Chapter 3

Modern Orthopaedic Implant Coatings — Their Pro's, Con's and Evaluation Methods

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1. Introduction

Metal implants in the field of orthopaedic and trauma surgery have been used for a long time to restore joint function, reduce pain or stabilize fractures [1-3]. Both prevention of infection as well as integration of the implant with the host-tissue (mostly bone) are factors of concern. The most frequently used implants for these purposes are made of metallic alloys (for plates and nails for fracture repair and for total joint arthroplasties) and polyethylene (PE) (used in the articulating parts of a prosthesis).

Infection of orthopaedic implants and prostheses is a medical issue that has intrigued people since their very first use over two-and-a-half millennia ago. Since that early beginning, lack of fixation and infection has been the major problem with such medical devices. In many cases, it may result in serious discomfort, limb amputation, illness and in many cases it may have even resulted in death of the patient. Even after 2500 years of medical progression we are still not able to fully conquer these major health risks. Since they are not isolated to the field of orthopaedics and trauma, their multi-factorial character indicates the complexity of the problems concerning healthcare-associated infections (HAI) [4, 5].

The purpose of this chapter is to give an insight in the quest to decrease the percentage of infections and increase the amount of osteointegration by coatings on metallic alloys in the field of orthopaedic and trauma surgery. Finally an overview will be provided of the available methods to examine and evaluate the properties of coatings *in vitro* and *in vivo*.

1.1. Healthcare-associated infections and orthopaedics

Healthcare-associated infections, also called nosocomial infections, are considered to be the biggest healthcare related complication worldwide. HAI annually affects over 600 million patients worldwide with approximately 4.1 million patients in Europe and about 1.7 million patients in the United States [6, 7]. These infections can be related to the cause of death of a considerable number of patients annually. Together with the tremendous economic burden of HAI, HAI is a major point of interest in medical research.

Area	# Patients (million/yr)	Prevalence (%)	Death (%)	Costs (billion)	Neonatal death rate (caused by HAI)
Europe	4.1	7.1	3.7%	(€) 7	< 5%
USA	1.7	4.5	5.8%	($) 6.5	< 5%
Worldwide	> 600	8.5 - 15.5			Up to 75% in South-East Asia and Africa

Table 1. Epidemiological data on HAI [6, 7].

With urinary tract infections as the most frequent implant related HAI in developed countries, orthopaedic implant infections is another major sub-populations within the multifactorial group of HAI (together with infections related to cardiovascular, neurological and gastrointestinal interventions). Infections due to implantation of total hip and total knee prostheses account for about 2% of the HAI, without taking trauma implants into account [7]. Trauma implants or implants for fracture fixation and stabilization, like plates, screws and stabilizing frames, have been described to have an even higher risk for infection, mainly due to the fact that they are used to repair complex injuries and open fractures. Infection together with the eventual loosening of an orthopaedic implant explains the limited lifespan of an orthopaedic device (generally up to 15 years for an artificial joint [5, 8, 9]).

Since the discovery of antibiotics, (implant) infections have been reduced and implant infections have become less lethal and can even be cured. Still, the extensive use of antibiotics has resulted in an increasing amount of resistant bacterial strains, which makes infections caused by those pathogens challenging. Medical device implantation remains troublesome also in the case of orthopaedic implants.

1.2. The race for the surface

After implantation of an orthopaedic device, a competition between bacterial colonization versus tissue integration takes place to conquer the surface of the implant. This phenomenon is often described as "the race for the surface" (Figure 1) [10, 11].

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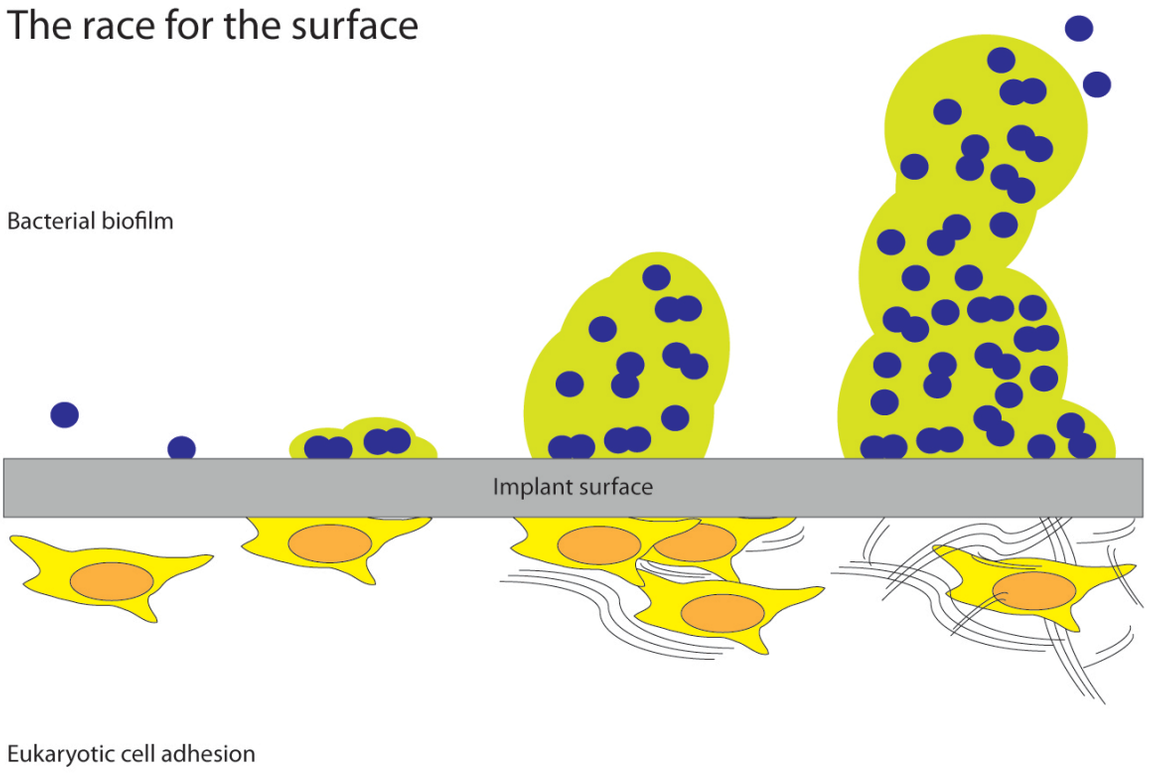

Figure 1. A schematic representation of "the race for the surface", between the bacterial biofilm colonization and eukaryotic cell adhesion with subsequent bone apposition on the implant surface.

