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Carlos André Passos Bastos

Study of $Cu(OH)_2$ nanoparticles with antibacterial properties

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Estudo de nanopartículas de $Cu(OH)_2$ com propriedades antibacterianas

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Nuno Faria, Investigador do Medical Research Council- Human Nutrition Research e do Doutor Tito Trindade, Professor Associado com Agregação do Departamento de Química da Universidade de Aveiro.

o júri

presidente

Prof. Doutor Manuel António Coimbra
Professor associado c/ agreg. do Departamento de Química da Universidade de Aveiro

Prof. Doutor Tito Trindade
Professor associado c/ agreg. do Departamento de Química da Universidade de Aveiro

Doutor Nuno Faria
Investigador do Medical Research Council-Human Nutrition Research

Prof. Doutor Brian Goodfellow
Professor associado do Departamento de Química da Universidade de Aveiro

Prof. Doutora Elisabete Costa
Professor auxiliar do Departamento de Engenharia Cerâmica e do Vidro da Universidade de Aveiro

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palavras-chave

Nanoparticulas, antibacterianos, cobre, hidróxidos de cobre, silica, *E.coli*. bacterias

resumo

Introdução: O uso de nanopartículas como antimicrobianos pode ter o potencial de superar algumas limitações associadas aos antibióticos, tal como o aparecimento de estirpes bacterianas resistentes a estes. Nanopartículas de prata e, em menor extensão, de cobre tem sido amplamente usadas com esta finalidade. Apesar de a prata apresentar maior actividade antimicrobiana, esta não tem qualquer função biológica no organismo, sendo potencialmente mais tóxica que o cobre. Embora as nanoparticulas de cobre sejam menos eficazes, estas são uma potencial alternativa devido ao baixo custo de produção e ao facto de serem mais biocompatíveis.

Objectivo: Síntese de novas nanopartículas de hidróxidos de cobre, de tamanho pequeno (<10nm) e desenvolvimento de um método de analise da actividade antimicrobiana das nanopartículas.

Parte Experimental: As nanopartículas de hidróxidos de cobre foram sintetizadas usando ligandos, tais como o ácido málico, cítrico, tartárico e adípico. Tentativas de modificar as nanopartículas originais foram feitas usando um tratamento térmico e sílica. A actividade antimicrobiana foi testada num ensaio usando *E.coli* NCTC 11100 em placas de 96 poços.

Resultados e discussão: As nanopartículas de hidróxidos de cobre foram sintetizadas usando ácido tartárico e ácido adípico com um elevado rendimento (>90%), tamanho pequeno (3-5nm) e elevada dispersibilidade. Em baixas concentrações no meio de cultura bacteriano, as nanoparticulas mostraram estar solúveis, sendo que as modificações feitas não foram capazes de ultrapassar este problema. Um método para testar a actividade antimicrobiana de diferentes compostos foi desenvolvido, mostrando que tanto as nanopartículas de hidróxidos de cobre como cloreto de cobre mostraram semelhantes reduções no crescimento de *E.coli*.

Conclusão: Estes resultados sugerem que as nanopartículas de hidróxidos de cobre estavam solúveis no meio de cultura bacteriano e possivelmente, a actividade bacteriana observada deveu-se a cobre solúvel. Ainda assim, foi desenvolvida uma novo metodologia de síntese de nanopartículas de hidróxidos de cobre que poderá permitir no futuro a síntese de nanopartículas mais estáveis. Por último, foi desenvolvido um método para testar a actividade antibacteriana de diferentes compostos, que permite obter um grande número de resultados no mesmo ensaio.

keywords Nanoparticles, antibacterials, copper, copper hydroxides, silica, *E.coli*, bacteria

abstract

Introduction: The use of nanoparticles as antimicrobials has the potential to overcome some limitations associated with antibiotics, such as the appearance of antibiotic resistant bacterial strains. Silver and, in lesser extents copper nanoparticles have been widely used nanomaterials for this purpose. Silver has shown higher activities, but it has no biological role in the organism and appears to be more toxic than copper. Copper-based nanoparticles, although less effective are a potential alternative as they incur a lower manufacturing cost and should be more biocompatible.

Aim: Synthesis of novel copper hydroxides nanoparticles, of a small size (<10nm) and development of an antimicrobial activity assay to test the efficacy of nanoparticles.

Methods: Copper hydroxides nanoparticles were synthesized using ligands, such as malic, citric, tartaric and adipic acid. Heat treatment and silicon modifications were attempted to modify the original synthesized nanoparticles. The antibacterial activity was tested in a 96 well plate assay using *E.coli* NCTC 11100.

Results and discussion: Copper-based nanoparticles were synthesized using tartaric and adipic acid at a high yield (>90%), small size (3-5nm) and well disperse. The nanoparticles appeared mostly soluble in broth at low concentrations, and the modifications attempted did not overcome this problem. An assay to test antimicrobial compounds was developed showing that copper chloride and the synthesized nanoparticles affected *E.coli* growth similarly.

Conclusion: The results suggested that copper hydroxides nanoparticles were soluble in bacterial culture, and probably the antibacterial activity was due to soluble copper, but a synthetic methodology for the synthesis of copper hydroxide nanoparticles was established and will allow further refinement to generate more stable nanoparticles. Also, a high throughput assay to test antibacterial compounds was developed.

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Abbreviations / Acronyms

Ad – Adipic acid

APS – (3-aminopropyl)trimethoxysilane

Cit – Citric acid

CFU – Colony Forming Units

DAP – Diaminopimelic acid

DLS – Dynamic Light Scattering

DNA – Deoxyribonucleic Acid

HRTEM –High Resolution Transmission Electron Microscopy

ICP-OES – Induced Coupled Plasma –Optical Emission Spectrometry

LPS – Lipopolysaccharides

LPT – Lipoteichoic acid

Mal – Malic acid

MBC – Minimum Bactericidal Concentration

MIC –Minimum Inhibitory Concentration

MRC-HNR – Medical Research Council Human Nutrition Research

NAG - N-acetylglucosamine

NAM - acid N-acetylmuramic

OD – Optical Density

PEG – Polyethylene glycol

ROS –Reactive Oxygen Species

Tart – Tartaric acid

TEM – Transmission Electron Microscopy

TEOS – Tetraethyl orthosilicate

TMOS – Tetramethoxysilane

XRD – X-Ray Diffraction

1. Introduction

1.1. Placement at Medical Research –Human Nutrition Research

Medical Research Council is a medical research organisation created in the beginning of the XX century. Since that time, the MRC has grown and, currently is composed by 3 institutes, 27 units and 28 centres all over United Kingdom. The mission of MRC comprises the improvement of human health, developing skilled researchers and supporting research in different areas, such as cancer, obesity, ageing, stem cells and DNA. From a long time ago, MRC scientists have been awarded with the highest distinctions in their areas, including 29 Nobel Prizes. Frederick Hopkins, Alexander Fleming, Hans Krebs, Frederick Sanger, Francis Crick, James Watson, Maurice Wilkins are examples of worldwide famous scientists responsible for great findings in areas such as medicine, physiology or chemistry. Recently, in 2009, Venkatraman Ramakrishnan won the Chemistry's Nobel Prize for his work with ribosomes at MRC Laboratory of Molecular Biology in Cambridge. Cambridge arises as one of the main locations of MRC with more than 10 units and centres, such as the MRC-Human Nutrition Research (HNR). MRC-HNR was established in 1998 and is based on Elsie Widdowson Laboratory. The unit aims to advance knowledge between human nutrition and health and, for that reason is divided in 5 sections: Population Nutrition Research that assesses and analyses dietary intakes and relationships to biomarkers and disease; Nutrition and Health that studies the role of dietary factors in the prevention and treatment of obesity and related metabolic diseases; Nutrition and Bone Health, that looks at nutrition and lifestyle factors for the optimisation of peak bone mass and reduction of osteoporosis risk; Bioanalytical discovers and measures physiological markers in nutrition and health and lastly Biomineral Research which investigates the uptake, distribution, cellular handling and functional role of micronutrients. During my placement I worked in the last group, headed by Dr. Jonathan Powell, which focus on five essential topics, (i) the development of novel mineral structures, (ii) the study of iron metabolism and the development of strategies to correct iron deficiency anaemia, (iii) the study of exogenous and endogenous particles of the gut lumen and their cellular target in gut tissue, (iv) also the characterisation of a silicon deficiency and its dietary requirements, and finally, (v) the development of methodology to analyse different circulating components in human body samples. Biomineral Research group is composed by a group of 10 scientists, 7 PhD students from the University of Cambridge, and several placement students form different universities in Europe. This project was aimed at synthesize novel mineral

structures, and in particular copper hydroxides nanoparticles to be potentially used as antimicrobials. These novel materials should ideally be easy to prepare, at low cost, should remain stable and, also have the potential for production at a large scale. Also, during the project was aimed the development of a method to test the antimicrobial activity of different compounds, including the nanoparticles.

1.2. Reasons behind the project

Nanotechnology is an area in a great development and the number applications of nanoproducts have been increased quickly. One of these applications is the use of metal nanoparticles as antimicrobials. Since a long time ago that has been known that bacteria are able to acquire resistance against antibiotics. Also, some of the effective antibiotics can present harmful side effects, especially when over-used, which is a reason for health concern. Alternatives to some antibiotics are currently under development, in which copper and silver arise as very well-known antimicrobials and recently has been shown that their activity may be increased by using nanoparticulated forms of these metals. This finding may lead to the use of copper and silver in lower concentrations than they have been used so far, which may avoid health safety issues. In particular, silver has been the most studied compound, because it presents higher antimicrobial activity than copper, but some studies have shown that silver can be toxic for several cell lines in human tissues, even at very low concentrations. Furthermore, silver is not apparently used by the organism, being less tolerable than copper and tends to be accumulated in the tissues, which can be a reason of concern after long exposure times. In contrast, copper is considered a micronutrient, because the organism requires its uptake daily, due to the important role in human metabolism, such as showed by the large group of copper-dependent enzymes in the organism. Also, human tissues have mechanisms able to control the copper homeostasis, by regulation of uptake and excretion, which may reduce the risk of accumulation and toxicity, even after long exposure times. In a different perspective, copper is also much cheaper than silver. Finally some studies comparing copper and silver reported that the difference between antimicrobial activities were not very remarkable. The synthesis of copper nanoparticles is very well-reported and different copper compounds can be synthesized at nanoscale. Also, nanoparticles can be modified to obtain desirable properties to improve their effectiveness. For that reasons, the aim of the work was the synthesis of stable copper-based nanoparticles, in particular copper hydroxides, with antibacterial activity. The topics introduced here will be more extensively described during the background.

2. Background

Particles that have typical dimensions between 1-100 nm are often called nanoparticles and show distinct properties from those of the bulk analogues. Although these particles have always occurred naturally, the nanotechnology only arose few years ago. One of the main reasons of these recent developments was the lack of previous suitable characterization techniques. Their study has grown because their size leads to a very high surface-volume ratio that may be one of the most important factors for their special properties, such as the increasing in chemically reactive and catalytic. For example, a decreasing of 10μm to 10nm represents an increase of 10^9 in surface area. The higher reactivity of nanoparticles due to the smaller size, may be explained by different reasons, such as the higher proportion of atoms at the surface, the influence of thermodynamics of chemical reactivity by different surface free energy, modifications in atomic structures, different bond lengths or bond angles, and finally modifications on the electronic structure of the materials [1]. These dramatic changes, when compared with microparticles allow new applications, and currently there are a large range of uses in areas such as medicine, food industry, clothing, hygiene and many others [2,3]. To put in perspective human cells are typically 10μm and most proteins are around 5 nm.

Nanoparticles may be included in the large concept of nanotechnology, which have arisen in the last years as one of the main areas of research. Nanotechnology is briefly defined by "engineering at nanoscale", including the development of nanomaterials to be applied in different areas [4]. The importance of nanotechnology emerges, since the development of new materials associate the understanding of the main scientific area, such as physics, chemistry, biology and materials (Figure 1). The development of nanoparticles includes not only the knowledge required in materials for the synthesis and manipulation, but also the understanding of physical and chemical properties of synthesized materials to know how to apply in different areas, such as biology. In particular, medicine has been one of the most relevant areas in which nanomaterials have been developed to be applied. The use of nanoparticles in medicine has been especially associated with diagnosis and therapy, being used in the development of analytical techniques and also in drug delivery methods to increase the efficacy of the treatments used [5]. Some examples of nanoparticle-based products that are currently commercialized for medicine purposes are described in the Table 1. In particular, relating the increase of interest of nanoparticles in medicine, copper nanoparticles are one great example of that, as seen in Figure 2, which showed the increase of publications about copper nanoparticles

as antimicrobials in the last years. Only, in 2010 and 2011 were released 49% of the total publications in this topic.

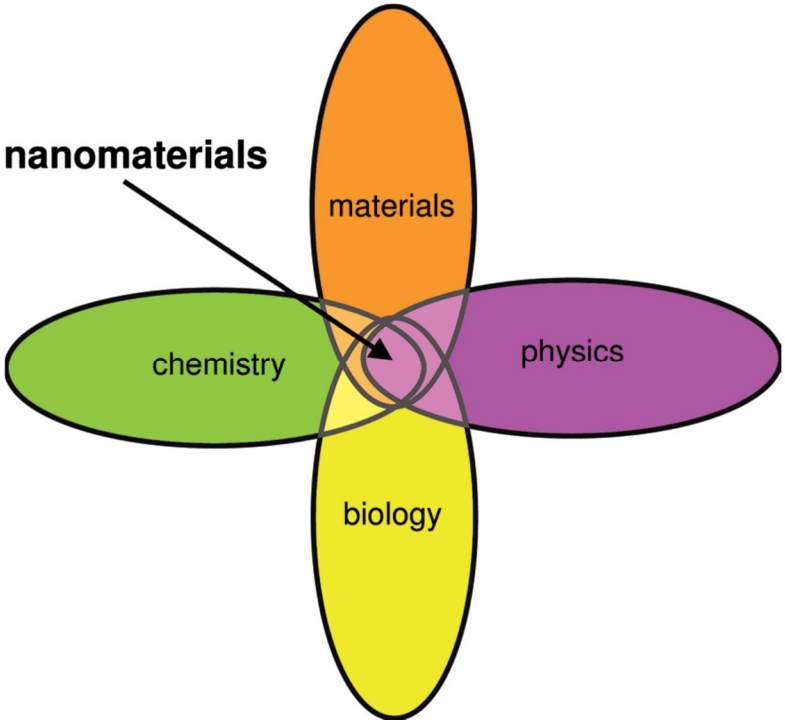

Figure 1- The development of nanomaterials involves the knowledge of different areas, such as chemistry, biology, physics and materials [6].

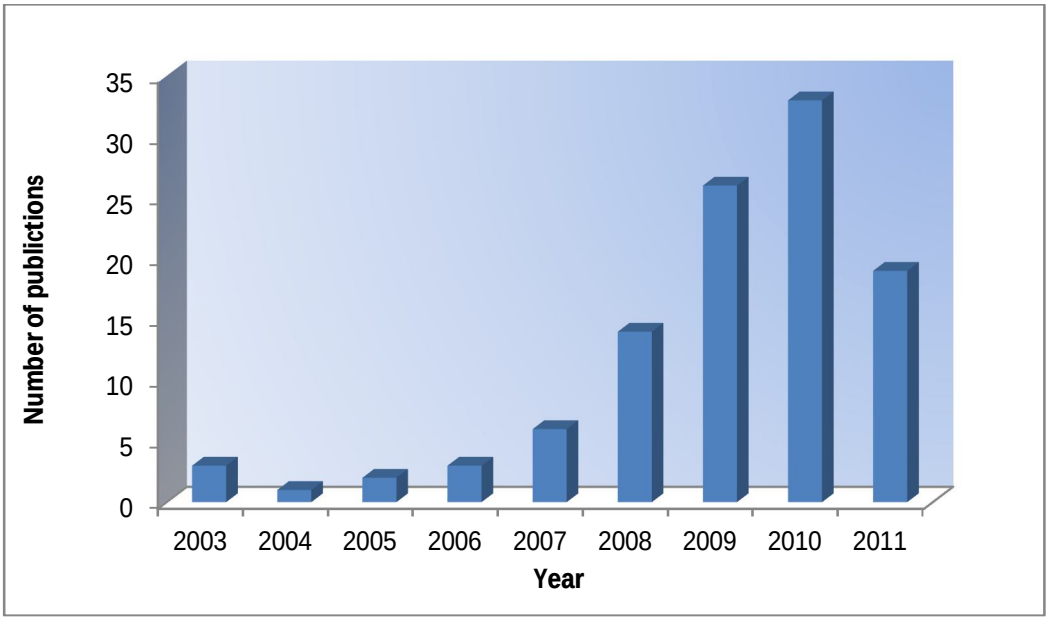

Figure 2- Number of publications that were published in ISI Web of Knowledge database, reporting the antimicrobial activity of copper nanoparticles in the last years. Numbers of publications in 2011 was obtained only for the first 6 months of the year, using the keywords: i) copper + nanoparticles + antibacterial or ii) copper + nanoparticles + antimicrobial.

Table 1- Examples of industrial companies actually commercialising nanomaterials for biomedical applications [2].

Company	Major area of activity	Technology
Advectus Life Sciences Inc.	Drug delivery	Polymeric nanoparticles engineered to carry anti-tumour drug across the blood-brain barrier
Alnis Biosciences, Inc.	Bio-pharmaceutical	Biodegradable polymeric nanoparticles for drug delivery
Argonide	Membrane filtration	Nanoporous ceramic materials for endotoxin filtration, orthopaedic and dental implants, DNA and protein separation
BASF	Toothpaste	Hydroxyapatite nanoparticles seems to improve dental surface
Biophan Technologies, Inc.	MRI shielding	Nanomagnetic/carbon composite materials to shield medical devices from RF fields
Capsulution NanoScience AG	Pharmaceutical coatings to improve solubility of drugs	Layer-by-layer poly-electrolyte coatings, 8–50 nm
Dynal Biotech		Magnetic beads
Eiffel Technologies	Drug delivery	Reducing size of the drug particles to 50–100 nm
EnviroSystems, Inc.	Surface desinfectsant	Nanoemulsions
Evident Technologies	Luminescent biomarkers	Semiconductor quantum dots with amine or carboxyl groups on the surface, emission from 350 to 2500 nm
Immunicon	Tarcking and separation of different cell types	magnetic core surrounded by a polymeric layer coated with antibodies for capturing cells
KES Science and Technology, Inc.	AiroCide filters	Nano-TiO2 to destroy airborne pathogens
NanoBio Cortporation	Pharmaceutical	Antimicrobal nano-emulsions
NanoCarrier Co., Ltd	Drug delivery	Micellar nanoparticles for encapsulation of drugs, proteins, DNA
NanoPharm AG	Drug delivery	Polybutilcyanoacrylate nanoparticles are coated with drugs and then with surfactant, can go across the blood-brain barrier
Nanoplex Technologies, Inc	Nanobarcodes for bioanalysis	
Nanoprobes, Inc.	Gold nanoparticles for biological markers	Gold nanoparticles bio-conjugates for TEM and/or fluorescent microscopy
Nanoshpere, Inc.	Gold biomarkers	DNA barcode attached to each nanoprobe for identification purposes, PCR is used to amplify the signal; also catalytic silver deposition to amplify the signal using surface plasmon resonance
NanoMed Pharmaceutical, Inc.	Drug delivery	Nanoparticles for drug delivery
Oxonica Ltd	Sunscreens	Doped transparent nanoparticles to effectively absorb harmful UV and convert it into heat
PSiVida Ltd	Tissue engineering, implants, drugs and gene delivery, bio-filtration	Exploiting material properties of nanostructured porous silicone
Smith & Nephew	Acticoat bandages	Nanocrystal silver is highly toxic to pathogenes
QuantumDot Corporation	Luminescent biomarkers	Bioconjugated semiconductor quantum dots

2.1. Bacteria: structure, resistance and occurrence

The development of antibacterial particles requires the understanding of how they can act to avoid the growth or even kill the microorganisms, thus, the understanding of the basic structure or vital mechanisms is essential. Bacteria are prokaryotic organisms, and one of the characteristics of these organisms is their cell wall that is very important when studying the bacteria structure, as it contains some components only present in these microorganisms. Of these components, the cell envelope is of particular interest, as it includes not only the cell wall but also plasma membrane and usually a capsule outside the cell wall composed by polysaccharides [7]. Overall, this structure acts as a protection barrier against external damage, avoiding the cellular rupture and consequently death. The constitution of the cell wall varies depending on the strain, for example, gram-negative bacteria have a thin layer of peptidoglycan

between two membranes, an inner membrane composed by phospholipids and an outer membrane containing lipopolysaccharides that are located to the outer leaflet [7]. Gram-positive have only one plasma membrane protected by a thick peptidoglycan layer that surrounds it. This difference in structure is responsible for the different staining properties of bacteria, since purple crystal violet is retained by the peptidoglycan layer of gram positive in the gram-staining procedure [8]. Also, these bacteria normally have teichoic acids and teichuronic acids covalently linked to peptidoglycan. The Figure 1 schematically represents the cell envelope of gram-negative and gram-positive bacteria.

Although it provides protection to bacteria, the cell wall is a preferential place for the action of antibiotics due its specific components which are not found in any other organisms. Murein is the main component responsible for this specificity, being one of the most important differences between bacteria and *Archaea* cell wall structure. This peptidoglycan is composed by a disaccharide polymer cross-linked by peptides, such as D-alanine, L-alanine and L-lysine. N-acetylmuramic (M) acid and N-acetylglucosamine (G) are linked between them, forming the basic unit of peptidoglycans. The degree of cross-linking is higher in gram-positive cells, which occurs between meso-Diaminopimelic acid (mesoDAP) and D-alanine, as seen in Figure 3. The bacterial cell wall presents a large number of functional groups in its surface that are able to interact with external agents and many antibacterial agents, such as nanoparticles, have been reported to inhibit the growth of bacteria through mechanisms associated with the cell wall. For example, lysozyme is an enzyme that is present in animal fluids, such as human tears, and acts as an antibacterial protector, by breaking down the linkage between NAM acid and NAG, thus damaging in the cell wall structure. Also penicillin, a well-known antibiotic, inhibits the formation of the cell wall by binding to bacterial transpeptidases responsible for the formation of NAG-NAM linkages, thus preventing the normal bacterial growth [8,9].

Recently, the resistance of many bacterial strains to antibiotics has been a reason of concern for human health. The mechanisms of resistance can be intrinsic or acquired. An example of intrinsic resistance occurs in *Pseudomonas aeruginosa* that has a very low permeability and consequently, prevents the entrance of most antibiotics [10]. The outer membrane of gram-negative bacteria is semi-permeable, and only small antibiotics are able to cross this membrane by porin channels, normally used for the uptake of water by bacteria [11]. The size of this porin channel excludes, automatically, the larger antibiotics. In particular, the porins of *Pseudomonas aeruginosa* are extremely small, being a strong mechanism of resistance for drugs. In addition, these bacteria present another mechanism of resistance related with the efflux of the drugs. The cell membranes are able to pump out the antibiotics that had

crossed the membrane. Together, both mechanisms are responsible for the high resistance of *pseudomonas aeruginosa* to antibiotics [10].

