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Green synthesis and *in vitro* evaluation of silver nanoparticle embedded antibiofilm coatings

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Abstract : Biofilm growth is widespread in medical and industrial settings with serious health and economic consequences. Bacterial growth on surfaces is problematic because bacteria within biofilms show enhanced to antimicrobials. Silver-based antimicrobial agents received much attention due to better efficacy and broad-spectrum activity. Here, we report a novel and straight forward preparation of silver-embedded coatings for antibiofilm applications. Rosin, a natural exudate from pine trees, enabled reduction of ionic silver (+) to nanoparticulate silver (0) and formation of a silver nanoparticle-embedded composite coating onto material surfaces at ambient conditions. Formation of Ag(0) nanoparticles was confirmed by UV-Vis spectroscopy, X-ray absorption spectroscopy, transmission electron microscopy and field emission scanning electron microscopy. Silver-nanoparticle-embedded coatings prevented adhesion and biofilm formation by Gram-positive and -negative bacteria. These coatings could find applications in controlling bacterial colonization in a variety of settings.

Keywords : Antibiofilm coatin; Biofilm control; Green chemistry; Silver nanoparticles.

INTRODUCTION

Bacterial colonization and biofilm formation on material surfaces is the most widespread growth mode for a vast majority of bacteria[1]. Biofilm growth is ubiquitous in medical and industrial settings with serious health and economic consequences[2,3]. In industry, biofilm growth can impede heat transfer, flux across membranes, and cause food spoilage and equipment contamination[4-7]. In health, biofilms are problematic because they tend to grow on teeth and on implanted medical devices and cause persistent infections[8]. Biofilm development involves attachment of bacterial cells to a surface, proliferation of attached cells and production of complex extracellular polymeric substance (EPS) matrix[9,10]. Bacterial cells in a biofilm exhibit two fundamental characteristics such as production of EPS matrix and enhanced resistance to antimicrobials[11]. The

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EPS is often composed of polysaccharides, proteins and DNA and dependent on type of bacteria[12]. Biofilm-bacteria are more resistant to antimicrobials as compared to their planktonic-counterparts and their elimination is a big challenge in medical and industrial settings[13]. Novel approaches are needed to prevent bacterial colonization and biofilm growth[14-17].

In this context, silver based antimicrobial agents received much attention for controlling bacterial growth and biofilm formation[18-25]. There is good evidence that nano-sized silver exhibit broad-spectrum and enhanced antimicrobial activity[20]. Various chemical (in situ reduction of metal salts or mixing preformed metal nanoparticles with polymers) or physical methods (i.e., sputtering, plasma deposition and layer-by-layer deposition) have been reported for developing silver embedded antimicrobial surfaces[26]. But, most of these methods involve multi-step synthesis, often use toxic chemicals and require high temperature and/or pressure. To overcome some of these drawbacks, there is a need to develop simple and environmentally benign processes for synthesis of metal nanoparticles[27,28] and development of antimicrobial coatings[26].

In this communication, we report a single-step procedure for synthesis of silver nanoparticle embedded composite coating at ambient conditions for antibiofilm applications. Gum rosin, a plant product was used for reduction of Ag(+) to Ag(0) nanoparticles in methanol medium at ambient conditions. A composite coating was developed on material surfaces upon incubation. We have characterized the formation of Ag(0) nanoparticles and silver nanoparticle embedded coating using various spectroscopic and imaging techniques and evaluated for bacterial biofilm prevention in vitro assays.

MATERIALS AND METHODS

Chemicals

Gum rosin (Colophony) was obtained from Sigma-Aldrich. Abietic acid (75% HPLC), silver benzoate and silver nitrate were obtained from Sigma, USA. Methanol was of analytical grade, obtained from Merck, India. Commercial grade pale-yellow colored rosin was purchased from the market and used directly by dissolving in methanol. Commercial grade rosin is manufactured by heating fresh liquid resinous material exuded from pine plants to vaporize the volatile liquid terpene components. It chiefly consists of different resin acids especially abietic acid. Initial experiments were formed using commercial rosin. Subsequently, all the experiments were carried out using Gum rosin obtained from Sigma-Aldrich.

Bacterial strains and growth conditions

Gram-positive bacterium, *Staphylococcus aureus* V329 and Gram-negative bacterium, *Pseudomonas aeruginosa* PAO1 were used for evaluating anti-biofilm activity. *S. aureus* V329 is a very efficient biofilm forming bacterium isolated from bovine mastitis[29]. *P. aeruginosa* PAO1 is commonly found in environmental and medical settings and one of the commonly used model organism in biofilm assays[30]. These bacteria were cultured in half-strength tryptic soy broth (TSB) supplemented with 0.25% (w/v) glucose. After inoculation, culture flasks were incubated at 30°C in an orbital shaker at 100 rpm. Overnight grown cultures were used as the inoculum in biofilm experiments.

Synthesis of silver nanoparticles by commercial rosin/gum-rosin/abietic acid

Rosin was used as the reducing and stabilizing agent. Typical reaction mixture consisted of 100 ml of methanol, 0.5 g of rosin, and 0.023 g of silver benzoate or silver nitrate. Synthesis was carried out under stirring at ambient conditions without heating. The concentration of rosin and silver benzoate / silver nitrate was varied. Synthesis was also carried out by replacing commercial grade rosin with gum-rosin and abietic acid.

