Università degli Studi di Sassari

Doctoral course in

Life Sciences and Biotechnologies

Director Prof. Sechi L.A.

Cycle XXXI

Nanotechnology: development of nanotools to counteract human diseases

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To…

Prof. Proto Pippia

"EARTH IS THE CRADLE OF HUMANITY,

BUT ONE CANNOT REMAIN IN THE CRADLE FOREVER"

Kostantin Eduardovitch

TSIOLKOVSKY

Agli scienziati che mi hanno

trasmesso la conoscenza

e l'amore per la scienza:

Prof. Serenella Medici

Dr. Roberto Cabizza

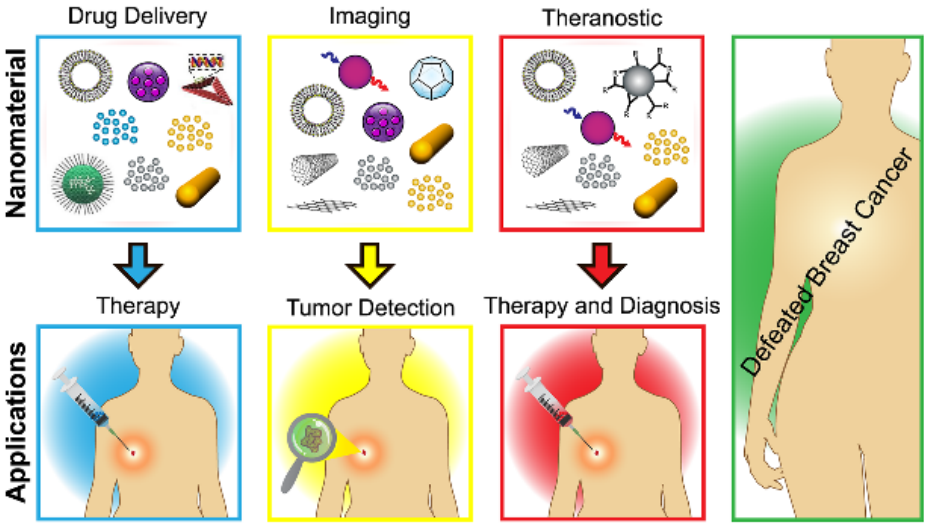
Giuseppe Delogu

Contents of PhD thesis

Abstract

Nanotechnology represents a set of methods and techniques, which allow the manipulation of matter on the atom-molecular scale, with the goal of creating radically new products and processes. It is well know that the materials in the nanoscale present unique chemical and electrostatic properties linked to a useful large surface of functionalization for molecular interactions or biologically relevant conjugates. Nowadays, the research is deeply focused on the translational applications of nanotools in medicine. In this context, nanomedicine appears as a new field of nanotechnological sciences based on the development of nanomaterials for biomedical investigation. At the same time, most nanomaterials are raising the attention of scientists thanks to the possibility of using them as therapeutic substitutes to improve current diagnostic agents or drugs. In terms of health safety, it is necessary to evaluate the chemical and physical properties of nanomaterials, in particular when they are synthesized for biomedical purposes. Nanomaterials, when carefully synthesized, remain stable preserving their unique nanoscale properties.

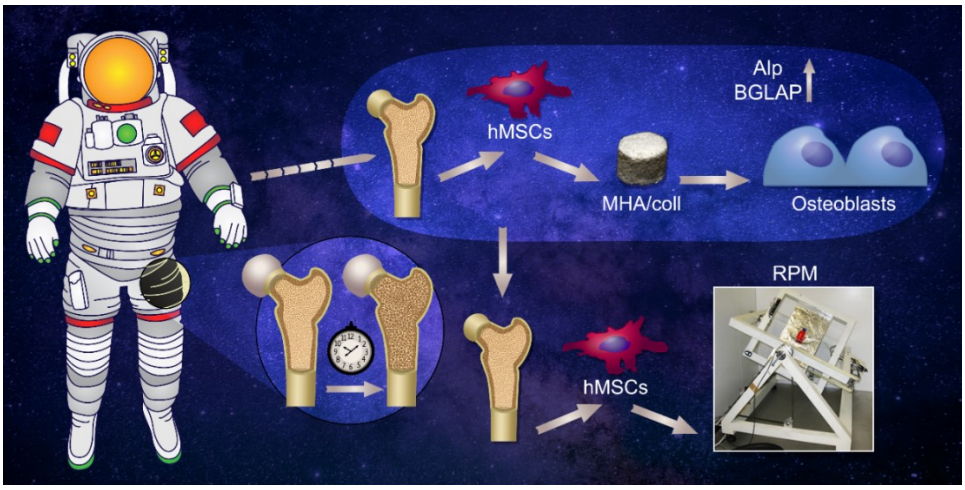
In this work, a critical review has been performed to understand how nanotechnology has helped the fight against one of the most important human health conditions like breast cancer disease in the last nine years. In this review, it was highlighted that the purpose of scientists is to identify new nanomaterials that can be tolerated by biological environments displaying no toxic-effects and their biocompatibility on cells.



Graphical abstract 1. Different nanomaterials developed against breast cancer.

Before any translational application of nanotechnology in medicine, the critical step that can be done is represented by the evaluation of biological activity in *in vitro* studies. In this study, different nanomaterials were explored to counteract two important human health conditions, such as bone loss dysfunction and malaria, using *in vitro* experiments.

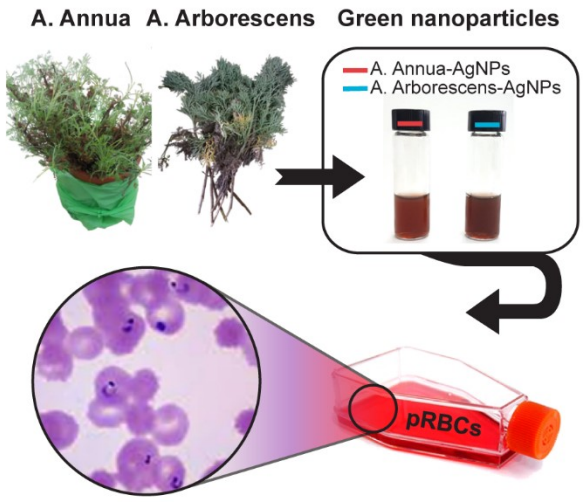
In the first part of this work, a novel material with nano-mineralization is presented as a bone regeneration application to counteract the bone loss dysfunction. This problem has been reported during spaceflights missions connected to a significant bone loss inducing an osteoporosis like condition. In this context, the Random Positioning Machine (RPM) was applied to simulate long duration microgravity conditions (M, 0 x g). The osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) cultured on nanocrystalline magnesium-doped hydroxyapatite/type I collagen composite scaffold (MHA/Coll) has been investigated under simulated microgravity. Results indicated that the amorphous nanostructured apatite of MHA/Coll niche (3D culture) is able to promote the hBM-MSCs differentiation in osteoblastic lineage in response to the transfer of key cues at the nanoscale to h-MSC in long-duration microgravity simulation.



Graphical abstract 2. Schematic diagram of MHA/Coll function in microgravity condition to counteract bone loss dysfunction.

In the second part of this work, a novel nanomaterial is presented as an anti-malarial application in order to evaluate its anti-protozoal activity. Different silver nanoparticles obtained using *Artemisia annua* and *Artemisia arborescens* extracts were prepared by a "green" synthesis approach to assess their possible use as anti-malarial agents. The "green" AgNPs have thus been tested in *Plasmodium falciparum* infected red blood cells. Results evidenced a high anti-malarial activity for *A. annua*-AgNPs in *in vitro* experiments against *P. falciparum* cultures compared to *A. arborescens*-AgNPs.

In conclusion, considering the results gathered by the two nanotechnological applications here presented, it emerges that the use of nanomaterials has improved the osteoinductive potential of the scaffold and the antimalarial activity against *P. falciparum*.



Graphical abstract 3. Green synthesis of *Artemisia*-silver nanoparticles.

Finally, data collected *in vitro* open a new path for further studies to investigate the potential of these nanomaterials as a possible new nanotechnological strategy against bone loss dysfunction and malaria diseases.

Chapter 1. Introduction

1.1 Historical concept of nanotechnology

Nanotechnology is defined as a novel approach to engineering of innovative materials and functional systems at the nanoscale, generally between 1 and 100 nanometers in size.

The concept of "*Nanotechnology*" was introduced for the first time by Dr. K. Eric Drexler in the 1980's, described as one of the most founding father of the nano science. In his work published in PNAS in the 1981, he outlined the ability to design protein molecules that make possible the construction of molecular machines.[1] This molecular engineering underline the future development paths in molecular manipulation and nanotechnology applications.[1] Furthermore, in his book entitled "*Engines of Creation: The Coming Era of Nanotechnology*" (Drexler K. E., 1986), he described to a wide audience the importance of nanotechnology in the construction of molecular machines working on a molecular scale in order to manipulate matter from the bottom up.[2] Drexler's research in the field of application of advanced nanotechnologies has been the basis of "*molecular nanotechnology*" and physics-based analysis in "*nanosystems*" as "*computation and molecular machinery*".[3] In spite of the large part of science that is defined today as "nanotechnology", the meaning of this word is not the original one. The primary term of "Nanotechnology" refers to engineering ability to design and construct nano-structures and devices using the bottom-up approach with atomic precision. This theoretical capabilities were predicted by the famous physicist Richard Feynman in 1959: "*I want to build a billion tiny factories, models of each other, which are manufacturing simultaneously. . . The principles of physics, as far as I can see, do not speak against the possibility of maneuvering things atom by atom. It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done because we are too big". — Richard Feynman,* Nobel Prize winner in physics.

Feynman's vision of nano scale factories represents the main objective of the advanced nanotechnology using molecular machine systems.[3] He was the first physicist who launched the challenged of molecular machines, collected by Jean Pierre Sauvage, Sir J. Fraser Stoddart and Bernard L. Feringa, the winners of the 2016 Chemistry Nobel Prize, during the famous talk in 1959 known as "*There's plenty of room at the bottom*".[4]

1.2 Cultural perception of nanotechnology

In the last decades, science history has observed the birth of new and innovative disciplines, which now influence the everyday life. Presently, the new disciplines like nanotechnology and nanomedicine are emerging as powerful applications aimed at improving the quality of life. It was described that the perception and correct scientific communication combined with public participation would have a significant impact on the greater growth of these new disciplines.[5] In the last decade, the concept of manipulation of matter at the atomic or molecular level and the possible use of nanotechnology in real life has given rise to a debate of a cultural nature. The concept of cultural conditions represents the tendency of people to base their cultural appraisal according to their factual beliefs of a probably dangerous activity.[6] An interesting study has described the problem of the cultural cognition of the concept of risks and benefits related to the use of nanotechnology involving 1,862 Americans divided in 2 groups: no-information condition and information-exposed condition.[7] As reported in **Figure 2**, the results showed that information exposure does not have a relevant effect of probability that either a subject can perceive the benefits of nanotechnology to be greater than its risks (**Figure 1a**).[7] On the contrary, information exposure showed statistically significant impact on subject defined with reference relate to their cultural worldviews (**Figure 2b**).[7]

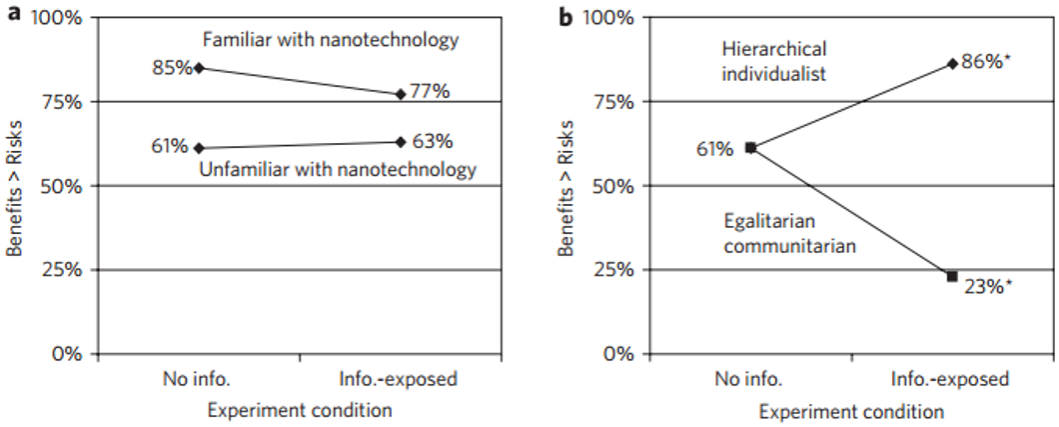


Figure 2. "Effect of information on risk–benefit perceptions of subjects defined by self-reported familiarity with nanotechnology and cultural worldviews. a) Likelihoods of response for the benefits of nanotechnology exceeding the risks in the no-information and information-exposed conditions when cultural worldviews are controlled (set to their means) for respondents who are unfamiliar (bottom line) and familiar (top line) with nanotechnology. **b)** Likelihoods of response across conditions when familiarity is controlled for (set to its mean) and the culture variables are set at values that reflect the worldviews of modestly hierarchical and individualistic subjects (top line), and modestly egalitarian and communitarian ones (bottom line). N ¼ 1,672. *Change in likelihood across conditions significant at P ≤ 0.05**"**, (Kahan *et al*., 2009).

These findings suggest–a model of how cultural predisposition and exposure to information about nanotechnology effects act on risk–benefit perceptions (**Figure 3**).[7]

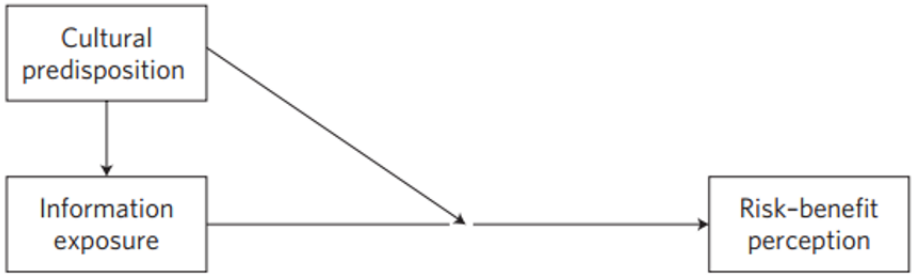


Figure 3. "Relationships between cultural worldviews, information exposure and risk–benefit perceptions. The study results suggest that cultural worldviews influence perceptions of the risks and benefits of nanotechnology both by influencing how likely subjects were to be exposed to information (or report being exposed to information) about nanotechnology, and by determining what effect—positive or negative—they gave to that information**"**, (Kahan *et al*., 2009).

Several works published in *Nat. Nanotechnology* have reported that analyzing various risk nanotechnology perception surveys, people are able to perceive more benefits than risks[8] and that non experts are more concerned with nanotechnology risks compared to scientific community[7, 9, 10] Basically, people who have a protechnology cultural orientation are thus more likely to draw positive conclusions related to nanotechnology contrary to individuals who lack that predisposition, showing a high probability that they can react negatively.[7] Many researchers have highlighted the possibility that spontaneous and continuous scientific information can play an important role on the public opinion on a balanced conception of the risks and benefits of nanotechnology.[7-11] In general, public perception of nanotechnology applications is basic for their realistic growth in everyday life. Nowadays, several pilot study have shed light on the positive public's perception of nanotechnology, due to the high interest for science and technology.[11-13] Furthermore, several websites and social networking services could offer advantages for knowledge sharing, promoting the fast science dissemination compared to the traditional methods.[14]

1.3 Manufacturing at the nanoscale

The world of nanotechnology acting directly on the natural connections between atoms, represents a future perspective of new materials through the assembly of atoms and molecules in an automatic way, as happens in the living world.[15] In this context, the goal of nanotechnologists is therefore to manipulate the matter at the nanometer scale to build new materials and products with special physical and chemical characteristics, better than existing materials.[16] The great success of matter manipulation is to be found in the excellent properties shown by the nanomaterials compared to the bulk structures.[17] It is well known that when the particle sizes are getting close to the nanometer they acquire new properties and just for this reason, it is necessary to revaluate the current knowledge under such a new perspective.[17]

It has been observed that while approaching the nanoscale size, the percentage of surface atoms

increases considerably, which are often much more reactive than the atoms located inside the particle.[17] Accordingly, as the particle size decreases, its surface area increases. Furthermore, thanks to the knowledge of the fundamental properties up to the atomic or molecular scale, nowadays it is possible to design "artificial materials" with excellent properties according to the prefigured application.[18, 19]

There are two main ways to create nano structures:

- the "*top down*" approach which consists in reducing the size of the smaller structures using physical methods;
- the "*bottom up*" approach allows starting from small components, normally molecules or atoms, to realize nanostructures both of inorganic and organic type.

