Aus dem Institut Berlin-Brandenburger Centrum für Regenerative Therapien
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DISSERTATION

THE CONTROLLED RELEASE AND ACTIVITY OF ANTIMICROBIALS FROM BIOMATERIALS

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ZUSAMMENFASSUNG

ABSTRACT

The increasing use of medical indwelling devices has triggered the development of difficult-to-treat infections due to biofilm formation on the implant surface. Nowadays, infection treatment consists of invasive surgical intervention combined with long-course systemic antimicrobial infusion. However, treatment failure due to ineffective surgical debridement and inappropriate antibiotic therapy is a troublesome reality. Hence, the development and formulation of novel smart biomaterials as scaffolds for the local release of drugs gained increasing interest.

The aim of this work was to investigate the anti-biofilm and anti-persister activity of selected antimicrobials, to fine-tune the isothermal microcalorimetry (IMC) as antimicrobial susceptibility method and to study the formulation, release characteristics and antimicrobial activity of different biomaterials (including recently developed hydrogels) loaded with therapeutically relevant antimicrobials for the management of orthopedic implant-associated infections.

Results showed that high concentrations of vancomycin enriched a *Staphylococcus aureus* biofilm in persister cells, which are mainly responsible for infection recalcitrance. It was reported that a deep analysis of IMC data enables the detection and identification in real-time of persister cells, proving the suitability of this method for the characterization of new anti-persister compounds and biomaterials. Interestingly, the combined use of the glycopeptide with daptomycin proved highly bactericidal against persisters. The lipopeptide was then successfully loaded into soft and fully degradable thermosensitive hydrogels, which released high concentrations of active drug (widely exceeding the minimum bactericidal concentration) in a controlled manner against *S. aureus* for at least 15 days. Similarly, high titers of bacteriophages were released from smart thermoresponsive hydrogels in a controlled manner for at least 7 days. As a comparison, also the gentamicin elution profile from bone graft substitutes was investigated, revealing a timely burst release of bactericidal concentrations.

This work demonstrated that high doses of commonly used antibiotics may select for persister cells in biofilms. In fact, recalcitrance and extreme resistance of biofilm-associated infections affecting the musculoskeletal system are deeply influenced by metabolically inactive cells that, to the best of our knowledge, were here identified and characterized for the first time using IMC. Smart bioscaffolds may serve as drug reservoirs and offer optimal conditions for the release of high doses of anti-biofilm and anti-persister molecules *in situ*, providing relevant progress to the fast-growing field of biomaterials and advances towards their clinical application.

ABSTRAKT

Die zunehmende Verwendung von medizinischen Verweilvorrichtungen hat die Entwicklung von schwer zu behandelnden Infektionen durch Biofilmbildung auf den Oberflächen von Implantaten bestimmt. Heutzutage besteht die Behandlung von Infektionen in einer Kombination aus operativen Eingriffen und systemischen antimikrobiellen Infusionen. Behandlungsversagen aufgrund ineffizienten chirurgischen Debridements und unpasssende antibiotische Therapie sind jedoch eine problematische Realität. Daher hat die Entwicklung und Formulierung neuartiger intelligenter Biomaterialien als Gerüst für die lokale Freisetzung von Arzneimitteln zunehmendes Interesse gefunden.

Das Ziel dieser Arbeit war die Untersuchung der Anti-Biofilm- und Anti-Persister-Aktivität ausgewählter antimikrobieller Mittel, die Feinabstimmung der isothermen Mikrokalorimetrie (IMC) als antimikrobielle Suszeptibilitätsmethode und die Untersuchung antimikrobieller Potenziale diverser Biomaterialien, die mit therapeutisch relevanten Mitteln zur Behandlung von orthopädischen implantatassoziierten Infektionen beladen sind.

Die Ergebnisse zeigten, dass hohe Konzentrationen von Vancomycin einen *Staphylococcus aureus*-Biofilm in Persisterzellen angereichert haben, die hauptsächlich für die Problemhaftigkeit der Infektionen verantwortlich sind. Eine gründliche Analyse von Daten der isothermen Mikrokalorimetrie (IMC) ermöglicht den Nachweis und die Identifizierung von persistenten Zellen in Echtzeit, was die Eignung dieser Methode für die Charakterisierung neuer anti-persistenter Verbindungen und Biomaterialien belegt. Interessanterweise erwies sich die kombinierte Verwendung des Glycopeptids mit Daptomycin als stark bakterizid gegen Persister. Das Lipopeptid wurde erfolgreich in vollständig abbaubare wärmeempfindliche Hydrogele geladen, die nachweislich auf kontrollierte Weise hohe Konzentrationen an Wirkstoff freisetzen, die die minimale bakterizide Konzentration gegen *S. aureus* für mindestens 15 Tage weit überschreiten. In ähnlicher Weise wurden hohe Titer von Bakteriophagen mindestens 7 Tage lang kontrolliert aus intelligenten thermoresponsiven Hydrogelen freigesetzt. Zum Vergleich wurde auch das Gentamicin-Elutionsprofil von Knochentransplantatersatzmitteln untersucht, was eine zeitnahe Freisetzung bakterizider Konzentrationen aufzeigt.

Diese Arbeit zeigte, dass häufig verwendete Antibiotika in hohen Dosen persistente Zellen in Biofilmen selektieren können. Tatsächlich werden Rekalzitranz und extreme Resistenz von Biofilm-assoziierten Infektionen im Bewegungsapparat stark durch metabolisch inaktive Zellen beeinflusst, die hier zum ersten Mal mit IMC identifiziert und charakterisiert wurden. Intelligente Bioscaffolds können als Reservoir für Arzneimittel dienen und bieten optimale Bedingungen für die Freisetzung hoher Dosen von Biofilm- und Anti-Persister-Molekülen *in situ*, was dem schnell wachsenden Biomaterialbereich und der klinischen Anwendung wichtige Fortschritte bringt.

1. INTRODUCTION

The raising use of medical indwelling devices, such as orthopedic joint prosthesis and artificial cardiac valves, has strongly improved life quality of patients and fostered longevity. However, this also carries the threatening evidence of the development of difficult-to-treat infections [1]. In the orthopedic field, the increasing number of implanted joint arthroplasties and fixation devices is associated to frequent cases of surgical infections, which represent a significant clinical, socio-economic and public burden [2]. Orthopedic implant-associated infections are mainly due to microbial colonization of abiotic surfaces [2] , which eventually evolves into biofilms (Figure 1), causing the onset of chronic infection. Within a biofilm, microbes develop into a complex community with functional and structural heterogeneity [3]. The early microbial attachment is usually mediated by cell surface components and, afterwards, when the newly-formed aggregate is growing in density and dimensions, further extracellular components (such as polysaccharides, DNA, proteins and lipids) foster microbial aggregation and stimulate the development of a slimy extracellular polymeric substance [4]. Therefore, slime-enclosed cells acquire extreme resistance to the action of host immunity and antimicrobial killing [5], as compared to free-floating cells. The most common bacteria isolated in bone and implant-associated infections are *Staphylococcus aureus* and coagulase-negative staphylococci, which account for more than 50% of periprosthetic infections [6], fracture fixation-associated and spinal implant-associated infections [7]. Other clinically relevant microorganisms include streptococci, enterococci and gram-negative rods like *Escherichia coli*, *Pseudomonas* spp. and *Enterobacter* spp. [8].

