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Antibacterial mucin and BMP-2 coatings for titanium implant surfaces

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Title (English)

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Title (Swedish)

Abstract

Bacterial infections are always a threat to hip or knee implant procedures. One way of reducing this risk is to make the titanium implant surface less hydrophobic. This project intended to combine the antibacterial properties of mucin with the osteoinductive properties of rhBMP-2. The surfaces were rigorously analyzed and the amount of adhered *Staphylococcus aureus* was detected with a confocal scanner. Physisorption of mucin to the surfaces seems to be more efficient than covalent attachment. rhBMP-2 was found to be bioactive in cooperation with mucin, and the latter was shown to keep its ability to deter proteins and bacteria when combined with other surface functionalizing agents.

Keywords

Titanium, implant, mucin, biomaterial, coating, BMP-2, jacalin, Staphylococcus aureus, ESCA, surface chemistry, bacterial adherence

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Antibacterial mucin and BMP-2 coatings for titanium implant surfaces

Martin Larsson

Sammanfattning

Titan är ett material som används ofta vid tillverkning av implantat. Det används till skruvar, knä- och höftleder, m.m. Materialet är tåligt, relativt billigt och framförallt så fungerar det bra tillsammans med den mänskliga kroppen; titan är vad man brukar kalla för biokompatibelt. Trots detta så uppstår det ofta komplikationer vid implantatoperationer, även när det inte är några större fel på implantatet i sig. Det finns alltid en risk för att man får in bakterier i operationssåret. Om dessa får fäste i skelettvävnaden så leder det ofta till elakartade och svårbehandlade infektioner. För att komma till rätta med detta problem så kan man modifiera implantatets yta.

I detta examensarbete har ett nytt sätt att modifiera implantatsytor utforskats. Mucin är ett i slemhinnor ofta förekommande protein som påvisats ha vissa antibakteriella egenskaper. BMP-2 är en tillväxtfaktor som kan stimulera tillväxt av benvävnad. Tanken är att kombinera dessa två egenskaper och på så vis skapa ett snabbläkande implantat med låg risk för infektion.

För att påvisa detta så skapades en flödeskammare i vilken en lösning med bakterien Staphylococcus aureus tilläts strömma över ett antal små titanplattor (10x5x1 mm stora). Titanplattorna analyserades och behandlades med olika ytbeläggningskombinationer; med olika komponenter och olika inbindningsmetoder. För att mäta bakteriemängden så färgades bakterierna med en fluorescent markör och stoppades in i en så kallad konfokalscanner; en maskin i vilken provets belyses med laserljus och det reflekterade fluorescenta ljuset kan mätas.

Denna inledande studie lyckades påvisa att det går att kombinera mucin med BMP-2 och samtidigt någorlunda bibehållna de enskilda egenskaperna. Fortsatta studier måste dock göras innan det går att börja använda för implantat till människor.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet December 2006

Contents

1. Introduction

The market for biomaterials is vast and with a growing aging population of the developed world, the number of procedures related to for instance hips and knees will continue to increase even further. A recent American estimate predicts the number of knee implants to reach nearly 3.5 million and the number of hip replacements to reach 572 000 by the year 2030, in the United States alone (Ptacek, 2006). Today, both these types of implants, together with many others, are often made from titanium or titanium alloys. The material has been used successfully for a long period of time and is considered to be one of the most biocompatible materials known today (Walpole AR, et al. 2003). But there are still some fundamental problems associated with implants in general. The healing process and the compatibility between implant and tissue are seldom as smooth as that of the natural state with original tissue. If not complicated by an acute incompatibility such as toxicity, physical material deficiencies or immunogenic induced rejection of the material, one always have to beware of the risk of infections received through the wound.

Bacterial infections are a potential threat to all patients undergoing surgery. The human immune system is not optimized to work with the deep wounds generated by implantation. The skin is usually the main barrier for keeping pathogens out and with orthopedic implants this risk is further induced by the deficient antibacterial properties of the immune system at skeletal level. This can be seen in the seriousness of bone disorders such as bacterial osteomyelitis. 70-80 % of this particular disease is internationally considered to be induced by the bacterium *Staphylococcus aureus*, a bacterium commonly found on our skin without there normally causing any problems. These implant infections are relatively hard to treat due to the therapeutical resistance of this particular bacterium. Two of the complicating factors are the capability for intracellular hiding and the production of biofilms. Healthy bone tissue is very resistant to infections, but once the bacteria have become established treatment is difficult (Ciampolini & Harding, 2000). Implant related bacterial osteomyelitis is cured in about 80 % of the cases, by a combination of surgery and orally taken antibiotics. The remaining 20 % lead to either the recurrence of infection or chronic osteomyelitis. This and other implant related infections cost society a lot of money and resources. Thus, a reduction in the number of cases ought to yield great benefits for both society and for the individual patients.

The purpose of this project was to develop a new type of coating for implants, primarily for titanium implants, but the concept is most likely applicable on other materials and devices. The idea is to combine two previously investigated coating materials into a new one whilst maintaining the advantageous properties of the individual components. Mucin has been shown to significantly reduce the bacterial adherence of surfaces thus decreasing the risk of pathogen colonization within the implant wound (Shi, et al, 2000). BMP-2 is a growth factor which has been shown to reduce the bacterial adhesion as well as induce bone growth (Jennissen, 2000). The combined effect will hopefully result in an orthopedic implant material that decreases the wound healing time as well as the risk for infections; thus resulting in a lesser probability for other complications such as material rejection.

1.1. Effects of implantation

Implantation is not a procedure free of risks. Any surgical procedure risks complications related to the effects of anesthesia, infection, swelling, redness, bleeding, and pain. With implants one can further add the risks of something being wrong with the device itself. There exists a wide range of possible errors, and the potential faults are specific to the different types of implants. However, in one way or another they are all associated with the implants biocompatibility; a word that unfortunately is very vaguely defined. But as one can imagine it has to do with how well a material interacts with living tissue. The human body constitutes a very hostile environment to foreign objects and therefore, to a varying degree, all materials interact with it. Depending on the purpose of the implant, the requirements are different.

What happens when you implant something into the human body? Tissue has to be damaged in order to get something to go inside the body; independent of the choice of procedure. This immediately initiates a response to protect the body and heal the wound; an inflammation occurs. The healing procedure results in the regeneration of parenchymal cells and/or formation of fibroblastic scar tissue at the site of implantation. The nature of the immunogenic response is dependent on the extent of the damage; the physical extent as well as the degree and persistence of the inflammation. An acute inflammation will induce recruitment of e.g. white blood cells such as neutrophils, monocytes and platelets. Over time, the monocytes will differentiate into macrophages, which further can aggregate to form foreign body giant cells. (Karlsson, 2004, p19). The dead space that is created as a result of implantation attracts macrophages to the implant-tissue interface (Silver, 1984). These macrophages, together with the fibroblasts of the scar tissue, often form a definable layer of cells that can surround implants. (Spector *et al*., 1992). Bacterial adherence is the primary molecular mechanism for bacterial infections in humans and animals (Gristina & Naylor, 1996, p206) and the presence of orthopedic implants has been shown to cause a local reduction in the ability to kill phagocytosed bacteria. This is caused by the exhaustion of the local polymorphonuclear cells (Ciampolini & Harding, 2000). This creates a suitable environment for colonization of bacteria such as *Staphylococcus aureus*. It might be possible, however, to stop this from being a problem by preventing the bacteria from colonizing the implant in the first place. For polymeric surfaces it has been shown that one way of reducing bacterial adhesion is to reduce the surface hydrophobicity with e.g. mucin (Lei Shi, et al., 2000).

1.2. The aim of the project

This project was a cooperative effort to combine two individually promising biomaterial related surface modification strategies. The antibacterial properties of mucin, and the antibacterial properties and bone growth induction properties of BMP-2. The aim was limited to studying the degree of bacterial surface colonization of modified titanium surfaces. The experimental setup was to coat small rectangular shaped titanium ingots with a combination of 3-aminopropyltriethoxysilane (APTES), Mucin and BMP-2, and then submit them to identical conditions for bacterial surface colonization.

