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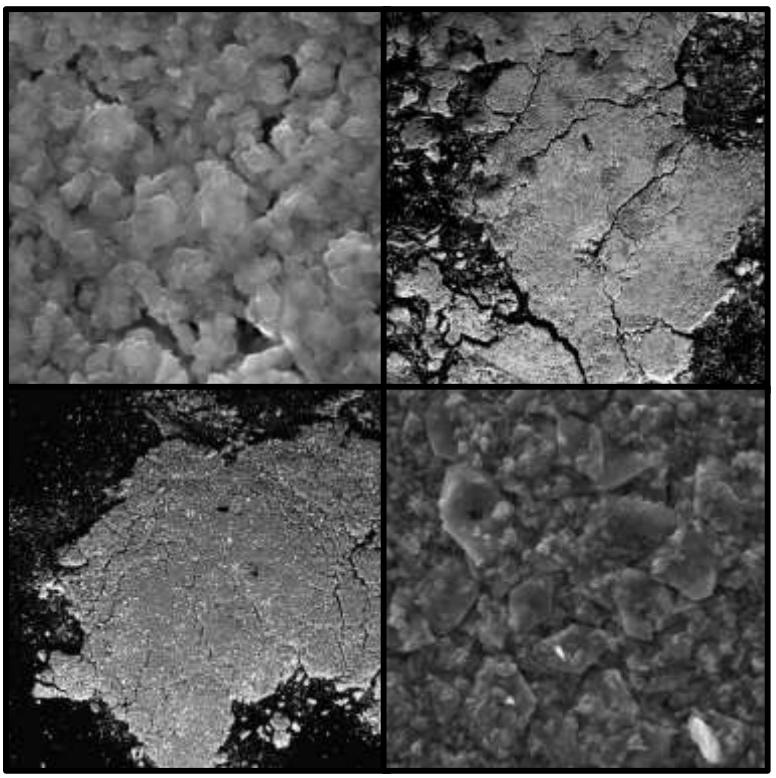
Master Thesis

Antibacterial activity of Calcium/Phosphate glass nanoparticles doped with zinc



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Abstract

Main constituents of bones and hard tissues are Hydroxyapatites [$Ca_{10}(PO_4)_6(OH)_2$, Hap]. Due to their high biocompatibility and biodegradability, calcium phosphate (CP) have been playing for years a role in human hard tissues bio-engineering, more specifically in bones regenerative therapy. Nowadays, we are focusing on combining metals for antibacterial activity with CP particles properties. Zinc is thoroughly present inside human body which makes it a serious candidate for the present application. In this report we synthetize different Calcium/phosphate glass (CaP) nanoparticles (Nps) containing a certain amount of ZnO (0%-28%). We also investigate, phosphorus/zinc Nps with high concentration of zinc (50%,60%). Both of particles were made via sol-gel process. We tested the antibacterial activity of these particles against Staphylococcus aureus and Pseudomonas aeruginosa. Each powders structures and compositions were investigated with Scanning Electron Microscope (SEM) and Energy Dispersive X-Rays Spectroscopy (EDS). We also characterized zinc releases of NPs inside Hepes (7,4 pH) medium in order to quantify the amount of zinc which is liberated depending on ZnO concentration. Both types of nanoparticles showed a satisfying result against bacteria for the most concentrated in ZnO.

Acknowledgment

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1 Introduction

1.1 Bones composition

From a chemical point of view bones are in their major part made of calcium and phosphorus which are both included in Hydroxyapatites. However, various others elements are also present in more or less high quantity. At microscopic scale, it is composed of a mineral phase (70% in wieght) and an organic phase (30%) which is made of 90% of collagen fibers itself. The organic matrix which is called osteoid is synthetize by osteoblast cells where takes place on its surface, the mineralization process. This mineral is mainly Hydroxyapatite (Hap) and gives to the bone its hardness and rigidity. Bones are constantly remolded thanks to osteoblast or osteoclast. Activated in part under mechanical stress, this remolding allows bone to be more resistant to stresses which are submitted. During bone remolding, osteoclast releases enzymes and decrease pH to dissolves Hap and hydrolyse collagen. After that, osteoblast will produce collagen again and Hap will precipitate (mineralization). The formula of stoichiometric HAp is $Ca_{10}(PO_4)_6(OH)_2$ and has a high chemical stability at physiological pH but lower at acidic pH to able its dissolution during bones remodeling. In fact the mineral which is formed by the bone is lowly crystalline. It has a lake of calcium and some phosphate are substituted by carbonate in few places. It promotes its low crystallinity and becomes more soluble and degradable. This degradability is also encouraged by the porosity of the bone. However there is not only substituted carbonate but also many other components which makes harder the definition of the composition of the bone. Because of all these substitutions the bone composition change from a person to person, depending of his sex, his age or his diet. In table 1 we referred different range of concentration for many elements which can vary the composition of the bone. [1]

Elements	From 30 to 80 years old		From 60 to 82 years old
	Mean	Range	Mean
Br	0,67± 0,25µg/g	0,29 -1,12 µg/g	4,1 ± 4 µg/g
Ca	22,2±2,6%	16,9 – 26,7 %	21 ± 4%
Cl	538 ±162 µg/g	322-941 µg/g	-
K	572 ± 205 µg/g	237 – 956 µg/g	-
Mg	2379 ± 314 µg/g	1951 – 3147 µg/g	2600 ± 400 µg/g
Na	5342 ±496 µg/g	4554 – 6172 µg/g	5400 ± 1000 µg/g
P	10 ± 2,8%	4,49 – 16,9%	8,8 ± 2,2%
Sr	112 ± 37 µg/g	49,8-184,6 µg/g	62 ± 18 µg/g
Zn	114 ± 16 µg/g	85-140,8 µg/g	180 ± 44 µg/g

Table 1: Range of composition of elements inside the bone [1]

If we look at the composition of the modified Hap in table 1, we see that zinc is already present. This is a first reason of why we are interested to incorporate zinc in Calcium/Phosphate particles. Zinc have been used for anti-inflammatory properties, and bone

formation [1]. Furthermore zinc ions have an impact on osteoblast proliferative properties but as well an inhibitory effect on bones resorption. [2] It could also improve the osteoconductivity and the absorption capacity of proteins by Hap.[3] Above all, in this study, we were focused in its antibacterial effect.

1.2 Antibacterial activity

The antibacterial capacity of ions are not completely clear. Nevertheless, the literature refers three hypothetical mechanisms [2]. The first mechanism talk about ions which penetrate inside bacteria and reduce the production of intracellular ATP (Adenosine Triphosphate). These molecules bring the required energy to perform the synthesis of membrane and proteins and their decrease disturb the process of DNA replication. The second hypothesis of mechanism is that, due to accumulation of ions inside the membrane, it changes its permeability. It also blocks the transport of ions through the membrane and as a result, the death of bacteria. Finally the third mechanism and more accepted is based on the ion induction of reactive oxygen species (ROSs). The membrane and cell wall of bacteria can react with oxygen radicals which change their shape and lead to its death.

According to the same study focused on silver ions, the mechanism of bacteria growth inhibition seems to be varying between the doping metallic ions. They supposed two mechanisms, one suggest that Hydroxyapatites doped with silver can attract bacteria to their surface due to electrostatic forces, and interact with the membrane of the bacteria. The second hypothesis is that silver ions are released from the inside of Hap and “discloses its bactericidal activity throughout the material surrounding it”. Hap/copper ions have a different mechanism. Even if it is not completely understood, they supposed that these ions could form resistant bonds with the constituents of bacteria and increase its permeability. It will disrupt the transport inside the membrane and lead to the death of the bacteria.[4] It was suggested that zinc could have the same mechanism as copper ion [2] and so a pretty similar antibacterial activity.

1.3 Usual treatment

A usual treatment for bones regenerative therapy is the application of the “Autogenous cancellous bone” graft. It is named as the “gold standard treatment in bone loss” due to its many advantages which are osteogenic, osteoconductive and osteoinductive properties of autograft and its low transmission of disease. However there are many issues, as the limited availability and its variable quality, infection, a long operative time and bleeding. It could also happened that the patient suffers from chronic pain located on the donor site which can induces additional difficulties. [5] It exists an antibiotic therapy in order to introduce an antibacterial assistance, which helps the body during the defense mechanism. This treatment is performed via oral way and has a poor efficiency. In fact, the concentration of antibiotics needs to be high to ensure a proper inhibition. It is possible to deliver drugs at the local place of infection but it requires a high concentration too, in order ensure its activity and it cannot be cytotoxic.[2] People started to investigate biomaterials which can be used as substitute to this kind of surgery and their related problems.

1.4 Calcium/phosphate materials

We will focus on calcium/phosphate ceramics such as Hydroxyapatites, TCP (tricalcium phosphate) or BCP (biphasic calcium phosphate), which are mainly used for bones regeneration, dental or drugs delivery, like Calcium/Phosphate (CaP) glass ceramics. These glasses as biomedical target were firstly investigated in the 80s by Burnie et al. [6] They studied P_2O_5 glass, as network former, mixed with CaO and Na_2O. They made a range of composition of glasses with different degradation rates and show that it can be tuned by modifying the ratio between Ca and Na. Increasing CaO content improved the stability of the glass and increasing Na_2O percentage improved its degradability [7].

The aim of this project is including zinc ions inside CaP nanoparticles (NPs). Many metallic ions were used as substitutes in hydroxyapatite. In [2] we can find a whole array on different ions which were used for their antibacterial properties among other aspects. We can talk about the most common of them, beginning by the well-knon: Silver (Ag+). It is well studied that silver has an important antibacterial activity covering a wide range of bacterias. [8] Ag-Hap showed impressive results against bacteria, viruses and fungi. Silver had the highest efficiency, for a concentration lower than 35 ppb [2]. At this concentration silver has no toxic effect on mammal's cell. It suggests that silver could have a toxic effect on cells above a certain concentration. In addition, it appears that silver-NPs are between 5 and 18 times more toxic than silver ions. [9] We can also cite copper ions, however as silver, they could be cytotoxic. Furthermore, other less-known metals were investigated as Selenium, Cerium or Europium. They reveal a helpful capacity for the development of the human body or metabolism. [10-11]

We started our work from calcium phosphate ormoglasses (organic modified glass) (CaP) previously studied in the laboratory [12]. In order to success the bone tissue regeneration, we need to precisely regulate angiogenesis and osteogenesis which are closely dependent.[12] It is possible to make a composite with calcium phosphate NPs and a well-chosen biodegradable polymer. Coupling particles with a polymer able to design various shapes and offers a broad range of applications. Based on previous studies we chose to work with polylactic acid, regarding its biodegradable and biocompatible properties [13]. One of the issues is that, PLA has low osteoconductive and angiogenic characteristics. Adding CaP particles could improve the bioactivity of the polymer and preserved its mechanical properties. We have demonstrated that Calcium phosphate ormoglass exhibit angionesis in vivo. [12] To go further, our goal is to add a third characteristics to this material: antimicrobial activity. We first tried to doped "P30" nanoparticles which had a composition of 30 %mol of Phosphorus and 70%mol of Calcium oxides. As it was hard to predict the capacity of incorporation of zinc inside CaP glass, we chose to begin with a composition which was relatively simple. We possibly could have studied the same for G5 particles [14] but to the sol gel process required 4 precursors (Ca,P,Ti and Na) and a much more complicated protocol. We thought that it would be easier to incorporate zinc inside P30-NPs. Moreover, we knew that P30 could be electrospun in PLA nanofibers.

1.5 Sol-Gel

There is many ways to synthesis doped HAp according to [3]. We can use for example hydrolysis, hydrothermal, wet chemical and sol–gel. In our study, we used sol gel method to prepare CaP biomaterials with different concentrations of ZnO. The incorporation of zinc should not modify the properties of the material: biocompatibility, degradability and electrospinnability. As P30 were synthetize via sol gel, we choose to work with the same process.

Using the sol gel as method of synthesis enables to form products with high purity. The result is homogeneous particle composition and can be performed at low temperature. This process allows to vary the shape and the morphology of nanoparticles [15]. It was also quick and easy to set up inside a laboratory. The use of sol gel requires the selection of the right precursors but also the right conditions of the experiments (working temperature, time of reaction, atmosphere, precursor, water, catalyst concentration, solvent). Most of the time, the sol is a colloidal suspension which is a dispersion of solid particles into a liquid where particles remains separated [16]. With this process we can achieved from 1 to 100nm size nanoparticles having amorphous or crystalline structures [17] or even bigger. The basis is to mix water into precursors. This first step is called hydrolysis (figure 1) and then the reaction continue forming a gel which is called the condensation step (figure 2). The size and shape of our particles depends on of the media.

1) Hydrolysis

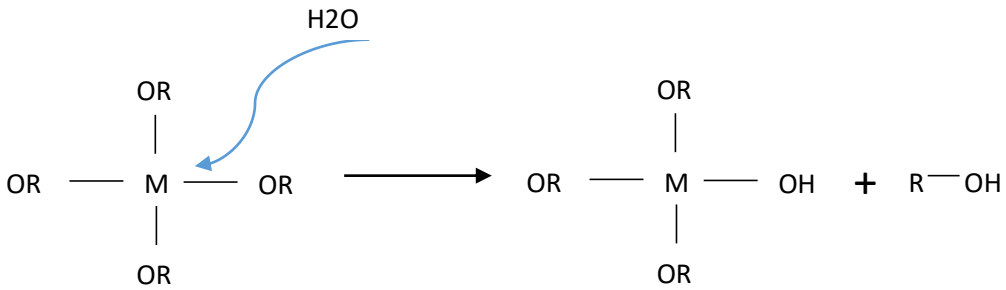


Figure: 1 Mechanism of Hydrolysis

2) Condensation

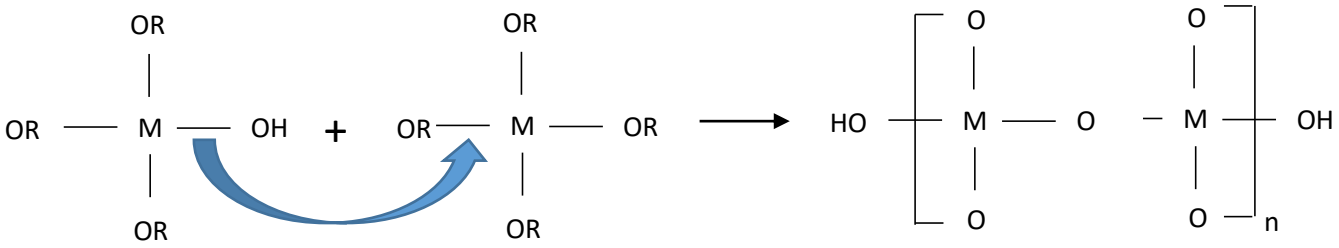


Figure 2 : Mechanism of Condensation

As it was studied on ZnO particles, the solvent and the pH modify its size and shape. For example it was noticed that using aqueous ammonia (NH_4OH) as a solvent, induces spherical particles. When sodium hydroxide is used, it produces wire-like particles. [18]

1.6 Precursors investigation

As we said, choosing the right precursors is a crucial point of the sol gel process. We choose to work with alkoxydes precursors because they present many advantages. First of all, alkoxyde allows to perform the reaction at room temperature. For example, metalorganic compounds and inorganic salt like zinc acetate that are commonly use as precursors in the preparation of ZnO nanoparticles via sol gel. Unfortunately, using zinc acetate required a final step at high temperature (calcination) to form the particles of ZnO due to its insolubility in polar media. [19] That is why we were interested in organozinc compounds such as diethylzinc. Combined with 2-Methoxyethanol we can form more a stable and easy to handle zinc alkoxyde. Alkoxydes are sensible to be hydrolysed which justify their manipulation in free water atmospheres. We supposed that during the sol gel we would have a fast hydrolysis step and slow condensation kinetics [19]. Calcium nitrate were mainly used as calcium precursor. Nonetheless, some issues were exposed during a study of calcium silica bioactive glass [20]. They found that calcium nitrate can cause heterogeneity. Nitrates also have to be burned due to their toxicity. Hence, as we were performing sol gel at room temperature, both of these precursors cannot be used. We used methoxyethanol in the preparation of the precursors and as a solvent during the sol gel process.

2 Material and methods,

2.1 Precursors preparation

2.1.1 Calcium precursor

We made the calcium precursor by mixing 10,121g of metallic calcium (99%, Sigma Aldrich) with 250 mL of 2-methoxyethanol anhydrous (99,8%, Sigma Aldrich) into a round-bottomed flask. We heated the batch to 134°C under reflux and Argon atmosphere during 24h. We filtered the solution with a syringe filter (45 µm pore size) before putting the liquid into a bottle full of argon.

2.1.2 Zinc precursor

To prepare the precursor of zinc we mixed 50mL of 2-Methoxyethanol and 50mL of diethylzinc (15 wt% in toluene, Sigma Aldrich) inside a 250mL round-bottomed flask. We work under argon (Ag) atmosphere and we used an ice bath because the reaction is highly exothermic.

2.1.3 Phosphorus precursor

To prepare the phosphorus precursor we had to mix phosphorus pentoxide (99,9%, Sigma Aldrich) with distilled ethanol . Phosphorus Pentoxide powder is highly reactive with water, so

we weight it in Ag atmosphere and used distilled absolute ethanol (99,5%, Panreac). We prepared 100mL of precursor into a 250mL round-bottomed flask. To perform this reaction we just mixed 28,3908g of phosphorus pentoxide with 100 mL distilled ethanol. We let the reaction all night long and pick up the final solution into a bottle full of argon for the storing.

The three precursors are protected from light and due to their sensitivity to water, stored under Ag atmosphere inside freezer at -20°C.

2.2 Nanoparticles synthesis

We prepared all nanoparticles batches into a Round-bottomed flask of 25 mL (figure 3). Before mixing all precursors and 2-Methoxyethanol, we had to well dry and clean the dishes to avoid any trace of water inside the batch. We also worked with inert atmosphere using argon. After replacing all air inside the flask we can introduce the solvent (Methoxyethanol) and cool it with an ice water bath. During the research of a solvent which could accommodates the mix of the precursors without any interactions ("Particles experimental pathway" in annex), we discover that zinc alkoxide was precipitating in ethanol so it was not an appropriate solvent. When the solvent is cold enough, we can introduce all precursors. We proceeded first by the introduction of zinc precursors, then calcium and finally phosphorus precursor. We can now start by adding gradually a mix of 50% Ethanol (99,5%) and 50% Ammonia (30% of NH_3, Panreac) to lounch the sol-gel process (figure 3). When the reaction is over we had to wash the particles. To separate the liquid and nanoparticles we centrifuged the solution inside tubes at 20000 rpm for 10 min. To wash particles we used absolut ethanol and hexane (99%, Sigma Aldrich) for the final step. To well disperse the particles inside the washing solvent, tubes were placed into an ultrasonic bath during 5 min. We dried the particles into a drier during 2 hours at 70°C. Helped with a mortar we reduced the particles into powder for analysis.

