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Pulsed Laser Ablation in liquid: top-down approach against *Staphylococcus aureus* infection in vitro

MARIA VITTORIA MORONE[1] – ANNALISA CHIANESE[1] – CARLA ZANNELLA[1]
ANNA DE FILIPPIS[1] – MASSIMILIANO GALDIERO[1] – ANTONIO MORONE[2]*

[1] Dipartimento di Medicina Sperimentale, Università della Campania "Luigi Vanvitelli", 80138 Napoli, Italy
[2] Consiglio Nazionale delle Ricerche, Istituto Struttura della Materia U. O. di Tito Scalo, 85050, Italy. E-mail: antonio.morone@cnr.it

*corresponding author

Abstract – In the following work we used the physics methodology of Pulsed Laser Ablation in Liquid (PLAL) to form nanoparticles (NPs) using a magnetic target of Samarium-Cobalt (SmCo). The magnetic nanoparticles thus produced, have been characterized using some chemical-physical strategies including: Total X-Ray Fluorescence (TXRF), Electron Transmission Microscopy (TEM) and to characterize the magnetic functionality we made magneto-optical measurements using the Kerr Effect (MOKE). However, in order to investigate the antibacterial potential of SmCo-NPs against the bacterium *Staphylococcus aureus* (S. aureus), which is primarily responsible for nosocomial infections, we conducted the MTT assay on human keratinocyte cells (HaCaT cells) to assess potential cytotoxicity. After that, using a non-toxic number of SmCo-NPs for the cells, we examined the antibacterial activity by the method of the Minimum Inhibitory Concentration (MIC), and we also monitored the bacterial growth in the time after exposure to SmCo-NPs through the use of a Time Killing to understand how SmCo-NPs act on bacterial culture.

Keywords: PLAL, SmCo-NPs, Nosocomial infections, S. aureus, antibacterial potential

Riassunto – Nel seguente lavoro abbiamo utilizzato la metodologia fisica dell'Ablazione Laser Pulsata in Liquido (PLAL) per formare nanoparticelle (NPs) utilizzando un target magnetico di Samarium- Cobalt (SmCo). Le nanoparticelle magnetiche così prodotte, sono state caratterizzate utilizzando alcune strategie chimico-fisiche tra cui: la Fluorescenza Totale indotta da Raggi X (TXRF), la Microscopia Elettronica a trasmissione (TEM) e per caratterizzare la funzionalità magnetica abbiamo effettuato misure magneto-ottiche utilizzando l'Effetto Kerr (MOKE). Tuttavia, al fine di indagare il potenziale antibatterico delle SmCo-NPs contro il batterio *Staphylococcus aureus* (S. aureus), principale responsabile delle infezioni nosocomiali, in primis, abbiamo condotto il saggio MTT su cellule di cheratinociti umani (HaCaT cells) per valutare la potenziale citotossicità. Dopo di che, utilizzando un numero di SmCo-NPs non tossico per le cellule, abbiamo esaminato l'attività antibatterica mediante il metodo della Minima Concentrazione Inibente (MIC) ed inoltre abbiamo monitorato la crescita batterica nel tempo dopo l'esposizione alle SmCo-NPs attraverso l'utilizzo di un Time Killing per comprendere in che modo le SmCo-NPs agiscono sulla coltura batterica.

Parole chiave: Ablazione Laser in liquido, Nanoparticelle di Samario Cobalto, Infezione Nosocomiale, Staphylococcus Aureus, Antibatterico

1. Introduction

The Pulsed Laser Ablation in liquid has been used to formed the magnetic nanoparticles of SmCo (SmCo-NPs). This technique has been used in last years to produce different materials, thin films and nanoparticles. Laser ablation strategy began in 1980 with Hight Temperature Transition Superconductors, materials such as YBCO and BISCO [1]. In last time, many materials for biosensors and for medical applications have been crafted by PLAL in many laboratories in the world [2]. In this study we report the results on antibacterial activity of SmCo-NPs against S. aureus.

