



Pulsed laser ablation of magnetic nanoparticles as a novel antibacterial strategy against gram positive bacteria



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ABSTRACT

Nanotechnologies comprise approaches able to synthesize, manipulate and visualize matter at the nanometer scale offering numerous advantages, especially in the biomedical field. In particular, nanoparticles represent a promising tool in the medical field to overcome antimicrobial resistance, which represent current serious problem. Furthermore, nanoparticles use multiple mechanisms to target microorganisms, rendering difficult the development of antimicrobial resistance. In the present, we used a Samarium-Cobalt (SmCo) target, permanent magnet, to synthesize nanoparticles (Nps) by Pulsed Laser Ablation in Liquid (PLAL). To evaluate their possible antimicrobial effect against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Streptococcus mutans*, we performed minimal inhibitory concentration (MIC) assays. In addition we evaluated their ability to affect human keratinocyte cells viability. By time killing assay, we monitored bacterial growth after exposure to Nps-SmCo. PLAL is an innovative, cheap, fast and safe nanotechnology to allow the synthesis of small nanoparticles that could be exploited for applications as novel antimicrobials.

1. Introduction

Bacterial infections represent one of the leading causes of death worldwide [1,2]. Currently, antibiotics are considered the main treatment against bacterial infections, but their excessive use has led to the rapid emergence of multidrug resistant bacteria. Longer periods of hospitalization with increasing health costs and higher mortality rates are a direct consequence of the continuous spreading of antimicrobial resistance [3,4]. It has been estimated that approximately 33.000 and 35000 people die yearly in Europe and the United States, respectively, as a direct result of antimicrobial resistance [5]. This scenario led to an increase in public health costs and the drastic reduction of therapeutic treatments, causing high mortality in infected patients [4,6]. The World Health Organization (WHO) anticipates that by 2050 none of the antibiotics currently available will be effective for some bacterial infections treatment [7,8]. Several bacterial species are already resistant to many

currently available antibiotics, including the latest generation ones [9, 10]. Markmark *et al.* reported an increased incidence of *Staphylococcus aureus* and *Enterococcus faecium* strains resistant to linezolid and daptomycin. In particular, the linezolid resistance rate between 2014 and 2018 was 1.6% and 0.28%, for *E. faecium* and *S. aureus*, respectively. While daptomycin resistance was about 1.1 %, for both strains [11]. Cabrera-Contreras *et al.* monitored multi-resistant *S. epidermidis* strains for eight years (2006 to 2013) in a children's health care unit in Mexico City. During the period under observation a large number of *S. epidermidis* multi-resistant strains with resistance to methicillin, beta-lactam, fluoroquinolones, and macrolides was detected [12].

In addition to antibiotic resistance, also the ability of bacteria to form biofilms has shown to be a limiting factor for use of empirical therapies. The worrying scenario described, highlights the need to search for new antibacterial agents and innovative therapeutic strategies. Contextually, nanotechnologies offer numerous advantages to overcome compliances

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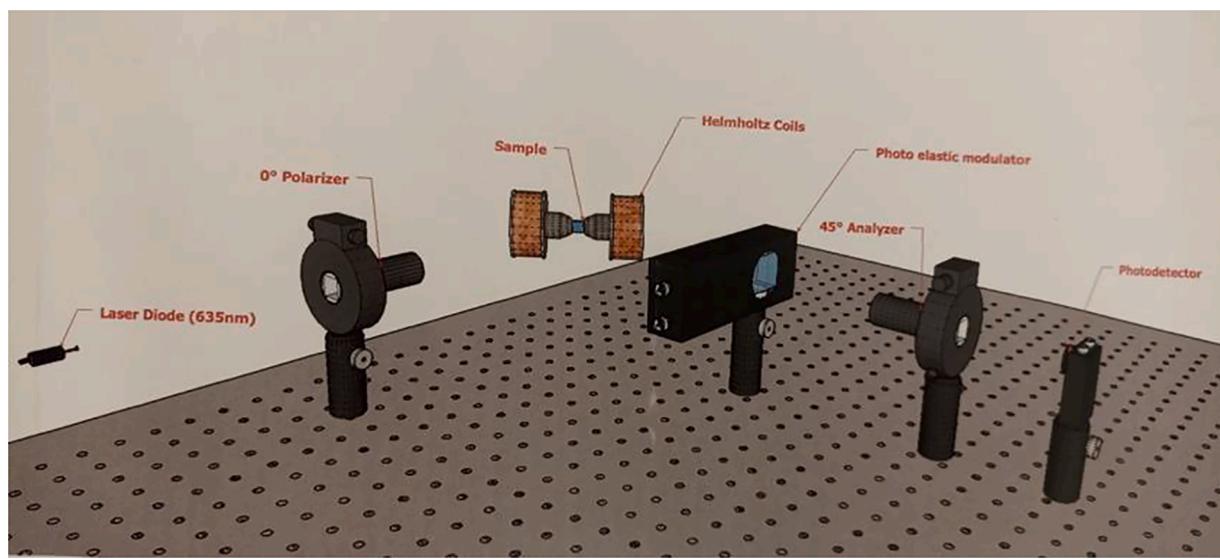


Fig. 1. Magneto-optic measurement set-up.

associated with the development of multidrug-resistant pathogens [13, 14]. Nanotechnologies are considered a great scientific advance of the 21st century, with a deep social and economic impact [15, 16].

