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Scuola di Ingegneria Industriale e dell' Informazione

Laurea Magistrale in Ingegneria Biomedica

SURFACE CHARACTERIZATION AND BACTERIAL CONTAMINATION OF DIFFERENT BIOMATERIALS FOR ARTIFICIAL JOINTS: CORRELATION BETWEEN SURFACE PROPERTIES AND BACTERIAL RESPONSE

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“Portate tenacia, tempismo e tolleranza in ciò che fate.
Guardate la vita con ironia, annusate il futuro con la luce negli occhi.
Andate dritti per la vostra strada, e sarete sorpresi di trovarla sgombra”

Beppe Severgnini, Italiani di domani

Il ringraziamento più grande va ai miei genitori e a Matteo. Questo traguardo è grazie a voi, è per voi. Siete stati una Squadra, una carica di fiducia ancora più forte nei momenti in cui questo percorso mi ha messo alla prova. Siete le mie radici, il mio riconoscimento non può essere che enorme. Un pensiero affettuoso è anche per i miei due zii e per la nonna, sempre aggiornati sull'andamento dei miei studi.

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Un pensiero speciale è per Diego, che con la sua determinazione contagiosa, non molla mai.

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Acronyms and abbreviations

2D	Bi dimensional
3D	Three dimensional
ACL	Anterior cruciate ligament
AES	Auger Electron Spectroscopy
AFM	Atomic force microscopy
Al_2O_3	Alumina
BAI	Biomaterial associated infection
BCA	Colorimetric bicinconinic acid assay
CoCrMo	Cobalt-chromium-molybdenum alloy
DBM	Decalcified (or demineralized) bone matrix
ECM	Extracellular matrix
EO	Ethylene oxide
ESCA, XPS	Electron Spectroscopy for Chemical Analysis,X ray Photoelectron Spectroscopy
FA	Fluoroapatite
FBS	Foetal bovine serum
FMS	Fresh measurement solution
FT-IR	Fourier Transform -Infrared Spectroscopy
HA	Hydroxyapatite
LMMA	Laser microprobe mass analysis
LS	Lysing solution
Mg	Magnesium
NPs	Nanoparticles

OD	Optical density
OM	Optical microscopy
OP	Optical profilometry
OWLS	Optical Waveguide Light mode Spectroscopy
PBS	Sterile buffered saline solution
PCL	Posterior cruciate ligament
PCL	Polycaprolactone
PE	Polyethylene
PEEK	Thermoplastic polyether ether ketone
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PJI	Periprosthetic joint infection
PLA	Polylactic acid
PMMA	Polymethyl methacrylate
PP	Polypropylene
PS	Polystyrene
PTFE	Polytetrafluoroethylene
QCM	Quartz Crystal Microbalance
SEM	Scanning Electron Microscopy
SM	Stereo microscopy
SPM	Scanning probe microscopy
SS	Stainless steel
SSIs	Orthopaedic surgical site infections
STM	Scanning Tunneling Microscopy

Ta	Tantalum
THR	Total hip replacement
Ti	Titanium
TOF	TimeofFlight – mass analysis
TZP	Tetragonal zirconia polycrystals
UHMWPE	Ultrahigh molecular weight polyethylene
WHO	World Health Organization
WR	Working reagent
XLPE	Cross-linked polyethylene
Zr	Zirconium

Summary

1- INTRODUCTION

The World Health Organization (WHO) has recently recognized antimicrobial resistance as one of the most important problems facing human health. Infections related to biomaterials implantation now constitute a significant clinical problem, and are the most common cause of implant failure, bringing with them high rates of morbidity and mortality. Profound knowledge about the effects of the different surface features on microbial inhibition response of biomaterials is required both for better understanding of *in-vivo* behavior of implants and surface engineering of biomaterials.

Surface characteristics, such as surface topography and wettability, affect material ability to adsorb water and proteins, and to interact with bacteria.

The purpose of this experimental work is to clarify whether surface characteristic could influence bacteria adhesion and biofilm development in order to study the resistance of the biomaterials to bacterial proliferation and to identify the material with the best antibacterial performances.

A selection of biomaterials (CoCrMo alloy, XLPE and 4 different types of ceramics), currently used in orthopedic applications, has been considered in the present work in order to verify intrinsic antibacterial adhesion properties towards the two biofilm formers strains *Staphylococcus aureus* and *Staphylococcus epidermidis*. The aim was to determine the effect of the surface characteristics, peculiar for each material, on the bacterial response.

Surface roughness (both micrometric analysis by means of profilometer, and nanometric observation by means of AFM) , surface wettability and protein absorption have been determined in the same conditions for all the materials. To correlate the data of surface topography characterization to the microbiological behaviour, a simulation of bacterial infection on the surfaces has been performed, followed by a metabolic analysis about the bacterial viability by means of MTT colorimetric assay, and a morphological analysis about the biofilm thickness through SEM analysis.

The thesis is organized in order to provide initial sections about the faced biomedical topic, a detailed materials and methods section that describes experimental set-up and procedures followed to perform experiments, and finally results and discussion sections aimed to describe and discuss obtained data. The work is a collaboration between the Department of Health Sciences – University of Novara, and the Department of Chemistry, Materials and Chemical Engineering-Politecnico di Milano.

2- EXPERIMENTAL TESTS : MATHERIALS AND METHODS

All the materials assayed in this work were selected and supplied in single-sterile packages by CeramTec; disks of 1.4cm diameter and 3mm thickness were provided as representative for CoCrMo metal, XLPE polymer, pink ceramic (BIOLOX®delta –alumina and zirconia ceramic) , yellow ceramic (BIOLOX®forte -alumina ceramic) , white ceramic (untreated ceramic), grey ceramic (silicon nitride) (fig. 1). No changes or further treatments were applied to the specimens surfaces prior to experiments in order to avoid any surface harm.

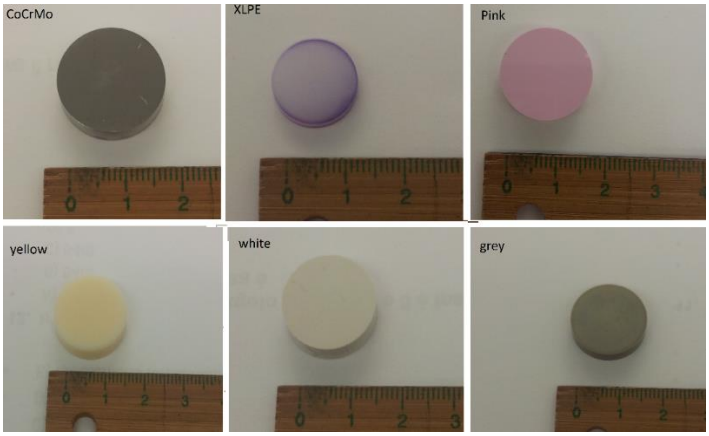


Figure 1 Biomaterial samples

2.1 **Profilometry**

Specimens surface roughness analysis on micro-scale level were performed using a laser optical profilometer (UBM-Microfocus Compact, NanoFocus AG, Germany). It is a non-contact method that provides 2D and 3D images of a surface, numerous roughness statistics, and feature dimensions. Specimens were gently fixed onto machine plate; each specimens surface was laser-scansioned by randomly selecting 5 representative areas. Three specimens of each tested materials were analyzed and Ra, Ry and Rmax values were extracted. Data are expressed as means ± standard deviations of the total 15 areas laser-scansioned. Finally, considering the values obtained, software reconstructed the surface 3D topography.

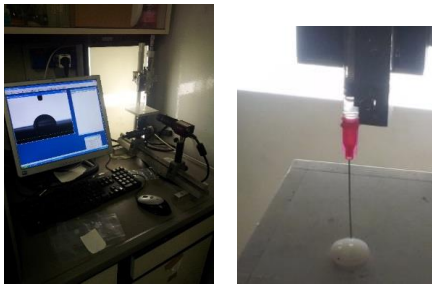
2.2 **Atomic-force microscopy**

Specimens surface roughness analysis on nano-scale level were performed using the atomic-force microscopy (AFM). Samples viewed do not require any special treatments (such as metal coatings) that would irreversibly change or damage the sample, and does not typically suffer from charging artifacts in the final image.

Using AFM technique it is possible to measure a roughness of a sample surface at a high resolution (lower than the nanometer). The AFM used (NT-MDT, Moscow, Russia.) is in contact mode (static mode). Two scans were performed for each sample (20 μm and 50 μm), and the parameter of interest has been extracted (Arithmetic mean height Sa). For each kind of biomaterial, 3 samples were analyzed, to perform comparisons about the surface morphology with nanometer resolution, between the different materials. The AFM provided a three-dimensional surface profile of the scanning area.

2.3 **Contact angle**

The contact angle allows to evaluate the wettability of a surface: the lower is the contact angle, the higher is the degree of wettability of the surface. The equipment consists of a horizontal stage to mount a sample, a micrometer pipette to form a liquid drop, an illumination source, and a telescope connected to the software (fig. 2) The measurement was achieved by simply aligning the tangent of the drop profile at the contact point with the surface and reading the protractor. A camera can be integrated to take photographs of the drop profile so as to measure the contact angle at leisure [166]. The use of relatively high magnifications enables a detailed examination of the intersection profile.



a. Camera and software b. Needle

Figure 2 *Experimental set-up used in the analysis*

The interaction was characterized by the static contact angle. A drop of constant volume is created and rested on the surface of the sample. 3 samples of each type of biomaterial of the study were subjected to the analysis of wettability; on each sample, five measurements are performed, because 5 drops have been deposited in different parts of the surface. The

software is Drop Shape Analysis for Windows, which reconstructs the two dimensional image and provides the contact angle. The liquid used for this test is distilled water.

2.4 **Bacterial contamination: MTT and SEM analysis**

The different biomaterials has been considered in order to verify intrinsic antibacterial adhesion properties towards the two biofilm formers strains *Staphylococcus aureus* and *Staphylococcus epidermidis*. Specimens surface colonization has been metabolically investigated by MTT assay, while biofilm morphology investigated by Scanning Electron Microscopy (SEM).

Two strong biofilm-producing bacterial strains were obtained from International PBI (International PBI Spa, Milan, Italy). The broth culture had a final concentration of $1x10^7$ cells/ml according to McFarland 1.0 standard, to simulate the bacterial infection (figure 3).

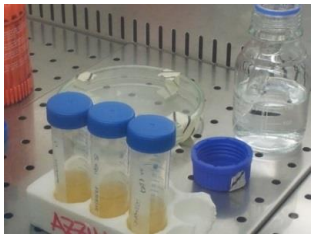


Figure 3 Bacterial strains in culture medium

Specimens disks were placed into the wells of a 12 multiwell plate and infected. Plate was then incubated in agitation to allow biofilm cells adhesion.

Quantification of viable biomass (MTT):

The quantification of viable biomass has been performed at the end of each time-point (24-48 hs), by the metabolic MTT assay. Absorbance was measured using a spectrophotometer at a wavelength of 550-620 nm; results were expressed as optical density (OD) units. The analysis is performed on samples and on PS, to obtain the control data. Then normalization on areas of the samples was necessary to obtain comparison data between test specimens and polystyrene controls; finally, the conversion of Optical Density into the percentage of contamination compared to the control was performed.

Scanning Electron microscopy (SEM):

One sample of each kind of contamination were processed for SEM analysis, to value the biofilm thickness. Specimens were observed with a scanning electron microscope

(Cambridge Stereoscan 360, Leica, Basel, CH) at 500, 2000 and 10000 magnifications, using secondary electrons at 12Kvs.

3D image processing:

The quantitative assessment of the biofilm thickness after SEM analysis were performed using the ImageJ software (NIH) plug-in for surface 3D-plot analysis, to obtain a computational model of biofilm.

2.5 Protein adsorption evaluation : BCA and Western Blot analysis

In order to better clarify whether surface characteristic could influence bacteria adhesion and biofilm development, proteins amount adsorption was investigated by BCA and Western Blot Analysis. The purpose of this analysis is to investigate quantitatively the adsorbed protein on the different surfaces, and subsequently to identify biomaterials able to selectively adsorb some adhesive and anti-adhesive proteins, through qualitative analysis (Western blot analysis).

Protein amount quantification (BCA):

To determine the different absorption capacity of the samples, disks were incubated in foetal bovine serum (FBS, Sigma) Then, the total amount of adsorbed proteins was quantified by the colorimetric bicinconinic acid assay (BCA, Thermo Scientific). To determine the amount (expressed as μg/cm2) of protein in each sample, a standard curve was generated using bovine serum albumin and mixed with BCA kit reagents The absorbance of all samples and standard curve was measured at 570 nm by spectrometer (SpectraCount, Packard Bell, USA) and test samples protein amount calculated as function of the standard curve.

Selective protein adsorption - Western blot analysis:

This analysis is used in research to identify proteins thanks to the possibility to separate them by the different molecular weight (mw). According to a commercial standard cocktail of known mw proteins, it is possible to distinguish different proteins adsorbed onto the surfaces. so it is possible to detect the presence of specific proteins in comparison with the standard.

2.6 Statistical analysis of data

Analysis of data was performed to value statistically significant difference between each material and the control (polystyrene) , and to compare the behavior between different biomaterials (ANOVA one-way followed by Sheffè test) .

3- RESULTS

All the presented data are expressed as means ± standard deviations of the replicates used to perform experiments. Replicates were used in order to get statistical analysis of the results and provide statically significances between specimens within performances evaluation.

3.1 Profilometry

Surfaces roughness analysis results are reported in figure 4a-c.

In figure 4 a, it's shown that XLPE specimens reported the higher average roughness Ra resulting as significant towards metal and ceramic materials (indicated by §). Between ceramic surfaces, white and pink resulted as the less rough ones, obtaining significant results towards both grey and yellow (indicated by * for white and ^^ for pink respectively). Results obtained for Ra analysis were very similar to those obtained for Ry (fig. 4b) and Rmax (fig. 4c).

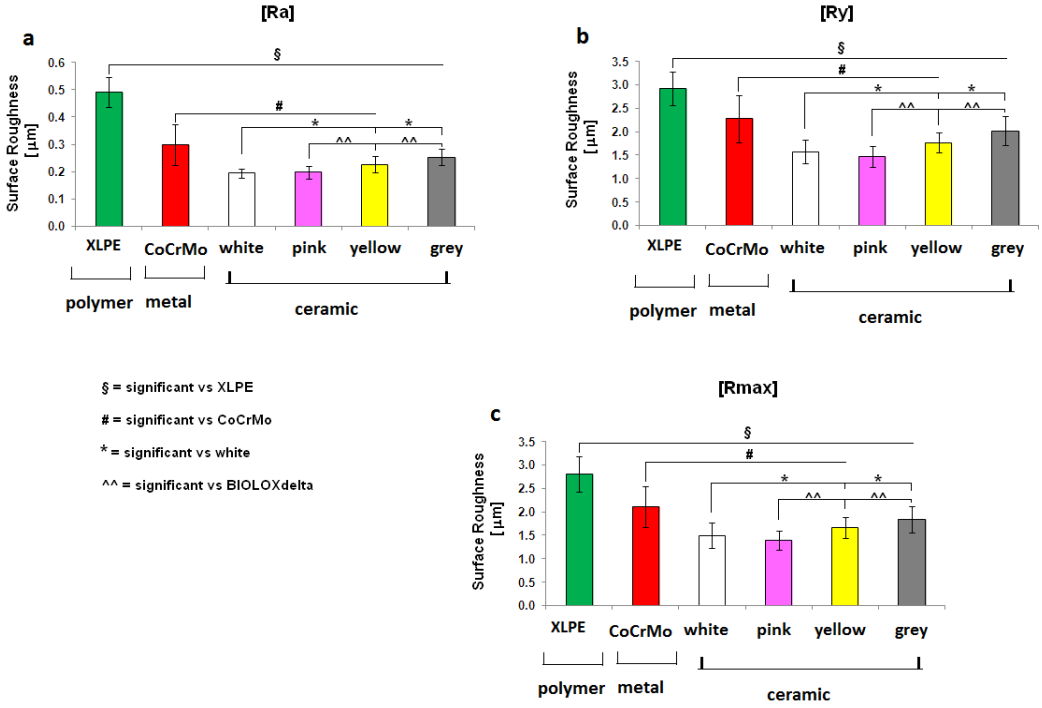


Figure 4 a-c. Specimens surface roughness evaluation by laser profilometry. Graphics represent Ra (a), Ry (b) and Rmax (c) scores. Data are expressed as means ± standard deviations.

The materials with the higher peaks are XLPE and CoCrMo surfaces; on the opposite, white and pink specimens revealed the lower presence of peacks, confirming that they represent the best materials concerning surface roughness.

3.2 Atomic-force microscopy

In fig 5a , the Sa of the analyzed samples is reported considering a scanning area of 20 µm, while results obtained considering a scanning area of 50 µm are presented in fig. 5b. Ceramic yellow reported the highest peaks, but at 20 µm, it was not result as significant towards other ceramics , metal and polymeric materials, while at 50 µm, it resulted as significant towards grey and white, which are the less rough ones (indicated by #). Between ceramic surfaces, no differences were noticed comparing pink with the other ceramics.

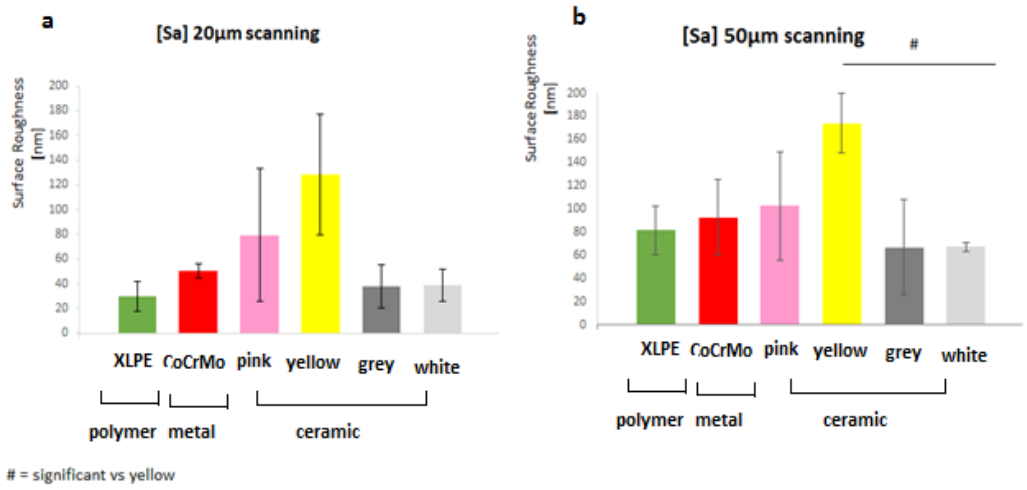


Figure 5 a-b. Specimens surface roughness evaluation by AFM. Graphics represent Sa 20 µm scanning (a), Sa 50 µm scanning (b) scores. Data are expressed as means ± standard deviations.

3.3 **Contact angle**

Figure 6 reports the contact angles values on the considered materials. XLPE and CoCrMo are the less wettable surfaces, while grey presents the lower contact angle, so it is more hydrophilic . Between ceramic surfaces only the grey material were significant towards the other ceramics (indicated by #), while no differences were noticed between yellow, pink, and white.

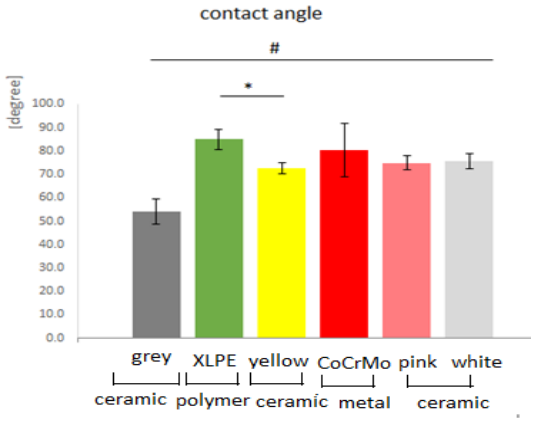


Figure 6 Static contact angles on the considered materials. Data are expressed as means ± standard deviations

3.4 **Bacterial contamination: MTT and SEM analysis**

S. aureus biofilm:

Figure 7 a-d represents the comparison in terms of bacterial viability between the *S. aureus* biofilm colonized XLPE, CoCrMo and ceramics surfaces after 24 (fig. 7a) and 48 (fig. 7b) hours of incubation. Significant difference were noticed between ceramic groups: yellow is more contaminated after 24 hs (fig. 7 a, indicated by #) and 48 hs (fig. 7 b, indicated by #) while the grey one is more contaminated after 48 hs (fig.7b, indicated by #). Significant differences between the ceramics and XLPE were noticed after 24 and 48 hours (indicated by §): XLPE resulted in general more contaminated (fig.7b, indicated by §).

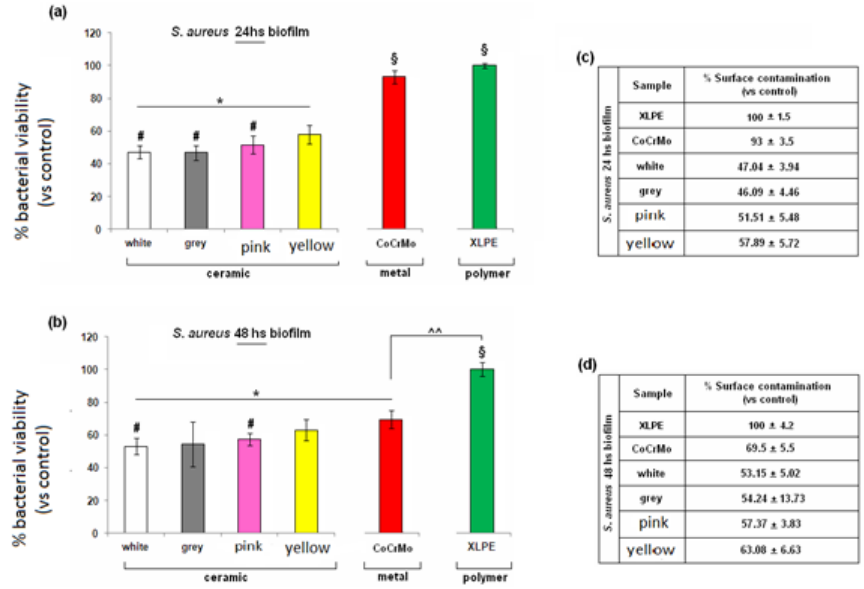
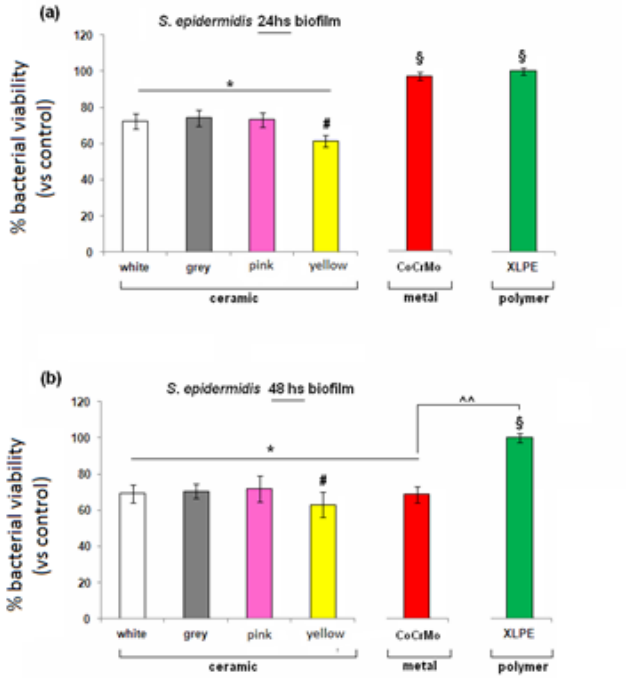


Figure 7 a-d. *S. aureus* viable biomass colonizing the surfaces of the ceramics materials (white-grey-pink-yellow), metal alloys (CoCrMo) and polymeric materials (XLPE) after 24 (a) and 48 (b) hours of incubation. Surface colonization % summary is reported in (c) for 24 hs data and in (d) for 48 hs; data are expressed as means ± standard deviations.

Subsequently a biofilm morphology investigation through Scanning Electron Microscopy has been performed, to compare the biofilm thickness. The SEM comparison in terms of surface colonization of ceramic, metallic (CoCrMo) and polymeric (XLPE) specimens, after 48 hours of incubation with *S. aureus* biofilm is reported.

S. epidermidis biofilm:

Figure 8 a-d represents the comparison in terms of bacterial viability between the *S. epidermidis* biofilm colonized XLPE, CoCrMo and ceramics surfaces, after 24 (fig. a) and 48 (fig. b) hours of incubation. Significant difference were noticed between ceramic groups: yellow is less contaminated after 24 hs (figure a, indicated by #) and 48 hs (fig. b, indicated by #). Significant difference between the ceramics and XLPE were noticed after 24 and 48 hours (indicated by §): XLPE resulted in general more contaminated (figure b, indicated by §).



(c)

S. epidermidis 24 hs biofilm	Sample	% Surface contamination (vs control)
	XLPE	100 ± 2.1
	CoCrMo	96.76 ± 2.1
	white	72.48 ± 4.25
	grey	74.08 ± 4.45
	pink	73.14 ± 3.86
	yellow	61.32 ± 3.30

(d)

S. epidermidis 48 hs biofilm	Sample	% Surface contamination (vs control)
	XLPE	100 ± 2.5
	CoCrMo	68.72 ± 4.4
	white	69.13 ± 4.88
	grey	70.54 ± 3.82
	pink	72.02 ± 7.18
	yellow	63.01 ± 7.02

Figure 8 a-d. *S. epidermidis* viable biomass colonizing the surfaces of the ceramics materials (white-grey-pink-yellow), metal alloys (CoCrMo) and polymeric materials (XLPE) after 24 (a) and 48 (b) hours of incubation Surface colonization % summary is reported in (c) for 24 hs data and in (d) for 48 hs; data are expressed as means and standard deviations.

Subsequently a biofilm morphology investigation through SEM has been performed, to compare the biofilm thickness of ceramic, metallic (CoCrMo) and polymeric (XLPE) specimens after 48 hours of incubation with *S. epidermidis.*

3.5 Protein adsorption evaluation : BCA and Western Blot analysis

Figure 9a represents the total amount of protein adsorbed onto the surface specimens (BCA assay), while in fig. 9 b is reported the western blot analysis of the absorbed proteins. The total amount of adsorbed proteins resulted as higher onto pink, yellow and CoCrMo but no statistical significant differences were noticed between different groups. Wester blot analysis showed that only pink adsorbed all the investigated proteins (b) .

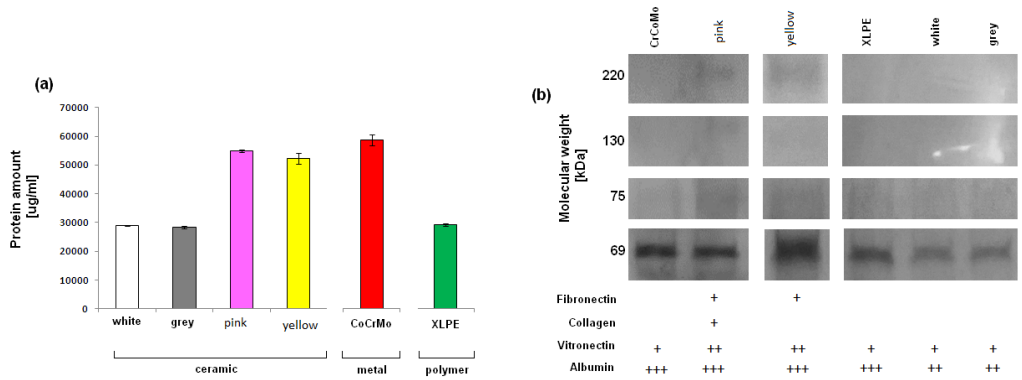


Figure 9a-b Protein absorption measurements. a) BCA quantification, b) Western Blot analysis. Data are expressed as means ± standard deviations.

4- DISCUSSION AND CONCLUSIONS

The increasing clinical use of implanted orthopedic medical devices, owing partly to the aging population, has lead to the emergence of new diseases and to rising public-health costs. Infections related to biomaterial implantation are a major clinical problem, bringing high rates of morbidity and mortality , mainly owing to the formation of biofilm, which is recalcitrant to antimicrobial agents. It is thus a clinical imperative to prevent bacterial colonization and subsequent biofilm adhesion. This work describes the ability of ceramic, metal and polymer materials to prevent *S. aureus* or *S. epidermidis* biofilm surface contaminations. The aim of this work is to summarize and link together obtained results regarding surface properties, antibacterial properties, and protein adsorption of ceramic, metal and polymer biomaterials for orthopaedic applications, in order to compare the different biomaterials performances.

Surface roughness analysis through the profilometer revealed that XLPE had the highest roughness at micro-scale level. Surface roughness is a key factor to influence bacteria adhesion: more roughness is normally alias of more bacteria adhesion. Therefore, considering that smoother materials are unfavorable to the bacterial adhesion, it's possible to affirm that ceramics are the best materials concerning surface roughness on micro-scale level. Looking at AFM measurements it's observed that ceramic yellow presents the greater roughness compared with the other materials. It's a more detailed investigation, which highlights nanometric characteristics of each surface. For instance it's noted that the ceramic materials at the nanometer level are the most wrinkled compared with the others materials, while they have a smoother surface at microscopic level analyzed by profilometer. Profilometry and AFM showed different morphological features, on two different scales of magnitude, but data provided by profilometry are more indicative concerning bacterial adhesion. As far as the contact angle measurements, it's noted that the highest wettability characterizes the grey substrate, while the lowest one characterizes the XLPE and CoCrMo. These results suggest that outermost surface layer is the most significant in order to determine the wetting behavior.

To correlate the data of surface characterization to the microbiological behaviour, a simulation of bacterial infection on the biomaterial surfaces has been performed, followed by a metabolic analysis about the bacterial viability by means MTT colorimetric assay, and a morphological analysis about the biofilm through SEM analysis. In general, ceramic materials showed the best performances in biofilm prevention, resulting as always less contaminated than metal and polymers ones for both strains. These data indicated that ceramics display a better ability to decrease bacteria adhesion in comparison with CoCrMo and XLPE.

Possible explanations to antibacterial activity could also be obtained by protein adsorption (albumin, fibronectin, collagen, vitronectin) assays results. The adsorption of cells pro-adhesion proteins including fibronectin, collagen and vitronectin, plays an important role in cell adhesion to artificial materials, and this fact could benefit an effective osseointegration; whereas adsorption of anti-adhesive protein, including albumin, could determine the inhibition of cell and bacteria adhesion, representing an important advantage from bacteriological point of view .

Western blot analysis showed that ceramics materials selectively absorbed fibronectin, collagen (pink and yellow) and vitronectin, that are pro-adhesion proteins. These results could be considered as positive interpreting them as good osteointegrative properties; in fact cells will be probably helped in adhesion and ECM production, supporting an effective osseointegration. On the opposite, the adhesive promotion properties could also enhance bacteria attachment.

Having regard to the tests performed in the present work and the correlations discussed, it's possible to affirm that ceramic materials prove to be advantageous to minimize the risk of infections in orthopedic applications. They could be considered superior in comparison with metal and polymers in terms of bacteria preventive anti-adhesion activity thanks to their smooth surfaces and the ability to selectively adsorb anti-adhesion proteins.

It’s possible to speculate that ceramics materials present surfaces with intrinsically antibacterial properties.

Sommario

1- INTRODUZIONE

L'Organizzazione mondiale della sanità (WHO) ha riconosciuto la resistenza antibatterica dei biomateriali come uno dei principali obiettivi della ricerca biomedica nel campo dei dispositivi medici impiantabili. Le infezioni relative ai biomateriali costituiscono al giorno d'oggi un problema clinico rilevante, e costituiscono una causa comune del fallimento dell'impianto, provocando un alto tasso di morbilità e mortalità. A questo proposito è sempre più necessario approfondire le conoscenze riguardo all'influenza delle proprietà superficiali sull'adesione e sulla colonizzazione batterica dei biomateriali sia per meglio comprendere il comportamento di un impianto *in-vivo,* sia per sviluppare tecniche di ingegneria delle superfici sempre più innovative ed efficaci contro la contaminazione batterica.

Le caratteristiche superficiali, come la topografia e la bagnabilità, influenzano l'interazione con i batteri e l'adsorbimento proteico. Lo scopo di questo lavoro sperimentale è proprio quello di chiarire in che modo le caratteristiche superficiali possano influenzare l'adesione batterica e lo sviluppo del biofilm, lo strato creato dai batteri al di sotto del quale proliferano proteggendosi dall'azione degli antibiotici. L'obiettivo del lavoro è quello di studiare la resistenza dei biomateriali alla proliferazione batterica e identificare il substrato che presenta intrinsecamente le migliori prestazioni antibatteriche.

Sono stati considerati differenti biomateriali attualmente in uso per le applicazioni ortopediche (lega metallica in CoCrMo, polimero XLPE and 4 diversi tipi di ceramiche),

per studiare le proprietà antibatteriche a seguito della contaminazione con *Staphylococcus aureus* and *Staphylococcus epidermidis*.

La rugosità superficiale (sia a livello microscopico tramite profilometro, sia nanometrico tramite AFM) , la bagnabilità superficiale e l'adsorbimento proteico sono state determinate nelle stesse condizioni per ogni campione. Per correlare i dati di caratterizzazione superficiale al comportamento microbiologico, è stata eseguita una simulazione di infezione batterica sulle superfici, seguita da un' analisi metabolica mediante saggio colorimetrico MTT per la valutazione della vitalità batterica, e da un' analisi morfologica tramite osservazione al SEM per valutare lo spessore del biofilm.

La tesi è strutturata in modo da presentare le sezioni introduttive riguardanti la problematica biomedica di interesse , un capitolo che descrive nel dettaglio le procedure sperimentali seguite e il set-up sperimentale di ogni prova, ed infine i risultati e le discussioni dei dati ottenuti, seguite da un capitolo di chiusura che mostra i limiti e le prospettive future di questo ramo dell'Ingegneria Biomedica.

Questo lavoro è frutto della collaborazione tra il Dipartimento di Scienze della Salute dell'Univesità di Novara, e il Dipartimento di Chimica, Materiali e Ingegneria Chimica del Politecnico di Milano.

2- PROVE SPERIMENTALI: MATERIALI E METODI

Tutti i materiali sono stati selezionati e forniti in confezioni singole sterili da CeramTec; si tratta di dischi di 1,4 cm di diametro e 3 mm di spessore (fig.1) in lega di CoCrMo, polimero XLPE, e 4 tipi di materiali ceramici: rosa (BIOLOX®delta –alumina e zirconia), gialla (BIOLOX®forte–alumina) ,bianca (ceramica di base non trattata) , grigia (nitruro di silicio) . Prima degli esperimenti non è stato applicato ai campioni alcun tipo di modifica o trattamento superficiale.

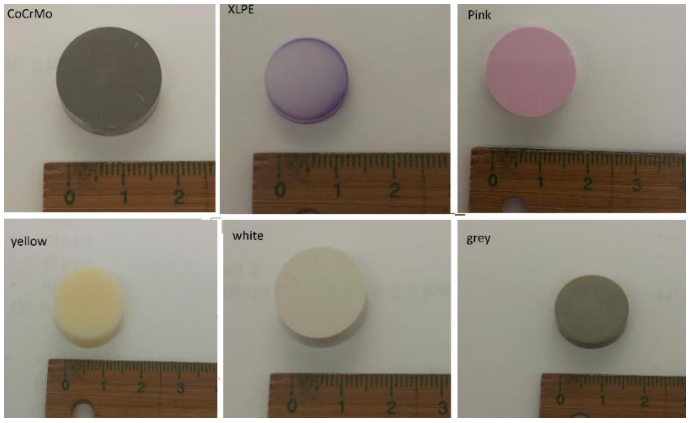


Figura 1 Campioni

2.1 **Profilometria**

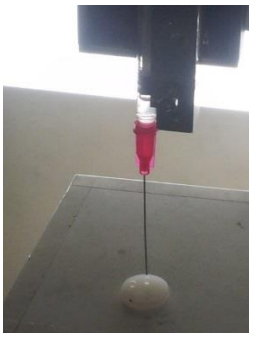
L’analisi della rugosità superficiale a livello microscopico è stata eseguita utilizzando un profilometro ottico laser (UBM-Microfocus Compact, NanoFocus AG, Germania), un metodo a non-contatto, che fornisce immagini 2D e 3D, valori statistici, e i parametri di rugosità. I campioni sono stati fissati sulla lastra di appoggio della macchina; ogni superficie è stata scansionata considerando 5 aree rappresentative. Tre campioni di ogni materiali sono stati analizzati e successivamente sono stati estratti i valori Ra, Ry e Rmax. Il software per la ricostruzione dell’immagine 3D ha riportato la topografia tridimensionale della superficie.

2.2 **Microscopia a forza atomica**

L’analisi della rugosità a livello nanometrico è stata eseguita utilizzando il microscopio a forza atomica (AFM). I campioni non richiedono trattamenti speciali (come rivestimenti metallici) che potrebbero modificare o danneggiare il campione. Con la tecnica AFM è possibile misurare una rugosità di superficie del campione ad alta risoluzione (inferiore al nanometro). L'AFM utilizzato (NT-MDT, Mosca, Russia.) è in modalità di contatto (modalità statica). Sono state eseguite due scansioni per ciascun campione (area di scansione 20x20 μm e 50x50 μm), e successivamente è stato estratto il parametro di interesse (Rugosità media Sa). Per ogni tipo di materiale, sono stati analizzati 3 campioni, per confrontare la morfologia superficiale dei diversi materiali con risoluzione nanometrica . L'AFM ha fornito anche un profilo superficiale 3D dell'area di scansione.

2.3 **Angolo di contatto**

L'angolo di contatto consente di valutare la bagnabilità di una superficie: minore è l'angolo di contatto, maggiore è il grado di bagnabilità. L'apparecchiatura consiste in una lastra di appoggio per il campione, una pipetta micrometrica per formare la goccia liquida, una sorgente di illuminazione, e un telescopio collegato al software (fig. 2). Una telecamera può essere integrata al sistema per fotografare il profilo della goccia e misurare l'angolo di contatto. L'uso di eventuali ingrandimenti consente un esame più dettagliato del profilo della goccia.

a. Telecamera e software b. Ago

Figura 2 Set-up sperimentale

E' stata effettuata una misurazione dell'angolo di contatto statico. E' stata creata una goccia di volume costante, appoggiata sulla superficie del campione. Sono stati sottoposti all'analisi di bagnabilità 3 campioni per ogni tipo di materiale in studio; su ciascun campione, sono state eseguite 5 misurazioni, dato che 5 gocce sono state depositate in diverse zone della superficie. Il software (Drop Shape Analysis for Windows) ricostruisce l'immagine a due dimensioni e fornisce l'angolo di contatto. Il liquido utilizzato per questo test è acqua distillata.

2.4 **Contaminazione batterica: MTT e SEM**

Queste analisi sono state eseguite al fine di verificare le proprietà antibatteriche intrinseche verso i due ceppi di *Staphylococcus aureus* e *Staphylococcus epidermidis*. L'analisi metabolica è stata svolta tramite saggio colorimetrico MTT, mentre l'analisi morfologica per valutare lo spessore del biofilm è stata svolta tramite microscopia elettronica a scansione (SEM). I due ceppi batterici sono di provenienza International PBI (International PBI Spa, Milano, Italia). Il brodo di coltura presenta una concentrazione finale di $1x10^7$ cellule / ml in base allo standard, in modo tale da simulare l'infezione batterica (figura 3).

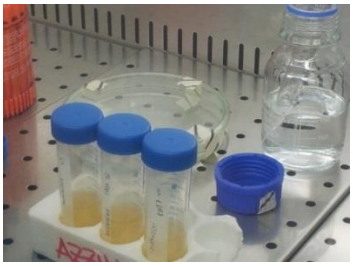


Figura 3 Ceppi batterici nel mezzo di coltura

I dischi sono stati collocati nei pozzetti di una piastra e infettati. La piastra è stata quindi incubata in agitazione per consentire l'adesione dei batteri e lo sviluppo del biofilm.

Quantificazione della vitalità batterica (MTT):

Questa analisi è stata eseguita al termine di ogni fase (24 hs and 48 hs) tramite saggio metabolico MTT. L'assorbanza è stata misurata utilizzando uno spettrofotometro (LP200, Diagnostic Pasteur, Milano, Italia) alla lunghezza d'onda di 550-620 nm; i risultati sono stati espressi come Optical Density (OD). L'analisi viene eseguita sui campioni e su PS, per ottenere i dati di controllo. La normalizzazione sulle aree dei campioni è necessaria per ottenere i dati di confronto tra i provini e i controlli in PS; infine, è stata effettuata la conversione di Optical Density in percentuale di contaminazione rispetto al controllo.

Microscopia elettronica a scansione (SEM):

Un campione per ogni tipo di contaminazione è stato osservato al SEM (Cambridge Stereoscan 360, Leica, Basilea, CH), per valutare la morfologia e quindi lo spessore del biofilm. I campioni sono stati osservati con ingrandimenti 500 X, 2000 X e 10000 X .

Modello computazionele 3D del biofilm:

La valutazione quantitativa dello spessore del biofilm dopo l'analisi al SEM è stata svolta utilizzando il software ImageJ (NIH, plug-in for surface 3D-plot analysis) , per ottenere un modello computazionale di biofilm.

2.5 Valutazione dell'adsorbimento proteico : BCA e Western Blot

Per chiarire in che modo le caratteristiche superficiali potrebbero influenzare l'adesione batterica, l'adsorbimento di proteine è stato studiato tramite analisi BCA e Western Blot. Lo scopo è quello di studiare quantitativamente l'adsorbimento proteico su diverse superfici, e successivamente identificare i biomateriali in grado di adsorbire selettivamente proteine adesive e antiadesive, mediante l' analisi qualitativa (Western Blot).

Analisi quantitativa - BCA:

Per determinare le diverse capacità di adsorbimento dei campioni, i dischi sono stati incubati in siero fetale bovino (FBS, Sigma). La quantità totale di proteine adsorbite è stata valutata mediante il saggio bicinconinic acid assay (BCA, Thermo Scientific).

Analisi qualitativa - Western Blot:

E' possibile identificare le proteine adsorbite selettivamente dai diversi substrati poiché esse vengono separarate in base al loro peso molecolare. Facendo riferimento allo standard

commerciale dei pesi molecolari proteici noti, è possibile distinguere differenti proteine adsorbite sulle superfici e rilevare la presenza di proteine specifiche pro-adesione o anti-adesione.

2.6 Analisi statistica dei dati

L'analisi dei dati è stata effettuata per valutare differenze statisticamente significative tra ogni singolo substrato e il controllo (polistirene), e per confrontare tra loro i differenti biomateriali (ANOVA unidirezionale seguito da Sheffè test).

3- RISULTATI

Tutti i dati presentati sono espressi come media ± deviazioni standard sul totale dei dati raccolti. I replicati sono stati considerati al fine di svolgere l'analisi statistica dei risultati e fornire differenze staticamente significative tra i campioni per la valutazione delle proprietà.

3.1 Profilometria

In figura 4° si nota che i campioni XLPE riportano la più alta rugosità media Ra, e mostrano differenze significative rispetto al metallo e i materiali ceramici (indicato da §). Tra le superfici ceramiche, la bianca e rosa sono risultate le meno ruvide, mostrando risultati significativi rispetto alla ceramica grigia e gialla (indicata rispettivamente da * per la bianca e ^^ per la rosa). I risultati ottenuti per l'analisi Ra sono molto simili a quelli ottenuti per Ry (fig. 4b) e Rmax (fig. 4c).

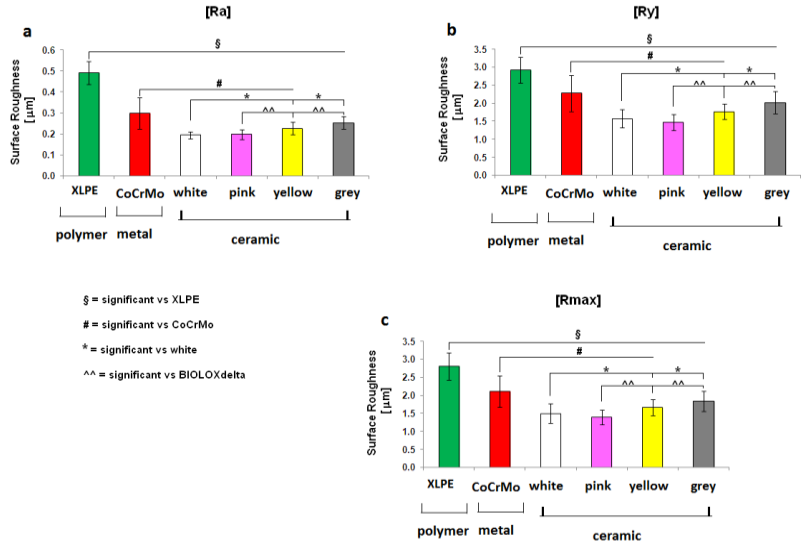


Figura 4 a-c. Valutazione della rugosità superficiale con profilometro laser. Sono rappresentati i valori di Ra (a), Ry (b) e Rmax (c). I dati sono espressi come come media ± deviazione standard.

