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Application of Scanning Electron Microscopy for the Morphological Study of Biofilm in Medical Devices

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1. Introduction

The widespread use of medical devices has caused a great advance in the management of many diseases. Indwelling medical devices are being increasingly used for the treatment of functional deficits in numerous medical fields. Urinary tract infections (UTIs) represent the most commonly acquired bacterial infection. The risk of developing a urinary tract infection increases significantly with the use of indwelling devices such as catheters and urethral stents/sphincters. Although these catheters are valuable, they also have complications, the major complications are: encrustation, stone formation and biofilm formation. Microbial biofilms may pose a public health problem for persons requiring catheterization as the microorganisms in biofilms are difficult or impossible to be treated by antimicrobial agents.

Several approaches have been studied to prevent the formation of biofilms or to eradicate biofilm associated microorganisms. Some of these depends on coating medical devices with silver, antiseptics or by producing radio-opacity in catheters by the silicone material and some depends on the use of antimicrobial agents or non antimicrobial agents.

One approach to overcome the antimicrobial resistance of biofilm bacteria would be to enhance the penetration of agents through the biofilm matrix. Many trials were done to increase the efficacy of antimicrobial agents using some agents such as protamine sulfate (anticoagulant), EDTA, sodium citrate and penicillamine.

This work was done to determine whether N- acetylcysteine could aid ciprofloxacin in penetrating biofilm formed by some microorganisms on ureteral stents.

The use of biomaterials in the urinary tract dates back to ancient times, when the Egyptians described using lead and papyrus to construct urinary catheters (Bitschay and Brodny, 1956). Today, the majority of biomaterials used in urology are made from synthetic polymeric compounds, which were originally developed in the plastic injury. In the process of endourological development, a great variety of foreign bodies have been invented besides urethral catheters like ureter, prostatic stents, percutaneous nephrostomy, penile, testicular implants, and artificial urinary sphincters (AUS). Although the tendency of patients being predisposed to infections due to foreign bodies has been recognized since the fourteenth

century, the mechanisms of device-related infections are still not completely understood (Tenke *et al.*, 2006).

Bacterial adherence and the growth of bacteria on solid surfaces as biofilm are both naturally occurring phenomena. Biofilm formation affects many aspects of our life and also plays an important role in medicine involving the field of urology. It is able to build up under natural circumstances, for instance on the urothelium or prostate stones and in the presence of temporarily or permanently implanted foreign bodies. The frequently used urethral catheters, double J stents and transrenal drains provide just as perfect surfaces to bacteria to adhere. Biofilms can have a positive impact as well, namely lining the healthy intestine and the female genito-urinary tract. Biofilms have significant implications for clinical pharmacology, particularly related to antibiotic resistance, drug adsorption onto and peeling off devices, and minimum inhibitory concentrations of drugs required for effective therapy (Mardis and Kroeger, 1988).

2. What is a biofilm

2.1 Definition

Biofilm is defined as structured communities of microbial species embedded in a biopolymer matrix on either biotic (living tissues) or a biotic (inert non living material) substrata. The general theory of biofilm predominance states that the majority of bacteria grow in matrix-enclosed biofilms adherent to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ greatly from their planktonic counterparts (Costerton *et al.*, 1978). The reason for this ubiquity is that the protective layer and distinct metabolic states of bacteria within biofilms provide them with special resistance to host defences and antimicrobials, including natural antibiotics.

2.2 Estructure

The basic structure unit of the biofilm is the microcolony. A mature biofilm is composed of cells (10-15% by volume) and of glycocalyx (85-90%). The cells embedded in glycocalyx form gross structure resembling towers and mushrooms, sometimes as high as a few millimeters. Open channels are interspersed between the microcolonies resembling a primitive circulatory system. Water and nutrients enter these channels and contribute to nutrition and formation of mature biofilms. Waste products are also removed through this system.

The cells composing a biofilm can be of single species or more commonly are heterogenous species of bacteria and fungi. In the latter case the metabolic by-products of one organism might serve to support the growth of another, and the adhesion of one species might provide ligands allowing the attachment of others. A mature biofilms contains thousands of bacteria (Dunne, 2002).

The glycocalyx is mainly composed of bacterial exopolysaccharides. Other components are nucleic acids, minerals and proteins. When fully hydrated, the glycocalyx is predominantly water, with an anionic charge that creates a scavenging system for trapping and concentrating essential nutrients from the environment. Glycocalyx also provides a protective layer against biocides and this is the most prominent morphologic feature addressed in this chapter.

2.3 Development

2.3.1 Reversible attachment

Once at the surface, different physical, chemical and biological processes take place during this initial interaction between bacteria and the surface. On the abiotic surface, primary attachment between bacteria and the surface is mediated by non-specific interactions such as electrostatic, hydrophobic, or vander waals forces. On biotic surfaces such as tissues, Primary attachment is through specific molecular adhesion by lectin or adhesin. The surface appendages and structures that bear adhesins include fimbriae, flagella, capsule, outer membrane and other appendages. Bacteria bearing adhesins can approach receptors at a distance, form complexes, and ultimately settle onto substratum (Ofek and Doyle, 1994).

In this stage, The organism must be brought into close approximation of the surface either randomly by a stream of fluid flowing over a surface or by a directed fashion via chemotaxis and motility. Once the organisms reach critical proximity of the surface (usually $< 1\text{nm}$) the final determination of adhesion depends on:

The net sum of attractive or repulsive forces generated between the two surfaces. These forces include electrostatic and hydrophobic interactions, steric hindrance, vander waals forces, temperature and hydrodynamic forces. Electrostatic interactions tend to favor repulsion, because most bacteria and inert surfaces are negatively charged. Hydrophobic interactions probably have greater influence on the outcome of primary adhesion depending on the molecules in the conditioned film. Repulsive forces can be over come by specific molecular interactions mediated by adhesins located on structures extending from the cell surface such as pili (Carpentier and Cerf, 1993).

It was observed that flagellar and twitching motility are necessary for *pseudomonas aeruginosa* biofilm development. As when biofilm formation to a biotic surface of polyvinyl chloride plates (PVC) by *P. aeruginosa* and its 2 mutants is compared, one mutant is defective in flagellar mediated motility and the other is defective in biogenesis of polar type of iv pili. The biofilm of wild type is followed using phase contrast microscopy. First, the strain formed a monolayer of cells on the a biotic surface (PVC) followed by the appearance of microcolonies that were dispersed through the monolayer of cells. Then using time lapse microscopy, it showed that microcolonies were formed by aggregation of cells present in the monolayer. As observed in the wild type, the strain defective in type iv pili formed a monolayer of cells on the PVC but cells failed to develop microcolonies suggesting that these structures play an important role in microcolonies formation, while very few cells of flagellar defective non-motile mutant are attached to PVC surface even after 8 hours incubation showing that the role of flagella and/ or motility in initial cell-surface adhesion is very important (Arora *et al.*, 1998).

2.3.2 Irreversible attachment

After binding to the surface through exopolymeric matrix, bacterial cells start the process of irreversible adhesion, proliferation and accumulation as multilayered cell clusters. These extracellular matrices, composed of a mixture of materials such as polysaccharides, proteins, nucleic acids and other substances are considered to be essential in cementing bacterial cells together in the biofilm structure, in helping to trap and retain nutrients for biofilm growth

and in protecting cells from dehydration and the effects of antimicrobial agents (Davis and Geesey, 1995).

2.3.3 Maturation of biofilm formation

Once having irreversibly attached to a surface, bacterial cells undergo phenotypic changes, and the process of biofilm maturation begins. Bacteria start to form microcolonies either by aggregation of already attached cells, clonal growth (cell division) or cell recruitment of planktonic cells or cell flocs from the bulk liquid. The attached cells generate a large amount of extracellular components which interact with organic and inorganic molecules in the immediate environment to create the glycocalyx (Jiang and Pace, 2006).

Mature biofilms consist of differentiated mushroom and pillar like structures of cells embedded in copious amounts of extracellular polymer matrix or glycocalyx, which are separated by water-filled channels and voids to allow convective flows that transport nutrients and oxygen from the interface to the interior parts of the biofilm and remove metabolic wastes (Stoodly *et al.*, 2002).

There are many environment within a biofilm, each varying because of difference in local conditions such as nutrient availability, PH, oxidizing potential (redox) and so on. Cells near the surface of the biofilm are exposed to high concentrations of oxygen, while near the center oxygen is rapidly depleted to near anaerobic levels (Lewandowski, 1994). The steep oxygen gradients are paralleled by gradients for other nutrients or metabolites from the biofilm (de Beer *et al.*, 1994). Apparently, biofilms display both structural and metabolic heterogeneity which provide this community the capability to resist stresses, whether from host defense systems or antimicrobial agents (Kumar and Anand, 1998).

2.3.4 Detachment

At some point the biofilm reaches a critical mass and the the outermost layer begins to generate planktonic organisms that may escap from the biofilm and colonize other surfaces. Dispersion of planktonic cells can be facilitated by digestion of glycocalyx by enzymes and quorum-sensing might be required for this phenomenon (Dagostino *et al.*, 1991).

