Cellulose Nanocrystals Show Anti-adherent and Anti-biofilm Properties Against Oral Microorganisms

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Article

Cellulose Nanocrystals Show Anti-Adherent and Anti-Biofilm Properties against Oral Microorganisms

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Abstract: Cellulose nanocrystals (CNCs) are cellulose-derived nanomaterials that can be easily obtained, e.g., from vegetable wastes produced by circular economies. They show promising antimicrobial activity and the absence of side effects and toxicity. This study investigated the ability of CNCs to reduce microbial adherence and biofilm formation using in vitro microbiological models reproducing the oral environment. Microbial adherence by microbial strains of oral interest, *Streptococcus mutans* and *Candida albicans* was evaluated on the surfaces of salivary pellicle-coated enamel disks in the presence of different aqueous solutions of CNCs. The antibiofilm activity of the same CNC solutions was tested against *S. mutans* and an oral microcosm model based on mixed plaque inoculum using a continuous-flow bioreactor. Results showed an excellent anti-adherent activity of the CNCs against the tested strains from the lowest concentration tested (0.032 wt.%). Such activity was significantly higher against *S. mutans* than *C. albicans*, suggesting a selective anti-adherent activity against pathogenic strains. At the same time, there was a minimal albeit significant antibiofilm activity (at least 0.5 and 4 wt.% CNC solution for *S. mutans* and oral microcosm, respectively). This makes CNCs particularly interesting as anticaries agents, encouraging their use in the oral field.

Keywords: cellulose nanocrystals; bacterial adherence; biofilms; *Streptococcus mutans; Candida albicans*; oral microcosm; bioreactors

1. Introduction

Bacterial adhesion and biofilm formation inhibition are increasingly promising topics because of the urgent need for strategies alternative to conventional antimicrobial principles [1]. In the oral environment, active principles must interact positively with the surfaces of natural and artificial hard tissues, modulating the biofilm behavior instead of eliminating them. In this context, antiadhesion and biofilm inhibition strategies could effectively restore the plaque ecosystem's balance [2–4]. From this point of view, one of the most promising strategies is based on using a wide variety of nanoparticles, taking advantage of their ability to interact with both the microorganisms and their colonization surface [5].

In this sense, cellulose nanocrystals (CNCs) represent a valid possibility. They are cellulose-derived nanomaterials that can be easily obtained [6] because cellulose is one of the most diffused vegetable wastes produced by circular economies [7]. CNCs are widely available and easily produced through acidic hydrolysis from many widely available cellulose sources, including agricultural and agro-industrial residues, such as wood, non-wood fibers, and algae [8–10].

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CNCs show many desirable properties, such as excellent mechanical strength, absence of toxicity, and, above all, biocompatibility [9,11]. These characteristics make it worthwhile for several biomedical applications.

As stated above, the most interesting property of CNCs is their antibacterial potential [12,13], expressed against bacterial adherence and biofilm formation, without any notable side effects. These characteristics fit the general requirements of an active principle that can be used to prevent biofilm-related oral diseases. In particular, the absence of known side effects and toxicity [13–16] seems ideal for oral hygiene products such as toothpaste and mouth rinses. For this reason, CNCs have attracted the curiosity of those in the oral field who are looking for antimicrobial agents.

Such agents target a broad spectrum of oral microorganisms, with particular attention to cariogenic species, such as *Streptococcus mutans,* and, for different reasons, related to mucosae infections, *Candida albicans*. *S. mutans*, in particular, is the species that exhibits intense cariogenicity and biofilm formation capability, being, for this reason, a model microorganism for in vitro caries testing [17,18]. Furthermore, growth conditions represent a crucial parameter to provide in vitro data with good translatability to clinical conditions. For these reasons, when testing the antimicrobial properties of new active principles for the oral environment, it is of utmost importance that the two main phases of microbial colonization (adherence and biofilm formation) are considered and tested in an environment with hydrodynamic stress similar to the natural one [19].

