Bacterial response to silica nanoparticle induced nano-dimensional surface topography

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Abstract

Bacterial adhesion and subsequent biofilm formation are the common pathogenic events associated with the severity (symptoms) and duration (chronic) of an infection, inferring a heavy socio-economic loss. Several factors including surface charge, surface topography, and physicochemical property play a crucial role in bacterial attachment and its subsequent biofilm formation on any biotic or abiotic surface. This work exploits the use of nano-dimensional surface topography generated using silica nanoparticles in hindering bacterial proliferation and attachment thereby preventing biofilm formation. The effect of nanoscale surface topography generated by 7 nm, 14 nm, and 21 nm sized silica nanoparticles are experimented on two types of bacterial strains, gram-positive *Staphylococcus epidermidis* and gram-negative *Escherichia coli.* Our results suggest the bactericidal effects of nanoscale surface topographies were effective in reducing proliferation and attachment in both the test strains and, we also demonstrate a size-specific toxicity of silica nanoparticle-induced topographies on each of the bacterial strains. We suggest this as a result of two complementary factors, (a) silanol groups (hydroxyl interactions) on the silica nanoparticle surface which interacts with the bacterial membrane lipoproteins and (b) surface topography roughness induced stress, due to the proximity effect, affects the membrane structure integrity leading to cell death. The encouraging data obtained from this study advocate that surface topography modifications using silica nanoparticles is an effective alternative approach towards chemical/drug-free antibacterial surface. This work has relevance in biomedical applications particularly nosocomial infections related to contaminated medical device surface, catheter, implants colonized with persistent multidrug resistant bacteria, simply by modifying the surfaces with non-metallic nanoparticles.

Résumé

L'adhérence bactérienne et la formation subséquente de biofilms sont les événements pathogènes communs associés à la gravité (symptômes) et à la durée (chronique) d'une infection, ce qui laisse supposer une lourde perte socioéconomique. Plusieurs facteurs, dont la charge superficielle, la topographie superficielle et les propriétés physicochimiques, jouent un rôle crucial dans la fixation bactérienne et sa formation subséquente de biofilm sur toute surface biotique ou abiotique. Ce travail exploite l'utilisation de la topographie de surface nano dimensionnelle générée à l'aide de nanoparticules de silice pour empêcher la prolifération bactérienne et l'attachement, empêchant ainsi la formation de biofilm. L'effet de la topographie de surface à l'échelle nanométrique générée par les nanoparticules de silice de taille 7nm, 14nm et 21nm est expérimenté sur deux types de souches bactériennes, *Staphylococcus epidermidis* à Gram positif et *Escherichia coli* à Gram négatif. Nos résultats suggèrent que les effets bactéricides des topographies de surface à l'échelle nanométrique ont été efficaces pour réduire la prolifération et l'attachement sur les deux souches d'essai et, nous démontrons également une toxicité spécifique à la taille des nanoparticules de silice, topographies induites sur chacune des souches bactériennes. Nous suggérons ceci en raison de deux facteurs complémentaires, (a) groupes de silanol (interactions hydroxyles) sur la surface de silice nanoparticule qui interagit avec les lipoprotéines de la membrane bactérienne et (b) la rugosité de la surface a induit le stress, en raison de l'effet de proximité, affecte l'intégrité de la structure membranaire menant à la mort cellulaire. Les données encourageantes tirées de cette étude préconisent que les modifications topographiques de surface utilisant des nanoparticules de silice constituent une approche alternative efficace vers une surface antibactérienne sans produits chimiques. Ce travail est pertinent dans les applications biomédicales, en particulier les infections nosocomiales liées à la surface contaminée du dispositif médical, au cathéter, aux implants

Colonisés par des bactéries multirésistantes persistantes, simplement en modifiant les surfaces avec des nanoparticules non métalliques.

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I would like to express my sincere gratitude and humbly thank my mentor, Professor Dan V. Nicolau for giving me the opportunity to join his research group as a master student and providing me invaluable guidance in developing my research skills. His dynamic vision and dedication has inspired me to develop analytical skills and carry out my research explicitly. I would also like to extend my sincere thanks to Professor Robert Funnell for the encouragement and support he provided me throughout the program as my GPD. The completion of my project would have not been possible without the constant guidance and unconditional support of Dr. Ayyappasamy, Post-Doc of our team. I would also like to thank my friend and teammate Monalisha for constant support and encouragement and also my teammate Giulia. I would like to thank the Professors whom I have credited courses with, they not only helped me through the course work but also supported me in applying for grants by providing recommendation letters: Professor Amine Kamen, Professor Robert Funnell, and all other professors. I would like to thank the administrative team in the biological and biomedical engineering department; especially Pina Sorrini and Sabrina Teoli for all the support, help, and making themselves available. I would like to thank the Bioengineering Department; Antonella Fratino, Aimée Jabour, Luisa Seidl, Dr. Horia Roman, Xavier Elisseeff, and Andy Catalano for all the wonderful support and guidance. Last but not the least, I would like to thank my mother Punitha, father Rajendran, my sisters, my aunt Dr. Dakshayini, and my partner for their prayers, love, sacrifices, and their constant support and encouragement in my academic endeavor without which I would have not been able to make it this far. My heartfelt gratitude and many thanks for everyone who has supported me throughout and made this a smooth experience.

Contribution of authors

Prof. Dan V. Nicolau conceived the idea, provided critical feedback, and directed the experiments, research analysis and manuscript. Initial experimental set up, instrument handling and training, SEM imaging, AFM imaging was jointly carried out with Dr. Ayyappasamy. Surface modifications, confocal microscopy experiments, live and dead cell assay, crystal violet assay, resazurin assay, sample preparation for SEM and AFM was done by Kavya Rajendran. Result analysis was done by Kavya Rajendran under Prof. Dan V. Nicolau and Dr. Ayyappasamy's supervision and support.

Thesis Composition

This is a manuscript-based thesis and Chapter 1 - Outlines the motivation and background for the current project; Chapter 2 - Contains the draft manuscript with experimental details, results, and discussion; Chapter 3 – Conclusion and future work.

Chapter 1 – Introduction

Antimicrobial surfaces are critical to a wide range of applications including medical facilities, medical devices and implants, water treatment facilities, and food processing industries. Scientific innovations have leveraged several routes to inhibit initial bacterial attachment or reduce bacterial viability in pre-formed biofilm. Despite the rigorous stratagem to develop anti-colonizing, anti-reflective material, overuse of antibiotics, and emergence of multi-drug resistant bacteria or super bugs pose a challenging threat in eradicating biofilm-mediated infections. Although these routes have been extensively pursued, biofilm-mediated outbreaks and infections have still been a cause for severe socio-economic loss, claiming several lives, and preventing or eradicating biofilm has been infeasible. Bacterial attachment and subsequent biofilm formation fundamentally depend on surface chemistry, topography, nutrient availability, physicochemical property of the substrate. The advancements in the inter-disciplinary research have led to remarkable development in understanding nano-scale interactions. The understanding of biological interactions at a nanoscale is particularly essential as most biological molecules and their bio stimuli functions are nano dimensional. This study is focused on the configuration of bacterial proliferation and their attachment on nano-patterned substrate. I started my master's thesis project by studying bacterial response to nano-dimensional topographies. Exploring the influence of nanoscale topography on bacterial proliferation and adhesion was executed by modifying glass surface (base substrate) using 7 nm, 14 nm and 21 nm sized colloidal silica nanoparticles. This study employs wild-type *Escherichia coli* and *Staphylococcus epidermidis* as model organisms. We hypothesize that surface topography modification at a nanoscale level may be a salient contributing factor in progressively declining bacterial proliferation, consequently reducing bacterial attachment. To study the hypothesis the following project-specific aims were followed;

Objective 1: Surface modification and characterization.

Objective 2: Determine the effect of silica nanoparticles-induced nanoscale topography on bacterial proliferation, bactericidal activity, substrate-cell interface interaction, and biofilm mediated study.

Bacterial prevalence and their habitats are ubiquitous, thanks to their eons of intricately evolved traits to adapt and colonize diverse environmental niches. As an adaptation strategy, bacterial species have the ability to respond to surface topographies, to the precision of nano-dimensions by producing a specific type of protein or carbohydrate components on the cell surface to a more complex three-dimensional architecture of sessile community forming biofilms [1]. Bacteria can also exponentially procreate in these microcosms shunning from antibiotics and antagonistic molecules [3, 7, 8, 12]. Understanding these response mechanisms and molecular processes are vital to devise stratagems to control and treat several bacterial-born acute and persistent infections. Bacterial infections, either at the lower respiratory regions caused by bacterial pneumonia, tuberculosis, or enteric infections and urinary tract infections, were prevalent since pre-medieval periods, and are still the top three causes of infections worldwide with high mortality and morbidity rates [2, 3]. Biofilm mediated colonization and virulence mechanisms are one of the major sources of bacterial-born refractory infections. Biofilm formation is a severe economic and technological concern in medical facilities, food processing industries, medical implants, and water treatment facilities [1-6]. Additionally, the efficacy of eradicating preformed biofilm is limited due to the low diffusion of any drug molecules, high physical and mechanical resilience on the surface, persistent attachment, and in many scenarios a non-specific multidrug resistance property exhibited by the bacterial cells in the biofilm matrix [4, 18, 19, 21, 22]. Therefore, the prevention and eradication of bacterial biofilm formation in its initial stages is a critical parameter towards preventing infection. We use the surface topography as a solitary parameter in preventing

bacterial attachment by fabricating nano-scale topographies using silica nanoparticles.

Silica (SiO_2) is an abundant, ubiquitous inorganic compound that has excellent receptiveness to interact with biological materials. Silica nanoparticles are employed in several biomedical applications like imaging, drug delivery, therapeutics, bioelectronics, and biosensor due to their relatively low toxicity towards eukaryotic cells [37]. Due to their abundance availability, GRAS status, versatility, and low toxicity silica is chosen as an ideal candidate to fabricate nano-scale topographies [36, 37]. Most studies involving silica nanoparticles as an antibacterial agent employ mesoporous silica nanoparticles, or solid silica nanoparticles functionalized and/or conjugated with drugs or other metallic nanoparticles. This study distinguishes itself in using solid silica nanoparticles as an effective antibacterial surface. Moreover, several studies dictating the toxicity of nanoparticles rely on traditional setups like colony forming units (cfu), quantification of minimum inhibitory concentration (MIC) or calorimetric assay, that takes several hours to days in understanding the efficacy of nanoparticle in eradicating bacterial biofilm. This work periodically studies the effect of silica nanoparticles induced surface topographies on bacterial cells at 3, 6, 12, 24, and 48 hours of incubation time and the effect is pronounced even at 3 and 6hr incubation time in both the bacterial strains.

