

Article

Multifunctional Hydroxyapatite–Barium Titanate Coatings with Green-Synthesized Silver Nanoparticles for Orthopedic Implants: Piezodynamic, Biological, and Antibacterial Evaluation

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Highlights

- Atmospheric plasma spraying (APS) preserved Hydroxyapatite (HA), barium titanate (BT), and silver nanoparticles (nAg) phases, achieving >43 MPa adhesive strength.
- BT imparted bone-like piezoelectricity ($d_{33} \sim 13$ pm/V), unaffected by nAg.
- Coatings exhibited high protein affinity, non-cytotoxicity, and osteogenesis.
- 1% nAg completely inhibited *E. coli*, *S. aureus*, and *P. aeruginosa* in 24 h.

Abstract

Infection associated with orthopedic implants remains a major challenge in the development of biomimetic materials for bone tissue engineering. Surface modification strategies are widely employed to transform bioinert metallic substrates into bioactive interfaces that promote tissue integration while preventing bacterial colonization. This study investigated the effect of incorporating silver nanoparticles (nAg) into hydroxyapatite–barium titanate (HA–BT) coatings on their structural, mechanical, piezoelectric, biological, and antibacterial properties. Raw materials were characterized by X-ray diffraction (XRD) and transmission electron microscopy (TEM), whereas coatings were evaluated by XRD, scanning electron microscopy (SEM), tensile adhesion testing, electrochemical analysis, piezoresponse force microscopy (PFM), cell viability, and antibacterial assays. The coatings exhibited secondary phases, including β -tricalcium phosphate (β -TCP), tetracalcium phosphate (TTCP), and calcium oxide (CaO), generated during atmospheric plasma spraying (APS), while no silver oxide phases were detected after nAg incorporation. SEM observations revealed homogeneous phase distribution and strong coating–substrate adhesion. The addition of nAg did not significantly affect adhesive strength (44.58 ± 2.2 MPa for HA30BT and 43.28 ± 2.8 MPa for HA30BT–nAg). Protein adsorption studies indicated



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moderate albumin affinity ($K_{ads} = 0.45$) for nAg-containing coatings. Both coatings exhibited piezodynamic activity with d_{33} values of approximately 13 pm/V. MTT assays confirmed non-cytotoxic behavior, with viability reductions below 2%. Osteocalcin expression demonstrated comparable osteogenic activity in coatings with and without nAg, regardless of low-intensity pulsed ultrasound stimulation. These findings highlight the potential of HA–BT–nAg coatings as multifunctional surfaces for bone regeneration and infection prevention.

Keywords: surface functionalization; Ti6Al4V; coating; biomimetic; hydroxyapatite; piezoelectricity; LIPUS; barium titanate; antibacterial efficacy; silver nanoparticles

1. Introduction

Orthopedic implants play a crucial role in the treatment of bone defects and tissue regeneration. Titanium-based alloys such as Ti6Al4V are widely used because of their excellent mechanical strength, corrosion resistance, and biocompatibility [1,2]. However, these alloys are biologically inert, showing limited interaction with surrounding tissues, which may compromise long-term osseointegration [3–5].

A critical determinant of biomaterial acceptance is the initial adsorption of proteins onto the implant surface, which governs integrin activation and subsequent intracellular signaling [6–8]. This process directly influences cell adhesion, proliferation, and differentiation, thereby modulating the host response and reducing the implant rejection. As reported in the literature, surfaces with higher protein affinity provide a favorable biochemical environment that enhances tissue integration and supports cell activity [9–12].

Nevertheless, bacterial adhesion and biofilm formation remain major challenge, as they can lead to chronic inflammation, implant loosening, and eventual implant failure. Therefore, considerable efforts have focused on developing multifunctional coatings capable of simultaneously enhancing bone regeneration and preventing bacterial colonization [13,14].

Among bioactive coating materials, hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) has attracted great attention because of its chemical similarity to bone mineral, excellent osteoconductivity, and high biocompatibility [15–17]. HA coatings are commonly applied to improve bone bonding and accelerate osseointegration [18,19]. More recently, intrinsically piezoelectric synthetic HA has gained interest, enabling the development of piezodynamic coatings capable of mimicking the electromechanical behavior of natural bone under mechanical stimulation [20–23].

Bone tissue naturally exhibits piezoelectricity, generating endogenous electrical signals under mechanical loading that regulate bone remodeling and regeneration [24]. The direct piezoelectric coefficient of bone ranges from 0.5 to 2.3 pC/N, while the converse response ranges from 0.1 to 10 pm/V [25–27]. Consequently, piezodynamic biomaterials capable of generating electrical charges under mechanical stress have emerged as promising systems for enhancing tissue regeneration. In this context, barium titanate (BaTiO_3 , BT) has been incorporated into HA coatings because of its ability to provide electromechanical stimulation and improve osteogenic activity [28]. In addition, BT-containing biomaterials exhibit low cytotoxicity and favorable biocompatibility, particularly within 10–40 wt% BT content [29–31].

Furthermore, low-intensity pulsed ultrasound (LIPUS) can non-invasively induce cyclic mechanical stresses within the bone microenvironment, promoting localized electromechanical activation of piezoelectric materials [32]. These electromechanical signals enhance cellular mechanotransduction, activate osteogenic pathways such as MAPK/ERK

and Wnt/ β -catenin, and stimulate the expression of osteogenic markers including ALP, osteocalcin, and type I collagen, ultimately promoting extracellular matrix mineralization and tissue repair [33].

Despite these promising properties, HA-BT systems still exhibit limited intrinsic antibacterial activity. To address this limitation, silver nanoparticles (nAg) have been widely investigated because of their broad-spectrum antimicrobial activity [34]. Their antibacterial effects involve membrane disruption, oxidative stress induction, and interference with bacterial DNA replication [35–37]. However, excessive silver incorporation may induce cytotoxicity, making it necessary to balance antibacterial efficacy and biocompatibility [38,39]. HA coatings containing approximately 1 wt% nAg deposited by APS have demonstrated antibacterial efficiencies above 99% against *Escherichia coli* and *Pseudomonas aeruginosa* while maintaining favorable cytocompatibility [40]. Likewise, HA/Ag-NPs coatings containing 1% silver nanoparticles effectively inhibited *Staphylococcus aureus*, *E. coli*, and *P. aeruginosa* without affecting osteoblast-like cells [41].

Building upon these findings, green synthesis of silver nanoparticles using plant extracts emerges as a strategic approach to enhance biomedical applicability. This eco-friendly and cost-effective route avoids toxic reagents and harsh synthesis conditions but also ensures that the antibacterial benefits of nAg are achieved without compromising biocompatibility. Plant-derived biomolecules act as reducing and stabilizing agents, improving nanoparticle stability, minimizing agglomeration, and facilitating homogeneous dispersion within HA matrices [42,43]. Thus, the integration of green-synthesized nAg represents a promising pathway to maximize antibacterial performance while maintaining compatibility, reinforcing the multifunctional potential of HA-BT coatings.

Although HA-BT composites have attracted considerable attention because of their piezoelectric and osteogenic potential, most studies have focused on bulk ceramics or porous scaffolds for bone tissue engineering, where the emphasis has been placed on improving cellular response and bone regeneration through piezoelectric stimulation [44–47]. Consequently, there is limited information regarding HA-BT coatings, particularly those produced by atmospheric plasma spraying (APS), and the few reported HA-BT coating systems have been developed without incorporating antibacterial agents such as silver nanoparticles [30,31]. In contrast, HA-Ag coatings have been extensively investigated for their antibacterial performance [40,48–50]; however, these systems lack a piezoelectric phase and, therefore, do not provide ultrasound-responsive functionality. Thus, these functionalities have been explored independently. To date, no study has systematically investigated a plasma-sprayed HA-BT coating incorporating green-synthesized silver nanoparticles to simultaneously achieve piezoelectric, osteoconductive, and antibacterial functionality. Moreover, the influence of silver incorporation on the piezoelectric response, piezodynamic behavior under LIPUS stimulation, and the relationship between these properties and antibacterial performance remains unknown. Therefore, the development of a multifunctional HA-BT/nAg coating and the comprehensive evaluation of its structural, mechanical, electrochemical, piezoelectric, biological, and antibacterial properties constitute the main scientific novelty of the present work.

2. Materials and Methods

2.1. Raw Powder and Process

Pure synthetic hydroxyapatite (HA, CAS No. 1306-06-5), with a particle size of approximately 35–60 nm, was commercially obtained from CaP Biomaterials, LLC (Fort Worth, TX, USA). Barium titanate powder (BT, CAS No. 12047-27-7), with a particle size of approximately 180–220 nm, was supplied by US Research Nanomaterials, Inc. (Houston, TX, USA).

These materials were used to prepare HA–BT composite powders employed as feedstock for coating fabrication.

According to our previous studies, the incorporation of 30 wt% BT resulted in the highest piezoelectric response while maintaining favorable cellular viability under LIPUS stimulation [31]. Therefore, the formulation containing 30 wt% BT, designated as HA30BT, was selected for the present study.

To evaluate the effect of silver nanoparticle incorporation, 1 wt% of silver nanoparticles (nAg) was incorporated into the HA–BT matrix. Silver nanoparticles (nAg) were synthesized via a green-synthesis approach following the methodology previously reported by Ledezma et al. [42]. For the synthesis, an aqueous *Opuntia* sp. extract was used as both reducing and stabilizing agent for the conversion of Ag⁺ ions from a 10 mM solution of silver nitrate (JTBaker, Avantor, US) into silver nanoparticles under controlled stirring and temperature conditions. The extract solution with Ag nanoparticles was dried using a freeze drier (Freezone 4.5L, Labconco Corporation, Kansas City, MI, USA).

The HA30BT composite powder matrix was homogenized by high-energy ball milling (HEBM) using an SPEX 8000M mill (Metuchen, NJ, USA). Ytria-stabilized zirconia (YSZ, 8 mol%) grinding media with diameters of 1 cm and 0.5 cm were employed at a ball-to-powder mass ratio of 2:1. Milling was carried out in a nylamide vial for 30 min using 0.2 mL of alcohol as a process control agent. After that, nAg was added to the matrix and mixed by mechanical agitation for 1 h to homogenize the matrix producing the composite powder designated as HA30BTnA.

2.2. Coatings Fabrication

The coatings were fabricated following the methodology previously reported in our earlier studies [31,51]. Medical-grade titanium alloy substrates (Ti6Al4V ELI, Titanio ABC, Guadalajara, Jalisco, MX, USA) were employed in the present work. Prior to coating deposition, the substrates were surface-treated by grit blasting using corundum particles (Al₂O₃, 700 µm, ANSI G-24 grit size; Sigma-Aldrich, St. Louis, MO, USA) to increase surface roughness and enhance coating adhesion. Subsequently, the samples were ultrasonically cleaned in 99% ethanol (JT Baker (Avantor), Phillipsburg, NJ, USA) to remove residual contaminants. The HA, HA30BT, and HA30BTnAg coatings were deposited onto Ti6Al4V substrates by atmospheric plasma spraying (APS) using an air plasma spray torch (TAFA Inc., Concord, NH, USA). The deposition parameters used to obtain coating thickness in the range of 150–200 µm were selected based on the optimized conditions previously established for HA-based coatings in our earlier studies [31,51]. This thickness range is consistent with ISO 13779-2 [52], which recommend nominal thickness between 50 and 200 µm to ensure adequate mechanical stability, adhesion strength, and long-term performance under physiological loading. The parameters employed to ensure the study complied with these requirements are summarized in Table 1.

Table 1. APS deposition parameters.

Parameter	Value
Voltage (V)	32
Current (A)	650
Work distance (cm)	8.5–10
Powder feeding rate (lb/h)	2
Primary gas Ar (psi) *	50
Secondary gas He (psi) *	110
Carrying gas Ar (psi) *	55

* Ultra high-purity argon grade 5.0 (99.999%) and high-purity helium grade 4.5 (99.995%) supplied by Praxair, Danbury, CT, USA were used during the APS process.

2.3. Crystal Structure and Phase Identification

Phase identification of the HA30BT and HA30BTnAg composite powders, as well as their corresponding coatings, was carried out by X-ray diffraction (XRD). Diffraction patterns were recorded using a Bruker D8 Advance diffractometer (Bruker AXS GmbH, Karlsruhe, Baden-Württemberg, Germany) equipped with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$). Data acquisition was performed over a 2θ range from 20° to 60° , employing a step increment of 0.02° and a counting time of 100 s per step. Phase identification of the composite powders and coatings was carried out using Match! software (version 3.11, Crystal Impact, Bonn, Germany) [53].

