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Infection of orthopedic implants with emphasis on bacterial adhesion process and techniques used in studying bacterial-material interactions

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Staphylococcus comprises up to two-thirds of all pathogens in orthopedic implant infections and they are the principal causative agents of two major types of infection affecting bone: septic arthritis and osteomyelitis, which involve the inflammatory destruction of joint and bone. Bacterial adhesion is the first and most important step in implant infection. It is a complex process influenced by environmental factors, bacterial properties, material surface properties and by the presence of serum or tissue proteins. Properties of the substrate, such as chemical composition of the material, surface charge, hydrophobicity, surface roughness and the presence of specific proteins at the surface, are all thought to be important in the initial cell attachment process. The biofilm mode of growth of infecting bacteria on an implant surface protects the organisms from the host immune system and antibiotic therapy. The research for novel therapeutic strategies is incited by the emergence of antibiotic-resistant bacteria. This work will provide an overview of the mechanisms and factors involved in bacterial adhesion, the techniques that are currently being used studying bacterial-material interactions as well as provide insight into future directions in the field.

Introduction

Bone and joint degenerative and inflammatory problems affect millions of people worldwide. In fact, they account for half of all chronic diseases in people over 50 years of age in developed countries. In addition, it is predicted that the percentage of the population over 50 years affected by bone diseases will double by 2020.[1]

An artificial implant must possess both structural and surface compatibility with the host tissue. With particular reference to bone implants, mechanical and physico-chemical compatibility is required. Each type of material used in orthopedic devices has its own advantages particularly suitable for specific applications.[2] Orthopedic implant devices are intended to restore the function of load-bearing joints which are subjected to high levels of mechanical stresses, wear and fatigue in the course of normal activity. These devices include prostheses for hip, knee, ankle, shoulder and elbow joints. They also include the fracture fixation devices such as wires, pins, plates, screws, etc. Metals (Ti-6Al-4V, Co-Cr-Mo and stainless steel), polymers [poly(methyl methacrylate) (PMMA) and ultrahigh-molecular-weight polyethylene (UHMWPE)] and ceramics (alumina, zirconia and hydroxyapatite) are the three classes of materials that are most commonly used for fabricating orthopedic implants.[3] Although Ti alloys, Co-Cr alloys, and stainless steel alloys are commonly used in orthopedic devices, Ti alloys and Co-Cr alloys are the most common metals used in total-joint arthroplasty (TJA) devices.[3] For example, Co-Cr alloys and ceramics are best suited for bearing surfaces, such as femoral heads, because of their superior hardness and resistance to wear.[3] Ti alloys are commonly used for nonbearing surface components (femoral necks, stems and porous coatings) instead of Co-Cr or stainless steel because of their superior resistance to corrosion and because their torsional and axial stiffness are closer to those of bone, resulting in less stress shielding of bone compared with other alloys.[3] Greater ductility (3-fold better percentage of elongation at fracture) of stainless steel relative to titanium and Co-Cr makes stainless steel ideal for fixation cables used in total-knee arthroplasty procedures.[3] Polymers are commonly used in orthopedics as articulating surfaces of joint replacements and as interpositional cementing material between bone and implant surfaces. The most common polymers used in TJA products are ultrahigh molecular weight polyethylene (UHMWPE) and polymethylmethacrylate (bone cement or PMMA). PMMA is used for fixation of joint replacement implants.[4] The most important application of bioactive ceramics such as hydroxyapatite has been the coating of orthopedic metal implants, at locations where a strong interface with bone is required (i.e., femoral stems and acetabular metal-backs for the hip joints and tibial and femoral components for the knee joints).[5] Alumina and zirconia are primarily used in the fabrication of femoral heads.[6] On the other hand, the introduction of an implant in the body is always associated with the risk of microbial infection, particularly for the fixation of

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open-fractured bones and joint-revision surgeries.[7] Infection is a major problem in orthopedics leading to implant failure. It is a challenging task to treat orthopedic implant infections that may lead to implant replacement and, in severe cases, may result in amputation and mortality.[8] Sources of infectious bacteria include the environment of the operating room, surgical equipment, clothing worn by medical and paramedical staff, resident bacteria on the patient's skin and bacteria already residing in the patient's body.[5] Implant-associated infections are the result of bacteria adhesion to an implant surface and subsequent biofilm formation at the implantation site.[9] Formation of biofilm takes place in several stages, starting with rapid surface attachment, followed by multilayered bacterial cell proliferation and intercellular adhesion in an extracellular polysaccharide matrix.[10] The formation of biofilms on medical devices presents three major problems. First, bacterial communities on these surfaces represent a reservoir of bacteria that can be shed into the body, leading to a chronic infection. Second, biofilm bacteria are highly resistant to treatment with antibiotics; therefore, once these bacterial communities form, they are extremely difficult to eliminate with conventional antimicrobial therapies. Finally, because host responses and antimicrobial therapies are often unable to eliminate bacteria growing in a biofilm, a chronic inflammatory response at the site of the biofilm may be produced.[11]

If bacterial adhesion occurs before tissue regeneration takes place, host defenses often cannot prevent surface colonization for certain bacterial species that are capable of forming a protective biofilm layer. Therefore, inhibiting bacterial adhesion is essential to prevent implant-associated infection, because biofilm are extremely resistant to both the immune system and antibiotics.[12,13] Therefore, to succeed in orthopedic implants, implant materials must be habitable by bone-forming cells (favoring adhesion of osteoblasts), hinder formation of soft connective tissue (hindering adhesion of fibroblasts) and be anti-infective (discouraging bacterial adhesion).[14]

Bacterial Infections of Orthopedic Implants

Tens of millions of medical devices are used each year and, in spite of many advances in biomaterials, a significant proportion of each type of device becomes colonized by bacteria and becomes the focus of an implant-related infection.[15] A very large proportion of all implant-related infections are caused by staphylococci (roughly four out of five), and two single staphylococcal species, respectively *Staphylococcus aureus* and *Staphylococcus epidermidis*, account together for two out of three infection isolates.[16] They represent, in absolute, the main causative agents in orthopedics.[16,17] While this review relates to bacteria in general, more emphasis is given to *S. aureus* and *S. epidermidis* since they are the main causative agents of implant-related infections in orthopedics.

Staphylococcus. Bacteria of the genus Staphylococcus are Gram-positive, nonspore forming facultative anaerobes that grow by aerobic respiration or fermentation, with diameters of 0.5–1.5 μm. They are characterized by individual cocci, which divide in more than one plane to form grape-like clusters.[18]

Staphylococcus comprises up to two-thirds of all pathogens in orthopedic implant infections and they are the principal causative agents of two major types of infection affecting bone, septic arthritis and osteomyelitis, which involve the inflammatory destruction of joint and bone; these infections are difficult to treat because of the ability of the organisms to form small colonies and to grow into biofilms. Many Staphylococcus strains, particularly *S. epidermidis* and some *S. aureus* strains, produce biofilm[18-20]

Staphylococcus aureus. Staphylococcus aureus is an important nosocomial pathogen, able to cause a variety of human disease conditions. It can often be found as a commensal and a transient or persistent part of the resident flora of the skin and anterior nares in a large proportion (20–50%) of the human population. However, when cutaneous/mucous barriers are breached, severe and at times life threatening infections can develop. Nosocomial infections by *S. aureus* are particularly frequent in immunocompromised and severely debilitated patients, and prevail in the presence of indwelling medical devices.[18,21-23]

Treatment of *S. aureus* infections is often complex, namely due to the emergence of methicillin-resistant *S. aureus* (MRSA) strains and resistance to other classes of antibiotics. Because of its pathogenic potential and complexity of its treatment, MRSA has received more attention than its methicillin-sensitive counterpart (MSSA). MRSAs are resistant to β-lactam antibiotics (oxacillin, penicillin and amoxicillin), including third generation cephalosporins, streptomycin, tetracycline and sulfonamides; and upon exposure to vancomycin and other glycopeptide antibiotics, certain MRSA strains become less susceptible to these antibiotics.[18,21]

S. aureus possesses several cell-surface adhesion molecules that facilitate its binding to bone matrix. Binding involves a family of adhesins that interact with extracellular matrix (ECM) components and these adhesins have been termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Specific MSCRAMMs are needed for the colonization of specific tissues and for the adhesion to biomaterials and to the ECM proteins deposited on the biomaterial surface. Particular MSCRAMMs include fibronectin-binding proteins, fibrinogen-binding proteins, elastin-binding adhesin and collagen-binding adhesin. A number of these adhesins have already been thoroughly investigated and identified as critical virulence factors implicated in various phases of infection, including early colonization, invasion, tissue localization and cell internalization.[18,24,25]

