



# All-green wound dressing prototype based on Nile tilapia skin impregnated with silver nanoparticles reduced by essential oil

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## Abstract

The development of eco-friendly prototypes for wound dressing systems represents an important source of investigation about alternatives for the treatment of severe infections. The production of antibacterial agents free of antibiotics, based on natural products and biological supports is a hot topic for science applied in circular economy concepts. Herein, the green synthesis of silver nanoparticles was assisted by essential oil and the following impregnation of products in tilapia skin has been conducted as a strategy to produce all-green wound dressing systems to be applied as biofilm protective layers and active antibacterial agents. The use of these wound dressing systems against *Staphylococcus aureus* was evaluated in terms of biofilm adhesion, inhibition halo assays, and time-kill assays as a function of the nature of reducing agent applied in the production of silver nanoparticles.

**Keywords** Silver nanoparticles · Essential oil · Tilapia fish skin · Wound dressing · Antibacterial · Biofilm

## Introduction

The increasing interest for investigation about smart dressings paves way for the development of therapeutic solutions to treat chronic infections (Ferrag et al. 2021; Hussain et al. 2017; Mao et al. 2021; Ochoa et al. 2020; van Wamel 2017). The skin is considered the major surface organ of the human body and is responsible by protecting the inner tissues from physical, chemical, and biological dangers (Chambers and Vukmanovic-SteJic 2020). In some cases, in which the skin integrity is lost, the healing of chronic wounds is a complex and slow process, that involves multiple cellular mediators and a set of well-organized phases in the coagulation and anti-inflammatory processes, and in the reconstruction of new tissues (Asati and Chaudhary 2017; Ciecholewska-Juško et al. 2021; Hussain et al. 2017; Roy et al. 2021). This is a serious health problem and is a life-threatening disease with rough prevalence and mortality (Sharifi et al. 2021).

Examples of innovation in wound dressings are the incorporation of silver nanoparticles into hydrogel matrices with enhanced antimicrobial and mechanical properties (Ferrag et al. 2021), electroanalytical flexible dressing based on conductive nanocomposites for monitoring bacterial infections (Roy et al. 2021), natural polymer-based films with antioxidant properties (Bergonzi et al. 2020), up to the use of cross-linked superabsorbent bacterial cellulose-based biomaterials for the treatment of chronic wounds (Ciecholewska-Juško et al. 2021).

Nile tilapia skin is an innovative natural product with the potential for application in cell regeneration and proliferation (Hu et al. 2017; Miranda and Brandt 2019; Ouyang et al. 2018; Song et al. 2021). With a similar morphological structure to human skin, non-infectious microbiota, and a large amount of type I collagen, tilapia skin has been applied to the treatment of burns (Lima et al. 2020; Ouyang et al. 2018; Song et al. 2019) control of bleeding in consequence of blood clotting ability/ hemostatic efficiency (Sun et al. 2020), and wound healing (Wang et al. 2021).

In terms of wound healing applications, it is important to evaluate the antibacterial activity of different materials. Chronic wounds are susceptible to infections, mainly caused by *Staphylococcus aureus*, a Gram-positive bacteria that impairs the healing process (Abele-Horn et al. 2000; Fazli

