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DEVELOPMENT OF RISUG® DERIVATIVE AS AN EFFECTIVE ANTIMICROBIAL COMPOUND THAT CAN BE COATED ONTO URINARY CATHETERS FOR PREVENTION OF BACTERIAL BIOFILMS

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Abstract: Bacterial colonization on implant surface leads to implant associated infection. These infections limit the long term benefits of medical implanted devices and also act as a source of continues chronic microbial load in catheterized individual. Alteration of surface properties has been a lucrative option for prevention of development of microbial aggregates on implant materials. In the present study application of styrene maleic anhydride (SMA) in combination with DMSO has been carried out for coating the Foley's catheter for prevention of bacterial adhesion. Drug coated polymer was found to be highly hemo-compatible in nature. A significant decrease in numbers of adhered bacteria was observed after microscopic evaluation of the surface. Further, SMA-DMSO complex led to bacterial killing, induced by blebbing and disintegration of bacterial cells. Lipid peroxidation of drug treated bacterial cells was also observed. The present study reports safe and effective coating of SMA-DMSO complex onto urinary catheter leading to prevention of bacterial adhesion.

Key Words: Biofilm, DMSO, Hemo-compatibility, LPO, Plasma

INTRODUCTION

"United we stand divided we fall" nature microorganisms have a tendency to go from unicellular lifestyle to a more organized and systematic life form called biofilm. Biofilms are microbial aggregations that are adhered to a solid substratum and are embedded in the capsular polysaccharide (CPS) secreted by the microorganism itself (Costerton 1999). Organisms in biofilm show several times increased resistance to antibiotics to which the same organism in planktonic state is susceptible, hinting towards biofilm specific mechanisms that might be playing role in antimicrobial resistance (Stewart *et al.,* 2001). According to one estimate more than 65% of microbial infections in humans are biofilm associated and there is a desperate need to look for newer methods of biofilm prevention and eradication. Owing to thick CPS and other intrinsic factors such as nutritional deficit and presence of persister cells, eradication of established biofilms is extremely difficult (Vu *et al.,* 2009). Hence, there is a renewed interest in prevention of biofilms on substrata of medical importance. In this direction variable outcomes have been documented by researchers working with antibiotic lock system and covalent linking of antimicrobials (Khare *et al.,* 2007).

Styrene maleic anhydride (SMA) is a polymer with known antimicrobial properties (Sharma *et al.,* 2003). SMA in conjugation with dimethyl sulphoxide (DMSO) has been used as a male contraceptive under the brand name RISUG® (Reversible inhibition of sperm under guidance) (Guha 1996). Some anti-HIV effects of the compound RISUG® have also been reported (Guha 2005). Further, SMA has also been studied for its self-assembly into nano liposome and their transport to prostate epithelial cells, where this nano drug may act as prostate cancer preventive (Guha *et al.,* 2011).

Recently there is an interest in surface coating properties of SMA along with DMSO. In the present study we have evaluated the antibiofilm properties of SMS-DMSO complex coated on to the surface of Foley's urinary catheter. We demonstrate with experimental evidence that SMA+DMSO complex acts as an anti-adhering and antibacterial agent.

MATERIAL AND METHODS

Bacterial sample preparation

Lyophilized cultures of two bacterial strains, *Escherichia coli* (ATCC 29213) and *Staphylococcus aureus* (ATCC 25923) were used in the present study. *S.aureus* was procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, Maharashtra, India while *E.coli* was purchased from Microbial Type Culture Collection, IMTECH, Chandigarh, India. Both the strains were revived in Tryptone Soy broth (TSB) (HiMedia Inc. Mumbai, India) and lawn cultured on TSA (Tryptone soy agar) plates. After two subsequent passages cells were scraped from the plates and suspended in TSB (Tryptone soy broth) to desired concentration.