In recent years, the polysaccharide intercellular adhesin (PIA) has been found in many *S. aureus* strains, and is required for biofilm formation and bacterium-bacterium adhesion.[17] This adhesin is responsible for the production of the extracellular polysaccharide matrix that makes up the biofilm. It is known that once a biofilm has formed, the bacteria within the biofilm are protected from phagocytosis and antibiotics.[18]

S. aureus produces virulence factors to facilitate disease causation, and rapidly develops antimicrobial resistance. The cell-surface virulence factors include the microbial surface components recognizing adhesive matrix molecules

(MSCRAMMs) as receptors in the human host, other surface proteins, polysaccharide intercellular adhesin and capsular polysaccharides. The cell-surface MSCRAMMs typically are produced during exponential growth phase. The role of these various virulence factors is to provide nutrients required for survival in the host, and microbial cell protection from the host immune system during lesion formation. The secreted virulence factors, typically produced during the post-exponential and stationary phases, include a large group of exoenzymes, such as proteases, glycerol ester hydrolase (lipase) and nucleases that make nutrients available to the microorganism.[18]

Staphylococcus epidermidis. Staphylococcus epidermidis is the most frequently isolated member of the group of coagulase-negative staphylococci (CoNS) from implant-associated infections and they are associated with nosocomial or hospital-acquired infections, and have been found to be more antibiotic resistant than *S. aureus*.[17] This group is diagnostically distinguished from *S. aureus* by its inability to produce coagulase.[18,26,27]

S. epidermidis very often becomes the major infective agent in compromised patients, such as drug abusers and immunocompromised patients (patients under immunosuppressive therapy, AIDS patients and premature newborns). The entry door into the human body in all of these infections is usually an intravascular catheter.[27] The pathogenesis of implant-associated *S. epidermidis* infections is characterized by its ability to colonize a surface and form a thick, multilayered biofilm, often referred to as slime. This biofilm is composed of an extracellular polysaccharide known as polysaccharide intercellular adhesin (PIA), which is essential for *S. epidermidis* biofilm formation. PIA production is also known to protect *S. epidermidis* from phagocytosis and other major components of the host defense system. Generally, the success of *S. epidermidis* as a pathogen has to be attributed to its ability to adhere to surfaces and to remain there, under the cover of a protecting extracellular material, in relative silence.[18,26-29]

S. epidermidis does not produce many toxins and tissue damaging exoenzymes, as does *S. aureus*. To date, few ECM recognizing adhesins have been identified for *S. epidermidis*; however, adhesins to fibronectin, fibrinogen, vitronectin and collagen have been identified.[18,27]

Bone tissue infections. Bone tissue infections, namely osteomyelitis, septic arthritis and prosthetic joint infections (PJI), still represent the worst complications of orthopedic surgery and traumatology. The main pathways of infection for osteomyelitis, septic arthritis and PJI are either hematogenous, resulting from bacteremia; contiguous, when the infection is transmitted from local tissue; or direct, resulting from infiltration of bone, often following injury, surgery or implantation of a foreign body, such as joint replacement.[20,30-33]

Osteomyelitis. Osteomyelitis describes a range of infections in which bone is colonized with microorganisms, with associated inflammation and bone destruction. The occurrence, type, severity and clinical prognosis of osteomyelitis depend on the interplay of a triad of factors, including the characteristics and virulence of the infecting pathogen, the properties of the host and the source of infection.[20,30]

The most common etiologic agents causing osteomyelitis are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis* and *Escherichia coli*. Historically, *S. aureus* has been the dominant pathogen for all classes of osteomyelitis, accounting for 45% of infections; however, the appearance of the microorganism dropped to 27% by 1988.[30,34]

The establishment of osteomyelitis begins with the infiltration of microorganisms into the body. Early infections are usually related to trauma or contamination during surgery; however, a number of improvements in surgical procedures have been responsible for reducing the infection rate. Late infections, which may not occur until after a number of months postoperatively, can also result from bacterial contamination during trauma, surgery or via remote infections. In many of these cases, bacteria introduced during trauma or surgery became dormant for an extended period of time.[30,34]

Haematogenous osteomyelitis most frequently affects children and the elderly.[35] In children, the incidence is typically between 1 in 5,000 and 1 in 10,000.[36] It has been argued that the incidence of hematogenous osteomyelitis is decreasing with an annual fall in childhood cases of 0.185 per 100,000 people recorded in Glasgow, Scotland between 1970 and 1997.[36-38] Conversely, osteomyelitis resulting from direct infection is reported as being increased.[38,39] This is probably due to motor-vehicle accidents and the increasing use of orthopedic fixation devices and total joint implants.[38]

Implanted biomaterials can act as an avenue for both bacterial contamination and colonization toward the development of osteomyelitis. The mechanisms of infection are quite complex and vary with the species of bacteria. If the conditions are favorable, bacteria create an initial attachment to the surface. A permanent attachment develops as protein adhesin-receptors form along with a polysaccharide film after the distance between the cell and the surface is sufficiently reduced. Because biomaterials do not elicit an antiphagocytic reaction toward bacteria after adhesion, these are able to multiply and colonize freely on implant surfaces.[34]

Septic arthritis. Septic arthritis is a joint disease typified by bacterial colonization and rapid joint destruction and it manifests as a serious infection characterized by pain, fever, swelling and even loss of function in one or more affected joints.[20,31] The most commonly involved joints are the knees and hips.[31]

In all age and risk groups, the most frequent causative organisms identified are *Staphylococcus aureus* followed by other gram-positive bacteria, including streptococci.[40,41]

Numerous different factors have been identified for developing of septic arthritis. These factors include rheumatoid arthritis or osteoarthritis, joint prosthesis, low socioeconomic status, intravenous drug abuse, alcoholism, diabetes, previous intra-joint corticosteroid injection and cutaneous ulcers.[41]

The yearly incidence of septic arthritis is between 2 and 10 in 100,000 in the general population but it may be as high as 30–70 per 100,000 in rheumatoid arthritis patients or recipients of prosthetic joints[42-44] and is more common in children than in adults, and in males rather than in females.[45] The incidence of septic arthritis seems to be rising, and this increase is linked to augmented orthopedic-related infection[46] and an aging population,

more invasive procedures being undertaken and enhanced use of immunosuppressive treatment.[46]

Mortality for septic arthritis varies in different studies, but seems to be around 11% for monoarticular sepsis.[47] In view of the 11% mortality rate for septic arthritis, patients should be admitted to hospital for prompt assessment, supportive care and intravenous antibiotic treatment, along with measures to aspirate pus from the joint.

Prosthetic joint infections. The implantation of prosthetic joints along with the use of other implantable orthopedic devices (e.g., pins, screws, plates and external fixators) has improved the quality of life greatly and restored function to patients suffering from debilitating bone and joint disease or injury. Based on conservative estimates, millions of people worldwide have some form of prosthetic joint or other implantable orthopedic device. Among the possible complications associated with implantation, infection is the most serious and occurs in 1 to 13 percent of the cases; the resulting consequences include postoperative prosthesis failure, chronic pain and immobility.[32]

Prosthetic joint infections (PJIs) occur less frequently than aseptic failures but represent the most devastating complication. These infections are a major threat, as therapy is difficult, resulting in a significant increase in hospitalization-related morbidity and mortality.[48,49]

The most common agents are *Staphylococcus aureus* and *Staphylococcus epidermidis*, which account for close to 65% of PJIs. They are the most commonly reported microorganisms both in early and late infections and in total knee and hip arthroplasty.[48]

Table 1 summarizes the classification of prosthetic joint infection according to the route of infection and the time of symptom onset after implantation.[50]

Numerous different factors have been identified as increasing a patient's risk for developing an infection of a prosthetic joint or orthopedic implant. These factors include rheumatoid arthritis, immunocompromised states, diabetes mellitus, poor nutritional status, obesity, psoriasis, long-term urinary catheterization, extreme age, surgical site infection and human immunodeficiency virus (HIV).[32,48]

