PROJECT FINAL REPORT

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1. Final publishable summary report

1.1. Executive summary

Certain microorganisms are capable of growing in layers, forming biofilms on medical surfaces, like implants, heart valves, etc... Biofilm-associated infections on implants are responsible for 15-25 % of implant failures as biofilms are resistant to most of the conventional anti-microbial agents. To improve integration of bone implants novel porous materials have been used as scaffolds that ironically serve as an ideal niche for microbial growth.

So far, implant coatings with biocidal properties have been generated, which release silver ions or conventional antibiotics to inhibit biofilm formation. The EU-funded project COATIM (**http://www.coatim.eu**, Development of antibiofilm coatings for implants) initiative aims to develop the next generation of implant coatings containing novel potent proprietary anti-biofilm molecules (ABMs) without releasing them.

During COATIM, five molecules showing the most promise in inhibiting bacterial and fungal growth along with two standard anti-microbial compounds (vancomycin and caspofungin) were selected by the consortium members. These selected molecules were grafted on small titanium implant substrates and their anti-microbial activity was evaluated *in vitro* and *in vivo*, alongside their *in vitro* osseointegration capacity. Researchers also evaluated their remaining activity following sterilisation procedures. Finally, the molecule-coatings were applied on complex orthopaedic and dental implants and osseointegration was assessed *in vivo* using rat and rabbit models, respectively.

The need to resolve biofilm-associated infections is urgent to reduce the necessity for revision surgery. Besides higher medical costs, such infections lead to significant pain and distress in patients. Study outcomes are expected to help avoid or reduce this unnecessary suffering.

1.2. Summary description of project context and objectives

Project context

Biofilm-associated implant infections result in costly and burdensome implant revision. Up to a quarter of **orthopaedic implants** are subject to revision surgery due to infection. The overall implant-associated infection rates in for example Canada and the USA are 5% for fracture-fixation devices, 2% for primary joint replacements and at least 14% of the total hip and knee implants. Because of the increasing use of implants, compounded by an ageing population, the burden of implant failure and subsequent surgical revision is expected to increase by more than 100% over the next 25 years. The cost and resource utilization for such revision procedures are substantially higher than for the primary procedures, and are estimated to be at least $50,000 per patient in the USA and €15,000 per patient in Europe. The implant surfaces can serve as a site of attachment for various pathogens, either acquired at the time of surgery or at a later stage (via a haematogenous route), thereby nucleating infections and resulting in the formation of complex biofilms. Additionally, also in patients receiving **oral implants**, complications and failures occur that are mainly related to biofilm-associated infections of the peri-implant tissues.

Biofilms consist of dense layers of microorganisms that are surrounded by a self-produced extracellular polymer matrix, and are resistant to most of the currently used antibiotics/antimycotics. This is largely due to poor penetration of antimicrobial agents into the biofilm and the lack of active growth of the microorganisms within the biofilm. Antibiotics/antimycotics that do display activity against microbial biofilms often result in only partial killing of the biofilm cells, leaving a subpopulation of the biofilm cells alive - so called “persisters”. Persisters are tolerant cells that survive treatments with high concentrations of antimicrobial agents. They are considered as one of the most important causes of the recurrence of biofilm-associated infections.

The presence of biofilms and the limited vascularization of the bone/implant interface, make for example prosthetic joint infections extremely difficult to treat. Treatment options include two-stage radical debridement with implant removal, antibiotic therapy, and delayed reimplantation. Therefore, biofilm formation should be avoided by prophylaxis including **(i)** conducting the surgery as aseptically as possible; **(ii)** adapting the implant material in such a way that the pathogens cannot readily attach and **(iii)** by impregnating or coating the implant with antimicrobials or antibiofilm molecules. **COATIM focuses on the development and application of novel antibiofilm implant coatings, thereby aiming at reducing the need for implant revisions due to infections. This problem is well recognized by the implant manufacturers and a major concern for the future.**

Reducing biofilm-associated implant infections via antibiofilm implant coatings. The new generation of cementless implants contain biocompatible and bioactive porous coatings that, on one hand, enable fast peri-implant bone growth of the implant (osseointegration), but on the other hand, result in an increasing risk of microbial contamination due to the high porosity of the surface coating on the implant. Hence, the application of antibiofilm coatings is of strong interest for this type of implant. The market share of open porous titanium (Ti) layer coated implants is expected to increase further in conjunction with progress in antibiofilm-coatings. To reduce biofilm-associated infections on implants, biocidal coatings can be applied based on **(i)** the use of metal ions like silver, which is toxic when accumulated, or **(ii)** the release of standard antibiotics/antimycotics to prevent infection at the site of the implant. A serious concern regarding such continuous antibiotic pressure however is an increased incidence of **clinical drug resistance**, such as observed for the methicillin-resistant *Staphylococcus aureus* (MRSA). In this respect, the development of antibiotic-resistant infections can lead to devastating effects in the absence of any valid medical treatment to control the infection, and has become a serious public health problem. Another important challenge of biocidal implant coatings is to achieve antimicrobial activity without impairing osseointegration caused by general cytotoxicity.

In COATIM, we developed **antibiofilm coatings** on porous titanium layer coated implant material **using <u>novel small molecules and peptides</u> with inhibitory activity against microbial biofilms, that are owned by specific consortium partners including SMEs.** We focused on implant coatings that **<u>do not release the antibiofilm</u>**

compounds, as this type of coating represents longer-lasting antibiofilm activity and garantuees topical activity. An important part of the COATIM project was to assess the effects of the antibiofilm molecules on various aspects of **osseointegration** using ***in vitro* and *in vivo* techniques**.

Objectives

The COATIM project aimed at **the development and application of the next generation of antibiofilm coatings for dental and orthopaedic implants**, thereby reducing the need for implant revisions due to infections. In a combinatorial approach, coatings containing novel proprietary small molecule and peptide antibiofilm molecules were developed for implants. The coated implants were evaluated for *in vitro* and *in vivo* efficacy and for fixation by osseointegration. The proprietary antibiofilm molecules were identified in drug screening programs conducted by COATIM partners. These molecules were selected for **(i)** inhibitory activity against bacterial and/or fungal biofilms (either inhibiting their formation or eradicating biofilms including persisters), or for sensitization of biofilm cells to currently used antibiotics/antimycotics; and **(ii)** lack of cellular toxicity on non-osteogenic cell lines.

The consortium focused on the following objectives (O):

O1. Identification of the five best antibiofilm molecules (ABMs) out of 100 ABMs belonging to 35 distinct structural small molecule or peptide classes, based on five **criteria**: **(i)** *in vitro* potency and activity to prevent biofilm formation of several species (axenic or polymicrobial biofilms); **(ii)** non-toxicity for model organisms*;* **(iii)** non-toxicity for osteoblasts; **(iv)** remaining activity upon gamma-irradiation; and **(v)** the presence of specific chemical groups to enable covalent binding on functionalized titanium substrates.

O2. Elucidation of the antibiofilm mode of action of the five best ABMs, based on a combinatorial genetic and biochemical approach.

O3. Development of antibiofilm coatings (Ac) on smooth titanium (SmTi) and osseointegrative porous titanium (OPTi) substrates. Based on the compound characteristics, a **tailored ABM-specific coating** was developed on various Ti substrates. Small ABM molecules were covalently bound to the substrates, whereas larger peptide molecules were grafted on the substrate surface by means of electrophoretic deposition (EPD).

O4. *In vitro* analysis of the AcSmTi/AcOPTi substrates. The development of AcSmTi substrates is an intermediate stage to develop more advanced AcOPTi substrates. Both AcSmTi and AcOPTi substrates were characterized regarding their **(i)** *in vitro* biofilm inhibitory activity; **(ii)** *in vitro* safety using ISO 10993-5 cytotoxicity assays; and **(iii)** *in vitro* analysis of their osseointegration potential. Next, the best SmOPTii and AcOPTi substrates were selected for subsequent *in vivo* osseointegration testing.

O5. Analysis of the *in vivo* efficacy and osseointegration of the AcSmTi/AcOPTi substrates. *In vivo* efficacy testing of AcSmTi/AcOPTi substrates was assessed in a biofilm mouse colonization model, developed specifically in the project. For assessing osseointegration of AcOPTi cylindrical components and SmOPTi dental implants, a rat and rabbit model were used, respectively.

O6. Antibiofilm coated complex implants and upscaling feasibility study. The developed and assessed ABM coating technologies were applied on commercially available complex shaped dental and orthopaedic implants and the exploitation of the results of this research project towards industrial implementation was prepared through a planning and cost calculation excercise for an industrial process.

O7. Dissemination and exploitation of the knowledge and ABM-tailored coatings generated by the COATIM project was described in a plan for the use and dissemination of knowledge (PUDK).

1.3. Description of the main S&T results/foregrounds

In this section, the main results obtained during the COATIM project are summarized. To give a general overview of the COATIM project, a schematic overview of the COATIM workflow is presented in the figure below (Figure 1). In addition, 7 key publications of COATIM (published or final draft for submission) and the corresponding work packages are shown in Table 1. Throughout the text, we have referred to these key-publications (in bold). The total number of publications based on the COATIM project is 21, see further TABLE 3.1.1: LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS.

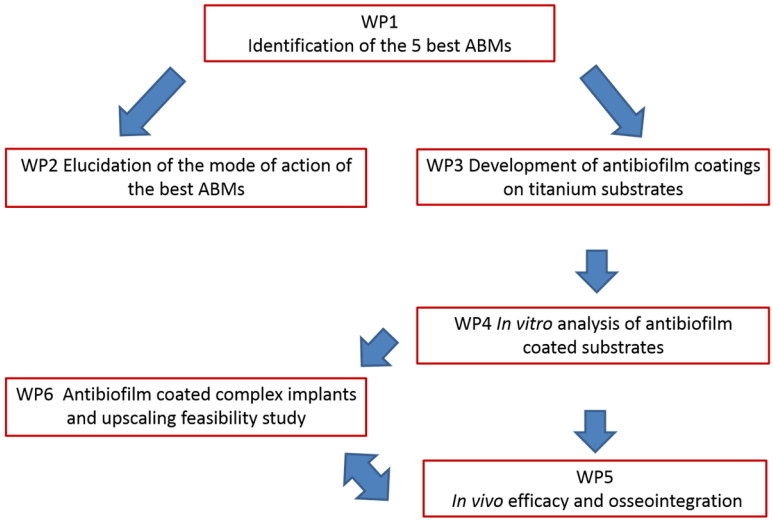


Figure 1. Overview of the COATIM workflow

Table 1. Key publications of COATIM

Key publications		
Title	Reference	Corresponding work package(s)
Derivatives of the Mouse Cathelicidin-Related Antimicrobial Peptide (CRAMP) Inhibit Fungal and Bacterial Biofilm Formation.	De Brucker *et al.*, AAC, 2014 doi: 10.1128/AAC.03045-14	1
Identification and characterization of an anti-pseudomonal dichlorocarbazol derivative displaying anti-biofilm activity.	Liebens *et al.*, BMCL, 2014 No doi available	1
Evaluation of the toxicity of 5-aryl-2-aminoimidazole-based biofilm inhibitors against eukaryotic cell lines, bone cells and the nematode Caenorhabditis elegans.	Steenackers *et al.*, Molecules, 2014 doi:10.3390/molecules191016707.	1
Covalent immobilization of antimicrobial agents on titanium prevents *Staphylococcus aureus* and *Candida albicans* colonization and biofilm formation	Kucharíková, Gerits *et al.*, JAC, 2016 doi: 10.1093/jac/dkv437	3, 4, 5, 6
Modulation of the substitution pattern of 5-aryl-2-aminoimidazoles allows to fine-tune their anti-biofilm activity spectrum and toxicity	Peeters *et al.*, AAC, submitted	1

Antibiofilm coatings for metallic implants by alternating current electrophoretic deposition	Braem *et al.*, in preparation	3
Elucidation of the mode of action of a new antibacterial compound active against *Staphylococcus aureus* and *Pseudomonas aeruginosa*	Gerits *et al.*, Plos One, major revision	2
Antibacterial activity of a new broad-spectrum antibiotic covalently bound to titanium surfaces	Gerits *et al.*, J Orthop Res, submitted	4

1.3.1. Description of the main S & T results

WP1 Identification of the five best antibiofilm molecules (ABMs)

Activity and toxicity testing of 100 ABMs

During this work package, five ABMS were selected out of 100 previously selected ABMs, in two steps and based on several characteristics. An overview of this selection procedure is shown in Figure 2.

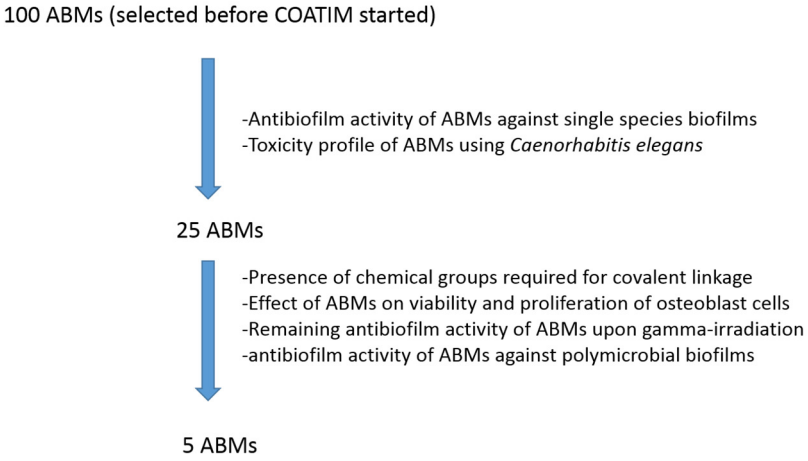


Figure 2. Selection of the 5 best ABMs out of 100 previously selected ABMs

To select the 25 best ABMs out of 100 ABMs, different experiments were performed. First, the antibiofilm activity of the different ABMs against several pathogens, including *Candida albicans*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*¸ *Pseudomonas aeruginosa* and *Escherichia coli*, was determined. Second, toxicity profiles of the 100 ABMs were determined using survival of the nematode *Caenorhabditis elegans* upon treatment with the different ABMs as a readout. Based on these data, the 25 best ABMs were selected. Next, we determined the antibiofilm activity of these 25 ABMs against polymicrobial biofilms, i.e. biofilms consisting of more than 1 species.

Analysis of the effect of ABMs on viability and differentiation potential of osteoblasts

Since it is of great importance that ABMs are not cytotoxic for bone tissue and do not negatively affect the osseointegrative potential of the implant, the effects of the ABMs on cell viability, and functional behavior (differentiation potential) were tested. Three most relevant cell types represented in bone tissue were used in these experiments, namely, human osteoblasts (OB), human bone marrow derived stem cells (MSC), and human endothelial cells (EN). Effects of ABMs on cell viability were tested according to ISO 10993-5 cytotoxicity standard.

Analysis of the effect of ABMs on cell viability

Two methods were employed to assess the cytotoxicity, MTT test and Trypan blue exclusion test. Three time points were chosen to assess the cytotoxic effect: 2 hrs, 48 hrs, and 6 days. 23 substances at concentration of 12,5 µM were tested and their effects ranged from non-cytotoxic to cytotoxic. Results are shown in table 1. (**De Brucker *et al.*, AAC, 2014**; **Delattin *et al.*, AAC, 2014; Steenackers *et al.*, Molecules, 2014; Liebens *et al.*, BMCL, 2014**).

Table 1. Viability of Mesenchymal stem cells (MSC), osteoblasts (OB), and endothelial cells (EN) after exposure to compounds according to Trypan blue exclusion test.

	2 hrs			48 hrs			6 days		
Compound	**MSC**	**OB**	**EN**	**MSC**	**OB**	**EN**	**MSC**	**OB**	**EN**
control	98.3	98.2	98.3	97.7	97.2	98.4	96.8	90.2	99.6
control+DMSO	92.6	/	97.3	96.9	97.8	84.5	94.3	92.2	4.2
0,005% Ph	99.6	91.1	96.3	95.5	98.3	92.5	82.0	87.6	59.7
0,05% Ph	99.2	91.7	97.4	78.1	86.7	20.8	12.9	38.2	0.0
0,5% Ph	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8339	94.8	95.8	95.9	23.6	6.4	52.4	0.0	0.0	1.1
2745	93.8	87.9	92.0	77.4	45.5	9.4	7.3	0.0	0.0
42170	53.2	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
P2-1	96.6	94.1	67.9	97.4	95.9	26.0	94.1	83.7	2.2
P2-5	97.7	94.9	91.7	94.0	91.2	11.5	83.2	10.2	0.0
P2-8	96.5	94.7	96.7	97.7	94.1	96.5	89.2	82.5	98.9
P2-14	97.8	98.0	94.4	97.4	95.3	38.1	86.5	16.2	0.0
P1a-pep1	95.5	80.2	96.6	94.6	92.8	95.6	87.8	83.7	99.6
OSIP 108	96.4	95.7	96.9	96.5	97.1	96.8	86.7	94.1	96.5
202611	95.4	97.9	91.6	95.4	95.1	54.8	11.7	44.1	0.0
4-29	98.5	95.6	98.1	97.3	97.6	96.0	91.8	87.0	99.0
BS-342	96.2	97.9	97.9	98.3	97.4	99.7	90.5	88.8	98.2
4-45	96.8	95.4	89.5	30.6	5.9	0.0	0.0	0.0	0.0
4-122	96.0	96.0	95.2	0.0	2.7	0.0	0.0	0.0	0.0
5-21	95.3	88.3	25.9	0.0	0.0	0.0	0.0	0.0	0.0
5-59	98.2	94.8	96.9	97.2	96.5	97.3	95.2	89.6	99.1
5-102	93.9	96.6	97.7	96.6	96.2	93.4	88.6	82.9	88.8
P1a-CIM02	99.7	91.6	96.0	96.6	96.9	96.3	87.3	92.0	99.0
P1a-pep7	92.9	76.1	96.5	94.8	83.7	97.8	78.7	80.1	91.8
CIM006387	95.0	97.0	98.0	4.0	40.7	21.8	0.0	0.0	0.0
CIM007844	96.7	94.3	70.4	97.9	95.8	3.8	92.6	91.2	0.0
CIM008405	81.1	81.8	47.1	0.0	0.0	0.0	0.0	0.0	0.0
CIM003592	98.1	98.9	94.0	97.7	93.9	9.4	95.6	42.8	0.0
5-86	96,9	96,6	98.5	97,9	98,4	95.2	97,8	96,4	95.3
JN016	96,7	98,2		98,3	97,7		97,2	95,1	
JN017	97,7	96,7		98,3	97,7		97,5	94,9	
JN018	98,6	97,0		97,9	98,4		97,2	97,3	
JN019	95,5	97,0		97,5	98,3		98,6	93,5	
LC0022	97,0	96,6		95,9	98,3		97,3	92,5	
LC0023	98,0	97,4		95,4	97,2		98,0	95,0	
LC0024	96,4	96,0		97,1	98,4		98,4	96,0	
LC0025	96,6	96,4		97,9	98,6		97,3	96,0	

Ph: phenol

Analysis of the effect of ABMs on osteogenic differentiation potential of cells

The effect of the ABMs on osteogenic potential of MSC and OB was further assessed, as those two cell types are responsible for the production of new bone matrix within bone tissue. Calcium was chosen as an indicator of osteogenic phenotype as the final and functional marker of osteoblast differentiation. Only the ABMs that allowed survival of cells for more than 3 weeks (which is a time needed for mature osteogenic differentiation) were tested. MSC and OB were cultured in osteogenic medium for 3 and 5 weeks, respectively in the absence or presence of the ABMs (12,5 µM). Tested ABMs (P2-1, P2-8, p1a-pep1, OSIP108, BS-342, 5-59, 4-29, JN016, JN018, JN019, LC0024, LC0025, 5-86, 5-102), did not inhibit the deposition of calcium, indicating that osteogenic differentiation potential of MSC and OB was not negatively affected (**Steenackers *et al.*, Molecules, 2014**).

Analysis of the effect of ABMs on endothelial differentiation potential of cells

Vasculature presents an important component of bone tissue, since it assures nutrient exchange and it is involved in proper bone development and remodeling. Hence, the effect of the ABMs (12,5 µM) was tested with respect to functionality of endothelial cells, more specifically, their tube formation potential and the average length of the formed tubes was chosen as a parameter. To this end, EC were seeded onto Matrigel coated surface. After 4, 8 and 24 hours tube formation was assessed. Results showed that tested substances had different effects on tube formation potential: P1a-Pep1 (**De Brucker *et al.*, AAC, 2014**) and Osip108 (**Delattin *et al.*, AAC, 2014)**, did not have any effect, while the substances 202611 and 4-45 had a negative effect on the tube formation process. While CIM008405 negatively affected endothelial cells in non-assembled stage in monolayer culture, more differentiated endothelial cells in assembled stage were not affected (**Liebens *et al.*, BMCL, 2014**).

