

Streptococcus mutans biofilm inhibition using antisense oligonucleotide to glucosyltransferases B and C

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Background. Biofilm formation by *Streptococcus mutans* bacteria on teeth leads to dental caries, which still remains one of the most prevalent human diseases strongly related to increase of dietary sucrose consumption in modern society. In the biofilm, sucrose is metabolized by *S. mutans* to acids causing tooth decay. *S. mutans* also produces glucosyltransferases (Gtfs) for synthesis of sticky glucan polymers from sucrose that provides matrix for biofilm formation on teeth. For reducing biofilm build-up, one preventive measure could be blocking of Gtf synthesis. The aim of this study was to test antisense phosphorothioate oligodeoxyribonucleotide (PS-ODN) targeting simultaneously *S. mutans gtfB* and *gtfC* mRNAs in order to inhibit biofilm formation *in vitro*.

Materials and methods. *S. mutans* bacteria were grown anaerobically on glass slides inserted vertically in 24-well cell culture plates containing Todd Hewitt broth with sucrose under exposure to antisense or missense PS-ODNs at the final concentration of 10 µM. Untreated bacteria served as controls. After 24 h of incubation, glass slides were removed, air-dried and further used for the quantitative evaluation of the streptococci biofilm applying an optical profilometry technique.

Results. It was revealed that antisense PS-ODN considerably reduced the most critical biofilm surface roughness parameter S_a (average difference between the peak height and valleys) inhibiting the biofilm development by 46% and 77% in comparison to untreated ($p = 0.06$) and missense PS-ODN-treated bacteria ($p < 0.05$), respectively.

Conclusions. The results demonstrate that antisense PS-ODN considerably decreases streptococci-induced biofilm development on glass slides, and might therefore significantly suppress dental biofilm formation through simultaneous inactivation of *S. mutans gtfB* and *gtfC* mRNAs.

Key words: *Streptococcus mutans*, biofilm, dental caries, glucosyltransferase, antisense oligonucleotide

INTRODUCTION

The main etiologic agent of human dental caries is recognized to be an oral bacterium *Strepto-*

coccus mutans (1). This streptococcus is capable to form a biofilm on the surface of teeth (2). The structural matrix of the dental biofilm consists of water-insoluble glucans synthesized from sucrose by several isoforms of glucosyltransferase (Gtf) enzyme present in *S. mutans* bacteria (2, 3). In this respect, *S. mutans* produces water-insoluble

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and partly water-soluble glucans using GtfB and GtfC enzymes encoded by *gtfB* and *gtfC* genes, respectively (2). Importantly, in the formed biofilm, dietary carbohydrates (such as sucrose, galactose) are metabolized by *S. mutans* and other oral streptococci (e. g. *S. sobrinus*) to organic acids leading to cavitation and dental caries because of the demineralization of tooth enamel (3, 4). In this context, the epidemiological studies clearly show a trend to global increase in dental caries despite the availability of fluoride toothpastes, water fluoridation, dental sealants, oral health educational programs and various antiseptic mouthrinses (5, 6). Thus, it is important to design new pharmaceuticals in order to suppress dental biofilm formation through the mechanism involving the specific inhibition of water-insoluble glucans production.

The currently available antisense technology provides the novel approach that can be applied for blocking the production of glucan polymers in *S. mutans* bacteria (7). It is based on the use of chemically modified single-stranded nucleic acid molecules (usually 20 nt in length), which can selectively hybridize to their target complementary mRNA through Watson-Crick base pairing, consequently leading to degradation of the bound mRNA by RNase H endonuclease, steric hindrance of ribosomal activity, inhibition of mRNA splicing or destabilization of pre-mRNA (8, 9). This results in specific down-regulation of the target gene and subsequent protein expression. A large number of studies have demonstrated the efficacy of antisense oligonucleotides for silencing of various genes in bacteria, including *S. mutans* (10–12). Importantly, Guo et al. (7) showed that phosphorothioate oligodeoxyribonucleotides (PS-ODNs) designed to antisense *gtfB* mRNA in *S. mutans* reduces the expression and activity of GtfB enzyme as well as water-insoluble glucan production and biofilm formation. The PS-ODNs represent DNA molecules containing a phosphorothioate-modified backbone, where one of the non-bridging oxygen atoms in the phosphodiester linkage is replaced by a sulphur atom in order to increase the resistance to endo- and exonucleases (13). Hence, in the present study, we aimed to test the PS-ODN targeting specifically and simultaneously *S. mutans gtfB* and *gtfC* mRNAs in order to inhibit biofilm formation.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The *S. mutans* strain UA159 (American Type Culture Collection No. 700610) was chosen for this experimental study because it preferably colonizes humans. Stocks of this bacterial strain were stored in 10% skim milk (Difco) at -70°C . Before experiments, *S. mutans* bacteria were cultured in Todd Hewitt (TH) broth (Difco) under anaerobic conditions (95% N_2 and 5% CO_2) at 37°C for 18 h. Purity of the culture was checked on Mitis salivarius agar (Difco) and Columbia agar with 7% sheep blood (E & O Laboratories).