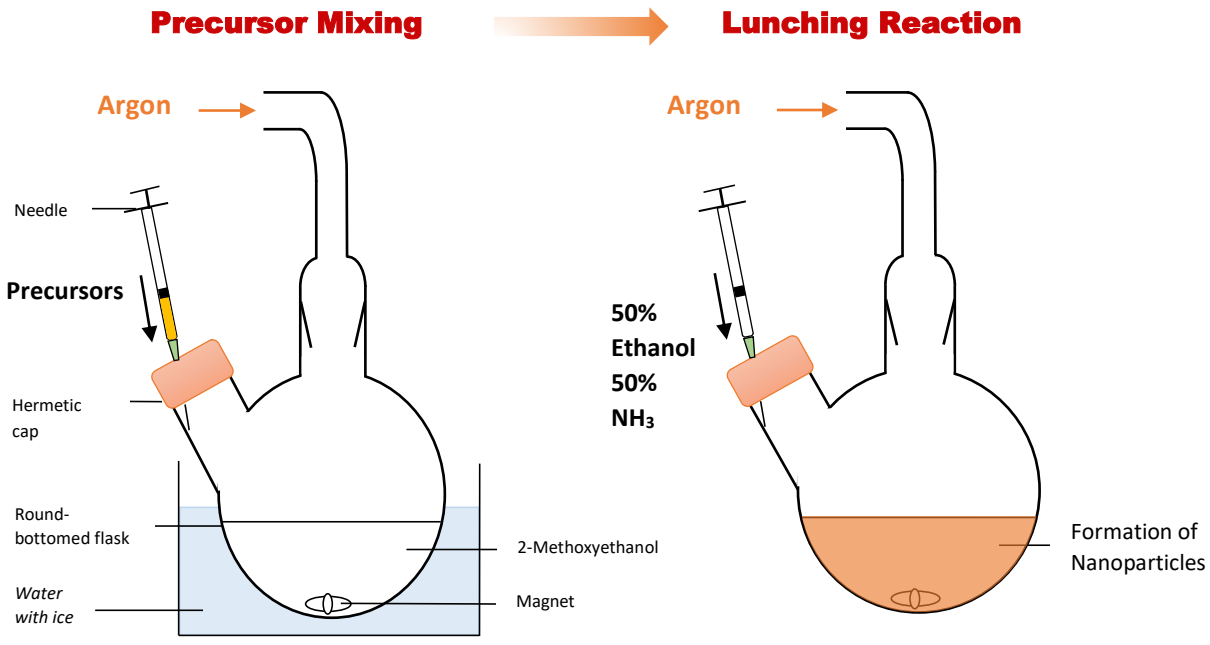


Figure 3: Schema of the batch

We used exactly the same protocol to synthesize P/Zn-NPs but we used only the precursors of zinc and phosphorus. You can find the concentrations we used in the same part as P30-Zn-NPs ("Particles experimental pathway" in annex). The main difference between both batches is that when we synthesize P/Zn-NPs it required less catalyst to form the particle.

For more details you can check the part dedicated to the resulting composition of particles in annex. These tables gives informations about all volumes we used, how we varied the composition of the particles by modifying the concentration of precursors at the begging of the batch.

2.3 Antibacterial Testing

Wild-type *Pseudomonas aeruginosa* PAO1 strain CECT 4122 (ATCC 15692) and *Staphylococcus aureus* CECT 86 (ATCC 12600) were obtained from the Spanish Type Culture Collection (CECT). To obtain inocula for examination, the strains were cultured overnight in Luria Bertani (LB) (Pronadisa, Spain) liquid medium for P.*aeruginosa* and tryptic soy broth (TSB) (Sharlab, Spain) medium for *S.aureus* at 37°C.
To determine the survival of the different strains in the presence of different nanoparticles, 100 µl of bacteria at a density of 5×10^5 CFU/ml in TSB or LB medium were inoculated into the wells of 96-well assay plates (tissue culture-treated polystyrene; Costar 3595, Corning Inc., Corning, NY) at different concentrations. The inoculated microplates were incubated at 37°C at 150 rpm for 8 h in an Infinite 200 Pro microplate reader (Tecan) and A_{550} was read every 15 minutes.

2.4 SEM

All pictures from Scanning Electron Microscope (SEM) and Energy Dispersive X-Rays Spectroscopy (EDS) analysis were performed on FEI Quanta 200 at 20kV as acceleration voltage. The samples were coated with a thin layer of carbon to improve the conductivity.

2.5 EDX

For X-ray powder diffraction (XRD), the sample were prepared by manual pressing of some of the powder, by means of a glass plate to form a flat surface, in cylindrical standard sample holders (16 millimetres of diameter, 2.5 millimetres of height). Patterns were recorded using a PANalytical X'Pert PRO MPD Alpha1 Powder Diffractometer in Bragg-Brentano θ/2θ geometry of 240 millimetres of radius.

2.6 Zinc Release

To measure the quantity of zinc which is released from our nanoparticles we decided to use a colorimetric assay. We used the same protocol as the study of improved Colorimetric Determination of Serum Zinc [21]. We have adapted their protocol to our needs. To start we prepared all the solutions for each assays. They were prepared with metal-free water as the original protocol and they were stored in the fridge. We had to adjust our volumes.

Stock guanidine reagent: to prepare this solution we mixed 14,32 g of Guanidine Hydrochloride (99%, Sigma Aldrich) and 0,7960g of tris(hydroxymethyl)aminomethane (99%, Sigma Aldrich) into 25 mL of water.

Stock Pyridylazo reagent: we add 0,02525g of 4-(2-Pyridilazo)resorcinol (95%, Fluka) inside 25 mL of water. However we had some difficulties to solubilize all the powder inside water. To help it we added few drops of Sodium Hydroxide solution 1M (98%, Panreac)

Stock Chloral hydrate reagent: we diluted 15,15 g of chloral hydrate (98%, Sigma Aldrich) in water.

Stock Ascorbic acid/Cyanide reagent: In the study they add ascorbic acid and cyanide to stock guanidine reagent on the day of analysis. It means that they weighed both products each time when they wanted to work with guanidine reagent. To save some time we decided to prepare a solution with these two components. Moreover we used cyanide which is very dangerous, you can find information about the use and safety of this product in "Cyanide Safe Use Guidelines" made by the University of Columbia [22]. Finally we put 0,3866g of Sodium Cyanide (97%, Sigma Aldrich) and 1,2563g of ascorbic acid (99,5, Sigma Aldrich) in 25mL of water. All the residues were treat specifically as it is indicate in the protocol.

The solutions were protected from light and stored in a fridge at 4°C. These four solutions are used to color the zinc solutions that we wanted to quantify by absorbance. We also needed standards, these solutions were made from a 0,1M zinc chloride (Sigma Aldrich) diluted in water. The concentration were 1mM, 0,75mM, 0,5mM, 0,25mM, 0,1mM, 0,075mM, 0,05mM and 0mM.

We tested zinc released from our particles in Hepes solution, an organic buffer. This solution mimic the buffer effect of physiological media and keep the pH near to neutral values. To prepare this solution we diluted 0,2994g of Hepes (99%, Fluka) into water. Sodium Hydroxide 1M was added until a pH of 7,4

On the day of analysis we had to prepare the working guanidine reagent by mixing the stock guanidine reagent with ascorbic acid/cyanide solution. For 100mL of working guanidine solution we took 10mL of ascorbic acid/cyanide reactive.

Procedure: We performed this test inside microplates Nuclon tm Delta Surface (Thermo Scientific). For each zinc solutions tested we took respectively, 22,5 µL of zinc solutions (From Nanoparticles and Reference), 111 µL of working guanidine reagent, 7,5 µL of chloral hydrate reagent and 10,5 µL of pyrodilazo reagent. We mixed all reactants in any order but the final one always needs to be the pyrodilazo, which gives the color to the solutions and it is sensitive to light. After adding the coloring agent, we waited 5 min before measuring Absorbance. We assessed the absorbance with Infinite M200pro (Tecan) plate reader at 497nm. You will find more details about the procedure to draw the reference line in the part which talk about results of zinc release.

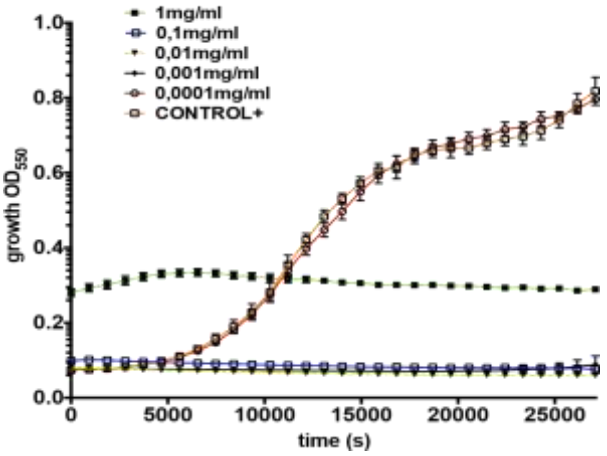
3 Results and Discussion

3.1 Preliminary results of different compounds against bacteria

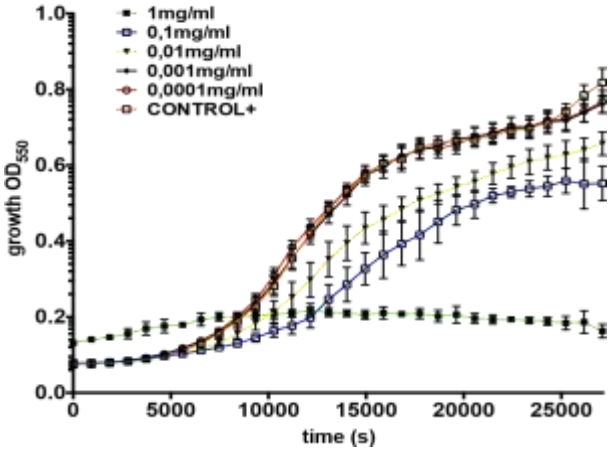
Before talking about our results, we will show a preliminary study of six different metallic salts and nanoparticles and their antibacterial activity against two bacteria performed in the laboratory before my master started: *Staphylococcus Aureus* (*S.aureus*) and *Pseudomonas Aeruginosa (PAO1).* You will find results about $AgNO_3$, $ZnCl_2$, ZnO, P15, Gallium Chloride and Cerium Chloride. This short presentation will provide a starting point to compare the antibacterial activity of different materials. During this test we looked for the minimum concentration of compounds which inhibit the bacteria growth : the minimum inhibitory concentration (MIC). To find it, we just had to prepare different concentration of antimicrobial agent, in presence of bacteria and measure their quantity. By increasing the concentration of inhibitors and measuring the growth of bacteria, we can find the limit where they have antibacterial properties On figure 4, which referred to the whole study, you can see the results for these compounds against PAO1.

Pseudomonas aeruginosa

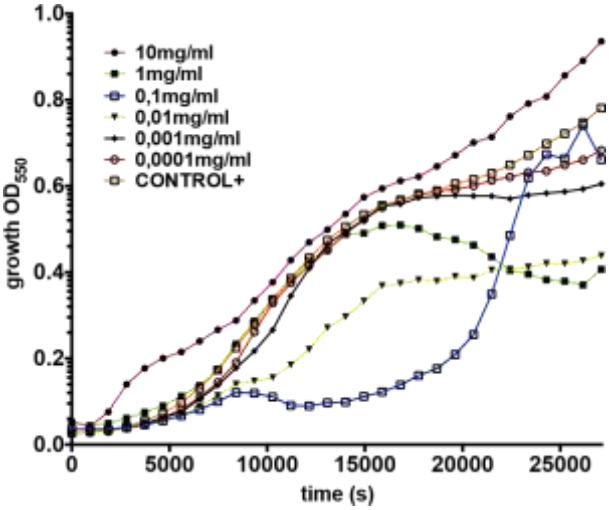
(A) **$AgNO_3$**_*PAO1*



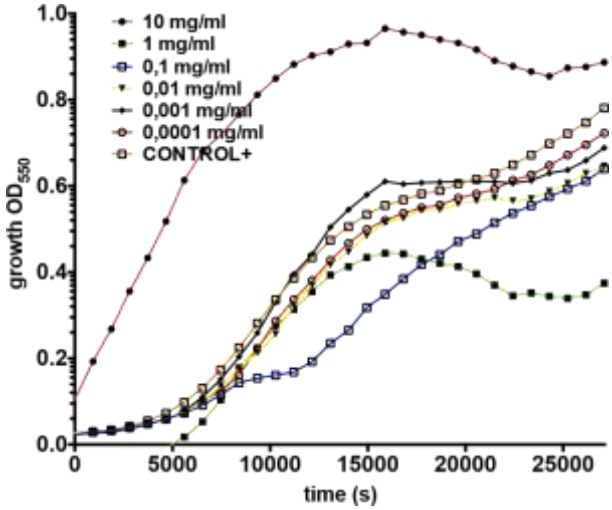
(B) **$ZnCl_2$**_*PAO1*



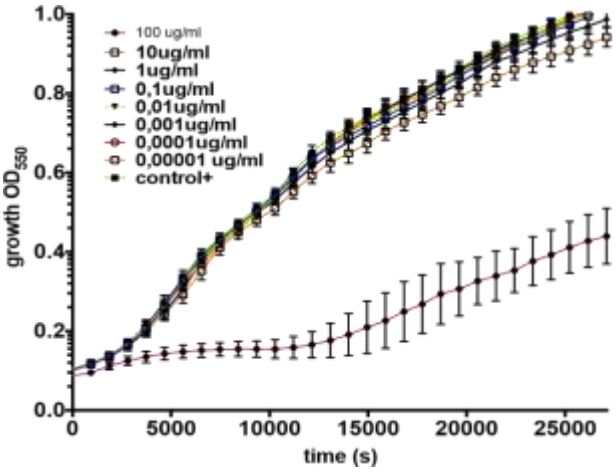
(C) **ZnO-NP**_*PAO1*



(D) **P15-NP**_*PAO1*



(E) Gallium_chloride_*PAO1*



(F) Cerium_chloride_*PAO1*

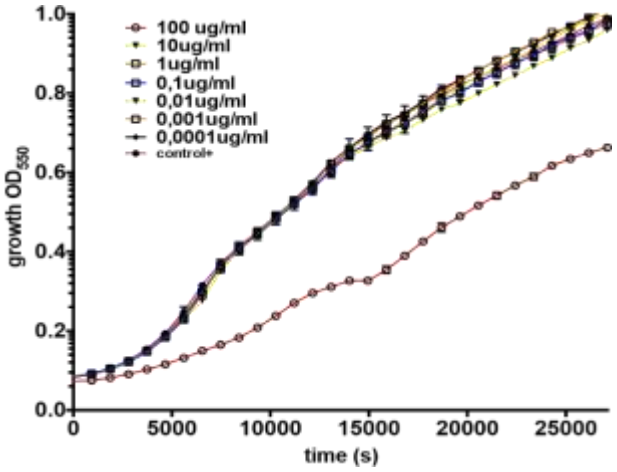


Figure 4: Antibacterial activity of salts and nanoparticles against PAO1, (A) $AgNO_3$,(B) $ZnCl_2$,(C) ZnO-NPs,(D) P15-NPs, (E) Gallium Chloride, (F) Cerium Chloride

On these graphs we draw the growth of bacteria Vs time (s). The different curves correspond to different concentrations of antibacterial compound. We can see that against *Pseudomonas Aeruginosa*, $AgNO_3$ has a significant antibacterial activity for a concentration higher than 0,001 mg/ml. If we take $ZnCl_2$ it is 1mg/mL, for ZnO we have 0,1 mg/mL. For P15 particles which are calcium/phosphate NPs, there is not a significant inhibition of the growth of particles. Maybe just a small effect at 1 mg/mL (probably due to basification of the media). For gallium and Cerium chlorides we can see a small beginning of inhibition for a concentration of 100 µg/mL. Between these six compounds we can conclude that AgNO3 seems to be the best inhibitor. The same study was performed against *Staphylococcus Aureus* bacteria (Annex). In this study we see that against *Staphylococcus Aureus,* $AgNO_3$ has a significant antibacterial activity for a concentration higher than 0,01 mg/ml. If we take $ZnCl_2$ it is 0,1 mg/mL, for ZnO we have 0,1 mg/mL. For P15 particles and Cerium chlorides there is not a significant inhibition of the growth of particles. Finally, for gallium we can see a small inhibition for a low concentration of 100 µg/mL. On both studies we have clearly demonstrated the efficiency of silver ions against bacteria. However, zinc salts and ZnO-NPs shows the highest inhibition after $AgNO_3$ and possess much more bones healing properties. This first study demonstrates the potential of zinc ions as antibacterial agents.

3.2 Structure

You can find in table 2 different information about the particles which are cited in this part.

Types of Particles	Abbreviation	Composition
CaO, P_2O_5, ZnO	P30/Zn2 2%ZnO-CaP-NPs	✓ Ca0 : 67,04% ✓ P2O5 : 31,18% ✓ ZnO : 1,78%
CaO, P_2O_5, ZnO	P30/Zn5 5%ZnO-CaP-NPs	✓ Ca0 : 65,45% ✓ P2O5 : 29,43% ✓ ZnO : 5,12%
CaO, P_2O_5, ZnO	P30/Zn8 8%ZnO-CaP-NPs	✓ Ca0 : 63,50% ✓ P2O5 : 28,30% ✓ ZnO : 8,20%
P_2O_5, ZnO	P50/Zn50	✓ Ca0 : 3,12% ✓ P2O5 : 43,01% ✓ ZnO : 53,86%
P_2O_5, ZnO	P40/Zn60	✓ P2O5 : 43,46% ✓ ZnO : 56,54%
CaO, P_2O_5, ZnO	P30/Zn14	✓ Ca0 : 59,73% ✓ P2O5 : 26,20% ✓ ZnO : 14,09%
CaO, P_2O_5, ZnO	P30/Zn10	Heterogeneous: ✓ Ca0 : 63,22% ✓ P2O5 : 26,7% ✓ ZnO : 10,08% Homogeneous: ✓ Ca0 : 59,97% ✓ P2O5 : 29,82% ✓ ZnO : 10,21%
CaO, P_2O_5	P30	✓ Ca0 : 69,48% ✓ P2O5 : 30,52%
CaO, P_2O_5, ZnO	P30/Zn1.5	✓ Ca0 : 67,36% ✓ P2O5 : 31,10% ✓ ZnO : 1,54%
CaO, P_2O_5, ZnO	P30/Zn3.5	✓ Ca0 : 65,52% ✓ P2O5 : 30,91% ✓ ZnO : 3,57%

Table 2: Abbreviation and composition of the cited NPs in the part of results and discussion

3.2.1 P30/ZnO Particles

In this part we will show all pictures obtained from SEM to see in details what we really formed. Using back-scattered electrons detector enables to investigate the homogeneity of our powders. We noticed that different phases with two clearly distinct compositions were present. With this detector matter appears with different intensity of colors depending on the atomic weight. In our case we can notice small brighter crystals with a very high content of zinc. You can see on figure 5 and figure 6 which have different ZnO concentrations, whiter points which correspond to high zinc content crystals as it is the heaviest atom.