2.4. Microscopy and Phase Distribution

The morphology of the nAg particles in the as-received powder, prior to incorporation into the HA30BT matrix, as well as that of the resulting composite powder (HA30BTnAg) after the high-energy ball milling (HEBM) process, was examined by transmission electron microscopy (TEM) using a JEOL JEM-2200FS + Cs instrument (JEOL Ltd., Tokyo, Japan) operated at 200 kV with a point-to-point resolution of $1.9 \text{ \AA}$. Particle size distributions of the nAg powders were obtained from TEM micrographs by manually measuring at least 100 particles per sample using ImageJ software (version 1.53t) [54]. The experimental data were fitted to a lognormal distribution using the Levenberg–Marquardt iterative method implemented in OriginPro software (version 2024, OriginLab Corporation, Northampton, MA, USA) to determine the average particle size. Additionally, nano-beam electron diffraction (NBD) was performed on an individual nAg particle to confirm its crystalline nature and crystallographic structure. The diffraction pattern was analyzed and indexed using the CrysTBox software package (version 2.0, Institute of Physics, Czech Academy of Sciences, Prague, Czech Republic) [55], enabling the identification of the corresponding crystallographic planes and the determination of the particle's crystal structure. The elemental composition of the HA30BTnAg composite powder was analyzed by energy-dispersive X-ray spectroscopy (EDS) mapping using an Oxford Ultim Max Infinity EDS detector (Oxford Instruments, Abingdon, Oxfordshire, UK) attached to a TEM microscope to confirm the presence of the constituent phases: Ca, P, and O corresponding to the hydroxyapatite (HA) phase; Ba, Ti, and O corresponding to the barium titanate (BT) phase; and Ag corresponding to the silver nanoparticles (nAg).

Surface and cross-sectional microstructural analyses were performed using a scanning electron microscope (SEM, SU3500, Hitachi, Naka, Japan). Phase distribution was examined using backscattered electron (BSE) imaging, while elemental composition and distribution were determined by energy-dispersive X-ray spectroscopy (EDS, AZtec, Oxford Instruments, Abingdon, Oxfordshire, UK).

For cross-sectional analysis, the coated samples were sectioned using a low-speed precision cutting machine equipped with a diamond blade (MetLab Co., Niagara Falls, NY, USA). The specimens were subsequently mounted in epoxy resin and subjected to standard metallographic preparation. Grinding was carried out using SiC abrasive papers with grit sizes of 240, 400, 600, 1000, and 2000. Final polishing was performed using alumina suspensions with particle sizes of 1.0, 0.3, and $0.05 \text{ }\mu\text{m}$. The final polishing step with a $0.05 \text{ }\mu\text{m}$ alumina suspension produced a mirror-like surface finish suitable for microstructural characterization.

2.5. Mechanical Properties by Adhesive Strength Analysis

The adhesive strength was performed by INSTRON 10 TON universal test machine (Instron Corporation, Norwood, MA, USA); the assay and failure analysis were carried off to the standard ASTM C633 [56]. For the adhesion test, titanium fixtures with a diameter of

1 inch were coated and subsequently bonded using Armstrong™ A-12 Epoxy Adhesive (ResinLab®, Germantown, WI, USA). The assembled specimens were allowed to cure for 24 h at room temperature. The adhesion strength was evaluated by applying a tensile load at a crosshead speed of 0.03 in s^{-1} until failure occurred. Five specimens were tested for each coating condition to ensure reproducibility of the results.

2.6. Protein Affinity by Electrochemical Analysis

Protein affinity was evaluated through electrochemical analysis using the Potentiodynamic Polarization (PDP) technique. Coated samples were immersed in phosphate-buffered saline (PBS; J.T. Baker, Phillipsburg, NJ, USA) either with or without protein supplementation. To investigate the influence of protein adsorption on the electrochemical behavior of the coatings, bovine serum albumin (BSA) was added to the PBS solution at concentrations of 12, 25, and 50 mg L^{-1} . PDP measurements were performed using a potentiostat/galvanostat (Autolab, Metrohm, Utrecht, The Netherlands) in a conventional three-electrode electrochemical cell consisting of an Ag/AgCl reference electrode, a platinum wire counter electrode, and the coated titanium specimen as the working electrode. Each experiment was conducted using 50 mL of electrolyte solution maintained at 36.5°C to simulate physiological conditions. Prior to polarization measurements, the samples were allowed to stabilize in the electrolyte for 2 h under open-circuit conditions. Potentiodynamic Polarization scans were subsequently performed within a potential range of $\pm 200 \text{ mV}$ with respect to the open-circuit potential (E_{corr}) at a scan rate of 10 mV min^{-1} . The electrochemical data were analyzed using CView software (version 3.5h, Scribner Associates Inc., Southern Pines, NC, USA) [57] to determine the polarization resistance (R_p) and estimate the corresponding corrosion rates. The changes in polarization resistance were used as an indirect indicator of protein adsorption on the coating surfaces. The corrosion current density (I_{corr}) was calculated using the Stern–Geary relationship [58], which correlates the polarization resistance (R_p) with the corrosion current density.

The calculation was performed according to Equation (1):

$$I_{\text{corr}} = \frac{B}{R_p} \quad (1)$$

where B is the Stern–Geary constant. The value of B was determined according to Equation (2):

$$B = \frac{(B_a * B_c)}{2.303(B_a + B_c)} \quad (2)$$

In Equation (2), B_a and B_c represent the anodic and cathodic Tafel slopes, respectively.

To evaluate the protein affinity of the coating surfaces, the experimental LPR data obtained at different bovine serum albumin (BSA) concentrations were analyzed using the correlation of adsorption species by Langmuir monolayer adsorption model and electrochemical analysis to determinate the adsorption isotherms [59]. This model assumes monolayer adsorption on a homogeneous surface with a finite number of adsorption sites and was employed to describe the interaction between BSA molecules and the coating surface. The adsorption parameters were determined by fitting the experimental data to the Langmuir equation, as shown in Equation (3).

$$\theta = \frac{K_{\text{ads}} * C}{1 + K_{\text{ads}} * C} \quad (3)$$

where θ represents the fractional surface coverage, K_{ads} is the adsorption equilibrium constantly associated with the affinity of the protein for the coating surface, and C corresponds

to the concentration of bovine serum albumin (BSA) in the phosphate-buffered saline (PBS) solution.

In electrochemical studies, θ represents the fraction of the surface effectively covered by adsorbed molecules. Its value can be estimated from the reduction in the corrosion current density I_{corr} observed in the presence of bovine serum albumin (BSA) relative to that measured in the BSA-free electrolyte. This approach assumes that adsorbed protein molecules partially block the active surface sites involved in the electrochemical reactions. Mathematically, the surface coverage parameter can be expressed as:

$$\theta = \frac{I_{\text{corr}}^0 - I_{\text{corr}}}{I_{\text{corr}}^0} \quad (4)$$

where I_{corr}^0 is the corrosion current density measured in PBS without BSA, and I_{corr} is the corrosion current density measured in the presence of BSA.

Furthermore, the Langmuir adsorption equation (Equation (5)) was linearized to determine the adsorption equilibrium constant (K_{ads}), which was used as a quantitative measure of the affinity between the coating surface and bovine serum albumin (BSA). The linearized form of the equation is presented below:

$$\frac{C_{\text{BSA}}}{\theta} = \frac{1}{K_{\text{ads}}} + C_{\text{BSA}} \quad (5)$$

2.7. Piezoresponse

The piezoelectric properties of the coatings were investigated using Switching Spectroscopy Dual AC Resonance Tracking Piezoresponse Force Microscopy (SS-DART-PFM) with an atomic force microscope system (MFP-3D, Asylum Research, Oxford Instruments, Oxfordshire, UK) operating in vertical mode at room temperature. Conductive Ti/Ir-coated silicon probes (5/20 nm coating thickness; ASYELEC-01, Asylum Research, Oxfordshire, UK) were employed. The cantilever possessed a nominal spring constant of 0.2 N/m and a free resonance frequency of approximately 70 kHz. Electrical contact between the conductive tip and the Ti6Al4V substrate, used as the bottom electrode, was established using silver paint (SPI Supplies, Dotite D-550 Silver Colloid, #05003-AB, West Chester, PA, USA).

An AC excitation voltage of 5 V was applied throughout the measurements, while the contact resonance frequency was maintained at approximately 295 kHz, well above the free resonance frequency of the cantilever. Local ferroelectric switching behavior was analyzed acquiring displacement–voltage curves and local ferroelectric hysteresis loops under a DC voltage sweep from -60 V to $+60$ V using the SS-DART-PFM mode. To minimize electrostatic artifacts commonly associated with DC “ON” measurements, hysteresis loops were acquired exclusively in the DC “OFF” state, following the methodology reported by Qiao et al. [60].

For each coating composition, three independently prepared coatings were analyzed. Five PFM measurements were acquired from different regions of each coating to account for the microstructural heterogeneity characteristic of APS deposits. The piezoelectric coefficient (d_{33}) was calculated from the slope of the displacement–voltage response according to Equation (6) [61]. Accordingly, the reported d_{33} value for each coating composition corresponds to the average of 15 independent local measurements.

$$d_{33} = \frac{D - D_1}{V - V_1} \quad (6)$$

where D and V are the displacement and applied voltage values obtained from the PFM measurements, respectively, and D_1 and V_1 correspond to the displacement and voltage coordinates of the intersection point of the displacement–voltage curve.

2.8. In Vitro Evaluation by Human Cell Line

For cell culture experiments, RPMI-1640 medium (Roswell Park Memorial Institute medium, McCoy's 5A modification; Sigma-Aldrich, St. Louis, MO, USA) was used and supplemented with 10% fetal bovine serum (FBS; Corning, Corning, NY, USA) and 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells and all in vitro assays were maintained in a humidified incubator (Heracell Vios 160i, Thermo Fisher Scientific, Waltham, MA, USA) under a 5% medical-grade CO₂ atmosphere at a controlled temperature of 36.5 ± 0.5 °C.

2.8.1. Viability and Cytotoxic Behavior

To assess cell viability and the potential cytotoxic effects of the coatings by indirect contact, the samples were immersed in RPMI culture medium and incubated at 36.5 °C for four different extraction periods (1, 3, 7, and 21 days) to allow the release of any potentially cytotoxic species. Following each extraction period, the supernatants were collected and serially diluted with fresh culture medium at dilution ratios of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128.

The cytotoxicity assay was performed using 96-well tissue culture plates. Initially, each well was seeded with 2.5×10^4 cells suspended in 50 µL of supplemented culture medium and incubated for 24 h to allow cell attachment. Subsequently, the corresponding extract dilutions were added to the wells and incubated for an additional 24 h.

After the exposure period, the culture medium was carefully removed, and 20 µL of MTT solution (5 mg mL^{−1} in PBS) was added to each well. The plates were then incubated at 36.5 °C for 3 h to promote the formation of formazan crystals. Following incubation, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). To ensure complete dissolution, the plates were incubated at 36.5 °C for an additional hour. The absorbance was subsequently measured at a wavelength of 595 nm using a microplate reader (iMark™ Microplate Reader, Bio-Rad, Hercules, CA, USA).

The percentages of cell viability and cytotoxicity were calculated according to Equations (7) and (8), respectively.

$$Viability(\%) = \frac{OD_S}{OD_C} * 100 \quad (7)$$

$$Cytotoxicity(\%) = \frac{OD_C - OD_S}{OD_C} * 100 \quad (8)$$

where OD_S and OD_C represent the optical density values of the sample and the control, respectively.

2.8.2. Cell Activity

To evaluate cellular activity, an immunofluorescence assay was performed. As previously reported in our work [31], a lab-on-a-chip cell culture platform was employed to investigate the piezoelectric response induced by low-intensity pulsed ultrasound (LIPUS). LIPUS is a non-invasive stimulation technique that delivers low-energy acoustic waves, generating cyclic mechanical stresses capable of activating piezoelectric materials and producing localized electrical stimuli. The lab-on-a-chip device is a microfluidic system that integrates cell culture and stimulation within microscale channels, enabling precise control of the cellular microenvironment, fluid flow, and exposure conditions [62].

