Antimicrobial Activity of Compounds Containing Silver Nanoparticles and Calcium Glycerophosphate in Combination with Tyrosol

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Abstract Nanocomposites containing antimicrobial agents and calcium phosphates have been developed. Thus, this study assessed the effects of two compounds containing silver nanoparticles (AgNPs) and β -calcium glycerophosphate (CaGP), associated or not with tyrosol (TYR), against planktonic cells and biofilms of *Candida albicans* and *Streptococcus mutans*. The nanocompounds were synthesized through chemical and ‘green’ processes and characterized by scanning electron microscopy. The minimum and fractional inhibitory concentrations of each compound were determined for planktonic cells. Next, 24-h single biofilms of *C. albicans* and *S. mutans* were treated for 24 h with the nanocompounds alone or in combination with TYR, and the antibiofilm effect was assessed through enumeration of colony forming units. Biofilm data were statistically examined using one-way ANOVA and the Kruskal–Wallis test ($\alpha = 0.05$). The chemically synthesized nanocompound in combination

with TYR demonstrated a synergistic effect against planktonic cells of *C. albicans* and *S. mutans*. For the nanocompound obtained through the ‘green’ route associated with TYR, a synergistic effect was observed only against *C. albicans*. For biofilms, only the combination obtained through the ‘green’ route + TYR demonstrated a synergistic effect against *C. albicans*. Our results may contribute to the development of oral care products containing AgNPs–CaGP and TYR to combat oral infections.

Keywords Metal nanoparticles · Polyphosphates · Biofilms · *Candida albicans* · *Streptococcus mutans*

Introduction

Streptococcus mutans is highly prevalent in dental biofilms and is considered the main etiologic agent in the development of dental caries in children and adults. Acidogenic properties, resistance to acidic environments, and the capacity to synthesize extracellular polysaccharides are the major virulence factors of *S. mutans*, contributing to enamel surface colonization and the development of pathogenic biofilms [1]. The progression of established dental caries lesions can also include other microorganisms such as *Candida* species, particularly *Candida albicans*. A preponderance of *C. albicans* was found in dentine caries lesions of children, with frequencies ranging from 66 to 97% [2].

In order to control infectious oral diseases, new strategies have focused on the prevention of biofilm formation [3]. Among the therapeutic approaches studied, the use of nanoparticles (NPs) can be highlighted, which have a higher ratio of surface area per volume and reactivity than microparticles. Various NPs have been tested against the

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microbes for their antimicrobial efficacy e.g. silver, gold, zinc and out of these silver nanoparticles (AgNPs) have been found to be the most effective [4]. In this sense, AgNPs have been incorporated into different dental materials, including adhesives, implants, and polymers, to promote caries arrestment, prevent biofilm formation and for osteogenic induction [5]. Different approaches using chemical or physical processes have been employed to obtain AgNPs, including chemical reduction, electrochemical reduction, photochemical reduction, radiation, and heat evaporation. However, in these methods, the chemical agents used are often costly, toxic, and not eco-friendly. The ‘green’ synthesis of metal nanoparticles has received great attention recently, as it is a cost-effective, clean, environmentally friendly and non-toxic alternative. Biological entities and non-hazardous chemicals have been used for the synthesis of nanomaterials; plant-mediated synthesis has received much attention, as it is very easy and rapid compared with other processes [6]. *Punica granatum* L., or pomegranate, has been used for AgNPs synthesis as it is a relatively non-toxic plant known for its antioxidant properties due to the presence of polyphenols (ellagic acid, gallic acid, and punicalagin), which are present in the fruit peels [7].

In recent years, organic or inorganic polyphosphates have been incorporated into dentifrices [8], fluoridated gels [9] and varnishes [10] in order to improve their anticaries and anti-erosion properties. Calcium glycerophosphate (CaGP) is an organic polyphosphate capable of adsorption to enamel surfaces [11], leading to the release of calcium (Ca) ions, which act on the remineralization process. Nanocomposites containing AgNPs and CaGP have been developed and demonstrated antimicrobial and remineralizing effects, attributed to the AgNPs and CaGP, respectively [12, 13]. However, there is concern regarding the use of AgNPs, since a concentration-dependent toxicity has been documented [14]. Thus, the combined use of these nanocomposites (AgNPs–CaGP) with other compounds at low concentrations could increase the antimicrobial efficacy and reduce the side effects of these agents [15].