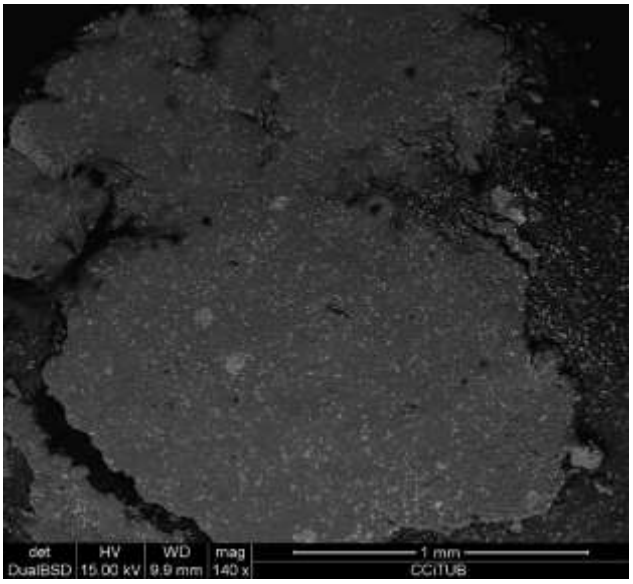


Figure 5: SEM picture of 5% ZnO-CaP-NPs

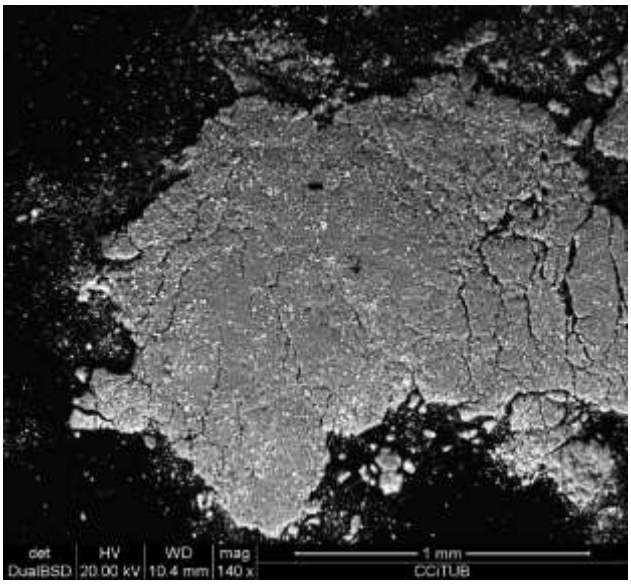


Figure 6 : SEM picture of 8% ZnO-CaP-NPs

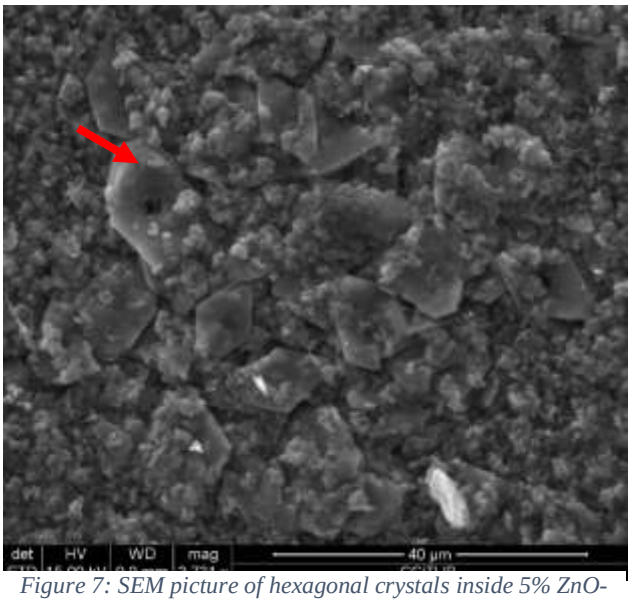


Figure 7: SEM picture of hexagonal crystals inside 5% ZnO-CaP-NPs

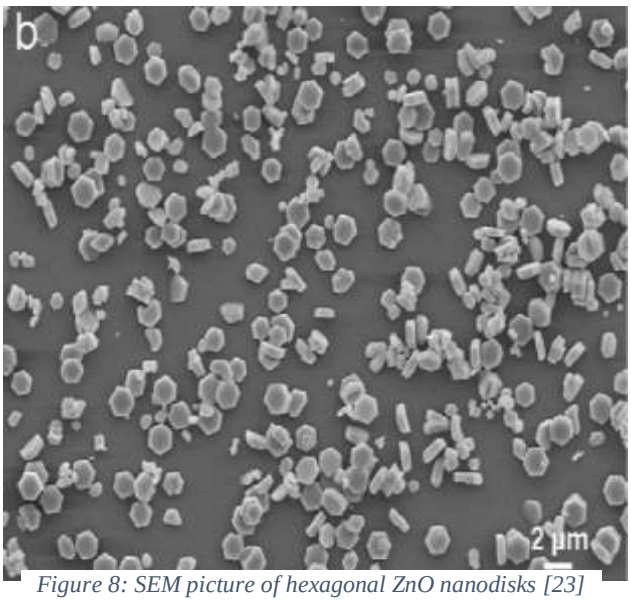


Figure 8: SEM picture of hexagonal ZnO nanodisks [23]

On figure 7, we increased the magnification of figure 5. It shows diamond-shaped crystals which are formed inside the powder. These crystals have a size included between 10 and 20

µm in size according to figure 7. They are few microns bigger than pure ZnO crystals that we see on figure 8[23], found in the literature. As we can see on their picture, ZnO crystalizes in hexagonal structure. If we look at the crystals inside 5%ZnO-CaP-NPs, they have a hexagonal shape but there were some changes of the lattice parameters. This document provides also the figure 9, of the hexagonal mesh of ZnO crystals with its system of axes. If we look at the initial shape (in black) and compare it to the final shape (in red) of the crystals inside the doped CaP particles, we see that the mesh is stretched following the direction [01$\bar{1}$0] according to figure 9.

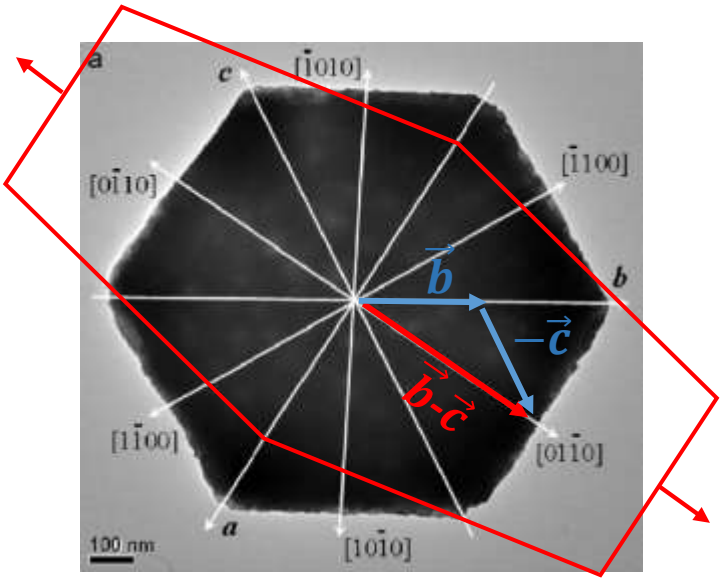


Figure 9: Hexagonal mesh of ZnO crystals with its system of axes [23]

These diamond-shaped crystals are mainly made of ZnO. If we take the example of 5%ZnO-CaP doped NPs powder and look at the composition of the darkest zone, we get particles which are composed of 65,75%M CaO, 30,92%M P_2O_5 and 3,33%M ZnO. Now if we look at the composition of the modified hexagonal crystals we have 36,83%M CaO, 8,83%M P_2O_5 and 54,34%M ZnO. The problem is that we cannot really investigate the antibacterial activity for this concentration which is supposed to be 5% ZnO CaP-NPs, as the composition of the crystals highly influence the final composition. To be accurate we need to test particles which are completely homogeneous. It could be interesting to compare powder containing hexagonal crystals and particles without crystals at the same concentration, to see if they have a similar effect against bacteria. We can find a similar case of a synthesis of ZnO nanoparticles via sol gel process [24]. They confirm that ZnO crystallize in a hexagonal wurtzite structure called zincite. In a solution of water and ethanol in a proportion of 1:1 molar ratio, the growth rate of ZnO crystals falls down due to ethanol properties such as its higher viscosity or lower polarity. Under these reduced rate conditions it is supposed that crystals adopt a convex polyhedral shape as it offers the minimum surface energy.[25] During the sol gel we are incorporating water (inside NH3 30% solution) and ethanol. As it was reacting during approximately 18h crystal had enough time to grow.

Hence to avoid the formation of crystals. We reduce the time of reaction to 1h30 instead of 18h. In that way crystals did not have the time required to grow. Figure 10 shows that crystals disappear to form homogeneous particles. On picture 7 crystals are between 10 and 20µm large and we did not noticed any crystals at this size on 2% ZnO-CaP-NPs (figure 10). We see here that reducing the time of reaction able to avoid the formation of crystals. In compensation we have to introduce more zinc precursor at the beginning of the reaction. At bigger scale, we should find a more appropriate time which is close to time corresponding to the formation of crystals but which stays below it. This could save some zinc precursor and reduce the cost in reactant to perform the sol-gel synthesis.

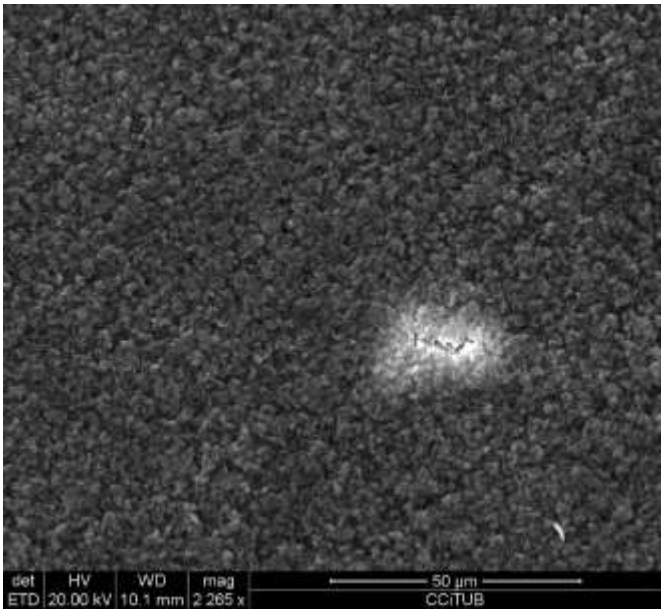


Figure 10: SEM picture of 2% ZnO-CaP-NPs whitout crystals

Why crystals appear in calcium/phosphate-NPs ?

We could suppose that during the reaction between zinc, calcium and phosphorus, we are firstly creating particles which have the lowest concentration of zinc, consuming a high quantity of reactive. We had to increase quantity of zinc precursor after the first batch because zinc was barely incorporated inside the NPs. We also know that hexagonal crystal required a certain time to grow, hence they could only be formed at the end of the reaction. As zinc is highly reactive and due to the excess of precursor, it is reacting in a higher proportion and forms crystal with high content of zinc. Furthermore if we add some drops of ethanol into the liquid phase after the first centrifugation. It reveals that after 1h30, appears a white precipitate but not after 18h. We can conclude that after 1h30, it lefts zinc inside the batch so zinc had not completely reacted. Moreover during our synthesis we highlight that for the same volumes of reactant, the quantity of zinc inside the particles is higher after 3 days of reaction than 18h. So, the more zinc is reacting, the more concentrated are the nanoparticles. We showed that after 1h30 crystals are not yet formed, and it supports our hypothesis in the sense that, we only form low concentrated particles after 1h30 without consuming all the zinc precursor. So after few hours, maybe this rest of zinc leads to the formation of diamond-shaped crystals with a high content of zinc.

3.2.2 Phosphorus/Zinc particles

In order to increase the concentration of zinc inside the particles and its release, we tried particles without calcium. In fact, calcium and zinc are both cations and they are reacting with phosphorus anions. The two powders (figure 11, 12) showed that we could obtain high concentrated zinc particles using only two precursors. P/Zn-NPs are more stable than the particles which contains calcium, as increasing content of zinc decrease the degradability. We found that we had a satisfying degradability for concentration between 40 and 60% of zinc. As

we measure (3.4 Zinc release), the release of these particles is sufficient to consider the particles as an antibacterial material.

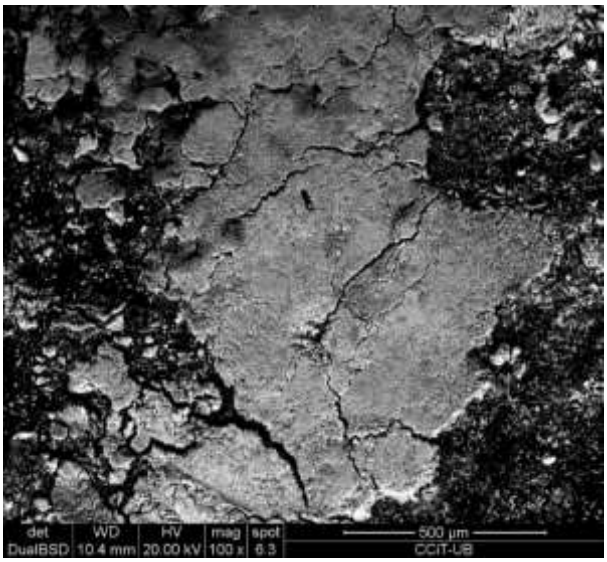


Figure 11: SEM picture of P50/Zn50 NPs (18h of reaction)

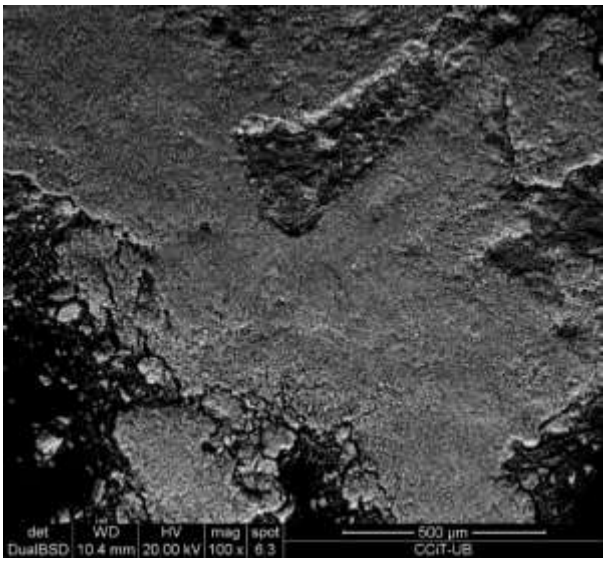


Figure 12: SEM picture of P60/Zn40 NPs (1h30 reaction)

This conclude the part about the structure of the particles. To go further and have a real control on the reaction, we can look for the exact time before crystals formation and work out the condition of their growing. This can lead to a better optimization of reactivity and time of reaction. In fact, as the reaction last only 1h30 we have to introduce more zinc precursor at the beginning of the experiment. Moreover, zinc is reacting as time goes by, finding that point would helped to find the best quantity of zinc precursor needed to perform the reaction, during this appropriate time.

3.3 DRX pattern

It is important to compare with the literature our CaP/Zn NPs glasses with other calcium/phosphate. As they have a really close composition, they would exhibit the same major properties keeping their own specificities. Knowing this, it would be easier to choose each CP material for a precise and appropriate application. The DRX pattern of P30/Zn10 nano-powder shows some similitudes with the pattern which was recorded, during the investigation of insertion of zinc and silver in Hap doped with 2,5% of ZnO. On figure 15 we can see some patterns from the Hap that they tested [26]. We are interesting to the X-Ray spectrae of Z25 as referred on figure 13.