Resistance of ABMs against gamma-irradiation

To select the 5 best molecules among the selected 25, their resistance to gamma irradiation, a commonly used technique to sterilize medical devices, was determined. To this end, AMBs were treated with gamma-irradiation (product dose ranged from 45kGy to 55kGy, according to the following standards: ISO 9001, ISO 13485, ISO 11137 and 21 CFR Part 820 (FDA c GMP) and their antibiofilm activity, before and upon gamma-irradiation, was compared.

In addition, also the presence of chemical groups, required for covalent binding of the ABM to the titanium discs, was taken into account for the selection of the 5 best ABMs.

Conclusion WP1

For the selection of the 5 best ABMs following parameters were taken into account: (1) Ability of the ABMs to prevent biofilm formation of various single and mixed species: ABMs that can inhibit biofilm formation of several bacterial/fungal species were preferably selected. (2) Toxicity against osteoblasts and mesenchymal stem cells, (3) Presence of functional groups that enable covalent binding, (4) Remaining antibiofilm activity upon irradiation of the ABMs. Also the following remark of our advisory board member Phil Stewart was taken into account: 'toxicity testing against the very sensitive osteoblasts and mesenchymal stem cells is very important after the compounds are covalently bound to the discs – toxicity testing of the unbound compounds might be less relevant'. Hence, we included various molecules that were scored as 'toxic' but characterized by high anti-biofilm activity in different setups, in the selection for the best 5 ABMs. Toxicity of these ABMs, upon coating to the Ti discs, was performed in WP4. Based on these parameters, the following 5 ABMs were selected:

- 202611
- 4-45
- CIM008405
- P1a-pep1
- P2-5

Specific data regarding several of these compounds can be found in published articles, such as De Brucker *et al.*, 2014; Liebens *et al.*, 2014 and Steenackers *et al.*, 2014.

WP2. Elucidation of the mode of action of the five best ABMs

Initially the 4-45 compound (a *N1*-substituted 2-aminoimidazole) was selected as one of the most active compounds with a broad activity spectrum. However, it was shown to be toxic against the tested bone cells and *C. elegans*. In contrast the related compound 5-102, one of the initial 100 compounds and a *2N*-substitued 2-aminoimidazole, only showed activity against Gram-negative bacteria and fungi, but was not toxic. Therefore, 8 structural analogues with a combination of features of both compounds were synthesized and tested. This resulted in the selection of LC0024, a 1,2,5-substituted 2-aminoimidazole, with a highly reduced toxicity (as compared to 4-45) and a high increase in activity against Gram-positive bacteria (as compared to 5-102). Since Gram-positive bacteria are much more common in implant related infections LC0024 was chosen to replace the 4-45 compound since it showed the most favorable activity and toxicity profile.

Mode of action of LC0024

Even though *Salmonella* is only rarely observed in implant related infections, its close relation to *E. coli* (also in relation to biofilm formation makes it a relevant bacterial pathogen for the study of the effect of the anti-biofilm compounds on gram-negative bacteria. Additionally, most of the expertise and experimental setups at the CMPG-S&P group (P1a) are developed for *Salmonella enterica* serovar Typhimurium (including the reporter fusion library, mutant libraries, natural isolates, transcriptome analyses and phenotypical tests. Therefore, this pathogen was further used for studying the mode of action of this compound.

By using a combined approach of microarray, RNAseq and reporterfusion analysis we could identify several important pathways involved the mode of action of LC0024, and closely related imidazoles, in *Salmonella* Typhimurium.

The first of two major pathways involved include activation of the PhoPQ regulon, which is known to affect *i.a.* motility, LPS modification and biofilm formation. The interaction of the imidazole with PhoPQ was studied at the molecular level during a research stay at the lab of prof. S. Miller (University of Washington), by making use of a specialized *in vitro* kinase assay. No direct interaction between the compound and PhoQ could be observed under the previously optimized environmental conditions for the assays. However, as these conditions are different from those at which biofilm inhibition and PhoPQ activation were observed, these experiments need to be redone at the relevant conditions, after further optimization of the assays for these conditions, which are currently ongoing.

The second major pathway involved in the mode of action of LC0024 is the down-regulation of the biofilm matrix production, via repression of the biofilm master regulator CsgD. The effect on *csgD* can be attributed to a delay in RpoS activity (stress sigma factor, needed for *csgD* transcription), as revealed by a delayed transcription of several RpoS regulated genes (tested by reporter fusions). However, the transcription nor protein levels (tested by time lapse western blotting) of RpoS itself were affected by the compound. Further analysis revealed that the compound reduces the expression of *crl*, encoding a chaperone needed for RpoS activity. Reduction of *crl* transcription can possibly also be attributed to activation of the PhoPQ system since reporter fusion analyses indicated (i) that the compound increases the transcription of many PhoPQ regulated genes and (ii) PhoPQ activation (by means of the constitutive *pho24* mutant) results in *crl* and *csgD* down regulation and biofilm inhibition.

Additionally, a screening of 243 natural *Salmonella* variants to determine hyper-sensitive and/or insensitive strains was conducted. RNAseq analysis was performed to compare gene expression in the presence of imidazoles in a hyper-sensitive vs. an insensitive strain. Application of 'Molecular interaction networks' and PheNetic indicated a strong overlap between processes affected in (i) wild type lab strain vs. wild type labs strain treated with compound (see above) and (ii) insensitive strain treated with compound vs. sensitive strain treated with compound, further pointing at the role for the above mentioned processes in the mode of action.

Finally, we provided a link between 'public goods', the biofilm matrix and the inhibitory effect of the imidazoles on the biofilm matrix with the linked lack of resistance development. Public goods (like the biofilm matrix) are products that are shared between individuals, costly for the individual to produce, but provide a benefit for the total population. We showed (like initially hypothesized) that interference with public good cooperation (in this case matrix inhibition) weakens the biofilm population. Additionally, we delivered proof that public good inhibitors (like the LC0024 and related imidazoles), select against resistance by showing that strains sensitive to a matrix inhibitor (thus non-matrix producers) would outcompete emerging resistant strains (matrix producers). Subsequently, via experimental evolution, we showed that no resistance was developed against the compound when not used in high growth-inhibiting concentrations (which are far removed from the active concentration against *Salmonella* biofilms). This indicates the potential of LC0024, and public good inhibitors in general, as an evolution-proof antimicrobial strategy, for which resistance will be unlikely to develop.

Mode of action of P1a-pep1

In a first attempt to unravel the mode of action of P1a-pep1, we screened 300 *C. albicans* deletion strains in which genes encoding for cell wall proteins or transcription factors were deleted. These strains were tested for sensitivity to P1a-pep1, using 1,56 µM (resulting in 10% biofilm mass relative to control treatment) and 0,095 µM P1a-pep1 (resulting in 100% biofilm mass relative to control treatment) to screen for resistant and hypersensitive *C. albicans* mutants, respectively. We identified seven deletion strains that were resistant to P1a-pep1, compared to the wild type. These deletion strains are *cmp1Δ/cmp1Δ* (*CMP1* encodes the catalytic subunit of calcineurin,) *orf 19.2476Δ/orf19.2476Δ* (uncharacterized ORF), *ece1Δ/ece1Δ* (Ece1 has a potential role in adhesion), *pga45Δ/pga45Δ* (*PGA45* encodes for a biofilm-induced cell wall protein with unknown function), *adh1Δ/adh1Δ* (*ADH1* encodes an alcohol dehydrogenase), *wor3Δ/wor3Δ* (Wor3 is a transcription factor involved in white/opaque switching) and *ada2Δ/ada2Δ* (Ada2 is a zinc finger and homeodomain transcriptional coactivator). We could not identify deletion strains that were characterized by an increased susceptibility towards P1a-pep1, compared to the wild type.

Secondly, RNAseq was performed on *C. albicans* biofilms grown in presence or absence of P1a-pep1 to identify differentially expressed genes. As P1a-pep1strongly interferes with the biofilm formation process without killing the *C. albicans* cells, RNA of all cells, including the small amount of attached biofilm cells and cells that did not properly integrate in the attached biofilm structure, was isolated. Although a significant amount of genes were found up or downregulated in the presence of P1a-pep1, the latter did not induce great changes in expression levels of these genes. At the 6h time point, fatty acid metabolism was upregulated while carbohydrate metabolism was downregulated by P1a-pep1 treatment. Furthermore, the upregulation of lipid metabolism and downregulation of carbohydrate metabolism could point to potential changes in the cell wall or membrane that might affect biofilm formation. The observed upregulation of fatty acid metabolism seemed less important at this time point while also at 24h, carbohydrate metabolic processes were downregulated. Additionally, processes related to host responses/interactions were downregulated as well. Strikingly, no processes related to biofilm formation were selected in this analysis. Comparison of the up- or downregulated genes with the screening results of the homozygous CWP and TF deletion mutants in the biofilm setup showed an overlap of 2 genes. *ADH1* is the third most downregulated gene after 24 h of biofilm formation in the presence of P1a-pep1. In addition, *ECE1* is downregulated in the 6-h P1a-pep1treatment.

Based on homology between LL-37 and P1a-pep1 variants with antibiofilm activity, a core sequence of 14 amino acids (CRAMP15-28, KKIGQKIKNFFQKL) was identified (De Brucker *et al*., 2014). This peptide was tested for antibiofilm activity and a whole amino acid scan of CRAMP15-28 was performed, thereby replacing each amino acid individually with the 19 remaining amino acids. This amino acid scan gives us information regarding important structural determinants such as net charge, flexible/rigid parts in the structure,… The antibiofilm activity of all these peptides was determined against *C. albicans*. These results indicated that substitution of leucine on position 14 with the negative charged glutamic acid or aspartic acid improved antibiofilm activity. Also replacement of glutamine on position 5 with glutamic acid, substitution of glycine

with glutamine on position 4 and phenylalanine on position 11 with lysine improved biofilm activity. In addition, we also performed this whole amino acid scan against planktonic cultures of *Fusarium oxysporum*, a human and plant filamentous pathogen, causing fusariosis in humans. In general, the antifungal activity of CRAMP15-28 increased when positively charged amino acids were introduced at positions 3-6, 8, 9 or 11-13. Also the introduction of amino acids with a hydrophobic side chain at positions 3-11 or 14 resulted in increased antifungal activity of CRAMP-15-29.

P1a-pep1 also acted synergistically with the clinically used antifungals amphotericin B and caspofungin.

Mode of action of CIM008405

To gain insight into the mode of action of CIM008405, we first monitored the ability of the compound to inhibit bacterial macromolecular synthesis processes in *S. aureus.* No specific inhibition of DNA, RNA, or protein synthesis could be observed, suggesting that CIM008405 displays membrane damaging activity. To further investigate the effect of CIM008405 on the cell membranes of *S. aureus* and *P. aeruginosa*, several membrane damage assays were performed. Bac Light™, $DiSC_3(5)$, NPN, SYTOX green and haemolysis assays all confirmed that CIM008405 causes rapid loss of bacterial membrane integrity. Furthermore, it was demonstrated that the compound is able to induce carboxyfluorescein leakage from liposomes, suggesting that it causes membrane permeabilization through direct physical interaction with the phospholipid bilayer (Gerits *et al.*, in preparation). In addition, by using the fluorescent membrane dye FM4-64, we observed that *S. aureus* and *P. aeruginosa* cells treated with CIM008405 have distinct membrane accumulations, which is in corroboration with the results obtained in the biophysical studies mentioned above.

Next, we performed a transcriptome analysis using RNA sequencing to uncover the cellular responses to CIM008405. The transcription profile was determined from *P. aeruginosa* cells treated for 5 min with a sub-inhibitory concentration of CIM008405 (0.2x MIC). 304 genes were differentially expressed upon treatment with CIM008405. Subsequently, a network analysis was conducted using PheNetic (De Maeyer *et al.*, Nucleic Acids Res, 2015). To investigate the regulatory pathways altered by CIM008405, a GO enrichment analysis was performed on the resulting subnetwork. This analysis demonstrated that CIM008405 alters expression of genes involved in fatty acid biosynthesis and lipid metabolic processes. We hypothesize that in this way the cell tries to compensate for the membrane defects induced by CIM008405, which supports the results described above. In addition, CIM008405 also affects expression of genes involved in alginic acid metabolism, peroxidase activity, cobalamine and tetrapyrrole metabolism, translation and proline biosynthesis and metabolism (**Gerits et al., PLosOne**).

To gain more information about the primary target of CIM008405, spontaneous CIM008405-resistant mutants of *P. aeruginosa* were generated and three of them were subjected to whole genome sequencing. Mutational analysis of the resistant mutants revealed mutations in *nfxB*, which codes for the repressor of the MexCD-OprJ multidrug efflux pump. These mutations probably cause a loss of function of NfxB, which leads to up-regulation of *mexCD-oprJ* and increased resistance (Stickland *et al.*, J Proteome Res, 2010). In addition, mutations were found in *htrB* and PA14_23400, two genes that are involved in outer membrane synthesis. HrtB is an acyltransferase involved in lipid A biosynthesis and PA14_23400 presumably plays a role in O-antigen biosynthesis (Clementz *et al.*, J Biol Chem, 1996; Windsor *et al.*, Nucleic Acids Res, 2011). We assume that these mutations confer alterations in outer membrane composition, thereby leading to increased resistance. Therefore, these results indicate a direct effect of CIM008405 on the outer membrane (**Gerits et al., PLosOne**).

In addition, a library of transposon insertion mutants of *P. aeruginosa* (Liberati *et al.*, PNAS, 2006) was screened to identify genes associated with altered sensitivity to CIM008405. This led to the recovery of two CIM008405-hypersensitive mutants affected in *minC* and *minD*, which are genes encoding proteins that are involved in cell division (de Boer *et al.*, Cell, 1989). These results suggest that cell division contributes to tolerance mechanisms against CIM008405 and are in agreement with a study showing that a mutant lacking

a gene that regulates septum formation is more susceptible to a membrane-targeting antibiotic (Blake & O'Neill, JAC, 2013).

Finally, using Biolog Phenotype Microarray plates, we detected synergy between CIM008405 and chromium(III)chloride ($CrCl_3$). Cr^{3+} is a metal required for proper sugar and fat metabolism. However, it also causes DNA damage and inhibits topoisomerase DNA relaxation activity, probably by preventing the formation of the covalent link between enzyme and double helix (Plaper *et al.*, Chem Res Toxicol, 2002). We hypothesize that the observed synergism can be explained by the membrane-damaging activity of CIM008405, thereby facilitating uptake of $CrCl_3$ by the cell (**Gerits et al., PLosOne**).

In summary, we have demonstrated that the membrane is the primary target of CIM008405, making it an excellent candidate molecule for the treatment of infections caused by biofilm-forming bacteria.

Mode of action of P2-5

To determine the mode of action of P2-5 we performed RNAseq analysis on *C. albicans* cells in the presence and absence of the compound. In parallel, we performed genetic screens using overexpression and deletion collections.

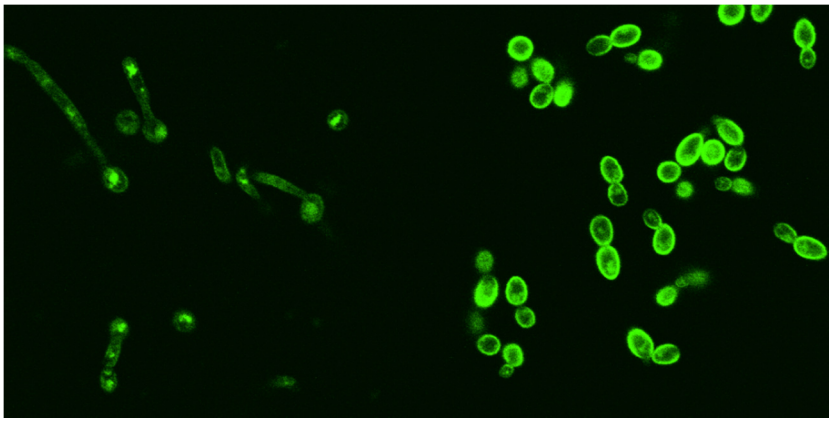
Genetic screens

P2 generated a transcription factor (TF) overexpression library (232 overexpression strains). In addition, a collection of 165 TF deletion strains is available. These collections were screened in a MIC-like assay (liquid YPD for the KO and SC-MET for the OX library, using 15 µM). Deletion strains *nrg1Δ*, *rbf1Δ*, *tac1Δ* and *upc2Δ* are more susceptible to P2-5, indicating that Rbf1, Nrg1, Tac1 and Upc2 may be involved in resistance/tolerance to this compound. We did not find any TF that upon overexpression resulted in increased susceptibility. We did not perform a screen for increased resistance. The role of the four TF was also tested in a biofilm assay. Absence of Tac1 and Upc2 resulted in increased sensitivity to P2-5 in biofilms whereas absence of Rbf1 made the biofilm more resistant to P2-5 compared to the wild type strain.

RNAseq analysis

Samples for RNA isolation were taken from 6h old biofilms treated with 8 µM P2-5 throughout this period as well as from cells that were pre-grown for 6 hours and then incubated for 90 min with the compound at a concentration of 10 µM. DMSO was used as the control. Overall, 1116 genes were differentially regulated by at least 2-fold in response to P2-5 of which 107 in both conditions. Subsequent analysis of the datasets revealed an over-representation of the Hap43-regulated genes among those regulated by P2-5. Three groups of genes were of particular interest due to their role in ergosterol homeostasis, drug response and iron homeostasis. Interestingly, *TAC1* and *UPC2* were upregulated by the compound.

Combination of the genetic and gene expression analysis seems to indicate that P2-5 results in the upregulation of efflux pumps as Tac1 is an important transcription factor required for upregulation of efflux pumps. This could explain why a *tac1Δ* strain is more sensitive to the compound. To confirm upregulation of efflux pumps we generated a Cdr1-GFP fusion strain. In the figure below (Figure 3) it is clear that P2-5 induces the expression of Cdr1. It also seems that the compound has an effect on morphogenesis, but that needs to be investigated further.



A **B**

Figure 3. *C. albicans* cells expressing Cdr1-Gfp, treated with DMSO (A) or P2-5 (B)

Mode of action of 202611

202611 is an antistaphylococcal agent capable of inhibiting the growth of planktonic (free-living) staphylococci with a minimum inhibitory concentration of 0.125 μg/ml, a level of antibacterial potency comparable to antibiotics currently in clinical use. In contrast to existing antibacterial drugs, however, 202611 is capable of eradicating pre-formed biofilms of *S. aureus* and other staphylococci (minimum biofilm eradication concentration of 8 μg/ml).

The mode of action (MOA) of 202611 was initially interrogated using a classic approach to antibiotic MOA that monitors incorporation of radiolabelled precursors in macromolecules. This revealed that 202611 mediates simultaneous and non-preferential inhibition of DNA, RNA and protein biosynthesis, a signature often seen for compounds that exert their antibacterial effect through perturbation of the bacterial membrane. Action of 202611 on the membrane was subsequently confirmed using assays that report on membrane integrity/ membrane potential, respectively (the *Bac*Light™ and $DiSC_3(5)$ assays), which revealed a substantial loss (55%) of membrane integrity and complete (100%) loss of membrane potential upon challenge with 202611. To examine whether 202611 directly acts to disrupt the physical integrity of the lipid bilayer of the staphylococcal cytoplasmic membrane, we assessed the ability of 202611 to compromise the integrity of carboxyfluorescein-filled liposomes formulated with a lipid composition to match that of *S. aureus*. At a concentration of 0.5 μg/ml, 202611 caused essentially no perturbation of staphylococcal liposomes after 3 hours, indicating that the MOA does not involve direct targeting of the phospholipid component of the cell membrane, and suggesting that the compound probably exerts its antibacterial effect through interaction with membrane proteins. A common and undesirable property of reported antibacterial agents that are membrane-perturbing is that they also perturb mammalian membranes (i.e. they are non-selective). To evaluate the selectivity of 202611, it was tested for its ability to cause haemolysis of equine erythrocytes. After 60 minutes at 4 x MIC, erythrocytes retained over 60% integrity, comparable to levels seen for the licensed antibacterial drug, ciprofloxacin.

Compound-mediated eradication of biofilms may occur through two possible mechanisms; disruption of the biofilm matrix or comprehensive killing (sterilization) of bacteria within the biofilm. Visual assessment of *S. aureus* biofilms after treatment with 202611 indicated that the matrix of the biofilm was largely unaffected by exposure to this compound, a finding that was corroborated by more quantitative studies using the matrix-specific stain, SYPRO Ruby. By contrast, SYTO 9 staining of bacterial cells within the biofilm revealed that 202611 led to a substantial loss of bacterial viability. In subsequent experiments, 202611 was shown to exhibit potent bactericidal activity against staphylococci independent of their growth state, including nutrient-depleted, non-dividing cells, and cells in the persister state – both cell types ordinarily present in biofilms. These results imply that 202611 eradicates biofilms as a consequence of its ability to kill bacteria regardless of growth state, a property that we have previously observed for compounds that perturb the bacterial membrane.