The first approach guarantees better results in terms of reproducibility, however it requires the use of special and often expensive equipment.[20] On the contrary, the second approach leads to higher performances under many points of view, because by starting from solutions in liquid phase, at controlled temperature and pressure, it allows to regulate factors as shape and dimensions of the nanomaterials, for instance. However, the latter approach requires a knowledge and a very fine control of the synthetic protocols, which has not yet been completely achieved.[21]

In general, the matter on earth, under the impulse of self-regulating forces, has assumed complex shapes over the course of billions of years. The possibilities of application of living matter are sometimes limited, because of its low stability and toxicity in biological environments.

The goal of nanotechnologies is based on the creation of artificial nanostructures able to withstand much more aggressive operating conditions, such as extreme acidity / basicity or temperatures of hundreds of degrees, high stability and biocompatibility. Furthermore, the creation of nanostructures and the possible future use of nanoparticles in the field of science

will result in the introduction of nanotechnology into daily life without radical changes. In general, today the revolutionary "*Nano-science*", among the various sectors in which it has been introduced, finds its application above all in the biomedical field, but it is also very developed in physics/chemical science and in the environmental field to look for a solution against pollution.

1.4 Nanotechnology application in medicine

The recent advances in nanoscale science and engineering have led to the application of nanotechnology in medicine with the development of new biomedical nanotools.[22] Besides industry and combinatorial chemistry applications, nanomaterials have been explored for future nanomedicine therapy.[22] Manipulation with macromolecular and molecular precision have led to the manufacture of innovative nanomaterials, which are able to interact with and often mimic biological systems.[23] In particular, nanotechnology can create human-made materials in the nanoscale range, the same scale where cellular and biological processes take place.[24] The potential adverse toxicity, biosafety and clinical opportunity of nanotools should be evaluated carefully in *ex vivo* preclinical models before any application in the human body. Precisely nanoparticles, nanostructured tissue scaffolds and nanobiomaterials, overcoming the limitation found in conventional agents, have been investigated for the development of alternative therapeutic treatment for human diseases.[25, 26] Over time, nanomedicine have found its primary application in the treatment of several serious human conditions including cancer and metastases, allergies, viral and microbial infections, bone resorption and brain disorders.

Nowadays, cancer represents a global health problem related to an uncontrolled malignant cells proliferation that overcrowd normal cells around in the body.[27] Until now, cancer nanotechnology represents a new field of science focusing on the use of nanomaterials for enhancing cancer detection, treatment and diagnosis through the development of new nanotools to be applied in oncology.[28, 29] Cancer nanotechnology has demonstrated excellent approaches

to cancer detection, diagnosis and treatment with limited toxicity compared to the traditional cancer therapy.[30, 31] One of the most important aspects of cancer nanotechnology represents the ability to engineer nano carriers with multiple molecules that, because of their small size, can penetrate tumors with high specificity, consequently with significantly fewer side effects.[32-34] Moreover, nonsurgical tumor ablation techniques have been developed with the complete destruction of tumor cells by the direct application of thermal and chemical therapies using nanomaterials, composed of metals, lipids, or polymers.[35] To this end, during the last years, nanotechnology approaches have provided innovative promising strategies in order to efficiently detect and treat—cancer and metastatic conditions.[36] The importance of nanotechnology in drug delivery application is addressed to the ability of molecular structure manipulation in order to create nanotools directed towards programmed target.[37] Of particular interest, manufacturing at the nanoscale has accelerate the development of nanoparticles complexes to improve the drug loading with precision, helping the possibility to become a real medical strategy.[38,39] In this context, nanomaterials can be designed to transport chemotherapeutic drugs directly to the tumor cells using specific antibodies to target the cancer site.[40] Anyway, before initiation of cancer treatment, it is essential to carry out diagnostic imaging procedures to understand the type of cancer lesion. In this context, a new field of nanomaterials as theranostic agents is emerging, which are able to combine diagnostic and therapeutic strategies into one procedure.[41]

In the last years, the scientific community is aiming to investigate the properties of nanoparticles-based biological molecules to induce stem cells proliferation in damaged tissue like bone, liver, spinal cord and heart, for regenerative medicine applications.[42-44] On the other hand, for human diseases based on bacterial or parasitic infections, scientist are pointing their attention towards metal nanomaterials.[45,46] Metal nanoparticles and in particular silver nanoparticles have been studied as antibacterial agents, and they could be used to counteract

viral or parasite human infections.[47, 48]

Indeed, thanks to their dual properties of drug delivery and imaging together, their unique properties make the metal nanoparticles a possible future candidate against human infections. Nevertheless, the risks connected to the presence of nanomaterials inside the human body should be balanced with their beneficial aspects in the field of nanomedicine application. However, an increased knowledge of nanotools mechanism effects will provide new less toxic therapies with safer nano-complex to specifically treat human diseases.

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Chapter 2. Aim of the thesis

Recent advances in technology and engineering have led to the application of nanotechnology in medicine with the development of new nanoscale biomedical systems. Nowadays, nanotechnology could represent one of the best promises to develop new therapies in medicine. Compared to traditional diagnostic agents or drugs, nanomaterials can be engineered to improve multiple functions in a single system. Moreover, in the recent years, large varieties of nanomaterials have been investigated for the control of drug release and gene therapy being of hope for the next generation treatments in the near future. However, despite the still limited results on the non-toxic effect of nanomaterials, the main step before any biological application is represented by their safety assessment. For all these reasons, scientists are researching new less toxic nanotools for new diagnostic strategies.

This thesis aims to shed light on the possibility of using innovative nanomaterials as future therapeutical agents. The research work consists of three parts that include several innovative nanotechnology applications to counteract three types of serious human diseases. The purpose is to present new nanoscale solutions to develop-nanotools able to fight human health problems. In this work, breast cancer, bone loss dysfunction and malaria have been taken into consideration as they represent three of the most studied human diseases in recent years.

In the first part of this work, breast cancer (BC) has been taken into consideration because it represents one of the most common types of cancer affecting women (and in fewer cases also men) worldwide. BC is one of the most studied tumors as it is considered one of the most aggressive forms of cancer and is the second leading cause of death in the world. In this perspective, a broad review of the current research on BC was necessary in order to point out what are the most promising nanomaterials that can perform a breakthrough revolution in the scenario of breast cancer for diagnosis, therapy and theranostics. It was thus necessary to

examine an extremely vast amount of works carried out in the last few years related to nanotechnology approaches against BC and discuss all the specific potentialities of the nanotechnology tools to overcome current diagnostic barriers, reduce side effects during cancer treatment and toxicity on healthy tissues.

In the second part of this work, bone loss has been taken into consideration because it represents one of the major age-related health problems, causing bone dysfunction and fractures that significantly impair the quality of life. In this context, it has been reported that also spaceflights lead to dysregulation of the osteoblast function connected to a significant bone loss inducing an osteoporosis like condition. The bone loss dysfunction under microgravity conditions (MG) is related to an impairment of osteoblast and an increase of bone resorption with a significant decrease in osteogenic gene expressions, which are ordinarily connected to a normal bone resorption. The purpose of this work was to perform an integrative study evaluating the effect of simulated microgravity on human bone marrow-derived mesenchymal stem cells (hBM-MSCs) cultured in nanocrystalline magnesium-doped hydroxyapatite/type I collagen composite scaffolds (MHA/Coll) to promote the osteogenic differentiation. In this study, the potentiality of nanostructured MHA/Coll scaffold (3D culture) to hinder osteoblast dysfunction under long duration simulated microgravity, as osteoporosis like condition is discussed.

Finally, in the third part of the work, malaria has been taken into consideration because it represents one of the most common infectious disease and one of the most serious public health problem worldwide. In this context, metal nanoparticles have been given increasing interest in the scientific community due to their chemical stability and biological activity against bacteria and parasites. Moreover,–silver nanoparticles (AgNPs) could represent new nano-tools for biomedical applications thanks to their promising antibacterial properties. The purpose of this

work was to perform the synthesis and characterization of different types of silver nanoparticles (AgNPs) using a "green synthesis" approach, and to assess their potential antimalarial efficacy. In this study the hemocompatibility and the antimalarial activity of such AgNPs against malarial parasite *Plasmodium falciparum* is also discussed.

Chapter 3. Literature review: nanotechnology and breast cancer

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3.1 Introduction

BC represents a malignant tumor where breast cells grow out of control and overcrowd normal cells. BC represents the most common type of cancer affecting women worldwide, and it is the second leading cause of death in the United States with 253 000 new cases estimated in 2017.[1] When BC occurs, it is crucial, for the prognosis, to achieve early detection, followed by opportune treatments including surgery and chemotherapy. Nowadays, the most important clinical analyses comprise mammograms, ultrasound examinations, and nuclear magnetic resonance imaging (MRI). Moreover, biopsy and blood chemistry studies help for a more accurate diagnosis of BC. Around 4.9 million breast biopsies are performed every year in the world, and 3.2 million of them are checked for screen detection of non-palpable breast lesions, of which a third are found to be malignant.[2] However, by considering the current methods of BC diagnosis, any doctor can ensure a survival rate close to 100%. On the other hand, if one focuses on BC therapy, many current treatments are invasive, involving several breast biopsies, wire-guided localization, and eventually surgical removal. All current treatments including chemotherapy and prophylactic strategies are disfiguring, invasive, and associated with significant side effects.[3]

For all these reasons new diagnostic strategies and new effective and less toxic therapies are urgently needed. The recent advances in technology and engineering have led to the application of nanotechnology in medicine with the development of new nanoscale biomedical systems.[4] Nanomaterials have been explored for biomedical research because of their extraordinary physicochemical characteristics. In particular, cancer nanotechnology has proposed excellent approaches to cancer detection, diagnosis, and treatment with limited toxicity compared to the

traditional cancer therapy.[5] In this context, nanotechnology can create human-made materials in the nanoscale range, the same scale where cellular and biological processes take place.[6] The major potential of cancer nanotechnology includes the possibility to engineer nanovehicles with multiple molecules that, because of their small size, can penetrate tumors with high specificity, consequently with significantly fewer side effects.[7-9] Furthermore, techniques for nonsurgical ablation of tumors have been developed, leading to the complete destruction of tumor cells by the direct application of thermal and chemical therapies using nanomaterials, composed of metals, lipids, or polymers.[10] Therefore, cancer nanotechnology brings in the scenario of BC oncology huge expectations, and nanomaterials can be adapted for the different BC forms and disease status. Because of the high degree of control, the characteristics of human-made nano-tools can ensure new perspectives. Nanomaterials in BC can act as: i) drug nanocarriers, ii) nanodiagnostic tools, and iii) theranostic tools.

Regarding drug delivery, nanomaterials can be designed to transport chemotherapeutic drugs directly to the breast cells using specific antibodies to target the cancer site.[11] Doxorubicin (Dox) linked to nanomaterials is the most investigated drug for cancer therapy. Very recently, the group of Ferrari has described an injectable nanoparticle generator (iNPG), consisting of a discoidal micrometer-sized particle that can be loaded with Dox conjugated to poly(L-glutamic acid) (pDox).[12] Intravenously injected iNPG-pDox accumulates in the tumor region and shows enhanced efficacy in mouse models of metastatic BC.

In the context of the development of diagnostic tools, there are many successful examples of nanomaterials applied to visualize BC [*e.g.* superparamagnetic iron oxide nanoparticles (SPIONs) and magnetic nanoparticles (MNPs)]. Different studies have reported a sensitivity of 73-100% and a specificity of 92-98% in the lymph nodes using SPIONs.[13] Among other advantages, SPIONs are useful for the early detection by MRI, displaying also a good immune-compatibility and echogenic properties.[14] Today other nanotechnology-enabled systems are

under clinical trials. For example, [^{18}F]-FAC isodeoxycytidine analogue for deoxycytidine kinase (DCK) labeled with fluorine ^{18}F, was proposed as a novel PET imaging probe.[15] Nanoparticle MRI contrast agents that bind the αvβ3-intregrin, expressed on the surface of the newly developing blood vessels associated with early tumor growth, were realized.[16] Lymphotrophic superparamagnetic nanoparticles developed by the MIT-Harvard Center for Cancer Nanotechnology Excellence (CCNE) were used to identify small, otherwise undetectable, lymph node metastases.[16]

Before initiation of a cancer treatment, it is essential to carry out diagnostic imaging procedures to understand the type of cancer lesion. In this context, theranostic agents can combine diagnostic and therapeutic strategies into one procedure.[17] Hosoya H. *et al.*[18] described a hydrogel-based nanoplatform conjugated with Dox that enables ligand-directed tumor targeting and multimodal imaging. The data obtained using this strategy suggest that targeted hydrogel photothermal therapy represents a functional theranostic clinical application such as image-guided approaches for diagnostic and therapeutic monitoring. Many carbon based nanomaterials have been studied for medical applications such as tissue engineering, drug delivery, and gene transfection explaining their potential as molecularly-targeted and dual-modality imaging agent.[19]

In this Review, the purpose was to point out what are the most promising nanomaterials that can perform a breakthrough revolution in the scenario of BC.

A thorough overview of nanomaterials that have been so far investigated against BC, analyzing the most interesting publications present in the literature, has been carried out. The applications on drug delivery, imaging, and theranostics have been scrutinized and all specific potentialities of the nanotechnology tools to overcome current barriers, to reduce toxicity, and to avoid suffering from anticancer treatments have been discussed, in order to shed light on the challenges and hope offered by the different nanomaterials in the treatment of BC.

3.2 Overview and study selection criteria

Initially,-three main nanomaterial applications for BC have been analyzed in details: i) drug delivery, ii) imaging, and iii) theranostics.

For this analysis, a PubMed search was performed using the following keywords: breast cancer, nanotechnology, nanomedicine, nanoparticles, nanomaterials, drug delivery, theranostics, and imaging. Keyword searching was also performed in different combinations. High impact review articles served as additional tools. The list of reported studies includes all retrieved publications from 2009 to December 2017. A full and deep characterization of all applications was carried out based on type of materials, conjugated drugs, imaging modialities, applications other than imaging, model, type of species examined (human, mouse and their combination), other types of cancer other than BC.

Figure 1 presents the number of publications over the years, the types of applications on therapy and diagnosis and the types of species examined in the cited works.

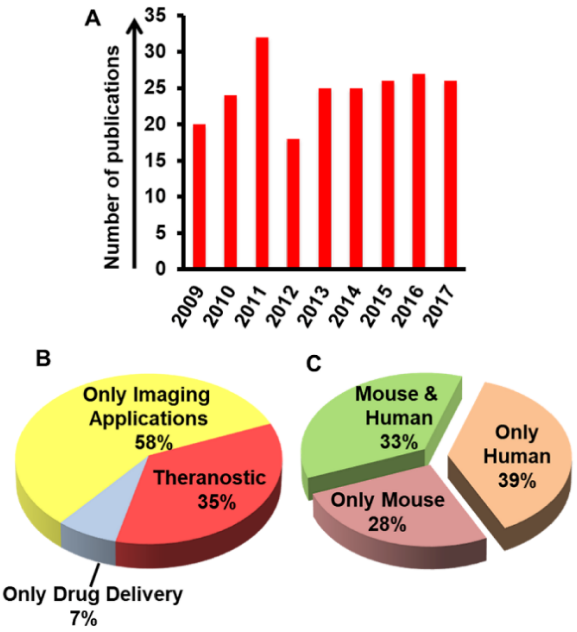

Figure 1. Status of applications used on BC studies. A) Analysis of publications in the last 9 years (2009 to 2017). **B**) Relative percentages of publications for imaging, theranostic and drug delivery applications. **C**) Species examined in each publications (human, mouse and combination of human and mouse).