Antimicrobial properties associated with silver, copper, zinc and gold nanoparticles have been widely described in literature. The mechanisms of action were related to the formation of reactive oxygen species (ROS) [17], enzymatic inhibition [18], protein degradation, DNA damage and bacterial cell wall destruction [19]. In particular, as reported by Pelgrift *et al.*, different types of nanoparticles use multiple and simultaneous mechanisms to combat microbes [20]. An example are nitric oxide-releasing nanoparticles (NO Nps), which exert their antimicrobial action through largely reactive nitrogen oxide intermediates (RNOS) that react with amino acid residues, Cys, Met, Tyr, Phe of bacterial proteins. In addition, RNOS causes direct nitrosative damage to DNA including breakage of the filament, formation of abasic sites, and deamination of cytosine, adenine and guanine. RNOS, also, causes increased generation of hydrogen peroxide (H_2O_2) and alkylating agents that themselves damage bacterial DNA. All these multiple mechanisms exerted by NO Nps make it unlikely the development of resistance, as multiple and simultaneous genetic mutations would be needed in the same bacterial cell.

Loo *et al.* have shown that the synthesis of silver nanoparticles (Ag-Nps) using plant extracts is a green, valid and easy alternative to chemical synthesis; it does not require the use of cell cultures and asepticity, showing advances respect to chemical methods. These suitable nanoparticles synthesized from pu-erh tea leaves extract showed minimum inhibitory concentration (MIC) against *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Salmonella enteritidis* of 7.8, 3.9, 3.9, 3.9 $\mu\text{g}/\text{ml}$, respectively [21]. Samarium-cobalt (SmCo) is a rare earth magnet [22]. Currently, no knowledge related to SmCo nanoparticles (Nps-SmCo) applied to microbiology has been reported. Therefore, our study described the synthesis and physical-chemical characterization of Nps-SmCo. Pulsed Laser Ablation in Liquid method is considered one of the promising techniques for the production of particles from the nanometric dimension monodisperse. PLAL exhibits several features such as stability of the product, low cost, high purity, simplicity, good control on the size and morphology of the product [23]. The size, shape, and morphology of the nanoparticles produced by laser ablation in liquid depend on several ablation parameters, such as laser fluence, pulse width, repetition rate, temperature, ablation time and wave-length [24]. The PLAL synthesis of SmCo Nps will be discussed using X-Ray Diffraction Data, SEM images, Zeta Potential results and magnetic characterizations (MOKE). Finally, we reported, for the first

time, the antimicrobial activity of Nps-SmCo against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Streptococcus mutans*. These findings pay the way for the development of new drugs to counteract Gram-positive bacterial infections.

2. Materials and methods

2.1. Nps-SmCo synthesis

We used to synthesis Nps-SmCo the PLAL method with these experimental conditions:

Laser Nd:Yag operating at $\lambda = 532 \text{ nm}$, 10 ns pulse width, frequency 10 Hz, 40 mJ/shot energy and the laser beam is focused on area (3-5) mm^2 of the target, the fluence used is $0,8 \text{ J/cm}^2$. Geometric set-up: the laser beam impinges on target surface forming an angle of 90° with respect to the surface of the SmCo target. The target material is insert in a quartz-cuvette and it is filled with 1 ml Phosphate Buffer Saline (PBS) 1x liquid (Gibco-BRL-Thermo Fisher Scientific, Waltham, MA, USA, 02451). During PLAL process is used atmospheric pressure, ambient temperature and the duration time is of 30 minutes.

The target material to produce Nps is SmCo and the same chemical-physical measurements are reported.

2.2. Nps-SmCo characterization

In situ measurements with a Total X Ray Fluorescence (TXRF) have been realized to check chemical homogeneity of SmCo Target. The system for the fluorescence measurements is constituted by a X ray Tube, with Ag anode and Be windows with a thickness of 125 μm , operating at 30 Kv and 10 A. The X-Ray collimator focuses the beam at 3 mm diameter on the surface sample. The TXRF data are collected on the surface of 3 mm x 3 mm area. Transmission Electron Microscopy (TEM) is used to assess Nps-SmCo morphology (TEM FEI TECNAI G12 TWIN 120 kV). Other measurements have been conducted to understand if the Nps-SmCo show magnetic characteristics. Magneto-Optic Kerr Effect (MOKE) system, has been used to control the Nps magnetic properties. MOKE system is based on the Kerr Effect. The polarization plane of linear polarized light rotates after reflection on a magnetized surface as a consequence of the Spin-orbit interaction. MOKE measurement system shows different advantages, such as high sensitivity (up to monolayer resolution), spatial and temporal resolution, straightforward in situ implementation, relatively easy installation and use. MOKE experimental system is realized with: $\lambda = 635\text{nm}$, 4.5 mW laser diode, Glan-

Table 1
Nps-SmCo number used for experimental procedures.