A common approach to further understanding an antibacterial agent's MOA is to select mutants resistant to the agent of interest for subsequent genetic characterization. Low-level resistance to 202611 (<1 μg/mL) was selected over 25 days' continuous exposure to 202611. Whole genome sequencing revealed multiple mutations in the resistant strains, often in hypothetical genes, and did not, at this stage, provide any additional insights into the MOA of 202611.

In summary, 202611 is a potent anti-staphylococcal agent, with low resistance potential, and capable of eradicating pre-formed *S. aureus* biofilms. The MOA of 202611 involves perturbation of the bacterial membrane, enabling the compound to kill staphylococci independent of growth state. Although 202611 exhibited a modest haemolytic effect, the compound nonetheless exhibits selective toxicity towards bacteria.

Effect of ABMs on biofilm microstructure

The effect of the antimicrobial compounds (ABMs) was evaluated by confocal laser scanning microscopy (CLSM) analysis of biofilms grown on titanium discs.

Biofilm formation on smooth titanium discs was analyzed after 19 hours incubation at 37 °C in the presence of the ABMs in TSB medium. The ABMs used were: 202611, CIM00805, 4-45, LC0024, P2-5, P1a-pep1. The following microorganisms were used: *Candida albicans* SC5314, *Pseudomonas aeruginosa* UCBPP-PA14, *Staphylococcus aureus S*H1000 and *Escherichia coli* TG1. Biofilms grown on the titanium discs were investigated with a CLSM (Leica TCS SP5) in an inverted microscope using a LIVE/DEAD®BacLight™ viability kit from Molecular Probes. Mathlab was used to calculate the area covered by viable and dead microorganisms in a thin visual section. Three replicates of each combination of ABM and microorganism as well as reference discs without ABMs were quantified using CLSM and image analysis.

The results showed that CIM00805 was capable of preventing biofilm formation of *P. aeruginosa*, already at a concentration of 10 μM. The total area fraction was reduced by approximately 50% at both concentration of 10 and 32.5 μM, compared to no addition of CIM00805 (**Liebens *et al.*, BMCL, 2014**)**.** Titanium discs with biofilm formed in the presence of CIM00805 comprised a greater proportion of dead cells within the biofilm compared to controls. This was most evident at the highest concentration (32.5 μM). This biofilm inhibitory effect was also confirmed when analyzing *S. aureus* treated with CIM00805 as the total area fraction was reduced with 60% in the presence of 10 μM of CIM00805. However, no effect was observed against *E. coli*. The ABM 202611 had a strong biofilm inhibitory effect on all three microorganisms. Most promising was the effect on *S. aureus*, since there were almost only dead bacteria, whereas the biofilm reduction of *E. coli* was 25% and of *P. aeruginosa* was 48%. The ABM 4-45 almost completely inhibits the growth of the *S. aureus* biofilm in our study. It was also effective against *P. aeruginosa* with a reduction of 76%, but had no effect on *E. coli*. Unfortunately it also showed to be toxic for human cells, therefore it was be replaced by LC0024 at a later stage in the project. Samples treated with P2-5 showed a small decrease in biofilm formation of *C. albicans* at the lower concentration (2.5 μM) and a nearly complete inhibition at the higher concentration (12.5 μM). No antibiofilm effect was seen for *C. albicans* treated with P1A-pep1.

WP3. Development of antibiofilm coatings (Ac) on smooth titanium (SmTi) and osseointegrative porous titanium (OPTi) substrates

Development and supply of state-of-the-art implant substrates

Smooth titanium discs of 0.5 and 1 cm were manufactured , washed with isopropanol, packed and distributed to the different partners. Also open porous titanium discs, produced from Ti6Al4V material, sandblasted and Ti-coated, were manufactured, washed in isopropanol, packed and distributed to the different partners.

A qualitative topographic evaluation of both surfaces was performed by scanning electron microscopy, (SEM, XL30 FEG, FEI). Figure 4 displays top views of SmTi and OPTi at different magnifications. At low magnification SmTi exhibits a smooth leveled surface whereas at higher magnification a non-directional submicron level roughness can be observed. OPTi shows an irregular surface consisting of rounded Ti agglomerates of over 100 μm in size with large open surface pores (> 100 μm). A 3D surface roughness analysis by white light

interferometry (Wyko NT 3300 Optical Profiler, Veeco Instruments) confirmed these observations showing an average roughness, Sa, of 0.69 ± 0.10 µm and 54.82 ± 6.47 µm, a ten point height, Sz, of 8.90 ± 2.00 µm and 476.03 ± 33.01 µm and a root mean square roughness, Sq, of 0.88 ± 0.14 and 69.01 ± 7.51, for SmTi and OPTi respectively. It can be seen that SmTi has a smooth surface finish with an average roughness below 1 µm, while OPTi presents a very rough surface with height differences over 400 µm in range. Image analysis of SEM images of polished cross-sections for OPTi coatings indicated an average total porosity of 13.01 ± 3.53%.

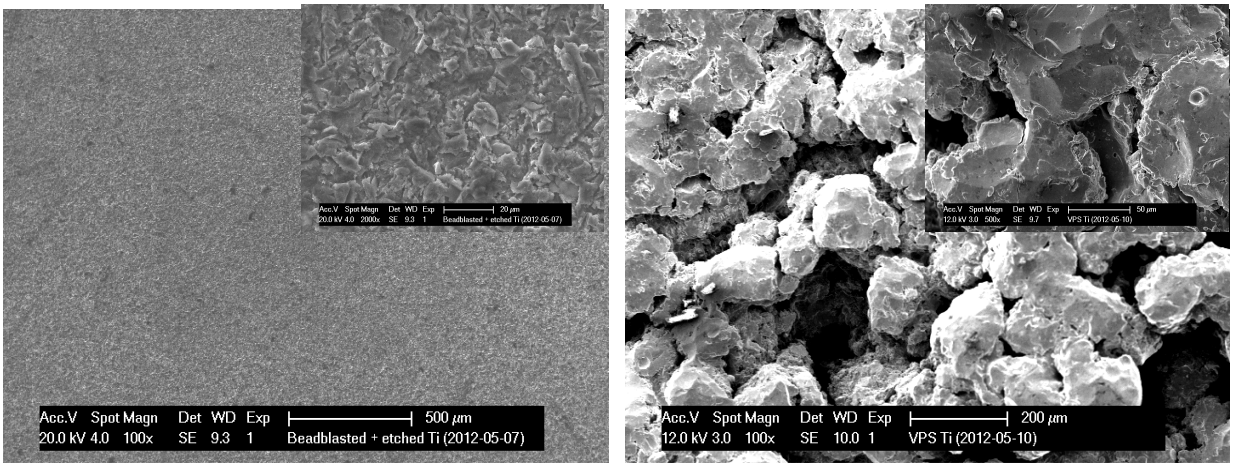


Figure 4. SEM micrographs of a surface top view for SmTi and OPTi.

Development and characterisation of coatings consisting of small molecule ABMs

Coating of smooth discs

Coating procedure

Discs were functionalized by coupling them with Fmoc-protected 3-aminopropyl-triethoxy silane and were deprotected by piperidine (90:10) in tetrahydrofuran. Discs were first placed in a hydrolysis vessel containing 45 mL n-heptane / hexamethylene diisocyanate (85:15) for 3 h at room temperature. Subsequently, discs were rinsed with n-heptane and dried. The covalent linkage was performed by one of the following procedures:

Procedure A:

The discs were placed in a solution containing 100 mL DMSO and a certain amount of ABM for 16 h with gentle agitation. Finally, the covalently linked ABM -Ti discs were washed three times with DMSO, three times with demineralised, pyrogen-free water and finally acetone, and were allowed to dry.

Procedure B:

The discs are placed in a certain volume of $NaHCO_3$- solution (9,6 g $NaHCO_3$/100 mL water) and a certain amount of ABM for 16 h with gentle agitation. Finally, the covalently linked ABM -Ti discs were washed three times with demineralised, pyrogen-free water and finally acetone, and were allowed to dry.

The detailed reaction conditions are summarized in the following table:

Compound ID	Procedure A/B	Amount in reaction solution	Volume reaction solution
CIM008405	A	0,512 g	100
P2-5	A	0,75 g	100
202611	A	0,43 g	100
Vancomycin	B	0,520 g	100
Caspofungin	B	0,305 g	50
P1a-Pep	B	0,25 g	50

Determination of surface content
Surface modified Ti discs were immersed in vessels containing demineralized water (1 mL), isopropanol (0.5 mL) and triethylamine (1 mL). The vessels were closed and heated at 60 °C for 1 h in a drying cabinet. Next, the solvents were removed from the vessels by evaporation at 60 °C. Finally, the residues were dissolved in demineralized water (0.5 mL) and were analyzed by HPLC on a C18 column (50 x 2.1 mm). Following results were obtained:

Compound ID	Sterilization	Amount [pmol/cm²)
CIM008405	Yes	54,8
P2-5	Yes	315,2
202611	Yes	104,3
Vancomycin	Yes	0
Vancomycin	No	35,2
Caspofungin	No	2191,4
P1a-Pep	No	0

LC0024 was covalently coated to the carboxyl modified discs via amide bond synthesis. The primary amine of the compound LC0024 reacted with the carboxylmoiety on the surface. Reaction was performed by shaking the reaction for 16 hours at room temperature. As coupling reagents a combination of DIC (3 equivalents) and Oxyma (2 equivalents) were employed in a dichloromethane/dimethylformamide (95/5) solvent mixture. After completion the solid supports were washed with a DCM/DMF (50/50) mixture (5 times) and with DCM (3 times). Solid supports were dried under an argon stream.

Coating of OPTi discs
Coating procedure
Discs were functionalized by coupling them with Fmoc-protected 3-aminopropyl-triethoxy silane and were deprotected by piperidine (90:10) in tetrahydrofuran. Discs were first placed in a hydrolysis vessel containing 25 mL n-heptane / hexamethylene diisocyanate (85:15) for 3 h at room temperature. Subsequently, discs were rinsed with n-heptane and dried.
The discs are placed in a solution containing 100 mL DMSO and a certain amount of ABM for 16 h with gentle agitation. Finally, the covalently linked ABM -Ti discs were washed three times with DMSO, three times with demineralised, pyrogen-free water and finally acetone, and were allowed to dry.

The detailed reaction conditions are summarized in the following table:

Compound ID	Amount in reaction solution
CIM008405	0,595 g
P2-5	0,531 g
202611	0,511 g
Vancomycin	0,525 g
Caspofungin	0,250 g

Determination of surface content
Surface modified Ti discs were analysed as described above.

Compound ID	Amount [pmol/cm²)	Sterilization
Vancomycin	169,87	No
P 2-5	2126,11	No
202611	1011,38	Yes
CIM008405	390,05	Yes
Caspofungin	5468,26	No

Coupling of LC0024 to carboxylated OPTi discs was performed using the same protocol as described for SmTi discs.

Determination of potential ABM release upon covalent linkage
For this investigation, three release experiments were carried out per test compound, for each setup two discs per compound were placed in a vessel with 1 mL demineralized water and stored at room temperature for 24 h without agitation. Upon this release procedure, the discs were analysed for their substance content. As a reference, 6 discs per compound (grouped in pairs of two discs each) without previous release procedure were analysed for their substance content as described in section 1.2.

Compound ID	Amount [pmol/cm²)	StDev [picomol/cm²]	Amount [pmol/cm²) after 24 h Elution	StDev [picomol/cm²] after 24 h Elution
Vancomycin	169,87	4,59	70,40	7,01
P 2-5	2126,11	598,89	1830,59	455,65
202611	1011,38	512,95	1734,35	327,83
CIM008405	390,05	195,87	228,22	68,56
Caspofungin	5468,26	1595,69	2122,97	817,57

Compared to the reference discs, all ABM coated discs showed a lower average value of surface amount after elution except the substance 202611 coated discs, which showed a higher average surface coverage after elution.

An analysis of variance (95% confidence, Fisher test) showed only for vancomycin and caspofungin coated discs a significant difference between surface amount of discs with and without elution. For all small ABMs the average difference between eluted and reference samples was not significant. From the limited number of samples and with the given limits in accuracy for the quantification method, it can be stated that the vancomycin and caspofungin coated discs release certain amounts of substance whereas the discs coated with small molecules do not release their coating.

Development and characterisation of peptide ABM coatings
The conventional route for biomolecule grafting, i.e. by activation of the surface using coupling agents followed by immersion of the substrate in the compound solution, is a time-consuming process. It can take several hours up to days to establish a monolayer of the biomolecule as the immobilization rate depends on the (passive) diffusion of molecules to the surface. Alternatively, we aimed to establish a proof-of-principle for a faster processing route through the application of alternating current electrophoretic deposition (AC-EPD) as a coating technique for antibiofilm peptides.

Initial experiments were performed using the model molecule bovine serum albumin (BSA). AC-EPD was carried out under ambient conditions using disposable 1 ml polystyrene cuvettes as the deposition cell in which the titanium electrodes were placed vertically at a fixed distance of 8 mm using a polymer spacer (Braem *et al.*, in preparation). It was shown that when applying an asymmetric AC-field with (a frequency of 50 Hz, an asymmetry of 4 and) a peak-to-peak voltage of 100 V/cm superimposed with a 2 V/cm DC-field during 40 min, a selective deposition of BSA on the electrode serving as the anode during the high amplitude peak could be realized. Figure 5a compares SEM micrographs of the pristine Ti surface and after AC-EPD coating with BSA, a thin (organic) layer covering the topographical features of the Ti surface can be observed in the latter. Using a Bradford reagent to visualize the protein (Figure 5b), it is evident that the negatively charged BSA homogeneously deposits on the AC-EPD anode (blue) and not on the cathode (no color). Additionally, the colorimetric detection of BSA using a bicinchoninic acid (BCA) formulation shows a significantly higher protein content on AC-EPD treated anodes than on Ti electrodes dipped in a BSA suspension for the same duration (Figure 5**Figure** c). Time-of-flight secondary ion mass spectrometry (ToF-SIMS) confirms the presence of BSA at the anode surface. No significant differences with dropcoated BSA are detected, indicating that AC-EPD does not affect the protein folding. In addition, the weaker substrate signals

detected in samples with longer deposition times point at a higher coating thickness (Figure 5**Figure** d) (**Braem *et al.*, in preparation**).

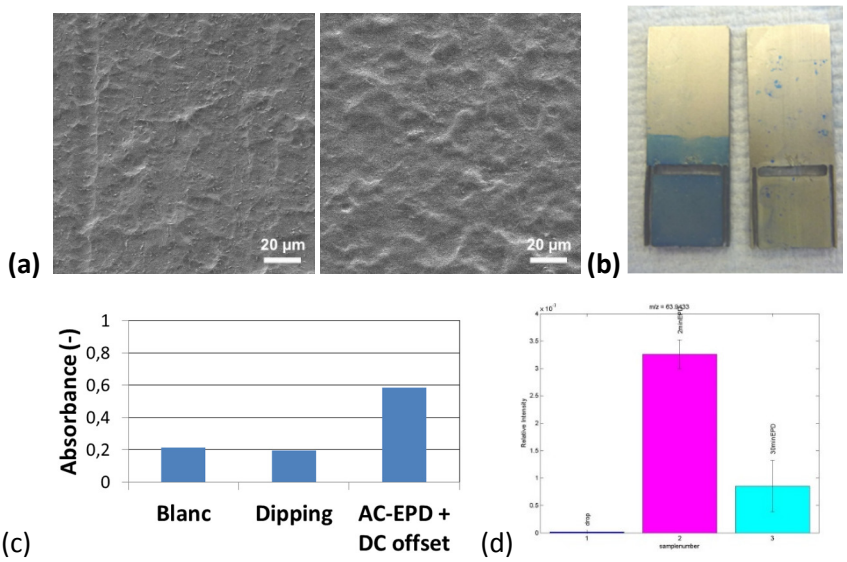


Figure 5. Selective deposition of bovine serum albumin on Ti electrodes serving as AC-EPD anode. (a) SEM micrograph of the pristine Ti surface (left) and BSA coated AC-EPD anode (right), (b) AC-EPD anode (left) and cathode (right) treated with Bradford reagent, (c) BCA protein assay absorbance for a pristine Ti electrode, after 40 min dipping or 40 min AC-EPD in a BSA suspension and (d) ToF-SIMS intensity of the TiO^+ mass fragment (m/z = 63.94) for BSA coated Ti substrates obtained by dropcoating or after 2 min and 30 min AC-EPD, respectively.

In a next step, AC-EPD was used for the application of caspofungin coatings on Ti substrates. In order to avoid Joule heating of the suspension, which might lead to denaturation of the protein, AC-EPD was now performed under galvanostatic (controlled current) conditions. Moreover, prior to AC-EPD, the Ti electrode surface was functionalized to establish a covalent bonding of caspofungin with the substrate. This was done by silanization and activation with isocyanate groups as described above (**Braem *et al.*, in preparation**). It was shown that when applying an asymmetric AC-signal with an amplitude of 10 mA and superimposed with a mild 0.1 mA DC offset during 10 min, a selective deposition of caspofungin on the functionalized electrode serving as the cathode during the high amplitude peak could be realized. A significantly higher protein content can be observed for AC-EPD cathodes compared to anodes as determined by a BCA protein assay (Figure 6a). Indeed, using SEM, a thick network of protein aggregates covering the whole electrode surface is found on the cathode surface (Figure 6b). ToF-SIMS analysis allowed to identify caspofungin at the cathode surface (Figure 6c). Moreover, substrate signals are significantly weaker for functionalized AC-EPD cathodes, then for AC-EPD anodes or non-functionalized AC-EPD cathodes (Figure 6d). This confirms the selective deposition of caspofungin on functionalized AC-EPD cathodes. In addition, it was shown that the biofilm formation for these caspofungin coated Ti electrodes is strongly reduced compared to pristine Ti surfaces (**Braem *et al.*, in preparation**).

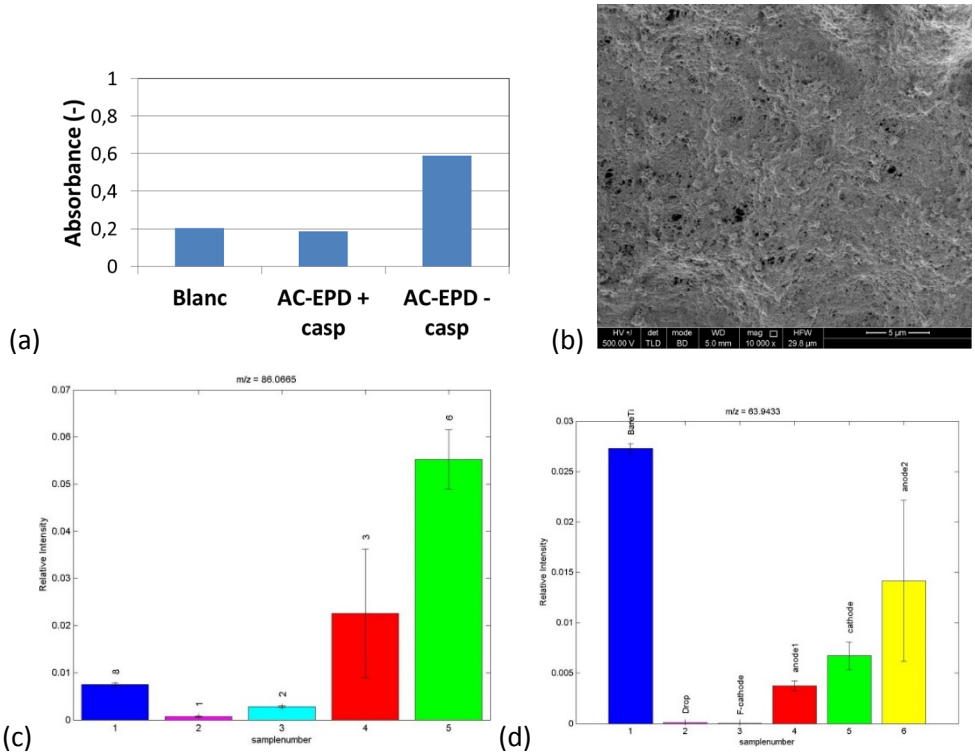


Figure 6. **Selective deposition of caspofungin on functionalized Ti electrodes serving as AC-EPD cathode.** (a) BCA protein assay absorbance for a pristine Ti electrode or as anode respectively cathode after 10 min of AC-EPD in a caspofungin suspension, (b) SEM micrograph of a caspofungin coated AC-EPD cathode, (c) ToF-SIMS intensity of a protein mass fragment (m/z = 86.0665) for pristine Ti, silanized Ti, silanized Ti with isocyanate linkers, functionalized Ti (silanized + isocyanate) with caspofungin applied by AC-EPD and Ti with dropcoated caspofungin, respectively, and (d) the TiO^+ mass fragment (m/z = 63.94) for pristine Ti or caspofungin coated Ti substrates obtained by dropcoating, as functionalized cathode, anode, non-functionalized cathode or anode, respectively, after 10 min AC-EPD.