Bioinformatical analysis and selection of target sequence. In order to find the appropriate target sequence, at first the conserved homologous regions among the *gtf* genes of oral streptococci were identified. For this purpose, we primarily conducted a comparative analysis of amino acid sequences between *S. mutans* UA159 GtfB and other Gtf proteins present in oral streptococci. Using *S. mutans* UA159 GtfB protein as a query sequence, the BLAST (Basic Local Alignment Search Tool; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) search of homologs among all known proteins was performed. Then, the proteins with high identity amino acid sequences were collected and multiple sequence alignment was produced employing the MAFFT online server at the Max-Planck Institute for Development Biology (<http://toolkit.tuebingen.mpg.de/mafft>). The conserved amino acid sequence was determined within the GtfB protein of *S. mutans* UA159, which is encoded by the respective region in *S. mutans* UA159 *gtfB* gene consisting of 26 nucleotide (nt) sequence: 5'-GTTAAGATTAAGCAATGGTCTGCCAA-3'. Applying such approach as described herein, it was identified that this segment of 26 nt has 100% homologous fragments in *S. mutans* UA159 *gtfC*, *S. criceti gtfI*, *S. dentirousetti gtfI*, *S. dentisuis gtfI* and *S. orisuis gtf* genes.

PS-ODN optimization and preparation. For the PS-ODN optimization, the Primer3 service (<http://primer3.ut.ee/>) was used to analyze thermodynamics as well as other aspects of the heteroduplex forming, and then a complementary antisense sequence of 19 nt (PS-ODN1: 5'-GCAGAC-CATTGCTTAATCT-3') was picked out for the target region in genes and their encoding mRNAs

of *S. mutans* UA159 *gtfB* and *gtfC* (GenBank accession No. AE014133). For a negative control purpose, the missense sequence of 19 nt (PS-ODN2: 5'-ACTCGTATGCTACAGCTAT-3') was designed, and it differed from the antisense molecule by the scrambling nucleotide sequence. These PS-ODNs were synthesized by the Metabion International AG (Germany) with all internucleotide linkages phosphorothioated. Stock solutions of the PS-ODNs were prepared in sterile nuclease-free distilled water (Thermo Fisher Scientific, Fermentas) and stored at -20°C until use.

Experimental design. For the experiments, optical density (OD) of the bacterial culture was adjusted to 0.2 at 630 nm employing a microplate reader spectrophotometer (Dynex MRX). Prior to inoculation of the bacteria, 24-well flat-bottomed polystyrene cell culture plates (Sarstedt) were filled with the TH broth, and then the PS-ODNs were added to the plate wells at the final concentration of $10\ \mu\text{M}$ in combination with the transfection reagent composed of cationic polymer – TurboFect™ (TF) (Thermo Fisher Scientific, Fermentas) prepared according to the manufacturer's instructions. Afterwards, *S. mutans* bacteria were inoculated to the wells at the final dilution of 1:100. Immediately, the sterile glass slides of 1 mm thickness cut from standard microscope slides ($76 \times 26\ \text{mm}$; Thermo Fisher Scientific) were vertically inserted into wells, and the plates were incubated anaerobically at 37°C for additional 2 h. Afterwards, a sterile solution of sucrose was added to the appropriate wells at the final concentration of 1%, and the plates were incubated anaerobically at 37°C for another 22 h. Wells without bacterial cells were used as blank controls, and untreated *S. mutans* bacteria served as experimental controls. Following 24 h of the total incubation time, the glass slides were removed from wells, dried and further used for the profilometric analysis of *S. mutans* biofilm.