The lab-on-a-chip used in the present study was developed by three layers of polytetrafluoroethylene (PTFE, Levinson, Chihuahua, Mexico), polydimethylsiloxane (PDMS, Dowsil™ 184, DOW Chemical Company, Midland, Miami, FL, USA) and polymethylmethacrylate (PMMA, Levinson, Chihuahua, Mexico). The PTFE and PMMA layers were fabricated using a laser cut machine (Epilog Fusion Pro, Epilog Laser, Golden, CO, USA). For the PDMS layer, a negative mold was used into which the polymer was poured and left to cure for 24 h. As shown in Figure 1, this platform allowed the evaluation of the biological response of cells subjected to piezoelectrically mediated electromechanical stimulation under well-controlled experimental conditions.

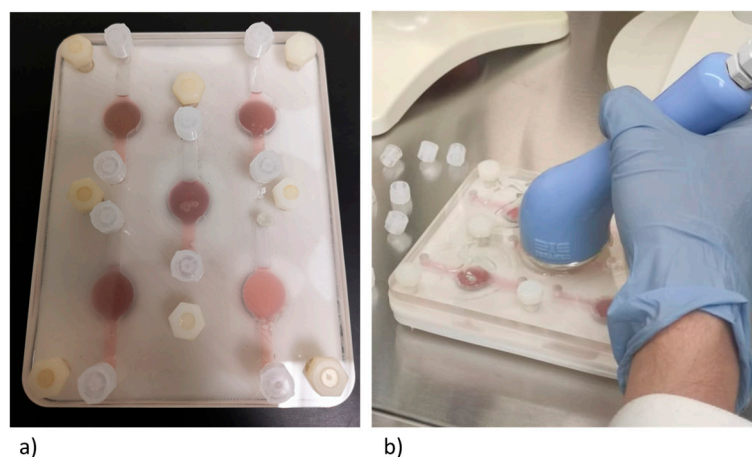


Figure 1. Lab-on-a-chip developed with samples for piezodynamic stimulus by LIPUS treatment. (a) Lab-on-a-chip with samples previous to LIPUS treatment and (b) samples with piezodynamic stimulus by LIPUS treatment. The design of the device allows the simultaneous testing of up to 5 samples under identical experimental conditions.

Samples and control groups were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at an initial seeding density of 1.5×10^4 cells per sample. The cultures were maintained for 7 days under standard incubation conditions.

For the LIPUS stimulation a therapeutic ultrasound device was used (Sonopulse III, IBRAMED, Amparo, São Paulo, Brazil). In the treatment group, the lab-on-a-chip platform enabled the activation of the piezoelectric response of the coatings. Samples were exposed to ultrasound for 3 min at a frequency of 1 MHz, an intensity of 0.1 W cm^{-2} , and a pulse repetition frequency of 100 Hz. The LIPUS treatment was applied every 48 h throughout the culture period.

Following the incubation period, with and without LIPUS treatment, the cells were fixed in 2.5% (*v/v*) glutaraldehyde prepared in 0.1 M cacodylate buffer for 1 h. To reduce autofluorescence, the samples were subsequently immersed in 0.2% (*w/w*) sodium borohydride solution. Cell permeabilization was then carried out using 2% (*v/v*) Triton X-100 in PBS for 30 min. Blocking was performed for 1 h at 36.5°C using PBST (PBS containing 0.05% (*v/v*) Tween 20) supplemented with 10% (*w/w*) milk protein.

Osteocalcin expression was evaluated using a primary polyclonal anti-osteocalcin antibody (OCN, rabbit anti-human IgG, Invitrogen, Carlsbad, CA, USA) at a dilution of 1:1000, followed by incubation with an FITC-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA). Both antibody incubations were performed at 36.5°C . Subsequently, F-actin was stained using rhodamine-phalloidin (Abcam, Cambridge, UK) at a dilution of 1:1000 and incubated for 30 min. All antibodies and staining reagents were diluted in PBST supplemented with 1% (*w/w*) milk protein.

Finally, the samples were mounted using a DAPI-containing nuclear staining medium (Abcam, Cambridge, UK), covered with glass coverslips, and sealed with resin. Fluorescence images were acquired using a laser scanning confocal microscope (LSM T-PMT, Carl Zeiss, Oberkochen, Germany). Image processing and analysis were performed using ZEN Blue Edition software (version 3.0, Carl Zeiss Microscopy GmbH, Jena, Germany) [63].

2.9. Antibacterial Efficacy

To evaluate the antibacterial activity of the coatings, three bacterial strains were employed: *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Bacterial cultures were maintained in tryptic soy broth (TSB) and tryptic soy agar (TSA) media.

The coated samples were totally immersed in 2 mL of PBS solution with a bacterial charge of 2.5×10^5 CFU mL⁻¹, samples were incubated for 24 h at 36.5 °C. Following incubation, the bacterial suspensions were recovered and serially diluted in sterile saline solution. From each sample, a 10⁻⁶ dilution was prepared, and a 50 µL aliquot was spread onto TSA plates. The inoculated plates were subsequently incubated for 24 h to allow colony formation and bacterial enumeration.

The antibacterial activity of the coatings was determined by comparing the number of viable bacterial colonies recovered from each sample with those obtained from the control group. Antibacterial efficiency was calculated according to the JIS Z 2801 standard [64] using Equation (9).

$$R = \log\left(\frac{B}{C}\right) \quad (9)$$

where R represents the antibacterial activity value. B and C correspond to the number of viable bacteria, expressed as colony-forming units (CFU), recovered from the control surface and from the coated samples (Ti, HA30BT, and HA30BTnAg), respectively. According to the JIS Z 2801 standard [64], a material is considered to exhibit antibacterial activity when ($R \geq 2$), corresponding to a reduction of at least 99% in the number of viable bacteria after the specified incubation period.

Finally, the antibacterial efficacy (%) was calculated using Equation (10):

$$\%_{\text{efficacy}} = \left(\frac{B - C}{B}\right) * 100 \quad (10)$$

where B is the number of viable bacteria (CFU) recovered from the control surface, while C is the number of viable bacteria (CFU) recovered from the bacterial suspensions exposed to the test samples.

3. Results

3.1. Crystal Structure and Phase Identification

The XRD patterns of the HA30BT and HA30BTnAg composite powders, together with their corresponding coatings, are presented in Figure 2. The main diffraction peaks observed for the composite powders in Figure 2a were indexed to the hexagonal crystal structure of hydroxyapatite (HA) with space group $P6_3/m$ (JCPDS card no. 09-0432, $\blacklozenge$). In addition, characteristic reflections corresponding to the tetragonal phase of barium titanate (BT) (space group $P4mm$, No. 99) were identified, in agreement with the reference pattern reported in JCPDS card no. 79-2264 ($\blacksquare$). For the HA30BTnAg composite powder, additional diffraction peaks located at 38.15° and 44.35° were detected and assigned to the (111) and (200) crystallographic planes of metallic silver (Ag), respectively. These reflections are consistent with a face-centered cubic (FCC) crystal structure with space group $Fm\bar{3}m$ (No. 225), according to PDF card no. 00-004-0783 ($\circ$). No diffraction peaks other than those corresponding to the HA, BT, and nAg phases were observed, indicating that the

incorporation of 30 wt.% BT and nAg into the HA matrix, as well as the high-energy ball milling process, did not promote the formation of secondary phases such as additional calcium phosphate compounds, oxides, or other metallic species.

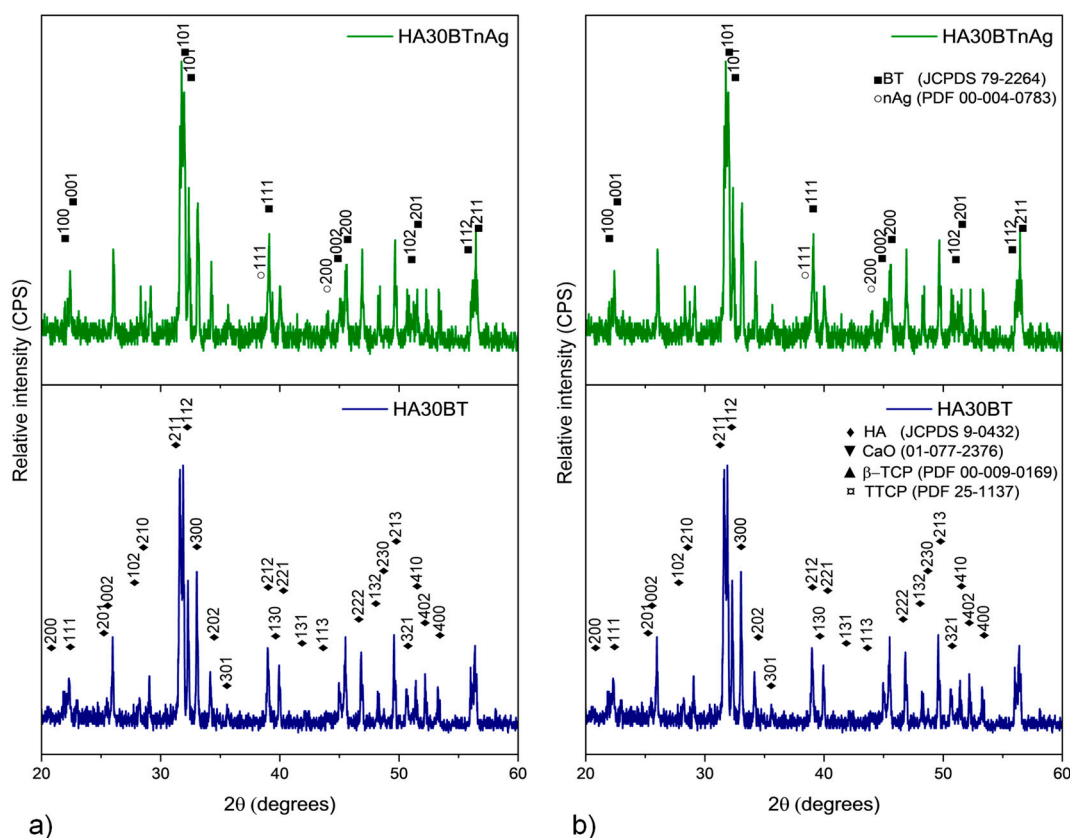


Figure 2. X-ray diffraction (XRD) patterns of the HA30BT and HA30BTnAg materials: (a) composite powders and (b) corresponding coatings deposited on Ti6Al4V substrates.

Figure 2b shows the XRD patterns of the HA30BT and HA30BTnAg composite coatings deposited onto Ti6Al4V substrates by atmospheric plasma spraying (APS). The diffraction patterns confirmed that the characteristic HA, BT, and nAg phases were retained after the coating deposition process, demonstrating the successful incorporation of all constituents into the coatings. However, additional reflections associated with secondary calcium phosphate phases were detected. These phases originated from the partial thermal decomposition of HA during exposure to the high-temperature plasma jet and were identified as β -tricalcium phosphate (β -TCP, $\text{Ca}_3(\text{PO}_4)_2$, $\blacktriangle$), tetracalcium phosphate (TTCP, $\text{Ca}_4(\text{PO}_4)_2\text{O}$, $\circ$), and calcium oxide (CaO, $\blacktriangledown$), according to JCPDS cards no. 00-009-0169, 25-1137, and 01-077-2376, respectively. The formation of these phases is commonly reported for plasma-sprayed HA-based coatings and is associated with the thermal stability of HA under APS processing conditions [31]. The absence of additional reflections associated with decomposition products or oxide phases derived from BT or nAg suggests that both phases retained their structural stability during plasma spraying and did not undergo significant phase degradation.

3.2. Microscopy and Phase Distribution

The morphology and crystallographic characteristics of the nAg particles were investigated using transmission electron microscopy. Representative TEM images and crystallographic analyses are presented in Figure 3. In Figure 3a, the bright-field TEM image of the as-received nAg powder shows particles with a predominantly semi-spherical

morphology. Figure 3b presents the corresponding particle size distribution. The nAg size distribution displays a right-skewed profile, likely due to the non-uniform nature of nanoparticle nucleation and growth processes. The particle size is represented by the median value and interquartile range (IQR), with a median size of 30.24 nm and Q1–Q3 values of 20.91–51.96 nm (IQR = 31.05 nm).

