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BIOFILM INHIBITORY EFFECT OF SILVER NANOPARTICLES COATED CATHETER AGAINST *STAPHYLOCOCCUS AUREUS* AND EVALUATION OF ITS SYNERGISTIC EFFECT WITH ANTIBIOTICS

S.Karthick Raja Namasivayam*, M.Preethi, Arvind Bharani.R.S, George Robin, Bhavani Latha

Department of Biotechnology, Sathyabama University, Chennai 600119, Tamil Nadu, India.

ABSTRACT

The present study was undertaken to evaluate pharmacological inhibition of biofilm of clinical isolate of *Staphylococcus aureus* on silver nanoparticles coated catheter under *in vitro* condition and the synergistic effect of nanoparticles with antibiotics was studied. Silver nanoparticles synthesized by chemical reduction method were coated on the catheter adopting simple dispersion method. Initially biofilm inhibitory effect was studied with spectrophotometric method and the biochemical composition of biofilm matrix mainly total carbohydrates and protein studied. Silver nanoparticles synthesized by chemical reduction of silver nitrate with sodium borohydride were coated on the catheter was characterized by surface topography of catheter by scanning electron microscopy which reveals complete dispersion of the nanoparticles on the fibre surface of the catheter and the size, shape of the particles shows uniform spherical particles with the size of 40-60 nm. Distinct effect of biofilm inhibition was recorded in the nanoparticles coated catheter and maximum inhibition was observed during 24^{th} hour of incubation Surface topography of nanoparticles and nanoparticles with antibiotics coated catheter with scanning electron microscopy reveals the complete degeneration of the biofilm whereas biofilm matrix biochemical composition mainly total carbohydrates and total protein was highly reduced. Biofilm inhibition rate and reduction of biofilm matrix biochemical composition was increased in nanoparticles with all the tested antibiotics treatment which suggests the synergistic effect.

Key words:- Biofilm, Silver Nanoparticles, Catheter, *Staphylococcus Aureus.*

INTRODUCTION

Traditional treatment of infectious disease is based on compounds that aim to kill or inhibit microbial growth. But most of the pathogenic micro organisms develop resistant to that compound by producing various virulent factor (Donlan, 2001) Among them, biofilm represents the most prevalent type of virulent factor and involved in crucial development of clinical infection and exhibit resistance to antimicrobial agents (Joseph, 2003). Now the biofilm is considered as major target for the pharmacological development of drugs. A biofilm serves to promote bacteria persistence by resisting antibiotic treatment and host immune responses. Antibiotics are rendered ineffective when biofilms form due to their relative impermeability, the variable physiological status of microorganisms, subpopulations of persistent strains, and variations of phenotypes present (Hall Stoodley *et al.*,

Corresponding Author

S.Karthick Raja Namasivayam
Email: biologiask@gmail.com

2004). Microbial bio films develop when microorganisms irreversibly adhere to a submerged surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix (Kadurugamuwa *et al*., 2001). This surface may be inert, nonliving material or living tissue. Biofilm-associated microorganisms behave differently from freely suspended organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem Protection of bacteria in biofilms from the innate immune system is mediated through electrostatic repulsion or proteolysis of antimicrobial peptides and prevention of phagocytosis (Vuong, 2004). The organisms most commonly isolated from catheter bio films are Staphylococcus epidermidis, S. aureus, Candida albicans, P. aeruginosa, K. pneumoniae, and Enterococcus faecalis. These organisms originate from patient's skin micro flora, exogenous micro flora from health-care personnel, or contaminated infusates (Abraham *et al*., 2004). They gain access to the catheter by migration externally from the skin along the exterior catheter surface or internally from the catheter hub or port. Colonization of these devices can occur rapidly within 24 hours and may be a function of host-produced conditioning films platelets, plasma, and tissue proteins Several studies have examined the effect of various types of antimicrobial treatment in controlling biofilm formation on these devices (Sanders *et al*., 2000), Parelma *et al*., 2006). Catheters impregnated with sodium metabisulfite minocycline and rifampin cationic surfactant tridodecylmethylammonium chloride. Many workers reported the prevention of biofilm on central venous catheters, including using aseptic technique during implantation, using topical antibiotics, minimizing the duration of catheterization, using an in-line filter for intravenous fluids, creating a mechanical barrier to prevent influx of organisms by attaching the catheter to a surgically implanted cuff, coating the inner lumen of the catheter with an antimicrobial agent, and removing the contaminated device. 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 (Pareta *et al*., 2008) 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 (Suci *et al*., 2007). 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 (Massonet et al, 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 Karthick Raja Namasivayam et al (2011) Anti biofilm effect of super para magnetic particles against biofilm of *Staphylococcus epidermidi* studied by Taylor and Webster. In the present study, silver nanoparticles coated catheter evaluated against biofilm of clinical isolate of *Staphylococcus aureus* and the synergestic effect with antibiotics studied.

