Master´s Thesis

Local Delivery of Bisphosphonates from FibMat Matrix

Henrik Aronsson

LiTH-IFM-Ex-1910
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Improving the functionality and reducing revision rates are important driving forces in the development of orthopaedic implants. FibMat is a fibrinogen based matrix developed towards commercialisation by the company Optovent AB. This matrix can be coated on implants and act as a local drug delivery system for bisphosphonates (BPs). BPs are drugs inhibiting bone resorption, and applied with FibMat to improve stability of implants in bone, e.g. when fixing bone fractures. In this thesis, FibMat loaded with BP (FibMat/BP) was coated on stainless-steel screws and titanium screws in order to investigate some technology properties relevant to its clinical applicability. Bone-mimicking materials were used to study scrape-off effect upon insertion. The coagulation properties of fibrinogen as well as the structural properties of BPs were studied after exposure to gamma radiation. The screws were coated with FibMat and BP (alendronate and $^{14}$C-alendronate) using standard coupling techniques. The total amount and distribution of BP after insertion was measured by liquid scintillation and autoradiography. Coagulation assays were performed in order to determine the coagulation properties of fibrinogen, exposed to doses up to 35 kGy, mixed with thrombin. The structural properties of four different BPs (alendronate, pamidronate, zoledronate and ibandronate), exposed to doses up to 35 kGy were analysed by transmission infrared spectroscopy. The results show that FibMat/BP coating on porous stainless-steel screws is virtually unaffected by insertion into bone materials. The anodised, planar titanium screws are more affected by the insertion process, but an even BP distribution in the cancellous material is indicated. The coagulation assays show that gamma-irradiated fibrinogen has a slower coagulation process compared to non-irradiated fibrinogen and form interrupted network unable to clot. The chemical structures of the BPs seem unaffected by exposure to gamma irradiation. In conclusion, the FibMat/BP is a promising technology for local distribution of BP in conjunction with bone implants.
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Local Delivery of Bisphosphonates from FibMat Matrix

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ABSTRACT

Improving the functionality and reducing revision rates are important driving forces in the development of orthopaedic implants. FibMat is a fibrinogen based matrix developed towards commercialisation by the company Optovent AB. This matrix can be coated on implants and act as a local drug delivery system for bisphosphonates (BPs). BPs are drugs inhibiting bone resorption, and applied with FibMat to improve stability of implants in bone, e.g. when fixing bone fractures. In this thesis, FibMat loaded with BP (FibMat/BP) was coated on stainless-steel screws and titanium screws in order to investigate some technology properties relevant to its clinical applicability. Bone-mimicking materials were used to study scrape-off effect upon insertion. The coagulation properties of fibrinogen as well as the structural properties of BPs were studied after exposure to gamma radiation.

The screws were coated with FibMat and BP (alendronate and $^{14}$C-alendronate) using standard coupling techniques. The total amount and distribution of BP after insertion was measured by liquid scintillation and autoradiography. Coagulation assays were performed in order to determine the coagulation properties of fibrinogen, exposed to doses up to 35 kGy, mixed with thrombin. The structural properties of four different BPs (alendronate, pamidronate, zoledronate and ibandronate), exposed to doses up to 35 kGy were analysed by transmission infrared spectroscopy.

The results show that FibMat/BP coating on porous stainless-steel screws is virtually unaffected by insertion into bone materials. The anodised, planar titanium screws are more affected by the insertion process, but an even BP distribution in the cancellous material is indicated. The coagulation assays show that gamma-irradiated fibrinogen has a slower coagulation process compared to non-irradiated fibrinogen and form interrupted network unable to clot. The chemical structures of the BPs seem unaffected by exposure to gamma irradiation. In conclusion, the FibMat/BP is a promising technology for local distribution of BP in conjunction with bone implants.
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<th>Description</th>
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<tbody>
<tr>
<td>APTES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton (Da) is a small unit of mass</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FibMat/BP</td>
<td>A fibrinogen based matrix with immobilised bisphosphonates</td>
</tr>
<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray (Gy) is a unit that measures the deposited energy of radiation</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrogen fluoride</td>
</tr>
<tr>
<td>KBr</td>
<td>Potassium bromide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>PCSA</td>
<td>Polarizer component sample analyzer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>U</td>
<td>One enzyme unit (U) is defined as that amount of the enzyme that catalyses the conversion of 1 micro mole of substrate per minute</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström (1Å=1x10^-10 m)</td>
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1 INTRODUCTION

1.1 Background

Orthopaedic and dental implants are often subjected to high levels of mechanical stress. Therefore, improving implant functionality and reducing unwanted side-effects are driving forces to reduce complications which can lead to implant failure.

Bisphosphonates (BPs) are drugs that inhibit bone resorption by inactivating the osteoclasts [1]. Bone resorption commence in the vicinity of an inserted implant. BP shifts the balance between bone formation and bone resorption during the bone remodelling. This effect is desirable since reducing bone resorption may play an important role for early implant stability [2]. Clinical tests have shown that systemic administration of BP improves implant fixation in cancellous bone [3]. However, with systemic distribution of the drug unnecessary high amount is required for treatment which increases the risk for side-effects in the body.

The FibMat matrix from Optovent AB (Sweden) acts as a local drug delivery system, and can therefore effectively release BP in the vicinity of the implant. The FibMat matrix is a cross-linked fibrinogen-derived matrix into which BPs can be embedded (FibMat/BP). The matrix is coated on the bone implant device, producing a thin layer invisible to the eye. Promising results show an improved mechanical fixation of implants in rats, with FibMat/BP coated on stainless-steel screws [2,4,5,6] and on titanium screws [7].

By use of the technology platform FibMat, local release of BP at the surgical site can be achieved, leading to reduced resorption and increased mechanical support for the implant device during the critical initial phase.

Prior to clinical trials of the FibMat technology, several preclinical tests need to be made. Certain practical matters have to be considered in order to achieve a smooth transition from experimental testing, to clinical testing and industrial production.
1.2 Aims

The aims in this thesis were set to clarify some of the practical considerations regarding the FibMat/BP for future surgical applications.

A scrape-off study was performed to investigate to what extent FibMat/BP material is redistributed during implant insertion.

Biological consequences to gamma irradiation, such as used when sterilising implant devices, of fibrinogen were studied by implementing different coagulation assays.

The tolerance of different BPs to gamma irradiation was evaluated by investigating structural properties of the molecules.

A real-time study was made to construct and improve the properties of the FibMat/BP formation.
2 THEORY

2.1 Fibrinogen

Fibrinogen is a glycoprotein and plays an important role in blood coagulation. When a blood vessel is damaged the first response is the formation of a blood clot in order to prevent excess blood loss. Fibrinogen is cleaved by thrombin resulting in activation of fibrin monomers which start to self-assemble. This polymerisation forms a fibrin network that results in a clot. [8]

Fibrinogen has a plasma concentration of 2-4 mg/ml and a molecular weight of 340 kDa. The protein is composed of three different polypeptide chains α, β and γ, each present in two copies (figure 1). The chains wrap around each other to form a coiled-coil triple helix. The fibrinogen molecule consists of two regions. The central region which is composed of the N-terminal (N) ends, forms the E domain, and the distal region, which is composed of the C-terminal (C) ends, forms the D domain. [8,9]

Fibrinogen is soluble in blood plasma and the molecules show very little tendency to aggregate to form blood clots. Aggregation is prevented in large part by the fibrinopeptides A and B located on the N-terminal tails. Blood clotting is initiated when these fibrinopeptides are cleaved by the specific protein-cutting enzyme thrombin. The removal of the fibrinopeptides leads to spontaneous polymerisation of the formed fibrin monomers into fibrin network, resulting in a clot. [8,9]

Fibrinogen is the carrier structure in the FibMat matrix for bisphosphonate delivery. Fibrinogen is a building block from blood and is therefore not recognised as foreign to the body, which could provoke inflammation and/or coagulation. Results from [10] indicate that fibrinogen has close to no complement or coagulation activity when applied for matrix construction and fibrinogen is better then other proteins for building layer by layer structures. This gives the FibMat matrix high biocompatibility and an adjustable matrix to the drug bearing capacity desired.
2.2 Bisphosphonates

