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FEUP Faculdade de Engenharia

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Study of nanostructured hydroxyapatite based surfaces to prevent biofilm formation associated to implant infections

Tese submetida à Faculdade de Engenharia da Universidade do Porto
para candidatura à obtenção do grau de Mestre em Engenharia Biomédica

Julho 2011

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Acknowledgments

I would like to express my sincere gratitude to the persons who helped me during this project, and made this thesis possible.

Firstly, I would like to express my acknowledgements and gratitude to my supervisor, Professor Dr. Maria Pia Ferraz, who maintained her believe and hope in my work with all her enthusiasm and affection, availability and all the sincere support.

I would also like to express my acknowledgements to my co-supervisor, Professor Dr. Fernando Jorge Monteiro for giving me strength and support during this hard journey.

I extend my thanks to everyone of the Biocomposites Group for a good working environment, for valuable suggestions and friendship during the development of this work. Special thanks to Liliana Grenho, which with great patience and accuracy helped me in the materials preparation and in the bacterial adhesion studies; and to Nilza Ribeiro for her help in the protein adsorption studies.

I would like to thank to Dr. Ana Paula Pacheco, who collaborated in this project with three interesting and important bacterial strains. I also want to thank Ricardo Silva from CEBIMED for his support, patience and availability; Ricardo Vidal from INEB for his kind assistance and availability in the Contact Angle equipment; and Daniela Silva from CEMUP for her assistance in SEM analyses.

Finally, I would like to thank my parents and Patrick for the love, caring and understanding that surely contributed for the success of this work.

Abstract

In orthopedics as in many other medical fields the vast majority of post-surgical infections is associated to the presence of implant materials, which represent a site of weakness for the host defenses where even bacteria with a low level of virulence can easily establish. Initial bacterial adhesion to biomaterial surfaces is a crucial step in the pathogenesis of prosthetic infection that can lead to biofilm formation, which enhanced resistance of bacteria against host defense mechanisms and antibiotic treatments. Unfortunately, the lack of a suitable treatment often leaves extraction of the contaminated device as the only viable option for eliminating the biofilm. Nanohydroxyapatite (nanoHA) is a calcium phosphate that has been studied aiming at being used as bone regeneration material due to its chemical similarities to the inorganic component of the body's hard tissues.

When biomaterials are implanted in the body, proteins immediately adsorb onto the implant surfaces. These host matrix proteins, which *in vivo* cover the implants, have been indicated as substrates for adhesion by specific bacterial adhesins. The study of the interaction between matrix proteins and their corresponding binding proteins in bacteria is important for understanding the mechanism of bacterial colonization with a view towards preventing or controlling infection at sites of injury. In this regard, bacterial adhesion studies performed in an environment with proteins may give important insights on the behavior of bacteria in an *in vivo* environment.

This work aimed at evaluating the initial adhesion of *S. aureus* and *S. epidermidis* strains to nanoHA sintered at 725ºC and 1000ºC. Adhesion studies of bacteria on nanoHA in the presence of fetal bovine serum (FBS), that is a mixture of serum proteins, were conducted in order to mimic *in vivo* conditions and determine how proteins presence affects bacterial adhesion. The effects of fibronectin (FN) and human serum albumin (HSA) on bacterial adhesion were also studied.

The evaluation of initial bacterial adhesion was performed for 60 minutes after which adhering bacterial cells were observed by scanning electron microscopy (SEM) and released by gentle sonication to be quantified by colony forming units (CFU). The wettability of nanoHA surfaces was determined through contact angle measurements. The distribution of FN, HSA and bacteria on nanoHA substrates was assessed by an immunofluorescence assay.

All strains adhered at a higher extent to nanoHA sintered at 725ºC than to nanoHA at 1000ºC. Adsorption of FBS on the nanoHA surface produced a decrease in adhesion of all strains on both materials. The bacterial adhesion patterns in the presence of FN

were different for both nanoHA substrates studied, where the adherence of the bacterial strains, except for *S. epidermidis* ORT strain, was significantly better on nanoHA 1000. The effect of HSA on bacterial adhesion was concentration and strain dependent. Two concentrations were tested and in this study the greater efficacy was obtained with the concentration of 200 μg/ml. The analysis of the biofilm formation ability in the presence of these proteins showed that the *S. epidermidis* strains exhibited differing profiles.

Resumo

Em ortopedia como em muitas outras áreas médicas a grande maioria das infecções pós-cirúrgicas está associada à presença de implantes, os quais representam um local de fraqueza para as defesas do hospedeiro e onde mesmo as bactérias com baixo nível de virulência podem facilmente estabelecer-se. A adesão bacteriana inicial à superfície de um biomaterial é uma etapa importante na patogénese das infecções protéticas que pode conduzir à formação do biofilme. As bactérias organizadas em biofilme são mais resistentes aos mecanismos de defesa do hospedeiro e a tratamentos com antibióticos. Infelizmente, a falta de tratamentos adequados leva frequentemente à extracção do dispositivo contaminado como a única opção viável para a eliminação do biofilme. A nanohidroxiapatite (nanoHA) é um fosfato de cálcio que tem sido estudada para o uso na regeneração óssea devido às suas semelhanças químicas com os tecidos duros do corpo.

Quando um biomaterial é implantado, as proteínas adsorvem imediatamente à sua superfície. Estas proteínas da matriz do hospedeiro, que *in vivo* recobrem os implantes, têm sido apontadas como substratos para a adesão através de adesinas bacterianas específicas. O estudo da interacção entre as proteínas da matriz e as suas proteínas de ligação correspondentes nas bactérias é importante para compreender o mecanismo de colonização bacteriana com vista a prevenir ou controlar a infecção nos locais da lesão. Desta forma, os estudos de adesão bacteriana realizados num ambiente com proteínas podem permitir a aquisição de informações relevantes acerca do comportamento das bactérias num ambiente *in vivo*.

Este trabalho teve como objectivo a avaliação da adesão inicial de estirpes bacterianas clinicamente importantes, pertencentes às espécies *S. aureus* e *S. epidermidis* a materiais de nanoHA sinterizados a 725ºC e 1000ºC. Estudos de adesão bacteriana a nanoHA na presença de soro bovino fetal, que é uma mistura de proteínas, foram realizados a fim de simular as condições *in vivo* e determinar como a presença de proteínas afecta a adesão bacteriana. A influência da fibronectina e albumina na adesão bacteriana também foi estudada.

A adesão bacteriana inicial foi avaliada aos 60 minutos de contacto após os quais as células bacterianas aderidas foram observadas no microscópio electrónico de varrimento e libertadas do material por sonicação para quantificação por contagem das unidades formadoras de colónias. A molhabilidade da superfície dos materiais foi determinada através da medição do ângulo de contacto. A ditribuição da fibronectina, albumina e bactérias pela superfície da nanoHA foi avaliada através de um ensaio de imunofluorescência.

Os resultados obtidos demonstraram que a nanoHA sinterizada a 1000ºC foi menos susceptível à adesão bacteriana comparativamente com a nanoHA a 725ºC. A adsorpção de soro bovino fetal na nanoHA produziu uma diminuição na adesão para todas as estirpes estudadas bem como em ambos os materiais. Os padrões de adesão bacteriana na presença de fibronectina foram diferentes para os materiais testados, onde a adesão das estirpes, excepto para a *S. epidermidis* ORT, foi significativamente melhor na nanoHA 1000. O efeito da albumina na adesão bacteriana foi dependente da concentração utilizada bem como da estirpe. Foram testadas duas concentrações de albumina e neste trabalho com maior eficácia obtida numa concentração de 200 μg/ml. A análise da capacidade de formação de biofilme na presença destas proteínas mostrou que as estirpes pertencentes à espécie *S. epidermidis* apresentaram perfis diferentes.

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List of abbreviations

AFM	Atomic force microscope
BHI	Brain heart infusion
BSA	Bovine serum albumin
CA	Contact angle
CCD	Charge-coupled device
CFU	Colony forming units
CoNS	Coagulase-negative staphylococci
CSLM	Confocal scanning laser microscopy
DAPI	4', 6-diamino-2 phenylindole
ECM	Extracellular matrix
EDS	Energy dispersive spectroscopy
FBS	Fetal bovine serum
FN	Human plasma fibronectin
FTIR	Fourier transformed infrared spectroscopy
HA	Hydroxyapatite
HIV	Human immunodeficiency virus
HSA	Human serum albumin
IFM	Inverted epi-fluorescence microscope
KCl	Potassium chloride
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NA	Nutrient agar
nanoHA	Nanohydroxyapatite
NaCl	Sodium chloride
PBS	Phosphate buffered saline
PIA	Polysaccharide intercellular adhesin
PJI	Prosthetic joint infections
RGD	Arg-Gly-Asp sequence
SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscopy
TEM	Transmission electron microscope
TSB	Tryptic soy broth
XPS	X-ray photoelectron spectroscopy

Chapter I – Introduction

1.1. Biomaterials

Biomaterials, an exciting field with steady, strong growth over approximately half century of existence, encompasses aspects of medicine, biology, chemistry, and materials science [1].

A definition of “biomaterial” endorsed by a consensus of experts in the field is: “A biomaterial is a nonviable material used in a medical device, intended to interact with biological systems” [1].

Biomaterials are synthetic or natural materials intended to function appropriately in a bio-environment. After the invention of first generation of materials during 1960–1970 for use inside a human body, synthetic biomaterials became a subject of interest. Often, the use of biomaterials focuses on the augmentation, replacement, or restoration of diseased or damaged tissues and organs. The use of biomaterials to restore the function of traumatized or degenerated connective tissues and thus improve the quality of life of a patient has become widespread [2-3].

The physical structure of a biomaterial is now known to be a key factor that determines cellular responses and hence the range of biomedical applications suitable for a material [4].

Several types of material, including synthetic polymers, metals, and ceramic materials, have been developed and used as biomaterials [5].

1.1.1. Ceramics

One of the most exciting and rewarding research areas of materials science involves applications of materials to health care, especially to reconstructive surgery. During the past decades, there has been a major advance in development of biomedical materials including various ceramic materials for skeletal repair and reconstruction [6].

Ceramics are inorganic compounds of metallic or nonmetallic materials, with interatomic bonding as ionic or covalent and which are generally formed at elevated temperatures. A class of such materials used for skeletal or hard tissue repair is commonly referred to as bioceramics [2].

In terms of their interaction with biological tissues, ceramics can be classified into several types: bioactive, bioinert, and biodegradable ceramics. Bioinert ceramics have almost no influence in the surrounding living tissue, and their finest example would be

alumina. Bioactive ceramics, by contrast, are capable of bonding with living osseous tissues; several calcium phosphates and certain compositions of glasses and ceramic glasses exhibit such feature. Bioactive ceramics, when in contact with physiological fluids, react chemically towards the production of newly formed bone. When dealing with repairing of a skeletal section, two extremely diverse routes could be initially considered: to replace the damaged part or to substitute it regenerating the bone. Biodegradable ceramics are ceramics that can be degraded and absorbed in a biological system. These include calcium phosphate, aluminum calcium phosphate, coralline, zinc-calcium-phosphorous oxide, and zinc sulfate–calcium phosphate. The absorbed ceramic material can be replaced by growing tissue, eventually restoring the natural structure and function of the damaged tissue [5, 7, 8].

For many years, bioceramics, especially hydroxyapatite (HA) have shown significant interest for the biomedical industry as a material to improve the quality of human life. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is a bioceramic with excellent biocompatibility and bioactivity with the human body which makes it a material of considerable interest for medical applications. It is the most important member of phosphate mineral group; mainly because it is the prototype of the inorganic material in hard tissues such as bone and teeth and is also regarded as an important implant material with significant clinical potential [9-10].

1.1.1.1. Hydroxyapatite

Many biomaterials have been studied to repair bone defects or restore bone tissue functionality [11].

During the last two decades, extensive research on ceramic materials, like calcium phosphates, has shown that these materials are suitable as bone substitutes due to their biocompatible, bioactive, biodegradable, and osteoconductive characteristics, and when implanted *in vivo*, they are nontoxic and do not induce any antigenic response [11].

Bone exhibits natural hydroxyapatite (HA) crystals with needle-like or rod-like shapes, well arranged within the polymeric matrix of collagen type I. These natural nanoparticles formed in physiological environment have a more dynamic response when compared with synthetic material with larger particle size [11-14].

Natural bone structure

Bone tissue has very interesting structural properties. The bones of all vertebrates are natural composite materials, where one of the components is an inorganic solid, carbonated hydroxyapatite. It amounts to 65% of the total bone mass, with the remaining mass formed by organic matter and water. Most of this organic matter is collagen. Collagen is the major extracellular matrix of bone and acts as the scaffold for crystallization of calcium hydroxyapatite crystals causing the unique flexibility and strength of bone tissue (Fig. 1.1). Inorganic components are mainly responsible for the compression strength and stiffness, while organic components provide the corresponding deformation and energy absorption properties [7, 15, 16].

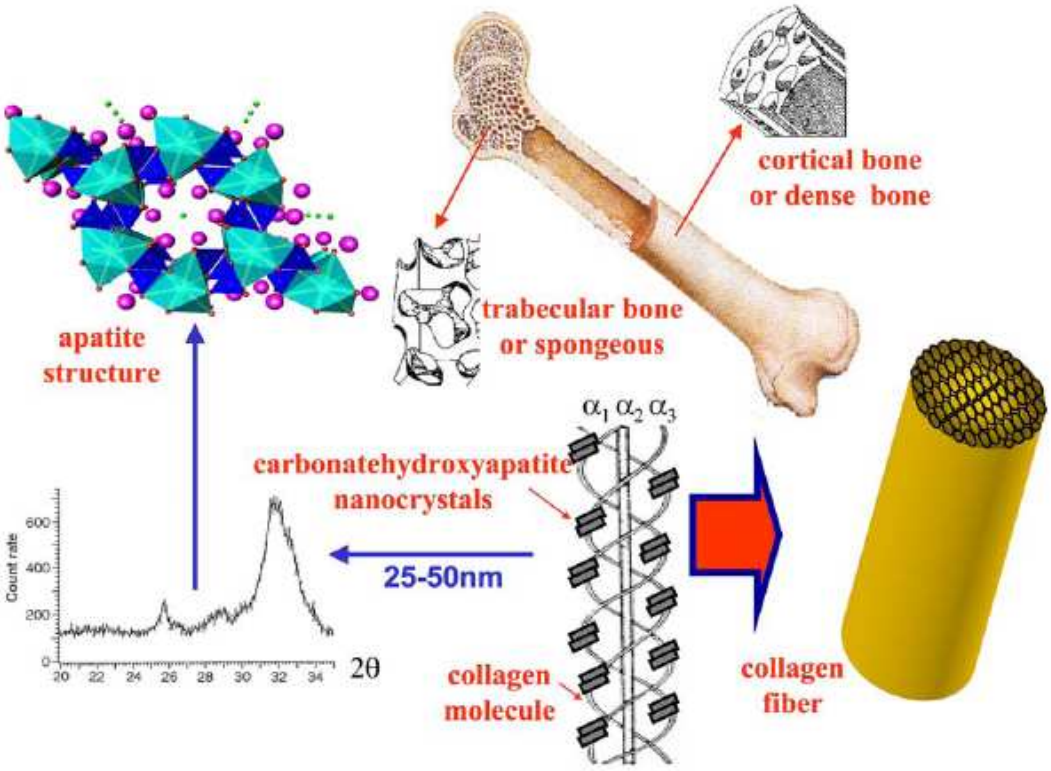


Figure 1.1. Cortical or compact bone, and trabecular or spongy bone. Arrangement of carbonate hydroxyapatite and collagen in the formation of hard tissues [7].

From a macroscopic point of view, bone tissue is non-homogeneous, porous and anisotropic. Although porosity can vary continuously from 5 to 95%, most bone tissues have either very low or very high porosity. Accordingly, we usually distinguish between two types of bone tissue. The first type is trabecular or spongy bone with 50–95% porosity. The second type is cortical or compact bone with 5–10% porosity and different types of pores (Fig. 1.1) (Fig. 1.2) [16].

Trabecular bone consists of a loosely organized, porous matrix of interconnected lamellar bone rods and plates (trabeculae). Cortical bone refers to the dense hard, calcified bone that forms the hard outer “shell” of bone that surrounds the marrow cavity. This type of bone also is present within its Haversian and Volkman canal systems for vascular supply. Cortical bone is surrounded externally and internally by membranes, periosteum and endosteum, respectively (Fig. 1.2) [17-18].

Differences between cortical and trabecular bone are both structural and functional. Differences in the structural arrangements of the two bone types are related to their primary functions: cortical bone provides mechanical and protective functions and trabecular bone provides metabolic functions [17-18].

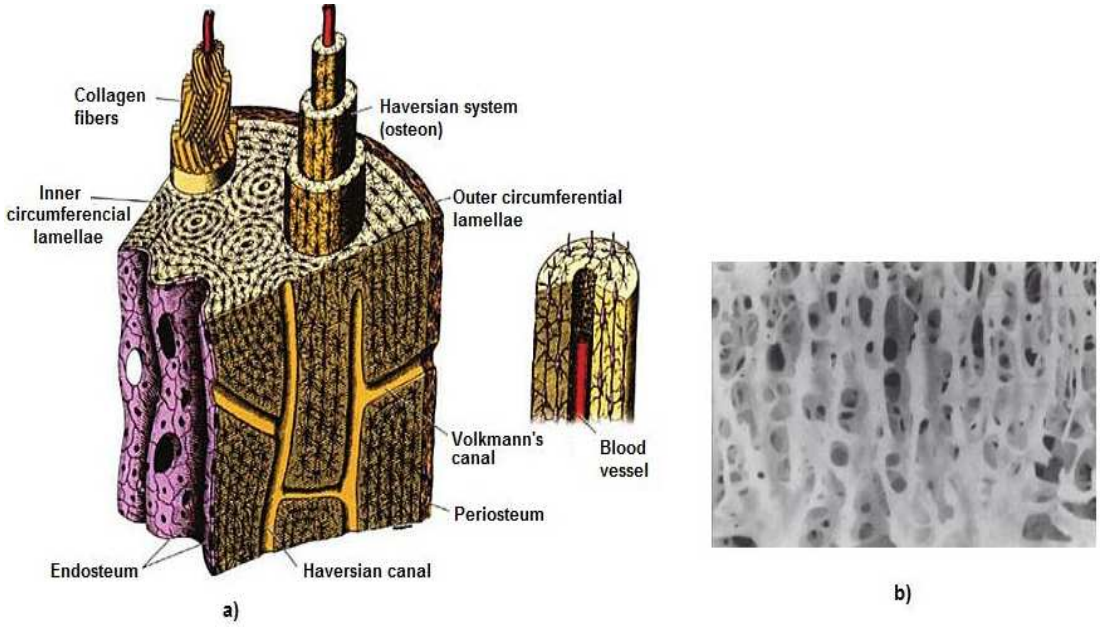


Figure 1.2. a) Schematic drawing of the cortical bone. Sectioned cortical bone showing the tubular and circumferential arrangement of osteon; in the center of each osteon is a canal, called the Haversian canal; each Haversian canal contains blood vessels. b) Trabecular bone consists of horizontal and vertical interconnecting plates called trabeculae [19].

Bone is the only tissue able to undergo spontaneous regeneration and to remodel its micro- and macrostructure. This is accomplished through a delicate balance between an osteogenic (bone forming) and osteoclastic (bone removing) process. Old bone is removed by special cells called osteoclasts[1], and new bone is deposited by osteoblasts[2]. Bone remodeling occurs during bone growth and to regulate calcium availability [3, 20].

[1] Osteoclasts remove bone, demineralizing it with acid and dissolving collagen with enzymes. These cells originate from the bone marrow.

[2] Osteoblasts are the differentiated mesenchymal cells that produce bone. They are created at the periosteum layer or stromal tissue of bone marrow.

HA is a major constituent of bone materials and is resorbed after a long time of residence in the body, showing its biocompatibility. HA is a preferred material for bone repair because of its stability under *in vivo* conditions, compositional similarity, excellent biocompatibility, osteoconductivity, and ability to promote osteoblasts functions. HAp elicits specific biological responses at the interface, which results in the formation of a strong bond between the bone tissues and the material [13, 21, 22].
Clinically, HA has been used in a variety of physical forms; as a sintered ceramic, such as in granular, block or porous form, as a deposited coating, such as bioactive coating on a bioinert implant, or as filler [10, 22].

1.1.1.2. Nanohydroxyapatite

Nanomaterials represent a promising application in a variety of areas due to their high surface area and reactivity and their ability to become dispersed in aqueous solution. A nanomaterial usually displays higher reactivity and sorption ability than the same material of micrometric particles size [23].
In addition to the dimensional similarity to bone tissue, nanomaterials also exhibit unique surface properties (such as surface topography, surface chemistry, surface wettability and surface energy) due to their significantly increased surface area and roughness compared to conventional or micrometric crystal size materials. As it is known, material surface properties mediate specific protein (such as fibronectin, vitronectin and laminin) adsorption and bioactivity before cells adhere on implants, further regulating cell behavior and dictating tissue regeneration. Furthermore, an important criterion for designing orthopedic implant materials is the occurrence of sufficient osseointegration between synthetic materials and bone tissue. Studies have indicated that nanostructured materials with cell favorable surface properties may promote greater amounts of specific protein interactions to more efficiently stimulate new bone growth compared to conventional materials (Fig. 1.3). This may be one of the underlying mechanisms why nanomaterials are superior to conventional materials for tissue growth [23-24].
Nanostructured HA materials can improve sinterability and densification due to their greater surface area, which could improve fracture toughness and other critical mechanical properties. Consequently nano HA ceramics are expected to have a better bioactivity than that of coarser HA crystals [9].