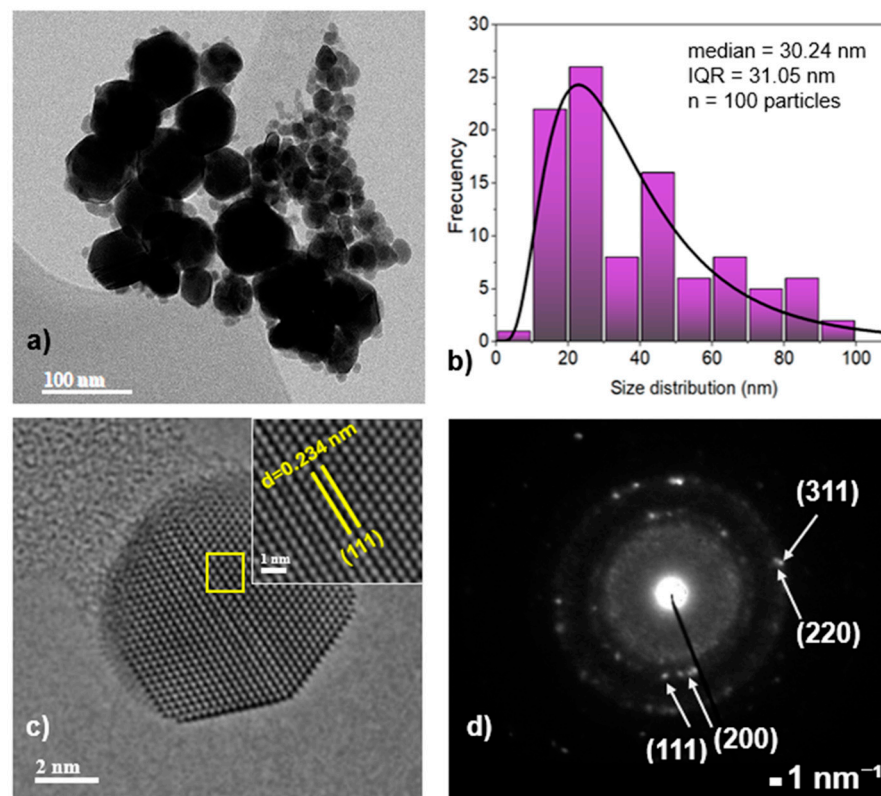


Figure 3. TEM and NBD analysis of as-received green-synthesized nAg particles: (a) bright-field TEM image; (b) particle size distribution; (c) high-resolution TEM (HRTEM) image of an individual nAg nanoparticle, with the inset showing the FFT-filtered image of the selected area, confirming lattice spacings of 0.234 nm corresponding to the (111) planes of face-centered cubic (FCC) silver; (d) nano-beam electron diffraction (NBD) pattern confirming the crystalline structure of metallic silver.

High-resolution transmission electron microscopy (HRTEM) analysis of an individual nAg particle (Figure 3c) further confirmed the high crystallinity and phase purity of metallic silver. The measured interplanar spacing of 0.34 nm was indexed to the (111) lattice planes of face-centered cubic (FCC) silver, in excellent agreement with reported values in the literature [65]. Furthermore, the nano-beam diffraction (NBD) pattern (Figure 3d) exhibited diffraction spots corresponding to the (111), (200), (220), and (311) planes of FCC silver. The absence of additional diffraction features attributable to silver oxide phases confirms the preservation of the metallic silver structure.

Transmission electron microscopy (TEM) was also employed to evaluate the morphology and confirm the presence of the three phases (nAg, HA, and BT) in the HA30BTnAg composite powder. As shown in Figure 4a, the bright-field TEM image reveals a heterogeneous microstructure composed of sub-rounded particles and fibrous-like features. Based on the elemental mapping presented in Figure 4b, these morphologies are associated with the BT and HA phases, respectively.

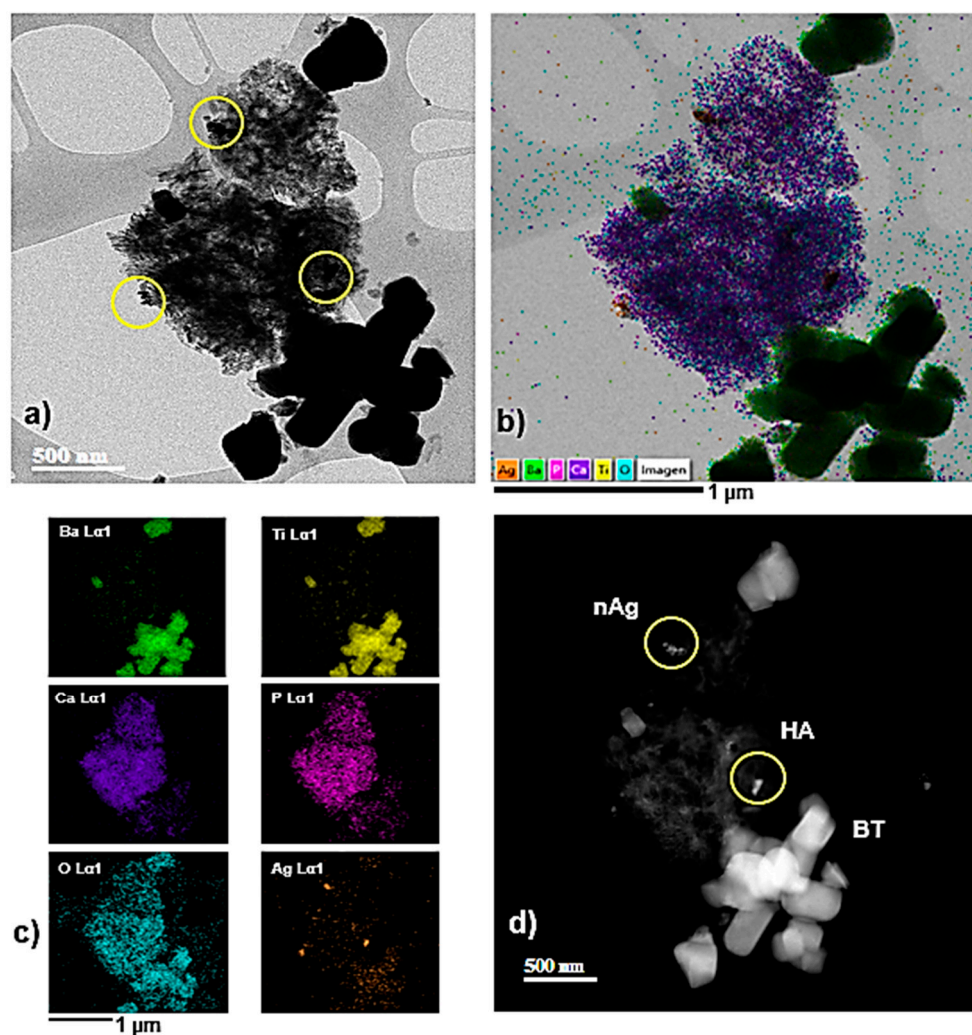


Figure 4. TEM analysis of the HA30BTnAg composite powder: (a) bright-field TEM image showing distinct morphologies associated with the different phases; (b) elemental mapping of BT, HA, and nAg distributions; (c) elemental maps corresponding to the individual signals of each constituent element in the HA30BTnAg composite powder; (d) Z-contrast (HAADF-STEM) image highlighting compositional differences, where the brighter regions enclosed in yellow circles correspond to nAg particles due to their higher atomic number compared to the HA matrix.

In addition, several nanosized features highlighted with yellow circles in Figure 4a can be observed, consisting of significantly smaller particles compared to the surrounding structures. These regions correspond to the Ag signal detected in the elemental map (Figure 4c), confirming their association with the nAg phase.

Furthermore, Figure 4d shows Z-contrast (HAADF-STEM) imaging, where differences in brightness are attributed to variations in atomic number. In this image, the brighter regions (also indicated with yellow circles) correspond to elements with higher atomic number associated with the nAg and BT particles, in contrast to the lower-intensity regions attributed mainly to the HA matrix.

Figure 5 presents backscattered electron imaging (BSEI) micrographs showing the surface and cross-sectional morphologies of the HA30BT (Figure 5a,b) and HA30BTnAg (Figure 5c,d) coatings. In both systems, the constituent phases are uniformly distributed throughout the HA matrix, with only limited agglomeration of BT and nAg particles, identified by their brighter contrast due to the higher atomic number of these phases. The relatively homogeneous dispersion of these functional components is expected to favor

a uniform piezoelectric and antibacterial response across the coating thickness, which is beneficial for the overall performance of the material [66].

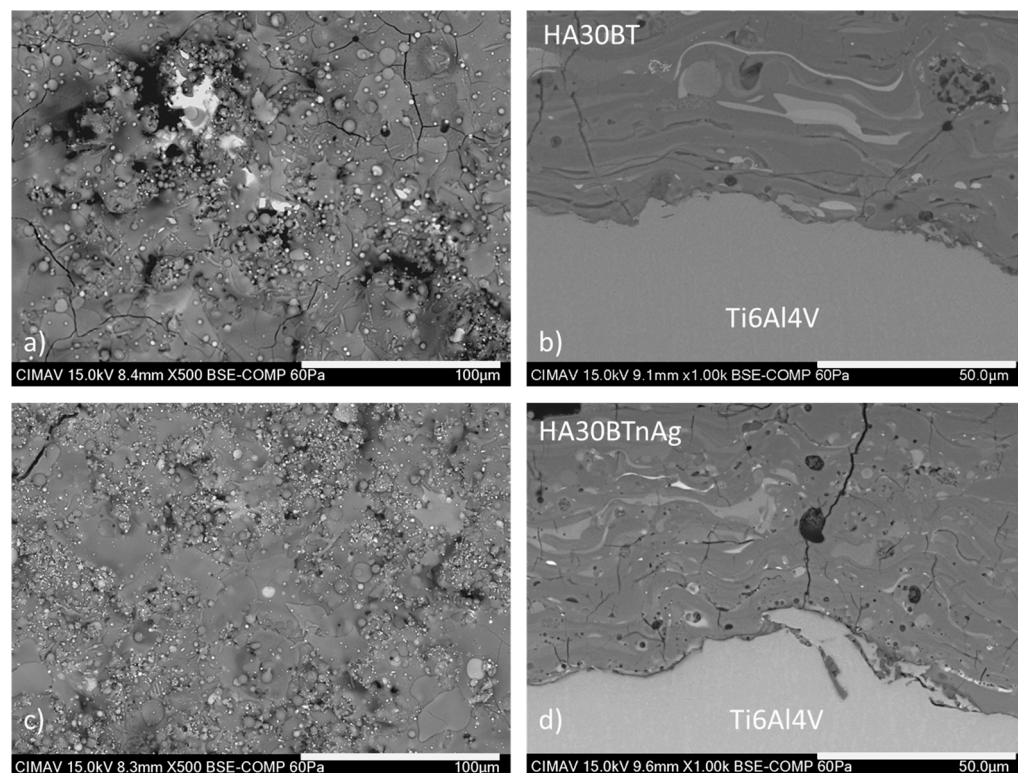


Figure 5. BSEI micrographs of the HA30BT and HA30BTnAg coatings: (a,b) HA30BT and (c,d) HA30BTnAg surface and cross-sectional views. Both coatings show homogeneous phase distribution and strong adhesion to the Ti6Al4V substrate.

Cross-sectional micrographs revealed dense coatings exhibiting intimate contact with the Ti6Al4V substrate. The absence of cracks, interconnected porosity extending to the interface, and delamination features indicates excellent interfacial cohesion and effective mechanical anchoring achieved during plasma spraying. In addition, the incorporation of nAg did not result in discernible changes in coating morphology or interface quality, confirming that the silver nanoparticles were successfully incorporated into the HA–BT matrix while preserving coating integrity.

3.3. Mechanical Properties: Adhesive Strength

Figure 6 presents the adhesion strength of HA, HA30BT, and HA30BTnAg coatings deposited on Ti6Al4V substrates, as measured by tensile adhesion testing using a 10-ton Instron universal testing machine in accordance with ASTM C633. The dashed line represents the minimum adhesion strength criterion of 15 MPa established by the FDA for plasma-sprayed hydroxyapatite coatings [67,68].

Table 2 summarizes the adhesion strength values of the HA30BT and HA30BTnAg coatings obtained from tensile adhesion tests conducted in accordance with ASTM C633 [56]. As shown in Table 2, the incorporation of BT resulted in a substantial increase in adhesion strength, from 21 ± 1.9 MPa for the pure HA coating to 44 ± 1.8 MPa for the HA30BT coating. The HA30BTnAg coating exhibited a comparable adhesion strength of 43 ± 2.1 MPa, indicating that the incorporation of BT and nAg did not adversely affect the coating–substrate bonding. These results suggest that the addition of silver nanoparticles preserved the mechanical integrity of the composite coating while maintaining its adhesion performance.