The trend, from 2009 to December 2017, indicates an oscillating tendency in the studies on BC (**Figure 1A**); *e.g.*, the number of retrieved publications in 2011 was 1.5 higher compared to 2009. After a clear decrease in 2012, the state of publications in all the following years was higher than 2009. Imaging is the first application (58%), while 35% of the articles referred to theranostic applications combined with drug delivery (**Figure 1B**). Besides, drug delivery, a single application, is reported in 7% of the studies. In **Figure 1C** the relative percentage of publications describing human cell lines (*in vitro* and *ex vivo)*, mice (in *vivo, vitro,* and *ex vivo*), or both has been shown. Although there are not many differences in the percentage of publications of the examined species, it was evident that the majority of the studies has been carried out in humans cells (39%), 28% in mice, and 33% in both of them. International variations in BC incidence rates reflect differences in the availability of early detection tools as well as risk factors.[20] In this context, the countries where BC studies were carried out have been analyzed, taking into consideration the affiliation of the corresponding author (**Figure 2**).

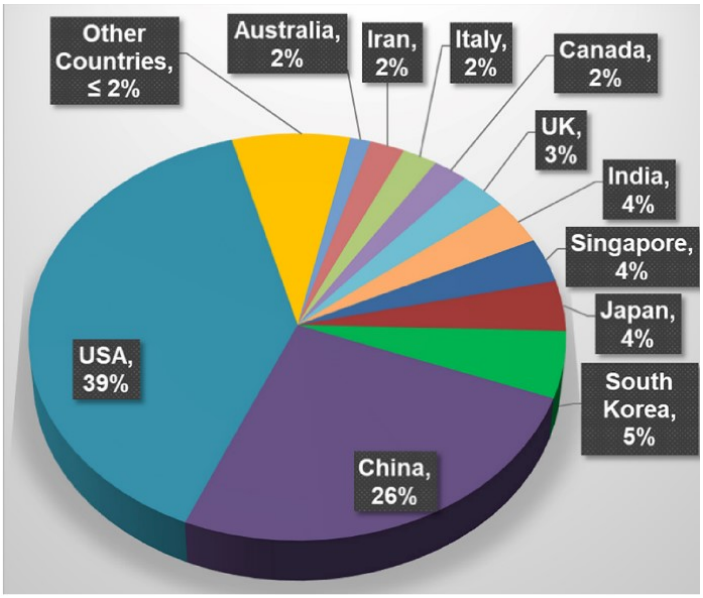

Figure 2. Percentage of publications carried out on nanomaterials fight against BC per Countries.
The piece of the cake in yellow-reported the studies in a percentage of <2 % conducted in other countries such as: New Zealand, Greece, Brazil, Taiwan, Malaysia, Riyadh-Saudi Arabia, The Netherlands, Israel, Germany, France, Georgia, Poland.

It emerged that the majority of the studies (39%) were conducted in USA, 26% in China, 5% in South Korea, 4% in Japan, Singapore and India, 3% in UK, 2% in Canada, Italy, Iran and Australia; very few studies were conducted in other countries (< 2%). By the analysis of these percentages, it was possible to conclude that there is no correlation between countries and incidence of BC. In fact, considering countries like United States where there is a high number of scientists, it is obvious to expect a larger amount of published studies. A careful analysis of these data showed that there is not a strong correlation between the number of studies published and the relative incidence recorded. More developed countries represent about one-half of all BC cases with 38% of mortality. In fact, as reported in the pie graph (**Figure 2**), USA, China, South Korea have the highest percentage of studies compared to the other countries, but it was estimated that the higher mortality from BC occurs in Asian countries, as Qatar, India and Iran. Since BC may lead to metastasis in other regions of human body, many scientists focused at the same time on other cancer forms together with BC. Thus, many authors studied the BC-specific ligand/cell surface also identified in other type of cancers, or BC overexpressed receptors using other cancers as negative controls. Therefore, other types of cancer investigated with BC have been also reported (**Figure 3**).

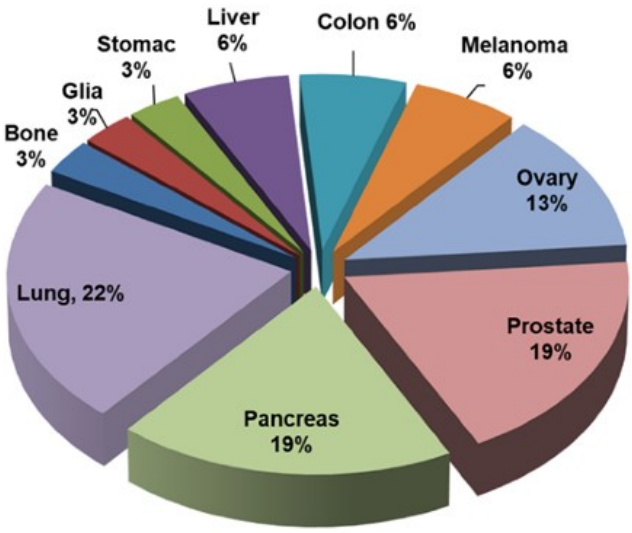

Figure 3. Percentage of publications focusing on other types of cancer together with BC.

Lung cancer was studied in 22% of the papers, pancreatic and prostatic cancer in 19% of the papers, and ovarian cancer in 13%. Melanoma, colon and liver cancers were studied in 6% of the publications and the other types of cancer including gastric cancer, glioblastoma and bone cancer were studied in 3% of the cases (**Figure 3**).

3.3 Drug Delivery

Drug delivery is a key nanotechnology application. This type of application alone is reported in only the 7% of the cases considered. Indeed, in many examples, drug delivery applications refer to theranostic applications (corresponding to 35% of the studies). Analyzing the different publications, it emerged that several types of drugs are used in drug delivery or in combination leading to theranostic nanomaterials (**Figure 4**).

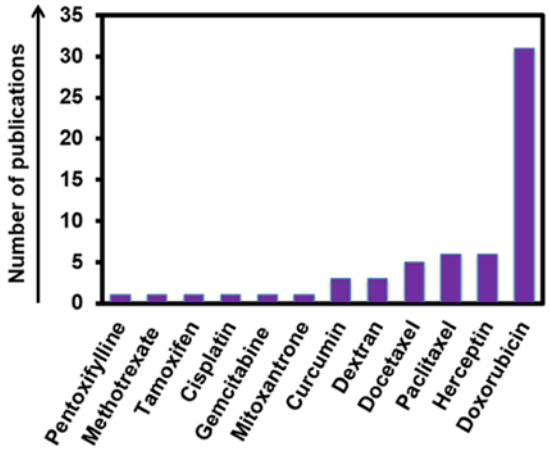

Figure 4. Conjugated drugs to nanomaterials. Number publications in the last 9 years based on the type of drugs conjugated to nanomaterials and nanoparticles against BC.

These drugs comprise Dox, herceptin, paclitaxel (PTX), dextran, curcumin, mitoxantrone, tamoxifen, methotrexate, pentoxifylline, and docetaxel**.** Dox represents the most important anticancer chemotherapeutic drug.[21] Indeed, its ability to intercalate DNA bases, inhibiting the topoisomerase II enzyme during DNA transcription, was widely demonstrated.[22] Many studies

used nanomaterials conjugated with Dox as an innovative cancer therapy.[22-27] Herceptin was used for the treatment of metastatic BC, thanks to its properties of blocking cell proliferation.[28-33] Recently, Wang *et al.*[34] reported a synthesis of a particular multifunctional anti-cancer complex based on functionalized magnetic nanoparticles (MNPs) and quantum dots (QDs) with a dual-drug combination. In detail, PTX/MNPs/QDs@Biotin–PEG–PCDA nanoparticles have shown a high uptake by BC cells (MCF-7/ADR) and good drug release. These nanoparticles are able to combine various proprieties useful for imaging (QDs), targeted delivery and uptake (MNPs), and dual drug treatment using two drugs (*e.g*. PTX and curcumin). Curcumin, a natural compound extracted from *Curcuma longa*, helped to obtain a high PTX accumulation in the tumor target and induced a down-regulation of drug efflux transporters. Moreover, PTX has shown excellent efficacy in a wide spectrum of cancer treatments, but its formulation has led to serious side effects in patients, as neurotoxicity, nephrotoxicity and allergic reaction. Modified PTX as nanomicelles was developed to overcome these obstacles and multidrug resistance.[35]

Zhao *et al*.[36] described hybrid paclitaxel nanocrystals that integrated fluorescent molecules for therapy and imaging in a breast tumor. The authors observed a more efficient anticancer effect of this system in mice with breast tumor than in mice treated with pure PTX. The hybrid PTX nanocrystals have shown the ability to easily accumulate in the tumor area following intravenous administration. Others described PTX release directly into the tumor sites for theranostic nanomedicine application [34-37] and drug delivery.[38] In the third position of the most important drugs used against cancer there is curcumin, a compound endowed with interesting properties including an anti-inflammatory action.[39] Curcumin can specifically modulate the expression of proteins in proliferating cells, in adhesion and in migration, and it is used as an anticancer drug to prevent metastatic formation or to limit cancer progression.[40, 41] Furthermore, other types of drugs such as dextran[33, 42, 43] or docetaxel[44, 45] were loaded onto nanoparticles for

cancer detection, while mitoxantrone,[46] tamoxifen,[47] methotrexate,[48] pentoxifylline,[49] docetaxel,[50, 51] cisplatin and gemcitabine were combined for the development of theranostics materials.[52] Recently, the group of Chan provided quantification of the delivery efficiency of nanoparticles at the tumor site. The authors reported a bombshell work whose meta-analysis suggested that very few "targeted" nanoparticles reach the target. According to this analysis, only 7 out of 1000 engineered nanoparticles are able to accumulate into the tumor *in vivo*.[53]

3.4 Imaging

Nowadays, nanotechnology based on imaging represents a very promising solution for non-invasive investigations of cancer lesions. We found that in 58% of the examined studies the first approach against BC is based on the use of nanotools for imaging (**Figure 1B**). Breast imaging can be undertaken using MRI, the most commonly available modality, thanks to its rapidity and high resolution [54](**Figure 5A**).

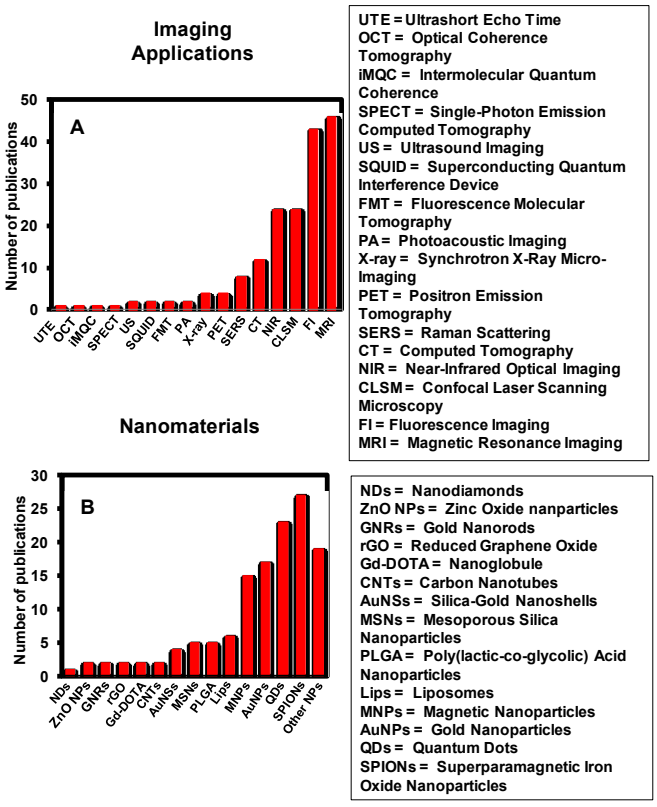

Figure 5. **Overview on different types of techniques and nanomaterials for imaging.**

A) Analysis of the number of publications based on the different kind of Imaging Applications for BC.

B) Types of nanomaterials used for imaging.

Different nanoparticles with appropriate surface modification have been used *in vivo* as MRI contrast agents because of their high magnetization and nano-size.[55] In particular, the surface coating was exploited to create non-toxic and biocompatible nanomaterials.[56-78] For example, Medarova *et al*.[79] have modified SPIONs with Cy5.5 dye and conjugated them to specific peptides. This tumor-specific contrast agent was able to successfully target the under-glycosylated MUC-1 (uMUC-1) tumor antigen, present in over 90% of the cases of BCs.

MRI is followed by two other techniques, namely fluorescence imaging (FI)[5, 64, 78, 80-119] and confocal laser scanning microscopy (CLSM) (**Figure 5A**).[58, 64, 67, 73-75, 120-134] Both of them have shown to be excellent imaging tools for many *in vitro* studies on murine and human cancer cells. Pan *et al*.[120] described *in vitro* cancer detection of human cells (MCF-7) using fluorescent quantum dots (QDs) as luminescent probes for targeted imaging. The authors described a new strategy to prepare QDs formulated in folate-decorated nanoparticles (PLA-TPGS/TPGS-COOH) (PLA-TPGS, poly(lactide)-D-α-tocopheryl polyethylene glycol succinate) for BC detection and diagnosis at its early stage. They demonstrated that functionalization with a copolymer was able to improve imaging sensitivity with reduced side effects on normal cells. Another imaging technique used in BC studies is the near-infrared (NIR) optical imaging, [56, 79, 83, 96, 97, 99, 107, 112, 127, 132, 135-143] which represents the fourth most exploited type of modality in the total of the examined studies. Through NIR fluorescence images, the authors analyzed directly *in vivo* the biodistribution of many nanomaterials in different organs and their elimination. Bardhan *et al*.[56] used modified gold nanoshells (AuNSs) with fluorophores to enhance the fluorescence in live mice grafted with human cancer cell lines over 72 h. The nanocomplex, conjugated with specific antibodies to target human epidermal growth factor receptor 2 (HER2) overexpressed in BC, provided significant information regarding the distribution of nanomaterials, and represents a new approach for cancer therapy and non-invasive treatment for soft-tissue tumors.[56]

Moreover, it was found that computed tomography (CT)[70, 113, 139, 144-152] is at the fifth position in terms of number of studies related to BC, while other techniquews are less used, including superconducting quantum interference device (SQUID),[94, 153] surface enhanced Raman scattering (SERS),[81, 154-159] synchrotron X-ray micro-imaging (X-ray),[150, 152, 160, 161] positron emission tomography (PET),[106, 115, 142, 162] and fluorescence molecular tomography (FMT).[70, 73] Single-photon emission computed tomography (SPECT),[163] ultrasound imaging (US),[71] intermolecular quantum coherence (iMQC),[164] optical coherence tomography (OCT),[165] ultrashort echo time (UTE)[166] are in the last positions as imaging techniques used (**Figure 5A**). Focusing on the imaging tools, it emerged that the first most studied nanomaterials are SPIONs (**Figure 5B**).

Many publications have shown the interesting potential of SPIONs for tumor detection, cancer therapy and drug delivery.[13, 54, 57, 59, 61, 64, 67, 68, 74-79, 94, 100, 104, 145, 148, 164, 166-170] SPIONs are applied as molecular imaging probes due to their monodisperse size distribution, but for biomedical applications, a surface modification [i.e., with poly(2-hydroxyethyl aspartamide)] is necessary to make them stable under physiological conditions and to avoid the uptake by phagocytic cells.[57] A recent publication has shown their use for gene therapy. Lin *et al.*[74] discovered that SPIONs conjugated with small interfering RNA (siRNA) were able to silence the target messenger RNA, consequently reducing the expression of P-glycoprotein (P-gp), a cell membrane protein responsible for multidrug resistance. Through this gene therapy, the authors demonstrated an excellent down-regulation of P-gp in MCF-7/ADR human BC cell lines in orthotopic mouse model.

In the same way as that of SPIONs, other small size (5-8 nm) magnetic nanoparticles (MNPs) have also shown the same characteristics in terms of biodistribution, and the ability to carry more compounds thanks to their high surface availability.[58, 69, 70, 72, 73, 83, 102, 125, 126, 131, 134, 139, 153, 171] In this context, Yigit *et al.*[171] used MNPs linked to microRNA (miRNA) for gene therapy.