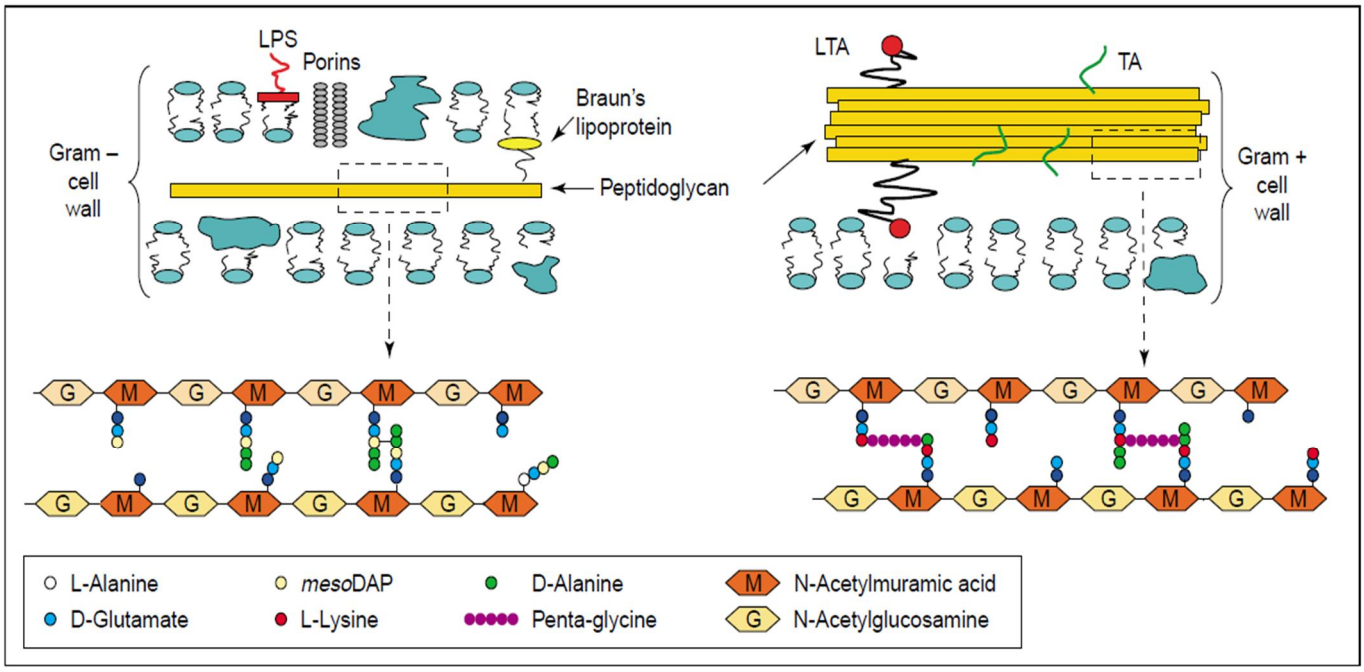

Figure 3- Schematic structure of gram-negative (left) and gram-positive (right) cell wall and respective peptidoglycan composition (left). Abbreviations: LTA, lipoteichoic acid; LPS, lipopolysaccharide; TA, teichoic acid; M, N-acetylmuramic; G, N-acetylglucosamine. Adapted from[12].

Acquired resistance is commonly related to genetic mechanisms. The development of enzymes that is able to inactivate antibiotics, such as β-lactamase that degrades the β-lactams, inhibiting their activity against bacteria [13]. Furthermore, the bacteria are able to acquire new genes, on plasmids, that lead to the modification of metabolic enzymes. These modifications prevent the binding of antibiotics to the enzymes, avoiding their inactivation, thus keeping the normal bacterial growth [13]. Besides *Pseudomonas aeruginosa*, also *Streptococcus pyogenes*, *Staphylococcus aureus* and *Escherichia coli* are examples of resistant bacteria, which have been studied to develop new effective antibiotics, to overcome serious health problems related to their pathogenicity.

The understanding of the metabolic pathways of some important pathogenic bacteria, which are responsible to cause diseases in the human body, is important to develop effective metal nanoparticles against these microorganisms. Both, skin and gut are main possibilities for the application of antibacterial nanoparticles and they present a variety of microorganisms in the normal flora. The human flora can be defined as the microorganism present in healthy tissues, such as gut or skin surface. The skin is the largest organ in the human body and is the

first physical barrier to the entrance of pathogenic organisms, which can cause infections. The pH of the skin surface (5-6) is important in this protection, as it prevent the growth of most of the microorganism. The low pH is due to the presence of acids, normally fatty acids, produced by bacteria of skin flora. Also, some bacteria of normal flora, such as *Staphylococcus epidermis* and *Propionibacterium acnes*, are able to produce some antibiotics that prevent the infection with pathogens [14]. The most common bacterial skin infections are related with *Staphylococcus aureus* and different forms of *Streptococcus*. In the gut, especially in colon, the flora present is very large and diverse. This flora is very important for the human body as it prevents infection by pathogenic bacteria, through the formation of a barrier in intestinal mucosa that avoid the invasion of pathogens. Also, controls the proliferation and differentiation of epithelial cells, is responsible for fermentation of carbohydrates that are not degradable by the human enzymes, and finally can interfere in immune response [15]. One of the most dangerous pathogens in the intestine is the *Clostridium difficile,* which can be responsible for serious infections of the colon. Also, the contamination of food products can lead to bacterial infections in the intestine. *Salmonella, Listeria monocytogenes, Campylobacter or E. coli* O157 are examples of strains, which usually cause disease in humans.

As described previously, many microorganisms have developed resistance mechanisms to antibiotics in short periods of time [16]. The effort involved in the development of new effective antibiotic shows that copper is a great alternative, due to its easy interaction with bacteria [17], and although it has used by mankind for antimicrobial purposes since thousands of years ago and it is still extremely rare to find any tolerant microorganisms. In particular, copper nanoparticles possess the advantages of copper salts but and may be able to reduce the toxicity risks associated to metallic copper while enhancing their antimicrobial activity.

2.1.1. *Escherichia coli*

E.coli was chosen as a model bacterium due to being well reported in the literature, making result comparison possible and, also, to adapt existing microbiological methods to the current project. *E.coli* is a very well-studied gram-negative bacterium that is a member of the family Enterobacteriaceae, which is composed by facultative anaerobic bacteria that generally compose the human flora. Thus, *E.coli* is able to grow whether in the presence of oxygen or under anaerobic conditions, such as in the human intestine, where this bacteria have been identified as one of the main populations. Most of the strains are not pathogenic, especially the ones present in the human intestine, which may present advantages to the hosts, such as the synthesis vitamin K and another important molecules for human compounds [18]. Also, the

fixation in the intestinal mucosa may lead to prevent pathogenic bacteria to access the human mucosa to attach and colonize there [19]. In contrast, some strains of bacteria can be highly pathogenic, being responsible for several diseases in human, such as diarrhoeas, gastrointestinal and urinary infections. In some cases, an infection with *E.coli* may lead to death, for example, by production of endotoxins that are released in the human body or by invasion and infection of the cells causing their death. Furthermore, these bacteria are normally very resistant even in hostile environments, as seen in some parts of intestine, where *E.coli* is able to grow even with lower amounts of nutrients [20]. Overall, E Coli is a good test bacterium and was used in the antimicrobial efficacy assays.

2.2. Copper, nanoparticles and antimicrobial activity

Metal ions such as silver, copper or zinc have been largely reported as antimicrobials and highly used for this purpose. The bactericidal effect of these metals is very well known and has been studied from a very long time ago, due to many possible applications. Not only the metal ions, but also different metal compounds have been used as antimicrobial in usual daily products. Associations with polymers, fixation in gels matrixes or even some crystalline forms are common examples of the occurrence of these metals that are used in medical (e.g. burns treatment) or feed applications (e.g. packaging). Despite a reasonable understanding of antimicrobial action of metals, most mechanisms of action still remain partially unknown.

The high activity of nanoparticles against microorganisms has been proposed to be due to their small size and high surface volume that leads to a closer interaction with bacterial membranes, generally better than the effect produced by isolated metal ions in solution [21,22]. This fact may be the key for the success of metal nanoparticles against the corresponding ionic forms. Many papers have been published demonstrating that same metals could present different activities depending on the characteristic of the particles, such as shape or size. For example, a study realized with silver nanoparticles against *E.coli* revealed that spherical and triangular shapes were the most effective [23]. Also, size is very important, and smaller particles have higher activity, probably due to the high surface area, and closer interaction, as mentioned above. The use of smaller nanoparticles can result in the same antimicrobial effectiveness with lower concentrations of metal, which is very important, not only economically, but also decreasing the possible toxicity and environmental risks involved with metal compounds.

Silver nanoparticles have been the most widely studied antimicrobial nanoparticles, as they present the highest activity against most microorganisms [24-28]. Nevertheless their use

presents some limitations such as potential toxicity and high cost. Environmental risks that are associated with silver are also of concern and may result in greater control of its utilization [29,30]. Silver has not any known utilization in human body, which reduces the potential for biocompatibility. As the silver is not metabolized, its intake can lead to the accumulation in human tissues, being a great concern for long exposition times. Silver is mostly absorbed in organism in intestine and then transported into blood, where the silver can be transported by albumin or as a free ion. Often, some silver salts are able to corrode the intestinal mucosa, increasing the permeability and consequently absorbing higher amounts of compounds including silver. High amounts may lead to silver precipitation in blood, or can be transported to different tissues, such as brain, skin, nails, kidney or spleen. Silver is not metabolized by the organism and tends to be accumulated in these tissues, and may be toxic for the cells [31]. Studies in mammal cells have shown cytotoxicity, mainly related with mitochondrial damage, by generation of reactive oxygen species, which may affect the production of ATP and activate the cell apoptosis regulators [32]. Also, DNA can be damaged, being silver considered cytotoxic and genotoxic [33,34]. The authors of these studies considered that silver nanoparticles can enter in the cell by diffusion or endocytosis causing the production of reactive oxygen species (ROS) that will affect the mitochondria. Also, some silver ions may be released and cross the nuclear membrane causing DNA damage and inducing cell apoptosis mechanisms. An example of consequence of extreme intake of silver is argyria, which is a pathological effect caused by exposure of silver, which is accumulated in different tissues of the organism, particularly in the skin, forming granules that confer the grey appearance to the patients. The constitution of granules is mainly silver sulphides and normally argyria did not show physiological problems for some tissues, which may mean that this is a detoxification mechanism of the body, because it avoids the negative effects of silver in cells. Argyria has no efficient treatment known, so far. [35]. Also, some topical deliver of nanoparticulated silver showed toxicity to skin tissues, including keratinocytes and fibroblasts when absorbed through the skin [36]. Furthermore, silver can cross the blood-brain barrier and accumulate in the central nervous system. Generally, there are several reports of toxicity of silver in different tissues and cell lines, as seen in Table 2. Although the high potential use as bactericidal compounds, a different strategy is required.

Table 2 –Toxicity of nanosized silver compounds in mammalians cells, including some human cells types [32].

Target cell/organism	Key aspects	Reference
Rat lung cells	Reduction in lung function and inflammatory lesions	(Sung et al. 2008)
Sprague-Dawley rats	Silver nanoparticles accumulation in olfactory bulb and subsequent translocation to the brain	(Kim et al. 2008a, b)
Mouse stem cells	Cell leakage and reduction of mitochondrial function	(Braydich-Stolle et al. 2005)
Rat liver cells	Cell leakage and reduction of mitochondrial function	(Hussain et al. 2005)
Human fibrosarcoma and human skin/carcinoma	Oxidative stress. Low doses produced apoptosis and higher dose necrosis	(Arora et al. 2008)
Mouse fibroblast	50 µg/mL induced apoptosis to 43.4% of cells	(Arora et al. 2009)
Human colon cancer	100 µg/mL produced necrosis to 40.2% of cells	
Human glioblastoma	Silver nanoparticles were found cytotoxic, genotoxic and antiproliferative	(Asharani et al. 2009)
Human fibroblast	Silver nanoparticles were found cytotoxic, genotoxic and antiproliferative	(Asharani et al. 2009)

To avoid these problems the use of lower toxic metallic particles might be an alternative. Copper arises as an essential micronutrient, being required a daily intake for the normal functionality of the body. Copper's intake is especially taken by ingestion of food containing copper. Also, copper can be absorbed in very low amounts through the skin [37]. The copper is not soluble at physiological pH, which leads to appear in very lower concentrations in the free ionic form, Cu^{+} and Cu^{2+}. Thus, copper appears normally associated with transport molecules, commonly bound by proteins, or smaller chelating agents, such as aminoacids. In the organism, copper is absorbed especially in stomach and small intestine. Particularly, in the intestine, copper diffuses through the mucosa into interstitial fluid and blood. If present in higher concentrations, copper can be absorbed by carrier-mediated systems used for different transition metal ions, such as zinc or iron. This competitive mechanism of absorption may lead to deficiency in some compounds due to the presence of one in higher amounts. On the other hand the efficacy of copper absorption in intestinal mucosa can be controlled by the body when the amount of copper ingested exceeds the normal concentration. The absorption of copper is normally between 55% and 75%. Once absorbed across the intestinal mucosa, copper binds to proteins, in particular to albumin and transcuprein, to be taken to the liver. Transcuprein has higher specificity for copper than albumin, which is the main protein present in blood, responsible for the transport of several components, so not specific for copper. In the liver, copper will be used for several purposes, such as synthesis of different copper-containing proteins, such as superoxide dismutase and cytochrome oxidase

and metallothionein. The role of copper in organism is partially related with these three proteins, because their functionality requires copper. Superoxide dismutase is an important enzyme in the control of oxidative homeostasis, being responsible for conversion of superoxide in hydrogen peroxide and oxygen Cytochrome oxidase is an enzyme of the electron transport chain in mitochondria which is involved in the reduction of oxygen to water, using electrons coming from the previous complexes. Lastly, metallothionein is a low-molecular weight protein that is used s oxidative stress scavenger, especially by degradation of superoxide, but can also be used for copper storage, when this metal is present in higher amounts than required and was not utilized by the organism. Also, other copper-dependent proteins can be synthesized in the liver as shown in the Table 3 [38]. Special emphasis is given to ceruloplasmin, which is an important transport protein synthesized in the liver, where that copper is added during this process. This protein transports copper to different body tissues, such as brain and muscles. Ceruloplasmin is also important in the iron metabolism by oxidizing Fe^{2+} to Fe^{3+}, which enables the binding of transferrin. If copper did not reach the liver and was not absorbed at all, it is taken directly to faeces. Often, copper can also be taken to different tissues in body by albumin and transcuprein. Copper that leaves the liver, transported by ceruloplasmin, can be excreted directly to urine, but mostly, copper is excreted by bile fluids, which present the highest amount of copper in the body, and are able to excrete copper by faeces. If required, copper can be reabsorbed in the intestinal tract and taken back to liver, being this mechanism very important in copper homeostasis [39]. The overall metabolism of dietary copper is schematized in Figure 4.

As seen, copper has an important role in organism, also some mechanisms of excretion that have emerged as possible protection against toxicity [40] Comparing with copper, it is important to consider the cost, being the copper much cheaper than silver, which allows its use at industrial scale at lower cost. Consequently at commercial level becomes much more promising too. Finally copper compounds present stable physicochemical properties and can be easily modified as will be seen below [18].

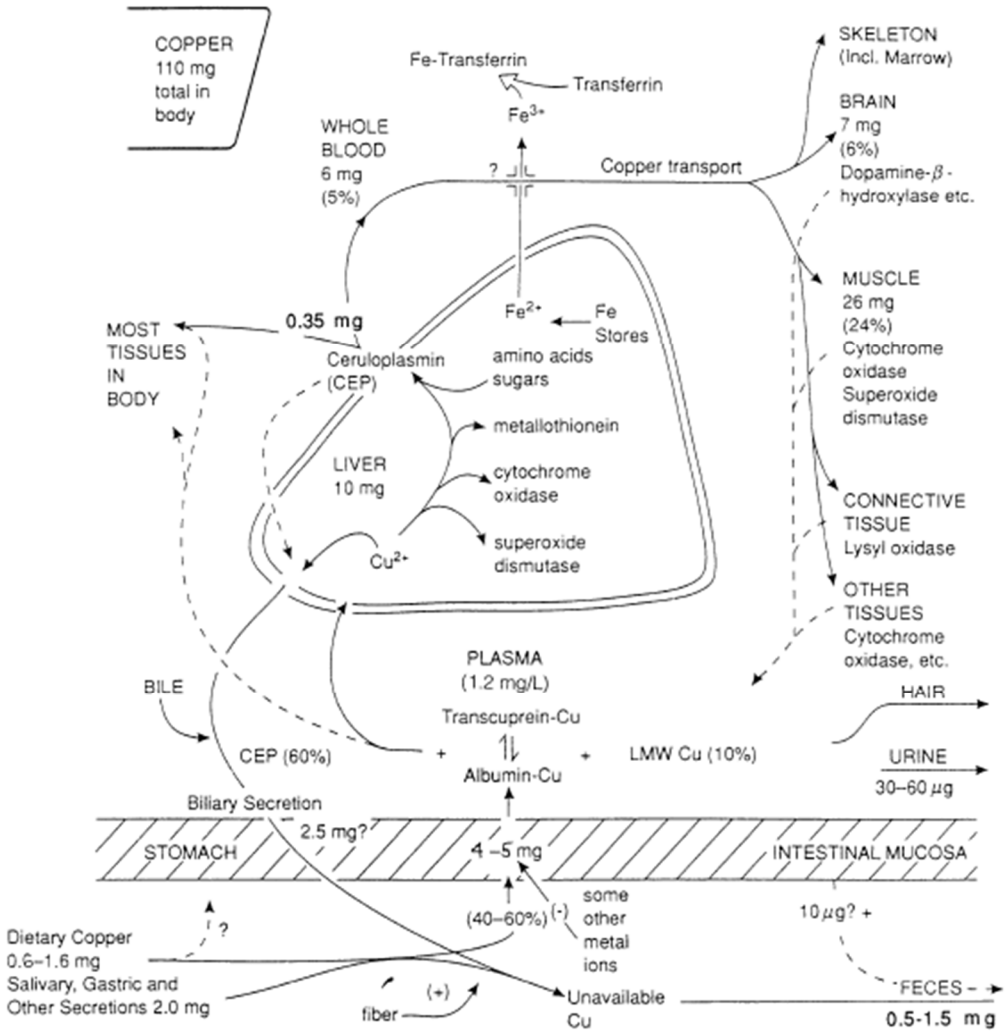

Figure 4 - Dietary copper metabolism in human body [39].

Table 3- Copper-dependent proteins in mammals [38].

Enzyme	Function
Cytochrome-*c* oxidase	Electron transport in mitochondria
Cu/Zn-SOD	Free radical detoxification
Metallothionein	Storage of excess Cu and other divalent metal ions [not Fe(II)]. Possible donor of Cu to certain apoproteins
Ceruloplasmin (extracellular)	Ferroxidase, promotes flow of Fe from liver to blood Scavenger of ROS, acute-phase reactant. Cu transport
Protein-lysine-6-oxidase	Cross-linking of collagen and elastin
Tyrosinase (catechol oxidase)	Formation of melanin
Dopamine-β-monooxygenase	Catecholamines production
α-Amidating enzyme	Modifies C-terminal ends of hypothalamic peptide hormones ending in glycine, leaving the COOH of the next to last AA amidated (necessary for hormone maturation)
Diamine oxidase	Inactivation of histamine and polyamines? (cellular and extracellular)
Amine oxidase (extracellular)	Inactivation of histamine, tyramine, dopamine, serotonin?
Peptidylglycine monooxygenase	Bioactivation of peptide hormones
Hephaestin	Ferroxidase, in trans-golgi of enterocytes; aids iron absorption homology to ceruloplasmin
CMGP	Ferroxidase/amine oxidase, homologous to ceruloplasmin (chondrocytes and eye ciliary epithelia)
β-Amyloid precursor protein	Normal function currently unknown
Prion protein (PrPC)	Copper binding properties suggests that it may protect against ROS; has SOD-like activity; may return copper to neurons at synapses (many cells)
S-Adenosylhomocysteine	Sulfur amino acid metabolism hydrolase
Angiogenin	Induction of blood vessel formation
Blood clotting factors V and VIII	Blood clotting

2.2.1. Antimicrobial compounds

As mentioned above, copper is an antimicrobial compound and to understand its mechanism of action and efficacy will be briefly described about antimicrobials already used and including metal nanoparticles. Antimicrobials are compounds that kill or inhibit the growth of microorganisms, and understanding the physiological mechanisms of these microorganisms is the first step in development of an agent that is effective against them. Therefore, to kill or inhibit bacteria the antimicrobial must be able to disrupt some vital components of the metabolic pathways of bacteria, thus hindering their homeostasis. Table 4 shows some antibiotics currently used and their physiological target in bacteria that could lead to their growth inhibition or even death.

Antimicrobial agents must also be delivered in the target place, and also they must act on the target bacteria. Some antimicrobials have a reduced range of bacteria against which they are very effective. The metal nanoparticles can be the alternative to overcome this limitation of antibiotics, due to their activity against some drug resistant bacteria. For example, silver nanoparticles showed bactericidal effect against methicillin-resistant *Staphylococcus aureus*, ampicillin-resistant *Escherichia coli* O157:H7, erythromycin-resistant *Staphylococcus pyogenes* and multidrug-resistant *Pseudomonas aeruginosa*. Also, strains of these types of bacteria that are susceptible to antibiotics were tested. Comparing the antibacterial activity, there are no remarkable differences between drug-resistant and drug-susceptible strains, which can indicate that the resistance mechanism did not affect the efficacy of nanoparticles [27].

Table 4- Various antimicrobials and their mechanisms of action in bacteria.

Antimicrobial	*Effect produced in bacteria*
β-lactams (Penicillin, cephalosporins)	Inhibit of bacteria cell wall synthesis
Tetracyclines, macrolides, clindamycin	Inhibit protein synthesis
Quinolones and metronidazole	Inhibit nucleic acid synthesis
Sulphonamides and Trimethoprim	Inhibit enzyme synthesis

The mechanism of copper nanoparticles is partially unknown, although it has been compared with the mechanism proposed for silver nanoparticles. The silver nanoparticles have been proposed to act via three different pathways, uptake of free silver ion released from nanoparticles, by generation of ROS or direct damage of cell membranes by nanoparticles (Figure 5). The first proposal requires the release of silver ions in the cell membrane of bacteria, which sometimes cannot occur in physiological conditions, because it requires strongly oxidative environments. Once released, the Ag^+ ions may bind proteins, through the thiols groups of some aminoacids, which may lead to the disruption of activity of proteins, including some enzymes that may lose completely their catalytic activity. In particularly, respiratory chain enzymes may be one of the most affected proteins in cell, with serious effects on ATP synthesis. Also, Ag^+ may affect the homeostasis of phosphate, inhibiting the uptake of this compound [41]. Although similar to action of silver salts, the prolonged release of silver (I) ions from nanoparticles may lead to longer exposure times and subsequently greater affinity.

A second proposal is the generation of ROS by the presence of silver compounds, whether nanoparticles or ions. In the presence of oxygen and silver can be produced free radicals responsible for the oxidative stress in cells. Furthermore silver may be also responsible for the inhibition of oxidative scavengers, such as glutathione. Increasing the ROS and decreasing the oxidative defenses will expose the cell to a hostile environment that probably leads to several damages in important structures, such as mitochondria and cell membrane, which may cause the cell death [32].

The third proposed mechanism is related with the direct damage of cell membrane by nanoparticles, which can be tackled by the interaction and penetration of nanoparticles in the bacterial envelope, creating pits or even holes, which cause the cell death [42]. Often, smaller nanoparticles were detected intracellularly after damaged the cell membrane [43]. The interaction between cell and nanoparticles is possibly due to electrostatic interaction between the positive nanoparticles and the negative bacterial membrane, which may explain some reports of coated nanoparticles that presented reduced activities, probably due to the decreased affinity between nanoparticle and bacteria caused by the incorporation of a coating [42]. Another effect caused by the presence of particles in the surface of bacteria is an increase in permeability of membrane [43].

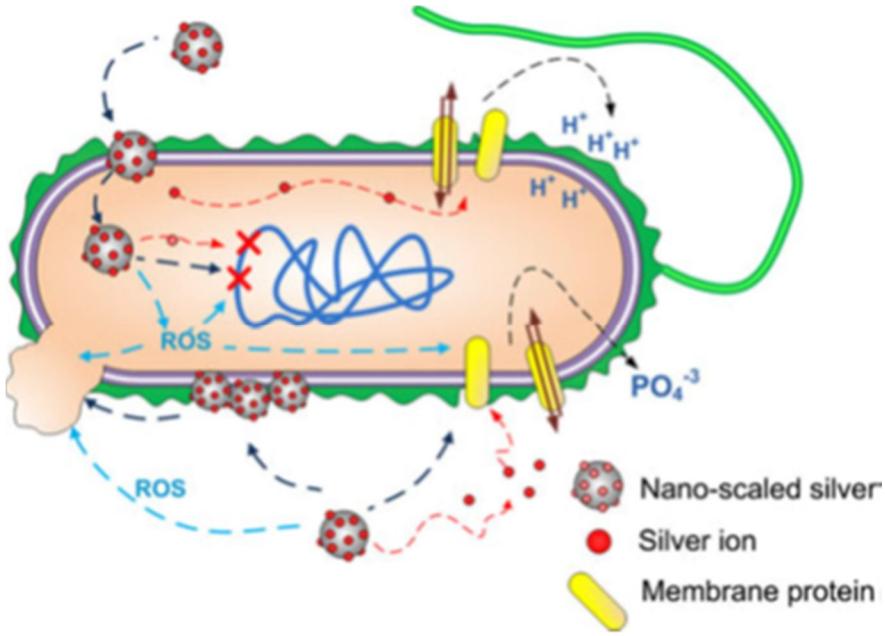

Figure 5- Three different proposed mechanism of silver nanoparticles action: Release of silver ions, generation of ROS and direct cell membrane damage [32].