In order to study the surface effect of silica induced nano-topography on bacterial colonization, negatively charged silica nanoparticles were ionically adsorbed on the glass substrate coated with positively charged polycationic polymer. Colloidal silica nanoparticles was ionically adsorbed on glass substrate using polycationic polymer, and the drying process leads to condensation of nanoparticles forming continuously dispersed nanoscale topography. To probe the effect of silica nanoparticle-induced surface topographies as a sole contributing factor for the antibacterial effect and preclude other potential factors, substrates like clean glass, polycationic polymer and plasma treated glass (PTG) were used as controls in this study. Surface

characterization of all substrates were performed to obtain insights on the dispersion of nanoparticles, surface roughness and hydrophilic/hydrophobic properties. SEM analyses confirmed the uniform deposition and distribution of silica nanoparticles on surface establishing successful topography modification which was further validated by high resolution images obtained from AFM, confirming the surface roughness of substrates subjected to plasma treatment and substrates modified with silica nanoparticles. All the coverslips were subjected to bacterial studies such as proliferation, bactericidal activity, and high-resolution scanning electron microscopy (SEM) imaging to understand the effect of topography on bacterial proliferation and at cell- substrate interface. The size-dependent, surface topography associated toxicity on bacterial cells has been studied here using two different gram stain bacteria namely, gram-positive – *Staphylococcus epidermidis* and gram-negative – *Escherichia coli* k12 bacterial species. *S. epidermidis* is a pathogenic biofilm forming bacteria, associated with nosocomial bacteremia induced by catheter bloodstream infections, endocarditis, urinary tract infection, surgical site infection, endophthalmitis [42]. *E. coli* k12 although a non-pathogenic bacterium, has the ability to form biofilm and certain strains of the same species can cause illness of varying degrees of fatality particularly in immunocompromised individuals, children, and pregnant women [43, 44]. Also, *E. coli* k12-wt used here is a model organism to various pathogenic variants of the same genus and species.

Even if the bactericidal activity is a key parameter, the cell-substrate interaction, understanding the presence of bactericidal compounds is also crucial to examine and comprehend the underlying toxicity mechanisms. To investigate the underlying bactericidal mechanism, surface characterization studies including confocal microscopy, live and dead cell assay, biofilm mediated study using crystal violet was employed. Furthermore, the cell-surface interface was studied using high resolution scanning electron microscopy. We explored the effect of surface

topography and available reactive silanol groups on the silica nanoparticle surface on bacterial attachment to the substrate particularly in the initial phases of biofilm formation. These reported observations were corroborated by our findings on the surface effects of silica nanoparticle induced topographies.

In this study, we were able to reduce the propensity of bacterial attachment to the glass substrate by modifying the surface topography using silica nanoparticles, demonstrated in both gram stains of bacteria establishing its broad-spectrum application. Furthermore, we attempted to understand the size- dependent silica nanoparticle-induced toxicity on bacterial candidates. While all the three sized nanoparticle had strong bactericidal effect of varying degrees for both gram-positive and gram-negative bacteria, 7 nm and 14 nm silica nanoparticles are more effective in controlling cell proliferation in both the types of bacteria. Specifically, the 14 nm silica nanoparticles showed slightly better efficiency in cellular damage dictating the bactericidal effects compared to 7 nm and 21 nm silica nanoparticles in both the strains. Although the topography modification produced different size-dependent effects on each of the test organisms by effectively reducing the bacterial proliferation, the surface adsorption due to silanol groups furthers the membrane damage, which can act as a dual effect on bactericidal effects on the surface. The structural organization of gram-positive and gram-negative bacterial cell membranes may also influence the mechanism and the subsequent mode of action of nanoparticles on bacteria. Overall, cumulative of confocal and the SEM analysis, the cell-surface interaction between bacteria and the surface modified with each sized nanoparticle, all three sized nanoparticles make for a hostile surface for bacterial attachment and proliferation. 7 nm is antagonistic in bacterial proliferation accounting for lower cell density, 14 nm exhibited interference in bacterial adhesion, multiplication and inferred cellular

damage in both the bacteria. This work has relevance in biomedical applications particularly nosocomial infections related to contaminated medical device surface, catheter, implants colonized with persistent multidrug resistant bacteria, simply by modifying the surfaces with non-metallic nanoparticles.

Chapter 2 – Bacterial response to silica nanoparticle induced nano-dimensional surface topography

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(Manuscript in preparation)

2.1 Abstract

Hypothesis

Bacterial adhesion and subsequent biofilm formation are the key steps in bacterial pathogenesis, leading to the onset of chronic infections and serious outbreaks, directly affecting human health, productivity, thus causing severe socioeconomic losses. Several factors associated with the surface colonization like surface chemistry, topography, and physicochemical properties play a crucial role in bacterial attachment and biofilm formation. Tuning surface topography at a nanoscale dimension may negatively influence the bacterial proliferation and attachment to the surface.

Experiments

This work studies the effect of nanoscale surface topography on reducing bacterial proliferation and adhesion using three differently sized silica nanoparticles namely 7 nm, 14 nm, and 21 nm bound ionically on to the glass cover slips. The nanoparticle-modified cover slips are

subjected to bacterial colonization studies for evaluating the surface colonization efficiency, nanoparticle interactions with bacterial cell surfaces, bacterial viability and biofilm production using two bacterial test strains, namely gram-positive *Staphylococcus epidermidis* and gram-negative *Escherichia coli* K12.

Findings

The studies performed here show silica nanoparticle induced size-specific bactericidal effects. We propose that these bactericidal effects are as a result of two complementary factors, (a) silanol groups on the surface of the nanoparticles that brings the bacteria close to the surface due to hydroxyl interactions with the membrane lipoproteins and (b) the surface topography roughness that induces stress, mechanically affecting the membrane structures leading to cell death.

2.2 Introduction

Biofilm mediated colonization and virulence mechanisms is one of the major sources of bacterial born refractory infections. Biofilm formation is a severe economic and technological concern in medical facilities, food processing industries, medical implants, and water treatment plants [1-6]. Biofilm formation is the ability of either a single species of bacteria or a bacterial consortium to colonize and adapt on a surface by forming an adhesive matrix, also defined as microbially derived sessile community of cells that attach to biotic or abiotic substratum [6-9]. The biofilm matrix is a three-dimensional architecture with pores, channels surrounded by hydrated extracellular polysaccharide substances (EPS) that are intricately matted for the sustainability of structured microbial communities inside [9-11]. This biofilm matrix physically and chemically shuns various bactericidal factors like physical stress (ultraviolet radiation), biocides (antibiotics, antibacterial, antifouling agents), host immune system responses from affecting the colonizing bacterial species, thus enabling their resilience and increasing their virulence leading to clinically significant infections [3, 7, 8, 12]. Centre for Disease Control and Prevention (CDCP) projects that about two thirds of bacterial infection are due to biofilm formation [7, 13, 14]. From an economic perspective, in the United States alone foodborne illness caused by pathogenic biofilm forming bacteria claims thousands of lives causing a financial loss of $78 billion/year [15]. Several studies have developed strategies to trigger biofilm disassembly by killing bacterial cells inside the preformed biofilms [16-20]. However, the efficacy of eradicating preformed biofilm is limited due to the low diffusion of drug molecules, high physical and mechanical resilience on the surface, persistent attachment and in many scenario a non-specific multidrug resistance property exhibited by the bacterial cells in the biofilm matrix [18, 19, 21, 22]. Therefore, the prevention and eradication of bacterial biofilm formation in its initial stages is a critical parameter towards preventing outbreaks and infections. Several factors including surface chemistry, the topography

of the substrate, and physicochemical property of bacterial cell to the surface influences bacterial adhesion and proliferation [23-26]. The role of surface roughness on bacterial attachment has been investigated in different contradicting aspects by several groups. For example, Scheuerman et al concluded that rougher surfaces offer higher surface area for attachment and protects bacterial cells from sheer forces, thus promoting favorable environment for bacterial attachment [27]. In another study on metallic surfaces, Boyd et al. found that surface roughness on a stainless steel promoted bacterial adhesion on to the surface compared to smooth stainless-steel surface [28]. On the contrary, another study by Woodling et al, showed electropolished smooth stainless steel enhanced bacterial colonization on the surface [29]. Other established strategies exploiting surface chemistry were altering surface hydrophilicity to hinder bacterial adhesion or grafting the surfaces with functional groups [28-30]. The effects were transient due to masking of surface chemistry by proteins and EPS secreted by bacteria and desorption of functional group molecules over a period of time [31, 32]. These lead to conclusions that, altering surface roughness or the net surface charge alone cannot suffice as an alternative strategy in preventing or reducing bacterial attachment to the surface.

Several groups reported that physically altering the surface with dynamic nanoscale features may reduce bacterial interaction with the surface, thereby significantly disrupting bacterial multiplication and adhesion on the surface [21, 22, 30, 33-35]. In this study, colloidal silica nanoparticles are selected as the substrate material to develop nanoscale topographies, as they have been by large, considered as safe (GRAS) status [36], and are used in various industries including, implants, pharmaceutical applications, electronics, biosensors [37]. Silica is an abundant, ubiquitous inorganic compound that has excellent receptiveness to interact with biological materials, it is found in connective tissues in the human body and is essential for bone growth [38]. Additionally, colloidal silica nanoparticles are low in toxicity, biocompatible, and can be

synthesized at a low cost [39]. Two typical studies by Cousins et al and Lord et al, showed silica nanoparticle induced topographies reduces the adhesion and proliferation of adherent mammalian cells [40, 41]. Adherent mammalian fibroblast cells exhibited two to three-fold reduction in proliferation, altered cellular orientation, and decreased adhesion on surfaces modified with silica nanoparticles [40]. Furthermore, reduced cellular attachment, spreading, and proliferation of human endothelial cells on substrate modified with silica nanoparticles was observed by Lord et al.

The work described here explores the effect of surface topography and available reactive silanol groups on the silica nanoparticles surface on bacterial attachment to the substrate particularly in the initial phases of biofilm formation. We studied here the bacterial proliferation and adherence to the substrate by modifying the surface topography at nanoscales using three different sized silica nanoparticles namely 7 nm, 14 nm, and 21 nm. The nanoparticles modify the surface topography of the substrate, here silicon dioxide (glass cover slips) by ionically binding with the glass surface in a continuous fashion. The size-dependent, surface topography associated toxicity on bacterial cells has been studied here using two different gram stain bacteria namely, gram-positive – *Staphylococcus epidermidis* and gram-negative – *Escherichia coli* K12 bacterial species. *S. epidermidis* is a pathogenic biofilm forming bacteria, associated with nosocomial bacteremia induced by catheter bloodstream infections, endocarditis, urinary tract infection, surgical site infection, endophthalmitis [42]. *E. coli* K12 although a non-pathogenic bacterium, has the ability to form biofilm and certain strains of the same species can cause illness of varying degrees of fatality particularly in immunocompromised individuals, children, and pregnant women [43, 44]. Also, *E. coli* K12-*wt* used here is a model organism to various pathogenic variants of the same genus and species.

