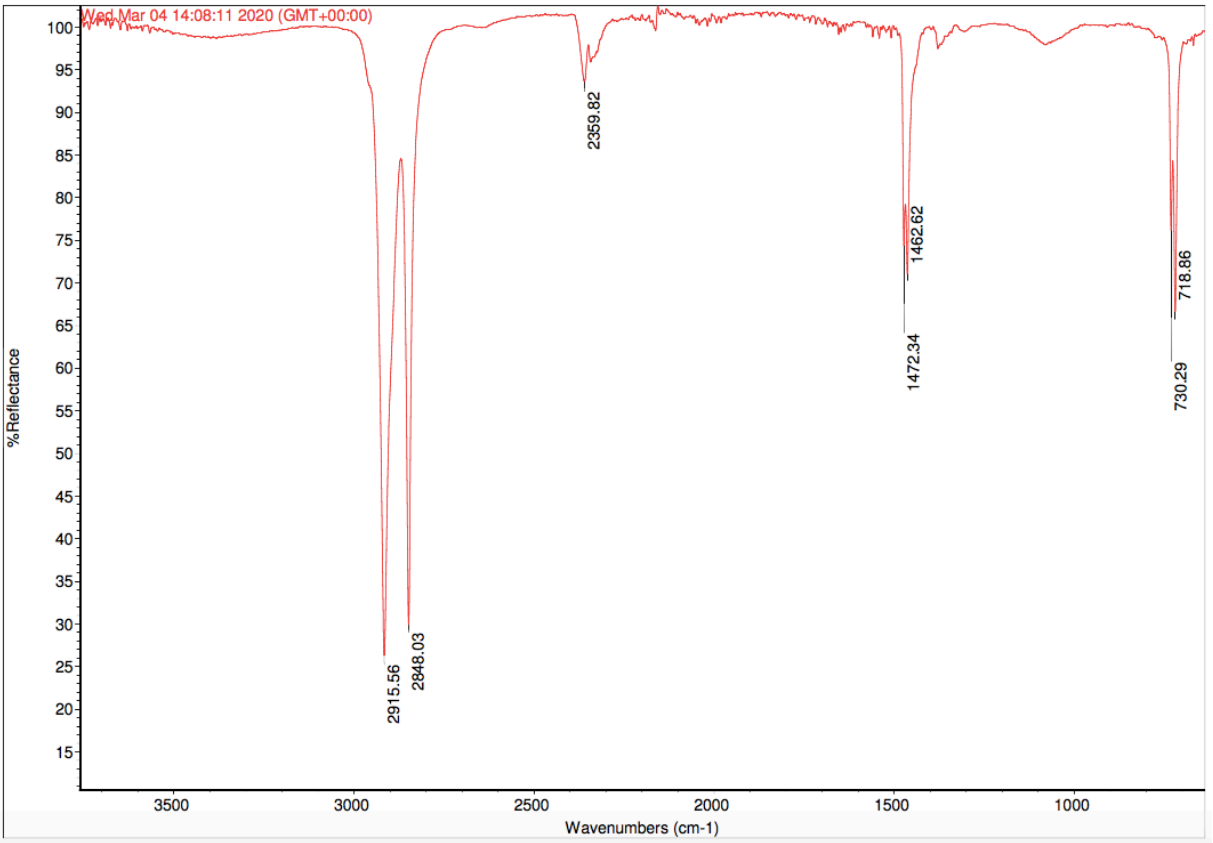
IR-spectra of APTMS treated silicone sheet.



