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Revelation of susceptibility differences due to Hg(II) accumulation in *Streptococcus pyogenes* against CX-AgNPs and Cefixime by atomic force microscopy

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ABSTRACT

Solution based method for the formation of chemically modified silver nanoparticles (CX-AgNPs) using Cefixime as stabilizing and reducing agent was developed. The CX-AgNPs were characterized by AFM, UV–visible, FT-IR and MALDI-TOF MS. Bactericidal efficiency of CX-AgNPs and Cefixime against *Streptococcus pyogenes* was evaluated. Afterwards, susceptibility differences of *Streptococcus pyogenes* due to accumulation of Hg(II) against CX-AgNPs and Cefixime were estimated and validated through Atomic force microscopy. Selectivity and sensitivity of CX-AgNPs against Hg(II) was evaluated in a systematic manner. The CX-AgNPs was titrated against optically silent Hg(II) which induced enhancement in the SPR band of CX-AgNPs. The increase in intensity of SPR band of CX-AgNPs was determined to be proportionate to the concentration of Hg(II) in the range of 33.3–700 μM obeying linear regression equation of $y = 0.125x + 8.962$ with the detection limit of 0.10 μM and the coefficient of determination equals to 0.985 ($n = 3$). The association constant Ka of CX-AgNPs-Hg(II) was found to be 386.0095 mol^{-1} dm^3 by using the Benesi Hildebrand plot.

1. Introduction

Cefixime belongs to third generation of cephalosporin, and as compare to cephalosporins of first-generation it is defiant against hydrolysis by the β-lactamases generated by gram-negative bacteria to a greater extent; the core structure of Cefixime is a fused β-lactam thiazole ring system. Moreover, its distinguishing features are the existence of two non-esterified carboxylic functional groups and vinyl group on the cephen nucleus which imparts resistance towards acid hydrolysis (Duverne et al., 1992; Faulkner et al., 1988; Roche, 1988). Therefore, numerous microorganisms invulnerable to cephalosporins and Penicillin are defenseless against Cefixime. Over the last few decades, various drug-metal complexes demonstrated transformed therapeutic activities (Brown et al., 1980; Sorenson, 1976; Williams, 1971). Similarly, complexation of Cefixime with various metals and their therapeutic potential has also been proclaimed (Anacona and Estacio, 2006; Kaushal et al., 2014). Integration of biological molecules with metallic nanoparticles embodies hybrid systems which ultimately paves the path from cell markers to bio-sensing (Bruchez et al., 1998; Lahav et al., 1999), bio-imaging (Djalali et al., 2002) and targeted drug delivery (Li et al., 1999), hence coating of Cefixime around metal nanoparticles is a valid approach.

Moreover, the study of morphological changes of bacterial strains after treatment is an effective tool for determining the potency of antibiotics which disrupt the morphology of that strain as an epiphenomenon of their mechanism of action (Braga et al., 1997; Braga and Ricci, 2002; Gemmell and Lorian, 1996). In this domain, Atomic Force Microscopy provides substantial advantages such as accession of quantifiable data in 3D, minimum time for specimen preparation, operating under atmospheric condition, doesn't require either vacuum or gold sputtering and efficacious enlargement of data (Braga and Ricci, 1999, 1998). *Streptococcus pyogenes* is a gram positive bacterium and is susceptible to Cefixime (Camara et al., 2013; Todar, 2005). *Streptococcus pyogenes* is one of the most frequent pathogens of humans. Moreover, it is also known for the speciation of Hg(II) and had also demonstrated sorbent capacity of 4.8 mg/g for Hg(II) (Tuzen et al., 2009). Mercury toxicosis leads to impairment of the nervous system, immune system, genetic and enzyme systems hence eventually threatens the life (Langford and Ferner, 1999). Due to bioaccumulation of mercury, from earth, running streams and atmosphere and biological magnification by consuming mercury enriched seafood, humans readily absorb mercury unconsciously. Thus, mercury is an important pollutant that continues to be under close scrutiny. At present, techniques for examining mercury levels in aqueous environments include colorimetry