Bacteria communicate with one another through QS system resulting in the regulation of several physiological activities (biofilm formation, competence, virulence, etc.). Due to the increased resistance of biofilms to conventional drugs, Quorum-Sensing (QS) molecules and QS inhibitors have been tested as antimicrobial agents [16, 17]. In this regard, the QS molecule Tyrosol (2-[4-hydroxyphenyl]ethanol) (TYR) demonstrated antibiofilm properties against *Candida* species and *S. mutans* in single and mixed cultures [3]. Moreover, when TYR at 80 µM was associated with 4 mg/L amphotericin B, a synergistic effect on *Candida* biofilm formation was observed [15].

Considering all the above-mentioned aspects, the aim of this study was to evaluate the antimicrobial effects of AgNPs–CaGP-containing solutions combined with TYR against planktonic cells and biofilms of *C. albicans* and *S. mutans*.

Materials and Methods

Synthesis of Test Solutions

Two AgNPs–CaGP-containing solutions were synthesized using chemical and ‘green’ processes. For both syntheses, β-calcium glycerophosphate (CaGP, 80% β-isomer and 20% rac-α-isomer, CAS 58409-70-4, Sigma-Aldrich Chemical Co, St Louis, Missouri, USA) was nanoparticulated using a ball mill for 24 h at 120 rpm, as described by Danelon et al. [8].

For the chemical process (compound C), sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, Merck KGaA, Germany) was used as a reducing agent of silver nitrate (AgNO_3 , Merck KGaA), and the stoichiometric ratio between these compounds was 3:1, respectively. This chemical route was based on the protocol proposed by Gorup et al. [18] with some modifications. Briefly, 5 g of CaGP was added to a flask containing 100 mL of deionized water, followed by 0.5 mL of ammonium salt of polymethacrylic acid (NH-PM, Polysciences Inc., Warrington, Pennsylvania, USA) and 1.4 g of $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$. This mixture was heated and kept under magnetic stirring. After reaching 95 °C, 0.85 g of AgNO_3 was added, and the suspension was stirred for an additional 30 min until the color of the solution changed to brown, which qualitatively indicated the formation of AgNPs. The solution was then dried overnight at 70 °C.

For the ‘green’ route (compound G), 0.42 g of AgNO_3 was dissolved in 100 mL of deionized water and heated to 90 °C. Next, 2.5 g of CaGP, 0.5 mL of NH-PM, and 0.7 g of aqueous extracts of peels of *Punica granatum* L. were added to the flask. The reaction occurred under constant agitation at 95 °C for 10 min. For both routes, the amount of silver was equivalent to 10% of the CaGP weight [12, 13].

In sequence, TYR (Sigma-Aldrich, CAS 501-94-0) was directly diluted with the nanocompounds (C and G) in deionized water, for the minimum inhibitory concentration test, and in artificial saliva (AS) [3], for the biofilm experiments.

In order to observe the particle morphology of the nanocompounds, a drop of each sample was placed on a silicon substrate, dried at 40 °C for 12 h, and characterized by scanning electron microscopy (SEM), using a Zeiss Supra 35VP microscope with a field emission gun electron effect (FEG-SEM) operating at 10 eV, with magnification

from $20,000 \times$ to $60,000 \times$. To determine the nanoparticles size, the high-resolution transmission electron microscopy (HRTEM) was performed, using a FEI TECNAI F20 microscope operating at 200 kV; for this, the samples were dripped on copper grids of 400 mesh (PELCO®), and the solvent evaporated at room temperature. After, histograms were constructed using the public domain ImageJ image processing software (version 1.50, National Institute of Health, Bethesda, MD, USA).

These nanocompounds were previously characterized using the UV–visible spectrum, X-ray diffraction, and scanning electron microscopy followed by energy-dispersive X-ray spectroscopy with mapping in 2D, as described by Fernandes et al. [12] and Souza et al. [13].