2.4 Factors affect the adherence of microorganisms to a device surface

2.4.1 Device related factors

Certain materials used in the design of Indwelling medical devices (IMDs) are more conducive to microbial adherence/biofilm formation than others. In vitro studies performed by many laboratories have determined that microbial adherence to biomaterials occurs in the following order: latex > silicone > PVC > Teflon > Polyurethane > stainless steel > titanium (Darouiche, 2001).

Surface characteristics determining the adherence properties of specific materials include: (a) surface texture, (b) surface charge, and (c) hydrophobicity.

a. Surface texture:

Materials with irregular or rough surfaces tend to have enhanced microbial adherence compared to smooth surfaces. It is documented that surface irregularities in central

venous catheters (CVC) varied with different polymer materials so that bacteria preferentially adhered to surface defects within minutes after infusing the catheters with contaminated buffer solution (Locci *et al.*, 1981). Another study examined the surface of five commercially available polyurethane CVCs by scanning electron microscopy and found that the catheters with the most surface irregularities had significantly more adherent bacteria compared to catheters with smoother surfaces (Tebbs *et al.*, 1994).

- b. Surface charge:
Biomaterial surface charge greatly influences adherence of microorganisms. Most microorganisms exhibit a negative surface charge in an aqueous environment. Therefore, a negatively charged biomaterial surface should lead to decreased adherence of microorganisms due to a repulsion effect between both negatively charged surfaces (Jansen *et al.*, 1988).
- c. Hydrophobicity:
Bacterial cells, which tend to have hydrophobic cell surfaces, are attracted to the hydrophobic surfaces of many biomaterials currently used in IMDs (Schierholz and Beuth, 2001). This hydrophobic interaction between the microorganisms and the biomaterial leads to increased adherence and subsequent biofilm formation. An increase in the surface hydrophilicity of the polymers leads to weakened hydrophobic interactions and decreased adherence (Jansen *et al.*, 1988).

2.4.2 Host factors

The biomaterials used in IMDs result in the activation of the host immune response leading to local tissue damage and the development of an immuno-incompetent, fibro-inflammatory zone that increases the susceptibility of the IMD to infection (Schierholz and Beuth, 2001). The deposition of proteinaceous layer (including fibronectin, fibrinogen, fibrin, albumin, collagen, laminin) on the surface of the device forming conditioning film leads to the alteration of surface properties of the biomaterial and the increase of microbial adherence (Pascual, 2002).

2.4.3 Microbial factors

The cell surface of a bacterium possesses many structures and properties that contribute to bacterial adhesion including fimbriae (pili), the cell wall (teichoic acid in gram-positive bacteria) and outer cell membrane (lipopolysaccharides in gram negative pathogens). These characteristics influence the surface charge and hydrophobicity of the bacterial cell, thereby directly affecting adherence (Bonner *et al.*, 1997). The physico-chemical characters of microbial cell surface, i.e., hydrophobicity and charge will influence adherence to biomaterial surfaces since the process is strongly governed by hydrophobic and electrostatic interactions (Martinez-Martinez *et al.*, 1991).

2.4.4 The suspending medium

The absorption of components from the suspending fluid can affect the adhesive properties of microorganisms. The ionic strength, osmolarity, and pH all influence the initial attachment of bacteria (Denstedt *et al.*, 1998). In the process of adherence of microorganisms

to an implanted device, one or both entities will be exposed to a biological secretion or body fluid of host origin. The subsequent conditioning of microbial cell or/and biomaterial surface will modify the nature of both surfaces, thereby determining the outcome of the adherence process. It is observed that prior colonization of endotracheal tubes, microorganisms preferentially adhere to a biological film of human origin rather than to the constituent biomaterial itself (Poisson *et al.*, 1991). Adherence of *E. coli* and *E. faecalis*, grown in Mueller- Hinton broth, was shown to increase after the biomaterial was exposed to human urine (Bonner *et al.*, 1997).

2.4.5 Bacteria- biomaterial interaction

The adhesion of microorganisms to biomaterial surfaces has been shown to require both non-specific reversible interactions and highly specific irreversible interactions. First, reversible adhesion of microorganisms to biomaterial surface is dependent upon the physical characteristics of the microorganisms, biomaterial and the surrounding environment (Gristina, 1987). Microorganisms randomly reach the surface of the biomaterial by several mechanisms: direct contamination, contiguous spread, hematogenous spread. Once near the surface, initial adherence of the microorganism depends upon microorganism-biomaterial interactions including van der waals forces and hydrophobic interactions (pascual, 2002). The common charges of the microorganisms and the IMD surfaces will repel each other, however the effect of van der waals forces overcome this repulsion beginning about 10 nm from the IMD surface keeping the microorganisms near the biomaterial surface (Gristina, 1987).

It has shown that hydrophobic forces are 10 to 100 times stronger than van der waals forces at 10 nm from the biomaterial surface. The hydrophobic forces easily overcome electrostatic repulsion and position the organisms 1-2 nm from IMD surface then allows irreversible adhesion to occur (Pashley *et al.*, 1985). Second, irreversible adhesion occurs with the binding of specific microorganism adhesins to receptors expressed by the conditioning film . i.e., *S. aureus* and *S. epidermidis* which are the most common microorganisms causing IMD-related infections relies on specific cell surface proteins called "microbial surface component recognizing adhesive matrix molecules" (MSCRAMM) which bind to specific host ligands that are found in the conditioning films. The most important MSCRAMMS are the fibronectin-binding proteins (FnBPs), the fibrinogen-binding proteins (clumping factors, CIf) and the collagen (Darouiche *et al.*, 1997).

Cell surface proteins also play an important role in *S. epidermidis* adhesion to IMDs. Proteinaceous autolysin and polysaccharide adhesin (PSA) are two surface proteins that play an early role in the irreversible adhesion of *S. epidermidis* to IMD surfaces. Once adherent to the biomaterial surface, cell accumulation and early biofilm formation are dependent upon the polysaccharide intercellular adhesin (PIA), which promotes intercellular adhesion (Rupp *et al.*, 1999).

2.5 Defense mechanisms

The use of antibiotics is currently one of the possibilities for the prevention of biofilm formation. However, even in the presence of antibiotics bacteria can adhere, colonize and survive on implanted medical devices as has been shown for urinary catheters and ureteral

stent surfaces in-vitro and in-vivo (Caldwell, 1995). In addition, resistance to antimicrobial agents and other chemicals is one of the greatest problems in the age of widely used medical devices. The problem in conventional clinical microbiology is how to treat patients in the best way when choosing antibiotics is based on bacterial cultures derived from planktonic bacterial cells which differ very from bacteria in the biofilm mode. This can stand behind the clinical failure rate of treating chronic bacterial infection (Choong and Whitfield, 2000).

The failure of antimicrobial agents to treat biofilms has been associated with a variety of mechanisms (Brown *et al.*, 1990):

1. The glycocalyx restricts access and diffusion of antimicrobial agents to the deeper lying bacteria (extrinsic resistance). In situ studies have shown that the surface film influences the transport of nutrients and interferes with the transport of antimicrobials (Nivens *et al.*, 1993).
2. The growth rates of bacteria within a biofilm vary widely. Slow-growing bacteria are particularly resistant to antimicrobial agents (Brown, 1997). The limitation of diffusion of nutrients in a biofilm results in spatial gradients of growth rate leading to a plethora of phenotypes within the biofilm. In general, the faster-growing, more susceptible bacteria lie superficially but the slow-growing, less susceptible bacteria being placed more deeply. The failure of antimicrobial agents to eradicate these slow-growing bacteria may exert selection pressures on the least susceptible genotype to select for a resistant population. Furthermore, antimicrobial binding proteins are poorly expressed in the slow-growing bacteria, rendering the antimicrobial agents ineffective (Cozens *et al.*, 1986). Commonly, the entire biofilm is coated with a complex of a hydrophilic polymer, the glycocalyx that is typically anionic in nature where the antimicrobial agents reacts chemically with exopolymer or is adsorbed to it, then the net effect is that of having the appearance of a penetration barrier. There will be a similar effect if antimicrobials adsorb onto cells, perhaps dead ones, in the outer parts of the biofilm (Sutherland, 2001).
3. Bacteria within a biofilm are phenotypically so different from their planktonic counterparts that antimicrobial agents developed against the latter often fail to eradicate organisms in the biofilms. Bacteria within a biofilm activate many genes which alter the cell envelope and molecular targets, and alter the susceptibility to antimicrobial agents (intrinsic resistance). Current opinion is that phenotypic changes brought on by a genetic switch, when 65-80 proteins change, play a much more important role in the protection from antimicrobial agents than the external resistance provided by the exopolysaccharide slime (Anonymous, 1999).
4. Bacteria within a biofilm can sense the external environment, communicate with each other and transfer genetic information and plasmids within biofilms (Trieu-Cuot *et al.*, 1987).
5. Bacteria in a biofilm can usually survive the presence of antimicrobial agents at concentrations 1000-1500 times higher than the concentrations that kill planktonic cells of the same species (Costerton, 1999).