Characterization of silver nanoparticles

The composite material was dried at 100°C and characterized using ultraviolet (UV)-visible spectroscopy, X-ray diffraction (XRD) and high resolution transmission electron microscopy (HRTEM). The UV-vis absorption spectra of the nanoparticle solutions were recorded using Shimadzu spectrophotometer (Japan), from 300 to 800 nm, against water blank. Aqueous dispersion was prepared by centrifugation at 10000 rpm for 5 min and re-suspending in ultra-pure water. Dried powder was for obtained XRD pattern. The HRTEM images were acquired using JOEL3010 (Tokyo, Japan), operating at 200 KV. The particle size distribu-

tion of was determined using *ImageJ*1.4 freeware.

Silver nanoparticle embedded coatings on material surfaces

After 2 h of stirring at ambient conditions, the reaction mixture was aliquoted into 50 ml falcon tubes. A clean glass slide was placed vertically into each falcon tube containing the reaction mixture. A pale yellow coating was evident on glass slides or falcon tube surfaces. At the end of 24 h, the glass slide and the falcon tubes were air dried, washed and used for biofilm formation studies. Glass slide with pale yellow coating was used for recording UV-vis spectra and X-ray photoelectron spectra (XPS). Coating was developed on a silicon wafer for field emission scanning electron microscopy (FESEM) - energy dispersive x-ray spectroscopy (EDAX) analysis.

Biofilm formation assays

Bacterial colonization and biofilm development on silver nanoparticle embedded coatings was determined. Plain material surfaces or material surfaces received rosin-coating was used as blanks in biofilm experiments. Biofilm formation was studied by exposing material surfaces to Gram-negative bacteria *Pseudomonas aeruginosa* PAO1 and Gram-positive bacteria *Staphylococcus aureus.* Biofilm assays were performed according to already described protocols[16]. Briefly, 20 mL of half-strength TSB was dispensed into 50 mL falcon tubes and inoculated with 50 µl of overnight grown culture (OD 600_{nm} = 0.2). Each glass slide with or without silver embedded coating was transferred to a falcon tube and was incubated at 30°C in an orbital shaker at 100 rpm. At 24 h of incubation, the glass slides were retrieved, rinsed with dimeneralised water and stained for imaging as described below.

Biofilm staining and confocal laser scanning microscopy

Biofilms were stained using LIVE/DEAD® *Bac*Light™ bacterial viability kit (L-7012, Molecular Probes, USA) as described earlier[7,16]. The slides were rinsed with dimineralised water, covered with a glass cover slip and sealed using a nail polish. Stained biofilms were imaged with a confocal laser scanning microscopy system (Leica TCS SP2 AOBS) equipped with an inverted microscope DMIRE2 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany). A 63x/1.2 numerical aperture water immersion lens was used for acquiring all images. Argon 488 nm laser was used for excitation. Emission was collected by setting the detection bandwidth between 500 - 520 nm for SYTO 9 and 600 - 630 nm for propidium iodide. Images stacks were acquired using 1.2 air pinhole with two-frame averaging. Images are presented using Leica confocal software 2.0 without any processing.

RESULTS AND DISCUSSION

Rosin, a natural exudate from pine and fir trees, is widely used in adhesives, paints and a traditional binder in antifouling assays[31-33]. The chemistry of rosin is complex and variable due to its origin from natural systems[32,33]. Abeitic-type resin acid is the predominant in commercial rosins and rosin are often classified according to the color for commercial purpose[33]. Incidentally, we found that rosin can catalyze the reduction of silver (+) ions and silver (0) nanoparticles very efficiently at room temperature. This finding is novel as the procedure is straightforward (single step) and it is according to green chemistry principles. In the synthetic procedure described here, rosin acted as the reducing agent; while methanol was used as the solvent. The synthesis of silver nanoparticles was straightforward. In a typical procedure, $AgNO_3$ and of rosin were added to methanol and the mixture was stirred on a magnetic stirrer for 2 hours and kept idle at room temperature for 24 h. The formation of silver nanoparticles was confirmed using UV-visible absorption spectroscopy, X-ray diffraction, X-ray absorption spectroscopy, and electron microscopy.

Visual changes were evident when silver nitrate was added to rosin dissolved in methanol during the course of stirring at room temperature. The solution turned pale yellow and brown within 2 h of stirring at room temperature. The UV-vis absorption spectrum of the sample, showing the surface Plasmon resonance (SPR) of Ag (0) nanoparticles, is presented in Figure 1a. An absorbance peak with a maximum at 420 nm was evident due to surface plasmon resonance of Ag (0) nanoparticles (Figure 1a). When silver nanoparticles were transferred to aqueous medium, they were stable during storage at room temperature. The average size