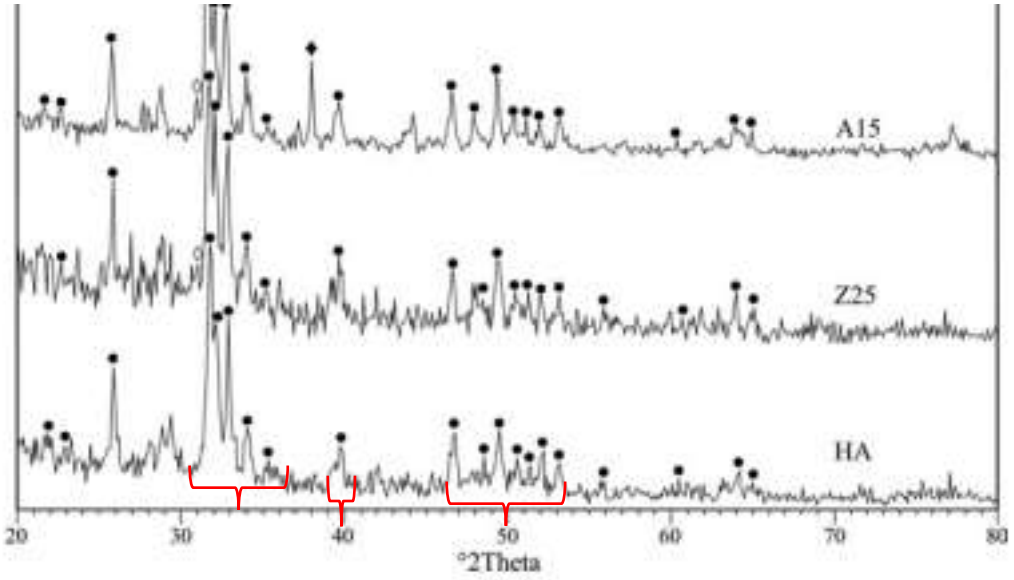


Figure 13: DRX spectra of doped Hydroxyapatites [26]

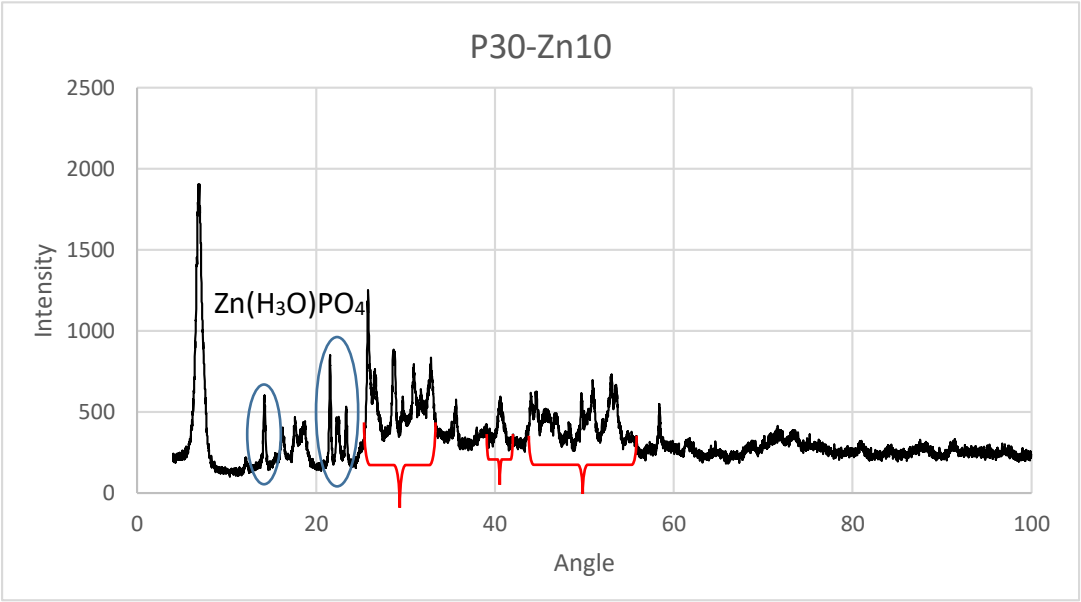


Figure 14: DRX spectra of 10%ZnO-CaP-NPs

If we compare both pattern from figure 13, 14 and 15, we can see that the global pattern of both material are very close. They have many series of peaks in common. If we look at the few peaks around 30°, 40° and 50° they match pretty much together between all the figures. This proximity allows us to compare the behaviors of CaP-NPs against bacteria after an inclusion of zinc with the other doped calcium/phosphate glass that were in the literature. However we were not able to identify precisely the composition of the phases for each peak. According to [26], the phases that the three patterns have in common are phases of hydroxyapatite.

From previous study in the laboratory, we had recorded the pattern of P15 particles (figure 15) which are made of 15 mol% of P_2O_5 and 85 mol% of CaO. In the study [26] of zinc and silver doped Hap, the two curves (HA, ZN25) on figure 13, present not many differences. On the

contrary, they found a new peak which appears for Ag-HAp sample at ≈38,2° and which is characteristic of Ag phases. However they have not notified any apparition of a new peak for ZnO sample which theoretically appears at ≈36,3°. They only saw a decrease of the degree of crystallinity of the Hap [29]. We obtained the same result between the two particles we recorded. As we can see on figure 14, the characteristic peak of ZnO did not appear. Nevertheless, four new peaks appeared at 14.3°, 21.6°, 23.2° and 23.4° which correspond to a phase of . We learned that zinc is included inside this zinc phosphate and not as a phase of ZnO. We can also conclude that we are forming crystalline phases in our material.

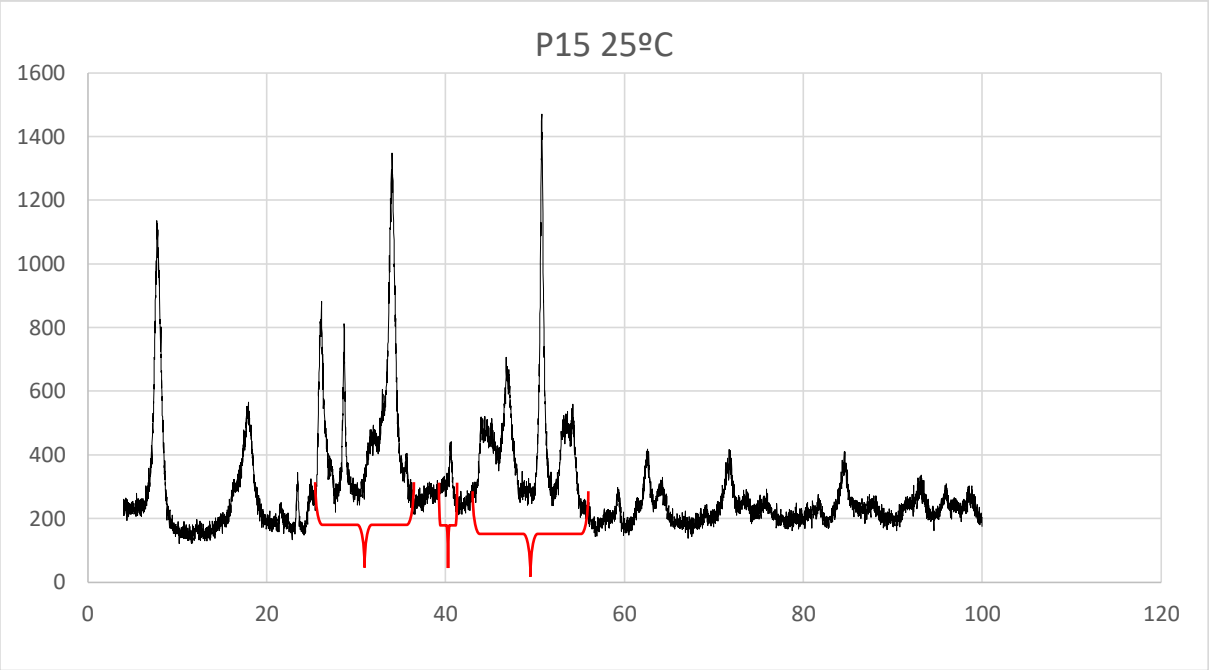


Figure 15: XRD spectra of P15-NPs (15%mol P_2O_5, 85%mol CaO)

3.4 Zinc release

To rapidly measure the quantity of zinc that particles release in a media during a period of time, we chose to use absorbance to immediately have a result, which can be converted in concentration, using a reference straight line that we preliminary made. (Method in annexe "9.1 Construction of the reference line (Absorbance)")

We tested 5 particles: three P30/Zinc nanoparticles with 2% (P30/Zn2), 5% (P30/Zn5) and 10% (P30/Zn10) as concentration of zinc and two Phosphorus/Zinc nanoparticles with respectively have 50/50% (P50/Zn50) and 40/60% (P40/Zn60) as concentration. The mean results are sum up in table 2. We talk about mean because we made 3 sample of each Phosphorus/Zinc particles and only one sample of each P30/Zinc. Moreover, it is important to make several samples in order to avoid pipetting errors. The result of absorbance for Hepes solution indicates that its value is really close to water. It means that Hepes has no influence on others values and we measure only the absorbance of zinc ions. We chose to measure zinc after 1h30 and 12h30 of release at 37°C.

		Mean weight					
		1,13 mg	1,3 mg	19,7 mg	19,9 mg	20,2 mg	
Time	***Samples of nanoparticles***	**40/60 P/Zn**	**50/50 P/Zn**	**P30/ Zn2**	**P30/ Zn3.5**	**P30/ Zn10**	**Hepes**
1h30	**Absorbance**	1,08	0,97	0,80	0,93	0,93	0,64
12h30		1,46	1,38	0,73	0,77	0,90	0,58
1h30	***Zinc release Concentration*** *(Using the equation of the reference slope) for 1 mg of particles. (mM/mg) Considering dilutions**	0,87	0,56	4,09.10^{-3}	8,06.10^{-3}	7,96.10^{-3}	
12h30		1,96	1,48	1,89.10^{-3}	3,35.10^{-3}	7,83.10^{-3}	

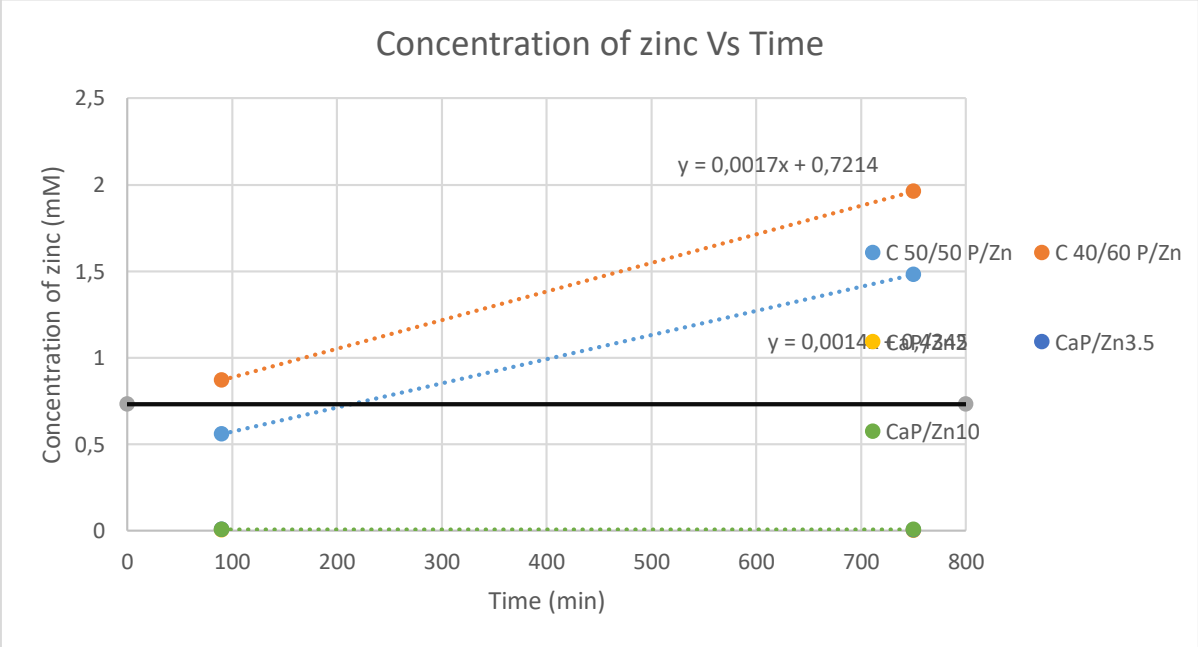
Table 3: Results of zinc release in Hepes for different powders

* We had to dilute the solution with P/Zn-NPs because there were releasing too much zinc in the medium and the absorbance was too high to fit into the range of values of the reference straight line. We recorded an absorbance which was around 3. If we look at the reference line, the maximal absorbance is 2,20 or 2.01 depending on the reference. By diluting the solution by 4 into Hepes, we reached to record absorbance in the range.

The conclusion of this test is that P30/Zinc NPs, release a really small quantity of zinc. For 1mg of powder, it just releases 10^{-3} mM of metals which is around a thousand times lower than P2O5/ZnO NPs. As we expected, more zinc we have into the composition of powders, more zinc is released inside solutions. These results are in agreement to the result of antibacterial activities. P/Zn particles had the highest antibacterial effect and they also release the highest quantity of zinc for the highest composition of zinc. Knowing this capacity, we can say that P/Zn particles have to be further deeply investigated. When tested the decomposition of the Nps inside water, we noticed that it remains a rest of white particles inside the solution. Obviously, a component of particles was degraded to release zinc but the velocity of degradation is maybe slow and still remains some part of particles. The degradation rate seems to decrease as far as we increase the concentration of zinc. We can also think that there

was another phase, which is formed by the degradation of particles and precipitate in water as white residues.

In the preliminary studies of salts and NPs against S.Aureus (annexe "9.5 Study of six elements against *S.Aureus"*) we can see that zinc chloride has an antibacterial activity for a concentration of 0,1mg/mL. As it is a salt it is possible to calculate the quantity of zinc which released inside the media. Hence we can draw the concentration of zinc inside the solution Vs the time and use zinc chloride ($ZnCl_2$) as reference. In fact, if the concentration of the particles is above that line, it means that the particles released the required concentration to inhibit bacteria growth. The result on graph 1 clearly shows that P/Zn-NPs are the most efficient between the two kinds of particles due to their high concentration of zinc. We saw that CaP doped NPs are far away to have an antibacterial activity. This quick study, gives an overview of the result which can be expected against *S.Aureus.* In the next part, we confirmed the antibacterial behaviors of P50/Zn50 and the inactivity of CaP doped nanoparticles. It appeared that a high ZnO concentration is required to show antibacterial capacity.



Graph 1 : Zinc releases concentration for different concentration of particles (P50/Zn50, P40/Zn60, CaP/Zn2, CaP/Zn5, CaP/Zn10)

3.5 Antibacterial activity

It has been proved that it is possible to improve the biological ability of HAp by adding a trace of metal ions like silver, zinc or manganese. [4] A study was made especially for silver, copper and zinc ions insertion. Sometimes it happens that we succeed the incorporation of metal ions in Hap, unfortunately without any improvement of antimicrobial activity.[27] In this case we clearly see that against *E.Coli,* AgHAp has an important impact on bacteria growth compare to the other ions (Cu and Zn). This result is according to the preliminary studies of

salts and NPs that we performed before the beginning of my project, where we demonstrated the strong capacity of silver against bacteria.

Silver is well-known for its properties against bacteria and it is widely used as a coating to reduce bacteria growth. For example, implants surfaces needs to attach bacteria as less as possible. As we are using more and more implants, it increases cases where we had complications due to implants. From this observation, antibacterial and osteogenic properties of silver-containing hydroxyapatite coatings were investigated in [28]. They evaluated a thin film hydroxyapatite/Ag deposition on titanium (Ti) using sol-gel. They tested the bacterial growth inhibition on coated implant surfaces against *Staphylococcus Aureus* and *Staphylococcus Epidermidis*. In addition they studied the proliferation and differentiation of osteoblast precursor cells. They work with two samples AgHA1.0 and AgHA1.5 which correspond to a concentration of 1,0 and 1,5wt% of $AgNO_3$. They found that doping HAp with Ag, helped to decrease the number of *S.epidermidis* and *S.Aureus*. However it seems that AgHA surfaces have the same osteoconductity than HAp surfaces. AgHA and HAp have an equivalent biological activity regarding the proliferation and differentiation of bones cells. In this part we will demonstrated the capacity of zinc to disturb the proliferation of *S.Aureus* and *POA1*. Against *S.Aureus*, it could be advantaging to use zinc instead of silver for bones applications, knowing all the properties of zinc that we already presented in the introduction and especially its impact on osteoblast production. Moreover, we also talked about the improvement of osteoconductivity of Hap by the inclusion of zinc [3] which silver seems to not present according to the previous study [28].

Silver shows positives results against bacteria but we are going to see that zinc could also be a bacteria growth inhibitor. As we already said zinc is present as a trace element in the bones. Moreover, zinc could favor the density of bones but also prevent bones loss. [29] Most of the time, the antibacterial activity of zinc ion is involved in three processes which are protein deactivation, microbial membrane interaction and thereby structural change and permeability. [30] We can take the example of similar case [30]. To incorporate zinc into HAp molecules, they used directly ZnO powder mixed with solutions that they used to create bare HAp-NPs. In the opposite way, we do not incorporate ZnO powder as reactant but a precursor containing zinc which is going to react and form ZnO. They incorporated different proportions of ZnO from 0 to 75 wt%. After getting their mix of particles they studied the antibacterial activity of each powder against *Klebsiella pneumonia* by disc diffusion method. Their results are recapitulated in table 4. It shows that the source of zinc needs to be well chosen and studied because the incorporation of ZnO had modified the structure of HAp and lead to reduce the antimicrobial activity [30]. Contrary to what we thought, raising the fraction of zinc inside the composition of HAp do not increase necessarily its antibacterial capacity. We can also conclude that using ZnO powder as precursor do not lead to improve the antibacterial activity following this protocol.

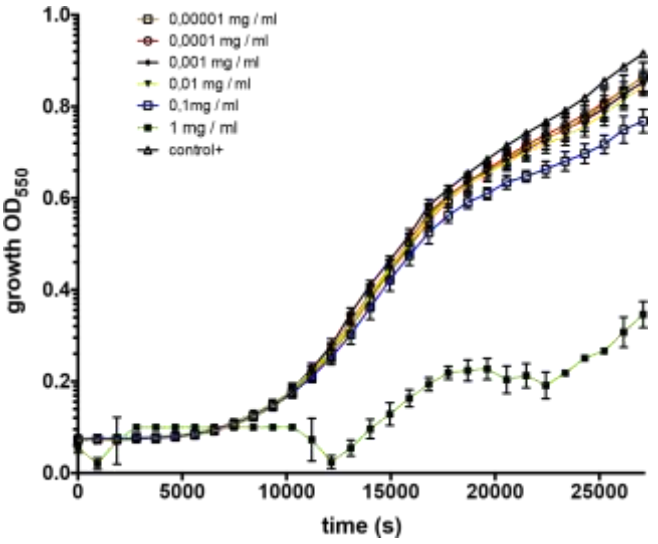
Organism	Sample name (representation of Zone of inhibition (diameter in mm))				
Klebsiella pneumonia	Z_0H	$Z_{10}H$	$Z_{25}H$	$Z_{50}H$	$Z_{75}H$
	200 μg/ml	200 μg/ml	200 μg/ml	200 μg/ml	200 μg/ml
	15	14	12	12	11

Table 4: Inhibition zone of different zinc doped Haps against Klebsiella pneumonia [30]

Fortunately we are able to find many studies where succeeded the improvement of antibacterial activity after addition of ZnO in calcium/phosphate species. When we talk about succeeded testes, we always taking into account that it is against a specific bacterium. We make the difference between a broad-spectrum compounds which are efficient against a wide range of bacteria and a narrow-spectrum compounds, which act only against a specific family of bacteria. We can mentioned again a study on Hap doped with zinc which reveals efficiency of ZnHAp against *E.Coli* but any positive results concerning *S.Aureus* inhibition. [4] Actually, we did not encountered any documents talking about positive results against *S.Aureus* excepted in the case of the study [29]. However, all of the studies on Hap we found, were introducing a really small quantity of zinc.

With these references we can now talk about our results and try to compare them with the previous study we just present. We first tested P30/Zn14, P30/Zn10, ZnO and P30 ("control") particles. You will find the antibacterial activity for each particles against S.Aureus and PAO1 in the following figures 16, 17, 18 and 19.