2. Theoretical context – Materials

2.1. Titanium

Titanium has been known to humanity since the late 1700s, but it wasn't until 1947, when the Kroll process was discovered (reduction of titanium tetrachloride with magnesium or sodium), that it became industrially and thus commercially available.

Titanium is known to be chemically very resistant at normal temperatures. It is capable of withstanding corrosion in water, air, and most acids. This resistance is caused primarily by the easy formation of an inert oxide layer. When the metal is introduced into living tissue, the titanium surface always oxidizes, thus making the oxides the primary counterpart that interacts with the organism on a microscale. The surface consists of a thin gradient layer; from the inside perspective ranging from elemental titanium into oxides and further into hydrophilic hydroxide phases. (Nationalencyclopedin, 2005) There are virtually no toxic effects for titanium or its oxidized forms, thus making it very suitable in medical applications. It is also rather inexpensive compared to other methods or materials. (Socialstyrelsen, 2005)

Other than its inertness, titanium is also a very strong and light metal ($\rho = 4.54 \cdot 10^3$ $kg \cdot m^{-3}$). Pure titanium also has a rather low conductivity of heat and electricity compared to other metals. (Nationalencyclopedin, 2005) When it comes to the suitability of a material as an implant, the Young's and Shear modulus has to be considered. If deviating too much from that of the surroundings, the steep gradient will create a potential that might cause fracture and material breakdown. The Young's modulus of pure titanium is $11 \cdot 10^{10}$ Pa and the Shear modulus is $4 \cdot 10^{10}$ Pa, both relatively close to that of living tissue. (Hillborn, 2004)

The Swedish National Board of Health and Welfare (Socialstyrelsen) concludes that the choice of implant material depends on the application but, in general, consider the positive aspects of titanium to outweigh the negative. (Socialstyrelsen, 2005)

2.2. Bone morphogenetic protein-2, BMP-2

Human bone morphogenetic protein-2 (BMP-2) is a relatively small basic dimeric protein with a total molecular weight of 25.8 kDa. It consists of two identical polypeptide chains of 114 amino acids each (Jennissen et al., 1999). The N-terminal region is clearly hydrophilic and the rest is fluctuating between slightly hydrophilic and slightly hydrophobic. Most of the latter are inaccessibly located in the natural structural conformation (Chatzinikolaidou, *et al.*, 2003). The protein is considered to be an osteoinductive factor and the coating of titanium implants with BMP-2 has been shown to significantly increase the mechanical strength of the bone as well as accelerate the growth rate in treatment of fractures. There is a risk of ectopic bone formation as a result of locally high BMP-2 concentrations or improper implantation. However, this risk is limited due to the short half-life of growth factors. Ectopic formation requires the factors to be applied directly and continuously in to the specific area. (G. Schmidmaier, et. al, 2002)

2.3. Mucin

Mucin is a naturally occurring substance in animals as well as humans and it has been shown that it reduces bacterial adhesion (Shi, et al., 2000). It is a very large glycoprotein, $M_r = 10^6 - 10^7$, built up by a thin linear polypeptide core and densely packed oligosaccharide side chains (Bansil, et al. 1995). The protein is one of the major constituents of mucus, the highly hydrated gel that covers many of the internal surfaces of the human body. The exact properties and the functionality vary depending on the location but it is associated with transportation and protection. (Malmsten, *et al.,* 1991)

The mucin backbone can easily adsorb to hydrophobic surfaces, and the carbohydrate segments prefer an aqueous environment. Thus, the oligosaccharide clusters will lower the overall hydrophobicity of the surface.

There are several available methods for keeping away bacteria from surfaces, but not all of them are kind towards living tissue. Adding mucin coating to the surfaces has the potential of reducing bacterial colonization and increasing biocompatibility. (Shi, et al., 2000)

2.4. Jacalin

Jacalin is a lectin (i.e. carbohydrate binding protein) originating from the Jackfruit, in this case *Artocarpus integrifolia*. Jacalin constitutes more than 50 % of the plant seeds proteins. The molecular weight is 65 kDa. It is a tetramer organized into two non covalently linked chains, an α- and a β-chain; the later with 3 subunits. Jacalin is a glycoprotein containing 7-10 % carbohydrates. The α-chain consists of 133 amino acid residues, organized in 9-11 β sheets, and about one third of it is glycosylated. There is a slight variation between the primary sequences of the β subunits, but they all consist of 20-21 amino acids, arranged in a prism-like structure of three four-stranded antiparallel β -sheets. No cysteine has been found in jacalin, indicating an absence of disulphide chains.
(Kabir, 1998)

Jaclin has been shown to have an affinity towards MeαGal and is therefore considered to be a carbohydrate binding protein. This characteristic can be utilized to advantage by using jacalin as a specific cross-linker between e.g. mucin and some other molecules.

2.5. *Staphylococcus aureus*

Staphylococcus aureus was chosen as a model organism for the project since it together with *Staphylococcus epidermidis* can be considered to be one of the most relevant implant associated bacterium. *Staphylococci* are gram positive bacteria and exist naturally in our environment. *Staphylococcus aureus* is pathogenic, invasive and quite pyrogenic. It usually enters the human body either through the respiratory system or by wounds. Among the conditions are: food poisoning, acne, cellulitis, pneumonia, meningitis, arthritis, osteomyelitis, endocarditis, urinary tract infections and toxic shock syndrome (Antigenics, 2006). It is also often the cause of biomaterial related infections world wide; more commonly so internationally than in Sweden, where *Staphylococcus epidermidis* more often causes the trouble. (Guss, 2004)

One major problem with *Staphylococcus aureus* is its high resistance to antibiotics. Another problem is that the cells surrounding an implant surface are not able to dislodge the bacteria once it has attached it self (Harris, et al., 2003). *Staphylococcus aureus* is known to express high affinity receptors for e.g. fibronectin, fibrinogen and lamin. These molecules have all been shown to be responsible for the adhesion to implant surfaces in animal models. (Ciampolini & Harding, 2000). It has also been shown that pathogenic bacteria often have a greater number of conditioning film receptors than similar strains of non-pathogenic bacteria (Gristina & Naylor 1996, p 210) Suggesting that relevant testing of surfaces for tendencies in bacterial adhesive properties ought to be made with pathogenic strains.

3. Theoretical context – Methods

3.1. ESCA

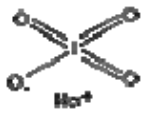
Electron transfer for Chemical analysis, ESCA, is a surface analytical technique providing compositional information about the outmost layer of an investigated surface. It and can provide information that can't be obtained by other means. The method is an application of the photoelectric effect, and utilizes the fact that a surface emits material characteristic electrons as it is bombarded with high energy X-rays. The interacting electrons are primarily inner shell electrons. The energy balance for an arbitrary electron can be described as follows:

$$E_{bond} = h\upsilon - E_{kinetic} \quad (1)$$

Where E_{bond} represents the binding energy, $h\upsilon$ is the energy of the X-rays and $E_{kinetic}$ represents the kinetic energy of the specific electron. (Ratner, 1996)

3.2. Oxidation with periodate

Sodium periodate, $NaIO_4$, can be used to create aldehyde groups on compounds containing internal or terminal adjacent diol groups or N-terminal serine peptides. The periodate catalyses the reaction by oxidation of a hydroxyl group. This results in cleavage of the carbon-carbon bond that is situated between the substituents and the release of formaldehyde (and in the serine case ammonia as well).(Greg T. Hermanson, 1996)



The selectivity of the oxidative process can be controlled by using a 1mM solution of sodium meta-periodate at 0°C. This results in periodate oxidizing mainly sialic acid groups. By increasing the concentration and temperature (~10 mM and RT) other sugar diols will be oxidized as well, thus creating more formyl groups. (Pierce, 2004)

Glycoproteins often have the required hydroxyl groups. Creation of aldehydes on the carbohydrate regions might be used covalently link it to some hydrazide, hydrazine or amine group by Schiff base formation or reductive amination. This makes it possible to cross-link glycoproteins to other proteins, support matrices, etc. (Pierce, 2004)

3.3. Confocal scanning microscopy

Confocal Scanning Microscopy usually requires tagging of the substrate of interest with appropriate dyes/probes and then measures the relative fluorescence intensity of the compared samples. This is a function of absorbance, fluorescence quantum yield of the dye, the intensity of the excitations source and the fluorescence detection capabilities of the instrument. The absorbance is defined by the Beer-Lambert law, making it dependant on the optical path length, the concentration of the light absorbing substance and the material dependent absorption coefficient.

$$A = \log (I_0/I) = e \cdot l \cdot C \qquad (2)$$

Where I_0 and I are the intensities from monochromatic light before and after passage through the system.