This study aimed to evaluate the ability of a CNC solution to reduce bacterial adherence and biofilm formation using in vitro microbiological models reproducing different aspects of the oral environment. The null hypothesis was that CNC solutions would not reduce microbial adherence and biofilm formation by *S. mutans* and *C. albicans* and that such solutions would not reduce biofilm formation by a multispecies oral biofilm.

2. Materials and Methods

2.1. Preparation of CNCs

According to a previously reported procedure [20], CNCs were obtained from Whatman filter paper by acid hydrolysis in 64% of H_2SO_4 for 1 h at 55 °C. CNCs were recovered in about 40 % yield, giving a suspension at around 1 wt. %. They were characterized by DLS measurements, and we found a ζ-potential of −39.0 ± 1.0 mV, a dimension of 92.4 ± 1.8 nm, while AFM and TEM analyses confirmed the rod-like structure, as previously reported [21]. Among the different characteristics, particular attention was devoted to the surface of the CNCs. This is characterized by different hydroxyl groups of cellulose and negatively charged sulfate half-ester groups introduced during the extraction, as indicated by the negative ζ-potential [22]. The CNCs were recovered by centrifugation (12.000 × g, 4 °C, 30 min), and a 12 wt. % stock solution was prepared.

2.2. Procedures

Serial dilutions were prepared from stock solution as 4%, 2%, 1%, 0.5%, 0.25%, 0.125%, and 0.063% by dilution using filter-sterile water, while a control solution was filter-sterile water.

2.3. Preparation of enamel disks

Anterior adult bovine teeth were used to prepare a total of 256 round enamel-dentin slabs with a diameter of 6.0 mm and a thickness of 2.0 mm. Disks were cut from the labial surfaces using a water-cooled trephine diamond bur (INDIAM, Carrara, Italy). Dentin bottoms were removed, and the enamel surfaces were subjected to a standardized polishing protocol, including polishing with 1000/4000-grit grinding paper (Buehler, Lake Bluff, IL, USA) using a polishing machine (Motopol 8; Buehler, Düsseldorf, Germany). All disks were sterilized before the experiments using a chemiclave with hydrogen peroxide gas plasma technology (Sterrad; ASP, Irvine, CA, USA). Limiting the maximum temperature to 45 °C prevented heat-related damage to the specimens [23].

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2.3.1. Saliva preparation

Stimulated whole saliva was collected by expectoration from the experimenters (ACI, EB, BM). They refrained from oral hygiene for 24 h, had no active dental disease, and did not have antibiotic therapy for at least 3 mo before the experiment. Procedures followed the protocol published by Guggenheim et al. 2001 [24]. Saliva was collected in chilled tubes, pooled, heated to 60 °C for 30 min to inactivate endogenous enzymes, and centrifuged (12.000 × g, 4 °C, 15 min). The supernatant was transferred into sterile tubes, stored at -20 °C, and thawed at 37 °C for 1 h before beginning the experiments. To obtain the artificial oral microcosm inoculum, whole human saliva was additionally collected from the same experimenters after 24 h, pooled, and immediately used to inoculate the plates [25].

2.3.2. Bacteria

Streptococcus mutans ATCC 35668 was cultured according to a previously published protocol [26]. Briefly, *Mitis Salivarius* Bacitracin agar was inoculated with *S. mutans* and incubated at 37 °C in a 5% supplemented CO_2 environment for 48 h. A pure suspension of the microorganism in Brain Heart Infusion additioned with 1 wt.% sucrose was obtained from these plates after incubating at 37 °C in a 5% supplemented CO_2 environment for 12 h (early log phase). *S. mutans* cells were harvested by centrifugation (2.200 × g, 19° C, 5 min), washed twice with sterile phosphate-buffered saline (PBS), and resuspended in BHI 1 wt. % sucrose. The suspension was subsequently subjected to low-intensity ultrasonic energy (Sonifier model B-150; Branson; Danbury, CT, USA; operating at 7-W energy output for 30s) to disperse bacterial chains. The suspension was then adjusted to a 1.0 on the McFarland scale, corresponding to a microbial concentration of approximately 3.0×10^8 cells/mL.

