Local Antibiotic Delivery for Prevention of Biomaterial Infection

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract: Biomaterial- and implant-associated infections (BAI/IAI) remain a major complication in orthopedic surgery. Although systemic antibiotic administration and local antibiotic delivery systems are commonly used, they give limited protection, systemic side effects, insufficient coverage of residual biomaterials, and the emergence of resistant bacteria due to subinhibitory antibiotic concentration. Following an initial burst release, polymeric materials can be repopulated with bacteria and form biofilm, losing “the race for the surface.” In the present thesis, two complementary strategies were investigated to protect biomaterials and implants from early infection: (1) local antibiotic delivery using calcium sulfate/hydroxyapatite (CaS/HA) as carrier and (2) biomodulation i.e. systemic antibiotic giving targeted local recruitment through antibiotic to HA accretion. Tobramycin (TOB) an aminoglycoside was evaluated as a locally delivered antibiotic, while tetracycline (TET), was selected for systemic recruitment due to high accretion to HA.

In Study 1 and Study 2, a biphasic CaS/HA biomaterial demonstrated controlled and sustained TOB release both in-vitro and in-vivo, with antibacterial activity against *Staphylococcus aureus* maintained for at least 19 days in-vitro. CaS/HA+TOB exhibited longer release duration and prolonged antibacterial activity for up to 35 days compared with combined delivery of TOB and vancomycin (VAN), and no synergistic effect was observed for the TOB+VAN combination. In a rabbit posterolateral lumbar spinal fusion model, CaS/HA+TOB used as a bone graft extender did not impair bone regeneration. CaS/HA+TOB resulted in fusion rates and bone formation comparable to CaS/HA+BMP-2 and high local TOB concentrations did not negatively affect bone healing. In Study 3 and Study 4, the accretion of TET to Hydroxyapatite (HA) particles and HA-coated implants was evaluated with different particle sizes, biological interferences, and systemic administration regimens. TET exhibited higher affinity to nano-HA compared with micro-HA, and accretion was influenced by protein passivation and competing HA binding drugs. In-vivo experiments demonstrated preferential recruitment of systemically administered TET to pre-implanted HA materials. The optimal biomodulation regimen consisted of one perioperative dose followed by a daily dose on postoperative days 1 and 2. TET was effectively bound to HA and reduced bacterial adhesion on the tested biomaterial and apatite coated implant.

In conclusion, this thesis presents two complementary approaches for early infection control in orthopedic surgery: (1) controlled local antibiotic delivery via CaS/HA biomaterials and (2) systemic antibiotic recruitment via HA-mediated biomodulation. These findings offer novel strategies to protect biomaterials and implants from bacterial adhesion while preserving bone regeneration. Collectively, the materials and methodologies developed in this work establish a translational framework for improving infection prevention in orthopedic surgery.

Key words: Bone, Infection, Biomaterials, Antibiotics, Local delivery

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To My Family and My Teachers....

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Popular science

Bone and joint infections are serious complications that may occur after orthopedic surgery and can lead to prolonged treatment or additional surgical procedures. Orthopedic implants are foreign materials in the body and cannot be fully protected by the immune system. The implant surface is rapidly covered by proteins. This creates an opportunity for bacteria to attach to the implant surface to form biofilms, which protect them from antibiotics and immune response.

Conventional treatment relies mainly on systemic antibiotic administration. However, it is sometimes difficult to achieve sufficiently high local antibiotic concentrations at the infection site in bone tissue, and high systemic doses may increase the risk of side effects. To overcome this problem, local antibiotic delivery systems have been developed, which at site can leak antibiotics in high concentrations. Since the 1970s, bone cement polymethylmethacrylate (PMMA) has been widely used for this purpose. However, PMMA has several limitations, including an initial burst release of antibiotics and the need for a secondary surgery to remove the material because it is non-degradable. Insufficient control of infection at an early stage may reduce the chances of successful treatment and increase the risk of antibiotic resistance.

The aim of this thesis was to investigate two strategies to improve local infection prevention and treatment. The first strategy was to use a biodegradable biomaterial composed of calcium sulfate/hydroxyapatite (CaS/HA) as a carrier for local delivery of the antibiotic tobramycin (TOB). Because this biomaterial gradually degrades in the surgical area, it does not require a second surgery for removal. The second strategy explored the possibility of recruiting systemically administered tetracycline (TET), to the surgical site, using pre-implanted hydroxyapatite (HA) known to bind chemically to TET. In this approach, HA acts as a recruitment platform that can attract HA-binding antibiotics like TET from the bloodstream, thereby enhancing local antimicrobial protection in a simple and minimally invasive way.

The results of this thesis showed that CaS/HA can serve as an effective carrier for TOB, providing sustained antibiotic release both in-vitro and in-vivo, thereby helping to eradicate bacteria without negatively affecting new bone formation. Furthermore, HA particles and HA-coated implants were shown to function as recruitment agents for systemically administered TET, helping to reduce the adhesion of *Staphylococcus aureus* on implant surfaces. The optimal timing for systemic TET administration was also investigated to maximize this protective effect.

Abstract

Biomaterial- and implant-associated infections (BAI/IAI) remain a major complication in orthopedic surgery. Although systemic antibiotic administration and local antibiotic delivery systems are commonly used, they give limited protection, systemic side effects, insufficient coverage of residual biomaterials, and the emergence of resistant bacteria due to subinhibitory antibiotic concentration. Following an initial burst release, polymeric materials can be repopulated with bacteria and form biofilm, losing "the race for the surface". In the present thesis, two complementary strategies were investigated to protect biomaterials and implants from early infection: (1) local antibiotic delivery using calcium sulfate/hydroxyapatite (CaS/HA) as carrier and (2) biomodulation i.e. systemic antibiotic giving targeted local recruitment through antibiotic to HA accretion. Tobramycin (TOB) an aminoglycoside was evaluated as a locally delivered antibiotic, while tetracycline (TET), was selected for systemic recruitment due to high accretion to HA.

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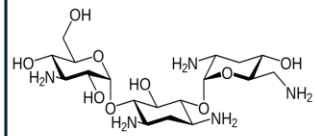
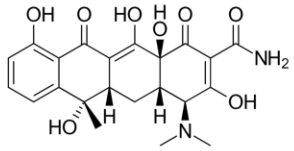
Thesis at a glance

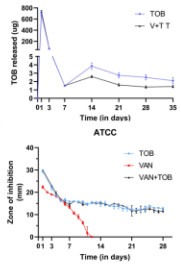


TOB
A broad-spectrum aminoglycoside antibiotic for infection control in clinic
(Study 1 and Study 2)



TET
A broad-spectrum tetracycline antibiotic with high affinity to hydorxyapatite
(Study 3 and Study 4)

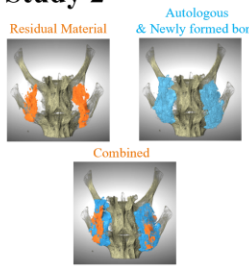
Study 1



Aim: Evaluate TOB release kinetics and antimicrobial efficacy of CaS/HA.

Conclusion: CaS/HA enabled sustained TOB release with prolonged antimicrobial activity

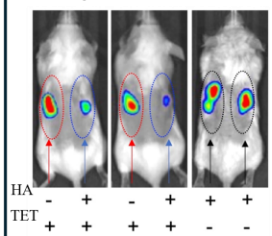
Study 2



Aim: Evaluate CaS/HA+TOB as a bone graft extender and assess TOB release.

Conclusion: CaS/HA+TOB provided controlled release without compromising bone healing.

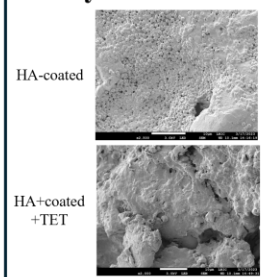
Study 3



Aim: Investigate the affinity of systemically administered TET to HA particles.

Conclusion: Systemically administered TET bound to pre-implanted HA particles and reduced bacterial adhesion.

Study 4



Aim: Identify the optimal timing for TET biomodulation.

Conclusion: Postoperative dosing on day 0–2 achieved maximal implant protection.

TOB: Tobramycin; TET: tetracycline; CaS/HA: Calcium sulfate/hydroxyapatite

List of Papers

1. A calcium sulphate/hydroxyapatite ceramic biomaterial carrier for local delivery of tobramycin in bone infections: Analysis of rheology, drug release and antimicrobial efficacy.
   Huang, J., Sebastian, S., Collin, M., Tägil, M., Lidgren, L., Raina, D. B.
   Ceramics International 2023, 49 (21), 33725-33734.
2. A Pre-Set Calcium Sulfate/Hydroxyapatite Biomaterial as an Antibiotic-Eluting Bone Extender and a Carrier for BMP-2: A Pilot Study in a Rabbit Posterolateral Spinal Fusion Model.
   Huang, J., Lukoševičiūtė, G., Mrkonjic, F., Alidadi, H., Jakstas, D., Sebastian, S., Lidgren, L., Tägil, M., Raina, D.B.
   J. Funct. Biomater. 2026, 17, 118.
3. Hydroxyapatite: An antibiotic recruiting moiety for local treatment and prevention of bone infections.
   Sebastian, S., **Huang, J**., Liu, Y., Tandberg, F., Collin, M., Puthia, M., Raina, D. B.
   J Orthop Res 2024, 42 (1), 212-222.
4. Systemic Administration with Antibiotics Having Accretion to Hydroxyapatite Prevents Infection of Hydroxyapatite-Coated Implants.
   Huang J., Tägil M, Lidgren L, Raina, D. B.
   In Manuscript

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Abbreviations

BAI/IAI	Biomaterial- and implant-associated infections
CaS/HA	Calcium sulfate/hydroxyapatite
TOB	Tobramycin
VAN	Vancomycin
PJIs	Periprosthetic joint infections
DAIR	Debridement, Antibiotics, and Implant Retention- a method of revising an infected joint replacement
HA	Hydroxyapatite
MMPs	Matrix metalloproteinases
TRAcP	Tartrate-resistant acid phosphatase
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
FnBPs	Fibronectin-binding proteins
SEM	Scanning electron microscopy
ClfA/B	Clumping factors
Cna	Collagen adhesin
TNF-α	Tumor necrosis factor-α
IL-1	Interleukin-1
IL-6	Interleukin-6
MSCs	Mesenchymal stem cells
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
PIA	Polysaccharide intercellular adhesin
EPS	Extracellular polymeric substances
SCVs	Small colony variants
PMMA	Polymethylmethacrylate
GEN	Gentamicin
CaP	Calcium phosphate
rhBMP-2	Recombinant human bone morphogenetic protein 2

β-TCP	Beta-Tricalcium phosphate
MRSA	Methicillin-resistant Staphylococcus aureus
RIF	Rifampicin
TGF-β	Transforming growth factor-beta
PBS	Phosphate-buffered saline
n-HA	Nano-hydroxyapatite
m-HA	Micro-hydroxyapatite
HAD	Hyaluronic acid
micro-CT	Micro-computed tomography
CFU	Colony-forming unit
LC-MS	Liquid chromatography–tandem mass spectrometry
MHA	Muller–Hinton agar
ZOI	Zone of inhibition
ZA	Zoledronic acid
IVIS	In-vivo imaging system
H&E	Hematoxylin and eosin

1. Introduction

1.1 Epidemiology and Clinical Burden

Since the term orthopedics was first coined in 1741 by French physician Nicolas Andry de Bois-Regard, the field significantly advanced over the past centuries. Pasteur identified bacteria, and Lister reduced mortality by protecting open fractures from secondary contamination and paved the way for fracture surgery and the development of internal fixation starting in the 1930s[1-3]. Aseptic and antiseptic measures have markedly improved healing outcomes and substantially reduced infection rates in orthopaedic surgeries[4, 5]. However, biomaterial- or implant-associated infections (BAI/IAI) remain a persistent and serious challenge[6, 7]. These infections are notoriously difficult to treat using conventional methods and with surgical failure, prolonged hospitalization, and the need for repeated revision surgery.

Periprosthetic joint infections (PJIs) and spinal infections after fusion surgery are common postoperative complications. The incidence of PJI has been brought down dramatically from 20-30% in the 1970's to approximately 1–2% today with the use of prophylactic antibiotics as well as antibiotic eluting bone cements. The infection rate in spinal surgery is considerably higher and can reach as high as 10%[8, 9] depending on surgical approach. These infections may result in implant loosening and damage to the surrounding soft tissues[10-12]. Although the definition of "early-stage infection" may vary depending on the type of orthopedic procedure, early intervention is commonly recognized to halt bacterial colonization and prevent progression[13]. Strategies such as Debridement, Antibiotics, and Implant Retention (DAIR) protocol are commonly employed to manage early-stage infections without revision. Nevertheless, if these initial treatments fail, revision surgeries become necessary, and are associated with substantially higher costs than primary surgeries and with a higher risk of reinfection, reported between 8–16%[14, 15].

1.2 The cellular composition of bone tissue

Bone tissue is primarily composed of three types of cells: osteoblasts, osteoclasts, and osteocytes. Although developmentally of different origins, these cells play distinct but interrelated roles in maintaining skeletal structure and function. Osteoblasts are responsible for bone formation. They actively deposit bone matrix and facilitate its mineralization, particularly through the production of hydroxyapatite (HA), the main inorganic component of bone[16, 17]. In contrast, osteoclasts mediate bone resorption. They form a specialized, sealed compartment called the resorption lacuna, which acidifies SEthe environment by secreting H^+ ions[18, 19]. This acidic microenvironment enables the action of lysosomal enzymes, including cathepsin K, matrix metalloproteinases (MMP-9 and MMP-13), and tartrate-resistant acid phosphatase (TRAcP), to degrade the bone matrix[20]. Osteocytes, which make up 90–95% of all bone cells, serve as key regulators of bone remodeling. These cells are derived from osteoblasts that have become embedded in the bone matrix they secrete, undergoing morphological transformation into mature osteocytes[21]. Interconnected via canaliculi, osteocytes form a complex three-dimensional signaling network that is crucial for mechanical sensing[22]. They transduce mechanical signals into biochemical responses, detect microdamage, and coordinate bone remodeling by influencing both osteoblasts and osteoclasts[23].