Resistance may be due to:

- production of inactivating enzymes as it is found that a relatively large amounts of antibiotic-inactivating enzymes such as β -lactamase which accumulate within the glycocalyx produce concentration gradients can protect underlying cells (Bagge *et al.*, 2000).

- Efflux pumps were also believed to play a role in the resistance of biofilms; however expression of the pumps decreases within the biofilm bacteria dependent on time and location of the cells within the community, as compared to planktonic bacterial form (De Kievit *et al.*, 2001).
- Recent work has highlighted the contribution of oxygen deprivation and anaerobic growth to antibiotic resistance, it is indicated that oxygen penetrate approximately to 25% of the depth of the biofilm, when challenged with antimicrobials, 4h old colony biofilms growing in the presence of air were susceptible, however similar aged biofilms grown anaerobically were much less susceptible. The authors calculated that oxygen limited could explain 70% or more of the protection of old biofilm cells (Borriello *et al.*, 2004).

2.6 Treatment and prevention of biofilms

Strategies for prevention of these infections include: (i) minimizing tissue destruction and removal of all extraneous biomaterials and devitalized tissues during surgery. (ii) development of biomaterials that resist the initial adherence of bacteria by surface characteristic or by promoting bactericidal, bacteriostatic or phagocytic activity at their surfaces. (iii) further study of the microstructure and chemical nature of the adherence mechanism and development of analogs and enzymes that might block the initial adherence by modification of receptors and ligands (Khardori and Yassien, 1995) .

Several approaches have been studied to prevent the formation of biofilms and to eradicate biofilms associated bacteria. Some of that depends on the use of antimicrobial agents or non antimicrobial agents.

a. Antimicrobial agents:

In the case of the use of antimicrobial agents, it was found that some antibiotic at sub-MIC inhibit the initial adherence. Dicloxacillin is the antibiotic that found to prevent the adherence to the greatest extent when it is used alone at 1/2 of the MIC (Cerca *et al.*, 2005). Also clindamycin at subinhibitory concentrations inhibits the adherence of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacteroids* spp., *Escherichia coli* to bone surfaces (Lambe *et al.*, 1987).

Norfloxacin, ciprofloxacin, ofloxacin and azithromycin at sub inhibitory concentrations reduced the glycocalyx production and inhibited the adherence of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Pézer-Giraldo *et al.*, 1989 ; Yassien *et al.*, 1995). Ciprofloxacin was reported to eradicate the performed biofilms of *P. aeruginosa* (Reid *et al.*, 1994). It was reported also that 1/2 MIC of ciprofloxacin, 1/4 MIC of ofloxacin and 1/32 of levofloxacin caused significant inhibition of adherence of some uropathogenic strains of *E. coli* to periurethral epithelial cells (Baskin *et al.*, 2002).

Macrolides are generally bacteriostatic in-vitro and in-vivo, and have useful activity versus gram-positive bacteria. Macrolides have been evaluated to affect the adherence of gram-negative bacteria at sub-MIC concentrations by 50 to 70% as it is found to affect the production of virulence determinants such as secreted virulence factor, motility, quorum sensing and biofilm production (Vranes, 2000). It is discovered that sub-MIC level of clarithromycin inhibits the twitching motility of *P. aeruginosa*, they do not affect the production of pili but inhibit their assembly on the surface of bacteria that should affect some steps in biofilm formation (Wozniak and keyser, 2004).

It is investigated that the antibiofilm effects by incubating ciprofloxacin with *P. aeruginosa* or in combination with macrolides. At twice the minimum bactericidal concentration of ciprofloxacin, 85% of the population of *P. aeruginosa* within the biofilm survived. In contrast, the killing effect of ciprofloxacin was greatly enhanced when combined with clarithromycin, erythromycin and azithromycin, but not with the 16-membered ring macrolides. It is speculated that the 14-membered and 15-membered ring macrolides possess an ability to increase the permeability of biofilms, thereby facilitating the penetration of quinolone antibiotics. Tigecycline was observed to inhibit the growth of *S. epidermidis* which indicates that tigecycline is able to diffuse through the biofilm and act normally against its cellular target (Labthavikul *et al.*, 2003).

b. Non-antimicrobial agents:

It is observed that some drugs other than antimicrobial agents such as anti-inflammatory or antiseptic compounds reduce the adherence of bacteria. Coating the catheter with acetylsalicylic acid or sodium salicylate reduces or inhibits microbial adherence, Bandazac lysine (non steroidal anti-inflammatory) was found to prevent the adherence of bacteria to contact lenses (Arciola *et al.*, 1998).

Some mucolytic substances such as EDTA, sodium citrate and penicillamine may disperse the biofilms formed by *P. aeruginosa* (Gordon *et al.*, 1991). It is observed also that N-acetylcysteine (NAC) (a non antibiotic drug that has antibacterial properties (bacteriostatic) and a mucolytic agent that disrupts disulphide bonds in mucus and reduces the viscosity of secretions) decreases biofilm formation and therefore may be an effective alternative for preventing infections by *S. epidermidis* and other coagulase negative staphylococci (Pérez-Giraldo *et al.*, 1997).

It is observed that NAC not only reduced the adhesion but in fact also detached adhered cells from a steel surface. This has some importance since the initial adhesion often develops into a stronger interaction with time (bond ageing) (Meinders *et al.*, 1995). The reduction in the amount of exopolysaccharides (EPS) in the presence of NAC may have many explanations. The direct effects of NAC include a possible reaction of its sulfhydryl group with disulfide bonds of enzymes involved in EPS production or excretion, which renders these molecules less active, or competitive inhibition of cysteine utilization. Also, the possibility of interference of NAC with control or signaling systems that direct the EPS production at translation or at the enzymatic level cannot be excluded. The fact that NAC is an anti-oxidant may have indirect effects on cell metabolism and EPS production. NAC increases the wettability of surfaces. Moreover, NAC detached bacteria that were adhering to steel surfaces. Growth of various bacteria, as monocultures or in multi-species community, was inhibited at different concentrations of NAC. It is also found that there was no detectable degradation of EPS by NAC, indicating that NAC reduced the production of EPS in most bacteria tested, even at concentrations at which growth was not affected (Olofsson *et al.*, 2003).

Aspirin (acetylsalicylic acid) has a short half life in circulating blood (about 20 min) and is rapidly deacetylated to form salicylic acid in-vivo. Sodium salicylate and related compounds such as aspirin are known to have a variety of effects on microorganisms. Growth of certain bacteria in the presence of salicylate can induce multiple resistance to

antibiotic. Paradoxically, it can also reduce resistance to some antibiotics (Price *et al.*, 2000). *E. coli*, for example exhibits increased resistance to chloramphenicol, ampicillin, naldixic acid and tetracycline after such treatment. On the other hand *E. coli* cells grown in the presence of salicylate are more sensitive to aminoglycosides (Aumercier *et al.*, 1990).

Sodium salicylate inhibits biofilm formation by *P. aeruginosa* and *S. epidermidis* on contact lenses and medical polymers such as polyethylene and polystyrene. It also decreases bacterial adhesion in a dose-dependent manner. Some strains of *S. epidermidis* secrete mucoid extracellular polymers (polysaccharides, proteins and teichoic acid) that promote biofilm formation and become important components of the biofilm matrix. Salicylate can inhibit the production of some of these components by as much as 95%. It has been suggested that the use of salicylate into contact lens solutions might decrease the incidence of some device- related infections (Farber and Wolff, 1992).

Chlorohexidine gluconate and silver sulfadiazine coated vascular catheter has been shown to be highly effective in decreasing catheter related infections (George *et al.*, 1997).

Protamine sulfate (a surface active, basic polypeptide presently used to reverse the anticoagulant effects of heparin) could aid antibiotics in penetrating a *P. aeruginosa* biofilm (Richards, 1976). Parsons and coworkers have shown that protamine sulfate penetrates and disrupts the protective glycosaminoglycan layer. There is a significant, synergistic effect observed between protamine sulfate and ciprofloxacin as protamine sulfate may have denatured the complex extracellular polymeric structure of the *P. aeruginosa* biofilm enhancing penetration of ciprofloxacin through the biofilm (Soboh *et al.*, 1995).

Gendine solution (a novel antiseptic solution) formed of gentian violet and chlorohexidine has the ability to coat various polymers and devices. It has also a broad spectrum antiadherence activity and antimicrobial activity which decreases the risk of device colonization, which may in turn decrease the rates of nosocomial infection and their associated morbidity and mortality (Chaiban *et al.*, 2005).

3. Techniques for the study of biofilm

This work was done to detect biofilm formed on ureteral stents and to determine whether N-acetylcysteine could aid ciprofloxacin in penetrating biofilm formed by some microorganisms on ureteral stents. Several techniques were used in this study first Stents were removed by physicians and collected in sterile screw capped tubes, then cut into segments to be examined by Scanning electron microscope (SEM) and to be cultured on different media.