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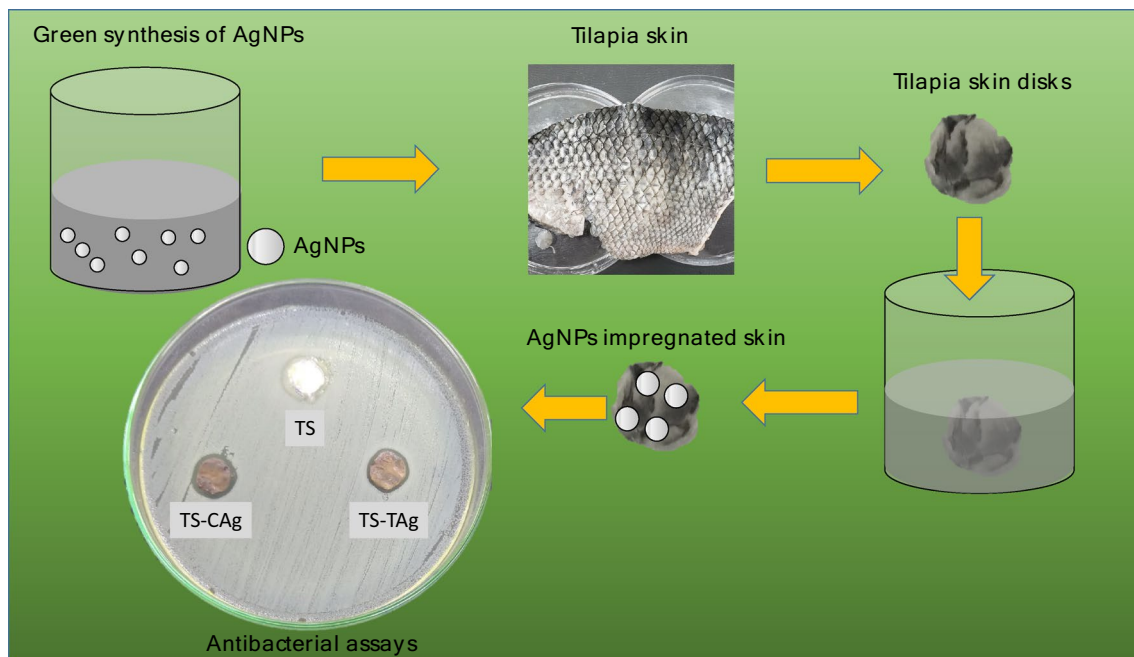
et al. 2009; James et al. 2008; Roy et al. 2021). Biofilms are associated with chronic nonhealing wound infections and have been considered a big challenge for the development of conventional therapy, stressing the necessity of new alternative antimicrobial strategies (Evelhoch 2020). An efficient way to increase the antibacterial activity of tilapia skin and its functionality as a material for biomedical application is based on its association with silver nanoparticles. Silver nanoparticles (AgNPs) play an important role as alternative antibacterial agents against *S. aureus*, and *Streptococcus pyogenes* (Gram-positive bacteria), as well as efficiency against Gram-negative bacteria, such as *Escherichia coli*, (Schierle et al. 2009) *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Maghimaa and Ali 2020; Nishanthi et al. 2018).

The synthesis of silver nanoparticles is divided into conventional methods (chemical, phytochemical and physical) and unconventional (biological) methods. Ion reduction by conventional methods is based on microemulsions, thermal degradation, electrochemical synthesis, evaporation–condensation, etc. (Tulinski and Jurczyk 2017) The biological method (or green synthesis) is based on the use of plant extracts (Guimarães et al. 2020), essential oils (Maciel et al. 2019), bacteria, fungi, algae (Abdelghany et al. 2018) and organic biomolecules (Salaheldin et al. 2017) for the reduction of silver ions.

The green synthesis of metal nanoparticles has been progressively reported in the literature, representing one

of the most important eco-friendly alternatives for the production of AgNPs. The overall process of metal nanoparticle synthesis can be mediated by essential oils, that offers a diversity of chemical components (phenolic acids, flavonoids, terpenoids, and anthocyanins) applied in the steps of nucleation, growth, and stabilization of nanoparticles, with the possibility of incorporation of synergistic processes in antibacterial activity of synthesized AgNPs (Mukundan et al. 2005; Vilas et al. 2016, 2014). The association of eco-friendly silver nanoparticles and natural materials as supports for wound dressing prototypes represents a promising strategy to reach desirable antibacterial activity against severe infections.

Herein, an environmentally friendly method is proposed for the production of silver nanoparticles followed by a step of impregnation of these nanostructures in Nile tilapia skin, which has been applied as a smart wound dressing system against *S. aureus*. For this, the optimization in the essential oil (type and concentration) was evaluated in terms of the response of synthesized samples (morphology, structure, and optical response), while the association of these nanoparticles with Nile tilapia was evaluated in terms of effective activity as an antibacterial and anti-biofilm agent. The general scheme for procedures explored in this work is summarized in Fig. 1, in which is possible to identify the green synthesis of silver nanoparticles, the impregnation in tilapia skin for the following step of the use of resulting material as support in antibacterial assays.



**Fig. 1** General scheme for the synthesis and impregnation of silver nanoparticles into tilapia fish skin and the following step of use for in vitro assays against bacteria

## Materials and methods

### Materials

Silver nitrate (Sigma Aldrich), sodium hydroxide, and acetone (Dinâmica, Brasil) all PA degrees were used as received. Ultrapure water was produced from the Millipore system (Millipore, USA) with a resistivity of 18 MΩcm. The essential oil of *Cymbopogon nardus*, *Melaleuca alternifolia*, *Eucalyptus globulus*, *Eugenia caryophyllus*, and *Cinnamomum zeylanicum* were acquired from Akã Óleos Essenciais (Bahia, Brazil), with a high degree of purity.

### Green synthesis of silver nanoparticles

Silver nanoparticles were biosynthesized according to the method described by Maciel et al. (2019) with some modifications. An initial group of five essential oils (*Cymbopogon nardus*, *Melaleuca alternifolia*, *Eucalyptus globulus*, *Eugenia caryophyllus*, and *Cinnamomum zeylanicum*) was explored as active agents for the reduction of the silver nanoparticles. An effective activity for the silver reduction was observed for tea tree oil (*Melaleuca alternifolia*) and citronella oil (*Cymbopogon nardus*). As a general standard method, aliquots of both essential oils (100 μL) were diluted in 17 mL of acetone at pH 8 which was adjusted from the incorporation of NaOH aqueous solution (0.1 M). Following this step, an aqueous solution of silver nitrate (1 mM) was prepared from which an aliquot of 30 mL was removed and kept under intense stirring and heating (95 °C).