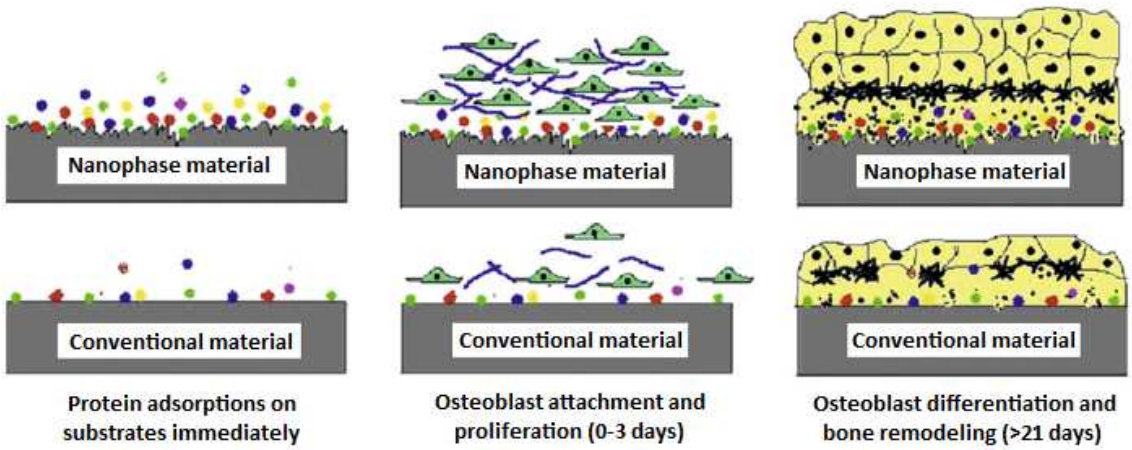


Figure 1.3. Schematic illustration of the mechanism by which nanomaterials may be superior to conventional materials for bone regeneration. The bioactive surfaces of nanomaterials mimic those of natural bones to promote greater amounts of protein adsorption and efficiently stimulate more new bone formation than conventional materials [24].

1.2. Bacterial Infections

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 a device-related infection. Implanted devices may be colonized by bacteria at the time of surgery, or they may be colonized by organisms that gain access to their surfaces by a hematogenous route, from a distant source [1].
Infectious diseases caused by pathogenic bacteria have been the leading cause of morbidity and mortality in human history. Bacterial adherence to biomaterial surfaces is an important step in the pathogenesis of prosthetic infection. The exact mechanism of prosthetic infection remains unclear [25-26].
Based on several reports, *Staphylococcus* species are the most important pathogens of implant-associated infections (Fig. 1.4) [13].

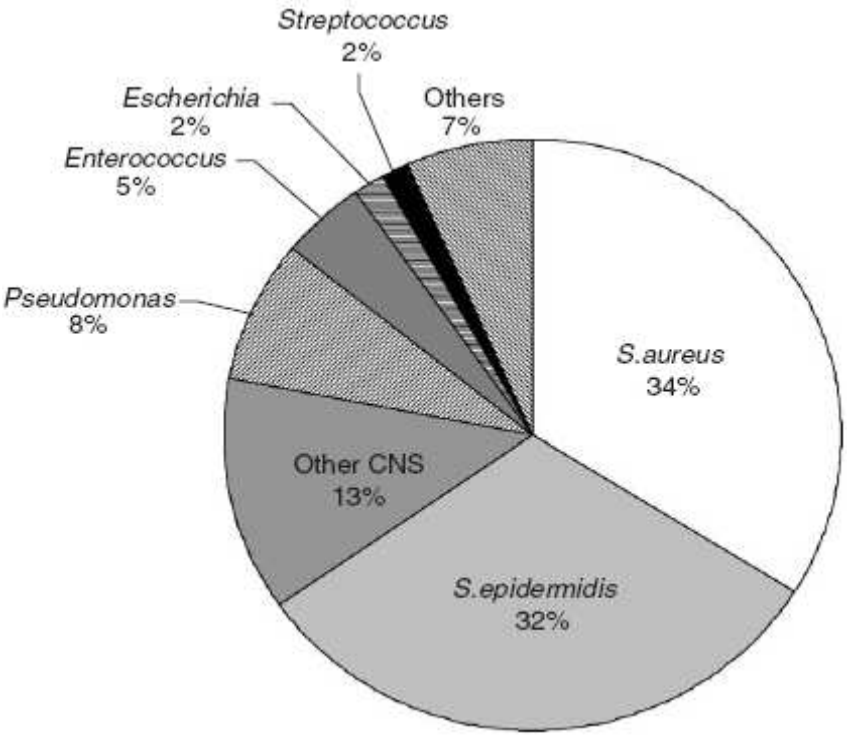


Figure 1.4. Frequency of main pathogenic species among orthopedic clinical isolates of implant-associated infections [13].

1.2.1. *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 [26].

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 colony and to grow into biofilms. Many *Staphylococcus* strains, particularly *S. epidermidis* and some *S. aureus* strains, produce a biofilm [26-28]

1.2.1.1. *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 immuno-compromised and severely debilitated patients, and prevail in the presence of indwelling medical devices [26, 29, 30, 31].

Treatment of *S. aureus* infections is often complicated, among others due to the emergence of methicillin-resistant *S. aureus* (MRSA) strains and resistance to other classes of antibiotics. Because of its pathogenic potential and the complexity of its treatment, MRSA has received more attention than its methicillin-sensitive counterpart (MSSA) [29].

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 [26].

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, these including early colonization, invasion, tissue localization and cell internalization [26, 32, 33].

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. 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 [26].

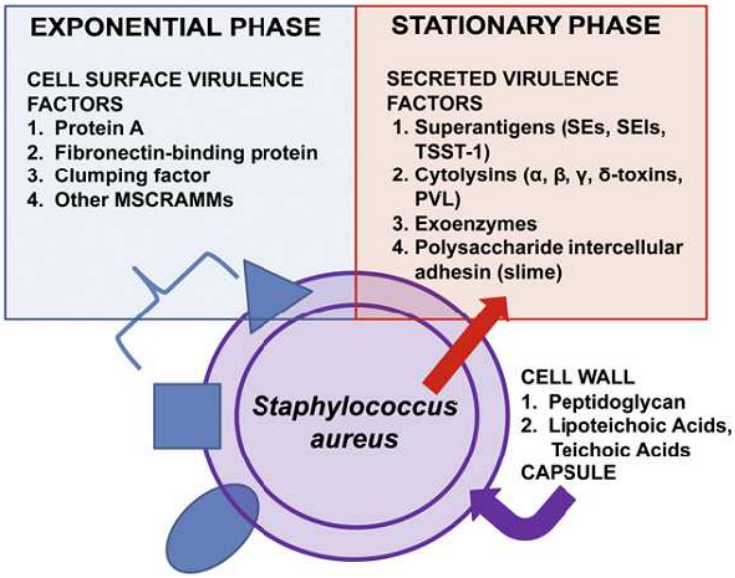


Figure 1.5. Virulence factor production by *S. aureus*. The organism produces cell surface virulence factors (MSCRAMMs) during the exponential phase and exoproteins and exopolysaccharides during the post-exponential/stationary phase [34].

S. aureus produces virulence factors (Fig. 1.5) 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 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 (Fig. 1.5), 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 [26].

1.2.1.2. *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*. This group is diagnostically distinguished from *S. aureus* by its inability to produce coagulase [26, 35, 36].

S. epidermidis very often becomes the major infective agent in compromised patients, such as drug abusers and immuno-compromised 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 [36]. 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 [26, 35-38].

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 [26, 36].

Treatment of *S. aureus* and *S. epidermidis* infections is generally difficult because of increasing resistance against many antibiotics and because the slime capsule of staphylococci represents an almost impermeable barrier to many antibiotics. For many years, the number of strains with resistance to methicillin, an antibiotic of first choice against staphylococci, has been increasing. Approximately 80% of *S. epidermidis* strains from nosocomial infections are resistant to methicillin and most are resistant to other antibiotics as well. The resistance patterns of *S. epidermidis* are similar to those of *S. aureus* strains and it can be presumed that resistance occurring in one species can be quickly transferred to the other one [36].

1.2.2. Bone tissue infections

Bone tissue infections, namely osteitis, spondylodiscitis, osteomyelitis septic arthritis and prosthetic joint infections (PJI), still represent the worst complications of orthopedic surgery and traumatology. Infection at the site of implantation of a biomaterial remains a major complication hindering the long-term use of implanted materials. The sources of bacteria that cause the vast majority of implant-associated infections include perioperative contamination, exit site contamination for percutaneous devices and hematogenous spread out from locations distal to the implant area [39-40].

Bacteria of the genus *Staphylococcus* 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 cause serious morbidity and are often difficult to manage [28].

1.2.2.1. Spondylodiscitis

Spondylodiscitis, or septic discitis, is infection of the intervertebral disc space. However, the vertebral endplates are frequently involved leading to infection of the vertebral body as well as the disc [41-42].

Many cases of spondylodiscitis are preceded by infection elsewhere, most commonly the genitourinary tract but also the skin, soft tissues, respiratory tract, gastrointestinal tract or oral cavity [41-42].

1.2.2.2. Osteomyelitis

Osteomyelitis describes a range of infections in which bone is colonized with microorganisms, with associated inflammation and bone destruction, usually caused by spread of bacteria through the blood. However, some bone or joint infections result from an adjacent focus of infection or from a penetrating injury (Fig. 1.6). 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 [28, 43].

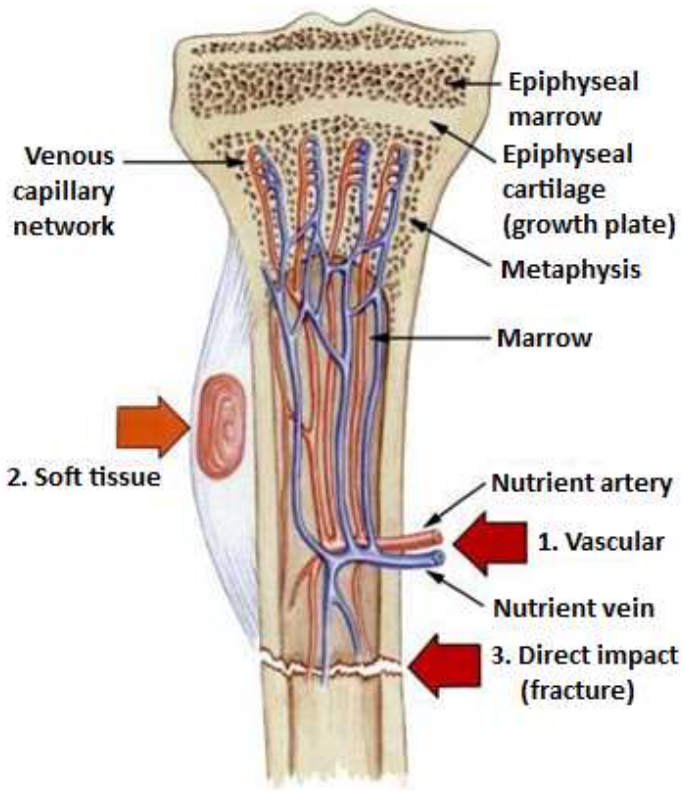


Figure 1.6. Osteomyelitis. Bone infections may be caused by contiguous spread from soft tissue infections; by fractures, especially those involving open wounds; or by hematogenous spread through vascular routes [44].

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 percent of infections; however, the appearance of the microorganism dropped to 27 percent by 1988 [43, 45].

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 [43, 45].

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-receptor form along with a polysaccharide filme after the distance between the cell and the surface is sufficiently reduced. Because biomaterials do not

elicit an antiphagocytic reaction toward bacteria after adhesion, they are able to multiply and colonize freely on implant surfaces [45].

1.2.2.3. Septic arthritis

Symptoms and signs of septic arthritis are an important medical emergency, with high morbidity and mortality [46].

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 [28, 47].

Septic arthritis arises from either hematogenous spread of microorganisms through the highly vascularized synovial membrane or from the direct extension of a contiguous infection (Fig. 1.7). The most commonly involved joints are the knees and hips [47].

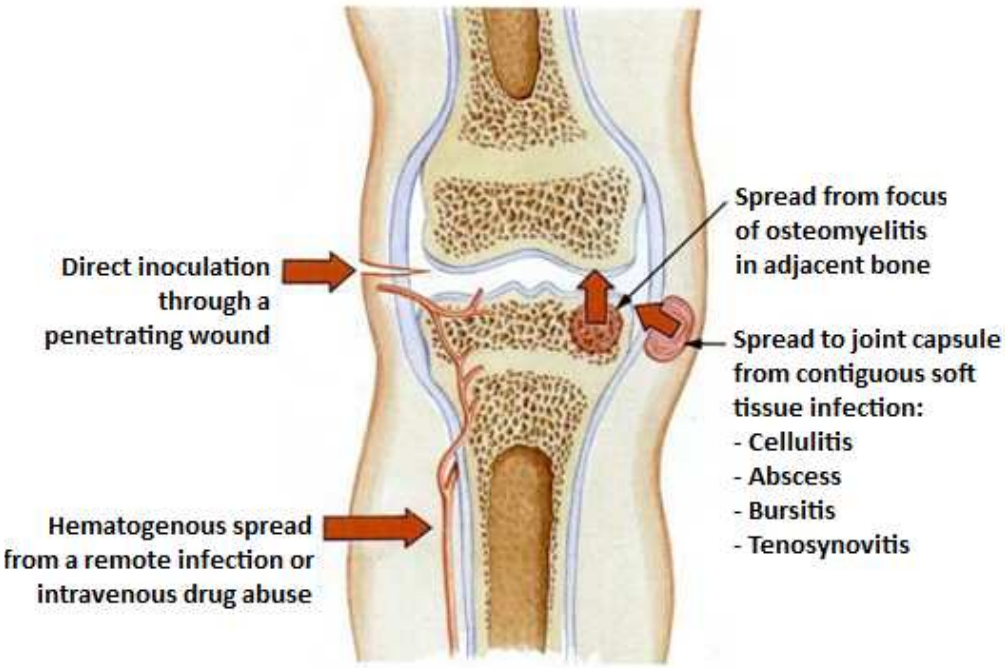


Figure 1.7. Sources of septic arthritis [44].

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 [46].

1.2.2.4. Prosthetic joint infections

The implantation of prosthetic joints along with the use of other implantable orthopedic devices (e.g., pins, screws, plates, 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. Of the possible complications associated with implantation, infection is the most serious and occurs in the 1 to 5 percent of cases; the resulting consequences include postoperative prosthesis failure, chronic pain and immobility.

Prosthetic joints and implantable orthopedic devices may become infected by two major mechanisms: (1) the prosthetic device may be contaminated by microorganisms at the time of implantation either as a result of airborne contamination in the operating room or through direct inoculation at the time of surgery, or (2) the prosthetic device may become infected through hematogenous seeding from bacteremia or by direct contiguous spread from an infection adjacent to the prosthesis [48].

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 [49-50].

The most common agents are *Staphylococcus aureus* and *Staphylococcus epidermidis*, which account for close to 65% of PJIs (Table 1.1). They are the most commonly reported microorganisms both in early and late infections and in total knee and hip arthroplasty [49].

Table 1.1. The common bacteriological findings in infected prosthetic joints [40].

Bacteriological findings in infected prosthetic joints	
Staphylococcus aureus	+++
Coagulase-negative staphylococci	+++
Haemolytic streptococcus	++
Other streptococci	+
Skin anaerobes	++
Gram-negative cocci	+
Haemophilus influenzae	+
Gram-negative aerobes	++
Pseudomonas aeruginosa	+
Salmonella	+
Intestinal anaerobes	+
Mycobacteria	+

+++, very common (30% or more); ++, common (5–30%); +, occurs in some circumstances (age, underlying disease).

Table 1.2 summarizes the classification of prosthetic joint infection according to the route of infection and the time of symptom onset after implantation [51].

Table 1.2. Classification of prosthetic joint infections [51].

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, preexisting osteomyelitis, skin and soft tissue lesions).
According to the onset of symptoms after implantation	
Early infection (<3 months)	Predominantly acquired during implant surgery or the following 2 to 4 days and caused by highly virulent organisms.
Delayed or low-grade infection (3-24 months)	Predominantly acquired during implant surgery and caused by less virulent organisms.
Late infection (>24 months)	Predominantly caused by hematogenous seeding from remote infections.

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 catheterisation; extreme age; surgical site infection; and human immunodeficiency virus (HIV) [48-49].

1.3. 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 [52-54].

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 [53].

During development of micro-colonies, some strains of bacteria, particularly S*taphylococcus epidermidis*, secrete a layer of 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 of mainly 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 [52-55].

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 [55].

An accumulated biomass of bacteria and their extracellular materials (basically slime) on a solid surface is called a biofilm (Fig. 1.8) (Fig. 1.9) [53, 55].

Figure 1.8. Staphylococcal biofilm; multiple layers of bacteria covered with a polysaccharide matrix [56].

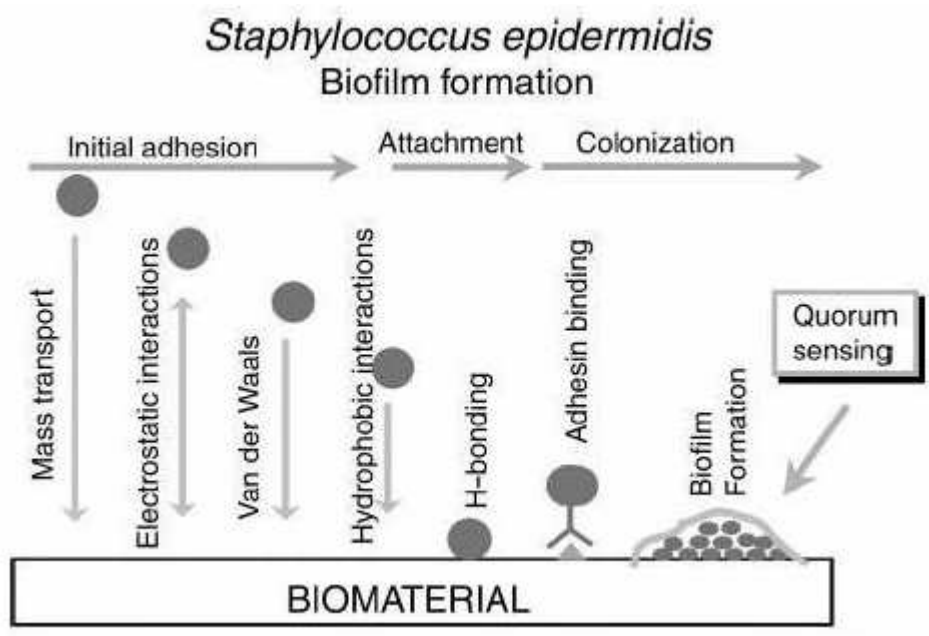


Figure 1.9. Factors involved in the colonization of a biomaterial by *S. epidermidis* [57].

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 signalling molecules (quorum sensing) induces bacteria within a population to respond in concert by changing patterns of gene expression involved in biofilm differentiation [51].

1.3.1. 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 [58].

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) [53, 55, 59].

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 (Fig. 1.10). 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 [53, 55].

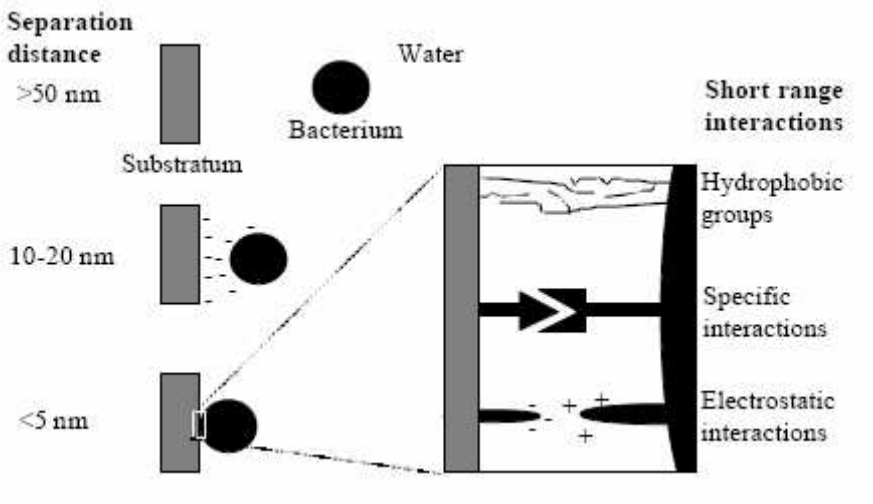


Figure 1.10. At separation distances of > 50 nm, only attractive Van der Waals forces occur. At 10 to 20 nm, Van der Waals and repulsive electrostatic interactions influence adhesion. At < 5 nm, short-range interactions can occur, irreversibly binding a bacterium to a surface [57].

1.3.1.1. 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 (Fig. 1.11). These physical interactions are further classified as long-range and short-range interactions [53, 55, 60].

The long-range interactions (nonspecific, 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 (Fig. 1.10) [53, 55, 60].

This initial attachment[3] of bacteria to surfaces is the initial part of adhesion, which makes the molecular or cellular phase of adhesion possible [53, 55].

[3] Attachment can be defined as the initial stage of bacterial adhesion, referring more to physical contact than complicated chemical and cellular interactions, and is usually reversible [53].

1.3.1.2. 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[4], fimbriae[5], 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 (Fig. 1.11) [53, 55, 60].

