Journal of Dental Research
2020, Vol. 99(2) 182–188
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DOI: 10.1177/0022034519894583
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Functionalized Graphene Oxide Shields Tooth Dentin from Decalcification

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Abstract

This in vitro study assessed the efficacy of functionalized graphene oxide (f-GO) nanocomposites on the decalcification of dentin, because dental caries of the root surface is becoming one of the new problems in aged society. Hydroxyapatite plates (HAP) and dentin slices were coated with f-GO nanocomposites by comparing them to silver diamine fluoride as a positive control, then treated with decalcification solutions such as ethylenediaminetetraacetic acid and citrate at 37°C for 24 h. Scanning electron microscopy (SEM) revealed significant protection of the surface morphology of HAP and dentin. On the other hand, a cariogenic *Streptococcus mutans* growth was inhibited by f-GO nanocomposites. In addition, cytotoxicity of them to epithelial cells was much less than that of povidone-iodine, which is commonly used for oral disinfectant. We synthesized 5 different f-GO nanocomposites such as GO–silver (Ag), GO-Ag–calcium fluoride (CaF_2), GO-CaF_2, GO-zinc, and GO–tricalcium phosphate ($Ca_3(PO_4)_2$). They were standardized by evaluating under SEM, transmission electron microscopy (TEM), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), thermogravimetry analysis (TGA), and Raman spectra after being synthesized in an aseptic technique. The abilities of GO-Ag, GO-Ag-CaF_2, and GO-CaF_2 nanocomposites were most preventive for decalcification. In addition, GO-Ag and GO-Ag-CaF_2 almost completely inhibited *S. mutans* growth. However, they did not exhibit cytotoxicity to epithelial cells except at the highest concentration (0.1 w/v%) of GO-Ag and GO-Ag-CaF_2. Furthermore, these f-GO nanocomposites exhibited less or no discoloration of dentin, although commonly used silver diamine fluoride causes discoloration of dentin to black. Thus, these f-GO nanocomposites are useful to protect dental caries on the tooth root that becomes a social problem in aged society.

Keywords: nanocomposites, hydroxyapatite, demineralization, sealing, antimicrobial, cytotoxicity

Introduction

Dentin is the main component of the human tooth, which consists of approximately 70% inorganic material and 30% organic material and water. Its inorganic part is calcium and phosphate ions that form hydroxyapatite (HAp) crystals (Goldberg et al. 2011). This HAp is easily dissolved by acids, resulting in tooth demineralization such as dental caries and dental erosion (Lussi 2009). This demineralization of dentin is a chronic irreversible loss of tooth tissue (Soares et al. 2012), thus causing one of the worldwide problems for humans, although the prevalence of it is decreasing (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators 2018). Dental caries is associated with demineralization of the tooth structure, so-called softening of dentin, by organic acids produced by microorganisms, but it can be prevented or arrested. Oral hygiene maintenance, fluoride application, pit and fissure sealants, xylitol substitutes, and vaccine (Smith 2012) have been described for caries prevention. Dental erosion is the irreversible loss of the tooth exterior due to acids from foods, environment, and even from the host body but not from microbiological sources (Kreulen et al. 2010). Dental erosion does not cause tooth softening, but both dental caries and erosion cause dentin hypersensitivity and pain (Addy 2005). The aging population suffers more gingival recession and root exposure, which causes dental caries and erosion on the exposed root surface (Carey and Brown 2017).

The treatment of this problem is to protect dentin from demineralization to prevent external stimulation to pulps via dentinal tubules. There have been several agents for this treatment such as fluoride and oxalate-based compounds (Zhong et al. 2015); however, these treatments are impermanent and cause discoloration of dentin. For example, Saforide (silver diamine fluoride) can help remineralization of dentin by long-term effects of intratubular deposition of mineral crystals (Markowitz and Pashley 2008); however, the formed calcium fluoride crystals are small and unstable and consequently

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A supplemental appendix to this article is available online.

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dissolve when exposed to acidic saliva, foods, and beverages (Ganss et al. 2001). In addition, the prime drawback is the discoloration of dentin. Therefore, the development of new agents is strongly needed in dental practice; thus, we were looking for antibacterial chemicals that adhere to the dentin surface to facilitate remineralization.