Because fluorescence quantification is dependent on the instrument, fluorescent reference standards are essential when calibrating measurements made at different times or using different instrument configurations. Fluorophores also bleach over time making it important to choose a relatively photostable probe and to not submit the samples to unnecessary light.

One sensitivity limiting factor is that of the occurrence of background signals. These distortions are often caused by autofluorescence of the sample constituents or from excess probes (unbound or nonspecifically bound). Autofluorescence of cells, tissues and biological fluids is easily reduced by using probes that can be excited at wavelengths above 500 nm. The light scattering from these relatively dense media is greatly reduced by longer wavelengths, resulting in a better penetration of the excitation light. Another method for increasing the sensitivity is by choice of the properties of the light filters.

3.4. Modified Robinson Device

The Modified Robinson Device, MRD, allows comparison under identical conditions of flow, temperature and carrier constituents (Linton, et al. 1999). It can be used to wash, or prepare multiple samples in identical manners. Essentially it is just a flow trough chamber with two openings, one for entering and one for exiting flows. The speed of the flow is controlled by a pump. In this study this is also relevant since the studied bacteria, *Streptococcus aureus* lack flagella. By controlling the flow of the media one controls the movement of the bacteria (Guss, 2003). The flow was set to be laminar in order to further control the environment. The device was built solely in Teflon, chosen due to the materials inertness. It can withstand a rather wide range of temperatures, and it is chemically resistant to most of the commonly used substances and organisms of microbiology.

4. Material and methods

The titanium pieces used in the experiments were manufactured in Germany. They measured 1.00 x 5.00 x 10.00 mm, ±0.1 mm. The oxidative surface enhancing process was also carried out in Germany; by Dr. Maria Chatzinikolaidou at the Institut für Physiologische Chemie, Universität Duisburg-Essen. After cleaning the surfaces in methanol, the ingots were submerged in heated stirring chromosulfuric acid (92% H_2SO_4, 1.3 % CrO_2, ρ = 1.84 g/cm3, T = 230-240°C) for 60 minutes. They were then cleaned in H_2O, boiled first in EDTA (2 %, pH = 7.0) and afterwards in pure water, 30 min in each. The ingots were then dried and stored in methanol. (Jennissen, *et al*., 1999 and Jennissen, *et al*., 2000)

Half the batch were then chemically functionalized by silanization with 3-aminopropyltriethoxysilane (APTES). APTES-silanization of titanium surfaces has been shown to increase the amount of adsorbed proteins, up to 20-30 times for e.g. ubiquitin (Chatzinikolaidou, et al, 2002). This was done by adding dry toluene and APTES to the substrates and boiling the mixture for ~4 h. Afterwards they were washed with toluene and ethanol, followed up by drying in methanol. (Jennissen, et al., 1999) The substrates were then put into individual Ependorf tubes and shipped to Sweden.

4.1. Electron Spectroscopy for Chemical Analysis

Electron Spectroscopy for Chemical analysis, ESCA, was carried out on a Scienta ESCA 300 instrument at the Ångström laboratory at Uppsala University. A total of four sample measurements were run, two with just titanium (sample 1 and 2), and two with added APTES (sample 3 and 4). The plate pairs were picked out randomly.

The surfaces were pretreated with methanol in order to simulate the washing procedure of the rest of the plates. The surfaces were rinsed by HPLC grade methanol in an air flow hood. They were then stored in fresh methanol for 36 hours, in order to sterilize. Before ESCA analysis, they were again rinsed by methanol and then dried with argon gas flow before put into the ESCA instrument.

The experiments were done with two different Take Off Angles (TOA) for each plate, 90° and 10°; where TOA was defined as the angle between the surface and the emitted beam, thus varying the penetration depth.

4.2. Addition of mucin

One part of this study was to measure the amount of adhered bacteria to different types of ingots. The idea was that a reduction of surface hydrophobicity ought to lower the amount of bacteria. The mucin used in this study was derived from Bovine Saliva Mucin. Mucin coating was achieved via two separate ways, by physisorption and by covalent linking to the surfaces.

The covalent linkage was done by creating a Schiff-base coupling. This was done by first activating the mucin with periodate thus creating reactive aldehydes on its surface. The final concentrations for the covalently bonding mixtures were 1-2 mg/ml. The concentration of the adsorption mixture was 1 mg/ml. The ingots were placed in the mucin containing solution in a plastic well for 3 hours under gentle agitation.

The mucin adhesion was confirmed by using fluorescently labeling Cy3 probes in a SA 5000 Confocal Scanner. The methods validity was verified by doing a comparative concentration series of Cy3/jacalin on known concentrations of mucin.

Fluorescently labeled jacalin was used as a mucin specific probe. The fluorescent molecule, Cy3, was mixed with jacalin and then run trough a PD-10 gel filtration column. The ratio of probe/protein was found by measuring the absorbance in a spectrophotometer.

4.3. Addition of BMP-2

Recombinant human BMP-2 (rhBMP-2) was added to some of the titanium ingots with adhered mucin. The BMP-2 adhesion was carried out at the Institut für Physiologische Chemie, Universität Duisburg-Essen, Germany. The rhBMP-2 was immobilized on to the ingot surfaces by incubating the substrates at room temperature for 12-14 hours in a borate buffer with ~0.25 mg/ml rhBMP-2. The procedure has previously been shown to be valid for surfaces containing a mixture of Ti, APTES

and rhBMP-2. The validity of the mucin containing system was tested in two ways. First, ^{125}I-labelled rhBMP-2 was added to the surfaces. Measures of the radioactive intensity yielded the amount of adhered rhBMP-2. A pilot study was also carried out to confirm the biological activity of the new material combination. An alkaline phosphatase bioassay was done on murine osteoblastic cell line MC3T3-E1 (as described in Chatzinikolaidou, *et al*. 2003). The cells were cultivated on titanium plates coated with APTES, physisorbed mucin and rhBMP-2. After adding the BMP-2, the ingots were stored and shipped in Eppendorf tubes containing PBS (pH 7.4).

4.4. Addition of Staphylococcus aureus

A new customized Modified Robinson Device, MRD, was constructed and used for controlled addition of *Staphylococcus aureus* to the ingot surfaces. The device was calibrated and designed to yield a robust laminar flow at the site of the substrates. A peristaltic pump was used to power the MRD.

A clinical strain of *Staphylococcus aureus* (R 445-7001) was taken from a shoulder puncture of a local Swedish patient. The bacterial experiments were carried out at the Swedish University of Agricultural Sciences (SLU). The bacteria was grown logarithmically over night at 37°, taken when at stationary phase and then centrifuged and washed with PBS-D (pH 7.3) for 3x15 min at 6000 RPM (5523 G). The final optical density was found to be 0.74 at a wavelength of 600 nm.