The treatment of human BC cells (MDA-MB-231) *in vitro* and *in vivo* with the nanocomplex down-regulated a pro-metastatic microRNA (miR-10b) arresting the metastatic process, and thus preventing the formation of lymph node metastases. Regarding QDs, it was found that they are in the third position as nanomaterials for imaging. Thanks to their fluorescence properties upon excitation, their high brightness and photostability, they represent unique nanomaterials ideal for *in vivo* imaging in animal cancer models, as shown in the measurement of the receptor expression level of type I insulin-like growth factor receptor (IGFIR) involved in BC proliferation and metastasis.[122] QDs have been coated with polymer to enhance biocompatibility,[64, 89, 92, 95, 101, 114, 120, 133, 172] or conjugated with antibodies to detect overexpressed receptors.[85, 88, 90, 91, 98, 99, 107-110, 116, 122, 135]

The fourth position is held by gold nanoparticles, used for tumor detection, diagnosis, and cancer therapy, due to the possibility of an easy surface modification.[173] The advantages of these nanomaterials include non-cytotoxicity, chemical stability, and high affinities for biomolecules.[121] Indeed, they can scatter visible and near-infrared light through surface plasmon resonance, and thus they have been used in many microscopic techniques including CLSM,[121, 130, 174] CT,[113, 147, 149, 150, 152] FI,[5, 87, 103, 113] and other imaging techniques like X-ray,[150, 152, 160] NIR imaging[141] and SERS.[155, 175] Finally, other imaging nanomaterials (**Figure 5B**) such as poly(lactic-co-glycolic) acid nanoparticles (PLGA),[71, 80, 97, 176] mesoporous silica nanoparticles (MSNs),[75, 86, 140, 142] liposomes,[93, 100, 144, 146, 177, 178] silica- AuNSs,[56, 137, 154, 165] gold nanorods (GNRs),[78, 84] carbon nanotubes (CNTs),[82, 129] nanoglobules [62, 66], graphene oxide,[115, 163] and nanodiamonds (NDs)[179] have been studied for breast tissue imaging and cancer therapy.

3.5 Theranostics

Recently, nanotechnology has provided new strategies that combine therapy and diagnosis approaches. The introduction of the word "*theranostics*" represents a well-established field of nanotechnology where multifunctional materials can be used for the detection and treatment of

cancer disease in a single procedure. Of particular importance is the simultaneous combination of contrast agents and therapeutic functions using chemically-modified nanoparticles or fluorescent probes.[180, 181] A characterisation of all theranostic applications was found in the literature for BC. Regarding nanoparticles, MNPs are in the first position in terms of theranostic materials investigated, followed by calcium phosphosilicate composite nanoparticles (CPSNPs), liposomes, AuNPs and GNRs, AuNSs, CNTs and polymers (**Figure 6**).

THERANOSTIC MATERIAL	Abbreviation
Magnetic Nanoparticles	MNPs
Calcium Phosphosilicate Composite Nanoparticles	CPSNPs
Liposomes	Lips
Gold Nanoparticles	AuNPs
Gold Nanorods	GNRs
Gold Nanoshells	AuNSs
Carbon Nanotubes	CNTs
Hydrotropic oligomer-conjugated nanoparticles	HO-CNPs
Nanoparticles	NPs
Mesoporous Silica Nanoparticles	MSNs
Quantum Dots	QDs
Copper(II) sulfide nanoparticles	CuS NPs
Superparamagnetic Iron Oxide Nanoparticles	SPIONs
Tungsten oxide Nanoparticles	WO3-x
Thiol-functionalized hyaluronic acid	HS-HA
Mesoporous Magnetic Gold "nanoclusters"	MMGNCs
Heparinefolic acid nanoparticles	HFNPs
Reduced Graphene Oxide	rGO
Carbon Nano-Onions	CNOs

Figure 6. List and acronyms of nanomaterials used as theranostic in the treatment of BC

Theranostic MNPs have demonstrated excellent performances in tumor detection,[182-185] drug delivery,[184, 185] and cancer therapy in mice model studies.[186, 187] Part of the works examined were carried out *in vitro* on MDA-MB-231,[40, 41] MCF-7,[41, 47, 188, 189] H1299 human cell lines,[188]

as preliminary studies to evaluate the response to cancer therapy. Basuki *et al.*[188] described the theranostic application of MNPs loaded with polymers and Dox in *in vitro* experiments using MCF-7 on H1299 human cell lines. The authors demonstrated the accumulation of MNP-Dox in lungs and BC cell lines through MRI and Dox release to cancer cells using CLSM and FI techniques. For theranostics, GNPs, AuNSs have raised interest in photodynamic therapy,[190, 191] photothermal therapy,[180, 192-194] ultrasonography[192] and gene therapy.[180]

On the other hand, in the context of imaging, AuNPs have shown the main applications regarding tumor detection.[18, 29, 195-200]

In the third position other known theranostic materials can be found, such as MSNs used for tumor targeting and drug delivery,[30, 201-203] GNRs for tumor detection and drug delivery,[31, 32, 204] gene therapy,[205] and photothermal therapy.[206, 207] Liposomes have been used for tumor detection and drug delivery,[46, 50, 208, 209] and other new theranostics systems like CNTs[48, 210-213] and CPSNPs.[214, 215] In particular, CNTs have good echogenic properties like contrast agents, with a promising future in the field of theranostic applications.[216] Even though positioned in the fourth position, QDs have acquired more importance in the field of theranostic applications. Rizvi *et al*.[217] reported an *in vitro* experiment using QDs loaded with antibodies for HER-2 localization in fixed and live cells (SK-BR-3 and MCF-7 cells). This study underlines how QDs coated with mercaptoundecanoic acid appeared to be non-toxic up to 24 h of exposure, and an excellent *in vitro* imaging agent. For this reason, QDs can be potentially used for targeted therapy in image-guided surgery and cancer therapy to directly destroy tumor cells. In addition to traditional nanomaterials, the combination of polymeric materials has opened a new way for theranostic nanomaterials, known as nano-complexes (**Figure 6**). Their properties have allowed a controlled release of drugs in addition to many medical applications, like photoacoustic tomography,[218] photothermal therapy,[219-221] photodynamic therapy,[222] drug delivery[37, 49, 223-229] and cancer therapy.[230-234]

During the last years, graphene-based materials have been investigated in biomedical applications thanks to its unique intrinsic chemical and physical properties.[235] Excellent electrical conductivity, ideal photothermal response, large surface area, and versatile chemistry have stimulated researchers to explore graphene based materials for applications in tissue engineering, drug delivery, molecular imaging and others. For example, Shi *et al*.[236] reported reduced GO (rGO) as an excellent photothermal agent that enabled *in vivo* tumor ablation. rGO could be also used as theranostic materials to integrate imaging and therapeutic components to fight cancer.[236] Recent studies of nanotechnology based on other carbon nanomaterials such as NDs, CNTs, and fullerenes have provided good results about their possibility to become theranostic agents in the different field of nanomedicine such as drug delivery, regenerative medicine, bioimaging. Carbon nano-onions (CNOs) showed the same vectorization characteristics possessed by CNTs, as described in a previous work.[237] Recently Bartelmess *et al*.[238] demonstrated the simple cell-penetration capability of CNOs in an *in vitro* MCF-7 human BC cell line. Boron dipyrromethene (BODIPY) functionalized CNOs exhibited high fluorescence intensity for high-resolution imaging and did not show significant toxicity effects. These results prove that modified CNOs can be used as new theranostic materials that are able to combine imaging, targeting and therapeutic modalities.

3.6 Conclusions

A thorough and detailed review of nanoscale innovations against BC proposed in the last nine years has been carried out, evidencing increasing interest in the study of nanotechnological applications to BC. Nanotechnology offers a possibility for early breast lesion detection and searching for more efficient therapies to significantly impact the degree of mortality of BC patients. Despite numerous studies on the application of nanotechnology in medicine, the hypothetical benefits still need to be clarified. Most of the nanomaterials tested have not been able to provide high efficiency for clinical use. Considering the intrinsic physicochemical

properties of nanomaterials and all studies analyzed here, superparamagnetic iron oxide nanoparticles, quantum dots and liposomes represent the best choice as advanced drug carriers for BC therapy. On the other hand, gold silica nanoparticles, nanoshells, nanorods, nanocages, and nanotubes were especially studied as photothermal agents under radiofrequency or magnetic field activation in non-invasive imaging and cancer therapy.[239] Regarding drug delivery, nanoparticles have been engineered as drug vehicles to bring the drug directly to the tumor site, to reduce toxic side effects of antineoplastic agents, and to enhance combinatorial drug delivery. Among all the reported studies, the most promising ones have been highlighted. Recent studies have focused on new strategies based on a combination therapy through a co-administration of multiple drugs using a single treatment. For example, this approach was carried out by Murugan *et al*.[240] to describe nanocarrier mediated inhibitory effects of topotecan and quercetin on BC cells like a new targeted therapeutic strategy to treat cancer. These revolutionary nanocarries have shown an excellent intracellular release of loaded drugs with important molecular-induced modifications leading to structural changes in the endoplasmic reticulum, nucleus and mitochondria of tumor cells.[240]

Nanotechnology can offer potential nanomaterials for creating new methods for detecting, targeting and killing BC cells at different stages. Several authors reported the problematic use of many nanomaterials because of their non-specific toxic effects in *in vivo* animal models. One of the major advantages of using nanoparticles is based on the possibility to modify their characteristics to face physical and biological barriers after injection. However, the analysis of the recent publications disclosed that the delivery efficiency has not improved during the last nine years.

Regarding innovative approaches that are able to accelerate nanotool integration into the clinic, it is important to mention single cell techniques and in particular single cell mass cytometry. Being aware that single cell mass cytometry can be useful in the context of BC as proven

recently by the group of Bodenmiller,[241] the interest in the application of this approach on nanotools to validate their effect on BC treatment becomes clear.

In conclusion, despite the numerous studies found in the literature, only a few nanomaterials or nano-compounds will move on from the pre-clinical phase and will be selected for clinical trials. Indeed, the research on biocompatibility is still at an early stage. At present, the possibility to control the transport of nanoparticles transport and the real delivery efficiency in the body for cancer treatment remains the real challenge for nanotechnology-based tools against BC.

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Chapter 4. Nanotechnology and bone loss dysfunction under microgravity condition

4.1 Introduction

Bone loss is one of the main problems associated to long spaceflight missions, quantified as total bone mineral density (BMD).[1, 2] Several experiments of Russian MIR, American and European missions conducted by astronauts or simulation models have described the variation of both pre-flight and post flight bone loss.[3-6] During the missions on the International Space Station (ISS) it was found that such loss is usually in the range between 3.0 and 10.0%. [7, 8] Many authors have described the negative effect of microgravity that acts by altering the bone structure of *ex-vivo* models.[9-11] Indeed, it is well known that microgravity (MG) is able to induce bone loss in terms of a mineral density decrease of about 2% in 30 days, while a similar decrease occurs in women affected by osteoporosis in about 1 year.[12] Many researchers described that the primary symptoms appearing while in orbit are loss of calcium within the first 10 days after the beginning of the space flight and bone changes within 20 days during short missions [13, 14]. Many of the experiments evaluating the effect of microgravity on cells were studied using short-duration microgravity simulation.[15-18] Moreover, many studies are now assessing the effects of microgravity on cells and physiology under long-duration MG simulation experiment.[19-23] Recently, Tavella *et al.*, investigated the alterations of bone microarchitecture using animal models during 91 days on the ISS. The authors described that microgravity is able to induce bone loss due to a decrease of bone deposition and an increase of bone resorption in wild type (Wt) and pleiotrophin-transgenic (PTN-Tg) mice.[24] Numerous study have demonstrated that short and long spaceflight cause the dysregulation of stem cells functions which leads to the inability of cells to repair and regenerate lesions.[25, 26] These findings indicate that microgravity leads to several modifications in osteoblasts and osteoclasts in terms of cellular morphology, proliferation and differentiation.[27-29] Experiments carried out on osteoblastic cell cultures, using

a tridimensional clinostat as the Random Positioning Machine (RPM) to simulate microgravity, are focused on the investigation of spaceflight-related osteoblastic dysregulation.[30, 31] Recently, Wengui *et al.* reported in an innovative study that primary cilia (key sensor and functioning organelles) of rat calvarian osteoblasts (ROBs) vanished after microgravity exposure.[32] In addition, the authors underlined that the differentiation and mineralization of ROBs were associated with the abrogation of primary cilia after simulated microgravity. Reconstruction of primary cilia may become a potential therapeutic approach against bone loss during space missions but also in human disease-related bone loss. To address the problem of the modification of osteoblasts and osteoclasts in microgravity, several groups have used different approaches, such as the use of RPM or Rotary wall vessel bioreactors (RWV) to simulate microgravity conditions[33, 34]. Furthermore, many authors have established that experiments conducted using RPM to simulate microgravity have clarified the gene expression dysregulation of important osteogenic-related osteoblastic genes, such as osteocalcin (BGLAP) and alkaline phosphatase (ALP).[31, 35] Nevertheless, the mechanisms of microgravity action on osteogenesis inhibition are actually unclear and need to be clarified. A recent review pointed out the attention of the current knowledge on the use of stem cells and specialized cells under simulated microgravity (RWV and RPM) for tissue engineering applications.[36] Many studies on the effects of microgravity on bones are carried out using human bone marrow-derived mesenchymal stem cells (hBM-MSCs) cultures, which are multipotent cells present in the bone marrow.[37] Their potential to differentiate into osteoblastic lineage and their applications in the studies of bone regeneration are also well known.[38, 39] As already reported, MHA/Coll particularly enhanced the early expression of crucial osteogenesis associated genes, such as ALP and BGLAP *in vitro*, and the early formation of *de novo* bone mass in an ectopic model in rabbit. [40] Under this perspective, the osteoinductive potential of 3D scaffold to induce human bone marrow-derived mesenchymal stem cells (hBM-MSCs) differentiation under long-

duration microgravity condition was evaluated. Indeed, it was previously demonstrated that MHA/Coll thanks to its particular nanostructure and biomimicry could induce the expression of osteogenesis associated marker genes in the hBM-MSCs.[40] Following this line of research, the present work aims to investigate the osteoinductive potential of MHA/Coll in counteracting microgravity dysregulation. The viability and differentiation of hBM-MSCs cultured on scaffold were determined following the exposure to the osteo-differentiation inducing media. Furthermore, the expression of osteoblastogenesis-associated genes was analysed in comparison to un-induced controls under MG.

4.2 Materials and methods

Scaffold fabrication and structural characterization

The biomineralization of MHA/Coll scaffolds have showed mineralized nanofibers (89 ± 1,2 nm) as described elsewhere (Minardi *et al*, 2015).[40] The group of Minardi *et al*, have used nanomaterials during the synthesis phase of MHA/Coll.[40] Thanks to the nanomineralization, the 3D scaffold is considered a nanomaterial because of the presence of nano porosity due to the nanostructured fibers as already described (Minardi *et al*, 2015).[40]

Briefly, an acidic solution of bovine type I collagen (Nitta Casing Inc.) was prepared at a concentration of 10 mg/mL in acetate buffer at pH 3.5. H_3PO_4 was added to 100 g of the acetic collagen solution (40 mM), and dropped in a solution of $Ca(OH)_2$ (40 mM) and $MgCl_2 \cdot 6H_2O$ (2 mM) in DI water. The material was crosslinking in an aqueous solution of 1,4-butanediol diglycidyl ether (BDDGE) (2.5 mM). The resulting slurry was molded in 48-well plates at a thickness of 3 mm. Finally, the slurry was lyophilized through an optimized protocol, to generate the desired porosity and pore size. Non-mineralized collagen scaffolds (Coll) were also synthesized, and used as controls [40, 41]. All chemicals were purchased from Sigma Aldrich. The scaffold was imaged by scanning electron microscopy (FEI Quanta 400 SEM). The scaffolds were sputter coated with 10 nm of Pt/Pd, via a Plasma Sciences CrC-150 Sputtering

System (Torr International, Inc), and imaged at a voltage of 7.5 kV. Fourier-transformed Infrared spectroscopy (FTIR) was performed through a Nicolet 4700 Spectrometer. 64 runs were performed per sample. Spectra were analyzed by the software EZ OMNIC (Nicolet). The amount and thermal properties of mineral phase nucleated on the organic template (type I collagen) was assessed by thermal gravimetric analysis - differential scanning calorimetric (TGA-DSC). The samples (n=3) were placed in alumina pans and subjected to a heating ramp from 25 ºC to 800 ºC, at 10 ºC/min. A Q-600 TGA was used (TA Instruments).

MHA/Coll characterization under MG

MHA/Coll structure and hBM-MSCs morphology after long duration microgravity exposure was observed using scanning electron microscopy (SEM). For SEM morphologic investigation, the samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer (pH 7.2) and post-fixed in 1% Osmium Tetroxide (OsO_4), dehydrated in a graded acetone series and dried by critical point method in an Polaron Jumbo apparatus (Polaron Equipment, Watford, UK) coated with gold in an Edwards S150A Sputter Coater unit (Edwards, Crawley, UK). The specimens were examined with a Zeiss DSM 962 scanning electron microscopy (Zeiss, Oberkochen, Germany).