Among generic elements, we have focused on carbon. Recently, significant efforts have been focused on 2-dimensional carbons due to their excellent chemical, physical, and electronic properties (Castro Neto et al. 2009). Graphene oxide (GO) is a graphene analogue mainly composed of sp^3-bonded carbon atoms, possessing extraordinary physical and chemical properties (Novoselov et al. 2004). Notably, dental application is one of the hot topics in GO application; within a few years, a lot of interesting works have been carried out such as drug/gene delivery, antibacterial agents, a biocompatible scaffold, physiochemical properties, and improvement of dental biomaterials (Xie et al. 2017). These applications are derived from GO properties, such as high specific surface area (>1,000 m^2/g), mechanical strength (Young's modulus of ~1,100 GPa), biocompatibility, low cost, and facile biological/chemical functionalization. Besides, GO has a strong affinity with metal species (Kulshrestha et al. 2014; Peng et al. 2017; Zhang et al. 2016). These outstanding properties motivated us to use GO as the treatment agent with a prolonged performance for dental issues. In this study, we aimed to synthesize functionalized GO (f-GO) nanocomposites for the materials to prevent demineralization of tooth dentin by covering the dentin surface, especially orifices of dentinal tubules.

The effects of f-GO nanocomposites should be evaluated from the viewpoints of antidemineralization of dentin such as the sealing orifice of the dentinal tubule and formation of a protective layer on dentin, as well as dentin discoloration and bactericidal and cytotoxic effects. Hence, the surface conditions of HAp and tooth dentin covered by f-GO nanocomposites after soaking in demineralizing solutions were analyzed. In addition, the effects on the cariogenic oral bacteria and epithelial cells were also analyzed.

Materials and Methods

Synthesis of f-GO Nanocomposites and Their Characterization

We synthesized and characterized GO and 5 different f-GO-nanocomposites by scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), thermogravimetry analysis (TGA), and Raman spectra (Appendix Sections 1–3).

Effects of the Nanocomposites on the Prevention of Demineralization

The conditions of HAp plates and the orifice of the dentinal tubule in the dentin slice (Appendix Section 4) were observed under SEM before and after being coated with f-GO nanocomposites. Saforide (silver diamine fluoride; Bee Brand Medico Dental), a reagent commonly used to prevent demineralization of tooth dentin and dental caries, was used as a positive control. These coated samples were incubated at 37°C in ethylenediaminetetraacetic acid (EDTA; 0.5 mM, pH 7.0) and citrate buffer (0.5 mM, pH 6.0) for 24 h and 72 h (Appendix Section 5).

Biological Effects of the Nanocomposites

Colony-forming units (CFUs/mL) of *Streptococcus mutans* were counted on an agar plate inoculated for 48 h after treatment with f-GO nanocomposites at various concentrations for 6 h (Appendix Section 6). Viability of human epithelial HeLa cells cultured with f-GO nanocomposites at the different concentrations for 24 h was determined by the MTS assay (Appendix Section 7). Povidone iodine (0.1%) was used as a positive control, and medium and phosphate-buffered saline (pH 7.0) were used as negative controls. We performed 3 individual experiments, each of which was carried out in triplicate.

Statistical Analysis

Statistical analyses were performed for normality and lognormality tests with the Anderson-Darling test, D'Agostino and Pearson test, Shapiro-Wilk test, and Kolmogorov-Smirnov test. In addition, Dunn's multiple comparisons test was also performed.

Results

Effects on Prevention of Demineralization of the HAp Plate

HAp plates (10 × 10 mm) were coated with GO and GO nanocomposites and soaked in EDTA buffer (0.5 mM, pH 7.0) for 24 and 72 h at 37°C. We observed the surfaces of all samples under SEM (Fig. 1). The surface of the intact HAp plate, which was not treated with anything, looked smooth with some pores (approximately 2 µm in diameter; Fig. 1a). Once the HAp plate was soaked in EDTA buffer, its surface turned rough, and the pores became remarkable (approximately 5 µm in diameter; Fig. 1b). When the HAp plate was coated with Saforide, its surface looked smooth but wavy, as covered with aggregated crystals (Fig. 1c). The pores were less prominent than those seen in the noncoated HAp plate. Even though the HAp plate was coated with GO alone (Fig. 1d), the surface looked very similar to the one without any coating. EDTA treatment might have removed residues from the pores.