Together, these three cell types maintain bone as a highly dynamic tissue, constantly undergoing remodeling to adapt to mechanical demands and repair damage. The dynamic balance between osteoblast-mediated bone formation and osteoclast-driven resorption is fundamental for maintaining skeletal integrity, bone strength, and microarchitectural stability[16]. Disruption of this tightly regulated coupling process results in pathological bone remodeling and contributes to the development of skeletal disorders[24, 25]. Furthermore, pathogenic bacteria such as *Staphylococcus aureus* (*S. aureus*) can invade this cellular system, particularly osteocytes, triggering inflammatory responses that disrupt homeostasis, impair remodeling, and contribute to infection-related bone disorders[26, 27].

1.3 The mechanism of *S. aureus* causing infection

Staphylococci, particularly *S. aureus* and coagulase-negative staphylococci, remain the most common pathogens in orthopedic implant-associated infections.[28-30]. As a natural resident of human skin and mucous membranes, *S. aureus* usually exists as a harmless commensal organism. However, during surgical procedures, particularly those involving open wounds or the insertion of implants, the bacteria can easily gain access to deeper tissues. This typically occurs through breaches in

the skin barrier or via perioperative contamination of surgical instruments, gloves, or even airborne particles in the operating room[31]. Upon entry into the surgical site, *S. aureus* encounters a local microenvironment characterized by exposed extracellular matrix components and implanted biomaterials, conditions that facilitate bacterial adhesion, colonization, and subsequent biofilm formation[29].

After implantation, plasma proteins and tissue fluid components from the host are rapidly and spontaneously adsorbed onto the implant surface, forming a protein adsorption layer, also known as a conditioning film[32]. This layer consists mainly of fibronectin, fibrinogen, collagen, albumin, and other extracellular matrix proteins, which act as biological bridges between the foreign material and host tissue. The conditioning film provides ideal anchoring sites for bacterial adhesion. *S. aureus* expresses a wide array of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which specifically bind to these host proteins[33]. Through MSCRAMMs such as fibronectin-binding proteins (FnBPs), clumping factors (ClfA/B), and collagen adhesin (Cna), the bacteria can firmly attach to both the implant surface and the adjacent bone tissue, initiating colonization[34]. This early adhesion is a critical first step that facilitates subsequent biofilm formation, where the bacteria become embedded in a self-produced extracellular polymeric matrix that protects them from antibiotics and host immune attack[35, 36].

Beyond surface attachment, *S. aureus* can also interact directly with host cells. FnBPs not only mediate bacterial adhesion to fibronectin but also allow *S. aureus* to be internalized by non-phagocytic cells such as osteoblasts[26, 34]. Once internalized, the bacteria can persist in a dormant state within the cytoplasm, effectively evading immune detection and antimicrobial treatments[37]. Over time, when the infected osteoblasts undergo lysis, viable bacteria are released back into the surrounding tissue, leading to recurrent or chronic infections that are difficult to eradicate[26].

The infection process is accompanied by a robust inflammatory response characterized by the release of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). These cytokines have been shown to play pivotal roles in the pathogenesis of bone infection and destruction[25, 27]. TNF-α and IL-6 can stimulate the proliferation and differentiation of osteoclast precursors into mature osteoclasts, thereby enhancing bone resorption. Meanwhile, these inflammatory mediators inhibit the osteogenic differentiation of mesenchymal stem cells (MSCs), suppress matrix synthesis, and impair mineralization, resulting in a net loss of bone-forming osteoblasts and osteocytes[38, 39]. Importantly, these detrimental processes can also be initiated by bacterial-derived components or virulence factors released from *S. aureus*, even in the absence of direct bacterial invasion or osteoblast involvement, suggesting that bacterial components alone can trigger bone degradation pathways.

Apart from the inflammatory cascade, *S. aureus* can directly induce osteoblast apoptosis through contact-dependent mechanisms. The adhesion of *S. aureus* to osteoblasts via FnBP–fibronectin interactions upregulate the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which activates apoptotic signaling cascades within the host cells[40]. As a result, osteoblast death and decreased bone formation further exacerbate local bone loss and compromise the integrity of the skeletal tissue. The combination of inflammatory cytokine-driven osteoclast activation, suppression of osteoblast differentiation, and TRAIL-mediated apoptosis creates a vicious cycle that ultimately leads to progressive bone destruction and chronic infection.

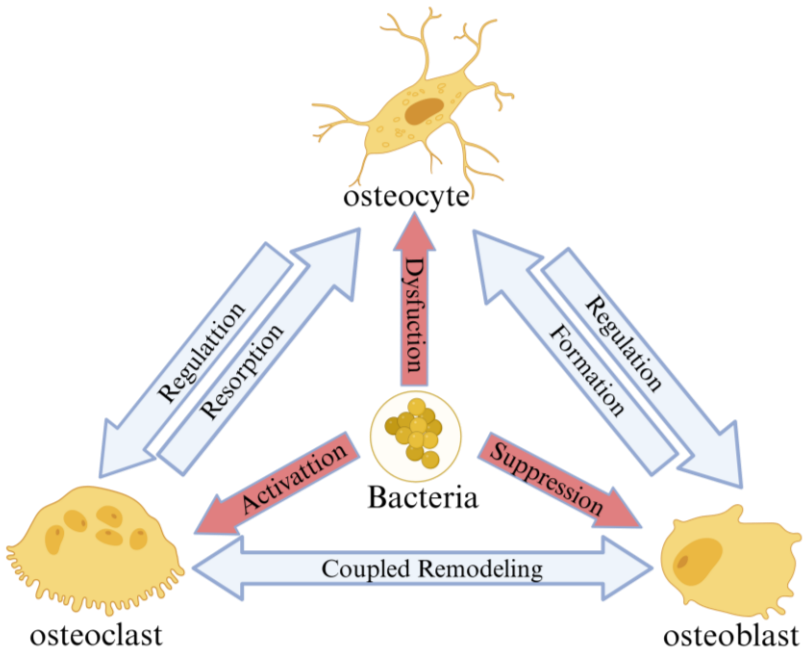

Figure 1: Bacterial infection disrupts bone homeostasis.

1.4 Biofilm Development and Structural Organization

In addition to the intracellular invasion of *S. aureus* and the tissue damage driven by inflammation, biofilm formation represents another critical mechanism by which the bacterium establishes infection in bone. Biofilms are widely regarded as the underlying cause of many persistent and chronic bacterial infections, primarily due to their extraordinary resistance to both antimicrobial agents and host immune

defenses[29, 35]. Although many aspects of biofilm biology, such as population heterogeneity, metabolic cooperation, quorum sensing dynamics, and host-pathogen interactions, remain incompletely understood, the early phases of biofilm development have been extensively characterized and are generally divided into four sequential stages: 1) initial attachment of bacterial cells; 2) cell aggregation and accumulation into multilayered clusters; 3) biofilm maturation; and 4) detachment or dispersal of bacterial cells into a planktonic state, which initiates new cycles of colonization elsewhere[41, 42].

The precise time point at which a biofilm is considered "mature" remains subject to experimental context and bacterial species. In many in-vitro models of *S. aureus*, however, the 24-hour time point is commonly used to represent established biofilm formation[43, 44]. Beyond this period, the biofilm becomes more structurally complex, metabolically heterogeneous, and significantly more difficult to eradicate with antibiotics[45, 46]. During the initial attachment stage, *S. aureus* cells are passively adsorbed onto the implant or bone surface through nonspecific physicochemical forces such as hydrophobic interactions, electrostatic attraction, and Lifshitz–Van der Waals forces. Once a few bacteria adhere, specific interactions mediated by MSCRAMMs strengthen this attachment. Subsequently, *S. aureus* actively recruits additional cells by secreting polysaccharide intercellular adhesin (PIA), which promotes intercellular aggregation and the formation of small multilayered bacterial clusters[47]. This early structure marks the transition toward a developing biofilm.

As the biofilm matures, it thickens and evolves into a highly organized community encased in a dense extracellular polymeric substances (EPS) matrix, which can constitute 80–90% of the total biofilm dry mass[48]. The EPS is a complex mixture of polysaccharides, proteins, lipids, and extracellular DNA, providing both mechanical stability and a formidable barrier to immune cells and antimicrobial agents. Mature biofilms display extreme antibiotic tolerance and immune evasion, allowing *S. aureus* to persist on implant surfaces and within bone tissue for prolonged periods[49]. In the final dispersion phase, parts of the biofilm release planktonic cells or cell aggregates that disseminate to new locations, seeding additional infection sites and perpetuating cycles of reinfection[50].

A mature *S. aureus* biofilm can be described as a highly structured microbial consortium in which bacteria adopt a lifestyle fundamentally distinct from that of free-living planktonic cells. Within the biofilm, oxygen and nutrient gradients lead to the formation of spatially stratified bacterial subpopulations. Cells in the outermost layer exhibit active metabolism and rapid proliferation, consuming most available resources, while cells in the middle and basal layers enter slow-growing or dormant states[51]. This metabolic heterogeneity is a critical survival strategy that enables the bacterial community to withstand environmental stress, nutrient deprivation, and antibiotic exposure. Indeed, studies have shown that even under antibiotic treatment, approximately 1% of the bacterial population within a biofilm

can survive due to their transition into a stationary or dormant phase[52, 53]. Over time, this proportion increases as more cells adopt low-metabolic or "persister" phenotypes, further enhancing the resilience of the biofilm.

Collectively, these observations underscore the pivotal role of biofilm formation in the pathogenesis of *S. aureus* bone infections. The presence of a mature biofilm not only confers protection against antimicrobial eradication but also acts as a persistent stimulus for host immune activation, thereby contributing to chronic inflammation and progressive tissue damage[54]. Therefore, preventing biofilm formation at the earliest stage, by minimizing bacterial adhesion to bone and implant surfaces, is a key strategy for reducing postoperative infection risk and improving the longevity of orthopedic implants.

1.5 Clinical Challenges in Implant-Associated Infection

When bacteria invade the body and infection begins, the host immune system is rapidly activated, recruiting neutrophils, macrophages, and other immune cells to the site of infection[55]. However, not only the invading bacteria but also the implanted materials themselves are recognized as foreign entities, triggering an immune response. Activated immune cells release inflammatory cytokines and degradative enzymes that, while intending to eliminate pathogens, also contribute to collateral tissue damage, including degradation of the bone matrix and trabecular structures[37]. Many of the innate immune responses that participate in antibacterial defense simultaneously disrupt normal bone homeostasis. The persistent release of inflammatory mediators at the infection site suppresses osteoblast activity and bone formation, while stimulating osteoclast differentiation and bone resorption[25]. This imbalance accelerates bone loss and delays osseointegration around the implant.

Because of the time required for immune activation and cell recruitment, some bacteria can survive this initial inflammatory phase. Once established, *S. aureus* can invade host cells to evade immune clearance or adhere to the implant surface and form biofilms that collectively resist both immune attack and antibiotic exposure. In addition, studies have identified a phenotypic variant of *S. aureus* known as small colony variants (SCVs), which display enhanced persistence and a remarkable ability to cause recurrent infections due to their reduced metabolism and increased tolerance to antibiotics[56, 57].

Based on these observations, researchers have concluded that the host immune response alone is often insufficient to protect implants and eliminate invading bacteria[58, 59]. To explain this early-stage competition, Gristina proposed the concept of the "race for the surface", which highlights the dynamic struggle between bacterial colonization and host-cell integration[60, 61]. If bacteria win this race, they rapidly adhere, replicate, reach quorum sensing, and form a mature biofilm, leading

to a persistent, treatment-resistant infection. Conversely, if host cells successfully colonize the surface first and establish a stable biological interface, the risk of infection and surgical failure is significantly reduced[62]. This concept highlights the importance of protecting both bone and implant surfaces by preventing bacterial colonization at the earliest stage. Effective approaches include the systemic administration of antibiotics for early bacterial eradication, and the use of biomaterial-based local delivery systems that enable sustained release of antimicrobial agents. The continuous development of these strategies reflects ongoing efforts to strengthen local antibacterial defense, support tissue integration, and ultimately reduce the risk of chronic and recurrent infections.

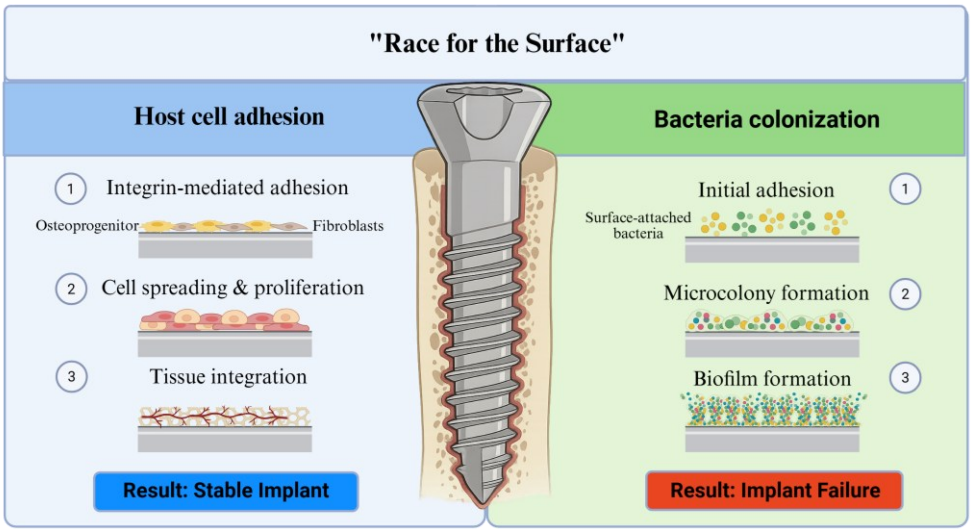

Figure 2: Conceptual model of “the race for the surface”.