Before and in between runs, the system was washed with PBS-D and autoclaved. The system was also cleaned with 1% acetic acid in order to efficiently remove contaminating proteins (Larsericsdotter, et al, 2004).

The bacteria flowed through the MRD for 2 hours at a speed of 50 RPM. This corresponds to a mass flow of $3.71 \cdot 10^{-5}$ kg/s or 0.037 ml/s, equivalent to 133 ml/h. The system volume is 6.61 ml which means that it takes roughly three minutes to run one volume through the system.

After rinsing the surfaces by flowing PBS-D trough the system, the bacteria were fixated by a 5 min flow through of 2% glutardialdehyde. The device was then opened up in a laminar flow hood and the ingots were rinsed in PBS-D (pH 7.3), and then fluorescently labeled with 0.005% Acridine orange (pH 4.0) for 5 min.

Several different methods of application with acridine orange as a fluorescence marker exist. An acetate buffer solution was considered to be optimal for the system (Rosengren, 2004). It is a slightly modified version of that used at the Avdelningen för klinisk mikrobiologi, Centrum för LaboratorieMedicicin, Akademiska sjukhuset, Uppsala, Sweden (document code: S303AA003). Since the starting acridine orange was in a 1 wt % aqueous solution, and not solid compound, the procedure had to be altered slightly to achieve the desired 0.005 %.

After this, the fluorescence intensity was measured with a SA 5000 Confocal Scanner; thus enabling for comparison of the bacterial coverage between the different surface types.

5. Results

5.1. Analysis of starting materials

ESCA was the primary method to characterize the initial surfaces (see Fig 1). The high energy of the X-rays in ESCA always constitutes a degenerative effect on the material that is being analyzed. In order to measure this effect on our particular materials the intensity peaks at the initial and the final scans were compared. The degeneration was found to be at an acceptable level.

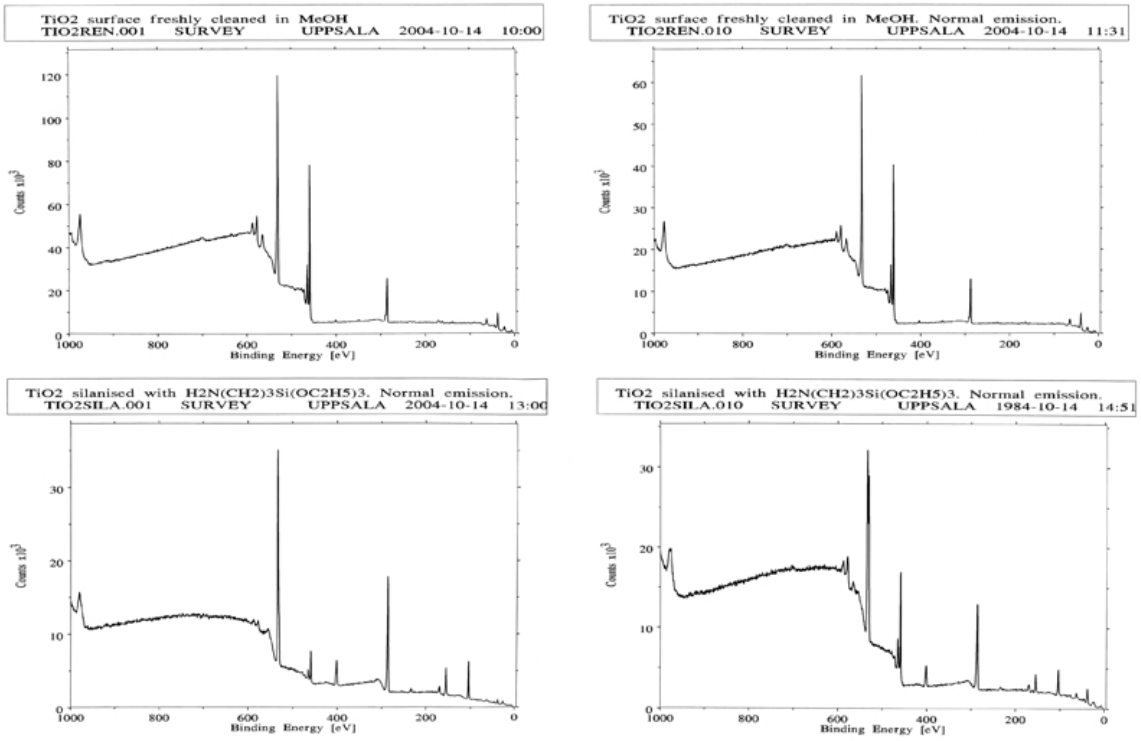


Fig 1 – Images from Electron Spectroscopy for Chemical Analysis
Top images depicting sample 1 and 2: CSA treated titanium ingots. The dominating peaks are that of 1s and $2p_{⅔}$. The bottom images depicts sample 3 and 4: CSA treated Titanium with an APTES coating. The dominating peaks in sample 3 are the 1s of oxygen and the 1s of carbon. Sample 4 is similar to sample 3 but with higher titanium peaks. All of the above sample images were taken with normal emission angle.

Information about the thickness of the oxide layer was received from Essen, supposedly circa 30-40 nm. This was neither confirmed nor further investigated, since the penetration depth of the runs were approximately 100 Å with normal emission angle and 25 Å with a 10 degree grazing angle.

The surfaces were, as expected, found to be conductive. Several impurities were found. In the first sample these included chromium, sulfide, sulfite/sulfate, carbon, calcium and lead-compounds. In the second sample no lead or sulfide were detected. The Sulfur compounds are most likely related to the oxidative treatment in chromosulfuric acid (CSA). The carbon peak is probably a combination of remaining methanol residues, from the sterilization/cleaning process, and naturally occurring atmospheric hydrocarbons. The origin of the calcium is unclear and the origin of the lead compound is unknown. Calcium could be the result of contamination of the impurities of water/buffers, or it might be an impurity of the HPLC grade methanol. However, the later is not very likely since neither sample 3 or 4 had this impurity. The probing depth was changed by decreasing the TOA. This resulted in a detectable decrease in the signals from both titanium (~75 %) and chromium (~83 %). The slight decrease in chromium intensity suggests that the chromium mainly where present at the top of the rough surface, further contributing to the theory of it being remnants of the CSA treatment rather than from the bulk metal it self.

In sample 3 and 4 no lead, calcium, nor sulfide could be detected. The height of the titanium peak was, as expected, reduced and the oxygen and carbon peaks were increased. This, in combination with the occurring silicon peaks proves that these plates have had an aminosilane layer added. The thickness of this layer could be estimated to roughly 100 Å thick. The thickness varies by a factor three between sample 3 and 4. Since the “tail” of an APTES monolayer tends to lie down close to the surface, the number of molecules that this thickness actually represents is unknown; but it is definitely greater than five. This means that the surface most likely had an unevenly polymerized aminosilane layer rather than the anticipated APTES monolayer. This should however not interfere too much with the chemical functionalization that was carried out within this study, but it will have to be accounted for in further studies.

5.2. Addition of mucin

Mucin was adsorbed to the titanium ingots: Covalently bonded to most of the APTES surfaces and physisorbed to half of the pure titanium surfaces. Adsorption was confirmed by measuring the fluorescence intensity of the constructed Cy3/Jacalin probe, with a confocal scanner as described earlier (se Fig 2). The probes Cy3/Jacalin ratio was determined by measuring the absorbance of the separated product (see table 1). The ratio was found to be 2.46 mol Cy3 per 1 mol Jacalin.

