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Original Article

Characteristics of an alternative antibacterial biomaterial for mouthwash in the absence of alcohol

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Abstract *Background/purpose:* The purpose of this study was to investigate whether poly-gamma-glutamic acid (γ-PGA), a naturally derived biomaterial, was suitable as an alternative antibacterial mouthwash in the absence of alcohol.
Materials and methods: Three bacterial strains, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, were used for testing the antibacterial activity of mouthwashes. In addition, cell viability, cytotoxicity, and genotoxicity experiments were conducted for testing the toxicity of mouthwashes.
Results: We demonstrated that 10000 ppm of γ-PGA without alcohol could efficiently inhibit 99% of bacterial growth. In addition, γ-PGA did not cause any cytotoxicity or genotoxicity.
Conclusion: 10000 ppm of γ-PGA in an alcohol-free mouthwash is an alternative biomaterial for mouthwashes.

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Introduction

Oral health is a foundation of a person's general good health and well-being. According to a World Health Organization fact sheet, 60—90% of school children and nearly 100% of adults have dental cavities worldwide. Hundreds of species of bacteria are present in the oral cavity, and some can cause various diseases such as dental caries, periodontal diseases, and even oral cancers.[1] The majority of people have a habit of brushing their teeth in ways that only cleans ~50% of a tooth's surface, thus a mouthwash may be used as an additional oral health care product. Table 1 summarizes the advantages and side effects of some materials that are used in commercially available mouthwashes. Although there is no perfect product, users can choose an appropriate mouthwash according to their needs.

It has been debated whether mouthwashes are helpful for oral care, because mouthwashes are made of chemicals that can cause side effects.[2—4] Regular brushing and flossing is still recommended. However, mouthwashes are still beneficial especially for patients who are unable to brush their teeth. Radiation causes damage to the salivary glands, thus patients who undergo head and neck radiation are at high risk for the development of dental caries.[5] Oral mucositis is another adverse event for some cancer patients who are treated with chemotherapy or radiotherapy.[6] Due to the pain these patients encounter in the oral area, mouthwashes are a quick and efficient method of reducing oral diseases and maintaining oral health.[7] However, most commercial mouthwashes usually contain alcohol to inhibit bacterial growth despite the irritation it causes to the users. Therefore, it is critical to use materials that can function as an antibacterial agent in the absence of alcohol.

Poly-gamma-glutamic acid (γ-PGA) is derived from *Bacillus anthracis*, and is known to be a biodegradable material. It is also difficult for proteases to catalyze γ-PGA, making it a potentially suitable antibacterial material.[8] In this study, we investigated whether γ-PGA could be applied as a mouthwash in the absence of alcohol. We optimized the most suitable concentration of γ-PGA for inhibiting bacterial growth without causing cytotoxicity or genotoxicity.

Materials and methods

Preparation of γ-PGA-containing prospective mouthwash

Different concentrations of γ-PGA (Vedan Enterprise Corporation, Taichung, Taiwan) were dissolved in distilled deionized water. Green peppermint essential oil (Lixin Ltd., Taichung, Taiwan) was dissolved in glycerol (PanReac, Barcelona, Spain) and added into the prospective mouthwash. A trace of Brilliant Blue FCF (food grade dye, PanReac) was added to distinguish the mouthwash from water.

The composition of commercial mouthwashes

Three commercial mouthwash products were used in this study. CHX mouthwash (Day and Night mouthwash, Day and Night company, Taipei, Taiwan) contains 0.1—0.2% (weight/volume) CHX, menthol, and 4.6% of alcohol. Sodium fluoride mouthwash (Oral-B mouthwash, P&G, Taipei, Taiwan) includes 0.022% of sodium fluoride. HOCl mouthwash (Water god antibacterial mouthwash, Want—Want Ltd., Taipei, Taiwan) mainly contains 10—30 ppm of HOCl.