I materiali con i picchi più alti sono XLPE e CoCrMo; al contrario, i campioni bianchi e rosa presentano i picchi inferiori, confermando che essi rappresentano i migliori materiali per quanto riguarda la rugosità superficiale.

3.2 Microscopia a forza atomica

In figura 5a, è riportato il valore della rugosità media Sa dei campioni analizzati, considerando un'area di scansione di 20 micron, mentre i risultati ottenuti considerando un'area di scansione di 50 micron sono presentati in figura 5b. I campioni in ceramica gialla hanno riportato la più alta rugosità superficiale, senza mostrare differenze statisticamente significative verso gli altri materiali, mentre a 50 micron, la ceramica gialla presenta differenze significative rispetto alla ceramica grigia e bianca, che sono le meno rugose (indicato da #).

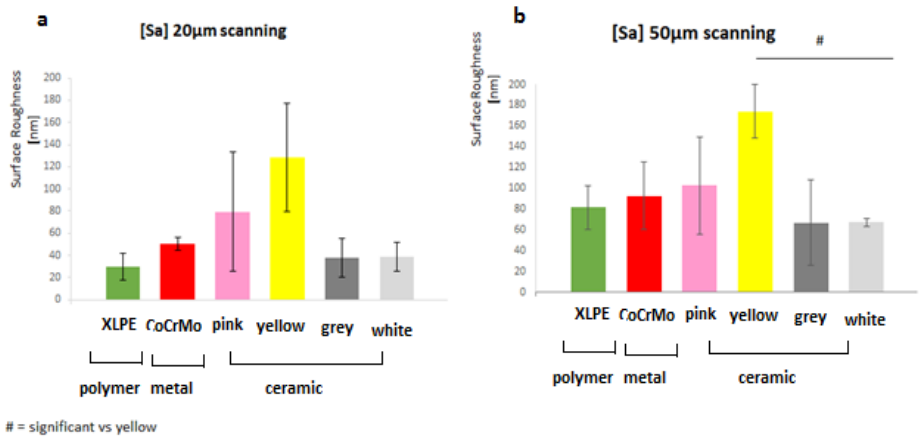


Figura 5 a-b. Valutazione della rugosità superficiale tramite AFM. Sono rappresentati i valori di rugosità media Sa su un'area di scansione 20x20 µm (a), e 50x50 µm (b) . I dati sono espressi come media ± deviazione standard.

3.3 **Anglolo di contatto**

La figura 6 riporta i valori di angolo di contatto per i materiali considerati, da cui si ricavano informazioni riguardo alla bagnabilità delle superfici. XLPE e CoCrMo sono le superfici meno bagnabili, mentre la ceramica grigia presenta l'angolo di contatto minore, quindi è una superficie più idrofilica. Tra le superfici ceramiche solo la grigia è statisticamente significativa nei confronti delle altre ceramiche (indicato da #), mentre nessuna differenza è stata notata tra la gialla, rosa e bianca.

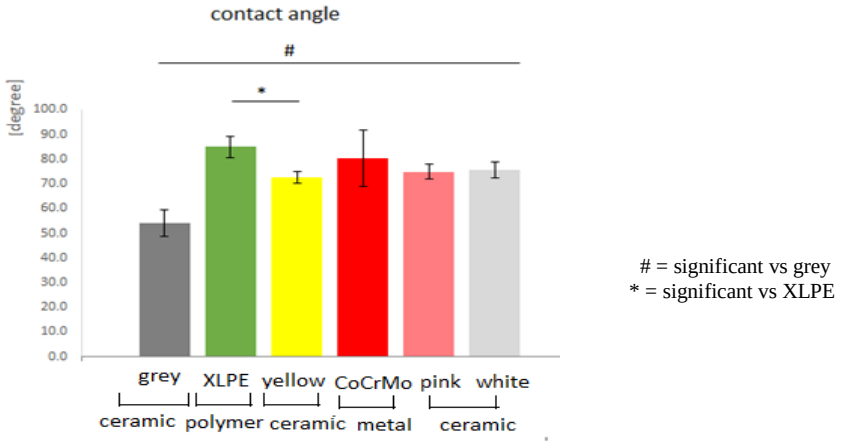
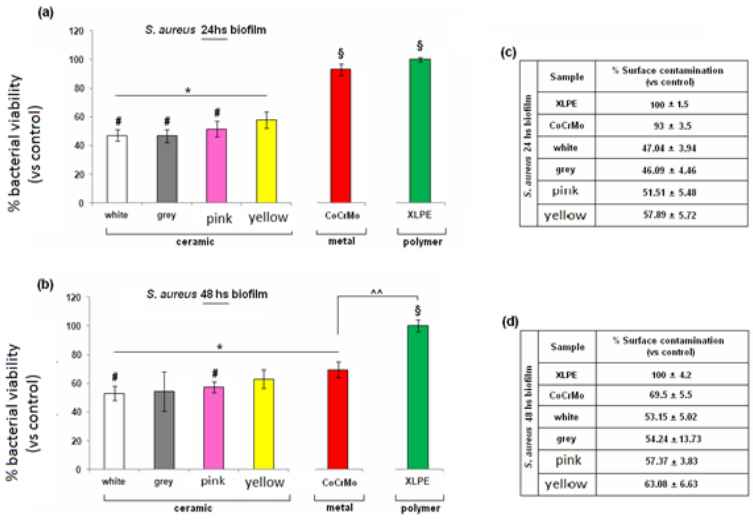


Figura 6 Angolo di contatto statico misurato sulle superfici considerate**.** I dati sono espressi come media ± deviazione standard.

3.4 **Contaminazione batterica: MTT e SEM**

S. aureus biofilm:

La figura 7 a-d riporta il confronto in termini di vitalità batterica tra le superfici ceramiche, CoCrMo e XLPE colonizzate da *S. aureus* dopo 24 (fig. 7 a) e 48 (fig. 7 b) ore di incubazione. Tra i materiali ceramici si notano differenze statisticamente significative: la ceramica gialla è più contaminata dopo 24 hs e 48 hs (fig. 7 b, indicato da #.), mentre la grigia è più contaminata dopo 48 hs (fig.7b, indicato da #). Si notano differenze significative tra le ceramiche e XLPE dopo 24 e 48 ore (indicati dal §): XLPE risulta in generale più contaminato (fig.7b, indicato da §).



Sample	% Surface contamination (vs control)
XLPE	100 ± 1.5
CoCrMo	93 ± 3.5
white	47.04 ± 3.94
grey	46.09 ± 4.46
pink	51.51 ± 5.48
yellow	57.89 ± 5.72

S. aureus 24 hs biofilm

Sample	% Surface contamination (vs control)
XLPE	100 ± 4.2
CoCrMo	69.5 ± 5.5
white	53.15 ± 5.02
grey	54.24 ± 13.73
pink	57.37 ± 3.83
yellow	63.08 ± 6.63

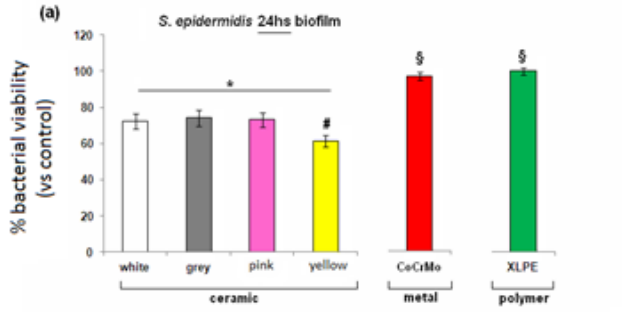
S. aureus 48 hs biofilm

Figura 7 a-d. Valutazione della vitalità batterica sulle diverse superifici contaminate con *S. aureus* dopo 24 (a) e 48 (b) ore di incubazione. Una sintesi dei dati in % riguardanti la colonizzazione è riportata in (c) per 24 hs e in (d) per 48 hs; i dati sono espressi come media ± deviazione standard.

Successivamente è stata effettuata un' indagine morfologia attraverso il microscopio elettronico a scansione, per confrontare lo spessore del biofilm. Il confronto tra le immagini al SEM delle superfici ceramiche, polimeriche (XLPE) e metalliche (CoCrMoMo) dopo 48 ore di incubazione con *S. aureus* è stato riportato.

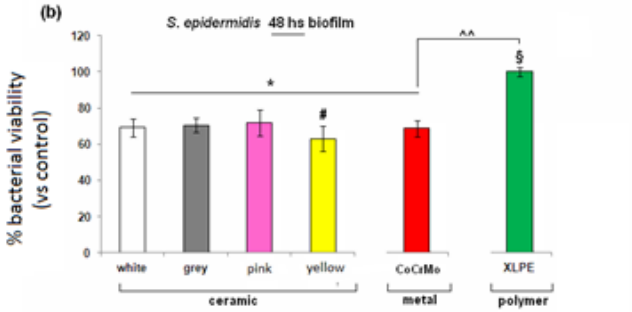
S. epidermidis biofilm:

La figura 8 a-d riporta il confronto in termini di vitalità batterica tra le superfici ceramiche, CoCrMo e XLPE colonizzate da S. epidermidis dopo 24 (fig. 8 a) e 48 (fig. 8 b) ore di incubazione.Tra i materiali ceramici si notano differenze statisticamente significative: la ceramica gialla è la meno contaminata sia dopo 24 hs (fig. a, indicato da #) sia dopo 48 hs (fig b, indicato da #.). Differenze significative tra le ceramiche e XLPE sono state notate dopo 24 e 48 ore (indicati dal §): XLPE risulta in generale più contaminato (figura b, indicato da §).



(c)

S. epidermidis 24 hs biofilm	Sample	% Surface contamination (vs control)
	XLPE	100 ± 2.1
	CoCrMo	96.76 ± 2.1
	white	72.48 ± 4.25
	grey	74.08 ± 4.45
	pink	73.14 ± 3.86
	yellow	61.32 ± 3.30



(d)

S. epidermidis 48 hs biofilm	Sample	% Surface contamination (vs control)
	XLPE	100 ± 2.5
	CoCrMo	68.72 ± 4.4
	white	69.13 ± 4.88
	grey	70.54 ± 3.82
	pink	72.02 ± 7.18
	yellow	63.01 ± 7.02

* = polystyrene VS ceramics
#= yellow VS other ceramics
§ = CoCrMo or XLPE VS ceramics
^^ = CoCrMo VS XLPE

Figura 8 a-d. Valutazione della vitalità batterica sulle diverse superifici contaminate con *S. epidermidis* dopo 24 (a) e 48 (b)ore di incubazione. Una sintesi dei dati in % riguardanti la colonizzazione è riportata in (c) per 24 hs e in (d) per 48 hs; i dati sono espressi come media ± deviazione standard.

Successivamente è stata effettuata un' indagine morfologia attraverso il microscopio elettronico a scansione, per confrontare lo spessore del biofilm. Il confronto tra le immagini al SEM delle superfici ceramiche, polimeriche (XLPE) e metalliche (CoCrMo) dopo 48 ore di incubazione con *S. epidermidis* è stato riportato.

3.5 Valutazione dell'adsorbimento proteico : BCA e Western Blot

La figura 9a rappresenta la quantità totale di proteine adsorbite dalle superfici (saggio BCA), mentre in figura 9b è riportata l'analisi qualitativa Western blot. La quantità totale di proteine adsorbite risultata più elevata sulla ceramica rosa, gialla e sul metallo CoCrMo ma senza differenze statisticamente significative tra i diversi gruppi. L'analisi Wester blot ha mostrato che solo la ceramica rosa ha adsorbito tutte le proteine esaminate (b).

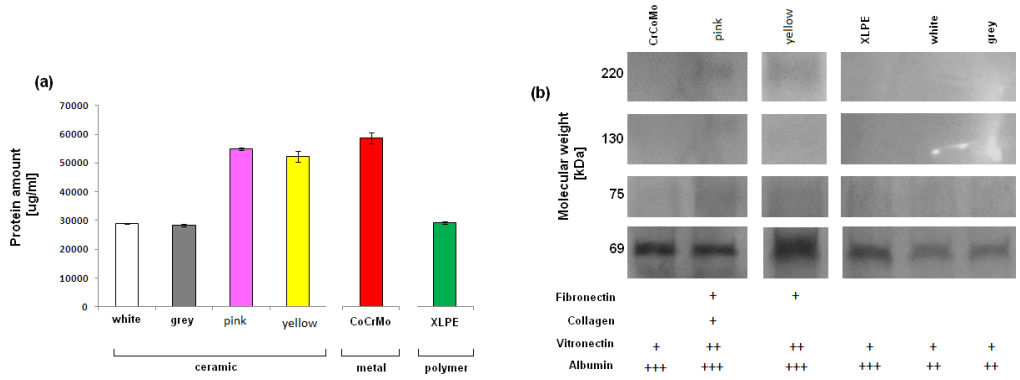


Figura 10a-b Adsorbimento proteico. a) analisi quantitativa BCA,b) analisi qualitativa Western Blot. I dati sono espressi come media ± deviazione standard.

4- DISCUSSIONI E CONCLUSIONI

L'uso clinico crescente di dispositivi medici ortopedici impiantabili ha portato allo sviluppo di nuove malattie e di conseguenza all'aumento dei costi nella sanità pubblica. Le infezioni correlate all'impianto di un biomateriale costituiscono un rilevante problema clinico, comportando alti tassi di morbilità e mortalità a causa della formazione di biofilm, lo strato batterico resistente agli agenti antimicrobici. In questo senso al giorno d'oggi è indispensabile minimizzare il rischio di infezioni per i biomateriali impiantabili ed impedire la colonizzazione batterica e il successivo sviluppo del biofilm.

Lo scopo di questo lavoro è quello di riassumere e correlare le proprietà superficiali dei biomateriali ortopedici alle loro proprietà antibatteriche , al fine di confrontare le prestazioni dei diversi materiali.

L’ analisi della rugosità attraverso il profilometro ha rivelato che il polimero XLPE ha la maggiore rugosità a livello micrometrico. La rugosità superficiale è infatti un fattore chiave che influenza l’adesione batterica: normalmente le superfici più rugose comportano una maggiore aderenza per i batteri. Pertanto, considerando che superfici più lisce sono sfavorevoli all'adesione batterica, si può affermare che le ceramiche costituiscono in questo studio i migliori materiali relativamente alla rugosità osservata a livello micrometrico. Tramite analisi AFM è emerso che la ceramica gialla presenta rugosità maggiori rispetto agli altri materiali. Si tratta di un'indagine più dettagliata, che mette in risalto le caratteristiche nanometriche di ogni superficie. I materiali ceramici a livello nanometrico sono duqnue più rugosi rispetto agli altri materiali, mentre hanno una superficie più liscia a livello microscopico, come analizzato dal profilometro. Questo significa che i campioni presentano diverse caratteristiche micrometriche e nanometriche: profilometria e AFM mostrano infatti differenti caratteristiche morfologiche, su due diverse scale di grandezza. Comunque per quanto riguarda l’adesione batterica i dati forniti dalla profilometria sono più indicativi; infatti perchè i batteri possano aderire, i picchi di rugosità devono essere dell’ordine dei micron.

Tramite le prove di angolo di contatto è emerso che la superficie più bagnabile è la ceramica grigia, mentre le meno bagnabili sono XLPE e CoCrMo. Questi risultati suggeriscono che lo strato più esterno è il più significativo per determinare il comportamento di bagnabilità.

Per correlare le caratteristiche superficiali al comportamento microbiologico, è stata eseguita una simulazione di infezione batterica sulle superfici dei biomateriali, seguita da un'analisi metabolica per la valutazione della vitalità batterica mediante saggio MTT, ed un’ analisi morfologica per valutare lo spessore del biofilm mediante osservazione al SEM . In generale, i materiali ceramici hanno mostrato le migliori performance per la prevenzione del biofilm, poiché risultano meno contaminati rispetto al metallo e al polimero per entrambi i ceppi (S. *aureus* e S. *epidermidis*). Questi dati indicano che le ceramiche mostrano una migliore capacità nella riduzione dell’adesione batterica in relazione al CoCrMo e XLPE.

Possibili spiegazioni circa il comportamento antibatterico possono essere ricercate anche nella valutazione dell’adsorbimento proteico (albumina, fibronectina, collagene, vitronectina). L'adsorbimento di proteine pro-adesione come la fibronectina, collagene e

vitronectina, svolge un ruolo importante per la adesione cellulare sui materiali artificiali, e questo potrebbe comportare un' efficace osteointegrazione; al contrario, l' adsorbimento di proteine anti-adesione, come l'albumina, potrebbe determinare l'inibizione dell' adesione batterica, rappresentando così un importante vantaggio dal punto di vista batteriologico.

Dall'analisi Western blot è emerso che i materiali ceramici adsorbono selettivamente fibronectina, collagene (rosa e giallo) e vitronectina, che sono proteine pro-adesione. Questi risultati possono essere considerati in un'ottica positiva, se interpretati a favore delle proprietà osteointegrative; infatti l'adsorbimento di proteine pro-adesione stimola l'adesione cellulare e quindi la produzione di matrice extra-cellulare (ECM), favorendo così un'efficace osteointegrazione. Tuttavia, bisogna anche considerare che questa proprietà potrebbe favorire l'adesione batterica sulla superficie.

A seguito di queste analisi e discussioni, si può affermare che i materiali ceramici sono vantaggiosi nella riduzione del rischio di infezioni in campo ortopedico. Essi possono essere considerati migliori rispetto ai metalli e ai polimeri per quanto riguarda la prevenzione di infezioni batteriche.

Alla luce di questo studio è possibile ipotizzare che i materiali ceramici presentano superfici con caratteristiche intrinsecamente antibatteriche.

Introduction

The present work analyses the recent advances in the design of bone implants, pointing the attention on biomaterials, in particular on the prevention of biomaterial-associated infections.

The thesis identifies major obstacles in orthopedic devices in term of infections and proposes a solution to contrast these phenomena. It's also underscored the need for rigorous performance criteria in the choice of the biomaterial used in hip and knee implants, to prevent and to contrast bacterial infection. It's highlighted the need to develop biomaterial with the best antibacterial performances.

Introduction to the problem

Bone tissue has a remarkable intrinsic ability to remodel and spontaneously regenerate; it exists in a dynamic state of homeostasis mediated by bone cells, namely osteoblasts, osteoclasts and osteocytes. As a consequence of osseous trauma, a powerful cascade of osteogenic events occurs to produce the fracture repair, which is the desired outcome. It's possible to categorize a spontaneously healing fracture as a subcritically sized defect for which the typical outcome is the natural restoration of form and function of the defect, and bone insufficiencies greater than a critical size, to which the intrinsic fracture healing as the spontaneous bone regeneration, does not extend [1].

The aim of the research in orthopedics is to design implants with specific structure, form and optimal surface treatments, able to extend intrinsic bone regeneration of a fracture to discontinuities that are critically sized [1]. In fact, the critically sized osseous deficiency will not regenerate spontaneously over the patient's lifetime and requires surgical intervention. The quest for regeneration in bone defects has inspired a search for clinically efficacious tissue-engineering therapeutics that has spanned over two decades.

This thesis analyses the recent advances in the design of bone implants pointing the attention on biomaterials; it highlights the need to develop biomaterial with the best antibacterial performances, and identifies major obstacles in terms of infections.

Primarily it's underscored the need for rigorous performance criteria in the choice of the biomaterial used in hip and knee implants, to prevent and to contrast bacterial infection. Goal of this work is to identify the biomaterial with the best antibacterial properties through *in vitro* experiments, in order to study the biomaterial resistance to bacterial proliferation.

It's known that with aging of the population, the prevalence of joint degenerative diseases is continuously rising. Joint replacement is one of the most successful surgical interventions alleviating pain, improving joint function, but also restoring general mobility and personal independence. The number of implanted hip and knee prostheses increases exponentially, which is followed by an increase of revision surgeries several years later .

The number of arthroplasties and consequently also the absolute number of complications is steadily increasing. In patients with primary hip arthroplasty, the most frequent local complications, that mostly required revision surgery, are hematomas (4 %), perioperative fracture (2.3 %), dislocation (2.2 %), paresis (2.1 %), and infection (0.4 %) . Thus,

periprosthetic joint infection is a rare complication compared to other postoperative complications, but it causes a considerable morbidity for the patient and accounts for a substantial proportion of health care expenditures.

Therefore, it's possible to affirm that the drawback of the technological advance in biomaterial field is the high susceptibility of implants to infection. This increase in infection susceptibility appears to be present across all biomaterials classes, regardless of form or function and has remained to this day as one of the most common, yet unresolved problems associated with the use of implanted biomaterials.

Whereas acute infection generally needs only one single antimicrobial treatment course to eradicate microorganisms, chronic infection may require sophisticated diagnostic procedures, long-term antimicrobial therapy and repetitive surgical interventions. In the case of orthopedic device-associated biofilm infection, cure of infection should be aimed at in the first attempt, because each treatment failure results in a worse functional result [2, 3] . Thus, the prerequisite for correct treatment of device associated infection is a rational concept for the optimal surgical and antimicrobial therapy.

Medical devices and the biomaterials from which they are composed are clearly central players in the pathogenesis of BAI. The presence of an implanted biomaterial causes a local defect in host immune defenses that contributes to the failure of the host to efficiently clear contaminating bacteria in a certain percentage of cases.

The processes of BAI then progress from bacterial adhesion to the biomaterial and colonization of adjacent tissues, through to formation of an antibiotic recalcitrant bacterial biofilm.

Modern biomaterials science has provided a vast array of modification and activation strategies to impart anti-infective properties upon biomaterials.

In this thesis anti-infective biomaterials and their properties are studied, and some of the most commonly applied approaches to contrast infections are discussed, such as antibiotic loaded materials and coatings, anti-adhesive surfaces, surface-functionalization.

With clinicians and insurance providers demanding reductions in BAI incidence, research into new anti-infective biomaterials and the clinical introduction of devices with anti-infective properties represent the front line in modern translational research.

An alternative to traditional approaches represented by the implant, concerns Tissue Engineering, which uses living cells and innovative biomaterials to develop 'bioactive'

tissue substitutes, as an alternative to artificial implants. The substantial progress in recent years in biology, and biomedical engineering,has lead to the creation of materials with similar properties to biological tissues, and has enabled the development of technologies to improve tissue regeneration in vivo.

This means new possibilities to restore the integrity and the function of the original tissue, but also to stimulate regeneration in situ, that is, the in vivo production of new tissue to replicate and replace the lost one. A bone substitute of new generation is then designed not only as a support (scaffold) to the neo-tissue formation in situ, but also as a vehicle of a biomolecular stimulation to induce the regeneration. In this sense it is increasingly strengthened the contribution of biomimetic approach of devices for bone tissue engineering, with the aim of reproducing the architecture of the physiological substrates, such as extracellular matrix (ECM), and the signals that lead to the integration of the implant in pre-existing tissue.

The biomimetic strategies aim to increase the biological activity of the surface of biomaterials, with physical changes (topography) or chemical changes (adhesives signals), so as to promote cell adhesion to the substrate and induce colonization of the scaffold.

The biomimetic strategies combine engineering and medical skills (respectively for the project and for clinical application) to make structures that imitate the biological microenvironment, to promote regenerative and reparative mechanisms.

Tissue engineering and regenerative medicine focus on the restoration of form and function to tissue insufficiencies; it concerns the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells, through a systematic combination of molecular and mechanical signals.

In the context of bone, a clinical osseous insufficiency is defined as a discontinuity in bone integrity resulting from trauma, congenital malformation or surgical resection [1] ,

that regenerative medicine has the task of solving. It’s a therapy that aims to induce the regeneration of tissues or organs following disease or injury, or in the presence of birth or developmental deformities. Such regeneration may be achieved through gene therapy alone, by cell therapy or tissue engineering, either of which may be assisted by concurrent gene transfer or pharmaceutical intervention.

In orthopedics, the reconstruction of bone are needed in the event of large losses of substance, as a consequence of serious birth defects, trauma, hypoplasia, ischemic necrosis, primary neoplastic lesions (osteosarcomas, benign bone tumors) or secondary (metastases). Currently, surgical treatment in case of bone loss refers to two alternatives: grafting of autologous tissue and tissue engineering.

The first approach is considered the 'gold standard' because it represents a safe solution in terms of compatibility and absence of immune response, but it also involves a second surgery to replace the missing portion with the correct amount of tissue. Thanks to the existence of the bone banks, the grafting of allogeneic tissue it's possible, but it is often subject to little remodeling, insufficient revascularization, rejection and the risk of infection.

The second one is represented by tissue engineering, whose potential has determined, as mentioned above, a significant boost to research in recent years.

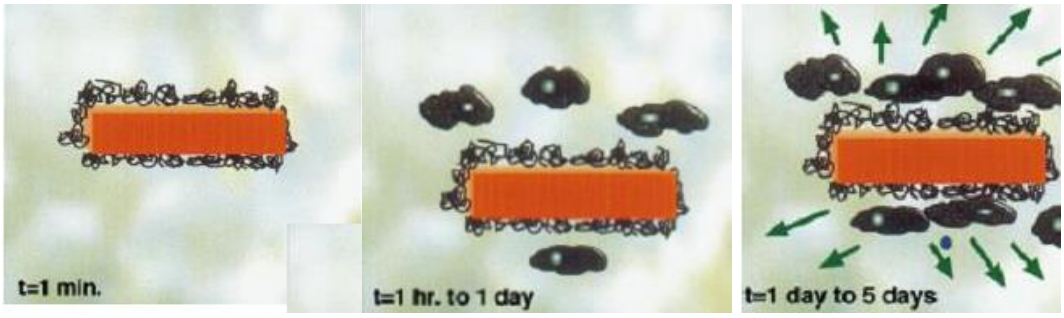
Bioactive materials are able to locally induce host cells to release growth factors, which in turn stimulate cells involved in the regeneration of tissue in situ.

Biomaterials which interface with biological systems are integral part of a process designed to evaluate, monitor or treat tissues of the body; they must replaces tissues and facilitates their regeneration and, in tissue engineering, they act as a temporary support, a surrogate for the extracellular matrix which degrades when new tissue is formed.

They interacts with tissue following these steps (figure 1):

1. The biomaterial adsorbs a layer of proteins
2. Cells (neutrophilis and macrophages) interrogate the biomaterial
3. Cells fuse to form giant cells and secrete protein signaling agents (cytokines)

If the material is recognized as ‘non-self’, it is encapsulated in an acellular collagenous bag (fibrotic capsule); if the material is recognized as ‘self’, it is integrated in a neo-formed tissue.



Step 1 Step 2 Step 3

Figure 1. (M.C. Tanzi, 2014, Approccio integrato per la medicina rigenerativa)

The overall objective in tissue engineering is to avoid a chronic inflammatory reaction to a foreign body that results in the formation of the fibrotic capsule, called biofilm, by isolating the biomaterial or the implants, so as to achieve integration of the implant into the surrounding tissue.

Therefore understanding the mechanisms and sequences of biochemical signals between living cells and the surrounding environment, is essential to designing material systems that encourage appropriate cellular behaviours.

Specific surface engineering strategies are required to encourage appropriate biological interactions with biomaterials, and to prepare surfaces that are more functional and controllable, so as to achieve functionality and specificity.

The surface of a biomaterial that interacts with bone tissue should also control the conformation and orientation of proteins with precision so that the body will specifically recognize them, to promote cellular differentiation, migration and proliferation. In this way the surface obtains the ability to perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signalling systems, in order to optimise tissue regeneration, without eliciting any undesirable effects in those cells, or inducing any undesirable local or systemic responses in the eventual host.

The nature of the absorbed protein layer depends on properties and topography of the material surface, the properties of the proteins present in the surrounding fluids, and the organization of the absorbed proteins, while cell adhesion and activation depend on type of

adsorbed proteins, initial protein conformation, and subsequent rearrangement of the absorbed protein layer.

This work presents an overview of the problem of biomaterial associated infection (BAI) with a particular emphasis on orthopaedic field; it's a contribution to research in the field of biomaterials to reduce infections incidence and to identify the material with the best antibacterial performances.

The work is a collaboration between the Department of Health Sciences – University of Novara, and the Department of Chemistry, Materials and Chemical Engineering-Politecnico di Milano.

Chapter 1

Biomechanical design of osseointegrative implantable devices

Clinical results in orthopaedics have demonstrated the need to find biomaterials that will help satisfy the minimum requirements for orthopaedic devices, to perform correctly on a long-term basis. This chapter highlights innovative biomaterial for bone implants, discussing the need for rigorous performance criteria in their design, and the key for the choice of new biomaterials.

The main fundamental requirements that orthopaedic devices must fulfill in order to function adequately, are summarized in this section.

1.1. Bone composition and structure

The purpose of the skeletal system is to protect internal organs, provide rigid kinematc links and muscle attachment sites, and facilitate muscle action and body movement. Bone has unique structural and mechanical properties that allow it to carry out there roles.

Bone is among the body's hardest structures; it is one of the most dynamic and metabolically active tissues in the body and remains active throughout life. It has an excellent capacity for self-repair and can alter its properties and configuration in response to changes in mechanical demand. Changes in bone density are commonly observed after periods of disuse and of greatly increased use; changes in bone shape are noted during fracture healing and after certain operations.

The bone tissue is a connective tissue present in all vertebrates, the harder tissue of the organism. It 's the predominant constituent of the skeleton, with the function of mechanical support. It protects the internal organs, allowing the movement of the joints and allows the mineral homeostasis and skeletal homeostasis.

It consists of two components: an extracellular matrix, which is hard and mineralized, and a component of cells, osteocytes (figure 1.1). The matrix, rich in calcium, forms a complex three-dimensional network that contains the cells that have produced within small gaps. The mineral component is made up of crystals of calcium salts, mostly calcium phosphate in addition to minor amounts of calcium carbonate and other salts, such as calcium fluoride or magnesium phosphate. The calcium phosphate is present in the form of apatite crystals. The extracellular matrix is constituted by a amorphous component, essentially proteoglycan, and a fibrous component, primarily made up of fibers of collagen type I.

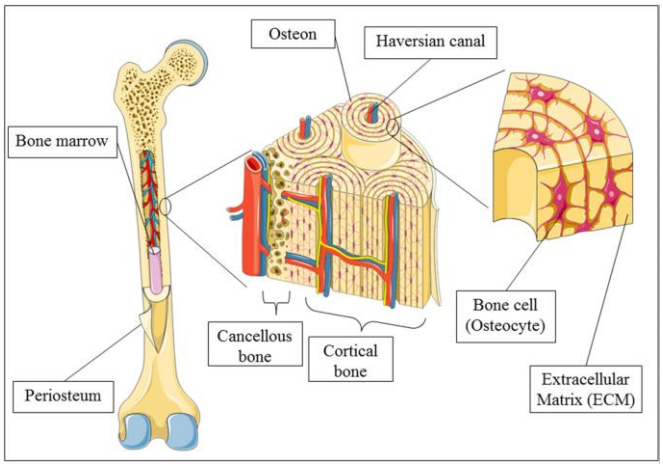


Figure 1.1 Bone composition
(*from http://anatomy-medicine.com/musculoskeletal-system/11-osteology-osteologia-the-science-of-the-bones.html)*

The bone tissue and the extracellular matrix are characterizes by organic components (30-35%) and extra organic or minerals components (65-70%).

The bone is considered an organic composite material, because it consists of two different phases, which contribute, with its own characteristics, to generating the properties of the resulting integrated system.

The lamellar bone is a composite material, constituted by fibers of collagen and crystals apatite; here the collagen fibrils have a diameter of about 60 nm and form a felt homogeneous; It constitutes almost the totality of compact bone and most of cancellous bone. It is characterized by the ordinate arrangement of the collagen fibrils, which are arranged in layers, called bone lamellae.

The collagen fibrils are aggregated to form collagen fibers with a diameter of 5-10 pm; it is the first layer to be deposited, both during the physiological development that in the repair of fractures.

The bone mechanical properties depend on the composition, structure, bonding between the constituents. The hydroxyapatite, which is the "stiff" component, prevents the yielding of collagen, while the collagen, which is the "soft" component prevents the brittle fracture of apatite.

Collagen fibers are responsible of the flexibility of bone, while the inorganic fraction is associated to their hardness.

In addition to collagen and proteoglycans, among the organic components are present some non-collagenous proteins, cytokines and growth factors. The most abundant element is

collagen type I, which is organized into fibers, which act as support (matrix) for the sedimentation of salts during the process of mineralization. Other protein components (osteocalcin, osteonectin, osteopontin) have the function of modulating this process of formation, mineralization and adhesion between the cells and the bone matrix.

Collagen fibers are aligned on a regular basis, giving origin to an organic matrix known as osteon, conferring a remarkable resistance and compactness to the bones

Among the inorganic components, there are minerals like calcium, phosphorus, fluorine and magnesium, which give the characteristic bone hardness.

Calcium is found as di-calcium phosphate, deposited in the form of hydroxyapatite crystals similar and anchored on a fibrous support of collagen. In the human body, the bones differ in size and shape, covering different functions. Therefore the bone is classified:

- Long bones, when the length prevails over the other dimensions;
- Bones flat or wide, when width or length prevail over thickness;
- Bones short, when the three dimensions are almost equal.
- Sesamoid bones are bones embedded in tendons. Since they act to hold the tendon further away from the joint, the angle of the tendon is increased and thus the leverage of the muscle is increased.
- Irregular bones do not fit into the above categories. They consist of thin layers of compact bone surrounding a spongy interior. As implied by the name, their shapes are irregular and complicated. Often this irregular shape is due to their many centers of ossification or because they contain bony sinuses. The bones of the spine, pelvis, and some bones of the skull are irregular bones.

At the macroscopic level, all bones are composed of two types of osseous tissue: cortical, or compact bone, and cancellous or trabecular bone.

Cortical bone forms the outer shell, or cortex, of the bone and it haa a dense structure similar to that of ivory.

Cancellous bone within this shell is composed of thin plates, or trabeculae, in a loose mesh structure; the interstices between the trabeculae are filles with red marrow. Cancellous bone tissue is arranged in concentric lacunae-containing lamellae but does not contain haversian canal.

On a microscopic level, bone consists of woven and lamellar bone. Woven bone is considered immature bone, found in the embryo, in the newborn and in metaphysical

region of growing bone as well as in tumors, osteogenesis imperfecta and pagetic bone. Lamellar bone begins to form one month after birth and it is secondary bone created by remodeling of woven bone. Lamellar bone has a regular parallel alignment of collagen into sheets (lamellae) and is mechanically strong. It is highly organized in concentric sheets with a much lower proportion of osteocytes to surrounding tissue. Lamellar bone is stronger and filled with many collagen fibers parallel to other fibers in the same layer (osteons). In cross-section, the fibers run in opposite directions in alternating layers. This kind of structural arrangement assists in the bone's ability to resist torsion forces.

The primary responsibility of the skeleton is to provide structural support for the body. In this role, the skeleton is the basis of posture, opposes muscular contraction conveying locomotion, withstands the daily challenges of load bearing, and protects internal organs. The structural success of the skeleton, is determined both by genetic determinants of bone architecture and the tissue's sensitivity to mechanical signals.

The quality of the tissue is as important to bone structure as quantity [4]; connectivity and orientation of trabeculae, as well as cortical bone's resistance to microdamage, helps define a bone's resistance to failure independent of a need to increase bone mass.

The success of the skeleton as a structure is jeopardized by genetic disorders such as osteogenesis imperfecta, metabolic diseases such as osteoporosis, the bone loss that parallels aging or menopause. Importantly, any decrease in the structural quality of the skeleton is accompanied by a proportional increase in skeletal fragility, whereas even subtle improvements in bone quality can result in significant reductions in fracture occurrence.

Whereas the great majority of therapies for bone disease work through modulation (inhibition or promotion) of a specific bone cell function, the success of any particular intervention is evaluated as a biomechanical outcome—the ability to reduce the incidence of fractures. In summary, to appreciate the structural consequences of bone and mineral disorders and to understand how new interventions may ameliorate the impact of bone loss or reduced bone quality, it is essential to understand how the mechanical parameters responsible for the skeleton's structural success, how metabolic conditions and/or aging compromise these features, and how mechanical signals influence those cells responsible for achieving and maintaining the mechanical integrity of bone tissue.

1.2. Bone as a composit material

Bone's composite structure allows it to withstand compressive and tensile loads, as well as bending and torsional moments to such a degree that its overall strength approaches that of cast iron while remaining, essentially, as light as wood [5]

The inorganic phase of bone, with hydroxyapatite crystals arrayed in a protein matrix, provides the ability to resist compression. Individual calcium phosphate crystals of multiple sizes are imbedded in and around the fibrils of the collagen type I [6] . Hydroxyapatite crystals, while effectively resisting compressive loads, have a poor ability to withstand tensile loads. As in concrete, a material that excels at resisting compression but is poor in resisting tension, tensile elements (e.g., steel reinforcing rods) are added to create a composite material that can cope with complex loading environments. In the case of bone, this tensile strength arises from collagen fibrils organized into lamellae (figure 1.2).

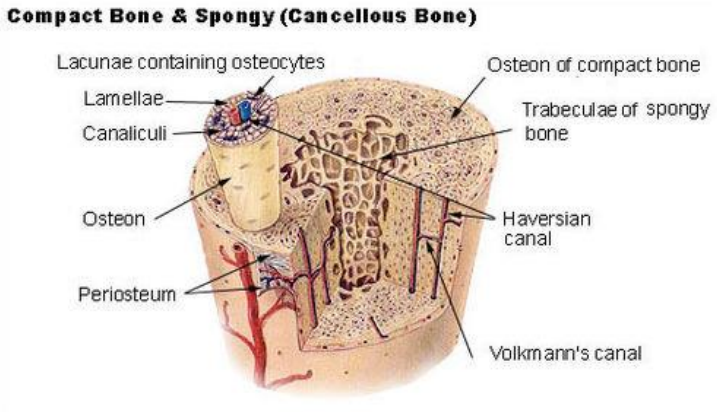


Figure 1.2 Compact bone and spongy (cancellous) bone
(from http://onceinawhale.com/2013/06/03/down-to-the-bone/).

The collagen orientation between adjacent lamellae can rotate by as much as 90°, permitting the tissue to resist forces and moments acting from several different directions, much like the added strength in plywood realized by the distinct orientation of the fibers. While the ultrastructural organization of the skeleton is defined by the genome, the functional environment also contributes to the organization and distribution of lamellae as well the osteons that house them [7].

Given that 80% of functional strains are caused by bending (and thus a high percentage of strain is tensile), the structural quality of the bone may ultimately be determined by the quality of the collagen and the organization of the microarchitecture. Thus, the age-related

deterioration of collagen would directly contribute to the declining material properties of the skeleton, just as a compromised inorganic phase of bone, which might occur under conditions such as rickets, may compromise the structural strength of bone.

Alterations in either the organic (collagen) or inorganic (hydroxyapatite) matrix components can bring about changes in bone strength. Mutations in the collagen gene give rise to several genetic skeletal problems, some of which increase fracture risk.

In some forms of osteogenesis imperfecta, mutations in the primary structure of type I procollagen lead to brittle bone [8] Another disorder of collagen resulting in excessively fragile bone is fibrogenesis imperfecta ossium(15); a rare disease where remodeling results in a disorganized, collagen-deficient tissue. While the number of hydroxyapatite crystals contributes to the ability to resist compression, density is not everything. Fluoroapatite, which incorporates into the mineral phase of bone during fluoride poisoning, is denser than hydroxyapatite, but is brittle and shatters easily under load [9]

1.3. Orthopaedic biomaterials and implants : main design requirements

Biomaterials are defined as materials used in a device intended to interact with biological systems. A biomaterial is a material that interacts with human tissue and body fluids to treat, improve, or replace anatomical elements of the human body. Biomaterial devices used in orthopaedics are commonly called implants; these are manufactured for a great number of orthopaedic applications. Biological materials such as human bone allografts (transplants of tissue between genetically different individuals) are considered to be biomaterials because they are used in many cases in orthopaedic surgery.

The history of biomaterials is related to the history and development of new healing techniques; new materials and biomaterials were introduced to answer to specific needs related to the development of individual medical devices.

Until the '50s -' 60s the development of new devices and materials was prerogative of the only surgeon or doctor; subsequently, the development of biomaterials and devices had involved engineers, chemists and materials experts. Still later the clinicians and engineers have been joined biologists, and biochemists, geneticists, chemical and materialists , and experts in nanotechnology and nanomedicine.

In orthopaedic field, it's possible to affirm that the first prosthetic replacement probably dates from 1881, when the German orthopedic Theodore Gluk used without success a ball

of ivory cemented. Smith-Petersen in 1925 tried to use a ball glass, but it proved too brittle. In 1938 Robert and Jean Judet resorted to the use of acrylic resins, which, however, they tended to wear out quickly. In 1956 McKee-Farrar and Watson developed the first total prosthesis, with the use of an acetabular cup made of metal alloy (CrCo) cemented to the pelvis.

Sir John Charnley in the early 60s designed the prosthesis with low friction at the Center for Hip Surgery at Wrightington (England). New concept of joint replacement:

- Stem and femoral head cobalt alloy
- Articulation of low diameter, low friction with the acetabular cup made of UHMWPE
- PMMA bone cement, to fix the prosthesis to the structure of the femur and pelvis.

During the past decades, medical devices have gained growing importance due to considerable medical and technical progress [10, 11] . In particular, biocompatibility, functionality, and durability of various implants have been considerably improved. Therefore, many implanted devices can properly function for decades.

The introduction of implantable devices represents a major advance of modern human medicine. Implants are used to improve impaired function, replace missing anatomic structure, or optimize physical appearance [10]. They significantly improve the quality of life in children (e.g., neurosurgical shunts, spinal implants for correction of scoliosis), adults (e.g., dental implants, arti fi cial heart valves, breast prostheses) and in the elderly (joint prostheses, fracture- fi xation devices, cardiac pacemakers).

Medical devices are made out of abiotic materials such as metals, polymers, ceramic but may also contain biological materials such as devitalized bone, blood vessels, muscle fascia from autologous (venous bypass), allogeneic (processed bone), or xenogeneic sources (e.g., porcine heart valve). Regardless of the type of material (synthetic or devitalized biological origin), it represents foreign material, and is therefore labeled as a biomaterial. Thus, the affix “bio” does not refer to a biological origin, but to the fact that “foreign” material is implanted in a living organism.

Interestingly, neither synthetic nor devitalized biological devices are rejected by the body, despite the fact that the host reacts to such implants in different ways depending upon the biocompatibility of the device [12, 13] . However, no implant is completely inert after implantation.

Medical prostheses can be used either as extracorporeal devices (e.g., limb prosthesis after amputation), intracorporeal devices (e.g., vascular prosthesis), or as implants that cross the anatomic cutaneous or mucosal barriers (e.g., dental implants).

Intracorporeal implants can be classified according to their localization as intravascular or extravascular devices; the two different types of devices interact very differently with the host. Whereas intravascular implants mainly interact with coagulation factors and circulating blood cells, extravascular implants interact with surrounding tissue, interstitial fluid and attracted phagocytes.

Permanent implants cannot be removed without compromising the replaced function. Therefore, the primary goal is to prevent implant failure due to mechanical reasons or infection. In this thesis the discussion is limited to joint prostheses.

The main fundamental requirements that orthopaedic devices must fulfill in order to function adequately, are summarized in this section.

First of all, during a discussion related to the choice of the biomaterial, it's important to define the concept of biocompatibility: biocompatibility is the primary characteristic that a medical device should have in any orthopaedic application; that is, it must not adversely affect the local and systemic host environment of interaction (bone, soft tissues, ionic composition of plasma, as well as intra- and extracellular fluids).

Orthopaedic devices are extravascular devices: they are localized in different compartments of the host and have no direct interaction with the circulating blood. Foreign materials such as bone grafts (devitalized bone) and bone substitutes (e.g., calcium phosphates) are often used in orthopaedic surgery for filling bone defects [14] . Despite the fact that joint replacement is a so-called clean procedure [15] , implant-associated infections are observed in 0.5–1 % (hip or knee arthroplasty) to >5 % (elbow or ankle arthroplasty) of primary implant surgeries [16, 17] . In general, all types of revision surgery increase the risk for wound infection due to less favorable soft tissue conditions, more extensive tissue damage, and longer operation times. In addition to intraoperative infections, implanted devices are at life-long risk of hematogenous or lymphogenous seeding during bacteremia or local skin infection.