Prosthetic joint infections (PJIs) of total hip arthroplasty (THA) or total knee arthroplasty (TKA) occur with an incidence of 1.5–2.5% for primary THA or TKA, respectively, whereas revision THA or TKA carries a respective infection risk of 3.2% or 5.6%.[51] Additionally, prosthetic joint infection is an economic burden; the estimated cost of treating an infected prosthetic joint in the US is $50,000 to $60,000.[52] The attendant mortality was estimated, in the 1970s and 1980s, to be between 2.7% and 18%[53] in older patients. Fisman et al. have estimated the mortality attendant to surgical intervention for PJI to be 0.4–1.2% for 65-y-old patients and 2–7% for 80-y-old patients.[54] The mortality reported since 1989 has ranged between 1% and 2.7%.[55] In patients with primary joint replacement, the infection rate in the first two years is usually $< 1\%$ in hip and shoulder prostheses, $< 2\%$ in knee prostheses and $< 9\%$ in elbow prostheses.[56] In addition, infection rates after surgical revision are usually considerably higher (up to 40%) than after primary replacement.[56] In two studies in patients with prosthetic hip and knee associated infection, 29–45% had an early, 23–41% had a delayed, and 30–33% had a late infection.[57,58]

In the future, it is expected that the incidence of prosthetic joint infections will further increase due to (1) better detection methods for microbial biofilms involved in prosthetic joint infections, (2) the growing number of implanted prostheses in the aging population and (3) the increasing residency time of prostheses, which are at continuous risk for infection during their implanted lifetime.[59]

Bacterial Adhesion

The research of bacterial adhesion and its significance is a large field covering different aspects of nature and human life. Adhesion of bacteria to human tissue surfaces and implanted biomaterial surfaces is an important step in the pathogenesis of infection, whereby the bacteria can divide and colonize the surface.[60-68]

Generally, any structures responsible for adhesive activities can be called adhesins. Bacteria may have multiple adhesins for different surfaces (different receptors). A receptor is a component on the surfaces of biomaterials or host tissue that is bound by the active site of an adhesion during the process of specific adhesion.[61]

During development of micro-colonies, some strains of bacteria, particularly S*taphylococcus epidermidis*, secrete a layer of

Table 1. Classification of prosthetic joint infections

Classification	Characteristic
According to the route of infection	
Perioperative	Inoculation of microorganisms into the surgical surgery or immediately thereafter
Hematogenous	Through blood or lymph spread from a distant focus of infection
Contiguous	Contiguous spread from an adjacent focus of infection (eg, penetrating trauma, pre-existing osteomyelitis, skin and soft tissue lesions)
According to the onset of symptoms after implantation	
Early infection ($<$ 3 mo)	Predominantly acquired during implant surgery or the following 2 to 4 d and caused by highly virulent organisms
Delayed or low-grade infection (3–24 mo)	Predominantly acquired during implant surgery and caused by less virulent organisms
Late infection ($>$ 24 mo)	Predominantly caused by hematogenous seeding from remote infections

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slime after adhering to the implant surface, making themselves less accessible to the host defense system and significantly decreasing antibiotic susceptibility. Slime, an extracellular substance (exopolymers composed mainly of polysaccharides) produced by the bacteria, may protect the bacteria from antibiotic therapy, physiologic shear, and possibly host cell-mediated defenses. Bacterial strains that do not produce slime are less adherent and less pathogenic.[60-62,69]

These bacteria can remain quietly on the material surface for a long period of time until the environment allows them to overgrow, such as with decreased host immune activity or poor tissue in-growth around the prosthesis, and a clinical infection then occurs.[69]

An accumulated biomass of bacteria and their extracellular materials (basically slime) on a solid surface is called a biofilm.[61,69-71] Biofilms contain interstitial voids (water channels). Within biofilms, bacterial cells develop into organized and complex communities with structural and functional heterogeneity resembling multicellular organisms in which water channels serve as a rudimentary circulatory system. Release of cell-to-cell signaling molecules (quorum sensing) induces bacteria within a population to respond in concert by changing patterns of gene expression involved in biofilm differentiation.[61,69,72]

Mechanisms of bacterial adhesion. Initial adhesion of bacteria to biomaterial surfaces is believed to be the critical event in the pathogenesis of foreign body infections.[73]

Bacterial adhesion to a material surface can be described as a two-phase process including an initial, instantaneous and reversible physical phase (phase one) and a time-dependent and irreversible molecular and cellular phase (phase two).[61,69,74]

From an overall physicochemical viewpoint, bacterial adhesion can be mediated by non-specific interaction forces, with a long-range character, and specific interactions forces acting in highly localized regions of the interacting surfaces, over distances smaller than 5 nm. Both specific and non-specific interactions may play an important role in the ability of the cell to attach to (or to resist detachment from) the biomaterial surface.[61,69]

Physicochemical interactions between bacteria and material surfaces: phase one. Bacterial adhesion to surfaces consists of the initial attraction of the cells to the surface followed by adsorption and attachment. Bacteria move to or are moved to a material surface through the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, surface electrostatic charge and hydrophobic interactions. These physical interactions are further classified as long-range and short-range interactions.[61,69,75]

The long-range interactions (non-specific, distances > 50 nm) between cells and material surfaces are described by mutual forces, which are related to the distance and free energy. Short-range interactions become effective when the cell and the surface come into close contact (< 5 nm), these can be separated into chemical bonds (such as hydrogen bonding), ionic and dipole interactions and hydrophobic interactions. Bacteria are transported to the surface by the so-called long-range interactions and upon closer contact, short-range interactions become more important.[64,69,75]

This initial attachment of bacteria to surfaces is the initial part of adhesion, which makes the molecular or cellular phase of adhesion possible.[61,69]

Molecular and cellular interactions between bacteria and material surfaces: phase two. In the second phase of adhesion, molecular-specific reactions between bacterial surface structures and substratum surfaces become predominant. This implies a firmer adhesion of bacteria to a surface by the selective bridging function of bacterial surface polymeric structures, which include capsules, fimbriae or pili and slime. In fact, the functional part of these structures should be the adhesins, especially when the substrata are host tissues. Beyond phase two, certain bacterial strains are capable of forming a biofilm if provided with an appropriate supply of nutrients. During biofilm formation, bacteria secrete an exopolysaccharide layer that retains nutrients and protects the microorganisms from the immune response.[61,69,75]

Factors influencing bacterial adhesion. Bacterial adhesion is an extremely complex process that is affected by many factors including the environmental factors, such as the presence of serum proteins or antibiotics, the bacterial properties and the material surface characteristics. A better understanding of the unique behavior of certain bacteria, the surface characteristics of the material and the relevant environment would make it possible for one to control the adhesion process by changing these factors.[61,65,69,76-79]

Environment. Certain factors in the general environment, such as temperature, exposure time length, bacterial concentration, chemical treatment, the presence of antibiotics and the associated flow conditions affect bacterial adhesion.[61,69]

Flow conditions are considered dominant factors that strongly influence the number of attached bacteria[80] as well as the biofilm structure and performance.[81] The decreased bacterial adhesion at higher flow rates is clearly established. Katsikogianni et al.[74] showed the effect of flow conditions on bacterial adhesion to several substrates, and in most material, except diamond-like carbon (DLC) coated poly(vinyl chloride) (PVC) deposited by Atom Beam (A.B.), the number of adherent bacteria significantly decreased with the increase of shear rate from 150 sec^{-1} to 1,500 sec^{-1}. DLC (A.B.) was the only material that exhibited a different behavior, and this difference appears to be associated with the significantly higher surface roughness values. Bacteria preferentially stick to rough surfaces and especially to irregularities that conform their shapes in order to maximize bacteria-surface contact area and probably protect themselves from shear forces.[74] In another study, Katsikogianni et al.[82] also found that the number of adherent *S. epidermidis*, for several materials and for two bacterial strains, decreased with increasing shear rate, from 50 to 500 or 1,000 sec^{-1}, and especially when it reached 2,000 sec^{-1}.[82] Therefore, it is generally considered that higher shear rates result in higher detachment forces that decrease the number of attached bacteria.[74,82] However, there is an optimum flow rate for bacterial attachment reflecting the balance between the rate of delivery and the force acting on the attached bacteria.[83] Mohamed et al.[84] showed that in the case of higher number of receptors/cell *S. aureus* adhesion to collagen coated coverslips increased for shear rates between 50–300 sec^{-1} and decreased for

shear rates above 500 sec^{-1}. However, in the case of lower number of receptors/cell this optimum flow rate was not clear.[84]