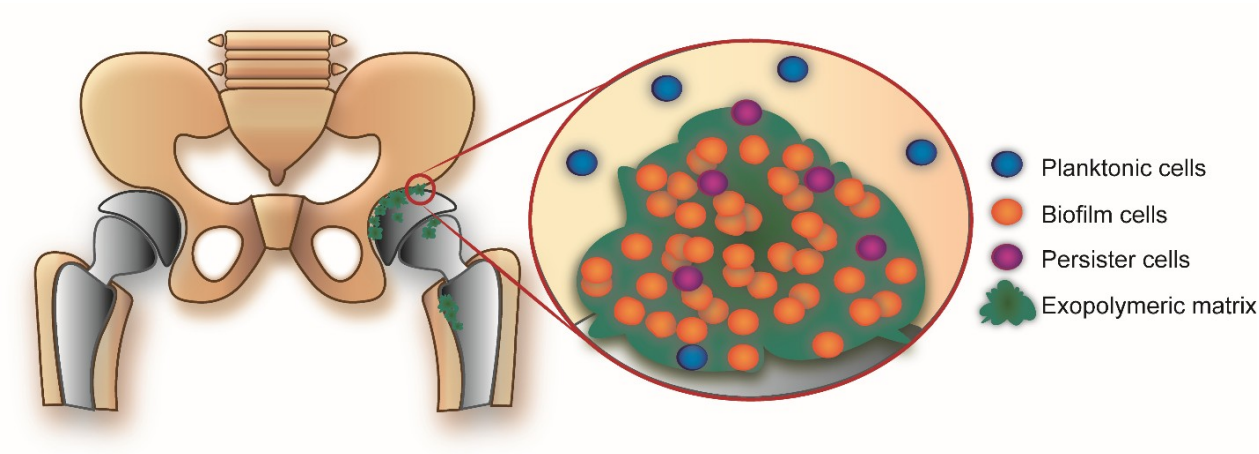

Figure 1 Schematic representation of a biofilm formed on a hip prosthesis. Biofilms are aggregates of different microbial cells, including susceptible and persister cells, embedded within a self-produced exopolymeric matrix. Planktonic cells are free-floating non-sessile cells that can spread and contaminate surrounding tissues and surfaces.

Along with normal-growing cells, bacterial biofilms host cells characterized by non-growing phenotypes, which are able to tolerate higher concentrations of bactericidal antibiotics [9], thus contributing to the recalcitrance of infection. In fact, even though the bactericidal treatment kills most of the susceptible cells in the sessile community, persisters survive and, upon treatment discontinuation, repopulate the biofilm [10]. Persister cells account for a small fraction of a microbial population, which is genetically susceptible but phenotypically tolerant to antimicrobials due to the non-growing quiescent state [11, 12]. In fact, persistence differs from resistance, which is a characteristic inherited by the whole microbial population and it entails the ability of bacteria to replicate in the presence of antimicrobials [13]. Although persisters mostly arise stochastically [14], their induction may also be promoted as protective response to particular stress conditions, like high antibiotic doses [15], as well as nutritional restrictions and oxygen depletion [16]. Indeed, levels of persisters frequently increase during stationary growth phase and in biofilms [17].

Bacterial tolerance and resistance [13] are key players in infection recalcitrance and therapeutic failure [18], along with the physical protection offered by the extracellular matrix. Hence, for an effective eradication of orthopedic biofilm-associated infections the resection of infected necrotic tissue through surgical debridement is associated to a long course systemic pharmacological therapy [19]. However, systemic antibiotic therapies expose patients to a number of severe side effects, besides offering low local concentrations and scarce tissue penetration. Hence, the combined use of local antimicrobials allows reaching high drug concentrations at the infection site, typically exceeding the minimum inhibitory concentrations (MICs) for most of bacteria [20], with low systemic toxicity [21]. Indeed, local drug release from bioscaffolds results in the optimization of the therapeutic effects associated to the drastic reduction of drug toxicity.

Extensive research has targeted degradable biomaterials as drug releasing vehicles [22], which do not require follow-up surgeries after complete drug elution. For instance, collagen implants [23], bone graft substitutes [24] and bioactive glasses [25] have been investigated. Of different chemical composition and structure are hydrogels, highly hydrophilic cross-linked three-dimensional networks which proved to be excellent candidates for tissue engineering [26] and for the controlled release of antibiotics and other therapeutic agents [27, 28]. Injectable thermosensitive hydrogels can be delivered in a minimally invasive manner [29], then jellifying upon temperature to body temperature [30]. Additional to antibiotics, alternative antimicrobial agents like bacteriophages (virus exhibiting lytic activity against bacteria) [31] and antimicrobial

peptides [32] can be encapsulated and efficiently released in therapeutically relevant quantities at the site of infection.

Taken together, the smart combination of surgical approach, systemic infusion and local administration of antimicrobials via degradable bio-friendly scaffolds reflects the paramount effort needed for the prevention and treatment of complicated of bone and implant-associated infections. As many novel technological biomaterials still undergoes pre-clinical tests, it is predictable that a clinical revolution in the management of biofilm-associated infections is to come in the forthcoming years.

2. AIM OF THE THESIS

The main aim of this work is to study the release and the *in vitro* activity of therapeutically relevant antimicrobial agents (*i.e.* antibiotics, bacteriophages) from novel biomaterials for the management of orthopedic implant-associated infections. Therefore, the main objectives are:

1. To establish an accurate, non-destructive and highly sensitive *in vitro* analytical technique for assessing biofilm susceptibility to antimicrobials in real-time by monitoring bacterial viability (Study A: Butini et al., Real-time antimicrobial susceptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry. *Advances in experimental medicine and biology* (2018));
2. To investigate and characterize persister cells in a *S. aureus* biofilm after anti-biofilm treatment using isothermal microcalorimetry (IMC) and standard antimicrobial susceptibility methods (Study B: Butini et al., Isothermal microcalorimetry detects the presence of persister cells in a *Staphylococcus aureus* biofilm after vancomycin treatment. Manuscript submitted to *Frontiers in Microbiology* and currently under revision);
3. To analyze release kinetics and anti-biofilm activity of antimicrobial and anti-persister agents eluted from novel fully degradable hydrogels (Study C: Casadidio[1], Butini[1] et al., Daptomycin-loaded biodegradable thermosensitive hydrogels enhance drug stability and foster bactericidal activity against *Staphylococcus aureus*. *European Journal of Pharmaceutics and Biopharmaceutics* (2018)) and resorbable bone graft substitutes (Study D: Butini et al., *In vitro* anti-biofilm activity of a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute. *Colloids and Surfaces B – Biointerfaces* (2018)).

