UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Development of new biomaterials with antibacterial properties for future application in regenerative medicine

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Master Degree Thesis in

Biomedical Sciences

(2nd cycle of studies)

Supervisor: Prof. Ilídio Joaquim Sobreira Correia (PhD)

Covilhã, June 2012

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Dissertação para obtenção do Grau de Mestre em

Ciências Biomédicas

(2º ciclo de estudos)

Orientador: Prof. Ilídio Joaquim Sobreira Correia (PhD)

Covilhã, Junho de 2012

"Valeu a pena? Tudo vale a pena
Se a alma não é pequena.
Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu."

Fernando Pessoa

Acknowledgments

First of all, I would like to thank my supervisor Professor Ilídio Correia for the opportunity to develop this project and for all the support, guidance and help. Furthermore, I would like to thank him for whatever he did to ensure all the necessary conditions for the development of this project.

I would like to thank to Eng. Ana Paula from the Optics center of Universidade da Beira Interior for the help in acquiring the lots of scanning electron microscopy images of the produced nanoparticles.

In addition, I would like to thank to all of my group colleagues for all the teaching and support during these last months. Their friendship was really important to overcome all the difficulties faced.

I also thank to all my friends that always give me support and advices and that had lots of patience with me in the bad or in the good moments of life, not only during the academic life, but also during my entire life. They all marked in a special way my life.

Finally, special thanks from the bottom of my heart to my parents and sister for granted me the possibility to make this master degree thesis. I thank for all the education, the advices, the support and the love. Thank you for always believe in me even when myself did not. Thank you for always help me in discover my way.

Abstract

Bacterial infections have been a constant threat to human health throughout the history. Bacterial colonization of biomedical devices and implants causes enormous problems for healthcare systems worldwide, costs and increases patient's suffering. Silver has been known, since the antiquity, by their antimicrobial properties and was used to produce reservoirs of food and with medical purposes. With the development of nanotechnology, silver nanoparticles have attracted the attention of different researchers due to their properties, as antimicrobial properties and high surface to volume ratio. However, these nanoparticles can form aggregates, which have toxic effects to the human cells. Recently, silver nanoparticles have been stabilized with several polymers and surfactants in order to avoid these problems.

In this work, silver nanoparticles were produced and stabilized with chitosan/dextran. The produced nanoparticles were characterized by Scanning Electron Microscopy, Ultraviolet-Visible and Fourier Transform Infrared spectroscopy. Furthermore, the antibacterial activity of the produced nanoparticles was evaluated and it was found that they are effective in the prevention of the growth of *Escherichia coli* through a minimum inhibitory concentration. These particles were also studied in contact with human osteoblast cells in order to ascertain if the particles that had an antibacterial effect to the bacteria do not have a toxic effect for human cells. The results herein obtained revealed that the nanoparticles can be used in a near future as a coating material of medical devices in order to avoid their bacterial colonization.

Keywords

Antibacterial mechanism; Chitosan/dextran nanoparticles; *Escherichia coli*; Nanotechology; Silver nanoparticles.

Resumo

As Infecções bacterianas têm constituído uma preocupação constante para a saúde humana. A colonização da superfície dos dispositivos biomédicos e implantes pelas bactérias é a causa de algumas infecções sofridas pelos pacientes, contribuindo para o agravamento dos custos e do sofrimento do paciente. A prata é conhecida desde a antiguidade pelas suas propriedades antibacterianas e foi utilizada na produção de dispositivos de armazenamento de comida e para o tratamento de algumas doenças. Com o desenvolvimento da nanotecnologia, as nanoparticulas de prata têm atraído a atenção de diferentes investigadores devido às propriedades que apresentam, como propriedades antimicrobianas e elevada razão entre a área de superfície e o volume. Contudo, estas nanoparticulas podem sofrer um processo de agregação e produzir efeitos tóxicos para as células humanas. De modo a superar estes problemas, as nanoparticluas de prata têm sido estabilizadas por diversos polímeros e surfactantes.

Neste trabalho, as nanoparticulas de prata foram produzidas e estabilizadas com quitosano/dextrano de modo a evitar a agregação das mesmas. As nanoparticulas produzidas foram caracterizadas por Microscopia Electrónica de Varrimento, Espectroscopia do Ultravioleta-Visível e Espectroscopia de Infravermelho. A actividade antibacteriana das nanoparticulas produzidas foi avaliada na prevenção do crescimento de *Escherichia coli*, através dos valores da determinação da concentração inibitória mínima. O perfil de citotoxicidade das nanoparticulas foi caracterizado através da utilização de osteoblastos humanos. Estas partículas na concentração inibitória mínima não apresentam efeito tóxico para os osteoblastos humanos, devido ao crescimento e proliferação das células na presença destas nanoparticulas. Os resultados obtidos revelam que estas nanopartículas podem ser usadas no revestimento de dispositivos médicos, prevenindo a sua colonização por bactérias.

Palavras-chave

Escherichia coli; Mecanismo antibacteriano; Nanoparticulas de prata; Nanoparticulas de quitosano/dextrano; Nanotecnologia.

Table of Contents

List of Figures

Chapter I – Introduction

Chapter III – Results and Discussion

List of Tables

Chapter I – Introduction

Chapter III – Results and Discussion

List of Acronyms

Ag^0	Metallic silver
Ag^+	Silver ions
$[Ag(NH_3)_2]^+$	Diamminesilver ions
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
CFU	Colony-forming unit
$C_6H_8O_6$	Ascorbic acid
DMEM-F12	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EtOH	Ethanol
FBS	Fetal bovine serum
FT-IR	Fourier Transform Infrared
HIV	Immunodeficiency virus
K^-	Negative control
K^+	Positive control
LB	Luria Bertani
LPS	Lipopolysaccharides
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
$NaBH_4$	Sodium borohydride
$Na_3C_6H_5O_7$	Sodium citrate
NCCLS	National Committee for Clinical Laboratory Standards
PBS	Phosphate buffered saline
PEG	Poly(ethylene glycol)
PEI	Poly(ethylene-imine)
PMS	Phenazine Methosulfate
PVA	Poly(vinyl alcohol)
PVP	Poly(vinylpyrrolidone)
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecylsulfate
SEM	Scanning Electron Microscopy

SPR	Surface Plasmon Resonance
UV-Vis	Ultraviolet-Visible

Chapter I
Introduction

1. Introduction

1.1. Bacterial infections that affect human beings

Bacterial infections have been a constant threat to human health throughout history (Vasilev *et al*. 2010). Human beings are often infected by different microorganisms such as bacteria, molds, yeasts, and viruses (Shahverdi *et al*. 2007; da Silva Paula *et al*. 2009). In the beginning of the 20th century, infectious diseases were the main cause of death worldwide (Huh *et al*. 2011). Examples of these diseases are the bubonic plague, tuberculosis, malaria, and the acquired immunodeficiency syndrome pandemic caused by the human immunodeficiency virus (HIV) that have affected a substantial number of patients worldwide, causing significant morbidity and mortality (Tenover 2006). In the middle of the 20th century, the development of new antibiotics and other methods to control infections helped the humans to prevent and treat from several diseases (Tenover 2006). All these advances began when Flemming, in 1928, discovered the first antibiotic that he called penicillin (Ligon 2004). Antibiotics are defined as chemical substances that are produced by a microorganism, that have the capacity, in dilute solutions, to selectively inhibit the growth of or to kill other microorganisms (Collier 2004). The penicillin was extracted from a plant of the genus *Penicillium* (Ligon 2004) and their commercial production began in the late 1940s (Kalishwaralal *et al*. 2010; Huh *et al*. 2011). The use of antibiotics had a great success in 70th and 80th decades of the 20th century (Huh *et al*. 2011; Prucek *et al*. 2011), when newer and even strong antibiotics were developed (Huh *et al*. 2011). However, the development of antimicrobial drugs contribute to the current crisis in fighting against multi-drug resistance bacterial strains (Huh *et al*. 2011), where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent, leading to the need of development of new antibiotics (Tenover 2006; Xu *et al*. 2011). The mechanisms of antibiotics resistance are spread in a variety of bacterial genera (Tenover 2006). These mechanisms are the result of the acquisition of genes encoding enzymes by the organism, such as ß-lactamases, that destroy the antibacterial agent before it can have an effect. Furthermore, the bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell, before it can reach its target site and exert its effect. Finally, bacteria may acquire several genes for a metabolic pathway which produces altered bacterial cell walls that do not present the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit the access of the antimicrobial agents to the intracellular target site via downregulation of porin genes (Stewart *et al*. 2001; Tenover 2006; Xu *et al*. 2011). Currently, the treatment of bacterial infections using classical antibiotics is becoming a serious global health problem (Sondi *et al*. 2004; Kim *et al*. 2007; Ghosh *et al*. 2010; Liu *et al*. 2010; Potara *et al*. 2011; Prucek *et al*. 2011) due to the fact that most of the prominent infectious disease agents are resistant to all the antibiotics presently available (Xu *et*

al. 2011). As an example, almost all known antibiotics are ineffective against the MDM-1 bacteria, which was discovered recently (Prucek *et al*. 2011). So far, many efforts have been done to develop effective and safe antibacterial drugs against bacteria (Potara *et al*. 2011). Although a large number of natural and synthetic antibiotics have already been reported in the literature (Liu *et al*. 2010), most of them are not effective against bacteria or have safety concerns (Liu *et al*. 2010). As an example, the Grepafloxacin and Trovafloxacin were removed from the market in several countries due to their secondary effects to humans (Liu *et al*. 2010).

1.1.1. Bacterial infections caused by biomaterials implantation

Over the past half century, advancements in the use of natural and synthetic biomaterials and the improvement of surgical techniques have led to an increase in the demand of biomaterials to be used in implants and medical devices production (Simchi *et al*. 2011). The biomaterials market is estimated to be worth more than 300 billion US Dollars and to be increasing 20% per year (Simchi *et al*. 2011). Medical specialists treat millions of patients every year using different implanting devices, like pacemakers, artificial hip joints, breast implants, dental implants, hearing devices and skin substitutes (Simchi *et al*. 2011). In orthopedic surgery, different implant materials have been used (Campoccia *et al*. 2010). This implanted materials ranging from internal to percutaneous and from resorbable to long-term ones (Campoccia *et al*. 2010). They included prostheses, soft moldable or injectable cavity-fillings, hard and heavy weight-bearing metallic, ceramic or polymeric biomaterials, allogeneic bone, viable tissue grafts, and also include the recent tissue engineering products, such as scaffolds, drug delivery systems, among others (Campoccia *et al*. 2010). In 2010 more than 4.4 million people had one internal fixation device, and 1.3 million people have an artificial joint (Simchi *et al*. 2011).

Even though very significant advances in microbiology field (Vasilev *et al*. 2010), one problem that is common to all medical devices (Campoccia *et al*. 2010; Fernebro 2011), is their colonization with bacteria or fungi, which cause infections in the host (Nava-Ortiz *et al*. 2010). Depending upon the location and type of the medical device, bacterial infections may result in morbidity or even in patient dead (Jones *et al*. 2008; Nava-Ortiz *et al*. 2010). Thus, sometimes prosthesis removal and replacement is the only option to definitively eradicate severe infections and avoid patient dead (Campoccia *et al*. 2006). An additional problem is the risk of re-infection in a second implantation, which usually occurs in 1-2% of the patients, depending on type of prosthetic implant, patient condition, clinical setting, and surgical procedure (Campoccia *et al*. 2010). As an example, the infection rate for total hip arthroplasties has been reported to occur in 0,5-3% of the cases, and the rate of reinfection, after revision of infected hip prostheses, is up to 14% (Campoccia *et al*. 2010). These drastic interventions bear obvious implications in terms of attendant patient trauma, prolonged hospitalization as well as in terms of health and social costs (it has been estimated that the treatment of each single episode of infected arthroplasty costs more than $50,000) (Campoccia *et al*. 2006; Vasilev *et al*. 2010).

Implant-associated infections are the result of bacteria adhesion to an implant surface and subsequent biofilm formation at the implantation site (Jones *et al*. 2008; Simchi *et al*. 2011). Biofilms are communities of microbial cells that attach to a surface and secrete a hydrated extracellular polymeric matrix (Gurjala *et al*. 2011). The organisms become embedded in this matrix, which is composed of polysaccharides, proteins, glycoproteins, glycolipids, and extracellular deoxyribonucleic acid (DNA) (Gurjala *et al*. 2011). The matrix polymers support microcolonies of cells, allows cell–cell communication, forms water channels, retains and concentrates nutrients, and can support gene transfer through conjugation, transformation, and transduction (Edwards *et al*. 2004; Martin *et al*. 2009). The matrix is thereby responsible for the maintenance of the structural integrity of the biofilm and provide an ideal matrix for bacterial cell growth (Monteiro *et al*. 2009). The biofilm formation is described as a sequence of different steps. In the first one, microbial cells adhere to the biomaterial surface, through exopolysaccharides that are synthesized by the bacteria (Speranza *et al*. 2004; Kalishwaralal *et al*. 2010). Surface adhesion is a critical step in the pathogenesis of implant-related infections and represents the beginning of the colonization of biomaterial surfaces (Montanaro *et al*. 2011). Thereafter, it follows the accumulation in multiple cell layers, biofilm maturation, and detachment of cells from the biofilm into a planktonic state to initiate a new cycle of biofilm formation elsewhere (Speranza *et al*. 2004; Montanaro *et al*. 2011).

The biofilm allows microbes to survive to host immune defenses and systemic antibiotic therapies (Campoccia *et al*. 2010), which is the main reason for the high prevalence of infections (Fernebro 2011). Therefore, extraordinary antibiotic resistance is a general feature of biofilm which is caused by several factors (Simchi *et al*. 2011). These factors include the compact nature of biofilm structures, the presumed reduced rates of cellular growth and cellular respiration of the bacteria in the biofilm and the protection conferred by biofilm matrix polymers (Simchi *et al*. 2011). The antibiotic resistance of biofilms is also related to the fact that in the extracellular polymeric substance cells are allowed to change their proteome (up to 50% of the proteome may differ from the same microorganism in a planktonic state) to their existence in a sessile state (Nava-Ortiz *et al*. 2010). This state has low metabolic levels and downregulated cell activity, which leads a decreased antimicrobial susceptibility compared with planktonic cells (Nava-Ortiz *et al*. 2010). Furthermore, the alteration of the proteome makes that bacteria in a biofilm express different sets of genes than those that are expressed in its planktonic form (Martin *et al*. 2009). This have important implications for clinical therapeutics, since the antimicrobials does not reach the bacterial cells in the biofilm (Martin *et al*. 2009). Thus, in the biofilm, after bacteria colonization, the resistance to antimicrobial agents is dramatically increased (up to 1000-fold) and even the antimicrobial agents that are effective against planktonic cells are ineffective against the same bacteria growing in a biofilm (Jones *et al*. 2008; Martin *et al*. 2009; Monteiro *et al*. 2009; Simchi *et al*. 2011). Thereby, biofilm leads to an undesirable and deteriorative impact to several fields, such as in medicine, industry, and commercial products (Inphonlek *et al*. 2010).

Due to these facts, it is important the use of antibacterial agents to inhibit bacterial adhesion, in order to prevent implant-associated infections (Kim *et al*. 2007; Inphonlek *et al*. 2010; Simchi *et al*. 2011). In order to achieve this purpose, medical devices with different antibiotics incorporated such as gentamicin, norfloxacin, nitrofurazone, minocycline, and rifampin have been produced, to avoid biofilm formation (Dave *et al*. 2011). However, most of these coatings only allow short release profiles, making them inappropriate for relatively long-term use (Dave *et al*. 2011). Furthermore, some antimicrobial agents are extremely irritant and toxic to the human being (Sondi *et al*. 2004), which emphasizes the need to develop antimicrobial materials to be applied in health and biomedical device, food, and personal hygiene industries (Häntzschel *et al*. 2009; Vasilev *et al*. 2010). These materials must be cost-effective, avoid bacterial resistance for them, have ability to act against a wide spectrum of bacteria, have high levels of bactericidal and bacteriostatic activity, be safe for the environment and be biocompatible for eukaryotic cells (Kim *et al*. 2007; Chaloupka *et al*. 2010; Fayaz *et al*. 2010; Inphonlek *et al*. 2010).

Since the antibiotic resistance is growing up (Nagy *et al*. 2011) and this has become a major issue in public healthcare (Mohammed Fayaz *et al*. 2009), there is a renewed interest in the development of products containing silver, since these have antimicrobial properties (Arora *et al*. 2008; Monteiro *et al*. 2009; Madhumathi *et al*. 2010; Nagy *et al*. 2011). In fact, the antibiotic-resistant pathogens has led to the resurgence of silver-based materials with antibacterial agents purpose(da Silva Paula *et al*. 2009; Fayaz *et al*. 2010; Kalishwaralal *et al*. 2010; Huh *et al*. 2011; Nagy *et al*. 2011), due to their antimicrobial activity against a large number of microorganisms and far lower propensity to induce microbial resistance than that of antibiotics (Arora *et al*. 2008; Fayaz *et al*. 2010). Thus, the incorporation of silver in topical dressings or as coating material on medical products may therefore play an important role in the era of antibiotic resistance (Ip *et al*. 2006). However, silver has high toxicity for the human being and in order to solve this problem, nanoscale materials have emerged as novel effective alternative to be used as antimicrobial agents (Sondi *et al*. 2004; Rai *et al*. 2009).

1.2. Nanotechnology

Nanotechnology is an area of science that appeared in the 20th century (Lu *et al*. 2008). It is emerging as a rapid growing field with applications in Science and Technology at the nanoscale level (Rai *et al*. 2009). The term Nanotechnology was created by Professor Norio Taniguchi of Tokyo Science University in 1974, to describe precision of manufacturing materials at the nanometer level (Rai *et al*. 2009). But the concept of Nanotechnology was given previously, in 1959, by physicist Professor Richard P. Feynman in his lecture "There's plenty of room at the Bottom" (Rai *et al*. 2009). The term Nanotechnology is derived from the word "nano" (Rai *et al*. 2009). "Nano" is a Greek word synonymous to dwarf meaning extremely small, used to indicate one billionth of a meter or 10^{-9} m (Rai *et al*. 2009). Nanoscale is taken to include active

components or objects in the size range of 1–100 nm (Cumberland *et al*. 2009; Rai *et al*. 2009; Kurek *et al*. 2011).