Although some mechanisms of copper ions have been already described, there are a limited number of studies reporting the mode of action of copper nanoparticles, and may be speculated that mechanisms of silver nanoparticles may be applicable for copper nanoparticles [25]. The biocide effect of copper ions is well known and has been related with the inactivation of biological functions of microorganisms. The envelope is the first part of bacteria in contact with copper, being the point of adhesion via electrostatic forces of copper ions. The extensive amount of copper leads to disruption of membrane and consequently loss of viability [16]. Also, copper can be transported inside the cell, and can interact with proteins and nucleic acids. The interaction with proteins can occur by binding directly to aminoacids of protein or by displacement of metal from their native binding sites in metallic proteins, which normally leads to structural modifications. These changes can cause the loss of biological activity of proteins, by inactivation of important metabolic routes and often with cell death. Also, DNA can be damaged by copper, which is able to create cross linkages in DNA strands leading to denaturation. Mechanisms involving free radicals generated by copper have been proposed, in particular, the catalysis of hydroxyl radical formation ($^{\cdot}OH$), which is responsible for several damages in important biomolecules, such as proteins and nucleic acids [44,45]. Although the antimicrobial effect of copper ions, the lower efficacy, comparing with nanoparticles, may be due to bacterial resistance mechanism proposed specific for copper ions. Some bacteria, such as *E.coli*, are able to control the uptake and excretion of copper. The mechanism of copper

intake by bacteria is still not completely known, but some relevant homeostasis mechanisms have already been proposed, in which copper can be taken up by bacteria through an ATPase system that is specialised for the transport of cations, such as copper, magnesium or silver, across the cell membrane. This ATPase is being called by P-type ATPase due to the phosphorylated intermediary that forms during the mechanism [46]. The ATPase's are generally a system of proteins located in the cell membranes that are responsible for the transport of compounds through the membrane and involves the use of ATP. ATPase specialized for the transport of copper, normally, have several transmembranar protein domains and some extracellular domains responsible for the binding of copper. CPx motif is one of these extracellular domains that are responsible for the binding of copper, and was shown a similar structure in humans which has an important role in copper deficiency diseases in humans, if not working properly [47]. There are two transmembranar ATPase channels responsible for the transport of copper, CopA, where the copper is uptaked and CopB that excretes copper out of the cell [48]. The homeostasis is controlled by the level of copper, if in excess, copper could cross the cell membrane through CopA or porins, which is coupled to an intracellular protein, CopZ, that binds copper, and transfer the copper to a zinc-containing protein that binds DNA (CopY). When CopY binds copper, zinc is released, losing the affinity to bind DNA, and is not able to bind the promoter and repress the expression of CopB to excrete copper (Figure 6). Then copper may be transported to CopB by molecules, such as glutathione (GSH). Lower concentrations of copper do not enable the transfer of copper from CopZ to CopY, which binds the promoter, and represses the expression of CopB, keeping the copper intracellularly [49].

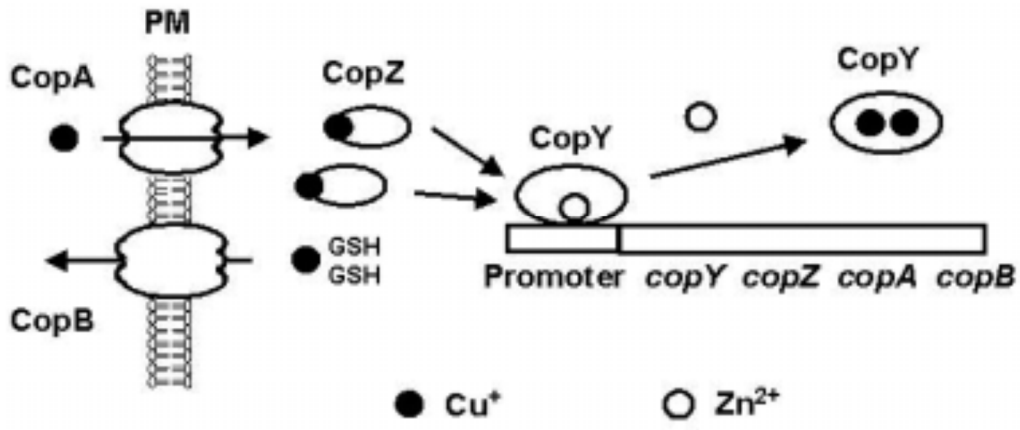

Figure 6 – Copper homeostasis mechanism in bacteria [49].

The mechanism uses preferentially Cu^{+}, a reduced ion of copper ions, which tends to oxidize to Cu^{2+}, but some mechanisms have been reported where copper reduction allows uptake and transport in bacteria, by a cupric reductase called NDH-2 [50]. Furthermore, silver seems to be able to use the same mechanism to be uptaked by bacteria, by the possible lower affinity may explain the high toxicity caused by silver. Copper can be taken up by bacteria using other transporters, but the mechanisms remain mostly unknown, and also seem to have a small influence in the overall homeostatic mechanism [48]. These mechanisms are directly associated with bacterial resistance against ionic copper, and it could be the reason for the lower activity of ionic copper, comparing with copper nanoparticles that may present a different mechanism of uptake by bacteria, these resistance mechanisms cannot protect bacteria. As mentioned above, these mechanisms have been proposed to be related with the bacterial cell wall, and often the death of bacteria is associated with damages in this structure in a similar fashion of silver nanoparticles. The metal catalysis of reactive oxygen species (ROS) is also involved sometimes, where the metals interacts with the cell membrane initiating a cascade of intracellular reactions affecting the permeability of the membrane. A different theory reports that metal nanoparticles are able to interact with thiol groups of proteins, due to the high affinity of metal to sulphur compounds, which leads to their inactivation and consequently loss of functionality of proteins, including enzymes. This mechanism is especially effective if the respiration or transport across the membrane is affected [17]. Finally, a mechanism of slow release of metal ions from nanoparticles was proposed, but the activity of metal nanoparticles has been reported to be higher than the corresponding metal ions what leads to believe that the mechanism is not merely involving the soluble metal ions, which is supported by the resistance mechanism against copper ions described [51].

2.2.2. Copper nanoparticles

Copper is a transition metal that has been used by mankind for more than six thousand years. Its use is related with the properties of copper, such as high electrical conductivity, resistance, recyclability and durability, being applied in several areas, such as constructions, transports, electrical applications or even as a coinage metal [52]. Copper compounds, such as copper sulphate or copper chloride and copper hydroxide, are largely used in agriculture as herbicides, fungicides and pesticides, being considered safe, because they do not penetrate the plant tissues [53]. Antimicrobials are one of the main copper applications, being this metal used as biocide since the ancient Egypt, at 2000 BC [16].

The antimicrobial activity of copper may appear initially surprising, since this element is used by most organisms and it seems in fact an essential micronutrient, for example in human body, where it has an important role as a catalytic cofactor in different metabolic pathways, such as mitochondrial electron transport chain, free radicals scavenging and iron absorption, as mentioned above [38]. Despite the copper, gold and silver belong to the same group in periodic table, copper is the only one that has an important role biochemically.

Copper exists in the oxidation states ranging from +1 to +4, being the cuprous (+1) and cupric (+2) by far the most commons forms. Cu^{+} is not stable is aqueous solutions, generating Cu^{2+} ions and Cu. Normally, Cu^{2+} ions generate coloured solutions, due to the absorption of radiation in the range of 600-900 nm. Different oxidation states of copper have different colours, which allow their use as a sensor of oxidative reactions, such as the Benedict reagent that is used to detect the presence of reducing sugars. These sugars are able to reduce Cu^{2+} (blue) to Cu^{+} (red), and the changing in the colour of the solution give an indication of the presence of reducing sugar. Copper oxidizes easily when exposed to oxygen sources, and to prepare elemental copper nanoparticles techniques that avoid the oxidation must be used, such as synthesis in organic solvents or reduction of produced copper nanoparticles oxidized. The use of copper oxides avoids these problems, as their synthesis does not require removing oxygen or use of hydrogen or even the utilization of organic solvents to prevent the oxidation [52,53].

2.2.3. Synthesis of different nanoparticulated forms of copper

The preparation of copper oxide nanoparticles involves the basic chemical reactions of copper. These nanoparticles are normally synthesized from copper salts, such as copper chloride ($CuCl_2$) and copper nitrate $Cu(NO_3)$ [54]. The reaction of these salts with NaOH produces copper hydroxides and sodium chloride, as seen in equation 1 and equation 2. The obtained solution with copper hydroxides can be heated to obtain copper oxide (CuO), as shown in equation 3. These basic, fast and easy reactions are the basic steps used in most of the synthesis of copper oxides nanoparticles by wet-chemical reactions. The formation of nanoparticles can occur by heating copper hydroxides to obtain the copper oxides. The copper hydroxides normally precipitate and the temperature and time of exposition applied influence the size and shape of nanoparticles obtained. The transformation of copper hydroxides in copper oxide occurs easily by thermal dehydration, due to the formation of a more stable oxidative phase. To avoid the use of high temperatures, this reaction takes place in alkaline

solutions, where the presence of hydroxyl ions (OH^-) can react with copper hydroxide producing an anion that easily converts in copper oxide (equation 4) [55].

Equations:

$$CuCl_2 + 2NaOH \rightarrow Cu(OH)_2 + 2NaCl \quad (1)$$

$$Cu(NO_3)_2 + 2NaOH \rightarrow Cu(OH)_2 + 2Na(NO_3)_2 \quad (2)$$

$$Cu(OH)_2 \xrightarrow{heat} CuO + H_2O \quad (3)$$

$$Cu(OH)_2 + 2OH^- \rightarrow Cu(OH)_4^{2-} \rightarrow CuO + H_2O + 2OH^- \quad (4)$$

Low temperatures lead to an incomplete reaction, obtaining heterogeneous particles. On the other hand, high temperatures may agglomerate the particles [56]. The production of copper nanoparticles in organic solvents is sometimes used to prevent the normal oxidation of copper nanoparticles during the synthesis in aqueous solvents. Often, the temperature required in the production is lower than the temperatures used in aqueous solutions [57]. For example, 1,2-propanodiol was used as a solvent, but the synthesis was difficult to control, and the resulting particle size was more than 100 nm [58].

The synthesis of nanoparticles with controlled size and higher stability can be done using templates. Templates are polymeric matrices that provide a good environment for the controlled growth of nanoparticles. Copper nanoparticles have been synthesized in different templates. Polyethylene glycol was used to prepare copper oxides nanorods from copper chloride salts, which were mixed with the polymer. The remaining of the method was similar to the wet-chemical techniques described above, addition of NaOH and heating. PEG was reported to have an important role in control of the shape of copper oxides. As a non-ionic surfactant, PEG permits the growth of nanoparticles along their chains, leading to the typical nanorod shape [59]. Also, polysaccharides have been used to prepare the nanoparticles in a synthesis defined as “green”, because uses non-toxic chemicals, including organic solvents and use renewable materials. The nanoparticles were synthesized in a starch matrix, which was mixed with the metal salt and heated up to obtain the nanoparticles. The particle size was very small, showing the capacity for size control of the starch matrix [60].

The nanoparticles can be synthesized using surface modifications, which provide suitable properties for specific applications and also stabilize them, avoiding agglomeration. The modifications normally occur by addition of organic groups, containing, for example, thiols, amines or carboxylic acids [61]. The surface modifications can incorporate functional groups in the surface of nanoparticles, providing the desirable effects. Thiols (R-SH) are

molecules containing atoms of sulphur, such as cysteine, that are strongly coordinate by metals, including copper. When exposed to these metal, the thiol groups are automatically adsorbed by the nanoparticles. The thiols can be used to control the growth of nanoparticles. While the nanoparticles are forming and growing, the surface area is decreasing, till the coordination of ligands becomes favourable and stops the growth of nanoparticles. Different concentrations of ligands allow different particle size [62]. Another, modification are the adsorption of amines by metal nanoparticles and is usually used to stabilize the nanoparticles [63]. The interaction of amines with metals is weaker than thiols, and therefore originates bigger particles. Compounds containing the ammonium ion can also be used, having similar effects of amines. Also, carboxylic acids are able to interact with nanoparticles in solution through the negatively charged carboxylate in a similar fashion of thiols. Multiple attachments can increase the stability of the nanoparticle. Fatty acids such as palmitic acid and oleic acid, are examples of carboxylates ligands that have been used to modify the surface of nanoparticles, allowing the control of the size and improving the stability [62,64].

As described, the copper nanoparticles can be synthesized by a large range of techniques, depending on the size or shape desired. The synthesis of nanoparticles of different metals, such as silver, zinc and iron, can also be adapted to produce copper nanoparticles. The use of ligands has also been reported to modify the surface of nanoparticles. An example of copper-based nanoparticles is shown in Figure 7.

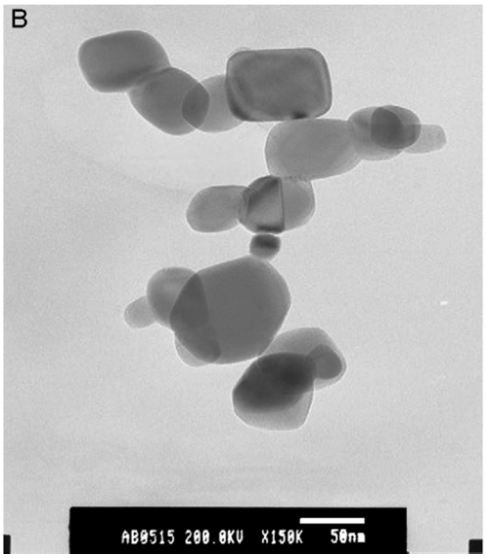

Figure 7- Copper oxide nanoparticles visible in TEM [21].

2.2.4. Antibacterial activity of copper nanoparticles

The antibacterial activity of copper oxide nanoparticles has been studied against a large range of bacteria. For example, nanoparticles prepared by a new method using thermal plasma were tested against different bacteria, such as *S.aureus, S.epidermis, E. coli* and *P. aeruginosa*. These bacteria are normally associated with hospital acquired infections and some of them are multi-resistant against some antibiotics, including strains of *S. aureus* methicillin-resistant. The results showed the effectiveness of copper oxide nanoparticles against all of them, with different minimal bactericidal concentrations. The highest activity occurred against *S. aureus* and *E.coli*. The authors proposed the use of copper nanoparticles in wall coverings, equipment, clothing and bedding in hospital [21].

Antibacterial effect of copper and silver nanoparticles was compared using different bacterial species [25,65]. Both copper and silver nanoparticles present a great inhibition of the bacterial growth against *E. coli, S. aureus* and *B. subtillis*, as seen in Figure 8. The antibacterial activity increases with the increasing of metals concentration in bacterial culture, being the silver nanoparticles those who present the highest antimicrobial activity, although the difference is not too much significant, and in some strains, copper nanoparticles presents higher inhibitory growth, as shown in Figure 9. Another interesting finding comes from energy-dispersive X-ray spectroscopy analysis, where the presence of oxygen was detected, that leads to believe that were used copper oxide nanoparticles [25]. The particle size reveals that the silver nanoparticles used were smaller than the copper nanoparticles. The silver nanoparticles measured around 3 nm, while the copper ones were around 9 nm, which could affect the antimicrobial capacity as explained above. Another report shows a higher activity of copper nanoparticles than silver nanoparticles against *E. coli* and *B. subtillis* [65]. These reports showed that the difference of silver and copper nanoparticles was not remarkable, although is known that silver presents much higher activities than copper. The synthesis of small copper nanoparticles may improve the antibacterial activity and reduce the difference between silver and copper activities.

Finally, metal nanoparticles including copper were tested against a different microorganism, *Saccharomyces cerevisiae*, a type of yeast, showing high biocide activity. In this experiment was tested the toxicity of copper in different forms such as copper oxide nanoparticles, ionic copper and bulk copper oxide. The nanoparticles presented the highest toxicity [44].

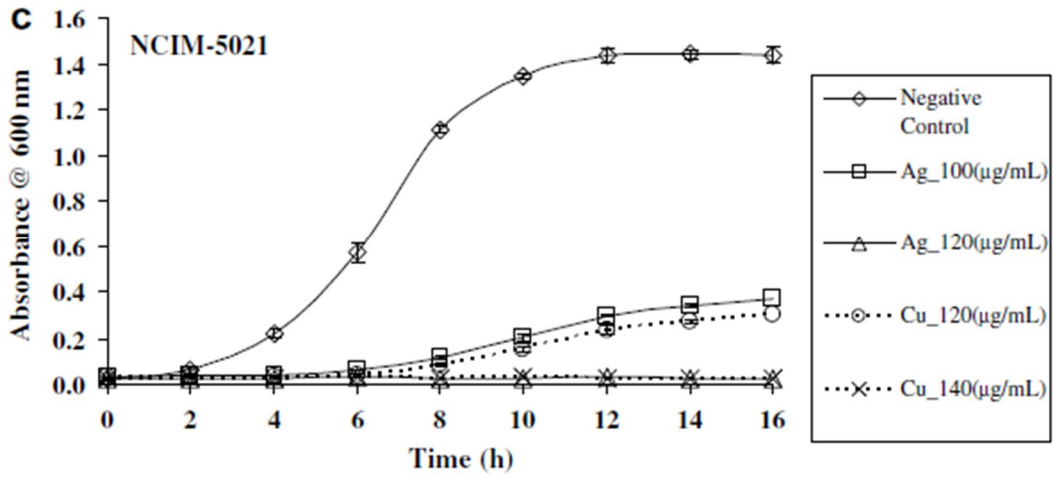

Figure 8- Representative batch growth profile in presence of varying concentration of silver/copper nanoparticles for (a) *E. coli* (MTCC 1302), (b) *B. subtilis* (MTCC 441) and (c) *S. aureus* (NCIM 5021). [25].

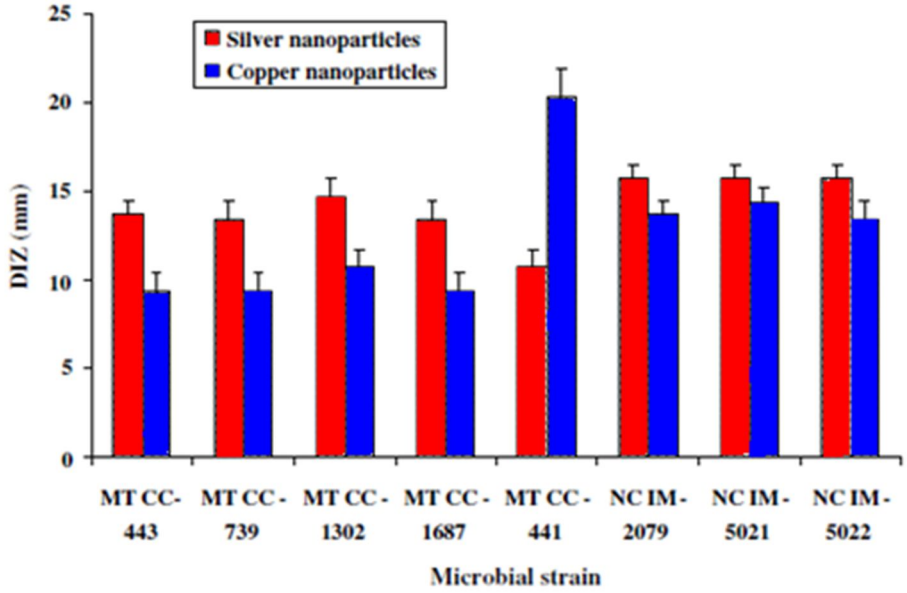

Figure 9- The graph shows the Diameter of Inhibition Zone (DIZ) surrounding silver and copper nanoparticles impregnated disks of 6 mm of diameter in presence of various microorganisms [25].

2.3. Silicon compounds and metal nanoparticles

Silicon is an atom from the same group of carbon, and presents very special physicochemical properties [66]. Silicon has already been used with metal nanoparticles, such as, for the formation coatings to protect the nanoparticles [64,67-70]. This compound is a very reactive element and is present in many compounds, including silica, whose chemical name is

silicon dioxide, SiO_2. Also, it is the most common mineral in the Earth's crust. Both, gel formation and coating are the main utilizations of silica with metal nanoparticles due to the high affinity of silica for some metals forming complexes with them. Iron, copper and zinc nanoparticles are examples of metal nanoparticles already used with silica [64,69-71].

Metal nanoparticles must be stable to allow their effectiveness in different applications, such as catalysis or antibacterial, and modifying the surface of these particles has been reported as a successful approach [66,70]. Since the first studies in stability of nanoparticles, silica was used to avoid problems related with low stability. For example, the surface of zinc oxide nanoparticles (ZnO) was coated with silica improving the dispersibility and reducing the agglomeration of nanoparticles [64]. Also, mineral ferrous oxide, in particular magnetite, was coated to avoid the formation of different oxidized species, such as the mineral ferric oxides hematite and maghemite, showing that silica was able to protect these nanoparticles, even in hard conditions, such as very high temperatures [71]. Silica-coated iron oxide nanoparticles can be seen in Figure 10. Copper nanoparticles have already been reported, such as zinc and iron nanoparticles, to be coated with silica [66]. In this work, the aim was to improve chemical stability of the nanoparticles, because during the storage period, the nanoparticles are unstable, leading to oxidation or aggregation, which represents an increase in particle size. To avoid both problems, the nanoparticles can be prepared in organic solvents, which minimize the surface oxidation. Nevertheless, organic solvents are hazardous and possibly very toxic to biological systems. Another strategy to prepare stable nanoparticles is the use of polymers or surfactants, which enable the interaction of oxygen atoms with the metal particles surface [72]. In addition, silica acts with a similar mechanism, creating a shell around the particle that prevents the contact of metal core with the outside, forming particles with a great stability in aqueous dispersions [66]. Copper nanoparticles with these coatings can be easily prepared by adding of a silica source, such as tetraethyl orthosilicate (TEOS), sodium silicate and (3-aminopropyl)trimethoxysilane (APS) [64,66,70]. Often, the coating preparation requires the addition of two compounds containing silica, in a two-step production. The first step is the absorption, by the surface of metal nanoparticles, of the first silica compound added. The second step is the formation of the coating around the metal core. APS is commonly used during the first step to create this interface due to have amino group, which interact with metal particles easily [70]. In the second step, to obtain the silica coating is normally used either sodium silicate or TEOS that can be hydrolysed and condensed to allow the growth silica layer around the nanoparticle [66].

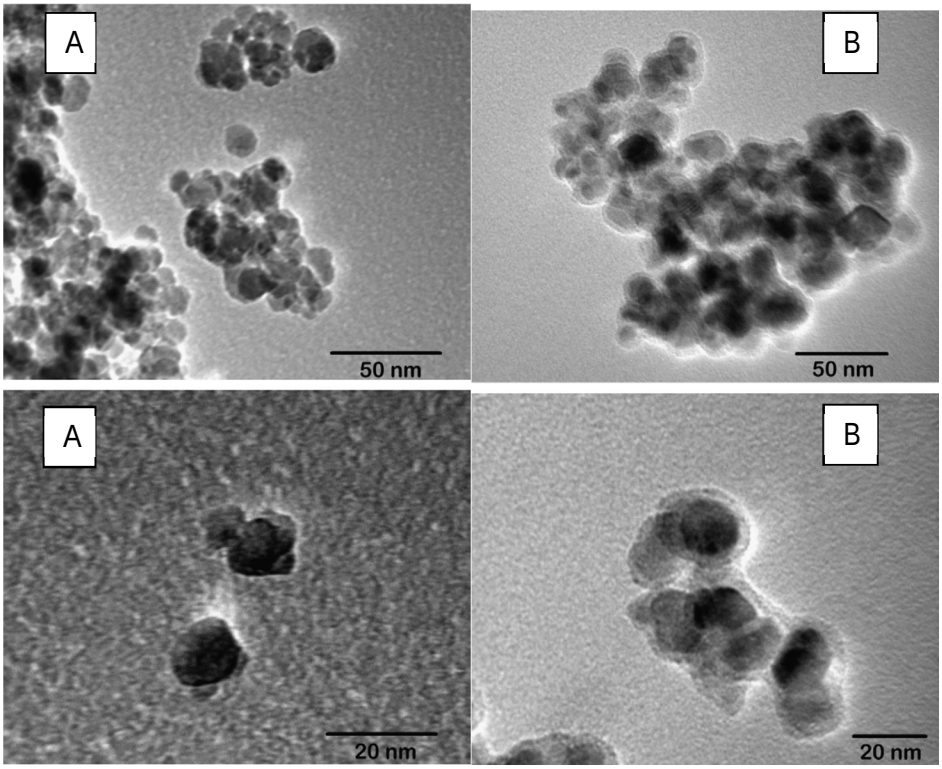

Figure 10- TEM images of Fe_3O_4 nanoparticles (A). Fe_3O_4 nanoparticles coated with SiO_2 (B) – Adapted from [71].

