Application of the response surface methodology for the evaluation of *Staphylococcus aureus* inhibition with Ag/TiO₂ nanoparticles

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Abstract

The present work shows the implementation of the Response Surface Methodology (RSM), fed by an experimental Central Composite Design (CCD) to find the conditions that allow maximizing the inhibition of the microorganism *Staphylococcus aureus* with nanoparticles of TiO₂ silanized with 3-Aminopropyltriethoxysilane (APTES) and doped with Ag. In addition, Poly(lactic) acid composites were prepared with these Ag/TiO₂ nanoparticles with the aim to confer their antimicrobial effect. The independent variables considered were pH, AgNO₃/TiO₂ ratio (% w/w), and TiO₂ nanoparticles concentration (g/250 mL), and as the variable of response, the length of the diameter of the halo or zone of inhibition presented by the microorganism (mm). Statistical analysis found that maximization of *S. aureus* inhibition occurs at intermediate levels with a value of 10 for pH and 5 g of TiO₂ solids, while for the concentration of AgNO₃ high levels are required, greater than 10% w/w. Likewise, the statistical significance was determined using the Student's t-test and the p-value; it was found that the significant effect corresponds to the concentration of AgNO₃, so a second experimental CCD design equirradial with two factors was considered, estimating AgNO₃ concentration and TiO₂ amount, the pH at constant 10 value. The second experimental design indicated that maximization in *S. aureus* inhibition occurs at an AgNO₃ concentration between 20-25% w/w with high amounts of TiO₂ solids (7-8 g), with a resulting zone of inhibition between 26-28 mm. The quadratic model obtained, which represents the relationship between the length of the zone of inhibition with the variables considered, shows an adjustment of experimental data with a coefficient of determination (R^2) of 0.82.

Introduction

In recent years, the overuse of synthetic polymers has generated one of the significant environmental problems; consequently, research in the development of materials using natural polymers has responded to a global need, seeking an ecological alternative to reduce environmental impact, whose properties being biodegradable and antimicrobial, contribute to mitigating the amount of waste and environmental pollution [1-4]. Furthermore, with the growing consumer demand and concerns about the environment and limited natural resources, renewable resources to produce edible or biodegradable packaging materials that maintain product quality and safety while reducing waste are widely explored. Consequently, a variety of renewable polymers such as polysaccharides (starch and chitosan), proteins, lipids, and their compounds, as well as Polylactic Acid (PLA) and Polydimethylsiloxane (PDMS) are being developed.

The biocide properties of metal nanoparticles (MNPs) have generated significant interest for their application as new antimicrobial agents [5-6]. Nanogold has presented an excellent antibacterial efficacy towards different pathogenic bacterial species (gram-positive and gram-negative) [7-8]. The antimicrobial activity of Ag-nanoparticles was investigated against yeast, *Escherichia coli*, and *Staphylococcus aureus*, making them applicable to various medical devices and antimicrobial control systems [9-10]. Ag-Au bimetallic nanocomposite is another strategy to study the antipathogenic effect [11-12]. Carbon quantum

dots (CQDs) and ZnO/CQDs Ncs, have recently been investigated and considered in the family of carbon nanostructures that exhibited excellent antimicrobial potency against *Bacillus cereus*, *E. coli*, *S. Aureus*, *S. Typhi*, and *Candida albicans* [13-15].

Titanium dioxide (TiO_2) is a mineral that finds naturally in three crystalline phases: anatase and rutile (both tetragonal) and brookite (rhombohedral) [4]; it is not toxic and is applied in environmental treatments such as air and water purification, disinfection, and sterilization of water due to its properties and chemical stability [16-17]. It is one of the most studied semiconductors and is an innocuous material with high resistance to photo corrosion. It is stable in aqueous solutions. It is not expensive, abounds in nature, and has good photocatalytic activity under ultraviolet irradiation [16-30]. Different routes can synthesize TiO_2 ; these include flame synthesis, chemical vapor deposition, precipitation, and the alkoxide sol-gel method [12]. In recent years, interest in TiO_2 -based nanomaterials has been steadily growing because of their unique physicochemical properties. They are used for photocatalysis, solar energy engineering, air and water purification of organic pollutants, removal of heavy metal ions, detoxification of solutions, antibacterial agents, and medical devices and medicine since it is compatible with human tissues [22,31-34].

TiO_2 has been used for the inhibition of microorganisms, especially Gram-positive ones such as *S. aureus*. However, there are limitations with the practical use of TiO_2 , since this material is only photoactive under ultraviolet irradiation ($\lambda < 380 \text{ nm}$), which restricts the use of TiO_2 as an active catalyst for the light since most of the sunlight consists of invisible light and infrared radiation, and only about 5% of UV light [25, 31]. Various methods have been used to improve its absorption of visible light; these techniques include surface modification through sensitization of organic or semiconductor materials, modification of the band space creating oxygen vacancies or oxygen sub-valence through the doping with some nonmetals (like nitrogen) or metals (like silver) [20, 31]. Modification of TiO_2 with transition metals (including silver, platinum, ruthenium, and palladium) has been reported to decrease the recombination process by forming structures and new interfaces that improve photocatalytic efficiency [17-18, 27]. An attractive solution is creating nanosystems with an Ag- TiO_2 structure [33], not only because TiO_2 is a material with desirable properties but also because Ag shows some unique chemical and biological detection activities compared to other noble metals.

Compared to other metal ions, silver-deposited TiO_2 has a broad spectrum of antibacterial activity and is safe due to its non-toxic nature [35]; and has also received special attention for its low cost of synthesis and high availability [36]. Therefore, the antibacterial activity applications of silver and TiO_2 generate great interest since they can be applied in the manufacturing of coated sanitary products, medical devices, surfaces for food preparation, air conditioning filters, among others. In addition, the deposition of Ag promotes the transformation of anatase to rutile, which is attributed to the increase in the specific surface area that causes the improvement in the photocatalytic activity and improves the separation of the electron-hole pair [30]. Furthermore, Ag nanoparticles photo catalytically deposited on individual rutile TiO_2 crystals exhibit multicolor photo-chromism and dissolution in different sizes, and Ag deposition

causes multicolor spectral changes [37]. Therefore, the study of Ag-TiO₂ modified has a significant practical value [38].