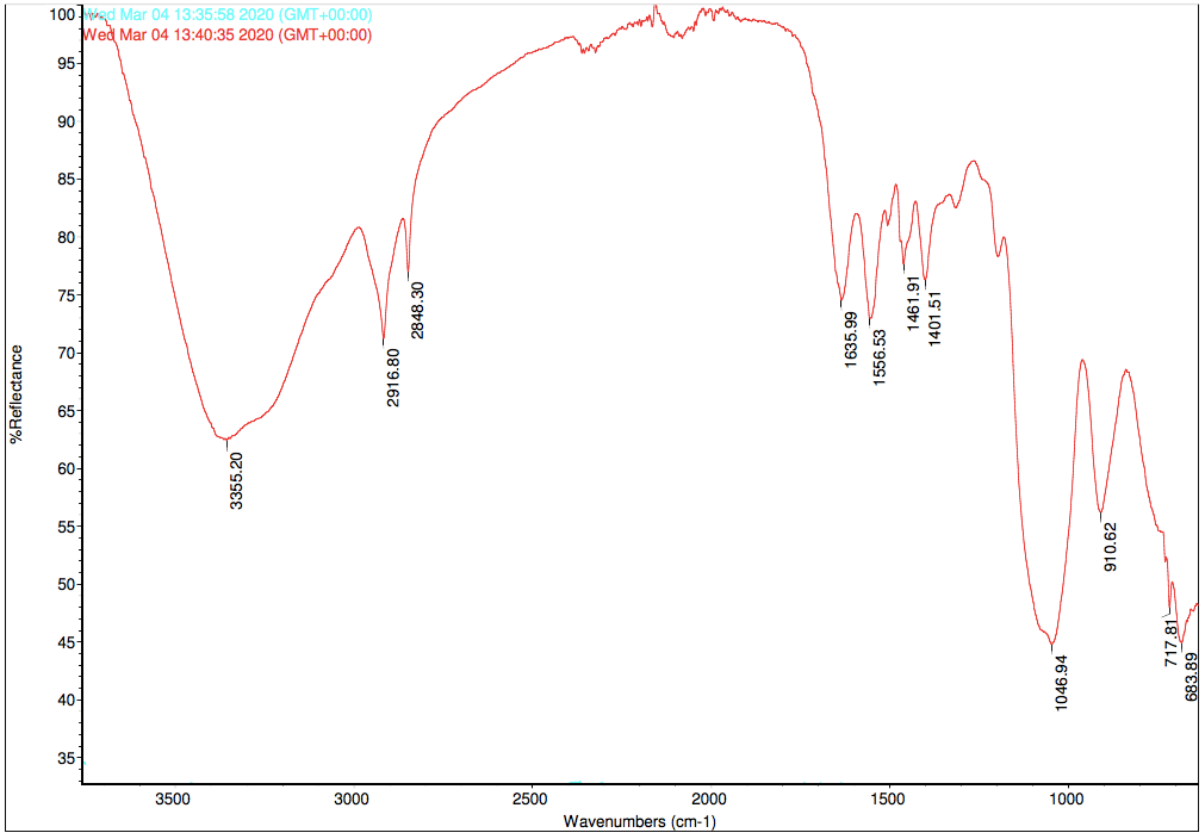
IR-spectra of GPTMS treated silicone sheet.



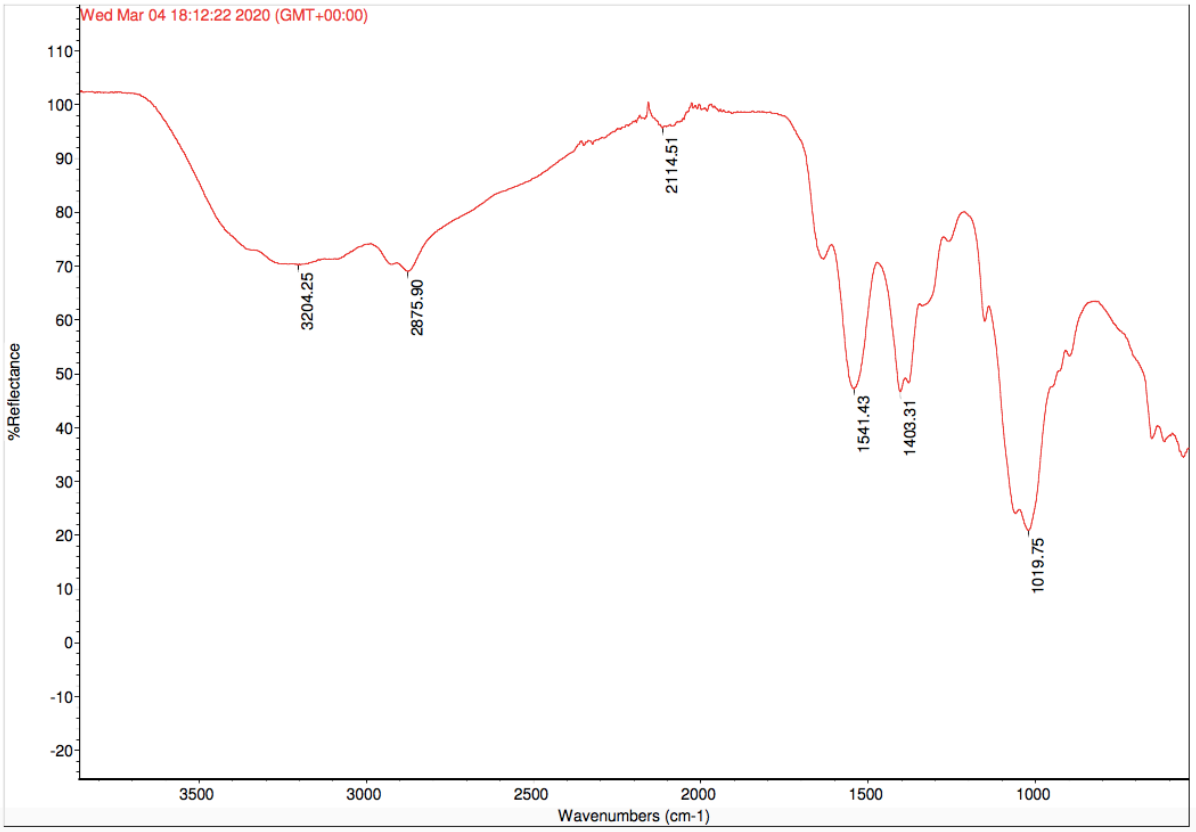
IR-spectra of silicone sheet A (GPTMS + glucosamine).