3. Materials And Methods

3.1. Storage and culture of bacterial strains

Stocks of *S. agalactiae* ATCC 13813, methicillin-resistant *S. aureus* (MRSA) ATCC 43300, methicillin-susceptible *S. aureus* (MSSA) ATCC 29213, *S. epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615, *E. faecalis* ATCC 19433 and *E. coli* Bj HDE-1 were stored in cryovial bead preservation system at -80 °C. Bacterial strains were cultured on Columbia Blood Agar for 24 h at 37 °C in an ambient air incubator, except for *S. agalactiae* and *S. pyogenes*, which were cultivated on Trypticase Soy Agar (TSA) supplemented with 5% defibrinated sheep blood for 18 h at 37 °C under 5% CO_2 atmosphere. All bacterial liquid cultures were prepared in Trypticase Soy Broth (TSB). *S. pyogenes* and *S. agalactiae* were inoculated in TSB supplemented with 2.5% lysate horse blood (Study A and D). Antimicrobial assays were performed in Cation Adjusted Müller Hinton broth (CAMHB), whereas Brain Heart Infusion (BHI) broth was used to investigate bacteriophage lytic activity.

3.2. Antimicrobial agents and biomaterials

Daptomycin was obtained as a powder and dissolved in sterile Phosphate Buffered Saline (pH 7.4, 10 mM) (Study B and C). Vancomycin was obtained as powder and reconstituted using sterile pyrogen-free water (Study B). Levofloxacin used in Study A was supplied in liquid form and the other antibiotics tested in Study B were supplied either as a powder or in liquid form from the Suppliers. Anti-staphylococcal bacteriophage Sb-1 were stored at 4 °C in 10 ml-vials as received from the suppliers. The titer of the bacteriophage solution was determined by plaque assay, as reported below.

Vinyl sulfonated poly(*N*-(2-hydroxylpropyl)methacrylamide mono/di-lactate) (p(HPMAm-$lac_{1,2}$)-PEG-poly(HPMAm-$lac_{1,2}$)) triblock co-polymers with a 10% degree of substitution (DS (%)) (VinylSulfTC_n) were synthesized by the research group of Prof. Di Martino (University of Camerino, Italy) and provided in lyophilized form, as well as hyaluronic acid with different DS (HASH_n´). Polymers were used to formulated daptomycin-loaded hydrogels (Study C).

A freeze-dried form of hyaluronic acid/poly(*N*- isopropylacrylamide) (pNIPAm) hydrogel was provided from AO Research Institute (Davos, Switzerland) and reconstituted with a bacteriophage solution (anti-staphylococcal Sb-1 bacteriophage) to formulate bacteriophages-loaded hydrogels.

Four different types of sterile beads of gentamicin-loaded bone graft substitute (CERAMENT™|G), containing increasing amounts of gentamicin were provided by BONESUPPORT AB (Lund, Sweden) (Study D).

3.3. Real-time anti-biofilm activity assay by isothermal microcalorimetry (IMC)

Study A reports on the convenient use of IMC as a real-time antimicrobial susceptibility assay. This non-conventional technique is an accurate and highly sensitive method that enables the continuous monitoring of microbial viability in terms of instantaneous heat produced due to the microbial metabolic activity. Moreover, IMC is a non-destructive method which do not require the use of toxic reagents and harsh mechanical manipulation, thus allowing the retrieval of tested samples for further analysis (plating/colony counting of biofilm cells after treatment). Besides validating the antimicrobial efficacy of drugs, IMC provided important advances also in the study of antimicrobial-loaded biomaterials, allowing undemanding sample preparation and fast monitoring of the microbial response to the released agent. Isothermal microcalorimetric analysis was performed using a TAM III-48 microcalorimeter (TA Instruments, New Castle, DE, USA) with a detection limit of heat production of 0.2 μW and equipped with 48 minicalorimeters. Sterile glass ampoules (4 ml-volume) were used and sealed for air tightness prior to introduction into the minicalorimeters. After 15 minutes of equilibration, ampoules were lowered in the measuring position. Heat flow (μW) and total heat (J) were measured in real-time against time (h) as measure of the instantaneous heat produced at any time point and as cumulative amount of heat produced during the experiment, respectively. Microbiologically relevant information, such as growth rate (μ, J/h) and lag phase (λ, h), were derived according to growth models [33]. (Study A)

Porous glass beads were used as material support for biofilm formation during static incubation in inoculated rich medium for 24 h at 37 °C. After incubation, beads were washed with sterile saline and exposed to different concentrations of antibiotic or to antimicrobial-loaded biomaterial for 24 h in glass ampoules. A growth control consisting in untreated biofilm was included, as well as a sterile bead as negative control. Next, beads were retrieved, rinsed and placed in sterile ampoules containing fresh medium for IMC.

To proceed with further analysis, treated biofilm beads were eventually collected, sonicated and analyzed by plating and colony counting of sonication fluids.

3.4. Isolation and characterization of persister cells from *S. aureus* biofilms after vancomycin treatment and anti-persister activity of daptomycin

To isolate staphylococcal persister cells (Study B), a 24 h-old biofilm grown on glass bead was exposed to high antibiotic concentrations (1024 μg/ml vancomycin) for 24 h. Next, rinsed beads were sonicated as described above to dislodge cells present after the treatment. Bacteria were diluted to a final concentration of ~$1x10^5$ CFUs/ml in PBS/1%CAMHB (to avoid any metabolic

re-activation, in case of persister cells), as previously described [11], and incubated at 37 °C with and without 100x minimum inhibiting concentration (MIC) of the tested antibiotic (100 μg/ml vancomycin). Staphylococcal cells dislodged from an untreated biofilm were inoculated (final concentration ~1x10^5 CFUs/ml) either in PBS/1%CAMHB or in CAMHB and used as cell viability controls. After 1, 3 and 6 h-incubation, samples were serially diluted and plated on TSA for colony counting. The time needed for persisters to revert into normal-growing cells was monitored by real-time IMC. Briefly, bacterial biofilm was formed and treated as reported above. After sonication, bacteria were diluted to ~1x10^5 CFUs/ml and incubated in glass ampoules filled with fresh CAMHB. Untreated biofilm was sonicated as well and diluted free-floating bacteria incubated in CAMHB, as control. Heat flow (μW) and total heat (J) were monitored for 15 h at 37 °C.

A 24 h-old MRSA biofilm treated with high vancomycin concentrations (1024 μg/ml) for 24 h was further exposed to sub-eradicating concentrations of daptomycin (16 μg/ml). After treatment suspension and sonication, serial dilutions of the sonication fluids were plated for colony counting. (Study B)

3.5. Daptomycin-loaded hydrogels formulation and daptomycin release *in vitro*

In Study C, two different formulations of daptomycin-loaded hydrogels were prepared at a VinylSulfTC_10 final concentration of 15% w/v and at a HASH_n′ (DS 31 and 53%) final concentration of 5 and 3%, respectively. The concentration of active daptomycin released was determined by the agar well diffusion assay against MRSA (ATCC 43300) [34] and tested against a 24 h-old biofilm using IMC.