Silica can also be used as a gel, where the nanoparticles can be encapsulated. Nanoparticle compounds in a solid matrix can be defined as nanocomposites [73]. Silica gel is commonly used, and easy to prepare in a three steps reaction, involving hydrolysis, condensation and polymerization. These reactions can be called sol-gel processing. Alkoxysilanes, such as APS, hydrolyse when present in water, forming an alcohol and a hydroxyl alkoxide containing silica, as seen in equation 1. If the reaction is very extensive, i.e. till completion, all the alkoxy groups are replaced by hydroxyl groups, as represented in equation 2. Next, the condensation occurs, in which the hydroxyl alkoxides link together to form dimeric structures, and afterwards large polymeric structures (polymerization). Water or alcohol molecules form during the condensation and polymerization, as represented in equations 3 and 4 [74]. The oxo connections (Si-O-Si) between silicon atoms are responsible to form the gel matrix. Since $Si(OH_4)$ is considered to be the monomeric unit of silica polymer, the four hydroxyl groups can condensate with another molecules to form a branched polymer. As seen in equation 2, water is the limiting factor in the formation of a branched polymer, because in low water environments the formation of $Si(OH_4)$ is reduced, which leads to a reduction in branch degree, and consequently, in gel quality [74].

Sol gel processing reactions [74]**:**

$$Si(OR)_4 + H_2O \rightarrow HO\text{-}Si(OR)_3 + ROH. \quad (1)$$

$$Si(OR)_4 + 4H_2O \rightarrow Si(OH)_4 + 4ROH \quad (2)$$

$$(OR)_3Si\text{-}OH + HO\text{-}Si(OR)_3 \rightarrow (OR)_3Si\text{-}O\text{-}Si(OR)_3 + H_2O \quad (3)$$

$$(OR)_3Si\text{-}OR + HO\text{-}Si(OR)_3 \rightarrow (OR)_3Si\text{-}O\text{-}Si(OR)_3 + ROH. \quad (4)$$

Different techniques can be applied using nanoparticles and silica gels. Pre-prepared nanoparticles can be fixed and/or modified inside the gel matrix, or synthesized inside the matrix [73]. To encapsulate, previously prepared nanoparticles, they can be added into a gel that is being formed. While the matrix is being polymerized, a three-dimensional network can be formed around the nanoparticles, which enables the use of nanoparticles, with desirable properties, directly in the matrix. A previous work showed that iron oxide nanoparticles can be impregnated in a silica matrix, preserving their properties [75]. However, sometimes the entrapment of unstable nanoparticles can cause changes in the properties of nanoparticles, such as agglomeration, which leads to particle size increasing. Also, the uniform dispersion of the particles in the gel is difficult to control. Such problems can be due to the increase in pH and ionic strength during the formation of gel [75]. Modification of the nanoparticles surface to create a stable interface, between particles and matrix, has been proposed to reduce the instability of nanoparticles [75]. Once again, silica coating demonstrated, that was effective to preserve nanoparticle properties, which were impregnated in a silica matrix. This matrix was prepared using two different silica sources: Sodium silicate and tetramethoxysilane (TMOS) [75]. Synthesis of nanoparticles within a silica matrix has also been proposed to overcome the difficulties associated with the fixation of already formed nanoparticles. It has the potential to produce nanoparticles without agglomeration and homogeneously distributed in the matrix. The synthesis can occur using the precursor of nanoparticles linked to the matrix, which will react to produce the nanoparticles, and organic functional groups have been reported to achieve this. These functional groups are able to create a link between the metal ions and silica matrix. For examples, the organic molecules may contain amino groups, which can chelate the metal cations and link them to the silica network. Then, the nanoparticles are obtained by degradation of the organic groups with controlled thermal treatment, as schematized in Figure 11. The thermal treatment involves very high temperatures (500°C) to degrade the organic groups and may also oxidize elemental nanoparticles, obtaining powders of their metal oxides. If the aim is not produce the metal oxide nanoparticles, it can be used hydrogen to reduce the metal oxides [73,76]. The nanoparticle size is directly influenced by the dispersion in gel, decreasing the

size distribution, with the increasing of the uniformity of dispersion [77]. The size distribution can be improved synthesizing the nanoparticles in a uniform matrix, where the growth of nanoparticles is controlled by the remaining space between polymeric branches. If the gel is uniform, the spaces are all similar, and consequently the growth of nanoparticles is limited, obtaining a narrow size distribution [76].

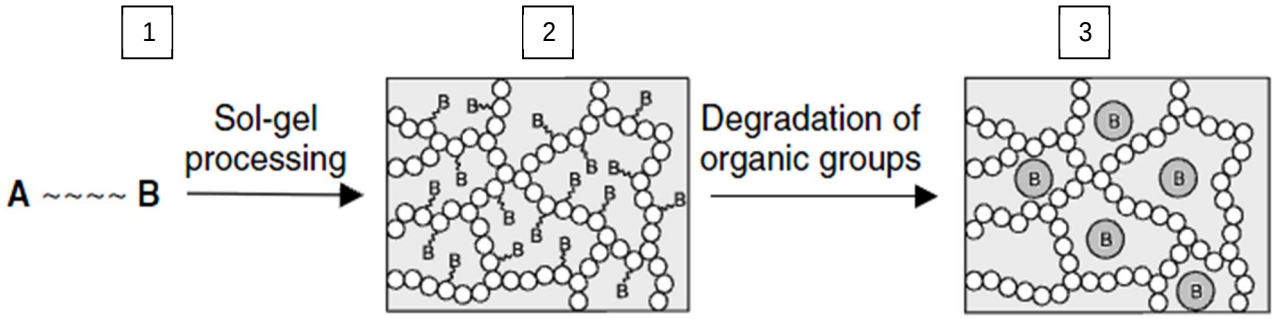

Figure 11- Preparation of nanocomposites by sol-gel processing of organofunctional single-source precursors. The connected spheres represent the gel network formed from precursor A. In step 1, A are the silica precursor linked to B that is the metal source, for example a metal salt (~ is the organic group connecting A and B). In step 2, the sol-gel processing formed a gel, while the metal (B) kept attached to the matrix. In step 3, silica matrix was heated up and the organic groups that are linked to A and B were degraded, forming the nanoparticles [73].

Zinc oxides have been already produced inside a silica matrix. Comparing with zinc oxides produced without the use of a silica matrix, the size changes were not significant. Also, the particles were highly uniformed dispersed. The stability of these particles was obtained by using a surfactant that stabilized the surface. The structure proposed for the ZnO and silica polymer is represented in Figure 12 [78].

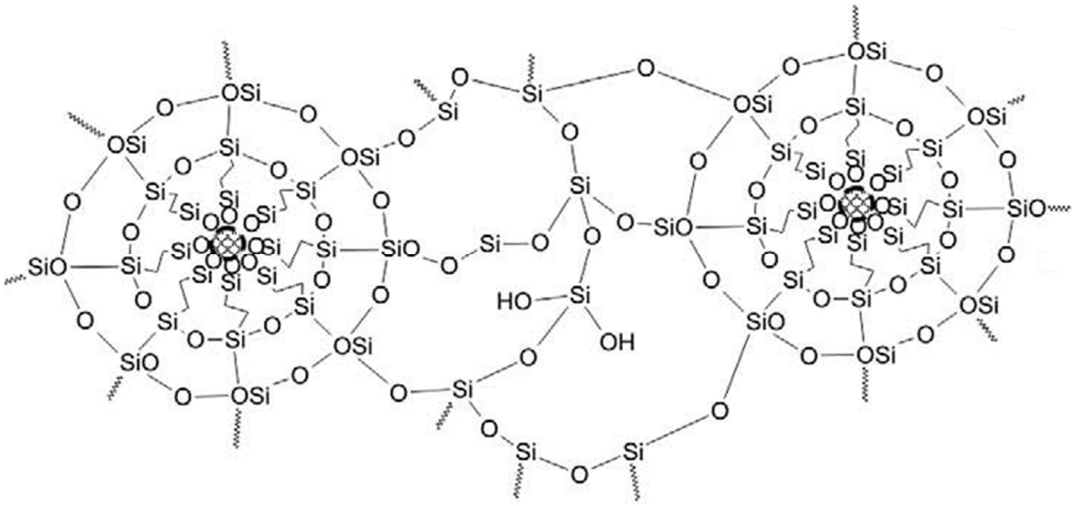

Figure 12- Scheme of ZnO nanoparticles incorporated inside a silica matrix [78].

Silica with metal nanoparticles has already been tested for antimicrobial purposes. Silver nanoparticles coated with silica were tested against some microorganisms in seawater, which are responsible for the corrosion of all material immersed in these environments. Silica-coated nanoparticles showed to be very effective protecting the surface from oxidative microorganisms in water [79]. Also, the release of metal ions was tested during the exposition of seawater, showing that the amount of ions released is much lower than the released by the metallic materials commonly used, which can cause environmental problems, due to the high concentrations of metals generated after long times of exposition in water [79]. A different work, reported that copper-based nanoparticles, with 20 nm, were prepared in a silica matrix by production of a thin film under high temperatures. Both, copper and copper oxide nanoparticles were produced and tested against E*scherichia coli*. A solution containing these bacteria was spread in the silica film with copper nanoparticles and it was seen a great reduction of their viability [69]. Such results suggest that it is possible to apply copper nanoparticles, immobilized in silica matrixes, in surfaces, where the bacterial contamination has to be prevented, such as food packing or hospital surfaces.

2.4. Analytical techniques for characterization of nanoparticles

This section introduces some of the analytical techniques that could be used during a study of the development of copper nanoparticles with antibacterial activity. To study nanoparticles is important to characterize them, and Dynamic Light Scattering (DLS) can determine the size distribution of nanoparticles in a solution. Transmission Electronic Microscopy (TEM) is also useful not only for the particle size but also to obtain images that enable a better morphological characterization of nanoparticles such as possible agglomeration, coatings and shape. The Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES) is a very sensitive technique used in elemental speciation that is able to determine the concentrations of some elements such as copper down to ppb values. These will be the three main analytical techniques used in the experimental part of the project.

2.4.1. Dynamic Light Scattering (DLS)

DLS is a technique to measure the particle size distribution of particles suspended in a solution. This technique measures the Brownian Motion, the random movement of particles in a solution, and establish a relation with the particle size because the increasing of the particle

size leads to a proportional decrease to the Brownian Movements, i.e. slower particles. The particles diameter is calculated from Stokes-Einstein equation:

$d(H) = (kT / 3 \pi \eta D)$

d (H) - Hydrodynamic diameter

D - Translational diffusion coefficient

k - Boltzmann's constant

T – Temperature

η – viscosity

This equation shows the relation between the hydrodynamic diameter and other physical parameters: Temperature, viscosity and translational diffusion coefficient. The last one is used to calculate the hydrodynamic diameter, so it is important that temperature and viscosity of the solutions are known and are constant during analysis, to obtain meaningful particle sizes. The speed of particles is obtained by measuring the different scattered light fluctuation rates of particles in solution. The scattered light is detected in the instrument and correlated to calculate the translational diffusion coefficient to obtain the particle size. The results are obtained in a graph representing the distribution sizes of the particles in solution. Figure 13 shows a graph representing the size distribution of an aqueous solution with nanoparticles.

The DLS enables the measurement of nano-sized particles depending on the range of particles that is optimized to analyse. This nano-sizer allows the study of nanoparticle coatings, if these coatings influence the behaviour of the particle in solution. On the other hand if the particles are not spherical the correlation of particle size and the movement in solution is not the same and the obtained values could carry a bias [80,81].

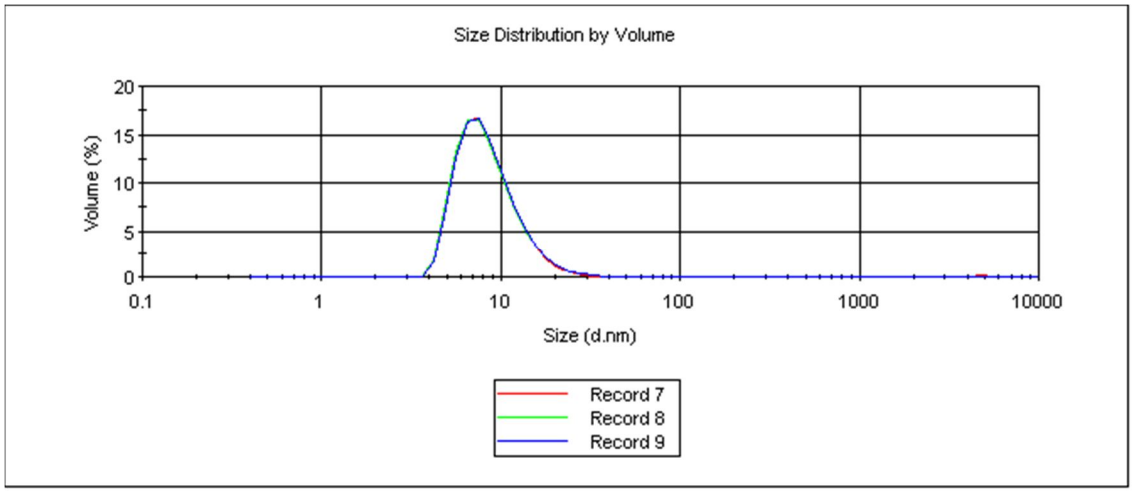

Figure 13- Size distribution by volume obtained using DLS for nanoparticles in aqueous system.

2.4.2. Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES)

The ICP-OES is an analytical technique developed to identify and quantify elements in different chemistry systems. The ICP-OES is characterized by a plasma source which is responsible for atomization of samples. The plasma can be defined as a gas electrically neutral composed by ions, electrons and atoms, whose temperature is incredibly high and can reach values near the temperature on the sun's surface, 8000K. In ICP-OES analysis the sample is pumped into the instrument, where the sample will be nebulised. Next, the water is removed and the sample is atomized in the torch, where the atoms become excited, emitting light of a characteristic wavelength. The ICP torch is composed by a plasma flame generated by a flow of argon. This light will be detected and measured in the spectrometer, to obtain the concentrations of each element. A scheme of an ICP-OES analyser is represented in Figure 14. During this project this technique can be used to quantify copper with a great sensitivity, range of ppb.

The limitations of this technique could be related with the emission at same wavelength of different elements. Also the formation of different species for the same element, such as different ions and neutral species that have different emissions could be a problem in analysis [82].

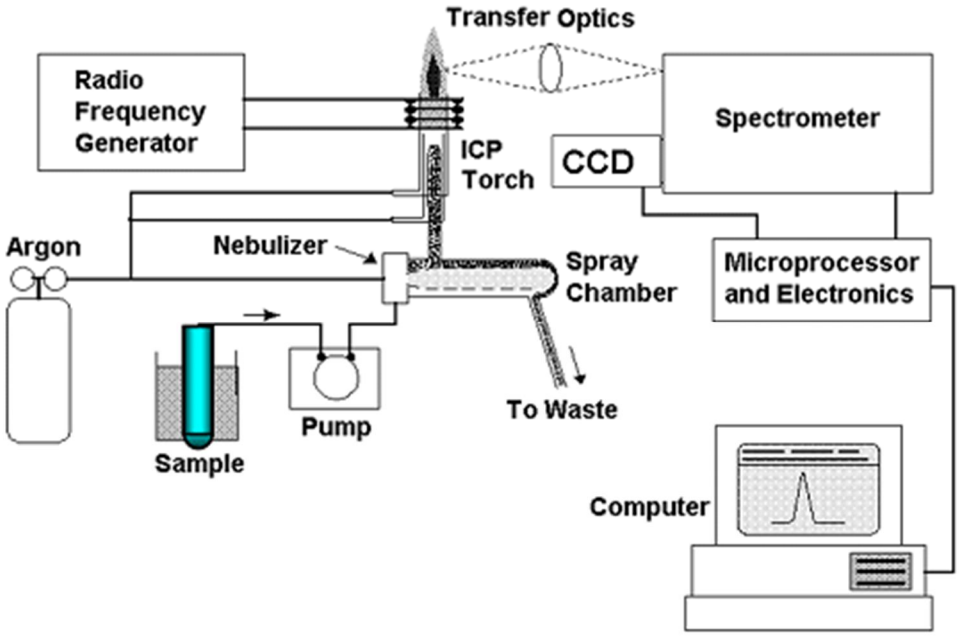

Figure 14- Schematic of the composition of an ICP-OES analyser [108][1].

[1] Image adapted from http://www.aandb.com.tw/Page0001/icp_oes_01_optima_7x00_dv.html

2.4.3. Transmission Electron Microscopy (TEM)

TEM is a microscopic technique with great definition that consists in an electron beam crossing through an ultra-thin sample that is processed to obtain the image. Information about the chemical composition, morphology and structure is obtained through the variety of signals that result from the interaction between electron beam and specimen. As the electrons pass through the specimen, the sample has to be very thin. The maximal thickness can be obtained increasing the acceleration voltage and decreasing the atomic number of the atoms in the sample [30]. The electron microscopy was developed to overcome the limited resolution of light microscopy, due to the wavelength of visible light. The detection limit of TEM leads to their use in nanotechnology because it allows the characterization of nano-sized material. Shape, particle size, agglomeration and surface modifications can be observed in TEM images. This technique is able to analyse either solid or liquid samples, such as suspensions, and requires a very small amount. Some figures showed above in this monograph were obtained by TEM (Figure 7, 10, 22 and 32).

The limitations of this technique can be related with the difficult preparation of some samples, especially due to the thinness required. Often the sample can also be altered during the analysis. The high cost involved not only in equipment but also in sampling could limit their use. Finally the detection limit sometimes is not enough to observe very small structures [83].

2.4.4. X-Ray Diffraction

X-ray diffraction is a technique used to identify materials, in particular crystalline substances. X-ray diffraction analysis considers that the materials can be characterized in two forms, crystalline or amorphous, which are distinguished by the order of their structures. A crystal is composed by an ordered structure, with its atoms arranged in a regular pattern that may be a repetition of a smaller unit, whereas an amorphous compound presents a disordered structure, with atoms distributed randomly. X-ray diffraction consists in the analysis of a diffraction pattern that is obtained by incidence of an x-ray beam through a crystal that diffracts the beam in many directions. The different angles of diffraction and corresponding intensities can be detected to determine the atomic structures present in the crystal. For different crystals are obtained different diffraction patterns, which means that can be used as a “fingerprint” of a substance. This method is only possible due to the X-rays have a wavelength in the order of 1 Angstrom (0.1 nm), which is in the same order of the size of an atom [84].

Braggs's law can explain this phenomenon of interference pattern of X-rays scattered by crystal (Figure 15), and is represented by the following equation:

Bragg's Law: $n\lambda = 2d \sin \theta$

λ – Wavelength of the x-ray beam
θ – Incident angle
d – Distance between atomic layers in a crystal
n – Order of the diffraction peak (integer number)

Bragg's law establishes a relationship between an X-ray beam with a specific wavelength (λ), the refracted angle (θ), and the distance between atomic plans (d). The wavelength is known and the angle θ can be determined experimentally, which allows the calculation of the distance between atomic layers (d). the interpretation of the calculated d values should allow the identification of materials by comparison and matching with standard reference patterns, once each compound presents a unique patterns.

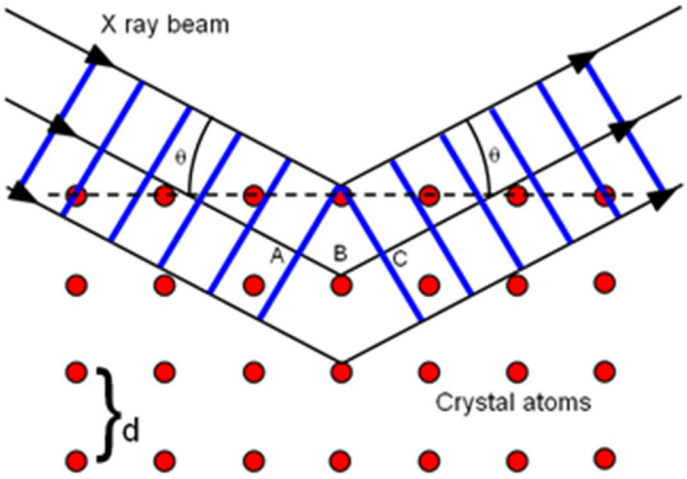

Figure 15 – Schematic of an incident X-ray beam being diffracted by the atoms present in the different layers of the crystal[2].

In particular, the method used was X-ray powder diffraction, to identify crystalline solids. The results are normally analysed in a diffractogram representing the diffracted intensity as a function of angle 2θ, that is obtained during the scan of the sample and the peaks

[2] Image obtained from http://tap.iop.org/atoms/xray/530/page_47297.html

detected correspond to the angles in which the X-ray beam were diffracted by the crystal. This method can be used not only to determine mineral structure of compounds, but also is able to determine the degree of crystallinity [85].

In conclusion, X-ray powder diffraction should be able to identify the materials synthesized, even if there is a multiplicity of components in a mixture. Also, sample preparation is easy and the analysis is very quickly.

3. Material and Methods

3.1. Materials

Chemical Compound	Chemical Formula	Supplier
Copper (II) Chloride di-hydrate	$CuCl_2.2H_2O$	Sigma
L-Tartaric acid	$C_4H_6O_6$	Sigma
Adipic acid	$C_6H_{10}O_4$	Sigma
Citric acid	$C_6H_8O_7$	Sigma
Malic acid	$C_4H_6O_5$	Sigma
Sodium Hydroxide	NaOH	Sigma
Iso-sensitest broth CM 0473	Not applicable	Oxoid
Iso-sensitest agar CM 0471	Not applicable	Oxoid
Sodium silicate solution	SiO_2.NaOH	Sigma

3.2. Methods

3.2.1. Copper hydroxides ligand-modified nanoparticles synthesis

$Cu(OH)_2$, $Cu(OH)_2$Ad, $Cu(OH)_2$Mal, $Cu(OH)_2$Cit, $Cu(OH)_2$Tart and $Cu(OH)_2$Tart-Ad were prepared from copper chloride as described in patent WO/2008/096130. $Cu(OH)_2$Tart-Ad nanoparticles synthesized were dried at 45°C overnight and then resuspended into the same mass of water to obtain initial concentration.

3.2.1.1. Identification of synthetic materials

The materials synthesized were completely dried at 45ºC and the powders were milled and analysed by X-Ray Diffraction at the Earth and Environmental Department at the University of Leeds.

3.2.1.2. Copper hydroxide ligand-modified nanoparticles in broth:

The copper nanoparticles suspension prepared previously and the dried and resuspended solution of copper nanoparticles were diluted in broth on a range of concentrations between 1 and 100 ppm. Then, the samples were analysed by ICP-OES (JY2000, Horiba Jobin Yvon). Particles size analysis was not carried out, due to the low concentration of nanoparticles which is below to their detection limit.

3.2.2. Nanoparticles modifications

3.2.2.1. Heat treatment

An attempt to synthesize copper oxide nanoparticles was carried out from copper chloride as described in patent WO/2008/096130. This synthesis took place under a boiling solution, closer to 100°C. Tartaric acid and adipic acid were used as ligands.

3.2.2.2. Silicon modified copper hydroxide nanoparticles

The procedure was the same as used in the synthesis of copper hydroxide ligand-modified nanoparticles (3.2.1). The sodium silicate solution with a concentration of 10, 20, 30 and 40 mM of silicon was added into the solution containing copper chloride and the ligands which were then titrated with NaOH 5M. The silicon modified copper hydroxide nanoparticles were diluted in broth on a range of concentrations between 1 and 100ppm of copper.

3.3. Entrapment of nanoparticles in a silica gel matrix

$Cu(OH)_2$Tart-Ad were prepared from copper chloride as described in patent WO/2008/096130. Instead of use sodium hydroxide, was used a concentrated solution of sodium silicate (7M). The final gel with copper was dried to be analysed by TEM.