Bionanotechnology has emerged up as integration between biotechnology and nanotechnology, to allow the development of biosynthetic and environmental-friendly technology for synthesis of nanomaterials (Rai *et al*. 2009). Nanotechnology allowed the development of several materials, devices and systems (Öztürk *et al*. 2008; Türkmen *et al*. 2009). Among the three areas mentioned above, the area of nanomaterials is the most advanced at present, both at the scientific level and for commercial applications (Öztürk *et al*. 2008; Cumberland *et al*. 2009; Türkmen *et al*. 2009). Nowadays nanomaterials are used for the development of novel devices that can be used in various physical applications as biophotonics, biosensors, fuel cells, photovoltaic devices, semiconductor nanowires, solar energy conversion, and also in catalysis, water treatment, and biological, biomedical and pharmaceutical applications (Raffi *et al*. 2008; Babu *et al*. 2010). Nanomaterials display unique and superior properties, like higher surface to volume ratio, increased percentage of atoms at the grain boundaries and the predominance of quantum effects instead of gravitational ones. This distinct properties are unavailable in conventional macroscopic materials (Raffi *et al*. 2008).

In the medical field, nanomaterials may provide a reliable and effective tool to treat diseases at a molecular level (Chouhan *et al*. 2009). This fact is important since their dimensions are close to that of the cellular components and biological molecules (Hung *et al*. 2007). Among the various types of nanomaterials, nanoparticles have attracted much attention in the present century due to the defined chemical, optical and mechanical properties (Rai *et al*. 2009). They need to be formulated with improved bioavailability and release rates, which can decrease required dosages while increasing safety and reducing side effects (Wong *et al*. 2009). However, on the other hand, several studies suggested that nanoparticles can cause injuries in the biological systems (Yen *et al*. 2009), since the similar size of nanoparticles to the cellular components make them bypass the natural barriers, such as the cell membranes, causing harmful effects to living cells (Wong *et al*. 2009; Yen *et al*. 2009). There is no concern, until now, about the interaction of nanoparticles with the living cells and the results in this field are very controversial (Arvizo *et al*. 2012). Nevertheless, nanoparticles have been used as drug delivery systems, as biomolecular sensing molecules, as targeted imaging, and as thin film coatings (Hung *et al*. 2007). The advances in the use of nanostructured materials for medical applications are possible due to the availability of novel techniques of processing, characterization and modeling and also the technology for manipulation and manufacturing of the nanostructured materials (Brigmon, Berry et al. 2010). Moreover, the production of devices with specific functionalities is obtained by the specific interactions between biological structures (e.g., tissues and other cellular processes) and nanostructured materials (Brigmon *et al*. 2010).

Inorganic nanoparticles, of either simple or composite nature, are a type of nanoparticles that have been receiving considerable attention as a result of their unique properties like chemical, electronic, magnetic, optical, and physical, including also antimicrobial and catalytic activity (Shahverdi *et al*. 2007; Guzman *et al*. 2011), which are different from those of the bulk

materials (Yin *et al*. 2005; Guidelli *et al*. 2011). These special and unique properties could be attributed to their small sizes and large specific surface area (Guzman *et al*. 2011). All of this makes the inorganic nanoparticles adequate for applications in biomedicine, catalysis, electronics, energy science, magnetic, mechanics, optics, and so on (Shahverdi *et al*. 2007; Fayaz *et al*. 2010). A number of recent works in this field, describe the possibility of generating new types of nanostructured inorganic materials with designed surface and structural properties (Sondi *et al*. 2004). Thus, the preparation, characterization, surface modification, and functionalization of nanosized inorganic particles open the possibility to formulate a new generation of bactericidal materials to avoid microbial biofilm formation on biomaterials surface (Sondi *et al*. 2004; Lipovsky *et al*. 2011). Nanoparticles with antibacterial properties offer many distinctive advantages with respect to the therapy with antibiotics since they allow the reduction of *in vivo* toxicity, overcoming the problem of resistance to the antibiotics, and lowering the cost associated with their production (Huh *et al*. 2011).

Among the different types of nanoparticles, the metallic ones are the most promising candidates for this purpose, since they show good antibacterial properties (Ruparelia *et al*. 2008; Rai *et al*. 2009), due to their high specific surface area, high fraction of surface atoms (Hung *et al*. 2007; Shahverdi *et al*. 2007; Martin *et al*. 2011), and small size. These properties allow nanoparticles to interact closely with cellular membranes of the bacteria. In addition to these characteristics, this kind of nanoparticles release metal ions in solution, which increases the antibacterial properties (Ruparelia *et al*. 2008). Another properties of these nanoparticles are the long life and the heat resistance (Potara *et al*. 2011). Well-known metallic nanoparticles with these properties are the silver nanoparticles (AgNPs).

1.3. Silver Nanoparticles

The unique antimicrobial properties of silver in the treatment of infections have been known for a long time (Gurunathan *et al*. 2009; Häntzschel *et al*. 2009; Mohammed Fayaz *et al*. 2009). Since 1000 BC, Egyptians, Greeks, Romans and other ancient civilizations used silver vessels to store perishable foods, to produce silver cutlery, glassware and dishes (Vasilev *et al*. 2010). Silver was also used with medical purposes for rheumatism, tetanus, gonorrhea and wound healing treatment (Vertelov *et al*. 2008). In the 18th century, silver nitrate ($AgNO_3$) was used for the treatment of venereal diseases, fistulae from salivary glands, bone abscesses (Rai *et al*. 2009), and ulcers (Neal 2008). Dilute solutions of $AgNO_3$ have been used since the 19th century in treatment of infections and burns (Ip *et al*. 2006). Due to the successful of the registered cases, in 1920s silver was recognized by the United States Food and Drug Administration for its antimicrobial activity and was regulated for wound management (Neal 2008). In 1940s, after penicillin was introduced in the market, the use of silver for the treatment of bacterial infections was reduced (Rai *et al*. 2009). Silver reappear again in the 1960s when Moyer introduced the use of 0.5% $AgNO_3$ for the treatment of burns as previously done in the 19th

century (Rai *et al*. 2009). In 1990s, silver was introduced in a colloidal form (i.e. AgNPs) in ointments that could be applied to open wounds, in order to kill bacteria (Arora *et al*. 2008). Recently, due to the emergence of antibiotic-resistant bacteria and limitations associated with the use of these medicines, doctors have restarted to use silver, mainly in the form of AgNPs in order to fight different types of infections affecting humans (Rai *et al*. 2009).

AgNPs are nano-sized structures formed from silver atoms that are metallically bonded together and have a size from approximately 1 nm to 100 nm (Chaloupka *et al*. 2010; Songsilawat *et al*. 2010). It has been shown that they can be used in medicine and health-related areas (Cumberland *et al*. 2009), due to their interesting optical and catalytic properties, high resistance to oxidation and high thermal conductivity (Vertelov *et al*. 2008; Wong *et al*. 2009; Prucek *et al*. 2011). Furthermore, their tunable size, shape and surface chemistry allow them to be designed with specific properties that are critical for several applications (Potara *et al*. 2011), most typically antimicrobial and sterile applications (Marambio-Jones *et al*. 2010). Taking into account the antibacterial activity of silver, it is better for AgNPs than for other silver forms, as bulk silver (Potara *et al*. 2011; Xu *et al*. 2011), silver ions (Ag^+) and other silver salts (Li *et al*. 2011), even when applied in lower concentrations (da Silva Paula *et al*. 2009; Domingos *et al*. 2011). The higher antibacterial activity is due to high specific surface area and high fraction of surface atoms (more than 1000 atoms in one 5 nm particle (Vertelov *et al*. 2008)) in AgNPs (da Silva Paula *et al*. 2009), which allows a better contact with microorganisms (Ghosh *et al*. 2010; Juan *et al*. 2010; Li *et al*. 2010; Guzman *et al*. 2011). Therefore, smaller-sized particles with 1/1000 of the bacterium size (since AgNPs have a nanometer size and bacteria a micrometer size) show stronger antibacterial activity (Guzman *et al*. 2011; Kurek *et al*. 2011; Shameli *et al*. 2011). Furthermore, the nanoparticles release Ag^+ in aqueous solutions, which enhance their bactericidal activity (da Silva Paula *et al*. 2009; Chaloupka *et al*. 2010; Juan *et al*. 2010).

AgNPs due their antimicrobial properties, are capable of kill several microorganisms responsible for 650 types of different diseases (Raffi *et al*. 2008). These nanoparticles have revealed bactericidal activity against as many as 16 bacteria species (Sondi *et al*. 2004) either gram-positive or gram-negative (Monteiro *et al*. 2009; Sheikh *et al*. 2010), including *Escherichia coli (E. coli)*, *Staphylococcus aureus (S. aureus)*, *Bacillus subtilis*, *Streptococcus mutans*, *Staphylococcus epidermidis* (Li *et al*. 2011) and highly multiresistant strains such as *methicillin-resistant S. aureus* (Fayaz *et al*. 2010). Moreover, they also showed antifungal activity (Kacarevic-Popovic *et al*. 2007; Häntzschel *et al*. 2009; Monteiro *et al*. 2009) against *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Trichophyton mentagrophytes* (Li *et al*. 2011). More recently, it has also been reported that AgNPs can inactivate virus (Kacarevic-Popovic *et al*. 2007; Monteiro *et al*. 2009; Li *et al*. 2010) like hepatitis B virus, herpes simplex, monkeypox, respiratory syncytial virus (Li *et al*. 2011), and also exhibit antiviral properties against HIV infected cells (Panáček *et al*. 2006; Shameli *et al*. 2010; Shameli *et al*. 2011), via preferential binding of the AgNPs to the gp120 glycoprotein knobs through the sulfur-bearing residues of glycoprotein amino acids, thus inhibiting the virus from binding to the target cell membrane receptor (Thomas *et al*. 2007).

Besides its antimicrobial activity, it has been recently found that AgNPs reduce cytokine release (Guidelli *et al*. 2011), decreasing lymphocyte and mast cell infiltration and also induce apoptosis of inflammatory cells (Chaloupka *et al*. 2010). These characteristics of AgNPs are responsible for the anti-inflammatory effect and contributes for accelerating the epithelialization by over 40% and, as a consequence, accelerate wound healing (Lu *et al*. 2008; Chaloupka *et al*. 2010; Kalishwaralal *et al*. 2010).

1.3.1. Applications of silver nanoparticles

The properties presented by AgNPs make them good candidates to be used in different applications, like in electronics and in sensor design based on the surface-enhanced Raman spectroscopy (SERS) (Badawy *et al*. 2010; Parashar *et al*. 2011). Moreover, these nanoparticles have been used in a number of medical applications (Arora *et al*. 2009), due to the antimicrobial activity owned by the silver-based compounds containing ionic silver or metallic silver (Panáček *et al*. 2006; Arora *et al*. 2008).

Some applications of silver and AgNPs are presented in figure 1. In the case of silver, it is used in the form of $AgNO_3$ solutions to perform cauterization, in order to stop epistaxis and the growth of post-traumatic granulomas (Chaloupka *et al*. 2010). $AgNO_3$ is also used to prevent some infections and to promote an anti-inflammatory effect in the procedure of pleurodesis (Chaloupka *et al*. 2010). Silver is also used in the form of silver sulfadiazine cream to apply in ulcers and burns to promote the skin regeneration (Chaloupka *et al*. 2010).

AgNPs have been used as coating material for medical purposes, orthopedic, vascular or dental graft materials (Panáček *et al*. 2006; Ruparelia *et al*. 2008; Vertelov *et al*. 2008), indwelling catheters (Vasilev *et al*. 2010), and arthroplasty (Panáček *et al*. 2006). AgNPs can be impregnated in wound dressings (Xu *et al*. 2011), in diabetic ulcers (Ip *et al*. 2006; Li *et al*. 2011), in chronic ulcers, and in traumatic injuries in order to prevent infections and enhance wound repair (Ip *et al*. 2006). Some medical products containing AgNPs available in the market, as wound dressings and catheters, are presented in table 1 (Arora *et al*. 2009; Chaloupka *et al*. 2010).

Moreover, silver can also be employed to eliminate microorganisms on textile products, food storage containers, cosmetics in the form of nanogels and nanolotions, contraceptive devices, and they can be used for water filtration too (Monteiro *et al*. 2009; Shameli *et al*. 2010; Vasilev *et al*. 2010; Mirzajani *et al*. 2011).

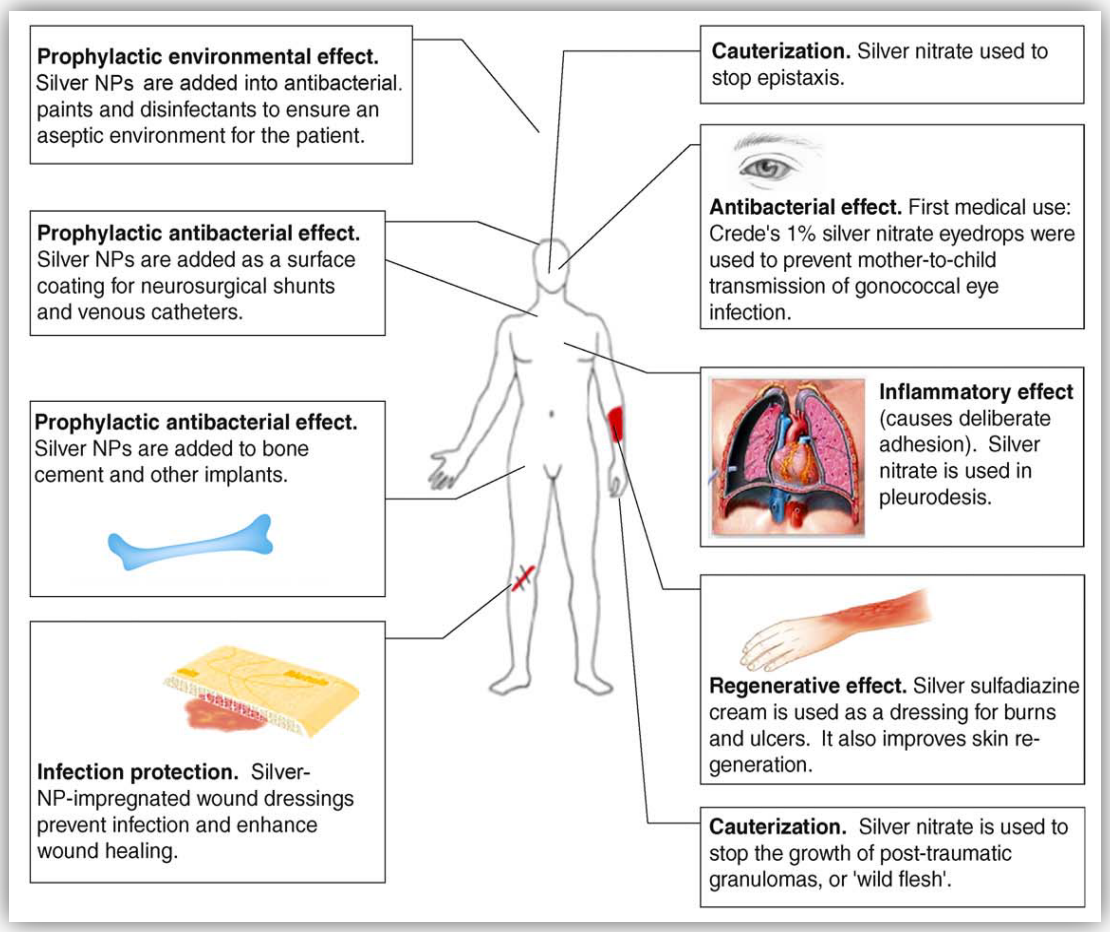

Figure 1 – Applications of silver (right-hand size) and AgNPs (left-hand size) in medicine (Chaloupka *et al*. 2010).

Now, silver is an additive of consumer products (Chaloupka *et al*. 2010), like socks, shirts, shoes, water filters, antiperspirants, combs, paints, washing machines (Chaloupka *et al*. 2010; Nagy *et al*. 2011), dishwashers, refrigerators, toilet seats (Li *et al*. 2011), antibacterial sprays, cosmetics, dietary supplements, cell phones, laptop keyboards, and children's toys, among other products, which purportedly exploit the antimicrobial properties of silver nanomaterials (Marambio-Jones *et al*. 2010).

Table 1 – Commercially available medical products containing AgNPs (Chaloupka *et al*. 2010).

Product	Company	Description	Clinical uses
Acticoat™	Smith & Nephew	Nanocrystalline silver wound dressings	Dressing for a range of wounds including burns and ulcers; prevents bacterial infections and improves wound healing.
Silverline®	Spiegelberg	Polyurethane ventricular catheter impregnated with AgNPs	Neurosurgical drain of cerebrospinal fluid for hydrocephalus. Also can be adapted for use as shunts. Antibacterial AgNPs coating prevents catheter associated infections.
SilvaSorb®	Medline Industries and AcryMed	Antibacterial products: hand gels, wound dressings, cavity filler	Wound dressings and cavity filler prevent bacterial infection. Hand gels used to disinfect skin in clinical and personal hygiene purposes.
QN-Q Silver Soaker ™	I-Row Corporation	AgNPs coated catheter for drug delivery	Delivery of medication (e.g. local anesthetics or analgesics) per-, peri-, post-operatively for pain management or for antibiotic treatment.

Thus, a quite a large amount of AgNPs are manufactured worldwide to be used in several different applications from research, academia and industry to even households (Parashar *et al*. 2011).

Due to all properties and applications of AgNPs, it is fundamental to know their mechanisms of action and their issues related with resistance and toxicity of them.

1.3.2. Mechanisms of action of silver nanoparticles

The mechanism of action of AgNPs against the bacteria has not been fully elucidated (Martinez-Castanon *et al*. 2008; Li *et al*. 2010; Fuertes *et al*. 2011; Mirzajani *et al*. 2011; Xu *et al*. 2011). However, their mechanism and the mechanism of Ag^{+} (Nagy *et al*. 2011), which are

released by AgNPs in aqueous solution (Martinez-Castanon *et al*. 2008; Vertelov *et al*. 2008; Juan *et al*. 2010) that enhance their bactericidal activity (Rai *et al*. 2009; Juan *et al*. 2010; Kurek *et al*. 2011), have been explored extensively (Nagy *et al*. 2011). Several mechanisms of how AgNPs act against bacteria and allow their destruction have been proposed (Ruparelia *et al*. 2008).