Antimicrobial Activity

Minimum Inhibitory Concentration (MIC)

The TYR and both nanocompounds (C and G) were tested alone or in combination (C + TYR and G + TYR). The minimum inhibitory concentration (MIC) assay against *S. mutans* ATCC 25175 and *C. albicans* ATCC 10231 was performed using the broth microdilution method, as previously reported [3]. First, C, G, TYR, C + TYR, and G + TYR were serially diluted (2–1024 times) in deionized water. Each previously obtained concentration was diluted (1:5) in RPMI 1640 medium (Sigma-Aldrich) or BHI broth (Difco, Le Pont de Claix, France), respectively for *C. albicans* and *S. mutans*. Inocula of both strains were adjusted to a turbidity equivalent to 0.5 McFarland standards in 0.85% saline solution. These microbial suspensions were diluted in saline solution and, posteriorly, in RPMI 1640 or BHI broth. Next, a volume of 100 μ L of each microbial suspension was added to the wells of 96-well plates (Costar, Tewksbury, USA) containing 100 μ L of each specific concentration of compounds. The final drug concentrations were in the range of 1.56–1600 μ g/mL for nanocompound C, 1.2–624.8 μ g/mL for nanocompound G, and 5.35–2763.2 μ g/mL for TYR. The microtiter plates were aerobically incubated at 37 °C for 48 h for *C. albicans*, while for *S. mutans* the plates were incubated in 5% CO₂ for 24 h. MICs were determined by measuring the optical density at 570 nm. The background optical density was subtracted from that of each well.

MIC values were used to calculate the fractional inhibitory concentration index (FICI), which is defined as the sum of the ratio of the MIC of a compound used in combination divided by the MIC of the compound tested alone. FICI values ≤ 0.5 indicate synergism; between 0.5 and 4.0, no interaction; and > 4.0 , antagonism [19]. The MIC and FICI calculations were conducted in triplicate on three different occasions.

Biofilm Killing

C. albicans ATCC 10231 and *S. mutans* ATCC 25175 were cultivated at 37 °C on SDA (in aerobiose) and BHI agar (in 5% CO₂), respectively. Subsequently, *Candida* cells were grown in Sabouraud Dextrose Broth (SDB; Difco) medium for 20 h at 37 °C (120 rpm), while *S. mutans* was inoculated in BHI broth (Difco) and statically incubated at 37 °C for 18 h in 5% CO₂. Next, the fungal and bacterial cells were harvested through centrifugation (8000 rpm, 5 min) and washed twice in phosphate-buffered saline (PBS, pH 7, 0.1 M). *S. mutans* cells were spectrophotometrically adjusted to 1×10^8 cells/mL in AS, while the number of *C. albicans* cells was adjusted to 1×10^7 cells/mL using a Neubauer chamber.

Based on the MIC results obtained, only the associations that presented synergism were tested against biofilms. Mono-species biofilms of *C. albicans* and *S. mutans* were developed for 24 h (37 °C) in 96-well microtiter plates containing 0.2 mL of *C. albicans* cell suspension (1×10^7 cells/mL) or 0.2 mL of *S. mutans* (1×10^8 cells/mL), both prepared in AS. Subsequently, the biofilm-containing wells were washed with 0.2 mL of PBS to remove the nonadherent cells, and 0.2 mL of the drug association C + TYR (20 $\times$ MIC for *C. albicans* and 10 $\times$ MIC for *S. mutans*) and G + TYR (20 $\times$ MIC for *C. albicans*) was added to the wells. All plates were re-incubated at 37 °C for 24 h. The compounds alone (C, G, and TYR) were also evaluated, and AS was added to the wells designated as negative controls.

After the treatment period, the resulting biofilms were scraped from the wells with 1 mL of PBS and placed into microcentrifuge tubes. The suspensions obtained were vortexed, serially diluted in PBS, and plated on SDA (for *C. albicans*) or BHI agar (for *S. mutans*). After incubation at 37 °C (24–48 h), the total number of colony forming units (CFUs) was counted and the Log₁₀ per unit area calculated (Log₁₀ CFU/cm²).

The effect of drug association on biofilms was interpreted according to Miceli et al. [20]. When the drug combinations showed a significant reduction in CFUs compared to each compound alone ($p < 0.05$), the association was considered synergistic. When the combinations reduced the number of CFUs compared to each drug alone, but without significant difference between them ($p > 0.05$), the effect was additive. An antagonistic effect was observed when the drugs alone were significantly more effective in reducing CFUs than the drug combinations ($p < 0.05$).

Statistical Analysis

SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA) was used for statistical analysis, and the significance level was set at 5%. The CFU results did not pass the normality test for either microorganism. Therefore, the Kruskal–Wallis test was applied followed by the Student–Newman–Keuls (for the combination G + TYR) and Tukey (for the combination C + TYR) tests.