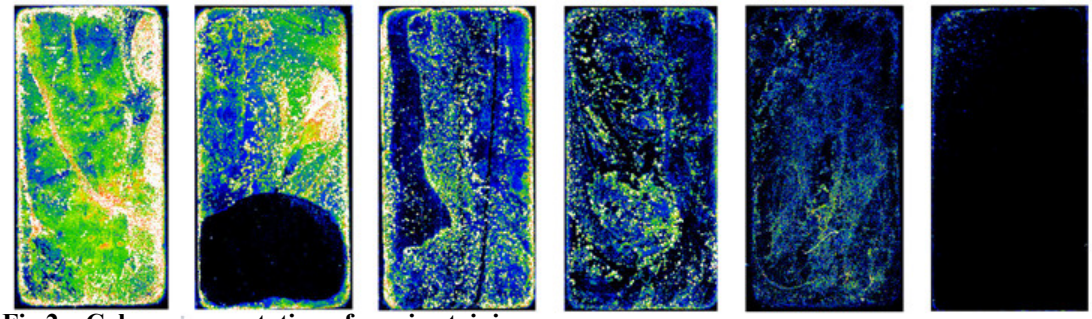


Fig 2 – Color representation of mucin staining.
The figure displays a concentration series of mucin (0.25, 0.10, 0.025, 0.010, 0.0025, 0 mg/mL). The presence of mucin on the titanium surfaces was confirmed by using a confocal scanner. A jacalin/Cy3 probe was used for detecting purposes. The relative light intensity in each pixel is transformed into a color representation: Dark and cold colors meaning a low intensity and warm and light meaning high concentrations.

Table 1 – Table over Cy3 and Jacalin Spectrophotometric data

	Λ_{ex} (nm)	Mw (g/mol)	ε	Abs	Concentration (M)
Cy3	550	1296	150 000	0.8234	$5.49 \cdot 10^{-6}$
Protein (Jacalin)	280	65 000	74 200	0.2313	$2.23 \cdot 10^{-6}$

5.3. Addition of BMP-2

After the mucin treatment, rhBMP-2 was adsorbed to some of the surfaces. Radioactively labeling with ^{125}I-rhBMP-2 confirmed that a previously investigated adhesive procedure was valid for the mucin-BMP-2 combination as well (see table 2); however, a higher amount of immobilized ^{125}I-rhBMP-2 was detected on the mucin surfaces that were covalently bonded compared to that of the physisorbed mucin surfaces. This ought to be investigated further. The initial pilot study of the rhBMP-2 bioactivity for the surface constellations within the project frame was promising (see Fig 3). This indicates that the chemotactic and juxtacrine properties of BMP-2 might be utilized by implant materials, thus possibly reducing the inflammatory response. The physisorbtion of rhBMP-2 can therefore be considered to be successful. By not bonding rhBMP-2 covalently to the surfaces, fewer compounds are required to functionalize the surfaces, thus reducing the risk for toxic effects as well as increasing the industrial value of the discovery.

Table 2 – rhBMP-2 immobilization data BMP-2 was shown to be more abundant on the surfaces with covalently immobilized mucin, but with less specific biological activity; compared with physisorbed mucin surfaces. The amounts were determined with radioactively labelled ^{125}I-BMP-2 and the bioactivity with a fluorescent alkaline phosphatase bioassay.

Surface Modification	^{125}I-BMP-2 amount immobilized [ng/cm2]	Specific activity FU* cm2/ng]
Ti-CSA-APTES physisorbed mucin BMP-2	149	155
Ti-CSA-APTES covalent mucin BMP-2	484	3

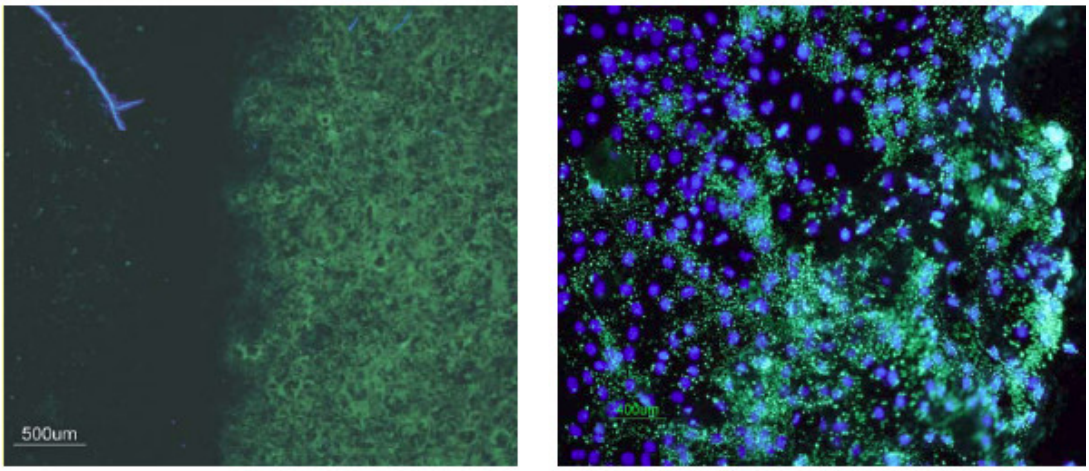


Fig 3 – BioActivity assay. Fluorescent alkaline phosphatase bioassay in the mouse osteoblastic cell line MC3T3-E1. It was cultured on modified titanium surfaces. A direct comparison with the controls demonstrates a significant increase in the induction of alkaline phosphatase on BMP-2 coated titanium surfaces (**A1** is without BMP-2 and **A2** with, the latter is a 2-fold magnification) in a 6 d test. Image **B** shows a 20-fold magnification, with the labelled cell nuclei in blue and the alkaline phosphatase in green induced from cells grown confluently on Ti-CSA-APTES-Mucin (physisorbed)-BMP-2 coated plates. (Chatzinikolaidou, *et al*. 2005)

5.4. Adhesion of *Staphylococcus aureus*

The adhesion of *Staphylococcus aureus* to the substrate surfaces was confirmed by a confocal scanner, using acridine orange staining as a marker (see figure 4). Absorbed mucin seems to have better repelling properties than the covalently bond, as can be seen in Figure 5 and Table 3. The cause for difference in the effect between the two covalently linked mucin surfaces is unknown. These surfaces had lower concentrations of mucin, and were carried out on surfaces from different batches of both mucin and titanium/APTES. The results suggest that mucin imparts bacterial repellent properties for biomaterials and implants, functional also when combined with other bioactive components. These results should be repeated to confirm the initial findings and extended in order to make a greater statistical analysis possible.

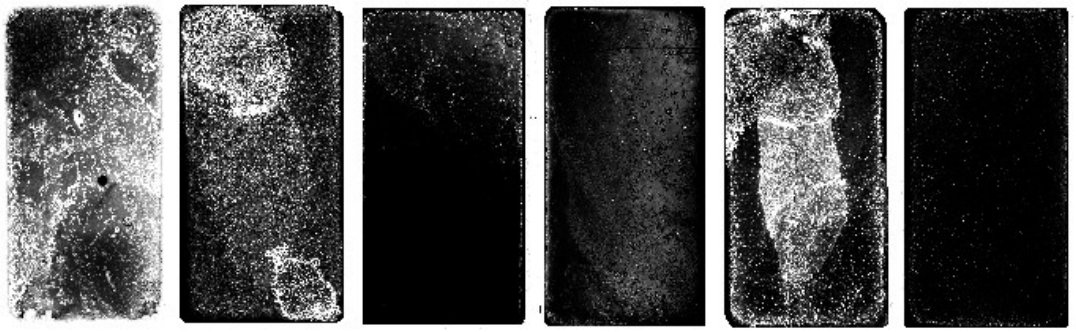


Fig 4 – Confocal scanner image of bacterial staining
The image portrays the different surfaces described in table 3 (surface 2-7). The intensity of the light is proportional to the amount of bacteria on the surface. The detected light of the confocal scanner is translated into an image with intensities ranging from 0 to 65k in each pixel: Black meaning zero amounts and white meaning maximum concentration.

Table 3 – Average pixel intensity. Column 1-6 corresponds to the: (1) Ti; (2) Ti + physisorbed mucin; (3) Ti + APTES + covalent mucin, (4) Ti + APTES + physisorbed mucin; (5) Ti + APTES + covalent mucin (2mg/mL) + BMP-2; (6) Ti + APTES + covalent mucin (1mg/mL) + BMP-2.

