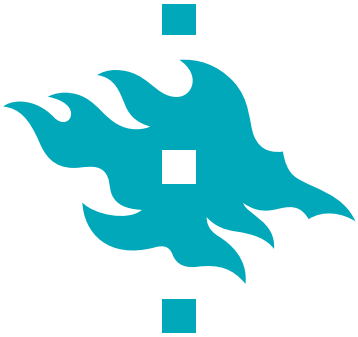

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
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RAMÓN PÉREZ TANOIRA

"RACE FOR THE SURFACE"
COMPETITION BETWEEN BACTERIA AND HOST CELLS IN IMPLANT COLONIZATION PROCESS

DEPARTMENT OF OTORHINOLARYNGOLOGY– HEAD AND NECK SURGERY
HELSINKI UNIVERSITY HOSPITAL
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UNIVERSITY OF HELSINKI

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"RACE FOR THE SURFACE" COMPETITION BETWEEN BACTERIA AND HOST CELLS IN IMPLANT COLONIZATION PROCESS

Ramón Pérez Tanoira

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture hall 3, Biomedicum-Helsinki, on 7th June 2019, at 12 noon.

Helsinki 2019

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To my parents Ramón and María

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I **R. Pérez-Tanoira, M. García-Pedrazuela, T. Hyyrynen, A. Soininen, A. Aarnisalo, M.T. Nieminen, V.M. Tiainen, Y.T. Konttinen, T. J. Kinnari.** "Effect of S53P4 bone substitute on staphylococcal adhesion and biofilm formation on other implant materials in normal and hypoxic conditions". *J Mater Sci: Mater Med. 2015 Sep;26(9):239.*

II **R. Pérez-Tanoira, T. J. Kinnari, T. Hyyrynen, A. J. Soininen, L. Pietola, V. M. Tiainen, Y. T. Konttinen, A. Aarnisalo.** "Effects of S53P4 bioactive glass on osteoblastic cell and biomaterial surface interaction". *J Mater Sci: Mater Med. 2015 Oct;26(10):246.*

III **R. Pérez-Tanoira, X. Han, A. Soininen, A. Aarnisalo, V. M. Tiainen, K. K. Eklund, J. Esteban, T. J. Kinnari**. "Competitive colonization of prosthetic surfaces by *Staphylococcus aureus* and human cells". *J Biomed Mater Res A. 2017 Jan;105(1):62-72.*

IV **R. Pérez-Tanoira, A. Aarnisalo, K. K. Eklund, X. Han, A. Soininen, V. M. Tiainen, J. Esteban, T. J. Kinnari.** "Prevention of Biomaterial Infection by Pre-Operative Incubation with Human Cells". *Surg Infect (Larchmt). 2017 Apr;18(3):336-344.*

ABBREVIATIONS

Al_2O_3	Aluminium oxide
BAG	Bioactive glass bone substitute granules
C	Carbon
Cu	Copper
CFU	Colony forming unit
AOM	Acute otitis media
FBS	Fetal bovine serum
hOB	Primary osteoblasts
Ir	Iridum
LDH	Lactate dehydrogenase
MEM	Minimum essential medium
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide tetrazolium dye
PA	Acrylate polymers
PBS	Phosphate-buffered saline
PDS	Polydioxanone
PDMS	Polydimethylsiloxane
PE	Polyethylene
PET	Polyethylene terephthalate (Dacron)
PLA	Poly(lactic acid)
PMA	Methacrylate polymers
PMMA	Polymethylmethacrylate
PP	Polypropylene
PS	Polystyrene
Pt	Platinum
PTFE	Polytetrafluoroethylene
PU	Polyurethane
ROS	Reactive oxygen species
SaOS-2	Human osteosarcoma cells
Si	Silicone
SS	Stainless steel
TSB	Tryptic soy broth
Ti	Titanium
UHMWPE	Ultra-high molecular weight polyethylene

ABSTRACT

Prosthetic infection represents a major problem in the outcome of patients after implantation of a foreign body. The presence of biomaterial in the body provides a substratum to host either tissue-cell integration or bacterial colonization. In obliteration of an infected bone, artificial bone substitutes and rigid fixation materials are usually necessary to fill bone cavity and to restore the properties of the bone respectively. This study attempted to discover the effect of bioactive glass bone substitute granules (BAG) S53P4 on bacterial and human-cell adhesion on other implant used simultaneously (I, II). During development of new infection-resistant biomaterials, adherence and colonization of either bacterial cells or tissue cells on biomaterials must be evaluated in parallel. A methodology allowing study of the simultaneous growth of bacteria and tissue cells on the same biomaterial surface was developed. This will allow discovery of the effect of various bacterial concentrations on host-cell viability and integration with an implant surface, and their relation to increasing reactive oxygen species (ROS) levels and cell apoptosis (III). Finally, considering our first results and that microorganisms frequently infect an implant surface during surgery and start to compete for the surface before tissue integration, it was hypothesized that incubation of implants with host cells before implantation may be one way to reduce the bacterial living space available and would prevent bacterial adhesion and consequently the infection of biomaterials (IV).

Bacterial and human osteoblast-like osteosarcoma cells (SaOS-2) or primary osteoblast (hOB) cells were incubated for 4.5 hours, 2 days, or 4 days at 37°C. As substratum, titanium (Ti), polytetrafluoroethylene (PTFE), polydimethyl-siloxane (PDMS), or bioactive glass plates (IV) were used. The study was done separately (I, II), in competition with SaOS-2 or hOB (III), or in competition with SaOS-2 after 24-hour pre-incubation with SaOS-2 (IV). The effect of BAG S53P4 on bacteria (I) and cell (II) adhesion was studied in either a normal atmosphere or in hypoxia-simulating atmospheric conditions of the middle ear, mastoid cavity, or sinuses. Human osteoblast-like SaOS-2 cells or primary osteoblast (hOB) cells (III) (both, $1x10^5$cells/mL), and collection strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* (I) [10^8 colony forming units (CFU) (I) or (serial 1:10 dilutions of 10^8 CFU (III, IV)] were employed.

The bacteria and cell proliferation, cytotoxicity (III, IV), and production of reactive oxygen species (ROS) (III) were evaluated by colorimetric (MTT, LDH, and crystal violet) (III, IV) as well as by fluorometric methods (fluorescent microscopy and flow cytometry) (III). Bacterial cell viability was studied by use of a drop-plate method after sonication. Effects of BAG S53P4 on cell adhesion were linked intimately with modifications of cellular

attachment organs (vinculin containing focal adhesions), rearrangement of the actin cytoskeleton, and cellular spreading.

The presence of bioglass under normoxic and hypoxic conditions prevented bacterial and biofilm adhesion for most of the materials and promoted integration of SaOS-2 cells with various biomaterial surfaces, especially under hypoxic conditions, in which S53P4 granules cause increased pH (I, II). In the competitive study, the presence of bacteria resulted in reduced adherence of human cells to the surface of the biomaterials, increased production of ROS, and increased apoptosis. The presence of either type of human cell was associated with a reduction in bacteria compared with that for the materials incubated with *S. aureus* only (III). Pretreatment with human cells was also associated with a reduction in bacterial colonization of the biomaterial compared with that of the non-pretreated materials, but the presence of bacteria produced a decrease in viable human cells for all materials (IV).

In conclusion, the presence of S53P4 granules may both protect implants from bacterial colonization and promote their osteointegration. In the presence of bacteria and cells, colonization of the surface by one reduces colonization by the other. The bacteria produce cellular oxidative stress in human cells, which may be related to the cellular death. The preoperative incubation of prostheses with host cells could be a new way to prevent infection of biomaterials and lessen the risk for bacterial antibiotic resistance.

1 INTRODUCTION

Prosthetic infection stills remains a major challenge to physicians and biomedical researchers despite the effective prophylactic measures adopted [1-3]. The number of implantations is rising, but meanwhile, the focus population needing the prosthetic devices or implant materials is aging and suffering from comorbidities. Medical implants are highly susceptible to infections, because the implant surface itself naturally has no active defense mechanisms. Furthermore, there occurs a localized immunological deficit at the interface between the implant and the host so that individual microorganisms may attach and persist mainly because of the rapid formation of a biofilm resistant to host defense and to antimicrobial agents [1, 4-8]. Implant-related infections are associated not only with important clinical consequences and patient suffering but also with a high economic burden. Their treatment requires a combination of long periods of wide-spectrum antibiotic therapy and repeated surgical procedures, which involve extended stays in hospital [4, 9].

Considering the ability of bacteria to persist in multicellular biofilm communities, the best way to prevent prosthetic infections is by inhibition of biofilm formation. The search for antimicrobial surfaces and materials that can resist biofilm formation focuses on incorporating anti-adhesive/antibacterial substances into the substratum [6, 10]. The disadvantage is the spread of antimicrobial substances which may induce bacterial resistance or cytotoxicity in neighboring tissues [11]. The presence of a foreign body automatically initiates a “race for the surface” between bacteria and host cells to colonize the surface of the implant. Competition occurs between integration of the implant into the host tissue and biofilm formation [12].

When new biomaterial-coating strategies are under development, tissue adaptation and prevention of bacterial adhesion and subsequent infections should be explored as separate phenomena. Promotion of tissue integration as a means to protect against infection has been poorly studied [13, 14]; this is understandable, because bacterial and human cell cultures are traditionally kept separate from each other, and simultaneous work with cells and bacteria is demanding.

After medical obliteration of an infected bone, artificial bone substitutes such as bioactive glass (BAG), and rigid fixation materials are usually means to restore the properties of the bone [15-17]. BAGs of different compositions elicit a specific biologic response at the interface of the material, forming direct chemical bonds with tissue and enhancing bone tissue formation due to their dissolution products, which stimulate osteoprogenitor cells at the genetic level [17-19]. The BAG S53P4 is indicated as a bone graft substitute for reconstruction of bone defects in treatment of osteomyelitis and craniofacial defects caused by mastoidectomy and frontal sinus surgery [20-22]. S53P4, as well as other BAGs, possesses antibacterial properties based on several factors, including high surface reactivity and ion-release capability. This leads to an

alkaline environment and osmotic effects in the surrounding tissues [23, 24]. Furthermore, the osteointegration and the bone remodeling form a bone-like layer on the surface of S53P4, which inhibits bacterial adhesion and consequent biofilm formation [25].

As a part of the host defense, reactive oxygen species (ROS) are generated and released from macrophages and polymorphonuclear granulocytes. ROS react against microorganisms, inflicting macromolecular damage on vital cellular components. ROS may also react with human cells and extra-cellular molecules, inducing apoptosis or even necrosis [26-28].

The research project presented here studied the outcome of the race for the surface between bacteria and tissue cells and its relation to increasing ROS levels and cell apoptosis. The bacterial living space available is reduced on the implant through the presence of a bone-like layer on its surface. This is a way to prevent bacterial colonization and to avoid use of antibiotic-loaded biomaterials, which confers a risk for bacterial antibiotic resistance. This may be achieved through the osteoconductive effect of S53P4, which increases the bone growth on the implant surface located near to the bioactive glass. Finally, considering that microorganisms frequently infect an implant surface during surgery and start to compete for the surface before tissue integration, pre-incubation of biomaterial with host cells before implantation could create an antiadherent coating.

The concept of competition for the surface has been embraced by some researchers in the field, but hitherto few in vitro experimental methodologies have begun to study the concept thus far.

2 REVIEW OF THE LITERATURE

2.1 OSTEOMYELITIS AND INFECTION OF BONE CAVITIES

2.1.1 OVERVIEW

Osteomyelitis is an inflammatory process that implies bone destruction and necrosis caused by progressive infection of bone. The source of bacterial colonization may be an infection in nearby tissue either of post-traumatic or post-operative origin, secondary to vascular insufficiency, which occurs predominantly in people with diabetes, or due to hematogenous spreading, when osteomyelitis has originated from bacteremia [29-31]. The bacterial contamination may cause a fulminant infection, but the patient may also be symptomless in cases where the replication of the adhered bacteria is hindered. After months or even years, acute osteomyelitis can progress to a chronic and persistent state, which is characterized by the presence of dead bone (sequestrum) and fistulous tracts to the skin. Osteomyelitis leads to a serious clinical and economic burden, as it causes thousands of hospital admissions each year worldwide. It is often difficult to diagnose and always hard to manage due to the heterogeneity of its origin, pathophysiology, and clinical manifestation. Successful management of osteomyelitis often requires prolonged antibiotic therapy and surgical procedures [32].
The cranial air cell system, paranasal sinuses, and mastoid air cells are developed by gradual pneumatization of the bone. The cranial air cell system provides acoustic properties for hearing, participates in cranial resonance of the human voice, reduces the mass of the head, and protects the central nervous system from physical damage. Paranasal sinuses also participate in aeration in humans beings [33, 34]. Sinusitis and mastoiditis are infections of the paranasal sinuses and the mastoid cavity, and both, without early detection and adequate treatment, are potential causes of important intracranial complications and sepsis [35, 36]. Colonization of the paranasal sinuses by bacteria may lead to chronic sinusitis and even lung infections [37], especially in cases with compromised local host resistance such as in cystic fibrosis.

The risk for mastoiditis in typical otitis media has been reduced from 5000-10 000/100 000 to 5-3.8/100 000/year with the use of antibiotics [35, 38]. In developing countries, however, due to the absence of antibiotic treatment, in children aged under 5 years, mastoiditis is the most common intratemporal complication of acute otitis media (AOM), and mastoiditis is an important cause of death [39]. This is the most important fact, considering that AOM is the most common localized infection.

The common cold is related to the spread of bacterial infection to the surrounding cavities, causing bacterial infection of the paranasal sinuses and middle ear. One of the complications of middle-ear infection is mastoiditis, infection of the mastoid cavity. Sinusitis, infection of the paranasal sinuses, may spread to the surrounding soft tissue, most often to the orbita. Infection of human body cavities is associated with various symptoms including swelling, pressure, and pain in the mastoid process, maxilla, forehead, nose, and around the eyes [38]. Acute osteomyelitis often displays an acute suppurative inflammation, which reduces local vascular supply and causes development of an ischemic area which contributes to bone necrosis in osteomyelitis [30, 31] (Figure 1). Osteomyelitis is associated with the presence of clinical symptoms such as relapses and fever for longer than 10 days [29-31].

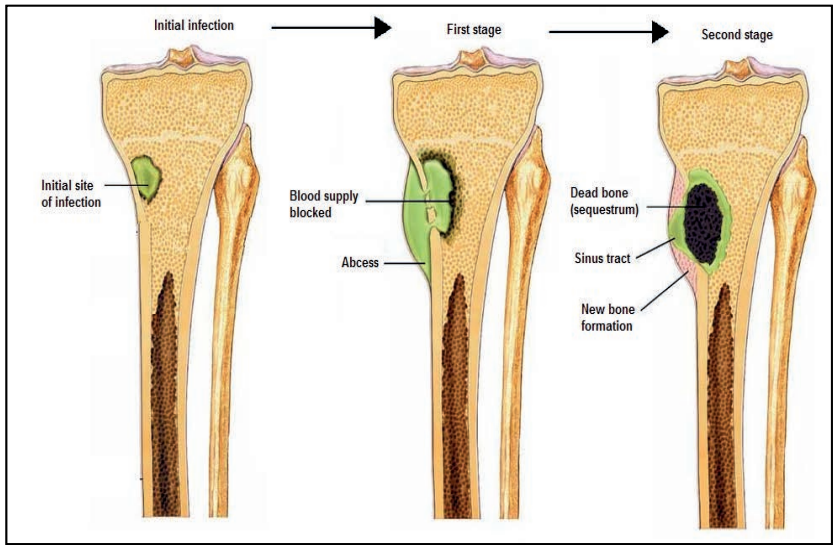


Figure 1: Steps in the process from acute to chronic osteomyelitis. At the initial site of infection, an intracapsular infection develops, accompanied by an area of devascularized dead bone. This infection progresses towards a subperiosteal location, which leads to a massive periosteal elevation resulting in new bone formation. Finally, a sequestrum progresses through the cortical bone and creates a cutaneous sinus tract, a fistula. "Illustration by author."

2.1.2 OBLITERATION OF A SEQUESTRUM

Obliteration is a surgical procedure intended to debride the necrotic and infected bone, mucosa, or air cells. In mastoidectomy, the mastoid air cells are debrided, and the empty mastoid cavity thus formed is filled with autologous

material or bone substitutes. The result is a safe and trouble-free cavity or bone (Figure 2). Mastoidectomy implies the formation of an open cavity, allowing complete disease visualization and removing the chronically infected bone sequester that has resisted other treatments [17, 22, 40-43].

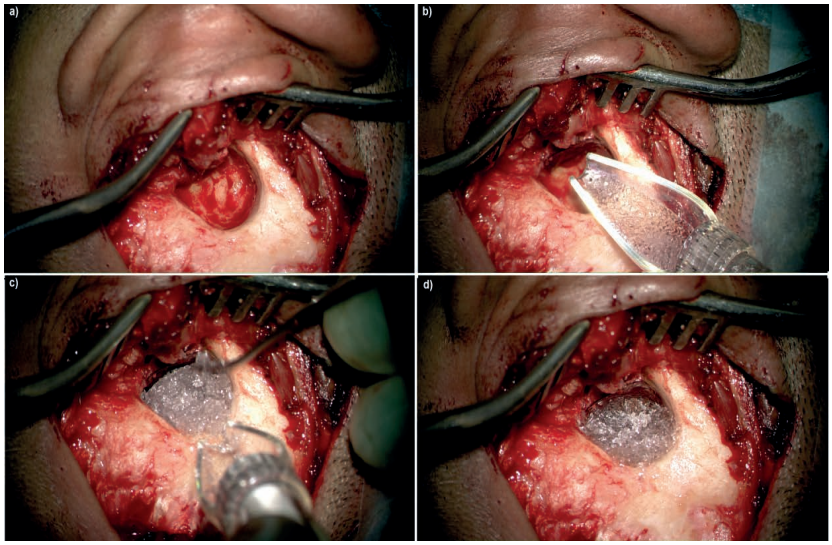


Figure 2. Mastoidectomy and mastoid obliteration with bioactive glass S53P4: a) a post-auricular incision where the temporalis muscle was dissected from the underlying mastoid bone from the mastoid tip superiorly to the temporal line. All the skin lining the mastoid cavity was removed so as not to risk burying a cholesteatoma during the mastoid obliteration; b-d) BAG S53P4 were used to obliterate the mastoid cavity. "Illustration by author."