Healthcare-associated or nosocomial infections appear in patients with medical care [3]. Since these infections occur in hospitals, they are responsible for prolonged stays, disability and economic burden. Studies in different parts of the world show that nosocomial infections account for about 7% in developed countries and about 10% in developing countries [4]. Among the pathogens responsible for nosocomial infections we first distinguish bacteria, considered the most common pathogens, followed by fungi and viruses [5]. Infection occurs when the pathogen spreads into a susceptible host patient. In today's healthcare, invasive procedures, surgery, internal medical devices, and prosthetic devices are associated with nosocomial infections. Different are the bacterial strains responsible for nosocomial infections, among them we distinguish the S. aureus, it is gram positive coccus, catalase positive, not motile, not spore forming, and facultative anaerobe, arranged in cluster [6]. S. aureus is both commensal and pathogen. Approximately 20% of individuals are persistently nasally colonized with S. aureus, and 30% are intermittently colonized. S. aureus can cause many diseases such as skin infections, abscesses, impetigo, necrotizing pneumonia, atherosclerosis, catheter-induced endocarditis septicemia, osteomyelitis and in particular opportunistic infections in hospitals which represent a serious problem in today's society [7]. In this scenario, it is important to find a valid alternative to common antibiotic therapies because their disproportionate use, to date, is increasing the phenomenon of resistance [8, 9].

In this work, we evaluated potential antibacterial of SmCo-NPs produced by PLAL, against infection of S. aureus in vitro. The SmCo-NPs were characterized through the use of different strategies including, TXRF, TEM, and MOKE. Thus characterized, SmCo-NPs have been used in our cytotoxicity and antibacterial experiments. In particular, the evaluation of cytotoxicity was carried out on human keratinocyte cells (HaCaT cells) using MTT test. While, to assess the antibacterial properties of SmCo-NPs, we used two assays: MIC and Time Killing. PLAL has proven to be a valuable approach for nanoparticles synthesis thanks to stability, high purity and good control of size and morphology of the nanoparticles produced [10].

2. Materials and Methods

2.1 Production and Characterization of SmCo-NPs

The Pulsed Laser fabrication system used in this research makes use of an Nd-Yag laser working at λ=532 nm, 10 ns pulse width, and 120 mJ/shot energy. The laser was focused on a rotating target having a spot area of 3÷5 mm^2. The SmCo-NPs were formed in 10 ml of Phosphate Buffer Saline (PBS) 1x liquid (Gibco-BRL-Thermo Fisher Scientific, Waltham, MA, USA, 02451) and the time of treatment was in the range 15-60 minutes. During the manufacturing process of SmCo-NPs, room temperature was used. The target material was a commercial alloy of SmCo.

The equipment of Total X-Ray Fluorescence (TXRF) included an X-Ray Tube, with Ag anode and Be windows with a thickness of 125 μm, operating at 30 KV with a current of 10 A. A collimator was used to focus the X-Ray beam on a surface of 7×10^{-3} m^2. The X-Ray beam hit the surface forming a 45° angle with the sample. The distance between the collimator exit and the sample surface was 5×10^{-3} m.

The symmetry and size of the SmCo-NPs produced by PLAL was investigated by Transmission Electron Microscopy (TEM) analysis. In particular, the magnetic nanoparticles were deposited on a carbon-coated copper grid on which 2% Phosphotungstic acid was gently added. With the help of a filter paper, the acid solution was aspirated and the grid was left to dry overnight. The next day, the microscopy analysis was conducted, using the instrument TEM FEI TECNAI G12 TWIN 120 kV.

To control the magnetic properties of product nanoparticles we performed Magneto-Optic measurement that used Kerr effect. The polarization of light is a function of magnetic field and using this property it was possible to measure a very small magnetic field such as that of our nanoparticles product by PLAL.

2.2 Quantification of SmCo-NPs produced by PLAL

The SmCo-NPs thus produced and characterized, before use in microbiological practices, they have been quantified through the Scanning Electron Microscopy

(SEM) counting method. Specifically, using a micropipette, 5 µl of SmCo-NPs sample were deposited on specific stabs for SEM measurements, and the PBS 1x was moved away. 20 images were captured by the tool for each stab, and they were processed using Image J.

All SmCo-NPs in the image were counted and the total volume was calculated by Image J. From the volume of a single nanoparticle, we were able to get the number of nanoparticles present in 5 µl. We got 10^8 nanoparticles in 1 ml. With the help of a mathematical proportion, we calculated the number of SmCo-NPs present in the sample microliters to be used in our microbiological procedures, as shown in Table 1.

Table 1 – SmCo-NPs number used in microbiological procedures.

