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Mesoporous silica films as a novel biomaterial: applications in the middle ear†

Nina Ehlert,[ab] Peter P. Mueller,[c] Martin Stieve,‡[d] Thomas Lenarz[d] and Peter Behrens*[ab]

In this tutorial review we present the process of the development of functional implants using mesoporous silica. The different steps from chemical synthesis and physicochemical characterization followed by *in vitro* testing in cell culture assays to clinically relevant *in vivo* animal studies are examined. Since the end of the 1990s, mesoporous silicas have been considered as biomaterials. Numerous investigations have demonstrated their non-toxic and biocompatible properties. These qualities in combination with the unique properties of high surface area and pore volume, uniform and tunable pore sizes and chemical modifiability are the reasons for the great scientific interest in this field. Here we show that besides bulk materials or mesoporous silica nanoparticles, mesoporous silica films are highly promising as coatings on medical prostheses or implants. We report on the development of functionalized mesoporous silica materials specifically for middle ear applications. Middle ear prostheses are used to restore the sound transmission through this air-filled cavity when the small bones of the middle air (the ossicular chain) have been destroyed by disease or by accidents. In addition to optimal restoration of sound transmission, this technique bears several challenges, *e.g.* an ongoing bacterial infection or the displacement of the prosthesis due to insufficient fixation. To improve the healing process, a mesoporous silica coating was established on ceramic middle ear prostheses, which then served as a base for further functionalizations. For example, the bone growth factor BMP2 was locally attached to the coating in order to improve the fixation of the prosthesis by forming a bony connection to the remainder of the ear bones. Further, an implant-based local drug delivery system for the antibiotic ciprofloxacin was developed with the aim of fighting bacterial infections. Further possibilities using mesoporous silica nanoparticles as part of a composite on an implant are briefly discussed.

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Key learning points

The way from clinical problems to improved implant materials: materials development, *in vitro* and *in vivo* testing.
Establishment of mesoporous thin silica films on ceramic surfaces.
Degradation of mesoporous silica films in physiological medium.
Implant-carried mesoporous silica films as drug delivery systems.
Implant-based delivery of biomolecules using mesoporous silica coatings.

Introduction

Since the discovery of mesoporous materials in the early 1990s,[1–3] the family of mesoporous silica materials has aroused great interest which is still growing rapidly. These materials are created by condensation of amorphous silica around ordered aggregates of amphiphilic molecules, also designated as structure-directing agents (SDAs). These molecules are then removed by a calcination or extraction step leading to a porous network.[4] This synthesis strategy can yield pore sizes from 2 to 10 nm with various pore topologies like cubic or hexagonal

[a] *Institut für Anorganische Chemie, Leibniz Universität Hannover, Callinstr. 9, 30167 Hannover, Germany. E-mail: peter.behrens@acb.uni-hannover.de; Fax: +49 511 762 3006; Tel: +49 511 762 3697*
[b] *Center for Solid-State Chemistry and New Materials, Leibniz Universität Hannover, 30167 Hannover, Germany*
[c] *Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany*
[d] *Hals-Nasen-Ohren-Klinik, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany*

† Part of the mesoporous materials themed issue.

‡ New address: Klinik für Hals-, Nasen- und Ohrenheilkunde, Universitätsklinikum Schleswig Holstein, 23538 Lübeck, Germany.

in 2D or 3D.[5] The most important properties of these materials are their high surface areas and pore volumes, the tunable and uniform pore size, and the convenience with which chemical modifications can be carried out due to the presence of surface silanol groups.[6]

These properties have been evaluated for a range of applications which are typical for porous materials. A novel application area for which mesoporous materials have been studied from the beginning of the 2000s is the area of biomaterials. Mesoporous silicas meet the basic requirements for biomaterials, as they are non-toxic and have a high biocompatibility.[7] In addition to many studies certifying these favourable properties, only a few examples for harmful reactions have been found *in vivo*.[8,9] Normal synthetic procedures for mesoporous silicas deliver a powder of aggregated particles which is difficult to apply in biomedical devices.[10] An alternative which is pursued by many scientists is the use of mesoporous or nanoporous silica nanoparticles.[6,11–13] As an example of the progress in this field, the FDA has recently approved the first in human trial of silica nanoparticles.[14] An alternative application form are coatings of mesoporous silica, which can be applied directly with a pre-formed implant or prosthesis. Thin films of mesoporous silica can be produced on various substrates using dip- or spin-coating procedures.[15,16] Their formation proceeds *via* the EISA (Evaporation-Induced Self-Assembly) process.[17] Applying this process in the dip-coating method, a substrate is immersed into a diluted synthesis solution. At the moment of extraction, the concentrations of the components increase and they form larger ordered aggregates (micelles from the amphiphilic SDAs) or undergo condensation (the silica precursors) due to the evaporation of the solvent at the border between liquid and air. Alternatively, spin-coating can be used. Considering the possibility to directly equip implants with the favourable properties of mesoporous silica, we have focused on establishing coatings of this material on known implant materials, on using the possible functions of such coatings and on testing them in cell culture and animal experiments.

Nina Ehlert

Nina Ehlert attended the Leibniz University of Hannover where she obtained a Diploma degree in 2005 while conducting research in the group of Prof. Peter Behrens at the Institute of Inorganic Chemistry. In 2009, she received a PhD degree again under the supervision of Prof. Peter Behrens joining a project within the collaborative research program 599 dealing with the functionalization of middle ear implants. Her research interests are focused on the development of implant materials with regard to their biological integration, especially in the field of hearing implants.

Peter P. Mueller

Peter P. Mueller received his PhD degree from the University of Basle in 1983. Until 1987 he was a visiting scientist at the NICHD, National Institutes of Health, Bethesda, MD, then a Research Associate at the University of Berne, Switzerland, and since 1994 at the Helmholtz-Center for Infection Research, Germany, where he became a Professor in 2008. He is a member of the European Society for Animal Cell Technology and Deutsche Gesellschaft für Biomaterialien. His interests are focused on molecular genetics of mammalian cells, fungi and bacteria and the interaction of these cells with implant materials in vitro and in vivo.

Martin Stieve

Martin Stieve studied medicine at the Hannover Medical School (MHH). After clinical education at the Clinic for Laryngology, Rhinology and Otology of the MHH he became a professor at the University of Lübeck in 2012. His research focuses on the development of animal models for the evaluation of implant materials. He is a member of the German Society of Oto-Rhino-Laryngology, Head and Neck Surgery and of the German Society for Biomaterials.

Thomas Lenarz

Thomas Lenarz received his Dr med. in 1982 after studies in Tübingen, Erlangen, Heidelberg and London. He was further educated to an otorhinolaryngologist at the University of Heidelberg and became an assistant medical director there in 1990. Since 1993, he has been the Director of the Ear–Nose–Throat Department at Hannover Medical High school. He is the speaker of the Collaborative Research Area SFB 599 of the DFG and the co-director of the Cluster of Excellence "Hearing4All". His research interests comprise all aspects of the auditory system as well as computer-assisted surgery and robotics.

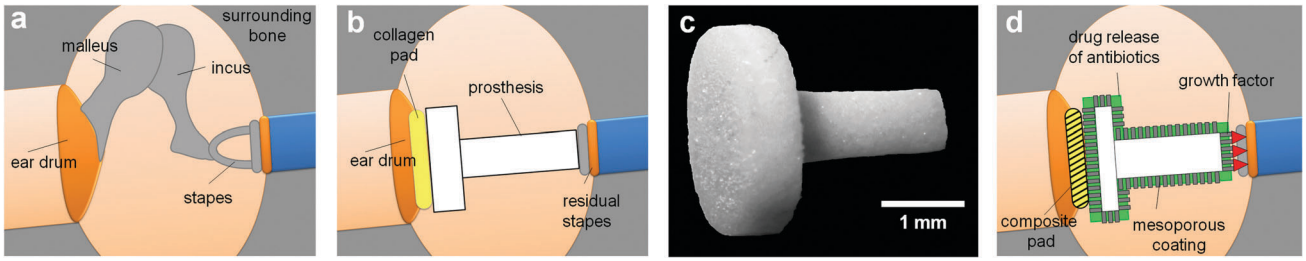

Fig. 1 (a) Anatomic situation in the middle ear with the ossicular chain consisting of three small bones, malleus, incus and stapes (or hammer, anvil, and stirrup); (b) replacement of the destroyed ossicular chain by a middle ear prosthesis; (c) middle ear prosthesis made of the glass–mica ceramic Bioverit® II, as used for animal experiments in rabbits (for humans the total length is *ca.* 8 mm); (d) specific functionalizations of a middle ear prosthesis with a mesoporous silica coating, which is used for a site-specific attachment of a growth factor and for local drug delivery.

