

Diplomarbeit

**Manual addition of antibiotics to PMMA bone
cement: Effects on mechanical and microbiological
properties**

eingereicht von

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Zusammenfassung

Einleitung: Die Verwendung von antibiotika-beladenem Knochenzement (ABKZ) stellt eine effektive adjuvante Therapieoption in der Bekämpfung periprothetischer Infektionen dar. Derzeit gibt es nur wenige Antibiotika, die zur lokalen Therapie von Infektionen erprobt sind, besonders wenn es um die Bekämpfung gramnegativer Bakterien geht. Ziel dieser Studie war es, PMMA-Knochenzemente, denen manuell Antibiotikapulver in verschiedenen Dosierungen beigemischt wurden, auf ihre mechanischen Eigenschaften hin zu untersuchen, sowie die Freisetzung und Wirksamkeit der enthaltenen Substanzen in Hemmhoftests gegen entsprechende Leitkeime zu beurteilen.

Material und Methoden: Meropenem, Imipenem und zwei Fosfomycin-Salze wurden den PMMA-Knochenzementpulvern beigefügt. Nach Zufügen der Monomer-Flüssigkeit wurden aus den antibiotikabeladenen Zementmischungen genormte Formkörper hergestellt. Zylinder aus beladenem Zement wurden in steriler phosphat-gepufferter Kochsalzlösung (PBS) versenkt und bei 37°C inkubiert. Zu pre-determinierten Zeitpunkten wurden Aliquote der Eluate für die weiteren Tests entnommen. Danach wurden die Zylinder jeweils in neue Behälter mit PBS überführt. Die Bioaktivität der Eluate wurde über 42 Tage gegen folgende Bakterien getestet: *Escherichia coli* (klinischer Stamm), *MRSA* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853 und *Proteus mirabilis* ATCC 12453. Mechanische Eigenschaften, darunter auch die Druckfestigkeit, die Biegefestigkeit, das Biegemodul und die Schlagzähigkeit, wurden mittels standardisierter Tests entsprechend den ISO 5833 und DIN 53435 Standards ermittelt.

Ergebnisse: Zement und Antibiotika ließen sich problemlos zu einem homogenen Teig vermengen. Die Zugabe von Calcium-Fosfomycin führte zu einer signifikanten Minderung der Schlagzähigkeit und der Biegefestigkeit. Alle anderen ABKZ entsprachen den ISO-Spezifikationen. Meropenem-beladener Zement war über den gesamten Zeitraum hinweg effektiv gegen *E. coli* und *Pseudomonas aeruginosa*. Imipenem-beladene Zemente waren gegen *MRSA* nur zwei Wochen lang wirksam. Fosfomycin-Trometamol zeigte sich effektiver gegen *Proteus mirabilis* als Ca-Fosfomycin.

Diskussion: Eine Empfehlung für die Verwendung von Meropenem, Imipenem und Fosfomycin-Trometamol in Spacern zur Bekämpfung empfindlicher

Erreger kann auf Basis der vorliegenden Untersuchungen abgegeben werden. Der Einsatz besagter Antibiotika insbesondere gegen gramnegative Keime stellt daher eine attraktive Therapieoption dar.

Abstract

Introduction: Use of antibiotic loaded acrylic bone cement (ALABC) represents an effective adjuvant treatment option when combating periprosthetic joint infection. There are few antibiotics available for local delivery, particularly when gram-negative bacteria are present. The objectives of our study were to evaluate the mechanical properties as well as the bio-activity of different PMMA bone cements after manual addition of one of four pulverulent antibiotics in different concentrations.

Materials and Methods: Meropenem, imipenem, fosfomycin trometamol and calcium fosfomycin were admixed to the polymer cement powder and subsequently combined with the liquid monomer to create antibiotic-loaded PMMA-cements. These cements were used to fashion standardized moulds for testing. ALABC-cylinders were immersed in sterile phosphate-buffered saline (PBS) solutions and incubated at 37°C. At pre-determined points aliquots of the eluate were extracted for further testing. The cylinders were then transferred into fresh tubes with PBS. The bio-activity of the eluate was tested over a period of 42 days against the following organisms: *Escherichia coli* (clinical strain), *MRSA (resistant to Gentamycin and Clindamycin)* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853 and *Proteus mirabilis* ATCC 12453. Mechanical properties, including compressive strength, flexural strength, flexural modulus as well as impact resistance were determined via standardized tests according to ISO 5833 and DIN 53435 standards.

Results: All of the antibiotics were easy to mix into the cement powder and each yielded a homogenous dough. All the ALABCs met the criteria defined by the International Organization for Standardization (ISO) with the sole exception of calcium fosfomycin which had a detrimental effect on impact and bending strength. Meropenem-impregnated cements proved particularly effective against *E. coli* as well as *Pseudomonas aeruginosa* throughout the measuring period. Imipenem-loaded cements were found to be biologically active against *MRSA* over a period of two weeks. Fosfomycin trometamol proved to be more effective than calcium fosfomycin against *Proteus mirabilis*.

Discussion: It is easy to amalgamate pulverulent antibiotics with acrylic bone cement. The mechanical properties were impaired by 2g of calcium fosfomycin, however this effect did not occur with carbapenems. On the basis of the present

study, the use of meropenem, imipenem and fosfomycin trometamol in spacers can be recommended as it represents an effective treatment option for periprosthetic infection particularly in the presence of gram-negative bacteria.

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Glossary and Abbreviations

ALABC	Antibiotic loaded acrylic bone cement
Aliquot	Portion of a larger whole; a sample
CFU	Colony Forming Unit
DHP-1	dehydropeptidase-I, a human renal enzyme that deactivates Imipenem
DIN	“Deutsches Institut für Normierung“; German Institute for Standardization
Eluate	Solution that results from elution
Elute (verb)	To wash out a substance by use of a solvent
ISO	International Organization for Standardization
MPa	MegaPascal: measurement unit for pressure
PBS	Phosphate buffered saline solution
PMMA	Polymethylmethacrylate
PPI	Periprosthetic infection
In vivo	Occurring within the living organism
In vitro	Outside or isolated from the living organism
kJ/m ²	Kilojoule per square-meter; unit of measurement
Imi	Imipenem
Mero	Meropenem
Ca.-Fos.	Calcium fosfomycin
Fos.-Trom.	Fosfomycin trometamol = fosfomycin tromethamine
E. coli	Escherichia coli
P.aeruginosa	Pseudomonas aeruginosa
P. mirabilis	Proteus mirabilis.
S. aureus	Staphylococcus aureus
MRSA	Methicillin-resistant Staphylococcus aureus

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1 Introduction

1.1 Arthroplasty

Destructive processes such as chronic arthritis or traumatic injury as well as age-related wear may cause irreversible damage to a joint. This kind of damage can then lead to severe pain and impaired mobility, thereby severely impacting a patient's quality of life. If the knee or hip joint is affected, a total joint replacement is often the only viable option. Nevertheless, it is a surgical intervention that puts considerable strain on the patient's body.

Eighty years ago, artificial joint reconstruction was still in its infancy. Today, the implantation of total or partial knee and hip prostheses is considered a standard orthopaedic procedure. The Austrian Federal Ministry of Labour, Social Affairs, Health and Consumer Protection recorded, that close to 19.000 knee joints and more than 20.000 hip joints were replaced in 2016 alone (1). Out of these, nearly 2% required a revision procedure within 12 months of the primary implantation. Infection and aseptic loosening were the two most common causes for revisions. Further reasons included dislocation, abrasion debris, fracture of the bone or prosthesis and others.

1.2 Periprosthetic Infection

The infection of a newly implanted joint is commonly referred to as "periprosthetic joint infection" or PJI, which illustrates that it affects not only the articulating segments but also the surrounding bone, soft tissue and synovial fluid.

PJI represents a rare but devastating complication in arthroplasty as it can have far reaching consequences. PJIs of the knee are most commonly caused by bacteria of the staphylococcus species (2–5). The pathogens may find their way into the joint either by continuous spread from a nearby soft tissue injury, via haematogenic dissemination or through iatrogenic infection. The latter may originate from a contamination during the surgery itself or may develop after a medical intervention such as an injection into the affected joint.

Predisposing factors for PJI are numerous and varied. For example, susceptibility to infection is increased in diabetic, malnourished, morbidly obese or immunocompromised patients (2,6,7). These conditions are often associated with either a weakening of the patient's immunological defences, an impairment of microcirculation or both. This can then potentially lead to wound dehiscence, thereby increasing the likelihood of pathogens entering the wound.

Additionally, there are studies which show that when a foreign material is present, the number of bacteria needed to cause an infection is significantly reduced (8,9). Once the bacteria have reached the prosthesis, they can adhere to the surface and organize themselves into microcolonies. They can then proliferate freely, and the formation of a biofilm may ensue (Figure 1). Members of the *Staphylococcus* family are particularly inclined to develop these biofilms (10). However, many commensal and iatrogenic microorganisms (including fungi) are capable of the same feat (11).

A biofilm is a complex structure, made up of the pathogens themselves, which are embedded in a polysaccharide matrix that they excrete (12). Typically, the environmental conditions within the film will prompt some of the enclosed pathogens to fall into a stationary state where they cease growing and thereby become resistant to growth-dependant antibiotics (13). This is further compounded by the fact that once a biofilm is fully formed, antibiotic substances are no longer able to adequately penetrate the matrix. At the same time, the embedded pathogens are also protected from the host's immune system.

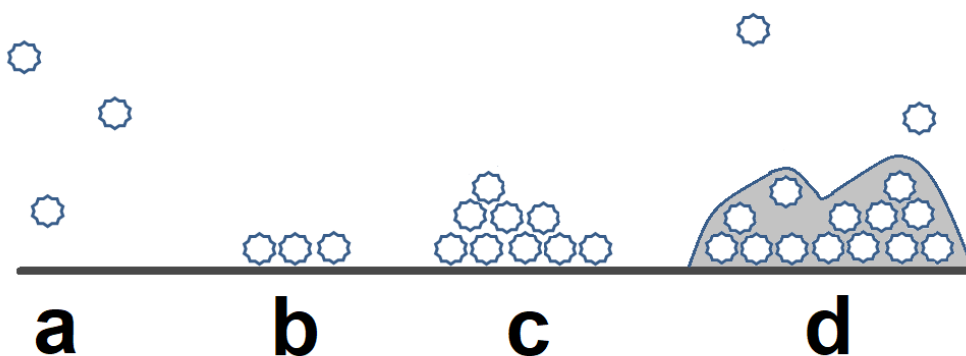


Figure 1: Biofilm Formation. Planctonic bacteria (a) adhere to the surface of the implant (b). Formation of microcolonies begins (c) until an adult biofilm is formed (d).

1.3 Classification

Generally, the categorization of PJI follows a tiered system, such as the one published by *Zimmerli and Ochsner* (14). It is a simple but effective classification scheme, which states that periprosthetic infections can be divided into three groups. These groups are differentiated according to the time between the implantation of the artificial joint and the presentation with signs and symptoms of infection in the patient.

- 1) Early: Indicators of infection appear within the first three months after the initial surgery
- 2) Delayed: Suspicion of present infection arises between three and 24 months post-operatively
- 3) Late: Time between implantation and first detection of symptoms exceeds 24 months

Early infections are thought to be caused by contamination of the surgical site at the time of implantation. The same is true for pathogens present in delayed-onset infections. It is believed that the virulence of bacteria causing delayed onset infections is lower than that of microorganisms found in early-onset infections (15).

Late-onset infections on the other hand are presumed to arise due to pathogens that spread via the bloodstream (16). The treatment regimen and the expected outcome for the three groups also differ greatly.

1.4 Diagnosis

In order to diagnose PJI, medical professionals rely on various tools. Clinical signs and symptoms, such as the five classical signs for inflammation - pain, swelling, heat, immobility and reddening of the joint – can be the first indicator for an infection of the joint. They are most likely to appear in cases of early-onset infections (Figure 2). In patients with late-onset infections on the other hand, persistent pain seems to be the primary complaint (17).

In a 2013 assembly of experts, an international consensus (18) was established, which focused on pertinent questions relating to the topic of PJI. Therein diagnostic criteria for those patients with conspicuous history or findings upon physical examination suggesting PJI, were identified. A fistula reaching the joint space was considered proof of infection.



