Master’s Thesis

Local release of lithium from sol-gel coated orthopaedic screws - an in vitro and in vivo study

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Sammanfattning


Syftet med detta examensarbete var att hitta en metod för att fästa litium på benimplantat. Litium skulle fästas på ett sådant sätt att frisläppning till omgivande vävnad blev möjlig.

Litiumklorid inkorporeras i en titanat-solgel och lager av detta lades på kiselytor och rostfria skruvar genom s.k. ”dip-coating”. Kiselytorna användes för initiala in vitro-studier av hur litium ändrade beläggningens egenskaper. Litium sitter antagligen fast på ytan av det tredimensionella nätverk som utgör solgelen, istället för att sitta inbundet i nätverket. Lagerstrukturen ändras ju mer litium som inkorporeras och vid stora mängder skapas inte de nanopartiklar som vanligtvis finns i en solgel-baserad beläggning. En följd av detta är reducerad bioaktivitet för beläggningen, dvs. en minskad förmåga för kalciumfosfatkrystaller att bilder på ytan. Litium frisläpps från beläggningen, dock sker denna frisläppning snabbt. Genom att belägga ytan med flera lager av solgel kan frisläppningskinetiken delvis ändras.

Solgelen kunde också med god vidhäftning appliceras på skruvar och frisläppningskinetiken från en skruv är liknande den från en kiselyta.

Abstract

In orthopaedic practice, fractures are usually stabilised with metal screws or rods. This is done in order to keep the fracture parts in place during the rather slow healing process. The healing time can potentially be reduced by local- or systemic treatment with different bone promoting drugs. In later years, lithium, otherwise used to treat bipolar disease, has shown promise to be such a drug.

The aim of this master thesis was to find a way to coat metal bone screws with lithium and to characterise the coating. The coating was to be designed in such a way that it could release lithium to the surrounding bone tissue.

Lithium chloride was incorporated into a titanate sol-gel and attached to silicon wafers and stainless steel screws by dip coating. Wafers were used for initial in vitro studies of how lithium changed coating characteristics. This was studied using ellipsometry, AFM and SEM. Lithium is most probably physisorbed and not incorporated into the network building up the sol-gel. Coating structure is changed as more lithium is incorporated. For large amounts of lithium, the nanoparticles normally formed when curing the sol-gel are inhibited. One effect of this is reduced bioactivity, seen as a reduced ability for calcium phosphate crystals to nucleate on the coating when immersed in simulated body fluid.

Lithium release was investigated using AAS. Lithium is released from the coating, showing a burst effect. By changing the number of coating layers used, the release profile can be partly altered. The coating was also applied to screws, showing good attachment, and the lithium release profile was similar to the one seen from wafers.

Finally, a screw model was used in rats to assess the effect of local lithium treatment from screws and systemic lithium treatment on fracture healing. In the model, a screw was inserted in tibia, mimicking a fracture. When the bone around the screw was healed, a pullout test was performed, giving information about the strength of the bone surrounding the screw. No significant difference could be found for either local- or systemic lithium treatment compared to control. However, when evaluating the strength of intact bone in a similar way, a positive effect of systemic lithium treatment could be seen. Therefore, it is still likely that lithium has a positive effect on bone and further studies are needed to fully evaluate its role in fracture healing.
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Abbreviations

AAS  atomic absorption spectroscopy
AFM  atomic force microscopy
APC  adenomatous polyposis coli
BMD  bone mineral density
Dvl  dishevelled
EDX  energy-dispersive X-ray spectroscopy
Fz   frizzled
GBP  GSK-3 binding protein
GSK-3β glycogen synthase kinase-3β
LRP5/6 low density lipoprotein receptor related protein 5/6
MSC  mesenchymal stem cells
PBS  phosphate buffered saline
SBF  simulated body fluid
SEM  scanning electron microscopy
TIO  tetraisopropyl orthotitanate
Introduction

1.1 Background

The fracture healing in long bones is a rather slow process, taking weeks to months. Clinically, fractured bones are in some cases fixated with screws of stainless steel or titanium. For several reasons, both regarding health and economy, one wishes to reduce the healing time. To promote fracture healing, various drugs have been suggested to work together with orthopaedic implants in order to do so. [1] Lithium has been used for treatment of bipolar disease and other mental illnesses since the late 1950ies. The effect of lithium in this aspect is complex but among other things it activates the wnt signalling pathway by inhibiting glycogen synthase kinase-3β (GSK-3β). [2] The wnt signalling pathway has multiple functions in cells, both in the embryonic development as well as later in life. Recently, it has also been shown to play an important role in bone formation. [3,4] Studies of patients being treated with lithium show a significant increase in bone mineral density (BMD). [5] The effect on lithium on bone growth and formation via the wnt pathway has been investigated in a number of animal studies, where it has shown a positive effect on bone when distributed systemically. [6,7] The findings mentioned above has led to the idea that it could be possible to influence bone repair locally by coating implants with lithium. If the coating slowly releases lithium, there might be a positive effect on bone cells in the surrounding tissue, which could lead to better healing.

1.2 Aim

The aim of this study was to find a method for immobilising lithium, first to reference wafers and later to metal screws (collectively called substrates). The most important property of the immobilised coating was lithium release, and selection of coatings should be based on these results. The wafers should also be evaluated in order to obtain information about the coating. If the results of the initial experiments were satisfactory, an animal model would be used to test the effect in vivo.

1.3 Choice of lithium immobilisation technique

The first task in the project was to decide on a technique to immobilise lithium onto a surface. Currently, lithium immobilisation is mostly used in the battery-industry where lithium-metal oxides are used as electrode materials. Extensive research is ongoing in this field, investigating new ways of incorporating lithium in order to increase performance and reduce cost. Examples of methods are ion exchange, hydrothermal reactions, vapour deposition and sol-gel processing. [8] The technique in question for this project needed to be accessible in the venues available and preferably used previously in medical applications. The sol-gel technique met these requirements. Sol-gel is a technique by which ceramic materials can be made and has, among other applications, been used to make bone-contacting materials. With this technique, it is possible to make both solids and coatings and to incorporate functional molecules. [9] Since the research group in which this project was made holds knowledge and experience of this method, the sol-gel technique was chosen to make the lithium coating.
2 Theory

2.1 Bone physiology

2.1.1 Bone cells

The cell content in bone is relatively low and consists primarily of three cell types: osteoblasts, osteocytes and osteoclasts. Osteoblasts are bone-forming cells and cover the bone surface. Their main task is to produce the organic part of the extracellular matrix, the osteoid, where the most abundant component is collagen I. It is secreted as procollagen and cleaved to tropocollagen, which forms collagen fibrils. Multiple fibrils finally form collagen fibres. Osteoblasts also release calcium, which stimulates nucleation and growth of mineral crystals in the matrix. [10,11]

Osteocytes are the most abundant cell in bone tissue. They belong to the same lineage as osteoblasts but have a different task. When osteoblasts become encased within the bone matrix, in spaces called lacunae, they evolve into osteocytes. This changes both their behaviour and phenotype. Osteocytes are star-shaped cells with extensions reaching out to other cells through channels called canaliculi. With these extensions they can communicate with osteoblasts and other osteocytes in the close region. The lacunae and canaliculi create a fluid network inside of bone allowing cells within it to respond to chemical and mechanical stimuli. The osteocytes are, because of this system, thought to function as mechanosensors. [12]

Osteoclasts belong to the same lineage as monocytes and macrophages and are responsible for bone resorption. When activated, osteoclasts attach to the bone surface by binding integrins in their cell membrane to vitronectin in the bone matrix. This creates a resorption zone into which hydrogen atoms are secreted. The acid lowers the pH in the region and dissolves the mineral in the bone matrix. When the mineral is removed, the organic components of the matrix can also be degraded by acid proteases. [13]

The above cells all work together to maintain or change bone structure and mass. During bone modelling, bone is either built-up, without subsequent resorption, or resorbed, with no build-up following. During bone remodelling, neither shape nor size of the bone is altered. A certain volume of the bone in a distinct region is resorbed, but deposition of new tissue of the same volume follows (Figure 1). Both modelling and remodelling prevent damage. When damage does occur, it is sensed by the osteocytes, damaged tissue is removed by the osteoclasts and new tissue is deposited by the osteoblasts. [14]

![Figure 1: Bone remodelling. Osteoclasts resorb bone, leaving a cavity (a). Osteoblasts fill the cavity (b), depositing new osteoid, which then becomes mineralised (c). Osteoblasts enclosed in the osteoid become osteocytes. When remodelling is finished, osteoblasts lining cells cover the bone surface once again (d).](image)
2.1.2 Extracellular matrix

The matrix can be divided into an inorganic and an organic phase. The inorganic phase is mainly composed of the calcium-containing mineral hydroxyapatite and the organic phase (osteoid) is made up of collagen and other proteins. This osteoid is produced by the osteoblasts and these cells also affect the inorganic phase by secreting calcium from intracellular compartments. This stimulates the two stages of mineralisation: nucleation and growth. Several matrix proteins are probably involved in this process. After initiation, more mineral is added to the collagen, primarily onto already existing crystals that grow, arranged along side the collagen fibres in the osteoid. When bone is fully mineralised, it contains about 70 % mineral but the content varies in different types of bone; woven (immature) bone contains less mineral than lamellar (mature) bone. Bone cells do not come in direct contact with the inorganic phase, but is surrounded by a thin layer of osteoid. [15]

