# Chemical and Biochemical Functionalization of Middle Ear Implants

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# Inhaltsübersicht

Die optimierte Funktion und Stabilität von Implantaten ist heute insbesondere auch aufgrund der ansteigenden Lebenserwartung der Bevölkerung ein wichtiges Forschungsthema. Um den Bedarf an optimierten Prothesen zu decken, wird nicht nur an der Verbesserung der Basismaterialien von Implantaten geforscht, sondern es werden auch bekannte Implantate durch chemische und biochemische Funktionalisierungen weiter verbessert. Die vorliegende Arbeit stellt Beispiele für solche Funktionalisierungen vor. Die hier vorgestellten Strategien wurden im Rahmen eines Kooperationsprojektes entwickelt, dass sich mit der Optimierung von Mittelohrprothesen beschäftigt. Ziel ist eine modular mit verschiedenen Funktionalisierungen bestückte Prothese, die in ihren unterschiedlichen Bereichen optimal auf ihre Aufgaben vorbereitet ist. Als Basismaterial diente Bioverit® II, eine für die Konstruktion von Mittelohrprothesen üblicherweise genutzte Glasglimmerkeramik. Für orientierende Vorversuche wurden Glassubstrate verwendet. Die entwickelten Systeme und die erhaltenen Ergebnisse sollten grundsätzlich auch auf andere Basismaterialien übertragbar sein.

Als erste biochemische Funktionalisierung sollte eine Anbindungsstrategie für das Signalhormon Bone Morphogenic Protein 2 (BMP2) entwickelt werden, das die Differenzierung zu knochenbildenden Zellen fördert. Hiermit soll im Falle der Mittelohrprothese eine bessere Anbindung an vorhandene Knochenreste erreicht werden, um so eine Extrusion der Prothese zu vermeiden. Dieser Ansatz ist aber auch generell zur Verbesserung der Integration von anderen Knochenersatzmaterialien von Interesse. Im ersten Teil der Arbeit wurde zur Testung zunächst als Modellsystem die Anbindung des Proteins Alkaline Phosphatase untersucht, besonders im Hinblick auf den Einfluss unterschiedlich strukturierter Silicatoberflächen in Kombination mit verschiedenen Linkern, die funktionelle Gruppen zur Anbindung tragen (Epoxy-, Harnstoff- und Aminfunktionen). Als beste Kombination stellte sich hier eine Aminopropyl-Funktionalisierung auf einer mesoporösen Oberfläche heraus. Im zweiten Teil der Arbeit konnte dieses Anbindungssystem erfolgreich auf das BMP2 übertragen werden, und zwar sowohl auf den gleichen silicatischen Substraten als auch auf Bioverit® II; dabei wurde auf Bioverit® II eine erhebliche Steigerung der gebunden BMP2-Menge erreicht. Die Materialien wurden im Rahmen der Zusammenarbeit im Projekt im Hinblick auf ihre biologische Aktivität in vitro und in vivo (Tierversuche im Kaninchenmodell) untersucht.

Als chemische Funktionalisierung wurde im dritten Teil der Arbeit die bereits etablierte mesoporöse Beschichtung zusätzlich als Reservoir für ein lokale Medikamentengabe (local drug release) genutzt, denn gemeinsam mit der Implantation einer Mittelohrprothese soll auch eine Infektionsbekämpfung erfolgen. Diese Untersuchungen wurden mit dem bei Mittelohrinfekten häufig systemisch angewendeten Antibiotikum Ciprofloxacin durchgeführt. Durch Modifikationen der Oberfläche des mesoporösen Materials konnte einerseits die Beladungsmenge erheblich gesteigert werden, andererseits auch eine kontrollierte Freisetzung des Medikaments über Zeiträume von bis zu 60 Tagen erreicht werden. Die Wirksamkeit des Systems wurde mittels Bakterienkulturen nachgewiesen, dessen Biokompatibilität in Zellkulturuntersuchungen. Erste positiv verlaufene Tierversuche fanden in der Maus statt.

**Stichworte:** mesoporöses Siliciumdioxid, Immobilisierung von Proteinen, Alkaline Phosphatase, Bone Morphogenetic Protein 2, kontrolliertes Drug Release, Mittelohr-prothesen.

# Abstract

Due to the increasing life expectancy of the population, the optimal function and stability of implants is an important topic today. In order to fulfil this need, current research not only improves the base materials for implants, but already existing implants are enhanced by chemical and biochemical functionalizations. This work presents examples of such functionalizations. These strategies were developed within a collaborative project that deals with the optimization of middle ear prostheses. The aim of the project is to develop a modularly functionalized prosthesis which in its different regions is optimally prepared for its tasks. As a base material, Bioverit® II was chosen, a glass-mica ceramic which is commonly applied for the construction of middle ear prostheses. Orienting experiments were carried out on glass substrates. The systems developed and the results obtained should in general also be transferable to other base materials.

As a first biochemical functionalization, a strategy for the immobilization of the signalling protein bone morphogenic protein 2 (BMP2) was developed. BMP2 supports the differentiation to bone-forming cells. In this way, a stronger fixation of the prosthesis shall be achieved by the attachment to bone residues, in order to avoid extrusion of the prosthesis. This approach should be generally valid also for the integration of other bone substitution materials. In the first part of this work, the immobilization of the protein alkaline phosphatase was studied as a model system, with a special focus on the influence of different structural properties of silicate surfaces in combination with different linkers, which carry functional groups for the attachment (epoxy, urea and amine functions). It was found that the most effective combination for immobilization is the functionalization of a mesoporous surface coating with aminopropyl residues. In the second part of this work, this immobilization strategy was successfully transferred to BMP2, which was attached on similar silicate surfaces, but also on Bioverit® II, where the amount of immobilized BMP2 was strongly increased. The materials were characterized biologically in vitro and in vivo (animal experiments in rabbits) within the framework of cooperation of the project.

In the third part of this work, a chemical functionalization is described, which uses the already established mesoporous silica coating as a reservoir for local drug release. Together with the implantation of a middle ear prosthesis, an infection shall be combatted. These investigations were carried out using the antibiotic ciprofloxacin, which often is applied systemically in the case of middle ear infections. By different modifications of the surface of the mesoporous material, the loaded amount was strongly increased and a controlled release of the drug, extending to up to 60 days, could be achieved. The efficacy of this system was demonstrated by bacterial culture tests, the biocompatibility by cell culture investigations. First positive animal experiments were carried out in the mouse model.



**Keywords:** mesoporous silica, protein immobilization, alkaline phosphatase, bone morphogenetic protein 2, controlled drug release, middle ear prosthesis.

## Erklärung

Hierdurch erkläre ich, dass ich die Dissertation „Chemical and Biochemical Functionalization of Middle Ear Implants“ selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 3. Juni 2009

Dipl. Chem. Nina Ehlert

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# 1 Introduction

Due to the increasing life expectancy of the population, the desired implantation times for implants increase. For that reason, sustainable implants need an adapted functionality and stability. In order to fulfil this need, not only the base material of implants is optimized, but existing implants are enhanced by further functionalizations, which can be classified into three categories: mechanical, chemical and biochemical. This work is an example of improving implants by innovative chemical and biochemical functionalization techniques.

Especially in the field of bone replacement materials an optimal function is essential. Hip or knee prostheses for example must bear high mechanical stresses. Besides optimal material performance, this requires the best possible integration of the prosthesis in the surrounding tissue. To achieve tight adhesion of the surrounding bone on such prostheses, these can for example be covered with hydroxyapatite or growth factors can be immobilized on the implants surface. Such growth factors, belonging to the family of bone morphogenetic proteins (BMPs), induce the formation of bone forming cells at the implant/tissue-interface. On the other hand, there are cases where not a very high, but an adapted bioactivity is required. An example is the special implantation site of the middle ear where middle ear prostheses are used to restore the sound-transmitting function of the ossicular chain. Here, high bioactivity can lead to the extensive formation of bone which overgrows the whole prosthesis, thus leading to “bony fixation” and inferior sound transmission.

A further challenge in the field of adapted implant functionality is to avoid the failure of implants due to accompanying infections. One idea in this area is to equip the prosthesis in such a way that the implant can “defend” itself against invading bacteria. Such a “self-defense” can for example be achieved by antibacterial coatings or by local drug release systems. The latter rely on the idea of loading a certain drug into a material which is to be released at the targeted tissue. Besides better efficacy, these systems have the advantage of a reduced stress for the body due to low doses as compared to systemic treatments. The challenge of designing such drug release systems lies in the control of the release kinetics. Often a very fast release of the inserted drug, the so called “initial burst” effect, is observed, but not always desired. With functioning “self-defense”, the time for the first healing could be shortened and many second or revision operations could be prevented.

The mentioned features of functionalized implants all play a role in the design of an optimized modularly constructed middle ear prosthesis (Figure 1–1). The major problems associated with the middle ear prostheses used nowadays are

- the tendency towards a strong bony fixation, leading to minor sound transmission properties, as described above;

- a high rate of extrusions, i.e. the prosthesis leaves its place between the tympanic membrane and the window to the inner ear due to weak connections to the surrounding tissue, making necessary a second implantation operation;

- associated infections, because the initial loss of the bones of the ossicular chain is often caused by a chronic middle ear infection and the implant has to be placed into an infected region.

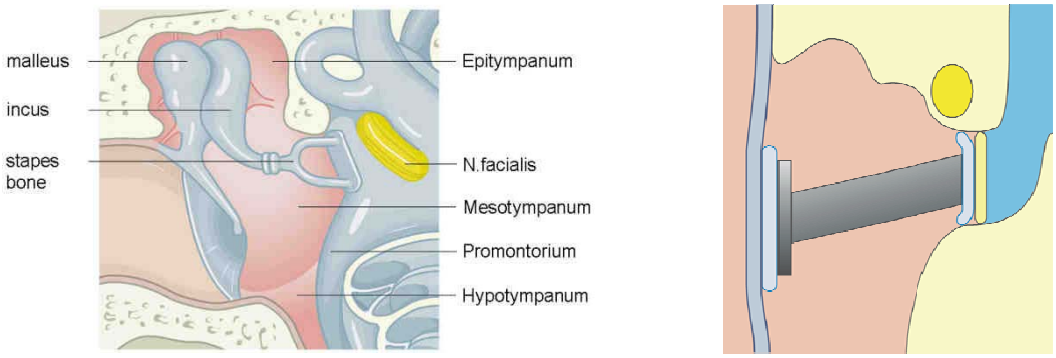


Figure 1-1. Normal middle ear structures (taken from [1] (left) and schematic example of a Total Ossicular Replacement Prosthesis (TORP) for establishing the connection between the drum and the inner ear (right).

In former investigations it had been shown that a coating of mesoporous silica [2] on implants made from the standard biomaterial Bioverit® II could enhance the implantation situation. In detail, the formation of a thin mucosa layer around the implant was increased, whereas the uncontrolled formation of spongiosa was decreased [3]. Such a mesoporous layer with its high surface area and its surface reactivity, which is based on the occurrence of silanol groups, appears to be a favorable basis to develop new concepts for the functionalization of middle ear implants. In the present work, the functionalization by the immobilization of proteins and the release of antibiotics were in focus.

Firstly, as a model system, a protein immobilization strategy was developed for the enzyme alkaline phosphatase, which is a cheap and robust enzyme with a well-

established detection assay. The binding of the enzyme to the surface is conducted by means of silanization procedures. Apart from finding a functional group suitable for the attachment of the alkaline phosphatase, a major focus of interest lies in the influence of differently structured silicate surfaces. Uncoated glass slides were tested in comparison with unstructured and with mesoporous silica coatings on standard glass slides.

In a second step the established immobilization procedure was transferred to the growth factor BMP2. In this case, in addition to glass, Bioverit® II is used as base material. The BMP2 functionalization is to be applied locally, in order to induce a controlled local bone formation between the middle ear prosthesis and the residual stapes bone on the window to the inner ear. The immunochemical and the biological activity of immobilized BMP2 were tested by ELISA and BRE-luc tests. These materials are currently being tested in animal experiments on rabbits.

The final part of this work uses the mesoporous silica coating as a reservoir for local drug release. Together with the implantation of a middle ear prosthesis, an infection shall be combatted. For this purpose, a drug release system was established for the antibiotic ciprofloxacin, which often is applied systemically in the case of middle ear infections. The efficacy of this system was demonstrated by bacterial culture tests, the biocompatibility by cell culture investigations. First positive animal experiments were carried out in the mouse model.

The work described here was carried out within the work package D1 "Functionalized Middle Ear Prostheses" within the Collaborative Research Area SFB 599 for "*Sustainable bioresorbable and permanent implants of metallic and ceramic materials*". It was a collaboration between the Medical School of Hannover (MHH), the Helmholtz Center for Infection Research (HZI) and the Leibniz University of Hannover (LUH)*. A strong contact was also established to the work package D7 and

*Members of the work-package D1: Prof. Dr. Peter Behrens, Dipl. Chem. Olga Kufelt, Anne Christel and Dipl. Chem. Nina Ehlert, Institut für Anorganische Chemie, Leibniz Universität Hannover; Prof. Dr. Thomas Lenarz, Dr. Martin Stieve, Dr. Hamidreza Mojallal, Dr. Julia C. Vogt, Dr. Julia Schöne, Iwa Hlozanek, Hals-Nasen-Ohren-Klinik, Medizinische Hochschule Hannover; Dr. Gudrun Brandes, Institut für Zellbiologie im Zentrum Anatomie, Medizinische Hochschule Hannover; Prof. Dr. Peter P. Müller, Dipl. Chem. Muhammad Badar, Helmholtz Zentrum für Infektionsforschung.

especially to PD Dr. A. Hoffmann from the Helmholtz Center of Infection Research (HZI)†.

The presentation of this doctoral thesis includes, apart from this introduction, a chapter giving a general description of the background of the work and a "Summary and Outlook" chapter, both framing the central part on "Results and Discussion". This part is presented in the way of three sections, each as a manuscript for submission to appear in a peer-reviewed journal. Before each "manuscript", a short introduction to the specific topic addressed is given, and the own contributions are delineated from those of the co-workers.

† PD Dr. Andrea Hoffmann, PD Dr. Gerhard Gross, Helmholtz Center for Infection Research.

# 2 General background

## 2.1. Middle ear anatomy and pathology

### 2.1.1. The anatomy of the middle ear and the principles of sound transmission

The ear consists mainly of three parts: the outer ear, the middle ear and the inner ear (Figure 2–1). The outer ear focuses the incoming sound. The middle ear translates these sound pressure waves to a mechanical motion, including an amplification of the signal. Finally, the inner ear translates it into neural signals to be transmitted to the brain. In the following a closer look at the structures of the middle ear and the principles of sound translation and amplification shall be given.

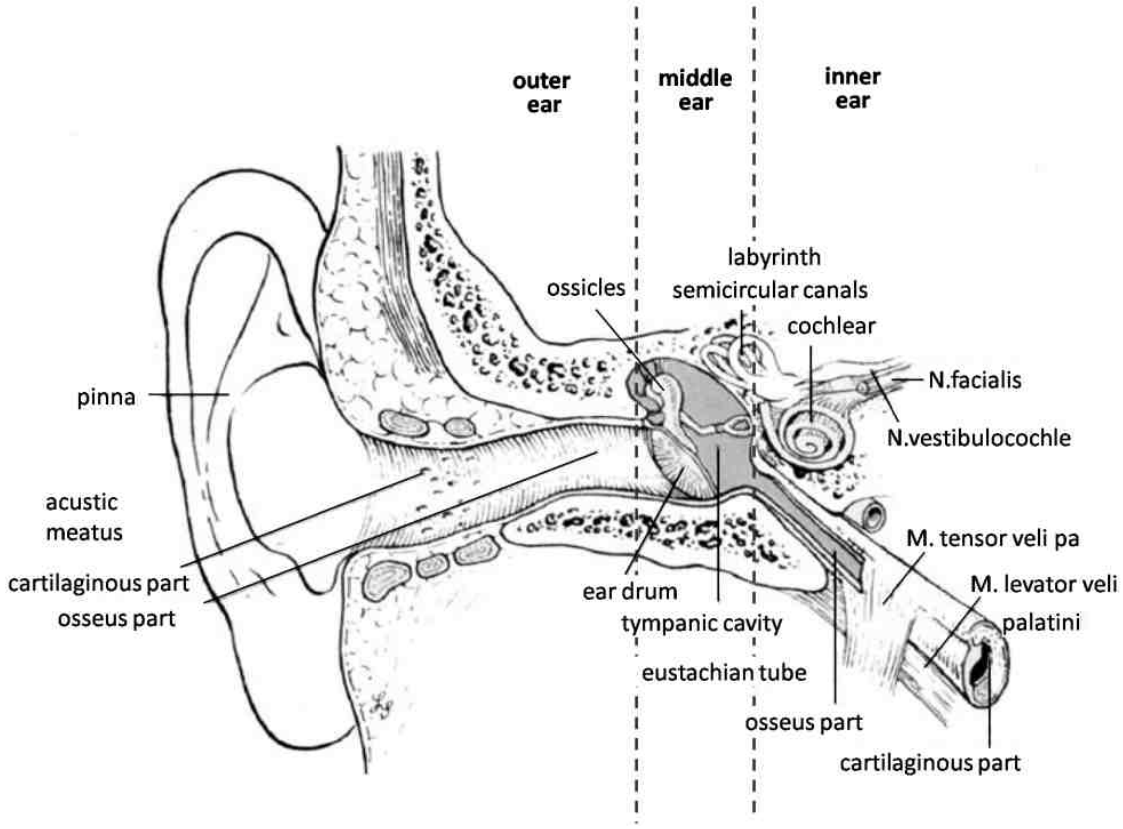


Figure 2-1. Anatomy of the ear. Structures of the outer, the middle and the inner ear (adapted from [4]).

The middle ear consists of the ear drum (tympanic membrane), the tympanic cavity, the bones of the ossicular chain, the eustachian tube as well as the associated articulations, tendons and muscles (Figure 2–2).

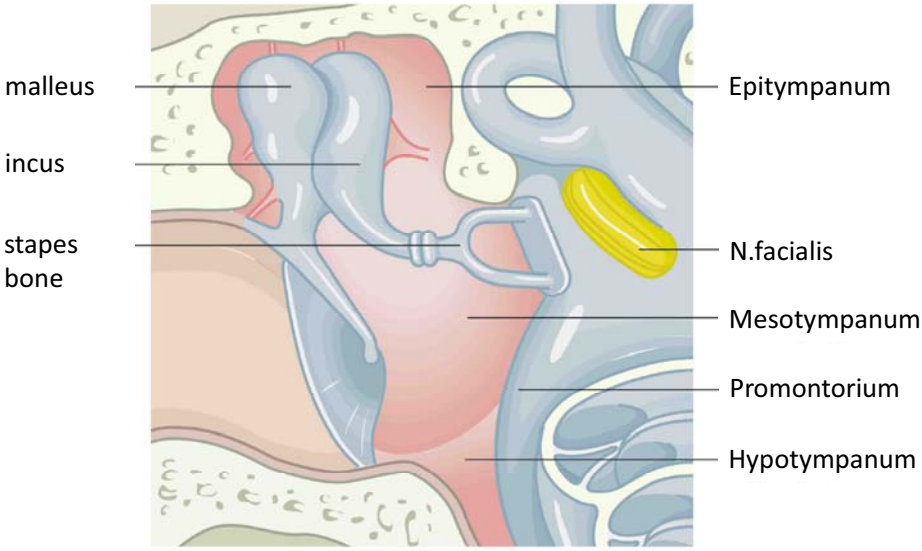


Figure 2-2. Normal middle ear structures (taken from [1]).

The ossicular chain is constructed from three bones the malleus, the incus and the stapes (Figure 2–3). These bones, also named ossicles, form the connection between the eardrum and the so called oval window to the inner ear. The malleus, resembling the shape of a hammer, is connoted with its shaft to the eardrum, and with its head it forms the joint to the short branch of the incus. The incus then is connected to the stapes at the end of its long branch within the second joint of the ossicles. Finally, the stapes is linked flexibly by the annular to the membrane of the oval window, which is the interface to the inner ear.

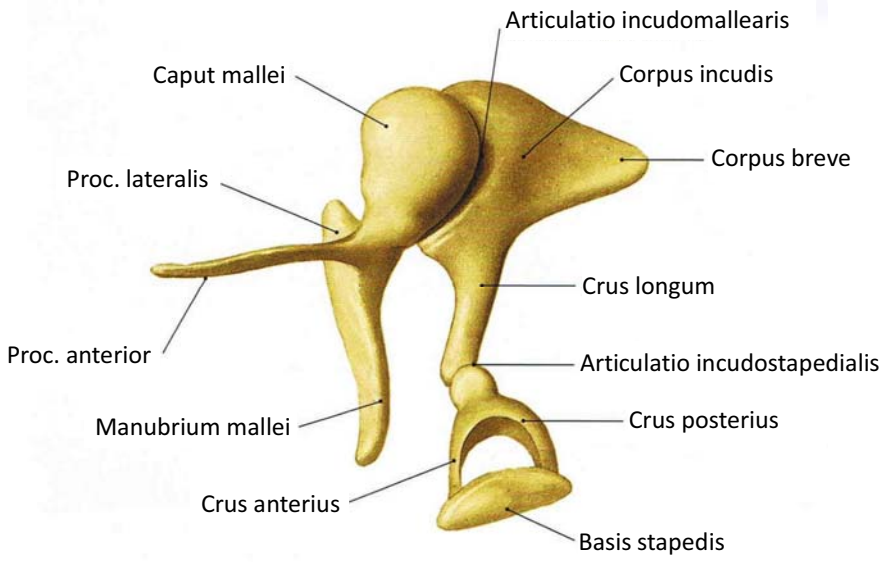


Figure 2-3. Detailed view of the ossicles: stapes, incus and malleus (taken from [5]).

The ossicles are hung up in the tympanic cavity by different tendons and two muscles, one connected to the malleus and one to the stapes (Figure 2–4). How these connections are constituted in detail has not yet been totally investigated. However, it is known that the anchorage of the middle ear is highly sophisticated, because mechanical disturbances, for example while walking, are not evoking an impression of noise [6]. Moreover, the muscles are playing an important role in the protection of the inner ear in case of high level of noise and pressure variations [7].

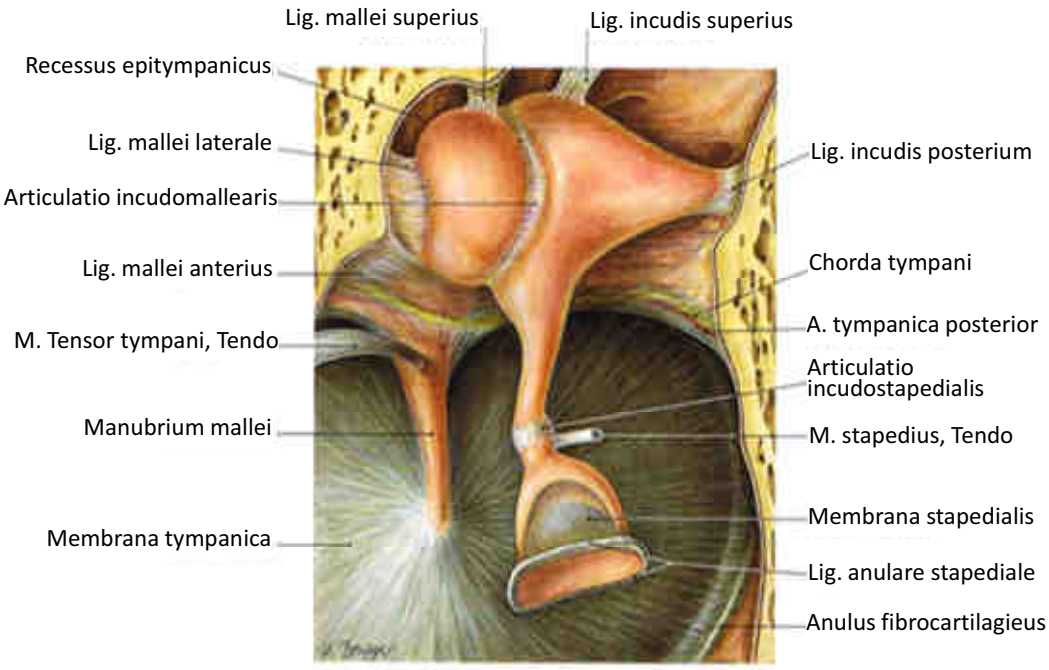


Figure 2-4. Joints and muscles in the middle ear (taken from [5]).

An incoming sound pressure wave leads to a vibration of the ear drum, creating motions of the ossicular chain which are transmitted via the oval window to the inner ear. The inner ear is filled with a liquid, so that by transmission via the ossicular chain, air waves become liquid waves. The middle ear is thus playing the role of an impedance converter, because air has much lower wave resistance than a liquid.

A simple mechanism is used to achieve amplification of the sound signal by the middle ear. The surface area of the tympanic membrane (60 $mm^2$) is many times that of the oval window (3 $mm^2$) which results in a sound concentration [8]. For a long time it was supposed that the ossicles are applying the "lever principle" for an additional sound amplification [6], but new investigations show that the ossicles are vibrating as one unit [7, 8]. The joint, tendons and muscles of the middle ear are acting as a complex adapting system to balance the static, ambient air pressure at the ear drum [7].

The amplification factor of the middle ear is 22-fold. Due to this amplification, only 40 % of the sound waves reaching the outer ear canal are reflected during the passage to the liquid of the inner ear. In addition, the amplification factor depends on the frequency. It is best at the natural resonance frequency of the ear drum lying between 1000 and 2000 Hz, which corresponds to the frequency of human speech [6].

### 2.1.2. Typical diseases of the middle ear

A typical disease of the middle ear is an inflammation like *otitis media* which can be caused by bacteria like *Streptococcus pneumoniae* or *Haemophilus influenzae*. Other bacteria occurring in the middle ear are *Pseudomonas aeruginosa* and *Staphylococcus* species [9, 10]. During an infection a fluid formation in the normally air-filled space interrupts the sound transmission. In most cases the inflammation can be healed by antibiotics. However, it some cases such inflammations can become chronically. If so, the bones of the ossicular chain can become destroyed and are then ineffective for sound transmission. A loss of the middle ear function leads not to complete deafness because of sound transmission by bone conduction, but to hardness of hearing with a reduction of ca. 26 dB [6].