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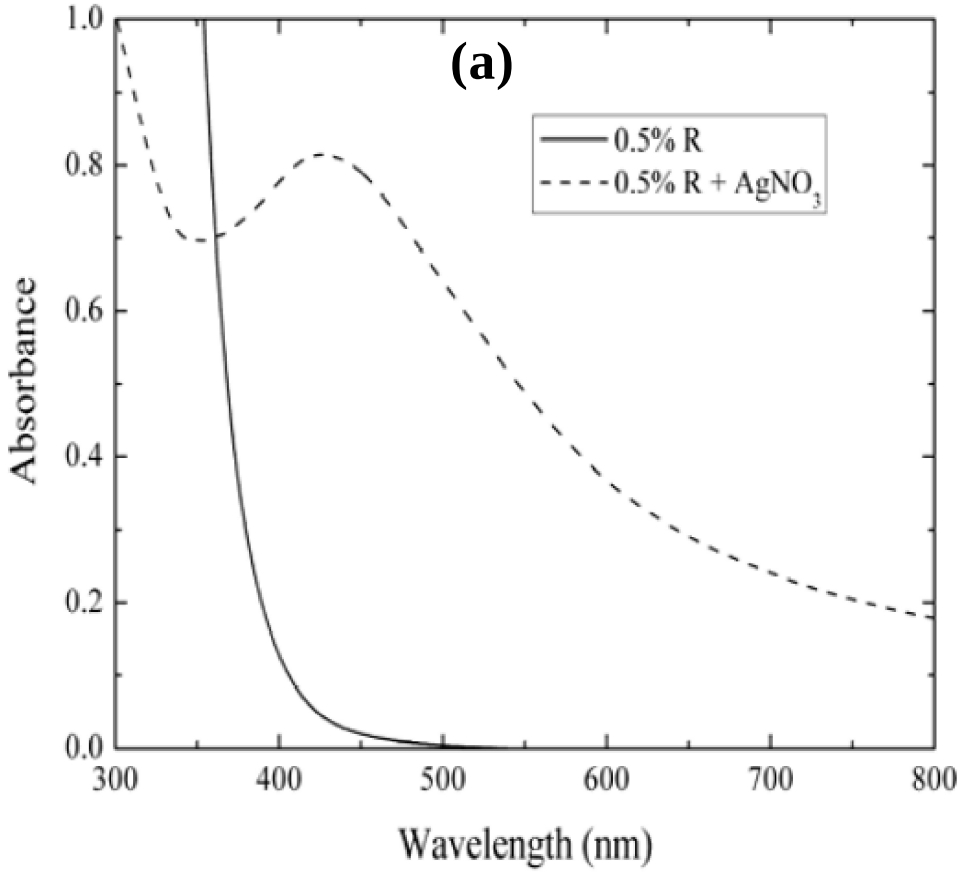