Stem cell culture in 2D condition

Human Bone Marrow-Derived Mesenchymal Stem Cells (hBM-MSCs) (ATCC®PCS-500-012™) were cultured in α-minimum essential medium (α-MEM), containing 10% of heat-inactivated fetal bovine serum (FBS), 2% of glutamine, and 1% of human Fibroblast Growth Factor-Basic human (bFGF) (all from Gibco). Adherent cells were passaged at 80% confluency using TripLE™ Express (Invitrogen) and reseeded for culture expansion.

Microgravity simulation

Microgravity was simulated by a random position machine (RPM). The RPM from Fokker, Netherlands is a tridimensional clinostat able to produce a multilateral gravitational simulation

when the samples are set in the center of the machine. A computerized program was used to create random movements and slow rotation of the two axes of the RPM in order to provide microgravity simulation (M; 0 x *g*). Static controls and the same samples were placed in the basement of the RPM to simulate the gravity condition (G; 1 x *g*).

hBM-MSCs viability assay

Cell viability was evaluated by Flow cytometry (FACS Canto II, BD Biosciences, Mountain View, CA, USA) using 7-AAD (7-amino-actinomycin D) staining (BD Bioscience, San Josè, CA, USA). hBM-MSCs cells were seeded into scaffolds as previously described and evaluated after 7, 14 and 21 days under microgravity conditions. To collect cell, the scaffold was washed three times in PBS and then digested using 2 mg/ml Collagenase I (Life Technologies) diluted in media without FBS. The cell pellet was washed in PBS to eliminate collagenase and afterwards cells were stained with 7-AAD, incubated for 20 min in the dark and suspended in PBS 1x solution for the flow cytometry analysis.

Induction of hBM-MSCs differentiation on MG conditions

Undifferentiated hBM-MSCs were harvested and prepared for 3D culture. A 30 µl drop containing 350,000 cells was seeded in the center of MHA/Coll scaffold and kept in incubator for 10 min. In order to evaluate the osteogenic potential of MHA/Coll, differential medium (StemPro® Osteogenesis Differentiation Kit, Gibco) supplemented with 25 mM of 1M HEPES buffer solution (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Gibco), as an organic chemical buffering agent, was added to each MHA/Coll and positive control. RPM-cultures were mounted horizontally in the center of the RPM at 37 °C. Identically the same number of cultures was placed in the same room of RPM at 37 °C in horizontal position to provide a control of samples grown under gravity condition (1 x *g*). Mesenchymal stem media was used as the negative control.

Gene expression analysis

Total RNA was isolated from cells using TriZol Reagent (TriZol, Invitrogen, Carlsbad, CA, USA). RNA purity and concentration were measured with Nanodrop Spectrometer (Nanodrop® ND1000). cDNA synthesis was performed using Superscript IV Reverse Trascriptase kit following the manufacturer protocol (Life Technologies). Amplification was performed using TaqMAN probes and TaqMan® Fast Advanced Master Mix (Applied Biosystems). The expression of alkaline phosphatase (ALP, Hs01029144_m1) and osteocalcin (BGLAP; Hs01587814_g1) was evaluated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991_g1) was used to normalize gene expression results respect to ctrl-MSC. The gene expression analysis of the osteogenic genes was performed comparing hBM-MSC cultured on MHA/Coll and hBM-MSC cells cultured with osteogenic media (induced MSC), or non-induced (Ctrl-MSC) under gravity and microgravity conditions. Polymerase Chain Reaction (PCR) was performed on plates using a CFX96 Real Time instrument (Bio-Rad).

Statistical analysis

Data analyses were performed using Prism GraphPad software. Statistics for experiments was performed using a One-Way ANOVA. In all cases * was used for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, and **** for $p < 0.0001$. Values were expressed as mean ± SD. Flow cytometry data were analyzed with FACS Diva software (BD-Bioscience Mountain View, CA, USA). Osteo-gene expression data were calculated by the comparative threshold cycle method. All experiments were performed at least in triplicate.

4.3 Results and discussion

Characterization of MHA/Coll

The architecture and structure of Coll and MHA/Coll were evaluated by SEM (**Figure 1**). The lower magnification images revealed the high porosity and regular pore size of Coll (**Figure 1A**), while it showed the anisotropic nature of MHA/Coll (**Figure 1B**). The higher

magnification micrograph of MHA/Coll confirmed the full mineralization of the collagen fibers with an amorphous apatite phase, accomplished during the bio-inspired process of synthesis (**Figure 1C**). The chemical interaction between the mineral phase and the type I collagen fibers of MHA/Coll was confirmed by FTIR spectroscopy (**Figure 1D**), where a shift from 1340 to 1337 cm^{-1} in the band corresponding to the stretching of $-COO^-$ group of collagen was observed. The TGA-DSC analysis, displayed that the overall mineral phase content in MHA/Coll was approximately 56 wt% (**Figure 1E**). Finally, MHA/Coll structure were evaluated by SEM on gravity condition (**Figure 1F**) and microgravity exposure (**Figure 1G**), showing the remodelling action played by microgravity long exposure.

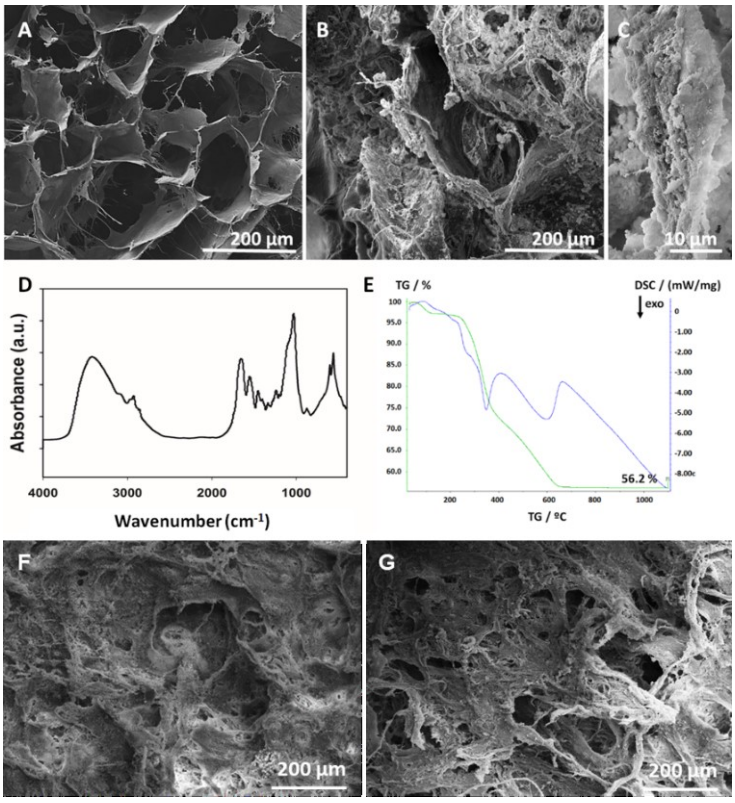

Figure 1. Scaffold architectures. Scaffold structure characterization by SEM of Coll **(A)** and MHA/Coll **(B)**. The higher magnification micrograph of MHA/Coll **(C)** shows the collagen fibers with a full mineralization and an amorphous apatite phase. **D)** FTIR spectra showing the chemical interaction between the mineral phase and the type I collagen fibers of MHA/Coll. **E)** Evaluation of the mineral phase on MHA/Coll by TGA-DSC analysis. **F)** SEM micrographs of MHA/Coll structure after 21 days of exposure (G, 100x). **G**) Disfiguring action of microgravity on MHA/Coll structure (MG, 100x).

MG effect on MHA/Coll structure and hBM-MSCs morphology

It was previously found that MHA/Coll was able to mimic the osteogenic niche of human trabecular bone.[40] MHA/Coll is synthesized through a sophisticated bio-inspired nanotechnological process, which recapitulates the chemical, physical, morphological and structural control mechanisms of the natural biomineralization process.[42] During the synthesis, a partial substitution of the calcium with magnesium ions in the apatite lattice allows for an amorphous nanostructured apatite, which more closely mimics that found in the early osteogenic niche.[43] Following the current findings, the microgravity effects on MHA/Coll structure were evaluated by SEM (**Figure 2**).

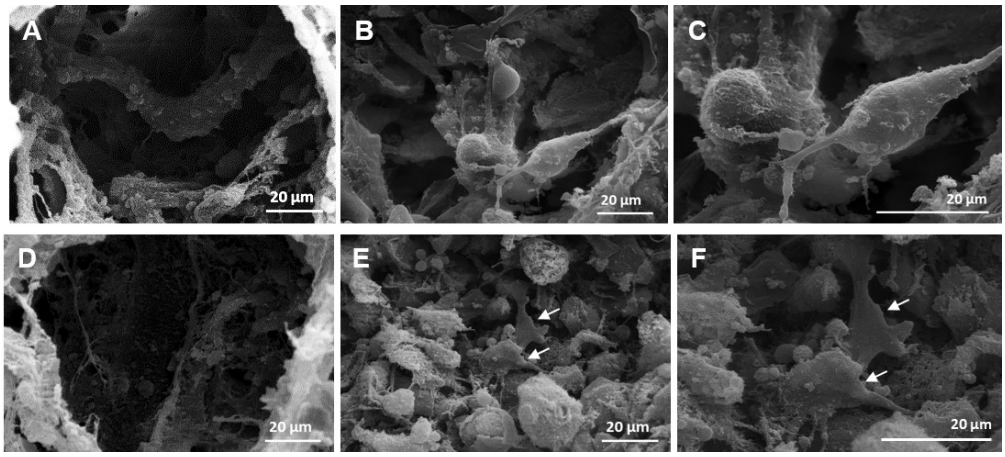

Figure 2. Evaluation of MG effect on cells morphology. SEM micrographs of MHA/Coll at 1000x on gravity **(A)** and disfigured structure of MHA/Coll after microgravity conditions **(D)**. **B)** Induced- hBM-MSCs attachment onto the fiber of the scaffolds after 21 days seeding taken on the center of the scaffold surface on gravity condition. **C)** Particular of Induced- hBM-MSCs at 2000x. **E)** Induced- hBM-MSCs on simulated microgravity condition. **F)** Particular of induced- hBM-MSCs at 2000x. White arrows indicate the change on cell morphology under microgravity.

MHA/Coll on gravity condition (**Figure 2A**) revealed a fibrous structure and a correct alignment of the fiber which forming the niche. On the contrary, MHA/Coll on microgravity long exposure (**Figure 2D**), displayed a disfiguring effect by MG on the fiber, providing a different structure of the niche. **Figure 2B** shows the induced- hBM-MSCs morphology after

21 days seeding taken on the center of the scaffold surface on gravity condition. At higher magnification, induced- hBM-MSCs appeared alive and in connection together attached onto the Nano mineralized fiber of the scaffolds. Morphology changes induce by long exposure of simulated microgravity are showed in **Figure 2E**. The induced- hBM-MSCs appeared in an altered morphology resulting to be flattened respect to the condition in gravity (white arrows, **Figure 2E** and **Figure 2F**). This effect is due to the remodelling action of the microgravity, which causes the partial destruction of the scaffold fibers and consequently the altered cellular morphology. **Figure 2F** shows the flattened cells on the linear surface of the scaffold. When three-dimensional niches are missing, the cells are not able to adhere to the fibers and appear to be flattened respect to the gravity condition (**Figure 2C**). A recent publication described that mesenchymal stem cells under RPM simulation respond to microgravity exposure inducing the upregulation of some osteogenic transcripts due to activate the differentiation process.[44] In detail, only the addition of particular osteogenic cocktail can complete the full cellular differentiation process both in gravity and microgravity conditions.[44] In accordance with this study, the microgravity simulation alone can act the hBM-MSCs differentiation toward a complete osteogenic phenotype only after a specific stimulus exposure.

According to these studies, observing the data collected in this work, it is possible to hypothesize that the altered morphology of differentiated cells showed in MG is due to the disfiguring action of microgravity on nanostructured fibers with remodelling effect on the 3D niche. These results supported such hypothesis and clearly underlined that the mineralization in the nanoscale of MHA/Coll structure (3D culture) is able to induce hBM-MSCs to differentiate in response to the transfer of key cues at the nanoscale to h-MSC in long-duration microgravity simulation.

hBM-MSCs viability assay on MHA/Coll

Viability analyses of hBM-MSCs were performed using 7-amino-actinomycin D (7-AAD) staining, a fluorescent chemical compound with strong affinity for DNA. Cell fluorescence was measured by flow cytometry (**Figure 3**). When excited by 488 nm laser light, 7-AAD fluorescence is detected in the far red range of the spectrum (650 nm long-pass filter). Late apoptotic and necrotic cells with compromised membranes allow the passage of this dye into the nucleus. Flow cytometry data did not exhibit damage in cell viability (**Figure 3**). Cells on MHA/Coll remained alive up to 21 days after MG stimulation. A non-significant difference between MHA/Coll in comparison to the control after 7, 14 and 21 days was found (**Figure 3**).

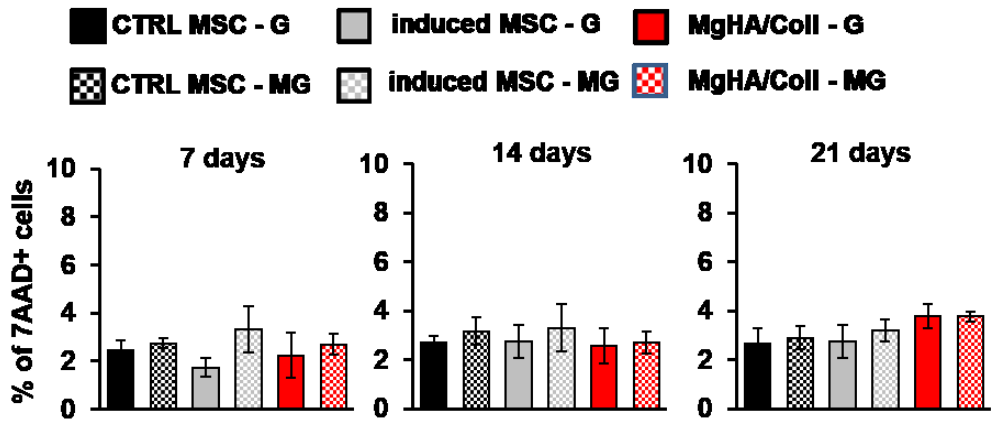

Figure 3. Viability assay of hBM-MSCs cultured on MHA/Coll scaffolds. Human BM-MSCs were cultured on MHA/Coll scaffolds (red) in gravity (G) and microgravity (MG) conditions. To assess the viability, the cells were stained with 7AAD marker. The histograms report the percentage of 7AAD positive cells (7AAD+) after 7, 14 and 21 days incubation compared to the un-induced-MSC (black). The experiments were performed at least in triplicate with flow cytometry.

Markers of differentiation hBM-MSCs under microgravity

To investigate the influence of microgravity simulation on the differentiation of hBM-MSCs, the expression surface CD- markers was determined in both induced 3D cultures and un-induced controls. Firstly, CD29 (a β1 integrin associated with late antigen receptors) and CD44

(a hyaluronic acid/fibronectin receptor involved in hematopoietic stem cell adhesion, mobilization and proliferation), were evaluated for mesenchymal stem cells surface markers expression by flow cytometry. The differentiation marker levels under gravity condition and microgravity simulation of hBM-MSCs and MHA/Coll cultures were determined after 7, 14 and 21 days. **Figure 4A** shows a significant decrease of surface markers expression of mesenchymal stem cells, CD29 and CD44, on MHA/Coll in both gravity and MG condition in comparison to the un-induced control (P value < 0.0001). It is well known that hBM-MSCs showed the capacity to differentiate along osteogenic lineage [45] and the consequent reduction in the expression of most surface markers is related to MSCs differentiation.[46] Surprisingly, a low expression of mesenchymal stem cells surface of these markers under MG was found, that showed the possible differentiation of hBM-MSCs on MHA/Coll-G and MG compared to the induced-MSC. In contrast, un-induced MSCs and induced MSCs showed no CD29 and CD44 expression when MG simulation was applied (**Figure 4A**). The loss of the hBM-MSc surface marker was explored in the MHA/Coll differentiation plot using flow cytometry (**Figure 4B**).