Bisphosphonate (BP) is a class of drugs that inhibit bone resorption by inactivating the osteoclasts. BP inhibits resorption by becoming engulfed in the osteoclast as it starts to resorb bone. BP then interferes with the intracellular metabolism killing the osteoclast. Inhibition also occurs by obstructing recruitment, adhesion and activity of the osteoclasts. [12]

In normal healthy bone there is a balance in bone remodelling, where osteoclasts constantly break down bone and osteoblasts replace old bone with new. Bone diseases and implant insertion can cause an increase in bone resorption, shifting that balance. BPs are used in treating diseases of excessive bone resorption, such as Paget’s disease, osteoporosis and bone metastasis. Clinical and animal tests are now conducted in order to improve implant stability after inserted into bone by systemic and local administration of BPs. [12]

The structure of BP consists of a P-C-P backbone which effectively binds to hydroxylapatite, the main mineral component of bone. The structure also has two side groups. One is commonly a hydroxyl group while the difference in the other group is what distinguishes the various BPs. BPs containing nitrogen has a more powerful antiresorptive activity compared to non-nitrogen BPs. The gradation in activity between the BPs is estimated from potency in relation to the non-nitrogen BP etidronate. [12,13]

The molecular structure of the BPs used in this thesis is shown in figure 2. They are all nitrogen containing BPs, also referred to as second generation BPs. They are investigated for
their effects in humans and are commercially available. Alendronate has the potency of \( \sim 500 \), pamidronate \( \sim 100 \), zoledronate \( \sim 10000 \) and ibandronate \( \sim 1000 \). [12]

\[
\begin{align*}
\text{Alendronate} & & \text{Pamidronate} \\
\text{Zoledronate} & & \text{Ibandronate}
\end{align*}
\]

\textbf{Figure 2}. The molecular structure of alendronate, pamidronate, zoledronate and ibandronate.

2.3 Gamma irradiation

Gamma irradiation denotes the process in which objects are exposed to gamma radiation for sterilisation or decontamination. Gamma radiation is one of the three types of natural radioactivity, together with alpha and beta radiation. Gamma rays are the most energetic form of electromagnetic radiation which consists of energy-bundles called photons. They originate from the nucleus of a radionuclide after radioactive decay. The emission of gamma rays does not alter the number of protons or neutrons in the nucleus but instead has the effect of moving the nucleus from a higher (unstable) to a lower (stable) energy state. Gamma ray emission frequently follows alpha decay, beta decay or other nuclear decay processes. A nucleus which is in an excited state, may emit one or more photons that can penetrate materials. The most
common source of gamma radiation for irradiation processing comes from the radioactive isotope $^{60}\text{Co}$. In a first step, $^{60}\text{Co}$ decays to excited $^{60}\text{Ni}$ by beta decay. Then $^{60}\text{Ni}$ drops down to the ground state by emitting two gamma rays in close succession. [14]

Gamma radiation causes ionisation of important cellular components, particularly nucleic acids, which results in the deaths of microorganisms. Gamma irradiation is therefore a popular method when sterilising implants and devices used in surgical applications. Gray (Gy) is a unit that measures the deposited energy of radiation. The validated absorbed dose used to sterilise medical equipment is 25 kGy. [15]

### 2.4 Coupling techniques

In order to achieve a strong covalent binding between the protein and the substrate, a chemical surface modification had to be carried out. Coupling techniques to form the fibrinogen multilayer matrix and the immobilisation of BPs are considered in this chapter.

#### 2.4.1 APTES silanisation

Silanisation allows proteins to be immobilised covalently to a surface by coupling of reactive groups on the surface to reactive groups of the proteins, via a linker molecule. In this thesis a thin film of 3-aminopropyltriethoxysilane (APTES) was coupled to a silica surface. The silica layer is instantly developed from a silicon surface when reacting with oxygen in the air. The silanes are hydrolysed, forming reactive silanes under the release of ethanol. Eventually these silanols undergo condensation reactions to form siloxanes at the hydrated silica surface, see figure 3. [16]

*Figure 3. Silanisation of a silica surface with APTES.* [16]
2.4.2 Glutaraldehyde

Glutaraldehyde (GA) (figure 4) is an amine-reactive homobifunctional cross-linker often used in biochemistry applications. GA has reactive aldehyde groups in both ends of the molecule and can therefore act as a linker molecule between two amine groups. In this thesis GA was used to link the amine groups of APTES with the amine groups of fibrinogen. [17,18]

![Figure 4. Schematic illustration of the attachment of an amine containing molecule to GA.](image)

2.4.3 EDC/NHS

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) is a cross-linker agent that together with N-hydroxysuccinimide (NHS) covalently couple carboxyl groups to primary amines, see figure 5. In this thesis EDC/NHS was used to form multilayer of fibrinogen and to immobilise BP to the protein. EDC reacts with the carboxyl group to form an amine-reactive O-acylisourea group that is hydrolysed. NHS is added to improve the process by transforming the unstable O-acylisourea group to an amine-reactive NHS ester, which is less susceptible to hydrolysis. The ester is then free to bind a primary amine containing molecule under the formation of a stable amide-bond. [19]

![Figure 5. Schematic illustration of EDC/NHS as a cross-linker between a carboxyl terminated group and a primary amine.](image)
2.5 Analysis techniques

2.5.1 Null ellipsometry

Null ellipsometry is an optical method often used to study adsorption of thin isotopic films on surfaces, by calculating thickness. Proteins can adsorb and form a multilayer that can range between 1-1000 Å and require methods with high sensitivity. This makes ellipsometry a suitable technique to analyse protein interaction on surfaces. [20]

When light is reflected at a surface, its phase and amplitude change. This change in polarisation state of the light depends upon the optical properties of the film, the surface and the ambient. The electric field component of the electromagnetic wave can be divided into two parts; one parallel (E_p) and one perpendicular (E_s) to the plane of incidence. The complex reflection coefficients for p- and s-polarised light can be described in equation 1 and 2, where i equals the incident light, r equals the reflected light and δ represents the phase-change after reflection. [20,21]

$$R_p = \frac{E_{ip}}{E_{ip}} = |R_p| \cdot e^{i\delta_p}$$  \hspace{1cm} \text{Equation 1}

$$R_s = \frac{E_{is}}{E_{is}} = |R_s| \cdot e^{i\delta_s}$$  \hspace{1cm} \text{Equation 2}

The ratio between the reflection coefficients is:

$$\rho = \frac{R_p}{R_s} = \frac{|R_p|}{|R_s|} \cdot e^{i(\delta_p - \delta_s)} = \tan \Psi e^{i\Delta}$$  \hspace{1cm} \text{Equation 3}

where Δ and Ψ are the ellipsometric angles, describing the change in phase and amplitude, respectively.
Figure 6 shows the Polarizer component sample analyzer (PSCA) null ellipsometer setup that was used in this thesis. Monochromatic light from a laser source passes a rotatable polarizer and becomes linearly polarized. The light’s phase changes through a fixed compensator to become elliptical polarized before reflecting onto the surface. Upon the surface the incident light changes phase and amplitude before reaching a rotatable analyzer and finally a photomultiplier detector. The ellipsometer varies the polarizer angle (P) until the reflected light is linearly polarized before it enters the analyzer where the angle (A) is changed until no light reaches the photodetector. Therefore the technique is called null ellipsometry. From the angles A and P the instrument can then calculate the amplitude change $\Psi$ and the phase change $\Delta$ caused by the film. [21]

From $\Psi$ and $\Delta$ the complex refractive index and the film thickness can be calculated by applying the Fresnel equations to the three-phase model (figure 7) and then use the McCrackin algorithm [22] to solve the equations. In transparent ambient, such as air, the refraction index is $N_{\text{air}}=1.000$ and refraction index for protein films are $N_{\text{film}}=1.465$. [20,21] 1 Å in ellipsometric thickness equals approximately 12 ng/cm$^2$ adsorbed molecules on the surface [4].
Ellipsometry is a method that has many advantages. It can be used both in situ and in air and it does not require any labeling. It is a fast and easy technique and also non-destructive to the sample. Antibodies can be used to receive information about interactions between different proteins during protein adsorption. Furthermore, the technique is restricted to surfaces that are flat and do not absorb the light, thus silicon and metals are suitable materials. [20,23]