2.3 Materials and Method

2.3.1 Surface modification and characterization:

2.3.1.1 Modification of surface topography with silica nanoparticles

Glass coverslip (22x22mm) purchased from Fisher brand were used as a substrate to deposit silica nanoparticles. Polycationic polymer solution of polyacrylamide-co-diallydimethyl-ammonium chloride (P(AAm-co-DADMAC)) purchased from Sigma Aldrich was used for ionic deposition of silica nanoparticles on glass substrate. Aqueous colloidal solution of silica nanoparticles with a diameter of 7 nm, 14 nm, and 21 nm were purchased from Sigma Aldrich and used for surface modification to produce nanoscale topography. Glass coverslips were cleaned by immersion in 100% methanol for 20 mins, coverslips were then rinsed with milli-Q for 3 mins and dried by passing nitrogen gas. To facilitate ionic adsorption of silica nanoparticles on the glass surface the cleaned coverslips were immersed in a 3.2 g/L dispersion of poly cationic polymer solution for 10 mins followed by rinsing with milli-Q for 1 min and drying with nitrogen gas. Coverslip deposited with polymer were completely immersed in 30% w/v suspension of colloidal silica nanoparticles for 5 mins. Coverslips were rinsed with milli-Q for 1 min and dried with nitrogen gas. Clean glass, polymer deposited, and plasma treated glass (PTG) coverslips were used as control. To establish the influence of surface chemistry does not contribute towards reducing bacterial adhesion and proliferation, substrate with similar or lower contact angle measurement to that of silica nanoparticles modified surface; here glass coverslips exposed to air plasma was employed as one of the controls. Clean glass coverslips after methanol treatment were washed using milli-Q and dried with nitrogen gas, coverslips were then subjected to air plasma treatment for 2 mins and immediately used for bacterial study. All coverslips except PTG were stored in desiccator overnight before use.

2.3.2 Surface characterization

2.3.2.1 Scanning electron microscopy (SEM)

To examine the distribution of nanoparticles deposited, the substrates were analyzed using scanning electron microscopy (SEM). Surfaces were coated with 4 nm Platinum by sputtering method prior to imaging to increase the conductivity and imaged using SEM under same conditions.

2.3.2.2 Atomic Force Microscopy

Atomic Force Microscopy (AFM) analysis was performed using Multimode 8 instrument and the images obtained were modified using Gwyddion software. Glass coverslips modified with silica nanoparticles and plasma treated glass coverslips were imaged under standard conditions, an automatic mode was used to study the topography of the surfaces.

2.3.2.3 Dynamic contact angle measurements

The dynamic contact angle measurements of all test substrates were obtained using Wilhelmy plate method. Distilled water with known surface tension was used as a solvent due to its polar nature and suitability to determine surface property [40]. The contact angle measurements account for the hydrophilic and hydrophobic nature of the substrates. Advancing and receding angles dictates the surface wetting behavior and its suitable for the experiments.

2.3.3 Characterization of bactericidal effects of surface deposited silica nanoparticle-induced topographies:

2.3.3.1 Effect of silica nanoparticles induced topography on bacterial proliferation

The effect of nanoscale topography induced by silica nanoparticles were studied on fluorescently labelled gram-positive and gram-negative bacteria namely *Staphylococcus epidermidis* (labelled with HOECSHT fluorophore) and *Escherichia coli* (labelled with mCherry expressing plasmid) respectively. All experiments were conducted in sterile 6 well TCPS plates. To each well 3 mL of sterile media was added, for example, Luria Bertani (LB) broth was used to culture *E. coli* and nutrient broth (NB) was used for *S. epidermidis*. 3 µl of overnight bacterial culture (0.1% inoculum strength) was added to each of the well and incubated at 37°C. Each of the controls and test substrates i.e., coverslips loaded with silica nanoparticles and their corresponding controls were placed in their respective well. The effect of silica nanoparticles on both the type strains were studied at regular time intervals of 3, 6, 12, 24, 48 hours of incubation. After the incubation period coverslips were washed with copious amount of 1X PBS followed by fluorescence microscopy analysis and image analysis. Blue fluorescent Hoechst stain was added to *S. epidermidis* for image analysis, as *E. coli* K12 expressed mCherry protein no external dye was added. 1ml of 1X PBS was added to each well of a sterile TCPS, to this 0.5 µl of Hoechst stain was added. Further, coverslips containing *S. epidermidis* were carefully placed in each of its respective wells and allowed to incubate at room temperature for 15 mins. All test substrate and control coverslips were imaged under confocal microscopy, images were analyzed, and the number of bacterial cells were quantified using ImageJ software.

2.3.3.2 Live and dead cell assay

To study the bactericidal effects of silica nanoparticles-induced nanoscale topography on bacteria, cell viability assay was performed using live:dead cell fluorescent staining using fluorescein diacetate (FDA) and propidium iodide (PI) [45]. All the coverslips incubated for different time points (3, 6, 12, 24, 48 hours) with bacterial cultures were thoroughly rinsed with 1X PBS, to each of the coverslips 1 µl of each of 4 mM FDA and 2 mM PI fluorescent dyes were added and incubated for 10 mins at room temperature. After the incubation time the coverslips were washed thoroughly using 1X PBS to remove any unbound dye. Following the fluorescent staining all the coverslips were imaged using a motorized inverted confocal microscope IX83 (Olympus) with a 20X objective (0.65 NA, 0.6 mm WD) fluorescence microscopy to obtain qualitative image and the micrographs were quantitative analyzed to understand the bactericidal effects of silica nanoparticles-induced topography in comparison to the control surfaces. For quantitative estimations, the number of living and dead cells on each of the test surfaces were quantified using Image J cell counter analysis functions.

2.3.3.3 Resazurin assay

To study the effect of silica nanoparticles on bacteria as a bulk phenomenon resazurin cell viability assay was performed. Resazurin assay was carried out in a 96 well, opaque, black Tecan microtiter plate. The wells were modified with silica nanoparticles in the similar fashion to that of the glass surface. 100 µl of 0.01% concentration of bacterial cells were added followed by 20 µl of freshly prepared resazurin solution (1.5 mg/ml) into each of the well. The fluorescence intensity of resazurin was obtained at 590 nm and 560 nm emission and excitation filter respectively.

2.3.3.4 Crystal violet

To study the effect of silica nanoparticles-induced nanoscale topography on biofilm

formation, crystal violet assay was performed. 1% (w/v) crystal violet solution was prepared in the lab by dissolving crystal violet dye in milli-Q. All test and control coverslips at 48 hours incubation time were obtained and thoroughly rinsed to remove unattached bacteria. Crystal violet dye was added ensuring to cover the surface of the coverslip and incubated for 10 mins. The coverslips were then rinsed with 1X PBS and dried. Images were obtained to analyze the effect of silica nanoparticles induced topography on bacteria qualitatively (S.6). The biomass accumulated on the coverslip at 48 hours incubation were quantified by solubilizing cells using 95% ethanol. Optical density at 580 nm was obtained to quantify the biofilm produced on control and test surfaces.

2.3.3.5 SEM imaging for bacterial cells on nanoscale topography

SEM was used to probe the effect of silica nanoparticle-induced topography on the bacterial cell structure at substrate surface-cell interface. All test surfaces were retrieved at 48 hours incubation with bacterial culture and rinsed using 1X PBS. Bacterial cells were fixed on the substrate using 2.5% (w/v) glutaraldehyde and incubated for 1 hour at room temperature. Fixed samples were dehydrated using gradient ethanol solutions followed by critical point drying with carbon dioxide. Dried substrate-bacterial coverslip surfaces were sputter coated with platinum for SEM imaging. The SEM images detailing the morphology and cell structures were examined for devising the effect of silica nanoparticles surface on structural integrity of bacterial cell and its subsequent effect on biofilm formation.

2.4 Results

2.4.1 Surface characterization

In order to study the surface effect of silica induced nano-topography on bacterial colonization, negatively charged silica nanoparticles were ionically adsorbed on the glass substrate coated with positively charged polycationic polymer (Fig 1A). We performed surface characterization of the substrates by three methods, Scanning electron microscopy (SEM) analysis, Atomic force microscopy (AFM), and Contact angle (CA) measurements. These characterization methods provided insights on the dispersion of nanoparticles on the surface of the coverslips, surface roughness and the hydrophilic/hydrophobic properties of the silica modified substrates.

SEM imaging:

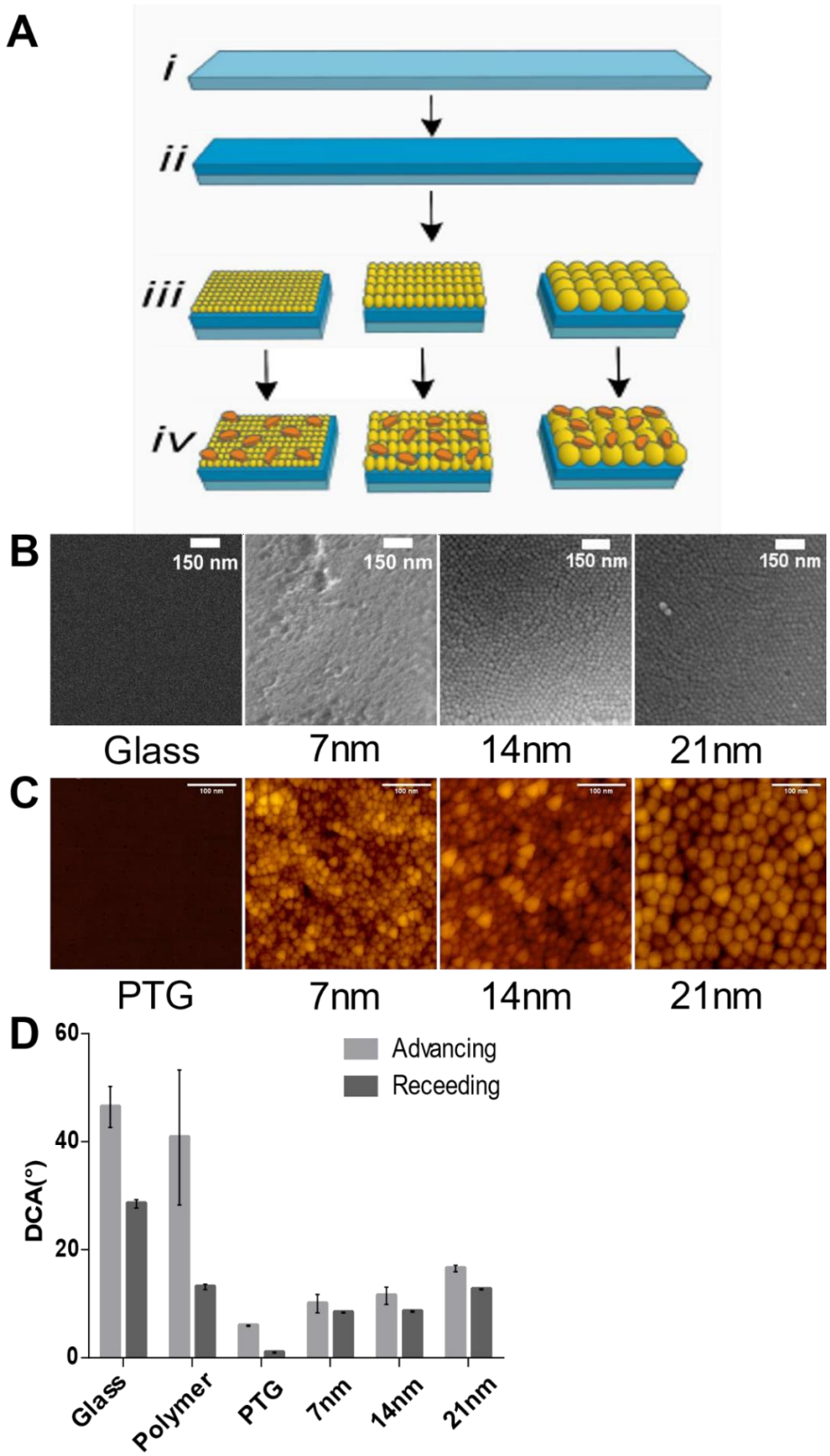
Surface topography images obtained from SEM showed the continuously dispersed three differently sized colloidal silica nanoparticles, 7 nm, 14 nm, and 21 nm throughout the ionically modified coverslip substrate (Fig 1B). It can be observed that, the silica nanoparticles, irrespective of the size, adsorbed ionically, without gaps or other aberrations, generating a continuous rough surface

Atomic Force Microscopy:

Coverslips exposed to plasma treatment as well the coverslips deposited with silica nanoparticles were subjected to AFM analysis. High-resolution images in Figure 1C depicts the surface treated with plasma is flat or void of any roughness whilst the substrate surface modified with silica nanoparticles showed more elevated, continuously bumpy surface leading to silica nanoparticles rendered surface roughness.