Catheter segment were fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco PBS (pH 7.2) for 1.5h, rinsed with Phosphate buffer saline (PBS), and then dehydrated through an ethanol series. Samples were critical point dried and gold-palladium coated. SEM examinations were made on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan).

Urine samples were collected and streaked onto the culture media and incubated at 37°C for 24 hours (Benson, 2002). The resultant colonies were streaked and examined morphologically, microscopically and biochemically.

Catheter samples: Each catheter was placed in 10 ml of tryptic soy broth (TSB), sonicated for 1 min and then vortexed for 15 s. 0.1 ml of the sonicated broth were surface plated by using

a wire loop on trypticase soy agar with 5% sheep blood and MacConkey agar. Organisms were then identified by routine microbiological techniques (Sherertz *et al.*, 1990).

Antibiotic susceptibility and MICs were determined for the isolated microorganisms by the agar dilution method, according to clinical laboratory standards institute (CLSI) (2007)

The isolated microorganisms were tested for their ability to form biofilm by tissue culture plate method (TCP). Effect of different concentrations of ciprofloxacin, N-acetylcysteine each alone and in combination on the bacterial adherence to plastic surfaces were determined by tissue culture plate assay (Christensen *et al.*, 1985). The Effect of different concentrations of ciprofloxacin, N-acetylcysteine each alone and in combination on the bacterial adherence to the surface of ureteral catheter were determined by Static adhesion assay (Reid *et al.*, 1994) and their effects were determined also using scanning electron microscope.

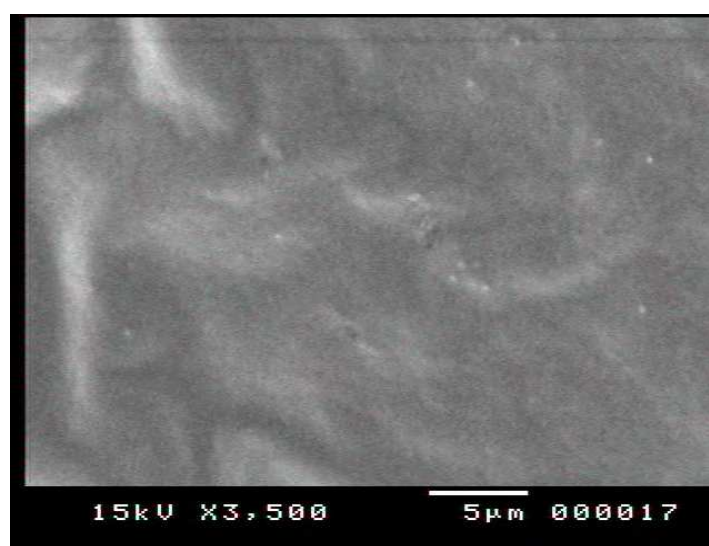


Fig. 1. Scanning electron micrograph of an empty ureteral stents incubated in saline for 24 h (control) ($\times 3500$).

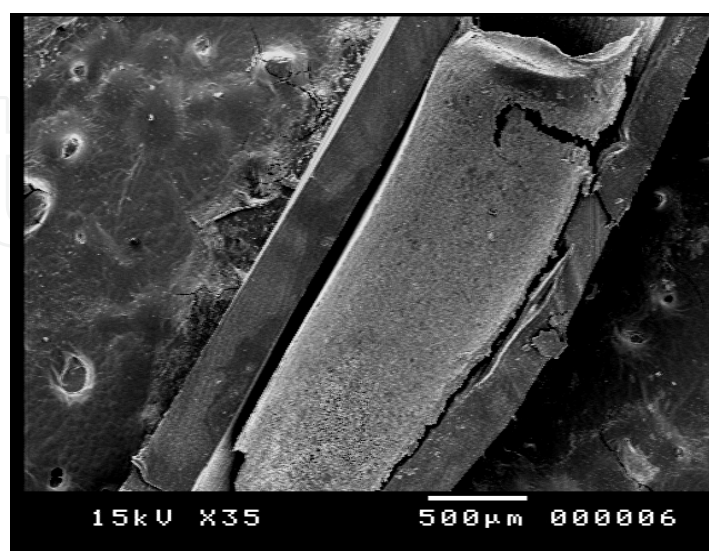


Fig. 2. Scanning electron micrograph showed the lumen of the ureteral stent ($\times 35$) blocked with a dense mass of biofilm containing bacteria.

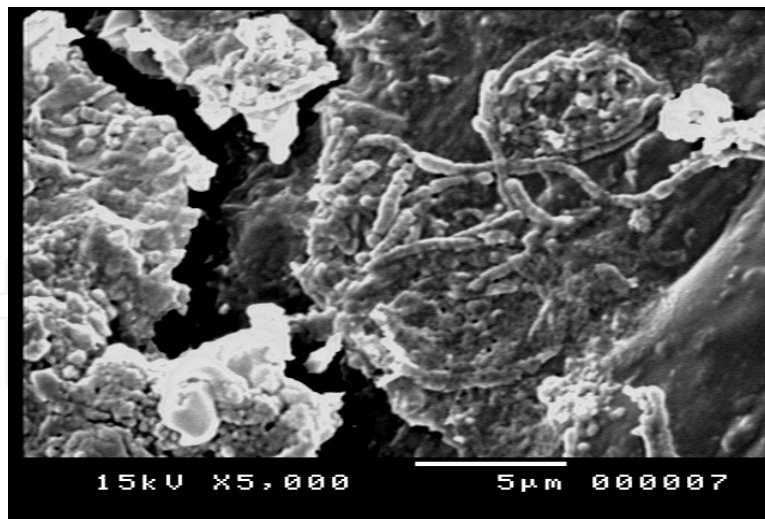


Fig. 3. Scanning electron micrograph showed the lumen of the ureteral stent covered with a densed mass of biofilm containing bacteria (*S. aureus* and *P. rettgeri*) and crystalline patches ($\times 5000$).

In the present work, 292 strains were isolated and identified from 284 samples. As out of 100 urine samples (before catheterization), 76 (76%) were positive for bacterial growth. Out of 92 urine samples (after stent removal), 80 (86.95%) were positive for bacterial growth and out of 92 stent samples, 84 (91.3%) were positive for bacterial growth. Stents collected from patients were examined for biofilm using SEM and it was found that all stents positive for microbial growth were showing biofilm upon their examination.

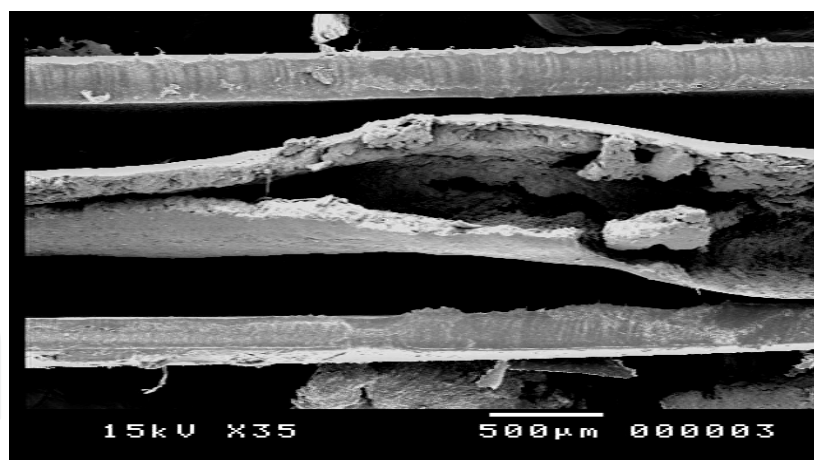


Fig. 4. Scanning electron micrograph showed the lumen of a ureteral stent obtained from patients treated with cefotaxime ($\times 35$). It showed a dense mass of biofilm and a high level of encrustation.

Klebseilla spp. was the most prevalent (21.9%) microorganism followed by *Pseudomonas* spp. (18.8%), *Staphylococci* spp. (18.2%), *E. coli* (17.8%), *Proteus* spp. (11.3%), *Providencia rettgeri* (4.8%) *Citrobacter freundii* (4.8%) and *Serratia marcescens* (2.8%). Mixed infection represented 22.9%. All *S. aureus* and coagulase negative staphylococci isolates were polymicrobial with *Klebseilla* spp., *Pseudomonas* spp., *Providencia rettgeri* and *S. marcescens*.