As a test field for this work we have chosen the middle ear. The middle ear is an air-filled cavity; it is separated from the outer ear by the ear drum (tympanic membrane) and from the inner ear by the oval window. Its main task is to transduct the air waves reaching the drum (which correspond to sound) to liquid waves in the cochlea in the inner ear, where the pressure fluctuations are further processed by hair and nerve cells and then sent as electrical signals to the brain. For this task, the middle ear is equipped with a mechanical transduction system consisting of three small bones, the malleus, the incus and the stapes (Fig. 1a). The malleus is directly attached to the drum and the stapes is fixed on the membrane of the oval window. This ossicular chain also provides a small amplification effect due to the leverage action of the bones. The flexible joints between the three bones can compensate changes in the atmospheric pressure and can reduce sound transmission in response to loud sounds to protect the inner ear.

As a consequence of diseases of the middle ear, the middle ear bones can be destroyed. Typical pathological manifestations are chronic bacterial infections or cholesteatoma, tumour-like aggregations of epidermal and connective tissues within the middle ear which do not belong there. The destruction of the middle ear bones constituting the ossicular chain disrupts sound transmission and consequently leads to hearing loss in the affected ear. Hearing can be restored using a prosthesis, the so-called total ossicular replacement prosthesis (TORP), which re-establishes the contact between the drum and the oval window, on which typically a residue of the stapes plate has remained undestroyed (Fig. 1b). As for any implant, the basic requirement for the prosthesis material is of course a good biocompatibility. In this special case, optimal sound transmission capabilities are also important. This is usually achieved using stiff materials such as metals, ceramics, composite ceramics or strong polymers.[18] Different prostheses made of such materials are commercially available and are chosen following the conviction of the particular surgeon, usually giving satisfying hearing results.

Although available middle ear prostheses are implanted frequently, there are still problems to solve in order to improve the quality of life for the patients.[19] Often, a revision surgery, which is of course straining for the patient, becomes necessary due to displacement of the prosthesis. When implanted the prosthesis is simply inserted between the eardrum and the residual stapes bone footplate with no additional fixation. In certain cases, the prosthesis may become dislocated. Another problem is that the construction of the middle ear prosthesis is inflexible. Due to the joints between the ossicular bones, the ossicular chain can deform, thus mediating pressure variations. This task cannot be accomplished by a stiff TORP. Therefore, a soft collagen pad (taken from another part of the body of the patient) is usually placed between the tympanic membrane and the head part of the prosthesis. Another crucial problem in middle ear surgery is infection, which can be brought to the implantation site during the operation, but is more often due to the persistence of the chronic infection which had originally caused the degradation of the middle ear bones.[20] Finally, when the destruction of the middle ear bones had been caused by a cholesteatoma, a relapse may occur after its surgical removal.

As satisfying hearing results can be obtained using prostheses made from stiff standard biomaterials, we have focused on developing measures against the above-mentioned healing disorders. For this purpose, we have chosen prostheses made from Bioverit® II. This is an advanced glass–mica ceramic implant material which is highly biocompatible, insoluble and

Peter Behrens

Peter Behrens studied chemistry at the University of Hamburg and performed the studies for his habilitation at the University in Konstanz. From 1994 to 1998, he was a professor at the Ludwig Maximilians University in Munich, and since 1998 he has been a full professor for Inorganic Chemistry at the Leibniz University Hannover. He is a member of the Collaborative Research Area SFB 599 of the DFG and of the Cluster of Excellence "Hearing4All". His major research interests are the controlled synthesis and preparation of materials, focusing on biomaterials, porous materials, nanocomposites and coatings.

corrosion resistant. Furthermore, Bioverit® II is clinically well-established, mainly as a bone replacement material in skull and middle ear reconstructive surgery.[21–25] A typical middle ear prosthesis made from this material is shown in Fig. 1c.

Our approach is based on coating Bioverit® II middle ear prostheses with a mesoporous silica layer which is then locally functionalized according to the different requirements (Fig. 1d). The idea to improve the fixation of the prosthesis relies on improving the connection between the prosthesis and the oval window by inducing the growth of new bone starting from the stapes footplate residue which is typically present on the membrane separating the middle from the inner ear. The formation of new bone can be induced by the bone growth factor BMP2 (Bone Morphogenetic Protein 2) which we attach selectively at the tip of the prosthesis pointing to the inner ear. For this purpose, we use the mesoporous silica film with its high surface area and reactive silanol groups.

Middle ear infections continuing after the implantation or triggered by the surgery are combated using antibiotics. Usually, these are applied systemically which is stressful for the body and can cause harmful side effects. Additionally, such a systemic treatment of the infection may be insufficient when the bacteria can form a treatment-resistant biofilm on the implant surface.[26] Therefore, it would be a great advantage to immediately fight the bacteria effectively before biofilm formation can take place, and thereby permit an undisturbed healing process. For this purpose, we used the pore system of the mesoporous silica coating as a reservoir for an antibiotic (Fig. 1d). In this way, the implant itself directly and locally delivers the drug. This strategy has the advantage to avoid possible side effects caused by a systemic administration of the drug, where much higher doses would affect the whole body.

In the following, we describe the preparation of mesoporous silica layers on glass slides (as a model system) and on Bioverit® II and their physico-chemical characterization.[27] Importantly, the stability of the coatings in a physiological medium is investigated and their biocompatibility is studied using cell cultures and animal experiments.[27–32] We then show how to immobilize the bone growth factor BMP2 onto the mesoporous coating,[31,33,34] focusing on methods how to quantify the amount of this protein bound.[33] For the reliable attachment of BMP2, the mesoporous silica has to be modified, and this is also the case in the construction of an efficient drug delivery system for an antibiotic. The development of this system and especially its comprehensive testing using *in vitro* cell and bacteria culture studies[32] as well as animal experiments will be described.[31] Finally, possible further improvements of middle ear prostheses are discussed, for example with regard to prevent the relapse of a cholesteatoma. Throughout this tutorial review, we will try to make clear how the interdisciplinary interaction between material chemists, biologists and clinicians on the path from chemical synthesis and physicochemical characterization to *in vitro* testing as well as biological and functional evaluation *in vivo* can lead to novel interesting biomaterials.

Mesoporous silica films on implant surfaces

The preparation of mesoporous silica films has been investigated thoroughly on a variety of smooth substrates and has been shown to proceed according to the EISA process.[17] For preliminary investigations, we have used glass slides as substrates, as Bioverit® II, an approved biomaterial, is rather expensive. The results obtained on glass slides were then transferred to this glass–mica ceramic, considering especially its much rougher surface.

For the generation of the mesoporous silica coatings, a standard reaction mixture consisting of tetraethoxysilane, ethanol, water, hydrochloric acid and an SDA similar to Pluronic® 123 was used.[27,32,33,35] The substrates were dip-coated, dried at high air humidity and at 60 °C, followed by a final calcination step at 415 °C. Because of the rough surface properties of the ceramic substrates, it was necessary to produce three layers of the film in order to achieve a total coverage of the surface.

X-ray diffraction (XRD) patterns show rather broad reflections at 1.1° 2θ for the mesostructured films which shifts to 1.6° 2θ for the calcined ones (Fig. 2), reflecting regular structures of 8.0 nm and 5.5 nm repeat size, respectively. As no higher-order reflections are present, but only one very broad additional peak at *ca.* 3° 2θ, the pores in this coating have no ordered arrangement, as a hexagonal, cubic or lamellar packing, and no long-range preferred orientation with regard to the substrate, as previously described.[32,33,35] Correspondingly, the pore topology in these coatings is disordered, similar to bulk mesoporous materials of the LMU-1[36] or KIT-1[37] type. The fact that the same reflections can be found on the coated glass slides as well as on the coated Bioverit® II samples shows that the porous structure of the silica film is present on the ceramic substrate, too. The Bioverit® II samples present another reflection at 9° 2θ in the investigated 2θ range; this is caused by the crystalline parts of the glass–mica material.[27]

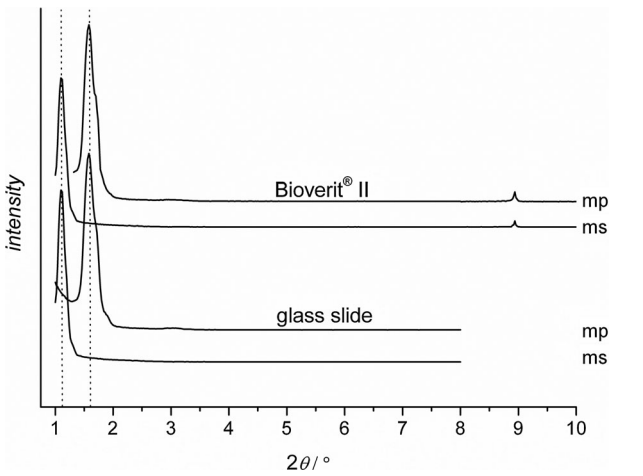

Fig. 2 X-ray diffraction patterns of silica coatings on Bioverit® II substrates (top two lines) and on glass slides (bottom two lines). ''ms'': as-synthesized mesostructured coatings still containing the amphiphilic SDA; ''mp'': mesoporous coatings after removal of the SDA. Adapted with permission from ref. 27.