2.5.2 Autoradiography

Autoradiography is a non-destructive technique for detecting radioisotope-labeled compounds within a solid sample, by producing an image on a radiographic film. The film is coated with photographic emulsion which consists of silver halide crystals (grains) suspended in a clear gelatinous phase. When the film is placed in close contact with the sample the silver halide crystals respond directly to beta particles or gamma rays emitted from the radionuclide in the sample. Each emission converts several silver ions to silver atoms to produce a stable latent image. When developing the film these silver atoms catalyse the reduction of the entire silver halide crystal to metallic silver to produce a dark visible image that is directly proportional to the amount of radiation reaching the film. Unexposed crystals are removed by dissolution in a fixer, giving an autoradiographic image of the radioisotope distribution in the sample. The energy from each beta particle or gamma ray is sufficient to render each grain it hits fully developable and many grains may be converted to the developable state by a single beta particle. The record provided by the photographic emulsion is sensitive and spatially accurate. It provides information on the localisation and distribution of radioactivity within a sample.
The method is best suited for detection of weak- to medium-strength beta-emitting radionuclides such as $^3$H, $^{14}$C and $^{35}$S. The problem when detecting highly energetic beta particles and gamma rays are limitations in sensitivity, because the emissions pass through the film resulting in energy loss. [24,25]

2.5.3 Liquid scintillation

Liquid scintillation is an analytical technique which can detect radioisotope-labeled compounds by converting the radioactive decay into light. Alpha and beta decay from radioactive samples can be measured and quantified when combined with a scintillation cocktail. The cocktail absorbs the energy emitted by radioisotopes and re-emit it as flashes of light. This is accomplished by the solvent and the phosphor (scintillator) in the cocktail. When a beta particle is emitted, the solvent assures efficient transfer of energy from the particle by solving the sample material for proper mixing. The solvent molecules absorb the beta energy and become excited. When returning to ground state, the energy of the solvent is emitted as UV light that excites other solvent molecules and scintillators. The light absorbed by the scintillators is emitted as blue light flashes which are converted to electrical signals in photomultiplier tubes. The light pulses produced are proportional to the amount of radiation present in the sample and data is presented as counts per minute (CPM). [26]

2.5.4 Transmission infrared spectroscopy

Infrared (IR) light is electromagnetic waves in the µm-mm range and is used in spectroscopic applications to examine compounds and thin molecular layers. When the frequencies of the incoming electromagnetic radiation and the vibrations correlate, energy is absorbed by the vibrating bonds within a molecule. When a molecule vibrates, the atomic distance changes, but leaves the position of the centre of mass unchanged. These vibration modes are independent vibrations that occur without affecting other atoms and exist mainly as stretching and bending. In order to become IR active, a vibration mode must change its dipole moment $\mu$, during motion. The direction and strength of this change, called the transition dipole moment $M_i$ for mode $i$ with the coordinate $r_i$, must be different from zero for IR activation, see equation 4.
\[ M_i = \frac{\partial \mu}{\partial r_i} \neq 0 \quad \text{Equation 4} \]

The absorption of energy increases the amplitude of the vibration without changing its frequency. This change in amplitude can be seen in an IR spectrum as an intensity peak at the frequency of the chemical bond that absorbed the energy. IR absorption positions are generally presented as either wavenumbers or wavelengths. [27,28]

Vibrational intensity:

\[ \text{Absorbance} \propto |M_i \cdot E|^2 \quad \text{Equation 5} \]

where \( E \) is the electric field strength.

In transmission infrared spectroscopy, light is passed through the sample and the absorbance is measured by a detector. It is possible to obtain an IR spectrum from samples in many different forms, such as liquid, solid, and gas. In this thesis we used solid samples that were grinded into a pressed pellet together with the IR transparent salt, KBr. The molecules in the pellet are randomly ordered and therefore give information about all vibration modes in the transmission spectrum. The majority of modern instruments use Michelson interferometer to produce interference patterns, along with Fourier transformation (FT) of the signal. This gives information of all frequencies simultaneously, allowing multiple samples to be collected and averaged together. This results in greatly improved sensitivity, much better frequency accuracy and time saving. [28,29]

2.5.5 Free oscillation rheometry

Free oscillation rheometry is a method for determining the viscoelastic properties of liquids. The principle is to set a sample into free oscillation while monitoring the frequency and the damping. The changes in frequency and damping are associated with the rheological characteristics of the sample. When the sample is repeatedly put in free oscillation the rheological properties can be measured as a function of time. Free oscillation rheometry is a
clinically used method for real-time studies of the coagulation of plasma or whole blood. [30,31]

![Image: The ReoRox4 instrument.](image)

In this thesis a ReoRox4 instrument (figure 8) from MediRox AB (Sweden) was used to determine the coagulation time by monitoring the changes in frequency and damping. The sample cup is placed on the sample holder which is connected to the instrument foundation by ultra-low friction bearings and a torsion wire, keeping the sample cup both radially and axially stable. The sample cup is then subjected to a forced turn towards a mechanical stop and thereafter released, initiating free oscillation around the vertical axis. Every 2.5 seconds another free oscillation is initiated [33]. An optical detector measures the frequency and the amplitude of the free oscillation continuously. [34]

The logarithmic damping (Ld) is given by equation 6, where $A_1$ is the amplitude in the first period and $A_n$ is the amplitude in the n:th period [34].

$$Ld = \frac{1}{n} \ln \frac{A_1}{A_{rel}}$$

Equation 6

At the initiation of clot formation in plasma or blood, the viscosity of the sample increases, which results in the damping increasing and the frequency decreasing [31].
The coagulation time is defined as the time when the alteration in damping and frequency exceeds a preset value [33].

2.5.6 Imaging coagulation

Imaging coagulation is a new method that visualises the blood coagulation process. The coagulation can be detected by image capture of light scattering from the developing fibrin network, because the network formed during the coagulation process scatters significantly more light than non-coagulated blood plasma. Samples are stored in cuvettes, fixed in special cuvette holders and illuminated by white light emitting diode arrays. Time-lapse images are captured and computer analysed to enhance the visibility of the fibrin formation. The images can be compiled and presented as time-lapse images, video sequences or converted to an intensity graph corresponding to fibrin formation. The method can be used to study the coagulation process in both time and space in bulk solutions as well as on surfaces. [35]

2.5.7 UV-visible absorbance spectroscopy

UV-visible absorbance spectroscopy is a common technique where a beam of ultraviolet or visible light is directed through a sample and intensity changes are measured with a detector. When a molecule absorbs light the additional energy causes electrons to be promoted to higher energy orbitals. The energy of the light being absorbed must match the energy required to promote the electron and therefore light with different wavelengths is absorbed differently by the sample. The fraction of the original light that passes through the sample is defined as transmittance (T) and the light absorbed by the sample is defined as absorbance (A). The relation between T and A is:

\[
A = - \log \left( \frac{T}{P_0} \right) = \log \left( \frac{P_0}{P} \right) \tag{Equation 7}
\]

where \( P \) is the light intensity after it has passed through the sample and \( P_0 \) is the initial light intensity. An absorption spectrum can be obtained with the absorbed light as a function of wavelength, which is useful for identifying compounds. The concentration of a sample can be
determined by measuring the absorbance at some wavelength and applying the Beer-Lambert law (equation 8).

\[ A = \varepsilon \cdot b \cdot c \]  

**Equation 8**

The quantity \( \varepsilon \) is called the molar absorptivity coefficient, \( b \) is the path-length of the sample and \( c \) is the sample concentration. [36]

In this thesis the fibrinogen concentration was investigated at a wavelength of 280 nm which is where proteins exhibit an absorption maximum [37].