The first stage of bacterial biofilm formation is the settling of a planktonic bacterium on the surface of the implant. After adhering to the surface, the bacterium starts to divide and encapsulate itself for protection against the host organism's immune response. This layer of protective matrix, mostly consisting out of polysaccharides, also shields the bacteria from effective antibiotic treatment. The first stage of the biofilm formation is complete and subsequently the present bacteria start to form colonies increasing the internal pressure in the biofilm, which starts to expand. At a certain point the bacterial load within the mature biofilm becomes so high that planktonic bacteria are released from the biofilm. These bacteria can then result in the infection of the surrounding tissue or in the expansion of the biofilm on a different location (Figure 1) [10-12]. Eukaryotic cell adhesion (e.g. adhesion of osteoblasts) on the other hand, can result in implant ingrowth by settling of the osteoblast on the implant surface, followed by cell division and collagen matrix production. Eventual calcification of the collagen matrix allows bone apposition on the implant surface (Figure 1) [10, 12]. In general the inability of the body and its immune system to cope with infected implants is one of the biggest issues when implants are used for medical treatment. Due to infection, local bone resorption takes place, leading to bone loss and implant loosening. As such it is essential to treat the infection, avoiding the risk of tremendous damage to the bone and the bony peri-implant tissue. After removal of an infected implant,

the accompanying bone fractures, soft tissue infection, and inflammation result in fixation issues and an increased infection risk during revision surgery [13].

1.3. Implant coatings

In order to decrease the amount of implant infections and prevent the implants from loosening, coatings can be applied to the surface of the implant. These coatings may vary from (antibiotic) releasing to non-releasing coatings. In general non-releasing coatings (like hydroxyapatite) are applied by thermal-processes, while releasing coatings (like RGD or antibiotic-containing coatings) are mostly applied to the surface by dip or spray coating, due to their limited thermal stability.

Since the principle of the "race for the surface" dictates that early tissue integration may also reduce the infection risk, a coating promoting tissue integration may also be regarded as a passive method to reduce the amount of infections. In order to promote this tissue integration, one of the biggest leaps forward in the improvement of implant fixation and "the race for the surface" in favour of eukaryotic cells might be the use of hydroxyapatite (HA) coatings on the surface of a metallic implant [12, 14-16]. Although in the beginning it was believed that uncemented prostheses, including the HA-coated implants, would have a higher infection percentage compared to implants fixated with an antibiotic-releasing bone-cement, long-term studies showed a comparable infection percentage and a longer survival in favour of the uncemented prosthesis [5, 17]. These HA-based coatings (and their derivatives) are still one of the most frequently used implant coatings in the field of orthopaedic surgery and trauma, resulting in improved implant ingrowth and a longer lifespan of the prosthesis [8]. A combined situation of a coating with both antimicrobial and osteoconductive properties, is yet to be found.

2. Active and passive implant coatings

2.1. Osteoconductive coatings

The definition of osteoconduction means that a material or coating "guides" the bone healing or formation. In case of coatings this means that the bone formation is "guided" to grow or attach to the coating surface (a passive coating) [18]. An orthopaedic implant with such a surface treatment or coating provides an ideal substrate for bone aposition which results in improved implant fixation and a possible prolonged lifespan, with a decreased risk of implant loosening and possibly infection [10, 12].

2.1.1. Apatite coatings

The initial idea of hydroxyapatite (HA) coatings originated from the use of calciumphosphates as a material to stimulate osteogenesis, like tricalciumphosphate (TCP). During the last decades, studies have shown that HA and TCP are suitable for the production of ceramic scaffolds to serve as a bone substitute. Studies on the stability of sintered TCP ($Ca_3(PO_4)_2$) and

HA ($Ca_{10}(PO_4)_6(OH)_2$) have shown that TCP ceramics dissolve over 10 times faster in acidic and alkaline environments compared to HA [19]. Explaining the rationale behind the current use of TCP for resorbable bone scaffolds and the use of HA for implant coatings.

Since the proposed use of HA as a coating, in the late 1980's by Geesink*et al.*[14-16], several implant designs have been used in the clinic, e.g. partially coated or fully coated hip implants. Fully coated implants achieved complete bone remodeling around the implant, with very good fixation properties. The major disadvantage of these fully coated implants was that in case of revision surgery (either for implant infection or component failure) removal of the implant resulted in massive bone trauma due to the fixation of the implant to the bone. By redefining the coating location to the taper only, a good fixation could be achieved with limited problems at the time of a revision surgery [15]. However this design allowed the formation of stress shielding due to the pressure of the stem against the cortical wall. Due to this strain an implant can get loose, resulting in bone loss or a cortical wall fracture. Still HA coatings sintered to an implant surface has proven itself to be the most successful implant coating made, with 20 years of clinical experience [8, 20].

2.1.2. Hydroxyapatite application methods for metallic surfaces

There are several ways to coat a metallic alloy, like titanium or stainless steel, with HA. The techniques to coat such a metallic implant include; dip coating [21], sputter coating [22], pulsed laser deposition [23], hot pressing and hot isostatic pressing [24], electrophoretic deposition [25], electrostatic spraying [26, 27], thermal spraying [28], and sol–gel [29]. Some of these techniques are still experimental, thermal spraying, in particular. Plasma spraying is the most accepted method for the production of HA coatings [30]. Plasma spraying requires high temperatures which may damage the HA crystallinity and create unwanted or amorphous phases, with HA ablation from the coated surface as a possible result [28]. Every technique has its advantages and disadvantages. For example, the thickness, the bonding strength and the properties of the HA-composition may be influenced by the application technique. Techniques such as thermal spraying and sputter coating are used for surfaces or substrates (e.g. porous titanium implants) which are difficult to coat. Techniques such as electrophoretic deposition and sol-gel may coat more complex substrates such as porous alloys, still the production of crack free coatings remains challenging (Table 2).