Dynamic contact angle measurements (DCA):

We performed DCA to test the surface charge and hydrophilicity/hydrophobicity of all the test substrates and assess its suitability for the experiments. Contact angle measurements reported in Figure 1D shows all surfaces were hydrophilic, with the mean value and standard deviation from 6 measurements (n=6). Glass and polycationic polymer exhibited similar advancing angle of 46.4 ± 3.8° and 40.795 ± 12.520° respectively, which ae relatively hydrophobic. These two substrates, namely glass and polymer treated surfaces were used as a negative control, due to its relatively hydrophobic natures (CA measurement close to 90°). The advancing angles of the surface modified with plasma, 7 nm, 14 nm and, 21 nm were always less than 10° meaning much hydrophilic compared to clean glass and polycationic polymer coated glass. 7 nm and 14 nm nanoparticles had similar advancing angle while 21 nm nanoparticles had a slightly higher advancing angle. Receding angles of the surfaces modified with nanoparticles were lower, and PTG was the most hydrophilic with an advancing and receding angle of 5.9 ± 0.1° and 3.2 ± 2.5° respectively. These contact angle measurements for the test substrates namely PTG and Silica-treated coverslips inferred that all surfaces are hydrophilic to a close range of comparison, has no obvious hydrophilic to hydrophobic differences. This also signifies the coverslips are thus suitable for our bacterial colonization studies on the surface. Here, PTG was used as another control, to mimic hydrophilicity with no detectable roughness variations compared to that of the glass coated with silica nanoparticles. The PTG control will help to convince that the bacterial colonization effects observed on silica nanoparticles coated surface are as a result of surface roughness only.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Glass
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PTG
7nm
14nm
21nm

Figure 1: A graphical process representation of modification of the surface topography using silica nanoparticles (7 nm, 14 nm, 21 nm). A(i) Clean glass coverslip as a substrate, A(ii) Polycationic polymer deposition, A(iii) Ionic adsorption of silica nanoparticles on the substrate, A(iv) Bacterial cell seeding. B. SEM image showing well dispersed distribution of silica nanoparticles on the modified surface. C. High resolution contact mode AFM imaging to study dispersed nanoparticles and the surface roughness of the substrate. PTG is used here as the control to that of nanoparticles deposited coverslips. D. Advancing and receding angles for the dynamic contact angle measurements of test and control surface.

2.4.2 Effect of silica nanoparticles induced topography on bacterial proliferation using confocal microscopy

Bacterial proliferation on silica nanoparticles deposited glass cover slips were studied at different time points, namely 3, 6, 12, 24, and 48 hours of incubation time points and proliferation imaged using fluorescence confocal microscopy (Figure 2A and Figure S1 & S2). The fluorescence micrographs were analyzed quantitatively by estimating the number of bacterial cells per unit area, that colonized/grew on the surface of the modified and unmodified substrates using ImageJ analysis tool [46]. Figure 2A (i and ii) shows the confocal microscopy imaging of the fluorescing bacterial cells on control and modified surfaces at 48-hour incubation time only. Irrespective of the incubation times, though studied up till 48 hours (Supplementary Figure S1 and S2), the surfaces modified with silica nanoparticles exhibited fewer bacterial cells colonizing for both the tested bacterial species *E. coli* and *S. epidermidis*. Thus, inferring all three sized nanoparticles negatively influenced bacterial cell multiplication and largely reduced biomass accumulation in both the gram-positive and gram-negative bacterial species. The controls, namely clean glass cover slips, poly-cationic polymer coated, and plasma treated glass coverslips showed normal growth

and the biomass accumulated at all time during the 48 hours experimental period, inferring the reduced bacterial colonization on silica modified coverslips as a result of silica nanoparticle-induced surface topography effects only. Highest bacterial colonization was observed on glass coverslips treated with air plasma in both the bacterial species, reassuring hydrophilicity does not contribute towards reducing bacterial growth or adhesion. The bacterial growths were higher than Silica coated coverslips for clean glass, polymer, and plasma treated glass coverslips. Consequently, the next observations were to assess any size- dependent antibacterial effect of the nanoparticles. Overall, the quantitative estimation of (Figure 2C) blue fluorescing *S. epidermis* that colonized the silica-modified substrates (irrespective of the nanoparticle sizes) were at the least three-folds lower than the controls. Hence, size dependent toxicity of silica nanoparticles was not distinctively pronounced in *S. epidermidis.* However, notably 14 nm silica nanoparticles had the most anti-colonizing effect, with lowest number of bacterial cells (Figure 2C). The gram-negative bacterial candidate, *E. coli* K12 exhibited a slightly different response to silica nanoparticles influenced topographic modifications (Figure 2Aii &2B). The number of bacterial cells on 7 nm nanoparticles was at least four folds lower than clean and treated glass coverslips (Figure 2B), the highest antibacterial effect among the three different sizes, thus deducing a size dependent toxicity on *E. coli* cells. Although 7 nm and 14 nm nanoparticles interfered with proliferation of *E. coli cells* (Figure 2Ai & 2B), exhibiting lower biomass density, interestingly higher cell density was observed on surface modified with 21 nm nanoparticles. The possible reasons for this observation are further discussed in the discussion section on adaptation or response of bacterial cells to surface topographies. To summaries, size-dependent toxicity of nanoparticles was distinctively observed in gram-negative bacteria, *E. coli* compared to the gram-positive, *S. epidermidis*, nevertheless, as a general trend, silica induced topographical roughness created a hostile surface of varying degree of hostility due to one of the following reasons, size of the nanoparticles, nature of the different

membrane composition and architecture of the bacteria cell walls.

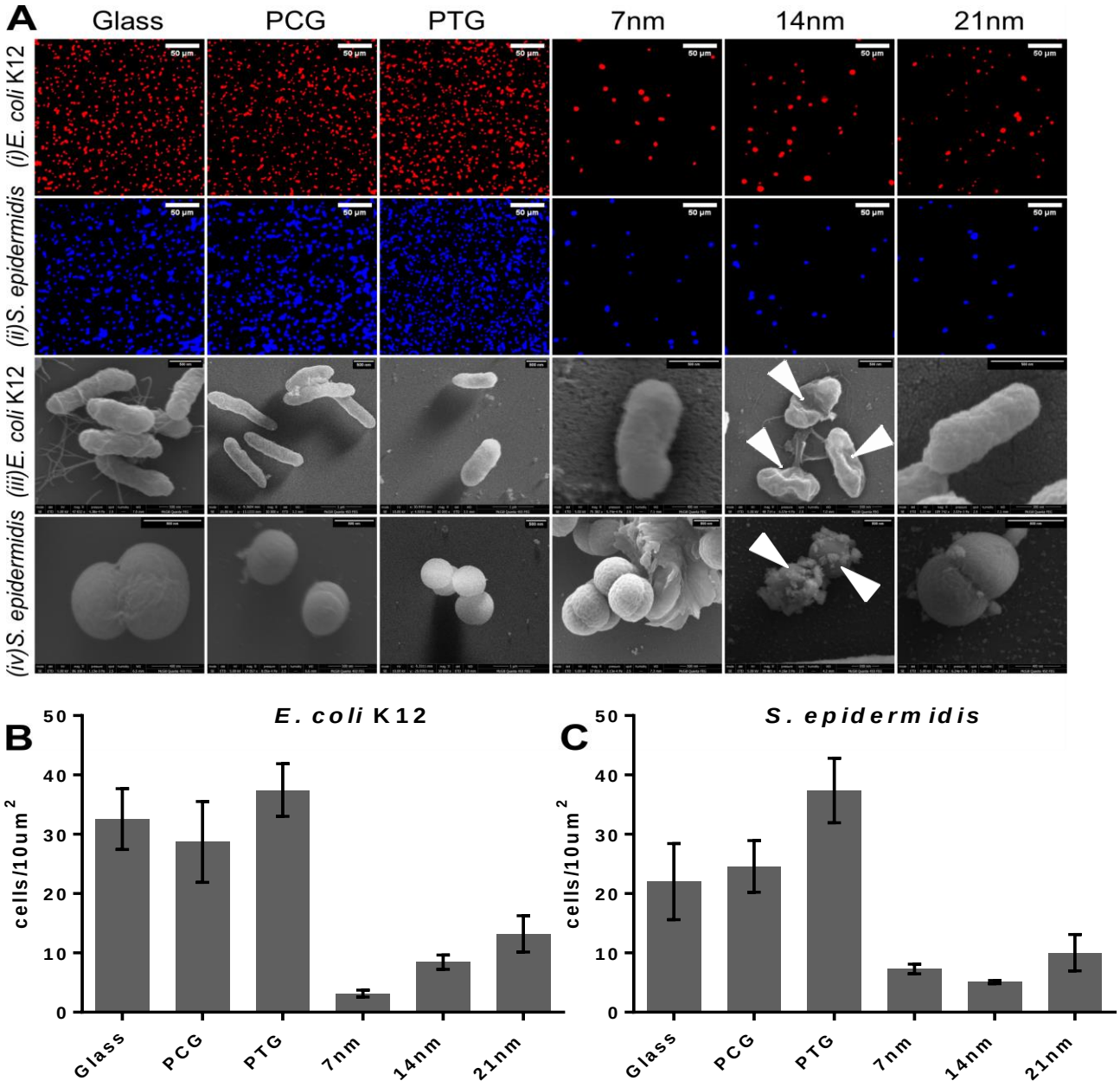

Figure 2. A. Confocal fluorescence microscopy image of (i) Gram negative - *E. coli* (red) and (ii) Gram positive – *S. epidermidis* (blue) on controls and test surfaces modified with silica nanoparticles. SEM image of (iii) *E. coli* and (iv) *S. epidermidis* for structural analysis of silica nanoparticles induced topography at substrate-bacterial interface. Graph representing quantitative data of (B) *E. coli* and (C) *S. epidermidis* on control and test surfaces. All values are a mean and standard deviation of 4 trial experiments and 40 measurements (n=40).