SmCo-NPs microliters (µl)	SmCo-NPs number (n)
100	1×10^7
50	5×10^6
25	2.5×10^6
12.5	1.2×10^6
6.2	6.2×10^5
3.1	3.1×10^5
1.5	1.5×10^5
0.8	0.8×10^5

2.3 Cell lines and Bacterial strain

Human keratinocytes (HaCaT cells) have been used to assess the cytotoxicity of SmCo-NPs. HaCaT cells were purchased at American Type Culture Collection (ATCC) and they were allowed to grow in Medium Dulbecco Modified Eagle (DMEM; Gibco; Thermo Fisher Scientific, Wal-Tham, MA, USA) with 4.5 g/L glucose, 2 mm L-glutamine, 100 IU/ml penicil-lin/streptomycin solution and supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) in humidified atmosphere with 5% CO 2 at 37 ºC for.

The bacterial strain used to assess the antibacterial potential of SmCo-NPs was *Staphylococcus aureus* (S. aureus 6538) purchased from ATCC. The bacterium was placed on a Muller-Hinton (M-H) agar plate (Oxoid, Hampshire, MA, USA) for 24 hours at 37° C. However, the antibacterial tests were conducted by inoculating a single colony of S. aureus 6538 into M-H broth for 24 hours at 37°C.

2.4 Cell viability assessment

First, evaluation of the cytotoxicity of SmCo-NPs on human keratinocytes, HACAT cells ATCC, was performed by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 24 hours. The cells (2×10^4 cells/well) were plated into a 96 well, and were left to grow in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% v/v of Fetal Bovine Serum (FBS), 100 U penicillin/100 µg streptomycin, and maintained at 37°C with 5% CO_2 overnight (ON). The following day, several number of SmCo-NPs ranging from 1×10^7 to 0.8×10^5, were added to the cell monolayer for 24 hours. Cells treated with 100% Dimethyl sulfoxide (DMSO) represented negative control (ctrl-), while cells not exposed to SmCo-NPs represented positive control (ctrl+). After 24 hours, the medium was removed and 0.5 mg/ml of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 3 hours. Formazano crystals were solubilized by supplementing 100% DMSO and cell viability was determined by reading the absorbance at 570 nm, using TECAN M-200 reader (Tecan, Männedorf, Switzerland). The percentage of cell viability has been calculated according to the following formula:

$$\textit{Cell viability}\ (\%) = \frac{\textit{absorbance of treated samples}}{\textit{absorbance of not treated cells}}\ x\ 100$$

2.5 Antibacterial potential of SmCo-NPs

2.5.1 MIC method

The antibacterial activity of SmCo-NPs is assessed by plate microdilution test. A colony of S. aureus 6538 has been inoculated in M-H broth at 37°C under orbital shaking (180 rpm) overnight. So, 300 l of inoculum was inserted in 15 ml of fresh M-H broth to ensure the logarithmic phase. The inoculum was diluted 1×10^6 CFU/mL (units formed colonies/ml) and a volume of 50 l was added in each well. Bacterial cells were exposed to SmCo-NPs (from 5×10^6 to 0.8×10^5), non-toxic for cells. The growth of S. aureus was evaluated after 20 hours by recording the OD_{600} nm through a microplate reader.

2.5.2 Time Killing

In order to monitor the growth of S. aureus 6538 in the time, after exposure to SmCo-NPs, Time Killing was conducted in accordance with the American Society for Testing and Materials International (ASTM) Standard guidelines. In particular, 1×10^7 (2×MIC), 5×10^6 (MIC)

and 2.5×10^6 (1/2×MIC) of SmCo-NPs and 1×10^5 CFU/ ml of bacteria cells were inserted in M-H broth in a final volume of 1ml/tube. Vancomycin (10 g/ ml) was used as positive control and untreated bacteria were used as negative controls.

The bacterial cells of S. aureus exposed to SmCo-NPs were incubated at 37°C in agitation for a precise time, in detail 0, 1, 4, 6 and 20 hours. After the incubation period, an aliquot of 50 µl of each suspension was plated on M-H agar plate and the rising colonies were counted the following day.

2.6 Statistical analysis

Statistical analysis was executed by One-way ANOVA followed by Dunnett's multiple comparisons tests, and graphs were engendered through GraphPad Prism ver. 8.2.1 program for macOS (GraphPad Software, San Diego, CA, USA, [4]). All tests were carried out in triplicate and displayed as ± Standard Deviation (SD).