Optical profilometry. 5 measurements (every measurement area of $200 \times 260\ \mu\text{m}$) were performed per slide halfway from bottom to top of the visible biofilm employing an optical profilometer MicroXAM™ (ADE Phase Shift) 50X objective with a zoom factor of 0.625. In addition, a Gaussian filter (size of $50 \times 50\ \mu\text{m}$) was selected to remove errors of the form and waviness. Several important biofilm surface roughness parameters were evaluated: S_a – the average difference between the peak height and valleys, S_t – the maximum difference between

the peak height and valleys, S_{dr} – the total surface area (if all roughness was stretched out) according to Wennenberg and Albrektsson (14). These roughness parameters reflect a maturity of the biofilm formation, that is, the amount of adherent bacteria and microcolonies.

Evaluation of bacterial aggregation. For this purpose, *S. mutans* bacteria were grown anaerobically at 37°C in 24-well cell culture plates filled with TH broth in the absence or presence of 1% sucrose and PS-ODNs combined with TurboFect™ (TF) reagent. After 24 h of the total incubation time, the biofilm in wells was scraped and everything (biofilm, bacteria) was mixed thoroughly with a pipette in the same medium within wells, and then the samples were taken from the plate wells in order to evaluate *S. mutans* aggregation applying Gram staining and light microscopy with a Leica DM500 microscope.

Statistical analysis. Data are expressed as means \pm standard deviation (SD). Statistical evaluation was performed using the One-Way ANOVA with the Tukey Post Hoc test of SPSS program (version 20.0). A p value less than 0.05 was considered statistically significant.

RESULTS

Effect of antisense oligonucleotide on the formation of *S. mutans* biofilm. Evaluation of the surfaces of glass slides with *S. mutans* biofilm applying optical profilometry revealed that the presence of 1% sucrose in TH broth significantly increased surface roughness parameters S_a and S_t in comparison to the clean glass surface therein used as a reference ($p < 0.05$) (Table). In this respect, it shows that 1% sucrose stimulated attachment of the bacteria to the glass surface and biofilm formation (Table, Fig. 1A and B). However, the test antisense oligonucleotide (PS-ODN1) with TurboFect™ (TF) reagent considerably prevented biofilm formation on the glass surface despite the presence of 1% sucrose resulting in *S. mutans* biofilm inhibition by 46% compared to the untreated bacteria ($p = 0.06$ for parameter S_a). Importantly, this effect was significant versus the bacteria exposed to missense oligonucleotide (PS-ODN2) in combination with TF reagent ($p < 0.05$ for parameters S_a and S_{dr}) as seen in the Table and Fig. 1C and D. In this respect, the PS-ODN1 in combination with TF reagent reduced the biofilm surface roughness