Conductive hearing loss can also be caused by other diseases. Occlusions may occur in the external ear canal, for example evoked by impacted earwax, or in the eustachian tube. These can be corrected by a simple removal procedure. Another common disease is otosclerosis, which affects the ear surrounding bone and can result in an immobility of the stapes. Moreover, when displaced outer skin (squamous epithelial cells) intrudes the mucosa of the middle ear and sheds dead skin cells, these can build up over a long time and form a cholesteatoma. It can expand and erode the middle ear structures [6].

In general, fixed, disconnected or missing ossicles or large holes in the tympanic membrane lead to a conductive hear loss. In these cases, grafting or implant surgery is applied to rebuild the sound transmission function of the middle ear. Holes in the tympanic membrane are normally closed by autogenous grafts taken from the ear surrounding skin or from muscle tissue. Ossicular chain reconstruction can be realized by autogenous, allogenic or alloplastic materials.

### 2.1.3. Ossicular chain replacement surgery – status quo

In general, two different types of reconstructive surgery for the ossicular chain exist. If the prosthesis can be attached to residual bone structures of the ossicles, it is called a PORP: partial ossicular chain prosthesis. When the whole connection between the stapes footplate and the ear drum has to be reinstalled, the prosthesis is named a TORP: total ossicular replacement prosthesis (Figure 2–5). Within this work all considerations are concentrated on TORPs.

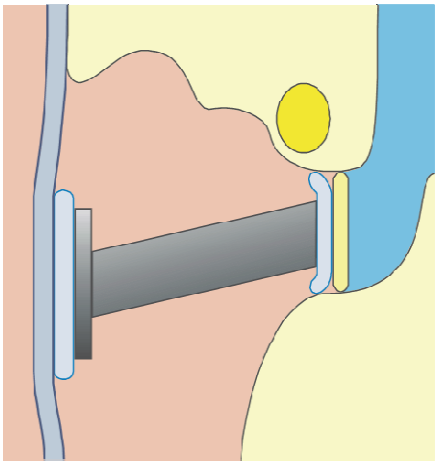

Figure 2-5. Schematic example of a Total Ossicular Replacement Prosthesis (TORP) for establishing the connection between the drum and the inner ear.

The middle ear is a special implantation site for bone reconstruction which has special requirements. It is an air filled area and a so called semi-open cavity, because it has a connection to the nose via the eustachian tube (Figure 2–1). Therefore it is possible that bacteria enter the normally sterile cavity. The surface of the middle ear structures is covered with a reactive mucosa. It would be favorable when this thin cell layer is also formed on an implant to avoid bacterial growth [9]. The property of sound conduction mainly depends on the implant stiffness, its mass, its shape and biocompatibility. A low weight of the prosthesis leads to better sound conduction at high frequencies; low frequencies are influenced more strongly by the stiffness of the middle ear after implantation, whereas a too strong fixation of the implant is disadvantageous [11]. Moreover implant materials for the middle ear should provide the possibility of shaping during the operation and have to be mechanically stable at time of implantation and for a long time in the body.

A variety of materials is applied for middle ear reconstruction. The use of autogenous ossicles for reconstructing the ossicular chain is restricted because in case of chronical inflammations they are normally subject to resorption or atrophy. Also the presence of

a cholesteatoma denies the replantation of the ossicles because the cholesteatoma may arise from these again [8]. Also, autogenous materials like cartilage or cortical bone are not stable enough [12]. The application of allogenic materials has decreased due to the possible transmission of diseases like immunodeficiency syndrome or Creutzfeldt-Jakob disease [12]. In addition these materials have the disadvantage of a low accessibility and a limited storage life [8]. On this account alloplastic materials are often needed for ossicular chain replacement surgery. Several different types of biomaterials are employed in the middle ear, mainly metals, ceramics and plastic.

Titanium has proved its good biocompatibility and has the advantage of good sound transmission properties at high frequencies due to its low weight [8, 12, 13]. Furthermore, titanium prostheses can be designed easily for difficult anatomical situations. They have the tendency to bend easily and are sometimes difficult to position. Although in literature an extrusion rate of only 0 to 2 % can be found [8], other sources give values of about 30 %. Gold prostheses can be formed as easily as titanium ones and have the benefit of the inhibition of bacterial growth, which can be an appropriate feature when implanting in a chronically infected area [8, 14]. However, due to its higher weight gold has worse sound conductive properties than titanium [8]. Typical titanium prostheses have a weight of about 4 mg, whereas gold prostheses have a mass of 56 mg.

Plastics used for middle ear reconstruction are polytetrafluorethylene in combination with carbon, aluminium or hydroxyapatite (Proplast®) or polyethylene, which was polymerized under high pressure and heat (Plastipore®). The latter is also called a HDPS (high density polyethylene sponge) and is a porous material with pore diameters between 20 and 40 µm. The porous nature of the material is supposed to enhance the anchorage of the materials by tissue ingrowth [8]. The suitability of plastic materials for middle reconstruction is discussed controversial. On the one hand foreign body reactions addressed to the implant were reported, for example in form of fibrous tissue around the implant [8, 12, 14]. This effect was observed in combination with an extrusion rate of over 60 % and poor hearing results after long term implantation. The results were improved significantly by adding a piece of cartilage between the prosthesis, but extrusion rates from 7 to 15 % remain [8, 12, 15]. On the other hand Plastipore® was stated to be second most applied material for middle ear reconstruction [8] and several work-groups reported satisfactory long-term hearing results [12, 16].

Typical ceramics applied in the middle ear are alumina, hydroxyapatite and glass ceramics. Alumina is a bioinert material when applied as a non-porous sintered ceramic and shows good biocompatibility and suitability for ossicular replacement surgery. Moreover, this material is producing good hearing results and exhibits an extrusion rate of less than 3 % after 4 years; extrusion occurs mainly in seriously infected middle ears. A thin cell layer, a so called mucosa, which is advantageous, is formed on the prosthesis after several weeks [8, 17]. In an investigation with typical micro-organisms of the middle ear aluminium oxide showed favorable preliminary inhibition effect on bacterial growth [9].

Hydroxyapatite provides a good biocompatibility in combination with a high bioactivity, which leads sometimes to fixation of the material to the middle ear boundaries. Furthermore, the material degrades in 4 % of cases, but does not show extrusions [8]. The material is brittle and therefore not easily being shaped during operation. On this account, hydroxyapatite is often used in combination with other materials as hybrid prosthesis. For example, the head may be formed from hydroxyapatite and the piston consists of Plastipore®, plastipore-covered steel or HAPEX® (a composite of hydroxyapatite crystals mixed with HDPS). These hybrid prostheses have the feature of a good fixation to the ear drum and the piston can be trimmed easily [12, 16, 15].

Glass ceramics consisting of various combinations of the oxides of silicon, calcium, phosphorus, sodium, potassium and magnesium are known to have a good biocompatibility, but can also have a poor biostability. In the case of Ceravital® middle ear prostheses, a complete dissolution of the material was observed after long time implantation [18]. Bioverit® II is another available glass ceramic. It has proved its biostability after 13 years of implantation [12]. In addition, it has shown the desired workability and bony fixation to other ossicles, which can be beneficial for a better fixation of the implant [8]. However, if uncontrolled, the formation of new bone may result in an overall bony fixation of the prosthesis, which prevents sound conduction. In an animal experiment in a rabbit model, Bioverit® II exhibited good results especially in combination with a nanoporous silica coating, which supported the formation of mucosa around the implant while reducing uncontrolled bone formation [3]. Moreover, Bioverit® II has a preliminary inhibitory effect on microorganisms appearing in the middle ear [9].

### 2.1.4. Development of innovative ossicular chain replacement prostheses

Different approaches exist to improve sound conduction of middle ear implants. Several researchers are investigating the enhancement of sound conduction by optimization of the implant geometry [19]. The effect of topographic structure is also in focus, in the micrometer range [19] as well as in the nanometer range [3, 20, 21]. Goldenberg states that the development of new materials is more important that innovative design [16], whereas Stieve and Lenarz are presuming that the question of interest is not the base material itself but its shape and functionalization [4]. Prostheses should be equipped with features like drug reservoirs, special surface structures or immobilized biomolecules for defending against infections, better fixation and avoiding uncontrolled formation of scar tissue. Jahnke and co-workers carry out investigations leading in this direction. They suggest that the fixation of the prosthesis shall be improved by immobilizing bone growth factor (BMP2 – bone morphogenetic protein 2) to the implant surface so that new bone can form a tight fixation towards the residual stapes bone. In general, they propose the attachment of cell surface receptors onto implant materials in order to mimic human tissue response. The attachment of bone growth factors is a strategy also followed by Zahnert and co-workers [19].

Within the work package D1 “Functionalized middle ear prostheses” of the collaborative research center 599 “*Sustainable bioresorbable and permanent implants of metallic and ceramic materials”* a strategy has been developed to create an optimized implant which carries adapted functionalities in a modular fashion (Figure 2–6).

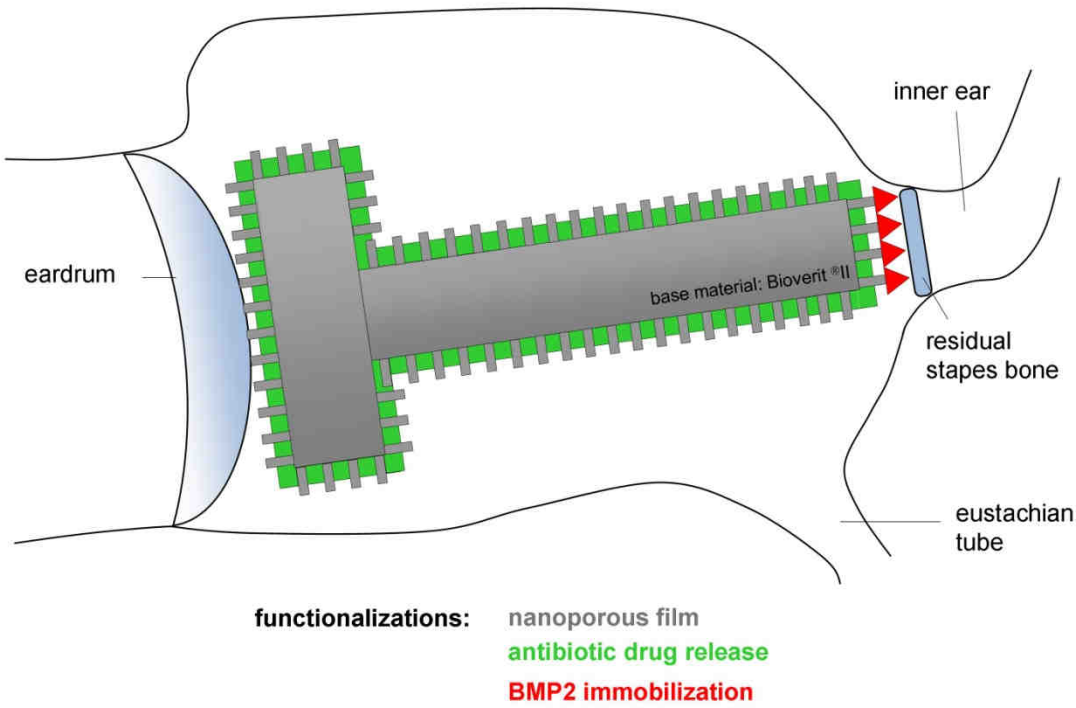


Figure 2-6. Concept of a modularly, functionalized middle ear prosthesis.

In a first step a mesoporous or as it is also called, a nanoporous silica coating has been applied to middle ear prostheses made from Bioverit® II .This coating has proved its ability in reducing the bioactivity of the base material Bioverit® II. In an animal experiment in the rabbit model, it was shown that the coating increases the formation of the desirable mucosa around the implant and decreases uncontrolled bone formation (Figure 2–7).

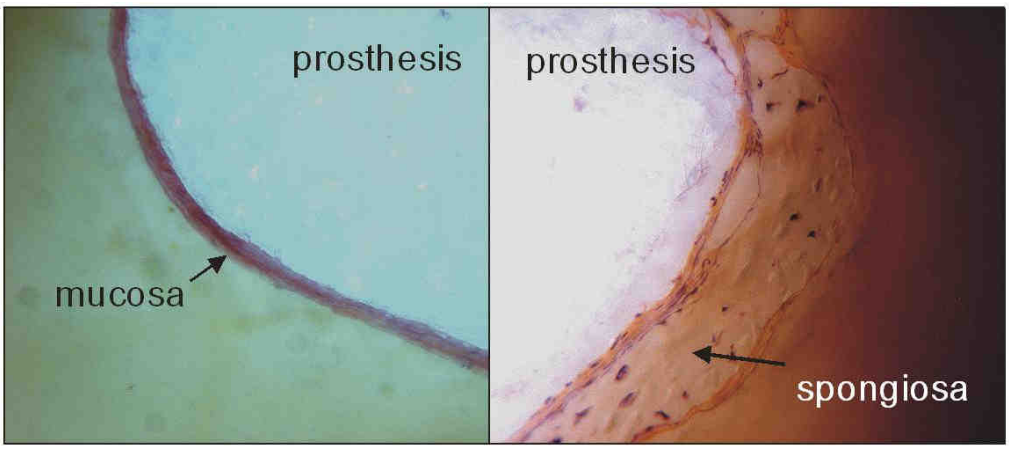


Figure 2-7. Formation of mucosa and spongiosa on the surface of Bioverit® II middle ear prostheses in the rabbit model [3].

Further biocompatibility testing of Bioverit® II implants spray-coated with a nanoporous silica film investigated in a special mouse model showed also good results [21]. The material was implanted for up to twelve weeks in the middle ear of mice. In this study the mesoporous silica film lead to an increased formation of new bone around the implant, but this was ascribed to the special implantation situation in the mice middle ear. The middle ear was nearly filled with the cylindrical implant and it had much more bone contact. In addition, the formation of new bone was activated by damaging the middle ear wall with a hole in order to position the implant in the cavity.

This nanoporous silica layer established in prior work forms the basis for carrying out further functionalizations, as described in this work. The immobilization of BMP2 at the end of the prosthesis directed to the residual stapes bone should generate a better fixation thus reducing extrusion rates. The pores of the mesoporous silica coating can be used as a drug reservoir for antibiotics. This would allow to fight infections present in the middle ear at the time of operation.

## 2.2. Mesoporous materials and their biomedical application

### 2.2.1. Mesoporous silica materials

Mesoporous materials have been in focus of interest since the Mobil Oil Corporation synthesized M41S materials in 1992 [22, 23]. The special structure of these materials results in outstanding features like very high surface area with a narrow pore size distribution. Mesoporous materials can exhibit pore sizes between 2 and 50 nm. Therefore the term “nanoporous“ is used for mesoporous materials as well. The walls of these pores are constructed from amorphous silica. The formation of the mesostructure can follow two different mechanisms (Figure 2–8). In both cases amphiphilic organic molecules (surfactants) act as structure-directing agents (SDAs), i.e. they influence the structure of the inorganic phase.

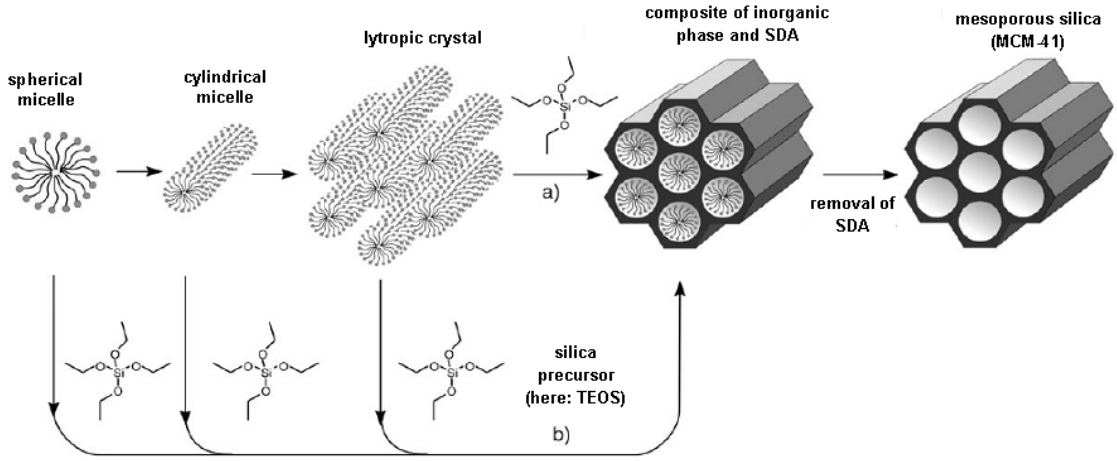


Figure 2-8. Formation of mesoporous materials using amphiphilic molecules as structure directing agents shown by the example of MCM-41. a) TLCT-mechanism (true liquid crystal templating), b) CSA-mechanism (cooperative self-assembly) (adapted from [24]).

Within the true liquid crystal templating-mechanism (TLCT) the inorganic phase is formed around a lyotropic liquid crystal which aggregates at a certain concentration of a self-organizing molecule (Figure 2–8, route a). The amphiphilic organic molecules self-assemble due to intermolecular noncovalent interactions (hydrogen bonding, VAN DER WAALS forces, electrostatic forces or π-π interactions) [25] and form larger aggregates, named micelles, which in turn can assume long range order resulting in a

lyotropic phase The shape of these micelles (spherical, cylindrical) depends on the concentration, temperature and type of surfactant.

However, more often the cooperative self-assembly-mechanism (CSA) is assumed because at standard synthesis conditions the concentration of the SDA is too low to form a lyotropic crystal (Figure 2–8, route b). Here, the added inorganic precursor forms condensed oligomers that interact with the SDA and aggregate. Due to the influence of the inorganic oligomers the self-assembling tendency of the SDA is increased so that they form lyotropic phases below the concentration required. So in simple aqueous solutions, the mesostructure is formed by a mutual cooperative assembly of organic and inorganic components. At the end, the removal of the SDA is carried out by calcination or extraction, for example with ethanol, to yield the mesoporous material.

The arrangement of the SDA aggregates influences the structure of the porous network. Mesoporous materials can exhibit hexagonal, cubic or lamellar structures. A typical example is the family of M41S-materials: MCM-41 (hexagonal), MCM-48 (cubic) and MCM-50 (lamellar) (Figure 2–9). The highly ordered structure of these materials can be demonstrated by X-ray measurements showing typically five to ten reflections. Further possible structures are combined in the family of MSU-materials [26, 27, 28]. These materials are constructed by pore channels of same size but with no further periodic arrangement. Other similar examples are materials of type LMU-1 [29] or KIT-1 [30]. The structure can also be described as worm-like and typically shows only one broad reflection in X-ray diffraction measurements.

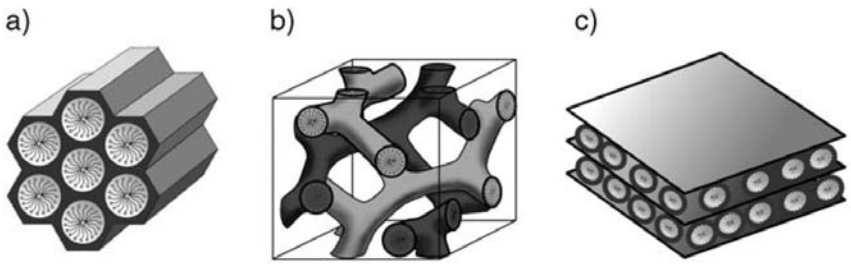


Figure 2-9. Structures of mesoporous M41S-materials, a) MCM-41 (hexagonal), b) MCM-48 (cubic) und c) MCM-50 (lamellar) (taken from [24]).

### 2.2.2. Preparation of mesoporous silica films: the EISA mechanism

The typical products of the synthesis of mesoporous materials are powders. In that form, an application as implant material is not possible. The use of mesoporous

materials in form of thin films on implant materials however, offers the general possibility of a biomedical application. A common method to deposit thin mesoporous silica films is the evaporation induced self-assembly (EISA) process. Within this process a substrate is dipped into a solution of a volatile solvent (e.g. ethanol), a silica precursor (e.g. TEOS), the surfactant, water and hydrochloric acid and withdrawn perpendicular to the surface. The concentration of the SDA in this solution is below the critical micelle concentration (CMC). The hydrochloric acid adjusts the pH value near the isoelectric point of colloidal silica to slow down further condensation of the inorganic phase during coating. The formation of the mesostructured film can now be correlated to a fixed position on the substrate as it is withdrawn from the dipping solution (Figure 2–10). At the point where the substrate leaves the reservoir, free SDA molecules and silica oligomers, or sol particles, are present. At higher positions, the evaporation of the volatile solvent occurs and the concentration of the surfactant increases progressively to form micelles, leading to a lyotropic phase. When all solvent is evaporated, the system is in the modulable steady state (MSS). At this point the liquid phase is equilibrating with its environment. The formed micelles obtain their final arrangement influenced by the relative humidity of the surrounding atmosphere [31]. Finally with further drying the condensation of the inorganic phase proceeds and the mesostructure becomes fixed.

Besides the composition of the dipping solution and the ambient conditions another crucial factor that influences the result of the dip-coating process is the dipping speed. The faster the speed is the thicker are the produced films. The thickness of the film has to be adjusted in such a way that the diffusion of the solvent leaving the film is faster than the condensation of the inorganic network, because otherwise the solvent would become trapped inside the film. A typical dip-coating film has a layer thickness of less than 1 μm [31].

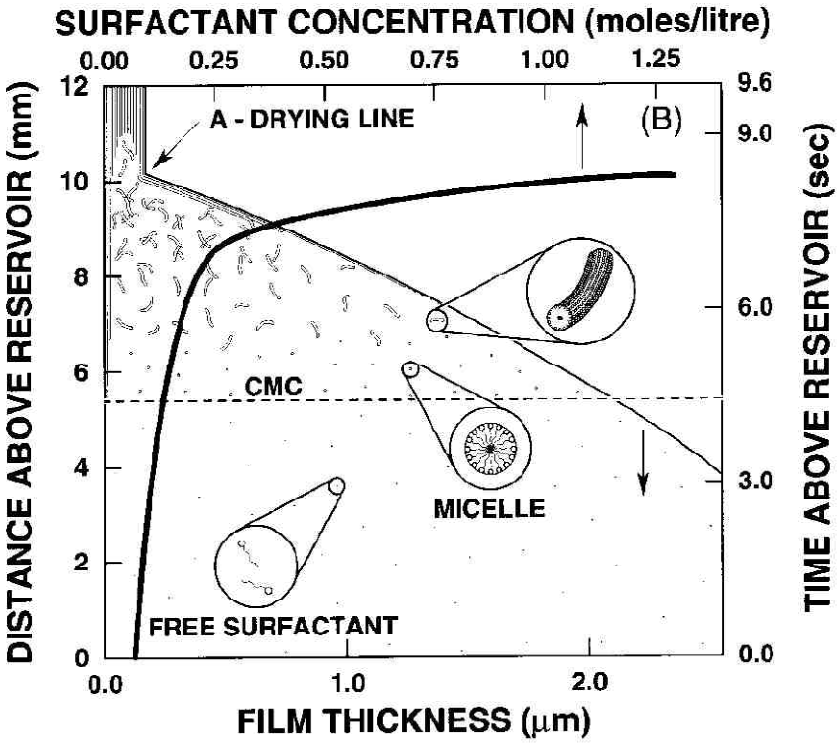


Figure 2-10. Steady-state film thickness profile during dip-coating (taken from [25] according to [32]).

### 2.2.3. Functionalization of silica surfaces

The first topic of the following part will be the organo functionalization of silica surfaces in general. In a second part a more detailed description of the reaction mechanisms of trialkoxysilanes with silanol groups of silica surfaces will be given.

Functionalization of mesoporous silica surfaces is a common tool of tailoring surface properties for desired applications, such as immobilization of proteins or drug delivery.

Basically two different approaches exist to modify silica surfaces with organic groups. The most applied one is a post-synthesis procedure also called grafting. Within this method mostly commercially available trialkoxysilanes from type $(R´O)_3SiR$, more rarely silazanes $HN(SiR_3)_2$ or chlorosilanes of various type, are allowed to react with the silanol groups of the silica surface. The advantage of this procedure is the preservation of the mesostructure which can be fixed by a preliminary calcination step. Various organic groups can be attached to the internal and external surface of the mesoporous network, for example thiol, amino, urea, epoxy, carboxyl, sulfonic acid, alkoxy or vinyl functions. One possible consequence of adding organic groups to the pore walls is a decrease of the materials porosity. Depending on synthesis

conditions, it is possible that the pore entrances are targeted firstly by the silane and thus, especially with large functional groups, pore blocking occurs. In this case the result is an unfavorable inhomogeneity of functional groups on the surface [24].

A selective grafting approach was shown by Ruiz-Hitzky and co-workers [33]. The first grafting step was carried out before the removal of the surfactant addressing to the external surface. After extraction of the surfactant a different functionality was grafted on the internal surface.

When grafting methods are applied to plane silica surfaces (e.g. glass), self-assembled monolayers (SAMs) can form [34]. A SAM consists of a 2-dimensional structural arrangement of a monolayer of silane molecules which form due to intermolecular interactions. Examples are alkylsiloxane monolayers or fatty acids on oxide materials [35].

The second functionalization method is co-condensation, a one-pot procedure. Here the organic functionality is added to the reaction mixture, typically as a trialkoxysilane, together with the tetraalkoxysilane and the SDA. A uniform surface coverage with organic groups, the prevention of pore blocking and the covalent fixation to the pore walls are advantages of this method. However, some disadvantages occur. The organosilanes can only be added to the reaction mixture up to 40 mol-%, because otherwise the formation of the mesostructure is disturbed. In general the ordering of the mesostructure degrades with increasing organosilane fraction. An even distribution of organic groups can only be achieved if the alkoxysilane has a similar hydrolysis rate as the tetraalkoxysilane [36]. Moreover, the removal of the SDA after the synthesis is hampered, because destruction of the established organic functions has to be prevented. By means of extraction, 20 % of the surfactant may remain trapped, probably inside the micropores within the silica walls [36]. A reduction of porosity is occurring within the co-condensation method as well [24]. Furthermore, some functionalities like epoxy, nitril, amides or ester groups are not stable at synthesis conditions and can therefore not be applied within this procedure [ 37 ]. The products of grafting and co-condensation methods are schematically depicted in Figure 2–11.