Results

The formation of AgNPs–CaGP occurred through the reduction of Ag^+ from AgNO_3 in a CaGP-containing solution. Figure 1 shows phosphate surfaces decorated with AgNPs, evidencing that both methods of synthesis (chemical and ‘green’) were effective in producing nanobiomaterials associating a nanoparticulated metal and an organic calcium phosphate. When the chemical and ‘green’ processes were employed, the AgNPs exhibited relatively uniform dispersion, a spherical shape, and diameters of approximately 20.3 and 12.2 nm, respectively (Fig. 1).

Table 1 shows the MIC and FICI values for the compounds evaluated. *C. albicans* and *S. mutans* were more susceptible to the nanocompound C, with MIC values of 800 $\mu\text{g/mL}$. For both microorganisms, the combinations tested (C + TYR and G + TYR) showed substantially lower MICs than those found for each compound alone, except for the combination G + TYR, which was not able to kill planktonic cells of *S. mutans*, even at the highest concentration tested. The combination of the nanocompound C with TYR exhibited a synergistic effect against *C. albicans* and *S. mutans*, with FICI values of 0.30 and 0.14, respectively. A synergistic killing effect on planktonic cells of *C. albicans* (FICI = 0.27) was also found when the nanocompound G (at 1/4 MIC) was associated with TYR (at 1/40 MIC). *S. mutans* was not susceptible to this association for any concentration tested.

Table 1 Minimum inhibitory concentration (MIC) of tyrosol (TYR) and nanocompounds obtained by chemical (C) and ‘green’ (G) routes against *Streptococcus mutans* and *Candida albicans*, and fractional inhibitory concentration index (FICI)

Groups	MIC ($\mu\text{g/mL}$)		FICI	
	<i>S. mutans</i>	<i>C. albicans</i>	<i>S. mutans</i>	<i>C. albicans</i>
C	800.0	800.0	–	–
TYR	12,434.4	6908.0	–	–
G	1560.2	1560.2	–	–
C + TYR	100.0/172.7	200.0/345.4	0.14	0.30
G + TYR	> 624.8/2763.2	390.0/172.7	–	0.27

The association was considered as synergistic when $\text{FICI} \leq 0.5$; indifferent when $0.5 < \text{FICI} < 4$, and antagonistic when $\text{FICI} > 4.0$

Regarding the effect on biofilms, when the combination C + TYR was tested against *S. mutans*, no significant reduction in the number of CFUs was observed ($p > 0.05$; Fig. 2a). For *C. albicans*, however, this drug combination significantly reduced the number of biofilm cells when compared to the negative control ($p < 0.001$), demonstrating an additive effect (Fig. 2a). In turn, the combination G + TYR was tested only against *C. albicans* biofilms as no MIC was found when this association was tested against *S. mutans* planktonic cells. G + TYR showed a significant reduction in the number of CFUs in comparison to the negative control, and this drug combination was classified as synergistic (Fig. 2b).

Discussion

Biofilm-associated diseases and their resistance to conventional antimicrobial agents employed are still a clinical challenge. Thus, there is growing interest in the development of new therapies to improve the antimicrobial properties of biomaterials and, at the same time, provide lower side effects. In this context, promising new agents containing AgNPs have been developed, presenting

Fig. 1 Scanning electron microscopy images and average size (histograms) of the AgNPs–CaGP nanocompounds: **a** Compound obtained by the chemical process (C); **b** Compound obtained by the ‘green’ process (G). Magnification: 30,000 x (1a), and 60,000 x (1b)

Fig. 2 **a** Mean values ($\pm$ SD) of the logarithm of colony forming units per cm^2 ($\log_{10}$ CFU/ cm^2) for single biofilms of *C. albicans* and *S. mutans*. NC = negative control; C = nanocompound obtained by the chemical process; TYR = tyrosol. Concentrations tested for *C. albicans*: C at 4000 $\mu\text{g/mL}$ and TYR at 6908 $\mu\text{g/mL}$. For *S. mutans*: C at 1000 $\mu\text{g/mL}$ and TYR at 1727 $\mu\text{g/mL}$. Different lowercase and uppercase letters indicate significant differences between the treatments for *C. albicans* ($p < 0.05$; Kruskal–Wallis test followed by the Tukey test) and *S. mutans* ($p < 0.05$; Kruskal–Wallis test followed by the Tukey test) biofilms, respectively. **b** Mean values ($\pm$ SD) of the logarithm of colony forming units per cm^2 ($\log_{10}$ CFU/ cm^2) for single biofilms of *C. albicans*. NC = negative control; G = nanocompound obtained by the ‘green’ route at 7810 $\mu\text{g/mL}$; TYR = tyrosol at 3454 $\mu\text{g/mL}$; G + TYR = 7810 $\mu\text{g/mL}$ of G and 3454 $\mu\text{g/mL}$ of TYR. Different lowercase letters indicate significant differences between the treatments ($p < 0.05$; Kruskal–Wallis test followed by the Student–Newman–Keuls test)

