Effect of MwCNTs functionalized gallic acid resin and its metal complex composites on biofilms of microbial pathogens

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Abstract

MwCNTs functionalized gallic acid resin and its metal complexes of Zn, Cu, Ni and Co (M_PGaMwCNT) were successfully synthesized and characterized. The impact of M_PGaMwCNT at 100 µg/mL on preventing and controlling biofilms produced by Streptococcus mutans, Enterococcus faecalis and Escherichia coli was investigated. The influence of M_PGaMwCNT on biofilm mass was measured through crystal violet assay and the extracellular polymeric substance (EPS) was quantified using phenol sulphuric acid assay. All the selected bacterial pathogens exhibited a maximum level of bacteriostatic activity at 100 µg/mL. The impact of M_PGaMwCNT, MwCNTs and polygallic acid on bacterial adhesion has revealed Zn_PGaMwCNT and Cu_PGaMwCNT as potential candidates for preventing bacterial adhesion and biofilm formation by selected bacterial strains.

Effect of M_PGaMwCNT on established biofilms of test organisms has shown Cu_PGaMwCNT as the most active agent with 91% and 85% reduction in the EPS concentration of biofilm formed by E. coli, S. mutans and E. faecalis. Zn_PGaMwCNT showed 85, 82 and 62% reductions in EPS of E. coli, S. mutan and E. faecalis. Polygallic acid (PGA) exhibited an 86% reduction of EPS in E. faecalis biofilm. This study further confirmed the enhancement of the antibiofilm efficacy when MwCNTs functionalized with polygallic acid metal complexes.

Keywords: Biofilm inhibition, Gallic acid, Extracellular polymeric substance, Pathogenic bacteria, Polygallic acid metal complex, MwCNTs.

Introduction

Bacterial biofilm production is a significant source of worry in various fields, posing substantial health, medical and industrial risks. The establishment of biofilm is a solemn and ongoing problem in the healthcare industry, as it increases patient morbidity and mortality. External agents including antibiotics, chemicals and disinfectants, resist bacterial pathogens in biofilms.[25] Biofilms aggravate bacterial infections to chronic diseases and are a prevalent trend worldwide.[23,27] Biofilms formed by bacteria can attach to several surfaces including metals, glass, plastics, organs and medical devices.

Bacterial populations form biofilms on various medical devices like prosthetic joints, catheters, sutures, vascular grafts, heart valves, pacemakers and corrective lenses, causing severe infections.[16] Bacteria in biofilms can tolerate extreme circumstances and store secreted polymers such as polysaccharides, proteins, extracellular DNA and amyloidogenic proteins.[23]

The biofilm matrix comprises a variety of biopolymers secreted by the organisms called EPS.[9] These EPS act as a protective layer around the cells. Hence, bacteria in the biofilm are resistant to medications, environmental stresses and even the host's immunological response, posing severe challenges in both industrial and clinical settings.[12,19] The rise of multidrug-resistant (MDR) bacteria has accelerated the search for biofilm inhibitors, specifically obtained from or modified from natural products.[8] Scientists around the globe are concentrating on novel alternatives such as plant extracts and many physiologically active plant-derived medicinal compounds for dissociation of existing biofilms or inhibition of biofilm formation.

Nanotechnology is considered a novel strategy for combating and eliminating biofilm-forming microbes.[21] Carbon-based nanomaterials with unique mechanical, electrical and biological properties have elevated them to the level of exciting platforms for various applications.[1] Multi-wall carbon nanotubes(MwCNTs) have been added to nanoporous solid-state membranes to offer substantial antibacterial and self-cleaning properties, allowing them to be employed in various biomedical devices to tackle biofouling.[2] MwCNTs are increasingly used in polymer matrices to control bacterial adhesion to surfaces, limiting biofilm formation on water pipelines, food containers and medical equipment.[15]

Polydimethylsiloxane (PDMS) is used in manufacturing urinary system-related devices.[18,24] But in biomedical applications, PDMS, like any other silicone material, is sensitive to bacterial biofilms which could be a disadvantage. The inclusion of MwCNTs into PDMS has recently been reported to improve the material's antifouling capabilities.[14]

Considering the proven advantages of MwCNTs, we have employed them as the primary component for synthesizing a new nanocomposite with gallic acid resin (PGaMwCNT) and its metal complexes of Zn, Cu, Ni and Co (M_PGaMwCNT). A comparative study of antibacterial and biofilm quenching capabilities of newly synthesized nanocomposites, MwCNTs and gallic acid resin has been carried out.