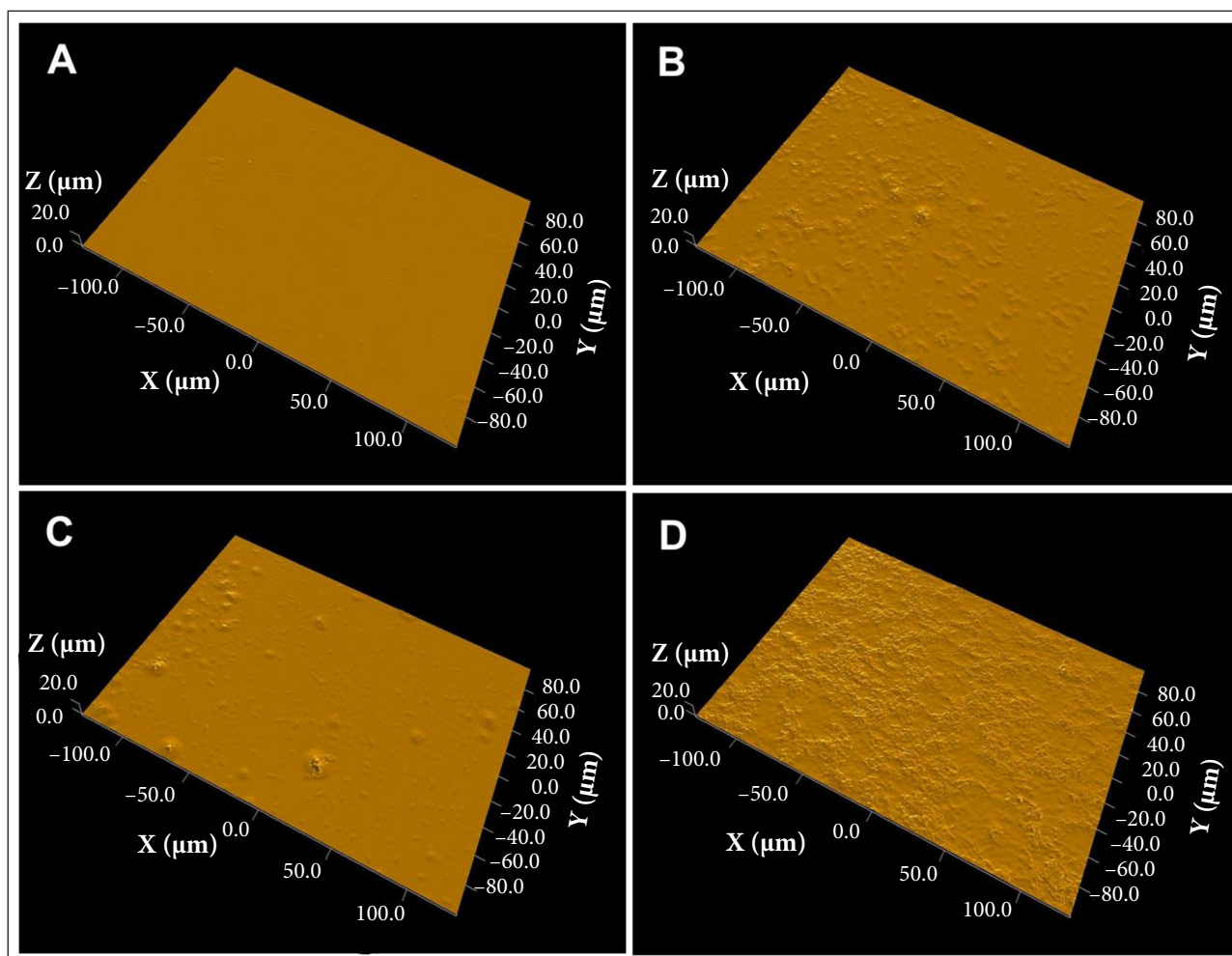


Fig. 1. Optical profile of the glass slides with *S. mutans* biofilm after 24 h of incubation under different treatments within Todd Hewitt broth. A is the clean glass slide surface (reference) without bacteria and treatment with PS-ODNs; B is the glass slide surface with bacteria incubated without treatment with PS-ODNs in the presence of 1% sucrose; C is the biofilm under treatment with PS-ODN1 + TurboFect™ in the presence of 1% sucrose; D is the biofilm under treatment with PS-ODN2 + TurboFect™ in the presence of 1% sucrose. Magnification, $\times 50$

parameters S_a and S_{dr} by 77% and 89%, respectively, in TH broth containing 1% sucrose. On the other hand, in our initial experiments, the test antisense oligonucleotide (PS-ODN1) without TF reagent did not decrease *S. mutans* biofilm formation in comparison with the PS-ODN1 conjugated to TF reagent because the biofilm surface roughness parameters were as follows: $S_a = 0.27 \pm 0.01 \mu\text{m}$; $S_t = 7.27 \pm 1.91 \mu\text{m}$; $S_{dr} = 10.09 \pm 0.9\%$. These parameters are close to the values produced by the treatment with PS-ODN2 in combination with TF reagent (Table). The transfection reagent TurboFect™ even combined with missense oligonucleotide (PS-ODN2) had no effect on *S. mutans* biofilm formation.

Antisense oligonucleotide treatment effect on the formation of bacterial aggregates in *S. mutans*

cultures. Gram staining of the samples taken after 24 hours of the total incubation time revealed that the addition of sucrose caused a substantial formation of *S. mutans* aggregates as compared to the bacteria growing without sucrose (Fig. 2A, B). It is an indication that 1% sucrose caused the formation of bacterial aggregates because of glucan production. However, treatment of *S. mutans* bacteria with the test antisense oligonucleotide (PS-ODN1) combined with TF reagent attenuated the bacterial aggregation and clustering in comparison with the untreated bacteria at the time point of 24 h (Fig. 2B, C). In contrast, the missense oligonucleotide (PS-ODN2) in combination with TF reagent did not exhibit such effect on the bacteria growing in TH broth with 1% sucrose (Fig. 2C, D).