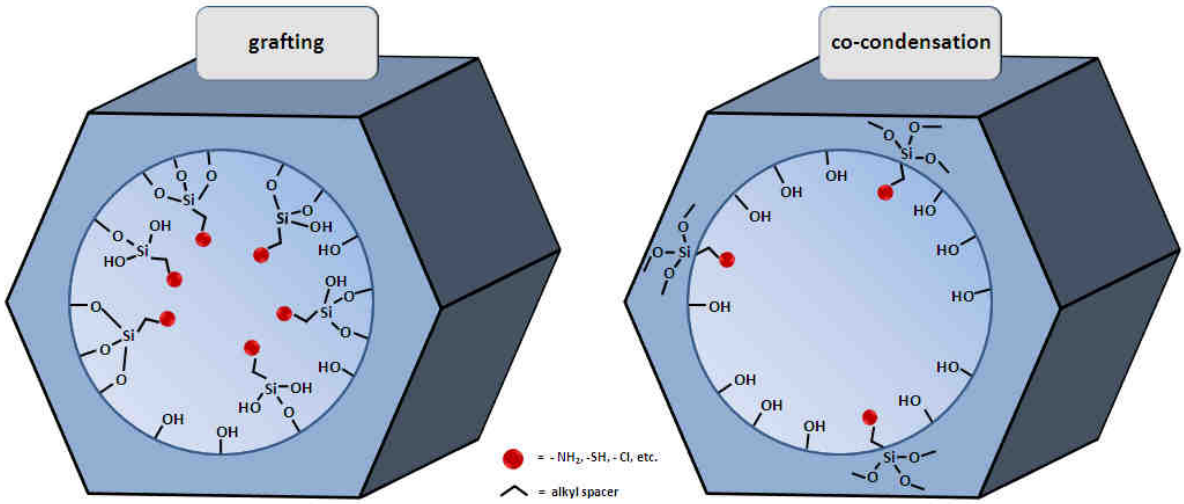


Figure 2-11. Surface functionalization products of mesoporous silica by grafting (left) and co-condensation (right) (adapted from [24]).

A further approach of establishing organic groups in mesoporous silica is the construction of the mesoporous framework with organosilica precursors of type $(R'O)_3Si\text{-}R\text{-}Si(OR')_3$. These periodic mesoporous organosilicas (PMOs) exhibit a defined mesostructure with a narrow pore size distribution in which the organic functions become an essential part of the material [24].

In the following part the modification of silica surfaces by coupling of trialkoxysilanes is focused. The modification of silica surfaces via grafting methods with organosilanes is well established. The connection mechanism of the silane to the silica surface depends on synthesis conditions (type of solvent, silane and substrate, concentration of the silane, temperature, humidity and time). The common mechanism suggested for the reaction in dry toluene at low concentrations of silane and minimum concentration of water is shown in Figure 2–12. First, the trialkoxysilane becomes hydrolyzed by water present on the silica surface. This hydrolysis is self-catalyzed when amines are the functional group. The hydroxyl groups formed in this way then connect to the surface silanol groups by hydrogen bonding. By heating, this attachment can be transformed into a covalent bonding by condensation, leading to siloxane bridges. The condensation can again be catalyzed by amine functions within the molecule [35]. In case of a lack of water in the system, this will lead to the formation of an incomplete monolayer [38].

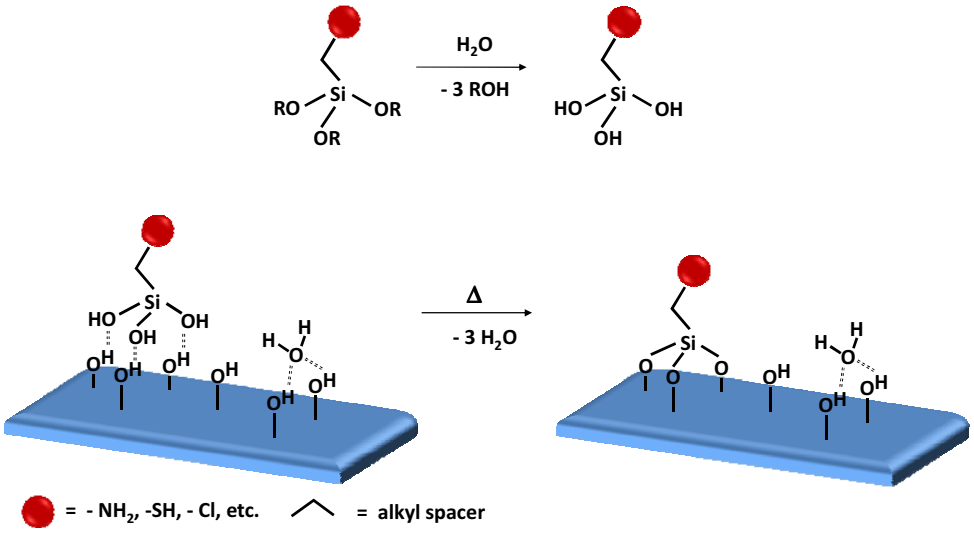


Figure 2-12. Silanization in case of low concentration of silane and with minimum water content on a mesoporous silica surface (adapted from [38]).

Smirnov and co-workers stated that two types of polycondensation reation occur, horizontal (intermolecular condensation of the trialkoxysilane) and vertical (formation of siloxane bridges to the surface) [39]. They studied the silanization of quartz slides with an amino trimethoxysilane in acetone and showed that the amino group density can be increased by an intermittent water treatment. They presumed that first the vertical condensation occurs, thereafter water proceeds to hydrolyze remaining methoxy residues; then, after a second silane treatment the horizontal polymerization is favored (Figure 2–13).

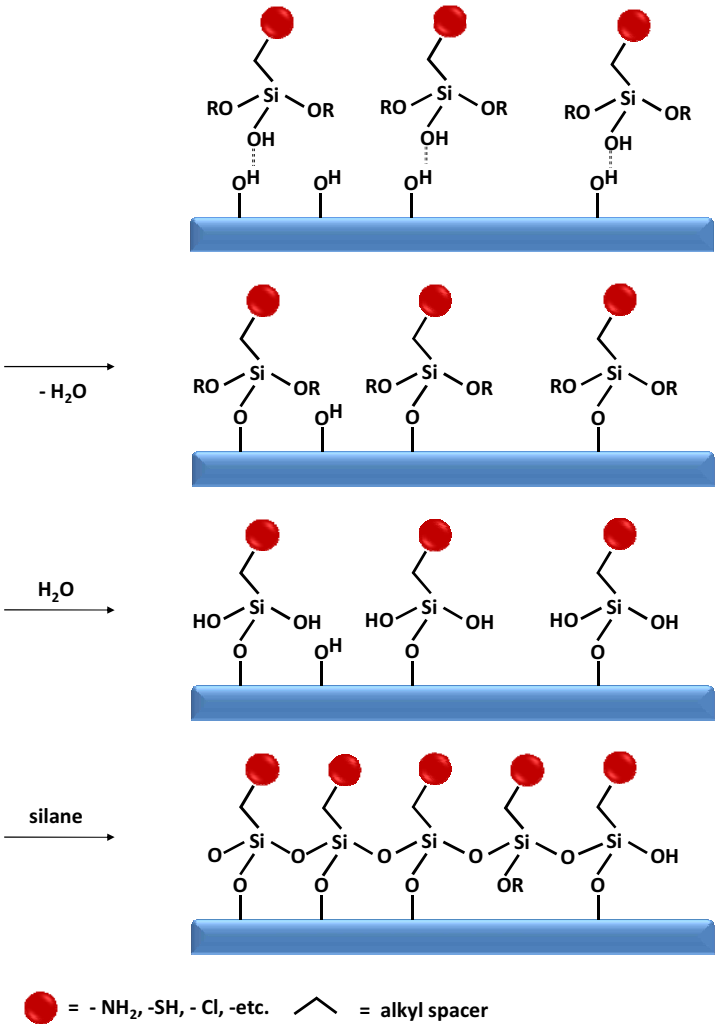


Figure 2-13. Two-step silanization with intermediate water treatment (adapted from [39]).

Concerning highly ordered monolayer formation two different models exist. First is the deposition of a liquid-like, non-oriented film that remodels to an ordered structure and grows continuously. The second theory presumes the initial formation of close-packed small areas, "islands", which grow together to a dense film. This mechanism is favored by increasing the concentration of the silane, because the intermolecular condensation becomes more probable. Also with increasing water content, the formation of islands is preferred [40]. In general, increasing water content also leads to intermolecular condensation reactions in solution (Figure 2–14a). The formed aggregates then deposit on the surface (Figure 2–14b) [39]. The tendency of polymerization strongly depends on the synthesis conditions for each silane. In addition, the effect of the functional group of the silane on the silanol groups has to be considered. One example are amine functions, which can form hydrogen bonds to

silanol groups; in this case protonated ammonium groups were found to be oriented towards the surface and neutral ones away from it [41, 42].

When the hydrolysis of the trialkoxysilane is carried out in water excessive polymerization will proceed [38]. Isolated monomers, cyclic oligomers, and larger branched oligomers are formed by alkoxysilanes (Figure 2–14c) in water depending on the type of silane, concentration, pH value, temperature, storage condition, and time [41]. In contrast, some silanes are stable in water for several hours (e.g., glycidyl-trimethoxypropylsilane at pH 7). Multilayer formation can occur in case of high concentrations of silane or long reaction times. It is possible that multilayers deposit on top of the monolayer which was formed in the early state of reaction (Figure 2–14d). Another alternative is direct fixation of higher condensed silica oligomers to the surface silanol groups in case of fast uncontrolled condensation in solution (Figure 2–14e).

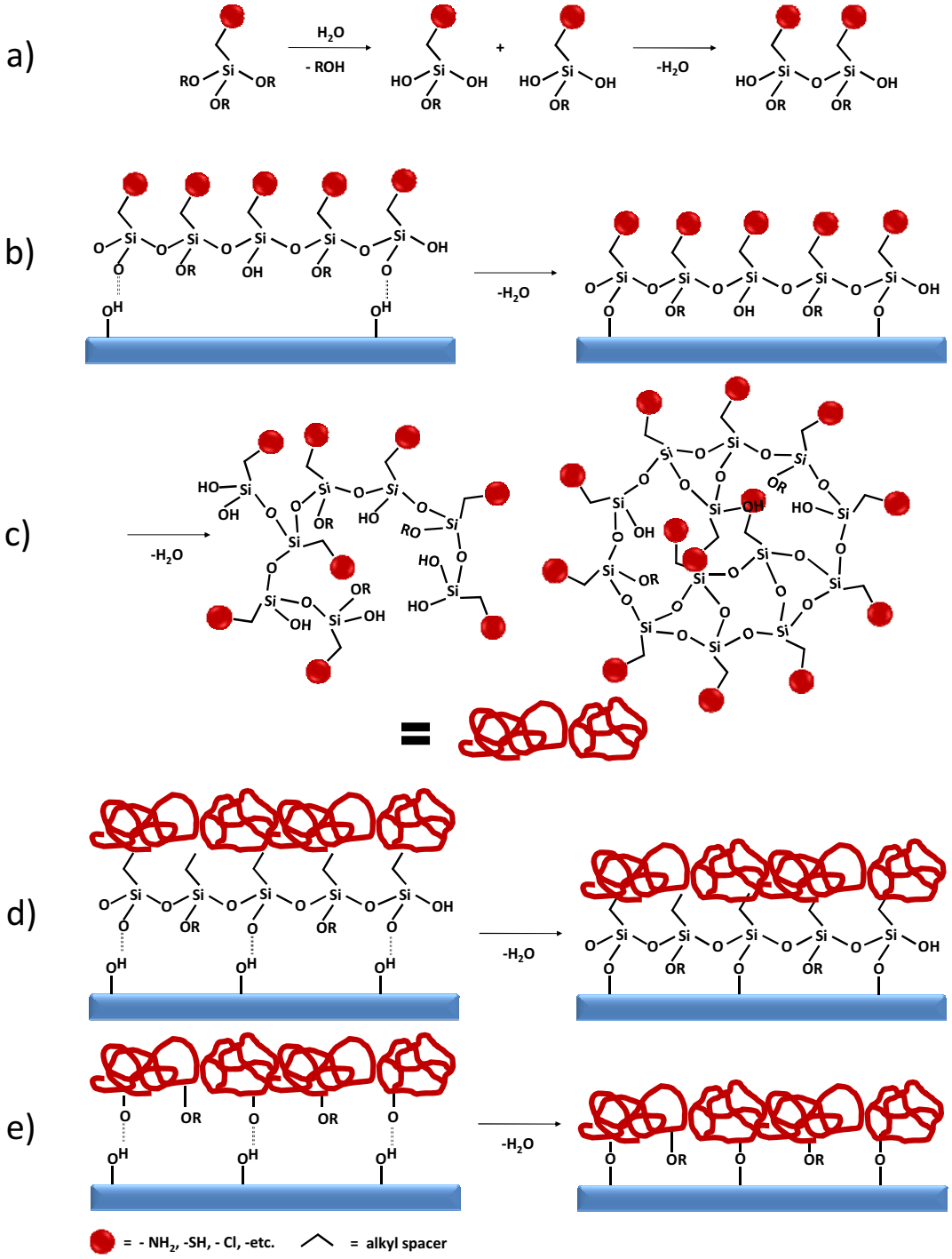


Figure 2-14. Silanization in case of high water or silane content. a) condensation of the silane forming larger molecules, b) deposition of higher silica oligomers at increased concentrations of silane, c) uncontrolled condensation of the silane in solution in case of extended reaction time or high concentration, d) Formation of a disordered multilayer on top of the monolayer at longer reaction times (right) or high concentration of silane (left), e) deposition of higher silica oligomers on the surface in case of fast intermolecular condensation in solution.

### 2.2.4. Mesoporous materials for immobilization of proteins

Biomolecules can be immobilized by entrapment in polymeric capsules or gels, by physical adsorption (via VAN DER WAALS, electrostatic or hydrogen bonding interactions) or covalent attachment (for example, formation of imine bonds or disulfide bridges) to a support [43]. Another possibility is cross-linking of the protein itself [44]. Often, polymers bearing functional residues are used as immobilization supports, but inorganic supports are also considered as immobilization material. Clays, layered double hydroxides, silica gels, controlled pore glasses, amorphous aluminium phosphate, alumina and zeolites are examples of utilizing an inorganic material as a host for biomolecules [45, 46]. For applications in living systems, general biocompatibility is an important issue which has been proven for silica materials in previous work [3, 20, 21, 47, 48].

Mesoporous silica was first used as support for enzyme immobilization in 1996 [49]. It is suitable for protein immobilization because of its high surface area, defined pore geometry, connectivity and adjustable size [44, 46]. In addition, the presence of microchannels allows faster diffusion [49]. Further factors controlling protein adsorption are particle shape and size, concentration of the protein and temperature during adsorption [50]. The advantage of protein immobilization on solid supports like mesoporous silica lies in their application. The possibility of separation, storage, reuse and stability, especially in organic media, are crucial factors for industrial processes.

In general, a protein can develop three different types of interaction with a silica surface: electrostatic interactions, hydrogen bonding and VAN DER WAALS interactions. All these depend on the chemical nature of the silica support and protein at the surrounding conditions. On this account three different strategies are followed concerning protein immobilization in mesopores.

Firstly, the protein can simply be physically adsorbed on the unmodified surface (Figure 2–15). Here larger pores are leading to higher amounts adsorbed, but always bear the disadvantage of leaching. However, if the further reaction is conducted in organic media the water soluble protein is trapped inside the pores and leaching does not occur. Moreover it is possible that the activity of an enzyme is even increased by adsorption in mesopores. On the one hand the adsorption of reactants can be facilitated by the hydrophilic surrounding of the silica, and on the other the adsorption of enzymes on the surface prevents enzyme aggregation [49, 51]. A

detailed strategy for adsorption of enzymes on mesoporous materials was developed by Hodnett and co-workers [52].

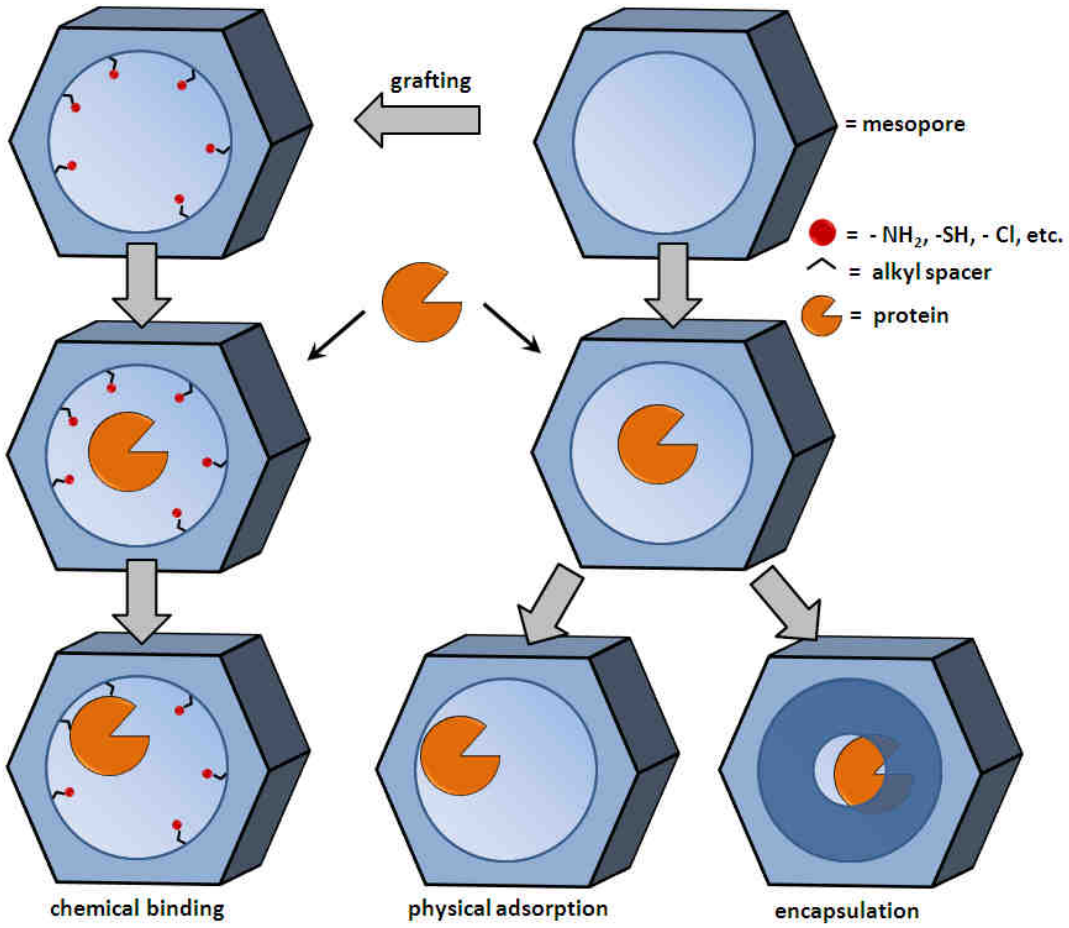


Figure 2-15. Protein immobilization strategies (adapted from [49]).

The second possibility of protein immobilization is encapsulation. This is a two step procedure. First the protein is adsorbed into the porous system and then the pore entrances are closed in a second silanization step (Figure 2–15). This is an effective way of keeping the protein in the pore, but has the disadvantage of a possible destruction of the protein during the synthesis.

The third and most effective way of controlling protein immobilization is chemical binding to the surface. This can be established by electrostatic or covalent binding. The functional groups necessary for this kind of strong fixation are introduced by the mentioned grafting and co-condensation methods. Possible organic functions are thiol-, carboxyl-, alkyl chloride-, epoxy- and amino-functions. Hydrophobic interactions can be utilized with alkyl-, phenyl-, or vinyl-functions. Epoxy residues

can react with amino functions of the protein to form covalent bonds [53] (Figure 2–16a). Aldehyde groups offer the possibility of forming imine bonds (Figure 2–16b) with amines present in proteins. In order to achieve bond formation between amino functions and carboxyl residues, often additional cross-linkers or activators have to be used. Besides carbodiimides [54] (Figure 2–16c), homo or hetero-bifunctional cross-linkers carrying succinimidyl esters [55] (Figure 16d, e) or maleimide functions are most common [56] (Figure 2–16f). Furthermore, covalent coupling of proteins can be achieved by means of click chemistry [57] and formation of disulfide bridges [58].

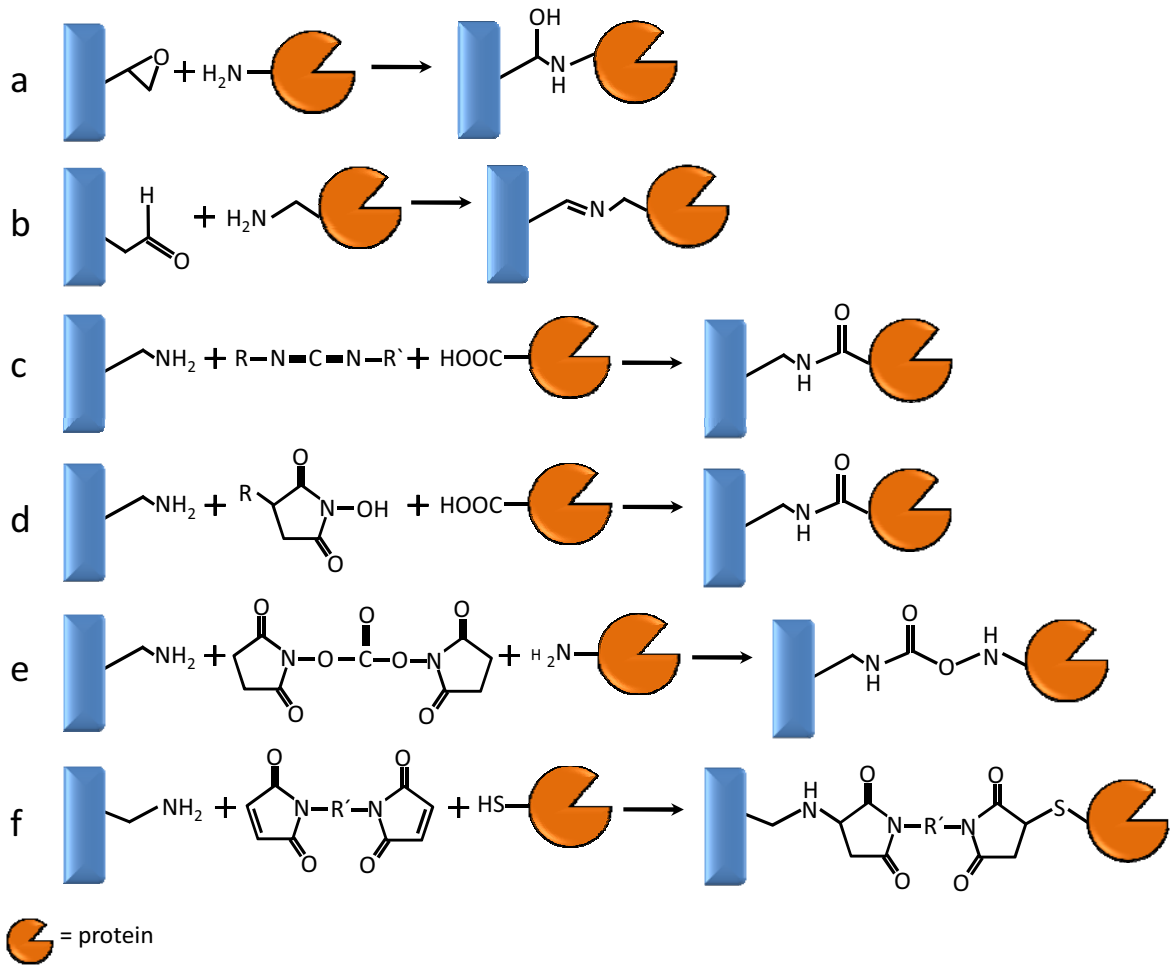


Figure 2-16. Possible reactions for the covalent immobilization of proteins on silica surfaces (adapted from [44, 53, 56, 58]).

### 2.2.5. Mesoporous materials for controlled drug delivery

Controlled drug delivery systems are tools for the transportation of drugs to the targeted tissue and for keeping a therapeutic drug level for a desired time. Often polymers are used for this purpose [59]. However, because of some disadvantages in

biocompatibility and degradation behaviour new materials are constantly being investigated as drug delivery systems.

Three different types of silica have been considered as drug delivery devices: silica based xerogels, mesoporous silica (also in form of nanoparticles) and mesoporous hollow silica spheres [60]. In this part a short overview of mesoporous drug delivery materials shall be given. Detailed information can be found in recent reviews [61, 62, 63].

Mesoporous silica is a promising candidate for drug delivery systems because of its unique properties. The size and shape of the pores of the well-defined porous network and their connectivity are important factors in delivery control. The ratio of pore to drug size is supposed to be adjusted properly. Whether the pores are much larger than the drug molecules, or the pores are much smaller, the adsorption of large drug amounts is inhibited [61]. Normally, a pore size just a bit larger than the drug molecule is optimal. Due to the variability in mesoporous silica synthesis, the pore size can be adapted to the size of drug molecule. In addition, larger pores connected through smaller pore windows are supposed to have slower release behaviour. In case of similar particle morphology, it can be found that smaller pores exhibit a slower release profile. Concerning particle size, one can state that the smaller the particles are the faster is the release, due to diffusion control. Another quality of mesoporous materials is their high surface area. A higher surface area accounts for a higher amount of drug loaded.