Concentrations of electrolytes, such as KCl, NaCl and pH value of the culture environment also influence bacterial adhesion.[61,69] Changes in pH can have a marked effect on bacterial growth and adhesion. Bacteria possess membrane-bound proton pumps that extrude protons from the cytoplasm to generate a transmembrane electrochemical gradient, i.e., the proton motor force.[85] The passive influx of protons in response to the proton motive force can be a problem for cells attempting to regulate their cytoplasmic pH.[86] Bacteria respond to changes in internal and external pH by adjusting the activity and synthesis of proteins associated with many different cellular processes.[86] Studies have shown that a gradual increase in acidity increases the chances of cell survival in comparison to a sudden increase by rapid addition of HCl.[87] This suggests that bacteria contain mechanisms in place which allow the bacterial population to adapt to small environmental changes in pH. However, there are cellular processes which do not adapt to pH fluctuations so easily. One such process is the excretion of exopolymeric substances (polysaccharides). Optimum pH for polysaccharide production depends on the individual species, but it is around pH 7 for most bacteria.[88] Hamadi et al.[89] investigated the adhesion of *Staphylococcus aureus* ATCC 25923 to glass at different pH values and observed that pH influenced bacterial adhesion. The images obtained by SEM showed that the adhesion behavior of *S. aureus* ATCC 25923 depended on the pH of the suspending medium and at highly acidic (pH 2 and pH 3) and alkaline conditions, the cells deposited in aggregate forms, while at pH 5 the aggregation phenomenon was absent. The quantitative adhesion (number of adhering cells to glass surface) showed that cells adhered strongly in the pH range 4 to 6 and weakly at highly acidic (pH 2 and pH 3) and alkaline conditions.[89] Kinnari et al.[90] studied whether the most common causative agents of orthopedic implant-related infections, *S. aureus* and *S. epidermidis*, can penetrate the ceramic pores and adhere particularly avidly to that surface at a slightly acidic pH, simulating conditions to which they may be exposed in vivo.[90] The isoelectric point of the materials at the surface-liquid interface changes with the decrease in pH following infection, surgery, trauma or aseptic implant loosening.[90,91] On these occasions, the pH of the bone tissue environment often falls below pH 7, whereas in healthy tissues this pH value varies in the range 7.35 to 7.45.[90] The ceramic materials used by Kinnari et al.[90] were hydroxyapatite (HA) and biphasic calcium phosphate (BCP) that are widely employed as bone substitutes.[90] Their porosity and the decrease in surrounding pH as a result of surgical trauma may, however, pre-condition these materials to bacterial infections. The authors showed that when pH decreased from 7.4 to 6.8, the adherence of staphylococci both to HA and BCP surfaces decreased significantly. Moreover, in this study they observed that HA and BCP ceramics did not have pores large enough to allow the internalization of staphylococci. Therefore, their anti-adherent properties seemed to improve when pH value decreased, suggesting that HA and BCP bioceramics are not compromised upon orthopedic use.

The presence of antibiotics decreases bacterial adhesion depending on bacterial susceptibility and antibiotic concentration.[69,78,92] Kohnen et al.[93] showed that *S. epidermidis* adhesion on catheters was reduced when catheters where impregnated with rifampin-sparfoxacin that were released slowly with time from catheter surface.[93] Stigter et al.[94] developed a biomimetic approach for coating titanium alloy (Ti6Al4V) implants with calcium phosphate containing an antibiotic. The authors showed that the coatings, containing tobramycin, were effective against the growth of *S. aureus* in a concentration-dependent manner. These results demonstrated the efficacy of the biomimetic coatings combined with tobramycin, to prevent local post-surgical infections in orthopedic surgery.[94] The most important fact is that bacteria normally grow as biofilms.[95] The bacteria in biofilms can be differentiated from free-floating planktonic forms by an extracellular polymeric substance, slower growth rate and the up- or down-regulation of certain genes. The extracellular polymeric substance acts as a filter and conduit for nutrients and minerals that are channeled to interior cells and protects cells from potentially harmful agents, including antibiotics.[96] The prevalence of biofilms in infections and on surfaces of medical implant devices has focused attention on the increased antibiotic resistance (10^3-fold) of biofilm-resident bacteria vs. the more commonly studied planktonic (free-floating) form.[97] It is postulated that biofilms contribute to antibiotic resistance by at least three mechanisms: reduced antibiotic penetration across the extracellular polymeric substance, a favorable (e.g., anaerobic) environment within the inner layers and bacteria cell differentiation and role specialization providing increased protection.[98] Even if antibiotic therapy is effective against some of bacteria in a colony, surviving bacteria can feed themselves of left behind nutrients.[99] As a result, bacteria in biofilms survive exposure to concentrations of antibiotics 10^3-fold higher than lethal values found for cells in suspension.[98] It has been suggested that if a low concentration of antibiotics or other drugs is able to prevent initial bacterial adherence to surfaces, the subsequent step of biofilm formation would also be inhibited.[100] However, it has previously been shown that in the case of staphylococcal strains the initial adherence and subsequent biofilm formation are two distinct phenomena.[101,102] Cerca et al.[26] evaluated the adherence of several clinical isolates of coagulase-negative staphylococci (CoNS) to acrylic and the effect of sub-minimal inhibitory concentrations (sub-MICs) of vancomycin, cefazolin, dicloxacillin and combinations of these antibiotics on adherence and biofilm formation. They showed that most of these antibiotics resulted in effective reduction of bacterial adherence to acrylic, in some cases reaching over 70% adherence inhibition, and when strains with a high biofilm-forming capacity were grown in sub-MICs of those antibiotics, there existed combinations of the drugs that significantly inhibited biofilm formation. However, they also saw that most of the antibiotic combinations that inhibited adherence did not have a profound effect on biofilm formation. In general, these results indicated that the effect on adherence inhibition was greater than the effect on inhibiting biofilm formation.[26] Prado et al.[103] evaluated the susceptibility of planktonic and biofilm-associated organisms of *Streptococcus pneumoniae* to antibiotics. The authors

also realized that amoxicillin, erythromycin and levofloxacin were less active against biofilm-associated organisms as compared with their planktonic counterparts.[103] Thus, the most promising anti-infective strategies seek to inhibit bacterial adhesion prior to biofilm formation. Reducing bacterial adhesion during the initial 6 h period following implantation is particularly important to avoid device-associated infection.[104] Pagano et al.[105] evaluated the differences between a prophylactic and therapeutic approach to the CoNS biofilm problem. These authors showed that by adding low concentrations of linezolid or vancomycin before the bacteria could reach the surface, they were able to inhibit biofilm formation. However, if the application of the drug was delayed just by 6 h after initial adherence occurrence, the inhibition of biofilm formation was less effective.[105]

All of these factors may influence bacterial adhesion by either changing physical interactions in phase one of adhesion or changing surface characteristics of bacteria or materials.[61]

Material surface characteristics. The factors influencing bacteria adherence to a biomaterial surface include chemical composition of the material, surface charge, hydrophobicity, surface roughness or physical configuration.[61,69]

Surface chemistry influences bacterial adhesion and proliferation. Materials with different functional groups change bacterial adhesion in a manner depending on material hydrophobicity and charge.[69]

Surface roughness is a 2-dimensional parameter of a material surface measured by roughness measuring systems. Biomaterials surface roughness is another relevant property for the bacterial adhesion process, with the irregularities of the material surfaces normally promoting bacterial adhesion and biofilm accumulation whereas an ultra-smooth surface does not favor bacterial adhesion and biofilm accumulation.[106] This is due to the increased surface area and depressions in the roughened surfaces that provide more favorable and additional sites for colonization.[61,69] Oztürk et al. investigated the adhesion of biofilm forming *S. epidermidis* strain YT-169a on nitrogen (N) ion implanted as well as on as-polished CoCrMo alloy materials and the adhesion test results showed that *S. epidermidis* strain YT-169a adhere much more efficiently to the N implanted surfaces than to the as-polished CoCrMo alloy surface. This was attributed mainly to the rougher surfaces associated with the N implanted specimens in comparison with the relatively smooth surface of the as-polished specimen.[107]

