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Original Article

Effects of adding functionalized graphene oxide nanosheets on physical, mechanical, and anti-biofilm properties of acrylic resin: In vitro- experimental study

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ABSTRACT

Background: Polymethyl methacrylate resin is widely used in orthodontic treatments. Graphene oxide (GO) has reactive functional groups on its surface that facilitate binding to various materials such as polymers, biomolecules, DNA, and proteins. This study aimed to investigate the impact of adding functionalized GO nanosheets on the physical, mechanical, cytotoxicity, and anti-biofilm properties of acrylic resin.

Materials and Methods: In this experimental study, fifty samples (for each test) were divided into groups of 10, in the form of acrylic resin discs with concentrations of 0, 0.25, 0.5, 1, and 2 weight percentage (wt%) of functionalized GO nanosheets and also the control group. Samples were evaluated in terms of physical properties (surface hardness, surface roughness, compressive strength, fracture toughness, and flexural strength), anti-biofilm properties (On four groups of micro-organisms, including *Streptococcus mutans*, *Streptococcus sanguis*, *Staphylococcus aureus,* and *Candida albicans*), and cytotoxicity. Data were analyzed using SPSS software version 22, descriptive statistics, one-way analysis of variance test, and Tukey *post hoc* test. The significance level was considered $P < 0.05$.

Results: No significant difference was observed between the different groups with weight percentages of 0.25, 0.5, 1, and 2% nano GO (nGO) and the control group (without nGO) in terms of surface roughness and toughness. However, compressive strength, three-point flexural strength, and surface hardness showed significant differences between the groups. Furthermore, the degree of cytotoxicity increased by increasing the weight percentage of nano-GO.

Conclusion: The addition of functionalized nGO in appropriate concentrations to polymethyl methacrylate can improve the anti-bacterial and anti-fungal biofilm properties without changing or increasing their physical and mechanical properties.

Key Words: Bacterial adhesion, graphene oxide, mechanical tests, polymethyl methacrylate, toxicity

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INTRODUCTION

The importance of cold-cure acrylic resin in dentistry is quite apparent.[1] These resins are often composed of methacrylates (especially polymethyl methacrylate [PMMA]) and PMMAs and added co-polymers.[2]

PMMA is a transparent synthetic polymer,[3] usually available as a powder and liquid system.[4] PMMA has been used in dentistry since 1937.[5] It also has several applications in dental laboratories (for the fabrication of orthodontic retainers, functional devices, dentures, and repair), dental clinics (for relieving dentures and making temporary crowns), and industry (such as making artificial teeth).[6,7]

PMMA is frequently used due to its sufficient compressive strength, low elastic modulus, low price, acceptable beauty, good color stability for several weeks, easy manipulation, repair, and biocompatibility of this material.[8-10]

Despite the many benefits and applications of PMMA, the use of this substance in dentistry is not without its drawbacks, including poor mechanical properties,[11] low abrasion and tear resistance,[12] volumetric shrinkage after polymerization,[13] low fatigue resistance,[14] porosity, water absorption, and solubility.[15] Another significant disadvantage is the deposition and formation of biofilms on the surface of PMMA resins,[16] which act as a reservoir for micro-organisms and contribute to oral diseases and tissue damage.[15] Therefore, different techniques have been proposed to improve the properties of PMMA,[3] and several studies have reported the improvement of PMMA properties using different types of fibers,[17] nanoparticles,[18] and nanotubes.[19]

PMMA resin is used in routine orthodontic treatments to make fixed and removable appliances.[20] The long-term presence of these orthodontic appliances with porous surfaces in the oral environment may cause the formation of biofilms and eventually lead to tooth decay, gingivitis, and periodontitis.[21,22] Using acrylic appliances can also affect the biofilm's metabolic activity and pathogenicity.[23] Therefore, recently, different types of particles of different sizes have been added to the acrylic resin to induce antimicrobial properties. Numerous nanoparticles, such as silver,[24] platinum,[25] silicon dioxide, and titanium dioxide,[26] have been successfully added to acrylic resins and have shown antimicrobial properties. Studies have also reported antimicrobial effects of carbon-based nanomaterials such as graphene oxide (GO) nanosheets, and carbon nanotubes when in direct contact with micro-organisms.[27]