Silver (Ag) is the most studied biocide agent, and numerous information is available regarding its antimicrobial activity mechanism [9-10; 39], which is much higher than other metals [32]. It is active against Gram-negative bacteria (*Escherichia coli* and *Pseudomona aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and some species of the *Streptococcus* family) [21, 26-27]. There are also reports on the antimicrobial activity of Ag nanoparticles obtained specifically against *Pseudomonas aeruginosa*, *Microbacterium foliorum*, *Bacillus subtilis* and *Rhodococcus equi* [40]. Although there is great controversy about the mechanism of action, these could be summarized in three main points. The first would be explained by the gradual release of Ag ions that inhibit the production of adenosine triphosphate (ATP) and DNA replication, fundamental factors for cell survival. The second mechanism could be attributed to the ability of nanoparticles to generate direct damage to the cell membrane. Then, the third to the generation of reactive oxygen species that generate oxidative stress and subsequent cell death [4]. The production of Ag particles at the nanoscale results in the generation of a high surface area, which is an important property to inhibit bacterial growth [17-18]

To prepare metal oxide nanocomposites suitable for antibacterial materials, it is necessary to disperse in organic binders the nanoparticles without aggregation. Nanoparticles tend to agglomerate strongly due to the surface/particle size ratio. At the same time, the use of different coupling agents is recommended for surface modification of the nanoparticles to achieve adequate dispersion of the nanoparticles. A suitable surface modification in the nanoparticles leads to better dispersion and compatibility between them, and the formation of physical and chemical interactions with the polymer matrix guarantees a lasting chemical bond between two incompatible phases. It is crucial to have a homogeneously dispersed metal oxide [41-42].

Poly(lactic acid) (PLA) is a new type of biodegradable material, which is made from renewable plant resources (such as sugar beets, corn, and potato starch) [45-46]. It has high thermal stability, biocompatibility, low cost, and low environmental impact, making it one of the most promising polymers in the 21st century [47-49]. However, pure PLA films have some disadvantages, such as their null antimicrobial activity and poor mechanical properties. In order to improve the functional properties of PLA films, different investigations have been carried out involving the combination of NPs of TiO₂ and Ag in PLA matrices to increase the effective inhibition of bacterial reproduction and mechanical properties [50-55, 17, 56-59]. In addition, researchers have worked to incorporate fillers in PLA matrices to broaden the applications of this polymer, which allows improving the antimicrobial properties [1]. The fillers that have been shown better microbicidal properties are silver, copper, zinc, titanium, and iron oxides [2, 3].

There are several works about the synthesis and use of Ag/TiO₂ nanoparticles as antibacterial agents [16-17, 19, 21, 43-44]. However, there are still some limitations related to the adequate deposition of the metal. The suitable dispersion of the nanoparticles is sought to maximize inhibition against *S. aureus*. Process variables are critical for successfully applying the deposition process of Ag nanoparticles on the

silanized TiO₂ with APTES to improve their antibacterial properties, which will be evaluated through the response surface methodology with an experimental Central Composite Design. Finding the optimal conditions for silver deposition based on the highest inhibition is essential to exploit the use and application of nanoparticles. Therefore, what is sought with this work is to maximize the inhibition of *S. aureus*, considering different variables such as pH, the precursor concentration, AgNO₃ (% w/w), and amount of TiO₂ in solution (g /250 mL). With this, resources would be saved, and the Ag/TiO₂ nanocomposites with outstanding antibacterial properties could be produced. This evaluation will be carried out by implementing a response surface methodology with a central compound type experimental design.

This research aimed to produce and characterize PLA-based high-performance biocomposites with Ag/TiO₂ particles to maximize the inhibition of the microorganism *Staphylococcus aureus*, using a DCC to obtain the best conditions. The effect of pH, AgNO₃/TiO₂ ratio (% w/w), and TiO₂ nanoparticles concentration (g/250 mL) in structural and thermal properties and materials behavior against bacterial growth and biofilm development of a strain of *E. Aureus* was investigated. Once the best values of the variables were found, the nanoparticles obtained by this optimization were immersed in a PLA matrix to evaluate if the resulting polymer will have adequate antimicrobial capacity.

Materials And Methods

Materials

Nanoparticles (NPs) of TiO₂ with a diameter of approximately 350 nm (R-104 DuPont, Mexico), 3-Aminopropyltriethoxysilane Coupling Agent (APTES) (99% Sigma Aldrich, Mexico), and silver nitrate (AgNO₃) (JT Baker).

Experimental design

A three-factor Central Composite Design (CCD) was proposed to study the process of Ag-nanoparticle deposition in TiO₂ in order to determine the influence of each of these considered factors. The CCD is a fractionated factorial or factorial design with central points, enlarged with a group of axial points that allow estimating the curvature, are used to estimate the first- and second-order terms efficiently and to model a response variable with curvature by adding central and axial points to a previously executed factorial design [60]. The variables evaluated were pH (X₁), AgNO₃/TiO₂ ratio (%w/w) (X₂), and TiO₂ nanoparticles concentration (g/250mL) (X₃) having as a response variable, the inhibition zone on the discs (halos measured in mm) of each of the experimental units, in order to demonstrate that there is a significant influence of these variables on the inhibition of *Staphylococcus aureus*. The matrix studied for this CCD is shown in Table 1.

Statistical analysis

MINITAB 2017 Statistical Software (version 17.1; MINITAB Inc., State College, PA, USA) was used for data analysis, prediction models, and construction of surface and contour plots. The accuracy of the polynomial equations was expressed by R^2 (coefficient of determination), and the F-test verified their statistical significance at a 95% confidence level.