In contrast, the surfaces of HAp plates coated with GO–silver (Ag), GO-Ag–calcium fluoride (CaF_2), GO-CaF_2, GO–zinc (Zn), and GO–tricalcium phosphate ($Ca_3(PO_4)_2$) (Fig. 1e–h) seemed smooth and with several clearly visible pores like the intact HAp plate but less than the ones seen in both noncoated and GO-coated plates. The surface views coated with Saforide and GO-Zn looked like they were covered with thick layers. Notably, the HAp plate coated with GO-Ag-CaF_2 seemed as smooth as the nontreated one, whereas the HAp plate covered with GO-$Ca_3(PO_4)_2$ looked as smooth as the one without any coating, but the pores seemed wider and deeper.

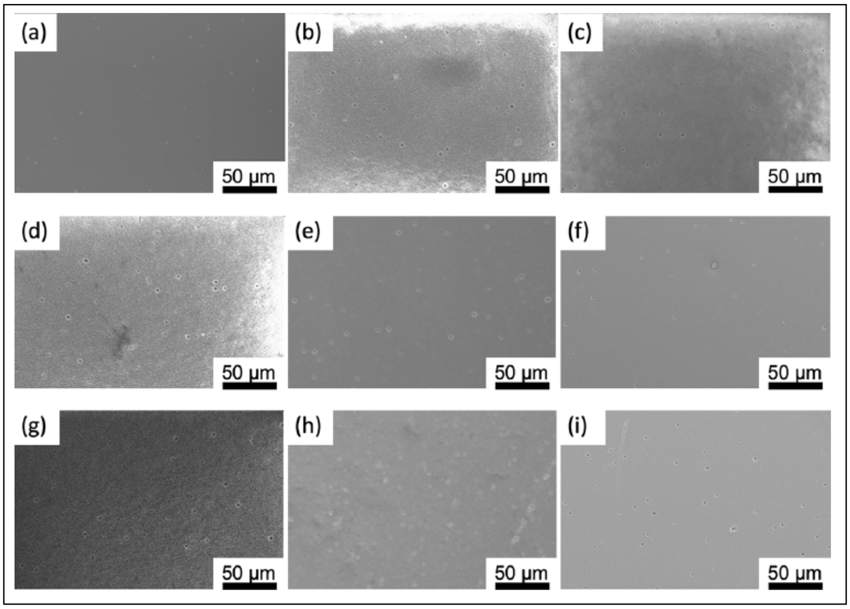

Figure 1. Scanning electron microscopy views of coated hydroxyapatite (HAp) plates after being soaked in ethylenediaminetetraacetic acid (EDTA) buffer. (**a**) Intact HAp plate (unexposed to EDTA), (**b**) none, (**c**) Saforide, (**d**) graphene oxide (GO), (**e**) GO–silver (Ag), (**f**) GO-Ag–calcium fluoride (CaF_2), (**g**) GO-CaF_2, (**h**) GO–zinc (Zn), and (**i**) GO–tricalcium phosphate ($Ca_3(PO_4)_2$). Magnification was ×500. Scale bar: 50 µm.

The HAp plates were protected by the Saforide, GO-Ag, GO-Ag-CaF_2, GO-CaF_2, and GO-Zn layer on the surface. The same tendency was observed in the 72 h of EDTA treatment.