After this step, solutions of essential oils (tea tree and citronella) were dropwise into silver nitrate aqueous solution until reaching a total volume of 32 mL (2 mL of essential oil solution). The kinetics of the formation of silver nanoparticles was monitored at a fixed interval of time (15, 30, 45, and 60 min) from the measurement of the characteristic peak of absorbance of silver nanoparticles in the UV–Vis spectrum. To avoid the effect of degradation induced by light, all of the procedures were conducted in dark conditions. The resulting samples of silver nanoparticles were identified as TAg (tea tree oil-based silver nanoparticles) and CAg (citronella oil-based silver nanoparticles).

### Preparation of tilapia fish skin (TS)

Nile tilapia fish skins were donated by Omega Pescados do Vale (Brazil) and treated as follows: fish scales and the

excess of residual fish meat were removed with a stiletto. In the following step, disks of TS with a 2 cm-diameter were separated and washed with ultrapure water in abundance and immersed in a phosphate saline solution (PBS) at 37 °C. These steps are based on the procedure reported by (Howaili et al. 2020) (with some modifications).

### Impregnation of silver nanoparticles on Nile tilapia fish skin

The impregnated Nile tilapia skin with different silver nanoparticles (TAg and CAg)—defined as TS-TAg and TS-CAg (respectively), were prepared as follows: circular disks of TS were immersed into as-prepared silver nanoparticle solution at a fixed interval of time, in a process that induces the adsorption of nanoparticles into the tilapia skin. The resulting material was dried at ambient temperature to eliminate residual water.

### Characterization methods

The absorption spectra of silver nanoparticles in water were measured by a UV–Vis HachDR500 spectrophotometer in the range of 300–800 nm (step of 1 nm). The nanoparticles' size, zeta potential, and dispersity index were determined in a zetasizer Nano-ZS90 Malvern. The structure of synthesized material was scrutinized by FTIR assays, using the KBr method in a Fourier Transform Infrared spectrometer IR Prestige-21 Shimadzu.

### Antibacterial assays

Bacterial cultures of *S. aureus* (ATCC 25923-reference for strong biofilm formation) were kept at 40 °C in stock solutions from which aliquots were removed to prepare saline aqueous solutions (0.5 in McFarland scale of turbidity). Aliquots of this solution were removed to be inoculated on Muller–Hinton agar plates ( $10^4$ – $10^8$  CFU). Qualitative evaluation of the activity of silver nanoparticles impregnated in tilapia skin was performed from inhibition halo experiments, in which circular disks of tilapia skin fish (negative control), TS-TAg, and TS-CAg samples were disposed on a Petri dish with Plate Count Agar (PCA) in the presence of *S. aureus* for 24 h at 37 °C. Biofilm formation quantification was evaluated according to (Da Silva et al. 2016) in which tryptic soy broth (TSB) solution + glucose aqueous solution (0.25 wt%) received *S. aureus* ( $10^7$  CFU/mL) and the disks of tilapia skin (negative control), TS-TAg and TS-CAg which were inoculated for 24 h at 37 °C. After this process, the resulting skin and reactor were immersed in a solution of crystal violet (0.25 wt% in water) to target the biofilm surface with the dye. Then, the skin and the reactor were washed with water to remove non-adhered species. The biofilm formation

was evaluated from absorbed (and colored) species that were removed from surfaces with a solution of 80:20 of alcohol: acetone. The intensity of absorbance at 570 nm was explored as a parameter for the identification of the number of adhered species.

The kinetics of bacterial growth was determined from the dispersion of viable cells into the saline solution and the time of interaction with an antibacterial agent (TAg and CAg). After a fixed interval of time (1 h, 2 h, 3 h, 4 h, and 5 h), an aliquot of 100 mL of the resulting dilution was inoculated in PCA for 24 h at 37 °C. After that, the remaining CFU was counted by direct inspection of the remaining viable cells, as reported in Ref. (da Silva et al. 2019).