Materials and Methods

Synthesis and characterization of silver nanoparticles:

Silver nanoparticles were synthesized by chemical reduction of 0.1 M silver nitrate with 0.1 M tri sodium citrate with 0.1 M sodium borohydride as reducing agent. Synthesis of silver nanoparticles was confirmed by the conversion of the reaction mixture into brown colour and further characterization of the synthesized silver nanoparticles was carried out with determination of Plasmon absorption maxima with UV vis spectroscopy and particle morphology with electron microscopy (SEM).

Evaluation of biofilm inhibition assay

Synthesized silver nanoparticles at different concentration viz 25, 50, 75 and 100 ug /ml was evaluated against clinical isolate of *Staphylococcus aureus* adopting biofilm inhibition spectrophotometric assay. An overnight culture *S.aureus* in Trypticase Soy broth was diluted 1:100 ratio in respective fresh medium and grown in for another hour. 100µl of diluted strains was added into 96 well titre plate and different concentration of nanoparticles 25, 50, 75 and 100 ug /ml was added and incubated 37º C 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 minutes the dye was removed and the wells were washed thoroughly, 95% ethanol was added and incubated for 15 minutes. The reaction mixture was read spectrophotometrically at 570nm. Inhibition mediated reduction of biofilm formation was calculated by the following formula

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

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 *S.aureus* was evaluated by anthrone and Lowry's method.

Evaluation of silver nanoparticles on biofilm inhibition on catheter

2000 ml capacity UROBAG (urine collection bag) fitted with NON-RETURN VALVE AND LONG TUBE STERLIZED-PYROGEN-FREE Ready ROMO-10 Obtained from medical centre, thambaram, Chennai was selected in the study and the sterility of the catheter

mainly microbial load was determined. The collected catheter was cut in to 1.5cm pieces and random sample of 10 cut pieces was immersed in 190ml of sterile saline solution and shake well and the suspension was serially diluted and aliquot was spread plated on nutrient agar and potato dextrose agar . The seeded plates were incubated at 37ºc for 24 to 48 hrs, PDA plates were incubated 28ºc for 3 to 4 days. After incubation the plates were observed for bacterial and fungal colonies, but in this study no bacterial and fungal growth was recorded which reveals that the sterility of tested catheter. The catheter was then used for further study.

Coating of silver nanoparticles on catheter

The cut pieces of the catheter completely immersed in mono dispersive colloidal silver nanoparticle suspension with the concentration of 100 µg and kept in 37ºCfor 24 hrs. Placed on blotting paper to remove excess suspension and allowed to dry at 50ºc. After complete drying. Surface topography of the dried catheter was carried out with Scanning Electron Microscopy

Biofilm inhibition assay

The silver nanoparticles coated catheter pieces were immersed in 10ml of 24hrs bacterial culture, incubated at 37ºc for 24hrs.After incubation period the treated catheter was stained with 0.1%weight by volume of crystal violet solution for 30min at room temperation, after staining the catheter was washed with 95% of ethanol for 3 times at room temperature,the washed solution was collected ,and read spectrophotometrically at 570nm. The percentage of biofilm inhibition was calculated by following formula.