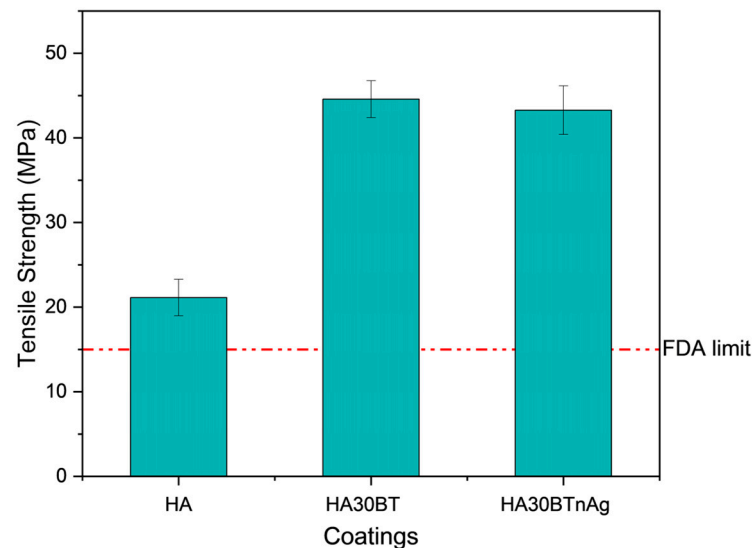


Figure 6. Nominal adhesion strength of HA, HA30BT, and HA30BTnAg coatings deposited on Ti6Al4V substrates and evaluated by tensile adhesion testing according to ASTM C633 [56]. The dashed line represents the minimum adhesion strength criterion (15 MPa) specified for plasma-sprayed hydroxyapatite coatings.

Table 2. Comparative values of adhesive strength and failure type analysis according to ASTM C633 [56].

Sample	Adhesive Strength (MPa)	Failure Type	
		Adhesive (%)	Cohesive (%)
HA	21 ± 1.9	84 ± 4	16 ± 4
HA30BT	44 ± 1.8	11 ± 2	89 ± 2
HA30BTnAg	43 ± 2.1	19 ± 4	81 ± 4

Compared with the pure HA coating, both HA30BT and HA30BTnAg exhibited an increase in adhesion strength of approximately 110%. This improvement is primarily attributed to the incorporation of BT, which enhances the coating microstructure and interfacial cohesion, thereby promoting more efficient load transfer across the coating–substrate interface. The addition of nAg preserved these benefits without compromising the mechanical integrity of the composite coating. Furthermore, the adhesion strength values of both composite coatings were well above the minimum FDA requirement for plasma-sprayed hydroxyapatite coatings [67,68].

Additionally, Figure 7 presents the fractographic analysis of the failure surfaces of the HA30BT and HA30BTnAg composite coatings following tensile adhesion testing. In the fractured specimens, the blue arrows highlight regions associated with cohesive failure within the coating layer. Table 2 also summarizes the percentage contribution of each failure mode, as determined by image analysis in accordance with ASTM C633.

In contrast to the pure HA coating, which exhibited predominantly adhesive failure (84% ± 4%), both HA30BT and HA30BTnAg coatings showed predominantly cohesive failure, with cohesive failure percentages of 89% ± 2% and 81% ± 4%, respectively. These results indicate that fracture occurred mainly within the coating rather than at the coating–substrate interface. Although the incorporation of nAg resulted in a slight increase in the adhesive failure fraction compared with the HA30BT coating, cohesive failure remained the dominant fracture mechanism in both composite systems.

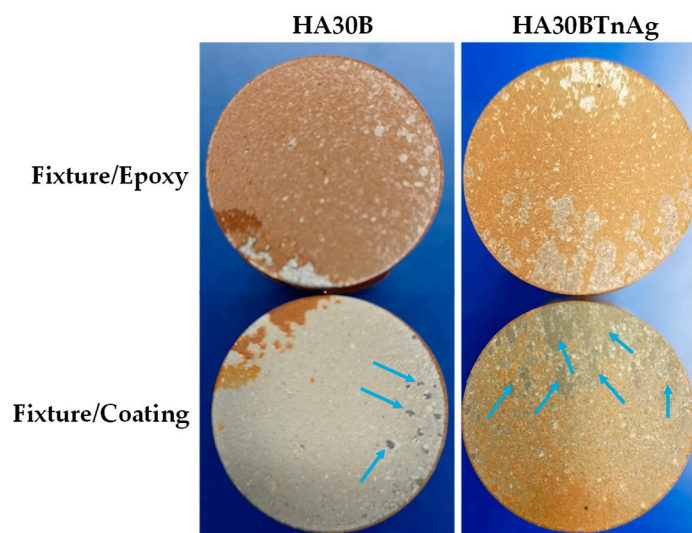


Figure 7. Representative failure surfaces of HA30BT and HA30BTnAg coatings after ASTM C633 tensile adhesion testing [56]. Blue arrows indicate regions of cohesive failure within the coating, demonstrating strong coating–substrate adhesion.

The predominance of cohesive failure indicates that the coating–substrate bond strength exceeded the internal cohesive strength of the coatings, confirming the strong interfacial adhesion achieved during the APS process. The slight increase in adhesive failure observed for the HA30BTnAg coating had no measurable effect on adhesion strength, which remained comparable to that of the HA30BT coating. Overall, these results demonstrate that the incorporation of BT significantly improved adhesion performance, while the addition of nAg preserved the mechanical integrity and interfacial bonding of the composite coatings.

3.4. Protein Affinity by Electrochemical Analysis

Figure 8 shows the Potentiodynamic Polarization (PDP) curves obtained from the electrochemical analysis of the uncoated Ti6Al4V substrate (Figure 8a), HA30BT coating (Figure 8b), and HA30BTnAg coating (Figure 8c) in electrolytes containing different concentrations of BSA.

The corresponding Langmuir adsorption isotherms are presented in Figure 8d and were derived according to the methodology described in Section 2.5. Table 3 summarizes the corrosion current density (I_{corr}), surface coverage parameter (θ), and adsorption equilibrium constant (K_{ads}) values calculated from the PDP measurements and the Langmuir adsorption model.

According to the results, in absence of bovine serum albumin, the addition of silver nanoparticles introduces additional electrochemically active sites on the coating surface, these phenomena observed by the increase of I_{corr} against coating without nAg (HA30BT). In the absence of proteins, these sites facilitate charge transfer and may induce localized galvanic interactions between metallic Ag and the surrounding HA/BT ceramic phases.

In contrast, in the presence of BSA, the results indicate that the incorporation of nAg into the HA30BT coating enhances the affinity of the coating surface toward BSA; this effect is due to the intrinsic affinity of nAg to different blood proteins, like albumin. For both HA30BT and HA30BTnAg coatings, the surface coverage parameter (θ) increased with increasing BSA concentration, indicating progressive adsorption of protein molecules onto the coating surface. At equivalent BSA concentrations, the HA30BTnAg coating exhibited higher θ values than the HA30BT coating. For example, the surface coverage increased

from 0.74 to 0.89, corresponding to an enhancement of approximately 20% following the incorporation of nAg.

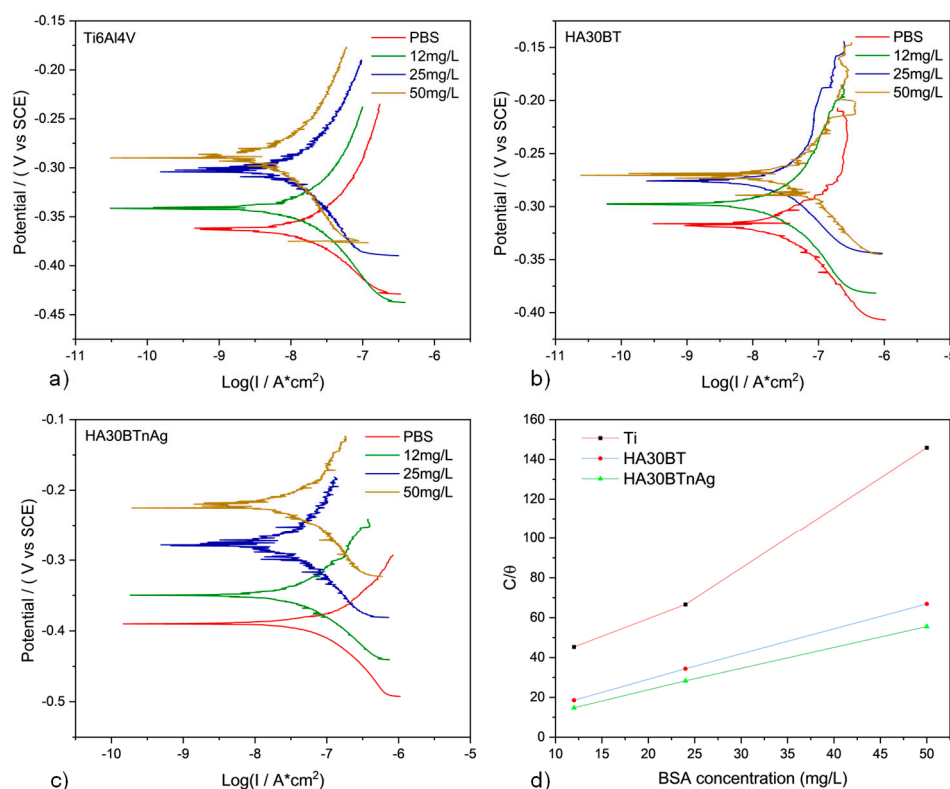


Figure 8. Potentiodynamic Polarization (PDP) curves of (a) Ti6Al4V substrate, (b) HA30BT coating and (c) HA30BTnAg coating in solutions containing different BSA concentrations; (d) Langmuir adsorption isotherms derived from the electrochemical measurements and used to calculate the surface coverage parameter (θ) and adsorption equilibrium constant (K_{ads}).

Table 3. Corrosion current (I_{corr}), surface coverage parameter (θ), adsorption equilibrium constant (K_{ads}) values calculated by LPR and Langmuir isotherms analysis.

Sample	[BSA]	I_{corr}	θ	K_{ads} (L/mol)
Ti	0	7.91×10^{-8}	-	0.12
	12	5.82×10^{-8}	0.26	
	25	5.06×10^{-8}	0.36	
	50	5.20×10^{-8}	0.34	
HA30BT	0	2.38×10^{-7}	-	0.28
	12	8.37×10^{-8}	0.64	
	25	6.48×10^{-8}	0.72	
	50	6.00×10^{-8}	0.74	
HA30BTnAg	0	3.70×10^{-7}	-	0.45
	12	6.88×10^{-8}	0.81	
	25	4.31×10^{-8}	0.88	
	50	3.66×10^{-8}	0.89	

This trend is further supported by the Langmuir adsorption analysis, which revealed higher K_{ads} values for the HA30BTnAg coating. The increase in K_{ads} indicates a stronger

interaction between BSA molecules and the coating surface, consistent with the well-established role of silver nanoparticles in increasing surface polarity and providing additional active sites for protein adsorption. These features enhance the overall affinity of the composite coating toward biological macromolecules.

The K_{ads} values obtained for the coatings fall within the range of $0.1 < K_{ads} < 1$, indicating a moderate affinity for BSA. Both HA30BT and HA30BTnAg coatings exhibit promising behavior as surface modifications for enhancing protein affinity. In contrast, the Ti6Al4V alloy, commonly used in orthopedic prostheses, exhibits a relatively low affinity for BSA, underscoring the relevance of these coatings for promoting improved biomaterial–tissue integration.

3.5. Piezoresponse

Figure 9 presents the piezoelectric characterization of the coatings performed by Switching Spectroscopy Dual AC Resonance Tracking Piezoresponse Force Microscopy (SS-DART-PFM). Figure 9a shows the amplitude–voltage and phase–voltage responses of the pure HA coating. The amplitude–voltage curve does not exhibit the characteristic butterfly-shaped loop typically associated with piezoelectric materials, while the corresponding phase response lacks the hysteresis behavior indicative of ferroelectric polarization switching. These results confirm the absence of measurable piezoelectric activity in the pure HA coating. In contrast, the BT-containing coatings exhibited a clear electromechanical response to the applied electric field. Representative amplitude–voltage butterfly loops for the HA30BT and HA30BTnAg coatings are presented in Figure 9b,c, respectively, demonstrating local piezoelectric displacement in the picometer range. Furthermore, the corresponding phase–voltage curves reveal polarization switching behavior, a characteristic feature of ferroelectric materials.

