PhD Thesis Research Plan

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<td><strong>Duration of study</strong></td>
<td>34 months from (2010/11) to (2013/10)</td>
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<td><strong>Title</strong></td>
<td>Use of Scanning Probe Microscopics for Accurate Measurements at Nanoscale of Properties of Nanomaterials and Nanosystems for Bio-application</td>
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**Research Background:**
Nanomaterials and nanosystems with bio-application are attracting much attention. The PhD program has combined the strong points in the host lab (nanomaterials and characterization techniques at micro and nanoscale) with the innovative ideas and need for biomedical applications. The previous experience in the material science is a good point for the PhD candidate. Nano-biomaterials refer to different nanosized and/or nanostructured materials engineered for interacting with biological materials. For example, organic or inorganic nanoparticles (NPs) can be used as surface for molecular assembly with membrane or nano-vesicle enclosed configuration. In most cases, the size distribution can be the major factor in controlling material properties when using quantum-sized effects. The size control also leads to light emission at different wavelengths, which is the basis for the realization of biomarkers. In nano-biomaterial the core particle is usually covered by monolayers already absorbed on the surface. The layer of linker molecules is a compound of reactive groups at two ends, where one of the ends works as the connection to attach the linker to nanoparticle surface and other ends is used to attach moieties, such as antibodies.

**Scanning Probe Microscopy (SPM)** is a wide branch of microscopy where images of samples surface is obtained using a physical probe scanning the specimen. Scanning probe microscopes (SPM) allow scientists to image, characterize and even manipulate material structures at exceedingly small scales including features of atomic proportions. A wide variety of material structures and properties can be studied, either natural or artificial systems, including biological systems. The type of interaction measured between the probe tip and the sample surface defines the type of scanning probe microscope being used. Since the invention of the first scanning tunneling microscope by Heinrich Rohrer and Gerd Binning in 1981, scanning probe microscopy has enabled a burst of nanotechnology achievements that includes the manipulation and arrangement of individual atoms on a surface. Wide areas in medicine and biological applications are affected by the use of nano biomaterials. For instance some of the recent developments for medicine applications are tissue engineering, detection of protein and cancer therapy.
drug delivery systems and medical imaging for diagnosis. One growing area is drug delivery system with benefit of targeting a specific cell for delivery with more therapy efficacy. Also, in medical imaging specific area is selected for imaging using quantum dots or chromophores synthesis.

In addition, tissue engineering and cancer therapy are other important applications for nano-biomaterial. All the mentioned areas are the most severe fields in medical applications that importantly need has to be improved, using nano-biomaterial. As an example, in the tissue engineering, it is evident the surface of the artificial bone is smooth, which will be rejected by the body, to overcome this problem the surface has been coated by the nanoparticles, but most of the existed suffers from the lack of bioactivity, like titanium. Therefore, the approaches were made to use apatite coating on titanium which resulted in thick non-uniform and poor adhesion surfaces. Hence the approach was made to construct an apatite from the simulated body fluid which has an advantage of strong adherent and uniform layers. Also in cancer therapy, for specially treatment of photodynamic cancer therapy, where cancer cells are attacked by the use of laser generated atomic oxygen and most of healthy cells are also exposed to the radiation and are destroyed. By using nanoparticles, dye molecule that is used to generate the atomic oxygen is kept inside an ormosil nanoparticle and is not spread out to the whole body cells.