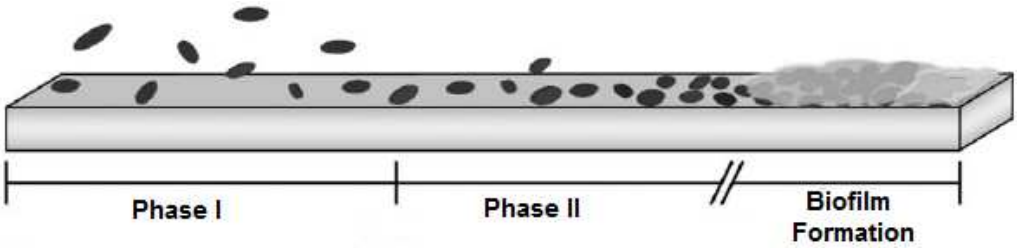


Figure 1.11. Representation of bacterial adhesion to a biomaterial substrate. Phase I adhesion involves reversible cellular association with the surface. During Phase II, bacteria undergo irreversible molecular bridging with the substrate through cell surface adhesin compounds. After this, certain bacterial species are capable of secreting a protective exopolysaccharide matrix (biofilm) that protects the adhered bacteria from host defenses and systemically-administered antibiotics [60].

1.3.2. 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 [53, 55].

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 [53, 55].

[4] Bacterial capsules are firmly adherent as a discrete covering layer with a distinct margin on the bacterial cells (at the outside of the cell wall) [53].

[5] Fimbriae (or pili) are a group of rigid, straight, filamentous appendages on a bacterial surface [53].

1.3.2.1. Environment

Certain factors in the general environment, such as temperature, time period of exposure, bacterial concentration, chemical treatment, or the presence of antibiotics, affect bacterial adhesion [53, 55].

Concentrations of electrolytes, such as KCl, NaCl and pH value in the culture environment also influence bacterial adhesion [53, 55].

The presence of antibiotics decreases bacterial adhesion depending on bacterial susceptibility and antibiotic concentration [55].

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 [53].

1.3.2.2. 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 [53, 55].

Surface chemistry influences bacterial adhesion and proliferation. Materials with different functional groups change bacterial adhesion in a manner depending on material hydrophobicity and charge [55].

Surface roughness is a 2-dimensional parameter of a material surface measured by roughness measuring systems. It has been found that the irregularities of polymeric surfaces promote bacterial adhesion and biofilm deposition whereas the ultra-smooth surface does not favor bacterial adhesion and biofilm deposition. This may happen since a rough surface has a greater surface area and the depressions in the roughened surfaces provide more favorable sites for colonization [53, 55].

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. It has been found that 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 the porous surface. Moreover bacteria adhere more to grooved and braided materials compared to flat ones, probably partially due to increased surface area. Physical configurations are routinely evaluated by scanning electron microscopy [53, 55].

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. 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. Hydrophilic materials are more resistant to bacterial adhesion than hydrophobic materials [53, 55].

The surface coating of substrates with proteins, such as bovine serum albumin (BSA), bovine glycoprotein, or fatty-acid free BSA decreases the hydrophobicity of the surface, leading to an inhibition of bacterial adhesion to surfaces [53].

1.3.2.3. Bacterial characteristics

For a given material surface, different bacterial species and strains adhere differently since they have different physicochemical characteristics [53, 55].

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. The hydrophobicity of bacteria varies according to bacterial species and is influenced by growth medium, bacteria age, and bacterial surface structure [53, 55, 61].

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 [53, 55].

1.3.2.4. 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. Firstly, transportation of the protein from the solution towards 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 [62-63].

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 [64-65].

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 [53, 55].

In this chapter brief description of fibronectin and albumin is presented since these proteins are an integral topic of this work.

Fibronectin (FN)

Human fibronectin (FN) is a large dimeric glycoprotein present in the ECM consisting of two almost identical polypeptide chains with a molecular weight around 250 kDa, linked by a pair of C-terminal di-sulphide bonds. Each sub-unit is composed of three types of repeating modules (type I, type II and type III) and contains discrete binding sites for a variety of other extracellular molecules, including fibrin, heparin and collagen, as well as for a number of integrins. This protein plays important roles in a variety of cellular processes including cell adhesion, growth, differentiation and migration. It plays an important role in cell attachment to biomaterials surfaces through its central-binding domain RGD sequence [66-70].

Fibronectin is a protein that seems to promote adhesion in certain strains. FN clearly promotes *S. aureus* adhesion to the substratum surface. The binding of FN to a strain of *S. aureus* is specific, time-dependent and irreversible. 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 [53, 55].

In the fibronectin molecule, two different binding sites are known for staphylococci: a first binding site in the N-terminal domain and a second near the C-terminus. A recent study by reports a higher affinity of *S. epidermidis* for the C-terminal fragment. FN has played a crucial role in the promotion of bacterial adhesion to biomaterial surfaces [71].

Fibronectin-binding proteins from *Staphylococcus*

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 [72].

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 [73-75].

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 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 [67, 76, 77].

S. epidermidis has been also 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 (Fig. 1.12). However, compared to *S. aureus* little is known about how *S. epidermidis* interacts with matrix proteins [74].

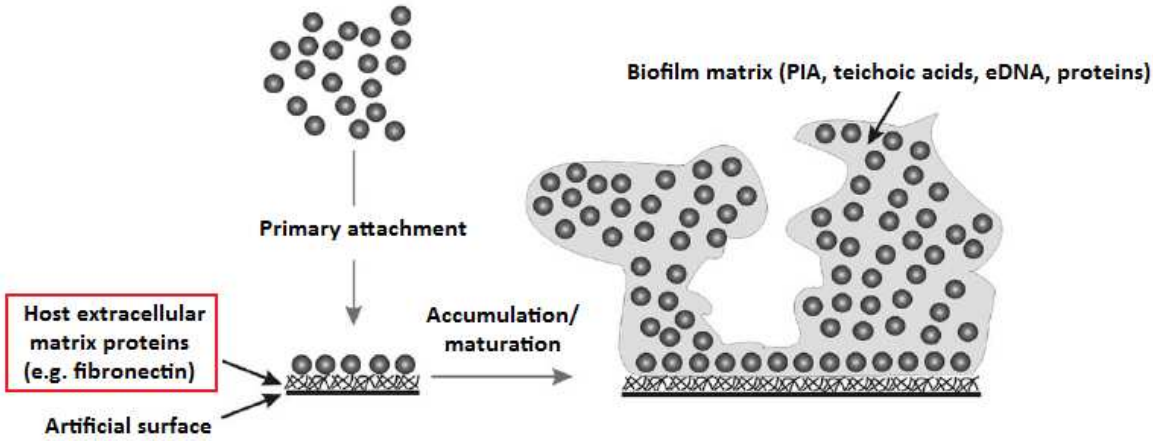


Figure 1.12. Schematic representation of *S. epidermids* biofilm formation. *S. epidermidis* surface colonization occurs in two steps. During primary attachment, bacteria (indicated by filled circles) get in contact with the surface to be colonized. Here, interactions with surface-deposited host ECM protein (e.g. fibronectin) mediate interactions *of S. epidermidis* with implanted material. Subsequently, via expression of intercellular adhesive surface properties, *S. epidermidis* organizes a multi-layered biofilm. This phase is referred to as the accumulative phase and crucially depends on synthesis of PIA. PIA forms an extracellular biofilm matrix embedding the bacterial cells [78].

It was found that in *S. epidermidis* 1585v over-expression 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 [78-79].

Albumin

Human serum albumin (HSA) is the most abundant plasma protein, which accounts for approximately 60% of the total protein. It is a single peptide chain composed of 585 amino acids with a largely-helical triple-domain structure that assemble to form a heart-shaped molecule. It is characterized by a repeating pattern of three a-helical homologous domains, derived from genic multiplication and numbered I, II and III, starting from the amino terminus. Each domain is divided into two sub-domains, A and B, composed respectively of six and four a-helices. It has a molecular weight of approximately 66 kDA [80-82].

Albumin plays important roles in the human body, including regulation of osmotic pressure between the blood and tissues; transport protein for a variety of endogenous and exogenous compounds such as fatty acids, hormones, calcium, drugs, and a large diverse of metabolites; anti-inflammatory effects; anti-apoptotic effects; anti-platelet effects; and anti-oxidant properties [80, 83, 84].

The most common and simple approach for albumin immobilization is physical adsorption onto biomaterials. Because albumin is adsorbed tightly onto hydrophobic surfaces but it has poor affinity for hydrophilic surfaces, it is easy modify hydrophobic surfaces with albumin (by simple exposure to the albumin solution) [85].

Albumin adsorbed on material surfaces has shown obvious inhibitory effects on bacterial adhesion to polymer, ceramic and metal surfaces. Albumin may inhibit the adhesion by means of binding to the bacterial cells or by changing the substratum surface to more hydrophilic [53, 55].

1.4. Techniques for studying bacterial attachment

Bacterial interactions are of prime importance in the many stages of the lifecycle of a bacterium. They play essential roles in pathology, ecology and biotechnology and contribute to phenomena as varied as bacterial infections, the subsequent immune response of the infected host, the formation of biofilms and adhesion of bacteria to surfaces [86].

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 [87-88].

The materials characterization and the study and quantification of the adhered bacteria and biofilm can be examined by a number of techniques, which are the following.

1.4.1. Techniques used in the materials characterization

Various techniques that can be used in the materials characterization are presented in Table 1.3. These techniques have been developed to analyze the materials surface and verify its influence on bacterial adhesion. The combined use of these techniques, investigating the materials at different depths and having various sensitivities, provides a complete set of information.

Table 1.3. Techniques used in the materials characterization [11, 89-92].

Techniques	Description
Fourier Transformed Infrared Spectroscopy (FTIR)	Chemical characterization and identification of functional groups on the surface.
Energy Dispersive Spectroscopy (EDS)	Semi-quantitative chemical analysis about very specific locations within the area of interest.
X-ray Photoelectron Spectroscopy (XPS)	XPS provides elemental information on the surface and it is suitable for chemical state identification of surface species.
Transmission Electron Microscope (TEM)	Determination of the particle size and morphology.
Scanning Electron Microscopy (SEM)	Analysis of the surface morphology of the samples.
Atomic Force Microscope (AFM)	Analysis of the surface topography of the samples.
Mercury porosimetry	Determination of the surface area and porosity.
Zeta potential	Determination of the surface charge.

The technique contact angle measurement is also used in the materials characterization, however it is discussed in greater detail in the next section since it was a technique used in the practical work.

1.4.1.1. Contact angle measurements

Wetting behaviour can be quantified by contact angle (CA) measurement. The contact angle is a very common measure of the hydrophobicity of a solid surface. Contact angle and the wetting behaviour of solid particles are influenced by many physical and chemical factors such as surface roughness and heterogeneity as well as particle shape and size. Two procedures are available for studying contact angles at the three-phase contact line of solid surface/water/air: a drop of water can be placed on a solid surface - sessile drop technique; alternatively, a submerged air bubble can be used to probe the surface of a solid immersed in water - captive bubble technique [92-93].
Protein adsorption at interfaces has been the focus of a significant body of research. Understanding the mechanism and processes involved when a protein interacts at the solid–liquid interface is essential for a number of medical applications. To study this critical phenomenon, control of the physico-chemical properties of the substrate is required. A key role is played by the surfaces physico-chemical status which can be determined by wetting techniques such as the sessile drop and the captive bubble [94].

1.4.2. Techniques used in the study and quantification of adhered bacteria

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. A brief description of these techniques is summarized in Table 1.4.

Table 1.4. Techniques used in the study and quantification of microorganisms attached to a surface [55, 87, 95-102].

Techniques	Description
Light microscopy	Technique for bacterial enumeration and observation. Normally bacteria are stained with dyes like crystal violet or fuchsin. The substratum surfaces have to be translucent to use light microscopy.
Epifluorescence microscopy	Possible to differentiate between live and dead bacteria. Image analysis systems are used for determining the number of cells

	adhered. It makes direct observation and enumeration possible for attached bacteria on an opaque surface. It is very quick.
Atomic Force Microscopy (AFM)	AFM has proved useful in imaging the morphology of individual microbial cells and bacterial biofilm on solid surfaces, both in dried and hydrated states. It is used for mapping interaction forces at microbial surfaces.
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.
Radiolabelling	This technique is useful in the study of bacterial adhesion to irregular material surfaces. It is very sensitive and very accurate and allowing rapid processing of a large number of samples. The disadvantage of radiolabelling is the need of special laboratory space and techniques for handling radioactive materials and the potential risk to performers.
Contact Angle Measurement	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.

The techniques colony forming units counting (CFU); scanning electron microscopy (SEM); and confocal scanning laser microscopy (CSLM) are also used to study and quantify bacterial adhesion on material surfaces. However these techniques are presented in greater detail in the subsequent sections since they were techniques used in the practical work.

1.4.2.1. Colony forming units counting

Colony forming units (CFU) plate counting is the most basic method for bacterial enumeration. After incubation with bacterial in study, the material is washed. The washing is a very important part of a bacterial adhesion study, and its purpose is to remove the unattached and loosely adhered bacteria from the material surface. Liquids commonly used for washing include sterile water, normal saline, and phosphate buffered saline (PBS). Methods for removing bacteria from substrata surfaces include homogenization, sonication, and the use of surfactants. According to the comparative study by *McDaniel and Capone* [95] sonication appears to be an efficient and safe way to remove bacteria from biomaterial surfaces [95].

There are two basic ways to perform plate counting, the pour plate method and the surface spread method. In the pour plate method, a measured volume of the sample is mixed with the appropriate molten agar medium in a sterile Petri dish. After incubation at 37°C overnight colonies are counted and the bacterial density in the original solution calculated. In the surface spread method, serial tenfold dilutions of the original sample are prepared. Commencing at the highest dilution, a measured volume from each dilution are pipeted onto the surface of each of agar plates. The drop is then spread over the whole surface using an inoculation loop or a flamed glass spreader. The plates are incubated at 37°C overnight and then the colonies are counted [55, 95, 103].

1.4.2.2. Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a well-established basic technique to observe the morphology of bacteria adhered on a material surface, the morphology of the material surface, and the relationships between them. It is also used to observe the morphology of bacterial biofilms on surfaces [95, 103, 104].

SEM produces its image by the differential analysis of backscattered and secondarily emitted electrons from the specimen. It provides a very useful topographic view of large areas of the surface of specimen, but it requires the specimen to be conductive (essentially “metal sputtered”). Once the surface of a bacterial biofilm has been made conductive, that exopolysaccharide surface constitutes the specimen surface; bacterial cells are seen only when they deform the biofilm or protrude when the polysaccharide matrix has been radically condensed during dehydration. It also requires specialist equipment, training and extensive preparation of samples. Even with these limitations, SEM is a very useful technique for the examination of the colonization of surfaces by bacteria and for the examination of biofilm development by these adhering microorganisms [1, 105, 106].

1.4.2.3. Confocal Scanning Laser Microscopy

Confocal scanning laser microscopy (CSLM) is a three-dimensional technique using fluorescent molecular probes and laser beams to study bacterial associations with surfaces *in situ*. It is used to visualize and count bacterial cells directly on transparent or opaque surfaces [104, 107].

The CSLM allows the examination of living fully hydrated biofilms in real time, and the simultaneous use of specific molecular probes allows determine the identity (oligonucleotide probes) and the physiological state (live versus dead) of the adherent

bacterial cells. This CSLM-based technique may be used to accurately assess the antibacterial properties of biofilm-resistant biomaterials [107-108].

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 [109].

1.5. Inhibition of bacterial adhesion

Persistent bacterial infections associated with medical implants remain a serious and costly drawback with both temporary and permanent consequences. Such infections have proven to be difficult to fight with antimicrobial therapy and have been identified as a major cause for device failure, explantation, and patient morbidity [110].

Clinical observations of implant-associated infections have revealed that the initial adhesion and growth of microorganisms on the surface of an implant may be the most critical event in the development of device-associated infection. Therefore, efforts have been directed to intervene in the early stages of bacterial colonization [110].

Despite several efforts to find medical therapies to treat biofilm infections, the physical removal of an infected medical device is often necessary, which carries an additional economic cost. Therefore, there is great interest in finding methods or strategies to inhibit biofilm formation. Several strategies have been proposed to inhibit biofilm formation on medical devices, including the administration of antibiotics, development of new anti-adhesive medical surfaces and coating medical devices with several different compounds, including antibiotics [110-111].

1.5.1. Antimicrobial agents

Antimicrobial agents are 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 growing in a biofilm (Fig. 1.13). Combined application 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. The combination of two kinds of antimicrobial agents, for example, silver and tobramycin has showed to enhance antimicrobial efficiency against biofilms, which might be an effective strategy for preventing biofilm formation [112-113].

The use of antimicrobial agents before the development of biofilms might also be an effective strategy, preventing adhesion of microorganisms [112].

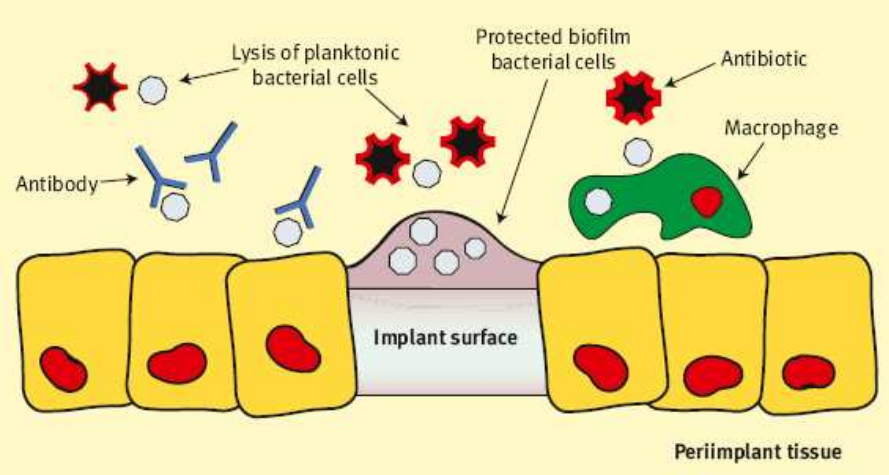


Figure 1.13. Schematic representation of planktonic bacterial cells killed by antibiotics and the immune system, and biofilm microorganisms, attached to a surface and protected in an extracellular matrix [114].

1.5.2. Surface modifications

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. New coatings with antibacterial properties have been developed to reduce bacterial adhesion by altering the physicochemical properties of the substrate so that conditioning films do not form and/or bacteria-substrate interactions are not favorable, using hydrophilic polymers, proteins, biosurfactants, metal ions, antibiotics, or bacterial quorum-sensing[6] inhibitors [115-118].

When a surface is reached, microorganisms will be attracted or repelled by it, depending on the resultant of the different nonspecific interaction forces. Most organisms are negatively charged and consequently a negatively charged surface exerts a repulsive electrostatic force. Controlling the charge and hydrophobic properties of substratum surfaces is likewise a way to influence bacterial interaction with the surface and must be taken into account when novel anti-infective biomaterials are to be developed [119].

Bacteria such as *S. aureus* and *S. epidermidis* are known to interact with adsorbed host proteins such as fibronectin, however, coatings based on hydrophilic proteins such as albumin or human serum have been shown to inhibit *S. aureus* and *S. epidermidis* adhesion to the surfaces. Albumin is believed to interfere with the hydrophobic interactions between the surface and bacterial cell wall, preventing initial bacterial adhesion [26].

[6] Quorum-sensing is a process in which bacterial communities detect their immediate population density and regulate biofilm formation and virulence [117].

1.6. Aims of the thesis

The presence of an implant within the body, particularly in open fractures is known to increase susceptibility to infection. *Staphylococcus aureus* and *Staphylococcus epidermidis* are a frequent cause of orthopedic infections and these bacteria express several adhesins that facilitate bacterial binding to the bone matrix and to bone implant biomaterials coated with host plasma constituents [26, 33].

After adhesion on the surface of the implant, bacteria produce an extracellular matrix leading to biofilm formation. Bacteria in biofilms are extremely resistant to host defense mechanisms or antibiotics because penetration or diffusion of antibodies and antimicrobial agents is hindered. Implant-related infections are thus a major clinical problem which may result in osteomyelitis, localized bone destruction, and implant failure, possibly necessitating its removal and substitution of the prosthesis [120].

Therefore, the aim of the present work was to study the ability of *S. aureus* and *S. epidermidis* strains to adhere to nanohydroxyapatite (nanoHA) sintered at 725°C and at 1000°C, a material commonly used in the bone regeneration, to contribute to a better understanding of the phenomena that involve biomaterial infection caused by these strains. In order to help mimic *in vivo* conditions, a model protein, fetal bovine serum (FBS), which is a mixture of serum proteins, was adsorbed onto the nanoHA samples to determine how proteins presence affects bacterial adhesion. The next objective was to evaluate how adsorption of two important proteins, namely fibronectin (FN) and albumin (HSA), on nanoHA surfaces affects *S. aureus* and *S. epidermidis* adhesion. The ultimate goal was to check if HSA adsorbed on nanoHA can be a possible method to minimize the adhesion and colonization of these strains on medical surfaces, thus reducing associated infections.