Graphene nanomaterials include ultrathin graphite, multilayer graphene, GO, reduced GO, and graphene nanosheets,[28] which differ in surface properties, number of layers, and size [Figure 1].[29] GO is one of the most important graphene derivatives that can be synthesized through active oxidation of graphite by the Hummers method using oxidative agents.[30] GO has reactive functional groups on its surface that facilitate binding to various materials such as polymers, biomolecules, DNA, and proteins.[31] So far, few studies have been conducted on the effects of adding this substance to PMMA. Furthermore, graphene nanoparticles' anti-bacterial effect has been controversial because this effect is strongly determined by the size, shape, stability, and distribution of these particles.[32]

Reports on the safety and biocompatibility of GO also show contradictions. For example, some studies have suggested that GO is nontoxic even at high doses (5–200 μg/ml).[33] However, Liu *et al*. stated that GO could be mutagenesis in both *in vitro* and *in vivo* conditions, and therefore, more attention is needed during its biomedical applications.[34] Due to the mentioned contradictions and lack of information, the present study aimed to investigate the effect of adding functionalized GO nanosheets on the physical, mechanical, and anti-biofilm properties of acrylic resin.

MATERIALS AND METHODS

This experimental *in vitro* study samples included acrylic resin discs containing concentrations of 0, 0.25, 0.5, 1, and 2 wt% functionalized GO nanosheets and

Figure 1: Sample of anti-adhesion test.

the control group. All groups were evaluated in terms of physical properties (surface hardness, surface roughness, compressive strength, fracture toughness, and flexural strength), anti-biofilm properties (on four groups of micro-organisms, *Streptococcus mutans*, *Streptococcus sanguis*, *Staphylococcus aureus,* and *Candida albicans*), and cytotoxicity. Ten samples were considered for each test in each group (a total of 50 samples for each test).

About 1 g of graphene nanoparticles was added to a chloroform-toluene solvent to prepare functionalized GO nanoparticles. A methacrylate trimethoxysilane compound was added to the mixture, and the whole complex was dispersed in an ultrasonic bath for 20 min. It was then stirred at 100°C for 48 h. The final product (functionalized GO nanoparticles) was then filtered and dried in an oven at 50°C. Graphene nano oxide powder was washed with 70% ethanol for cleaning and sterilization.[35] Chemically activated PMMA acrylic resins (Orthocryl resin, Dentaurum, Ispringen, Germany) were used to prevent thermal damage to nano-GO (nGO) during polymerization.

nGO was added in 0.25, 0.5, 1.0 or 2.0 wt% to PMMA powder. Each of these nGO concentrations was then dispersed in MMA fluid for 1 h using a sonicator. PMMA powder was mixed with the liquid in the ratio of powder (g) to liquid (ml) 1: 1.2. Afterward, the samples were cut and polished with Silicon Carbide paper (SiC) with 1200 grit. 3-point flexural test specimens were prepared and polished with 2400 grit SiC. All samples were sterilized with ethylene oxide (EO) gas for biological testing.[36]

Physical tests

The following physical tests were performed on the prepared samples (10 samples from each of the five groups.)

Surface hardness test

Was performed by a microhardness machine (Copa, Iran) on ten samples from each group (n = 50). Each sample had dimensions of 8 × 8 × 3 and was polished with a 1200 SiC. For each sample, the dimensions of the depression resulting from the index's landing were measured in 3 areas.

Surface roughness

An atomic force microscope (AFM) was used to examine the surface hardness of 10 samples from each group (n = 50). The level of surface hardness was determined by visually observing images obtained from the AFM.