Table. Surface roughness parameters of the glass slides with *S. mutans* biofilm after 24 h of incubation in the presence of 1% sucrose under different treatments

Treatments	S_a , μm	S_p , μm	S_{dr} , %
Clean glass slide (reference)	0.01 ± 0.00	0.92 ± 0.53	0.05 ± 0.02
Untreated bacteria	$0.13 \pm 0.04^*$	$8.44 \pm 4.03^*$	1.57 ± 0.95
PS-ODN1 + TurboFect™	$0.07 \pm 0.04^{**}$	6.94 ± 5.81	$0.99 \pm 1.37^{**}$
PS-ODN2 + TurboFect™	0.31 ± 0.02	7.99 ± 1.67	10.08 ± 1.13

Data are the means \pm SD of five measurements. * $p < 0.05$ for untreated bacteria compared to the clean glass slide (reference), ** $p < 0.05$ for PS-ODN1 + TurboFect™ compared to the PS-ODN2 + TurboFect™.

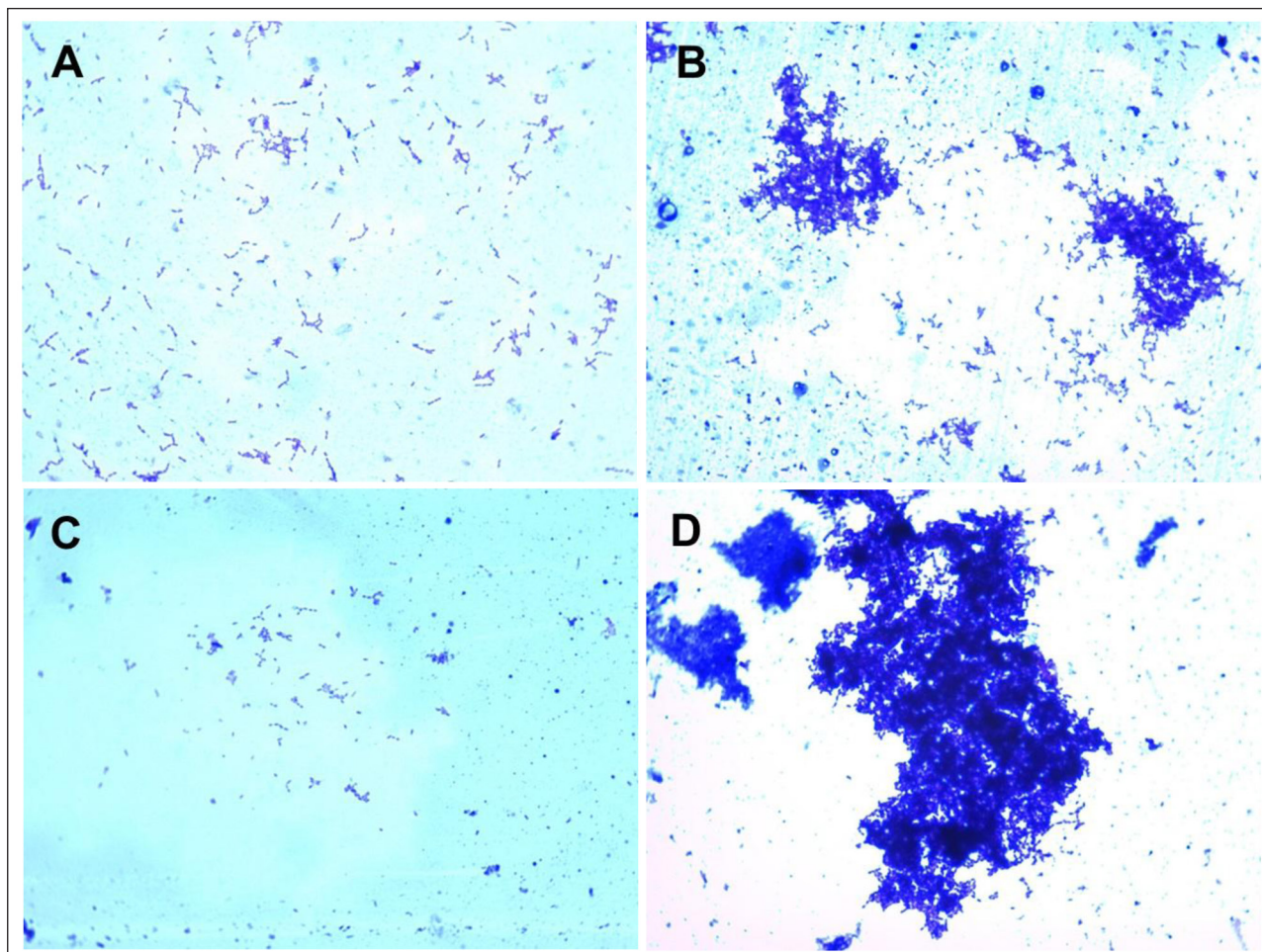


Fig. 2. Morphology of the Gram stained *S. mutans* bacteria after 24 h of incubation without or under the treatments with PS-ODNs in Todd Hewitt broth. A is bacteria growing in TH broth without 1% sucrose and treatments with PS-ODNs; B is bacteria growing in TH broth with 1% sucrose in the absence of PS-ODNs; C is bacteria under treatment with PS-ODN1 + TurboFect™ in TH broth with 1% sucrose; D is bacteria under treatment with PS-ODN2 + TurboFect™ in TH broth with 1% sucrose. Magnification, $\times 100$ (oil immersion)

DISCUSSION

This experimental work showed the ability of antisense phosphorothioate oligodeoxyribonucleotide targeting specifically *S. mutans* *gtfB* and

gtfC mRNAs to inhibit biofilm formation *in vitro* conditions. The most likely mechanism of action is that one antisense oligonucleotide of the same sequence can simultaneously suppress the expression of two target genes (glucosyltransferases B and

C) in *S. mutans* bacteria leading to reduction of the biofilm development on a solid surface (i. e. glass). In this respect, our investigation essentially differs from the study performed by Guo et al. (7), where the researchers used antisense oligonucleotides for inhibition of one target function only – *gtfB* mRNA in *S. mutans* bacteria. On the basis of results reported by Guo et al. (7), we selected 10 μ M as the final concentration of PS-ODNs for treatment of streptococci, and also applied the similar transfection reagent (TurboFect™) consisting of a cationic polymer in order to facilitate penetration of the PS-ODNs to the bacterial cells. The cationic polymer forms complexes with PS-ODNs via electrostatic interaction between negatively charged oligonucleotide molecules and positively charged reagent molecules (7, 15). Since such