Effects on Prevention of Demineralization of Tooth Dentin

The same treatment and observation performed for the HAp plate were applied on the 2-mm-thick dentin slices (Appendix Section 4.2) for 24 h at 37°C (Fig. 2A). We observed that the surfaces of the dentin slice, which were not treated with anything, had copious numbers of open dentinal tubules (approximately 2 µm in diameter; Fig. 2A-a) by SEM. Once the dentin slice was soaked in EDTA buffer, many tubule openings were visible in the noncoated dentin (Fig. 2A-b). Its intertubule spaces and tubules became irregular and wider (approximately 5 µm in diameter; Fig. 2A-b). We found many open dentinal tubules in the dentin slice coated with Saforide (Fig. 2A-c), although Saforide has been used as a protective barrier in clinical dental practice. In contrast, GO-coated dentin seemed to be covered by a thin layer, resulting in dentinal tubules that seemed to be partially sealed (Fig. 2A-d). On the other hand, the dentin slices coated with GO-Ag, GO-Ag-CaF_2, GO-CaF_2, GO-Zn, and GO-$Ca_3(PO_4)_2$ seemed to be covered entirely, resulting in no traces of prominent dentinal tubules (Fig. 2A-e–i). These results indicate that all f-GO nanocomposites can make up the layers covering the entire surface of dentin and orifices of the dentinal tubules.

In addition to EDTA buffer, citrate buffer (0.5 mM, pH 6.0) was used to assess the effects of f-GO nanocomposites in an acidic condition to resemble the condition for dental caries and erosion (Fig. 2B). We used the same experimental designs as mentioned above. The results were comparable to the ones with EDTA (Fig. 2A) except for the GO-Zn-coated dentin slice (Fig. 2B-h). After soaking in citrate buffer, the surfaces coated with GO-Ag, GO-Ag-CaF_2, GO-CaF_2, and GO-$Ca_3(PO_4)_2$ kept the views completely covered without traces of prominent dentinal tubules as seen in the results after soaking in EDTA buffer (Fig. 2A-d–g, i). However, the dentin coated with GO-Zn showed many open dentinal tubules and intertubule spaces. In addition, the surface of dentin covered with Saforide seemed rough. This may be the result of the aggregation of silver diamine fluoride coated on the dentin surface after citrate exposure to acidic conditions.

Since the results demonstrated that f-GO nanocomposites succeeded in sealing the orifices of dentinal tubules, Raman spectra analysis was performed to confirm that the protective layer was formed by GO. GO-characteristic spectra (2 peaks around 1,500 cm^{-1} Raman shift) were found in each specimen (Appendix Section 9), suggesting that the GO layer was formed on the dentin surface of dentin and the orifice of the dentinal tubules. The elemental existence on the dentin surface was satisfactory, as confirmed by SEM/energy dispersive spectroscopy (EDS) and XPS analysis (Appendix Sections 10, 11).

Biological Activities of Cariogenic Bacteria and Epithelial Cells

We examined the antimicrobial activity of f-GO nanocomposites against cariogenic bacteria *S. mutans* in liquid media. We observed that the CFUs in control (media alone) were approximately 470 CFUs/mL (Fig. 3A). This was reduced to about 45 CFUs/mL (90%) by positive control (0.1% povidone iodine solution). Unexpectedly, GO alone also showed antimicrobial activity, reducing to 65% to 80% (among 0.01–0.1 w/v% of GO). f-GO nanocomposites such as GO-Zn, GO-CaF_2, and GO-$Ca_3(PO_4)_2$ showed less antimicrobial activity than that of GO alone: 45% to 60% by GO-Zn, around 60% by GO-CaF_2, and 5% to 15% by GO-$Ca_3(PO_4)_2$. However, once GO conjugated with either Ag or Ag-CaF_2, the antimicrobial activity reduced to more than 90%. In particular, Ag-CaF_2 showed the most substantial reduction of more than 98%. These reductions were statistically significant (Fig. 3A). The tested f-GO nanocomposites can be categorized into 2 groups; one had a mild to moderate reduction of *S. mutans* growth, and another had a high reduction. These large reductions of the growth of *S. mutans* may be beneficial for saving the tooth from acidic conditions. However, no statistical difference was observed.