### 2.5.8 Surface plasmon resonance

Surface plasmon resonance (SPR) is a surface sensitive technique for studies of biomolecular interactions in real-time, without the use of labels. Interactions, such as protein-protein, antigen-antibody, and receptor-ligand, are frequently analysed to determine concentration, specificity, and selectivity on prepared gold surfaces. SPR is a phenomenon that occurs when light is reflected off thin metal films. The incident light can at a certain angle interact with the delocalised electrons in the metal film (plasmon), thus reducing the reflected light intensity, see figure 9. The angle where this resonance occurs is dependent of the refractive index of the metal. Because of the extremely thin metal, adsorption or binding of biomolecules can be detected by changes in refractive index. [38]

![Figure 9. Principal set-up of Biacore instrument using SPR technology.][39]
In this thesis, a Biacore 2000 instrument from Biacore AB (Sweden) was used to investigate the characteristics of the FibMat matrix on bare gold surfaces. The instrument consists of a microfluid system, with four separate channels, that allows the test solution to be passed over the sensor surface during controlled flow. The immobilised biomolecules produce changes in the refractive index which are detected as changes in the SPR signal. The signal is usually expressed in resonance units (RU) and is proportional to the mass concentration of the biomolecules at the surface. By monitoring the changes of RU as a function of time a sensorogram is obtained, see figure SPR1. [40]

A change of 1000 RU represents about 1 ng/mm$^2$ of protein immobilised on the surface [41].
3 MATERIALS AND METHODS

In all experiments involving FibMat matrix formation and coagulation assays, human plasminogen-depleted fibrinogen from Calbiochem (USA) was used. Alendronate, zoledronate, pamidronate and ibandronate, all from LKT Laboratories (USA), were used in the BP experiments.

3.1 Preparation of implants and surfaces

Screws and surfaces were prepared in order to construct the FibMat matrix to which BP could be immobilised.

3.1.1 Stainless-steel screws

Stainless-steel screws from Emag (Sweden), measuring 1.7 mm in diameter (type M 1.7) and 3 mm in length, were used in the scrape-off study, see figure 10. The screws were etched for 40 min in 40% hydrogen fluoride (HF) (Merck, USA) to create a micro-porous surface. They were then washed in a basic hydrogen peroxide solution (5:1:1 proportions of H₂O, 30% H₂O₂ and 25% NH₄OH) at 85°C for 5 min, in order to remove organic contaminants, and rinsed thoroughly in MilliQ water. Next step was silanisation in 99% xylene (Merck, USA) and 1% APTES (ABCR, Germany) for 2 h. In order to remove weakly attached silanes the screws were ultrasonicated in xylene for 30 s and rinsed in xylene. The screws were dried with flowing N₂ and incubated in 6% glutaraldehyde (GA) for 30 min in phosphate buffered saline (PBS) at pH 9. The screws were incubated in 1 mg/ml fibrinogen (Calbiochem, USA) and PBS, pH 7.4, followed by rinsing in PBS and incubation for 30 min in 37.5 mg/ml EDC (Sigma-Aldrich, USA) and 5.75 mg/ml NHS (Sigma-Aldrich, USA), containing PBS, pH 5.5. Then a new 1 mg/ml fibrinogen solution in PBS, pH 5.5 was prepared and screws were incubated 30 min and rinsed in PBS buffer, and again incubated in EDC/NHS solution. The EDC/NHS solution is unstable at room temperature and new solutions were prepared every second hour. This procedure was repeated 10 times before immobilising BP onto the matrix. This type of prepared screw has been used in several previous studies on implant fixation [2,4,5].
Before immobilising BP, incubation in EDC/NHS solution for 30 min and rinse in PBS buffer followed by MilliQ water, was carried out. The screws were then incubated in a 1 mg/ml solution of 95% alendronate (LKT Laboratories, USA) and 5% 0.5 mg/ml \(^{14}\)C-alendronate (Moravek, USA) to obtain radioactivity of 5 \(\mu\)Ci/ml, in MilliQ water. Finally the coated screws were dried in flowing \(N_2\) and stored at 4°C in plastic tubes, which were sealed with plastic film, until further use in autoradiography and liquid scintillation experiments.

![Image 1](image1.png)

**Figure 10.** Image to the left shows the stainless-steel screw. The SEM image to the right shows the HF-treated stainless-steel surface [4].

### 3.1.2 Titanium screws

Anodised Gamma\(^{3}\)\(^{\text{TM}}\) Lag Screws (Stryker, Germany), referred to as titanium screws in this thesis, are clinical implants used in hip surgeries, see figure 11. They have a diameter of 10 mm and a length of 30 mm. These screws were not etched and only the screw-threads were FibMat-coated. Otherwise the titanium screws were prepared in the same way as the stainless-steel screws.
3.1.3 Silicon surfaces

Flat silicon wafers (Okmetic, Finland) cut in 5x10 mm pieces in the (1 0 0) crystal direction, were used to measuring the thickness of the FibMat matrix and BP immobilisation, with the AutoEL III null ellipsometer from Rudolph Research (USA). Except for the etching procedure, the silicon surfaces were prepared in the same way as the stainless-steel screws, see figure 12. In the experiment investigating gamma-irradiated fibrinogen, 1 mg/ml pamidronate was immobilised to the FibMat matrix instead of alendronate and $^{14}$C-alendronate. Because of the difficulties in measuring thickness on the curved and rough screws, silicon surfaces were also used as references to reflect immobilisation of fibrinogen and BP on the titanium and the stainless-steel screws. Before each measurement the surfaces were rinsed in MilliQ water, to remove salts from the PBS-buffer, and dried with flowing N$_2$. 

![Figure 11](image)

Figure 11. Image to the left shows the Gamma3™ Lag Screw and the SEM image to the right shows an anodised titanium surface.
3.1.4 Gold surfaces

Plain gold surfaces from Biacore AB (Sweden) were used in the Biacore experiments.

3.2 Scrape-off of BP after implant insertion into bone material

By using autoradiography, BP distribution along coated screws and bone materials after insertion, was examined. Liquid scintillation was also used in order to estimate total amounts of BP left on the screws.

3.2.1 Preparation of screws and bone materials

Stainless-steel screws and titanium screws were prepared as described in section 3.1. Reference thickness measurements of the matrix were performed on silicon surfaces with ellipsometry.

Two polymer materials with characteristics corresponding to cancellous and cortical bone were used to mimic surgical insertion of the screws. The cortical bone forms a hard outer shell, protecting the more porous cancellous bone. The cortical material, Renshape BM 5166 50/750MM from Huntsman (Belgium) [42], and the cancellous material from Freiburg Saw Bones 1521-171, are cured polyurethane resins. The bone materials were prepared in the workshop at Applied Physics, Linköpings University. Each material piece was sawed through

![Figure 12. The principal of multilayer fibrinogen film (FibMat matrix) after repetitive incubations of APTES and GA treated silicon surfaces in fibrinogen and EDC/NHS solutions. [10]](image-url)
and the surfaces were smoothened with a milling machine to reduce friction. The pieces were then joined together and fixed in place by screw-plugs. This procedure was performed so that the material could be opened and screws removed after insertion without damaging the FibMat/BP coating. By using this method the distribution of \(^{14}\)C-labeled BP will reflect the presence of BP after a surgical insertion.

For the stainless-steel screws, 3 mm deep holes were drilled in the centre of the sawed joint prepared above, with a diameter of 1.2 mm in the cancellous material and with a diameter of 1.3 mm in the cortical material [4,5]. The coated screws were screwed into the materials, which were then slowly opened up by removing the screw-plugs. The screws were carefully removed leaving two identical halves of the material with notches from the screw-threads. \(^{14}\)C-alendronate from screws and material-halves were then detected by autoradiography. The screws were also used in the liquid scintillation experiment.

For the titanium screws, the materials were prepared in different ways. The cancellous material was prepared as described above. 30 mm deep holes with a diameter of 6 mm were drilled using a specialised surgical drill from Stryker, which makes an approximately 3 mm deep funnel-shaped entrance to fit the screw-threads. The coated screws were screwed into the material with a specialised screwdriver from Stryker. The material was then carefully opened and the screws removed as described for the stainless-steel screws. Because of the wider diameter of the titanium screws, the notches from the screw-threads will go deeper in the material. The autoradiography film needs to be close to the scraped off \(^{14}\)C-labeled BP on the material for good exposure. The surface of the material-halves hollowed out by the screw, was therefore cut down with a milling machine and thereafter exposed to the autoradiography film. The screws were used in liquid scintillation to count the remaining \(^{14}\)C-alendronate.

The cortical material was sawed to a thickness of 5 mm and drilled through with the specialised drill producing the 3 mm deep funnel-shaped entrance. The screws were then screwed in 30 mm and the cortical material was then sawed through and carefully split apart to extract the screws without harming the screw surface. Liquid scintillation was then used to detect \(^{14}\)C-alendronate on the screws.
3.2.2 Detection of $^{14}$C-labeled BP by autoradiography

Five stainless-steel screws were used for each of the two bone materials. Also five reference screws not inserted to any material was examined. The cancellous material-halves of three titanium screws were also investigated using autoradiograph techniques. All equipment used for developing came from Kodak (USA).