Figure 2: Infected Knee. Example of an early-onset infection after primary total knee arthroplasty. (Picture by courtesy of Prim. Mag. Dr. Gregor Kienbacher, MSc.)

Other indicators, including laboratory parameters such as C-reactive protein, histological findings in samples of periprosthetic tissue or positive cultures from either joint aspirate or blood can also be used to substantiate suspicions of PJI.

In cases where the implants contain ferromagnetic materials, neither magnetic resonance imaging nor computer tomography are able to provide valuable information, as the visual artifacts can dramatically decrease the quality of the images (19).

Research into the viability of using polymerase chain reaction (PCR) for the detection of bacterial DNA in the diagnosis of PJI is being conducted (20). Trampuz et al. (20) summarize that the problems with this method are twofold. Firstly, due to the sensitivity of the method, results could easily be falsified by contaminating organisms and secondly, it does not offer information regarding antibiotic sensitivity.

The step from an empiric to a definitive therapeutic approach is only possible once the causative microbe has been found. Furthermore, knowledge as to the antibiotic susceptibility of the present pathogen as well as potential allergies of the patient to certain antimicrobials are essential for designing a structured treatment approach.

1.5 Therapy

The common consensus is, that while antibiotics are essential to the eradication of infection, a treatment consisting solely of the administration of intravenous antibiotics is futile. Instead, a multidisciplinary approach, amalgamating the expertise of clinical microbiologists, surgeons and infectious disease specialists is expedient.

In most cases, a surgical intervention is mandatory. There are two main strategies for clinicians to choose from:

- 1) A single-stage procedure
- 2) A two-stage exchange

Both interventions require the removal of the infected prosthesis. In a further step all the surrounding tissue (including bone and soft tissue etc.) suspected of being infected is cleared away, which is also referred to as “debridement”. Extensive lavage is used to clean the joint cavity and wash out the synovial fluid and any planktonic bacteria it might contain. The difference between a single- and a two-step exchange lies in the time of implantation of the new prosthesis. For the single-stage approach the contaminated artificial joint is swapped with a new one in the course of a single procedure. In the case of a two-stage exchange, a second surgery with an interval of 6 weeks or more between interventions is necessary. Currently there seems to be a preference of a two-stage exchange over the single-stage approach (21–23) for the treatment of PJI.

It is common practice to take a number of tissue samples which are then used to determine the causative agent and its susceptibility to antibiotics. Even if a pathogen had previously been identified via blood or aspiration culture, the results

of the intra-operative tissue cultures take precedence and the therapeutic regime is adapted accordingly.

If the decision falls on performing a double stage operation, there is the further option of inserting a temporary implant, commonly referred to as a “spacer”, for the duration of the interval. The purpose of using an intermediate spacer is to keep the soft tissue from contracting, thereby preserving the joint-space. Furthermore, if a spacer can withstand the mechanical stress of movement, atrophy of the muscles involved can be reduced and mobility is preserved. Bone cements made up of polymethyl methacrylate are frequently used to form

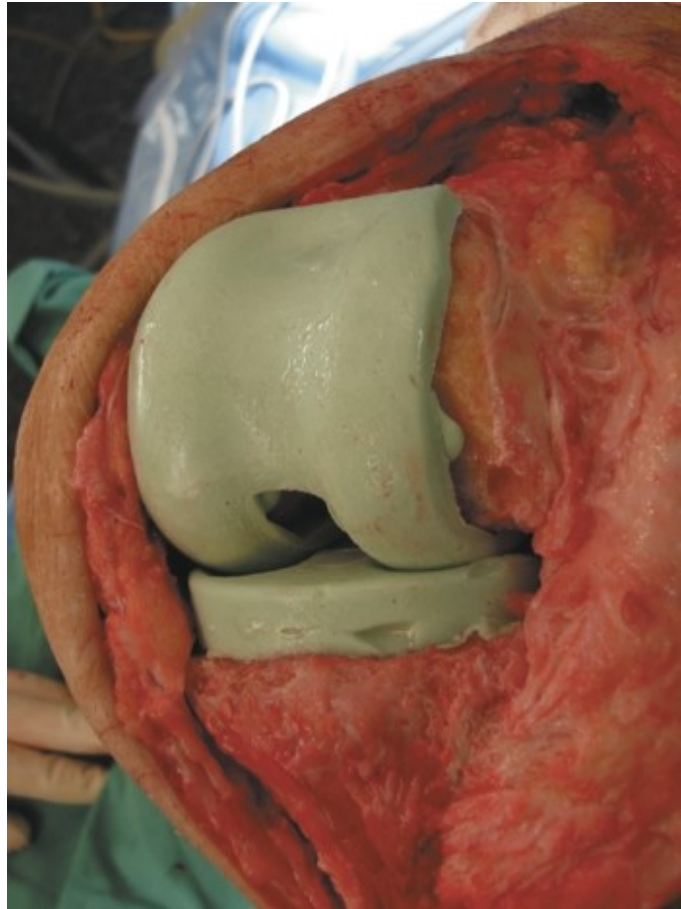


Figure 3: Articulating cement spacer of the knee (95)

intermediate spacers such as the one seen in Figure 3.

Generally, the surgical intervention is followed by a 2- to 6-week course of intravenous antibiotics, irrespective of whether an antibiotic-loaded spacer was used or not (13,14,21,24). To confirm that the infection has ceased, the patient is monitored for a two-week period, while no antimicrobials are administered. If no signs or symptoms of inflammation re-emerge, the infection can be considered as successfully treated and the pathogens completely eradicated. The new prosthesis is implanted in the second stage of the two-step approach.

1.6 Antibiotics

It is thanks to the efforts of scientist like Paul Ehrlich and Alexander Fleming that we can now efficiently combat bacterial infections. It was Paul Ehrlich who, at the beginning of the 20th century was the first to find a compound that was active

against *Treponema pallidum*, the bacterium causing syphilis (25). Later, Fleming's observation of a fungus affecting bacterial growth led to the discovery of the antimicrobial potential of penicillin (26).

Antibiotics are commonly understood as substances that can either outright kill bacteria or at least significantly inhibit their growth. Different antibiotic groups selectively target different vital processes or integral structures of the bacterial cell make-up. β -lactam antibiotics such as penicillin for example, aim to harm specific enzymes in the murein layer of the bacterial cell wall, thereby destabilizing it (27).

In an orthopaedic setting aminoglycoside antibiotics like tobramycin and gentamicin, glycopeptide antibiotics like vancomycin and cephalosporin antibiotics as well as combinations thereof are quite frequently used (28). However, the prevalence of multi-drug resistant organisms is steadily on the rise (29) and with it, the demand for antibiotics with alternative modes of action.

1.6.1 Carbapenems

Carbapenem antibiotics, together with penicillins, cephalosporins and monosporins make up the β -lactam class of antibiotics. Out of all these substances, carbapenems cover the widest range of bacteria as they are effective against a high number of gram-negative and gram-positive bacteria (30). Because the main point of attack of β -lactam antibiotics lies within the bacterial cell wall, they are inherently unsuitable in combating infections caused by intracellular microorganisms or bacteria without an exterior cell wall such as mycoplasma.

The discovery of thienamycin was a result of the efforts in finding a β -lactamase inhibitor (31). It represents the progenitor of the carbapenem family. However, it was discovered that thienamycin was increasingly unstable when dissolved in water and when confronted with a pH of 7 or higher (32).

Chemically, it is the 4:5 fused ring lactam as well as the double bond connecting the C-2 and C-3 atom in combination with the carbon (instead of sulfur) at the C-1 position that act as distinguishing characteristics for carbapenems (33).

In the treatment of internal ailments, meropenem and imipenem have proved to be popular, largely owing to their relative resistance to β -lactamases and their potency against common pathogens such as *Pseudomonas aeruginosa* (34). In orthopaedics however, they are largely relegated to the role of reserve antibiotics.

1.6.1.1 Imipenem

Imipenem (see Figure 4) was the first substance derived from thienamycin that was adapted for clinical usage. It is vulnerable to dehydropeptidase-I (DHP-1), an enzyme found in epithelial cells of the renal cortex in humans, which leads to the hydrolysis and thereby the inactivation of the compound (35). The metabolic deactivation can be prevented by combining imipenem with cilastatin at a 1:1 ratio (36). Cilastatin is an enzyme inhibitor with no antibacterial activity of its own. Since the introduction of imipenem to the market, very few adverse effects have been reported and it is generally considered to be well tolerated by patients (37).

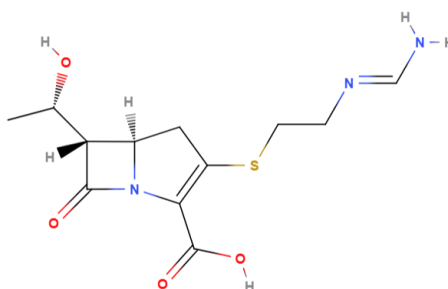


Figure 4: Chemical Structure of Imipenem (drawn with MolView)

1.6.1.2 Meropenem

Meropenem (Figure 5) shows a higher activity against gram-negative bacteria than most of its sibling compounds. Its ability to inhibit growth of the Enterobacteriaceae family (including *Proteus* and *E.coli*) far surpassed that of imipenem (38). Furthermore, it is less prone to inactivation by DHP-1 than imipenem (39) and it is therefore unnecessary to simultaneously administer cilastatin or similar enzyme-inhibitors. In its pulverulent form, meropenem was shown to be heat-resistant (40).

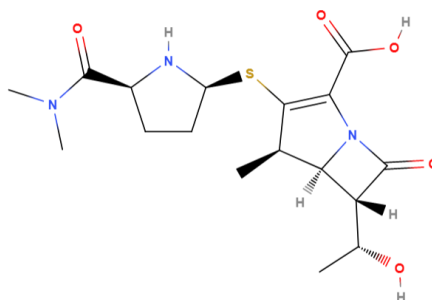


Figure 5: Chemical Structure of Meropenem (drawn with MolView)

1.6.2 Fosfomycin Trometamol & Fosfomycin Calcium

The discovery of the broad-spectrum antibiotic fosfomycin was first reported in 1969 (41). It is unrelated to any other known antibiotic and therefore constitutes its own class of antibiotic. For this reason, the occurrence of cross-resistances is rather unlikely. Fosfomycin inhibits a specific enzyme that synthesizes peptidoglycan, which is integral for the construction of the bacterial cell wall (42).

Fosfomycin demonstrates its microbiologically active against a diverse assortment of organisms, ranging from *Staphylococcus aureus* to *E.coli* and is furthermore able to adequately permeate soft tissue as well as bone (43). Concerning adverse events, a mild upset of the gastro-intestinal tract that manifests as diarrhoea, nausea or dyspepsia, seems to be the most prevalent (44). Few drug interactions have been reported. When administering an oral formulation, food may cause a delay in the uptake of the drug (45).

There are different drug formulations available. While fosfomycin represents the parent compound, fosfomycin trometamol (synonymous to fosfomycin tromethamine) and calcium fosfomycin constitute two variations intended for oral application. The two compounds show vast differences in structure (Table 1), resulting in divergent biochemical as well as physical properties.

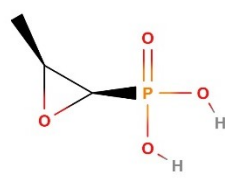
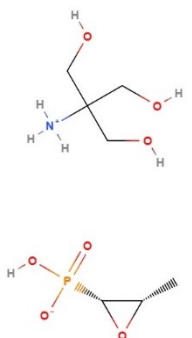
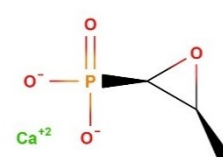
Compound:	Fosfomycin	Fosfomycin Trometamol	Calcium Fosfomycin
Structural Formula	$C_3H_7O_4P$	$C_3H_7O_4P + C_4H_{11}NO_3$ (1:1)	$C_3H_5CaO_4P$
Structure			
Molecular weight	138.059 g/mol	259.195 g/mol	176.121 g/mol

Table 1: Fosfomycin and derivatives. Structure and molecular weight. Pictures produced with MolView.

1.7 PMMA Bone Cements

Generally, PMMA-bone cements intended for medical use are made up of two central components. The first is a pulverulent polymer and the second a liquid monomer. These are combined to form PMMA, which is short for polymethylmethacrylate. When the two constituents come into contact, an exothermic polymerization process begins, which results in the setting of the acrylic cement. The preparation process can be divided into the following four stages, which transition seamlessly into one another (46).