When a joint is irreparably damaged owing to trauma or disease, it's necessary to supplement its function using an artificial joint .

Hip and knee are joints involved in the prosthesis field ; this kind of implants are discussed in the thesis, pointing the attention on the biomaterials.

The components of the artificial hip and knee are presented in figure 1.3 a-b.

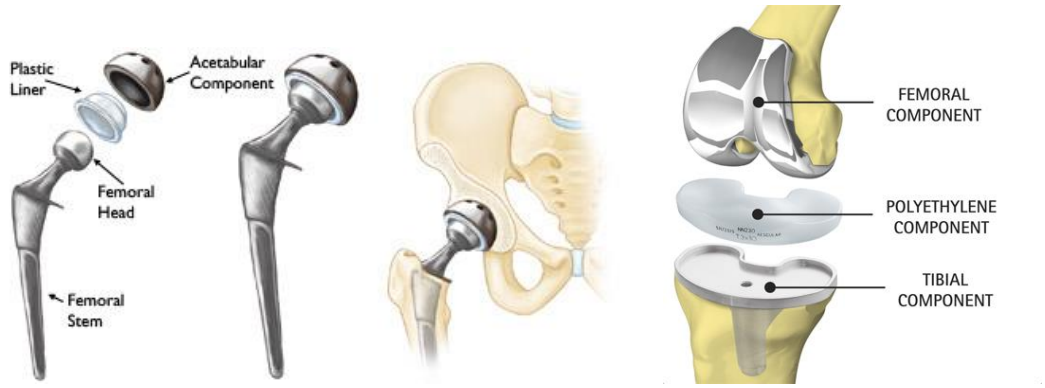


Figure 1.3

a. Components of artificial hip (Matthew Osso, 2014, Hip Replacements)

b. Components of artificial knee (*from https://www.soactivesofast.com/knee/types-of-knee-implants)*

It is important for orthopaedic surgeons to understand the nature of biomaterials, their structural configurations, and their properties, as well as the effects of their interaction with soft and hard tissues, blood, and intra- and extracellular fluids of the human body. The orthopaedics field has benefited from the great efforts of many orthopaedic surgeons, experimental surgery laboratories, and research centers and from research work at universities, academies, societies, scientific organizations, and many interdisciplinary groups. However, many challenges remain to be conquered in the development of new biomaterials that will improve the long-term performance of clinical results in orthopaedic surgery. The main biomaterials used in orthopaedic surgery are divided into two groups: metals and nonmetals.

The use of metals in therapeutic procedures dates back several centuries.

Metallic biomaterials have their main applications in load-bearing systems such as hip and knee prostheses and for the fixation of internal and external bone fractures. It is very important to know the physical and chemical properties of the different metallic materials used in orthopaedic surgery, as well as their interaction with the host tissue of the human body.

The metallic implants most widely used in orthopaedic surgery are:

• Low carbon grade austenitic stainless steels: 316L

• Titanium and titanium-base alloys: commercially pure titanium (CP Ti), Ti-6Al-4V, and other titanium-base alloys

• Cobalt alloys: Co-Cr-Mo, and other cobalt-base alloys

Three main subgroups make up the category of non metal: polymers, ceramics, and composites.

Polymers are organic materials that form large chains made up of many repeating units. Polymers are extensively used in joint replacement components. Currently the polymers most widely used in joint replacements are:

• Ultrahigh molecular weight polyethylene (UHMWPE)

• Acrylic bone cements

• Thermoplastic polyether ether ketone (PEEK)

• Bioabsorbables

Ceramics are polycrystalline materials. The great majority are compounds made up of metallic as well as nonmetallic elements; they generally have ionic bonds or ionic with some covalent bonds. The main characteristics of ceramic materials are hardness and brittleness. They work mainly on compression forces; on tension forces, their behavior is poor. The main ceramics in orthopaedic surgery and their applications are:

• Alumina, Al_2O_3, used for acetabular and femoral components

• Zirconia, ZrO_2, used for acetabular and femoral components

• Hydroxyapatite, used for coating stem femoral components to integrate the surface material to the bone.

Composite biomaterials are made with a filler (reinforcement) addition to a matrix material in order to obtain properties that improve every one of the components. This means that the composite materials may have several phases. Some matrix materials may be combined with different types of fillers. Polymers containing particulate fillers are known as particulate composites. The following composites are considered in the orthopaedic devices:

• Fiber-reinforced polymers

• Aggregates to polymethyl methacrylate (PMMA)

Bone Allografts Bone allografts are also commonly used as implants in orthopaedic surgery. They are procured using aseptic techniques and are preserved according to their storage needs:

• Freeze dried/lyophilized: tissue dehydrated for storage by changing the water content of frozen tissue to a gaseous state in a vacuum that extracts moisture

• Fresh: bone allograft stored for a maximum of 1 week at a temperature of 4 °C

• Frozen: bone allograft stored for up to 5 years at a temperature of 70 or 80 °C

• Cryopreserved: tissue frozen with the addition of, or placed in a solution containing, a cryoprotectant agent such as glycerol or dimethylsulfoxide

• Demineralized: demineralized bone matrix that is osteoconductive and is mainly used for fi lling bone and/or cavitary defects, not used for structural purposes 1

Joint Replacements Prosthetic devices are implanted in the human body to replace the affected joint in order to eliminate pain and restore its normal function.

It is well known that femoral stem joint replacements have a mean useful life which, among other factors, is intimately linked to wear particles.

In the first half of the 20th century, a total hip replacement was designed and used in patients; however, the initial results were not completely satisfactory. The main concerns at that time, besides the implant design, were the surface bearing materials of the metal-on-metal and the metal-on-polymer femoral-acetabular component types. Also, methods for implant fixation (cemented and cementless femoral stem components) needed to be established. Sir John Charnley did not use the metal-on-metal femoral acetabular component because of frictional torque in the bearing of metallic surfaces. In 1962, he found a high-density polyethylene to be a more adequate bearing surface. For the femoral stem fixation.

In 1979, Carl Zweymüller, M.D., started to use a cementless tapered titanium femoral stem. A great number of total hip replacements, cemented and cementless prosthetic devices, have been developed since the relevant early design of Dr. Charnley's total hip replacement prosthetic device.

The femoral-acetabular component types currently used are: • Metal-on-polyethylene • Metal-on-metal • Ceramic-on-polyethylene • Ceramic-on-ceramic Currently, persistent problems remain to be solved with total hip replacements, including implant wear, aseptic loosening, and osteolysis.

Regarding the knee joint replacement there are two types of this device: total and unicondylar, which is usually called half replacement. It is recommended when half of the damaged joint is to be replaced. The implant biomaterials used in total knee replacements are titaniumbase alloys, cobalt-chromium alloys, ceramics, and cross-linked ultrahigh molecular weight polyethylene.

The improvements on implant materials and manufacturing processes have made great contributions to the longterm performance of these prosthetic devices, but at present, strong requirements in orthopaedics are still to be met, both in bone and joint substitution, and in the repair and regeneration of bone defects.

In this framework, tremendous advances in the biomaterials field have been made in the last 50 years where materials intended for biomedical purposes have evolved through three different generations, namely first generation (bioinert materials), second generation (bioactive and biodegradable materials) and third generation (materials designed to stimulate specific responses at the molecular level).

Orthopaedic biomaterials are meant to be implanted in the human body as constituents of devices that are designed to perform certain biological functions by substituting or repairing different tissues such as bone, cartilage or ligaments and tendons, and even by guiding bone repair when necessary.

During most of the twentieth century, the availability of materials for the elaboration of implants was the same as for other industrial applications. Indeed, pioneer surgeons designed their implants using materials available and with a successful record of industrial use such as in chemistry, energy, mechanical and aerospace. Since the human body consists of a highly corrosive environment, very stringent requirements are imposed on the candidate materials' properties. Consequently, the first generation of biomaterials consisted of easily available materials of industrial use, that were required to be as inert as possible in order to reduce their corrosion and their release of ions and particles after implantation. Mechanical properties also play a leading role in the selection of candidate materials for implant manufacture. The concept of biocompatibility, associated with a set of *in vitro* and *in vivo* standardized tests, was introduced in order to assess the biological behaviour of synthetic materials.

When trying to understand the evolution of biomaterials research and their clinical availability during the last 60 years, three different generations seem to be clearly marked:

bioinert materials (first generation), bioactive and biodegradable materials (second generation), and materials designed to stimulate specific cellular responses at the molecular level (third generation).

These three generations should not be interpreted as chronological, but conceptual, since each generation represents an evolution on the requirements and properties of the materials involved. This means that at present, research and development is still devoted to biomaterials that, according to their properties, could be considered to be of the first or the second generation. The materials that each new generation brings in do not necessarily override the use of those of a previous one.

This evolutionary perspective, may provide a clearer insight into how biomaterials research and evolution set up the ground for the design and development of innovative devices for improved solutions to orthopaedic clinical problems. Third-generation materials will open new possibilities of treatments and applications, but they are not meant to substitute plainly the materials from previous generations.

The third-generation biomaterials are meant to be new materials that are able to stimulate specific cellular responses at the molecular level. For these biomaterials, the bioactivity and biodegradability concepts are combined, and bioabsorbable materials become bioactive and vice versa. These material's properties should merge with their ability to signal and stimulate specific cellular activity and behaviour. Temporary three-dimensional porous structures that stimulate cells' invasion, attachment and proliferation, as well as functionalized surfaces with peptide sequences that mimic the ECM components so as to trigger specific cell responses are being developed.

The third generation of biomaterials appeared approximately at the same time as scaffolds for tissue engineering applications started to be developed. Tissue engineering emergence is boosted as an alternative potential solution to tissue transplantation and grafting. The use of allografts, autografts and xenografts presents several limitations, namely donor site scarcity, rejection, diseases transfer, harvesting costs and post-operative morbidity .

Tissue engineering and regenerative medicine are recent research areas exploring how to repair and regenerate organs and tissues using the natural signalling pathways and components such as stem cells, growth factors and peptide sequences among others, in combination with synthetic scaffolds .

In addition to the combination of the basic tissue engineering triad (cells, signalling and scaffold), there are some processes such as angiogenesis and nutrients delivery that are crucial to stimulate tissue regeneration and must take place right after implantation.

Although tissue engineering has emerged as a very brilliant alternative to overcome many existing problems related to the current use of autografts, allografts and xenografts, its implementation as part of a routine treatment for tissue replacement is controversial. At present the angiogenesis problem has not been solved. Besides, tissue engineering involves cells' manipulation which is not a simple and straightforward issue, and represents a main drawback for the generalized use of this technique in a hospital. In spite of that, tissue engineering is a very promising strategy that opens numerous possibilities of study and research in the field of regenerative medicine.

Tissue engineering is a multi- and interdisciplinary field that involves the complementary effort of the engineering, chemistry, physics and biology fields. Engineers, chemists and physicists will have to focus on the improvement and development of new materials and processing technologies, new surface treatments and characterization techniques, bioreactors and cell seeding methods. From the biology side, new cell sources will have to be found as well as new isolation and expansion methodologies. Furthermore, new biomolecules such as growth factors and peptides involved in cell differentiation, angiogenesis and tissue formation processes will have to be developed. Finally, surgeons will have to enhance and develop new surgical procedures, probably minimally invasive, in order to overcome present limitations.

The combination of bioactivity and biodegradability is probably the most relevant characteristics that encompass third-generation biomaterials. The bioactivation of surfaces with specific biomolecules is a powerful tool that allows cell guidance and stimulation towards a particular response. The aim is to mimic the ECM environment and function in the developed scaffold by coupling specific cues in its surface. Thus, cell behaviour including adhesion, migration, proliferation and differentiation into a particular lineage will be influenced by the biomolecules attached to the material surface.

Metals have also been used in the development of porous structures for bone tissue engineering. The development of porous metallic scaffolds (metallic foams) for bone tissue engineering and drug delivery applications has mainly been focused on titanium and titanium alloys .

Despite the numerous studies on the manufacture and design of metallic foams works dealing with the *in vitro* or *in vivo* behaviour of this type of materials are still scarce. Titanium fibre meshes (86% porosity and a 250 μm average pore size) have been used for the *ex vivo* culture of rat bone marrow stromal cells, under static and dynamic conditions (flow perfusion bioreactor), and subsequent implantation in cranial defects in rats.

Titanium foams tested *in vitro* with human osteoblasts have shown osteoblast colonization and differentiation into mature bone cells, and consequently could be appropriate materials for spine fusion and other applications. Porous tantalum is also being successfully used clinically in several orthopaedic applications. Its high volumetric porosity, low elastic modulus (it can be as low as 3 GPa) and good frictional characteristics make tantalum foam an ideal candidate for weight-bearing applications such as total joint arthroplasty.

Moreover, tantalum has an excellent *in vivo* biocompatibility and can become bioactive via a simple chemical treatment.

The main concerns with these unresorbable scaffolds are related to their permanent implantation in the body that can trigger risks of toxicity caused by the accumulation of metal ions due to corrosion, the premature failure due to poor wear properties, and the higher elastic modulus compared with bone, which leads to heterogeneous stress distributions.

Bioresorbable foams of magnesium have been developed as a new alternative for bone graft substitutes. By adjusting their porosity, an elastic modulus similar to that of cancellous bone can be achieved. In addition, magnesium scaffolds have shown good osteoinductive properties.

Research on shape memory metallic foams is currently carried out, especially with NiTi alloys, so as to reduce stress shielding and increase the wear resistance of conventional porous titanium scaffolds.

All these metallic materials belong to the first generation. The innovation lies in the porosity, although a fibrous layer between the metal and the ECM growth inside the pores should be expected. Thus, as mentioned above, when discussing strategies for improving metal bioactivity, a proper treatment of the material surface may help to avoid this problem and create a direct bonding with the tissue.

Since cell adhesion is mediated by protein–cell interactions via biological recognition of protein sequences by transmembrane cell receptors, with specific sequences that enhance

cell attachment, the design of biomaterials is now focused on biological stimulation. Some of the most commonly employed strategies are the functionalization of biomaterial surfaces using proteins and peptides that mimic the ECM chemistry, in particular RGD sequences and the addition of growth factors into the scaffold composition for a controlled delivery to the surrounding cells.

Surface bioactivation can be achieved by functionalizing surfaces with different biomolecules by applying a variety of methods where both chemical bonding and physical adsorption take place. Some of these methods are an update and evolution of some of the methods already explored at the end of the second generation of biomaterials. During the third generation, dip-coating techniques are still used, but some more sophisticated 'bottom-up' and 'top-down' techniques are also developed to engineer surfaces with high specificity levels. Additionally, the synthesis and tailoring of new biomolecules for specific applications occur during this third generation. The development of more complex biopolymers and biomolecules such as elastin-like biopolymers including peptide sequences that induce mineralization and cell adhesion, or self-assembled amphiphilic peptides that include cell signalling cues could provide the answer.

Therefore, it is predictable that in the short and medium term, future trends in biomaterials will involve the active interaction between chemistry and biology and will be more and more focused on achieving their combination with biological entities in order to obtain a totally viable biological environment.

1.3.1. Metals: stainless steel, Ti and its alloys

Materials for bone replacement might mimic the architecture of the bone [18]. Most implants are metals: stainless steels, Co–Cr system alloys, and titanium alloys [19]. Actually, most of the metallic implants are produced with stainless steel (SS) because it has adequate bulk properties to be used as biomaterials for orthopedic implants and is less expensive than Ti and its alloys, but it is less biocompatible than them.

The Young's moduli of those biomaterials are much greater than that of cortical bone, then bone resorption occurs[19]. That is because when the bone is stressed, the bone-producing cells called osteoblasts are stimulated into generating more bone [20]. So if the bone is replaced by a metallic counterpart that is stiffer than the original bone, the replacement will

tend to bear a greater proportion of the load, shielding the surrounding skeleton from its normal stress levels.

The two most important criteria for the development of materials for implant applications are the presence of non-toxic elements and low modulus of elasticity. Low modulus Ti alloys for instance, can be developed by designing β-Ti alloys containing non-toxic alloying elements such as Nb, Zr, and Ta [21–23].

Therefore, in the choice of the material the elastic modulus is an important parameter, as a value close to that of the bone material leads to a better transfer of functional loads to the bone, enhancing the stimulation for new bone growth [24–26].

The surface characterization of implant materials is also a topic of main importance, since the surface plays a key role in the living tissue response to the metal presence; regarding wear, it is important that the implant surface has high hardness to prevent abrasion waste generated during functional loading to be released in the body.

In titanium and its alloys [27–30] it was found that both the topography and the chemical surface composition have a strong influence in the early stages of the osseointegration process. The surface–biological media interactions are a multiscale problem, with a broad range from a few microns, where the surface topography changes the effective contact area between the implant and the surrounding bone [27,28], to tens of nanometers, where the influence of chemical species present on the surface can modify the nucleation and growth of Ca–P compounds that precede the formation of hydroxyapatite [29,30].

To allow the formation of nanostructured thin films [31], in the biomedical field it's used a physical deposition technique called sputtering, that has been widely used in several industrial applications with great success.

This is a novel technique for which many potential applications are prompting extensive research [32–35]. Nanostructured surfaces are interesting when it comes to the bone/implant interface due to the fact that both the surface and the bone have nanoscale particle sizes and similar mechanical properties [36]. The high surface energy of nanostructured materials leads to desirable cellular responses since bone-forming cells generally attach themselves to surfaces with roughness in the nanometer range [36]. The combination of these characteristics causes an increase of fracture resistance and biocompatibility for the implants. In addition, the particles generated by nanostructured implants are not immunoreactive and therefore less harmful to the human body than the

microparticles of conventional implants. Stainless steel (SS) has adequate bulk properties to be used as biomaterials for orthopedic implants and is less expensive than Ti and its alloys, but it is less biocompatibility than them. For this reason, the coating of this SS implants with Ti alloy thin films by sputtering may be one alternative to improve the biomaterial properties at a relatively low cost: TiNbZr thin films can be deposited on both Si and stainless steel substrates: the TiNbZr/Si film is used as a model system, while the TiNbZr/SS film might improve the biocompatibility and extend the life time of stainless steel implants.

1.3.2. Biodegradable implants: magnesium alloys

The currently applied permanent metallic internal fixation devices have several negative aspects such as stress shielding, an inflammatory osteolysis caused by released toxic titanium particles , interference in radiological studies , and the need of a second surgery for implant removal.

In recent years, biodegradable biomaterials for medical use have gained interest and are intensively investigated; promising candidates are magnesium alloys. These biomaterials have to comply several requirements: (i) good biocompatibility and non-toxicity of degradation products, (ii) appropriate mechanical properties, and (iii) a moderate degradation rate adapted to the fracture healing process.

Although pure magnesium demonstrates generally suitable corrosion properties as an implant material for resorbable applications, it frequently possesses insufficient mechanical properties. One possibility of enhancing its mechanical properties is represented by the use of magnesium alloys, but it must be taken into consideration that such elements simultaneously modify the material's corrosion behavior, as rate and type of corrosion (figure 1.4).

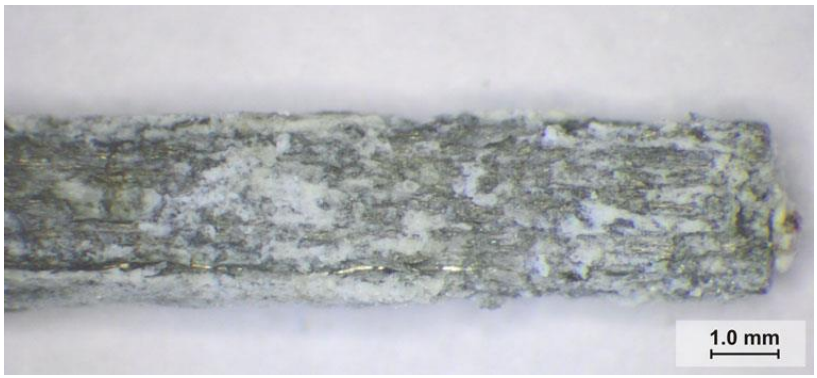


Figure 1.4 Pitting corrosion at the surface of an explanted magnesium alloy (stereo-microscopy) (H. Waizy, A. Weizbauer, F. Bach, B. Denkena, 2012, Biodegradable magnesium implants for prthopedic applications, p. 46)

Magnesium alloys were first developed as degradable metallic biomaterials for vascular and orthopedic applications. Despite the successful clinical application as degradable vascular stents, the development of Mg biomaterials as orthopedic implants is still hampered by unpredictable corrosion behavior and a limited understanding of the tissue response to Mg implants. The metal is lightweight with a density of 1.74 g/cm3, has a high strength to weight ratio, is thermally conductive, and can be easily cast. The propensity of Mg to corrode and its low elastic modulus prevented its continued use in a wide range of applications. However, it is precisely these characteristics, together with low relative density, which suggest Mg for application as a degradable metallic biomaterial for orthopedic applications.

The use of Mg would allow the production of lightweight implants with an elastic modulus much more comparable to bone than commonly used metallic materials such as titanium alloys and stainless steels. This feature, along with the corrosion of the implant, would reduce some of the pathological issues associated with the implantation of permanent metallic materials, such as the production of inflammatory wear particles and osteopaenia.

However, of the features mentioned above, the corrosion of Mg is of primary importance, as the function of a biomaterial is reliant on maintaining appropriate mechanical stability for specific periods of time. The rapid corrosion of Mg and Mg alloys has been the primary limitation in the use of these materials for a range of applications in which there is exposure to a corrosive environment. For this reason, the development of Mg-based materials for orthopaedic use is somewhat further behind: despite significant improvements

in the production of Mg since its historical application as a biomaterial, the commercially available pure metal cannot provide either the appropriate mechanical properties or corrosion resistance for application in a load bearing orthopedic environment.

Various techniques are currently being investigated to improve both these features, together with the biocompatibility and osseointegrative potential of Mg as a biomaterial. These primarily include alloying Mg, surface modification of the substrate, or various coating techniques.

Then there is significant promise in the field of biomaterials for the identification of Mg alloys that could be applied as orthopedic implants, as long research focuses on two factors: firstly, the development of a set of standardized protocols for both corrosion and biocompatibility assessment, that would allow the comparison of materials between experimental groups; secondly, it is imperative that the field encourages more extensive collaboration with clinicians, allowing the design and development of the materials at the earliest stages for specific clinical uses. Only then can the characteristics of a Mg-based biomaterial truly be tailored to a functional end point, rather than fitting an appropriate application to the characteristics of a material.

As a result of these considerations, it's possible to affirm that magnesium and magnesium alloys have been found to be excellent biomaterials for orthopedic applications. Magnesium degrades non-toxically in the body, thus allowing full bone regeneration in the implant site. Magnesium has also been shown to increase osteoconductivity in vivo compared with polymer rods.

Composites have been widely used for bone regeneration scaffolds and fixation devices, including organic/inorganic composites such as collagen/HA, and polymer/mineral composites such as PLGA/B-TCP and PLLA/HA . Magnesium salts have been embedded in PLGA microspheres and scaffolds, and were found to buffer the acidic pH degradation of the PLGA. This was found to increase the bioavailability of proteins and drugs encapsulated in the PLGA/Mg microspheres. Porous Mg/PLGA scaffolds could be synthesized to provide buffering of acidic PLGA by-products and long-term release of magnesium. These characteristics not only increase cell proliferation in vitro, but provide a safe and osteoconductive environment for bone regeneration in vivo. These findings show promise for the use of Mg/PLGA composite materials for a wide range of bone regeneration applications.

The development of bone substitute with controllable biodegradable properties and improved bone regeneration is a step toward personalized therapy that can adapt to patient needs and clinical situations.

1.3.3. Innovative composites of bioactive ceramics

The most widespread and consolidated ceramic material in orthopedic field is alumina (Al_2O_3) used to make heads in coupling with polyethylene, or for heads and cups in ceramic / ceramic. In 1980 has been created high purity alumina sintered in air, characterized by micron grain size, but it presented problems caused by frequent impingement and mobilization of the acetabulum.

In 1994 has been created a ceramic material of high purity alumina, in its α phase (better known as corundum), characterized by its particular structure and stability. The octahedron structure of corundum is formed to 2/3 from the cations of aluminum and for 1/3 from anions of oxygen.

The presence of oxygen octahedron creates strong ties that give alumina its characteristics of stability and biocompatibility. This material is composed of aluminum oxide and magnesium oxide (MgO) to control the growth of the grains during sintering (figure 1.5).

The processo of realization includes the following steps: preparation of the powder, pressing and turning, sintering, finish, inspection, washing and sterilization

Figure 1.5 Powder of aluminum oxide (Al_2O_3) with a small percentage of MgO
(A. Porporati, 2014, Ceramici per articolazioni)

The surface layer is composed of oxygen atoms that create a residual charge; this interacts with the polarized molecules of the lubricant binding it to the surface with the strong links of Van der Waals forces.

This ensures the presence of a fluid film that reduces the coefficient of friction between the two surfaces which constitute the articulation.

Even the zirconia has good characteristics in terms of fracture resistance, but in the production stage presents the risk of uncontrolled formation of porosity that can channel fluids.

Advantages of ceramic materials concerns chemical inertia, that are biocompatible and immune from corrosion; stability, as it does not degrade over time; stiffness; high abrasion resistance; hydrophilicity.

An innovative ceramic material in the orthopedic field, designed with the aim of raising the toughness, minimize the likelihood of fracture, keep the excellent resistance wear,keep the biocompatibility, and allow more flexibility in the forms, is a material based on alumina and zirconia, which couples the stability and the wear resistance of the first component, which constitutes about 75%, with good properties in terms of hardness and toughness of the second , presented in 25%. Any additives of chromium oxide or strontium oxide enhance the performance of the material . This materials presents best mechanical features mechanical compared with alumina, but it has similar wear rate in Ce / Ce coupling, it is biocompatible and it presents more flexibility.

a. Hip ceramic components **b.** Knee ceramic components

Figure 1.6 (A.Porporati, 2014, Ceramici per articolazioni)

1.3.4. Nanofluorapatite polymer-based composite

Hydroxyapatite (HA) biomedical materials have attracted extensive attention over the past decades because of the similarity in chemical composition and crystallographic structure to those of hard tissues (such as tooth and bone) in humans. Fluoroapatite (FA), was recognized as a promising biomedical material for bone implants because of its structural similarity to HA, and FA has the additional benefit of fluorine release. Similar to HA, FA itself cannot be used for heavy load-bearing applications for bone substitutes due to its poor mechanical properties. Thus, most studies were focused on the mechanical or biological properties of FA in the form of coating, which is stable and does not exhibit signs of dissolution and degradability; FA can develop chemical bonds with bone tissue, which is stronger than bone or bioceramics alone [37–39].

.The low solubility of FA is beneficial to improve osseointegration at implant–bone interface, which is considered a promising biomedical material for bone fixation.

This composite was prepared by cosolution method to improve the mechanical properties (such as compressive strength and elastic modulus) and hydrophilicity; it has good antibacterial properties, which could meet the needs of antibacterial implanted materials, preventing bacterial infections. The proliferation are signifcantly high indicating that composite could promote cell proliferation and differentiation. The histological evaluation results confirmed that it could stimulate new bone formation, it has good mechanical, hydrophilic, antibacterial properties, good biocompatibility and osteogenesis. For these reasons, it is expected that the composite as orthopedic implants and prostheses might be suitable for application in orthopedic surgery.

1.4. Hip joints as artificial implants: the choice of the biomaterial

A detailed understanding of the complex anatomy and biomechanics of the hip in conjunction with a focused physical examination, diagnostic injection, and appropriate radiographic studies can help the orthopaedic surgeon to successfully diagnose and treat complex pathologies of the hip. General anatomy of the hip is shown in figure 1.7.

This kind of analysis it's a base support to the bioengineering researches related to the hip implants.

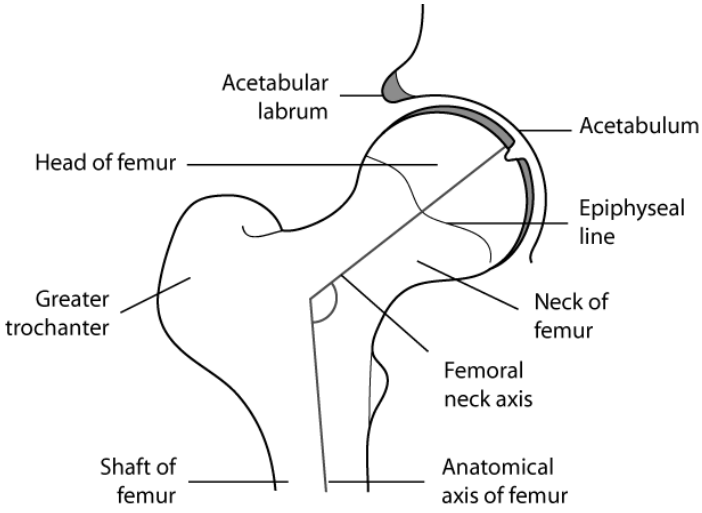


Figure 1.7 Cross-sectional view of the normal hip joint.
(Tom Goom, 2012, Femoro-acetabular Impingement)

1.4.1. The design of the device

A typical hip prosthesis is constituted by :

-an acetabular shell, which contains the acetabular cup and which is rigidly fixed to the bone of the pelvis;

-acetabular cup. In it the femoral head rotates, allowing the articulation of the prosthesis;

-femoral head, rigidly bound to stem means of conical coupling;

-stem, rigidly fixed to the inside of the channel diaphyseal femoral.

The acetabular shells wrap the acetabular cup (figure 1.8), and they are rigidly connected to the bone of the pelvis.

They can be cemented or uncemented. Some cups in UHMWPE or in ceramic, are directly cemented to the pelvis . In some metal cups, a layer of UHMWPE is interposed between two metal shells, to decrease the stiffness of the metal / metal or ceramic / ceramic coupling.

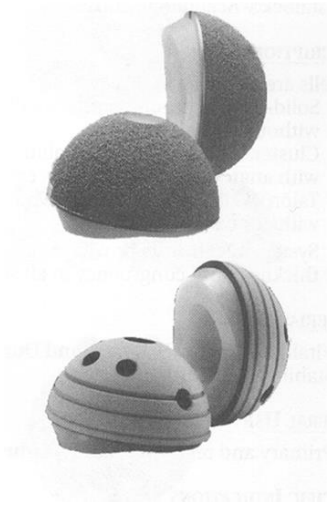


Figure 1.8. Acetabular cup or metal back
(R. Chiesa, 2014, Protesi ortopediche e materiali metallici)

The current state of materials systems used in total hip replacement is represented by metals, polymers, ceramics and composites.

In this section, the merits and demerits of these material systems are evaluated.

Many synthetic materials are used in the medicine for a variety of applications ranging from total replacement of hard or soft tissues (such as bone plates, pins, total joint replacement, dental implants, intra-ocular lenses), repair, diagnostic or corrective devices (such as pacemakers, catheters, heart valves). The two primary issues in materials science of new bone biomaterials are mechanical properties and biocompatibility. Although mechanical properties of biomaterials have been well characterized, the term bio compatibility is only a qualitative description of how the body tissues interact with the biomaterial within some expectations of certain implantation purpose and site [40]. The average load on a hip joint is estimated to be up to three times body weight and the peak load during other strenuous activities such as jumping can be as high as 10 times body weight. In addition hip bones are subjected to cyclic loading as high as 106 cycles in 1 year [41]. Materials scientists have investigated metals, ceramics, polymers and composites as biomaterials.

The general criteria for materials selection for bone implant materials are:

• It is highly biocompatible and does not cause an inflammatory or toxic response beyond an acceptable tolerable level

• It has appropriate mechanical properties, closest to bone

• Manufacturing and processing methods are economically viable.

Ideally, a bone implant such as a hip implant should be such that it exhibits an identical response to loading as real bone and is also biocompatible with existing tissue. These materials are also classified as bioactive (illicit a favorable response from tissue and bond well), bioinert and biodegradable.

The purpose of this section is to provide an overview of the various material systems currently being investigated as potential components of the total hip replacement (THR) implants. Replacement of joints such as a THR is a serious health concern.

The articulation of a human hip is simulated with the use of two components, a cup type and a long femoral type element. A typical hip implant fabricated from titanium is shown in Fig. 1. The head of the femoral element fits inside the cup to enable the articulation of human joint.

These two parts of the hip implant have been made using a variety of materials such as metals, ceramics, polymers and composites.

Typically polymeric materials alone tend to be too weak to be suitable for meeting the requirement of stress deformation responses in the THR components.

Metals typically have good mechanical properties but show poor biocompatibility, cause stress shielding and release of dangerous metal ions causing eventual failure and removal of implant.

Ceramics generally have good biocompatibility but poor fracture toughness and tend to be brittle.

Composite materials with engineered interfaces resulting in combination of biocompatibility, mechanical strength and toughness, is the focus of many current studies.

Total joint replacements generally involve implantation components held in place by a cement. Loosening of the components often occurs at the interface between the cement and bone due to failure of the fixation of the cement to the bone. Although deeper penetration of the cement into the interstices of cancellous bone should improve the mechanical interlock, subsequent bone resorption often results due to the modulus mismatch between cancellous bone and cement.

The bone cement interface is highly dynamic with degradation of the polymer in the cement and bone ingrowth. The nature of this interface is specific to the materials used in

implants. The following sections evaluate the different materials systems used in orthopaedic applications for total hip replacement.

1.4.2. The choice of the biomaterials

Metals and bioactive coating in total hip replacement

Metals have been the primary materials in the past for this purpose due to their superior mechanical properties [42], albeit dangerous ions that are released in vivo from these alloys. Originally femoral components of the THR were made of stainless steel that was replaced by a cobalt-chromium-molybdenum alloy [43,44].

Metallurgical heat treatments and resulting microstructures guide the resulting mechanical properties in metallic implant materials [45]. Most commonly, the long femoral element is made of stainless steel, Co–Cr alloys, or Ti alloys, and the cup component is made up of alumina or zirconia ceramic, polytertrafluoro ethylene (PTFE) or Co–Cr alloy.

The commercial metallic THR implants are five to six times stiffer than bone and result in significant problems associated with stress shielding. Ti alloys in the femoral elements of the THR have shown improvement in wear properties. The regenerative and remodeling processes in bone are directly triggered by loading. Thus, the effect of a much stiffer bone implant is to reduce the loading on bone resulting in the phenomenon called as stress shielding. The key problems associated with the use of these metallic femoral stems are thus release of dangerous particles from wear debris, detrimental effect on the bone remodeling process due to stress shielding and also loosening of the implant tissue interface. It has been shown that the degree of stress shielding is directly related to the difference in stiffness of bone and implant material [46, 47].

Titanium alloys are favourable materials for orthopedic implants due to their good mechanical properties. However, titanium does not bond directly to bone resulting in loosening of the implant. Undesirable movements at the implant-tissue interface results in failure cracks of the implant.

One approach to improving implant lifetime is to coat the metal surface with a bioactive material that can promote the formation and adhesion of hydroxyapatite, the inorganic component of natural bone. The application of bioactive coatings to titanium-based alloys enhance the adhesion of Ti-based implants to the existing bone, resulting in significantly better implant lifetimes than can be achieved with materials in use today.

Typically, several silicate glasses are used as bioactive coatings. An ideal bioactive coating would bond tightly both to the bone and the metal. Some ceramic coatings are known to be bioactive and have been tested on Ti implants. However, two problems arise when attempting to coat metals with ceramics. For one, the thermal expansion coefficients of the ceramic and metal are usually different, and as a result, large thermal stresses are generated during processing. These stresses lead to cracks at the interface and compromise coating adhesion. In addition, chemical reactions between the ceramic and metal can weaken the metal in the vicinity of the interface, reducing the strength of thecoated system. This problem is particularly important when coating Ti alloys, due to their high reactivity with most oxide materials. Since the modulus of the Ti alloys is lower than that of the Co–Cr–Mo alloys, they have been more suitable for THR components. The elastic moduli of the Ti alloys have been engineered to be more suitable by heat treatments resulting in microstructures that have a reduced elastic modulus.

The fundamental wear mechanisms of the Ti alloys is still not well understood. Bioglass coatings on Ti implants further improves the biocompatibility of these implants. The glasses are based on mixtures of the oxides of silicon, sodium, potassium, calcium, and magnesium. By adjusting the stoichiometry of the bioglass coating, the thermal expansion coefficient of the glass is made to match that of the Ti alloy, avoiding the generation of thermal stresses.

Also, the glasses become soft at the processing temperature, which is well below the melting point of the Ti alloy. Thus, they flow to uniformly coat the Ti surface. These coatings develop a layer of HAP on their outer surface upon exposure to simulated body fluid [48].

Metallic femoral head articulating inside a polymeric (PTFE or UHMWPE) acetabular cup has been one of the most favorable THR element structure [49,50]. Clinical results show that excessive wear and wear debris is the primary cause of failure of UHMWPE or metal implants. Thus, the use of materials with lower modulus and strength such as polymers appear to be more useful for use as bone biomaterials.

Polymers

For orthopedic applications in THR, polymers of very high strength and stiffness are required. The use of polymeric materials in bone biomaterials research is extensive due to many useful properties of polymers. For orthopedic applications, common polymers used are: acrylic, nylon, silicone, polyurethane, ultra high molecular weight polyethylene (UHMWPE), and polypropylene (PP) [20]. Highly stable polymeric systems such as PTFE, UHMWPE or poly(etheretherketone) (PEEK) have been investigated due to their excellent mechanical properties.

Acetabular cups made of ultra high molecular weight polyethylene have shown to exhibit superior properties. In the use of acetabular cups made of polyethylene, debris created by wear of polyethylene (PE) articulating surfaces is attacked by the body's immune system. This leads to bone loss, also known as osteolysis. Since the debris accumulates in the area close to the implant, the bone loss leads to loosening of the implant stem. Thus, the main problems associated with the use of PE as acetabular cups is not the wear of the cups themselves but wear of the interfacial adhesion between tissue and implant.

The use of degradable polymers in THR is rather limited due to their inadequate mechanical properties. Due to their degradation properties these polymers have extensive application in tissue engineering. The initial high strength of some degradable polymers such as PLLA has spurred interest in use of these polymers as composite systems with ceramic fillers.

The use of these materials for composites where stiffening agents are used to enhance mechanical properties is the subject of several current studies.

Ceramics

As compared to metals, ceramics often cause reduced osteolysis and are regarded as favorable materials for joints or joint surface materials. Several ceramics due to their ease of processing and forming and superior mechanical properties, were investigated as bone substitute materials. Conventional ceramics such as alumina were evaluated due to their excellent properties of high strength, good biocompatibility and stability in physiological environments [52]. Due to lack of chemical bonding between sintered alumina and tissue, its applications as a potential bone substitute are limited.

Alumina, because of the ability to be polished to a high surface finish and its excellent wear resistance, is often used for wear surfaces in joint replacement prostheses. Femoral heads for hip replacements and wear plates in knee replacements have been fabricated using alumina. In hip replacements, the alumina femoral head is used in conjunction with a metallic femoral stem and an acetabular cup made from UHMWPE for the opposing articulating surface. The wear rates for alumina on UHMWPE have been reported to be as much as 20 times less than that for metal on UHMWPE, making this combination far superior and producing less wear debris.

Other ceramic materials have also been investigated for potential applications in orthopedics. Considerable research has focused on zirconia and yttria ceramics that are characterized by fine grained microstructures. These ceramics are known as tetragonal zirconia polycrystals (TZP). Zirconia is the material of choice currently for ball heads.

A better match between the bulk material properties of the implant and the bone it replaces can decrease some of the problems associated with using coated metallic implants such as stress shielding. This is often achieved with coatings on implants.

Since calcium phosphates are present as apatites in natural bones, researchers have investigated calcium phosphates extensively. Calcium phosphate ceramics, are widely used for hard tissue replacement due to their biocompatibility and osteoconductive properties [53,54].

As bone defect fillers, these ceramics are utilized in powder and block forms. Porous forms with 100–300 _m pores are preferred since they allow bone to grow into the implant, promoting mechanical fixation with the natural bone.

The particulate form lacks cohesive strength and lends to dislodge and migrate under externally applied stresses during healing period.

In general, the applications of calcium phosphates in the body have been limited by the low strength and low fracture toughness of the synthetic phosphates.

Alumina and titanium dioxide have been used as nanoceramics separately or in nanocomposites with polymers such as polylactic acid or polymethlyl methacrylate. The nanoceramic formulations promote selectively enhanced functions of osteoblasts (bone-forming cells). These functions include cell adhesion, proliferation, and deposition of calcium-containing minerals.

Ceramics that elicit a favorable bonding to bone tissue are often called as bioactive ceramics. Some compositions of glasses containing SiO_2, Na_2O, CaO, and P_2O_5 bond to soft tissues as well as bone [55–57]. The practical use of bioactive glass for THR components has been limited to their use as bioglass coatings on the femoral and acetabular THR components.

Composites

Generally the use of composites for bone biomaterials have included three broad areas:

• functionally graded composites,

• polymer-ceramic composites (with and without fiber reinforcements),

• biomimetic composites or composites with biological macromolecules.

Composites are fabricated of HAP and zirconia to enhance the mechanical properties of HAP while retaining its bone bonding property. Functionally, graded composites are an important area in composites research.

The main feature of a functionally graded composite is the almost continuously graded composition of the composite that results in two different properties at the two ends of the composite. Powder metallurgy methods have been used to make HAP/titanium functionally graded composites offering the biocompatible HAP on the tissue side and titanium for mechanical property [58].

The research in this field is quite promising but currently, the mechanical properties of these composites are clearly in excess of the properties of bone.

1.5. Knee joints as artificial implants: the choice of the biomaterial

It's here presented a brief outline of the anatomy of the structures of the knee.

Movement of the knee joint can be classified as having six degrees of freedom—three translations: anterior/posterior, medial/lateral, and inferior/superior and three rotations: flexion/extension, internal/external, and abduction/adduction.

The movements of the knee joint are determined by the shape of the articulating surfaces of the tibia and femur, and the orientation of the four major ligaments of the knee joint: the anterior and posterior cruciate ligaments and the medial and lateral collateral ligaments as a four bar linkage system.

The primary function of the medial collateral ligament is to restrain valgus movement of the knee joint with its secondary function being control of external rotation. The lateral collateral ligament restrains against varus rotation as well as resisting internal rotation.

The primary function of the anterior cruciate ligament (ACL) is to resist anterior displacement of the tibia on the femur when the knee is flexed and control the screw home mechanism of the tibia in terminal extension of the knee. A secondary function of the ACL is to resist varus or valgus rotation of the tibia, especially in the absence of the collateral ligaments. The ACL also resists internal rotation of the tibia.

The main function of the posterior cruciate ligament (PCL) is to allow femoral rollback in flexion and resist posterior translation of the tibia relative to the femur.

The menisci are intra-articular structures made of elastofibrocartilage. They are important for reducing contact stresses on the articular cartilage, shock absorption, circulation of synovial fluid and joint stability. The medial meniscus is tethered to the deep part of the medial collateral ligament and so is more prone to injury than the lateral meniscus which is more mobile. The lateral meniscus is smaller than the medial and is sometimes discoid in shape.

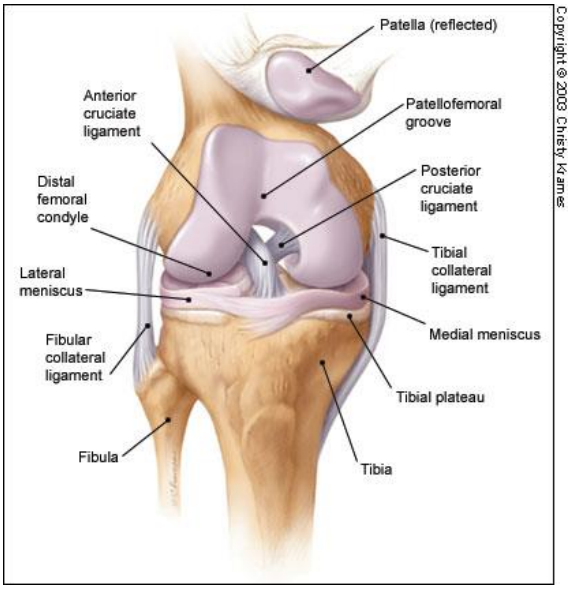


Figure 1.9 Anatomy of the knee.
(Tandeter HB, Shvartzman P, Stevens MA, 1999, Acute knee injuries: use of decision rules for selective radiograph ordering)

1.5.1. The design of the device

A typical knee prosthesis is made of:

-a metal femoral component that wheel on an insert UHMWPE;

-a metal tibial plateau, which supports the insert in UHMWPE.