complexes maintain the positive charge, therefore they can bind to the negatively charged bacterial cell wall, and then can be taken up by the cells. Indeed, a significant reduction of the biofilm surface roughness parameters under exposure to the test antisense oligonucleotide (PS-ODN1) in combination with TurboFect™ reagent strongly supports this mechanism of oligonucleotide transfer to bacteria. Furthermore, since the treatment of *S. mutans* bacteria with antisense oligonucleotide (PS-ODN1) without TurboFect™ reagent produced approximately the same values for biofilm surface roughness parameters as the exposure to missense oligonucleotide (PS-ODN2) conjugated with TurboFect™ reagent, hence it indicates that the transfection reagent is necessary for penetration of the antisense oligonucleotide into bacterial cells in order to reach the target mRNAs. In general, the present research confirms the results of Dufour et al. (11) and McLeod et al. (12) that antisense phosphorothioate oligodeoxyribonucleotides can successfully penetrate *S. mutans* bacteria causing downregulation of the selected target genes – fructose-biphosphate aldolase and enoyl-CoA hydratase, thereby inhibiting the bacterial growth.

Additionally, the finding that missense oligonucleotide (PS-ODN2) in combination with TurboFect™ reagent did not affect the biofilm development highlights that the activity of test antisense oligonucleotide (PS-ODN1) was very specific, i. e. the selective inhibition of *S. mutans gtfB* and *gtfC* mRNA functions resulting in the decreased biofilm production. The latter fact suggests an inhibition of water-insoluble glucans synthesis because these

polymers are essential for the biofilm development, and no glucans lead to no biofilm formation (3, 16). It should be noted that, in the presence of sucrose, *S. mutans* adhesion to solid surfaces (e. g. glass, tooth enamel) is mainly dependent on the activity of glucosyltransferases, especially those synthesizing water-insoluble glucans (16–18). This was evidently demonstrated by Ooshima et al. (16) using *S. mutans* GtfB/C-deficient mutant strains – their ability to adhere to the glass surface was significantly reduced in spite of the presence of sucrose within the medium. Therefore, in the context of these studies, we can state that the test antisense oligonucleotide (PS-ODN1) exhibited a highly specific effect on the biofilm formation, probably by simultaneous inactivation of *S. mutans gtfB* and *gtfC* mRNAs.