Culture of bacteria

Standard strains were used in this study: *Escherichia coli* (ATCC 23815), *Staphylococcus aureus* (ATCC 10832), and *Pseudomonas aeruginosa* (ATCC 10145). The bacteria were added into a centrifuge tube containing 10 ml of lysogeny broth in the laminar flow hood, and cultured at 150 rpm at 37 °C overnight. The next day, 1 ml of cultured bacteria was transferred into 9 ml of fresh broth and incubated at 37 °C. The optical density (OD) value of cultured bacteria was

Table 1 The comparison of CHX, sodium fluoride, and HOCl in commercial mouthwashes.

Antibacterial agent	Chlorhexidine (CHX)	Sodium fluoride	Hypochlorous Acid (HOCl)
Antibacterial mechanism	Damaging the cytoplasmic membrane to increase the inner permeability and to result in the membrane damage or loss of structural organization.[22]	Releasing F^- to create an over-acidification of the cytoplasm resulting in the disruption of bacteria.[23]	Inhibiting DNA synthesis and causing partial inhibition of membrane protein synthesis.[24]
Advantages	Bacteria will not develop to resist CHX.[25]	Good routine home care oral hygiene in children due to anti-carcinogenic properties.[4]	Dissolves in water, and does not cause damage to the environment.[3]
Side effects or disadvantages	Staining on teeth and dental mucosa; alternates taste perception.[2]	Risk of ingestion resulting in toxicity.[4]	Easily degraded and required to be prepared freshly.[3]

measured at 600 nm every hour to establish the growth curve of each bacterial strain.

The measurement of antibacterial activity

Monitoring bacterial growth by measuring the turbidity of bacterial cultures is a quick method, and it has been applied for testing antibacterial activity of materials.[9,10] 9 ml of mouthwash were mixed with 1 ml of bacterial suspension (OD value at 600 nm was 0.1, 10^6 CFU/ml) and the experiments were performed in triplicates. The mixtures were incubated at 37 °C for 8 h, and OD values at 600 nm were observed. The antibacterial activity (%) was calculated by the following formula:

[OD value in phosphate buffered saline (PBS)] − (OD value in prospective mouthwash or commercial mouthwash)/OD value in PBS.

Cell viability and cytotoxicity testing

The 3T3 cells were plated in a 96-well culture plate with a density of 3×10^4 cells per well and cultured overnight. For cell viability, 9 ml of mouthwash was mixed with 1 ml of medium and 200 μl of the mixture was added into each well and cells were incubated at 37 °C overnight. 15 μl of WST-8 reagent (water-soluble tetrazolium salts-8, Sigma, St Louis, MO, USA) was then added, and incubated at 37 °C for 90 min in the dark. After incubation, the OD value was measured at 450 nm by the ELISA reader (Tecan Austria GmbH, Sunrise Remote-F0393000). The viability was obtained by calculating the OD value of the mouthwash grown culture divided by the OD value of the control. For cytotoxicity, 50 μl of freshly prepared LDH reagent (lactate dehydrogenase, Clontech, Mountain View, CA, USA) was mixed with cells, and the OD value was measured at 490 nm by the ELISA reader after shaking at 100 rpm for 20 min. The cytotoxicity (%) was obtained according to the following formula:

[(OD value in mouthwash)-(OD value in control)]/[(OD value in total lysis)-(OD value in control)].