Teughels et al.[108] also found that an increase in surface roughness facilitated biofilm formation on implant surfaces.[108] However, the accumulation of bacteria in such locations depends largely on their size, cell dimension and division mode.[109] According to Katainen et al.,[109] surfaces may have roughness in several length scales, but due to the short range of the van der Waals interactions, roughness in the nanoscale ultimately determines the adhesion strength. This is corroborated by another study[110] where the impact of nanometer-scale roughness on bacterial adhesion was tested and according to which, a reduction in the nanoscale roughness (of R_a = 2.1 nm to R_a = 1.3 nm) lead to a strong increase in the number of adhered bacteria. Therefore, it seems that roughness at a nanoscale can strongly influence initial attachment of bacteria, probably by providing the presence of a greater number of contact points. Truong et al. have shown that the adhesion of bacterial cells on titanium surfaces is promoted by the presence of nanoscale topographical features.[111] Whitehead et al. have studied bacterial colonization on nanostructured titanium surfaces, and demonstrated improved colonization efficiency when surface roughness increases.[112] Webster et al.[113] evaluated the adhesion of *Pseudomonas fluorescens* on nanophased alumina, compared with conventional grain size alumina substrates. They observed greater *P. fluorescens* attachment to nanophased as compared with conventional alumina. Moreover, the ability of a nanostructured surface to influence irreversible adhesion, attachment of *P. fluorescens* to alumina was followed after fibronectin was allowed to adsorb to the surfaces. Results of this study indicated a greater adhesion of *P. fluorescens* in this environment as well.[113] Colon et al.[114] examined the functions of *S. epidermidis* (known to be detrimental to orthopedic implant efficacy) and osteoblasts (or bone-forming cells) on ZnO and titania (TiO_2), that presented nanostructured compared with microstructured surface features. ZnO is a well-known antimicrobial agent and TiO_2 readily forms on titanium once implanted. When normalized to the projected surface area, they observed significantly decreased *S. epidermidis* colony forming units on nanophase compared with microphase ZnO as well as TiO_2 (by 60% and 69%, respectively) and osteoblast adhesion increased by 146% and 200% on nanophase compared with microphase ZnO and TiO_2, respectively, leading to improved calcium mineral deposition on two nanophased ceramics: ZnO and TiO_2. Although the exact mechanism is not known, some comments for why *S. epidermidis* adhesion decreased on these nanophase ceramics were made by these authors. For example, it has been suggested that ZnO reduces bacterial activity through the release of ZnO ions to the local environment, which alters protein adsorption and intracellular mechanisms pertinent to bacteria activities. Following this line of thought, two important properties may be responsible for decreasing bacterial adhesion on nanophased as compared with microphased ceramics: increased surface area and greater numbers of surface grain boundaries. Higher surface areas of nanophased as compared with microphased ZnO may result in the increased presence of soluble ZnO ions to disrupt bacteria activities. Moreover, the increased presence of soluble ZnO ions that disrupt bacterial activities may have resulted from the fact that greater material dissolution occurs at grain boundaries and more grain boundaries are present on the surfaces of nanophased compared with microphased ZnO. The aspect ratio of ZnO nanoparticles used in that study may also have influenced bacterial adhesion. Therefore, this study suggests that nanophased ZnO and TiO_2 may reduce S. *epidermidis* adhesion and increase osteoblastic performance required to promote the efficacy of orthopedic implants.[114] Since clinically different prostheses or implant devices have different surface roughnesses that may play a role in bacterial adhesion and implant infection, more studies are needed to test the effects of a broader range of surface roughness values.

Physical configuration of a material surface is different from surface roughness and is rather complicated. It is a morphological description of the pattern of a material surface, such as a

monofilament surface, a braided surface, a porous surface or a grid-like surface, and it is a 3-dimensional parameter.[61,69] Merrit et al.[115] showed that porous materials had significantly higher infection rates than nonporous materials when implanted subcutaneously in mice that were challenged with *Staphylococcus aureus*. Therefore, the implant site infection rates are different between porous and dense materials with porous materials having a much higher rate. This implies that bacteria preferentially adhere and colonize on the porous surface.[115,116] Moreover, bacteria adhere more to grooved and braided materials compared with flat ones, probably partially due to increased surface area. Physical configurations are routinely evaluated by scanning electron microscopy.[61,69]

Metal surfaces have a high surface energy and are negatively charged and hydrophilic as shown by water contact angles, while polymers have low surface energy and are less electrostatically charged and hydrophobic.[61] The structure of water in the region near any surface (such as solid material surface or bacterial surface) is perturbed over distances of up to several tens of molecular layers. Near a hydrophobic surface the water is less structured in terms of intermolecular hydrogen bonding between the water molecules, while near a hydrophilic surface water is more structured. Water contact angle (WCA) is a good example of the hydrophobic or hydrophilic nature of a surface. A high WCA represents hydrophobicity and a low WCA represents hydrophilicity.[61] The hydrophobicity of a material surface has been evaluated mainly by contact angle measurement. Depending on the hydrophobicity of both bacteria and material surfaces, bacteria adhere differently to materials with different hydrophobicities.[61,69] A microorganism may adhere to a substratum via the hydrophobic effect if the associating sites possess sufficiently high densities of apolar areas.[117] In staphylococcal species, for instance, these hydrophobic areas are provided by proteins that are covalently bound to the cell wall.[118] Charville et al.[119] also showed that the pre-treatment of the PVC surface with bovine serum albumin (BSA) originated a decrease of surface hydrophobicity and reduced bacterial adhesion for each of the three species tested. Katsikogianni et al.[74] reported that diamond-like carbon coated PVC exhibited lower levels of *S. epidermidis* adhesion probably due to reduced hydrophobicity in comparison to uncoated PVC. Moreover, these authors also found that the fluorinated PVC presented a slightly higher level of *S. epidermidis* adhesion compared with the sterilized PVC, probably due to the increased hydrophobic properties of its surface.[74] In this study, Katsikogianni et al. also showed that *S. epidermidis* was a moderate hydrophobic bacterium; therefore adhesion was favored to the most hydrophobic substrate, which, in their case, was the fluorinated surface. However, the relatively small increase in *S. epidermidis* adhesion observed for the fluorinated surface may be due to the moderating effect associated with the reduction in surface roughness of the fluorinated PVC. The reduction in surface roughness would mean fewer sites for bacteria to adhere despite the more favorable hydrophobic surface.[74] The surface coating of substrates with proteins, such as BSA, bovine glycoprotein, or fatty-acid free BSA decreases the hydrophobicity of the surface, leading to an inhibition of bacterial adhesion to surfaces.[61]

Bacterial characteristics. For a given material surface, different bacterial species and strains adhere differently since they have different physicochemical characteristics.[61,69]

Surface hydrophobicity of bacteria is an important physical factor for adhesion, especially when the substrata surfaces are either hydrophilic or hydrophobic. Generally, bacteria with hydrophobic properties prefer hydrophobic material surfaces; the ones with hydrophilic characteristics prefer hydrophilic surfaces and hydrophobic bacteria adhere to a greater extent than hydrophilic bacteria.[61,69] The hydrophobicity of bacteria varies according to bacterial species and is influenced by growth medium, bacteria age and bacterial surface structure.[61,69] Walker et al.[120] found a decrease in adhesion and hydrophobicity of *Escherichia coli* during mid-exponential compared with the stationary phase. These observations were attributed to hydrophilic (acidic) proteins on the outer membrane of *E.coli* that decrease with the culture age, and consequently lead to a decrease in hydrophobicity and adhesion.[120] Kuntiya et al.[121] also found that cell surface hydrophobicity of *Pseudomonas* sp decreased with increasing cellular age. Moreover, Kuntiya et al.[121] showed that changing the medium composition by the addition of sodium chloride (0.5% w/v) resulted in a faster decrease in the cell surface hydrophobicity. The authors explained that there appear to be at least three possible reasons for the observed changes in hydrophobicity. First, the presence of salts has been reported to increase exopolysaccharide production although the mechanism is not completely understood; this may, however, account for a drop in hydrophobicity, if the exopolysaccharides are predominantly neutral or hydrophilic. Second, it has been reported that the production of exopolysaccharides is higher with aged cells. Third, nutrient starvation in the batch culture may be another reason for lowering the hydrophobicity since this also triggers the production of exopolysaccharides.[121] Therefore, these results demonstrated that the presence of sodium chloride in the medium and cellular age did affect cell surface hydrophobicity and consequently biofilm formation and growth. It was reported that marked differences in both the slime production and *S. epidermidis* adhesion were observed when comparing four culture media.[122] Slime production was notably poor in used peritoneal dialysis fluid (PUD). Adherent growth was markedly increased in a chemically defined medium (HHW) and synthetic dialysis fluid (SDF) but was poor in tryptic soy broth (TSB) and PUD when air containing 5% CO_2 was used. These findings emphasize the advantages in using chemically defined and biological fluids when studying slime production and adhesion of *S. epidermidis*.[122]

The surface charge of bacteria may be another important physical factor for bacterial adhesion. The surface charge attracts ions of opposite charge in the medium and results in the formation of an electric double layer. Most particles acquire a surface electric charge in aqueous suspension due to the ionization of their surface groups. Bacteria in aqueous suspension are almost always negatively charged. The surface charge of bacteria varies according to bacterial species and is influenced by the growth

medium, the pH and the ionic strength of the suspending buffer, bacterial age and bacterial surface structure.[61,69]