(A) **Zn10%-NPs**_*PAO1*



(B) **Zn10**%-NPs_*S. aureus*

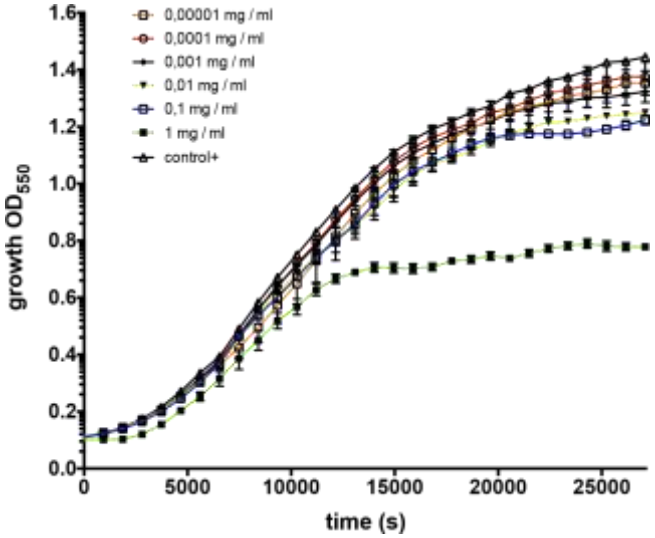
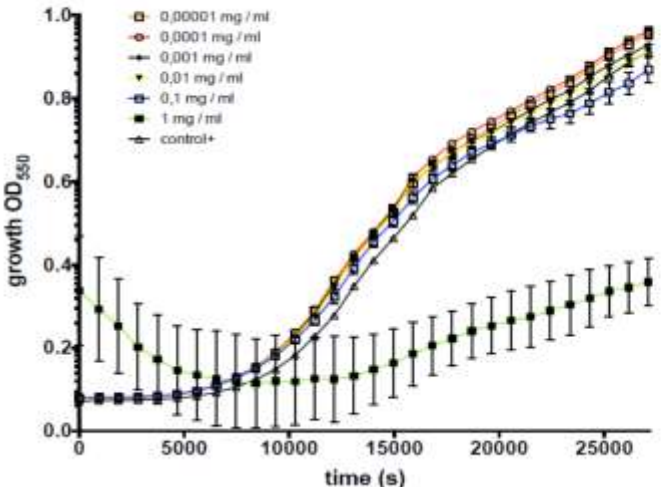


Figure 16: Antibacterial activity of P30/Zn10-NPs against PAO1 (A) and S.Aureus (B)

(A) **Zn_14%_NP**_*PAO1*



(B) **Zn_14%_NP**_*S. aureus*

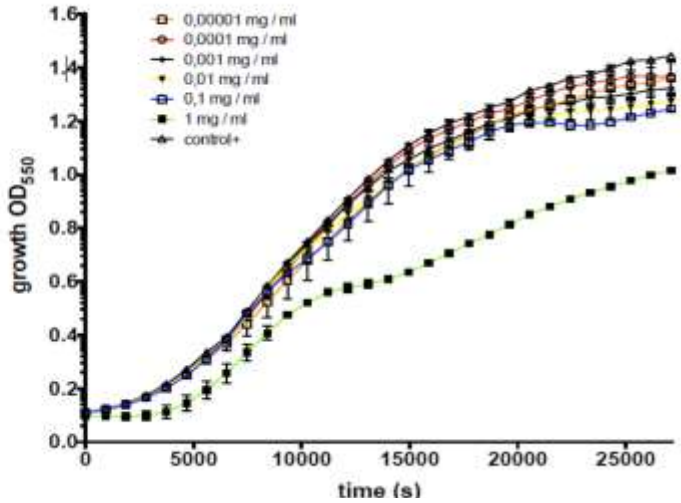


Figure 17: Antibacterial activity of P30/Zn14-NPs against PAO1 (A) and S.Aureus (B)

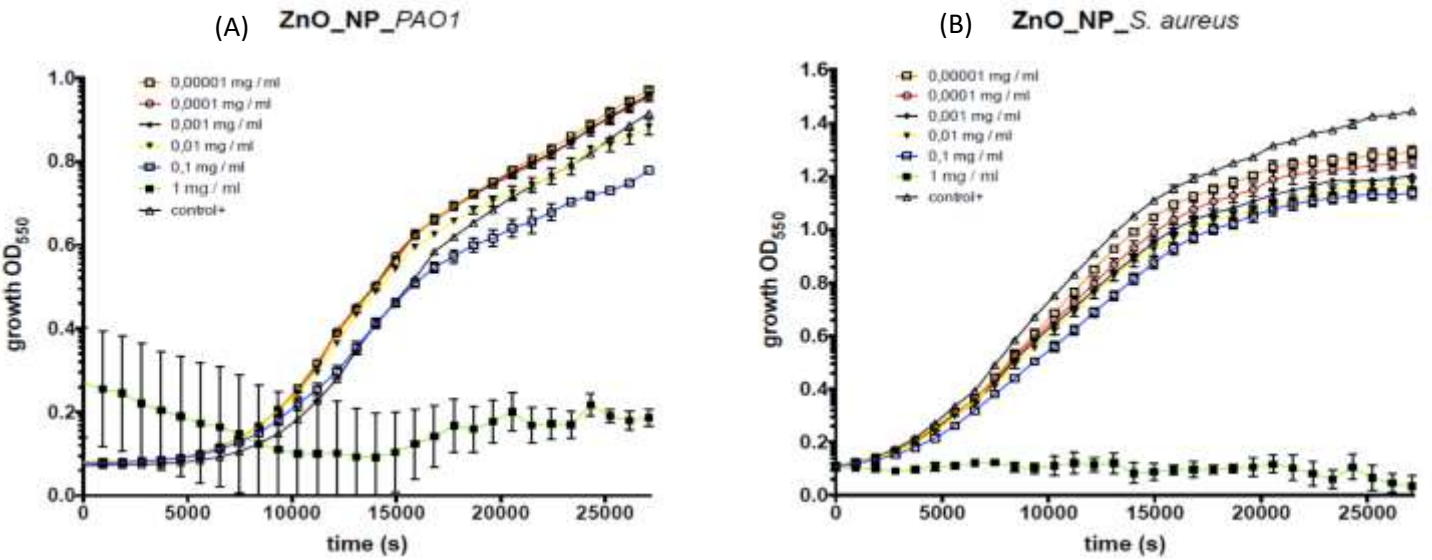


Figure 18: Antibacterial activity of ZnO-NPs against PAO1 (A) and S.Aureus (B)

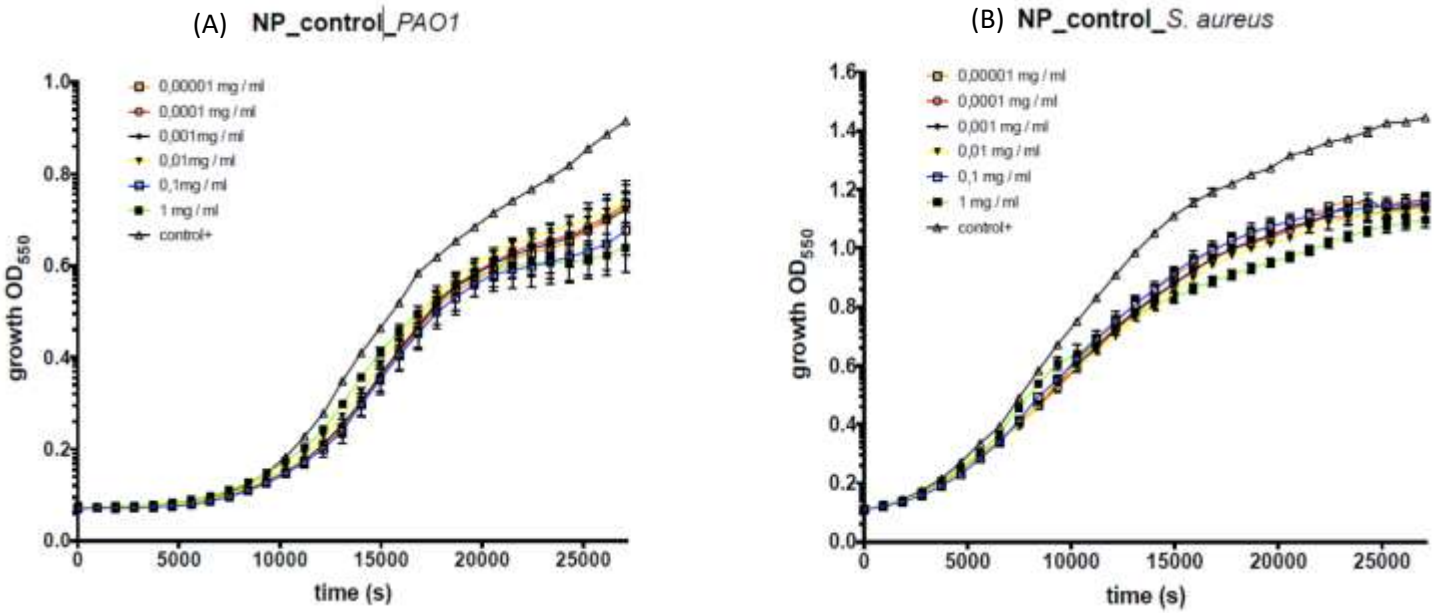


Figure 19: Antibacterial activity of P30-NPs against PAO1 (A) and S.Aureus (B)

If we look at the antibacterial effect of the different particles against *S.Aureus* we can see that for P30/Zn10-NPs we have a reduction of bacteria growth for a concentration of 1 mg/mL. We can also notice a smaller inhibition for P30/Zn14-NPs at the same concentration. Concerning the ZnO particles that we achieved by precipitation, we had antibacterial effect for 1 mg/mL. As expected there is no positive antibacterial effect from P30 particles which are the reference without zinc. We can come to the same conclusion for particles against POA1

where we get the same results with very few differences. All the same, it appears a small break on the curve of P30-NPs for a concentration of 1mg/mL, after 15000s, against *S.Aureus* (Figure 19). When we look at the literature, it is possible to find a similar case. Antibacterial activity against *E.Coli* was showed by biphasic calcium phosphate (BCP) nanopowder [31] but also for P15-NPs (table 4, D) previously studied. Their particles were synthetized via sol gel using phosphoric pentoxide as we also did. BCP were made by mixing Hydroxyapatites (Hap, $Ca_{10}(PO_4)_6(OH)_2$) and β-tricalcium-phosphate (β-TCP, β-$Ca_3(PO_4)_2$). For a composition of 50% Hap, 50% β-TCP (Ca: 38wt%, P: 19,1wt%) and a concentration of 300mg/mL in Triptic soy broth. They recorded after 72h, a decrease of *E.Coli* bacteria. It is explained by the modification of pH which raised form 7,3 to 8,32. Maybe P30-Nps had played the same role and produced a short increase of pH in the solution, which momentarily decrease the number of *S.Aureus*. In spite of the reduction of the bacteria growth we cannot qualify CaP/Zn-NPs as a bacterial inhibitor. In fact its inhibition is too low to be considered.

We observe that the concentration (1mg/mL), where particles have a partial antibacterial capacity against *S.Aureus,* is the same for all nanoparticles. As it was already proved, we also noticed the potential antibacterial effect of a CaP material after inclusion of zinc.Particles are made of oxides and we measure the inhibition of bacteria growth for CaP/Zn-NPs which are containing a maximum of 14% of ZnO. The more we are introducing Zn against bacteria, the more the growth is supposed to be inhibited. As we can see, pure ZnO particles are better inhibiters than P30/Zn14. In addition, calcium and phosphorus oxides have normally, no antibacterial effect. However, we found something which was interesting. If we compare P30/Zn14 and P30/Zn10 we can see that the 10% ZnO nanoparticles have an inhibition slightly superior than the 14% ZnO against *S.Aureus*. It completely contradicts the previous results but this conclusion led us to follow our experiments with lower concentrations, in order to confirm if by reducing the quantity if zinc we could have better antibacterial properties.

That is why for the second test we tested nanoparticles with a smaller content of zinc. We have also investigate the antibacterial activity of P50/Zn50. These particles seem to have a poor degradability in water too, so they could be used to inhibit bacteria growth. We also try our particles against a third bacteria *S.Mutans* but we did not noticed any kind of inhibition. We will still focus on the results against *S. Aureus* and *PAO1*. We investigated P30-NPs with a composition of 1,5%, 5% and 8% in ZnO. You will find all the results on figures 20, 21, 22 and 23.

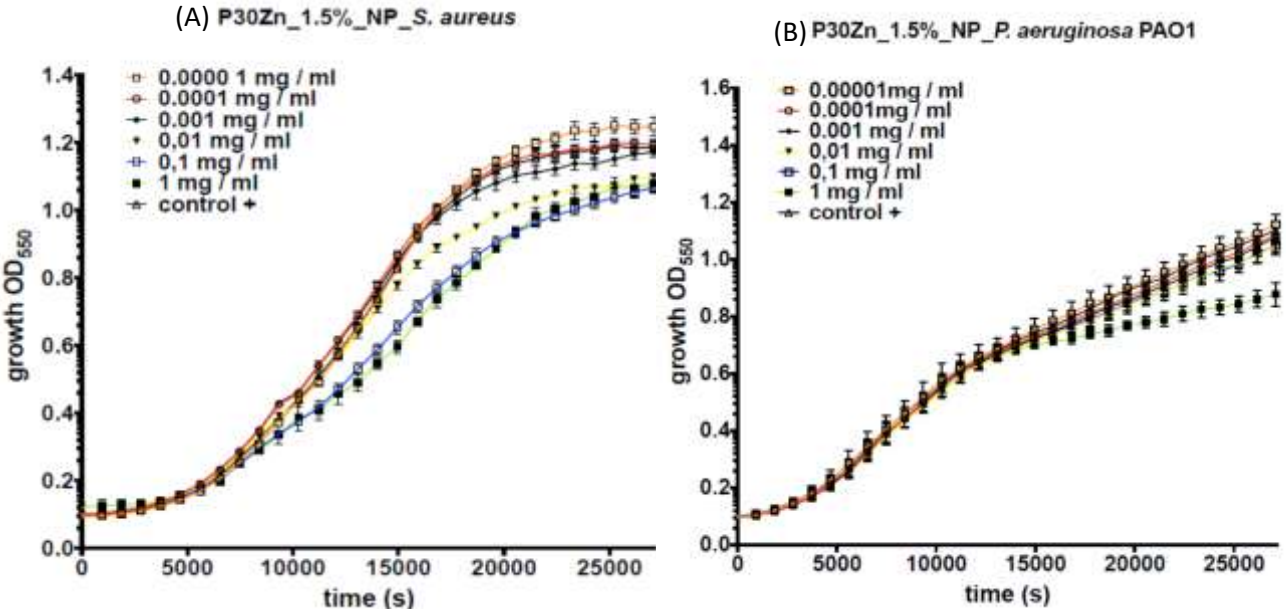


Figure 20 : Antibacterial activity of P30/Zn1.5-NPs against S.Aureus (A) and (B) PAO1

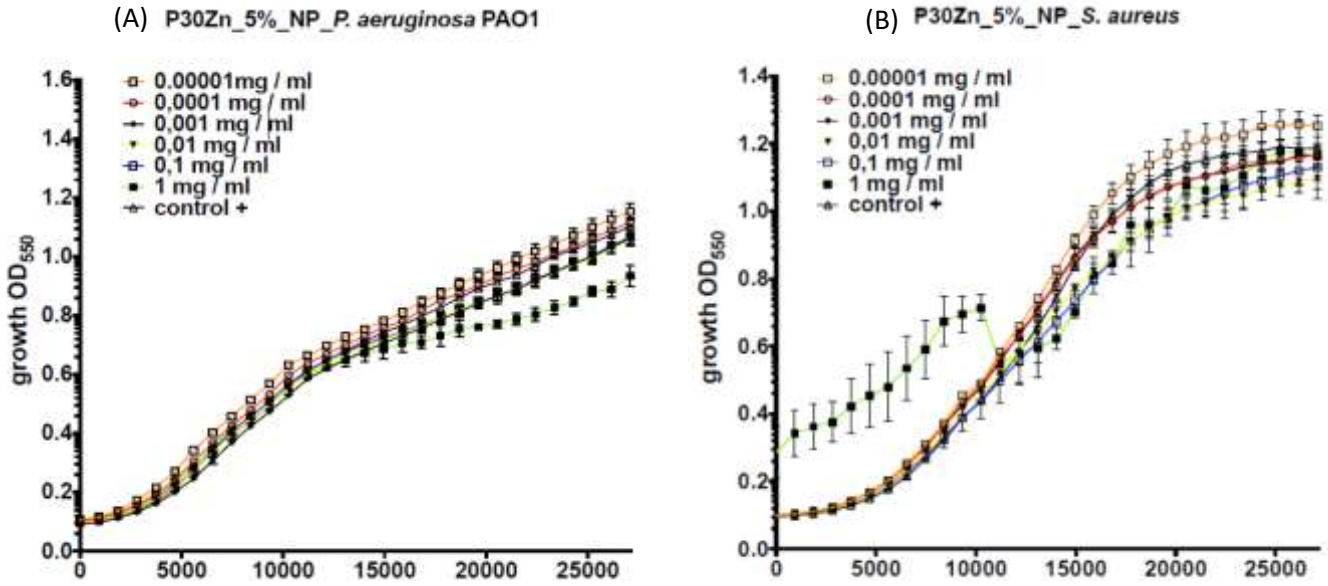


Figure 21: Antibacterial activity of P30/Zn5-NPs against S.Aureus (A) and (B) PAO1

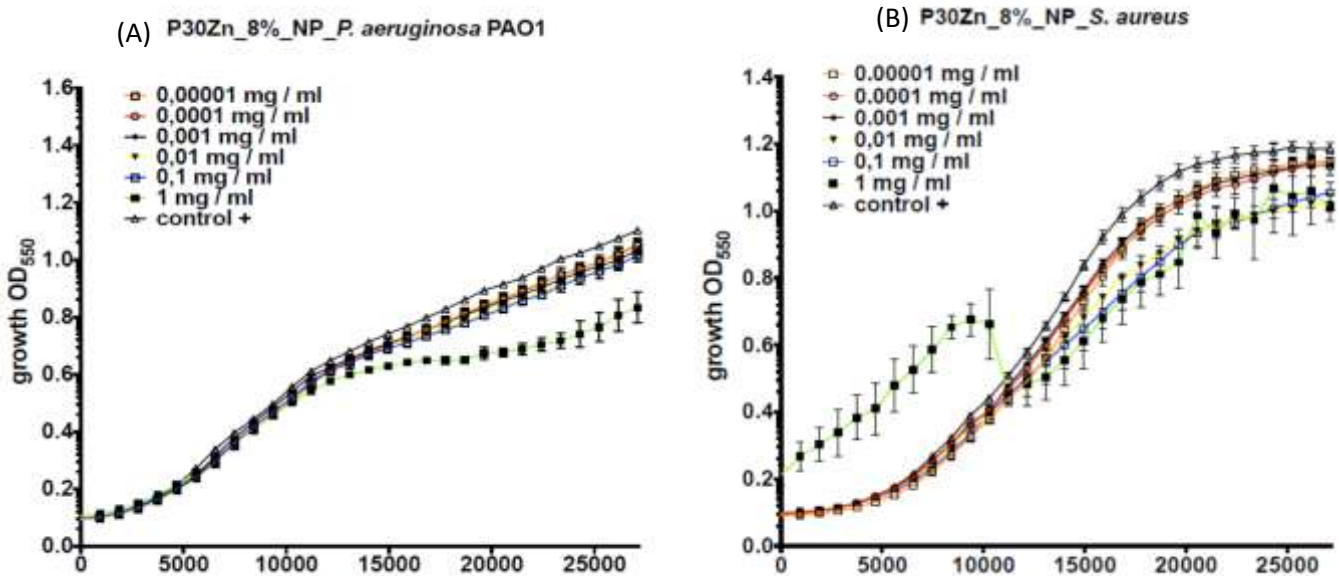


Figure 22: Antibacterial activity of P30/Zn8-NPs against S.Aureus (A) and (B) PAO1

