POLITECNICO DI TORINO

Master Degree in Biomedical Engineering

Role of LL37-AuNPs in Antimicrobial Activity and Dental Application

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Abstract

The aim of this thesis is to study the strategy for revitalization of damaged teeth, by eliminating infections and regenerating the dentine-pulp complex instead of traditional root canal treatment, such as the obturation of the root canal with biologically inert materials and the use of sodium hypochlorite (NaOCl), as an endodontic irrigant and antimicrobial agent. NaOCl can induce potential complications ranging from permanent bleaching of clothes to serious soft tissue damage. The main problem for a dental pulp damaged is linked to bacterial contamination of the root canals. Therefore in this work, a multifunctional nanoformulation has been developed as potential antimicrobial and differentiation agent to differentiate dental stem cells to odontoblast-like cells. The Human Cathelicidin LL37 shows important antimicrobial and pro-regenerative properties. Peptide can be chemically immobilized on nanoparticles in order to increase its stability, decrease toxicity, improve antimicrobial properties. Gold nanoparticles (AuNPs) have good compatibility with human body, low toxicity, small size and stability and it is easy to modify their properties, immobilize high concentrations of peptide per surface area. In this project, LL37-conjugated AuNPs have been prepared by a one step procedure. In the first part of the work, the antimicrobial activity of the LL37-Au NPs has been evaluated against Gram-negative (*E.coli* and *E.coli ML-35p*) and Gram-positive (*S.aureus*, *S.epidermidis*, *E.faecalis*) bacteria using Broth Microdilution Antibacterial Assay and spread plate technique. In order to improve the antimicrobial activity of the LL37 peptide a green laser was used.The thermal energy generated by AuNPs after exposure of laser light helps to damage bacterial membranes and allows the internalization and bactericidal action of LL37-AuNPs.In the second part of the work, it has been shown the effect of LL37-AuNPs on stem cells from the apical papilla (SCAPs). The apical papilla tissue is found in the root of an immature permanent tooth.They have the capacity to differentiate into adipocytes and osteoblasts/odontoblasts in vitro condition. SCAPs as well as mesenchymal stem cells (MSCs) have the following characteristics: self-renewal, proliferation, migration, differentiation and immunosuppression, they can be used in immunotherapy and in regeneration of dental tissues, neural, bone and vascular tissues.To determine potential cytotoxic effects of the AuNPs and peptide LL37, the CellTiter-Glo Luminescent Cell Viability Assay was conducted. The differentiation of SCAPs was induced by odontogenic differentiation media, which contains dexamethasone and β-glycerophosphate. Alkaline phosphatase and Alizarin Red staining were used to study the odontogenic differentiation of SCAPs. To investigate the effects of LL37, AuNPs and LL37-

AuNPs on the cellular proliferation of SCAPs, an In Cell Analyzer was used and the expression of epidermal growth factor (EGF) receptor on the surface of SCAPs was shown using flow cytometry. Moreover, LL37-AuNPs and Au-NPs were deposited on electrospun nanofibers of gelatin and antimicrobial properties have been tested. Further studies are necessary to understand how to optimize the parameters used in this work with the evidence that multifunctional nanoformulation could be further applied in clinical therapies.

1. Nanomedicine

Nanomedicine is the application of nanotechnology used for the prevention and treatment of disease. Ad defined by the Royal Society: *Nanotechnology is the design, characterisation, production and application of structures, devices and systems by controlling shape and size at nanometer scale* (The Royal Society & The Royal Academy of Engineering, 2004a) (1). In this scale, which is generally seen as going from 100 nm and down to a few nanometers, the materials have different properties compared to those at a larger scale. A nanometer (nm) is one thousand millionth of a meter, 10^{-9}. To visualize this, the diameter of a human hair is 80 000 nm and a virus is around 100 nm. Nanomedicine involves different array of devises come from biology, engineering, physics, chemistry using nanoscale materials, such as biocompatible nanoparticles, nanorobots used for different purposes, for detection, delivery, sensing or actuation reasons in human body *(2)*.

Nanoscale materials can be manufactured through two different ways: the top-down nanofabrication transforms a structure from a larger one to a smaller one through sequentialcuttings, like to cutting a block of wood, whereas the bottom-up technique is like to building a house brick by brick, it starts with singular atoms and builds them up to a nanostructure. Particles with dimensions of about 1–100 nanometers change their properties from those at larger scale. Properties such as melting point, fluorescence, electrical conductivity, magnetic permeability, and chemical reactivity depend on the size of the particle. To the nanoscale for example, some materials used for electrical insulations can develop into conductive and other materials can develop into transparent or soluble. For example, nano gold and bulk gold are different, the nanoscale particles can be orange, purple, red or green depending on the size of the particle because the particles are so small that electrons cannot move about as in bulk gold, the movement is limited and change the way in which particles interact with light (3).

Recently, nanotechnology-based strategies have been developed to solve dental problems, through the developing of nanomaterial used in the dental filling, polishing of the enamel surface to reduce the possibility to have caries, or used for the dental implants, they result to be more efficient than the traditional materials. Nanodentistry can became cheap, time-saving and less traumatic for the patients (4).

1.1 Nanoparticles and their properties

Nanoparticles (NPs) are small materials with size ranges from 1 to 100 nm. Due to their high surface area and nanoscale size, they have unique chemical and physical properties as compared to their bulk counterparts. Generally their size, structure and shape influence their properties such as reactivity, toughness, catalytic, electronic and magnetic properties. For instance a 20-nm gold (Au) NPs, platinum (Pt), silver (Ag), and palladium (Pd) NPs have wine red, yellowish gray, black and dark black colour, respectively. The colour of the Au NP solution changes according to the variation of the aspect ratio, shell thickens and the percentage of Au. Thanks to these characteristics, they can be used in multidisciplinary fields, such as medical or environmental applications, imaging, energy-based research. NPs are made of three layers, the first is the surface layer, it can be functionalized with various small molecules, polymers, metal ions and surfactants, the second one is the shell layer, which chemically differs from the core and the last one is the core which the centre of the NP (5).

1.1.1 Classification of NPs

NPs can be dived into various categories according to their size, chemical properties, morphology and compositions.

Inorganic NPs:

- Carbon-based NPs: Carbon nanoparticles are applied at different fields. For example, polymer materials can be enhanced by inclusion of conductive fillers such as carbon nanoparticles. Filler inclusion in molten polymers can cause an important change in viscoelastic behaviors since they are sensitive to the concentration, structure, particle size, shape and surface changes of the fillers. Nanofillers can be used for the fabrication of nanocomposites such as orfanoclays, CB, CNTs, metal oxide and recently graphite oxide (GO) and grapheme. The main feature of Carbon nanoparticles (CB, CNT and graphene derivatives) is to improve electrical conductive properties of polymer nanocomposites, their morphology influence the final properties (6).

- Metal NPs: Metal nanoparticles are broadly used in biomedical and engineering applications. They can be synthesized and functionalized with different chemical groups which allow them to be conjugated with ligands, antibodies or drugs. They have a broad range of important applications in biotechnology, magnetic separation,

drug delivery and diagnostic imaging. Magnetic nanoparticles Fe_3O_4, silver and gold nanoparticles, nanoshells, nanocages can be used in the diagnostic imaging and therapy of cancer (7).

- Ceramics NPs:Ceramics nanoparticles are composed of oxides, carbides, phosphates and carbonates of metals and metalloids such as silicon, titanium, calcium. Thanks to their properties, these nanomaterials have good heat resistance and chemical inactivity, which can be applied in many fields for example in biomedical area, they can be used like carriers for genes, drugs, proteins and imaging agents. It is essential to control the size range, porosity, surface area to volume ratio, etc to have optimal outcomes. They can be utilized as drug delivery systems against bacterial infections, glaucoma, and cancer (8).

- Semiconductor NPs: Semiconductor nanoparticles also known as colloidal quantum dots are small (<10 nm)fluorescent semiconductor nanocrystals, with singular luminescent properties. Their fluorescence emission has strong stability and is linked to the particle size. Specifically, the structure is composed of a semiconductor core, covered by a semiconductive shells such as cadmium selenide, cadmium telluride, zinc cadmium selenide, and zinc sulfide to enhance their optical properties, and a cap to facilitate aqueous solubility for biology applications. The quantum dots can be used in vitro real-time bioimaging or monitoring of intracellular processes with time, diagnostic and imaging because of their properties such as narrow emission, great ultraviolet excitation or bright fluorescence. For example, cadmium selenium-zinc suphide quantum dots can be utilized to visualize cerebral vasculature (9).

Organic NPs:

- Polymeric NPs: Polymeric nanoparticles are colloidal particles (from 10 nm to 1 um) (10), made of biocompatible and biodegradable polymers of natural (such as alginate, albumin, chitosan)or synthetic (such as polylactide, polylactide–polyglycolide copolymers, polycaprolactones, and polyacrylates)origin. According to fabrication process (solvent evaporation, solvent diffusion, polymerization or spontaneous emulsification) , two types of structures can be obtained, nanosphere and nanocapsule. In the nanospheres the drug is uniformly diffused, while the nanocapsules are composed of a polymeric membrane and the drug is incorporated in

a cavity. Polymeric nanoparticles made from natural polymers are mostly used as drug carriers, for example they have been successfully used to deliver compounds such as acyclovir (antiviral), retinol (vitamin A), and quercetin (antioxidant). Synthetic polymers can be biodegradable such as (d,l-lactic-co-glycolic acid) (PLGA), which has been used for the transdermal delivery of ketoprofen, Spantide II and indomethacin. No biodegradable NPs such oilyacrylatescan be used for dermal and transdermal delivery of drugs (11).

- Lipid-based NPs: Lipid-based nanoparticle (LBNP) systems (for example liposomes, solid lipid nanoparticles and nanostructured lipid carriers) are one of the best hopeful colloidal carriers for bioactive organic molecules. LBNPs have many advantages such as high temporal and thermal stability, excellent loading capacity, low fabrication costs, easy of fabrication, and ability to monitor the release of the drug and to induce low or no toxicity. They can transport hydrophobic and hydrophilic molecules. Lipid nanosystems can be chemically modified in order to improve the solubility of the drug and to escape from the immune system. They have shown excellent results in vitro cancer therapy but also in vivo, improving the antitumor activity of various chemotherapeutic agents. Additionally, they can in specific conditions be sensitive to the pH and therefore to stimulate the drug release in acid environment and can also linked to antibodies that identify tumor cells or their receptors (such as folic acid (FoA) (12).

1.1.2 Nanoparticles in dentistry

Nanoparticles can be used in the field of dentistry and the most common nanoparticles used are:

- Carbon nanotubes (CNTs): they are characterized by C22C covalent bond and hexagonal orientation. These can be used for teeth filling and different applications due to their good electrical and mechanical properties such as het stability, efficiency, high strength, lower density and transmission efficiency (4);

- Graphene: it is an allotrope of carbon in the shape of a single layer of atoms placed in an hexagonal arrangement. Acrylic teeth covered with graphene are utilized for its cost-

effective, resistance to fracture and low-density. Anti-biofilm tests express biofilm reduction using Graphene/zinc oxide nanocomposite (GZNC) (4);

- Hydroxy apatite (HAp): $Ca_{10}(PO_4)_6(OH)_2$, generally it has the same composition of teeth and bone and mostly present in enamel. HAp NPs can be incorporated into dental tubules helping to reduce the dental hypersensitivity. They can be liked to proteins or bacterial due to their great surface area. If they are absorbed by the enamel of the teeth they can delay erosive demineralizarion. The main biomimetic capacity of hydroxyapatite is to create an artificial film on the tooth in order to protect it (4);

- Iron oxide ($Fe3O_4$): magnetite and maghemite, two typical examples of iron oxide nanoparticles are used in clinical applications due to their biocompatibility and no-toxic effects on the human beings. Iron oxide NPs can be used to remove biofilms on dental implants (4);

- Zirconia (ZrO_2): it owns analogous metallic characteristics and color as tooth, for its biocompatibility, high resistance to corrosion, low cytotoxicity, the ability to be osseo conductive is mostly used in dental applications. Moreover it is insoluble in water and this decreases the bacterial attachment. Studies have demonstrated that Zirconia oxide nanoparticles contrast bacteria as *E. faecalis* (4);

- Silica (SiO_2): silica-based NPs are commonly used in dentistry for its properties such as low density, biocompatibility, low cost ,surface area and size, usually used as dental filler. They can be utilized for the polishing of enamel, helping to inhibit caries (4);

- Titania(TiO_2): titanium nanoparticles are diffused in dentistry, physical modification (through silica/titania nanoparticle coating) reduces microbial attachment moreover they have important characteristics such as high strength, durability and biocompatibility (4);

- Silver: AgNPs are an excellent antimicrobial agent, they reduce microbial activity on tooth and improve oral health, they can penetrate the bacterial cell membrane and kill bacteria due to their small sizes. They show other advantages such as biocompatibility, low toxicity, long antimicrobial activity but they tend to change the color of the tooth (4).

1.2 Gold Nanoparticles (AuNPs)

Gold nanoparticles (also known as colloidal gold) can be utilized in various fields and one of the most interesting is the biomedical one. They show different colours (brown, orange, red and purple) in aqueous solution as the size goes from 1 to 100 nm and usually present a size-relative absorption peak from 500 to 550 nm (13).

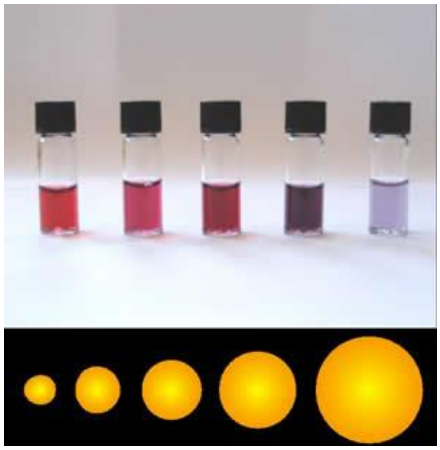


Figure 1-Suspensions of AuNPs of different sizes (14)

They have excellent properties such as good compatibility with human body, low toxicity, small size and stability that allow them to be used for cancer treatment, drug delivery, and diagnosis. Gold nanoparticles can be used as drug delivery systems since they can be coated with different therapeutic agents and for their large surface. They can absorb near infrared (NIR) light due to their optical properties and emit thermal energy locally to kill tumours. In the recent years, studies have been showed their ability to cross the blood-brain barrier, and interact with the DNA, which may cause genotoxic effects (15). Generally gold nanoparticles have been chosen in biomedical field because it is easy to modify their properties, immobilize high concentrations of peptide per surface area (16).

1.2.1 Properties of AuNPs

Optical property, Surface plasmon resonance (SPR)

Gold nanoparticles have good optical absorption and scattering of the light from the visible to near IR (NIR) spectral range, this effect is known as the surface plasmon resonance (SPR) band (17), because of the resonant oscillation of the conduction electrons at the interface with incident light (18), therefore when metallic NPs are hit by the light, the oscillations produced by electromagnetic field of the light cause a collective oscillation of the free electrons of the

metallic NPs. The highest amplitude of the oscillation is at specific frequency, called surface plasmon resonance (SPR). At this frequency, an great amount of the incident light is absorbed and thus can be evaluate through a UV-Vis absorption spectrometer. The oscillating electrons interacting with the crystal lattice of AuNPs produce and transport thermal energy to the lattice. Thus, AuNPs are warmed up and dissipate their thermal energy to the surrounding medium to obtain the heating effect (14).For plasmonic nanoparticles (Au and Ag nanoparticles) this effect is much more evident than other metals (19). The size and the shape of the nanoparticles and the dielectric properties of the surrounded environment influence the peak wavelength and the linewidth of the SPR band (17). AuNPs express the SPR band about 520 nm in the visible region. Small gold nanoparticles (<10 nm) show the dumped SPR band due to the high rate of electron-surface collisions compared to wider particles. Large particles (>100 nm) show a broader band because of higher order electron oscillations and the ratio of the scattering to absorption increases a lot. Larger particles, thanks to the higher scattering efficiency are used for imaging instead smaller nanoparticles are mostly used for photothermal therapy since light primarily absorbed by the particles is converted to heat for cell and tissue demolition (19).

Shape and Crystallinity

According to the fabrication method, it is possible to create gold nanoparticles with different sizes and shapes. Usually anisotropic shapes are produced using a stabilizing polymer that tend to links to one crystal face and leads to have one crystal direction growing faster than others. The size of the crystalline domains depends on the fabrication method (20).

Stability

It is complicated to control the nanoparticle aggregation. Nanoparticles are charge stabilized or sterically stabilized. In the first case the NP's stability can be determined by the zeta potential and usually, nanoparticles with zeta potentials higher than 20 mV or less than -20mV have enough electrostatic repulsion to stay stable in solution. Particle stability can be traced using UV-Visible spectroscopy or Dynamic Light Scattering(14). The lack of colloidal stability a long time can become a problem in real world applications, especially the production of irreversible aggregates when they are undergone chemical and physical changes such as the interaction with biofluids or freeze-drying. Many approaches have been considered to improve the NP stability, one of the most successful approach is to functionalize the AuNPs with non-ionic poly(ethylene glycol) (PEG) (21).

1.2.2 Biomedical application of AuNPs

Au NPs have been widely used in nanomedicine due to their low toxicity and biocompatibility.

Photodynamic therapy

Gold nanoparticles can be functionalized with both tethering ligands and photosensitises for photodynamic therapy(PDT) of cancer, and targeting over-expressed recpetors on the surface of tumour cells (22). This therapy takes advantages of two features of AuNPs, efficient fluorescence extinction and surface Plasmon resonance (SPR) absorption, moreover the conjugation of AuNps with biomolecules promotes intracellular penetration for its ability to link to thiols, amines and disulfides (23).

Photothermal therapy

Gold nanoparticles can be introduced to tumor cells through an intravenous or intratumoral injection and to be exposed to light and generate heat that kill cancerous cells, this treatment is known as photothermal therapy (PTT) or thermal ablation; this therapy can be a valid alternative to traditional treatments such as chemotherapy, surgery and radiotherapy (24). AuNPs larger than 50 nm, thanks to strong NIR absorption, are mostly used in PTT (23).

X-ray imaging

Gold nanoparticles can be used as an x-ray contrast agent thanks to showing a high x-ray attenuation (25), nontoxicity, surface fictionalization for colloidal stability and targeted delivery. They can overcome the limits due to common vascular agents as to show a longer vascular retention time (23).

