A graphene/zinc oxide nanocomposite film protects dental implant surfaces against cariogenic Streptococcus mutans

Shatavari Kulshrestha, Shakir Khan, Ramovatar Meena, Braj R. Singh and Asad U. Khan

Introduction

Oral biofilms play a crucial role in the development of dental caries and other periodontal diseases. Streptococcus mutans is one of the primary etiological agents in dental caries. Implant systems are regularly employed to replace missing teeth. Oral biofilms accumulate on these implants and are the chief cause of dental implant failure. In the present study, the potential of graphene/zinc oxide nanocomposite (GZNC) against the cariogenic properties of Streptococcus mutans was explored and the anti-biofilm behaviour of artificial acrylic teeth surfaces coated with GZNC was examined. Acrylic teeth are a good choice for implants as they are low cost, have low density and can resist fracture. Microscopic studies and anti-biofilm assays showed a significant reduction in biofilm in the presence GZNC. GZNC was also found to be nontoxic against HEK-293 (human embryonic kidney cell line). The results indicate the potential of GZNC as an effective coating agent for dental implants by efficiently inhibiting S. mutans biofilms.

Keywords: graphene/zinc oxide nanocomposite; dental caries; acrylic teeth; dental implants; cytotoxicity; anti-biofilm

Oral biofilms are complex three-dimensional structures with adherent multispecies bacterial communities which can contribute to dental caries and numerous periodontal diseases (Selwitz et al. 2007; Nance et al. 2013). These common infectious diseases can lead to a major public health concern (Falsetta et al. 2014). Biofilms have a greater tendency to resist antibiotics and create an environment that enhances microbial resistance with respect to their planktonic counterparts. Streptococcus mutans is one of the most frequently detected microorganisms on the tooth surface and is a major etiological agent of human dental caries (Hasan et al. 2014). This bacterial species has also been recognized as a causative agent of endocarditis (Abranches et al. 2011). S. mutans grows in the oral cavity by means of several unique mechanisms (Dmitriev et al. 2011). Acidogenicity and aciduricity play a major role in the increase in the severity of infection along with the ability to produce extracellular polysaccharides (Koo et al. 2003; Krol et al. 2014).

At present implant systems are utilized extensively to replace missing teeth. Oral biofilms consisting mainly of Streptococcus spp. accumulate on implants (Nakazato et al. 1989). The formation of biofilm on these implants is one of the major causes of implant failure (Costerton et al. 2005). The inflammatory changes in the soft tissues surrounding the implant induced by infection give rise to progressive destruction of the supporting bone (Zitzmann & Berglundh 2008). Nanoparticle-based implant coatings may well offer useful antimicrobial and anti-biofilm functionalities to prevent dental implant failure.

Nanoparticle based approaches are expected to open new horizons for preventing biofilm based infections by their unique mode of action (Ruparelia et al. 2008; Raghupati et al. 2011). Zinc oxide nanoparticles (ZnO NP) have already been found to have antibacterial activity against a wide range of microorganisms (Huang et al. 2008; Xie et al. 2011), but aggregation is one of the drawbacks since it makes them toxic against mammalian cells (Yuan et al. 2010).

Graphene oxide (GO) has unique physical and chemical properties (Wu et al. 2013). GO contains a single layer of sp² carbon atoms with hydroxyl and epoxy functional groups on the surface and the carboxyl groups at the edges (Dai et al. 2014). These functional groups offer active sites for hybridization with metals and metal oxides and thus act as a supporting surface for growing metal and metal oxide nanoparticles (Ocsoy et al. 2013). Graphene in its functionalized state has been used for biosensing, photothermal therapy, drug delivery as well as imaging (Wang et al. 2011). Recently the use of graphene and graphene based nanocomposites as potential antimicrobial agents has gained substantial interest in the field of nanomedicine (Ma et al. 2011; Xu et al. 2011; Tang et al. 2013; Fariaa et al. 2014). Moreover graphene oxide has been reported to show good biocompatibility

*Corresponding author. Email: asad.k@rediffmail.com

© 2014 Taylor & Francis
compared to other nanoparticles (Chang et al. 2011). To the best of the authors’ knowledge no study has yet examined the anti-biofilm action of graphene/zinc oxide nanocomposites (GZNC) and GZNC coated tooth surfaces on *S. mutans*.

The objective of this present study was to evaluate the antimicrobial, anti-biofilm and anti-adherence activity of GZNC against *S. mutans*, a major cause of caries infection, and to access the applicability of GZNC as a coating for dental implants.

**Materials and methods**

**Microorganisms**

*S. mutans* MTCC 497, an ATCC analogue of the UA159 strain of *S. mutans* (purchased from IMTECH, Chandigarh, India) was used in this study. The clinical isolates of *S. mutans* used in this study (SM 497, SM 34 and SM 06) were isolated and characterized earlier in the authors’ laboratory (Islam et al. 2008). The clinical samples were collected from the Department of Conservative Dentistry, Dental College, AMU, Aligarh, India. CLSI guidelines were followed for the isolation and characterization of *S. mutans* from samples. The strains of *S. mutans* were grown in a CO2-rich environment using candle jar incubation. The isolates were confirmed by PCR amplification of conserved regions of the GTF B and GTF C genes. All the strains were grown in Brain Heart Infusion broth (BHI) (Hi Media Laboratories, Mumbai, India) at 37°C. The cultures were stored at –80°C in BHI containing 25% glycerol.

**Synthesis of GZNC**

Graphene oxide (GO) was prepared according to the method described by Hummers and Offman (1958) with little modification. H2SO4 (100 ml) was added to 2 g of graphite powder (Loba Chemie, Mumbai, India) while stirring in an ice-water bath. KMnO4 (25 g) was slowly added to the solution. The mixture was kept on a stirrer at room temperature until it became pasty brown. It was then diluted with the slow addition of 200 ml of water. Finally, 10 ml of a 30% aqueous solution of H2O2 were added. The impurities were removed from the graphene oxide (GO) using 3% HCl with repeated washing (Hummers & Offman 1958). For the synthesis of the GZNC, 100 mg of zinc acetate and 200 mg of GO were dispersed into 200 ml of absolute ethanol followed by sonication and harvested by centrifugation at 5,000 rpm for 5 min. It was then washed with 80% ethanol. The pellet was vacuum dried and 100 mg of this dried sample were mixed with 100 ml of ethylene glycol using sonication for 10 min. The resulting mixture was heated to 140°C with vigorous stirring on a magnetic stirrer (REMI, Mumbai, India; Model: 1MLH) for 3 h. The synthesized GZNC suspension was centrifuged, washed with 80% ethanol and dried in a vacuum oven at 60°C.