1.6 Limitations of Systemic Antibiotic Therapy

Since the discovery of penicillin by Alexander Fleming in 1928 and its later development for clinical use in the early 1940s, antibiotics have saved countless patients from life-threatening infections[63, 64]. Following this milestone, numerous other antibiotics have been developed and introduced into clinical use, each with distinct mechanisms of action and antibacterial spectra. Among these, several antibiotics have played particularly important roles in orthopedic infection management, such as tobramycin (TOB), vancomycin (VAN) and tetracycline (TET). TOB is a broad-spectrum aminoglycoside antibiotic primarily targeting Gram-negative bacteria, with additional activity against *S. aureus*, and is widely used for both systemic treatment and local prophylaxis of infections[65, 66]. VAN

is a glycopeptide antibiotic regarded as one of the “last lines of defense” against multidrug-resistant Gram-positive bacteria, owing to its unique mechanism of inhibiting bacterial cell wall synthesis[67]. TET is a broad-spectrum bacteriostatic antibiotic that binds to the 30S ribosomal subunit, blocking protein synthesis, and it is also known for its strong affinity to HA, which enables potential local delivery applications in bone-related infections[68, 69]. Therefore, different antibiotics are selected according to the clinical situation and the bacterial species involved, targeting Gram-positive, Gram-negative, or both types of pathogens.

Early formulations of penicillin were unsuitable for oral administration, and rapid attainment of therapeutic plasma concentrations was required; therefore, antibiotics were initially administered primarily via parenteral routes, particularly intravenous injection[64]. This systemic approach proved to be effective and remains the mainstay of treatment for severe infections today. In theory, the ideal systemic antibiotic should be able to reach bactericidal concentrations within both organic and inorganic bone components, on implant surfaces, and even within intracellular compartments. It should also be active against slow-growing biofilms and metabolically quiescent SCVs, while exhibiting low toxicity and minimal potential for resistance development[70, 71]. Unfortunately, no antibiotic with such comprehensive properties has yet been discovered.

In clinical reality, the complex structure of bone and its limited vascularization result in poor antibiotic penetration, and reported bone concentrations often reach only 10–50% of corresponding serum levels, depending on the specific agent[70]. To compensate, higher systemic doses or prolonged treatment durations are often required, both of which increase the risk of adverse effects and may promote bacterial persistence. Oral antibiotics are therefore commonly used as a step-down or supplementary therapy following intravenous administration. They are more convenient and safer for patients, especially when long-term treatment over several months is needed[72]. However, oral therapy has intrinsic limitations, including slower onset of action, variable gastrointestinal absorption, and the same fundamental problem of inadequate delivery to poorly vascularized bone tissue[71, 72].

Another major limitation of systemic antibiotic therapy lies in its poor efficacy against biofilm-associated bacteria and intracellularly hidden pathogens, which often remain undetected during the asymptomatic early stages of recurrence[73]. Consequently, regardless of the route of administration, systemic antibiotic therapy alone remains insufficient to fully eradicate bone and implant-associated infections.

1.7 The development of local drug delivery systems

The limitations of systemic antibiotic therapy encouraged researchers to explore alternative approaches capable of achieving higher local concentrations around the surgical site. The first generation of biomaterials, developed and introduced into clinical practice since the 1940s, included stainless steel, titanium, polymethylmethacrylate (PMMA), polyethylene, and others[74, 75]. These materials were primarily designed for mechanical fixation, fracture stabilization, or tissue replacement, and not for local drug delivery. Nevertheless, many of them remain widely used in orthopedic surgery today due to their mechanical reliability and biocompatibility. Among them, PMMA stands out as a landmark material that bridged the transition between inert structural implants and functional local drug delivery systems. PMMA was the first synthetic polymer produced by the chemical industry adopted as a biomaterial for implant fixation in orthopedic surgery. In the 1970s, clinicians began incorporating gentamicin (GEN) into PMMA to create the first local antibiotic delivery system[76]. Gentamicin was chosen for its thermal stability, allowing it to withstand the exothermic polymerization of PMMA during curing. This innovation represented a milestone in the development of local drug delivery and was soon widely applied for the prevention and treatment of infections in arthroplasty, trauma, and osteomyelitis. However, in the early years, the pharmacokinetics and release characteristics of antibiotic-loaded PMMA were poorly understood, which may have contributed to bacterial resistance[77]. Over time, several inherent drawbacks of PMMA became evident. As a bioinert and non-degradable material, PMMA cannot resorb, promote bone cell adhesion, or integrate with host tissue. Consequently, antibiotic release occurs mainly from the superficial layers, providing only a short-term burst, while the remaining drug remains entrapped within the cement matrix[78, 79]. Moreover, residual PMMA often requires surgical removal after infection has healed[80]. It can even act as a substrate for late bacterial adhesion and biofilm formation.

The limitations of PMMA have prompted researchers to search for biodegradable and bioactive alternatives capable of providing sustained antibiotic release, supporting bone regeneration, and eliminating the need for secondary surgery. Consequently, the second generation of biomaterials was developed, including bioactive glass, calcium phosphate (CaP), calcium sulfate (CaS), HA, and others[75]. Both CaS and HA are classified as ceramic biomaterials, characterized by excellent biocompatibility, biodegradability, and osteoconductive potential[81, 82]. Notably, CaS is fully biodegradable in vivo, whereas HA exhibits slower resorption and long-term structural stability, as its degradation is primarily mediated by osteoclast activity. Osteoclasts could adhere on the implants and generate an acidic microenvironment (pH ~4–5), which promotes dissolution of the calcium–phosphate mineral phase and contributes to the gradual remodeling of HA in vivo[83]. The CaS typically resorbs within several 6-8 weeks after implantation.

During dissolution, the released calcium ions can promote osteoconduction by providing a favorable ionic environment for osteoblasts[84]. CaS exhibits good plasticity and can be mixed with liquid, such as saline, to form an injectable paste, which allows easy molding to fill irregular bone defects in clinical use[81]. However, this same property makes CaS highly sensitive to moisture, resulting in faster degradation, lower mechanical strength, and increased fragmentation. Due to its brittle structure and limited osteoconductivity, CaS is mainly used as a supplementary filler rather than a load-bearing material, especially in large bone defects. Moreover, the release of Ca^{2+} and SO_4^{2-} ions during resorption may occasionally induce serous drainage causing local inflammation[85]. HA, the main inorganic component of bone, exhibits excellent biocompatibility and osteoconductivity because it can physicochemically bind to host bone[86, 87]. A bone-like carbonated apatite layer formed on the HA surface can adsorb proteins, thereby facilitating osteoblast attachment and proliferation[88]. Following CaS dissolution, the exposed HA particles create a porous structure that facilitates vascularization and new bone ingrowth, providing a favorable surface for osteoblast adhesion and proliferation[89]. HA is also widely used on uncemented implant surfaces to enhance osseointegration, highlighting its strong physicochemical affinity to native bone[90, 91].

In recent decades, biomaterials have been conceptually categorized into successive generations, reflecting a gradual shift from passive structural support toward increasingly biofunctional and adaptive systems[92]. Materials often described as third-generation biomaterials represent a transition from inert or merely bioactive scaffolds to systems designed to actively modulate cellular behavior at the molecular level[75]. Through molecular modifications of resorbable polymers and ceramics, these materials can interact with cell surface integrins and associated signaling pathways, thereby influencing cell adhesion, proliferation, differentiation, and extracellular matrix organization[93]. Two principal strategies have emerged within this framework: tissue engineering, in which biomaterials function as scaffolds for seeded progenitor cells, and in situ tissue regeneration, where the material itself provides biochemical and biophysical cues that recruit and activate endogenous cells[94]. To further enhance osteogenesis, bioactive ions (such as Ca^{2+}, Sr^{2+}, or Si^{4+}), growth factors including recombinant human bone morphogenetic protein 2 (rhBMP-2), or mesenchymal stem cells have been incorporated into these systems[95-97]. These approaches can significantly improve osteoconduction and osteoinduction, bridging the gap between conventional graft substitutes and more advanced regenerative therapies. Building upon these developments, biomaterials with intelligent and responsive properties, sometimes referred to as fourth-generation biomaterials, are increasingly being explored. Rather than functioning solely as passive implants or controlled-release matrices, these systems are designed to exhibit adaptive and context-dependent behavior[98, 99]. They may incorporate controllable release mechanisms capable of delivering growth factors, drugs, or therapeutic ions in response to specific physiological stimuli, as well as feedback-

responsive elements that modulate activity according to local conditions, such as infection, inflammation, or mechanical loading. In addition, emerging concepts such as immunomodulatory design and artificial intelligence (AI)-assisted structural optimization aim to tailor material architecture and biological performance to patient-specific needs[100, 101]. Although these advanced biomaterial strategies demonstrate considerable promise in preclinical studies, long-term clinical validation and large-scale translational evaluation remain necessary before widespread implementation in routine orthopedic practice can be achieved.

Table 1. Commercially available antibiotic-loaded bone graft substitutes. (Adapted from Armbruster et al., 2025)

Product name (Manufacturer)	Form	Composition	Antibiotic Loading
Cerament (Bonesupport)	Paste	• 60% calcium sulphate • 40% hydroxyapatite	• Gentamicin • Vancomycin
Osteoset T (Stryker)	Beads	• Calcium sulphate alpha-hemihydrate	• Tobramycin
PerOssal (Osartis)	Beads	• 48.5% calcium sulphate • 51.5% nanocrystalline hydroxyapatite	• Vancomycin • Gentamicin • Tobramycin • Rifampicin
StimulanR (Biocomposites)	Beads/Paste	• Calcium sulphate hemihydrate	• Vancomycin • Gentamicin • Tobramycin
Cerasorb (Curasan)	Beads/Paste	• Beta-Tricalcium phosphate (β-TCP)	• Vancomycin • Gentamicin • Meropenem

1.8 Biphasic CaS/HA as a local drug delivery platform

During the exploration of biomaterials such as CaS and HA, researchers found that their combination could provide a more effective and balanced solution for local drug delivery[102-104]. The osteoconductivity and structural stability of HA help maintain the material architecture during degradation, while the rapid dissolution and moldability of CaS facilitate drug incorporation and controlled release. When combined, HA particles are evenly dispersed within the CaS matrix, creating a biphasic composite that degrades in a controlled manner. As CaS dissolves, it releases calcium ions that stimulate osteoblast activity and promote bone matrix mineralization, while the remaining HA phase forms an interconnected porous network that allows vascular infiltration and new bone ingrowth. The presence of HA moderates the burst release of HA-affinity drugs by providing additional binding sites, thereby extending the drug release period from less than two weeks to over four weeks[104, 105]. In addition to improving the release kinetics, HA also provides a favorable surface for molecular adsorption. Since HA is the major

inorganic component of natural bone, its residual surface offers high affinity for drugs capable of chelating calcium or phosphate ions, such as bisphosphonates and tetracyclines[106]. The optimized formulation, typically containing 60% CaS and 40% HA, exhibits balanced biodegradation and mechanical strength in the range of cancellous bone. This biphasic CaS/HA system has been incorporated into several FDA-approved or CE-marked products and is currently a clinically approved injectable ceramic composite[107]. Its versatility and injectability allow minimally invasive treatment of irregular bone defects, and it has been successfully applied in bone regeneration, tumor resection reconstruction, and infection management[102-104].

GEN was among the first antibiotics incorporated into PMMA for local infection control and was subsequently adapted for use in resorbable carriers such as CaS/HA composites. However, prolonged and widespread use of gentamicin-loaded polymeric systems has been associated with the emergence of resistant bacterial strains, potentially compromising long-term therapeutic efficacy[108]. To overcome these limitations, and because of the lower processing temperature, CaS/HA composites allow incorporation of other thermolabile antibiotics, TOB, a related aminoglycoside with enhanced activity against Gram-negative pathogens such as Pseudomonas aeruginosa, became a preferred alternative. However, TOB alone was insufficient to eliminate highly resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). To address this challenge, VAN was introduced into CaS/HA systems in the early 2000s, targeting MRSA and other Gram-positive pathogens[109, 110]. VAN's large molecular size results in slower diffusion and extended local retention, matching the degradation kinetics of CaS/HA. In more recent studies, rifampicin (RIF), known for its potent antibiofilm activity, has been combined with VAN or GEN to enhance bacterial eradication and prevent recurrence[104]. Together, these developments illustrate the evolution of CaS/HA-based drug delivery systems from simple biodegradable carriers to multifunctional therapeutic platforms capable of simultaneously achieving infection control and guided bone regeneration.

1.9 Current Limitations and Emerging Strategies for CaS/HA

Although numerous commercial products based on CaS/HA composites have been developed, diverse clinical conditions continue to highlight the need for further optimization of their use as local drug delivery systems[111]. Incorporating different antibiotics or biological agents can significantly alter the physical and chemical properties of CaS/HA, including its degradation rate, release kinetics, surrounding pH, and even mechanical strength. At the same time, to win the so-

called “race for the surface”, antibiotic protection alone may not be sufficient. Rapid coverage of the implant surface by host cells is essential, and this process could be accelerated by incorporating osteogenic growth factors that promote early osteoblast adhesion and proliferation[112].

Among these biological molecules, bone morphogenetic proteins (BMPs), a subgroup of the transforming growth factor-beta (TGF-β) superfamily, play a critical role in bone formation and remodeling[113]. BMP-2, in particular, is a potent osteogenic and chondrogenic protein that induces the differentiation of mesenchymal stem cells into osteoblasts and stimulates angiogenesis. Both osteogenesis and vascularization are essential processes for bone regeneration and are closely linked to the outcome of the “race for the surface”. Previous studies have demonstrated that delivering BMP-2 via CaS/HA composites can significantly enhance bone healing and improve defect bridging[114-116]. Furthermore, some reports suggest that BMP-2 may also contribute to faster recovery by improving local vascularization and immune responses, which may indirectly enhance local host defense mechanisms and potentially reduce susceptibility to infection[117]. Therefore, the combination of antibiotics with BMP-2 within CaS/HA composites represents a promising strategy for achieving both infection prevention and bone regeneration.

However, the long-term presence of residual HA particles after CaS resorption introduces another important consideration. Owing to its high affinity for certain molecules, HA can act as a passive reservoir or recruitment platform for a number of drugs with strong HA-binding capability, such as bisphosphonates (e.g., zoledronic acid)[106, 118], but interestingly, TET, a broad-spectrum antibiotic known for its strong affinity to HA, may display similar behavior. This property suggests that HA could potentially attract TET molecules to the surgical site even after systemic administration, thereby prolonging local antibacterial protection. Such interactions could transform the slow resorption of HA from a disadvantage into a functional advantage, providing sustained antimicrobial effects and long-term protection against bacterial persistence or recurrence.