Obliteration can be combined with other surgical treatments where rigid fixation materials are intended to reconstruct the bone or the facial contour or to restore conductive hearing properties of the middle ear such as in surgical cholesteatoma treatment [16, 41, 44, 45].

2.1.3 ARTIFICIAL BONE SUBSTITUTES

In an obliteration, the frontal sinus, skull bone, or other bones are filled with suitable material after the complete surgical removal of the pathologic mucosa, air cells, or bone tissue. There exists a demand for adequate obliteration materials in otolaryngology, as well as in all bone-reconstructive surgery. Otolaryngologists and traumatologists have traditionally selected transplanted

autologous tissues such as demineralized cranial bone, bone matrix, allogenous cancellous bone chips, and cartilage. Each of these, however, shows different donor site related problems including morbidity, wound complications, immunological rejection, release of calcium into the bloodstream, postoperative infections, fat necrosis, and recurrent chronic sinusitis [17, 22]. Other possible complications comprise infections related to bank tissues, prolongation of surgical procedure time, and complexity of surgical technique. All these disadvantages have led to increased interest in synthetic materials such as metals (titanium), synthetic polymers such as polymethyl methacrylate (PMMA), Proplast or Polytef, ceramics (hydroxyapatite), and glasses (bioactive glass S53P4) to induce osteoneogenesis in bone defects [42, 43, 46, 47].
The prerequisites for biomaterials used in the head and neck area differ from those used for joints and long bones, where load-bearing properties are required [43]. In the restoration of facial and skull defects that suffer slower bone curing, the implant has to fix and maintain the anatomical profile of the reassembled area.

2.1.4 BIOACTIVE MATERIALS

Bioactive biomaterials induce a specific action in the surrounding cells, promoting a binding between tissues and the biomaterial surface, which enhances their bioactivity. In bone replacement, they stimulate the osteoblasts and bonding of bone tissue leading to the deposition of a bone mineral calcium phosphate layer on the implant surface [17, 18, 48, 49]. Bioactive materials are served in multiple clinical applications and can be incorporated into the structure of such different materials as glasses or ceramics and attaching them onto the surface of inert materials such as titanium. Other methods, such as to modify the biomaterial surface with biological molecules, can induce a similar specific cell behavior [48].

2.1.4.1 Bioactive glass

Bioactive glass is a biocompatible, non-toxic, and osteostimulative synthetic material, which releases into its environment the ions required for new bone formation. Bioactive silica-based glass materials are based on a SiO_2–Na_2O–CaO–P_2O_5–Al_3O_2–MgO–K_2O structure. They serve as bone substitutes or fillers, which are implanted into a bone defect [17, 22, 48, 50, 51]. Due to their osteostimulative properties, they promote the enrollment and differentiation of osteoblasts, they activate osteoblasts to create new bone, and they trigger specific osteoblast genes as a response to ion dissolution from the material [20, 52-54]. In vivo, bioactive glass achieves a natural amorphous hydroxyapatite

surface similar to the mineral structure of the bone surface. A silica-rich layer deposited on its surface enables direct binding to the bone without any fibrous connective tissue interface [20, 22, 48, 50]. Bioactive glass is osteoconductive and partially osteoinductive (Figure 3). It acts as a platform for orthotopic bone formation but not for ectopic bone formation [20].
Commercially available BAG compositions are directed toward several clinical applications such as bone: the two main products are S53P4 and 45S5. These BAGs show different antibacterial, osteoconductive, and angiogenic properties as well as different resorption rates in vivo. S53P4 presents a significantly slower resorption rate with respect to 45S5 [18, 51, 55, 56].

2.1.4.2 BAGS53P4

The biologically active response from bioactive materials depends on their structural properties and composition [49]. BAG S53P4 is a synthetic silica-based bone substitute. The name "S53P4" is based on its specific chemical composition of 53% SiO_2 and 4% P_2O_5, the rest of the components are 20.0% CaO and 23.0% Na_2O. It has served in different clinical bone reconstructions and obliterations: In otorhinolaryngology it has served in craniofacial reconstructions, frontal sinus obliteration after severe chronic sinusitis, in mastoid cavity obliteration, and in nasal septum operations [17, 20]. In orthopedics, it has served as bone-cavity filling material in the treatment of chronic osteomyelitis [17, 20, 40, 50, 57]. In several experimental studies, evaluating the use of different artificial bone substitutes in obliteration of frontal sinus and other skull defects, BAG S53P4 has produced more new bone than does either synthetic hydroxyapatite or tricalcium phosphate [17, 42, 58].

When a S53P4 bioactive glass surface is exposed to body fluids, sodium, silica, calcium, and phosphate ions are released from it inducing the formation of a silica gel layer on the surfaces of the glass granules and leading to increased local pH and osmotic pressure. This silica gel is based on silanol (Si-OH) groups, which are produced by reaction of bioglass silica and protons (H+) from the surrounding tissue (Figure 3). Amorphous structures of calcium phosphates precipitate and crystalize on natural hydroxyapatite on this layer, stimulating migration, replication, and differentiation of osteoblasts for the formation of new bone [20, 25, 40, 59]. On the other hand, this ion-dissolution process which causes an increase in pH and in osmotic pressure in the environment, confers antimicrobial properties on BAG S53P4, as it is more harmful and often lethal to prokaryotic structures but not to eukaryotic cells [20, 25, 60-65]. BAG S53P4 proved important antibacterial properties against 46 clinically relevant aerobic and anaerobic bacteria, including methicillin-resistant *Staphylococcus aureus* [24, 64, 66]. Lindfors et al. [57] and Sarin et al. [22] showed that BAG S53P4 was a good treatment for 11 patients with chronic osteomyelitis and a highly effective material in the mastoid obliteration

of 25 patients with chronic otitis media. Similar results were obtained by Stoor et al. when they evaluated BAGS53P4 in the repair of nasal septum perforations in 49 patients and in mastoid obliteration of 7 patients with chronic otitis media [15, 67]. S53P4 bioactive glass thus could become a safe and adequate alternative to antibiotic-containing PMMA beads in a one-stage procedure in treatment of osteomyelitis, avoiding the use of local antibiotics [20, 68].

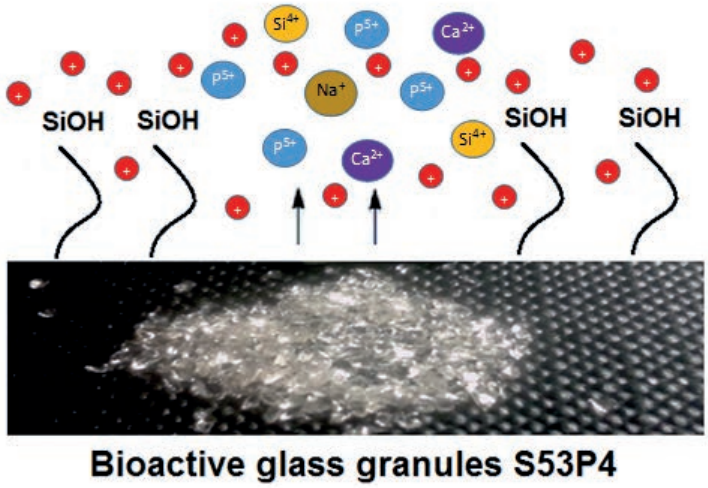


Figure 3. Summary of bioactive glass reaction mechanisms when it is exposed to body fluids. Alkaline ions are released causing a) an increasing in pH and in osmotic pressure producing antimicrobial effect b) production of silanol (Si–OH) functional groups on the bioactive glass surface with osteoconductive effect c) an activation, enrollment and differentiation of osteoblasts with osteostimulative properties.

2.2 IMPLANTATION OF BIOMATERIALS

2.2.1 MEDICAL BIOMATERIALS

2.2.1.1 History of medical biomaterials

Just 60 years ago the word "biomaterial" was not used, however since the beginning of civilization, man has implanted into human bodies non-biological materials in one form or another. Development of material and biological science has come a long way. Ancient Egyptians used ligature wire made of gold for stabilizing affected teeth (2500 BC). The Mayan population employed

pieces of shells as a replacement for mandibular teeth (600AD) [69]. In 1860, Adolf Gaston Eugen Fick invented the glass contact lens and tested them on both animals and humans. Biomaterials were conceived first by Leonardo DaVinci in 1508 and later by Rene Descartes in 1632. Two key innovations to support the clinical use of biomaterials occurred in the late 1800s with the implementation of aseptic operative techniques and the use of x-ray for radiography [70].

In 1829, the first studies assessed in vivo bioreactivity of metallic implant materials including gold, silver, lead, and platinum and further studies were performed evaluating iron, steel, magnesium, aluminum alloy, zinc, and nickel. In 1947, a cobalt-chromium-molybdenum (CoCrMo) alloy was used successfully in dentistry after it had shown itself to be well tolerated and strong some decades before. In 1947, a possible medical use of titanium and alloys was discussed as well. The history of biomaterials was mainly linked to metals, and the development of other materials such as plastics and other polymers for implant materials does not extend as far back as metals, simply because there were few of them prior to the 1940s.

The biomaterial field has seen accelerating growth since World War II, as newly developed high-performance materials invented and manufactured for airplanes or other war equipment were applied to medicine. Materials appeared such as silicones, polyurethanes, polytetrafluoroethylene (Teflon®), nylon, methacrylate, titanium, and stainless steel. During the World War II, Sir Harold Ridley, based on observations of the biological effects of shards of airplane canopy on the eyes of pilots, developed ocular lens implants from polymer materials [70, 71].

In 1952, Per Ingvar Brånemark screwed a titanium cylinder into a rabbit bone for observing healing reactions, he found this device tightly integrated after several months and named this phenomenon “osseointegration”. He promoted the use of titanium and its alloys as implants to surgical and dental procedures. By 1961, John Charnley developed the first successful low-friction hip prosthesis with metallic femoral stem, ball head, and ultra-high molecular weight polyethylene (UHMWPE) acetabular cup resulting in good long-term results. During 1982–1985, an aluminum and polyurethane artificial heart was designed and successfully implanted in a human. Patients lived up to 620 days with this Jarvik7® device. From 1990s, nonmetallic implants, part of them biodegradable, have taken place and been used first as screws and later even as weight bearing implants. At present, metallic alloys, mainly of titanium, as well as polymers and ceramic materials are used separately or in combination for manufacturing great variation of implants for different tissue types [70, 71].

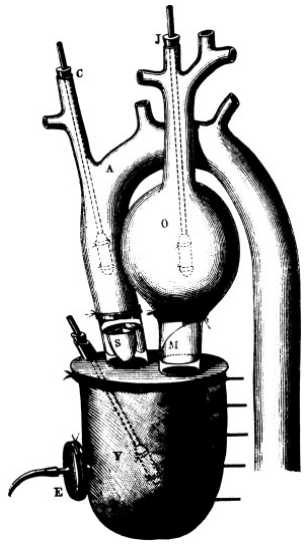


Figure 4. An artificial heart by Étienne-Jules Marey, Paris, 1881. Figure from Ratner B, Hoffman A, Schoen F, Lemons J, editors. Biomaterials Science. A Introduction to Materials in Medicine. 3rd ed. Oxford,UK.: Academic Press Elsevier.; 2013 [71].

2.2.1.2 Definition

Biomaterials science integrates interdisciplinary research approaches in which engineers and physical scientists converge with biologists and clinicians in multidisciplinary thinking and analysis. Here interaction of materials with the biological environment can solve highly complex problems, enhancing our capacity to solve previously untreatable medical conditions [71, 72]. Over the years there have been several efforts to define biomaterials science and choose the goal of their study. Medical biomaterials are nonviable materials, intended to interface with biological systems; they serve to evaluate, treat, augment or replace any tissue, organ, or function of the body [70, 71, 73]. Due to the fact that most biomaterials induce a foreign body reaction, it is important to consider the term "biocompatibility", which is defined as the ability of a material to perform with an appropriate host response in a specific application.

Table 1. Key applications of synthetic materials and modified natural materials in medicine. Source: Buddy D. Ratner, A.S.H., Frederick J. Schoen, Biomaterials Science: An Introduction to Materials in Medicine. third edition ed. 2013: Elsevier [71].

Application	Biomaterials used
Skeletal	
Joint replacements	Ti, SS, PE
Bone fixation plates screws	Metals, PLA
Bone cement	PMMA
Dental implant/tooth fixation	Ti
Cardiovascular	
Pacemaker	Ti, PU
Stent	SS, other metals, PLA
Catheter	PTFE, Si, PU
Organs	
Heart assist device	PU, Ti, SS
Hemodialysis	Polysulfone, Si
Skin substitute	Collagen, cadaver skin, nylon, Si
Otorhinolaryngological	
Cochlear prosthesis	Platinum, platinum-iridium, Si
Ear tubes	Si, PTFE, Ti, Au
Tracheostomy tubes	Si, Ag
Voice prostheses	Si
Ossicular chain	Ti, PMMA
Other	
Contact lens	PA/PMA/Si polymers
Contact lens	PA/PMA/Si polymers
Intraocular lens	PA/PMA
Breast implant	Si
Mesh	Si, PP, PTFE
Sutures	PLA, PDS, PP, SS

Ti: titanium; SS: stainless steel, PE: polyethylene, PLA: poly(lactic acid), PMMA: polymethylmethacrylate, PU: polyurethane, Si: silicone, PTFE: polytetrafluoroethylene, Au: gold, Ag: silver, PA: acrylate polymers, PMA: methacrylate polymers, PP: polypropylene, PDS: polydioxanone.

Biomaterials cannot be explored without considering medical devices and the biological response to them, as they are integrated into devices or implants. One example is titanium in conjunction with UHMWP, which becomes the device, a hip prosthesis. The success of a device depends on interactions of the material: the device has an impact to the recipient (patient), and the patient's host tissue has an impact to the device. Table 1 shows medical devices implanted in different medical applications [71].

Four general categories of materials are available as biomaterials: a) metals (based on the metallic bond and including pure metals and alloys), b) polymers (based on covalent bonds and including glasses, glass–ceramics, and carbons), c) ceramics (based on ionic bonds, they are polymers and include ceramics, polymers including thermosets, thermoplastics, elastomers and textiles) and d) their composites [70, 74].

2.2.1.3 Metals

This unique atomic arrangement and bonding enhances the mechanical, thermal, and electrical conductivity properties of metals, making them ideal for prostheses for hard tissue replacement, fixation devices, and active devices such as stents, guide wires, and electrodes. Among metallic biomaterials, titanium (Ti) and its alloys are generally preferred to stainless steels and cobalt-based alloys because of their reduced elastic modulus, enhanced corrosion resistance, superior biocompatibility, and low toxicity [75, 76]. The good biological properties of Ti and elevated ossointegration are due to the formation of the native oxide (TiO_2) when Ti is exposed to oxygen [77], otherwise a connective tissue capsule would be formed on the bioinert titanium surface [49].TiO_2 induces formation of a biologically active layer of calcium phosphate, which is crucial for integration of prostheses with bone.
Numerous surface coatings and porous designs from stainless steel, cobalt–chromium (CoCr), or titanium alloys have been developed as biomaterial implants showing excellent clinical results due to enhanced biological fixation to bone. However, all of them have several inherent limitations (low volumetric porosity, relatively high modulus of elasticity and low frictional characteristics). A new totally inert porous tantalum biomaterial, with an appearance similar to cancellous bone allows for almost unlimited uses in design, shown by the wide variety of available implants and multiple medical devices, including pacemaker electrodes, foils, and meshes for nerve repair, radio-opaque markers, and cranioplasty plates [78].

2.2.1.4 Polymers

Polymer materials show an array of unique physicochemical properties. The number of polymeric materials has increased enormously over the past decade and to date they represent the most important class of biomaterials used in a large variety of medical applications [79]. These include a huge variation of materials and indications such as:
Polypropylene for sutures, abdominal wall prostheses and intraocular lenses, PMMA used in cranioplasties [80-82], silicone in drainage tubes and orthopedic surgery [6, 83], composite prostheses of hydroxyapatite-polyethylene for ossicular chain reconstruction [84], UHMWPE in orthopedic

prostheses [85], and polyglycolic acid in mesh reinforcement of pancreaticojejunostomy [86].
Polymers are large molecules composed of linear chains in 3-dimensional form. The main characteristic of polymer molecules is their high molecular weight. The linear polymer chains are constituted of many covalently linked units of alkanes. This carbon chemistry helps to couple them to biological interfaces, better than to inorganic materials do, and it can be used for targeted interaction to the body. They can be classified as synthetic polymers such as the family α-hydroxyacid, which includes poly lactic-co-glycolic acid, polyanhydrides, naturally occurring polymers, such as polysaccharides including starch, cellulose, chitin, pectin, alginic acid, and inorganics such as hydroxyapatite. They can also be classified by function or structure as for example hydrogels, injectable, or capable of drug delivery [79].