2.4.3 Characterization of the bacterial cell structure on silica nanoparticle modified substrate interface using electron microscopy

The structural integrity of bacterial cells that colonized the control and silica nanoparticles coated surfaces were evaluated using high resolution scanning electron microscopy on 48 hours incubation cultures with the different substrates-end point surface effects analysis (Figure 2A *iii* & *iv*) on bacteria. SEM results of the bacterial density on different sized silica induced surface modifications and the controls surfaces (plain glass cover slips, poly-cationic polymer modified, and PTG cover slips) corroborated well with that of the population density observed using the fluorescence microscopy based, biomass estimation on the corresponding substrates for both the bacterial species (Supplementary Figure S5). Healthier bacterial cells with intact membrane structures and in most cases with intact flagella (for *E. coli* only since these are motile) were observed on clean glass, polymer coated, and plasma treated glass in both *E. coli* and *S. epidermidis* (Fig 2A iii, iv). However, on the surface modified with 7 nm, 14 nm and, 21 nm nanoparticles, *E. coli* bacterial cells exhibited change in cellular morphology such as under-developed flagella, membrane dents and punctures, cellular irregularities, flattened cells tracing to stress induced response phenomenon. Here, the stress is induced due to surface topographic roughness generated by different sized nanoparticle dispersed on the surface. The membrane damages were in fact phenotypically severe on surface modified with 14 nm nanoparticles in both the bacteria. Bacterial cell integrity was distinctively compromised, in *E. coli*, cells appeared to be punctured and emptied of cellular components (Figure 2A iii, marked by white arrows). Cell membrane damage and probably the subsequent release of intercellular components out of the cells was observed in *S. epidermidis* as well. Although all sized silica nanoparticles pronounced interference with bacterial proliferation, 14 nm nanoparticles evidently had a stronger interaction and cellular damage on both

gram-positive and gram-negative bacteria (Figure 2 A – both confocal studies and SEM studies corroborated these points). In some instances (Figure 2A(iv) – 14 nm and 21 nm and S3(i) 14 nm and 21 nm) the surface was covered with micelle like cellular debris and several other cellular debris that are bound on the surface of the bacteria. We expect these to be the cellular components oozing out of the cells that precipitated on to the surface or possibly biofilm, as a response to topographical surface aberrations. We have further discussed the possible reasons for variations among the gram-positive and gram-negative species, size-specific bactericidal effects in the discussion section.

2.4.4 Bactericidal activity of silica nanoparticles

Topography induced bactericidal activity of silica nanoparticles were further analyzed using live and dead cell assay for *E. coli* and *S. epidermidis*. This study was performed to estimate the severity of the bacterial cell viability by the substrates with surface aberrations. Figure 3 shows the qualitative and quantitative proof for live: dead bacterial cell cultures studies at 48 hours of culture only, while a time point study was documented starting from 3, 6, 12. 24, and 48 hours incubation (Supplementary Figure 3, 4). Bacterial cells cultured on control and test substrates were stained with live-dead cell specific fluorophores namely fluorescein di-acetate (FDA) and propidium iodide (PI), imaged using fluorescence confocal microscopy. Images were analyzed and quantified using Image J software. Viable cells with intact cell wall fluoresce green and dead cell with damaged cell membrane fluoresce red, based on the classical live dead cell assay performed with FDA and PI. Higher cellular viability (green living bacteria) were observed on clean glass, polymer deposited, and plasma treated control substrates only, while on the contrary, substrates modified with silica nanoparticles showed higher number of dead bacterial cells in both the bacterial species studied here (Figure 3). The ratio of green to red fluorescent cells in the Figure

3A provides a qualitative index of bacterial viability on test and control surfaces. The number of live and dead cells of the same mean surface area for each of the substrate were quantified using Image J software. The quantified data obtained on both the test strains are represented as a cell viability percentage and is a ratio of number of live to total bacterial cells (Figure 3B and C). The number of living cells on glass, polymer, and PTG surface were at the least three folds higher than surfaces modified with silica nanoparticles. In both the test strains number of living cells were higher on PTG surface, *S. epidermidis* exhibited a five- fold increase in living cells compared to surfaces modified with silica nanoparticles. There was no significant difference observed in antibacterial effect among the three sized nanoparticles in both *E. coli* and in *S. epidermidis*. However, in *E. coli* the number of living cells were lowest on the surface modified with 7 nm nanoparticles with a slight increase in living cell ratio with increase in nanoparticles size. On the other hand, the number of living cells were lowest on surface modified with 14 nm nanoparticles in *S. epidermidis,* 7 nm and 21 nm nanoparticles exhibited similar bactericidal effects. All three sized nanoparticles exhibited a significantly higher rate of cell death in both the bacterial species. The probable mechanism and necessary explanation for such effects are detailed in the discussion below.

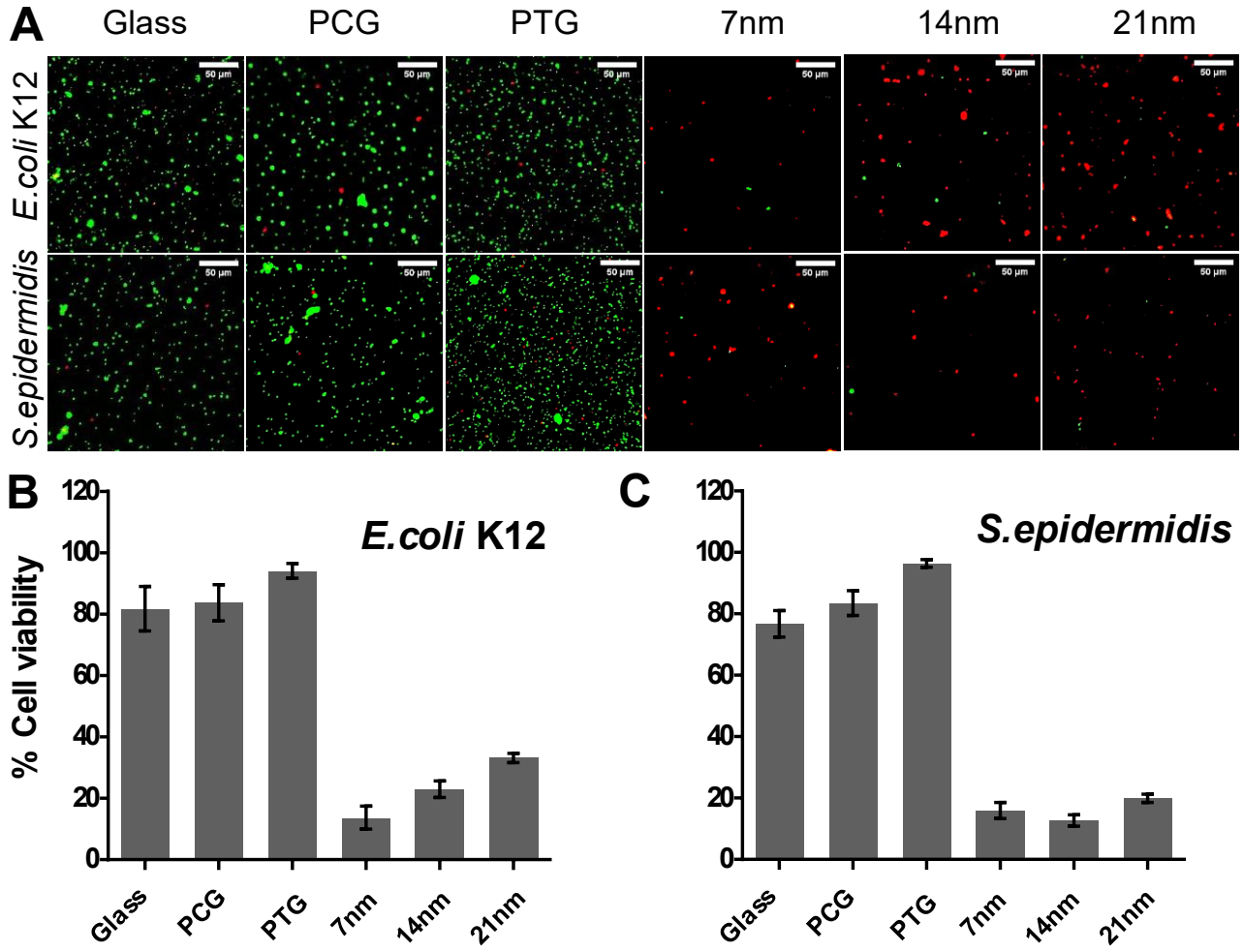

Figure 3: A. Bactericidal activity of control and silica nanoparticles-induced nanoscale topography on (i) *E. coli* K12 and (ii) *S. epidermidis* at 48-hour incubation using live and dead cell assay. Live cells stain green and dead cells stain red. Quantified data of live to dead cell ratios for *E. coli* (B) and *S. epidermidis* (C) bacterial cells on control and test surfaces. Images were obtained using confocal microscopy, analyzed and quantified using Image J software. Quantified data are represented in cell viability percentage with a mean and standard deviation of 4 trial experiments and 40 measurements (n=40).

The live:dead cell assay was complemented by testing if the surface dispersed nanoparticles had any effect on the bacterial growth in bulk solution by coating the surface of the wells with silica nanoparticles. To perform this, we modified the walls of the 96 well plates with silica nanoparticles (7 nm, 14 nm & 21 nm) by a similar process followed for modifying the surface of glass. Bacterial strains were grown in control and modified wells of a 96 well plate. Overnight bacterial culture was added with resazurin as a bacterial growth indicator into each of control and test wells and incubated for a duration of 5 hours. Fluorescence intensity was measured at 1-hour intervals for the total incubation period (Figure 4). In an ideal scenario, when bacteria grow faster in the liquid broth, the fluorescence intensity due to resorufin increases faster until all the substrate i.e., resazurin is used up. This test was performed to understand the bacterial cell viability in the bulk solution at initial stages of the growth cycle, to see if there is any negative effect of the ionically bound silica nanoparticles on the bacterial growth in the bulk solution. Hence, plasma treatment was not included as a control, besides the fact that the 96 well titer plate is polystyrene. The fluorescence intensity was high in the control wells (absence of silica nanoparticles) for both the bacterial strains. In the wells modified with silica nanoparticles of three sizes, the growth of bacteria in the bulk solution was slightly affected over the incubation period but was not striking (Figure 4) like observed in Figure 2. As the fluorescence intensity is directly proportional to the concentration of viable cells, it can be noted from Figure 4 that both on *E. coli* K12 and *S. epidermidis* there is a linear increase in growth over the 5hour incubation period. The inset image provided for *E. coli* K12 shows all control and test wells had similar colorimetric change indicating high cell viability in all the wells irrespective of modifications with silica nanoparticles. However, in both visual colorimetric evidence from inset image of the wells and the increase in fluorescence intensity *S. epidermidis* exhibited slight difference in fluorescence intensity, which could be as a result of longer doubling time associated with *S. epidermidis*. Cells were relatively

less viable in the wells modified with silica nanoparticles compared to the control wells but are not striking. Although the nanoparticles had an effect in reducing bacterial proliferation their bactericidal activity was low allowing multiplication and gradual but exponential increase in cellular density in the bulk solution (Fig 4). It is therefore evident that the strategy to reduce bacterial multiplication and its subsequent biofilm formation is very effective as a surface phenomenon than as a bulk phenomenon. Consequently, the above points buttress the bactericidal activity induced primarily as a result of surface deposited silica nanoparticles only.