Taken together, these insights highlight the evolving potential of CaS/HA as a multifunctional biomaterial that integrates local drug delivery, osteoinduction, and infection control. However, the molecular mechanisms underlying antibiotic–HA interactions remain poorly understood. Therefore, further research is warranted to investigate how specific antibiotics, such as tobramycin and tetracycline, interact with CaS/HA composites and how these interactions influence drug release, antibacterial efficacy, and implant protection in orthopedic applications.

1.10 A historical perspective on orthopedic infection research in Lund

In 1963, a 5-year prospective register infection study started covering all patients at that time in the only single orthopaedic clinic in a major city with 250 000 inhabitants (Malmö) who were diagnosed with an infection. The study included surgical procedures and bacteriology with a minimum 5-year follow-up. The study ended in 1972. Several reports on bone and joint infections including epidemiology and health economic data based on the register study have been published. A total of 5,724 clean operations were performed on inpatients during the 5-year period. Of these, 185 (3.2 percent) became infected and in 107 (1.9 percent) the infection was classified as major with complications spoiling the result. It was clear that operations in the hip region together with insertion of osteosynthesis not only significantly increased the incidence of postoperative infection but also accounted for no less than 60 percent of all postoperative infections and half of the spoiled results after operation[119].

In 1969 the first patients with total hip replacement (THR) in Lund /Malmö were operated. It soon became clear that as expected, early infection rates skyrocketed and with no solution THR risked being halted and immediate action was deemed necessary. A double-blind randomized controlled trial with a placebo arm comparing preoperative systemic antibiotic prophylaxis was decided, a controversial study at that time. The hypothesis was that the placebo group was expected to be noninferior to the antibiotic group. Cloxacillin was selected as antibiotic prophylaxis based on the bacteriology and resistance patterns from the register hip infection study showing *S. aureus* to be the dominating bacteria. The statistical advisers wisely insisted on having an independent board to sequentially analyse infection rates at predetermined intervals to not unnecessarily prolong the study if no difference was found within the first few years (100 patients recruited per year). The study was financed by the Swedish medical research council.

The study was stopped by the external board after 171 hip operations for ethical reasons. The cloxacillin group had no postoperative infections, while the placebo group had twelve (in eighty-eight patients). Ten of the twelve infections were caused by *S. aureus*.

The study “Cloxacillin in the prophylaxis of postoperative infections of the hip” was rapidly published 1973 in American Journal of Bone and Joint Surgery concluding that antibiotic prophylaxis with Cloxacillin significantly reduced the number of early and late infections in major hip surgery[120]. This study had a major impact and changed infection prevention globally establishing antibiotic prophylaxis as routine in joint prosthetic surgery. It is important to note that all hips were cemented without adding local antibiotics, performed in a standard ventilated operating theatre and with a surgical procedure which at that time took on average 3.5 hours.

A review from the same clinics with a longer follow up period investigated the prophylactic value of cloxacillin against also late diagnosed infections after total hip replacements. At 6-year follow up of 1065 THR the cumulative infection incidence with prophylaxis was 2.0 per cent. During the period, several aseptic and antiseptic preventive measures had in parallel been introduced. Haematogenous deep infection was estimated to have contributed to less than 1% of the infections[121].

During the next 60 years hundreds of studies on prophylactic systemic antibiotics have been published focusing on risk factors, optimal antibiotic dosage, timing and treatment duration. Only a few studies have looked at temporal change in bacteria and resistance patterns.

PJI has in the new millennium again increased. The risk of revision at a 5 year follow up due to infection after primary THR was almost doubled, both in absolute cumulative incidence and in relative risk, throughout the period 2004–2018 reported in a study from the Nordic countries.

Uncemented hip joint implants were reported with infection levels mirroring 1970 cumulative incidence rates and today to nonantibiotic containing cemented implants with similar follow up. Historical comparisons are difficult and if the increase may reflect frailer patients or more use of uncemented implants was not answered in the study and additional research was suggested[122].

Several more questions could be addressed based on the study. Are we using the right preventive antibiotic in uncemented apatite coated implants. It seems that second generation cephalosporins 60 years later are still being offered irrespective of individual microbiome, patient risk factors and selected implant. “The patient can get whatever antibiotic prophylaxis they want as long as it is a cephalosporin”.

There are a few antibiotics with proven local accretion to HA that could be given systemically. Even more compelling would be to impregnate an uncemented apatite coated implant preoperatively.

2. Research questions

In this thesis, the research aims are to investigate the following questions:

1. What are the in-vitro material characteristics of antibiotic-containing CaS/HA composites, including injectability, setting behavior, degradation, and antibiotic release kinetics? Furthermore, how do different antibiotics or antibiotic combinations affect the antibacterial efficacy of CaS/HA against *S. aureus* in vitro? These aspects were investigated in **Study 1**.

2. How does the use of tobramycin-impregnated CaS/HA as a bone graft extender influence systemic TOB pharmacokinetics and the material's bone-regenerative performance. Can the same CaS/HA material be combined with BMP-2 to serve as a viable alternative to autologous bone grafting in spinal fusion? These questions were evaluated in **Study 2**.

3. Can synthetic HA particles serve as a recruiting moiety to capture systemically administered TET, a HA-binding antibiotic, at the target site, thereby enhancing local antibacterial activity through in situ enrichment? This question was explored in **Study 3**.

4. What is the preventive effect of systemically administered TET in reducing *S. aureus* colonization on HA-coated implants, and what dosing regimen optimizes this preventive effect? This was examined in **Study 4**.

3. Experimental design

3.1 Biomaterials

During this thesis work, two types of CaS/HA biomaterials, powder and pre-set beads, were evaluated for antibiotic delivery in both in-vitro and in-vivo settings. In addition, micro- and nano-sized HA particles were investigated for their biomodulatory and antibiotic-recruiting properties.

1. The CaS/HA biomaterial used in **Study 1** was a commercially available product that has been used clinically for several decades (purchased from Bonesupport AB, Sweden). The composite consisted of 60% CaS and 40% HA by weight. For material preparation, 1 g of CaS/HA powder was mixed with 430 µL of an iodine-based mixing solution (Iohexol, C-TRU; Bonesupport AB) together with the antibiotic solution to form a homogeneous paste. The paste was subsequently transferred into a 1 mL syringe and injected into hemispherical molds (Ø = 4.8 mm). After setting, antibiotic-loaded pellets were collected and used for in vitro experiments.
2. A second CaS/HA biomaterial investigated in **Study 2** was provided by Moroxite AB (Sweden) and consisted of 60% CaS and 40% HA by weight. For in-vitro experiments, the material was supplied as CaS/HA powder. To prepare TOB-loaded beads, 400 µL of TOB solution (16 mg total amount, 40 mg/mL) was applied to 1.5 cc of pre-set CaS/HA beads (Ø = 3 mm, approximately 70 beads) and allowed to soak and dry at room temperature for 10 min. For in-vivo implantation, the material was pre-manufactured as hemispherical beads (Ø = 2-3 mm, length = 2-4 mm) for mixing with tobramycin or BMP-2 prior to implantation.
3. Nano-hydroxyapatite (n-HA; <50 nm) and micro-hydroxyapatite (m-HA; 10 µm) powders used in **Study 3** were purchased from Fluidnova. For in vitro experiments, 100 mg of n-HA or m-HA powder was prepared in microcentrifuge tubes. For in vivo experiments, HA pellets were prepared by mixing 100 mg of n-HA or m-HA powder with 60–80 µL of hyaluronic acid (HAD, 1 mg/mL).
4. In **Study 4**, m-HA was used as a coating on titanium implants. HA-coated pins (0.6 mm diameter DePuy K-wires) were coated with microparticulate

HA by Plasma Biotal Ltd. U.K. HA-coated screw was purchased from Smith and Nephew.

3.2 Animal Models

During this thesis work, four different animal models in mice, rats, and rabbits were established to address distinct research aims across the different studies.

1. A posterolateral spinal fusion model was established in female New Zealand White rabbits: Briefly, the transverse processes at the L4–L5 level were surgically exposed and decorticated using a 1.5 mm round burr until punctate bleeding was observed. Subsequently, CaS/HA beads loaded with TOB or BMP-2, with or without 1.5 cc of morselized autologous bone graft harvested from the iliac crest, were implanted between the L4 and L5 transverse processes together. It was used to evaluate if the addition of TOB to a CaS/HA material affects the material ability to act as a bone graft extender and if CaS/HA can combine with low dose BMP-2 to replace autologous bone grafting in spinal fusion surgery in **Study 2**.
2. An ectopic abdominal muscle pouch model was established in Sprague–Dawley (SD) rats: Briefly, a small incision was made in the abdominal musculature, and blunt dissection was performed to create a muscle pouch between the muscle layers on both the left and right sides. Nano-HA (n-HA) and micro-HA (m-HA) pellets were implanted into the pouches. It was used to evaluate the binding effect of TET to pre-implanted HA in **Study 3**.
3. A dorsal subcutaneous infection model was established in BALB/c mice: Briefly, after shaving the dorsal hair, bilateral skin incisions were made using sterile scissors. N-HA pellets were implanted subcutaneously. After TET administration, mice were subcutaneously inoculated with a bioluminescent strain of *S. aureus*. It was used to evaluate the anti-bacterial effect of TET with pre-implanted HA in **Study 3**.
4. A tibial implant model was established in SD rats: Briefly, two titanium implants, either coated with m-HA or left uncoated, were implanted into the right tibia of each rat, with one implant placed in the cortical bone and the other in the metaphyseal bone. TET was administered via intraperitoneal injection according to different dosing regimens. It was used to evaluate the optimal timing of systemic TET administration on HA-coated implants in **Study 4**.

3.3 Experimental techniques

This thesis employs a range of in-vivo and in-vitro experimental techniques, including biomaterial functional evaluation, microbiological assays, imaging-based analyses, and animal models. Given the breadth of methodologies applied, only the key experimental approaches central to addressing the research questions are described in detail in this section, while more specialized or auxiliary techniques are presented where relevant in the corresponding studies

3.3.1 Drug release profile

Drug release profile experiments were primarily conducted in **Study 1** and **Study 2**. For the in-vitro experiments, TOB release from CaS/HA was evaluated by immersing CaS/HA+TOB pellets in 1 mL of sterile phosphate-buffered saline (PBS) in 2 mL microcentrifuge tubes. The tubes were maintained at 37°C to mimic physiological conditions. At predetermined time points, the release medium was collected and centrifuged to remove CaS/HA particulate residues originating from CaS/HA degradation, thereby preventing interference with subsequent quantitative analyses. A total of 1 mL of fresh PBS was added to the material-containing tubes to initiate the next collection interval. The resulting supernatants were carefully collected and stored at −20°C until further analysis. For the in-vivo experiments, serum samples were collected at predefined time points. TOB concentrations in the collected samples were quantified using either a homogeneous enzyme immunoassay or liquid chromatography–tandem mass spectrometry (LC–MS), depending on the experimental design and sensitivity requirements.

3.3.2 Kirby-Bauer disk diffusion assay

The Kirby–Bauer disk diffusion assay was used to evaluate the antibacterial effects of biomaterials in **Study 1-4**.

Bacterial suspensions were prepared by resuspending isolated *S. aureus* colonies in sterile saline, and the optical density was adjusted to $OD_{600} = 0.10 \pm 0.005$ using a spectrophotometer. Bacterial lawns were established by evenly spreading the suspension onto Muller–Hinton agar (MHA) plates using sterile cotton swabs. After inoculation, the plates were left at room temperature for 5 min to allow the suspension to absorb and dry.

In **Study 1** and **Study 2**, CaS/HA pellets mixed with antibiotics in the paste phase or pre-set pellets impregnated with TOB, respectively were placed directly onto the surface of freshly inoculated MHA plates. The plates were incubated at 37 °C for 24 h. The diameter of the zone of inhibition (ZOI) surrounding each biomaterial was measured three times using a standard ruler, and the mean value was recorded. After

ZOI measurement, the biomaterials were aseptically transferred to newly prepared MHA plates inoculated with fresh *S. aureus* using sterile forceps. This procedure was repeated daily until no detectable ZOI was observed or until the predefined experimental endpoint was reached. MHA plates exhibiting ZOI were imaged daily using a ChemiDoc MP imaging system, while representative time points were selected for presentation. In **Study 3**, TET-bound m-HA or n-HA particles, with or without prior protein passivation or zoledronic acid (ZA) treatment, were placed onto MHA plates inoculated with *S. aureus* and incubated for 24 h. ZOI diameters were measured and documented photographically.

For in-vivo experiments in **Study 3** and **Study 4**, HA pellets, harvested organs, or HA-coated implants retrieved from animals were placed onto *S. aureus* inoculated MHA plates and incubated for 24 h. Among the in-vivo derived samples, only tissues collected for antibacterial analysis were subjected to ZOI testing. ZOI diameters and images were recorded after incubation. In contrast to the in-vitro assays, these samples were not transferred to new agar plates after measurement.

3.3.3 Imaging-based analyses

In **Study 2**, micro-CT imaging combined with image analysis using Dragonfly (version 2025.1, Comet Technologies Inc., Montreal, QC, Canada) software was used to evaluate bone regeneration and bone volume. Rabbit spine samples were harvested together with one vertebra above and one vertebra below the L4–L5 fusion segment to accommodate the scanning field of the micro-CT system. The specimens were scanned to obtain full 360° projections. Image datasets were reconstructed with an isotropic voxel size of 30 µm. Reconstructed two-dimensional (2D) images were generated in the sagittal, coronal, and axial planes, together with three-dimensional (3D) renderings of the spinal fusion region. Quantitative analysis was subsequently performed using Dragonfly to calculate newly formed bone volume and to distinguish mineralized bone from remaining biomaterial. Segmentation thresholds were applied to differentiate newly formed bone from residual biomaterial.

In **Study 3**, an in-vivo imaging system (IVIS Spectrum, PerkinElmer) was used to monitor *S. aureus* activity and to evaluate the antibacterial effects of the biomodulation strategy. Animals were subcutaneously injected with a bioluminescent strain of *S. aureus* at the dorsal implantation site containing HA particles, as well as at a contralateral site serving as an internal control. At predefined time points after bacterial inoculation, animals were anesthetized and imaged using the IVIS system. Bioluminescence signals at the injection sites were quantified as a surrogate marker for bacterial proliferation. Signal quantification was performed using Living Image 4.0 software.