Mechanical tests

Compressive strength

To perform this test, ten samples of acrylic resins from different groups were placed into Teflon molds with dimensions of 6 mm × 3 mm (length × diameter). Then the samples underwent a compressive load applied by a Universal Testing Machine (UTM) (Copa-Iran) at a speed of 0.5 mm per min, and the force required for fracture was recorded and placed in the formula. The compressive strength was calculated and reported in MPa.

Fracture toughness

Each group prepared ten rectangular molds (2 mm × 5 mm × 30 mm). The molds were made of an alloy of aluminum and brass and contained a central slit. The molds were filled with the samples and covered with a transparent matrix strip. A glass slab was used to remove the excess material, and the extra material was removed by hand pressure. Then, the glass slab was removed to obtain a smooth surface. The samples' edges were gently sanded using 1000-grit sandpaper (silicon carbide) followed by 1500-grit sandpaper; afterward, the samples were dried.

A scalpel was used to create a small crack in the slit of the sample under hand pressure. A stereomicroscope measured the crack length (d) with a magnification of 80, and a digital caliper measured the width and height of each sample. Four-point bending method was used to measure the fracture toughness. The samples were pressured using a UTM at a 0.5 mm/min speed. The maximum fracture force was measured and calculated by the formula KIC.

$$K_{IC} = \frac{Lmax}{w\sqrt{h}} \times \frac{L0 - L1}{h} \times \frac{3TM\sqrt{\frac{d}{h}}}{2(1-\frac{d}{h})^{\frac{3}{2}}}$$

Lmax = Maximum pressure

l0, l1 = Internal, and external span length

W, h = Sample width and height

d = Crack length

Three-point flexural strength

In each group, by placing acrylic resins in Teflon molds with dimensions of 2 × 2 × 25 (length × width × height), ten samples were subjected to bending load by a UTM machine at a speed of 0.5 mm/min. The amount of the fracture force was

obtained, and by placing it in the formula, the amount of flexural strength was reported in megapascals. The anti-adhesion effect of adding GO to acrylic resin was investigated by the direct contact method.

Anti-biofilm tests

Direct contact test

Three sterile 500 µl microtubes were selected for each experimental group. Two hundred µl of the acrylic resin in each experimental group was added to the microtubes. Then, a Teflon jig was inserted into each microtube. After being placed, it was far enough away from the microtube walls so that the acrylic resin inside the microtube formed a layer with a constant thickness inside the microtube. After setting the acrylic resin, 50 µl of the standard half-McFarland microbial or fungal suspension of *S. mutans*, *Streptococcus sanguinis*, *S. aureus,* and *C. albicans* prepared by Pasteur Institute of Iran (108 × 1/5 bacteria or fungi) were poured inside each microtube. Then each microtube was shaken for the suspension to cover the surface of the acrylic resin. The microtubes were incubated in a sterile medium for one hour to evaporate the solution. After that, 200 µl of BHI (heart and brain extract) was added, and the microtubes were kept in an incubator at 37°C for 24 h under sterile conditions. Afterward, 10 µl of the solution was removed. The colony count was performed in the blood agar medium for *S. aureus*, *S. mutans*, *S. sanguis,* and Saboro dextrose agar medium for *C. albicans*. The number of counted colonies and an average of 3 microtubes in each group were reported[14] [Figure 1].

Cytotoxicity tests

To show the toxicity of GO, gingival fibroblast-like cells were selected. After culturing the cells (1.6×10^4 cells) in 96-well plates and incubating at 37°C with 5% CO_2 and 95% relative humidity, the sample extract (3 cm^2/ml in DW) was added to 2X DMEM/(3: 1) containing 20% fetal bovine serum, and 2% penicillin/streptomycin (Invitrogen, Waltham, MA, USA). After 24 h of incubation, a Mean transit time assay (Optical Density 450 nm) was performed.[35]

Statistical analysis

SPSS software version 22 (SPSSS Ins., Chicago III.,USA) was used for data analysis. Descriptive statistics (mean and standard deviation) were used to describe the data, and One-way analysis of variance (ANOVA) and Tukey *post hoc* test was used to compare the groups. The significance level was considered $P < 0.05$.