3.4. Antibacterial activity of different copper hydroxide nanoparticles synthesized

3.4.1. Copper stock suspensions

Copper suspensions with concentrations of 1, 10 and 100 ppm were prepared in broth by diluting the original copper nanoparticle suspension. The suspensions were filtered through a 0.2 μm membrane to sterilize the solution.

3.4.2. Preparation of broth and agar media

The broth solution was prepared in ultrapure water and then autoclaved at 121°C during 15 minutes. Agar plates were prepared to assess a possible contamination of *E.coli* culture. The agar medium was prepared in ultrapure water and then autoclaved. Next, the solution was cooled down to 50°C and distributed in sterile petri dishes to form the agar gel. The agar plates were kept on fridge upside down.

3.4.3. Determination of antibacterial activity

To determine the amount of bacteria in solution was used a turbidimetric assay for quantitation of viable bacterial density, using *Escherichia coli* NCTC11100. Bacterial cells present a higher refractive index than water, thus scattering incident light, which can be

measured using a visible wavelength. The bacteria cultures were prepared in the day before to grow overnight at 37°C with shaking. Different decimal dilutions of bacterial culture were prepared in a sterile 96 well-plate and the optical density was measured at 595 nm using a plate reader (Multiskan RC 351, Labsystems). The same concentrations of bacteria were used for each concentration of copper tested in the sterile 96 well-plate. The plate was kept at 37°C with shaking and the measurements were carried out at 0, 2, 4 and 6 hours after prepared the plate.

To every sample the blank was corrected, by subtraction of the baseline (without bacteria) to every well on the correspondent row in the 96 well-plate.

3.5. Copper phase distribution analysis:

All the samples were diluted in 5% nitric acid to dissolve completely the copper and then analysed by ICP-OES, using a wavelength of 324.754 nm. Different standard calibrations were used, with copper concentrations ranging between 0 and 150 ppm. To study the fractions of copper, i.e. soluble, nanoparticulated and precipitated, three aliquots were collected for each pH. One aliquot was used to calculate the total copper in solution, a second one was centrifuged and the supernatant was analysed and the last sample was ultrafiltered through a membrane of 3KDa and then analysed to determine the soluble copper. The calculations used were:

Copper Precipitated % :	$[(Cu_{total} - Cu_{supernatant})/ Cu_{total}] * 100$
Copper Soluble % :	$(Cu_{soluble} / Cu_{total}) *100$
Copper Nanoparticulated %	$100 - Cu_{soluble}$ (%) $- Cu_{precipitated}$ (%)

3.6. Particle Size analysis:

Dynamic Light Scattering (DLS) (Zetasizer, Malvern Instruments) was used to obtain the particle sizes distribution. A 0.5 ml sample was transferred to a cuvette to be analysed. All the samples were analysed in DLS immediately after have been prepared. The error bars shown in the particles size distribution were obtained from 3 measurements.

3.7. Transmission Electron Microscopy analysis

TEM analysis was carried out by Dr. Emma Thomas-McKay at the Multi Imaging Centre of the Department of Physiology, Development and Neuroscience at The University of Cambridge.

4. Results and Discussion

4.1. Copper hydroxides nanoparticles synthesis

The first part of this work consisted in the synthesis of nanoparticulated copper materials, in which different ligands were tested in the synthesis of copper hydroxide nanoparticles. Also, the materials were characterized by Dynamic Light Scattering (DLS), to obtain the size distribution of particles in suspension, and by ICP to determine the copper phase distribution[3], with a particular interest in the percentage of nanoparticulated copper.

Copper is a hydrolytic metal, that forms ions in aqueous solution that act as a Lewis, i.e. receive electrons from the solution decreasing its pH (Equation 5 and 6). Thus, when copper chloride was dissolved in water, the final pH was below 2. The addition of sodium hydroxide into the copper solution increased the pH and, above pH 4 copper started precipitating, as seen in the Figure 16. The precipitation seen was due to the formation of copper hydroxide that was not soluble (Equation 7). The overall reaction of copper hydroxide synthesis in aqueous solution may be explained by the Equation 8. This reaction was the basis of the copper nanoparticles formulation and the control of this agglomeration of copper hydroxides can lead to the synthesis of the respective nanoparticles.

Equations:

$$CuCl_2.2H_20\ (s) \rightarrow Cu^{2+}(aq) + 2Cl^-(aq) + 2H_20\ (l) \quad (5)$$

$$Cu(H_20)_n^{2+} + H_20 \rightarrow Cu(H_20)_{n-1}(OH)^{1+} + H_30^+ \quad (6)$$

$$Cu^{2+}(aq) + 2OH^-(aq) \rightarrow Cu(OH)_2\ (s) \quad (7)$$

$$CuCl_2.2H_2O + 2NaOH \rightarrow Cu(OH)_2 + 2NaCl + 2H_2O \quad (8)$$

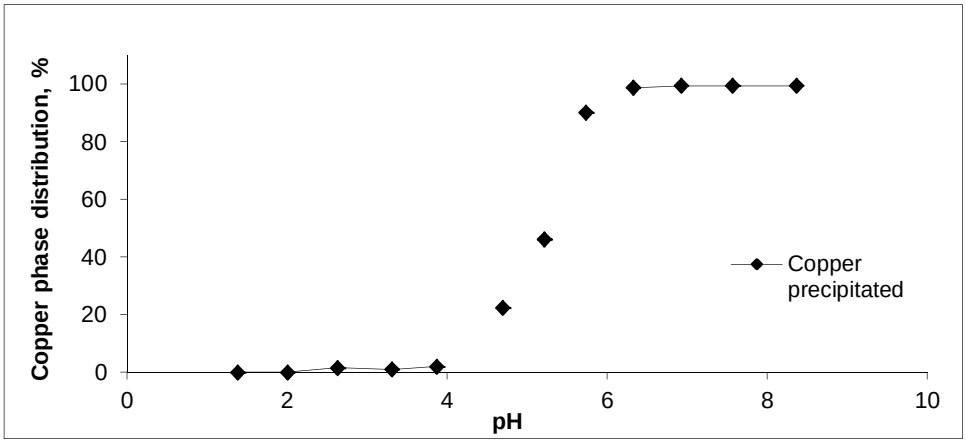

Figure 16- Copper precipitation curve at different pHs.

[3] Copper phase distribution refers to the differences between soluble, nanoparticulated and precipitated copper and not mineral phases of this compound.

The synthesis of copper hydroxide nanoparticles in the current work consists in mixing copper ions and organic ligands at a low pH and then increase to a higher pH, using a strong alkaline solution, such as sodium hydroxide, to cause the solid precipitate of solid-ligand modified copper ions materials. The manipulation of ratio, initial and final pH and the rate of change between the initial and final pH are essential for the formation of nanoparticles. The starting pH should be below the polymerisation pH of copper, between pH 3 and 4, also the final pH should be above this range of pH to produce solid copper hydroxides. Malic acid and citric acid were tested in the formulation of copper hydroxide nanoparticles as they are protonated at low pH and have the potential to be incorporated into the solid phase of the ligand modified materials.

Malic acid is a dicarboxylic acid that is deprotonated (malate) in aqueous solution at physiological pH, and consequently, becomes negatively charged. It is present fruits, such as apples and it is used as a chelating agent in magnesium supplements. Malic acid is not only able to transport this mineral to different tissues, especially brain, but also to bind aluminium to be excreted out of the organism, preventing its accumulation in the tissues [86]. The use of this organic acid in the synthesis of copper-based nanoparticles resulted in the production of nanoparticulated copper in low amounts, only 30% at pH 7, as seen in Figure 17A. These experiment indicates that possibly malic acid is a strong chelator that keeps the metals bound in solution, being inadequate for the synthesis of nanoparticles, as showed the high soluble fraction of copper obtained even at higher pH. The particle size distribution of copper hydroxides nanoparticles synthesized with malic acid showed particles around 30 nm, as seen in Figure 17B.

Citric acid is a tricarboxylic acid that is also deprotonated in aqueous solution (citrate) at physiological pH. This molecule can be used as a chelating agent to keep metals in aqueous solution, and it is also very important in the human metabolism, therefore should not present safety issues. The addition of citric acid showed again that nanoparticulated copper was synthesized as seen in Figure 18A. Also, copper was not completely soluble, at higher pH, being mostly nanoparticulated, especially above pH 6. At pH 8, about 63% of the copper was nanoparticulated, but the particle size analysis showed that most of these nanoparticles were not stable and possibly agglomerated quickly, being detected only very large microparticles (Figure 18B). DLS is not appropriate for the analysis of large particles, and great error bars were determined due to sedimenting particles which are known to affect the measurements.

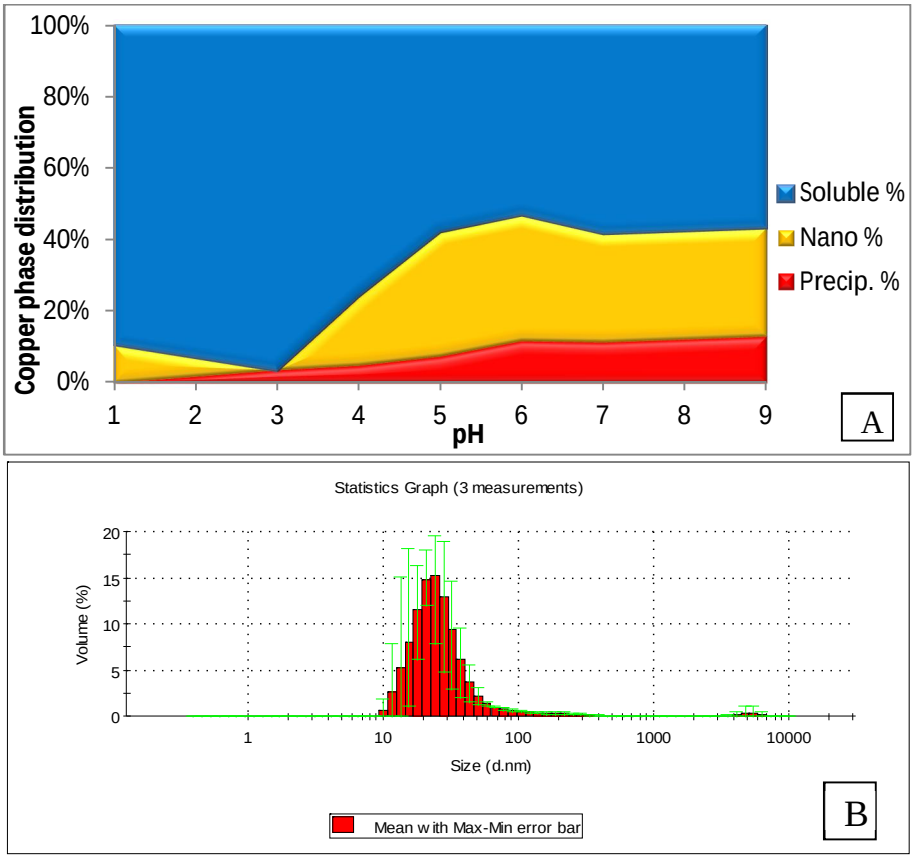

Figure 17- Copper phase distribution as a function of pH (A) and particle size distribution of $Cu(OH)_2Mal$ (B).

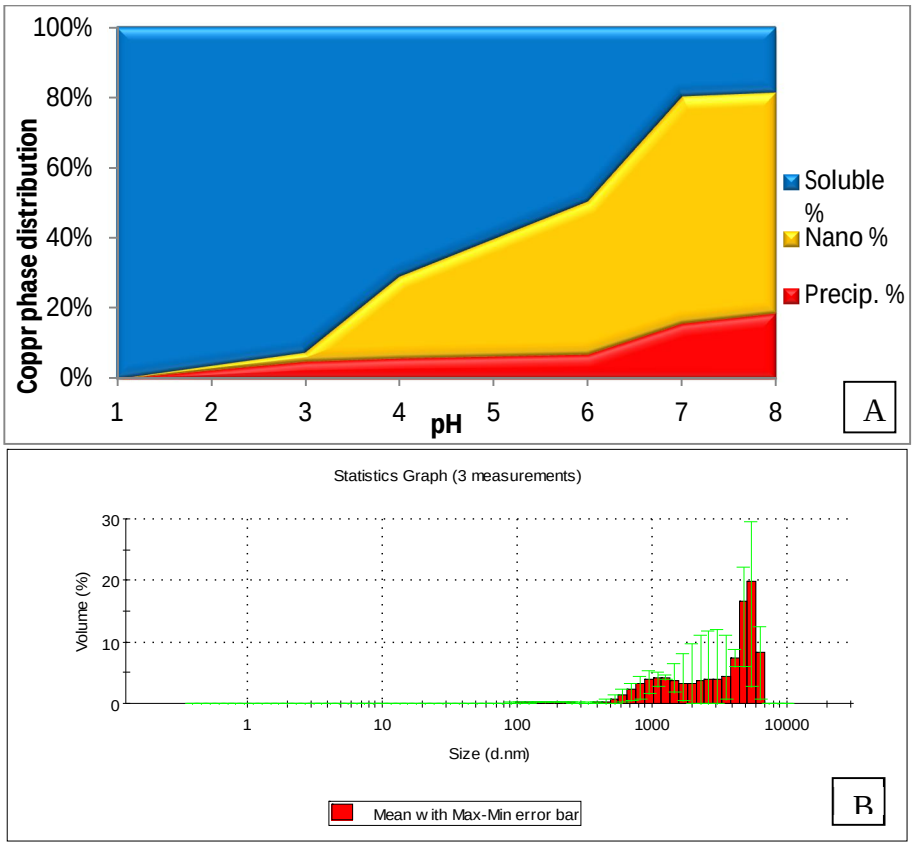

Figure 18- Copper phase distribution as a function of pH (A) and particle size distribution of $Cu(OH)_2Cit$ (B).

The results using malic acid showed that only 30 % of the total copper was nanoparticulated, which was too low yield of nanoparticles production. The use of citric acid improved the amount of nanoparticles obtained to 70%, but in the particles size distribution were detected only large particles, probably due to the quickly agglomeration of smaller nanoparticles. Thus, the following step was to test a different ligand, and tartaric acid was used using the same procedure as used previously. The synthesis with this ligand improved the amount of nanoparticulated copper to 70% of the total copper, and reduced the precipitated copper (Figure 19A), comparing with the synthesis using malic acid and citric acid. Also, an improvement in particle size distribution was seen, in which were detected very small nanoparticles of 1 nm with a narrow distribution, which means that the size of the particles did not present a great variation (Figure 19B). Although the improvement in the amount of nanoparticulated copper and also in particle size in the synthesis of nanoparticles using tartaric acid, the amount of copper soluble was still higher than 20%, and the pH was difficult to control during the experiment, due to the low buffer capacity of the solution. For that reason, adipic acid was added to improve the buffer capacity of the solution.

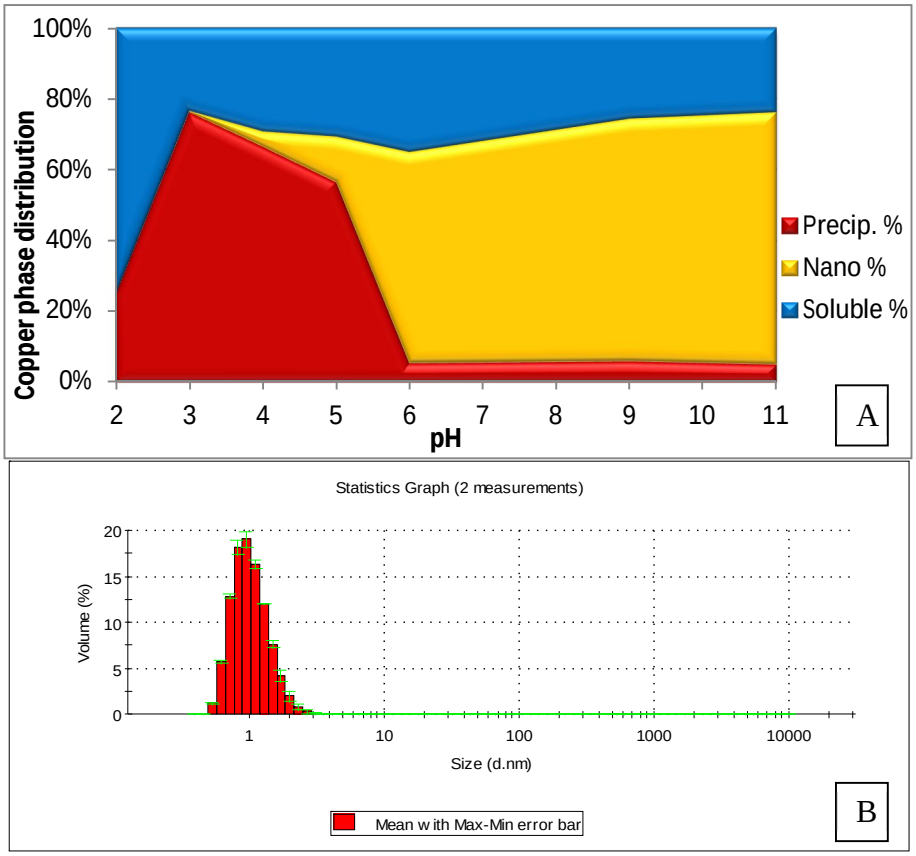

Figure 19- Copper phase distribution as a function of pH (A) and particle size distribution of $Cu(OH)_2Tart$ (B).

Tartaric acid and adipic acid were then used as ligands, which led to the production of copper nanoparticles, as already expected (Figure 20). Comparing with the experiment using only tartaric acid, the amount of nanoparticulated copper increased from pH 5 till pH 8, where it reached more than 90% of the total copper. Also, the precipitation at higher pH was reduced. A peak of precipitation was observed at pH 4, which may be due to the beginning of the formation of copper hydroxides that were not soluble. Although there was a continuous formation of copper hydroxide during the titration, copper hydroxides nanoparticles were formed above pH 4 only. Tartaric acid was the main responsible to the formation of copper hydroxide nanoparticles, because the same experiment using, only adipic acid, showed that there was any formation of nanoparticulated copper, more than 99% was precipitated copper. The higher interaction between copper and tartaric acid may increase the repulsion between particles, especially when tartaric acid was charged (tartarate). Its pKa_2 is 4.25, which leads us to believe that can be associated to the formation of nanoparticles observed above pH 4. The pH influenced the amount of nanoparticles obtained and also their size. At pH 7 the nanoparticles were larger (~70 nm) than at pH 8 (3-4 nm). This decrease in particle size may be explained by the disaggregation that occurred after the precipitation of copper. An evidence of the end of the disaggregation process can be seen in the Figure 21A where the size of nanoparticles was reducing with the increasing of pH. Drying and resuspending the copper nanoparticles suspension did not modify the size of nanoparticles obtained, as seen in Figure 21 B and C. Also, the peaks obtained were narrow, which means that the population of nanoparticles is can be well defined by size. The nanoparticle size and the narrow size distribution obtained by DLS at pH 8 can be confirmed in the TEM images, Figure 22, where the particles were very small, well-dispersed and seemed to present a very similar size between them.

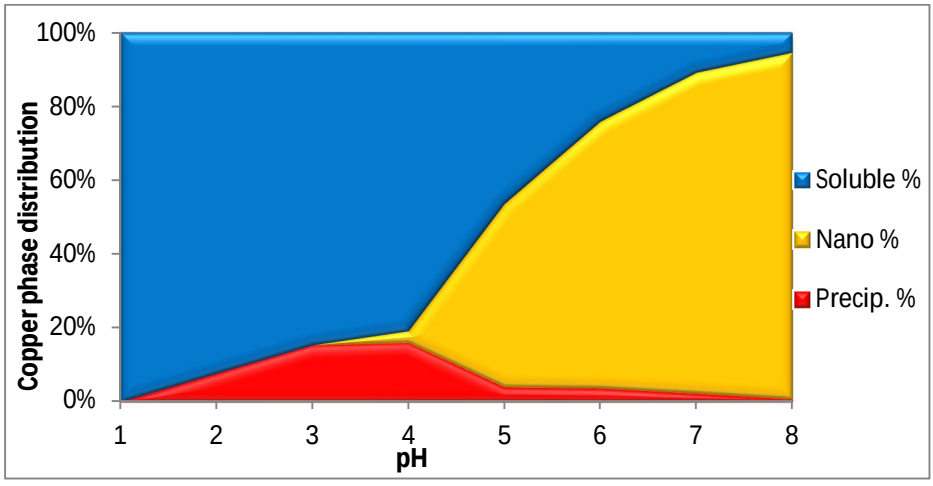

Figure 20- Copper phase distribution of $Cu(OH)_2$Tart-Ad.

Comparing with the previous experiment, using only tartaric acid, great improvements were obtained, such as high nanoparticulated copper at physiological pH (6-8), and consequently a reduction in precipitated and soluble copper, was also obtained narrow sizes distribution of nanoparticles. Thus, the effect of adipic acid may be due to the increase of buffer capacity that increased the amount of NaOH added during the titration to reach the same pH, which lead to the production of nanoparticulated copper, decreasing the solubility.

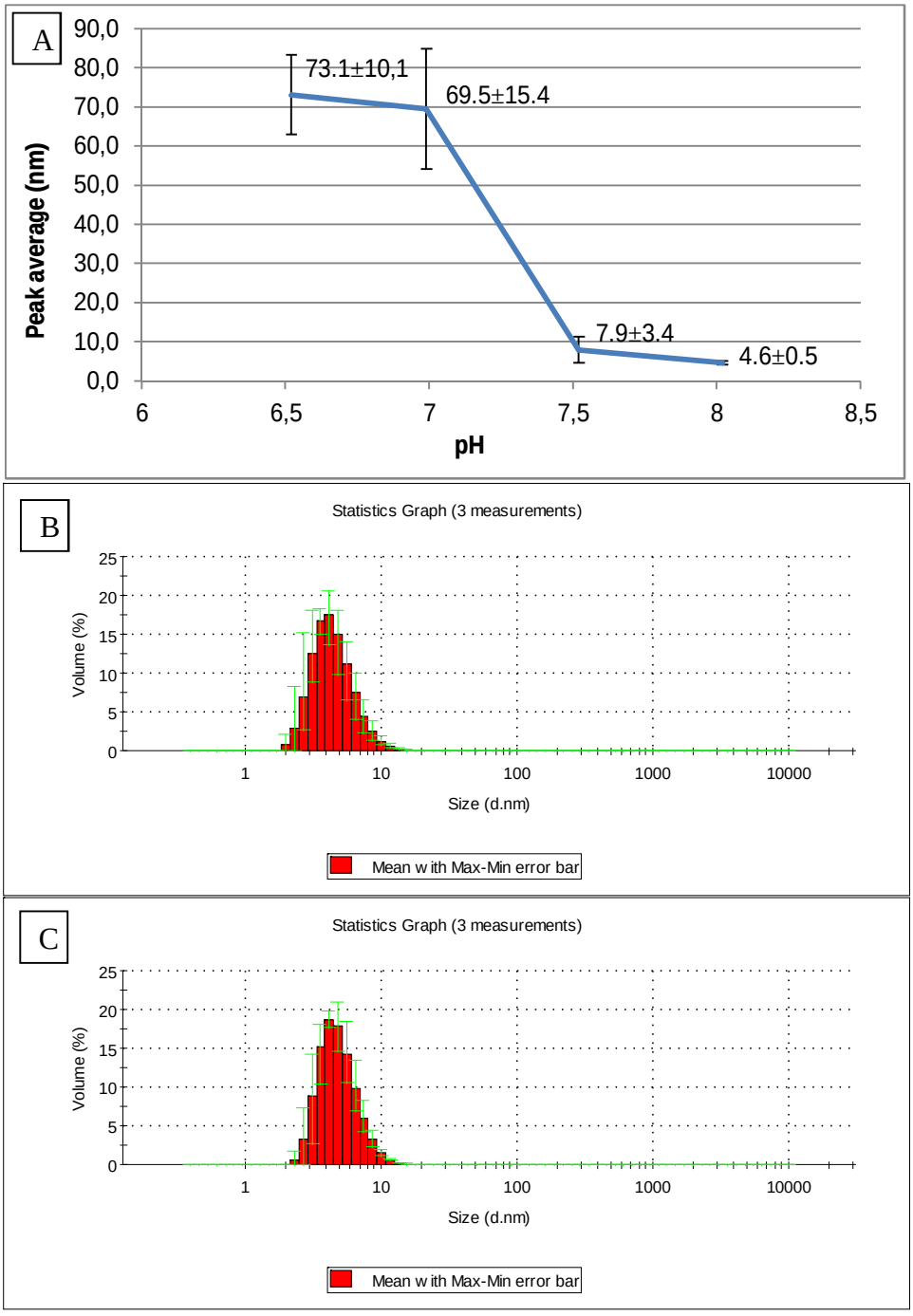

Figure 21- Particle size distribution of copper nanoparticles synthesized using the ligands tartaric acid and adipic acid, $Cu(OH)_2$Tart-Ad, A) as a function of pH; B) at pH 8.2 and C) pH 8.2 dried and resuspended nanoparticles.