3.6. Bacteriophages-loaded hyluronan hydrogels formulation and bacteriophages release *in vitro*

Bacteriophages-loaded hydrogels were formulated with a concentrated bacteriophage solution (Sb-1 initial titer ~8.5 x 10^8 Plaque Forming Units (PFUs)/ml) to a final concentration of 15% w/v. Then, PBS was added on top of the gels (gel:PBS volume ratio = 1:4) and gels were incubated at 37 °C. The titer of released bacteriophages was determined by plaque assay against MRSA [35] and expressed as PFUs/ml.

4. RESULTS

This chapter contains key results published in *Advances in experimental medicine and biology* (2018) (Study A), *European Journal of Pharmaceutics and Biopharmaceutics* (2018) (Study C) and *Colloids and Surfaces B – Biointerfaces* (2018) (Study D). Key findings from Study B are reported in a manuscript that has been submitted to *Frontiers in Microbiology* and is currently under revision.

IMC allows for the real-time evaluation of the anti-biofilm activity of antimicrobials against *in vitro* biofilm

As reported in Study A, IMC enables the real-time monitoring of exothermic reactions associated to microbial metabolic activities in the presence or absence of an antimicrobial agent. As depicted in Figure 2A, the co-incubation of a 24 h-old MRSA biofilm with high concentrations of vancomycin (ranging from 256 to 1024 µg/ml) inhibited staphylococcal metabolism, as no heat flow was detectable during 24 h-incubation. By contrast, the untreated biofilm produced a first peak in heat flow (µW) within the first 4h. As soon as the antibiotic treatment was suspended (Figure 2B), the re-incubation of beads in fresh rich medium revealed the presence of residual biofilm cells, which re-started replicating. In fact, a heat flow related to cells' metabolic reactivation was detected, although delayed in time. Indeed, as shown in Figure 2B, increasing vancomycin concentrations correlated to more delayed onsets of biofilm exponential metabolic activity, as compared to the untreated control. (Study B).

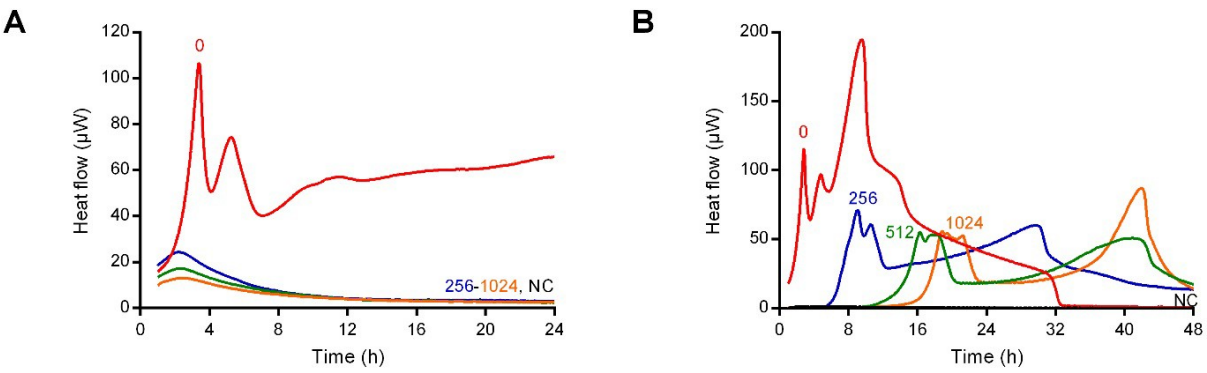

Figure 2 IMC evaluation of anti-biofilm activity of vancomycin against MRSA biofilm in real-time (A) during and (B) after 24 h-treatment. Numbers represent vancomycin concentrations (in µg/ml) ranging from 256 to 1024 µg/ml. Zero represents an untreated control NC; negative (sterility) control. (Study B: Figure 5 in Butini et al., Manuscript submitted to *Frontiers in Microbiology* and currently under revision)

High concentrations of vancomycin select for persister cells in MRSA biofilm and daptomycin exerts an anti-persister activity *in vitro*

Study B revealed that free-floating cells dislodged from treated biofilms and exposed to 100xMIC vancomycin (P+$V_{100xMIC}$) were not affected by high drug concentrations, as indicated by the steady number of colonies (~10^5 CFUs/ml) observed during 6h-incubation, similarly to the same bacterial sample derived from pre-treated biofilms, which were not challenged with 100xMIC vancomycin (P) (Figure 3). By contrast, cells derived from biofilm without any drug pre-treatment (GC) showed an increase of 1.5log_{10} CFU/ml in comparison to the initial inoculum after 6h-incubation (from 4.75±1.30x10^4 to 2.47±3.98x10^6 CFUs/ml) in PBS/1% CAMHB.

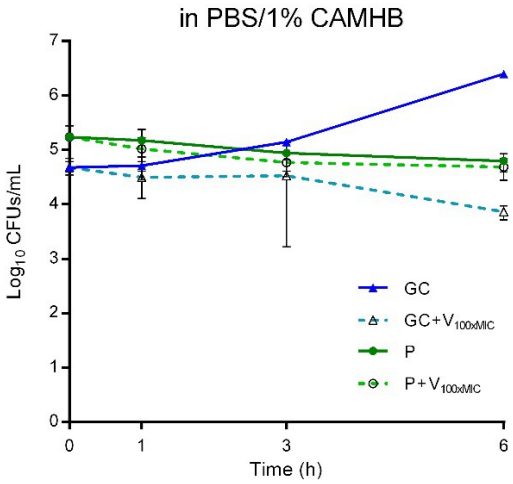

Figure 3 Characterization of persister cells isolated from MRSA biofilm after 24h-treatment with 1024 µg/ml vancomycin. The effect of exposure to high bactericidal vancomycin concentrations (100xMIC_{VAN} = 100 µg/ml) was assessed by colony counting (Log_{10}CFUs/mL) of persister cells (P; P+$V_{100xMIC}$) during 6 h-incubation in PBS/1% CAMHB. GC, growth control; GC+$V_{100xMIC}$, growth control treated with 100xMIC vancomycin. Data are expressed as mean ± SD, 3≤ n ≤6. (Study B: Figure 6 in Butini et al., Manuscript submitted to *Frontiers in Microbiology* and currently under revision)

The same samples exposed to 100xMIC vancomycin (GC+$V_{100xMIC}$) showed a reduction of ~ 2.5log_{10} CFU/ml after 6h in comparison to the untreated GC (7.25±2.10x10^3 *vs* 2.47± 3.98x10^6 CFUs/ml respectively), indicating a susceptibility to 100xMIC vancomycin higher than that observed P+$V_{100xMIC}$ sample.

To deepen the investigation in Study B by evaluating persisters' reversion to normal growth, dislodged treated and untreated biofilm cells (P and GC, respectively) were incubated in fresh

rich medium. As shown in Figure 4, GC rapidly reached the exponential growth phase (λ =3.8 h), as suggested by the timely increase in heat flow, whereas P showed the characteristic delayed growth curve (λ =7 h) resembling the GC heat flow/time curve. Specifically, a Δλ = 3.2 h was calculated between the first peaks of GC and P, suggesting that P cells remained in a metabolically inactive status and, after 6 hours (time needed for the heat flow to exceed the detection limit), they reverted into metabolically active cells.