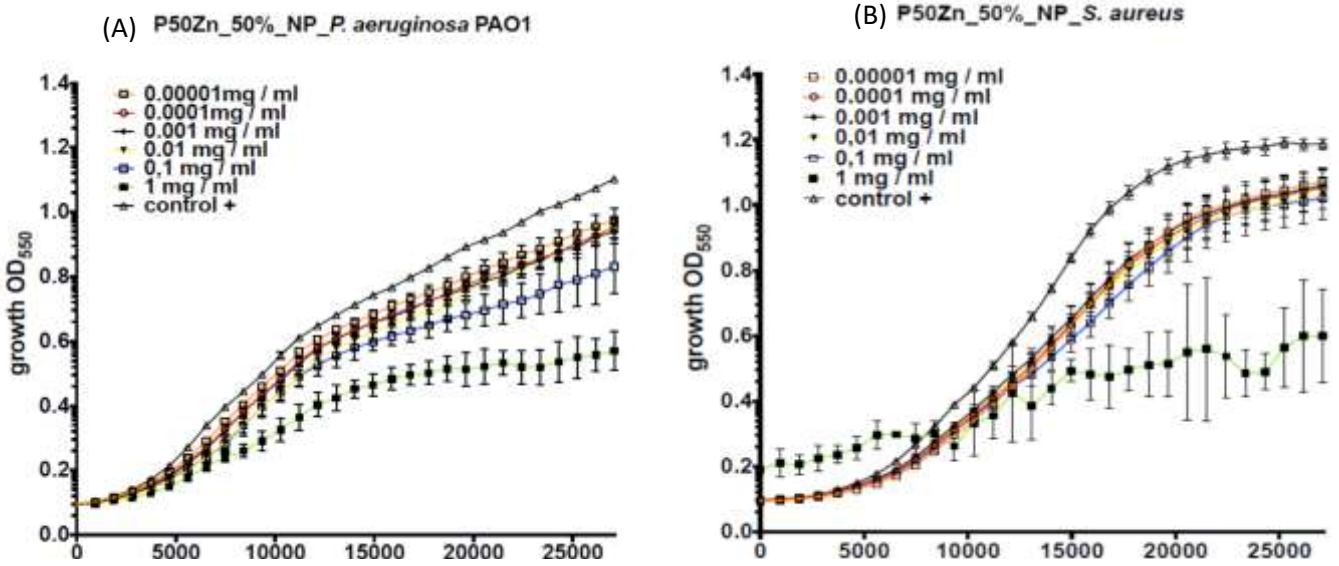


Figure 23: Antibacterial activity of P50/Zn50-NPs against S.Aureus (A) and (B) PAO1

When we look the results for P30/Zn particles (Figure 20, 21 and 22), it barely shows results of inhibition. We had no satisfying antibacterial effect against *S.Aureus* and a really weak one against *PAO1*. If we had to classify, it seems that for P30/Zn8, the growth rate of bacteria cells seems a little bit higher than for P30/Zn5 and P30/Zn1.5 for a concentration of 1 mg/mL. This time, it is the most concentrated in CaP-Zn-NPs which shows the highest inhibition. P50/Zn50 on figure 25 are the only particles which showed positive results again both bacteria. We saw an inhibition against *PAO1* and *S.Aureus* for a concentration of 1 mg/mL. We can conclude that reducing the composition of zinc does not induce antibacterial properties. As we clearly see, there is a limited or a minimum quantity of zinc, required to have antibacterial activity.

We concluded that the result is only satisfying for zinc/phosphate nanoparticles. Introducing a small quantity of P50/Zn50 particles could drastically improve the antibacterial properties of a material. However we have to work with CaP based powder in order to have all the properties required for bones regeneration process. It is important to underline that, all the particles which were tested with bacteria, were particles which were heteregeneous. However, we tested the release of homogeneous particles and as we could see on graph 1, CaP/Zn-NPs released a too small quantity of zinc to have antibacterial properties. As the quantity of zinc which is liberated is not relevant, half-composed hexagonal zinc crystals could not have an influence on the released quantities. The antibacterial activity will not be significantly improved by the removal of the crystals. The antibacterial activity of P50/Zn50-Nps can be linked to the quantity of zinc which was released in Hepes media after 1h30 or 12h30. Futhermore, 1mg of nano-powder liberates between 0,8mM and 2mM of zinc, hence it means that at this concentration there are enough ions to inhibit the bacteria growth. According to graph 1, we see that P50/Zn50 start to inhibit the growth of bacteria from 200 min.

We demonstrated the antibacterial activity of our glass CP-NPs doped with zinc. It did not showed significant results for CaP/ZnO particles. We only noticed a reducing of the growth for concentration higher than 8%ZnO. As it was studied, at low concentration there is no inhibition of S.Aureus bacteria. Nevertheless, results are encouraging for particles, which contained a high concentration of zinc. We have proved that it is possible to synthetize phosphate based nanoparticles with high content of zinc (more than 50%) via sol gel. These particles showed positive results against both bacteria. However if we compare P30/Zn doped particles with other calcium phosphate Nps doped with silver, we see that zinc remains less effective than silver. However, silver present many disadvantages as its toxicity and does not possess whole benefits that zinc has on bones regeneration process. We suggest that zinc is suitable for an antibacterial application by a simple sol gel process, which can be performed at room temperature. This was a first investigation and it has to be completed in many points that we will enumerate in the “Future works” section.

4 Applications

One of the document we already present in this report [2], provides a schema with the possible applications of substituted hydroxyapatites. These applications can be transposed to our particles and so it is interesting to look at figure 24.

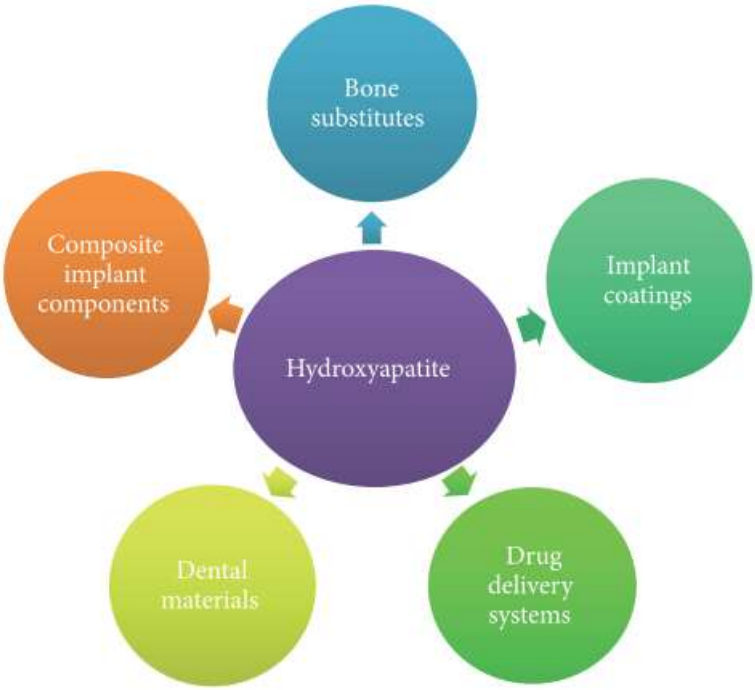


Figure 24 : Schema of different applications for doped Hydroxyapatites

Among these applications we find the composite implant components. As we already talked about, we are studying the introduction of P30-Nps into PLA nanofibers to be able to implant them. As it was demonstrated with silver [28], zinc could be further investigate to be used instead of Ag, as implants coating.

5 Conclusion

During this project, we demonstrated the potential of Calcium/Phosphate glass nanoparticles doped with zinc. We had synthetized via sol gel, two different kinds of particles: CaO/P_2O_5/ZnO-NPs and P_2O_5/ZnO-NPs and proved their antibacterial activity against *S.Aureus* and *PAO1.* The composition was controlled by SEM and EDS and XRD patterns confirmed the crystalline structure of the nanoparticles and the apparition of a zinc phosphate phase. CaP/Zn particles showed an irrelevant antibacterial activity. XDR patterns unlighted crystalline phases and the apparition of peaks corresponding to the phase of a zinc phosphate species. P/Zn particles exhibited an even stronger activity against both bacteria. We also measured the release of zinc from the particles inside Hepes. As expected, we learned that, the more concentrated were the particles the more they released in the medium. P/Zn-NPs released around, 1mM of zinc after 1h30 and 2 mM after 12h30, for 1mg of powder. This result is a thousand time higher than CaP/Zn-NPs. When we look at the structure of the powders it appears that, after 18h of reaction, could appeared high concentrated zinc crystals and caused heterogeneity inside the powders. As they had a slow growth rate, we decrease the time of reaction to 1h30 and the crystals disappeared. Investigations have to be pursued, in order to see if we can make a viable nanocomposite between NPs and PLA using electrospinning. We proved the encouraging antibacterial activity of Zinc/Phosphate-NPs and demonstrated its

capacity against *S.Aureus* and *PAO1*. In table 5 we sum up the results against bacteria and the release of zinc.

Particles	Zinc release (mM/mg)		Antibacterial effect
	1h30	12h30	
CaP/Zn2	$4{,}09.10^{-3}$	$1{,}89.10^{-3}$	NO
CaP/Zn3.5	$8{,}06.10^{-3}$	$3{,}35.10^{-3}$	NO
CaP/Zn10	$7{,}96.10^{-3}$	$7{,}83.10^{-3}$	NO
P50/Zn50	0,56	1,48	YES
P40/Zn60	0,87	1,96	YES

Table 5: Summary of the results of the antibacterial activity and zinc release of NPs

6 Future works

A first point is that, we should have to disperse particles into a proper medium to isolate some of them and determine precisely the size of nanoparticles. On pictures from SEM we see that agglomerates are at the nanometric scale but isolated particles could be useful to investigate their shape. It could be possible that the introduction of zinc inside the calcium/phosphate glass NPs changes its shape. Deep investigations of the modification of the structure of doped HAp were made *[3]*. They noticed different shape of nanoparticles depending of their concentration in Zn. They choose to study 2, 4, 6 and 8 mol% of zinc substituted HAp, made by precipitation of diammonium hydrogen phosphate, hydrate calcium acetate and dehydrated zinc acetate. SEM picture (figure 25), reveal that 0%Zn (a) is passing from what they called “cauliflower –shape” to an “irregular rough-shape” by increasing zinc content to 6% (d). It appears that from 8%Zn (e), particles came back to this ‘cauliflower-shape”.

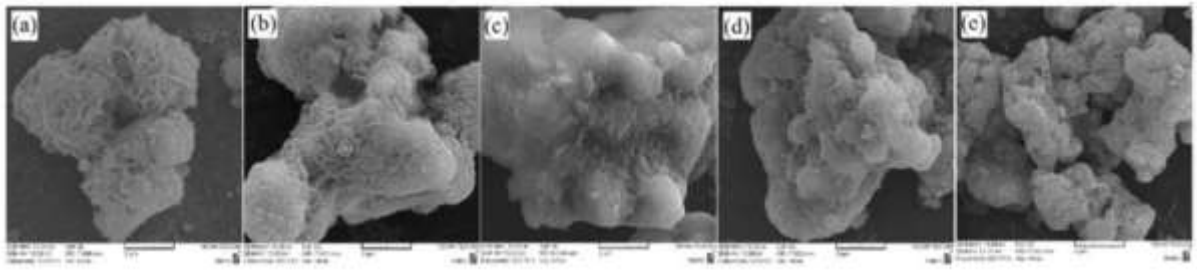


Figure 25 SEM picture of substituted Hydroxyapatites with different concentration of zinc, (a)0%, (b)2%, (c)4%, (d)6%, (e)10%

These results are according to the behaviors of the crystallinity. They noticed that the crystallinity was decreasing from 0 to 6%Zn but was starting to increase from 8%Zn. Here the crystallinity is directly linked to the changing of shape. This phenomena could also appears into P30/Zn particles and so it has to be investigate.

Second point, we already mentioned that to save some reagents and reduce the cost of precursors, we could find the time were appears high zinc content hexagonal crystals that we discovered. We only try for 1h30 of reaction and so had to introduce more zinc precursor due to its progressive reactivity. Hence optimizing this time able to introduce less reactive at the beginning of the batch.

Thirdly, one of the objective to complete this study is to see if the particles which are formed remains electrospinnable. Also proved the antibacterial activity of the composite which is make between PLA and CaP/Zn NPs and P/Zn-NPs.

Fourthly, we have to continue the investigations of P/Zn-NPs and start by performing its XRD spectra and find its structure.

Finally we should evaluate the cytotoxicity of particles. It was revealed on BCP material that for an inclusion which is superior to 1,2wt% in zinc, doped BCP show cytotoxicity. [32]

7 Economic analysis of the project (table 6)

Material	Price/unit	Quantity	Price total €
Flask	1.69€	15	25,35
96-wells plate	108.5€/50 plates	6	12,96
Nitryl gloves	0.55€/pair	100 pairs	55,00
Parafilm	22€/tape	2/10 tape	4,4
Blue pipette tip (100-1000µL)	32,6€/1000 units	250	8,15
Yellow pipette tip (2-100µL)	31,10€/1000 units	250	7,775
Syringes	1,15 €	70	80,5
Tips	10 €	70	700
XRD	20,40/H	10	204
SEM	19,06/H	15	285,9
TOTAL MATERIAL COST			1384

Product	Price/unit	Quantity	Price total €
Ethanol	17.95€/L	1L	17,95
2-Methoxyethanol	165,36€/1L	700mL	17,96
Metallic calcium	56,43€/100g	10,121g	115,752
Diethylzinc	157,14/109mL	100mL	144,16
Phosphorus pentoxide	372,17€/250g	28,4g	42,25
Ammonia	55,68€/5L	75mL	0,83
Hexane	118,96€/1L	300mL	35,68
Aminomethane	27,84€/100g	0,7960g	0,22
Guanidine	58,21€/100g	14,3g	8,32
Resorcinol	93,90€/5g	0,2525g	4,741
Sodium hydoxide	37,97€/50mL	10mL	7,594
Chloral hydrate	35,54€/50g	15,15g	10,76
Sodium Cyanide	14,20€/50g	0,3866g	0,11
ascorbic acid	51,04€/25g	1,2563g	2,56

Zinc chloride	76,14€/100mL	30mL	22,84
Hepes	112,45€/100g	0,2994g	0,3
TOTAL PRODUCT COST			432,03

Global price: rent of locals, energies and resources, devices, personal	13000€/month

TOTAL COST OF THE PROJECT	**53816€**

Table 6: results of the economic analysis of the project

8 Environmental impact

The particles we created are biocompatible and degradable in water, therefor, they have a low impact on the environment. Nevertheless, a majority of the products we used are toxic and in order to limit their impacts, we always work under a fume hut. It possesses filters which avoid any contamination of the air released in the atmosphere.

We had some training about security and environmental risks and we learned to separate our wastes inside specific containers depending of the solution. (Basic Aqueous, Acid Aqueous, Bio-waste, Halogenated, Non-Halogenated, Specific wastes …). Then, a qualified person treat each containers to avoid any kind of pollution. We applied a special protocol concerning the residues of Cyanide as it had to be treated separately due to their high toxicity.

The most important impact were the consummation of energy, due to the equipment and the materials which we used to prepare the batch as gloves or syringes that we used ones then we threw away. Even if they are especially treated, they had an impact as there are not renewable and used in large quantities.

9 ANNEXE

9.1 Particles experimental pathway

We wanted to test the antibacterial activity of zinc, we made several particles with different composition of calcium, phosphorus and zinc. We vary the composition in zinc inside the P30 particles but also try to see what happened with only phosphorus and zinc nanoparticles. In this part you will find many tables resuming conditions of experiment, time of reaction and reactive that we used to get different compositions.

Before giving you the composition, we will explain everything about what we made before starting the first batch of the first nanoparticles synthesis. As we already said, zinc alkoxides is a component which his highly reactive, especially with water and air. We had to find a solvent which could contain all our precursors. We knew that calcium and phosphorus precursors were not reacting with ethanol. So ethanol was the first solvent that we tried. We put a drop of zinc precursor into absolut ethanol (99,9%) in air atmosphere at room temperature. Zinc precipitated in ethanol, due to traces of water. We managed to reduce the reactivity of zinc and reduce the amount of water. By cooling the batch with iced water, using argon

atmosphere, distillation and finally using 2-methoxyethanol. Table 7 resume all the condition to finding the solvent.

Solvent	**Temperature**	**Atmosphere**	**Comments**
Absolut Ethanol (99,9%)	Room temperature	Air	Zinc precipitation (reacting with water ?) Solution: Distillation
Distilled Absolut Ethanol	Room temperature	Under Argon	Still precipitate Solution: Slow reaction with ice
Distilled Absolut Ethanol	Cooled with ice	Under Argon	Still precipitate Solution: Try another solvent
2-Methoxyethanol	Cooled with ice	Under Argon	Can dissolve all precursors inside

Table 7: Summary of the solvent that we tried for the sol gel

After optimizing the solvent we also had to find a catalyst to start the reaction. As we need water to start the sol-gel process, we tried different concentrations of solution mixed with water. We experimented all these solutions during the first batch. Table 8 resume all the catalyst that we tried to finally choose a mix of 50% of absolute ethanol and 50% of ammonia (30%). We choose to mix ammonia with ethanol because it disperses easier in the mix of precursor. In fact, we used only ammonia which, when it was introduced too fast, it could produces bigger particles due to the local concentration of ammonia.