Moreover, we also performed the experiment seeking to find out whether the PS-ODNs affect the bacterial aggregation. According to Gram staining results, in the medium with sucrose, the exposure of *S. mutans* to test antisense oligonucleotide (PS-ODN1) in combination with TurboFect™ reagent caused a substantial decrease in bacterial aggregation as compared to the untreated bacteria, whereas missense oligonucleotide (PS-ODN2) did not possess such effect. Since the aggregation or clustering of *S. mutans* bacteria are also dependent on the water-insoluble glucans produced from sucrose due to enzymatic activities of GtfB and GtfC as indicated in the review by Koo et al. (3), therefore the latter finding supports and corresponds to the main results outlined herein proving that the test antisense oligonucleotide (PS-ODN1) selectively inhibited *S. mutans gtfB* and *gtfC* mRNA functions.

CONCLUSIONS

On the basis of the obtained data, we conclude that the phosphorothioate oligodeoxyribonucleotide designed to antisense simultaneously *S. mutans gtfB* and *gtfC* mRNAs suppresses biofilm formation through the specific inactivation of the respective glucosyltransferase mRNAs function. We also conclude that this phosphorothioate oligodeoxyribonucleotide decreases the aggregation of *S. mutans* bacteria. These results are important for the designing and development of new pharmaceuticals with the aim to prevent dental caries via the inhibition of *S. mutans* biofilm formation.

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**STREPTOCOCCUS MUTANS BIOPLĖVĖS
SLOPINIMAS NAUDOJANT PRIEŠPRASMINĮ
OLIGONUKLEOTIDĄ PRIEŠ
GLIUKOZILTRANSFERAZES B IR C**

Santrauka

Įvadas. *Streptococcus mutans* bakterijų formuojama bioplėvė ant dantų lemia ėduonies raidą, kuri tebėra viena iš labiausiai paplitusių žmogaus ligų, glaudžiai susijusių su padidėjusiu maisto sacharozės vartojimu šiuolaikinėje visuomenėje. Bioplėvėje sacharozė yra metabolizuojama *S. mutans* iki rūgščių sukeltant dantų irimą. *S. mutans* taip pat gamina gliukoziltransferazes (Gtf), reikalingas lipnių glukano polimerų sintezei iš sacharozės sudarant matricą bioplėvei formuotis ant dantų. Siekiant sumažinti bioplėvės susidarymą, viena iš prevencijos priemonių galėtų būti Gtf sintezės slopinimas. Šio darbo tikslas – išbandyti priešprasminį fosforotioato oligodeoksiribonukleotidą (FT-ODN), veikiančią tuo pačiu metu *S. mutans gtfB* ir *gtfC* iRNR, siekiant nuslopinti bioplėvės formavimąsi *in vitro*.

Medžiagos ir metodai. *S. mutans* bakterijos buvo auginamos anaerobinėmis sąlygomis ant objektinių stik-

liukų, įdėtų vertikaliai į 24 šulinėlių plokštelę, užpildytą *Todd Hewitt* sultiniu su sacharozė, esant 10 μ M galutinės koncentracijos priešprasminiam arba išmaišytos sekos FT-ODN poveikiui. Kontrolei pasirinktos nepaveiktos bakterijos. Po 24 val. inkubacijos objektiniai stikliukai buvo ištraukti, išdžiovinti ore ir panaudoti vertinant kiekybinę streptokokų bioplėvę taikant optinės profilometrijos metodiką.

Rezultatai. Atskleista, kad priešprasminis FT-ODN labai sumažino svarbiausią bioplėvės paviršiaus šiurkštumo parametras S_a (vidurkio skirtumas tarp paviršiaus viršutinių ir apatinių taškų), slopindamas bioplėvės susidarymą 46 ir 77 %, palyginti atitinkamai su nepaveiktomis ($p = 0,06$) ir išmaišytos sekos FT-ODN paveiktomis bakterijomis ($p < 0,05$).

Išvados. Rezultatai rodo, kad priešprasminis FT-ODN labai sumažina streptokokų sukeltą bioplėvės susidarymą ant objektinių stikliukų ir galbūt galėtų smarkiai slopinti dantų bioplėvės formavimąsi vienu metu inaktyvinant *S. mutans gtfB* ir *gtfC* iRNR.

Raktažodžiai: *Streptococcus mutans*, bioplėvė, dantų ėduonis, gliukoziltransferazė, priešprasminis oligonukleotidas