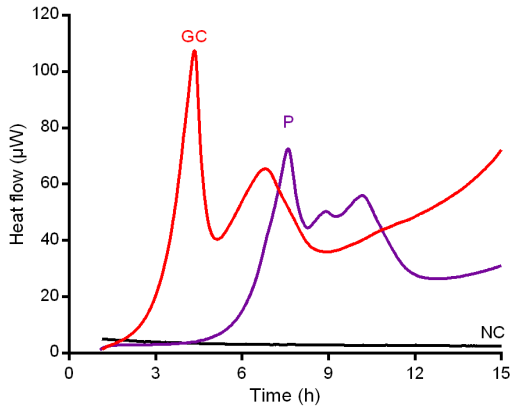

Figure 4 Characterization of persister cells isolated from MRSA biofilm after 24 h-treatment with 1024 µg/ml vancomycin. Revival assay in CAMHB by IMC monitoring the heat flow (µW) plotted against time (h) produced during the experimental time (h). GC, growth control; P, persister cells; NC; negative (sterility) control. (Study B: Figure 7 in Butini et al., Manuscript submitted to *Frontiers in Microbiology* and currently under revision)

Since vancomycin anti-biofilm monotherapy did not eradicate a 24 h-old staphylococcal biofilm, a staggered use of high doses of vancomycin (1024 µg/ml) and sub-eradicating concentrations of daptomycin (16 µg/ml) was tested (Figure 5).

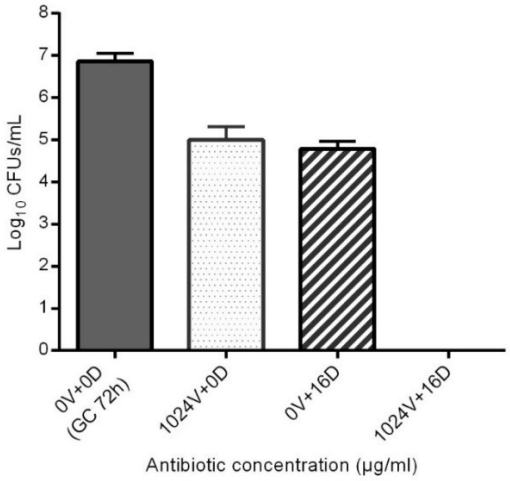

Figure 5 Evaluation of the eradicating activity of the combined staggered treatment vancomycin+daptomycin against a 24 h-old MRSA (ATCC 43300) biofilm by sonication and colony counting (Log_{10}CFUs/mL). Numbers and letters indicate vancomycin (V) and daptomycin (D) concentrations (in µg/ml). GC 72h; growth control of untreated 72 h-old biofilm. Data are expressed as mean ± SD, n = 3. (Study B: Figure 8 in Butini et al., Manuscript submitted to *Frontiers in Microbiology* and currently under revision)

The bactericidal activity of daptomycin resulted in the complete killing of biofilm viable cells (0 colonies on plate counts), highlighting a synergistic eradicating activity against a persister-enriched MRSA biofilm. By contrast, both vancomycin and daptomycin monotherapies failed in eradicating staphylococcal biofilm, since only a mild bactericidal activity resulted in ~2log_{10} CFUs/ml reduction.

Thermosensitive fully degradable hydrogels release high concentrations of daptomycin with retained anti-biofilm activity in a controlled manner

Study C reports on the successful drug loading using daptomycin-saturated solutions (28 mg/ml, pH 4.5 and 8) to reconstitute the lyophilized polymers. During incubation at 37 °C, the material fully jellified, showing no flow behavior after approximately 4 h.

Then, when gels were formulated using HASH_53 (3% w/v solid polymer content), a prominent and timely release of high amounts of lipopeptide, outreaching the minimum biofilm eradicating concentration against MRSA, was obtained (~1.3 mg/mL after 24 h), until hydrogel full degradation after 8 days of incubation (Figure 6A). Differently, hydrogels formulated with HASH_31 (5% w/v solid polymer content), consisting of higher cross-link density and lower network mesh size, retained higher drug loads but still releasing high concentrations (~600 µg/mL) that widely exceeded the MBC during 15 days of incubation. Taken together, a

cumulative release of ~60% and 40% of the initial amount of loaded drug was calculated for gels of 3% and 5% w/v of polymer content, respectively (Figure 6B).

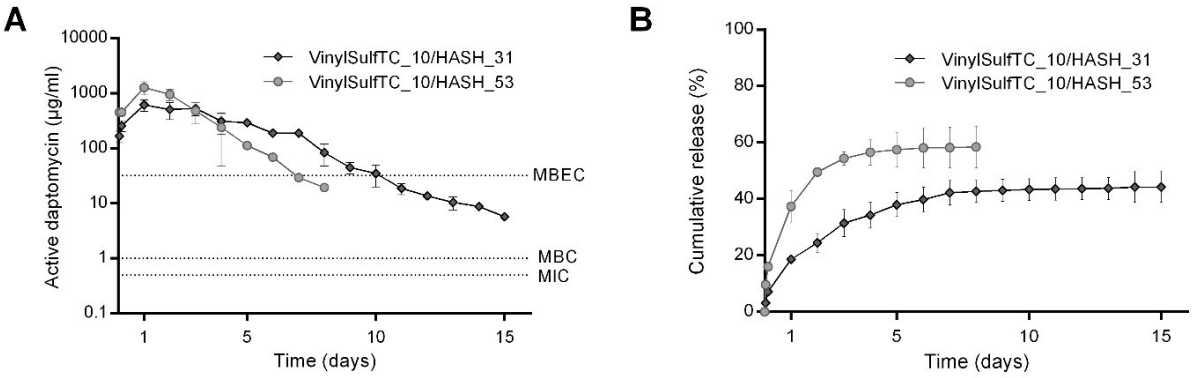

Figure 6 (A) Released active daptomycin and (B) cumulative release from hydrogels containing 10% vinyl sulfonated triblock copolymer (VinylSulfTC_10) (15% w/v) and a varying amount of thiolated hyaluronic acid (3 and 5% w/v of HASH_31 (1) and 53 (□), respectively) assayed by agar well diffusion method. Active daptomycin concentration (µg/mL) is plotted versus time (days), whereas the cumulative daptomycin release is expressed as percentage (%) over time (days). MBEC, minimum biofilm eradication concentration; MBC, minimum bactericidal concentration; MIC, minimum inhibiting concentration; (mean ± SD, *n*=3). (Study C: Figure 8 in Casadidio[1], Butini[1] et al., *European Journal of Pharmaceutics and Biopharmaceutics* (2018))

During co-incubation of MRSA biofilm with daptomycin-loaded hydrogels in microcalorimetric ampoules, a complete inhibition of biofilm metabolism-associated heat was observed, as compared to the growth control (~7x10^6 CFUs/mL by sonication/colony counting). Although a complete killing of biofilm cells was not achieved, as demonstrated by the detection of a delayed heat flow (maximum peak ~140 µW with a 7.5 h delay compared to the untreated biofilm), a major reduction in cell viability was monitored. Indeed, a 36% decrease of metabolism-related heat flow was detected in real-time (Study C: Figure 9 in Casadidio[1], Butini[1] et al., *European Journal of Pharmaceutics and Biopharmaceutics* (2018)).