Drug delivery

Gold nanoparticles are considered excellent nanocarrier in drug delivery systems due to their singular chemical, optical, biological and physical properties. Their surface can be functionalised in order to improve their circulation, decrease aggregation rates, improve the link to therapeutic molecules, transfer different drugs such as proteins, peptides, plasmid DNAs, chemotherapeutic agents. Moreover they are able to cross cell membranes and reduce cytotoxicity due to their smaller size (26).

Sensing

Gold nanoparticles can be used as chemical or biological sensors for detecting various analytes such as anions, metal ions or molecules like nucleotides, proteins, toxins and saccharides. Different AuNPsnanobiosensors can be used such as colorimetric (visible colour changes because of the aggregation of nanoparticles), fluorescence-based, electrical and electrochemical, SPR and Quartz Crystal Microbalance-based sensors (23).

1.2.3 Peptide-nanoparticle conjugates (PNCs)

Peptide can be conjugated to nanoparticles with the aim to overcame limitations of the single materials. Synergy between these two types of material allows to better control over their biological performance. PNCs have been used in biomedical applications such as drug delivery, molecular imaging, liquid biopsy (27).

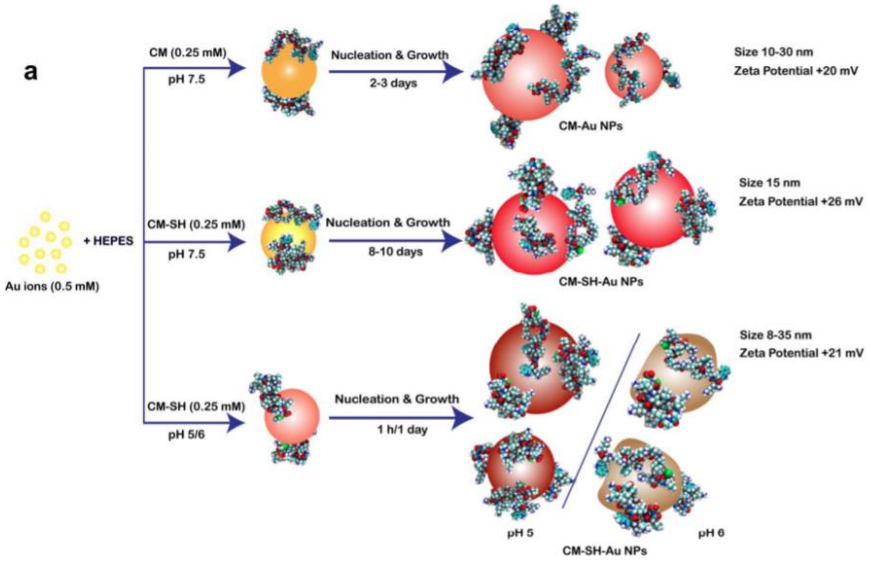


Figura2-Synthesis of CM-AuNPs and CM-SH-AuNPs (28)

2. Peptides

Peptides are short chains of amino acids generally made of 2-50 amino acids. The amino acids are connected to each other in a sequence by peptide bonds. Peptide are different from proteins for their shorter length, proteins are long molecules formed by one or more polypeptides, which are composed of long and continuous strings or peptide while dipeptides, tripeptides and tetrapeptides are shorter chains. Peptides (expect for cyclic peptides) are constituted of an N-terminal (amine group) and C-terminal (carboxyl group) residue at the end (29) .

2.1 Antimicrobial peptides (AMPs)

Penicillin, the first antibiotic was discovered by Alexander Fleming in 1928, since then different antibiotics have been used to treat human infectious diseases. The large amounts and the abuse of traditional antibiotics have led to the bacterial drug resistance and bacterial mutation therefore the developing of a new antibiotic is necessary. Antimicrobial peptides were discovered in the 1980's, they are essential components of the immune system in human, plants and animals and they act first in the human body against bacteria, virus and fungi. They have many advantages over the traditional antibiotics, owing many antimicrobial activities such anti-virus, anti-fungi, anti-cancer. AMPs are small peptides (usually below 40 aminoacids) and are also called the host defence peptide, most of them have cationic and amphiphilic characteristics. The amphiphilic peptide molecules are α-helices with both hydrophilic and hydrophobic sides. The cationic AMPs interact strongly with the negatively charged bacterial cell membranes through electrostatic interactions, and they change the electrochemical potential on bacterial cell membranes and lead to the cell membrane damage, allowing the permeation of AMPs and the bacterial death. Compared to the conventional antibiotics they are not able to induce bacterial drug resistance. Natural peptides can be replaced by synthetic ones in order to limit the short half-life and toxic effects (30).

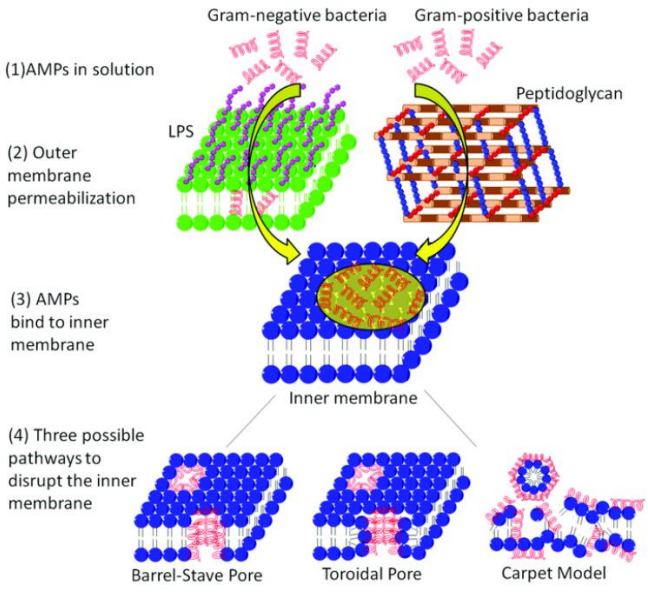


Figura3- Mechanism of interaction between AMP and bacterial membrane (30)

2.3 The Human Cathelicidin, LL37

Cathelicidins are antimicrobial, cationic and small peptides found in human body and animals such as cattle, pigs, sheep, goats, chickens, rabbits and some fishes. They show antimicrobial activity against bacteria, virus and fungi and also generate defensive responses in the host (31).LL-37 is a 37 amino acid cationic peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and fold into amphipathic α-helices.The unique cathelicidin-derived antimicrobial peptide is found in human, especially in human skirt, digestive tract, leukocytes like monocytes, neutrophilis, T cells, NK cells and B cells (32). This peptide has a net charge of +6 in physiological conditions (pH 7.0) since the number of basic residues (5 Arg, 6Lys) is greater than the acidic ones (3 Glu, 2 Asp). Around 35% of residues are hydrophobic therefore it shows an amphipathic helical structure. These characteristics are important for its oligomerization in aqueous salt solutions and for interactions with membranes (33).

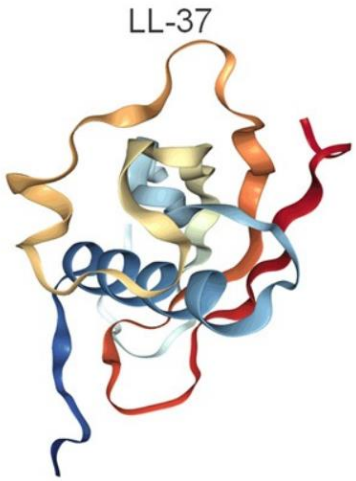


Figura4 The three-dimensional structure of (30)

LL-37 peptide is produced by extracellular cleavage of the C-terminal end of the 18KDa hCAP18 protein by serine proteases of the kallikrein family in keratinocytes and proteinase 3 (PR3) in neutrophils. Its name derives from the first two residues at the N-terminus (Leucine,Leucine) and it is constituted of 37 amino acids. Thanks to its positive charge, it can strongly interact with negative charge of phospholipid membranes (34).

LL37 can control inflammation, angiogenesis and wound healing, it shows important antimicrobial and pro-regenerative properties. Peptide can be chemically immobilized in nanoparticles in order to increase its stability, decrease toxicity, improve antimicrobial

properties (more positive charges and peptide mass) and a better controlled target compared with the AMP alone. It has been shown that LL37-conjugated NPs improve wound-healing properties than free LL37 since the biological activity of the peptide lasts for more time (16).

3. Bacteria

Bacteria, (singular noun bacterium), are single biological cell that live in any type of environment on earth, soil, oceans, rocks, and in symbiotic and parasitic relationships with plants and animals. Their cell structure is simpler than that of other organism since they do not own nucleus and membrane bound organelles, therefore they are part of prokaryotic microorganisms (35). According to their shapes, they can be classified in spherical (cocci), rod (bacilli), spiral(spirilla), comma(vibrios) or corkscrew (spirochaetes) (36). Some bacteria are used in the preparation of foods, chemicals and antibiotics. Microbiology is the science which studies bacteria.

Bacteria can provoke diseases in animals, plants and humans but the most part of them are innocuous and they are fundamental for survival of many animal life on earth because only bacteria and some archea owns genes and enzymes to synthesize vitamine B_{12} that is involved in the metabolic activities of all the human cells. Vitamine B_{12} , also called cobalamin, is a cofactor in DNA synthesis and it is involved in the synthesis of myelin for a correct functioning of the nervous system.

There are around $5x10^{30}$ bacteria on Earth, for example 40 million bacterial cells live in a gram of soil and a million bacterial cells in a millilitre of tap water. They play an important role in the nutrient cycle by transforming atmospheric nitrogen into fixed nitrogen (inorganic compounds used by plants). They are involved in the decomposition of dead bodies and are able to convert components such as hydrogen, sulphide and methane to energy.

Mainly bacteria live in the gut and on the skin in humans and most animals, thanks to the immune system the most part of them are harmless. At the same time they can cause infectious diseases like tubercolosis, a respiratory infection that causes the death of about 2 million living beings per year (35).

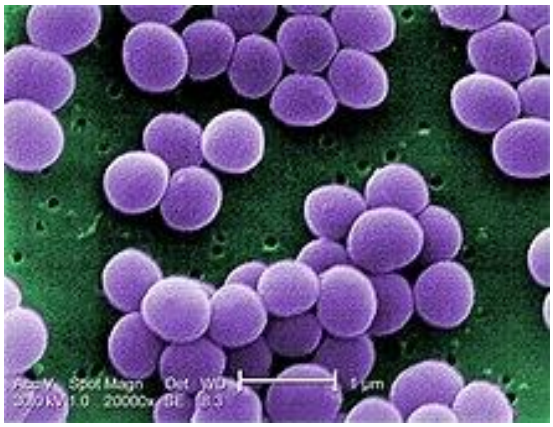


Figura5- Scanning electron micrograph of S.Aureus (37)

3.1 Bacterial structure

Bacteria are composed of plasma membrane and cell wall. The bacterial cell wall surrounds the cytoplasmatic membrane and is constituted of peptidoglycan. In prokaryotes the main role of the cell is to control the turgor pressure because concentration of proteins and other molecules inside the cell is higher than the external part. Peptidoglycan is a polymer made up of carbohydrates and amino acids, which gives rigidity and determines the characteristic shape of different bacteria. The plasma membrane, also known as cytoplasmic membrane is constituted of a lipid bilayer and it is a selective permeability barrier for most substances that pass into and out of the cell. It does not allow passage of charged molecules but there are some channels (porins) in the external membrane that permit for passive transport of many sugars, amino acids and ions through the external membrane (38). Although the bacterial interacts with host tissues. The cell molecular architecture is important in the pathogenesis of bacterial infection (39).

3.2 Classification of Bacteria

Bacteria can be divided in two categories, Gram positive and Gram negative, depending on their cell wall composition and how they react to the Gram stain test. Particles of around 2 nm can cross the peptidoglycan of both the types of bacteria. The inventor of the Gram-staining test is Hanks Christian Gram and it is based on the response of the bacterial cell walls to specific chemicals and dyes (40).

Gram-negative

Gram-negative bacteria have an internal cytoplasmic cell membrane, surrounded by a thin peptidoglycan cell wall and a bacterial external membrane composed of lipopolysaccharides

and phospholipids and there are some porins that allow the passage of substances. Their cell wall is not able to maintain the crystal violet stain utilized in the Gram staining method and therefore they show a pale reddish colour if they are checked with the microscope. Because of their external membrane that protect them from some antibiotics (also penicillin) many studies have been developed about Gram-negative bacteria such as Escherichia coli, or pathogenic bacteria such as Pseudomonas aeruginosa, Neisseria gonorrhoeae, Cblamydia trachomatis (41).

Gram-positive

Gram-positive bacteria are composed of a cytoplasmatic lipid membrane and a thick peptidoglycan layer without an external membrane. They show a purple color visualized under a light microscope because the thick peptidoglycan layer of the cell wall maintains the purple crystal violet stain. Due to the lack of an external membrane Gram-positive bacteria are more sensitive to antibiotics compared to Gram-negative ones (42).

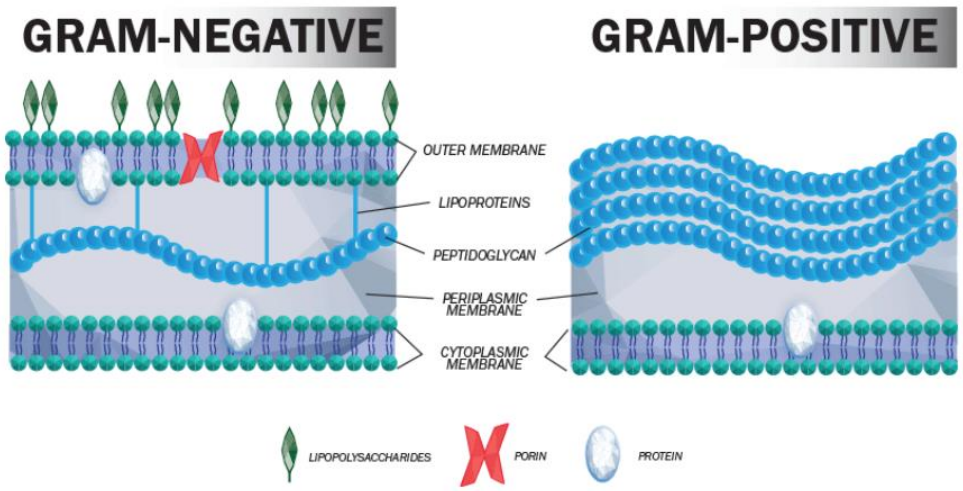


Figura6- Gram-negative and gram-positive bacteria (43)

3.3 Growth of bacterial populations

During the reproduction one bacterial cell divides into two cells via binary fission, daughter cells are genetically equally to the mother cell. To define the typical growth curve for a bacterial populations the microorganisms need to be placed in a flask, under controlled conditions, this technique is known as batch culture. Different growth phases can be observed within a growth curve:

- Lag phase: slow growth or absence of growth because bacteria adapt themselves to culture conditions, during this phase, the synthesis of RNAs, enzymes and other molecules can be observed. Bacteria are not dormant but are not ready to divide. In this phase the curve has not evidently changed because cells need time to reproduce in a new medium and this period can last for 1 hour to many days.
- Exponential or log phase: the cell numbers and the rate of population double; during this period a straight line can be observed and its slope represents the growth rate of the organism. This phase lasts for a limited time because the medium is soon exhausted of nutrients and full of wastes.
- Stationary phase: growth rate and death rate are identical therefore the cell numbers does not increase due to a lack of nutrients or/and the presence of toxic constituents such as an organic acid. An horizontal linear trend can be observed.
- Death phase: bacteria die due to higher death rate compared to growth rate (44).

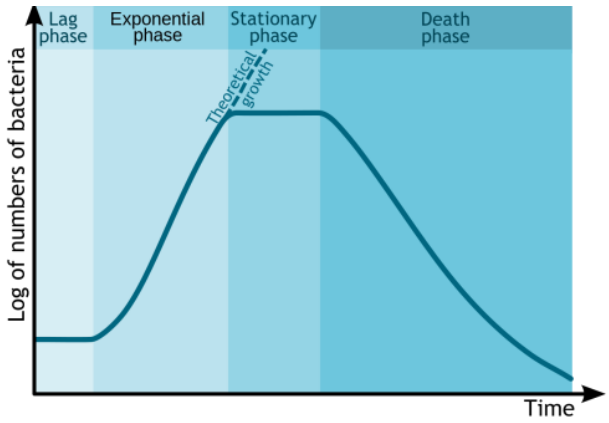


Figura7-Bacterial growth curve (40)

3.4 Antimicrobial resistance

Antimicrobial resistance (AMR) is one of the main public health problems of 21st century. Traditional medicine used to treat infections due to bacteria, parasites, virus and fungi seem to not be efficient against these microorganisms because they have become resistant to antibiotics (45). AMR is the capacity of a microbe to be resistant to medication that in the past could treat the microbe. These microbes are called multidrug resistant and they are complicated to treat and require high doses of antimicrobial drugs and therefore development of new medicines is urgently required (46).

In the recent years, The World Health Organization (WHO) has recognized the need to develop new strategies such as new drugs and new vaccines to contrast AMR (45). All

microbes can develop resistance such as antifungal resistance due to fungi, antiviral resistance due to virus, antiprotozoal resistance due to protozoa and antibiotic resistance due to bacteria.

A strong spread of antibiotics has caused more bacteria resistant, especially in developing countries. They are used in human being, animals and agriculture. In the United States, 2.8 million people are infected with bacteria resistant to antibiotics and around 35,000 million deaths result per year. In order to minimize the spread of resistant infections, it is important to adopt some preventive measures, antibiotics and antimicrobials should be used only when it is absolutely necessary(38).

4. Endodontics

Endodontics is the branch of dentistry concerned with the study and treatment of the dental pulp. “Endo” is the Greek word for “inside” and “odont” is the Greek word for “tooth”. Endodontic involves the treatment of diseases and injuries of the soft pulp tissue inside the tooth (containing nerves, arterioles, venules, lymphatic tissue, and fibrous tissue) (47).

4.1 Dental structure

The human teeth cut and crush the food preparing for swallowing and digesting. Human beings have different types of teeth: the incisors to cut the food, the canines to tear the food and the molar and premolars to crush the food. The roots of teeth are incorporated in the maxilla or the mandible and lie under the gums. Teeth are composed of various tissues with different density and hardness (48).