μL	Number Nps-SmCo
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
0.78	0.78×10^5

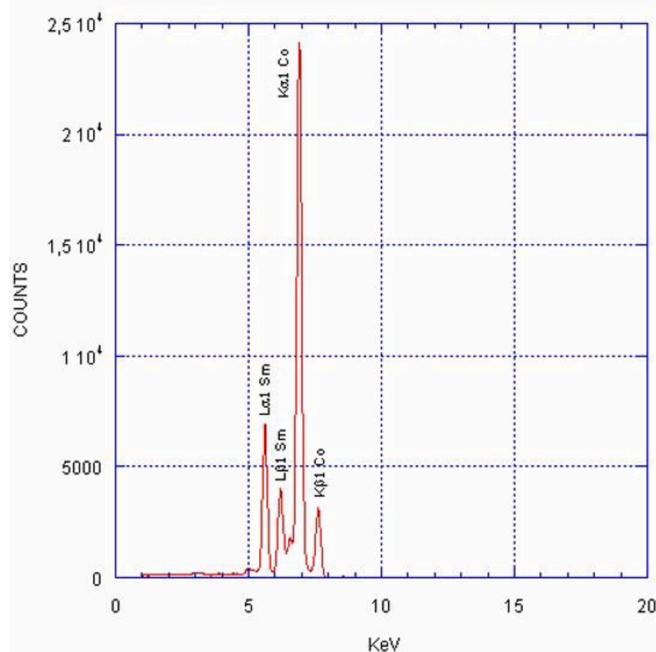
Thompson polarizers, Self- made Helmholtz coils (or solenoid), Photo elastic modulator (PEM), fast photo detector and Lock-in amplifier is used to collect the dates. Experimental MOKE setup is reported in Fig. 1.

X-Ray Diffraction analysis using a Siemens D5000 with Cu Anode X-Ray tube is reported to control crystallographic structure of the Nps-SmCo. Finally, the charge present on the surface of nanoparticles was determinate using the Zetasizer Nano S Malvern Panalytical instrument. The Zeta Potential analysis was measured at 25°C.

2.3. Quantitation of Nps-SmCo

Nps-SmCo were counted by Scanning Electron Microscopy (SEM). 5 μL of the PBS 1x solution containing our Nps-SmCo were deposited on several stab for SEM measurements. About 20 SEM images were captured and for each stab, one single drop was deposited on stab surface using a calibrated micropipette and after that the PBS is removed. This method we can avoid Nps aggregation on SEM stab. The images were processed via Image J. All the nanoparticles present in the images were then counted and the total volume calculated by Image J. Starting by volume of the single nanoparticle we were able to derive the number of nanoparticles in a volume of 5 μL. We have computed that 10^8 nanoparticles are present in the 1 ml volume sample. For experimental test we used a number of nanoparticles reported in Table 1.

a)



2.4. Cytotoxicity evaluation

Produced Nps-SmCo were sterilized for 30 minutes by UV lamp. As demonstrated by Dutz *et al.*, ultraviolet radiation does not alter the average size of nanoparticles, particle size distribution as well as zeta potential [25]. In the present we tested the Nps-SmCo to evaluate their antimicrobial activity. First of all, their toxicity was assessed by 3-(4, 5-dimethylthiazol-2yl)-2,5- diphenyltetrazolium bromide (MTT) assay after 24 hours on Human keratinocyte (HaCaT) cells (American Type Culture Collection, ATCC). Cells were plated in a 96- well flat- bottomed plate (10^4 cells/well) and cultured 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 % CO₂ [26]. All reagents were purchased from Gibco-BRL (Thermo Fisher Scientific, Waltham, MA, USA). Then, cells were treated with different number of Nps-SmCo as previously reported in Table 1. Dimethyl sulfoxide (DMSO) was used as a negative control.

After 24 h, the MTT was added for 3 h and cells converted it into formazan. Finally, DMSO was used to re-solubilize the formazan and the cell viability was assessed by recording the absorbance at wavelength of 570 nm with a Bio-Rad microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Bacterial strains

The bacteria strains used to evaluate the antibacterial activity were *Staphylococcus aureus* strain ATCC 6538, *Staphylococcus epidermidis* strain ATCC 12228, *Enterococcus faecalis* strain ATCC 29212 and *Streptococcus mutans* strain ATCC 35668. Each strain was plated on Brain Heart infusion agar plates (BHI-agar) (Oxoid, Cheshire, UK) and incubated overnight at 37°C.