2.1.3 Structure

On the microscopic level, two types of bone exist: woven and lamellar. Woven bone is immature and its collagen fibres are unorganized. In lamellar bone, the collagen fibres are arranged and the bone therefore has anisotropic properties. These two types are arranged into different types of bone structures, the two main types being cortical- and trabecular bone. Cortical bone is dense and has a higher mass whereas trabecular bone is spongy. Generally, trabecular bone makes up the insides of bones and cortical bone the outside. Cortical bone is made up of smaller structural units called osteons. It consists of a haversian canal, containing blood- and lymphatic vessels and sometimes nerves. Surrounding the canal are osteocytes lying in the calcified matrix. Periosteum covers the outermost part of the bone and consists e.g. of fibroblasts and progenitor cells. (Figure 2) [11,15]

![Figure 2: Section showing structural parts of the bone. Edited from Wikipedia.](image-url)
2.1.4 Fracture healing

In contrast to other types of tissue, damaged bone will regenerate without the formation of scar tissue. Fracture healing is often divided into primary- and secondary healing. Primary healing refers to repair of the cortex, and seems to occur only when the fracture is internally fixated with e.g. screws and plates. Secondary healing involves the periosteum and is a more complex process than primary healing. It is stimulated by motion of the fracture and inhibited by rigid fixation. Secondary healing can be divided into two different processes: intramembranous- and endochondral ossification. Endochondral ossification occurs closest to the fracture site and is brought about by the formation of a soft, cartilage-containing callus. The fracture attracts undifferentiated mesenchymal stem cells from the blood and surrounding tissues. The cells differentiate into chondrocytes that are responsible for making the callus and also for calcifying it. The calcified callus stimulates chondroclasts and osteoblasts, recruited from newly formed blood vessels, to resorb the cartilage and to lay down new bone, respectively. Intramembranous ossification occurs further away from the fracture site, beneath the periosteum. This process does not involve cartilage formation, but osteoprogenitor cells from the periosteum forms bone directly by forming a so-called hard callus. In intramembranous- and endochondral ossification, bone is laid down as woven, immature bone and later replaced by lamellar bone.

Usually, healing of a fracture site involves both intramembranous- and endochondral ossification and the whole healing process can be said to occur in 3 phases.

1) Acute inflammation – haematoma (blood collection outside of vessel) and angiogenesis (Figure 3a)
2) Repair – cartilage formation and resorption, bone formation (Figure 3b)
3) Bone modelling (Figure 3c)

The inflammatory cells in the haematoma provide signalling molecules required to initiate the cellular responses necessary for healing e.g. differentiation of MSC and angiogenesis. The inflammatory phase recruits cells to the fracture site, making callus formation possible by either of the two processes explained above. The two types of calluses, hard and soft, begin to form at approximately the same time within the first week. Even after the bone is united, modelling and remodelling continues in order for the bone to regain its shape and mechanical stability. [16,17]

Figure 3: Schematic image of fracture healing. After trauma, bleeding occurs around the fracture site, later forming a blood clot (a). Soft- and hard callus then form, causing bridging of the bone parts (b). Bone modelling restores the bone to its initial shape (c).
2.2 The screw model

In this project, a screw model was used to study fracture healing. In this model, a screw is inserted in intact bone of a rat, causing necrotic bone and a small cavity around the screw. This mimics the situation found at a fracture site. When left to heal, bone will grow around the screw, creating a bony “screw nut”. The strength of the new bone (the nut) is then analysed by a pullout test. By coating the screw with an active agent, or by affecting the bone by other means, change in fracture healing can be studied. However, if a difference in pullout force between control and test groups is seen, the model does not state why. It could be caused by either an increase of bone mass, improvement of bone quality or increased healing speed.

2.3 The wnt signalling pathway

The wnt family is a collection of glycoproteins functioning in embryonic development and in several stages of cell development later in life. The wnts are currently known to be involved in three different signalling pathways: the wnt/β-catenin pathway (also called canonical pathway), and the Ca\(^{2+}\) dependent- and the planar cell polarity pathway. [3] All three pathways are dependent on the frizzled (fz) receptor family, structurally similar to the G-protein-linked receptors. [18] The pathways all play a role in the formation of bone, but the canonical pathway is particularly important. [3,19] The focus of this section will therefore be on the canonical pathway.

The final functional part of this pathway is β-catenin. β-catenin is a protein involved in cell adhesion by connecting the cadherins spanning the cell membranes of two adjacent cells with the actin in the cytoskeleton inside the cells. It also functions as an important gene regulatory protein. [18] When the wnt signalling pathway is inactive (Figure 4a), β-catenin located in the cytosol is subjected to phosphorylation, leading to degradation of the protein. The phosphorylation is carried out by glycogen synthase kinase-3β (GSK-3β) in complex with adenomatous polyposis coli (APC) and axin. APC and axin serves as a scaffold to enable reaction between GSK-3β and β-catenin. [20]

When wnt proteins are present in the extracellular space (Figure 4b), they can bind to a receptor complex made up of a fz protein and low density lipoprotein receptor related protein 5/6 (LRP5/6). Both of these receptor parts are transmembrane proteins. However, the exact mechanism that follows this binding is not clear. The receptors are thought to activate the protein dishevelled (dvl), which inhibits the active form of the GSK-3β-complex mentioned above. Dvl binds GSK-3 binding protein (GBP), a protein that binds GSK-3β and inhibits its activity. Possibly, dvl also binds to axin and APC, furthering disrupting the breakdown-complex. [21,22] Because of this, β-catenin will not be phosphorylated and not broken down. The level of β-catenin therefore rises in the cytosol and when levels are high enough, β-catenin is transported to the nucleus. In the nucleus, it interacts with transcription factors lymphoid enhancer binding factor/T-cell factor (Lef1/Tcf) and regulates the activity of certain genes.
Figure 4: Non-active and active wnt signalling. With no wnt proteins present, β-catenin is degraded in the cytosol by GSK-3β (a). When wnt proteins bind to the receptors, the GSK-3β complex is inhibited, causing a rising concentration of β-catenin in the cytosol and finally its action in the nucleus (b).
The effect of wnt signalling in bone is complex and highly debated. The noticed macroscopic effect is often affected bone mass; [23] a reduced bone mass is seen in LRP5/6-deficient mice [24], and an increase is seen with augmented wnt signalling. [6] The cause for this is not yet fully established but there is evidence that the pathway:

- inhibits chondrocyte differentiation and stimulates osteoblasts differentiation of mesenchymal progenitor [25]
- enhances the proliferation of osteoblasts [4]
- inhibits apoptosis of both osteoblasts and osteocytes [26]
- inhibits osteoclast differentiation, leading to reduced bone resorption [27]
- works as a switch, deciding whether a cell should become osteogenic or adipogenic [28]

Chen and co-workers performed an animal study in order to investigate the role of wnt signalling in fracture repair. β-catenin seems to play different roles in undifferentiated mesenchymal cells and in osteoblasts and hence could have varying functions in different stages of fracture repair. [7]

### 2.3.1 Response to lithium

Lithium affects the wnt signalling pathway by inhibiting GSK-3β. Since GSK-3β is responsible for targeting β-catenin for degradation, inhibition of it will increase the amount of β-catenin, mimicking an active wnt signal. [2] This, combined with the apparent effect of wnt signalling on bone, has led to the idea to increase bone strength by the use of lithium. Multiple studies have been performed in order to investigate the effect of lithium on osteoblasts and on bone as a whole. The results are diverse, and sometimes conflicting, showing both positive [7] and negative [29,30] effects. However, most of the negative results come from older studies, usually with few experimental animals. An increase in bone mineral density has been seen, both in animals and in humans. [5,6] The cell response for lithium has also been studied, primarily its impact on proliferation and differentiation of MSC and osteoblasts. Inhibition as well as stimulation of MSC differentiation has been seen in different studies. [7,31] β-catenin seems to be upregulated in osteoblasts and lithium stimulate their proliferation. [7,32] The response of the wnt signalling pathway to lithium is most likely dose-sensitive and could explain the conflicting results obtained by the different studies. The time of lithium regulation in bone development is probably another important factor, since there is an indication that MSC and osteoblasts respond differently. According to Chen and co-workers, one should not affect the cells with lithium until they are committed to the osteoblasts lineage. When stimulating with lithium at this later stage, a positive effect on bone healing is seen. However, increased signalling in mesenchymal stem cells seems to hinder further differentiation of the cells and repair of the fracture. [7] It is also possible that lithium can affect other signalling pathways than wnt and in this way affect bone. [32]
2.4 The sol-gel process

The traditional methods to make glasses and other ceramic materials require high temperatures due to the high melting point of silica (~1650 °C), a principal component in many types of glasses. By using the sol-gel process, which has been used since the 1950ies, ceramic materials can be made at much lower temperatures, around 500 °C. The process can be divided into several steps that can roughly be described as: (M indicates metal and R indicates an arbitrary alky group.)