WP4. *In vitro* analysis of the AcSmTi/AcOPTi substrates

In vitro activity analysis of the AcSmTi/AcOPTi substrates

Upon coating of the selected ABMs to AcSM/AcOPTi substrates, the *in vitro* activity profile of these coated substrates was determined. Results are shown in the tables below. Please note that the originally selected compound 4-45 was replaced with the LC0024 analogue, a derivative that has a similar antibiofilm activity but is less toxic. The antibiofilm activity of P1a-pep1 smooth discs, produced using AC-EPD was not significantly different from the control discs. In contrast, P1a-pep1 was still active upon HMDI coating. However, no P1a-pep1 could be detected upon HMDI coating. As such, we do not know if this observed antibiofilm activity is due to P1a-pep1 concentrations below the minimal detection limit or due to the coating procedure. As AC-EPD coating does not results in active P1a-pep1 and P1a-pep1 coatings using HMDI are not detectable, it was decided to stop our work with P1a-pep1-coatings.

Table 1. Percentage biofilm growth relative to uncoated SmTi discs.

Discs coated with	Used strain	% biofilm growth relative to uncoated SmTi discs
202611	*S. aureus* SH1000	66%
LC0024	*S. aureus* ATCC6538	75%
CIM008405	*S. aureus SH1000*	13%
P2-5	*C. albicans* SC5314	<15%
Caspofungin	*C. albicans* SC5314	<0.5%
Vancomycin	*S. aureus* ATCC6538	12.63%
P1a-pep1 (HMDI coating)	*C. albicans* SC5314	31% ± 4%
P1a-pep1 (AC-EPD coating)	*C. albicans* SC5314	80% ± 8%

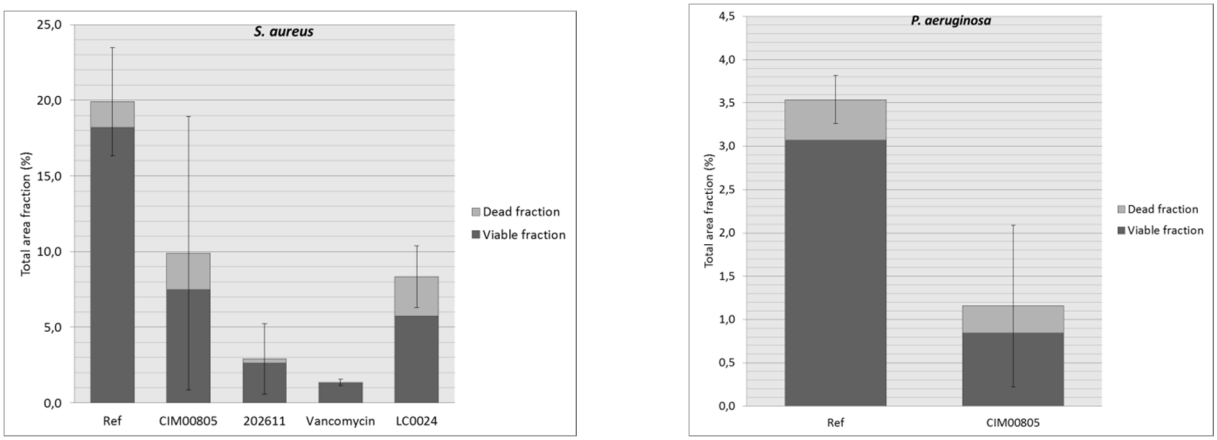
In conclusion, coating of all ABMs on smooth titanium discs resulted in significant inhibition of microbial biofilm growth when assessed in vitro.

In contrast, coating of most ABMs on coated open porous discs did not result in significant reduction of microbial biofilm formation as compared to control discs when assessed via CFU determination (data not shown). Note that HPLC analysis (see WP3) showed efficient coating of the molecules to the open porous discs and this to the same extent as in case of smooth discs.

This apparent absence of *in vitro* efficacy of the coating is probably due to the fact that CFUs determination might not be the best method for quantification of biofilm formation on these substrates, as microbial attachment and biofilm formation to such surfaces might be too strong. Hence, quantification of biofilm formation via CFU's (necessitating dispersal of the cells from the substrates by vortexing and sonication) is probably hampered. A quantification method relying on a metabolic dye is probably more suitable.
In addition, the increased surface area of the open porous discs, might result in relative lower molecule loads on open porous discs as compared to smooth discs. As the increase in surface area of such open porous discs is difficult to estimate, the actual number of ABM molecules per μm^2 might be lower than anticipated .Further follow up projects should be dedicated to increase the number of ABM molecules coated to such open porous discs, resulting in equal ABM molecule load between smooth and open porous titanium discs.

Microscopic analysis of the AcSmTi/AcOPTi substrates
The biofilm formation was analyzed on smooth titanium discs coated with different antimicrobial compounds (ABMs) by confocal laser scanning microscopy (CLSM) and image analysis. *Pseudomonas aeruginosa* UCBPP-PA14 was tested on discs coated with CIM00805, whereas the biofilm formation of *Staphylococcus aureus* SH1000 was tested on discs coated with either CIM00805, 202611, LC0024 or vancomycin. Finally, biofilm formation of *Candida albicans* SC5314 was tested on discs coated with either P2-5, LC0024 or caspofungin. The method used is described in detail in our publication (**Kucharíková, Gerits *et al.*, JAC, 2016**).
The area covered with viable cells on the CIM00805 coated discs was decreased with 72 % when incubated with *P. aeruginosa* and by 59 % when incubated with *S. aureus* compared to the reference discs (figure 7a and b) (**Gerits et al., J Orthop Res**). The thickness of the *S. aureus* biofilm was also reduced with 40%. There was no major change in the thickness of the biofilm with *P. aeruginosa*.
The amount of viable *S. aureus* covering the discs coated with 202611 was decreased with 86% (Figure 7a). whereas for discs coated with LC0024, the area fraction of viable *S. aureus* decreased with 68% (Figure 7).
Vancomycin was the most effective compound and decreased the area fraction of viable *S. aureus* with 93% (Figure 7) (**Kucharíková, Gerits *et al.*, JAC, 2016**). In contrast, the area fraction of dead cells did not decrease with CIM00805 and LC0024 compared to the reference. Discs coated with 202611 and vancomycin had less dead cells attached to the surface compared to the reference.

All ABMs were effective against bacteria in these tests and gave a major reduction of the biofilms. As such, these are all promising candidates for implant coatings. The only compound that had major effect on *C. albicans* in these tests was caspofungin. This compound almost totally inhibited the biofilm formation when coated on discs (**Kucharíková, Gerits *et al.*, JAC, 2016**).



A B

Figure 7. Total area fraction of ABM-coated discs covered with *S. aureus* (A) or *P. aeruginosa* (B) cells.

***In vitro* safety analysis of the AcSmTi/AcOPTi substrates**

Cytotoxicity of the discs was evaluated in the direct contact test according to International Standard ISO-10993: "Biological Evaluation of Medical Devices" guidelines. In the direct contact test we tested how the cells responded to substances that are now coupled to the scaffold/disc (and are not free in the medium as described in work package 1). In this experiment, the subject of interest was the lateral surface of the discs, which is identical in AcSmTi and AcOPTi ones. Therefore, in this test, only one type of discs was tested, namely the smooth discs. Human bone marrow derived stromal cells were used as a cell source as they represent a highly relevant cell type in bone tissue turnover and regeneration processes. Cell growth was observed for 6 days and photographed at day 1, 3 and 6. The vicinity of the discs was monitored for the zone of growth inhibition and cell culture morphology. The following samples were tested: Uncoated smooth discs, smooth discs coated with Vancomycin, Caspofungin, CIM008405, P2-5, 202611 and LC0024. Non-cytotoxic and cytotoxic reference material was used for positive and negative control.

Cell culture morphology remained unchanged and no zone of growth inhibition was observed for all discs tested. As such, according to the direct contact test, all discs/coated molecules are considered non-cytotoxic.

***In vitro* analysis of the osseointegrative potential of the AcSmTi/AcOPTi substrates**

To assess the osseointegration potential of AcSmTi and AcOPTi substrates, osteogenic (human bone marrow derived stromal cells – MSC) and vasculogenic (human microvascular vein endothelial cells – HMVEC) were used, and were seeded directly on the top surfaces of AcSmTi and AcOPTi substrates. Attachment and growth of cells was observed using Phalloidin/DAPI stain at day 5 and 12. The following samples were tested: uncoated smooth discs, smooth discs coated with vancomycin or caspofungin (**Kucharíková, Gerits *et al.*, JAC, 2016**), CIM008405 (**Gerits *et al.*, J Orthop Res**), P2-5, 202611, LC0024; and uncoated open porous discs, open porous discs coated with vancomycin, caspofungin, CIM008405, P2-5, 202611, or LC0024.

All tested smooth discs supported attachment and growth of MSC, as observed by Phalloidin/DAPI stain at day 5. Moreover, discs' surfaces enabled growth of MSC also for extended periods of time, i.e. 12 days. For all smooth discs tested, an obvious increase in cell numbers was observed at day 12 in comparison to day 5, except for smooth discs coated with caspofungin, where MSC were less numerous, indicating, that cells detached from the surface.

With respect to HMVEC, all tested smooth discs supported attachment and growth of cells, as observed by Phalloidin/DAPI stain at day 5. However, smooth discs coated with carboxyl-linker and HDMI-linker yielded less cells than other discs at day 5. While uncoated smooth disc, smooth discs coated with CIM008405, P2-5,

202611, and LC0024 still supported growth of HMVEC at day 12, very few or no cells were detected on smooth discs coated with vancomycin and caspofungin.

All tested open porous discs, except caspofungin coated ones, supported attachment and growth of MSC, as observed by Phalloidin/DAPI stain at day 5. Moreover, discs' surfaces enabled growth of MSC also for extended periods of time, i.e. 12 days. For all open porous discs tested, obvious increase in cell numbers was observed at day 12 in comparison to day 5.

For HMVEC, all open porous discs supported cell growth at day 5. At day 12, increased cell numbers were observed on uncoated open porous discs, vancomycin, and CIM008405 coated open porous discs. On the contrary, caspofungin, P2-5, 202611, and LC0024 didn't provide supportive environment for long-term culture of HMVEC.

Taken together, all discs tested, supported attachment and growth of osteogenic and vasculogenic cells (at least in one time point, and for one cell type), indicating a good osseointergation potential. Generally, MSC performed better than HMVEC; they attached on more substrate types and proliferated more, which is expected due to the differences in characteristics between the cell types.

WP5. Analysis of the in vivo efficacy and osseointegration of the AcSmTi/AcOPTi substrates

Development of a mouse biofilm colonization model

We developed a new model to study *in vivo S. aureus* and *C. albicans* biofilm development using a murine model of biomaterial-associated infections. The development of the model was performed using SmTi discs (height: 2 mm, diameter: 0.5 cm; beadblasted and etched, washed in isopropanol) as OPTi discs could not be used in this model due to their strong attachment to the surrounding tissue (as a consequence of their open porosity) causing a lot of distress to the mice.

All animal experiments performed in this study were approved by the Animal Ethical Committee of the KU Leuven (project number P125/2011). Pathogen-free BALB/c female mice (20 g, 8 weeks old) were housed in groups of 4 in individually ventilated cages. Briefly, one day prior to the surgery all animals were immunosuppressed by adding dexamethasone (0.4 mg/L) to the drinking water. Based on our experience, immunosuppression results in higher reproducibility of the number of biofilm-forming cells retrieved from implanted devices. Suppression of the immune system was carried out throughout the entire experiment (4 days in total). At the day of implant, animals were anesthetized using an intraperitoneal injection of a mixture of ketamine and medetomidine. The lower back of the animals was shaved and disinfected with iodine isopropanol (1 %). Prior to the incision, local anesthesia was performed with xylocaine gel directly on the skin. A small incision was made and the subcutis was carefully dissected to create a space (approximately 2 cm long and 1 cm wide) for 1 smooth disc. The incision was closed with surgical staples, disinfected and locally anesthetized with xylocaine gel. Anesthesia was reversed with intraperitoneal injection of atipamezole. Twenty four h post implant, animals were anesthetized with a mixture of ketamine and medetomidine as indicated above and inoculated with the pathogens. For inoculation of the discs with *S. aureus* or *C. albicans*, microbial overnight cultures were washed and resuspended in sterile saline (0.9 %) to a concentration of 1 x 10^8 cells/ml. 100 µl of the bacterial or fungal inoculum was injected subcutaneously into the area around the disc (resulting in final concentration of 1x10^7 cells). The final concentration of microorganisms injected was adapted based on our preliminary experiments where animals were challenged with a subcutaneous injection of different amount of *S. aureus* cells (1 x 10^5 cells, 1 x 10^6 cells and 1 x 10^7 cells) or *C. albicans* cells (2.5 x 10^4 cells, 2.5 x 10^5 cells and 1 x 10^7 cells), alongside the implant. Only in the mice challenged with the highest inoculum, biofilms developed on control SmTi discs. Subsequently, anesthesia was reversed with an intraperitoneal injection of atipamezole as indicated above. Bacterial and fungal biofilms were left to develop for 4 days or 2 days, respectively. These time points are known to provide enough time to bacterial or *Candida* cells to proliferate and create biofilm structures on the implants. For discs explant, the animals were euthanized by cervical dislocation. The skin was disinfected with 0.5 % chlorhexidine in 70% alcohol; discs were removed from under the subcutaneous tissue and washed twice with PBS before further quantification

of biomass. The bacterial and fungal burden was assessed by cfu quantification. Additionally, we analyzed the dissemination of *S. aureus* or *C. albicans* to the tissue surrounding the discs.

***In vivo* efficacy testing of the AcSmTi/AcOPTi substrates**

In the next step we used the above described biomaterial-associated infection model to test the ability of *S. aureus* to develop biofilms on vancomycin (VAN)-coated or *C. albicans* to develop biofilms on caspofungin (CAS)-coated SmTi (Figure 8).

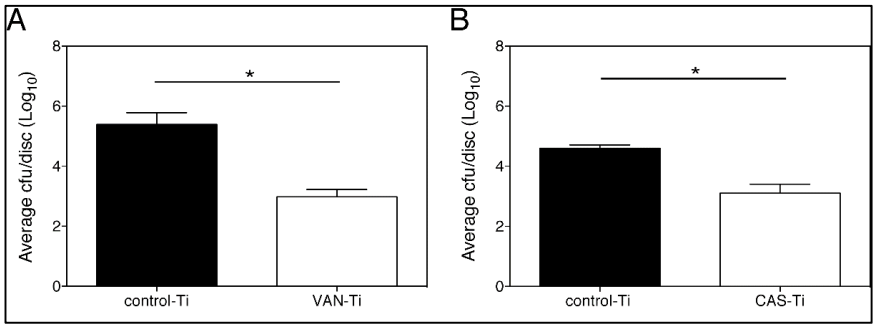


Figure 8. *In vivo* efficacy of VAN-Ti and CAS-Ti discs against *Staphylococcus aureus* and *Candida albicans* biofilm formation, respectively. (A) Quantification of *S. aureus* and (B) *C. albicans* biofilm formation developed on control-Ti, VAN-Ti and CAS-Ti in a murine model of biomaterial-associated infection (**p*<0.05), via cfu counting. All data represent means ± SEM from 2 independent experiments.

We found that *S. aureus* biofilm formation on the VAN-Ti discs was reduced by approximately 99.9 % (Figure 8a), whereas *C. albicans* biofilm development on the CAS-Ti was inhibited by 89 %, as compared to the control-Ti discs (**p*<0.05) (Figure 8b) (**Kucharíková, Gerits *et al.*, JAC, 2016**).

Finally, we analyzed the amount of *S. aureus* and *C. albicans* cells in the tissue surrounding the implant. No significant difference was found between the number of bacterial or fungal cells colonizing the tissue surrounding the VAN-Ti or CAS-Ti discs, respectively, as compared to control-Ti, indicating that release of vancomycin or caspofungin from the discs to the surrounding tissue is negligible.

Next, we assessed whether a novel compound CIM8405 covalently bound to SmTi would diminish *S. aureus* biofilm formation *in vivo* and dissemination into the surrounding tissue. After 4 days of biofilm development, CIM-8405 coated discs contained significantly less *S. aureus* cells in comparison with control-Ti discs. This difference corresponds to a significant reduction of 98 % (p<0.05) (Figure 9). Additional analysis of the tissue surrounding the implanted discs revealed a similar amount of bacteria colonizing the tissue surrounding the control-Ti and CIM8405-Ti discs, indicating that CIM-8405 was not released from the discs.

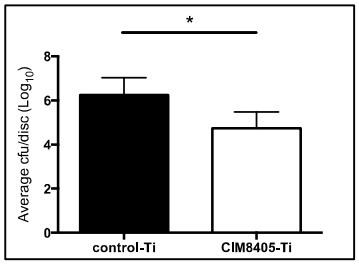


Figure 9. *In vivo* characterization of biofilm formation of *S. aureus* on CIM8405-Ti discs. Growth of *Staphylococcus aureus* biofilms on control-Ti and CIM-8405 Ti discs. Data represent the mean ± SEM of 2 independent experiments (**p*<0.05).

Furthermore, we determined the anti-biofilm activity of LC0024 on *S. aureus* biofilm development. Mice were challenged with control-Ti and LC0024-coated Ti. One day post implant these implants were infected with *S. aureus* cells by subcutaneous injection into the surrounding tissue. After biofilm development (in total 4 days), LC0024 coated discs contained significantly less *S. aureus* cells in comparison with control-Ti discs (*p*<0.05) (Figure 10).

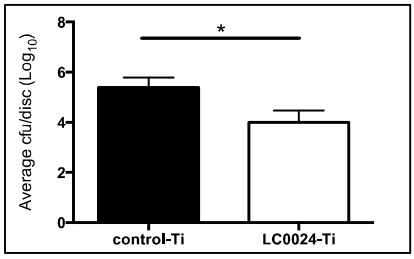


Figure 10. *In vivo* characterization of biofilm formation of *S. aureus* on LC0024-Ti discs. Growth of *Staphylococcus aureus* biofilms on control-Ti and LC0024 Ti discs. Data represent the mean ± SEM of 2 independent experiments (*p<0.05).

In addition, also the antibiofilm activity of 202611- and P2-5- coated Ti discs was tested in mice injected with *S. aureus* and *C. albicans* cells, respectively, at the implantation site. However, no significant differences in biofilm formation between non-coated and coated discs could be observed *in vivo* for the 202611 coated discs. Similarly, also no significant decrease in *C. albicans* biofilm formation between non-coated and P2-5-coated discs could be observed 2 days after infection. In contrast, P2-5 coated Ti discs did show significantly less *C. albicans* biofilm formation 7 days after infection, compared to non-coated discs (Figure 11).

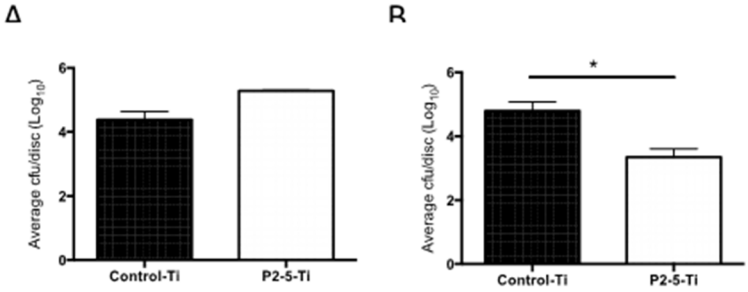


Figure 11. *In vivo* antifungal activity of P2-5 coated discs. Fungal biomass retrieved from discs, two days (A) and seven days (B) post infection with *C. albicans*. Data represent the mean ± SEM of 2 independent experiments (*p<0.05).

***In vivo* analysis of osseointegration of the AcOPTi substrates**

An open porous Ti surface (OPTi), which is of clinical relevance in orthopaedic implant applications, was coated with vancomycin (VAN-OPTi) or LC0024 (LC0024-OPTi) and the osseointegration profile was evaluated in an *in vivo* rat model. Small cylindrical implants (ø 1.5 mm, l = 6 mm) were placed inside the femoral medulla and the bone response was measured 8 and 12 weeks after implantation. A thorough qualitative and quantitative analysis was performed by combining the gold standard 2D histology and histomorphometry by light microscopy and SEM with a 3D µCT image analysis. The results are summarized in Figure 12 and 13, respectively. Bone tissue sections with the implants were stained with Stevenel's blue and VanGieson pichorfuchsin and were initially closely examined under light microscope for potential differences in the contact between bone tissue and the implants from different groups. No differences in the proximity of bone tissue to the implant was observed between groups as all implants regardless of the coating had good, tight contact with the bone tissue. This qualitative analysis was followed by quantitative analyses using ImageJ software package. All sections were subjected to color threshold to highlight the red color (bone tissue) followed by the measurement of the size of the bone tissue in tissue sections. Total area of the bone (in mm2) and percentage of the section filled with the bone tissue were measured. All data were subjected to ANOVA to determine statistically significant differences between groups of implants. No statistically significant differences were found in the amount of bone tissue surrounding the implant, suggesting that both Vancomycin and LC0024 do not have any negative effect on bone growth in the proximity of the implant.