	1	2	3	4	5	6	7
Relative pixel intensity (average)	-	29650	11733	4491	12361	23467	3994
Relative index	∞	7,4	2,9	1,1	3,1	5,9	1

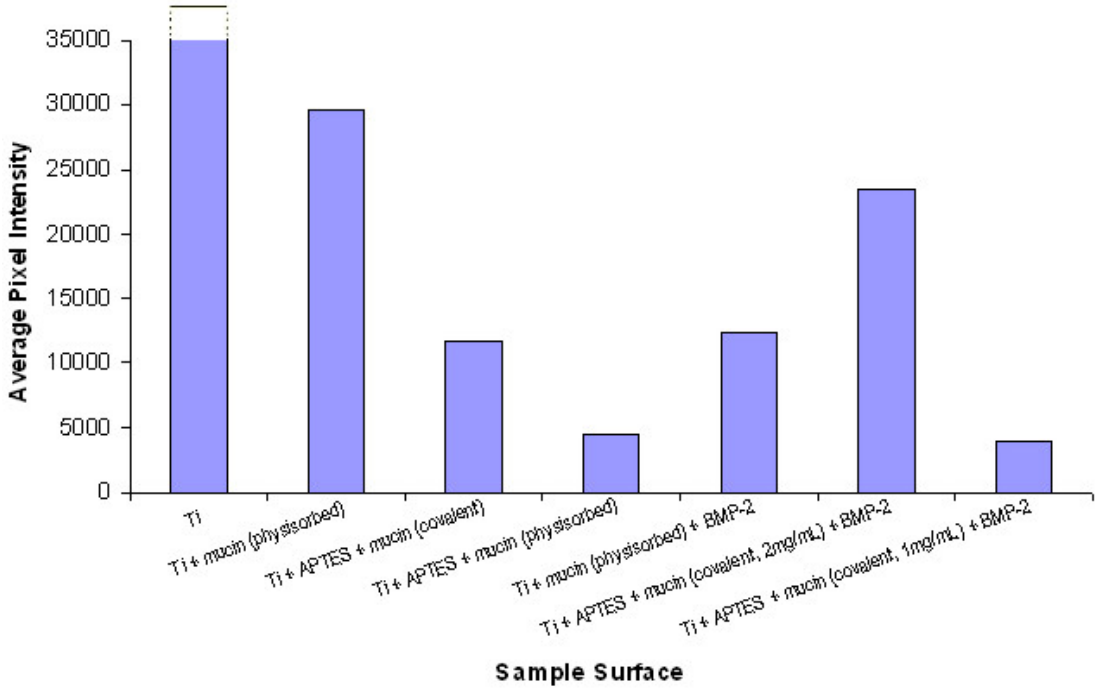


Fig 5 – Average pixel intensity
Depicting how fluorescence clearly differs with varying surface modifications, thus indicating different tendencies for bacterial adherence. The average pixel intensity level is relative and not absolute. And the titanium substrate with no modification peaked off scale in intensity. Indicating much more bacteria than the rest (Had to be run at different laser settings, etc, to be detected). Surfaces not containing any added bacteria had relative pixel intensity similar to that of the background.

6. Discussion

A distinct difference in the level of bacterial colonization could be noticed between the different types of surface modifications. The study was a first trial on the possible combined or even holistic, effects of mucin together with titanium, APTES and BMP-2. A distinctly lower level of bacterial colonization could be noticed with mucin treated surfaces. The results indicate that the antibacterial properties of mucin are still effective when incorporating growth factors such as BMP-2. It was also indicated that rhBMP-2 is biologically active in the presence of mucin.

All titanium ingots were pre-treated with the oxidative surface enhancing chromosulfuric acid (CSA), this made the initial surfaces extremely hydrophilic, and thus possibly in it self effecting adhesion to the surfaces.

One reason for the discovered variation in properties between different mucin surfaces could be found in how the molecules bond. Adsorption seldom fully denatures adhered proteins (Horbet, 1996). Covalently linking the mucin to the surfaces will probably radically change the conformation and denature it. The jacalin targets the mucin backbone, thus forcing the mucin into contact with the APTES surface. This could yield conformational tension and change; it could result in a change of the distribution of molecules across the surface. It could also expose more hydrophobic regions of the mucin.

In its natural state, the mixture of added proteins will probably adhere in a patch-like manner, with small clusters of mucin and/or BMP-2; thus enabling for the added substances to interact independently with the environment. Mucin is much larger than the other molecules used in this study. An even distribution of it would most likely mean that the adhered BMP-2 would become hidden within the large mucin network. The same effect ought to be seen with very high surface concentrations of mucin; which was also indicated in the pilot study. The seemingly superior repellant properties of the natural conformation of mucin, compared to covalent adhered mucin, could be one explanation for the lower amounts of immobilized ^{125}I-rhBMP-2 on the surfaces with physisorbed mucin. An interesting finding was that these surfaces also have a higher specific biological activity.

Physisorption of the proteins to the implant surface will be a largely irreversible process. Compared with covalently bond mucin, the physisorbed mucin will yield a state closer to the molecules natural state in living tissue. This will likely yield a more hydrophilic characteristic for the surfaces and will make better use of mucin antibacterial properties. If it can be shown that it is located in a cluster-like pattern it is also more probable that other adhered functionalizing molecules will be more effective.

Another advantage of Physisorbed mucin is that it reduces the number of required chemicals for functionalizing the implant surface, thus increasing the industrial value of the discovery.

6.1. Experimental issues

The surfaces of the titanium ingots were relatively rough; this did for instance make it impossible to use techniques such as elipsometry; due to the high scattering of laser light striking the surface. The effects of the variation in APTES layer thickness are unknown. The discovered occurrence of a polymerized APTES layer changes the parameters of the initial setup, the system might now not be as easily transferred to in vivo testing as first anticipated.

The construction of the flowchamber and the suggested advantages of its use could not clearly be seen. It might therefore have been better if it had been omitted from these first trials, thus saving a lot of time. Simply immersing the samples in bacterial solution might have done the job equally well, and at a lower cost.

The logistics of doing laboratory work at several different places was time consuming; locally as well as with the transports between Germany and Sweden. Reducing these would increase the efficiency of further studies.

A more advanced image analysis on the files received from the confocal scanning microscopy could result in better and more usable results. Mathematic filtering the images with an algorithm created in e.g. Matlab™ could reduce the background noise and more clearly present the effect in differences between materials. In future experiments high-precision fluorescent microspheres should be used as a reference, thus creating a standard in signal intensities making comparisons between different runs more reliable.

6.2. Conclusion

Three major discoveries were done during this pilot study. First, the APTES layer was shown to be unevenly polymerized. Secondly, physisorption of mucin seems to be superior to covalent bonding of mucin to the surfaces. Third, it was shown that different functionalizing agents can be used simultaneously, combining the individual properties of the constituents. In this case the biological activity of rhBMP-2 was combined with the antibacterial properties of mucin and both were found to be effective in the presence of the other.

6.3. Future

There are issues that still have to be solved, and there is a lot of room for expansion of the project: How does one optimize the effect of the combination of mucin and rhBMP-2? How and with what concentrations should these be bonded to the surfaces? Several interesting findings have been made which indicates that further studies in this area would be fruitful. The market still craves for implants with improved wound healing properties and reduced risk for bacterial infections and mucin seems to be a promising route, both by itself and in cooperation with growth factors.

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Great thanks to Prof. Dr. Karin Caldwell for presenting the possibility for me to participate in the project, Prof. Dr. Herbert Jennissen and Dr. Maria Chatzinikolaidou for their material support and the pleasant stay in Germany, Prof. Dr. Bengt Guss for the bacterial support, Dr. Jonas Jarvius for the Fluorescence equipment, Prof. Dr. Ulrik Gelius for the ESCA expertise and the Ministerium für Wissenschaft und Forschung des Landes NRW, through the "Sweden-Northrhine-Westphalia Research Initiative 'Molecular Medicine'", for their sponsorship of the project.

