Chitosan nanoparticles loaded with Epigallocatechin-3-gallate: synthesis, characterization, and effects against *Streptococcus mutans* biofilm

Short title: Epigallocatechin gallate loaded chitosan nanoformulation: effects against *Streptococcus mutans* biofilm

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Abstract

This study evaluated the effects of chitosan nanoparticles loaded with epigallocatechin-3-gallate (EGCG) against *Streptococcus mutans* biofilm. EGCG-loaded chitosan (Nchi+EGCG) nanoparticles and Chitosan (Nchi) nanoparticles were prepared by ion gelation process and characterized regarding particle size, polydispersion index, zeta potential, and accelerated stability. *S mutans* biofilms were treated twice daily with NaCl 0.9% (negative control), Nchi, Nchi+EGCG, and chlorhexidine (CHX) 0,12% (positive control). After 67 h, the biofilms were evaluated for acidogenesis, bacterial viability and dry weight. Biofilm morphology and structure were analyzed by scanning electron microscopy. The nanoformulations presented medium to short-term stability, size of 500 nm, and polydispersion index around 0.400. Treatments affected cell morphology and biofilm structure. However, no effects on microbial viability, biofilm dry weight, and acidogenesis were observed. Thus, the nanoformulations disassembled the biofilm matrix without affecting microbial viability, which makes them promising candidates for the development of dental caries preventive and therapeutic agents.

Keywords: *Streptococcus mutans*, chitosan, Epigallocatechin-3-gallate, dental caries, biofilm

Experimental

Experimental design

An experimental in vitro study was conducted to evaluate the effects of EGCG-loaded chitosan nanoparticles against *S. mutans* UA159 biofilm. The biofilm was cultivated on bovine enamel slabs using ultrafiltered tryptone-yeast extract broth (TYE). The biofilm was treated for 1.5 min with NaCl 0,9% (NaCl - negative control), Nanoparticles of chitosan (Nchi), Nanoparticles of chitosan loaded with EGCG 4 mg/ml (Nchi+EGCG) (concentration based on Vilela et al., 2020) (Vilela et al. 2020) and chlorhexidine CHX 0,12% (positive control) (Figure S1). After 21 hours of biofilm cultivation, the biofilm was treated two times a day according to the groups previously described. The culture medium was changed twice daily during biofilm growth and the pH of the spent medium was measured as an indicator of biofilm acidogenicity. After 67 hours of growth, the biofilm was collected to analyze viable cells (counting of colony forming unities (CFU)) and biofilm dry weight. The architecture of the biofilms was evaluated through scanning electron microscopy (SEM). SEM was also performed to evaluate the effects of the treatments on the morphology of the biofilm cells and structure. Two independent experiments were performed in at least triplicates. All data are available upon request to the first author.

Preparation of chitosan nanoformulation

The chitosan nanoparticles were prepared by ionic cross-linking using tripolyphosphate. Low molecular weight chitosan (#448869, Sigma-Aldrich, Darmstadt, Germany) was dissolved in 0.33% (vol/vol) glacial acetic acid to prepare a 2 mg/mL stock solution. The pH was adjusted to 5 using 0.1 N sodium hydroxide. Then, the chitosan stock was filtered through a 0.45 µm filter (#C045A047A, Analitica, São Paulo, Brazil). Under mild stirring, the tripolyphosphate solution

(1 mg/mL) was slowly added, drop by drop, to the chitosan solution. The proportion in volume of chitosan to tripolyphosphate was 2.5:1 (Cánepa et al. 2017).

Preparation of EGCG-loaded chitosan nanoformulation

EGCG (#4143, Sigma-Aldrich, Darmstadt, Germany) was dissolved in purified water to obtain the final concentration of 4 mg/mL. After complete solubilization, the 4 mg/mL. EGCG solution was added to the chitosan solution. After 5 minutes of stirring, tripolyphosphate was added to the mixture according to the previously described protocol. The final preparation of Nchi +EGCG was kept at -4 °C until its use.

Characterization of chitosan-based nanoformulations (Nchi and Nchi+EGCG)

Size distribution, polydispersity index, and zeta potential of the nanoformulations were determined using Zetasizer Nano Series (Malvern Instrumentation Co, Westborough, MA). To do so, 10 µL of each solution was diluted in 1 mL of purified water in a clear plastic cuvette of 1cm optical path lenght. The size and polydispersity index were determined using photon correlation spectroscopy at 25 °C and a scattering angle of 173°. Zeta potential was determined using a disposable capillary zeta potential cell (Malvern DTS1060) through the electrophoretic mobility of the particles. The dynamic light scattering measurement for an average size, polydispersion index, and zeta potential were monitored over a window of 90 days after preparation. Nchi and Nchi + EGCG were observed over 75 days.