**Characterization techniques:** Characterization can take the form of actual materials testing, or analysis, for example in some form of microscope. Analysis techniques are used simply to magnify the specimen, to visualize its internal structure, and to gain knowledge as to the distribution of elements within the specimen and their interactions. Atomic Force Microscopy (AFM) is a particular type of SPM that emerged as one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale. The information is gathered by “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. In some variations, electric potentials can also be scanned using conducting cantilevers. In newer more advanced versions, currents can even be passed through the tip to probe the electrical conductivity or transport of the underlying surface. AFM provides a three-dimensional surface profile. Additionally, 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. While an electron microscope needs an expensive vacuum environment for proper operation, most AFM modes can work perfectly well in ambient air or even a liquid environment. This makes it possible to study biological macromolecules and even living organisms. In principle, AFM can provide higher resolution than SEM. It has been shown to give true atomic resolution in ultra-high vacuum (UHV) and, more recently, in liquid environments. High resolution AFM is comparable in resolution to scanning tunneling microscopy and transmission electron microscopy. AFM can also be combined with a variety of optical microscopy techniques, further expanding its applicability. AFM-based techniques have been developed and are still being studied to retrieve, simultaneously to morphology, maps of
**Experimental objects:** Humans have always been exposed to a wide variety of nanoparticles (NPs) naturally produced and present in the environment. Moreover, due to the enormous progress of the scientific and technological research, synthetic NPs have been produced and proposed for a number of applications (e.g., biomedical or food industry applications) which increase the exposure to NPs of humans, either nanomaterials researchers and producers or people under medical treatments which use NPs or simply food products customers. In particular, nanosystems (NSs) have been studied extensively and intensively, due to their potential applications in many fields of technology; for example, magnetic NSs can be used in biomedicine and bioengineering for magnetohypertermia treatments, magnetically assisted drug delivery, cell isolation, molecular recognition, biological macromolecules purification, biosensors, and magnetic resonance imaging (MRI) or positron emission tomography (PET) enhancement. Functionalized NSs have useful applications in the field of MRI and drug delivery for their ability to bind drugs and/or biological macromolecules. Multi-shell Fe@Cu@Au and Fe3O4@Cu@Au are new multifunctional carriers (MRI/PET contrast agents, drug delivers and hyperthermia treatment agents), which have been developed for biotargeting aims. They represent a new level of multifunctional NSs, since they combine the properties of the different materials they are built with (magnetic properties for MRI and thermo ablation, linkage of organic molecules due to the gold shell, positron emission for PET for the eventual presence of 64Cu atoms in the copper shell by synthesis of these NSs in presence of 64Cu salts). The advent of ceramic nanomaterials in the field of medicine has a key role in both diagnosis and therapy due to their stability and versatility. In particular, silica-based mesoporous materials have received increasing attention as carrier for the delivery of different species (e.g. drugs, antibodies, nanoparticles and small molecules). The ease of silica surface functionalization and the possibility to tune the size and shape of pores make these systems as promising candidate for targeting and drug delivery. In the case of magnetic and/or metallic NPs for diagnostic imaging, it is expected that their confinement into inorganic carriers can reduce eventual toxic effects deriving from the use of NPs.

**Scientific preparations of the PhD candidate:**

1. I have learnt atomic force microscopy (AFM), and AFM will be the main technique I will use in my project.
2. Just a basic knowledge in experimental material science, physic and chemistry is required here; however, my background in the physics is very important, as well as my experience with characterizing the structure of sample: Xray Diffraction (XRD); X-ray Photoemission Spectroscopy (XPS); Brunauer Emmett Teller (BET); Fourier Transform Infrared Spectroscopy (thermal gravity-ultra red chromatography).

**Expected goal, research methods and arrangements:**

The nano biomaterial with an advantage of nano-sized particles and compatibility with the human body has been applied extensively in biomedical application and in medicine. In a word, the preparation and structural characterization of the nano-materials have to
be studied, together with the potential application in bone tissue engineering. At the present time, there are industries involved in developing the commercial applications of nano biomaterial for biomedical industries. For example, magnetic NPs will be prepared and their pathways in cells will be investigated. Such a study is finalized to verify the suitability of magnetic NPs for drug delivery and/or as contrast agents for magnetic imaging. We will investigate the occurred reactions and in what conditions, and, if possible, what is the role played by nanomaterials. First, the preparation and structural characterization have to be undertaken. Then, the bio-activity and bio-degradation have to be revealed, mainly by the in vitro test of the nano-materials immersed in simulated body fluid (SBF). My PhD project is to study the preparation and characterization of the bioactive materials with novel biomedical applications, and to study the possibility to develop scanning probe-based microscopies that could be implemented in rapid and early diagnostic methods for the biological effect of NPs. My PhD thesis concerns the study of interaction between nanoparticles (NPs) and biological systems using Scanning probe microscopes (SPM) based techniques.

For two aims: 1) obtaining novel nanostructured surfaces for the prevention or enhancement of the growth of cells; 2) seeing where NPs accumulate in biological systems.

For the 1st aim, novel nanostructured surfaces are the key points. The nano biomaterial with an advantage of nano-sized particles and compatibility with the human body has been applied extensively in biomedical application and in medicine. In a word, the preparation and structural characterization of the nano-materials have to be studied, together with the potential application in bone tissue engineering. At the present time, there are industries involved in developing the commercial applications of nano biomaterial for biomedical industries. As an example, magnetic NPs will be prepared and their pathways in cells will be investigated. Such a study is finalized to verify the suitability of magnetic NPs for drug delivery and/or as contrast agents for magnetic imaging. Two steps are designed for the 1st aim. First, the preparation and structural characterization have to be undertaken to secure the novel surface properties. Then, to verify the bio-activity and bio-degradation in biological systems, the in vitro test of the nano-materials immersed in simulated body fluid (SBF) will be studied.