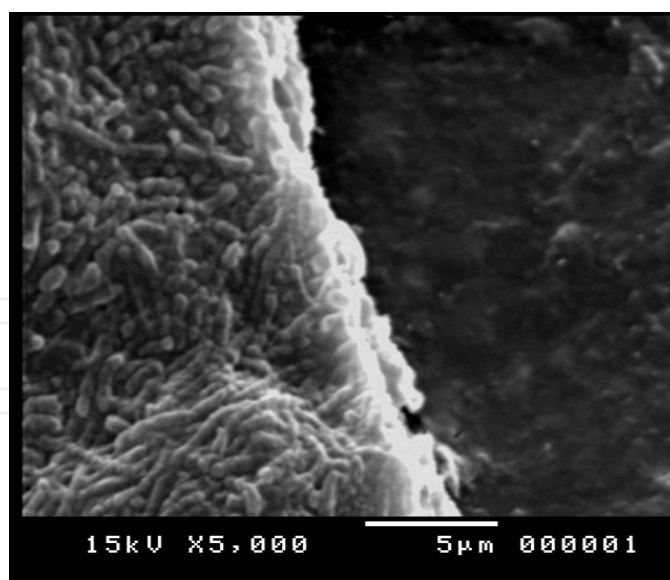


Fig. 5. Scanning electron micrograph showed the lumen of the ureteral stent ($\times 5000$). It showed a dense mass of biofilm (rods and cocci bacteria).

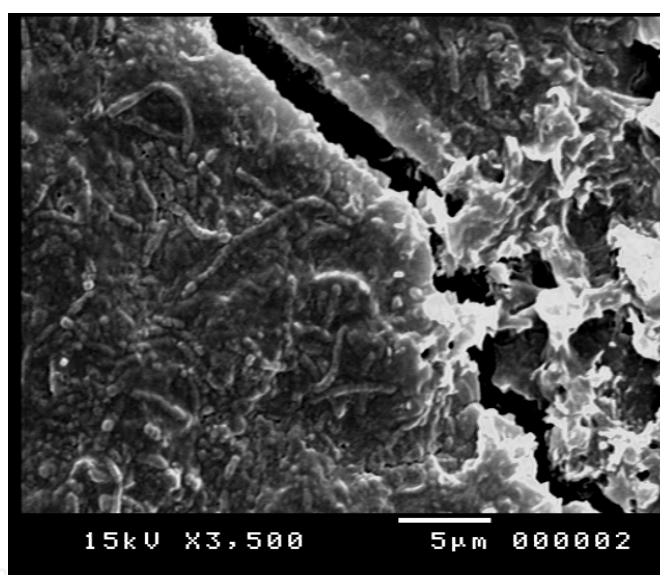


Fig. 6. Scanning electron micrograph showed the surface of the ureteral stent ($\times 3500$). It showed a dense mass of biofilm containing microorganisms and a high level of encrustation.

The resistance pattern to cefotaxime, augmentin, ciprofloxacin, levofloxacin and ofloxacin revealed that the highest incidence of resistance to cefotaxime was shown by *K. oxytoca* (54.2%). Also the highest incidence of resistance to augmentin and levofloxacin was shown by *Pseudomonas* spp. (80 and 72.7%, respectively), while the highest resistance to ciprofloxacin and ofloxacin was shown by *C. freundii* (78.6% each).

Biofilm production was found in 84.6% of the isolates using TCP. *Pseudomonas* spp. were the highest biofilm producing microorganism. A dose related decrease in biofilm formation was observed by both ciprofloxacin and N-acetylcysteine. This was detected by a decrease in the optical density of the biofilm layer on microtiter plates and the number of viable cells attached to the catheter surfaces in comparison to controls. It was found

also that CIP/NAC combinations have the highest inhibitory effect on the initial adherence (84-100% of the controls) and the highest disruptive effect to mature biofilms (87-100% of the controls).

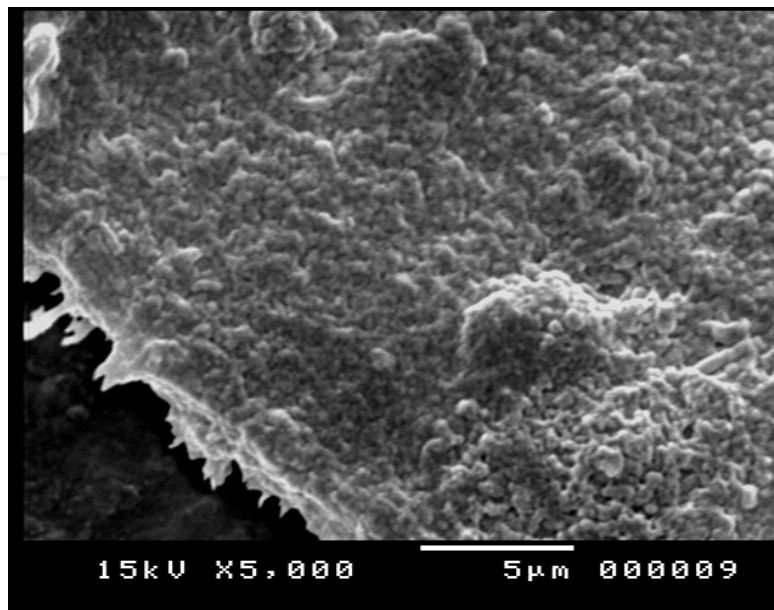


Fig. 7. Scanning electron micrograph showed the surface of a ureteral stent covered with high dense crystalline biofilm ($\times 5000$).

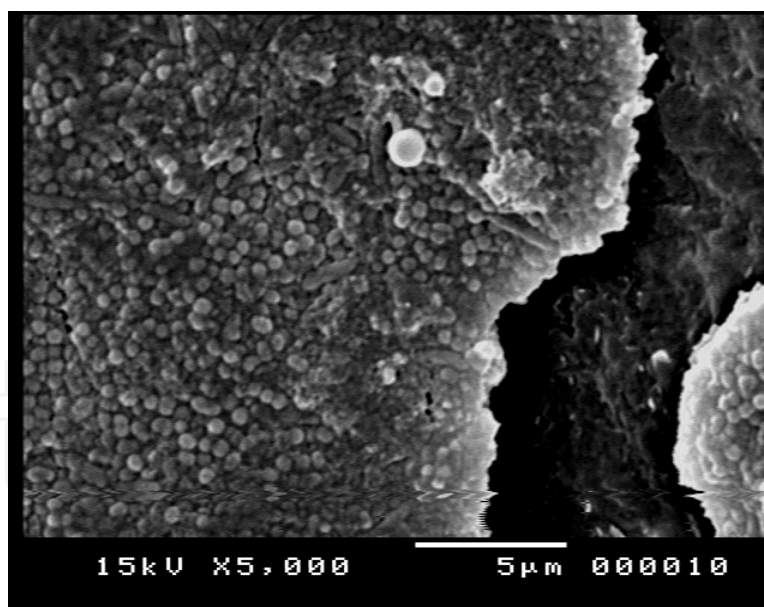


Fig. 8. Scanning electron micrograph showed the lumen of a ureteral stent covered with a big mass of biofilm containing bacteria (rods and cocci) (*K. pneumoniae* and *S. aureus*) ($\times 5000$).

The inhibitory effects of the tested agents were also verified by (SEM). Scanning electron micrographs showed the morphological response of the tested organisms to ciprofloxacin and N-acetylcysteine. They showed also the decrease in the extent of biofilm formation in the presence of the tested agents.

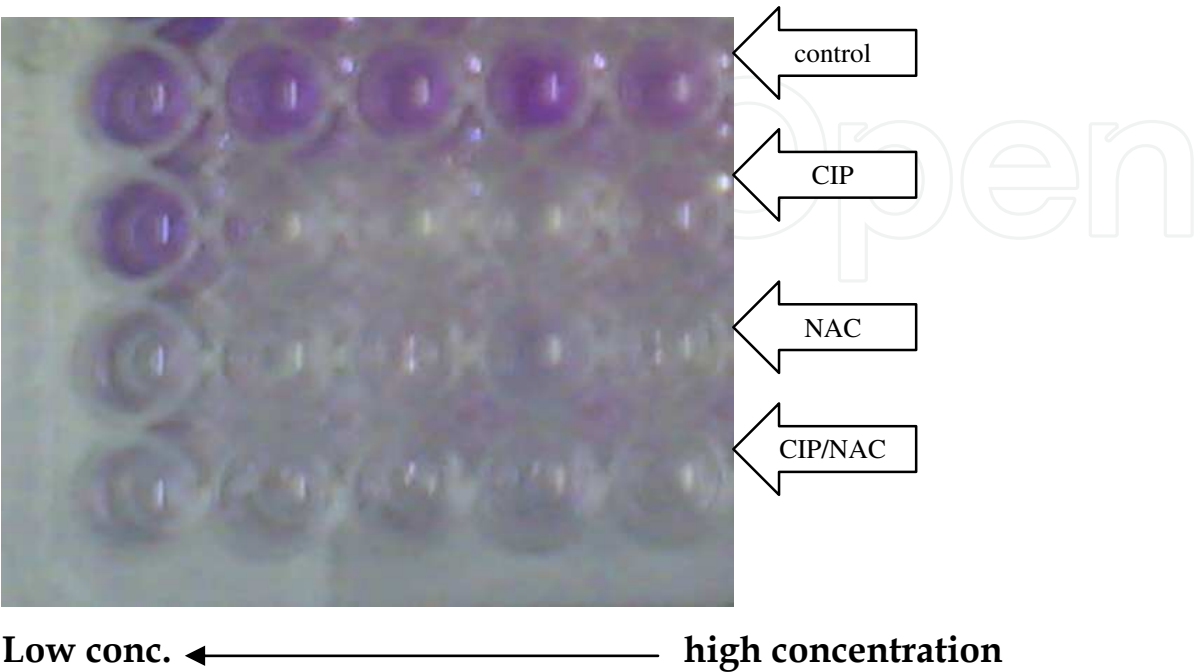


Fig. 9.