**Characterization of GZNC**

The synthesis of GZNC in solution was monitored by measuring absorbance using a UV-visible spectrophotometer (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA) in the wavelength range 200 to 800 nm. Transmission electron microscope (TEM) analysis was performed using a JEM-2100F TEM (Jeol, Tokyo, Japan) operating at 120 kV. The X-ray diffraction (XRD) patterns of the powdered sample were recorded on a Mini-Flex™ II benchtop XRD system (Rigaku Corporation, Tokyo, Japan) operating at 40 kV. For the Fourier transform infrared (FTIR) spectroscopic measurements, graphene/ZnO powder was mixed with spectroscopic grade potassium bromide (KBr) in the ratio of 1:100 and spectra were recorded in the range 400–4,000 wave number (cm⁻¹) on a Perkin Elmer FTIR Spectrum BX (PerkinElmer Life and Analytical Sciences) in the diffuse reflectance mode at a resolution of 4 cm⁻¹ in KBr pellets. The thermal stability of the graphene/ZnO was investigated by thermogravimetric analysis (TGA) at a heating rate of 10°C min⁻¹ under a nitrogen atmosphere.

**Determination of bacteriostatic (MIC) and bactericidal (MBC) concentrations**

The minimum inhibitory concentration of GZNC against the strains MTCC 497 and clinical isolates was determined using the micro-dilution method (Khan et al. 2010). Overnight grown cultures (50 μl) were diluted to 10⁻⁵–10⁻⁶ cfu ml⁻¹ and inoculated into fresh BHI (50 μl) containing various concentrations of serially diluted GZNC, starting at an initial concentration of 1 mg ml⁻¹. The MIC was determined as the lowest concentration that totally inhibited visible bacterial growth. The MBC, on the other hand, was determined by sub-culturing the test dilutions on tryptic soya agar plates and incubating for 24 h. These determinations were performed in triplicate and the means of three independent experiments were calculated.

**Effect on adherence to smooth glass surfaces**

The effect of sub-MIC concentrations of GZNC on all three strains was performed as the inhibition of adhered cells on a smooth glass surface (Hamada & Torii 1978). The bacteria were grown at 37°C for 24 h at an angle of 30° in a glass tube containing 10 ml of BHI with 5% (w/v) sucrose (Aires et al. 2006) and sub-MIC concentrations of GZNC. After incubation, the glass tubes were slightly rotated and the planktonic cells were decanted. The adhered cells were then removed by adding 0.5 mM
sodium hydroxide followed by agitation. The cells were washed and suspended in saline. Adherence was quantified spectrophotometrically at 600 nm. These determinations were performed in triplicate, using untreated BHI medium as control.

The percentage adherence was calculated as the OD of adhered cells divided by the OD of total cells divided by 100.

**Biofilm formation assay (crystal violet assay)**

Biofilm formation was assessed by using the protocol of Loo et al. (2000) with a few modifications. Briefly, 50 ml of overnight grown cultures of *S. mutans* strains (MTCC 497 and clinical isolates) diluted to 10^5–10^6 cfu ml^{-1} were inoculated into fresh BHI (150 ml) with 5% sucrose containing various sub-MIC concentrations of GZNC with respective controls and used to inoculate the wells of microtitre plates. After incubation for 24 h at 37°C, the medium and unattached cells were decanted from the microtitre plates. The remaining planktonic cells were removed by gentle rinsing with sterile water. The wells with adhered biofilms were fixed with formalin (37%, diluted 1:10) plus 2% sodium acetate, and each well was stained with 200 ml of 0.1% crystal violet (CV) for 15 min at room temperature. After two rinses with distilled water, bound dye was removed from the cells using 100 ml of 95% alcohol. Plates were then set on a shaker for 5 min to allow full release of the dye. Biofilm formation was quantified by measuring the OD\textsubscript{530} using a BIO-RAD iMark TM Microplate reader, Gurgaon, India.

**Assessment of cellular viability**

This assay was performed as described previously with slight modifications as performed earlier (Islam et al. 2008), 2,3-Bis(2-methoxy-4-nitro-5-sulphonyl)5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) (Sigma-Aldrich, New Delhi, India) was dissolved in distilled water, bound dye was removed from the cells using 100 ml of 95% alcohol. Plates were then set on a shaker for 5 min to allow full release of the dye. Biofilm formation was quantified by measuring the OD\textsubscript{630} using a BIO-RAD iMark TM Microplate reader, Gurgaon, India.

**Effect on growth**

The effect of sub-MIC concentrations of GZNC was tested on the growth of all strains of *S. mutans*. Overnight grown broth cultures of *S. mutans* were inoculated into tubes to obtain a final inoculum of 1.56 × 10^6 CFU ml^{-1} followed by the addition of GZNC. The tubes were incubated at 37°C. Growth was monitored spectrophotometrically (UV mini 1240, UV-VIS Spectrophotometer Shimadzu, New Delhi, India) by taking the absorbance of the culture at 600 nm for 24 h. All determinations were performed as triplicates using untreated growth controls.

**Inhibition of water-insoluble glucan synthesis**

A crude GTFase was assayed to estimate the effect of GZNC on glucan synthesis (Hasan et al. 2012). Cell-free enzymes were precipitated from culture supernatant of *S. mutans* by adding solid ammonium sulphate to 70% saturation (an ammonium cut). The mixture was stirred at 4°C for 1 h and allowed to stand for another 1 h under cold conditions. The precipitate was collected by centrifugation at 12,000 g at 4°C for 20 min, dissolved in a minimum volume of 20 mM phosphate buffer (pH 6.8) and then dialysed against 2 mM phosphate buffer (pH 6.8) at 4°C for 24 h. The crude enzyme was stored at –70°C for further experiments. A reaction mixture consisting of 0.25 ml of crude enzyme and varying concentrations of GZNC in 20 mM phosphate buffer (pH 6.8) containing 0.25 ml of 0.4 M sucrose was incubated at 37°C for 18 h. The fluid was removed post incubation and the tube contents were washed with sterile water. The total amount of water-insoluble glucan was measured by the phenol–sulphuric acid method (Dubois et al. 1956). Three replicates were performed for each concentration of the GZNC.