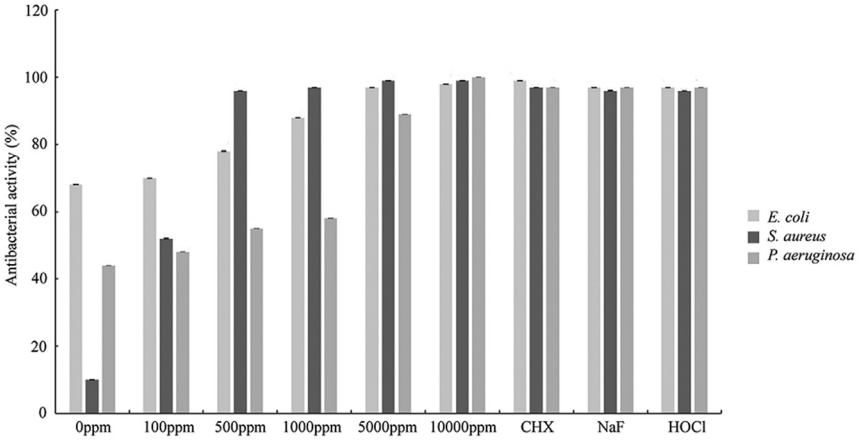
Genotoxicity test

5 ч 10^5 of Chinese hamster ovary cells were plated and incubated at 37 °C for 4 h 15 ml of the mixture (mouthwash: medium = 1:9) was then added, and cells were incubated at 37 °C for 24 h. At 20 h, 200 μl of colchicine solution (Sigma) was added. At 24 h, cells were collected and fixed with 3 ml fixation solution (methanol: acetic acid = 3:1) at 4 °C for 15 min. Cells were placed on a 75% ethanol pretreated glass, and dried at 55 °C in an oven. The glass was soaked in 5% Giemsa (Sigma) for 10 min, and the stained chromosomes were observed after rinsed and dried at 55 °C.

Results

In order to evaluate the antibacterial effect of γ-PGA, different concentrations of γ-PGA were investigated (Fig. 1A). We discovered that the antibacterial activity in the absence of γ-PGA was 67.56%, 9.52%, and 67.56% for *E. coli*, *S. aureus*, and *P. aeruginosa* (Fig. 1B), respectively, suggesting that green peppermint essential oil may play a role in inhibiting bacterial growth. However, the antibacterial activity increased dramatically when γ-PGA was added and there was a dose-dependent effect. These results indicated that γ-PGA could efficiently inhibit the

A



B

Mouthwash / Bacterial strain	0 ppm γ-PGA	100 ppm γ-PGA	500 ppm γ-PGA	1000 ppm γ-PGA	5000 ppm γ-PGA	10000 ppm γ-PGA	CHX	NaF	HOCl
E coli.	68 ± 0.016	70 ± 0.018	78 ± 0.004	88 ± 0.021	97 ± 0.003	98 ± 0.002	99 ± 0.001	97 ± 0.002	97 ± 0.003
S. aureus	10 ± 0.029	52 ± 0.026	96 ± 0.002	97 ± 0.006	99 ± 0.004	99 ± 0.003	97 ± 0.004	96 ± 0.005	96 ± 0.002
P. aeruginosa	44 ± 0.013	48 ± 0.030	55 ± 0.005	58 ± 0.007	89 ± 0.008	100 ± 0.002	97 ± 0.001	97 ± 0.002	97 ± 0.002

Figure 1 The inhibition of *E. coli*, *S. aureus*, and *P. aeruginosa* by different concentrations of γ-PGA compared with different commercial mouthwash products. (A) The x-axis shows the concentration of γ-PGA from 0 to 10000 ppm and mouthwashes containing chlorhexidine (CHX), sodium fluoride (NaF), and HOCl. The y-axis shows the antibacterial activity (%) of each concentration of γ-PGA or commercial mouthwash product. (B) The table lists the mean ± standard deviation for each testing condition.

growth of the three tested bacterial strains. To investigate whether the antibacterial activity of γ-PGA was as effective as the commercial mouthwashes, we compared 10000 ppm γ-PGA mouthwash with mouthwashes that contained CHX, sodium fluoride, or HOCl (Fig. 1). All products showed higher than 95% antibacterial activity regardless of the strain of bacteria, indicating that the antibacterial activity of 10000 ppm of γ-PGA was as effective as the common known antibacterial materials.