Among the hypotheses that have been proposed to explain the mechanism of antimicrobial activity of AgNPs, it is believed that Ag^+ interact with the bacterial cell wall peptidoglycans (sulfate, oxygen and nitrogen), promoting bacterial lysis through the potassium release from bacteria (Rai *et al*. 2009). AgNPs can be incorporated through the cell membrane by the same mechanism of Ag^+ (Lu *et al*. 2008; Maneerung *et al*. 2008; Ruparelia *et al*. 2008; Rai *et al*. 2009). Nanoparticles may attach on the surface of the cell membrane and disturbs its power function, such as electron transport chain and permeability (Martinez-Castanon *et al*. 2008; Raffi *et al*. 2008; Gurunathan *et al*. 2009; Li *et al*. 2010; Fuertes *et al*. 2011). A damage in the membrane permeability affects the transport through the plasma membrane, like the efflux of reducing sugars and proteins as well as the depletion of the levels of intracellular adenosine triphosphate (ATP) (Raffi *et al*. 2008; Xu *et al*. 2011). This makes the bacterial cells incapable of properly regulate the transport through its membrane, resulting in cell dead (Ruparelia *et al*. 2008). In Gram-negative species, like *E. coli*, AgNPs are responsible for the formation of irregular shaped "pits" in the outer membrane of the bacteria. Such "pits" are accountable for the increase of the cell wall permeability by progressive release of lipopolysaccharides (LPS) molecules and membrane proteins (Raffi *et al*. 2008; Mirzajani *et al*. 2011) resulting in the collapse of the cell membrane potential (Xu *et al*. 2011). In addition, it is believed that silver binds to functional groups of proteins, resulting in protein desnaturation (Raffi *et al*. 2008).

In addition, cell membrane disruption also allows the passage of AgNPs into cytoplasm (Ruparelia *et al*. 2008; Li *et al*. 2010; Kurek *et al*. 2011; Potara *et al*. 2011). Subsequently, AgNPs interact with phosphates of DNA (Thomas *et al*. 2007) and it loses its replication ability (Martinez-Castanon *et al*. 2008; Raffi *et al*. 2008; Vertelov *et al*. 2008). In a study performed by Raffi and colleagues, they reported that DNA may have lost its replication ability and cellular proteins became inactive, after cells being treated with AgNPs (Raffi *et al*. 2008). The entrance of such nanoparticles inactivate their enzymes, generate hydrogen peroxide and cause bacterial cell death (Raffi *et al*. 2008).

Other important factor that is involved on antimicrobial mechanism of AgNPs is the formation of reactive oxygen species (ROS) (Lu *et al*. 2008; Kurek *et al*. 2011; Potara *et al*. 2011). The formation of ROS is one of the primary mechanisms of nanoparticle toxicity, and these are thought to result in damage of proteins and DNA, as well as perturb cell membrane integrity (Kurek *et al*. 2011; Nagy *et al*. 2011). Furthermore, the ROS facilitate the interactions of AgNPs with the bacteria through the membrane lipid peroxidation (Kurek *et al*. 2011).

A short summary of AgNPs mechanisms of action against bacteria are presented in figure 2.

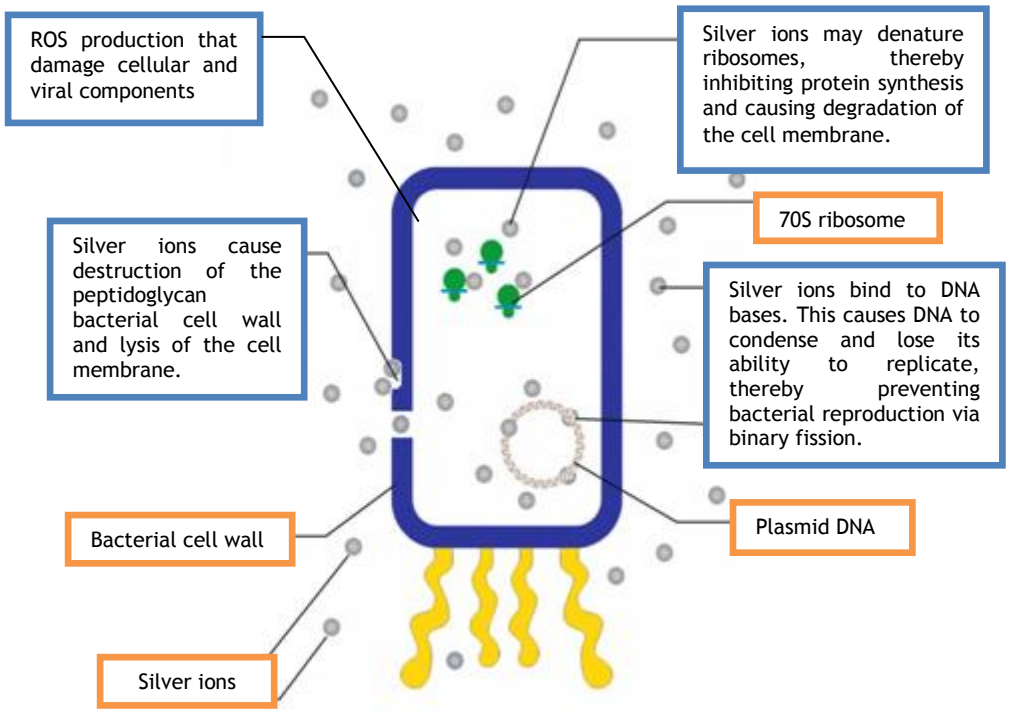

Figure 2 – Different mechanisms of action of AgNPs against bacteria. In general, these mechanisms include: photocatalytic production of ROS that damage cellular and viral components, compromising the bacterial cell wall/membrane, interruption of energy transduction, and inhibition of enzyme activity and DNA synthesis. Adapted from (Chaloupka *et al*. 2010; Huh *et al*. 2011).

1.3.3. Bacterial silver nanoparticles resistance

The probability of AgNPs induce microorganism resistance is much lower than that of conventional antibiotics (Xu *et al*. 2011) or than other antimicrobial materials (Li *et al*. 2011). This ability to promote minimal (Ip *et al*. 2006) or no resistance in microorganisms (Prucek *et al*. 2011) allows to postulate their use for replace some of the antibiotics presently in use (Sheikh *et al*. 2010). This ability is due the fact that the metal attacks a broad range of targets in the organisms, which means that they would have to develop a host of mutations simultaneously to protect themselves from the AgNPs (Pal *et al*. 2007). Furthermore, the presence of this multiple bactericidal mechanisms that act in synergy against bacteria, makes more difficult the acquisition of resistance by bacteria to AgNPs (Chaloupka *et al*. 2010).

In fact, resistance to silver is rare, but not unknown (Atiyeh *et al*. 2007). In the literature, there are two forms of resistance described: cells can bind to silver and form an intracellular complex, or they can be excreted from microorganisms, by using cellular efflux systems (Atiyeh *et al*. 2007). Li and collaborators showed that resistance was induced using low concentrations of silver (Li *et al*. 1997). Bactericidal levels of silver do not produce resistance, however, minimum inhibitory concentration (MIC) (2–4 mg Ag^+/L) and sub-MIC levels can allow the development of resistance by bacteria (Atiyeh *et al*. 2007). This occurs due to halide ions that act as precipitating agents, for example, the chloride remove Ag^+ by precipitating in the form of silver

chloride (Silver 2003), which decreases silver bioavailability and increases bacterial silver resistance (Marambio-Jones *et al*. 2010). Resistant bacteria also have modified plasmids that confer this resistance (Silver 2003), in this case, resistant cells appeared to develop reduced permeability to silver combined with an upgraded active efflux mechanism that pumps silver out of the cell and protecting the cytoplasm against toxic concentrations of silver (Parikh *et al*. 2008). It is therefore clear that non-controlled use of silver in sublethal levels may result in development of resistance by the bacteria, like to other antibiotics (Atiyeh *et al*. 2007).

1.3.4. Toxicity of silver nanoparticles

Silver toxicity for different organisms is known for a long period (Cumberland *et al*. 2009) and has been described by different researchers (Huh *et al*. 2011). Silver toxicity can cause an irreversible skin pigmentation (arygria - a permanent disorder caused by silver deposition in the skin's micro vessels in patients who are exposed to high quantities of silver, 50,000-300,000 ppm) and pigmentation in the eyes (agyroses) (Rai *et al*. 2009; Huh *et al*. 2011). In addition, other toxic effects include organ damage, (e.g., the deposition of silver in liver and kidney), irritation (e.g., eyes, skin, respiratory, and intestinal tract), and changes in the blood cell counts (Arora *et al*. 2008; Huh *et al*. 2011; Kurek *et al*. 2011). Beyond the toxicity for the several organisms, heavy metal accumulation in the environment has been mentioned by the United States Agency for Toxic Substances and Disease Registry (ATSDR) as well as by the European Commission as a concern (Monteiro *et al*. 2009). Nevertheless, silver has not been cited amongst the most prevalent heavy metals, and it is not in the priority list of hazardous substances for public health (Monteiro *et al*. 2009).

Conversely, the citotoxicity of AgNPs is not fully characterized (Sheikh *et al*. 2010). AgNPs have an advantage over ionic silver because of their efficacy at low concentrations (Pal *et al*. 2007; Gurunathan *et al*. 2009) show reduced toxicity and higher antibacterial potential (Kurek *et al*. 2011; Prucek *et al*. 2011). The toxicity of AgNPs is concentration-dependent (Monteiro *et al*. 2009), and therefore, it is prudent to incorporate a minimum amount of silver in the organism, for example on implant surfaces in order to reduce bacterial adhesion as well as minimizing tissue cytotoxicity (Chen *et al*. 2006). Moreover, several studies reported that AgNPs significantly decreased the function of mitochondria and induced cell necrosis or apoptosis in several cell types (Yen *et al*. 2009; Huh *et al*. 2011), via the production of ROS, which leads to cell death (Atiyeh *et al*. 2007). It was also observed liver function abnormalities, following acute silver toxicity (50 µg/mL) due to nanocrystalline silver (Atiyeh *et al*. 2007).

A consensus about the detailed molecular mechanism of action of AgNPs that is responsible for its toxicity is still missing (Mohammed Fayaz *et al*. 2009; Arvizo *et al*. 2012). It is possible to state that a lack of physical barriers for nanoparticle diffusion into cells, determines their generalized (bio)availability, with the risk of a massive uptake by eukaryotic cells, which eventually leads to their death (Mohammed Fayaz *et al*. 2009). In fact, the issue of possible adverse effects and toxicity of nanoparticles for the human body is progressively recognized as a

central issue and, although the increasing number of studies, they are still limited (Mohammed Fayaz *et al*. 2009; Arvizo *et al*. 2012). Li and collaborators reported that the AgNPs with the size from 6 to 20 nm can induce the mitochondrial dysfunction and ROS production (Chaloupka *et al*. 2010; Li *et al*. 2011) that can induce DNA damage and chromosomal aberrations (Li *et al*. 2011). Another study published by Rosas-Hernández and colleagues reported that the AgNPs with a distribution size from 10 to 90 nm, have selective and specific effects on the vascular endothelium in a concentration-dependent manner (Rosas-Hernández *et al*. 2009). In general, the toxicity is associated with the size of the AgNPs. The small AgNPs (<5 nm) are more toxic than the largest ones (Atiyeh *et al*. 2007) and than any other form of silver, as metallic silver or silver solutions (Songsilawat *et al*. 2010). Other studies demonstrated that, beyond the size, morphology, aggregation and surface functionality are also critical factors that influence the toxicity and the biologic responses to the presence of these type of nanoparticles (Li *et al*. 2011).

1.3.5. Combination of silver nanoparticles with other materials

At the nanometric scale, due to small interparticle distances, aggregation of the nanoparticles can occur owing to van der Waals forces (Domingos *et al*. 2011). Furthermore, the high surface area to volume ratio of the nanoparticles results in high reactivity that can also lead to particle aggregation (Badawy *et al*. 2010). This on the other hand, affects their toxicity (Kvítek *et al*. 2008; Songsilawat *et al*. 2010), since in a non-agglomerated and well dispersed form, AgNPs do not present toxicity for cells (Tomsic *et al*. 2009) and do not lost their antibacterial activity (Kvítek *et al*. 2008). Beyond of agglomeration, AgNPs can also undergo precipitation and oxidation (Radziuk *et al*. 2007). Therefore, all these phenomena make AgNPs to lose their peculiar properties associated with the nanoescale, producing toxic effects to the human being and make them to lose their strong antibacterial activity (Mohammed Fayaz *et al*. 2009).

Consequently the preparation and stabilization of metal nanoparticles represents a great challenge (Mohammed Fayaz *et al*. 2009; Domingos *et al*. 2011). For this purpose, different polymers and surfactants in small concentration, like polyphosphate, polyacrylate, sodium dodecyl sulfate, poly(vinyl-sulfate), poly(ethylene-imine) (PEI), poly(allylamine), poly(vinylpyrrolidone) (PVP), poly(ethylene glycol) (PEG), poly(vinyl-alcohol) (PVA), polyacylamides, polyurethanes, poly(oxyethylene–oxypropylene)-monoamine, and chitosan have been used, for nanoparticles stabilization and preventing of the formation of aggregates (Radziuk *et al*. 2007; Kvítek *et al*. 2008; Mohammed Fayaz *et al*. 2009; Marambio-Jones *et al*. 2010; Lee *et al*. 2011; Lin *et al*. 2012). Particularly, in the case of AgNPs, the most prevalent capping agents are citrate, PVP, chitosan, and PVA (Badawy *et al*. 2010). The stabilization of metal nanoparticles is explained by the electronic interaction of the polymer functional groups with the metal particles (Mohammed Fayaz *et al*. 2009) providing electrostatic, steric, or electrosteric repulsive forces between particles, avoiding particles aggregation (Levard *et al*.

2012). Protective polymers can coordinate metal ions before reduction (Mohammed Fayaz *et al*. 2009), forming a polymer-metal ion complex, which can then be reduced to form zerovalent metal colloids (Radziuk *et al*. 2007; Domingos *et al*. 2011). This process allows the production of nanoparticles with a narrower size distribution, than those obtained without protective polymers (Mohammed Fayaz *et al*. 2009). Once the reduction occurs, particles are attached to the much larger protecting polymers that cover or encapsulate the metallic particles and thus stabilize them to be used in biomedical field (Mohammed Fayaz *et al*. 2009). In order to be applied in the referred field, is also required that both the stabilizing and the reducing agents must not represent a biological hazard (Mohammed Fayaz *et al*. 2009).

Besides the problem of aggregation, as already described, typically AgNPs are likely to be toxic for cells under physiological conditions, which limits their applications in biological systems (Potara *et al*. 2011). The polymers and surfactants, used to stabilized AgNPs, are also used to solve the cytotoxic problems (Ghosh *et al*. 2010) and can also lead to synergistic antibacterial agents with new, improved optical, electrical and catalytic properties, unavailable in the individual components themselves (Maneerung *et al*. 2008; Potara *et al*. 2011). Thus, the use of the stabilizing agents in order to avoid aggregation, also provide a protective interfacial barrier between the metal core and cells, which is especially important for preventing damage to the surrounding healthy tissues (Schrand *et al*. 2008). Moreover, it was demonstrated that the incorporation of AgNPs into polymers create a protective interfacial barrier that do not affect the antibacterial properties of the nanoparticles and may increase them, as mentioned before (Schrand *et al*. 2008). All these advantages have been widely employed in a vast number of engineering and technical areas, especially in medical field, to produce biomedical devices with specific properties (Prashantha *et al*. 2006; Liu *et al*. 2008; Maneerung *et al*. 2008).

Chitosan and dextran were used in this work to stabilize AgNPs in order to widening its applications.

1.3.5.1. Dextran

Dextran (Figure 3) is a bacterial-derived polysaccharide generally produced by enzymes from certain strains of Leuconostoc or Streptococcus (Xiao *et al*. 2009), with good biodegradability and biocompatibility (Wang *et al*. 2011). It is built by glucose molecules containing 17-20% sulfur coupled into long branched chains, mainly through 1,6-glucosidic and some through 1,3-glucosidic linkages (Tiyaboonchai *et al*. 2007; Hwang *et al*. 2010; Anitha *et al*. 2011; Saboktakin *et al*. 2011; Wang *et al*. 2011).

Moreover, it is also colloidal, water-soluble, and inert to biological systems (Hwang *et al*. 2010; Jeong *et al*. 2011). Due to these properties, dextran has been studied to be used as a carrier system for a variety of therapeutic agents including antidiabetic, antibiotic, anticancer, peptides, and enzymes (Hwang *et al*. 2010). It has been also investigated to be used as an antiviral agent, in the treatment of hypolipidemia, and for the prevention of free radical damage, among other applications (Saboktakin *et al*. 2011).

Furthermore, it is the most widely used polysaccharide since it is cheap (when compared to hyaluronan or heparin), available (when compared to glucomannans for instance) and the presence of the sulfate groups ensures strong electrostatic interactions with other the positive polymers, like chitosan (Delair 2011).

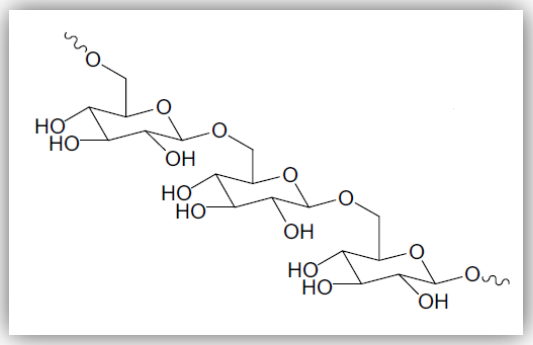

Figure 3 – Representation of the dextran chemical structure(Liu *et al*. 2009).

1.3.5.2. Chitosan

Chitosan (figure 4) is a deacetylated form of chitin, that is the major compound of the exoskeletons of crustaceans shells such as crabs, shrimps and lobsters and is also found in some microorganisms, as yeasts and fungi (Sarmento *et al*. 2007; Tiyaboonchai *et al*. 2007; Nagpal *et al*. 2010). It is composed of randomly distributed ß-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Sundar *et al*. 2010; Anitha *et al*. 2011; Saboktakin *et al*. 2011). The commercially available chitosan has a deacetylation degree between 66 and 95% and has molecular weights ranging from 3.8 to 2000 kDa (Sundar *et al*. 2010).