2.2.1.5 Ceramics, glass and glass-ceramics

This group includes an extensive spectrum of compositions, useful in different medical applications. Insoluble glasses have served as carriers for enzymes, antibodies, and antigens, and one designed as a microinjectable drug-delivery vehicle for radioactive isotopes [87, 88]. Ceramics and glass-ceramics are required in routine dental practice in fabrication of dental prostheses [89]. This group of materials is frequently used to repair skeletal hard connective tissues and as bone substitutes because of their osteoinductive properties. Some examples of this group are aluminium oxide (Al_2O_3), bioactive glasses, hydroxyapatite, calcium sulfate, and tricalcium phosphate.
The use of porous ceramic implants is limited to non-loadbearing applications, because the porosity of the material is inversely proportional to its strength. These materials serve as structural scaffolds due to their inertness and the mechanical stability of the intricately developed bone-interface in the pores of the ceramic [71].

2.3 INFECTION OF PROSTHESES

During recent years, the number of patients who have improved their clinical situation because of the implantation of prosthetic devices or implant materials is rising, and the targeted population of this medical treatment is aging and enduring more comorbidities. For this reason it is logical that the number of complications related to the use of these biomaterials also increases, and within these complications, the prosthetic infections play a special role due to the devastating difficulties they bring about and how difficult they are to treat [1-3]. Biomaterial infections entail important clinical and economic consequences such as prolonged antibiotic treatment, long hospitalizations, and multiple surgeries leading in the worst cases to the patient's death. Despite all the

prophylactic measures adopted to avoid these nosocomial infections, they still remain as an important clinical challenge for physicians and researchers of other areas of science including physics, chemistry, and microbiology [2].
The presence of a foreign body offers an adequate surface for bacterial adherence, and consequently for development of an infection of the surrounding tissue. This situation is favored by implantation surgery in differing ways [1]: tissue damage produced by the surgical procedure, impairment of the host immune response in the wound tissue, entrapment of bacteria by fibrin in the wound (and this protects them against the action of antibiotics), seclusion of bacteria in prosthesis interstices, and the development of a biofilm on the implant surface [1, 2, 90, 91].

2.3.1 RACE FOR THE SURFACE

In 1988, Anthony Gristina explained clearly that the problem of infections related to biomaterials was a rising problem and published his theory: "Race for the surface" by which he argued that the presence of a foreign body provokes a rivalry between bacteria and host cells to conquer the implant surface [12, 92]. Tissue integration on the one hand, and bacterial adherence and development of a biofilm on the other are the goals of host cells and bacteria respectively (Figure 5). These two phenomena are in conflict, because after the adherence of either one, the surface is less vulnerable to colonization by the other. Attachment of bacteria to medical materials sparks an inflammatory response by the host tissue [93], however, a slimy layer known as a biofilm develops that defends the pathogen against host defenses and antimicrobial agents [94]. This complicated process is divided into two phases: the initial, instantaneous, and reversible bacterial adherence, which is time-dependent, and biofilm formation, which is irreversible, molecular, and independent of time [90, 95, 96].
This process is influenced by different aspects, including the properties of the a) microorganism themselves; in each situation, different bacteria may adhere differently to the same biomaterial, b) the target-material surface, as the same bacteria may adhere differently to different biomaterials, and c) environmental factors, thus same bacteria may adhere differently to the same biomaterial depending on circumstances such as medium, pH or temperature [6, 10, 80, 90, 97-100]. On the other hand, antimicrobial properties and inhibition of bacterial colonization of a certain biomaterial in vitro does not ensure antimicrobial effect in vivo.

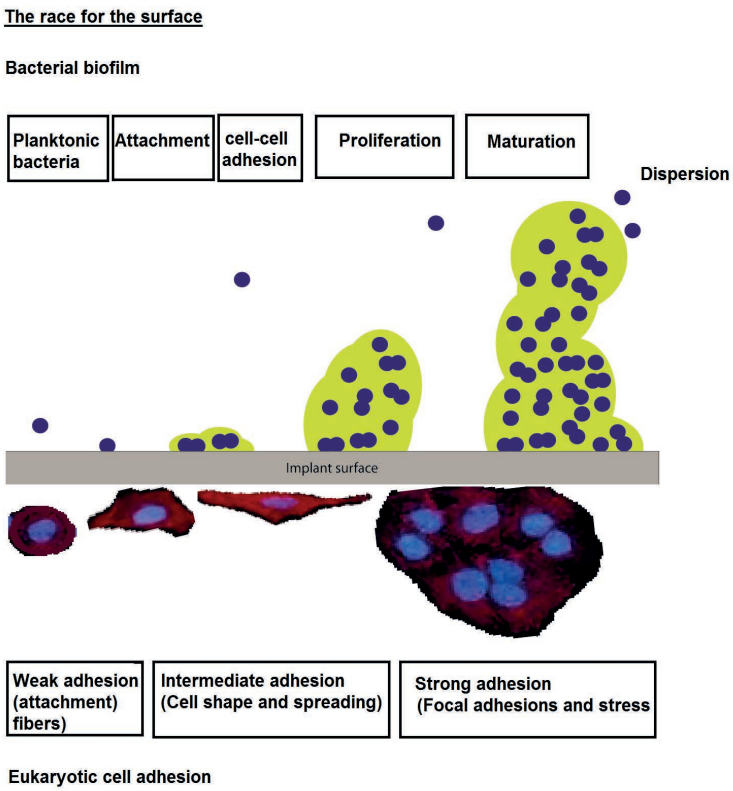


Figure 5. Representation of bacterial and human cells adhesion, two processes competing according the theory of the race for the surface. "Illustration by author."

2.3.1.1 Effects of biomaterial and microenvironmental properties

With respect to the properties of the biomaterial, we have to consider chemical composition, surface charge, hydrophobicity, and the surface roughness or physical configuration. These properties can be altered by adsorption of proteins and micro environmental properties.

2.3.1.2 Host defenses and antimicrobial agents

A biofilm protects bacteria against the host defense, antibiotics, and biocides. Macrophages and polymorphonuclear granulocytes, through the metabolism of oxygen during cell respiration, generate ROS including superoxide-anion radicals ($\bullet O_2^-$) and hydroxyl radicals ($\bullet OH$) which are capable of inflicting macromolecular damage on vital cellular components [101, 102]. ROS are the first line of defense against microorganisms, used to kill the microorganisms within phagocytic cells, as they bear highly reactive species due to their single unpaired electron. However, as ROS are also released outside the granulocytes, their production may be detrimental, because they may react with host cells and with extra-cellular molecules: An elevation in ROS that is not controlled by antioxidant defenses, may result in oxidative stress, which may stimulate pathways leading to apoptosis or even to necrosis [26, 103].
Research related to treatment or prevention of prosthesis infections are mainly targeted at the development of materials loaded or coated with anti-adhesive/antimicrobial substances called active coatings. They release pre-incorporated bactericidal agents such as antibiotics, antiseptics such as silver ion or growth factors, chemokines, or peptides that prevent the infection [10, 60, 104-107]. This method has a very important disadvantage, because the dissemination of these bactericidal agents into adjacent tissues could promote bacterial resistance or cytotoxicity [11]. A more recent line of research, what is called passive coatings, appear promising, since they reduce or prevent biomaterial infection by surface chemistry and/or structure modifications, enhancing tissue compatibility and integration, or by directly inhibiting bacterial adhesion [12, 108-111]. On the other hand, considering that microorganisms frequently infect an implant surface during surgery and start to compete for the surface before tissue integration some research is focused on protection of the prostheses through antimicrobial carriers or coatings administered during the surgical procedure [112-114].
Tissue integration and bacterial contamination of prostheses and medical devices have been mainly investigated as independent phenomena [58, 60, 104, 115, 116] . These two topics need to be simultaneously investigated during the development of new biomaterial-coating strategies because both aspects are indispensable in order to achieve a successful long-term outcome [117]. Tissue integration in the presence of perioperative bacterial contamination has been poorly studied.

3 AIMS OF THE STUDY

Presence of any foreign material in the human body provokes a competition between host cells and bacteria to colonize the biomaterial surface, with tissue integration and development of infection being their respective goals. These infections are very difficult to manage, because the development of a biofilm protects the pathogen against the host defenses and antimicrobial agents.

Specific questions to be answered:

1: Does the presence of BAG S53P4 affect bacterial adherence and biofilm formation on the surfaces of different biomaterials in vitro?

2: Do the hypoxic conditions, resembling the low oxygen levels of human bone cavities and the presence of BAG S53P4 affect human cell adhesion on different biomaterials?

3: Does the presence of bacteria affect human cell viability and adhesion to different biomaterials, and what are the correlations between the number of bacteria, the production of reactive oxygen species, and the number of dead cells?

4: Does preoperative incubation of prostheses with human cells prevent bacterial infection of the biomaterials?

4 MATERIAL AND METHODS

4.1 BIOMATERIALS

4.1.1 S53P4 BIOACTIVE GLASS GRANULES (STUDIES I, II)

S53P4 bioactive glass was used in granules sized 0.5 to 0.8 mm and <45 mm (BonAlive Biomaterials Ltd., Turku, Finland)(Figure 6). The composition of S53P4 by weight is: 53% SiO_2, 23% Na_2O, 20% CaO and 4% P_2O_5.

Figure 6. Sterile syringe full of bioactive glass granules bone substitute for filling, replacement or reconstruction of bone defects.

4.1.2 IMPLANT PLATES

Samples were tailored to the surface roughness average (Ra) by use of the same SiC abrasive papers (120grit)(Table 2).

Table 2. Properties of the different materials used in each study.

Material	Size	Roughness (Ra)	Study
S53P4 plates	5×5×1.5 mm	500–630 nm	*I, II*
Titanium	9×9×2 mm	300–400 nm	*I, II, III, IV*
PDMS	9×9×2 mm	300–400 nm	*I, II, III*
PTFE	9×9×1 mm	300–400 nm	*I, II, III, IV*

PDMS: polydimethylsiloxane, PTFE: Polytetrafluoroethylene

S53P4 bioactive glass
Bulk bioactive glass plates prepared from S53P4 bioactive glass were cut by means of a low speed diamond saw provided by the Process Chemistry Centre of Åbo Akademi (Turku, Finland).

Titanium
Titanium plates were produced using titanium deposition by the magnetron sputtering system (Stiletto Series ST20, AJA International Inc., North Scituate, MA, USA) onto 2-mm-thick 8×10-cm polished glass plates. Samples were cut by the EXAKT cutting and grinding system (EXAKT-Apparatebau, Hamburg, Germany).

PDMS and PTFE
PDMS and PTFE plates were cut from industrial polymers (ETRA, Helsinki, Finland) of 1-mm and 2-mm thick sheets, respectively.

4.2 STUDY CONDITIONS

4.2.1 THE pH STUDY (STUDIES I, II)

BAG S53P4 granules were added to the different media, and the pH of each solution was measured at time-points mentioned in Table 3. The study was done in normoxia (0.035% CO_2 and 20.9% O_2) and hypoxia (7% CO_2 and 6%

O_2) at 37 °C, resembling the situation in the normal middle ear [118-121]. The Invivo 2 Hypoxia Workstation (Ruskinn Technologies, Ltd., Sanford, ME, USA) (Figure 7) was used to produce the hypoxia. The pH study was done in triplicate.

Table 3. Description of the different media used in Studies I and II

Medium	Ratio (concentration)	Time of measurement	Study
PBS pH 7.4	1/10 (100 mg/mL)	1, 2, and 24 hours	*I*
McCoy's 5A medium*	1/5 (200 mg/mL)	4.5 hours, 2 and 4 days	*II*

*containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.
PBS: phosphate-buffered saline

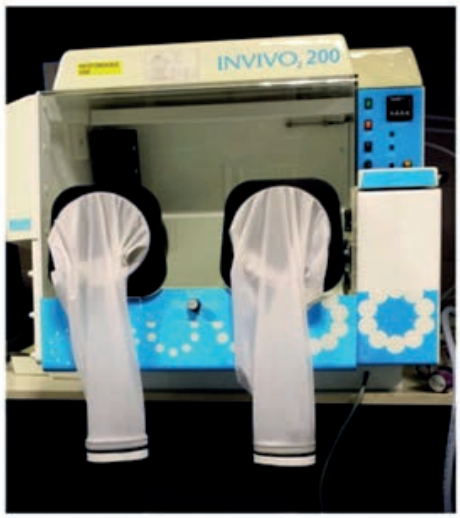


Figure 7. The Invivo 2 Hypoxia Workstation (Ruskinn Ltd., Sanford, ME, USA).

4.2.2 CULTURE OF STAPHYLOCOCCI

Two strains of *Staphylococcus* spp. were used with well-known antibiotic susceptibility profiles and a strong biofilm-forming phenotype. *Staphylococcus aureus* 15981 [122] (I, III, IV), isolated at the microbiology department of the University Clinics of Navarra, Spain, and the collection strain *Staphylococcus*

epidermidis ATCC 35984 (I) were separately cultured overnight in tryptic soy broth (bioMerieux, Marcy l'Etoile, France) at 37º C in a 5% CO_2 atmosphere. Then the solution with the bacteria was centrifuged for 10 min. at 350 g at room temperature, the pellet obtained was washed three times with PBS, and the supernatant was removed. The bacteria were suspended and diluted in 10 mM sterile PBS to obtain a 10^8 colony forming units (CFU)/mL suspension. This bacterial density was checked according to the McFarland standard by measuring the optical density of the bacterial suspension with a spectrophotometer at a wavelength of 550 nm (BioMerieux, SA Lyon, France).

4.2.3 CELL CULTURE (STUDIES II, III, IV)

Human osteosarcoma SaOS-2 cells (ECACC 890500205, Salisbury, Wiltshire, UK) (Studies II, III,IV) or primary osteoblasts (hOB) collected aseptically during orthopedic knee surgery (Study III) [123] were cultured in 10-cm-diameter Petri dishes (Corning Inc., Corning, NY, USA) using: a) McCoy's 5A culture medium containing GlutaMAX (Gibco BRL/Life Technologies Inc., Gaithersburg, MD, USA) (Study II) or b) minimal essential medium (MEM) (Studies III, IV). Both of the media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing 500 IU/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained at +37ºC in 20% or 6% O_2 in a humidified incubator in cell-culture flasks.

4.2.4 CO-CULTURE OF HUMAN CELLS AND STAPHYLOCOCCI (STUDIES III, IV)

To be able to compare the number of human and bacterial cells attached on the substrata in co-culture, a suitable media, supporting both eukaryote and prokaryote cells, had to be chosen. Three different media were tested, MEM, MEM:PBS (1:1) and PBS based on comparison of the numbers of viable SaOS-2 and *S. aureus* cells present after 48 h when incubated separately. In order to analyze viable human cells the reduction of 3–(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) dye was studied, measuring optical density at 570 nm, and for bacteria the drop plate method was used. MEM:PBS was chosen for two reasons: PBS resulted to be a hostile environment for human cells and compared to the other media, there was an increase of the number of viable bacteria when *S. aureus* was incubated in MEM.

4.2.4.1 Simultaneous co-culture of staphylococci and human cells (studies III, IV)

Ti, PDMS, and 24-well PS cell culture plates were incubated with 10^5 (SaOS-2 or hOB) cells per milliliter and with different *S. aureus* dilutions (10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU/mL) in a total volume of 2 mL of MEM:PBS (1:1) 5% FBS, 0.5% L-glutamine, and maintained in co-cultures for 4.5 hours or 48 hours. The different bacterial densities were obtained from consecutive 1:10 dilutions from a 10^8 CFU/mL *S. aureus* suspension.
A total of 10 samples of titanium, PDMS, and well plates were incubated with each dilution for 4.5 h at 37ºC to analyze cell and bacterial adhesion, or for 48 hours to study biofilm formation and cell adhesion in a static model. In each experiment, a negative control(1 mL of MEM and 1 mL of PBS), bacterial control (1 mL of 10^8 CFU/mL of *S. aureus* on PBS + 1 mL of MEM), and a cellular control (1 mL of 10^5cells/mL on MEM +1 mL of PBS) were also included.

4.2.4.2 Co-culture of staphylococci and pre-attached human cells (study IV)

First, Ti, PDMS, and 24-well PS cell-culture plates were incubated with 10^5 SaOS-2 cells/mL in 2mL of MEM supplemented with 10% heat-inactivated FBS and 1% l-glutamine containing 500 IU/mL penicillin and 0.1mg/mL streptomycin for 24 hours at +37ºC in 20% or 6% O_2 in a humidified incubator. Then, the medium was removed, and samples were washed three times with PBS to remove any non-adherent human cells. Later, samples with attached SaOS-2 cells were incubated with the different dilutions of *S. aureus* (10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 CFU/mL) in a total volume of 2 mL of MEM:PBS (1:1) 5% FBS, 0.5% L-glutamine and maintained in co-cultures for 4.5 hours or 48 hours.
A total of 10 samples of titanium, PDMS and well plates were studied in each experiment. Each bacterial dilution was covered and incubated at 37°Cfor 4.5 hours to allow bacterial adhesion or 48 hours of biofilm formation, respectively. Each experiment included a negative control, bacterial control, and cellular control with 1 mL of 10^5 cells/mL on MEM + 1 mL of PBS after 4.5 hours, (24 + 4.5) hours, 48 hours, or (24 + 48) hours.