i) A solution of precursors in an organic solvent is made

ii) The solution is turned into a “sol”, a colloidal suspension of nanometer sized particles in liquid

iii) The sol is turned into a gel by:
   1) Hydrolysis, M-OR + H₂O → M-OH + ROH
   2) Alcohol condensation, M-OR + M-OH → M-O-M + ROH
   3) Water condensation, M-OH + M-OH → M-O-M + H₂O

iv) Aging at room temperature

v) The gel is formed into the appropriate shape

vi) Drying turns the gel into a xerogel

vii) The gel is converted to a ceramic material by curing in an oven

The precursor (i) can be any molecule that can be cross-linked and turned into a network (e.g. metal alkoxides, acetylacetonate). Metal alkoxides (M-OR, e.g. titanium- or silicon alkoxides) are often used to create oxide ceramics because they are easy to purify, dissolves easily in organic solvents and are easily cross-linked. They also react readily with water, which is used when turning the solution into a sol (ii). When mixed with water, often with a bit of acid added, the alkoxides are hydrolysed (iii, 1) and can then easily be polycondensated into a gel. Both alcohol- (iii, 2) and water condensations (iii, 3) are taking place. The reactions mentioned above do not actually occur stepwise, but rather simultaneously. When the gel has formed, it is allowed to “age” for some hours (iv), extending the network throughout the gel. The shaping of the gel (v) can be made by several methods. It can simply be cast into a ready-made shape, spin- or dip coated. In air, the gel dries and becomes a xerogel (dry gel) (vi). Finally the gel is cured in an oven at varying temperatures and times (vii). This evaporates the solvents and further densifies the gel, leaving a porous network of the precursor molecules. [33]

2.4.1 Sol-gel derived ceramic materials in bio-applications

Ceramic materials have been used in medicine for many years, primarily because of their mechanical properties. The use of sol-gel derived materials for orthopaedic applications has proven to be successful, both as solid materials and as coatings, and used e.g. as scaffolds for bone tissue engineering. [9] Sol-gel derived coatings can be synthesised for specific in vivo responses (e.g. inert, in growth of tissue, degradable) by changing factors such as network pore size and coating thickness. By coating a bioinert material with a sol-gel having the appropriate characteristics, it can be made bioactive. The bioactivity of a sol-gel ceramic is often evaluated as the ability to induce formation of hydroxyapatite crystals on the surface. The formed crystals function as a binding interface between the implanted material and the surrounding bone, improving fixation. For sol-gel derived titania, one often wishes to resemble the bone bonding ability of titanium. [34] Both in vitro and in vivo studies have
been made for several sol-gel materials, proving that hydroxyapatite can nucleate on sol-gel derived silica- and titania. [35] Just as for conventional titanium implants, the presence of hydroxyl groups and surface structure of the sol-gel derived coating, seem to be important factors for hydroxyapatite formation. If the particles forming the sol-gel derived material are nanostructured (<100 nm), crystal formation can come about. Particularly particles between 5-50 nm in size promote extensive crystal formation. Sol-gel derived titania have also showed good soft tissue attachment. [36,37]

2.4.2 Nomenclature

It is important to note that the word sol-gel refers to the liquid gel formed in the beginning of the process. The resulting material appearing after curing is not a sol-gel, but a ceramic material. Often, this is also referred to as a sol-gel derived material. In this report, the word sol-gel derived coating, or just coating, will be used when referring to cured coatings on wafers or screws. Lithium sol-gel is a sol-gel containing lithium chloride whereas pure sol-gel states that there is no lithium present.

2.5 Analysis techniques

2.5.1 Ellipsometry

Ellipsometry is an optical method that can be used to investigate thin films, particularly film thickness. Light with a known polarisation is reflected on a surface. Depending on the thickness of the coating and its refractive index, the polarisation will change. By measuring the size of this change and by knowing the refractive index of the film, the film thickness can be calculated. A popular version of ellipsometry is null ellipsometry. Here, a compensator affects the light in such a way that it is linearly polarised when leaving the surface. The analyser is then set such as no light can pass through it. (Figure 5) Ellipsometric parameters Ψ and Δ represent angle adjustments in the polariser and the analyser needed to accomplish this. Information about these parameters is obtained when measuring, and used in the Mccrackin algorithm to calculate the film thickness. [38-40]

Ellipsometry was used to study how lithium incorporation changed the thickness of sol-gel derived coatings.

Figure 5: Ellipsometric setup. Light from a source is passed through a polariser, making it linearly polarised. A compensator further changes the state of the light. After reflection on the coating the polarisation has changed. The light passes through an analyser before reaching the detector monitoring the intensity.
2.5.2 Atomic Force Microscopy

Atomic force microscopy (AFM) is a type of scanning probe microscopy and is mainly used to retrieve information about the surface topography of a sample. This is done by moving a nanometre-sized tip mounted on a cantilever close to, or in contact with the sample. Because of the close proximity between the tip and the surface, a force is created. By keeping this force constant with a feedback loop, the cantilever deflects according to the topography when moved over the sample. A laser beam registers the deflections of the cantilever and an image of the surface is created (Figure 6). [41] Different measuring modes can be used depending on the wanted information and the sample in question. [42]

![Diagram of Atomic Force Microscope](image)

**Figure 6**: Atomic force microscope. The reflected laser beam monitors the deflection of the tip caused by the surface structure.

As mentioned in 2.4.1, surface topography is an important factor when it comes to trying to predict how implants will be accepted by tissue. AFM was in this study used to investigate how incorporation of lithium changed the surface structure of the sol-gel derived coatings.

2.5.3 Simulated Body Fluid

Ability to bind to living bone tissue is often a desirable property when it comes to material used in orthopaedic applications. It has been shown that surface apatite formation is a requirement for implants in order to bind to bone. An in vitro technique created to evaluate this property is based on placing the material in a simulated body fluid (SBF). The solution contains multiple ions at concentrations also present in blood plasma. The calcium and phosphate rich layer that forms between an artificial material and bone in vivo also forms on a material surface in SBF. [43] Apatite formation can be evaluated using scanning electron microscopy (SEM). [44]
2.5.4 Scanning Electron Microscopy

In conventional microscopy, light is used to view a sample at large magnification. As the name implies, in scanning electron microscopy, electrons are used to retrieve an image with even higher resolution. High-energy electrons are emitted from an electron gun and focused on the sample. The atoms in the surface region of the sample will interact with these electrons and emit various types of radiations. Because of the impact with the electrons, the atoms in the surface region emit secondary electrons and by measuring the intensities of these, an image of the surface can be created. Emitted secondary electrons cause electron holes in the atoms that can be filled by outer electrons. This creates x-ray radiation, and since every element has a specific atomic structure, this provides element information. The technique for element analysis is called energy-dispersive X-ray spectroscopy (EDX) [45].

During this project, SEM was used to study the bioactivity of the sol-gel derived coating, and to investigate its quality.

2.5.5 Atomic Absorption Spectroscopy

In Atomic Absorption Spectroscopy (AAS), atoms specific response to light is used in order to determine the concentration of a certain metal (analyte) in a liquid sample. First, the sample is atomised by heating in a flame, furnace or plasma. A hollow-cathode lamp is used as the light source, with the cathode being of the same element as the analyte. The element in the cathode is excited, causing emission of light with an element specific wavelength. When the light passes the atomised sample, the analyte will absorb the light, allowing only a fraction of the light to pass to the detector. The light is absorbed in accordance with Lambert-Beers law, which makes it possible to use the amount of light absorbed to calculate the analyte concentration in the sample. A monochromator is used to prevent light from the heater to reach the detector (Figure 7). [46]

AAS was in this study used to quantify the amount of lithium released from the sol-gel derived coatings.

Figure 7: Schematic image of atomic absorption spectroscopy. Light, specific for a certain analyte, is absorbed by the atomised analyte in the flame. The amount of absorbed light is correlated to the analyte concentration in the sample.
3 Materials and methods

A flow chart of the laboratory work can be found in Appendix A.

3.1 Materials

3.1.1 Substrates

To simplify evaluation of coating thickness and coating structure, light microscopy, ellipsometry, AFM and SEM measurements were made on flat silicon wafers. These were also used for initial lithium release studies. When using screws with a more complex surface structure, coating properties will most likely change. However, the variations between different coatings are probably the same. Stainless steel screws were used for SEM measurements, lithium release- and in vivo studies.

3.1.2 Chemicals

Details of chemicals used are presented in Appendix B. Milli-Q water (purified water, resistivity 18.2 MΩcm) was acquired from a Millipore system (Milli-Q Academic).

3.2 Preparation of lithium sol-gel derived coatings

3.2.1 Sol-gel recipe

Extensive research on developing sol-gels for medical applications has been made by Areva and co-workers. [47] The following recipe is a modification of sol-gels used by them.

Solution 1: 10.22 g of tetraisopropyl orthotitanate (TIO) was dissolved in 15 ml of 99.5 % ethanol during stirring and placed in an ice-bath. (Figure 8)

Solution 2: 840 µl of 65 % HNO₃ and 170 µl of Milli-Q water was added to 15 ml of 99.5 % ethanol during stirring.