The solubility of chitosan (pKa 6.5) is dependent on protonation of the amino groups of their molecules; therefore it is often solubilised in acids at pH lower than 6.5 including formic, acetic, tartaric, and citric acid (Chen *et al*. 2003; Tiyaboonchai *et al*. 2007). It is a weak base and has a positive charge (Chen *et al*. 2007), which allows chitosan to react with negatively charged surfaces (via mucoadhesion) and materials, including polymers (alginate, dextran, PVA) and DNA (Chen *et al*. 2007; Sundar *et al*. 2010).

These features, and other properties like its biodegradability *in vivo* by lysozyme (Sundar *et al*. 2010) and others hydrolytic enzymes (Carmen Rodríguez-Argüelles *et al*. 2011), low toxicity, good biocompatibility, improving wound healing and blood clotting, absorption of liquids in order to form protective films and coatings, make it versatile and attractive to be used in biomedical and pharmaceutical formulations (Chen *et al*. 2007; Babu *et al*. 2010). Chitosan has been used for the preparation of microparticles and nanoparticles (Sundar *et al*. 2010) to be used in the regeneration of different types of tissues, especially skin, bones and in many other

biomedical and pharmaceutical applications (Carmen Rodríguez-Argüelles *et al*. 2011). Furthermore, it also presents very important biological properties among which antimicrobial, anti-inflammatory, and antioxidant (Carmen Rodríguez-Argüelles *et al*. 2011).

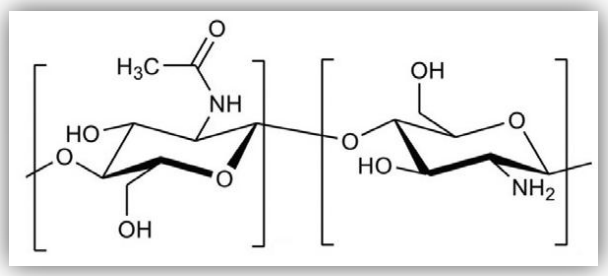

Figure 4 – Representation of the chitosan chemical structure(Kumirska *et al*. 2011).

In this work, the chitosan aqueous solution was added to dextran sulfate polyanion aqueous solution. The formation of polycation–polyanion (polyelectrolyte) complex was mainly driven by an electrostatic mechanism where charge neutralization and possible local bridging (such as hydrogen bounding, Coulomb forces, van der Waals forces, and transfer forces) occurs (Yu *et al*. 2005; Meng *et al*. 2010). The advantages of chitosan/dextran nanoparticles are enhanced stability and increased mechanical strength compared with chitosan/tripolyphosphaste microparticles, whose lower stability and mechanical strength limit their application for drug delivery (Chen *et al*. 2007). It has also been reported that DNA and insulin structures are protected when dextran is used in the formulation of polyethylenimine/dextran nanoparticles (Chen *et al*. 2007). Dextran was also described as being capable of reducing the cationic charge-related cytotoxicity of PEI nanoparticles in vitro (Chen *et al*. 2007). Therefore, it is possible that the combination of chitosan and dextran as matrix materials, in an optimal charge ratio, may act synergistically to incorporate and protect proteins and drugs, in order to reduce the toxicity of chitosan caused by its cationic charge (Kean *et al*. 2010).

1.4. Objectives

In the present study different nanoparticles with antibacterial properties were produced in order to be applied in several biomedical products like bone implants or skin substitutes. The specific objectives of the workplan herein presented are the following:

- Development of AgNPs stabilized with chitosan/dextran;
- Determination of the antibacterial activity of the produced nanoparticles;
- Characterization of the different nanoparticles by different methods: Fourier Transform Infrared (FT-IR), Ultraviolet-Visible (UV-Vis), and Scanning Electron Microscopy (SEM);
- Evaluation of the cytotoxic profile of the nanoparticles.

Chapter II
Materials and Methods

2. Materials and Methods

2.1. Materials

Human fibroblasts cells (Normal Human Dermal Fibroblasts adult, criopreserved cells) were purchased from PromoCell (Spain), bacterial strain *Escherichia coli* DH5α (ATCC 68233) was purchased from ATCC (United States).

Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium reagent (MTS) and electron coupling reagent phenazine methosulfate (PMS) were purchased from Promega. Amphotericin B, ascorbic acid ($C_6H_8O_6$), Dulbecco's Modified Eagle Medium-F12 (DMEM), ethanol (EtOH), high molecular weight chitosan, L-glutamine, LuriaBertani (LB) Broth, penicillin G, phosphate-buffered saline (PBS), rezazurin sodium salt, sodium borohydride ($NaBH_4$), sodium citrate ($Na_3C_6H_5O_7$), and trypsin were purchased from Sigma. Dextran sulfate 500,000 was purchased from Amresco. $AgNO_3$ was purchased from Panreac (Spain). LB agar was purchased from Pronadise.

2.2. Methods

2.2.1. Preparation of silver nanoparticles

The AgNPs were produced based on the method previously developed by Mafune and collaborators (Mafune *et al*. 2000). Briefly, the procedure consists on the rapid injection of 0.5 ml of $NaBH_4$ (10 mM) into an aqueous solution, with continuous stirring, containing 0.5 ml of $AgNO_3$ (0.1 M, 0.01 M, and 0.001 M) and 20 ml of $Na_3C_6H_5O_7$ (0.001 M). The resultant solution was stirred for 1 h and aged for 2 h. Moreover, the $NaBH_4$ solution was replaced by $C_6H_8O_6$ (10 mM) and this component was added to a 0.01 M $AgNO_3$ solution. The resulting nanoparticles were washed three times with distilled water and centrifuged at 75000 g, during 30 min. Subsequently, a dried powder of particles was obtained by freeze-drying the particles overnight.

2.2.2. Preparation of the Chitosan/Dextran nanoparticles

Chitosan/dextran nanoparticles were prepared by the ionotropic gelation of chitosan and dextran as described before by Chen and colleagues (Chen *et al*. 2007). A 0.1 % (m/v) chitosan solution was prepared by dissolving chitosan in aqueous acetic acid 0.2 % (v/v) and the pH was adjusted to 3.5. A 0.1 % (m/v) dextran solution was prepared by dissolving the dextran in water.

Briefly, the dextran solution was added dropwise to chitosan solution under stirring. This process was repeated with different ratios of dextran/chitosan (1:6, 1:3, 2:3 (v/v)) and for high and low molecular weights of chitosan and dextran. The resulting nanoparticles were left to rest for 30 min and then, rinsed three times with destilled water and centrifugated at 17000 g, for 30 min. A dried powder of particles was obtained by freeze-drying the particles overnight.

2.2.3. Preparation of the Chitosan/Dextran nanoparticles with silver

To the solution of chitosan/dextran nanoparticles already prepared was added to 50 µL of 0.01M of $AgNO_3$ solution. After 5 min of stirring, 50 µL of 0.01M of $NaBH_4$ were added dropwise and left aged for 30 min. The same procedure was performed by replacing $NaBH_4$ for the same concentration of $C_6H_8O_6$. Then the particles were washed three times with destilled water and centrifugated at 17000 g, during 30 min. A dried powder of particles was obtained by freeze-drying the particles overnight.

The formed AgNPs and chitosan/dextran nanoparticles were also produced by the dropwise addition of 150 µL of AgNPs solution on the chitosan/dextran nanoparticles under stirring and left aged for 30 min. Formerly, the particles were washed three times with destilled water and centrifugated at 17000 g, during 30 min. A dried powder of particles was obtained by freeze-drying the particles overnight.

2.2.4. Nanoparticles Scanning Electron Microscopy Analysis

The morphology of the produced nanoparticles was analyzed by scanning electron microscopy (SEM). First, the produced nanoparticles were resuspended in 100 µL of ultrapure water and then, one drop was added to a 16 mm cover glasses and subsequently mounted in aluminum board using a double-sided adhesive tape and covered with gold using an Emitech K550 (London, England) sputter coater. Then, the prepared samples were analyzed using a Hitachi S-2700 (Tokyo, Japan) scanning electron microscope, operated at an accelerating voltage of 20 kV with variable magnifications (Gaspar *et al*. 2011).

2.2.5. Nanoparticles Ultraviolet-Visible Spectroscopy Analysis

The produced AgNPs were analyzed by UV-Vis specroscopy. The UV-Vis spectra were recorded using UV-1700 PharmaSpec from Shimadzu at 300 nm/min scanning rate, with a wavelength range from 200 to 700 nm, and then analyzed with UVProbe Shimadzu 2.0 software.

2.2.6. Nanoparticles Fourier Transform Infrared Spectroscopy Analysis

The different nanoparticles were also analyzed by FT-IR. This spectroscopic tool gives information about the molecular structure of chemical compounds, and it is useful for the characterization of biopolymers (Lawrie *et al*. 2007). The spectra was acquired in a Fourier transform infrared spectrophotometer Nicoletis 20 (64 scans, at a range of 4000 to 400 cm^{-1}) from Thermo Scientific (Waltham, MA, USA) equipped with a Smart iTR auxiliary module.

2.2.7. Determination of antibacterial activity

2.2.7.1. Minimum Inhibitory Concentration and Minimal Bactericidal Concentration

The antimicrobial activity of the synthesized AgNPs was assessed through a standard microdilution method, in order to determine the Minimum Inhibitory Concentration (MIC), which is defined as the lowest concentration of material that inhibits the growth of an organism. This assay was performed in agreement with the standards recommendations from the National Committee for Clinical Laboratory Standards (NCCLS). The antimicrobial activity of AgNPs was scrutinized using *E. coli* as a model of Gram-negative bacteria, in a 96 wells plate. The AgNPs were diluted several times with 100 µL of culture medium (LB Broth). After that, 100 µL of culture medium inoculated with the tested bacteria at a concentration of 2.5×10^5 colony-forming unit (CFU)/mL was added to the plate. A negative control was prepared with 200 µL culture medium and a positive control was set by using 100 µL of culture medium inoculated with 100 µL of the bacteria at a concentration of 2.5×10^5 CFU/mL. The plate was incubated 24 h at 37 °C and then the MIC of the tested substance was determined (Panáček *et al*. 2006). This test was done in triplicate.

The Minimal Bactericidal Concentration (MBC) may be characterized as the minimum concentration of the sample required to achieve bacterial dead after 24 h of incubation. The MBC was examined by a modified imprinted method. 5 µL of the tested samples with defined concentrations were transferred from the plate wells and imprinted on the surface of a plate of LB agar without antimicrobial agents. Then the petri plate was incubated for 24 h at 37 °C and the MBC was determined as the lowest concentration that inhibited the bacterium visible growth (Panáček *et al*. 2006). The nanoparticle concentration causing bactericidal effect was determined based on the absence of colonies on the agar plate (Ruparelia *et al*. 2008).

2.2.8. Proliferation of cells in the presence of the produced nanoparticles

Human osteoblast cells were cultured with Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with heat-inactivated FBS (10% v/v), penicillin G (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL). Cells were seeded in 75 cm^3 T-flasks until confluence was obtained. Detachment of confluent cells was achieved by a 3 min incubation in 0.18% trypsin (1:250) and 5 mM EDTA. Then, an equal volume of culture medium was added to the free cells in order to stop the reaction. The cells were centrifuged, the pellet was resuspended in culture medium, and the cells were then seeded in new 75 cm^3 T-flasks (Ribeiro *et al*. 2009). To verify the influence of the presence of the produced nanoparticles in cell adhesion and proliferation, cells were seeded with nanoparticles in a 96-well plate at a density of 15×10^3 cells/well, for 24 and 48 h. Before this procedure, plates and the materials were UV sterilized for 30 min. Cell growth was monitored using an Olympus CX14 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.

2.2.9. Evaluation of the cytotoxic profile of the produced nanoparticles

The MTS assay was performed in order to evaluate nanoparticles toxicity. Human osteoblast cells, at a density of 15×10^3 per well, were seeded in a 96-well plate and cultured with DMEM-F12. At the same time, in another 96-well culture plate, DMEM-F12 was added to the produced nanoparticles that were previously irradiated with UV light for 30 min, in order to be sterilized. The nanoparticles were left in contact with medium for 24 and 48 h. After the period of incubation, the cell culture medium was removed and replaced with 100 µL of medium that was in contact with the nanoparticles. Then, cells were incubated at 37°C, in a 5% CO_2 humidified atmosphere for more 24 h. The cell viability and proliferation was assessed through the reduction of MTS into a water-soluble brown formazan product. Briefly, the medium of each well was removed and replaced with a mixture of 100 µL of fresh culture medium and 20 µL of MTS/PMS reagent solution. Then, cells were incubated for 4 h at 37 °C, in a 5% CO_2 atmosphere (Ribeiro *et al*. 2009). The absorbance was measured at 490 nm using a Biorad Microplate Reader Brenchmark (Tokyo, Japan). Wells containing cells in the culture medium without biomaterials were used as negative control (K^-). EtOH 96% was added to wells containing only cells and was used as positive control (K^+).

2.2.10. Statistical Analysis of MTS results

Statistical analysis of the results of cell viability assays was performed using one-way analysis of variance (ANOVA) with Bonferroni t-test, with at least three independent results. A value of $p<0.05$ was considered statistically significant. Results of different nanoparticles in contact with cells were compared with the positive control.

Chapter III
Results and Discussion

3. Results and Discussion

In the following sub-sections are presented and discussed the results related to the different assays of nanoparticles production and analysis. Furthermore, the antibacterial and citotoxicity assays are also presented and discussed in order to evaluate the properties of the produced nanoparticles.

3.1. Characterization of Particles Morphology

In order to avoid the formation of AgNPs aggregates they were stabilized with chitosan/dextran nanoparticles. Firstly, AgNPs were synthesized at room temperature using $AgNO_3$ with different concentrations (0.1 M; 0.01 M and 0.001 M) and two reducing agents ($C_6H_8O_6$ and $NaBH_4$). During chemical reaction, the reducing agent donates electrons to Ag^+, leading to the reversion of Ag^+ to its metallic form (Ag^0) (Chaloupka *et al*. 2010). This was verified by the appearance of a yellow-brownish color in the solution, in the case of $NaBH_4$ (strong reducing agent), and yellow-greenish in the case of $C_6H_8O_6$ (weak reducing agent). Such color change indicates the formation of AgNPs (Mallick *et al*. 2004; Nam *et al*. 2011). By controlling the experimental conditions (e.g. temperature, energy input, presence of capping agents), the reaction kinetics can be manipulated in order to produce AgNPs (Chaloupka *et al*. 2010).

The produced AgNPs were visualized through SEM. It could be stated that no AgNPs were formed when higher concentrations of $AgNO_3$ (0.1 M) were used (figure 5A). Conversely, for the lowest concentration of $AgNO_3$ (0.001 M), only a small amount of AgNPs were observed (figure 5B). The higher number of AgNPs was obtained with 0.01 M of $AgNO_3$ (figure 5C, D, E and F). Such nanoparticles presented larger sizes (between 100 and 400 nm) than that previously produced by Mafune and co-workers, with a size around 10 nm (Mafune *et al*. 2000) or the ones produced by Guzman and collaborators, that had a size around 20 nm (Guzman *et al*. 2011). Nevertheless, the size of silver nanoparticles depends both on the process used for their production and the reagents used in the procedure. Therefore, it is difficult to establish a comparison between this work and others. Moreover, in the present study nanoparticles aggregates were also observed which can lead to a loss of the antibacterial activity (Kvítek *et al*. 2008).

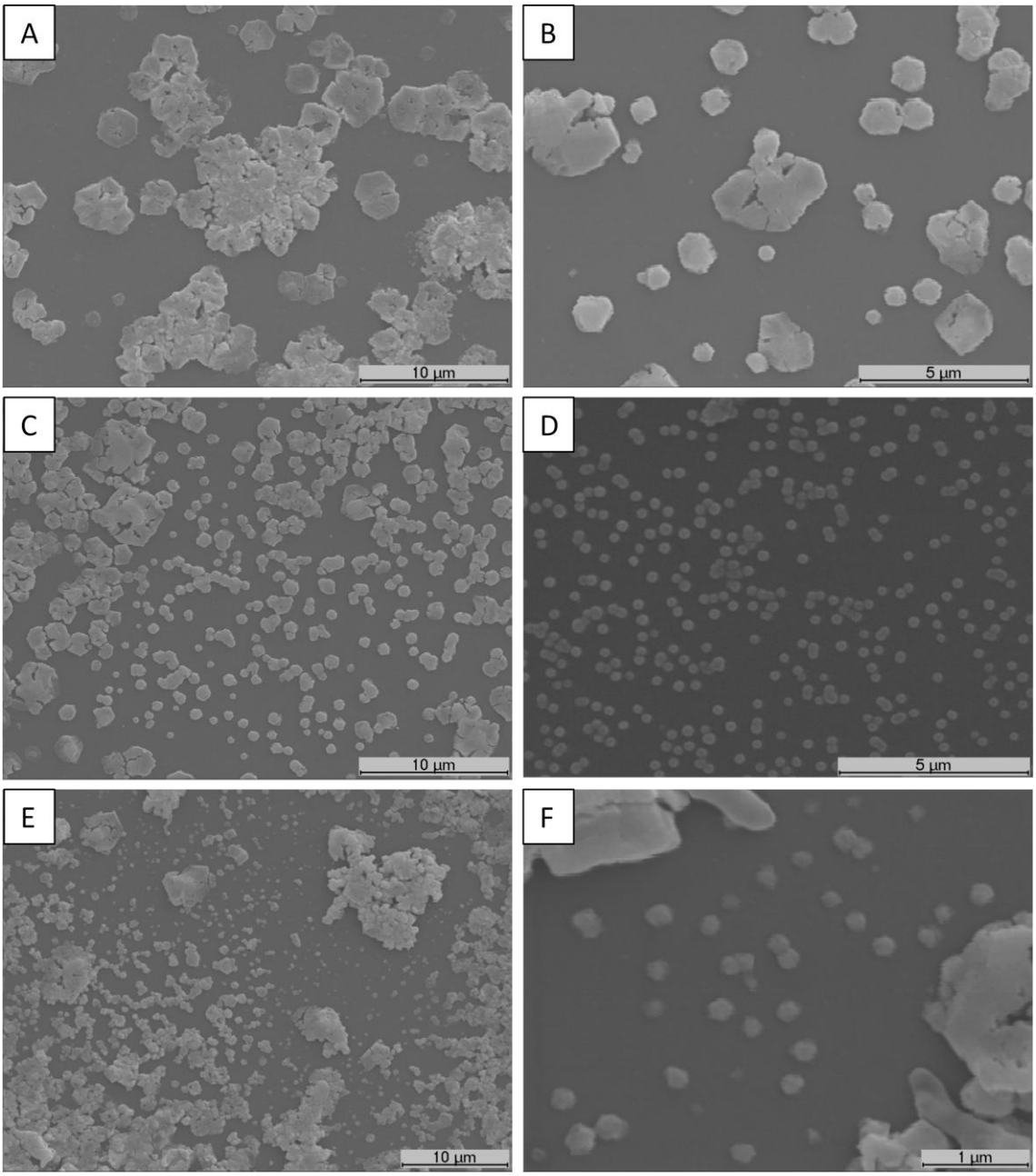

Figure 5 – SEM images of AgNPs produced by chemical reduction with $NaBH_4$, with a concentration of $AgNO_3$ of 0.1 M (A); 0.001 M (B); 0.01 M (C and D); and AgNPs produced by chemical reduction with $C_6H_8O_6$ with a concentration of $AgNO_3$ of 0.01 M (E and F).