Contrary to hip replacement, the most of the knee prosthesis are similar in shape, differing for the way in which the two tibial and femoral components are fixed to the bone.

Total knee arthoplasty may include femoral, tibial and patellar parts. If only one part of the knee joint is damaged, the unicompartmental knee replacement can be done in order to save the healthy part. The biomaterials of the total knee prosthesis are presented in figure 1.10.

In total knee arthoplasty, the joint surface of diseased femoral condyle is replaced with a metallic round ended femoral component mimicking the curvature of the natural femoral condyle; fixation of the femoral component may be achieved with bone cement or using cementless method with the help of porous surface structure or hydroxyapatite coating of the implant.

A flattened or slightly dished tibial component made of polyethylene replaces the destroyed articulating surface. Patellar components are also made of polyethylene. The tibial component often contains also a metallic back-up plate which minimizes the deformation of the polymer component under loads. The metallic back-up plate may have

an intramedullary stem which further stabilizes the structure. All-polyethylene tibial components are also available in the market. Cemented fixation or cementless fixation with screws and pegs can be used with the tibial component.

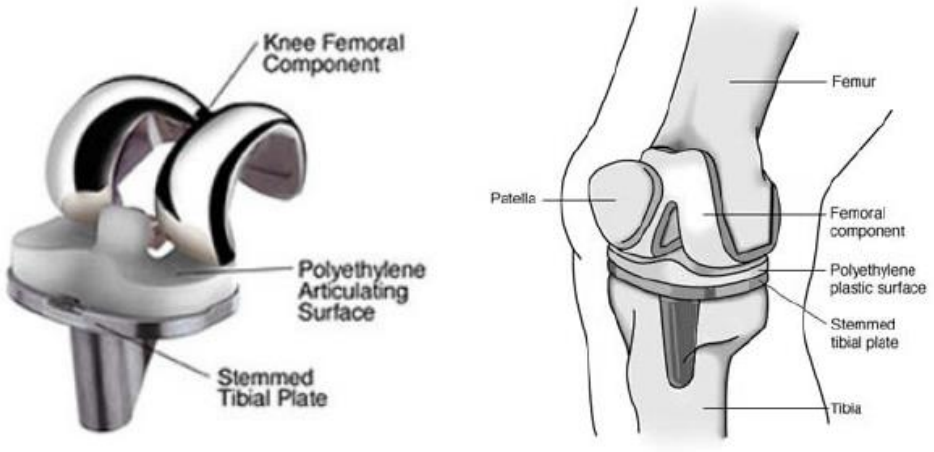


Figure 1.10 The components of the total knee prosthesis
(from https://www.hss.edu/conditions_understanding-implants-in-knee-and-hip-replacement.asp)

The fixed-bearing total knee arthoplasty is suitable for most of the patients. The polyethylene tibial component is firmly attached to the metallic back-up plate. The femoral metallic component rolls on this cushioned surface. In some cases, excessive activity can cause more rapid wear down of the fixed-bearing prosthesis.

The mobile-bearing knee prosthesis may be a choice for younger and more active patients, because the polyethylene insert can rotate short distances inside the metal tibial back-up plate and this rotation reduces stress and wear on the implant and allows for a more natural range of knee motion. Mobile-bearing knee implants require more support from soft tissues, as the ligaments surrounding the knee, than fixed-bearing design. If the soft tissues are not strong enough, mobile-bearing knees are more likely to dislocate.

The replacement knee joint is comprised of a flat metal plate and stem implanted in your tibia, a polyethylene bearing surface and a contoured metal implant fit around the end of the femur. The use of components made from metals and polyethylene allow for optimum articulation (or joint mobility) between the joint surfaces with little wear. Because the knee

implant has a flatter bearing, wear is less of a problem than in a hip implant which has a very deep bearing.

1.5.2. The choice of the biomaterial

Cobalt-chromium Alloys

Cobalt-chromium alloys are hard, tough, corrosion resistant, bio-compatible metals. Along with titanium, cobalt chrome is one of the most widely used metals in knee implants. There is no consensus as to which material is better and more suitable. Although the percentage of patients having allergic reactions related to the use of cobalt-chromium alloys to is very low, one area of concern is the issue of tiny particles (metal ions) that may be released into the body as a result of joint movement. These particles can sometimes cause reactions in the human body, especially in case of those patients who have allergy to special metals like nickel.

Titanium and Titanium Alloys

Pure titanium is generally used in implants where high strength is not necessary. For example, pure titanium is sometimes used to create fiber metal, a layer of metal fibers bonded to the surface of an implant which allows bone to grow into the implant or allows cement to better bond to the implant for stronger fixation. Titanium alloys are bio-compatible in nature. They commonly contain amounts of vanadium and aluminum in addition to titanium. The most used titanium alloy in knee implants is Ti_6Al_4V. Titanium and titanium alloys have great corrosion resistance, making them inert biomaterial (which means they will not change after being implanted in the body). Titanium and its alloys have a lower density compared to other metals used in knee implants. Additionally, the elastic nature of titanium and titanium alloys is lower than that of the other metals used in knee implants. Because of this, the titanium implant acts more like the natural joint, and as a result, the risk of some complications like bone resorption and atrophy are reduced.

Uncemented implants

Knee implants may be “cemented” or “cementless” depending on the type of fixation used to hold the implant in place. The majority of knee replacements are generally cemented into place. There are also implants designed to attach directly to the bone without the use

of cement. These cementless designs rely on bone growth into the surface of the implant for fixation. Most implant surfaces are textured or coated so that the new bone actually grows into the surface of the implant. Surface of the titanium is modified by coating the implant with hydroxyapatite, a bioactive surfacing agent that will ultimately bond as the bone grows into it.

Polyethylene

The tibial and patellar components in knee replacements are made of polyethylene. Though standard polyethylene surfaces traditionally suffered from wear in hip implants, wear is less of a problem in knee implants as the bearing surfaces are flatter and do not result in the same kind of wear. The use of Ultra Highly Cross Linked PolyEthylene (UHXLPE) or Ultra High Molecular Weight PolyEthylene (UHMWPE) reduces even the minimal wear enabling the knee implants to last for a much longer time.

Zirconium alloy and all plastic tibial component

Zirconium alloy is used in a new ceramic knee implant. The zirconium alloy is combined with an all-plastic tibial component, replacing the metal tray and plastic insert used in other knee replacements. It is believed that this new knee could last for 20-25 years, substantially more than the 15-20 years that cobalt chromium alloy and polyethylene implants are effective. The new combination can be lubricated, which results in a smoother and easier articulation through plastic.

Another important characteristic of this material is that it is biocompatible, meaning that people who have nickel allergies and cannot have knee implants made of cobalt chromium alloy. Zirconium alloy implants eliminate the risk to nickel-allergic patients because this new material contains no nickel.

Oxinium oxidized zirconium

Oxinium oxidized zirconium is a new material used in knee implants since 2001. It is basically a transformed metal alloy that has a ceramic bearing surface. It contains zirconium and niobium alloy that was oxidized to convert the surface of the material into zirconia ceramic. The advantage of this metal is that just the surface has been changed, so the rest of the implant component is a high tensile metal. Although it is twice as hard as cobalt chromium alloys, it provides half the friction thus performs with higher quality and lasts for a longer time.

1.6. The tissue-engineering approach

The building blocks to assemble tissue-engineered therapies that fulfill the demanding biological performance standards required for bone regeneration include cells, signaling molecules and scaffolds, but the simple statements championing the amalgamation of cells, growth factors and matrix elements have not yet translated into direct clinical success. Multidisciplinary teams of engineers, doctors, biologists, chemists and material scientists are working together to understand what combinations of cells, biologicals and matrix elements are necessary to achieve the common goal, what are the required physiological and therapeutic doses for cells and biologicals, what temporal and spatial distribution of triad elements is required for tissue regeneration, and what are the physiological dynamics and kinetics associated with these distributions. They must achieve the precise, predictable coordination of cell responses with matrix scaffolds and biological signaling molecules to match the dynamics of physiological bone fracture repair.

1.6.1 Scaffolds and biomaterials

A primary purpose of biomaterials engineered for tissue regeneration is to support and facilitate the requisite physiological functions at the injury site. Broadly, this includes providing an extended framework for regenerative cell population migration and specialization, as well as the sequestration of extracellular matrix (ECM) components and growth factors. In the context of cellular content, support is multidimensional and can be defined as providing the capability for cell attachment, anchorage, differentiation, proliferation and function. The physiological role of bone tissue also demands that biomaterials at defect sites be capable of withstanding loads associated with compressive loading of bone. These functional properties are paramount in the function of biomaterials for bone tissue regeneration that are, first and foremost, determined to be biocompatible and patient safe.

The contemporary biomaterials for bone tissue regeneration can be classified broadly into inorganic and organic materials, which include both naturally derived and synthetic components.

Inorganic materials, such hydro-xyapatite (HA) and bioactive glasses, have long been used for bone tissue-engineering purposes because of their similarities in structure and composition to the inorganic elements of bone itself [59].

Among the benefits of inorganic biomaterials are their compressive strength (which is often equal to, or greater than bone tissue) and potential for osteoconductivity [60]. The main deficiency in these materials is their brittle nature, which poses a concern in high load-bearing biological applications. The alternatives to inorganic materials are organic polymers, which can be either naturally occurring or chemically synthesized. These materials provide an alternate set of characteristics that encourage their use for tissue-engineering applications.

This description begins with biomaterials derived from natural sources, such as collagen [61], hyaluronic acid [62], cellulose [63], silk, alginate [64] and chitosan.

Generally, naturally derived biomaterials are characterized by biocompatibility, enabling the adhesion and migration of cells within their structures. Collagen sponges, in particular, have long been used to deliver growth factors to promote bone regeneration. The major limitations of naturally derived polymers include difficulties in processing and purification as well as concerns regarding immunogenicity. The potential also exists for batch-to-batch variability in materials, diminishing the predictability of results in the clinic. Finally, no naturally derived organic biomaterial is capable of matching the mechanical properties of bone tissue, which contains both organic and inorganic components.

The field of organic polymer synthesis for tissue engineering has grown considerably as a consequence of the limitations associated with naturally derived polymeric materials. Through advances in polymer synthesis technologies, particularly with regards to controlled radical polymerization and scaffolding techniques, synthetic biomaterials with tunable micro- and macroscale features are being developed. Microscale features include composition, architecture and binding groups, whereas macroscale features include porosity, stiffness and elasticity. With respect to polymer composition, polymers frequently used for bone tissue regeneration include polylactic acid (PLA), polyglycolic acid (PGA), PLGA, polycaprolactone (PCL), polyethylene (PE), polyethylene glycol (PEG) and poly(methyl methacrylate) (PMMA), among others. Biologically inspired synthetic polymers derived from amino acids, such as tyrosine-derived polycarbonates, polyethers and, to a lesser extent polyarylates, have also been investigated for tissue repair and regeneration [65,66]. Despite the high degree of versatility available with synthetic polymer synthesis, they too have shortcomings as platforms for tissue engineering. Their lack of bioactivity restricts positive biomaterial–host interactions, particularly in

comparison to naturally derived polymers that have ECM-binding domains. Additionally, the degradation products of synthetic polymers often include acidic byproducts (e.g. PLA or PGA) that might hinder regenerative processes. The clinical success of scaffold-based approaches for bone regeneration relies on overcoming the limitations associated with single-phase biomaterials by developing synergistic combinations of inorganic and organic biomaterials. The field of bone tissue regeneration has already made progress on this quest for hybrid bio-materials. Intelligent scaffold design is achieved by combining organic and inorganic materials, enabling the creation of biocompatible scaffolds with the compressive strength required in osseous defect sites (figure 1.11).

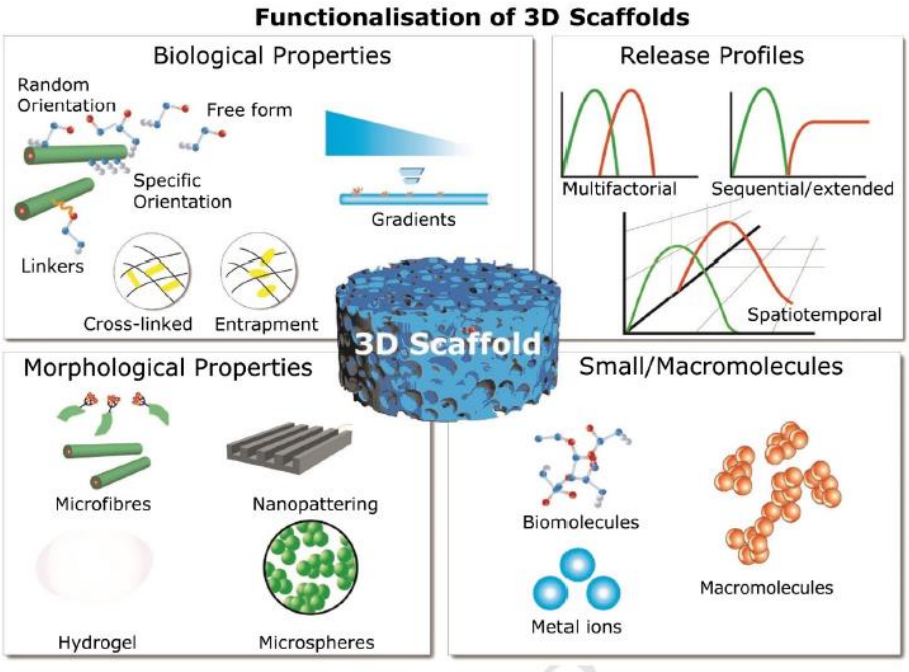


Figure 1.11 Scaffold functionalization: various biofunctionalization strategies have been explored to alter the topographical and biochemical properties of implantable materials as well as localize and control the delivery of bioactive factors (Kim, J. et al. ,2012, Bone regeneration in a rabbit critical-sized calvarial model using tyrosine-derived polycarbonate scaffolds. Tissue Eng. A 18, 1132–1139)

Combining electrospun collagen nanofibers with PCL microstrands, for example, has been achieved without compromising the cell adhesive properties of collagen or the mechanical strength of PCL [67]. The blending of chitosan and hydroxyapatite in scaffolds has resulted in materials with mechanical properties, porosity and bioactivity to support ingrowth of cells and new bone formation [68]. Other examples of recent ingenuity with combinatorial biomaterial platforms include collagen and HA [69], PGA and bTCP [70], as well as a

particularly novel combination of PEG, PCL, collagen and nano-HA [71]. Scaffolds, in particular, might be instrumental to success of both growth factor and stem cell-based tissue-engineering therapeutics. In fact, the quest to produce a comprehensive tissue-engineering approach to bone regeneration in the clinic has given rise to several novel approaches that might be clinically impactful.

Scaffolds have three-dimensional porous structures, need to fulfil the following criteria in order to be used in tissue engineering:

- The material must be biocompatible and its degradation by-products non-cytotoxic;
- The scaffold must be biodegradable and should resorb at the same rate as the tissue is repaired;
- The scaffold must possess a highly interconnected porous network, formed by a combination of macro- and micropores that enable proper tissue ingrowth, vascularization and nutrient delivery.

The mechanical properties of the scaffold must be appropriate to regenerate bone tissue in load-bearing sites. Moreover, the material must keep its structural integrity during the first stages of the new bone formation.

Both natural and synthetic polymers have been used in the development of new three-dimensional scaffolds for bone, cartilage, ligament, meniscus and intervertebral disc tissue engineering. In particular, synthetic biodegradable polymers have attracted special attention because they enable a better control of their physico-chemical properties and also because they have been successfully used in clinical applications. PLA, PGA, PCL and PHB are the most widely studied polymers for bone tissue engineering purposes. PLA, collagen and silk have been studied as potential materials for ligament tissue engineering .

Hyaluronic acid, polyglactin ,collagen, fibrin, alginates, chondroitin sulphate photocrosslinked hydrogels and glycosaminoglycans are also under study for cartilage and intervertebral disc (nucleus pulposus) tissue engineering applications. In addition, decalcified (or demineralized) bone matrix (DBM) is currently being used successfully in various clinical applications as an alternative to autografts.

It is commercially available from different manufacturers as an allergenic human freeze-dried bone graft, and is generally used for filling bone defects. Moreover, due to its similarity with natural bone, DMB has been demonstrated to be an efficient carrier for

bone morphogenetic proteins (BMP), which are growth factors that enhance bone formation.

Biodegradable composite scaffolds combining biodegradability and bioactivity offer unique advantages in the tissue engineering field. The incorporation of an inorganic phase into a bioabsorbable polymer matrix modifies the mechanical behaviour of the porous structure modifies the degradation pattern of the polymer and also enhances the bioactivity of bone tissue engineering scaffolds.

Numerous elaboration techniques and approaches to the development of three-dimensional scaffolds that combine biodegradability and bioactivity are currently under study.

Each preparation technique confers particular and different structural characteristics to the scaffold. Therefore, the choice of the technique depends on the requirements of the final application. Some of the most promising techniques for the processing of such scaffolds are gel casting, solvent casting and particulate leaching, laminated object manufacturing, phase separation, gas saturation, fibre bonding and membrane lamination among others.

The micro- and macrostructure of the scaffolds depend strongly on the processing technique. Pore distribution, interconnectivity and size are of paramount importance in order to guarantee proper cell proliferation and migration, as well as tissue vascularization and diffusion of nutrients. A comprehensive assessment of the ability of a three-dimensional scaffold to allow cell viability and capacity of ECM production is to evaluate its permeability. Moreover, it seems that it would be advisable to evaluate the transport properties of oxygen and nutrients inside the scaffold in order to assess whether they will be able to reach cells seeded inside them.

Some ceramics and glasses have been used for the elaboration of porous scaffolds. As in the case of polymers, a high degree of macro-, micro- and nanoporosities is needed.

Recently, the introduction of undifferentiated bone marrow stromal cells into a hydrogel containing CaP particles in suspension has resulted in an injectable cement composite mixed with living cells. The *in vivo* results obtained after the implantation of this material in mouse have shown a good vascularization and integration into the host tissue.

There exist several commercially available materials, both inorganic and organic, that enable bone ingrowth in clinical scenarios, but these successes to date, although notable, have not surpassed the autograft and allograft in their abilities to treat critically sized bone defects.

The next generation of biomaterials for bone regeneration must physically support osseous defects, as well as chemically and biologically sustain growth factors and stem cells. The convergence of organic and inorganic materials for bone tissue engineering has already begun. Further advancements in technologies that combine scaffolding with growth factors and stem cells might ultimately dictate whether tissue-engineering approaches to bone regeneration will be clinically impactful.

Chapter 2

Bacterial contamination and infections on orthopaedic implants

Increasing number and types of implants used in patients have resulted in increasing numbers of biomaterial-associated infections. For this reason it is necessary to combine engineering , medical, biological and chemical skills with the common goal to reduce infections. Researchers and medical device manufacturers are innovating device designs, surgical implantation protocols, and biomaterials to minimize infection opportunities.

In this section it's underscored the need to develop biomaterial with the best antibacterial performances, to prevent and contrast the phenomena of biomaterial-associated infections. This point represents the interest of this experimental work, and it's discussed in detail in the next sections.

2.1. Adverse events and complications consequent to the implant

Medical devices are increasingly used worldwide for an expanding repertoire of patient clinical needs. Biomaterials and medical device designs have become progressively more complex to accommodate diverse demands for performance and safety in vivo, but in their specific applications, they can induce serious adverse events with substantial health and economic consequences.

One recognized challenge is the growing clinical problem with implant associated infections. Increasing number and types of implants used in patients have resulted in increasing numbers of biomaterial-associated infections. For this reason it is necessary to combine engineering , medical, biological and chemical skills with the common goal to reduce infections. Researchers and medical device manufacturers are innovating device designs, surgical implantation protocols, and biomaterials to minimize infection opportunities. Medical devices with claims to limit microbial adhesion and colonization using combinations of pharmacological, topological, and materials chemistry approaches have been brought into clinical use with the intent of reducing device-related infections.

Approaches include different biomaterials chemistries that intrinsically resist microbial colonization or that deter active growth on contact, surface modifications that produce topologies observed to limit pathogen attachment, medicinal, antiseptic or bioactive coatings, direct antimicrobial attachment to surfaces, or drug impregnation within the biomaterial, and extended release strategies that control antimicrobial agent release from the device over time after implantation.

Many categories of anti-infective biomaterials are currently available and new ones are rapidly advancing. They certainly represent powerful and valuable tools.

Only with evidence-based data there will be a real advancement in this field, enabling the identification of the most effective anti-infective solutions and the correct circumstances of use. Myriads of new technologies will be introduced, but the choice of anti-infective biomaterials will mostly remain a matter of subjective “good sense” and “faith”, with little support of reliable robust data.

Implant-associated infection occurs frequently during and after surgery, and is caused mainly by *staphylococci* and *streptococcus*.

Complications can lead to implant failure necessitating removal by a second surgery.

Formation of a biofilm is one of the main reason for infection, because it develops quickly after bacteria attach onto the implant surface often contains a macromolecular layer formed under physiological conditions. The macromolecular layer constitutes the so-called "conditioning film" rendering the surface more hospitable for bacteria to colonize.[72,73] Various kinds of proteins from surrounding biological fluids like blood, interstitial fluids, salivary proteins, and plasma proteins adsorb on the implants before the first germs appear.[73,74] However, when the conditioning film is formed, bacteria can adhere and subsequently colonize to form a biofilm that possesses a complex architecture by accumulation.[73,75] A biofilm can resist the host immune response via host defense mechanism consequently destroying the host immunity ability on the implant. Furthermore, it can withstand antimicrobial challenge in two ways. The first one is failure of the antimicrobial agent that penetrates into the biofilms due to neutralization reactions between the antibacterial agent and some components in the biofilms. The second one is reduced susceptibility of the biofilm to antibacterial agents, resulting in loss of activity of antibiotics against some slowgrowing bacteria in the biofilm.

Although contamination during surgery can induce infection, most infection occurring on implants is not related to surgeries.[76,77] In the early stage after implantation, the local immune system is affected by the surgical trauma, and consequently bacterial infection often occurs during this stage.[78] Even after tissue integration, the local host immune ability is poor and restricted by a small quantity of blood vessels at the interface between the implant and surrounding tissues. If biofilm-related infection becomes chronic, implant failure normally ensues because both surgical debridement and conventional systemic antibiotic therapy have no effects at that time.34 Hence, it is critical to prevent the formation of biofilms by inhibiting initial bacterial adhesion or killing bacteria directly [79,80] and a good understanding of the general antimicrobial mechanism is crucial to the fabrication of functionalized antibacterial surfaces.

The most frequent microorganisms recovered in orthopedic biomaterial infections belong to endogenous commensals of the skin such as *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis (S. epidermidis*).

These bacteria are not virulent in planktonic form, but they become pathogens when arranged in biofilm (figure 2.1).

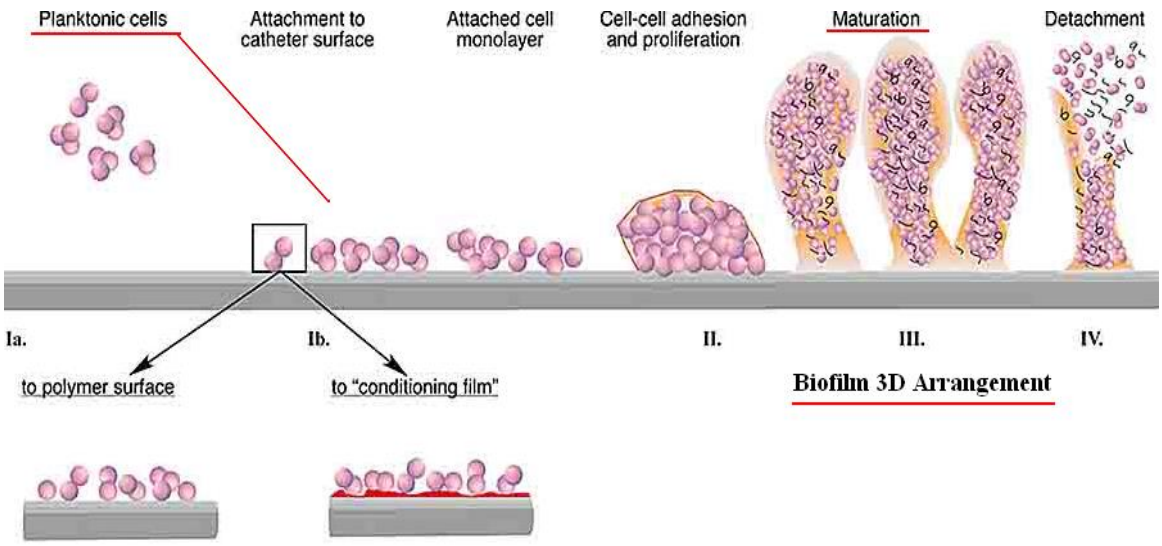


Figure 2.1 From planktonic single cells to biofilm 3D arrangement development of *S. epidermidis and S. aureus.*

Biofilm is a complex protected arrangement self developed by bacteria to survive in a hostile environment with respect to planktonic forms. Particular, it allows them to optimize nutrient uptake, to be sheltered from the removal forces, and to be protected from the host defense mechanisms and from the potential toxic or harmful including antibiotics.
The bacterial growth curve (figure 2.2) provides an indication of the temporal growth in a bacterial population; it is divided into four sections:

- Lag time: it's the time it takes for the organism to adapt to the environment; this time interval is longer or shorter depending on the conditions
- Exponential growth phase (16hs): the organism multiplies rapidly; it's possible to keep the crops at this stage by transferring the bacteria in new lands (continuous culture).
- Stationary phase: the micro-organism stops its growth, because nutrients are completed, and it will form the matrix of protection. Bacteria that are divided and those who die are in equilibrium, some cells enter a state of latency waiting for better conditions. Some bacteria at this stage start to produce secondary metabolites, hindering the vitality of competitors microorganisms.

- Decline and death: the number of microorganisms drops, because dead cells exceed those in division or latency phase.

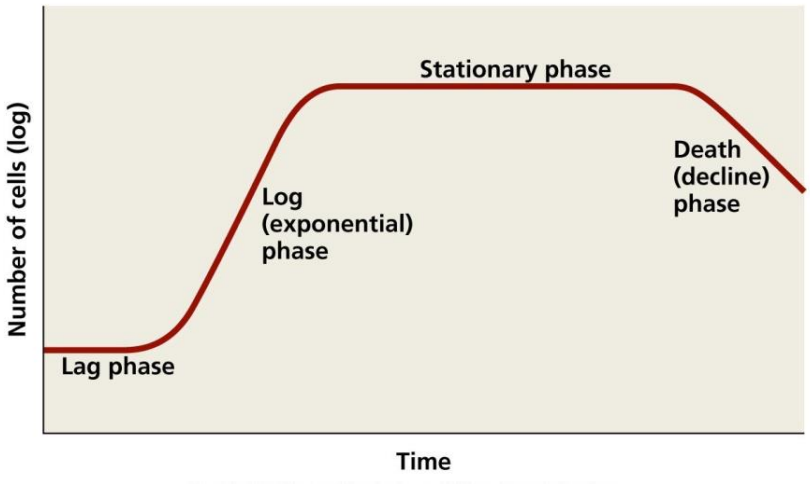


Figure 2.2 Bacterial growth curve

The surface of the materials plays a pivotal role just in this early phase of biofilm formation.

Particular, surface energy, micromorphology, roughness and stability of the material have been proven to affect susceptibility of biofilm adhesion and early development. Therefore, biomaterials surface characteristics could promote or repress bacterial adhesion and colonization; for this reason, in this experimental work, (to study the antibacterial performances of the biomaterials,) the surface characterization has been developed in parallel to the evaluation of the biofilm colonization on the samples .

2.2. Biomaterial-associated infection: general overview

With aging of the general population, the prevalence of joint degenerative diseases is continuously rising. Joint replacement is one of the most successful surgical interventions alleviating pain, improving joint function, but also restoring general mobility and personal independence. The number of implanted hip and knee prostheses increases exponentially, which is followed by an increase of revision surgeries several years later [81] .

In addition, shoulder and ankle joint replacements have been increasingly performed during the last two decades [82, 83] , but also other joint replacement surgeries are increasingly performed, such as elbow .

The number of arthroplasties and consequently also the absolute number of complications is steadily increasing. In our cohort of patients with primary hip arthroplasty, the most frequent local complications, that mostly required revision surgery, were hematomas (4 %), perioperative fracture (2.3 %), dislocation (2.2 %), paresis (2.1 %), and infection (0.4 %) [84] .

Thus, periprosthetic joint infection is a rare complication compared to other postoperative complications. However, infections cause a considerable morbidity for the patient and accounts for a substantial proportion of health care expenditures [85] .

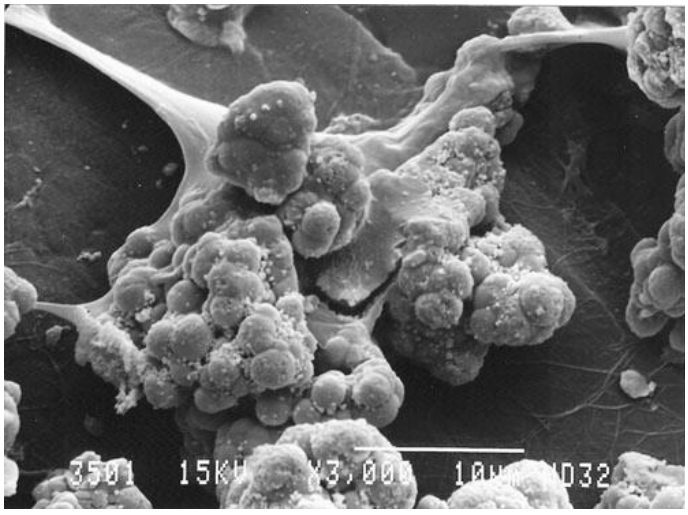


Figure 2.3 SEM from the surface of an explanted hip cup from a patient with hematogenous periprosthetic infection. (W. Zimmerli and A. Trampuz, 2013, Biomaterial Associated Infection, p.10)

Device-related infections often persist until the device is removed, even if the microorganism is highly susceptible to the antibiotic *in vitro*.

Biomaterial associated infection compromises the quality of life, has a high morbidity and is even associated with mortality. In addition, it has a high economic impact, since treatment of infection costs many times more than the primary implantation of the device. Therefore, major efforts should be invested to minimize susceptibility of implants to infection. Coating of the implant surface with antimicrobial substances such as antibiotics, antimicrobial peptides, or silver is an option. This strategy has been tested *in vitro* and in experimental models [86– 88] .

Biomaterial-associated infection is a disastrous complication of modern orthopaedic surgery that often leads to prolonged patient pain and functional losses. While international

efforts to minimize the risk of these infections are underway [89], orthopaedic surgical site infections (SSIs) continue to occur in staggering numbers. Current estimates suggest that up to 2.5% of primary hip and knee arthroplasties and up to 20% of revision arthroplasties are complicated by periprosthetic joint infection (PJI) [90].

Staphylococcus aureus is the leading cause of both the SSIs and PJIs, and deep infection leads to implant removal and ensuing increased morbidity and even mortality [91].

Moreover, therapy of PJI is associated with enormous costs [92].

As the majority of operating rooms are contaminated within the first few hours of service [93,94], most surgeries are not performed in a bacterial-free environment. Within a certain operating room all patients are exposed to the same environment. The question therefore arises as to why some patients go on to have infections and others do not.

Several recent scientific forums have recommended that researchers should focus on the development of effective antibacterial surfaces that prevent bacterial adhesion, colonisation and proliferation into the surrounding tissues [89].

Aim of this work is to summarize current knowledge in this field, with particular emphasis on orthopaedic biomaterials that have intrinsic antibacterial properties, that could be suitable for prevention of PJI in total joint arthroplasty.

Any type of artificial implant is highly susceptible to infection, due to an inefficient host defense in the vicinity of an implanted foreign body. Microorganisms rapidly form biofilms on the artificial surface. Such infections resist host defense and most antimicrobial agents. In case of periprosthetic joint infections, successful management requires a combination of surgery and antibiotic treatment. In view of the difficult treatment, future research should focus on prevention of implant associated infections.

The processes of biomaterials associated infection (BAI) progress from bacterial adhesion to the biomaterial and colonization of adjacent tissues, through to formation of an antibiotic recalcitrant bacterial called biofilm; then a good knowledge of BAI from microbiological and immunological point of view is required to achieve a effective reduction in its incidence, in addition to the engineering approach of surface modification. For this reason multidisciplinary teams of engineers, doctors, biologists, chemists and material scientists are working together, to understand what combinations of cells, and matrix elements are necessary to achieve the common goal of tissue regeneration, to design anti-infective surfaces that are functional and controllable.

The surface of a biomaterial that interacts with bone tissue should also control the conformation and orientation of proteins with precision so that the body will specifically recognize them, to promote cellular differentiation, migration and proliferation. In this way, the surface obtains the ability to perform as a substrate that will support the appropriate cellular activity, in order to optimize tissue regeneration, without eliciting any undesirable effects in those cells, or inducing any undesirable local or systemic responses in the eventual host.

Therefore anti-infective biomaterials need to be tailored according to the specific clinical application. All their properties (surface properties and topography) have to be tuned to achieve the best anti-infective performance together with safe biocompatibility and appropriate tissue interactions.

Biofilm formation is part of a biological cycle, which includes four main stages:

initiation, maturation, maintenance, and dissolution .

Bacteria have a high ability to adhere and survive on virtually all natural and synthetic surfaces [95,96]. Bacterial cell membranes contain various types of adhesins for a wide range of biomaterial surface receptor sites.

Environmental and surface characteristics of a biomaterial such as surface roughness, hydrophobicity, and electrostatic charge play only conditional roles [97]. A reservoir of receptors for bacterial adhesive ligands mediating adhesion of free-floating bacteria to the surface of the biomaterial offers a conditional protein film covering an implant immediately after its placement into the host body [20–23]. Complement and albumin are considered the main components of this conditional protein film [98]. However, the protein spectrum extends much beyond complement and albumin and depends at least in part on a particular type of biomaterial attracting an exact set of host proteins and lipids [99–101].

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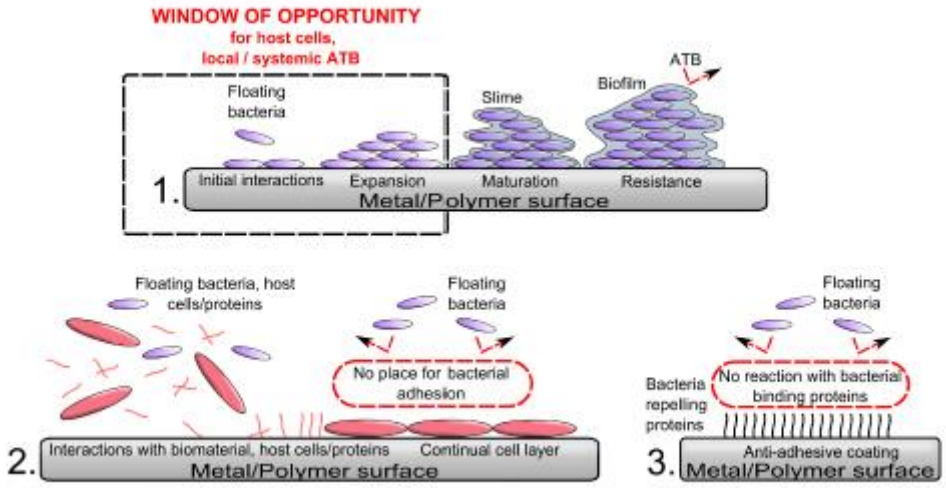


Figure 2.4 Schematic illustration of the process of biomaterial colonization starting from individual bacteria adhesion across micro-colonies towards formation and maturation of biofilm (1); Bacteria cannot activate the biofilm-related phenotype before they firmly attach to the substrate. After attachment and change in the phenotype they are able to produce the matrix of extracellular polymeric substances that protect them against host immune response and antibiotics. If host cell achieves irreversible attachments on the biomaterial surface first it is difficult for bacterial cells to start with biofilm formation (2); The period before firm attachment and phenotypic change is therefore the window of opportunity for almost all antibiofilm strategies (3). During that time these strategies compete with bacteria for implant surface attachment and microenvironment

(Gallo J. Holinka M, Moucha CS, 2014, Antibacterial surface treatment for orthopaedic implants)

Bacteria appear to initiate biofilm development in response to specific environmental cues, such as nutrient availability. Biofilms continue to develop as long as fresh nutrients are provided, but when they are nutrient-deprived, they detach from the surface and return to a planktonic mode of growth. This starvation response is thought to allow the cells to search for a fresh source of nutrients, and is driven by well-studied adaptations that bacteria undergo when nutrients become scarce.

The biofilm is also a complex protected arrangement self-developed by the bacteria to enable them to survive in a hostile environment more easily than can planktonic forms. In particular, it enables them to optimize nutrient uptake, shelters them from removal forces, protects them from desiccation, from host-defense mechanisms, and from potential toxic or harmful agents, including antibiotics.

Bacteria cells in the biofilm community coordinate efforts with their neighbors, to accomplish cooperative activities such as bioluminescence production, biofilm development. The bacteria that lives under the biofilm cannot be killed by antibiotics

because they are at a low metabolic stage, which contributes to maintaining the drug's ineffectiveness.

Biofilm infections have the following distinct characteristics :

- bacteria are adhering to a surface
- bacterial clusters are encased in a matrix,
- infection is confined to a local site,
- adherent bacteria may escape routine diagnostic procedures
- infection generally persists despite susceptibility of plank-tonic bacteria to the antimicrobial agent host defences are unable to eradicate microorganisms, i.e., spontaneous cure does not occur .

Microorganisms within a biofilm are protected against antimicrobial chemotherapy as well as against the host immune system. As a consequence, in the case of implant- associated infections the removal of the medical device frequently is necessary to eradicate the infection.

Therefore BAI it is difficult to treat, as the biofilm mode of growth protects the infecting organisms against the host immune system and antibiotic treatment ; for this reason, the future line in orthopaedics research is to design biomaterials with infection-resistant surfaces.

2.3. Antibacterial strategies to minimize the risk of infections

Strategies relying on decreased bacterial load and creating bacteria-free environment around an implant during the perioperative period, are widely implemented in clinical practice [102–105].

Attempts at formulating evidence-based standards for good clinical and logistic practice in orthopaedic operating rooms have been utilized [106–108]. Furthermore, while there is certainly room for improvement,educational programs aimed at educating orthopaedic surgeons in perioperative strategies of periprosthetic joint infection (PJI) prevention are under way.

A wide spectrum of technological approaches has been proposed and tested, to improve antibacterial performances of implantable devices. Among them, different kind of thin surface modifications have been applied. A change in the surface chemistry and/or structure of the bulk implant can be achieved either by chemically or physically altering

the surface layer in the existing biomaterial (e.g., oxidation or mechanical modifications like roughening/polishing/texturing).

A different method involves over-coating the existing surface with a new thin layer of material having a different composition (e.g., hydroxyapatite coating on titanium alloys, antibiotics bound covalently to the substrate, fixation of other antimicrobial compounds) [109].

A critical step in progress lies in the demonstration that newly developed biomaterials possess antibacterial efficacy [110], but to date there is no widely accepted methodology available that could precisely and reproducibly demonstrate antibacterial behaviour of the proposed anti-infective technologies.

Major criticisms lie around static "closed" testing system, whereas *in vivo* the implant has to facea dynamic, continuously changing, mechanically unstable and predominantly fluid environment [111].

As a result, the majority of studies to date have used inappropriate and insufficient protocols.

Controllable, standardized testing conditions that closely mimic the human *in vivo* environment are needed in order to overcome the aforementioned issues [111]. PJIs develop at low shear conditions and under multidirectional low-pressure fluid flow.

Protocols for cultivation of particular species (multispecies) biofilms at controllable, constant and reproducible conditions have also been described [112].

Finally, representative *in vitro* and *in vivo* models for each particular clinical situation should be further developed and appropriately validated. Given the large variability of antibacterial strategies it is likely that testing methods must be better tailored to match the specific proposed strategy at hand [113].

By an engineering point of view, it's possible to treat biomaterial surfaces in order to prevent the critical step of bacterial biofilm formation .

Hydrophilic, highly hydrated, and non-charged surfaces could be a good choice. These surfaces have been shown *in vitro* to prevent many bacterial species from biomaterial adhesion, by limiting the contact between bacterium and potential surface placement sites [114]. Host cells attachment, however, may also be negatively affected by certain surface treatments.

However, it should be mentioned that the basic concept favouring the hydrophilic over the hydrophobic forces might be criticised from both the bio-physicochemical misunderstanding of these terms and the complexity of biological interactions around an implant [111]. As a result, much more attention has been focused recently on hydrophobic and superhydrophobic surface treatment technologies and their repellent antibacterial effects.

Treating protein-surfaces and/or protein-bacteria interactions may be a good strategy of inhibiting bacterial adhesion to a specific biomaterial [115].

Proteins such as albumin, fibronectin, fibrinogen,denatured collagens, and some plasma/tissue lipids are the first host substances that interact with the surface structure of the biomaterial [101]. Reduction of conditional lipid-protein layer formation can be achieved by changing surface physico-chemical characteristics, and/or surface micro-morphology [116].

2.4. Surfaces with intrinsically antibacterial properties

Historically, two main strategies have been proposed for effective antibacterial surface treatment either "contact killing" or drug eluting. The majority of them are not suitable for surface treatment of orthopaedic implants due to problems with cytotoxicity, immunoreactivity, and genotoxicity [117–120].

In killing bacteria they rely on diverse mechanisms of action, which may interfere with a cell respiration, cell division, or formation of a cell wall. Another very promising approach involves interference of the bacterial signalling network or inhibition of the transition of planktonic phenotype of bacteria into a sessile type [121]. This tactic could prolong the window of opportunity for both prophylactic antibiotic activity and the host immune response.

Antibacterial surface technologies can employ metals (silver, zinc, copper, zirconium *etc.*), non-metal elements (e.g., selenium), organic substances (antibiotics, anti-infective peptides, chitosan, other substances), and their combinations.

Antibacterial activity of the majority of metal coatings is closely linked to the ionic or nano form rather than to the bulk material [122].

Creating a coating-substrate interface robust enough to sustain the mechanical stresses involved in surgical implant insertion and ultimate loading once *in vivo* remains a

challenge [123]. Lastly, the risk of bacterial resistance to metallic coatings, a phenomenon common to all antibacterial strategies, remains a concern.

Silver is the most prevalent metal used in biomedical applications. Dissolved silver cations are biochemically active agents that interfere with bacterial cell membrane permeability and cellular metabolism. Silver also contributes to formation of reactive oxygen species and other mechanisms that potentially influence prokaryotic cells [124]. There has been concern, however, about the toxicity of silver ions.

Research efforts have focused on the development of silver coating technologies that reduce or even eliminate toxicity while maintaining constructive antibacterial effects [125,126].

Cobalt-chrome and titanium alloys are the most commonly used materials in total joint arthroplasty implants. Several technologies have been proposed to expand the antibacterial properties of these implants. Functionalization of biomaterial surfaces with silver and copper ions is one such method [123,126,127].

The anti-infective potential of titanium dioxide layers has also been widely investigated both alone [128,129] or in combination with other substances [130].

Great expectations are associated with polyetheretherketone (PEEK) implants.

These implants could become immune to bacterial colonization by employing the chelate bonding ability of inositol phosphate to immobilize silver ions on the hydroxyapatite film of the PEEK substrate [131]. Such implants, however, are not currently available in the joint arthroplasty field.

Non-metal elements like hydrogen, chlorine, iodine, or oxygen are commonly used in biomedicine for their anti-infective properties. They have been rarely indicated as antibacterial coating technologies in orthopaedic implants due to their general softness and brittleness. Selenium bound covalently onto the surface of titanium or titanium alloy implant discs have been shown to prevent *Staphylococcus aureus* and *Staphylococcus epidermidis* attachment without affecting osteoblast viability

A large number of studies have investigated the efficacy of surfaces coated with covalently linked antibiotics [132–135]. Clinical effectiveness of such implants is most likely limited to infections caused by bacteria that are sensitive to the specific antibiotic that has been coupled.

In addition, strong forces such as covalent binding are insufficiently sensitive to react to weak external stimuli . To overcome these issues, combinations of antibiotics with other compounds have been proposed either alone or in association with a particular mechanism of controlled release [136].

A promising new approach for prevention of implant-related infection involves coating implants with antimicrobial peptides, cytokines or other molecules critical for host response to bacteria invasion [137, 138]. This heterogeneous group of substances has proven experimentally their efficacy against a wide range of pathogens [139].