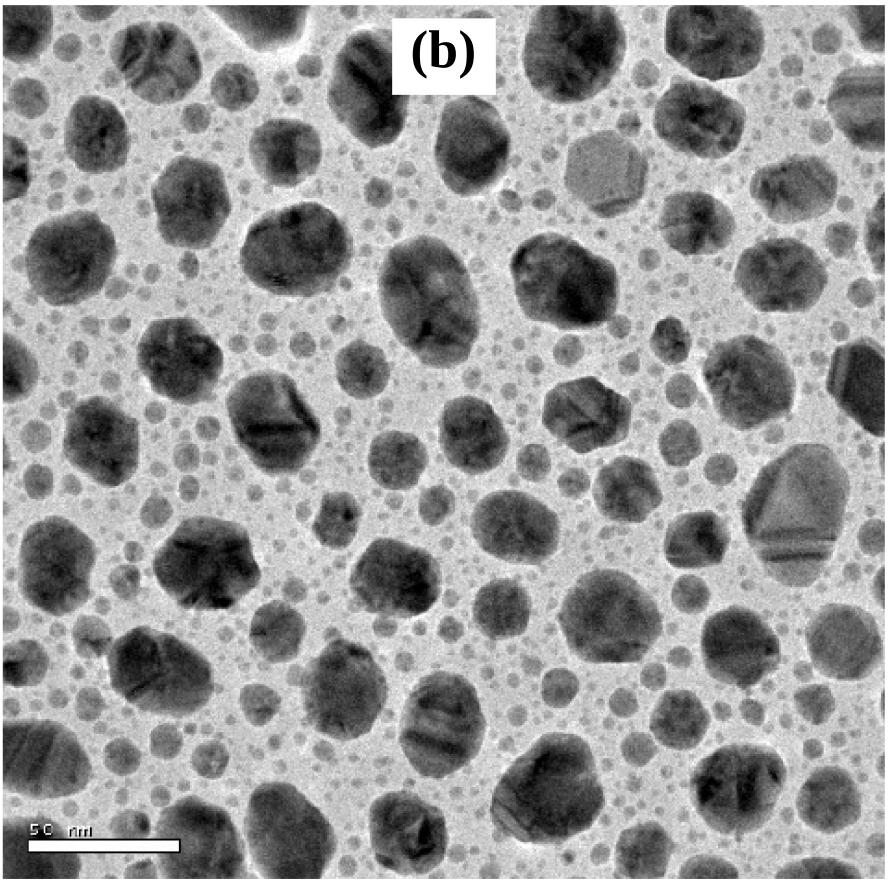

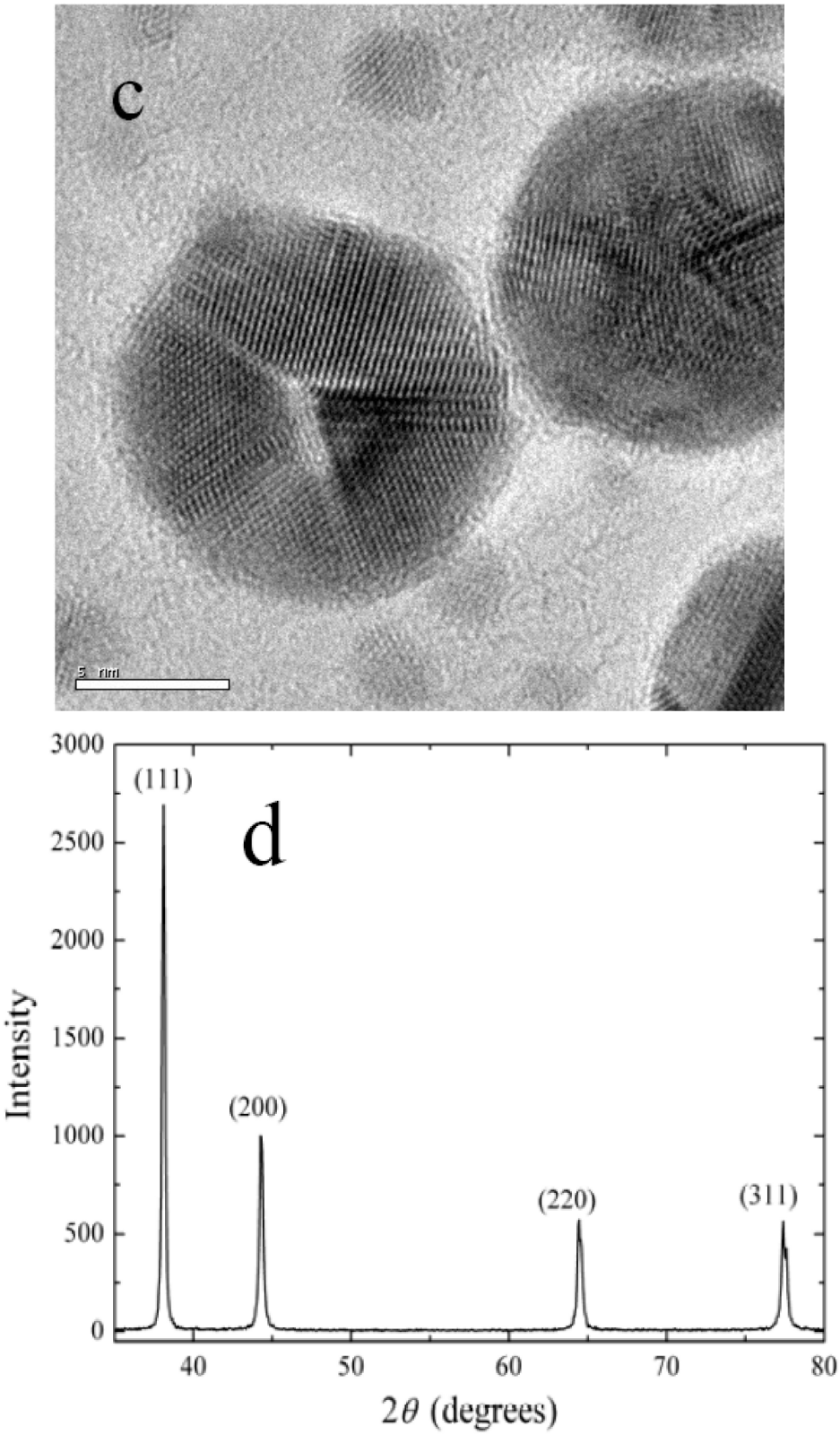

Figure 1 : Characterization of Ag(0) nanoparticles formed by rosin. a) UV-Vis spectra of silver nanoparticles. Synthesis mixture 1 mM of $AgNO_3$ and 0.5% rosin in methanol was centrifuged at the end of 2 h reaction. The silver nanoparticles were recovered by centrifugation and the pellet was re-suspended in 50 ml demineralized water for recording UV-vis spectra. b and c) high-resolution transmission electron microscope image of silver nanoparticles. d) X-ray diffraction spectra of silver nanoparticles formed by rosin.