## Results and discussion

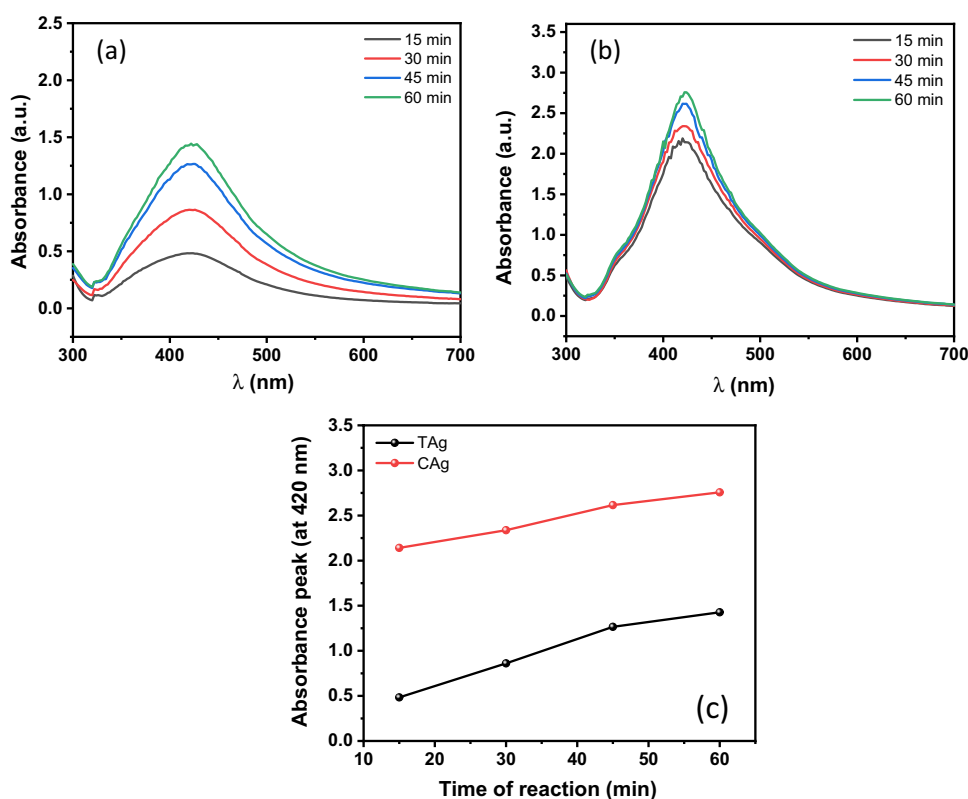
### Surface plasmon resonance band formation

The surface plasmon resonance (SPR) phenomena observed in metal nanoparticles make use of the confinement of electromagnetic field near the metal nanoparticles surface as an important tool for the in-situ monitoring of the synthesis of silver nanoparticles by UV–visible spectroscopy. The intensity and the typical band shift of the characteristic peak around 410 nm depend on several factors, such as the size of

nanoparticles, shape, and aggregation level (Granbohm et al. 2018; Guimarães et al. 2020).

Based on these relevant properties, the kinetics of silver nanoparticle formation was evaluated at a fixed interval of 15 min from the measurement of the UV–Vis spectrum of the resulting solution. As shown in Fig. 2a, b, the intensity of the SPR band (observed at 420 nm for both samples) increases with the time of reaction, indicating the progressive nucleation and silver nanoparticles formation with the progressive reaction that is dependent on the action of a reducing agent component (Guimarães et al. 2020). The absorbance for TAg-based samples presents a general behavior indicating a lower amount of silver nanoparticles at the beginning of the reaction (see Fig. 2a), while for CAg-based samples, it is observed a stronger reaction for nanoparticles reduction (the initial absorbance level for CAg-based samples is in order of 4 × higher than TAg-based samples)—see Fig. 2b. The corresponding variation level in the absorbance of CAg is limited in the range of 2.14–2.76, while the variation for TAg-based samples is observed in the range of 0.48–1.43. The variation in the absorbance peak (at 420 nm) is plotted as a function of the time of reaction in Fig. 2c. As shown, despite a higher slope for TAg-based reaction, the more effective synthesis (in terms of density of synthesized nanoparticles) is observed for the CAg-based system in consequence of initial faster kinetics of nanoparticles nucleation and growth.

**Fig. 2** Absorbance of silver nanoparticles solution in the UV–Vis region as a function of time of reaction (black—15 min, red—30 min, blue—45 min, and green—60 min) for TAg-based nanoparticles (a), CAg-based nanoparticles (b) and comparison of characteristic SPR band for TAg- and CAg-based nanoparticles as a function of time of reaction (c)



## Morphology and size distribution of synthesized nanoparticles

The size of nanoparticles, measured from dynamic light scattering (DLS), is summarized in Fig. 3. As can be seen, a unique distribution of the size of particles is observed for TAG-based silver nanoparticles that are centered around 39.41 nm with a zeta potential of -35.6 mV, while a two population of particles with specific size distribution is observed for CAG-based silver nanoparticles, that are centered at 1.81 nm and 45.64 nm with a zeta potential of -27.8 mV. As a consequence of this distribution, the polydispersity index (PDI value—which is a dimensionless measurement of the amplitude of the particle size distribution) is higher for CAG-based samples (0.554) than observed for TAG-based samples (0.287). An important aspect to be considered from these results concerns the available surface area for the synthesized nanoparticles, that present size in the order of nanometers, and favor the attachment of these structures on bacterial cell wall for the following step in which the number of viable cells tends to be strongly reduced (diffusion of ions into cells).