The accelerated stability studies were carried out on the shelf-life equipment LUMiSizer® analyzer (612 LUMiSizer, LUM GmbH, Berlin, Germany). The data were obtained through an optical system working in the near-infrared region (NIR) coupled with a centrifuge system that

records transmission profiles for each point between the top and the bottom of the analysis container. Particles on top migrate to the bottom of the container during the centrifugation process, resulting in differences between the transmission profiles as a function of time. For this analysis, 0.4 mL of Nchi and Nchi + EGCG were placed in cuvettes with a maximum of 129.5-mm radial position and centrifuged at 3500 rpm and 25 °C for seven hours. The results were analyzed with the V.5.1 SEPView (LUMGmbH, Berlin, Germany) (Tedesco et al. 2021).

Preparation of enamel slabs

Crowns of bovine incisors were sectioned using a low-speed diamond blade to obtain enamel slabs (4 x 7 x 1 mm) (Fernández et al. 2016). The slabs had their surfaces ground using aluminum oxide abrasive papers (#400, #600, and #1200) and were polished with one μm diamond paste in a grinder machine (Phoenix Beta, Buehler, USA) (Fernández et al. 2016). Then, the enamel slabs were mounted in a metallic holder and autoclaved for 15 minutes at 121 °C.

Biofilm preparation

A previously described *S. mutans* biofilm model was used (Rocha et al. 2018) with the following modification: adjustment of the inoculum to OD600 1.6±0.05(Ccahuana-Vásquez & Cury, 2010) . Briefly, a strain of *S. mutans* UA 159 was grown on BHI agar plates (48 h / 37 °C / 5% CO_2). Five to ten colonies were inoculated into 10 ml of TYE + 1% glucose and incubated (37 °C / 5% CO_2). After 16 hours, the starter culture was standardized in a spectrophotometer at OD600 1.6±0.05 to obtain the bacterial inoculum. Thus, 1.5 ml of the adjusted inoculum was distributed to each well of a 48-well plate. Then, the enamel slabs were vertically placed into this inoculum, left undisturbed, and incubated (37 °C/ 5% CO_2) to allow initial biofilm formation. After 19 hours of incubation, the culture medium was changed, and the pH of the spent medium was evaluated.

This procedure was repeated twice daily (at 8 a.m. and 4 p.m.). The biofilm was treated twice daily, two hours after culture medium change (at 10 a.m. and 6 p.m.) until the end of the experiment (67 hour-old biofilms).

Treatments

The biofilms were exposed to the test agents by individually immersing the enamel slabs containing the biofilm into 0.5 mL of the treatment solution for 1.5 min according to each experimental group. After the immersions, each enamel slab/biofilm was washed in 0,9% saline solution to remove any excess treatment. After being washed, the enamel slabs/biofilm were placed back into the culture medium, where they remained until the following medium change.

Biofilm analyses

After 67 h of growth, biofilms were harvested in saline solution by sonication for 15 s at 20% potency and the suspension was used for biofilm analyses. For the bacterial viability analysis, an aliquot of 100 µL of the biofilm suspension was ten-fold serially diluted in saline solution until $1:10^7$. Two drops of 20 µL of each dilution were plated on BHI agar and incubated (37˚C/5% CO_2/48 h) C. The count of CFU was performed with a stereoscopic microscope (Ccahuana-Vásquez and Cury. 2010; Fernández et al. 2016). For the biofilm dry weight analysis, an aliquot of 200 µL of the biofilm suspension was added to pre-weighed microcentrifuge tubes and lyophilized. The biofilm dry weight was determined by the difference between the final and initial weight of the microcentrifuge tubes (Ccahuana-Vásquez and Cury. 2010, Fernández et al. 2016).

Culture medium analysis

The culture medium was collected twice daily and immediately used to determine the acidogenicity of the biofilm through the analysis of the culture medium pH. The pH was measured directly from each well of the 48-well culture plate using a pH microelectrode (Alberto Ccahuana-Vásquez Jaime Aparecido Cury in Cariology et al. 2010; Fernández et al. 2016; Rocha et al. 2018) (Orion, Thermo Scientific, MA, USA) coupled to a pHmeter (Thermo Scientific, MA, USA) previously calibrated with pH standards of 4.0 and 7.0.