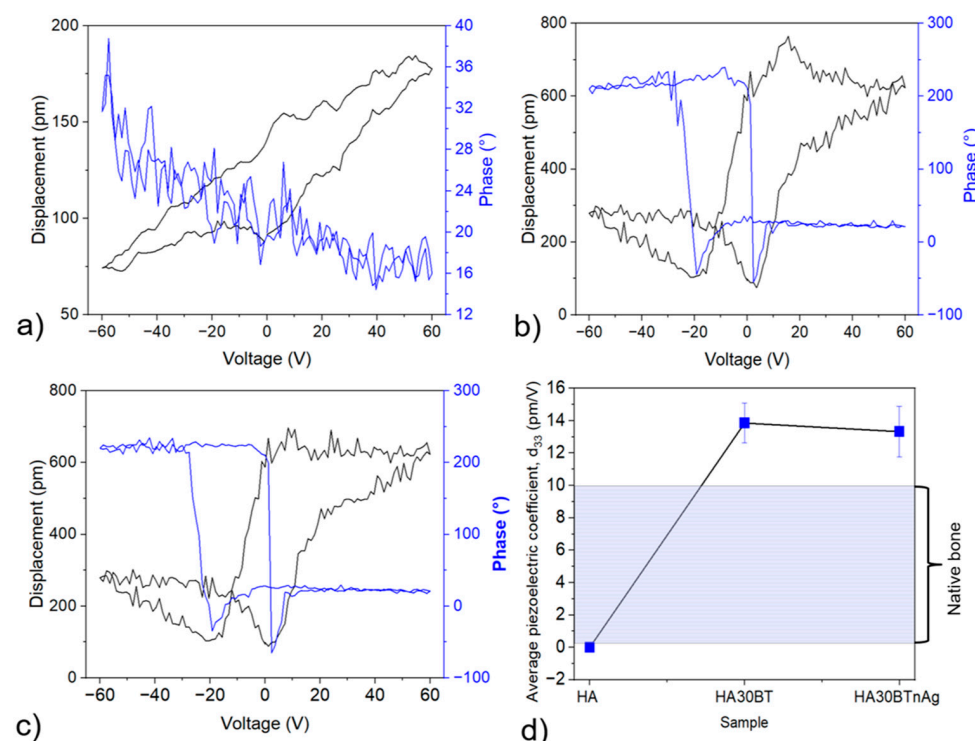


Figure 9. SS-DART-PFM characterization of the coatings. (a) Amplitude–voltage and phase–voltage responses of the HA coating. Representative butterfly loops and corresponding phase hysteresis curves of the (b) HA30BT and (c) HA30BTnAg coatings. (d) Average piezoelectric coefficient (d_{33}) of the coatings, compared with the range reported for native bone, indicating that the obtained values fall within a physiologically relevant range.

The average piezoelectric coefficient (d_{33}), calculated for each coating composition according to Equation (6), is presented in Figure 9d together with the range of values reported for native bone (~ 0.1 – 10 pm/V). As expected, the pure HA coating exhibited a negligible piezoelectric response. In contrast, the incorporation of BT into the HA matrix generated a pronounced electromechanical response, yielding an average d_{33} value of 13.85 ± 1.23 pm/V. The subsequent addition of nAg resulted in a comparable d_{33} value of 13.32 ± 1.57 pm/V. Considering the overlap of the standard deviations, the incorporation of nAg did not significantly affect the piezoelectric performance of the HA30BT coating, indicating that the piezoelectric functionality imparted by the BT phase was successfully preserved.

Within this framework, the d_{33} values of approximately 13 pm/V obtained for the HA30BT and HA30BTnAg coatings represent a favorable compromise between piezoelectric functionality and biofunctionality. In our previous study, HA/BT coatings containing 30 wt.% BaTiO₃ and exhibiting a d_{33} value of approximately 15 pm/V significantly enhanced osteogenic activity under low-intensity pulsed ultrasound (LIPUS) [31]. Therefore, although the d_{33} values obtained in the present work are slightly lower, they remain within a range expected to provide effective electromechanical stimulation while avoiding excessive cellular stress associated with higher piezoelectric responses. In contrast, pure conventional piezoelectric ceramics such as bulk BaTiO₃ (typically ~ 100 – 300 pm/V) and PZT (~ 250 – 700 pm/V) exhibit piezoelectric coefficients well above the physiological range of native bone [25–27]. Although these materials possess outstanding piezoelectric properties, the presence of lead in PZT raises significant concerns regarding cytotoxicity, limiting its use in biomedical applications [69]. Consequently, the moderate d_{33} values together with the inherent biocompatibility of the HA-based matrix make both the HA30BT and HA30BTnAg coatings promising candidates for piezodynamic stimulation and ultrasound-assisted bone regeneration.

3.6. Cell Viability and Biocompatibility

Figures 10 and 11 present the cell viability and cytotoxicity results, respectively, calculated according to the equations described in Section 2.7. The values were obtained from optical density measurements following the MTT-formazan reduction assay.

As reported in our previous work [31], the incorporation of BT into the coating matrix did not induce adverse cellular responses at any of the evaluated extraction periods. Cell viability values consistently exceeded 100%, indicating that the HA30BT coating promotes cellular proliferation and exhibits excellent cytocompatibility.

Furthermore, the HA30BTnAg coating did not exhibit significant cytotoxic effects (see Figure 11). Cytotoxicity values remained below 10% throughout the evaluation period, while negative cytotoxicity values were observed in several cases, indicating that coatings enhanced cellular activity relative to the control group (untreated cells). These results suggest that the incorporation of nAg at the evaluated concentration does not impair the biocompatibility of the coating and may contribute to maintaining a favorable cellular response.

When the effect of nAg incorporation was analyzed, a slight reduction in cell viability was observed at shorter extraction periods. However, as the incubation time increased, the cellular response became comparable to that of the HA30BT coating, indicating that the presence of silver nanoparticles did not adversely affect long-term cell behavior.

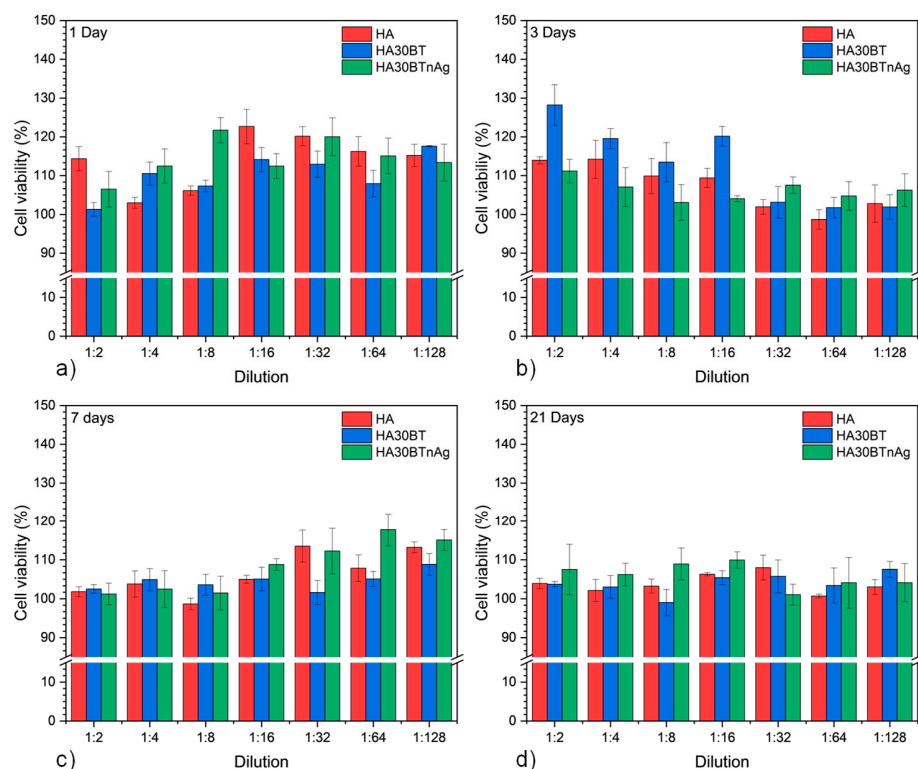


Figure 10. Viability (%) determined by the MTT-formazan assay for HOS ATCC CRL-1543 cells exposed to different dilutions of ion-release supernatants obtained from HA, HA30BT, and HA30BTnAg coatings after (a) 1, (b) 3, (c) 7, and (d) 21 days of incubation. All coatings exhibited high cell viability, indicating good cytocompatibility.

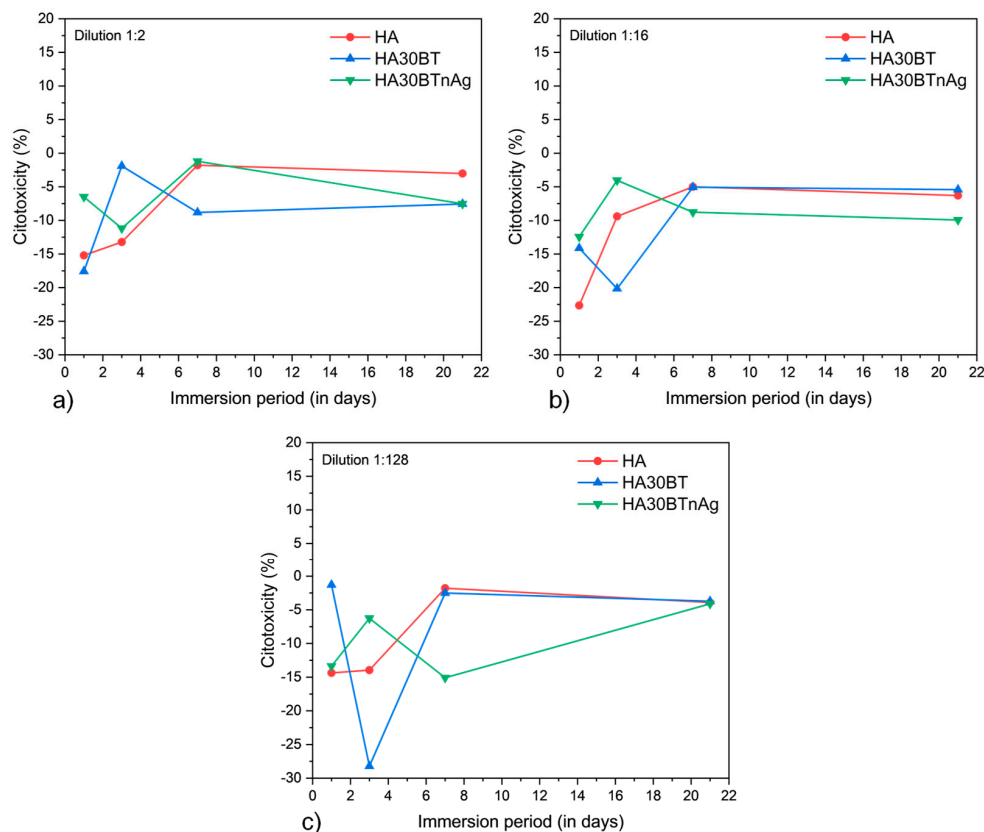


Figure 11. Cytotoxicity (%) determined by the MTT-formazan assay for HOS ATCC CRL-1543 cells exposed to ion-release supernatants from HA, HA30BT, and HA30BTnAg coatings at dilutions of

(a) 1:2, (b) 1:16, and (c) 1:128 after 1, 3, 7, and 21 days of immersion. All coatings exhibited cytotoxicity values below the 10% threshold, confirming their non-cytotoxic behavior according to ISO 10993 and ASTM F895 standards [70,71].

Overall, both HA30BT and HA30BTnAg coatings promoted cell proliferation and exhibited no cytotoxic effects. Cytotoxicity values remained below the 10% threshold established by ISO 10993 [70] and ASTM F895 [71] for the classification of non-cytotoxic biomaterials. These findings demonstrate that the incorporation of 1 wt.% nAg into the HA-BT coating does not compromise cytocompatibility and supports its potential application in orthopedic implant systems.