2.6. Determination of minimum inhibitory concentration (MIC)

Antimicrobial activity assays were performed using the broth

b)

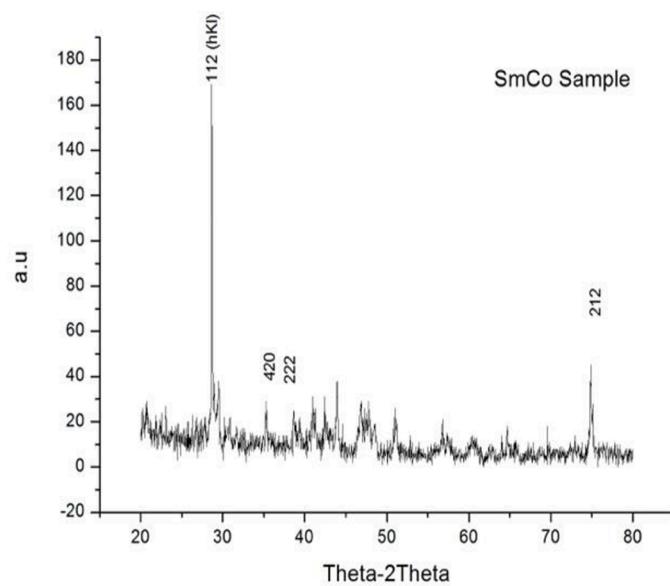
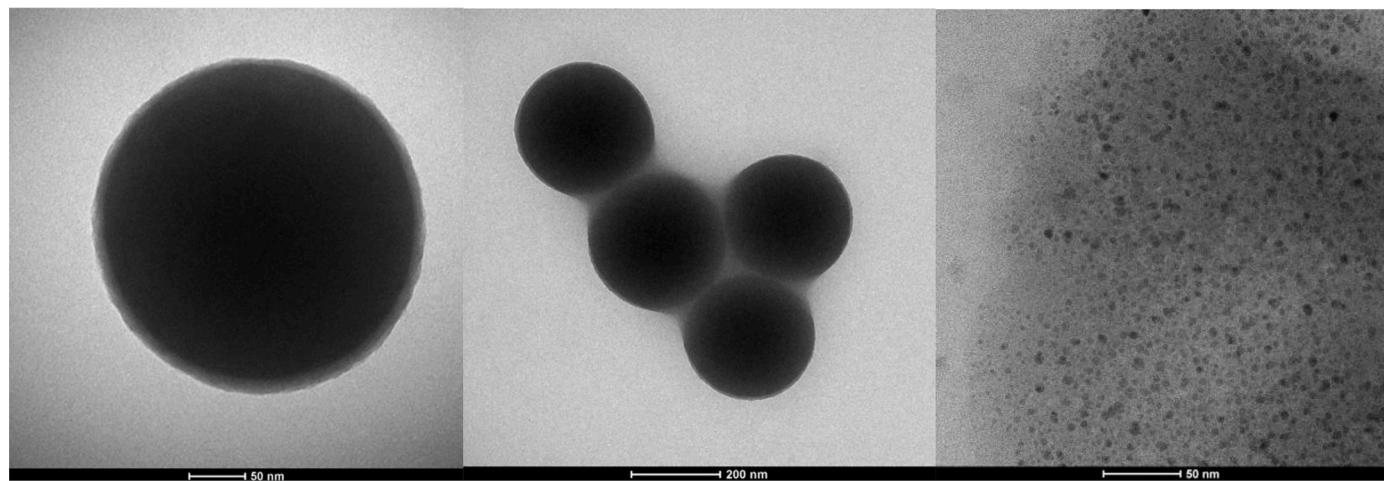


Fig. 2. a) Total X-Ray Fluorescence spectra of SmCo target material used to form nanoparticles, the data showed the presence of only SmCo X-ray emission lines from the surface of the target material. b) Theta-2Theta X-Ray diffraction spectra of Nps-SmCo.

a)



b)

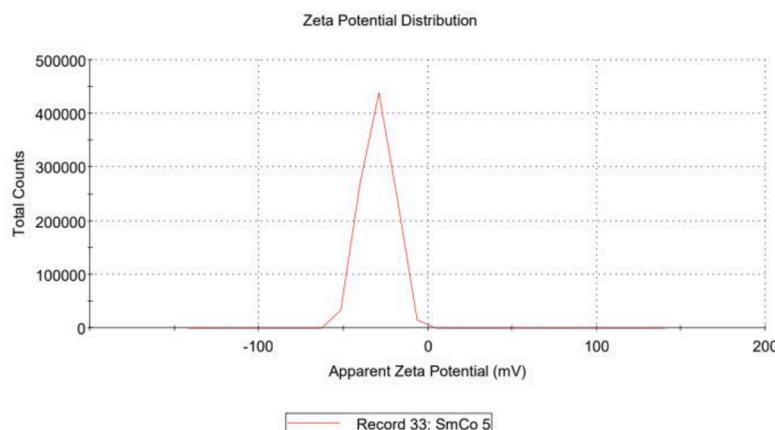


Fig. 3. a) Transmission Electron Microscopy images of SmCo nanoparticles. **b)** Zeta potential analysis of Nps-SmCo.