They employ a number of mechanisms, pathways, and targets that participate in implant bacterial invasion including those related to local deficiency in immune response induced by surgical approach or the implant insertion method itself. Antimicrobial peptides, like antibiotics, function via damage of the cell wall and inhibition of key bacterial protein synthesis.

A very promising set of new molecules called biofilm disruptors has been discovered recently [140]. They might not only protect an implant surface from biofilm formation but also disrupt existing biofilms. However, in contrast to local delivery of antibiotics, the optimal doses and surface pharmacokinetics of above-mentioned substances need to be determined.

2.4.1. Nanostructured Surfaces and Coatings

Nanostructured surfaces and coatings are currently of great interest [141–143]. Consequently, nanoscale surface patterning methods have been applied to fabricate different nanopatterns. Several studies have demonstrated that nanopatterning in conjunction with other surface treatment could inhibit bacterial adhesion.

Another example of nanotechnology application is fabrication of polymers containing antibacterial nanoparticles and substances that inhibit both the quiescent and sessile bacteria. Synthetic polymers, natural polymers, and their derivatives (e.g., gelatin, chitosan) have potential to be used as implant surface scaffolds and delivery vehicles of antibacterial agents [144–147].

The antibacterial effect of silver nanoparticles (NP) is not fully understood to date. It might be based on the release of silver cations from nanostructured surfaces (Figure 6). These

cations permanently disrupt bacterial cell wall, inactivate essential proteins, cause DNA condensation, and lead to reacting oxygen species generation [148].

The antibacterial activity of the silver NPs is dependent on both size and shape. Differences in the mechanism of action of diverse forms of silver may explain why to date there have been no reports of resistance to this type of antibacterial treatment [147].

As compared to non-nanoscale silver applications, a nanoscale form offers simultaneously greater solubility, chemical reactivity, and strong antibacterial activity even at low concentrations (units of milligrams per liter), [148,149]. Silver NPs have been shown to cover a wide spectrum of causative bacteria [150,151]. Moreover, *in vitro* and *in vivo* experiments have shown long-lasting antibacterial protective effects of nanostructured titanium coating incorporated with silver NPs [151].

Intense research is being done to combine both the antibacterial effect of silver NP with osteointegrative properties and improved biocompatibility of materials such as titanium alloys [130,153–155].

Lastly, nanoparticles of selenium, copper, zinc, and other elements have also demonstrated strong antibacterial efficacy [156–158].

Taken together, nanotreatment of biomaterial surfaces offers new opportunities for periprosthetic joint infection prevention. Early studies have shown high biocompatibility of such approaches and therefore great potential for use in surface treatment of orthopaedic implants.

It should be cautioned, however, that nanotechnologies can also induce unintended inflammatory responses related to activation of dendritic cells and macrophages [159]. Concern also exists about the mechanical properties of implant nanocoatings since damage may occur during surgical implantation, especially in cementless implants inserted via press-fit methods [160].

Implants covered by a nanosilver coating are not currently available in clinical practice.

2.5. Correlation between bacterial adhesion and surface properties

A number of studies have demonstrated that the biological response to biomaterials can be controlled via alterations in surface chemistry and structure.

It has been demonstrated that implants with rough and porous surface structure are prone to greater bacterial adhesion in comparison to smooth surfaces. This is due to much larger surface area available for adhesion and subsequent higher number of anchor points.

Porous implants have much larger surface available for bacterial adhesion; some studies report their usage is associated with increased risk of infection .

However, other investigators found similar risk for infection in relation to the type of total hip arthroplasty fixation.

This could point paradoxically to the complexity of the clinical situation where a myriad of factors participate in PJI pathogenesis and surface roughness is only one of many, implant related characteristics.

At the nanometre scale, bacterial adhesion does not simply follow the roughness of the surface but also is dependent on other variables like the quantity of adsorbed proteins. When roughness increases, the formation of a thick protein layer on such implant surface could suppress bacteria adhesion.

In addition, the adhesion process can be different among the materials with different surface structure in terms of short-range van der Waals interactions and surface energy (Figure 2.4), [97]

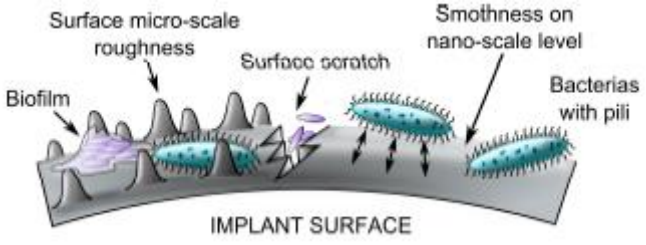


Figure 2.5. The relationship between biomaterial surface roughness and bacterial attachment is intricate. Bacterial attachment is facilitated by increased surface microscale roughness since larger surface areas (especially when irregular) provide binding sites and protection. Increased smoothness of the surface should prevent bacterial colonization. (Gallo J, Holinka M, Moucha CS ,2014, Antibacterial surface treatment for orthopaedic implants)

To date a number of anti-adhesive tactics have been proposed for different purposes. Only a few, however, have met the elementary features required for bone implant usage.

More recent strategies include production of self-assembled mono- or multilayers, surface grafting, or hydrogels. Importantly, the level of anti-adhesive properties has to respect the purpose of a particular type of orthopaedic implant surface.

Specifically, a strong anti-adhesive layer cannot be used for coating of fixation surfaces of total joint arthroplasty because it could also prevent host bone osseointegration and lead to early mechanical failure. The solution lies in a coating technology that retains required host cell interactions while selectively inhibiting bacterial adhesion.

It was found that specific changes of a surface morphology at the micro- and nanometre scales might influence not only the bacterial adhesion but also biofilm phenotype conversion. As a result, nanopatterning and other surface treatment nanotechnologies can offer new opportunities for development of effective anti-adhesive treatment in orthopaedic implants.

Taken together, anti-adhesive technologies offer attractive opportunities for engineers and collaborating researchers to develop a prosthetic surface that should ultimately diminish PJI rates. This approach does however have some important limitations. Cementless arthroplasty implants that require host bone integration may not be amenable to such coatings. The process of unifying ongrowth or ingrowth with antibacterial anti-adhesive functionality as part of a surface coating is technically demanding and has not been fully elucidated. Another challenge of designing antiadhesive technologies relates to the current inability to design a universal surface treatment that can be applied to all surfaces, all bacterial species, and under all (ingrowth and noningrowth) implants.

Chapter 3

Surface characterization and bacterial culture on biomaterials samples

The drawback of the technological advance in biomaterial field is the high susceptibility of implants to infection that chronically persist, if no comprehensive treatment concept is applied.

In this section the purpose of the work is presented: the effect of surface properties on bacterial adhesion is analysed, to study the microbiological behavior related to the different surface features. The aim is to study the resistance of the biomaterials to bacterial proliferation, through a simulation of infection, in order to compare the behavior of different surfaces.

3.1. Purpose of the experimental work

Whereas acute infection generally needs only one single antimicrobial treatment course to eradicate microorganisms, chronic infection may require sophisticated diagnostic procedures, long-term antimicrobial therapy and repetitive surgical interventions.

In the case of orthopedic device-associated biofilm infection, cure of infection should be aimed at in the first attempt, because each treatment failure results in a worse functional result. Thus, the prerequisite for correct treatment of device associated infection is a rational concept for the optimal surgical and antimicrobial therapy.

With aging of the general population, the prevalence of joint degenerative diseases is continuously rising. Joint replacement is one of the most successful surgical nterventions alleviating pain, improving joint function, but also restoring general mobility and personal independence. The number of implanted hip and knee prostheses increases exponentially, which is followed by an increase of revision surgeries several years later [81] .

The number of arthroplasties and consequently also the absolute number of complications is steadily increasing. In patients with primary hip arthroplasty, the most frequent local complications, that mostly required revision surgery, were hematomas (4 %), perioperative fracture (2.3 %), dislocation (2.2 %), paresis (2.1 %), and infection (0.4 %) [84] . Thus, periprosthetic joint infection is a rare complication compared to other postoperative complications. However, infections cause a considerable morbidity for the patient and accounts for a substantial proportion of health care expenditures [85] .

In this section, the effect of surface properties of different kinds of biomaterial on bacterial adhesion are analysed, to study the microbiological behavior in relation to the different substrate characteristics.

It is actually well known that surface properties determine the interactions of biomaterials with the biological environment [161, 162, 163]. The first contact between implant surface and biological fluids has been described as a sequence of interactions between biological entities and the artificial surface: water molecules, in the first nanoseconds, then ions, and after few seconds proteins. Finally, in a time interval typically comprised between minutes and hours different kind of cells will approach the protein modified material [161].

Surface characteristics, such as topography and wettability, affect material ability to adsorb water and proteins, and to interact with bacteria. (Figure 3.1)

A selection of biomaterials (CoCrMo alloy, XLPE and 4 different types of ceramics), currently used in orthopedic applications, has been considered and compared with polystyrene surfaces used as a control. The aim was to determine the effect of the surface characteristics, peculiar for each material, on the bacterial response.

Surface roughness (both macroscopic analysis by means of profilometer, and microscopic observation by means of AFM) , surface wettability and protein absorption have been determined in the same conditions for all the materials in the present work. A protocol for samples surface preparation has been developed in order to obtain the same cleaning degree on all the materials; in fact it has been evidenced that surface properties (e.g. wettability) can vary in a considerable way depending on the surface conditions.

To correlate the data of surface topography characterization to the microbiological behaviour, a simulation of bacterial infection on the biomaterial surfaces has been performed, followed by a metabolic analysis about the bacterial viability by means MTT colorimetric assay, and a morphological analysis about the biofilm through SEM analysis.

Therefore, the aim of the work is to study the resistance of the biomaterials to bacterial proliferation, to identify the material with the best antibacterial performances.

In the next sections, the procedure of each experimentation is presented; the experimental set-up, materials and methods, and the samples are described in detail.

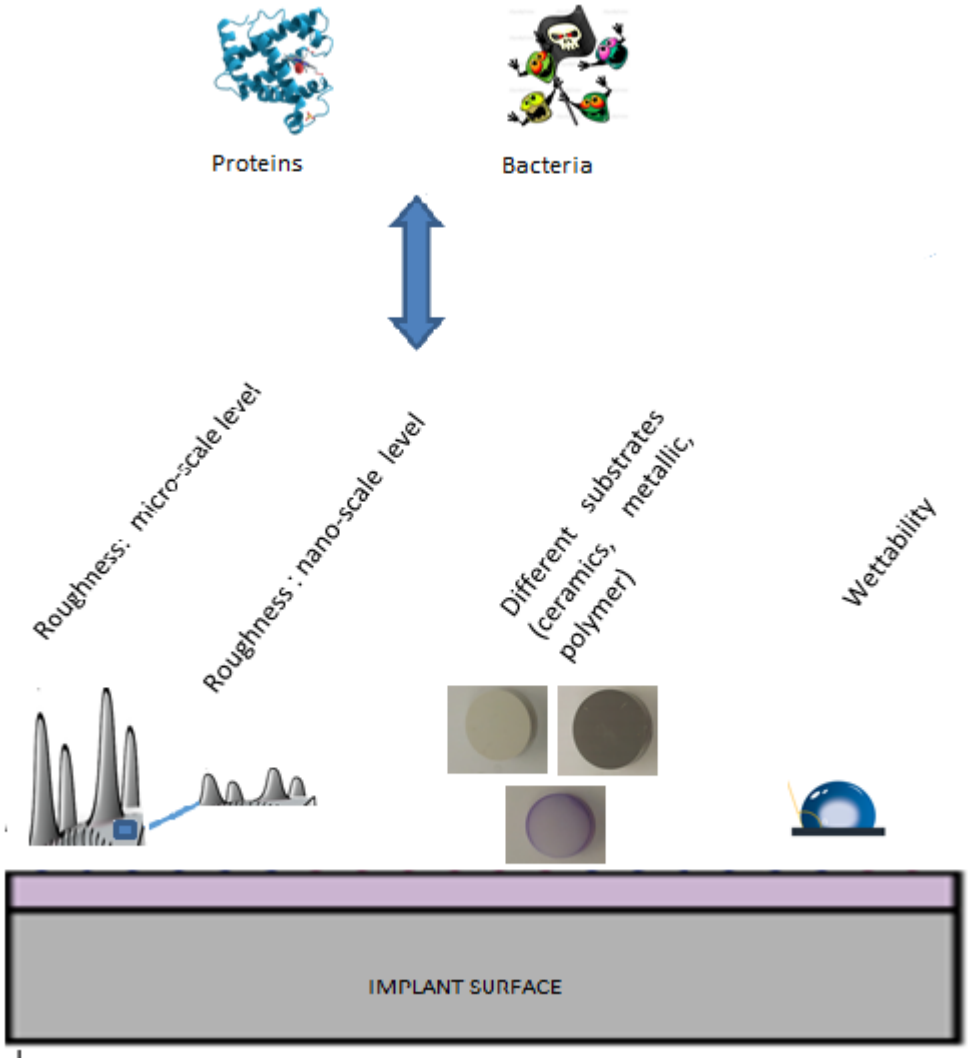


Figure 3.1 Effect of surface characteristics on bacterial adhesion and protein adsorption

3.2.Surface analysis methods

The intrinsic properties depend on the internal atomic or molecular structure and this affects the physico-mechanical behavior of the material itself.

However, the interface with the environment in which the material must operate plays a crucial role in determining the subsequent events, and in this context the properties of the material surface are of fundamental importance.

Specifically, following implantation in the human body, the material surface is in contact with the fluids and biological tissues. After an initial absorption of liquids and low molecular weight substances (water, ions, lipids) the adsorption of proteins occurs: the type of protein and the mode of adsorption determine the future events of response of the human organism to the implanted device.

Possible strategies to increase the performance of devices include:

- change the chemical structure of the material while maintaining the desired mechanical properties;
- change the morphology ;
- modify the surface;
- modify the shape or construction technology of a device.

The goal is to maintain the physical properties of the material by changing only its surface to modify the interactions at the interface between device and tissue and direct themfavorably.

Many parameters characterize surface structure:

- surfaces can be rough, smooth, or stepped;
- surfaces can be composed of different chemistries (atoms and molecules);
- surfaces may be structurally or compositionally inhomogeneous in the plane of the surface;
- surfaces may be inhomogeneous with depth into the specimen;
- surfaces may be covered by an overlayer;
- surfaces may be highly crystalline or disordered.

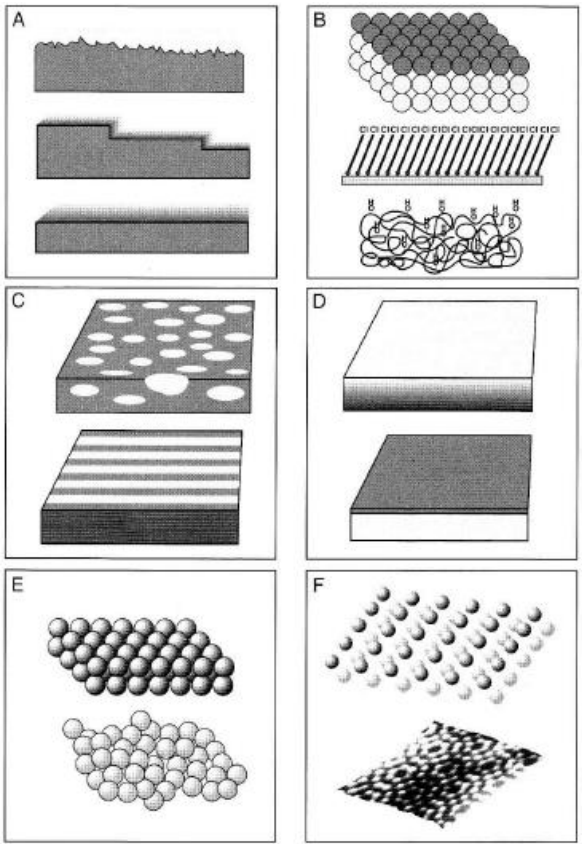


Figure 3.2 Surface properties of materials. (A) Surfaces can be rough, smooth, or stepped. (B) Surfaces can be composed of different chemistries (atoms and molecules). (C) Surfaces may be structurally or compositionally inhomogeneous in the plane of the surface. (D) Surfaces may be inhomogeneous with depth into the specimen. (E) Surfaces may be covered by an overlayer. (F) Surfaces may be highly crystalline or disordered. (Biomaterials Science 1996, p. 24)

Obviously, the more parameters it's possible to measure, the more it's possible to fully describe a surface.

A complete characterization requires the use of many techniques to gather all the necessary information.The most important parameters include roughness, wettability (critical surface tension), surface mobility, chemical composition, crystallinity on the interaction with the biological environment.

All the methods used for surface analysis can potentially alter the surfaces themselves.

So, because of the ability to create artifacts, it is necessary to collect as much information as possible with different investigation techniques.

A typical classificationof surface analysis methods is the following:

- methods that evaluate a particular property of the surface, such as wettability (throught contact angle analysis), roughness (throught laser profilometry), uniformity, thikness, optical constant;
- spectroscopic techniques, which evaluate the surface chemistry as ESCA (Electron Spectroscopy for Chemical Analysis) also named XPS(X ray Photoelectron Spectroscopy), AES (Auger Electron Spectroscopy), SIMS(Secondary Ion Mass Spectroscopy), X rays, FT-IR(Fourier Transform -Infrared Spectroscopy);
- methods to perform mass measurements to match or complement the spectroscopic techniques as TOF (TimeofFlight –*mass analysis*), QCM (QuartzCrystal Microbalance), OWLS (OpticalWaveguideLightmodeSpectroscopy), LMMA(Laser microprobe mass analysis) ;
- microscopy techniques, which assess the topography and surface morphology as OM (opticalmicroscopy), SM (stereo microscopy), SEM andTEM (Scanning e TransmissionElectron Microscopy), STM(Scanning TunnelingMicroscopy), AFM(AtomicForceMicroscopy).

3.3. Analysis of roughness on micro-scale level : profilometry

Roughness is a property of the surface, measured by profilometers.

The measurement of roughness is the recording the surface profile obtained along a given line of measurement (or scanning); this profile is analyzed by defining a numeric parameter which is the measure of the roughness.

The roughness is the surface properties of a body, formed by geometric micro imperfections, that are present on the surface, or resulting from machining.

Such imperfections can be grooves and fissure, with different shape, depth, or direction .

The measurement procedure consists in recording the profile of the surface, obtained along a certain line of measurement; that profile is analyzed by defining a numerical parameter which is the measure of the roughness.

While the historical notion of a profilometer was a device that measures a surface as the surface is moved relative to the contact profilometer's stylus, this notion is changing with the emergence of numerous non-contact profilometery techniques.

The profilometer is the instrument for the measurement and evaluation of micro-irregularities of a surface; it is able to perform measurements with a precision that can reach the thousandth of a micron.

The measurement methods may vary and can be “direct” or “indirect”.

A method of direct measurement is one that utilizes a probe profilometer, positioned at the end of a stylus (figure 3.3)

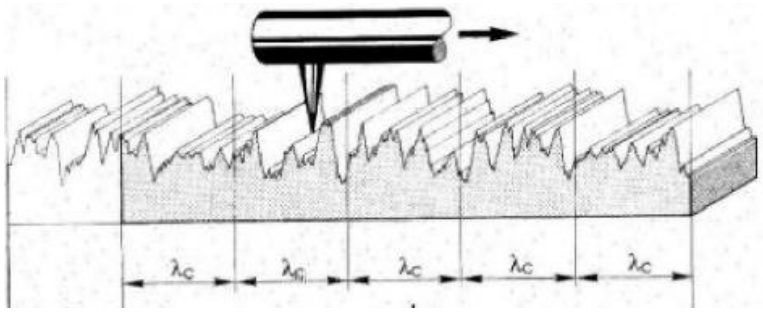


Figure 3.3 Probe profilometer
(M.C. Tanzi , Ingegneria Tessutale e Biomimetica)

A method of indirect measuring is one that uses a sensor that interprets the variations in height measured along the axis of acquisition as changes in position of the reflected beam on the surface to be probed.

An optical profilometer represents a method of non-contact measurement.

There are different techniques that can be used today, such as: laser triangulation, confocal microscopy (for very small objects), low coherence interferometry, and digital holography.

3.3.1. Definition of the experimental set up

The profilometer (figure 3.4) is an instrument for the measurement and evaluation of the micro irregularities of the surface. It is made by the following parts:

- probe: is the part that comes into contact with the surface to be measured. It can be inductive, that interprets the variations in height, measured along the axis of acquisition, as voltage variations, or optical which is equipped with a sensor that interprets the height variations as variations of the position of the reflected beam on the surface to be probed.
- sideshift: it is a motorized unit fixed by means of appropriate support to the probe that provides to move the latter along the measuring axis, in order to acquire the data of the surface.
- Electronic unit: manages the movement of the traversing units and the processing of measured data, using the touch probe. Using an inductive probe, the values of tension detected in analog format is first converted into digital format, in order to be subsequently processed and analyzed.

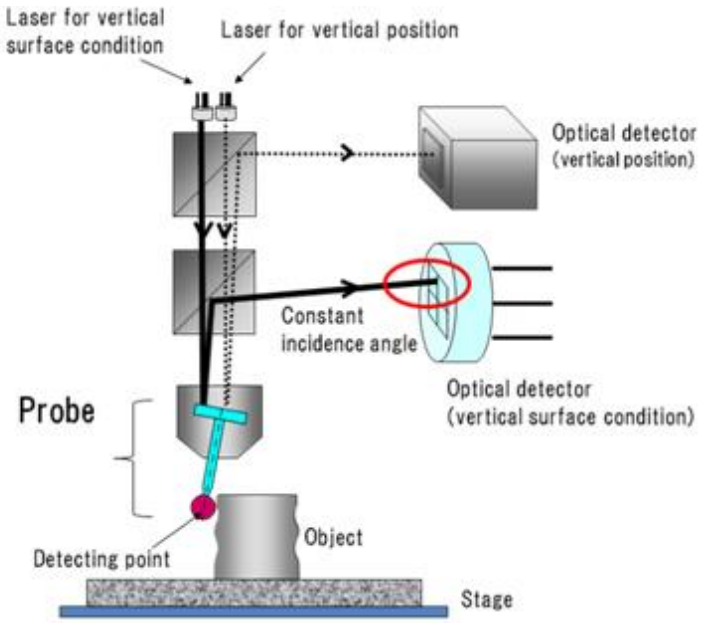


Figure 3.4. Optical system in ultrahigh accurate 3D profilometer
(*from http://news.panasonic.com/press/news/official.data/data.dir/en090717-5/en090717-5.html)*

Specimens surface roughness analysis were performed using a laser optical profilometer (UBM-Microfocus Compact, NanoFocus AG, Germany, figure 3.5). Optical profilometry (OP) is a non-contact interferometric-based method for characterizing surface topography. A typical OP analysis provides 2D and 3D images of a surface, numerous roughness statistics, and feature dimensions.

OP is suitable for numerous applications and sample types because it can accommodate many sample geometries, offering a wide range of possible analysis dimensions and a versatile Z-range, covering a broad range of potential surface roughnesses.

In this work, a laser profilometer was used (figure 3.5) ,allowing to perform measurements of roughness on all kinds of material.

Some applications include:

- measures of rugosity ;
- measures of depth ;
- measurements of steps (eg. thickness deposits);
- measures of curvature.

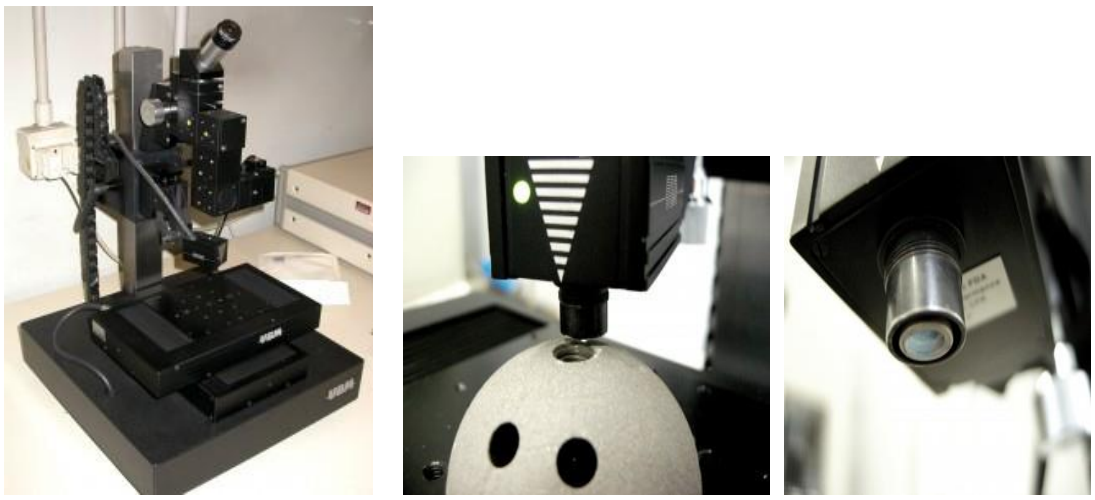


Figure 3.5 Laser profilometer : UBM-Microfocus Compact, NanoFocus AG, Germany.

It is a profilometer "no contact" based on dynamic focusing. It calculates the variations in height relative to the plane of focus, going to hit the surface of the sample via a laser beam and obtaining the reflected beam.The automatic movement of the table allows to perform a two-dimensional scan in x, y (5 cm x 5 cm).

The application software manages the automated displacement of the sample, the automatic calculation of the various parameters of roughness on a linear profile and on a surface, the calculation of thicknesses and of various parameters of curvature.

It has the advantage to be a non-destructive method of measurement for delicate surfaces, to acquire easily 3D images, and to obtain measurements on non-reflective surfaces.

The profilometer is composed of a matrix camera and a laser. The camera evaluates the bending of the laser projected on the area under test. In this way it is possible to reconstruct the 3D image starting from a 2D image, through an algorithm of triangulation. The output of the sensor is the profile of the tested object.

Over the past decade, profilometers have started using laser technology in order to improve the degree to which a surface can be measured without having to physically touch the profile being measured. The most recent laser profilometers have been able to map surfaces in 3D, thereby displaying form and texture over a large area.

The laser profilometers are fast and accurate, and display their results in both 3D and 2D outputs. They use a laser displacement sensor that measures surface topography down to

the sub-micrometer level over an area 500 mm x 500 mm in size. These profilometers digitize surfaces for mold texturing, conduct in-tray inspections, and provide information for any application that requires accurate large area surface profiling. They use precision linear motors to help measure a surface area at a rate of one meter per second. While conducting the scan, the device also collects the sample data at a rate of a micrometer per sample.

3.3.2. Materials and methods

A fundamental part of the process of calculation of the various parameters of roughness, is the filtering operation, which allows to obtain a measure of surface quality, purified from the effects that errors in workpiece geometry have on the measured profile.

The measurement of the surface roughness Ra expressed in microns, is the arithmetic value of the deviations of the real profile of the surface with respect to the midline.

Ra is therefore an average value that does not say anything about the type of irregularity (figure 3.6)

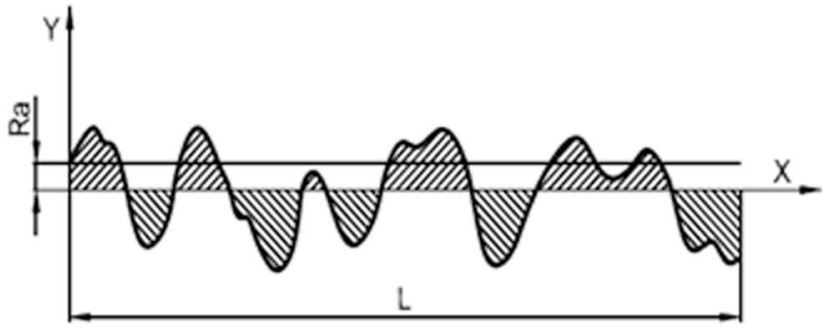


Figure 3.6 Average roughness Ra

The Ra value is not sufficient to define the morphological characteristics of the surface, because profiles with different trend that have the same average deviation, will present the same value of Ra. For this reason other parameters have been defined:

- Rq: quadratic mean deviations of the profile from the mean line ;
- Rz: distance between two straight lines parallel to the mean line drawn at a distance equal to the average of the five highest peaks and the average of the 5 lowest valleys in the range of the base length; Rz is then the average of the maximum roughness of 5 peaks and five valleys;

- Rmax: distance between two lines parallel to the midline, the first tangent to the highest peak and the second tangent to the lower valley. It is the maximum irregularity of the profile and it is a not very significant parameter, since it may be strongly influenced by an irregularity of the surface accidental.
- Ry,Rt: it is defined as the maximum distance between the highest peak and the deepest valley along a general line L

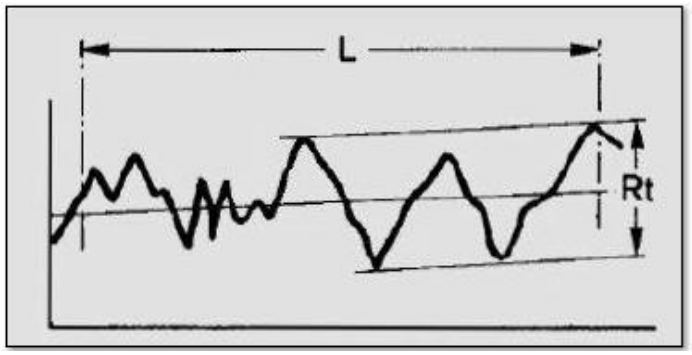


Figure 3.7 Rt

The laser profilometry allows to reconstruct the profile of an object and to measure all its parameters in order to control the dimension, position, inclination and deformation of the object under test.

The parameters of interest for the evaluation of roughness are Ra, Ry, Rmax; these parameters have been analysed to identify any differences or similarities between the various biomaterials of interest.

Specimens surface roughness analysis were performed using a laser optical profilometer (UBM-Microfocus Compact, NanoFocus AG, Germany). Briefly, specimens were gently fixed onto machine plate; each specimens surface was laser-scansioned by randomly selecting 5 representative areas. Three specimens of each tested materials were analyzed; data are expressed as means ± standard deviations of the total 15 areas laser-scansioned. Finally, considering the values obtained, software reconstructed the surface 3D topography.

Specimens

3 samples of each type of biomaterial of the study were subjected to the analysis of profilometry: CoCrMo, XLPE, Ceramic Pink (BIOLOX®delta –alumina and zirconia ceramic),Ceramic Yellow (BIOLOX®forte - alumina ceramic), Ceramic White (untreated ceramic), Ceramic Grey (silicon nitride). Samples are shown in figure 3.8. Specimens were provided by CeramTec.

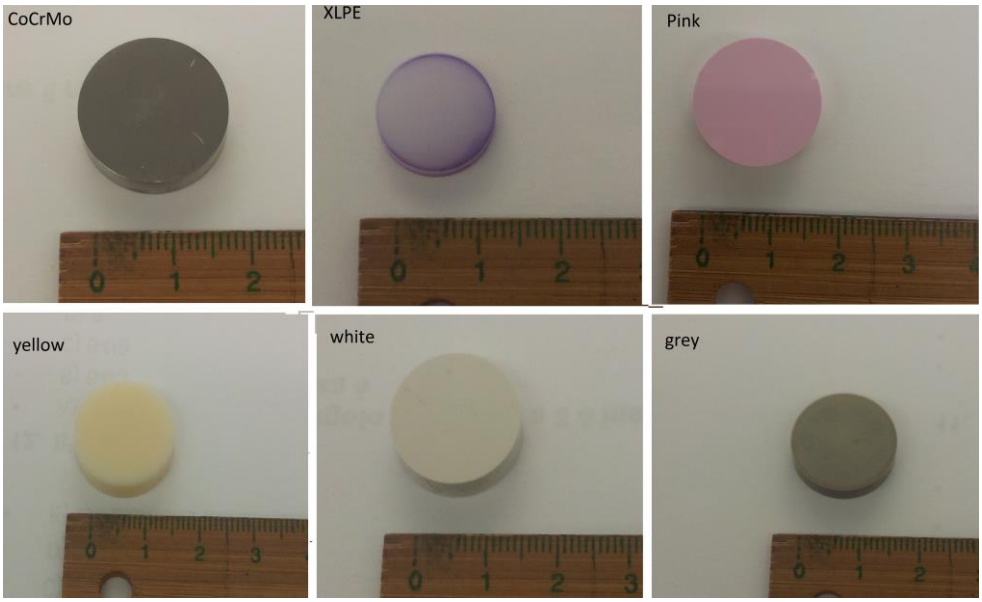


Figure 3.8 Biomaterials samples

Samples are clean and free of processing residues , powders or surface deposits.

They are analyzed considering the smoother surface finish.

These substrates have been chosen because they are widely employed for orthopaedic applications; they present different surface chemistry (metals/ceramics/polymer), they are known to be non toxic and to present a negligible ion release (at least at short times).

Specimens size: 14mm diameter and 3mm thickness disks

In order to obtain clean and comparable surfaces for analyses, washes in acetone and Millipore water in an ultrasonic bath have been performed, to remove any dirt or processing residues

Specimens sterilization and storage: CoCrMo specimens were autoclaved (121°C, 20 minutes) and stored in sterile packaging at RT until use, while XLPE disks were gas sterilized using ethylene oxide (EO) and sterile packaged until use at RT.

For each sample, five measurements were performed, and each parameter was extracted considering the mean value.

Statistical analysis of data.

Results were compared by the SPSS v20 software (IBM, Chicago, USA) using the ANOVA-one way analysis and the Sheffé test as pot-hoc. Significance was fixed at $p<0.05$.

3.4.Analysis of roughness on nano-scale level : atomic-force microscopy

The atomic force microscope is a powerful scanning probe microscope invented in 1986 by Binning, Quate and Gerber.

Using an atomic force microscope (AFM), it is possible to measure a roughness of a sample surface at a high resolution, to distinguish a sample based on its mechanical properties (hardness and roughness) and, in addition, to perform a microfabrication of a sample, as an atomic manipulation.

In the AFM technique the signal is based on the atomic forces developed between the probe and the surface to be analyzed. A feature that makes the microscope very useful in the field of surface analysis is its high resolving power, which is lower than the nanometer.

Atomic-force microscopy or scanning-force microscopy (SFM) is a very high-resolution type of Scanning probe microscopy (SPM), with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit.

It is used to investigate the surface of a material, especially after a modification with a molecular layer through different methodologies such as the use of plasma and radiation.

Compared with the SEM, the atomic force microscope has the advantage of allowing non-destructive analysis of the untreated samples; it can adapt to samples of non-conductive material, supplying a three-dimensional mapping, with an area and a scan depth limited, and with a long time required to the investigation.

The information is gathered by "feeling" or "touching" the surface with a mechanical probe. Piezoelectric elements that facilitate tiny but accurate and precise movements on (electronic) command enable the very precise scanning.

The AFM can be operated in a number of modes, depending on the application.

In general, possible imaging modes are divided into static (also called *contact*) modes and a variety of *dynamic* (*non-contact or "tapping"*) modes where the cantilever is vibrated or oscillated at a given frequency.

AFM operation is usually described as one of three modes, according to the nature of the tip motion:

- contact mode, also called static mode;
- tapping mode, also called intermittent contact, AC mode, or vibrating mode, or, after the detection mechanism, amplitude modulation AFM;

- non-contact mode, or, again after the detection mechanism, frequency modulation AFM.

In *contact mode*, the tip is "dragged" across the surface of the sample and the contours of the surface are measured either using the deflection of the cantilever directly or, more commonly, using the feedback signal required to keep the cantilever at a constant position. Because the measurement of a static signal is prone to noise and drift, low stiffness cantilevers are used to boost the deflection signal. Close to the surface of the sample, attractive forces can be quite strong, causing the tip to "snap-in" to the surface. Thus, contact mode AFM is almost always done at a depth where the overall force is repulsive, that is, in firm "contact" with the solid surface below any adsorbed layers.

In *tapping mode*, the cantilever is driven to oscillate up and down at its resonance frequency by a small piezoelectric element mounted in the AFM tip holder similar to non-contact mode.

The interaction of forces acting on the cantilever when the tip comes close to the surface, Van der Waals forces, dipole-dipole interactions and electrostatic forces, cause the amplitude of this oscillation to decrease as the tip gets closer to the sample.

A tapping AFM image is produced by imaging the force of the intermittent contacts of the tip with the sample surface.[164]

In non-contact atomic force microscopy mode (figure 3.9) the tip of the cantilever does not contact the sample surface. The cantilever is instead oscillated at either its resonant frequency (frequency modulation) or just above (amplitude modulation) where the amplitude of oscillation is typically a few nanometers (<10 nm) down to a few picometers.[165]

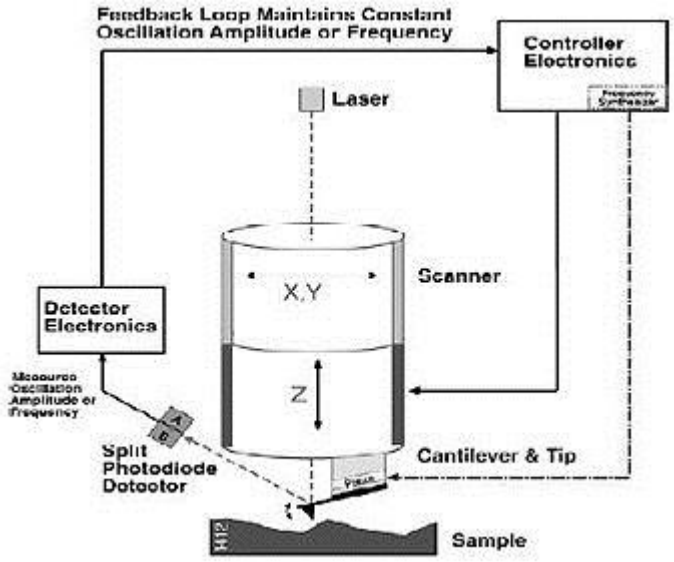


Figure 3.9 AFM – non-contact mode

Non-contact mode AFM does not suffer from tip or sample degradation effects that are sometimes observed after taking numerous scans with contact AFM. This makes non-contact AFM preferable to contact AFM for measuring soft samples, such as biological samples and organic thin film.

In the case of rigid samples, contact and non-contact images may look the same. However, if a few monolayers of adsorbed fluid are lying on the surface of a rigid sample, the images may look quite different. An AFM operating in contact mode will penetrate the liquid layer to image the underlying surface, whereas in non-contact mode an AFM will oscillate above the adsorbed fluid layer to image both the liquid and surface.

3.4.1. Definition of the experimental set up

The atomic-force microscopy used in this experimental thesis (NT-MDT, Moscow, Russia. Figure 3.10) is in contact mode (static mode). Then the tip touches the surface of the samples with horizontal direction of scan.

Figure 3.10 AFM NT-MDT, Moscow, Russia

Tip characteristics:

- standard size (1,6mm x 3,4mm) and thikness (0,3mm) of cantilever chip;
- Au high reflectivity coating : reflective property is increased by 3 times in comparison with uncoated cantilevers;
- typicalcurvature radius of the tip is 10nm;
- tip height 14-16μm

The Data Sheet is shown in figure 3.11

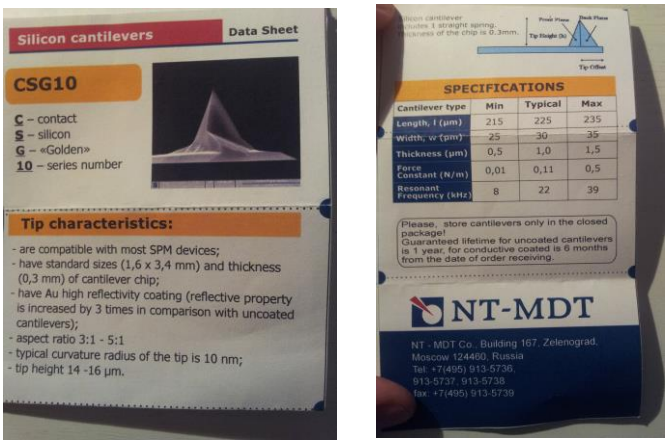


Figure 3.11 Data Sheet

The principal element of an atomic force microscope is the *cantilever*, which is a flexible lever of which the elastic constant is known; on it, a very thin tip is mounted, the *tip*, with a radius of curvature of nanometers , made of silicon nitride or silicio.

The tip is placed very near the sample surface , at a distance of a few nanometers. The Van der Waals forces acting between the tip and the sample, cause a deflection of the cantilever, in accordance with Hooke's law.

Following the interactions between the tip and the surface of the sample, the deflection of the cantilever it's recorded, by detecting a laser beam reflected from the lever and sent to a photodetector. From the photodetector, an electrical signal proportional to the interaction force between the tip and the sample is obtained.

A system of piezoelectric positioners allows movement of the probe in the 3 dimensions. During scanning, a system back-action makes the applied force constant; therefore the probe follows the profiles of the object surface.

The micro deflections are translated in a tridimensional topographic image of the sample surface.

As shown in figure 3.12 AFM is typically consisted of following features:

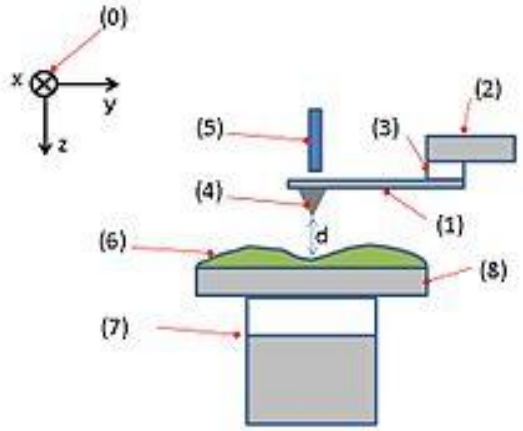


Figure 3.12 Typical configurations of AFM: (1):Cantilever ,(2):Support, (3): Piezoelectric element, (4):Tip, fixed to open end of a cantilever ; (5):Detector ; (6): Sample; (7):xyz-drive, to move the sample and sample stage to be displaced in x, y, and z directions ; (8):Stage.

- the cantilever : small spring-like cantilever is supported on the support by means of a piezoelectric element, so as to oscillate the cantilever at its eigen frequency. The tip is fixed to open end of a cantilever .

-the detector: it is configured to detect the deflection and motion of the cantilever . Sample measured by AFM are mounted on Sample stage .

-The xyz – drive: it permits a sample and sample stage to be displaced in x, y, and z directions with respect to a tip apex.

The detector of AFM measures the deflection (displacement) of cantilever and converts it into an electrical signal. So, during the oscillating (that is to say a change in the defection of cantilever with respect to time) motion, the output of the detector of AFM is a Time-Intensity curve, the Intensity will be proportional to the displacement of cantilever.

The motion of cantilever is regarded mostly as a sinusoidal vibration in the steady state. So, the output will approximately regarded as a sine wave. As the sinusoidal vibration is characterized by three factors : frequency, amplitude, and phase.

The image formation is a plotting method as a color mapping through changing the x-y position of the tip by scanning and corresponding some particular amount (a measured variable/the intensity of control signal) to each x-y coordinate. In this respect, the color mapping is a method which corresponds a value to each coordinate and shows it. It means the image which especially expresses the intensity of a value as a hue.

AFM scanners (figure 3.13) are made from piezoelectric material, which expands and contracts proportionally to an applied voltage. The scanner is constructed by combining independently operated piezo electrodes for X, Y, and Z into a single tube, forming a scanner that can manipulate samples and probes with extreme precision in 3 dimensions. Independent stacks of piezos can be used instead of a tube, resulting in decoupled X, Y, and Z movement.

Scanners are characterized by their sensitivity, which is the ratio of piezo movement to piezo voltage, by how much the piezo material extends or contracts per applied volt.

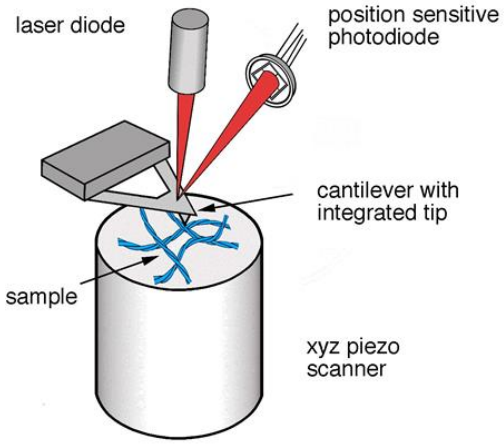


Figure 3.13 AFM scanner

The sensitivity of piezoelectric materials decreases exponentially with time. This causes most of the change in sensitivity to occur in the initial stages of the scanner's life. Piezoelectric scanners are run for approximately 48 hours before they are shipped from the factory so that they are past the point where they may have large changes in sensitivity. As the scanner ages, the sensitivity will change less with time and the scanner would seldom require recalibration, though various manufacturer manuals recommend monthly to semi-monthly calibration of open loop AFMs.