Polymer processing and drug preparation

Commercially available Foley's catheter was used in the study. 1 cm long pieces of catheter were cut

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and treated with argon plasma in M-PECVD-1A [S] (M/S Milman Thin Film Systems, Pune, India) plasma reactor (Gomathi *et al.*, 2008). The process conditions applied were, flow rate 20 SCCM, pressure 150mTorr, power 100 W and time 5- 6 min (Chauhan *et al.*, 2012). Four different concentrations of SMA (5mg, 10 mg, 20mg and 50mg) were dissolved in 1 ml of DMSO and used for dip coating of plasma exposed catheter segments in a sterile environment.

Static biofilm adhesion assay and scanning electron microscopy

Biofilm was grown with no fluid shear. Sterile filter paper discs (Whatman Qualitative Grade 2, 70-mm diameter) lying on TSA plates were inoculated with 1.5 ml of *S. aureus* and *E. coli* inoculums ($0.5x10^3$ - $2.5x10^3$). Drug Coated and uncoated polymers were placed on top of the bacteria inoculated filter paper discs on separate TSA plates, with coated side of catheter facing the inoculated side of the filter paper. The plates were incubated for 48 h at 37 °C (Charaf *et al.*, 1999). The efficacy of coated SMA in prohibiting the formation of biofilms on outer surface of catheter was checked visually by electron microscopy and the extended efficacy of coated SMA in inhibiting the growth of biofilms was checked by determining the cfu/ml of treated catheter after ultra-sonication (piezo-u-sonic, ultrasonic cleaner, Korea 1700). For electron microscopy polymer samples were fixed with 3% glutaraldehyde followed by osmium tetraoxide post fixation. Then, series of ethanol gradation steps were performed and samples were sputter coated with gold and examined by using JEOL JSM-5800 Scanning microscope using 20 kV acceleration voltage

Lipid peroxidation (LPO) assay

LPO assay was performed by the method of Hodges *et al.*, (1999). The stock solution contained equal volumes of trichloroacetic acid 15% (w/v) in 0.25N HCl and 2-thiobarbituric acid 0.37% (w/v) prepared in 0.25N HCl. One volume of bacterial sample and two volumes of stock reagent were vortexed and heated for 15 mins in a boiling water bath. After cooling on ice, the flocculent precipitate was removed by centrifugation at 1000g for 10 min and absorbance measured at 535nm (OD_{535}) against blank. The values were expressed as nM malondialdehyde produced / min / mg protein.

Hemocompatibility

Hemocompatibility tests were carried out on the basis of ASTM (American Society of Testing and Materials) standard. In this test, physiological tolerance of the implant coating with blood is evaluated by calculating its hemolysis percentage with blood. The hemolysis percentage is defined as:

$$\% \text{ Hemolysis} = \frac{[OD(Test) - OD(Negative)]}{[OD(Positive) - OD(Negative)]} \times 100$$

Here optical density (OD_{545}) indicates optical density of the samples. For this purpose human blood was taken in a beaker containing sodium citrate (3.8 mg/ml) to avoid coagulation. The anti-coagulated blood was diluted with normal saline (N-saline) in the proportion of 8:10. For evaluating hemolysis, 10 ml of 0.01N HCl solution (HCl causes large scale rupture of RBC) was added to 0.2 ml of diluted blood and the OD_{545} count of this solution was taken as for positive control referred to as OD (positive). Similarly, for negative control 10 ml of N-saline (N-saline solution does not rupture RBC) was added to 0.2 ml of diluted blood and OD_{545} of this solution was referred to as OD (negative). Drug coated polymer samples were taken in a standard test tube containing N-saline and incubated at 37°C for 30 min for providing thermal equilibrium. Then 0.2 ml of diluted blood was added to the test tube and mixed gently. This process is referred as OD (test). The positive control, negative control and OD (test) were incubated for 60 min at 37°C before OD_{545} count (Roychowdhury *et al.*, 2004).

Statistical analysis of data

All data were analyzed statistically by Student's T-test with $P<0.05$ as significance level.