The most important feature of mesoporous silica in control of drug delivery is the possibility of surface functionalization as already mentioned above. The silanol groups present on the silica surface can be modified by means of grafting or co-condensation methods as described in section 2.2.3, whereas post-synthesis grafting methods have shown better results so far [61]. A wide range of functionalities is possible, allowing host guest interactions like electrostatic attractions (positively or negatively charged), hydrogen bonding, aromatic or hydrophobic interactions or formation of labile ester functions, tailored for the desired drug [61, 64]. Here again, the density of these functionalities has to be adjusted carefully because not the type of charge is the crucial factor but the net charge of the surface at a defined pH value [65].

These functionalizations have not only the purpose of increasing the amount of inserted drug, but also to control the release behaviour. In most cases an initial burst release profile can be observed (Figure 2–17a). This release behaviour can be useful if

an acute infection is present and a high dose of drug is needed at the beginning. Unfortunately the small doses released later are often too low to have a therapeutic effect. Release profiles controlled by hindered diffusion or dissolution of the drug are slowed down (Figure 2–17c) Therefore, covering the inserted drug in the porous system with an additional layer is a strategy for controlled release [60]. A favorable case for many applications would be a constant drug release over the necessary time (Figure 2–17d) maintaining the therapeutic drug levels for a long time period. A combination of large initial release dose with sufficient doses released later could also be effective in surgery (Figure 2–17b). A more sophisticated approach is a stimulus-responsive drug release system (Figure 2–17e). Here drug doses are released after a stimulus from outside or changes of surrounding conditions such as temperature change, illumination or change of pH value. These systems are to be applied especially when the drug carrier has to reach the targeted tissue before the release of the drug, or when the need for the application of the drug itself triggers the drug release (e.g., infections cause acidic pH values that would stimulate the release of drug).

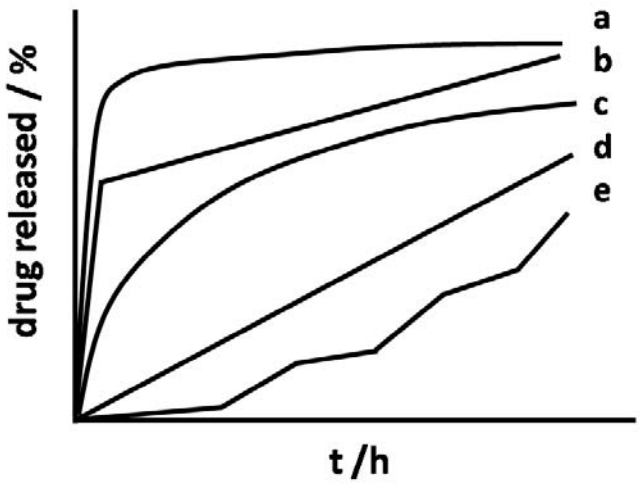


Figure 2-17: Drug release profiles (adapted from [61]).

## 2.3. Detection methods for immobilized proteins

### 2.3.1. Chemical detection – *para*-nitrophenylphosphate assay for alkaline phosphatase

Alkaline phosphatase is a hydrolyzing enzyme which catalyzes the hydrolysis of phosphoric acid esters. It cleaves phosphate groups of molecules like proteins, nucleotides and alkaloids and to produce alcohols. Alkaline phosphatase is present in most organisms. In the human body, alkaline phosphatase is found prodominantly in the liver, kidney, bile duct and in bones in the form of different isoenzymes. It can be detected in the blood serum where an increased amount of the enzyme indicates diseases of liver and bones, mainly. For example, bone defects cause elevated levels of alkaline phosphatase.

Alkaline phosphatase is a metalloenzyme which is made up of two subunits. Three divalent cations are necessary (three zinc ions or one magnesium and two zinc ions) at its catalytic center. The metal ions facilitate the covalent binding of the substrate-phosphate group at the active center [66, 67].

The most frequently used methods for the detection of enzymes are based on fluorogenic and chromogenic substrates. During the application of these assays, a nonfluorescent or colorless substrate turns into a fluorescent or colored product. Often electron-poor conjugated aromatic phenols carrying a functional residue that can be cleaved during the enzyme-catalyzed reaction are used as substrates [68].

One method to determine the activity of the alkaline phosphatase is based on the reaction with the substrate *para*-nitrophenylphosphate. After dephosphorylation, the yellow product *para*-nitrophenolate anion or *para*-nitrophenone respectively is formed, which can be detected spectrophotometrically at 405 nm (Figure 2–18) [69]. The amount of *para*-nitrophenone formed is directly proportional to the activity of the enzyme. The measurement of enzymatic activities is given in “units” with one unit being defined as the amount of enzyme that transforms one micromole of substrate per minute at given conditions ( 1 U = 1 µmol $min^{-1}$).

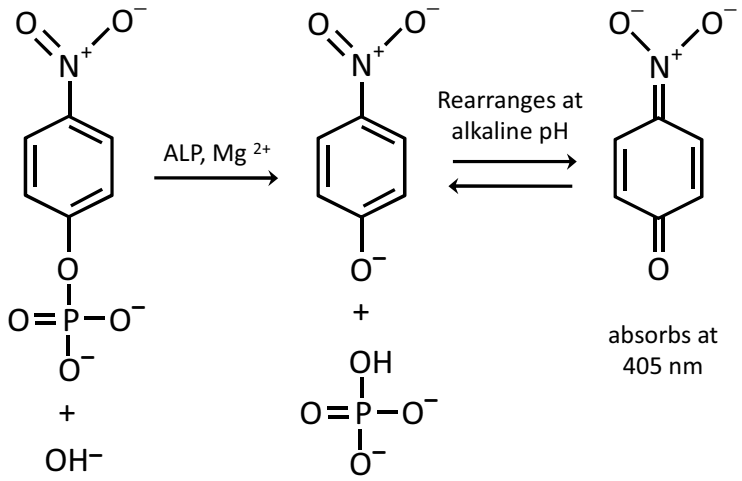


Figure 2-18. Reaction of the *para*-nitrophenylphosphate assay for quantification of alkaline phosphatase.

In this work the *para*-nitrophenylphosphate assay is used as an indirect method to determine the amount of immobilized active enzyme on the surface. The activities detected with the enzyme-bearing glass substrate are compared to calibration curves, which rely on the relation between activity and free enzyme in solution. In this way, the activity of immobilized enzyme can be correlated with an amount of free enzyme in solution. This value corresponds to the amount of active alkaline phosphatase on the surface. It is possible that more enzyme has been immobilized, but is inactive, for example due to steric hindrance. It is important to carry out the calibration measurement at the same time as the measurement of the immobilized enzyme using identical solutions, because surroundings and synthesis conditions are crucial factors for enzyme activity.

### 2.3.2. Biochemical detection – enzyme-linked immunosorbent assay for BMP2

For the detection of the immobilized BMP2 an enzyme-linked immunosorbent assay (ELISA) can be applied. This method relies on the specific recognition of an antibody by an antigen. An antigen is an **anti**body **gen**erating substance that evokes an immune response in vivo. Antibodies can be proteins or polysaccharides, for example. In the simplest set-up, an enzyme is covalently linked to an antibody, which is specific for the substance to be quantified. In case of antigen being present this enzyme-linked antibody forms an antigen-antibody-enzyme complex. After removal of unbound antibody, the corresponding enzyme substrate is added and the enzyme is allowed to react with the substrate. The amount of the product correlates with the amount of protein. Often used enzymes include horseradish peroxidase or alkaline phosphatase.

They convert colorless substrates into colored products, which can be determined spectrophotometrically.

The method for BMP2 detection is based on an indirect ELISA. In this set-up, a specific antigen is immobilized on a plate (optimized for high protein adsorption) and the antibody is allowed to bind to the antigen. In a subsequent step the enzyme-linked antibody is applied and can form colored products to be detected (Figure 2–19).

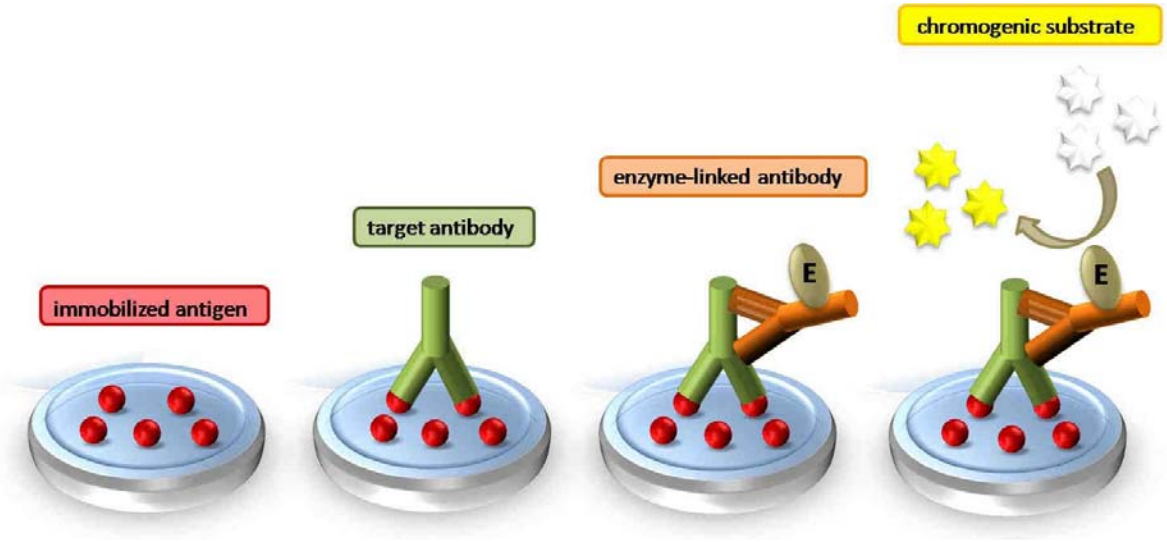


Figure 2-19. Principle of an indirect ELISA; after each step of the procedure washing occurs.

In the special case of the detection of immobilized BMP2 the sample contains antigen (namely, BMP2) already immobilized on the desired substrate. After blocking of nonspecific binding sites over night with 10 % fetal calf serum in phosphate buffered saline and washing, a monoclonal mouse anti-human BMP2 antibody (the antibody is produced in mice and recognizes human BMP2) is added. After washing, the sample is combined with a goat anti-mouse antibody peroxidase conjugate. This enzyme-linked antibody is raised in goats against the mouse antibody and is allowed to react with the horseradish peroxidase substrate 3,3',5,5'-tetramethylbenzidine. After addition of sulfuric acid, a stable yellow diimine is formed which can be detected spectrophotometrically at 450 nm (Figure 2–20) [70].

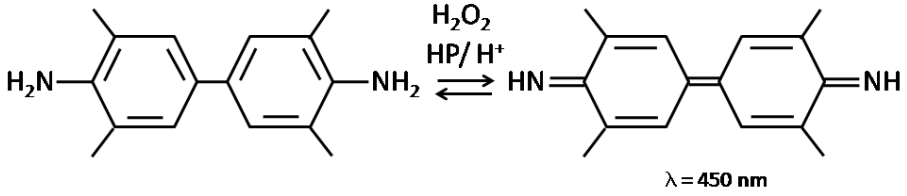


Figure 2-20. Reaction of the substrate 3,3',5,5'-tetramethylbenzidine for the colorimetric detection of BMP2 (according to [70]).

The washing steps within this procedure are carried out with Tween-20® in tris-buffered saline. Tween-20®, a polyoxyethylene derivative of sorbitan monolaurate, is a nonionic polysorbate surfactant (Figure 2–21). Tris-buffer (tris-hydroxymethyl-aminomethane) can be used in a pH range between 7 and 9.2 (here: 7.5) and is therefore often used to simulate physiological conditions [71].

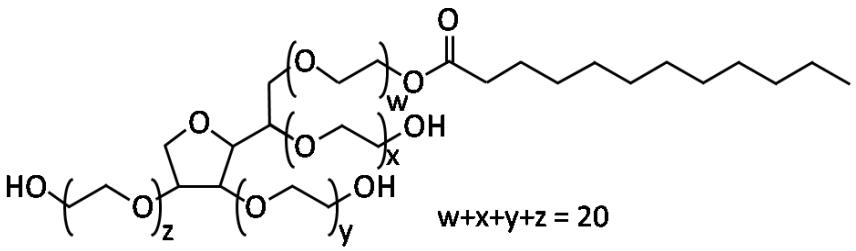


Figure 2-21. Chemical structure of Tween-20®.

### 2.3.3. Biological detection – BRE-luc test for the detection of immobilized BMP2

For the detection of immobilized and biologically active BMP2, a recently developed cellular test system was applied [72]. The principle of this BRE-luc test is as follows. Suitable mouse cells (C2C12) were stably transfected with an expression construct containing a specific DNA sequence (called promoter which contains the **B**MP-**r**esponsive **e**lements, cf. below) and the luciferase reporter gene (**luc**). This means that a plasmid was introduced into the mouse cells and stably inserted into their genomes. Due to this expression construct, the cell is able to produce the desired gene (luciferase from fireflies) under certain conditions which relate to the specific DNA sequence.

The expression vector is carrying a promoter with BMP-responsive elements. A promoter is a sequence of DNA positioned near a certain gene (here: the luciferase gene) that allows for the transcription of this gene. The transcription in a cell is

carried out by the enzyme RNA polymerase. As the name implies, BMP-responsive elements are activated only by the presence of BMP. Once the extracellular BMP protein is binding to the surface of the cells, within the cells the BMP-related Smad signalling pathway is induced. Smads target the BRE-responsive elements and therefore the luciferase reporter gene is switched on, which leads to production of luciferase. Similar to alkaline phosphatase, the activity of the produced enzyme is measured, in this case using luciferin as substrate. During this reaction photons are emitted that can be detected as chemoluminescence in the cell lysates 24 to 48 hours after BMP exposure. The signal is dose-dependently related to the amount of BMP which has interacted with the cells and can be applied for the detection of BMP2, BMP4 and BMP7. The assay with a detection range of 0.5 to 1 ng $ml^{-1}$ is at least 100-fold more sensitive than the classical alkaline phosphatase activity assay (about 1000 ng $ml^{-1}$). And, because the alkaline phosphatase is an indirect BMP target gene, the assay needs three to six days to give reliable information. The alkaline phosphatase is present when fully developed bone cells occur, whereas the BRE-luc test is conducted with undifferentiated precursor cells.

This general method has recently been adapted to the detection of immobilized BMP2 on inorganic substrates [71].

# 3 Results and discussion

## 3.1. Immobilization of alkaline phosphatase on modified silica coatings

***Preface***

This section deals with the immobilization of the enzyme alkaline phosphatase on structurally different silicate substrates. Uncoated glass sides as well as an unstructured and a mesoporous silica film, both coated on glass slides, were investigated as substrates. The main objective was to develop an immobilization strategy for proteins which can be transferred to implants. A major question was whether the structural properties of the surface exert an influence on enzyme immobilization, especially whether the high surface area of the mesoporous film increases the amount of bound enzyme. Furthermore, a suitable linking agent had to be identified among from different trialkoxysilanes carrying different functional groups like epoxy, urea and amino. Another aim of this investigation is the optimization not only of type of functional group, but also of its density, which is influenced by the concentration of the silane during the modification step.

This section will be submitted as an original research article to the journal Microporous and Mesoporous Materials. The authors are Nina Ehlert, Dr. Peter P. Müller, Dr. Martin Stieve and Prof. Peter Behrens.

The idea to develop a general immobilization strategy for proteins which can in principle be transferred to implants was developed within the work package D1 consortium by Prof. Peter Behrens, Dr. Peter P. Müller and Dr. Martin Stieve. The approach to test this strategy with alkaline phosphatase as a cheap and robust model enzyme was developed by the author of this thesis, Prof. Peter Behrens and Dr. Peter P. Müller. The author of this thesis has designed the experiments in agreement with the supervisor and has carried out all experimental work, including the preparation of the samples, their characterization and the adaptation of the nitrophenyl phosphate assay. Furthermore, the results have been interpreted with the aid of the supervisor.

**Abstract**

The influences of silica surface structures and various silane linker molecules on the capacity to bind active proteins were investigated. For this purpose, microscope glass slides were coated with unstructured or mesoporous silica films. The binding of the protein to the surface was mediated by trialkoxysilanes with different functional groups like amino, epoxy, and urea functions. As a model protein, alkaline phosphatase (ALP) was chosen. Enzyme assays showed that all the functionalized trialkoxysilanes tested were able to immobilize active ALP on silica surfaces. Using the 3-aminopropylsilyl modification resulted in the highest activity of bound ALP, especially in combination with a mesoporous surface coating. Interestingly, unstructured silica films had only low capacity to immobilize active enzyme whereas both mesoporous silica coatings and plain glass bound higher amounts of active ALP. By using mesoporous coatings functionalized with 3-aminopropylsilyl residues maximal binding capacities for active ALP were achieved. This combination appeared most promising for further development of bioactive surfaces for practical applications such as industrial enzymatic applications or surgical implant functionalization.

**Keywords:** mesoporous coating, protein immobilization, alkaline phosphatase (ALP), silanization.

**Introduction**

The immobilization of biomolecules on solids plays a decisive role for a variety of applications, and improvements are made continually for example in the fields of biosensors [73, 74], drug delivery [75-77] or for optimal tissue integration of implants into living systems. Especially the latter topic is getting ever more important, as there is an increasing demand of implants due to higher life expectancy. Apart from other important approaches, as the application of specific surface structures in the micro- or the nanometer range [3] or chemical modification of implant surfaces [78, 79], coatings with biochemically active substances like drugs or proteins cannot only improve the biocompatibility of implants, but can be employed to promote the healing process and enhance implant functionality. For example, a coating with antibiotics can avert the occurrence of infections after implantation [80-82]; the modification of an implant surface with BMP2 (Bone Morphogenetic Protein2) can induce the differentiation from precursor to bone-forming cells, thus allowing better fixation of bone replacement implants [83, 84]. Here we present the use of mesoporous silica

coatings for implant materials which can be used as a base system for further functionalization, especially for the immobilization of proteins. We have recently shown in several animal experiments that such mesoporous silica coatings are biocompatible [3, 20, 21].

Protein immobilization has been developed for various materials [78, 85]. Due to its high surface area [24], mesoporous silica materials have shown promising results, using various immobilization strategies [43, 46, 49, 86-89]. A major problem is the stable attachment of the protein to the surface. Biomolecules only adsorbed physically are easily removed, for example by body fluids. Therefore, fixation of proteins by strong covalent or ionic bonds is usually preferred, although the strong fixation can influence the conformational properties of the proteins or render their active parts inaccessible, thus reducing their activity. Nevertheless, industrial processes have shown that the immobilization of enzymes in mesopores can have the advantage of easy separation and increased stability due to protection from denaturation in the pores [49]. The activity of bound proteins generally can be influenced by multiple parameters, such as the surface structure (topography, roughness, porosity) [90, 91] of the substrate, the material surface properties [49, 86] and the chemical nature of the bonding [49].

The present approaches of immobilizing proteins on glass or silica surfaces [92] or on mesoporous silica [43, 49, 86-89] are following different routes like the mentioned simple adsorption [86, 88] or the use of reactive functional groups which are attached to the substrate by the reaction of trialkoxysilanes with surface silanol groups [43, 49, 89, 92]. However, it has not yet been convincingly demonstrated that mesoporous silica exhibits true advantages in comparison with other silica surfaces (e.g. plain glass or unstructured sol-gel-derived coatings). In spite of the large surface area of mesoporous silicas, protein immobilization inside mesopores as well as the access of the substrate molecules might be hampered by diffusion limitations.

Here we present a basic study on the strong immobilization of alkaline phosphatase (ALP) onto differently structured silica surfaces, namely plain glass slides, glass slides coated with an unstructured or with a mesoporous silica layer. The design of our study is summarized in Figure 3–1. We tested different functional groups attached to the silica surface by a silane like amino, epoxy and urea functions. Judging from the activity of the immobilized ALP, we can thus compare the influence of the different surfaces and the different linking agents. As the protein to be

immobilized we chose alkaline phosphatase (ALP). ALP is a favorable model protein, as it is a cheap and robust enzyme, the activity of which in an immobilized state on a surface can be tested by a simple assay [69, 93]. The immobilization of ALP on various materials has been described [73, 74, 85, 94-97]. In spite of this extensive work on ALP, its attachment on glass surfaces has not yet been systematically investigated [93, 98].

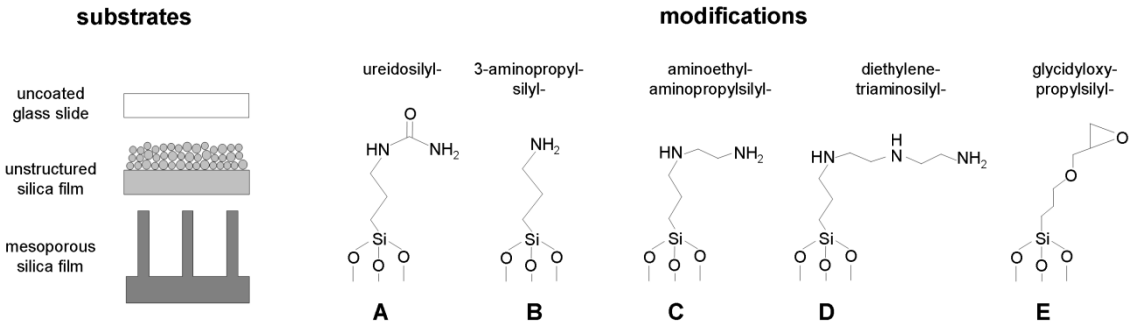


Figure 3-1. Immobilization strategy for binding of ALP on different silica substrates: uncoated glass slides (white), amorphous silica (grey) and mesoporous silica (dark grey) coatings were modified by means of silanization with 3-aminopropyl-trimethoxysilane (A), [3-(2-aminoethylamino)-propyl]trimethoxysilane (B), 3-[2-(2-aminoethylamino)ethyl-amino]propyl-trimethoxysilane (C), (3-glycidyloxypropyl) trimethoxysilane (D) and *N*-[2-(trimethoxysilyl)-proply]urea (E).

## Experimental

### *Preparation of the substrates*

All chemicals except for the ethanol were of analytical grade, purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) and were used without further purification. Glass slides (No. 2400, Elka, Sondheim, Germany) were cleaned by ultra-sonification for 10 min each in ethanol (Merck, Darmstadt, Germany) and acetone prior to use.

The solution used for the preparation of mesostructured silica coatings contained ethanol, water, hydrochloric acid, tetraethoxysilane (TEOS) as a silica source and poly(ethylene glycol)-poly(propylene glycol)-*block*-co-polymer (Sigma-Aldrich, $EO_{20}PO_{70}EO_{20}$, average $M_n$ ~ 5.800, similar to Pluronic® P-123 from BASF) as the structure-directing agent. The molar ratios of the constituents were TEOS : EtOH : $H_2O$ : HCl : $EO_{20}PO_{70}EO_{20}$ = 1 : 48.9 : 26.9 : 0.06 : 0.0135, following the literature [99].

The solution was prepared by adding TEOS to a solution of $EO_{20}PO_{70}EO_{20}$ in ethanol, water and hydrochloric acid. The solution was stirred for about 10 minutes before coating the specimens. The glass slides were coated using a dip-coating procedure (DC Small Dip-Coater from NIMA, Coventry, England) taking place at constant air humidity of 80 %, adjusted by 50 m% glucose solution [25, 31]. They were dipped into the solution for 30 seconds, withdrawn with approximately 1 mm/min and left at constant air humidity for five minutes. Afterwards, the specimens were dried at 60 °C over night. The organic material was subsequently removed by calcination at 415 °C for 4 h to yield mesoporous silica. The amorphous coatings were synthesized in a similar procedure, but omitting the $EO_{20}PO_{70}EO_{20}$ surfactant. In this case the glass slides were left in the dipping solution for 2 minutes.

***Chemical modification of the substrates***

The cleaned or coated glass slides were incubated in aqueous solutions of 3-aminopropyl-trimethoxysilane with different concentrations between 0.1 to 10 m% or in 10 m% solutions with other linker molecules, namely [3-(2-aminoethylamino)-propyl]trimethoxysilane, 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxy-silane, (3-glycidyloxypropyl)trimethoxysilane or *N*-[2-(trimethoxysilyl)-proply]urea (Figure 3–1). Prior to incubation, the silane solution was allowed to hydrolyze for 15 min. After an incubation time of 2 minutes the glass slides were rinsed sufficiently with water to remove the excessive silane and were then transferred directly into the solution containing ALP.

***Characterization methods***

X-ray diffraction patterns were recorded on a Stoe (Darmstadt, Germany) $\theta/\theta$-diffractometer in reflection geometry using Cu$K\alpha$ radiation and a secondary beam monochromator (graphite).

The thickness of the mesoporous silica film was measured with a stylus profiler DETAK6M from Veeco instruments Inc. (Plainview, USA) with a force of 8 mg, duration of 200 seconds and length of 10 000 µm per measurement.

Static contact angle measurements were performed on a Surftens universal contact angle goniometer (OEG, Frankfurt/Oder, Germany) with water as the probing liquid. On every glass slide, the contact angle was measured at ten different positions, all at least five millimeters away from the edges of the glass slide. Each experiment was

repeated ten times and the average was calculated. The standard deviation did not exceed 4°.

### *Immobilization of alkaline phosphatase*

The modified glass slides were incubated in a 0.026 mg $ml^{-1}$ solution of the ALP (from porcine kidney, 123 Units per mg protein, one unit will hydrolyze 1.0 µmole of *p*-nitrophenylphosphate per min at pH 9.8 at 37 °C, Sigma-Aldrich no. P4439) for 4 min at 0 °C and afterwards washed three times with a solution of magnesium chloride and diethanolamine (same concentrations as in the substrate assay) to remove physically adsorbed ALP.