Catalyst	**Pure Ethanol**	**10% Water / 90% Ethanol**	**50% Water / 50% Ethanol**	**Water**	**10% NH3 (30%) / 90% Ethanol**	**50% NH3 (30%) / 50% Ethanol**	**NH3 (30%)**
Particle formation	No	No	No	No	No	Yes slowly	Yes

Table 8: Summary of the catalyst that we tried for the sol gel

9.1.1 Heterogeneous Particles:

P30/zinc nanoparticles:

We will first start with the modified zinc/P30 nanoparticles. At the beginning we choose to study 3,5 and 8% ZnO P30 particles. From the first synthesis we would be able to adjust the volume of zinc precursor to get the concentration we need. As we are going to see sometimes it does not give the expected result. You can find the result of the first synthesis in table 9.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 22,1 mL 2-Methoxyethanol ➢ 1,75 mL CaO precursor ➢ 0,75 mL P2O5 precursor ➢ 0,4 mL Zinc precursor	Cooled with ice	Under Argon	Every solutions we tried in "slides 2" Quantity? because first try	≈ 18h	0,29 g	✓ Ca0 : 64,90% ✓ P2O5 : 34,52% ✓ ZnO : 0,893%

Table 9: Reaction's parameters and volumes for P30/Zn0.8-NPs

We found that the composition of nanoparticles helped with SEM and more especially with EDS technique. We wanted to achieved a 8% ZnO nanoparticles but we only got 0,9%. As it was the first synthesis we didn't know how zinc would react. At least we knew that the three precursors were reacting together and we just had to adjust the volume of precursors. To find this composition we made a ratio between the theoretical values of composition with the one we get with EDS. To finish we multiplied the volume of reactive we put for the first synthesis with this ratio (table 10).

	%R with EDS (%M)	%T (%M)	V (mL)	V$\frac{\%T}{\%R}$= New volume (mL)
CaO	64,90	27,6	1,75	1,74
P2O5	34,52	64,4	0,75	0,6
ZnO	0,893	8	0,4	3,58

Table 10: Method to calculate the new volumes necessary for the next batch

As we have the new volume we are able to create new particles and so on to get the composition that we wanted to achieve. We always work with a final volume of 25 mL.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 19 mL 2-Methoxyethanol ➢ 1,74 mL CaO precursor ➢ 0,6 mL P2O5 precursor ➢ 3,58 mL Zinc precursor	Cooled with ice	Under Argon	➢ 3,2 mL 50/50% Ethanol/NH3 ➢ 2,7 mL pure NH3	≈ 18h	0,21g	✓ Ca0 : 59,73% ✓ P2O5 : 26,20% ✓ ZnO : 14,09%

Table 11: Reaction's parameters and volumes for P30/Zn14-NPs

We can see in table 11 that the final content of zinc is too high this time. With the same calculus we can adjust the different volumes. But for the next reaction as it was the week end we tried to see what happened if we let the reaction reacting during 3 days. You will find the final composition and volumes in table 12.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 20,46 mL 2-Methoxyethanol ➢ 1,88 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 2,03 mL Zinc precursor	Cooled with ice	Under Argon	3,6 mL pure NH3	≈ 3 Days	0,32g	✓ Ca0 : 57% ✓ P2O5 : 23,36% ✓ ZnO : 20%

Table 12: Reaction's parameters and volumes for P30/Zn20-NPs

We almost reduce the volume of zinc by two and it gave 20% ZnO P30 particles. It indicates that the more is reacting the zinc, the higher is the concentration. We will try to let batches reacting during the same time as much as possible. In table 13 you will find the composition for same volumes but we only let the reaction 18 hours.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 20,46 mL 2-Methoxyethanol ➢ 1,88 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 2,03 mL Zinc precursor	Cooled with ice	Under Argon	3,5 mL pure NH3	≈ 18h	0,27g	✓ Ca0 : 63,22% ✓ P2O5 : 26,7% ✓ ZnO : 10,08%

Table 13: Reaction's parameters and volumes for P30/Zn10-NPs

Finally we are getting closer to our objective of 8%ZnO. At this point we tried our particles against bacteria. In fact, if a 10% ZnO particles or higher composition were not affecting bacteria growth, there were no reasons that a 5% worked. For this test we had to make what we called a "control" which are basically P30 particles. You will find its composition in table 14.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 22,49 mL 2-Methoxyethanol ➢ 1,88 mL CaO precursor ➢ 0,63 mL P2O5 precursor	Cooled with ice	Under Argon	8 mL 50/50% Ethanol/ NH3	≈ 18h	0,23g	✓ Ca0 : 69,48% ✓ P2O5 : 30,52%

Table 14: Reaction's parameters and volumes for P30-NPs

Thanks to this test (let's see the part about results of antibacterial activity) we decided to continue to decrease the content of zinc. We wanted to see what happened for 8% and lower concentrated zinc particles. We kept the same protocol and continue to decrease the volume of zinc.

From the 10% ZnO P30 particles we could calculate the volume to create an 8%. After antibacterial testing we were running out of zinc precursor, so we had to prepare a new one. We used to be working with precursor which were prepared before the beginning of this

study. It means that our new precursor could not react as the same way as the previous one. We kept all volumes that we calculated for an 8% even if we used a newer zinc precursor. It was really hard to work out this composition because it appears that this new zinc precursor batch is reacting differently. We made three times EDS analysis to be sure about the composition of these particles. First we get 8% and finally 5%. We noticed different phase inside particles which make harder to find the composition. You will find the volume and composition in table 15.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 21 mL 2-Methoxyethanol ➢ 1,91 mL CaO precursor ➢ 0,65 mL P2O5 precursor ➢ 1,5 mL Zinc precursor	Cooled with ice	Under Argon	9 mL 50/50% Ethanol/NH3 (30%)	≈ 18h	0,281 g	✓ Ca0 : 65,45% ✓ P2O5 : 29,43% ✓ ZnO : 5,12%

Table 15: Reaction's parameters and volumes for P30/Zn5-NPs

As we thought it was a 8% ZnO particles we wanted to calculate the volume to achieve a 5%, so of course we get something lower than 5% as we actually calculated it from a 5% ZnO. From this point we also decided to fix the volume of calcium and phosphorus precursor. We will use always 1,94 mL of calcium precursor and 0,63 mL of phosphorus precursor. That is how we made a 1,5% ZnO, you will find volumes and composition in table 16.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 21,5 mL 2-Methoxyethanol ➢ 1,94 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 0,917 mL Zinc precursor	Cooled with ice	Under Argon	8,5 mL 50/50% Ethanol/ NH3 (30%)	≈ 18h	0,218g	✓ Ca0 : 67,36% ✓ P2O5 : 31,10% ✓ ZnO : 1,54%

Table 16: Reaction's parameters and volumes for P30/Zn1.5-NPs

With 1,54% ZnO we tried to make a 3%. The problem is that with our technique of calculating the volume by correcting the previous one, when we are getting closer to low zinc content, the coefficient of correction is getting bigger. It induces higher volumes and we calculated them, to make a 3% from 1,54% we work out that we have to introduce 1,83 mL of zinc precursor. We tried with this volume and the volume of phosphorus and calcium that we already fixed. It gives us a 17% ZnO P30 particles. This content was out of our study so we decided to use a volume which was between the one we took for 5% and 1,5%. We put 1,5 mL of zinc to create a 5%, 0,917mL for 1,5% so we decided to introduce 1,15 mL to see what we get. The result was not the one we expected but it was good all the same as we get 8% ZnO particles. We had not tested 8% ZnO particles and it was the first we wanted to create so we decided to test these three particles with bacteria. You will find the composition and volumes in table 17.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 21,28 mL 2-Methoxyethanol ➢ 1,94 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 1,15 mL Zinc precursor	Cooled with ice	Under Argon	9 mL 50/50% Ethanol/ NH3 (30%)	≈ 18h	0,247g	✓ Ca0 : 63,50% ✓ P2O5 : 28,30% ✓ ZnO : 8,20%

Table17: Reaction's parameters and volumes for P30/Zn8-NPs

9.1.2 Homogenious Particles :

We have made two parts for P30 ZnO particles because we noticed small crystals inside ours powders of particles after two months. At the beginning we were focused on particles concentration and we only used EDS. After getting several composition and find a way to control the concentration, we get interested in the composition of our particles. By changing SEM detector we were able to identify two different phases into particles. It appears small crystals with high concentration of zinc (50%M). To avoid this problems we just let our reaction during 1h30 instead of 18h. As we knew that zinc concentration is depending of the time, we choose to start with a high volume of zinc which give us a high content zinc nanoparticles. We chose the same volume of zinc precursor that we took to get a 10% ZnO. As you can see in table 18 , let the reacting during only 1h30 decrease a lot the final zinc content but it does not decrease so much the yield.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 20,4 mL 2-Methoxyethanol ➢ 1,94 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 2,03 mL Zinc precursor	Cooled with ice	Under Argon	8,5 mL 50/50% Ethanol/ NH3 (30%)	≈ 1h30	0,201g	✓ Ca0 : 67,04% ✓ P2O5 : 31,18% ✓ ZnO : 1,78%

Table 18: Reaction's parameters and volumes for P30/Zn2-NPs

For the next batch we calculate the volume of zinc precursor from 1,78% and continue to let it only 1h30. You will find volume and final composition in table 19.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 19,01 mL 2-Methoxyethanol ➢ 1,94 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 3,42 mL Zinc precursor	Cooled with ice	Under Argon	7 mL 50/50% Ethanol/ NH3 (30%)	≈ 1h30	0,221g	✓ Ca0 : 65,52% ✓ P2O5 : 30,91% ✓ ZnO : 3,57%

Table 19: Reaction's parameters and volumes for P30/Zn3.5-NPs

Finally we wanted to achieve 5% without high concentrated zinc crystals. We also calculated the volume from 1,78% ZnO P30 particles because at this time we did not know the concentration the previous particles (3,5%). Finally we get something more concentrated with also different phase (see the part about the morphology). You will find the final compositions and volume in table 20.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 16,73 mL 2-Methoxyethanol ➢ 1,94 mL CaO precursor ➢ 0,63 mL P2O5 precursor ➢ 5,7 mL Zinc precursor	Cooled with ice	Under Argon	7 mL 50/50% Ethanol/ NH3 (30%)	≈ 1h30	0,237g	✓ Ca0 : 59,97% ✓ P2O5 : 29,82% ✓ ZnO : 10,21%

Table 27: Reaction's parameters and volumes for P30/Zn10-NPs

Phosphorus/Zinc Particles:

Aside from doing P30/Zinc particles we also tried to make degradable particles with the highest zinc content as possible. To do it, we chose to keep the same protocol but only working with phosphorus and zinc. As calcium is a cation, like zinc, they are reacting together with phosphate which is the anion. Hence, to get something with a high content of zinc we had to remove calcium ions, to let zinc react as much as possible with phosphorus and create nanoparticles. As you are going to see these particles have interesting properties. During the drying process, we need to disperse our particles into ethanol or hexane to wash them. We noticed that it was harder to disperse phosphorus/zinc nanoparticles than P30/zinc particles into these two media. It is also faster to start the reaction because we only need to introduce between 1 and 2 mL of the mix of 50% ethanol and 50% ammonia (30% in NH_3).

We tried to make a 50%/50% P2O5/ZnO particles. Always with the same calculus calculated (table 21) from 10% P30/Zinc particles.

	%R with EDS (%M)	%T (%M)	V (mL)	$V\frac{\%T}{\%R}$= New volume (mL)
P2O5	26,7	50	0,63	1,18
ZnO	10,08	50	2,03	10,06

Table 21: Method to calculate the new volumes necessary for the new batch

For these particles we did not calculate the final composition because there were not soluble in water, so there were no reason to study them deeper. We directly tried to make a 30%M zinc and 70%M phosphorus. From the volume we just calculated we adjust the volume of the two precursor as we always did. You will find the final composition and volumes in table 22. When we tested them we work out that there were degradable in water after a day. Unfortunately it seems that the dishes was not well cleaned and we introduce a small quantity of calcium inside the batch. We also get something more concentrated in zinc compare to concentrations we expected.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 17,35 mL 2-Methoxyethanol ➢ 1,65 mL P2O5 precursor ➢ 6,0 mL Zinc precursor	Cooled with ice	Under Argon	1 mL 50/50% Ethanol/ NH3 (30%)	≈ 18h	0,602g	✓ Ca0 : 3,12% ✓ P2O5 : 43,01% ✓ ZnO : 53,86%

Table 22: Reaction's parameters and volumes for P50/Zn50-NPs

So we tried to make again this particles to make ones without calcium and also reduce again the content of zinc. For the next one we have already notice the small zinc crystals into P30/zinc particles. To avoid the formation of crystal into phosphorus/zinc particles, we also applied the same protocol and let reacting only 1h30. You will find final composition and volumes of these new particles in table 23.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 19,15 mL 2-Methoxyethanol ➢ 2,76 mL P2O5 precursor ➢ 3,09 mL Zinc precursor	Cooled with ice	Under Argon	1,2 mL 50/50% Ethanol/ NH3 (30%)	≈ 1h30	0,28g	✓ P2O5 : 43,46% ✓ ZnO : 56,54%

Table 23: Reaction's parameters and volumes for P40/Zn60-NPs

As we just let the reaction 1h30 we get nanoparticles with high content of zinc. We needed to adjust volumes again, reduce the one of zinc twice and increase the volume of phosphorus. You will find the final volumes and composition is table 24.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 19,15 mL 2-Methoxyethanol ➢ 4,44 mL P2O5 precursor ➢ 1,63 mL Zinc precursor	Cooled with ice	Under Argon	1,2 mL 50/50% Ethanol/ NH3 (30%)	≈ 1h30	0,28g	✓ P2O5 : 54,89% ✓ ZnO : 45,11%

Table 24: Reaction's parameters and volumes for P60/Zn40-NPs

This conclude the part about the nanoparticles composition and how do we made them. For P30/Zn particles, we have seen that sometimes it is harder to get the composition we wanted to achieve at the beginning of the experiment. This is due to the high reactivity of zinc which make the composition less predictable. There is some conditions which are indispensable to get homogeneous nanoparticles, as the fact to introduce slowly the catalyst and use 50/50% ammonia/ethanol for it. Moreover, it is very important to well choose the time of reaction because it has a huge influence on the different phase we could form at the end.

ZnO particles:

For the first antibacterial characterization we wanted to compare ours modified P30 particles with Zinc oxide. So we just precipitated zinc precursor into distilled ethanol. You can find volumes of reactive and conditions of experiment in table 25.

Reaction's Parameters						
Reactant	**Temperature**	**Atmosphere**	**Starting solution(s)**	**Time of reaction**	**Yield**	**Final composition (%M)**
➢ 25 mL Distilled Ethanol ➢ 0,4 mL Zinc precursor	T°amb	In Air	No	≈ 18h	Small quantity	✓ ZnO : 100%

Table 25: Reaction's parameters and volumes for ZnO-NPs

9.2 Construction of the reference line (Absorbance)

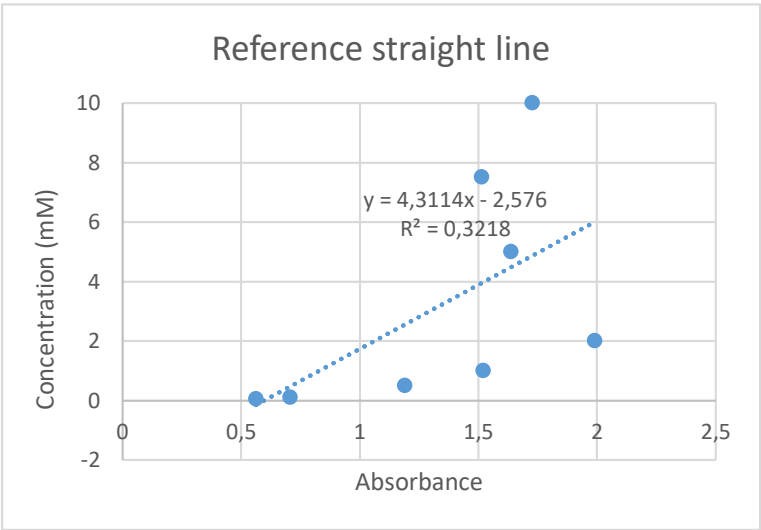
To find this line we had to proceed step by step. There is a maximal amount which can be released to quantify the release of zinc. It will be more explicit if we look at the result step by step of the construction and selection of values for the reference.

We first made 8 different solutions of zinc chloride with concentration from 0,05mM to 10mM. We prepared 1mL of each solution and mix a few quantity of each solution to various reactive (for details see the protocol of zinc release). The more concentrated is the solution, the more colored it is and higher is the absorbance. The result of the absorbance are recapitulated inside table 26.

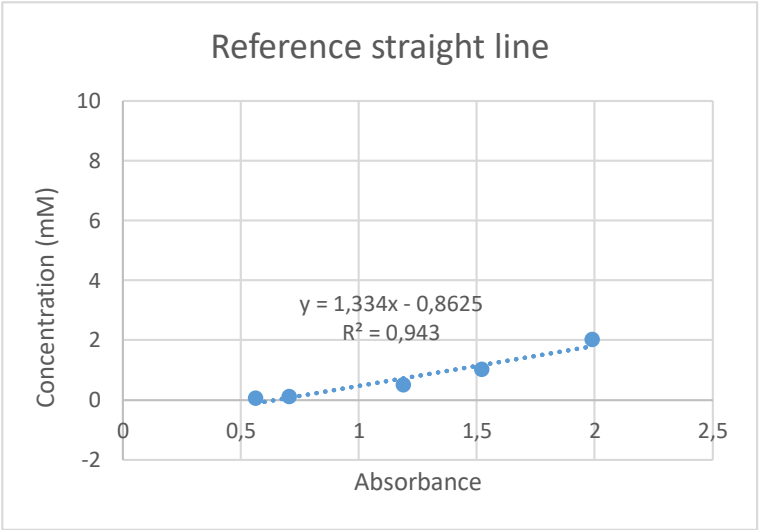
Concentration (mM)	0,05	0,1	0,5	1	2	5	7,5	10
Absorbance	0,56	0,71	1,19	1,52	1,99	1,64	1,51	1,73

Table 26: Different concentrations of zinc chloride and its assimilated absorbance

If we look at graph 2, we can see that after the point corresponding to 2mM, the corresponding absorbance is lower than 1,99. If we remove all the point after 2mM we see that we get a linear slope on graph 3. We can conclude that after 2mM we get the point of saturation. With this first graph we know that our reference line has to be between 0mM and 2mM.