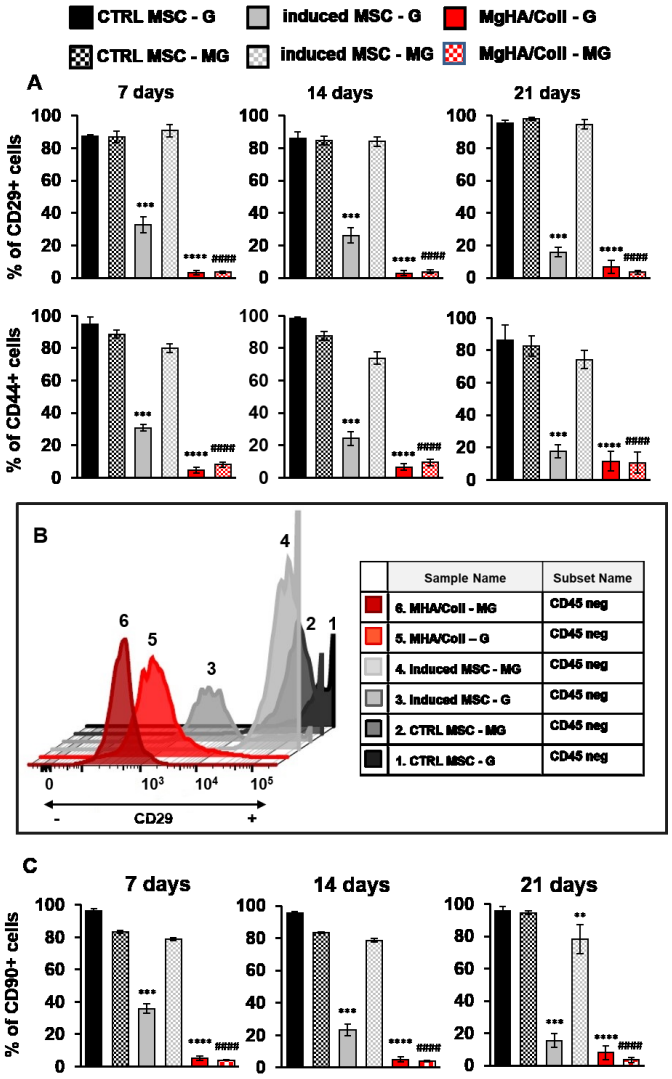

Figure 4. MHA/Coll induced hBM-MSCs differentiation under MG conditions. Human BM-MSCs osteo-differentiation was investigated in MHA/Coll scaffolds under G and MG conditions. **A)** CD29 and CD44 were used as differentiation markers. Un-induced-MSC were used as negative control (black) and induced-MSC were used as positive controls (grey) for induced hBM-MSCs on scaffolds. **B)** Differentiation plot were displayed the hBM-MSCs wich are negative for CD45 marker and the differentiated cells, which showed the loss of CD29 marker. The osteogenic differentiation assay was assessed in triplicate by flow cytometry on G and MG simulation (***P < 0.001 or ****P < 0.0001 vs Ctrl of G group; $^{###}$P < 0.001 or $^{####}$P < 0.0001 vs Ctrl of MG group). **C)** The differentiation assay was assessed looking at the expression of CD90 as a differentiation marker towards osteogenic lineage. A significant difference compared to the uninduced-MSC control was observed (**P < 0.01, ***P < 0.001, ****P < 0.0001) vs Ctrl of G group; ##P < 0.01, ###P < 0.001 or ####P < 0.0001 vs Ctrl of MG group).

It is known that hBM-MSc are suggested to be negative of CD45 and positive to CD29 surface markers. This is the criterion used to analyze the hBM-MSCs differentiation after 21 days. **Figure 4B** shows the group of CD45-/CD29+ hBM-MSCs in both G and MG condition

compared to the controls. More in details, MHA/Coll showed high level of differentiation status (red; plot 5 and 6 in G and MG respectively) even after microgravity stimulation in comparison to induced MSC-G (gray, plot 3) as negative CD29 cells (**Figure 4B**). On the contrary, induced MSC-MG (gray; plot 4) suggested the dysregulation on the MSC differentiation potential. Similarly, un-induced MSC-MG (black; plot 2) displayed no differentiated level equally to the un-induced MSC-G control (black; plot 1) as positive CD29 cells.

Furthermore, to evaluate the osteogenic potential of MHA/Coll under microgravity (M, 0 x g), the expression of CD90 (thymocyte differentiation antigen-1, Thy-1), a well-known differentiation marker toward osteogenic lineage, was measured. Indeed, it has been reported that the expression of CD90 decreases while there is a cell differentiation towards osteoblast-like cells.[47] CD90 was assessed on MHA/Coll incubated with induced media under G and MG simulation. Induced-MSC and un-induced Ctrl-MSC were used as a positive and negative control, respectively. Induced MSC-G suggested a significant decrease level of CD90 expression (P value < 0.001) in comparison with the induced MSC-MG, which confirmed the dysregulation on the osteogenic differentiation due to microgravity exposure (**Figure 4C**). Intriguingly, the CD90 marker suppression of MHA/Coll was more significant compared to the induced MSC-G (P value 0.001) after 21 days in both conditions (G, P Value 0.0001 and MG, P value 0.0001), evidencing the passage towards the osteogenic lineage compared to the undifferentiated control (**Figure 4C**). The decrease of CD90 level of induced MSC-G was observed between 14 and 21 days (P value < 0.001), which demonstrates the hBM-MSCs differentiation during induced media exposure.

The results here discussed demonstrated that the low expression of CD29, CD44 and CD90 level on both MHA/Coll G and MG, indicating that the structure of the scaffold with nanostructured niche may help the differentiation into osteogenic cells under microgravity simulation.

hBM-MSCs osteogenic differentiation on MHA/Coll under MG

To investigate the osteogenic differentiation under microgravity, it was decided to assess the next gene expression experiment using the time point of 21 days on MHA/Coll. Firstly, the molecular changes of induced hBM-MSc were investigated, performing a genomic analysis on the expression of two highly representative osteo-differentiation genes. The purpose was to evaluate the expression of osteoblastic genes onto MHA/Coll scaffolds under microgravity simulation (**Figure 5**).

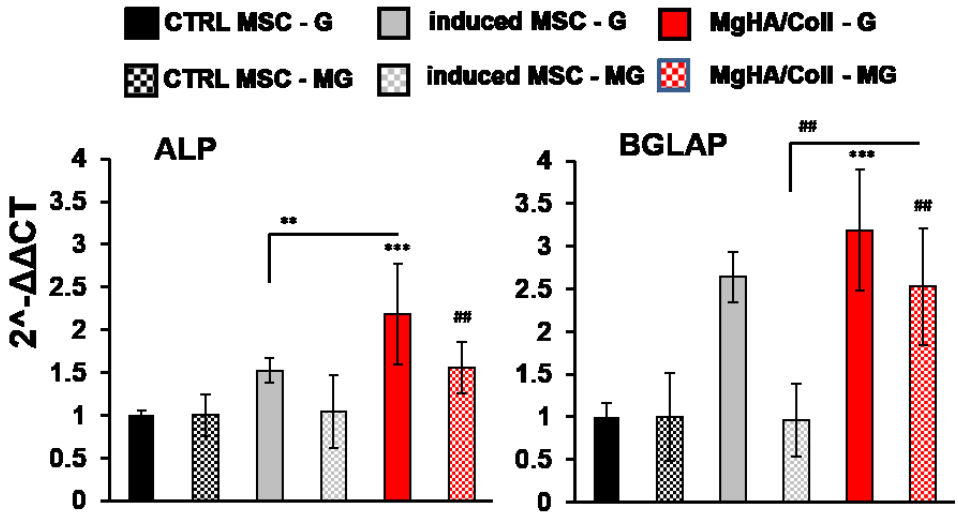

Figure 5. Osteogenic differentiation on MHA/Coll after MG. The main genes involved in osteoblast differentiation were analyzed using Real-Time PCR: alkaline phosphatase (ALP) and osteocalcin (BGLAP). Statistical significance (ANOVA test) is indicated by *** = $p < 0.001$ or ** $p = < 0.01$ for G group vs Ctrl G; by ### = $p < 0.001$ or ## = $p < 0.01$ for MG group vs Ctrl MG. Bars indicate the compared samples under different conditions.

The gene expression analysis on MHA/Coll MG, showed an increase expression level of ALP (P value < 0.01) and BGLAP genes (P value < 0.01) compared to the un-induced MSC-MG (ctrl) and a significant difference between MHA/Coll MG vs induced MSC-MG (P value < 0.01). On the contrary, induced MSC-MG showed the lower expression gene levels under microgravity condition, underlining the dysregulation effect played by microgravity. The

osteogenic gene expression profiling played in MG suggested that MHA/Coll can influence hBM-MSCs at the molecular level also in microgravity. Together with current findings, this suggests that MHA/Coll is able to transfer key cues at the nanoscale to h-MSC, which overally results in an increased osteo-differentiation in microgravity conditions. Unlike MHA/Coll, it was found that microgravity is able to cause a dysregulation of induced- hBM-MSCs with a downregulation of osteogenic markers and genes. These findings are consistent with many other studies conducted on microgravity. In fact, many authors described that MG causes a dysregulation of osteoblast and osteoclasts with a consequent decrease of bone growth and mineralization.[48-50] MHA/Coll instead, showed its osteoinductive properties on induced-MSCs on gravity and under microgravity stimulations. These results are further confirmed by the MHA/Coll osteogenic properties under microgravity which displayed the expression of ALP and BGLAP, a group of growth factors associated with the development of bone mineralization.

4.4 Conclusions

In this work, an integrative study evaluating the osteoinductive potential of MHA/Coll scaffold to promote osteogenic differentiation under long-duration microgravity simulation was proposed. The osteogenic differentiation of induced hBM-MSCs cultured into nanocrystalline magnesium-doped hydroxyapatite/type I collagen composite scaffold (MHA/Coll) was investigated under microgravity simulation. The osteogenic potential of MHA/Coll was evaluated using the Random Positioning Machine to simulate microgravity (M, 0 x g). Data showed that MHA/Coll with its particular nanostructured niche was able to induce osteogenic differentiation of hBM-MSCs even under microgravity simulation, despite the remodeling effect played by MG. These results contribute to the understanding of the osteogenic capabilities of nanostructured scaffold under long duration microgravity simulation and may help counteracting the bone loss dysfunction after prolonged space flights and weightlessness but also bring new knowledge for the development of scaffolds and materials able to counteract

human bone loss disease. Despite the MG negative effect on the structure of scaffold, the collected data emphasize the importance of osteogenic capabilities of nanostructured MHA/Coll such as future excellent candidates for bone regeneration in microgravity studies. Furthermore, this study provides a significant contribution to evaluate new nano-materials for regenerative medicine and tissue engineering for use in spaceflight mission and Earth-based disease research.

4.4 References

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Chapter 5. Nanotechnology and malaria

5.1 Introduction

Malaria represents one of the most common infectious diseases, which has become one of the most impellent public health problem worldwide.[1] Malaria disease is due to the mosquito bite that is able to infect humans introducing different species of *Plasmodium* into the host.[1] Among various species, *P. falciparum* represents the most dangerous and lethal parasite that infects humans.[2] It was estimated that *P. falciparum* is one of the leading causes of death in the tropical and subtropical countries with 429.000 deaths and 212 million new cases in 2016.[3] However, by considering the current drugs accessible for the prevention and treatment of this disease, it seems that, despite the effectiveness of available antimalarial treatments, the main threaten connected to malaria is the emergence of drug resistance.[4, 5] In this context, nanotechnology could represent a possible future solution against malaria by introducing the control of drug release at the nanoscale or building active nanoparticles to be used against the parasite. In the last decades, the applications of nanotechnology have been mainly focused on the development of a large variety of nanoscale tools designed for their use in therapy [6, 7] and malaria can be one of their targets. Recent advances in nanotechnology have led to the improvement of drug delivery strategies in order to overcame the barriers found in several conventional therapeutic drugs.[8-11] Among different nanomaterials, metal nanoparticles have been significantly studied thanks to their particular physical and chemical properties as an alternative theranostic tool for treating human diseases.[12-14] In this context, among several noble metals, silver nanoparticles (AgNPs) represent an ideal material for biomedical applications, and it has been largely investigated during the last years.[15-17] Its use became less frequent with the introduction of antibiotics in the 1940's, but it is slowly regaining popularity as antibiotic resistance became a serious threat to public health[18]. In fact, bacterial resistance to silver is very rare, and documented in just a few particular cases.[18] In the past two decades a huge number of silver

compounds, mainly coordination complexes, have been prepared and showed promising antibacterial or, more interestingly, anticancer activity, sometimes higher than the reference compound for cancer treatment, cisplatin.[18] Unfortunately, such activity was evident only in *in vitro* experiments, while the applications *in vivo* suffered the limits deriving from scarce bioavailability of the active species (Ag^+) inside the body once the drug was administered to animal models.[18] Researchers are trying to overcome these problems, and the route leading to successful silver-based drugs seems to pass through the preparation of silver nanoparticles.[18] According to the literature, silver nanoparticles are toxic for prokaryotic organisms, but relatively safe for eukaryotic species, including humans.[18] Their cytoxicity is probably associated to several characteristics of the nanoparticles, including reactivity in solution, size distribution, shape, coating/capping, all factors depending on the method of synthesis used to prepare them.[18,19] Indeed, conventional physical and chemical methods for AgNPs synthesis employ reagents and solvents that can be toxic, and are rather expensive.[20, 21] Thus, the synthesis of eco-friendly metal nanoparticles using biological fluids represents a new growing field of nanotechnology for nanomedicine applications.[22] Generally, plants extracts, plant-based phytochemicals, bacteria, algae and other biological sources are used as reducing agents in the preparation of nanoparticles. According to the published literature, biologically-synthesized AgNPs seem to be relatively soluble, showing high stability and less toxic effects.[23-25] Despite the synthetic routes and reaction conditions, the advantages of the biosynthesis approach are the high efficiency and inexpensiveness of the methods and the production of relatively non-toxic and safe nanoparticles for translational research.[26-28] The biological activity of AgNPs depends on different characteristic such as particle reactivity in solution, size distribution, coating/capping of the nanoparticles that influence their cytotoxicity.[29] As previously mentioned, plant extracts are highly effective in reducing silver ions into AgNPs possessing biological activity, and among them many species of the genus *Artemisia* have been

investigated for their action against bacterial and cancer cells with promising results.[30, 31] This effectiveness is undoubtedly linked to factors like size and shape of the AgNPs, but their capping is at least as much important. Several studies have reported that biomolecules contained in the plant extracts such as phenols, flavonoids, enzymes and other proteins not only lead to the reduction of silver ions to form nanosized particles, but also provided a useful capping on the nanoparticle surface.[32] Such capping is able to influence the particle size and avoid aggregation;[33] moreover, it allows a better internalization of the AgNPs by the cells and determines a lower cytotoxicity.[34] A large variety of compounds have been extracted from *Artemisia annua* plants, in particular artemisinin, which is used as an anti-malarial drug.[35] Considering all the advantages of "green" AgNPs and *Artemisia* properties, the synthesis of silver nanoparticles has been carried out using two different types of *Artemisia* (*annua* and *arborescens*) extracts, and the AgNPs thus prepared have been assessed for their activity against *P. falciparum* parasite.

5.2 Materials and methods

Cell cultures

The Palo Alto (PA) strain represents a reference parasite strain to study various antimalarial drugs in *P. falciparum*.[36] PA strain was isolated from a Ugandan patient and is considered as a reference strain due to its high genetic stability. *P. falciparum* PA strain (mycoplasma-free) was cultivated in RPMI 1640 medium containing HEPES (R5886 Sigma Aldrich), supplemented with 20 mM glucose, 2 mM glutamine (59202C Sigma Aldrich), 0.025 mM adenine, and 32 mg/L gentamycin at 2% hematocrit. Parasite cultures were synchronized as described by Lambros and Vanderberg.[37]

Synthesis of silver and green nanoparticles

A. *annua* plants were purchased from an accredited plant grower in Puglia. *A. arborescens* leaves were collected in a growth spot in the countryside around Sassari. Fresh *A. annua* and *A. arborescens* leaves were collected in the months of February and March.