Surface modification of TiO₂ nanoparticles with APTES

Incorporating the coupling agent (APTES) superficially on the TiO₂ surface is known as silanization. The best coupling route for the modification of TiO₂ NPs [41] requires 1 mL of APTES and 100 mL of ethanol for every 5 g of TiO₂ to be modified. For incorporating TiO₂ in ethanol, 30 minutes of stirring were needed using a stirring magnetic grid (Thermo Scientific, Cimarec) with 30 minutes of sonication at 60 Hz in an ultrasonic bath (Bransonic, model 5510R-MT) until completing a total time of 2.5 hours. Subsequently, the APTES was added, and in a 10-minute interval, 1 mL of water was added to allow hydrolysis in the reaction. This mixture was left stirring at 400 rpm for 6 more hours. The solution was placed in 50 mL falcon tubes for centrifugation and was washed twice with distilled water and five times with methanol to remove the reaction medium. Each washing was performed at 300 rpm for 10 minutes with approximately 30 mL of solvent. The silanized sample was dried at 80 °C for 3 hours. The dry material was stored in vials for subsequent characterization and use.

Fourier Transform Infrared Spectroscopy (FTIR) and Thermo-gravimetric Analysis (TGA)

The FTIR spectra of unmodified silanized TiO₂ NPs were measured using a Fourier Transform Infrared spectrometer (FTIR-ATR Perkin Elmer, Spectrum 100 model). The analyses included the wavenumber range of 3000 to 1000 cm⁻¹ with 80 scans or sweeps per spectrum, placing the NPs in granular form for a better reading [52]. The thermo-gravimetric analysis was carried out on a TGA (TA Instruments Model Q500), performed in a temperature range of 25 to 600°C, with a heating ramp of 10°C per minute in a nitrogen atmosphere with a constant flow of 60mL per minute. A sample of about 10 mg was placed in platinum baskets [41].

Electron Transmission Microscopy (TEM)

The size and morphology of the NPs were obtained by transmission electron microscopy (TEM) on a 200 kV microscope (JEOL model JEM 1230). The samples consisted of 0.005 g of each nanocomposite suspended in 2 mL of ethanol and sonicated for 5 min, and a drop of 10 µL was placed on a copper grid [61].

Z Potential

The samples were sonicated and mixed with a Vortex-Genie 2 Scientific Industries vortex shaker To make colloidal solutions [41]. The surface charge (Zeta potential) of the two different TiO₂ nanoparticle systems was characterized by a Delsa Nano C Particle Analyzer Z-Potential meter (A53878), using electrophoretic light scattering (ELS). This equipment has a dual source of 30 mW and a laser diode of

659 nm. The method consisted of suspending the NPs in water at a concentration of 0.01% w/v by scanning pZ at different pH (measured in a Thermo Scientific potentiometer, model Orion Star A211) modified with HCl 0.1N and NaOH 0.1N [62].

Nuclear magnetic resonance (NMR)

The NMR spectra (^{29}Si and ^{13}C) of the NPs were obtained from the Bruker Ascend equipment (400 MHz), which operates at 400 MHz for ^{29}Si and 100 MHz for ^{13}C . The parameters established for the ^{13}C case were a scan number of 16 and a pulse length of 8.5 μs .

Deposition process of TiO_2 with Ag by reduction of AgNO_3

The preparation of metallic silver on the TiO_2 surface was based on the AgNO_3 chemical reduction route proposed by [44]. The amount of TiO_2 (total solids), the pH, and the Ag/ TiO_2 ratio (% w/w) followed the experimental design proposed in Section 2.2. The aqueous suspension of TiO_2 NPs was prepared by adding the required amount of it to 250 mL of deionized water followed by sonication for 10 minutes, heating it to 80 °C with vigorous agitation on a magnetic grid. Once the temperature was reached, the required amount of AgNO_3 was added to the suspension to obtain the desired concentration, maintaining heating and stirring. The required pH of the suspension was adjusted with NaOH 0.5 M, the suspension at 80 °C was mixed for 2 hours. The solution was placed in falcon tubes for later centrifugation, performing two washes with distilled water to remove unreacted agents. Each wash was performed at 300 rpm for 10 minutes with approximately 30 mL of water. The mixture obtained was dried in a convection oven (Lab-Line Instruments, model 3471) at 80 °C for 12 hours.

Antibacterial test

The Inhibition test was performed by the disk diffusion method, a modification of the technique described by Bauer, Kirby, Sherris, and Turk; it is described in the following sections [63]. Medium. Mannitol Salt Agar was used. The medium was prepared according to the instructions on the package. Because the temperature of the medium was high at the end of the sterilization time, it was necessary to place it in a water bath until a temperature of approximately 48-50 ° C was reached. Finally, the medium was distributed in approximately 25 cm^3 in Petri dishes, previously sterilized.

Filter paper discs for nanoparticle impregnation. Filter paper discs were prepared for the different experimental units. First, they were cut out with a commercial hole punch; these discs were each 6mm in diameter, 6 to 8 discs were placed in number in Eppendorf tubes of 2 mL capacity (1 tube for each system). Once this was done, the tubes were sterilized in an autoclave at 121 ° C for 15 min. Once labeled, approximately 1.5 mL of the nanoparticle solution at 1000 ppm from each system were added to each tube; before this, the tubes remained in a UV light chamber for 15 min. Finally, they were stored at room temperature for 24 h to allow the nanoparticles to soak into the discs.

Inoculum. 4 to 5 colonies of the microorganism were selected from a pure culture. The colonies were transferred by touching the top of each with a bacteriological loop to a tube containing 3-5 cm³ of sterile Mueller-Hilton broth. It was incubated at 35°C for 2 to 8 h until moderate growth occurred. This growth was determined by comparing the pure broth with the one containing microorganisms; a change in turbidity was attributed to the growth of microorganisms. The culture was diluted with sterile broth at 5: 9 until obtaining a turbidity equivalent to a 0.5 tube on the McFarland scale, which corresponds to approximately 108 viable microorganisms per mL.

Turbidity standard. To prepare the standard, 0.5 mL of BaCl₂ dihydrate (1.175% w / v) was added to 99.5 mL of H₂SO₄ 0.36N (1% w/v). It was mixed perfectly and distributed in screw cap tubes in a quantity of 6-8 mL; were hermetically sealed and stored in a dark place at room temperature. To make a comparison with the culture, the standard was vortexed.