Graph 2: Reference line with all the points



Graph 3: Reference line without unnecessary points

We can now, do the same measurement with our particles. We just have to introduce nanoparticles inside a solution where the can release zinc. After one hour at 37°C, we can take some solution from the mix between particles and Hepes. The tubes had to be well-centrifuged to avoid particles inside the final solution, otherwise it can distort the results of absorbance. To be used as reference we need to work inside the range of values which characterized the reference line. With the equation of the straight line we can calculate the concentration corresponding to the absorbance of solutions. Every time we made a new test of absorbance we had to prepare new solutions for the reference straight line. It means that

every time the coefficients of the line are changing. This ensure an utilization of a reference which was made at the same conditions than the experiment.

9.3 SEM theory (source [33-36])

Scanning electron microscope (SEM) is widely used to get images of the surface of samples. These images are mainly formed using electronic emissions from surfaces (secondary and backscattered electrons) due to the impact between a very fine brush of primary electrons and surfaces. Different contrasts can be observed, bringing a wide variety of information about the sample. Especially its relief and the distribution of the phases (contrast "atomic number" using backscattered electrons). It's also possible to observe chemical or crystalline contrast. Scanning electron microscope can also give a local precise chemical analysis with collection of X-Rays which are emitted. We will present the different type of electron which are emitted and see how to exploit them to identify and characterise samples.

Different types of electrons collected: Secondary electrons: a primary electron thrown on the sample may transfer energy to an electron on the conduction layer of a sample atom. This electron will then be ejected and ionized: it's a secondary electron and it has a relatively low energy (Figure 27) Because of this low energy, secondary electrons are emitted in the surface layers near the surface (10nm) (Figure 38). These electrons are numerous and simple to be collected, they give information on the topography of the surface of the sample. If there are hollows or depression on the surface of the sample, the number of secondary electrons that are emitted is low and so these places appear darker on images. At contrary if there are inclined surfaces such as peaks or hills on samples, more electrons can be reemitted and these places appear brighter on images. We can call this the edge effect (Figure 26).

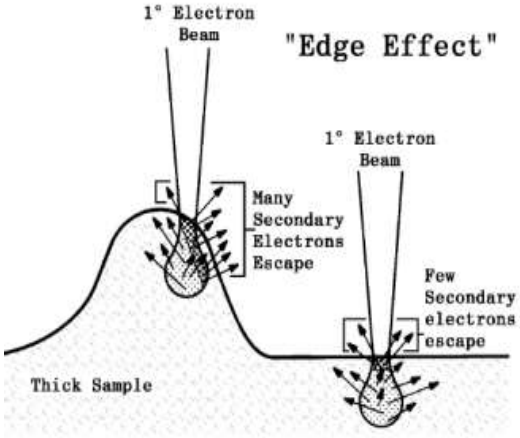


Figure 26: Edge effect of secondary electrons [33]

Backscattered electrons: these electrons are the result of an interaction between a primary electron and a nucleus of a sample atom. The electron will be reemitted in a direction close to the original while losing a small amount of energy (Figure 27). They have therefore a higher energy than secondary electrons so they penetrates deeper in the sample as we can see on Figure 28. However, they are less numerous. They enable to qualitatively analyse the chemical homogeneity of a sample, as the heaviest atoms reemit more electrons than the lightest, heavy atoms appears brighter.

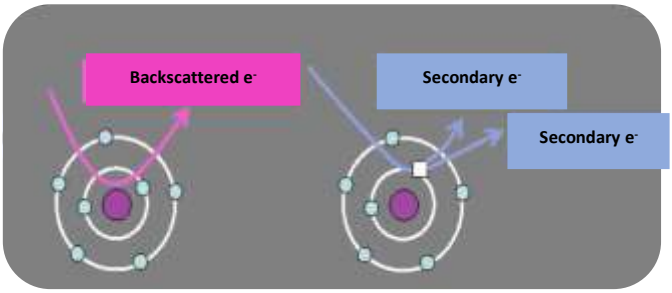


Figure 27: Interactions electrons-atom [35]

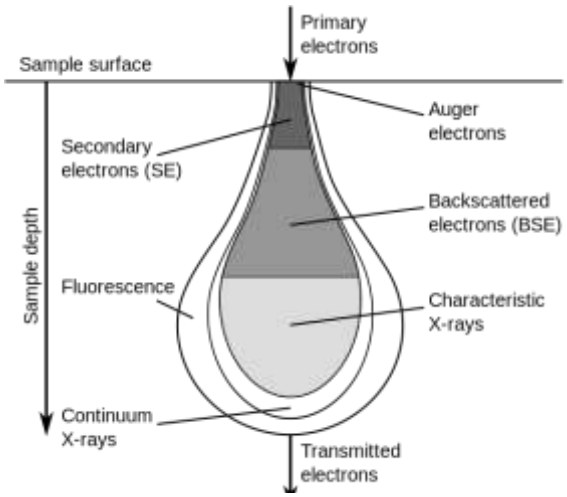


Figure 28: Interaction pear [36]

By choosing the appropriate detectors we can investigate the morphology of the surface of ours particles (secondary electrons) or its composition (backscattered electrons).

9.4 EDS: Energy Dispersive X-ray Spectroscopy

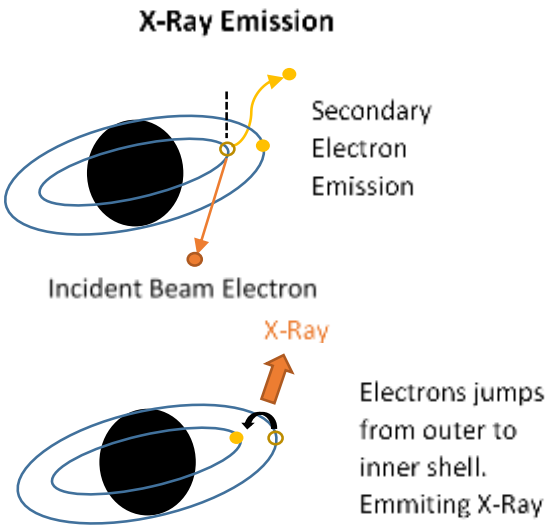


Figure 29 : Emission of X-Rays due to incident electron beam

We used EDS (Energy Dispersive X-Ray Spectroscopy) in the way to find compositions of every nanoparticles we achieved. As indicate the name of the technique we are going to use X-Ray to characterise our material. We already saw that different types of electrons are emitted (backscattered and secondary) when we hits the surface of a material with electron beam. Why X-Rays are emitted?

When the incident electron beam hits the sample and create a secondary electron it produce holes in electron shells where secondary electrons took place. (Figure 29)

If these holes are located inside the shell, atoms are not in a stable state. To stabilised, electrons from the outside of the shell jump into that gap because electrons on the outskirt have a higher energy state. So to fill the gap, electrons need to lose energy and do it emitting X-Rays. (Figure 29)

X-Rays emitted from the atom have its proper energy and wavelength. But each shells as we are going to see, emit its proper kind of X-Ray, depending of which shell is losing its electron and which shell is going to replace it. For example if we take an atom:

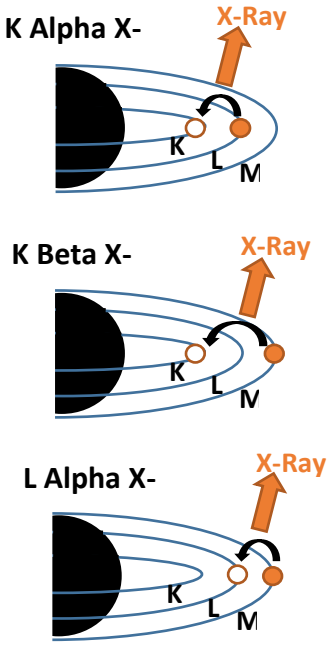


Figure 30 : Different emissions of X-Rays depending on the shell

If the secondary electron is emitted from the K shell and if it is an electron from the L shell which replace it, it produces X-Ray with a specific energy to this jump. (Figure 30)

If the secondary electron is emitted from the K shell and if it is an electron from the M shell which replace it, it produces again X-Ray with a specific energy. In this case we called Beta-X-rays because there is two shells between the gap en the electron.(Figure 30)

If the secondary electron is emitted from the L shell and if it is an electron from the M shell which replace it. Again we produce X-ray with another specific energy because the energy required to jump between L and M shells and K and L shells in not the same.(Figure 30)

We can show an example of EDS graph on figure 31, using the one we recorded for ours nanoparticles. On this graph you can

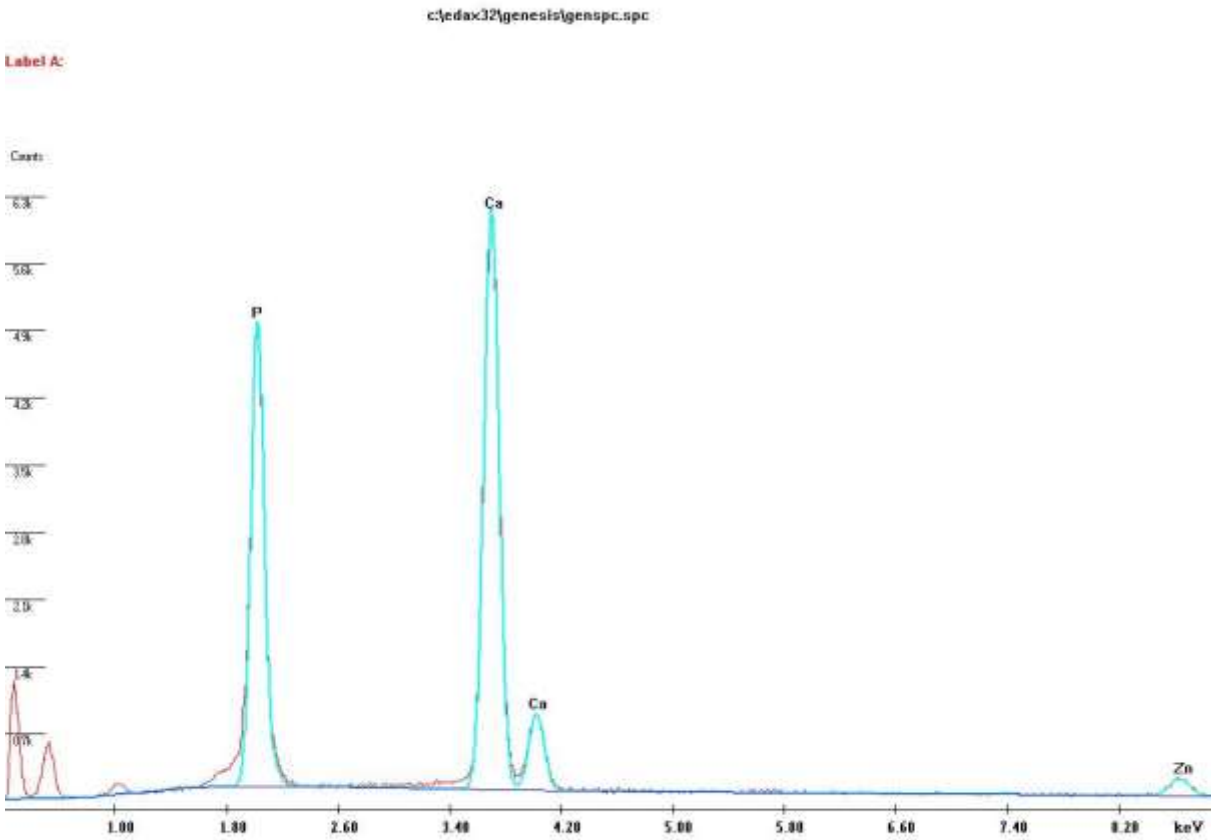


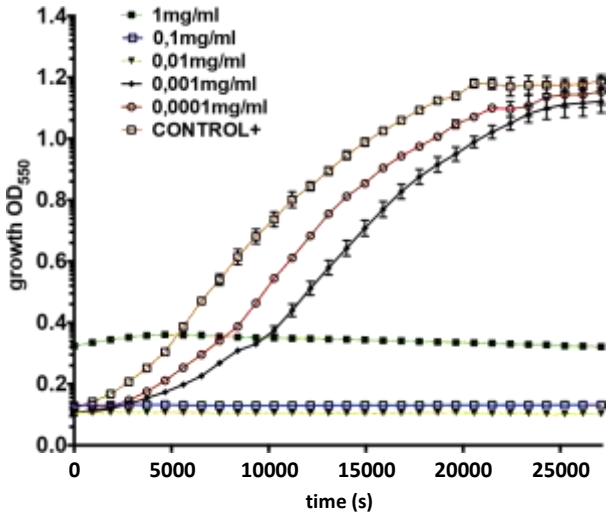
Figure 31: EDS spectra of P30/Zn-NPs

see that we work out 3 peaks which correspond to three elements which composed ours particles. When we gets peaks, we can use a device which calculate directly the composition (%M) of samples, considering the intensity of peaks.

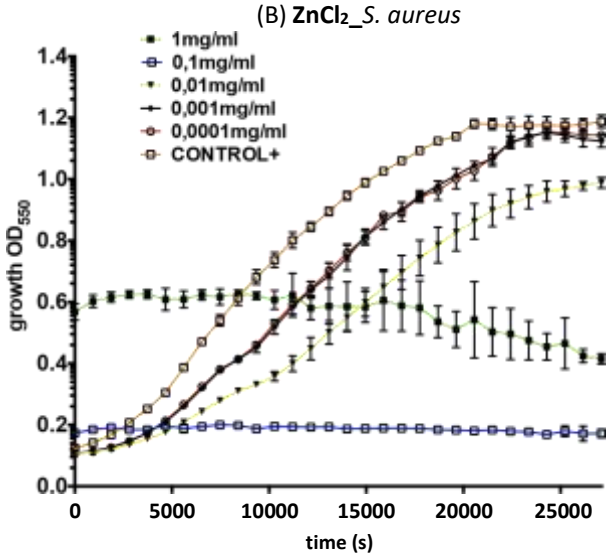
9.5 Study of six elements against *S.Aureus* (Figure 32)

Staphylococcus aureus

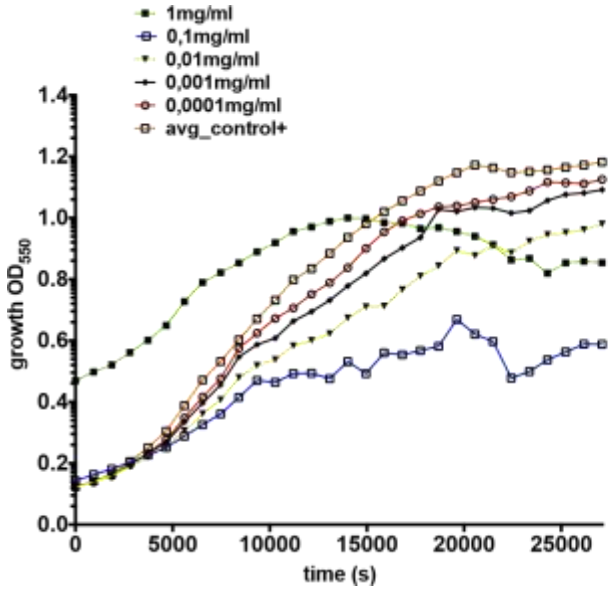
(A) **$AgNO_3$** _*S. aureus*



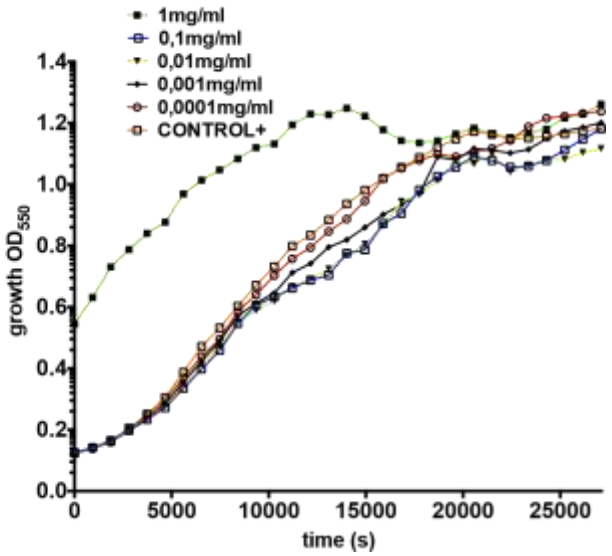
(B) **$ZnCl_2$**_*S. aureus*



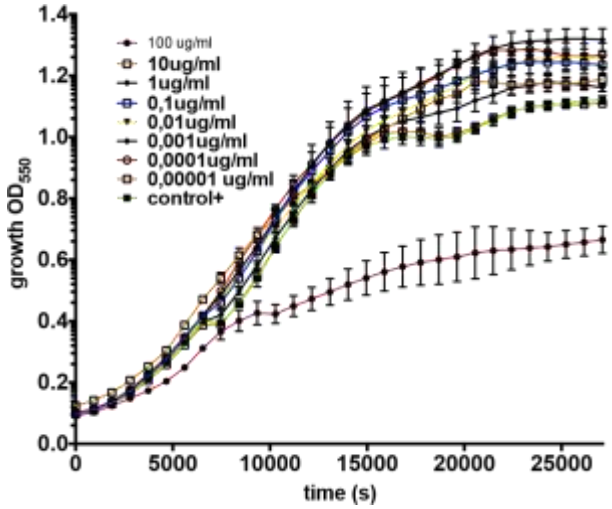
(C) **ZnO_NP**_*S. aureus*-ETOH_effect



(D) **P15_NP**_*S. aureus*-ETOH effect



(E) **Gallium_chloride**_*S. aureus*



(F) **Cerium_chloride**_*S. aureus*

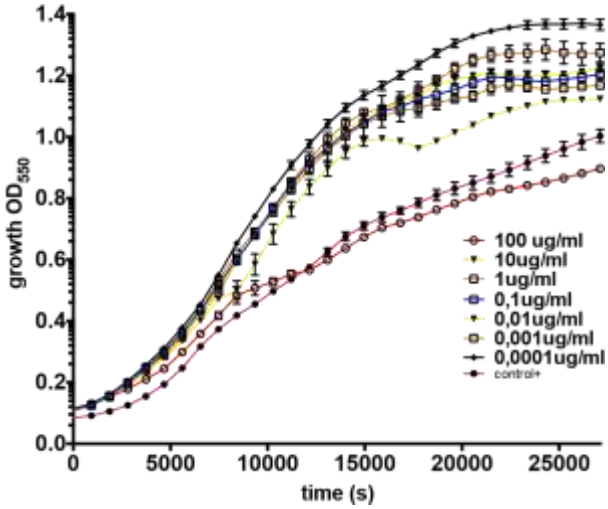


Figure 32: Antibacterial activity of salts and nanoparticles against PAO1, (A) $AgNO_3$,(B) $ZnCl_2$,(C) ZnO-NPs,(D) P15-NPs, (E) Gallium Chloride, (F) Cerium Chloride

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