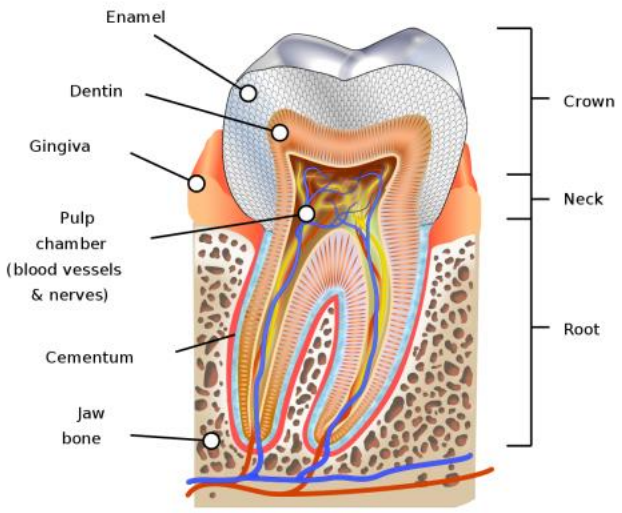


Figura8-Dental structure (48)

A tooth is composed of four tissues:

- enamel: the white and hard tissue constituted of mineralized substances that protect the tooth. It covers the surface of the dental crown, it can be damaged by drink and food (49);
- dentin: the yellowish tissue constituted of mineral hydroxyapatite, organic material and water represents the bulk of all teeth. It supports the enamel but it is softer, sensitive and is produced throughout life compared to the enamel (50);

- cementum: the calcified substance that covers the surface of the tooth root and it links the alveolar bone (the jaw who supports the tooth) to the tooth by the periodontal ligament (fibrous tissue that links the tooth root to the alveolar bone) (51);
- dental pulp: the central part cavity constituted of living connective tissue and odontoblasts (52).

4.2 Common diseases in dentistry

Caries: the most diffused diseases in the world, it is an infectious dental disease which destroys the structure of tooth. Caries are caused by acids produced by bacteria when fermentable carbohydrates such as fructose, glucose and sucrose react with bacteria located in the dental biofilm (plaque) on the tooth surface (48);

Gum disease: It is an infection of the gums surrounding the teeth due to bacteria. The two stages of gum disease are gingivitis when gums are red, inflamed and can be followed by a severe stage, known as periodontitis, teeth can fall down. In some cases antibiotics or dental surgery are necessary (53).

5. Mesenchymal stem cells (MSCs)

Stem cells are cells that can differentiate into other types of cells and have the ability to self-renew producing the same type of stem cells. They are used for the treatment of many diseases such as the mesenchymal stem cells (MSCs). MSCs can differentiate into tissues such as bone, fat, muscle and cartilage and they can be take from bone marrow, blood, placenta, skeletal muscle, umbilical cord. New studies have shown that MSCs can be used to treat infections due to their ability to produce antimicrobial peptides (54). MSCs are multipotent adult stem cells (55). MSCs can be isolated from dental tissues like dental pulp, apical papilla, periodontal ligament and gingiva (56).

5.1 Stem cells from the apical papilla (SCAPs)

Stem cells from the apical papilla (SCAPs) are MSCs situated in the apical papilla of permanent tooth. The apical papilla tissue is only situated into the root of an immature permanent tooth before the tooth emerges into the mouth or better on the tips of growing tooth roots. They create adherent clonogenic cluster and have the capacity to differentiate into adipocyes and osteoblasts/odontoblasts in vitro (57). SCAPs as well as MSCs have the following characteristics: self-renewal, proliferation, migration, differentiation and immunosuppression, they can be used in immunotherapy and in regeneration of dental tissues, neural, bone and vascular tissues.

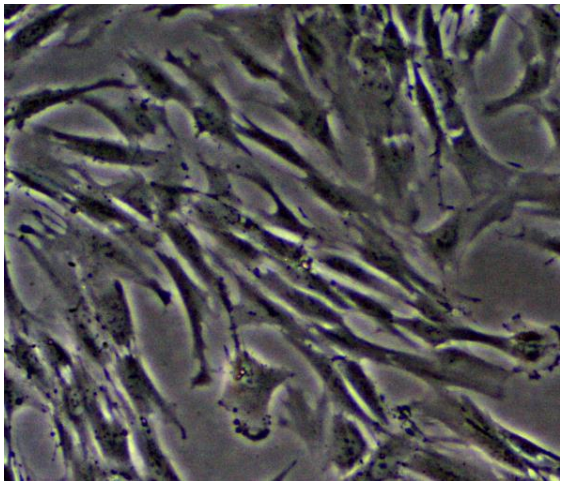


Figura9- SCAP culture at day 3 after initial seeding

Papilla contains a lower quantity of cellular and vascular elements than the pulp and more MSCs than mature dental pulp tissue. SCAPs can be isolated following two approaches:

- the enzyme digestion: the apical papilla tissue is detached from the tip of the root, crashed into fragments and digested in a collagenase type I and replaced with slight stirring. After that, the lumps of tissue are raised and pass by a cell strainer to get single cell suspension of SCAPs, which is at the end seeded in culture dishes;
- the explant culture: from the apical papilla tissue samples around 1 mm^3 in size are obtained and plated on culture dishes.

SCAPs extracted during the developing of the tooth so before that apical papilla tissue matures into dental pulp they can be stored by the cryopreservation and be used for future applications, as witnessed by Ding et al (58).

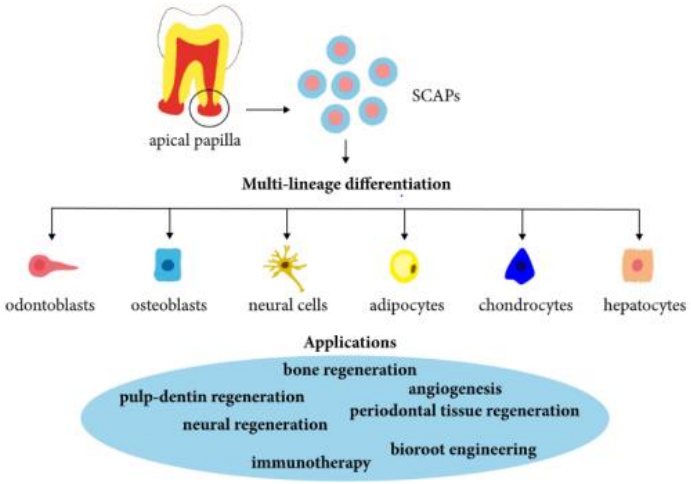


Figura10-SCAPs (53)

5.2 LL37 peptide in dental application

Periodontitis and rheumatoid arthritis are diffused bone diseases due to inflammation and characterized by loss of bone matrix components, causing resorption and bone loss. It is still a clinical challenge to understand how to repair and regenerate bone damaged by an inflammation. Many studies have shown that the degradation of collagen is caused by activation of the proteolytic enzymes such as matrix metalloproteinase (MMP) in inflammatory disease, causing a pathologic disruption of the extracellular matrix. The innate immune response is activated by inflammation in order to build a physical and functional barrier against microorganisms. Neutrophilis are effector cells of the innate immune system, which show antimicrobial effector mechanisms at the site of infection. LL37 is an effector molecule of the innate immune system, it shows a bactericidal activity and acts as an immunomodulator under physiological conditions. After the formation of a wound, LL37

increases and shows many activities such as cell proliferation, vascularization, cytokine production. Migration of stem cells to the damaged site and stimulation of vascularization are important for bone regeneration. Studies have shown that LL37, mainly joined to bone morphogenetic protein 2 (BMP2), increased MSC proliferation, migration, and osteogenic differentiation and stopped osteoclast production and bacterial infection in vitro. This can be seen as a new therapeutic strategy to use MSC to stimulate bone regeneration in infected areas (59).

6. Materials and methods

The following chemicals have been purchased from Sigma-Aldrich: tetrachloroauris acid (HAuCl4·3H2O), sodium citrate (Na3C6H5O7) HEPES, NaOH, Poly(ethylene glycol), they were used as received. Lyophilized LL37 peptide with a purity of 96% modified witha C-terminal cysteine (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPR TESC) was received from Caslo Laboratory, Denmark. The water used for the experiments was milli-Q.Human serum was purchased from Invitrogen, components for bacterial media were purchased from Frilabo, Portugal. Bacteria were purchased from DSMZ, German Collection of Microorganisms and Cell Culture.

6.1 Synthesis of AuNPs

6.1.1 LL37- AuNPs preparation

The first step was to dissolve LL37 (0.5 mM) in DMF (100 μL) and then 900 uL of HEPES at 100 mM and pH 7,5 was added. The pH was modified using NaOH. $HAuCl_4 \cdot 3H_2O$ (10^{-2} M, 50 μL) was put in 950 mL of a peptide solution at 0.25 mM; obtaining the final concentration of $HAuCl_4$0.5 mM) and the NP synthesis was performed at 25 °C in the water-bath for about 7 days, until the solution became wine red color. The synthesized LL37-Au NPs were centrifuged two times at 11,000 rpm for 10 min at 4°C and at 14,000 rpm for 10 min at 4°C followed by washing with Milli-Q water to eliminate residues of peptides and HEPES, frozen and freeze-dried at 223 K with a Snijders Scientific freeze-dryer (28).

6.1.2 AuNPs preparation

AuNPs were made by citrate reduction of $HAuCl_4.3H_2O$. An aqueous $HAuCl_4$ solution (0.5 mM, 100 mL of water) was brought to a boil in a 250 ml round bottom flask while stirring and then an aqueous sodium citrate solution (2%, w/v, in water) was added quickly. The citrate ions are the reducing, stabilizing and capping agent, they reduce the Au^{3+} ions to neutral Au atoms. The color changes from pale yellow to wine red, suggesting the presence of gold nanoparticles. The solution was cooled to room temperature(57). The AuNPsare usually functionalized with PEG in order to improve the stability both in vitro and in vivo conditions. PEG was added to the AuNPs solutions at room temperature. 1 mg of PEG was added in 40 mL of Au NPs, the solution was put on the orbital shaker at room temperature for 2 h in order to totally exchange citrate molecules with PEG (60). The synthesized AuNPs were

centrifuged at 12,000 rpm for 15 min at 4 °C followed by one washings with Milli-Q water. The pellet generated after centrifugation was redispersed in Milli-Q water and then freeze-dried. (61).

6.2 Antimicrobial activity testing

All culturing and experimental work was carried out under sterile conditions, either on the bench with a Bunsen burner or in a laminar flow hood. Many types of bacterial growth media are used to culture bacteria in the laboratory. A growth medium can be solid, liquid or semi-solid used to allow the growth of microorganisms (62).
Common medium used:

- TSY Agar (Tryptone Soy Yeast Extract) supports the growth of different microorganisms. Enzymatic digest of casein, yeast extract and papaic digest of soyabean meal give essential elements for growth: nitrogen, vitamins, minerals and amino acids. For example, glucose gives carbon and energy, dipotassium hydrogen phosphate is a buffer system and bacteriological agar is used to solidify (63). It was obtained adding at the Trypticase soy broth (TSB) yeast extract and agar. TSB is a culture broth to grow aerobic bacteria and it is used for bacteria that require high nutritional elements (64).
- Brain heart infusion (BHI) is a nutrient-rich medium used to grow different types of microorganisms, it is composed of an infusion from boiled bovine or porcine heart and brain (65).

6.2.1 Agar plate preparation

Staphylococcus aureus (ATCC 6538), *Escherichia coli* (ATCC 25922), Escherichia *Coli ML 35p*,*Staphylococcus epidermidis* (ATCC 19433) were grown at 37° and maintained on TSY agar plates or in TSB medium. *Enterococcus Faecalis* was grown on Brain Heart Infusion *(BHI)* Agar plate or BHI broth medium.

Trypticase Soy Yeast Extract Agar Medium is composed of:

- 30 g of Trypticase Soy Broth
    - 17 grams (0.60 oz) of Tryptone.
    - 3 grams (0.11 oz) of Soypepton.
    - 5 grams (0.18 oz) of NaCl.

    - 2.5 grams (0.088 oz) of dipotassium phosphate (K_2HPO_4).
    - 2.5 grams (0.088 oz) of glucose.
- 3.0 g of Yeast Extract.
- 15.0 g of Agar.
- 1000 ml of DI Water.

All of these previous components were mixed and the medium was sterilized into autoclave at 121ºC. After that the medium was cooled to 50°C and then poured into plates and left it to solidify at room temperature (64).

6.2.2 Growing Bacteria

S. *aureus*, E. *coli*, S. *epidermidis*, and E. *faecalis* were grown at 37°C and maintained on agar plates. After that, few isolated colonies from the plates were placed into 4 ml of broth medium and grown for overnight at 37°C at 150 rpm into the shaking incubator. After about 16 hours, during the lag phase, cell counts were done at OD 600 nm using the plate reader (Bioteck).

6.2.3 Serial Dilutions and Plating in microbiology

A serial dilution consists of successive dilutions utilized to decrease a dense culture of cells to a more operable concentration. Each time the concentration of bacteria is reduced by an exact amount. Through the plate technique bacteria in a solution were quantified, plating a liquid sample containing bacteria so that the bacteria can be easily counted and isolated. Through a sterilized spreader with a smooth surface a specific amount of bacteria is widespread over a plate. The plate is left to dry at room temperature under the hood and the agar adsorbs the bacteria readily (66).

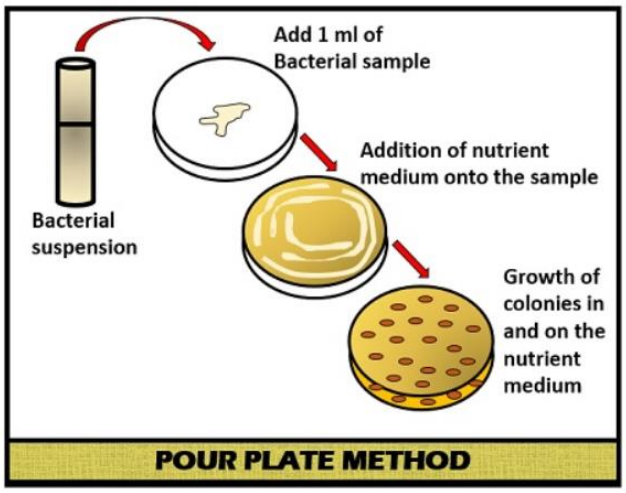


Figura11- Spread Plate Technique **(67)**

6.2.4 Testing the effect of LL37-AuNPs on bacteria

After the cell count bacteria were diluted to 10^5 CFU (colony formation unit)/mL in human serum (10%, v/v) and specific amount of LL37-AuNPs (60 µg/mL) and AuNPs (60 µg/mL) were added into bacterial suspensions and incubated for 24 h at 37° and 150 rpm. At 0, 5 , 24 h bacterial suspensions were diluted to 100 times in PBS buffer and aliquots (50µL) were removed from the respective suspensions and widespread over the surface of agar plates using a sterile glass spreader and incubated for 24h at 37°. The number of colonies growing on plates were estimated after 24 h for each time considered. All antibacterial activity tests were performed in triplicate and were performed at different times to certify the reproducibility of the data.

6.2.5 Testing the Effect of green laser on bacteria

In order to improve the antimicrobial activity of the LL37-AuNPs,a green laser is used (CNI Model with PSU-H-LED). All solid state 532nm green laser is commonly used for medical treatment, scientific experiment, analysis of optical properties, etc. Localized surface plasmon resonance (LSPR) is an important optical property of AuNPs. Laser excitation of the LSPR of gold nanoparticles (AuNPs) can cause photon–electron and electron–electron interactions and then produce heat. The thermal energy generated by AuNPs after exposure of laser light helps to damage bacterial membranes and allows the internalization and bactericidal action of LL37-AuNPs. It is difficult to understand the optimal conditions by comparing previous studies (68), parameters for this experiment were chosen remaining in a safety region for eventual studies in vivo.

To determine the laser-activated photothermal effects of AuNPs and LL37-AuNPs on bacteria behaviour, different experimental groups were exposed to the green laser: bacteria, bacteria treated with AuNPs, bacteria treated with LL37-AuNps, they were exposed to laser (500 mW/cm^2) at 0 h .The laser wavelength was 532 nm, and the irradiation lasted for 3 minutes at 500 mW/cm^2 stopping the irradiation each minute for some seconds in order to stabilize the solution. After laser irradiation, bacteria suspensions were incubated for 24 h at 37° and 150 rpm. At 0, 5 , 24 h bacterial suspensions were diluted to 10 or 100 time in PBS buffer and aliquots (50µL) were removed from the respective suspensions and widespread over the surface of TSY or BHI agar using a sterile glass spreader and incubated for 24h at 37°C. The number of colonies growing on plates were estimated.

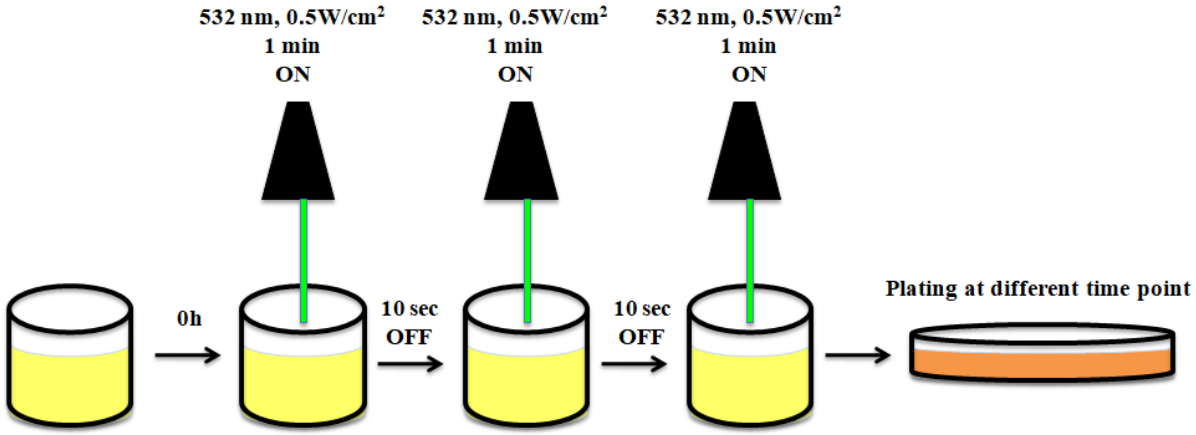


Figura12-Green laser irradiation

6.2.6 Permeabilization test on *E. coli* ML35p

To study inner membrane (IM) and outer membrane (OM) permeability of bacteria by external agents it has been engineered a specific E.coli ML 35p strain.