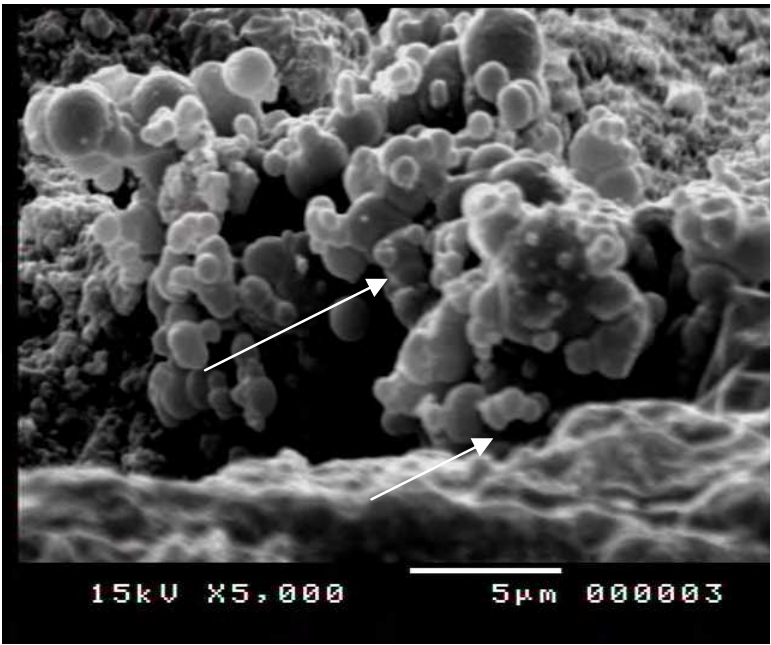


Fig. 10. a. Scanning electron micrograph of *S. aureus* biofilm on the surface. (a uretral stent incubated with *S. aureus* suspension for 24h as a control) ($\times 5000$).

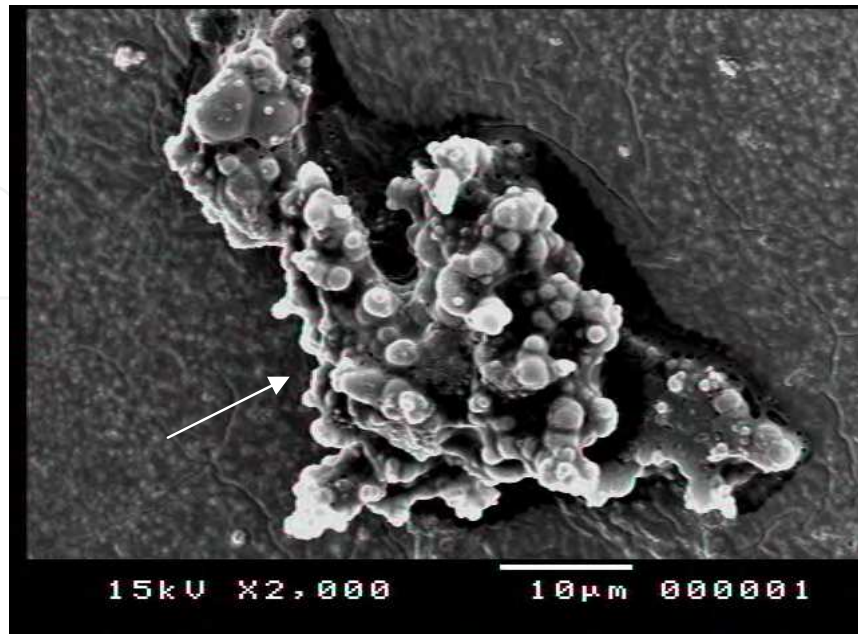


Fig. 10. b. Scanning electron micrograph showed the morphological response of *S. aureus* performed biofilm on the surface of a uretral stent exposed to sub-MIC concentration (CIP 4 µg/ml). there was a decrease in the amount of biofilm mass adhered to stent surface.

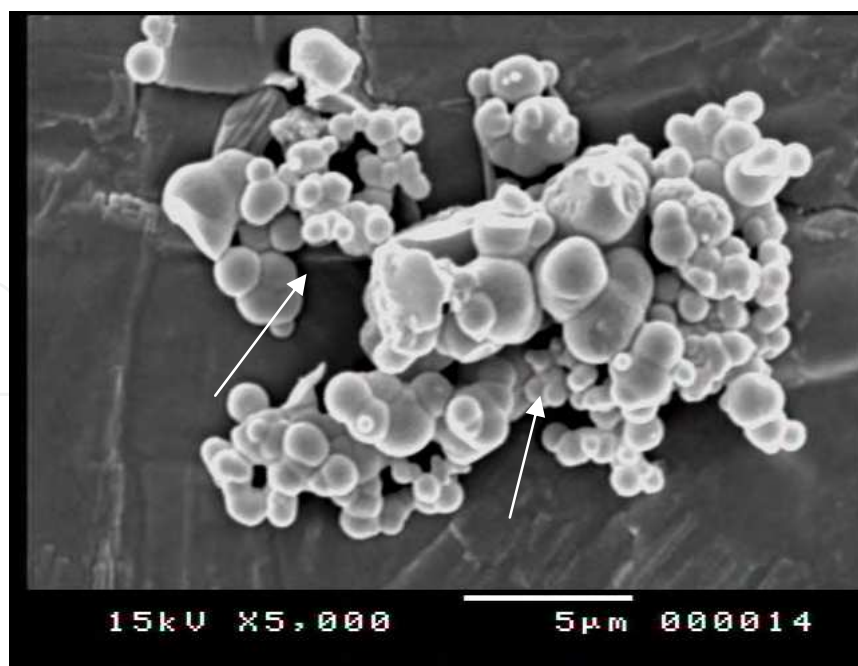


Fig. 10. c. Scanning electron micrograph showed the effect of N-acetylcysteine on a performed *S. aureus* biofilm. Cotton like mass disappeared and cells appeared swollen with disrupted cell wall ($\times 5000$).

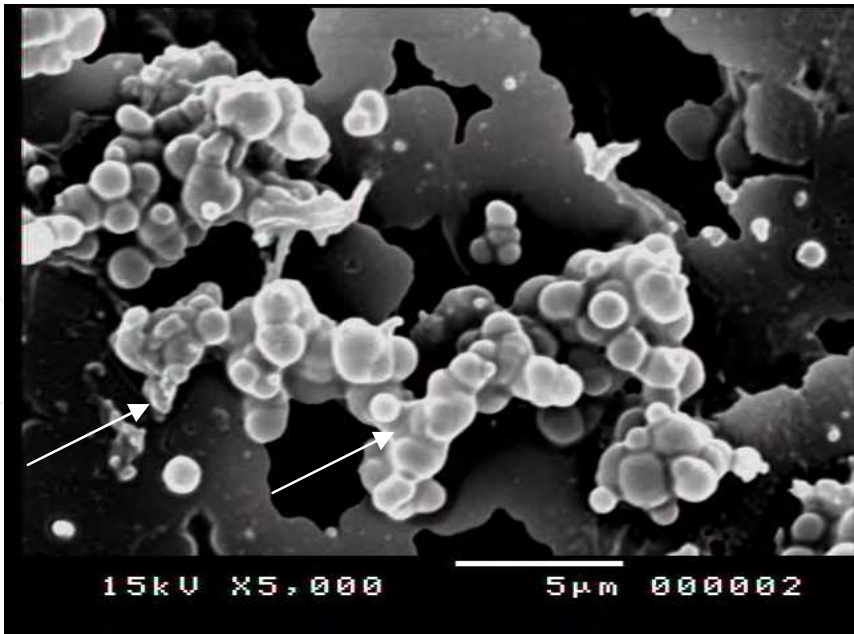


Fig. 10. d. Scanning electron micrograph showed the effect of ciprofloxacin-N-acetylcysteine combination on a performed *S. aureus* biofilm. Cell appeared swollen, disrupted and scattered ($\times 5000$).

Scanning electron micrographs showed the effect of Ciprofloxacin, N-acetylcysteine each alone and in combination on a performed *S. aureus* biofilm developed *in-vitro* on stent surface.