**Influence of GZNC on EPS production**

The Congo red (CR) binding assay which detects glucose containing polymers was used to evaluate exopolysaccharide (EPS) production, as previously reported (Friedman et al. 2001; López-Moreno et al. 2014). Fifty microlitres of overnight growth culture of *S. mutans* strain (MTCC 497) diluted to 10^5–10^6 cfu ml^{-1} were inoculated into fresh BHI (50 ml) with 5% sucrose containing sub-MIC concentrations of GZNC. These solutions as well as untreated controls were used to inoculate microtitre plates. After incubation for 24 h at 37°C, the medium was removed and biofilms were washed with PBS and then fresh medium (100 µl) was added to each well including the respective controls. Then 50 µl of CR (0.5 mM) were added to each well. Medium (100 µl) along with 50 µl CR were added to another well for blank measurements (Blank CR). Plates were incubated for 60 min. The medium in each well was transferred to 200 µl micro centrifuge tubes and centrifuged at 10,000 × g for 5 min. The supernatant was transferred...
to empty wells in microtitre plates. Absorbance was measured at 490 nm. The absorbance value of the supernatant was subtracted from the absorbance value of the ‘blank CR’. The resultant value represents the amount of bound CR or EPS produced. This experiment was conducted in triplicate.

**CR agar method**
An alternative method of screening biofilm formation and EPS production as described by Freeman et al. (1989) was also used in this study. Solid agar medium was prepared using BHI (37 g l\(^{-1}\)), sucrose (5%), agar No. 1 (2%), and CR stain (0.8 g l\(^{-1}\)). CR was prepared in the form of a concentrated aqueous solution and was autoclaved at 121°C for 15 min, separately. After autoclaving, CR was added to the agar which had been cooled to 55°C. For treated samples, sub-MIC concentrations of nanoparticles were added to the medium. Plates were inoculated and incubated aerobically for 24 h at 37°C. EPS production was indicated by black colonies with a dry crystalline consistency.

**Effect on acid production**
The effect of GZNC on the acidogenicity of *S. mutans* and its clinical isolates was assessed using a previously published protocol (Khan et al. 2010). Five millilitres of BHI broth containing 5% (w/v) of sucrose and sub-MIC concentrations of GZNC were inoculated with SM 497, SM 06 and SM 34 and incubated at 37°C for 24 h. The pH of the treated samples and the controls was assessed at 0 h and after incubation for 24 h. All determinations were performed in triplicate.

**Scanning electron microscope analysis of biofilms**
The effects of GZNC on biofilms was visualized using scanning electron microscopy (SEM) using saliva-coated glass coverslips. Saliva was collected under masticatory stimulation (chewing a piece of paraflm), from a healthy individual who abstained from tooth brushing and eating for 5 h prior to collection. The collected saliva was centrifuged at 8,000 g for 15 min to obtain clarified saliva and was stored at −80°C (Hasan et al. 2012). Clarified saliva (100 ml) was added to each well of a 12-well microtitre plate equipped with glass coverslips. The plate was then incubated at 37°C for 2 h to coat the coverslips with a salivary pellicle. After incubation, these coverslips were rinsed three times with PBS before adding the bacterial culture. The experiment was performed in triplicate. Sub-MIC concentrations of GZNC were used as treatment while the control was untreated *S. mutans*. The wells were inoculated (10\(^7\)–10\(^8\) cfu ml\(^{-1}\)) and incubated at 37°C for 24 h. The coverslips were removed after 24 h and washed three times in sterile PBS. The resultant samples were fixed with 2% formaldehyde and 2.5% glutaraldehyde. The samples were rinsed three times with PBS after fixing, followed by an ethanol dehydration series. Samples were then completely dried, coated with gold, and observed using a scanning electron microscope.

**Biofilm reduction seen through confocal microscopy**
Bacterial biofilm was grown in the presence of sub-MIC concentrations of GZNC at 37°C in covered glass bottom confocal dishes (Genetix Biotech Asia Pvt. Ltd, New Delhi, India) with a dish size of 35 mm, 22 mm coverglass, 9.4 cm\(^2\) growth area and a working volume of 3 ml. The dishes were inoculated (10\(^5\)–10\(^6\) CFU l\(^{-1}\)) and incubated at 37°C for 24 h. Then medium was removed and attached biofilms were washed with PBS and treated with SYTO-9 (5 μM; excitation and emission wavelength; 488 nm, 498 nm) and propidium iodide (PI) (0.75 μM; excitation and emission wavelength: 536 nm, 617 nm). The stained bacterial biofilm was observed with a FluoView FV1000 (Olympus, Tokyo, Japan) confocal laser scanning microscope equipped with argon and HeNe lasers.

**Coating of acrylic teeth with GZNC and biofilm assays**
GZNC coating on the tooth surface was done using sonochrome as previously described (Eshed et al. 2012). A GZNC coating on artificial acryl teeth (obtained from Dr Ziauddin Dental College at the Aligarh Muslim University) was performed by placing the tooth in a nanoparticle suspension in a sonicator. The tooth was kept at a constant distance from the sonicator tip throughout the process. The nanoparticle surface coating was characterized by SEM (EVO 40; Zeiss, Jena, Germany). Artificial teeth were assayed using a static biofilm assay. Teeth were placed in a 24-well plate. Each well contained 2 ml of a suspension of *S. mutans* at a final concentration of ~1.5 × 10\(^8\) CFU ml\(^{-1}\) in BHI medium. After incubation for 24 h at 37°C, biofilm formation was assayed using the CV assay as described above. To examine biofilm morphology, teeth samples were further exposed after incubation in fixative (glutaraldehyde + paraformaldehyde) for 4 h. Finally, samples were dehydrated using increasing concentrations of ethanol. Samples were then air dried and imaged by SEM.