Permeabilization of OM and IM was monitored by the chromogenic reporter molecules nitrocefin and ortho-Nitrophenyl-ß-galactoside (ONPG) respectively, on E. coli ML 35p in a single assay. Nitrocefin is not able to pass through the OM but the permeabilization of OM allow the passage of nitrocefin where it cleaved by β-lactamase to generate color change that can be observed at 486 nm using UV-vis spectrophotometer. ONPG cannot pass through IM because of lack of lactose permease in this bacteria but the permeabilization of IM causes passage of ONPG where it cleaved by cytoplasmic β-galactosidase to o-nitrophenol that can be determined at 420 nm. Typically, E. coli ML-35p bacteria were grown in TSB media from a single colony, overnight at 37 ºC and 150 rpm . Bacteria was washed 3 times with PBS (pH

7.2). LL37-AuNPs and Au-NPs were incubated with 10^6 CFU/mL of bacteria with 30 μM nitrocefin and 2.5 mM ONPG. At specific time amount of bacterial solutions were removed and absorbance was measured at two different wavelengths, 486 and 420 nm to identify nitrocefin and ONPG, respectively, using a BioTek synergy MX microplate reader for 24h (69).

6.3 Cell culture

The human MSCs cultures used were derived from the apical papilla (SCAP) of normal and premature tooth of 1 donor. Donor was healthy without any known disease.The molar was surgically extracted respecting orthodontic considerations and a specific protocol. SCAPs were cultivated into two different medium: complete growth medium (non differentiation) and odontogenic medium (differentiation).

The vial with the cells was stored in liquid nitrogen. Under a laminar flow hood, the complete growth medium was prepared, it contains KO-DMEM , 20% FBS(it contains proteins, lipids, and growth factors that support cell growth), 1% Pen-Strep(it acts as an antibiotic and prevente bacterial contamination), 1% 2-Mercaptoethanol (it serves as an antioxidant and protect cells and proteins from oxidative harm). Basal media (KO-DMEM) provides the basic nutrients that cells need, such as amino acids, ions, and a pH buffer. After the complete thawing of FBS, it was heated for 30 min at 56°C to inactivate complement (unwanted blood clotting) proteins in the water bath. The vial containing the frozen cells was washed with medium to thaw the cells. The cells were transferred into a 15 ml centrifuge tube (PAA) and centrifuged for 3 min at 300 g. The supernatant was aspirated, the pellet resuspended and then transferred into a T-75 cell culture flask (PAA); it was supplemented with 20 ml of complete growth medium and incubated at 37°C and 5%CO_2. The cells were further maintained until they were approximately 80% confluent and ready for the experiments.

The cells were rinsed with PBS to remove the remaining serum of medium, PBS was aspirated and Trypsin/EDTA was added in order to detach the cells. The cell were trypsinated for 3 min at 37°C. Fully trypsinized cells should appear rounded up and no longer attached to the bottom surface of the flask. The cells were centrifuged for 3 min at 300 g and room temperature, the supernatant was aspirated and the cells were resuspend in the fresh medium. Cell counting was done with an hemocytometer. The cells used for further assays were frozen

with 10% Dimethyl sulfoxide/ 90% FBS, the remaining cells were used for different experiments.

The cells in this study were cultivated in single use sterile polystyrene flasks with ventilated caps with surface area of 75 cm^2, 6 well plates, 24 well plates and 96 well plates.

6.3.1 Cell culture assays

6.3.1.1 Mycoplasma detection in the cell culture

Generally, all stuff like cell cultures or cell banks that enter for the first time in a laboratory need to be tested for the presence of Mycoplasma. Mycoplasma is a prokaryotic organism that can contaminate cell cultures and modify cell physiology. Mycoplasmas are very small and they can cross filters used to contrast bacterial and fungal contamination and therefore can spread to all the cultures in a laboratory. (70)

Mycoalert assay procedure

1. Take 1ml from the culture flask
2. Centrifuge at 200xg for 5 min
3. Remove 50 uL of cleared supernatant and put it in 96 six well plate
4. Add 50ul of MycoAlert Reagent and remove the balls with a needle
5. Measure luminescence (read A)
6. Add 50 uL MycoAlert Substrate
7. Measure luminescence (Read B)

Following parameter was considered:

$$R = Read\ A\ /Read\ B$$

$$R < 0{,}9 \text{ negative}$$

$$0{,}9 < R < 1{,}2 \text{ Borderline and repeat after 24 h}$$

$$R > 1{,}2 \text{ positive}$$

This procedure is indicated in the Uc-Biotech (Canthanede,Portugal) and the SCAPs were used at Passage 9.

6.3.1.2 Cytotoxicity assay

To determine potential cytotoxic effects of the LL37-AuNPs and peptide LL37, the CellTiter-Glo Luminescent Cell Viability Assay was conducted. It is utilized to check the number of viable cells in culture based on quantification of the ATP present, which shows the presence of metabolically active cells.

Cells were seeded at 2x10⁴ cells/well at passage 5 in 24-well plates using KO-DMEM, 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep and incubated at 37°C in 5% CO_2. After 24 h, cells were incubated with LL37 (8 , 20 μg/mL), LL37-Au NPs and AuNPs (both 30, 60 μg/mL) for 24 h at 37°C in 5% CO_2. 1 mg of LL37 was weighed in an eppendorf and under the hood in the cell culture room was added 1 ml of PBS buffer(LL37 can precipitate in the DMEM medium). Gold nanoparticles were sterilized under the UV light for 30 min before to be used for the cell culture. After 24 h, cells were washed with PBS to remove non-internalized NPs, at this time the cytotoxicity assay was made. The experiment was performed with3 replicates.

Luminescent CellTiter-Glo® Assay

100 μL of reconstituted CellTiter-Glo Cell Viability reagent (Promega cat # G7572) and 100 μL of cell culture medium were added in 96-well plates. The plate was incubated in the dark at room temperature and shaken on an orbital shaker for 10 minutes until the cell lysis. Spectrophotometer-Microplate reader Synergy H1 was used to record the luminescence (71).

ATP Standard Curve

It's necessary to generate an ATP Standard Curve with the cells used. Cells were seeded at $5x10^2, 1x10^3, 2x10^3, 4x10^3, 1x10^4, 2x10^4, 5x10^4$ cells/well in 24-well plates using KO-DMEM , 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep and incubated at 37°C in 5% CO_2. The experiment was performed into 3 replicates. After 24 h, the CellTiter-Glo Luminescent Cell Viability Assay was conducted.

6.3.1.3 Cell proliferation Assays

To investigate the effects of LL37, AuNPs and LL37-AuNPs on the cellular proliferation of SCAPs, an In Cell Analyzer was used. Cells were treated with different concentrations of LL37, AuNPs and LL37-AuNPs at different time points.The cell viability assay performed involves the simultaneous application of two fluorescent dyes directly to cells in culture

medium, subsequent live-cell image acquisition, and analysis on the IN Cell Analyzer. Hoechst 33342 is used as a blue fluorescent marker of the nuclei of all cells and propidium iodide is used as a red fluorescent indicator of cells with compromised membranes.

Cells in log-phase growth were seeded into 24-well plate (8000 cells/well) and incubated in culture medium using KO-DMEM, 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep for 24 h at 37 °C, 5% CO2. Culture medium was removed from the cells, washed with PBS and replaced for 4 h with new culture medium (500 µl) containing KO-DMEM and 1% pen/strep and with LL37 (5,10,20,40 µg/mL), AuNPs (30, 60 µg/mL) and LL37-AuNPs (30,60 µg/mL). The experiment was performed with4 replicates. For this treatment FBS was not used.

Culture medium containing KO-DMEM and 1% pen/strep was removed and the cells were washed with PBS. A solution containing Hoechst 33342 and propidium iodide, was prepared in culture medium using KO-DMEM , 20% FBS, 1% Pen-Strep, 1% 2 Mercaptoethanol and 1% Pen-Strep and added directly to the contents of each well to provide final concentrations of 25 µg/mL Hoechst 33342 and 25 µg/mL propidium iodide. Cells were incubated for 24 and 48 h at 37 ºC, 5% CO2 .Three time points were considered, 24,48 and 72 h after the cell seeding images were acquired on the IN Cell Analyzer.

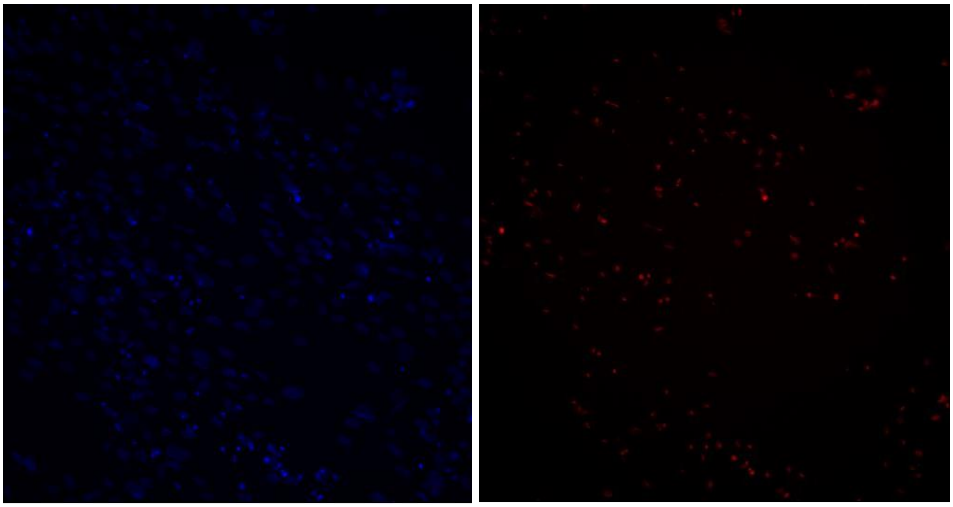


Figura13-Fluorescence images of SCAPs obtained from IN Cell Analyzer 48 h after the treatment.

6.3.2 Odontogenic differentiation in vitro

SCAPs were induced with odontogenic medium (KO-DMEM culture medium supplemented with 10 mM beta-glycerophosphate [Sigma], 50 mg/mL ascorbic acid [Sigma] and 10^{-8} M dexamethasone[Sigma] for 7 days (for ALP activity assay) or 18 days (for alizarin red staining) (59).

SCAPs were prepared as previously described. Cells were seeded at $1x10^4$ cells/well at passage 5 in 2 different 24-well plates using KO-DMEM , 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep and incubated at 37°C in 5% CO_2. After 24 h, cells were incubated with LL37 (8 , 20 µg/mL), LL37-Au NPs and AuNPs (both 30, 60 µg/mL) for 24 h at 37°C in 5% CO_2 , after cells began to adhere they were cultured both in complete medium containing KO-DMEM , 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep and in odontogenic medium. Odontogenic medium was added to induce differentiation. The experiment was performed with3 replicates.

In the first 24-well plates, complete medium and odontogenic medium (both 800 µl) were changed every three days. In the other one complete medium and odontogenic medium supplemented with LL37 (8 , 20 µg/mL), LL37-Au NPs and AuNPs (both 30, 60 µg/mL) were changed every three days.

With the aim to understand better the influence of LL37, AuNPs and LL37 AuNPs on the cells was made the following experiment. SCAPs were prepared as previously described. Cells were seeded at $1x10^4$ cells/well at passage 5 in 24-well plates using KO-DMEM , 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep and incubated at 37°C in 5% CO_2.After 24 h, cells were incubated with LL37 (8, 20 µg/mL), LL37-Au NPs and AuNPs (both 30, 60 µg/mL) for 4 h at 37°C in 5% CO_2 in KO-DMEM and 1% Pen-Strep. After 4 h cells were washed with PBS and incubated with both complete and odontogenic medium.The experiment was performed into 3 replicates. Complete medium and odontogenic medium (both 800 uL) were changed every three days.

6.3.2.1 Alkaline phosphatase (ALP) bioactivity assay

Phosphate disodium salt (1-step PNPP) is used as substrate for the detection of alkaline phosphatise in ELISA-based applications and the reaction generates a water-soluble yellow product, the colour adsorbs at 405 nm.

SCAPs in 24-well plates were washed twice with PBS and incubated with 200 µL of P-nitrophenyl phosphate disodium salt (1-step PNPP). 100 µL was added to stop the reaction and the absorbance at 405 nm was measured with a spectrophotometer (72).

6.3.2.2 Mineralization assays

Mineralization assays of SCAPs cultured in 24-well plates. For Alizarin red S staining, the cultures were washed three times with PBS and fixed in 10% formalin and incubated with 1% Alizarin red S (Sigma-Aldrich) (pH 4.1) for 20 min at room temperature on orbital shaker. Cells were then washed three times with Milli-Q to reduce non-specific staining. Plates were photographed using a microscope and scanner.

6.3.3 Flow cytometry analysis of EGF receptors

SCAPs were prepared as previously described. Cells were seeded at $4x10^5$ cells/well at passage 5 in 6-well plates using in each well 3 ml of KO-DMEM , 20% FBS, 1% Pen-Strep, 1% 2-Mercaptoethanol and 1% Pen-Strep and incubated at 37°C in 5% CO_2. After 24 h, cells were incubated with LL37 (8 µg/mL), LL37-Au NPs and AuNPs (both 30 µg/mL) for 4 h at 37°C in 5% CO_2 with KO-DMEM and 1% Pen-Strep. Cells without any treatment were used

as control. The experiment was performed in 2 replicates. After 4 h, cells were washed with PBS and complete medium was added and was adopted the following protocol taken from Abcam (73).

PROTOCOL FOR THE FLOW CYTOMETRY ANALYSIS OF EGF RECEPTORS:

1. Aspirate media using pipette.
2. Wash gently with 1 ml of PBS.
3. Add 1 ml of Trypsin-EDTA and mix gently.
4. Put back in incubator at 37° for 3 min.
5. Add 3 ml of maintaining medium, mix gently.
6. Collect cells cells ad pipet into falcon tube.
7. Centrifuge cells for 3 min at 300xg and room temperature.
8. Aspirate media above the cell pellet using glass Pasteure.
9. Add 200 ul of PBS and put into Eppendorfs.
10. Centrifuge cells for 5 min at 300xg and room temperature.
11. Remove PBS.
12. Add 200 ul of 4%PFA (parafolmaldehyde) and incubate at room temperature for 10 min.
13. Add 500 ul of PBS.
14. Centrifuge for 5 min at 300xg and room temperature.
15. Remove pbs and PFA.
16. Add 400 ul of PBS and centrifuge for 5 min at 300xg and room temperature.
17. Remove PBS and add 200 ul of PBS and store at 4° overnight.
18. Centrifuge for 5 min at 300xg and room temperature.
19. Remove PBS.
20. Add 90%Metanol and incubate for 30 min at room temperature.
21. Add 500 ul of PBS.
22. Centrifuge for 5 min at 300xg and room temperature.
23. Remove PBS, add 400 ul of PBS.
24. Centrifuge for 5 min at 300xg and room temperature.
25. Remove PBS.
26. Add 200 ul of 1%BSA (Bovine Serum Albumin) and incubate for 1 h at room temperature (cover with aluminum sheet) to block non specific protein-protein interactions.

27. Split each condition in 2 different eppendorf, putting 100ul in each new eppendorf.
28. Take the half of the total number of eppendorf and add the primary antibody ab52894 at 1:20 dilution, incubate for 30 min.
29. Incubate the second half of the total number of eppendorf without adding nothing and incubate for 30 min.
30. Add in all the eppendorf Goat anti rabbit IgG (Alexa Fluor 488) secondary antibody at 1:2000 dilution and incubate for 30 min.

BD ACCURRI C6 FLOW CYTOMETER was used to monitor the mean fluorescence intensity of each sample.

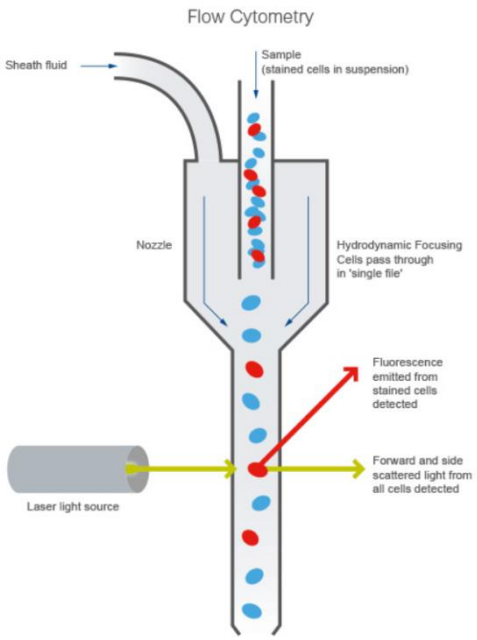


Figura 14-Flow Cytometry (73)

7. Results

7.1Synthesis and characterization of LL37-AuNPs

This part was not developed during execution of this project but the purpose to highlight the properties of LL37-AuNPs, which is used in the current project (74). LL37 peptide with a cysteine at C-terminus conjugated to AuNPs have been prepared by a one step procedure. Researchers have demonstrated that AuNPs with diameter below 10 nm show high toxicity against mammalian cells therefore nanoparticles with diameters 12±3 nm have been obtained. LL37-AuNPs show important physico-chemical properties such as: low size, stability in aqueous solution, low polydispersity and significant incorporation of LL37. HEPES buffer at pH 7.5 and 5 were selected and LL37-AuNPs at pH 7,5 and 5 were formed in 7 and 1 days respectively. Importantly LL37-AuNPs formed at pH 7.5 showed some aggregation while no significant aggregation was found at LL37-AuNPs (pH 5) over time.LL37-AuNPs had a SPR band centered at 530 nm and show some irregular shapes at pH 7.5 compared to spherical morphology at 5, monitored by transmission electron microscopy (TEM) (74). Fourier transformed infrared (FTIR) analyses showed that LL37-AuNPs had a random coil structure and Zeta potential analyses showed that NPs had a charge of -3.5±1.1 mV. The rate of reduction of Au ions depends on the aqueous conditions and it is slower for pH 7.5 that for pH 5.0 (74). LL37 immobilizes on AuNPs presents a compact structure, some cationic residues of peptide(LYS8, LYS10, LYS12, LYS15, ARG19 ,ARG34 and the terminal residue CYS38 were close the surface of the Au-NP while other cationic residues (LYS18, ARG23, LYS25 and ARG29) were not in contact. During the immobilization period the parts of LL37 that are distant from the surface of the nanoparticle show an alpha-helix structure that is not present at the end (16).