The process of impregnation of silver nanoparticles into TS samples results in a progressive change in color of the tilapia skin, that turns from grayscale to a brown aspect, as observed in Fig. 4a in which are compared the aspects of pure tilapia skin (TS) with the impregnated skin with TAG-based nanoparticles (TS-TAG) and modified skin with CAG-based nanoparticles (TS-CAG). The identification of the impregnated silver nanoparticles into tilapia skin was evaluated from EDS measurement. For comparison, it is shown in Fig. 4b, d, f the SEM images for tilapia skin, TS-TAG samples, and TS-CAG samples, respectively. The corresponding EDS map is shown in Fig. 4c, e, g, respectively.

As shown, the typical wrinkled surface of the tilapia skin (Fig. 4b) is preserved under impregnation with silver nanoparticles (Fig. 4d, f), that are identified from small metallic aggregates (regions in white) scrutinized in the

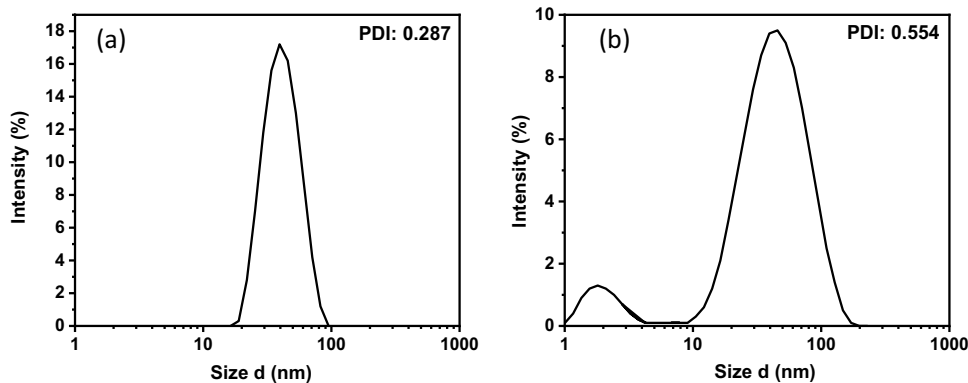
EDS spectrum (Fig. 4e, g), confirming the impregnation of tilapia skin with silver nanoparticles.

## Characterization of wound dressing systems (impregnated silver nanoparticles into tilapia skin)

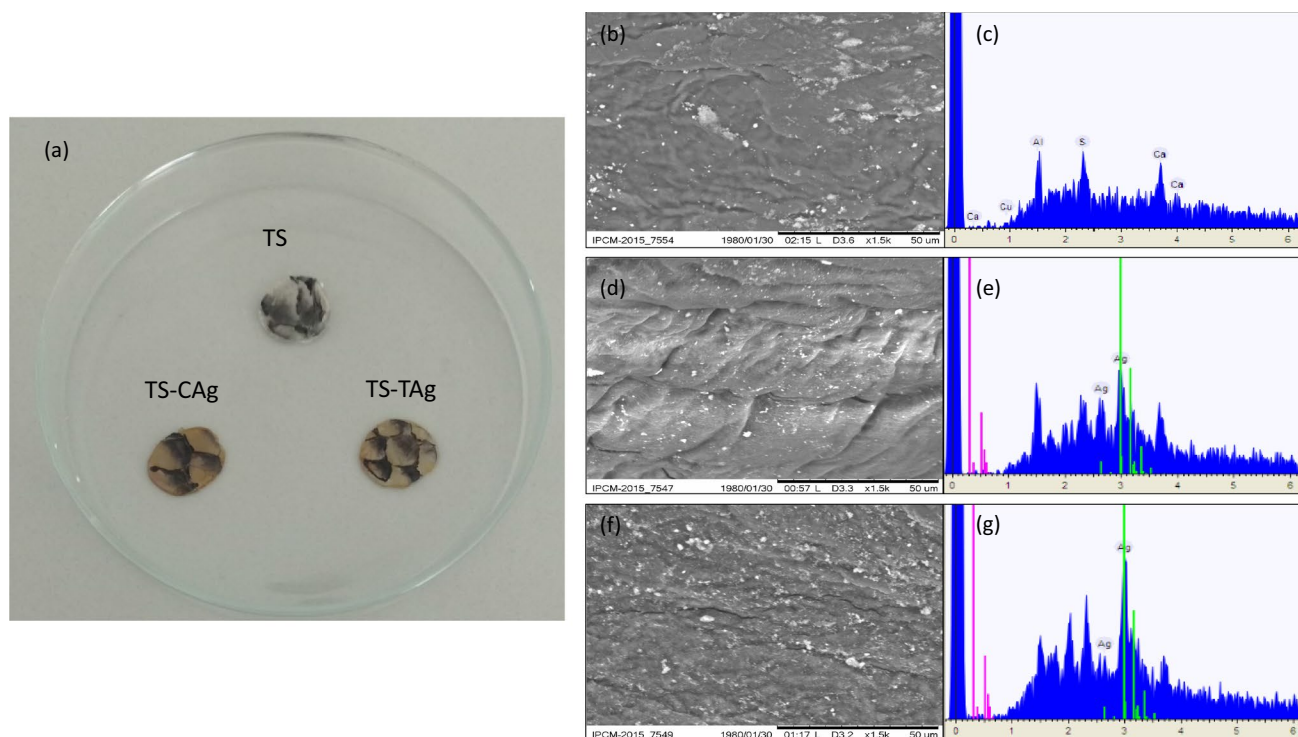
The amount of adsorbed silver nanoparticles on tilapia skin was estimated from the direct measurement of SPR peak intensity of solution in the reactor before and after the process of nanoparticle adsorption on the skin. As shown in Fig. 5a, a variation in order 67.59% is observed for TAG-based samples, while 61.42% of the variation is observed for CAG-based samples. Despite higher performance in relative adsorption of silver nanoparticles for TAG-based nanoparticles, due to the higher amount of synthesized CAG-based nanoparticles (a higher value for SPR band), it is expected a higher density of CAG-based nanoparticles impregnated into tilapia skin samples.