**Detection of reactive oxygen species (ROS)**
The ROS formed by GZNC were identified using 2′,7′-dichlorofluorescein diacetate (DCFDA) (Cui et al. 2012). The sub-MIC concentration of GZNC was used and the number of bacterial cells was adjusted to 10\(^8\) CFU ml\(^{-1}\). All cultures were incubated at 37°C for 4 h and then centrifuged at 4°C for 30 min at 8,000 g. Then each
supernatant was treated with 100 μM DCFDA for 1 h. The ROS formed in the sample were detected at a fluorescence excitation wavelength of 485 nm and an emission wavelength of 528 nm. The same procedure was used to determine ROS after incubation for 12 h.

**Cytotoxicity assay**
A human embryonic kidney cell line (HEK-293) obtained from National Centre for Cell Science (NCCS) Pune, was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Biological Industries, Beit HaEmek, Israel), supplemented with 10% heat inactivated foetal calf serum and IX Penstrept antibiotic solution, incubated at 37°C and 5% CO₂. HEK-293 cell viability was used to determine ROS after incubation for 12 h. The ROS formed in the sample were detected at a fluorescence excitation wavelength of 485 nm and an emission wavelength of 528 nm. The same procedure was used to determine ROS after incubation for 12 h.

**Intracellular uptake of GZNC in HEK-293 and bio-imaging**
HEK-293 cells were cultured in DMEM supplemented with 10% heat inactivated foetal calf serum (Biological Industries) and IX Penstrept antibiotic solution (Biological Industries) and then incubated in a fully humidified 5% CO₂ incubator at 37°C. All cells were seeded in culture flasks and were divided into treatment group and control group. When ~70% of growth occurred, the cells were washed with 0.1 M PBS (phosphate buffer saline) and old medium was replaced with fresh medium. Culture plates were treated with various concentrations of GZNC (100, 200, 300 and 400 μg ml⁻¹) and incubated at 37°C and 5% CO₂ for 24 h. All plates were washed with 0.1 M PBS and cells were collected by trypsinization (0.05% trypsinase). The cell pellets were dissolved in 1 ml of 0.1 M PBS and were imaged under bright field, UV-excitation and blue excitation with an Olympus Fluo View™ FV1000 laser scanning confocal microscope.

**Statistical analysis**
All experiments were executed in triplicate. For each assay, data are presented as mean ± standard deviation (SD). The values were calculated as the mean of individual experiments in triplicate and compared with those of the control groups. Differences between two mean values were calculated by the Student’s test. Data with p-values < 0.05 were considered statistically significant.

**Results and discussion**

**Characterization of GZNC**
TEM images (Figure 1A) of GZNC showed ZnO nanocrystals dispersed on the surface of the graphene sheets. The average size of the ZnO nanoparticles in the composite was found to be in the range of 20–40 nm (Figure 1B). TEM results confirmed the attachment of pure ZnO with GO platelets and ZnO nanoparticles. The UV visible spectrum of graphene, ZnO and GZNC is shown in Figure 1C. The presence of a sharp characteristic absorption peak at ~370 nm clearly indicated the formation of pure crystalline ZnO nanostructures. In addition, the red shift in the GZNC curve as compared to pure ZnO shows an increased π-electron concentration and structural ordering and may be ascribed to the chemical bonding (Zn–O–C bond) of zinc oxide with GO platelets and ZnO nanoparticles. The UV visible spectrum of graphene, ZnO and GZNC is shown in Figure 1C. The presence of a sharp characteristic absorption peak at ~370 nm clearly indicated the formation of pure crystalline ZnO nanostructures. In addition, the red shift in the GZNC curve as compared to pure ZnO shows an increased π-electron concentration and structural ordering and may be ascribed to the chemical bonding (Zn–O–C bond) of zinc oxide with GO platelets and ZnO nanoparticles. The UV visible spectrum of graphene, ZnO and GZNC is shown in Figure 1C. The presence of a sharp characteristic absorption peak at ~370 nm clearly indicated the formation of pure crystalline ZnO nanostructures. In addition, the red shift in the GZNC curve as compared to pure ZnO shows an increased π-electron concentration and structural ordering and may be ascribed to the chemical bonding (Zn–O–C bond) of zinc oxide with GO platelets and ZnO nanoparticles.

**FTIR spectrum of GZNC**
The FTIR spectrum of GZNC with strong absorption bands around 3468.5, 2342.07, 1547.47, 1059.65, 648.9 and 482.9 cm⁻¹. The band at 3468.5 cm⁻¹ may be assigned to O–H bond stretching and δ(OH) vibrations for adsorbed water molecules.

**XRD analysis**
Powder X-ray diffraction (XRD) analysis confirmed that GZNC consists of cubic ZnO (Figure S1A). [Supplementary information is available via a multimedia link on the online article web page.] The average crystalline size of ZnO NPs was calculated following the Debye–Scherrer formula (Cullity 1978). The calculated average particle size was found to be ~14.76 nm. The thermogravimetric (TG) measurement was carried out in order to determine the mass ratio of ZnO to graphene in the composite. The TGA curve of...
GZNC is shown in Figure S1B. A schematic representation of nucleation of zinc oxide nanoparticles on functionalized graphene oxide is illustrated in Figure 2.

**Enhanced antibacterial activity of ZnO anchored to graphene sheets (GZNC)**

The minimum inhibitory concentration (MIC) of GZNC against *S. mutans* and its clinical isolates was found to be 125 μg ml⁻¹ whereas the minimum bactericidal concentration (MBC) was found to be 250 μg ml⁻¹. The antibacterial effect of GZNC is much greater than that of ZnO nanoparticles alone where the MIC and the MBC are reported to be 500 ± 306.18 μg ml⁻¹ and 500 μg ml⁻¹ respectively (Hernández-Sierra et al. 2008), indicating that graphene is enhancing the antibacterial property of ZnO nanoparticles. It is also clear from the results that the killing of planktonic cells of *S. mutans* is not dependent on clinical vs reference strains. In view of the above results, it is evident that the nanostructure formed by the interaction of ZnO with graphene provides a unique nano-interface for interacting with microbes as compared to ZnO alone.