microdilution method, in accordance with National Committee on Clinical Laboratory Standards (NCCLS) guidelines, using sterile 96-well plates. Briefly, fresh colonies of each strain grown on BHI-agar plates were inoculated in BHI-broth (Sigma-Aldrich, USA) and incubated at 37°C under orbital shaking (180 rpm) overnight. The following day, bacterial suspension was transferred into fresh BHI-broth and incubated at 37°C under orbital shaking (180 rpm) up to the OD₆₀₀ value = 0.5 (1 × 10⁸ CFU / mL). Three serial dilutions were performed to determine the final bacteria concentration (1 × 10⁶ CFU / mL). Established number of Nps-SmCo (Table 1) were by inoculation of each dilution into a well with 50 µL of bacterial suspension (obtaining a final concentration of 1 × 10⁵ CFU / mL). Vancomycin was used as positive controls for Gram-positive bacteria. The antibacterial activity was assessed after 20 hours of incubation at 37°C, measuring the optical density at 600 nm [27].

2.7. Time killing

In order to monitor bacterial growth over time after exposure to Nps-SmCo, the time-killing assay was performed in accordance with the American Society for Testing and Materials International (ASTM) Standard guidelines. 1 × 10⁷ (2 × MIC), 5 × 10⁶ (MIC) and 2.5 × 10⁶ (1/2 × MIC) of Nps-SmCo and 1 × 10⁵ CFU / mL of bacteria were added to BHI

broth in a final volume of 1mL/tube. Vancomycin (10 µg/ mL) and untreated bacteria were used as positive and negative controls, respectively. The bacterial suspension was incubated at 37°C under orbital shaking (180 rpm). After the incubation period (0, 1, 3, 6 and 20 hours) an aliquot of 50 µL of bacterial suspension was subjected to serial dilutions and plated on BHI-agar. Plates were incubated at 37°C for 20 hours and the following day colonies were counted to define CFU / mL values.

2.8. Statistical analysis

Statistical analysis was performed by One-way ANOVA followed by Dunnett's multiple comparisons tests, ad graphs were generated using GraphPad Prism ver. 8.2.1 for macOS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). All tests were performed in triplicate and expressed as ± Standard Deviation (SD).

3. Results and discussion

3.1. Characterization of Nps-SmCo

To assess the chemical properties of the target used to produce Nps-

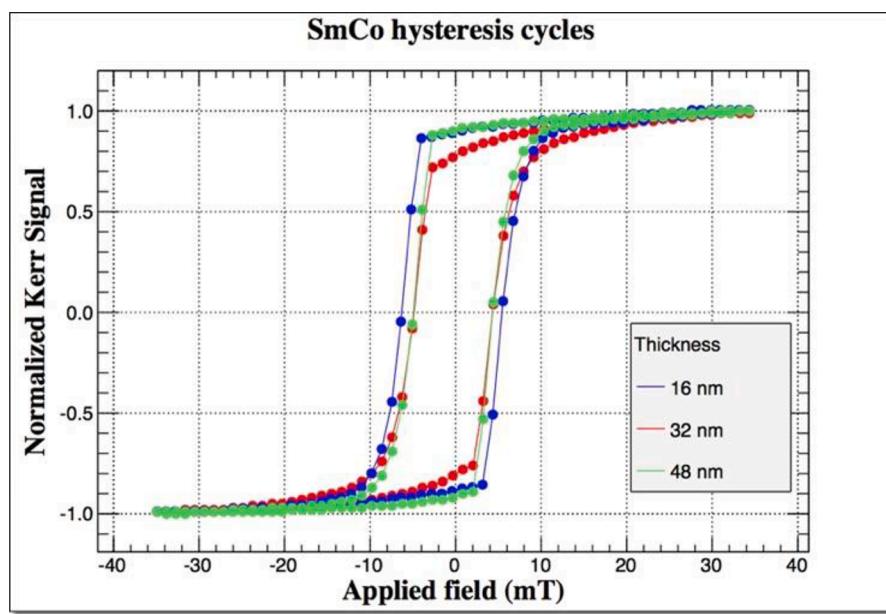


Fig. 4. The graph shows Hysteresis curve of Nps-SmCo, by MOKE measurements.

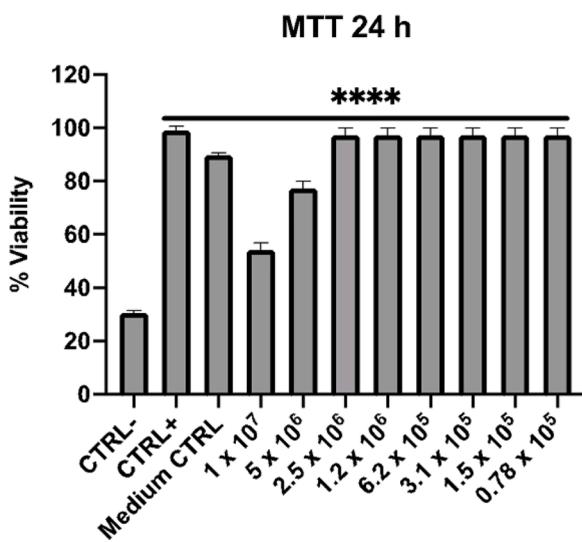


Fig. 5. Effect of Nps-SmCo on cell viability. Statistical significances are referred to the negative control and were determined by analysis of variance (ANOVA). Dunnett's test was used for multiple comparisons with the control (**** p value < 0.0001; *** p-value < 0.0002; ** p-value < 0.002; ns: not significant).