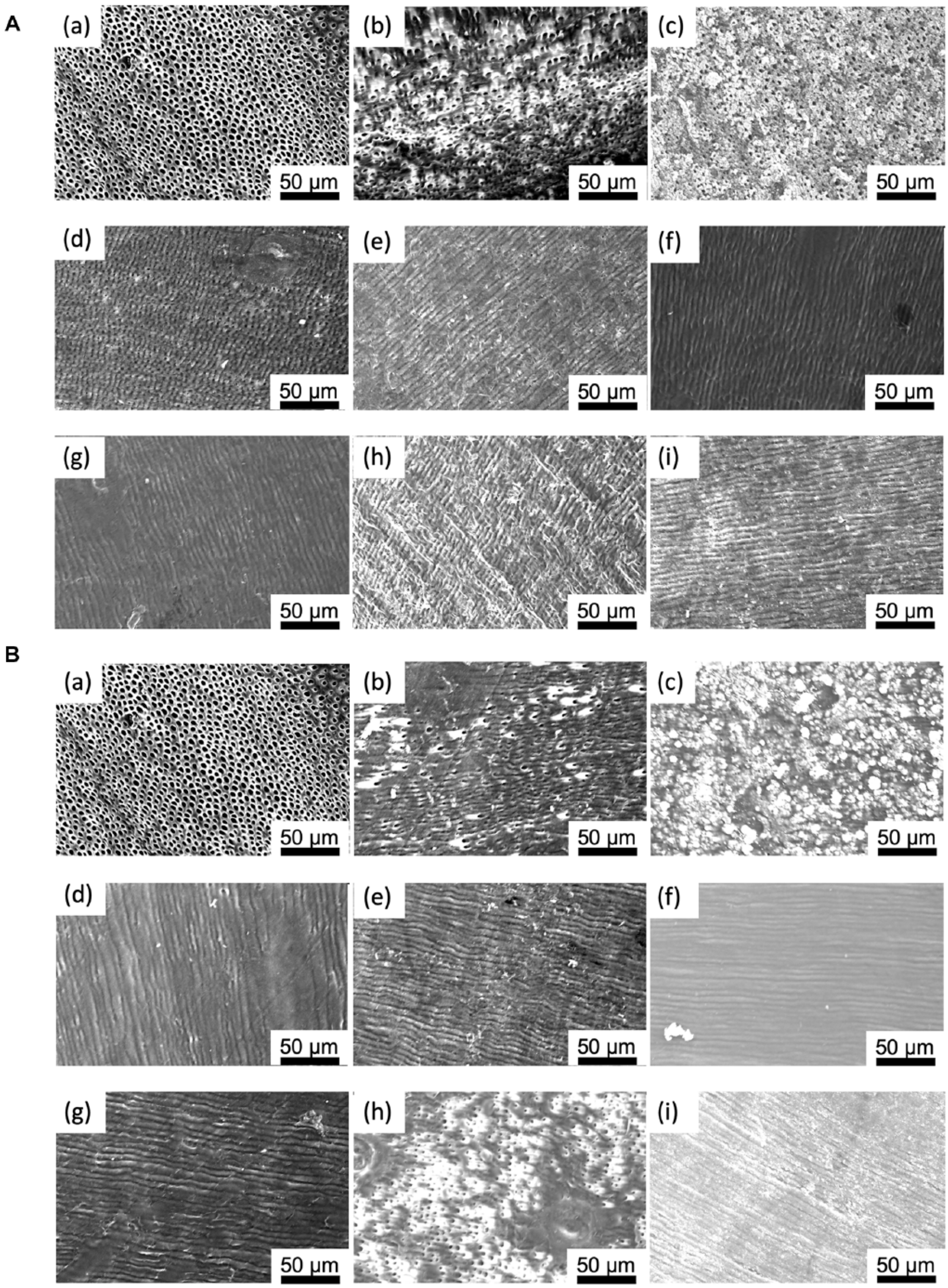

Figure 2. Scanning electron microscopy views of dentin slices after being soaked in ethylenediaminetetraacetic acid (EDTA) buffer (**A**) and citrate buffer (**B**). (a) Intact dentin slice (unexposed to EDTA), (b) none, (c) Saforide, (d) graphene oxide (GO), (e) GO–silver (Ag), (f) GO-Ag–calcium fluoride (CaF_2), (g) GO-CaF_2, (h) GO–zinc (Zn), and (i) GO–tricalcium phosphate ($Ca_3(PO_4)_2$). Magnification was ×500. Scale bar: 50 µm.