**Effect on cariogenic properties and biofilms of *S. mutans***

Adherence of bacteria to the tooth surface is an important step in biofilm formation and a reduction in adherence could serve as preventive biofilm formation (Hasan et al. 2012). The effect of sub-inhibitory concentrations.
of GZNC on sucrose dependent adherence of all three strains is represented in Figure 3A. The sub-inhibitory concentration (62.5 μg ml⁻¹) of GZNC reduced the adherence of SM 497, SM 34 and SM 06 by 46, 68 and 69%, respectively (results were statistically significant with p ≤ 0.05). There was a considerable decrease in adherence on treatment with sub-inhibitory concentrations of GZNC in all three strains.

S. mutans secretes exopolysaccharide (glucans) in the presence of sucrose which helps in the clumping and adherence of cells. This implies that the composite reduces the polysaccharide mediated adherence of bacterial cells.

The reduction in biofilm and the inhibition of biofilm formation was observed to occur in a dose-dependent manner (Figure 3B). The inhibition of biofilm in SM 497 was 80% while in the case of SM 34 and SM 06, the reduction was found to be 44% and 29%, respectively (results were statistically significant with p ≤ 0.05). The XTT assay was performed to detect the amount of viable cells present after treatment with the composite. SM 497, SM 34 and SM 06 showed only a 37, 31 and 38% decrease in viability, respectively, when treated with 62.5 μg ml⁻¹ GZNC (Figure 3C), which is not statistically significant. No significant differences were observed in the growth curves of all strains compared to the control (Figure 4). XTT and growth curve data indicated that the nanocomposite may be inhibiting virulence traits without affecting bacterial viability.

Effect on acid production and glucan formation

Acid production and acid tolerance are considered to be primary physiological factors linked with the cariogenic potential of S. mutans (Krol et al. 2014). GZNC reduced acid production in both the reference and clinical strains (Table 1). Reducing acid production is a cariostatic effect and may also influence the biofilm forming abilities of S. mutans (Welin-Neilands & Svensater 2007). Water insoluble glucans play a significant role in adhesive interactions compared to water soluble glucans. Further, the effects of different concentrations of GZNC on the synthesis of water insoluble glucans were evaluated (Figure 3D). There were reductions of almost 90, 85 and 60% in the case of SM 497, SM 34 and SM 06, respectively (the results were statistically significant with p ≤ 0.05). A considerable reduction in insoluble glucans was observed in the presence of GZNC. Reducing the amount of insoluble glucans could potentially influence the process of biofilm formation by disturbing the physical integrity and stability, affecting the diffusion properties and reducing the binding sites for S. mutans. The malformed exopolysaccharide (EPS) matrix containing less insoluble glucans may also be more susceptible to the influences of antimicrobials and other environmental attacks (Wang et al. 2013).

Significant reduction in EPS

The production of EPS is one of the key virulence factors of cariogenicity as it is produced by bacteria for the formation, spread and maintenance of biofilms. EPS mediates the adhesion of biofilms to surfaces, provides mechanical stability and transiently immobilizes cells (Flemming & Wingender 2010). EPS production in the presence and absence of GZNC was evaluated by using CR dye which binds to glucose containing polymers. Biofilm formation by S. mutans was tested by growing the organism on BHI agar supplemented with CR in the presence and absence of GZNC. When the colonies were grown without GZNC in the medium, the organisms appeared as dry crystalline black colonies, indicating the production of EPS (Figure 5A). Whereas when the organisms were grown in
the presence of sub-MIC concentrations of GZNC, they continued to grow, but GZNC treatment decreased the synthesis of EPS, as indicated by the decrease in dry crystalline black colonies. Further, the reduction in the amount of EPS of attached cells was calculated by the CR binding assay which directly relates to the amount of EPS formed. The results showed a reduction of almost 51% in EPS production on treatment with sub-MIC concentrations of GZNC (the results were statistically significant with $p \leq 0.05$). Figure 5B clearly indicates that the nanocomposite is reducing EPS production which is a prerequisite for the formation and maintenance of biofilm.

**Microscopic exploration of the effect of GZNC on S. mutans biofilm architecture**

Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) were performed to examine the architecture of the biofilm in the presence of GZNC. In SEM images of control samples (Figure 6A), *S. mutans* cells can be seen embedded in EPS, while in treated samples (Figure 6B) cells were highly dispersed,

![Graph](image1.png)

Figure 3. Inhibitory effect of sub-MIC concentrations of GZNC on: (A) sucrose dependent adherence; (B) biofilm formation; (C) viability (XTT assay); (D) glucan formation.

![Graph](image2.png)

Figure 4. The growth curves of treated and untreated SM 497, SM 06 and SM 34 cells over 24 h.

![Graph](image3.png)

Figure 5. Inhibitory effect of sub-MIC concentrations of GZNC on: (A) sucrose dependent adherence; (B) biofilm formation; (C) viability (XTT assay); (D) glucan formation.
indicating a reduction in EPS. In CLSM images, the majority of cells were observed to be alive (green) in non-treated samples; a green mat was visible with chains of *Streptococcus* cells interacting with each other (Figure 6C). In treated samples, cells were observed to be scattered all around with poor interaction, representing a reduction in biofilm (Figure 6D, E).

**Characterization and anti-biofilm properties GZNC coated tooth surfaces**

Dental implants are widely accepted as a replacement for natural teeth. A considerable percentage of medical implants are the cause of device related infections (Costerton et al. 2005). These infections are difficult to eradicate because the bacteria that cause them live in well-developed biofilms. *S. mutans* biofilm is one of the causes of failure of dental implants (Busscher et al. 2010). Oral implant related biofilms can cause inflammation of peri-implant tissues which may be a direct cause of periodontal disease (Heuer et al. 2007). In general, the most effective way of preventing biofilm formation on implants is to prevent initial bacterial adhesion as biofilms are relatively difficult to remove after formation. Therefore, these infections may be greatly reduced by improving the antimicrobial and anti-biofilm properties of the implant surface by means of surface modification (Zhao et al. 2009).