Solution 2 was carefully dripped into solution 1, still kept in ice-bath and during stirring. The resulting solution was continuously stirred during one hour, after which 100 µl poly(ethylene glycol) 400 (PEG 400) was added. The resulting gel was left overnight for aging.

Figure 8: Schematic image of TIO. Alcohol and water condensations (indicated by the arrow) link the molecules to each other, creating a network.
3.2.2 Lithium incorporation into sol-gel

In a first attempt to incorporate lithium into the sol-gel, lithium titanate was used in order to resemble the titanate used in the original sol-gel recipe. In order to make the sol-gel, the precursor molecules need to be soluble in ethanol. Lithium titanate could not be dissolved in ethanol, not even when mixed 50/50 with TIO. A second try was made with lithium chloride instead of lithium titanate. Lithium chloride cannot replace TIO in the sol-gel since TIO is needed to build up the sol-gel network, but could potentially be mixed with it. Lithium chloride is easily dissolved in ethanol, but when TIO was added, a white precipitate formed. A clear solution is required to make the sol-gel; hence this route was also abandoned. Finally, lithium chloride was dissolved in ethanol and added to an already prepared sol-gel base. This trial was successful and therefore used in the project. The protocol for this was as follows: Sol-gels containing lithium were made by dissolving lithium chloride in 10 ml of 99.5 % ethanol and mixing this with 10 ml of sol-gel base. The amount of lithium chloride in the sol-gel was varied and correlated to the amount of TIO. Content of lithium chloride in a particular sol-gel is specified as a molar percentage, n(LiCl)/n(TIO) x 100.

3.2.3 Substrate preparation

Before coating, organic contaminants were removed from the substrates by washing them in a solution containing Milli-Q water, hydrogen peroxide 30 % and ammonia 25 % in a 5:1:1 ratio at 85 °C for 10 minutes (TL-1 wash). Wafers were subsequently cleaned by sonication in ethanol for 5 minutes. Screws were sonicated in acetone and ethanol, 5 minutes respectively, to resemble usual treatment of screws used for sol-gel coating before in vivo experiments, in order to remove hydrophobic contaminants. [47] Substrates were rinsed in Milli-Q and dried in nitrogen gas before use.

3.2.4 Dip coating

A dip coater (KSV Instruments, Finland) was used to coat the substrates with sol-gel (Figure 9). The substrates were fastened on an arm moving at a certain speed, predefined by a computer programme (KSV Instruments, Helsinki, Finland). The substrates were immersed in sol-gel and immediately pulled out at the same speed.

![Figure 9: Dip coater used to coat substrates with sol-gel.](image)

When pulled out completely, substrates were left to dry for one minute before curing in an oven (Heraeus, Germany) at 500 °C. After curing, the substrates were once again cleaned by sonication in the same way as stated above. This was done to ensure that if a second layer was used, it was properly fixed to the first one (Figure 10). When finished, the substrates were stored for further studies.
Coating speed and curing time varied for different coatings (Figure 11). When the substrate is drawn out of the container at a lower speed, more sol-gel will have time to flow off the substrate, resulting in a thinner coating. Curing times were chosen in relation to the number of coatings used, total curing time for a sol-gel coating was always 30 min.

<table>
<thead>
<tr>
<th>Type of coating</th>
<th>Coating speed [mm/min]</th>
<th>Curing time [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>single layer on wafer</td>
<td>49.7</td>
<td>30</td>
</tr>
<tr>
<td>multiple layers on wafer</td>
<td>29.7</td>
<td>30/number of layers</td>
</tr>
<tr>
<td>multiple layers on screw</td>
<td>0.7</td>
<td>30/number of layers</td>
</tr>
</tbody>
</table>

Figure 11: Coating speed and curing time for different types of coatings

### 3.2.5 Sol-gel derived coatings on wafers

Initial studies were made on silicon wafers. To investigate the effects of lithium on the sol-gel derived coating and to establish how much lithium it was possible to incorporate, four different types of lithium sol-gels were made. The content of lithium chloride in the sol-gels were 5, 20, 50 and 80% respectively. Initially, these sol-gels were used to make single coatings on wafers and studies were made. In an attempt to alter the release profile of lithium from the wafers, additional samples were made using multiple sol-gel layers. Two types of these multiple coatings were made; one was a lithium containing sol-gel was combined with a coating without lithium and one with multiple layers of a lithium containing sol-gel. The coatings used can be seen in figure 12.

![Figure 12](image)

**Figure 12**: Sol-gel derived coatings used on silicon wafers: single layers with varied amount of LiCl (a), double layers with varied amount of lithium + pure sol-gel (b), two layers with 50% LiCl (c), three layers with 20% LiCl (d)

### 3.2.6 Sol-gel derived coatings on screws

After evaluating the different lithium sol-gel derived coatings on wafers, selected coatings were applied to stainless steel screws. For this, an additional lithium sol-gel was made, containing 35% lithium chloride. Because of the more complex surface structure of screws compared to smooth silicon surfaces, a thinner sol-gel was required. 5 ml of lithium sol-gel was diluted in 15 ml of 99.5% ethanol. When coating screws with sol-gel for the in vivo study, the washing procedure was revised. The washing step in between coatings was limited to ethanol only (5 min), since evaluation proved that lithium was lost during the wash.
3.3 Characterisation of sol-gel derived coatings

3.3.1 Surface appearance
To study how the lithium content affected the general appearance of the coating, a light microscope (BX60, Olympus, PA USA) was used. Images were taken through the microscope with a digital camera (E-410, Olympus, PA USA).

3.3.2 Coating thickness
To establish the thickness of the coatings made, an ellipsometer (Rudolph Research AutoEL, NJ USA) was used ($\lambda = 632.8$ nm). Ellipsometric parameters $\Delta$ and $\Psi$ were obtained for the sol-gel coated surfaces as well as for a clean silicon surface. These parameters were then used to compute the coating thickness in a computer program based on the McCrackin algorithm.

3.3.3 Surface structure
Images of the topography of the sol-gel derived coatings were obtained using tapping mode on an atomic force microscope (Digital Instruments, NY USA).

3.3.4 Bioactivity in vitro
In vitro-evaluation of bioactivity of the coatings were made by immersing wafers in SBF. The recipe for the SBF-solution was obtained from Kokubo and co-workers [44] and can be found in Appendix C. Silicon wafers coated as described above were immersed in SBF in a test tube and placed in a 37 °C water bath for 14 days. The SBF-solution in each test tube was renewed twice a week. After two weeks, the wafers were taken out, rinsed cautiously and dried in nitrogen air. Presence of calcium phosphate crystals was subsequently evaluated with SEM.

3.3.5 Coating quality on stainless steel screws
The quality of the coating when applied to screws was studied. Cracks in the sol-gel derived coating usually occur when applied to complex structures and SEM was used to investigate the extent of this. Too many cracks could cause the coating to peel off. Based on this study, certain coatings were selected and used for lithium release studies. To further study the coating stability, these coatings were also observed in SEM after lithium release.

3.4 Lithium-release from sol-gel derived coatings
To study the release of lithium from the coating, the substrates were immersed in phosphate buffered saline (PBS), pH=7.4, to mimic the ionic environment in the body. (PBS recipe can be found in short in Appendix C) Substrates were put in test tubes, one substrate in each tube, and placed in a 37 °C bath. For every coating type, three replicates were used. Tubes containing sol-gel coated wafers ($A=2x(1x1.5)$ cm$^2$) were filled with 5 ml of PBS. Tubes with sol-gel coated screws ($A=0.25$ cm$^2$) were filled with 3 ml. 2 and 1.5 ml of fluid, for wafers and screws respectively, was drawn from the test tubes at different time points. At each time point, additional PBS was added, leaving the total volume constant (Figure 13).
Figure 13: Image illustrating sample handling at the first sample time point. Substrates were left in a heated bath between $t=0$ and $t=1$, allowing release of lithium. A sample volume was taken out (2 or 1.5 ml) for AAS measurements. An equal volume of PBS was added to keep the volume constant. The procedure was repeated at the subsequent sample time points.

Liquid samples taken from the test tubes were analysed with AAS (Perkin Elmer 1100, MA USA) to determine the lithium concentration. (Air-acetylene flame, lithium lamp, wavelength 670.8 nm used when measuring.) First, a calibration curve was made with known lithium concentrations in PBS. 100, 500, 1000 and 2000 µg/l were used as standards. For each sample, the instruments made three measurements. Between each measurement, a blank sample was run. Milli-Q water with 0.2 % nitric acid was used as blank since the difference in absorbance between PBS and milli-Q was considered negligible.