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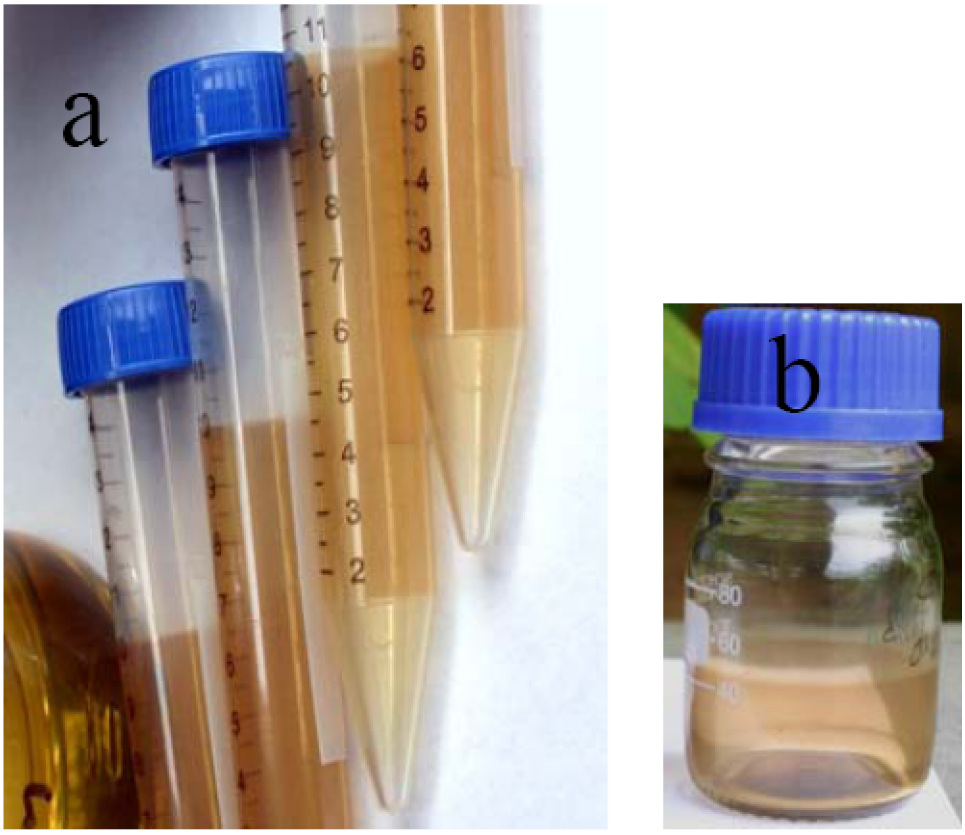
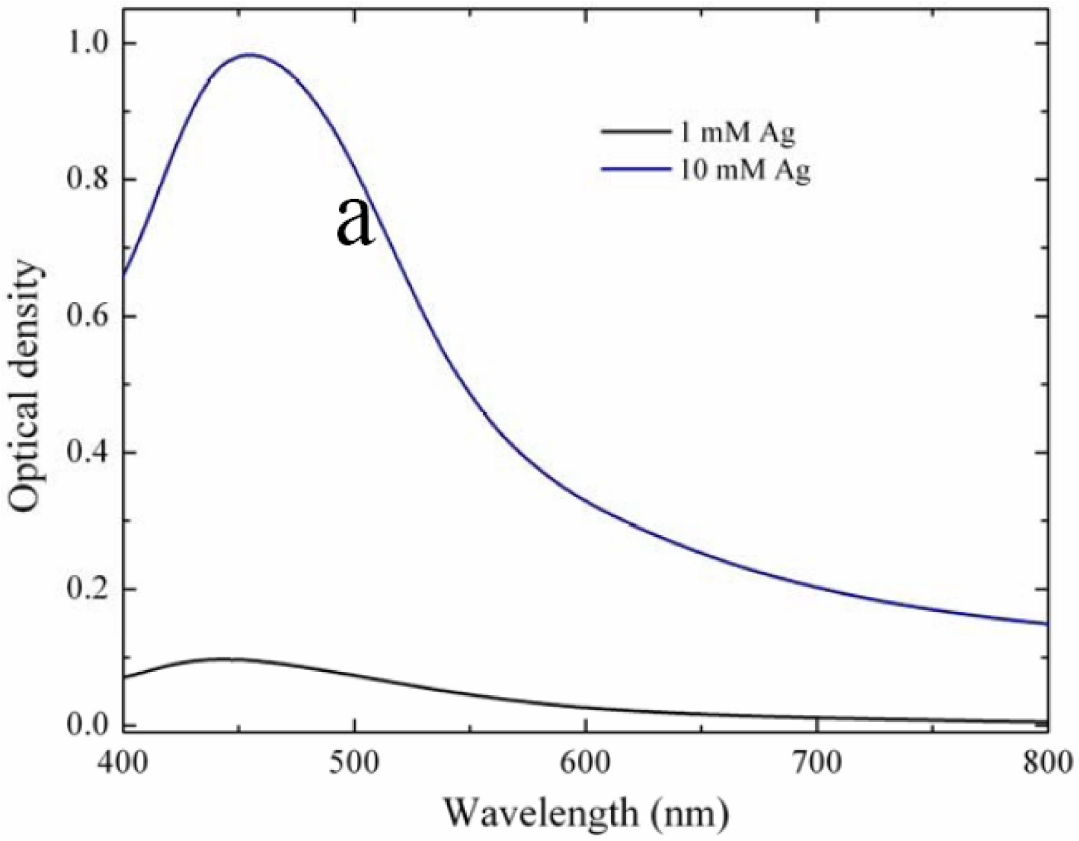


Figure 2 : Images of silver-nanoparticle-embedded coatings formed on polyvinyl chloride (a) and glass (b) surfaces. Yellow color indicates the deposition of silver-nanoparticle-embedded rosin composite.

of silver-nanoparticles was derived from the TEM images and found to be 7.5 ± 6.1 nm (Figure 2). Majority of the silver nanoparticles were in hexagonal shape (Figure 1b and c). XRD analysis of the powder sample confirmed the crystalline structure of silver nanoparticles (Figure 1d). The choice of the solvent medium, the selection of reducing agent and the stabilizing agent are according to green chemistry principles. The synthetic procedure described here used methanol and rosin, as the solvent medium and reducing agent, respectively. Methanol was used as the solvent medium as rosin is sparingly soluble in water. Rosin is commercially available, non-toxic and renewable. Importantly, this method does not require any external heating and the synthesis occurs at room temperature (30°C). Overall, the method used for synthesis of silver nanoparticles is straight-forward, and uses inexpensive, commercially available renewable material as the reducing agent. Excepting the solvent medium, the procedure fully complies with the green chemistry principles. Moreover, the use of methanol facilitated the coating of the silver nanoparticle embedded composite onto material surfaces.