### *Alkaline phosphatase activity assay and quantification of immobilized ALP*

The enzymatic activity of ALP was tested by a simple colorimetric assay with the substrate *p*-nitrophenylphosphate. The ALP immobilized on glass slides was allowed to react for five minutes with the substrate (1.25 mM) in a buffer of diethanolamine (1060 mM) and magnesium chloride (0.53 mM). The reaction was stopped by adding 3 M sodium hydroxide followed by the extinction measurement 405 nm. For this purpose, a spectrophotometer UV mini 1240 (Shimadzu, Duisburg, Germany) was used. All values given are an average of five measurements. The baseline correction was carried out with the unreacted nitrophenylphosphate substrate solution.

The extinction values were compared to a calibration curve which was established individually for each series of experiments. Different soluble equivalents of an ALP solution (0.026 mg $ml^{-1}$) were added to the substrate solution and allowed to react for five minutes. Extinction values were determined and a linear calibration curve was calculated. The amount of ALP immobilized determined in this way was referred to the macroscopic surface area of the glass slide and these values are then given in ng $cm^{-2}$. Values given are the result of five independent experiments. For positive control experiments a nitrocellulose membrane (blotting-cellulose nitrate membrane, Sigma-Aldrich no. 15363) was cut into same size as standard glass slides and the incubation and assay were done under identical conditions.

## Results

### *X-ray diffraction*

As expected, the uncoated glass slides and the unstructured films do not show any X-ray reflections, due to the absence of a periodic arrangement in their structures. In

contrast, the mesoporous films show a reflection at $2\theta = 1.6°$ (Figure 3–2). This reflection is evoked by the electron density contrast between the empty cavities after removal of the organic material and the silica wall material. As no additional peaks appear, the pores in this coating have no ordered arrangement as a hexagonal, cubic or lamellar packing, and no preferred orientation with regard to the substrate. The mesopore structure is thus disordered, similar to bulk mesoporous materials of the LMU-1 [29] or KIT-1 [30] type. Therefore, pore mouths are present on the surface of the coating, as has also been shown by us in related work by high-resolution scanning electron microscopy [100], and the pore system can be accessed from the surface. Animal experiments [3, 20, 21] have shown that such mesoporous coatings on standard biomaterials (Bioverit® II) are biocompatible and that they can alter tissue reactions in living beings.

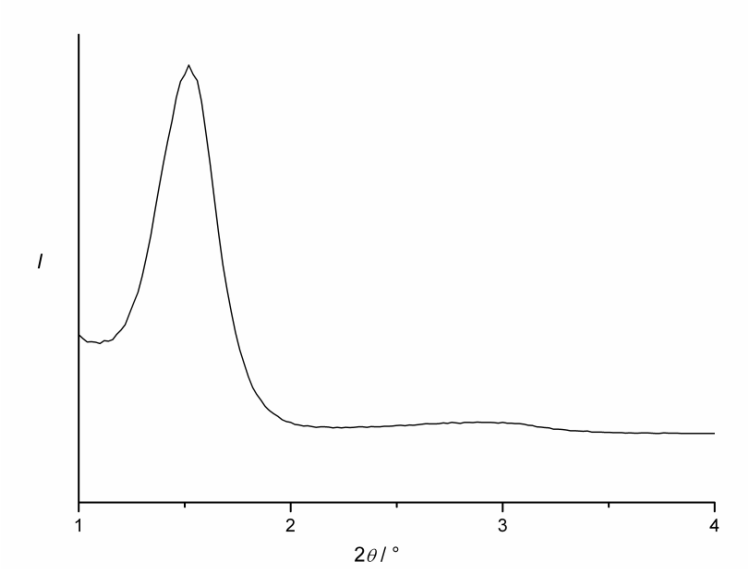


Figure 3-2. XRD pattern of a mesoporous coating on a glass slide.

***Contact angle measurements***

To investigate the changes of surface properties due to silanization, contact angle measurements were performed. The static water contact angles after modification were compared to glass slides treated the same way but without the silane (Figure 3–3). The efficiency of silanization can be monitored by an increase of the contact angle. The mesoporous film had the most hydrophilic surface with a contact angle of 8°, which is in line with published results [101] where the strong hydrophilicity of mesoporous silicas was inferred from determinations of the number of surface silanol groups (Sears number). This can be explained with the high surface area due to the inner pores of the film. The unstructured coating had a more hydrophobic contact

angle of 30° and uncoated glass slides showed the typical value of about 18°. The fact that the amorphous film is more hydrophobic than the mesoporous one can be understood as a consequence of its nature, consisting of aggregated nonporous silica particles.

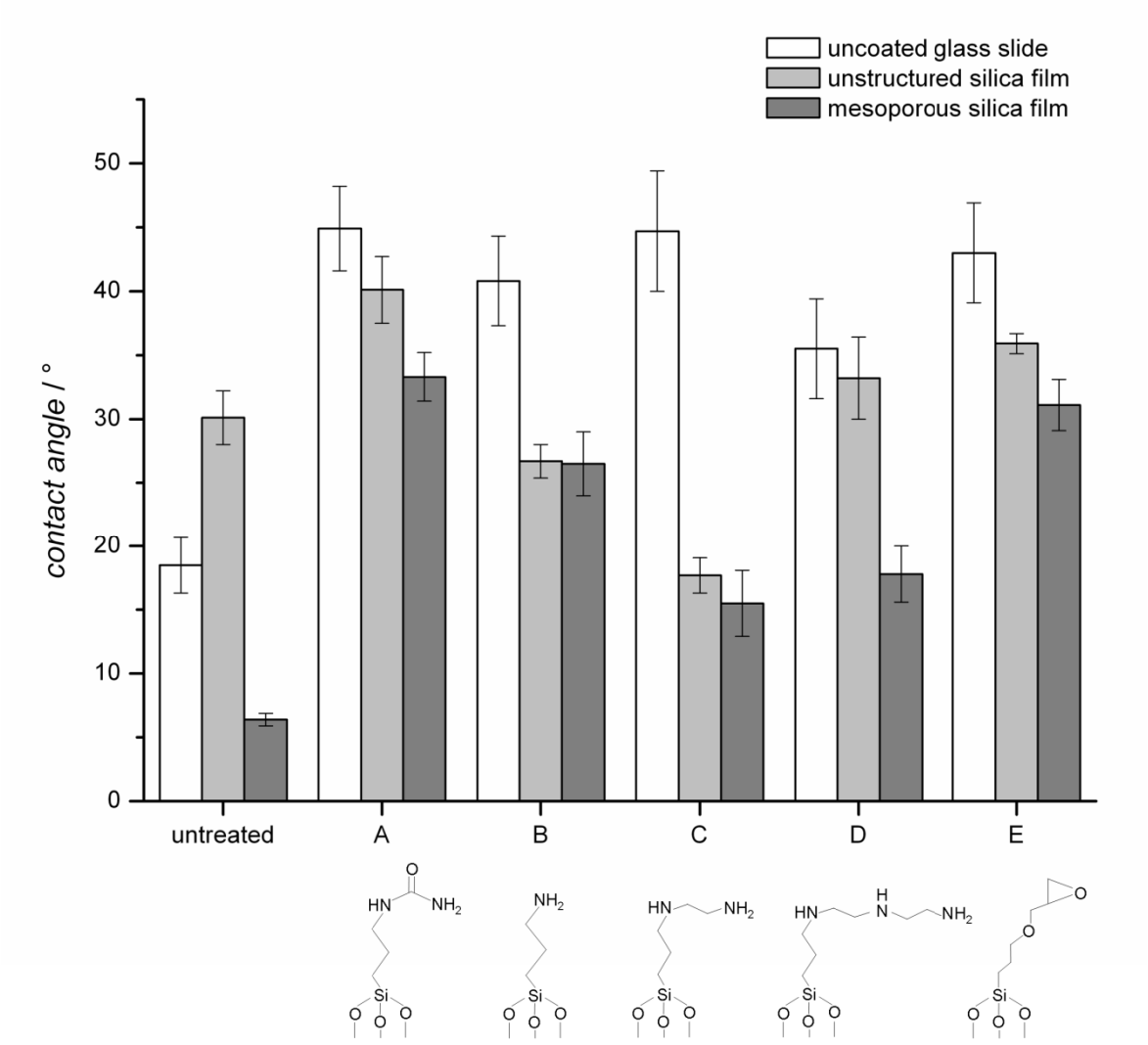


Figure 3-3. Results of contact angle measurements on different surfaces with different chemical modifications.

Silanization generally resulted in a more hydrophobic character, as expected. This was observed for example for the plain glass surfaces, which showed a strong increase of contact angles, from 18° to values varying between 35° to 44°. The hydrophobizing effect of silanization was also seen for the mesoporous coatings, although it was less pronounced (from 8° to 15° to 33°). Especially surfaces bearing groups containing several amino functions (C and D in Figure 3–3) were still quite hydrophilic. The results were less clear for the unstructured coating, which was already quite

hydrophobic in its pristine state. Ureido and epoxy functionalization lead to a further increase in hydrophobicity, whereas amino functionalization tended to decrease the contact angle. Recently, it was shown that surface topographies, i.e. nanostructures and nanopores, can directly influence contact angles [102]. Little is known so far about these influences so that they have been omitted from the discussion above, which relates mainly to the chemical character of the modifications.

***Activity of surface-immobilized alkaline phosphatase***

The results of the nitrophenylphosphate assay are shown in Figure 3–4. Because of the rather large standard deviations of the measurements all conclusions must be drawn with care. All types of silane linkers are able to immobilize ALP on silica surfaces and at least the part detected by the assay is biochemically active. For comparison, on the uncoated glass surface only 4 ng $cm^{-2}$ of ALP were found; without modification by a silane, the unstructured silica layer as well as the mesoporous ones were able to adsorb only negligible amounts of about 2 ng $cm^{-2}$ of the immobilized ALP. For all substrates, the simple aminopropyl linker appears to be the most effective immobilization agent. Comparing the different substrates, it is interesting to note that the unstructured silica coating consistently gave lower activity values than the plain glass slides and than the mesoporous coating. More importantly, with the most effective linking agent, the aminopropylsilane, the activity measured for the samples based on a mesoporous coating were considerably higher than those for the other substrate surfaces. Therefore, further investigations were carried out using the 3-aminopropyl-silane system.

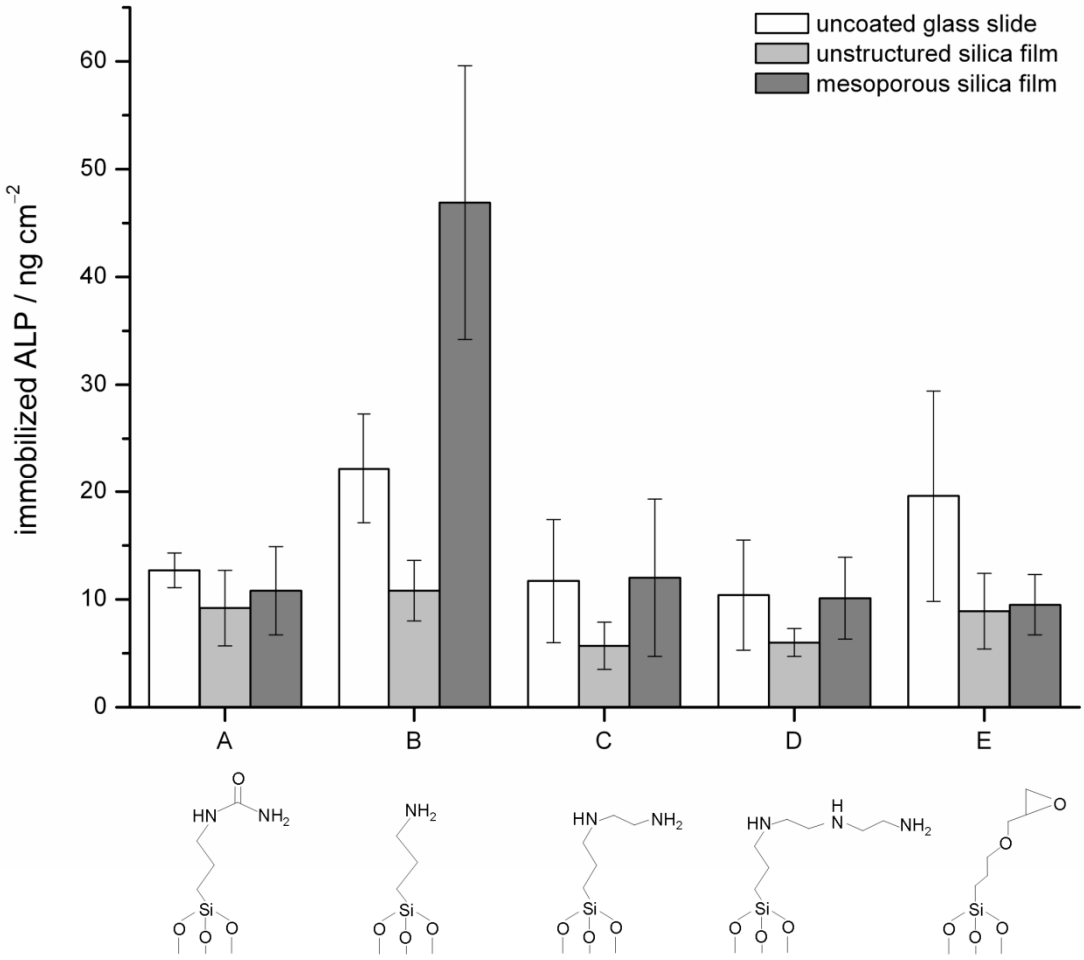


Figure 3-4. Activity of immobilized ALP on different substrates modified with different silanes according to the results of the nitrophenylphosphate assay.

Furthermore, we tested the effectiveness of the immobilization of active ALP in dependence of the concentration of the aminosilane. Results are shown in Figure 3–5. Noteworthy, when comparing the different substrates, the ranking of their immobilization efficiency is the same for all concentrations, i.e. the mesoporous coatings store more active ALP than the plain glass slides which in turn are able to immobilize larger amounts than the unstructured silica films. However, there is no directly obvious correlation between the concentration of the silane and the amount of immobilized enzyme in this range of concentrations. In addition, contact angle measurements showed that the surface properties vary dependent on the concentration of the aminosilane. The angles increase with increasing concentrations, but attain saturation already at low concentrations, for the uncoated glass slides at 0.1 m% and for the mesoporous film, probably due to its higher surface area, at 1 m% of the aminosilane.

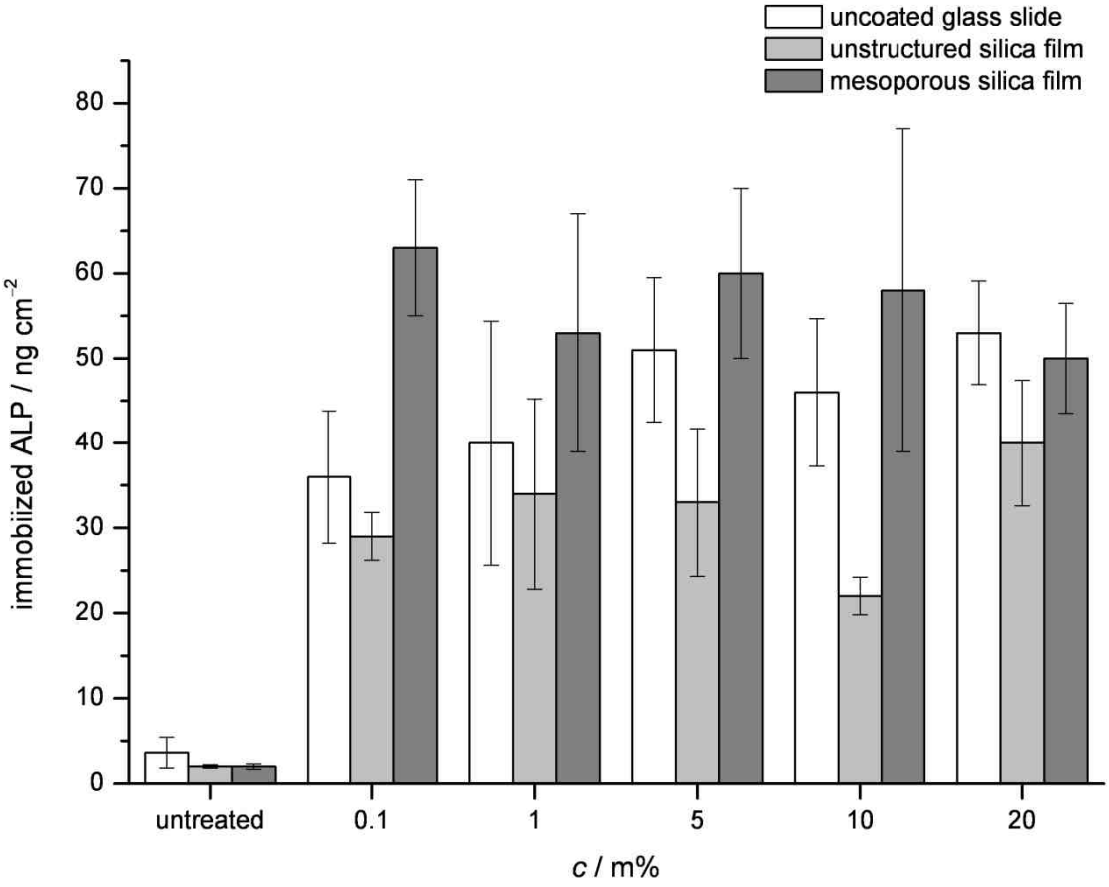


Figure 3-5. Activity of immobilized ALP on mesoporous silica coatings modified with different concentrations of the 3-aminopropyltrimethoxysilane solution.

Interestingly, storing the silane-treated samples under argon atmosphere for five minutes before the incubation in the enzyme solution lead to a slight increase in the amount of active immobilized ALP, amounting to increments of 22 ± 5 ng cm$^{-2}$ to 46 ± 9 ng cm$^{-2}$ for the plain glass slides, of 11 ± 3 ng cm$^{-2}$ to 22 ± 2 ng cm$^{-2}$ for the unstructured silica coatings and of 47 ±13 ng cm$^{-2}$ to 58 ± 19 ng cm$^{-2}$ for the mesoporous coatings (for the values obtained without argon storage, compare columns "B" in Figure 3–4 and column "10" in Figure 3–5).

**Discussion**

The immobilization of ALP on glass surfaces has been reported in previous studies [35,41]. Comparing the immobilized amounts is difficult because of the different substrate shapes. In former studies glass beads [98] or glass fibre filter discs [93] were used and the immobilized amount of ALP was calculated per gram of glass, whereas we used plain glass slides or thin coatings and refer to the amount of immobilized

ALP per $cm^{-2}$ of the substrate. However, we can compare the amount of 63 ± 8 ng $cm^{-2}$ ALP obtained by our optimized method (applying an aminopropylsilyl modification to a mesoporous coating) with a control experiment using a nitrocellulose membrane, which is known to effectively bind proteins and, especially, to have a very high capacity for binding ALP. Applying the same concentrations and conditions as in our other experiments, a nitrocellulose membrane bound 116 ± 7 ng $cm^{-2}$ of ALP. Comparing the mean thickness of the membrane of about 140 µm (specification from Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) to that of the mesoporous silica film, which lies between is 30 to 150 nm, suitably modified mesoporous silica coatings can effectively bind ALP and, presumably, other proteins.

The main result of this study lies in the influence of surface structural properties on the efficiency of binding active protein. The amorphous silica coating showed the lowest capacity in binding ALP, whereas the mesoporous surface coating is able to bind similar and in some cases higher amounts of active ALP as the uncoated glass slides. This finding could appear unexpected in that the increase of surface area from plain glass to the amorphous coating has a negative effect on the ability to immobilize the protein, although tentatively an increase of surface area, for example by increasing the roughness of the substrate surface by means of a coating with nanoparticles [90], should evoke an increase in the binding capacity. When discussing possible reasons, one has to keep in mind that here we determined the amount of *active* enzyme bound to the surface. Many peculiar properties of the binding systems then come into play. For example, the irregular pore geometry of the unstructured coating might inhibit the formation of specific conformations of the enzyme molecules. On the other hand, the regular and defined geometry of the pores of the mesoporous coating might induce a higher activity when the fit of the enzyme in the pores is appropriate. Plain glass slides might actually bind much less enzyme than the mesoporous coatings, but leave the enzyme intact and easily accessible so that the measured activity of bound ALP is similar.

The binding mechanism between amino functions on a surface and a protein has not been clarified yet. The type of binding is possibly electrostatic, between protonated amino groups and anionic groups of the protein; covalent bonds, however, cannot be excluded regarding the nucleophilicity of unprotonated amino groups. Hydrophobic interactions may also play a role, as it was recently proposed for BMP2 (bone morphogenetic protein 2) [103]. Such interactions could occur with the propyl residues of our linking agent or with hydrophobic parts of the silica surface (regions where only

fully interlinked [$SiO_{4/2}$] tetrahedra and no silanol groups are present). The elucidation of the binding mechanism was not the aim of this study. It is then also difficult to tell why one specific combination (aminopropylsilane modification of mesoporous coatings) yields much higher values of active immobilized ALP. However, these results were consistently reproduced and we have been able to successfully transfer this binding mode to the immobilization of BMP2 (bone morphogenetic protein 2), a signaling molecule of importance in bone regeneration and implant construction [104, 105].

It has to be mentioned that mesoporous silica films exhibit only low stability under conditions of relevance for biological applications [106]. We have investigated this stability problem in another context [100]. However, with regard to the short time scales used in the investigations presented here, the films can be considered as stable.

**Conclusions**

In our studies, all functionalized trialkoxysilanes tested were able to immobilize ALP on silica surfaces. Especially, it was shown that mesoporous coatings were more effective in binding ALP than unstructured sol-gel coatings. The combination of a mesoporous coating equipped with amino functions resulted in the highest activity of bound ALP. In fact, using this optimized binding method, mesoporous silica layers gave results which compare favourably with a standard substrate (nitrocellulose membrane). Meanwhile, this protein attachment strategy has successfully been transferred to the immobilization of BMP2 [107].

**Acknowledgements**

This work was supported by the DFG within the Collaborative Research Area SFB 599 „*Sustainable bioresorbable and permanent implants based on metallic and ceramic materials*". We thank our colleagues from the work package D1 for interesting discussions. Furthermore we thank Hendrik Fullriede for support by carrying out the alkaline phosphatase assay and the group of Professor Jürgen Caro from the Institut für Physikalische und Elektrochemie for the possibility to perform measurements at the Veeco stylus profiler.

## 3.2. Amino-modified silica surfaces efficiently immobilize Bone Morphogenetic Protein 2 (BMP2) for medical purposes

***Preface***

In this second section of the chapter on Results and Discussion, the results dealing with the immobilization of BMP2 on different glass and ceramic surfaces are presented. The immobilization strategy established for the alkaline phosphatase was transferred successfully to the attachment of BMP2. Again, glass substrates (plain, with unstructured or with mesoporous coating) were used; in addition, the glass-mica ceramic Bioverit® II was employed as a typical biomaterial established in bone replacement and especially in middle ear surgery. The BMP2 is supposed to induce a controlled local bone formation between a middle ear prosthesis and the residual stapes bone (which typically remains after removal of the ossicular chain) in order to achieve a stronger fixation.

The procedure for BMP2 attachment should be as simple as possible, so that the reaction can be carried out directly before or even during the operation. This is due to the low storage stability of proteins and to the fact that a middle ear prosthesis may have to be shortened during the implantation to adapt it to the space of the specific middle ear; in this case an unfunctionalized surface would be presented.

This section will be submitted as an original research article to the journal Biomaterials. The authors are Nina Ehlert, Dr. Andrea Hoffmann, Dr. Gerhard Gross, Dr. Peter P. Müller, Dr. Martin Stieve, Britta Hering, and Prof. Peter Behrens.

The idea to develop an immobilization strategy for BMP2 and to apply this strategy to middle ear implants was developed within the work package D1 consortium by Prof. Peter Behrens, Dr. Peter P. Müller, Dr. Martin Stieve and Prof. Thomas Lenarz. The author of this thesis has designed the experiments in agreement with the supervisor and has carried out the experiments concerning the preparation of the samples and their basic characterization. SEM investigations were carried out together with Britta Hering from the Institute of Inorganic Chemistry in Hannover. The immunochemical (ELISA) and specific biological in vitro tests (BRE-luc) were carried out by PD Dr. Andrea Hoffmann and Dr. Gerhard Gross from the Helmholtz Center for Infection Research in Braunschweig. The results have been discussed and interpreted with her and with the supervisor of this thesis. The manuscript presented here was developed

in cooperation with Dr. Andrea Hoffmann, Dr. Peter P. Müller and Prof. Peter Behrens.

The positive results of this work have encouraged the D1 consortium to test implants with attached BMP2 in animal experiments on rabbits, using two sites, namely a subcutaneous location and the functional site in the middle ear. For this purpose, the author of the present thesis has produced large numbers of partially or fully BMP2-coated implants (30 pieces). These implant samples were always prepared directly before the operation which took place on 18 different days. Great care was taken with regard to the reproducibility of the procedure. First results from the histological evaluation of the explanted samples from the subcutaneous site (performed by Iwa Hlozanek, Dr. Gudrun Brandes and Dr. Martin Stieve) clearly show a distinct biological reaction induced by the BMP2. The results of these animal studies are evaluated further and will be published in two papers.

**Abstract**

Due to its ability to induce differentiation of bone-forming precursor cells, the growth factor Bone Morphogenetic Protein 2 (BMP2) is often used for better enhanced integration of bone implants. With the aim to reduce possible high dose side effects and to lower the costs thereby targeting an affordable implant, we developed a simple and fast method for the immobilization of BMP2 on silica-based surfaces using silane linkers (carrying amino or epoxy functions). We put special emphasis on the influence of the nanoscale surface topography of the silica layer. Therefore we chose glass and Bioverit® II as base materials and coated these substrates with unstructured or nanoporous amorphous silica layers for comparison. Immobilized BMP2 was quantified by two different methods: a standard ELISA and a cell-based test which probe for immunologically and biologically active BMP2, respectively. The results show that the amino function has the highest capacity of immobilizing the protein and that the immobilized amounts of BMP2 on unstructured and nanoporous silica surfaces are similar. Strikingly, a considerably higher amount of BMP2 can be immobilized on coated Bioverit® II surfaces as compared to coated glass substrates, which is probably due to the macroscopic roughness of the Bioverit® II substrates.