Chapter II - Materials and methods

2.1. Preparation and characterization of nanoHA samples

2.1.1. NanoHA samples

Nanohydroxyapatite (nanoHA) was kindly supplied by Fluidinova S.A. (Maia, Portugal), (nanoXIM·Hap202). According to Ribeiro *et al*. [121], cylindrical nanoHA discs were obtained using 75 mg of dry powder under uniaxial compression stress of 20 bar (Mestra Snow, P3, Talleres Mestraitua). The discs, when prepared, were submitted to a sintering[7] cycle inside the furnace Commodore II (VPF, Jelenko). As used by Ribeiro *et al*. (2009), the sintering temperatures were 725°C (nanoHA 725) and 1000°C (nanoHA 1000) during 15 minutes with a heating rate of 20°C/min. The sintering cycle was completed with a cooling process inside the furnace (9°C/min). Sintering temperatures and cycle were selected based on previous work on materials characterization [121]. The mass of the nanoHA discs was assessed by using an analytical balance (Metler, AG285).
The sterilization of the disks was made by dry heat in the oven (FP115, Binder) at 180°C, during 2 hours at atmospheric pressure.

2.1.2. Contact angle measurements of nanoHA samples

The hydrophobicity/hydrophilicity of a solid surface is usually expressed in terms of wettability which can be determined by contact angle measurement. Contact angle measurements were performed on untreated and protein-treated (0.5 mg/ml of FBS, 20 μg/ml of FN or 20 μg/ml of HSA in PBS, for 60 min) nanoHA by the captive air-bubble technique (hydrated samples), using an automated contact angle measurement apparatus (Data physics measurement system, model OCA 15, Germany) (Fig. 2.1). It includes a video charge-coupled device (CCD) camera, a liquid container, a solid surface, a nozzle connected with an electronic syringe and SCA 20 software. Glass slides were used as solid surface and phosphate buffered saline (PBS) (pH7.4, Sigma)

[7] The processing of powders to form solid products necessarily involves the packing together of fine powders, their consolidation and heating to form bonds. This process of diffusion and bonding is referred to as solid state sintering. In the process of sintering the powder particles are compacted together, after this the particles beginning to bond together, and finally we have a fully sintered ceramic [122].

was used as liquid. The samples were attached to a glass slides with histofluid and the slides were horizontally placed above the nozzle and immersed in a cuvette full of PBS. Measurements were made at room temperature and ambient humidity. The deposition of an air bubble of 5 μl, by injecting air through the syringe, on each sample was recorded in a video and analyzed to obtain the contact angles. Reported results are the average ± standard deviation (SD) of at least 10 measurements (two air bubbles per sample).

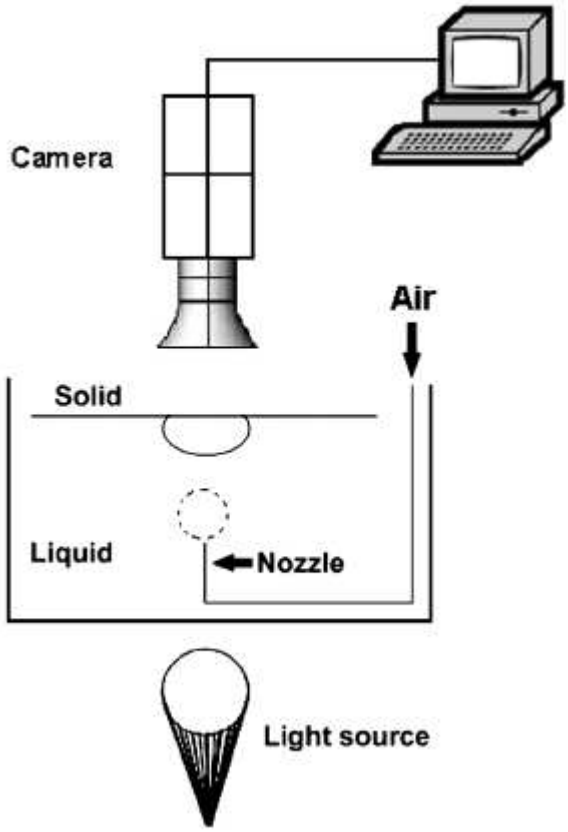


Figure 2.1. Ilustration of the automated contact angle measurement apparatus [123].

2.2. Bacterial strains

The strains *Staphylococcus aureus* and *Staphylococcus epidermidis*, which have been identified as major causative agents for bone infections, were used in this study. The strains of *S. aureus* that were used in the experiments were two clinical strains with phenotype MSSA (Methicillin-sensitive *Staphylococcus aureus*) and MRSA (Methicillin-resistant *Staphylococcus aureus*) isolated from bone infections, both kindly provided by Dr. Ana Paula Pacheco. The strains of *S. epidermidis* used were the reference type culture RP62A (ATCC35984) slime producing provided by CEBIMED (Centro de

Estudos em Biomedicina, Universidade Fernando Pessoa, Portugal) and a clinical strain isolated from a bone infection, identified as *S. epidermidis* ORT, kindly provided by Dr. Ana Paula Pacheco.

2.2.1. Growth and storage conditions

Microorganisms were kept at −70°C, in a solution containing 70% tryptic soy broth (TSB, Difco, USA) and 30% diluted glycerol (glycerol/water: 1/1) [124].
S. aureus cultures were growth in Nutrient Agar (NA, Liofilchem, Italy) and *S. epidermidis* in Brain Heart Infusion agar (BHI, Liofilchem, Italy) for 24h at 37°C.
All media were prepared according to the manufacturer's instructions.

2.3. Bacterial adhesion studies

2.3.1. Adhesion assay

For bacterial adhesion studies, bacteria were cultured in 15 ml of tryptic soy broth (TSB) and placed to incubate during 24h at 37°C. After 24h, 50 ml of culture was transferred to a tube containing 30 ml of sterile TSB and it was incubated for 18h at 37°C with gentle shaking, in order to obtain cells in stationary phase. After that the bacterial suspensions were harvested by centrifugation (Labofuge 400R, Heraeus) during 5 min at a centrifugal force of 1050 *g*. Bacterial cells were washed twice and finally re-suspended in saline solution (0.9% NaCl prepared in distilled water) at a final concentration of $1{,}5\times10^{8}$ colony forming units cells/ml. Bacteria cell concentrations were measured using a densitometer (Densimat, BioMérieux, France) and its scale McFarland [124].
The material samples were placed in glass tubes with 2 ml of the bacterial suspension, previously prepared, and incubated in a bath at 37°C with gentle shaking during 60 min. Negative controls were obtained by placing the materials samples in a saline solution without bacterial cells. Three replicates were used for each experiment. After adhesion, each sample was gently washed twice with PBS to remove non-adherent or loosely adherent bacteria.

2.3.2. Colony forming units counting

After the washes, the samples were transferred to glass tubes containing 5 ml of sterile PBS. All the tubes were sonicated for one second, at a frequency of 20 kHz in a

sonicator (Sonopuls HD 2200, Bandelin, Germany) with a probe MS 73. After the release of the adhered cells serial dilutions of the sonicated solutions in PBS were prepared and inoculated in appropriate culture plates according the bacterial strain in study. After 24h of incubation at 37°C, the number of adherent bacterial colonies was counted. As a control, material samples were also plated after the sonication procedure in order to check if all bacteria were removed by sonication [124].

Initially, the number of adhered bacteria was expressed as CFUs/ml, calculated according with the equation 1.

$$CFU/ml = \frac{number\ of\ colonies \times dilution\ factor}{inoculated\ volume}$$

After that, the values of CFUs/ml were adjusted to the area of the samples in order to be expressed in CFUs/mm^2.

2.3.3. Scanning electron microscopy

After adhesion assays, each sample was gently washed with PBS to remove non-adherent or loosely adherent bacteria and then fixed in 1.5% (v/v) glutaraldehyde (Fluka, Germany) for 20 min at room temperature. After fixation, the samples were dehydrated by several passages in ethanol-water solutions increasing concentrations of ethanol to 50, 60, 70, 80, 90 and 100% (v/v) at 10-minute intervals and air dried at room temperature overnight [124].

Finally, the samples were sputter-coated (SPI-Module) with a thin gold/palladium film and analyzed by SEM using a scanning electron microscope FEI Quanta 400 FEG/ESEM (FEI, USA) at an accelerating voltage of 15 kV.

In order to assess the extent of bacterial adhesion in each sample five SEM micrographs of randomly selected areas were taken of each sample and all photographs were examined using a magnification of 5000×. To evaluate the interactions between bacteria as well as the interactions between bacteria and material higher magnifications (30000×) were used.

2.4. Protein adsorption studies and its influence on bacterial adhesion

2.4.1. Protein solutions

Three proteins were used as models, fetal bovine serum (FBS) (Invitrogen), which is a mixture of serum proteins; human plasma fibronectin (FN) (Sigma, Ref. F0895) and

human serum albumin (HSA) (Sigma, Ref. A9511). The FBS solution was prepared by dilution in phosphate buffered saline, (PBS, Sigma, USA) at a concentration of 0.5 mg/ml [125]. The FN and HSA solutions were also prepared by dilution in PBS at concentrations of 20 μg/ml for FN [121] and of 20 μg/ml [116] and 200 μg/ml for HSA.

2.4.2. Adsorption of FN and HSA to nanoHA

Samples corresponding to nanoHA discs were placed in glass tubes with 2 ml of protein solution, previously prepared, and incubated in a bath at 37°C with gentle shaking during 60min. Three replicates were used for each experiment. Following incubation in the protein solution, each sample was gently washed twice with PBS to remove non-adherent or loosely adherent protein. After removal from the protein solution, the samples were immersed in 2 ml of a bacterial suspension and incubated in a bath at 37°C with gentle shaking during 60 min [116].

2.4.3. Adhesion assay

Bacterial adhesion assays were performed as previously described in sub-chapter 2.3.1.

2.4.4. Colony forming units counting

The enumeration of adhered bacterial cells was performed as described in sub-chapter 2.3.2.

2.4.5. Scanning electron microscopy

After the studies of the adsorption of FBS, FN and HSA to nanoHA a procedure similar to the one followed for bacterial adhesion studies described in sub-chapter 2.3.1 was used. Subsequently, the samples were fixed in 1.5% (v/v) glutaraldehyde (Fluka, Germany) for 20 min and the procedure described in 2.3.2. was repeated.

2.4.6. Immunofluorescent staining of FN, HSA and adherent bacteria

The distribution of FN, HSA and bacteria on nanoHA substrates was assessed by immunofluorescence. For FN detection, the samples corresponding to nanoHA were incubated in the FN solution (20 μg/ml; 60 min) and following incubation the samples were washed twice with PBS to remove non-adherent or loosely adherent proteins, and

then fixed in 3.7% methanol-free formaldehyde for 20 min. Samples corresponding to nanoHA discs incubated in PBS without FN were also processed to provide controls for non-specific antibody binding. After this, the samples were incubated with 1% heat denatured BSA for 60 min, to block non-specific adsorption, and washed again with PBS to remove non-adsorbed BSA. Subsequently, samples were incubated at 37°C for 60 min with the mouse anti-human monoclonal antibody HFN7.1 directed against the major integrin-binding RGD site of human FN (Developmental Studies Hybridoma Bank, Iowa City, IA) diluted 1:10,000 in blocking buffer (PBS, 0.25% bovine serum albumin (BSA), and 0.05% Tween-20). The unbound antibodies were removed by washing with a buffer containing PBS and 0.05% Tween-20. After that, the samples were incubated at 37°C for 60 min with a Alexa fluor 488-conjugated rabbit anti-mouse IgG, (Invitrogen, Molecular Probes), diluted at 1:400 in blocking buffer, to visualize the primary antibody and therefore to allow the identification of the protein location by fluorescent imaging. After incubation, the samples were washed with buffer (PBS with 0.05% Tween-20) to remove the unbound antibodies [121].

For HSA detection, the nanoHA samples were incubated in the HSA solution (20 μg/ml; 60 min) and the procedure was equal to the one followed for FN using a mouse anti-human/mouse albumin monoclonal antibody (CITOMED, Ref. MAB1455), diluted at 1:50, and a Alexa fluor 488-conjugated rabbit anti-mouse IgG, (Invitrogen, Molecular Probes), diluted at 1:400 in blocking buffer (PBS, 0.25% BSA, 0.05% Tween-20). Samples corresponding to nanoHA discs incubated in PBS without HSA were also processed to provide controls for non-specific antibody binding. Finally, the samples were examined in an inverted epi-fluorescence microscope (IFM), (Zeiss Axiovert 200M) using Micro-Manager 1.3 software.

For the assays involving bacteria labeling, the nanoHA samples with adsorbed FN and HSA were incubated in the bacterial solution (60 min) and following incubation the samples were washed twice with PBS to remove non-adherent or loosely adherent bacteria. Subsequently the samples were fixed in 3.7% methanol-free formaldehyde for 20 min. After this period of time, the samples were washed with PBS, label with the specific antibodies for FN and HSA as described above, and finally label with DAPI (10 μg/mL in PBS) for 20 min to allow the identification of the bacteria location. Samples corresponding to nanoHA incubated in the bacterial solution without protein were also processed to provide controls. The procedure applied in this immunofluorescence analysis was similar to the one followed above.

2.5. Statistical analyses

Results from all the assays obtained were analyzed using SPSS (Statistical Package for the Social Sciences Inc., USA). The comparison was performed through one-way analysis of variance (One-way ANOVA), followed by post-hoc comparisons for all possible combinations of group means applying the Tukey HSD multiple comparison test. The difference was considered statistically significant for $p<0.05$.

Chapter III - Results

3.1. Contact angle measurements of nanoHA samples

Contact angle measurements were performed by the captive air-bubble technique The results regarding the contact angles of nanoHA sintered at 725ºC and 1000ºC both with and without adsorbed FBS, using PBS as testing liquid, are present in Table 3.1.

Table 3.1. Contact angles measurements (deg) (average ± standard deviation) for nanoHA materials sintered at 725ºC and at 1000ºC with and without (control) adsorbed FBS.

	Control θ (deg)	FBS θ (deg)
NanoHA 725°C	40,2 ± 3,9	32,7 ± 3,4
NanoHA 1000°C	38,3 ± 1,8	26,1 ± 1,8

Contact angle measures the wettability of the surface, with values higher than 65°C indicating more hydrophobic surfaces [126], and it in turn affects the adhesion of the bacteria. As it can be seen in Table 3.1, from the low contact angles values obtained for all surfaces, it can be inferred that the substrates have hydrophilic surfaces. However, the contact angles for nanoHA sintered at 725°C and 1000°C both with adsorbed FBS were 32,7 ± 3,4 and 26,1 ± 1,8, respectively, indicating more hydrophilic surfaces when compared to control nanoHA.

The influence of the FN (20 μg/ml) and HSA (20 μg/ml) on contact angles carried out in this study is given in Table 3.2. The contact angle measurements were also performed by the captive air-bubble technique, using PBS as testing liquid.

Table 3.2. Contact angles measurements (deg) (average ± standard deviation) for nanoHA materials sintered at 725ºC and at 1000ºC and for nanoHA with adsorbed FN and HSA.

	Control θ (deg)	FN θ (deg)	HSA θ (deg)
NanoHA 725°C	40,2 ± 3,9	41,3 ± 4,1	37,5 ± 1,9
NanoHA 1000°C	38,3 ± 1,8	40,0 ± 2,2	41,9 ± 4,1

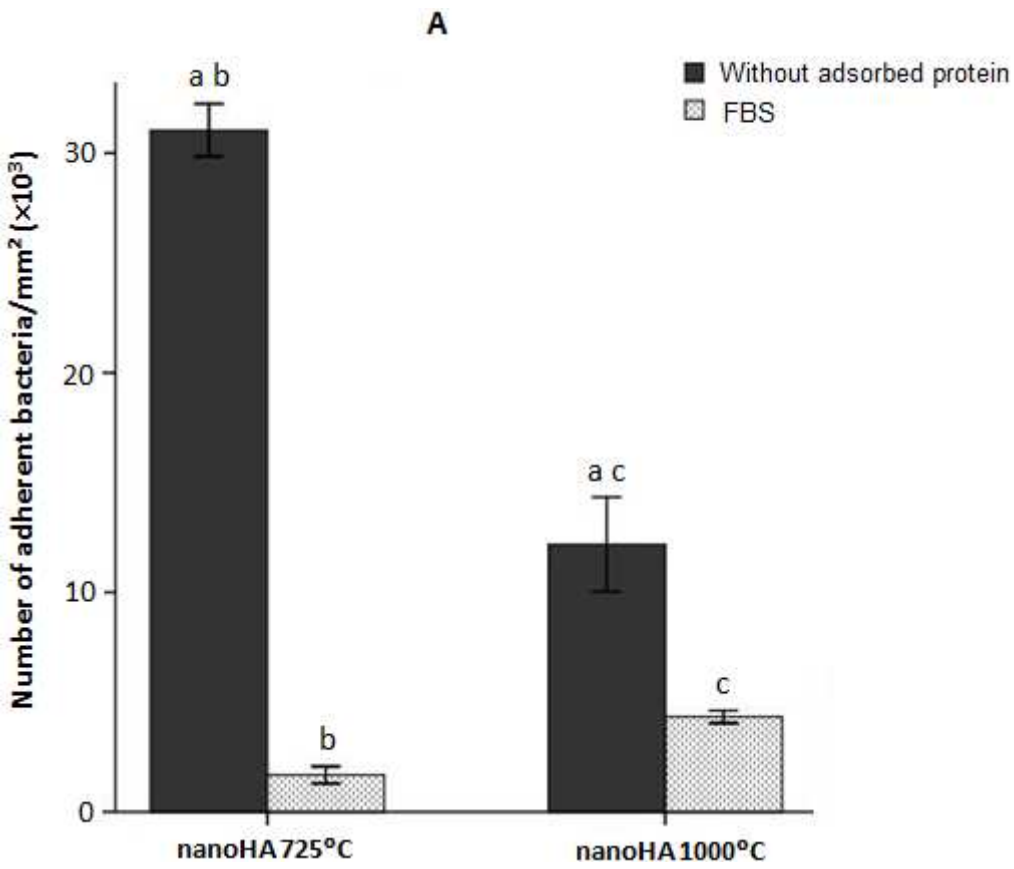
The contact angles for the samples with adsorbed FN and HSA were similar to that of the control nanoHA (without adsorbed FN and HSA). No statistical differences were found in the contact angles acquired, indicating that the degree of hydrophilicity of the nanoHA surfaces with adsorbed FN and HSA was similar to those obtained for nanoHA surfaces without adsorbed protein.

3.2. FBS-mediated bacterial adhesion

The bacteria adhered on nanoHA materials were evaluated by CFU plate counting and by SEM after 60 min of incubation. Results of the initial adhesion of Methicillin-Sensitive *Staphylococcus aureus* (MSSA), Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* RP62A and *Staphylococcus epidermidis* ORT to nanoHA sintered at 725°C and sintered at 1000°C are presented.

The influence of fetal bovine serum (FBS) adsorbed on nanoHA in the bacterial adhesion was evaluated. Bacterial adhesion to control surfaces (nanoHA without adsorbed protein) was compared to surfaces with adsorbed FBS under the same conditions.