Silver nitrate, ethanol and all the chemicals used were purchased from Sigma-Aldrich Europe. Silver nanoparticles have been prepared using either a protocol reported in the literature by Khatoon *et al*.[33] or a personally modified version of the former. In the first protocol, fresh *A. annua* and *A. arborescens* leaves (10 g) were extracted in 100 ml of 50% ethanol at 60° C. Then, the extracts were filtered, diluted with 50% milliq water and 100 mL of this solution were slowly added to 900 ml of aqueous $AgNO_3$ (2 mM) at room temperature to obtain the desired nanoparticles within a couple of hours. In the modified protocol, *Artemisia* leaves were extracted at a lower temperature, 50° C, to avoid cleavage of the peroxo bridge in artemisin. 100 ml of the hydroalchoolic extracts were diluted to 500 mL with milliq water and slowly added to 500 mL of 2 mM $AgNO_3$ under magnetic stirring at room temperature. Reaction was complete within a couple of hours. To label the *A. annua*-AgNPs synthesized with the two different methods, (1) will be used to indicate the modified protocol and (2) for the first method reported in the literature, as previously described. Silver nanoparticles have also been synthesized through a classical chemical approach (0.1 M $AgNO_3$, 0.3 mM ascorbic acid as the reductant and 0.3 mM sodium citrate as the stabilizer at pH 10.5 (method 3) and 13 (method 4), respectively) at 30° C and used as the control.

Characterization of nanoparticles

The Transmission Electron Microscopy analysis (TEM) observations were performed on FEI TECNAI G2 F20 TWIN instrument using an accelerating voltage of 200 kV. The Energy Dispersive X-ray Spectroscopy was used to collect the spectrum of elemental analysis. Samples for TEM and EDAX analysis were prepared by dispersing a small amount of nanoparticles in

ethanol, sonicated for 20 or 30 min followed by the deposition of one or two drops of the suspension on a holey carbon/copper supported grid. The determination of the average size distribution of the nanoparticles was performed using the spectroscatterer LB-550, Horiba.
The dried powder (1 mg) was dispersed in 10 ml of distilled water. The aqueous solution of each sample was filtered and then used for dynamic light scattering analysis.

Evaluation of nanoparticles green capping

Samples for Fourier Transform Infrared spectroscopy were prepared as KBr pellets using nanoparticles (2 mg) and potassium bromide (200 mg) by compressing the dried powders in a KBr press. FTIR measurement were performed recording the signals in the 400-4000 cm^{-1} range with a resolution of 4 cm^{-1}. FTIR spectra were recorded using a Vertex 70 Bruker spectrophotometer and analysed with OPUS 7 software. UV-visible spectrum of the nanoparticles dispersed in acqueus buffer samples was taken to check the absorption bands. The spectra were recorded by a Nicolet Evolution 300 UV-vis spectrophotometer.

In vitro hemo-compatibility assay on parasitized Red Blood Cells

Fresh human heparinized whole blood was obtained from healthy volunteer donors. RBCs were purified from blood by centrifugation at 200 g for 5 min to remove plasma and leukocytes. RBCs were then washed three times in sterile complete growth medium as previously described. Parasitized RBCs cultures were maintained at 2–5% parasitemia (1% haematocrit) at 37˚C in a 95/5% (vol/vol) air/CO_2-atmosphere. All assays were performed at this parasitemia and hematocrit. Ultrapure water was used as the positive control (Ctrl+). To determine the hemolytic activity on pRBCs, nanoparticles suspension (stock = 1 mg/mL) prepared with sterile isotonic PBS 1X and 5 mM glucose was added to diluted pRBC culture (0.1 ml, ~ 2 X 10^8 cells/mL) at different concentrations (1.25, 2.5, 5 μg/mL) and 24 h times by vortexing. Then, samples were centrifuged at 2000 rpm for 1 minute and a microplate reader (Thermo Scientific) was used to measure the absorbance of hemoglobin release in the supernatant at 405 nm.

Evaluation of growth inhibition of P. *falciparum* in Palo Alto strain

Parasite viability and parasitemia of PA strain were determined using Diff-Quick stained thin blood smears and light microscopy (Carl Zeiss Standard Microscope Lamphouse 467230). Parasitemia was defined as the number of parasites/number of RBCs counted, for a total of 5000 RBCs. Two thin smears per condition were counted 3 separate times by each of three operators. Cultures were synchronized weekly using Percoll separation or 5% sorbitol solution treatment[37] in order to obtain the first parasite stage (rings) to start the experiments at 0 h.

Statistical analysis

Data analyses were performed using Prism GraphPad software. Statistics for experiments were performed using a t-test. In all cases * was used for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. Values were expressed as mean ± SD. All experiments were performed at least in triplicate.

5.3 Results and discussion

Product and purification of "green" nanoparticles

At a first time, AgNPs have been prepared using Kathoon protocol[33] using the leaf extract of *A*. *annua* plants. Later, a modification of this method was introduced in the attempt to prepare particles with smaller size. **Figure 1** shows the "green" synthesis process using two different types of *Artemisia* plant (i.e. *A. annua* and *A. arborescens*). The choice to use *A. annua* was based on the excellent medicinal properties of its extracts, while *A. arborescens* was chosen as a common species in Sardinia with traditional uses in medicine, and an ancient remedy against malaria. In particular, *A. annua* is an aromatic annual herb that grows endemically in China. The major compounds isolated from *A. annua* have shown different biological activity as antimalarial, antibacterial, antitumor and anti-inflammatory agents.[38] Among them, Artemisinin is a compound with a potent antimalarial effect as it is very active against *P.*

falciparum and other malaria-parasites, its activity being attributed to the alkylation of malaria-specific proteins.[39]

The second plant chosen for this study, *A. arborescens*, grows in ruderal environments on calcareous soils in the Mediterranean area[40] and is very frequently found on the island of Sardinia. Several studies have reported the potential of *A. arborescens* compounds as antibacterial, anti-inflammatory, antioxidant and anticancer agents.[40-42] Following these studies, an idea emerged as to investigate and to compare the effects of AgNPs grown from *A. annua* (a species from China) and *A. arborescens* (a species from Sardinia) against malaria.

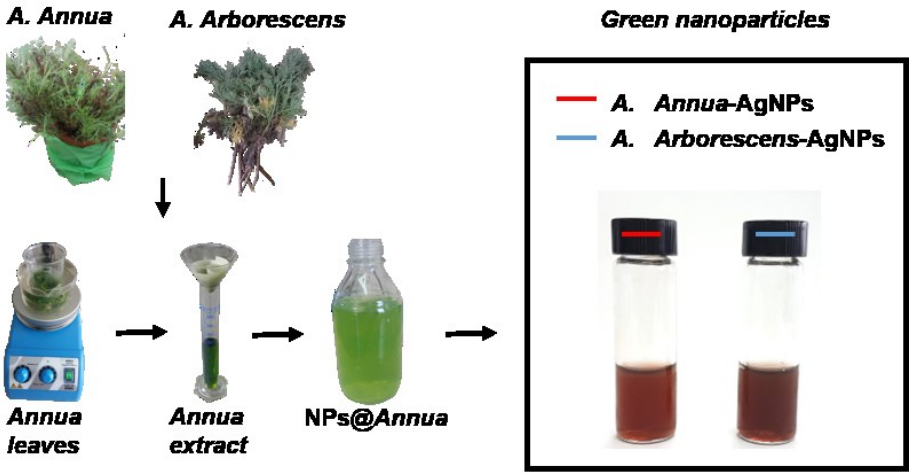

Figure 1. Nanoparticles green synthesis. Schematic representation of "green" AgNPs synthesis using *A. annua* and *A. arborescens* extracts.

Characterization of nanoparticles

A critical step in biomedical applications of nanoparticles is to study their chemical composition and physical characteristics for a correct characterization in order to correlate the biological activity to specific parameters of the nanoparticles (composition, size, shape, capping, etc.).

Therefore, to investigate the nanoparticle effects on PA cultures, firstly their chemical structure has been studied in order to correlate it to their biocompatibility.

The biosynthesized silver nanoparticles were characterized using TEM analysis.

Figure 2A shows the TEM images of AgNPs which were close to spherical shape and not homogenous in the size range, lower than 20 nm in diameter. Dynamic light scattering analyses were used to evaluate the size distribution of AgNPs in aqueous dispersion. The average size of "green" nanoparticles was 13.12 nm for *A. annua*-AgNPs (1), 11.45nm for *A. annua*-AgNPs (2), and 10.0 nm for *A. arborescens*-AgNPs (1), respectively (**Figure 2B**).

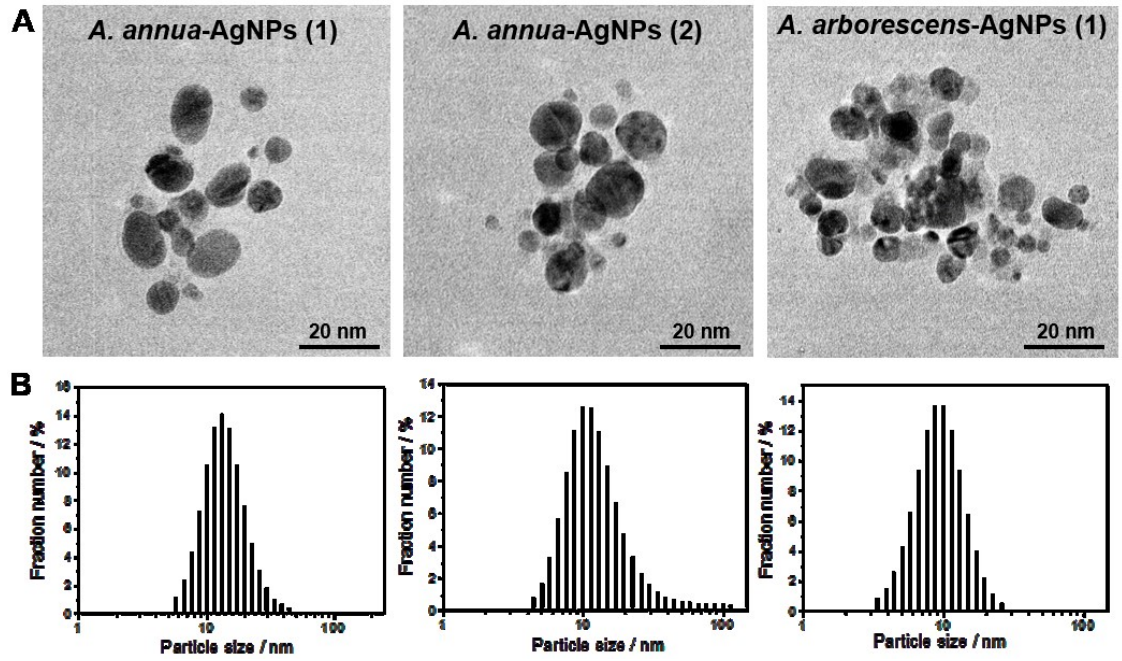

Figure 2. Green nanoparticles characterization. A) TEM micrographs of *A. annua*-AgNPs (1) and (2) and *A. arborescens*-AgNPs (1). **B)** Evaluation of nanoparticles size distribution using dynamic light scattering analysis.

All "green" nanoparticles dispersed well in aqueous medium displaying no aggregation in water while both silver nanoparticles from the "classical" chemical synthesis appeared to be slightly aggregated. EDX profile displayed the elemental composition of the synthetized nanoparticles.

EDX of "green" AgNPs (**Figure 3**) shows the silver peak and organic signal due to the capping agent/stabilizing agent on the surface of each nanoparticle. The Cu peak is due to the grid.

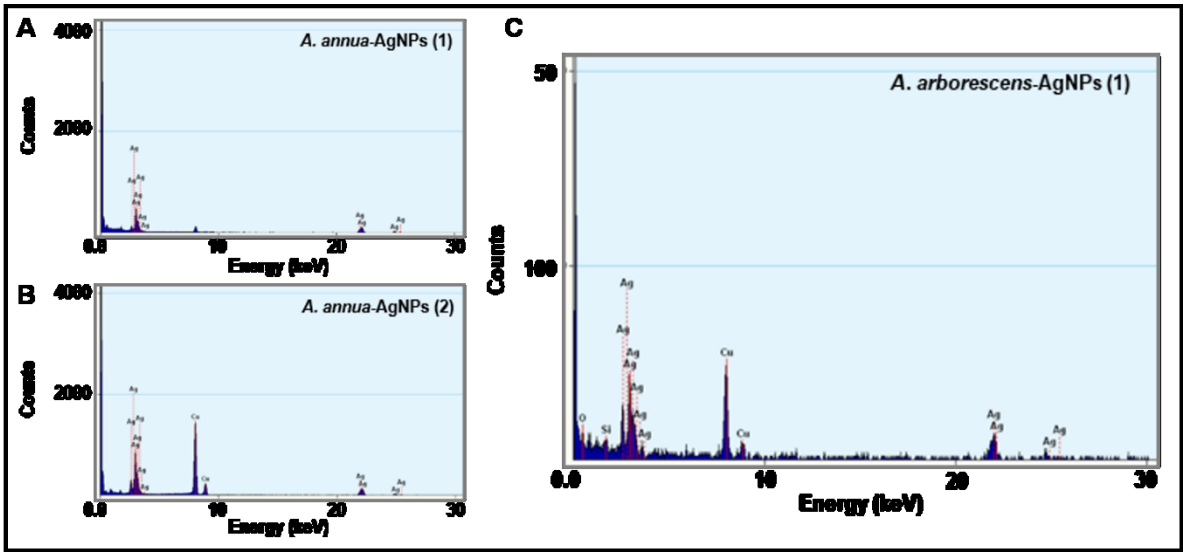

Figure 3. Elemental composition of "green" AgNPs. Evaluation of nanoparticles silver signal using EDX profile: **A)** *A. annua*-AgNPs (1), **B)** *A. annua*-AgNPs (2) and **C)** *A. arborescens*-AgNPs (1). Cu and Si peaks presented in the graphs are due to the grid used.

Evaluation of "green" nanoparticles surface

The The evaluation of the structural features of the surface of nanoparticles plays a significant role in the understanding of the possible effect of AgNPs on the cell membrane for biological applications. FT-IR spectra (**Figure 4A**) were performed to evaluate the surface composition of the "green" nanoparticles compared to "classical" nanoparticles.

The peak in the range 2800-3200 cm^{-1} (**Figure 4A, panel a,b,c**) may be attributed to methanol and to phenolic compounds of *Artemisia* leaf extracts, while it does not appear in "classical" AgNPs (**Figure 4A; panel d,e**). The signals at 1600 and 1000 cm^{-1} are due to the stretching of organic compounds (i.e amide I). On the contrary, the stretching in the range 1400 cm^{-1} may be attributed to silver nanoparticles in accordance with the literature.[32, 33] The structural characterization of silver nanoparticles synthetized using both *Artemisia* leaf extract was

performed using Uv-vis spectroscopy analysis. In **Figure 4B**, the image of the nanoparticles solution color using the different methods of synthesis considered is shown. The initially green solution rapidly changed to a brown color when *Artemisia* extracts were used (**Figure 4B; panel a, b, c**), while it became yellow and then gray during the synthesis of both AgNPs (3) and (4) (**Figure 4B; panel d,e**), respectively. To monitor the synthesis of silver nanoparticles, the Uv spectrometer was used.

The Uv-vis spectra displayed the surface plasmon resonance recorded as a function of the nanoparticles methods of synthesis used and their consequent size. Data results indicate that the Uv-vis spectra obtained in the range of 400-450 nm wavelength was increased in *A. annua*-AgNPs (1) compared to *A. annua*-AgNPs (2) and *A. arborescens*-AgNPs (1). Contrariwise, the AgNPs (3) and (4) displayed a lower absorbance intensity, due to their high aggregation in solution (**Figure 4B**). Considering the same concentration of nanoparticles used for the analysis, the difference in the spectra signal depends on the size, the aggregation and the different capping (or absence of capping) of nanoparticles. Indeed, *A. annua*-AgNPs (1) and (2) have demonstrated a lower aggregation in aqueous medium and good shape and dimension compared to the other nanoparticles taken into consideration.