RESULTS

In this experimental study, the physical, mechanical, anti-biofilm, and toxicity properties of graphene nano oxide groups were evaluated, and the results were obtained as follows.

Physical tests analysis

Table 1 shows the mean and standard deviation of surface hardness and surface roughness in the five groups with different nGO concentrations. The results of the ANOVA test showed that the surface hardness in the experimental groups were significantly different from each other ($P = 0.001$), and the difference in mean hardness between the control and the 0.5% wt% group ($P = 0.03$), the control and the 0.25% wt% group ($P = 0.003$), the 0.25 wt% and the 1% wt% group ($P = 0.02$) and the 0.25 wt% and the 2% wt% group ($P = 0.02$) were significant. However, the surface roughness level in the different groups did not differ significantly ($P = 0.15$).

Mechanical tests analysis

Table 2 shows the mean and standard deviation of three-point compressive strength, toughness, and flexural strength in 5 groups with different nGO concentrations. The ANOVA test results showed that the compressive strength in the experimental groups differed significantly from each other ($P < 0.001$). According to the results

Table 1: Physical test status (hardness and surface violence) in the different groups

Groups	Number of samples	Surface hardness (HV0.3)		Surface roughness (Ra)	
		Mean	SD	Mean	SD
Control	10	10.5330	1.59799	544.8080	132.82851
0.25 weight percentage nGO	10	13.3787	2.13970	512.4650	104.42104
0.5 weight percentage nGO	10	12.6927	1.85891	557.1270	197.19118
1 weight percentage nGO	10	11.0710	1.29538	525.9500	132.51435
2 weight percentage nGO	10	11.0317	1.05188	408.2820	122.49849
Total	50	11.7414	1.91842	509.7264	145.71213
P		0.001		0.15	

SD: Standard deviation; nGO: Nano-graphene oxide

of the Tukey *post hoc* test, significant differences were observed between the control group and the 1 wt% group ($P = 0.03$), the 0.5 wt% group with the 1 wt% group ($P = 0.00$), and the 0.5 wt% group, and the two wt% group ($P = 0.00$). The three-point flexural strength was significantly higher in the control group compared to other groups ($P = 0.001$). But the degree of toughness of different groups was not significantly different ($P = 0.40$).

Anti-biofilm tests

Table 3 shows the mean and standard deviation of the number of counts of *S. aureus*, *S. mutans, S. sanguis,* and *C. albicans* colonies in five groups with different concentrations of nGO. The results of the ANOVA test showed that the number of *S. aureus* colonies in the studied groups were significantly different from each other ($P = 0.03$), and the difference between the mean number of colonies between the control group and the 1% wt% group was statistically significant ($P = 0.04$). Furthermore, the number of *S. mutans* colonies in the experimental groups differed significantly from each other ($P < 0.001$). Furthermore, when comparing two groups, it was found that the control group and the 1% wt% group, the control group and the 2% wt% group, the 0.25% wt% group and the 1% wt% group, the 0.5% wt% group and the 1% wt% group have significant differences with each other ($P < 0.05$). The number of *S. sanguis* ($P = 0.07$) and *C. albicans* ($P = 0.41$) colonies in the study groups did not differ significantly.

Cytotoxicity tests

Table 4 shows the toxicity mean and standard deviation in five groups with different nGO concentrations. The ANOVA test results stated that the studied groups' toxicity differed significantly ($P < 0.001$). Comparing the groups according to the Tukey *post hoc* test results concluded a significant difference between all groups ($P = 0.00$).