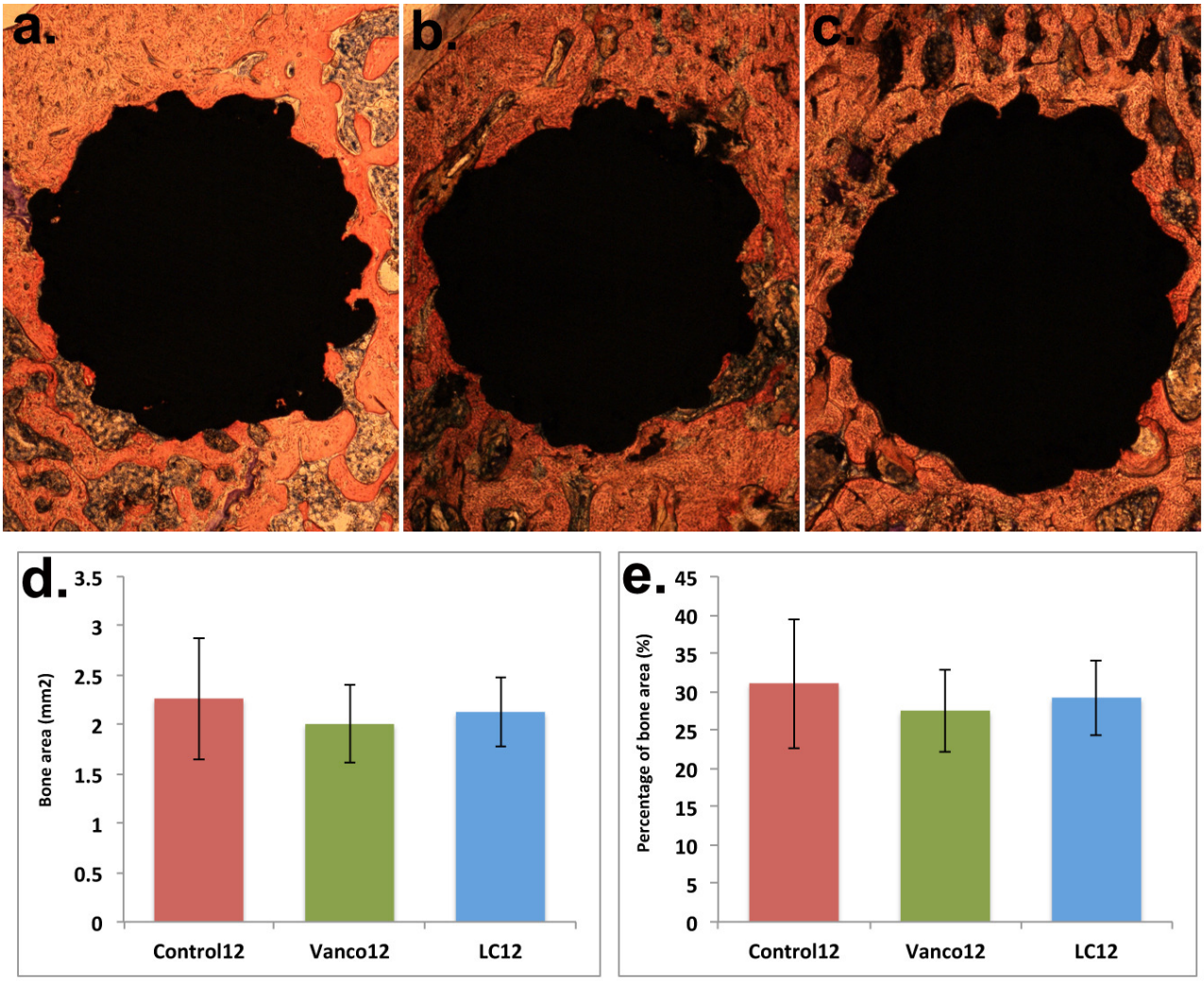


Figure 12: Tissue sections of femurs with the implants (black). Red colored tissue represents the bone tissue, and there was no obvious difference between three kinds of implants (a – control, b – LC0024, c – vancomycin). Statistical analyses revealed no difference in the amount of bone tissue surrounding the implant (d – total area covered by bone in one tissue section, e – percentage of are covered by bone in one tissue section). Images were taken under 40-x magnification with Nikon microscope.

Qualitative analysis of the 3D osseointegration after 8 weeks of implantation shows that the volume and distribution of the bone phase within 150 µm around the VAN-OPTi or LC0024-OPTi surface is comparable to that around control-OPTi surfaces. Quantification of the bone ingrowth in the porous implant surface based on the SEM data in 2D, BIF-2D, after 12 weeks does not show any significant differences between the experimental coatings and control samples.

In summary, the presented results allow to conclude that the bone growth at the bone/implant interface around the experimental vancomycin or LC0024 coatings does not differ from that around pristine OPTi implants, suggesting that the experimental anti-infective coatings do not interfere with osseointegration after implantation.

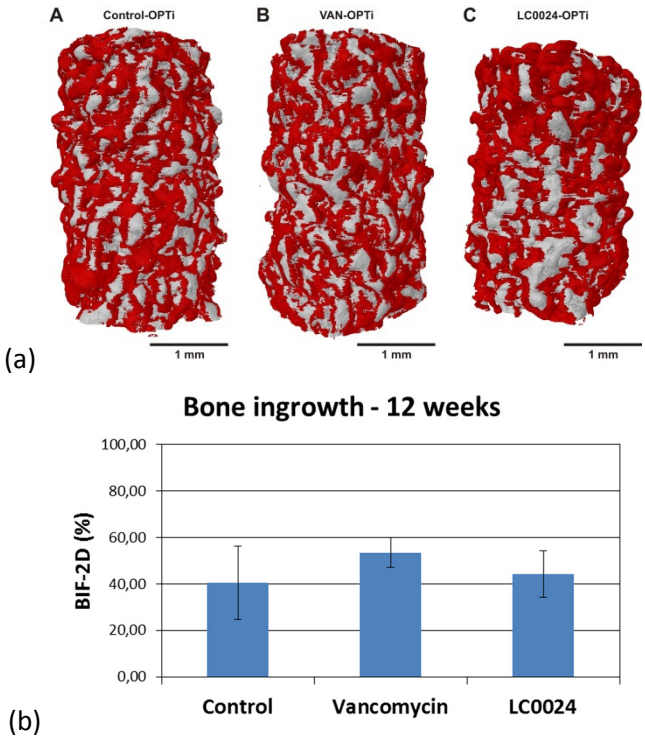


Figure 13. (a) µCT-based 3D visualization of the bone growth (red) within a volume of interest of 150 µm from the implant surface (grey) for control-OPTi (A), VAN-OPTi (B) and LC0024-OPTi (C) implants following 8 weeks of implantation and (b) SEM based bone ingrowth fraction (BIF-2D, %) after 12 weeks of implantation. Results are presented as mean values ± standard deviation.

***In vivo* analysis of osseointegration of the AcSmTi substrates**

The hypothesis that the antifungal compound caspofungin, covalently attached to the titanium implant surface, does not compromise titanium implant osseointegration, was tested *in vivo* in the rabbit. Adult New Zealand white rabbits were used and commercially available screw-shaped conical-shaped implants 'KONTACT' (Biotech Dental, Salon de Provence, France) with 3,0 mm diameter and 10 mm length, either coated or uncoated, were installed, randomly, bilateral in the tibia (Figure 14).

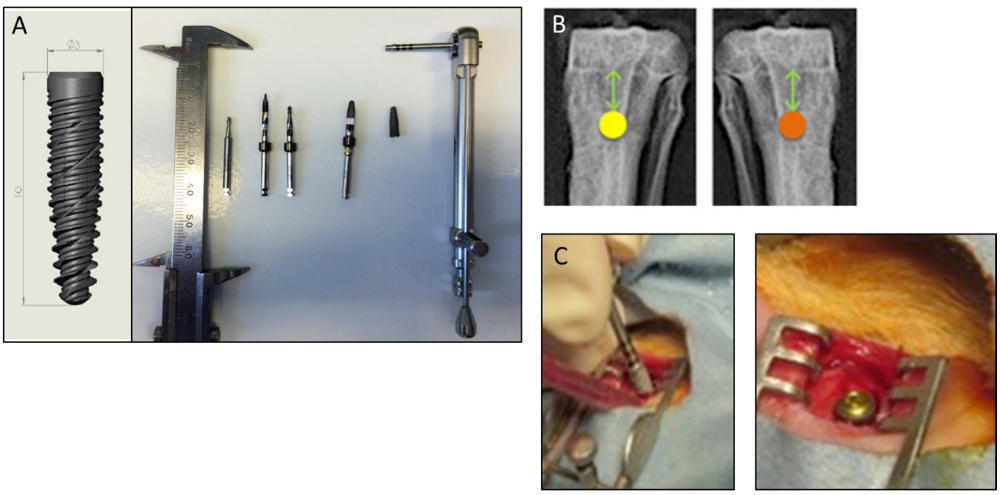


Figure 14. Biotech 'KONTAKT' implant (A); Surgical site of implantation (B); Implant surgery (C).

Healing was allowed for either 1 or 4 weeks. The 1-week time point had been selected in order to observe the early events occurring during implant osseointegration. Based on the differences in bone remodelling

period in humans (3 to 6 months) and in rabbits (6 weeks), it is anticipated that by 4 weeks of implant healing, implant osseointegration has been established though bone remodeling is still ongoing, thereby enabling us to identify potential differences between the caspofungin-coated and the uncoated implant. Expert statistical advice was obtained prior to the start of the study (L-Biostat KU Leuven). We aimed to compare the experimental implant with antifungal coating to the uncoated control implant group using a two-tailed paired t-test to check the null hypothesis that the rate and degree of osseointegration are the same for both groups. Based on previous studies, we anticipated a standard deviation of up to 11% (Braem et al., 2014). For an effect size of 20%, Cohen's standardized effect size equals 20/11=1.82. Based on an unpaired two-tailed t-test with alpha=0.05, a minimum sample size of 6 was required in each group to have at least 80% power in this setting. As 2 healing time points were considered, the total number of required animals was 12. Note that the sample size calculation is performed assuming no correlation within each animal between the conditions. In reality, a correlation is very plausible, so the actual power of the study is expected to exceed largely 80%.

Surgery was uneventful, with no signs of infection. All implants were clinically stable at the time of animal euthanasia. After either 1 or 4 weeks of healing, the animals were euthanized and implants and surrounding bone tissues were isolated, fixed in a CaCO3-buffered formalin solution, dehydrated in an ascending series of ethanol concentration and embedded in methylmethacrylate resin. Qualitative histology on stained sections was performed using light microscopy (Leica Laborlux, Wetzlar, Germany) for assessing bone tissue response in the implant vicinity and at the implant interface. The histological results revealed close contact of the bone tissue with the implant for both the uncoated and the caspofungin-coated implants for both healing times (Figure 15), revealing no adverse effect of the caspofungin coating on implant osseointegration.

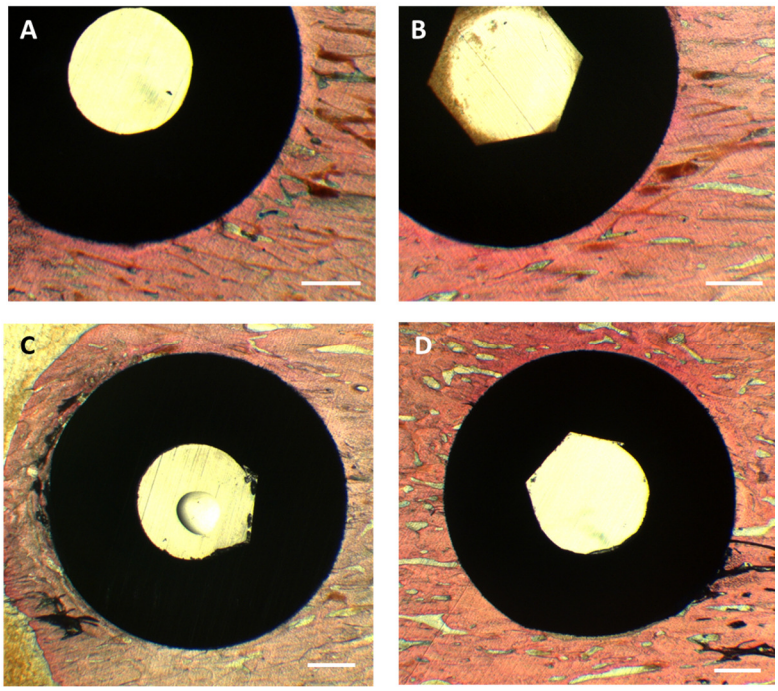


Figure 15. Representative Stevenel's blue and picrofuchsin red stained section, displaying bone apposition onto the implant for (A) 1 wk healed uncoated implant, (B) 1 wk healed coated implant, (C) 4 wks healed uncoated implant and (D) 4 wks healed uncoated implant. Scale bar: 500 µm.

WP6. Antibiofilm coated complex implants and upscaling feasibility study

Development and supply of ABM coated implants

Open porous cylindrical implants were produced for the *in vivo* rat experiments. These implants were coated with an open porous titanium layer using vacuum plasma spray technology, resulting in a coating thickness of 261 µM and a mean porosity of 22%. Upon isopropanol cleaning, the implants were individually vacuum-

packed and distributed to the different partners. Smooth dental implants of the range Kontact (diameter 3mm, length 10 mm), made with titanium TA6V ELI, were supplied by Biotech dental upon sterile packaging, and distributed to the different partners. These implants were coated with ABMs and used in *in vivo* rat or rabbit experiments (WP5).

Coating of complex implants

Implants for rat model

Covalent vancomycin coating was applied to small cylindrical OPTi-implants by the same procedure as described earlier for the OPTi discs. The cylinders had the same OpTi surface as the discs but a small surface compared to the 1 cm OpTi discs. So it was decided to run 6 OpTi discs instead of cylindrical implants in all reaction steps as control samples to do the analytical quality control. The aminated samples (6 OPTi discs, 17 implants) were placed into a 50 mL hydrolysis vessel. An amount of approx. 25 mL n-hexane / hexamethylene diisocyanate (85:15) solution was added to each hydrolysis vessel. The samples were then agitated with the aid of a laboratory roller mixer for three hours at room temperature. The solution was discarded afterwards. The samples were then rinsed thoroughly with n-hexane and stored under n-hexane.

An amount of approx. 0.529 g vancomycin hydrochloride was diluted in 100 mL dimethyl sulfoxide resulting in a clear colourless solution. The solution was then carried over into a 50 mL hydrolysis vessel. The isocyanated samples (stored under n-hexane) were then directly carried over into the hydrolysis vessel containing the vancomycin solution. The samples were then agitated with the aid of a laboratory roller mixer for 16 hours (overnight). The solution was discarded afterwards. The samples were then rinsed 3 times thoroughly with DMSO and 3 times with demineralised, pyrogen-free water. The rinsed samples were then dried at room temperature for 1 hour. Analysis was carried out as described in section 1.2 with OPTi discs instead of the implants because the implant surface is too small. Vancomycin amount was 82,1 pmol/cm².

A different approach was used to coat LC0024. An LC0024 analogue containing a primary amine was synthesized (LC0024-NH2). Upon carboxylation, LC0024 was immobilized (6 OPTi discs, 17 implants, Figure 16). The primary amine of the compound was reacted with the carboxyl moiety on the surface. Reaction was performed by shaking for 16 hours at room temperature. As coupling reagents a combination of DIC (3 equivalents) and Oxyma (2 equivalents) were employed in a dichloromethane/dimethylformamide (95/5) solvent mixture. After completion the solid supports were washed with a DCM/DMF (50/50) mixture (5 times) and with DCM (3 times). Solid supports were dried under an argon stream.

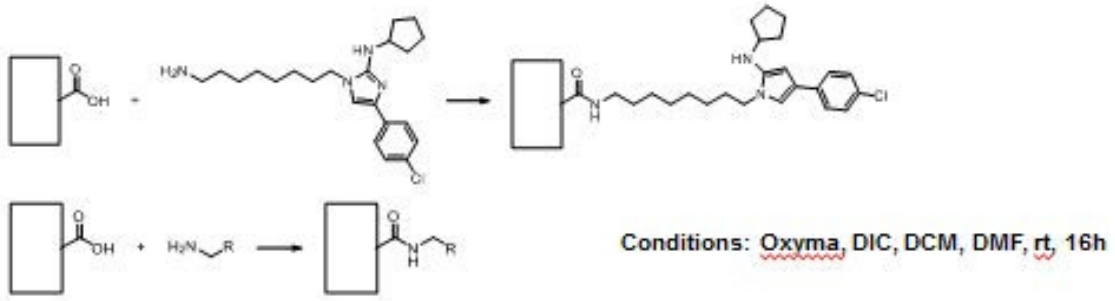


Figure 16. Coating of LC0024 to titanium.

Implants for rabbit model
Covalent caspofungin coating was applied to dental implants by the same procedure as already described earlier for the OPTi discs. The aminated samples (2 OPTi discs, 27 implant screw parts) were placed into a 50 mL hydrolysis vessel. An amount of approx. 30 mL n-hexane / hexamethylene diisocyanate (85:15) solution was added to the hydrolysis vessel. The samples were then agitated with the aid of a laboratory roller mixer for three hours at room temperature. The solution was discarded afterwards. The samples were then rinsed thoroughly with n-hexane and stored under n-hexane.

An amount of approx. 0.250 g caspofungin acetate was diluted in 30 mL dimethyl sulfoxide resulting in a clear colourless solution. The solution was then carried over into a 50 mL hydrolysis vessel. The isocyanated samples (stored under n-hexane) were then directly carried over into the hydrolysis vessel containing the caspofungin solution. The samples were then agitated with the aid of a laboratory roller mixer for 16 hours (overnight). The solution was discarded afterwards. The samples were then rinsed 3 times thoroughly with DMSO and 3 times with demineralised, pyrogen-free water. The rinsed samples were then dried at room temperature for 1 hour.

Analysis was carried out as described above with original implants and discs processed in the same manner. Caspofungin amount on the discs was 3204 pmol/cm², on the implants 12577 pmol/cm².

Upscaling feasibility study for ABM coated implants
We performed a feasibility study on each of the successful coatings identified during COATIM, aiming to produce 100 coated implants/day. The main production steps of the manufacturing process were identified and time, related costs and resources in term of personnel and capital investment were defined.

Costs to coat hip and dental implants with vancomycin, caspofungin and LC0024 were calculated, assuming the production of 100 implants/day in a one 8h shift. These costs include the full process from manufacturing of the implant until the final sterilization process, upon which the product is ready to be implanted. Based on these calculations, the cost to produce and coat hip implants with vancomycin is 30.2 euro. The target sales price would be 40 euro, resulting in a 35% margin. Required capital investments are 3 220 000 euro and certification costs would be 410 000 euro. Production and coating of dental implants with caspofungin would also cost 30.2 euro. Also here, the target sales price would be 40 euro. The required capital investments and certification costs are identical for caspofungin and vancomycin.

Also the costs for manufacturing and coating of hip implants with LC0024 are in the same range: it would cost 30.12 euro and target sales price would also be 40 euro. The slightly decreased cost is due to the low production costs of LC0024. Also capital investments would be identical. However, costs for certification would drastically increase, as a CE mark has to be obtained for this newly identified molecule, resulting in an estimated certification cost of 12 300 000 euro.
To bring these innovative products to the market, it is imperative to qualify and afterwards certify these products. Major costs are attributed to drug approval (12 000 000 euro) and the required clinical tests (2 000 000 euro). Design verification will cost around 250 000 euro. All processes and final application on the implants must be validated according to the latest standards (250 000 euro). In addition there are also costs associated to the work performed by the notified bodies in the different countries where the product will be distributed (100 000 euro).

Based on these calculations, we can conclude that the price of the compound is irrelevant for the product price. The required capital investments are identical for the three coatings. However, whereas certification costs for vancomycin and caspofungin are around 400 000 euro, the certification costs of LC0024 are significantly higher, as this new drug still needs to be approved. The target market price is expected at 40 euro per coated implant, as higher prices will not have commercial success in Europe.

1.4. Potential impact, main dissemination activities and exploitation of results

Impact

Up to five percent of implants are subject to costly and burdensome revision surgery due to infection. The formation of bacterial and/or fungal biofilms on implants, that are resistant to current antibiotics/antimycotics, is the major factor responsible for these implant infections. Therefore, there is an urgent need to develop novel antibiofilm implant coatings. COATIM focused precisely on this need, because it aimed to develop coatings for orthopaedic and dental implants containing novel proprietary antibiofilm molecules or standard antimicrobial agents, thereby reducing the need for costly and burdensome implant revisions due to infections.

Within COATIM, we demonstrated that by carefully engineering the antibiofilm coatings, biofilm prevention against bacterial or fungal pathogens can be established. Our research will open many opportunities in the field of biomedical implants as our technology is not limited to dental and orthopaedic implants, but can also be implemented to other implants.