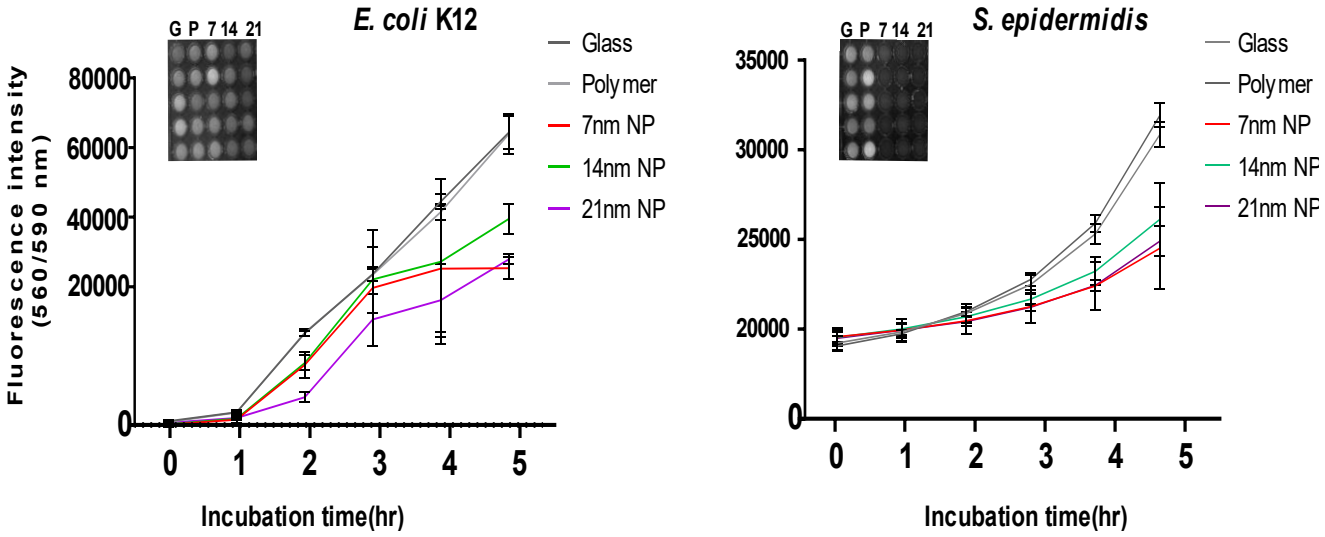

Figure 4: Resazurin assay to monitor cell viability of *E. coli* K12 and S. *epidermidis* in the bulk solution. Clean wells and wells modified with polycationic polymer were controls and wells modified with 7 nm, 14 nm, and 21 nm silica nanoparticles as test wells. Fluorescence intensity was measured at 590 nm emission and 560 nm excitation filter sets, using multimode microplate reader. Inset image indicates a qualitative end point analysis of resazurin assay un both the test bacterial strains. Fluorescence intensity was obtained for 5 replicates and mean measurements in represented in the graph.

2.4.5 Biofilm quantification using crystal violet assay:

Crystal violet assay was performed to study the biomass accumulation of bacterial cells on clean glass, polymer-coated, plasma treated glass, and silica nanoparticles treated substrates. At 48-hour incubation, the coverslips were stained using 1% crystal violet staining solution and incubated for 10 minutes at room temperature. All test samples were allowed to dry, and images were obtained (Figure SI 6(ii and iii)) to qualitatively study the degree of biofilm formed on the coverslips. Furthermore, the crystal violet stain was eluted with ethanol and absorbance was measured at 580 nm (Figure 5A). The ethanol solution acts as surrogate for biofilm growth. Clean glass, polymer, plasma treated cover slips, and silica nanoparticles coated test coverslips without any bacterial growth immersed in media for 48 hours were used as the controls for the biofilm formation studies. The control coverslips were later stained with 1% crystal violet solution, it was observed that the CV dye stained silica nanoparticles (Figure S6(i)). Therefore, the control coverslips were also subjected to ethanol wash followed by measurement of optical density at 580 nm. The obtained OD was subtracted from the OD obtained from coverslips treated with bacteria. Figure 5B shows the quantified biomass on glass, polymer, and surfaces modified with silica nanoparticles on both the test strains. It can be observed that the biofilm formed on coverslips modified with silica nanoparticles shows two-fold lower biomass for *E. coli* K12 and three-fold lower biomass for *S. epidermidis*. It can therefore be inferred that silica nanoparticles induced topography is effective in reducing bacterial attachment and thus the subsequent biofilm formation.

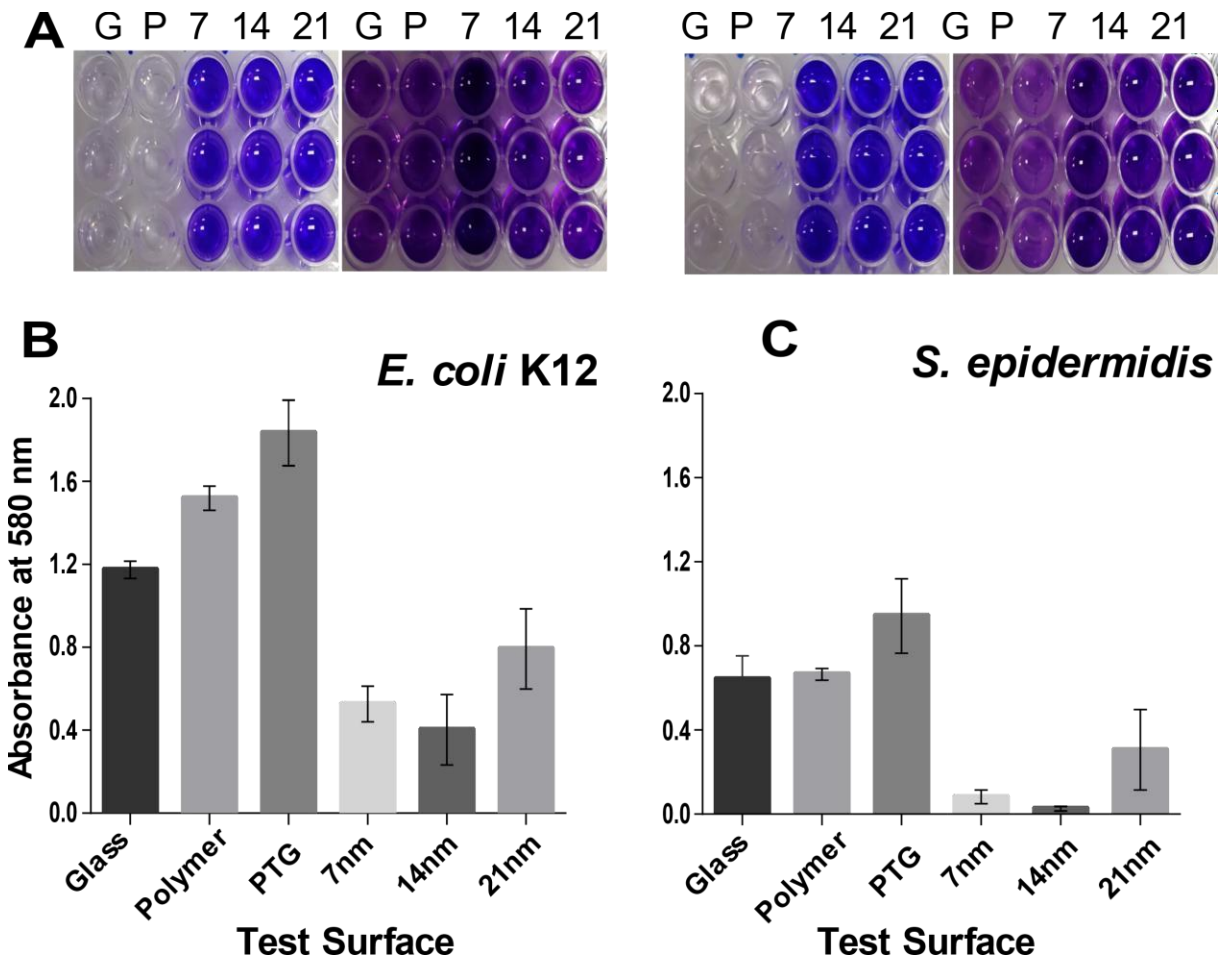

Figure 5: A. Biofilm assay using 0.1% crystal violet at 48-hour incubation, the crystal violet bound to biomass was washed using 95% ethanol and transferred to 96 well plate. Absorbance was measured at 580 nm using multimode micro plate reader. B. Biomass quantification of control and test coverslips from crystal violet assay for both the test strains *E. coli K12* and *S. epidermidis.* Biomass accumulation was relatively low in both the test strains indicating surface modified with silica nanoparticles hinders biofilm formation.

2.4.6 Nanoparticle-induced morphological changes:

Gram-negative *E. coli* K12 exhibited interesting phenotypic response to each sized nanoparticle modified surface. On 7 nm nanoparticles, few bacterial cells with null or underdeveloped flagella was observed, few cells appeared to be submerged on to the surface indicating nanoparticles penetration into the cell membrane. Bacterial cells on 14 nm nanoparticles exhibited compromised cell integrity, punctured and void on cellular components. Interestingly, cells exposed to 21 nm nanoparticles expressed reshaping strategy where cells appeared coccus-like morphology (VNBC state) [47, 48] to adapt and colonize the surface (Figure 5A). Furthermore, bacterial cells on 21 nm nanoparticles appeared shorter in length compared to healthier cells on glass (Figure 5B, data represents a mean and standard deviation of 46 measurements (n=46).