The silver nanoparticles generated in the methanol tend to form a coating on the surface of the container during incubation at room temperature (Figure 2). Such silver nanoparticle embedded rosin coating on surfaces was observed during overnight exposing of material specimens at ambient conditions (Figure 2). The coating formation was dependent on the initial concentration of Ag (+) ions and rosin. This method produced reproducible coating formation on the material surfaces. UV - vis spectroscopy and FESEM imaging confirmed the presence of silver nanoparticles in the coating (Figure 3). The coating contained significant amounts of silver as confirmed by the EDAX analysis. XPS confirmed the presence of both Ag(+) and Ag(0) ions in the coating. Based on the analysis of XPS spectra, it was concluded that Ag nanoparticles of dimension 7 – 10 nm are covered with a thin layer of Ag_2O with an approximate thickness of 13 Å.

Subsequent to the material characterization, the silver nanoparticle embedded coating was evaluated for antibiofilm activity in vitro using Gram-negative (*Pseudomonas aeruginosa* PAO1) and Gram-posi-



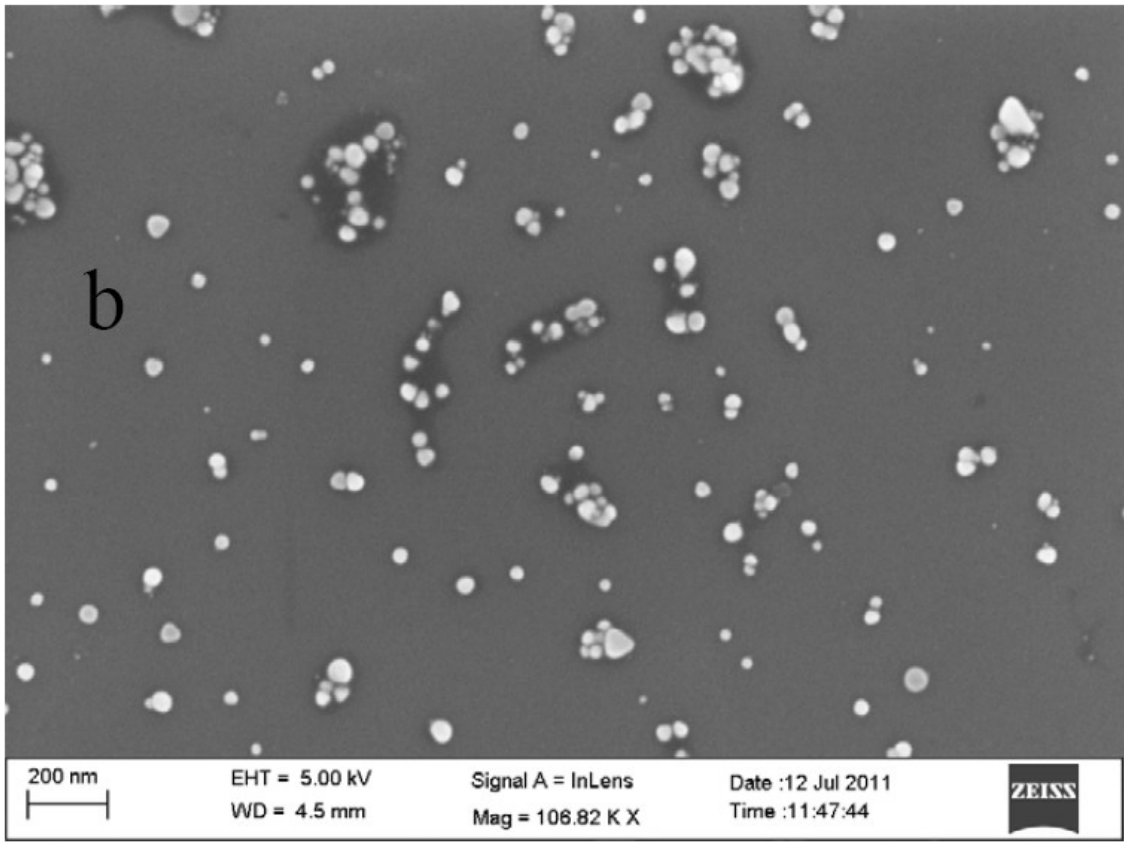


Figure 3 : UV-Vis spectra (a) and field-emission scanning electron microscope image (b) of silver-nanoparticle embedded coating.