3.2.1. Colony forming units counting



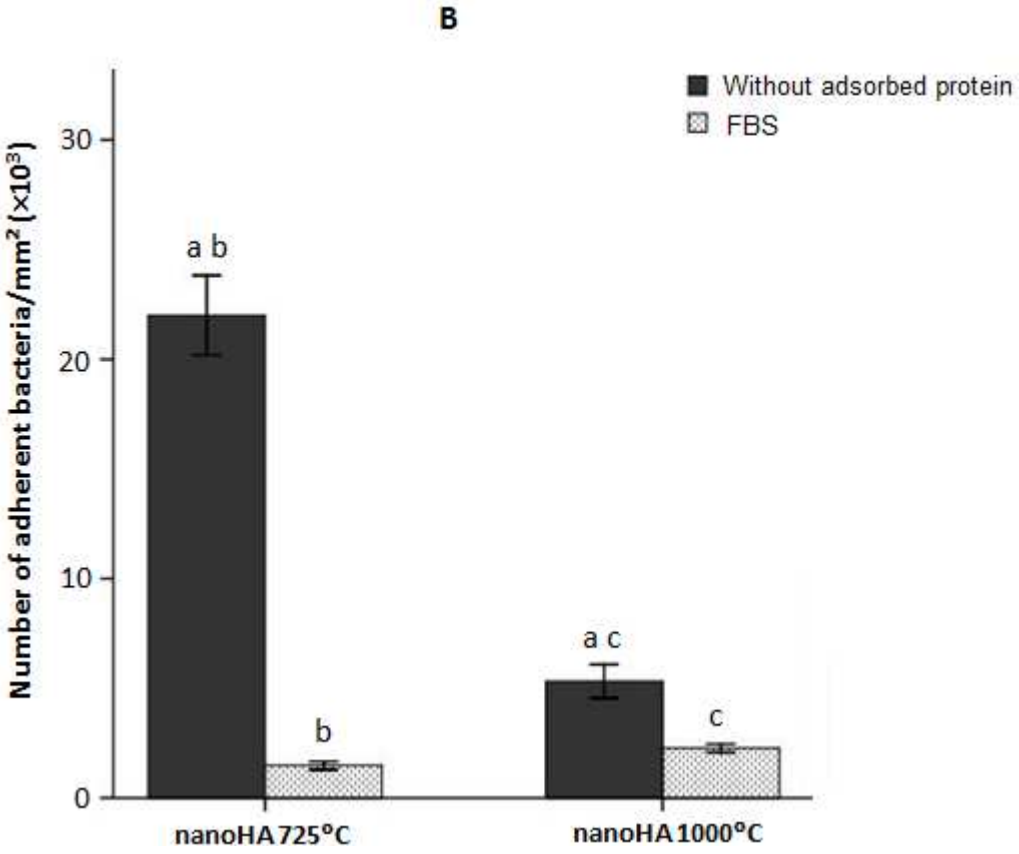
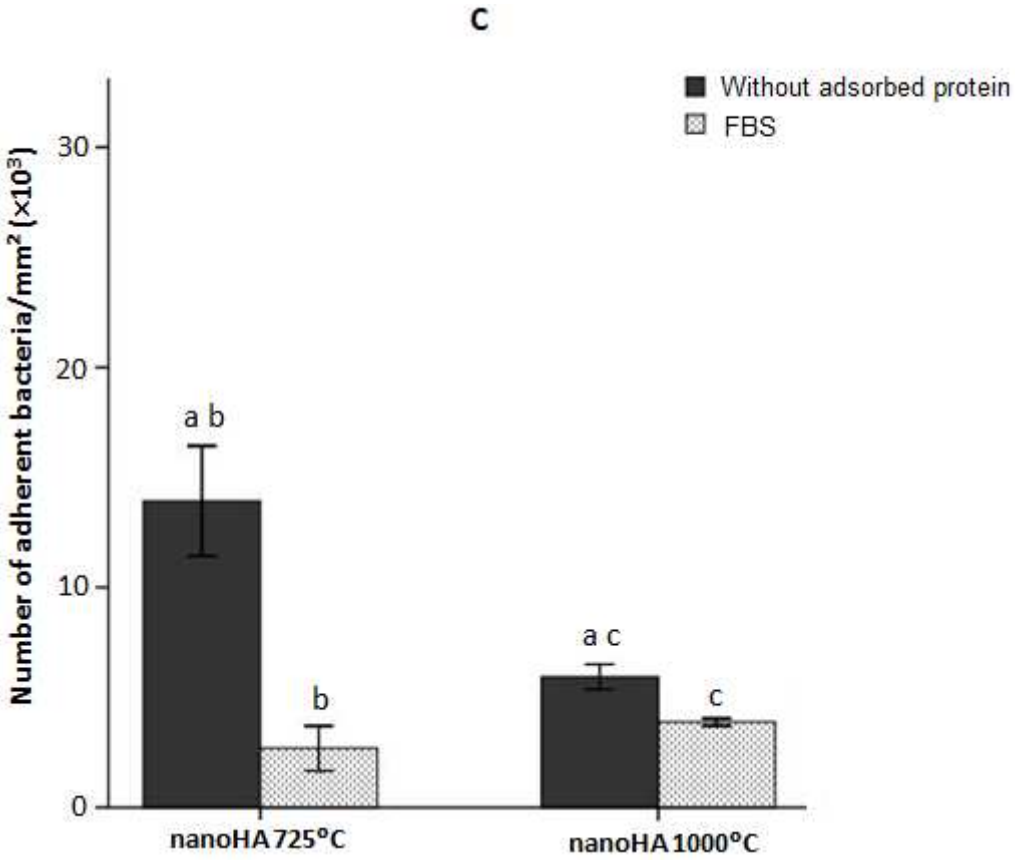


Figure 3.1. Graphs showing the influence of adsorbed FBS on the adhesion of *S. aureus* MSSA (A) and *S. aureus* MRSA (B) to nanoHA sintered at 725ºC and at 1000ºC. Results presented are the average ± SD. Same lowercase letters indicate significant differences (p<0.05) according to Tukey HSD.



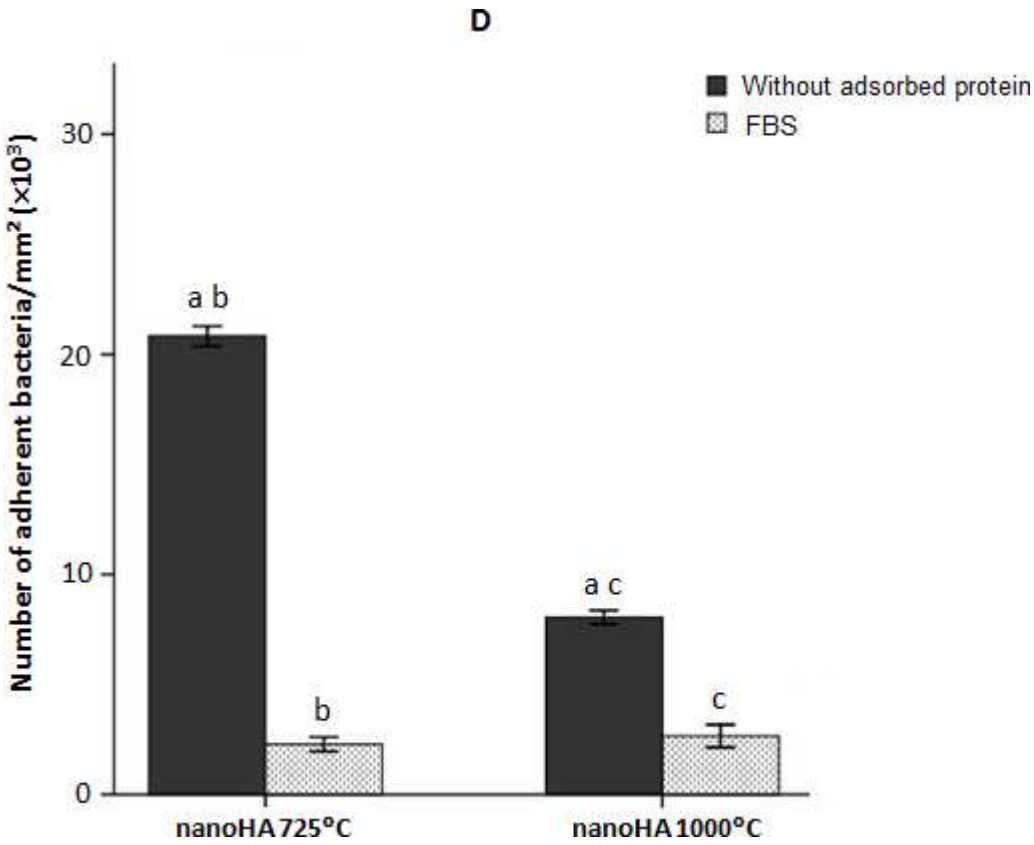


Figure 3.2. Graph showing the influence of adsorbed FBS on the adhesion of *S. epidermidis* RP62A (C) and S. epidermidis ORT (D) to nanoHA sintered at 725ºC and at 1000ºC. Results presented are the average ± SD. Same lowercase letters indicate significant differences (p<0.05) according to Tukey HSD.

As it may be seen, both *S. aureus* and *S. epidermidis* strains adhered at a significantly higher extent to nanoHA 725 than to nanoHA 1000.

In the case of nanoHA with adsorbed FBS, a significant decrease in the extent of bacterial adhesion was observed in comparison to nanoHA without protein, for all strains studied, in both nanoHA materials. No statistical differences were found between the extent of bacterial adhesion on nanoHA 725 and nanoHA 1000 with adsorbed FBS.

3.2.2. Scanning electron microscopy

SEM images of bacteria adhered to control nanoHA at 725°C and at 1000°C and to nanoHA with adsorbed FBS are presented.

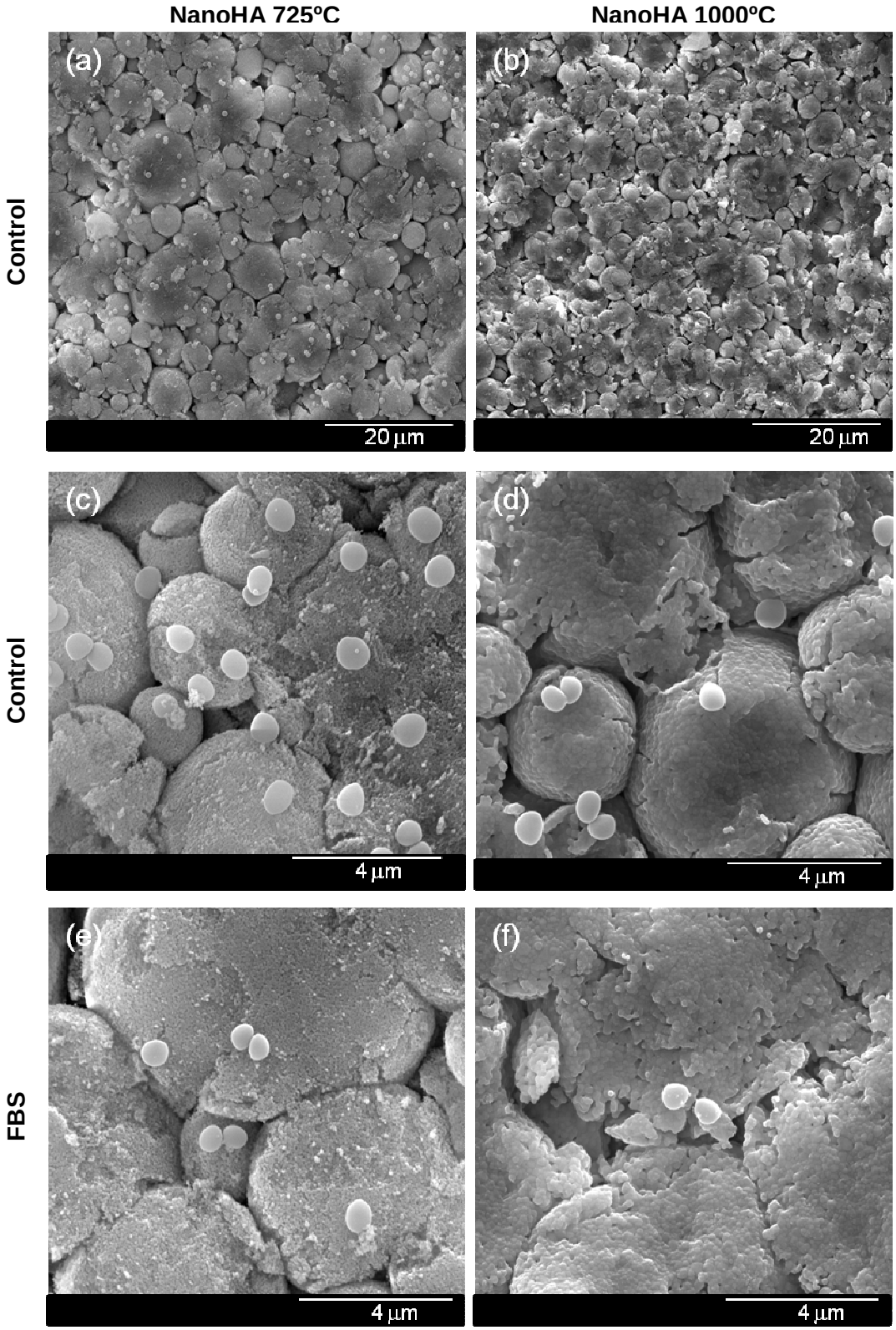


Figure 3.3. SEM images of methicillin-sensitive *Staphylococcus aureus* (MSSA) adhesion on nanoHA at 725ºC (a, c); nanoHA at 1000ºC (b, d); nanoHA at 725ºC with adsorbed FBS (e) and nanoHA at 1000ºC with adsorbed FBS (f). a, b - magnification ×5000; c, d, e, f - magnification ×30000.

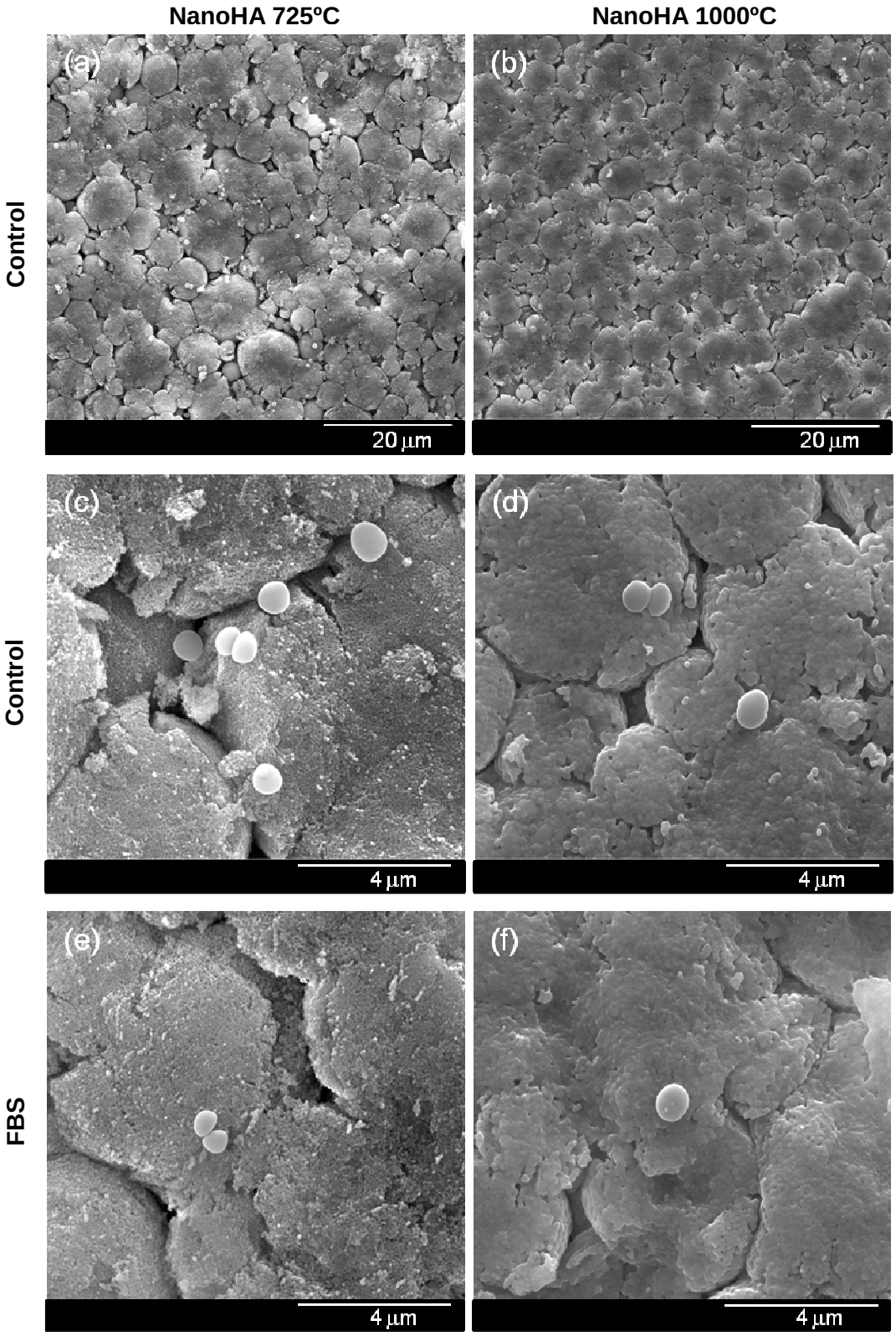


Figure 3.4. SEM images of methicillin-resistant *Staphylococcus aureus* (MRSA) adhesion on nanoHA at 725°C (a, c); nanoHA at 1000°C (b, d); nanoHA at 725°C with adsorbed FBS (e) and nanoHA at 1000°C with adsorbed FBS (f). a, b - magnification ×5000; c, d, e, f - magnification ×30000.

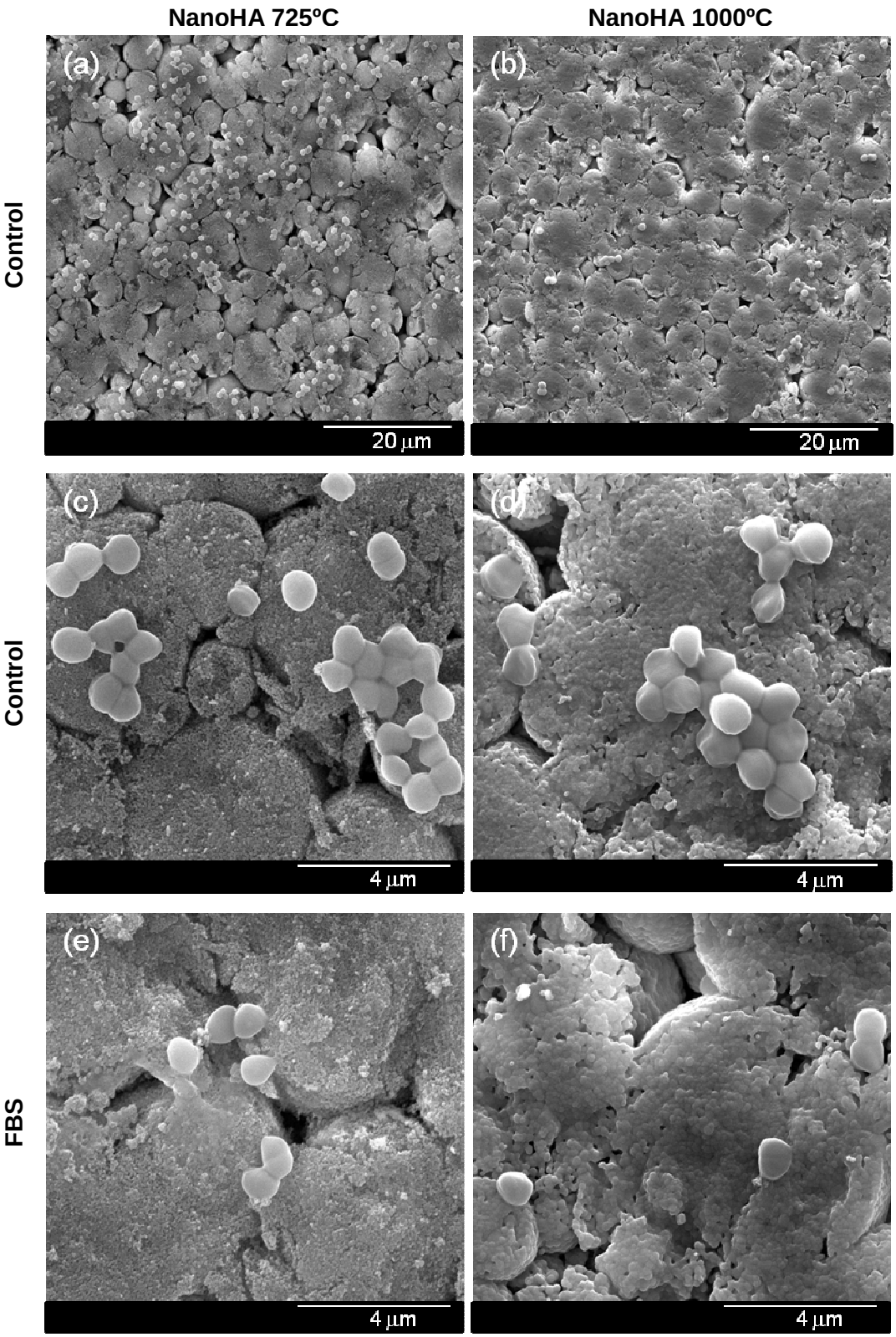


Figure 3.5. SEM images of *Staphylococcus epidermidis* RP62A adhesion on nanoHA at 725°C (a, c); nanoHA at 1000°C (b, d); nanoHA at 725°C with adsorbed FBS (e) and nanoHA at 1000°C with adsorbed FBS (f). a, b - magnification ×5000; c, d, e, f - magnification ×30000.

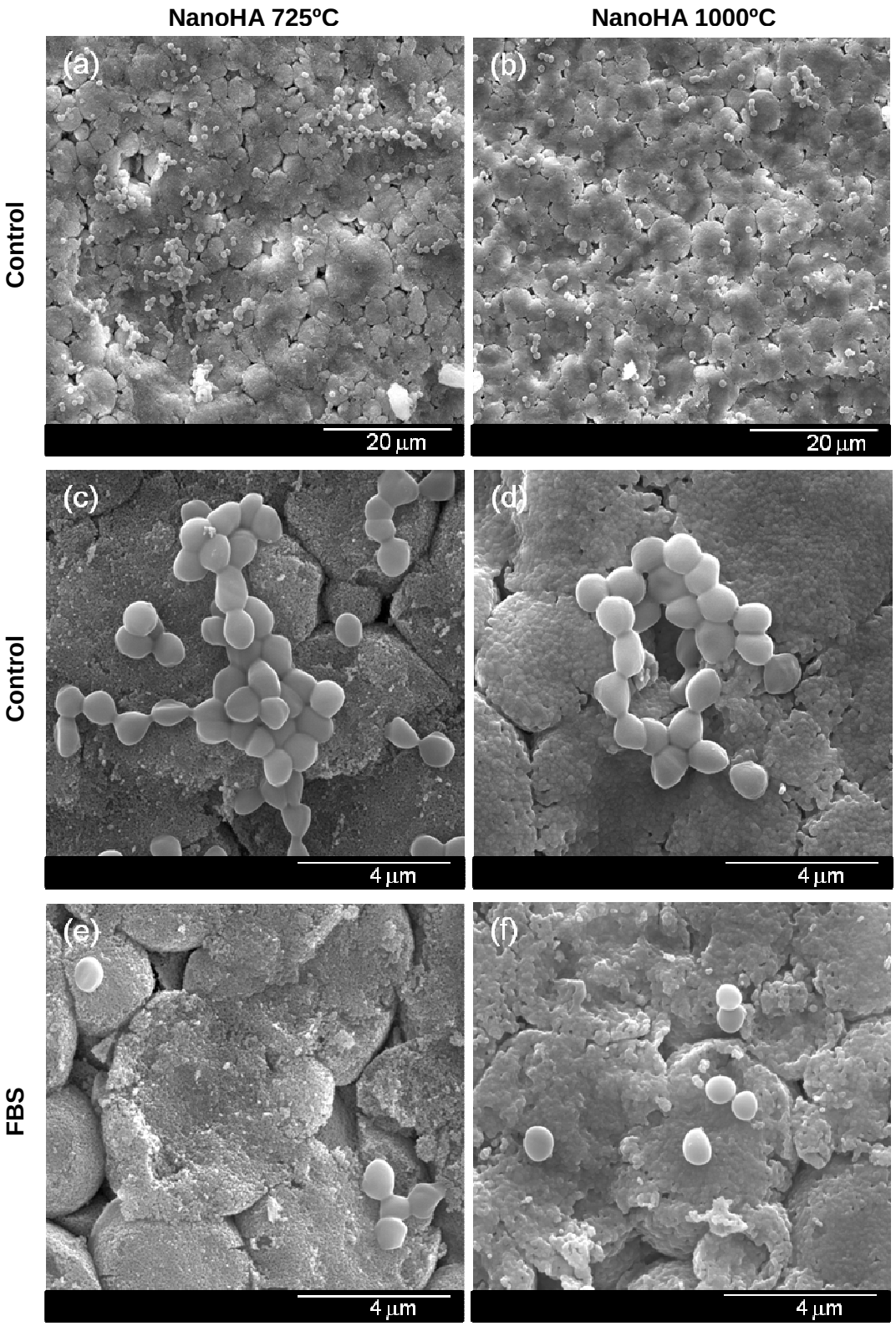


Figure 3.6. SEM images of *Staphylococcus epidermidis* ORT adhesion on nanoHA at 725°C (a, c); nanoHA at 1000°C (b, d); nanoHA at 725°C with adsorbed FBS (e) and nanoHA at 1000°C with adsorbed FBS (f). a, b - magnification ×5000; c, d, e, f - magnification ×30000.