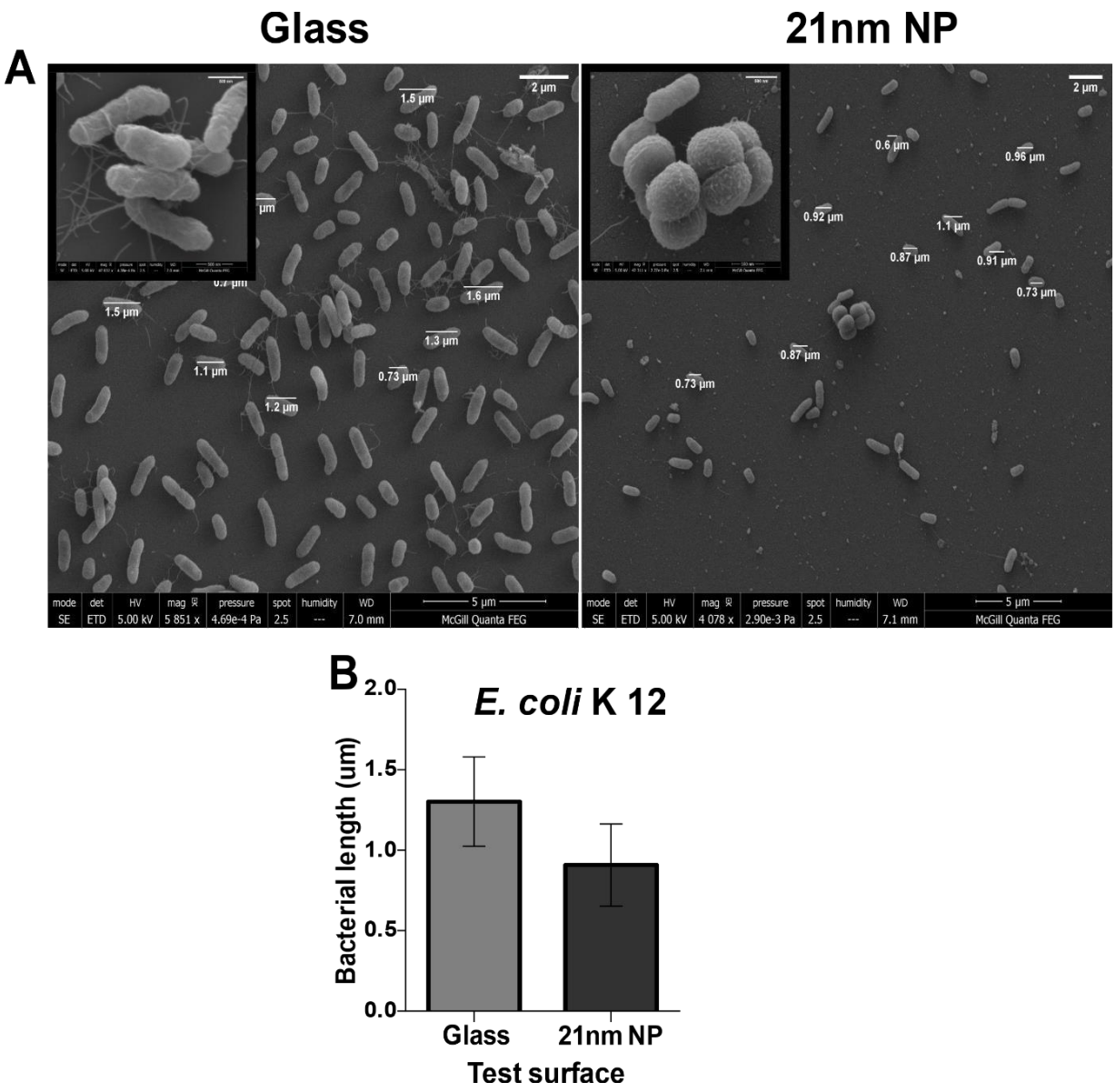

Figure 6: A. SEM image analysis of *E. coli* adopts the reshaping strategy to survive stress induced by 21 nm nanoparticles and enter a viable but non culturable (VBNC) state. B. *E. coli* cells expressing phenotypic mutation by decreasing their length compared to healthier cells on glass.

2.5 Discussion

2.5.1 Silica nanoparticles-induced super hydrophilicity and reactivity of silanol moieties:

Bacterial attachment and biofilm formation to any biotic or abiotic surface is a complex process involving bacterial proliferation [49], surface sensing, production of EPS [10, 13, 14], and attachment to the surface [44]. The surface property, including surface charge, topography, and physicochemical factors, play a crucial role in bacterial sensing and subsequent attachment to form biofilm [18, 33]. In this study, we were able to reduce the propensity of bacterial attachment to the glass substrate by modifying the surface topography using silica nanoparticles in both gram stain of bacteria. Furthermore, we attempted to understand the size-dependent silica nanoparticles induced toxicity on bacterial candidates. Colloidal silica nanoparticles were ionically adsorbed on glass substrate using polycationic polymer, and the drying process leads to condensation of nanoparticles forming well-dispersed nanoscale topography. SEM analyses confirmed the uniform deposition and distribution of silica nanoparticles on surface establishing successful topography modification (Figure 1B). High resolution images obtained from AFM confirmed surface roughness of substrates subjected to plasma treatment and substrates modified with silica nanoparticles. Plasma treatment removes organic contaminants rendering the surface more even, whereas, surface coated with nanoparticles showed rougher topography and distinguished nanoparticle arrangements. Dynamic contact angle measurements were conducted to characterize the surface property of glass, polymer, plasma treated glass, and surfaces modified with silica nanoparticles. All surfaces were hydrophilic with a contact angle lower than 90º. The hydroxyl groups on the glass surface contributes to its hydrophilicity [16, 40]. Similarly, cationic functional parts of poly cationic polymer contribute to the hydrophilic nature of the surface deposited with the polymer. PTG was the most hydrophilic of all the substrates due to deposition of polar groups on

the surface through air plasma treatment. The nanoparticles exhibit unique physiochemical properties compared to their bulk counterparts. The physicochemical properties of nanoparticles are highly size dependent. The concentration of silanol groups (Si-OH) on specific surface area increases with the decrease in the nanoparticle size [50, 51]. Due to the high number of silanol groups, 7 nm was the most hydrophilic among the three sized nanoparticles, and the contact angle increased slightly with smaller sized nanoparticles, while the hydrophilicity decreased slightly with an increase in nanoparticle size due to the decrease in the number of silanol groups. The difference in the contact angles between the three sized nanoparticles could be an effect of its surface roughness, as well as the surface area to silanol groups distribution.

The mechanism of silica nanoparticle damaging the cell membrane can be explained based on two interrelated phenomena, i.e., silanol group reactivity on cell membrane components and as well the surface morphology induced stress. The organic silanol groups present on the nanoparticles surface interact with the lipophilic portion of the bacterial cell membrane, and thus effectively disorienting the cell membrane by increasing lipid solubility that causes membrane damage and cell death in bacteria [52]. Also, the chemical reactivity/adsorption efficiency of biomolecules are directly proportional to the number of available silanol groups on the surface and the number of lipophilic membrane components, hence, higher the chance for bacterial interaction with such groups. Here, due to high volume of surface silanol groups, 7 nm nanoparticles facilitate more substantial interaction with the lipophilic portion of bacterial cell membrane, provoking irreparable membrane damage. It was observed that the cell density was at least three-fold lower for 7 nm nanoparticles compared to 14 nm and 21 nm. Although, PTG has high hydrophilicity that could attract bacteria on the surface, they lacked nanoparticles-induced surface roughness to provide the mechanical stress that would tear the bacterial cell membrane. For, silica-induced nano topography, had the advantage of both surface attraction due to high hydrophilicity and surface

topography induced stress that could break the bacterial cells.

2.5.2 Size specific bactericidal effects affect the gram-positive and gram-negative bacterial cells differently:

Qualitative confocal microscopy observations demonstrated that clean glass and polymer-coated glass supports bacterial proliferation and surfaces modified with silica nanoparticles negatively interfered with bacterial proliferation in both *E. coli* and *S. epidermidis*. SEM imaging provided insights on cell surface interaction induced by surface topography. Gram-positive bacteria have a multi-layered thick peptidoglycan cell wall structure that is more porous with teichoic acid arrangements [53]. Gram-negative bacterial cell wall on the other hand have single peptidoglycan layer made of dense lipopolysaccharides followed by an outer membrane [53]. Although cell wall structure can contribute for difference in effect of silica nanoparticles on each of the bacteria, various other factors like size, charge of the nanoparticles may also affect the mechanism of interaction with bacterial cell wall components [18]. Silanol treatment on gram-negative *E. coli* showed detached plasma membrane and compromised cell integrity, and gram-positive bacteria exhibited disorganized cytoplasmic membrane leading to cell death [52]. As the silica nanoparticles surface contain a high volume of silanol groups, cellular damage is initiated through surface chemistry interaction of silanol groups with the bacterial cell membrane [52]. These reported observations were corroborated by our findings here on the surface effects of silica nanoparticles induced topographies.

While all the three sized nanoparticle had strong bactericidal effect of varying degrees for both gram- positive and gram-negative bacteria, 7 nm and 14 nm silica nanoparticles are more effective in controlling cell proliferation in both the types of bacteria. Specifically, the 14 nm silica nanoparticles showed slightly better efficiency in bactericidal effects compared to 7 nm and 21 nm silica nanoparticles. Bacteria showed adaptive effects to overcome the surface topographies and

proliferate slightly better, explained below. Considering SEM analysis, the cell-surface interaction between bacteria and the surface modified with each sized nanoparticle, 14 nm exhibited interference in bacterial adhesion, multiplication and inferred cellular damage in both the bacteria. Although the topography modification produced different size-dependent effects on each of the test organisms by effectively reducing the bacterial proliferation, the surface adsorption due to silanol groups furthers the membrane damage, which can act as a dual effect on bactericidal effects on the surface.

Further, the resazurin test in a 96 well plate shows the observed effects of bacterial proliferation due to surface topographies are as a result of the surface-induced bactericidal phenomenon and does not affect much the bacteria in the bulk of the culture media, despite the presence of surface bound silica nanoparticles. Comparing the viability of the cells on the surface (<three to five times lower compared to controls, Figure 3, for both the strains) to that of the viability in wells coated with silica nanoparticles (around 0.5 times lower to that of controls) clearly shows the effect as a result of surface modification coupled with silanol group effect. Bacteria in bulk could still survive, reproduce, and contribute towards the optical density/resazurin reduction. With sufficiently high initial inoculum strength, bacteria could easily overcome the surface induced effects when provided a bulk environment. However, when used as a surface material, it could easily be detrimental to both gram-positive and gram-negative bacterial candidates. Our evidence here on the bactericidal effects of nanoparticles modified surfaces supports the use of silica-coated nanoscale topographic materials on catheters, food manufacturing facilities, implants, etc. however, the coating of the silica nanoparticles on to these materials might require a much stronger interactions like covalent crosslinking of nanoparticles on to the substrates.

2.5.3 Bacterial adaptation to overcome the nanoscale topographic stress:

The structural organization of gram-positive and gram-negative bacterial cell membranes may influence the mechanism and the subsequent mode of action of nanoparticles on bacteria. Interestingly, on surface modified with 21 nm nanoparticles, *E. coli* expressed phenotypic mutation by changing its shape to more spherical like shapes (Figure 6A). *E. coli*, when exposed to stress adapt the reshaping strategy to survive various environmental stress and enter a viable but nonculturable (VBNC) state [48, 54, 55]. Reshaping from rod-shaped cells to spherical-shaped cells reduced the contact area with surface topographies, thereby reducing the stress thus incurred when the cells are elongated rods. The coccus like phenotypic mutation of *E. coli* is its VBNC state.