**Keywords:** BMP2, immobilization, nanoporous silica, silanization, Bioverit® II, mesenchymal progenitor cells

**Introduction**

Enhancing the surface properties of implants for a better integration into their biological environment is in focus of biomaterial research these days. Especially, the improvement of the cell-surface interactions of bone replacement implants by tailoring surface properties is elaborated. Due to their high potent osteoinductive property which induces mesenchymal progenitor cells to differentiate into osteoblasts and chrondroblasts, Bone Morphogenetic Proteins (BMPs) play a key role in bone formation and repair. On this account, these growth factors are often used for a better integration of bone replacement prostheses and for inducing a fast healing process of critically sized defects. In particular, BMP2 has proved its high ability for the induction of bone formation in both, ectopic [108, 109] and orthotopic sites [104, 105, 110-115].

Although BMP2 must be present in a certain minimum local concentration to induce new bone formation, excess doses of free BMP2 are not amenable to practical surgery

because BMP2 possibly has dangerous side effects in other regions of the body, including induction of immune responses [116]. In addition, high doses of BMP2 are very costly. Also, BMP2 loses its bioactivity in solution after a short time in vivo [117, 118].

Two approaches have been developed in order to achieve a low but sufficient supply of active BMP2. The protein may either be incorporated into a polymer or mineral carrier phase which may be degradable or non-degradable (within a certain period of time). [83, 105, 108, 110, 113, 114, 118, 119] The delivery then is controlled either by the diffusion of the BMP2 within the carrier or by its degradation. The other approach consists in connecting the BMP2 firmly and persistently to the implant surface, which has the advantage of preventing the BMP2 from affecting untargeted tissue. This approach can be realized by chemically attaching the protein to the implant surface, either by covalent or by strong ionic bonding. Several techniques are based on the modification of the surface by an aminosilane as aminopropyl trimethoxysilane; the reactive amino function is then used for the coupling to the protein to the surface by using succinimidyl 4-(N-maleidomethyl)cyclohexane-1-carboxylate (SMCC) [120], hexamethylene diisocyanide [121] or carbonyldiimidazole (CDI) [111, 112, 122, 123] as linking agents. Another complex procedure starts with a phosphonic acid monolayer on a titanium surface and applies polymer chemistry for the fixation of the protein [71]. BMP2 can also be bound to dextran-coated titanium surfaces by a reductive amination method [124]. Additionally, strategies have been described based on the strong physisorption of BMP2 on nanocrystalline diamond [125, 126] or the immobilization of the protein on plasma activated polystyrene [127].

Recently, it has been discussed controversially whether strongly bound BMP2 can still be biologically effective. The group of Shiba showed that the BMP2 activity as assessed by induction of BMP2-dependent signalling cascades and induction of cellular differentiation is enhanced when the binding to the surface is reversible [128]. However, their system involved a genetically altered, non-naturally occurring BMP2 variant that had been created by molecular-biological techniques. In contrast, some promising work has been presented showing that BMP2 fixed on a surface still can be biologically active [111, 112, 120, 121, 124, 127].

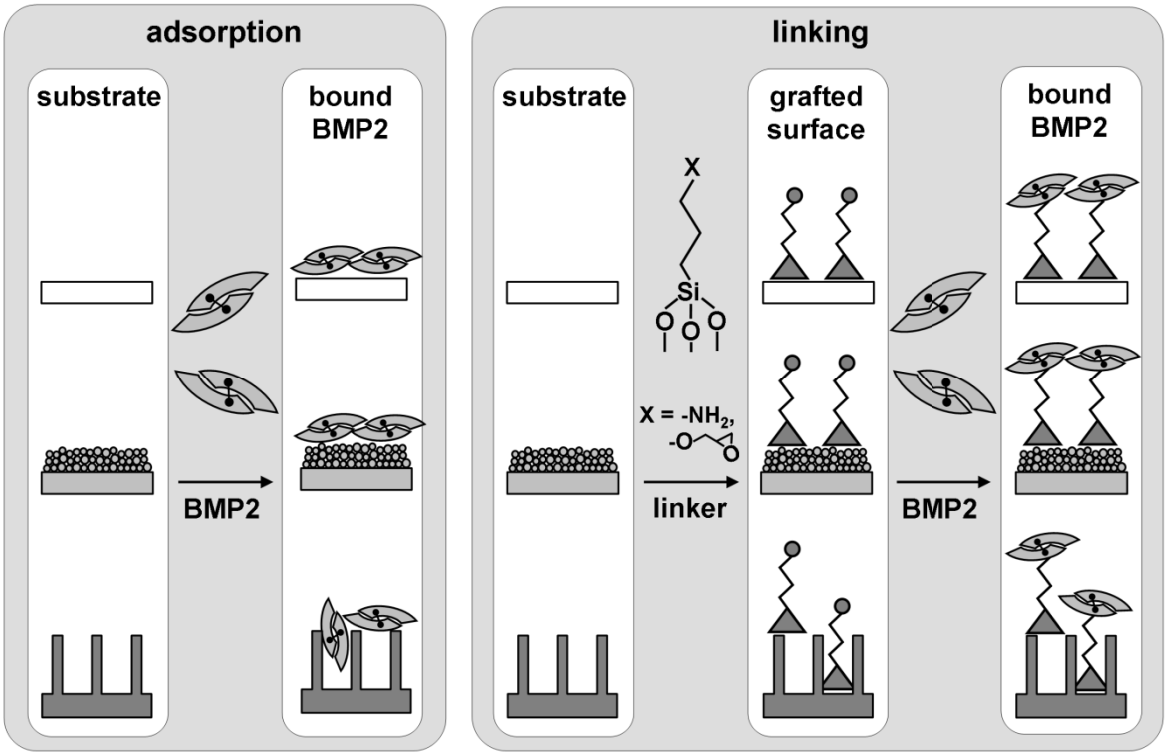


Figure 3-6. Scheme of immobilization strategies for BMP2 on silica surfaces.

In this study, we describe the immobilization of recombinant human BMP2 on structurally different silica surfaces by means of a silane linker without the need for using a further linking agent like SMCC or CDI (Figure 3–1). These silica surfaces are either an unstructured amorphous silica layer or an amorphous silica layer containing regular nanopores, both on plain glass disks. For this reason, we were especially interested in the properties of the nanoporous layer to mediate binding of BMP2 addressing both the amount of protein that can be immobilized and its resulting bioactivity. Such nanoporous layers (or, as they are also often called mesoporous layers) are currently being studied with regard to their applications as biomaterials [3, 20, 21, 129-131]. Our former studies on the immobilization of the enzyme alkaline phosphatase [132] on similar surfaces had shown promising results with regard to increasing the amount of active immobilized protein. The results obtained with the silica layers on glass slides were then transferred to Bioverit® II substrates [2], which were also coated by unstructured and nanoporous silica layers and silanized in order to bind BMP2. Bioverit® II is a glass-mica ceramic with various applications as a non-resorbable bone substitute material [133]. The present study was undertaken with special regard to the modification of Bioverit® II middle ear prostheses to locally induce bone formation in order to provide a strong fixation of the prostheses to remaining bone structures [3]. The amount of immobilized BMP2 was quantified by

two different methods: a standard ELISA and a cell-based test. ELISA measures immunologically active BMP2, i.e. total BMP2 bound, whereas the cell test quantifies biologically active BMP2 only. This cell test relies on the signalling cascade initiated by biologically active BMP2.

## Materials and Methods

*Nanoporous and unstructured silica films*

In this study two different types of base materials were used, namely Bioverit® II (3di GmbH, Jena, Germany) and glass (Glasbearbeitung Henneberg & Co., Martinroda, Germany), both in shape of square disks (10 mm x 10 mm) with a height of 1.0 to 1.3 for the Bioverit® II and 0.95 mm for the glass disks. The substrates were coated with unstructured or nanoporous silica layers. Prior to use, all specimens were cleaned in absolute ethanol (Merck, Darmstadt, Germany) and acetone. All chemicals except for the ethanol were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany) and were used without further purification.

The solution used for the preparation of nanostructured silica coatings contained ethanol, water, hydrochloric acid, tetraethoxysilane (TEOS) as a silica source and poly(ethylene glycol)-poly(propylene glycol)-*block*-co-polymer, PEG-PPG-PEG, (Sigma-Aldrich, $EO_{20}PO_{70}EO_{20}$, average $M_n$ ~ 5.800, similar to Pluronic® P-123, BASF) as the structure-directing agent [99]. A solution with the molar composition TEOS : EtOH : $H_2O$ : HCl : $EO_{20}PO_{70}EO_{20}$ = 1 : 48.9 : 26.9 : 0.06 : 0.0135 was prepared by adding the TEOS to the $EO_{20}PO_{70}EO_{20}$ dissolved in the mixture of ethanol, water and hydrochloric acid and was stirred for about 10 minutes before coating the specimens. The unstructured silica coatings were prepared by using similar solutions, but without the $EO_{20}PO_{70}EO_{20}$.

The Bioverit® II and the glass disks were coated using a dip-coating procedure, employing a DC Small Dip-Coater with 75 mm travel from NIMA (Coventry, England), operated in a climate box at a constant humidity adjusted by 50 m% glucose solution. The samples were immersed in the coating solution and then withdrawn perpendicular to the surface of the solution with a speed of approximately 1 mm/min. The samples were then left at constant humidity for five minutes, followed by a drying step at 60 °C for 30 minutes. For the Bioverit® II samples, this procedure was repeated twice, resulting in three layers of silica. The multiple coating of the Bioverit® II substrates is necessary in order to fill the cavities present on the material

surface and to create a continuous layer. For the glass substrates only a single coating is needed. Afterwards, the specimens were dried at 60 °C overnight, followed by calcination at 415 °C for 4 h (rate of heating/cooling 1 °C $min^{-1}$).

*Characterization*

The presence of the nanostructured layers was confirmed by X-ray diffraction (XRD) and scanning electron microscopy (SEM). The samples were measured on a Stoe (Darmstadt, Germany) $\theta$-$\theta$-diffractometer in reflection geometry. A secondary beam monochromator (graphite) was applied to produce CuKα radiation. SEM images were collected on a field-emission scanning electron microscope type JSM-6700F from Jeol (Eching, Germany) with an acceleration voltage of 2 kV and a working distance of 15 mm. The thickness of the mesoporous silica film was measured with a stylus profiler DETAK6M from Veeco instruments Inc. (Plainview, USA) with a force of 9 mg, duration of 100 seconds and a length of 3 000 µm per measurement.

*Surface modification*

The calcined samples were modified by a chemical functionalization step. After cleaning with absolute ethanol the specimens were immersed for 2 minutes in 10 m% aqueous solutions of the silanes 3-aminopropyl trimethoxysilane or glycidyloxypropyl trimethoxysilyl silane, respectively. They were then rinsed with water and dried under a flowing argon atmosphere for 10 minutes.

*Immobilization of the BMP2*

After surface modification the substrates were covered with a solution of recombinant human BMP2 produced by and purified from *E.coli* bacteria [ 134 ] with a concentration of 250 µg $ml^{-1}$ in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer pH 5.0 and left overnight at 4 °C under gentle shaking. The washing procedure consisted of eight washings with 0.125 M sodium tetraborate buffer (pH 10.0) containing 0.066 v% sodium dodecyl sulphate, followed by one washing step with phosphate buffered saline (PBS). For negative control experiments, the procedure was identical, except that MES buffer solution was used instead of protein.

*Quantification of the amount of bound BMP2*

Two quantification methods were applied: An indirect enzyme-linked immunosorbent assay (ELISA) was applied according to reference [71]. In brief, non-specific protein binding sites were blocked by incubation with 10 v% fetal calf serum in phosphate-

buffered saline ("10 % FCS"). Monoclonal mouse anti-human BMP2 antibody was added (R&D, cat.no. MAB3551) followed by washing with tris-buffered saline containing 0.1 v% Tween-20, and incubation with goat-anti-mouse antibody, peroxidase-conjugate. After removal of unbound antibody detection was performed with 3,3´,5,5´-tetramethylbenzidine (TMB Plus; KemEnTec) and stopped with 2 M sulphuric acid. Absorbance was read at 450 nm versus 620 nm. In addition, the so-called BRE-luc (BMP2 responsive element-luciferase) assay for the determination of biologically active bone morphogenetic proteins was used. This was carried out according to ref. [72]. The assay used a mouse muscle satellite cell line, C2C12, transfected with an inhibitor of differentiation promoter-luciferase construct [135], resulting in a BMP2-dose-dependent increase in luciferase activity in the cell lysates.

C2C12 mesenchymal progenitor cells were stably transfected with the luciferase reporter plasmid (kindly provided by Peter ten Dijke, Leiden) using DOSPER™ according to the manufacturer's protocol (Roche, Mannheim, Germany). A selection plasmid conferring G418-resistance (pAG60) was cotransfected and cells were selected with 750 µg $ml^{-1}$ G418. Individual clones were picked, propagated, and tested for presence of the reporter by stimulation with BMP2 and detection of luciferase activity (cf. below). For the test, C2C12-BREluc cells were seeded at 35,000 c/well of 24-well plates in medium containing 10 % FCS without G418. 2 hours after seeding, medium was removed and each well was washed once with 500 µl medium containing 2 % FCS, 4 mM glutamine and penicillin/streptomycin ("test medium"). Thereafter, 500 µl of test medium was added. 2 µl of BMP2 diluted in test medium was added for the standards to give final amounts of 200, 50, 20, 5, and 1 ng BMP2 per well. 2 µl test medium was added for the negative control. To correct for putative influence of the different surface coatings on cell behavior, these standards were performed in parallel with all materials tested. Cells were incubated for 2 days and harvested by washing once with PBS then frozen at – 70 °C. 70 µl of CAT lysis buffer (from CAT ELISA kit, Roche, Mannheim, Germany) with protease inhibitors (Roche, Mannheim) was added. The lysates were centrifuged for 10 min at 20,000 g, 4 °C and 7.5 µl were used for detection of luciferase activity (Promega, Mannheim, Germany: E1500: 25 µl/ sample). The amounts of BMP2 bound to the surfaces of glass or Bioverit were calculated from the standards by linear regression analysis.

For both methods, all experiments were carried out in triplicate. The values observed were corrected for the values obtained on blanks, which again were an average of three measurements. The samples for the blank values were exposed to the same

preparation procedure, but without BMP2. Due to the small number of experiments, which are due to the high costs in preparing the BMP2, a full statistical treatment of the observed values was not performed. Values obtained in repetitive experiments in different experimental campaigns are given separately in order to obtain an idea of the reproducibility of both the preparation and the detection methods.

## Results

*Stability testing of the silica coating*

To make sure that the nanoporous silica coating is stable throughout the whole coupling procedure with the BMP2 and especially during the washing steps at pH 10, we carried out X-ray diffraction investigations (Figure 3–2). We measured standard glass slides with a nanoporous coating before and following incubation in MES buffer plus washing with 0.125 M sodium tetraborate buffer (pH 10.0). After calcination, the nanoporous film exhibits one peak at 1.5 °2$\theta$ and a broad reflection at 2.8 °2$\theta$, corresponding to a disordered arrangement of nanopores [100]. The intensity of the peak at 1.5 °2$\theta$ in just slightly decreased after the washing procedures. Intensity reduction of this reflection was supposed to be caused by a reduction of the layer thickness of the nanoporous coating or it could be ascribed to a structural rearrangement within the layers, leading to a reduction of the number of regular pores (for example by relocation of silicate units).

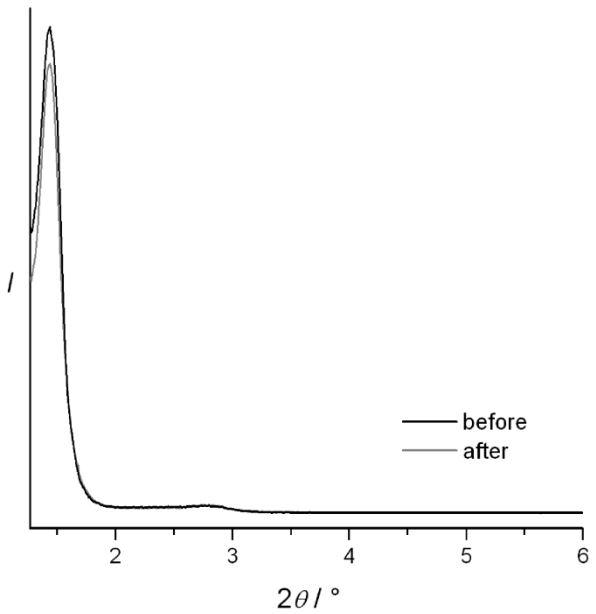


Figure 3-7. X-ray diffraction patterns of standard glass slides dip-coated with nanoporous silica layer. Comparison of glass slides before (black) and after (grey) the immobilization treatment (incubation in MES buffer and washing with borate buffer).

In addition, SEM pictures were taken before and after the whole treatment of the glass and Bioverit® II specimens. As shown in Figure 3–8, differences between the two films on the glass surface can be recognized. The glass surface before the treatment is very plain with few small defects. After the treatment some larger damages appear. However, despite these obvious defects, it can be concluded that the silica layer is affected but as a whole still existent on the surface of the glass substrate. The surfaces of the Bioverit® II-based samples look similar before and after the treatment. Here, both images demonstrate the presence of a covering silica layer as can be concluded from the observable small cracks which result from calcination. Combining the results from X-ray diffraction and SEM, it is likely that the pore system of the nanoporous layer has been partially destroyed (either by pore filling or by partial dissolution), but that there is still a silica layer present.

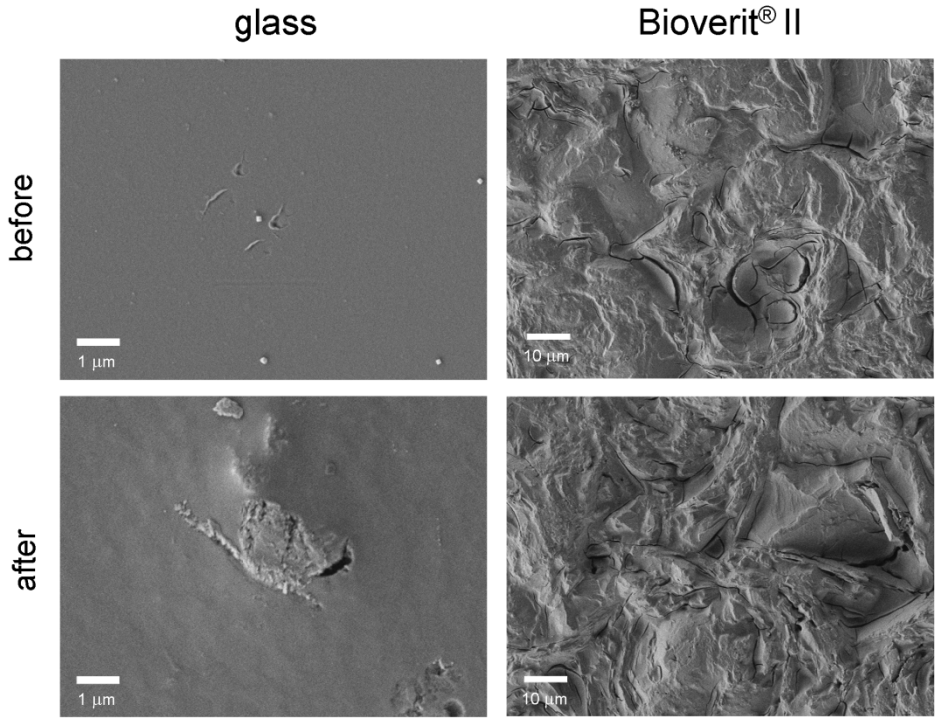


Figure 3-8. SEM images of glass disks and of Bioverit® II substrates coated with nanoporous silica, before and after the washing and incubation treatment involved in the immobilization of BMP2.

Further stability tests in 10 % FCS at 37 °C revealed that the mesoporous structure of film is stable during the first 12 hours under these conditions (The mesoporous film was modified with the aminosilane and the enzyme alkaline phosphatase was immobilized according to ref. [132] as a substitute protein). Afterwards, profilometer

measurements and microscopic control showed that a film with reduced thickness is still present on the surface for at least 24 hours.

*Quantification of the amount of immobilized BMP2*

To quantify the amounts of immobilized BMP2, two complementary tests were carried out: the ELISA [71] and the BRE-luc [72] test. The results are shown in Figure 3–9. The results depicted there reflect the results obtained in one experimental campaign on glass disks and in another one on Bioverit® II-based samples. Results from repetitive experiments are given in the text.

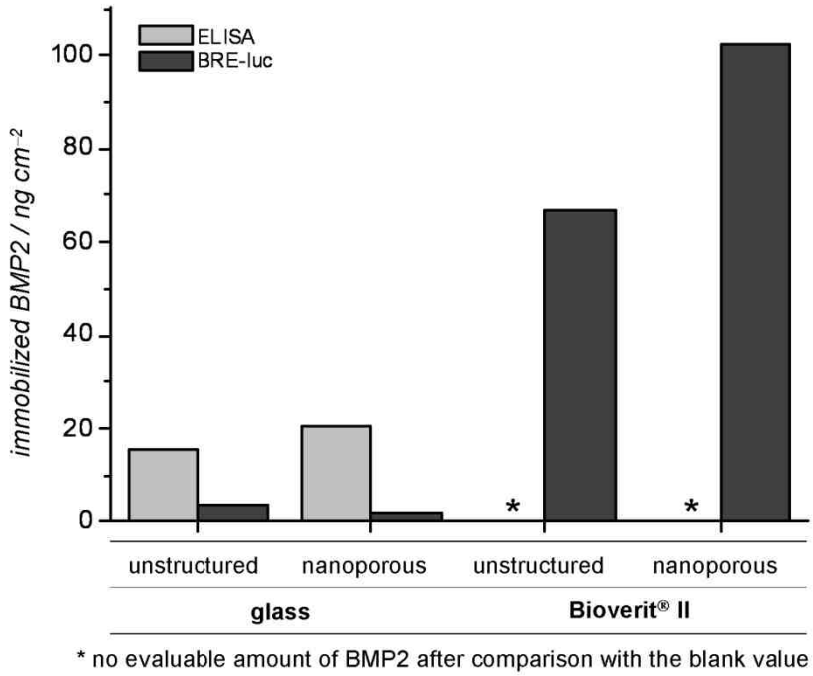


Figure 3-9. Results of ELISA and BRE-luc tests for detecting the immobilized amounts of BMP2 on 3-aminopropyl-modified nanoporous and unstructured silica layers on glass and on Bioverit® II substrates.

Both detection methods show that it was possible to immobilize small amounts of BMP2 on the glass substrates using the aminosilane linker. According to the ELISA test, the amino-modified unstructured and the amino-modified nanoporous silica layer on glass surfaces bind about 15 to 20 ng $cm^{-2}$ BMP2. In contrast, neither the amino modification nor the silica coatings alone were suitable for binding BMP2 (data not shown). In comparison, the BRE-luc test detected considerably lower amounts of biologically active BMP2, about 3 to 4 ng for the amino-modified unstructured and 2 ng for the amino-modified nanoporous glass surface. Here, the blank samples prepared without the addition of BMP2 gave signals below the detection limit of

< 1 ng $cm^{-2}$ BMP2. With the epoxysilyl modification, conflicting results were obtained. Whereas in the BRE-luc test, the amount of BMP2 was below the detection limit of < 1 ng $cm^{-2}$, the ELISA detected 120 ng $cm^{-2}$ in case of the unstructured and 100 ng $cm^{-2}$ in case of the nanoporous silica surface. We were able to reveal by further experiments that this discrepancy is caused by a malfunction of the ELISA which is possibly caused by a direct binding of antibody molecules to the epoxy functions of the silanized surface despite previous blocking with fetal calf serum. Due to the low biological activity detected by the cellular test system this chemical modification strategy was abandoned.

On Bioverit® II substrates, considerably higher amounts of immobilized BMP2 can be achieved compared to the glass surfaces. The bioactivity test detected about 70 ng $cm^{-2}$ for the amino-modified unstructured and more than 100 ng $cm^{-2}$ for the amino-modified nanoporous surface. To validate this result, the experiment was repeated twice for the nanoporous surface; in both cases even higher amounts of 150 and 154 ng $cm^{-2}$ bound BMP2 were detected. All tests carried out for comparison (omitting the silane or the silica layer or both) gave values below 4 ng $cm^{-2}$ (data not shown). In contrast, the ELISA test was not able to detect any BMP2. Obviously, this test is disturbed by the presence of Bioverit® II due to unknown reasons.

**Discussion**

In the present study we have addressed three topics concerning BMP2 immobilization: firstly, the question whether there is a simple route of immobilizing BMP2 to the surface of a silica-based material, secondly, whether the immobilized amount of BMP2 can be increased by applying a nanoporous layer on the ceramic surface. And finally, we wanted to know whether the BMP2 fixed to the surface is still biologically active. The biological effectiveness of immobilized BMP2 is not clear yet. Although there are some positive results [111, 112, 120, 121, 127], it was also found that the BMP2 has to be coupled reversibly to surface and needs to be released into solution to have biological effects [128]. The group of Shiba made first experiments on the release behaviour of immobilized BMP2 on titanium and found more prominent activation of BMP signalling and induction of differentiation in cells in vitro on samples with reversibly bound but genetically modified BMP2. In contrast, Wang et al. [124] were able to immobilize BMP2 on dextran-grafted titanium surfaces, which showed not only significantly higher promoted osteoblast spreading, alkaline phosphatase activity and calcium mineral deposition, but also a reduced bacterial

adhesion due to the dextran-grafting. In our case, it is not quite clear whether the BMP2 acts in the immobilized state or from solution. Although apparently the BMP2 is fixed strongly to the support (otherwise it would not survive the extensive washing procedures) the stability of the mesoporous silica layer appears to be quite low as has also been discussed in the recent literature [106] The stability of mesoporous silica films will depend crucially on the conditions of preparation and calcination and possibly on further modifications. Our own results described above indicate a somewhat higher stability as described in ref. [106] Especially, the X-ray diffraction features from the mesostructure may disappear before the silica layer has been dissolved. Obviously, when in our system the BMP2 acts only in a dissolved state, then the rate of release as resulting from the combination of strong fixation to and dissolution of the support appears to be quite favorable. We have started experiments investigating a possible release of the immobilized BMP2 from our mesoporous films in cell culture medium at different time intervals up to one week.