3. Results and discussions

3.1 Characterization of SmCo-NPs

SmCo-NPs were produced by a physical approach called PLAL, as described in paragraph 2.1. Different strategies have been used for their characterization. First, TXRF analysis was conducted in order to investigate the chemical composition of SmCo-NPs. As shown in Fig. 1, from the nanostructures produced by PLAL, it is possible to observe only the X-ray fluorescence lines of 5 KeV and 8.5 KeV characteristics of Sm and Co, respectively.

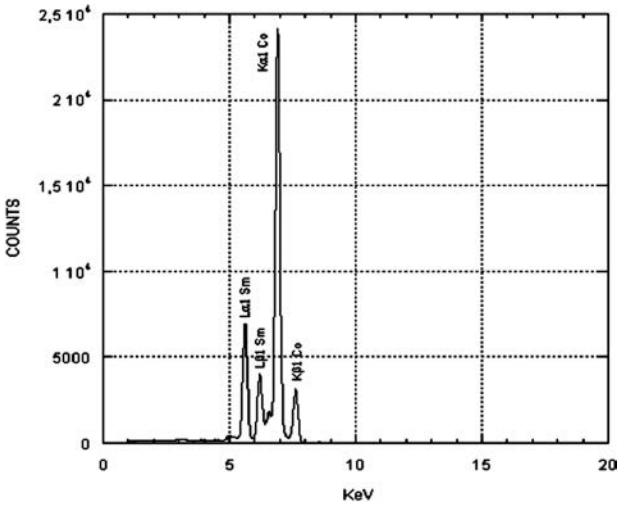

Fig. 1. TXRF spectra of SmCo Nanoparticles product by PLAL.

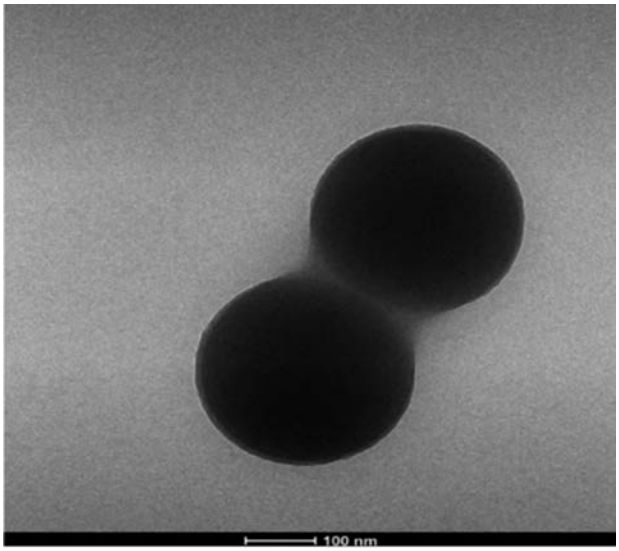

Fig. 2. TEM analysis of SmCo-NPs produced through PLAL methodology.

Therefore, we can say that SmCo nanoparticles produced by PLAL have a good chemical homogeneity.

However, in order to examine the symmetry and size of SmCo-NPs was performed TEM analysis, as shown in Fig. 2. The nanoparticles produced by us, through the PLAL strategy, appeared with spherical symmetry and their average radius size was about 100 nm.

To ensure that the magnetic properties of SmCo-NPs were retained after the PLAL process, the MOKE measurement was performed. Fig. 3, in fact, represented the magnetic hysteresis obtained with a magnetic-optical analysis that used the Kerr effect of light, when a sample of SmCo nanoparticles was inserted within a magnetic field.

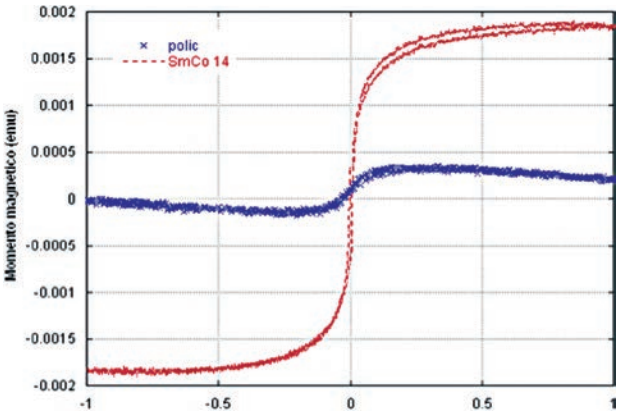

Fig. 3. Magnetic Hysteresis of SmCo-NPs.

3.2 Cell viability assessment

Before carrying out antibacterial assays, the assessment of cell viability was conducted on human keratinocyte cells (HaCaT cells) through the MTT test, after 24 hours. Cytotoxicity was expressed as a viability percentage compared to the untreated cells monolayer (ctrl+).