Acrylic teeth are a good choice for dental prosthesis. The basic ingredient of acrylic teeth is polymethyl methacrylate (PMMA) resin. PMMA resins are a resilient plastic formed by joining multiple methyl methacrylate molecules. A cross linking agent is added which serves as a bridge that unites two polymer chains (Stoia et al. 2011). In this manner it yields a net-like structure that provides increased resistance to deformation, thus the teeth have greater fracture toughness and are easier to adjust.

Thus, the focus of the present study was to observe the ability of nanoparticle coated acrylic teeth to inhibit biofilm formation. Teeth were coated GZNC using sonochemistry. Sonochemical irradiation has been demonstrated to be a successful technique for the synthesis and deposition of nanoparticles on/into glass, polymer supports, and fabrics as well as tooth surfaces (Pol et al. 2009).

|                  | pH ± SD (onset) | pH ± SD (control after 24 h) | pH ± SD (treated after 24 h) |
|------------------|----------------|-----------------------------|-----------------------------|
| SM 497<sup>a</sup> | 7.37 ± 0.09    | 4.87 ± 1.14                 | 5.27 ± 0.06                 |
| SM 06<sup>b</sup>  | 7.38 ± 1.03    | 4.41 ± 0.07                 | 5.06 ± 1.27                 |
| SM 34<sup>c</sup>  | 7.37 ± 0.08    | 4.39 ± 1.23                 | 6.67 ± 0.09                 |

<sup>a</sup>SM 497 (MTCC strain)
<sup>b</sup>SM 06
<sup>c</sup>SM 34 (clinical isolates) (Islam et al. 2008).
Figure 6. Effect of GZNC on biofilm architecture: SEM images of *S. mutans* biofilm in the absence (A) and presence (B) of GZNC, and CLSM images of *S. mutans*. (C, F) control biofilm; (D, G) 62.5 μg ml\(^{-1}\) GZNC treated biofilm; (E, H) 32.2 μg ml\(^{-1}\) GZNC treated biofilm.

2005; Eshed et al. 2013). Figure 7A and C shows photographs of uncoated and coated teeth. The deposition of GZNC was characterized by SEM. Figure 7B represents the uncoated acrylic tooth surface while in Figure 7D, a uniform coating was observed over the entire tooth surface. Further, biofilm biomass on the tooth surface was quantified using the CV assay. GZNC coating of teeth reduced biofilm formation by 85% (Figure 7I), compared to the uncoated tooth surface. Photographic images of CV stained tooth surfaces clearly show the inhibition of biofilm formation (Figure 7J–M). These results were also supported by SEM imaging (Figure 7E, F). A considerable reduction in *S. mutans* biofilm was observed on coated teeth (Figure 7E, F) compared to the control tooth in which dense colonization was observed (Figure 7G, H).
Production of reactive oxygen species

The antibacterial properties of many nanoparticles have been attributed to the production of reactive oxygen species (ROS), such as TiO$_2$ (Su et al. 2009) and Ag NPs (Vecitis et al. 2010). GZNC produced a noticeable increase in cellular ROS (Figure S2). The internalization of zinc oxide nanoparticles in bacteria induces the production of ROS and this can affect DNA and may also affect the total cellular machinery of bacteria (Xie et al. 2011).

The present study confirms that GZNC is very effective against some of the main caries causing virulence factors of *S. mutans*. The anti-biofilm properties of GZNC may be attributed to the wrapping of graphene sheets on the bacterial surfaces which reduces cell to cell interaction and further causes the deposition of ZnO nanoparticle on the surfaces, leading to high concentrations of zinc ions in the cell. Moreover, the leaching of Zn$^{2+}$ ions from nanoparticles may inhibit the active transport and metabolism of sugars. Zinc has also been reported to reduce acid production by *S. mutans* and has the ability to inhibit glucosyltransferase activity (Sevinc & Hanley 2010). Based on the above discussion, a mechanism of anti-biofilm activity of GZNC against *S. mutans* is proposed (Figure 8). Biofilm formation in *S. mutans* is a complex process and more investigation is needed to further understand the mechanisms of biofilm inhibition in the presence of the GZNC.

Cytotoxicity

Despite potential antibacterial activity, the therapeutic use of nanoparticles is limited because of their cytotoxicity against mammalian cells (Yen et al. 2009). Graphene oxide has already been shown to be non-toxic even at very high concentrations (Chang et al. 2011). On the other hand, due to aggregation and other factors, zinc oxide nanoparticles have been reported to be toxic to
mammalian cells even at low concentrations (Yuan et al. 2010). Figure S3 shows the dose dependent effect of GZNC on the viability of HEK 295 cells. There was almost 80% viability during the treatment of cells with 200 μg ml⁻¹ of GZNC, which is three times higher than the concentration used for the anti-biofilm experiment. Even at very high concentrations of GZNC (up to 400 μg ml⁻¹), the cell viability was found to be > 50% after incubation for 24 h. The results clearly indicate that GZNC causes much lower toxicity compared with zinc oxide nanoparticle alone. CLSM images of the internalization of GZNC in HEK-293 cells (Figure S4) showed an increase in blue fluorescence compared with the control with increasing GZNC concentrations. Membrane damage was observed at a much higher concentration of GZNC (400 μg ml⁻¹). These observations may indicate the promising antibacterial and anti-biofilm activity of GZNC.

Conclusions
This study indicates that GZNC may be an effective antibacterial and anti-biofilm agent against S. mutans. Moreover, sub-MIC GZNC treatment resulted in a significant reduction in biofilm and the cariogenic properties of S. mutans. This is the first study where GZNC has been investigated as a potential coating material for dental implants. GZNC coated acrylic tooth surfaces successfully inhibited S. mutans biofilm (85%) formation. Furthermore, the decreased toxicity of the nanocomposite makes it an effective coating agent for dental implants. However, future work on the stability and practical
implication of this nanocomposite is clearly required before it can be implemented.