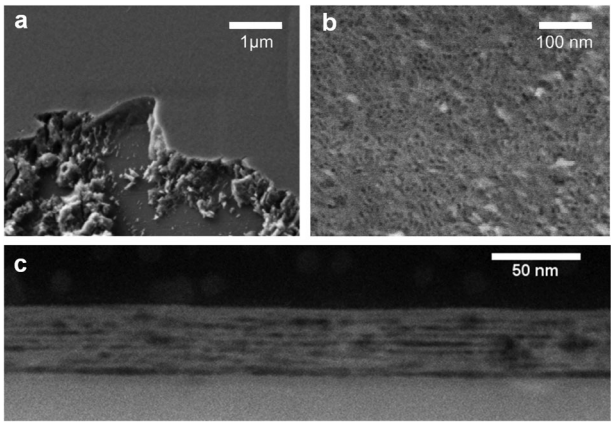

Fig. 3 Electron microscopic characterization of a mesoporous silica layer on a glass slide. (a) Low-resolution SEM image corresponding to an inclined sight on an intentionally broken layer; (b) SEM top view onto the surface of the film showing open pore mouths; (c) STEM image of a cross section of the film revealing the pore topology. Adapted with permission from ref. 27.

In accordance with the XRD results, electron microscopic investigations reveal a disordered arrangement of nanopores within the silica film. In Fig. 3 images of silica films on glass are presented. With low resolution, the scanning electron microscope (SEM) image in Fig. 3a shows a side-view of an intentionally broken layer, where the direct attachment of the film on the glass substrate can be observed. Whereas at this magnification the coating appears smooth, a closer look onto the surface (Fig. 3b) reveals pore mouths on the surface from which the pore system can be accessed. The pore mouths, however, do not form a regular pattern. The disorder in the pore system is clearly revealed in Fig. 3c, which shows a scanning transmission electron microscope (STEM) high resolution image of a cross section of a mesoporous silica film. The channels run primarily parallel to the substrate, but do so in all directions. These results are in good agreement with the presented XRD results.[27] The thickness of the film can be determined as *ca.* 40 nm. Further investigations showed that the layer thickness for one silica film lies between 30 and 150 nm.[32]

In order to use mesoporous silica layers as a surface modification on implants, we transferred the synthesis procedure to the ceramic implant material Bioverit® II as a substrate. In contrast to glass substrates, this material has a rather rough surface structure. In order to safely fill the surface cavities and potholes of the Bioverit® II surface, the coating procedure was performed three times in total. The procedure was successful as can be seen in Fig. 4, and by a STEM cross section of the mesoporous silica film on Bioverit® II (Fig. 5). Mesoporous silica material can be detected in the cavities of the rough surface of the glass-ceramic. Similar to the coatings on glass slides, an irregular system of mesopores can be observed.[27] An orientation of the channels parallel to the substrate can only be observed very close to the surface of the support (Fig. 5b, arrows). The lower orientational order as compared to the findings with glass slides as substrates (see Fig. 3c) is probably due to the more irregular liquid flow during dip-coating on rough Bioverit® II substrates as compared to the smooth glass surfaces. Furthermore, SEM investigations showed that the adhesion of the mesoporous silica film was good on glass substrates as well as on Bioverit® II.

Furthermore, krypton sorption experiments revealed a surface area of about 11.2 cm^2 cm^{-2} of the macroscopic substrate.[32]

Stability tests

It is well-known that thin films of mesoporous silica are not stable in water and in biological media.[38] In order to investigate the stability of our coatings, we exposed films prepared on glass slides in a typical cell culture medium (10% v/v fetal calf serum in 0.01 M phosphate-buffered saline solution) at 37 °C for different time intervals. After this treatment, the presence of the mesoporous structure was checked by XRD measurements as presented elsewhere.[33] After having determined the point of

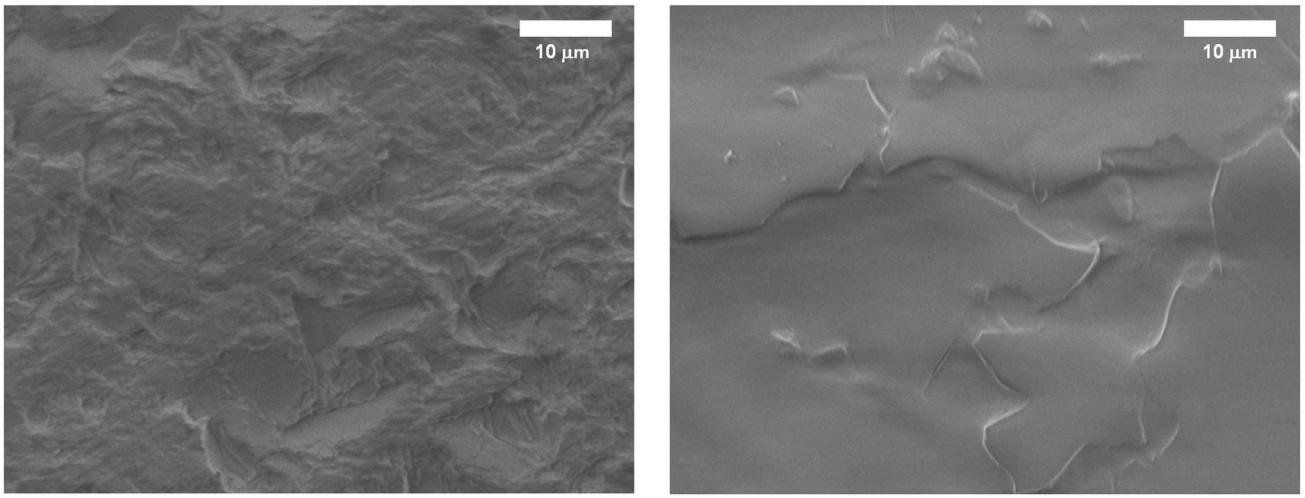

Fig. 4 SEM pictures of a (left) rough surface of native Bioverit® II, and (right) of a smooth surface covered with a mesostructured silica coating.

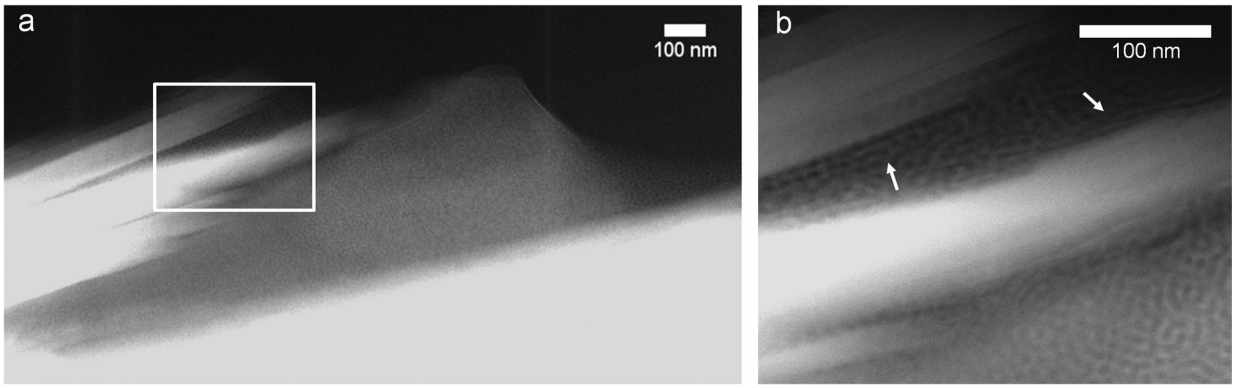

Fig. 5 STEM images of a cross section of a mesoporous silica coating on Bioverit® II substrates at lower (a) and higher magnification (b), showing the disordered mesoporous structure of the coating. Arrows point to orientationally ordered pore channels close to the substrate surface.

time where the XRD-visible mesostructure collapses, further investigations on such samples were performed by FE-TEM and STEM in order to clarify the rearrangement of the film structure.

XRD patterns measured on the films after different exposure times revealed that the mesoporous structure of the film is destroyed, showing different successive phases discussed in detail elsewhere.[33] The intensities of the reflections at 1.6° 2θ and at 3.0° 2θ decreased within the first six hours of exposure, continuing up to an exposure time of twelve hours. Afterwards the reflection at 3.0° 2θ is no longer present, but a broad reflection in the range of 2.0 to 2.5° 2θ can be seen (Fig. 6). After longer periods of time, no diffraction peaks appear anymore. These changes in the XRD patterns are indicative of different states passed through during a structural rearrangement of the silica layer. The disordered pore structure of the original coating collapses and a material with a lower degree of ordering is formed. Finally also this lower-order phase is not detectable anymore in the XRD pattern.[27]

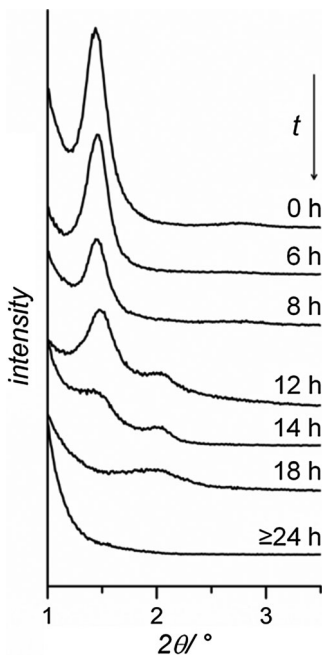

Fig. 6 X-ray diffraction patterns of a mesoporous silica layer on glass. Samples were exposed to a typical mammalian cell-compatible medium (10% v/v fetal calf serum in 0.01 M phosphate-buffered saline solution) for different times. Reprinted with permission from ref. 33.