Biofilm morphology analysis using scanning electron microscopy

One enamel slab/biofilm of each experimental group was randomly chosen for the scanning electron microscopy analysis. The biofilm of each group was individually stabilized in 1 mL of 2.5% glutaraldehyde for two h (Weber et al. 2014) and dehydrated in serial dilutions (50, 70, 80, 90, 95, 100%v/v) of ethanol for 20 min. Then, the enamel slabs/biofilms were immersed in 100% ethanol for one hour. After that, the biofilm was air-dried for one day and then mounted on copper disks, transferred to the camera for a critical drying point, and sputter-coated with gold. The biofilms were analyzed using scanning electron microscopy (EVO® 50 series, Carl Zeiss AG, Koln, Germany) and photographed at 10 and 20,000 times magnification.

Statistical analysis

Data analysis was performed using the Statistical Package for Social Science software for Windows (version 21; SPSS Inc, IL, USA). The evolution of the pH acidogenesis was recorded as the mean and standard deviation of the culture medium pH through the 67 hours of biofilm formation (19, 27, 43, 51,67 hours). Then the pH area under the curve (AUC) for each experimental

group was calculated. The distribution of errors of the AUC data, CFU/mg of biofilm, and biofilm dry weight was evaluated using the Shapiro-Wilk's test. As AUC and dry weight data presented normal distribution of errors and were analyzed by one-way ANOVA followed by the Tukey HSD test. Data of CFU/mg of biofilm were analyzed by Kruskal-Wallis test followed by Chi-square multiple comparisons since the CFU data did not present normal distribution. The significance level was set at 0.05.
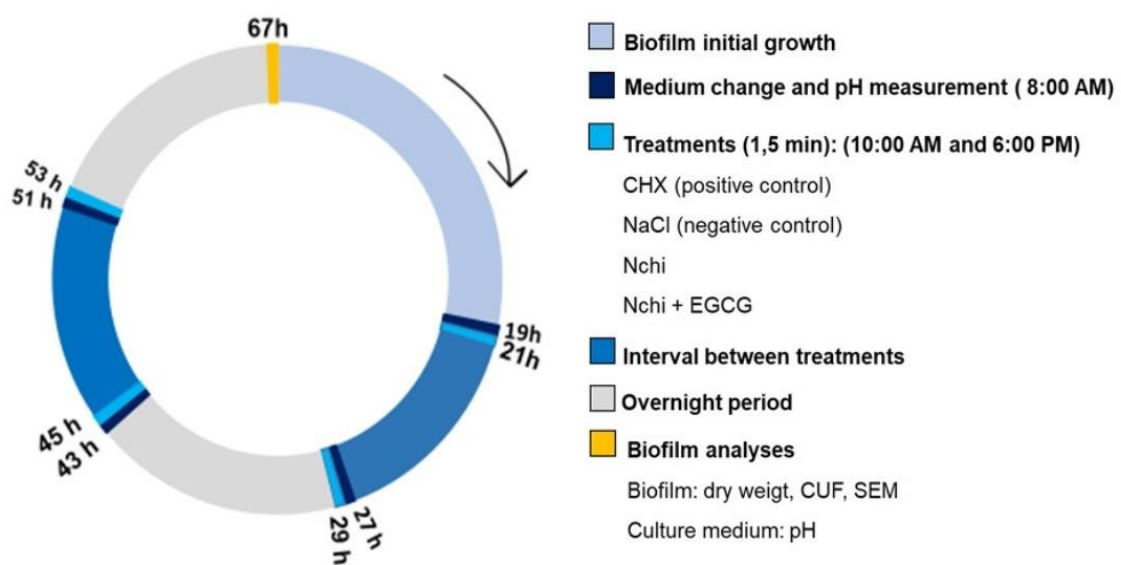
Figure S1: Flowchart of the experimental design.
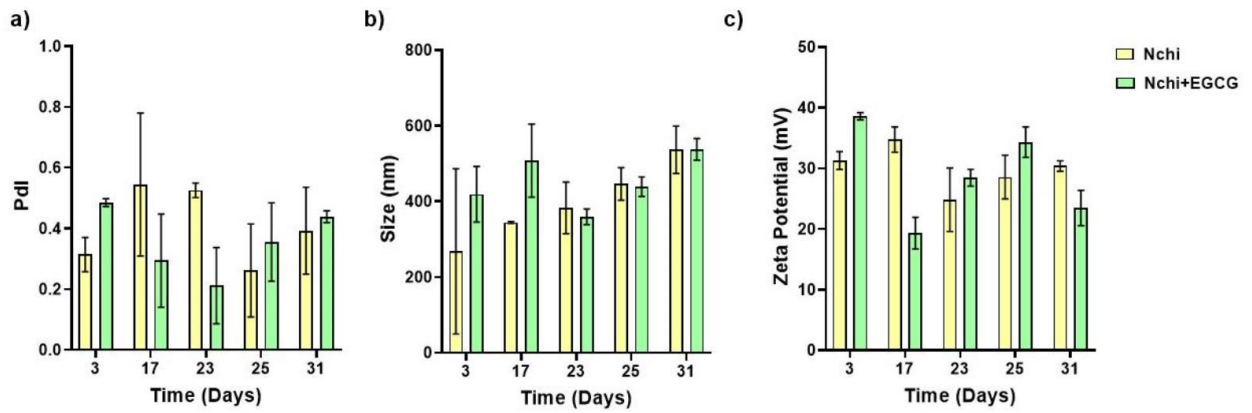
Figure S2: Characterization of Nchi and Nchi+ EGCG (, Polydispersity index, Size distribution, and Zeta potential).