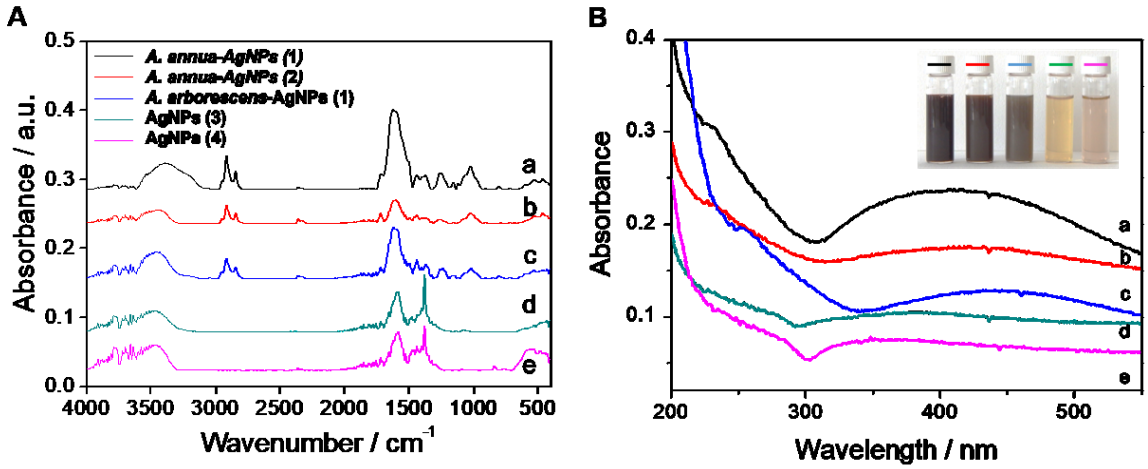

Figure 4. Structural characterization of nanoparticles. A) FT-IR spectra of "green" nanoparticles (panel a,b,c) and "classic" silver nanoparticles (panel d, e). **B)** Structural characterization of nanoparticles analyzing the surface plasmon resonance by Uv-vis spectra.

Biocompatibility of "green" nanoparticles

To study the effect of "green" AgNPs on RBCs biocompatibility, several *in vitro* experiments have been performed on human RBCs infected by *P. falciparum*. Firstly, PA culture was treated with increasing doses of AgNPs (1.25, 2.5 and 5 µg/mL, respectively) at 24 h in order to evaluate the effect of toxicity. The supernatant color indicates the release of hemoglobin from pRBCs and the pellet at the bottom of the 1.5 mL tubes corresponds to intact RBCs precipitated after centrifugation. The Hemolysis was evaluated by spectrophotometry at the fixed absorbance wavelength of 405 nm. Unlike the *A. arborescens*-AgNPs (1) that have demonstrated a significant haemolytic effect after 24 h of treatment (**Figure 5A**; 2.5 µg/mL $p < 0.01$; 5 µg/mL $p < 0.001$), the *A. annua*-AgNPs (1) have shown a dose-dependent haemolytic effect (**Figure 5A**; 2.5 µg/mL $p < 0.05$; 5 µg/mL $p < 0.001$), compared to the negative control. Results evidenced that pRBCs treated with low concentrations of *Artemisia*-AgNPs demonstrated good hemocompatibilty displaying low hemolysis after 24 h. (**Figure 5B**). The different hemolysis effect could be assigned to the difference in the size of nanoparticles.

In fact, it has been reported that the variety of shape, size and chemistries of nanoparticles produce different effects on the biological environment.[43]

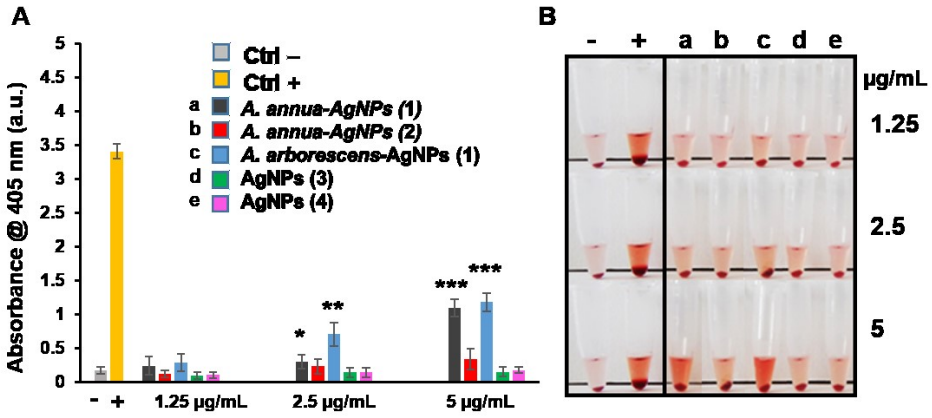

Figure 5. Hemo-biocompatibility assays on human RBCs. A) Hemolysis assay on human pRBCs (PA strain) with increasing doses (1.25, 2.5, and 5 µg/mL) of "green" nanoparticles treated for 24 h. **A)** Samples were analysed by spectrophotometer. The hemolysis value is reported in Absorbance (405 nm). **B)** Pictures of human pRBCs at the different doses and time points. RBCs is the negative (-) and DDW the positive (+) control.

Growth inhibition of parasite *P. falciparum* by *A. annua*-AgNPs

To further evaluate the effects on parasite maturation and death, a morphology investigation was performed on PA strain. PA culture was treated with increasing doses of nanoparticles (1.25, 2.5 and 5 µg/mL) at 24 h in order to evaluate the effect of parasite growth inhibition. *A. annua*-AgNPs (1) and (2) have demonstrated significant *in vitro* activity against *P. falciparum* in pRBCs, showing a decrease of parassitemia (**Figure 6A)** compared to AgNPs (3) and (4), synthesized through a classical chemical approach. Following the data results, it was decided to assess the next experiments using the intermediate dosage of nanoparticles (2.5 µg/mL). The IC^{50}, IC^{90}, IC^{99} values determined at 24 h using 2.5 µg/mL of nanoparticle dose display the best antiplasmodial activity played by *A. annua*-AgNPs (1) (**Figure 6B)**.

A	*A. annua*-AgNPs (1)	*A. annua*-AgNPs (2)	*A. arborescens*-AgNPs (1)	AgNPs (3)	AgNPs (4)
CTRL 24h	8.8%	8.8%	8.8%	8.8%	8.8%
1.25 µg/mL	3.1%	5.6%	2.9%	7.0%	4.8%
2.5 µg/mL	2.6%	5.6%	2.9%	6.5%	7.8%
5 µg/mL	1.4%	4.1%	1.6%	4.1%	5.1%

B	*A. annua*-AgNPs (1)	*A. annua*-AgNPs (2)	*A. arborescens*-AgNPs (1)	AgNPs (3)	AgNPs (4)
IC^{50} (µg/mL)	0.05	2.56	1.17	26.25	10.28
IC^{90} (µg/mL)	0.08	12.44	1.45	32.70	12.81
IC^{99} (µg/mL)	0.01	69.90	1.85	41.57	16.28

Figure 6. Antiplasmodium effect of nanoparticles. A) Percentage of parasitemia on PA strain treated for 24 h with increasing doses (1.25, 2.5, and 5 µg/mL) of nanoparticles. **B)** IC^{50}, IC^{90}, IC^{99} values determined at 24 h using 2.5 µg/mL of nanoparticles dose response.

Unlike the *A. arborescens*-AgNPs (1) that have demonstrated haemolytic effect after 24 h of treatment (**Figure 5)**, the *A. annua*-AgNPs (2) have shown antiplasmodial activity by blocking the parasite maturation stage from trophozoite to rings (**Figure 7**).

Instead, *A. annua*-AgNPs (1) have shown a dose-dependent haemolytic effect (**Figure 5)** connected to the parasite death (**Figure 7**).

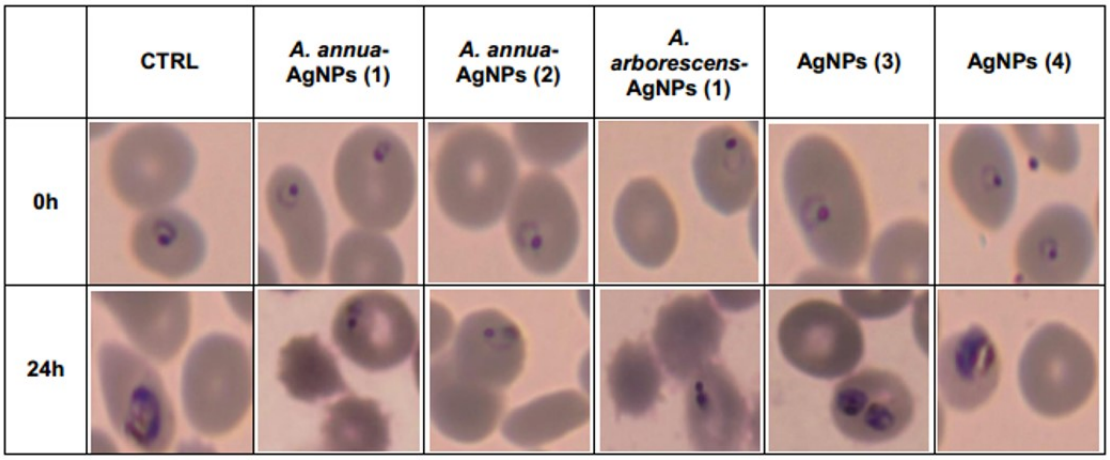

Figure 7. Nanoparticles effect on parasite maturation. The morphology of pRBCs and parasite stage was determined in PA strain treated with the intermediate dose of nanoparticles (2.5 µg/mL) after 24 h.

The striking morphological alteration observed in fixed blood smears of PA strain treated with *A. arborescens*-AgNPs (1) is linked to the small size and the different molecules in the two *Artemisia* extracts involved in the capping of the nanoparticles. Unlike the "classical" AgNPs, the "green" nanoparticles show a dose-dependent antiparasitic activity as reported in **Figure 6** and an antimalarial effect based on their size and surface capping. Furthermore, *A. arborescens*-AgNPs (1) appear to be more haemolytic and reactive than the *A. annua*-AgNP (1) and (2), because of their smaller size and *A. arborescens* capping, compared to *A. annua* capping.

5.4 Conclusions

This work is based on the synthesis and characterization of different types of silver nanoparticles (AgNPs-) using a "green synthesis" approach. Data results have demonstrated that *Artemisia* extracts derived from *A. annua* and *A. arborescens* plants can be used to create a bio-capping on the AgNPs useful to modulate their biological activity.

This type of “green” nanoparticles has demonstrated an antiplasmodium activity in *in vitro* experiments against *P. falciparum* malaria parasite. The present work was performed as a pilot study in order to evaluate the nanoparticles potential antimalarial efficacy in parasitized human red blood cell and to understand their efficacy against *P. falciparum* as a new nanotool against malaria.

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Chapter 6. General conclusions

The recent advances in technology have led to the application of nanotechnology in medicine, starting a new discipline called nanomedicine. Nanotechnology represents a new field of science focused on the use of nanomaterials for enhancing detection, treatment and diagnosis of pathologies through the development of new nanotools. The present research work reviewed the applications of nanotechnology in the field of nanomedicine in order to shed light on the possibility of using innovative nanomaterials as future therapeutic agents. The aim is to present new nanoscale solutions to develop nanoagents able to counteract serious problems related to human health. Thus, breast cancer, bone loss dysfunction and malaria have been taken into consideration as they represent three of the most studied human diseases in the recent years.

In the field of cancer therapy, the major potential of nanotechnology includes the ability to engineer nanovehicles with multiple molecules that, because of their small size, can penetrate tumors with a high specificity, therefore with significantly less side effects. The first phase of this work has been focused on the review of the literature about the main nanomaterials used in nanotechnology against breast cancer, because it represents the second most common form of neoplasia afflicting women all over the world. In particular, a deep overview has been provided on nanomaterials that have been so far investigated for the fight against BC, analysing all publications presented in the literature in the last nine years. In general, analysing the last decade of literature, the most nanomaterials studied to counteract breast cancer are the metal-nanoparticles. Moreover, it has been put in relation the major nanoparticles which have been engineered to generate heat upon activation and lead to tumor cell destruction in non-invasive cancer therapy. Moreover, the review has been focused on theranostic materials for human applications, to inhibit tumor growth and realize the benefits for patients. The goal of this state of the art is to provide a useful guide depicting how nanotechnology can be used to overcome current barriers in breast cancer clinical practice, and how it will shape the future scenario of

breast cancer treatment, prevention and diagnosis i.e. reducing suffering related to chemotherapy and revolutionizing the current surgical approaches. Furthermore, the aim of the work is to inform the whole nanoscience-nanomedicine community on the research and latest advances on the nanotech BC -related field.

Instead, in the context of bone loss diseases, the nanotechnology is used to develop a wide variety of nanotools to counteract the bone loss dysregulation. It has been reported that human spaceflights lead to the dysregulation of the osteoblast functions like an osteoporosis like conditions that are ordinarily connected to a normal bone resorption. In this context, the nanotechnology applications in the field of bone regeneration are focused on the development of nanomaterials able to counteract the osteoblastic dysfunction. Following this scenario, the attention of the second study has been focused on the role of nanotechnology on bone loss dysregulation. The aim was to evaluate the osteoinductive potential of induced human stem cells cultured in nano-scaffolds to promote osteogenic differentiation under microgravity simulation. Finally, during the last years, metal nanoparticles have been raising increasing interest in the scientific community due to their chemical stability, good conductivity and biological activity. In this context, silver nanoparticles (AgNPs) could represent new nanotools for biomedical applications thanks to their promising properties and their efficient anti-inflammatory and antibacterial activity. Thus, the purpose of the last study was to focus the attention on metal nanoparticles to counteract malaria disease. In particular, silver nanoparticles have been synthetized in order to investigate their antiparasitic activity against P. *falciparum* using a novel green synthesis approach.

In general, the results obtained have shown that the use of structures acting on the nanoscale allow to overcome some limitations found in conventional agents, showing promising results for the development of future alternative therapeutic treatments against human diseases. The investigation of the effects of these nanomaterials in *in vitro* experiments has shown a good

stability and biocompatibility in the interaction with the different cells used. Despite nanomaterials have confirmed their good osteogenic potential and anti-plasmodium activity played in the nanoscale context, further investigations into specific interactions between nanomaterials and *in vivo* models are needed to confirm this hypothesis. Finally, the data collected in *in vitro* experiments open a new path for further studies aimed at investigating the potential of these nanomaterials as a possible new nanotechnological strategy in the fight against the serious human diseases considered and tissue regeneration.

Chapter 7. List of publications and presentations

Part of this thesis has been prepared as manuscripts for publications in refereed scientific journals:

- **Elisabetta Avitabile**, Davide Bedognetti, Gianni Ciofani, Alberto Bianco and Lucia G. Delogu, "*How can nanotechnology help the fight against breast cancer*?" Nanoscale, 2018, 10, 11719-1173.
- **Elisabetta Avitabile**, Nina Senes, Antonella Pantaleo and Serenella Medici, "*Green synthesis of silver nanoparticles using Artemisia extract and its antiparasitic activity*", (Status: *under preparation*).
- Julian Schütt, **Elisabetta Avitabile**, Gleb Milyukov, Lucia G. Delogu, Martina Rauner, Larysa Baraban and Gianaurelio Cuniberti, "*Nanocytometer for detection and smart analysis of acute myeloid leukemia: a pilot study*". (Status: *submitted*).

Part of this thesis has been presented in seminar and scientific conferences:

- 24–27/09/2018, 1st NanoBio Conference, Heraklion, Crete, Greece; *"A novel characterization of silver nanoparticles using Artemisia Annua: green synthesis, characterization and anti-malarial activity"*, (Poster presentation).
- 02/09/2017, Bone lab Seminar, Dresden, Germany; *"Nanotechnology for Biomedical Applications: immune system and bone regeneration"*, (Oral presentation).
- 07–10/06/2017, SIF- Scuola di Fisiologia e Biofisica, XII corso residenziale 2017, I metodi e i limiti della ricerca nello spazio, Alcatraz, Perugia, Italia; *"Space scaffolds and bone regeneration in microgravity"*, (Oral presentation).
- 24–27/06/2017, 1st NanoBiomedSardinia workshop, Alghero, Sardinia, Italy; *"Space scaffolds and bone regeneration in microgravity"*, (Poster presentation).
- 07–10/03/2017, NanoSpainConference, San Sebastian, Basque Country, Spain; *"Molecular impact of functionalized nanodiamonds on ex vivo human immune cells response"*, (Poster presentation).

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