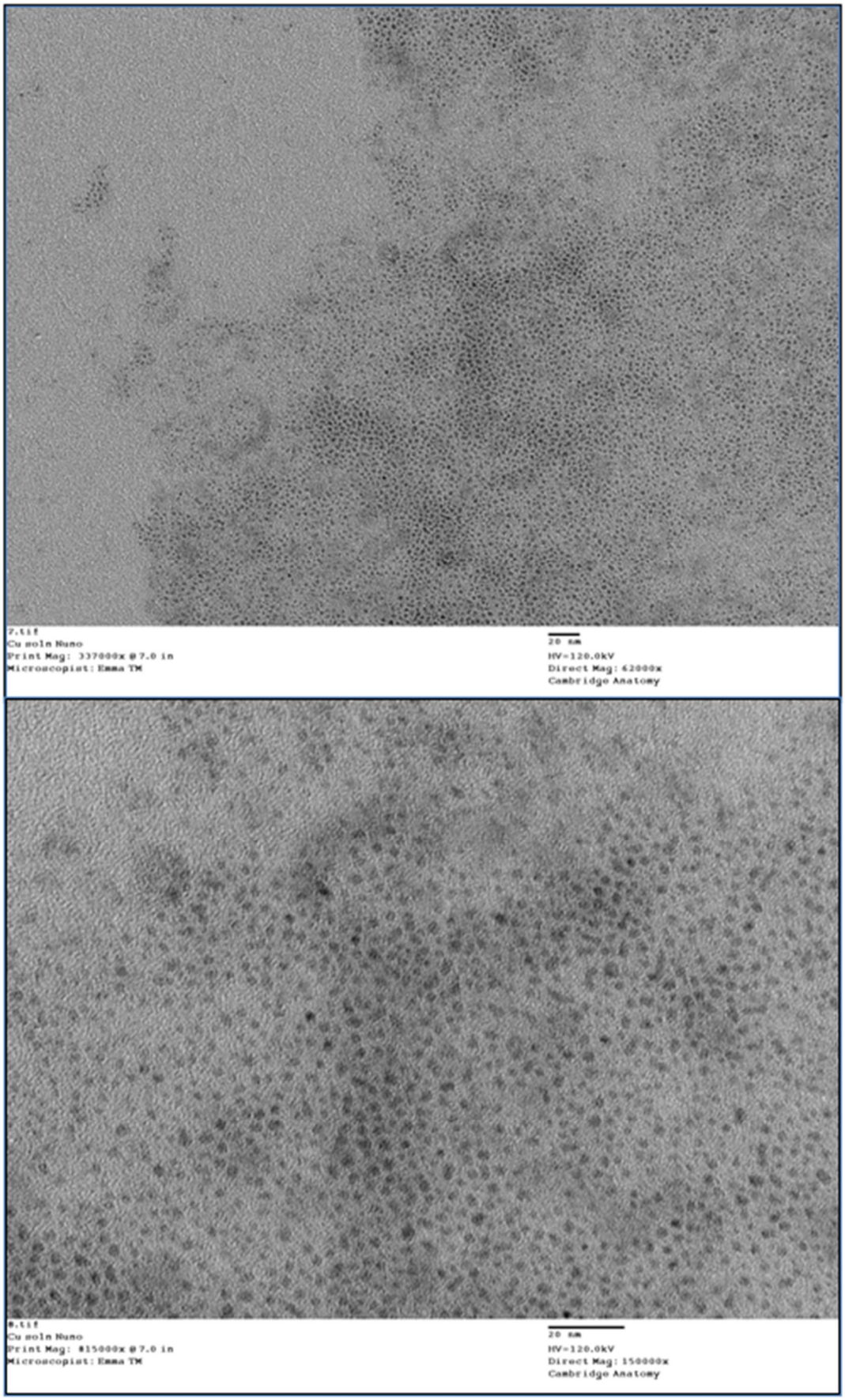

Figure 22- TEM image of copper hydroxide nanoparticles, $Cu(OH)_2$Tart-Ad (scale 20 nm).

4.1.1. Identification of synthetic materials

The identification and characterization of synthesized materials was carried out by X-Ray Diffraction (XRD), to determine the mineral phase of copper materials synthesized, in particular the precipitated materials obtained from the mixture of copper chloride with sodium hydroxide and $Cu(OH)_2$Tart-Ad nanoparticles synthesized. This method is able to provide a pattern of a crystalline material which is a "fingerprint" of the substance.

The copper mineral phase detected by XRD and shown in Figure 23A was paratacamite. This mineral phase is one of the four possible polymorphs of copper hydroxides with chloride incorporated($Cu_2(OH)_3Cl$) and is composed of two simple copper salts, copper chloride ($CuCl_2$) and copper hydroxide ($Cu(OH)_2$) in a ratio of 1:3, respectively [87]. Paratacamite presents a rhombohedral crystalline structure with a pale green colour and is the most stable phase of the $Cu_2(OH)_3Cl$ forms, which means that normally other polymorphs, easily tend to recrystallize to form paratacamite [88]. The synthesis of paratacamite was easily carried out by direct precipitation, when sodium hydroxide was added to an aqueous copper chloride solution, and this procedure has been reported as allowing the synthesis of pure paratacamite [89]. Nevertheless, different copper hydroxides can be prepared using the same protocol, only by manipulating the initial concentrations of copper and the ratio of copper, chloride and hydroxide. Paratacamite was reported to be obtained when $CuCl_2$ and NaOH were mixed in the same ratios and the mixture was independent of the order of addition [90]. Furthermore, to obtain only solid copper hydroxide ($Cu(OH)_2$) the hydroxyl ions (OH^-) should be present in excess, and this reaction produces a blue precipitate, typical of spertiniite, contrasting with the green colour of paratacamite [88,89].

The Figure 23B shows the XRD spectrum of $Cu(OH)_2$Tart-Ad nanoparticles, being this method inconclusive to determine the mineral phase, due to the high amorphous phase detected. The two peaks represented in the spectrum at position $2\theta = 32°$ and 46° corresponded to halite, a crystal constituted of sodium chloride that was present during the synthesis[4]. As seen above, in the absence of ligands, the mineral phase formed was a copper hydroxide with chloride incorporated, and consequently, the nanocrystalline copper hydroxides synthesized could also be paratacamite.

The characterization of mineral phase of copper hydroxides nanoparticles synthesized showed an amorphous phase at micro-scale. The characterization of a possible crystalline nanomaterial, as suggested by TEM, would require electron diffraction of High Resolution

[4] Information obtained in the data report from the University of Leeds.

Transmission Electron Microscopy (HRTEM) where the phase of individual small nanoparticles can be determined[5].

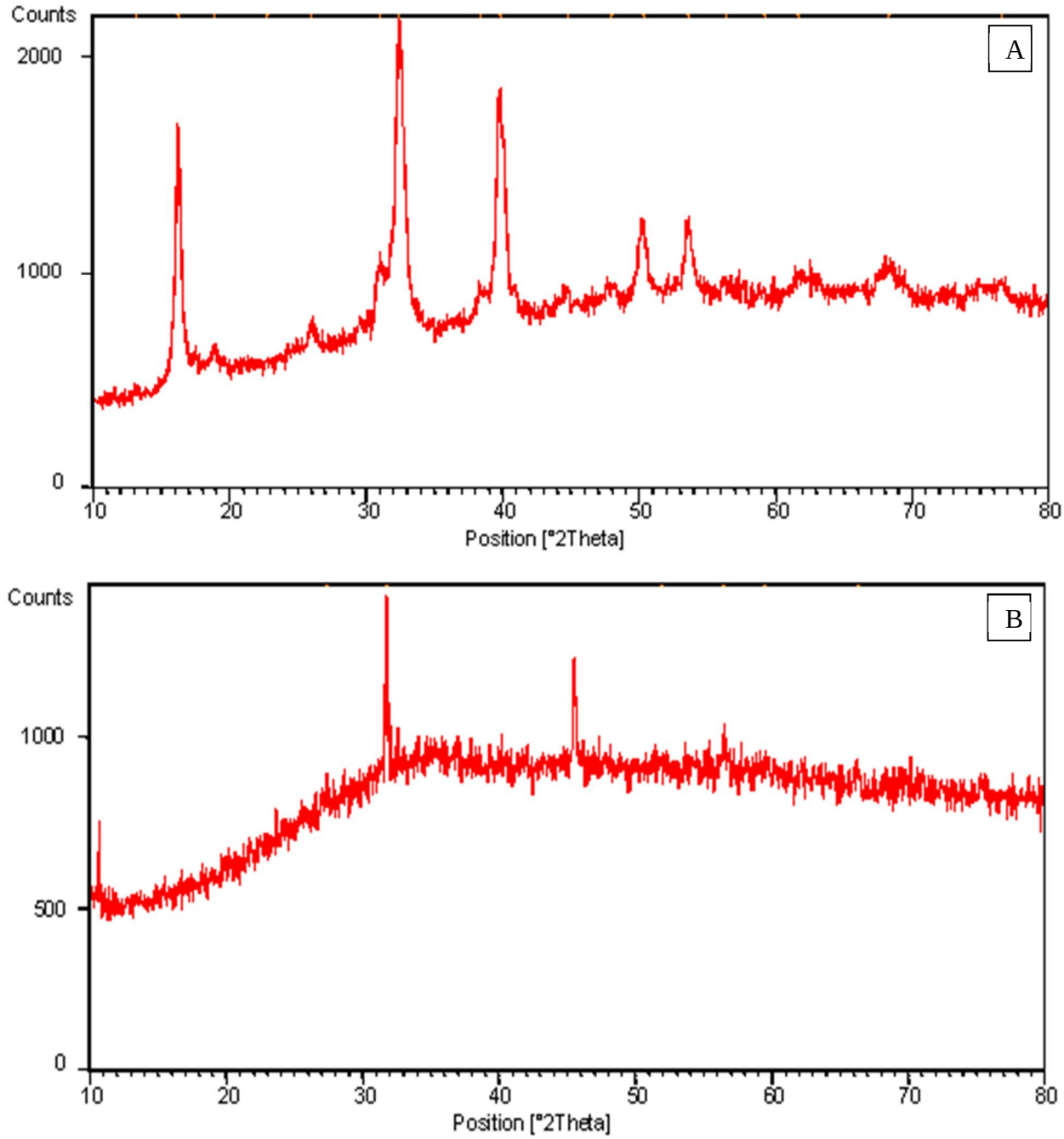

Figure 23- XRD characterization of copper hydroxides particles synthesized by direct addition of Sodium hydroxide into a solution of copper chloride (A) and $Cu(OH)_2$Tart-Ad nanoparticles (B).

[5] Personal communication by Dr. Andrew Brown from the Institute of Materials Research, University of Leeds

4.1.2. Copper phase distribution analysis in broth

The aim of this experiment was to characterize the copper phase distribution in broth, which was the medium used in the antibacterial assays. The results showed that most of the copper was soluble in the different concentrations tested, as seen in Figure 24, which may mean that copper hydroxide nanoparticles were very labile, dissolving easily at lower concentrations. Another possible explanation is that these were due to an analytical artefact. As smaller the nanoparticles may be able to cross through the 1.9 nm (3KDa) pore diameter filter being detected as copper soluble, which would justify the obtained results. Increasing the concentration of copper in broth was also showed to decrease the amount of nanoparticulated copper. This finding was not very well understood as the opposite was expected, i.e. an increase in nanoparticulated copper fraction with the increase of copper concentration, as seen in a previous experiment carried out by Tin Wong at MRC-HNR laboratory, in which the nanoparticles were dissolved in water, using the same concentrations (data not shown).

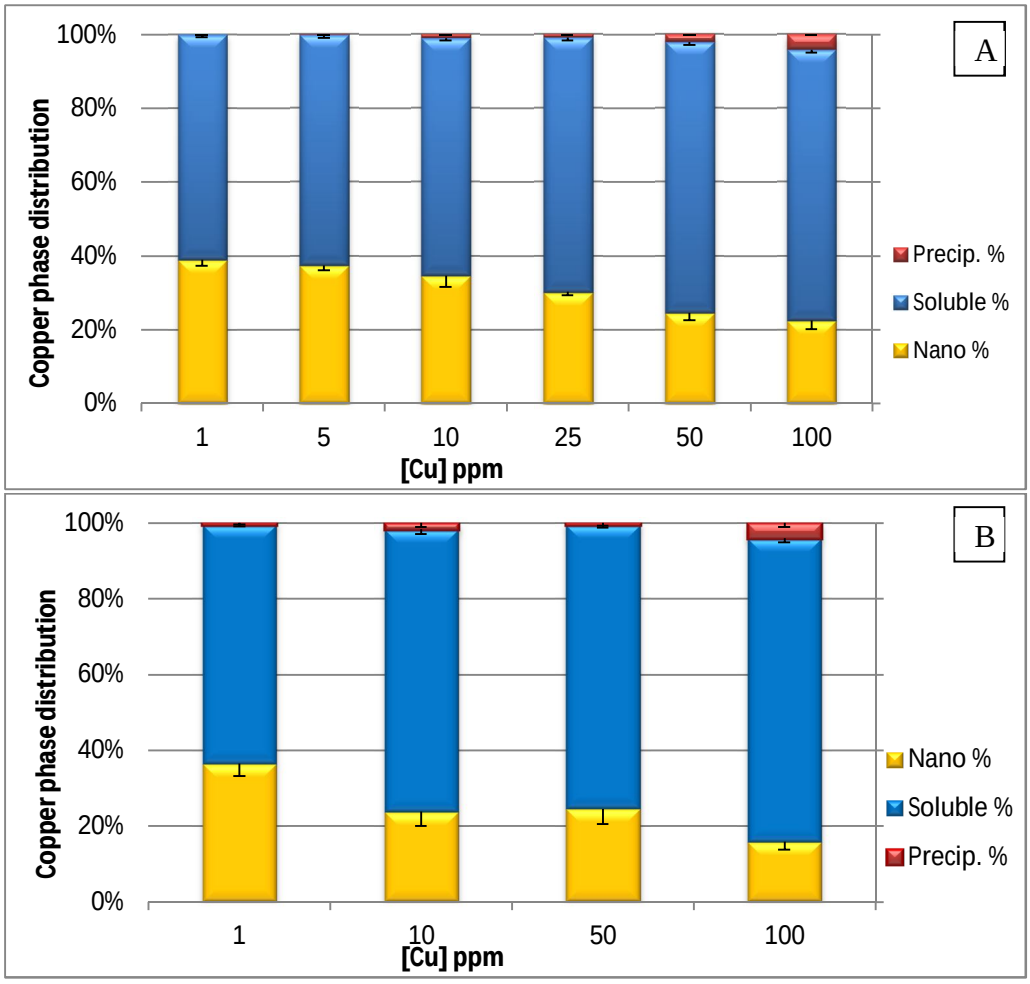

Figure 24- Copper phase distribution at different concentrations of copper in broth. A) Copper nanoparticles solution prepared with the described method. B) The same solution, but dried and resuspended. The errors bars are standard deviations of 2 independent replicates.

4.2. Nanoparticles modifications

The solubilisation of copper hydroxide nanoparticles in broth may have been an analytical artefact, but also the nanoparticles possibly were not very stable at low concentrations in complex environments. Thus various strategies were tested to develop more stable copper-based nanoparticles.

4.2.1. Heat treatment

The synthesis of copper oxide nanoparticles (CuO) was attempted since these nanoparticles should be more stable than the previously synthesized copper hydroxides nanoparticles [55]. Copper oxides can be prepared from copper hydroxides by heating, thus boiling conditions should be able to provide the ideal conditions for the synthesis of copper oxides. The equations shown below represent the proposed reactions of formation of copper hydroxide (9), and its conversion in copper oxide under high temperatures (10). The equation 11 represents the overall reaction.

Equations:

$$CuCl_2.2H_2O + 2NaOH \rightarrow Cu(OH)_2 + 2NaCl + 2H_2O \quad (9)$$

$$Cu(OH)_2 \xrightarrow{Heat} CuO + H_2O \quad (10)$$

$$CuCl_2.2H_2O + 2NaOH \xrightarrow{Heat} CuO + 2NaCl + 3H_2O \quad (11)$$

The synthesis was attempted by repeating the same method used to synthesize the copper hydroxides nanoparticles, but at boiling temperature (~100ºC). During the experimental work was obtained a green boiling solution, which contrasted with the blue suspension of copper hydroxide nanoparticles synthesized previously, which indicates that probably was formed a different copper phase. However, after centrifugation a green precipitate at the bottom of the tube was seen, and the supernatant was blue. In spite of the presence of a precipitate, the amount of copper nanoparticles obtained was higher than 50%, as seen in Figure 25A. The nanoparticles presented a size between 10 and 30 nm at pH 7, as seen in Figure 25B. The precipitated copper was 40% of the total copper and may correspond to agglomeration caused by the heat treatment [55].

In this experiment the pH was difficult to control, due to the low buffer capacity of the solution. Finally, the synthesis was carried out again with same amount of copper and tartaric acid, but in the presence of adipic acid, which increased the buffer capacity of the solution,

allowing a better control of pH. Also, adipic acid is a weak ligand that can bind copper to prevent the precipitation seen in the Figure 25A.

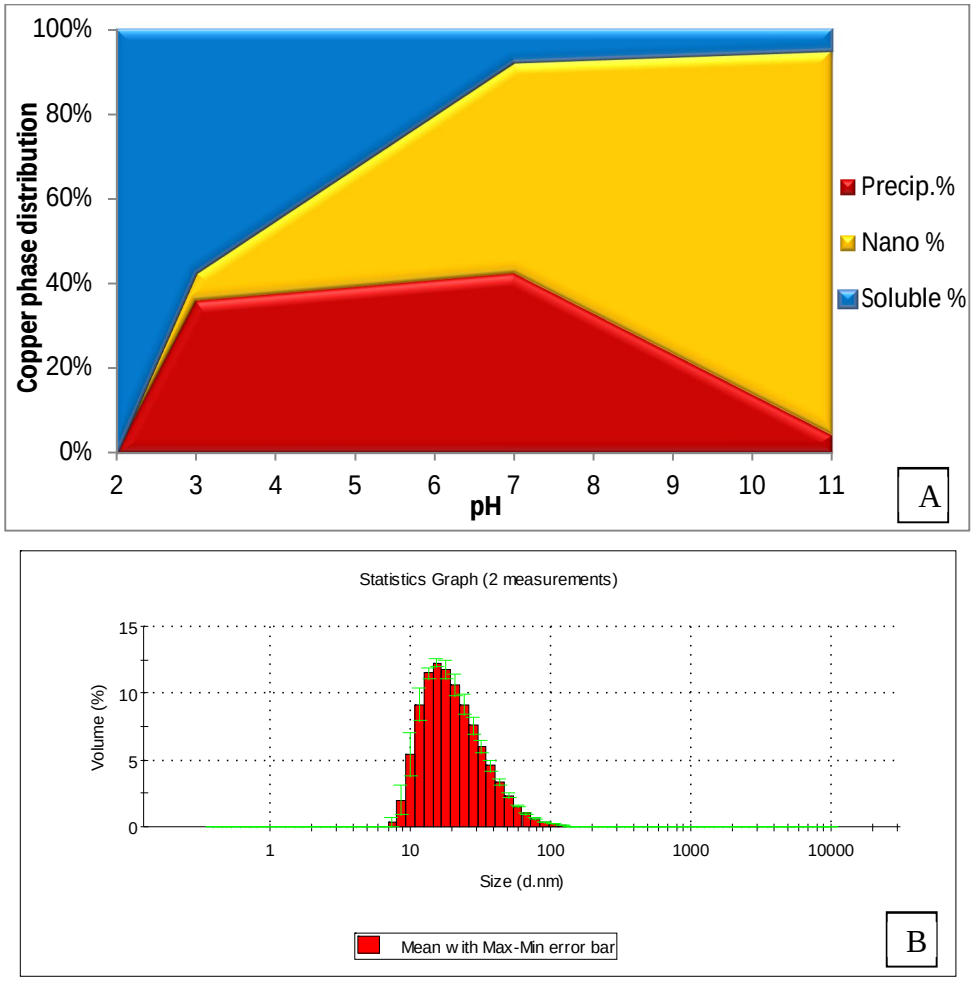

Figure 25- Synthesis of $CuO_x(OH)_y$-Tart at boiling temperature; A) Copper phase distribution during the titration. B) Particles size distribution obtained at pH 7 (centrifuged before analysis).

The results shown in Figure 26A confirmed the initial expectations using adipic acid, the precipitated copper fraction decreased comparing with the synthesis of $CuO_x(OH)_y$-Tart (Figure 25A), also the soluble copper decreased, and consequently the nanoparticulated fraction at pH 8 was higher (73%). The particle size distribution at pH 8 showed nanoparticles mostly of 3-4 nm (Figure 26B), which were of similar size to the $Cu(OH)_2$Tart-Ad nanoparticles synthesized, as shown in Figure 21B, but the comparing with the copper phase distribution (Figure 20) the amount of copper nanoparticulated was still below the target level, i.e. above 90%.

The highest amount of nanoparticulated copper obtained was 70% only, which was too low to the desired amounts and, experimental evidences also indicated that copper oxide

nanoparticles were not synthesized, such as the blue colour of the suspension and the size of the nanoparticles synthesized were very similar to the copper hydroxides synthesized. The main difference of the synthesis at boiling temperature was the green precipitate obtained, which may be a copper oxide chloride, due to the excess of chloride in solution which can be incorporated inside the structure [87]. Also, the greenish colour is coincident with the colour described for copper oxide chloride compounds, and differs to copper (II) oxide (CuO), which is a black compound. The characterization and identification of this compound may be done by XRD.

The method used was not ideal for the synthesis of copper oxide nanoparticles and further approaches might be done in the future to improve the amount of nanoparticulated copper. Then, the characterization of the synthesized compounds and the analysis of copper distribution phase in broth should be done to identify the copper mineral phase of nanoparticles synthesized and also to test the dissolution in broth.

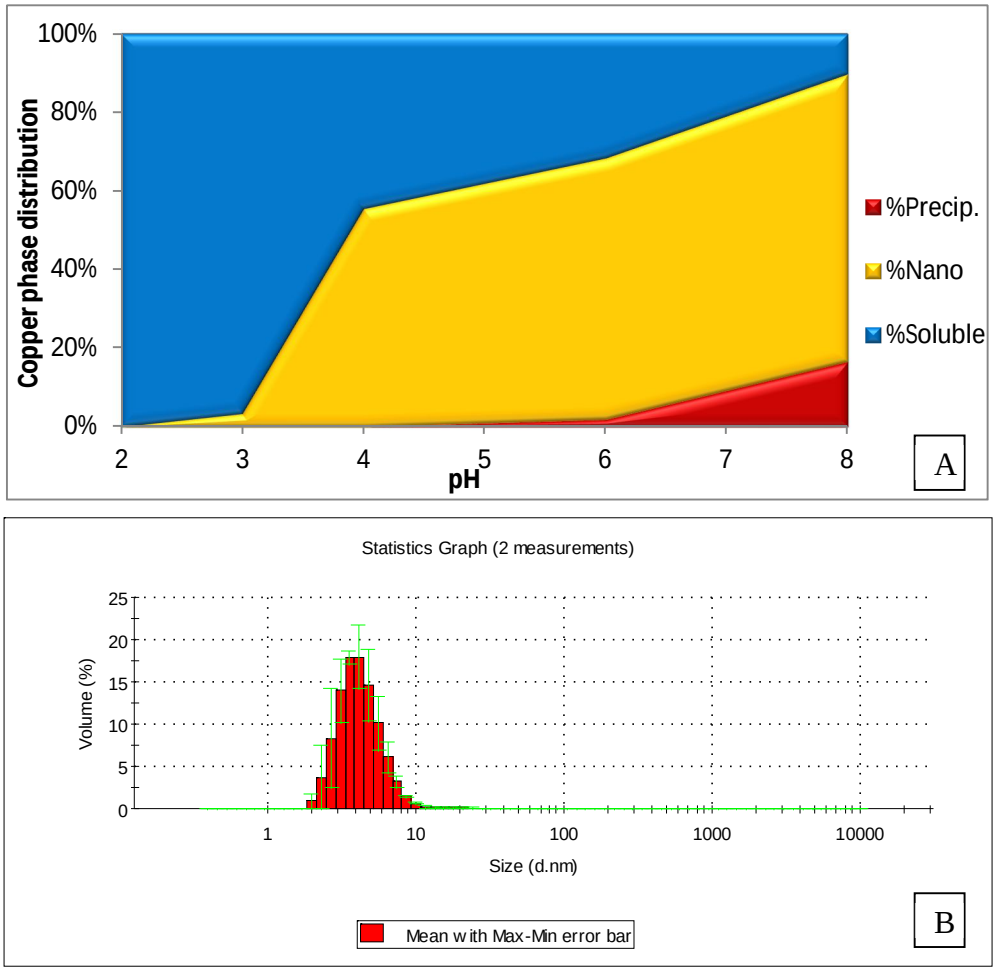

Figure 26- Synthesis of $CuO_x(OH)_y$-Tart-Ad at boiling temperature; A) Copper phase distribution as a function of pH. B) Particles size distribution obtained at pH 8 (centrifuged before analysis).