Serum or tissue proteins. It is well accepted that the protein adsorption is the first event following blood-material contact. The process of protein adsorption from an aqueous solution onto a solid surface is typically described in three steps. First, transportation of the protein from the solution toward the solid surface occurs. This is followed by attachment of the protein to the surface, and finally the protein structure undergoes a conformational change after adsorption.[123,124]

When an implant is inserted into host tissue, small biomolecules including extracellular matrix (ECM) proteins adsorb onto the material surface to form a conditioned protein layer conducive to the adherence of free floating planktonic bacteria. The adhered bacteria then rapidly proliferate, recruit other cells and produce sticky secretions to form dense communities of attached cells called biofilms.[125,126]

Serum or tissue proteins, such as albumin, fibronectin, fibrinogen, laminin, denaturated collagen and others, promote or inhibit bacterial adhesion by either binding to substrata surfaces, binding to the bacterial surface or by being present in the liquid medium during the adhesion period. Most of the bindings between bacteria and proteins are specific ligand-receptor interactions. Proteins may also change the adherent behavior of bacteria by changing bacterial surface physicochemical characteristics.[61,69]

Fibronectin. Fibronectin (FN) is a protein that seems to promote adhesion of certain strains. FN clearly promotes *S. aureus* adhesion to the substratum surface.[61,69] The binding of FN to a strain of *S. aureus* is specific, time-dependent and irreversible.[61,69] Therefore, in the presence of FN, the adherence of *S. aureus* to foreign surfaces is significantly increased. Most studies showed that adsorbed FN promotes adherence of bacteria, especially *staphylococci* to biomaterials.[61,69]

In the fibronectin molecule, two different binding sites are known for staphylococci adhesion: a first binding site in the N-terminal domain and a second near the C-terminus.[127] A study by surface plasmon resonance reported a higher affinity of *S. epidermidis* for the C-terminal fragment.[128] FN has played a crucial role in promoting bacterial adhesion to biomaterial surfaces.

In the last few decades, it has become clear that many bacteria possess fibronectin-binding proteins and that such proteins can bind to a growing number of sites in fibronectin.[129]

S. aureus produces a number of surface proteins that are likely to be involved in the initial attachment to host tissues. These proteins, which have been termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) bind specifically to components of the ECM. One such component of the ECM is fibronectin and it has binding sites for several pathogens.[130-132] *S. aureus* has been shown to specifically bind to adsorbed fibronectin. This bacterium has two fibronectin-binding proteins, FnBPA and FnBPB, encoded by the closely linked genes *fnbA* and *fnbB*, both of which contribute to the adherence to fibronectin-coated surfaces. At least one of two genes coding for the very similar surface proteins FnBPA and FnBPB is found in almost all clinical isolates of *S. aureus*. This fibronectin-binding proteins (FnBPs) are involved in the pathogenesis of infection.[133-135]

S. epidermidis has also been reported to bind to a number of host cell extracellular matrix proteins, including fibronectin. In vitro studies have shown that *S. epidermidis* can bind to biomaterials coated with fibronectin.[131] However, compared with *S. aureus* little is known about how *S. epidermidis* interacts with matrix proteins.[131] It was found that in *S. epidermidis* 1585v overexpression of a 460 kDa truncated isoform of the ECM-binding protein (Embp) is necessary for biofilm formation. This *S. epidermidis* cell surface-associated protein termed Embp is a giant fibronectin-binding protein. Studies using Embp-expressing strains adhered significantly stronger to the fibronectin-coated surface compared with Embp-negative strains, indicating that Embp mediates *S. epidermidis* adherence to fibronectin. Furthermore, a quantitative association between fibronectin amounts used for plate coating and *S. epidermidis* adherence was found, indicating that here, fibronectin is essential for bacterial binding. These findings suggest that Embp plays a role during primary attachment to conditioned surfaces.[136,137]

Albumin. Albumin adsorbed on material surfaces has shown obvious inhibitory effects on bacterial adhesion to polymer, ceramic and metal surfaces.[61,69] An et al. showed that human serum albumin (HSA) inhibited *S. epidermidis* adhesion to cpTi surfaces by more than 95% after treatment of the cpTi sample with 200 mg/mL of HSA at 37°C for 2 h.[62] Kinnari et al.[138] also showed that the level of adherence of both *S. aureus* and *P. aeruginosa* was significantly lower on the HSA-coated titanium surface than on the uncoated surface, with overall bacterial adhesion dependent on bacterial concentration. Adhesion of *S. aureus* on HSA-coated surfaces was significantly inhibited (from 82% to 95% depending on the concentration) and the adhesion of *P. aeruginosa* was inhibited from 29% to 37%. However, the inhibitory effects of HSA seem to depend on bacterial strain and species, as indicated by a previous study,[138] due to differences between bacterial strains in terms of their cell surface properties.[139] Albumin may inhibit adhesion through binding to the bacterial cells or by changing the substratum surface to more hydrophilic character.[139,140]

In a study, a cross-linked albumin coating reduced the prosthetic infection rate in a rabbit model. Animals with albumin-coated implants had a much lower infection rate (3/11 animals, 27%) than those with uncoated implants (8/13 animals, 62%).[141] This finding may represent a new method for preventing prosthetic infections.

However, in a recent study, Prado et al.[142] showed that the effect of HSA on ability of *Streptococcus pneumoniae* strains to form biofilms on polystyrene plates was concentration dependent and HSA at concentrations from 40 to 25,000 μg/mL stimulated bacterial growth, while higher concentrations produced bacterial inhibition. The activity of HSA to prevent biofilm formation was concentration and strain dependent with the greater efficacy at concentrations 0.5 × minimum inhibitory concentration (MIC).[142] Although the use of HSA for preventing biofilm formation on abiotic material has been proposed, these results

showing that certain concentrations of this compound produced stimulation of bacterial growth and even significantly increased biofilm formation by 2 out of the 11 strains tested could be a serious drawback of such approach.[142] Naves et al.[143] also showed that HSA inhibited biofilm formation by all *E. coli* strains on polystyrene plates, but as a possible drawback, it stimulated bacterial growth.

Fibrinogen. Fibrinogen (Fg) is another important protein that mediates bacterial adhesion to biomaterials and host tissues.[144] Fibrinogen promotes bacterial adhesion by bridging the biomaterial surface with bacterial cell membrane receptors specific to Fg.[145] Such interactions are responsible for bacterial adhesion to medical devices in vivo, and bacteria possessing the ability to specifically bind surface-adsorbed Fg have been found to be responsible for significantly more clinical orthopedic device-associated infections than those without Fg-binding proteins.[146]

Charville et al. showed that the extent of *S. aureus*, *S. epidermidis* and *Escherichia coli* adhesion was greater to PVC substrates with pre-adsorbed Fg compared with substrates without protein. However, the most significant increase was observed in the case of *S. aureus*, where adhesion to Fg-coated substrates was more than 5 times that of uncoated controls.[119] Baumgartner et al.[147] also reported similar results for *S. aureus* adhesion to polyurethane surfaces with pre-adsorbed Fg. Pei et al.[148] found that *S. epidermidis* adhesion to control catheters without pre-adsorbed functional Fg was approximately half that observed at Fg-coated catheters. Collectively, the data indicate that the increase in bacterial adhesion in the presence of Fg is the result of specific Fg-mediated interactions between the bacterial cells and the substrate.[149-153]

Laminin. Laminin has a promoting effect on *S. aureus* and CNS adhesion to PMMA coverslips but to a lesser extent compared with the effects of FN and fibrinogen.[145] The presence of laminin receptors in *S. aureus* has also been reported.[154]

Serum. The role of serum proteins in mediating bacterial adhesion has also been evaluated. Some studies have revealed a strong inhibition of adherence of bacteria to biomaterials in the presence of whole serum.[155,156] Ardehali et al.[157] observed a marked, up to 5-fold, reduction in bacterial adhesion to polyurethane (PU) surfaces in the presence of bovine/human serum or plasma at 0.5% or higher concentration. Moreover, the authors reported that the inhibition of bacterial adhesion by serum is to a large extent due to apo-transferrin.[157]

Techniques Used in Studying Bacterial-Material Interactions

Bacterial interactions are of prime importance in the many stages of the lifecycle of a bacterium.[158] Specific bacterial interactions are mediated by polymeric substances which are present on the outside of the cell wall. Highly diverse classes of surface constituents have been implicated in bacterial interactions, such as slime, surface proteins, lipopolysaccharides, lipoteichoic acids, capsules, lectins and fimbriae or pili.[158] These specific interactions between bacterial surface structures and substratum surfaces imply a firmer bacterial adhesion to a surface by the selective bridging function of bacterial surface polymeric structures. In fact, the functional part of these structures should be the adhesins, especially when the substrata are host tissues.[159-162] Subsequently, certain bacterial strains are capable of forming a biofilm if provided with an appropriate supply of nutrients. During biofilm formation, bacteria secrete a slime layer that retains nutrients and protects the microorganisms from the host immune response.[61,69,75] Biofilms are the most common mode of bacterial growth in nature and are also important in clinical infections, especially due to the associated high antibiotic resistance.[163-165] Investigations of phenomena such as irreversible cell adhesion (i.e., the initial stage of the biofilm formation) to surfaces and an understanding of factors affecting spatial arrangement of biofilms, including the distribution and composition of microorganisms within the biofilm matrix and characterizing properties of this matrix, are recognized as essential in understanding the function of biofilms.[166,167]

A fundamental aspect of the study of bacterial adhesion and attachment to surfaces is the need for reliable quantification of the microbiological population that attaches to the surface. Several experimental techniques have been developed to study and quantify bacterial adhesion on material surfaces. Since this is a very exhausting topic, only the commonly used techniques and the basic principles will be summarized in **Table 2**.