2.2. Osteoinductive coatings

Although biomimetic HA coatings improve the osteoconductivity of metal implants, they do not influence the osteoinductivity. In general osteoinductive coatings are described as coatings which induce bone formation of undifferentiated cells in the surrounding tissue to ultimately promote osteointegration of bone to the coating (active coatings). In order to promote the differentiation of immature progenitor cells to an osteoblastic lineage, attempts to integrate functional biological agents such as growthfactors into biomimetic coatings have been realized [33, 34]. Several of these coatings have been studied extensively, the most important coatings are described below.

Technique	Advantage	Disadvantage	Thickness	Ref
Sol-Gel	• Coats 3D complex porous substrates • Low processing temperatures • Relatively cheap • Very thin coatings	• Processing in controlled atmosphere	< 1 µm	[29]
Sputter coating	• Uniform coating thickness on flat substrates	• Only coats visible area • Expensive and time consuming • Unable to coat complex 3D porous substrates • Risk for amorphous coating	0.02 - 2 µm	[22]
Pulsed Laser Deposition	• See sputter coating	• See sputter coating	1 - 10 µm	[23, 25]
Electrostatic Spray Deposition	• Uniform coating thickness on flat substrates • Relatively cheap	• Only coats visible area • Fragility	1 - 10 µm	[26, 27, 31]
Electrophoretic Deposition	Uniform coating thickness • High deposition rates • Coatscomplex 3D poroussubstrates	• Cracks in coating • High sintering temperatures	0.1 - 200 µm	[25]
Thermal spraying, Plasma spraying	• High deposition rates	• Only coats visible area Coating decomposition due to high temperature • Rapid cooling may result in amorphous coating	30 – 200 µm	[25, 28]
Dip Coating	• Inexpensive Coatings applied quickly • Coatscomplex 3D poroussubstrates	• High sintering temperatures • Thermal expansion results in amorphous coating • Fragile due to thickness	0.05 - 2 mm	[21, 25]
Hot Pressing, Hot Isostatic Pressing and Sintering	• Dense coatings	• Unable to coat complex 3D porous substrates • Differences in coating elastisity • Expensive • Interaction with or changes due to the encapsulation material	0.1 – 10 mm	[24, 25, 32]

Table 2. Different coating techniques to apply hydroxyapatite (HA) on an implant.

2.2.1. RGD coatings

Extracellular matrix proteins contain a short functional domain of three aminoacids, arginine (R), glycine (G) and asparagine (D), the so-called RGD-domain. This domain plays an impor-

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tant role in cell adhesion, cell proliferation and differentiation. RGD peptides coated to a surface can initiate these processes in their direct vicinity. The major advantage of using peptide coatings instead of protein coatings is that peptides are smaller and more stable compared to proteins. This allows more peptides to be coated to a surface, which results in a more dense coating. Studies have shown that the flanking amino acids in a RGD containing peptide are of great importance for their efficiency. *In vitro* studies show promising results, where RGD enhances (human) cell adhesion, proliferation and differentiation in the osteogenic lineage [35]. An *in vivo* study of an HA/HA-RGD coating with antibiotic release showed that the HA-RGD coating performed at least equally well as the HA-only coating [33]. Still these coatings remain experimental by application.

2.2.2. BMP coatings

Bone morphogenic proteins (BMP's), which belong to the transforming growth factor-β (TGF-β) superfamily, are generally accepted osteoinductive additives for per-operative use to enhance bone remodeling. Due to the lack of a local delivery system, capable of a sustained release, relatively high dosages of BMP's (e.g. BMP-2) are being used in the clinic. The use of BMP's has a locally higher incidence of tumorigenesis as a major disadvantage. Other osteogenic BMP's, such as BMP-4 [36, 37] and BMP-7 [38], are also potent inducers of bone regeneration. Knippenberg et al showed that BMP7, in contrast to BMP2 may be a more chondrogenic growth factor in contrast to BMP2 which was described as a more osteogenic growhtfactor [39]. BMP-2 works in 2 concentration dependent directions, at low dosages it promotes bone remodeling, while at high dosage it promotes bone resorption. Therefore a low-dose releasing BMP-2 coated implant may be the most optimal [40]. While many techniques to incorporate BMP's result in a burst release, they can also be incorporated into the crystal latticework of coatings to establish a gradual release system [34, 40]. As such the incorporated proteins can be released gradually and steadily at a low pharmacological level; not rapidly as in a single high-dose burst [41]. In conclusion, incorporation of osteoinductive coatings may seem attractive but, release rate, potential carcinogenesis, inactivation of the compound (e.g. due to changes in temperature, pH), and bonding to the implant remain of concern.

3. Antimicrobial coatings

Since vascularization of infected tissue is often compromised and a bacterial biofilm is formed, which results in poor penetration of antibiotics, systemic antibiotics are not sufficient to treat a bone infection properly [10-12]. To achieve a local dose high enough to treat the infection, this would involve a systemic overdose of the antibiotic (possibly resulting in kidney and liver failure and damage to the function of the inner ear). The best solution to this problem is to have a local delivery system, this suggests an approach for the treatment of orthopaedic infections. Still in many cases the prosthesis can be rescued by infection treatment *in situ*, without a surgical procedure [13, 42, 43]. The use of local antibiotics by antibiotic-loaded bone cement, either as beads or spacers often placed after implant removal in the remaining infected cavity. The general treatment procedure requires at least two surgical procedures, one to

remove the infected implant and the surrounding affected tissue, combined with the placement of a spacer or antibiotic-loaded beads to fill up to void [44-46]. The second operation is required to remove the spacer or beads after a couple of weeks or months. Once the infection is regarded as treated sufficiently, a new implant or prosthesis is implanted. If the treatment was not successful, new beads can be placed, which will require a third operation for the removal of the beads [44-46]. Due to the high costs and the tremendous burden for the patient a one-step procedure would be preferable. An antimicrobial coating directly on the surface of the newly placed implant, in case of revision surgery after infection, could prevent the infection form re-occurring, but such a coating may also work as a prophylactic in the case of the placement of a primary hip.