Since new PBS was added at each sampling time, this obviously diluted the concentration of lithium in the test tube. The correct total concentration at each time was therefore calculated using the following equation:

$$C_t = C_{(t-1)} + A_t - \left( \frac{V_{\text{total}} - V_{\text{sample}}}{V_{\text{total}}} \right) \times A_{(t-1)}$$

- $C_t$ = lithium concentration at sampling time point $t$ [g/l], $C_0=0$
- $A_t$ = value obtained from AAS at sampling time point $t$ [g/l], $A_0=0$
- $V_{\text{total}}$ = total PBS volume in test tube [l]
- $V_{\text{sample}}$ = volume of sample drawn from test tube for AAS measurements [l]

To analyse how much lithium that was lost during the washing step when preparing coated screws, ethanol and acetone used in this procedure was also analysed with AAS. Results were then evaluated before coating screws for in vivo experiment.
3.5 *In vivo study*

Male Sprague-Dawley rats with a mean weight of 350 g were used. The animals were kept three per cage with free access to food and water. The regional ethics committee approved the study and the animals were treated in accordance with institutional guidelines for treatment of laboratory animals. Surgical equipment were sterilised in an autoclave.

3.5.1 Pilot study

For patients receiving lithium treatment for bipolar disease, a suitable serum lithium concentration has been established. Lithium is used clinically in a narrow band between 0.4 and 0.9 mmol/l. Therefore, we wished to study the effect of this serum lithium concentration on fracture healing. To establish the lithium concentration in the drinking water needed to obtain an adequate serum concentration in rats, a pilot study was made. Two groups were used (N=3), receiving 900 and 1200 mg/l respectively of lithium chloride in the drinking water during one week. Water bottles were weighed each day, ensuring that the rats were drinking the lithium-containing water. Since the water consumption during the first days was low, particularly for the group receiving higher concentrations, sugar was added to the water to make it tastier. After one week, serum samples were sent to the centre for laboratory medicine at Linköping University hospital for analysis of lithium concentration.

Serum lithium concentrations for the group receiving 1200 mg/l of lithium chloride were the highest, with a mean value of 0.53±0.13 mmol/l, which is within the therapeutic region. The group receiving 900 mg/l had a mean concentration of 0.27±0.04 mmol/l. 1200 mg lithium chloride/l water was therefore used in subsequent studies. Receiving lithium in these concentrations did not seem to affect the animals’ general health or behaviour.

3.5.2 Effect of lithium *in vivo*

Thirty rats were used and divided into three groups (N=10). The rats were acclimatised in the laboratory for one week before surgery. During this time they received 1200 mg/l of sodium chloride with ~1800 mg/l of sugar added in their drinking water. This treatment was continued throughout the experiment for two of the groups. For one group, sodium chloride was switched to 1200 mg/l lithium chloride, investigating the effect of systemic lithium treatment.

10 stainless steel screws were prepared with a coating of two layers of sol-gel containing 50 % lithium chloride, as described above. Previously described washing procedures were used before coating. The screws were sonicated in 99.5 % ethanol for 5 minutes in between coatings. These screws were used in one of the groups receiving sodium chloride. 20 screws were TL-1 washed, left uncoated and autoclaved before use. These were used in the two remaining groups (Figure 14). Tweezers used to handle screws for *in vivo* experiments were TL-1 washed and kept in ethanol. Containers were cleaned with 99.5 % ethanol before use.
<table>
<thead>
<tr>
<th>Group</th>
<th>Screw</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lithium sol-gel coated</td>
<td>NaCl + sugar</td>
</tr>
<tr>
<td>2</td>
<td>uncoated</td>
<td>LiCl + sugar</td>
</tr>
<tr>
<td>3</td>
<td>uncoated</td>
<td>NaCl + sugar</td>
</tr>
</tbody>
</table>

**Figure 14:** Table of groups used in *in vivo* study, stating type of screw and drinking water.

Rats were anesthetised with isofluoran gas and received pre and postoperative analgesica. The right hind leg was shaved and cleaned with chlorhexidine. The rat was placed in a sterile glove in which a hole was made and the shaved leg pulled out. Sterile tape was wrapped around the paw and the leg was once again cleaned with chlorhexidine. A 5-6 mm longitudinal incision was made along the medial aspect of the rat tibia and the periosteum was deflected dorsally to the physis. A hole (Ø 1.2 mm) was made through the cortex, a screw was inserted and the skin sutured.

The amount of water consumed each day was monitored to ensure lithium consumption. Additionally, the rats were weighed to ensure weight gain. After 6 days of trial, the concentration of lithium in drinking water was lowered from 1200 to 900 mg/l since water consumption was low and the rats showed a slight weight loss. Serum samples from this group were sent for analysis.

All rats were euthanized after 14 days using carbon dioxide. The tibiae were harvested and the screws tested for pullout strength in a computerised material testing machine (100 R, DDL, MN USA), at a speed of 0.2 mm/s. Before pullout, the distance from the tibia physis to the screw was measured. The tibiae were mounted with the screw head pointing out through a hole (diameter 3.5 mm) in a metal plate and the screw head was fixed in a connector. Maximum force during pullout was regarded as the pullout force and the energy was determined from the area under the force-deformation curve from sampling start at 0.2 N until the load had dropped to 10% of the maximal value. Stiffness was measured as the slope of the force-deformation curve (Figure 15).

**Figure 15:** Force – deformation curve obtained for each pullout test.
To test whether systemic lithium treatment had an effect on bone as a whole, and not on fracture healing in particular, the contra lateral tibia was also harvested from each rat. A screw was inserted in the same way as during the surgical procedure. Immediately after, the screw was pulled out using the material testing machine. This test can be seen as an investigation of the strength of the intact bone. Since local treatment with lithium from the coated screw can be assumed to have no effect on the contra lateral tibia, this group was regarded as part of the control group.

To determine the serum lithium concentration in the animals receiving systemic lithium, serum samples from this group, as well as control samples from the other groups, were sent for analysis.

### 3.6 Statistics

SPSS 15.0 was used for statistical analysis. One-way analysis for variance was used with Tukey’s post-hoc test for pairwise group comparisons. Student’s t-test was used for comparisons between two groups. Results were considered significant when p<0.05.
4 Results

4.1 Characterisation of sol-gel derived coatings

4.1.1 Surface appearance

As an initial study, the coatings on silicon wafers were viewed in a light microscope. As can be seen in Figure 16, there is a great change in surface appearance as more lithium is incorporated. Images were also obtained after lithium release, that is, after immersion in PBS for 13 days (Figure 17). There is not a great difference between images in Figure 16 and 17. Five times magnification was used on all images.

Figure 16: Single layer of sol-gel with 5 (a), 20 (b), 50 (c) and 80 % (d) LiCl before lithium release. Inset: one layer of pure sol-gel.

Figure 17: Single layer of sol-gel with 5 (a), 20 (b), 50 (c) and 80 % (d) LiCl after lithium release during 13 days.
4.1.2 Coating thickness

Thicknesses of coatings were established using ellipsometry. Measurements were made on three wafers for each surface type, displayed in the graph as dots (Figure 18). Increased lithium concentration resulted in a thicker coating.

![Thickness of single sol-gel layers](image)

**Figure 18:** Thickness of single layers of sol-gel with 0, 5, 20, 50 and 80 % LiCl.

4.1.3 Surface structure

The differences in topography between pure sol-gel derived coatings and coatings containing varied amount of lithium were studied using atomic force microscopy. Both single (Figure 19) and multiple layers (Figure 20) were examined. The average peak-to-peak distance and peak height was calculated for each image, using three peaks for each calculation (Figure 21). Coatings with low amounts of lithium displayed surface structures similar to that found for a pure sol-gel derived coating. For coatings with higher amounts of lithium, the surface structure was greatly altered. Generally for multiple layers where the outer layer was pure sol-gel, this layer caused a reduction in peak-to-peak distance and peak height compared to single layers (image not shown). Three layers of sol-gel with 20 % lithium chloride had a surface structure similar to that for a single sol-gel layer with 20 % lithium chloride.
Figure 19: AFM images (1x1 µm) for single layers of sol-gel. In the reference surface (pure sol-gel without LiCl) (a), titanate nanoparticles can be seen. A sol-gel derived coating with 5 % LiCl (b) seems to have a finer grain size than a coating made from pure sol-gel. Coating with 20 % LiCl (c) appears to have rougher base structure, with a nanostructure still present on top. The surface structure of a coating with 50 % LiCl (d) is even coarser with significant change in particle size compared to the reference surface. Sol-gel derived coating with 80 % LiCl (e) has little resemblance with coating without lithium (a) and the surface structure cannot be considered nanostructured.
Figure 20: AFM images (1x1 µm) showing multiple layers of sol-gel. Two layers of sol-gel with 50 % LiCl (a), displays a directionality dependence, being nanostructured in one direction but not in the other. The same behaviour is seen for a coating with 80 % LiCl + coating without lithium (b).