7.2 Antimicrobial activity of LL37-AuNPs

LL37-AuNPs have excellent antimicrobial activity in the presence of human serum and green laser

The antimicrobial activity of the LL37-Au NPs (60 µg/mL, which corresponds to an immobilized concentration of about 20 µg/mL of LL37) was evaluated against gram-

negative (*E. coli*) bacteria and gram-positive (*S. aureus, S. epidermidis, E. faecalis*) in 10% (v/v) Human Serum. At different time interval (0, 5 and 24h), aliquots were removed and plated on agar plates and bacteria colonies were counted after 24 h. Bacteria were used as a control. Au NPs (60µg/mL) had an insignificant antimicrobial activity. Au NPs exposed to green laser show a slight antimicrobial activity probably due to thermal effects. The LL37-AuNPs had good antimicrobial activity, killing:

- About 20% and 40 % of 10^5 CFU gram-negative *E.coli* in 5 h and 24 h respectively;
- About 20% and 40 % of 10^5 CFU gram-positive *S.epidermidis* in 5 h and 24 h respectively;
- About 20% and 50 % of 10^5 CFU gram-positive *S.aureus* in 5 h and 24 h respectively;
- About 20% and 20 % of 10^5 CFU gram-positive *E faecalis* in 5 h and 24 h respectively.

The thermal energy generated by AuNPs after exposure of laser light helps to damage bacterial membranes and allows the internalization and bactericidal action of LL37-AuNPs.The LL37-AuNPs exposed to green laser had excellent antimicrobial activity, killing:

- About 40% and 80% of 10^5 CFU gram-negative *E.coli* in 5 h and 24 h respectively;
- About 40% and 80 % of 10^5 CFU gram-positive *S.epidermidis* in 5 h and 24 h respectively;
- About 45% and 85% of 10^5 CFU gram-positive *S.aureus* in 5 h and 24 h respectively;
- About 30% and 70 % of 10^5 CFU gram-positive *E.faecalis* in 5 h and 24 h respectively.

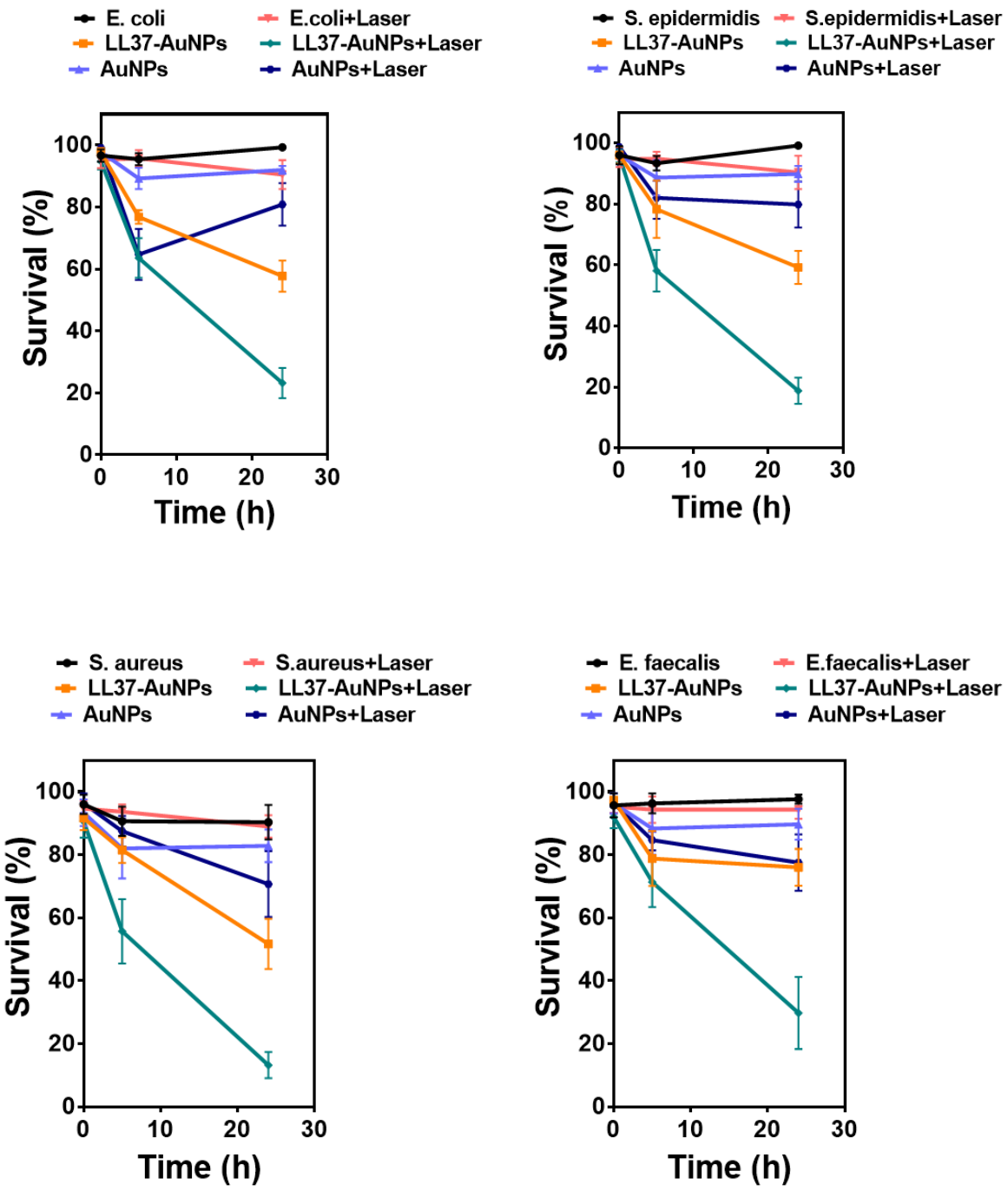


Figura15- Antimicrobial activity of LL37-AuNPs against E.coli, S.epidermididis, S.aureus, E.faecalis in 10% human serum.

LL37-AuNPs show an higher ability in inducing the permeabilization of bacterial cell membrane than AuNPs

In order to have more information about the destabilization of outer and inner bacterial membrane, *E. coli* strain ML-35p that was engineered especially to monitor the permeability of outer (OM) and inner (IM) membranes was used. No significant increase in OM and IM permeability of the bacteria was shown after incubation with AuNPs and AuNPs exposed to the green laser. In contrast, both LL37-AuNPs and LL37-AuNPs exposed to the green laser induced either OM or IM permeability. LL37-AuNPs exposed to the green laser were more efficient than LL37-AuNPs in inducing OM and IM permeability. These results showed that antimicrobial effect of the LL37-AuNPs is fast and characterized by the destabilization of the bacterial membrane, however no such signature was observed with bacteria alone bacteria exposed to laser.

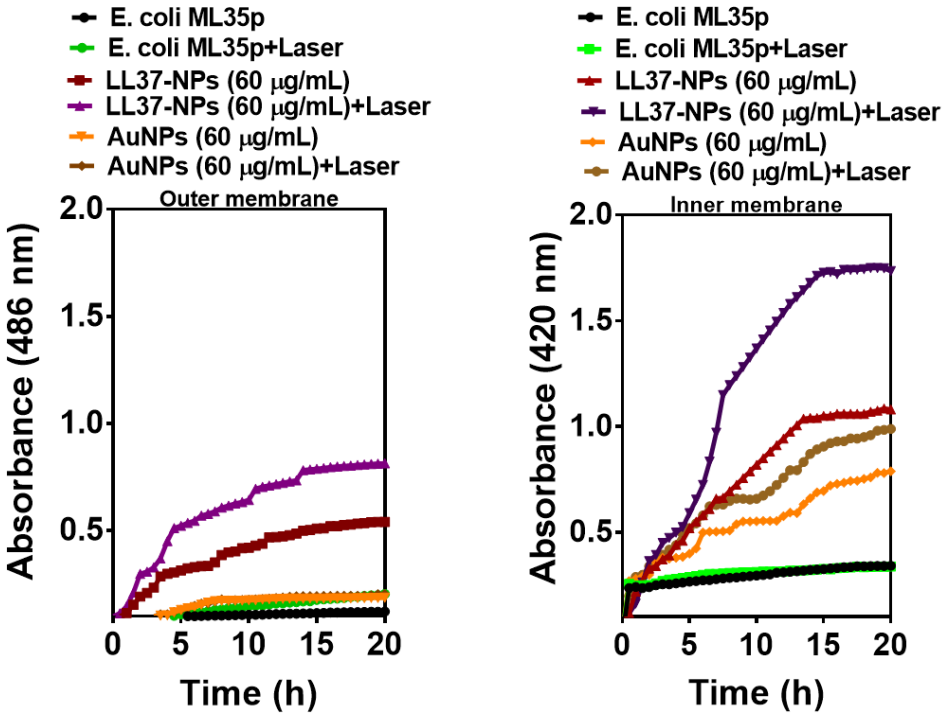


Figura16-Permeabilizing effect of LL37-AuNPs on E. coli ML35p outer and inner membranes.

7.3 Morphology and growth characteristics of SCAPs

On day 1 after initial seeding, isolated single cell could be seen in all cultures, along with some cell debris derived from the tissues' digestion process. After 2-3 days,

colonies of high density were observed in cultures and SCAPs cultures reached confluence by day 5-6. SCAPs, are small in size, fibroblast-like or stellate in shape, showing many cytoplasmatic projection or filopodia. They have high proliferation rate and the capacity to produce dense monolayers (75).

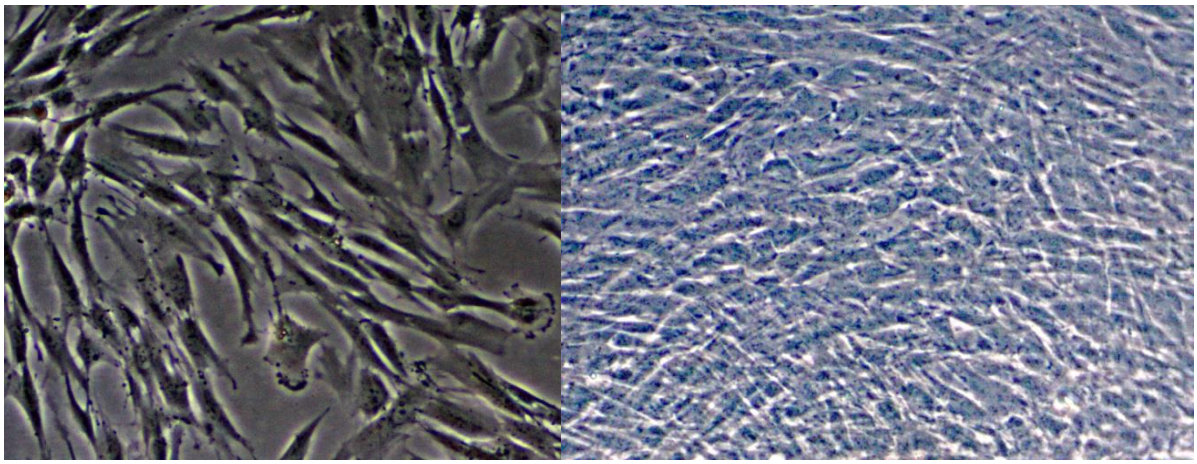


Figura17-Microscopy photographs of SCAP cell culture 3 days and 10 days after initial seeding

7.4 Cell viability

LL37-AuNPs and LL37 are Not Cytotoxic to SCAPs

It was found that 8 and 20 μg/mL of LL37 and 30 μg/mL of LL37-AuNPs were not cytotoxic and generally increased cell viability compared to the cells, used as a control. Concentration of 60 μg/mL of LL37-AuNPs was slightly cytotoxic. On the other hand, 30 and 60 μg/mL of AuNPs were not cytotoxic and showed unexpected high number of cells probably due to the influence of red color of the AuNPs with the luminescence during quantification of ATP.

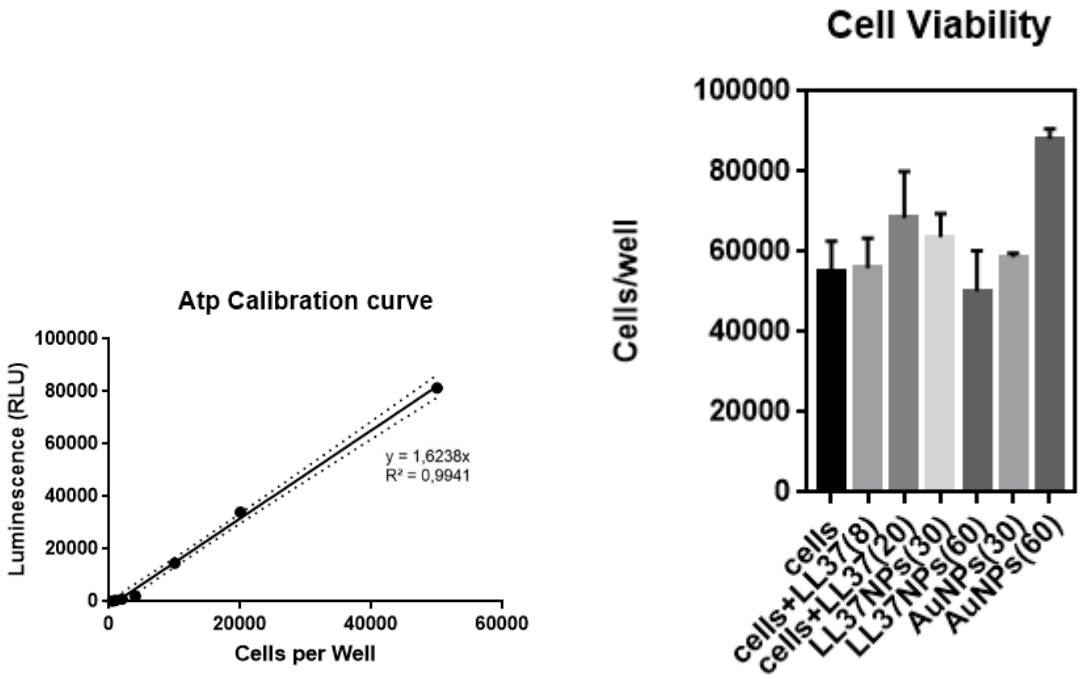


Figura18- Cell number correlates with luminescent output (on the left) and Effects of LL37,LL37-AuNPs and AuNPs on cell viability (on the right).

7.5 Effect of LL37-AuNPs on differentiation activity

LL37-AuNPs enhance the odontogenic differentiation potential of SCAPs in vitro

SCAPs were cultured under odontogenic-inducing conditions in the presence of 8 and 20 µg/mL of LL37, 30 and 60 µg/mL of LL37-AuNPs. After about 10 days in SCAP cultures, cell migration started generally from the walls towards the middle of the well. Generally SCAPs can create both rounded aggregates and elongated 3D-organized structures, these structures are the first to be mineralized, usually shown by the calcium-specific AR-S staining, also some single mineralized nodules can occur on the whole adherent monolayer (75).

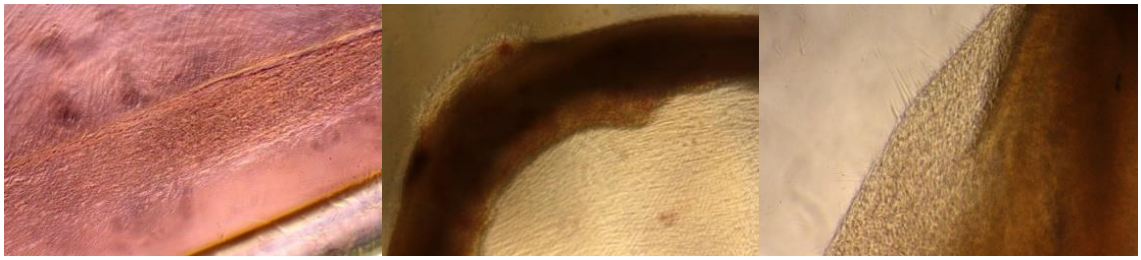


Figura19-Microscopy photographs of SCAP cell culture 10 days after induction of differentiation.

Three different test were performed:

1. In the first test the induction of odontogenic differentiation cells was made changing complete medium and odontogenic medium every three days.

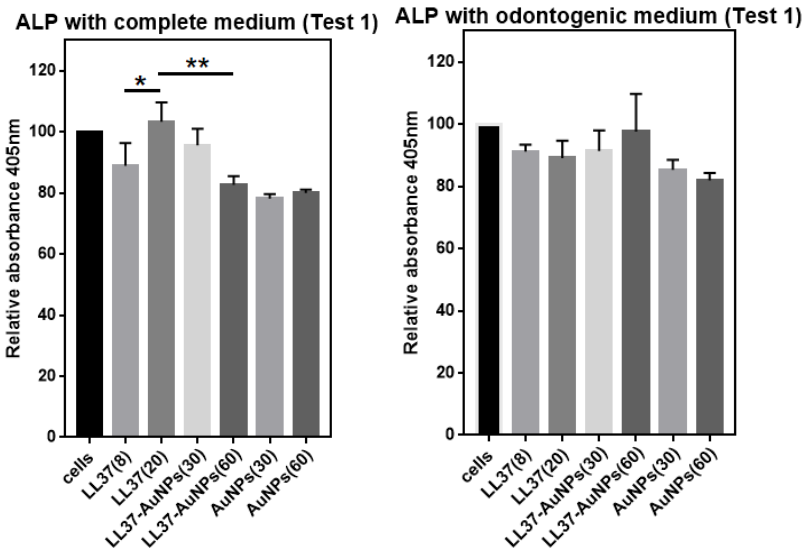


Figura20- Alkaline phosphatase staining Test.

In the first test, ALP activity was measured at day 7 but LL37 and LL37-AuNPs were not found to increase the ALP activity. The level of calcium mineral deposition was displayed by alizarin red staining with about 18-day odontogenic induction. In line with ALP activity results, pictures took after the alizarin red staining have not shown great intensity of red color.