The autoradiography films are highly sensitive to light. Therefore, all procedures are performed in complete darkness except for a specialised dark room lamp. X-Omat LS films were cut and carefully fixed in close vicinity to the sample surfaces. One film was fixed on each half of the material and two films were fixed on each side of the screw threads, producing a sandwich model. The samples were wrapped in aluminium foil, sealed in black plastic bags and put in a -20°C freezer for two weeks of exposure to the films. The films were then gently removed from the materials and screws before placed in a tray with GBX Developer and Replenisher for developing during 5 min with intermittent agitation. Agitation allows fresh developer solution to contact the film emulsion and maintain a constant development rate. The films were then removed and placed in running water for 30 s to remove unexposed developer. The films were moved to a tray with GBX Fixer and Replenisher for fixing, with moderate agitation during 8 min. The films were then washed in a tray of running water with a rate of 8 volume changes per hour for 8 min, before dried in a drying cabinet. The films were then digitalised with a scanner and the ImageJ software was used to calculate intensity profile from pixel values along the film, produced by the $^{14}$C-alendronate on the screw-threads.

3.2.3 Detection of $^{14}$C-labeled BP by liquid scintillation

The same 15 stainless-steel screws used in the autoradiographic experiment were used for the liquid scintillation tests. Because the whole screws were incubated when immobilising the FibMat/BP, the screw-heads was sawed off in order to detect only $^{14}$C-alendronate present on the screw threads. The three titanium screws from the autoradiographic test were used for the scintillation experiments. Three titanium screws prepared from the cortical material, together with three reference screws that had not been inserted to any material, were also used.

The screws were placed in scintillation cups and incubated in the scintillation cocktail Optiphase Supermix from Perkin Elmer (USA) for 2 h. Because of their size, the titanium
screws had to be removed from the cups before measurements could be made. The screws were therefore rinsed in scintillation cocktail and MilliQ water to minimize the loss of scintillators. Measurements were performed with the LKB Wallac 1217 Rackbeta liquid scintillator counter. The data are mean values collected during 300 s. The amount of $^{14}$C-alendronate was calculated from a standard curve with known $^{14}$C-alendronate concentrations in MilliQ water and the released amount of alendronate was assumed to be proportional to the released amount of $^{14}$C-alendronate.

3.3 Gamma irradiation of fibrinogen and BP

To investigate the impact of gamma sterilisation, fibrinogen, alendronate, pamidronate, zoledronate and ibandronate, were sent to ARTIM spol s r.o. (Czech Republic) for gamma irradiation. Fibrinogen and each BP had five samples that were irradiated with the doses (kGy): 0, 5, 15, 25 and 35. The samples were placed in eppendorf tubes, wrapt in parafilm and sealed in plastic bags before sent for exposure.

3.4 Properties of fibrinogen after gamma irradiation

Gamma-irradiated fibrinogen was analysed with different coagulation assays in order to investigate biological changes of the protein. The gamma irradiation induced effects for the construction of the FibMat matrix was also examined. In the coagulation experiments, fibrinogen solution with concentration 1 mg/ml, human thrombin solution from Sigma-Aldrich (USA) with enzymatic activity 0.5 U/ml, and PBS buffer with pH 7.2 was used.

3.4.1 Coagulation process

The fibrinogen coagulation process was examined by two methods.

- Imaging coagulation was used to observe the fibrin network formation of the irradiated fibrinogens. Fibrinogen solution with doses of 0, 5, 25 and 35 kGy was transferred to 1.5 ml polymethyl methacrylate (PMMA) spectrophotometric cuvettes from Kartell (Italy) and thrombin was added to initiate coagulation. The cuvettes were quickly fixed in the cuvette holder and the developed fibrin network was detected by images taken every five seconds with an EOS 400D camera from Canon (Japan).
Images of 0 kGy and 35 kGy irradiated fibrinogen was also captured after the coagulation process had stabilised, in order to investigate the clottability of the fibrin network.

- A ReoRox4 rheometer from Medirox (Sweden) was used to compare the coagulation time of the irradiated fibrinogens. Three samples of each irradiated fibrinogen solution were prepared in polyamide cups. Thrombin was mixed to every sample and quickly transferred to the rheometer for detecting the coagulation times.

3.4.2 Clottability

The effect of gamma irradiation on fibrinogen clottability was analysed by three different methods.

- By using the absorbance spectrometer Multiscan spectrum from Thermo Fisher Scientific (USA), the optical density at 280 nm was measured before and after coagulation. Samples of dissolved fibrinogen from each dose was transferred to PMMA cuvettes (Kartell, Italy) and measured three times, with PBS buffer as a blank. Then three samples for each gamma dose were mixed with thrombin to initiate coagulation and incubated for 30 min at room temperature. The formed gelatinous mass was then gently torn with a wooden stick, in order to get good separation of the mass and the supernatant after centrifuging at 4000 g for 15 min. The supernatant was removed and measured three times, alongside a PBS-thrombin solution as a blank. The quotient fibrinogen not participating in the network formation was compared between the different doses.

- Using the optical microscope Axio Observer D1 from Zeiss (USA), the network formation was examined in more detail. The formed network of fibrinogen irradiated with 0 kGy and 35 kGy was sampled onto microscopic slides for exposure. Captured images of the network were analysed with the Axio Vision software.

- Visual inspection was performed in most coagulation assays mentioned above. The sample cups/cuvettes were tilted and held upside-down, in order to detect changes in consistency of the sample. When the sample has transformed to a gelatinous clump and is unaffected of the upside-down treatment, the sample is said to be clotted. [43]
3.4.3 FibMat matrix

The AutoEL III null ellipsometer from Rudolph Research (USA) was used to investigate the impact of gamma irradiation on the FibMat matrix formation process. The FibMat matrix was constructed on silicon surfaces as described in section 3.1.3 with ten layers cross-linked fibrinogen to which pamidronate was immobilised. The 0 kGy and 35 kGy fibrinogen samples were examined on three surfaces each. After every fibrinogen layer the surfaces were rinsed in MilliQ water and dried with flowing N2, before measured in at least five distinct points.

3.5 Structural properties of BP after gamma irradiation

By using the transmission infrared spectrometer Bruker Vertex 70 from Bruker Optics (USA), structural changes of alendronate, pamidronate, zoledronate and ibandronate, due to gamma irradiation were examined and compared. 0.6 mg of each BP with doses of 0, 5, 15, 25 and 35 kGy, were grinded together with 300 mg potassium bromide (KBr). The samples were then pressed into a pellet with 7 tons of pressure for 10 min. Before measuring each sample, a reference spectrum was measured in order to nullify the possible presence of IR active air-molecules in the spectrometer. Each sample was then scanned 200 times resulting in a spectrum with information about the chemical bonds in the molecules.

3.6 Construction and improvement of FibMat/BP

With the Biacore 2000 instrument from Biacore AB (Sweden), a study of the FibMat/BP formation in real-time was performed. Untreated gold surfaces from Biacore AB were fixed to a chip-holder to fit the instrument. The first layer fibrinogen was directly adsorbed to the gold surface and the following fibrinogen layers and BP was immobilised using EDC/NHS coupling techniques. Different parameters such as fibrinogen concentration, BP concentration, flow-rate and flow-time, were tested in order to receive more information on the FibMat/BP.
3.7 Statistics

Results from coagulation process and amount BP on screws were analysed using one-way ANOVA and controlled with Bonferroni correction for multiple comparison. The data were expressed as mean values ± standard deviation (SD). Statistical differences marked with an asterisk * were defined at a 95% confidence level (p<0.05).
4 RESULTS

4.1 Scrape-off of $^{14}$C-labeled BP on stainless-steel screws

The stainless-steel screws and bone materials were prepared as described in section 3.2.1. Autoradiography was used to obtain the results of $^{14}$C-alendronate distribution on bone materials and screws after screw insertion. The quantitative results of alendronate on the screws were acquired by counting the $^{14}$C-alendronate with liquid scintillation.