3.4.2. Materials and methods

Using an atomic force microscope (AFM), it is possible to measure a roughness of a sample surface at a high resolution.

In this analysis, two scans were performed for each sample (20 μm and 50 μm), and the parameter of interest has been extracted (Arithmetic mean height Sa). For each kind of biomaterial, 3 samples were analyzed, to perform comparisons about the surface morphology with nanometer resolution, between the different materials.

The operation modes of AFM are generally classified into image formation and the others. The image formation, , is a plotting method as a color mapping through changing the x-y position of the tip , by scanning and corresponding some particular amount (a measured variable/the intensity of control signal) to each x-y coordinate. In this respect, the color mapping is a method which corresponds a value to each coordinate and shows it. It means the image which especially expresses the intensity of a value as a hue. Usually, the correspondence between the intensity of a value and a hue is shown as a color scale in the explanatory notes. The other group includes various matters such as force spectroscopy and potential mapping.

The AFM provides a three-dimensional surface profile. In addition, samples viewed by AFM do not require any special treatments (such as metal/carbon coatings) that would irreversibly change or damage the sample, and does not typically suffer from charging artifacts in the final image.

AFM can also be combined with a variety of optical microscopy techniques such as fluorescent microscopy, further expanding its applicability. Combined AFM-optical instruments have been applied primarily in the biological sciences but have also found a niche in some materials applications, especially those involving photovoltaics research[164] .

A disadvantage of AFM compared with the scanning electron microscope (SEM) is the single scan image size. In one pass, the SEM can image an area on the order of squaremillimeters with a depth of field on the order of millimeters, whereas the AFM can only image a maximum height on the order of 10-20 micrometers and a maximum scanning area of about 150×150 micrometers. One method of improving the scanned area

size for AFM is by using parallel probes in a fashion similar to that of millipede data storage.

The scanning speed of an AFM is also a limitation. Traditionally, an AFM cannot scan images as fast as a SEM, requiring several minutes for a typical scan, while a SEM is capable of scanning at near real-time, although at relatively low quality.

As with any other imaging technique, there is the possibility of image artifacts (figure 3.14), which could be induced by an unsuitable tip, a poor operating environment, or even by the sample itself, as depicted on the right. These image artifacts are unavoidable; however, their occurrence and effect on results can be reduced through various methods.

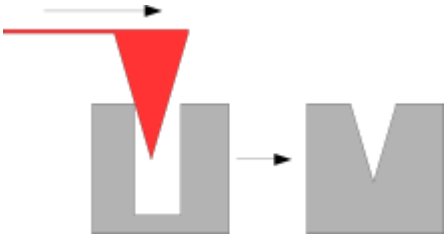


Figure 3.14 AFM artifact, steep sample topography

Specimens

3 samples of each type of biomaterial of the study were subjected to the analysis of AFM: CoCrMo, XLPE, Ceramic Pink (BIOLOX®delta –alumina and zirconia ceramic),Ceramic Yellow (BIOLOX®forte-alumina ceramic), Ceramic White (untreated ceramic),Ceramic Grey (silicon nitride). Samples are shown in figure 3.15. Specimens were provided by CeramTec.

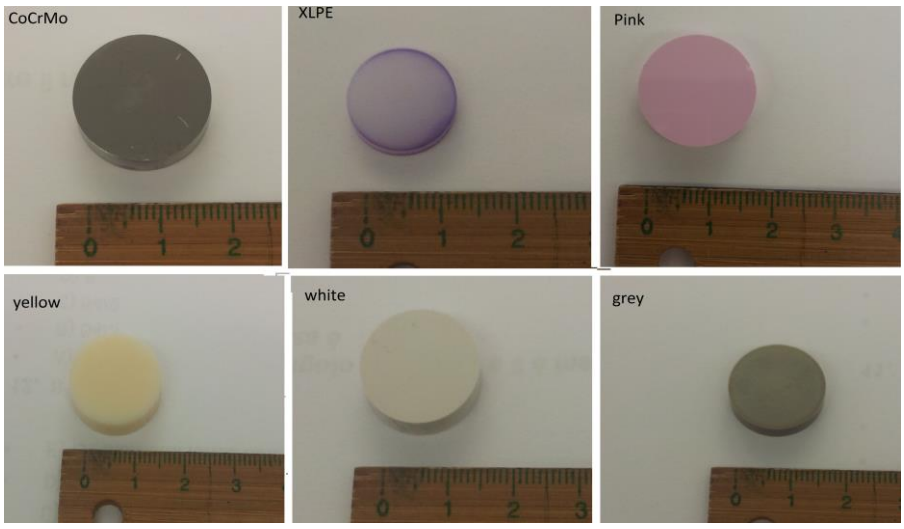


Figure 3.15 Biomaterials samples

Samples are clean and free of processing residues , powders or surface deposits.

They are analyzed considering the smoother surface finish.

Two scans were made: 20 microns and 50 microns; for each scans, the 2D and 3D image has been formed and the value of average rugosity Sa has been extracted ; subsequently an analysis of the parameters has been performed, to compare different biomaterials.

Specimens size: 14mm diameter and 3mm thickness disks

These substrates have been chosen because they are widely employed for orthopaedic applications; they present different surface chemistry (metals/ceramics/polymer), they are known to be non toxic and to present a negligible ion release (at least at short times).

Specimens sterilization and storage: CoCrMo specimens were autoclaved (121°C, 20 minutes) and stored in sterile packaging at RT until use, while XLPE disks were gas sterilized using ethylene oxide (EO) and sterile packaged until use at RT.

3.5. Analysis of surface wettability: contact angle

The contact angle is one of the most simple techniques of surface analysis. It allows to evaluate the wettability of a surface: the lower is the contact angle, the higher is the degree of wettability of the surface.

The contact angle is defined as the angle between the tangent to the profile of the drop in the point of contact between the phases, and the line of contact between the liquid phase and the solid substrate.

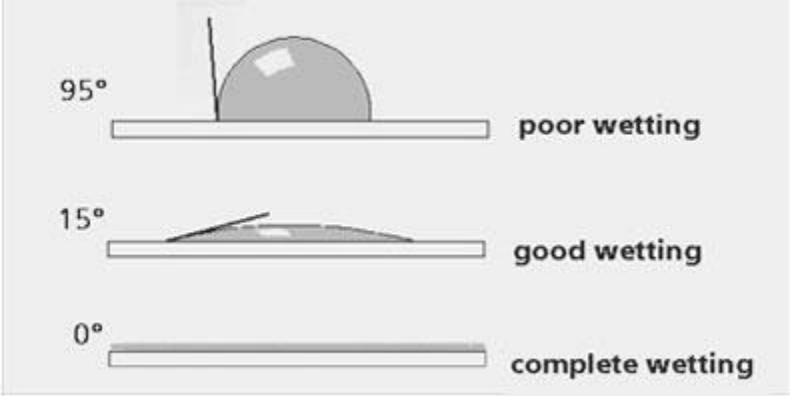


Figure 3.16. Contact angles for hydrophobic and hydrophilic surfaces.

Wetting refers to the study of how a liquid deposited on a substrate spreads out or the ability of liquids to form boundary surfaces with solid states. The wetting is determined by measuring the contact angle, which the liquid forms in contact with the solids or liquids.

The angle is determined by both properties of the solid and the liquid and the interaction and repulsion forces between liquid and solid and by the three phase interface properties (gas, liquid and solid). Those interactions are described by cohesion and adhesion forces which are intermolecular forces.

- The smaller the contact angle - cohesive forces are weaker then adhesive forces and molecules of the liquid tend to interact more with solid molecules then liquid molecules.

- The larger the contact angle - cohesive forces are stronger then adhesive forces and the molecules of the liquid tend to interact more with each other then with the solid molecules.

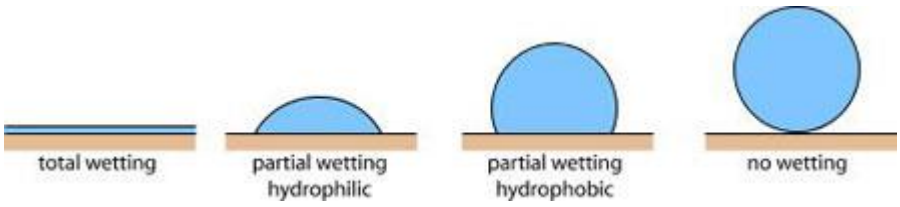


Figure 3.17 On a hydrophilic surface a water drop spreads out to increase the contact surface. On a hydrophobic surface a water drop contracts to minimize the contact surface.

The strength of the attractive or repellant force is closely related to the "contact angle" between the water drop and the surface (see Figure 4). On a hydrophilic surface, the contact angle will be less than 90°; the water drop tends to spread out and "wet" the surface. On the other hand, if the surface is hydrophobic, the contact angle will be greater than 90°, and instead, the water drop tends to bead up on the surface.

An obtuse contact angle occurs when the liquid adheres to the solid less than with itself.

Low values of contact angle θ indicates that the liquid spreads, or wets well, while high values indicates poor wetting.

If the angle θ is < 90°, the liquid is said to wet the solid; if it > 90° it is said to be non-wetting; a zero contact angle represents complete wetting.

Thomas Young, treating the contact angle of a liquid with a surface as the mechanical equilibrium of a drop resting on a plane solid surface, has developed the theory underlying the formation of the contact angle, according to which a thermodynamic equilibrium is reached owing to the interaction of three phases.

The parameters present in the equilibrium condition, known as the Young equation, are:

- γ_{lv} : energy at the interface of the liquid and vapor phases
- γ_{sl} : energy at the interface of the solid and liquid phases)
- γ_{sv} : energy at the interface of the solid and vapor phase).

This lead to *Young's equation*:

$$\gamma_{sv}-\gamma_{sl}=\gamma_{lv}\cos\theta \qquad (1)$$

In general, knowing the energy at the interface, it's possible to define the contact angle θ.

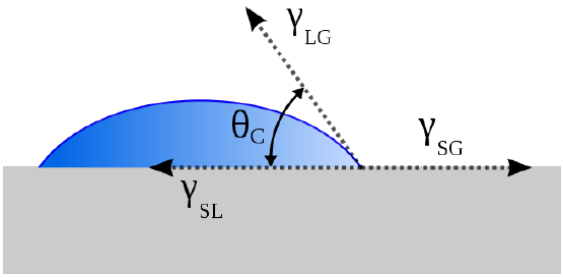


Figure 3.18 Contact action for the wetting of a surface

Comparing the wettability with the measure of theta, it's possible to examine four borderline cases, shown in figure 3.19:

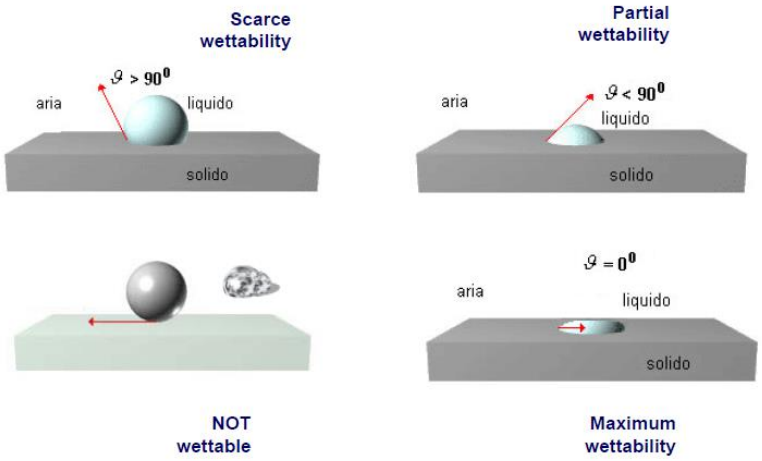


Figure 3.19 Wettability compared with the measure of angle
(M.C. Tanzi , 2014, Ingegneria Tessutale e Biomimetica)

3.5.1. Definition of the experimental set up

The most widely used technique of contact angle measurement is a direct measurement of the tangent angle at the three-phase contact point on a drop profile.

The equipment consists of a horizontal stage to mount a sample, a micrometer pipette to form a liquid drop, an illumination source, and a telescope connected to the software. The measurement was achieved by simply aligning the tangent of the drop profile at the contact point with the surface and reading the protractor.

A camera can be integrated to take photographs of the drop profile so as to measure the contact angle at leisure [166]. The use of relatively high magnifications enables a detailed examination of the intersection profile [167].

This direct optical method is advantageous because of its simplicity and the fact that only small amounts of liquid (a few microliters) and small surface substrates (a few square millimeters) are required. On the other hand, there is a relatively higher risk/impact of impurities due to the small size of the liquid and substrate.

A background light is used to assist observation, while a specific light source is selected to avoid undesired heating of the liquid or substrate.

To establish an advancing contact angle, it is best to slowly grow the drop to a diameter of approximately 5 mm using a micrometer syringe.

The needle diameter should be as small as possible so it does not distort the drop profile shape.

For a relatively large substrate, contact angles should be measured at multiple points to give an average value that is representative of the entire surface.

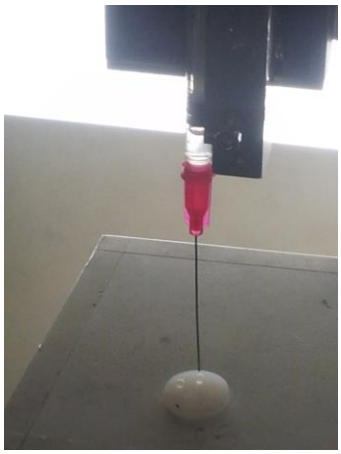
The dependence of the contact angle on the drop size can causes a systematic problem [168, 169] and surface heterogeneity or roughness could well cause variations of the contact point along the three-phase contact line.

This method is considered to be the most convenient method if high accuracy is not required [170]. Ideally, contact angle measurements should be made inside an enclosed chamber to exclude airborne contamination and establish an equilibrium vapor pressure of the liquid tested, which is especially preferable when the test liquid is volatile.

It has been observed that evaporation can cause the liquid front to retract, and that a retreating or an intermediate contact angle is recorded unintentionally. However, the

inherent inaccuracy of the direct measurement technique and the use of liquids with high boiling points make the enclosed chamber unnecessary in many cases.

a. Camera and software



b. Needle

Figure 3.20 Experimental set-up used in the analysis

3.5.2. Materials and methods

Before the tests of wettability a rigorous surface cleaning protocol for all the tested materials (washes in acetone and Millipore water in an ultrasonic bath) has been performed, to remove any dirt or processing residues.

The contact angle can be measured according to two different methodologies:

- Static contact angle:

A drop of liquid with a nominal volume constant is placed on the surface examined and the angle θ is measured.

The angle θ varies over time because the volume of the droplet changes due to different causes:

• Evaporation of the liquid;

• Migration of substances from the solid surface to the liquid;

• Migration of substances present in the drop towards the surface;

• Chemical reactions between solid and liquid;

• Dissolution of part of the solid surface in the liquid;

- Dynamic contact angle:

If the three phase (liquid/solid/vapor) boundary is in actual motion, the angles produced are called Dynamic Contact Angles and are referred to as ‘advancing’ and ‘receding’ angles.
The difference between ‘advanced’ and ‘advancing’, ‘receded’ and ‘receding’ is that in the static case motion is incipient in the dynamic case motion is actual.
Dynamic contact angles may be assayed at various rates of speed. Dynamic contact angles measured at low velocities should be equal to properly measured static angles.
In general, when the liquid droplet does not penetrate into the substrate (eg. water on glass) the interaction can be characterized by the static contact angle, if the surface is smooth and homogeneous; while, when the liquid penetrates into or expands along the surface of the sample, the interaction should be characterized by the dynamic contact angle, as a function of time.

The phenomenon of wetting is more than just a static state. The liquid moves to expose its fresh surface and to wet the fresh surface of the solid in turn. The measurement of a single static contact angle to characterize wetting behavior is no longer adequate. If the three-phase contact line is in actual motion, the contact angle produced is called a “dynamic” contact angle. In particular, the contact angles formed by expanding and contracting the liquid are referred to as the advancing contact angle θa and the receding contact angle θr , respectively. These angles fall within a range, with the advancing angles approaching a maximum value, and the receding angles approaching a minimum value. Dynamic contact angles can be measured at various rates of speed.
At a low speed, it should be close or equal to a properly measured static contact angle.
In this experimental work, the interaction has been characterized by the static contact angle.
A drop of constant volume is created and rested on the surface of the sample.
On each sample, five measurements are performed, because 5 drops have been deposited in different parts of the surface.
The software (figure 3.21) is Drop Shape Analysis for Windows, which reconstructs the two dimensional image and provides the contact angle.
The liquid used for this test is distilled water.

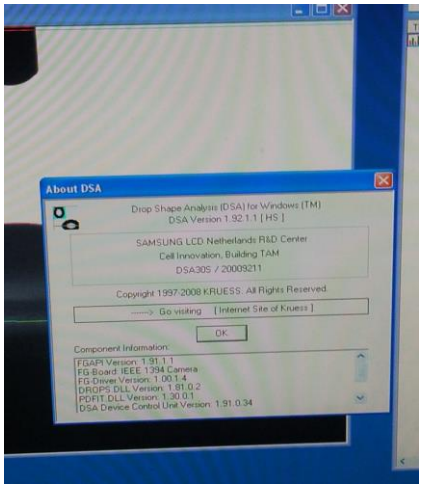


Figure 3.21 Software for the image reconstruction

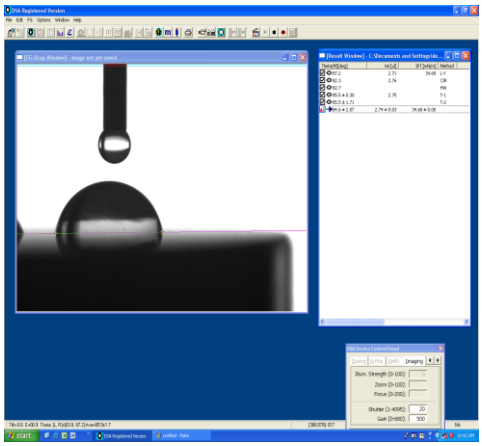


Figure 3.22 Exemple of image reconstruction

Specimens

3 samples of each type of biomaterial of the study were subjected to the analysis of wettability: CoCrMo, XLPE, Ceramic Pink (BIOLOX®delta –alumina and zirconia ceramic), Ceramic Yellow (BIOLOX®forte - alumina ceramic), Ceramic White (untreated ceramic), Ceramic Grey (silicon nitride). Samples are shown in figure 3.23. Specimens were provided by CeramTec.

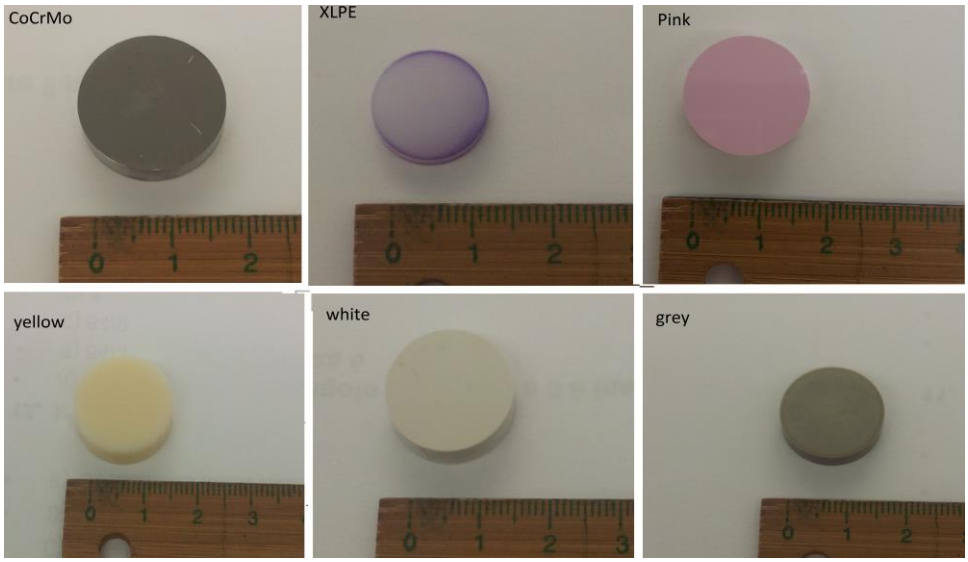


Figure 3.23 Biomaterials samples

Samples are clean and free of processing residues , powders or surface deposits.

They are analyzed considering the smoother surface finish.

Specimens size: 14mm diameter and 3mm thickness disks

In order to obtain clean and comparable surfaces for analyses, washes in acetone and Millipore water in an ultrasonic bath have been performed, to remove any dirt or processing residues.

These substrates have been chosen because they are widely employed for orthopaedic applications; they are different biomaterials (metals/ceramics/polymer), they are known to be non toxic and to present a negligible ion release (at least at short times).

Specimens sterilization and storage: CoCrMo specimens were autoclaved (121°C, 20 minutes) and stored in sterile packaging at RT until use, while XLPE disks were gas sterilized using ethylene oxide (EO) and sterile packaged until use at RT.

3.6. Simulation of bacterial infection: *in vitro* tests

The drawback of the technological advance in biomaterial field is the high susceptibility of implants to infection. In addition, such infections chronically persist, if no comprehensive treatment concept is applied.

Whereas acute infection generally needs only one single antimicrobial treatment course to eradicate microorganisms, chronic infection may require sophisticated diagnostic procedures, long-term antimicrobial therapy and repetitive surgical interventions. In the case of orthopedic device-associated biofilm infection, cure of infection should be aimed at in the first attempt, because each treatment failure results in a worse functional result. Thus, the prerequisite for correct treatment of device associated infection is a rational concept for the optimal surgical and antimicrobial therapy.

In this section, through an *in vitro* bacterial culture, a simulation of bacterial infection on metal and cross-linked polyethylene samples is performed, in order to observe the microbiological behavior in relation to the different surface properties, and to compare different biomaterials used in orthopaedics; the aim is to identify which biomaterial presents the best antibacterial performances.

This section reports on progress microbiological experiments on disks provided by CeramTec.

The experiments were aimed to study the early phase of bacteria biofilm formation onto specimens in comparison with polystyrene, considered as gold standard.

Staphylococcus aureus (S. aurues) and *Staphylococcus epidermidis (S. epidermidis)* were considered as contaminant bacteria separately.

3.6.1. Definition of analysis methods : MTT and SEM analysis

This section analyses the metabolic activity and the morphological observation of the biofilm; it reports the valuation of biomaterials surfaces biofilm colonization through MTT and SEM analysis.

Biofilm is a complex protected arrangement self developed by bacteria to survive in a hostile environment with respect to planktonic forms.

Particular, it allows them to optimize nutrient uptake, to be sheltered from the removal forces, and to be protected from the host defense mechanisms and from the potential toxic or harmful including antibiotics.

The bacterial growth curve (figure 3.24) provides an indication of the temporal growth in a bacterial population; it is divided into four sections:

- Lag time: it's the time it takes for the organism to adapt to the environment; this time interval is longer or shorter depending on the conditions
- Exponential growth phase (16hs): the organism multiplies rapidly; it's possible to keep the crops at this stage by transferring the bacteria in new lands (continuous culture).
- Stationary phase: the micro-organism stops its growth, because nutrients are completed, and it will form the matrix of protection. Bacteria that are divided and those who die are in equilibrium, some cells enter a state of latency waiting for better conditions. Some bacteria at this stage start to produce secondary metabolites, hindering the vitality of competitors microorganisms.
- Decline and death: the number of microorganisms drops, because dead cells exceed those in division or latency phase.

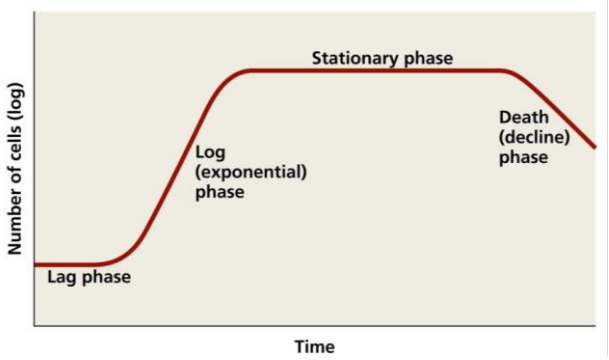


Figure 3.24 Bacterial growth curve

The study of biofilm development in direct contact with the biomaterials is the best way to evaluate the effect of the materials itself and its surface on the adhesion of microbial cells and develop of bacteria community.

Available methodologies to study biofilm formation range from the quantification of the slime produced by bacteria, to morphological evaluation of the structure using electron scanning microscopy.

Biofilm quantification may be performed by indirect evaluation of metabolic activity: it's evaluated the number of viable cells using a colorimetric assays, for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a violet color.

The method allows to evaluate the metabolic activity of cells, and indirectly the amount of viable cells grown onto the whole surface of the materials, providing a reliable quantitative data of the degree of contamination of any sample.

The following figure (figure 3.25) shows the reaction of the MTT assay and the color changing due to the reduction of the reagents.

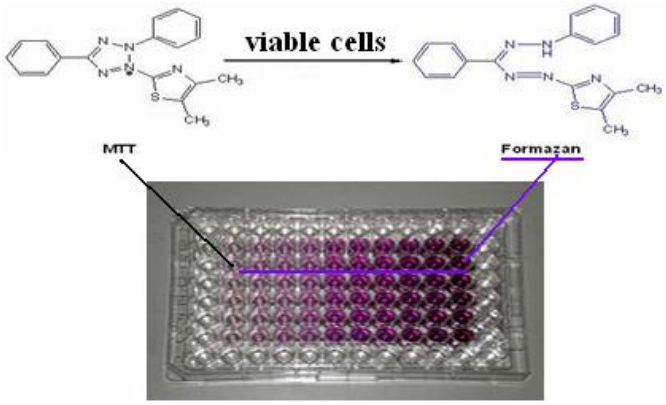


Figure 3.25 MTT Assay. MTT solution is reduced only by viable cells into violet crystals by a metabolic process related to cells respiration.

The morphological observations of the biofilm is performed using scanning electron microscopy (SEM) in order to verify if the biofilm covered uniformly or not the surface of the materials , and if the bacteria are concentrated in correspondence of some particular area of the surface. This approach provide useful information particularly regarding materials, but any conclusions are limited to the fields observed. Actually, the observation is qualitative and limited to some fields, and does not describe the whole specimens.

3.6.2. Materials and Methods

Experiments were carried to study the intrinsic antimicrobial properties of the material.

The tests were performed using 14 mm wide – 3 mm thick disks in comparison with polystyrene (commercial, Nunc Delta Surface, Themo-Scientific, considered as control) using *S. aureus* and *S. epidermidis* strains.

Metallic materials received from CeramTec were autoclaved and stored in sterile packs at RT till to the beginning of experimentation. Polymeric XLPE materials were provided by CeramTec, sterilized by EO gas exposition and stored at RT until use.

The aim of the research is to study the antifouling properties of the materials. The analyses are focused on the evaluation of bacterial adhesion to the surface of the materials in comparison with those of polystyrene.

This experimentation is addressed to evaluate the amount of viable biomass adhering to materials surfaces using *S. aureus* and *S. epidermidis* strains, often responsible of the failure of implanted materials used in orthopedic prosthetic rehabilitation.

Goal of the experimentation was to study:

- Bacterial viability on the surfaces (metabolic analysis) by MTT colorimetric assay;
- Biofilm thickness (morphological analysis) by SEM analysis and 3D images elaboration by ImageJ software.

Finally, obtained results were compared with historical data of ceramic materials providing a statistical comparison to determine which of the tested materials presents the best intrinsic antibacterial performance for orthopedics applications.

Specimens:

- Each assays are performed using 4 specimens (CoCrMo , XLPE, ceramic) (figure 3.26) . Specimens were provided by CeramTec.
- Specimens size: 14mm diameter and 3mm thickness disks
- Specimens sterilization and storage: CoCrMo specimens were autoclaved (121°C, 20 minutes) and stored in sterile packaging at RT until use, while XLPE disks were gas sterilized using ethylene oxide (EO) and sterile packaged until use at RT.

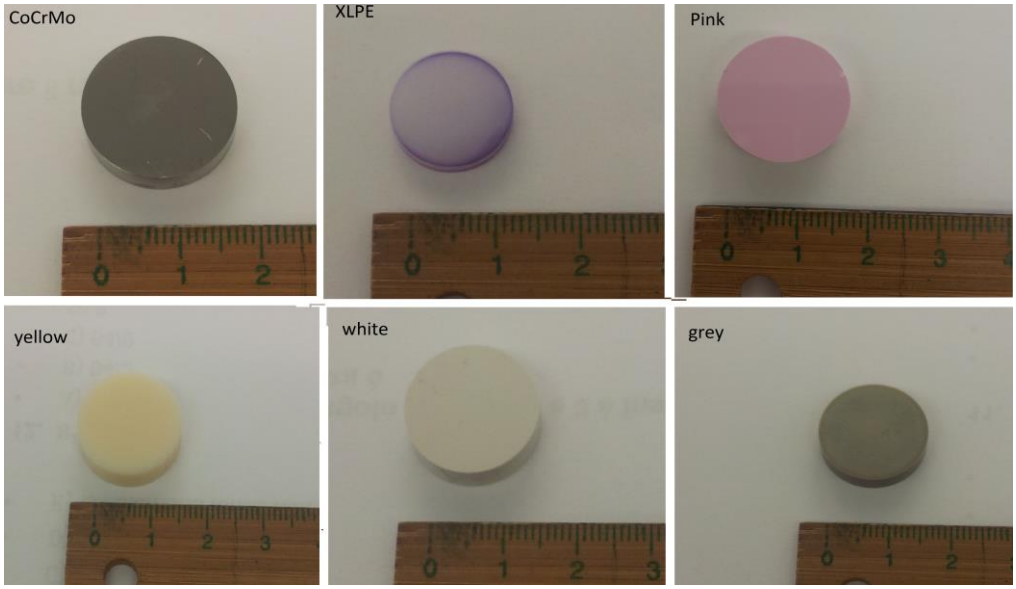


Figure 3.26 Biomaterials samples

These substrates have been chosen because they are widely employed for orthopaedic applications; they are metals and polymer material), they are known to be non toxic and to present a negligible ion release (at least at short times).

Bacterial strains and growth conditions:

All culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, NJ, USA), and all reagents for the microbiological procedures including biomass assessment were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Two strong biofilm-producing bacterial strains (*Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* ATCC 25923) were obtained from International PBI (International PBI Spa, Milan, Italy). Bacterial strains were frozen at -80°C, kept one night in Luria Bertani (LB) broth culture at 37°C into an incubator, in thermal agitation (120rpm). Afterwards, bacteria suspension were collected and stored at 4°C. Prior to each experiments, and adequate amount of bacteria were collected, suspended in sterile buffered saline (PBS) solution at pH = 7.4, and diluted to obtain a broth culture with a final concentration of $1x10^7$ cells/ml according to McFarland 1.0 standard to simulate the bacterial infection. Fresh broth culture were prepared prior each experiments.

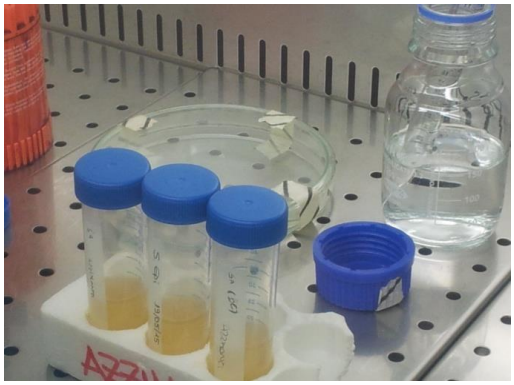


Figure 3.27 Bacterial strains in culture medium

Laboratory instrumentation :

- Nipper
- Pipette
- PBS sterile buffered saline solution at pH = 7.4, diluted to obtain culture broth with a final concentration of 1 * 10^7 bacteria / mL, to simulate the bacterial infection
- Incubator
- Chemical hood
- Polystyrene wells as control
- PS plates

Figure 3.28 Nipper

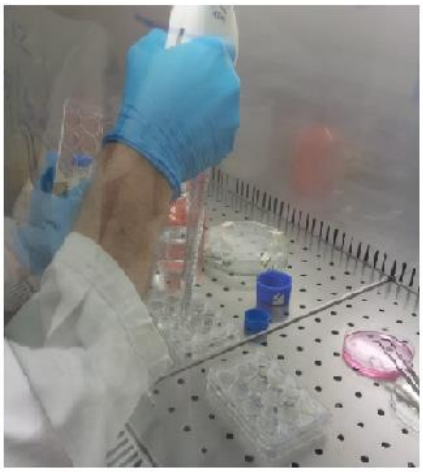


Figure 3.29 Pipette

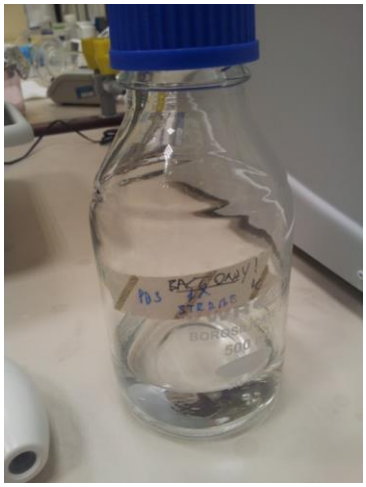


Figure 3.30 PBS sterile saline solution

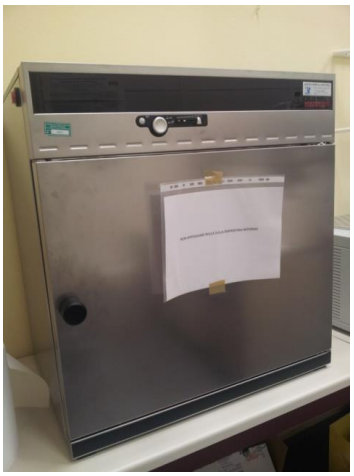


Figure 3.31 Incubator

Figure 3.32 Chemical hood

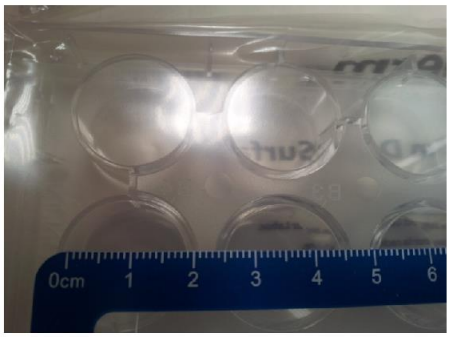


Figure 3.33 Polystyrene wells

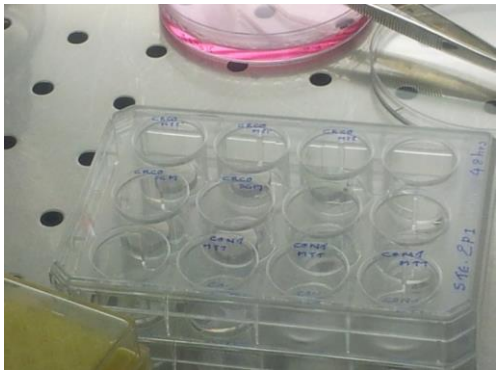


Figure 3.34 PS plates , with 12 wells, in which disks are placed

Biofilm formation onto specimens:

Specimens disks were placed into the wells of a 12 multiwell plate (Nunc Delta, Thermo-Scientific) and infected with 1 ml/well of *S. aureus* or *S. epidermidis* bacteria suspension prepared as previously described. Plate was then incubated 90 minutes in agitation (90 rpm) to allow biofilm cells adhesion (separation phase) while planktonic cells remain in the supernatant. Then, surnatants containing planktonic cells were removed and samples submerged with 1 ml of fresh LB medium and cultivated at 37°C for 24 and 48 hours (biofilm growth phase).

Quantification of viable biomass:

At the end of each time-point (24-48 hs), biofilm viable mass was evaluated by the metabolic MTT assay. MTT stock solution was prepared by dissolving 3 mg/mL of [3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide] powder in sterile PBS; PMS stock solution was prepared by dissolving 0.3 mg/mL of N-methylphenazonium methyl sulphate powder in sterile PBS. All solutions were stored at 4°C in light-proof vials until utilization. Before quantification experiments, a fresh measurement solution (FMS) was prepared by mixing 1 mL of MTT stock solution, 1 mL of PMS stock solution, and 8 mL of sterile PBS. After incubation period (24 hrs), samples were carefully removed and immediately placed into new 12-well plates and washed carefully three times with 3 ml of sterile PBS to remove non-adhered cells. 2 ml of FMS were added to each well and the plates were incubated at 37°C in the dark. A lysing solution (LS) was prepared by dissolving 10% v/v of sodium dodecyl sulphate and 50% v/v dimethylformamide in distilled water. After 1 h, FMS was carefully removed and 1 ml of LS were added to each well. Finally, 80 µL were collected from each well and transferred to a 96-well plate. Absorbance was measured using a spectrophotometer (LP200, Diagnostic Pasteur, Milan, Italy) at a wavelength of 550-620 nm; results were expressed as optical density (OD) units. The analysis is performed on metal samples, XLPE samples and on PS, to obtain the control data. Then normalization on areas of the metal and polymer samples was necessary to obtain comparison data between test specimens and polystyrene controls; finally, the conversion of Optical Density into the percentage of contamination compared to the control was performed.

Scanning Electron microscopy (SEM):

One sample of each kind of contamination were processed for scanning electron microscope (SEM) analysis (figure 3.35) , to perform morphological analysis and to value the biofilm thickness. After incubation samples were washed carefully three times with 3 ml of sterile PBS to remove non-adhered cells and fixed with 2.5% glutharaldeide for 2 hours in 1M Na cacodylate buffer, washed with the latter, dehydrated with 70%, 80%, 90% and twice with 100% ethanol (10 min each) and finally treated with CO_2 at top critical point. Specimens were fixed on aluminium stubs using a conductive carbon tape, covered with a 10-nm platinum layer (Coating Unit 5100, Polaron Ltd., Hertfordshire, UK) and observed with a scanning electron microscope (Cambridge Stereoscan 360, Leica, Basel, CH) at 500, 2000 and 10000 magnifications, using secondary electrons at 12Kvs.

Figure 3.35. Scanning electron microscope

Statistical analysis of data.

Analysis of data was performed to value statistically significant difference between CoCrMo and control, and to compare the metallic behavior with ceramic materials. ANOVA one-way followed by Sheffè test was used for statistical analysis of data. Statistical significance was considered for score with p values < 0.05.

3.6.3. 3D image processing: computational model of biofilm

The quantitative assessment of the biofilm thickness after SEM analysis were performed using the ImageJ software (NIH) plug-in for surface 3D-plot analysis.

3.7. Protein adsorption on biomaterials surface: quantitative and qualitative analysis

This section reports on progress the selective protein adsorption obtained by using metal (CoCrMo), polymer (XLPE), and ceramic specimens(figure 3.35).Specimens were provided by CeramTec.

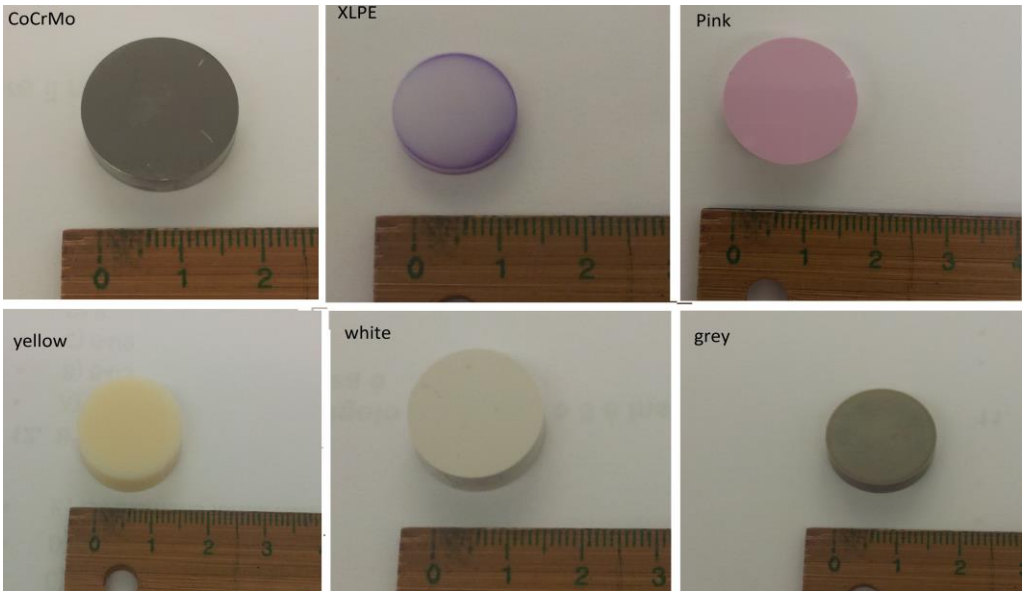


Figure 3.36 Biomaterials samples

- Samples were provided as 14 mm wide – 3 mm thick disks.
- Specimens were autoclaved and stored in sterile packages at room temperature (RT) until use.

Experiments were carried on metal (CoCrMo alloy), polymer (XLPE) and ceramic (White, Grey, Yellow, Pink) specimens in order to quantify the total amount of adsorbed protein and identify if some of the protein was selectively adsorbed. The selective protein adsorption could be an important advantage for cells adhesion and tissue repair; on the opposite, it could also promote bacteria adhesion. Admittedly, the adsorption of cells pro-

adhesion proteins including fibronectin and collagen plays an important role in cell adhesion to artificial materials, either eukaryotic or prokaryotic, and this fact benefits a quick and effective osseointegration ; whereas adsorption of anti-adhesive protein, including albumin, could determine the inhibition of cell and bacteria adhesion, representing an important advantage from bacteriological point of view .

Obviously, from a bacteriological and microbiological point of view, the biomaterial shows better performances in case of adsorption of anti-adhesive proteins, although this fact could represent disadvantage for an efficient and rapid osseointegration.

The purpose of this analysis is to investigate quantitatively the adsorbed protein on the different surfaces, and subsequently to identify biomaterials able to selectively adsorb anti-adhesive proteins, through qualitative analysis (Western blot analysis).

Based on these premises, the total amount of surface-adsorbed proteins were quantified by BCA assay using foetal bovine serum as protein source; afterwards, selective protein adsorption was investigated by Western Blot analysis.

3.7.1. Definition of the analysis methods: BCA assay and Western Blot analysis

Protein amount quantification (BCA)

The bicinchoninic acid assay (BCA assay), also known as the Smith assay, is a biochemical assay for determining the total concentration of protein in a solution (0.5 μg/mL to 1.5 mg/mL). The total protein concentration is exhibited by a color change of the sample solution from green to purple in proportion to protein concentration, which can then be measured using colorimetric techniques.

The Bicinchoninic Acid (BCA) Protein Assay, also known as the Smith Assay, is a highly sensitive colorimetric assay that is compatible with detergent solubilized protein solutions.

The BCA Protein Assay primarily relies on two reactions. Firstly, the peptide bonds in the protein sample reduce Cu^{2+} ions, in a temperature dependent reaction, from the copper solution to Cu^{+}. The amount of Cu^{2+}reduced is proportional to the amount of protein present in the solution. Next, two molecules of bicinchoninic acid (BCA) chelate with each Cu^{+} ion, forming a purple-colored product that strongly absorbs light at a wavelength of 562 nm that is linear for increasing protein concentrations between the range of 0.02-2mg/ml. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions with known concentrations.

The Bicinchoninic Acid (BCA) Protein Assay is suitable for quantifying protein solutions in 1ml assays or in micro-wells.

The assay is supplied with a traditional bovine serum albumin (BSA) protein standard or a non animal protein standard.

The BCA assay is very objective, since the universal peptide backbone contributes to color formation.

One disadvantage of the BCA assay is that it is susceptible to interference by some chemicals present in protein samples, including reducing agents, copper chelators, and buffers with high concentration, which can be avoided by generating diluted samples.

Materials and reagents to perform the assay are:

- Bovine serum abumin
- BCA protein assay reagents
- BCA working reagent (WR)
- Spectrophotometer

The BCA Protein Assay Kit is a two-component, high-precision, detergent-compatible assay reagent set to measure total protein concentration compared to a protein standard.