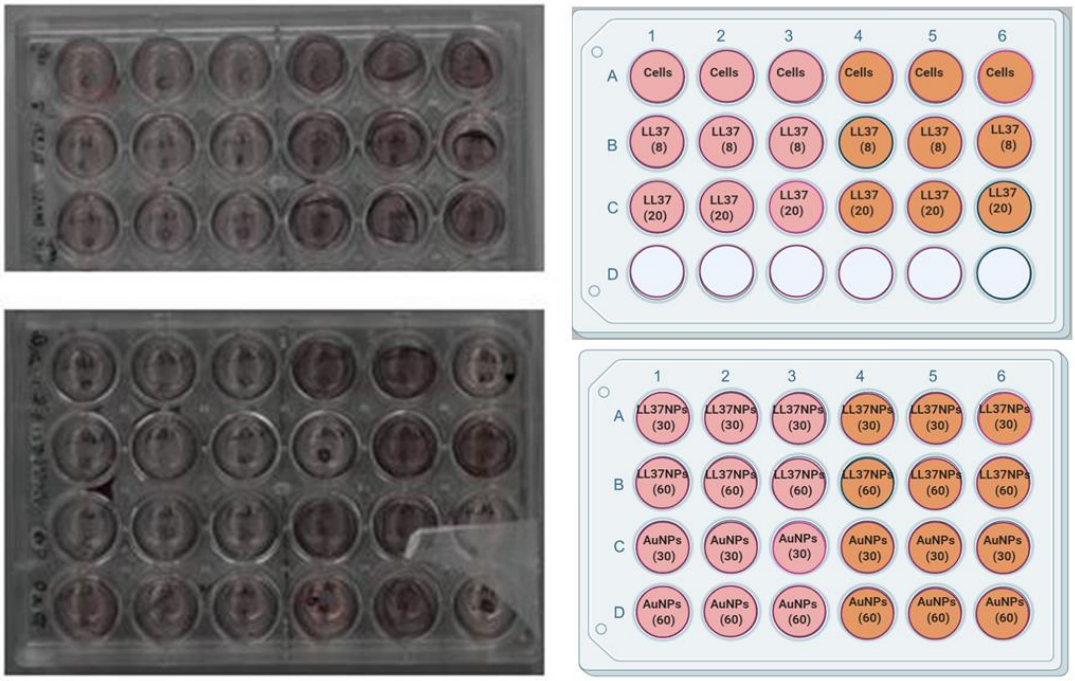


Figura21-Alizarin red staining Test 1.

2. In the second test the induction of odontogenic differentiation cells was made changing complete medium and odontogenic medium supplemented with LL37 (8 , 20 µg/mL), LL37-Au NPs and AuNPs (both 30, 60 µg/mL) every 3 days.

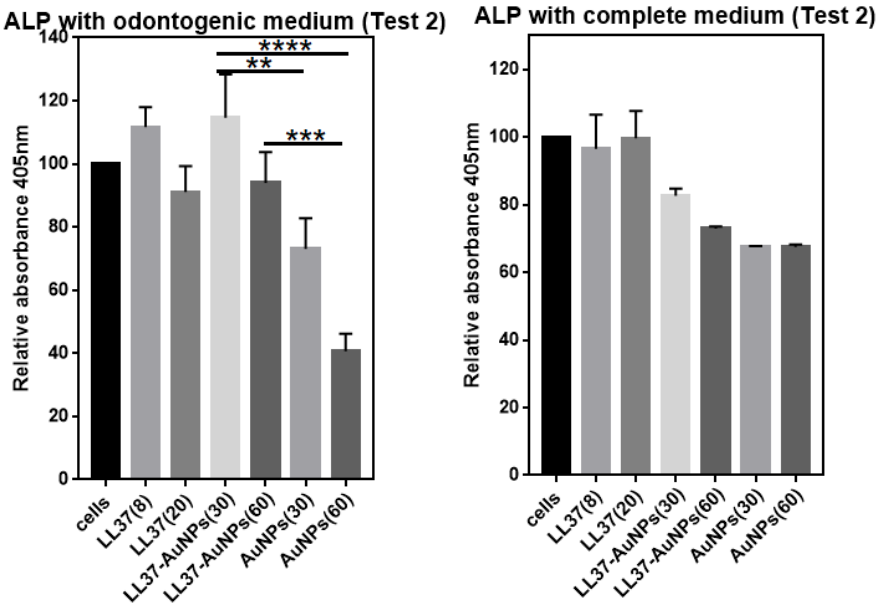


Figura22-Alkaline phosphatase staining Test2

In the second test, the effect of LL37 and LL37-AuNPs on odontogenic differentiation was examined by alkaline phosphatase (ALP) activity assay and alizarin red staining. ALP activity was measured at day 7 supplemented the cell culture with LL37 (8 , 20 µg/mL) and LL37-Au NPs every three days. LL37-AuNPs enhanced the ALP activity. The level of calcium mineral deposition was displayed by alizarin red staining after 16-day odontogenic induction. In line with ALP activity results, LL37-AuNPs(30 µg/mL) increased calcium mineral deposition, detected by higher intensity of red color.

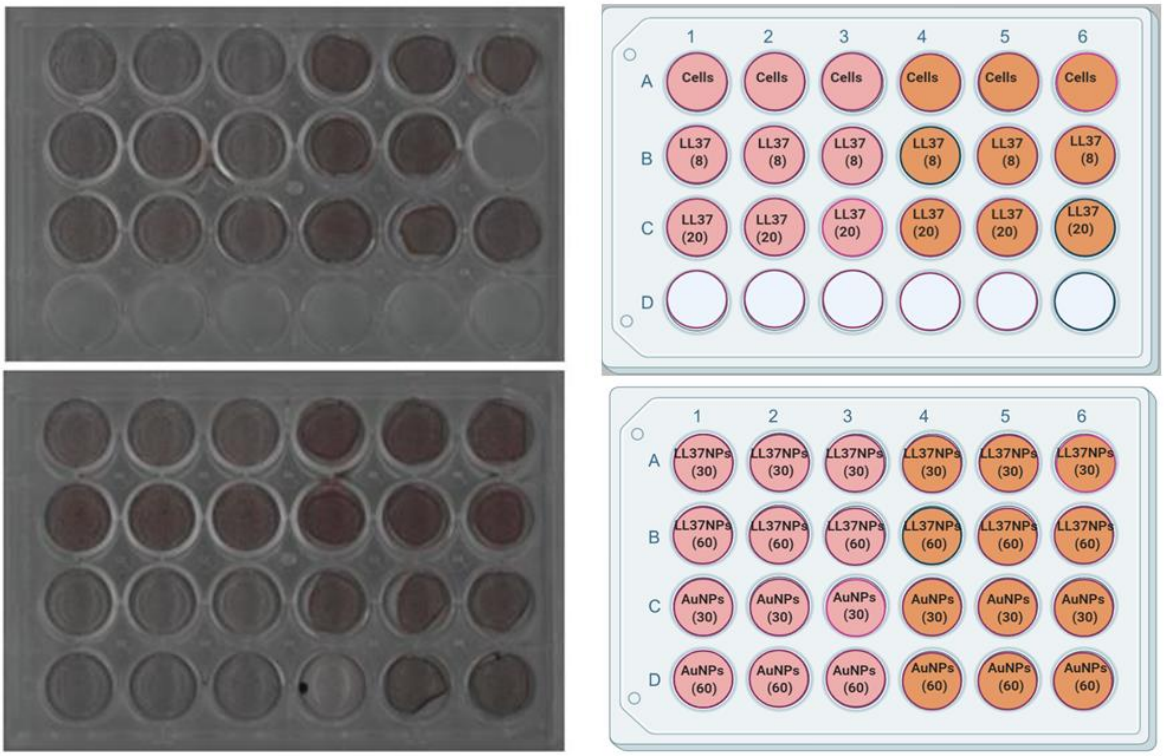


Figura23- Alizarin red staining Test2.

3. In the third test the induction of odontogenic differentiation cells was made incubating the cells without FBS for 4 h at 37°C in 5% CO_2 and change of complete and odontogenic medium every 3 days.

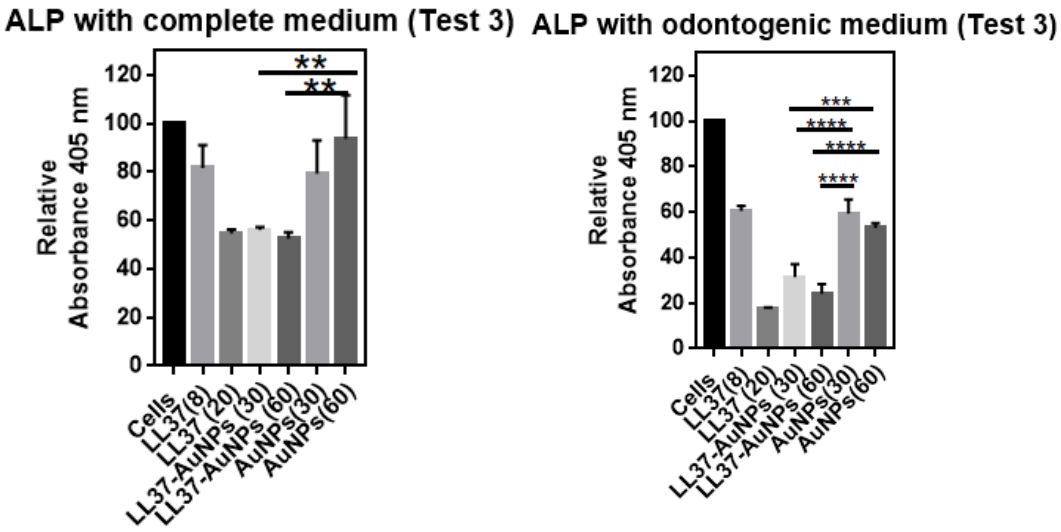


Figura24- Alkaline phosphatase staining Test3.

This test was made to understand if in the absence of FBS, the activity of LL37 could be effective on SCAPs. However, it was shown that medium without FBS can affect the overall number of cells.FBS contains many nutrients and macromolecular factors important for cell growth and a lot of small molecules such as amino acids, sugars, lipids and hormones. ALP activity was measured at day 7, all the conditions tested reduced the ALP activity, probably due to the absence of essential nutrients containing in FBS that can affect the interaction between cells and peptide or NPs. The level of calcium mineral deposition was displayed by alizarin red staining at 18-day odontogenic induction. In line with ALP activity results, pictures, took after the alizarin red staining, have not shown great intensity of red color.

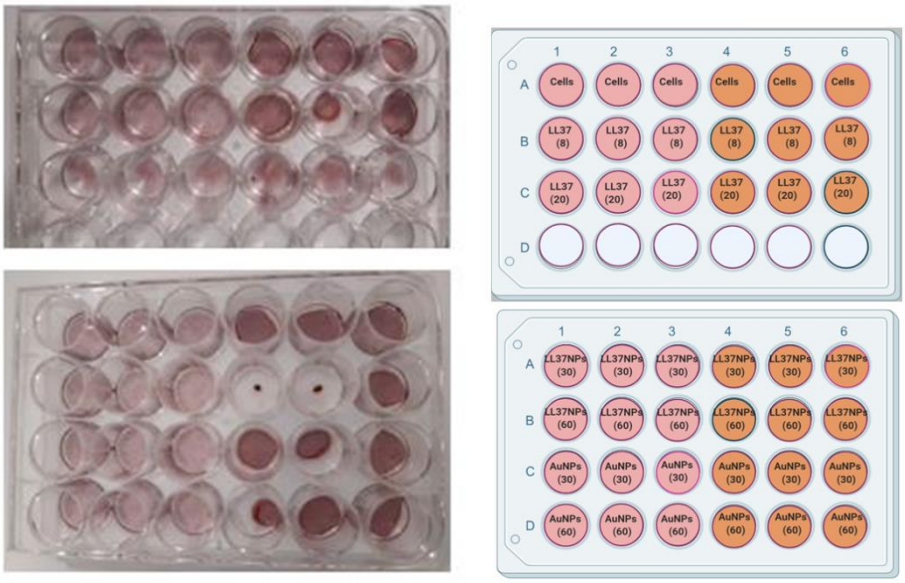


Figura25- Alizarin red staining Test3.

7.6 Cell proliferation

LL37 and LL37-AuNPs promote cell proliferation

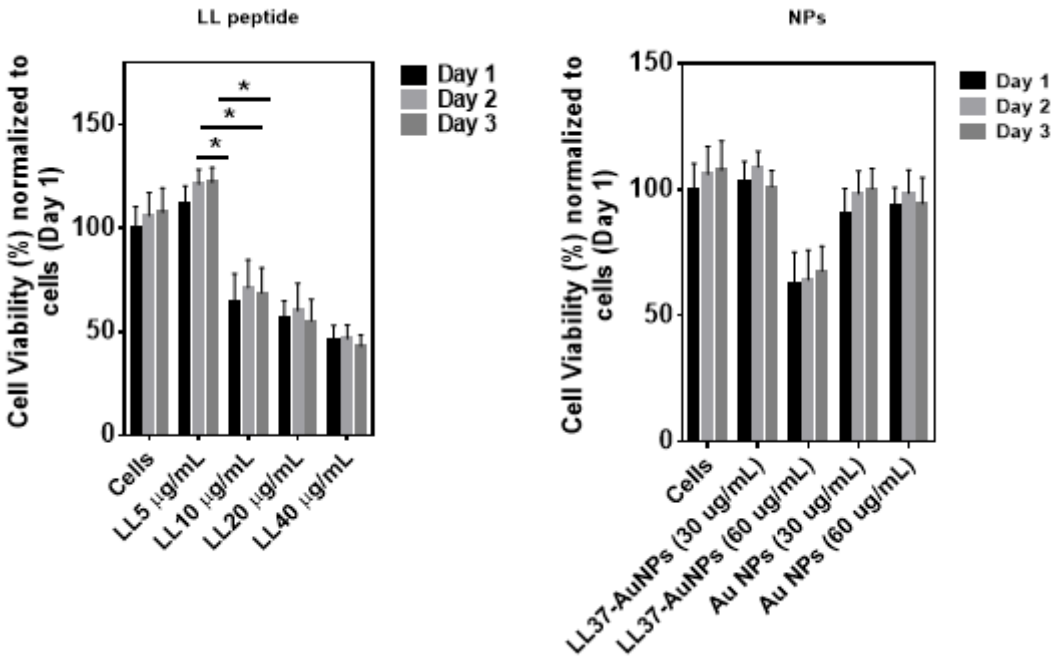


Figura26-Effects of LL37,LL37-AuNPs and AuNPs on cell proliferation.

Like in the previous test, the aim was to understand if the absence of FBS for 4 h could affect the cell proliferation. The proliferation activity of LL37, LL37-AuNPs and AuNPs were tested at different dosages. The result demonstrated that 5 μg/mL increased the proliferation rate of SCAPs at day 2 and 3. LL37 (10, 20 and 40 μg/mL) and LL37-AuNPs (60 μg/mL) significantly decreased the proliferation rate of SCAPs, suggesting that at these dosages they can be cytotoxic. Previous study have shown that the proliferation activity of LL37 (10 μg/mL) on SCAPs significantly increased at day 7 (59), suggesting that probably the absence of FBS reduced the activity of LL37.

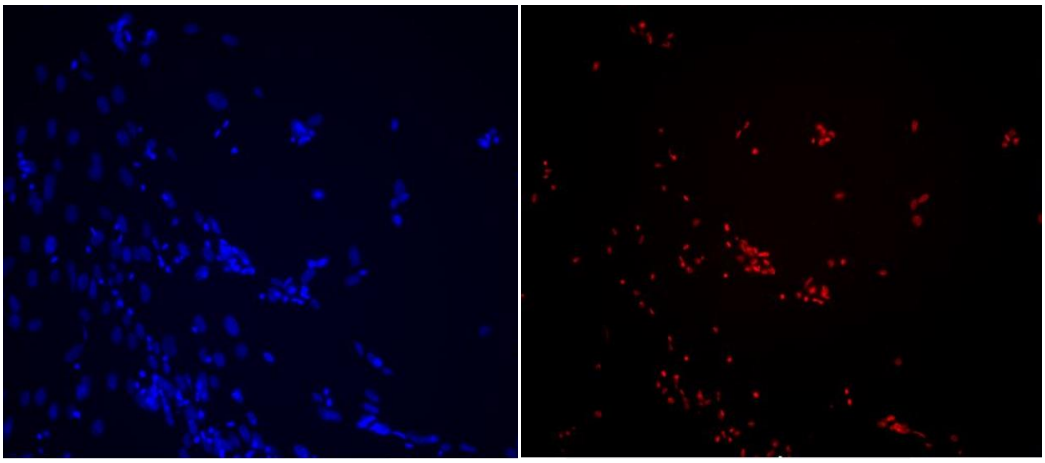


Figura 27-Fluorescence images of SCAPs incubated with 20 µg/mL of LL37 obtained from IN Cell Analyzer 48 h after the treatment.

7.7 Flow cytometry Analysis of EGF Receptors

Previous studies have shown that both LL37-Au NPs and LL37 peptide encourage keratinocyte migration by transactivation of EGFR. In the specific, the activation of metalloproteinases (probably ADAM10 and/or ADAM17) occurs and releases EGF linked to cell membrane into a heparin-binging EGF (HB-EGF), which sequentially binds to EGFR. This involves the phosphorylation of ERK1/2 and STAT3, translocation of STAT3 into the nucleus and at the end, the initiation of transcription of target genes. Studies have shown that keratinocytes expressed high level of EGFR, while FPRL-1, and P2X7 receptors mediate the bioactivity of LL37 in fibroblasts and monocytes respectively. The phosphorylation of EGFR by LL37 peptide was fast and lasted for 10 min, moreover the phosphorylation of EGFR by LL37-Au NPs reached the peak at 10 min of contact and lasted for about 60 min (16). On this line, this work has shown that SCAPs expressed good level of EGFR and SCAPs incubated with LL37-AuNPs have shown an higher level of EGFR than SCAPs alone , evaluated by flow cytometry.