3.1. Antibiotic releasing coatings

Already in clinical use in other medical specialties (e.g. in sutures and central venous and urinary tract cathethers), antibiotic releasing coatings remain mainly experimental in the field of orthopaedic and trauma surgery. For orthopaedic applications gentamicin, vancomycin, rifampicin, and tobramycin are the most frequently used local antiobiotics in case of an implant infection. There are several published *in vitro* and *in vivo* studies based on the use of these antibiotic drugs for an orthopaedic implant coating. Poly-L-lactide (PLLA) coatings with rifampicin on a fracture fixation plate, placed on the tibia of rabbits, showed good results on both antimicrobial properties and acceptance of the host-tissue within 28 days after surgery [47]. Also the direct application of minocycline and rifampicin on titanium, placed in the distal femur of a rabbit, lead to good results on prevention of device colonization and infection prevention within a week after surgery [48]. A combined osteoconductive/antimicrobial coating (HA/tobramycin) on titanium, evaluated in the proximal tibia of a rabbit indicated the potential of a combined coating for infection prevention as well as implant incorporation [49]. A recent study on a combined osteoconductive/osteoinductive/antimicrobial coating (HA/RGD/gentamicin) on stainless steel showed promising results on bone integration and antibiotic release characteristics [33]. Furthermore antibiotic releasing coatings on biodegradable substances could replace antibiotic containing PMMA-beads, in this case no implant coating would be necessary. A study on gentamicin coated poly(trimethylene carbonate) (PMTC), a biodegradable polymer, showed good results on antibiotic release, biofilm inhibition and biodegradability, suggesting to be a good substitute for PMMA-beads [50]. A recent report on a prospective study of the first antibiotic releasing tibial nail has shown promising clinical results with no deep surgical wound infections within the first six months after implantation [51]. The major disadvantage for these coatings which they will face in the near future is the increasing number of antibiotic-resistant bacterial strains. This is the main reason why antimicrobial coatings, based on disinfectants or non-traditional antibiotics, are of great interest in the research and development of such coatings.

3.2. Silver-based coatings

Silver is (amongst copper, lead and mercury) a potent antimicrobial heavy metal which has been related to medicine for many centuries. Instead of its metallic state, only the ionic state

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of silver is considered to be antimicrobial and its mode of function is multifactorial. Ionic silver not only reacts easily with amines and microbial DNA to prevent bacterial replication, but also with sulfhydryl groups of metabolic enzymes of the bacterial electron transport chain, resulting in their inactivation [52, 53]. This also forms its treat to large scale clinical applications, since it can also inhibit eukaryotic metabolic function in a patient. Therefore a local release of silver ions is preferable. In contrast to lead and mercury silver does not appear to have cumulative toxic effects in the body, suggesting its potential as a coating component. The use of silver in releasing coatings currently spans from central venous catheters to urinary tract catheters and coated orthopaedic implants, with limited *in vivo* antimicrobial effectiveness as a main problem. While some studies show that a silver coated surface can minimize the infection risks by lowering the bacterial load [54-57], to date, pre-clinical studies and randomized controlled trials of silver coated catheters, implants and external fixation pins were not able to prove its antimicrobial efficacy [52, 58-61].

4. Coating evaluation

Newly developed coatings need evaluation before implementation in the clinic to prevent possible adverse reactions to the coating. This evaluation includes mechanical testing and cytotoxicity and biocompatibility tests. In general these tests can be subdivided in two categories: *in vitro* and *in vivo* testing.

4.1. *In vitro* evaluation

This is defined as all testing modalities performed in controlled laboratory conditions, so outside of a living organism or its natural setting (Table 3).

4.1.1. Cytotoxicity tests

Cytotoxicity tests can be subdivided in cell viability, cell adhesion and cell spreading assays and are usually performed with fibroblastic cell lines (e.g. A529 [62], MC3T3-E1 [62-65], L929 [66], MG-63 [67, 68]). Cell viability assays evaluate the toxicity of a compound present in the vicinity of the cells either in solution or in a solid state. During these tests the material to be tested is incubated in cell culture medium. The resulting pre-conditioned culture medium is then used for cell-culture to evaluate the viability of the cells after exposure to the extracted medium from the material to be tested. Depending on the material, also direct cell culture on the material surface can be performed. The viability of the cells can e.g. be assessed by performing an MTT-assay.

- **The MTT-assay** is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, or another tetrazolium salt) to formazan by the enzyme succinate dehydrogenase in the mitochondria of living cells. The formed purple product can be measured spectrophotometrically and provides a direct measurement of the cell viability based on mitochondrial activity, hence energy metabolism [55, 64-70].