Interestingly, further investigations on glass substrates showed that a silica film with reduced thickness is still present on the surface for at least 24 hours. When the mesoporous silica film is somehow protected, for example by silanization or the attachment of a protein,[35] reflections from the nanoporous structure can be observed for longer time periods.[27]

We were interested in the structure of the intermediate phase with the characteristic broad peak in the range of 2.0 to 2.5° 2θ and performed a corresponding sample high-resolution FE-TEM/STEM analysis. Fig. 7 shows corresponding images. The pictures can be interpreted in terms of transformation of the mesoporous silica layer to a packing of silica nanoparticles with a size of about 7–8 nm. Their packing appears to be partly ordered, as can be seen from the striations visible in the pictures in Fig. 7. The transformation process probably involves the destruction of the original pore network, *e.g.* by Ostwald ripening-type processes, and a rearrangement of the silica material.[27]

Biocompatibility tests

The initial test for biocompatibility normally consists of cell culture experiments. Although such *in vitro* tests have only limited significance for the situation in living beings, they can detect toxicity and may help to reduce the number of animal experiments. General biocompatibility tests are usually performed with well-defined standard cell lines such as fibroblasts, HeLa or hepatoma cells. For specific applications, the informative value can be increased by using more specialized cells, *e.g.* primary human cells, Schwann cells for nerve regeneration or osteoblasts for bone reparation. Also stem or precursor cell lines can be used, for example pluripotent mesenchymal stem cells, which can under appropriate conditions be differentiated to osteoblasts.

In the case of our mesoporous silica coatings, samples for cell culture tests can be easily prepared on circular glass slides with diameters corresponding to the size of the containers

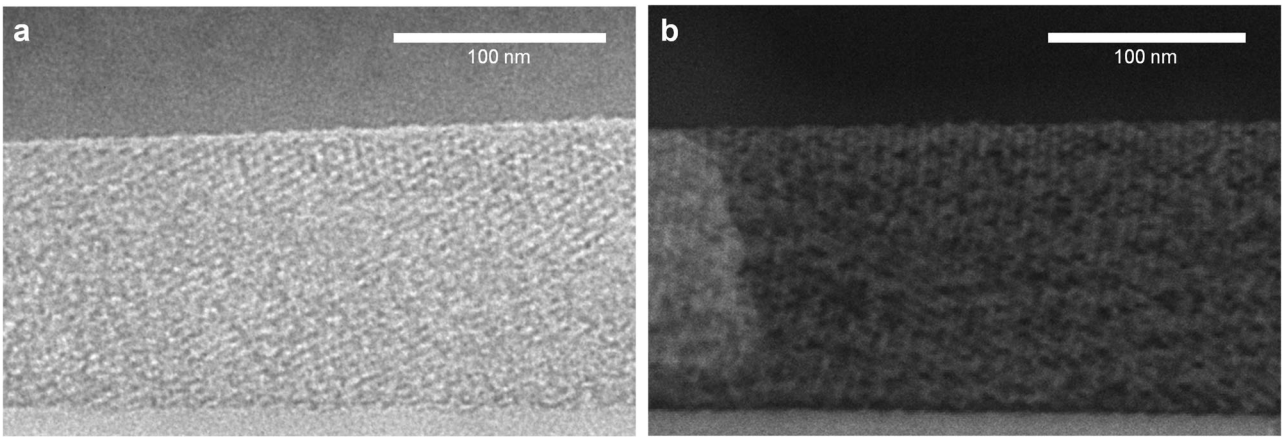

Fig. 7 FE-TEM (a) and STEM (b) images of a cross-section of a mesoporous silica film exposed for 12 h to a typical cell culture medium (10% v/v fetal calf serum in 0.01 M phosphate-buffered saline solution). After exposure to the medium, the mesoporous structure of the silica film rearranges to a partly ordered packing of spherical silica nanoparticles with a size of seven to eight nanometers. Adapted with permission from ref. 27.

wherein these tests are usually performed, the so-called wells (typically 10 or 13 mm). Initial tests of the biocompatibility of our silica were performed with the murine fibroblast cell line NIH3T3 and with the epithel-derived cell line HEK293. Both showed excellent biocompatibility of the coating. Here we show our results obtained with a cell type representative of bone tissue–implant interactions, namely the murine mesenchymal precursor cell line C3H10T1/2. Different mesoporous silica variants prepared with different amphiphilic block copolymers and differing pore sizes were tested. For comparison, bare standard glass slides and glass slides coated with an unstructured silica layer were also tested. The unstructured silica layer was prepared in a similar way to the mesoporous coating, but without using a surfactant. Cells were seeded onto the samples and the interactions were monitored microscopically. During the initial 3 to 5 hours of the cell culture experiment, cell attachment takes place; to exclude any interference by proteins adhering to the surfaces, serum was omitted from the cell culture medium during this phase. The cells adhered well to unstructured as well as to nanoporous silica layers and also to glass. During the subsequent incubation phase under standard cell culture conditions, the cells spread out on the coated slides. Similar to the attachment phase, no differences were observed in the cell response for the different materials; also different pore sizes of the mesoporous silicas did not influence the behavior of the cells. Cell proliferation on these materials was almost as high as on the cell culture-optimized polystyrene surface. The results of cell culture tests can for example be quantified by (automatically) counting the cells (Fig. 8).

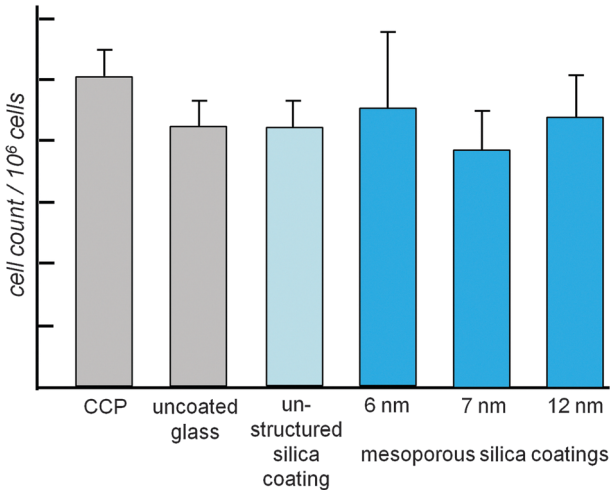

Fig. 8 Results from cell culture tests. Mesoporous coatings with different pore sizes support cell proliferation as well as an unstructured silica coating and as plain glass, and nearly as well as polystyrene used for cell culture containers. The *d* values indicated in nm in the figure were calculated from the most intense reflection in the XRD patterns of the mesoporous silica coatings. Murine mesenchymal precursor cells (C3H10T1/2) were seeded on the corresponding substrate and were incubated with a standard cell culture medium (Dulbecco's Modified Eagle's Medium, DMEM) with 10% fetal calf serum (FCS) for four days at 37 °C in a humidified atmosphere containing 5% CO_2. Adapted with permission from ref. 27.

Whereas glass slides are a convenient substrate for the deposition of mesoporous silica for cell culture testing, further development of these coatings for implant applications requires a substrate which has been established as a biomaterial and which is used for the construction of prosthesis. We have chosen Bioverit® II, a glass–mica ceramic, for this purpose due to the chemical compatibility of the silicate systems. Bioverit® II is a commercially available implant material which is highly bioactive especially for bone-forming cells which is used for a variety of medicinal applications, for example as bone replacement for temporal or skull bones or as middle ear prostheses.[39] Especially the middle ear is a convenient location for carrying out animal studies which poses only limited strain for the animals and offers a control at hand, namely the other ear which is usually left untreated.[40–42]

The combination of a mesoporous silica layer on a Bioverit® II again has to be tested for biocompatibility. Corresponding cell culture tests showed that the surface covered with nanoporous silica is somewhat less bioactive than the plain uncoated, highly "bone-friendly" Bioverit material. In Fig. 9 it can be seen that the cells on uncoated Bioverit® II have spread out,

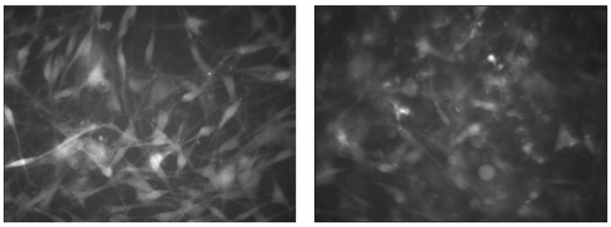

Fig. 9 Results of cell culture tests, comparing uncoated Bioverit® II (left) with Bioverit® II coated with a mesoporous silica layer (right). Recombinant murine mesenchymal precursor cells marked with green fluorescent protein were incubated in a cell culture medium for 3 days. Adapted with permission from ref. 27.

whereas on the coated samples the cells are more roundish, indicating that cellular adhesion is somewhat less favoured.[27]

After successful *in vitro* cell culture tests, *in vivo* animal studies are the next step in the examination of biocompatibility. In addition to the biocompatibility, specific reactions of the surrounding tissue can be studied and also a functional evaluation can be performed. We tested the mesoporous silica film in the middle ear of rabbits, comparing plain Bioverit® II middle ear prostheses with ones which were covered by a mesoporous silica layer. The general results confirmed the high biocompatibility of the mesoporous silica. Histological studies were performed in order to define the type of tissue which had formed on the prosthesis. A tendency was found that on coated prosthesis, somewhat less new bone (Fig. 10a and b) was formed, in agreement with the reduced bioactivity of such samples observed in cell cultures. Instead, a thin cell layer of mucosa had formed directly around the prosthesis (Fig. 10c and d).[28] The formation of a thin mucosa corresponds to an excellent biological situation and therefore a good implant healing.