To examine the effect of the f-GO nanocomposites on epithelial cells, a human epithelial cell line, including HeLa cells derived from human cervical cancer, was used. Cells were cultured on a tissue plate with f-GO nanocomposites dispersed in liquid media for 24 h and 48 h. The proliferation rate of the cells was measured by using the MTS assay (Fig. 3B). GO

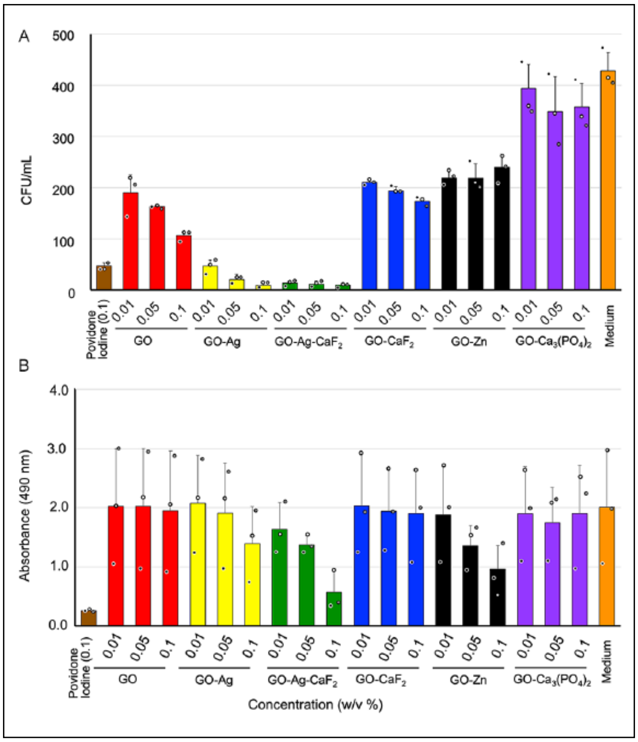

Figure 3. Biological effects on bacteria and epithelial cells. Graph shows mean and standard deviation of 3 individual experiments, each of which was carried out in triplicate. Each dot represents an individual experiment. (**A**) Growth of *Streptococcus mutans*. Bacteria were cultured in the medium with a specific functionalized graphene oxide (f-GO) nanocomposite, then inoculated on an agar plate. After incubation, colony numbers were counted, and colony-forming units were calculated. (**B**) Proliferation of human epithelial cells. Cells were cultured in the medium with specific f-GO nanocomposites, and then MTS reagent was added. After incubation for 24 and 48 h, absorbance at 490 nm was measured.

exhibited cell proliferation in a dose-dependent manner but no effects at 0.01 w/v%. This tendency was found with GO-Ag, GO-Zn, and GO-$Ca_3(PO4)_2$ composites, but the inhibition of GO-Ag at 0.1 w/v% was stronger than that of GO alone. However, GO-CaF_2 did not exhibit any inhibition up to 0.1 w/v%. In contrast, GO-Ag-CaF_2 also did not exhibit inhibition at 0.01 and 0.05% w/v%, but it showed the inhibition to 33% of the control with media alone at 0.1% w/v%, which was the most active inhibition among f-GO nanocomposites tested in this study. This inhibition was like the one with povidone iodine (24% proliferation at 0.1 w/v% concentration). However, no statistical difference was observed.

Discussion

We conjugated GO with different nanoparticles to evaluate their effects on bactericidal activity and antidemineralization activity to use in dental applications. We used silver (Ag), zinc (Zn), calcium fluoride (CaF_2), and tricalcium phosphate ($Ca_3(PO_4)_2$). These 4 nanoparticles were used in direct conjugation with GO. As the fifth one, we conjugated GO with both Ag and CaF_2 together to evaluate the combined effect. Ag nanoparticles are characterized by high bactericidal activity (Bhardwaj et al. 2009) and thus have been used with success in numerous medical and dental fields (Cheng et al. 2012; Samiei et al. 2013). Zn nanoparticles also possess broad-spectrum bactericidal activity (Finney et al. 2003) against several bacteria, including *S. mutans* (Tsai et al. 2013). In addition, it inhibits collagen degradation in acid-etched dentin (Oh et al. 2018). Zn has been used for dental cement, in restorative materials, and in toothpaste and mouthwash (de Souza et al. 2000). Fluoride has been applied in dentistry to prevent tooth decay by strengthening the HAp structure (Yamazaki et al. 2007). The CaF_2 powder is highly soluble and a good source of fluoride ion (Matthias and Christian 2013). Amorphous $Ca_3(PO_4)_2$ has been used as a remineralization agent for enamel and dentin lesions (Bailey et al. 2009) and scaffolds for bone and tissue engineering (Schwarz et al. 2006). The nanoparticles described above can act as active ingredients to inhibit demineralization of dentin and cariogenic bacteria. GO is expected to act as carriers, adhesives, and stabilizers.