DISCUSSION

Investigation of physical properties

The samples' surface roughness was measured using AFM, and their hardness was examined with a microhardness device. The present study results showed that the experimental groups' level of surface roughness (0.25 wt%, 0.5 wt%, 1 wt%, and 2 wt% of nanographene) and the control group (without nanographene) was not significantly different. Accordingly, it appears that the presence of nanographene particles does not increase the surface roughness of PMMA. This is very important

Table 2: Mechanical tests status (MPa) (compressive strength, toughness and flexural strength) in the different groups

Groups	Number of samples	Compressive strength (MPa)		Toughness (MPa)		Flexural strength (MPa)	
		Mean	SD	Mean	SD	Mean	SD
Control	10	39.3060	6.07992	53.8900	0.60083	3.9850	2.04361
0.25 weight percentage nGO	10	42.9540	13.38476	53.3600	1.00797	1.8900	0.79242
0.5 weight percentage nGO	10	33.1650	8.40107	53.2300	1.06254	2.0120	0.88990
1 weight percentage nGO	10	51.1340	9.57782	53.9700	1.71727	1.6920	1.59479
2 weight percentage nGO	10	47.5380	3.37785	53.1200	1.42579	1.3240	1.01866
Total	50	42.8194	10.57358	53.5140	1.22458	2.1806	1.60361
P		<0.001		0.40		0.001	

SD: Standard deviation; nGO: Nano-graphene oxide

Table 3: Evaluation of anti-biofilm tests (number of colonies of different micro-organisms) in the experimental groups

Groups	Number of samples	*Taphylococcus aureus*		*Streptococcus mutans*		*Streptococcus sanguinis*		*Candida albicans*	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	10	1.510×10^8	0.0316	1.480×10^8	0.0632	1.500×10^8	0.0000	1.510×10^8	0.0316
0.25 weight percentage nGO	10	1.550×10^8	0.5681	1.880×10^8	0.4566	1.670×10^8	0.1567	1.580×10^8	0.2781
0.5 weight percentage nGO	10	2.330×10^8	0.9978	1.710×10^8	0.4067	1.760×10^8	0.4274	1.680×10^8	0.1932
1 weight percentage nGO	10	2.900×10^8	1.4780	2.480×10^8	0.3853	1.860×10^8	0.1776	1.660×10^8	0.2633
2 weight percentage nGO	10	2.760×10^8	1.9811	2.230×10^8	0.6360	1.750×10^8	0.3951	1.650×10^8	0.2415
Total	50	2.210×10^8	1.3097	1.956×10^8	0.5496	1.708×10^8	0.2954	1.616×10^8	0.2207
P		0.03		<0.001		0.07		0.41	

SD: Standard deviation; nGO: Nano-graphene oxide

Table 4: Evaluation of cytotoxicity (percentage of living cells) in the experimental groups

Groups (weight percentage)	Number of samples	Mean (%)	SD	*P*
Control	10	1	0.03680	<0.001
0.25	10	0.841	0.01684	
0.5	10	0.739	0.04358	
1	10	0.638	0.03979	
2	10	0.504	0.02744	
Total	50	0.742	0.1755	

SD: Standard deviation

in bacterial retention; changes in the level of surface roughness can prevent caries, gingivitis, periodontitis, peri-implantitis, and stomatitis.[37] However, in Lee's study (2018), the level of surface roughness in the 2 wt% nanographene group was significantly higher than in the control group. However, the difference between the other groups (control, 0.25 wt%, 0.5 wt%, and 1 wt% of nanographene) was not statistically significant. They attributed this increase in surface roughness in the 2% group to the greater amount of graphene nano-oxide exposed on the outer surface after polishing.[36]). The homogenization method, nanoparticle distribution, and good polishing can affect the level of surface roughness. Also, the surface roughness assessment method is helpful in measuring the accuracy of this rate. In Lee's study, SEM was used to investigate the surface roughness, and as we know, the image detail in AFM is more accurate than in SEM.[38] However, there need for further research seems essential, as there is very little evidence in this regard. In the present study, the 0.25 wt% nanographene group's hardness was significantly higher than the 1 wt%, the 2 wt%, and the control groups. Also, the hardness of the 0.5 wt% group was considerably higher than the control group. This may be due to the accumulation of graphene nanosheets and defects in the PMMA matrix.