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(Li et al., 2009), spectrofluorometry (Xia and Zhu, 2008), electrochemistry (Cesarino et al., 2008) and ICP-MS (i.e., inductively coupled plasma mass spectrometry) (Jackson et al., 2009). However almost all the assays cited above, rely either on well equipped laboratories to work under constrain conditions or based on prolonged procedures. Furthermore, these assays are susceptible to intermeddling effects of other species even the minute interferences can make the sensor ineffective especially when transduction of signal is in the form of optical responses (i.e., emission or absorption).

Solution to the above stated problem lies in the utilization of nanoparticles as a sensor as their optical properties can be tailored by regulating self-assembling processes (El-Sayed, 2001; Haynes et al., 2003; Haynes and Van Duyne, 2001; Kelly et al., 2003; Kneipp et al., 1999; Stolz et al., 1997).

Herein, we are demonstrating a facile and swift method for the synthesis of nanoconjugates of silver with Cefixime (CX-AgNPs). Moreover, bactericidal efficiency of CX-AgNPs against *Streptococcus pyogenes* was evaluated which is known to be susceptible against Cefixime (Camara et al., 2013). Afterwards due to the bioremediation capability of *Streptococcus pyogenes*, the differences in susceptibility of *Streptococcus pyogenes* due to accumulation of Hg(II) against CX-AgNPs and Cefixime were also assessed. Morphological studies of control, Hg (II) accumulated, Cefixime treated and CX-AgNPs incubated bacteria were also conducted by Atomic Force Microscopy.

2. Materials and methods

2.1. Synthesis of CX-AgNPs

About 6.8 milligrams of $AgNO_3$ was dissolved in 40 mL of water, and subsequently 10 mL of a solution of Cefixime in methanol (4.5 milligrams Cefixime dissolved in 10 mL of methanol; previously stirred for 10 min) was added into it. Afterwards, we added triethylamine primarily working as an accelerating agent (Hsu and Wu, 2007; Rasheed et al., 2016; Wu and Hsu, 2008) and kept stirring for 30 min.

2.2. Characterization of CX-AgNPs

UV–visible spectra were recorded with Evolution 300 UV–visible spectrophotometer by Thermo-vision with a quartz cuvette (path length ≈ 1 cm). Vector 22 FTIR Spectrophotometer was deployed for recording of infra red spectra. Mass measurements were conducted on Ultraflex MALDI TOF-TOF. Specimen for mass measurement was formulated by dispensing 1:1 (v/v) of CX-AgNPs with matrix (i.e., 2.5 mg/mL 4-Hydroxy-2-cyanocinnamic acid in acetonitrile: water: trifluoroacetic acid (50:50:0.1)) on to a target plate. Subsequently, irradiation of the sample was performed by nitrogen laser and the spectrum was recorded in reflection mode for the attainment of higher resolution. Morphological studies were conducted by using Atomic Force Microscope (AFM) Agilant-5500, operated in tapping mode.

2.3. Mineral and bacterium specimens

Selection of mineral specimen was based on their surface charge and hydrophobicity. For muscovite point-of-zero-charge (pzc) = 2–3 and contact angles (θ) = near 0°. Since muscovite exhibits perfect basal cleavage hence it was peeled instantaneously prior to usage (Adamson and Gast, 1997; Stumm and Morgan, 1996). In vitro bactericidal activities of silver nanoparticles (CX-AgNPs) and its organic precursor, Cefixime were evaluated against *Streptococcus pyogenes ATCC 700294*.