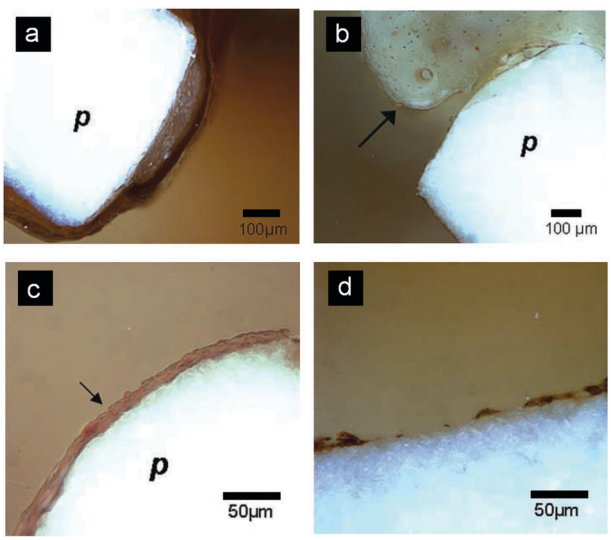

Fig. 10 Results from animal experiments in the middle ear of rabbits. (a) Bioverit® II prosthesis (p) coated with mesoporous silica (the coating is not observable at this resolution): minor ossification (the formation of new bone) has taken place at the tip of the prosthesis. (b) On an uncoated Bioverit® II prosthesis the ossification is slightly increased (arrow). (c and d) On other parts of the prosthesis, mucosa has formed directly on the implant material. On a prosthesis coated with mesoporous silica a complete and regular mucosa (arrow) layer is present (c), whereas the mucosal coverage is partly incomplete on uncoated Bioverit® II (d). Reprinted with permission from ref. 28.

Mesoporous silica films as a base for attachment of biomolecules

As detailed in the Introduction, one of the major problems in middle ear surgery is the displacement of the prosthesis which is simply inserted between the tympanic membrane and the oval window. Our idea to improve the anchoring of the prosthesis is based on establishing a bony connection between the prosthesis and the stapes footplate (a bone residue which typically remains on the oval window membrane after the destruction of the ossicular chain). The formation of new bone can be induced by the bone growth factor BMP2 (Bone Morphogenetic Protein 2). When BMP2 is attached selectively at the tip of the prosthesis, bone formation may occur locally at this spot without a full bony enveloping of the prosthesis which would impede sound transmission.

In order to develop a procedure for binding BMP2 to mesoporous silica, we initially worked with the cheaper protein alkaline phosphatase (ALP), the activity of which can easily be determined by a colorimetric enzyme assay.[35] Results of this initial study – mainly the use of an aminosilane linker to bind the protein to the surface – were then transferred to the much more expensive growth factor BMP2.[33] As substrates we used glass or Bioverit® II. For comparison, we also tested the immobilization capability of a plain substrate surface and of an unstructured silica layer; the latter was prepared in a similar fashion to the mesoporous film, but by omitting the amphiphilic structure-directing agent. For both types of substrates, all three samples – the plain substrates, the unstructured and the mesoporous silica coatings – were studied with and without the linker. Fig. 11 shows the construction of the different samples.

The quantification of small amounts of protein attached to a surface is a delicate task, especially when the specific activity of the immobilized BMP2 is to be evaluated, for the immobilization procedure may reduce part of this activity. This can for example happen due to conformational changes of the protein upon attachment to the surface. Also, a growth factor like BMP2 does not act by catalyzing an easily detectable chemical reaction (as an enzyme does), but it acts by binding to a receptor protein on the outer cell membrane and thereby induces a specific signaling pathway within living cells bearing this specific receptor. If an attached BMP2 molecule is misfolded or sterically hindered, the activity is impeded. However, when one makes use of this interaction, a highly specific determination of the amount of active protein can usually be attained. This can be done for example using a so-called ELISA (enzyme-linked immunosorbent assay). Here, an epitope of the protein is recognized by a primary antibody which carries another antibody which again is linked to an enzyme. This enzyme catalyzes a reaction which yields a product which is easily detectable, for example colorimetrically (Fig. 12). In this way, the amount of BMP2 can be determined which – after binding to the substrate – still shows the correct epitope to the antibody,

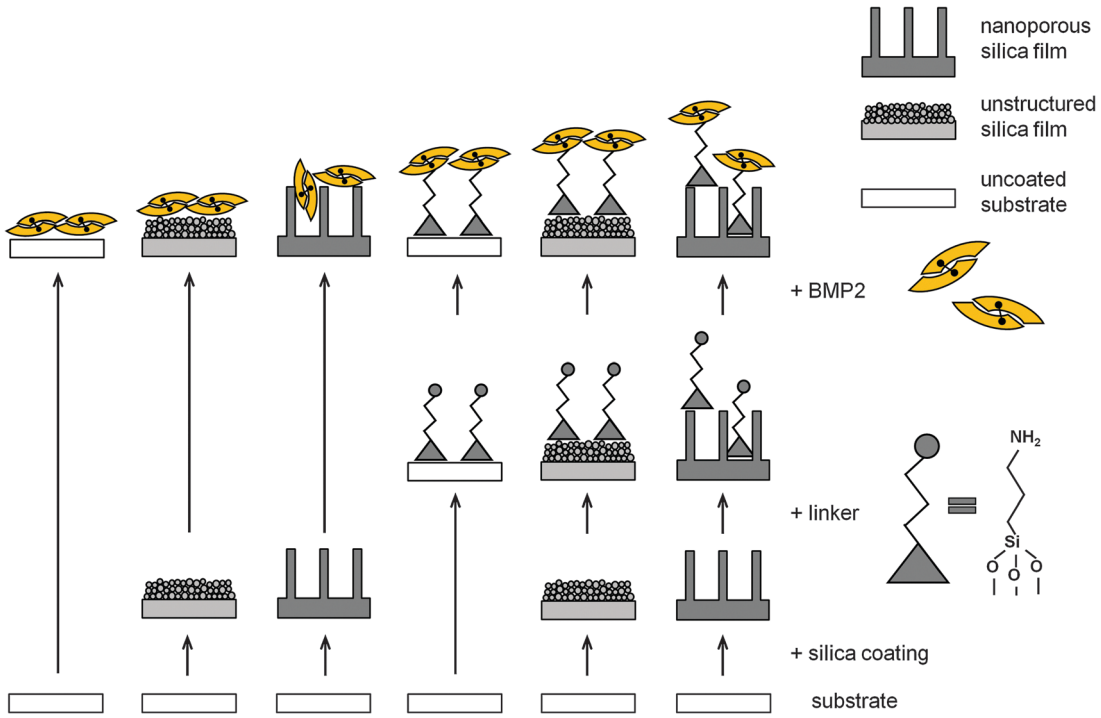

Fig. 11 Scheme for the construction of the different samples studied for the attachment of BMP2. The substrate is either glass or Bioverit® II. Reprinted with permission from ref. 33.

i.e. the amount of BMP2 which is immunologically active. The ELISA, however, cannot determine whether this BMP2 can still fulfill its normal biological function. In order to perform such a more stringent determination, a reporter cell line was constructed (Fig. 12) which is used in a special assay, the so-called BRE-luc test (BRE: BMP2-responsive element; luc: luciferase).[43,44] When BMP2 binds to the cognate receptor of these cells, a signal pathway is activated which ends in the nucleus of the cell. In unmodified cells this would lead to the expression of genes typical of bone-forming cells (osteoblasts). In the genetically altered cells, the gene for the production of luciferase is activated instead. Luciferase is an oxidative enzyme which shows bioluminescence. The intensity of the resulting bioluminescence is then proportional to the amount of biologically active BMP2.