We decided to use a simple silanization method for the immobilization procedure and tested two different functional groups. Tests on silica coated glass and ceramic surfaces showed that the aminopropyl silanization is a successful method of binding BMP2 to the silica surface. This result is in line with those of Jennissen and co-workers [112]. They were able to immobilize BMP2 on titanium surfaces using an elaborate and laborious method. Titanium surfaces treated with chromosulfuric acid were first boiled at reflux in a solution of aminopropyl triethoxysilane in toluene under an inert gas atmosphere for several hours. BMP2 was coupled to this surface either directly or employing carbonyldiimidazole (CDI) as an intermediate agent. In comparison to that our route for the modification of the silica surface consists of a simple dipping method carried out in aqueous solution within 2 minutes. With this method, we were able to immobilize repeatedly 100 to 150 ng $cm^{-2}$ BMP2. In the work of Jennissen and co-workers, 596 ng $cm^{-2}$ bound BMP2 were achieved without the use of CDI as a coupling agent, a value that increased to 819 ng $cm^{-2}$ when CDI was employed [112]. In this work, the determination of the immobilized amount of BMP2 was conducted based on radiolabeled $^{125}$I-BMP2. This method detects all BMP2 molecules on the surface, whether these are still immunologically and biologically active or not. In contrast, we can safely conclude that the values given for the immobilized BMP2-amount of our samples denotes biologically active protein since the employed BRE-luc test relies on the activation of intracellular signalling protein cascades which are only induced by biologically active BMP2. In fact, our samples

probably contain more immobilized BMP2, when biologically inactive BMP2 molecules are included. Jennissen and co-workers further showed that at least some of the immobilized BMP2 on their samples is biologically effective in vivo. In animal experiments on dogs, they found that samples with immobilized BMP2 can induce bone formation around a dental implant [112].

As another option for the immobilization of BMP2 on biomaterials, we cite the work of Matzuzaka et al., who detected 44.2 ng $cm^{-2}$ BMP2 on plasma-activated polystyrene [127] as determined by a frequency shift method, and that of Park et al., who bound 800 ng $cm^{-2}$ BMP2 (detected by radio-labeling) to a chitosan matrix by using the crosslinking agent succinimidyl 4-(N-maleidomethyl)cyclohexane-1-carboxylate (SMCC) [136].

The type of binding between the substrate and the BMP2 is not clear. We consider first the interaction between the silica layer and the aminosilane. In order to covalently bind aminopropylsilyl function by establishing siloxane bonds, the material has to be heated [38, 36] which is not part of our dipping procedure. It is more probable that a layer of intermolecular condensed aminopropylsilane is formed and connected to the silanol groups of the silica surface by hydrogen bonds with its amino residues [36]. With regard to the interactions between the aminopropylsilyl linkers and the BMP2, several binding modes are possible. In neutral or slightly acidic solutions (the pH of the MES-buffer used for coupling is 5.0), the amino groups will be partially protonated and electrostatic interactions can occur with negatively charged or polarized elements of the BMP2 molecules. Recently, it was also proposed that BMP2 interacts strongly with alkyl residues placed on a surface by hydrophobic interactions [122]. Such interactions could occur with the propyl residues of our linking agent or with hydrophobic parts of the silica surface (i.e. areas which contain only fully interlinked [$SiO_{4/2}$] tetrahedral). The formation of amide bonds between the surface amino groups and carboxylic groups of the BMP2 appears improbable. Jennissen and co-workers postulate that such a true covalent binding occurs after activation of with CDI, so that a linkage is formed from the surface amino groups to the ε-amino groups of lysine residues within the protein [111].

ELISA tests are a well-established method to determine the concentrations of proteins in solution with high specificity. However, our results show that results from ELISA tests have to be evaluated carefully when solid surfaces (of implant materials) come into play. When conflicting results between the ELISA for BMP2 and the BRE-luc test

are observed, the latter is surely more reliable. Also, the BRE-luc test provides evidence for biologically active BMP2 and is very sensitive since up to 1 ng BMP2 per sample can be detected. This contrasts favourably with other methods for detection of BMP2 biological activity like, e. g., determination of Alkaline Phosphatase activity. In contrast, other methods detect either immunologically active BMP2 (ELISA), all BMP2 molecules present (radiolabelling methods) or simply a mass increase (frequency shift method).

With regard to the structural influence of the substrate, our study showed that on silica-covered Bioverit® II large amounts of BMP2 can be immobilized (70 ng $cm^{-2}$ on the unstructured silica layer versus 100-150 ng $cm^{-2}$ on nanoporous silica coatings). These are considerably larger amounts than on plain glass (2 ng $cm^{-2}$ for the nanoporous coatings, 3-4 ng $cm^{-2}$ for the unstructured coating). This could be due to several reasons: Firstly, the effective surface area per square centimetre is larger for the Bioverit® II material due to its strong roughness in the micrometer range (compare Figure 3–3); secondly, the silica layer is thicker, as it was prepared by three subsequent dipping procedures in order to cover the whole surface. The fact that the nanoporous layer binds slightly more BMP2 than the unstructured silica coating can be traced back to its higher roughness in the nanometer range and to the larger number of silanol groups present on this material [101]. The finding that on glass substrates this tendency is supposed to be reversed has to be considered carefully because of the small masses of BMP2 detected are near the detection limit.

To clarify whether the immobilized BMP2 can induce bone formation in vivo, the samples have to be tested in animal experiments. These are currently being evaluated both in rabbits and in mice.

**Conclusions**

We have developed a simple, fast and effective binding system for the immobilization of BMP2, based on the established bone reconstruction material Bioverit® II. Coating of Bioverit® II with a nanoporous silica layer and subsequent modification with 3-aminopropyl trimethoxysilane reliably gives BMP2-functionalized surfaces which carry 100 ng $cm^{-2}$ to 150 ng $cm^{-2}$ of biologically active BMP2, as judged by the highly BMP2-specific cellular in vitro test. It is noteworthy that the BMP2 coupling procedure employed is so simple that after further optimization it could potentially also be carried out by a surgeon in the operation room. This could be advantageous to

avoid long-term storage of coupled BMP2 or when implants have to be reshaped during operation, making it necessary to carry out the BMP2 functionalization on the "fresh" implant surface.

**Acknowledgements**

This work was supported by the DFG within the Collaborative Research Area SFB 599 „*Sustainable bioresorbable and permanent implants based on metallic and ceramic materials*". We thank our colleagues within work package D1 ("Functionalized Middle Ear Prostheses") for valuable discussions and the group of Professor Jürgen Caro from the Institut für Physikalische und Elektrochemie for the possibility to perform measurements at the Veeco stylus profiler.

## 3.3. Mesoporous silica films for controlled release of ciprofloxacin from Iimplants

The last part of this work deals with the use of mesoporous silica coatings for drug delivery, again with regard to the application on implants. Here, the mesoporous silica coating acts as a reservoir for local drug release. Together with the implantation of a middle ear prosthesis, an infection shall be combatted. These investigations were carried out using the antibiotic ciprofloxacin, which often is applied systemically in the case of middle ear infections. This work shows how different modifications of the silica coating act together to obtain a high loading the antibiotic and a controlled release of it.

The idea to develop a strategy for local antibiotic delivery and to apply this feature to middle ear prostheses was implants was developed within the work package D1 consortium by Prof. Peter Behrens, Dr. Peter P. Müller, Dr. Martin Stieve and Prof. Thomas Lenarz. The author of this thesis has designed the experiments in agreement with the supervisor and has carried out the experiments concerning the preparation of the samples and their basic characterization. Also, an assay for the detection of released ciprofloxacin was established. STEM investigations were carried out by Dr. Armin Feldhoff from the Institute of Physical and Electrochemistry Chemistry in Hannover. In vitro cell and bacteria culture experiment were performed by Mohammad Badar and Dr. Peter P. Müller at the Helmholtz Center for Infection Research in Braunschweig. The latter also has developed the mouse model used in a first in vivo test. The manuscript presented here was developed in cooperation with Prof. Peter Behrens and Dr. Peter P. Müller.

**Abstract**

To generate bioactive coatings for medical implants a novel procedure has been developed using a coating of mesoporous silica for controlled drug delivery. Mesoporous silica layers were coated on plan glass slides and on a commercially available glass mica ceramic implant material. The mesoporous material was loaded with the antibiotic drug ciprofloxacin. The drug release characteristics were investigated using mesoporous coatings on glass slides. The drug capacity was low initially but could be increased nearly ten-fold (to about 2 μg $cm^{-2}$ of the macroscopic surface) by functionalizing the mesoporous surface with sulfonic acid groups. In cell culture assays the surfaces showed good biocompatibility. The antibacterial efficacy was proven in experiments using luminescent bacteria. A first in vivo test in a newly developed mouse model demonstrated high antibacterial efficacy. To achieve a controlled drug release over an extended time period further coatings were added. Covering the surface of the drug loaded mesoporous silica layer by dip-coating with bis(trimethoxysilyl)hexane resulted in a retarded release for up to 31 days. By an additional evaporation coating with dioctyltetramethyldisilazane, the release of ciprofloxacin was prolonged for up to 63 days.

**Introduction**

Mesoporous silica materials are currently being investigated as controlled drug release systems due to their unique properties as high surface area, tunable pore size with narrow distribution in the nanometer range and adaptable surface chemistry based on functionalization of the silanol groups present on the silica surface. Another important feature of mesoporous silica is its good biocompatibility, as shown for example in several animal experiments [3, 20, 21]. Mesoporous silicas are usually studied in the form delivered by solution-phase syntheses, namely as powders, or, in some cases, as mesoporous hollow silica spheres [63, 137, 138]. These material forms are, however, difficult to apply in combination with a pre-formed implant or prosthesis. For this purpose, mesoporous silica films, deposited on the surface of the implant, present a promising practical approach. For example, ossicular replacement prostheses, substituting the ossicular chain, often have to be implanted into an infected middle ear. Local delivery of an antibiotic could help in combating the infection and thus assist in the healing process.

The loading and the release of drugs from mesoporous silica can be controlled in different ways, as shown in many investigations. In addition to the size and the shape of the pores and particles [139, 140, 141], the chemical interaction of the materials surface with the drug is the most important factor [61]. Silica surfaces can be equipped with functional residues by grafting, i.e. the post-synthetic reaction with the silanol groups on the surface, or by co-condensation in a one-pot synthesis; both methods are currently being discussed in view of their advantages and disadvantages [61, 142-144]. The different modifications can be used to optimize the amount of drug taken up and delivered by mesoporous silica-based drug delivery systems. Furthermore, they can influence the kinetics of drug release. A variety of modifications is possible. For example, a hydrophobic character can be imparted to the silica surface by the attachment of unreactive groups (e.g. trimethylsilyl [145-147]). In addition, negatively charged groups (like carboxyl [148, 149]), positively charged ones (as protonated amino groups [143, 144, 150]) or reactive groups (as epoxy [151] or thiol residues [152]) can be used to tailor the surface properties in order to match the properties of the drug to be delivered. The density of these functionalities has to be adjusted carefully. Especially in the attachment of charged groups, the net charge of the surface is a crucial factor, but also the unreacted silanol groups retain their influence [65]. For that reason the pH value at adsorption and release conditions has always to be considered in aqueous media. For example, the loading of a drug consisting of negatively charged molecules can be enhanced when positive charges are placed on the surface [150, 153, 154]; drug molecules with extended hydrophobic parts can be attracted to the surface by hydrophobic residues [145, 155]. The release of a drug to an aqueous medium can be retarded by a protective outer hydrophobic layer which hinders the surrounding aqueous media from entering the pores [60, 147].

Here we report a practical approach for fighting bacterial infections by local delivery of ciprofloxacin, a broad spectrum antibiotic, from a specially developed mesoporous silica coating (see scheme in Figure 3–10). Such silica coatings can be applied to different implant materials, e.g. Bioverit® II, thus providing the implant with the ability to defend itself against a bacterial infection. We use a sulfonic acid modification to increase the amount of loaded ciprofloxacin in a mesoporous silica layer and show its biocompatability and its effectiveness in combating bacteria in vitro. A first in vivo test in a mouse model with unmodified ciprofloxacin loaded material was carried out to prove its suitability in living systems. Furthermore, we

are able to avoid the typical initial burst release behavior of drug delivery systems and retard the delivery of the drug by a two-step procedure involving dip- and evaporation-coating of the silica films with hydrophobic substances.

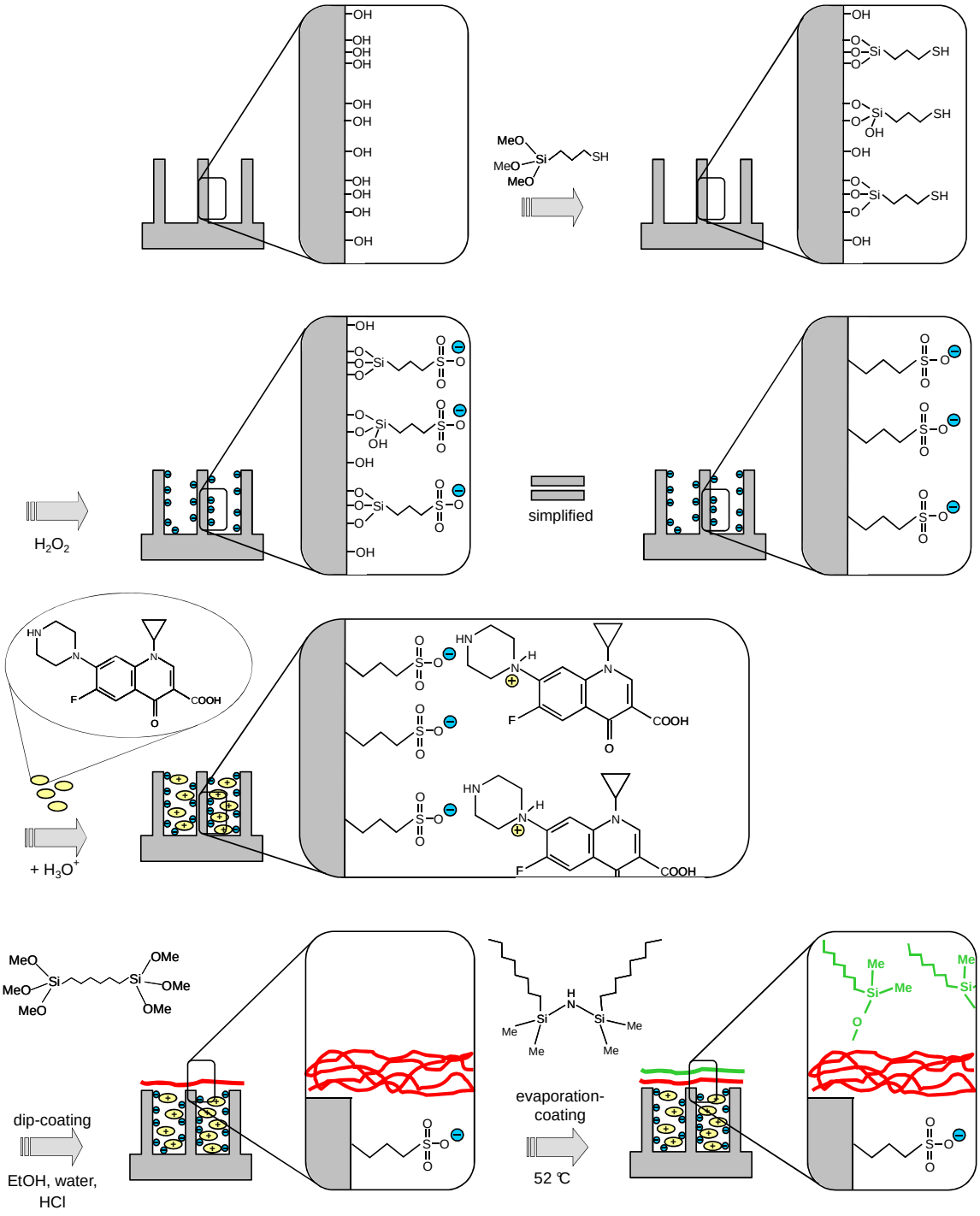


Figure 3-10. Scheme for the different modifications carried out to achieve a high loading and a controlled release of ciprofloxacin from a mesoporous silica film.

**Experimental**

*Mesoporous silica layer*

Two different types of base materials were used as substrates for the coatings, namely glass (Glasbearbeitung Henneberg & Co., Martinroda, Germany) and Bioverit® II (3di GmbH, Jena, Germany). The 3-mercaptopropyltrimethoxysilane, 1,3-di-*n*-octyltetramethyldisilazane and the bistrimethoxysilylhexane were ordered from ABCR GmbH & Co. KG (Karlsruhe, Germany). Absolute ethanol was purchased from Merck (Darmstadt, Germany) All other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany).

For the compilation of in vitro release profiles standard glass slides (76 x 26 mm), for cell culture experiments glass disks (10 mm x 10 mm) with a height of 0.95 mm, and for the in vivo experiments Bioverit® II cylinders with a diameter of 1 mm and a height of 1.0 to 1.3 mm were employed. The different substrates were first coated with nanoporous silica layers. Prior to the coating, all specimens were cleaned in an ultrasonic bath, first in acetone and then in absolute ethanol for ten minutes each. All chemicals were used without further purification.

The solution used for the preparation of nanostructured silica coatings contained ethanol, water, hydrochloric acid, tetraethoxysilane (TEOS) as a silica source and poly(ethylene glycol)-poly(propylene glycol)-*block*-co-polymer, (Sigma-Aldrich, $EO_{20}PO_{70}EO_{20}$, average $M_n \approx 5.800$, similar to Pluronic® P-123, BASF) as the structure-directing agent [99]. The dip-coating solution had a molar composition of TEOS : EtOH : $H_2O$ : HCl : $EO_{20}PO_{70}EO_{20}$ = 1 : 48.9 : 26.9 : 0.06 : 0.0135. It was prepared by adding TEOS to the $EO_{20}PO_{70}EO_{20}$ dissolved in the mixture of ethanol, water and hydrochloric acid and was stirred for about 10 minutes before coating the specimens. The glass samples were coated using a dip-coating procedure, employing a DC Small Dip-Coater with 75 mm travel from NIMA (Coventry, England), operated in a climate box at a constant humidity adjusted by 50 w/w glucose solution. The samples were immersed in the coating solution and then withdrawn perpendicular to the surface of the solution with a speed of approximately 1 mm/min. The samples were then left at constant humidity for five minutes. For the Bioverit® II samples, a spray-coating procedure was applied using the same solution. The samples were sprayed lying on the plane side and spray-coated with a thin layer of the solution followed by a drying step at 60 °C for 30 minutes. A specimen was exposed to this procedure three times, lying on the plane circular side each time. The multiple coating

of the Bioverit® II substrates is necessary in order to fill the cavities present on the rough surface of this biomaterial and to create a continuous layer. For the glass substrates, a single coating is sufficient. After coating, the specimens were dried at 60 °C over night, followed by calcination at 415 °C for 4 h (rate of heating/cooling 1 °C $min^{-1}$).

*Sulfonic acid modification*

The sulfonic acid modification was carried out according to ref. [156]. The following synthesis was applied in parallel for five glass slides. The glass slides were cooled to 0 °C in 45 ml of dichlormethane before 5.9 ml of 3-mercaptopropyltrimethoxysilane were added and the solution was gently stirred for 22 h without renewing the ice bath. Then the glass slides were washed with dichlormethane and absolute ethanol and dried at 100 °C for 5 h. 50 ml of hydrogen peroxide (30 m%) were added and allowed to react for 48 h followed by washing with water and absolute ethanol. Finally the glass slides were dried at 60 °C for 2 h and cooled to room temperature before the insertion of the ciprofloxacin.

*Drug insertion procedure*

The insertion of the ciprofloxacin was carried out in a 60 mM solution at pH 4 at 37 °C for 3 days. The solution was prepared as follows. 10 g of ciprofloxacin were added to about 300 ml of water and the pH value was decreased with hydrochloric acid (2M) until a clear yellow solution was formed (pH ~ 2). Then the pH was adjusted to 4 with sodium hydroxide solution (1M), thereby approaching a volume of 500 ml. Finally the solution was filled up to 500 ml. A volume of 45 ml was used for the insertion procedure of 5 glass slides.

After the insertion, the glass slides were rinsed with 50 ml of water for each side to wash of the high concentrated solution at the outer surface of the glass slide. Afterwards the glass slides were dried for 2 hours at room temperature at constant air humidity adjusted with 50 m% glucose. Then, they were either modified further or transferred to the release experiment.

*Controlled release modifications*

To produce a hydrophobic layer from the reaction of bis(trimethoxysilyl)hexane on the surface, a dip-coating procedure, following ref. [60], was used. For this purpose, a solution containing bis(trimethoxysilyl)hexane, ethanol, water and 0.1 M hydrochloric acid was stirred for 30 minutes. Glass slides were dipped into the solution

individually and withdrawn with an approximate speed of 2 cm $s^{-1}$ to avoid leaching of the inserted ciprofloxacin. The samples were dried for 12 h at room temperature at constant air humidity adjusted with 50 m% glucose. Then, they were either modified further or transferred to the release experiment.

For the further modification with 1,3-di-n-octyl-tetramethyldisilazane (97 %) the glass slides were coated by an evaporation procedure. 6 ml of dioctyltetramethyldisilazane (1,3-di-n-octyltetramethyl-disilazane) were put into a 300 ml Erlenmeyer flask (broad) and heated to 52 °C for 10 h. During this time, the five glass slides were hanging above the liquid at the upper end of the closed flask with a distance of about 15 cm [147]. After drying for 10 minutes at ambient conditions, the samples were transferred to the release experiment.

*Release experiments*

The release measurements were conducted as follows. Five glass slides were put into a preheated solution (37 °C) of 45 ml of 0.1 M phosphate buffered saline (PBS). The samples were kept at 37 °C. The measurements took place after fixed time intervals of 15, 35, 55, 75, 135, 195 and 315 minutes. Afterwards, the measurements were performed ca. every 24 h.

The quantitative determination of the ciprofloxacin in the release solution was carried out on a spectrophotometer UV-mini 1240 (Shimadzu, Duisburg, Germany) at 275 nm. The whole medium was replaced after each measurement to simulate the dynamic fluidic conditions in the body.

*In vitro testing*

For biocompatibility testing mesoporous coated, sulfonic acid modified and ciprofloxacin loaded glass discs were transferred to a 24-well plate. Plane glass discs were used as a control. Briefly, a near to confluence culture of NIH3T3 cells was diluted to 1:5 and 1 ml of this cell suspension was added to each of the wells containing glass discs. These discs were incubated with these cells at 37 ºC in an incubator. Pictures were taken with an *Axio Observer.A1* microscope (Carl Zeiss, Oberkochen, Germany) at 24 and 72 h intervals after inoculation of the cells. The experiment was repeated three times.

For testing of efficacy against bacteria the glass discs with the mesoporous coating, the sulfonic acid modification and with the loaded ciprofloxacin were transferred to a 24-well plate. Plane glass discs were used as control. Each of these discs was

incubated with 100 µl of bacterial $OD_{600}$ =0.2 suspension of *Pseudomonas aeruginosa* (PAO1 CTX-lux) in PBS for 15 min at room temperature. After 15 min, 0.9 ml of LB (Luria broth [157]) was added into each well and the multiwell plate was placed on a shaker at 60 rot $min^{-1}$ at 37 ºC. The plate was observed under the IVIS (Xenogen Corp., Alameda, CA) after six hours and the luminescence for each well was measured individually.

*Mouse model*

First animal experiments of ciprofloxacin-loaded Bioverit® II-based implants were carried out in a mouse model. Cylindrical Bioverit® II implants were spray-coated to produce a mesostructured silica layer and then calcined to give a mesoporous coating. For insertion of the antibiotic, they were kept in a 60 mM ciprofloxacin solution at pH 4 at 37 °C for 1 week for insertion. After washing and drying they were immersed into a bacterial suspension. Each sample was incubated separately in an Eppendorf tube containing 10 µl of a bacterial suspension with optical density $OD_{600}$ =0.2 of *Pseudomonas aeruginosa* with a recombinant lux operon (PAO14 CTX-lux and PAO14 PQS-lux) in PBS for 15 min at room temperature and then implanted subcutaneously into a BALB/c mouse. One sample of plain Bioverit® II (as a control) was incubated in the same way with PBS only. A total of four Bioverit® II samples with a nanoporous coating were implanted, two of which carried ciprofloxacin. In addition a control sample of plain Bioverit® II was implanted as well. Implantations were carried out in Balb/c mice. The details of the implantation procedure will be published elsewhere (M. Badar et al, in preparation). The experiments were done under the permission of the official authorities, Bezirksregierung Braunschweig, application number 33.42502/07-10.05.

*Characterization methods*

The nanostructured layers were investigated by X-ray diffraction (XRD) and scanning transmission electron microscopy (STEM). The samples were measured on a Stoe $\theta$-$\theta$-diffractometer (Darmstadt, Germany) in reflection geometry. A secondary beam monochromator (graphite) was applied to produce CuKα radiation. STEM images were collected on a field-emission transmission scanning electron microscope type JEM-2100F from Jeol (Eching, Germany). Static contact angle measurements were performed on a Surftens universal contact angle goniometer (OEG, Frankfurt/Oder, Germany) with water as the probing liquid. On every glass slide, the contact angle

was measured at 5 different positions, all at least five millimeters away from the edges of the glass slide, and a representative image was chosen.