RESULTS AND DISCUSSION

Static adhesion assay and colony forming units

SEM micrographs of polymer samples, coated with different concentrations of drug and exposed to *E. coli* and *S. aureus* respectively are shown in Figure 1 and Figure 2. *E. coli* at a drug concentration of 10 mg/ml showed slight membrane disintegration (Figure 1a) while significant membrane disintegration and ultrastructure alterations like membrane blebbing were observed at 20 mg/ml of coated drug (Figure 1b). However, highest bacterial disintegration and membrane degeneration was observed at a drug concentration of 50 mg/ml (Figure 1c).

Table 1: Adhesion assay of uncoated and drug coated polymer under static condition (strains *E. coli* and *S. aureus*)

Drug conc.	*E. coli* (cfu/cm²)	*S. aureus* (cfu/cm²)
0 mg/ml	$3.7 X 10^6$	$3.4 X 10^6$
10 mg/ml	116	102
20 mg/ml	16	20
50 mg/ml	<1	< 1

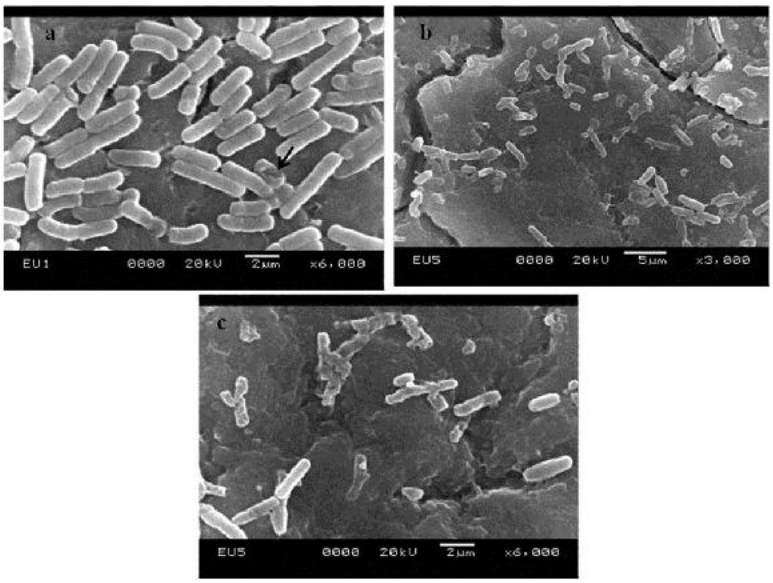

Figure 1: *E. coli* adhesion on drug coated polymer sample (a) membrane degeneration shown by black arrow on 10mg/ml coated polymer (b) membrane blebbing and breakage on 20mg/ml coated polymer (c) membrane blebbing and high disintegration shown on 50mg/ml drug coated polymer

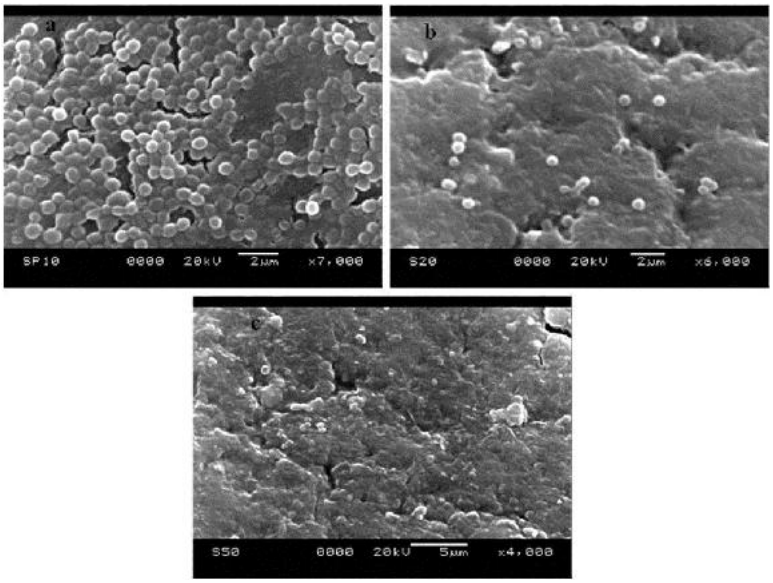

Figure 2: *S. aureus* adhesion on control and drug coated polymer: (a) adhesion on 10mg/ml coated polymer (b) adhesion on 20mg/ml coated polymer (c) adhesion on 50mg/ml coated polymer