Table 3. *In vitro* analytical methods – part 1

Analytical method	Detection method	Detects	Ref.
Eukaryotic cultures			
Tetrazolium based assay • MTT • XTT • MTS	Spectrophotometric	Cell viability by metabolic activity • Reduction of a tetrazolium salt (yellow) to formazan (purple) by metabolically active cells	[55, 64-70]
FDA based assay • Fluorescein diacetate • DAPI	Fluorescence	Cell adhesion • Fluorescein diacetate→ cytoplasm of healthy cells (green) • DAPI → nuclei of every cell (blue)	[64, 70, 71]
Crystal violet	Spectrophotometric	Cell viability by DNA content • DNA staining, released dye indicates level of cell viability compared to control situation	[62]
SRB • Sulforhodamine B	Spectrophotometric	Cell density based on protein content • protein staining, released dye indicates amount of cells present compared to control situation	[72]
Phalloidinbased assay • Rhodamine • DAPI	Fluorescence	Actin staining • Rhodamine → actin cytoskeleton (red) • DAPI → nuclei (blue)	[64, 68, 69, 73]
Alizarin Red S	Fluorescence	Osteogenic cells → staining of calcium deposition (red)	[62]
ALP • Alkaline phosphatase	Spectrophotometric • Enzymatic assay	ALP activity is a marker for osteogenic potential of a cell • Enzymatic turnover of p-nitrophenyl phosphate to p-nitrophenol	[62, 63, 69, 74]
Live/dead staining • Fluorescein diacetate • Ethidium bromide	Fluorescence	Cell viability • Fluorescein diacetate → cytoplasm of healthy cells (green) • Ethidium bromide → nuclei of death cells (red)	[65, 70]

Analytical method	Detection method	Detects	Ref.
Prokaryotic cultures			
JIS Z 2801	Bacterial growth	Bacterial growth inhibition	[70, 73]
ASTM E-2810	Bacterial growth	Bacterial growth inhibition	[70]
Agar diffusion	Bacterial growth • Colony formation	Zone of inhibition, antibiotic potential of test compound • Distance antibiotic containing object to colony defines potency of antibiotic compound and its release system	[65]
MIC-MBC-assay	Bacterial growth • OD 600 measurements (MIC) • Quantitative bacterial culture (MBC)	Bacterial growth inhibition • Elevation in OD 600 indicates bacterial growth • Colony formation	[54, 55, 65, 69]
Other			
Immunocytochemistry	Light microscopy	Tissue specific staining	[62, 75]
Optical imaging	Fluorescence or bioluminescence • Fluorescence → emission after excitation • Bioluminescence → auto-emission	Presence of light emitting cells (e.g. cell growth or biofilm) • Fluorescence → GFP • Bioluminescence → luciferase	[76]
PCR	Fluorescence • SYBR-green related dyes	DNA/RNA expression	[62, 63]
Western blot	Chemiluminescence	Protein expression	[63]
SEM/TEM • Scanning electron microscopy • Transmission electron microscopy	Electron microscopy • Sputtering with gold or carbon for visualization	Evaluation of bacterial biofilm or extracellular matrix formation • SEM → Cell to surface interactions • TEM → Cell to cell interactions	[62-64, 66, 67, 73, 74]

Table 3. *In vitro* analytical methods – part 2

- **Cell adhesion assays** evaluate the potential of an implant surface to allow cell adhesion by culturing cells directly on the surface of the material to be tested. After allowing the cells to adhere to the surface, non-adhering cells are washed of the implant surface after which the adhering cells are double-stained with fluorescein diacetate (FDA) and ethidium bromide. In this live/death staining, FDA will stain the cytoplasm of intact cells green, while ethidium bromide will stain the DNA of dead cells red. The cell adhesion can be assessed by fluorescence microscopy. The ratio between the FDA-positive and ethidium bromide-positive cells provides insight into the live-dead percentage and thus biocompatibility of the culture surface. If the cells are only incubated with FDA, cell lysis allows quantification by fluorescence spectrophotometry. The level of fluorescent signal is an indication of cell adhesion on the material surface [64, 70, 71].
- **Cell spreading assays** evaluate the potential of a surface to allow cell adhesion and proliferation including matrix formation in the case of e.g. osteocytes. There are multiple ways to assess this surface property. One of the methods described is the use of cell staining directly on the surface after cell culture on the material surface by e.g. crystal violet staining or by an actin staining based on phalloidin. The crystal violet staining is a DNA staining in which cells are fixed on the cultured surface, then incubated with crystal violet to stain the cellular DNA. After washing the stained cells the dye is released by the incubation in a weak acid. The released dye can be measured on a spectrophotometer and providing a quantitative measure for the amount of cells present on the surface. A phalloidin-based staining allows staining of the actin cytoskeleton and cellular organization on the surface of a material. This is a direct cell staining which is visualized by fluorescence microscopy. In most cases the phalloidin based stainings are counterstained with DAPI to stain the cells nuclei, which allows visualization of the individual cells and their cytoskeleton [62, 64, 68, 69, 73].
- **Assays to assess the osteogenic potential**, quantify the osteogenic potential of a coating. This can be determined by using cultured cells on the coating surface for an alkaline phosphatase assay (ALP). The ALP assay determines the ALP activity within the tissue, which is related to osteogenesis and bone deposition on the coating surface. Another method to assess the osteogenic potential of a coated surface is an alizarin red s staining, which stains calcified tissue [62, 63, 69, 74].

4.1.2. Antimicrobial coating tests

In the case of antimicrobial coatings the effect of the coating on bacteria can be assessed with a wide variety of assays, with the most well-known being the agar diffusion test where the release of an antimicrobial compound into the agar leads to an inhibition zone around the releasing material.

- **Bacterial viability** can be assessed by a minimal inhibitory concentration (MIC)/ minimal bactericidal concentration (MBC) assay. In this assay the releasing material is allowed to release its effective compound into a buffer or culture medium over a certain time span. The acquired pre-conditioned buffer/medium is then used in a bacterial culture setting in which

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a standardized amount of bacteria is exposed to the preconditioned buffer/medium. After 24 hours of incubation the optical density can be measured at 600 nm, the lowest concentration which shows no increased optical density compared to the uncultured control condition defines the MIC, while the lowest concentration which shows no bacterial growth after incubation of the MIC-cultures on agar plates for another 24 hours defines the MBC [55].

- **In vitro biofilm formation** on a surface can be confirmed by incubating the surface in a bacterial suspension, rinsing the surface with an isotonic buffer (PBS) and use sonication to release the bacteria from the surface for quantitative culture. Or fix the bacteria on the surface with 2.5% glutaraldehyde/PBS for evaluation with SEM. This method can easily be transferred to the *in vivo* situation.