Sample seeding. The seeding of the sample was carried out in an extraction hood, previously sterilized with UV light. A sterile cotton swab was immersed into the tube with the suspension of the microorganism in turn, in a 5: 9 dilution (this dilution does not exceed the standard turbidity equivalent to 0.5 on the McFarland scale). Once the swab was inside, it was placed above the level of the contents of the tube and rotated against the tube walls to remove excess inoculum. The inoculum was then evenly seeded on the medium surface of the Petri dish with the swab. This seeding was carried out in four directions, rotating the Petri dish 90 degrees. Thus, it was avoided that the inocula were very concentrated or diluted and that parts of the medium remained uninoculated. Once the boxes of all the systems were inoculated, it was necessary to allow the surface of the seeded medium to dry for 15 minutes, keeping the Petri dish closed. After a lapse of time, the Eppendorf tubes were labeled with each system (which rested for twenty-four hours to impregnate the nanoparticles), discs were placed on the surface of the agar with sterile forceps. The discs were slightly pressed on the agar to ensure uniform contact. Each box corresponding to each Eppendorf tube of each system was labeled. A total of 3 discs were placed on the surface of the medium, trying to leave a uniform space between each disc to observe the results of the inhibition halos better. Once the Petri dishes already contained the discs on the surface, they were wrapped with parafilm paper to avoid sudden movements of the medium. Finally, in a drying oven (Lab-Line Instruments, model 3471), all the boxes were incubated at 35 °C for 36 h to allow the microorganism to grow.

Measurement of inhibition halos

To measure the zone of inhibition of each disk, the samples were kept inside the extraction hood on the dark surface, with good lighting and lights reflected on the boxes. Next, electronic photos with 12MP resolution were taken of each of the systems. Finally, these stored photos were analyzed with ImageJ version 1.52a software to obtain the average diameter of the experimental units.

Characterization of TiO₂ particles deposited with Ag

The characterization of TiO₂ particles deposited with Ag was performed within the experimental units that presented the highest and lowest halo/zone inhibition. The samples were analyzed by transmission electron microscopy (TEM) to confirm the superficial modification and the Ag-deposition, using a JEOL equipment (model JEM 1230 microscope) with a resolution of 0.4 nm and an acceleration voltage of 100 kV.

Preparation of PLA composites with Ag-deposited TiO₂ nanoparticles

PLA composites at 0.1% w/w were prepared. First, 500 mg of PLA were dissolved in 20 mL of chloroform; once a homogeneous solution was obtained, 0.5 mg of NPs were added and then stirred and sonicated for 30 min at 60 Hz in an ultrasonic bath. Finally, the suspension obtained was placed on a glass container (silica), and the solvent was evaporated at room temperature. This procedure was made for each system of nanoparticles.

Antibacterial analysis of PLA films by drop-test method

PLA composites were tested against *S. aureus* using the antibacterial drop-test method with some modifications [4]. First, a film of each respective composite was introduced in a different test tube, in duplicate, each containing nutrient broth inoculated with the bacteria. Moreover, a culture of the microorganism was grown without any treatment as a control. Then the tubes were incubated for 24 h, and the solutions were read with a spectrophotometer. The optical absorptions [A] were recorded, and the inhibition rate (%) was calculated according to the following equation:

$$\text{Inhibition rate(\%)} = \frac{[A]_i - [A]_c}{[A]_i} \times 100 \quad (1)$$

where [A]_i is the optical absorption of control bacteria, and [A]_c is the absorption of each composite.

Characterization of the TiO₂ nanoparticles and PLA composites by X-ray diffraction (XRD)

The XRD-measurements for the TiO₂ nanoparticles and PLA composites were used to investigate the crystalline structure of the pristine and Ag-deposited TiO₂ and the influence of these nanoparticles in the crystalline structure of the PLA composites by using a Bruker Model D8 ADVANCE diffractometer with Lynx Eye detector and Copper tube (Wavelength 1.5406Å). The scanning of samples (2θ) was performed at 20-80° with a 0.02° increment.

Results And Discussion

Experimental design and response surface methodology

The application of the experimental CCD design and the response surface methodology allowed the researchers to know the conditions that had a better inhibition of *Staphylococcus aureus* by using nanoparticles of TiO₂ modified and deposited with Ag as an antibacterial agent. The zone of inhibition,

the halos present in the *S. aureus* plate (mm), obtained by CCD design established are shown in Table 2. The sizes of the halos are in a range of 7.32 to 11.26 mm.

The application of the RSM generated the second-order regression polynomial (Eq.1), which represents an empirical relationship between the response (length of the inhibition halos with *Staphylococcus aureus*) and the evaluated variables. The statistical significance of each of the coefficients was evaluated by the Student's t-test and the p-value. Table 3 shows the variables and interactions that have the highest incidence on the deposition process in *Staphylococcus aureus* inhibition. In Table 3, a significant variable is found when the p-value is less than 0.05, which indicates that the effect is significant with 95% confidence. Therefore, the significant effect corresponds to AgNO₃ (x₂) concentration, while the TiO₂ solids in solution and pH are not significant in the process, being pH the least influential. The model shows a good fit with the experimental data, having a coefficient of determination (R²) of 0.86, implying that 86.00% of the variables fit the model and that it only does not explain 14% of the total variation.

$$y = -13.45 + 4.468x_1 - 0.818x_2 + 1.472x_3 - 0.2347x_1^2 + 0.0018x_2^2 - 0.1479x_3^2 + 0.0636x_1x_2 - 0.0434x_1x_3 + 0.0650x_2x_3 \quad (\text{Eq. 1})$$

The analysis of the response surface, illustrated in Fig. 1, shows the behavior of the different combinations of effects on the size of *S. aureus* inhibition halos, in which the largest size occurs at intermediate levels, pH of 10 and 5 g of TiO₂ solids in solution. In contrast, for AgNO₃ concentration, high levels are required (10% w/w). The most suitable working ranges were determined from the contour graph, Fig. 2; the results obtained were pH 9 to 11, a TiO₂ amount between 4 and 6 g, while the range of AgNO₃ concentration covers a region above 11% w/w. From the second-order model (Eq. 1), the canonical model (Eq. 2) was obtained, and the characteristic values, with alternating signs ($\lambda_1 = -0.2459$, $\lambda_2 = -0.1464$, $\lambda_3 = 0.0115$), determine that the results show a saddle point.