According to Badway and colleagues, the high surface area to volume ratio of the nanoparticles, results in a high reactivity which leads to particle aggregation and settling. Such events can be avoided if particles are protected by a capping agent, like surfactants and polymers, that provide colloidal stability through electrostatic or steric repulsion (Badawy *et al*. 2010). Therefore, the second step of this work was to produce chitosan/dextran nanoparticles. These type of nanoparticles were produced by ionotropic gelation between the negatively

charged sulfate groups of dextran and the positively charged amine groups of chitosan (Anitha *et al*. 2011).

The chitosan/dextran nanoparticles were first produced by using chitosan of low molecular weight combined with dextran of both low and high molecular weight. Then, chitosan of high molecular weight was also combined with dextran of low and high molecular weight. In both conditions different ratios of dextran/chitosan were used.

All the produced particles were analyzed by SEM and it was possible to verify that when chitosan of low molecular weight was used, no particles were obtained, independently of the dextran's molecular weight or the ratio of chitosan/dextran used. Such events can be observed in figure 6.

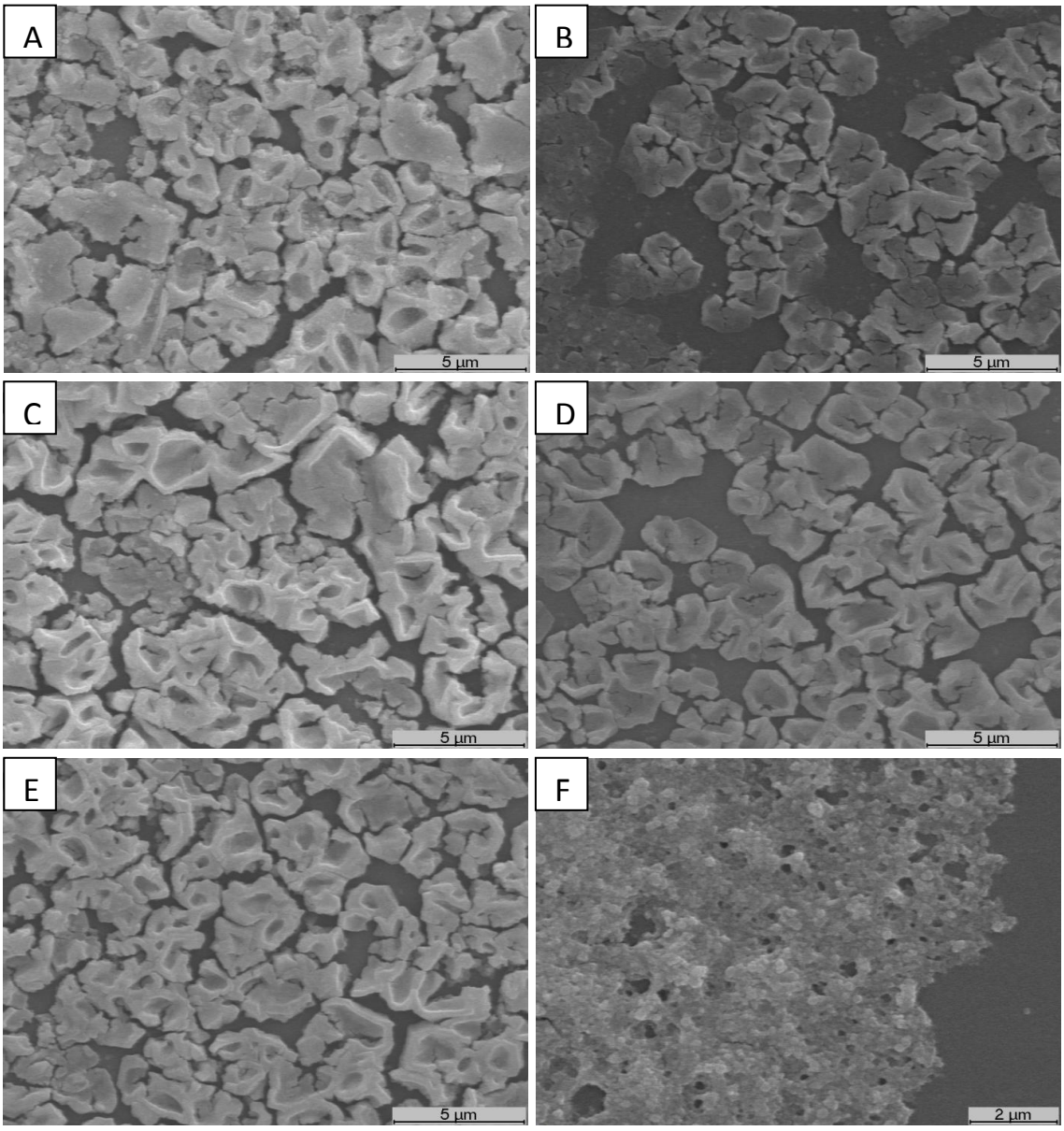

Figure 6 – SEM images of chitosan/dextran nanoparticles produced with low molecular weight chitosan and with high and low molecular weight dextran respectively, using different proportions: 1:6 (v/v) ratio (A and B); 1:3 (v/v) ratio (C and D); 2:3 (v/v) ratio (E and F).

When using chitosan of high molecular weight, several particles were formed for some specific dextran/chitosan ratios like 1:6 and 1:3 for high molecular weight dextran (figure 7 A and C) and 2:3 for both high and low molecular weight dextran (figure 7 E and F). In figures 7 E and F are depicted the nanoparticles with a highly aggregated structure. Such assembly was caused by a strong interaction between polyions that occur rapidly by random incorporation of different polymeric chains into the particle structure (Sarmento *et al*. 2007). The finest particles were obtained with high molecular weight dextran at 1:6 (v/v) ratio of dextran/chitosan (figure 7A). These particles were polydispersed and presented a spherical morphology with diameters ranging from 150 nm to 300 nm. The use of polymers with high molecular weight to perform nanoparticles can be an advantage for the posterior production of stabilized AgNPs. A study reported by Shkilnyy and colleagues showed that increasing the molecular weight of the polymer facilitates the formation of AgNPs, stabilizing therefore the colloids (Shkilnyy *et al*. 2009). A summary of the chitosan/dextran ratios where the production of nanoparticles was achieved is presented in the table 2.

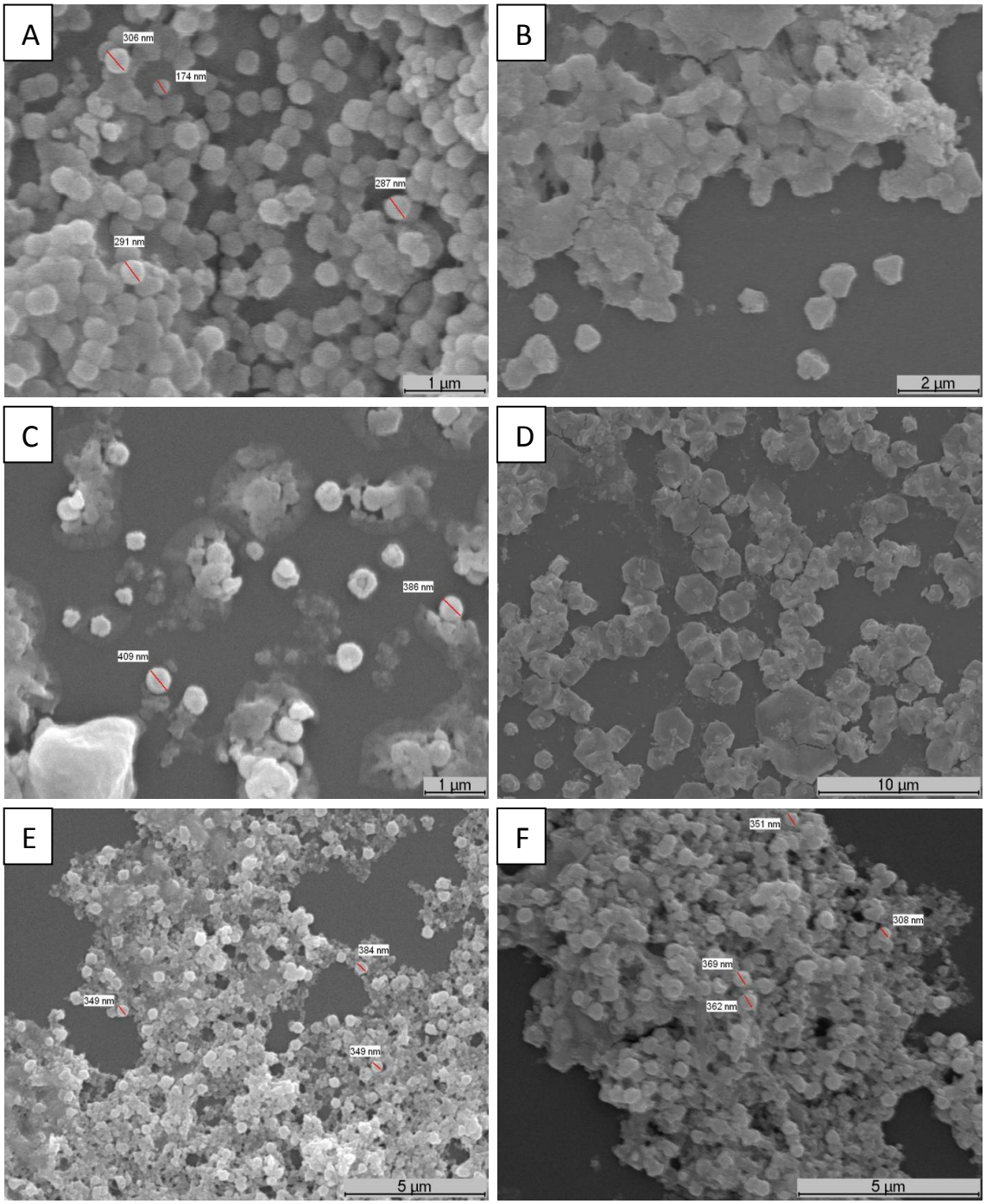

Figure 7 – SEM images of chitosan/dextran nanoparticles produced with high molecular weight chitosan, with high and low molecular weight dextran respectively, using different proportions:1:6 (v/v) ratio (A and B); 1:3 (v/v) ratio (C and D); 2:3 (v/v) ratio (E and F).

Table 2 – Formation of chitosan/dextran nanoparticles for different ratios.

Chitosan (molecular weight)	Dextran (molecular weight)	Ratio (v/v)	Formation of nanoparticles
Low	High	1:6	No
Low	Low	1:6	No
Low	High	1:3	No
Low	Low	1:3	No
Low	High	2:3	No
Low	Low	2:3	No
High	High	1:6	Yes
High	Low	1:6	No
High	High	1:3	Yes
High	Low	1:3	No
High	High	2:3	Yes
High	Low	2:3	Yes

After performing all this evaluations, the next step was the stabilization of the AgNPs with chitosan/dextran nanoparticles through the combination of the two previous nanoparticles (AgNPs and chitosan/dextran nanoparticles). The AgNPs synthesized either with $C_6H_8O_6$ or with $NaBH_4$ were added to the recently synthesized chitosan/dextran nanoparticles with high molecular weight chitosan and dextran (1:6 (v/v) of dextran/chitosan). AgNPs were also synthesized in the chitosan/dextran nanoparticles, where $AgNO_3$ was incorporated into chitosan/dextran nanoparticles and then it was reduced either with $C_6H_8O_6$ or with $NaBH_4$ in order to produce the AgNPs. In order to verify particles formation and morphology, SEM analysis were performed (figure 8). In figure 8A and B it can be observed images of the nanoparticles that were produced by the combination of chitosan/dextran nanoparticles and AgNPs produced with $NaBH_4$ and $C_6H_8O_6$, respectively. These particles presented a spherical morphology with diameters around 200-300 nm. In this case, the stabilization effect was achieved due to the presence of the chitosan/dextran nanoparticles that sterically hindered the formation of aggregates (Shkilnyy *et al*. 2009). In figures 8 C and D are represented the AgNPs produced in the chitosan/dextran nanoparticles reduced with $NaBH_4$ or with $C_6H_8O_6$, respectively. It can be observed that particles in figure 8 C and D were not polydispersed and appeared as aggregates. Notwithstanding, in figure 8 C is depicted bigger particles surrounded by smaller ones. Such feature can be caused by the formation of AgNPs on the surface of chitosan/dextran nanoparticles. However, additional experiments are necessary to prove this idea and to improve the production of these particles while avoiding their aggregation.

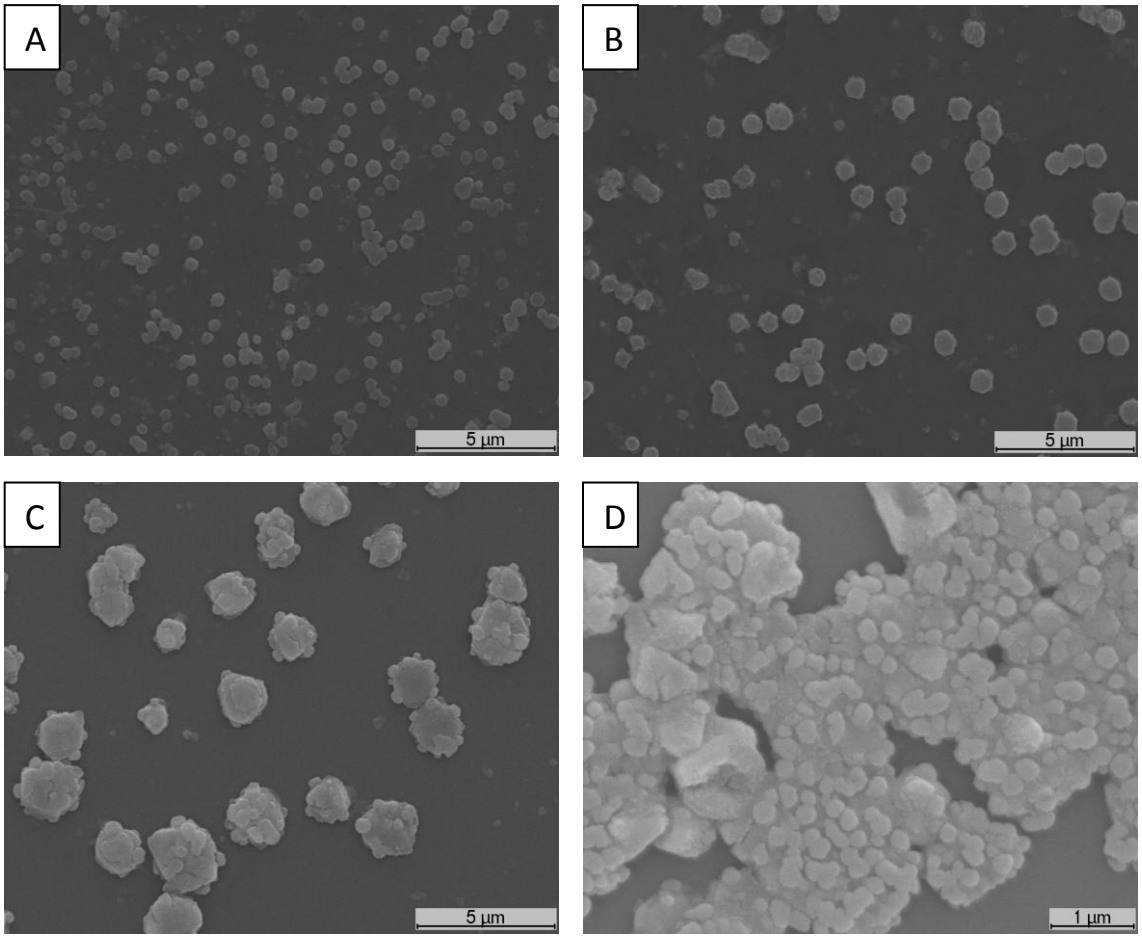

Figure 8 – SEM images of: chitosan/dextran nanoparticles with AgNPs produced with $NaBH_4$ (A) and with $C_6H_8O_6$ (B); AgNPs produced in chitosan/dextran nanoparticles with $NaBH_4$ (C) and with $C_6H_8O_6$ (D).

3.2. Nanoparticles Ultraviolet-Visible Spectroscopy Analysis

UV-Vis spectroscopy is a valuable tool for structural characterization of AgNPs (Guzmán *et al*. 2009; Shameli *et al*. 2010; Guzman *et al*. 2011). It is well known that the optical absorption spectra of metal nanoparticles are dominated by surface plasmon resonances (SPRs) that shift to higher wavelengths with increasing particle size (Guzman *et al*. 2011). The dispersions of AgNPs display intense colors due to the plasmon resonance absorption (Guzmán *et al*. 2009). SPR is a collective excitation of the electrons in the conduction band near the surface of the nanoparticles when they absorb energy of a certain wavelength (Guzmán *et al*. 2009; Guidelli *et al*. 2011). Electrons are limited to specific vibrations modes by the particle's size and shape (Guzmán *et al*. 2009) therefore, metallic nanoparticles have characteristic optical absorption spectrums in the UV-Vis region (Guzmán *et al*. 2009). The characteristic of the SPR bands of AgNPs appear around 400 nm (Shameli *et al*. 2010; Fuertes *et al*. 2011; Guzman *et al*. 2011), which corresponds to AgNPs with a size around 10 nm (Shameli *et al*. 2010) and with spherical shape (Guzman *et al*. 2011). In general, the number of SPR peaks decreases as the symmetry of the nanoparticle increases (Guzman *et al*. 2011). The position and shape of the plasmon

absorption depends on particles size, shape and the dielectric constant of the surrounding medium (Guzmán *et al*. 2009).