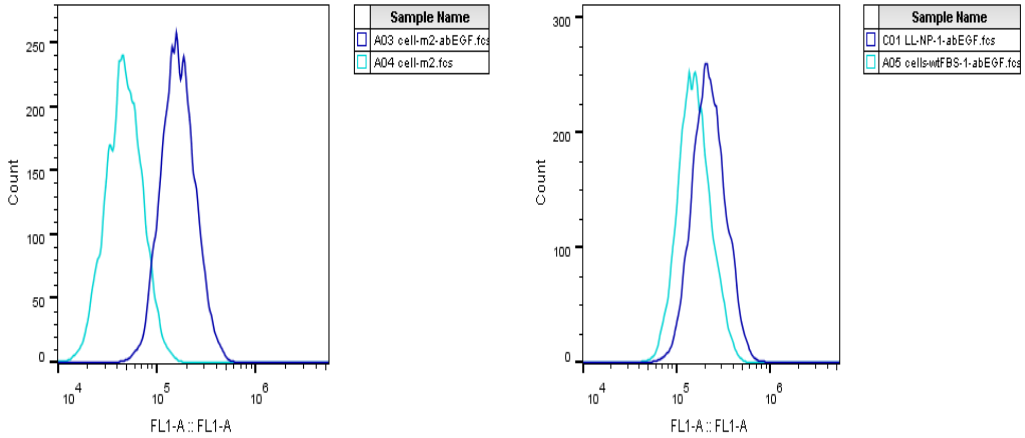


Figura28-Flow cytometry analysis of EGF receptors on SCAPs and SCAPs incubated with LL37-AuNPs.

The phosphorylation of EGFR in SCAPs mediated by LL37 (8 µg/mL), LL37-AuNPs (30 µg/mL) and AuNPs (30 µg/mL) was evaluated after having exposed the cells for 4 h without FBS. LL37-Au NPs (30 µg/mL) expressed higher mean fluorescence intensity compared to LL37, it means an higher expression of EGFR. This parameter was evaluated using FlowJo software.

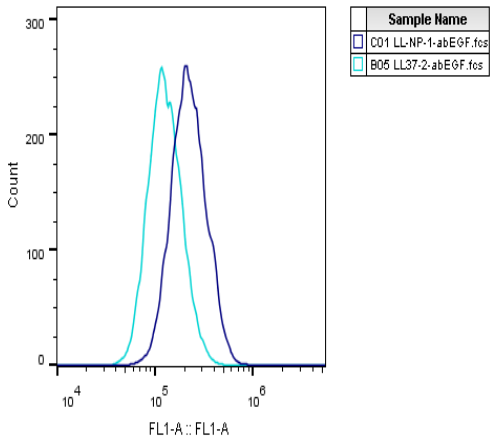


Figura29-Flow cytometry Analysis of EGF receptors on LL37 and LL37-AuNPs.

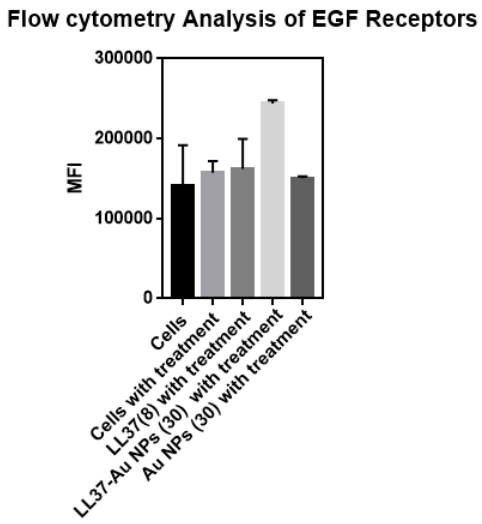


Figura30-Flow cytometry Analysis of EGF Receptors. MFI, mean fluorescence intensity.

8 Scaffold for teeth tissue engineering

Tissue engineering (TE) is a promising multidisciplinary fields and combines cells, engineering, materials and appropriate biochemical and physicochemical factors to restore or improve biological tissues using momentary structures, known as scaffolds (76). The main aim is to mimic the extra-cellular matrix providing suitable stimuli to cells in order to favorite tissues growth. In specific, in this part of the thesis LL37-AuNPs and Au-NPs were deposited on electrospun nanofibers of gelatin and antimicrobial properties have been tested.

Recent studies have shown the possibility to design bioengineered 3D scaffold to regenerate tooth organogenesis. The design of scaffold is complicated and it needs to be able to support cell attachment and proliferation and have adequate mechanical properties. Tooth loss or damage for periodontal diseases, trauma, dental caries is a common problem among young and old population, since adult teeth cannot regenerate, artificial approaches such as dental implants or tooth transplantation are used to restore lost mastication. Mesenchymal and epithelial cells are necessary for the tooth growth; they stimulate the formation of mineralized dentin, enamel, cementum and its roots. Advantages of scaffold in dental application can alleviate suffering, eliminate infection, and prevent the loss of the entire tooth. In dental field, the types of scaffolds range from long-term porous hydroxyapatite ceramis, to middle-term such a collagen and chitosan or short-term such as polyglycolic acid (PGA), polylactic acid (PLA), polyglycolic acid-poly-L-lactic acid (PGA-PLLA) and polylacticpolyglycolic acid (PLGA). Studies have shown that collagen sponge scaffolds and gels can be used for tooth regeneration. Scaffolds promote adhesion, proliferation, differentiation of the cells and stimulate development of calcified tissues (77).

8.1 Gelatin nanofibers by electrospinning

Gelatin is a biopolymer, which is biocompatible and biodegrade similar to collagen. It can be extracted from animal tissues such as skin, muscle and bone. Due to its properties such as its biodegradability in physiological environments and its abundance it can be used in different fields, in food, cosmetic and medical applications. Gelatin-based scaffolds can be prepared by electrospinning, their 3D structure mimics extracellular matrix. The nanofibers show exceptional properties: great surface-to-volume ratio and high porosity with small pore size (78). . Electrospining can fabricate random or aligned fibrous matrices and fiber diameters range from tens of nanometers to many microns. Fibre size and orientation can influence the

morphology, proliferation rate, phenotype and function of cell types. GL can be easily electrospun by use of organic solvent like 2,2,2-trifluorothanol, acetic acid, hexafluoroisopropanol and formic acid . Organic or acid solvents can cause GL denaturation and degradation, residues of un-removed solvent can cause toxic effects in vivo and the cost of the processing is high and not eco-friendly. During cell adhesion, proliferation and deposition of natural ECM, GL nanofibres should not dissolve or degrade in vivo. GL is water-soluble therefore GL needs a crosslinker to make stronger fiber. Typical crosslinkers are aldehydes (formaldehyde, glutaraldehyde, glyceraldehydes), carbodiimides, and enzymatically or naturally derived crosslinking agents.

Electrospinning process parameters were set to fabricate GL porous matrices with a pore size of 1-2 μm and diameter of 300 nm. Water is not toxic ad cheap, not induce protein denaturation and is eco-friendly so water can be used as solvent for GL electrospinning. Processing is made at high temperature of around 50°C due to the formation of 3D interconnected network of GL chains under the sol-gel transition temperature of around 30°C. Before processing, a silane-coupling agent (γ-glycidoxypropyltrimethoxysilane, GPTMS) was added into GL water solutions. In the GPTMS crosslinling action, the oxirane rings on the GPTMS molecules and amino groups on the GL chains react and due to hydrolysis of the trimethoxy groups on the GPTMS, pendant silanol groups (Si-OH) are formed thanks to an acid catalyzed reaction (equation 1).

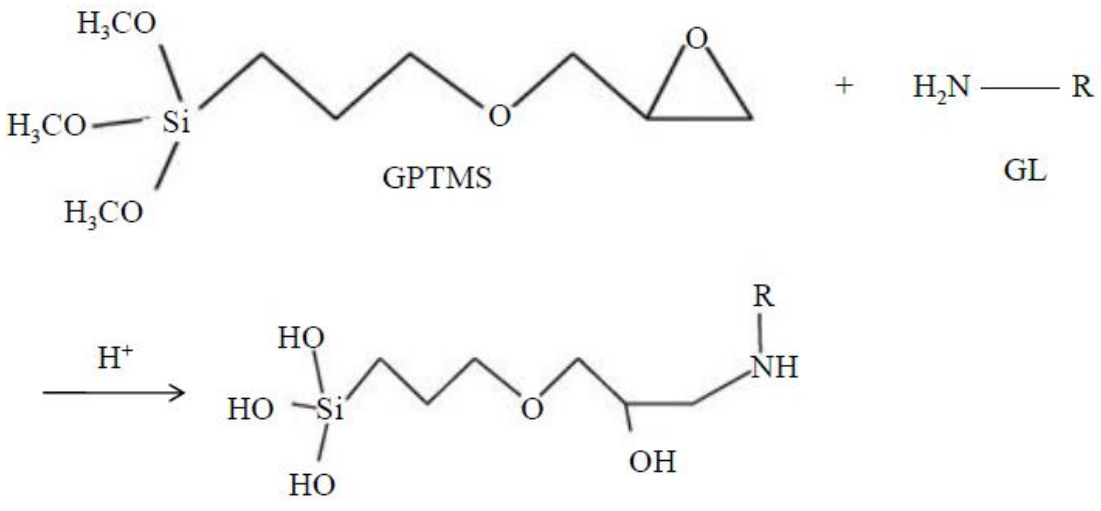


Figura31-Equation 1 (79).

Si-O-Si bonds are generated after the condensation of two Si-OH, in the course of solvent evaporation, they allow inter-chain covalent bonds forming a crosslinked structure (equation 2) (79).

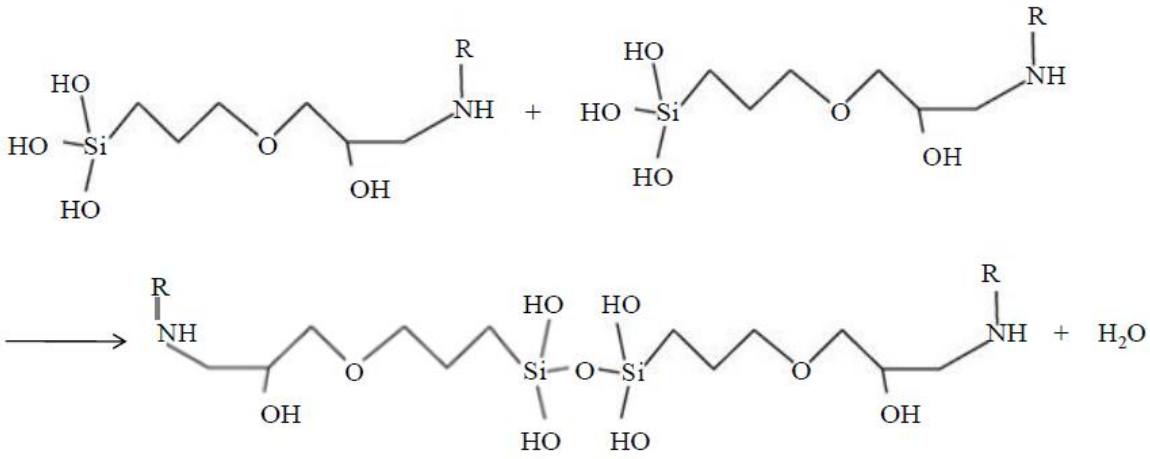


Figura32-Equation 2 (79).

8.1.1 GL Solution preparation

GL type A derived from porcine ski and GPTMS were purchased from Aldrich. GL was dissolved in Milli-Q at 50°C with solution concentration of 15% w/V to obtain homogeneous and electrospun nanofibers with high porosity. After about 3h an appropriate amount of GPTMS was added (92 µl of GPTMS per GL gram) to the GL solution and mixing for 30 min before spinning (79).

Square nanofibrous membranes (2 cm^2) were picked up from the aluminum sheets using tweezers.

8.1.2 Electrospinning technique

The elecrospinning instrument is situated inside a vented hood able to extract solvent vapors; it is composed of a high voltage source situated out the hood to not compromise the electric field, an electrode, a syringe with 21 Gauge needle, a volumetric pump support and a collector. During the electospinning technique an electric field is created by an applied voltage that produces surface charges on the polymer solution. This causes the formation of a Taylor cone on the polymeric solution drop at the end of the spinneret. If the electric potential on the drop surface overcomes a critical value, the electrostatic forces exceed the solution surface tension and the polymeric jet stream occurs and nanofibers are collected on

grounded collector as the solvent evaporates. The topography and orientation of nanofibers can be controlled by modifying the electrospinning setup.

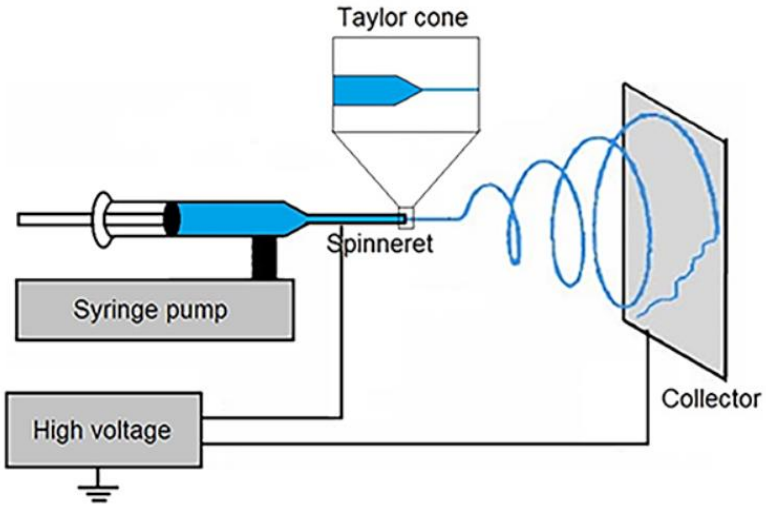


Figura33- Typical electrospinning system (80).

The high voltage generator used was the PS/EL30R01.5-22 Glassman High Voltage, providing a voltage 30 kV. The volumetric pump (KDS210 of KDScientific) was set to have a flow rate of 1,5 ml/h. The distance between nozzle and collector was 12 cm and the collector was covered by aluminum sheets of about 1.5 mm thickness, connected to a cable for grounding. In order to obtain uniform samples the nozzle was put parallel to the support base (79).

8.1.3 Conjugation of LL37-AuNPs with GL nanofibers

NPs can be conjugated with GL nanofibers in different ways. In this work, three test were performed:

1. Gelatin nanofibers were functionalized with AuNPs, prepared by electrospinning using solutions of gelatin mixed with LL37-AuNPs or AuNPs (1 mg/mL). Nanofibers were examined with the aid of scanning electron microscopy (SEM) and have shown similar results with GL nanofibers.

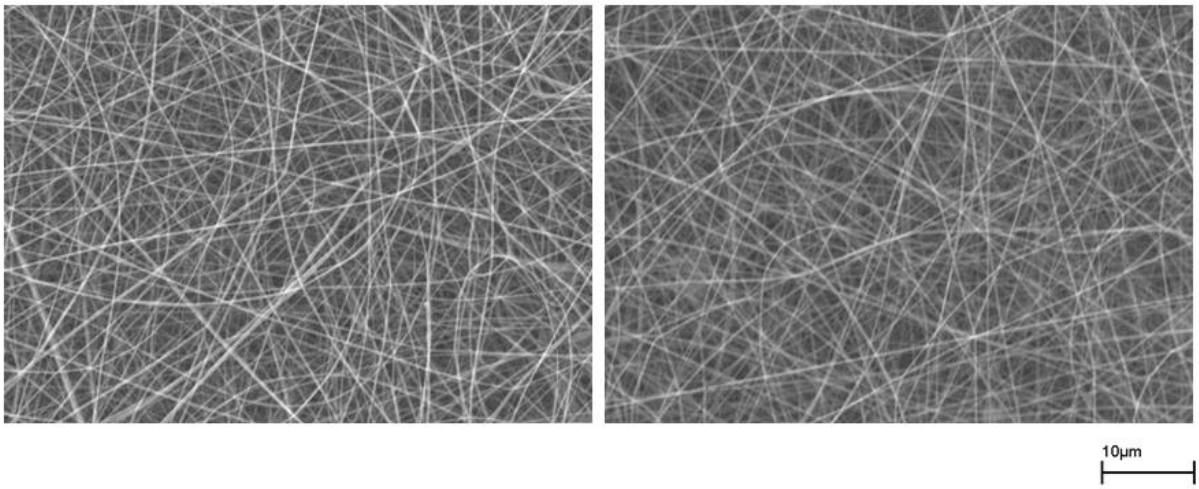


Figura34-SEM micrographs of AuNPs_NF (on the left) and LL37-AuNPs_NF (on the right).

Previous studies have shown that GL/GPTMS_NF in PBS at 37°C after 15 days were completely dissolved (79). This test was not performed because was in contrast with the time of the antimicrobial test. Antimicrobial activity of LL37-AuNPs-GL/GPTMS_NF was evaluated against *S.aureus* bacteria but it was not relevant because the test were made after 24 h from the incubation and NPs were not released yet from GL_NF and probably the concentration of nanoparticles on the nanofibers surface was not enough to induce bactericidal activity moreover it is not known the effect of electrospinning on the peptide(results not shown).

2. LL37-AuNPs and AuNPs were conjugated with GL_NF by using NHS/EDC chemistry. Gelatin is a very convenient polymer for immobilization of biomelucules covalently for the presence of carboxylic acid group –COOH. This immobilization involves the preparation of a succinimidyl ester (-COOSuc)-terminated surface and its reaction with an amino (-NH2) group of peptide. This activation is done by reacting carboxyl end groups with N-hydroxysuccinimide (NHS), in the presence of carbodiimide like 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) (81). EDC (1mg) was put in PBS (pH=7.2) and mixed properly, NHS (1 mg) was added to the solution. Nanofibers were put into the obtained solution and were mixed properly on the orbital shaker. After 15 min, the solvents were removed and the nanofibers were washed with PBS and Milli-Q water, 0.5 mL LL37-AuNPs or AuNPs (pH=5 and pH=7) solution were added forming covalent bonds with gelatin and incubated overnight.

3. LL37-AuNPs and AuNPs were conjugated with GL_NF by using electrostatic interaction of surface charge between AuNPs and GL_NF, forming non-covalent bonds between the amino groups of GL and the Au NPs. 0.5 mL of LL37-AuNPs or AuNPs (pH=5 and pH=7) were incubated overnight with GL_NF.

The membranes were washed with milli-Q water dried under a stream of N_2 gas. They were sterilized under the UV lamp for 30 min each side. In order to not change the conformation of the membranes they need to dry on a flat surface. Different conditions were tested for the antimicrobial tests and the best results suggesting to use LL37-AuNPs and AuNPs at pH=7 conjugated with GL_NF by using electrostatic interaction. Nanofibers were put in tubes and used for the antimicrobial activity testing.