This is the first study to test the series of f-GO nanocomposites to cover the dentin surface and to seal the orifice of the dentinal tubule under demineralization conditions (summarized in the Table). The results obtained from this study of substantial tubule sealing by f-GO nanocomposites are encouraging. Although it is unclear if these nanocomposites could seal dentin permeability completely, they may effectively seal the orifices of dentinal tubules and significantly reduce dentin demineralization and might reduce its permeability. It is well known that sealing the orifices of dentinal tubules is one of the essential factors for reducing dentin demineralization for the treatment of dentinal hypersensitivity on the surface of the tooth root. In regular dental practice, the topical application of dentin adhesives on the root surface is widely performed (Fu et al. 2007). In this study, we found that the protective layers with f-GO nanocomposites were stronger against demineralization conditions than that of Saforide. In addition, there was less color change of the dentin surface with f-GO nanocomposites; only GO-Ag and GO-Ag-CaF_2 provided a slight yellowish coloration. These findings strongly suggest that these f-GO nanocomposites are superior for protecting the tooth dentin exposed to the oral environment compared with Saforide.

Several acidogenic streptococci and lactobacilli are causing the demineralization of the tooth surface, initiating dental caries (Marsh et al. 2011). Thus, inhibiting the initial stage of biofilm formation can be a strategic target for protecting demineralization and erosion of the tooth. GO composites (i.e., composted with metals such as zinc, silver, and titanium) have been investigated as antimicrobial agents (Kulshrestha et al. 2014; Kim et al. 2017; Peng et al. 2017). It is better for antidemineralizing coating reagents to have antimicrobial activity because oral bacteria produce acids, causing dental caries and erosion. GO can accelerate the antimicrobial activity of the original reagent once GO makes a composite with it (Kulshrestha et al. 2014; Kim et al. 2017; Peng et al. 2017). In our study, GO-Ag, GO-Ag-CaF_2, and GO-CaF_2 exhibited

Table. Summary of Our Findings.

Nanocomposite	GO Fraction (w/v), %	GO Layer over the Dentin (analyzed by Raman Spectra)	Orifice Closing (observed by SEM)	Color Change (Naked Eye Visibility)	Antibacterial Activity (CFU Reduction[a]), %	Cytotoxicity (MTS Reduction)
GO alone	1	Yes	Almost complete	No visible color change	65–80	No
GO-Ag	1	Yes	Almost complete	Slight yellowish	90	No
GO-Ag-CaF_2	1	Yes	Complete	Slight yellowish	98	35% (at high concentration) No (at low concentration)
GO-CaF_2	1	Yes	Almost complete	No visible color change	60	No
GO-Zn	1	Yes	Partial	No visible color change	45–60	No
GO-$Ca_3(PO_4)_2$	1	Yes	Almost complete	No visible color change	5–15	No

CFU, colony-forming unit; GO, graphene oxide; SEM, scanning electron microscopy.
[a]Results of measurement but not statistically analyzed.

reduced CFUs, and they did not show any cytotoxicity to the epithelial cells, except at high concentrations of GO-Ag and GO-Ag-CaF_2. There are reports regarding the cytotoxicity by Saforide, and the activities of it were stronger than those of f-GO nanocomposites (Marsh et al. 2011). These reports used 6 different human oral cells (3 kinds of squamous cell cancer cell lines, pulpal cells, gingival fibroblasts, and periodontal ligament cells) and found severe toxicity in them (most of the cells were dead at 0.039 mM) (García-Contreras et al. 2013). Another study reported the inhibition of bacterial growth (Scarpelli et al. 2017). In summary, the substantial toxicity of Saforide is an issue in clinical dental practice, but f-GO nanocomposites are expected to solve the problem.