A pure culture of *Candida albicans* strain ATCC 90028 in BHI + 1 wt. % sucrose was obtained at 37 °C in a 5% supplemented CO_2 environment after 24h of incubation. Cells were harvested by centrifugation (2200 × g, 19° C, 5 min), washed twice with sterile PBS, and resuspended in BHI + 1 wt. % sucrose, the suspension adjusted to a turbidity equivalent to a 1.0 McFarland standard.

2.3.3. Adherence

Enamel disks were placed with sterile tweezers, one into each well of 48-well plates (Nunc, Kastrup, Denmark), and 50 µL of sterile thawed saliva was added on the surface of each disk, and plates were incubated at 37 °C in a 5% supplemented CO_2 environment for 24 h to allow for the development of the acquired salivary pellicle. After that, excess saliva was discarded, and the surface of the disks was washed with sterile PBS. A total of 250 µL of each concentration of CNCs was added to each of n=8 wells. Then, 250 µL of either *S. mutans* or *C. albicans* microbial suspension was added to each well. Thus, the final concentration of CNCs in each well was 2.00%, 1.00%, 0.50%, 0.25%, 0.12%, 0.06%, 0.03%, and 0% (negative control). A total of 64 disks for each strain were used. After 2 h of incubation, the enamel disks were washed with sterile PBS, and biomass viability was assessed.

2.3.4. Biofilm formation

Biofilm formation under shear stress conditions was evaluated using a modified-drip flow reactor (M-DFR) according to Ionescu et al. 2019 [26]. The modified design allowed the placement of customized specimen trays on the bottom of the flow cells and the complete immersion of the surfaces of the enamel disks into the surrounding flowing medium. All tubing and specimen-containing trays were sterilized before the experiments using the Sterrad chemiclave. The M-DFR was assembled inside a sterile hood and transferred into an incubator to operate at 37 °C. To simulate salivary pellicle formation, the enamel disk surfaces in each flow cell were exposed to the thawed sterile saliva for 24 h. Subsequently, excess saliva was discarded. Biofilm formation was obtained on the surfaces of the enamel disks by inoculating 10 mL of either *S. mutans* or pooled whole saliva suspension into each flow cell. A total of 64 disks for each strain were used. After 4 h, a multichannel, computer-controlled peristaltic pump (RP-1; Rainin, Emeryville, CA, USA) was turned on to provide a constant flow of nutrient broth through the flow cells. The sterile nutrient broth was enriched with 10.0 g/L sucrose

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and consisted of 2.5 g/L mucin (type II, porcine gastric), 2.0 g/L bacteriological peptone, 2.0 g/L tryptone, 1.0 g/L yeast extract, 0.35 g/L NaCl, 0.2 g/L KCl, 0.2 g/L $CaCl_2$, 0.1 g/L cysteine hydrochloride, 1 mg/L hemin, and 0.2 mg/L vitamin K_1. The flow rate was set to 9.6 mL/h. After 24 h, the flow of nutrient broth was stopped, the flow cells were opened, and the trays containing the specimens were carefully removed and immediately placed in dishes containing sterile PBS at 37 °C. The specimens were gently removed from the tray and transferred into 48-well plates. Each specimen was exposed to 500 µL of CNC solution previously diluted (2%, 1.00 %, 0.50%, 0.25%, 0.12%, 0.06%, 0.03%, and 0%, n = 8 disks for each dilution). Plates were incubated at 37 °C in a 5% supplemented CO_2 environment for 2 h. Then, the solutions were discarded, the wells were washed with sterile PBS, and biomass viability was assessed on the surface of the enamel disks.

2.4. Biomass viability assay

The viable biomass was conducted as previously described [26]. Briefly, two stock solutions were prepared by dissolving 5 mg/mL 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) and 0.3 mg/mL of N-methylphenazinium methyl sulfate (PMS) in sterile PBS. The solutions were stored at 2 °C in light-proof vials until the day of the experiment, when a fresh measurement solution (FMS) was prepared by diluting 1:1:8 the MTT stock solution, PMS stock solution, and sterile PBS, respectively. A lysing solution (LS) was prepared by dissolving 10 vol % of sodium dodecyl sulfate and 50 vol % dimethylformamide in deionized water.