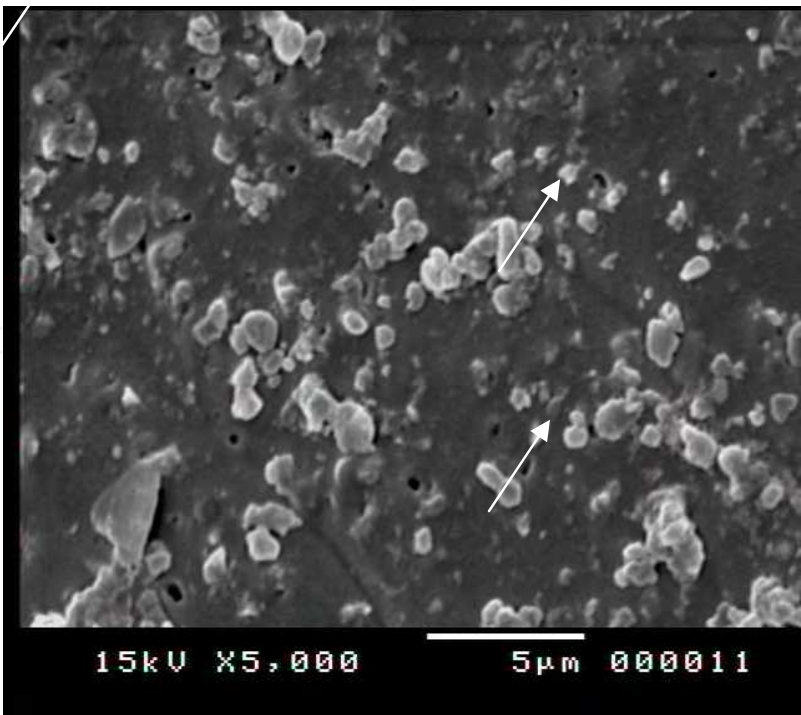


Fig. 11. a. Scanning electron micrograph showing the morphological responses of *Pseudomonas* spp. and *S. epidermidis* grown in the presence of sub-MIC concentration of ciprofloxacin. Cells appeared swollen and scattered with no biofilm mass.

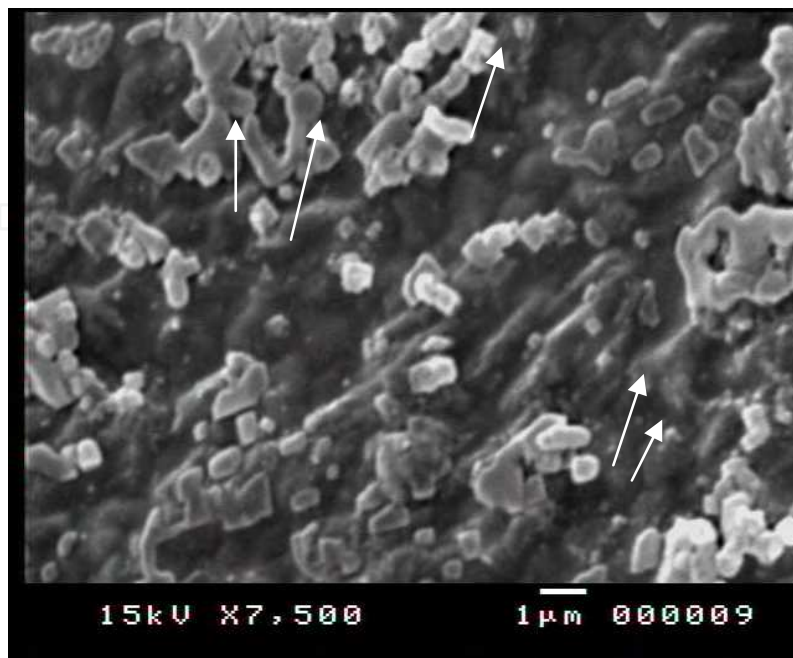


Fig 11. b. Scanning electron micrograph showing the effect of N-acetylcysteine on the biofilm formed by *S. epidermidis* and *pseudomonas* spp.. Cells showed membrane disorganization, appeared swollen and with disrupted outer membrane.

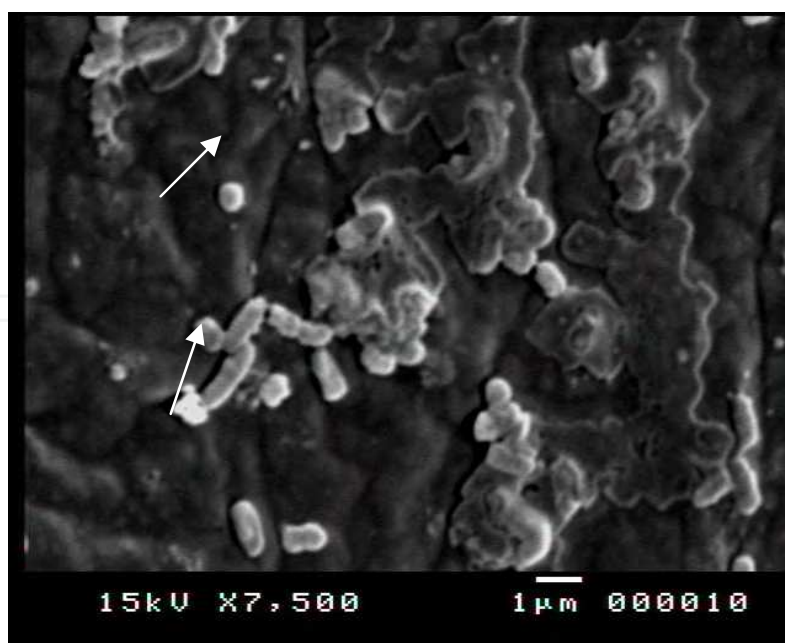


Fig 11. c. Scanning electron micrograph showed the effect of CIP/NAC (MIC/4_{mg/ml}) on the ability of *S. epidermidis* and *Pseudomonas* spp. to form biofilm. Cells appeared scattered, elongated, swollen, with disorganized (irregular) membrane and with no cotton like mass (biofilm) around cells.

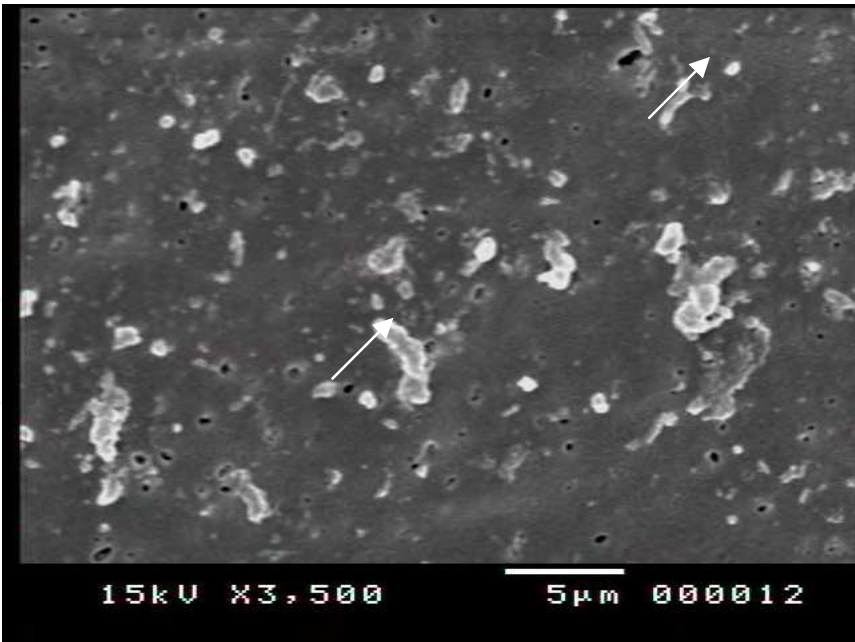


Fig 11. D. Scanning electron micrograph showed the effect of CIP/NAC combination (2 MIC/ 8 mg/ml) on the ability of *S. epidermidis* and *pseudomonas* spp. To form biofilm. A high decrease in the number of adherent cells observed. Cells appeared large, swollen and with disrupted cell wall.

Scanning electron micrographs showed the morphological response and the ability of *S. epidermidis* and *Pseudomonas* spp. grown in the presence of Ciprofloxacin, N-acetylcysteine and their combinations to form biofilm on stent surfaces.

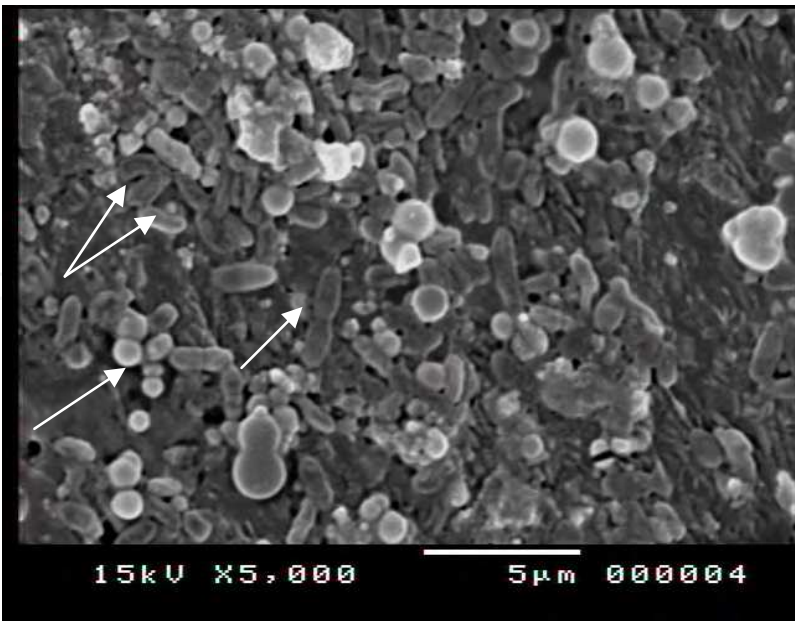


Fig. 12. a. Scanning electron micrograph showed the morphological response of *S. aureus* and *pseudomonas* spp. cells grown in the presence of ciprofloxacin at sub-MIC concentration. Cells appeared swollen, enlarged, with irregular cell wall, some showed v-shaped cells and small amount of biofilm mass observed.