- 1) mixing
- 2) waiting
- 3) working
- 4) curing

The PMMA-bone cements which are currently available on the global market differ notably in regard to these processing characteristics. Blending of the polymer with the monomer can be done manually or with the help of a vacuum mixing system. The “working” stage describes the stage at which the cement forms a dough that is still easily pliable but will hold a shape it is given. Only during this step can the surgeon mould and then position the cement. As such, the duration of this stage represents the limiting factor in the manipulation of bone cement.

The various commercially available products come with a wide array of different additives. Often, compounds that make the cement radio-opaque and thereby visible on x-ray images are worked into the polymer powder. In the case of Palacos®R+G, chlorophyll was added, which has the effect of staining the cement a light green colour (47). As a consequence, the cement is more easily discernible from other structures (such as bone) at the surgical site. Catalysts and stabilizing agents are also commonly found additives.

1.7.1 Antibiotic loaded bone cement

It was Buchholz and Engelbrecht (48) who first published their ideas of integrating antibiotics into acrylic bone cement. PMMA that contains an antimicrobial substance is often referred to as an “antibiotic-loaded” bone cement. The idea was, that the soluble antibiotic contained in the cement detaches when coming into

contact with the synovial fluid. The antibiotic from the surface is then slowly leached out of the cement and eluted into the joint space where it can take full effect. The release of the antibiotic out of the cement follows the law of diffusion. The increase in the porosity allows the fluid to penetrate deeper into the cement to release yet more of the antibiotic.

One advantage when using this particular mode of transportation to deliver the antibiotic into the joint is that even though local concentrations are high, the systemic levels of the substance stay low (49–51).

Factors that influence the mechanical stability of the cement are numerous and varied. Some of the most commonly referenced are (52,53):

- The amount of antibiotic in the cement
- Mixing technique
- Density of the cement

Factors that can impact the elution rate of the antibiotic from the cement include (51):

- pH-value
- Porosity of the cement
- Biochemical structure and related properties

Antibiotics with a high solubility in water such as fosfomycin (54) offer the extra benefit of being able to reach and penetrate the surrounding tissues after their release into the joint space (55). Some antibiotics like the carbapenem-progenitor thienamycin (30) or disodium fosfomycin (56) are susceptible to hydrolysis, in particular under either alkaline or acidic conditions.

1.7.2 Clinical applications

Acrylic bone cement can be used to aid the fixation of joint prostheses. There it assumes the role of a “grout”, connecting the prosthesis to the bone. This ensures a reliable anchoring and a balanced transfer of load from the foreign body to the bone.

Cements that are impregnated with antimicrobials play an important role in arthroplasty. In fact, their use has evolved into a standard of care procedure for the

eradication of periprosthetic infection (28). DePuy-JJ, Stryker, Zimmer-Biomet and Heraeus Medical are some of the manufacturers that offer pre-blended ALABCs.

It is also possible to manually add antibiotic powders to the cement. Neut et al. (57) observed, that the antibiotic-release from these hand-mixed cements was inferior to their industrially pre-loaded counterparts. Nelson et al. (58) came to the same conclusion. However, the selection of antibiotics which are available as pre-loaded ALABCs is very limited indeed. In fact, commercially available impregnated cements use only six different antibiotics (59). Out of these, gentamicin is the most frequently added (60). As an aminoglycoside it is effective against a broad spectrum of gram-positive and gram-negative organisms. Additionally, it dissolves well in water and is fairly resistant to heat (61).

The quantity of antibiotic released from the spacer is linked to the amount contained within the cement (62). Interestingly, investigations by Kühn (63) found that a high amount of antibiotic in the cement did not always correspond to a high release. Rather, the cement matrix and related physical properties like hydrophilicity were crucial factors in determining antibiotic release. When manually admixing antibiotic powders, it is generally advisable that the amount of antibiotic added to any cement does not exceed 10% of the total pulverulent mass (64) to prevent a negative impact on mechanical stability.

1.7.3 Meropenem, Imipenem and Fosfomycin in PMMA

Only a scant number of papers pertaining to the use of carbapenems and fosfomycin in combination with acrylic bone cements are currently available.

Meropenem

A survey of scientific articles relating to the use of meropenem in PMMA bone cements showed that it was rarely evaluated alone and instead was often combined with vancomycin for research purposes (65–67).

Kristofer et al. (68) found that meropenem at 5% and 10% concentrations (2g and 4g in 40g of cement) demonstrated high antimicrobial activity against strains of *Klebsiella pneumoniae*, vancomycin-resistant *Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus*. An investigation by Samuel et al. (69) using the same antibiotic-to-cement ratio seconded these findings and added, that

meropenem eluates were active against *E.coli* and *P. aeruginosa* for a duration of three weeks. A study by Gálvez-López (70) using cement combined with 10% and 20% of meropenem, demonstrated an elution above the minimal inhibitory concentration for susceptible organisms for a duration of 30 days.

Imipenem

Chang et al. (71) observed, that the compressive strength of Surgical Simplex bone cement dramatically decreased when impregnated with 1g of imipenem (Merck Sharp and Dohme Ltd.) per 40g of cement. Additionally, they reported minimal bioactivity against *MSSA* and a complete lack of activity against *MRSA*.

A study by Bowyer et al. (72) which compared the elution of various antimicrobials from Plaster of Paris and Simplex P. (Stryker), found that the percentual release of imipenem (Merck Sharp and Dohme Ltd.) from the PMMA cement was inferior to that of the other five antibiotics. This may be attributed to the fact, that all other beads were loaded with triple the amount of antibiotic when compared to imipenem. A reasoning for this approach was not given.

Fosfomicin

Yuenyongviwat et al. (54) examined the bioactivity of fosfomicin. They added 4g of vancomycin hydrochloride (Vancogen, Alkem Laboratories, Daman, India) to 40 grams of Palacos[®]R (Heraeus Kulzer GmbH) and tested the ALABC against *MRSA*- strains obtained from tissue samples. They noted that the efficacy of fosfomicin against *MRSA* ceased after only three days. Conversely, experiments by Roth et al. (51) identified fosfomicin in combination with Palacos[®]R+G as the most effective agent against *MSSA* and *MRSA* (ATCC 33591).

Eick et al. (73) reported that fosfomicin in PMMA cement resulted in growth-inhibition for strains of *MSSA*, *S. epidermidis* and *E. coli*. However, although they mention that the cement was provided by Heraeus, it is unclear which of their products was used (i.e. Palacos[®]R, Palacos[®]R+G or Copal[®]G+C). Furthermore, although the authors disclose that the fosfomicin was manufactured by Sigma-Aldrich Chemie GmbH, it remains unclear which of their formulations was used (i.e. fosfomicin sodium / disodium / calcium / tromethamine).

1.8 Research Focus

Over the years, the emergence of multi-drug resistant microbes has steadily increased. The discovery of new antibiotics is a rare occurrence. Therefore, optimizing the dosage and delivery of well-known antimicrobials with proven efficacy and safety is of great importance.

In the case of periprosthetic infections, PMMA bone cement represents an interesting medium for the delivery of antibiotics directly into the affected joint.

When choosing to use a spacer made of antibiotic loaded bone cement, resistance to mechanical stress and adequate antibiotic elution from the implant are paramount to ensure an optimum outcome.

The aim of our study was therefore, to compare the in vitro efficacy of the two carbapenem antibiotics meropenem and imipenem, as well as two different fosfomycin salts, when combined with three widely used PMMA bone cements. *E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Staphylococcus aureus* were the chosen adversaries, all of which are pathogens of significant clinical relevance. Moreover, the mechanical stability of the resulting antibiotic loaded cements was investigated according to international standards.

2 Material and Methods

2.1 Overview (see Appendix for details)

a. Bone cements:

Palacos®R 40

Palacos®R+G 40

Copal®G+C 40

b. Tested bacterial strains

Escherichia coli - Clinical strain

Staphylococcus aureus ATCC® 43300™

Pseudomonas aeruginosa ATCC® 27853™

Proteus mirabilis ATCC® 12453™

c. Antibiotics

Meropenem, vial, Dr. Friedrich Eberth Arzneimittel GmbH®

Imipenem /Cilastatin, vial, 500mg/500mg, Fresenius Kabi GmbH®

Fosfomycin Trometamol, Zach System®

Calcium Fosfomycin, Ercros®

d. Chart of Test Specimens and Test Series

Antibiotic Doses	Palacos®R		Palacos®R+G		Copal®G+C	
1 x Imipenem		MB	M	MB	M	MB
2 x Imipenem						
1 x Meropenem			M	MB	M	MB
2 x Meropenem		MB	M	MB	M	MB
4 x Meropenem				MB		
1 x Fosfomycin Trometamol				MB		
2 x Fosfomycin Trometamol	M	MB	M	MB	M	MB
4 x Fosfomycin Trometamol				MB		
1 x Calcium Fosfomycin				MB		
2 x Calcium Fosfomycin	M	MB	M	MB	M	MB
4 x Calcium Fosfomycin				MB		

Table 2: Overview of produced cement bodies and executed testing. "M" indicating mechanical testing and "MB" indicating completion of microbiological activity assays

2.2 Antimicrobials and PMMA

Three different types of PMMA bone cement (Table 2) were investigated in this study (provided by Heraeus Medical GmbH, Wehrheim, Germany). Two high viscosity bone cements (Palacos®R and Palacos®R+G, the former a plain cement without antibiotic additives and the latter containing 0,5g of gentamycin) as well as Copal®G+C (containing 1g of gentamycin and 1g of clindamycin) were used.

Meropenem (1g vial, Dr. Friedrich Eberth Arzneimittel GmbH, Ursensollen, Germany), imipenem (Fresenius Kabi Germany GmbH, Bad Homburg, Germany) as well as fosfomycin trometamol (ZaCh System SpA, Almisano di Lonigo, Italy) and calcium fosfomycin (Ercros Industrial S.A., Madrid, Spain) were commercially purchased. All four of the antibiotics were applied in powdered form.

From heron forward, “1g” of the below listed substances is understood to stand for:

- Meropenem: 1000mg meropenem (active ingredient) and 208mg sodium carbonate
- Imipenem: 500mg imipenem (active substance) and 500mg cilastatin (enzyme inhibitor), together with 1,6 mEq sodium bicarbonate
- Fosfomycin trometamol: 1g (measured out) of the industrial blend
- Calcium fosfomycin: 1g (measured out) of the industrial blend

Meropenem and imipenem are carbapenem antibiotics that are active against gram-positive and gram-negative bacteria (65). Fosfomycin is an epoxide antibiotic and most commonly used to treat infections with *Staphylococcus aureus* (27). For orthopaedic purposes, all four of these are considered reserve antibiotics.

2.3 Bacterial strains

Most of the strains we used for testing were obtained via the “American Type Culture Collection” (ATCC), a non-profit institution committed to the acquisition, registration and distribution of biologic material for scientific and research purposes.

With the exception of *Staph. aureus* all strains were susceptible to gentamicin and clindamycin.

2.3.1 Pseudomonas aeruginosa ATCC 27853

Pseudomonas aeruginosa Migula is a gram-negative, rod-shaped bacterium, showing ideal growth under aerobic conditions. In humans it mostly acts as an opportunistic pathogen as it commonly affects immunocompromised patients.

2.3.2 Proteus mirabilis ATCC 12453

The German microbiologist Gustav Hauser was the eponym for this species of bacteria (74). *Proteus mirabilis* Hauser is a gram-negative, motile bacterium in rod shape that best grows at 37°C in an aerobic atmosphere.

2.3.3 Staphylococcus aureus ATCC 43300

Staphylococcus aureus Rosenbach is a ball-shaped, gram-positive bacterium and constitutes an *MRSA* (Methicillin-resistant *Staph. Aureus*). This strain is likewise resistant to gentamycin and clindamycin as well as oxacillin. The cocci typically form clusters resembling grapes.

2.3.4 Escherichia coli (clinical strain)

E. coli is a facultatively anaerobic gram-negative bacterium. It is generally considered “facultative pathogenic”, as it is a regular part of the intestinal microflora in humans. However, if it is displaced from its customary habitat it may realize its potential as a pathogen, causing, for instance, infections of the urinary tract or the meninges (75).