<u>High titers of anti-staphylococcal bacteriophages are easily loaded and locally released from thermosensitive hydrogels</u>

With the future perspective of formulating double-antimicrobial controlled release systems, preliminary tests on the formulation of bacteriophages-loaded hyaluronan hydrogels were

performed. In an experimental time of 7 days, a noteworthy prolonged and controlled release of Sb-1 was observed (Figure 7).

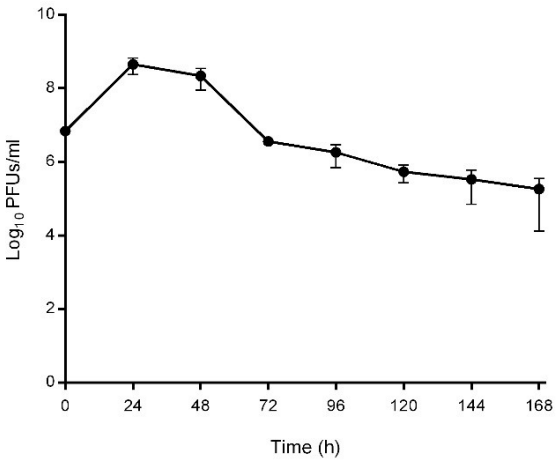

Figure 7 Anti-staphylococcal bacteriophage Sb-1 release from bacteriophages-loaded hyaluronan hydrogels. The titer of released phages was monitored during 168 h-incubation at 37 °C and assessed by plaque assay. Sb-1 titers are expressed as Log_{10}PFUs/ml against time (h). PFUs; plaque forming units. Data are expressed as mean ± SD, *n* = 5.

Indeed, from an initial titer of $6.95\pm1.64x10^6$ PFUs/ml upon buffer addition (T0), the concentration of released phages remained high throughout the whole experiment, decreasing to a minimum of $1.83\pm1.70x10^5$ PFUs/ml after 7 days of incubation. A peak in phages release was monitored during the first 24 h-incubation, reaching a maximum of $4.52\pm2.13x10^8$ PFUs/ml. Taken together, a minimum concentration of $3.59\pm4.41x10^6$ PFUs/ml was ensured in the first 72 h.

Resorbable gentamicin-loaded bone graft substitutes quickly elute high concentrations of active gentamicin, strongly inhibiting biofilm viability of Gram-negative bacteria

As observed with Study D, the incubation of biphasic hydroxyapatite/calcium sulfate beads gave a burst gentamicin elution up to ~2.5 mg/ml in the first 24 h (Figure 8).

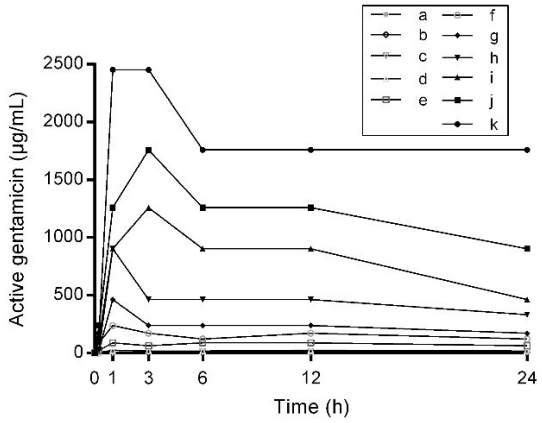

Figure 8 Release kinetic of active gentamicin from different combinations of gentamicin-loaded bone graft substitute beads (a–k) during 24 h-incubation at 37 °C under static conditions. Aliquots of release medium were sampled after 0, 1, 3, 6, 12 and 24 h-incubation. The concentration of active gentamicin (µg/mL) eluted in time (h) was calculated by agar diffusion assay. (Study D: Figure 1 in Butini et al., *Colloids and Surfaces B – Biointerfaces* (2018))

The observed high gentamicin concentrations released in the very first hours of incubation demonstrated to prevent, to different extent, the biofilm formation of different laboratory bacterial strains on glass bead. Indeed, *Escherichia coli* biofilm was prevented with remarkably low gentamicin concentrations (~12 µg/ml), considering that the bioavailability of the loaded drug is lower than that for free drug dissolved in aqueous solution, as conventionally tested. The loaded biphasic bone substitute also demonstrated a good anti-biofilm efficacy, in terms of reduction the metabolism-related heat of a 24 h-old biofilm of Gram-negative rods. Indeed, a strong bactericidal activity against *E. coli* biofilm was achieved with low gentamicin concentrations (~23 µg/ml). Differently, the preventive and bactericidal efficacy of released gentamicin against Gram-positive bacteria proved less effective, as the minimum preventing and bactericidal doses exceeded 171 µg/ml.

5. DISCUSSION

Chronic orthopedic infections are due to bacterial colonization of the surface of implanted devices, which evolves into biofilm, a microbial aggregate encased in an extracellular matrix and extremely resistant to the activity of host immunity and antibiotics [3]. The antibiotic susceptibility assay of biofilm-embedded cells is needed for the establishment of the best therapeutic regime. However, this test results rather laborious and time consuming. As demonstrated in Study A, the application of IMC allows for the real-time monitoring of bacterial viability in terms of heat production related to the cellular metabolic activity, proving to be a suitable method for the antimicrobial susceptibility of biofilm microorganisms. As expected, biofilm MRSA was tolerant to high concentrations of most of the tested antibiotics. In the past, the increased tolerance of biofilm bacteria to antibiotics was believed to be caused by the reduced permeability of the biofilm to drugs given by exopolymeric matrix [36]. Nevertheless, it was demonstrated that most antibiotics could efficiently penetrate biofilms *in vitro* [37], therefore supporting the hypothesis that a subpopulation of cells extremely tolerant to conventional antibiotics, referred to as peristers, could have a major role in treatment failure and relapse of infection [38]. To the best of our knowledge, with Study B we reported for the first time that a deeper analysis of IMC curves enables the detection and identification in real-time of persister cells within a biofilm after anti-biofilm treatment, proving that this technique is also suitable for the analysis and characterization of new anti-persister compounds and biomaterials.

In particular, Study B showed that the scarce efficacy of vancomycin, when administered alone against a *S. aureus* biofilm *in vitro*, might be due to the selection of persister cells. Our observation was further confirmed by the IMC real-time monitoring of persisters' growth reversion in fresh medium, which indeed revealed a diverse metabolic behavior, in terms of delayed metabolism resumption. If translated into clinical practice, this phenomenon might explain infection relapses leading to higher failure rates of vancomycin monotherapy [39]. Since persisters display a reduced replicating activity, the therapy should focus on compounds that are able to kill cells independently from the metabolic state [40]. Although the tolerance of *S. aureus* to daptomycin (as monotherapy) was reported in the literature [41], the lipopetide might exert a bactericidal activity against dormant cells by affecting the microbial outer membrane through rapid depolarization [42, 43]. As we observed, a sub-eradicating concentration of daptomycin (16 μg/ml) was able to clear a staphylococcal biofilm infection enriched in persisters after vancomycin treatment, supporting the use of daptomycin in the clinical practice against MRSA infections after vancomycin treatment failure.