Metabolic assays are also excellent candidates for quantification of bacterial viability in biofilms. These assays are indirect methods based on the detection of metabolic products produced by bacteria and have the advantage of being able to assess viability without sample manipulation since these assays generally do not require the removal of the biofilm from the adherent surface.[168] Moreover most assays are simple, fast and perfectly suitable for high-throughput quantification of biofilms grown in a microtiter plate.[169] Some of these assays, including colorimetric biomass (crystal violet), Syto 9, resazurin and fluorescein diacetate (FDA), will also be summarized in **Table 2**.

However, it is important to mention that several conditions during the biofilm formation process can affect the results obtained including growth conditions, the cultivation medium and the surface selection. Growth conditions are very different among the available literature, namely different physiological states influence adhesion and biofilm formation. The stationary growth phase is the most common among other works from the literature. The bacterial inoculums should be determined with caution since it is known that increased inoculums increase biofilm density. So the exact size of the inoculums should be determined by adjusting to a specific optical density or absorbance. In order to avoid error in the optical reading, cell clusters should be avoided using a brief agitation and/or filtration. The medium for biofilm cultivation is also known to be crucial to the biofilm formation ability.[170]

After the biofilm incubation step, the parameters that have been identified as being extremely important for biofilm quantification and which are not usually taken into account and/or omitted in the previously published work are: (1) bacterial removal and rinsing procedures of the wells as it assures the removal of non-adherent cells while keeping biofilm integrity. For

Table 2. Techniques to study and quantify the microorganisms attached to a surface and the bacterial viability in biofilms

Techniques	Advantages	Limitations
Colony forming units counting (CFU)	CFU plate counting is the most basic method for bacterial enumeration.[69,176,177] The washing is a very important part of a bacterial adhesion study using this technique, and its purpose is to remove the unattached and loosely adhered bacteria from the material surface. Methods for removing bacteria from substrata surfaces include homogenization, sonication, and the use of surfactants. According to the comparative study by McDaniel and Capone[176] sonication appears to be an efficient and safe way to remove bacteria from biomaterial surfaces. There are two basic ways to perform plate counting, the pour plate method and the surface spread method.[176,177]	This technique is time consuming and involves tedious work, indirect and complicated procedures that give more uncertainty.[177] It detects only viable bacteria.[177]
Light microscopy	Technique for bacterial enumeration and observation. Normally bacteria are stained with dyes like crystal violet or fuchsin. Some special staining methods allow the observation of bacterial surface structures such as capsules, or appendages.[176,177] Light microscopy has been combined with a bacterial flow chamber to observe living attached bacterial cells in real time.[176,177] A transparent material forms part of the wall of a cell flow chamber so that the bacteria attached to the inner side of the material might be directly observed. The advances in image analysis make bacterial counting by light microscopy much faster and more efficient.[176,177]	The substrata surfaces have to be translucent to be able to use light microscopy.[176,177]
Epifluorescence microscopy	It allows to differentiate between live and dead bacterial cells on the surface, if certain fluorochromes are used.[178,179] Image analysis systems are used for determining the number of cells adhered.[178] It makes direct observation and enumeration possible for attached bacteria on an opaque surface. Relatively fast, easy method for biofilm characterization that is especially suitable for a large set of samples.[177,179] Wirtanen et al.[180] evaluated the efficacy of various disinfectants against biofilms of *Pseudomonas aeruginosa* and *Pseudomonas fragi* on stainless steel surfaces using epifluorescence microscopy coupled with acridine orange.	Two-dimensional imaging only.[179] The use of fluorochromes is necessary for viewing bacteria.[179] Limited to macroscopic investigation of bacteria-surface interactions.[179]
Scanning electron microscopy (SEM)	SEM is a well-established basic technique to observe the morphology of bacteria adhered on a material surface, the material surface morphology, and the relationships between the two. It is also used to observe the morphology of bacterial biofilms on surfaces.[176,177,181] Environmental SEM or Low Vacuum SEM do not require metal or carbon sputtering and is less prone to damaging the bacteria adhered on a surface or alter the surface characteristics of the specimen, therefore overcoming the referred drawbacks. Chemical composition of samples can be determined by using energy-dispersive X-ray (EDX) for elements with $Z > 6$.[179] SEM has previously been used to visualize biofilm development of *S. epidermidis* on contact lenses,[182] extensive biofilms on endoscope tubing samples that had been sent for endoscope servicing[183] and the development of biofilms on catheters.[184]	SEM has been used for the enumeration of adhered bacteria, but, because of the small field and time-consuming work, it is less adequate for this purpose.[177] It requires samples preparation for observation and the procedure for preparation can be tedious and labor intensive.[179] It requires the specimen to be conductive (essentially "metal sputtered"). Cannot differentiate between live and dead bacterial cells.[179] During sample preparation the drying step is considered to cause noticeable cell shrinkage and it exacerbates other undesirable outcomes, like damage and distortion of the biofilm.[179] It also requires specialist equipment, training and extensive samples preparation.[178]
Confocal scanning laser microscopy (CSLM)	CSLM is a three-dimensional technique using fluorescent molecular probes and laser beams to study in situ bacterial associations with surfaces.[185] It is used to visualize and count bacterial cells directly on transparent or opaque surfaces. It allows the examination of living fully hydrated biofilms in real time, and the simultaneous use of specific molecular probes allows to determine the identity (oligonucleotide probes) and the physiological state (live vs. dead) of the adherent bacterial cells.[186,187] This CSLM-based technique may be used to accurately assess the antibacterial properties of biofilm-resistant biomaterials.[186] This technique offers several advantages, including the ability to control depth of field, elimination or reduction of background information away from the focal plane (that leads to image degradation), and the capability to collect serial optical sections from thick specimens.[187-189] Burnett et al.[190] observed the attachment of *E. coli* O157:H7 to apple tissue by confocal scanning laser microscopy. Lindsay et al.[191] visualized co-cultured biofilms of *Pseudomonas fluorescens* M2 and *Bacillus cereus* DL5 on stainless steel surfaces.	The bacteria need to be colored or labeled with oligonucleotide probes for visualization.[186,187] Requirement of a CLSM to obtain the requested image quality is expensive.[192]

Table 2. Techniques to study and quantify the microorganisms attached to a surface and the bacterial viability in biofilms (continued)