- **International standards** provide guidelines of how to assess coating stability and function, e.g. ISO 10993-5 provides guidelines for *in vitro* medical device evaluation. The Japanese Industrial Standard Z 2801 (JIS) describes a test for contact killing by the incubation of bacteria on a potential antimicrobial surface. Culturing of this surface provides insight on the antimicrobial properties of the evaluated coating [70, 73]. The American Standard E-2810 (American Society for Testing and Materials, ASTM)) describes a test for contact killing by the application of a bacteria loaded agar onto the coated surface, after incubation the number of viable bacteria is determined [70].

4.2. *In vivo* evaluation

This is defined as all testing modalities performed in a controlled group of living organisms, often including clinically relevant parameters and a broad range of imaging techniques (Table 4).

The first models concerning orthopaedic conditions date back to the late 19th century, primarily focusing on osteomyelitis [77]. Rodet described 2 basic methods to establish an osteomyelitis in a rabbit, the first one by inflicting a fracture and subsequent intravenous injection of the bacterium, resulting in an osteomyelitic leasion in the area of the fracture. The second method was performed by merely injecting bacteria intravenously, which resulted in a systemic infection with periosteal leasions leading to local osteomyelitis [77].

The most well-known model for osteomyelitis is the model by Norden *et al.*; this model describes the direct percutaneous injection, directly into the tibial intramedullary cavity of a rabbit, of both a scleroting agent (sodium morrhuate, bile salts from codfish) and *S. aureus* [78]. Andriole *et al.* however, established one of the first osteomyelitis models with a foreign object. Their model was based on a tibial fracture and subsequent tibial stabilization by a stainless steel pin, contaminated with *S. aureus* [79]. Together with the model by Norden, the model of Andriole mainly form the basis for current animal models used for the evaluation of implant coatings. In general, rabbits are still the most frequently used animal species for these experimental studies, but there have also been successful models in mice, rats, dogs and sheep. During the years, models increased in complexity and included multifactorial parameters.

The bone bonding properties of apatite-coated implants were first evaluated in dogs by Geesink *et al*. [16]. After the development of these apatite-coated implants, Vogely *et al.* described a rabbit proximal tibial model for the evaluation of hydroxyapatite coated titanium implants in an implant site infection [80]. Darouiche et al. were one of the first who described a rabbit lateral femoral condyle model for the evalauation of antimicrobial coatings on titanium [48]. Poultsides *et al*. described a haematogeneous implant contamination model by MRSA [81]. Moojen et al. evaluated a combined coating with both osteoconductive (periapatite) and antimicrobial (tobramycin) properties in a proximal tibial implant infection model in rabbits [49]. Also, Moskowitz *et al*. developed antibiotic multilayer implant coatings with an antibiotic release of over 4 weeks in a 2 stage rabbit distal femoral condyle infection model. The first surgical stage contained the initial infection with the insertion of a pre-colonized peg, the second surgical stage was the removal of the peg and implantation of the antibiotic coated implant [65]. Alt *et al*. was one of the first to describe a coating which combined osteoconductive (hydroxyapatite), osteoinductive (RGD) and antibiotic (gentamicin) properties in an experimental rabbit implant infection model [33].

4.2.1. Clinical parameters

Body weight and temperature provide general information about the animal's physical condition, with weight loss and fever in case of an infection. Leucocyte differentiation provides a detailed overview of the percentages of lymphocytes, neutrophillic granulocytes, monocytes, basophilic granulocytes and eosinophilic granulocytes in the total leucocyte population. An elevated number of leucocytes or a shift in differentiation indicates a bacterial infection. The ESR is based on the fibrinogen balance in the blood. In case of an inflammation or infection the fibrinogen levels increase, resulting in agglutination of erythrocytes with sedimentation as a result. CRP is an acute phase protein whose levels rapidly increase in case of inflammation or infection. ESR and CRP both only indicate the presence of inflammation or infection, never the cause or the location [82].

4.2.2. Imaging modalities

- **Optical imaging (based on fluorescence and bioluminescence)** is based on the detection of light emitted from the body. The use of fluorescently labeled antibodies results in a very specific signal, although resulting in a very local detection, it also requires a large amount of antibodies when used in humans. This renders large scale use in humans not yet profitable [76]. Also bioluminescence can be used to visualize infection. The main disadvantage of bioluminescence is the requirement of the luciferase gene in the cell to be detected, meaning the use of genetically modified organisms in case of detection by either autologous cells or bacteria. E.g. a bacterium expressing luciferase can be used to monitor an implant infection initiated with that bacterium [76]. Both methods are currently available in laboratory animal setting.
- **X-ray** is by far the oldest imaging technique and the most frequently used imaging technique to assess fractures, implant fixation, location and loosening, but also for the differential diagnosis of bone diseases like osteomyelitis. The use of X-rays is cost effective, they are

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easy to obtain and have a relatively low burden for the patient. An X-ray only provides detailed information about the mineralized tissue (or the lack thereof) and the disease related changes accompanied with it [15, 83, 84].