4.1.1 Remaining BP on screws

Figure 13 illustrates the amount alendronate present on the screw-threads of the stainless-steel screws. Approximately 0.15 µg alendronate was present on the reference screws and approximately 0.12-0.13 µg alendronate was still present on the screw-threads of the screws after being inserted into cancellous and cortical material. No difference of significance (p>0.05) in amount BP between the inserted screws and the reference screw was detected. 150 ng alendronate on the reference screws from the scintillation test suggests approximately 375 ng/cm$^2$ alendronate on the macroscopic surface of the screw.

![Alendronate coated on stainless-steel screws](image)

**Figure 13.** The results show the average amount of alendronate on stainless-steel screw-threads by using the LKB Wallac 1217 Rackbeta liquid scintillation counter. Five screws inserted into cancellous material, five screws inserted into cortical material and five reference screws not inserted, were measured two times each. The two-way error bars represent SD and no significance (p>0.05) in amount could be established between the different screws.
Figure 14 shows the intensity profile of distributed $^{14}$C-alendronate along the screw-threads of two stainless-steel screws, after having been inserted into cancellous and cortical material, together with a reference screw not inserted. The graph shows an even distribution of $^{14}$C-alendronate along the 3 mm length of all three screws. The results indicate virtually no $^{14}$C-alendronate lost in the cancellous and cortical screws compared to the reference screw. The presence of $^{14}$C-alendronate extending the 3 mm length of the screw may be explained by the $^{14}$C-alendronate present on the tip of the screw. Even though $^{14}$C-alendronate is not in the vicinity of the film during exposure, the high amount of $^{14}$C will still affect the film to some extent.

![$^{14}$C-alendronate on screws](image)

**Figure 14.** The graph illustrates $^{14}$C-alendronate distribution along the screw-threads of two stainless-steel screws after being inserted into cancellous and cortical material, together with a reference screw not inserted. The ImageJ software was used to calculate intensity profile from the inverted pixel values of the autoradiography films. The film image used for each screw is one typical image out of ten. The x-axis represents the length of the screw-threads. 0 mm indicates the end of the screw-head and the start of the screw-threads.

4.1.2 Scraped off BP on bone materials

Figure 15 illustrates the distribution of scraped off $^{14}$C-alendronate on bone materials. Figure 15a shows the $^{14}$C-alendronate present in the cancellous material from the screw. The vertical broad dark area to the left in the image represents the $^{14}$C-alendronate scraped off at the material surface. The dark area to the right of that area shows the distribution of $^{14}$C-
alendronate in the material after contact with the screw-threads. In figure 15b the faint dark areas indicate a lesser amount of scraped off $^{14}$C-alendronate in the cortical material compared to the cancellous material. An intensity profile of the two images was performed and the result is illustrated in figure 16. The graph shows that more $^{14}$C-alendronate is scraped off from the screws along the cancellous material compared to the cortical material. The screw-threads are 3 mm in length and most $^{14}$C-alendronate is located approximately 1 mm into both materials. Thereafter, the scraped off $^{14}$C-alendronate steadily decreases in the materials, leaving only small amounts 3 mm into the materials.

Figure 15. The results illustrate two scanned autoradiography film images of the silver grains (dark areas) that are directly proportional to the $^{14}$C-alendronate distribution on the bone materials produced by the stainless-steel screws. Figure 15a (left image) shows scraped off $^{14}$C-alendronate on cancellous material and figure 15b (right image) shows scraped off $^{14}$C-alendronate on cortical material. Each of the two images is one typical image out of ten.
4.2 Scrape-off of $^{14}$C-labeled BP on titanium screws

The bone materials and titanium screws were prepared as described in section 3.2.1. Liquid scintillation was used to count $^{14}$C-alendronate in order to get quantitative results of alendronate on the screws. Autoradiography was used to obtain the results of $^{14}$C-alendronate distribution on the cancellous material after screw insertion.

4.2.1 Remaining BP on screws

Figure 17 shows the amount alendronate present on the titanium screws. The average presence of immobilised alendronate was approximately 0.65 µg on the reference screws, 0.3 µg on the cancellous screws and 0.06 µg on the cortical screws. The result indicates a significant ($p<0.05$) difference in amount BP between the inserted screws and the reference screws. Approximately 50 % alendronate is scraped off after screw insertion into the cancellous material and 90 % alendronate is scraped off after screw insertion into the cortical

\[\text{Figure 16. The graph shows }^{14}\text{C-alendronate distribution along the bone materials after having been in contact with screw-threads. The ImageJ software was used to calculate the intensity profile from the inverted pixel values of the images seen in figure 15. The x-axis represents the distance into the bone materials.}\]
material. 650 ng alendronate on the reference screws from the scintillation test suggests approximately 39 ng/cm$^2$ alendronate on the macroscopic surface of the screw.

![Alendronate coated on titanium screws](image)

**Figure 17.** The results illustrate the average amount of alendronate on titanium screw-threads by using the LKB Wallac 1217 Rackbeta liquid scintillator counter. Three screws inserted into cancellous material, three screws inserted into cortical material and three reference screws not inserted, was measured two times each. The two-way error bars represent SD and the * indicates a difference of significance (p<0.05) in amount BP between all three screws.

4.2.2 Scraped off BP on cancellous material

Figure 18 shows the distribution of scraped off $^{14}$C-alendronate on cancellous material. The dark area shows the distribution of $^{14}$C-alendronate in the material having been in contact with the screw-threads. The $^{14}$C-alendronate is almost homogeneously distributed in the material, producing a fingerprint of the screw. An intensity profile of the image was performed and the result is illustrated in figure 19. The graph illustrates an even distribution of $^{14}$C-alendronate along the entire distance produced by the 30 mm long screw. The intensity-dip at the beginning of the material is probably due to the funnel-shaped entrance, resulting in lesser BP scraped off.
Figure 18. The result shows a scanned autoradiography film image of the silver grains (dark areas) that are directly proportional to the $^{14}$C-alendronate distribution on the cancellous material produced by the titanium screw. The image is one typical image out of six.

$^{14}$C-alendronate on cancellous material

Figure 19. The graph illustrates $^{14}$C-alendronate distribution along the screw-threads area-length of the cancellous material seen in figure 18. The ImageJ software was used to calculate intensity profile from the inverted pixel values. The x-axis represents the distance into the cancellous material.
4.3 Gamma-irradiated fibrinogen

In order to determine how gamma irradiation affects the coagulation properties of fibrinogen, different coagulation assays were used in the experiments. In every assay 1 mg/ml fibrinogen, 0.5 U/ml thrombin and PBS-buffer with pH 7.2 were used.

4.3.1 Coagulation process

To investigate the coagulation process, thrombin was mixed with fibrinogen in PBS solution and coagulation studied using rheometry and Imaging coagulation.

According to the pre-set parameters for coagulation-time in the ReoRox4 instrument, only non-irradiated fibrinogen formed a clot, see table 1. The irradiated samples did not form a proper clot within the experimental time of 60 min. This was also confirmed with visual inspection. After the coagulation time had the 0 kGy fibrinogen samples formed a gelatinous clump and were unaffected when the cups were held upside-down. The irradiated fibrinogen samples showed no change in consistency after 60 min and poured out when the cups were held upside-down. Just prior to coagulation time of the non-irradiated fibrinogen, a small change in damping was seen. This damping-change could also be seen in the graphs from the irradiated samples. The small change in damping occurred because of changes in viscoelastic properties of the samples, assumed to originate from the activated fibrinogens starting to self-assemble and initiate the coagulation process. The time-point of this damping-change is used to illustrate the differences in behavior between the samples in figure 20. The results indicate that it takes longer time for the coagulation to be initiated, with higher irradiation dose. 15, 25 and 35 kGy fibrinogen show a significantly later coagulation start (p<0.05) compared to 0 kGy fibrinogen.
Table 1. Coagulation time could only be obtained for non-irradiated samples with the ReoRox4 instrument.

<table>
<thead>
<tr>
<th>Irradiation dose (kGy)</th>
<th>Average coagulation time (min)</th>
<th>SD</th>
<th>Number of independent samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.16</td>
<td>0.16</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>&gt;60</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>&gt;60</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>&gt;60</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>35</td>
<td>&gt;60</td>
<td>-</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 20. The delay for damping change to occur increased with irradiation dose when measured with the ReoRox4 instrument. Three independent samples were used and the two-way error bars represent SD. The irradiation doses marked with * show a significant time difference (p<0.05) compared to the non-irradiated dose.