Techniques	Advantages	Limitations
Atomic force microscopy (AFM)	AFM has proved to be useful in imaging the morphology of individual microbial cells and bacterial biofilm on solid surfaces, both in dried and hydrated states.[193] It is used for mapping interaction forces at microbial surfaces.[194-199] AFM is a non-invasive microscopic technique capable of imaging surfaces at nanometer resolutions,[185] and three-dimensional images at high resolution.[179] Furthermore, as no stains or coatings are needed in this method, biofilms may be observed in situ.[185] Preparation of sample surface is not required.[179] AFM can be used preferencially to other methods, such as scanning electron microscopy, as the technique has several major advantages. Since the sample do not need to be electrically conductive, no metallic coating of the specimen is required. Unlike the case with the SEM, no dehydration of the sample is required, and biofilms may be viewed in their hydrated state. The resolution of AFM is higher than that of the environmental SEM, where images can also be obtained with hydrated samples, and extracellular polymeric substances may not be imaged with clarity.[179] Within the medical context, AFM has been used to observe the effect of modified catheter surfaces on bacterial biofilm development.[200,201]	The observation area is limited as compared with SEM.[164] It cannot differentiate between live and dead bacterial cells.[164] Imaging bacterial cells can be a time consuming task.[179,202]
Fourier transform infrared spectroscopy (FTIR)	Spectroscopic techniques provide a wealth of qualitative and quantitative information about a given sample. FTIR spectroscopy measures the vibrations of chemical bonds within all the biochemical constituents of cells (i.e., proteins, lipids, polysaccharides and nucleic acids) and thus provides quantitative information about the total biochemical composition of the intact whole microbial cell.[203-207] Differences in the structure and quantity of cell wall polysaccharide, lipids and protein are reflected in the FTIR spectra enabling differentiation between bacterial strains.[208] The FTIR method is rapid, non-invasive, accurate, automated, inexpensive and quantitative, allowing users to collect full spectra in a few seconds per sample.[208,209] FTIR spectroscopy has shown to be an effective tool for analyzing bacterial strains.[194] This technique has also been shown to have sufficient resolving power for differentiation between CNS and *S. aureus*.[210,211] Amiali et al.[207] observed that FTIR spectroscopy had considerable potential as a rapid (1 h) and simple method for MRSA strain typing and monitoring in clinical settings.	Its efficacy in differentiating metabolic changes of differentially induced bacteria or genetically identical bacterial strains on different growth substrates remains untested.[205]
Radiolabelling	This technique is useful in the study of bacterial adhesion to irregular material surfaces. It is very sensitive and very accurate, allowing for rapid processing of a large number of samples.[177,212] For example, it was shown that the radiolabelling of bacteria was very useful for the studies of bacterial adhesion to irregular material surfaces, such as the surfaces of particles or spheres.[213]	It requires special laboratory space and techniques for handling radioactive materials and it carries potential risk to performers.[177]
Contact angle measurements	In the contact angle technique, a water droplet is applied to the surface of a dried lawn of bacteria. The angle formed where the water contacts the organisms is proportional to the surface hydrophobicity of the bacteria.[214] Analysis is very quick to perform.[179] Fonseca et al.[65] evaluated the hydrophobicity of *S. epidermidis* RP62A (ATCC 35984) using contact angle measurements.	Contamination of test surface may cause error in the obtained values.[179]
Molecular biological techniques	It can identify the total community of bacteria attached to a surface. It offers a very sensitive method for detection of specific genes or species. A species of bacteria can be viewed in a heterogeneous community by fluorescently labeling by oligonucleotide probes.[179] Castonguay et al.[215] employed quantitative polymerase chain reaction (PCR) in their studies for confirmation of the presence of the two bacteria in a mature biofilm.	When using oligonucleotide probes, there is a requirement that they must bind specifically to the bacterial DNA sequence.[179]
Colorimetric biomass assay (crystal violet)	This assay is used for quantification of biofilm biomass and crystal violet (CV) is frequently used. CV is a basic dye that stains both living and dead cells, by linking to negatively charge surface molecules and polysaccharides in the extra-cellular matrix.[170,216] CV assay is cheap, straightforward and is commonly used for the quantification of biofilms formed by a broad range of microorganisms.[169]	It cannot differentiate between live and dead bacterial cells.[169] Moreover, because both living and dead cells, as well as matrix, are stained with CV, this method provides no information about viability.[217]

Table 2. Techniques to study and quantify the microorganisms attached to a surface and the bacterial viability in biofilms (continued)

Techniques	Advantages	Limitations
Syto 9 assay	The fluorogenic dye Syto9 is a nucleic acid stain, which diffuses passively through cellular membranes and binds to DNA of both viable and dead cells.[218] As DNA is also a substantial part of the extracellular matrix,[219] this staining will provide information on total biofilm biomass. Syto9 has previously been used in CLSM studies of biofilm composition and morphology.[220] This stain has also been used for the routine quantification of bacterial and yeast biofilm biomass.[221,222]	This assay includes high costs of Syto9.[169] It cannot differentiate between live and dead bacterial cells.[169]
Resazurin assay	Resazurin is a common metabolic activity indicator that has been shown to be effective in assessing bacterial viability[223] and in biofilm quantification.[169] Resazurin, the main component of Alamar Blue, is a blue redox indicator that can be reduced by viable bacteria in the biofilm to pink resorufin,[224] thus continued growth maintains a reduced environment (pink) and the extent of conversion from blue to pink is a reflection of cell viability.[225] Peeters et al. showed that resazurin viability assay is a good alternative for quantification of microbial biofilms grown in microtiter-plates.[169]	It is necessary to construct a calibration curve.[226]
Fluorescein diacetate (FDA) assay	Viable microbial cells are capable of converting non-colored, non-fluorescent fluorescein diacetate (FDA) into yellow, highly fluorescent fluorescein by non-specific intra- and extracellular esterases. FDA has been used for the quantification of biofilm biomass and viability.[221,227]	It is necessary to construct a calibration curve.[164]

this, special caution should be taken in terms of the number of washings (two or three times with PBS is the most often) and the washing technique, because if it is insufficient it may lead to false-positives and if it is excessive to false-negatives.[170] Extremina et al. recommend the three washing procedure and the careful pipetting of the wells to avoid compromising biofilm integrity. (2) Another important issue that is often omitted in the literature is the need to measure planktonic growth before washing. Extremina et al. showed that *E. faecium* 1162Δ*esp* had higher planktonic index compared with the other tested strains, although being a non-biofilm producer. This procedure allows you to measure the engagement of bacteria to form biofilms by normalizing biofilms formation by the growth index, thus obtaining the biofilm formation index (BFi). For this purpose, the easiest way is to transfer the bacterial suspension to a new microtiter-plate and measure the optical density in a microtiter-plate reader. (3) It is crucial that the selection of the method takes into consideration the specific target for quantification. (4) Finally, of utmost importance is the interpretation of results and evaluation of assay quality.[170]

In the study conducted by Extremina et al., the use of BFi confirmed that E1162Δ*esp* isolate is a good negative control for biofilm quantification and demonstrates the importance of knowing the growth index of different sets of strains or conditions in order to compare biofilm formation values. Cut-off values (ODc) separate biofilm-producing from non-biofilms producing strain,[171] which is in accordance with previously described work.[172,173] Z' factor[174,175] indicated high quality for the different assays during the optimization process, thus confirming a good repeatability and reproducibility of the experimental procedures. This study recognizes that parameters for classification of biofilm producers (cut-off values), evaluation of assay accuracy (BFi), and quality (Z' factor) are of utmost importance for evaluation, comparison and validation of biofilm screening assays.[170]

Concluding Remarks and Future Perspectives

Better understanding of the interaction between microorganisms, the implant and the host may improve our current approach to the diagnosis and treatment of implant-associated infections. Despite several efforts to find medical therapies to treat biofilm infections, the physical removal of an infected medical device is often necessary, thus carrying an additional economic cost. Therefore, there is great interest in finding methods or strategies to inhibit biofilm formation. Several strategies have been proposed to achieve this on medical devices, including the use of antibiotics, development of new anti-adhesive medical surfaces and coating medical devices with several different compounds, including antibiotics. Applying antimicrobial agents is an easy and frequently used way to control biofilms. However, many antimicrobial agents that are effective against planktonic bacterial cells turn out to be ineffective against the same bacteria when growing in a biofilm. Combined use of multiple antimicrobial agents with different chemistries and modes of action may be a strategy to improve the performance of these antimicrobial agents and circumvent bacterial adaptation. However, the tremendous resistance of biofilms to conventional antibiotic therapy has prompted a great deal of research on synthetic surfaces and coatings that resist bacterial colonization.

Several biomaterials used in orthopedic surgery show different susceptibilities to infection, because adhesion and growth of infecting bacteria are controlled by biomaterial surface properties, like hydrophobicity and roughness. Controlling the topography and hydrophobic properties of materials surfaces is likewise a way to influence bacterial interaction with the surface and must be taken into account when developing novel anti-infective biomaterials.

However, since bacterial adhesion is a very complex process affected by many factors, such as bacterial and material properties and environment, further studies are required to understand the

mechanisms of bacterial adhesion and implant infection, and to provide adequate methodologies to prevent them to occur. Future research must strive to better understand the pathogenesis of implant-related infections, with a special attention on the alarming phenomenon of antibiotic resistance. Future investigations should also focus on designing animal model systems to study in vivo-grown biofilms and infections.

All the above mentioned techniques provide us with an impressive array of tools for investigating bacteria-material interactions in vitro. Each one has certain advantages and limitations with respect to the others. However, although they cannot be routinely used because of the cost, complexity of the set up and time required to give results, are useful in studying bacteria-material interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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