8.2 Antimicrobial activity of LL37-AuNPs_NF

S. *aureus*, E. *coli*, S. *epidermidis*, and E. *faecalis* were grown at 37°C and maintained on agar plates. After that, few isolated colonies from the plates were placed into 4 ml of broth medium and grown for overnight at 37°C at 150 rpm into the shaking incubator. After about 16 hours, during the lag phase, cell counts were done at OD 600 nm using the plate reader (Bioteck).

After the cell count bacteria were diluted to 10^5 CFU (colony formation unit)/mL in human serum (10% v/v) and LL37-AuNPs_NF and AuNPs_NF were added into bacterial suspensions and incubated for 24 h at 37° and 150 rpm. At 0, 5 , 24 h bacterial suspensions were diluted to 100 times in PBS buffer and aliquots (50µL) were removed from the respective suspensions and widespread over the surface of agar plates using a sterile glass spreader and incubated for 24h at 37°C. The number of colonies growing on plates were estimated after 24 h for each time considered. All tests were performed 3 times and at different times to ensure the reproducibility of the data.

To investigate the laser-activated photothermal effects of AuNPs_NF and LL37-AuNPs_NF on bacteria behaviour, different experimental groups were exposed to the green laser: bacteria, bacteria with AuNPs_NF, bacteria with LL37-AuNps_NF, they were exposed to laser (500 mW/cm^2) at 0 h. The laser wavelength was 532 nm, and the irradiation lasted for 3 minutes at 500 mW/cm^2 stopping the irradiation each minute for some seconds in order to

stabilize the solution. After laser irradiation, bacteria suspensions were incubated for 24 h at 37° and 150 rpm. At 0, 5 , 24 h bacterial suspensions were diluted to 100 time in PBS buffer and aliquots (50µL) were removed from the respective suspensions and widespread over the surface of TSY or BHI agar using a sterile glass spreader and incubated for 24h at 37°C. The number of colonies growing on plates were estimated.

LL37-AuNPs_NF have excellent antimicrobial activity in the presence of serum and green laser

The antimicrobial activity of the LL37-Au NPs_NF was evaluated against gram-negative (*E. coli*) bacteria and gram-positive (*S. aureus, S. epidermidis, E. faecalis)* in 10% (v/v) Human Serum. At different time interval (0, 5 and 24h), aliquots were taken out and plated on agar plates and bacteria colonies were counted after 24 h. Bacteria were used as a control.

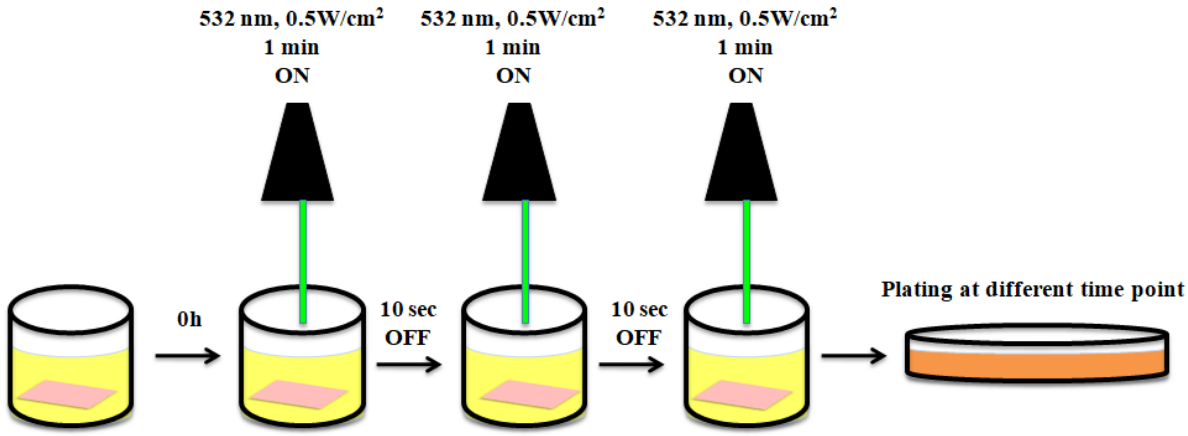


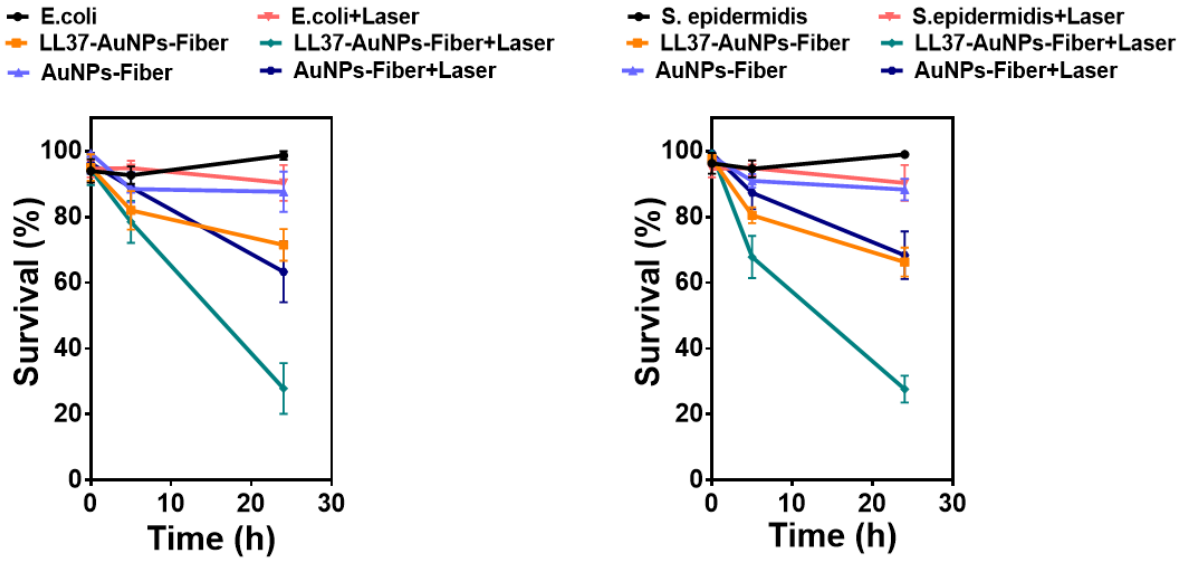
Figura35-Procedure for testing the antimicrobial activity of LL37-AuNPs_NF.

AuNPs_NF showed an insignificant antimicrobial activity. Au NPs exposed to green laser show a slight antimicrobial activity probably due to thermal effects. The LL37-AuNPs_NF exposed to green laser had good antimicrobial activity, killing:

- About 20% and 70 % of 10^5 CFU gram-negative *E.coli* in 5 h and 24 h respectively;
- About 30% and 70 % of 10^5 CFU gram-positive *S.epidermidis*in 5 h and 24 h respectively;

- About 40% and 80 % of 10^5 CFU gram-positive *S.aureus* in 5 h and 24 h respectively;
- About 40% and 70 % of 10^5 CFU gram-positive *E.faecalis* in 5 h and 24 h respectively.

The thermal energy generated by AuNPs after exposure of laser light helps to damage bacterial membranes and allows the internalization and bactericidal action of LL37-AuNPs.



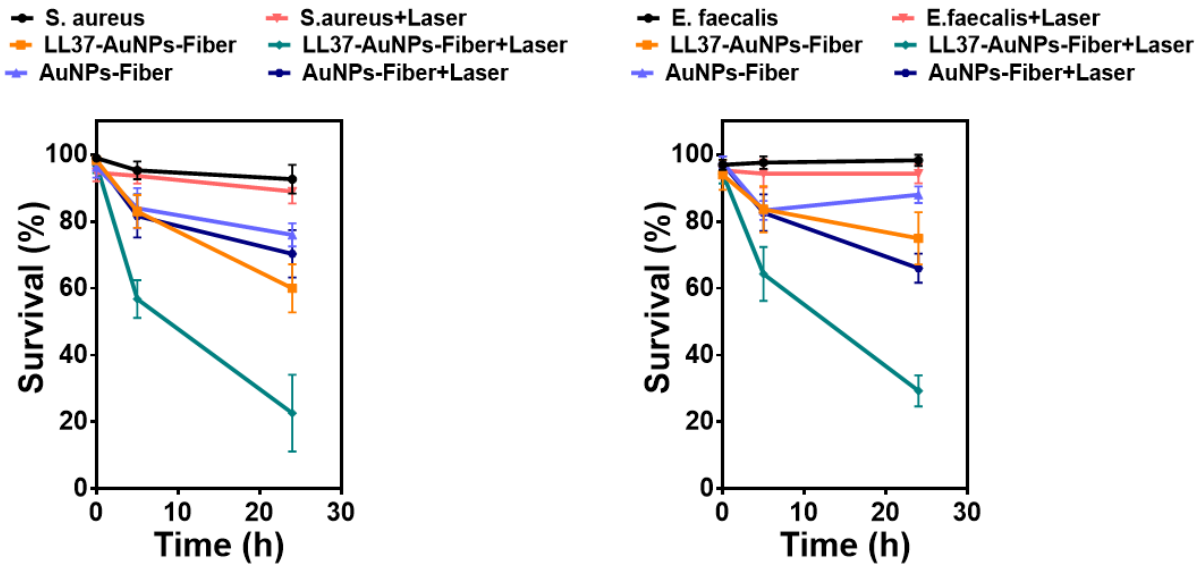


Figura36-Antimicrobial activity of LL37-AuNPs_NF against E.coli, S.epidermididis, S.aureus, E.faecalis in 10% human serum.

8.3 Permeabilization test on *E. coli* ML35p

E. coli ML-35p bacteria were grown in TSB media from a single colony, overnight at 37 ºC and 150 rpm . Bacteria were washed 3 times with PBS (pH 7.2), and the culture was diluted to 10^6CFU/mL. LL37-AuNPs_NF and Au-NPs_NF were incubated with 10^6CFU/mL of bacteria and 30 µM nitrocefin and 2.5 mM ONPG were added. They were exposed to green laser as previously described. At specific time specific amount of bacterial suspensions were removed and absorbance was measured at 486 and 420 nm to identify nitrocefin and ONPG, respectively, using a BioTek synergy MX microplate reader for 24h.

LL37-AuNPs_NF show an higher efficacy in inducing the permeabilization of bacterial cell membrane than AuNPs_NF

Both Au NPs_NF and LL37-AuNPs_NF interact with cell membrane and LL37-AuNPs_NF induce loss of bacteria cytoplasm. In order to have more information about the destabilization of outer (OM) and inner (IM) bacterial membrane *E.coli* bacterial strain ML-35p that was engineered especially to monitor permeability membranes was used. No significant increase in OM and IM permeability of the bacteria was shown after contact with AuNPs_NF and AuNPs_NF exposed to the green laser. In contrast, both LL37-AuNPs_NF and LL37-AuNPs_NF exposed to the green laser induced either OM or IM permeability. LL37-

AuNPs_NF exposed to the green laser were more efficient that LL37-AuNps_NF in inducing OM and IM permeability. These results showed that antimicrobial effect of the NPs is rapid and occurred due to the destabilization of the bacterial membrane.

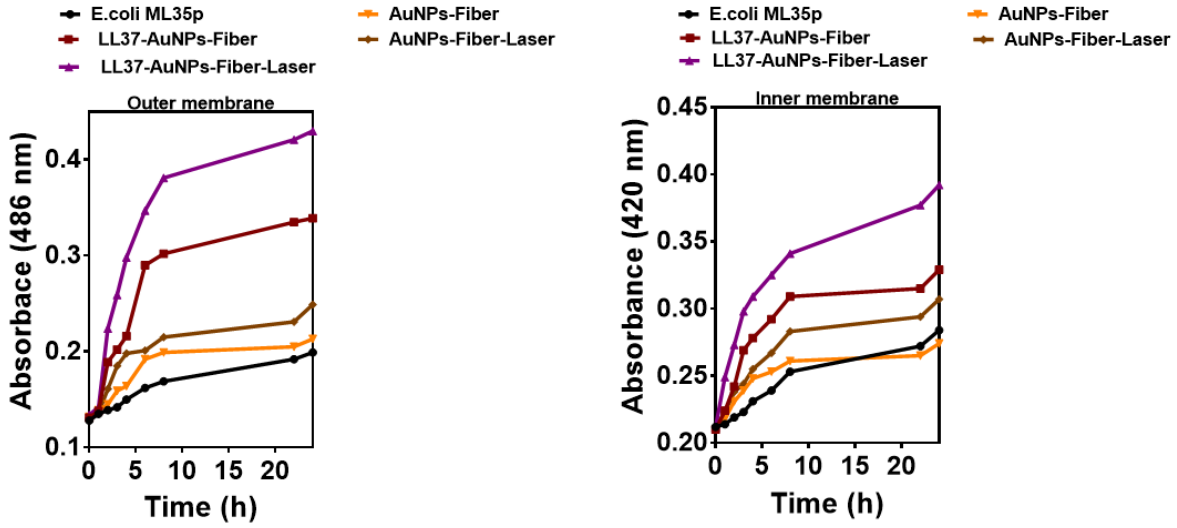


Figura37-Permeabilizing effect of LL37-AuNPs_NF on E. coli ML35p outer and inner membrane.

9 Conclusions and future work

Nanomedicine is a promising field due to the recent and useful applications of the nanomaterials and unique properties of the material in the nanoscale. The overall aim of the work is to find an effective and multifunctional nanoformulation as potential antimicrobial and differentiation agent to differentiate dental stem cells to odontoblast-like cells. The present study is related to an antimicrobial peptide conjugated with NPs, in particular the antimicrobial peptide LL37-conjugated Au NPs for use in dental application. Antimicrobial peptides protect the human body against bacteria, virus and fungi, these small peptides (usually< 40 aminoacids) can be used as potential anti-infective therapy seen like an alternative to conventional antibiotics and shown their pro-regenerative effects. It was shown thatLL37can be used in medicine or cosmetic, for example for the treatment of wound healing, ischemic and vascular diseases. LL37 modulates inflammation, angiogenesis and wound healing. LL37 is chemically immobilized on AuNPs in order to increase its stability, decrease toxicity, improve antimicrobial properties (because of anincrease in the local density of positive charges and peptide mass) and improve targeting compared with free AMP. The AuNPs were selected because they have good compatibility with human body, low toxicity, small size and stability and it is easy to modify their properties, immobilize high concentrations of peptide per surface area. LL37-conjugated AuNPs were synthesized using one-step procedure. Initially, antimicrobial properties of LL37-AuNPs were shown. They showed higher antimicrobial activity in human serum and using a green laser. Localized surface plasmon resonance (LSPR) is an important optical property of AuNPs. Laser excitation of the LSPR of AuNPs can cause photon–electron and electron–electron interactions and then produce heat. The thermal energy generated by AuNPs after exposure of laser light helps to damage bacterial membranes and allows the internalization and bactericidal action of LL37-AuNPs. It is hard to determine the optimal conditions by comparing previous studies (68), parameters for this experiment were chosen remaining in a safety region for eventual studies in vivo. Further studies are necessary, such as calorimetric test to better understand how the green laser affect the antimicrobial test. The antimicrobial activity of the LL37-Au NPs has been evaluated against Gram-negative (*E. coli* and *E. coli* ML-35p) and Gram-positive *(S. aureus, S. epidermidis, E. faecalis*) bacteria. LL37-AuNPs have not shown the 100% of killing, interesting results are shown against *S. aureus, S. epidermidis and E.coli* killing more than 80% of bacteria after 24 h after the green laser irradiation. More specific bacteria present in dental issues need to be

tested. Generally dental diseases, periodontal diseases and dental caries, are caused by bacteria of normal oral flora, there are more than 300 different types of bacteria. For example, mutans streptococci and Lactobacilli are the most common bacteria in dental caries, leading to pain, tooth loss and infection. Oral bacteria can spread into blood stream due to damaged tooth and be dangerous for immune compromised patients or patient with endoprotheses. The link between dental infections and myocardial or cerebral infarction have presented new important challenges for collaboration between dental and medical researchers. This work has shown that *E. faecalis*, often found in reinfected, root canal-treated teeth were more resistant than the other bacteria studied. Recent studies have shown that NaOCl or CHX had low capacity to eliminate *E. faecalis*, in this study LL37-AuNPs were able to kill about 70% of bacteria in combination with the green laser exposure. It was shown that green laser can improve bactericidal activities, more studies are necessary to understand how to optimize the parameter linked to the laser, such as the time of irradiation and the power used in order to not affect dental tissues. In the second part of the work, differentiation properties of LL37-AuNPs were tested to understand if this nanoformulation can be used to regenerate the dentine-pulp stimulating the differentiation of SCAPs in odontoblast-like cells instead of traditional root canal treatment, such as the obturation of the root canal with biologically inert materials and the use of sodium hypochlorite (NaOCl), as an endodontic irrigant and antimicrobial agent. NaOCl can induce potential complications ranging from permanent bleaching of clothes to serious soft tissue damage. It was shown that LL37-AuNPs enhanced the ALP activity and calcium mineral deposition detected by alizarin red staining, when the cell culture was supplemented with LL37-AuNPs (30 µg/mL) every three days. Alizarin Red S quantification is necessary to confirm the results. Moreover, LL37-AuNPs (30 µg/mL) increased the proliferation rate and the EGFR expression of the SCAPs. It is important to underline that the cells used have been taken from one tooth and so all the results are not statistically significant. In the last part of the thesis has shown the importance of scaffold in biomedical fileds, in specificallyLL37-AuNPs and Au-NPs were deposited on electrospunnanofibers of gelatin and antimicrobial properties have been tested. Similar antimicrobial activity gas shown between LL37_AuNPs and LL37_AuNPs_NF both exposed to green laser, although the presence of gelatin whichacts as nourishment for bacteria, so these results are better than expected. Probably the reasons are linked to the high exposed surface, the high concentration of nanoparticles and their possibility of leaching. It is not known the quantification of nanoparticles on nanofibers, it would be important to conduct specific characterizations on

LL37_AuNPs_NF to have relevant information and understand how to improve the antimicrobial activity and to conduct cell culture experiments. Further studies are necessary to understand how to optimize the parameters used in this work with the evidence that multifunctional nanoformulation could be further applied in clinical therapies. Overall, the data presented in this study provides evidence towards the efficacy of LL37-AuNPs and LL37-AuNPs_NF in antimicrobial activity, contributing to the improvement of dental tissue adopting engineering protocols trough stem cell-based approaches.

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