4.3 BACTERIAL ADHERENCE AND BIOFILM FORMATION

4.3.1 EFFECT OF BAGS53P4 GRANULES AND HYPOXIC CONDITIONS (STUDY I)

Discs of titanium, PDMS, PTFE, and BAG S53P4 were placed in a bacterial suspension diluted to 10^8 CFU/mL of *S. aureus* 15981 or *S. epidermidis* ATCC 35984 in unpretreated PBS or PBS pretreated for 2 h with S53P4 at a ratio of 1/10 (100 mg/ml) in the presence of S53P4 granules and in normoxic or hypoxic conditions. The bacterial adhesion or biofilm formation were studied under four different conditions:(1) unpretreated PBS under normoxia, (2) pretreated PBS under normoxia and in the presence of S53P4 granules, (3) pretreated PBS under hypoxia, and 4) pretreated PBS under hypoxia and in the presence of S53P4 granules.

4.3.1.1 Bacterial adhesion

In well plates, the biomaterial discs were inserted into polycarbonate membrane socks (Thermo Scientific Nunc, Goteborg, Sweden) to keep them out of direct contact with the S53P4 granules and were incubated in the staphylococcal suspension for 90 min at 37ºC under the four different conditions. Afterwards, the biomaterial plates were rinsed three times with sterile PBS to remove any non-adherent bacteria.

4.3.1.2 Biofilm formation

Biofilm formation was evaluated by the static biofilm method described by Buckingham-Meyer et al. [124]. A sample of each of the four materials (titanium, PDMS, PTFE, and BAG S53P4) was placed symmetrically on sterile filter paper (Whatman qualitative grade 2, GE Healthcare Life Sciences, Little Chalfont, UK), which was located on the surface of a tryptic soy agar (TSA) plate (Becton–Dickinson, Helsinki, Finland) and inoculated with 1.5 mL of a staphylococcal solution of 10^8 CFU/mL prepared in PBS or S53P4pretreated PBS. In the presence of S53P4 granules, these were recovered from the PBS pretreated solution and were symmetrically distributed from each plate on top of the inoculated filter paper as shown in Figure 8.

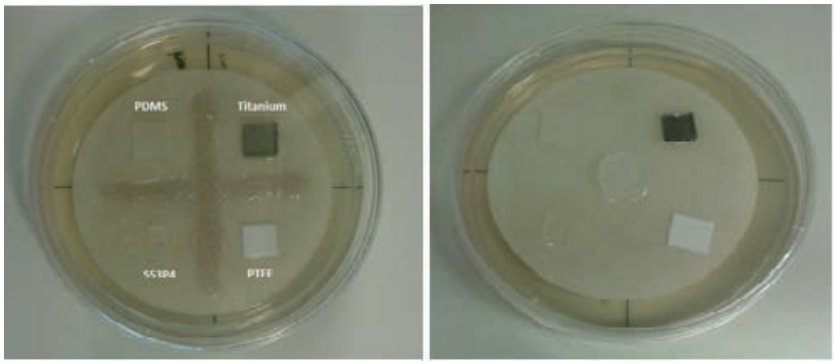


Figure 8. Static biofilm method: S53P4 granules were distributed in a cross shape in the middle of the inoculated filter paper, all at the same distance from samples of titanium, PTFE, PDMS, and S53P4 plate. The diameter of the petri dish is 10 mm.

After 24 hours of incubation at 37ºC, the filter paper was remoistened with 1.5 ml of 3 g/L (TSB). Plates were incubated for a total of 48 hours. Then, samples were rinsed gently in order to remove the non-adhered planktonic bacteria.

4.3.2 EFFECT OF THE PRESENCE OF HUMAN CELLS IN BACTERIAL ADHERENCE AND BIOFILM FORMATION

In Studies III and IV, bacterial adherence and biofilm formation on Ti, PDMS, and 24-well PS cell culture plates incubated only with *S. aureus* were compared with results from materials a) pre-incubated with SaOS-2 cells during 24 hours or b) in a co-culture with SaOS-2 cells or hOB cells [19]. After a 4.5-hour incubation time for bacterial adherence and 48 hours for biofilm formation, samples were washed three times with 200mL of PBS to remove non-adherent bacteria or cells.
Finally, to compare properly the results and to take into consideration the possible effect of trypsin, all the materials, incubated or not with human cells, were treated with 400 µL of trypsin.

4.3.3 DROP PLATE METHOD (STUDIES I, III, IV)

As Figure 9 shows, each material plate was placed in 2.5 mL of PBS and sonicated for 5 min. in an ultrasonic cleaning bath USC100T (VWR, Leuven, Belgium) at 45 kHz with a power output of 300 W. The protocols of Esteban et al.[125] and Kobayashi et al were used, which recommend that a sonication time of 1to 5 min. is ideal for dislodging biofilm bacteria without affecting bacterial viability [126]. After sonication, the number of viable bacteria was

determined by the drop-plate method [127]. Bacterial counts obtained under the differing conditions of Study I and the number of viable bacteria isolated from co-cultures and monocultures (Studies III and IV) were compared. All experiments were performed in triplicate for each condition in the comparisons.

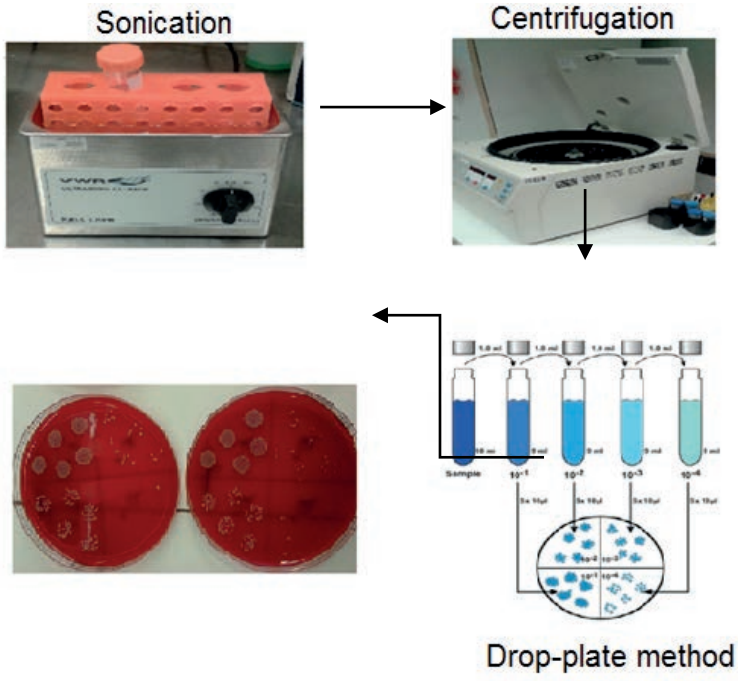


Figure 9. Schematic representation of sonication and drop plate method

4.3.4 Crystal violet (studies III, IV)

After 4.5or 48 hours incubation time, the attached bacteria were stained for 15 minutes with 1% crystal violet. Excess stain was washed off with water, and the dye bound to the adherent cells was redissolved in 300 mL of ethanol 95%. The absorbance, from aliquots of 200 mL of this solution in 96-well plates, was measured at 570 nm wavelength with a Chameleon-V microplate reader [128, 129].

4.3.5 FLUORESCENCE MICROSCOPY (STUDY I)

Once the plate surfaces were dry, they were covered with the fluorescence stain of the Live/Dead BacLight™ Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA) [130] for 15 min. A total of 10 fields per plate were photographed under a fluorescence microscope (AX70, Olympus, Tokyo, Japan) from identical locations according to a premade plan, at x40 magnification. All experiments were performed in triplicate. The surface area covered with adhered bacteria was calculated using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

4.4 ASSESSMENT OF CELL ADHERENCE, PROLIFERATION AND CYTOTOXICITY (STUDIES II, III, IV)

4.4.1 MICROSCOPIC ANALYSIS OF ATTACHED CELLS (STUDY II)

4.4.1.1 Staining of vinculin, actin, and nuclei

After incubation with SaOS-2 cells, the biomaterial plates were washed with sterile PBS to remove any nonadherent cells and were treated with 4% paraformaldehyde (ChemCruz™, Heidelberg, Germany) and TritonX-100 (Polyethylene glycol tert-octylphenyl ether) (Sigma, Steinheim, Germany)in PBS, in order to fix and permeabilize the cells. Then, Ti, PTFE, PDMS, and BAGS53P4 plates were covered first with monoclonal mouse anti-human vinculin IgG [131, 132] (1:400 in 0.1 % BSA in PBS), and second with Alexafluor 488-conjugated secondary antibody (1:400 in BSA-PBS; Molecular Probes, Eugene, OR, USA) for 1 hour and 30 min respectively. The addition of Alexafluor 568-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (blue color) (Invitrogen, Eugene, Oregon, USA) (1:40 dilution in PBS) led to subsequent visualization of the actin cytoskeleton and cell nucleus. Finally, the samples were mounted on objective slides.

4.4.1.2 Immunofluorescence analysis of cell numbers and focal adhesions

DAPI served as the marker for determining the number of attached cells, vinculin for focal contact, and actin for evaluating the cellular cytoskeleton and for quantifying the size and spreading of cells. Ten photographs were taken from the surface of each material incubated for4.5 hours, 2 days, or 4 days under the different conditions, using a CCD camera (Nikon Coolpix 8400 (Nikon, Melville, NY, USA) coupled to a fluorescence microscope (AX70, Olympus, Tokyo, Japan) at x20-x40 magnification [104]. Considering a total of 30 representative images per material and condition, 40 cells were outlined for measuring the cell area using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Unfortunately, after 4 days of incubation, it was detected that cells overlapped one another, making it impossible to differentiate and display these markers. All experiments were performed in triplicate.

4.4.1.3 Fluorescence microscopy

The attached bacteria and/or human cells on plates incubated at different proportions during 4.5 hours or 48 hours were stained with acridine orange (BD Diagnostics, Sparks, MD, USA) and then photographed from eight fields per material. Fluorescent microscope Leica DM6000 B/M (Leica Microsystems, Wetzlar, Germany), equipped with a Leica DFC420 digital camera (Leica Microsystems, Wetzlar, Germany) and with 20x objective magnification was used.

4.4.2 COLORIMETRIC METHODS

4.4.2.1 MTT assay for cell viability

SaOS-2 and hOB cell proliferation were analyzed by MTT assay considering the ability of viable cells with active metabolism to convert soluble MTT to its insoluble purple product, formazan. After incubation in a co-culture of bacteria and cells, the culture medium was removed, and samples were incubated for four hours in dimethyl sulfoxide (DMSO) (MP Biomedicals, LLC, Illkirch, France) containing 10 µL of 5 mg/mL MTT (EMD Millipore Corp., Billerica, MA, USA). Later, 300 µL of DMSO was added and, after 2 hours at room temperature, absorbance was measured with a CHAMELEON V Multilabel Microplate Reader (Hidex, Turku, Finland) with a 570 nm test and 690 nm reference wavelengths.

4.4.2.2 Lactate dehydrogenase (LDH) cytotoxicity assay

The percentage of damaged cells was evaluated with a CytoTox 96 non-radioactive cytotoxicity colorimetric assay (Promega, Madison, WI, USA). This measures lactate dehydrogenase (LDH) released into the supernatant medium from damaged cells as a biomarker for cellular cytotoxicity and cytolysis, through reading absorbance at 490 nm [133, 134].

4.4.3 FLOW CYTOMETRIC METHODS

4.4.3.1 Assessment of ROS production

ROS production was evaluated by studying the fluorescence shown by human cells, stained with Cell-ROXTM Deep Red Reagent (Life Technologies, Eugene, OR, USA), when subjected to oxidation by ROS (absorption/emission maxima at 644/665 nm). Fluorescence emission was analyzed by a BD Accuri™ C6 flow cytometer and fluorescence microscopy.

4.4.3.2 Apoptosis / necrosis detection kit

Human cells were stained with the dead cell apoptosis kit containing Alexa Fluor488 Annexin V and propidium iodide (PI) (Life Technologies) and fluorescence emission was analyzed with a BD Accuri™ C6 flow cytometer at 530 nm and 575 nm, selecting 1000 events in each sample.

4.5 STATISTICAL ANALYSIS

Statistical analyses were performed with four independent experiments per test condition. The data are reported as mean ± standard deviation (SD) and were analyzed with IBM SPSS Statistics for Windows Version 22.0 software (IBM Corp., Armonk, NY, USA). Non-parametric tests were used. Mann-Whitney or Wilcoxon tests were used for two samples, and the Kruskal-Wallis test for more than two samples. Experiments were performed by triplicate.

5 RESULTS

5.1 STUDY CONDITIONS

5.1.1 EFFECT OF BAG S53P4 ON THE PH OF THE ENVIRONMENT

As shown in Figure 10, measured pH values of both the bacteria and cell culture medium were measured in the presence of S53P4 granules in both atmospheric conditions at: a) 1 hour, 2 hours and 1 day for PBS, and b) 4.5 hours, 2 days, and 4 days in McCoy's 5A medium.

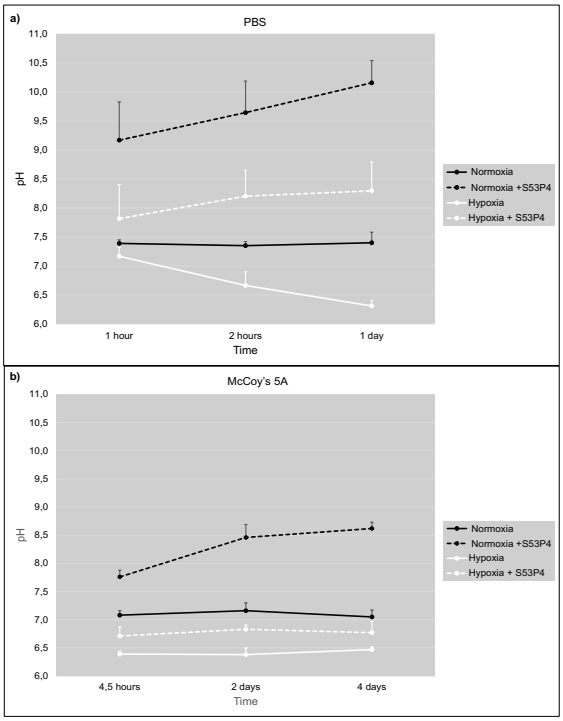


Figure 10. The pH of : a) bacteria culture medium (PBS) after 1 hour, 2 hours, and 1 day or b) cell culture medium (McCoy's 5A) at 4.5 hours, 2 days, and 4 days in normoxia and hypoxia, with or without S53P4 granules. Error bars represent the standard deviation

S53P4 granules raised the pH of both culture media compared with the other conditions ($P < 0.05$, Mann–Whitney test). In contrast, pH was lower in hypoxic conditions without any S53P4 granules compared to the other conditions ($P < 0.05$, Mann–Whitney test).

5.1.2 SELECTION OF CO-CULTURE MEDIUM

Based on the results of MTT, and the drop plate method, shown in Figure 11, MEM:PBS (1:1) was selected as the culture medium for these experiments. The percentage of viable SaOS-2 cells on the titanium surface in MEM:PBS (1:1) was reduced 11% compared with MEM.

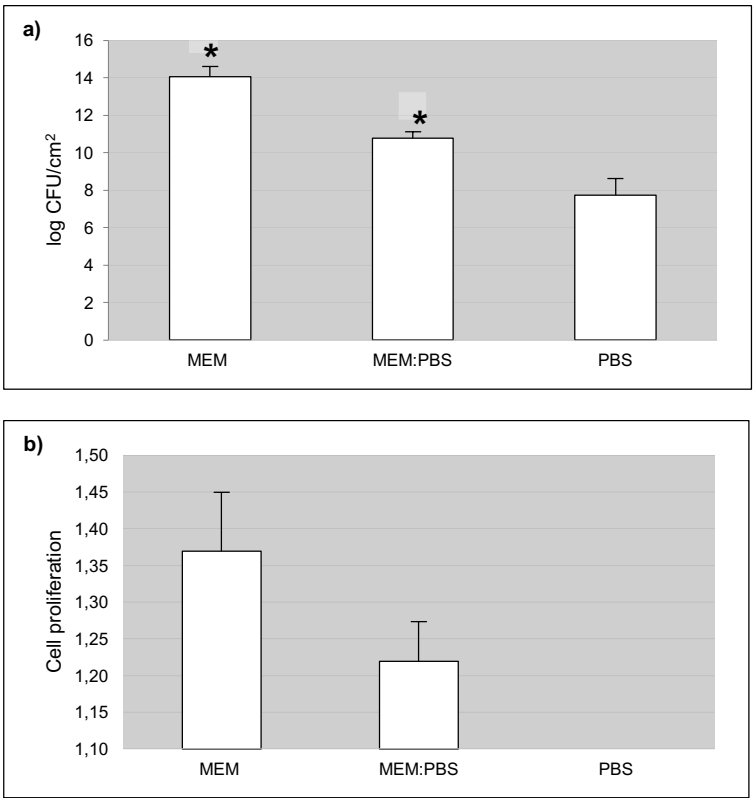


Figure 11 . Means ± SD of a) log CFU/cm^2 (drop plate method) or b) MTT assay to compare number of viable S. aureus or viable human cells, respectively, attached on titanium beyond incubation with 10^5 SaOS-2 cells or 10^8 CFU of S. aureus during 48 hour each. Comparison of different culture media: MEM, MEM:PBS, or PBS, evaluating. *P < 0.05 vs. all the other media.