In fact, by increasing the weight percentage of nGO, the average hardness decreased.[39] Other studies, such as Gonçalves *et al*. and Wang *et al*. have also confirmed the effects of percolation and aggregation of graphene nanosheets at higher weight percentages.[39,40] However, in Lee's study (2018), the surface hardness increased significantly in groups with more than 0.5 wt% nGO.[36] Perhaps, the difference in the results of the present study and Lee's study is due to the difference in the number of samples. More samples have been evaluated in the present study than in Lee's study. So far, very few researches have been conducted on measuring PMMA/nanographene particles' surface hardness, indicating the need for other similar studies to provide more definitive results.

Mechanical properties

The homogeneity of the composite and the strong bonds between the nanomaterials and the polymer matrix significantly affect the mechanical properties.[38] According to the present study results, the 1wt% group had significantly higher compressive strength than the control and the 0.5 wt% groups. Still, the 0.25wt% and the 2 wt% groups did not show a significant difference. In general, the 1 wt% groups and the 0.5 wt% group had the lowest and the highest compressive strength among all the experimental groups. The control group had a significantly higher three-point flexural strength than all the other groups, and there was no significant difference observed between the other groups. The levels of toughness did not show any significant difference between the groups. In the study of Alamgir *et al*. and Azevedo *et al*., the improvement of mechanical properties of PMMA nanocomposites containing GO was reported.[41,42] The study of Khan *et al*. showed that adding 0.024 wt% and 0.048 wt% GO to PMMA improves its abrasion resistance and flexural strength.[43] Furthermore, the results of Lee's study (2018) showed that only in the 0.5 wt% group of graphene nanoparticles the flexural strength increased significantly[36] and the flexural strength in the 1 wt% and 2 wt% groups was lower than the 0.5 wt% group. In Wang's study (2011), the tensile strength and the elongation at break reached their highest levels in the 1 wt% GO group, compared to the control group, but increasing the concentration of GO to 2 wt% led to a decrease in its mechanical properties.[39] In a review by Kausar, 1 wt% GO was considered the optimum amount to improve the nanocomposite's mechanical properties.[5] This is because GO plates (in amounts above 1 wt %) do not interface well with the PMMA matrix.[40] Therefore, no force is transferred from the matrix to the GO plates, clarifying the composite's mechanical behavior.[40] However, it seems that the amount of 1 wt% by or less of these nanoparticles can improve or not change the mechanical properties of acrylic resin, which is due to crack deflection and bridging of nGO in combination with the polymer matrix, which can also improve the mechanical properties.[44,45]

Anti-biofilm (anti-adhesion) properties

The present study showed no decrease in the colony count in all cases, and the bacterial load was equal

to or even greater than the zero concentration (initial inoculation) one hour after inoculation. This could be due to the lack of diffusion of nGO particles and its lack of release and bactericidal effect. These results also show that the microbe could not attach to the nGO-bound acrylic resin on the tube's inner surface, and therefore its load into the liquid was increased. On the other hand, the anti-biofilm results of this substance increase with increasing concentration, which indicates that this substance has a dose-dependent effect, and the best anti-dehydration effect occurs at high concentrations of this substance.