We next tested whether γ-PGA would affect cell survival or cause any toxicity in mouse embryonic fibroblasts (Fig. 2). The results showed that CHX mouthwash had the lowest viability (1.04%) and the viability of sodium fluoride mouthwash was 73.06% (Fig. 2A). Although CHX and sodium fluoride demonstrated efficient antibacterial activity, both materials did not promote cell viability. In contrast, the viability of HOCl— and γ-PGA-containing mouthwashes were higher than 98% indicating that both materials were sufficient for inhibiting bacterial growth and promoting cell viability. We then tested whether the high viability was due to low cytotoxicity. The results of the LDH assay demonstrated that indeed CHX caused high cytotoxicity (73.58%) while the cytotoxicity of sodium fluoride, HOCl, and γ-PGA was 0% (Fig. 2B). The results of the cell viability and cytotoxicity experiments indicated that γ-PGA did not cause any toxicity.

In addition, we investigated whether γ-PGA would cause any chromosomal abnormalities by observing the chromosomes in γ-PGA treated cells (Fig. 3). There were no abnormal chromosomes observed after cells were treated with 10000 ppm γ-PGA for 24 h (Fig. 3B) and there were no differences when compared to the negative control (Fig. 3A) indicating γ-PGA did not cause genotoxicity.

Discussion

We investigated whether γ-PGA could be an antibacterial agent in the absence of alcohol for mouthwashes, and we tested its ability to inhibit the growth of three different kinds of bacteria. In addition, we tested whether γ-PGA would cause any cytotoxicity or genotoxicity in vitro. Our results showed that γ-PGA could indeed be an alternative antibacterial biomaterial for mouthwashes in the absence of alcohol.

We demonstrated that bacterial growth could be inhibited in the absence of γ-PGA. It was not surprising green peppermint oil could inhibit bacterial growth, since some herbal extracts have been shown to reduce dental plaque levels.[11] However, the antibacterial activity was increased greatly in the presence of γ-PGA, especially when inhibiting the growth of *S. aureus*. Previous studies tested the antibacterial activity of γ-PGA when mixed with chitosan or magnetite nanoparticles, and the results showed that the mixtures could inhibit the growth of *E. coli*, *S. aureus*, or *P. aeruginosa* after culturing bacteria with the mixtures for 18—24 h.[12] In this study, we tested the antibacterial activity of γ-PGA after culturing with bacteria

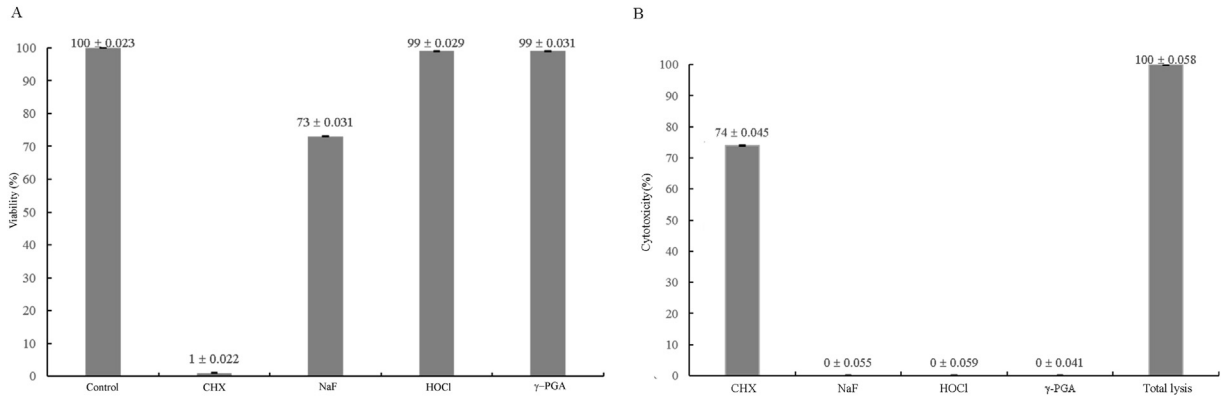


Figure 2 **γ-PGA shows high viability and low cytotoxicity.** Mouthwashes containing sodium fluoride (NaF), HOCl or 10000 ppm γ-PGA show good cell viability (A) and low cytotoxicity (B) as tested by WST-8 (A) and LDH (B) assays, respectively.