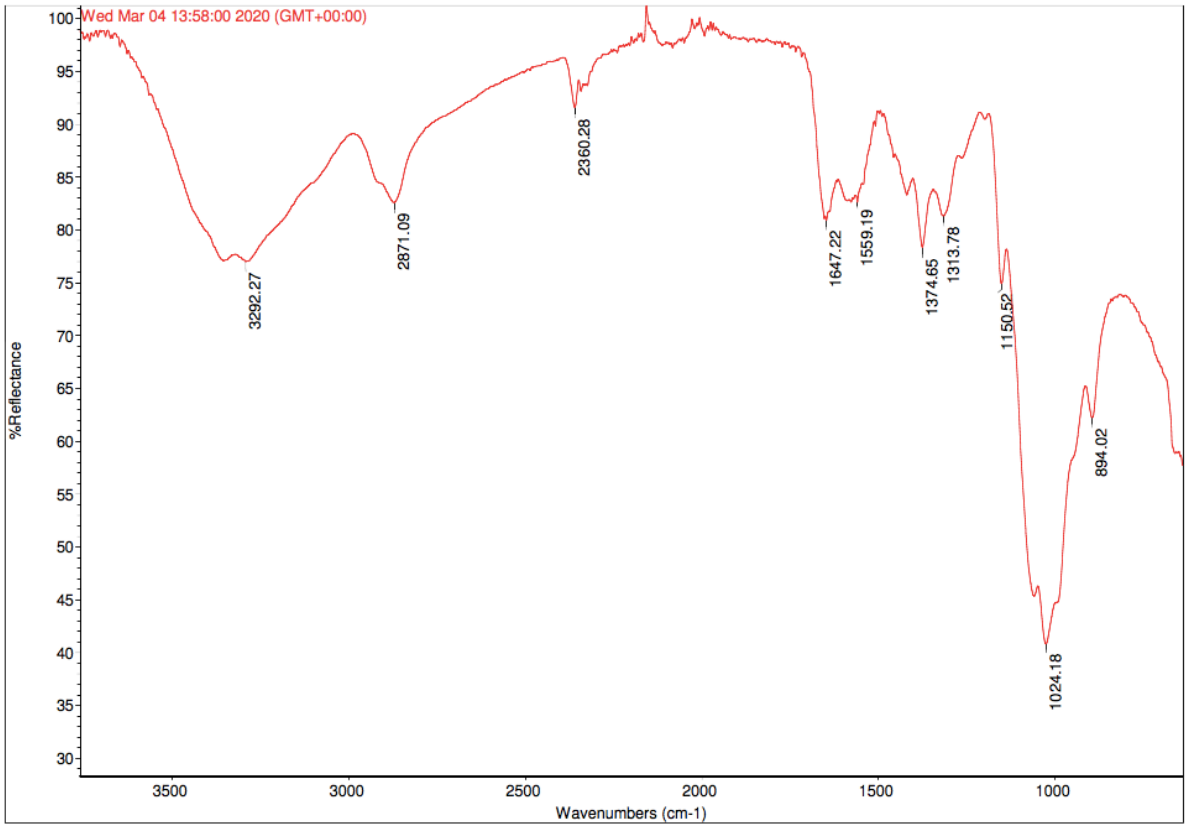
IR-spectra of silicone sheet B (GPTMS + chitosan).



