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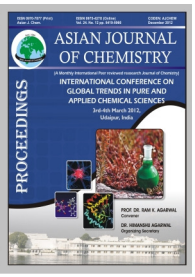

Biofilm Inhibitory Effect of Chemogenic Nano Zerovalent Iron Against Biofilm of Clinical Isolate of *Staphylococcus aureus*†

S. Ganesh and S. Karthick Raja Namasivayam*

Department of Biotechnology, Sathyabama University, Chennai-600 119, India

*Corresponding author: E-mai: biologiask@gmail.com

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Due to their sub colloidal size and unique molecular and/or atomic structures, many nanomaterials have been shown to possess distinctive mechanical, magnetic, optical, electronic, catalytic and chemical properties that contribute to promising applications in machinery, energy, optics, electronics, drug delivery and medical diagnostics and they have a wide range of potential applications in biomedical fields and in water treatment as disinfectants or antibiofilm agents. In the present study, nano-zerovalent iron was synthesized by aqueous-phase reduction of ferrous sulfate using sodium borohydride as the reducing agent and the synthesized particles characterized by transmission electron microscopy which reveals spherical shape of particles with the size range of 25-30 nm. Biofilm inhibitory effect of synthesized nano zerovalent iron particles against clinical isolate of *Staphylococcus aureus* reveals distinct inhibition of biofilm with 95 % of inhibition and the biofilm matrix derived from nano zerovalent treated biofilm of *S. aureus* weakened due to reduction of biochemical composition of biofilm.

Key Words: Nano zerovalent iron, Biofilm, *Staphylococcus aureus* inhibitory effect, Matrix.

INTRODUCTION

Nanotechnology is significant on account of its pre-eminence upon the comprehension, use and control of matter at magnitudes of a minute scale, akin to approaching atomic levels, with which to manufacture new substances, instruments and frameworks[1]. Nanoparticles possess exceptional physical and cd hemical properties, which lead to rapid commercial-ization. Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging[2] sensing[3], targeted drug delivery[4], gene delivery systems[5] and artificial implants[6]. Hence, nanosized organic and inorganic particles are catching increasing attention in medical applications due to their amenability to biological functionalization[7]. Based on enhanced effectiveness, the new age drugs re-nanoparticles of polymers, metals or ceramics can combat conditions like cancer and fight human pathogens like bacteria[8]. Nanotechnology may provide the answer to penetrate such biofilms and reduce biofilm formation. Silver nanotechnology chemistry can prevent the formation of life-threatening biofilms on medical devices. Silver nanoparticles are nanoparticles of silver, *i.e.* they are of between 1 nm and 100 nm in size[9]. While frequently described as being 'silver' some are composed of a large percentage of silver oxide due to their large ratio of surface to bulk silver atoms[10]. Silver is one of the oldest known antimicrobials. Antimicrobial silver is now used extensively to combat organisms in wounds and burns. It works because pathogens cannot mutate to avoid its antimicrobial effect[11]. Biofilm inhibitory effect of metallic nanoparticles against pathogenic bacteria has recently studied Synergestic effect of biogenic silver nanoparticles with various plant products and chemotherapeutics against the biofilm of *Staphylocooccus aureus* has recently reported by Namasivayam *et al.*[12]. In the present study, anti biofilm effect of nano zero valant iron synthesized by chemical method was evaluated against *Staphylococcus aureus*.

EXPERIMENTAL

Synthesis and characterization of nano zero valant iron: Synthesis and characterization of nano zero valent iron was carried out by chemical reduction method. Nanoscale zerovalent iron (nano ZVI) was synthesized by adding 1:1 volume ratio of $NaBH_4$ (0.8 M) into $FeCl_3.6H_2O$ (0.2 M) and mixing the solution vigorously under room temperature for 5 min. Ferric iron was reduced by borohydrate and nano zero-valent iron was formed according to the following equation:

$$4Fe^{3+} + 3BH^-_4 + 9H_2O \rightarrow 4Fe^0 + 3H_2BO_3^- + 12H^+ + 6H_2$$

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Nano-zerovalent iron was filtrated through 0.45 micron filter paper and washed several times with DI water to get rid of excessive borohydrate. Nano-zerovalent iron was dried by N_2 gas and was preserved from the oxidation by maintaining a thin layer of ethanol on the top of nano-zerovalent iron. The synthesized nano-zerovalent iron were characterized by transmission electron microscopy and energy dispersive X-ray spectroscopy.