4.2.2. Silicon modified copper nanoparticles

Silicate was added into the copper solution to prepare copper nanoparticles with silica incorporated or creating a layer around copper nanoparticles, to form a protective shell, to protect nanoparticles against environmental instability factors, as reported to be able to protect elemental copper nanoparticles to be oxidized [1]. Also, the high affinity of silica for metals allows their easy interaction and consequently easy complexation [2]. Furthermore, this modification would be able to lead to more stable nanoparticles, avoiding problems such as high solubility in broth. Sodium silicate was used as the silicon source in this experiments, as it had been previously used in synthesis of nanoparticles for antimicrobial applications [5,6]. This sodium salt of silicic acid presents an alkaline character, being more soluble above pH 9, due to the formation of silicate ions [91]. Silicate was used in low concentrations to avoid polymerization and consequent formation of silica gel. In the presence of silicate, copper were mostly in a nanoparticulated phase, especially at lower concentrations of silicate, such as 10, 20 and 30mM (Figure 27). At 30mM precipitated copper increased, indicating that larger particles were forming, and similarly at 40mM, 60% of the copper was precipitated. This result showed that the presence of silica influenced the formation of copper hydroxide nanoparticles, which may be explained due to copper complexation with silica forming large particles that agglomerate. Alternatively, silica polymerization may be responsible, since pH was kept in range of pH which silica is highly insoluble. At higher concentrations of silica, the particles growth is faster, probably by polymerization of monomeric species, and precipitates quickly attaching some copper [92]. Furthermore, the particle size distribution showed very large particles (Figure 28D), which were micron sized, and consequently, large enough to precipitate, confirming the copper phase distribution. At lower concentrations of silicon, the precipitated copper was very low or even inexistent (20mM), which supported the theory of growth of silica polymers. At these concentrations the copper was present as a nanoparticulated phase (>90% of the total copper) and characterization by DLS showed that the size was dependent on the silicon concentration used, as seen in Figure 28 and Figure 29. Furthermore, at higher concentrations the particle size showed large error bars, which may be explained by sedimenting particles present in suspension, which reduced the accuracy of measurement, especially at 30mM and 40mM, Figure 28C and D respectively.

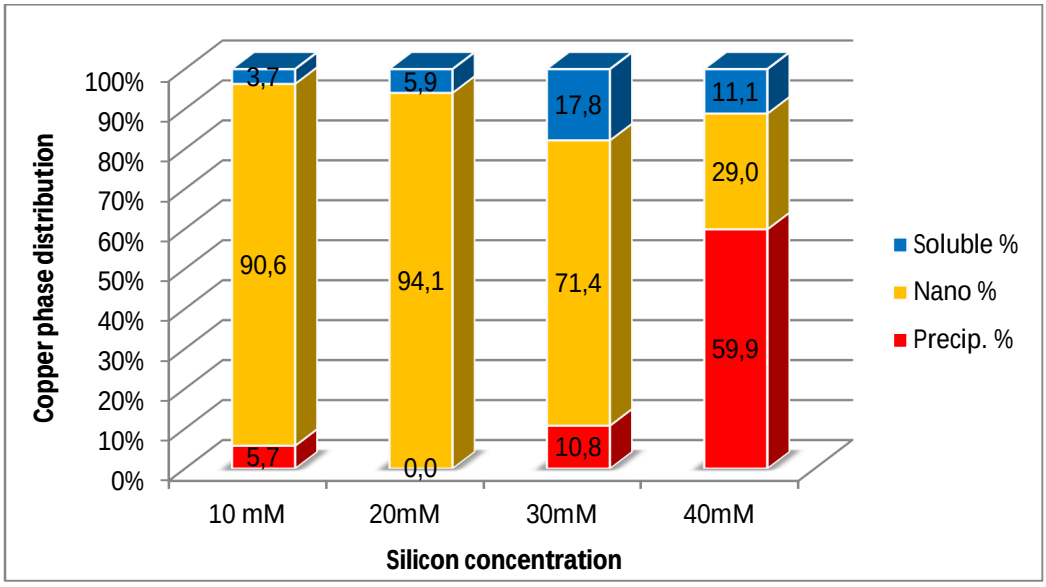

Figure 27- Copper phase distribution of $Cu(OH)_2$Tart-Ad nanoparticles prepared in the presence of different concentrations of sodium silicate.

Silica showed to influence the nanoparticles synthesized, this may have been due to silica incorporation into the copper hydroxide nanoparticles which is supported by the increase in particle size with the increasing in silica concentrations. Alternatively, silica may have formed a coating around copper hydroxide nanoparticles, an increase in silica led to an increase in the thickness of the coating. Further analysis must be done in order to understand the role of silica in this synthesis. TEM and XRD would be two techniques to identify and characterize the nanoparticles synthesized. The TEM could be a good tool to analyse the formation of a coating around the nanoparticles, and to compare how the different silicon concentrations affected the size of nanoparticles. The XRD would be able to identify the mineral phase present, and maybe indicate if silica changed the mineral phase of copper nanoparticles.

The nanoparticles synthesized in the presence of 10mM, 20mM and 30mM of silica, were tested in broth at low concentrations of copper in broth to investigate if there was any influence the solubility of copper. The synthesis using 40mM of silicon was not tested due to low amount of copper nanoparticulated, only 29%, as seen on Figure 27.

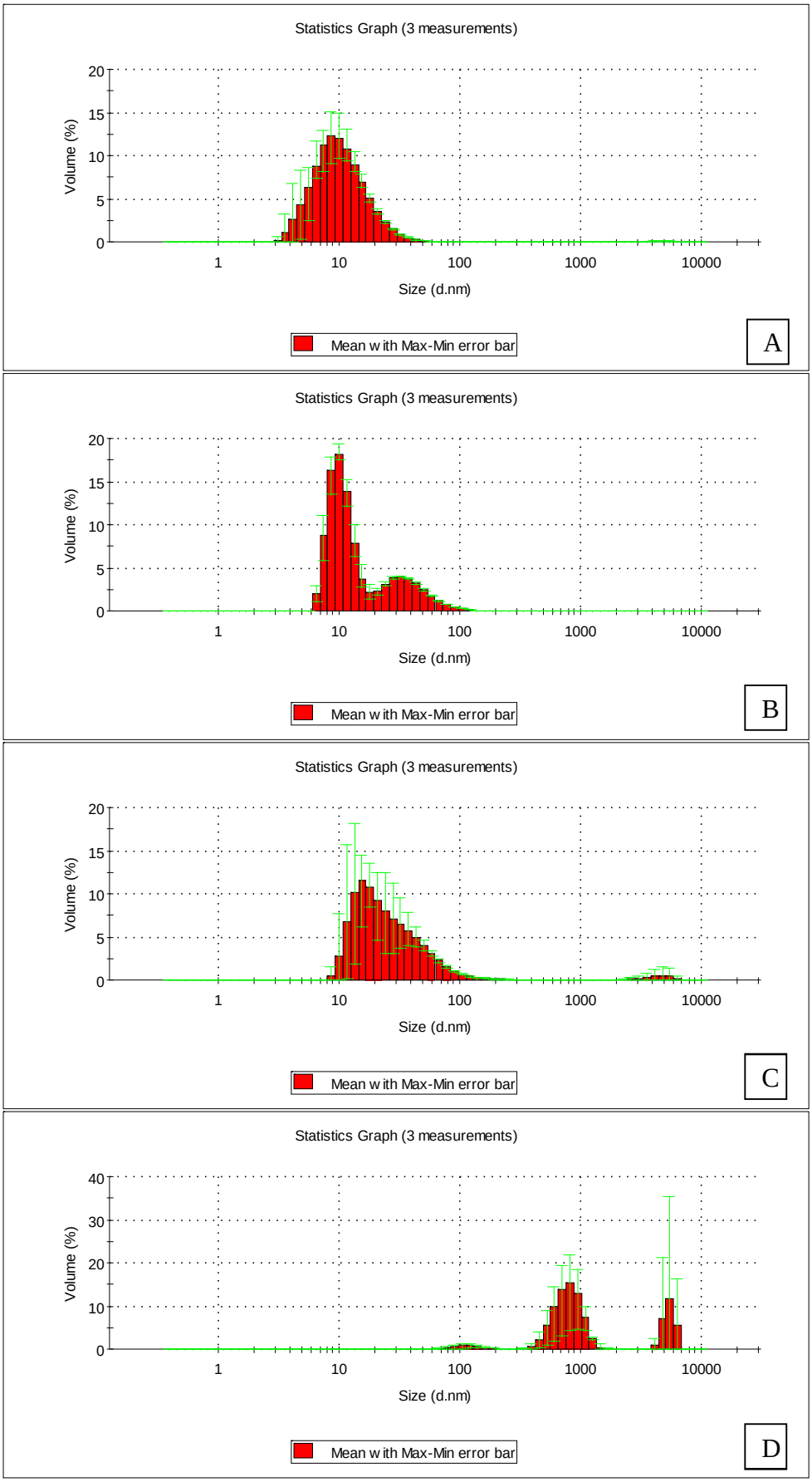

Figure 28- Particle size distribution of $Cu(OH)_2$Tart-Ad particles prepared in the presence of sodium silicate with different concentration of silicon: a) 10mM, b)20mM, c) 30mM and d) 40mM.

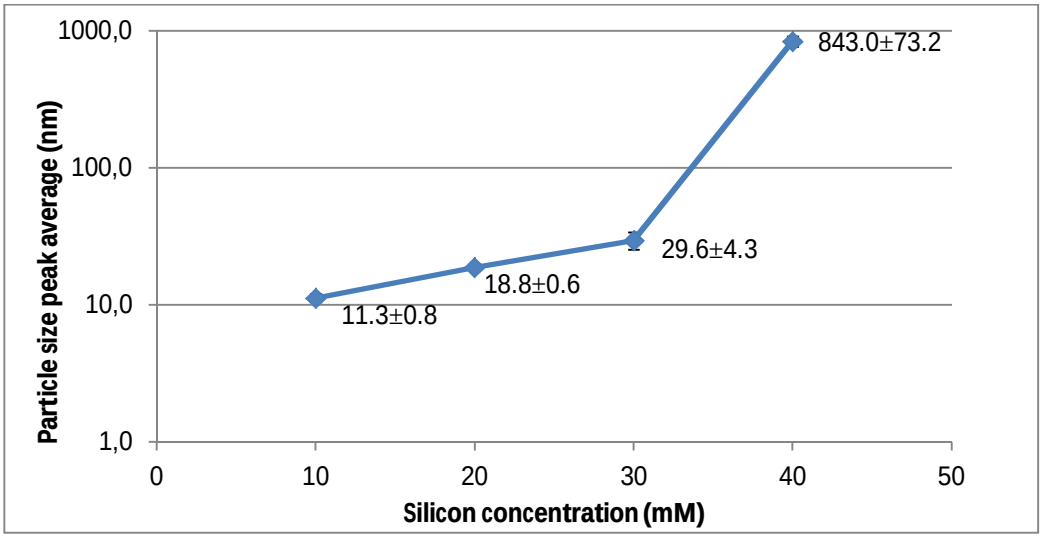

Figure 29- Particle size as a function of silicon concentration in the preparation of $Cu(OH)_2$Tart-Ad. The error bars are standard deviations of 3 measurements.

4.2.2.1. Silicon modified copper nanoparticles in broth

The presence of silicate during the synthesis of nanoparticles did not result in significant differences in the solubility of copper in broth, especially at 10mM and 30mM of silicate, as seen in Figure 30A and C. An increase in nanoparticulated copper was seen in the graph of 20mM of silica (Figure 30B). The solubility of copper in the presence of silicate may lead to wonder whether copper nanoparticles were not modified by silicate, or if the modification produced was not able to reduce the high lability of nanoparticles, and consequently their solubilisation. The hypothesis of copper nanoparticles were not modified by silicate was not supported by showed above that showed the increasing of particles size, with the increasing of silicate concentration. Although the influence of silicate is not understood using the analysis carried out so far, the silicate seemed to have an influence in copper nanoparticles. On the other hand, this possible modification did not show influence in the solubility of copper nanoparticles. Also, there was no correlation between copper solubility in broth and the concentration of silica.

Once again, as already seen above, the nanoparticulated copper tends to decrease with the increasing of copper concentration, especially it can be seen at lower concentrations of silica used, Figure 30A and B.

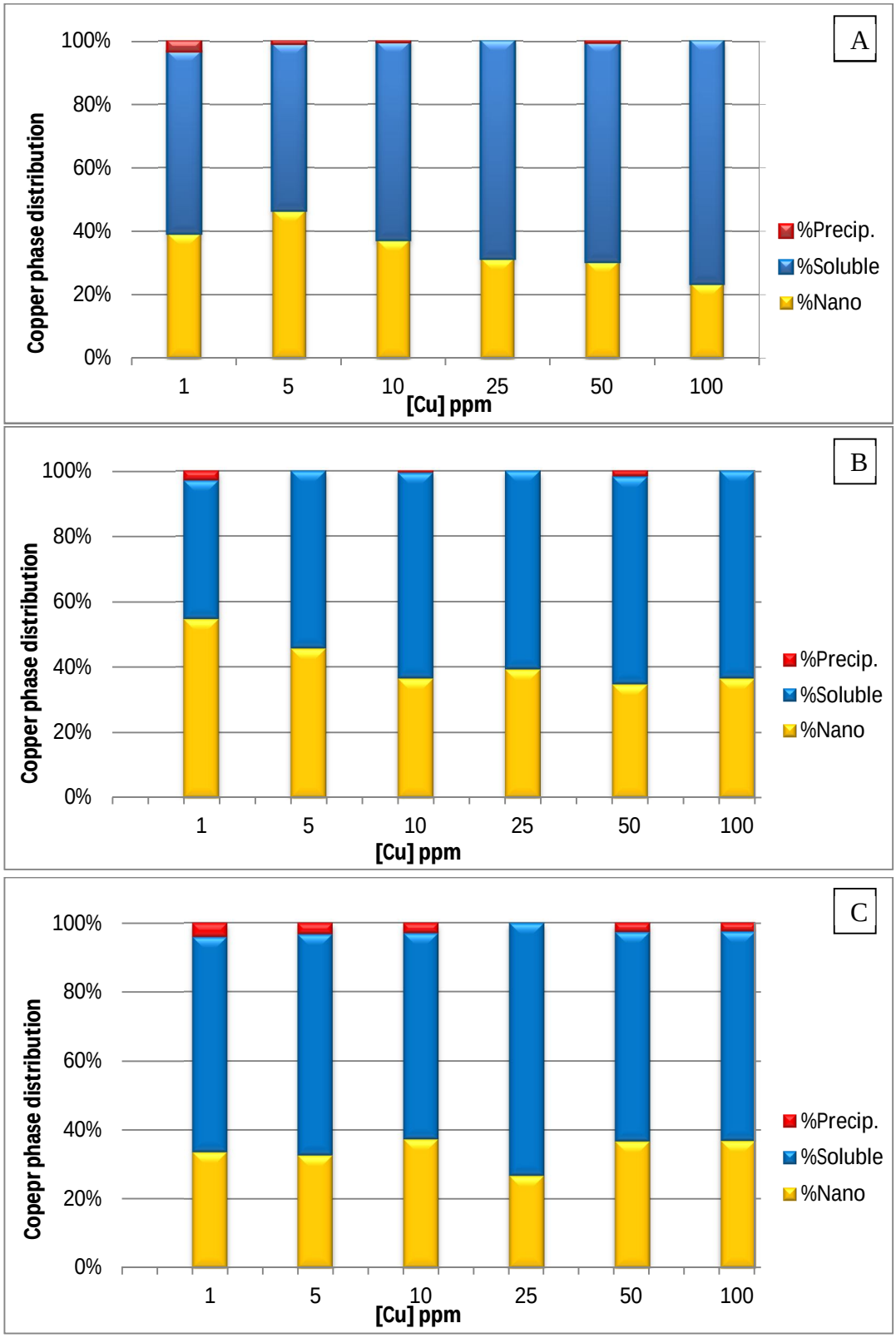

Figure 30- Copper phase distribution of $Cu(OH)_2$Tart-Ad nanoparticles synthesized in the presence of a)10mM, b)20mM and c)30mM of sodium silicate.

4.3. Entrapment of nanoparticles in a silica gel matrix

The entrapment of nanoparticles inside a silica gel matrix was developed in parallel to the synthesis of disperse nanoparticle suspension. This strategy was of interest because it allows the direct utilization of antimicrobial activity of copper nanoparticles in a matrix, to apply, for example, in covering of surfaces or in food packaging. Also, some studies reported the use of gel, such as silicon gels, as template for the synthesis of nanoparticles, because the growth of nanoparticles can be controlled by the pores size in the gel. The result of this synthesis showed that can be obtained nanoparticles without agglomeration, presenting a narrow size distribution [85]. Sodium silicate (SiO_2.NaOH) was used as silica source for the production of gel, and its gradual addition provided hydroxyl groups into solution that could be able to form copper hydroxides, which in the presence of ligands such tartaric acid and adipic acid can form nanoparticles as explained above in the synthesis of $Cu(OH)_2$Tart-Ad nanoparticles (section 4.1). Meanwhile, the concentration of silica was increasing and consequently the solution started to polymerize over the titration. In the end it was expected to obtain copper hydroxides nanoparticles, inside the matrix of the silica gel. To investigate that was determined the amount of copper that was present in the gel.

Experimentally, the gel formed was blue, probably meaning that some copper was entrapped in the matrix, which can be confirmed in the results obtained, shown in the Figure 31. Above pH 6 can be seen an increasing in copper precipitated, that probably is due to the formation of gel at this pH, as seen experimentally. The titration with sodium silicate was stopped at pH 7, and after one hour, the amount of copper in supernatant and in gel was the same, as seen in Figure 31. This result led to believe that polymerization of silica occurred quickly during the addition of silica into solution, and ended when the addition stopped. Also, copper was not released in the supernatant after stopped the polymerization.

At pH 7, 75% of the copper was fixed in the gel, but was important to know if the copper was nanoparticulated. The procedure to determine the copper phase distribution required ultrafiltration that was not possible with the gel. Although this problem, the copper in supernatant was analysed, showing that 66% of the copper was precipitated (Table 5). The proposed reason for that result was the presence of small particles of silica polymerized with copper attached that precipitated after centrifugation, which mat occurred because of the great affinity of copper and silica [93]. Although this precipitation observed, there was 34% of nanoparticulated copper in supernatant, which was an indication that during this method some nanoparticles can be produced, and probably the copper inside the gel may be nanoparticulated.

Thus, the investigation of the presence of copper entrapped in the gel was carried out by TEM, confirming that copper was nanoparticulated in gel, as shown Figure 32.

$Cu(OH)_2$Tart-Ad nanoparticles can be encapsulated inside a gel of silica, but to apply this new synthesized material the antimicrobial activity of copper should be tested. The method described above was used for copper in liquid environments, and obviously was not adequate to test a gel. Thus it is required the development of a method to assess the antimicrobial activity of copper hydroxides nanoparticles that are entrapped in the solution to evaluate the potential of this material.

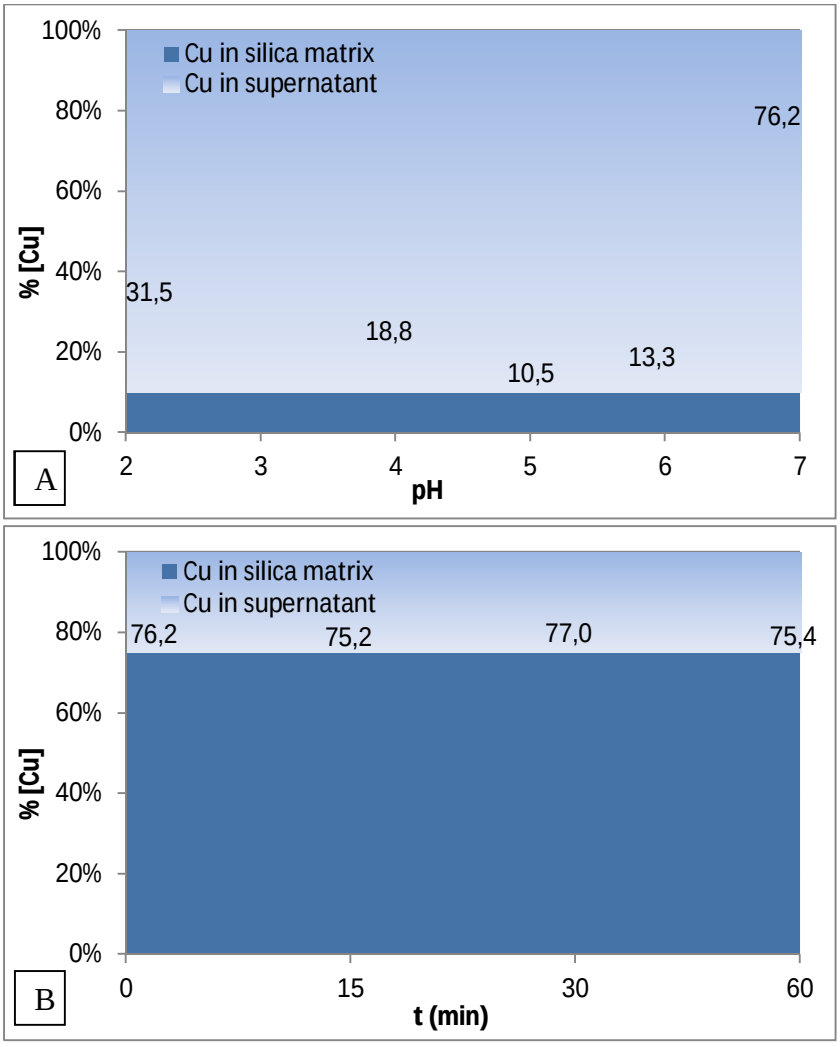

Figure 31- Percentage of copper retained in the gel as a function of pH (A) and then, the same gel with copper over the time (B).

Table 5- Copper phase distribution in supernatant of gel.