Exploitation

The final aim of COATIM was to license the developed coating techniques to implant manufacturers for dental and orthopaedic implants. Therefore, the final phase of COATIM involved the development of complex implant components with the selected antibiofilm coatings. These included dental implant screws and cylindrical implants as a model for orthopaedic implants. Data from the *in vivo* experiments conducted within COATIM showed *in vivo* efficacy of the coatings without hampering osseointegration, documenting proof-of-concept. In order to facilitate exploitation, a special Workpackage (WP6) was defined comprising a feasibility study on the upscaling of the coating processes in order to make a planning and cost calculation for an industrial process (described in work package 6). However, follow-up research projects to complete the necessary preclinical trials are needed before results can be transferred to industry.

Follow-up research projects include:
-more detailed *in vivo* preclinical studies, using various infection models, and demonstrating effectiveness and stability of the coating over longer periods of time, allowing complete integration of implant
-coating of combinations of active molecules as to inhibit biofilm formation of bacterial and fungal biofilms simultaneously

Successful application of antibiofilm coatings on complex implants will enhance the market penetration of implants with an osseointegrative porous Ti top coating such as vacuum plasma sprayed Ti coatings. In general, the Consortium expects all next generations of biomedical implants to have an integrated antibiofilm functionality in the future.

Dissemination

The start of the COATIM project was marked by a press release on the KU Leuven website. At the end of the project, a concluding COATIM workshop was organized, open to the public (see below for more information). In addition, on Dec 10, 2015, a EU delegation of 18 participants (headed by dr. Koen Van Dyck, head of AMR Task Force) attended a special COATIM meeting, highlighting COATIM results, interactions between academia and SMEs and the problem of antimicrobial resistance in general. This meeting took place on Dec 10, 2015 at the project coordinator's premises (CMPG, KU Leuven).

Following specific activities to disseminate COATIM results were taken up during the project:

• publication of COATIM-based research results and technological advances in peer-reviewed scientific journals: 21 publications in total of which 7 joint publications originating from research performed by at least 2 different COATIM partners.
• participation of COATIM partners in more than 50 workshops, national and international scientific meetings

• distribution of the COATIM flyer during the above workshops and meetings. This flyer, which explains the main objectives and results of COATIM in layman's terms to reach a broad public, is also available on our project website (www.coatim.eu).
• continuous updating of our project website
• organization of various networking events involving other consortia (BALI and BacAttack consortia) by COATIM. Partners of all these consortia attended the concluding COATIM workshop on Dec 18, 2015.
• organization of the concluding COATIM workshop, which was attended by 48 participants from 10 different nationalities. Also industry was present, namely Insitut Straumann, Educell, Layerwise, BioModics, Bactiguard, Hemoteq and Alhenia.
• participation of the scientific coordinator in the iProMedai COST action (TD1305), thereby promoting results from COATIM and providing reference materials developed within COATIM, to be tested by all partners from this COST network.

Address of the project public website

www.coatim.eu

2. Use and dissemination of foreground

2.1. Section A

2.1.1. List of all scientific (peer reviewed) publications

LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS										
NO.	Title	Main author	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Year of publication	Relevant pages	Permanent identifiers[1] (if available)	Is/Will open access[2] provided to this publication?
1	Identification of Fungicidal 2,6-Disubstituted Quinolines with Activity against Candida Biofilms.	P1b	Molecules	17			2012	12243-51	http://www.mdpi.com/1420-3049/17/10/12243/pdf	yes
2	Potentiation of Antibiofilm Activity of Amphotericin B by Superoxide Dismutase Inhibition	P1b	Oxidative Medicine and Cellular Longevity	2013			2013	704654	http://dx.doi.org/10.1155/2013/704654	yes

[1] A permanent identifier should be a persistent link to the published version full text if open access or abstract if article is pay per view) or to the final manuscript accepted for publication (link to article in repository).

[2] Open Access is defined as free of charge access for anyone via Internet. Please answer "yes" if the open access to the publication is already established and also if the embargo period for open access is not yet over but you intend to establish open access afterwards.

3	Recent insights into Candida albicans biofilm resistance mechanisms.	P2	Current genetics	59(4)			2013	251-264	DOI 10.1007/s00294-013-0400-3	yes
4	Peri- and intra-implant bone response to microporous Ti coatings with surface modification	P1d	Acta Biomaterialia	10			2014	986-995	10.1016/j.actbio.2013.10.017	yes
5	Bacterial colonisation of porous titanium coatings for orthopaedic implant applications – effect of surface roughness and porosity	P1d	Powder Metallurgy	56			2013	267-271	http://dx.doi.org/10.1179/0032589913Z.00000000000124	yes
6	Reactive oxygen species-inducing antifungal agents and their activity against fungal biofilms.	P1b	Future Medicinal Chemistry	6			2013	77-90	10.4155/fmc.13.189.	yes
7	The plant-derived decapeptide OSIP108 inhibits *Candida albicans* biofilm formation without affecting cell viability	P1b, P8	AAC	58			2014	2647-56	doi: 10.1128/AAC.01274-13	yes
8	Structure-Activity Relationship Study of the Plant-Derived Decapeptide OSIP108 Inhibiting Candida albicans Biofilm Formation.	P1b	AAC	58			2014	4974-7	10.1128/AAC.03336-14	yes

9	Derivatives of the Mouse Cathelicidin-Related Antimicrobial Peptide (CRAMP) Inhibit Fungal and Bacterial Biofilm Formation.	P1b, P1a, P1c, P8	AAC	58			2014	5395-404	doi: 10.1128/AAC.03045-14	yes
10	Protocol for determination of the persister subpopulation in *Candida albicans* biofilms	P1b	Methods in Molecular Biology	1333			2016	67-72	10.1007/978-1-4939-2854-5_6	yes
11	Identification and characterization of an anti-pseudomonal dichlorocarbazol derivative displaying anti-biofilm activity.	P1c, P1a, P1b, P3, P8, P4	Bioorg Med Chem Lett.	24			2014	5404-5408	doi: 10.1016/j.bmcl.2014.10.039.	yes
12	Evaluation of the toxicity of 5-aryl-2-aminoimidazole-based biofilm inhibitors against eukaryotic cell lines, bone cells and the nematode Caenorhabditis elegans.	P1a, P1b, P8	Molecules	19			2014	16707-23	doi: 10.3390/molecules191016707	yes
13	Peri-implant bone response to novel PM porous Ti coatings	P1d	Powder Metallurgy	57			2014	84-88	http://dx.doi.org/10.1179/0032589914Z.00000000000166	yes

14	Novel anti-infective implant substrates: controlled release of antibiofilm compounds from mesoporous silica-containing macroporous titanium	P1d	Colloids and Surfaces B: Biointerfaces	126			2015	481-488	Doi: 10.1016/j.colsurfb.2014.12.054.	yes
15	Fungal β-1,3-Glucan Increases Ofloxacin Tolerance of *Escherichia coli* in a Polymicrobial *E. coli/Candida albicans* Biofilm.	P1b	AAC	59			2015	3052-8	Doi: 10.1128/AAC.04650-14	yes
16	Combinatorial drug approaches to tackle *Candida albicans* biofilms	P1b	Expert Review of Anti-infective Therapy	13			2015	973-984	Doi: 10.1586/14787210.2015.1056162	yes
17	Antimicrobial Peptides as a Strategy to Combat Fungal Biofilms	P1b	Current Topics in Medicinal Chemistry	accepted					Accepted, awaiting publication	yes
18	Alternating current electrophoretic deposition of bovine serum albumin onto magnesium	P1d	Key Eng. Mater.	654			2015	139-43		yes

19	Electrophoretic deposition of porous Ti coatings for bone implants: in vitro and in vivo evaluation	P1d	Key Eng. Mate.	654			2015	144-148		yes
20	Gene expression variability in clonal populations: causes and consequences	P1a	Critical reviews in microbiology	Accepted, awaiting publication			2015		10.3109/1040841X.2015.1122571	yes
21	Evaluation of the antibacterial and antibiofilm activity of novel CRAMP-vancomycin conjugates with diverse linkers.	P1a	Organic and Biomolecular Chemistry	13			2015	7477-86	10.1039/c5ob00830a	Yes
22	The Effect of Antimicrobial Implant Coatings on Yeast Biofilm Formation	P4	Master thesis Sarah Jensen	06-09-2014	Linköping University, Sweden	Scientific community	2014			
23	Identification and mode of action analysis of new antibiofilm compounds against the fungal pathogen *Candida albicans*	P1b	PhD thesis Nicolas Delattin	October 2014	KU Leuven, Belgium	Scientific community	2014			
24	A novel antimicrobial in the fight against the nosocomial pathogen Pseudomonas aeruginosa	P1c	Master thesis, Eline Blommaert	June 2015	KU Leuven, Belgium	Scientific community	2015			

25	Novel approaches to prevent and treat infections involving staphylococcal biofilms	P3	PhD thesis Anna Lippell	To be submitted 31st December 2015	University of Leeds, UK	Scientific community	2015	UK		
27	Covalent immobilization of antimicrobial agents on titanium prevents *Staphylococcus aureus* and *Candida albicans* colonization and biofilm formation	P2, P1c, P1b, P1d, P5,P4, P6, P1e, P7, P8, P9	JAC	accepted	VIB, Belgium	Scientific community	2015		10.1093/jac/dkv437	yes
28	Biofunctionalisation of metallic surfaces	P1d	Master thesis Yexiao Chen	June 2013	KU Leuven, Belgium	Scientific community	2013			
29	Development of porous materials for localized drug delivery	P1d	Master thesis Dan Yang	June 2013	KU Leuven, Belgium	Scientific community	2013			
30	Experimental Evolution in Biofilm Populations.	P1a	FEMS Microbiology Reviews				2016		Minor revision	yes
31	FabR regulates Salmonella biofilm formation via its direct target FabB	P1a	BMC Genomics				2016		Revision	yes

32	Elucidation of the mode of action of a new antibacterial compound active against *Staphylococcus aureus* and *Pseudomonas aeruginosa*	P1c, P3, P1b	Plos One						revision	yes
33	Modulation of the substitution pattern of 5-aryl-2-aminoimidazoles allows to fine-tune their anti-biofilm activity spectrum and toxicity	P1a, P1b, P1c, P2 P8	AAC						Submitted	yes
34	Pyrimidine limitation inhibits *Salmonella* biofilm formation via csgD repression despite increased c-di-GMP levels.	P1a	Molecular Microbiology						Submitted	yes
35	Antibacterial activity of a new broad-spectrum antibiotic covalently bound to titanium surfaces	P1c; P2, P4, P6; P1b, P8	Journal of Orthopaedic Research						revision	
36	Identification And Characterization Of New, Broadly Applicable Small Molecule Inhibitors Of *Salmonella* Biofilm Formation	P1a	PhD Thesis, Stijn Robijns	September 2013	KU Leuven, Belgium	Scientific community				

37	Evolutionary dynamics of resistance development against a biofilm inhibitor in *Salmonella* Typhimurium	P1a	PhD Thesis, Akanksha Dubey	November 2014	KU Leuven, Belgium	Scientific community				
38	Study of specific metabolic processes as innovative targets for combating bacterial biofilms	P1a	Master Thesis, Elien Peeters	June 2013	KU Leuven, Belgium	Scientific community				
39	*Salmonella* biofilm inhibitors; mode of action and triggered release from MOF coatings	P1a	Hannes Campo	June 2013	KU Leuven, Belgium	Scientific community				
40	The study of specific metabolic processes as innovative targets for combating bacterial biofilms.	P1a	Master Thesis, Mizanur Rahman	August 2014	KU Leuven, Belgium	Scientific community				
41	Inhibition of Common Good Production in *Salmonella* Biofilms as an Antimicrobial Strategy that selects against Resistance	P1a	Master Thesis, José Francisco Bergua Canudo	June 2014	KU Leuven, Belgium	Scientific community				

42	Study of specific metabolic processes as innovative targets to combat bacterial biofilms	P1a	Master thesis, Bram Lories	June 2015	KU Leuven, Belgium	Scientific community				

2.1.2. List of all dissemination activities

LIST OF DISSEMINATION ACTIVITIES

NO.	Type of activities	Main leader	Title	Date	Place	Type of audience	Size of audience	Countries addressed
1	Press release	P1b	Onderzoekers ontwikkelen ontstekingswerende coatings voor implantaten	11/10/2011	Leuven, Belgium	Scientific community	10 000	Belgium
2	Conference 'Knowledge for growth': poster presentation	P1b	Coatim, development of antibiofilm coatings	24/05/2012	Ghent, Belgium	Scientific community, Industry	1000	Belgium
3	Newletter SIK	P4	Implant coatings to prevent biofilm formation and microbial infection	January 2012	Sweden	Industry	200	International
4	Conference 'Knowledge for growth': oral presentation	P1a		24/05/2012	Ghent, Belgium	Scientific community, Industry	1000	Belgium

5	Conference: International Powder Metallurgy Congress & Exhibition – EuroPM2012: oral presentation	P1d	Bacterial colonization of porous titanium coatings for orthopaedic implant applications – A comparison	16/9-19/9/2012	Basel, Switserland	Scientifc community, Industry	400	Switzerland, International meeting
6	Conference ' ASM conference on biofilms': poster presentation	P1b	Coatim, development of antibiofilm coatings	29/9-4/10/2012	Miami, USA	Scientific community	400	USA, international meeting
7	Conference: 5th Annual Symposium on Host Defence Peptides: Biology and applications of antimicrobial and related peptides: poster presentation	P1b	Coatim, development of antibiofilm coatings	25/10/2012	Utrecht, Netherlands	Scientific community, Industry	40	Netherlands, International meeting
8	Conference: 2nd International Conference on Antimicrobial Research - ICAR	P1d	Porous titanium coatings for implant applications: the role of surface properties in	21-23/11/2012	Lisbon, Portugal	Scientific community, healthcare professionals	500	Portugal, international meeting

	2012: poster presentation		controlling bacterial colonisation					
9	Seminar at KULeuven	P1b	Coatim, development of antibiofilm coatings	18-12-2012	Leuven, Belgium	Scientific community	40	Belgium
10	Website of CMPG-SPI	P1c			http://www.biw.kuleuven.be/dtp/cmpg/spi/translational.aspx			
11	Joint meeting of BALI, BacAttack and COATIM	P1b P1a		7-05-2013	Leuven, Belgium	Scientific community, industry	30	Belgium, international meeting
12	First Congress of Slovenian society of Experimental animals: oral presentation	P5	Laboratory mice in neuroendocrine and behavioral studies	24-01-2013	Ljubljana, Sovenia	Scientific community, pharmaceutical industry	100	Slovenia, Italy
13	KU Leuven MRC Annual Research Meeting: oral presentation	P1d	Biofunctionalisation of implant surfaces	26-02-2013	Leuven, Belgium	Scientific community, industry	50	Belgium
14	113th General Meeting ASM,	P1c	Small Molecules Targeting *Pseudomonas*	18-21/05/2013	Denver, USA	Scientific community	10000	USA, Internati

	poster presentation		*aeruginosa* Persisters					onal meeting
15	Conference 'Knowledge for growth': posterpresentation	P1b	Coatim: development of antifungal coatings II	30-05-2013	Ghent, Belgium	Scientific community, Industry	1000	Belgium
16	Press release	P4	COATIM, antibiofilm coatings for implants	June 2013	Gothenburg, Sweden	Industry	216	
17	Eurobiofilms conference, oral presentation and poster	P1b	Development of antibiofilm coatings for implants	September 2013	Ghent, Belgium	Scientific community	230	International meeting
18	Eurobiofilms conference, oral presentation and poster	P1b	The plant-derived decapeptide OSIP108 inhibits *Candida albicans* biofilm formation without affecting cell viability	September 2013	Ghent, Belgium	Scientific community	230	International meeting

19	Eurobiofilms conference, oral presentation	P1b	Repurposing as a means to increase the activity of Amphotericin B and Caspofungin against *Candida albicans* biofilms	9 – 12th September 2013	Ghent, Belgium	Scientific community	230	International meeting
20	Eurobiofilms conference, poster	P4	Evaluation of the ability of antimicrobial molecules to prevent biofilm formation on titanium implant materials using CLSM	9 – 12th September 2013	Ghent, Belgium	Scientific community	230	International meeting
21	Eurobiofilms, oral presentation	P2	Yeast biofilm models	9 – 12th September 2013	Ghent, Belgium	Scientific community	230	International meeting
22	Eurobiofilms, oral presentation	P2	Novel tools to study biofilm formation in live animals and Als3 peptides as novel	9 – 12th September 2013	Ghent, Belgium	Scientific community	230	International meeting

			antibiofilm molecules					
23	British Society for Medical Microbiology, oral presentation	P2	Molecular tools to investigate fungal pathogenicity	April 12-16, 2013	Newcastle, UK	Scientific community	60	International
24	FEBS course on human fungal pathogens, oral presentation	P2	Als3-specific peptides reduce epithelial adhesion and invasion of C. albicans cells and reduce biofilm development	May 29, 2013	La Colle Sur Loup, France	Scientific community	180	International
25	30th ISSY (international specialized symposium on yeast, oral presentation	P2	Development of in vivo Candida albicans biofilm model systems and Als3-specific peptides as novel antibiofilm molecules	Juni 21, 2013	Stara Lesna, Slovakia	Scientific community	100	International
26	Sinbio: International symposium on biodiversity, oral presentation	P2	Diversity of pathogenic Candida strains in biofilm formation	September 2, 2013	Lavras, Brazil	Scientific community	70	International

			and phenotypic switching					
27	Federal University of Minas Gerais, oral presentation	P2	Diversity of pathogenic Candida strains in biofilm formation and phenotypic switching	September 5, 2013	Belo Horizonte, Brazil	Scientific community	30	Brazil
28	14th International Conference on Pseudomonas, poster presentation	P1c	Oral presentation Novel tools to study biofilm formation in live animals and Als3 peptides as novel antibiofilm molecules	7-11.09.2013	Lausanne, Switzerland	Scientific community	400	International meeting
29	Conference: international Powder Metallurgy congress and exhibition – EuroPM20113, Oral presentation	P1d	Peri-implant bone response to novel PM porous Ti coatings'	15-18/09/2013	Gothenburg, Sweden	Industrial and scientific communityy	400	International meeting

30	Food & Drug Development from many perspectives, poster presentation, SP Technical Research Institute of Sweden	P4	Evaluation of the ability of ABMs to prevent biofilm formation on titanium implants using CLSM and TEM	27-11-2013	Södertälje, Sweden	Industrial community	40	International meeting
31	PhD seminar program CUSO	P2	Development and use of C. albicans in vivo biofilm systems and novel tools to prevent biofilm formation	February 19, 2014	Lausanne, Switzerland	Scientific community	15	Switzerland
32	Annual report SIK 2013	P4	New antimicrobial coatings reduce risk of implant infection	01-03-2014	Göteburg, Sweden	Scientific and industrial community	800	International
33	Conference Knowledge for growth: poster presentation	P1b	COATIM, development of antibiofilm coatings	08-05-2014	Ghent, Belgium	Scientific community, Industry	1000	Belgium
34	VIB seminar: poster presentation	P1b	*The plant-derived decapeptide OSIP108 inhibits Candida albicans*	28-30-04-2014	Blankenberghe, Belgium	Scientific community	800	Belgium

			biofilm formation without affecting cell viability					
35	International summer school on biofilms, oral presentation	P2	Fungal biofilms: structure, characteristics, antifungal drug resistance mechanisms	September 15-18, 2014	Vaalbeek, Belgium	Scientific community	60	Internati onal
36	First international Conference on Materials in Medicine, oral presentation	P8	Differential exhibition of cytotoxicity in mouse cell line L929 compared to human primary cell culture	8-11/10/201 3	Faenza Italy	Scientific and industrial community	200	Italy
37	Nanotechnology for heatlh, talk	P1b	Antimicrobial coatings for titanium, towards anti-infective implants	24/09/201 4	Imec, Leuven, Belgium	Scientific and industrial community	70	Belgium
38	IMAP conference, poster	P1b	Synergistic activity of peptides with standard antimycotics against biofilms	30/09/201 4	Graz, Austria	Scientific community	100	Internati onal