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

Evaluation of synergistic activity of nanoparticles with antibiotics

Synergistic effects of silver nanoparticles with antibiotics was carried out with cephaloxin , oflaxacin ,neoflaxin. Respective antibiotics of 100 dissolved in 10ml of deionised water and 5 ml of the respective antibiotics suspension was mixed with 5ml of the nanoparticles suspension kept ix under magnetic stirrer for 3 hours at 40 C. The homogenate thus obtained was dried at 50 ^{0} C, the dried particles thus obtained was collected dispersed in 100 ml of the deionised water. The cut pieces of catheter immersed in the suspension at 37ºCfor 24 hours. After the incubation period, catheter pieces were allowed to dry 50ºC. Dried pieces were used for biofilm inhibition assay as described earlier. Similarly biochemical composition of biofilm matrix total carbohydrate and total protein was also studied .

Results and Discussion

Silver nanoparticles synthesis adopting chemical reduction was primarily confirmed by colour change of the reaction mixture from pale yellow to brown clearly indicating the formation of silver nanoparticles (Fig. 1). The characteristic brown colour due to the excitation of Plasmon vibrations in the nanoparticles provides a convenient signature of their formation. Brown colour formation was observed within 10 minutes after the drop wise addition of sodium borohydride to the silver nitrate and tri sodium citrate solution. Synthesized silver nanoparticles characterized by UV-Vis spectroscope which reveals a strong broad surface Plasmon peak located at 420 nm (Fig. 2). Particle morphology size and shape with scanning electron microscopy reveals spherical particles with the size of 40-60nm (Figure 3). Moreover the particles were finely dispersed and no aggregation was observed which reveals the stability of the particle Biofilm inhibition with spectrophotometric assay reveals all the tested concentration of silver nanoparticles inhibited biofilm of *S. aureus*.(Table 1) Maximum inhibition of biofilm (95.5) was recorded in 100 µg/mg followed by 75(89.0). Biochemical composition of biofilm matrix mainly total carobohydrate and total protein was reduced in all the tested concentration of nanoparticles and nanoparticles coated catheter with antibiotics (Table 3). Karthick raja Namasivayam *et al* (2011) reported anti biofilm effect of biologically synthesized silver nanoparticles against *S. aureus* and *Candida tropicalis* and its synergestic effect with chemotherapeutics and plant products .Their studies reveals biogenic synthesized silver nanoparticles inhibited biofilm of both the tested organism and the inhibition was increased in nanoparticles amended with chemotherapeutic agents and plant products. Distinct effect on biofilm was also recorded in silver nanoparticles coated catheter. Silver nanoparticles coated by the simple dispersion method on the catheter were confirmed by scanning electron microscopy. Surface topography of the control catheter (without silver nanoparticles coating) was easily differentiated from catheter coated silver nanoparticles which reveals complete dispersion of the nanoparticles on the fibre surface of the catheter and the size, shape of the paricles shows uniform spherical particles with the size of 40-60 nm.(Figure 4).Distinct inhibition of biofilm was observed in nanoparticles coated catheter. Biofilm inhibition was noticed at 6th of incubation with 61.5% of inhibition and 78.5 % inhibition in 12 th hour of incubation whereas complete inhibition (100%) was recorded in 18th hour of inhibition (Table 3).Surface topography with SEM reveals complete degeneration of biofilm with weakened cell masses (Figure 4a,b). Similar observation was recorded in nanoparticles with oflaxacin coated catheter (Figure 4c) whereas control catheter reveals dense tightly packed masses of cells. 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 Karthick Raja Namasivayam *et al* (2011). Anti biofilm effect of super para magnetic particles against biofilm of *Staphylococcus epidermidi* studied by Taylor and Webster..Biochemical composition of biofilm matrix total carbohydrate and total protein was also highly reduced at the respective tested time. In respective concentration of nanoparticles treatment, 75.0, 55.0, 11.5 and 7.5 µg/mg of total carbohydrates was recorded. Distinct reduction of total carbohydrate as 0.21, 0.36, 0.43 and 0.52 µg/mg was observed in nanoparticle coated catheter with oflaxacin, cephalexin, neoflaxin and nanoparticles without antibiotics respectively (Table 3). 79.0, 45.0, 22.0 and 17.5 µg/mg and 34.1, 22.0, 18.3 and 9.0 µg/mg of protein were recorded in respective concentration of nanoparticles treatment and nanoparticles with neoflaxin, oflaxacin, cephalexin, nanoparticle without antibiotics. 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. Synergistic effects of silver nanoparticles with antibiotics were carried out with cephaloxin, ofloxacin and neoflaxin. All the antibiotics showed best compatibility with the silver nanoparticles on the biofilm inhibition Among the three antibiotics, maximum inhibition (99.1%) was recorded in oflaxacin followed by neoflaxin (95.0%) and cephalexin (90.1%) and the inhibition was observed during 12 t^h. hour of incubation. Karthick Raja Namasivayam *et al* (2011) reported the synergistic effect of antibacterial antibiotics choloroamphenicol with silver nanoparticles against biofilm of clinical isolate of *Pseudomonas aeruginosa*. The antimicrobial activity of nanoparticles (consisting of a mixture of silver nitrate and titanium dioxide) and nanoparticle-coated facemasks to protect against infectious agents *S.aureus* and *E.coli* reported by Li et al. Anti biofilm effect of NO releasing silica nanoparticles against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Candida albicans* revealed that ≥99% of cells from each type of biofilm were killed via NO release, with the greatest efficacy (99.999% killing) against gram-negative *P. aeruginosa* and *E. coli* biofilms . Polysulfone ultrafiltration membrane impregnated with silver nanoparticles showed distinct biofouling effect. The development of surface-attached bio film bacterial communities on medical devices like catheter is considered an important source of infections. Inhibition of biofilm on the devices now being considered for the prevention of microbial infection. Silver nanoparticles is now extensively used as anti microbial agents and utilized in development of anti microbial dressings, anti microbial medical devices etc. In the present study, anti biofilm effect of silver nanoparticles coated on the catheter against biofilm of *Staphylococcus aureus* and its synergistic effect with antibacterial antibiotics would suggests the development of effective anti microbial medical devices.