The results of the BMP2 quantification studies are presented in Fig. 13. For the samples based on glass substrates, both quantification methods showed that it was possible to attach small amounts of BMP2 on the glass substrates and different silica coatings when the aminosilane linker was used. According to the ELISA test, a native glass surface modified with the aminosilane linker bound about 5 ng cm^{-2} BMP2 (the area refers to the macroscopic surface of the samples). These values increased to about 13 to 15 ng cm^{-2} BMP2 when the unstructured or the mesoporous silica coating was applied. The cell-based BRE-luc assay detected considerably smaller amounts of biologically active BMP2: about 2 ng cm^{-2} for the amino-modified unstructured and 5 ng cm^{-2} for the amino-modified mesoporous coating. With this method, no BMP2 could be detected on the uncoated but amino-modified glass surface. With both methods, no BMP2 was observed for all types of surfaces when no amino modification was applied.

On Bioverit® II substrates, considerably higher amounts of immobilized BMP2 could be achieved as compared to the glass surfaces. The BRE-luc test detected about 67 ng cm^{-2} for the amino-modified unstructured and more than 100 ng cm^{-2} for the amino-modified mesoporous surface. To validate the latter result, the experiment was repeated twice for the mesoporous surface; in both cases even higher amounts of 156 and 160 ng cm^{-2} bound BMP2 were detected. All tests carried out for comparison (omitting the silane or a silica coating or both) gave values below 4 ng cm^{-2} BMP2. In contrast, the ELISA test was not able to detect any BMP2, except for the uncoated amino-modified surface where an amount of 24 ng cm^{-2} was observed. Obviously, this test is disturbed by the presence of Bioverit® II due to unknown reasons.

In similar experiments, but using an epoxy-bearing silane instead of the aminosilane as a linker, conflicting results were obtained. Whereas in the BRE-luc test, the amount of BMP2 was below the detection limit of <1 ng cm^{-2} in all cases, the ELISA detected 58 ng cm^{-2} in the case of an unstructured silica coating and 46 ng cm^{-2} in the case of the mesoporous silica coating, when these were epoxy-modified. We were able to reveal by further experiments that this discrepancy is possibly caused by a direct binding of antibody molecules to the epoxy functions of the silanized surface, although these should have been blocked by a previous treatment with fetal calf serum. This shows that results from biochemical methods which were originally developed for solutions or for simple surfaces must be treated with care when these methods are transferred to more complicated systems. Due to the low biological activity detected by the cellular test system, the use of the epoxy modification was abandoned.

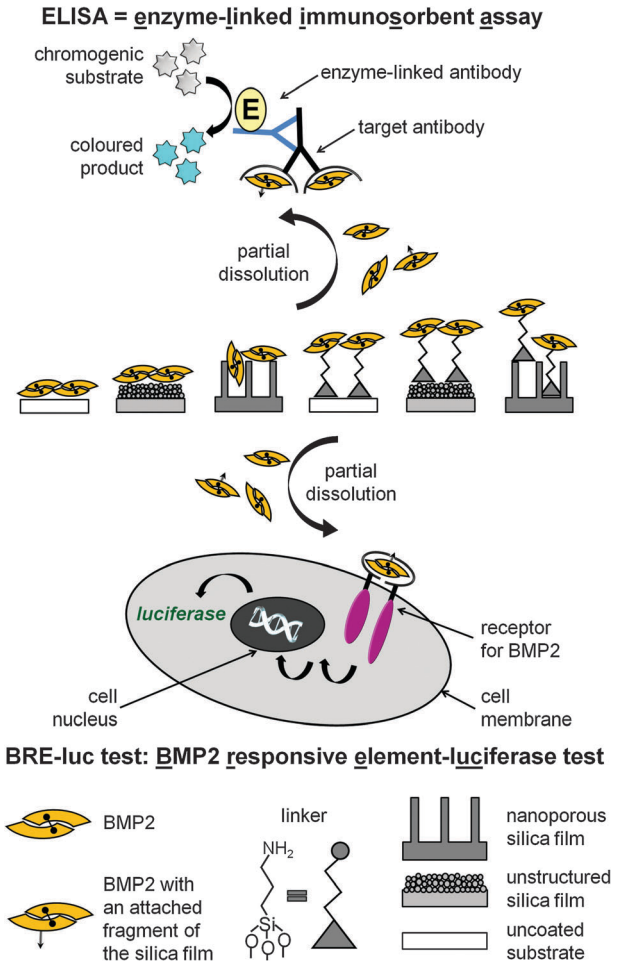

Fig. 12 Scheme explaining the methods for the quantification of surface-attached BMP2. The ELISA (top) determines the amount of immunologically active BMP2, whereas the cell-based BRE-luc test (bottom) recognizes biologically active BMP2.

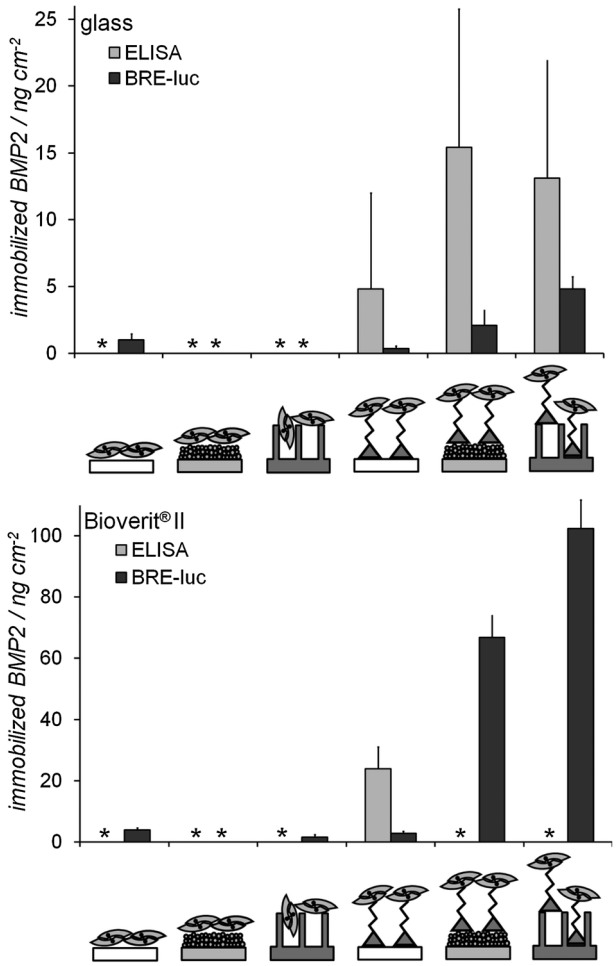

Fig. 13 Quantification of BMP2 detected on different supports based on glass (top) and on Bioverit® II (bottom) substrates. Native surfaces as well as substrates with an unstructured and with a mesoporous silica coating were employed, either in an unmodified form or with a modification derived from 3-amino-propylsilane. Results from ELISA and BRE-luc tests are given (*: no detectable amount of BMP-2 after comparison with the blank value; note the different scales of the ordinates). Reprinted with permission from ref. 33.

First *in vivo* tests of the construct Bioverit® II – mesoporous silica coating – aminosilane – BMP2 have shown that a local biological action of BMP2 can be achieved. These preliminary experiments were carried out with corresponding implants which were placed subcutaneously (below the skin). These cylindrical Bioverit® II implants were fully covered with a mesoporous silica layer, but only half of this coating was exposed to the aminosilane and thus able to bind BMP2. The biological reactions on these two halves of the implant were very distinct,[34] as schematically shown in Fig. 14. For further *in vivo* studies in the middle ear, the same procedure for a selective detachment of BMP2 to the tip of the prosthesis by immersing only this end into the solution containing the linker 3-aminopropylsilane is possible.

Establishing a mesoporous silica-based drug release system on implant surfaces

Another clinical problem in the healing of middle ear prostheses is bacterial infections. *Pseudomonas aeruginosa* (*P. aeruginosa*) is a bacterium which causes middle ear infections and which is able to form a biofilm on an implant surface. Following a stepwise strategy, we have developed several variants of a drug release system based on the pore system of the mesoporous silica film (Fig. 15).[32] We used the antibiotic ciprofloxacin (CFX) as a drug, which is active against *P. aeruginosa* and which is also normally applied to treat infections systemically.