In the current study, both *S. aureus* and *S. epidermidis* showed to have higher affinity to nanoHA 725 than to nanoHA 1000.

As one may see in Figure 3.3 (c, d) and 3.4 (c, d) the clinical strains of *S. aureus* MSSA and *S. aureus* MRSA appeared dispersed throughout the surface of the sample, without the establishment of links between bacterial cells, and therefore no slime production was observed on both nanoHA surfaces.

As expected, SEM images of the reference type culture *S. epidermidis* RP62A, a slime producer that express polysaccharide intercellular adhesin (PIA), show the presence of slime embedding the bacterial cells on both materials studied (Figure 3.5 c, d). The clinical strain of *S. epidermidis* ORT also shows the ability to produce slime (Figure 3.6 c, d).

Exposure of the nanoHA materials to FBS before incubation with the bacterial strains resulted in significantly altered adhesion patterns. By SEM images, adsorption of FBS on nanoHA reduced bacterial attachment for all strains as well as in both materials studied. Moreover, regarding the SEM images of *S. epidermidis* RP62A (Fig. 3.5 e, f) and *S. epidermidis* ORT (Fig. 3.6 e, f) we can see that they lost the capacity to produce slime.

It is clear that the adherent bacteria observed by SEM are in agreement with the results obtained by counting the viable organisms on the materials tested.

3.3. Influence of FN and HSA on bacterial adhesion

The influence of the adsorption of FN and HSA on bacterial adhesion to nanoHA substrates was analyzed by CFU plate counting and by SEM after 60 min of incubation. Results of the initial adhesion of *S. aureus* MSSA, *S. aureus* MRSA, *S. epidermidis* RP62A and *S. epidermidis* ORT to nanoHA sintered at 725°C and sintered at 1000°C are presented.

3.3.1. Colony forming units counting

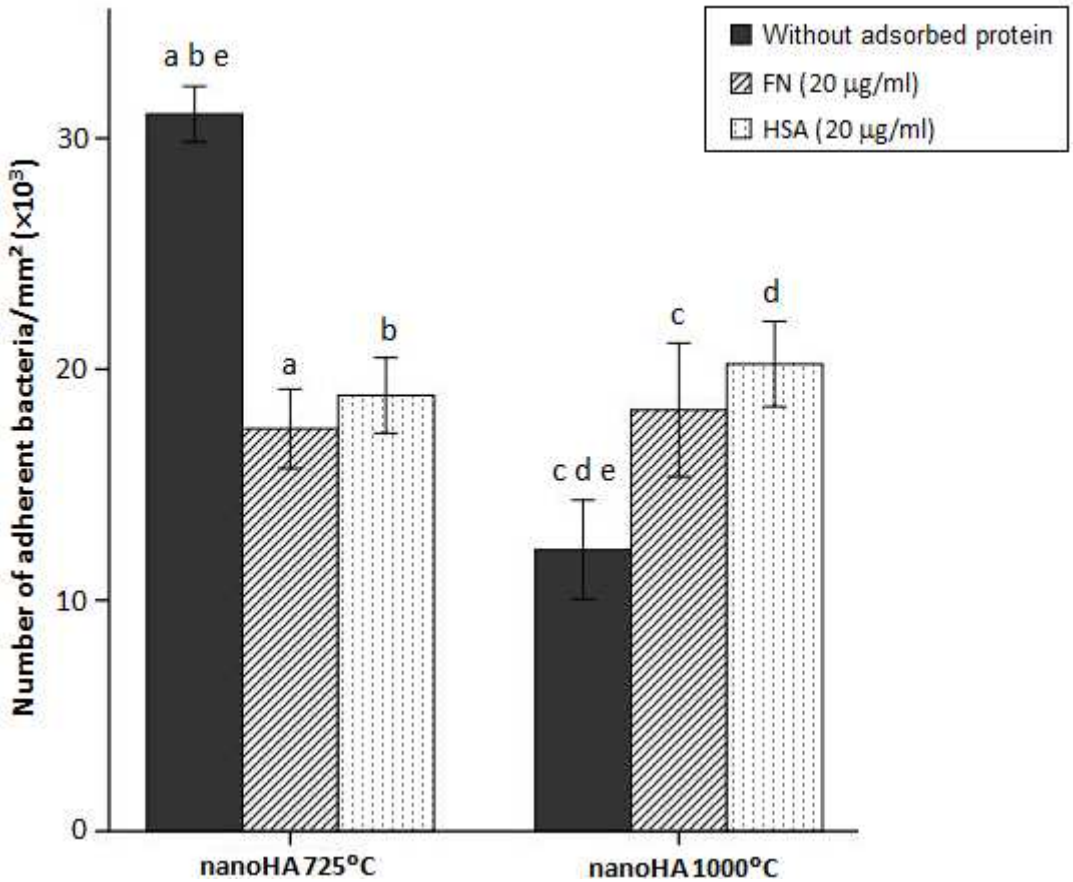


Figure 3.7. Influence of adsorbed FN and HSA on adhesion of *S. aureus* MSSA to nanoHA sintered at 725°C and at 1000°C. Results presented are the average ± SD. Same lowercase letters indicate significant differences ($p<0.05$) according to Tukey HSD.

The results concerning nanoHA 725 with adsorbed FN showed a decrease of the *S. aureus* MSSA attachment on material. On the contrary, an increase was observed in the bacterial adhesion on nanoHA 1000 with adsorbed FN.

Adsorption of HSA on nanoHA 725 resulted in a lower number of attached bacteria. Interestingly enough, nanoHA 1000 with adsorbed HSA showed an increase of the MSSA adhesion.

For this bacterial strain the results for nanoHA sintered at 725ºC with adsorbed FN and HSA resulted in a bacterial adhesion pattern similar to the one found for nanoHA 1000.

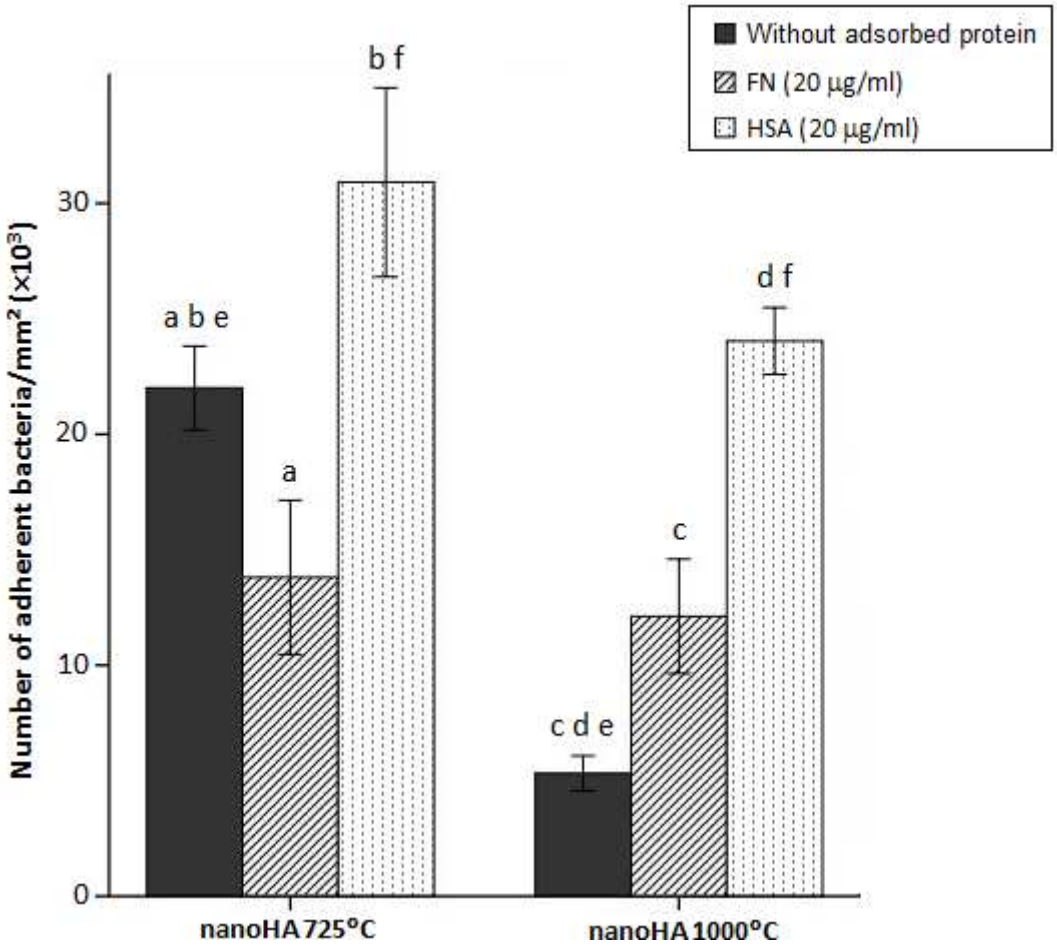


Figure 3.8. Influence of adsorbed FN and HSA on adhesion of *S. aureus* MRSA to nanoHA sintered at 725ºC and at 1000ºC. Results presented are the average ± SD. Same lowercase letters indicate significant differences ($p<0.05$) according to Tukey HSD.

The adhesion of *S. aureus* MRSA on nanoHA 725 with adsorbed FN was significantly lower than the adhesion on nanoHA 725 without protein. On the contrary, in the presence of HSA, the MRSA adhesion on nanoHA 725 increased.

The extent of the MRSA adhesion was higher for nanoHA 1000 with adsorbed FN and HSA as compared to nanoHA 1000 without protein.

The adhesion pattern was similar for both materials tested with adsorbed FN. On the other hand, a decrease was found in the MRSA adhesion on nanoHA 1000 with adsorbed HSA when compared to nanoHA 725 with adsorbed HSA.

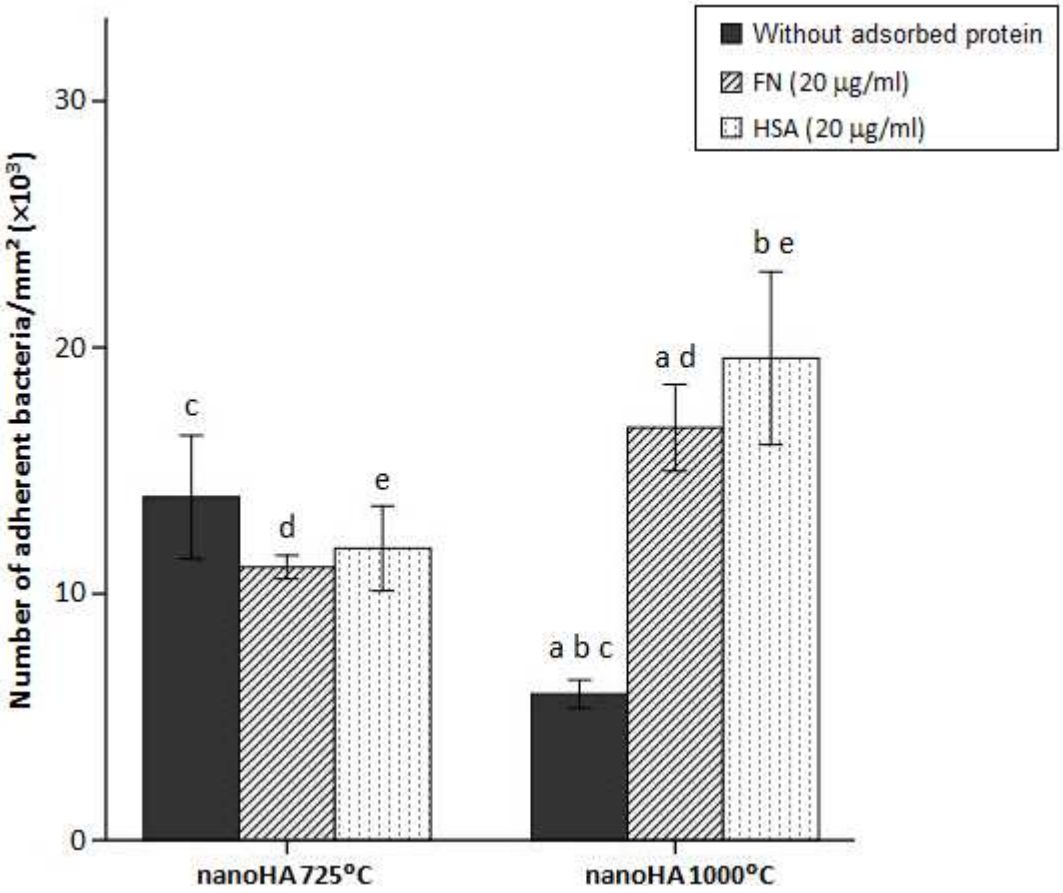


Figure 3.9. Influence of adsorbed FN and HSA on the adhesion of *S. epidermidis* RP62A to nanoHA sintered at 725°C and at 1000°C. Results presented are the average ± SD. Same lowercase letters indicate significant differences ($p<0.05$) according to Tukey HSD.

After adsorption of FN and HSA, the adhesion pattern of *S. epidermidis* RP62A on nanoHA 725 was similar to that of nanoHA 725 without protein. No significant differences in the amounts of adherent bacteria were found for this case.

The extent of the *S. epidermidis* RP62A adhesion was higher for nanoHA 1000 with adsorbed FN and HSA as compared to nanoHA 1000 without protein.

It is interesting to notice that the results for nanoHA 1000 with adsorbed FN and HSA showed that the attachment of the *S. epidermidis* RP62A strain was higher when compared to nanoHA 725 with adsorbed FN and HSA.

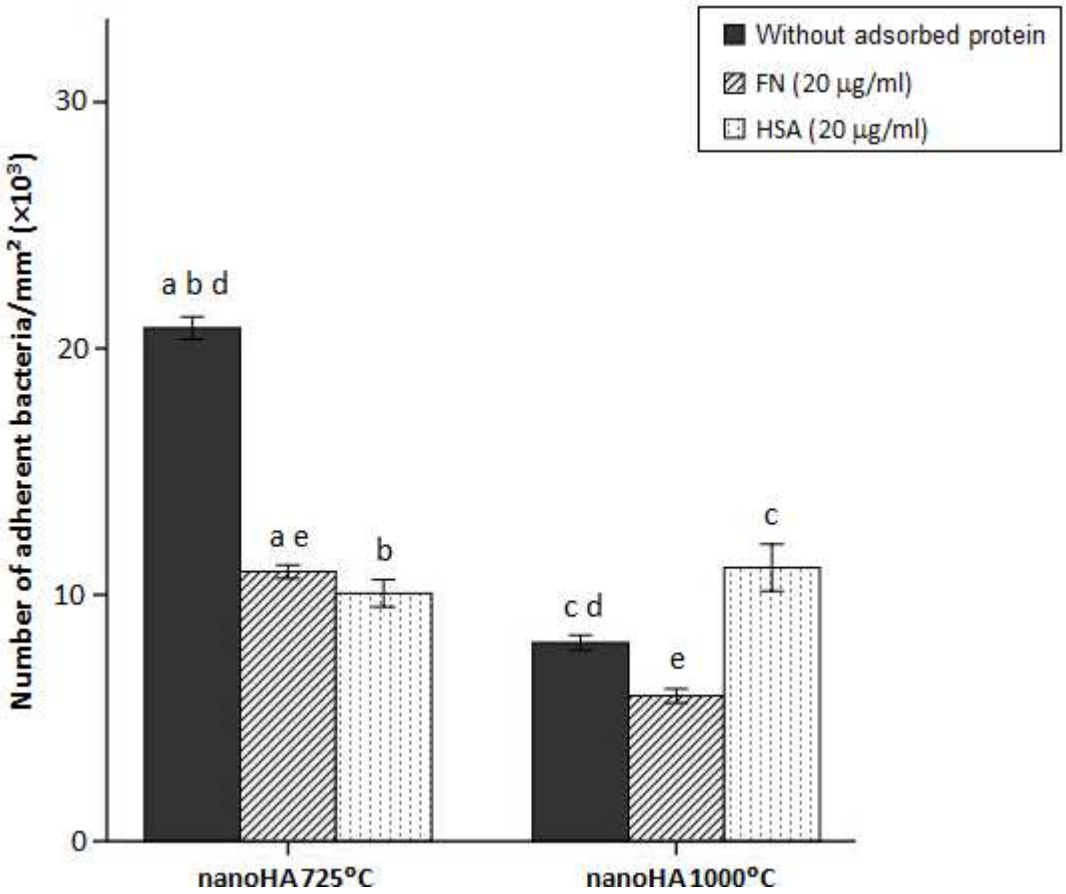


Figure 3.10. Influence of adsorbed FN and HSA on the adhesion of *S. epidermidis* ORT to nanoHA sintered at 725°C and at 1000°C. Results presented are the average ± SD. Same lowercase letters indicate significant differences ($p<0.05$) according to Tukey HSD.

The results concerning the clinical strain of *S. epidermidis* ORT showed that the extent of bacterial adhesion was lower for nanoHA 725 with adsorbed FN and HSA as compared to substrates without protein.

Adsorption of FN on nanoHA 1000 resulted in an adhesion pattern similar to the control nanoHA 1000. No significant differences were found in the amounts of adherent bacteria. On the other hand, the nanoHA 1000 with adsorbed HSA showed an increase of the adhesion of this bacterial strain.

The results for nanoHA 1000 with adsorbed FN indicated that the attachment of the *S. epidermidis* ORT strain was lower than for nanoHA 725 with adsorbed FN. The bacterial adhesion pattern in the presence of HSA was similar for both tested materials.

3.3.2. Scanning electron microscopy

SEM images of bacteria adhered to control nanoHA at 725°C and at 1000°C and to nanoHA with adsorbed FN (20 μg/ml) and HSA (20 μg/ml) are presented.

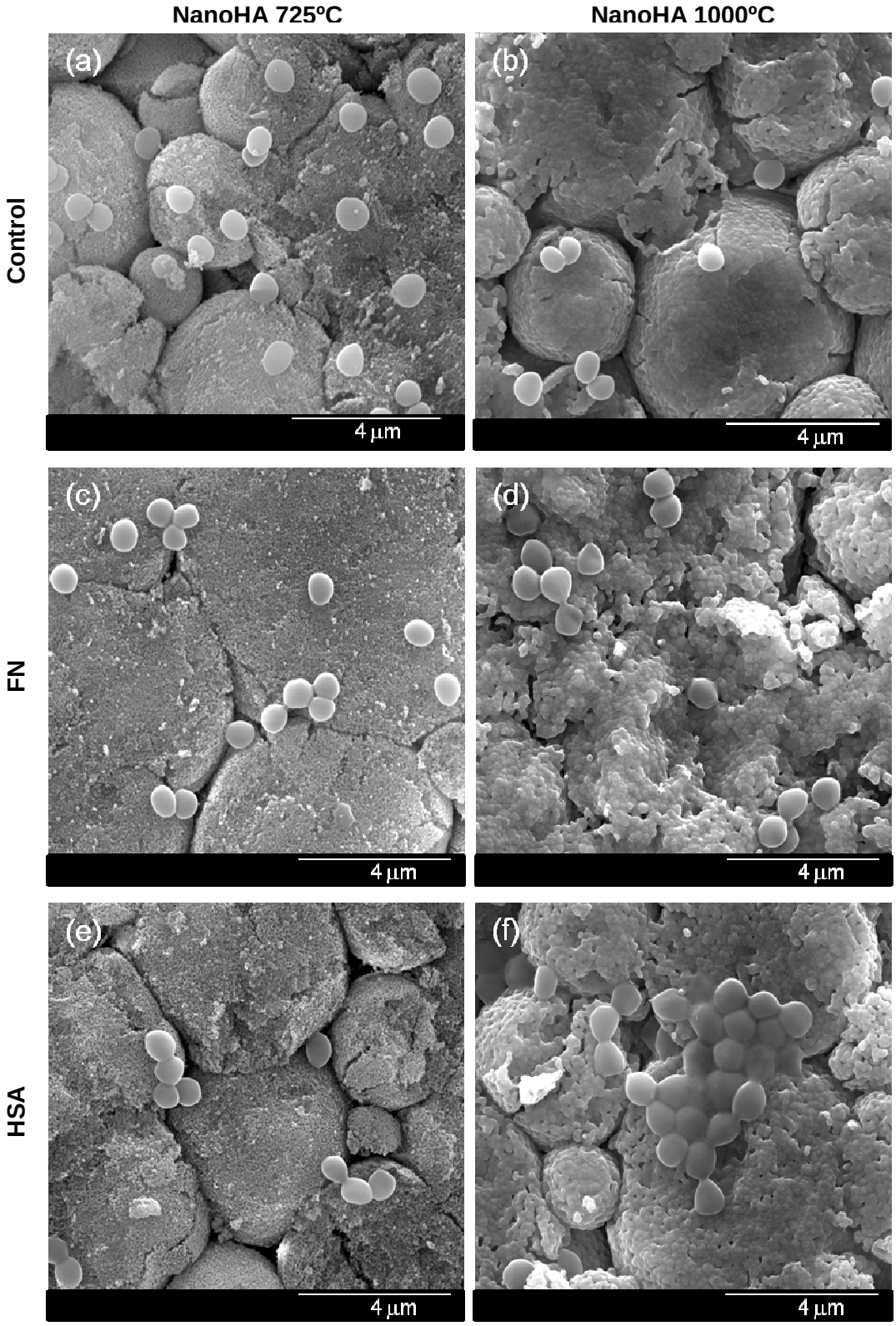


Figure 3.11. SEM images of *S. aureus* MSSA adhesion on nanoHA at 725°C (a); nanoHA at 1000°C (b); nanoHA at 725°C with adsorbed FN (c); nanoHA at 1000°C with adsorbed FN (d); nanoHA at 725°C with adsorbed HSA (e); nanoHA at 1000°C with adsorbed HSA (f). Magnification ×30000.