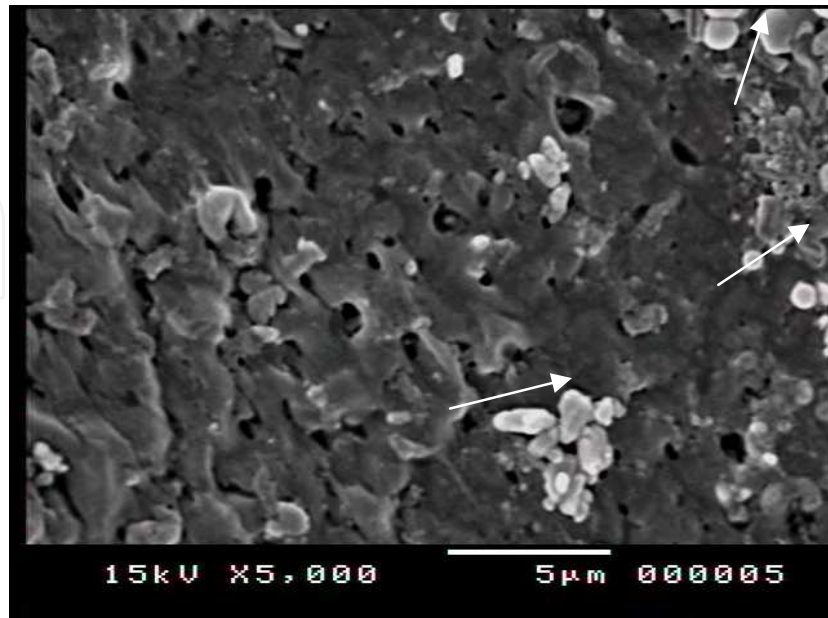


Fig. 12. b. Scanning electron micrograph showed the effect of N-acetylcysteine (4 mg/ml) on biofilm formation by *S. aureus* and *Pseudomonas* spp.. Cells appeared swollen, irregular in shape and small microcolonies observed scattered. A decrease in the number of adherent cells was observed.

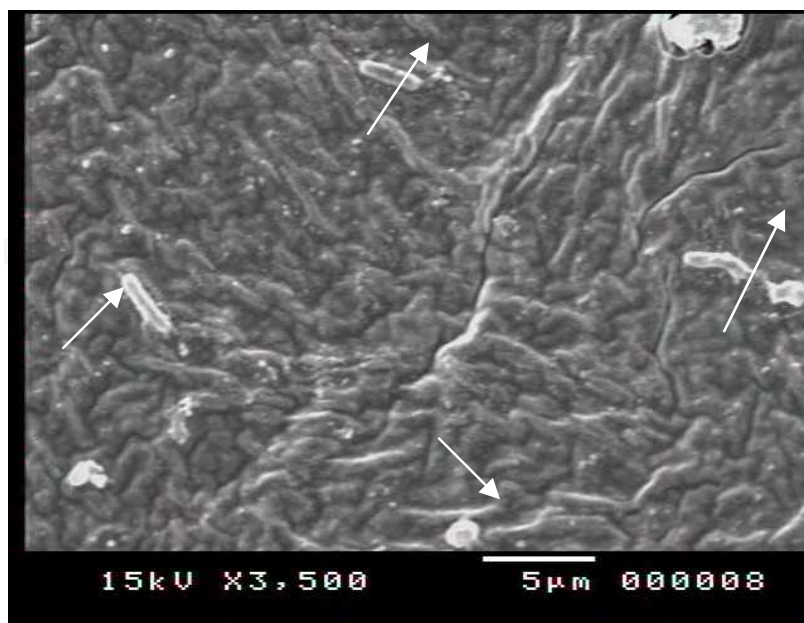


Fig. 12. c. Scanning electron micrograph showed the effect of CIP/NAC combination of (MIC/4_{mg/ml}) on *S. aureus* and *pseudomonas* spp. ability to form biofilm. Cells appeared elongated, enlarged and scattered with no biofilm mass observed on the surface.

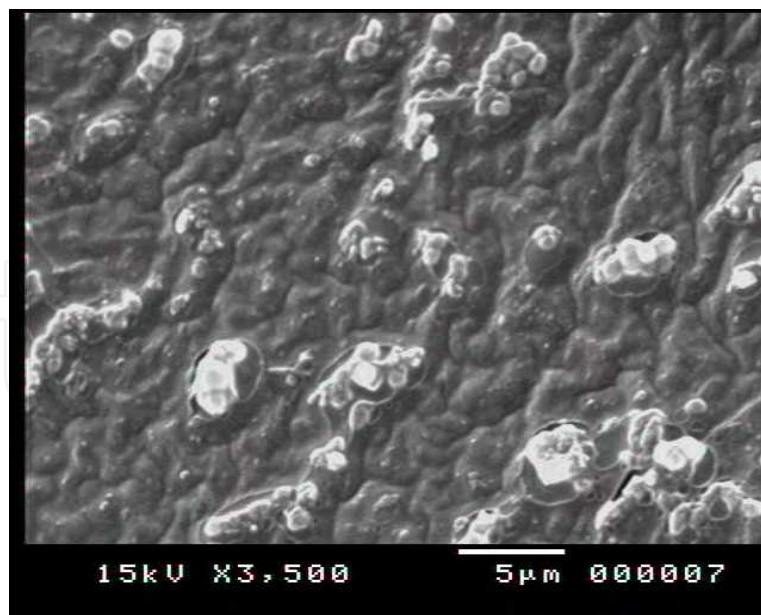


Fig. 12. d. Scanning electron micrograph showed the effect of CIP/NAC combination of (2 MIC/8 mg/ml) on *S. aureus* and *pseudomonas* spp. ability to form biofilm. No biofilm observed on the surface of stent.

Scanning electron micrographs showed the morphological response and the ability of *S. aureus* and *Pseudomonas* spp. grown in the presence of Ciprofloxacin (sub-MIC), N-acetylcysteine and their combinations to form biofilm on stent surfaces.

4. Conclusion

The presence of non antimicrobial agent such as N-acetylcysteine (NAC), caused significant decrease in biofilm formation by a variety of bacteria and reduces the production of extracellular polysaccharide matrix while promoting the disruption of mature biofilms. It was found that the inhibitory effect of both ciprofloxacin and N-acetylcysteine was concentration dependent.

CIP/NAC combinations were found to show the highest effect on bacterial adherence inhibition and on the disruption of the already formed biofilms. As N-acetylcysteine increase the therapeutic activity of ciprofloxacin when used in combination by degrading the extracellular polysaccharide matrix of biofilm.

In the chapter, Scanning Electron Microscope is used for the evaluation of medical implants, detection of biofilm and studying the effect of different biofilm inhibitory agents. This technique provides excellent visualization of glycocalyx, which is one of the most prominent features of biofilms and a crucial research subject in the searching for alternative antimicrobial and anti adherent agents treatments.

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6. References

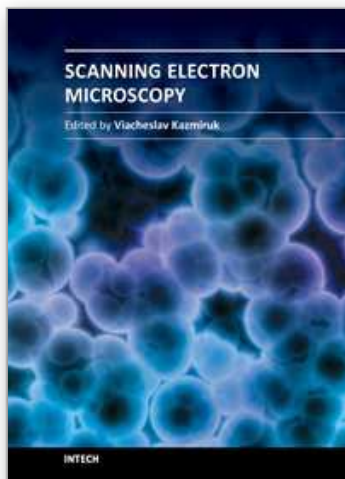
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Today, an individual would be hard-pressed to find any science field that does not employ methods and instruments based on the use of fine focused electron and ion beams. Well instrumented and supplemented with advanced methods and techniques, SEMs provide possibilities not only of surface imaging but quantitative measurement of object topologies, local electrophysical characteristics of semiconductor structures and performing elemental analysis. Moreover, a fine focused e-beam is widely used for the creation of micro and nanostructures. The book's approach covers both theoretical and practical issues related to scanning electron microscopy. The book has 41 chapters, divided into six sections: Instrumentation, Methodology, Biology, Medicine, Material Science, Nanostructured Materials for Electronic Industry, Thin Films, Membranes, Ceramic, Geoscience, and Mineralogy. Each chapter, written by different authors, is a complete work which presupposes that readers have some background knowledge on the subject.

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