2.4 Production of cement moulds

In a single-use plastic cup the appropriate amount of the pulverulent antibiotic (as indicated in Table 2) was added to the contents of a single bag of the respective cement powder. For meropenem and imipenem it was postulated that the content of a single vial corresponds to 1g of antibiotic. The desired amounts of fosfomycin trometamol and calcium fosfomycin were measured utilizing a precision scale (FRANK PTI GmbH®, Germany).

The cement powder and antibiotic powder were blended using a metal spatula. The two components were constantly stirred for 3 minutes to ensure even

dispersion of the antibiotic in the cement. All subsequent steps were completed according to the manufacturer's recommendations

In a separate cup the liquid monomer was measured out. The powder was added to the liquid to prevent the formation of powder nests. The components were then manually stirred for 30 seconds until a homogenous doughy compound formed, which was subsequently pressed into metal moulds.

The experimental design required specimens in four different shapes for each ALABC respectively. The compression test required cylinders (height: 12mm and diameter: 6mm). The four-point bending test called for rectangular planks (width: 10.00-10.10mm, height: 3.20-3.40mm and length: 75.00 mm). The Dynstat impact test demanded small rectangular plates (width: 10.00-10.10mm, height: 3.20-3.40mm and length: 12mm). The discs for the microbiological activity tests had a diameter of 25,00mm and a height of 9,90mm.

The ALABCs were put under a hydraulic press (Figure 6 Table 6). Three tonnes of pressure were applied for 30 minutes while they cement cured. The cement-bodies for mechanical testing were transferred into cups and stored in a standard climate at 23°C ($\pm 2^\circ\text{C}$) and 50% humidity ($\pm 3\%$) for 24h while the specimens for microbiological activity testing were put into sterile, air-tight plastic bags until testing resumed. Any specimens that showed signs of damage (e.g. cracks or macroscopic air pockets) in a first visual assessment were immediately eliminated.



Figure 6: hydraulic press

2.5 Mechanical tests

To assess the effect of the admixed AB on the mechanical properties of the cements, different characteristic parameters were determined. Mechanical testing was performed 24 hours after mould-preparation. Compressive strength, flexural strength and flexural modulus were determined according to ISO 5833:2002 standards. Impact strength was investigated according to DIN 53435. All mechanical tests were performed in air at environmental temperature. For the flexural and impact tests we used *Frank PTI Quality Testing Instruments*[®] while a *Zwick Roell*[®] materials testing machine was used for the compressive tests.

2.5.1 Impact Strength

The test for impact strength was conducted with the Dynstat Configuration and according to DIN 53435 specifications. As is the case with similar impact tests, the idea was to evaluate the ability of the materials to withstand sudden impact. For this test, the swing hammer was adjusted to swing with an energy of 0,5J and speed of 2,2m/s on impact, with the cement specimens placed into a mount and their front facing the pendulum.

Cement bodies that showed no signs of damage or flaws on visual inspection were measured with a digital calliper gauge and placed upright into a fitted groove on the machine. Since bone cement can be considered a brittle material, we forwent notching the specimens. For each cement preparation, eight test replicas were performed.

In this configuration (Figure 7) the pendulum swings with a pre-determined energy towards the cement platelet and beyond it, thereby shifting an indicator along a scale. With the help of this scale it is possible to read off how much of the energy was absorbed by the tested material. This also means, that this test requires the specimen to break in order to receive valid data. Furthermore, it is necessary to convert the results indicated on the scale from Joules to the actual impact strength [kJ/m²].

$$a_n = 1000 * \frac{A_N}{B * T}$$

A_N ... impact in J
 a_n ... Dynstat impact strength
 B ... breadth in mm
 T ... thickness in mm

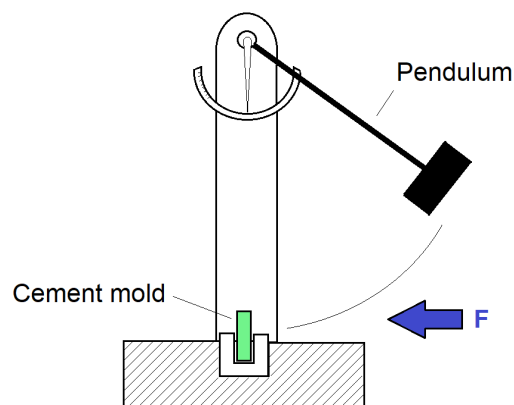


Figure 7: Pendulum Swing, Dynstat configuration for impact strength test

2.5.2 Compressive Strength

Compressive strength is an indicator of a material's ability to withstand axial compression. The cylinders for the compressive test were 12mm high and had a diameter of 6mm.

For this experiment, the testing machine lowered a metal plate towards the upright test-cylinder, gradually applying a load upon it (see Figure 8). The machine calculated and recorded the amount of stress that the cement was able to withstand. The maximum compressive strength was measured at the point at which failure in the form of fracture of the specimen occurred. Measurements were repeated seven times for each cement preparation.

Compressive strength is expressed with "Pascal" (Pa). Pascal is a dimensional unit which is defined as "force per area".

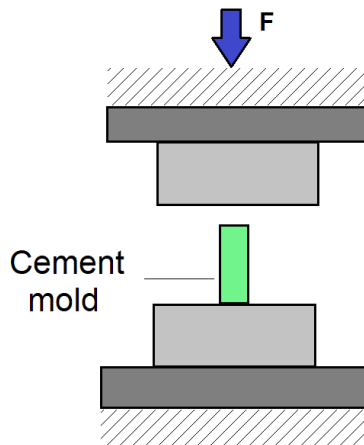


Figure 8: ISO configuration for compressive strength test. F...force.

2.5.3 Bending Tests

The flexural modulus and flexural strength correlate to the amount of weight and stress a material can bear while remaining structurally sound. Both are expressed in psi (MPa).

The setup of the 4-point bending test (Figure 9) according to ISO 5833 called for plates of 75mm length, 10mm width and 3,3mm thickness. The pre-load was set to two Newton. The specimens rest on two supports. Via two loading pins mounted on a beam, a load is applied at a constant rate.

Both, load and resulting displacement in the tested material, are recorded. Calculation of the bending strength happened at the same time as that of the bending modulus. For each antibiotic-loaded cement measurements were done in triplicate. Documentation and analysis of the results was carried out with the help of the testXpert®II software.

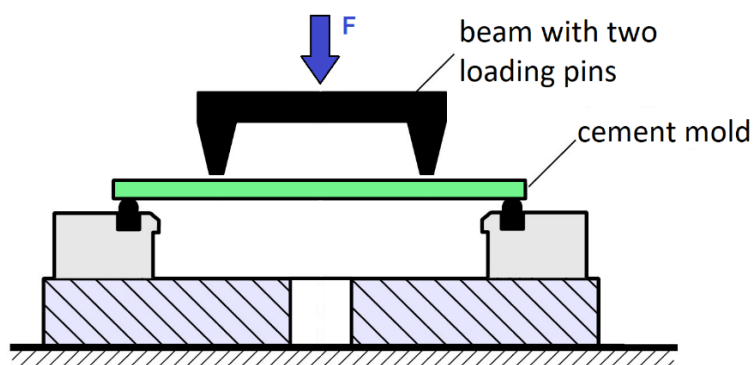


Figure 9: ISO configuration for bending tests

2.6 Antibacterial Activity Tests

The antibacterial activity tests called for ALABC-cylinders with a diameter of 25mm and a height of 9,0 mm. Each cylinder (three specimens per ALABC-mixture) was put in a sterile plastic falcon® tube containing 20ml of autoclaved PBS (prepared using amresco® Phosphate buffered saline tablets) and submerged therein. The filled vessels were placed upside down to ensure continuous complete immersion and stored at room temperature (Figure 10).



Figure 10: Filled falcons®

At specified points in time (1h, 24h, 48h, 7d, 14d, 28d, 42d), an aliquot of the eluate was removed from the vials for further testing, while the cylinders were removed from the tubes, patted dry with sterile swabs and transferred into new tubes containing fresh PBS. The thereby gathered eluate-samples served as the basis for the bio-activity tests.

The agar plates needed for the test were prepared on-site. The sterile petri dishes were commercially purchased. Müller-Hinton Agar was prepared according to the manufacturer's specifications (OXOID®) and autoclaved for 15 minutes at a temperature of 121°C. For each dish, 20ml of the liquid agar were poured using a

sterile glass pipette which was then left to set and dry. The finished plates were either used immediately or kept at 4°C until further use.

As mentioned in section 2.3, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis* were used for in-vitro antibacterial activity testing. Several colonies from a pre-prepared bacterial culture were gathered with the help of an inoculating loop and inserted into an Eppendorf tube filled with distilled water. The aim was to achieve a suspension with a visual turbidity equal to a 0,5 McFarland standard (corresponding to a bacterial concentration of 105 colony-forming units [CFU/ml]).

Using a sterile cotton swab the bacterial suspension was then spread across petri dishes holding Mueller Hinton agar to obtain a confluent lawn. Afterwards, wells measuring 6mm in diameter were punched into the agar. These wells were subsequently filled with 50µl of the eluates up for testing. The petri dishes were each sealed with a strip of Bemis® Parafilm® and then incubated at 37°C for 24h under aerobic conditions. Evaluation of the inhibition zones was performed after incubation was completed. The diameter of the zone of no bacterial growth was measured with a ruler and rounded to the nearest millimetre (the diameter was recorded as the mean of 2 measurements perpendicular to each other and included the diameters of the wells).

2.7 Statistical Analysis

The statistical analysis was done with the help of IBM SPSS Statistics v.25. As a first step we checked for variance homogeneity between the groups, followed by univariate ANOVA testing (Analysis of Variance) to check whether there was a significant variance between the means of the test groups and the reference.

For detailed post-hoc analysis of the mechanical test results we chose to use Dunnett's test to compare the pairwise differences of sample groups and their respective controls, since this method is already considering as well as correcting for the multiplicity error that arises with multiple comparisons. For the evaluation of microbiological activity, we decided to focus on descriptive statistical analysis as there was only a sample size of 3 available for each ALABC group.

3 Results

3.1 Mixing and Visual Assessment

Even though all antibiotics were added in their pulverulent form, some of them more readily blended with the cement powder than others.

Those ALABS containing fosfomicin showed a tendency to form small clumps during the dry ad-mixing with the cement powder. However, once the liquid monomer was added, visually discernible differences between doughs containing fosfomicin and those doughs containing carbapenems were minimal (Figure 11).

All hardened and set cement specimens were visually inspected before undergoing testing. Any sign of inhomogeneity in the material (cracks, air pockets, clumps of AB etc.) was considered an indication that the components were not uniformly distributed and consequently resulted in the exclusion of the pertaining samples.

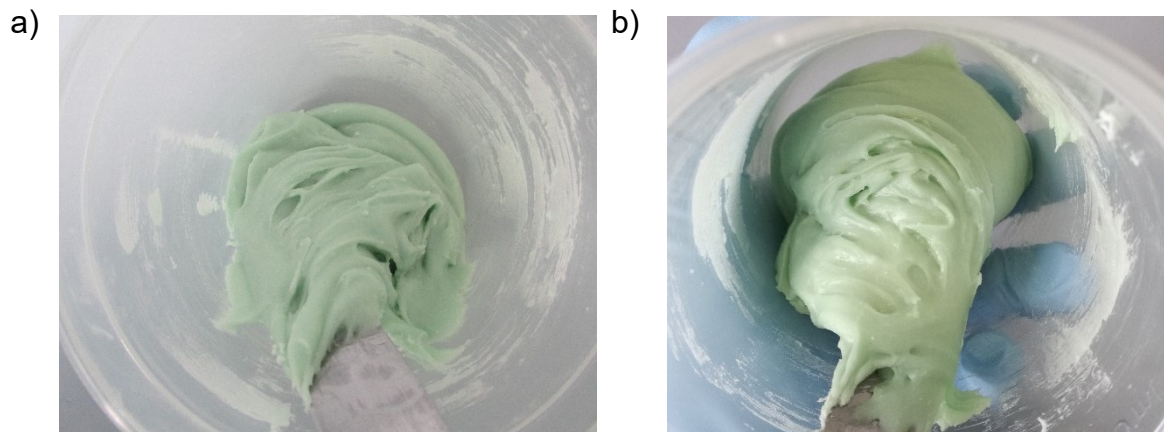


Figure 11: Comparison of dough phase. a: Palacos®R+G with 2g of fosfomicin trometamol. b: Palacos®R+G with 2g of imipenem/cilastatin.