SmCo, we used Total X-Ray Fluorescence measurements. As reported in Fig. 2 a, X-Ray fluorescence showed that SmCo peaks are emitted by the target material after exposure to X-Ray beam. These characterizations are important to establish absence of other material after laser treatment. During laser ablation treatment to produce Nps, we used high laser energy and this experimental condition can produce other nanoparticles inside the cuvette. Total X-Ray Reflection Fluorescence system showed SmCo purity, assessed the presence of only SmCo x-ray beam in the energy range 5–8 Kev. We collected the spectra in the range 2–20 Kev to control the chemical quality of the target material. The spectra showed only characteristic X-Ray emission peaks of SmCo material.

Theta-2Theta spectra reported in Fig. 2 b shows characteristic diffraction peaks and using JCPDS 30-457 and other SmCo peaks appear in literature [28], so we can assign the peaks at SmCo material and we conclude that Nps-SmCo are random orientation with same diffraction

peak of monoclinic structure.

Size and structural morphology of nanoparticles were characterized by using TEM as shown in Fig. 3 a. Our nanoparticles appeared perfectly spherical with a diameter between 10–60 nm. While in Fig. 3b we report Zeta Potential analysis, the data shown that nanoparticles have a negative charge value of -28.9 mV and we conclude that SmCo nanoparticles do not form aggregates but are evenly dispersed in liquid.

In order to verify magnetic properties of Nps-SmCo, we report the MOKE experimental measurement. The Nps have grown up on thin amorphous film substrate, the spectra showed Nps magnetic characteristic correlated with particles dimensions, the magnetic coercive Field is high about 4.5 mT for Nps-SmCo material, as shown in Fig. 4.

3.2. Effect of Nps-SmCo on cell viability

By MTT assay we assessed the effect of Nps-SmCo on cell viability of HaCaT cells. As reported in Fig. 5, we can confirm that Nps-SmCo are not toxic at lower number of Nps-SmCo tested, showed a percentage of cell viability of 100% between 2.5×10^6 and 0.78×10^5 Nps-SmCo. 5×10^6 Nps-SmCo reduced cell viability of 20%, while the highest number of Nps-SmCo tested (1×10^7) reduced viability of 50% respect to the positive control.

3.3. Antibacterial properties of Nps-SmCo

Antibacterial activity of the Nps-SmCo, against four species of Gram-positive bacteria, was evaluated using plate microdilution method. After 20 hours of treatment with 5×10^6 Nps-SmCo, the growth inhibition rate of *S. aureus* was of 83%, while an inhibition of 70%, 75% and 78% was observed for *S. epidermidis*, *S. mutans* and *E. faecalis*, respectively. At a lower amount (2.5×10^6), 60 and 58% of inhibition was detected against *S. epidermidis* and *E. faecalis*, while an inhibition of 37% and 39% was recorded against *S. aureus* and *S. mutans* (Figure 6).

Obtained results were confirmed by monitoring bacterial growth over time, for 20 hours. Time killing assay showed a similar trend against all analyzed bacterial species. In detail, the curve of untreated bacteria increased exponentially over time. The treatment with 2.5×10^6 Nps-SmCo (% × MIC) did not induce relevant change in bacterial growth, showing viable cell counts comparable to negative control. No increase in bacterial load was recorded at MIC and 2 × MIC value between 0 and 20 hours compared to the initial bacterial density,