2.4. Determination of threshold value/maximal non-cytotoxic dose of Hg (II) by MTT assay

Freshly harvested cells *Streptococcus pyogenes ATCC 700294* isolated from tryptone soya agar were seeded at 10^6 cells in each well of 96 wells plate. Distinct concentrations of Hg(II) (i.e., 500–5 μg/mL) were prepared by serial dilution method by utilizing Muller Hinton broth as a diluent. Afterwards, 200 μL of each concentration was added in duplicate well and the plate was incubated for 18 ± 2 h at 35.5 ºC. After incubation, in each well 20 μL of tetrazolium dye (MTT) (i.e., 3-(4, 5-dimethylthiazol-2-yl) − 2, 5-diphenyltetrazolium bromide) having a concentration of 5 mg/mL was added and the plate was incubated at 37 ºC for 4 h. Afterwards, for the dissolution of crystals of formazan salt formed inside each well, dimethyl sulfoxide was added. After dissolution, the absorbance was measured at a primary wavelength of 570 nm and a reference wavelength of 650 nm (Wang et al., 2010).

2.5. Morphological changes of Streptococcus pyogenes ATCC 700294 monitored by AFM

The negative surface charges that prevail on the surfaces of muscovite and *S. pyogenes* make the positively charged α-poly(L-lysine) (PLL) polymer an adequate adhesive as a result of which *S. pyogenes* remains adhered to surface of muscovite even under the action of lateral forces exerted by Atomic Force Microscope's tip. Additionally at low concentration, α-poly(L-lysine) is a harmless adhesive (Conte et al., 2007). We used "dry coating method" (Atabek et al., 2008; Bolshakova et al., 2001; Vadillo-Rodríguez et al., 2004), to achieve the desire thickness of α-poly(L-lysine) layer. The 100 μL of α-poly(L-lysine) was allowed to completely air-dry on the slide. In the meanwhile, a freshly incubated culture of *Streptococcus pyogenes* was diluted in sterilized water to constitute 10^6 CFU of *S. pyogenes* and 5–10 μL of the resultant culture was dispensed onto α-poly(L-lysine) coated muscovite surface and left to dry. After drying, morphological studies of resultant strain were conducted by AFM.

2.6. Minimum inhibitory concentration by Agar well diffusion method

Minimum inhibitory concentration (MIC) was determined by the Agar well diffusion method (Nathan et al., 1978). Distinct Mueller Hinton agar plates containing control samples of *Streptococcus pyogenes* and Hg(II)-accumulated *Streptococcus pyogenes* were prepared for CX-AgNPs and its organic precursor, Cefixime. In brief, Mueller Hinton agar was utilized as medium to cultivate a lawn of *Streptococcus pyogenes ATCC 700294 at* the concentration of 10^6 cells per mL. The 60 mm well was formed by using a borer. The 500 μg/mL stock solution of Cefixime and CX-AgNPs was utilized to evade nonspecific merged zones of inhibition. Duplicate dilutions were utilized to determine minimum inhibition zones. In each well, different concentrations of test compounds ranging from 500 to 5 μg/mL were added. For attainment of effective diffusion of test compounds within the plates, these plates were first incubated at room temperature for two hours. Afterwards, we maintained the temperature at 37 ºC ± 1 and continued the incubation for 24–48 h, followed by measurement of inhibition zones.

2.7. Thermogravimetric analysis (TGA)

CX-AgNPs was also subjected to thermogravimetric analysis in a TGA analyzer (Hi Res TGA 2950, TA). Weighed sample of CX-AgNPs was heated in a platinum pan at a rate of 10 °C/min under nitrogen purge.

2.8. Sensing protocol

The potential of CX-AgNPs as plasmonic sensor was investigated by mixing it (133 μM) with equimolar quantities of various metal cations. After mixing, signal transduction capability was observed by monitoring SPR band of resultant solutions. Sources of metal cations are as follows: thallium (III) trifluoroacetate, copper (II) sulfate, lanthanum (III) chloride, lithium perchlorate, magnesium perchlorate, palladium (II) chloride, antimony (III) trichloride, bismuth (III) nitrate, mercury