$$\hat{y} = 9.7357 - 0.2459w_1^2 - 0.1464w_2^2 + 0.0115w_3^2 \quad (\text{Eq. 2})$$

Based on the results obtained, a second equi-radial experimental CCD design was established with two factors (Design 2), considering as independent variables: AgNO₃ concentration concerning TiO₂ (X₁) (25, 35, and 45% w/w) and the amount of NPs of TiO₂ in solution (X₂) (3, 5 and 7 g/250mL), and now with constant pH in 10. The response variable remains the same, size of the halo or zone of inhibition of the disks (diameter of halos in mm). Table 4 shows the results obtained from Design 2 of the disk test. The ranges of the *S. aureus* halo of this second proposed design are between 13.16 and 22.09 mm, and compared to the first experimental design (7.32 to 11.26 mm), it presents an increase of 96.18%, which indicates that there is an enhancement of almost the double in halo size.

Statistical analysis of the results using the Student's t-test and calculation of the p-value provided the estimated coefficients for the quadratic model. Table 5 shows the variables and interactions present in the deposition process in *Staphylococcus aureus* inhibition. As a result of Design 2, the p-value shows

that the TiO₂ solids in solution remain as a non-significant variable, while the AgNO₃ concentration is the significant variable in the deposition process due to the p-value was found below 0.05.

The response function (length of the inhibition halos with *Staphylococcus aureus*) was represented using an RSM through a second-degree polynomial equation (Eq. 3), where the two independent variables were considered. As a result, the quadratic model shows an acceptable fit of experimental data, with a coefficient of determination (R²) of 0.82, which implies that 82.00% of the experimental data fit the model; therefore, 18% of the total variation was unexplainable.

$$y = 14.9 - 0.370x_1 + 1.07x_2 + 0.01678x_1^2 + 0.343x_2^2 - 0.1253x_1x_2 \quad (\text{Eq. 3})$$

In the response surface graph, Fig. 3, it was observed that the maximization of *S. aureus* inhibition occurs at a low concentration of AgNO₃ (20-25% w/w) with high amounts of TiO₂ solids. (7-8 g), observing the highest levels of inhibition (26-28 mm) highlights the importance of the addition of TiO₂ solids. In the statistical analysis, the X₁X₂ interaction was significant; this was observed in the curvature shown by the surface graph. The significance of this interaction indicates that within the experimental domain, synergistic interaction effects commonly attributed to the dispersion of TiO₂ particles were observed when they are dispersed in an aqueous medium in the Ag deposition process.

The most suitable working ranges were determined through the contour graph, Fig. 4, having a value between 21 and 24% w/w for the AgNO₃ concentration, with a TiO₂ quantity ranging from 7.5 to 7.8 g. The results obtained from the second experimental design allowed determining that an AgNO₃ concentration between 20-25% w/w with amounts of silanized TiO₂ between 7-8 g and pH of 10 achieves an inhibition zone of 28 mm in *Staphylococcus aureus*. This value has improved 300% regarding the zone of inhibition, reported by [64], who analyzed the antibacterial behavior of an Ag/TiO₂/PVC composite membrane tested in the same bacterial strain. Likewise, an increase of just over 200% was found in the inhibition of the same microorganism, given that they tested Ag and TiO₂ modified polycaprolactone nanofibers (PCL/TiO₂-Ag), showing an antibacterial impact of 9.2 mm in the diameter of the zone of inhibition [66]. In other research [36], a 40% increase in the zone of inhibition was obtained compared to unmodified Ag/TiO₂ nanoparticles since they showed a maximum zone of inhibition of 20 mm when working with a similar strain of *S. aureus* (methicillin-resistant).

The most surprising results emerging from the data are that these Ag/TiO₂ showed excellent inhibition against *S. aureus* since inhibition is better than previous reports. For example, Emam et al. [7] found no antibacterial activity against this gram-positive bacterium using AuNPs obtained using starch polymer. While Ahmed et al. [12] obtained nanoparticles using hydroxyethyl cellulose as nanogenerator and surfactant, they reported an inhibition zone of only 16 mm using AgNPs obtained, 15 mm with AuNPs, and 18 mm with Ag-Au bimetallic nano-alloy.

Characterization of TiO₂ nanoparticles

Surface-modified TiO₂ nanoparticles were used in the Ag deposition process and subsequently used in the antibacterial test. The modification of the surface of TiO₂ was carried out with APTES; these particles underwent a series of tests to confirm the presence of silica (present in APTES) on the oxide surface. One of the characterizations performed was the FTIR, which provides information about the functional groups present in the samples to be analyzed. Fig. 5 shows the results of the FTIR spectra for unmodified (black line) and modified TiO₂ (red line). It is essential to mention that new bands appeared in the particles modified with APTES; symmetric and asymmetric stretching vibration of the C-H bond was observed in the methylene group at 2960, 2927, and 2858 cm⁻¹ [29, 66-67].

The peak spanning between 1630 to 1640 cm⁻¹ is attributed to the stretching vibration of absorbed water and the hydroxyl (OH-) groups present on the surface of the nanoparticles [29, 66, 52, 68]. The small peak at 1641 cm⁻¹ corresponds to the stretching vibration of the (NH)C=O group [66]. Other evidence of the modification with APTES on the nanoparticle surface is the peak at 1560 cm⁻¹ that corresponds to the flexion of the amino-functional group (-NH₂) [66, 68]. The peak shown between 1420 and 1490 cm⁻¹ corresponds to the elastic band of the C-H organic group bond [29, 32, 52]. The peak observed at 1378 cm⁻¹ was assigned to the C-N aromatic amine group [65]. The stretch band of the C-O group bonds is also observed at 1259 cm⁻¹ [62] and the carbonyl group at 1300 cm⁻¹ [52]. Finally, the last peaks around 1120, 1130, and 1044 cm⁻¹ are attributed to the Si-O-Si bond, indicating polymeric siloxane as a product of the organosilane precursor [29, 66, 52, 68]. Similarly, peaks at 1045, 1075, and 1130 cm⁻¹ are attributed to Ti-O-C bonds [29, 66, 52]. The superficial modification of the TiO₂ nanoparticles with the coupling agent (APTES) is confirmed with the above described.