A total of 300 µL of FMS solution was added to each well, and the plates were incubated at 37 °C under light-proof conditions for 1 h. During incubation, electrons transported across the microbial plasma membrane, and, to a lesser extent, microbial redox systems convert the yellow salt to insoluble purple formazan crystals. The conversion at the cell membrane level was facilitated by the intermediate electron acceptor (PMS). The unreacted FMS solution was gently removed, and the formazan crystals were dissolved by adding 300 µL of LS to each well. The plates were stored under light-proof conditions at 37 °C for an additional 1 h, and 100 µL of the solution from each well was then transferred into 96-well plates. The absorbance of the solution was measured using a spectrophotometer (Genesys 10-S, Thermo Spectronic, Rochester, NY, USA) at a wavelength of 550 nm. The results were expressed as relative absorbance in optical density (OD) units corresponding to the amount of adherent, viable, and metabolically active biomass. The results were converted to % viability, considering negative control (filter-sterile water) as 100% and a 1 wt. % chlorhexidine digluconate solution in filtered sterile water (positive control) as 0%.

2.5. Statistical analysis

Analyses were performed using JMP 17.0 software (SAS Institute, Cary, NC, USA). The normal distribution of data was checked using Shapiro-Wilk's test ($p < 0.05$), and the homogeneity of variances was verified using Levene's test ($p < 0.05$). Means and standard errors were calculated from the raw data. One-way analysis of variance (ANOVA) was performed considering α value of 0.05. Tukey's HSD was employed as a post-hoc test ($p < 0.05$).

3. Results

S. mutans and C. albicans adherence in response to the CNC solution tested at different concentrations showed a similar trend. The viable mass results indicated a significant inhibition of adherence on both S. mutans and C. albicans, starting from the lowest concentration tested (0.03 %, $p < 0.0001$ and $p=0.008$, respectively). This anti-adherent effect was much more pronounced on S. mutans (- 70 %) than on C. albicans (- 30 %). The effect reached maximum values at 0.25 % and 0.5 % concentrations for S. mutans and C. albicans, respectively. Complete inhibition was obtained for S. mutans, while a maximum of 80 % inhibition was reached for C. albicans (Figure 1).

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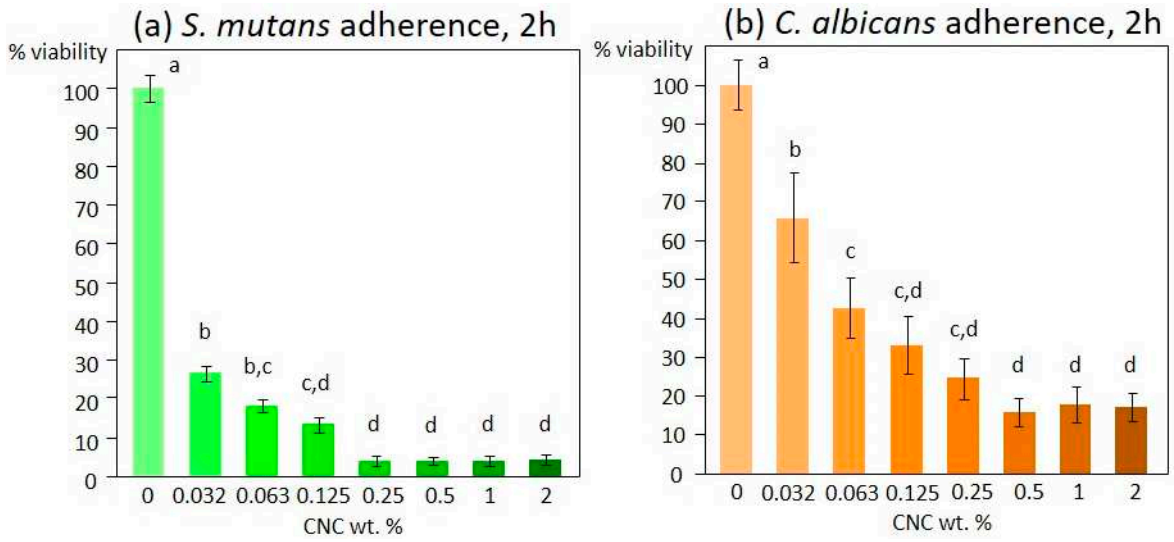