<table>
<thead>
<tr>
<th></th>
<th>Amount of LiCl [%]</th>
<th>Figure</th>
<th>Peak-to-peak distance [nm]</th>
<th>Peak height [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single layers</td>
<td>0</td>
<td>19a</td>
<td>26.0±3.7</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>19b</td>
<td>26.0±8.0</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19c</td>
<td>27.3±3.2</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>19d</td>
<td>84.6±17.6</td>
<td>5.7±4.4</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>19e</td>
<td>112.0±59.3</td>
<td>73.3±40.9</td>
</tr>
<tr>
<td>Single layers with outer layer of pure sol-gel</td>
<td>5+0</td>
<td></td>
<td>14.3±4.9</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td></td>
<td>20+0</td>
<td></td>
<td>18.2±4.9</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td></td>
<td>50+0</td>
<td></td>
<td>43.0±13.9</td>
<td>4.4±1.1</td>
</tr>
<tr>
<td></td>
<td>80+0</td>
<td>20b</td>
<td>157.6±82.7 (horizontal)</td>
<td>29.2±18.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33.9±12.1 (vertical)</td>
<td>7.5±0.9</td>
</tr>
<tr>
<td>Double layers</td>
<td>50</td>
<td>20a</td>
<td>156.3±22.3</td>
<td>17.6±5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33.9±15.1</td>
<td>7.0±1.7</td>
</tr>
<tr>
<td>Triple layers</td>
<td>20</td>
<td></td>
<td>18.3±8.1</td>
<td>1.1±0.1</td>
</tr>
</tbody>
</table>

Figure 21: Table showing peak values for each coating, correlated with the pictures above.
4.2 Bioactivity in vitro

SEM was used to evaluate the presence of calcium phosphate crystals on silicon wafers after immersion in SBF for two weeks (Figure 22). White bead-like structures are calcium phosphate crystals, confirmed with EDX (Data not shown). Single layers with low amounts of lithium (5 and 20 %) showed very limited amounts of crystals, as did multiple layers with 20 and 50 % lithium chloride. Coatings with higher amounts showed no crystal formation at all. Surfaces with an outer coat of sol-gel without lithium showed nearly restored levels of crystals.

![Figure 22: One layer of sol-gel with 5 % LiCl (a) shows a very limited amount of calcium phosphate crystals. One layer of sol-gel with 20 % LiCl + one layer of sol-gel base on top (b) show plenty of calcium phosphate crystals on the surface. Two layers of sol-gel with 50 % LiCl (c) show a limited amount of calcium phosphate crystals. Substrate coated with pure sol-gel is covered with crystals when immersed in SBF (d).](image)

4.2.1 Coating quality on stainless steel screws

To investigate how sol-gel derived coatings with 35 and 50 % lithium chloride (selected from release studies from wafers) appeared on the more complex surface structure of stainless steel screws, SEM was used. Different numbers of layers were evaluated in order to establish how many layers could be applied to a screw (Figure 23). This showed acceptable stability for one up to three layers of sol-gel with 50 % lithium chloride, and for two up to four layers of sol-gel with 35 % lithium chloride. After selection of number of layers and subsequent immersion in PBS for lithium release studies, coatings were viewed again using SEM (Figure 24). These results did not show any clear difference in coating quality.
Figure 23: Uncoated screw (a). Screw coated with pure sol-gel (b). One (c) and two (d) layer(s) of sol-gel with 50% LiCl show fine cracks in the coating on the edges of the screw. Three layers of sol-gel with 50% LiCl (e) shows cracks both on the edges and within the threads. Two (f), three (g) and four (h) layers of sol-gel with 35% LiCl show cracks on the edges.
Figure 24: No clear difference of the sol-gel derived coating is visible for two layers of sol-gel with 50 % LiCl (a) or four layers of sol-gel with 35 % LiCl (b) after lithium release during 8 days, compared to before (Figure 23:d and h).

4.3 Lithium-release from sol-gel derived coatings

Lithium release from different sol-gel derived coatings were analysed as described in 3.4. The first trial was made on silicon wafers coated with single sol-gel layers containing varied amounts of lithium chloride (Figure 25), later with multiple sol-gel layers (Figure 26) and finally with multiple layers on stainless steel screws (Figure 27). Results are presented as mean values from the three replicates used in the experiment. Bars indicate standard deviation. Area of the substrates and volume of PBS used in the experiments are compensated for in the plot. When viewing the results, the release kinetics for all layers show an obvious burst effect. For some of the coatings with multiple layers, a prolonged release after the burst can be seen. The release profiles for screws are similar to those from wafers, but the total release is higher. AAS studies were also made of ethanol and acetone used as washing liquids when coating screws. As seen in figure 28, more lithium is lost when using acetone than when using ethanol.
Figure 25: Lithium release from single layers with varied amount of LiCl. A burst effect of lithium is visible, with no significant release after 1 hour.

Figure 26: Lithium release from multiple layers with varied content of LiCl. A burst effect of lithium is seen, with a continued release after 0.5 h for some of the coatings.
Figure 27: Lithium release from multiple layers with varied content of LiCl on screw. A burst effect of lithium is visible, with a small, continued release after 0.5 hour for one of the coatings. Because of a mishap, one replica of two layers of sol-gel with 50% LiCl was discarded. Displayed values for this curve are therefore means of two replicas.

Figure 28: Because of the visible burst release (Figure 25-27), the amount of lithium lost from coated screws during wash was assessed for different numbers of layers of sol-gel containing 35 and 50% LiCl.
4.4 Effect of lithium in vivo

4.4.1 Effect on fracture healing

The effect of systemic- and local lithium treatment was evaluated using a pullout test of the screw. The group receiving systemic lithium treatment had a pullout force of $58.9 \pm 10.5$ N, local lithium treatment $53.7 \pm 14.6$ N and control $52.1 \pm 8.7$ N. No significant difference was seen between either of the groups at level 0.05. The pullout forces for the different groups are presented in a scatter plot (Figure 29). Values for force, stiffness, energy and distance can be found in Appendix D. No correlation was found between force and distance.

![Pullout force, fractured tibia](image)

**Figure 29:** Scatter plot displaying pullout forces for screw inserted in tibia for trial groups. Horizontal lines represent mean values.

After six days of trial, the group receiving systemic lithium (1200 mg/l) had a mean serum lithium concentration of $0.49 \pm 0.08$ mmol/l. In hopes of increasing the water consumption and rat weight, as well as keeping the serum lithium concentration high enough, water lithium concentration was lowered to 900 mg/l. After euthanisation, serum lithium concentrations were once again monitored (Figure 30). The group receiving systemic lithium treatment showed lithium concentrations below the therapeutic region.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean lithium conc. [mmol/l]</th>
<th>St. dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic lithium</td>
<td>0.35</td>
<td>0.13</td>
</tr>
<tr>
<td>Lithium sol-gel</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Control</td>
<td>0.03</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Figure 30:** Serum lithium concentrations at the day of euthanisation for the groups used. The mean value for the group receiving systemic lithium lies below the therapeutic region (0.4-0.9 mmol/l). No significant difference between the group receiving local lithium treatment and control group.
4.4.2 Effect on intact bone

The systemic effect of lithium on intact bone was investigated using a modified version of the screw model. A screw was inserted in the contra lateral tibia after euthanisation and the pullout force of the screw was established using the material testing machine. The group receiving local lithium treatment was considered a part of the control group. The pullout force for the group receiving systemic lithium (24.5±3.6 N) was significantly higher compared to the control group (17.7±6.1 N). Pullout forces are displayed in a scatter plot, horizontal line representing mean values (Figure 31).

![Pullout force, contra lateral tibia](image)

**Figure 31:** Scatter plot displaying pullout forces for screw inserted in contra lateral tibia for trial groups. Horizontal lines represent mean values.
Discussion

4.5 Lithium incorporation into sol-gel

In a first attempt to incorporate lithium into the sol-gel, lithium was added in the beginning of the sol-gel process (3.2.2). Trials were made with lithium in two different forms: lithium chloride and lithium titanate. Due to poor solubility of lithium titanate in ethanol, this compound was deemed unsuitable for use. Hence, a choice was made to use lithium chloride since it is highly soluble in both water and ethanol. However, when mixed with tetraisopropyl orthotitanate it precipitated. It was therefore decided to add lithium chloride at a later stage, into an already prepared sol-gel base.

In the sol-gel recipe used in this study, tetraisopropyl orthotitanate functions as the precursor molecule, building the network in the sol-gel. By adding lithium to the sol-gel when the titanium network is ready, lithium is not likely to be incorporated into the network. Lithium will rather interact with the network as a whole, for example through electrostatic interaction. Positively charged lithium ions can possibly interact with negatively charged oxygen atoms in the sol-gel network. If lithium were to have been added in the beginning of the sol-gel process instead, there is a greater chance it would have been incorporated into the network, replacing some of the titanium atoms.

The way lithium is included in the sol-gel probably affects its release kinetics. The hypothesis that lithium is not chemically bound to the sol-gel is supported by the fact that a burst effect is seen when studying lithium release. This reasoning is also supported by others using lithium in a scaffold (although not made by the sol-gel process). [49] Binding of lithium in the network could potentially slow down the release but this is difficult to state without further research.