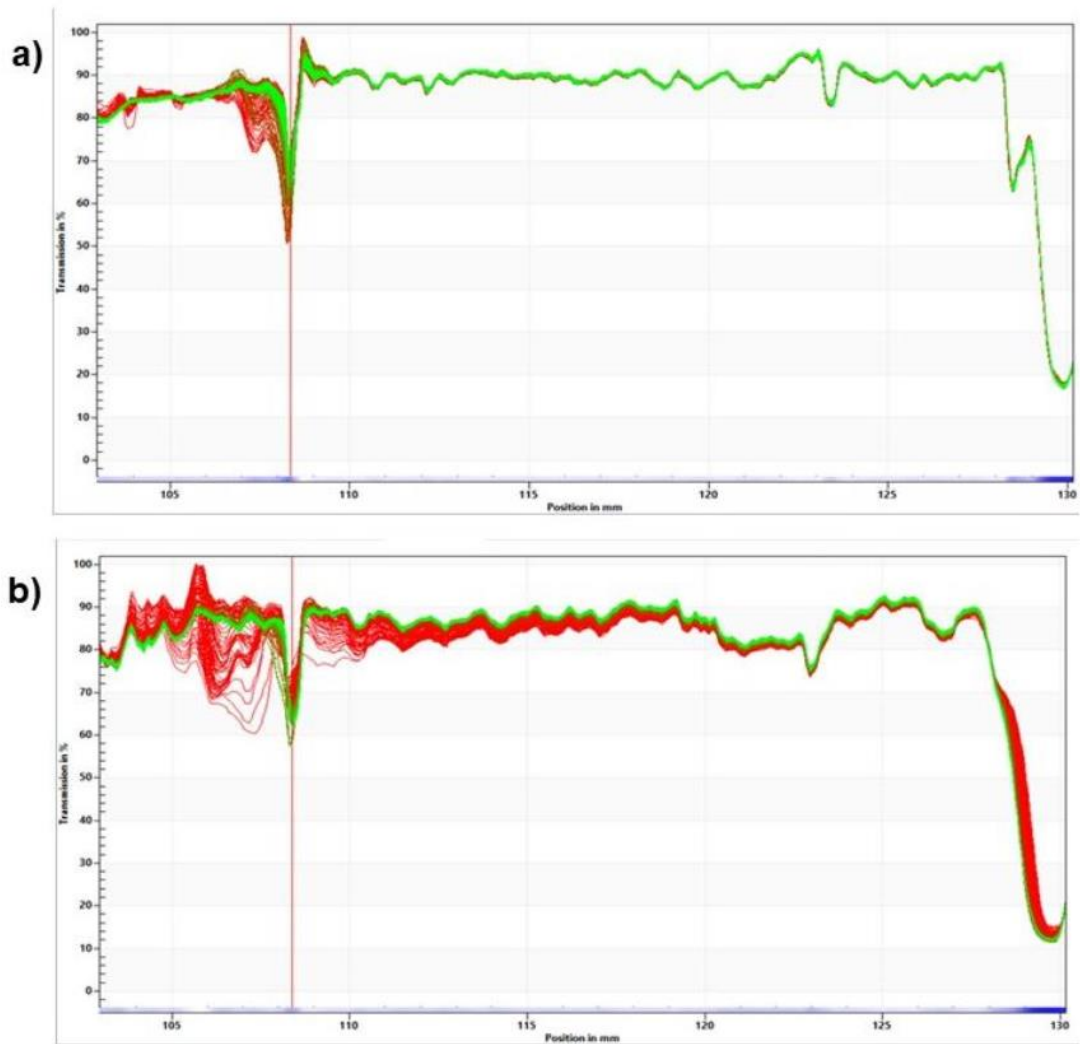
Figure S3: Evolution of the transmission profiles obtained in accelerated stability, the first profiles are in red and the last are in green. a) Nchi and b) Nchi + EGCG.
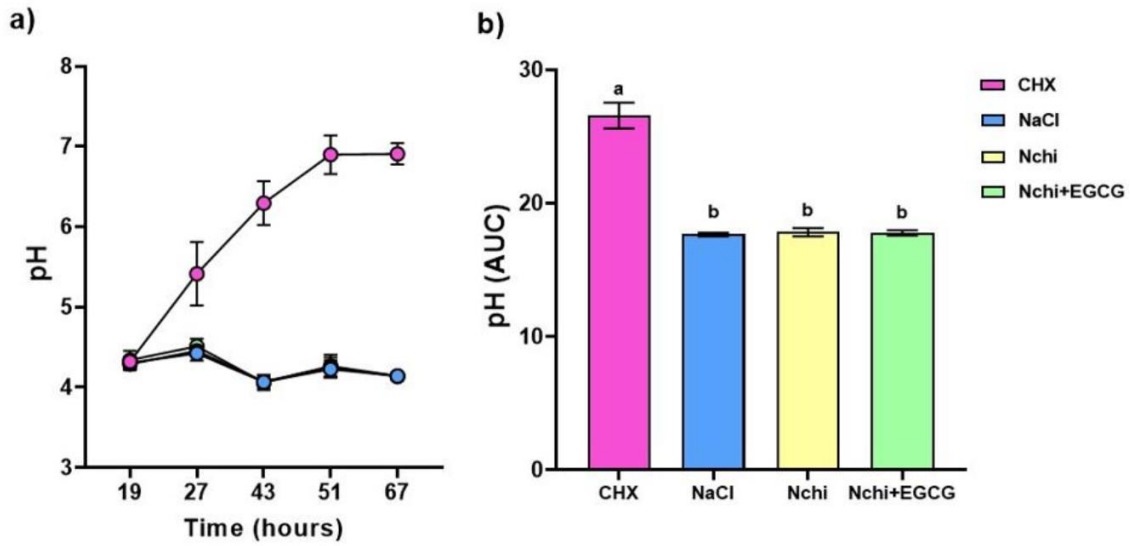
Figure S4: Acidogenesis of the treated biofilms. a) Represents the pH of the spent medium during the 67 hours of biofilm growth (mean ± standard deviation, n=9). b) Represents the AUC for the evolution of acidogenesis over time (mean ± standard deviation, n=9). Different lower-case letters indicate statistical difference (p<0,05).
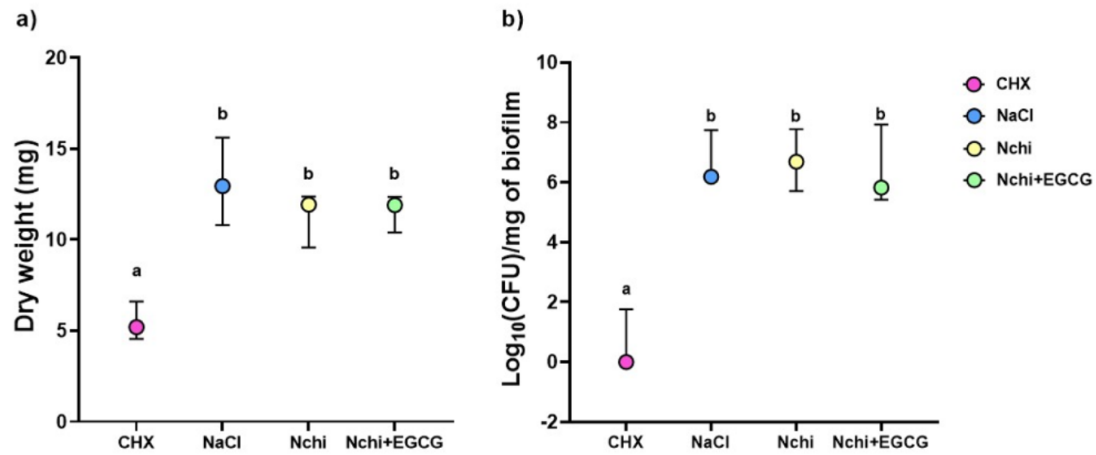
Figure S5: Biofilm biomass and microbial viability. a) Represents the dry weight of the treated biofilms (median ± interquartile range, n=7). b) Represents the microbial viability of *S. mutans* presented as $\log_{10}$ of CFU counts per mg of biofilm (median ± interquartile range, n=7). Different lower-case letters indicate statistical difference (p<0,05).