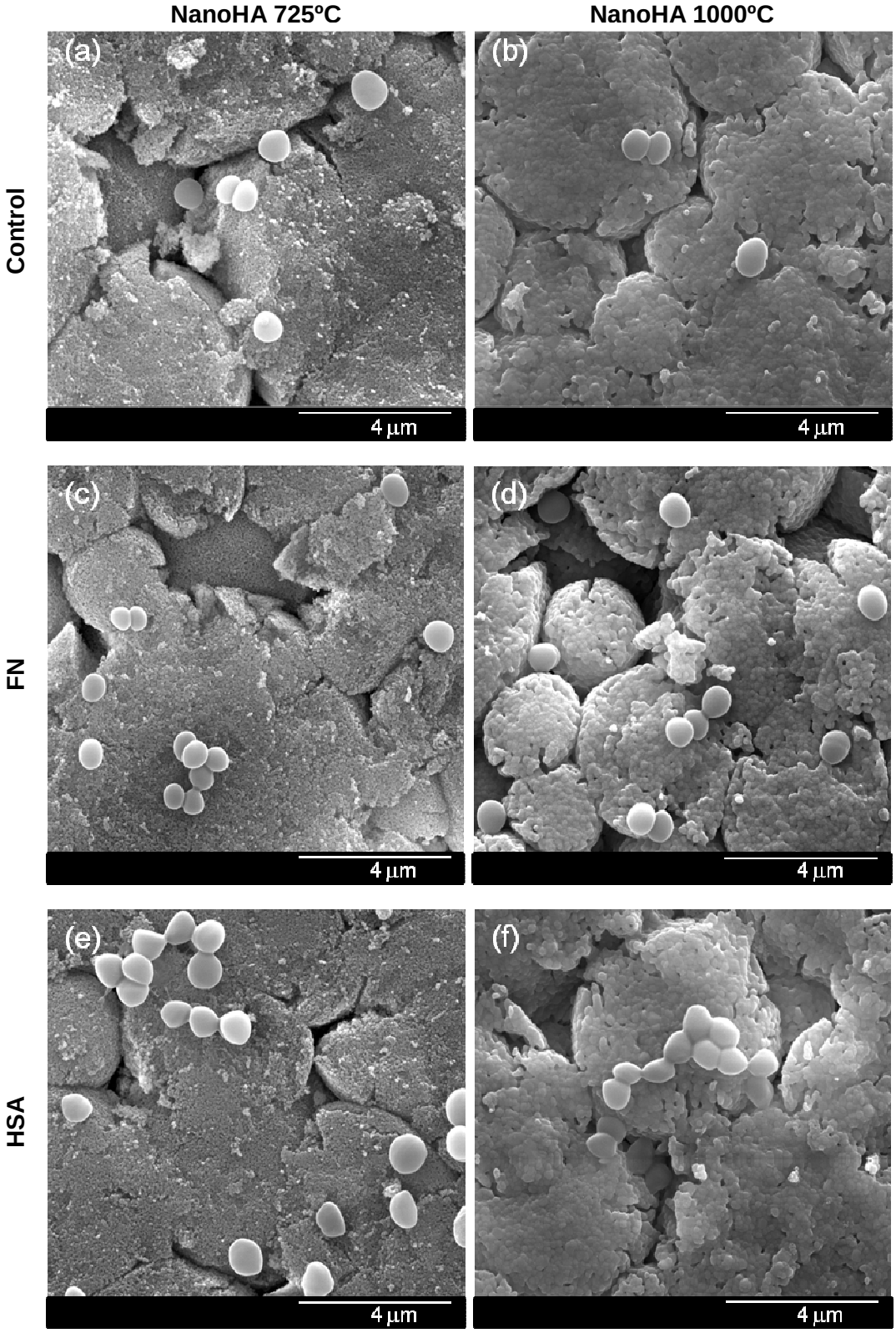


Figure 3.12. SEM images of *S. aureus* MRSA adhesion on nanoHA at 725ºC (a); nanoHA at 1000ºC (b); nanoHA at 725ºC with adsorbed FN (c); nanoHA at 1000ºC with adsorbed FN (d); nanoHA at 725ºC with adsorbed HSA (e); nanoHA at 1000ºC with adsorbed HSA (f). Magnification ×30000.

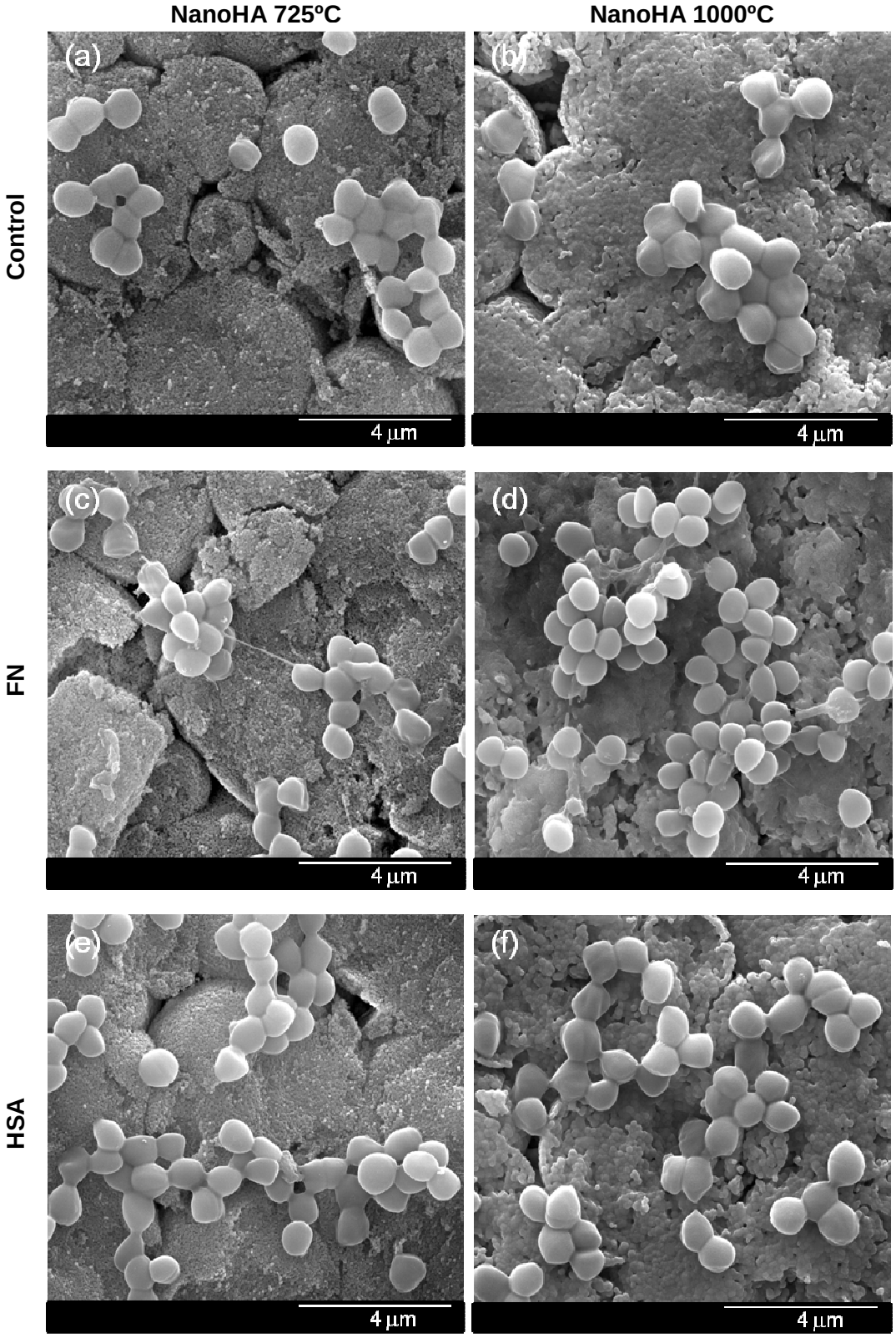


Figure 3.13. SEM images of *S. epidermidis* RP62A adhesion on nanoHA at 725°C (a); nanoHA at 1000°C (b); nanoHA at 725°C with adsorbed FN (c); nanoHA at 1000°C with adsorbed FN (d); nanoHA at 725°C with adsorbed HSA (e); nanoHA at 1000°C with adsorbed HSA (f). Magnification ×30000.

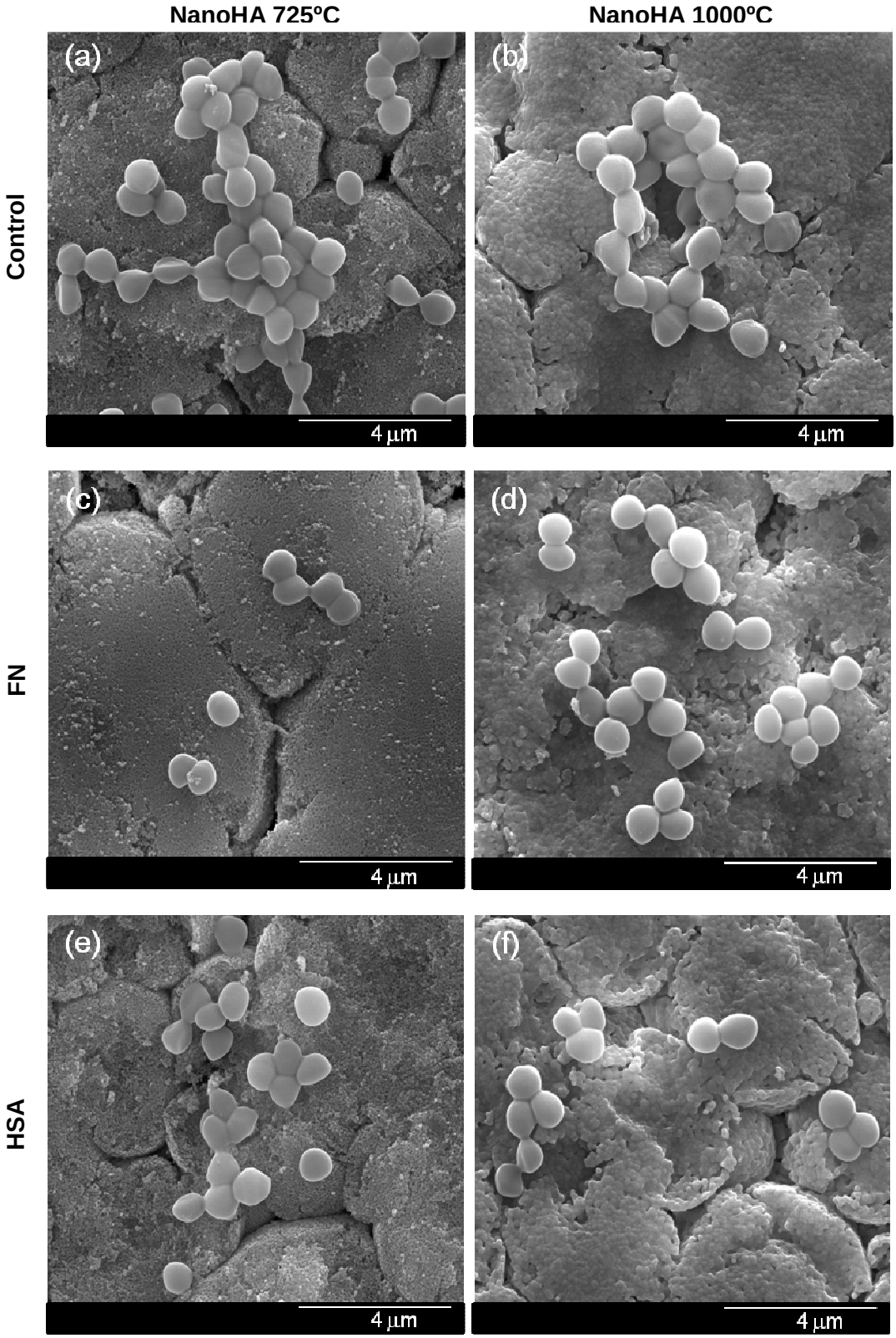


Figure 3.14. SEM images of *S. epidermidis* ORT adhesion on nanoHA at 725°C (a); nanoHA at 1000°C (b); nanoHA at 725°C with adsorbed FN (c); nanoHA at 1000°C with adsorbed FN (d); nanoHA at 725°C with adsorbed HSA (e); nanoHA at 1000°C with adsorbed HSA (f). Magnification ×30000.

Scanning electron microscopy was also used to study if the presence of adsorbed proteins on nanoHA surface influences the process of interaction between the bacteria and the material.

Based on the SEM images analysis it is possible to observe that the clinical strains of *S. aureus* in the presence of FN and HSA began to form small aggregates on the nanoHA surface (Figure 3.11 (c, d, e) for MSSA strain and Figure 3.12 (c, d, e, f) for MRSA strain). A large adherent aggregate, a more developed stage in biofilm formation, was observed for MSSA strain on nanoHA at 1000°C with adsorbed HSA (Figure 3.11 f).

According to the results, there is strain variability in terms of biofilm formation ability in the presence of proteins between these *S. epidermidis* strains studied. For the *S. epidermidis* RP62A strain the SEM images revealed distinct adherence behavior, with biofilm formation remarkably enhanced, compared with the adhesion pattern obtained for this strain in the absence of proteins (Figure 3.13). Analyzing the adhesion behavior of the *S. epidermidis* ORT strain in the presence of these proteins some differences were observed, with lower ability for biofilm formation as perceptible in Figure 3.14, where less bacterial aggregates were visible on the surface.

3.3.3. Effect of concentration of HSA on bacterial adhesion

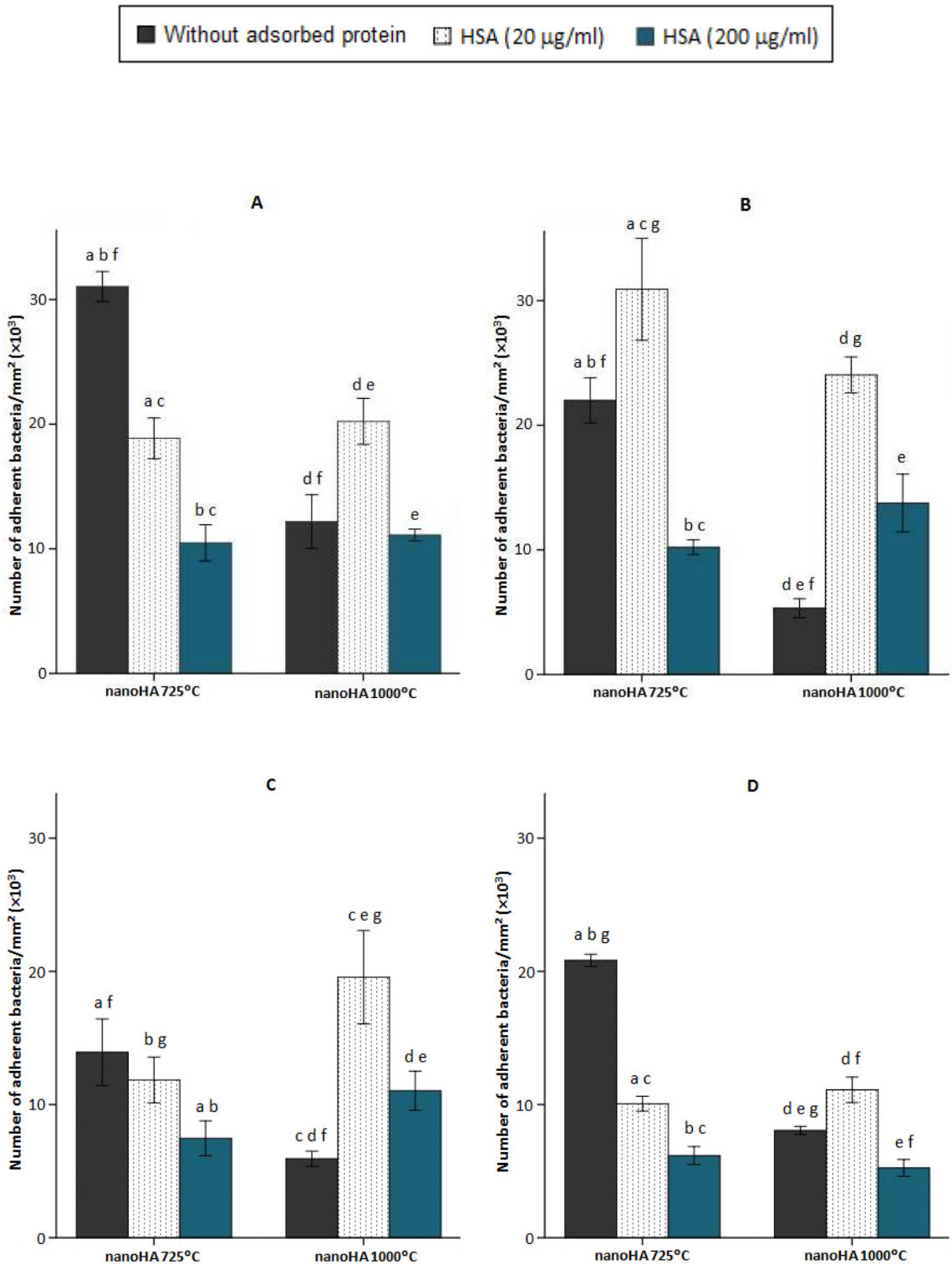


Figure 3. 15 – Effect of different concentrations of HSA on the adhesion of MSSA (A); MRSA (B); *S. epidermidis* RP62A (C) and *S. epidermidis* ORT (D) to nanoHA sintered at 725ºC and at 1000ºC. Results presented are the average ± SD. Same lowercase letters indicate significant differences (p<0.05) according to Tukey HSD.

Preliminary results about the effect of different concentrations of HSA on bacterial adhesion to nanoHA at 725ºC and at 1000ºC are presented in Figure 3.15. It was possible to observe that the level of adherence of both *S. aureus* and *S. epidermidis* strains decreased with increasing concentration of this compound. However, the concentration of 200 μg/ml was only effective in reducing the *S. epidermidis* ORT adhesion for both tested materials. For all other strains this concentration was only able to reduce the adhesion to nanoHA 725 substrates.

3.3.4. Distribution of FN, HSA and bacteria on nanoHA samples

The distribution of FN, HSA and *S. aureus* MRSA on nanoHA substrates sintered at 725ºC and at 1000ºC was followed by inverted fluorescence microscopy.

In Figure 3.16 it was possible to observe that the MRSA strain presented a homogeneous distribution on the nanoHA surface for both materials. NanoHA substrates with adsorbed FN also revealed a homogeneous distribution of this protein on the surface. The distribution of MRSA on both nanoHA surfaces with adsorbed FN doesn't seem to be as homogeneous as on nanoHA without protein.

In the presence of HSA the distribution of MRSA strain on both nanoHA substrates appears to be more concentrated on one side of the surface, which doesn't reveal a homogeneous distribution on the surface (Figure 3.17).