The aim of this project was not to optimise the incorporation of lithium into sol-gel, but merely to find a way to do it. Extensive studies of how lithium is incorporated have not been made. By using other lithium containing compounds, one can possibly find a way to incorporate lithium earlier in the sol-gel process and thereby alter the release kinetics. Many factors during sol-gel synthesis are important and have a great influence on the final properties for the sol-gel derived coating. Research has shown that mixing speed and mixing time during the synthesis affect the porosity of a sol-gel derived coating. Precursor and water concentration, stirring speed and time, curing time and temperature are examples. This will most likely also affect the release of an active agent within the sol-gel. [50]

4.6 Effect of lithium on sol-gel derived coating

The general appearance of the coating is visibly changed as more lithium is added to the sol-gel (Figure 16). Little difference is seen between pure sol-gel and 5 and 20 % lithium chloride. However, a greater difference is seen when the lithium chloride content is increased to 50 and 80 %. The change in colour is probably caused by different diffraction patterns due to an altered coating morphology. The three-dimensional structure of a coating derived from pure sol-gel does not seem to be greatly altered when adding small amounts of lithium (5 and 20 %). If lithium is primarily physisorbed to the titanate network and no complex chemical reactions take place, it is possible that the cavities in the sol-gel network can accommodate small amounts of lithium with only minor structural changes. When
increasing the lithium content, the network needs to expand to be able to house the new atoms. This hypothesis is also supported when viewing the results from the ellipsometry measurements (Figure 18). There is a little increase in thickness between pure sol-gel derived coating and coatings containing lower concentrations of lithium and no significant difference between coatings containing 5 and 20 % lithium chloride. There is a greater increase when raising the concentration to 50 and 80 %. However, it is important to point out that many factors influence surface thickness. These results should not be used to state total thickness but to study differences between coating types.

When studying the surface profiles from the AFM measurements (Figure 19), a greater variance is seen between coatings derived from pure sol-gel and from sol-gel containing small amounts of lithium. However, coatings with both 5 and 20 % lithium chloride, like coatings without lithium, seem to have a nanostructured surface, with a peak-to-peak distance around 26 nm and a peak height around 1 nm. When the lithium concentration is increased further, the nanostructure is altered. The larger amount of lithium affects the network to a greater extent and inhibits nanoparticles to take form during curing. For multilayered wafers (Figure 20), the final layer of pure sol-gel seems to reduce particle size. This layer seems to have the greatest influence on the sol-gel layer with 80 % lithium chloride. A directional dependence is created, probably caused by the dip coating. The same behaviour is seen for two layers of sol-gel with 50 % lithium chloride. If these surfaces are nanostructured or not is difficult to determine by calculations from a few peaks only. There seem to be a coarse and a fine structure present at the same time.

Lithium release did not greatly change the general appearance of sol-gel coated wafers (Figure 17). This indicates that lithium, and not the coating as a whole, is released when immersed in SBF. The network is intact and lithium merely leaves its cavities.

4.6.1 Bioactivity in vitro

The original sol-gel recipe, which was modified in this study, was developed with the aim to have a network with nanostructured dimensions in hopes that this would promote nucleation of calcium phosphate crystals. Although bioactivity was not the primary goal with the lithium containing sol-gel, we wished to test how lithium effected the promotion of crystal growth. It is obvious from SEM measurements of SBF-immersed coated wafers, that lithium drastically reduces the amount of crystals formed on the surface (Figure 22). Small amounts can be seen when low concentrations of lithium is used, whereas no crystals are visible when using high lithium concentrations. As mentioned earlier, deposition of calcium phosphate crystals depend on the surface profile being nanostructured and on the presence of hydroxyl groups [37]. When combining results from AFM and SEM, it is difficult to come to a conclusion. Coatings with low amounts of lithium are similar in surface structure to coatings without lithium, but still show a significant reduction when it comes to crystal formation. When coating with pure sol-gel on top of these layers (retaining nanostructure), crystal formation is regained to almost initial levels. Sol-gels containing higher amount of lithium show almost no crystals and the nanostructure for these surfaces is doubtful. Coating a sol-gel layer containing 80 % lithium chloride with pure sol-gel restores the crystal formation ability, even though the nanostructure is not obvious. These findings are probably evidence that the right surface structure alone is not enough to create nucleation of calcium
phosphate crystals. If hydroxyl groups are present in the pure sol-gel, it is possible that these are switched to lithium oxide groups and that this, rather than surface structure, limits crystal formation.

The importance of bioactivity for a specific coating depends on the application. The formation of calcium phosphate crystals is particularly important when firm attachment of bone to implant is necessary, e.g. for dental screws. The application intended for this project is a lithium releasing implant, with no specific demand on load bearing. This effect of the calcium phosphate crystals was neither tested during the pullout test in the in vivo experiments. Pullout primarily measures the strength of the bone surrounding the screw and not screw attachment.

4.7 Lithium-release from sol-gel coated wafers

The first studies were made from coatings consisting of single layers of sol-gel (Figure 25). The results prove that lithium is in fact released from the coating and is, not surprisingly, increased with increasing lithium concentration. However, a burst effect is clearly visible, with no significant release of lithium after one hour. This phenomenon is common for release of functional agents from scaffolds and has e.g. been showed in other lithium studies. [49] In order for the coating to have a considerable positive effect on bone cells when used in vivo, lithium release probably needs to continue for a longer period of time. This is because lithium is not stored in body tissue to any greater extent and to effectively influence cells in bone tissue, new lithium need to be distributed continuously. [51]

In order to manipulate the release profile of lithium, multiple layers of sol-gel was used for coatings (Figure 26). A hypothesis was that if a layer of pure sol-gel covered lithium-containing layers, this could delay the release of lithium. Instead, the results show that this layer blocks the release. The profile for these coatings and the ones in the earlier studies are the same (showing a burst release) but the coatings with a pure sol-gel layer on top display a much lower total release. However, when all layers contain lithium, a change in the release profile is seen. The burst release is still significant but the release is continued for a longer time, particularly for two layers of sol-gel with 50 % lithium chloride. Three layers of sol-gel with 20 % lithium chloride also show a prolonged release, even if it is not as high as for 50 %. After this, a new sol-gel containing 35 % lithium chloride was created and this, along with sol-gel with 50 % lithium chloride, was applied to stainless steel screws for further tests.

4.8 Coating quality on stainless steel screws

After curing, cracks can sometimes appear in the sol-gel derived coating. If too many cracks appear, it can affect the stability of the coating. The risk is particularly high when the coating is applied to complex structures, e.g. a screw. To be sure to use the most stable coating for in vivo experiments, coated screws were studied in SEM (Figure 23). Screw coated with pure sol-gel show a few cracks, which is common. One and two layers of sol-gel with 50 % lithium chloride, and two, three and four layers of 35 % all show fine cracks on the edges of the threads but overall the attachments of the coatings seem good. However, three layers of sol-gel with 50 % lithium chloride show cracks even inside the threads and the stability of this coating is therefore doubtful. Because of these results, two layers of sol-gel with 50 % lithium chloride and four layers of sol-gel with 35 % of lithium were chosen to use on screws.
Screws covered with these coatings were used in a lithium release study. To further monitor the stability, the coatings where viewed in SEM after lithium release (Figure 24). There is no apparent difference between the before and after images, which indicates that the lithium detected during the lithium release studies is due to lithium leaving the coating, and not to a collapse of the coating itself. To further assess the stability, the sample fluid from the release studies should be analysed in respect to titanium content. This can be done using AAS with a graphite furnace. In vivo, a stable coating is important not to cause an inflammatory response due to wear particles.

4.9 Lithium-release from sol-gel coated screws

The release profile for wafers and screws are similar, except that the total release from screws are higher (Figure 27). This is a bit surprising, considering that the sol-gel used is thinner than the one used for wafers. The dip coating speed is also slower and hence the layers probably thinner. However, there are several possible explanations for this: The area of a screw surface is difficult to calculate and can differ from the one stated in this report. It is also difficult to know how the sol-gel responds to such a complex structure. Sol-gel probably accumulates within the threads, resulting in higher lithium content. When calculating the release from coated wafers, equal amount of sol-gel is assumed to fasten to both the front and the backside of the silicon wafer. If this is not true (e.g. because one side is oxidised and one is not) the area in the calculations is too big, giving a smaller release and a greater difference between wafer and screw.

The large standard deviation for four layers of sol-gel with 35 % lithium chloride is probably an effect of the many layers used, giving room for greater variation. There is no large difference in the profile between the two coatings, except that two layers of sol-gel with 50 % lithium chloride show a greater total release of lithium than 35 %, during the eight days of trial. This coating was therefore used in the in vivo experiments.

Considering that a burst effect is visible for all coatings, lithium is likely to be lost during the washing procedure. To evaluate how much was lost, the washing liquids were analysed (Figure 28). For both coatings studied, more lithium is lost to acetone than to ethanol. This led to the exclusion of acetone sonication and the screws used for in vivo studies were washed in ethanol only.

4.10 Effect of lithium in vivo

4.10.1 Effect on fracture healing

The pilot study shows (as shown by others [7]) that it is possible to administer lithium to rats via drinking water. In general, the rats consumed more of the water with low lithium content. However, serum lithium concentrations were higher for the group receiving 1200 mg/l and this concentration was therefore used in the in vivo study.