3.2 DIN Impact Strength

The DIN 53435 does not stipulate a threshold value for the Dynstat impact strength test. For our experiments, we proposed that even with the introduction of additional pulverulent components to the cements, a decrease in impact strength of no more than 20% when compared to the respective reference was desirable.

In the group which used Palacos®R as the cement base (Figure 12), the addition of the fosfomicin salts resulted in a substantial drop in impact strength.

While the mean value for the ALABC containing 2g of fosfomycin trometamol decreased by 34% relative to the reference, that of the ALABC containing the same amount of calcium fosfomycin decreased by 47%. Both fosfomycin salts therefore undercut the 80% level of 3,37 [kJ/m²].

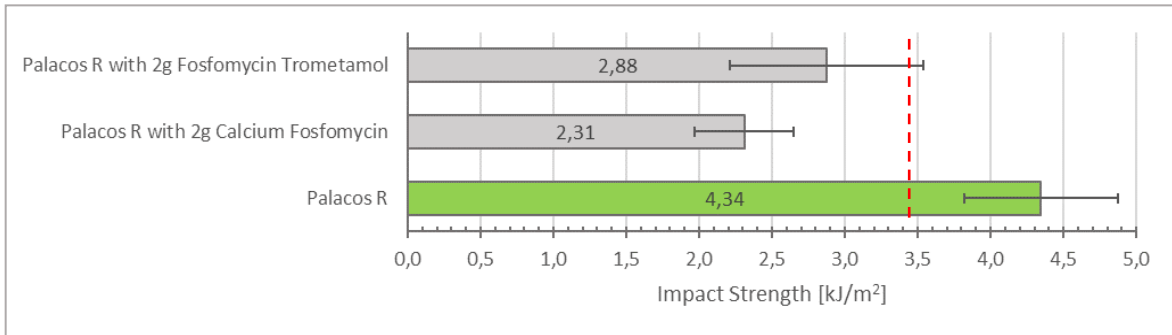


Figure 12: Impact Strength Palacos®R Group (red line indicates 80% benchmark). Green colour marks reference.

In the Copal®G+C-group (Figure 13) the addition of 1g of meropenem interestingly resulted in an infinitesimal increase of impact strength by 2% in comparison to the pure reference cement. The addition of 2g of the same substance resulted in a decrease of impact strength by 13%. Nonetheless, with the notable exception of calcium fosfomycin, which caused the impact strength to reduce by 41%, all ALABCs stayed within the 80% limit (=2,1 kJ/m²).

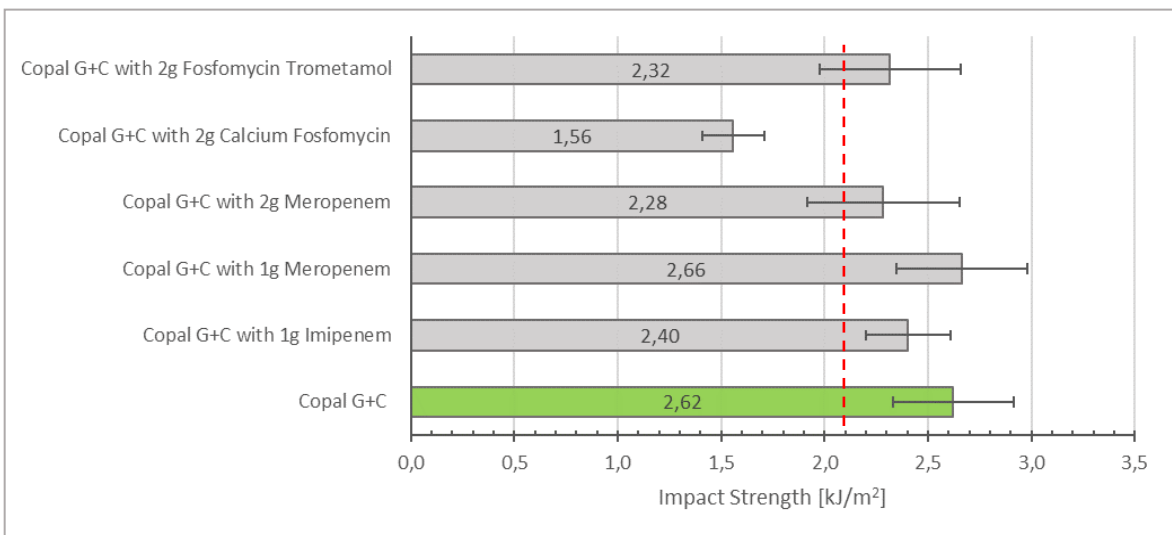


Figure 13: Impact Strength Copal®G+C Group (red line indicates 80% benchmark). Green colour marks reference.

Mixing Palacos®R+G with 1g meropenem lead to a 9% decrease in impact strength, while 2g of meropenem caused a further 9% decline (Figure 14). Imipenem prompted a 24% loss of impact resistance while 2g of calcium fosfomycin resulted in a more noticeable drop of 51%. These last two samples also fell short of the pertinent benchmark value of 2,7 kJ/m².

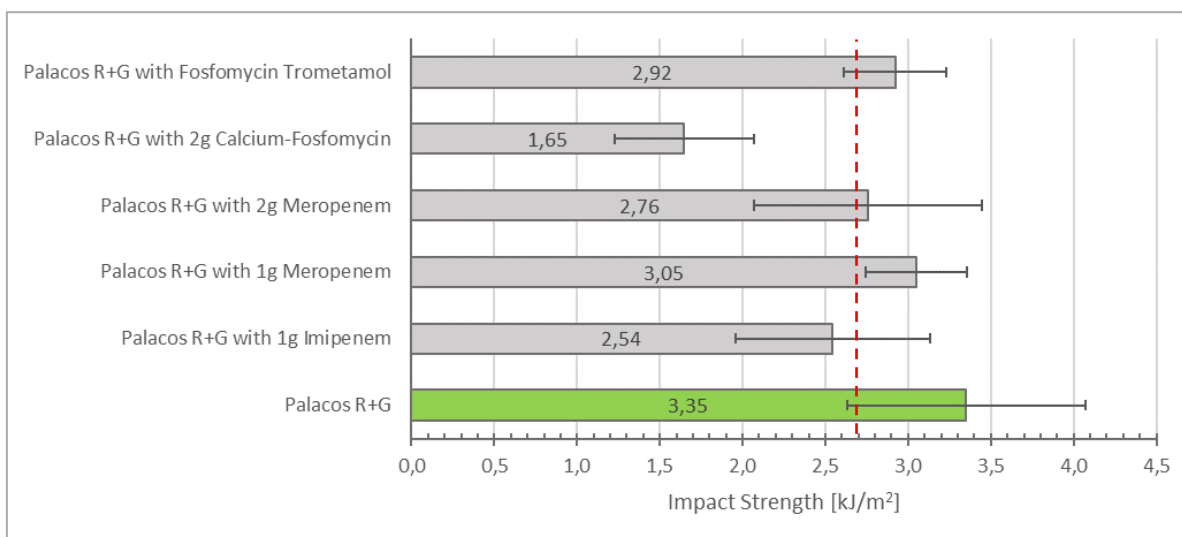


Figure 14: Impact Strength Palacos®R+G (red line indicates 80% benchmark). Green colour marks reference.

3.3 ISO Compressive Strength

To determine the compressive strength of the various ALABCs, seven specimens of each mix underwent testing. The experiment was conducted according to the ISO 5833 guideline, which also specifies a minimum value of 70 MPa for this test.

The results of the t-test are depicted in the right-most column in the tables shown below, with p-values of 0,05 or lower indicating a significant difference between the test group and the corresponding reference.

As can be seen in Table 3, neither the addition of calcium fosfomycin nor fosfomycin trometamol to Palacos®R had a noticeable effect on the target variable.

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Palacos®R reference	83,60	1,66	81,96	86,19	-	REF
Palacos®R + 2g ca-fos	83,63	2,05	80,45	85,76	0,0	0,999
Palacos®R + 2g fos-trom.	83,39	1,28	81,84	85,59	0,0	0,962

Table 3: Compressive strength Palacos®R Group; ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

In contrast to the previous group, Palacos®R+G was more noticeably affected by the addition of the antibiotic powders. The 2-sided Dunnett-T-test found that all supplements except for the single gram of meropenem had a statistically significant negative effect on mean values. However, attention should be paid to the fact that in the Palacos®R+G group even the largest decrease in compressive strength, albeit statistically significant, constitutes a percentual change of only 5,7% (Table 4).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Palacos®RG reference	86,54	2,24	83,81	90,01	-	REF
Palacos®RG + 1g imi	81,64	2,56	77,35	85,11	5,7	0,006
Palacos®RG + 1g mero	84,86	2,70	79,23	86,80	1,9	0,651
Palacos®RG + 2g mero	82,79	3,86	78,15	89,71	4,3	0,049
Palacos®RG + 2g ca-fos.	82,12	2,66	78,05	85,08	5,1	0,016
Palacos®RG + 2g fos-trom.	81,93	1,29	80,32	84,40	5,3	0,011

Table 4: Compressive strength Palacos®R+G group. Significant t-test results highlighted with yellow. Ca-fos.: calcium fosfomicin; fos-trom.: fosfomicin trometamol

Three ALABCs in the Copal®G+C group showed a significant decrease in compressive strength. By adding a single gram of imipenem, 2g of fosfomicin trometamol or 2g of meropenem, mean values dropped by 4%, 4,2% and 5,3% respectively, as compared to the reference (Table 5).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Copal®GC reference	82,16	0,93	81,08	83,41	-	REF
Copal®GC + 1g imi	78,89	1,86	76,29	80,90	4	0,009
Copal®GC + 1g mero	82,57	1,98	79,82	85,10	0,5	0,992
Copal®GC + 2g mero	77,79	3,16	73,87	81,92	5,3	0,000
Copal®GC + 2g ca-fos.	81,80	1,06	80,04	82,84	0,4	0,995
Copal®GC + 2g fos-trom.	78,71	0,89	77,30	80,09	4,2	0,005

Table 5: Compressive strength Copal®G+C Group. Significant t-test results highlighted with yellow. Ca-fos.: calcium fosfomicin; fos-trom.: fosfomicin trometamol

In summary this test found that cements containing either 1g imipenem, 2g meropenem or 2g fosfomicin Trometamol had a significantly lower impact strength than their corresponding references. Nevertheless, all specimens achieved values far superior to the minimum of 70 MPa required by the International Organization for Standardization as illustrated in Figure 15.