- **CT (computed tomography)** is a 3D-imaging technique which uses X-rays to construct a 3D image of the mineralized tissue in a patient. It generally provides more in-depth data about the density of the mineralized tissue and bone remodeling compared to X-rays, however imaging of metallic implants can result in scattering of the X-rays resulting in a blur around the implant, rendering data-analysis difficult [76, 85, 86].
- **DEXA (dual energy X-ray absorptiometry)** is often incorrectly stated as a bone scan. The use of 2 different energy levels of the X-ray beam allows accurate determination of the bone mineral density. DEXA is the most common imaging technique to diagnose osteoporosis and is seldomly used in *in vivo* coating assessment studies [86].
- **MRI (magnetic resonance imaging)** does, in contrast to other imaging techniques, not rely on ionizing radiation but on the magnetic spin of protons. Due to the high water content (and thus protons) of soft tissue, MRI is one of the main imaging techniques to assess the musculoskeletal tissues, like cartilage and tendons. MRI only allows indirect imaging of bony structures due to the limited water content of the bone. The main drawbacks for MRI are the duration of the imaging acquisition and the inability for it to be used in combination with metallic implants [76, 84].
- **PET (positron emission tomography)** is based on the detection of the annihilation event of a positron with an electron (beta-decay). Every annihilation-event results in 2 gamma-photons in an opposite direction from the point of decay. The detections of the photons on the detectorring of the scanner results in a 3D image [97]. ^{18}F is one of the most frequently used isotopes (connected to a carrier molecule) to serve as a PET-tracer in orthopaedic research. ^{18}F-fluorodeoxyglucose (FDG) is used for the detection of infection and inflammation (figure 2) and ^{18}F-fluoride as a tracer for bone remodeling [85, 94, 98]. With signal specificity as its advantage, PET does not provide anatomical information, merely the location of the tracer uptake. This is the main reason why PET and CT are often combined in the clinic.

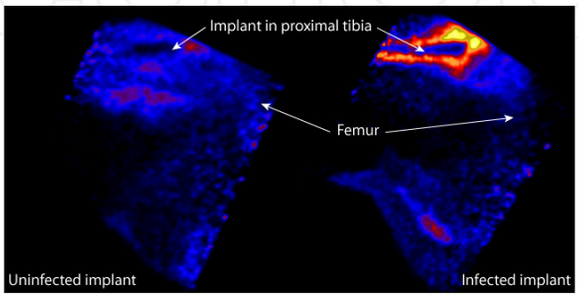

Figure 2. FDG PET of an uninfected implant versus an infected implant in the proximal part of a rabbit tibia, six weeks after surgery. The increased tracer uptake around the infected implant (black area) depicts the local osteomyeliticlea-sion.

Table 4. *In vivo* analytical methods – part 1

Analytical method	Detection method	Detects	Ref.
Clinical parameters			
Body weight	Weighing scale	General physical condition • Weight loss after surgical intervention, returns to pre-operative values within first weeks after intervention. • Persistent weight loss indicates animal discomfort, infection or another systemic event related to device or intervention.	[49, 78, 79, 81, 87]
Temperature	Thermal probe	General physical condition • Post-operative thermal elevation, returns to normal within days after surgery. • Persistent elevation indicates infection or another systemic event related to device or intervention.	[49, 81]
ESR • Erythrocyte sedimentation rate	Anticoagulated blood • Capillary tube	Infection by increase of erythrocyte sedimentation • Elevation in first post-operative week due to surgical intervention. • Remains elevated in case of infection.	[49, 78, 80, 81, 88]
CRP • C-reactive protein	Serum/plasma • ELISA	Infection by increase of CRP levels • Elevation in first post-operative week due to surgical intervention. • Remains elevated in case of infection.	[84]
Leucocyte count and Leucocyte differentiation	Anticoagulated blood • Cell count	Infection by shift in leucocyte distribution • ↑ Neutrophils and monocytes → bacterial infection • ↑ Lymphocytes → viral infection or tumor • ↑ Basophils and eosinophils → inflammatory processes and/or allergic reactions	[49, 65, 78, 80, 81, 84, 88]

Table 4. *In vivo* analytical methods – part 2

Analytical method	Detection method	Detects	Ref.
Imaging modalities			
Optical imaging	Fluorescence or bioluminescence • Fluorescence → emission after excitation • Bioluminescence → auto-emission	Presence of light emitting cells • Fluorescence → GFP • Bioluminescence → luciferase	[76]
X-ray	Electromagnetic radiation	Bone, bone pathology and metal objects	[33, 49, 76, 78-81, 84, 87-94]
CT • Computed tomography	Electromagnetic radiation • X-ray (3D)	Bone, bone pathology and metal objects	[76, 84, 86, 88, 91, 94]
DEXA • Dual energy X-ray absorptiometry	Electromagnetic radiation • Dual energy X-ray of same object	Bone density • Difference in signal between both X-rays allows calculation of bone density	[86]
MRI • Magnetic resonance imaging	Nuclear magnetic resonance • Proton magnetic spin resonance (3D)	Soft tissue • Detection of tissues with a high water content	[76, 84, 88]
Bone scintigraphy	γ-radiation • Single photon emission (2D)	Active bone remodeling • ^{99m}Tc –MDP → increased osteogenesis • ^{67}Ga-citrate → leucocyte activation (e.g. infection)	[84, 88, 95]
SPECT • Single photon emission computed tomography	γ-radiation • Single photon emission (3D)	Active bone remodeling • ^{99m}Tc –MDP → increased osteogenesis • ^{67}Ga-citrate → leucocyte activation (e.g. infection)	[76, 88]
PET • Positron emission tomography	γ-radiation • Positron mediated dual photon emission (3D)	Active bone remodeling and infection • ^{18}F-Fluorodeoxyglucose → inflammation and infection • ^{18}F-Sodiumfluoride → active bone remodeling	[76, 84, 88, 91-94]

Table 4. *In vivo* analytical methods – part 3

Analytical method	Detection method	Detects	Ref.
Other – *Ex vivo*			
Calcium binding fluorophores • Calcein blue • Calcein green • Tetracycline • Xylenol orange • Alizarin red S	Fluorescence (excitation/emission) • Calcein blue (370 / 435 nm) • Calcein green (470 / 530 nm) • Tetracycline (390 / 570 nm) • Xylenol orange (470 / 610 nm) • Alizarin red S (550 / 620 nm)	Calcium deposition during bone remodeling (color) • Calcein blue → blue • Calcein green → green • Tetracycline → yellow • Xylenol orange → orange/red • Alizarin red S → red	[96]
Histology • Paraffin • PMMA	Light microscopy	Tissue specific staining (including bacteria) • Haematoxylin/eosin staining (general) • Masson Goldnertrichrome staining (general) • Gram staining (bacteria) • Wear particles (e.g. polyethylene by polarized light) • Immunostaining (antibody specific)	[33, 49, 75, 79-81, 84, 87, 90-94]
SEM • Scanning electron microscopy	Electron microscopy	Surface treatment, bacterial biofilm, wear particles • Surface assessment of non-metallic substrates by gold or carbon sputtering • Metallic substrates can be assessed directly	[65, 80]
Bacterial/bone culture • Tissue swaps • Bone homogenates	(Selective) culture media • Tryptic soy agar/broth • Tellurite glycine agar (selective for coagulase negative Staphylococci)	Bacterial growth under specific circumstances • Quantification by colony count or OD 600 measurements	[48, 49, 65, 78-81, 84, 87, 89-94]