Figure 21 illustrates the network formation of the different fibrinogens using Imaging coagulation. The 0 kGy and 5 kGy irradiated fibrinogens show resemblance in the curves. They both quickly reach maximum network development, although 0 kGy is more concentrated. This could also be seen visually during the network formation in the cuvettes. 0
kGy emerged as a homogeneous milky mass (figure 23a), while 5 kGy had an almost transparent homogeneous appearance. The 25 and 35 kGy curves show a steady increase in intensity, implying it takes longer time for the network development to become saturated, compared to 0 and 5 kGy fibrinogens. During the experiment, irregular threads became visible in the 25 and 35 kGy fibrinogen solutions, see figure 23b. The thick threads floated freely in the solution and were seemingly not able to link together, in order to form a homogeneous network.

![Network formation](image)

**Figure 21.** The network formation process in irradiated fibrinogen was studied by Imaging coagulation. Changes in light scattered from the developing network is shown on the y-axis, and time on the x-axis. Images were captured every five seconds.

### 4.3.2 Clottability

Network formation was also studied by absorbance spectroscopy and imaging. Purpose was to study amount of fibrinogen able to participate in network formation, and to observe differences in network structures. To examine the fibrinogen network formation, thrombin was mixed with fibrinogen in PBS solution.

Figure 22 illustrates how the amount of fibrinogen not participating in the network formation (free fibrinogen) is changed by exposure to gamma radiation. The data was given by measuring fibrinogen absorbance at 280 nm with the Multiscan spectrum instrument, before
and after removal of the network. Except for 5 kGy, the results show an almost linear increase in free fibrinogen with higher irradiation dose. Fibrinogen irradiated with 25 kGy and 35 kGy show a significantly higher percentage of free fibrinogen (p<0.05) compared to non-irradiated fibrinogen.

**Figure 22.** The graph illustrates average percentage of irradiated fibrinogen not participating in network formation. Three independent samples were measured three times each by light absorbance at 280 nm with the Multiscan spectrum instrument. The two-way error bars represent SD and the irradiation doses marked with * show a significant higher percentage of non-coagulated fibrinogen (p<0.05) compared to 0 kGy fibrinogen.

Figure 23 shows the network formed after the coagulation process had stabilised. Close-up images were taken to detect scattered light from the network formed in the cuvettes. In figure 23a the non-irradiated fibrinogen solution has transformed to a jelly-like substance. This is due to the formation of a dense, fine-masked, homogeneous network, also shown at higher magnification in figure 24. Figure 23b on the other hand, shows an inhomogeneous network, like a faint mist of light threads. The network is interrupted, forming only sporadic clusters of threads. This is also shown at higher magnification in figure 25. The white spheres visible in figure 23b are air bubbles formed in the solution.
Figure 24 and 25 show images of the network formed, taken with the Axio Observer D1 microscope. The fibrin network construction after coagulation of non-irradiated fibrinogen is illustrated in figure 24. In figure 24a the homogeneous fibrin network can be seen, like an even carpet with no visual interruptions. Figure 24b show the fibrin network at higher magnification. The fine fibrin network can be seen as the light and dark grey spots throughout the image, with some thicker fibrin threads also present. The absence of fibrin in the lower corner is because the image was taken at the edge of the sample.

Figure 25 illustrates the network formation of 35 kGy fibrinogen. Both images in figure 25 show the interruption of the network and the empty gaps produced. Where the threads are present, they are more concentrated, forming thicker threads. This can be seen clearly in figure 25b as dark branches. The white areas in figure 24b and 25b arise from high concentrations of material in other layers than the layer focused on by the microscope in the sample.
4.3.3 FibMat matrix

The results of the FibMat matrix formation with immobilised pamidronate in figure 26, were obtained measuring the change in thickness on silicon surfaces, with the AutoEL III ellipsometer. The FibMat/BP construction was performed according to section 3.1.3. Non-irradiated and 35 kGy fibrinogen graphs show the build-up of a ten layer fibrinogen matrix. The result indicates a somewhat lower thickness of the 35 kGy FibMat matrix compared to the non-irradiated matrix. A thickness of 10 Å pamidronate corresponding to 120 ng/cm², was immobilised in both fibrinogen matrices.

Figure 24. Images of the fibrin network with the Axio Observer D1 microscope, 60 minutes after the coagulation of 0 kGy fibrinogen was initiated by thrombin. Figure 24a (left image) was taken using phase contrast while figure 24b (right image) was taken using differential interference contrast (DIC).

Figure 25. Images of the network with the Axio Observer D1 microscope, 60 minutes after the coagulation of 35 kGy fibrinogen were initiated by thrombin. Figure 25a (left image) was taken using phase contrast while figure 25b (right image) was taken using differential interference contrast (DIC).
FibMat matrix with immobilised pamidronate

Figure 26. The graph illustrates the construction of the FibMat matrix on silicon surfaces, as measured by the thickness of each layer with the AutoEL III ellipsometer. Irradiated fibrinogen with doses of 0 kGy and 35 kGy were examined and 1 mg/ml pamidronate was used for immobilisation to the ten layer thick matrix. Three samples for each dose were measured in at least five distinct points.

4.4 Gamma-irradiated BP

The BPs were prepared as described in section 3.5 and measured with the Bruker Vertex 70 IR spectrometer. Resulting spectra from alendronate, pamidronate, zoledronate and ibandronate, gamma-irradiated with 0, 5, 15, 25 and 35 kGy were obtained. The lower-dose irradiated BPs showed no differences in spectrum profiles compared to BPs irradiated with 35 kGy. Therefore, in order to better illustrate the differences between non-irradiated BPs and irradiated BPs, only 0 kGy and 35 kGy are compared in figure 27. The wavenumber range in the graphs comprises the characteristic peaks produced by the chemical bonds in the BPs. The spectra are very similar in each BP graph. There is no visible loss or displacements of peaks in the 35 kGy spectrum compared to the 0 kGy spectrum. Some differences in intensity can be found in certain peaks of the spectra.
Figure 27a. The graphs were obtained using the Bruker Vertex 70 IR spectrometer and show spectra of alendronate after irradiation doses 0 kGy and 35 kGy.

Figure 27b. The graphs were obtained using the Bruker Vertex 70 IR spectrometer and show spectra of pamidronate after irradiation doses 0 kGy and 35 kGy.
**Figure 27c.** The graphs were obtained using the Bruker Vertex 70 IR spectrometer and show spectra of zoledronate after irradiation doses 0 kGy and 35 kGy.

**Figure 27d.** The graphs were obtained using the Bruker Vertex 70 IR spectrometer and show spectra of ibandronate after irradiation doses 0 kGy and 35 kGy.
4.5 Construction and improvement of FibMat/BP

Unfortunately no results from the Biacore measurements could be obtained. The technique was not suitable for constructing multilayer of proteins. When building the matrix on the gold surface, fibrinogen layers were also formed inside the flow-system of the instrument, interrupting the flow and eventually plugging the system.
5 DISCUSSION

5.1 Scrape-off

The stainless-steel screws are virtually unaffected by insertion into the bone materials. This can be seen from both scintillation (figure 13) and autoradiography (figure 14). The scintillation experiments give values for the total amount of BP present on the screws. Insertion in cortical material gave a reduction of ~20%, in cancellous ~15%, none of them statistically different from the control screw (non-inserted). The large standard deviations in the scintillation results maybe explained by the screws having been used in the autoradiography experiments prior to the scintillation tests. Autoradiography is a non-destructive method, but handling of the screws might have influenced the results.

Autoradiography (figure 15) of bone materials confirms scintillation results; some BP being scraped off. It is seen in the figure that more BP is scraped off at 1 mm (in the beginning) than further in. The scrape-off is due to friction between the screw and the bone during insertion. The micro-porous surface character of the screw seems to protect the FibMat/BP from frictional forces and thus reduce the amount of material scraped off during insertion. The results show that the FibMat/BP matrix coated on the porous stainless-steel screws is well suited for local distribution of BP to the screw/bone interface.