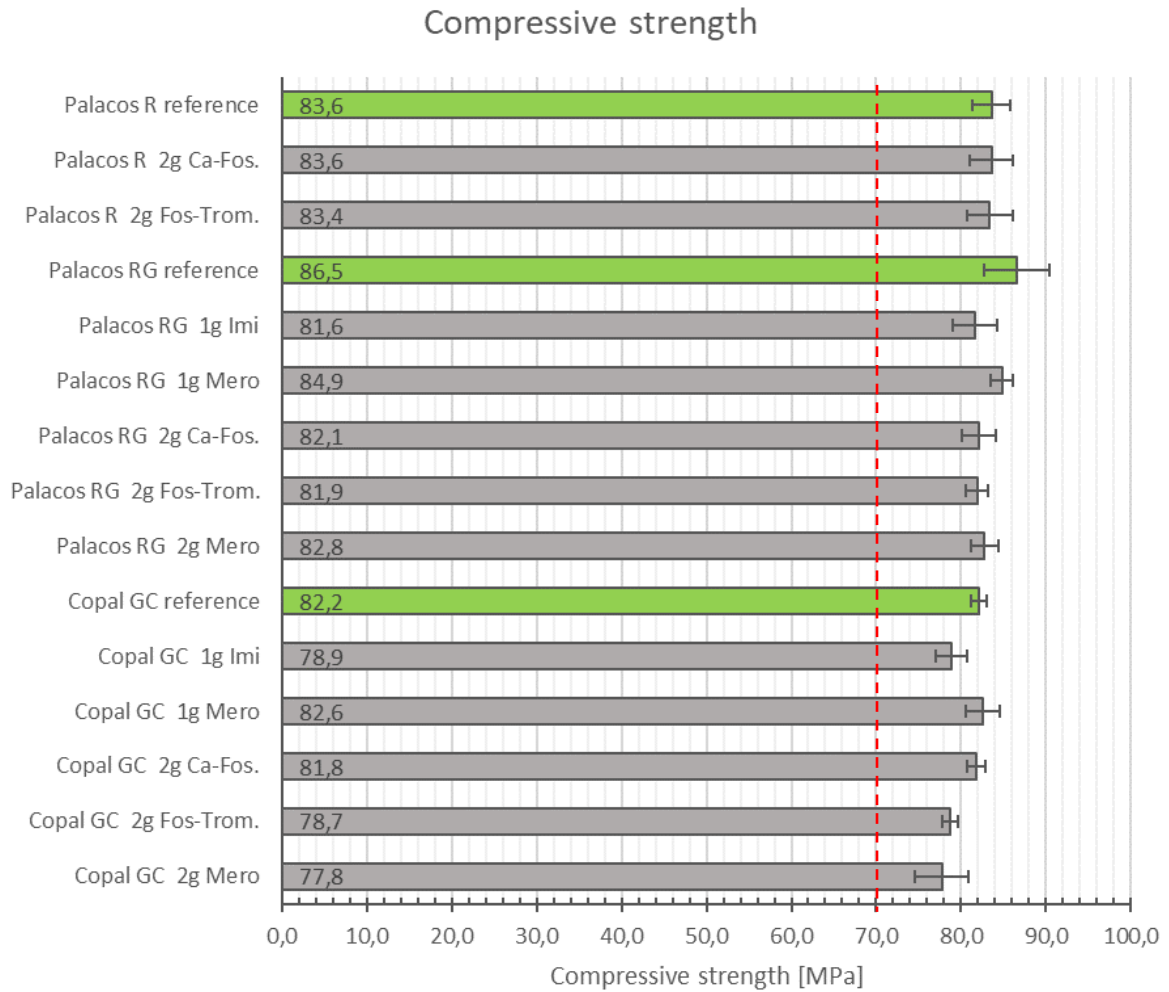


Figure 15: Compressive Strength. Red line represents ISO limit of 70 MPa. References are highlighted in green. Ca-Fos.: calcium fosfomicin; Fos-Trom.: fosfomicin trometamol

3.4 ISO Bending Modulus

The International Organization for Standardization requires acrylic bone cements to have a bending modulus of at least 1800 MPa.

In this experiment, Palacos®R when combined with calcium fosfomycin, attained significantly higher values than the unadulterated cement. Palacos®R containing fosfomycin trometamol on the other hand barely differed from the reference (Table 6).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Palacos®R reference	2827,67	136,841	2673	2933	-	REF
Palacos®R + 2g ca-fos	3042,00	44,508	2997	3086	+7,5	0,039
Palacos®R + 2g fos-trom.	2801,33	36,254	2777	2843	0,9	0,904

Table 6: Bending modulus Palacos®R. Significant t-test results highlighted with yellow. Ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

As shown in Table 7 the bending modulus of Palacos®R+G was significantly affected by the addition of 2g fosfomycin trometamol and 1g meropenem, which lowered the mean by 6,2 % and 12,2% respectively. Interestingly, the mean value of the ALABC containing 2g meropenem differed little from the reference. Overall the mean values in this group remained far above the required 1800 MPa mark.

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Palacos®RG reference	3106,33	64,508	3047	3175	-	REF
Palacos®RG + 1g imi	3011,00	33,867	2984	3049	3,1	0,236
Palacos®RG + 1g mero	2729,00	31,000	2705	2764	12,2	0,000
Palacos®RG + 2g mero	3014,33	68,792	2958	3091	3	0,262
Palacos®RG + 2g ca-fos.	3085,00	25,632	3061	3112	0,7	0,988
Palacos®RG + 2g fos-trom.	2914,67	96,862	2803	2976	6,2	0,008

Table 7: Bending modulus Palacos®R+G. Significant t-test results highlighted with yellow. Ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

The bending modulus of Copal®G+C was scarcely affected by the addition of the various antibiotics as can be seen in the tale below. The Dunnnett-t-test revealed

that only the ALABC containing 2g of fosfomycin trometamol had a significantly different mean to that of the reference (Table 8).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Copal [®] GC reference	3078,67	30,039	3044	3097	-	REF
Copal [®] GC + 1g imi	3083,33	32,470	3046	3105	+0,2	1,000
Copal [®] GC + 1g mero	2998,00	38,936	2968	3042	2,6	0,274
Copal [®] GC + 2g mero	3058,67	68,413	2981	3110	0,7	0,986
Copal [®] GC + 2g ca-fos.	3080,00	13,000	3067	3093	0,0	1,000
Copal [®] GC + 2g fos-trom.	2881,33	91,577	2825	2987	6,4	0,003

Table 8: Bending modulus Copal[®]G+C. Significant t-test results highlighted with yellow. Ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

3.5 ISO Bending Strength

Regarding the bending strength the ISO 5833 guideline specifies a minimum threshold of 50 MPa. In the Palacos[®]R group, admixing 2g of calcium fosfomycin caused the mean value to drop by 24,9%. The same amount of fosfomycin trometamol on the other hand only resulted in a 5% decline (Table 9).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Palacos [®] R reference	71,47	2,07	69,1	72,9	-	REF
Palacos [®] R + 2g ca-fos	53,70	0,52	53,4	54,3	24,9	0,000
Palacos [®] R + 2g fos-trom.	67,80	2,35	65,5	70,2	5,1	0,085

Table 9: Bending strength Palacos[®]R. Yellow highlights if t-test significant, red if for values below ISO limit. Ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

The arithmetic means for samples containing Meropenem and calcium fosfomycin was significantly lower than that of the pure Palacos[®]R+G cement. Adding 1g and 2g of meropenem provoked a 11,1% and 9,5% decrease respectively. The detrimental effect of calcium fosfomycin was even higher, amounting to a 40,8% drop in bending strength (Table 10).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Palacos®RG reference	71,13	1,74	69,8	73,1	-	REF
Palacos®RG + 1g imi	69,30	1,84	67,2	70,6	2,6	0,738
Palacos®RG + 1g mero	63,23	1,54	62,2	65,0	11,1	0,003
Palacos®RG + 2g mero	64,37	2,42	61,8	66,6	9,5	0,008
Palacos®RG + 2g ca-fos.	42,13	1,65	40,5	43,8	40,8	0,000
Palacos®RG + 2g fos-trom.	67,10	3,11	63,7	69,8	5,7	0,133

Table 10: Bending strength Palacos®R+G. Yellow highlights if t-test significant, red if for values below ISO limit. Ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

As with the two previous groups, we found that calcium fosfomycin had the most notable effect. Compared to the Copal®G+C reference, it caused a 36,1% drop. Adding 2g of meropenem led to a milder, yet significant 12,6% decrease (Table 11).

Group	Mean	Standard deviation	Min.	Max.	Change in %	t-test*
Copal®GC Reference	69,07	1,56	67,6	70,7	-	REF
Copal®GC + 1g lmi	67,47	2,25	64,9	69,1	2,3	0,890
Copal®GC + 1g Mero	65,10	1,25	63,7	66,1	5,8	0,243
Copal®GC + 2g Mero	60,37	4,06	55,7	63,1	12,6	0,004
Copal®GC + 2g Ca-Fos.	44,13	2,83	41,0	46,5	36,1	0,000
Copal®GC + 2g Fos-Trom.	63,77	1,89	61,6	65,1	7,7	0,083

Table 11: Bending strength Copal®G+C. Yellow highlights if t-test significant, red if for values below ISO limit. Ca-fos.: calcium fosfomycin; fos-trom.: fosfomycin trometamol

As demonstrated in Table 10 and Table 11, supplementing 2g of calcium fosfomycin had a deleterious effect on the mean bending strength of Palacos®R+G and Copal®G+C with mean values dropping below the ISO threshold.

3.6 Summary of Mechanical Test Results

Fosfomycin salts: The Dynstat impact strengths of all ALABCs containing fosfomycin trometamol were considerably higher than those of cements containing equal amounts of calcium fosfomycin. In fact, the impact strength of Palacos®R, Palacos®R+G and Copal®G+C when impregnated with 2g of calcium fosfomycin was reduced by 47%, 51% and 41% respectively, as compared to the controls.

The ISO compressive strength of the cements was not significantly altered by the addition of 2g of either fosfomycin trometamol or calcium fosfomycin. While the addition of 2g of the fosfomycin salts to Palacos®R had no discernible effect on the compressive strength at all, the values achieved when added to Palacos®R+G or Copal®G+C were only minimally lower as compared to the respective references.

The ISO bending modulus was only minimally affected by the addition of the fosfomycin salts. Overall, cements with 2g of calcium fosfomycin achieved slightly higher values than cements with 2g fosfomycin trometamol. Palacos®R containing 2g calcium fosfomycin even demonstrated a 7% higher bending modulus than the respective reference.

The bending strengths of the cements loaded with 2g fosfomycin trometamol were similar to the references. However, 2g calcium fosfomycin caused a drastic decrease of bending strength in all cements.

Carbapenems: Dynstat impact strength of Copal®G+C combined with 1g meropenem was slightly higher than that of Copal® mixed with 1g imipenem. The addition of 2g meropenem had a similar effect on impact strength as the addition of 1g imipenem. Dynstat impact strength for Palacos®R+G when impregnated with 1g meropenem was distinctly higher than when combined with 1g imipenem. The results of the cement containing 2g meropenem also surpassed those achieved by the 1g imipenem mix.

Compressive strength was only minimally affected by the addition of the carbapenems to the cements. Admixing either 1g imipenem, 1g meropenem or 2g meropenem to Palacos®R+G and Copal®G+C, resulted in a decrease in compressive strength of less than 6% as compared to the controls.

The bending modulus for Copal®G+C remained practically unchanged when loaded with either 1g imipenem, 1g meropenem or 2g meropenem. In Palacos®R+G

group, 2g of meropenem and 1g imipenem achieved values similar to the reference, while 1g meropenem comparatively had the lowest bending modulus.

The bending strengths of Palacos®R+G and Copal®G+C loaded with 1g imipenem closely resembled those of the references. The bending strengths of Palacos®R+G containing 1g and 2g of meropenem were nearly identical, while the addition of 2g meropenem to Copal®G+C comparatively achieved the lowest values.

3.7 Microbiological Tests

As stated before, the microbiological efficacy of the ALABCs against specified pathogens was tested with an agar diffusion assay. The diameters of the inhibition zones were measured and recorded. All tests were done in triplicate. All results are given in mean values. For easier visual appraisal they were color-coded. Green represents a large (>25mm), yellow represents medium (15mm<x<25mm), and red indicates small (<15mm) inhibition zones.

3.7.1 Meropenem

The eluate of meropenem-loaded ALABC showed consistent antimicrobial activity against *E. coli* over the entire testing period. Even on day 42, the smallest diameter spanned 26mm (Table 12).

Against *Pseudomonas aeruginosa* Palacos®R+G in combination with 4g of meropenem was the most effective. This is also the mix containing the highest overall amount of antibiotics (meropenem plus 0,5g gentamycin per 40g pack).

MRSA proved resistant to most samples from day 14 onwards.

	Cement Mix	1h	24h	7d	14d	28d	42d
<i>E. coli</i>	R + Mero 2g	33	37	32	31	28	29
	RG + Mero 1g	31	34	29	29	26	26
	RG + Mero 2g	32	37	32	31	28	28
	RG + Mero 4g	35	39	33	36	33	30
	GC + Mero 1g	31	33	29	27	27	28
	GC + Mero 2g	35	37	31	32	30	30
<i>MRSA</i>	R + Mero 2g	19	15	23	14	8	9
	RG + Mero 1g	14	12	11	0	0	0
	RG + Mero 2g	16	16	18	17	0	0
	RG + Mero 4g	21	23	28	26	0	0
	GC + Mero 1g	15	11	14	0	0	0
	GC + Mero 2g	17	17	22	20	9	9
<i>Pseudom. aeruginosa</i>	R + Mero 2g	29	30	30	27	20	21
	RG + Mero 1g	26	25	26	22	19	18
	RG + Mero 2g	31	30	29	22	22	21
	RG + Mero 4g	36	35	34	29	25	30
	GC + Mero 1g	26	25	26	23	22	25
	GC + Mero 2g	33	29	30	25	25	26

Table 12: Meropenem inhibition assay results

3.7.2 Imipenem

Against *Escherichia coli* Palacos®R+G as well as Copal®G+C containing imipenem produced large to medium sized inhibition zones. These inhibition zones were substantially smaller than those of their corresponding meropenem-counterparts however. Furthermore, Palacos®R plus imipenem was ineffective past day 14 (Table 13).