39	COA 2014	P7	COATIM, development of antibiofilm coatings, poster	21 Nov. 2014 to 23 Nov. 2014	Beijing, China	Industry and scientific community	10.000	Internati onal
40	Award / Conference: International Powder Metallurgy Congress & Exhibition – EuroPM2014: 1 prize for PhD with oral presentation in plenary session	P1d	Development of biofunctional porous coatings for bone implants	22/09/201 4	Salzburg, Austria	Scientific community, industry	800	Internati onal meeting
41	Conference: European Association for Osseointegration 23rd Annual Scientific Meeting (EAO 2014), poster presentation	P1d	Novel anti-infective implant substrates: controlled release of antibiofilm compounds to mesoporous silica incorporated in macroporous titanium	24/27/09/2 015	Rome, Italy	Scientific and industrial community	2000	Internati onal meeting
42	Newsletter SIK	P4	COATIM, antibiofilm	October 2014	Sweden	Industry	200	Internati onal

			coatings for implants					
43	Conference: 5th International Conference on Electrophoretic Deposition: Fundamentals and Applications (EPD 2014), oral presentation	P1d	Alternating current electrophoretic deposition (AC-EPD) of biomolecule coatings	5-10 October 2014	Hernstein, Austria	Scientific and industrial community	100	International meeting
44	Conference: 5th International Conference on Electrophoretic Deposition: Fundamentals and Applications (EPD 2014), oral presentation	P1d	Electrophoretic deposition of porous Ti coatings for bone implants, *in vitro* and *in vivo* evaluation	5-10 October 2014	Hernstein, Austria	Scientific and industrial community	100	International meeting
45	Conference: the 2015 IADR/AADR/CADR General Session & Exhibition, oral presentation	P1d	Novel anti-infective implant substrates: controlled release of antibiofilm compounds through mesoporous silica	March 2015	Boston, Massaachussetts, USA	Scientific and industrial community	6000	International meeting

			incorporated in macroporous titanium					
46	Newsletter SIK	P4	COATIM – Identifying and characterising an antipseudomonal compound with anti-biofilm activity	January 2015	Sweden	Industry	200	Internati onal
47	Graduation course in Biochemistry, Workshop on pathogenic fungi, oral presentation	P2	Molecular mechanisms determining azole tolerance in *Candida albicans*	April 15th, 2015	São Paulo, Brazil	Scientific community	85	
48	Workshop on fungal pathogens, oral presentation	P2	Treatment or prevention of Candida albicans and Candida glabrata biofilms present on implants in the host	April 15th, 2015	Ribeirao Preto, Brazil	Scientific community	30	
49	Hot topics in microbiology, oral presentation	P2	Development and use of *in vivo* fungal and mixed fungal-bacterial	April 25, 2015	Štrbské Pleso, Slovakia	Scientific community	100	

			biofilm model systems					
50	Seminar Hernieuwbare en biodegradeerbare materialen (LeuvenInc)	P1b	De nieuwe generatie implantaten: anti-infective coatings	April 22nd, 2015	Leuven, Belgium	Scientific and industrial community	30	Belgium
51	GRC on antimicrobial peptides, poster	P1b	Structure-activity relationship study of a 14-AA peptide fragment of the mouse cathelicidin-related antimicrobial peptide (CRAMP) that inhibits *Candida albicans* biofilm formation	May 3rd – 8th, 2015	Il Ciocco, Italy	Scientific and industrial community	150	International
52	CTESS Symposium 2015 (17th April 2015)	P8	Distribution of COATIM poster-prints	April 17th, 2015	Ljubljana, Slovenia	Scientific and industrial community	130	International
53	6th Congress of European Microbiologists	P1c	Elucidation of the mode of action of a new anti-	7-11 June 2015	Maastricht, the Netherlands	Scientific community		International

	(FEMS 2015), poster		pseudomonal compound					
54	6th Congress of European Microbiologists (FEMS 2015), distribution of COATIM flyers	P1c	COATIM flyer	7-11 June 2015	Maastricht, the Netherlands	Scientific community		Internati onal
55	ASM conference on mechanisms of interbacterial cooperation and competition	P1a	Oral presentation: Interference with Microbial Cooperation as an Antimicrobial Strategy that Selects Against Resistance : Proof of Concept on Salmonella Biofil m EPS	13-16 March 2015	Washington, USA	Scientific community	50	Internati onal
56	Eurobiofilms 2015	P1b	Oral presentation and poster: b-1,3-glucan increases Escherichia coli ofloxacin tolerance in a polymicrobial E.	23-26th June, 2015	Brno, Czech Republic	Scientific community	100	Internati onal

			coli / Candida albicans biofilm					
57	23rd Annual Meeting of the European Orthopaedic Research Society	P1b	Oral presentation Novel anti-infective implant substrates	3-4th September, 2015	Bristol, UK	Scientific community	450	International
58	55th *Interscience Conference* on Antimicrobial Agents and Chemotherapy (*ICAAC*) – poster presentation	P3	Poster: BPCH: An Antibiofilm Compound for Coating Medical Device Implants	17th-21st September 20125	California, USA	Scientific community, industry	10,000	International
59	Third workshop on bacterial and fungal biofilms, oral presentation	P1b	Oral presentation: b-1,3-glucan increases Escherichia coli ofloxacin tolerance in a polymicrobial E. coli / Candida albicans biofilm	September 4th, 2015	Antwerp, Belgium	Scientific community	40	International
60	Third workshop on bacterial and fungal biofilms, oral presentation	P1b	Distribution of COATIM flyer	September 4th, 2015	Antwerp, Belgium	Scientific community	40	International

61	7th ASM conference on biofilms, oral presentation	P1a	A covalent anti-biofilm coating for orthopedic implants	October 25-29 th	Chicago, USA	Scientific community	500	Internati onal
62	Project meeting at Radboud University Medical Center, oral presentation	P1d	Biofunctionalisati of implant surfaces	9/11/2015	Nijmegem, The Netherlands	Scientific community	10	Internati oanl
63	Seminar at Friedrich Alexander Universität Erlangen, oral presentation	P1d	Alternating current electrophoretic deposition (AC-EPD) of biomolecule coatings	December 2015	Erlangen, Germany	Scientific community	30	Internati onal
64	COATIM conference " Anti-infective strategies for medical devices" at KU Leuven, oral presentation	P1d	Anti-infective coatings for titanium implants by alternating current electrophoretic deposition	18/12/201 5	Leuven, Belgium	Scientific community and industry	50	Internati onal
65	10th World Biomaterials Congress	P1d	Anti-infective coatings for titanium implants by alternating current	17-22/05/201 6	Montreal, Canada	Scientific community	3-4000	Internati onal

	(WBC2016), poster		electrophoretic deposition					
66	COATIM conference " Anti-infective strategies for medical devices" at KU Leuven, oral presentation	P1b	Overview of FP7 project COATIM: development of antibiofilm coatings for implants	18/12/2015	Leuven, Belgium	Scientific community and industry	50	International
67	COATIM conference " Anti-infective strategies for medical devices" at KU Leuven, oral presentation	P8	Evaluation of cytotoxicity and osseointegration potential of antibiofilm molecules in vitro	18/12/2015	Leuven, Belgium	Scientific community and industry	50	International
68	Organization of COATIM conference " Anti-infective strategies for medical devices" at KU Leuven, open to public	P1b		18/12/2015	Leuven, Belgium	Scientific community and industry	50	International

2.2.Section B

2.2.1.List of applications for patents, trademarks, registered designs, etc.

LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP Rights[3]:	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant (s) (as on the application)
Patent	No		GB1218813.2	Antimicrobial peptides	Briers Y, Cammue B, De Brucker K, Delattin N, Landuyt B, Lavigne R, Luyten W, Robijns S, Schoofs L, Steenackers H, Thevissen K, Vandamme D, Vanderleyden J, Walmagh M, Boonen K
Patent	yes	27/04/2017	GB1518988.9	Compounds, components and methods for controlling biofilms	KU Leuven – specific inventors not yet identified

[3] Possible types of IP rights: Patents, Trademarks, Registered designs, Utility models, Others.

2.2.2.List of exploitable foreground

LIST OF EXPLOITABLE FOREGROUND					
N°	*Exploitable Results*	*Exploitable Result Manager*	*Partners Involved*	*Relevant WPs*	*Relevant Deliverables*
1	Mode of action of ABMs against fungal and bacterial biofilms		P1a,P1b, P1c, P2, P3, P4	WP2	D1.1, D1.2, D2.1, D2.2, D2.3, D2.4, D5.5
2	Antibiofilm coated Titanium substrates and implant components		all	WP3, 4	D3.1, D3.2, D3.3, D3.4, D5.5, D6.2

More details regarding the exploitable foreground can be found in the PUDF plan below.

Grant Agreement Number: 278425

Development of antibiofilm coatings for implants

Plan for using and disseminating of the foreground (PUDF)

SEVENTH FRAMEWORK PROGRAMME

COLLABORATION PROGRAMME

Project start date: 01.01.2012

Duration: 48 months
Deliverable 8.3 due date: M48 (December, 2015)
Actual submission date:
Dissemination leven: CO (confidential)
Work package: WP8 – Dissemination and exploitation.
Workpackage leader: Hemoteq (partner 6), Alhenia (partner 7)
Contact: Martin Erdtmann, Jasminka Kovac
Project URL: http://www.COATIM.eu

Project co-funded by the European Commission within the Seventh Framework Programme		
Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

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Section 2 - Risk Assessment and Action Plan ..

Section 3 - Dissemination of knowledge

Section 4 - Publishable results

Section 1 - Exploitable knowledge and its use

The expected exploitable results are given in the Table 1 below. IPR issues (background/pre-existing and foreground information) are reported in the two matrixes below (matrix 1&2). The exploitation claims of the partners are indicated in matrix 3.

Table 1: Overview of the exploitable knowledge

1: Mode of action of ABMs against fungal and bacterial biofilms

2: Antibiofilm coated Titanium substrates and implant components

Category number	Exploitable Knowledge (description)	Exploitable product(s) or measure(s)	Sector(s) of application	Time-table for commer-cial use	Patents or other IPR protection	Owner & other Partner(s) involved	Deliverable	Background/fore ground knowledge
2	Surface-treated smooth Ti (SmTi) substrates					Biotech	**D3.1**	**B**
1	A list of all antibiofilm molecules (ABMs) ranked according to potency, activity spectrum and non-toxicity for *C. elegans*					P1a,b,c P2, P3,10	**D1.1**	**B**

1	A list of the 20 best ABMs with their intracellular accumulation level in biofilm cells and indication of specific 2chemical groups					P1a,b,c P2, P3, P8, P10	**D1.2**	**F**
2	First generation of Antibiofilm coated SmTi (AcSmTi) substrates					HTQ, P1d,	**D3.2**	**B, F**
2	Design of osseointegrative specimen (cylindrical profile					P7	**D5.5**	**F**
1	A list of ABM biofilm target genes & tolerance genes					P1a, b,c, P2, P3	**D2.1**	**F**
2	Surface-treated osseointegrative porous Ti (OPTi) substrates					P7	**D3.3**	**B, F**
1	Report on the effect of ABMs on the attachment and biofilm formation and the					SIK	**D2.4**	**F**

	microstructure of microorganisms							
2	First generation of Antibiofilm coated OPTi (AcOPTi) substrates					P1d, P6	**D3.4**	**F**
2	Antibiofilm coated complex implant components					P1d, P6	**D6.2**	**F**
1	*In vivo* osseointegration profile of 2 types of AcOPTi substrates					P5	**D5.5**	**B, F**
1	Biochemical support for suspected ABM targets and validation of the genetic data of D2.1					P1a., b, c, P2, P3	**D2.2**	**F**
1	Effect of the ABM on essential cellular processes					P3	**D2.3**	**F**

Patent applications

See table 2.2.1

Table 2 – Summary table of exploitable results

N°	*Exploitable Results*	*Exploitable Result Manager*	*Partners Involved*	*Relevant WPs*	*Relevant Deliverables*
1	Mode of action of ABMs against fungal and bacterial biofilms		P1a, b,c, P2, P3, P4	WP2	D1.1, D1.2, D2.1, D2.2, D2.3, D2.4, D5.5
2	Antibiofilm coated Titanium substrates and implant components		all	WP3, 4	D3.1, D3.2, D3.3, D3.4, D5.5, D6.2

Characterisation of each Exploitable Results

The templates are presented in the same order as the list of Table 2.

Exploitable Result n° 1: Mode of action of ABMs against fungal and bacterial biofilms

Describe the innovation content of result	Mode of action of ABMs against fungal and bacterial biofilms
Who will be the customer?	Lead users will be basic and applied researchers who are involved in research aimed to increase understanding of the mode of action of antibiofilm molecules as well as of tolerance of biofilm cells against antibiofilm molecules.
What benefit will it bring to the customers?	The elucidation of the mode of action of the ABMs as well as identification of tolerance pathways will lead to a better understanding of fundamental aspects of biofilm formation, microbial physiology inside biofilms, intracellular signaling pathways and processes related to the defense of biofilm cells against the ABMs. Hence, the ABMs may be used as tools to study these pathways and processes in more detail. In this regard, data on the involvement of specific processes in the response of microbes to the ABMs will provide clues into the cellular function of these processes. Besides being essential for the claim(s) structure in patent applications, from a scientific point of view, the elucidation of the mode of action and target identification (result category 1) of the selected ABMs will lead to novel insights into the molecular mechanisms controlling biofilm formation and/or functioning in both bacteria and fungi. Knowledge on ABM biofilm targets may be used as a basis for the development of future anti-biofilm strategies.

When is the expected date of achievement in the project (Mth/yr)?	48/4
When is the time to market (Mth/yr)?	Not applicable
What are the costs to be incurred after the project and before exploitation?	Depending on the nature of the targets, targets may be patented for screenings. However, value of such IPR is rather low. Additional costs may pertain to IPR strategy (entry in PCT phase, national phase etc)
What is the approximate price range of this result / price of licences?	Not applicable
What is the market size in M€ for this result and relevant trend?	Not applicable
How this result will rank against competing products in terms of price / performance?	Not applicable
Who are the competitors for this result?	Scientific community including academia and industry
How fast and in what ways will the competition respond to this result?	Not applicable

Who are the partners involved in the result?	P1a,P1b,P1c, P2 and P3
Who are the industrial partners interested in the result (partners, sponsors, etc…)?	Screening companies, interested in the identified and patented antibiofilm targets. However, pursuing of exploitation of these data was not foreseen within COATIM
Have you protected or will you protect this result? How? When?	no screenable target was found - hence patent protection was not sought

<u>Exploitable Result n° 2:</u> Antibiofilm coated Titanium substrates and implant components

Describe the innovation content of result	**category 2: Antibiofilm-coated titanium substrates and implant components**
Who will be the customer?	SMEs in the coating field and the dental/orthopedics OEM
What benefit will it bring to the customers?	Successful application of antibiofilm coatings on complex implants will enhance the market penetration of implants with an osseointegrative porous Ti top coating such as vacuum plasma sprayed Ti coatings. The knowledge to apply antibiofilm coatings with proven activity on sand-blasted acid etched implant surfaces (SmTi) can be directly exploited in the dental field as well as on temporary implants. In general, the Consortium expects all next generations of biomedical implants to have an integrated antibiofilm functionality in the future.
When is the expected date of achievement in the project (Mth/yr)?	Sand-blasted acid etched implant surfaces: 36/3 Open porous implant surfaces: 48/4
When is the time to market (Mth/yr)?	5-10 years after project end, depending if active substances have CE or FDA approval
What are the costs to be incurred after the project and before exploitation?	Market research and regulatory compliance assessment will form the basis of further exploitation of the findings. In

	case of confirmed market demand and successful regulatory compliance, additional clinical testing will be necessary to further direct new product development. These costs can be covered by direct funding and exploitation by the SMEs or via joint ventures. **Take-up activities** include **(i)** the completion of the necessary (pre)clinical trials, **(ii)** the assessment, trial and validation, according to European and international standards, **(iii)** easier access to and the transfer of best practices for the early use and exploitation of technologies.
What is the approximate price range of this result / price of licences?	The targeted maximum price for the antibiofilm coating on an acetabular cup implant should be around 45€ per piece. This price is negligible compared to the high surgery cost for implant revision (15,000 €/revision). However there is an extremely high pressure in the price of medical devices and the national healthcare systems are cutting budgets all over Europe.
What is the market size in M€ for this result and relevant trend?	An increase in the aging population and increasing number of injuries are the major drivers that positively affect the demand for **orthopaedic implants**. In 2011, the market for orthopaedic implants was worth $20 billion worldwide. The CAGR from 2006 to 2013 is expected to be 7.1%. The dental implant market is expected to reach $1.94 billion by 2013 in Europe, with a Compound Annual Growth Rate (CAGR) of 13.7% from 2006 to 2013. The total U.S. dental implant market generated revenues of $606 million in 2007 and is to increase with an expected CAGR of 16.3% from 2007 to 2014

How this result will rank against competing products in terms of price / performance?	Competition results are silver or gentamicin based coatings. We anticipate that our coatings are not toxic in contrast to silver and do not lead to increased antibiotic resistance development in contrast to gentamicine based coatings.
Who are the competitors for this result?	Alhenia and Hemoteq coating service competitors in Europe and US and major orthopaedics OEM's that are intensively researching in the same field.
How fast and in what ways will the competition respond to this result?	-They will try to show to the market that their products are much more effective than COATIM invention. -Strategic pricing
Who are the partners involved in the result?	Partners owning IPR on bioactive molecules: P1a, P1b, P2 – Hemoteq/P1d – Alhenia/Biotech: consortium of bioactive molecules, coating and titanium implants.
Who are the industrial partners interested in the result (partners, sponsors, etc…)?	Alhenia, Hemoteq, Biotech Int.
Have you protected or will you protect this result? How? When?	No, data are too preliminary at the moment: more research is needed regarding how the compound load affects the *in vitro/ in vivo* performance. Also reproducibility and control over the amount of the ABM coating is required before SMEs want to engage. In addition, follow-up *in vivo* experiments are needed using a combined infection/osseointegration model.

IPR'S ON BACKGROUND INFORMATION 1/4

IPRs Issues

IPR'S ON BACKGROUND INFORMATION

Information, excluding foreground information, brought to the project from existing knowledge, owned or controlled by project partners in the same or related fields of the work carried our in the research project.

Result number	1	2
PARTNERS	Mode of action of ABMs against fungal and bacterial biofilms	Antibiofilm coated Titanium substrates and implant components
P1a	B	B *
P1b		B*
P1c		B*
P1d		B**
P2		B*
P3		B*

P4		**B***
P5		**B***
P6		**B****
P7		**B****
P8		**B***
P9		**B****
P10		**B***

B: Background information, brought to the project from existing knowledge, *: Knowledge of biological and functional testing of components with antibiofilm coating, **: knowledge of manufacturing of components and/or coating application

IPR'S ON BACKGROUND INFORMATION 2/4

IPRs Issues

IPR'S ON FOREGROUND INFORMATION

Information, excluding foreground information, brought to the project from existing knowledge, owned or controlled by project partners in the same or related fields of the work carried our in the research project.

Result number	1	2
PARTNERS	Mode of action of ABMs against fungal and bacterial biofilms	Antibiofilm coated Titanium substrates and implant components
P1a	F	F *
P1b	F	F*
P1c	F	F*
P1d		F**
P2	F	F*
P3	F	F*
P4	F	F*
P5		F*
P6		F**
P7		F**
P8		F*

P9		**F****
P10		

F: Foreground information, generated by parteners during project. *: Knowledge of biological and functional testing of components with antibiofilm coating, **: knowledge of manufacturing of components and/or coating application respectively

IPR'S ON BACKGROUND INFORMATION 3/4

IPRs Issues

EXPLOITATION CLAIMS

Information, excluding foreground information, brought to the project from existing knowledge, owned or controlled by project partners in the same or related fields of the work carried our in the research project.

Result number	1	2
PARTNERS	Mode of action of ABMs against fungal and bacterial biofilms	Antibiofilm coated Titanium substrates and implant components
P1a	U,L,O	U,L

P1b	**U,L,O**	**U,L**
P1c	**U,L,O**	**U,L**
P1d		**U,O,L**
P2	**U,L,O**	**U,L**
P3	**U,L,O**	**U,L**
P4	**U,O**	**U,O**
P5		**U,O**
P6		**M,U**
P7		**M,U,L,O**
P8		**U**
P9		**M,L,U,O**
P10		

Current application of the background knowledge in the same field or related to the work carried out in the project:, U: internal use to produce something different for sale, L: license to 3rd party,

O: provide services as consultancy, M: Manufacturing and selling as product

IPR'S ON BACKGROUND INFORMATION 4/4

IPRs Issues

IPR'S AND EXPLOITATION CLAIMS

Information, excluding foreground information, brought to the project from existing knowledge, owned or controlled by project partners in the same or related fields of the work carried our in the research project.

Result number	1			2		
PARTNERS	Mode of action of ABMs against fungal and bacterial biofilms			Antibiofilm coated Titanium substrates and implant components		
P1a	B	F	U,L,O	B *	F *	U,L
P1b		F	U,L,O	B*	F*	U,L
P1c		F	U,L,O	B*	F*	U,L
P1d				B**	F**	U,O,L
P2		F	U,L,O	B*	F*	U,L
P3		F	U,L,O	B*	F*	U,L
P4		F	U,O	B*	F*	U,O

P5				**B***	**F***	**U,O**
P6				**B****	**F****	**M,U**
P7				**B****	**F****	**M,U,L,O**
P8				**B***	**F***	**U**
P9				**B****	**F****	**M,L,U,O**
P10				**B***		

Intended application of the foreground knowledge from the project: U: internal use to produce something different for sale, L: license to 3rd party, O: provide services as consultancy, M: Manufacturing and selling as product, *: Knowledge about biological and functional testing of components with antibiofilm coating, **: knowledge about manufacturing of components and/or coating application respectively

Section 2 – Risk Assessment and Action Plan

Risk Assessment

Standard Definition

Risk: The uncertainty of an event occurring that could have an impact on the achievement of objectives.