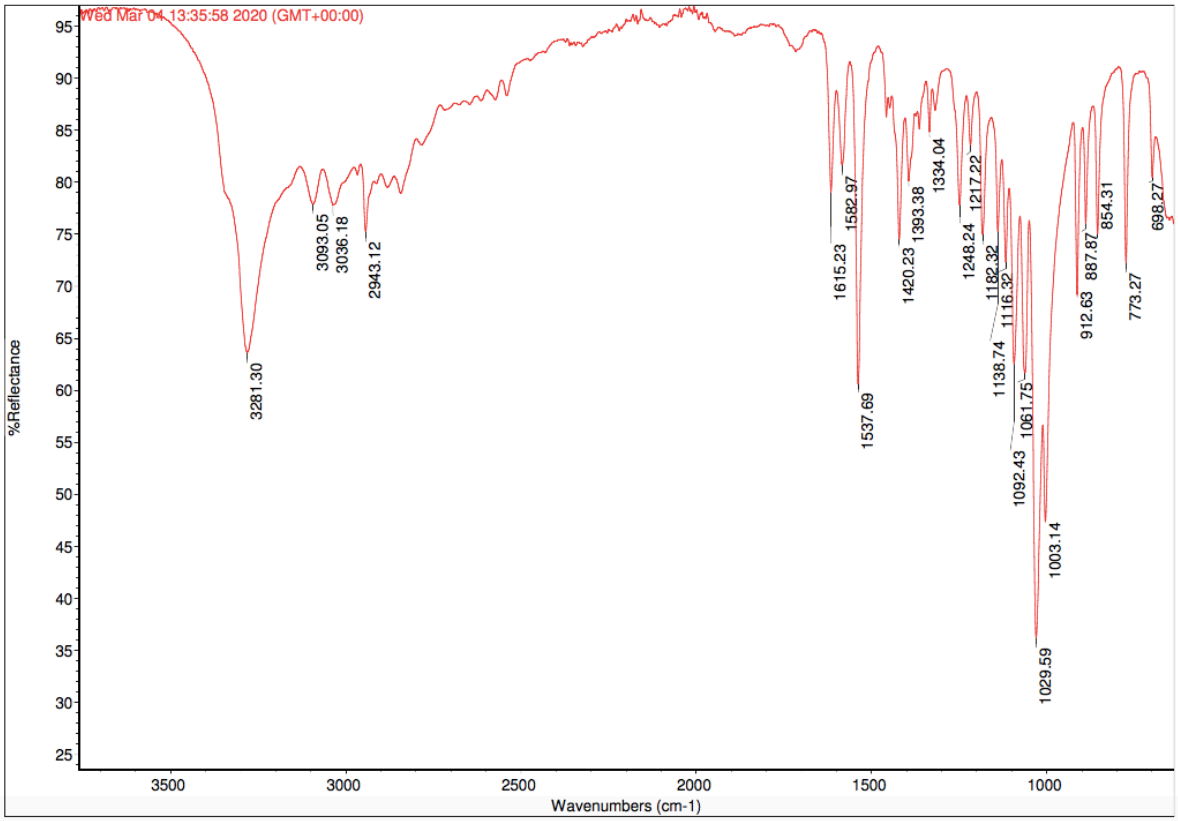
IR-spectra of silicone sheet C (APTMS + glutaraldehyde + glucosamine).



IR-spectra of silicone sheet D (APTMS + glutaraldehyde + chitosan).



IR-spectra of chitosan.



IR-spectra of glucosamine.