This, however, was not statistically significant, as Figure 11 shows, with the results of optical density for the MTT test (P=0.083, Mann–Whitney test). No viable SaOS-2 cells were detectable on titanium in PBS. A higher number of viable *S. aureus* cells was found, by using the drop plate method, on titanium incubated in MEM than with MEM:PBS (1:1) or PBS (P=0.021 for all comparisons, Mann–Whitney).

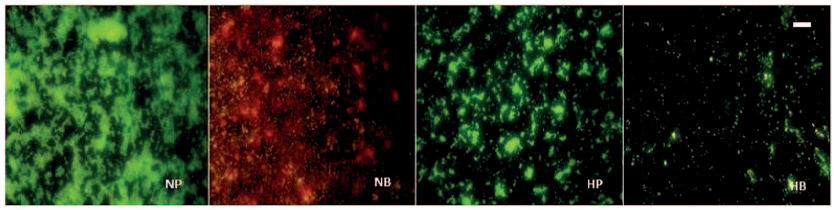
5.2 BACTERIAL ADHERENCE AND BIOFILM FORMATION

5.2.1 EFFECT OF BAGS53P4 GRANULES AND HYPOXIC CONDITIONS (STUDY I)

5.2.1.1 Bacterial adhesion

The presence of S53P4 granules reduced the number of both collection strains of *Staphylococcus* spp. attached to the different materials only with the exception of *S. aureus* incubated with titanium in normoxia (P = 0.061) and *S. epidermidis* with S53P4 plates (P = 0.4918 and P = 0.0712). On the other hand, hypoxic conditions reduced the attachment of bacteria to the materials except for *S. epidermidis* on S53P4 plates (P = 0.0992) (Fig.12).

a)



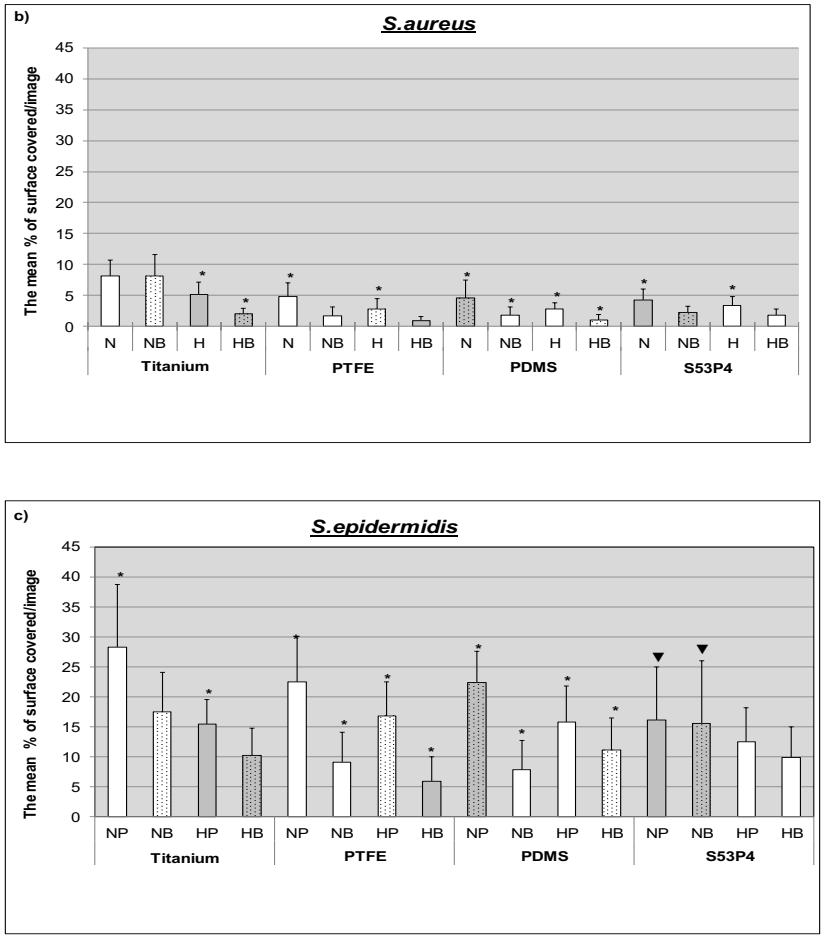


Figure 12. a) Fluorescent microscope images (×40 magnification) of S. epidermidis attached to titanium in the different conditions. Samples were stained with BacLight LiveDead stain (Molecular Probes). White bar represents 10 µm. b) The mean percentage of each biomaterial surface covered with S. aureus and S. epidermidis. Error bars represent the standard deviation. *P < 0.05 vs. all the other conditions. ▼ P < 0.05 vs. HB.

5.2.1.2 Percentage of dead bacteria

The proportion of dead bacteria on each biomaterial in different conditions is shown in Figure 13. S53P4 granules showed bactericidal effect against both collection strains of *Staphylococcus* spp., but they were effective only for: a) both collection strains attached to PDMS in both atmospheric conditions or to S53P4 plates in hypoxia and b) *S. epidermidis* incubated with Ti in normoxia.

Moreover, hypoxia induced a bactericidal effect against *S. aureus* incubated with PDMS ($P < 0.0001$) and *S. epidermidis* with PTFE ($P = 0.018$).

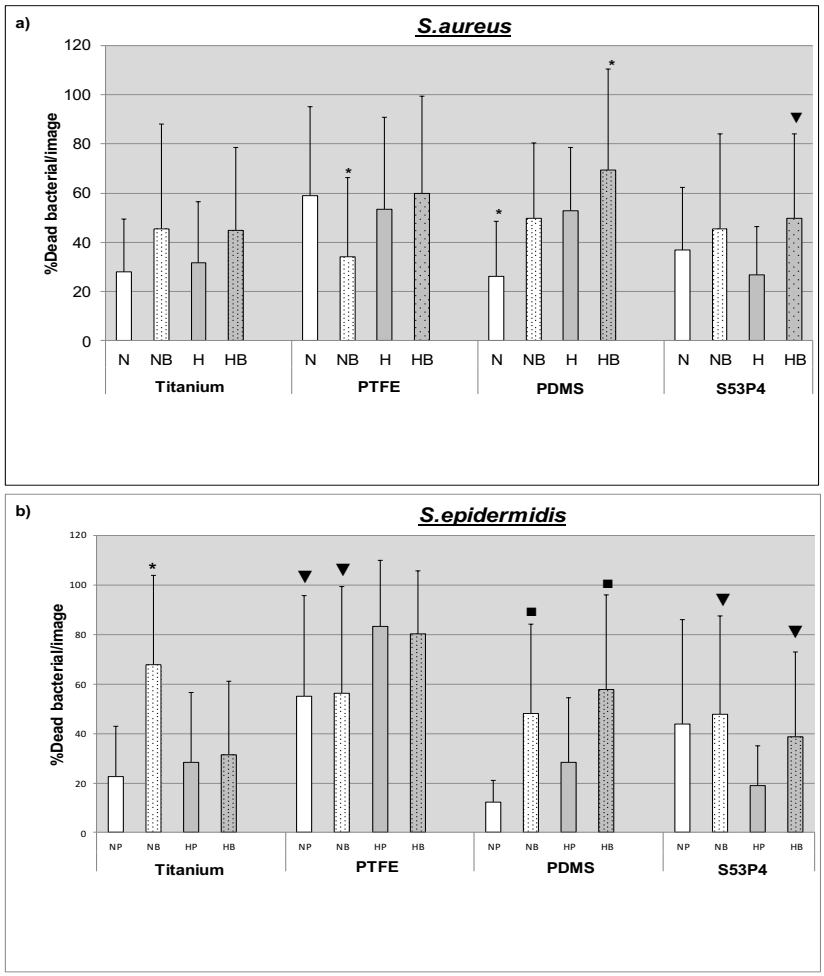


Figure 13. The mean percentage of dead bacteria adhered on each biomaterial in the different conditions, a) using S. aureus or b) S. epidermidis. Error bars represent the standard deviation. *P < 0.05 vs. all the other conditions. ■ P < 0.05 vs. N and H.▼ P < 0.05 vs. H.

5.2.1.3 Biofilm formation

Figures 14 and 15 show that S53P4 granules diminish biofilm for all materials, but reduction in CFU was statistically significant only for *S. aureus* attached to PTFE andS53P4 plates and for *S. epidermidis* attached to Ti, PDMS, or S53P4

plates, for both bacteria in normoxic conditions. This anti-biofilm effect was reduced in hypoxia. It occurred for both collection strains attached to Ti, and for *S. epidermidis* on PDMS.

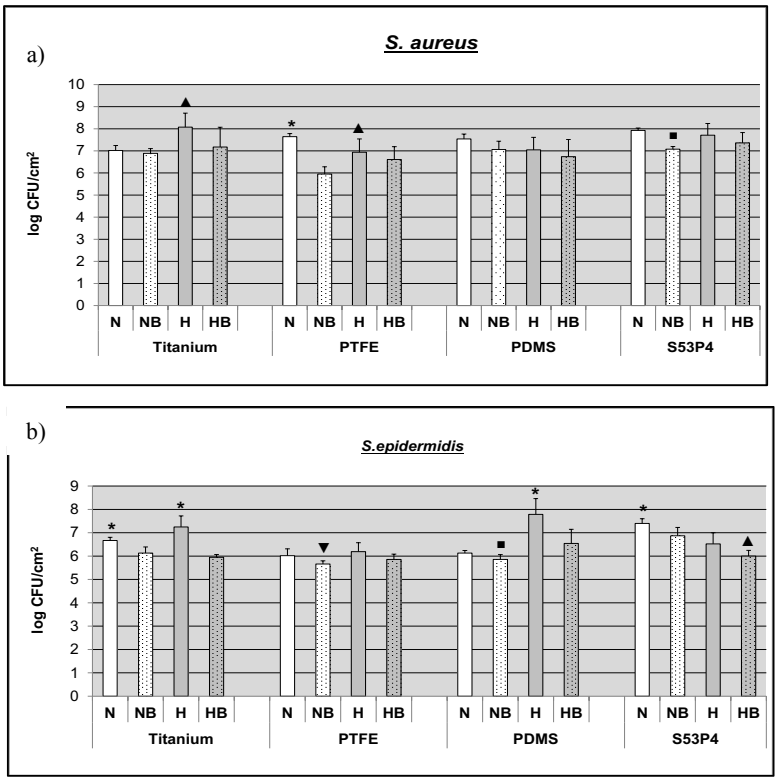


Figure 14. Biofilm formation on each material and atmospheric condition on a logarithmic scale (logCFU/cm^2) , a) using S. aureus or b) S. epidermidis. Error bars represent the standard deviation. *P < 0.05 vs. all the other conditions. ■ P < 0.05 vs. N and H. ▼ P < 0.05 vs. H. ▲ P < 0.05 vs. N and NB

Hypoxic conditions produced a decrease in biofilm formation only for *S. aureus* with PTFE (P = 0.0495), and for *S. epidermidis* with S53P4 plates (P = 0.0495). Hypoxia caused an increase in biofilm formation on titanium for both bacteria (P = 0.0495) and on PDMS for *S. epidermidis* (P = 0.0495) (Figure 15).

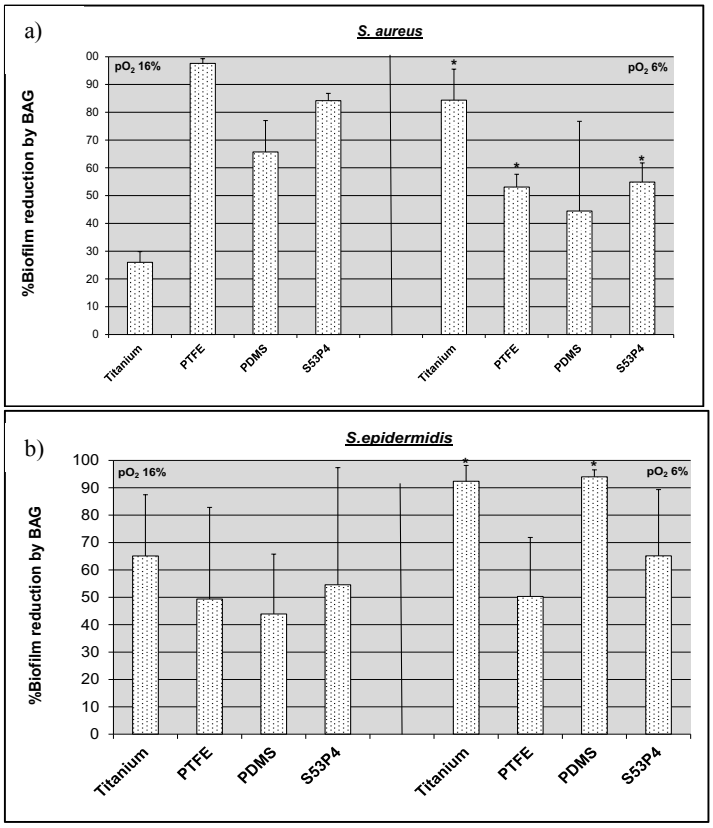


Figure 15. Biofilm-reducing effect of S53P4 on each material and atmospheric condition, a) using S. aureus or b) S. epidermidis. Error bars represent the standard deviation. ***P < 0.05 vs. pO_2 16%.**

5.2.2 EFFECT OF THE PRESENCE OF HUMAN CELLS IN BACTERIAL ADHERENCE AND BIOFILM FORMATION

The diminishing of bacterial adherence and biofilm biomass occurred when 10^2-10^8CFU/ml of *S. aureus* was incubated in co-culture with SaOS-2 cells or hOB cells. The only exceptions were a) PS with both human cell types at 4.5 hours, b) PS, and c) PDMS with hOB cells at 48 h. The diminishing effect was stronger with SaOS-2 cells on PE and PDMS than for to hOB cells. Moreover, pre-incubation of materials with SaOS-2 cells during 24 h diminished even more the bacterial adherence and biofilm biomass compared with the materials incubated simultaneously with SaOS-2 cells. This was except for simultaneous

co-culture on: a) PS with CFU/mL $\geq 10^7$ at 4.5 hours, b) titanium with CFU/mL = 10^8 at 4.5 hours, and c) PS with CFU/mL = 10^8 at 48 hours.

Furthermore, human cells reduced the number of viable bacterial cells in all materials incubated with 10^2-10^8 CFU/ml of *S. aureus* with the few exceptions including hOB cells on PDMS at 4.5 h. This effect was more pronounced for hOB cells than for SaOS-2 cells, with the exception of Ti after 4.5 h and PDMS after 48 h. On the other hand, as Figure 16 shows, the pre-incubation of SaOS-2 cells for 24 h reduced the number of viable bacteria cells in all conditions compared with findings for the materials in simultaneous co-culture.

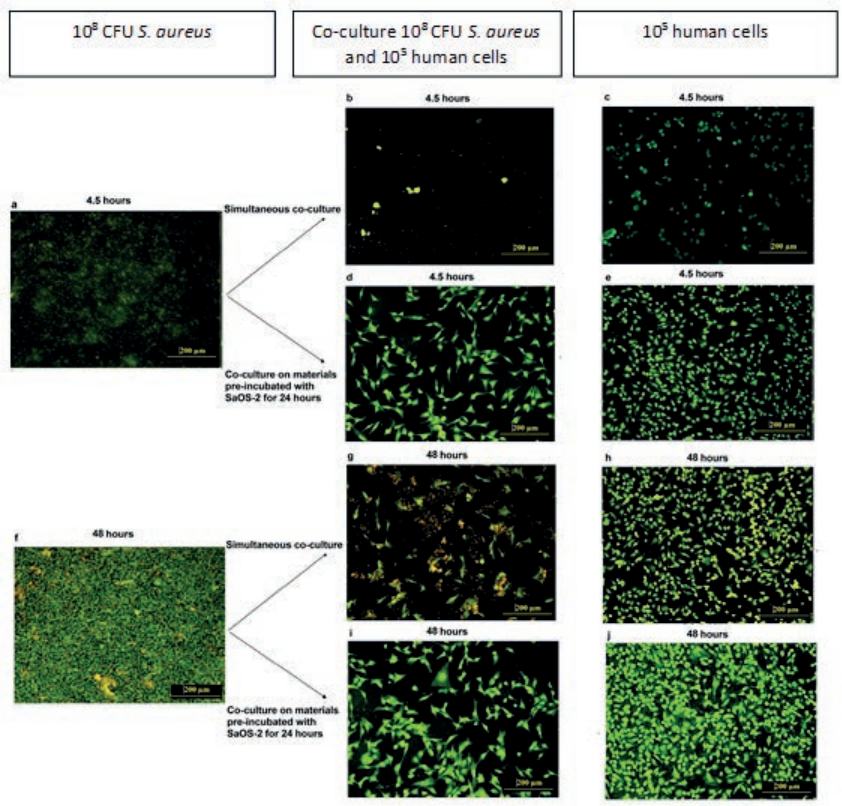


Figure 16. Fluorescence microscope images (×20 magnification) of titanium surface incubated with a) S. aureus, b) simultaneous co-culture of SaOS-2 cells and S. aureus, or c) with SaOS-2 for 4.5 hours or for 48 hours (f–h). In panels d, e, and i, j, SaOS-2 cells had been pre-incubated for 24 hours before addition of bacteria. Panels d, e show an incubation time of 4.5 hours and i, j of 48 hours. The samples were stained with acridine orange. S. aureus was incubated at a concentration of 10^8 CFU/mL and SaOS-2 cells at 10^5 cells/mL.