Also, special thanks to Dr. Tomas Sandberg for his continuous expertise and Assistant Professor Dr. Adam Feiler for his supportive and positive attitude. The project taught me a lot about research and what defines good science. On a more personal level it also stimulated independent and critical thinking. Despite a somewhat chaotic personal life at the time, it was still a very fun and challenging degree project.

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Appendix I - Work protocol

Work protocol, December 2004

Author: Martin Larsson

Day 1

- Reconstituate Jacalin in PBS-D
- Mix Cy5 stemsolution
- Mark Jacalin with Cy5

Day 2

- Reconstitute Mucin (BSM-2) over night
- Dissolve IPO_4

Day 3

- Modify Mucin

Day 4

- Start up bacterial growth
- Quantify the amount of adhered mucin by use of the Cy5/Jacalin-system.

Day 5

- Bacterial adhesion in MRD

Day 6

- Quantify the number of adhered bacteria.

Mucin

The majority of the plates will have mucin added to their surfaces. Avoid plastic surfaces in reaction where mucin is supposed to interact, since it adheres to the surface.

Preparation of Mucin

1. Sterile PBS-D (0,01-0,1 M), pH 7.3 is filtered through a 0.22 μm filter.
   (*If substituting the PBS, amine containing buffers such as Tris and glycine should be avoided*).

2. Mucin is taken out of the freezer and dissolved in the filtered sterile PBS-D, pH 7.3.

3. The mixture is allowed to stand for 24 hours, enabling for the protein to reconstitute.

4. The mixture is then filtered through a 0.45 μm filter.

Mucin addition to APTES/Ti

Covalent adhesion of mucin to Ti/APTES is done through a Schiff base coupling.

Periodate activation of Mucin

1. The mucin mixture is diluted with Mili-Q water (or buffer at physiological pH) to achieve a suitable concentration (For glycoproteins a concentration range of 1-10 mg/ml will produce acceptable results).

2. Prepare a container with ice, and put the mucin mixture there to cool; allowing for the 0°C needed in sialic acid modification.

3. Dissolve sodium periodate (MW 213.91) in deionized water at a concentration of 10 mg/ml (0.046 M).

4. Add 21.8 µl of the stock solution, to obtain ~1 mM sodium periodate in the reaction solution, maintain solution on ice. (For general oxidation of carbohydrates, other than just sialic acid, add 218 µl of the stock solution to achieve a concentration of about 10 mM).

5. Protect the reaction containing vial from light. Wrap the vial with aluminum foil or similar.

6. Let the vial react for ~30 min in room temperature.

7. Quench the reaction by addition of 0.1 ml of glycerol per milliliter of reaction solution. Alternatively the reaction may be stopped by immediate gel filtration on a Sephadex G-25 column. The dextran beads of the chromatography support will react with sodium periodate to quench excess reagent. To quench the reaction with cellular samples , wash the cells with buffer to remove remaining traces of periodate.

Mucin addition to Ti

Mucin is spontaneously adsorbed to the CSA treated titanium surfaces.

Mucin adhesion to pure CSA treated Ti

1. The plastic well are placed on a shaking board.

2. The plastic wells are filled up with the solution of PBS-reconstituted mucin.

3. The titanium plates are sunken down into the wells and left to adsorb for 3 hours on the shaking board.

4. The plates are then rinsed in filtered PBS-solution.

Quantification of the amount of adsorbed mucin

Preparation of Cy3

1. A correct concentration of Cy3 is mixed with jacalin.

2. The mixture is then filtered through a PD10 colon

3. A sample is taken and the adsorbance is measured to confirm binding and the ratio of the components.

4. The mixture is then added to the plates, and then rinsed from unspecifically bonded probes, followed up with drying under a laminar gas flow.

5. The plates are then placed on the specially designed aluminum tray, made for the confocal scanning microscope at the Rudbeck laboratory, Uppsala (i.e. a holder in the shape of a regular microscope glas slide).

The confocal scanner

6. The slide is loaded into the confocal scanner

Bacteria

Only 6 plates can be analyzed at the same time, which means that the device has to be thoroughly rinsed and autoclaved for each separate run.

Sterilization of Modified Robbins Device

1. The entrances (i.e. the silicon tubes) into the MRD are loosely sealed with aluminum foil.

2. The equipment is autoclaved.

3. The MRD and foil is stored away until experimental run.

S. aureus

4. *S. aureus* is grown logarithmically at 37°C for 24 h. OD counts are made as a confirmation for the initial hours. The bacteria are then left over night.

5. The suspension is then mildly washed twice;

   - 6000 RPM (5523 G) for 15 min at 4°C
   - Centrifugation for 15 min at 1200 x g at 4°C
   - The supernatant is taken away and the flask is refilled with PBS-D

   The washing procedure is repeated once.

6. The final concentration was adjusted to an optical density of 0.90, measured with a light source of 600 nm.

In future experiments a viable count ought to be made as a further confirmative step.

Bacterial adhesion

7. The system was set up and wetted by first filling up the system with distilled water.

8. It was then set to equilibrium by running filtered PBS-D, pH 7.3, through the system for ~45 min at a speed of 50 RPM (roughly 100 mL solution).

9. The bacterial culture is left to flow through the modified Robbins device for 2 hours at 50 RPM (about 265 mL bacterial solution).

10. Loosely or non-adhered bacteria is removed by washing with PBS-D for ~45 min at speed of 50 RPM (roughly 100 mL solution).

11. Fixation of bacteria was to at first done with running methanol through the system for 3 min. But in later tests this where substituted by glutaraldehyde fixation. This was done as the methanol was thought to be too aggressive. The later dissolves membrane bi-layers thus making it possible for DNA to dissipate, spreading out over the reaction chamber.

12. The system is then opened up as quickly as possible in order to minimize system self-contamination.

Acridine Orange staining

Making a batch solution
Staining washed disc with 0.005 % w/v Acridine Orange [10^{-5} M].

A 100 mL batch solution was made and aliquots were taken from this when staining.

- **Sodium acetate buffer, 0.2 M.**
  90 mL Mili-Q water, 18.2 MΩcm.
  2.7267 g Sodium acetate trihydrate (m_w: 136.08 g•mol^{-1})
- **HCl, 1 M**
  Add an amount of HCl such that pH ends up at 4.0 +/- 0.05. [~9 mL 1M]
- **Acridine Orange in aqueous solution**
  The original protocol demanded 5 mg of solid acridine orange, this corresponds to 500 µL of the aqueous 10 mg/mL solution.

Staining of samples
Recommending the use of 24 well plates when staining.

13. Make sure the appropriate LASERs are turned on
    (it takes ~15 min for them to warm-up)

14. When staining the plates, fill up a well with ~2 mL of 0.005% acridine solution.

15. Place the plates in the acridine orange filled wells for ~5 min.

16. Wash the plates by putting them in 10 min in a Mili-Q water.
    This step is repeated 3 times.

17. Dry the plates

18. Put on 0.5µL of internal calibration spheres in a corner on each plate.
    This step was partially abandoned due to technical difficulties. The concentration/intensity of the spheres should otherwise be chosen in accordance to a calibration curve

19. Load the alumina tray with the plates, and insert it into the microscope;
    start measuring.

Antibacterial BMP-2 Coatings for Implants in Bones Endangered by Infections

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Abstract

Antibacterial mucopolysaccharides (mucin) were immobilized by covalent or non-covalent methods to titanium surfaces, followed by a biocoat with adsorption of recombinant human BMP-2 (rhBMP-2). Surfaces with immobilized mucopolysaccharides have been shown to be antibacterial [1]. Implants coated with BMP-2 on the other hand have been shown to enhance bone growth and the integration of implants [2-5]. The combination of these two methods is expected to lead to a novel antibacterial bone-inducing surface. *In vitro* characterisation of the surfaces in the murine osteoblast cell line MC3T3-E1 showed that BMP-2 coated metal implants which have been pretreated with mucin remain their biological activity.