3.3.4 Histology

Histological analysis was conducted to assess fusion across the transverse processes. Hematoxylin and eosin (H&E) staining enables visualization of overall tissue architecture and cellular composition, allowing qualitative assessment of bone formation and fusion at the histological level. Following micro-CT analysis, spinal specimens were carefully cleared of surrounding soft tissues while preserving the integrity of the fusion mass. The samples were subsequently fixed in 4 wt.% neutral-buffered formalin. After fixation, specimens were decalcified using formic acid until complete decalcification was achieved, as verified by tissue softening. Decalcified samples were then processed through graded dehydration and embedded in paraffin according to standard histological protocols. Serial sagittal sections with a thickness of 5 μm were prepared using a semi-automated microtome (Thermo Scientific). Sections were stained with H&E. To ensure adequate coverage of the fusion region, sections were obtained from three different depths within each specimen. All stained sections were digitized using a bright-field slide scanner (Olympus) at 20× magnification. Histological evaluation was performed qualitatively to characterize the composition of the fusion mass and to determine the presence of continuous bone bridging indicative of successful fusion.

3.4 Design of animal studies

Animal studies have been performed in **Study 2**, **Study 3** and **Study 4** entailed in this thesis. Groups for comparison were selected as summarized in Table 2 below.

Table 2. Overview of the experimental framework of the animal studies included in this thesis, specifying the biomaterials evaluated, study aims, animal models, comparison groups, and sample size distribution.

Study	Biomaterial Used	Main Aim	Model	Groups for Comparisons	Sample Size/Group
2	CaS/HA	1. Evaluate whether addition of TOB to a CaS/HA material affects the material ability to act as a bone graft extender 2. Can CaS/HA combined with low dose BMP-2 replace autologous bone grafting in spinal fusion surgery.	Rabbit posterolateral lumbar fusion	1. CaS/HA+TOB +autograft 2. CaS/HA +BMP-2	G1-2 (n=6)
3	nHA mHA	The binding effect of TET to pre-implanted HA	Abdominal muscle pouch in rats	1. m-HA 2. n-HA	G1-2 (n=6)
	nHA	The anti-bacterial effect of TET with pre-implanted HA	Subcutaneous infection in mice	1. n-HA+TET 2. n-HA	G1 (n=5) G2 (n=3)
4	HA-coated pin	The optimal timing of systemic TET administration on HA-coated implants	Tibial implantation model in mice	HA-coated pins 1. One dose TET 1h pre-operation 2. TET at post-operation, day 1 and day 2 3. TET at day 5, 6 and 7 4.One dose TET at day 5 5. No TET No HA-coated pins 6. TET at post-operation, day 1 and day 2	G1 (n=2) G2 (n=2) G3 (n=2) G4 (n=2) G5 (n=1) G6 (n=1)

3.5 Methodological considerations: Animal models and ethics

Except for the in-vitro investigations in **Study 1**, this thesis employs four different animal models, ranging from mice, rats and rabbits, all aimed to investigate local antibiotic delivery and in-vivo recruitment in **Studies 2–4**. Each animal model was selected to progressively validate and extend findings from earlier in-vitro experiments before moving toward clinically relevant scenarios.

In **Study 2**, a rabbit posterolateral lumbar fusion model was used to investigate early antibacterial effects and bone regeneration. Rabbits were chosen because their spinal size allows for precise surgical manipulation and imaging, and their spinal

anatomy more closely resembles that of humans compared with smaller rodents. Moreover, the rabbit posterolateral lumbar fusion model is a well-established and widely recognized experimental model for evaluating spinal fusion strategies. In this model, no critical-sized bone defect was created. Instead, the transverse processes were decorticated using a 1.5 mm round burr until punctate bleeding was observed, and autologous bone graft was harvested from the iliac crest. CaS/HA beads were implanted between the decorticated transverse processes and stabilized by the surrounding paraspinal musculature, resulting in minimal postoperative impairment for the animals.

In **Study 3** and **Study 4**, mouse and rat models were employed, including an ectopic muscle pouch model, a subcutaneous infection model, and a tibial implant model. The muscle pouch and subcutaneous infection models were established in soft tissue sites, such as the abdominal musculature and dorsal skin, respectively. These locations were intentionally chosen to minimize the influence of surrounding bone tissue, thereby allowing clearer assessment of whether systemically administered TET could bind to pre-implanted HA particles. The tibial implant model was designed to more closely mimic a clinical implant scenario and to investigate whether HA-coated implants retained the ability to recruit TET when placed in both cortical and metaphyseal bone environments.

Although surgical procedures inevitably cause a certain degree of animal discomfort, all experimental models in this thesis were designed to minimize tissue damage and avoid unnecessary surgical interventions. In-vitro experiments alone were insufficient to fully replace in-vivo studies, as it is not possible to completely replicate the complex physiological environment ex vivo. Differences observed between in-vitro and in-vivo antibiotic release profiles further highlight the importance of using complementary in-vivo models.

All studies included in this thesis were conducted in accordance with the 3R principles, with the aim of reducing and refining the use of experimental animals without compromising the scientific objectives of the thesis. Experimental groups were excluded where appropriate to further reduce animal numbers. Sample sizes and power calculations were based on previous studies conducted and published by the research group. All measures to minimize animal suffering were followed, and animals had free access to standard food pellets and water throughout the experimental period. All mouse and rat experiments were approved by the Swedish Board of Agriculture (Jordbruksverket) under permit numbers 15288/2019, and all personnel involved in animal procedures had received appropriate training and certification in accordance with FELASA guidelines. Rabbit experiments were conducted in compliance with national and institutional regulations and were approved by the relevant Lithuanian governmental authority responsible for animal welfare (permit number G2-277). All in-vivo experiments were reported in accordance with the ARRIVE guidelines to ensure transparent and ethical reporting.

3.6 Statistical analysis

All data are presented as mean ± standard deviation (SD), unless otherwise explicitly stated. Normality of data distribution was assessed prior to statistical analysis. For comparisons between two unpaired groups, an unpaired student's t-test was used in **Study 1** and **Study 3** when data were normally distributed. For paired comparisons with normally distributed data, a paired student's t-test was applied in **Study 2**. Comparisons involving more than two groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. A p-value < 0.05 was considered statistically significant. All statistical analyses and data visualizations were performed using GraphPad Prism version 8 and 10 (GraphPad Software, USA).

4. Results

4.1 Study 1 – Controlled release and antimicrobial efficacy of TOB and VAN from CaS/HA

- TOB and VAN released from CaS/HA exhibited a pronounced burst release within the first 3 days (87% and 92%, respectively), followed by sustained release up to 35 days.
- TOB showed a faster release profile when co-loaded with VAN in CaS/HA, whereas VAN exhibited a slower release rate in the combined CaS/HA+VAN+TOB formulation.
- CaS/HA+TOB pellets maintained antibacterial activity against *S. aureus* ATCC 25923 and the clinical strain P-3 for up to 28 days, while CaS/HA+VAN+TOB showed no activity within 8 days and CaS/HA+VAN showed no activity after 11 days.
- Analysis of antibiotic release fractions demonstrated that CaS/HA+TOB retained antibacterial activity for up to 35 days against ATCC 25923 but only 7 days against P-3, whereas both CaS/HA+VAN+TOB and CaS/HA+VAN showed shorter activity durations.
- CaS/HA+TOB pellets retained antibacterial activity against both strains even after 35 days in PBS when mechanically disrupted, while CaS/HA+VAN+TOB showed activity only against ATCC 25923 and CaS/HA+VAN showed no detectable antibacterial effect.

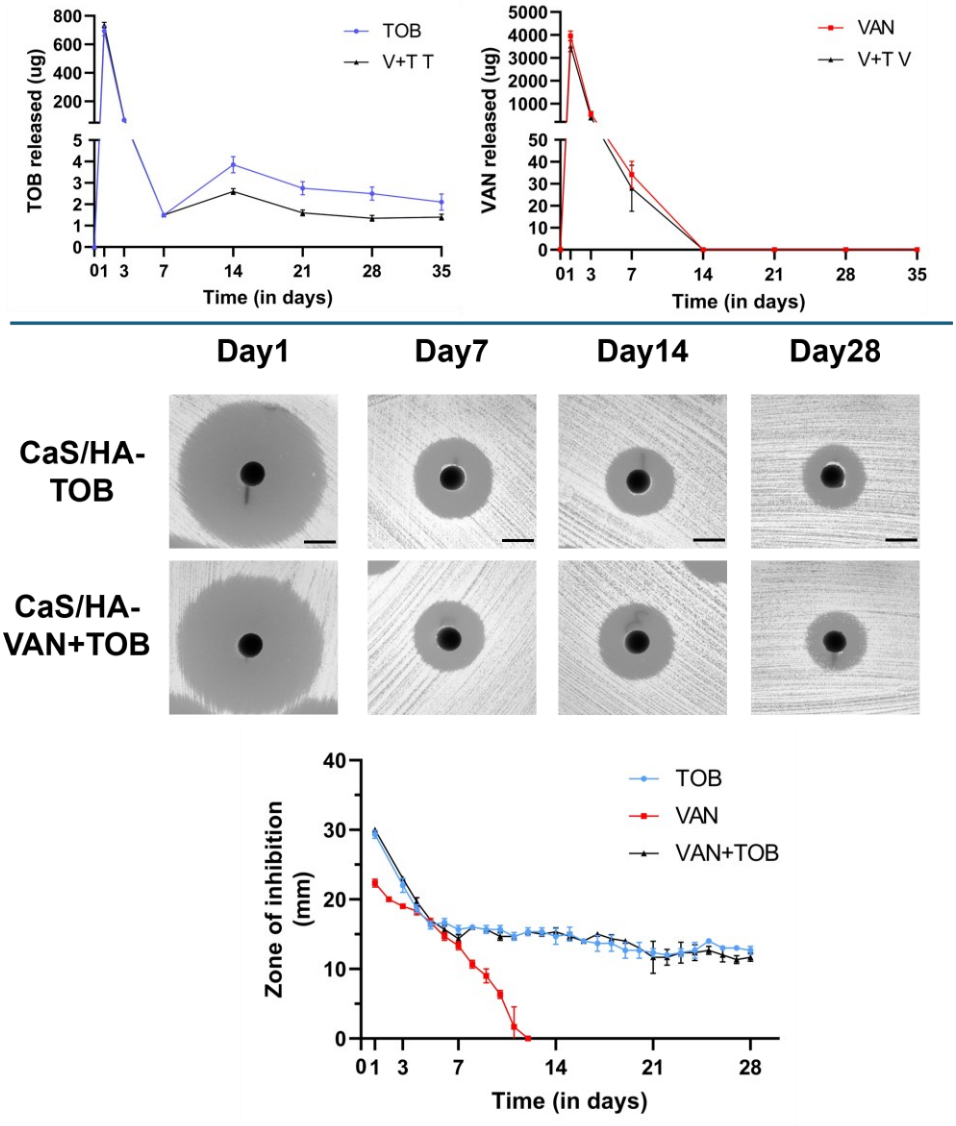

Figure 3: In-vitro release profiles of TOB and VAN from different CaS/HA-antibiotic combinations (top). Antibacterial efficacy of different CaS/HA–antibiotic combinations against *S. aureus* ATCC 25923 assessed by the Kirby–Bauer disk diffusion assay (bottom). Figure has been reproduced from [123].

4.2 Study 2 – CaS/HA loaded with TOB and BMP-2 in a rabbit posterolateral spinal fusion

- CaS/HA beads loaded with TOB exhibited a rapid initial release within the first 1–6 hours, both in-vitro and in-vivo, followed by a sharp decline, with TOB concentrations falling below the detection limit of 1 μg/mL after 48 hours.
- TOB-loaded CaS/HA beads demonstrated a strong antibacterial effect during the first 3 days and sustained activity for up to 19 days in-vitro.
- Radiographic evaluation demonstrated successful spinal fusion in 5 out of 6 animals in the TOB group and in all animals (6/6) in the BMP-2 group. Quantitative micro-CT analysis and manual mechanical palpation revealed no statistically significant differences in fusion outcomes between the two treatment strategies.
- Histological analysis further confirmed continuous bone bridging between the transverse processes in both treatment groups, supporting the radiographic and micro-CT findings.