3.7. Cell Activity

Figure 12 shows immunofluorescence micrographs obtained by confocal microscopy for different markers: osteocalcin (OCN, first column), DAPI for nuclei (second column), rhodamine-phalloidin for F-actin (third column), and merged images (fourth column).

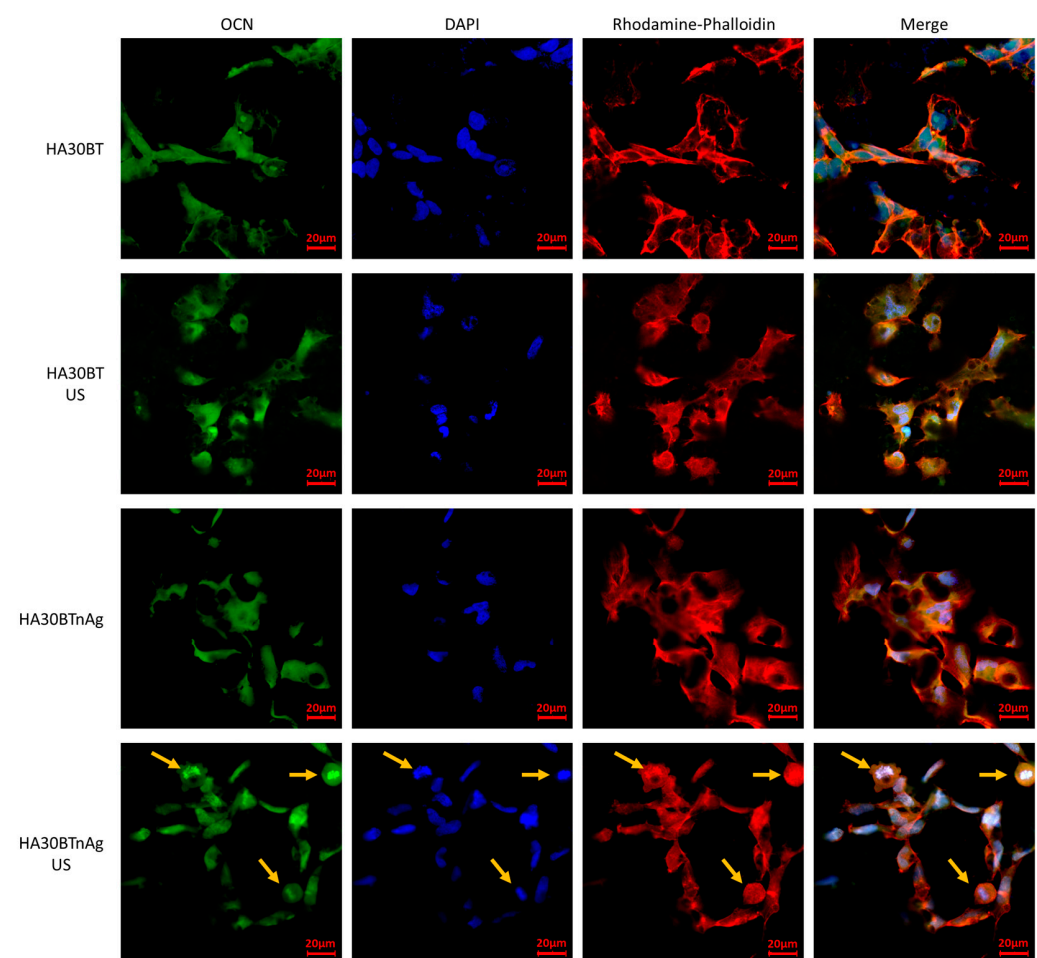


Figure 12. Immunofluorescent staining of HOS with and without LIPUS treatment for HA30BT and HA30BTnAg. The first column in green presents the FITC conjugate polyclonal Goat-Anti-rabbit/IgG for OCN, on the second column in blue DAPI labeling for nucleus, on the third column in red, Rhodamine-phalloidin for F-actin; and the fourth column represents the merge image of the three channels. The yellow arrows indicate some cells in mitogenic state possibly induced by the activity of the piezodynamic stimulus.

Immunodetection of osteocalcin (OCN) confirms that the incorporation of nAg does not exert a negative effect on osteogenic activity. Both HA30BT and HA30BTnAg coatings exhibit comparable OCN expression, indicating active osteogenic differentiation and sug-

gesting that nAg preserves the osteoconductive response of the system. OCN expression is closely associated with mineralization processes, supporting the ability of both coatings to promote osteogenic activity.

Analysis of the cytoskeletal F-actin staining further demonstrates that nAg incorporation does not adversely affect cell adhesion or morphology, as cells maintain their characteristic elongated shape. These results are consistent with the enhanced protein affinity observed in the PDP analysis, which plays a key role in mediating initial cell–material interactions.

As reported in previous studies [31,72–74], integrin activation is a critical step in cell–biomaterial interactions, triggering intracellular signaling pathways that regulate cell proliferation, differentiation, and the expression of osteogenic markers such as OCN. In this context, the increased protein adsorption observed on both HA30BT and HA30BTnAg coatings provides a favorable biochemical environment for integrin-mediated signaling and subsequent osteogenic differentiation.

In addition, LIPUS stimulation induces a piezoelectric response associated with the activation of the d_{33} coefficient, promoting osteocalcin expression and enhancing cellular activity. Importantly, this piezoelectric stimulation does not compromise cell adhesion, as cells maintain their elongated morphology. Moreover, in the presence of nAg, cells remain in a proliferative state, indicating that neither the piezoelectric response nor silver incorporation negatively affects cell proliferation.

3.8. Antibacterial Efficacy

Figure 13 presents a comparison of bacterial growth for *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. It can be observed that the HA30BTnAg coating containing 1 wt.% nAg exhibits high antibacterial efficacy against all three strains, as evidenced by the absence of colony formation after 24 h of incubation. In contrast, both the titanium substrate and the coating without nAg (HA30BT) did not exhibit antibacterial properties, showing significant bacterial colony growth for all tested strains under the same conditions.

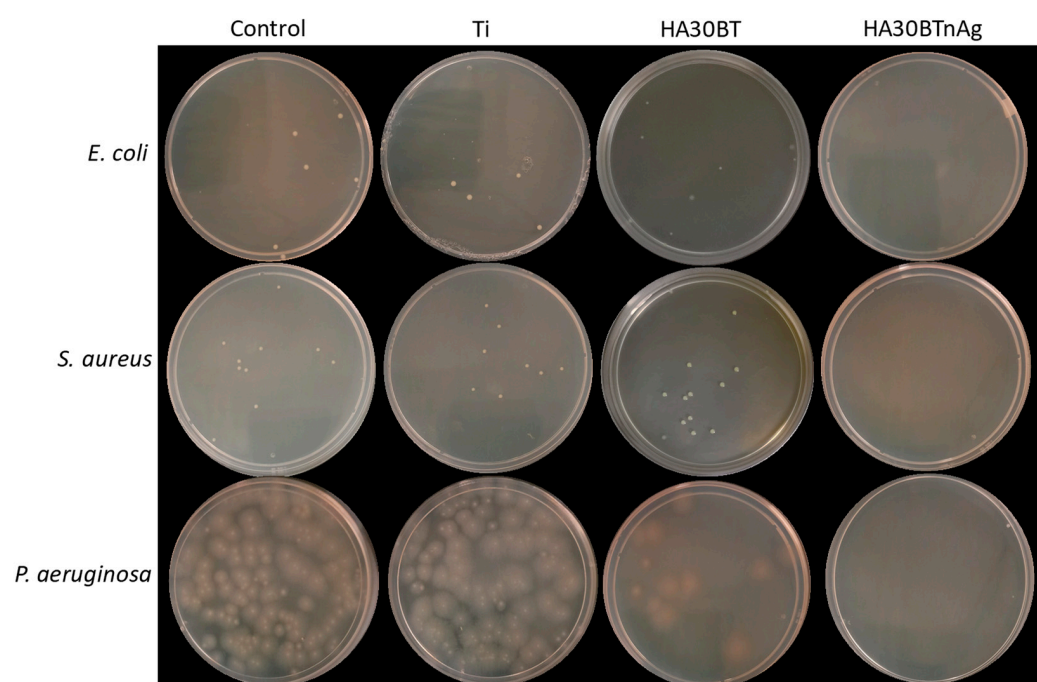


Figure 13. Comparative bacterial growth for control, Ti6Al4V, and coatings (HA30BT and HA30BTnAg). First row corresponds to *E. coli* growth; on the second row, the bacterial growth

of *S. aureus*; and on the third row, the bacterial growth of *P. aeruginosa*. The image presents the antibacterial efficacy of HA30BTnAg coatings against the different tasted stains.

Tables 4–6 summarize the antimicrobial activity (R) and antibacterial efficiency (%) against *E. coli*, *S. aureus*, and *P. aeruginosa*. According to the criteria established by the JIS Z2801 [64] standard, the HA30BTnAg coating exhibited R values of 10.2, 10.1, and 8.9 for *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively. These values significantly exceed the JIS threshold ($R \geq 2$), confirming strong antibacterial efficacy within 24 h of incubation and achieving 100% inhibition of bacterial growth for all tested strains.

Table 4. Summary of antibacterial efficacy against *E. coli* for uncoated Ti6Al4V, HA30BT, and HA30BTnAg coatings according to JIS Z 2801 standard [64].

<i>Escherichia coli</i> ATCC 4157			
Sample	CFU	Antibacterial Activity (R)	Antibacterial Efficacy (%)
Control	$1.4 \times 10^8 \pm 3.7 \times 10^7$	-	-
Ti6Al4V	$1.1 \times 10^8 \pm 2.3 \times 10^7$	0.13 ± 0.04	29.7 ± 6.4
HA30BT	$7.0 \times 10^7 \pm 6.3 \times 10^7$	0.23 ± 0.21	43.0 ± 25.2
HA30BTnAg	0	10.2	100

Table 5. Antibacterial activity of uncoated Ti6Al4V, HA30BT, and HA30BTnAg coatings against *S. aureus*, assessed using the JIS Z 2801 method [64].

<i>Staphylococcus aureus</i> ATCC 24213			
Sample	CFU	Antibacterial Activity (R)	Antibacterial Efficacy (%)
Control	$3.3 \times 10^8 \pm 1.1 \times 10^7$	-	-
Ti6Al4V	$1.8 \times 10^8 \pm 2.3 \times 10^7$	0.22 ± 0.12	39.6 ± 17.7
HA30BT	$1.6 \times 10^8 \pm 6.3 \times 10^7$	0.32 ± 0.15	47.5 ± 15.5
HA30BTnAg	0	10.1	100

Table 6. Comparative summary of the antibacterial efficacy of Ti6Al4V, HA30BT, and HA30BTnAg coatings against *P. aeruginosa* based on JIS Z 2801 evaluation [64].

<i>Pseudomonas aeruginosa</i> ATCC27833			
Sample	CFU	Antibacterial Activity (R)	Antibacterial Efficacy (%)
Control	$3.1 \times 10^7 \pm 7.5 \times 10^6$	-	-
Ti6Al4V	$1.5 \times 10^7 \pm 1.2 \times 10^7$	0.18 ± 0.11	31.6 ± 17.7
HA30BT	$1.4 \times 10^6 \pm 6.3 \times 10^5$	1.36 ± 0.31	94.6 ± 1.8
HA30BTnAg	0	8.9	100

In contrast, both the titanium substrate and the HA30BT coating exhibited R values below the antibacterial threshold ($R < 2$), indicating no significant antibacterial activity and confirming that both surfaces support bacterial proliferation under the tested conditions.

In the antibacterial analysis, Tables 4–6 present the calculated values of colony-forming units (CFU), the antimicrobial activity (R), and antibacterial efficiency percentage against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. According to the criteria established by the JIS Z2801 standard [64], the HA30BTnAg coating exhibited R values of 10.2, 10.1, and 8.9 for *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively. These values significantly exceed the JIS threshold ($R \geq 2$), confirming high antibacterial efficacy within 24 h of incubation and achieving 100% inhibition of bacterial growth for all tested strains.

In contrast, both the titanium substrate and the HA30BT coating exhibited R values below the antibacterial threshold ($R < 2$), indicating no antibacterial activity and confirming that both surfaces promote bacterial proliferation under the tested conditions.