On the other hand, to analyze size and morphology, transmission electron microscopy is a suitable test for this purpose. The images provided by TEM gave further evidence of the superficial modification of the TiO₂ NPs with the coupling agent APTES. Fig. 6 (a) shows TiO₂ without modification. These NPs do not have a uniform surface, and it is appreciated how they tend to agglomerate. This behavior is due to the surface/particle size ratio that causes strong agglomerates that limit its suspension stability. Besides, this is attributed to Van der Waals forces, and the attracting effect tends to decrease the dispersion of the nanoparticles. While in Fig. 6 (b), the micrograph is observed after performing the silanization process. It is possible to observe an organic coating due to the APTES bound to the TiO₂ surface with an average of 2.82 nm thick. All this information confirms the presence of the coupling agent on the TiO₂ surface.

However, it was necessary to verify that the superficial modification of the TiO₂ led to an improvement in the dispersion and colloidal stability, which is necessary to facilitate the interaction in the aqueous medium of these nanoparticles in the deposition process with Ag. The profiles of Z potential as a function of the pH of pure TiO₂ (unmodified) and silanized TiO₂ (modified) are observed in Fig. 7. After surface modification of the NPs, the Zeta potential of the silanized TiO₂ NPs increased considerably due to the amino groups (NH₂) that appeared in the outer layer of the surface of the NPs.

The increase in the positive charge generated by the protonation of the amino groups caused an increase in the positive Zeta potential in the acid region [67], increasing the repulsion between the NPs. Likewise, this increase in the Z potential is favorable because it prevents instability (IEP) and agglomeration [41]. The IEP value for the unmodified oxide is approximately at pH 3.5, lower than for modified TiO_2 , at pH 5.6. This shift in IEP is attributed to the alkaline characteristics of NH_2 groups [52] or the easy desorption of hydrogen (H^+) protons found on the NPs surface of silanized TiO_2 . All the aforementioned indicates that the surface behavior of the TiO_2 NPs changed very markedly and that at the same time, the dispersion of the nanoparticles favors the deposition process with Ag since there is a higher surface ratio of TiO_2 nanoparticles dispersed that come into contact and interact with the metal favoring the process and the amount of silver deposited on the surface. Values of Z potential from -30 to 30 mV indicate instability in the system; values below -30 and above 30 mV indicate an increase in stability, increasing with higher absolute values of the Z potential [41].

Another essential characteristic sought when developing these types of materials is that they acquire the ability to withstand high temperatures and that there is no degradation thereof, to such a degree that the material is not lost: at the same time, it maintains its characteristics and properties. The TGA analysis corresponding to unmodified TiO_2 is shown in Fig. 8 (black line), in which it is possible to observe that it remains practically stable from 25 °C to 800 °C, unlike the functionalized system present losses of weight. When analyzing the thermal degradation of the coupling agent APTES on the surface of the TiO_2 , Fig. 8 (blue line), it can be distinguished that the modified material has thermal stability at high temperatures, such as the temperatures used in industrial polymer processes, for example, since at approximately 98.5 °C only 0.15% by weight of the material is lost, at 563 °C a total of 0.38% is lost. Finally, until 734 °C, a total of 0.56% of material is lost in weight. This result is incredibly low since little more than 99.4% of the total is conserved; the loss in weight is related to the decomposition of the organic chains of the coating with APTES.

The characterization of the NMR spectrum for ^{13}C , Fig. 9, shows the existence of 3 peaks in the spectrum that correspond to signals 12.40, 27.84, and 45.76 ppm, they are identified in Fig. 9 as C_1 , C_2 , and C_3 , these signals refer to the three carbons of the propyl group (a group that is present in the coupling agent APTES), these signals are approximately the same indicating that the carbons are in the same amount on the surface of the TiO_2 . As already mentioned, the signals belong to carbons. However, the difference between each one is related to the type of group to which it is bond, Carbon 1 is the one that is bond to Silicon, Carbon 2 is linked to two other carbons, and Carbon 3 would be the one that is linked to the amino group (NH_2). On the other hand, in Fig. 10, the NMR spectrum for ^{29}Si denotes signals at -67.43 and -59.45 ppm, identified as T_3 and T_2 , respectively, attributed to the silica found on the surface of TiO_2 . T_3 is assigned to the bond between silica in the APTES and three oxygens on the TiO_2 surface. In comparison, T_2 silicon refers to silica bonds with only two oxygens on the TiO_2 surface and one remaining oxygen as hydroxyl (OH) group.

Characterization of TiO_2 particles deposited with Ag considering the highest and the lowest result of the response variable of the two-factor experimental CCD design.

The results obtained from implementing the experimental design on the microorganism inhibition allowed the researchers to know the highest and lowest size of the inhibition zone. The results of Table 4 made it possible to determine that the experimental unit 6 presented the maximum size of inhibition (22.09 mm). While sample 1 determined that the halo size (13.16 mm) was not favored under these conditions. Nevertheless, the inhibition of the microorganism exists in all the experimental units of the CCD. Therefore, the characterization was carried out using TEM/EDS to explain the behavior of these two samples.

Fig. 11 illustrates the TEM micrograph of experimental unit 6 of Table 4. This sample was made up of 49.14% w/w AgNO_3 concentration and 5g of TiO_2 , that is, the points (+1.4142, 0) of the CCD of two-factor, founding the maximum length of inhibition for *S. aureus* (22.09 mm). On the other hand, Fig. 12 shows the micrograph of experimental unit 1, which obtained the shortest length of the microorganism's inhibition halo (13.16 mm). This sample was prepared with 25% w/w AgNO_3 concentration and 3g of TiO_2 , that is, the axial points (-1, -1) of the two-factor CCD. Morphologically in both experimental units, TiO_2 nanoparticle with Ag dots on the surface can be seen; the difference is distinguished in the amount of Ag that is superficially distributed on the TiO_2 nanoparticle. This difference is mainly attributed to the AgNO_3 concentration that was used in each sample.