Evaluation of biofilm inhibition assay: Synthesized nanoparticles at different concentration *viz*. 25, 50, 75 and 100 µg/mL was evaluated against clinical isolate of *Staphylococcus aureus* adopting biofilm inhibition spectrophotometric assay (Table-1)[13]. An overnight culture *S. aureus* in trypticase soy broth was diluted 1:100 ratio in respective fresh medium and grown in for another hour. 100 µL of diluted strains was added into 96 well titre plate and different concentration of nanoparticles 25, 50, 75 and 100 µg/mL was added and incubated 37 ºC for 3 days. After the incubation the medium was removed and 100 µL of 1 % w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 min the dye was removed and the wells were washed thoroughly, 95 % ethanol was added and incubated for 15 min. The reaction mixture was read spectrophotometrically at 570 nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula.

$$\% \text{ of inhibition} = \frac{\text{OD in control} - \text{OD in treatment}}{\text{OD in control}} \times 100$$

TABLE-1
BIOFILM INHIBITION (%) OF *S. aureus*
WITH NANO ZERO VALANT IRON

S. No.	Nanoparticles concentraction (µg/mL)	Biofilm inhibition (%)
1	25	63.1
2	50	71.0
3	75	89.0
4	100	95.0

Effect of nanoparticles on the biochemical composition of biofilm matrix: Biochemical composition of biofilm matrix mainly total carbohydrates and total protein from the biofilm of *Staphylococcus aureus* was evaluated by anthrone and Lowry's method.

RESULTS AND DISCUSSION

The primary identification of synthesized zero valent iron was confirmed by the colour change of the reaction mixture from reddish orange to black colour (Fig. 1) after the addition of sodium boro hydride to the ferric chloride solution. The synthesized particle were further characterized byich reveals transmission electron microscopy and energy dispersive X-ray spectroscopy (EDX), which reveals spherical shape of particles with the size range of 25-30 nm (Fig. 2) and EDX analysis confirmed that nano-zerovalent iron was produced from the synthesis procedure employed (Fig. 3) and the concentration of Fe was found 71 %. The biofilm inhibitory assay with different concentration of chemogenic nano zero valant iron reveals that all the tested concentration of nanoparticles inhibited biofilm. Maximum inhibition was recorded at 100 µg/mL with