Actually, the 2nd aim is designed for biofilm targeting. We want to see in bacteria because they are a good experimental model and we are interested in the accumulation sites. Vesicles are interesting for drug delivery (if you put a magnetic NP in a vesicle with drug you can use a magnet for driving the vesicle in the body to the target organ). Drug delivery and targeting to the bacterial biofilms is receiving increasing interest. Vesicles are the highly investigated delivery and targeting devices designed and developed for biofilm targeting. However, the potential of drug delivery in the localization and/or targeting of biofilm still remains to be proved and adopted in the field of pharmaceutical research. Targeting could be obtained by the intrinsic and inherent distribution profile of carrier (passive targeting) or by using complex systems with carriers with suitable ligands to alter their distribution or uptake in the biological milieu and to release the drug in the proximity of bacteria within biofilm (active targeting).

In the 2nd aim, the spatial distribution and the quantitative localization of NPs, which
are delivered by suitable vesicles and labeled by magnetic NPs, will be studied for their biological features, such as their activation when bonded to antibodies or drugs, to produce the release of drugs to target cancer cells. We will investigate the occurred reactions and in what conditions, and, if possible, what is the role played by nanomaterials.

**Expected goal:**
The main objective of the project is the definition of innovative protocols/methodologies which give information about the localization of nanosystems (NSs) into biological systems (BSs) aiming at:
1) the study diffusion dynamics of specific natural substances with antimicrobial action (lactoferrin) loaded in NS (niosomes+magnetic nanoparticles) inside specific bacterial biofilm;
2) the study the possible anti-biofilm activity of loaded substances, their interactions and the development of potential therapeutic effects;
3) the study possible cytotoxic effects on appropriate cultured cell lines.
This will require the development of innovative multi-scale techniques for the functional imaging of NPs into biological materials (cells and bacteria) as well as the development/definition of protocols of preparation of the different samples to make them adequate for the experimental purposes and suitable for the observation by the different available techniques.

**Research Methods:**
1, **Design and preparation of nanomaterials and biological samples:** for example, Ti substrates with/without NPs (such as HAp, carbon NPs, magnetic NPs and others); as another example, NPs with magnetic properties, constituted either by a single material or with a core-shell structure, functionalized to increase their bio-compatibility.

2, **Material characterization:** The characterization of biomaterial is the essential first step for understanding their performance in the host. The characterization of internal and external interfaces of biomaterials is a central issue of this project, as these determine the biomaterial's mechanical performance and the interaction with the host.
AFM as a surface sensitive technique is a suitable tool to explore these phase boundaries. Advanced AFM-based techniques will be employed to study mechanical properties of samples with nanometrical lateral resolution, to image subsurfacial features of the samples such as the presence of NPs inside cells. Magnetic force microscopy (MFM) will be used to study both magnetic NPS on substrates and inside cells. AFM characterizations will be performed in air and in simulated biological environment (see below). Nanomaterials alone and in biological samples will be investigated by standard techniques like X-ray diffraction, electron microscopy (either scanning or transmission electron microscopy, SEM and TEM respectively) and related techniques (microanalysis, electron diffraction), Raman spectrometry. Mechanical/thermal properties as well as chemical stability will represent two aspects to be tested.

3, **Biological characterization** will be performed following classical methods used in labs, such as cell attachment/proliferation. Samples will be analyzed both in air and in SBF that will be prepared to simulate the human plasma.
Arrangements: we want to understand what reactions has occurred and in what conditions, and, if possible, what is the role played, during the interaction of Magnetic nanoparticles with bacteria and vesicles. At present involved in the development of AFM techniques for the imaging of nanomaterials into biological systems. The contrast in the images derives by different mechanical, electrical and magnetic properties of NPs and biological materials. It concerns the study of interaction between NPs and biological systems using SPM based techniques. For two aims: 1) nanostructured surfaces for prevent or enhance the growth of cells; 2) see where NPs accumulate in biological systems.

In detail:

1) The study of the dynamics of different nanosystems in bacterial biofilms will allow to select those that enable the prevention of biofilm formation and to comprehend the iron role in biofilms already formed. This is of great interest since pathogen bacteria in biofilm state are scarcely sensitive to antibiotics and are the main source of hospital infections and moreover, they form at pulmonary stage especially in subjects affected by cystic fibrosis. Besides the evident risk for human health, one should not forget the expenses for the society produced by such infections, for instance in terms of hospitalization days and prostheses removing and substituting (the costs for substituting a prostheses can be evaluated in 50,000 euro). The solutions that will be in particular studied are: 1) functionalized NPs for improving their internalization, that could have antibacterial properties due to the presence of metal oxides; 2) vesicles with Lf and magnetic NPs for the delivery of vesicles into biofilm; 3) mesoporous materials for the delivery of Lf and magnetic NPs; 4) mechanical eradication of biofilm by thermal ablation due to the heat produced by the agitation of NPs produced by a magnetic field.