Given the evidence in Study B that daptomycin exerts an anti-persister activity, our interest focused on the effective loading of this molecule into biodegradable carriers for a controlled local release. Indeed, Study C provided new insights into the use of fully degradable biomaterials, such as hydrogels, as reservoir for the local release of antimicrobials as a safe therapeutic option for achieving high local drug concentrations without using invasive or harmful approaches, improving patient compliance and clinical outcomes. Hyaluronic acid/p(HPMAm-lac$_{1,2}$)-based hydrogels are cross-linked networks of hydrophilic polymers that retain large amounts of water yet maintaining their three-dimensional structure. A first physical gelation is given by the hydrophobic interactions between the thermosensitive polymer chains (p(HPMAm-lac$_{1,2}$)). Then, this newly-formed structure is further stabilized via the Michael Addition reaction, which occurs spontaneously at physiological conditions, between vinyl sulfone and thiol groups upon addition of thiolated hyaluronic acid as a cross-linker [30]. These type of gels proved tunable biodegradability profiles, fast gelation kinetics, tailorable mechanical properties and excellent biocompatibility [30]. The particular polymeric composition of these thermosensitive hydrogels used as daptomycin carrier favored a bimodal release profile, where ~50% of loaded drug was released within the first 50 h-incubation following a diffusional release mechanism. Then, a second release phase followed a zero-order kinetic, providing a constant release of active daptomycin during the subsequent 13 days. This release mode based on an initial burst release followed by a sustained elution of high drug concentrations is remarkable. Indeed, differently from biomaterials characterized by a fast diffusion-driven bolus release (*e.g.* collagen-based gels [22]), the tested cross-linked hydrogels demonstrated to release the loaded compound with an initial burst release followed by a sustained drug elution up to polymer full degradation, possibly suppressing an early infection in the first stages of bacterial replication. Moreover, the hydrogel network exerted a protective activity on drug structure, limiting the formation of daptomycin degradation products and, consequently, fostering the retention of bactericidal activity, as observed by HPLC-MS analysis and microbiological assays performed against a laboratory strain of MRSA.

Similar features characterize also different hydrogel formulations, for example the hyluronan/p(NIPAm)-based hydrogels, which were simultaneously formulated and loaded with lytic bacteriophages. Conceived to be applied mostly for the management of spinal infections or as implant coating, these gels released high phage titers for several days in a controlled manner, providing outstanding advances as alternative anti-biofilm strategy. Poly(NIPAm)-based hydrogels are attractive candidates for biomedical applications thanks to their high biocompatibility and thermosensitivity, albeit a major limitation consists in the non-

biodegradability of the polymeric backbone [44]. Hence, depending on the final intended clinical application, the choice of biomaterial varies based on its chemical composition and physical structure. For instance, the soft nature and hydrophilicity of cross-linked hydrogels can provide neither a mechanical support nor a solid scaffold for bone ingrowth. Nevertheless, injectable loaded hydrogels may serve as optimal carrier for the management of spinal implant-associated infections.

As opposed to hydrogels, the mechanical stability and resistance offered by resorbable bone graft substitutes are essential characteristics for the application as bone void fillers. Thus, with Study D we aimed to compare the antimicrobial activity offered by loaded hydrogels to that of a commercially available gentamicin-loaded bone graft substitutes consisting of hydroxyapatite in a calcium sulfate matrix. Designed to fill bone voids and foster bone healing, the ceramic materials provide a stronger mechanical support by protecting and promoting a dynamic bone remodeling and formation, thus protecting the newly formed tissue from fractures and infections. In fact, together with gentamicin elution, the calcium sulfate matrix resorbs, being replaced by naturally regenerated bone within the hydroxyapatite scaffold. However, as observed during the 24h-release kinetic studies, the fast resorption of calcium sulfate might not allow for a controlled drug release comparable to what obtainable with hydrogels or other chemically cross-linked materials. Nevertheless, the combination of resorbable and soft fully degradable biomaterials may be applied, thus synergistically act for promoting the bone tissue healing and protecting the regenerative process via a rapid anti-persister activity. Indeed, although the mechanism of bactericidal action exerted by aminoglycosides is mostly effective against rapidly growing cultures, their ability to eradicate persister cells of Gram-positive and negative strains *in vitro* and *in vivo* has been enabled using specific metabolite stimuli [45].

With this thesis, the successful formulation and analysis of different biocompatible materials for orthopedic applications have been demonstrated. The bactericidal effect exerted by the combination of vancomycin with daptomycin was shown, revealing the anti-persister activity of the lipopeptide, which was then loaded and efficiently released from high-technology smart hydrogels. In addition, the investigation of thermosensitive hydrogels loaded with bacteriophages revealed a surprising long-lasting release of high titer and the convenient application of IMC was also demonstrated in many regards, deeply fostering the use of this technique in clinical microbiology and material science. Then, low doses of gentamicin eluted from a ceramic scaffold proved to prevent the formation of *E. coli* biofilm, besides also completely inhibit the growth of an established one.

Taken together, the reported findings suggest that the application of smart biomaterials such as antibiotic-loaded biphasic bone graft substitutes as bone void fillers, together with the local injection of thermosensitive hydrogels as degradable vehicles for the controlled release of antimicrobials (*e.g.* daptomycin and bacteriophages) may provide paramount improvements for the management of biofilm-associated infections affecting the musculoskeletal system, indeed promoting bone healing and protecting the tissue regeneration from (re)infections by killing any persister cells potentially left after surgical and/or pharmaceutical treatment.

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EIDESSTATTLICHE VERSICHERUNG

„Ich, Maria Eugenia Butini, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema „The controlled release and activity of antimicrobials from biomaterials“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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ANTEILSERKLÄRUNG AN DEN ERFOLGTEN PUBLIKATIONEN

Maria Eugenia Butini hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Butini ME, Gonzalez Moreno M, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M. Real-time antimicrobial susceptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry. Advances in experimental medicine and biology (2018); 1-17, doi: https://doi.org/10.1007/5584_2018_291.

Beitrag im Einzelnen:

- Planung und Organisation aller Experimente in Absprache mit Herrn PD Dr. Trampuz, Frau Dr. Mariagrazia Di Luca (Mentor).
- Durchführung eines Teils der Experimente, im Einzelnen: Tests zur Vorbeugung der Biofilmbildung, Formulierung von Levofloxacin-beladenen Hyaluronsäure Hydrogelen für Freisetzungskinetik-Assay und mikrokalorimetrische Experimente, Mitarbeit an den mikrokalorimetrischen Antibiofilmaktivitätstests.
- Mitarbeit an Versuchen zur Akkumulierung eines Anteils der Daten und Auswertung aller Daten, Interpretation aller Ergebnisse und Mitarbeit an der Durchführung der Datenanalyse. Aus meiner Auswertung sind die Abbildungen 4 und 5 sowie die Tabellen 2 und 3 entstanden.
- Verfassen des Manuskripts und Mitarbeit an der finalen Version des Papers.