Against *Pseudomonas aeruginosa* only the Copal®G+C combination may be considered viable since it yielded medium inhibition zones up until day 42. The efficacy of ®R+G mix steadily decreased, while that of the ®R did not extend past the first time point to begin with.

Against *MRSA*, cements impregnated with imipenem proved to be quite effective up to the 14-day point, with mean inhibition zones equal to or bigger than 20mm. However, *MRSA* was resistant from this point forward.

	Cement Mix	1h	24h	7d	14d	28d	42d
E. coli	R + Imi 1g	25	16	18	14	0	0
	RG + Imi 1g	25	24	19	19	19	18
	GC + Imi 1g	25	27	21	20	20	20
MRSA	R + Imi 1g	34	31	30	29	0	0
	RG + Imi 1g	29	28	29	28	0	0
	GC + Imi 1g	21	20	22	21	0	0
Pseudom. aeruginosa	R + Imi 1g	12	0	0	0	0	0
	RG + Imi 1g	16	16	16	12	12	9
	GC + Imi 1g	19	19	19	16	16	18

Table 13: Imipenem inhibition assay results

3.7.3 Fosfomycin Trometamol

As can be seen in Table 14, fosfomycin trometamol was largely ineffective against *MRSA*. Only a single ALABC – Copal®G+C combined with 2g of fosfomycin trometamol – retained an inhibitory effect past day one. This may well be attributed to the presence of gentamycin and clindamycin in the eponymous Copal®G+C base.

Against *Pseudomonas aeruginosa* the mix with a Copal®G+C base again proved the most effective, whereas the one using Palacos®R as a base was the least effective.

Against *Proteus mirabilis*, the eluates containing fosfomycin trometamol universally generated large inhibition zones. Furthermore, they were all effective throughout the whole test period.

	Cement Mix	1h	24h	7d	14d	28d	42d
MRSA	R + 2g F.-Trom.	10	0	0	0	0	0
	RG + 1g F.-Trom	0	0	0	0	0	0
	RG + 2g F.-Trom	15	0	0	0	0	0
	RG + 4g F.-Trom	18	0	0	0	0	0
	GC + 2g F.-Trom	21	18	24	0	0	0
Pseudom. aeruginosa	R + 2g F.-Trom.	23	19	21	0	0	0
	RG + 1g F.-Trom	20	19	22	24	14	9
	RG + 2g F.-Trom	25	20	26	24	19	10
	RG + 4g F.-Trom	29	25	30	28	21	18
	GC + 2g F.-Trom	27	22	27	24	22	24
Proteus mirabilis	R + 2g F.-Trom.	20	24	31	25	23	26
	RG + 1g F.-Trom	26	26	28	22	21	21
	RG + 2g F.-Trom	28	26	30	27	26	24
	RG + 4g F.-Trom	31	28	32	29	28	29
	GC + 2g F.-Trom	30	27	31	28	27	28

Table 14: Fosfomycin trometamol inhibition assay results

3.7.4 Calcium Fosfomycin

All ALABCs loaded with calcium fosfomycin were effective against *Proteus mirabilis* for the entire duration of the trial with the notable exception of the one using Palacos®R as a base (Table 15).

Pseudomonas aeruginosa was most susceptible to the combination of Palacos®R+G with 4g and Copal®G+C with 2g of calcium fosfomycin. Both produced medium sized inhibition zones for the duration of the test.

Interestingly calcium fosfomycin demonstrated no inhibitory effect on *MRSA* growth.

	Cement Mix	1h	24h	7d	14d	28d	42d
MRSA	R + 2g Ca.-F.	0	0	0	0	0	0
	RG + 1g Ca.-F.	0	0	0	0	0	0
	RG + 2g Ca.-F.	0	0	0	0	0	0
	RG + 4g Ca.-F.	0	0	0	0	0	0
	GC + 2g Ca.-F.	0	0	0	0	0	0
Pseudom. aeruginosa	R + 2g Ca.-F.	0	0	0	0	0	0
	RG + 1g Ca.-F.	9	14	17	14	14	16
	RG + 2g Ca.-F.	15	14	18	13	14	15
	RG + 4g Ca.-F.	17	16	22	16	18	20
	GC + 2g Ca.-F.	17	16	21	20	20	22
Proteus mirabilis	R + 2g Ca.-F.	0	0	0	0	0	0
	RG + 1g Ca.-F.	19	20	21	20	20	20
	RG + 2g Ca.-F.	22	22	23	24	21	22
	RG + 4g Ca.-F.	25	24	28	27	26	29
	GC + 2g Ca.-F.	21	25	27	26	26	29

Table 15: Calcium fosfomycin inhibition assay results

4 Discussion

Therapeutic intervention for periprosthetic infection is often laborious. In general, meticulous surgical debridement, extensive microbiological investigations and individually adapted antibiotic regimens are necessary to effectively combat infection. In cases that require a two-stage exchange of the joint prosthesis, the insertion of a temporary place-holder may be expedient. A PMMA spacer acting as a place-holder, can help to preserve the joint space and may additionally act as a carrier for antibiotic substances which are then released locally. Loading an acrylic bone spacer with an antimicrobial, facilitates the release of high concentrations at the site of infection (28). The ability to manually admix sterile antibiotic powders to PMMA bone cement, provides the opportunity of tailoring the local antibiotic to the individual causative agent and its respective susceptibility profile.

Only a limited number of antibiotics have been tested and proven suitable for local delivery, and of these, only few are suited to combat gram-negative bacteria. Therefore, the aim of this study was to assess two carbapenems and two fosfomycin salts, all of which are considered reserved antibiotic in an orthopaedic setting, for their potential use in antibiotic-loaded cements. The investigated antimicrobials included meropenem, imipenem, calcium fosfomycin and fosfomycin trometamol. They were admixed to the acrylic bone cements Palacos®R, Palacos®R+G and Copal®G+C respectively.

To assess the effect of the added substances on the mechanical properties of the cements, standardized testing according to DIN 53435 and ISO 5833:2002 specifications were carried out. DIN impact strength, ISO bending strength, ISO bending modulus and ISO compressive strength were used as indicators of mechanical stability. For clinical application, the elution of the antibiotics from the cement and the antibiotic efficacy was also of interest.

Tande et al. (15) summarised the data of 14 studies, spanning a total of 2435 cases of periprosthetic infection in hip and knee joints and found that *Staphylococcus aureus* and coagulase-negative *Staphylococcus* together accounted for more than half of all cases. When investigating 53 cases of primary PJI-cultures caused by gram-negative pathogens, Hsieh et al. (76) found that *E. coli* and *P. aeruginosa* were the most common organisms. A study by Zmistowski et al.

(3) which looked at 31 cases of gram-negative PJI, supported these findings and further mentioned *Proteus* as a “significant colonizer”.

Staphylococcus aureus (ATCC 43300), *Proteus mirabilis* (ATCC 12453), *Pseudomonas aeruginosa* (ATCC 27853) and a clinical strain of *Escherichia coli* were chosen as test organisms in this study since they can be considered adequate representatives of pathogens that are frequently encountered in revision arthroplasty. Inhibition zone assays were used to determine microbiological efficacy.

4.1 Consolidated Findings

A number of studies have reported, that mechanical properties of loaded cements may suffer with increasing amounts of antibiotic added, particularly when surpassing an amount of 4,5g antibiotic per 40g cement (77–79). Lilikakis et al. (62) consider the addition of 2g of antibiotic per 40g of polymer cement powder (amounting to 5% foreign substance in the cement) as a “gold standard in clinical practice”. Jiranek et al. (80) summarize that 2g or more of antibiotic per 40g cement pouch are feasible for revision arthroplasty while cautioning that admixing more than 4,5g might very well compromise the compressive strength of the ALABC.

Our own experiments showed, that from a stability standpoint, the addition of 2g of calcium fosfomycin to acrylic bone cement cannot be recommended without reservations. In comparison to the reference, the DIN impact strength of Palacos®R, Palacos®R+G and Copal®G+C containing 2g of calcium fosfomycin was reduced by 47%, 41% and 51% respectively (Figure 12Figure 4). Moreover, the ISO bending strength (Table 10 and Table 11) of Palacos®R+G and Copal®G+C containing 2g of calcium fosfomycin was also significantly reduced. In fact, these two combinations had a bending strength of 42,1 MPa and 44,1 MPa respectively and therefore did not fulfil the ISO 5388 requirement of a 50 MPa minimum bending strength.

The addition of the other fosfomycin salt or one of the carbapenems prompted only minor alterations to the characteristic mechanical parameters and summarily fulfilled the ISO 5388 requirements for flexural strength, flexural modulus and compressive strength. Contrary to the findings of Chang et al. (71) who reported that the addition of 1g of imipenem (Merck Sharp and Dohme Corp.) to 40g Surgical Simplex bone cement (Stryker) resulted in a compressive strength below 70 MPa,

this study found that the addition of imipenem to either Palacos®R+G or Copal®G+C caused a reduction in compressive strength of merely 5,7% and 4% respectively, with mean values remaining well above the minimum ISO requirement. This may in part be attributed to the fact that the imipenem used in the studies was manufactured by different companies (Merck Sharp & Dohle and Fresenius Kabi GmbH® respectively). Furthermore, although not explicitly stated, Chang et al. may have used imipenem as a pure substance, while this study used an imipenem/cilastatin (500mg/500mg) combination. Therefore, although comparable amounts of foreign substance were added to the cements, the differences in biochemical makeup may explain the disparity in compressive strengths. The difference in the bone cements that were used was doubtlessly also a contributing factor.

The test results of this study, gained via standardized testing according to ISO and DIN specifications, effectively illustrated, that the investigated mechanical properties remain largely unimpaired when adding up to 5% of meropenem, imipenem or fosfomycin trometamol. Since all antibiotics with the exception of calcium fosfomycin achieved values well above those required by the International Organization for Standardization, the addition of even higher amounts of these antibiotics might still be safe. Further research which focuses on the effects of different dosages in sufficient increments are necessary to validate this claim.

For the microbiological assay, the inhibition zone diameters correlate with the amount of eluted antibiotics and the respective antimicrobial efficacy. When interpreting the results, it is important to keep in mind that at each of the measurement-points, the cement specimens were transferred into new falcons containing fresh PBS. Each interval between measurements represents an elution process of its own. The inhibition zones measured on the last day therefore do not depict the cumulative effect of antibiotic elution for a duration of 6 weeks.

Continuous, uninterrupted elution over the 42-day period, would have resulted in a higher overall accumulation of antibiotic in the buffer. Therefore, the results depicted in the Tables of Chapter 3 may be considered conservative estimates.

4.1.1 Activity against E. coli

Only the carbapenems were tested against *Escherichia coli*. Meropenem demonstrated exceptional efficacy against this pathogen. In fact, all meropenem-loaded specimens produced inhibition zone diameters that continuously remained above 25mm throughout the entire 42-day test period

These findings are consistent with those of Sumant et al. (69), who studied the antimicrobial activity of Simplex P® (Howmedica International) cements impregnated with 5% and 10% meropenem (AstraZeneca) for a duration of 21 days. Cylinders of the ALABCs were submerged in bacterial culture media, aliquots of which were then used to seed agar plates to permit tallying of the bacterial colonies. Eluates of the samples proved bio-active against E. coli ATCC 25922 for a 3-week period.

The same antibiotic doses of meropenem than imipenem resulted in considerably larger inhibition zones for the former than the latter. However, while the addition of 1g of imipenem and 1g of meropenem resulted in equal amounts of foreign substance being added to the cement, there were different amounts of active substance present (as defined in section 2.2). The imipenem which was provided for this study was industrially premixed in equal parts with cilastatin, which puts these results into perspective. Since “1g imipenem” actually represents only 0,5g of active antibiotic, a direct comparison with 1g of meropenem would give biased results, while any extrapolations from the imipenem inhibition zone data at hand would be mere conjecture.