In order to verify the concentration of $AgNO_3$ that produced the highest number of AgNPs, the absorption spectra of the AgNPs were obtained and are presented in Figure 9. All samples presented the characteristic surface plasmon of AgNPs (peak between 400 and 420 nm) with the exception of the spectrum of high concentrations (green line) where no AgNPs were produced. It was also observed that for lower concentrations (black line) the absorbance is reduced indicating the presence of few AgNPs. Such was also confirmed through SEM images. Based on these results, it was concluded that the intermediate concentration of $AgNO_3$ was the best to produce the AgNPs. Recently, it was reported by Martinez-Castanon that the absorption spectrum of spherical AgNPs present a maximum absorbance band between 420 and 450 nm with a blue or red shift with particle size decreasing or increasing, respectively (Martinez-Castanon *et al*. 2008). These observations showed that the reducing agent $NaBH_4$ produced smaller nanoparticles than that produced with $C_6H_8O_6$. In the same study, Martinez-Castanon reported that the width of each plasmon is related to the size distribution. As referred above, large peaks indicate a large size distribution of nanoparticles, and for irregular particles (non spherical), two or more plasmon bands are expected depending on the symmetry of the particles (Martinez-Castanon *et al*. 2008). Therefore, the nanoparticles herein produced with $C_6H_8O_6$ were less uniform in their size and less symmetric compared to that produced, using $NaBH_4$.

The spectra of the samples obtained by UV-Vis spectroscopy are in agreement with those previously reported in literature (Kim *et al*. 2007; Guzmán *et al*. 2009; Vasilev *et al*. 2010; Guidelli *et al*. 2011). For instance, Kim and colleagues observed an absorption band at 391 nm for AgNPs (Kim *et al*. 2007). In the work of Guidelli and coworkers a characteristic absorption peak at 435 nm of AgNPs was observed (Guidelli *et al*. 2011). A study performed by Guzmán and collaborators demonstrated that the peak of the AgNPs was around 418 nm (Guzmán *et al*. 2009). Vasilev and collaborators observed a peak around 410 nm for AgNPs (Vasilev *et al*. 2010). In own study, an absorption peak around 400 nm was also observed, in accordance to previous studies.

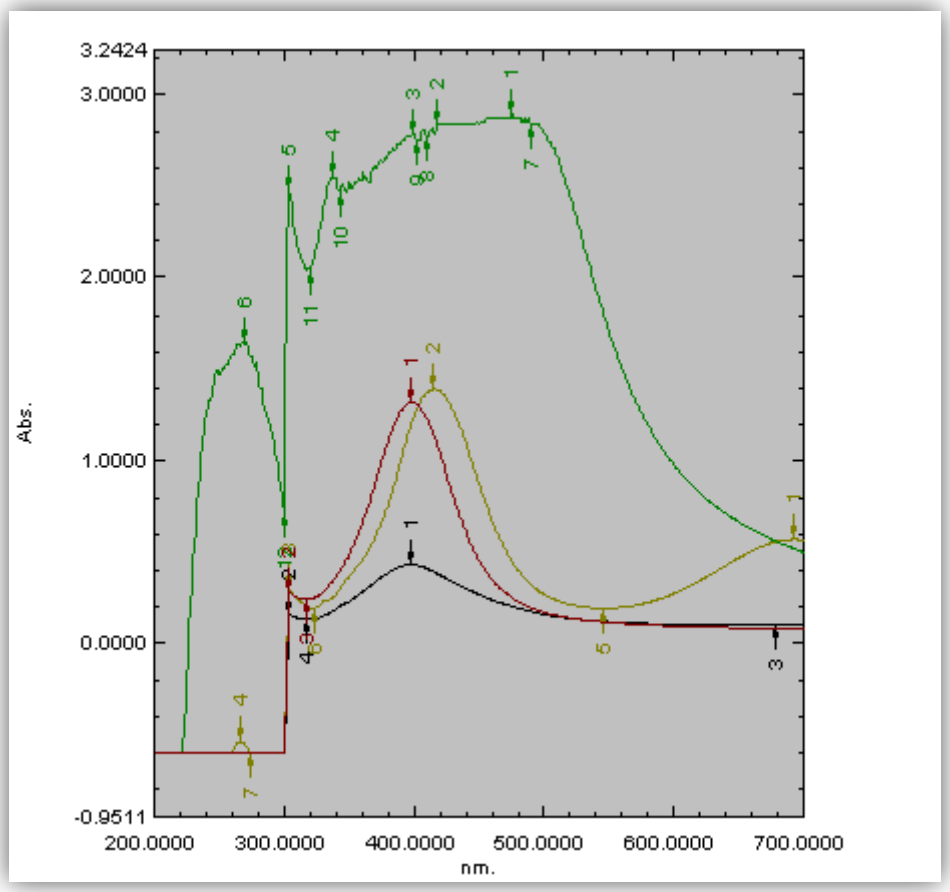

Figure 9 – UV-Vis spectra of the produced AgNPs with different concentrations of $AgNO_3$: black line – 0.001 M $AgNO_3$ with $NaBH_4$; red line – 0.01 M $AgNO_3$ with $NaBH_4$; yellow line – 0.01 M $AgNO_3$ with $C_6H_8O_6$; green line – 0.1 M $AgNO_3$ with $NaBH_4$.

3.3. Nanoparticles Fourier Transform Infrared Spectroscopy Analysis

FT-IR spectroscopy reveals information about the molecular structure of chemical compounds and is useful for the characterization of biopolymers (Lawrie *et al*. 2007). To further characterize the interaction of the chitosan with dextran in the produced chitosan/dextran nanoparticles different FT-IR spectra were performed on chitosan, dextran and chitosan/dextran nanoparticles.

In figure 10 the blue and green lines show the representative spectra of the chitosan and dextran, separately, featuring the main vibrational bands of chitosan and dextran respectively. In the case of chitosan, the spectra demonstrated a peak at 3354.09 cm^{-1}. This peak is within the range of 3300 and 3430 cm^{-1} which can be assigned for the O-H stretching vibration and the N-H extension vibration of the polysaccharide moieties of chitosan (Anitha *et al*. 2009; Das *et al*. 2010; González-Campos *et al*. 2010; Reicha *et al*. 2012). Another characteristic peak was found at 1651.73 cm^{-1} that belongs to amide I band that results of the C=O stretching of *N*-acetyl group

of chitosan (Anitha *et al*. 2009; Delair 2011; Potara *et al*. 2011). One peak at 1585.68 cm^{-1} was observed and corresponds to the NH_3^+ deformation in the chitosan (Delair 2011).

In dextran spectrum was verified a peak at 2950.07 cm^{-1} that corresponds to a C–H stretching (Kumar *et al*. 2012). The peak at 1636.30 cm^{-1} was also observed and corresponds to the sulfate asymmetric stretching (Delair 2011). Another peak was found at 1220.72 cm^{-1} that belongs to S=O vibrations (Delair 2011).

When examining FT-IR spectrum of chitosan/dextran nanoparticles, changes in the amine and sulfate absorption bands were detected (figure 10 red). These spectral changes were attributed to the electrostatic interaction between the chitosan amine and dextran sulfate groups (Anitha *et al*. 2011). The formation of the chitosan/dextran nanoparticles was noticed by the appearance of a specific band at 1531.68 cm^{-1} that was also observed by Delair and coworkers (Delair 2011). The band at 1220.72 cm^{-1} in the dextran spectrum, broadened and was separated into two peaks along with the complexation process, as already observed by Delair and coworkers (Delair 2011). Tyaboonchai and collegues reported that the complex formation by a shift of the N-H bending adsorption at 1652 cm^{-1} and 1599 cm^{-1} for chitosan, to 1623 cm^{-1} in the chitosan/dextran nanoparticles was consistent with the presence of electrostatic interaction (Tiyaboonchai *et al*. 2007; Delair 2011). Such phenomenon was also herein observed, the appearance of a band at 1633.41 cm^{-1} in the chitosan/dextran nanoparticles spectrum.

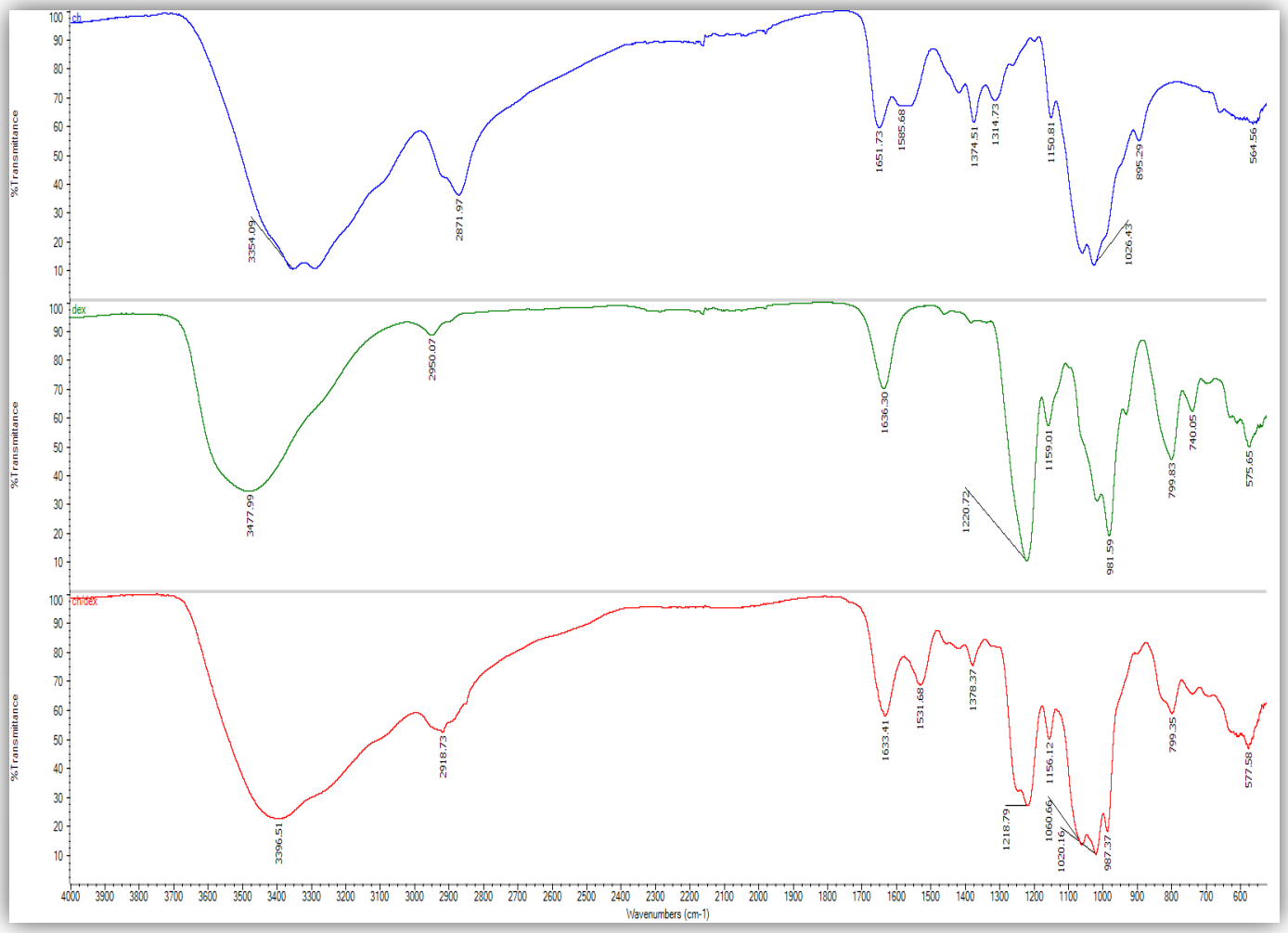

Figure 10 – FT-IR spectra of high molecular weight chitosan (blue), high molecular weight dextran (green), and chitosan/dextran nanoparticles (red).

FT-IR analysis was carried out to identify possible interactions between $AgNO_3$ and AgNPs with the produced chitosan/dextran nanoparticles, which could contribute for stabilizing AgNPs (Wei *et al*. 2008).

The spectra of the AgNPs and $AgNO_3$ with chitosan/dextran nanoparticles (figure 11) exhibited few alterations when compared to that of chitosan/dextran nanoparticles. Such feature was also observed by Reicha and colleagues when they produced AgNPs with chitosan (Reicha *et al*. 2012). This is probably due to the much weaker interaction between the AgNPs or Ag^+ with the chitosan/dextran nanoparticles (Bozanic *et al*. 2010).

In the spectra of the chitosan/dextran nanoparticles with AgNPs (figure 11 green and brown) and the spectra of the chitosan/dextran nanoparticles with $AgNO_3$ and $NaBH_4$ or $C_6H_8O_6$ (figure 11 red and yellow) a new peak appeared at 2849.79 cm^{-1}. By increasing $AgNO_3$ concentration, the peak became more intense, especially when using $NaBH_4$ as a reducing agent.

There was also reported an appearance of a peak at 1740 cm^{-1}, indicating that $AgNO_3$ was bounded to the functional groups of the chitosan (Saifuddin *et al*. 2011). Once again, this peak

was more intense with the increasing of the $AgNO_3$ concentration and if the reducing agent is, again, $NaBH_4$.

Another difference between the chitosan/dextran nanoparticles alone and these with silver in general (both $AgNO_3$ and AgNPs) was the change in the relative intensities of the infrared bands located around 1530 cm^{-1}. Such band appeared due to the specific interaction between chitosan and dextran. Therefore, considering a change in the intensity of such band, it can be depicted that silver (in general) interacts with the complex chitosan/dextran.

The peak at 3300–3430 cm^{-1} for the chitosan/dextran nanoparticles spectrum has showed a relative decrease of transmittance when $AgNO_3$ and AgNPs were attached to them, indicating that the N-H vibration of chitosan (characteristic of this spectrum) was affected due to the attachment of the NH_2 groups of chitosan with the Ag^+ during the electrochemical process (Reicha *et al*. 2012). Also, a relative reduction in the intensity of the peak at 1630 cm^{-1} has been noted due to the deformation vibration of the amine groups of chitosan (Ali *et al*. 2011; Reicha *et al*. 2012).

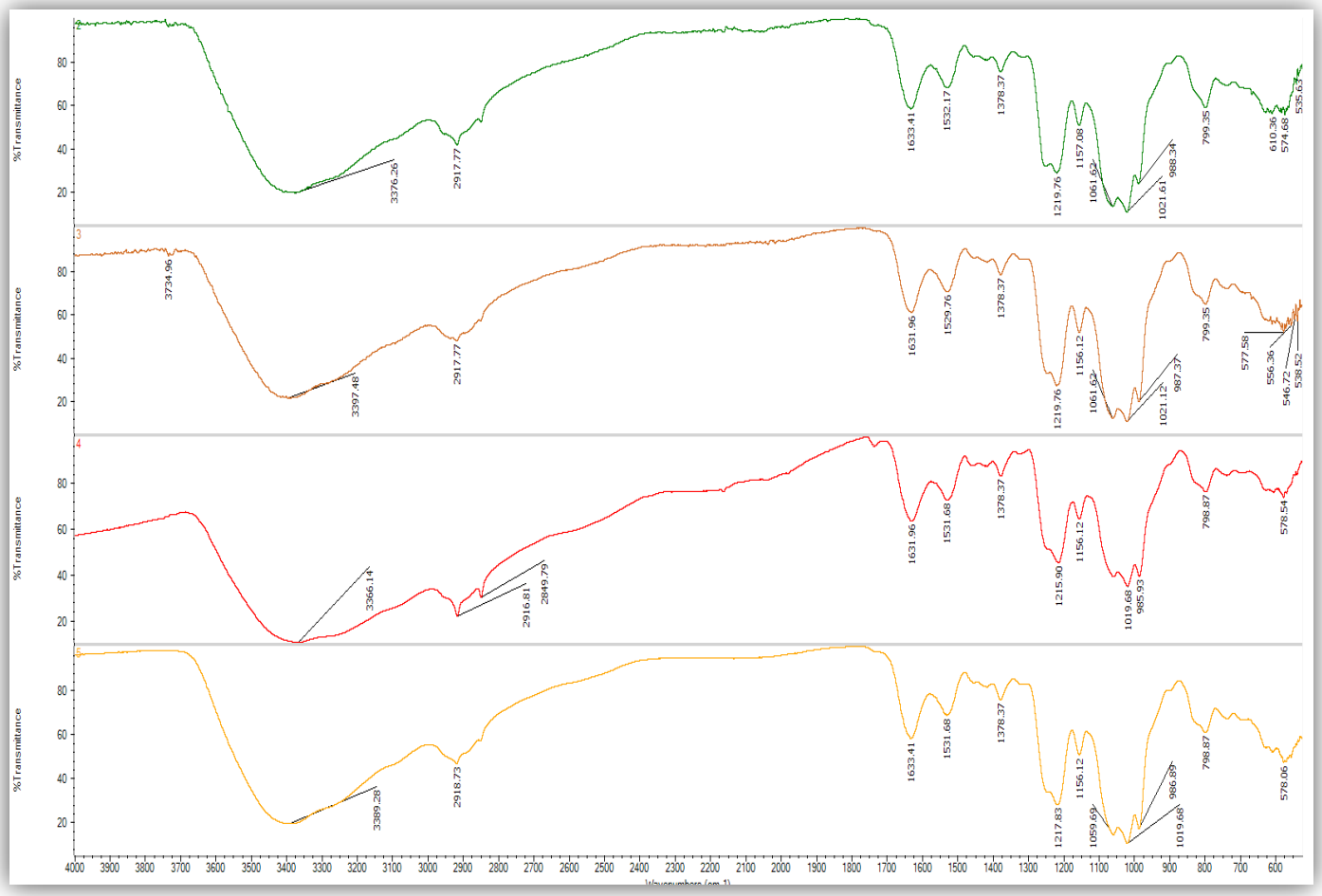

Figure 11 – FT-IR spectra of chitosan/dextran nanoparticles with AgNPs produced with $NaBH_4$ (green), chitosan/dextran nanoparticles with AgNPs produced with $C_6H_8O_6$ (brown), chitosan/dextran nanoparticles with $AgNO_3$ and $NaBH_4$ (red), and chitosan/dextran nanoparticles with $AgNO_3$ and $C_6H_8O_6$ (yellow).