Copper Precip. %	Copper Soluble %	Copper Nano %
66.04	0.00	33.96

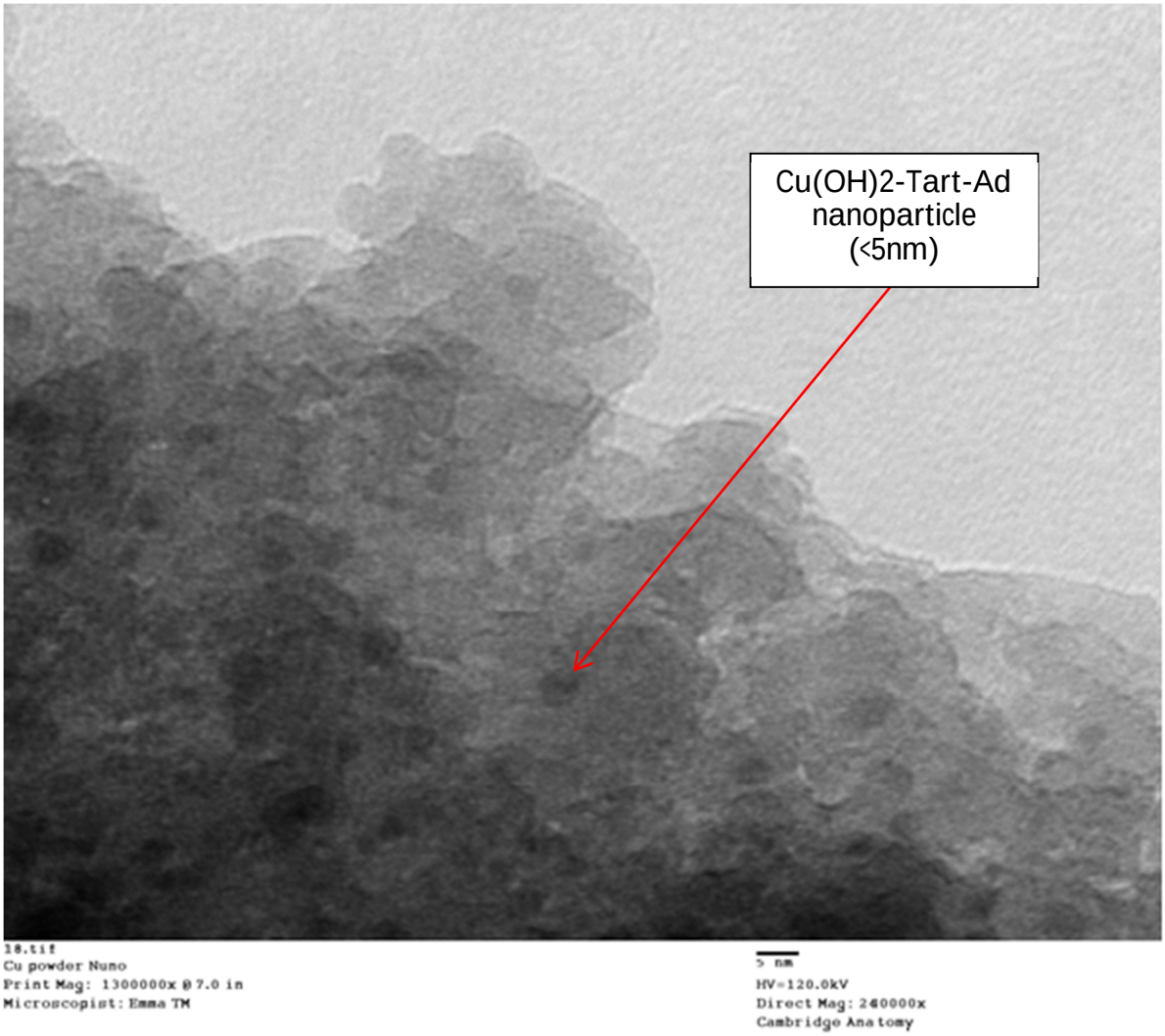

Figure 32- TEM image of $Cu(OH)_2$Tart-Ad nanoparticles encapsulated inside a gel of silica.

4.4. Determination of antibacterial activity

The antimicrobial assay method was developed to analyse the antibacterial activity of different metal compounds, whether ionic or nanoparticulated. This method was developed to compare the inhibition of different antimicrobial compounds with a normal bacterial growth. Thus, this is not a method to count Colony Forming Units (CFU) as used in most of the traditional microbiological assays. The bacterial counting was done by measurement of optical density (OD) at 595nm, which can provide a direct correlation with the bacterial concentration, as seen in Figure 33 [94]. The method using 96 well-plates allowed the use of a range of metal and bacterial concentrations in the same assay. The bacterial concentrations were obtained by decimal dilutions of the initial culture and, the ideal concentration to analyse the antibacterial activity was selected to allow the detection of bacteria when the test started (0 hours), and to avoid optical densities (OD) above 1 in the last measurement, in which the instrument would not be so accurate. The ideal concentration of analysis is 0.5 McFarland, which corresponds to 10^6 to 10^8 CFU of *E.coli* [96]. The initial bacterial culture presented an OD of 0.55, which corresponded to $5.3x10^7$ CFU that is inside the range of CFU proposed for 0.5 McFarland. To reach an OD about 0.5 in the end of the measurements, it was established that the initial OD values should be ranged between 0.05 and 0.1. Furthermore, if the analysis of the bacterial growth occur in between the established OD values (0,05-0,5) a linear correlation with cells number may be guaranteed, as showed in Figure 33. The bacterial concentration that satisfied these conditions was 10^{-2}.

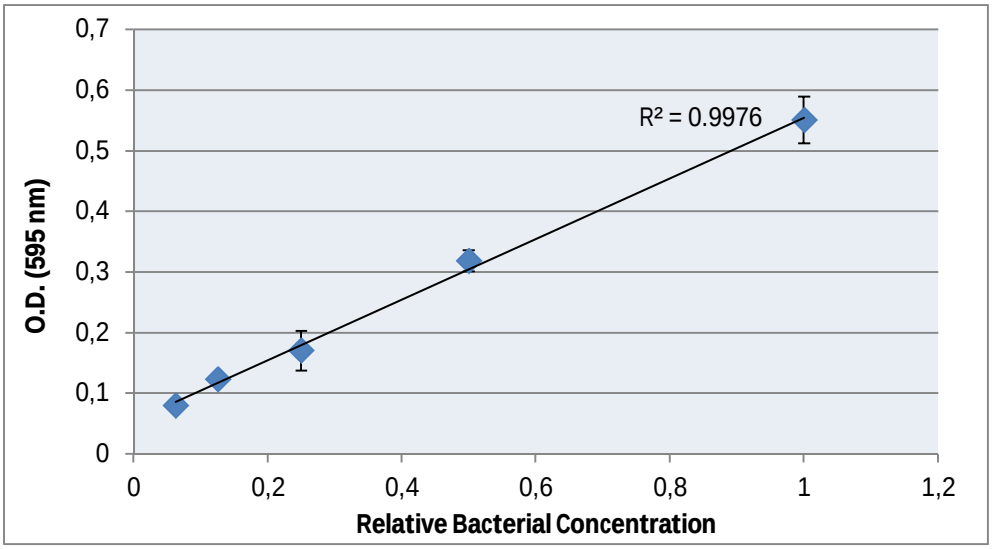

Figure 33- Optical density as a function of bacterial concentration. Error bars represent the standard deviation of 8 replicates. The highest concentration was considered 1, and the remaining concentrations were dilutions in a factor of 2.

The bacterial assay was not carried out at 24 hours due to the decrease of bacterial growth, probably due to the small volume of each well, which led to depravation of nutrients and consequent stop of growth, avoiding a comparison between the normal bacterial growth and the inhibition caused by the compounds tested. Also, at 6 hours can already be seen the typical bacterial growth curve (Figure 34B), as will be explained later. To every concentrations of copper were done 4 replicates without bacteria, representing a negative control, which were subtracted to the OD values of every well with the same concentration of copper. Silver nitrate was used as a positive control, because of its very well-known antibacterial properties and also having higher antimicrobial activities than copper, as explained in the background.

The development of this method involved the analysis of the normal bacterial growth in broth during the period of time analysed. Bacteria tend to grow faster initially, exponential growth, and then the growth becomes slower and tends to stabilize as represented by the logarithmic curve in Figure 34A. For that reason, the curve with the values obtained for the first hours of growth was linearized using the natural logarithm, base *e* ($y = \ln(x)$), and was obtained a great linear correlation as shown in Figure 34C. The curve containing the values for the last hours of measurement was linearized using the exponential function ($y = e^x$) and was obtained a strongly linear trend again (Figure 34D). The expected bacterial growth was confirmed by the developed method, which showed to have a good accuracy for the antibacterial tests. The control of contamination of the bacterial cultures was carried out regularly by plating the *E.coli* bacterial growth onto agar plates. No apparent different bacterial species were detected by visualization of colonies shape and colour. Although the method was not the most accurate to detect contamination, it was an easy procedure to control major contaminations.

The antimicrobial tests showed that all the compounds caused a reduction in bacterial growth, as expected initially. Copper chloride was tested to evaluate the antibacterial activity of copper ions (Cu^{2+}), and to provide a comparison between ions and nanoparticles. The results showed that both, $CuCl_2$ and $Cu(OH)_2$Tart-Ad, were able to cause a delay in the growth of *E.coli*, which was already reported in previous work, using similar concentrations of copper [95]. The same inhibition was obtained for both copper compounds, which probably may be explained by the dissolution of $Cu(OH)_2$Tart-Ad nanoparticles, and consequently, the inhibition seen was an effect of copper ions in both assays (Figure 35). It was already shown that $Cu(OH)_2$Tart-Ad nanoparticles may solubilize when present at low concentrations in broth (section 4.1.2).

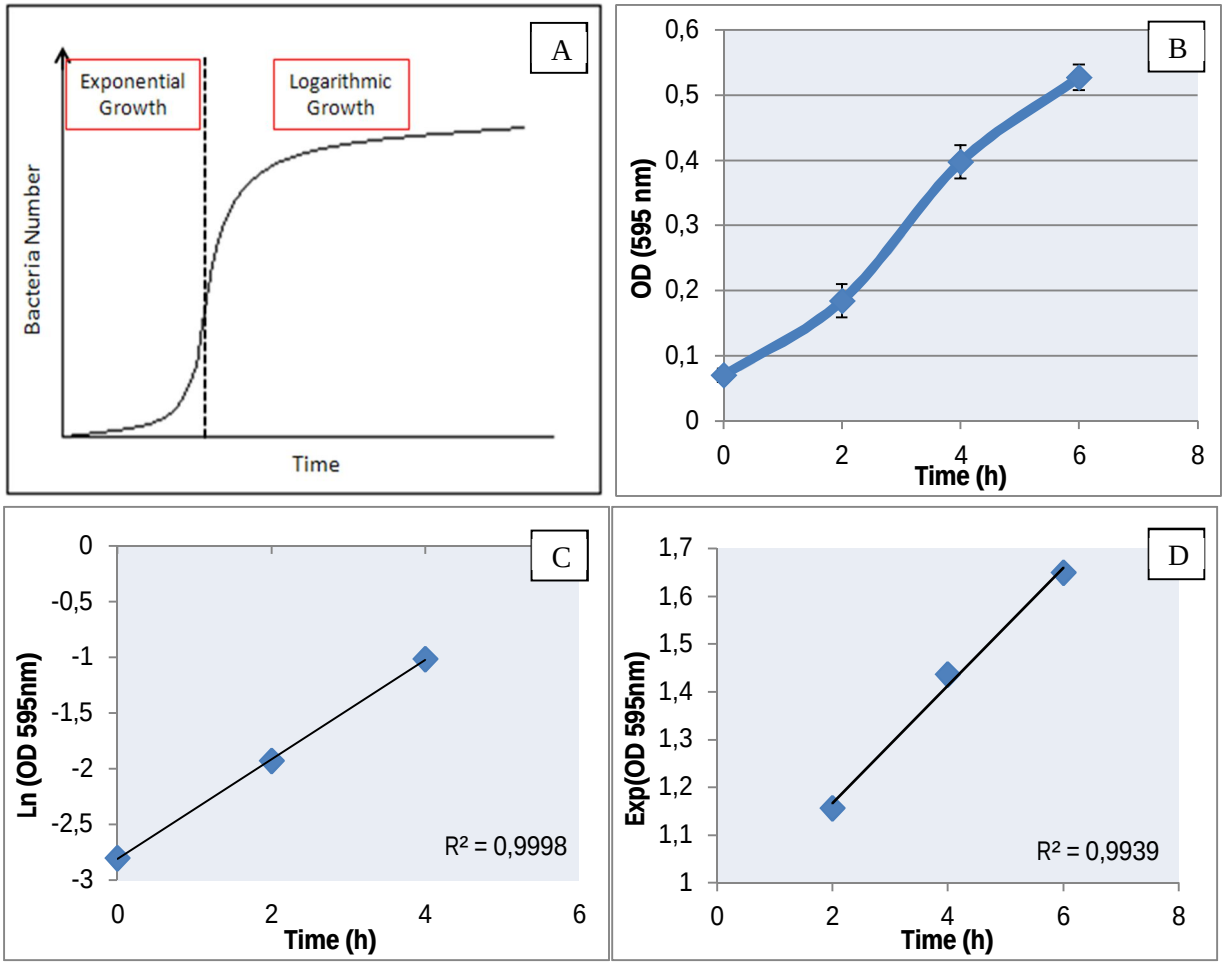

Figure 34– A) Expected bacterial growth curve as a function of time, showing initially a fast growth rate, represented by an exponential curve, and then a slower growth represented by a logarithmic curve. B) Bacterial growth curve obtained experimentally, with standard errors of 12 replicates. The linearization of the two parts of growth showed trendlines with a very high coefficient of correlation: Linearization of exponential growth curve (C) and logarithmic growth curve (D).

Although there was a possibility of an analytical artefact, these similar results provided another evidence about the dissolution of copper nanoparticles. The final inhibition of bacterial growth obtained for copper compounds was 58%, which means that copper did not inhibit completely the growth of bacteria (Figure 35D). At lower copper concentrations a bactericidal effect was not seen. At 1 ppm any differences in bacterial growth were not detected, and at 10 ppm a very low inhibition was already observed, which may mean that this concentration may be very near to the minimum inhibitory concentration (MIC) of copper. The minimum bactericidal concentration (MBC) of copper cannot be determined, because this concentration is above the highest concentration tested, which was 100 ppm. A further analysis using higher concentrations should be done to obtain this value. Also, this analysis showed that copper

delayed the normal bacterial growth, but was not understood if copper have an bactericidal or bacteriostatic effect, i.e. if copper killed the bacteria, or only inhibited the growth of bacteria but did not kill them completely, which may mean that bacteria may regrowth again. Agar plates can be prepared with the bacterial cultures that were exposed to copper. If the agar plate shows bacteria growth, this may mean that copper only inhibited the bacterial growth, but did not kill them. If there are not any bacterial growth, copper may be considered bactericidal.

A study about the antibacterial activity of copper against *E.coli* showed that using the same concentrations of metal, in liquid culture showed a delay in bacterial growth, whereas in solid medium copper inhibit completely the growth. The authors of that investigation proposed that in liquid medium the concentration of nanoparticulated copper gradually decreased, which allowed the regrowth of bacterial. A reason for that could be the interaction of copper with intracellular compounds that were released from the dead bacterial cells [95]. The same effect may have occurred during this experiment, and could be a reason for the delay in growth, instead of a complete inhibition.

Silver nitrate was used to provide Ag^+ ions in solution, and to establish a comparison with copper compounds tested. Ag^+ showed a very high antibacterial activity, even at lower concentrations than copper, as already expected. At 10 ppm silver was able to completely inhibit the growth of bacteria, almost 100%, while copper presented a very low antimicrobial activity at the same concentration (Figure 35C). Furthermore, at 1 ppm a very small reduction in bacterial growth was already obtained, showing that the MIC of silver nitrate should be near to 1 ppm. Also, a prediction of the MBC of silver which is between 1 and 10 ppm may be done.

The different activity of copper and silver was suggested to be due to different affinities to the cell membrane, leading to a closer interaction with bacteria and consequently a better efficacy [25]. These authors suggested that this affinity to cell membrane may be the responsible for the different bactericidal activities of copper and silver against different bacterial strains, because of their different cell membrane composition. This fact may lead to consider that silver presented a higher affinity for bacterial membrane of *E.coli* than copper, but this should not be the only factor to have in consideration. Also, the metabolism of copper and silver in bacteria is very important for the toxicity caused by these components, because it may determine the response of bacteria to high amounts of these metals in the environment, and a resistance mechanism for copper ions was already described, and was also proposed to be used for silver ions [49].

In summary, a method for high throughput determination of antibacterial compounds was tested effectively and can be used for antimicrobials in solutions or suspensions. Although, all the compounds tested presented an antibacterial effect against *E.coli*, the antimicrobial results showed that $Cu(OH)_2$Tart-Ad and $CuCl_2$ presented similar results, which may be an indication that copper hydroxides nanoparticles were dissolved, and consequently the activity of nanoparticles were not evaluated.

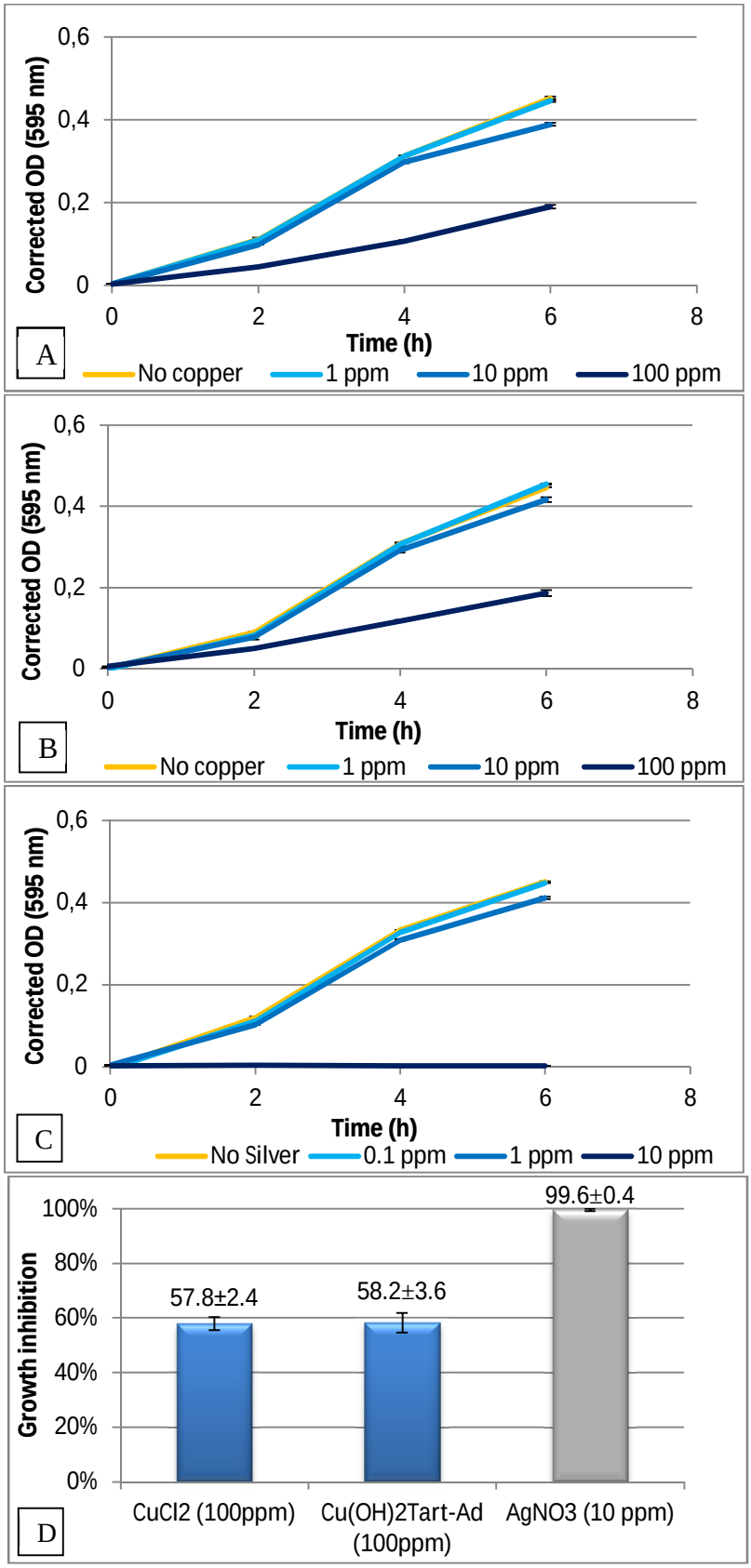

Figure 35- Bacterial growth of *E.coli* in the presence of $CuCl_2$ (A), $Cu(OH)_2$Tart-Ad (B) and silver nitrate (C). The graph D shows the growth inhibition 6 hours after exposition of bacterial culture of *E.coli* to $CuCl_2$, $Cu(OH)_2$Tart-Ad, both with copper concentration of 100 ppm, and $AgNO_3$ at 10 ppm of Ag. The error bars represent standard deviations calculated from 4 replicates.

5. Conclusion

In conclusion, copper hydroxides nanoparticles were synthesized using different ligands, which were carboxylic acids typically present in food. The synthesis with tartaric acid resulted in the highest production of nanoparticulated copper. This was further improved by including adipic acid in the synthesis and more than 90% of the total copper was then present as nanoparticles. The synthesized $Cu(OH)_2$Tart-Ad nanoparticles presented desirable properties, such as a very small size (3-5 nm), and being well disperse. The size could also be tailored depending on the final pH of synthesis. However the nanoparticles appear to be dissolved in broth when diluted to low concentrations of copper. Attempts were done to overcome this problem by synthesizing copper oxides, but the yields for the production of nanoparticulated copper were lower. Additionally, modifications of nanoparticles using silicate showed that the concentration of silicon was associated to the increase of particles size, which was an indication that silicon affected the nanoparticles. However, these silicon-modified nanoparticles were still susceptible to dissolution. The entrapment of copper nanoparticles in a silica matrix gel was tested as a different approach to the use of copper nanoparticles in suspension. Elemental and TEM analysis showed that $Cu(OH)_2$Tart-Ad nanoparticles were successfully entrapped and presented a very small size and were disperse. However, some methodological constraints prevented the testing of the activity of these materials. Finally, a high throughput method to measure antimicrobial activity of several compounds in liquid systems was developed. This allowed the assessment of the activity of $Cu(OH)_2$Tart-Ad nanoparticles and copper chloride, which were showed to be similar, and thus suggested again that $Cu(OH)_2$Tart-Ad nanoparticles dissolved when present in broth and probably most activity was due to soluble copper. Soluble silver was also tested as positive control and to establish a positive point for further development of antimicrobial agent.

In summary, this project was successful at establish an initial synthetic methodology for the production of copper-based nanoparticles, which allows the future development of new ones. Furthermore, a high throughput methodology was developed and implemented effectively, which was used to determine the antibacterial activity of nanoparticles and will allow the future development of new ones.

6. Future work

Future work will focus in increase the stability of the nanoparticles produced to avoid dissolution, while maintaining their appropriate small size. Possible strategies are the use of polymer and liposomes, since these modifications may protect the nanoparticles against the instability. Particle size, shape, capping agents, pH, ionic strength, specific ions and ligands may affect the stability of nanoparticle.

Once synthesized, the nanoparticles should have higher antibacterial activities than soluble copper, and then they should be characterized to determine the mineral phase of nanocrystalline structures. HRTEM may be used for that propose, since this technique allows the analysis of an individual nanoparticle. To complement the antibacterial profile of compounds synthesized, they can be tested against different species and antibiotic resistant strains to confirm the broad spectrum of action of nanoparticles.

The development of new strategies of application of nanoparticles, such as the entrapment in matrices, may also be an alternative to avoid the instability problems seen in liquid systems. Furthermore, should be developed a method to determine the antibacterial activity of these materials, such as the silica gel with nanoparticles entrapped in the matrix that was synthesized during this work.

7. References

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Attachments

Iso-sentist CM 0473 Bacterial broth composition:

Typical Formula*	gm/litre
Hydrolysed casein	11.0
Peptones	3.0
Glucose	2.0
Sodium chloride	3.0
Soluble starch	1.0
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-Cysteine hydrochloride	0.02
L-Tryptophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Guanine	0.01
Xanthine	0.01
Uracil	0.01
Agar	8.0

	1	2	3	4	5	6	7	8	9	10	11	12
A	[Cu]=0 No bacteria		[Cu]=0 10^{-5}		[Cu]=0 10^{-4}		[Cu]=0 10^{-3}		[Cu]=0 10^{-2}		[Cu]=0 10^{-1}	
B												
C	[Cu]=1 ppm No bacteria		[Cu]=1 ppm 10^{-5}		[Cu]=1 ppm 10^{-4}		[Cu]=1 ppm 10^{-3}		[Cu]=1 ppm 10^{-2}		[Cu]=1 ppm 10^{-1}	
D												
E	[Cu]=10 ppm No bacteria		[Cu]=10 ppm 10^{-5}		[Cu]=10 ppm 10^{-4}		[Cu]=10 ppm 10^{-3}		[Cu]=10 ppm 10^{-2}		[Cu]=10 ppm 10^{-1}	
F												
G	[Cu]=100 ppm No bacteria		[Cu]=100 ppm 10^{-5}		[Cu]=100 ppm 10^{-4}		[Cu]=100 ppm 10^{-3}		[Cu]=100 ppm 10^{-2}		[Cu]=100 ppm 10^{-1}	
H												

Original *E.coli* culture

10^{-x}: Dilution from original culture

Antibacterial test: 96 well plate scheme