Table 1. Biofilm inhibition (%) of Staph.aureus with silver nanoparticles with well plate assay at time period (hours)

S.No.	Nanoparticles	Biofilm inhibition (%)					
	Concentration (µg/ml)	12	24	36	48	60	72
1	25	0.0	0.0	31.0	42.5	45.2	46.0
2	50	0.0	0.0	67.2	45.3	50.1	50.0
3	75	0.0	0.0	73.4	72.3	81.0	89.0
4	100	0.0	0.0	73.4	80.4	91.0	100.0
5	Control	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. Biofilm inhibition (%) of *Staph.aureus* on silver nanoparticles coated catheter and its synergistic effect with antibiotics

S.No	Incubation time (hour)	Biofilm inhibition (%)			
		NP	NO +Oflaxacin	NP+ cephalexin	NP+ Neoflaxin
1	6	61.5	87.0	80.1	83.2
2	12	78.5	99.1	90.1	95.0
3	18	100.0	99.1	90.1	95.0
4	24	100.0	99.1	90.1	95.0
5	30	100.0	99.1	90.1	95.0
6	36	100.0	99.1	90.1	95.0
7	42	100.0	99.1	90.1	95.0
8	48	100.0	99.1	90.1	95.0
9	54	100.0	99.1	90.1	95.0
10	60	100.0	99.1	90.1	95.0
11	66	100.0	99.1	90.1	95.0
12	72	100.0	99.1	90.1	95.0

Table 3.Effect of total carbohydrates and protein of biofilm matrix of Staph.aureus

S.No	Treatment	Total carbohydrates(µg/mg)	Total protein(µg/mg)
1	NP- 25((µg/mg)	75.0	79.0
2	NP- 50((µg/mg)	55.0	45.0
3	NP- 75((µg/mg)	11.5	22.0
4	NP- 100((µg/mg)	7.5	17.5
5	NP coated catheter	0.52	34.1
6	NP + Neofloxin	0.43	22.0
7	NP=Cephalexin	0.36	18.3
8	NP+Oflaxacin	0.21	9.0
9	Control	123.4	101.2