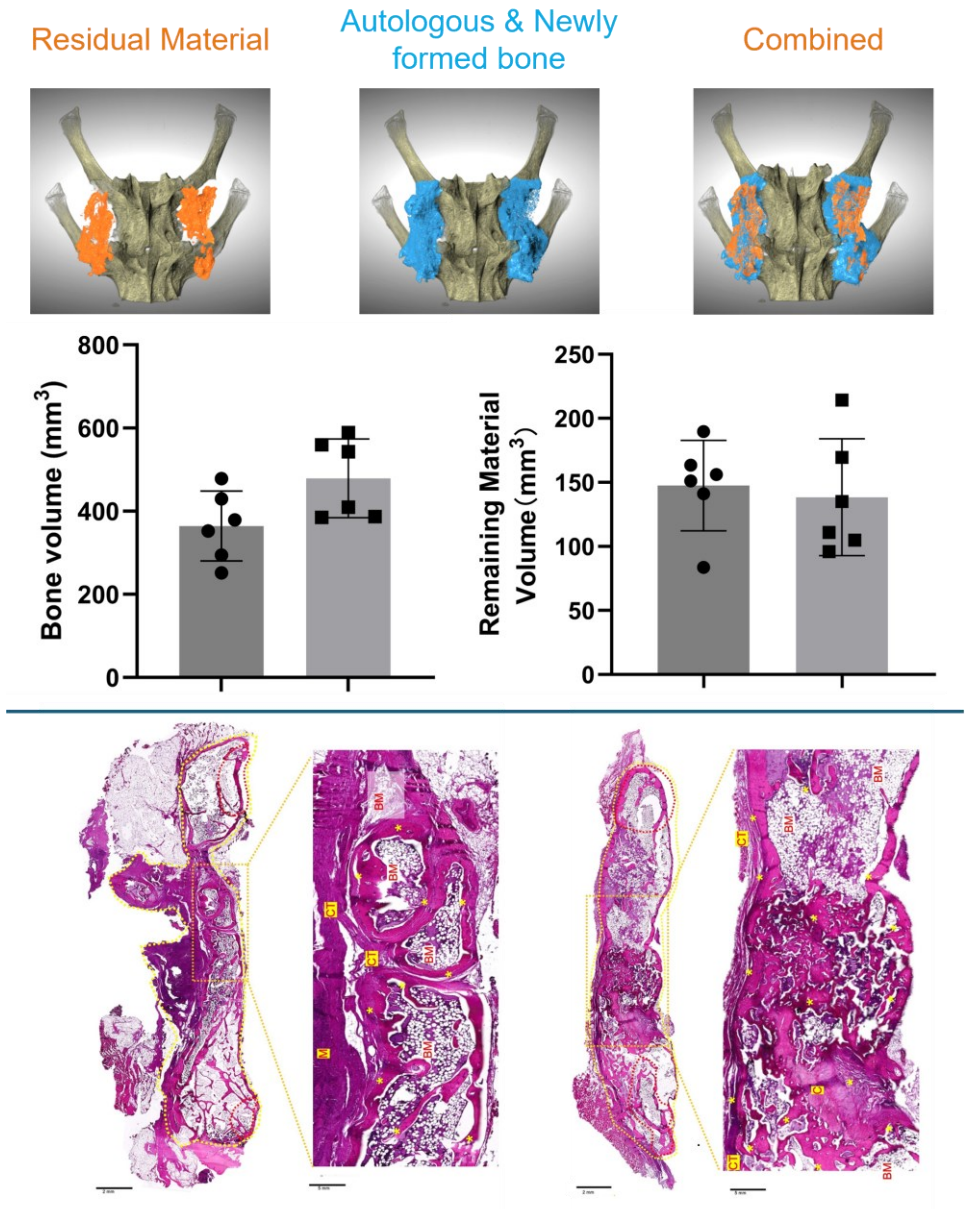

Figure 4: Representative 3D micro-CT reconstructions and quantitative analysis of bone volume and residual material volume using Dragonfly software for the evaluation of spinal fusion. Orange indicates residual material, whereas blue represents autologous bone and newly formed bone (top). Representative histological sections stained with H&E demonstrate bridging cortical bone formation between the L4 and L5 transverse processes in both groups. The dashed red circle delineates the original transverse process, while the dashed yellow circle outlines the entire fusion mass, including the original **transverse** processes. The lower panel highlights the region indicated by the orange dashed box. *Bone; BM, bone marrow; C, cartilage; CT, connective tissue; M, material (bottom). Figure has been reproduced [124].

4.3 Study 3 – Antibiotic recruitment ability of HA in-vitro and in-vivo

- TET was rapidly bound to both m-HA and n-HA particles in-vitro, with n-HA demonstrating a stronger binding capacity than m-HA.
- The binding of TET to HA was influenced by serum proteins and drugs with accretion to HA, indicating that the total binding sites on HA crystal can get affected depending on implantation time or drug exposure.
- Following systemic administration of TET, pre-implanted HA particles exhibited larger ZOI than liver, kidney, muscle, and bone tissues, with n-HA showing greater ZOI than m-HA in-vivo.
- Compared with control groups without TET or without HA, the combination of systemic TET and n-HA particles demonstrated a clear antibacterial effect against *S. aureus* in a subcutaneous mouse infection model.

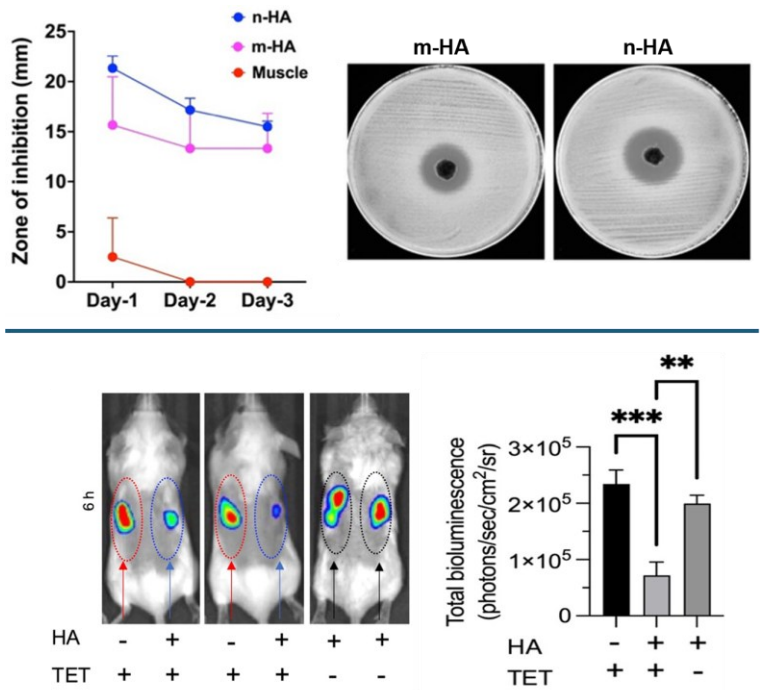

Figure 5: Antibacterial effects of HA particles retrieved from muscle tissue. ZOI determined by the Kirby–Bauer disk diffusion assay against *S. aureus* ATCC 25923 using different HA particles with TET biomodulation collected from a rat muscle pouch model (top). IVIS performed 6 h after local bacterial infection is shown below, with quantitative analysis of the measured luminescence intensity at the infection site presented on the right (bottom). Adapted and reproduced from Sebastian et al., 2023[125].

4.4 Study 4 – Optimal biomodulation timing of TET for HA-coated implants

- Compared with other dosing schedules, a single dose of TET administered immediately post-operation followed by once-daily injections on postoperative days 1 and 2 demonstrated the strongest antibacterial effect.
- No significant differences were observed between HA-coated pins implanted in cortical bone and those implanted in metaphyseal bone.
- Scanning electron microscopy (SEM) confirmed that systemically administered TET bound to HA-coated implants and reduced bacterial colonization compared with implants in the absence of TET.

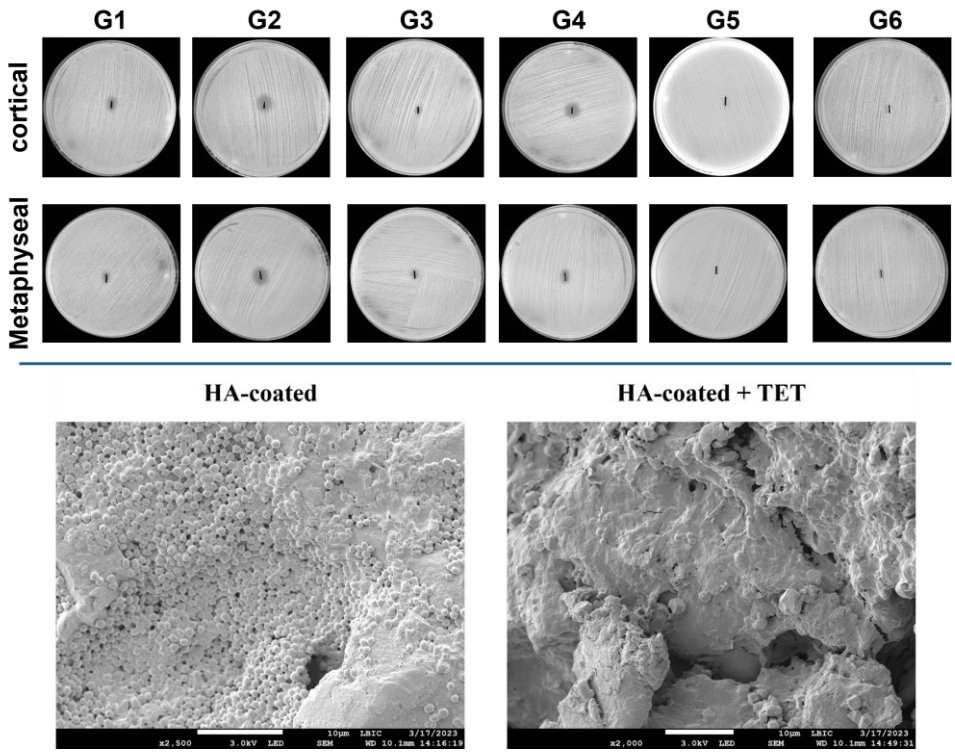

Figure 6: ZOI demonstrating the antibacterial activity of HA-coated and uncoated pins under different systemic TET treatment regimens (top). G1: single TET injection 1 h prior to surgery; G2: TET administered 15 min post-surgery followed by daily injections on postoperative days 1 and 2; G3: TET administered on postoperative days 5–7; G4: single TET injection on postoperative day 5; G5: no TET treatment; G6: uncoated implants with TET administered post-surgery and on postoperative days 1 and 2. Representative SEM images of HA-coated implant surfaces with and without systemic TET treatment are shown below (bottom).

5. Connections between the studies

All studies described in this thesis have a common goal, to protect implanted biomaterials from bacterial colonization. Lessons learned from preceding studies were implemented in the following studies.

In **Study 1**, CaS/HA was shown to be compatible with both TOB and VAN, enabling controlled and sustained antibiotic release with antibacterial activity lasting for approximately one month in-vitro. These findings raised the question of whether such antibiotic-loaded CaS/HA formulations would retain their efficacy in-vivo under more complex physiological conditions.

In **Study 2**, CaS/HA beads loaded with TOB were evaluated in a rabbit posterolateral spinal fusion model. It was observed that TOB exhibited an early burst release followed by sustained antibacterial activity, while radiography and histology results demonstrated that local TOB delivery did not adversely affect bone regeneration. In parallel, low-dose BMP-2 proved effective in promoting bone formation, suggesting that bone regeneration could be achieved without the need for harvesting autologous bone.

In **Study 3**, TET was found to exhibit affinity for both micro- and nano-scale HA particles, with binding influenced by protein passivation and the presence of other HA-affinity drugs such as zoledronic acid in-vitro. The binding affinity of TET was consistently higher for n-HA compared with m-HA, both in-vitro and in-vivo. Furthermore, pre-implanted HA pellets were able to recruit systemically administered TET and provided protection against bacterial infection. These observations raised the question of whether this biomodulation strategy could be translated to clinically relevant implant scenarios and how the timing of systemic antibiotic administration might affect its efficacy.

In **Study 4**, the biomodulation concept was extended to HA-coated implants in a rat tibial implant model. Systemic administration of TET was shown to bind to HA-coated implants in-vivo, with the most effective antibacterial outcome achieved by administering one dose immediately post-operation, followed by once-daily dosing on postoperative days 1 and 2. The binding efficacy was not affected by implant location, whether placed in cortical or metaphyseal bone, supporting the robustness of this approach across different bone environments.

6. Discussion

6.1 CaS/HA as a local delivery system for TOB and VAN

CaS/HA combined with aminoglycoside antibiotic GEN or a non-aminoglycoside VAN is commercially available [126, 127], but pharmacokinetics of the combination with two antibiotics have never been evaluated before. Combining antibiotics might be warranted in complex osteomyelitis, wherein single-antibiotic formulations are insufficient. For instance, a CaS/HA material containing GEN or TOB alone may not eradicate resistant Gram-positive bacteria, whereas VAN alone is ineffective against Gram-negative species. Failed antibiotic therapy does not only halt recovery but may also promote the emergence of multi-resistant pathogens, thereby exacerbating the infection severity[128]. Karr et al. reported that CaS/HA loaded with both TOB and VAN, used as a local antibiotic delivery system and combined with four weeks of systemic oral or intravenous antibiotics, provided a safe, reliable, and effective treatment for osteomyelitis[129]. Nevertheless, this approach raises an important question of whether a dual-antibiotic CaS/HA formulation alone, without prolonged systemic supplementation, could achieve a comparable therapeutic effect. Previous research has suggested that dissolution rate and material composition may influence the treatment outcomes[130]. In our study, all three formulations demonstrated a comparable degradation profile, losing approximately 50–60% of their weight within 35 days, corresponding well with the CaS content (≈ 60%). Such slow, steady degradation supports a sustained and locally elevated antibiotic concentration at the infection site, which is critical for effective bacterial eradication while limiting systemic toxicity. In one study evaluating TOB and VAN release from calcium sulfate beads (CSB) and PMMA, concentrations increased when CSB were combined with PMMA, yet the antibiotic levels remained above the MIC for only 48 hours[131]. In contrast, Vajra et al. demonstrated that most minimum biofilm eradication concentration values for bacterial pathogens associated with orthopedic infections, such as *S. aureus* and *S. epidermidis*, ranged from 100 to 750 µg/mL of VAN+TOB when sustained for at least 24 hours, concentrations typically unattainable through systemic therapy alone[132]. In **Study 1**, CaS/HA+TOB+VAN exhibited an initial burst release during the first three days. Following this, TOB maintained a stable and sustained release from day 7 to day 35, with concentrations consistently exceeding the MIC.

Such a release pattern, with an early high dose burst followed by long-term sustained delivery, is clinically desirable because the initial burst may also kill resistant bacteria while the subsequent sustained release suppresses residual bacteria and prevents recurrence[133]. VAN displayed a faster burst-type release lasting approximately two weeks, with most of the drug eluted within the first three days. Compared with the earlier CaS/HA+VAN formulation from our group, VAN release kinetics were similar, indicating that co-loading with TOB does not substantially alter its behavior[104]. The differing release rates may reflect differences in molecular size and material affinity. The smaller TOB molecule may penetrate deeper within the CaS/HA matrix and may exhibit stronger ionic interactions with HA, thereby producing a more prolonged release. Importantly, the release kinetics of TOB or VAN alone were not significantly different from those of the dual-antibiotic formulation, and both antibiotics demonstrated nearly 100% cumulative release across all tested groups.