2) On the contrary, by analyzing the target structures of NPs in biofilms and if suitable bacteria were identified which were able to efficiently absorb NPs, these could be used for creating bacterial biofilms that, immersed in the water flux, act as filters for NPs. This would be of the greatest interest for reducing the pollution from NPs in water that, directly drunken or accumulated in animals, may represent a risk for human health, which represents one of the so-called “emerging risks”, with which nano-toxicology deals.

3) The studies enabled by the developed techniques on animal cells will have an immediate spin-off on the knowledge of toxicity of NPs and on target organs. Nano-toxicology deals with these arguments, which is becoming a wide field of study since the tremendous increase of nanotechnologies puts human being in contact with nanomaterials whose toxicity has not yet been assessed, as detailed long-term studies are missing.

4) The possibility of delivering drugs and nanosystems may be used, analogously to what illustrate above for biofilms, for destroying cancer cells through a suitable choice of drugs and/or mechanisms of thermal ablation.

The research activities will be carried out within the framework of the scientific collaboration in which are involved the three research groups leaded by prof. Marco Rossi, prof. Mario Barteri and prof. Piera Valenti, and taking advantage of the
instrumentation at the state-of-art available at the EMINA (Electron Microscopy and Nanoscopies) Lab and at the SNN (Sapienza Nanotechnologies and Nanosciences) Lab.

Details about the experiments:

Period A—(from 2011/10 to 2012/03)
1. **AFM characterization on magnetic nanoparticles (MNPs) with bacteria**
   A. choosing floppy as a research object which has magnetic domains very clearly
   B. using polysaccharides with different concentration which simulate the polymeric produced by bacteria that was put on floppy, to check the magnetic domains
   C. using bacteria (staphylococcus aureus) put on floppy to find the topography and the change of magnetic phase
      (the above three steps are the preparation for using of magnetic nanoparticles)
   D. using magnetic nanoparticles instead of magnetic floppy, at first to check the topography and the magnetic domains of magnetic core and the one which was treated with APTES technique, to see the nanoparticles which accumulate in biological systems
      (the above four steps have been done, and get good results)

Period B—(from 2012/04 to 2013/10)
E. finding a suitable kind of magnetic nanoparticles which is biocompatible and have good measurements results
F. using the new kind of magnetic nanoparticles to measure with polysaccharides and staphylococcus aureus with different concentration and cultured time, to see the impact of the two elements and the accumulation of nanoparticles in biosystems.

Such a characterization will be aimed at the visualization and the identification of NSs into either the biofilm or the single cells. In such a context, we expect as final results that:
- the imaging of NSs into the biofilm will allow a better comprehension the transportation mechanisms of nutrients through the extra/intra-cellular channels;
- the imaging of NSs into the cells will enable us to study their interaction mechanisms, thus allowing us to comprehend the local nanotoxic mechanisms of the different NSs.

After this objective is reached, which is the first task of the project, further developments will be aimed at studying toxic effects of different nanosystems on the cells forming the biofilm. Such a study aims at individuating suitable nanosystems with antibacterial properties, thus allowing the inhibition of the biofilm growth.

(the above two steps have been prepared but not measured yet)

**Point of innovation:** All of the measurements will be taken in air, and some will be taken in liquid condition, it is deserved to say this is the first time to using magnetic force microscopy in liquid, it will provide experimental support for clinical application.

2. **AFM characterization on magnetic nanoparticles (MNP) with vesicles**
   preparation of vesicles and of the vesicle-polyion aggregates (NvPA) from the union of negative vesicles and a cationic polyelectrolyte (epsilon-PLL and chitosan with different
polymerization degrees); identification and localization of Nv and NvPA within biofilm and bacterial cells, to understand the mechanisms of interaction between vesicles and biofilm/bacterial cells, the obtained results will allow also the realization of composite nanosystems made by vesicles able to deliver active molecules for therapeutic purposes and magnetic NPs for allowing both the traceability of vesicles and their delivery to specific targets (active targeting), for example using magnetic NPs with a suitable external magnetic field. The delivery of the abovementioned species will be performed also using silica based mesoporous materials. The versatility of such systems allows the confinement of different typologies of species due to the possibility of controlling the porous structure and of functionalize the surface.

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