5.2.3 EFFECT OF PLATE MATERIAL ON BACTERIAL AND CELLULAR ADHERENCE

An increase in human cells adhered to Ti was observed when compared to other materials at 4.5 h ($p < 0.05$) with the exception of hOB cells in the presence of $\geq 10^3$ CFU/mL of *S. aureus* where there were no statistically significant differences. After 48 h, the situation changed: PS and PDMS showed an elevation in attached hOB cells or PDMS in SaOS-2 cells; the number of human cells adhered to different materials was however, similar with CFU/mL being $\geq 10^6$ ($P > 0.05$).

The increase in bacterial concentration leads to diminishes human cell adherence. Considering the SaOS-cells, with their increase in bacterial concentration up to CFU/mL $\geq 10^7$, the material that favors the most adherence always favors all adherence tested, including cells, bacteria, and biofilm mass. On the other hand, such correlation for hOB cells was not observed.

5.3 ASSESSMENT OF CELL ADHERENCE, PROLIFERATION AND CYTOTOXICITY (II, III, IV)

5.3.1 EFFECTS OF S53P4 BIOACTIVE GLASS ON OSTEOSARCOMA (SAOS-2) CELL AND BIOMATERIAL-SURFACE INTERACTION

5.3.1.1 Measurement of cell spreading and attached cells

Figure 17 shows cells cultured on titanium for each condition at 4 days, where arrangement of the actin cytoskeleton is stained with red phalloidin and nuclei stained with blue DAPI. On the other hand, in the same image the effect of S53P4 granules on the spreading and the attachment of cells on each biomaterial surface under normoxic and hypoxic conditions at 4.5 hours, 2 days and 4 days can be seen.

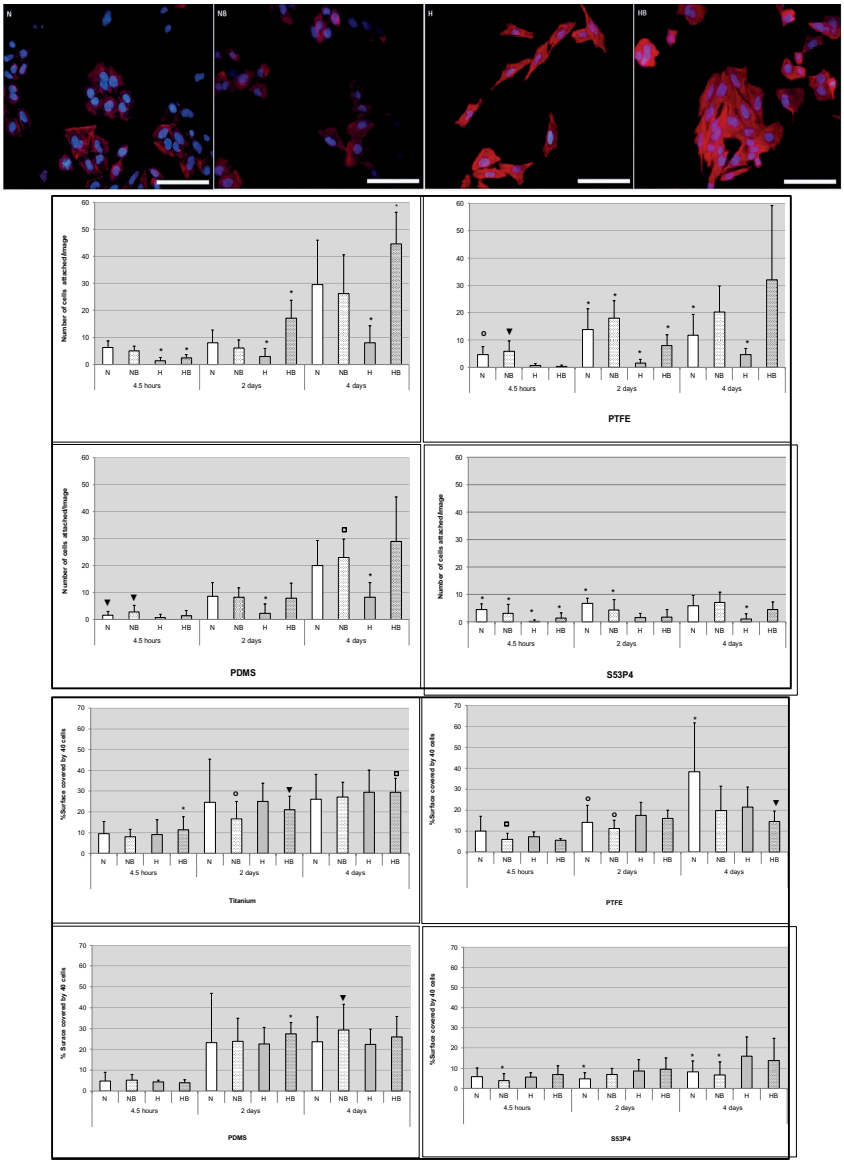


Figure 17. a) Fluorescence microscope images of titanium surface incubated with SaOS-2 cells (×20 magnification) at 4 days. Cells were stained with Alexafluor 568-conjugated phalloidin for actin (red) and with DAPI for nuclei (blue), white bar represents 100 µm, b) the mean number of cells attached to four different biomaterials and c) the mean percentage of surface covered by 40 cells measured per image in normoxia and hypoxia, with and without S53P4 granules, at 4.5 hours, 2 hours, and 4 days. Error bars represent the standard deviation. *P < 0.05 vs. all the other conditions. o P < 0.05 vs. H and HB. s P < 0.05 vs. N. ▼ P < 0.05 vs. H.

At 4.5 hours, the presence of S53P4 granules in normoxic conditions, diminished attachment of cells and cell spreading on S53P4 plates and increased numbers of attached cells in PTFE. After the same time, in hypoxia, the spreading and the attachment of cells increased on titanium and cell adhesion rose on S53P4 plates.
After 2 days, cell spreading increased on S53P4 plates and cell attachment on PTFE. In hypoxic conditions, increased cell spreading occurred on PDMS and increased attachment of cells in Ti, PTFE, and PDMS. Finally, at 4 days, in normoxia, a reduction in cell spreading on PTFE and S53P4 was found, on the other hand, the number of cells adhered on PTFE and PDMS increased.
In general, hypoxic conditions reduced the number of attached cells for all materials after different time periods. However, the surface area of cell spreading was not clearly affected. Table 4 shows all the P-values.

Table 4a. P-values for comparison of cell attachment using Mann–Whitney test.

a) Mean number of cells attached to material per image					
	Conditions	**Titanium**	**PTFE**	**PDMS**	**S53P4**
4.5h	N vs. NB	0.107	0.201	0.124	0.014
	H vs. HB	0.001	0.076	0.406	0.005
	N vs. H	<0.001	<0.001	0.008	<0.001
2 d	N vs. NB	0.066	0.037	0.843	0.07
	H vs. HB	<0.001	<0.001	<0.001	0.262
	N vs. H	<0.001	<0.001	<0.001	<0.001
4 d	N vs. NB	0.459	≤0.001	0.049	0.271
	H vs. HB	<0.001	<0.001	<0.001	<0.001
	N vs. H	<0.001	<0.001	<0.001	<0.001

Table 4b. P-values for comparison of percentage of surface covered by 40 cells using Mann–Whitney test.

b) Mean percentage of surface covered by 40 cells per image					
	Conditions	**Titanium**	**PTFE**	**PDMS**	**S53P4**
4.5 h	N vs. NB	0.584	0.032	0.052	0.021
	H vs. HB	0.013	0.074	0.277	0.571
	N vs. H	0.303	0.596	0.169	0.508
2 d	N vs. NB	0.451	0.283	0.139	0.002
	H vs. HB	0.030	0.491	0.019	0.400
	N vs. H	0.068	0.042	0.149	≤0.001
4 d	N vs. NB	0.169	<0.001	0.102	0.008
	H vs. HB	0.745	0.002	0.160	0.248
	N vs. H	0.139	<0.001	0.725	0.008

N: Normoxic conditions without S53P4 granules; NB: Normoxic conditions with S53P4 granules; H: Hypoxic conditions without S53P4 granules; HB: Hypoxic conditions with S53P4 granules.

5.3.1.2 Vinculin-containing adhesion junctions

Figure 18) shows vinculin stained focal contacts in the peripheral regions of cells attached only on titanium and PTFE surfaces, because of the autofluorescence of the PDMS and S53P4 surfaces. Figure 18b shows the number of vinculin-stained focal contacts per cell in different conditions. With a series of similar results for all time points, a higher number of vinculin-stained focal contacts at the edge of cells could be visualized: a) attached on titanium compared with PTFE, b) in normoxic conditions compared with hypoxic conditions ($P = 0.026$ for titanium and $P < 0.001$ for PTFE at 2 days; $P < 0.001$ for both materials at 4 days) and c) considering hypoxic conditions, in the presence of S53P4 granules ($P < 0.001$ for both materials at 2 days; $P = 0.004$ for Ti and and $P < 0.001$ for PTFE at 4 days).

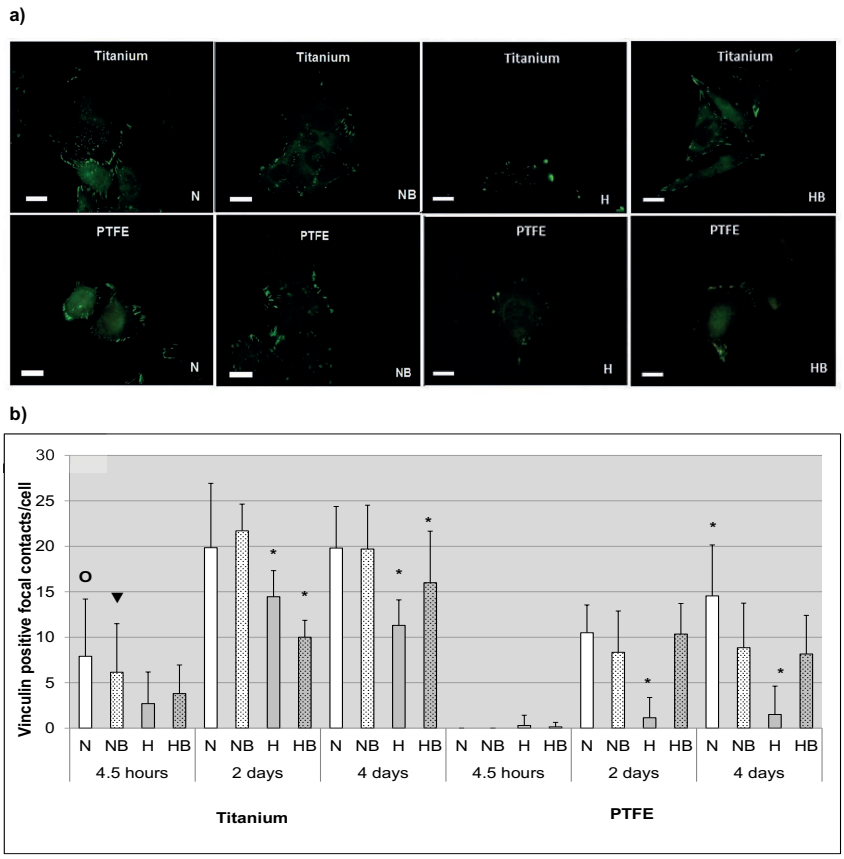


Figure 18. a) Distribution of vinculin-stained focal contacts in SaOS-2cells attached to titanium or PTFE in normoxic or hypoxic conditions and in the presence or absence of S53P4 granules at day 4 (×40 magnification). The white bar represents 20 µm length. b) The mean percentage of vinculin-stained focal contacts per each SaOS-2 cell adhered to titanium or PTFE surface. Error bars represent the standard deviation. Asterisk statistically significant difference. *P < 0.05 vs. all the other conditions. o P < 0.05 vs. H and HB. s P < 0.05 vs. N. ▼ P < 0.05 vs. H.

The number of vinculin-stained focal contacts was unaffected by the presence of S53P4 granules in normoxic conditions, with the exception that their presence produced a decrease in the number of vinculin-stained focal contacts on the PTFE surface at 4 days (P = 0.002).

At 4.5 h, vinculin-stained focal contacts were observable only at the periphery of the cytoplasm of cells cultured on Ti and mainly at the main edges of the cells but not in their cellular extensions. After 4.5 h, vinculin-containing focal contacts were more numerous and were visible throughout the entire cell contours, although they were still at a higher density at the periphery of cells at the end-contact points of the actin filament fibers.

5.3.1.3 Arrangement of the actin cytoskeleton

Figure 19 shows examples of the organization of the actin cytoskeleton in SaOS-2 cells attached to the different materials in different conditions and at different times. At 4.5 h, only cells attached to titanium, in normoxia, showed a polygonal shape and an actin cytoskeleton in the periphery of the cells, but without much evidence yet of active spreading. However, cells cultured on all other materials still had an immature round shape without any apparent organization of the actin cytoskeleton or spreading.

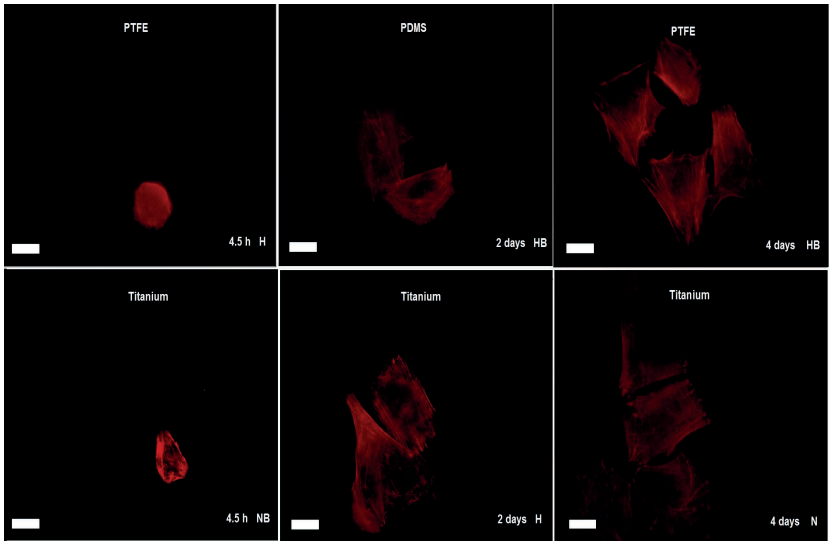


Figure 19. Arrangement of actin cytoskeleton of SaOS-2 cells cultured on polytetrafluoroethylene (PTFE), titanium and polydimethylsiloxane (PDMS) (×40 magnification). The samples were stained with Alexafluor 568-conjugated phalloidin. The white bar represents 20 µm length.

After 2 days, a parallel organization of actin filaments building a polygonal morphology of SaOS-2 cells was found, although it was most evident on cells attached to Ti compared with other materials and in normoxic conditions compared to hypoxia. At 4 days the main change compared with 2 days was that PTFE and PDMS surfaces showed a more parallel orientation and more polygonal morphology of the actin filaments and well-defined stress fiber bundles. This situation was less evident in hypoxic conditions but the presence of S53P4 may counteract this negative effect on arrangement of the actin cytoskeleton, produced by hypoxia, on the PTFE and PDMS surfaces.

5.3.2 EFFECTS OF BACTERIAL EXPOSURE ON CELL PROLIFERATION AND CYTOTOXICITY

5.3.2.1 Cell proliferation and viable cells

The incubation of *S. aureus* and human cells (SaOS-2 or hOB) in co-culture on Ti, compared with the same biomaterial incubated only with the human cells or the bacteria, reduced the proliferation of human cells and the attached *S. aureus* on Ti at 4.5 h and 48 h, as it can be observed in the fluorescence images in Figure 20. A significant reduction of cellular proliferation was confirmed by an MTT assay and flow cytometry in most of the cases (Figure 21).
Co-culture with *S. aureus* CFU/mL $\geq 10^2$ diminished the number of adhered SaOS-2 cells ($P = 0.021$) in all materials tested, and it was more evident when SaOS-2 cells were exposed to *S. aureus* CFU/mL $> 10^5$. Fluorescence microscopy showed that attachment of hOB cells supported better the presence of bacteria. Flow cytometry showed that CFU/mL $\geq 10^2$ produced decreased numbers of adhered hOB cells at 4.5 h, with the exception of Ti, where this reduction started at CFU/mL $\geq 10^3$. Finally, this decrease was also manifest on PS incubated with CFU/mL $\geq 10^2$, on Ti and PDMS with CFU/mL $\geq 10^3$, all when incubated for 48 h.
When the materials were pre-incubated during 24 hours with SaOS-2 cells and thereafter simultaneously incubated with added cells and bacteria, one could observe a reduction in the number of viable SaOS-2 cells in the same way that was observed in simultaneous co-culture, and, as compared with SaOS cells incubated alone (Figure 16); MTT assay showed that an increased bacterial density did not reduce cell proliferation, with PS and PDMS (Kruskal-Wallis test for PS: $P = 0.685$ and $P = 0.100$, 4.5 and 48h respectively, and for PDMS: $P = 0.158$ and 0.246). However, surface incubated with CFU/mL $>10^6$ diminished the number of viable SaOS-2 cells compared with CFU $= 10^2$. Finally, the number of viable SaOS-2 cells attached to Ti was unaffected by bacterial density in the range of CFU $= 10^3$-10^8 ($P = 0.187$ for 4.5 hours, and 0.550 for 48 h).