The identification of additional components than silver that is impregnated in the tilapia skin (subproducts from bioreduction provided by essential oils) was measured by FTIR assays. For this, the spectrum of tilapia skin, TS-TAG, and TS-CAG were analyzed as follows. Figure 5b shows the FTIR spectrum of TS (curve in black), TS-TAG (curve in red), and TS-CAG (curve in blue). Bands at  $2924\text{ cm}^{-1}$  and  $2850\text{ cm}^{-1}$  correspond to the stretching vibration of  $=\text{C}-\text{H}$  group and arise from primary and secondary amines (Alfuraydi et al. 2019; Veisi et al. 2019), while bands at  $1240$  and  $1550\text{ cm}^{-1}$  are assigned to the  $\text{N}-\text{H}$  vibration modes associated with  $\text{C}-\text{N}$  and  $\text{C}-\text{H}$  vibration from amide II and III from the collagen of Nile tilapia skin (Song et al. 2021). In addition, characteristic bands at  $1382\text{ cm}^{-1}$  observed for TS-TAG and TS-CAG (samples impregnated with silver nanoparticles) are assigned to the carbonyl group and carboxylic acid which have a high affinity to Ag ions, acting as reducing and stabilizing agents for produced silver nanoparticles (Vilas et al. 2016, 2014). The band at  $1175\text{ cm}^{-1}$  is associated with the stretching vibration of  $\text{C}-\text{OH}$ , which has been correlated with changes in the characteristic vibrations of

**Fig. 3** DLS measurement showing the distribution of size of nanoparticles and PDI value for **a** TAG-based silver nanoparticles and **b** CAG-based silver nanoparticles



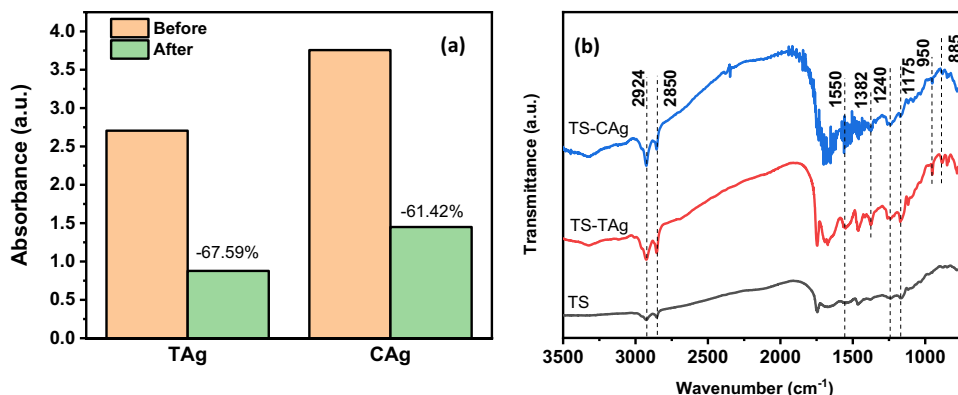




**Fig. 4** **a** Photos of pure tilapia skin (TS), impregnated with CAg-based silver nanoparticles (TS-CAg) and with TAg-based silver nanoparticles (TS-TAg); **b** SEM image of pure tilapia skin and **c** cor-

responding EDS spectrum; **d** SEM image of TAg-based modified tilapia skin and **e** corresponding EDS spectrum; **f** SEM image of CAg-based modified tilapia skin and **g** corresponding EDS spectrum