Material and Methods

Materials: The reagents utilized in this study were obtained from well-known commercial sources. Gallic acid, formaldehyde, NH_4OH and CH_3OH, were obtained from Spectrochem. MwCNTs and metal acetates of Cu, Zn, Co and Ni were obtained from Sigma –Aldrich. *Streptococcus mutans* MTCC 890, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* MG1586 were initially procured from MTCC, Chandigarh and maintained at the laboratory of Dextrose Technologies Pvt. Ltd., Bangalore and were used in the current study. At 37 °C, *E. faecalis* and *S. mutans* were cultivated in brain heart infusion broth and *E. coli* was grown on Luria Bertani agar/broth. Media used for culturing bacteria were procured from Himedia Laboratories.

Synthesis of MwCNTs functionalized gallic acid resin (PGaMwCNT): 2 m moles of gallic acid were dissolved in 25 mL distilled water in a 100 mL round-bottomed flask. The solution mentioned above was stirred until it became transparent. Next, 1.7 ml of 37 % formaldehyde was added with constant stirring along with 200mg of MwCNTs. The pH of the resulting reaction mixture was eventually adjusted to 8 by adding NH_4OH. Later the reaction mixture was agitated for 3 hours at 85 °C. Finally, the reaction mixture was filtered and rinsed with water before being washed with methanol to remove unreacted impurities. The product was dried in a vacuum oven at 100 °C for 10 hours.

Synthesis of transition metal complexes of gallic acid resin MwCNTs composite (M_PGaMwCNT): After bringing the previously synthesized PGaMwCNT reaction mixture to room temperature, one equivalent of metal acetate (Zn, Cu, Co and Ni) with respect to gallic acid was added with constant stirring for 5 hours. Next, the resultant reaction mixture was filtered, washed with water and then with methanol to remove unreacted impurities. Finally, the product obtained was dried in a vacuum oven at 100°C for 10 hours.

Characterization of M_PGaMwCNT: The structure analysis of **M_PGaMwCNT** was performed by SEM, EDX and FTIR instrumentation. The functional groups in newly synthesized samples were determined using an FT/IR-6000 FTIR spectrometer in the range 4000 to 400 cm^{-1}.

The surface topography of prepared M_PGaMwCNT was studied using a TESCAN-VEGA3 LMU ESEM with a 25 kV accelerating voltage. Each sample was placed on a stub glued with carbon tape and sputtered with gold to capture photos. The SEM was outfitted with an EDX system, which was used to evaluate the elemental composition of PGa, MwCNTs and M_PGaMwCNT on a qualitative level.

Evaluation of the antibacterial activity of M_PGaMwCNT by Well diffusion assay: The antibacterial activity of M_PGaMwCNT, PGa and MwCNTs was evaluated using the well diffusion method. After autoclaving, Petri plates were allowed to dry before being perforated with 6 mm wells. The agar plates were seeded with 100 µL of the inoculums and spread evenly over the agar medium with a sterile glass spreader. Newly synthesized samples and MwCNTs of different concentrations varying from 10–100 µg /mL were added to separate wells in the culture plates and incubated at 30 °C for 24 hours. At the end of the incubation period, the diameter of the zone of inhibition was measured to the nearest millimeter (mm) using a Vernier caliper and tabulated.

***In vitro* Biofilm formation:** For biofilm growth on the surface of microtitre wells, Tryptic Soy broth supplemented with 1% D-(+)-glucose (TSBg) (Sigma Aldrich) medium was utilized. First, 1:100 dilutions of overnight cultures of each bacteria were prepared by mixing 2 µL of a pure cell suspension of 10^7 $CFUmL^{-1}$ with 198 µL of TSBg medium in the wells of a sterile 96-well microtitre plate to set the concentration of the seeding inoculum to 10^5 $CFUmL^{-1}$. Next, a set of wells with 200 µL of TSBg was used as a negative control and all the samples were seeded in triplicate. The microtitre plate was kept for incubation at 37°C for 24-96 hours to induce biofilm formation.[4,17]

After each time, the plates were removed and gently cleaned twice with phosphate-buffered saline. The crystal violet assay was used to estimate the biomass of biofilms[7,11] by staining with 1% crystal violet dye. The excess dye was removed by washing colored wells with distilled water and the dye adhered to the biofilm in each well was dissolved by adding 200 µL of methanol and this sample was used to estimate biomass. The optical density of each sample was calculated and tabulated using a UV-spectrophotometer at 530 nm.

Quantification of EPS (Extracellular polymeric substances): As described earlier, the biofilm formation by bacterial strains was carried out on a microtitre plate. First, the microtitre plate was decanted to remove the free-floating contents of the wells and later, EPS was extracted from the biofilms by treating with 0.85% NaCl solution. The resulting NaCl solution containing EPS was centrifuged at 6000 rpm for 10 minutes at 4 °C. Next, the supernatant obtained was treated with methanol to precipitate EPS and the precipitate was incubated at -4°C overnight. After overnight incubation, the contents were centrifuged at 6000 rpm for 10 minutes at 4 °C to obtain the pellet. Finally, the pellet was subjected to phenol sulphuric acid assay to determine EPS using glucose as the standard by spectrophotometric method at 490nm.