3.4. Evaluation of the antibacterial activity of the produced nanoparticles

The antibacterial properties of the produced nanoparticles were evaluated against one bacterial strain a Gram-negative *E. coli*. The bacterial strain was deemed appropriate for testing the antibacterial properties of the nanoparticles since it has been reported to be the most common Gram-negative pathogen found in biomaterial-associated infections (Juan *et al*. 2010).

The antibacterial effects of the produced nanoparticles were evaluated using the MIC and MBC, which are the standard microbiological procedures used to evaluate the bacteriostatic and bactericidal properties of antimicrobial agents.

As already referred in the previous chapter, to determine MIC values, the nanoparticles were diluted 8 times with 100 µL of LB Broth inoculated with the tested bacteria at a concentration of 2.5×10^5 CFU/mL. The MIC was analyzed after 24 h of incubation, at 37 °C, based on culture turbidity. In cases where the nanoparticles affected the turbidity, rezazurin was used to determine the MIC value. The MIC was determined as the lowest concentration that inhibited the visible growth of the bacteria (Guzmán *et al*. 2009). Control bactericidal tests of chitosan and $AgNO_3$ solutions were also performed. To verify the reproducibility of the results, all antibacterial activity tests were performed in triplicate in three different days. The MBC was also determined by an imprinted method in an agar plate and it was also analyzed after 24 h of incubation, at 37 °C, based on the absence of colonies on the agar plate. The results of the tests of MIC and MBC of the nanoparticles against Gram-negative bacteria are listed in table 3 and 4, respectively. Figure 12 shows a representative experiment for the determination of the MIC and MBC.

Table 3 – MIC obtained for the different tested nanoparticles.

Materials tested	**MIC (µg/mL)**
Chitosan/Dextran nanoparticles	107.100
Chitosan solution	62.500
Chitosan/Dextran nanoparticles + AgNPs ($NaBH_4$)	1.500
Chitosan/Dextran nanoparticles + AgNPs ($C_6H_8O_6$)	3.000
Chitosan/Dextran nanoparticles + $AgNO_3$ + $C_6H_8O_6$	11.750
Chitosan/Dextran nanoparticles + $AgNO_3$ + $NaBH_4$	23.500
AgNPs ($NaBH_4$)	10.000
AgNPs ($C_6H_8O_6$)	>10.000
$AgNO_3$	15.625

Table 4 –MBC obtained for the different tested nanoparticles.

Materials tested	MBC (µg/mL)
Chitosan/Dextran nanoparticles	>107.100
Chitosan solution	>62.500
Chitosan/Dextran nanoparticles + AgNPs ($NaBH_4$)	3.000
Chitosan/Dextran nanoparticles + AgNPs ($C_6H_8O_6$)	3.000
Chitosan/Dextran nanoparticles + $AgNO_3$ + $C_6H_8O_6$	23.500
Chitosan/Dextran nanoparticles + $AgNO_3$ + $NaBH_4$	94.370
AgNPs ($NaBH_4$)	>10.000
AgNPs ($C_6H_8O_6$)	>10.000
$AgNO_3$	15.625

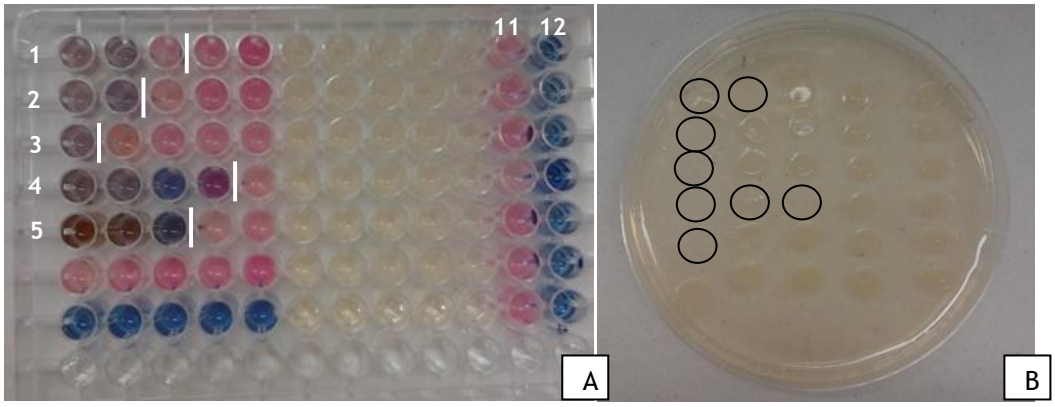

Figure 12 – Determination of MIC and MBC by microdilution method in microplate. (A) Image of the microplate for the determination of the MIC of the nanoparticles in different lines. Chitosan/dextran nanoparticles (line 1), chitosan/dextran nanoparticles with AgNPs ($NaBH_4$) (line 2), chitosan/dextran nanoparticles with AgNPs ($C_6H_8O_6$) (line 3), chitosan/dextran nanoparticles with $AgNO_3$ and with $NaBH_4$ (line 4), and chitosan/dextran nanoparticles with $AgNO_3$ and with $C_6H_8O_6$ (line 5). A positive control with *E. coli* and a negative control with the culture medium are also presented in the columns 11 and 12 respectively. (B) Image of the agar plate used to determine the MBC of the respective nanoparticles in the microplate.

Due to their small size, AgNPs produced by the reduction of $NaBH_4$ presented a MIC value of 10 µg/mL. Those produced by using $C_6H_8O_6$ as reduction agent presented higher values for this assay, due to their bigger size. This fact can be a disadvantage for the antibacterial activity, since smaller particles have a larger surface area available for interaction, which enhances the contact with the bacteria (Martinez-Castanon *et al.* 2008; Neal 2008; Raffi *et al.* 2008; Guzmán *et al.* 2009; Monteiro *et al.* 2009; Reicha *et al.* 2012). Nevertheless, $NaBH_4$ is toxic for human cells, which makes $C_6H_8O_6$ more adequate to be used in the organism. There are several reports in the literature referring to the antibacterial activity of AgNPs. For instance, Kvítev and collaborators prepared AgNPs by the reduction of diamminesilver ions ($[Ag(NH_3)_2]^+$) with D-maltose obtaining nanoparticles with 26 nm, with a MIC value of 1.69 µg/mL against *E. coli* (Kvítek *et al.* 2008). Another study reported that 10 nm AgNPs inhibit growth of *E. coli*, with a

concentration of 2.5 µg/mL (Vertelov *et al*. 2008). The results of antibacterial activities of AgNPs using $AgNO_3$ reduced with hydrazine hydrate against *E. coli* was 6.74 µg/mL (Guzman *et al*. 2011). The MIC value of AgNPs with 5 nm against *E. coli* was 10 µg/mL (Li *et al*. 2010). The AgNPs solution prepared by the reduction with glucose at the concentration of 3 µg/mL inhibited *E. coli* growth (Lkhagvajav *et al*. 2011). The different reported values for the antimicrobial activity of AgNPs can be influenced by the preparation method used as well as by particle size, strain employed and initial bacterial concentration (Raffi *et al*. 2008; Lkhagvajav *et al*. 2011). Furthermore, other fact that can affect the antibacterial activity of AgNPs is the culture medium used to perform the MIC assay. The medium used in this study was LB broth, which may be involved in the precipitation of the released Ag^+, in the form of insoluble silver chloride (AgCl). This form of silver is not available to interact with the bacteria reducing, therefore the antibacterial capacity (Guzman *et al*. 2011). Thus, the direct comparison between different studies is not totally feasible (Ruparelia *et al*. 2008). However, the MIC value of AgNPs produced with $NaBH_4$ was in agreement with the study previously reported by Li and collaborators (Li *et al*. 2010).

In this study, the AgNPs were also produced with chitosan/dextran nanoparticles, as stabilizers agents. Therefore, the antibacterial activity of these particles was also evaluated. The results showed that these nanoparticles presented antibacterial activity (MIC value of 107.1 µg/mL), which was due to the interaction of chitosan amine groups with the bacteria anionic components, such as LPS and bacteria surface proteins, resulting in the inhibition of bacterial growth (Kumirska *et al*. 2011). However, the MIC values determined were higher than those obtained for the chitosan alone. Such occurrence can be explained by the interactions between the negatively charged sulfate groups of dextran and the positively charged amine groups of chitosan, which is responsible for the reduction of the total positive charge available to interact with bacteria. Still, these results showed that the produced chitosan/dextran nanoparticles can also contribute to enhance the antibacterial activity of AgNPs.

Previous studies reported that several polymers have been used as stabilizer agents of AgNPs to prevent their aggregation which is responsible for the loss of the inhibitory effect on bacterial growth (Levard *et al*. 2012). In one study performed by Lkhagvajav and colleagues the antimicrobial activity of AgNPs prepared by using various stabilizers, such as sodium dodecylsulfate (SDS) and PVP, was also studied and the MIC values against Gram-negative (*E. coli*) were determined (10 µg/mL) (Lkhagvajav *et al*. 2011). Nevertheless, the effect of the stabilization of AgNPs with polymers could improve, worsen or not even affect the antibacterial activity of such particles. In a study of Potara and co-workers chitosan was used to promote stabilization and decreased the aggregation potential, which in turn increased the effective concentration of particles capable of interact with the bacterial cellular surface (Potara *et al*. 2011). In that study, the MIC values obtained against *S. aureus* were around 4 µg/mL. This low value resulted from the synergic effect between silver and chitosan (Potara *et al*. 2011). However, in another study, AgNPs with some capping agents showed to be less bioactive, since these agents hinder the release of Ag^+ (Marambio-Jones *et al*. 2010).

The combination of the two types of nanoparticles (AgNPs and chitosan/dextran nanoparticles) herein produced provided results for MIC as lower as 1.5 μg/mL with AgNPs produced by $NaBH_4$ reduction, and 3.0 μg/mL with AgNPs produced by $C_6H_8O_6$ reduction. Still, AgNPs produced into chitosan/dextran nanoparticles with $NaBH_4$ or with $C_6H_8O_6$ presented a higher MIC value, 23.50 μg/mL and 11.75 μg/mL respectively, compared to those produced by the combination of both types of nanoparticles. The AgNPs produced into chitosan/dextran nanoparticles were too big, which can be a disadvantage in terms of the antibacterial activity, as already reported for AgNPs. The combination of both nanoparticles presented a better antibacterial activity than AgNPs alone. Therefore, such combination was an advantage to improve the antibacterial activity of AgNPs.

MBC values also determined in this work were in all cases, except for the chitosan/dextran nanoparticles with AgNPs ($C_6H_8O_6$), above of those obtained for MIC. Thereby, a higher concentration of $AgNO_3$ was needed to kill the bacteria. In this study, chitosan was not able to eradicate the bacteria. However, it was able to inhibit bacteria growth as observed through MIC assay. Hereupon, the ability to completely kill bacteria is due to the bactericidal effect of $AgNO_3$.

As mentioned in chapter I, the mechanism by which the AgNPs are able to interact with the bacteria is not fully understood (Kim *et al*. 2007; Raffi *et al*. 2008; Guzmán *et al*. 2009; Potara *et al*. 2011). However, there are some reports that showed that the AgNPs may attach on the surface of the cell membrane and disturb its power function, such as permeability and electron transport chain (Kim *et al*. 2007; Guzmán *et al*. 2009). AgNPs can also penetrated inside the bacteria and caused damage by interacting with phosphate and sulfur containing compounds, such as DNA (Raffi *et al*. 2008). Finally, the production of ROS by the AgNPs is also responsible for their antimicrobial mechanism (Kim *et al*. 2007; Potara *et al*. 2011). Even though the antibacterial mechanism of AgNPs is not totally understood, the antimicrobial effect of Ag^+ has been quite well known and it is routinely used in the pharmaceutical industry for wound healing and similar anti-infection-related applications (Parashar *et al*. 2011). These new AgNPs with chitosan/dextran nanoparticles showed to have biocidal effect and potential to reduce bacterial growth for practical applications.

3.5. Evaluation of the cytotoxic profile of the produced nanoparticles

Nowadays orthopedic surgery is extremely important and several biomaterials are used for bone regeneration (Campoccia *et al*. 2010). The coating of orthopaedic implants with AgNPs is an interesting strategy to decrease the postoperative infection rates (Albers *et al*. 2011). However, silver induced cytotoxicity to bone cells has not been investigated in detail (Albers *et al*. 2011). Therefore, in this study the cytotoxic profile of the different types of nanoparticles, herein

produced, was characterized using human osteoblast cells, through optical microscopy images and MTS assay.

There are several studies reporting different results about citotoxicity of AgNPs in different eukaryotic cells. The toxicity of these particles is described as being dependent of their size, shape, agglomeration and concentration (Samberg *et al*. 2010). For instance, the toxic effects of AgNPs have been reported in mammalian cells, including alteration of the normal function of mitochondria, the increase of membrane permeability, and the generation of ROS (Martinez-Gutierrez *et al*. 2010).

In previous studies AgNPs have been coated and others hybridized with other materials to form nanocomposites (Chen *et al*. 2008). Moreover, nanoparticulate colloids may need different stabilizers to avoid aggregation and prevent toxicity (Chen *et al*. 2008). All these features combined may probably modify the intrinsic physicochemical properties of silver and, therefore, give rise to modifications in cellular uptake, interaction with biological macromolecules and translocation within the human body (Chen *et al*. 2008). Additionally, there are different results for toxicity of coated AgNPs. For example, Samberg and coworkers reported that uncoated particles caused significant toxicity to human cells, while the carbon-coated particles had no adverse effects (Samberg *et al*. 2010). In this case, the toxicity of AgNPs was reduced due to the carbon coating that prevented the direct contact of the particle surface with cellular components, avoiding toxicity (Samberg *et al*. 2010). Cao and co-workers verified that the addition of chitosan to silver/chitosan composites avoid the toxicity of AgNPs (Cao *et al*. 2010). On the other hand, Li and coworkers reported that PVP-coated AgNPs are more toxic than the uncoated AgNPs (Li *et al*. 2011).

In this study, human osteoblast cells were seeded at the same initial density in 96 well plates, with nanoparticles, herein produced, at different concentrations (high, low and the MIC concentration and with the supernatant) and without nanoparticles in order to access their cytotoxicity. The different nanoparticles and their concentrations used are presented in table 5.

Table 5 – Different nanoparticles and their concentrations to perform the cytotoxic assays.

Nanoparticles	Identification	Low concentration (µg/mL)	MIC (µg/mL)	High concentration (µg/mL)
1	Chitosan/dextran nanoparticles	20.0000	107.1000	428.6000
2	Chitosan/dextran nanoparticles with AgNPs ($NaBH_4$)	0.3000	1.5000	6.0000
3	Chitosan/dextran nanopartices with AgNPs ($C_6H_8O_6$)	0,1875	3.0000	6.0000
4	Chitosan/dextran nanoparticles with $AgNO_3$ and $NaBH_4$	1,6243	11.7500	42.5000
5	Chitosan/dextran nanoparticles with $AgNO_3$ and $C_6H_8O_6$	1,6243	23.5000	42.5000
6	AgNPs ($NaBH_4$)	2,5000	10.0000	40.0000
7	AgNPs ($C_6H_8O_6$)	2,5000	10.0000	40.0000

Cell adhesion and proliferation was observed by using an inverted light microscope after 24 and 48 h (figure 13 and 14). In the negative control (K^-) cells were viable, appearing with stellate geometry and showing slender lamellar expansions that joined neighboring cells. In the positive control (K^+), dead cells were seen with their spherical characteristic form. For the cells in contact with the different concentrations of nanoparticles, no toxic effects were observed when nanoparticles in low or MIC concentrations were put in contact with cells during 24 and 48 h. This can be depicted in figure 13, where cells were similar to that of the K^+. For the high concentrations of nanoparticles almost no toxic effects were obtained, except for the particles presented in figure 14 A, 4 (Chitosan/dextran with $AgNO_3$ and with $C_6H_8O_6$ as reducing agent) and 5 (Chitosan/dextran with $AgNO_3$ and with $NaBH_4$ as reducing agent) that presented some dead cells. In the figure 14 B are presented the results obtained for the cells in contact with the supernatants obtained from the nanoparticles washing, in which some adhered cells and some dead cells were observed, indicating some toxic effects on the cells, during the period of exposure to the supernatants.