## Results and discussion

*Characterization of the mesoporous silica layer*

The mesoporous structure of the silica layer is shown in Figure 3–11. The STEM investigations of a cross section of the mesoporous silica film on a glass substrate show a 40 nm thick layer of a mesoporous material with channellike pores which run mainly parallel to the surface. Pore diameters are ca. 5 nm. In former SEM investigations it was shown that pore mouths are present on the surface of the coating, so that the pore system can be accessed from the surface of the layer [2, 100]. X-ray diffraction measurements gave results similar to those as described in detail elsewhere [132], thus also confirming the presence of the mesostructure. Further investigations with a profilometer revealed that a silica film on a glass slide has a layer thickness varying between 30 and 150 nm.

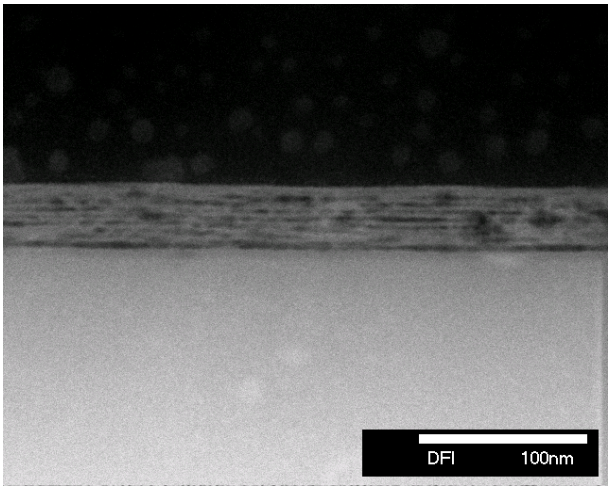


Figure 3-11. STEM image of a cross section of the mesoporous silica layer on a glass substrate (bottom).

The different surface modifications carried out during the whole procedure were monitored by static contact angle measurements. The results show drastic changes in surface properties (Figure 3–12). Initially, the hydrophilic surface of the mesoporous silica layer can be discerned from the decrease of the contact angle from about 20° (Figure 3–12a) for the cleaned glass substrate to less than 5° for the mesoporous silica layer (Figure 3–12b). After the first modification steps resulting in sulfonic acid groups on the surface, the surface stays hydrophilic with only a slight increase of the contact angle which remains below 5° (Figure 3–12c). Although propyl groups are also

introduced onto the surface, the hydrophilic sulfonic acid groups seem to dominate the surface chemistry. After dip-coating with bis(trimethylsilyl)hexane, the surface exhibits hydrophobic properties with contact angles between 50° to 55° (Figure 3–12d). The contact angle increases further upon evaporation coating with dioctyltetramethyldisilazane in the last step (Figure 3–12e), yielding values of more than 90°.

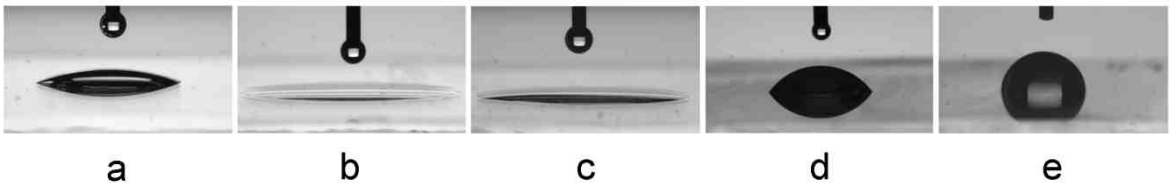


Figure 3-12. Static contact angle measurements from a) a cleaned glass substrate, b) equipped with a mesoporous silica layer, c) after modification with sulfonic acid groups, d) covered with a dip-coated layer derived from bis(trimethoxysilyl)hexane, e) in addition covered with a layer obtained by evaporation coating with dioctyltetramethyldisilazane.

*Controlled release profiles*

Spectrophotometrically monitored experiments in PBS gave information about the release profiles of different functionalized mesoporous silica films (Figure 3–13).

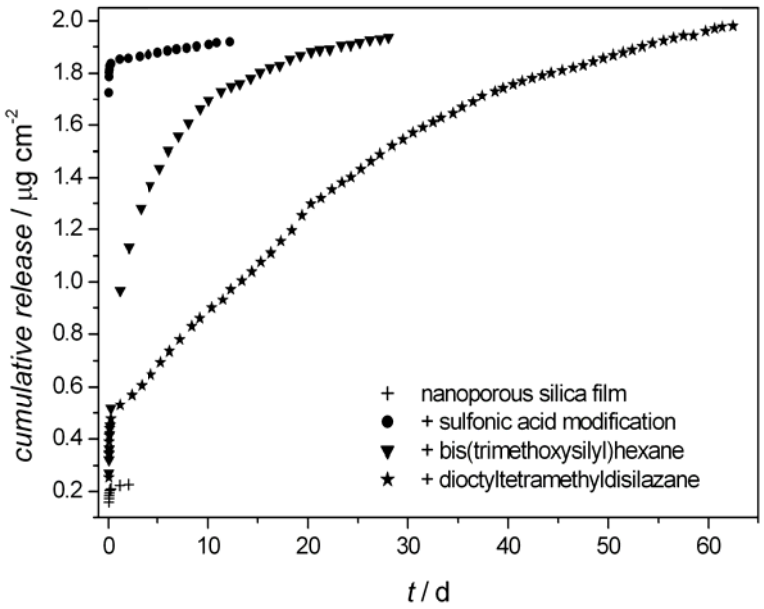


Figure 3-13. Release profiles of ciprofloxacin loaded mesoporous silica layers on glass substrate functionalized successively with sulfonic acid groups, dip-coated layer derived from bis(trimethoxysilyl)hexane and a layer derived from dioctyltetramethyldisilazane by evaporation.

From the unmodified mesoporous silica layer, 0.2 μg $cm^{-2}$ ciprofloxacin were released. Upon modification with sulfonic acid groups, this value increased nearly ten-fold to 1.9 μg $cm^{-2}$. The modified as well as the unmodified mesoporous layer both showed a typical initial burst release profile, where most of the drug was released within the first few hours, with just small amounts being released after the first 24 hours. After 12 days, practically 100 % of the ciprofloxacin from the sulfonate-modified layer had been released. However, the release profiles could be tailored by further functionalization steps. Samples which were dip-coated with bis(trimethoxysilyl) hexane show a slower release. After the first 12 days, ca. 90 % of the total amount had been released. The release rate then decreases so that quite similar doses are still obtained up to 31 days. With the additional surface coating produced by the evaporation of dioctyltetramethyldisilazane on top of the sample, an even more prolonged release profile is established. Here, after 12 days less than 50 % of the total amount is released. A constant release rate can be observed for more than 30 days, followed by regular smaller doses up to 63 days. The surface coatings did not influence the total amount of drug released, which in all cases is about 2 μg $cm^{-2}$ of ciprofloxacin. This fact demonstrates that only very small amount of the ciprofloxacin is lost during the additional functionalization steps. The release profiles depicted in Figure 3–13 all show different release rates in different regions. During the initial burst, the sample which was only sulfonated, discharged ca. 95 % of the total loading whereas the samples which were coated lost only ca. 30 %. The sample which was only equipped with the dip-coated layer produced from bis(trimethoxysilyl)hexane, showed a rather fast release in the first ten days and a slower release afterwards. The behavior of the sample which has an additional coating produced from evaporation of dioctyltetramethyldisilazane, is similar, but the release rates are smaller and the change from a medium to a slow release occurs only after ca. 30 days.

*In vitro testing*

Mesoporous coated, sulfonic acid modified and ciprofloxacin loaded sample showed good biocompatibility in cell culture assays using the standard murine fibroblast cell line NIH3T3. Microscopic observation indicated that the cells could efficiently adhere and proliferate on all modified surfaces.

Results of experiments with luminescent bacteria illustrate the antibacterial efficiency of ciprofloxacin-loaded samples. After six hours in LB medium, ciprofloxacin-loaded samples showed only about one eighth of radiance caused by

luminescent bacteria in comparison to blank values obtained with the mesoporous silica coating only, with the sulfonate-functionalized mesoporous silica and with a glass control (Figure 3–14) The strong antibacterial activity of ciprofloxacin-loaded sulfonated mesoporous silica layers presents a promising practical approach to effectively fight bacterial infections of implants with very high local drug concentrations that would not be possible by systemic administration.

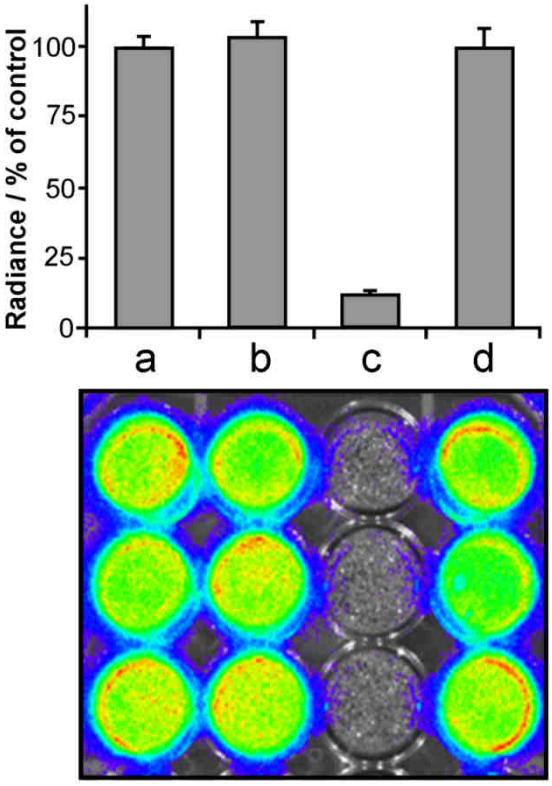


Figure 3-14. Antibacterial efficacy of ciprofloxacin loaded mesoporous silica in vitro. Glass substrates consecutively functionalized a) with a mesoporous silica film, b) after sulfonic acid modification, c) after loading with ciprofloxacin, d) glass control.

*In vivo testing in a mouse model*

A first in vivo experiment shows the practicability of the application of ciprofloxacin-loaded mesoporous layers to implants in a mouse model. For this purpose, a mesoporous coating was applied to a small cylindrical specimen of the standard biomaterial Bioverit® II, a glass-mica ceramic applied for bone substitution. Mesoporous silica coated-Bioverit® II specimen with and without ciprofloxacin were implanted subcutaneously into a mouse. Prior to this, the materials were coated with a film of *Pseudomonas aeruginosa* bacteria with a recombinant lux operon (PAO14 CTX-lux and PAO14 PQS-lux). The presence of these bacteria can be detected by their

luminescence even through the skin, allowing for the facile observation of the evolution of infection processes in vivo. After implantation, all implanted specimen show a strong luminescence signal (Figure 3–15). After one day, these signals are still present for samples which contained no ciprofloxacin, whereas no bacterial luminescence is observed from the samples loaded with ciprofloxacin. This indicates that the infection has been diminished by these implants. Therefore the drug binding and release capacity of the sulfonic acid modified meosporous surface is sufficient to be highly effective even against high concentrations of bacterial pathogens.

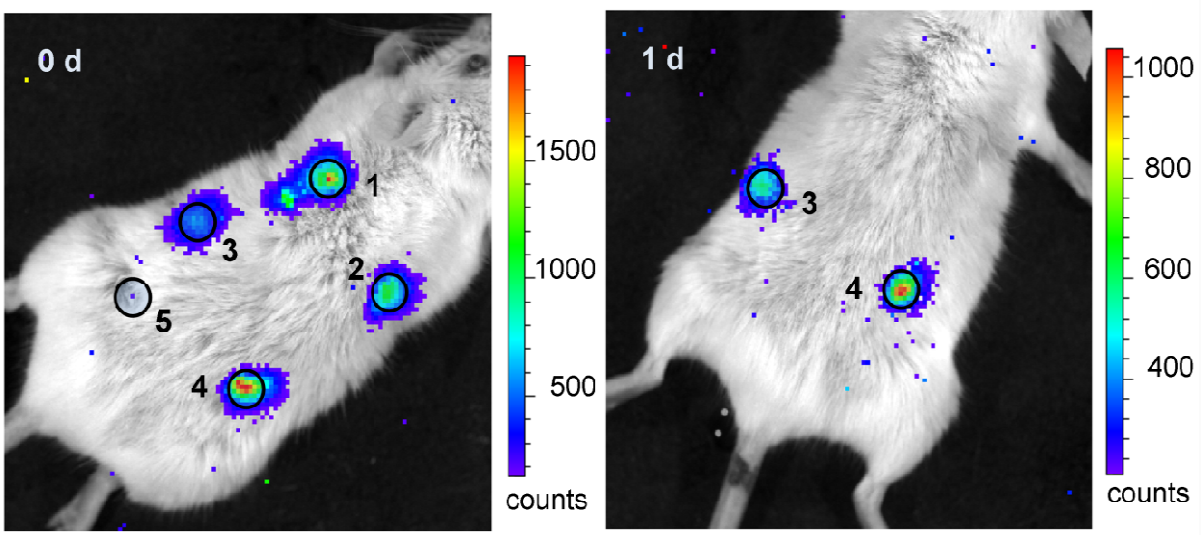


Figure 3-15. Antibacterial efficacy of ciprofloxacin loaded mesoporous silica. Result of a first experiment in a mouse model after one day. Type and position of implants:1) PAO14 CTX-lux on Bioverit® II loaded with ciprofloxacin, 2) PAO14 PQS-lux on Bioverit® II loaded with ciprofloxacin, 3) PAO14 CTX-lux on Bioverit® II, 4) PAO14 PQS-lux on Bioverit® II, 5) Bioverit® II. The two type of luminescent bacteria deposited on implant 1 and 2 are showing no infection after one day whereas the signal of implant 3 and 4 is still clearly visible.

**Conclusions**

We have presented a general practical approach for the antibiotic defense for implants. A sulfonated mesoporous silica film allows a high loading of the antibiotic ciprofloxacin. Controlled release profiles could be tailored using a dip-coating procedure, possibly in combination with an evaporation-coating approach. The general biocompatibility was shown in cell culture experiments. Antibacterial efficacy was proven in investigations with luminescence bacteria, both in vitro as well as in vivo.

**Acknowledgements**

This work was supported by the DFG within the Collaborative Research Program SFB 599 „Sustainable bioresorbable and permanent implants based on metallic and ceramic materials". We thank our colleagues within work package D1 ("Functionalized Middle Ear Prostheses") for valuable discussions.

# 4 Summary and outlook

The aim of this work was to develop new strategies for the functionalization of implant surfaces. By way of example of a middle ear prosthesis, the base material of which consists of Bioverit® II, two different strategies could be established, namely a route for the immobilization of biomolecules (especially the signaling protein BMP2) and a system for controlled local drug delivery.

Due to high costs for BMP2, immobilization techniques were first tested with the enzyme alkaline phosphatase, which is cheap and the activity of which can easily be determined by a well-established assay. As substrates for these investigations structural different silicate surfaces were chosen. Uncoated glass slides were used in comparison with unstructured and mesoporous silica films deposited on glass slides. Also, different silane linking agents were tested. The major questions of this investigation were whether structural properties of the surface have an influence on enzyme immobilization, specifically whether the high surface area of the mesoporous film increases the amount of bound enzyme, and which functional group on the silane linker is best suited for the attachment of a protein. We were able to show that in general it is possible to immobilize the alkaline phosphatase by means of all functional groups on the three different surfaces by a simple and fast dipping procedure. The best combination showing an outstanding amount of active immobilized enzyme was the mesoporous silica surface equipped with an aminopropylsilyl linker.

In a second step, this established immobilization strategy was transferred to the binding of the growth factor BMP2. This time not only (coated) glass surfaces were used as substrates, but also the standard biomaterial Bioverit® II. The quantification of active BMP2 was much more complex and was carried out in cooperation with the Helmholtz Center for Infection Research in Braunschweig. In addition to an ELISA test, a specially developed cell-based test (BRE-luc test) was applied. Only the latter gave reliable quantifications of the amount of biologically active BMP2 bound to the surface. Here again the aminopropylsilyl function was found to be the best linker, although the investigations were not as extensive as in the case of ALP immobilization. On Bioverit® II substrates, high amounts of immobilized BMP2 were found (150 ng $cm^{-2}$). In designing the procedure for BMP2 attachment, care had to be taken to make it as simple as possible, so that the reaction can be carried out directly

before or even during the operation. This is due to the low storage stability of proteins and to the fact that a middle ear prosthesis may have to be shortened during the implantation to adapt it to the space of the specific middle ear; in this case an unfunctionalized surface would be presented. So, it is noteworthy that the procedure developed here is so simple that it can in principle be carried out in the operating theatre.

In addition to the work presented here in this thesis which deals with the materials chemistry of implants, in vivo animal tests were performed in a rabbit model. Blank Bioverit® II samples as well as specimen partially or fully with BMP2 were implanted in two body regions, namely subcutaneously under the skin of the neck and at the functional site as a middle ear prosthesis. For these studies, large numbers of implants (15 blank Bioverit® II samples, 15 partially and 15 fully coated with BMP2) were prepared according to the route described in section 3.2. The results are still under evaluation. So far, it can be stated that at the subcutaneous site, a clear tissue reaction can be observed. The fibrous capsule surrounding the implant is significantly thicker in regions where BMP2 has been applied. This can especially be recognized very well in the borderline regions of partially coated samples. However, no ectopic bone formation was observed with BMP2-bearing samples, as was the case in other experiments, where collagen sponges impregnated with BMP2 were implanted into muscle tissue of mice [158]. Currently, our samples are also being tested in this site. The animal experiments were carried out in collaboration with Iwa Hlozanek, Dr. Gudrun Brandes and Dr. Martin Stieve (Medical School Hannover) as well as Dr. Andrea Hoffmann and Dr. Gerhard Gross (Helmholtz Center for Infection Research Braunschweig) and the results will soon be published.

The stability of thin mesoporous silica films in aqueous and especially in biological environments is currently being debated. Bulk MCM-41 and SBA-1 and SBA-3 (impregnated with ibuprofen) were found to dissolve about 10 – 15 % in simulated body fluid at 37 °C after 80 hours [147, 140], whereas mesoporous silica films showed complete dissolution within a timescale of approximately two hours in cell culture medium at 37 °C [106]. The more rapid dissolution of silica films in cell culture medium is supposed to occur as a result of the presence of active nucleophils in the medium. Preliminary own investigations have shown that the structural order of the coating is lost rapidly (within six to twelve hours) but that a silica layer still persists on the surface of a glass or Bioverit® II surface. Therefore, it cannot be excluded that the biologically active BMP2 found in cell and animal studies has dissolved from the

prosthesis and that the structural breakdown influences the release kinetics. Investigations which should further clarify these questions are currently being carried out together with Dr. Andrea Hoffmann from the Helmholtz Center for Infection Research The release behavior for BMP2 from mesoporous silica coatings is currently studied in cell culture medium (10 % fetal calf serum in phosphate buffered saline) in order to find out how much of the protein is released into solution at different stages within the first week.

A second strategy of implant functionalization was realized by using the mesoporous silica coating as a reservoir for the release of the antibiotic ciprofloxacin, which often is applied systemically in the case of middle ear infections. By functionalizing the silica surface with sulfonyl groups, the loaded amount of ciprofloxacin could be increased nearly tenfold (up to 2 μg $cm^{-2}$) as compared to the blank mesoporous layer. These materials were tested in vitro in bacterial cultures. A high effectiveness against *Pseudomonas aeruginosa* (PAO1 CTX-lux) was observed after 6 hours, and experiments with fibroblasts showed a good general biocompatibility of these materials. Microscopic observation indicated that the cells could efficiently adhere and proliferate on the materials surfaces. A first in vivo animal experiment carried out using an especially developed mouse model also provided evidence for the high efficacy of the drug delivery system.

By means of a combined dip- and evaporation-coating with hydrophobic substances, the release of ciprofloxacin could be prolonged up to over sixty days. In order to prove the anti-bacterial efficacy over that long period of time further bacterial culture experiment are planned. In this investigation, the ciprofloxacin-loaded materials equipped with different retarding coatings are every day placed in a distinct volume of fresh cell culture medium. The medium remoced is then tested for its efficacy against bacteria. The in vitro and the in vivo investigations on the drug delivery materials are carried out in collaboration with Mohammad Badar and Dr. Peter P. Müller from the Helmholtz Center for Infection Research in Braunschweig.

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# 6 Appendix

## A List of publications

**Articles, which represent the "Results and Discussion" part of this work and are to be submitted:**

N. Ehlert, P.P. Müller, M. Stieve, P. Behrens, *Immobilization of alkaline phosphatase on modified silica coatings*, Microporous and Mesoporous Materials.

N. Ehlert, A. Hoffmann, G. Gross, B. Hering, M. Stieve, P. Behrens, *Amino-modified silica surfaces efficiently immobilize Bone Morphogenetic Protein 2 (BMP2) for medical purposes*, Biomaterials.

N. Ehlert, M. Badar, M. Stieve , T. Lenarz, P.P. Müller, P. Behrens, *Mesoporous silica films for controlled release of ciprofloxacin from implants*, Journal of Materials Chemistry.

**Further articles**

J.C. Vogt, G. Brandes, N. Ehlert, P. Behrens, I. Nolte, P.P. Müller, T. Lenarz, M. Stieve, *Free Bioverit® II implants coated with a nanoporous silica layer in a mouse ear model – A histological Study*, J. Biomater. Appl., in press; DOI:101177/ 0885328208095469.

P. Behrens, R. Münnekhoff, A. M. Schneider, S. Münzer, K. Schaper, F. Heinroth, B. Beiße, K. Bokelmann, N. Ehlert, B. Hering, M. Jahns, S. Klingelhöfer, S.J. Lohmeier, C. Menneking, S. Noyun, S. Steinahaus, B. Ufer, *The Chemical Ghostbusters – Science Meets Drama*, *P*, Praxis Naturwiss. Chem. Schule 3 **57** (2008) 12 - 17.

**Conference contribitions**

**Oral presentation**

N. Ehlert, A. Hoffmann, G. Gross, P.P. Müller, T. Lenarz, M. Stieve, G. Brandes, I. Hlozanek, P. Behrens, *Biofunctionalization of mesoporous silica*, 21. Deutsche Zeolithtagung, 4 – 6 März 2009, Kiel, Germany.

**Poster presentation**

N. Ehlert, P. Behrens, A. Hoffmann, G. Gross, P.P. Müller, T. Lenarz, M. Stieve, G. Brandes, I. Hlozanek, *Tailored surface functionalization of middle ear prostheses: Nanoporous silica as a basis for biofunctionalization* , Jahrestagung der Deutschen Gesellschaft für Biomaterialien e.V. (DGBM), 20 – 22 November 2008, Hamburg, Germany.

N. Ehlert, P. Behrens, P.P. Müller, *Immobilization of alkaline phosphatase on glass surfaces modified with porous coatings*, Nanoday, 25 September 2008, Hannover, Germany.

N. Ehlert, P. Behrens, A. Hoffmann, G. Gross, P.P. Müller, T. Lenarz, M. Stieve, *Immobilization of the signalling protein BMP-2: A comparison of mesoporous and amorphous silica*, Nanoday, 25 September 2008, Hannover, Germany.

N. Ehlert , P.P. Müller, P. Behrens*, Immobilization of alkaline phosphatase on glass surfaces modified with porous coatings,* 6th International Mesotructured Materials Symposium (IMMS2008), 8 – 11 September 2008, Namur, Belgium.

N. Ehlert, P. Behrens, A. Hoffmann, G. Gross, P.P. Müller, T. Lenarz, M. Stieve, *Immobilization of the signalling protein BMP-2: A comparison of mesoporous and amorphous silica*, 6th International Mesotructured Materials Symposium (IMMS2008), 8 – 11 September 2008, Namur, Belgium.

N. Ehlert, P.P. Müller, P. Behrens, *Immobilisierung von alkaliner Phosphatase auf modifizierten Glasoberflächen*, Jahrestagung der Deutschen Gesellschaft für Biomaterialien e.V. (DGBM), 22 – 24 November 2007, Hannover, Germany, published in *Biomaterialien*, 2007, 8, 204.

N. Ehlert, P. Behrens, A. Hoffmann, G. Gross, P.P. Müller, H. Mojallal, M. Stieve, *Immobilisierung von BMP-2 auf amorphem und nanoporösem Siliciumdioxid*, Jahrestagung der Deutschen Gesellschaft für Biomaterialien e.V. (DGBM), 22 – 24 November 2007, Hannover, Germany, published in *Biomaterialien*, 2007, 8 192.

N. Witteck, I. Krueger, P. Behrens, H. Mojallal, M. Stieve, T. Lenarz, P.P. Müller, *Herstellung und Untersuchung von Biomaterialien für den Einsatz im Mittelohr*, 77. Jahresversammlung der Deutschen Gesellschaft für Hals-Nasen-Ohren-Heilkunde, Kopf- und Hals-Chirurgie e.V., 24 – 28 Mai 2006, Mannheim, Germany.

N. Witteck, I. Krueger, P. Behrens, F. Dimpfel, P.P. Mueller, M. Stieve, T. Lenarz, H. Mojallal, C. Turck, B. Süß, *Nanoporous silica coatings and their modification,* Interface Biology of Implants, 17 – 19 Mai 2006, Rostock, Germany, published in *Biomaterialien*, 2006, 7, 124.

N. Witteck, Ch. Menneking, P. Behrens, *Polysaccharide-ceramic composites for medical applications I. Chitosan and hyaluronic acid*, 16th Joint Meeting of the "Netherlands Society for Glycobiology", the "Studiengruppe Glykobiologie der Gesellschaft für Biochemie und Molekularbiologie", the "Groupe Lillois de Glycobiologie", and the "Belgian Working Group for Glycosciences", 27 – 29 Oktober 2005, Hannover, Germany.

N. Witteck, I. Krueger, H. Mojallal, M. Stieve, T. Lenarz, P.P. Müller, P. Behrens, *Herstellung und Untersuchung von Biomaterialien für den Einsatz im Mittelohr*, Jahrestagung der Gesellschaft Deutscher Chemiker (GDCh), 11 – 14 September 2005, Düsseldorf, Germany.

## B Curriculum vitae

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