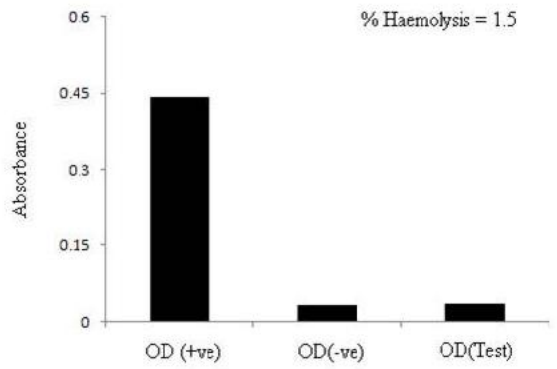

Figure 3: Percentage hemolysis of drug coated polymer

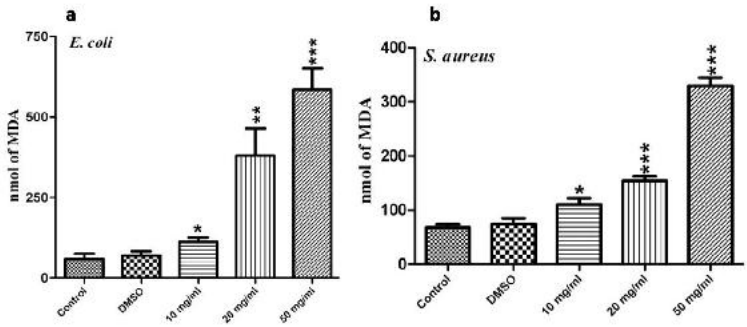

Figure 4: MDA release with 10mg/ml. 20mg/ml and 50mg/ml drug treatment in (a) *E. coli* (b) *S. aureus*

Likewise, *S. aureus* in Figure 2 (a)-Figure2 (c) showed significant reduction in adhesion to polymer coated with increasing drug concentrations. The electron micrographs clearly suggested that Figure 2c had less number of bacterial colonies present on the surface compared to Figure 2a and Figure 2b. The number of bacteria adhered to the polymer surface was evaluated and expressed in colony forming unit/ml recovered from each polymer sample.

The results are presented in table 1. The bacterial counts at a drug concentration of 5 and 10 mg/ml were not significantly different ($P<0.05$) from the bacterial numbers seen on untreated catheter surface. The reduction in bacterial numbers at 20 mg/ml of SMA in comparison to 10 mg/ml became significant ($P=0.001$). However, highest efficacy of drug in inhibiting the bacterial colonization was found at a concentration of 50 mg/ml where less than one bacteria/ cm^2 of catheter surface was observed ($P=0.0001$).

Hemocompatibility

The OD_{545} values of positive, negative and test sample are presented in Figure 3. A hemolysis of 1.5 was shown by drug coated (50 mg/ml) polymer. According to the ASTM, if the hemolysis percentage is less than 10, the test material is taken to be hemocompatible and if it is less than 5 then the

material is highly hemocompatible (Roychowdhury *et al.*, 2004). Therefore from the result it is clear that the drug coated polymer sample was highly hemocompatible in nature.

Lipid Peroxidation assay

Lipid Peroxidation study presented in Figure 4 showed a distinct pattern of increase in lipid peroxidation of bacterial cells with exposure to increased drug concentrations. Among different concentrations of drug 50mg/ml exhibited the highest MDA production in both *E. coli* (Figure 4a) and *S. aureus* (Figure 4b), thereby indicating highest antibacterial activity compared to either DMSO or lower concentrations of SMA (10, 20 and 50mg/ml).