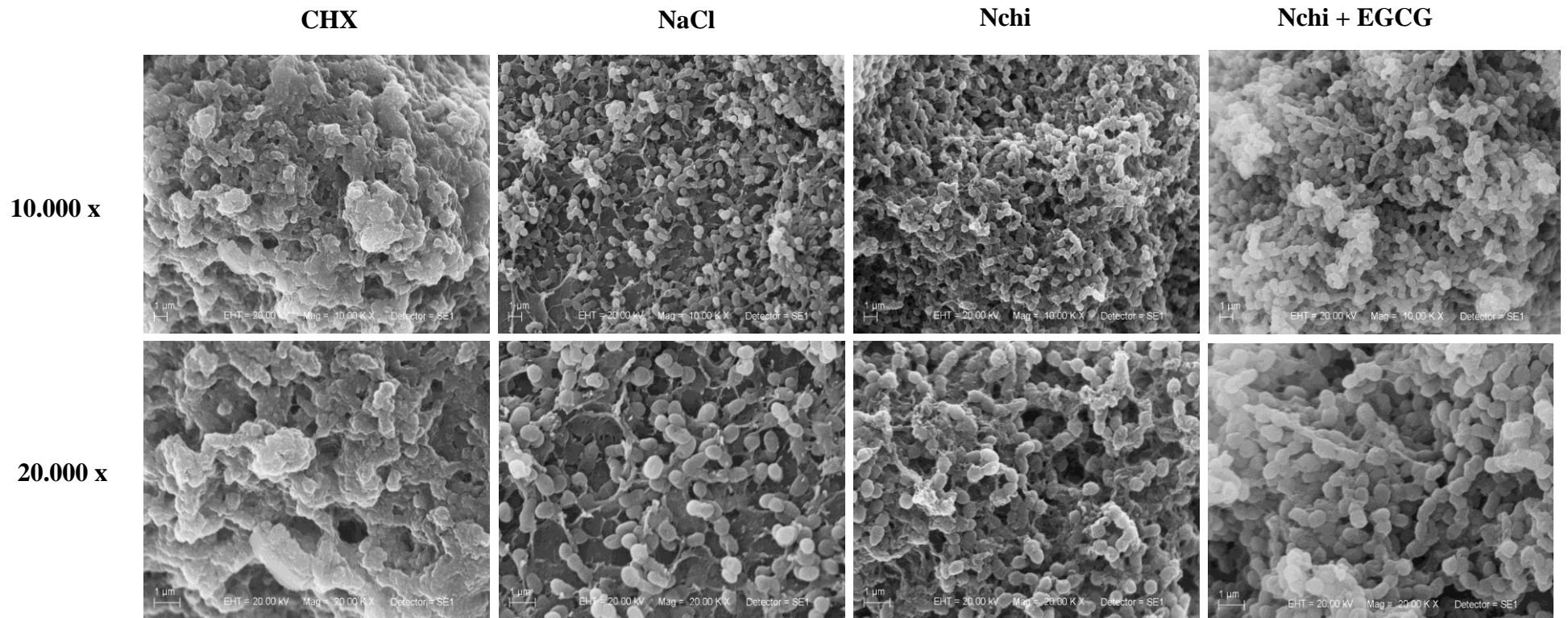|	CHX	NaCl	Nchi	Nchi + EGCG
10.000 x				
20.000 x				

Figure S6: Representative scanning electron microscopy images of 67 h-old *S. mutans* biofilms treated with different solutions.