When comparing pullout forces between groups, no significant difference was found (Figure 29). The hypothesised positive effect of lithium on fracture healing can therefore not be demonstrated in this study. There can be several reasons for lack of effect in this study. Firstly, it appears quite difficult to administer enough lithium through drinking water to reach therapeutic levels. It seems that lithium affects the taste of the water, causing the rats to drink less and to loose weight. When lowering the lithium concentration, the rats drank
more, causing a higher total intake of lithium. However, this was not enough to keep the serum levels high. Analysis of serum lithium concentrations at euthanisation showed lithium level below the therapeutic region, lower than those seen in the pilot study (Figure 30). This is probably due to the fact that lithium does not accumulate in the blood, and that a higher consumption of fluid with lower lithium concentrations cannot compensate the use of higher lithium concentrations. In other animal studies using lithium, different routes of administration have been used e.g. gavage-feeding [6], intraperitoneal injections [30] and in drinking water [7] with different lithium concentrations. Apart from a lowered water intake, lithium did not seem to affect the animals’ health in general. Secondly, it is possible that the timing and duration of the lithium treatment was wrong. Chen and co-workers suggests that β-catenin has different functions at different stages in fracture healing. If β-catenin levels are high in early fracture healing, it will inhibit mesenchymal stem cells to differentiate and hence block bone formation. If levels are high at a later stage in fracture repair, it will stimulate the proliferation of osteoblasts and promote bone formation. [7] It is possible that a difference between groups could have been seen if evaluation had been made at a different time point. If increased β-catenin levels and stimulated proliferation of osteoblasts causes a faster fracture healing, an effect of lithium treatment could have been seen at an earlier time point. If, as many other studies implies [5,6], wnt signalling positively affects BMD, pullout testing at a later time point could possibly have shown a difference. To test whether an effect of lithium could be seen on intact bone, an addition test was made. (4.9.2)

The advantage of administering lithium locally, in this case from a sol-gel coated screw, is to specifically target the tissue of interest, bone. Wnt signalling is involved in many processes in the body and systemic treatment often causes side effects [53]. Local treatment might allow higher local concentrations without side effects. If the reasoning above is true, that lithium in fact has a positive effect on fracture healing although, for the reasons stated, it was not seen in this study, modifications of the coating needs to be made. Since no increase in released lithium is seen after 8 days, this amount is considered to be max. No in vivo studies where lithium has been distributed locally from a scaffold have been found. The necessary amount of released lithium for a positive effect in such a trial is difficult to evaluate. In a study made by Wang and co-workers, a lithium-containing scaffold was made and used in cell culture studies. The release from the most potent scaffold in that study reached its plateau after 24 h and released a total amount of 0.62 mg of lithium in 50 ml of fluid (not stating how large the scaffold was). In the cell study, this scaffold had a positive effect on the proliferation of human mesenchymal stem cells. Because the release from the scaffold also showed a burst effect, an effect on proliferation was only visible during the first three days. [49] The screws used in this project released $0.63 \times 10^{-3}$ mg of lithium in total. To assess the amount needed for effect in vivo is hard since the volume of the created cavity and the fluid flow around the screw is unknown.

It is likely though, that more lithium needs to be incorporated and, as mentioned earlier, this is linked to the synthesis of the sol-gel. By systematically changing factors in the synthesis, a sol-gel with higher lithium content might be found. Another problem, even though not unusual, is the burst effect. Prolonged lithium delivery is probably needed instead of most lithium being lost in the early phase. As well as optimising the synthesis procedure, which
potentially could solve this problem also, cross-linked collagen could be used. Collagen is degraded \textit{in vivo} at a speed predefined by its synthesis parameters. [54] By using an outer coating of collagen, lithium release could be delayed.

\section*{4.10.2 Effect on intact bone}
Because no effect of lithium was seen on fracture healing, a test was made to see whether an effect could be seen on intact bone. The contra lateral tibiae were harvested, a screw was inserted and the system was analysed in the material testing device. Contrary to the fracture-healing test, systemic lithium treatment had a significant positive effect on bone strength (Figure 31). This is in accordance with several studies previously mentioned in this report and supports the hypothesis that lithium has a positive effect on bone. [6,7] The dissimilarity in results from the two studies made supports the hypothesis mentioned above, that positive effect on fracture healing is time dependent. When treating the rats with lithium systemically, the bone around the inserted screw is not affected. This is probably due to the fact that the bone cells are not yet committed to the osteoblasts lineage. However, intact bone is strengthened by systemic lithium treatment through the mechanism stated in (2.3.1). These findings are promising, and indicate that there is a need for further investigation of the effect of lithium on fracture healing.

Possible methods to further evaluate the effect on intact bone could be histology and ash weight. With histology one can see if there is a difference in cell types present around the fracture site between the different groups. By comparing ash weights for bones from the different groups, difference in BMD can be estimated. In this technique, a part of a bone is weighed and dried in an oven at high temperature. The remaining ashes are then weighed and compared to the initial bone weight. [52]
5 Conclusions

• It is possible to incorporate lithium in the form of lithium chloride into a titanate sol-gel and apply it to silicon wafers and stainless steel screws by dip coating.

• Lithium affects the structure of the sol-gel derived coating, causing a coarser surface structure as more lithium is incorporated.

• Lithium affects the ability of calcium phosphate crystals to nucleate on the sol-gel derived coating when immersed in simulated body fluid. Even small amounts of lithium reduce the number of crystals formed. Crystal formation can be regained by applying an outer coat of pure sol-gel.

• Lithium is released from the coating when immersed in PBS, showing a burst effect, and can be detected using atomic absorption spectroscopy. The release profile can be manipulated by using different concentrations of lithium chloride in the sol-gel and different number of layers.

• Lithium can be administered to rats via their drinking water, causing serum lithium levels in the therapeutic region. However, the intake of lithium containing water is low.

• When using a screw model in rats, no significant difference on pullout force was found between groups receiving local- and systemic lithium treatment compared to a control group, when testing a screw surgically inserted in tibia. However, lithium seems to have an effect on the strength of intact bone, shown by comparing pullout force for screws inserted in a harvested bone. This further establishes lithium treatment as a possible method for influencing bone strength and, in the future, hopefully also fractures healing.
6 Future studies

The two main areas in this project were lithium release from a sol-gel derived coating and studying lithium’s effect in vivo. For further evaluation and improvement of these two areas, continued research is necessary.

To reduce the burst effect seen from the coatings derived from lithium containing sol-gel, the following trials could be made:

- Incorporating lithium earlier in the sol-gel process, making it a part of the sol-gel network.
- Optimising of sol-gel synthesis parameters, creating a sol-gel network better designed for release of an active agent. This will also affect particle size and hence bioactivity.
- Applying of an outer coat of cross-linked collagen delaying the lithium release.

To further assess the effect of lithium in vivo, the following studies are suggested:

- Raising the animals’ lithium intake, e.g. through higher water concentrations or injections.
- Testing the effect of longer systemic lithium treatment on pullout force.
- Starting the systemic lithium treatment at a later stage.
- Further evaluating lithium’s effect on intact bone in other ways than pullout force, e.g. by measuring ash weight.
7 Acknowledgements

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8 References

25. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Developmental Cell 2005;8(5):739-750.
48. Centre for laboratory medicine, Linköping University Hospital, May 2009
Appendix A – Laboratory flow chart

Boxes to the left describe layer used on certain substrates. Boxes to the right indicate methods of analysis used on those substrates. Number in brackets correlates to figures found in the report. Sg indicates pure sol-gel without lithium. Li-sg indicates lithium-containing sol-gel.
## Appendix B – Chemicals

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<tr>
<td>titanium tetraisopropoxide)</td>
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Appendix C – Recipes

Simulated Body Fluid

1. Rinse all beakers and containers in ethanol.
2. Heat up 750 ml of purified water to 36.5°C during stirring.
3. Add the following chemicals, one at a time:
   - NaCl, 7.996 g
   - NaHCO₃, 0.350 g
   - KCl, 0.224 g
   - KH₂PO₄, 0.228 g
   - MgCl₂·6H₂O, 0.305 g
   - HCl 1 kmol/m³, 40 cm³
   - CaCl₂ 0.278 g
   - Na₂SO₄, 0.071 g
4. Add 6.057 g of (CH₂OH)₃CNH₂ carefully to the solution, no more than 1 g at a time.
5. Measure pH and adjust it to 7.25.
6. Adjust the volume to 1000 ml.
7. Rinse container for storing twice with SBF before filling.
8. Fill a small test tube with SBF and put in a 36.5°C heated bath.
9. After 24 hours, check the test tube. If precipitates can be seen, discard the solution.
   If not, it is ready to use.

Phosphate Buffered Saline

For 5 l of PBS:
1. Dissolve in Milli-Q water:
   - NaCl, 44 g
   - Na₂HPO₄ x 12H₂O, 18 g
   - KH₂PO₄, 7 g
   - 0.4 % NaN₃, 20 ml
2. Adjust pH to 7.4 with 1M NaOH (~25 ml).
3. Adjust the volume to 5 l with Milli-Q water.
## Appendix D – Statistics

### Pullout test of surgically inserted screw

#### Descriptives

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#### Multiple comparisons

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Confidence intervals are presented as percentage of improvement for group I compared with group J.


Pullout test of screw inserted in contra lateral tibiae

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<th>Std. Deviation</th>
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Comparisons between groups

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Confidence intervals are presented as percentage of improvement for group I compared to group J.