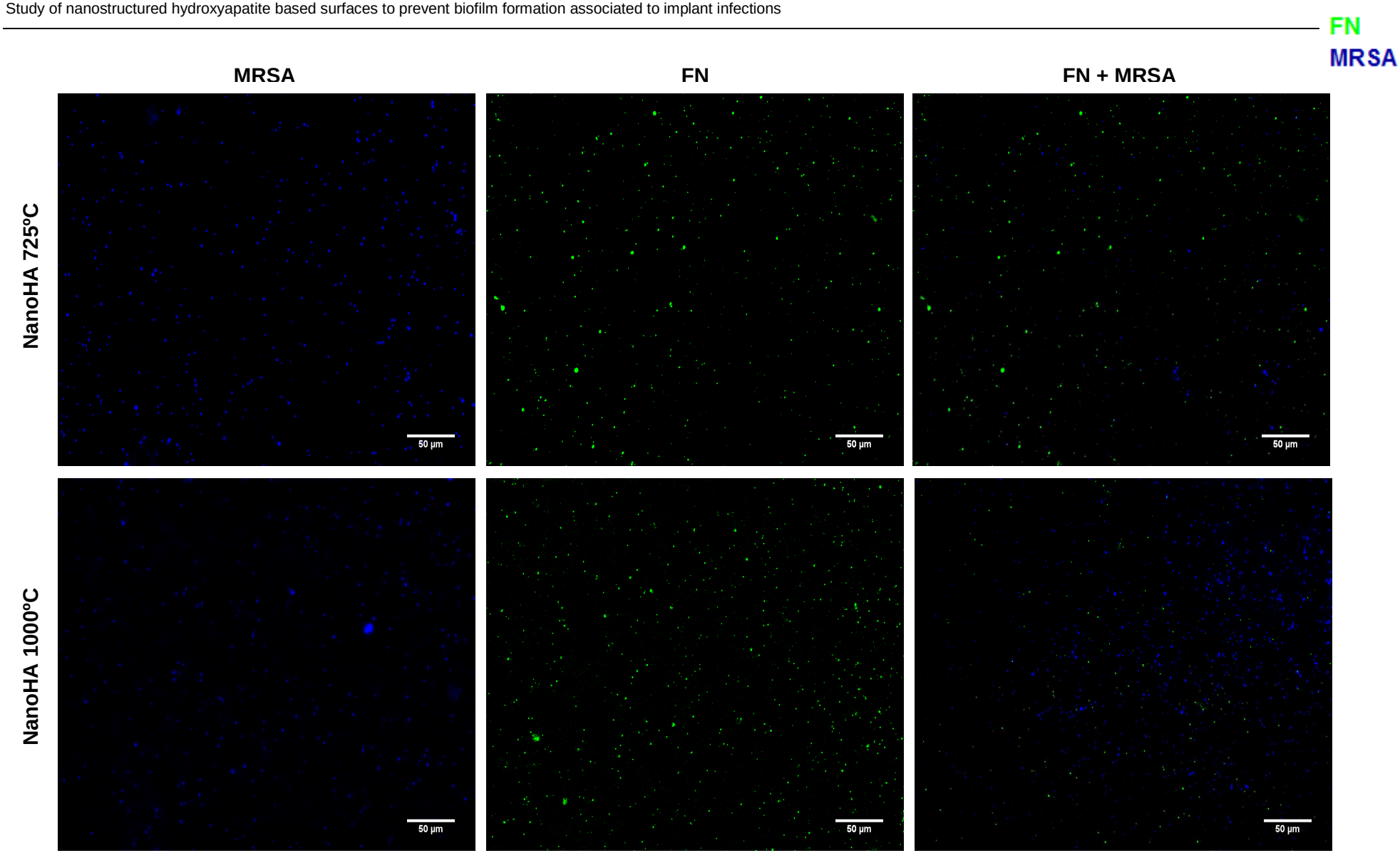


Figure 3. 16 - MRSA adhesion on nanoHA imaged with inverted epifluorescence microscope after 60 min without protein adsorption and with FN (20 µg/ml) adsorption. FN is indicated in green while bacteria were counterstained in blue with DAPI dye.

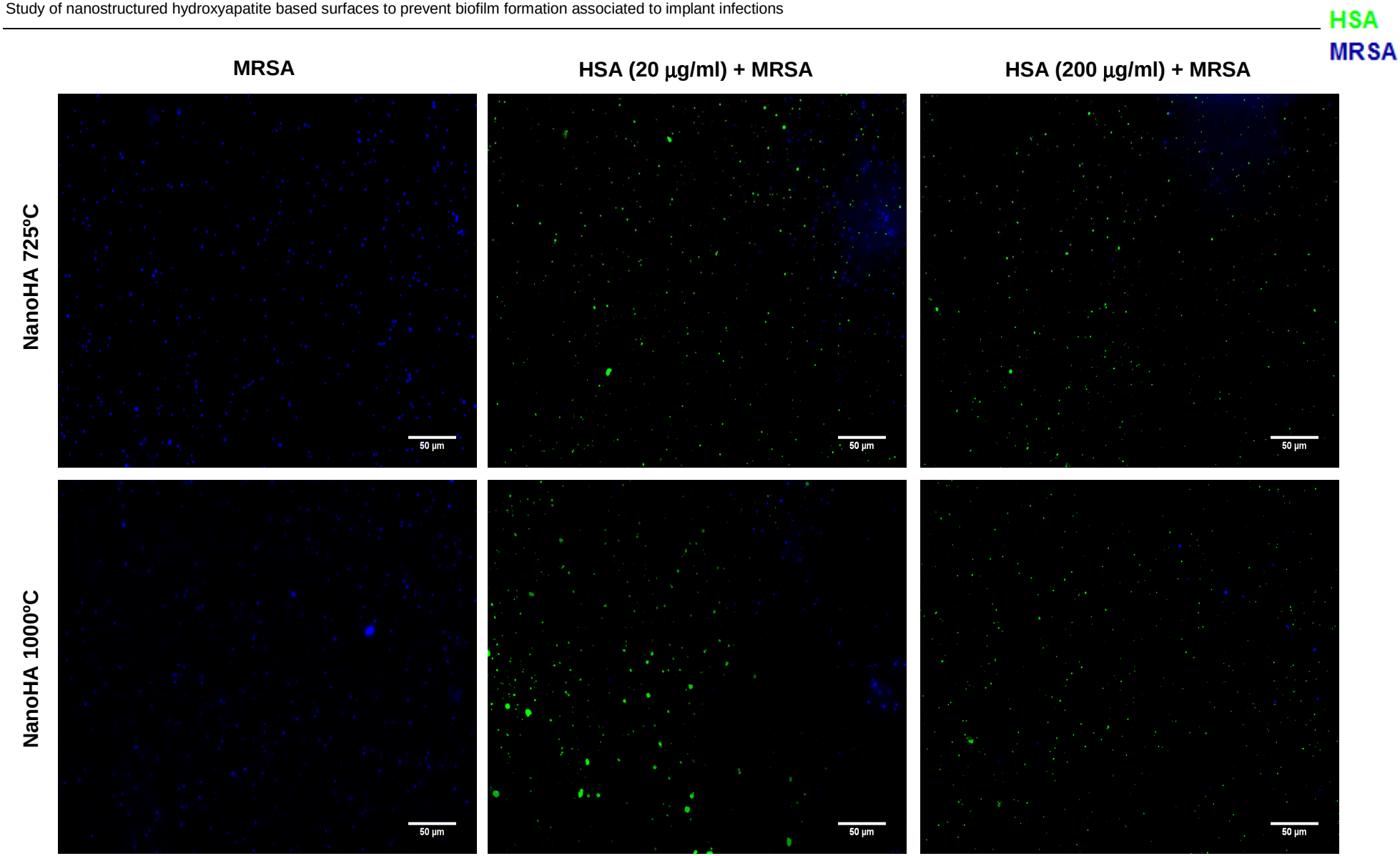


Figure 3. 17 - MRSA adhesion on nanoHA imaged with inverted epifluorescence microscope after 60 min without protein adsorption and with HSA adsorption in concentrations of 20 µg/ml and 200 µg/ml. HSA is indicated in green while bacteria were counterstained in blue with DAPI dye.

Chapter IV - Discussion

Any biomaterial that can be easily colonized by microorganisms, may act as a favorable medium for the growth of a bacterial biofilm. Such biofilms exhibit a strong resistance to any form of therapy and are difficult to eliminate, thus they can be a source of recurrent infections [26, 55]. Given the fact that nanoHA is a material applicable in devices for bone regeneration, it is of major relevance to study the adhesion of S*. aureus* and *S. epidermidis* to this material because they are main pathogenic species among orthopedic clinical isolates of implant-associated infections [13].

Bacterial adhesion onto a surface is a very complicated process that depends on several factors, including surface chemistry, surface wettability, and the presence of specific proteins on a biomaterial surface. It is important to mention that in a protein-rich environment, however, as it would be the general trend for most biomaterials at their site of implantation, the originally intended antibacterial properties of a given biomaterial surface can become considerably altered and in some cases abolished. Thus it is important to study the bacterial adhesion in an environment with proteins. Many proteins have been studied for their effects on bacterial adhesion to material surfaces, including fibronectin and albumin [127-129].

In the present work the ability of *S. aureus* and *S. epidermidis* strains to adhere to nanoHA sintered at 725ºC and at 1000ºC was evaluated, aiming at reaching a better understanding of the phenomena that involve material infection caused by these strains. In order to mimic *in vivo* conditions a model protein, fetal bovine serum (FBS), which is a mixture of serum proteins, was used to determine how proteins presence affects bacterial adhesion. It was also studied how the adsorption of fibronectin (FN) and human serum albumin (HSA) on nanoHA affect *S. aureus* and *S. epidermidis* adhesion.

As the present results indicated, all *S. aureus* and *S. epidermidis* strains adhered to a higher extent to nanoHA 725 than to nanoHA 1000. This difference observed for the nanoHA materials may be related with actual surface area, surface morphology and porosity of these materials sintered at different temperatures. According to Ribeiro *et al* [121], nanoHA 725 showed a significantly higher total surface area value than nanoHA1000, as well as, a higher total porosity when compared to nanoHA 1000. Thus, the higher surface area and porosity observed may validate the higher number of

adherent bacteria on nanoHA725 samples as compared to nanoHA1000 samples, since the two surfaces present similar chemical composition.

When biomaterials are implanted in the body, proteins immediately adsorb onto the implant surfaces, that in turn serve as attachment sites for bacterial strains [130-131]. In this regard, we chose to study the influence of proteins in the bacterial adhesion, mimicking the physiologic environment in the body, using FBS, that is a mixture of proteins. The presence of FBS on the nanoHA surface significantly reduced bacterial adhesion for all strains tested as compared with that measured on control nanoHA. This reduction is consistent with the observations of other authors [132], and may be due to the fact that the main component in FBS is bovine serum albumin, which has been shown to provide anti-adhesion abilities to bacteria. These inhibitory effects of t~~he~~ albumin are the result of the increased hydrophilicity of the substrate surface [128], and in this study the presence of FBS on the nanoHA surface resulted in a decrease in the contact angles indicating that the surfaces became more hydrophilic. These changes in the material surface properties can explain the significant decrease in bacterial adhesion, confirming previous reports [119], proposing that more hydrophilic materials are more resistant to bacterial adhesion, when compared to hydrophobic materials. Therefore the low adhesion between these bacteria (*S. aureus* and *S. epidermidis*) and nanoHA with adsorbed FBS could be due to an inhibitory effect of albumin by increasing the hydrophilicity of the material surface**.**
Hence, these results show the importance of the role played by proteins present in an *in vivo* environment during the initial stages of bacterial adhesion.

The experimental approach also involved two key proteins, FN and HSA. FN is an adhesive protein of the extracellular matrix (ECM) and it plays important roles in a variety of cellular processes including cell adhesion, growth, differentiation and migration. FN promotes attachment of cells to the biomaterial surface through its central-binding domain RGD sequence [121]. This protein seems to promote adhesion in certain strains. Most studies showed that adsorbed FN promotes adherence of bacteria, especially staphylococci to biomaterials [53, 55]. During the initial attachment the bacteria establish contact with the material, mediated in *S. aureus* and *S. epidermidis* by specific adhesins interacting with host extracellular matrix proteins deposited on the surface [133]. On the contrary, HSA is the predominant plasma protein and it is generally considered to “passivate” surfaces preventing cell adhesion and greatly reducing the acute inflammatory response to the material [130]. In addition, albumin adsorbed on material surfaces has shown inhibitory effects on bacterial

adhesion [53]. Therefore, these proteins are considered to be important in modulating the interactions between the bacteria and the material. In this matter, the adhesion behavior of *S. aureus* and *S. epidermidis* on nanoHA 725 and nanoHA 1000 in the presence of FN and HSA was analyzed and the ability of strains belonging to different species of *Staphylococcus* to bind to FN and HSA was examined.

Clinical isolates of *S. aureus* were examined for fibronectin affinity and the results were similar for both strains. The adsorption of FN on nanoHA 725 caused a decrease in the adhesion of MSSA and MRSA strains. On the other hand, the nanoHA 1000 with adsorbed FN showed that the MSSA and MRSA strains adhered significantly better than to nanoHA without protein. Therefore, the clinical strains of *S. aureus* exhibited bacterial attachment profiles different on nanoHA materials in the presence of FN as compared to nanoHA materials without protein. No correlation was found between surface wettability of the nanoHA with adsorbed FN and these adhesion patterns, since the contact angles for nanoHA with adsorbed FN were similar to that of the nanoHA without protein. This fact suggests that other factors can contribute to the initial attachment of bacteria on surfaces with adsorbed FN. Ribeiro *et a*l. [121] analyzed the adsorption of FN onto nanoHA sintered at 725ºC and nanoHA at 1000ºC, and reported that nanoHA 1000 samples allow a higher amount of FN to be adsorbed than nanoHA 725 samples. Moreover, in that study, protein adsorption was evaluated through of protein exchangeability by radiolabelling and by an antibodies-based assay specific for the RGD region, and it was showed that FN is less attached to nanoHA 725 surfaces, with a lower number of epitopes exposed as compared to nanoHA 1000 surface. These observations may explain the differences obtained in the present results. As expected, the presence of FN on nanoHA 1000 substrates favored the increase of adhesion of these clinical strains, while this was not found for nanoHA 725. In this regard, the increase of *S. aureus* adhesion on nanoHA 1000 may be related probably by expression of fibronectin-binding adhesins activities in these bacteria, since *S. aureus* has two fibronectin-binding proteins, FnBPA and FnBPB, both of which contribute to adherence to fibronectin-coated surfaces, [67, 76, 77] and the N-terminal domain of FN forms part of the binding site for FN-binding proteins from *S. aureus* [132]. Furthermore, the FnBP expression and FN recognition are found in most clinical isolates of *S. aureus* [134]. On the other hand, the results concerning the lower number of epitopes exposed on nanoHA 725 point towards the notion that some conformational changes of FN occurred upon adsorption on this surface [121]. These conformational changes may have led to the decrease observed for both *S. aureus* strains on this substrate. The conformation adopted by the protein probably modulates the bacterial

adhesion hiding binding sites of the FN to these *S. aureus* strains. Moreover, the FN may occupy sites at the surface to be established.

The adhesion behavior of *S. epidermidis* in the presence of FN was also evaluated and the results for the two strains studied showed that the extent of fibronectin affinity varied greatly between these strains. The adhesion pattern of *S. epidermidis* RP62A on nanoHA 725 with adsorbed FN was similar to that of nanoHA 725 without protein, whereas for the clinical isolate *S. epidermidis* ORT a decrease was observed. In contrast, adsorption of FN on nanoHA 1000 did not result in a significant adhesion-promoting effect for *S. epidermidis* ORT as compared to *S. epidermidis* RP62A, where FN significantly promoted attachment to the nanoHA 1000. The increase of *S. epidermidis* RP62A adhesion on nanoHA 1000 with adsorbed FN may also be related probably by expression specific adhesins that interacted with FN, and thus allowed the binding of this strain to the FN. In contrast to the *S. aureus*, it was reported that the binding between *S. epidermidis* and FN takes place at FN domains near the C-terminus [71] and it was found that *S. epidermidis* cell surface-associated protein termed ECM-binding protein (Embp) is involved in binding to fibronectin [78-79]. Moreover, the Embp is also widely distributed in clinical isolates [50, 78]. However, the results obtained for *S. epidermidis* ORT point towards the notion that this bacterial strain probably did not have the ability to bind to FN, since the presence of FN on nanoHA 1000 surface did not change the adhesion pattern of this strain. The adhesion pattern obtained for both *S. epidermidis* strains on nanoHA 725 may be related probably with conformational changes of FN occurring upon adsorption on this surface and with the occupation of the nanoHA surface by fibronectin in a way that doesn't allow some links between the bacteria and this material to be established. Therefore, it should, in this context, be pointed out that the capacity of binding to fibronectin varies from one strain to another within the same species.

The adhesion profiles of both *S. aureus* and *S. epidermidis* strains decreased with the increase of the HSA concentration. The activity of HSA on adhesion of *S. aureus* and *S. epidermidis* was concentration and strain dependent, and in this study with greater efficacy at a concentration of 200 μg/ml. However, this concentration was only effective in reducing *S. epidermidis* ORT adhesion in both materials, since for other strains this concentration was only able to reduce the adhesion onto nanoHA 725 substrate. Regarding the behavior of HSA on adhesion of these bacterial strains, the results of the present study are in agreement with data described in the literature, despite the difference in the studied organism [135]. Therefore, although the use of HSA to prevent bacterial adhesion to materials has been proposed [53, 116, 136, 137], these results

showed that certain concentration of this compound produced stimulation of the bacterial adherence. Therefore, exposure of nanoHA samples to HSA solutions before incubation with the bacterial strains resulted in significantly altered adhesion patterns, which are consistent with the notion that there is a significant correlation between proteins concentration and bacterial strain studied.

The analysis of SEM images provided information concerning the bacterial morphology, the production of slime on surfaces as well as the interactions between bacteria and material. The production of slime protects the bacteria from host immune defenses and clinical antibiotics [138]. Bacterial strains that do not produce slime are less adherent and less pathogenic [53]. In this study, the two clinical strains of *S. aureus* isolated from bone infections with phenotype MSSA and MRSA displayed ability to adhere on material but no slime production. With SEM it was possible to observe that the bacterial adhesion profiles on both materials were different. In fact, nanoHA 1000 samples were less susceptible to bacterial adhesion and the results were very similar between these strains. As expected, the reference type culture *S. epidermidis* RP62A exhibited a viscous appearance duo to slime production on both nanoHA surfaces (Figure 3.5 c, d) and therefore presented itself as an excellent slime producer, an important virulence factor for *S. epidermidis* in medical implant/device-related infections that contributes towards their patogenicity [139]. As one may see in Figure 3.6 (c, d) it was also possible to observe the production of slime in the clinical strain of *S. epidermidis* ORT isolated from a bone infection.

The effects of the adsorbed FBS on bacteria-nanoHA surface interactions were also analyzed by SEM. These results showed that the bacterial adhesion on nanoHA with adsorbed FBS decreased very significantly when compared with that on nanoHA without protein. The impossibility of *S. epidermidis* strains to produce biofilm was likely related to the reduction in the number of bacteria present on the surface.

With SEM images, it was also possible to observe that the presence of FN and HSA on the nanoHA surface affected bacteria-surface interactions. An interesting observation was that in the case of clinical strains of *S. aureus* the bacterial cells began to form small aggregates on the materials surface and a larger adherent aggregate, a more developed stage in biofilm formation, was observed for MSSA strain on nanoHA 1000 with adsorbed HSA (Figure 3.11 f). Moreover, results of the present work assured that biofilm formation by *S. epidermidis* RP62A was remarkably enhanced in the presence of these specific proteins (Figure 3.13). These data suggest that the presence of proteins may contribute to the virulence of this bacterial strain. For *S. epidermidis* ORT

strain the ability of biofilm formation in the presence of FN and HSA was lower as perceptible in Figure 3.14.

Chapter V - Concluding remarks and suggestions for future work

5.1. Concluding Remarks

Bacterial adhesion and the consequent biofilm production is an important cause of failure of orthopedic prostheses, and it is affected by the presence of proteins. This work aimed at better understanding how *S. aureus* and *S. epidermidis* adhesion was influenced by proteins adhered to nanoHA materials.

The results presented showed that the lower available surface area and porosity present in nanoHA 1000 surfaces make this material more resistant to bacterial adhesion.

The results concerning the adsorption of FBS on nanoHA surfaces showed that bacterial adhesion to these materials was influenced by the composition of the adsorbed proteins on surface. This study emphasizes the importance of the role played by presence of proteins at the initial stages of bacterial adhesion.

The adhesion profiles in the presence of FN were different for both tested materials, where the adherence of the bacterial strains, except for *S. epidermidis* ORT strain, was significantly lower on nanoHA 1000. The influence of HSA on adhesion was concentration and strain dependent. An important aspect to retain from these results is that they could have some clinical potential, showing that HSA in certain concentrations is effective in reducing bacterial adhesion. From these results it was possible to conclude that the bacterial response can drastically vary, depending of the amount, nature and conformation of the proteins adsorbed on the surface as well as of the bacterial strain. Moreover, in this work the *S. epidermidis* strains exhibited differing abilities of biofilm formation in the presence of proteins.

In conclusion, when evaluating bacterial adhesion behavior on the surfaces, it is important to consider the relationships between bacterial strains, material and proteins.

5.2. Future work

The present work represents an advance concerning the behavior of bacteria in an environment with proteins.

Further analysis of fibronectin behaviour regarding nanoHA surfaces and its influence on bacterial adhesion is still required. Analysis by PCR of *S. aureus* and *S. epidermidis* isolated from bone infections are required to assess which genes were expressed by these bacteria. Other key information may be obtained by inactivating the genes encoding the fibronectin-binding proteins to support the hypothesis that these proteins are involved in the binding of *S. aureus* and *S. epidermidis* to nanoHA substrates. Additional studies are needed to explore the conformation of FN on both nanoHA surfaces in terms of influence on bacterial adhesion.

With respect to the effect of HSA on bacterial adhesion more detailed studies are necessary, and the results obtained with HSA should encourage further studies using more bacterial strains and more compounds with the aim of finding new and effective alternatives both to prevent biofilm formation and enhance bacterial killing against biofilm-embedded organisms.

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