Introduction

The aim of the project is to develop an antibacterial BMP-2 containing metal implant surface. The antibacterial properties are bestowed to the surface by immobilizing mucopolysaccharides (mucin) which dramatically reduce bacterial adherence. The simultaneous immobilization of BMP-2 is intended to have two effects: (i) the additional reduction of infections, since the application BMP-2 has been shown to reduce infections in the treatment of open tibial fractures [6] and (ii) the induction of bone growth, which should rapidly reverse the osteomyelitis associated bone destruction, which at the same time would reduce bacteria supporting environments by closing the gaps between the bone and the implant. Thus such surfaces in connection with oral antibiotic therapy should lead to a significant reduction in the number of recurrent and chronic osteomyelitis cases. Antibacterial BMP-2 containing metal implant surfaces, as described in this proposal, have as yet not been reported and therefore pose a novel approach to the treatment of osteomyelitis. If these surfaces help to reduce the number of recurrent and chronic osteomyelitis patients, this will be a great benefit to the community.

Materials and Methods

Electrolytically polished titanium miniplates were treated by the chromosulfuric acid method (CSA) [7] leading to ultrahydrophilic surfaces [8] followed by chemical modification with 5% 3-aminopropyltriethoxysilane (APS) solution in toluene, endowing surfaces with hydrophobic properties.
In recent work, soluble high molecular weight proteoglycans (mucin) from saliva have been found to significantly reduce the bacterial adhesion to mucin coated surfaces. The titanum implant surfaces in the present project was treated in two ways, notably through adsorptive coating with mucin as well as through covalent attachment to surfaces pre-activated with 3-aminopropyltriethoxysilane (APS). The mucin concentration on surfaces prepared in these two ways is examined, and then recombinant human bone morphogenetic protein 2 (rhBMP-2) is coupled to the mucin coat e.g. by means of a jacalin (mucin specific lectin) affinity link. The treated titanium surfaces are incubated with a suspension of Staph. aureus, carefully washed and evaluated with respect to bacterial colonization, using an untreated surface as a negative control.
Biologically active BMP-2 is prepared by recombinant methods in E. coli in sufficient amounts. rhBMP-2 was adsorbed on the mucopolysaccharides coat. The biological activity of the immobilized rhBMP-2 was tested *in vitro* by an *in situ* alkaline phosphatase induction assay in the murine osteoblast MC3T3-E1 cell line. The *in vivo* investigations were curried out in a gap healing model in femur condylus of adult sheep.

Schematically the order of surface modification can be shown as following:
Titanium – CSA – APS – Mucin – rhBMP-2
All treating steps were carried out under sterile conditions in sterile agents.

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Results

Dynamic Contact angle

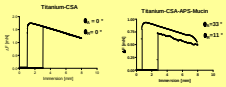


Fig. 1: Dynamic contact angle measurements according to Wilhelmy, indicating ultrahydrophilic surfaces after treatment with CSA (θ_A=0 °) become after APS-treatment hydrophobic (θ_A=75 °) and after mucin coating hydrophilic (θ_A=33 °).

Mucin coating on a hydrophobic polymer surface

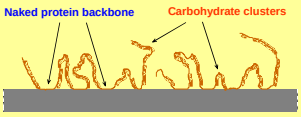
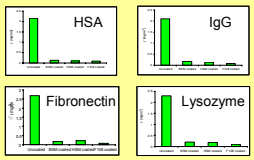


Fig. 2: Schematic view of protein backbone and carbohydrate clusters on a hydrophobic surface

Inhibition of Protein Adsorption on Mucin Coated Polystyrene



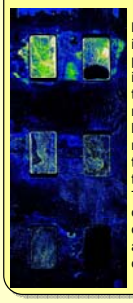


Fig. 3: Confocal scanning image of different titanium plates. The relative light intensity in each point is transformed into a colour representation: absence of bacteria in black and a maximum (ranging from 0 to ~ 65 k) in white. From left to right descending: Ti-mucin, Ti-mucin, Ti-APS-covalent mucin, Ti-APS-covalent mucin, Ti-APS-adsorbed mucin, negative control.

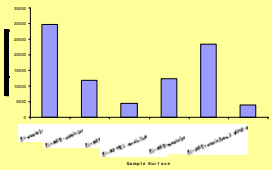


Fig. 4: The relative amount of bacteria present on different types of surfaces

Staphylococcus epidermidis adhesion to non-coated and mucin-coated PS surfaces

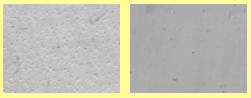


Fig. 5: Mucin coated surfaces indicate a marginal bacterial adhesion in comparison to non-coated surfaces.

Quantification of immobilized rhBMP-2 amount

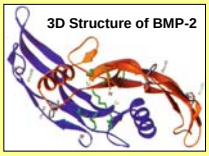


Fig. 6: 3D structure of BMP-2 according to Scheufler [9]

Table: Immobilized BMP-2 amounts and specific activity on different mucin-coated titanium plates determined with radioactively labelled ^{125}I-BMP-2

Surface Modification	^{125}I-BMP-2 immobilized amount [ng/cm^2]	Specific Activity [FU* cm^2/ng]
Ti-CSA-APS non-covalent mucin BMP-2	149	155
Ti-CSA-APS covalent mucin BMP-2	484	3

Fluorescence microscopy assay of immobilized BMP-2: proof of biological activity

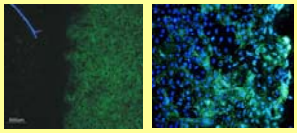


Fig. 7: Fluorescent alkaline phosphatase bioassay in mouse osteoblastic cell line MC3T3-E1 cultured on titanium plates Ti-CSA-APS-Mucin(ads.). A direct comparison with the controls (without BMP-2, A1 left lane, black part) demonstrates in a 2-fold magnification a high induction of alkaline phosphatase on BMP-2 coated titanium surfaces (green fluorescence, A2 right part) in a 6 d test. Image B shows in a 20-fold magnification both the labelled cell nuclei in blue and the alkaline phosphatase in green induced from cells grown confluently on Ti-CSA-APS-Mucin(ads.)-BMP-2 coated plates.

Osteoinductive potential of rhBMP-2 coat *in vivo*

Induction of cancellous (B) und cortical bone (C) by titanium implants biocoated with rhBMP-2 in a gap healing model in sheep after 9 weeks (own previous work)

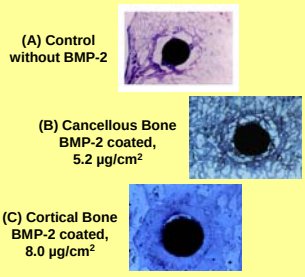


Fig. 8: Cross sections of titanium implants indicating a complete osteointegration (B, C)

Conclusions

In preliminary and present investigations we could show that:

- **mucin can be covalently and / or non-covalently immobilized as monolayer on titanium surfaces**
- **mucin retains its antibacterial properties after immobilization on titanium surfaces**
- **the amounts of adsorbed BMP-2 on covalently bound mucin lie in the range of 1 µg/cm² and on non-covalently immobilized mucin titanium-APS surfaces at 0.5 µg/cm²**
- **BMP-2 immobilized on mucin coated titanium surfaces is bacteria-repellent and indicates a high biological activity *in vitro***
- **BMP-2 immobilized on non-covalent mucin coat indicates a stronger specific biological activity by induction of alkaline phosphatase in MC3T3-E1 osteoblast cell line.**

Future Work

Based on these successful experiments we are planning to continue this very fruitful cooperation with the aim of making osteoinductive bacteria-repellent surfaces for medical application.