From the graph in Fig. 4, we can say that only 1×10^7 SmCo-NPs exhibited a minimum of cytotoxicity, showing however 60% of cell viability. SmCo-NPs amounts between 5×10^6 and 0.8×10^5 showed no cytotoxicity on human keratinocytes. For this reason, this SmCo-NPs range has been used in the next antibacterial tests.

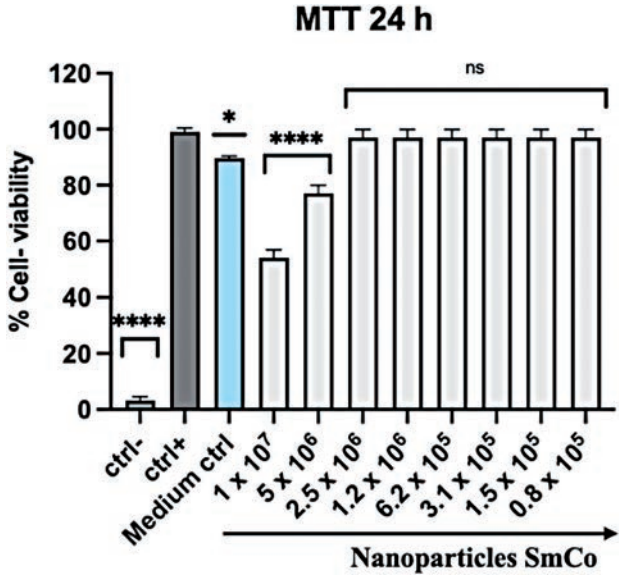

Fig. 4. The evaluation of the cytotoxicity of SmCo-NPs was conducted, through the MTT test, on HaCat cells, after the cell monolayer was treated with SmCo-NPs ranging from 1×10^7 to 0.8×10^5. The untreated cell monolayer was used as a positive control (ctrl+), the DMSO 100% as a negative control (ctrl-), and finally the PBS 1x (medium in which SmCo-NPs were dissolved) as a control of the medium. The statistical significance has been evaluated versus the ctrl+. Data represent the mean standard deviation (SD) of three independent experiments. ****: p-value < 0,0001, *: p-value = 0.0244 ns: not significant.

3.3 Antibacterial activity of SmCo-NPs

3.3.1 MIC method

In order to investigate the antibacterial activity of SmCo-NPs against S. aureus 6538 ATCC plate microdilution assay was conducted. The test was performed as described in paragraph 2.5.1. However, as shown in Fig. 5, after 20 hours of treatment 5×10^6 and 2.5×10^6 SmCo-NPs inhibited the growth of the bacterium by about 90% and 60% respectively. Smaller amounts of SmCo-NPs (1.2×10^6, 6.2×10^5, 3.1×10^5, and 0.8×10^5) inhibited the growth of S. aureus 6538 ATCC by about 40, 30, 20, and 10% respectively, in a dose-dependent manner. During the test procedure, bacterial cells of S. aureus 6538 ATCC (not treated with nanoparticles) were used as a negative control (ctrl-) instead vancomycin was used as a positive control.