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- **A bone scan** (bone scintigraphy) is based on the direct detection of gamma radiation originating from the injected tracer molecule (often ^{99m}Tc, ^{67}Ga or ^{111}In) connected to a specific ligand which allows tissue specific binding and thus imaging. A bone scan provides two-dimensional images of the patient, which are sufficient in the clinic for the diagnosis [84].
- **SPECT** (single photon emission computed tomography) on the other hand allows acquisition of three-dimensional images, providing more insight in size and localization of certain pathology. In general, bone scan/SPECT-tracers have a longer half-life than PET tracers making them more cost-effective to produce. Just like PET, SPECT provides limited anatomical information and is therefore often combined with CT in the clinic [76].

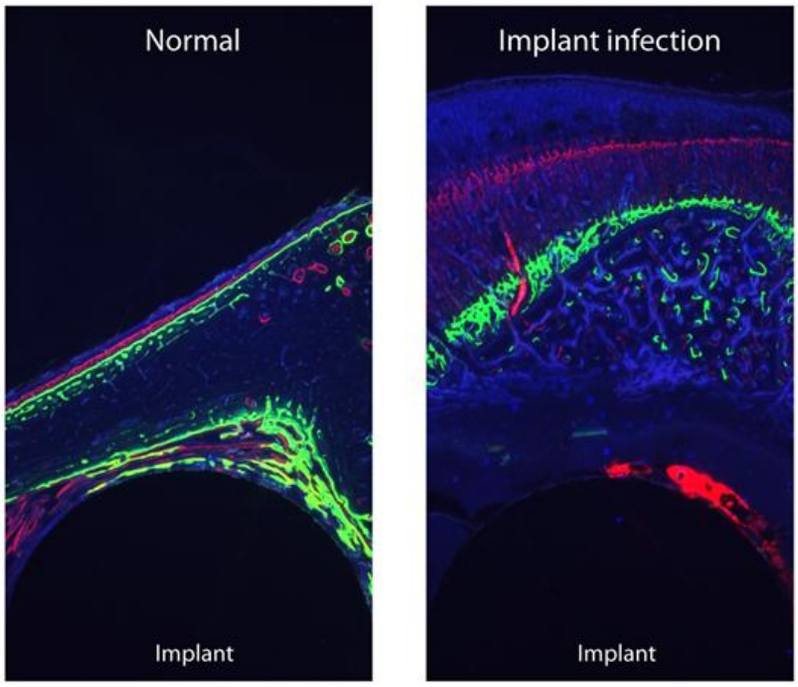

Figure 3. The use of calcium binding fluorophores, depicted in 50 micron PMMA sections, of a rabbit tibial intramedullary nail model, to address normal bone remodeling and bone remodeling in case of an implant infection. Calcein green was injected at 2 weeks, xylenol orange at 4 weeks and calcein blue at 6 weeks. In the case of normal bone remodeling, calcium deposition is detected around the implant, combined with bone remodeling of the cortical wall. In case of an implant infection the most calcium deposition is located in the outer cortical wall depicting the periosteal elevation and calcification during the 6 week follow-up.

4.2.3. Ex vivo analysis

- **Calcium binding fluorophores** (like calcein green, blue and xylenol orange) are being used for the *in vivo* labeling of the calcium deposition at the time of injection. The use of different fluorophores, emitting at different wavelengths, allow post-mortem visualization of the calcium deposition during the experimental follow-up [96]. This provides the opportunity to determine implant ingrowth and bone remodeling in a normal healthy situation and periosteal elevation and calcification during the progression of an osteomyelitis (Figure 3).

- **Histology** is a commonly used method to assess the tissue in the implant area on e.g. tissue morphology or bacterial presence. Tissue sections with metallic implants generally require embedding in methylmethacrylate, instead of paraffin, with the inability to allow immunostainings as a major drawback. Still it provides the unique opportunity to assess the tissue-implant interface [33, 49, 57, 80].
- **Electronmicroscopy** allows analysis of the implant surface (with or without coating) after distraction from the surrounding tissue. This can include analysis of the bone matrix-implant interface but also analysis of a formed biofilm [31].

5. Conclusion

Both osteointegration and infections are of concern in implants and prosthesis used in the field of orthopaedic and trauma surgery. Metallic alloys used for plating and nailing of fractures and joint replacements are the largest group of these implants. Hydroxyapatite coatings have proven to be successful to promote osseous integration of uncemented total hip prosthesis. During the last years the focus on coating development has shifted from osteoconductive coatings (like hydroxyapatite) towards osteoinductive coatings to support bone remodeling (like RGD and BMP coatings) and antimicrobial coatings for implant infection treatment and prophylaxis (like silver or antibiotic releasing coatings).

Plasma spraying is the most used and accepted method for hydroxyapatite coatings. Other coating techniques which do not require high temperatures are necessary for the application of bioactive coatings that promote osteogenesis and/or prevent infections.

With the current palette of *in vitro* (e.g. MTT, ALP and SEM), *in vivo* (e.g. ESR, CT and PET) and *ex vivo* techniques (e.g. bacterial culture, calcium binding fluorophores and histology), we can thoroughly evaluate novel implant coatings in a qualitative and quantitative fashion. The strength of such an evaluation will always lie in the combination of the individual methods, leading to a complete, broad-spectrum analysis on coating toxicity and efficacy.

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