Figure 1. Viable microbial cells of *S. mutans* (**a**) and *C. albicans* (**b**) adherent to the pellicle-coated enamel surfaces after 2 h. Each bar shows a different concentration of CNC in aqueous solution. Negative control (0% CNC) is set to 100% viability, while 0% viability was a 1 wt. % chlorhexidine digluconate solution. Different superscript letters indicate significant differences between groups (Tukey's test, $p < 0.05$).

S. mutans biofilm formation (Figure 2a) was significantly reduced by CNCs when in a concentration of at least 0.5 % ($p=0.01$), reaching about –15 %. Increasing concentrations did not produce additional reductions. Only the highest concentration of CNC tested (2 %, $p=0.01$) reduced the artificial oral microcosm biofilm by about 25 % (Figure 2b).

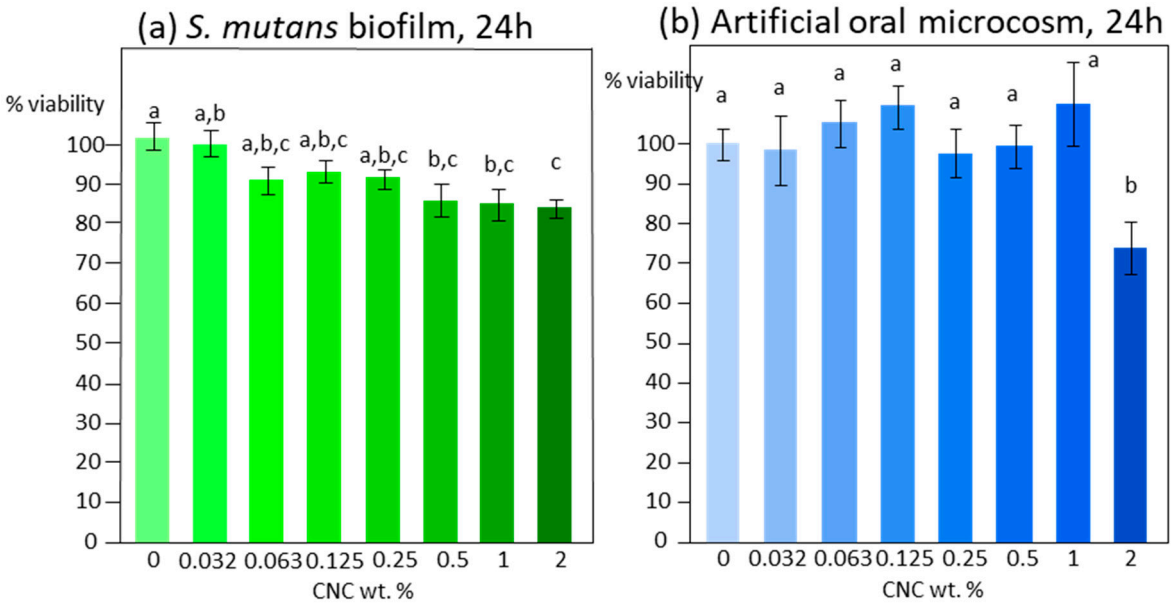

Figure 2. Viable biomass of *S. mutans* (**a**) and artificial oral microcosm from mixed plaque inoculum (**b**) developed under shear stress on the pellicle-coated enamel surfaces after 24 h. Each bar shows a different concentration of CNC in aqueous solution. Negative control (0% CNC) is set to 100% viability, while 0% viability was a 1 wt. % chlorhexidine digluconate solution. Different superscript letters indicate significant differences between groups (Tukey's test, $p < 0.05$).