**Fig. 5** **a** Comparison of the absorbance of silver nanoparticles dispersed in the reactor before and after the impregnation of silver nanoparticles into tilapia fish skin, **b** FTIR spectrum of pure tilapia skin (TS-curve in black), tilapia skin impregnated with TAg-based nanoparticles (TS-TAg-curve in red) and tilapia skin impregnated with CAg-based nanoparticles (TS-CAg-curve in blue)

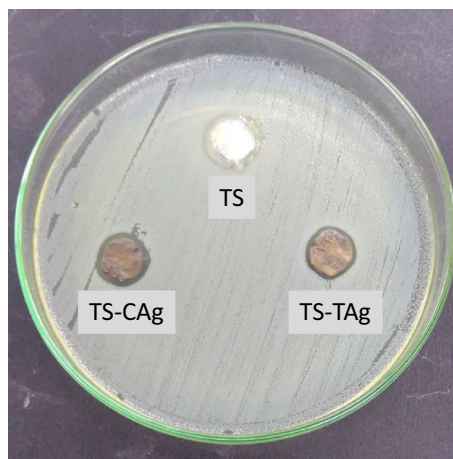


carboxyl, phenol, and proteins from essential oils (Gavade et al. 2015). Characteristic vibration of the group  $\text{CH}_3(\text{CO})$ , vibrations in the group  $\text{C}-\text{O}-\text{C}$ , and  $\text{CH}_2$  are observed at  $1087\text{ cm}^{-1}$  (Dzimitrowicz et al. 2016). The band at  $950\text{ cm}^{-1}$  is assigned to heterocyclic compounds (Sutthanont et al. 2019), while bands at  $885\text{ cm}^{-1}$  are assigned to  $\text{C}-\text{O}$  vibrations, and the band at  $846\text{ cm}^{-1}$  is associated with out-of-plane vibration of  $\text{N}-\text{H}$  from amides (Gavade et al. 2015; Vilas et al. 2014). These bands confirm the presence of alcohol, carboxylic acid, phenolic acid, and heterocyclic compounds, which are active components for the complexation,

nucleation, and growth of silver nanoparticles (Ahmad et al. 2013; Maghimaa and Ali 2020).

### Antibacterial assays

The qualitative evaluation about antibacterial activity of TS-TAg-based systems was performed by measurement of inhibition haloes, in which circular disks of TS-TAg and TS-CAg are disposed of in plates inoculated with *S. aureus*. As shown in Fig. 6, for both systems are observed a discrete inhibition halo (in comparison with negative control), characterizing



**Fig. 6** Inhibition halo for control experiment (tilapia fish disk), TS-CAg, and TS-TAg disks

the diffusion of antibacterial active agents in direction of the bacterial viable cells. Moreover, negligible inhibition halo was observed for pure tilapia skin, characterizing the relevance of impregnated species. The antimicrobial activity of silver nanoparticles is associated with membrane binding, DNA damage, silver ions liberation, and oxidative stress (Tang and Zheng 2018).

In general, the most accepted hypothesis to explain the cytotoxicity of the silver nanoparticles is the Trojan horse mechanism (Jorge de Souza et al. 2019), a concentration-dependent mechanism based on the toxicity of released ions that migrates to the intracellular environment and damage lysosomes, with subsequent lesions in the cell membrane and genetic material, that justify the antibacterial activity for this material.

As a consequence, the treatment of skin wounds with silver nanoparticles alone or functionalized with diverse

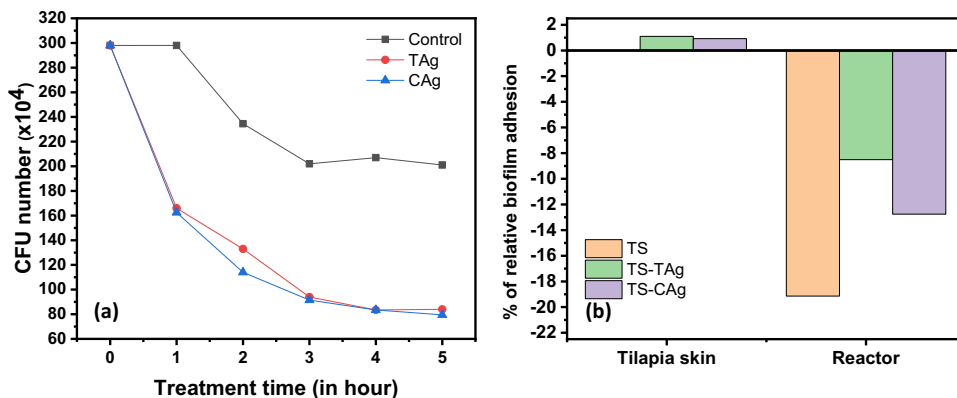
antimicrobial compounds has been demonstrated promising wound therapeutic effects (Li et al. 2019; Negut et al. 2018).