Acknowledgements

The authors would like to acknowledge the Advanced Instrumentation Research Facility, JNU, for providing instrumental support and the Department of Biotechnology (DBT), government of India, for the support and internal facilities of the department. The Council of Scientific and Industrial Research is acknowledged for grant sanction no. 37(1576)/13/EMR-II to AUK, and SK and S Khan thank BSR-UGC JRF and CSIR SRF respectively, for a fellowship.

References

Abranches J, Miller JH, Martinez AR, Simpson-Haidaris PJ, Burne RA, Lemos JA. 2011. The collagen-binding protein cnm is required for Streptococcus mutans adherence to and intracellular invasion of human coronary artery endothelial cells. Infect Immuno. 79:2277–2284.

Ahmed SA, Gogal RM, Walsh JE. 1994. A rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. J Immunol Methods. 170:211–224.

Aires CP, Tabchoury CP, Del Bel Cury AA, Koo H, Cury JA. 2006. Effect of sucrose concentration on dental biofilm formed in situ and on enamel demineralization. Caries Res. 40:28–32.

Bora T, Lakshman KK, Sarkar S, Makhal A, Sardar S, Pal SK, Dutta J. 2013. Modulation of defect-mediated energy transfer from ZnO nanoparticles for the photocatalytic degradation of bilirubin. Beilstein J Nanotechnol. 4:714–725.

Busscher HJ, Rinastiti M, Siswomihardjo W, van der Mei HC. 2010. Biofilm formation on dental restorative and implant materials. J Dent Res. 89:657–665.

Chang Y, Yang ST, Liu JH, Dong E, Cao YWA, Liu Y, Wang H. 2011. In vitro toxicity evaluation of graphene oxide on A549 cells. Toxicol Lett. 200:201–210.

Costerton JW, Montanaro L, Arciola CR. 2005. Biofilm in implant infections: its production and regulation. Int J Artif Organs. 28:1062–1068.

Cui Y, Zhao Y, Tian Y, Zang W, Lü X, Jiang X. 2012. The molecular mechanism of action of bactericidal gold nanoparticle on Escherichia coli. Biomaterials. 33:2327–2333.

Cullity BD. 1978. Elements of X-ray diffraction. Reading, MA: Addison-Wesley.

Dai K, Lub L, Liang C, Dai J, Zhan Q, Liua Z, Liua Q, Zhang Y. 2014. Graphene oxide modified ZnO nanorods hybrid with high reusable photocatalytic activity under UV-LED irradiation. Mater Chem Phys. 143:1410–1416.

Denizot F, Lang R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 89:271–277.

Dmitriev A, Mohapara SS, Chong P, Neely M, Biswas S, Biswas I. 2011. CovR controlled global regulation of gene expression in Streptococcus mutans. PLoS One. 6:e2012.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem. 28:350–356.

Eshed M, Lellouche J, Banin E, Gedanken A. 2013. MgF2–nanoparticle-coated teeth inhibit Streptococcus mutans biofilm formation on a tooth model. J Mater Chem B. 1:3985–3991.

Eshed M, Lellouche J, Matalon S, Gedanken A, Banin E. 2012. Sonochemical coatings of ZnO and CuO nanoparticles inhibit Streptococcus mutans biofilm formation on a teeth model. Langmuir. 28:12288–12295.

Falletta ML, Klein MI, Colonne PM, Scott-Anne K, Greigore S, Pai CH, Gonzalez M, Watson G, Krysan DJ, Bowen WH, Koo H. 2014. Symbiotic relationship between Streptococcus mutans and Candida albicans synergizes the virulence of plaque-biofilms in vivo. Infect Immun. 82:1968–1981.

Farria AFD, Martinez DST, Meira SMM, de Moraes ACM, Brandelli A, Filho AGS, Alves OL. 2014. Anti-adhesion and antibacterial activity of silver nanoparticles supported on graphene oxide sheets. Colloids Surf B: Biointerfaces. 113:115–124.

Fleming HC, Wingender J. 2010. The biofilm matrix. Nat Rev Microbiol. 8:623–633.

Freeman DJ, Falkiner FR, Keane CT. 1989. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 42:872–874.

Friedman LE, de Rossi BN, Messina MT, Franco MA. 2001. Phenotype evaluation of Bordetella bronchiseptica cultures by urease activity and Congo red affinity. Lett Appl Microbiol. 33:285–290.

Hamada S, Torii M. 1978. Effect of sucrose in culture media on the location of glucosyltransferase of Streptococcus mutans and cell adherence to glass surfaces. Infect Immun. 20:592–599.

Hasan S, Danishuddin M, Adil M, Singh K, Verma PK, Khan AU. 2012. Efficacy of E. officinalis on the cariogenic properties of Streptococcus mutans: a novel and alternative approach to suppress quorum-sensing mechanism. Plos One. 7:e40319.

Hasan S, Singh K, Danishuddin M, Verma PK, Khan AU. 2014. Inhibition of major virulence pathways of Streptococcus mutans by curcumin and deoxynojirimycin: a synergistic approach of infection control. PLoS One. 9:e91736.

Hernández-Sierra JF, Ruiz F, Cruz Pena DCC, Martínez-Gutiérrez F, Martinez AE, de Jesús Pozos Guillén AJP, Tapia-Pérez H, Martínez Castañón GM. 2008. The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold. Nanomedicine. 4:237–240.

Heuer W, Elter C, Demling A, Neumann A, Suerraum B, Hannig M, Heidenblut T, Bach FW, Stiesch-Scholz M. 2007. Analysis of early biofilm formation on oral implants in man. J Oral Rehabil. 34:377–382.

Hu Z, Li J, Li C, Zhao S, Li N, Wang Y, Wei F, Chen L, Huang Y. 2013. Folic acid conjugated Graphene-ZnO nanohybrid for targeting photodynamic therapy under visible light. J Mater Chem. 1:5048.

Huang Z, Zheng X, Yan D, Yin G, Liao X, Kang Y, Yao S, Huang D, Hao B. 2008. Toxicological effect of zinc oxide nanoparticle based bacteria. Langmuir. 24:4140–4144.

Hummers WS, Offman RE. 1958. Preparation of graphitic oxide. J Am Chem Soc. 80:13–39.