4. Discussion

The results demonstrate that the incorporation of BT plays a critical role in imparting piezoelectric functionality to HA-based coatings, whereas the addition of green-synthesized nAg provides antibacterial capability without compromising the structural integrity or electromechanical performance of the composite system. To the best of the authors' knowledge, this is the first study evaluating the influence of green-synthesized silver nanoparticles incorporated into an HA-BT composite coating on its piezodynamic response under low-intensity pulsed ultrasound (LIPUS) stimulation, as well as on its cytocompatibility and antibacterial performance for orthopedic implant applications.

The XRD analysis confirmed the successful preservation of the HA, BT, and nAg phases after atmospheric plasma spraying. The absence of secondary phases, decomposition products, or oxide-related reflections suggests that the selected APS parameters were suitable for maintaining the phase stability of both BT and nAg despite the high temperatures involved during deposition. This finding is particularly relevant because degradation of BT could significantly reduce the piezoelectric performance of the coatings, while oxidation or agglomeration of silver could adversely affect its antibacterial effectiveness.

Microstructural observations further revealed a homogeneous distribution of the constituent phases throughout the coatings, with only limited agglomeration of the heavier BT and nAg particles. Such a distribution is advantageous because it promotes a more uniform electromechanical response and facilitates the antibacterial behavior of the coating. Cross-sectional analysis showed dense coatings with intimate contact between the coating and the Ti6Al4V substrate, without evidence of interfacial cracks, delamination, or other structural defects like porosity and splat level that could compromise the mechanical integrity and stability of the coating.

According to the mechanical analysis by tensile strength, coatings demonstrate a clear improvement in mechanical performance compared with the pure HA coating. The incorporation of BT and nAg increased adhesion strength by approximately 110% against pure HA coatings, reaching values over the FDA requirements (adhesive bond strength $\geq 15\text{MPa}$) [67,68], and fall within the range reported for high-quality APS hydroxyapatite coatings that ensures the mechanical integrity and stability of the coatings [75–78].

Although some authors have reported that adding silver nanoparticles or silver oxide nanoparticles can reduce coating adhesion, occasionally to values at or below regulatory limits (approximately to $\leq 16\text{MPa}$) [48]. Our results suggest that the chosen APS parameters, as well as the homogenization protocol for the raw material mitigates this effect.

The failure analysis according to the ASTM C633 confirms the mechanical robustness of the composite coatings. Both HA30BT and HA30BTnAg coatings showed cohesive-dominant failure modes ($89\% \pm 2\%$ and $81\% \pm 4\%$, respectively). According to the literature, cohesive failure is the preferred and most reliable failure pattern for APS HA coatings because it indicates that the coating-substrate bond strength surpasses the internal cohesive strength of the coating. This behavior is associated with stable interfaces and reduced risk of delamination under mechanical loading. In contrast, the adhesive failure is mainly useful as a warning that the interfacial adhesion, residual stress or splat-level bonding needs improvement [78–80]. These observations indicate that the incorporation of BT and nAg did not adversely affect the coating bond strength or structural integrity, essential requirements that may play an important role in the long-term implant performance under physiological loading conditions.

PFM results clearly demonstrated that pure HA coatings exhibit negligible piezoelectric activity, whereas the incorporation of BT generated a pronounced electromechanical response characterized by well-defined butterfly loops and polarization switching behavior. These observations are consistent with the ferroelectric nature of BT and confirm that the piezoelectric functionality of the composite coatings is predominantly governed by the BT phase. The piezoelectric coefficients obtained for the HA30BT and HA30BTnAg coatings were statistically comparable, indicating that the addition of nAg did not significantly interfere with the polarization mechanisms or domain switching processes responsible for the piezoelectric response. This preservation of piezoelectricity is particularly important because it demonstrates that antibacterial functionality can be introduced while preserving the electromechanical properties of the coating.

Interestingly, both composite coatings exhibited piezoelectric coefficients close to those reported for native bone [25–27], suggesting that they can reproduce a physiologically relevant electromechanical microenvironment. This biomimetic electromechanical behavior may be highly beneficial for orthopedic applications, as bone tissue naturally generates electrical potentials in response to mechanical loading. Such endogenous electrical signals are known to influence cellular activities including adhesion, proliferation, differentiation, and extracellular matrix production.

As highlighted in the literature, protein adsorption plays a fundamental role in the host response, serving as the initial step for cell adhesion and the activation of intracellular signaling pathways that regulate cell proliferation and differentiation [9,10,12]. The combination of electrochemical data obtained from Potentiodynamic Polarization (PDP) measurements with the Langmuir monolayer adsorption model provides a quantitative framework for evaluating protein affinity toward biomaterial surfaces.

Based on the electrochemical analysis, the decrease in corrosion current density observed in protein-containing solutions suggests that adsorbed proteins form a protective layer on the surface, reducing the electrochemical activity of the substrate and promoting interfacial stabilization. Such stabilization may favor subsequent cellular adhesion and proliferation processes. This behavior is consistent with previous studies reporting that protein adsorption modulates surface reactivity and contributes to the formation of a stable biomaterial–tissue interface [81,82]. Although these studies demonstrate the influence of protein adsorption on surface properties and corrosion behavior, they do not establish a direct correlation with *in vitro* biological responses. Therefore, the present approach provides valuable insight into the relationship between protein adsorption, electrochemical performance, and the biological functionality of the coatings.

Importantly, the incorporation of nAg into HA30BT coating further enhances protein affinity due to the intrinsic tendency of nAg. Numerous studies have shown that silver nanoparticles readily adsorb abundant blood proteins, particularly albumin, forming a stable protein corona driven by hydrophobic interactions, electrostatic attraction, and van der Waals forces [83–86].

Reported association for nAg–albumin interactions confirming a spontaneous and thermodynamically favorable adsorption process promotes interfacial stabilization. This inherent affinity of nAg toward albumin is consistent with the increased surface coverage parameter (θ) obtained for HA30BTnAg coating, indicating that the presence of silver nanoparticles facilitates protein binding and contributes to the formation of a protective layer.

In particular, the adsorption constant (K_{ads}) and surface coverage factor (θ) derived from the Langmuir adsorption model confirm that the HA30BT and HA30BTnAg coatings exhibit a higher affinity for proteins than the bare Ti6Al4V alloy. The higher θ values indicate a greater extent of surface coverage by adsorbed proteins, which is associated with

lower corrosion current densities and enhanced surface stability. This protein-rich layer may facilitate the adsorption of cell-adhesive molecules and create a favorable microenvironment for cell attachment and proliferation. Consequently, protein adsorption not only represents the initial stage of host–material interactions but also acts as a functional barrier that regulates interfacial processes and promotes downstream cellular signaling. These findings further support the potential of HA30BT and HA30BTnAg coatings for orthopedic implant applications, where improved corrosion resistance, protein affinity, and biological performance are essential for successful osseointegration.

Immunofluorescence analysis confirmed the expression of OCN in both HA30BT and HA30BTnAg coatings, indicating that the incorporation of nAg does not compromise the osteogenic response. According to the literature, OCN plays a crucial role in the mineralization process [33,72–74]. Therefore, the maintenance of OCN expression across both coatings highlights their ability to support osteogenic differentiation. Furthermore, the induction of the piezoelectric effect by LIPUS contributed to enhancing cellular activity, promoting cell proliferation and OCN expression. The combination of protein adsorption, integrin activation, and piezoelectric stimulation provides a favorable microenvironment for cell adhesion, proliferation, and osteogenic gene expression, thereby reinforcing the potential application of these coatings for improving bone tissue regeneration.

From an application perspective, the integration of piezoelectric and antibacterial functionalities within a single coating system is particularly attractive. While BT provides the ability to convert external mechanical stimuli, such as LIPUS, into localized electrical cues, nAg provides antibacterial activity that may reduce the risk of implant-associated infections. The preservation of both functionalities after APS processing suggests that the HA30BTnAg coating represents a promising multifunctional platform for next-generation orthopedic implants. Furthermore, the ability to generate electrical stimulation under LIPUS exposure could enable non-invasive activation of the coating after implantation, potentially enhancing bone healing while simultaneously maintaining protection against bacterial colonization.

Beyond preserving piezoelectricity, the magnitude of the piezoelectric response is also a critical factor, as effective biological stimulation requires a balance between sufficient electromechanical cues and avoiding cellular overstimulation. The measured d_{33} value of approximately 13 pm/V for the HA30BT and HA30BTnAg coatings is slightly higher than the values commonly reported for native bone; however, it remains within a physiologically relevant range for bone electromechanical activity. HA/BT coatings exhibiting a d_{33} value of approximately 15 pm/V enhanced osteogenic activity under LIPUS, whereas higher values (~35 pm/V) were associated with cellular stress [31]. Similarly, Zheng et al., (2023), demonstrated that BaTiO₃ coatings with an effective d_{33} of approximately 10 pm/V promoted cell elongation and osteogenic differentiation, while substantially higher coefficients (60–180 pm/V) reduced cell proliferation and osteogenic performance [87]. These findings suggest that the d_{33} values obtained in the present study provide an electromechanical stimulus that is sufficiently strong to promote osteogenic responses while remaining below the threshold associated with adverse cellular effects. This favorable response is likely related not only to the magnitude of the piezoelectric coefficient but also to the composite design of the coatings. Both HA30BT and HA30BTnAg consist of a hydroxyapatite-rich matrix containing 30 wt% of BaTiO₃, allowing the coatings to retain the excellent biocompatibility and osteoconductivity of HA while introducing piezoelectric functionality. Consequently, the combination of a biomimetic d_{33} value, preserved antibacterial activity, and a highly biocompatible HA matrix further supports the potential of these multifunctional coatings for bone regeneration and orthopedic implant applications.

According to the antibacterial tests performed in accordance with JIS Z 2801 [64], the obtained data confirm that silver nanoparticles confer broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria when incorporated into the HA-BT matrix coating. Consistent with previous reports, low concentrations of nAg (1–5 wt.%) are sufficient to produce a strong antibacterial effect [40,41,48,49,88,89]. The incorporation of 1 wt.% nAg into the HA-BT matrix was sufficient to completely inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* within 24 h. Although excessive silver concentrations or ion releases have been reported to adversely affect mammalian cell viability and osteogenic activity [48,88,89], the present coating maintained excellent biocompatibility. Cytotoxicity and cellular activity assays demonstrated that the incorporation of nAg did not adversely affect osteoblast viability, while after 21 days of exposure all coatings exhibited cytotoxicity values below the accepted 10% threshold, remaining within the limits established for non-cytotoxic biomaterials [70,71]. Moreover, the incorporation of nAg did not compromise coating stability or adhesive strength (adhesive strength ≥ 15 MPa), as all evaluated parameters complied with the corresponding international standards [67,68]. Although these findings demonstrate an excellent balance between antibacterial efficacy, structural reliability, and long-term biocompatibility, further studies involving long-term Ag^+ ion release and XPS analyses before and after immersion are recommended to elucidate the stability, oxidation state, and antibacterial mechanism of silver within the coating. Overall, these findings highlight the potential of HA30BTnAg composite coatings as a multifunctional platform that combines piezoelectric functionality, antibacterial activity, and excellent biocompatibility to promote osteointegration while reducing the risk of implant-associated infections in orthopedic applications.

5. Conclusions

Under the experimental conditions employed, the APS system does not induce degradation of the BT and nAg phases. This structural stability ensures that both antibacterial and piezoelectric effects remain at optimal performance levels, consolidating the coatings potential as a multifunctional strategy for biomedical applications.

The results demonstrate that polarization curves could be an effective tool for assessing cell viability. The decrease in current corrosion, associated with the formation of a protein monolayer on the surface, directly affects the materials' ability to promote initial cell adhesion and intracellular signaling processes.

This work demonstrates that HA–BT composite coatings incorporating green-synthesized nAg can successfully combine piezoelectric and antibacterial functionalities without compromising structural, mechanical, or biological performance. BT is responsible for the piezoelectric response, while nAg provides strong antibacterial activity, and both phases are stably preserved after APS processing.

This study lays the groundwork for future in vivo test aimed at evaluating the behavior of the coating when exposed to animal models, representing a critical step toward its preclinical validations. Specifically, it was demonstrated that the HA-based coating with 1% of nAg and 30% of BT exhibits outstanding performance, combining structural stability with optimal antibacterial, compatibility, and piezoelectric properties to promote osteointegration. Overall these findings reinforced the potential strategy for the surface functionalization of Ti6Al4V metallic implants, simultaneously enhancing osteointegration and reducing infection risk.

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