4.1.2 Activity against MRSA

This study found the microbiological activity of the two fosfomycin variants against methicillin-resistant *Staphylococcus aureus* ATCC 43300 to be negligible. When added to a plain cement, calcium fosfomycin showed no microbiological activity, whereas its sibling compound still showed some, if minimal inhibitory potential.

These findings are concurrent with a study done in 2017 by Eick et al. (73). In their test series they determined MIC values, inhibition zones, as well as microbiological activity against planktonic bacteria, one-time added bacteria and repeatedly added bacteria when using combinations of fosfomycin and gentamicin

in bone cement. They noted that fosfomycin/gentamicin ALABCs showed only minimal growth inhibition against the same MRSA ATCC strain. Eick et al. do not specify which Sigma-Aldrich fosfomycin product was used (i.e. sodium or calcium).

Yuenyongviwat et al. (54) used 40 g of Palacos®R (Heraeus) and 4g of fosfomycin (Fosmicin®, Meiji Seika Kaisha Ltd.) to form articulating knee spacers. The evaluation of microbiological efficacy against a clinical MRSA strain was carried out via disc diffusion. The results of the bio-assay are congruent with our own. Yuenyongviwat et al. stated, that while inhibition zones were large on the first day, fosfomycin showed a bio-activity only for the first three days of the 42-day test period.

The addition of imipenem to cements led to the creation of substantial inhibition zones. Interestingly the addition of 1g imipenem to the plain Palacos®R cement, resulted in bigger halos than any meropenem-blend.

A publication by Charlton-Ouw et al. (68) evaluated antibiotic efficacy of Surgical Simplex P (Stryker) impregnated with 5% meropenem (AstraZeneca), via disk diffusion at a single point in time. They considered low-dose meropenem to be highly effective and reported inhibition zones against MRSA (ATCC 43300) with a 17mm diameter. In our own study, cements loaded with 2g of meropenem achieved similar results, with mean inhibition halos between 16mm and 19mm on day one.

Surprisingly, from day 14 onwards the carbapenems were no longer effective. A study by Samuel et al. (69) that was already mentioned in section 4.1.1, also observed the quick occurrence of meropenem-resistance in this particular MRSA strain. They found that eluates from meropenem-loaded ALABCs had no bio-activity post day 7.

Chang et al. (71) tested a Simplex Surgical (Stryker) cement loaded with imipenem (Merck Sharp & Dohle Corp.) against the same MRSA strain (ATCC 43300). However, they noted that imipenem showed no activity at all against MRSA and suggested that this was because it inherently lacked sufficient elution efficacy from acrylic bone cement. A possible explanation for this notable discrepancy in imipenem efficacy may lie in the different base cements. As summarised by Duncan et al. (79), the cement matrix and porosity have a notable impact on antibiotic elution. When comparing antibiotic elution from commercially available cements, a number of studies emphasized the eminent differences between products (77,81–84). Currently there is a lack of studies that directly compare Heraeus cements with

other commercially available products, not to mention studies that these cements in conjunction with manually added antibiotics.

None of the ALABCs retained their antimicrobial activity against *MRSA* post day 14 and they are therefore summarily unsuited to combat infections caused by this agent.

4.1.3 Activity against *Pseudomonas aeruginosa*

When impregnating Palacos[®]R with either of the fosfomycin salts, antibiotic efficacy peters off around the two-week mark. Interestingly, up until day 14 Palacos[®]R+G and Copal[®]G+C combined with fosfomycin trometamol was more effective against *Pseudomonas aeruginosa* ATCC 25973 than when combined with calcium fosfomycin. Past this point, both formulations – irrespective of the cement they were added to – achieved similar inhibition zone diameters. As expected, higher amounts of fosfomycin added, resulted in wider inhibition zones.

In their experiments, Roth et al. (51) also combined 2g of fosfomycin (Infectofos[®]) with Refobacin-Palacos[®]R40 (currently Palacos[®]R+G by Heraeus Medical). In their 10-day elution tests, the inhibition zones of the fosfomycin-sodium-loaded ALABC started off at 30mm diameter on day one, then gradually declined to around 10mm on the 10th and last day.

Meropenem showed excellent and long-lasting activity against *Pseudomonas* over the whole trial period, particularly when added to Copal[®]G+C or Palacos[®]R+G. This finding is analogous to that of Samuel et al. (69), who also reported microbiological activity against *Pseudomonas aeruginosa* ATCC 27853 for three weeks. Growth inhibition through imipenem was unsatisfactory. Although the study by Chang et al. (71) used imipenem (Merck Sharp & Dohme Corp.) in conjunction with Surgical Simplex (Stryker) cement, they reached a similar conclusion and noted that imipenem had no antibacterial activity against the ATCC 27853 strain.

When intending to treat an infection caused by *Pseudomonas aeruginosa*, this study found that three most effective combinations were:

1. Palacos[®]R+G with 4g of meropenem
2. Copal[®]G+C with 1g or 2g of meropenem
3. Copal[®]G+C with 2g of fosfomycin trometamol

4.1.4 Activity against *Proteus mirabilis*

Both fosfomycin salts were tested against the ATCC 12453 strain of *Proteus mirabilis*. Four of the ALABCs were particularly effective and managed to create inhibition zones of adequate size for the entire 42-day trial. Halo diameters for all four specimens were exceedingly similar. The group included:

1. Palacos®R+G with 4g of fosfomycin trometamol
2. (Palacos®R+G with 4g of calcium fosfomycin)
3. Copal®G+C with 2g of fosfomycin trometamol
4. Copal®G+C with 2g of fosfomycin trometamol

The combination of Palacos®R+G with 4g of calcium fosfomycin also resulted in large zones of inhibition. However, admixing even two grams of this substance had a considerable negative impact on mechanical stability. Therefore, the addition of even higher amounts of calcium fosfomycin requires careful consideration.

Interestingly the plain Palacos®R cement impregnated with calcium fosfomycin showed no inhibitory potential at all, while its sibling substance still produced adequate inhibition zones.

Although some studies have investigated the inhibitory effect of fosfomycin-loaded cements on various bacteria (51,54,73), to our knowledge this work is the first to include a strain of *Proteus* in their investigations.

4.2 Secondary Conclusions

Although not the primary focus of this study, the following paragraphs contain extrapolations from the data obtained in the experiments.

4.2.1 Synergistic Release

The elution rates from acrylic bone cement of some antibiotics are lower when they appear alone than when they are combined with another antibiotic. In other words, some substances can enhance one another's elution rates when combined. This effect is commonly referred to as "synergistic release". Interestingly, this effect occurs only in very specific combination of antibiotics.

Minelli et al. (85) described synergistic release when combining gentamicin with vancomycin. The inhibition zone assay against *B. subtilis*, *E. faecalis* and *E. coli* which is presented in their study, clearly illustrated, that the antibacterial activity of the vancomycin/gentamicin combination far surpassed those of the respective single-antibiotic cements.

Gentamicin is pre-mixed into both the Palacos®R+G and the Copal®G+C cement. When comparing the results derived from the microbiological assays against *E. coli*, *MRSA* or *Pseudomonas aeruginosa* for the combination of Palacos®R and Palacos®R+G with 2g of meropenem respectively, there is no indication of a synergistic effect.

The blend containing 2g of fosfomycin trometamol is of interest, however. The combination of 2g fosfomycin with the gentamicin-containing Palacos®R+G revealed a distinctly higher microbiological activity against *Pseudomonas aeruginosa* compared to that exerted by the fosfomycin alone (when added to the plain Palacos®R cement). There is no reason to rule out that passive opportunism from the combination of antibiotics is at play.

A comparison of unmodified Palacos®R+G and Palacos®R+G impregnated with fosfomycin trometamol would be necessary in order to make definitive assertions as to the presence of this effect in the present ALABCs, which was not within the intended purview of this study.

4.2.2 Thermal Stability

The process of polymerization which makes the bone cement harden is based on an exothermic reaction (86). A study by Meyer et al. (87) observed, that the heat released during the process could reach up to 70 degrees Celsius. However, Kühn et al. reason (88), that some of the heat would dissipate to the surrounding structures and that circulating blood would help to level off the temperature peaks in-vivo.

There was a concern, that these temperatures might lead to the degradation of the antimicrobials. The fact that all antibiotics showed inhibition halos against some if not all the tested strains, proves that a large enough fraction of the substances survived the chemical process to still be microbiologically active.

4.3 Limitations

4.3.1 Test setup

As detailed in in chapter 2, each antibiotic contained different quantities of active substance. The manufacturers of the carbapenems that were used in this study published information to this effect, which is included in section 2.2. However, no information as to the exact amount of the active ingredient contained in the two fosfomycin salts was available.

The measurements to assess stability in this study were primarily based on a single application of high stress upon the material. The ability of the materials to withstand numerous cycles with repetitive application of stress as may be encountered in vivo, when limb mobility is preserved and movement of the articulating knee spacer occurs, was not taken into consideration.

The in vitro results of this study may not correlate to those in vivo. In order to simulate in vivo conditions, the cement specimens would need to be immersed in saline (89,90) with mechanical testing taking place over the course of several weeks. It stands to reason that some of the antibiotic contained in the cement dissolves over time, resulting in a significant reduction of mechanical stability. Chang et al. (71) measured compressive strength of Surgical Simplex (Stryker) bone cement (40g) loaded with one of 5 different antibiotics (1g) and observed a decline in mean compressive strength from before to after the 14-day immersion. Research by Paz et al. (91) corroborated these findings. For their experiment they immersed Palacos®R+G cement loaded with different amounts of a vancomycin/cefazolin combination for a period of 4 weeks and reported a decrease in ISO compressive and bending strength in all ALABC groups as compared to the non-immersed control.

The in vivo elution may also notably differ from the rates found in this study, as an articulating spacer will offer a vastly different ratio of surface area to volume to that of our cylindrical moulds. This is supported by the findings of a study by Seligson et al (92), who noted that the size and shape of cement beads had a significant impact on the elution of the antibiotic.

Additionally, since antibiotic release is dependent on surface area and surface texture (93), specimens that more closely resemble the geometric design of spacer would be preferable.

It was beyond the scope of this study to incorporate these variables and explore their impact on the results. However, future scientific research may want to include pre- and post-immersion mechanical tests.

4.3.2 Fosfomycin

Fosfomycin has two pathways that allow its entry into the bacterium. One of those is reliant on the presence of glucose-6-phosphate (G-6-P). G-6-P in turn is a substance that is nearly universally present throughout the body, as it is an integral part of glycolysis as well as the storage of glucose in cells. According to Barry and Fuchs 1991 (94), fosfomycin shows a significant increase of antimicrobial activity in the presence of G-6-P which should therefore be added to the Muller Hinton agar for future testing, since this would more accurately emulate in vivo conditions.

Additionally, incorporation of fosfomycin disodium, which represents the intravenous formulation of fosfomycin, should be considered. Due to the slightly altered biochemical structure to its progenitor, differences in effects upon cement stability and elution are conceivable.

4.4 Conclusion

The aim of this study was to investigate the effect of the manual addition of meropenem, imipenem and fosfomycin on mechanical properties of cements and explore the antimicrobial activity of the resulting impregnated cements.

The results of our investigations lend support to the hypothesis that the addition of up to 4g of meropenem or fosfomycin trometamol to Palacos[®]R+G and up to 2g of either to Copal[®]G+C can be considered as safe, whereas calcium fosfomycin impacts the stability in such a way as to render it unsuitable for clinical use. Furthermore, meropenem proved highly active against *E. coli* and *P. aeruginosa*, while fosfomycin trometamol showed particular efficacy against *P. mirabilis*.

In summary, despite the fact that data concerning the in vivo efficacy of these antibiotics is lacking, the results of this study support their use in PMMA spacers to combat PJIs caused by susceptible bacteria.

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6 Appendix

Media, buffers and devices:

Name	Company / Manufacturer	Cat#	Lot / Batch
Palacos®R	Heraeus		8703
Palacos®R+G	Heraeus		7953
Copal®G+C	Heraeus		8168
Meropenem	Eberth		7DB0417
Imipenem / Cilastatin	Kabi		IDEA1346
Fosfomycin tromethamine	Zach Systems S.P.A.		14B15A3774
Calcium fosfomycin	Ercros		FC10184
Müller Hinton Agar	Oxoid	CM0337	2114571
PBS tablets	Amresco	E404-200 tabs	1034C412
Falcons			18217131
Petri dishes			