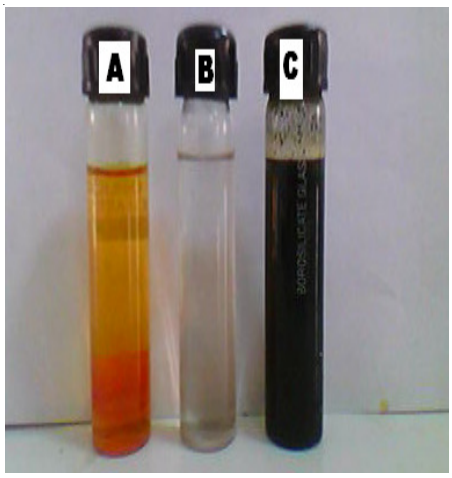

Fig. 1. Synthesized nnao-zerovalant iron

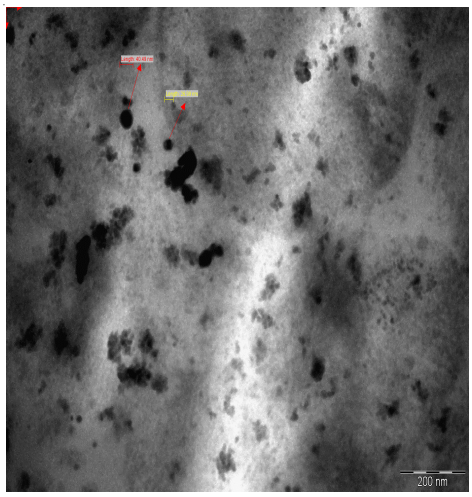

Fig. 2. TEM image of nano-zerovalent iron

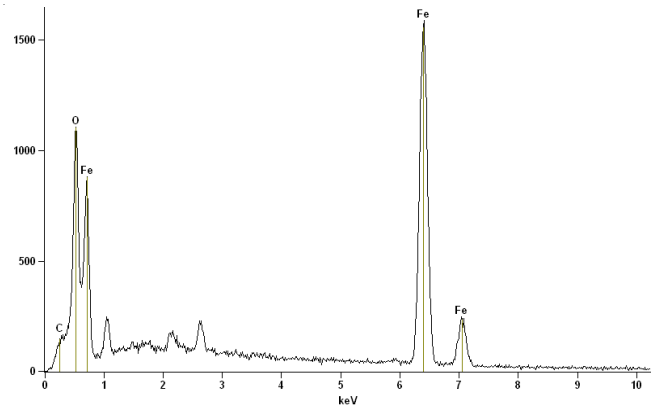

Fig. 3. Energy dispersive spectroscopic analysis of nano-zerovalent iron

95 % of biofilm inhibition followed by 75 and 50 µg/mL with 89.0, 71.0 and 63.1 % of biofilm inhibition respectively. SEM study of nanoparticle (100 µg/mL concentration) treated biofilm reveals complete degeneration of biofilm with weakened cell masses (Fig. 4b) whereas control reveals dense tightly packed masses of cells (Fig. 4a). Biofilm inhibitory effect of metallic nanoparticles against pathogenic bacteria has recently studied. Synergestic effect of biogenic silver nanoparticles with various plant products and chemotherapeutics against the biofilm of *Staphylocooccus aureus* has recently reported by

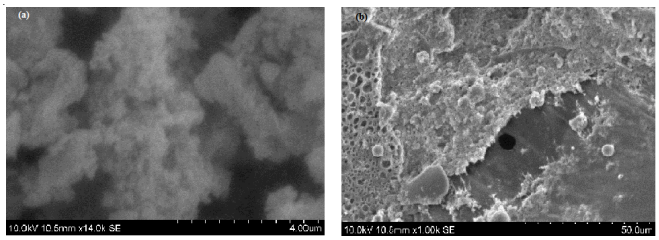

Fig. 4. Scanning electron microscopic analysis of biofilm treated with nano-zerovalent iron (a) control; (b) nano-zerovalent iron treated

Namasivayam *et al.*[12]. Anti biofilm effect of chemogenic silver nanoparticles coated on the catheter against pathogenic bacteria studied by Namasivayam *et al.*[14]. Biochemical composition of biofilm matrix mainly total carbohydrate and total protein was reduced in all the tested concentration of nanoparticles. Maximum reduction in total carbohydrates and protein was recorded in 100 µg/mL followed by 75 µg/mL. 125.0, 90.5, 85.0 and 51.5 µg/mg of total carbohydrate and 165.0, 121.0, 96.0 and 85.5 µg/mg of total protein was recorded in the respective concentration of nanoparticles treatment (Table-2). The matrix is one of the most distinctive features of a microbial biofilm. It forms a three dimensional, gel-like, highly hydrated and locally charged environment in which the microorganisms are largely immobilized. Matrix-enclosed micro colonies, sometimes described as stacks or towers, are separated by water channels, which provide a mechanism for nutrient circulation within the biofilm the composition of the matrix varies according to the nature of the organism and reduction of the biochemical composition of the biofilm matrix leads to weakening of the biofilm thus facilitate entry of the drugs. Improved anti biofilm effect was observed in antibiotics with nanoparticles treatment[15]. Since the initial field demonstration of the nano-zerovalent iron technology in 2001, significant progress has been made in research and development of iron nanoparticles for soil and groundwater treatment[16]. New research and development efforts should be directed toward enhancing real-world performance and minimizing potential economic and environmental risks. Iron nanoparticles may actually provide a valuable opportunity to demonstrate the positive effect on environmental quality. Iron is the fifth most used element; only hydrogen, carbon, oxygen and calcium were consumed in greater quantities[17]. It has been found at the active center of many biological molecules and likely plays an important role in the chemistry of living organisms. It is well documented that iron is an essential constituent of the blood and tissues. Iron in the body is mostly present as iron porphyrin or heme proteins, which include hemoglobin in the blood, myoglobin and the heme enzymes. The challenge is to determine eco- and human toxicity of highly reactive zerovalent iron nanoparticles. Reduction of iron ion with borohydride derivatives in solvent is a simple and mild reaction which can be done safely and the generated nano zero valant iron could be used as effective antimicrobial agent. The present findings proved the distinct biofilm effect of nano zero valant iron synthesized by easier and cost effective chemogenic method against *Staphylococcus aureus* and the present study suggests the possible use of nano-zerovalent iron as the anti microbial agent against pathogenic bacteria.

TABLE-2
EFFECT OF TOTAL CARBOHYDRATES AND PROTEIN OF BIOFILM MATRIX OF *Staphylococcus aureus*

S. No.	Concentration	Total carbohydrates (µg/mg)	Total protein (µg/mg)
1	25	12.5.0	165.0
2	50	90.5	121.0
3	75	85.0	96.0
4	100	51.5	85.5
5	Control	235.0	210.0

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