Risk is measured in terms of impact and likelihood

Note: In the risk assessment we look at the inherent risks before considering any controls

Risk Grading Matrix

Likelihood / Impact	Rare [1]	Unlikely [2]	Moderate [3]	Likely [4]	Certain [5]
Low [1]	Low	Low	Low	Low	Low
Slight [2]	Low	Low	Significant	High	High
Moderate [3]	Low	Significant	Very High	Very High	Very High
Serious [4]	Low	High	Very High	Unacceptable	Unacceptable
Very Serious [5]	Low	High	Very High	Unacceptable	Unacceptable

Risks at exploitable results level

Exploitable result N°1: Antibiofilm mode of action of antifungal ABM

Risks \ Groups					Comments
		Impact	Likelihood	Ranking	
	Technological risks:				
1	Disagreement on continuation: expenditures are increasing.	4	2	High	
2	Disagreement on further investments: some partners may leave.	n/a	n/a	n/a	
3	Worthless result: the project lasted too long.	1	3	Low	
4	Worthless result: ill-timed disclosure.	5	2	High	
5	Worthless result: earlier patent exists.	4	2	High	
6	Worthless result: better technology/methodology exists.	5	2	High	
7	Significant dependency on other technologies.	2	2	Low	
8	The life cycle of the new technology is too short.	n/a	n/a	n/a	
	Partnership risks:				

9	Industrialization at risk: no manufacturer for the prototype.	n/a	n/a	n/a	
10	Industrialization at risk: an industrial partner leaves the market.	n/a	n/a	n/a	
11	Industrialization at risk: a partner declares bankruptcy.	n/a	n/a	n/a	
	Market risks:				
12	Exploitation disagreement: partners on the same market.	n/a	n/a	n/a	
13	Exploitation disagreement: partners with divergent interests.	5	1	Low	
14	Worthless result: performance lower than market needs.	2	4	High	
15	Nobody buys the product. Nobody needs it.	2	4	High	
16	Nobody buys the product. Too expensive.	2	2	Low	
17	Nobody buys the product. Unsuitable sales force.	2	4	High	
18	Nobody buys the product. The project hits against a monopoly.	2	2	Low	
19	Nobody buys the product. Problems at the time of the first sales.	2	4	High	
20	Nobody buys the product. Rejected by end-users.	2	2	Low	
	Legal risks:				
21	Legal problems: proceeding against us.	4	2	High	

22	Legal problems: we are sued for patent infringement.	4	2	High	
23	Know- how risks: it is easy to counterfeit the patent.	n/a	n/a	n/a	
24	Know- how risks: a counterfeit cannot be proved.	n/a	n/a	n/a	
25	Know- how risks: the patent application is rejected.	2	4	High	
	Management risks:				
26	Nobody buys the product. Our licensee is not exploiting his exclusive license.	3	3	Significant	
27	Know- how risks: there are leaks of confidential information.	5	2	High	
28	Multiple change to original objectives.	n/a	n/a	n/a	
29	Lack of awareness of risk management.	4	1	Low	
30	Inadequate communication among partners.	4	1	Low	
31	Inadequate reporting procedures.	5	1	Low	
32	On time supply of financial means.	2	1	Low	
	Environmental/regulation/safety risks:				
33	Nobody buys the product. Does not comply with the standards.	n/a	n/a	n/a	

34	Nobody buys the product. Standards to make it compulsory don't yet exist.	n/a	n/a	n/a	
35	Research is socially or ethically unacceptable.	n/a	n/a	n/a	
36	Influence of laws and regulations.	n/a	n/a	n/a	

Risks at exploitable results level

Exploitable result N°2: Antibiofilm coated Titanium substrates and implant components

Risks / Groups					Comments
		Impact	Likelihood	Ranking	
	Technological risks:				
1	Disagreement on continuation: expenditures are increasing.	3	1	Low	
2	Disagreement on further investments: some partners may leave.	3	1	Low	
3	Worthless result: the project lasted too long.	5	1	Low	
4	Worthless result: ill-timed disclosure.	5	1	Low	

5	Worthless result: earlier patent exists.	3	2	Significant	
6	Worthless result: better technology/methodology exists.	1	1	Low	
7	Significant dependency on other technologies.	1	1	Low	
8	The life cycle of the new technology is too short.	3	1	Low	
	Partnership risks:				
9	Industrialization at risk: no manufacturer for the prototype.	1	1	Low	
10	Industrialization at risk: an industrial partner leaves the market.	5	1	Low	
11	Industrialization at risk: a partner declares bankruptcy.	5	1	Low	
	Market risks:				
12	Exploitation disagreement: partners on the same market.	3	1	Low	
13	Exploitation disagreement: partners with divergent interests.	2	2	Low	
14	Worthless result: performance lower than market needs.	5	3	Very high	see contingency plan
15	Nobody buys the product. Nobody needs it.	5	1	Low	
16	Nobody buys the product. Too expensive.	5	5	Unacceptable	Better understanding of pricing of health sector, see WP6

17	Nobody buys the product. Unsuitable sales force.	3	3	High	
18	Nobody buys the product. The project hits against a monopoly.	5	1	Low	
19	Nobody buys the product. Problems at the time of the first sales.	5	3	Very high	consultancy of key opinion surgeon advisory board
20	Nobody buys the product. Rejected by end-users.	5	2	High	
	Legal risks:				
21	Legal problems: proceeding against us.	5	1	Low	
22	Legal problems: we are sued for patent infringement.	2	2	Low	
23	Know- how risks: it is easy to counterfeit the patent.	2	3	Significant	
24	Know- how risks: a counterfeit cannot be proved.	1	2	Low	
25	Know- how risks: the patent application is rejected.	2	1	Low	
	Management risks:				
26	Nobody buys the product. Our licensee is not exploiting his exclusive license.	n/a	n/a	n/a	
27	Know- how risks: there are leaks of confidential information.	3	3	High	
28	Multiple change to original objectives.	1	1	Low	

29	Lack of awareness of risk management.	5	2	High	
30	Inadequate communication among partners.	5	1	Low	
31	Inadequate reporting procedures.	n/a	n/a	n/a	
32	On time supply of financial means.	n/a	n/a	n/a	
	Environmental/regulation/safety risks:				
33	Nobody buys the product. Does not comply with the standards.	n/a	n/a	n/a	
34	Nobody buys the product. Standards to make it compulsory don't yet exist.	n/a	n/a	n/a	
35	Research is socially or ethically unacceptable.	4	1	Low	
36	Influence of laws and regulations.	3	1	Low	

Section 3 – Dissemination of knowledge

List of scientific (peer reviewed) publications: see table 2.1.1.

List of dissemination activities : see table 2.1.2.

Detailed description of each major activity

1) Short messages, announcements and posters

During COATIM, more than60 dissemination activities took place, including more than 35 oral presentations, 7 press releases or announcements and 22 poster presentations.

2) Training of young people

Bachelor and Master thesis related or inspired by Coatim

Nine master theses, directly related to or inspired by COATIM, were performed.

Ph.D. thesis directly related to or inspired by Coatim

Four PhD's, directly related to or inspired by Coatim, were finished during COATIM or will be finished beginning of 2016.

3) Publications in Internationally reviewed Journals

At the moment, 21 publications in total, of which 7 joint publications originating from research performed by at least 2 different COATIM partner, based on COATIM research results and technological advances, were published or accepted in peer-reviewed scientific journals: Several other manuscripts are in the pipeline (see dissemination table).

4) Networking

-Scientific coordinator Dr. Ir. Karin Thevissen is a member of the management committee of iPromedai, COST action TD1305. In addition, COATIM has provided reference materials for the iPromedai Summer School "Antimicrobial Medical Devices", September 2015, The Netherlands, organized by dr. Bas Zaat (coordinator of the BALI consortium)

-Closing COATIM conference (December 18th, Leuven, Belgium) : 'Anti-infective strategies for medical devices' was attended by academy, industry, members of BALI and BacAttack as well as surgeons and students

-Joint meeting of the FP7 consortia COATIM, BacAttack and BALI was organised in Leuven, May 7th 2013.

5) Possible follow-up projects and exploitation

At the moment no suitable Horizon 2020 call are available. As stated above, data are too preliminary to warrant bilateral follow-up projects and/or further engagement of the SMEs at this moment.

Section 4 – Publishable results

Most obtained results are published. However, some articles are still in the pipeline and will be published in 2016. Unfortunately, data are still too preliminary for exploitation possibilities. Follow-up projects in which extensive preclinical and clinical tests will be performed, are required before we can determine a suitable exploitation trajectory for the COATIM results.

3. Report on societal implications

Replies to the following questions will assist the Commission to obtain statistics and indicators on societal and socio-economic issues addressed by projects. The questions are arranged in a number of key themes. As well as producing certain statistics, the replies will also help identify those projects that have shown a real engagement with wider societal issues, and thereby identify interesting approaches to these issues and best practices. The replies for individual projects will not be made public.

A General Information *(completed automatically when **Grant Agreement number** is entered.*	
Grant Agreement Number:	278425
Title of Project:	COATIM – Development of antibiofilm coatings for implants
Name and Title of Coordinator:	Dr. Ir. Karin Thevissen

B Ethics	
1. Did your project undergo an Ethics Review (and/or Screening)? • If Yes: have you described the progress of compliance with the relevant Ethics Review/Screening Requirements in the frame of the periodic/final project reports? Special Reminder: the progress of compliance with the Ethics Review/Screening Requirements should be described in the Period/Final Project Reports under the Section 3.2.2 *'Work Progress and Achievements'*	***0Yes No***
2. Please indicate whether your project involved any of the following issues (tick box) :	***YES***
RESEARCH ON HUMANS	
• Did the project involve children?	
• Did the project involve patients?	
• Did the project involve persons not able to give consent?	
• Did the project involve adult healthy volunteers?	
• Did the project involve Human genetic material?	
• Did the project involve Human biological samples?	
• Did the project involve Human data collection?	
RESEARCH ON HUMAN EMBRYO/FOETUS	
• Did the project involve Human Embryos?	
• Did the project involve Human Foetal Tissue / Cells?	
• Did the project involve Human Embryonic Stem Cells (hESCs)?	
• Did the project on human Embryonic Stem Cells involve cells in culture?	
• Did the project on human Embryonic Stem Cells involve the derivation of cells from Embryos?	
PRIVACY	
• Did the project involve processing of genetic information or personal data (eg. health, sexual lifestyle, ethnicity, political opinion, religious or philosophical conviction)?	
• Did the project involve tracking the location or observation of people?	

RESEARCH ON ANIMALS	
• Did the project involve research on animals?	yes
• Were those animals transgenic small laboratory animals?	
• Were those animals transgenic farm animals?	
• Were those animals cloned farm animals?	
• Were those animals non-human primates?	
RESEARCH INVOLVING DEVELOPING COUNTRIES	
• Did the project involve the use of local resources (genetic, animal, plant etc)?	
• Was the project of benefit to local community (capacity building, access to healthcare, education etc)?	
DUAL USE	
• Research having direct military use	0 Yes No
• Research having the potential for terrorist abuse	

C Workforce Statistics

3. Workforce statistics for the project: Please indicate in the table below the number of people who worked on the project (on a headcount basis).

Type of Position	Number of Women	Number of Men
Scientific Coordinator	1	0
Work package leaders	5	3
Experienced researchers (i.e. PhD holders)	9	8
PhD Students	4	2
Other	3	3

4. How many additional researchers (in companies and universities) were recruited specifically for this project?	
Of which, indicate the number of men: 6, of which 1 man	

D Gender Aspects

5. Did you carry out specific Gender Equality Actions under the project?

■ Yes
○ No

6. Which of the following actions did you carry out and how effective were they?

		Not at all effective				Very effective
■	Design and implement an equal opportunity policy	○	○	○	○	■
■	Set targets to achieve a gender balance in the workforce	○	○	○	■	○
❑	Organise conferences and workshops on gender	○	○	○	○	○
❑	Actions to improve work-life balance	○	○	○	○	■

○ Other:

7. Was there a gender dimension associated with the research content – i.e. wherever people were the focus of the research as, for example, consumers, users, patients or in trials, was the issue of gender considered and addressed?

○ Yes- please specify

■ No

E Synergies with Science Education

8. Did your project involve working with students and/or school pupils (e.g. open days, participation in science festivals and events, prizes/competitions or joint projects)?

■ Yes- please specify: Several master and PhD students were involved in the COATIM project

○ No

9. Did the project generate any science education material (e.g. kits, websites, explanatory booklets, DVDs)?

○ Yes- please specify

■ No

F Interdisciplinarity

10. Which disciplines (see list below) are involved in your project?

○ Main discipline[4]: Biological sciences
○ Associated discipline[4]: Other engineering sciences
○ Associated discipline[4]: basic medicine

G Engaging with Civil society and policy makers

11a Did your project engage with societal actors beyond the research community? *(if 'No', go to Question 14)*

■ Yes
○ No

11b If yes, did you engage with citizens (citizens' panels / juries) or organised civil society (NGOs, patients' groups etc.)?

■ No
○ Yes- in determining what research should be performed
○ Yes - in implementing the research
○ Yes, in communicating /disseminating / using the results of the project

[4] Insert number from list below (Frascati Manual).

11c In doing so, did your project involve actors whose role is mainly to organise the dialogue with citizens and organised civil society (e.g. professional mediator; communication company, science museums)?	○ ■	Yes No

12. Did you engage with government / public bodies or policy makers (including international organisations)

- ○ No
- ■ Yes- in framing the research agenda
- ○ Yes - in implementing the research agenda
- ○ Yes, in communicating /disseminating / using the results of the project

13a Will the project generate outputs (expertise or scientific advice) which could be used by policy makers?

- ○ Yes – as a **primary** objective (please indicate areas below- multiple answers possible)
- ○ Yes – as a **secondary** objective (please indicate areas below - multiple answer possible)
- ■ No

13b If Yes, in which fields?

Agriculture		Energy		Human rights	
Audiovisual and Media		Enlargement		Information Society	
Budget		Enterprise		Institutional affairs	
Competition		Environment		Internal Market	
Consumers		External Relations		Justice, freedom and security	
Culture		External Trade		Public Health	
Customs		Fisheries and Maritime		Regional Policy	
Development Economic		Affairs		Research and Innovation	
and Monetary Affairs		Food Safety		Space	
Education, Training, Youth		Foreign and Security		Taxation	
Employment and Social		Policy		Transport	
Affairs		Fraud			
		Humanitarian aid			

13c If Yes, at which level?

- ○ Local / regional levels
- ○ National level
- ○ European level
- ○ International level

H Use and dissemination

14. How many Articles were published/accepted for publication in peer-reviewed journals?	22
To how many of these is open access[5] provided?	22
How many of these are published in open access journals?	13
How many of these are published in open repositories?	9
To how many of these is open access not provided?	0
Please check all applicable reasons for not providing open access:	
❑ publisher's licensing agreement would not permit publishing in a repository ❑ no suitable repository available ❑ no suitable open access journal available ❑ no funds available to publish in an open access journal ❑ lack of time and resources ❑ lack of information on open access ❑ other[6]: ……	

15. How many new patent applications ('priority filings') have been made? *("Technologically unique": multiple applications for the same invention in different jurisdictions should be counted as just one application of grant).*		**2**
16. Indicate how many of the following Intellectual Property Rights were applied for (give number in each box).	Trademark	**0**
	Registered design	**0**
	Other	**0**
17. How many spin-off companies were created / are planned as a direct result of the project?		**0**
Indicate the approximate number of additional jobs in these companies:		

18. Please indicate whether your project has a potential impact on employment, in comparison with the situation before your project:

■ Increase in employment, or	❑ In small & medium-sized enterprises
■ Safeguard employment, or	❑ In large companies
❑ Decrease in employment,	❑ None of the above / not relevant to the project
❑ Difficult to estimate / not possible to quantify	

[5] Open Access is defined as free of charge access for anyone via Internet.
[6] For instance: classification for security project.

19. For your project partnership please estimate the employment effect resulting directly from your participation in Full Time Equivalent (*FTE = one person working fulltime for a year*) jobs:	*Indicate figure: 36*
Difficult to estimate / not possible to quantify	❑

I	**Media and Communication to the general public**		
20.	**As part of the project, were any of the beneficiaries professionals in communication or media relations?**		
	○ Yes	■	No
21.	**As part of the project, have any beneficiaries received professional media / communication training / advice to improve communication with the general public?**		
	○ Yes	■	No
22	**Which of the following have been used to communicate information about your project to the general public, or have resulted from your project?**		
■	Press Release	■	Coverage in specialist press
❑	Media briefing	■	Coverage in general (non-specialist) press
❑	TV coverage / report	❑	Coverage in national press
❑	Radio coverage / report	❑	Coverage in international press
■	Brochures /posters / flyers	■	Website for the general public / internet
❑	DVD /Film /Multimedia	■	Event targeting general public (festival, conference, exhibition, science café)
23	**In which languages are the information products for the general public produced?**		
❑	Language of the coordinator	■	English
❑	Other language(s)		

***Question F-10*:** Classification of Scientific Disciplines according to the Frascati Manual 2002 (Proposed Standard Practice for Surveys on Research and Experimental Development, OECD 2002):

FIELDS OF SCIENCE AND TECHNOLOGY

1. NATURAL SCIENCES

1.1 Mathematics and computer sciences [mathematics and other allied fields: computer sciences and other allied subjects (software development only; hardware development should be classified in the engineering fields)]

1.2 Physical sciences (astronomy and space sciences, physics and other allied subjects)

1.3 Chemical sciences (chemistry, other allied subjects)

1.4 Earth and related environmental sciences (geology, geophysics, mineralogy, physical geography and other geosciences, meteorology and other atmospheric sciences including climatic research, oceanography, vulcanology, palaeoecology, other allied sciences)

1.5 Biological sciences (biology, botany, bacteriology, microbiology, zoology, entomology, genetics, biochemistry, biophysics, other allied sciences, excluding clinical and veterinary sciences)

2 ENGINEERING AND TECHNOLOGY

2.1 Civil engineering (architecture engineering, building science and engineering, construction engineering, municipal and structural engineering and other allied subjects)
2.2 Electrical engineering, electronics [electrical engineering, electronics, communication engineering and systems, computer engineering (hardware only) and other allied subjects]
2.3. Other engineering sciences (such as chemical, aeronautical and space, mechanical, metallurgical and materials engineering, and their specialised subdivisions; forest products; applied sciences such as geodesy, industrial chemistry, etc.; the science and technology of food production; specialised technologies of interdisciplinary fields, e.g. systems analysis, metallurgy, mining, textile technology and other applied subjects)

3. MEDICAL SCIENCES
3.1 Basic medicine (anatomy, cytology, physiology, genetics, pharmacy, pharmacology, toxicology, immunology and immunohaematology, clinical chemistry, clinical microbiology, pathology)
3.2 Clinical medicine (anaesthesiology, paediatrics, obstetrics and gynaecology, internal medicine, surgery, dentistry, neurology, psychiatry, radiology, therapeutics, otorhinolaryngology, ophthalmology)
3.3 Health sciences (public health services, social medicine, hygiene, nursing, epidemiology)

4. AGRICULTURAL SCIENCES
4.1 Agriculture, forestry, fisheries and allied sciences (agronomy, animal husbandry, fisheries, forestry, horticulture, other allied subjects)
4.2 Veterinary medicine

5. SOCIAL SCIENCES
5.1 Psychology
5.2 Economics
5.3 Educational sciences (education and training and other allied subjects)
5.4 Other social sciences [anthropology (social and cultural) and ethnology, demography, geography (human, economic and social), town and country planning, management, law, linguistics, political sciences, sociology, organisation and methods, miscellaneous social sciences and interdisciplinary , methodological and historical S1T activities relating to subjects in this group. Physical anthropology, physical geography and psychophysiology should normally be classified with the natural sciences].

6. HUMANITIES
6.1 History (history, prehistory and history, together with auxiliary historical disciplines such as archaeology, numismatics, palaeography, genealogy, etc.)
6.2 Languages and literature (ancient and modern)
6.3 Other humanities [philosophy (including the history of science and technology) arts, history of art, art criticism, painting, sculpture, musicology, dramatic art excluding artistic "research" of any kind, religion, theology, other fields and subjects pertaining to the humanities, methodological, historical and other S1T activities relating to the subjects in this group]

1. FINAL REPORT ON THE DISTRIBUTION OF THE EUROPEAN UNION FINANCIAL CONTRIBUTION

This report shall be submitted to the Commission within 30 days after receipt of the final payment of the European Union financial contribution.

Report on the distribution of the European Union financial contribution between beneficiaries

Name of beneficiary	Final amount of EU contribution per beneficiary in Euros
1.	
2.	
n	
Total	