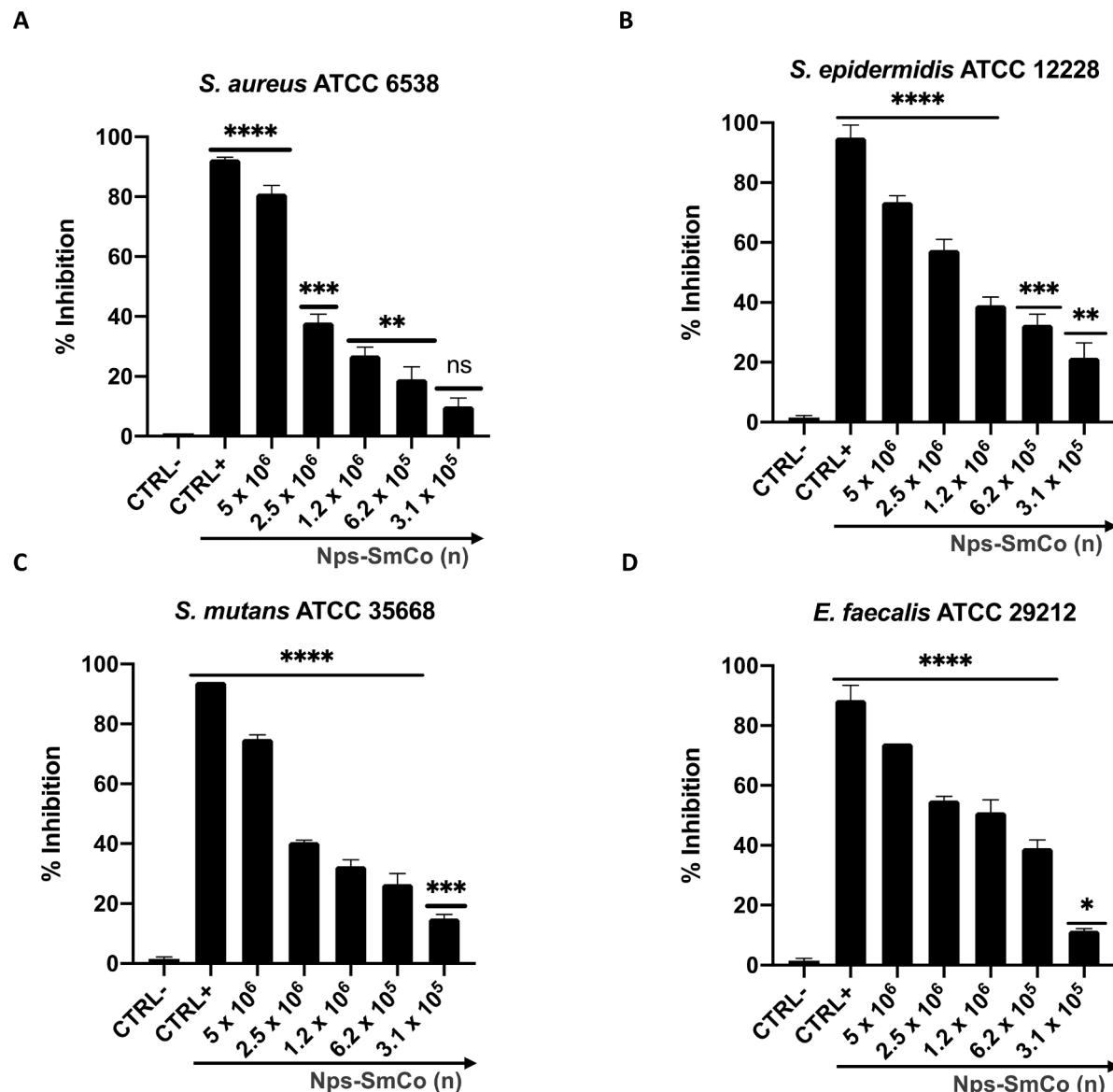


Fig. 6. Antibacterial activity of Nps-SmCo against *S. aureus*, *S. epidermidis*, *E. faecalis* and *S. mutans*. Statistical significances are referred to the negative control and were determined by analysis of variance (ANOVA). Dunnett's test was used for multiple comparisons with the control (**** p value < 0.0001; *** p-value < 0.0002; ** p-value < 0.002; ns: not significant).

indicating a bacteriostatic action of the nanoparticles (Fig. 6). A gradual reduction in bacterial load occurred after treatment with vancomycin (positive control) showing a decrease of 1×10^2 times for *S. aureus*, *S. epidermidis*, *E. faecalis* and 1×10^4 for *S. mutans*, respect to the initial bacterial density (Fig. 7).

Our finding highlighted for the first time the role of Nps-SmCo against Gram-positive bacteria. No data are currently available using this nanotechnology, to counteract bacterial infections. The mechanism of action could be due to the interaction of the Nps-SmCo with the cell wall surface, causing a structural damage. Other studies will be necessary in order to address more precisely the mechanism of action of the Nps-SmCo.

4. Conclusion

This study demonstrated that PLAL is an innovative and safe method to synthesize Nps from a target of SmCo, permanent magnet. As reported by R. A. Ismail et al., PLAL is a method that has many advantages over conventional procedures for the synthesis of nanoparticles [29]. It

is an economical system, it can produce high purity nanoparticles, it shows a good control of both the size and the morphology of the product, preserving the stoichiometry, all with short reaction times. In recent years, advances in Nanosciences and Nanotechnologies have led to incredible improvements in biology and medicine. Nanoparticles usually ranging in size from 1 to 100 nanometers have unique properties compared to their mass equivalent. They are small agents, whose size is similar to that of most biological molecules and structures. And it is precisely this characteristic that makes them excellent candidates for application in biomedical research *in vitro* and *in vivo*. In fact, nanoparticles have different uses mainly in the targeted administration of drugs, in imaging, and in their use as sensors [24]. In our study we decided to use them as an antimicrobial agent in order to target Gram positive bacteria for an innovative alternative therapy to common antibiotics. Indeed the Nps-SmCo showed significant antibacterial activity against Gram-positive bacteria *S. aureus*, *S. epidermidis*, *E. faecalis*, *S. mutans*. Through Time Killing experiments we have demonstrated that our nanoparticles are bacteriostatic agents, in fact they block bacterial replication through multiple and simultaneous mechanisms such as DNA