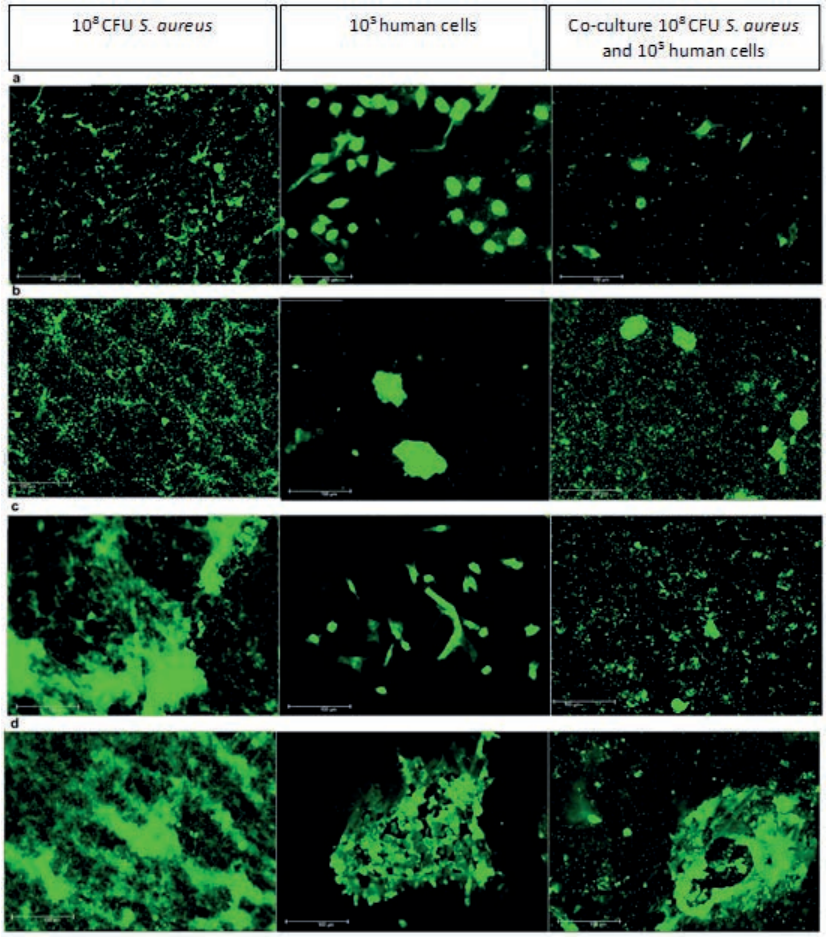
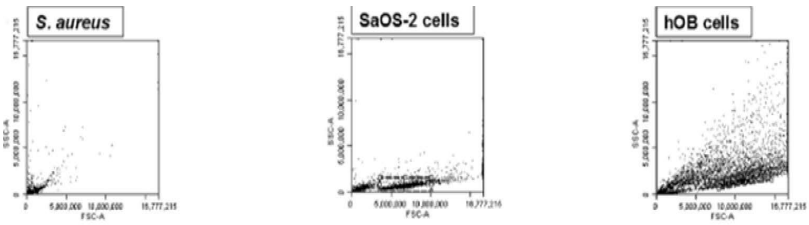


Figure 20. Examples of fluorescence microscope images of a) the titanium surface incubated with S. aureus only, with human cells only, and in co-culture with S. aureus and human cells at 4.5h: a) SaOS-2 cells, b) hOB cells and 48 h: c) SaOS-2 cells, d) hOB cells. The samples were stained with acridine orange. S. aureus was incubated at a concentration of 10^8 CFU/mL and human cells at 10^5 cells/mL.

a)



b)

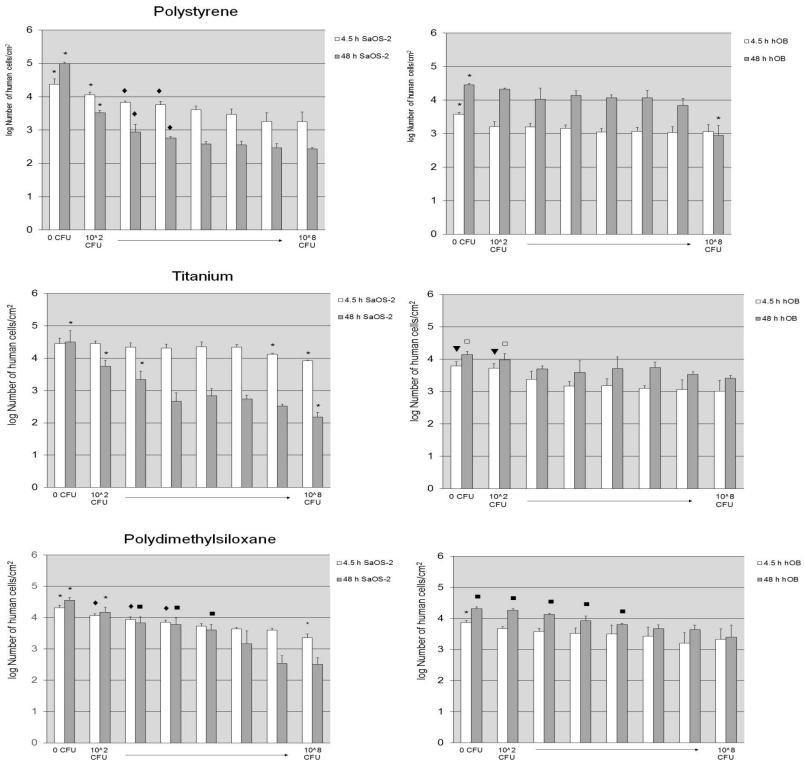


Figure 21. Fluorescence-activated cell-sorting (FACS) analysis of (a) S. aureus, SaOS-2 cells, and hOB cells after incubation with PS for 48 h. FACS analysis for (b) SaOS-2 and hOB cells with PS, titanium, and PDMS alone or incubated for 4.5 h and 48 h at a concentration of 10^2-10^8 CFU/mL of S. aureus. Results are means ± SD of experiments performed in quadruplicate. * P < 0.05 vs. all CFU. ♦P < 0.05 (≤ 10^4 CFU vs> 10^5 CFU). ○ P < 0.05 (0 CFU vs ≥ 10^3 CFU) (10^2 CFU vs 10^8 CFU). ▼P < 0.05 (≤ 10^2 CFU vs > 10^2 CFU). ● P < 0.05 (≤ 10^5 CFU vs ≥ 10^6 CFU). ■ P < 0.05 (≤ 10^2 CFU vs ≥ 10^3 CFU)(10^3 CFU vs > 10^4 CFU) (≤ 10^5 CFU vs ≥ 10^7 CFU)

5.3.2.2 Cytotoxicity induced by the presence of bacteria

The collection strain of *S. aureus* was cytotoxic to SaOS-2 and hOB cells on PS, titanium, and PDMS, as demonstrated by increased LDH levels (Figure 22). In SaOS-2cells, the increase in LDH was visible on PS and PDMS with a *S. aureus* concentration $\geq 10^6$CFU/mL and on Ti with a concentration $\geq 10^7$CFU/mL for 4.5 h. In the same way, *S. aureus* CFU/mL $\geq 10^7$ increased LDH in hOB compared to results with CFU/mL $\leq 10^5$ except on PS (P = 0.226). On the other hand, only hOB cells showed an elevation in LDH when incubated on PDMS with CFU/mL $\geq 10^5$.

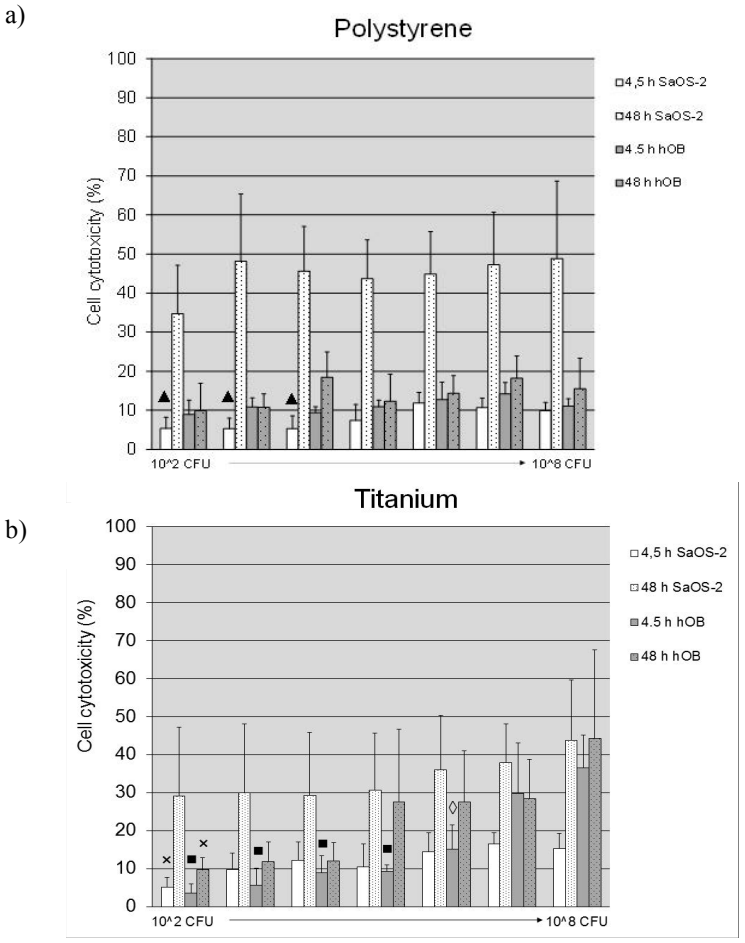


Figure 22 a and b: LDH values of human cells (SaOS-2 and hOB) on a) PS and b) Ti in the absence of bacteria or incubated for 4.5 h or 48 h with S. aureus at a concentration of 10^2-10^8CFU/mL. Results are means ± SD of experiments performed in quadruplicate. ▲ P < 0.05 (≤ 10^4 CFU vs ≥ 10^6 CFU). × P < 0.05 (10^2 CFU vs≥ 10^7 CFU). ■ P < 0.05 (≤ 10^5 CFU vs ≥ 10^7 CFU). ◊ P < 0.05 (10^6 CFU vs 10^8

c)

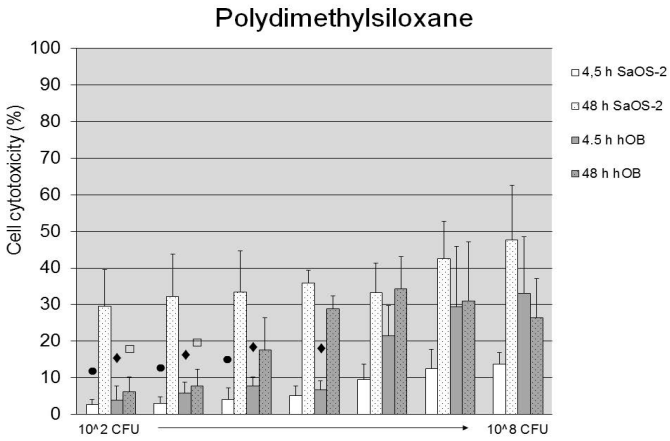


Figure 22 c. LDH values of human cells (SaOS-2 and hOB) on c) PDMS in the absence of bacteria or incubated for 4.5 h or 48 h with S. aureus at a concentration of 10^2-10^8CFU/mL. Results are means ± SD of experiments performed in quadruplicate. ● P < 0.05 (≤ 10^4 CFU vs ≥ 10^6 CFU). ♦ P < 0.05 (≤ 10^5 CFU vs ≥ 10^6 CFU). □ P < 0.05 (≤ 10^3 CFU vs ≥ 10^5 CFU)

Furthermore, there was an elevation in LDH produced by pre-incubated SaOS-2 cells attached to all materials due to the presence of bacteria. An increase in bacterial concentration was not correlated with an increase in cytotoxicity on PS ($P = 0.494$ and $P = 0.275$ for 4.5 hours and 48 hours, respectively) and PDMS ($P = 0.361$ and $P = 0.457$ for 4.5 hours and 48 hours). Only pre-incubated SaOS-2 cells attached to titanium for 4.5 hours showed an elevation in LDH in the presence of S. aureus$\geq 10^6$ CFU/mL ($P = 0.388$ for 10^2-10^5 CFU/mL for 4.5 hours and $P = 0.382$ for 10^2-10^8 CFU/mL for 48 hours). Cell cytotoxicity was more evident in the 48-hours experiment compared to 4.5 hours in the same conditions of staphylococcal density and of material except (on)¬ titanium incubated with $\geq 10^7$ CFU/mL ($P = 0.248$ for 10^7-10^8CFU/mL).

5.3.2.3 Apoptosis and necrosis of human cells provoked by the presence of bacteria

The collection strain of *S. aureus* caused signs of cellular damage in human cells (Figure 23). Co-culture with increasing bacterial density up to 10^5 CFU/mL caused an increase in the number of cells affected by apoptosis and necrosis, contrary to what happens on Ti, where *S. aureus* diminished the

early stage of apoptosis of hOB cells. At 48 h, the early-stage apoptosis of SaOS-2 cells on PS was unaffected by bacteria ($P = 0.327$); nevertheless it diminished for hOB cells in all materials at bacterial concentrations $> 10^5$ CFU/mL. The number of cells affected by apoptosis/necrosis increased, associated with rising bacterial density, but the number in early apoptotic state decreased.

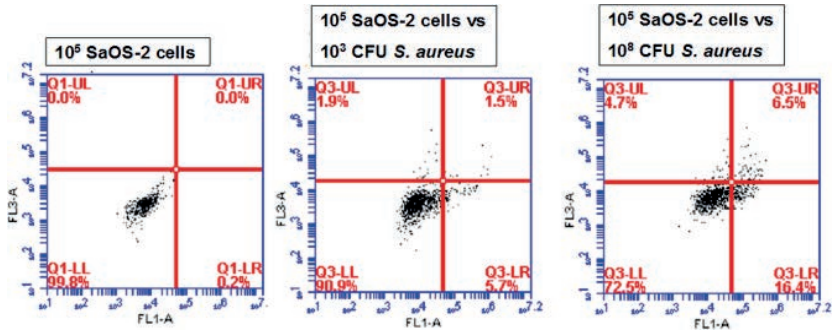


Figure 23. Fluorescence-activated cell sorting (FACS) analysis of SaOS-2 cells in the absence of bacteria and in co-culture with 10^3 CFU/mL or 10^8 CFU/mL of S. aureus.

5.3.2.4 Production of ROS

Bacteria provoked an increase in ROS production in both human cell types at 4.5 h and 48 h (Figure 24) with the unique exception of SaOS-2 cells attached to titanium which remained unaffected by bacteria at 4.5 h. Figure 26 shows that, after 48 h, there occurred an elevation in ROS released from SaOS-2 cells in co-culture with $\geq 10^3$ CFU/mL of *S. aureus* on titanium, with $> 10^6$ CFU/mL on PDMS, and with 10^8 CFU/mL on PS.

Considering hOB cells, after 4.5 h, an elevation in ROS was detectable when human cells were incubated with $\geq 10^3$ CFU/mL of *S. aureus* on titanium and with $\geq 10^7$CFU/mL on PDMS ($P = 0.021$ for all comparisons). After 48 h, the presence of *S. aureus* $\geq 10^7$CFU/mL produced an increase in ROS on both titanium and PDMS.

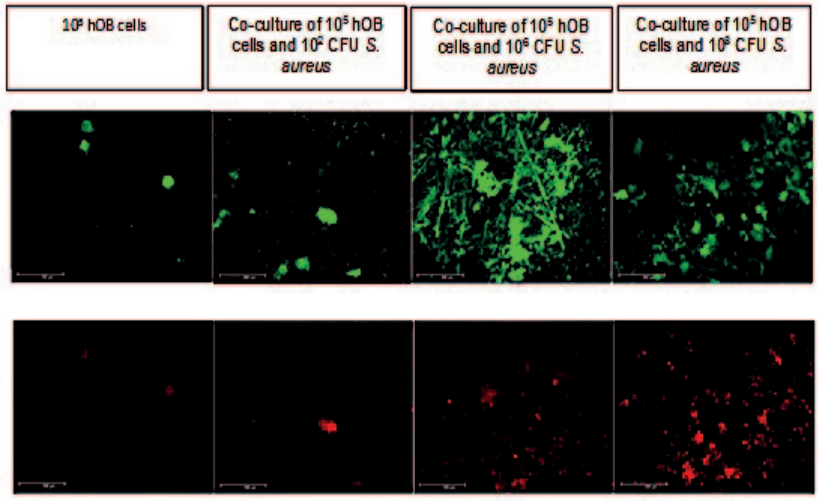


Figure 24. Fluorescence microscopy images of hOB (10^5cells/Ml) incubated on Ti in the absence or presence of S. aureus. Staining with (a) acridine orange or (b) CellROX™ Deep Red Reagent for detecting ROS.

5.3.2.5 Relation between apoptosis-necrosis and ROS

A negative correlation between ROS production and the number of SaOS-2 or hOB cells suffering from apoptosis and necrosis as a value of lower right (LR)-apoptosis occurred in the presence of *S. aureus* on all materials and at all bacterial concentrations, with the exception of hOB cells on PS. This correlation was visible by the end of the 48-hour experiment; however, after the first 4.5 hours it was detected only in SaOS-2 cells attached to titanium and hOB cells on PS.
Similarly, ROS produced by human cells corresponded well with LDH values of hOB cells on Ti and PDMS ($P < 0.01$ for both materials at 4.5 h and 48 h); however, this correlation did not appear for PS and for SaOS-2 cells adhered to PS or Ti at 4.5 h and for PDMS at 48 h.