The chemical analysis provided by TEM-EDS of the Ag-deposited TiO_2 is shown in Fig. 13 and Fig. 14 of the experimental units 6 and 1, respectively. From this analysis, the chemical composition of each sample was obtained. The results of this analysis confirmed the presence of silver (b), oxygen (c), and titanium (d), which interact during the deposition process with the metal. Furthermore, it is possible to observe that Ag is distributed on the surface of TiO_2 (same that is confirmed in Fig. 11 and Fig. 12), this indicates a correct dispersion of AgNO_3 during the deposition process with the oxide because an ideal chemical interaction was assumed by improving the dispersion of TiO_2 after the silanization process with APTES. The different compositions of the samples are found in Table 6.

The results of the drop-test method carried out to the composites against *S. aureus* are shown in Fig. 15. The values indicate inhibition activity in the PLA composites prepared with 0.1% TiO_2 nanoparticles deposited with Ag. As observed, better inhibition was obtained for the system with the highest result of the response variable of the two-factor experimental CCD design, M6, with an 18.75% of inhibition rate. Nevertheless, the lowest result, M1, also presented inhibition activity but with less efficiency, only 6.25%. Therefore, with this information, it is possible to report that it is possible to confer the antimicrobial property from the TiO_2 deposited with Ag to the PLA composites [1]. Last, with this antibacterial test was possible to corroborate the applicability of the experimental CCD design since the best inhibition result for the composite (PLA-M6) aggress with the highest value obtained for the TiO_2 deposited nanoparticles in the disc diffusion method.

Compared with the literature, although these results may look lower values than those reported previously, it is important to mention that the filler (Ag/TiO₂) in the PLA matrix is exceptionally low. For example, Emam [11] reported a high reduction percentage in *S. aureus* (96-100); nevertheless, he stated that the antibacterial efficacy was attributed to the bi-action of two nanoparticles: silver and gold; in addition, they worked with a high amount of nanoparticles (0.5 mL/20 mL). At the same time, Ahmed et al. [12] reported great inhibition rates against *S. aureus* of approximately 60%, but with approximately 0.3% of Au content in silk fabrics. With all this information, it is possible to state that the composites prepared at 0.1% of Ag/TiO₂ in PLA are consistent and even better than previous reports regarding the nanoparticle content.

XRD-patterns were used to know the crystalline structure of the TiO₂ particles deposited with Ag. Fig. 15A shows by X-ray diffraction (XRD) the results, and it can be observed the characteristic peaks for the structures of anatase and rutile in both samples; these results are due to both phases of titanium dioxide are mixed during their industrial origin [5]. Besides, it is possible to observe the characteristic peaks of the silver nanoparticles at 32.12° (101) and 38.01° (111) [5]. While Fig. 15B shows the structures of the PLA nanocomposite films were analyzed to study the effect of nanoparticle content on the crystallinity of the PLA matrix. Both XRD patterns showed an amorphous curve indicating a low degree of crystallinity of PLA and a maximum intensity at approximately 2θ = 17° attributed to the characteristic peaks of PLA [6] [7]. On the other hand, the absence of TiO₂ peaks or new diffraction peaks can be seen, which can be attributed to the few numbers of functionalized nanoparticles (only 0.1%) immersed in the PLA matrix. This information suggests that the structure of PLA was not changed by incorporating the TiO₂ particles deposited with Ag [8].

Conclusions

The superficial modification of the TiO₂ nanoparticles with APTES has a significant influence because they tend to agglomerate due to their high surface area to volume ratio. A correct superficial modification of the TiO₂ nanoparticles was achieved, which was demonstrated with the dispersion of the silanized material favoring colloidal stability; since electrostatic charges increased the repulsion between the nanoparticles present, the measurement of Z Potential gave a value that is outside the range of instability. Likewise, FTIR, TEM, TGA, and NMR measurements confirmed a correct surface modification and an approximate thickness of 2.82 nm of APTES obtained in the silanization process. Current research indicates that the conditions established from the second experimental design in the doping process of TiO₂ with Ag (Concentration of AgNO₃, Amount of TiO₂, and constant pH) can be applied to make an antibacterial material with a high impact on the inhibition of *Staphylococcus aureus*. Implementing the first experimental design and the response surface methodology indicated an increase in the AgNO₃ concentration causes an enhanced microorganism inhibition. However, at the same time, the statistical significance evaluated by the Student's t-test and the p-value showed that the same variable was significant in the process, while pH was the least influential variable. Due to the above, a second experimental design was established, leaving the pH constant at 10. For the inhibition of *S. aureus*, the AgNO₃ concentration range between 20-25% w/w is adequate together with 7 or 8 g of TiO₂ in

solution. The length of the *S. aureus* zone of inhibition can be well represented by the second-order model obtained from the application of the response surface methodology, with a determination coefficient R^2 of 0.82, so the length can be predicted of the inhibition zone with up to 82% precision.

Declarations

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Competing interests:

The authors declare no competing interests.