Features of the BCA Protein Assay Kit:

• Colorimetric- estimate visually or measure with a standard spectrophotometer or plate reader (562nm)

• Excellent uniformity—exhibits less protein-to-protein variation than dye-binding methods

• Compatible—unaffected by typical concentrations of most ionic and nonionic detergents

• Moderately fast—much easier and four times faster than the classical Lowry method

• High linearity—linear working range for BSA equals 20 to 2000 µg/mL

• Sensitive—detect down to 5 µg/mL with the enhanced protocol.

BCA provides accurate determination of protein concentration with most sample types encountered in protein research.

Selective protein adsorption - Western Blot analysis

Western blot is often used in research to separate and identify proteins. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein.

The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present.

Cell lysates are the most common form of sample used for western blot. Protein extraction attempts to collect all the proteins in the cell cytosol. This should be done in a cold temperature with protease inhibitors to prevent denaturing of the proteins.

Protein concentration is often measured using a spectrophotometer. Using this concentration allows to measure the mass of the protein that is being loaded into each well by the relationship between concentration, mass, and volume.

Western blot uses two different types of agarose gel: stacking and separating gel. The higher, stacking gel is slightly acidic (pH 6.8) and has a lower acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Protein is thus separated by their size more so in this gel, as the smaller proteins to travel more easily, and hence rapidly, than larger proteins.

Gels are usually made by pouring them between two glass or plastic plates.

After separating the protein mixture, it is transferred to a membrane. The transfer is done using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. The membrane is placed between the gel surface and the positive electrode in a sandwich. The sandwich includes a fiber pad (sponge) at each end, and filter papers to protect the gel and blotting membrane. Here two things are very important: the close contact of gel and membrane to ensure a clear image and the placement of the membrane between the gel and the positive electrode. The membrane must be placed as such, so that the negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer, and can be done in semi-dry or wet conditions. Wet conditions are usually more reliable as it is less likely to dry out the gel, and is preferred for larger proteins.

It is very important to be aware that the data produced with a western blot is typically considered to be semi-quantitative. This is because it provides a relative comparison of protein levels, but not an absolute measure of quantity. There are two reasons for this; first, there are variations in loading and transfer rates between the samples in separate lanes which are different on separate blots. These differences will need to be standardized before a more precise comparison can be made. Second, the signal generated by detection is not linear across the concentration range of samples. Thus, since the signal produced is not linear, it should not be used to model the concentration.

3.7.2. Materials and methods

Protein amount quantification (BCA)

To determine the different absorption capacity of the samples, disks were placed into the wells of a 6 multiwell plate (NuncDelta, ThermoScientific) and incubated in 6 ml/well of foetal bovine serum (FBS, Sigma) for 1h at 37°C. Then, the total amount of adsorbed proteins was quantified by the colorimetric bicinconinic acid assay (BCA, Thermo Scientific).

The BCA assay is based on the biuret reaction (protein-induced reduction of Cu^{2+} to cuprous cation Cu^{+} in alkaline medium). The cuprous cation can be easily detected by the chelation of two molecules of BCA whereas the complex product a purple-coloured reaction (as represented in figure 3.37).

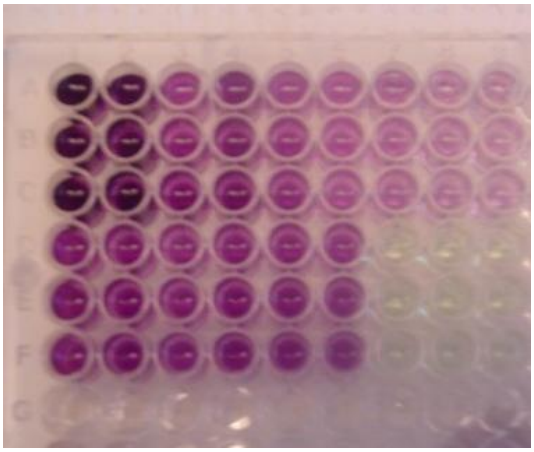


Figure 3.37 BCA revealing the presence of cuprous cation derived from Cu^{2+} reduced by protein

Briefly, after incubation in FBS, samples surface adsorbed proteins were lysed in 1 ml of Ripa Buffer (50 mM Hepes, 150 mM NaCl, 0,1% SDS, 1% Triton-X100, 1% sodium deoxycholate, 10% glycerol, 1,5 mM MgCl2, 1 mM EGTA, 1 mM NaF, 1% PMSF, 0,5% Na_3VO_4, 1% protease inhibitor mix) and gently collected using a cell scraper. To determine the amount (expressed as $\mu g/cm^2$) of protein in each sample, a standard curve was generated using bovine serum albumin (Albumin Standard, Thermo Scientific, 0-2 mg/ml) and mixed with BCA kit reagents (Thermo Scientific).

The absorbance of all samples and standard curve was measured at 570 nm by spectrometer (SpectraCount, Packard Bell, USA) and test samples protein amount calculated as function of the standard curve.

Selective protein adsorption - Western blot analysis

To investigate a possible selective adsorption of pro- or anti- cells adhesion proteins, 10 µg of each protein extracts were dissolved in Laemmli buffer 5x (62.5 mMTris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue), heated at 95°C for 5 min, resolved on 8% SDS-PAGE and transferred to a PVDF membrane. Finally, membrane was marked by Comassie blue and analyzed with Image j software (NIH) for protein quantification.

Western blot is often used to identify single proteins thanks to the possibility to separate them by the different molecular weight (mw). According to a commercial standard cocktail of known mw proteins (Precision plus protein All Blue standard, Biorad, Berkeley, California), it is possible to distinguish different proteins adsorbed onto the surfaces. In

fact, Fibronectin weight 220 KDa, Collagen 130 KDa, Vitronectin 75 KDa and Albumin 69 KDa; so it is possible to detect the presence of specific proteins in comparison with the standard.

Statistical analysis of data.

Experiments were performed in triplicate for each samples and assays. Data are expresses as means and standard deviations. Statistical analysis of data was performed using the SPSS v20 software (IBM, Chicago, USA); data were analyzed by ANOVA-one way followed by Sheffé post-hoc test. Significance level was set at $p<0.05$.

Chapter 4

Results of the experimental tests

In this chapter the results of all experimental analysis are summarized. A selection of biomaterials (CoCrMo alloy, XLPE and 4 different types of ceramics), currently used in orthopedic applications, has been considered. The effect of the materials surface characteristics on the bacterial response was studied to identify the best antibacterial biomaterial.

Surface roughness (both macroscopic analysis by means of profilometer, and microscopic observation through AFM), surface wettability and protein absorption, have been determined in the same conditions for all the materials.

To correlate the data of surface topography characterization to the microbiological behaviour,metabolic analysis about bacterial viability has been performed using MTT colorimetric assay, and morphological analysis of the biofilm thickness starting from SEM analysis has been performed.

The comparison between surface characteristics of the various substrates are here presented .

Deep knowledge about the effects of surface features of biomaterials on biological response is required to both understand clinical performances and to address engineeringand design of medical devices.

Results of all experimentations are here presented as a comparison between surface characteristics of the various substrates.

4.1. Profilometry

The profilometer provides a macroscopic analysis (order of microns) about the roughness peaks.

Surfaces roughness analysis results are reported in figure 4.1 a-c.

In Figure 4.1 a, the Ra of the analyzed samples is reported.

XLPE specimens reported the higher surface roughness resulting as significant towards metal and ceramic materials ($p<0.05$, indicated by §).

Metal CoCrMo surfaces showed significant differences in comparison with all the ceramics ($p<0.05$, indicated by #).

Finally, between ceramic surfaces, white and pink resulted as the less rough ones, obtaining significant results towards both grey and yellow ($p<0.05$, indicated by * for white and ^^ for pink respectively). No differences were noticed between grey and yellow and comparing white and pink ($p>0.05$).

Results obtained for Ra analysis were very similar to those obtained for Ry (figure 4.1 b) and Rmax (figure 4.1 c).

For both values, white ceramic and pink ceramic reported the lowest peaks while XLPE and CoCrMo the highest. As obtained for Ra, XLPE were significant towards metals and ceramics (b-c, indicated by §) and CoCrMo significant in compared with ceramics (b-c, indicated by #). Between ceramics, white and pink were significant towards grey and yellow (b-c, indicated by * and ^^ respectively) showing the lowest picks.

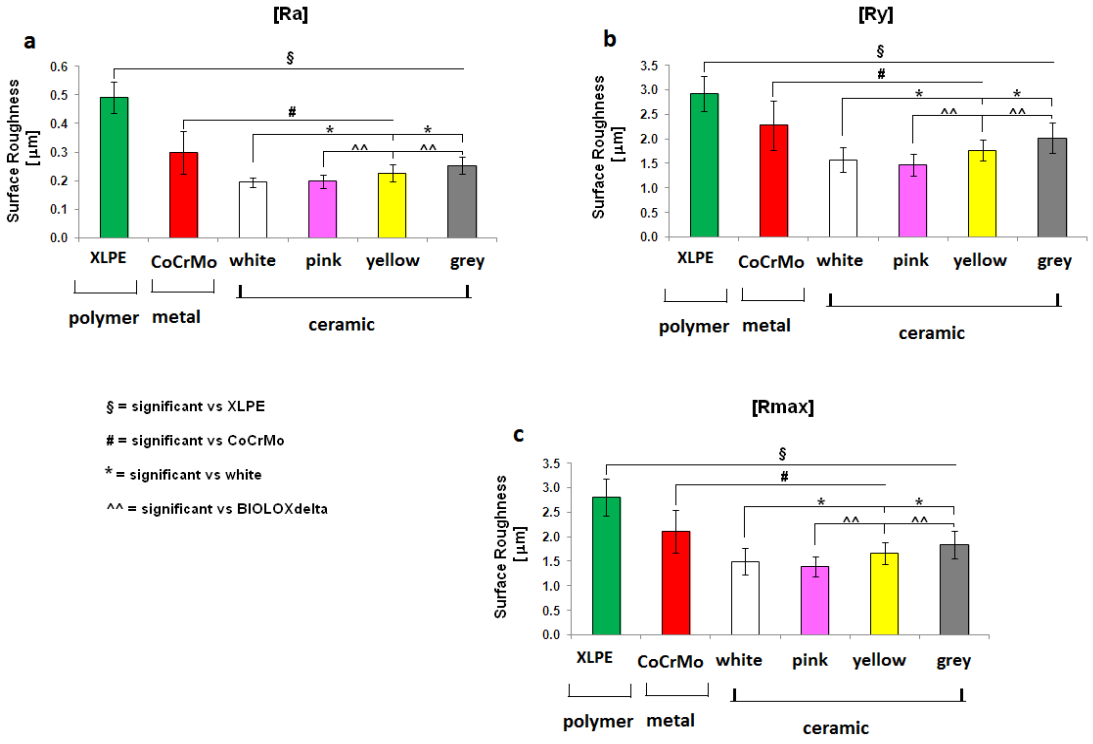


Figure 4.1 a-c. Specimens surface roughness evaluation by laser profilometry. Graphics represent Ra (a), Ry (b) and Rmax (c) scores. Data are expressed as means ± standard deviations.

Finally, in Figure 3 A-F, the surfaces 3D reconstruction are reported.

Reconstructed representative images showed that XLPE has rounded edges, while CoCrMo showed steep and narrow peaks . Pink and yellow ceramics presented peaks denser than XLPE. The 3D reconstruction also shows grooves machining and surface finish.

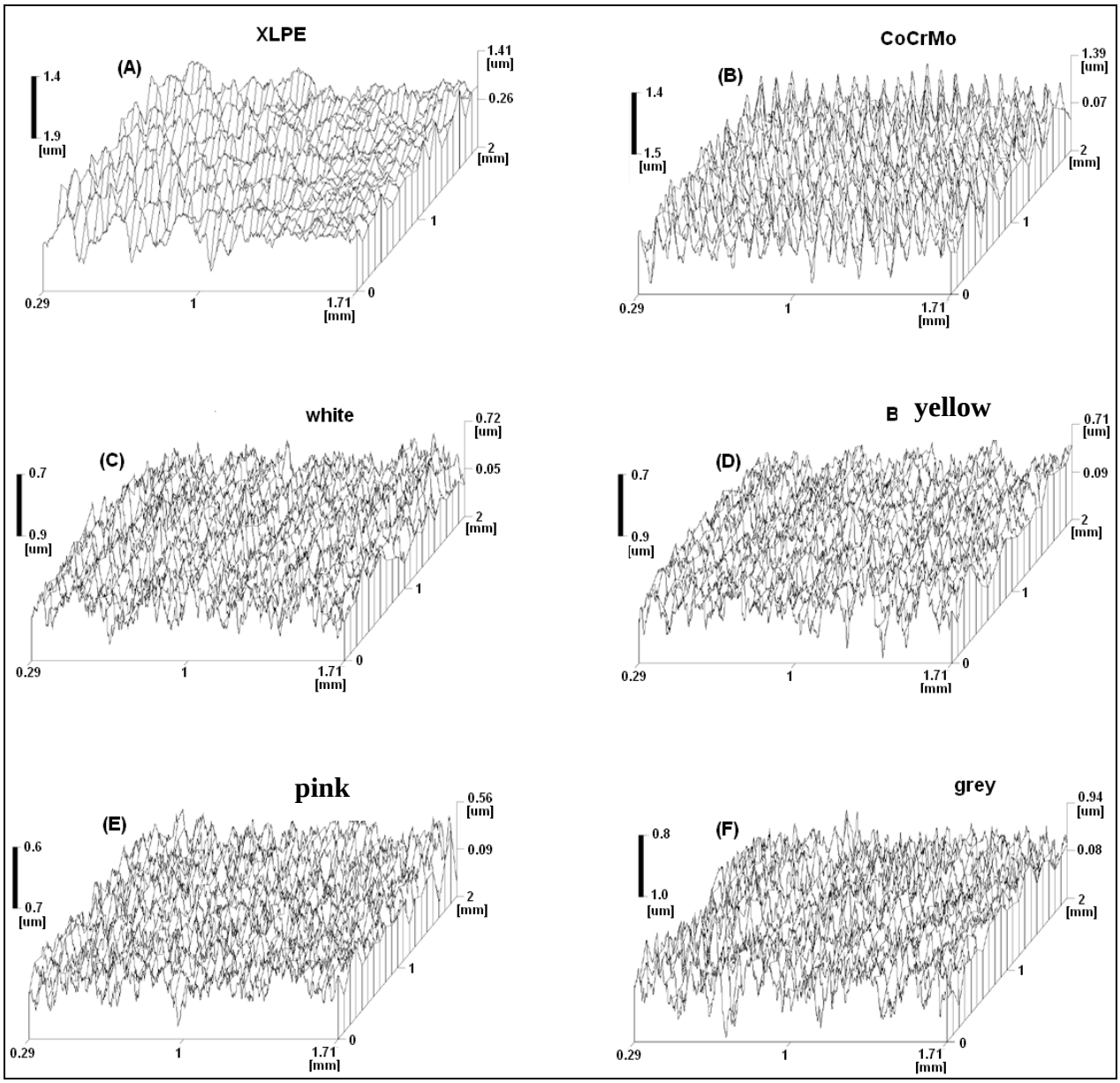


Figure 4.2 A-F. 3D surface reconstruction after profilometer analysis of XLPE (A), CoCrMo (B), white (C), yellow (D), pink(E) and grey (F) materials.

Based on this analysis, it's possible to conclude that the materials with the higher peaks are XLPE and CoCrMo surfaces; on the opposite, white and pink specimens revealed the lower presence of peacks, confirming that they represent the best materials concerning surface roughness

4.2. Atomic-force microscopy

The value of average roughness on nanoscale level is reported in figure 4.3 a-b. Data are expressed as means ± standard deviations.

In Figure 4.3 a , the Sa of the analyzed samples is reported, considering a scanning area of 20 μm.

Yellow specimens reported the higher surface roughness, but it's not result as significant towards other ceramics , metal and polymeric materials ($p>0.05$).

Results obtained considering a scanning area of 50 μm are presented in Figure 4.3 b. Ceramic yellow reported the highest peaks, resulting as significant towards grey and white, which are the less rough ones ($p<0.05$, indicated by #). No differences were noticed between XLPE and the other materials, and comparing CoCrMo with the other specimens ($p>0.05$).

Between ceramic surfaces, no differences were noticed comparing pink with the other ceramics. ($p>0.05$).

The atomic force microscopy has a high resolution, and the analysis is affected by the surface defects and surface treatment (turning). The instrument is very sensitive; for this reason data showed high variability and high standard deviation.

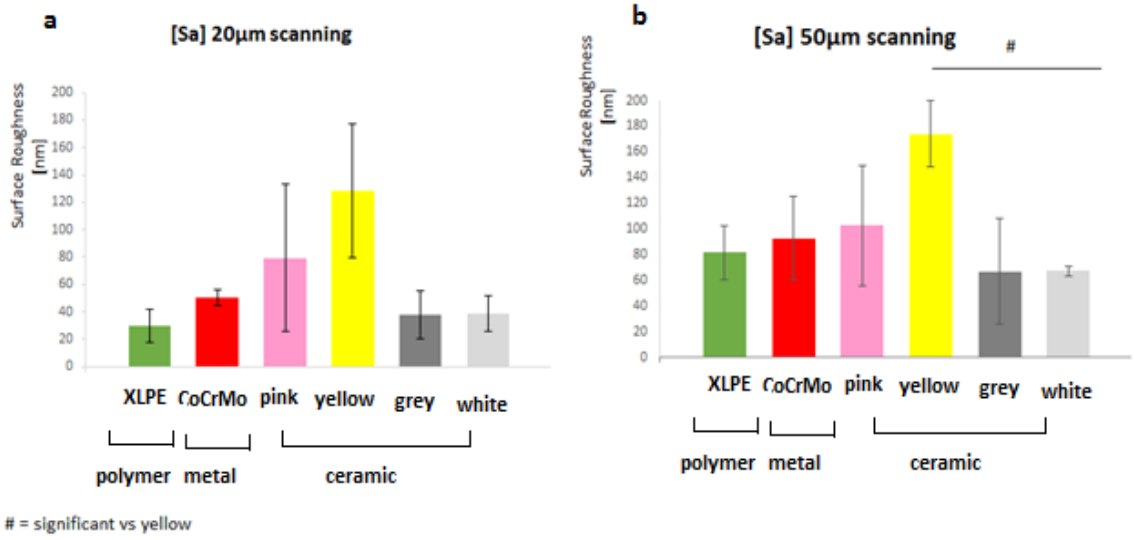
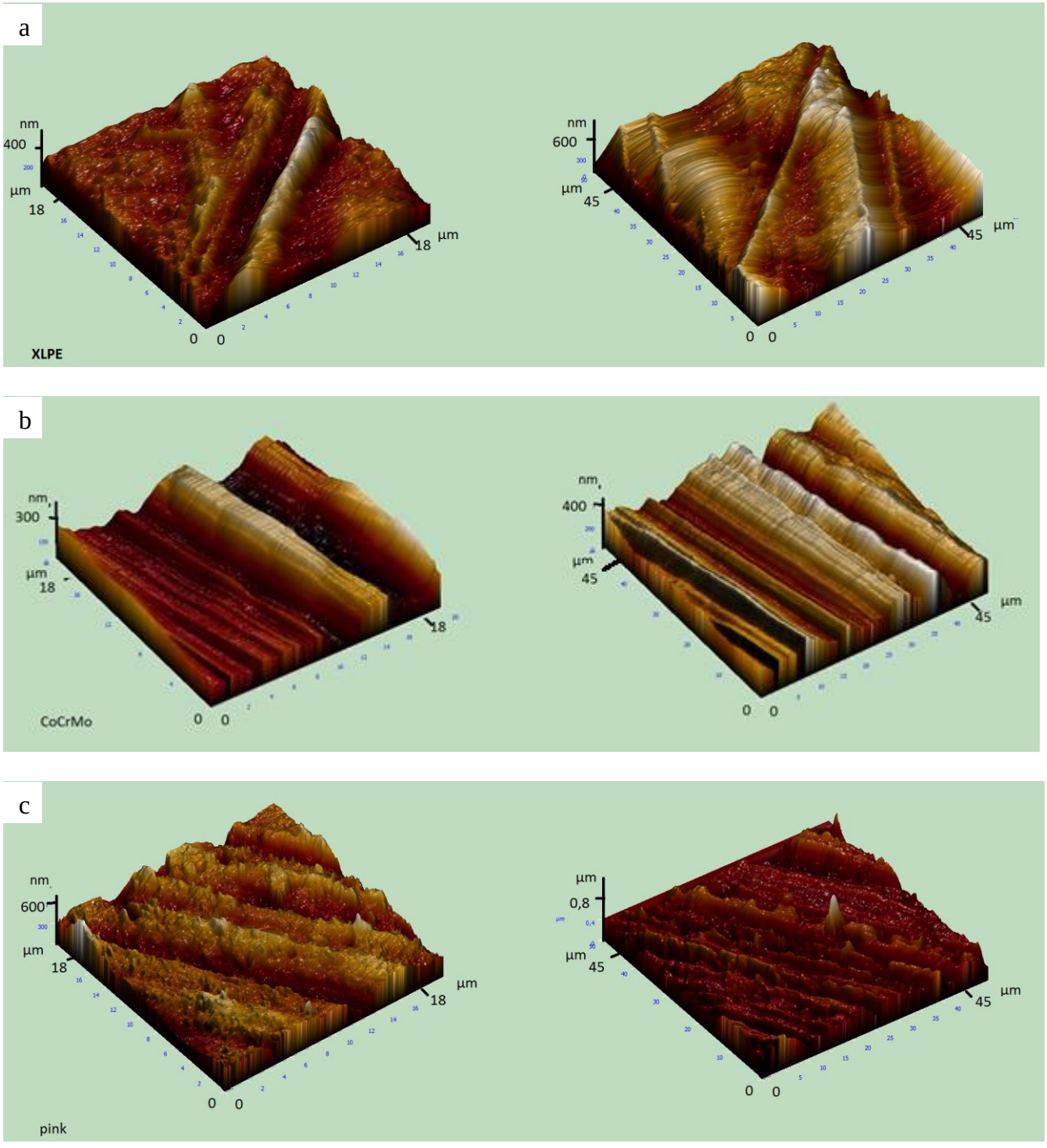


Figure 4.3 a-b. Specimens surface roughness evaluation by AFM. Graphics represent Sa 20 μm scanning (a), Sa 50 μm scanning (b) scores. Data are expressed as means ± standard deviations.

Finally, in figure 4.4 a-f, the surfaces 3D reconstruction are reported (20µm scan on the left, 50µm scan on the right)



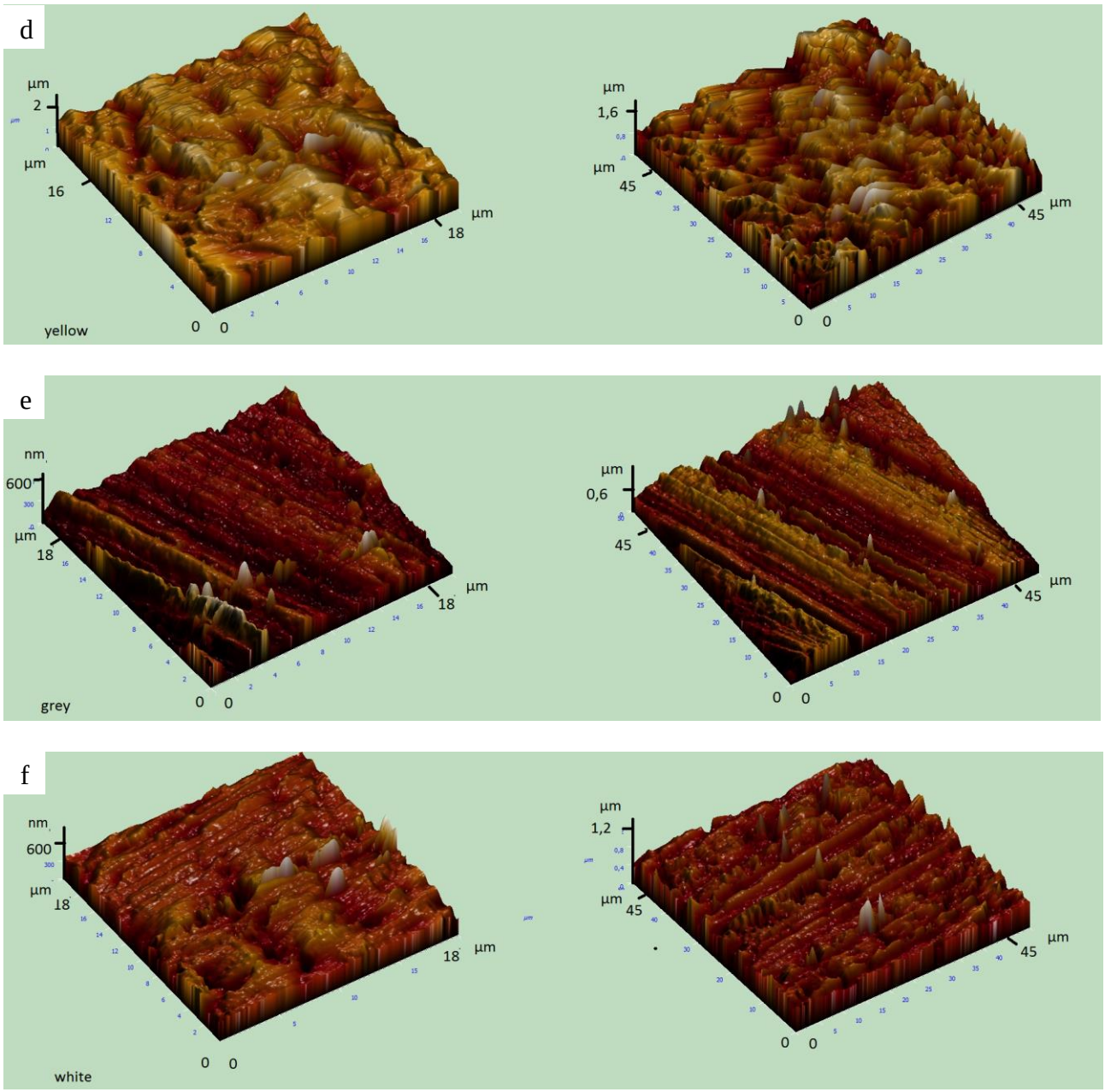


Figure 4.4 a-f. 3D surface reconstruction after AFM analysis of XLPE (a), CoCrMo (b), pink(c) , yellow (d), grey (e), and white (f) materials.

As noticed for Sa values, also reconstructed representative images showed the presence of high picks for yellow surfaces.

4.3. Contact angle

The contact angle allows to evaluate the wettability of a surface: the lower is the contact angle, the higher is the degree of wettability of the surface. On a hydrophilic surface, the contact angle will be less than 90°, while, if the surface is hydrophobic, the contact angle will be greater than 90°; a zero contact angle represents complete wetting.

Figure 4.5 reports the contact angles values on the considered materials. Data are expressed as means ± standard deviations.

XLPE and CoCrMo are the less wettable surfaces, while grey presents the lower contact angle, so it is more hydrophilic .

CoCrMo surfaces showed significant differences in comparison with grey, ($p<0.05$, indicated by #), and XLPE specimens reported the highest contact angle, also showing significant differences in comparison with yellow ($p<0.05$, indicated by *).

Between ceramic surfaces only the grey material were significant towards the other ceramics ($p<0.05$, indicated by #), while no differences were noticed between yellow, pink, and white($p>0.05$).

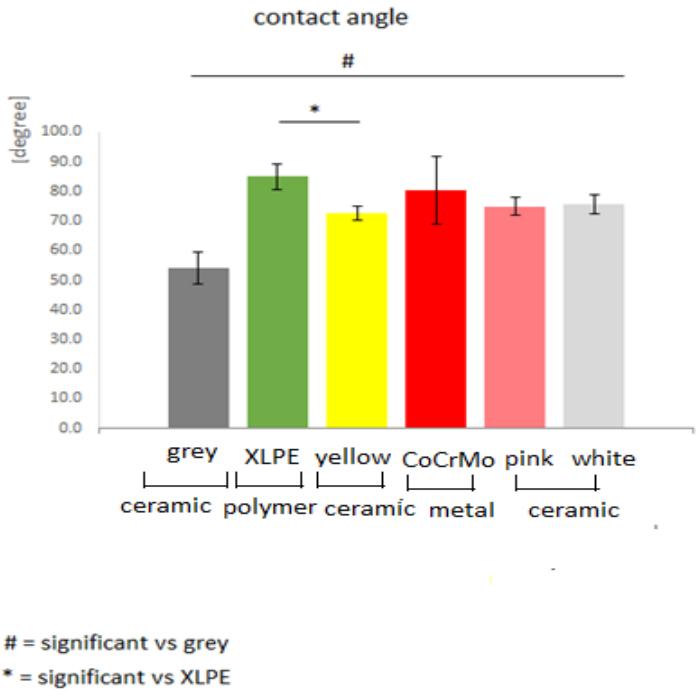


Figure 4.5 Static contact angles on the considered materials. Data are expressed as means ± standard deviations

4.4. Bacterial contamination: MTT , SEM and computational model

This section reports results of microbiological experiments to study the intrinsic antimicrobial properties of the material and to correlate, with a critical analysis, the microbiological behavior to the data of surface topography characterization.

In particular, results are presented as a comparison between ceramic, metallic (CoCrMo alloys) and polymeric (XLPE) materials.

The results present the amount of viable biomass adhering to materials surfaces using *S. aureus* and *S. epidermidis* strains, often responsible of the failure of implanted materials used in orthopedic prosthetic rehabilitation. Results related to metabolic and morphological analysis, with these two different bacterial strains, are presented separately.

The experiments are aimed to study the early phase of bacteria biofilm formation onto metallic and polymeric specimens in comparison with polystyrene, considered as control references.

Obtained results are compared with data of bioceramics, providing a statistical comparison to determine which of the tested materials presents the best intrinsic antibacterial performance for orthopedics applications.

S. aureus **biofilm metabolic analysis comparison: MTT**

Figure 4.6 represents the comparison in terms of bacterial viability between the *S. aureus* biofilm colonized XLPE and CoCrMo surfaces VS ceramics specimens (white-grey-pink-yellow), after 24 (figure 4.6 a) and 48 (figure 4.6 b) hours of incubation.

Significant difference between the ceramics and the control material are noticed: ceramics materials are less contaminated than the control after 24 hs ($p<0.05$, figure 4.6 a, indicated by *) and 48 hs ($p<0.05$, figure 4.6 b, indicated by *).

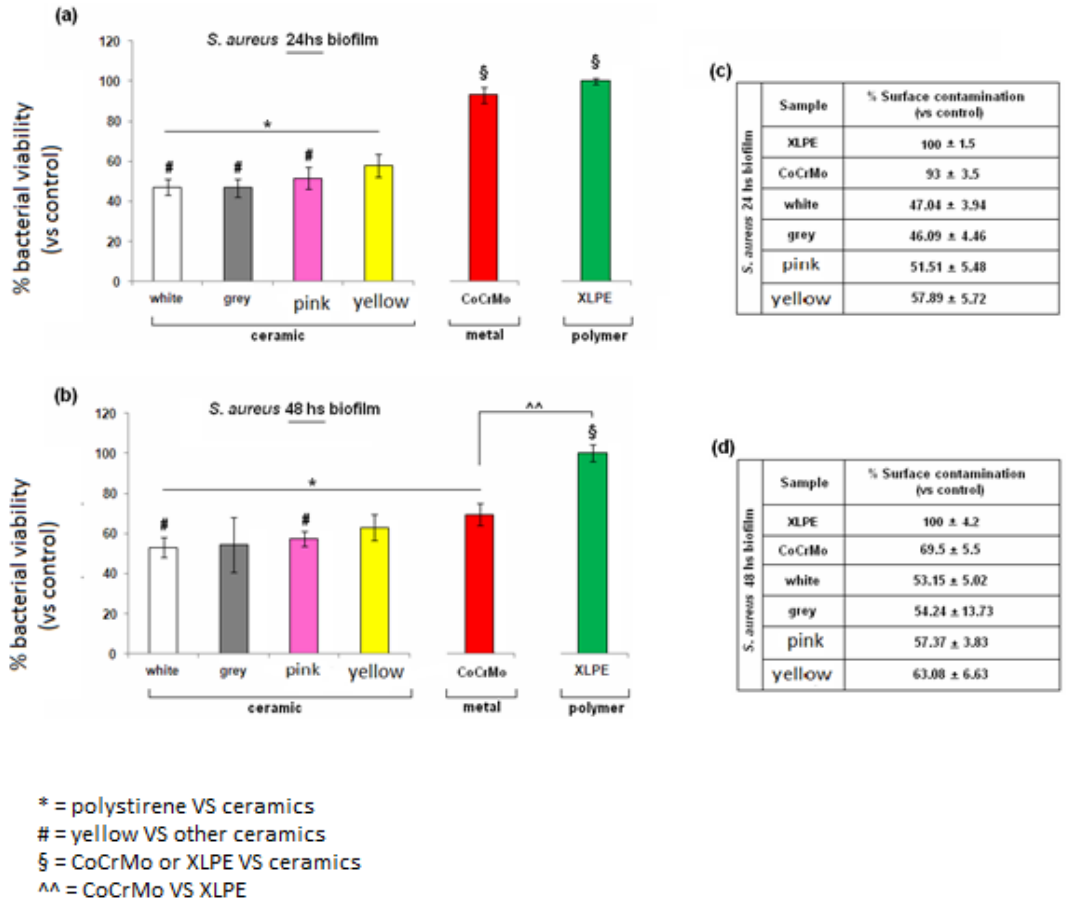
Significant difference are noticed between ceramic groups: the yellow material is more contaminated after 24 hs (figure 4.6 a, indicated by #) and 48 hs ($p<0.05$, figure 4.6 b, indicated by #) while the grey one is more contaminated after 48 hs ($p<0.05$, figure 4.6 b, indicated by #).

Significant differences are noticed between ceramics and CoCrMo or XLPE after 24 hours (a, $p<0.05$, indicated by §); at 48 hours, only XLPE remained more contaminated than ceramics.

Significant difference between the ceramic and XLPE are noticed after 24 and 48 hours ($p<0.05$, indicated by §): XLPE resulted in general more contaminated ($p<0.05$, figure 4.6 b, indicated by §).

By comparing XLPE and CoCrMo to each other, only after 48 hours there was a significant difference ($p<0.05$, indicated by ^^) with XLPE more contaminated than the metallic ones.

Finally, surface contamination ratio of each specimens after 24 and 48 hs are summarized in figure 4.6 c and d respectively; data are expressed as means ± standard deviations.



Sample	% Surface contamination (vs control)
XLPE	100 ± 1.5
CoCrMo	93 ± 3.5
white	47.04 ± 3.94
grey	46.09 ± 4.46
pink	51.51 ± 5.48
yellow	57.89 ± 5.72

S. aureus 24 hs biofilm

Sample	% Surface contamination (vs control)
XLPE	100 ± 4.2
CoCrMo	69.5 ± 5.5
white	53.15 ± 5.02
grey	54.24 ± 13.73
pink	57.37 ± 3.83
yellow	63.08 ± 6.63

S. aureus 48 hs biofilm

Figure 4.6 a-d. *S. aureus* viable biomass colonizing the surfaces of the ceramics materials (white-gray-pink-yellow), metal alloys (CoCrMo) and polymeric materials (XLPE) after 24 (a) and 48 (b) hours of incubation. Ceramics materials are less contaminated than the control after 24 hs (a, indicated by *) and 48 hs (b, indicated by *). Particular, yellow ceramics resulted as the most contaminated (a,b, indicated by #) after 24 hs and 48 hs together with grey ones. CoCrMo were more contaminated than ceramics only at 24 hours but not at 48 (a, indicated by §). Finally, XLPE specimens were always more contaminated than ceramics at 24 and 48 hours (a, b, indicated by §); significant differences were noticed at 48 hs also comparing XLPE and CoCrMo (b, indicated by ^^). No significant differences are noticed after 24 hs between CoCrMo and XLPE towards polystyrene controls. Surface colonization % summary is reported in (c) for 24 hs data and in (d) for 48 hs; data are expressed as means and standard deviations.

S. aureus biofilm morphological analysis comparison : SEM

Figure 4.7 represents the SEM comparison in terms of surface colonization of ceramic, metallic (CoCrMo) and polymeric (XLPE) specimens, after 48 hours of incubation with *S. aureus* biofilm. The regions of maximum, medium and minimum contamination of the samples are shown.

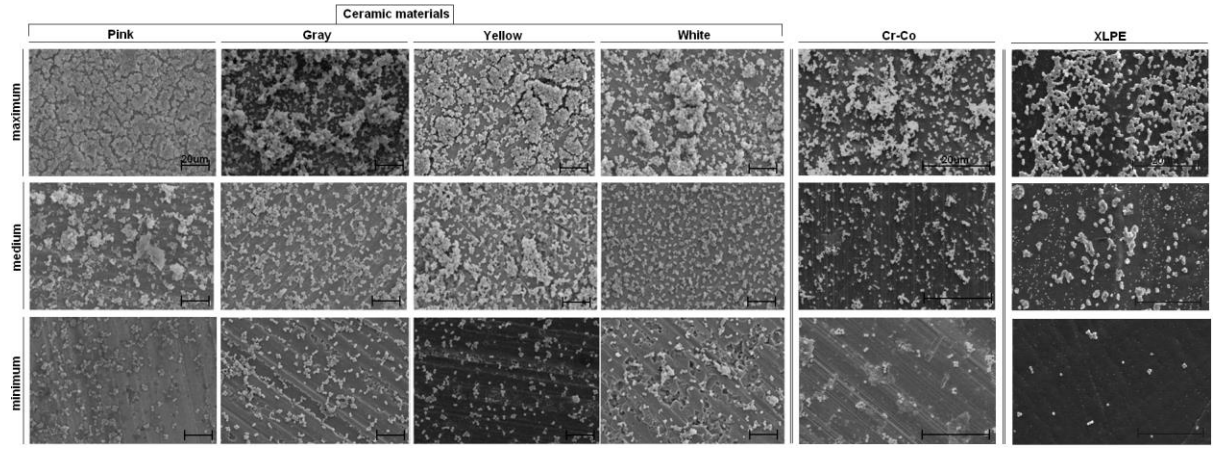


Figure 4.7. Comparison of *S. aureus* 48 hs biofilm colonization of ceramic (pink-grey-yellow-white, left panel), metallic (CoCrMo, right panel) and polymeric specimens (XLPE, right panel) by SEM images analysis. Magnifications: 2000X (bar scale = 20 um).

S. epidermidis biofilm <u>metabolic analysis</u> comparison : MTT

Figure 4.8 represents the comparison in terms of bacterial viability between the *S. epidermidis* biofilm colonized XLPE and CoCrMo surfaces VS ceramics specimens (white-grey-pink-yellow), after 24 (figure 4.8 a) and 48 (figure. 4.8 b) hours of incubation.

Significant difference between the ceramics and the control material are noticed: ceramics materials are less contaminated than the control after 24 hs ($p<0.05$, figure 4.8 a, indicated by *) and 48 hs ($p<0.05$, figure 4.8 b, indicated by *).

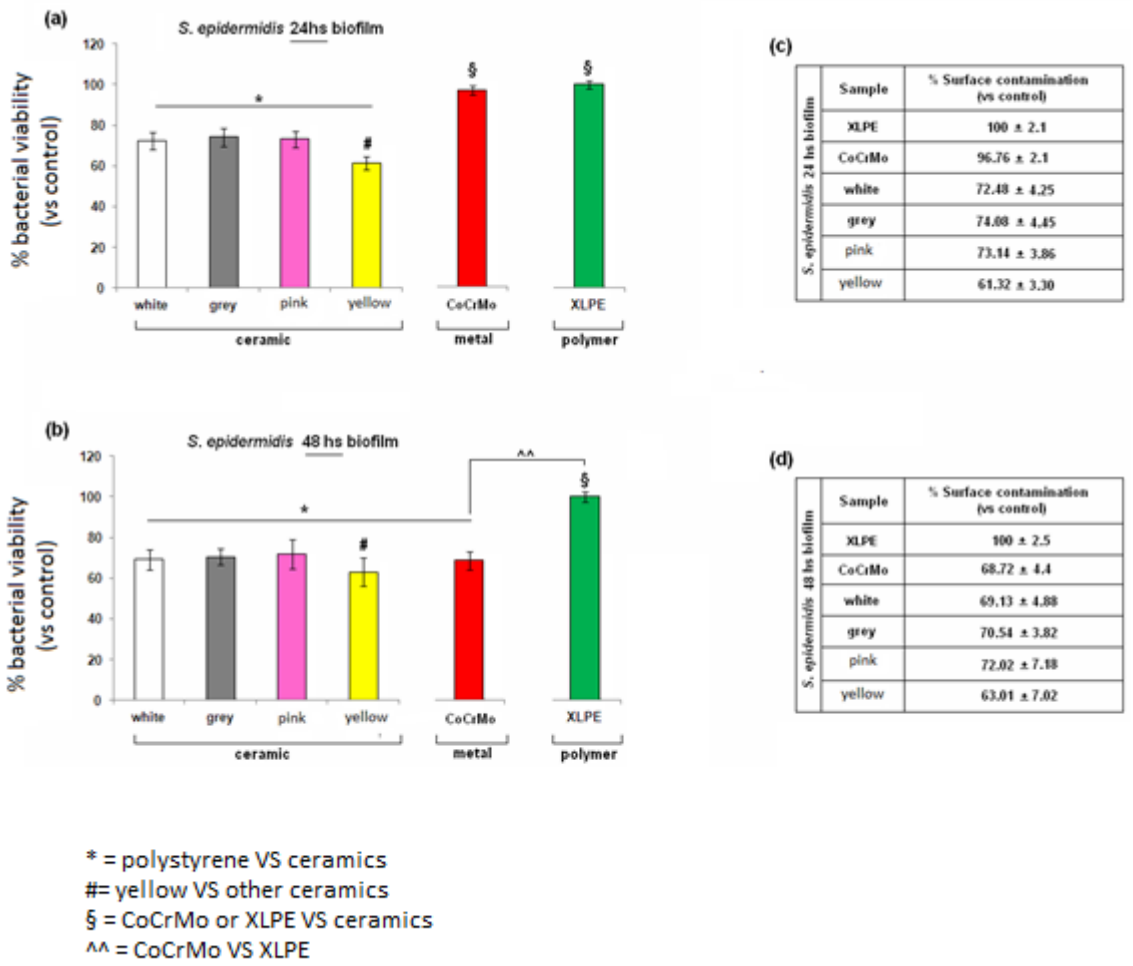
Significant difference are noticed between ceramic groups: the yellow material is less contaminated after 24 hs (figure 4.8 a, indicated by #) and 48 hs ($p<0.05$, figure 4.8 b, indicated by #).

Significant differences are noticed between ceramics and CoCrMo or XLPE after 24 hours (a, $p<0.05$, indicated by §); at 48 hours, only XLPE remained more contaminated than ceramics.

Significant difference between the ceramic and XLPE are noticed after 24 and 48 hours ($p<0.05$, indicated by §): XLPE resulted in general more contaminated ($p<0.05$, figure 4.8 b, indicated by §).

By comparing XLPE and CoCrMo to each other, only after 48 hours there was a significant difference ($p<0.05$, indicated by ^^) with XLPE more contaminated than the metallic ones.

Finally, surface contamination ratio of each specimens after 24 and 48 hs are summarized in figure 4.8 c and d respectively; data are expressed as means ± standard deviations.



(c)

S. epidermidis 24 hs biofilm	Sample	% Surface contamination (vs control)
	XLPE	100 ± 2.1
	CoCrMo	96.76 ± 2.1
	white	72.48 ± 4.25
	grey	74.88 ± 4.45
	pink	73.14 ± 3.86
	yellow	61.32 ± 3.30

(d)

S. epidermidis 48 hs biofilm	Sample	% Surface contamination (vs control)
	XLPE	100 ± 2.5
	CoCrMo	68.72 ± 4.4
	white	69.13 ± 4.88
	grey	70.54 ± 3.82
	pink	72.02 ± 7.18
	yellow	63.01 ± 7.02

Figure 4.8 a-d. *S. epidermidis* iable biomass colonizing the surfaces of the ceramics materials (white-gray-pink-yelow), metal alloys (CoCrMo) and polymeric materials (XLPE) after 24 (a) and 48 (b) hours of incubation. Ceramics materials are less contaminated than the control after 24 hs (a, indicated by *) and 48 hs (b, indicated by *). Particular, yellow ceramics resulted as the less contaminated (a,b, indicated by #) after 24 hs and 48. CoCrMo are more contaminated than ceramics only at 24 hours but not at 48 (a, indicated by §). Finally, XLPE specimens are always more contaminated than ceramics at 24 and 48 hours (a, b, indicated by §); significant differences were noticed at 48 hs also comparing XLPE and CoCrMo (b, indicated by ^^). No significant differences are noticed after 24 hs between CoCrMo and XLPE towards polystyrene controls. Surface colonization % summary is reported in (c) for 24 hs data and in (d) for 48 hs; data are expressed as means and standard deviations.