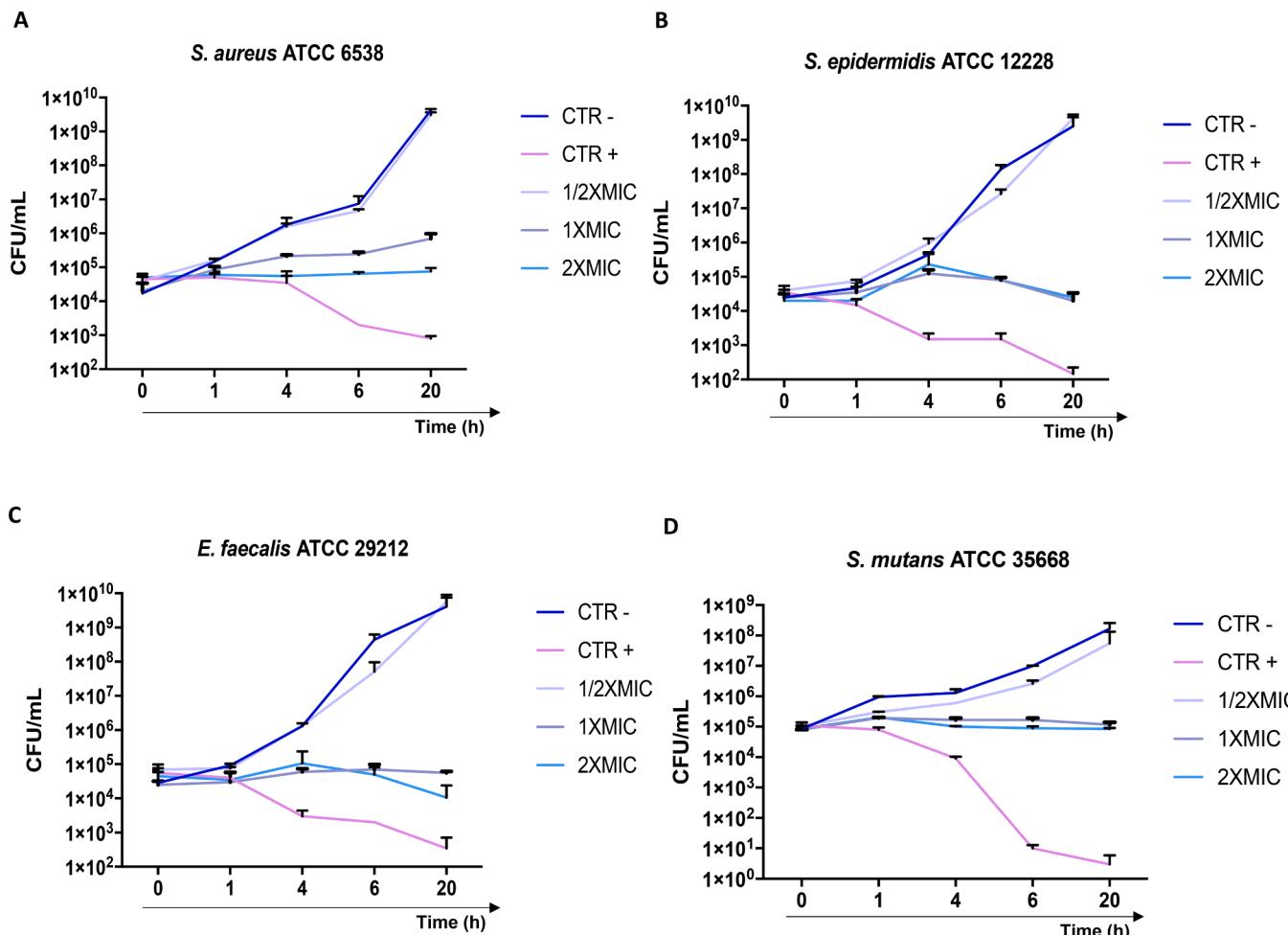


Fig. 7. Time killing kinetics of Nps-SmCo against *S. aureus*, *S. epidermidis*, *E. faecalis* and *S. mutans*.

damage, the formation of reactive oxygen species (ROS), the degradation of essential proteins, and the destruction of the bacterial wall. All these contemporary mechanisms exerted by nanoparticles make it impossible to develop resistance because multiple genetic mutations would be needed in the same bacterial cell [30]. For this reason, nanoparticles could be a valid alternative to common antibiotics, thus solving a worldwide problem such as bacterial resistance.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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