6 DISCUSSION

The introduction of a manufactured medical device into the human body represents the starting shot for a race where tissue cells and bacteria compete to reach the finish line, which implant-surface colonization represents. The outcome of the race is tissue integration or development of implant-related infection. Bacteria that colonize the surface build a protective wall known as biofilm, which protects them against the host defenses or against antibiotic treatment.
The aim of this thesis was to investigate this race, how the final result can be affected by the possible traps used by each of the two competitors, bacteria and cells: using more runners or eliminating lanes of the opposing team. This research project proposes creating an antiadherent coating on the biomaterial surface by placing more human cells in the race. This would reduce the bacterial living space available and avoid biomaterial-related infection. Osteointegration and infection of a biomaterial depends on attachment, adhesion, and spreading of human osteoblastic or bacterial cells on the biomaterial surface. These processes are governed by the physico-chemical characteristics of the implant and their extra-cellular surroundings [135-138]. The hypothesis was that the antiadherent property could be achieved by preoperatively incubating the biomaterial with host cells before implantation or when the biomaterial is already implanted by inducing the attachment of a host tissue layer influenced by the osteoconductive effect of S53P4 located around the implant.

6.1 EFFECT OF HYPOXIA ON BACTERIAL AND CELL ADHERENCE TO PROSTHESES (I, II)

Oxygen level and pH are interdependent, and both influence tissue conditions, cellular metabolism, and cell behavior. In this study, hypoxic conditions produced a significant decrease in pH of the solution due to the high CO_2 levels of the hypoxia chamber (7%), which acidifies the medium via formation of weak carbonic acid [24]. Hypoxia reduced attachment of both bacteria and human cells, reduced vinculin-containing focal adhesion contacts, and impaired the formation of a dynamic cellular fiber network of actin filaments. Cell spreading and biofilm formation are the next steps: respectively after initial attachment of human or of bacterial cells. After the first few hours, bacteria and cells adapted themselves to the alterations in the extracellular

environment through a metabolic switch which enabled them to adhere and that promoted cell spreading and biofilm formation [139].
These results go beyond those of previous reports, showing that adaptation to hypoxia among pathogenic microorganism such as *S. aureus* and other facultative aerobes is important in order for them to produce virulence factors and to develop biofilm in different and often hostile environmental niches [140-142]. This is not surprising, considering that hypoxia and inflammation are coincidental events in a number of chronic infectious diseases, due to the metabolic demands of host cells and pathogens. On the other hand, tissues and bone cavities are mainly filled with hypoxic air at an oxygen level of around 6% [120, 121] and at a high CO_2 level. Thus, it is logical to think that host cells and invading pathogens have developed the capacity to respond to low molecular oxygen levels [143, 144]. Furthermore, hypoxia induces gene expression that enhances adaptation of both host cells and bacteria, which has a significant impact on the development of both infection and inflammation.
Cell and bacterial culture experiments are normally performed at the atmospheric oxygen concentration; this factor may be considered as a potential source of error, because in most chronic infections such as in chronic mastoiditis, the O_2 level is assumed to be low [145, 146]. Thus, it is recommended that biomaterial-surface studies should be done also under hypoxic conditions.
The impact of hypoxia on tissue integration and infection of biomaterials requires more attention, as it could influence the race for the surface in many ways such as by modifying the expression of virulence and antibiotic resistance genes [147] or modifying the cellular metabolism [148].

6.2 EFFECT OF BIOGLASS TO BACTERIAL AND HOST CELLS (STUDIES I, II)

S53P4 bioactive glass in granule form has shown osteoconductive and antimicrobial properties, which may provide a successful strategy for the treatment of bone and prosthetic joint infections [20, 31, 149, 150]. The effectiveness of S53P4 as a bone substitute in cavitary bone defects, especially in craniofacial reconstruction like mastoid obliteration and orbital floor reconstruction, has been corroborated in various clinical studies [63, 68, 149,151-153].
In infected cavities, the acidic environment is devastating for the bone. Actually, the pH level and O2 level play a key role in clinical wound infection. The accumulation of bacteria leads to increased consumption of oxygen and increased hypoxia that in turn produces an acid environment in bone cavities. S53P4 neutralizes this acidic environment and exerts an antimicrobial effect, which inhibits accumulation of bacteria. In clinical use in infected cavities, it may help to prevent the development of bacterial resistance to antibiotics and

cytotoxicity or damage to surrounding tissues, because its constituent chemicals are all found already in the body [20, 24, 149, 154-156]
Bone cells are extremely strongly affected by decreased pH. Hypoxic conditions lead to an unbalanced acid-base equilibrium in bone because the OH(-) ions of hydroxyapatite contained in bone are insufficiently available to neutralize the excess of metabolic H(+). Mineral deposition by osteoblasts is inhibited by the local acidic environment as well. The negative impact of acidosis on the skeleton, because of physiochemical dissolution of bone mineral, is bone destruction [157-159]. In cultured osteoblasts, Arnett et al described acidosis causing the increased hydroxyapatite solubility and selective inhibition of alkaline phosphatase, which is required for mineralization. The present experiments show that S53P4 granules shift the acid-base balance in the alkaline direction, promoting mineralization through enhancing adherence of SaOS-2 cells. In contrast to hypoxic conditions, in normoxia, bioactive glass had no effect in vinculin-containing focal-adhesion contacts and the production of an actin filament network after the first hours. Brandao-Burch at al. have shown that uncorrected acidosis caused by hypoxia blocks the growth and differentiation of osteoblasts (and thus bone formation), due to the fact that deposition of alkaline mineral in bone by osteoblasts is reduced, and dissolution of hydroxyapatite is raised [160]. The result is that the bioglass effect was more evident on the initial attachment of bone cells, and cell spreading showed no effect from the presence of S53P4 granules in either atmospheric condition.
S53P4 granules have been able to reduce the bacterial adherence and biofilm produced by *S. aureus* and *S. epidermidis* on different substrates in vitro. This inhibition of bacterial growth is advantageous and has resulted in avoidance of infections in implantation of biomaterials in necrotic, chronically infected spaces [20, 161]. This agrees with Coraça-Huber et al, who found a clear growth-inhibitory effect on *S. aureus* biofilms by S53P4, although their study was conducted only in normoxia [25]. In the present study, S53P4 reduced bacterial adhesion in hypoxia. Bacterial cells may be more sensitive, and so they suffered damage in alkaline environments [162, 163]; however, bioglass shifted the pH close to neutral, and thus other properties may have accounted for the antimicrobial effect [164]. These include the increase in osmotic pressure [18, 24, 64, 65], physical damage to the cell wall by S53P4 debris, and an increase in the ionic strength [165], which is based on a combination of inert SiO2 and oxygen radicals generated from bioglass [166-167]. All these antimicrobial properties also affect the bacteria that are already adhered to the implant surface. Our findings show that the bioglass effect was more evident for the attachment of bacteria than for the consequent biofilm formation. Biofilm formation was reduced mainly in normoxia, while in hypoxia, the reduction of bacterial adhesion was not well correlated with the biofilm formation, the same as found by Hu et al. [162]. In some cases, a few bacteria were enough to proliferate and develop a mature biofilm due to the biofilm-promoting effect of the substratum.

6.3 EFFECT OF BACTERIA ON INTEGRATION OF HOST TISSUE CELLS INTO PROSTHESES (STUDY III)

The effect of bacterial adhesion and biofilm formation on the tissue integration of prostheses has been neglected until the last few years. Bacteria can attach to a biomaterial surface at different time points either during the implantation or thereafter [168, 169]. A perioperative bacterial contamination model has been the choice of most researchers, like Yue et al. In their model, bacterial adherence is allowed prior to human-cell adhesion [170-174, 14, 112, 169]. Zhao et al. have studied hematogenous spreading of bacteria, which could be another route by which cells are attached to a biomaterial surface before adhesion of bacteria [169, 171, 175]. However, some biomaterials are implanted in contaminated tissue such as an infected cavity, where simultaneous competitive adhesion of bacteria and host tissue cells occurs. This should, in fact, become an important design parameter for improving implanted devices [176, 177].One of the main objectives of this thesis was therefore to develop a plausible in vitro approach to the Anthony Gristinas concept of the race for the surface, by using a simple experimental setting. [9, 92, 133, 178]. Moreover, this model hopes for a fair race, without any advantage such as a runner previously located in the biomaterial or a surface doped because of favorable culture conditions. In contrast to other researchers such as Subbiahdoss et al, who used a modified culture media to avoid dead cells [112],this experimental model looks for a fair race, and the culture conditions were studied and selected in order to avoid any doping of bacteria or cells in the race.

It seems that in a simultaneous competition between bacteria and human cells, the attachment and the viability of either one, on the biomaterial surface, is diminished, and this reduction depends on bacterial density. Seven bacterial dilutions were tested starting from 10^2 CFU/cm^2 up to 10^8 CFU/cm^2, considering that < 10^2 CFU of S. *aureus* are needed to infect a prostheses, less than half the amount needed for a surgical infection without the presence of an implant, and that 10^8 CFU/cm^2 has been selected in several other studies. [80, 179][10, 180]]. Other researchers such as Subbiahdoss et al. studied only one (10^6 CFU/cm^2) or two bacterial densities (10^2 or 10^5 CFU/cm^2), which does not allow estimation of how the bacterial density influences this competition [112, 172, 181]. In an infection related to the use of biomaterials, less than 10^2 CFU of *S. aureus* is needed to develop a biofilm and to establish a prosthesis infection [179][180]. In multiple studies using high bacterial densities, $\geq 10^6$ CFU/mL, researchers have found that a certain biomaterial inhibits bacterial growth. However, the clinical importance of such a reduction is questionable because the bacterial densities are far from a clinical situation. The infection of a prosthesis and poor clinical outcome of a patient is related to the presence of biofilm, which can be formed from very low bacterial densities such as 10^2 CFU/mL. When enough incubation time is provided, the biofilm grows from a

fairly low inoculum. For that reason, studies have used serial 1:10 dilutions from 10^2 CFU/mL up to 10^8 CFU/mL.
Cell proliferation occurs at one point in tissue integration of prostheses, and the integrated host tissue builds the first line of host defense even in contaminated environments [30]. The presence of bacteria reduces human cell attachment and damages the cells that have managed to adhere to the biomaterial surface. This destructive effect increases with higher concentrations of adhering staphylococci, as found by Subbiahdoss et al. and Martinez-Perez et al. [112, 178]. In contrast to the results of Yue et al. [171] and Lee et al., [182] in the present study it was found that also low bacterial densities, as low as 10^2 CFU/mL, may diminish cell spreading. Host cells may have an inherent mechanism allowing them to compete more effectively against low staphylococcal densities, because they seem to be less defective in low concentrations [112, 171].
Moreover, all human aerobic cells generate ROS against microorganisms, through stress-activated pathways; this induces oxidative cell damage also in attached bacteria [183, 184]. The problem is that the toxic effect is unselective: it can react to any cell, even to the human cells themselves, [112]. This study shows a direct correlation between bacterial density, ROS production, cytotoxic reaction, and cell death. An overabundance of bacteria may produce an uncontrolled inflammatory response with excessive generation of ROS and consequent tissue destruction.

6.4 ANTIMICROBIAL EFFECT OF THE PREINCUBATED HOST CELLS (STUDY IV)

Considering the theory of Anthony Gristina and earlier results from a competitive study involving bacteria and human cells, it was logical to consider the promotion of tissue integration to levels beyond complete surface coverage as a way to prevent bacterial colonization of a biomaterial. Preincubation with host cells having functional host defense mechanisms provides an attractive alternative to avoid the devastating consequences of bacterial colonization of an implant, also taking into account the emerging problem of bacterial resistance to antibiotics [13, 185].
The promotion of tissue integration has been proposed by some researchers such as Dexter et al., who suggest that the presence of ligands on a surface will stimulate tissue integration [13]. Their study suggests that, by controlling the density of proteins or ligands on a surface, one can potentially optimize mammalian cell adhesion without stimulating bacterial adhesion, hence reducing the likelihood of an infection. Actually, something similar happens with BAG S53P4. Its presence promotes the formation of new bone matrix through the release of alkali ions that then become hydroxyapatite [154].
Adding the host cells directly on the biomaterial through pre-operative incubation before implantation sounds like a very reasonable way to reduce the

surface available for circulating planktonic bacteria and consequently to prevent infection and biofilm formation. Once osteoblast host cells have adhered to the implant surface, it is more difficult for bacterial cells to displace them. This is important because of two key points: bacterial cells frequently have already started the race for the surface before implantation, and before the host cells even reach the starting line; bacterial adhesion leads to formation of biofilm which provides resistance to conventional antibiotic therapies and necessitates the use of alternative agents [186, 187].

6.5 LIMITATIONS OF THE STUDY

All findings of this project have possible limitations; its methodology involves aspects that prevent this model's being considered identical to pathogenic processes of implant-related infections. Various questions, such as the effect of protein or another extracellular compound coating the biomaterial surface, are still unanswered. Once a biomaterial is implanted in the body, it is in contact with the patient's blood, and its surface is rapidly coated with proteins of extracellular fluid such as albumin or fibrinogen. These can inhibit or promote bacterial adhesion via specific ligand-receptor interactions [188, 189]. This was not considered, because the clinical setting of this project was mastoid-revision surgery, accompanied by middle-ear implants to restore conductive hearing. In those circumstances, perioperative bacterial attachment mainly occurs on a bulk biomaterial substratum located in a dry middle ear. Meanwhile, the infected mastoid cavity is revised and filled with bioglass granules. However, the addition of whole blood or serum in an in-vitro model of prosthetic infection could be interesting and closer to actual physiological conditions.

The main limitation of these experiments is their use of bacterial collection strains that are adapted to the laboratory. Wild-type strains would perhaps show more virulence against human cells. Martinez-Perez et al. have shown that clinical strains isolated from prosthetic infections show great variability and different pathogenic properties and adhere to the material surface at lower concentrations than do collection strains [178]. This suggests the need for studying as many as possible of clinical strains in order to obtain realistic results and to better learn bacterial behavior. Based on the promising findings presented here, biofilm formation should be studied also using clinical strains isolated from prosthetic infections, strains which possess a higher genetic load and more pathogenic factors.
It is important to extend our knowledge of the host immune system to the infection itself. The role of phagocytosis in the competition between bacteria and osteoblast cells was another aspect not considered. Future research should involve macrophages and other cell types such as soft tissue fibroblasts in co-culture with bacteria and osteoblasts. As PTFE is used more for soft tissue implantation, more experiments will be needed to show whether other

biomaterials show similar prevention of infection when pre-operatively incubated with fibroblasts.
Future research using other types of cells appears fully justified because it could show different types of susceptibility to the presence of ROS. Moreover, additional research seeking other signals of cellular stress related to bacterial presence remains to be performed. Finally, in vivo animal studies should be performed primarily to assess how the attached autologous cells affect the tissue integration of the implant and the efficiency with which the S53P4 produces a change in pH and in osmolarity.

6.6 ADVANTAGES AND DISADVANTAGES OF USING CELL THERAPY INSTEAD OF ANTIBIOTIC

In this thesis, the possible benefits of cell therapy have been proposed and future research should be directed to this field. Once host cells have adhered to the implant surface, it is more difficult for bacterial cells to displace them and consequently to form biofilm which provides resistance to conventional antibiotic therapies and necessitates the use of alternative agents [186, 187]. As hypothesized, preoperative incubation of prostheses with human cells prevents bacterial infection of the biomaterials since it reduces the surface available for circulating planktonic bacteria. With this method one can avoid the use of antibiotic or other antimicrobial substances, which could induce bacterial resistance or cytotoxicity in neighboring tissues [11].
These results need to be interpreted with attention towards standardization of the cell therapy and their clinical application considering economical aspects. Cell therapy could be a complicated and expensive process which should include the isolation of host cells, cultivation and freezing. However, it has to be considered that biomaterial infections entail important clinical and economic consequences such as prolonged antibiotic treatment, long hospitalizations, and multiple surgeries, which may in the worst case lead to the patient's death. The first clinical application could be revision operation where patient has already suffered a prosthetic infection and in whom it is necessary to replace the biomaterial. Bacteria may remain in the patient's tissues and could infect the new implant.
When a biomaterial is implanted into the body, it is cover with extracellular fluid containing proteins and cells. Their interaction with preincubated cells is still unknown. This cellular covering may promote the adherence of ligands, which can lead the race for the surface towards two different directions: infection by planktonic bacteria or tissue integration [188, 189].Finally, possible immune response from the host against pre-incubated cells should be studied.

7 CONCLUSIONS

- This study shows that S53P4 bioactive glass granules inhibit staphylococcal adhesion and biofilm formation on the surfaces of other biomaterials in simultaneous use. In vivo this would reduce the risk of implant infection.
- The use of S53P4 bioactive glass granules compensate the negative effects of hypoxic conditions supporting focal adhesion contacts, rearrangement of the actin cytoskeleton and cellular adhesion of osteoblast.
- This study shows that in the race for the surface between bacteria and tissue cells, the presence of either one reduces biomaterial adhesion and the viability of the other one; the outcome of this competition depends on the initial bacterial density. The presence of bacteria leads to enhanced release of ROS from human cells, which correlated well with cell death. Excessive inflammation and production of ROS leads to the destruction of host tissue.
- This study shows that pre-incubation with host cells inhibits the attachment of bacteria on implant surface in vitro. As microorganisms frequently infect implant surfaces during surgery and start to compete for the surface before tissue integration, pre-incubation of biomaterial with host cells before implantation could create an antiadherent coating.

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