Compared with mono-specie infections, multi-species infections typically have lower healing rates, require more revision surgeries, and result in longer hospital stays[134]. In addition to *S. aureus*, Gram-negative bacteria and resistant strains such as MRSA can frequently be isolated from osteomyelitis sites. The presence of multiple species increases the likelihood of biofilm formation, which further restricts antibiotic penetration[135]. Therefore, multispecies infections often require combination antibiotic regimens. Nan et al. demonstrated that a β-TCP/calcium sulfate system delivering VAN+TOB or gentamicin produced a clear ZOI for at least 20 days[136]. Our previous study showed that CaS/HA+VAN maintained antimicrobial activity for no more than 14 days[104]. In **Study 1**, both CaS/HA+TOB+VAN and CaS/HA+TOB exhibited antimicrobial activity for more than 28 days against *S. aureus* ATCC 25923, consistently producing ZOIs larger than 16 mm. Even after 28 days, crushed pellets generated ZOIs exceeding 20 mm, indicating that active antibiotic concentrations remained trapped within the material and continued to be released upon further degradation. Differences between laboratory reference strains and clinical isolates have been highlighted since the study by Christoph et al. in 2004[137]. Clinical strains often display enhanced resistance, stronger biofilm formation, and altered metabolic activity, requiring stronger antimicrobial effects for eradication[137]. In **Study 1**, we included a clinical isolate, *S. aureus* P-3, obtained from a case of periprosthetic joint infection. ZOI patterns differed substantially between P-3 and the ATCC 25923 strain. The CaS/HA+TOB+VAN combination rapidly lost antimicrobial activity against P-3, similar to the CaS/HA+VAN group, and effects disappeared by day 10. In contrast, TOB alone exhibited the strongest antimicrobial activity, maintaining ZOIs larger than 6 mm for approximately four weeks. This finding supports the possibility that TOB may have higher affinity for HA particles, resulting in prolonged release. Compared with ATCC 25923, all combinations generated smaller ZOIs and declined more rapidly against P-3, reflecting the greater resilience of clinical strains and the challenges faced in real clinical scenarios.

Liu et al. reported that the release of a HA-binding cytostatic drug (doxorubicin) differed significantly between in vitro and in vivo environments[103]. Likewise, we observed that ZOI patterns did not fully match the antibiotic release profiles obtained in PBS. For instance, CaS/HA+TOB+VAN exhibited a faster loss of effect against P-3 than TOB or VAN alone. To further investigate this discrepancy, ZOI assays were repeated using paper discs impregnated with previously collected release fractions. While results matched those obtained for ATCC 25923, they differed considerably for P-3[123]. Notably, these differences may better reflect clinical conditions, since CaS/HA pellets implanted in vivo are more likely to be exposed to dynamic environments such as blood or interstitial fluid rather than static PBS.

6.2 Win the "race for the surface" by promoting bone formation

Antibiotics are used in both infection treatment and prophylaxis to suppress bacterial proliferation in local drug delivery systems. However, regardless of the delivery mode, the fundamental objective remains the same, to secure an early advantage in the "race for the surface." In this context, promoting bone regeneration becomes equally important, because long-term protection of an implant depends on timely bone apposition and stable osseointegration, the period between implantation and the establishment of a close bone–biomaterial contact[138]. This process has been shown to proceed not only from native bone toward the implant, but also from the biomaterial surface, where biomaterial-bound bone-forming cells can initiate faster de novo bone formation[138]. As a potent osteogenic factor, BMP-2 enhances this process by accelerating bone–implant interface formation [139]. From this mechanistic perspective, antibiotics and BMP-2 act at complementary phases of the race for the surface. Antibiotics dominate the early phase by preventing bacterial adhesion or eradicating any organisms already present. Subsequently, BMP-2 governs the later phase by accelerating the recruitment, adhesion, and differentiation of osteogenic cells, ultimately enabling host tissue to achieve stable, long-term control of the surface.

Prolonged operative time has been associated with an increased risk of surgical-site infection, with some studies reporting more than a 10% rise in risk for every 15 minutes increase in surgery duration[140]. Traditional ceramic local-delivery systems often require mixing and molding, followed by approximately 15 minutes of initial setting[104]. In **Study 2**, we modified the CaS/HA-TOB preparation by impregnating TOB onto pre-set small CaS/HA beads, to increase surface area but also to meet the concerns regarding setting time and injectability of mixed CaS/HA during the operation.

The in-vivo environment introduces additional complexities that may reduce the efficiency of local drug-delivery systems. Serum proteins and other host macromolecules can rapidly adsorb onto biomaterial surfaces, altering surface chemistry and potentially modify the drug-binding interactions[141, 142]. In **Study 2**, serum TOB levels displayed a consistent release pattern over 48 hours, with the highest concentrations observed between 2 and 6 hours. Importantly, even at 48 hours, TOB remained above the MIC against *S. aureus*[143]. Although systemic concentrations appeared low, local antibiotic concentrations at the implant site are substantially higher than serum levels in local-delivery systems. In some reports, local concentrations have been shown to exceed systemic levels by several hundred- to thousand-fold during the early release phase, supporting the rationale for local delivery strategies[107, 144]. Clinically, systemically administered TOB exhibits a short half-life of approximately 2 hours, and its effective perioperative window is generally limited to 24 hours[145]. In contrast, the sustained release observed here suggests that TOB may maintain a protective concentration during the early postoperative period when bacterial adhesion is most likely.

Concerns have been raised regarding potential inhibitory effects of TOB on bone healing due to osteoblast cytotoxicity at high concentrations[146, 147]. However, Glatt et al. demonstrated that these inhibitory effects can be reversed when TOB is combined with BMP-2, and Rousseau et al. similarly reported that combining BMP-2 with antibiotics (including TOB) in bone-defect models resulted in periosteal reactions comparable to BMP-2 alone[148, 149]. Consistent with these findings, both sides of the fusion site in **Study 2**, CaS/HA + TOB + ABG and CaS/HA + BMP-2, exhibited robust bone regeneration with no evidence of infection. Radiographic interpretation alone can be misleading due to the radiodensity of ceramic materials. Therefore, micro-CT analysis and manual palpation were included. All three methods demonstrated successful fusion across all animals. Although the CaS/HA + TOB + ABG group showed slightly lower bone volume than the CaS/HA + BMP-2 group, no significant differences in fusion were observed. The observed fusion rates were 5/6 in the TOB group and 6/6 in the BMP-2 group. Notably, these rates appear favorable when compared with previously reported fusion rates of approximately 70% for autograft alone in a similar animal study[150]. In addition, bone volume outcomes in the CaS/HA + TOB + ABG group were benchmarked against findings from a parallel unpublished study performed within our group. In that study, the ABG-only cohort demonstrated substantially lower bone volume. This comparison reinforces the conclusion that TOB, when incorporated into the CaS/HA matrix, does not impair osteogenesis or compromise autograft mediated bone healing. Histological evaluation also confirmed clear bone bridge between two transverse processes. Collectively, these findings support the concept that a CaS/HA based local delivery system combining TOB and BMP-2 has the possibility to act synergistically to influence both phases of the race for the surface, that TOB provides early-phase antibacterial protection, while BMP-2

enhances subsequent host bone integration, ultimately promoting a favorable healing environment for spinal fusion.

6.3 TET binding to HA and influencing factors

Although CaS/HA functions effectively as a local drug delivery system and the CaS component degrades within a few weeks, the long-term presence of HA remains a potential concern because its limited degradability may provide a surface for bacterial colonization when antibiotic concentrations fall below effective levels[151]. This problem encourages researchers to consider strategies for managing the remaining HA and preventing reinfection.

TET is a broad-spectrum antibiotic that inhibits bacterial protein synthesis by preventing the attachment of aminoacyl-tRNA to the mRNA–ribosome complex. Owing to this mechanism, TET is bacteriostatic rather than bactericidal, but it remains effective against a wide range of Gram-positive and Gram-negative bacteria[152]. Importantly, TET also exhibits strong binding affinity to HA-containing materials, which has stimulated interest in how this property could be exploited for local antimicrobial strategies. As early as the 1960s, Perrin et al. evaluated and reported the mechanism of interaction between TET and bone mineral, particularly HA[153]. This early work was later supported by subsequent study demonstrating that TET adsorption onto HA is primarily mediated through calcium chelation, electrostatic interactions, hydrogen bonding, and weak van der Waals forces[154]. A previous study from our group initiated the concept of "biomodulation", which refers to the recruitment of systemically administered HA-binding drugs to implanted HA materials through their natural affinity, thereby enabling localized antimicrobial effects even after surgery[106]. In **Study 3**, TET was found to exhibit rapid and time-independent affinity for HA, with no significant differences observed across multiple timepoints within 24 hours. This finding aligns with the work of Cazalbou et al., who reported that TET–HA binding occurs rapidly and typically reaches saturation within approximately 30 minutes[155]. However, the binding of other biological macromolecules to HA has been widely reported[156-158]. An earlier work from Liu et al. indicated that protein passivation of HA surfaces did not significantly influence the ability of doxorubicin, an anticancer drug, to interact with HA either in vitro or in vivo[159]. In contrast, **Study 3** demonstrated that TET binding to HA was clearly affected by protein passivation. HA particles pre-treated with FBS showed significantly reduced ZOIs compared with non-passivated HA exposed to TET. This result suggested that not all HA-binding drugs share the same sensitivity to protein masking. The intrinsic properties of the interacting molecule, such as size, charge distribution, and the presence of specific functional groups, may determine the extent to which serum proteins interfere with binding[160]. Although FBS clearly reduced the binding of

TET to HA, the TET-bound HA still demonstrated strong antibacterial activity with a distinct ZOI. In addition to serum proteins, several pharmaceuticals commonly used by orthopedic patients, such as the modern bisphosphonates, including zoledronic acid (ZA), also exhibit HA-binding capacity[106]. The present study evaluated the effect of increasing ZA concentrations on TET binding to n-HA and demonstrated a dose-dependent competitive inhibition. Together, these in-vitro findings indicate that the biomodulation process can indeed be influenced by the biochemical environment surrounding implants, including serum proteins and competing HA-affinitive drugs. Nevertheless, even under such competing conditions, HA retains its ability to recruit TET and contribute to implant protection, underscoring the robustness of this interaction under biologically relevant circumstances.

6.4 The influence of size differences of HA particles and in-vivo biomodulation

HA can be produced in a wide range of particle sizes, from micrometer to nanometer scales, resulting in differences in structure, porosity, and surface characteristics that are known to critically affect drug loading and binding performance[161-163]. Previous studies have reported that m-HA (mHA, ~10 µm) and n-HA (nHA, <50 nm) may exhibit different affinity profiles toward various drugs, with some molecules binding preferentially to mHA while others show stronger binding to nHA[161]. In the in-vitro portion of **Study 3**, TET was incubated with either nHA or mHA at multiple timepoints, and significant differences were observed between the two particle types: nHA consistently produced larger ZOIs at all timepoints. This difference persisted even under protein-passivation conditions, indicating that nHA maintains a higher binding affinity for TET despite the presence of adsorbed serum proteins. The in-vivo biomodulation experiment further confirmed these findings where biomodulation refers to the recruitment of systemically administered TET by HA particles after implantation. After three days of systemic TET administration, nHA exhibited markedly greater antibacterial activity than mHA, demonstrating that nHA retains its superior affinity for TET not only in aqueous environments but also in soft-tissue surroundings. This may be attributed to the substantially larger specific surface area of nHA, as well as its increased density of accessible functional groups and higher surface energy, which collectively contribute to improved drug adsorption[164].

nHA has been extensively investigated and with applications in regenerative medicine and biomaterials engineering[165]. However, most studies have focused on its use as a drug-delivery system or as a platform for gene therapy rather than as a drug-binding substrate capable of pharmacological activation[166, 167]. It is well

established that preventing initial bacterial adhesion and colonization is more challenging than suppression[168]. After the early period of adherence to the material surface and the formation of biofilm, eradication becomes significantly more difficult due to reduced antibiotic penetration and decreased bacterial sensitivity[62]. In the subcutaneous infection model used in **Study 3**, the biomodulation of nHA with systemically administered TET proved effective in protecting the implantation site and preventing infection. After three days of systemic TET administration, tissue surrounding the nHA implantation sites demonstrated significantly lower infection activity and markedly reduced colony-forming unit (CFU) counts compared with groups lacking TET or HA. Notably, this difference became even more pronounced over time, particularly between 30 minutes and 6 hours after bacterial exposure. During this critical early window, when bacterial adhesion and microcolony initiation typically occur, bacterial activity remained almost unchanged in the TET+nHA group but increased substantially in the controls[125]. These findings suggest that biomodulated nHA forms an antimicrobial barrier capable of intercepting bacteria before stable attachment and colonization occur. Based on these results, nHA appears to play an important role in the biomodulation strategy, and implants may also be effectively protected through drug loading with systemically administered TET. Therefore, incorporating an nHA layer in combination with systemic TET administration may represent a promising approach for infection prevention.

6.5 Optimal time for biomodulation

Antimicrobial prophylaxis is generally recommended for surgical procedures associated with a high risk of infection, such as clean-contaminated or contaminated surgeries, but also in selected clean procedures, even when the overall likelihood of infection remains low but where the consequences of infection would be severe, for example, implantation of orthopedic prostheses[169]. A widely accepted principle is that achieving and maintaining an antibiotic concentration at the surgical site that exceeds MIC is critical for preventing early bacterial adhesion and colonization. Several clinical studies have investigated the timing of prophylactic antibiotic administration and consistently report that delivering antibiotics within 60 minutes prior to incision results in the lowest postoperative infection rates compared with administration at earlier timepoints or after surgery[170-172].

Despite these recommendations, the optimal postoperative antimicrobial administration window remains less clearly defined. Some studies suggest that postoperative antibiotics ideally should be administered within the first 24 hours, as extending treatment beyond this period does not significantly reduce infection rates and may instead increase the risk of antimicrobial resistance[173-175]. However, these recommendations are based largely on pharmacokinetic principles involving

systemic antibiotic levels, tissue perfusion, and MIC thresholds, all of which may be influenced by factors such as drug dosage, patient body weight, vascularity, and surgical manipulation. Importantly, these conventional models do not account for the presence of a drug-recruiting substrate within the surgical environment.

The concept of biomodulation introduces a fundamentally different paradigm. When a recruitment station such as HA is present, the local antibiotic concentration is governed not solely by systemic pharmacokinetics but by the ability of the material to selectively bind, retain and release the antibiotic. This shifts the focus from systemic exposure profiles to the interaction between drug and material surface chemistry. The findings from **Study 4** illustrate this distinction clearly. When HA-coated implants were used, administering systemic TET 15 minutes postoperatively, followed by two additional doses over the subsequent 48 hours, produced a superior antimicrobial effect compared with a single preoperative administration alone or with more delayed postoperative dosing. This suggests that early perioperative administration may coincide with an optimal window in which circulating TET can be effectively recruited by the HA coating before substantial bacterial adhesion or microcolony establishment occurs.