Release curves for these different materials A to E were determined spectrophotometrically (based on the UV absorption of ciprofloxacin) in the supernatant of a phosphate-buffered saline (PBS) release medium; released amounts are given in Fig. 16 in μg cm^{-2}, referring to the macroscopic surface of the sample. Using the CFX-loaded bare mesoporous silica film (corresponding to sample A + CFX in Fig. 16), a fast discharge, a so-called burst release, was observed, with a rather small total amount of CFX released. In order to enhance the amount of the released antibiotic, the mesoporous silica film was modified by establishing negatively charged sulfonate groups on the surface.[45] Ciprofloxacin molecules carry amino

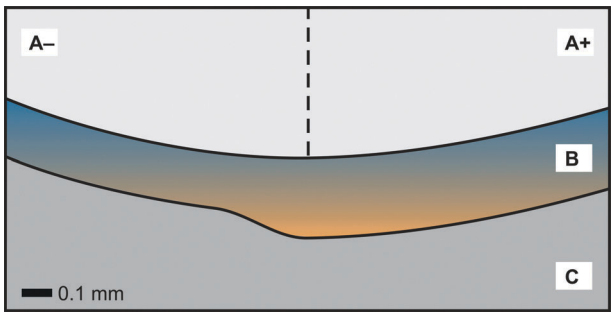

Fig. 14 Scheme of the histological results from animal experiments carried out subcutaneously in rabbits. A–: part of a Bioverit® II implant coated with mesoporous silica; A+: part of a Bioverit® II implant coated with mesoporous silica, modified with an aminosilane and loaded with BMP2; B: cell-rich layer of connecting tissue near to the implant; C: surrounding tissue. The layer of connective tissue is considerably thicker on the side where BMP2 is present, proving the local action of the growth factor.[31,34] Note the shape of the borderline between B and C which is indicative of a diffusive action of the BMP2. Therefore, it can be assumed that BMP2 does not act directly from the implant surface, but becomes detached (possibly also by dissolution of the mesoporous silica coating).

groups which can be protonated, lending a positive charge to them when acidic conditions (pH = 4) are used during the insertion process, thus supporting uptake. With glass slides as a substrate, this modification (sample C in Fig. 15) increased the amount of ciprofloxacin released eightfold. However, in spite of the electrostatic attraction, the samples still show a burst release behavior.[32]

In some cases, a slower and more continuous release of the drug could be preferable. Therefore, we tested different further modifications in order to establish different release rates for the antibiotic. These modifying reactions were performed on sulfonated mesoporous silica coatings already loaded with CFX, so that the chemical processing had to be adapted in such a way that no premature release of the antibiotic occurred. Therefore, we used a fast dip-coating process to establish a polymeric layer of bis(trimethoxysilyl)hexane on top of the drug-loaded silica film (sample D).[46] The use of a bis(trimethoxysilyl)-functionalized alkane also supplied additional silanol groups, which could then be used to add a further barrier effect by hydrophobizing the surface with tetramethyldioctyldisilazane, a reaction which could be carried out *via* the vapour phase.[47] These two treatments were able to considerably slow down the release (Fig. 16, glass substrates, samples D and E). With sample E, equipped with a polymeric barrier derived bis(trimethoxysilyl)hexane and a hydrophobizing layer prepared with tetramethyldioctyldisilazane, a prolonged delivery could be established: after an only small initial burst, a constant release rate was 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 was about 2 μg cm^{-2} of ciprofloxacin. This fact demonstrates that only very small amounts of ciprofloxacin were lost during the additional functionalization steps.

Interestingly, even the native Bioverit® II surface demonstrated a rather high drug release amount of 4.5 μg cm^{-2}, revealing a high drug storage capacity of the plain glass ceramic. Only a slight increase up to 5 μg cm^{-2} was achieved here by the mesoporous coating. As with glass-based samples, the effect of the sulfonic acid modification is pronounced. The total amount released was raised nearly twofold up to 9 μg cm^{-2}.

Again, the novel materials had to be tested for their biocompatibility. Fig. 17 shows relative cell densities determined on cell cultures of NIH3T3 fibroblast cells. Whereas the sulfonate-modified mesoporous silica shows excellent biocompatibility, also when loaded with ciprofloxacin, this property is somewhat compromised in the case of those samples (D and E) which had been additionally coated using silanization reactions to prolong the release period. Here, cell density is reduced to about half the value observed for cell culture plastic.[32] These additional coatings probably reduce cell adhesion and proliferation on their surfaces, without necessarily being toxic.

A functional test of antibiotic-loaded samples can also be carried out *in vitro*, namely in bacterial cultures. For this test a special stem of *P. aeruginosa* was used (PAO1 CTX::lux). Bacteria of this stem exhibit luminescence as long as they are alive. Fig. 18 shows the results obtained from experiments using these bacteria. After 6 h in LB medium (Luria Broth[48]), ciprofloxacin-loaded samples showed only about one-eighth of the bacterial luminescence in comparison to the values obtained with the mesoporous silica only, with the sulfonate-modified mesoporous silica and with a glass control. These results show that the amount of ciprofloxacin of the delivery system is in principle appropriate to locally curb bacterial proliferation.

Driven by these positive results, animal experiments were carried out, again in the middle ear of rabbits.[49] These posed special problems, because the middle ear of a rabbit does not normally show an infection, so that this disease had to be provoked by inoculating a suspension of *P. aeruginosa* bacteria into the middle ear to induce a middle ear infection. The infected ears were then supplied with Bioverit® II prostheses, which carried a sulfonate-modified mesoporous silica coating. For the study group, the implants were loaded with ciprofloxacin (corresponding to sample C as described above); the antibiotic was omitted in the control group. As typical in animal experiments, the size of the two groups, seven animals each, had to be kept small. Nevertheless, meaningful results could be obtained, by monitoring and evaluating various aspects of the clinical status and the behaviour of the animals and by collecting data from them after sacrificing them after seven days.[49]

Already the general behaviour of the animals showed obvious differences. Animals of the control group, where the infection was not treated locally, were severely impaired, showing increased neurological symptoms like head tilting and mechanical head motions as well as elevated body temperature. In contrast, animals of the study group barely showed any disorders of their general condition. When the middle ear was irrigated after the end of the experiment, *P. aeruginosa* bacteria were detected in high concentrations in every middle ear of the control group, but were almost completely eliminated in the study group. The examination of different organs revealed that the infection had spread throughout the body in the control group, whereas a bacterial spread was

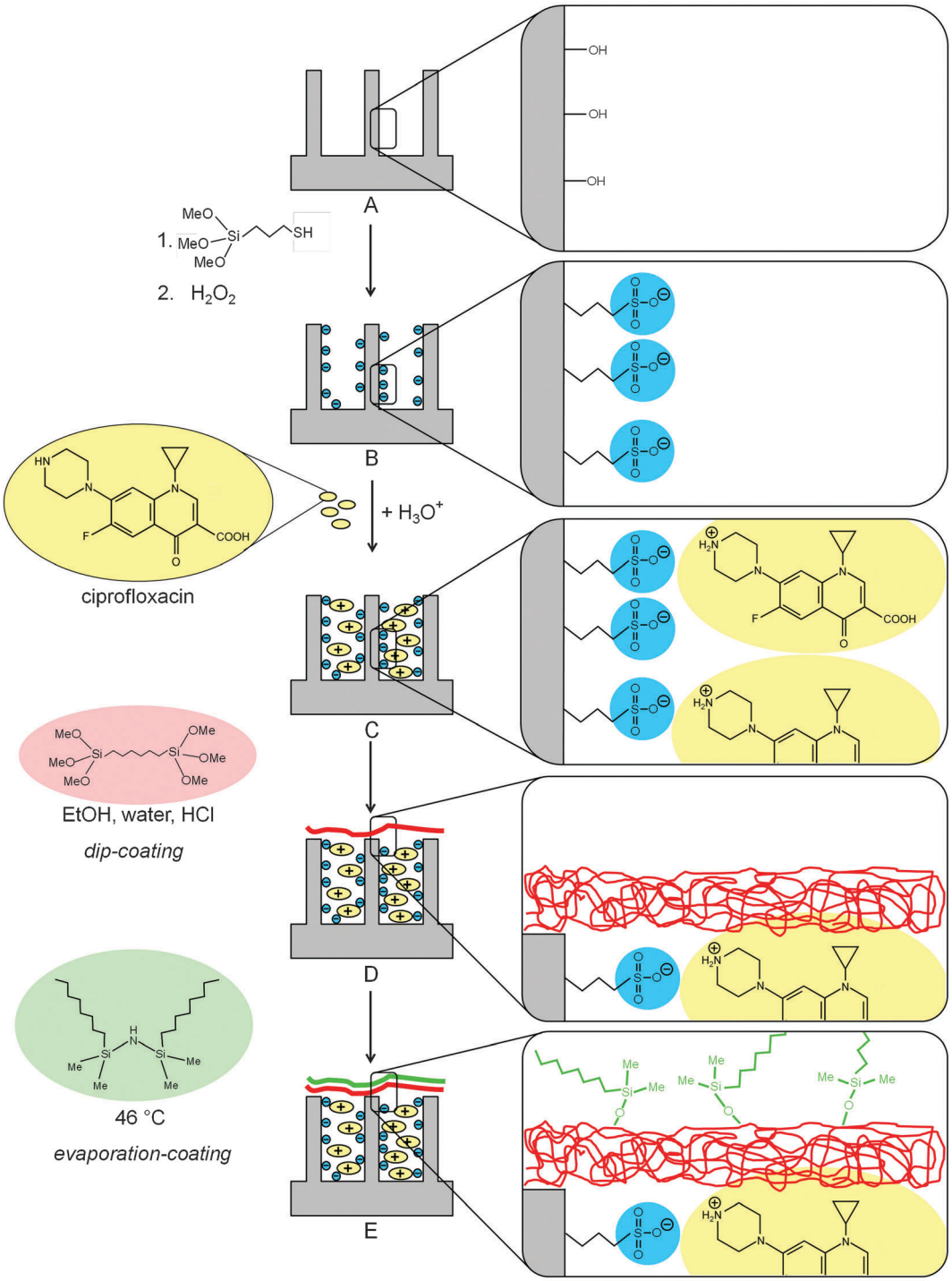

Fig. 15 Scheme showing the construction of the different materials used in this study. Different modifications were carried out to achieve a high loading and a controlled release of ciprofloxacin from mesoporous silica films. Glass substrates were functionalized successively with a mesoporous silica film (sample A), by the introduction of sulfonic acid groups (sample B), by loading with ciprofloxacin (sample C), by the application of a surface layer derived from bis(trimethoxysilyl)hexane by dip-coating (sample D), and by the additional application of a surface layer derived from dioctyltetramethyldisilazane by evaporation-coating (sample E). Adapted with permission from ref. 32.