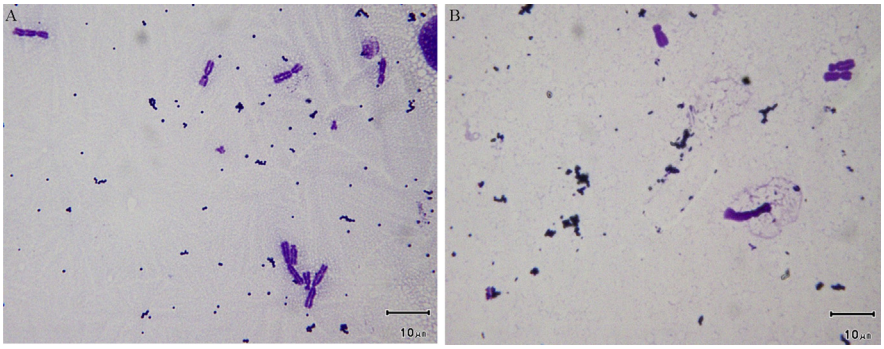


Figure 3 **No genotoxicity caused by γ-PGA.** Chinese hamster ovary cells were treated either with culture medium (A) or 10000 ppm γ-PGA (B), and all chromosomes observed were normal. The scale bar demonstrates 10 μm.

for 18 h and found that 10000 ppm of γ-PGA had the same antibacterial effect as commercial mouthwashes. Further investigation needs to be done in order to determine whether γ-PGA can inhibit bacterial growth under this concentration for a shorter period of time.

CHX has been shown to cause cytotoxicity in canine embryonic fibroblasts and non-cytotoxic concentrations allowed the survival of bacteria.[13] It has also been shown that CHX mouth rinses reduced the adhesion and proliferation of human gingival fibroblasts and keratinocytes.[14] Although the safety of CHX-containing mouthwashes has been proven for short-term use or usage at lower concentrations,[15,16] our results demonstrated that 10000 ppm of γ-PGA might be more suitable for mouthwashes due to its low cytotoxicity and genotoxicity.

When bacterial masses release metabolic products on the tooth surface, they are usually found on the margin of the gingival. The bacterial metabolic products then diffuse through the junctional epithelium and activate several host mechanisms including anti-inflammatory reactions.[17] Anti-inflammatory cytokines, including interleukins (ILs), prostaglandins, tumor necrosis factor alpha (TNF-α), and matrix metalloproteinases, are produced from the gingival epithelium and enter into the connective tissue. The cytokines are thought to form a gradient of chemoattractant signals that guide the leukocytes to the location of the bacterial plaque for the consequent repair processes.[18] It has been shown that γ-PGA can induce the production of IL-12p40 and IL-6 in mice when delivered as nanoparticles,[19] and an increase of IL-10 has been observed when γ-PGA nanoparticles were delivered to human allergen-derived dendritic cells.[20] Anti-inflammatory activities of γ-PGA have also been demonstrated when conjugated with L-phenylalanine ethylester in nanoparticles (γ-PGA-Phe-NPs), where the results showed that IL-1α, IL-1β, IL-6, and TNF-α were generated when γ-PGA-Phe-NPs were applied on rat middle ear mucosa.[21] The mechanisms of how the γ-PGA-containing mouthwash inhibits the growth of bacteria and whether it has anti-inflammatory activity are still unknown, thus further investigation should be focused on these topics. However, the efficacy and safety of γ-PGA in mouthwashes shown here should be supportive of the fact that it is an alternative biomaterial for oral care.

Our studies have shown that 10000 ppm of γ-PGA can efficiently inhibit the growth of *E. coli*, *S. aureus*, and *P. aeruginosa*. Unlike some commercial mouthwash reagents such as CHX, γ-PGA was characterized to promote high cell viability with low cytotoxicity while being non-genotoxic. Our results demonstrated that γ-PGA is an alternative antibacterial biomaterial that can be potentially applied in mouthwashes in the absence of alcohol.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2019.01.002.

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