Antimicrobial (Anti-adhesive) surfaces in PMMA are usually created through two strategies: 1-Formation of a hydration layer on the surface, which creates a physical or energetic barrier to prevent microbial adhesion[46] 2-Direct contact of nGO with microbes and antimicrobial effects such as phospholipid extraction, membrane rupture and isolation of microbes by wrapping.[47] When nGO enters the composite structure, the formation of a hydration layer on the surface is one of the main antimicrobial-anti-adhesive mechanisms.[48] A study by He *et al*. showed that GO nanosheets effectively inhibit the growth of dental pathogens. Examination of TEM microscope images also showed that the cell wall and bacterial membrane lost their integrity, and the intracellular contents leaked out.[49]

Bykkam *et al*. study stated that nGO has intense anti-bacterial activity against *Klebseilla* and *Staphylococcus* species, and higher concentrations of GO had a larger zone of inhibition.[50] According to Bregnocchi *et al*., after 24 h, the samples in the 0.2 wt% group of graphene nanosheets had high anti-bacterial activity, and only 28% of mutant streptococci were able to survive in these conditions. However, samples in the 0.1 wt% group did not affect mutant streptococci survival.[51] In Lee's study,(2018) samples with higher weight percentages of nGO showed more potent antihydrogen effects against the microbial species *Escherichia coli*, *C. albicans*, *S. aureus,* and *S. mutans*.[36] The results of the mentioned studies are similar to the results of the present study. Various antimicrobial substances and additives used in polymers' structure and composition may cause cytotoxicity.[4] Therefore, more *in vivo* clinical studies are needed to confirm these substances' antimicrobial effects, safety, and biocompatibility.[52]

Cytotoxicity

Given the widespread use of graphene-based materials, understanding their interaction with biological systems seems essential; because it can lead to local and systemic toxic effects.[53] The present study results showed that the higher the weight percentage of nGO, the higher the toxicity. In fact, with increasing GO concentration, the amount of unreacted monomer (methyl methacrylate) also increases, and cytotoxicity increases.[54] Studies evaluating nanoparticle cytotoxicity in the oral environment are minimal and show contradictions. The results of the study by Olteanu *et al*. showed that graphene nanosheets (especially GO) increased intracellular ROS (reactive oxygen species), which depends on time and concentration. The number of viable cells decreases at high concentrations (40 μg/mL), and the mitochondrial membrane potential changes.[53]

In contrast, low concentrations (4 μg/mL) show good safety. Of the three graphene materials studied in this study (GO, thermally reduced GO, and nitrogen-doped graphene), GO showed the lowest cytotoxicity and minimal damage to human follicle dental stem cells.[53] The results of studies by Wang *et al*. and Ding *et al*. also indicated that the concentration of 50 ug/ml dose threshold of toxicity of GO in human mammalian cells and concentrations higher than this value could lead to human fibroblasts and T lymphocytes damage.[31,55] In another study, Gamal *et al*. stated that the PMMA/graphene nanoparticles were biocompatible at concentrations of 0.05 wt%, 0.1 wt%, 0.15 wt%, and 1 wt%. The hemolysis rate was <5% for all groups. However, the percentage of hemolysis increased with increasing the nGO concentration. The hemolysis rate in the 0.05 wt % group was significantly lower than the 0.15 wt% and the 1 wt% groups.[3] The results of these studies were similar to the present study.

In the study of Lee *et al*., cytocompatibility tests showed that samples with different weight percentages of nGO did not significantly reduce keratinocytes' viability.[36] This study's results were inconsistent with the present study, which may be due to the small number of samples in Lee's study compared to the present study.

Although no systemic toxic effects have been reported in humans from fully polymerized PMMA products,[56] further *in vivo* studies are needed to confirm the biocompatibility of graphene nano/PMMA.

CONCLUSION

According to the present study results, adding functionalized graphene nanoparticles to PMMA in optimum concentrations improves the anti-bacterial and fungal biofilm properties. It does not significantly change or increase its physical and mechanical properties. Therefore, it can be used in patients with removable orthodontic appliances. Due to this material's toxicity in high concentrations, it is advised to add acrylic resin in concentrations <2 wt%.

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Conflicts of interest
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