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Tables

Table 1 Levels of the experimental design of the variables studied

N°	ENCODE VARIABLE			NATURAL VARIABLE			RESPONSE
	X1	X2 (% p/p)	X3 (g/250 mL)	X1	X2 (% p/p)	X3 (g/250 mL)	<i>S. aureus</i> halo (mm)
1	-1	-1	-1	8.00	4.00	3.00	8.56
2	1	-1	-1	12.00	4.00	3.00	8.27
3	-1	1	-1	8.00	10.00	3.00	8.25
4	1	1	-1	12.00	10.00	3.00	9.37
5	-1	-1	1	8.00	4.00	7.00	8.42
6	1	-1	1	12.00	4.00	7.00	7.32
7	-1	1	1	8.00	10.00	7.00	9.56
8	1	1	1	12.00	10.00	7.00	10.10
9	-1.68179	0	0	6.64	7.00	5.00	7.88
10	1.68179	0	0	13.36	7.00	5.00	7.75
11	0	-1.68179	0	10.00	1.95	5.00	9.86
12	0	1.68179	0	10.00	12.05	5.00	11.17
13	0	0	-1.68179	10.00	7.00	1.64	8.96
14	0	0	1.68179	10.00	7.00	8.36	8.63
15	0	0	0	10.00	7.00	5.00	10.50
16	0	0	0	10.00	7.00	5.00	9.58
17	0	0	0	10.00	7.00	5.00	9.15
18	0	0	0	10.00	7.00	5.00	10.88
19	0	0	0	10.00	7.00	5.00	11.26
20	0	0	0	10.00	7.00	5.00	10.62

Table 2 Results of the response variable for *Staphylococcus aureus*

N°	ENCODE VARIABLE			RESPONSE
	X ₁	X ₂ (% p/p)	X ₃ (g/250 mL)	<i>S. aureus</i> halo (mm)
1	-1	-1	-1	8.56
2	1	-1	-1	8.27
3	-1	1	-1	8.25
4	1	1	-1	9.37
5	-1	-1	1	8.42
6	1	-1	1	7.32
7	-1	1	1	9.56
8	1	1	1	10.10
9	-1.68179	0	0	7.88
10	1.68179	0	0	7.75
11	0	-1.68179	0	9.86
12	0	1.68179	0	11.17
13	0	0	-1.68179	8.96
14	0	0	1.68179	8.63
15	0	0	0	10.50
16	0	0	0	9.58
17	0	0	0	9.15
18	0	0	0	10.88
19	0	0	0	11.26
20	0	0	0	10.62

Table 3 Significance of the regression coefficients of the *Staphylococcus aureus* evaluation

Variable	Regression coefficient	T-student value	<i>p</i> value
Interception	-13.45	41.63	
X ₁	4.468	0.02	0.984
X ₂	-0.818	3.07	0.012
X ₃	1.472	0.17	0.867
X ₁ ²	-0.2347	-5.85	0.000
X ₂ ²	0.0018	0.10	0.921
X ₃ ²	-0.1479	-3.69	0.004
X ₁ X ₂	0.0636	1.77	0.107
X ₁ X ₃	-0.0434	-0.81	0.439
X ₂ X ₃	0.0650	1.81	0.100

Table 4 Results of the response variable for *Staphylococcus aureus* from the CCD experimental design of two factors

N°	ENCODED VARIABLE		NATURAL VARIABLE		RESPONSE
	X1 (% p/p)	X2 (g/250 mL)	X1 (% p/p)	X2 (g/250 mL)	y, <i>S. aureus</i> halo (mm)
1	-1.00000	-1.00000	25.0000	3.0000	13.16
2	1.00000	-1.00000	45.0000	3.0000	19.59
3	-1.00000	1.00000	25.0000	7.0000	20.40
4	1.00000	1.00000	45.0000	7.0000	16.80
5	- $\sqrt{2}$	0.00000	20.8579	5.0000	13.98
6	$\sqrt{2}$	0.00000	49.1421	5.0000	22.09
7	0.00000	- $\sqrt{2}$	35.0000	2.17157	18.34
8	0.00000	$\sqrt{2}$	35.0000	7.82843	16.50
9	0.00000	0.00000	35.0000	5.0000	15.26
10	0.00000	0.00000	35.0000	5.0000	14.93
11	0.00000	0.00000	35.0000	5.0000	14.29
12	0.00000	0.00000	35.0000	5.0000	14.13
13	0.00000	0.00000	35.0000	5.0000	14.17

Table 5 Significance of the regression coefficients of the evaluation of *Staphylococcus aureus* from the CCD experimental design of two factors

Variable	Regression coefficient	T-student value	<i>p</i> value
Interception	14.9	21.08	
X_1	-0.370	3.27	0.014
X_2	1.07	0.42	0.686
X_1^2	0.01678	2.87	0.024
X_2^2	0.343	2.34	0.052
X_1X_2	-0.1253	-3.25	0.014

Table 6 TEM / EDS chemical analysis of Ag-doped and modified TiO₂ particles from experimental unit 6 and 1

	Experimental unit (6)	Experimental unit (1)
Element	% Weight	% Weight
Silver (Ag)	21.7	16.2
Oxygen (O ₂)	28.6	37.7
Titanium (Ti)	49.7	46.1

Figures

Figure 1

Response surfaces of the evaluation of *S. aureus*, a) pH vs. AgNO₃ concentration (% w/w), b) pH vs. TiO₂ solids (g), c) AgNO₃ concentration (% w/w) vs. TiO₂ solids (g)

Figure 2

Contour graphs of the evaluation of *S. aureus*, a) pH vs. AgNO₃ concentration (% w/w), b) TiO₂ solids (g) vs. pH, c) AgNO₃ concentration (% w/w) vs. TiO₂ solids (g)

Figure 3

Response surface of the evaluation of *S. aureus*, AgNO_3 concentration (% w/w) vs. TiO_2 solids (g)

Figure 4

Contour graph of the evaluation of *S. aureus*, AgNO_3 concentration (% w/w) vs. TiO_2 solids (g)

Figure 6

TEM micrographs a) TiO₂, b) Silanized TiO₂ (modified)

Figure 7

Z potential of the NPs of TiO2 and modified TiO2

Figure 8

Thermo-gravimetric analysis of TiO2 and silanized TiO2 (modified)

Figure 9

NMR spectrum for ^{13}C

Figure 10

NMR spectrum for ^{29}Si

Figure 11

TEM micrograph of CDC Two-Factor experimental unit 6

Figure 12

TEM micrograph of CDC Two-Factor experimental unit 1

Figure 13

TEM/EDS analysis of the CCD experimental unit 6 of two factors: a) Ag/ TiO2 morphology, b) Ag distribution, c) O2 distribution, and d) Ti distribution

Figure 14

TEM/EDS analysis of the CCD experimental unit 1 of two factors: a) Ag/ TiO2 morphology, b) Ag distribution, c) O2 distribution, and d) Ti distribution

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