Publikation 2: Casadidio C[1], **Butini ME[1]**, Trampuz A, Di Luca M, Censi R, Di Martino P. Daptomycin-loaded biodegradable thermosensitive hydrogels enhance drug stability and foster bactericidal activity against *Staphylococcus aureus*. European Journal of Pharmaceutics and Biopharmaceutics (2018); 130:260-71, doi: https://doi.org/10.1016/j.ejpb.2018.07.001.

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Beitrag im Einzelnen:

- Planung und Organisation aller mikrobiologischen Experimente in Absprache mit Herrn PD Dr. Trampuz und Frau Dr. Mariagrazia Di Luca (Mentor).
- Durchführung aller mikrobiologischen Experimente, im Einzelnen: Bestimmung der Freisetzung von Daptomycin, antimikrobieller Aktivitätstest gegen Biofilme von *S.*

aureus, Formulierung von Daptomycin-beladenen temperaturempfindlichen Hydrogelen für Freisetzungskinetik-Assay und mikrokalorimetrische Experimente, Antibiofilmaktivitätstest von Daptomycin-beladenen Hydrogelen.

- Verarbeitung und Auswertung aller mikrobiologischen Daten, Interpretation aller mikrobiologischen Ergebnisse und Mitarbeit an der Interpretation der chemischen Ergebnisse. Aus meiner Auswertung sind die Abbildungen 4, 7, 8, 9 entstanden.
- Verfassen des Manuskripts und Mitarbeit an der finalen Version des Papers.

Publikation 3: Butini ME, Cabric S, Trampuz A, Di Luca M. *In vitro* anti-biofilm activity of a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute. Colloids and Surfaces B – Biointerfaces (2018); 161:252-60, doi: https://doi.org/10.1016/j.colsurfb.2017.10.050.

Beitrag im Einzelnen:

- Planung und Organisation aller Experimente in Absprache mit Herrn PD Dr. Trampuz und Frau Dr. Mariagrazia Di Luca (Mentor).
- Durchführung aller Experimente, im Einzelnen: Bestimmung der Freisetzung von Gentamicin, antimikrobieller Aktivitätstest gegen Planktonische Bakterien und Biofilme, Bewertung der Resistenz *in vitro*, Tests zur Vorbeugung der Bildung eines Biofilms an unbehandelten Materialien.
- Verarbeitung und Auswertung aller Daten, Interpretation der Ergebnisse, Erstellung der Abbildungen und Tabellen.
- Verfassen des Manuskripts und Mitarbeit an der finalen Version des Papers.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

Unterschrift der Doktorandin

SELECTED PUBLICATIONS

Publication 1 (Study A): Real-time antimicrobial suseptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry

Butini ME, Gonzalez Moreno M, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M

Advances in Experimental Medicine and Biology - Advances in Microbiology, Infectious Diseases and Public Health (2018)

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Impact factor (2017): 1.760

Publication 2 (Study C): Daptomycin-loaded biodegradable thermosensitive hydrogels enhance drug stability and foster bactericidal activity against *Staphylococcus aureus*

Casadidio C[1], **Butini ME[1]**, Trampuz A, Di Luca M, Censi R, Di Martino P

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European Journal of Pharmaceutics and Biopharmaceutics (2018); 130:260-71

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Impact factor (2017): 4.491

Publication 3 (Study D): *In vitro* anti-biofilm activity of a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute

Butini ME, Cabric S, Trampuz A, Di Luca M

Colloids and Surfaces B - Biointerfaces (2018); 161:252-60

doi: https://doi.org/10.1016/j.colsurfb.2017.10.050

Impact factor (2017): 3.997

CURRICULUM VITAE

My curriculum vitae does not appear in the electronic version of my thesis for reasons of data protection.

COMPLETE LIST OF PUBLICATIONS

Published peer-reviewed articles

1. **Butini ME**, Gonzalez Moreno M, Czuban M, Koliszak A, Tkhilaishvili T, Trampuz A, Di Luca M (2018). Real-time antimicrobial susceptibility assay of planktonic and biofilm bacteria by isothermal microcalorimetry
   Advances in Experimental Medicine and Biology - Advances in Microbiology, Infectious Diseases and Public Health; 1-17
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   Impact factor (2017): 1.760
2. Casadidio C[1], **Butini ME[1]**, Trampuz A, Di Luca M, Censi R, Di Martino P (2018). Daptomycin-loaded biodegradable thermosensitive hydrogels enhance drug stability and foster bactericidal activity against *Staphylococcus aureus*
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   Impact factor (2017): 3.997
4. Dubbini A, Censi R, **Butini ME**, Sabbieti M, Agas D, Vermonden T, Di Martino P (2015). Injectable hyaluronic acid/PEG-p(HPMAm-lac)-based hydrogels dually cross-linked by thermal gelling and Michael addition as potential cell/drug carrier with tunable gelation kinetics, degradation and mechanical properties
   European Polymer Journal; 72:423-37
   doi: https://doi.org/10.1016/j.eurpolymj.2015.07.036
   Impact factor (2017): 3.741

Submitted articles

1. **Butini ME**, Abbandonato G, DI Rienzo C, Trampuz A, Di Luca M (2018). Isothermal microcalorimetry detects the presence of persister cells in a *Staphylococcus aureus* biofilm after vancomycin treatment
   Submitted to **Frontiers in Microbiology**

Manuscripts under preparation

1. **Butini ME**, Gonzalez Moreno M, Maiolo EM, Sessa L, Trampuz A. The sensitivity of isothermal microcalorimetry in assessing the antimicrobial activity of bioactive glass S53P4
2. Karbysheva S, Di Luca M, **Butini ME**, Schütz M, Trampuz A. Comparison of sonication and chemical methods, including chelating and reducing agents, for the detection of biofilm-embedded bacteria involved in implant associated infections

Abstracts at international conferences

1. **Butini ME**, Maiolo EM, Trampuz A (2016). The activity of fosfomycin, daptomycin and vancomycin against planktonic and biofilm *Staphylococcus aureus* determined by microcalorimetry. Poster presentation at 26th European Conference of Clinical Microbiology and Infectious Diseases (9-12 April 2016), Amsterdam, The Netherlands
2. **Butini ME**, Trampuz A, Di Luca M (2016). Activity of a gentamicin-loaded bone graft substitute against different bacterial biofilm by microcalorimetry. Oral presentation at 35th Annual Meeting of the European Bone and Joint Infection Society (1-3 September 2016), Oxford, United Kingdom
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6. **Butini ME**, Casadidio C, Trampuz A, Di Martino P, Censi R and Di Luca M (2018). Daptomycin release from thermosensitive hydrogels: favourable release kinetics for the management of implant-associated infections. Poster presentation at 28th European Conference of Clinical Microbiology and Infectious Diseases (21-24 April 2018), Madrid, Spain
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10. **Butini ME**, Trampuz A, Di Luca M (2018). Chasing after persister cells in bacterial biofilms: bad conditions at the wrong time. Poster presentation at 9th BSRT Symposium (28-30 November 2018), Berlin, Germany

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