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4. Discussion

There is currently an overwhelming interest in medical science regarding nanoparticles showing antimicrobial properties [5,28]. However, many of the possibilities entail concerns about their biocompatibility and toxicity [10–12]. In this sense, cellulose nanocrystals have received much attention for their biocompatible and non-toxic properties in addition to promising antimicrobial properties. [12,13,29–31].

In this study, we evaluated in vitro the anti-adherent and anti-biofilm effect of CNCs against microbiological models of oral interest, including *S. mutans*, *C. albicans*, and the oral artificial microcosm made by mixed flora. Our results showed that the tested CNCs were already highly effective as an anti-adherent principle from the lowest tested concentration (0.03 wt. %). This activity was twice as high against *S. mutans* compared to *C. albicans*.

This finding is of utmost interest for an active principle in the oral environment. Adherence is an essential step for microbial colonization in the oral cavity [32]. Dental hard tissues are covered by an acquired salivary pellicle, which, from a microbial colonization point of view, makes them unique substrates in the human body. For this reason, the biofilm that permanently colonizes such an ecological niche possesses peculiar features [33]. Pioneer microorganisms that first adhere are equipped with adhesins that specifically bind to the acquired salivary pellicle, thus resisting shear stresses [34]. Colonization by other taxa allows biofilm formation and maturation, including extracellular matrix production and an increase in its complexity to harbor at least 2,000 taxa, with ~700 species residing in an individual's mouth at a lifetime [20,35]. Some species are typically commensal and spend their time symbiosis with the human host. Then again, some can cause oral diseases such as dental caries, gingivitis, and periodontitis [2,17,36]. Thus, control of microbial adherence in the oral cavity is essential for the control of biofilm and, ultimately, for the development of the above diseases [37]. For these reasons, our results open a very promising possibility to control and modulate the interactions of oral microorganisms with the host and its substrata. Furthermore, the fact that the anti-adherent activity was higher for a pathogenic microorganism such as *S. mutans* than the other tested strain suggests the promising possibility of a selective activity that could be directed mainly towards pathogenic species. Such a hypothesis will be the core of future studies on the activity of CNCs in the oral environment.

The literature regarding the anti-adherent properties of CNCs is still poor. [29] In 2017, it was demonstrated that 0.1 to 1 wt. % solutions of CNCs produced a very significant reduction of the adherence by *Escherichia coli* to the cell surfaces of an intestinal cell line. This effect was caused by a direct interaction of CNCs with microbial cells rather than a biocidal effect. In fact, a biocidal activity could be conferred to cellulose-based materials only by further functionalization with antimicrobial compounds [15,38]. Interestingly, Noronha et al. demonstrated a significant biocidal activity of CNCs against *E. coli* due to a disruption of the integrity of lipid bilayer vesicles, assuming, therefore, that CNCs penetrated the cells and inflicted irreversible damage to their membrane [39]. This result is in contrast with the findings of the present study. It must be noted that oral biofilms develop a thick layer of extracellular polysaccharides that protects microbial cells from the surrounding environment. For this reason, we never saw complete inactivation of microbial cells organized in biofilms to concentrations up to 2 wt. % CNC and only a slight reduction in viability was observed, possibly due to the inactivation of the outer layer of cells. This consideration highlights the importance of the experimental conditions to replicate the physiological conditions as closely as possible. The latter include, for the oral environment, testing microbial cell colonization to natural hard surfaces in the presence of acquired salivary pellicle and shear stresses that force adherent cells to present a biofilm structure and characteristics that resemble clinical ones [40]. In this sense, using a bioreactor allows the replication of most, if not all, of the clinically relevant biofilm behavior.

5. Conclusions

The results of the present study show that the tested CNCs exhibited excellent anti-adhesive activity against *S. mutans* and *C. albicans*. Such activity was significantly higher against *S. mutans* than *C. albicans*, suggesting a selective anti-adherent activity against oral pathogenic strains. At the same

time, there was a minimal albeit significant anti-biofilm activity against both monospecies *S. mutans* biofilm and oral microcosm multispecies biofilm. This makes CNCs particularly interesting as anticaries agents, encouraging their use in the oral field.

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