Islam B, Khan SN, Haque I, Alam M, Mushfiq M, Khan AU. 2008. Novel anti-adherence activity of mulberry leaves: inhibition of Streptococcus mutans biofilm by 1-deoxynojirimycin isolated from Morus alba. J Antimicrob Chemother. 62:751–757.

Khan R, Zakir M, Khanam Z, Shakil S, Khan AU. 2010. Novel compound from Trachyspermum ammi (Ajowan caraway) seeds with antibiofilm and anti adherence activities against biofilm formation on a tooth model. J Mater Chem B. 2405–2411.
Streptococcus mutans: a potential chemotherapeutic agent against dental caries. J Appl Microbiol. 109:2151–2159.

Koo H, Hayachara MF, Schobel BD, Cury JA, Rosalen PL, Park YK, Vacca-smith AM, Bowen WH. 2003. Inhibition of Streptococcus mutans biofilm accumulation and polysaccharide production by apigenin and tt-farnesol. J Antimicrob Chemother. 52:782–789.

Krol JE, Biswas S, King C, Biswas I. 2014. SMU.746–Liu X, Pan L, Zhao Q, Lv T, Sun Z, Sun C. 2012. UV-assisted photocatalytic synthesis of ZnO-reduced graphene oxide composites with enhanced photocatalytic activity in reduction of Cr(VI). Chem Eng J. 183:238–243.

Loo CY, Corliss DA, Ganeshkumar N. 2000. Streptococcus gordonii biofilm formation: identification of gene that code for biofilm phenotypes. J Bacteriol. 182:1374–1382.

López-Moreno A, Sepúlveda-Sánchez JD, Mercedes Alonso WB. 2014. Calcium carbonate precipitation by heterotrophic bacteria isolated from biofilms formed on deteriorated ignimbrite stones: influence of calcium on EPS production and biofilm formation by these isolates. Biofouling. 30:547–560.

Ma J, Zhang J, Xiong Z, Yong Y, Zhao XS. 2011. Preparation characterization and antibacterial properties of silver-modified graphene oxide. J Mater Chem. 21:3350–3352.

Nakazato G, Tsuchiya H, Sato M, Yamauchi M. 1989. In vivo plaque formation on implant materials. Int J Oral Maxillo-fac Implants. 4:321–326.

Nance WC, Dowd SE, Samarjan D, Chludzinski J, Delli J, Battista J, Rickard AH. 2013. A high-throughput microfluidic dental plaque biofilm system to visualize and quantify the effect of antimicrobials. J Antimicrob Chemother. 68:2550–2560.

Ocsoy I, Paret ML, Ocsoy MA, Kunwar S, Chen T, You M, Tan W. 2013. Nanotechnology in plant disease management: DNA-directed silver nanoparticles on graphene oxide as an antibacterial against Xanthomonas perforans. ACS Nano. 7:8972–8980.

Pol VG, Wildermuth G, Felsche J, Gedanken A, Calderon-Moreno J. 2005. Sonoochemical deposition of Au nanoparticles on titania and the significant decrease in the melting point of gold. J Nanosci Nanotechnol. 5:975–979.

Raghubathi KR, Koodali RT, Manna AC. 2011. Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. Langmuir. 27:4020–4028.

Ruparelia JP, Chatterjee AK, Duttagupta SP, Mukherji S. 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater. 4:707–716.

Selwitz RH, Ismail AI, Pitts NB. 2007. Dental caries. Lancet. 369:51–59.

Sevinc BA, Hanley L. 2010. Antibacterial activity of dental composites containing zinc oxide nanoparticles. J Biomed Mater Res Part B: Appl Biomater. 94:22–31.

Stoia AE, Sinescu C, Pielmasi M, Enescu M, Tudor A, Rominu RO, Rominu M. 2011. Tensile testing, a method used to demonstrate the effect of organic solvents on acrylic teeth denture base resin bond strength. Int J Bio Eng. 5:9–17.

Su HI, Chou CC, Hung DJ, Lin SH, Pao IC, Lin JH, Huang FL, Dong RX, Lin JJ. 2009. The disruption of bacterial membrane integrity through ROS generation induced by nanohybrids of silver and clay. Biomaterials. 30: 5979–5987.

Tang J, Chen Q, Xu L, Zhang S, Feng L, Cheng L, Xu H, Liu Z, Peng R. 2013. Graphene oxide-silver nanocomposite as a highly effective antibacterial agent with species-specific mechanisms. ACS Appl Mater Interfaces. 5:3867–3874.

Vecitis CD, Zodrov KR, Kang S, Elimelech M. 2010. Electronic-structure-dependent bacterial cytotoxicity of single-walled carbon nanotubes. ACS Nano. 4:5471–5479.

Wang H, Liu J, Wu X, Tong Z, Deng Z. 2013. Tailor-made Au@Ag core-shell nanoparticle 2D arrays on protein-coated graphene oxide with assembly enhanced antibacterial activity. Nanotechnology. 24:205102.

Wang Y, Li ZH, Wang J, Li JH, Lin YH. 2011. Graphene and graphene oxide: biofunctionalization and applications in biotechnology. Trends Biotechnol. 29:205–212.

Welin-Nelands J, Svensater G. 2007. Acid tolerance of biofilm cells of Streptococcus mutans. App Environ Microbiol. 73:5633–5638.

XU WP, Zhang LC, Li JP, Lu Y, Li HH, Ma YN, Wang WD, Yu SH. 2011. Facile synthesis of silver@graphene oxide nanocomposites and their enhanced antibacterial properties. J Mater Chem. 21:4593–4597.

Yen HJ, Hsu SH, Tsai CL. 2009. Cytotoxicity and immunological response of gold and silver nanoparticles of different sizes. Small. 5:1553–1561.

Yuan JH, Chen Y, ZHA HX, Song Li-Jun, Li CY, Li J, Xia XH. 2010. Determination characterization and cytotoxicity on HLEF cells of ZnO nanoparticles. Colloid Surf B. 76: 145–150.

Zhao L, Chu PK, Zhang Y, Wu Z. 2009. Antibacterial coating on titanium implants. J Biomed Mater Res B. 91B:470–480.

Zitmann NU, Berglundh T. 2008. Definition and prevalence of peri-implant diseases. J Clin Periodontol. 35:286–291.