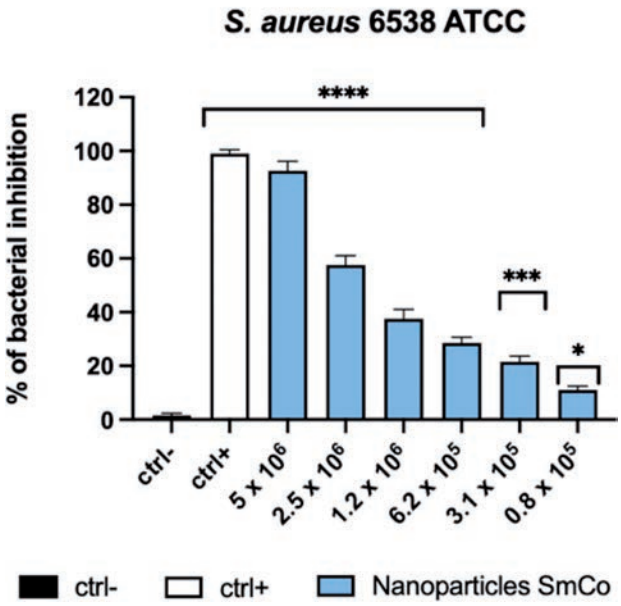

Fig. 5. Antibacterial activity was investigated through the MIC method. In particular, the S. aureus 6538 ATCC cells were exposed to SmCo-NPs ranging from 5×10^6 to 0.8×10^5. Untreated S. aureus 6538 ATCC cells were used as a negative control (ctrl-), and Vancomycin was used as a positive control (ctrl+). The statistical analysis was determined using ANOVA with Dunnett's test for multiple comparisons. Significance versus the ctrl- or untreated S. aureus 6538 ATCC cells have been evaluated. ***: p-value < 0,0001, ***: p-value = 0,0003, *: p-value = 0,0260.

3.3.2 Time Killing

However, to study how SmCo-NPs acted on the bacterial culture of S. aureus 6538 ATCC, we examined the growth of the bacterium over time, in particular at 0, 1, 4, 6, and 20 hours after exposure to different concentrations of SmCo-NPs (5×10^6) MIC, (2.5×10^6) 1/2 MIC and (1×10^7) 2×MIC. In detail, in Fig. 6, the curve of untreated bacteria ctrl- has increased exponentially over time. Treatment with 2.5×10^6 SmCo-NPs did not induce significant changes in the bacterial culture of S. aureus 6538 ATCC. No increase in bacterial load was recorded using 5×10^6 SmCo-NPs (MIC), and 1×10^7 SmCo-NPs (2x MIC), indicating a bacteriostatic action

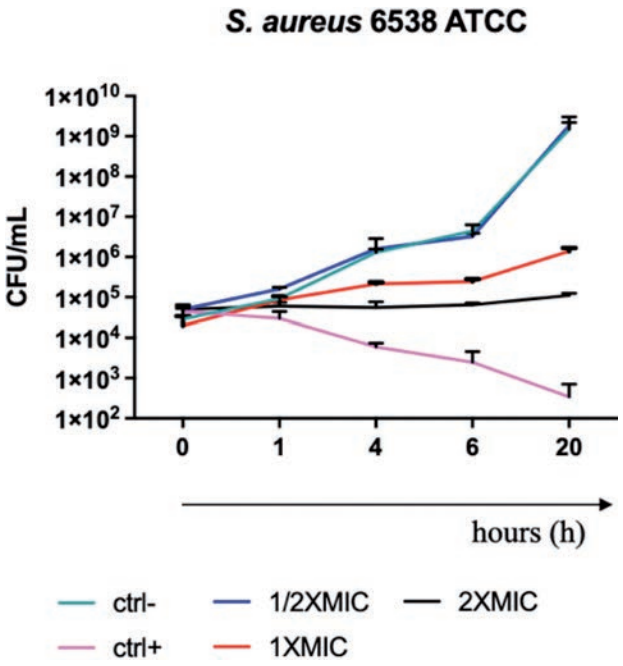

Fig. 6. The study of the growth of S. aureus 6538 ATCC over time (0, 1, 4, 6, and 20 hours) after exposure to different concentrations of SmCo-NPs, in particular 2.5×10^6 (1/2 MIC), 5×10^6 (MIC), and 1×10^7 (2x MIC) was conducted through time killing assay. S. aureus 6538 ATCC untreated cells were used as a negative control (ctrl-), and Vancomycin was used as a positive control (ctrl+).

of nanoparticles. Our nanoparticles acted on bacterial replication demonstrating as bacteriostatic agents.

4. Conclusions

Pulsed Laser Ablation in liquid (PLAL) has been shown to be a top-down approach for nanoparticles production [11]. Usually, the techniques mainly used for the synthesis of nanoparticles make use of some toxic chemicals, such as reducing or stabilizing agents. PLAL, however, has a low environmental impact as it does not require precursors or reducing chemicals and is able to produce high-purity colloids without generating harmful waste [12]. In this work we synthesized magnetic nanoparticles using PLAL, starting from a SmCo target, permanent magnet, in order to investigate their antibacterial potential against S. aureus, the main pathogen involved in the much-feared nosocomial infections [13]. From the results obtained, we can say that SmCo-NPs have not been shown to be toxic on human keratinocyte cells (HaCaT cells), being this type of cells a model for epidermal cells, our SmCo-NPs could be used as skin cleansers [14, 15]. However, through the MIC and Time Killing assays, we can say that SmCo-NPs have been able to inhibit the growth of S. aureus 6538 ATCC showing as bacteriostatic agents, in vitro.

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