Fig 1. Synthesized Ag nanoparticle

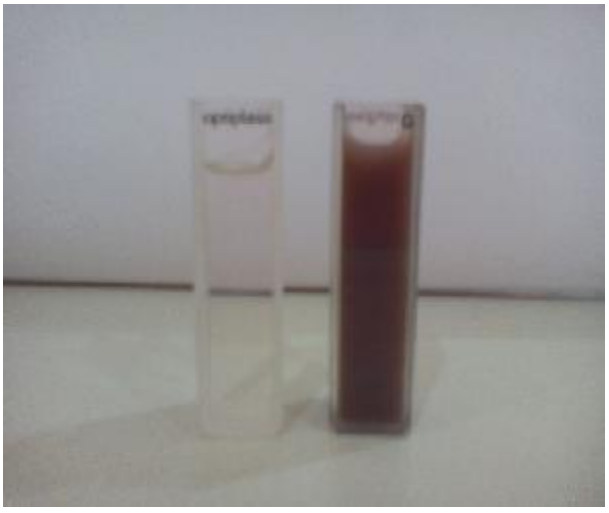


Fig 2. UV absorption spectra of Ag nanoparticles

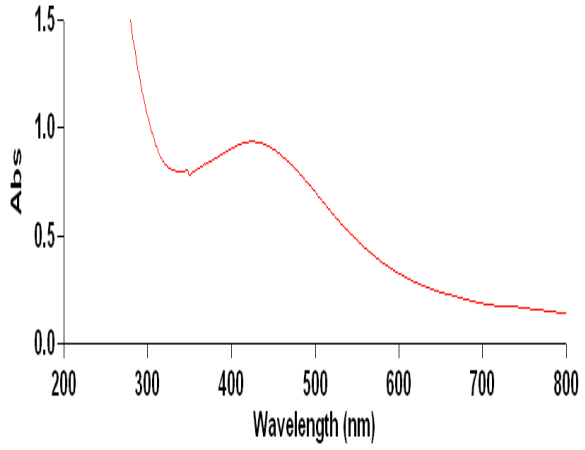


Figure 3.TEM micrograph of silver nnaoparticles

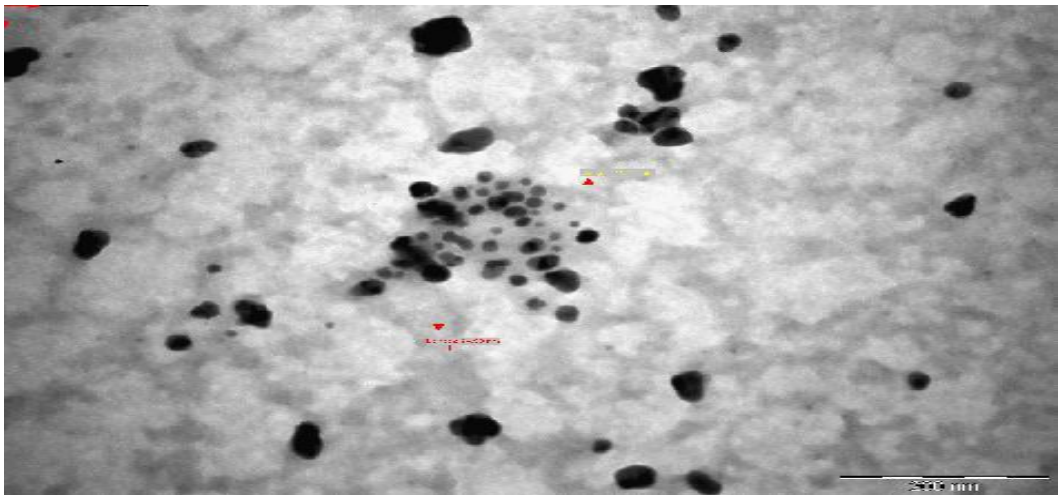
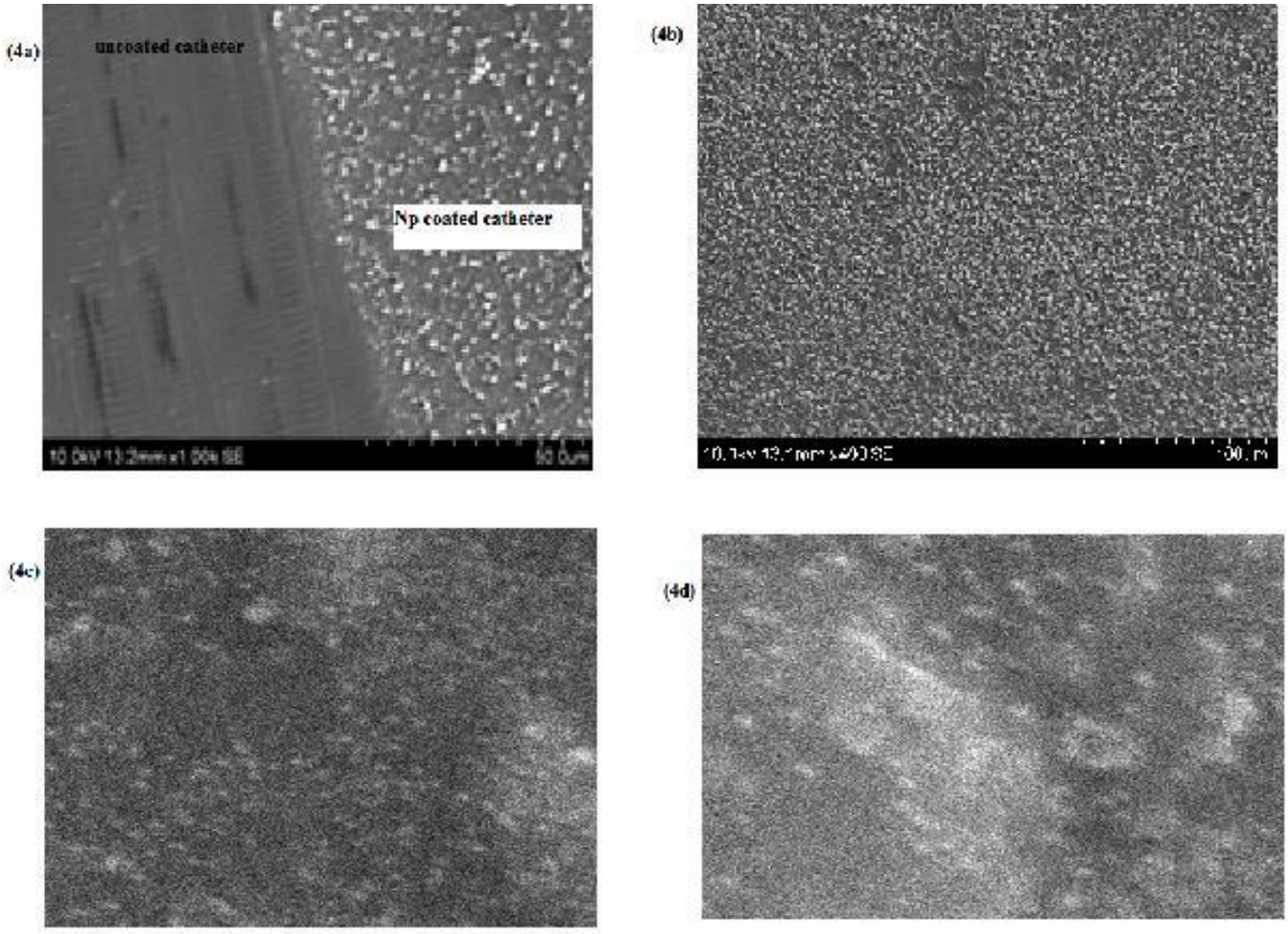


Figure 4. SEM of Ag nanoparticle coated catheter (4a); Biofilm formation on the catheter(4b) ;Degraded Biofilm on Ag nanoparticle coated catheter 4c; Complete degeneration of biofilm on NP+ oflaxacin coated catheter



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