Furthermore, HA-coated implants that received systemic TET demonstrated a markedly reduced tendency for biofilm formation. Only sparse colonies were observed on these biomodulated surfaces, whereas implants without TET biomodulation displayed extensive biofilm coverage. This highlights the capacity of HA-mediated drug recruitment to create a localized inhibitory microenvironment capable of disrupting the initial stages of bacterial colonization. The study by Caneva et al. reported that bone formation proceeds more rapidly when implants are placed in the metaphyseal region than in the diaphysis[176]. They attributed this difference to the denser trabecular architecture in the metaphysis compared with the tibial diaphysis, which provides a more favorable environment for early bone ingrowth and osseointegration. Interestingly, no significant difference was observed between implants placed in the cortical region and those placed in the metaphyseal region of the tibia. This indicates that the TET–HA interaction is robust and not substantially influenced by the anatomical location of implantation, likely because HA surface chemistry remains the dominant factor governing drug binding. These findings emphasize that the timing of antibiotic administration in the presence of HA-containing implants may differ from conventional prophylaxis guidelines. Early postoperative systemic dosing, when recruitment capacity is high and bacterial adhesion is still reversible, appears particularly effective.

6.6 Future work

6.6.1 CaS/HA as a local drug-delivery system

The limited vascularity and highly compartmentalized microarchitecture of bone make it difficult to achieve therapeutic antibiotic concentrations using systemic administration alone, underscoring the importance of local drug-delivery strategies[177]. Beyond infection control, biomaterial-based delivery systems can also function as structural supplements in bone defects, for example, reducing the demand for autograft in spinal fusion, and can contribute to bone regeneration either intrinsically or through co-delivery of osteoinductive factors such as BMP-2 or ZA[178, 179]. In this thesis, CaS/HA was evaluated as a carrier for TOB in vitro and as a dual-delivery system for TOB and BMP-2 in vivo in a rabbit spinal fusion model. The system demonstrated stable release kinetics and no detrimental effects on bone regeneration within the controlled, non-infected environment tested here. However, it remains unclear how this system performs in a true infectious environment or clinically relevant scenario. Infection introduces a series of challenges, including immune activation, elevated inflammatory cytokines, biofilm formation, altered pH, enzymatic activity, and changes in material degradation, that could modify the release kinetics and bioactivity of both TOB and BMP-2. Consequently, the effective antimicrobial window and the ability of BMP-2 to support bone regeneration may differ substantially from those observed in the present studies. To better approximate in vivo conditions, future in vitro work should incorporate more physiological testing environments, such as protein-rich media, inflammatory media, enzymatically active solutions, or perfusion systems, rather than relying solely on PBS.

In addition, although serum TOB concentrations were measured in **Study 2** to confirm systemic release, the local antibiotic concentration at the implant, bone interface remains unknown. This represents a major limitation, as local concentration, rather than serum level, is the primary determinant of antimicrobial efficacy in a local-delivery system. Detailed quantification of local TOB concentrations, along with time-dependent analysis of bone coverage over CaS/HA surfaces, would help to identify potential gaps in the “race for the surface” where bacterial adhesion might occur before sufficient bone formation is established. Importantly, **Study 2** evaluated the antimicrobial and osteogenic functions of the TOB + BMP-2 combination separately, and the full synergistic or competitive behavior of this dual-delivery strategy still requires validation in an established infection animal model. A future clinical project within our group will also investigate the translational performance of CaS/HA combined with TOB and BMP-2 in real surgical settings.

6.6.2 Hydroxyapatite and biomodulation

HA possesses several advantageous properties, including osteoconductivity, biocompatibility, surface reactivity, and inert characteristics, that make it attractive for bone engineering applications[180]. However, its slow degradation remains a limitation, particularly regarding the potential risk of secondary bacterial colonization on long-lasting material surfaces. Although HA has been widely evaluated as a recruitment site for bisphosphonates, its interactions with HA-affinity antibiotics have not been fully elucidated[106]. In this thesis, the binding behavior of TET to nano- and micro-sized HA particles was evaluated under protein and ZA competition, both in vitro and in vivo. While these studies demonstrate the feasibility of biomodulation, the protective antimicrobial effect of TET-mediated recruitment still needs to be confirmed in a bone infection model using HA-coated implants.

Study 4 functioned as a pilot investigation to identify the optimal systemic administration timing for biomodulation, laying the groundwork for the next phase of research. Future studies should include a more detailed pharmacokinetic assessment of TET concentrations at the implant surface under various dosing regimens and in the presence of active infection. Furthermore, given the known osteogenic potential of ZA, its role as a supplementary factor following TET recruitment warrants investigation. Determining the remaining HA-binding capacity after TET occupancy, as well as defining the optimal timing for ZA administration, will be essential to maximize osteogenesis. Furthermore, AI tools and supercomputer-based simulations should be used to predict HA-antibiotic molecular docking based on known models including antibiotics like TET and rifampicin and their model of interaction (physical or chemical) with HA. By doing so, a library of potential antibiotics with accretion to HA could be created, giving surgeons a wide array of options in their fight against orthopedic infections.

7. Conclusions

- The CaS/HA biomaterial was demonstrated as an effective carrier for VAN and TOB, enabling prolonged and sustained antibiotic release.
- No pronounced synergistic antibacterial effect was observed between VAN and TOB against *S. aureus*. CaS/HA containing TOB exhibited more sustained release profiles and superior antibacterial efficacy.
- Pre-set CaS/HA beads pre-loaded with TOB showed substantial antibiotic release both in-vitro and in-vivo, maintaining antibacterial activity against *S. aureus* at concentrations exceeding the MIC.
- Local delivery of TOB using CaS/HA as a bone graft extender did not adversely affect bone regeneration and resulted in fusion outcomes comparable to those achieved with CaS/HA combined with BMP-2.
- Interactions between TET and HA occurred rapidly and were partially influenced but not silenced by serum proteins and HA-affinity molecules.
- Pre-implanted m- and n-HA particles functioned as effective recruiting moieties for systemically administered TET, conferring antibacterial activity against both reference and clinical *S. aureus* strains.
- In an infection model in mice, systemic administration of TET in the presence of pre-implanted n-HA particles resulted in reduced bacterial colonization.
- For HA-coated implants, systemic administration of TET consisting of one dose immediately post-operation followed by once-daily dosing on postoperative days 1 and 2 achieved optimal antibiotic binding, with no observed differences between cortical and metaphyseal implantation sites.

8. Summary in English

Orthopedic implant-associated infections remain a major clinical challenge. The eradication of bacteria is difficult due to biofilm formation and limited antibiotic penetration. Conventional systemic antibiotic administration requires high doses to achieve sufficient concentrations at the surgical site, which increases the risk of systemic side effects and often provides only transient protection. Local antibiotic delivery systems, such as PMMA, are associated with an initial burst release and leave behind non-degradable foreign material once the antibiotic has been released. New biomaterials have been developed to overcome these limitations, but several challenges remain: 1) limited duration of antibiotic release; 2) subtherapeutic concentrations that may promote bacterial resistance; and 3) residual materials left unprotected after drug depletion.

In this thesis, biphasic CaS/HA biomaterials are demonstrated to represent a promising platform for local antibiotic delivery in early infections. In addition, HA can function as a recruitment agent for systemically administered HA-affinity antibiotics, thereby protecting implants through a biomodulation strategy. Together, these complementary approaches enable the reutilization of residual HA remaining after CaS degradation, effectively extending the duration of antimicrobial protection. This dual strategy addresses both early infection control and prolonged implant protection.

In Study 1, the clinically used CaS/HA biomaterial was shown to function as a controlled and sustained local delivery system for VAN and TOB. Although no pronounced synergistic antibacterial effect was observed when VAN and TOB were combined, CaS/HA loaded with TOB demonstrated prolonged antimicrobial activity in-vitro.

Based on these in-vitro findings, CaS/HA+TOB was evaluated in a rabbit posterolateral spinal fusion model in Study 2. Pre-set CaS/HA beads were used to enhance surgical handling, as antibiotic concentration influenced setting and injectability. TOB exhibited an initial burst release followed by sustained antibacterial activity. BMP-2 was included to assess whether high local concentrations of TOB would adversely affect bone regeneration. The results indicated that TOB could be combined with BMP-2 without compromising fusion outcomes, suggesting that this strategy may contribute to preventing early infection while preserving bone healing.

In Study 3, the affinity of TET to micro- and nano-scale HA particles and the antimicrobial efficacy of the biomodulation strategy were investigated. TET demonstrated higher affinity to n-HA than m-HA, and this interaction was influenced by biological macromolecules such as serum proteins and bisphosphonates. In-vivo experiments showed that pre-implanted HA particles preferentially recruited systemically administered TET, with stronger effects observed for n-HA. The biomodulation approach reduced bacterial adhesion to HA particles compared with control tissues.

In Study 4, the biomodulation strategy was extended to HA-coated implants to better mimic clinical conditions. The optimal timing of systemic TET administration was identified as one dose immediately post-operation followed by once-daily dosing on postoperative days 1 and 2. SEM analysis confirmed reduced bacterial colonization on HA-coated implants following this regimen. These findings demonstrate that systemic administration of HA-affinity antibiotics can effectively load pre-implanted HA biomaterials in-vivo and prolong implant protection.

Overall, this thesis presents complementary strategies for infection prevention through local antibiotic delivery and systemic antibiotic recruitment via HA-based biomaterials. These approaches protect implants without compromising bone regeneration and provide a potential strategy to win “the race for the surface” in orthopedic surgery.

9. Sammanfattning på svenska

Infekterade ortopediska implantat (ben/implantatassocierade infektioner; BAI/IAI), utgör fortfarande en betydande klinisk utmaning. Konventionell antibiotikabehandling sker via blodbanan och kräver höga doser för att uppnå tillräcklig lokal koncentration vilket ökar risken för systemiska biverkningar. Det är svårt att fullständigt eliminera alla bakterier då dessa tidigt bildar en biofilm som begränsar antibiotikapenetrationen. Bencement (PMMA)innehållande antibiotika används idag vid artroplastik vilket ger hög tidig frisättning men lokalt infektionsskydd är begränsat till första dygnen.

I denna avhandling används ett nedbrytbart keramiskt material innehållande kalciumsulfatdihydrat (CSD), i kombination med hydroxyapatit (HA), vilken utgör den huvudsakliga mineralfasen i ben. Härdad CSD löses relativt snabbt upp i kontakt med kroppsvätskor och tillsatt läkemedel frisätts. Hydroxyapatit är stabilt och nedbrytningen sker långsamt men snabbare i sur miljö exempelvis vid infektion. Vissa läkemedel/antibiotika binder kemiskt till HA som på så sätt fungerar som en läkemedelsreservoir tills hydroxyapatiten upplöses. Kombinationen av de två biomaterialen utgör således en lovande läkemedelsplattform, som dels kan frisätta antibiotika lokalt kontrollerat via CSD, men som dessutom via HA binder in vissa systemiskt givna antibiotika och på så sätt kan fylla på materialet efter hand.

I studie 1 undersöktes hur CSD/HA-materialet frisätter vancomycin (VAN) och tobramycin (TOB). Vi fann ingen synergistisk antibakteriell effekt när VAN kombinerades med TOB, men CSD/HA laddat med TOB gav en förlängd antimikrobiell aktivitet in-vitro.

I studie 2 utvärderades CSD/HA+TOB in-vivo i kaninmodell med steloperation mellan två ryggkotor (posterolateral spinal fusion). Eftersom tillsats av antibiotika påverkar stelning och injicerbarhet, användes redan stelnade kulor av CSD/HA. TOB frisattes snabbt initialt men uppvisade också en långvarig antibakteriell aktivitet. För att undersöka om de höga lokala koncentrationerna av TOB påverkar benregenerationen negativt blandades TOB innehållande material med bentransplantat på ena sidan som jämfördes med enbart material kombinerat med ett potent beninducerande protein BMP-2. TOB innehållande CSD/HA kunde kombineras med transplantatet och var lika effektivt som material utan TOB kombinerat med BMP-2. Resultaten visar att tillsättning av antibiotika till CSD/HA

inte påverkade fusionen negativt vilket indikerar att vi kan förebygga tidig infektion samtidigt som god benläkning bevaras.

I studie 3 undersöktes biomaterial med tetracyklin (TET) vilken i motsats till VAN och TOB binder starkt till HA. Vi använde två storlekar av HA-kristallen, mikro- respektive nano-HA. Vi undersökte även här CSD/HA utan tillsats av antibiotika och lät i stället antibiotika efter injektion cirkulera i blodbanan och bindas kemiskt till hydroxyapatiten i det implanterade materialet (biomodulering). In-vitro band TET bättre till n-HA än till m-HA. In-vivo visades att förimplanterade HA-partiklar selektivt kunde binda in och rekrytera systemiskt administrerad TET. n-HA band bättre än m-HA. TET som band till HA-partiklarna gav effektivt skydd mot infektion av det HA-baserade materialet i subkutan musmodell.

I Studie 4 undersöktes HA-belagda implantat med syftet att bättre efterlikna kliniska förhållanden. Den optimala tidpunkten för systemisk administrering av TET identifierades som en dos given perioperativt följt av daglig dosering under postoperativ dag 1 och 2. SEM-analys bekräftade minskad bakteriell kolonisation på HA-belagda implantat enligt detta schema. Resultaten visar att systemisk administrering av HA-bindande antibiotika effektivt kan ladda förimplanterade HA-biomaterial in-vivo och förlänga implantatskyddet.

Sammanfattningsvis visar vi i denna avhandling kompletterande strategier för infektionsprevention genom dels lokal antibiotikaleverans men också systemisk antibiotikarekrytering via HA-baserade biomaterial. Resultaten indikerar vidare att dessa metoder kan skydda implantat utan att påverka benregeneration negativt och öppnar för nya och förbättrade strategier för att hantera implantatrelaterade infektioner inom ortopedisk kirurgi.

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