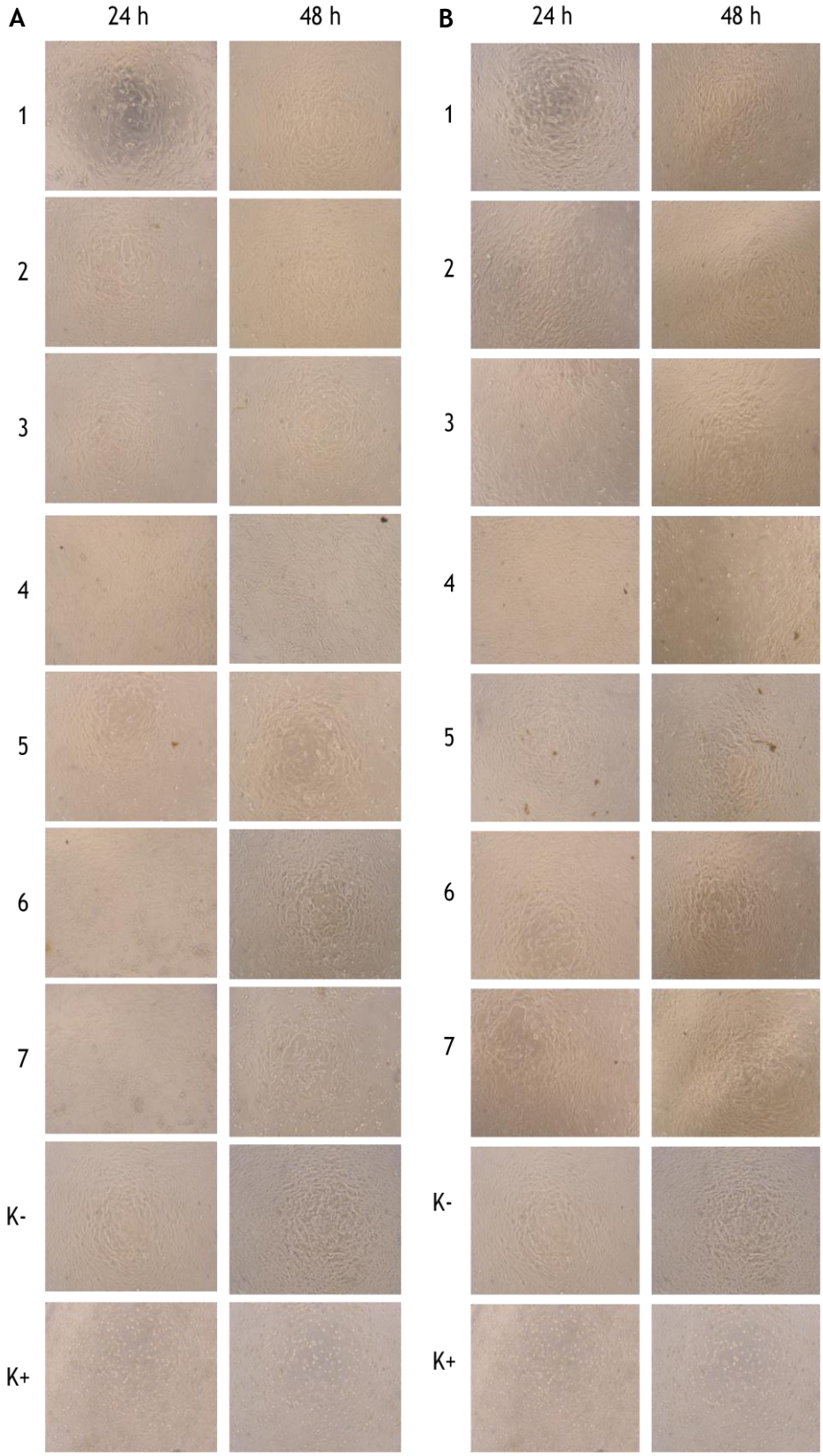

Figure 13 – Inverted Light Microscope Images of human osteoblast cells in contact with: chitosan/dextran nanoparticles (1); chitosan/dextran nanoparticles with AgNPs produced with $NaBH_4$ (2); chitosan/dextran nanoparticles with AgNPs produced with $C_6H_8O_6$ (3); chitosan/dextran nanoparticles with $AgNO_3$ and with $NaBH_4$ (4); chitosan/dextran nanoparticles with $AgNO_3$ and with $C_6H_8O_6$ (5); AgNPs produced with $NaBH_4$ (6); AgNPs produced with $C_6H_8O_6$ (7); in lower concentrations (A) and at MIC concentrations (B) after 24 and 48 h. Negative control (K^-) and positive control (K^+) are also presented for both cases. (×10)

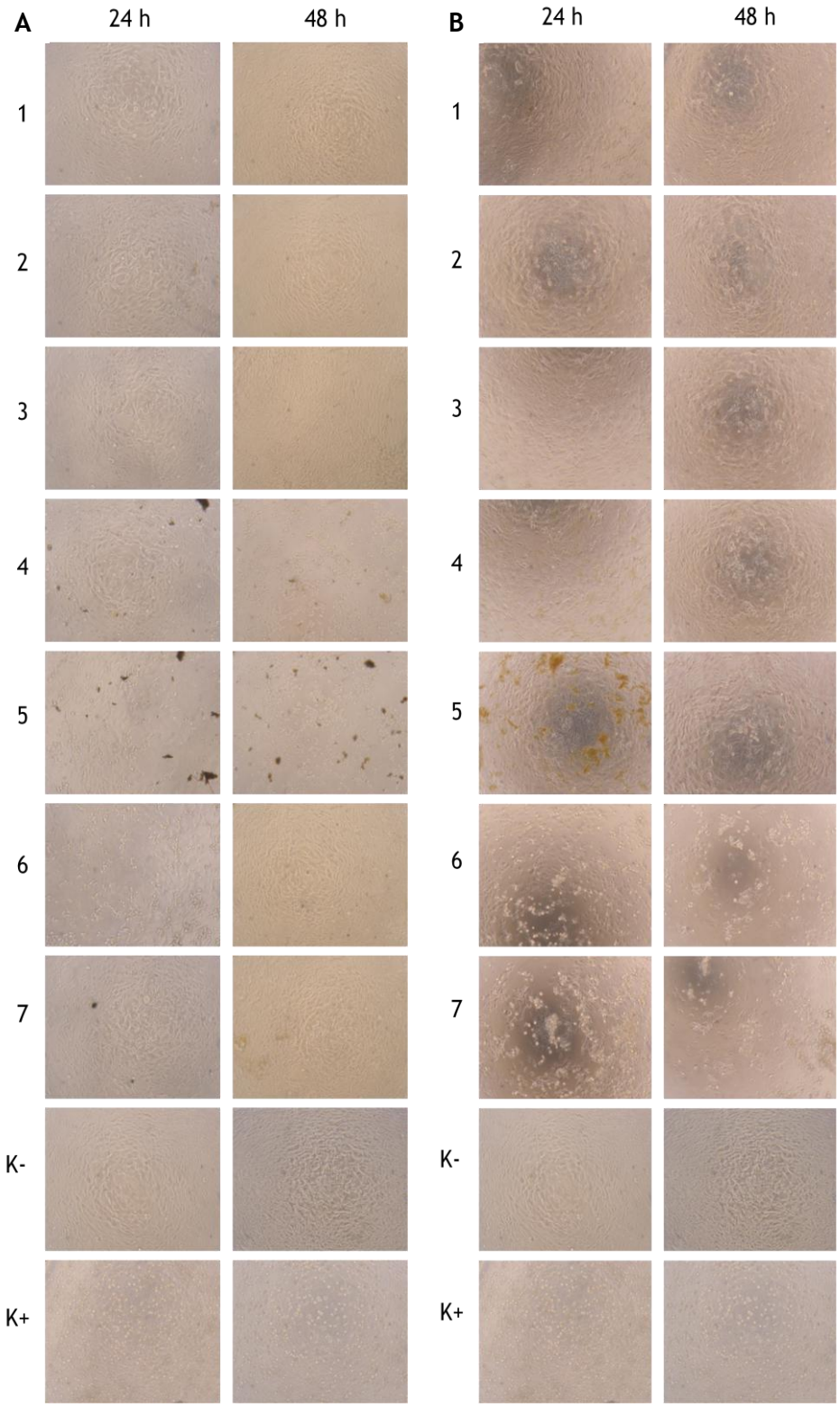

Figure 14 – Inverted Light Microscope Images of human osteoblast cells in contact with: chitosan/dextran nanoparticles (1); chitosan/dextran nanoparticles with AgNPs produced with $NaBH_4$ (2); chitosan/dextran nanoparticles with AgNPs produced with $C_6H_8O_6$ (3); chitosan/dextran nanoparticles with $AgNO_3$ with $NaBH_4$ (4); chitosan/dextran nanoparticles with $AgNO_3$ with $C_6H_8O_6$ (5); AgNPs produced with $NaBH_4$ (6); AgNPs produced with $C_6H_8O_6$ (7); in higher concentrations (A) after 24 and 48 h. The supernatants of the referred nanoparticles in contact with cells during 24 and 48 h are also presented (B). Negative control (K^-) and positive control (K^+) are also presented for both cases. (×10)

The MTS assay was also performed in order to quantify the cytotoxic effect of the different nanoparticles produced. The reagent MTS was reduced into a water-soluble brown formazan product. The absorbance of the formazan produced, is proportional to the number of cells whose mitochondrial metabolism is intact, after their exposure to nanoparticles (Mukherjee *et al*. 2011).

For all the experiments the negative control (K^-), in which cells were only seeded with culture medium, presented a high percentage of viable cells. Conversely, the positive control (K^+), in which ethanol solution was added to the cells, showed no cellular viability. The MTS assay performed on the chitosan/dextran nanoparticles alone (1), in different concentrations, showed that cells' viability was similar to the negative control, proving that this type of particles did not affect cell viability. These results are in agreement with a study performed by Anitha and collaborators, in which was shown that the chitosan/dextran nanoparticles had no toxicity for normal eukaryotic cells (Anitha *et al*. 2011). The toxic effects of AgNPs produced either by the reduction with $C_6H_8O_6$ (6) or $NaBH_4$ (7), were also evaluated. For these particles in low, MIC and high $AgNO_3$ concentrations (figure 15 A, B and C, respectively) the percentage of viable cells was similar to that of the K^-, reinforcing the idea that these $AgNO_3$ concentrations in the nanoparticles did not have toxic effects for the cells. The same was also observed for the remaining nanoparticles, but only for low and MIC concentrations of $AgNO_3$ (figure 15 A and B, respectively). However, for the high $AgNO_3$ concentrations (figure 15 C), nanoparticles labeled as 4 (Chitosan/dextran with $AgNO_3$ and $C_6H_8O_6$) and 5 (Chitosan/dextran with $AgNO_3$ and $NaBH_4$) showed toxic effects for the cells. Furthermore, nanoparticles labeled as 2 (chitosan/dextran nanoparticles with AgNPs produced with $NaBH_4$) and 3 (chitosan/dextran nanoparticles with AgNPs produced with $C_6H_8O_6$) showed no toxic effects. Such features are explained by the fact that the $AgNO_3$ concentration used to reach the MIC value was lower in particles 2 and 3, than in the particles 4 and 5. Such results confirm that nanoparticles toxicity is dependent on $AgNO_3$ concentration. This relation, of $AgNO_3$ concentration *versus* toxicity was already reported in two studies performed by Arora and collaborators, where they observed a decrease of the mitochondrial function of cells exposed to AgNPs (1.56–500 μg/mL) in a dose-dependent way (Arora *et al*. 2008; Arora *et al*. 2009). The analysis of the supernatants of the different nanoparticles (figure 15 D) showed that they were extremely toxic for cells, since the obtained values are quite similar to those of the K^+, after two days of test. Therefore, it can be depicted that some toxicity could actually be due to the presence of contaminants in solution instead of the nanoparticles *per se* (Samberg *et al*. 2010). Based on this results, particles' washing may represent a major issue in order to decrease some of the toxicity, that was previously reported has being caused by the nanoparticles.

The MTS assay showed a significant difference between the K^+ and the K^- cells exposed to the different nanoparticles after 24 and 48 h of incubation, for the low and MIC concentrations. These results confirmed that nanoparticles in low and MIC concentrations did not have any cytotoxic effect. For high concentrations, there was observed a cytotoxic effect for nanoparticles labeled as 4 and 5, after 48 h, since no significant difference was observed

between themselves and the K^+. For the supernatant of the nanoparticles there was no significant difference between themselves and the K^+, demonstrating that the supernatant was toxic for the cells.

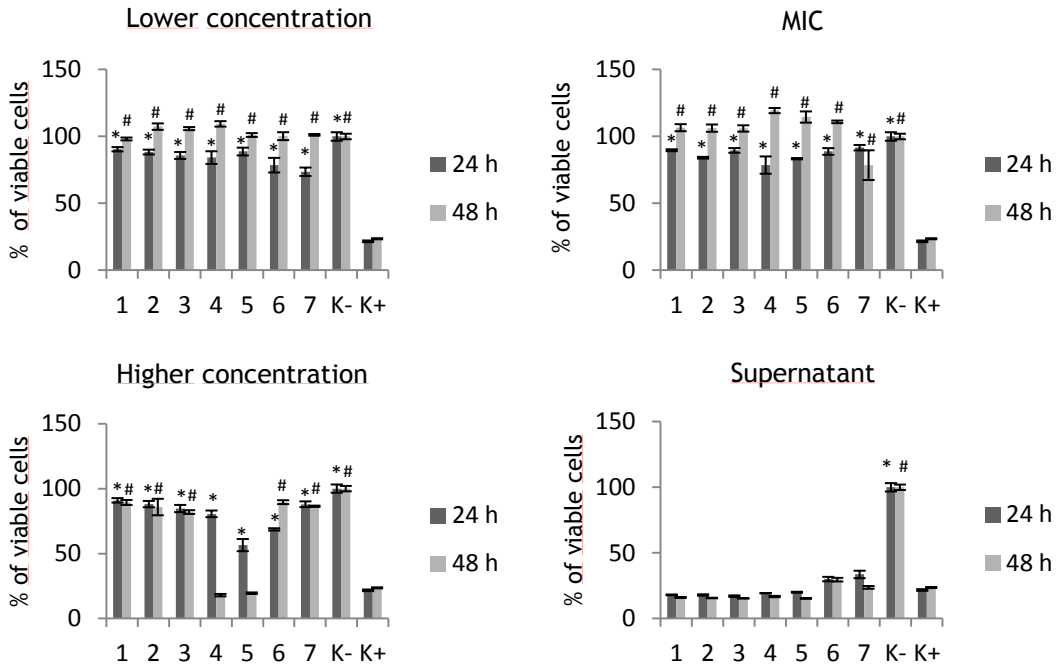

Figure 15 –Cell viability measured by the MTS assay after 24 and 48 h in contact with chitosan/dextran nanoparticles (1); chitosan/dextran nanoparticles with AgNPs ($NaBH_4$) (2); chitosan/dextran nanoparticles with AgNPs ($C_6H_8O_6$) (3); chitosan/dextran nanoparticles with $AgNO_3$ and with $NaBH_4$ (4); chitosan/dextran nanoparticles with $AgNO_3$ and with $C_6H_8O_6$ (5); AgNPs with $NaBH_4$ (6); AgNPs with $C_6H_8O_6$ (7); in lower concentrations (A), at MIC concentrations (B) and in higher concentrations (C). The cellular activity was also measured for supernatants of the referred nanoparticles after 24 and 48 h (D). Negative control (K^-) and positive control (K^+) are also presented for both cases. Each result is the mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Bonferroni t-test (*, $^\#p<0.05$).

Chapter IV

Conclusions and future perspectives

4. Conclusions and future perspectives

Silver is kwon for a long time as an agent with antimicrobial activity. However, with the appearance of antibiotics, silver's use decreased due to the high efficiency of antibiotics. With the onset of new bacterial strains resistant to several antibiotics, silver has regained its former usage when nanotechnology techniques started to be used. AgNPs are now considered promising candidates to be used in the combat of the several infections caused by different bacterial strains.

There are several methods to produce AgNPs. In this study, AgNPs were produced by chemical reduction using two different reducing agents, namely the $NaBH_4$, which is a strong reducing agent, and the $C_6H_8O_6$, which is a weak reducing agent. However, the AgNPs produced without stabilizing agents could aggregate and subsequently be toxic for human cells. To overcome this problem, chitosan/dextran nanoparticles were added to AgNPs in order to promote their stabilization. Chitosan was used for this purpose, due its known antibacterial activity and also its capacity to stabilize AgNPs. However, the positive charge of chitosan, which is responsible for its antibacterial properties, can be harmful to the normal cells. In order to avoid this problem, dextran, a polymer negatively charged, was used to reduce the positive charge of chitosan and to decrease the probability of cell damage occurrence.

In this work were produced several types of nanoparticles, chitosan/dextran nanoparticles combined with the AgNPs, previously formed, and AgNPs produced in chitosan/dextran nanoparticles. Both AgNPs were produced by using $C_6H_8O_6$ or $NaBH_4$ as reducing agents. The morphology of the produced nanoparticles was analyzed by SEM, and additionally characterized by UV-Vis and FT-IR spectroscopy. In order to fulfill the objectives of this work, the antibacterial activity of the nanoparticles (MIC and MBC assays) were tested against *E. coli*. Through this assay, chitosan/dextran nanoparticles combined with the AgNPs produced by the reduction with $C_6H_8O_6$ and with $NaBH_4$ presented a noticeable antibacterial activity (3.0 and 1.5 μg/mL, respectively). Furthermore, the AgNPs produced in chitosan/dextran nanoparticles with $C_6H_8O_6$ and with $NaBH_4$ presented higher MIC values (11.75 and 23.50 μg/mL, respectively), than those produced by the combined ones.

Moreover, the produced nanoparticles were placed in contact with human osteoblast cells at MIC concentrations and in concentrations above and below the MIC, in order to verify if the nanoparticles that disturb bacteria activity, do or do not affect the viability of normal human cells. Chitosan/dextran nanoparticles combined with the AgNPs, produced by the reduction with $C_6H_8O_6$ or $NaBH_4$, did not show toxic effects to the human osteoblast cells. However, $NaBH_4$ is a toxic agent for human cells and for that reason $C_6H_8O_6$ was preferred to use as the reducing agent. Regarding to the AgNPs produced in chitosan/dextran nanoparticles with $C_6H_8O_6$ or $NaBH_4$, they also showed no toxic effects at MIC concentrations. Nevertheless, they showed toxic effects to cells for higher $AgNO_3$ concentrations, indicating that the toxicity of $AgNO_3$ was concentration-dependent. Taking all this in account, the chitosan/dextran nanoparticles combined with the

AgNPs produced by the reduction with $C_6H_8O_6$ are the preferred ones, due to their low MIC against *E. coli* and the absence of toxic effects for human osteoblast cells. The supernatants resulting from the washing of the several nanoparticles were also put in contact with cells, in order to evaluate their cytotoxicity. The supernatants of all the produced nanoparticles were extremely toxic for human osteoblast cells. Hereby, particles' washing decreased the toxicity of the nanoparticles for human cells.

Chitosan/dextran nanoparticles combined with the AgNPs produced by the reduction with $C_6H_8O_6$, would be a good choice for coating an orthopedic implant to avoid the bacterial colonization, i. e., the biofilm formation. Moreover, these particles may also be valuable candidates to be applied in skin regeneration, since AgNPs also possess an anti-inflammatory effect. Additionally, chitosan/dextran nanoparticles are currently used as drug delivery systems due their high efficiency in drug encapsulation. Therefore, it would be interesting to study the incorporation of growth factors in these systems for bone and skin regeneration. In a near future, *in vivo* studies will allow to validate the results herein produced.

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