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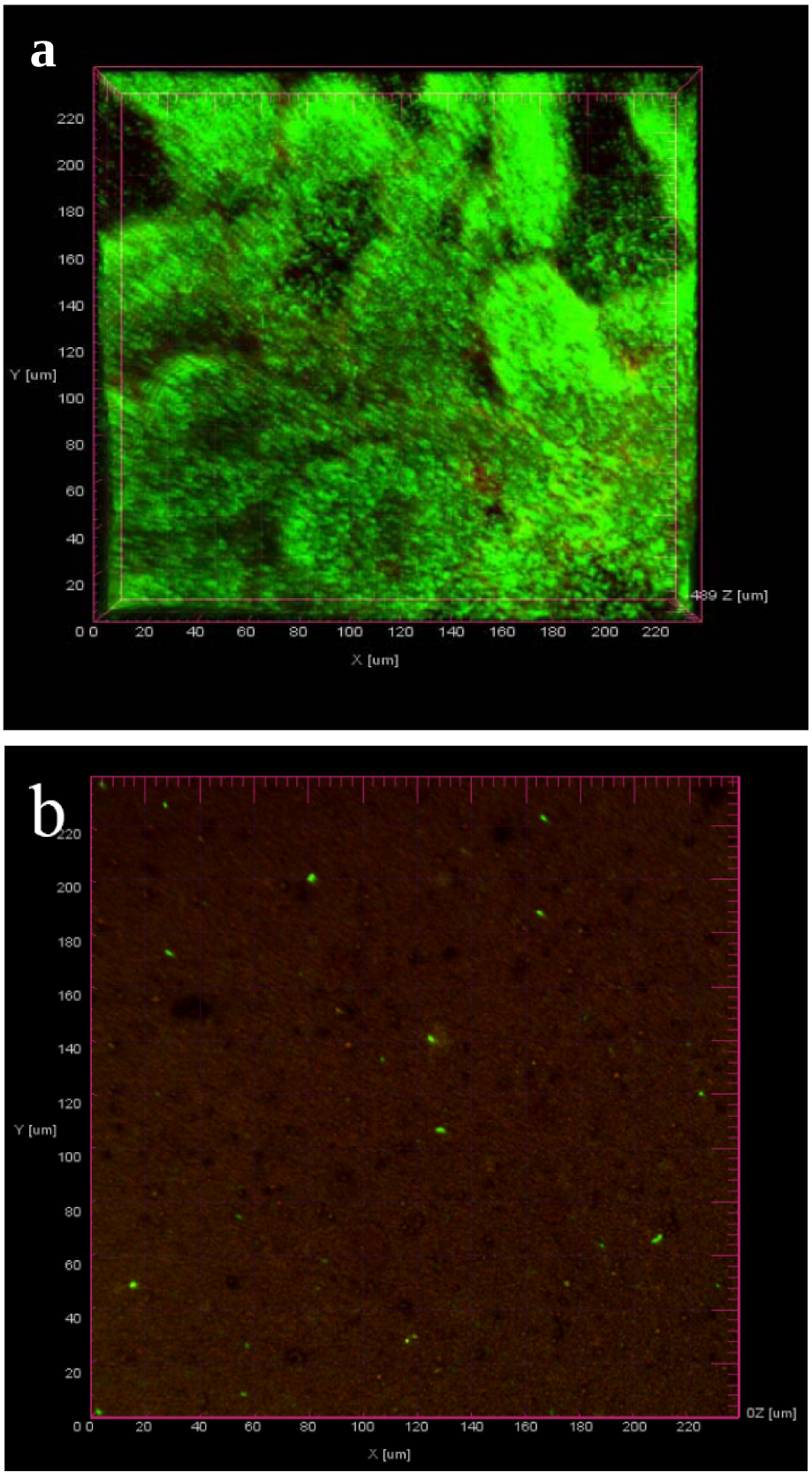

Figure 4 : Biofilm growth of *P. aeruginosa* PAO1 on control glass surface (a) and silver-embedded-coating (b). Attachment and biofilm growth was negligible on glass slides with silver-embedded coating. Control biofilm image was reconstructed with 16 xy-slices collected at a z-interval of 2 microns.