The quantification of the antibacterial activity of synthesized nanoparticles was evaluated from time-kill assays, in which an aqueous solution with  $10^4$  colonies of *S. aureus* received aliquots of synthesized nanoparticles at a determined time of reaction in a saline solution in the presence of silver nanoparticles. The resulting material was inoculated into plates at 37° C for 24 h for the posterior counting of viable cells. The treatment was fixed in the interval of time of 1 h, 2 h, 3 h, 4 h, and 5 h—for comparison, a negative control experiment was evaluated in the absence of antibacterial materials. As shown in Fig. 7a, a decrease in the order of 27% is observed in the number of viable cells for the control experiment. However, the reduction in the number of viable cells after treatment with both experimental systems (TAg and CAg) is in order of 73% after 5 h of contact with antibacterial material. The general behavior of the curves is the same, with an exponential decay that reaches saturation for a time in order of 5 h, characterizing a relevant reduction in the number of viable cells that confers an important application for synthesized nanoparticles.

On the other hand, *S. aureus* biofilm producers are involved in the pathogenesis of chronic wound infections (Mottola et al. 2016). These opportunistic pathogens live in skin biofilms and the use of conventional antimicrobial agents induces bacterial survival and the development of persistent infection (van Wamel 2017). Moreover, an important parameter to be evaluated is the antibiofilm activity of silver nanoparticles impregnated in tilapia fish skin. This activity was evaluated for negative control (tilapia fish), TS-TAg, and TS-CAg samples which were applied as a support for the removal of biofilm that could be attached to the reactor.

The measurement of variation in the relative biofilm adhesion for both surfaces (tilapia skin and reactor) was performed according to the comparative variation in the

**Fig. 7 a** Time-kill assays performed to evaluate the characteristic time of action of synthesized nanoparticles (CAg and TAg) and **b** the variation in the biofilm adhesion degree on the sample (tilapia skin coated with nanoparticles) and on the reactor



absorbance of released dye molecules that target biofilm species. The percentage of variation in the biofilm adhesion (BA) is given by Eq. 1 (da Silva et al. 2019; dos Santos et al. 2018).

$$\%BA = \left( \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}} \right) * 100\% \quad (1)$$

where the negative control for tilapia skin is the absorbance of adhered targeted species on pristine tilapia skin, while the control for the reactor is measured in the absence of antibacterial agents. As shown in Fig. 7b, the relative variation in the biofilm adhesion on tilapia skin substrates is minimally affected by the incorporation of silver nanoparticles. A positive variation in the order of 1% in biofilm adhesion degree is attributed to the impregnation of silver nanoparticles on tilapia skin. On the other hand, a strong reduction in the biofilm formation on the reactor is observed as a result of interaction with pristine tilapia skin (control experiment) in order of 19%. The impregnation of tilapia skin with silver nanoparticles reduces this efficiency to values in order of 9% (TS-TAg) and 13% (TS-CAg). This process is a consequence of the filling of active sites for biofilm adsorption with silver nanoparticles that favor the antibacterial activity against planktonic form (Estevez et al. 2020; Le Ouay and Stellacci 2015). These results suggest that impregnated silver nanoparticles in tilapia skin associate its intrinsic antibacterial activity with antibiofilm properties, characterizing an important combination of mechanisms for this prototype of wound dressing system.

## Conclusion

The impregnation of “green” silver nanoparticles into tilapia skin as prototypes of wound dressing systems introduces a promising procedure that associates the antibacterial activity of silver nanoparticles with the antibiofilm activity of chemically modified tilapia fish skin on reactor, in a system that can be considered as a candidate for the treatment of severe infections. The use of natural materials for the preparation of silver nanoparticles and the development of the tilapia skin-based wound dressing represents an important trend preconized by the science for a circular economy that is favored by the use of free-antibiotic and low-cost systems. The reduction in the viable cells of 73% in association with the reduction in the biofilm formation (in order of 19%) represents two important parameters that can be synergistically explored from this new platform for all-green wound dressing systems.

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## Declarations

**Conflict of interest** The authors have declared no conflict of interest.

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