The titanium screws are more affected by the insertion process. The scintillation data (figure 16) shows that ~50% and ~90% is scraped off upon insertion in cancellous and cortical material, respectively. This may be partly explained by the practical difficulties in screwing into the materials (with the specialised equipment). The procedure might have added to the friction between the screw surface and the material, especially in the cortical material. For practical reasons, 5 mm thick cortical material was used, whereas clinically relevant thicknesses are 0.5-1.5 mm. The cortical material used is much harder than real cortical bone, but relevant for testing bone-devices according to FDA standards. The relevance of scrape-off results in the cortical material might therefore be questioned. It can be speculated that much less is scraped off in a clinical situation of thinner cortical layer with a lower density than in the present tests. On the other hand, if most BP is scraped off in the cortical bone, a strong effect on resorption prevention in this area will be expected. It has been indicated [44] that screw interaction and anchoring with cortical bone during the initial phase is important for
implant stability. In the clinical applications using the titanium screw, the cortical shell is entirely removed before insertion, into cancellous bone in the femoral head. The BP is evenly distributed in the cancellous material, shown in (figure 17), after insertion. Thus, scraped off BP will reduce bone resorption around/along all of the screw. The results indicate that the FibMat/BP matrix is also applicable to non-porous materials, but that the thickness of the cortical material to be penetrated is of importance. For local distribution of BP in cancellous bone the coating seems very promising.

The proportion BP scraped off is less on the stainless-steel screws compared to the titanium screws. The screw surfaces were treated differently prior to the FibMat immobilisation. The stainless-steel screws were etched in HF to produce a more rough (porous) surface (figure 10) protecting the FibMat/BP from friction. The titanium screws, on the other hand, are anodised and relatively planar, as seen in (figure 11). A planar surface will expose more of the immobilised material to friction forces. The design of the screws and the geometry of the pre-drilled holes also play a significant role to the abrasion during insertion.

The absolute amount of BP on the screws is almost ten times more in the stainless-steel screw (375 ng/cm$^2$) (macroscopic area unit) compared to the titanium screw (39 ng/cm$^2$). The amount BP on the stainless-steel screw agrees well with [45] using the same quantification method. The results show that the surface topography is important for the amount BP on the implants. For the stainless-steel screw the microscopic surface area is much bigger than the macroscopic, as opposed to the titanium screw. The porosity created from etching gives cavities which increase the surface area available for FibMat/BP immobilisation. Increasing implant surface porosity thus increases the amount BP possible to immobilise.

The stainless-steel screw was loaded with a total amount of 150 ng BP. This coating has been shown [4,5,6] to improve screw fixation in rats. 650 ng BP was loaded on the titanium screws. This can be compared to [3] where 70 mg/week alendronate was administrated orally to patients for 3 months, after hip surgery, improving screw fixation. By using FibMat/BP for local drug delivery, BPs can effectively be distributed directly to the screw/bone interface, requiring a much lower total dose. BPs administrated systemically requires much higher doses, and increase the risks of unwanted side-effects.
5.2 Gamma irradiation of fibrinogen

Coagulation assays show that gamma-irradiated fibrinogen requires longer time to coagulate, and does not form a clot, in the common sense of the word.

The coagulation assays show that gamma-irradiated fibrinogen has a slower coagulation process, the time increasing with radiation dose (figure 20 and 21). From figure 23, 24 and 25 it can be seen that 35 kGy form an inhomogeneous interrupted network of thick fibers compared to the non-irradiated fibrinogen. [46] suggests that the fibrin network structure is determined by the initial coagulation rate of the sample, which is determined by the thrombin and fibrinogen concentrations. Higher thrombin concentration activates more binding sites (nucleation sites) from which the fibrin monomers can start the polymerisation, thus giving a higher polymerisation rate compared to lower thrombin concentration. According to [46], fewer initial nucleation sites characterise a slower coagulation process, resulting in a network with thicker fibers, whereas many nucleation sites characterise a quick coagulation process leading to more and thinner fibers. Results presented here agree well with results from [41] which show a network of thicker fibers when using a low thromboplastin concentration compared to a high thromboplastin concentration.

In this thesis, constant thrombin and fibrinogen concentrations were used. Effects comparable to such resulting from reducing number of nucleation sites were observed. This implies that gamma irradiation causes changes to the fibrinogen thrombin binding/interactions. Lack of nucleation sites for polymerisation to be initiated explains the slower coagulation process of gamma-irradiated fibrinogens in figure 20 and 21.

In gamma-irradiated fibrinogen, less than all fibrinogen is included in network formation. Percentage not included increases with irradiation dose, as is shown in figure 22. Almost 35 % of fibrinogen exposed to 35 kGy does not take part in network formation compared to 8 % of non-irradiated fibrinogen. The effects from gamma irradiation on the appearance of the formed networks are shown in figure 23, 24 and 25 as fine-masked homogeneous fibrin network from the non-irradiated fibrinogen, and thick, irregularly branched network from fibrinogen exposed to 35 kGy. This is consistent with theories cited above on relation between time required for coagulation and structure of network formed.
Coagulation time of irradiated fibrinogen samples could not be obtained by standard rheometric measurement device (table 1). A coagulation time could only be obtained for non-irradiated fibrinogen, and only this sample did by visual inspection have the consistency of a gelatinous clump expected. The gamma-irradiated fibrinogens differ in coagulative properties compared to the non-irradiated fibrinogen to the degree that the network formed is not referred to as a fibrin network in this thesis. It is clear from this study that gamma-irradiated fibrinogen does not form a normal fibrin network, and networks formed from this fibrinogen will not be able to stop flowing liquid, like blood.

5.3 Gamma irradiation of BP

The IR spectrometer results indicate that BPs are unaffected by exposure to gamma irradiation. The molecular structures of alendronate, pamidronate, zoledronate and ibandronate were studied after exposure to gamma irradiation with doses up to 35 kGy, and as shown in figure 27, virtually no differences can be seen. The graphs show no displacements or absence of peaks which would have indicated changes in the chemical bonds altering the molecular structure. Some differences in intensity can be found in certain peaks, though the relations in the spectra remain unchanged. These differences in intensity are related to small variations in amount of the samples prepared in the tablets. The results indicate that BPs can tolerate gamma irradiation doses relevant for clinical applications.

5.4 Matrix formation of gamma-irradiated fibrinogen

The fibrinogen exposed to 35 kGy was used to build a FibMat matrix, and as compared to the non-irradiated fibrinogen, there was no difference in the resulting matrix with regard to BP-binding capacity. As much BP could be loaded into the matrix built from irradiated fibrinogen as from the control, as is shown in figure 26. In general there is a lack of reproducibility in final thickness of FibMat/BP matrices with the procedures used at the institute (IFM/Appl.Phys). This inconsistency makes it difficult to draw any conclusions on matrix forming ability from the small difference observed in the present experiment. The same amount of BP was immobilised in the matrices even if there was a difference in thickness. These results show that gamma-irradiated fibrinogen can be used for constructing the FibMat matrix and loading of BP.
The effects on fibrinogen and BP taken together also indicate that a device coated with BP-loaded FibMat matrix may be gamma-sterilised without changing the essential properties of the coating. In [7] no impairment of mechanical fixation in rat tibia was detected when the stainless-steel screws with immobilised FibMat/BP were gamma-irradiated with 25 kGy. Taken together with the present results, it can be claimed that BP-loaded FibMat is an appropriate and suitable technology for local BP distribution in conjunction with bone implants.
6 CONCLUSIONS

In this thesis, some aspects of the FibMat matrix of relevance to its clinical applicability have been investigated. It can be concluded that:

- Porosity is of importance. The surface topography of the screw is important for protection of the FibMat/BP from frictional forces during screw insertion and for the amount BP immobilisable to the implant surface. More material is scraped off from a planar surface than from a porous.
  - The porous stainless-steel screws are virtually unaffected by insertion into the bone materials.
  - The planar titanium screws seem promising, though for local distribution of BP in cancellous bone.
- Gamma irradiation is well tolerated by both coating components
  - Gamma-irradiated fibrinogen requires longer time to coagulate, and does not form a normal fibrin network. The networks formed from this fibrinogen will not be able to stop flowing liquid, like blood.
  - The BPs in this thesis are unaffected by exposure to the gamma irradiation which indicate that BPs can tolerate gamma irradiation doses relevant for clinical applications.

It is therefore concluded that FibMat loaded with BP is an appropriate and suitable technology for local BP distribution in conjunction with bone implants after gamma sterilisation.
7 FUTURE ASPECTS

This work has given improved understanding of the FibMat technology and the application of it. Next steps include further preparative actions for the industrial production set-up and commercialisation of it.
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