prevented in the study group. All the middle ears in the control group showed an abscess, whereas only one of the seven animals in the study group showed this symptom (Fig. 19).[49]

This animal experiment clearly proved the efficacy of the implant-supported local drug delivery system which we have developed in the course of our work. It is especially noteworthy that the estimated total released amount of ciprofloxacin is extremely small, namely only *ca.* 1.44 μg (with a calculated surface of the prosthesis of 0.16 cm^{-2} and the total release amount of 9 μg of ciprofloxacin per cm^2 of the macroscopic surface) and

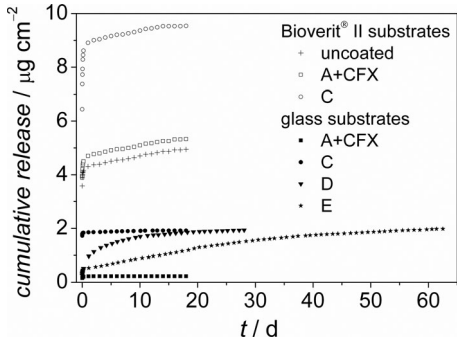

Fig. 16 Ciprofloxacin (CFX) release profiles conducted in 0.01 M PBS of drug delivery systems based on glass substrates (filled marks) and Bioverit® II substrates (unfilled marks), and of native Bioverit® II (cross marks). The drug delivery systems are described in Fig. 15. A + CFX (squares): mesoporous silica coating loaded with CFX; C (circles): sulfonate-modified mesoporous silica coating loaded with CFX; D, triangles: as C, with an additional polymeric layer derived from bis(trimethoxysilyl)hexane; E (stars): as D, with an additional hydrophobizing layer derived from tetramethyldioctyldisilazane. Adapted with permission from ref. 32.

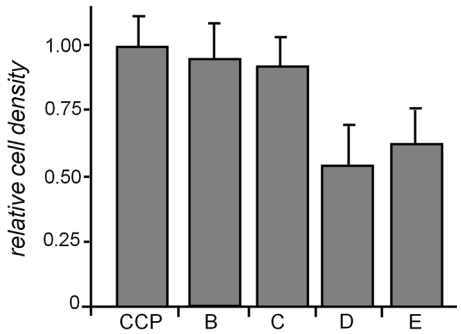

Fig. 17 Results of biocompatibility tests of drug release samples based on glass slides as a support.[32] NIH3T3 fibroblast cells cultured under standard cell culture conditions at 37 °C for 72 h. Cell densities are given in respect to the density of cells which had grown on standard cell culture plastic (CCP); this value was set to 1. B: sulfonate-modified mesoporous silica coating; C: sulfonate-modified mesoporous silica coating loaded with CFX; D: as C, with an additional polymeric layer derived from bis(trimethoxysilyl)hexane; E: as D, with an additional hydrophobizing layer derived from tetramethyldioctyldisilazane. Adapted with permission from ref. 32.

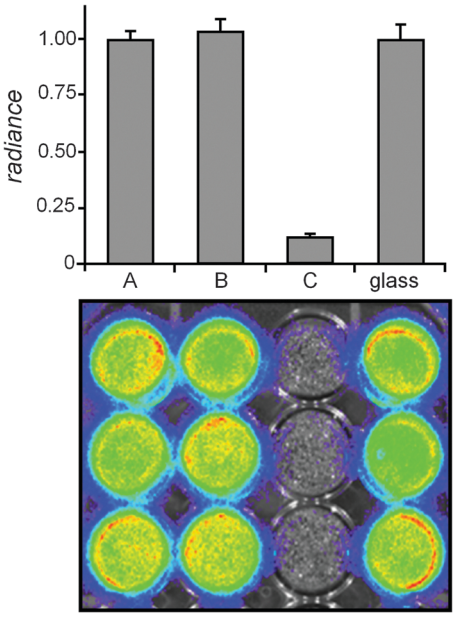

Fig. 18 Antibacterial efficacy of a ciprofloxacin-loaded mesoporous silica film against *P. aeruginosa* bacteria (luminescent PAO1 CTX::lux) after 6 h *in vitro*. A: mesoporous silica film on a glass substrate; B: as A, but with sulfonic acid modification; C: as B, but loaded with ciprofloxacin. A plain glass sample (glass) is used as a reference; radiance is given relative to the value of this sample which was set to 1. Reprinted with permission from ref. 32.

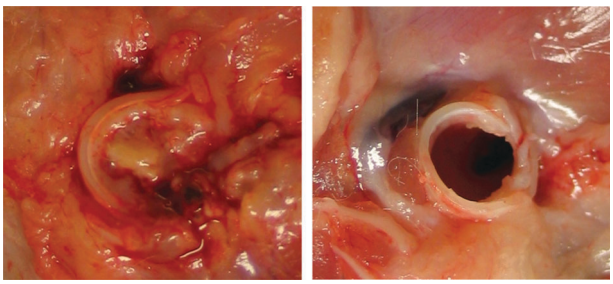

Fig. 19 Results from animal studies in intentionally infected middle ears of rabbits. Left: infected tissues and an abscess as observed in all the middle ears of the animals of the control group, but in only one of the animals in the study group; right: healthy situation as observed in most of the animals of the study group.

would have practically no effect when applied systemically. The local application of such a small amount, however, can effectively curb a bacterial infection in the middle ear. The middle ear is an especially favourable place for the application of such a local drug delivery device, as by its anatomy, it is concluded and there is no through flow of body fluids.

Outlook: mesoporous silica nanoparticles as drug carriers in the middle ear

Apart from bacterial middle ear infections, cholesteatomas are another disease of the middle ear, consisting of a destructive and expanding growth of epithelial cells in the middle ear. Cholesteatomas often have the harmful property to arise again after surgical removal. To prevent this relapse, our next aim is to establish a local drug delivery system for retinoic acid in the middle ear. Retinoic acid is an antiproliferativum (a growth-inhibiting agent) and has been shown to counter the formation of cholesteatoma relapses in animal experiments on guinea pigs.[50] In addition, the nowadays common practice to use collagen pads as interponates to soften the transition between the tympanic membrane and the head part of the prosthesis (Fig. 1b) seems to be in need of improvement in our view. Therefore, we aim to develop a composite pad of silicone and mesoporous silica nanoparticles (Fig. 1d) where the viscoelastic properties of the silicone can be adapted to guarantee optimal sound transmission. The silica nanoparticles shall be loaded with retinoic acid to prevent the relapse of a cholesteatoma.

Conclusions

In this tutorial review we present our scientific results obtained during the construction of functional middle ear prostheses based on mesoporous silica layers. Additionally, we describe step-by-step the path from chemical synthesis and physicochemical characterization *via in vitro* testing in cell cultures to *in vivo* animal experiments. Already at the start, when planning the chemical synthesis, problems with biocompatibility should be kept in mind. An elaborate physico-chemical characterization allows for the interpretation of further results obtained. *In vitro* cell culture experiments provide a first test for biocompatibility. Care must be taken in this step, as there are different types of test conditions and cells which have variable sensitivity to their surrounding (*i.e.* a biomaterials surface). *In vitro* tests may also provide first clues about the efficacy of an implant with a specific function; an example is the testing of antibacterial biomaterials in bacterial cell cultures. Finally, animal studies allow us to evaluate the function of an implant and its interactions with the surrounding living tissue. It has to be noted that this development from chemical synthesis to the application in a living body is only possible through a close interdisciplinary collaboration between chemists and biochemists, biologists and surgeons, requiring cooperativeness and the willingness to learn the specific scientific ''languages'' of the other disciplines.

The use of a mesoporous silica coating on a prosthesis, as described in this review, appears to us as an especially favourable medicinal or pharmaceutical form to use this novel and interesting biomaterial. Our work has shown that sensitive biomolecules like growth factors can be attached to such coatings spatioselectively (*i.e.* only on a certain part of an implant) and that the pore system can be used for a local drug delivery system. The results obtained should be easily transferable to other types of prostheses and implants, as long as these support the preparation of a mesoporous silica film on their surface.

Acknowledgements

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