encouraging results [5]. Furthermore, TYR was shown to significantly reduce the adhesion and biofilm formation of *C. albicans* and *S. mutans* in single and mixed biofilms [3, 16]. Therefore, the current study was designed to evaluate the antimicrobial effect of two nanocompounds containing AgNPs–CaGP (C and G), combined or not with TYR, against *S. mutans* and *C. albicans*, which could allow their use at lower concentrations.

These nanocompounds were obtained through physico-chemical and ‘green’ routes. ‘Green’ synthesis of AgNPs has emerged as a more viable alternative, as it is a simple, environmentally friendly, and cost-effective method. Plant extracts are the most advantageous biological materials since they are free from toxic chemicals, present lower biohazards, and provide natural capping agents for AgNPs stabilization. The synthesis of compound G was performed with polyphenols-rich pomegranate peel extract [13],

which is responsible for Ag^+ ion reduction and AgNPs stabilization, and has excellent anti-oxidant properties [21].

Both nanocompounds tested in this study (C and G) were effective against *C. albicans* and *S. mutans* in a planktonic state, however these microorganisms were more susceptible to the nanocompound C (Table 1). Pomegranate peel is an important source of polyphenolic substances including ellagic and gallic acids. Thus, after reaction to obtain compound G, the functional groups of polyphenols may be connected to the Ag^+ ions [22], hindering the antimicrobial effect of the nanoparticles.

Regarding the effect of drug associations on planktonic cells, both nanocompounds (C and G) combined with TYR were effective against *C. albicans*, but only the combination C + TYR was effective against *S. mutans* (Table 1). MIC values for TYR alone against the studied microorganisms were similar to those found by Monteiro et al. [16] and Arias et al. [3]. However, when the nanocompounds C and G were combined with TYR, the TYR concentrations capable of inhibiting the microorganism growth were much lower, especially for the association with compound C. The synergistic effect observed on planktonic cells by combining AgNPs–CaGP and TYR may be due to the disruption of the cell membrane by TYR, since this molecule interferes with ergosterol biosynthesis [23], facilitating the entry of AgNPs into the cytoplasm. Thereby, AgNPs interact with both intracellular proteins (especially sulfur rich-proteins) and DNA, causing further damage to cells [24].

For biofilms, a synergistic effect was found only for the combination G + TYR against *C. albicans*. This result could be related to the silver concentration present in the tested combinations (in the combination G + TYR, Ag concentration was 781.0 $\mu\text{g/mL}$ and, in the combination C + TYR, Ag concentration was 400.0 $\mu\text{g/mL}$; these values correspond to 10% of tested concentration in the biofilms), considering the AgNPs action mechanisms discussed above. TYR concentrations that presented an antibiofilm effect in previous reports [3, 21] were higher than those used in the current study for the treatment of biofilms with both associations (between 25 and 50 mM approximately). In this study, an antimicrobial effect was obtained using a lower concentration of TYR, reinforcing the importance of drug combination therapy to reduce drug concentration. It is possible the synergism occurred through the different action mechanisms of the TYR and AgNPs. Another point to be considered is the stage of biofilm formation, which affects the drug effectiveness. Within this context, Monteiro et al. [25] demonstrated that treatment with TYR led to no significant reductions in the number of CFUs of mature biofilms (96 h) of *C. albicans*, *C. glabrata*, and *S. mutans*. In our study, however, biofilms were formed for 24 h and maintained for an additional 24 h

in the presence of nanocompounds; thus, the stage of biofilm formation tested in the present study may have contributed to the significant reductions observed for nanocomposites alone or in combination with TYR.

In conclusion, the AgNPs–CaGP-containing nanocompounds alone or in combination with TYR act synergistically, inhibiting planktonic cells of *C. albicans* and *S. mutans* with lower concentrations of both drugs. For biofilms, a synergistic effect was observed only when the compound obtained through the ‘green’ route was associated with TYR. Finally, these findings might be of interest for the development of new antimicrobial strategies against bacterial and fungal oral infections.

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