The results mentioned above, we assume f-GO composite's capability in creating a proactive layer on HAp plate and dentin, though GO and GO-$Ca_3(PO_4)_2$ on HAp plate in EDTA buffer and GO-Zn on dentin in citrate buffer failed to show such efficiency. We know that the surface deposition of materials affects changing the orifices of dentinal tubules. An amount of fluid in the dentinal tubules is proportional to the fourth power of the diameter of dentinal tubules, and the surface characteristics of dentin determine 86% of the tubular fluid flow resistance. A slight reduction of the dentinal tubule diameter can momentously reduce the liquid flow in the dentinal tubules, which later alleviates dentinal sensitivity (Yuan et al. 2012). As we observed that these f-GO nanocomposites sealed the tubules and created the protective layer on the dentin surface, we assume that it will help to avoid the dentin surface in the exposed oral condition. Fluid dynamics in dentinal tubules induce the nerve activity in dentinal tubules, which causes dentin hypersensitivity (West et al. 1998). Desensitizer has been used to seal dentinal tubules mechanically (Schüpbach et al. 1997) or physiologically decrease the excitability of the nerve in dentinal tubules (Markowitz and Kim 1992).

Although complete relief from demineralization, erosion, and hypersensitivity are nearly impossible in clinical cases. Early studies have shown the effectiveness of fluoride, bioactive glass, and HAp-containing dentifrices in sealing dentinal tubules and reducing sensitivity (Farooq et al. 2015). A wide-range literature search did not reveal any similar studies that could be compared to the results of this research in terms of tubule-sealing competence of f-GO nanocomposites. The multiwalled carbon nanotube/graphene oxide hybrid carbon-based material combined with nanohydroxyapatite was found to be a protective coating for dentin erosion (Nahorny et al. 2017). In this study, all nanocomposites showed considerable tubule sealing after 24 h, even with vigorous brushing. This could be a direction of the remineralization and sealing potential of these f-GO nanocomposites. This shows excellent retentive properties of f-GO nanocomposites. Analyzing the extent of the penetration of particles inside dentinal tubules was not part of the present study, but it could form a solid basis for future work in this area. The effects of these f-GO nanocomposites are more than the impact of Saforide, which is commonly used for the same purposes. In addition, there is no visible color change on the dentin with f-GO nanocomposites apart from slight yellowish coloration with GO-Ag and GO-Ag-CaF_2 but not black like Saforide. In the future, quantitative and clinical studies analyzing the effect of these nanocomposites in preventing demineralization-related problems such as tooth decay and dentin hypersensitivity over a period are still required.

As a limitation of this in vitro study, we have concerns about the condition of dentinal tubules and the dynamic changes of fluid movement in them whether dental pulp is vital or not. Thus, future studies are needed to better elucidate these early findings. These studies may help us to understand the mechanism of how the GO nanocomposites work and the possibility of a clinical application of them.

Conclusion

f-GO nanocomposites such as GO-Ag, GO-Ag-CaF_2, and GO-CaF_2 were preventive for demineralization without discoloring. Among them, GO-Ag and GO-Ag-CaF_2 inhibited *S. mutans* growth and were not toxic to epithelial cells except at a high concentration (0.1 w/v%). Therefore, we may conclude that these 3 f-GO nanocomposites may be suitable for the prevention of tooth decalcification caused by cariogenic bacteria. Further in vivo animal studies are needed to test safety and effectiveness.

Author Contributions

M.Z.I. Nizami, contributed to conception, design, data acquisition, analysis, and interpretation, drafted the manuscript; Y. Nishina, contributed to design, data analysis, and interpretation, drafted and critically revised the manuscript; T. Yamamoto, contributed to conception and data interpretation, critically revised the manuscript; Y. Shinoda-Ito, contributed to conception, design, data acquisition, and interpretation, critically revised the manuscript;

S. Takashiba, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

The authors thank Ms. Tomoko Ohkubo for performing XPS analysis, Mr. Yusuke Tomita for assisting in XRD and SEM EDS analysis, and Dr. Shin Nakamura for assisting in statistical analysis. The authors received no financial support and declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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