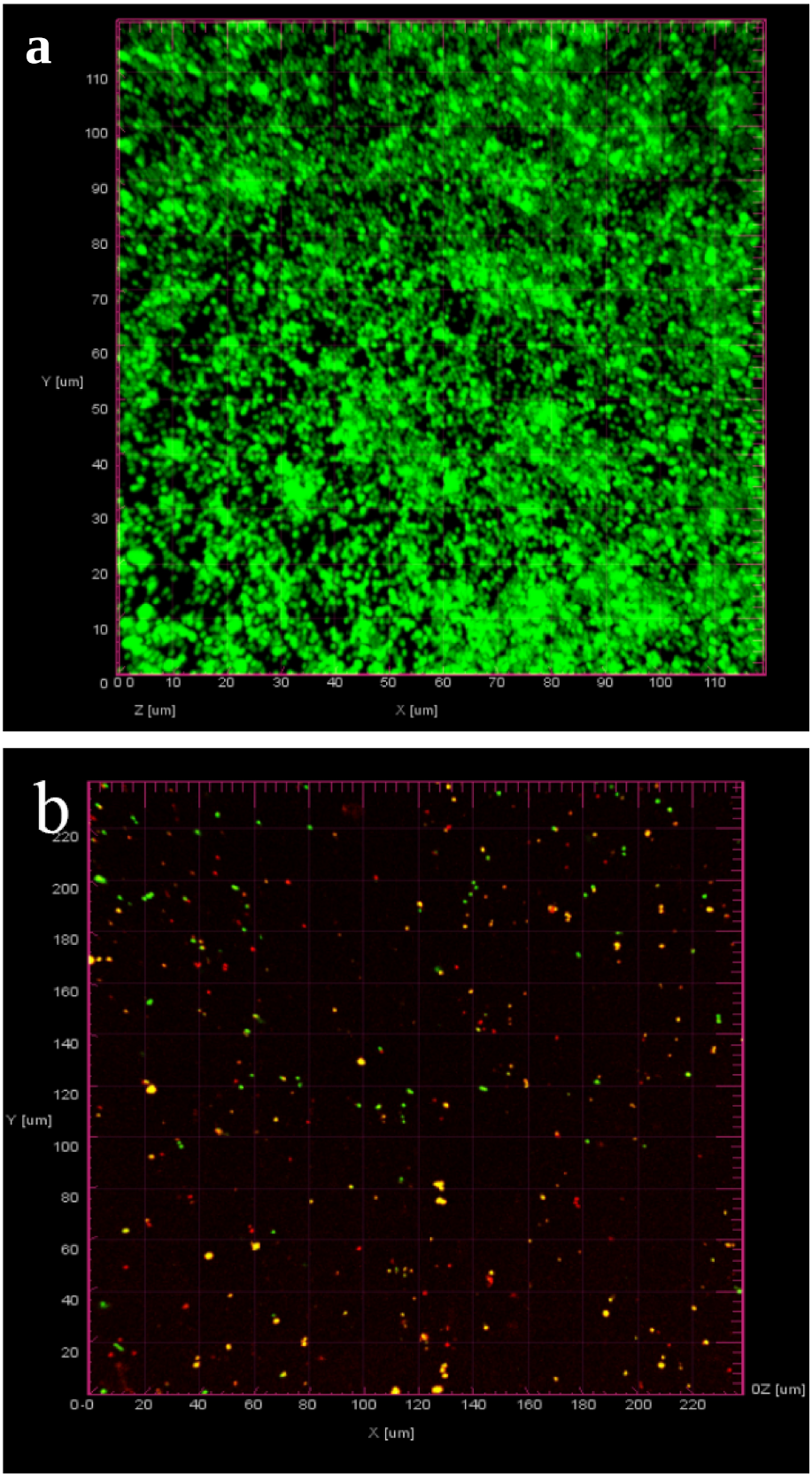

Figure 5 : Biofilm growth of *Staphylococcus aureus* on control (a) and silver-nanoparticle-embedded composite coated (b) glass surfaces. *S. aureus* colonization and biofilm growth was inhibited on glass slides with silver-embedded coating. The control biofilm image was reconstructed with 14 xy-slices collected at z-interval of 2 microns.

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tive (*Staphylococcus aureus* SAV329) bacteria. Microscopic glass slides with and without coating were exposed to *P. aeruginosa* or *S. aureus* cultures. Glass slides exposed to rosin without silver did not inhibit bacterial adhesion and growth. The surfaces without coating were almost fully colonized by the bacterial cells and microcolonies. The biofilms consisted of 3-dimentional structure as visualized by the confocal fluorescence microscopy, often found in majority of biofilms[10]. While the glass slides with coatings embedded with silver nanoparticles inhibited bacterial adhesion and growth. The coatings prevented biofilm formation by *P. aeruginosa* and *S. aureus* (Figure 4 and 5).

Attachment was sparse with isolated bacterial cells on surfaces covered with silver nanoparticle embedded coatings. Moreover, majority of the attached bacterial cells were propidium iodide stained (yellow or red) indicating that they have compromised cell membrane[16]. Release of silver into the medium was not observed in biofilm experiments. However, the release and long-term activity of the coatings requires further experimentation. Silver nanoparticles are known to interact with the cytoplasmic membrane, causing permeabilization and eventually killing of bacterial cells[34-36]. Silver nanoparticles of smaller size (i.e. 15 nm) were reported to have efficient bactericidal activity[34] as compared to larger particles. The silver nanoparticles observed in the coating were of smaller in size (~10 nm) and presumably detrimental to bacterial colonization and biofilm-growth. Earlier studies have shown the presence of Ag(+) ions in Ag(0) nanoparticles[26]. In our study, XPS confirmed the presence of both Ag(0) and Ag(+) in the coatings. Therefore, it is conjectured that both Ag(0) particles and Ag(+) ions contribute to antibiofilm activity. In conclusion, the synthetic method described here enabled straight-forward formation of silver nanoparticles and development of anti-biofilm composite coatings on surfaces using a simple and completely green approach.

CONCLUSIONS

We have reported a novel straightforward method for the preparation of Ag(0) nanoparticles and silver embedded coatings. To the best of our knowledge, this is the first report on the use of rosin for synthesis of Ag(0) nanoparticles and silver nanoparticle embedded coatings. The silver nanoparticle embedded composite coatings were characterized using various spectroscopic and microscopy techniques. The silver nanoparticle embedded coatings have prevented colonization and biofilm formation by Gram-positive and –negative bacteria in vitro assays.

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