Bacterial adhesion to biomaterials and their capability to form biofilm on foreign bodies are well established steps in the pathogenesis of implant associated infections. Growth of these microorganisms in direct contact with the implant surface plays a crucial role in biofilm formation as they link the entire biofilm formation to the implant surface, making the long term use of implants a challengeable task (Stover *et al.*, 2009). Hence, therapeutic manipulation of implant surface is a researchable issue that needs to be addressed urgently. Various research groups have tried different compounds for coating the surface of catheter to make them microbe resistant (Donlan *et al.*, 2009; Johnson *et al.*, 2006; Regev-soshani *et al.*, 2012), but not much has been gained and a great deal still remains to be explored.

SMA is a synthetic polymer that is built-up of styrene and maleic anhydride monomer units. The main characteristics of SMA copolymer are its transparent appearance, high heat resistance, high dimensional stability, and the specific reactivity of the anhydride groups. The latter feature results in the solubility of SMA in alkaline (water-based) solutions and dispersions. Several derivatives of SMA have been synthesized and demonstrated to possess antimicrobial activities (Jeong *et al.*, 2002). At a particular concentration, in association with DMSO, SMA has been used as a male contraceptive and its application as an anti-HIV agent is of special interest (Guha 2005). However, the antimicrobial properties of SMA have been underestimated. In the present study we have applied SMA-DMSO complex as an antibiofilm agent that can be coated onto the catheter surface and demonstrated its safety to the host and its efficacy against the two most common urinary tract pathogens, *E. coli* and *S. aureus*.

An essential part in the development of any manipulated surface for human use is analytic evaluation of its safety and efficacy. In the present study, drug coated polymer was found to be completely hemocompatible thus emphasizing the fact that even in close contact SMA-DMSO coated urinary catheters will not have any deleterious effect and can be allowed to stay in contact with host tissue for longer durations, if not contaminated by the microorganisms.

Microscopic evaluation demonstrated substantial antibacterial action of SMA-DMSO complex against biofilm producing strains of *E. coli* and *S. aureus*. Most significant changes that were observed in bacterial cells included membrane blebbing and disintegration of cell wall. The cell wall and cell membrane is the defensive barrier of bacteria and hence is the most important target for antibacterial action. Polyelectrolyte nature of the drug leads to generation of static charge at the site of action which leads to peroxidation of lipids and cause membrane damage (Sharma *et al.*, 2003). Gram positive bacteria such as *S. aureus* have a cytoplasmic lipid membrane and a thick peptidoglycan layer whereas, Gram-negative bacteria like *E. coli* have a cytoplasmic membrane, a thin peptidoglycan and an outer membrane containing lipopolysaccharide (LPS). Disintegration of the lipopolysaccharide layer, the peptidoglycan layer and the cytoplasmic membrane would lead to cell death. Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, of which the most abundant is malondialdehyde (MDA). MDA release from the bacterial strain with SMA-DMSO complex treatment indicated high level of lipid peroxidation of the membranes. Oxidation of the membrane lipids lead to cell lysis and loss of membrane integrity (Halliwell *et al.*, 1993). Moreover, DMSO did not seem to have any antimicrobial effect of its own as only DMSO coated polymer did not result in decreased bacterial colonization (results not shown).

CONCLUSION

Hence, through the present study we have been able to demonstrate that SMA is an effective and safe drug that could be incorporated onto the polymer surface to give local drug delivery system. SMA acted as an anti-adhesion and antibacterial agent. The initial in vitro experimentation with SMA looked very promising but its in vivo evaluation in mouse model of UTI would ultimately help in establishing the SMA-DMSO as a viable option for coating the surface of catheter that would minimize the initial bacterial adhesion and also result in eradication of established biofilms.

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