S. epidermidis biofilm morphological analysis comparison: SEM

Figure 4.9 represents the SEM comparison in terms of surface colonization of ceramic, metallic (CoCrMo) and polymeric (XLPE) specimens, after 48 hours of incubation with *S. epidermidis* biofilm. The regions of maximum, medium and minimum contamination of the samples are shown.

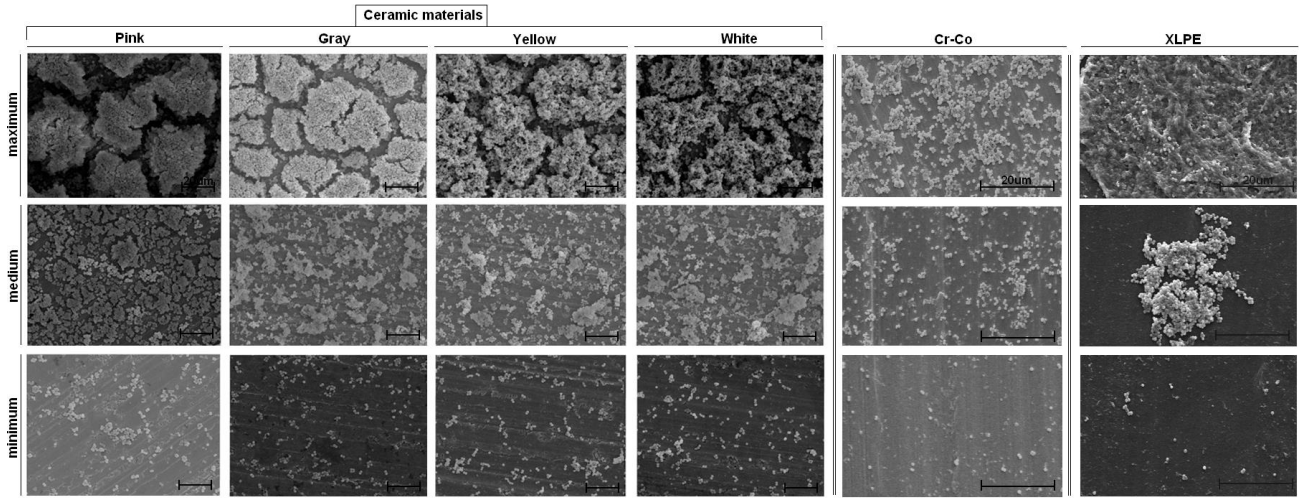


Figure 4.9 Comparison of *S. epidermidis* 48 hs biofilm colonization of ceramic (pink-grey-yellow-white, left panel), metallic (CoCrMo, right panel) and polymeric specimens (XLPE, right panel) by SEM images analysis. Magnifications: 2000X (bar scale = 20 um).

CoCrMo biofilm 3D analysis

3D analysis of *S. aureus* bioflm obtained by SEM pictures is reported in figure 4.10, while in figure 4.11 the same analysis is reported for *S. epidermidis* biofilm.

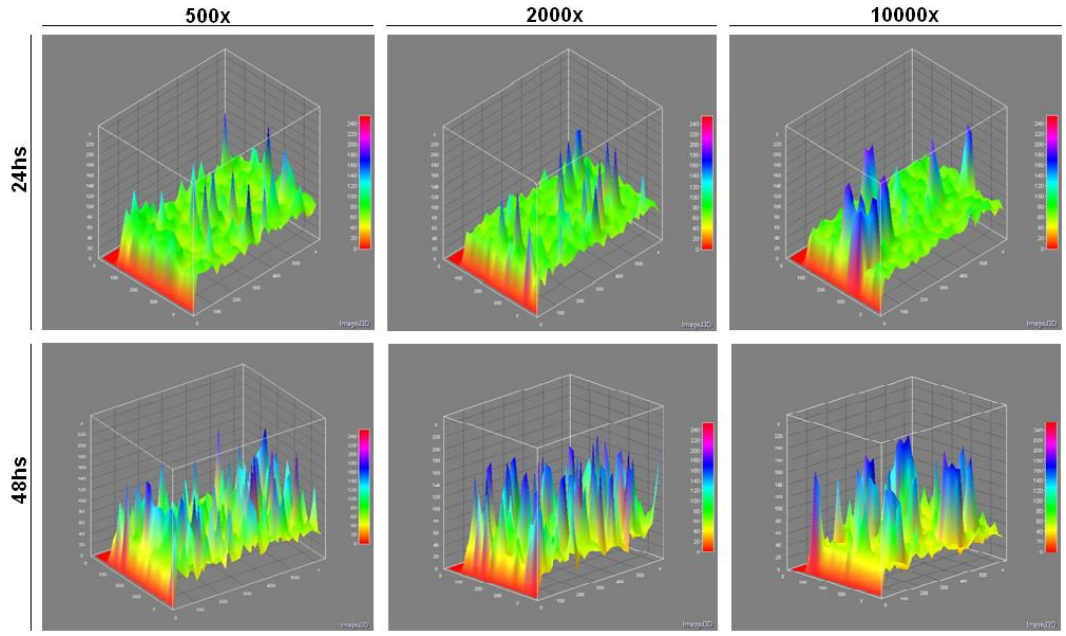


Figure 4.10 3D analysis (by ImageJ surface plot plug-in) of CoCrMo specimens colonized by 24 hs (upper panel) and 48 hs (lower panel) *S. aureus* biofilm.

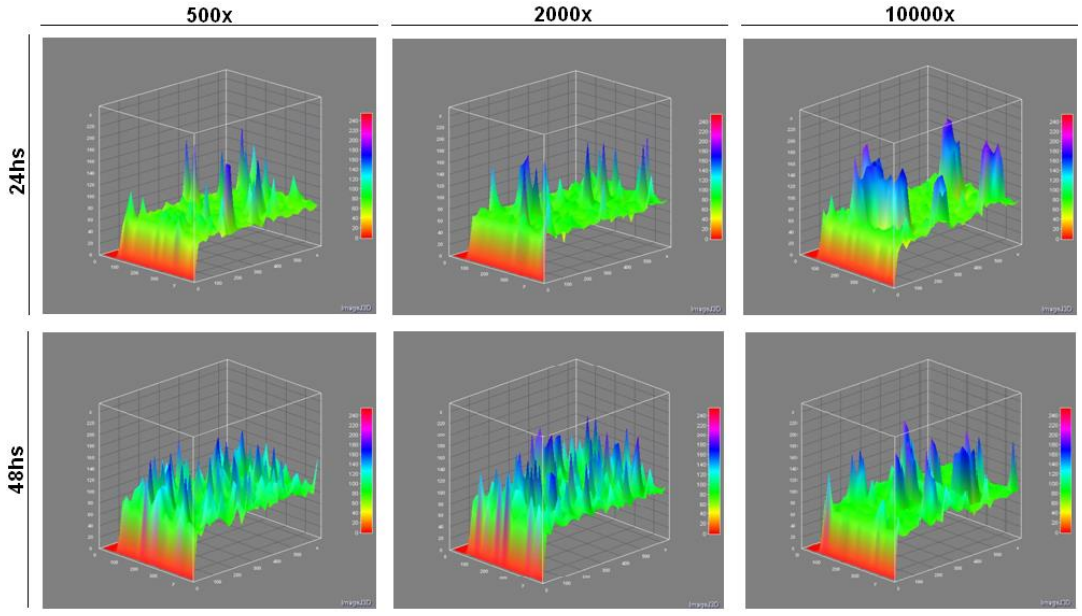


Figure 4.11. 3D analysis (by ImageJ surface plot plug-in) of CoCrMo specimens colonized by 24 hs (upper panel) and 48 hs (lower panel) *S. epidermidis* biofilm.

XLPE Biofilm 3D analysis

3D analysis of *S. aureus* bioflm obtained by SEM pictures is reported in figure 4.12, while in figure 4.13 the same analysis is reported for *S. epidermidis* biofilm.

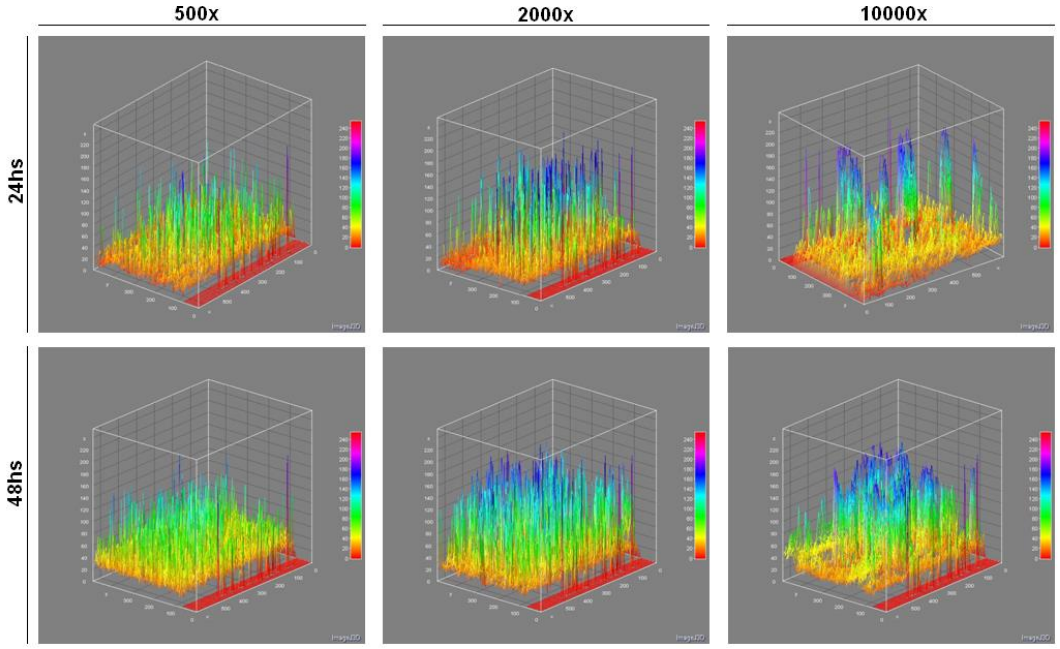


Figure 4.12. 3D analysis (by ImageJ surface plot plug-in) of XLPE specimens colonized by 24 hs (upper panel) and 48 hs (lower panel) *S. aureus* biofilm.

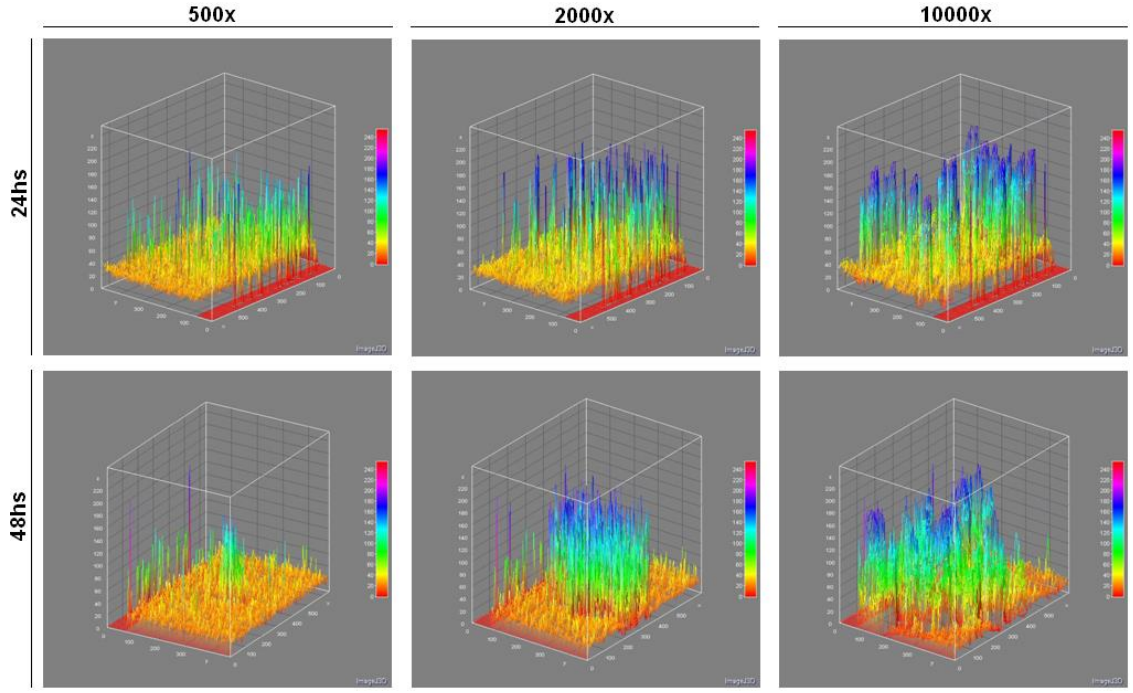


Figure 4.13. 3D analysis (by ImageJ surface plot plug-in) of XLPE specimens colonized by 24 hs (upper panel) and 48 hs (lower panel) *S. epidermidis* biofilm.

The computational model shows the biofilm thickness: it's evident that XLPE presents higher and more dense peaks than CoCrMo.

Ceramics Biofilm 3D analysis

3D analysis of *S. aureus* bioflm obtained by SEM pictures is reported in figure 4.14, while in figure 4.15 the same analysis is reported for *S. epidermidis* biofilm.

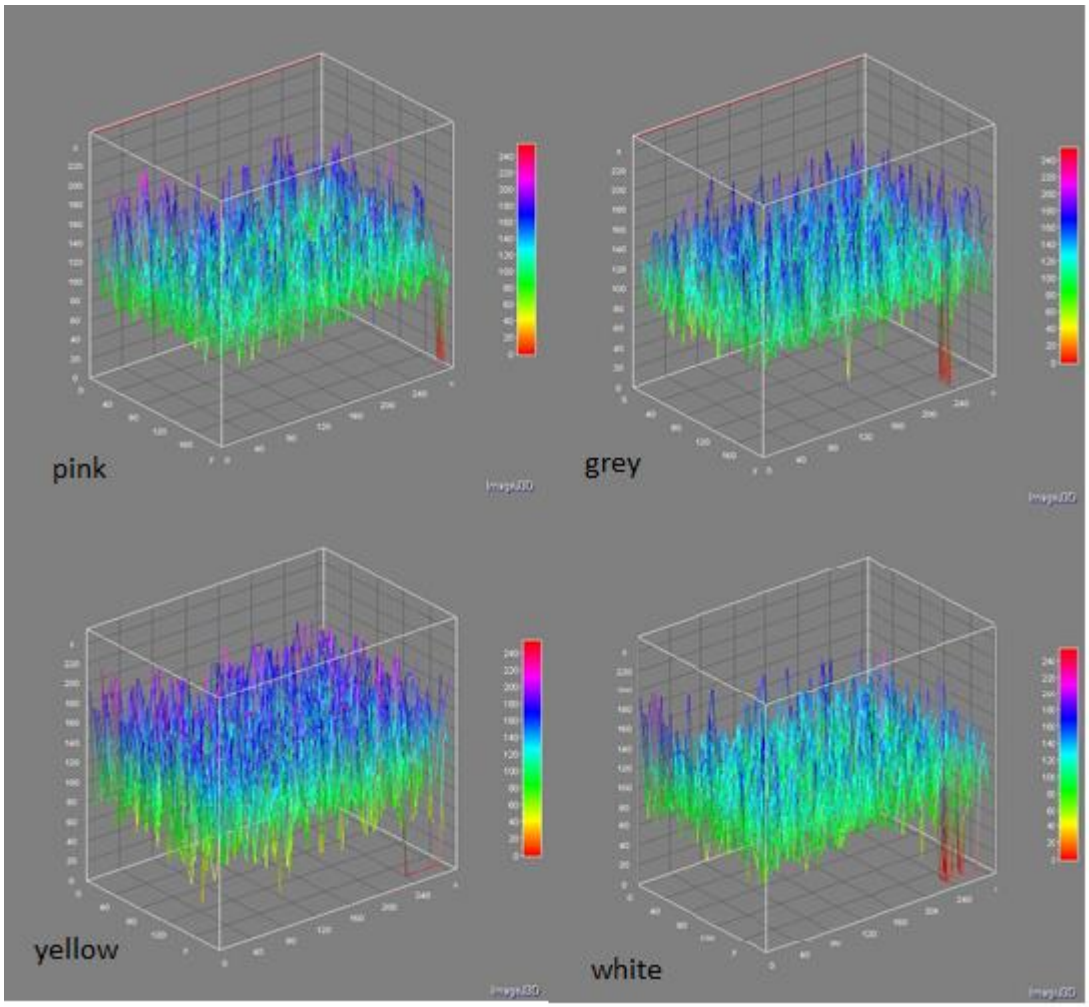


Figure 4.14 3D analysis (by ImageJ surface plot plug-in) of ceramics specimens colonized by 48 hs *S. aureus* biofilm

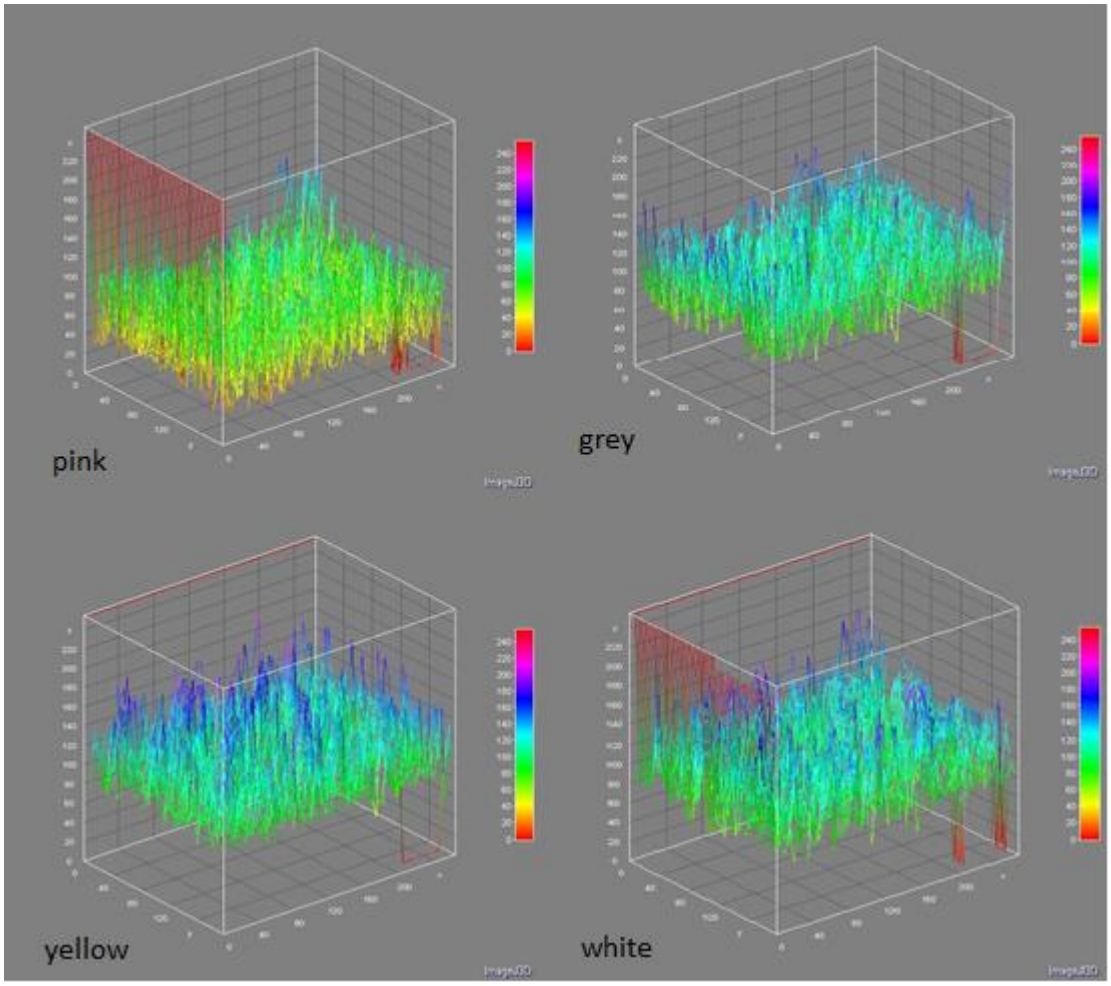


Figure 4.15 3D analysis (by ImageJ surface plot plug-in) of ceramics specimens colonized by 48 hs *S. epidermidis* biofilm

Based on this analysis, it's possible to affirm that ceramic materials allow a greater bacterial inhibition compared with CoCrMo and XLPE. In fact, bacterial viability results lower, as just described.

4.5. Protein adsorption: BCA and Western Blot analysis

The purpose of this analysis is to investigate quantitatively the adsorbed protein on the different surfaces, and subsequently to identify biomaterials able to selectively adsorb some adhesive and anti-adhesive proteins, through qualitative analysis (Western blot analysis). This analysis is used in research to separate and identify proteins.

This section reports results about total adsorbed protein amount and specific adsorption obtained by using metal (CoCrMo), polymer (XLPE), and ceramic specimens, in order to discuss which material presents the best antibacterial properties.

Results are reported in figure 4.16 a-b.

Figure 4.16 a represents the total amount of protein adsorbed onto the surface of metal, bioceramic, and XLPE specimen (BCA assay) while in figure 4.16 b is reported the western blot analysis of the absorbed proteins.

BCA showed that the total amount of adsorbed proteins resulted as higher onto pink, yellow and CoCrMo but no statistical significant differences were noticed between different groups (a); statistical analysis (ANOVA-one way and Sheffé post-hoc) did not revealed significant differences between samples ($p>0.05$).

More interesting, western blot assay reported that pink materials are able to adsorb all the investigated proteins (b). In general, albumin (69 kDa band) was well adsorbed by all the samples; vitronectin (75 kDa) is detected in CoCrMo, pink and yellow. Collagen (130 kDa) is detected only in pink samples; finally, fibronectin (220 kDa) is observed for pink and yellow.

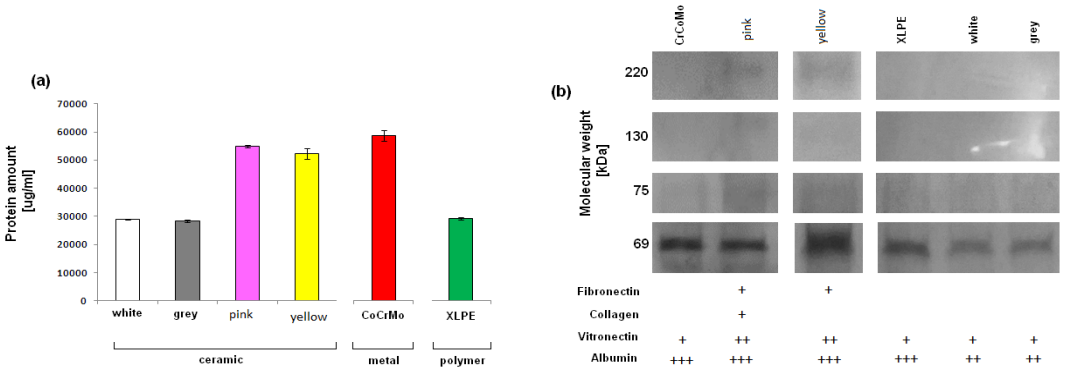


Figure 4.16. Protein absorption measurements. a) BCA quantification, b) Western Blot analysis. The total amount of adsorbed proteins resulted as higher onto pink, yellow and CoCrMo but no statistical significant differences were noticed between different groups. Wester blot analysis showed that only pink adsorbed all the investigated proteins. Data are expressed as means ± standard deviations.

It's possible to conclude that ceramics materials selectively absorbed albumin, which is anti-adhesive, but also fibronectin, collagen (pink and yellow) and vitronectin, that are pro-adhesion proteins.

Chapter 5
Critical discussion

This section reports the discussion about all experimental tests, in view to develop strategies that reduce medical device-associated infection.

The comparison between surface characteristics of the various substrates and the correlation with their bacterial response are here critically discussed.

5.1. Critical correlation between bacterial adhesion and surface features

In the present work a selection of biomaterials (CoCrMo alloy, XLPE and 4 different types of ceramics), actually used in orthopedic applications, has been considered, to determine the effect of the surface characteristics on the bacterial response, and to identify the best antibacterial biomaterial.

To achieve this purpose surface roughness (both macroscopic analysis by means profilometer, and microscopic observation through AFM) , surface wettability and protein absorption, have been determined in the same conditions for all the materials.

To correlate the data of surface topography characterization to the microbiological behaviour, a metabolic analysis about bacterial viability has been performed through MTT colorimetric assay, and a morphological analysis about the biofilm thickness has been performed by means SEM analysis.

Here it's discussed how surface characteristics, such as topography and wettability , affect material ability to adsorb proteins, and to interact with bacteria.

Surface roughness analysis through the profilometer (figure 4.1) revealed determinant materials properties. In fact, surface roughness at micro-scale level is a key factor to influence bacteria adhesion: more roughness is normally alias of more bacteria adhesion. By discussing Ra values, it is possible to determine that XLPE showed the highest roughness. Values (Ra-Ry-Rmax) were always significant in comparison with other materials. The profilometry showed that white and pink ceramics reveal the lower presence of peaks. Therefore, considering that smoother materials are unfavorable to the bacterial adhesion, it's possible to affirm that ceramics are the best materials concerning surface roughness on micro-scale level. On the opposite the most uneven surfaces are more favorable to bacterial adhesion compared to the smoothest ones. This is due to much larger surface area available for adhesion and subsequent higher number of anchor points.

Looking at AFM measurements it's observed that ceramic yellow presents the greater roughness compared with the other materials (figure 4.3). It is a complementary analysis to profilometry; it analyzes in detail the surface, because the instrument has a higher resolution. It's a more detailed investigation, which highlights nanometric characteristics of each surface.

Actually differences between the macroscopic and microscopic features are emerged; in fact, comparing microscopic and nanometric analysis performed by profilometry and AFM respectively, it's noted that the ceramic materials have a smoother surface at microscopic level, while at the nanometer level, they are the most wrinkled compared with the others materials.

However, as regards bacterial adhesion, data provided by profilometry are more significant than AFM data, because the most favorable surface for bacterial proliferation is represented by high peaks in the order of micrometers.

Therefore, profilometry and AFM showed different morphological features, on two different scales of magnitude , but data provided by profilometry are more indicative concerning bacterial adhesion.

As far as the contact angle measurements, it's noted that the highest wettability characterizes the grey substrate (figure 4.5), while the lowest one characterizes the XLPE and CoCrMo. These results suggest that outermost surface layer is the most significant in order to determine the wetting behavior. XLPE and CoCrMo presents the higher contact angle , while grey presents the lower contact angle. It means that XLPE and CoCrMo are the less wettable surfaces (hydrophobic) , while grey is more wettable and than hydrophilic . In fact the greater the angle of contact, the lower the wettability of the surface. In particular, CoCrMo surfaces showed significant differences in comparison with grey, ($p<0.05$, indicated by #), and XLPE specimens reported the highest contact angle, also showing significant differences in comparison with yellow ($p<0.05$, indicated by *).

Between ceramic surfaces only the grey material were significant towards the other ceramics ($p<0.05$, indicated by #), while no differences were noticed between yellow, pink, and white($p>0.05$).

This work describes the ability of ceramic, metal and polymer materials to prevent *S. aureus* or *S. epidermidis* biofilm surface contaminations. For this reason, a metabolic analysis to evaluate the bacterial viability has been performed by means MTT colorimetric assay, and a morphological analysis about the biofilm thickness has been performed through SEM analysis.

MTT and SEM performed on the samples during the simulation of bacterial infection *in vitro* showed that ceramic materials present the best performances in biofilm prevention,

resulting as always less contaminated than metal and polymers ones for both strains (Figures 4.6 and 4.8). Particular, significant differences ($p<0.05$) were noticed after 24 hours of cultivation between all the ceramics and metals or polymers. These data indicated that ceramics display a better ability to decrease bacteria adhesion in comparison with CoCrMo and XLPE. However, after 48 hours cultivation, ceramics were significant only towards XLPE specimens but not in comparison with CoCrMo; thus, it can be speculate that ceramics were able to firstly reduce bacteria adhesion but not further proliferation. If compared to each other, ceramics specimens reported different behaviors in relation with the two strains. In fact, considering *S. aureus* biofilm, pink and white ceramic resulted as the less contaminated at both 24 and 48 hours. On the opposite, yellow and grey ceramic resulted as the most bacteria colonized with a similar trend after 24 or 48 hours. Differences between ceramics were significant figure 4.6, $p<0.05$). However, when the *S. epidermidis* biofilm was considered, yellow resulted as the less contaminated in comparison with other ceramics (figure 4.8, $p<0.05$); same results were obtained after 24 and 48 hours. Regarding CoCrMo and XLPE, their performances were not strain-dependent; in fact, metal specimens were always contaminated in a significant manner in comparison with ceramics at 24 hours (Figures 4.6 and 4.8, upper panels) but not at 48 hours. XLPE resulted as always the most contaminated ones for both strains and time-course.

MTT results were confirmed by SEM images (figures 4.7 and 4.9). In fact, XLPE surfaced showed a large presence of seeded bacteria prevalently in complex 3D-biofilm structures. Looking at *S. aureus* SEM images (figure 4.7), pink and white ceramic showed the presence of prevalently single colonies rather than organized communities that are appreciable onto yellowand CoCrMo surfaces. Conversely, *S. epidermidis* SEM images (Figure 4.9) reported that yellow specimens were the less contaminated even if 3D-like structure were observed in almost all the samples. Again, XLPE presented the most compromised surfaces.

These data are linear to those obtained by the roughness analysis [171, 172] : the presence of high peaks could be the most probable explanation of the easy bacterial adhesion. In fact, Lorenzetti et al. demonstrated that macroscopic grooves provided a preferential site

for bacteria deposit within the valleys, while the microscopic roughness of the valleys determined the actual interaction surface between bacterium and substrate.

It means that rough surfaces (XLPE) are prone to greater bacterial adhesion, as demonstrated by Oder et al [173] : the intensity of adhesion is in positive correlation to the increase in surface roughness ,while smoother materials on micro-scale level are unfavorable to the bacterial adhesion, because they provide a smaller surface for bacteria adhesion. CoCrMo were the second specimens considering the presence of roughness-related peaks. So surface roughness could explain the bacteria colonization differences showed by MTT. In fact, Ra, Ry and Rmax of CoCrMo were always significant in comparison with pink, yellow and white ceramics (but not grey ones). Finally, if compared to each others, white and pink ceramics resulted as the most smooth ones. This could justify good results obtained against bacteria adhesion.

Therefore, ceramic materials prove to be advantageous to minimize the risk of infections in orthopedic applications. They present surfaces with intrinsically antibacterial properties, because any surface treatment, antibiotics coating or drugs administration have not been performed.

Possible explanations to antibacterial activity could also be obtained by protein adsorption (albumin, fibronectin, collagen, vitronectin) (figure 4.16) assays results. In fact, the selective protein adsorption could be an important advantage for cells adhesion and tissue repair; on the opposite, it could also promote bacteria adhesion . Particular , the adsorption of cells pro-adhesion proteins including fibronectin, collagen and vitronectin, plays an important role in cell adhesion to artificial materials, and this fact could benefit an effective osseointegration. In fact, as shown by Lee et al [174] , the surface properties govern the affinity and the binding mechanisms between biological macromolecules (e.g., proteins) and the surface, which indirectly determines the interactions between bone cells and implanted biomaterials. These surface properties ultimately play a pivotal role in determining the success of the bone implants; protein adsorption affects cells/materials interactions [174]. On the other hand**,** adsorption of anti-adhesive protein, including albumin, could determine the inhibition of cell and bacteria adhesion, representing an important advantage from bacteriological point of view .

Obviously, from a bacteriological and microbiological point of view, the biomaterial shows better performances in case of adsorption of anti-adhesive proteins, although this fact could represent disadvantage for an efficient and rapid osseointegration.

In this way it's possible to identify which kind of proteins are absorbed by the surfaces, and so it's possible to analyse if the material is favorable or not favorable to bacterial adhesion.

For protein adsorption, BCA displayed that yellow, pink and CoCrMo specimens were able to retain the higher amount of proteins (in terms of ug/ml) in comparison with other samples. However, these data were not statistically significant (figure 4.16, $p>0.05$).

The highest total protein absorption has been observed on yellow and pink, and the lowest one on ceramic grey. Interestingly, yellow and pink are less wettable compared with ceramic grey (figure 4.5) . The obtained result is in accordance to the literature consensus on a preferential protein absorption on hydrophobic substrates compared to hydrophilic ones. [175, 176].

This phenomenon can be explained considering that highly hydrophilic surfaces creates hydrogen bonds with water molecules in the first steps of surface-fluid interaction (hydration) and that proteins, in order to adsorb to the surface, must displace water molecules with a certain energy consumption. The reduced protein absorption on hydrophilic surfaces has been exploited in the realization of super-hydrophilic anti-adhesive and anti-fouling materials [177].On the other hand protein absorption on hydrophilic surfaces can be induced by electrostatic interactions between the surface and the protein [178].

More interesting, western blot analysis (figure 4.16 b) showed that of the total amount of adsorbed proteins, only pink was able to retain all the investigated proteins (fibronectin-collagen-vitronectin-albumin).

In fact, all specimens showed high amount of albumin and vitronectin, while only pink and yellow presented fibronectin. Collagen was exclusively found in pink. These are interesting data because not all the investigated proteins are favorable for bacteria adhesion; albumin is normally reported as in competition for surface adhesion if used with bacteria culture. Thus, the ability to retain albumin could be a good antibacterial step. Pink, yellow, and CoCrMo were the best ones to retain albumin. Fibronectin and

vitronectin are normally considered as pro- cells adhesion proteins while collagen is probably the most cells adhesion and spread promoting protein.

Having noted that ceramics materials selectively absorbed fibronectin, collagen (pink and yellow) and vitronectin, that are pro-adhesion proteins, the results could be considered as positive interpreting them as good osteointegrative properties [174]. In fact cells will be probably helped in adhesion and ECM production, supporting an effective osseointegration. On the opposite, the adhesive promotion properties could also enhance bacteria attachment.

Having regard to the tests performed in the present work and the correlations just discussed, it's possible to affirm that ceramic materials prove to be advantageous to minimize the risk of infections in orthopedic applications. They could be considered superior in comparison with metal and polymers in terms of bacteria preventive anti-adhesion activity thanks to their smooth surfaces and the ability to selectively adsorb anti-adhesion proteins.

It’s possible to speculate that ceramics materials present surfaces with intrinsically antibacterial properties.

Chapter 6
Conclusions and future perspectives

The most important goal in this field is the development of biomaterials that present the best antibacterial performance for orthopedics applications. The goal can be achieved, evaluating the antimicrobial properties for infection-resistant surfaces, to cope with the increased susceptibility to infection, that has remained to this day one of the most common, yet unresolved problems associated with the use of implanted biomaterials.
In this section the conclusive notes concerning the present work are reported.
Furthermore, limitations and future research to develop innovative anti-infective biomaterial are presented.

Anti-infective biomaterials have progressively become a primary strategy to prevent medical device-associated infections. The requirements that antibacterial biomaterials need to cover depends on the type of biomaterial application , so a variety of alternative approaches have been adopted to achieve biomaterials with antibacterial properties, and to reduce the vulnerability of medical devices to the development of infections.

Medical devices are increasingly used worldwide for an expanding repertoire of patient clinical needs, and their designs have become progressively more complex to accommodate diverse demands for performance and safety *in vivo*. While a majority of these implants satisfy their clinical expectations with safety and efficacy in their specific applications, a minority of implants induce serious adverse events with substantial health and economic consequences.

Increasing number and types of implants used in patients have resulted in increasing numbers of biomaterial-associated infections. Researchers and medical device manufacturers have responded to this challenge with intensified attention to innovating device designs, surgical implantation protocols, and biomaterials to minimize infection opportunities.

Medical devices with claims to limit microbial adhesion and colonization using combinations of pharmacological, topological, and materials chemistry approaches have been brought into clinical use with the intent of reducing device-related infections.

Approaches include different biomaterials chemistries that intrinsically resist microbial colonization or that deter active growth on contact, surface modifications that produce topologies observed to limit pathogen attachment, medicinal, antiseptic or bioactive coatings, direct antimicrobial attachment to surfaces, or drug impregnation within the biomaterial, and extended release strategies that control antimicrobial agent release from the device over time after implantation.

Typical implant infection rates are 1% for primary hip implants, 4% for knee implants, and more than 15% for trauma-associated implants. Types of infections associated with orthopedic implants vary widely; however, infection risk is rising as number of implants increases and bacterial attachment on the device is a lifetime risk. Revision surgeries, due to both septic and aseptic complications, represent an even more substantial risk for

infection with a 5–40% infection rate. Common causative microbes include commensal bacteria, but especially *Staphylococcus aureus.*

Biomaterial-associated infections are typically caused by microorganisms that grow in biofilms.

If no comprehensive treatment concept is applied, infections chronically persist leading surgical intervention and long-term antimicrobial therapy directed against biofilms. Whereas acute infection generally needs only one single antimicrobial treatment course to eradicate microorganisms, chronic infection may require sophisticated diagnostic procedures, long-term antimicrobial therapy and repetitive surgical interventions. In the case of orthopedic device-associated biofilm infection, cure of infection should be aimed in the first attempt, because each treatment failure results in a worse functional result. In fact, in case of failure, permanent implants cannot be removed without compromising the replaced function. Therefore, the primary goal is to prevent implant failure due to infection.

In order to reach this goal, management should not be limited to antimicrobial therapy alone. The therapeutic approach is always interdisciplinary, including surgical revision and antimicrobial therapy. The cornerstone of successful treatment is early diagnosis, since surgical intervention is less invasive in patients with a short history of infection. The first treatment attempt is the most decisive in order to avoid tissue damage and loss of function. Therefore, a well-defined treatment strategy is required.

In general, any type of artificial implant is highly susceptible to infection, due to an inefficient host defence in the vicinity of an implanted foreign body. Microorganisms rapidly form biofilms on the artificial surface. Such infections resist host defence and most antimicrobial agents.

Microorganisms within a biofilm are protected against antimicrobial chemotherapy as well as against the host immune system. As a consequence, in the case of implant- associated infections the removal of the medical device frequently is necessary to eradicate the infection.

It's clear that the risk of infection for persons with medical devices (so called BAIs) is substantial and, with antibiotic resistant pathogens increasing without effective mitigation, the probabilities for infections are increasing.

Biomaterial associated infection compromises the quality of life, has a high morbidity and is even associated with mortality. In addition, it has a high economic impact, since treatment of infection costs many times more than the primary implantation of the device.

Clinical approaches to this challenge are multifactorial, involving surgical techniques, operating room protocols and facilities, pre- and post-surgery hospital procedures, patient-personnel contact, device designs, patient profiling and personalization, and antimicrobial innovations. Current clinically approved methods for reducing BAI include many types of antimicrobial device coatings that either intrinsically limit pathogen colonization through topology and chemistry, or actively kill through antibiotic release or immobilization. Other contributions to the problem include improved sterile surgical techniques and methodologies, and altered device designs.

While these approaches demonstrate some metric of effectiveness in reducing infection risk, all current devices including those with antimicrobial technologies remain susceptible to BAIs. In many cases, existing clinical data for improved antimicrobial device performance are under-powered and inconclusive, or outright contradictory.

In almost all cases, *in vitro* efficacy for a given strategy is produced in bacterial assay test beds that have little relevance to in vivo operating conditions for infections and provide little predictive reliability to in vivo expectations for BAIs.

The US Center for Disease Control has outlined several key research needs for better addressing clinical issues that link biofilms, devices and BAIs. First, the ability to collect, assay and quantify reproducible features of biofilms must be advanced for each type of device and pathogen. Second, development of model biofilm systems should be better developed to closely simulate in vivo conditions encountered by the device. Third, improved understanding for how biofilms relate to antimicrobial drug resistance and how biofilms spread to seed other types of infections must be acquired. These variables must then be correlated to device designs and materials aspects to guide rational device-based design innovations to address BAIs.

Therefore BAI it is difficult to treat, as the biofilm mode of growth protects the infecting organisms against the host immune system and antibiotic treatment; for this reason, the future line in orthopedics research, by the engineering point of view, is to design biomaterials with infection-resistant surfaces to prevent the risk of infections, as proposed

in this thesis. Obviously, works and research are still long in this field, to develop innovative materials that limit the risk of bacterial contamination, but many progress have been achieved.

In general, major efforts should be invested to minimize susceptibility of implants to infection; commercial investment and government agencies should provide incentives to help protect, select and direct the more practical promising antimicrobial device applications into key validation protocols to certify better technologies against BAIs and bridge the research-to-product gap.

Engineering strategies of coating of the implant surface with antimicrobial substances such as antibiotics, antimicrobial peptides, or silver is an option.

However, the best protection against BAI is a rapid and complete tissue integration of an implant surface, although not all implants and devices require or allow tissue integration.

In order to stimulate tissue integration it is important to control protein adsorption to biomaterial surfaces.

This thesis proves that it may be possible to design a surface that intrinsically discourages bacterial adhesion and at the same time encourages tissue integration.

Research has developed innumerable solutions currently proposed either to reduce the susceptibility of medical devices to bacterial colonization and infection, or to clear the tissues from existing contaminations/infections. Each of the different strategies pursued to achieve systems endowed with anti-infective properties have generated diverse candidate biomaterials or biomaterial surfaces.

It's important to have an idea about the newly developed surfaces with anti-infective activity; for this reason, the present work proposed principle and potential of innovative anti-infective biomaterial, pointing out expected advantages and pitfalls.

Several surface treatments exist, but the aim of this work is the development of biomaterial that are intrinsically anti-infective, because there is no doubt that prevention is the best response to the growing problem of orthopedic implant infections.

Research in the field of antibacterial surface treatment has demonstrated *in vitro* and *in vivo* effectiveness of several potentially promising technologies. Some interfere with bacterial adhesion and with the initial phases of the biofilm formation. Others exhibit direct

antibacterial properties. Strategies incorporating nanopatterning and other nanotechnologies have also shown great promise.

In the future, multifunctional smart surfaces could open new avenues for prevention of bacterial attachment while simultaneously enhancing healing and restoration of tissue homeostasis.

Issues related to the mechanical properties of these technologies and the potential for detrimental side effects such as toxicity and interference with osseointegration require further investigation. It is of utmost importance to realize, however, that some of the aforementioned technologies have already shown strong enough evidence of antibacterial efficacy, safety, and endurance. The time is here for more efficient development and testing of these technologies in the clinical setting.

However, it must also take into account that an important consideration in designing implants with antibacterial coating relates to the characterization of reasonable and justifiable cost [179]. Theoretically all patients undergoing total joint arthroplasty are at risk for periprosthetic joint infections (PJI). Revision cases carry an increased risk in part due to the suboptimal local tissue environment [180,181–183]. Moreover, several studies emphasize that the risk of PJI across the board in orthopaedic surgery is on the rise [184,185]. As a result, one could argue that all patients should benefit from implants coated with a proven anti-infective surface. On the other hand, the risk for PJI is not homogenously distributed among the arthroplasty patients: it is stratified into the specific groups [186–189]. Therefore, it might be convincing to implant "biofilm resistant" prostheses only in patients at increased risk of PJI.

A validated tool for screening patients for increased risk of PJI does not currently exist. Despite attempts to identify and stratify patients at risk of PJI specific clinical algorithms are not routinely used. In addition, we have no data relevant for determining the potential costs associated with wide range usage of such a screening strategy. Taken together, the preventative strategy involving all patients undergoing primary and revision total joint arthroplasty seems to be more justifiable than a more restrictive approach targeting high risk patients.

While research in this field is promising, there appears to be a great discrepancy between proposed and clinically implemented strategies, and there is urgent need for translational

science focusing on this topic. In fact, clinical data are still scarce, but the task of biomedical engineering, for the achievement of the purposes about infections reduction, implant integration and tissue regeneration seems a feasible challenge in the future.

Of course, it's necessary the synergistic interdisciplinary work of engineering, materials science, biology, chemistry, physics and medicine.

Therefore, careful attention to the clinical results associated with the use of antimicrobial devices is expected to provide the necessary direction for future biomaterials research.

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