Furthermore, it was observed that the average length of *E. coli* was smaller on 21 nm nanoparticles surface than the bacteria that colonized glass coverslip, indicating the bacterial adaptation to the surface topographies and reactive groups by morphological changes. This phenotypic change could be due to distress on bacterial cells and their inability to divide [55]. Bactericidal activity of silica nanoparticles induced topography was well pronounced in both the bacterial species tested using live and dead cell assay. It was observed that the number of living cells were increasing with increasing nanoparticle size, particularly in *E. coli.* This could possibly infer that the physical/mechanical stress of each sized nanoparticle on bacterial cell and their attempt to mutate to VBNC state. However, the metabolic activity of bacteria in its VBNC state is very low, and thus multiplication is relatively slow [56]. The presented results showed *S. epidermidis* didn't undergo much of morphological changes, probably due to its original spherical morphology that already reduced the stress due to surface topographies induced effects and the presence of thick peptidoglycan layer thereby reducing the stress induced due to nanoscale topographies. However, the topography modifications did hinder bacterial proliferation in all our

tested conditions. As another effect, to overcome the surface topography effects, bacteria produced biofilm that we predict could negate the bactericidal effects of the surface topographies. The SEM images show small debris like particle on the surface around the bacteria, more typically the broken pieces of the cells, and importantly biofilm (Figure 2). Unlike the reports of mammalian cell response to surface coated silica nanoparticles, where there was a complete lack of growth of adherent cells on the surface, here with bacteria we observed a resilient/adaptive behavior. This is due to bacterial response to nanoparticles either by forming spherical cells or by producing biofilms or by entering a non-replicable dormant vegetative state. These various response phenomena by a pro/eukaryote show bacteria overcome such effects given enough time, while cell lines are very fragile for such modifications under *in vitro* conditions [40].

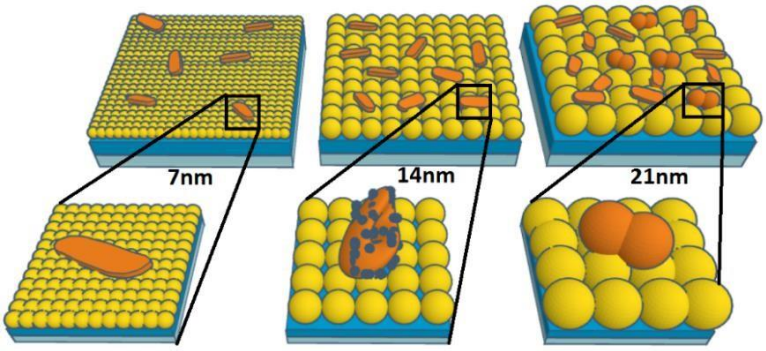

Figure 7: Schematic representation of highly pronounced size specific interactions of silica nanoparticles induced surface topography effects on bacterial cells particularly gram-negative *E. coli* K12 in this study.

2.6 Conclusion

Overall, our work here examines various microbial aspects related to the effects of silica nanoparticles on bactericidal activity. Followed by that, the various adaptive strategies to overcome the surface topographic effects. While this work requires further investigation on how the observed bactericidal phenomena could be used for real-world silica nanoparticles based coated surface applications. Further, the bacterial mutants with biofilm production or with other adhesive production could show how such topographies are effective. Most studies on silica nanoparticles used free silica in solution to enumerate the antibacterial activity [29]. However, here we have used surface bound silica nanoparticles, i.e., ionically adsorbed silica nanoparticles. This work, as described before, focusses only on the surface induced antibacterial effects, which can be either silanol group-based reactivity on cell membranes or surface topography induced stress on cell membranes or both. This study has opened new sections that could target using silica like new types of nanoparticles for surface topography modifications. Our work here will find relevance to the use of nanoparticles for antimicrobial surface applications and, for designing surface-modified, antibacterial medical equipment that is used for probing the internal organs or mucous glands of an organism. These will primarily find ways to prevent the incidence of nosocomial infections after surgery or other medical procedures.

2.7 Supplementary figures and information

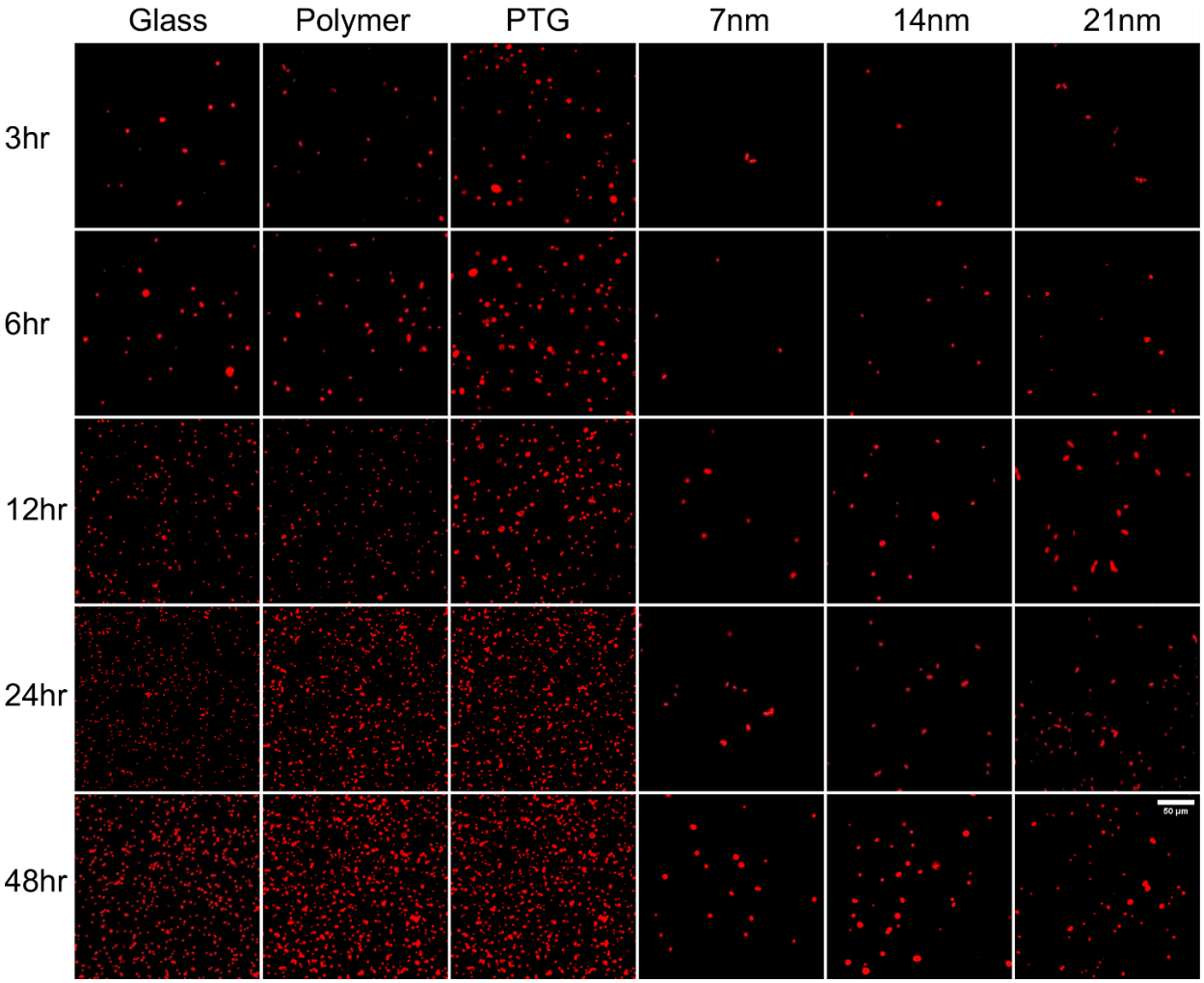

Figure S1. Effect of silica nanoparticles-induced topography on *E. coli* K12 at different incubation time. Images were obtained using confocal microscopy and analyzed using Image J software.

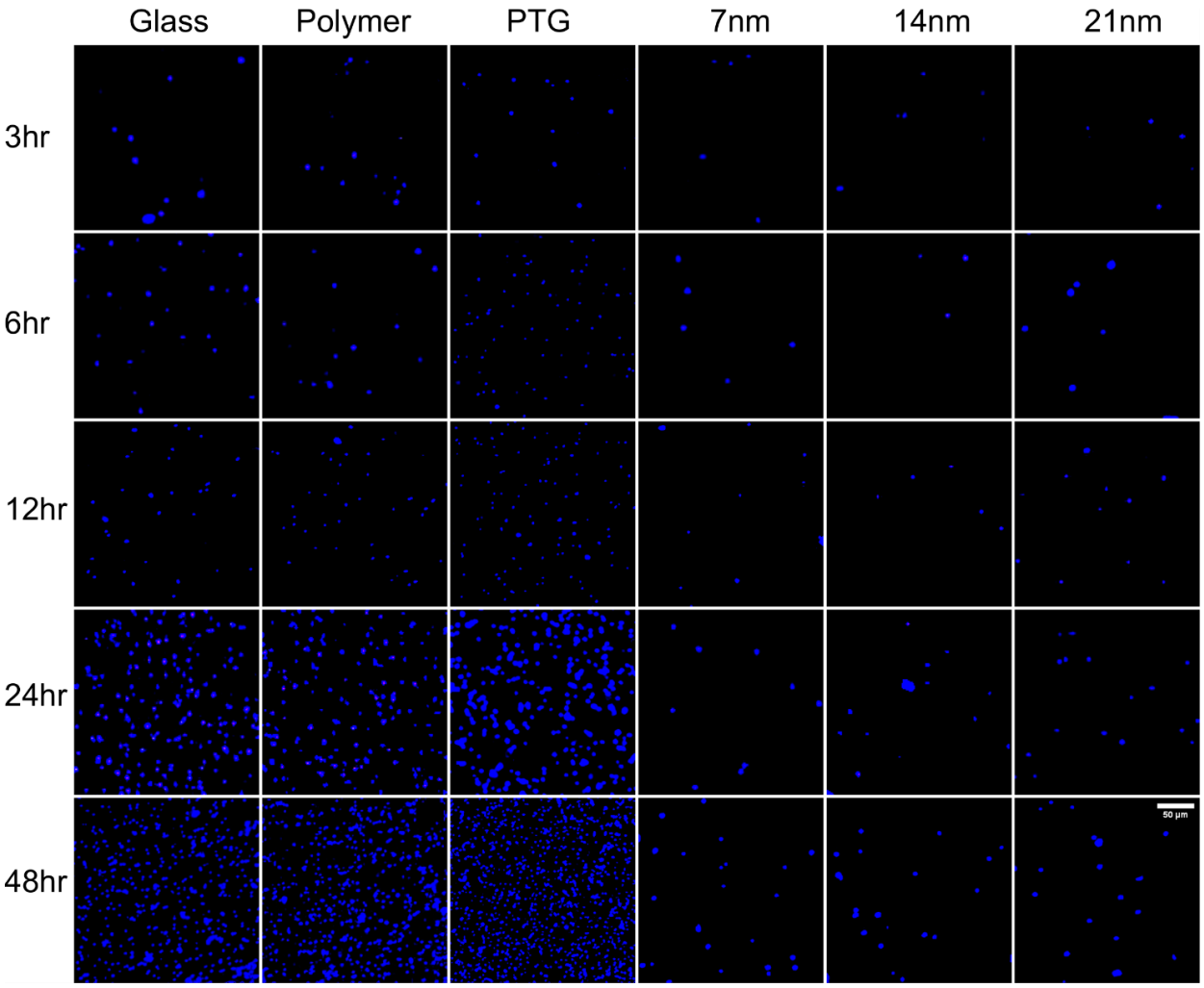

Figure S2. Effect of silica nanoparticles-induced topography on *S. epidermidis* at different incubation time. Images were obtained using confocal microscopy and analyzed using Image J software.

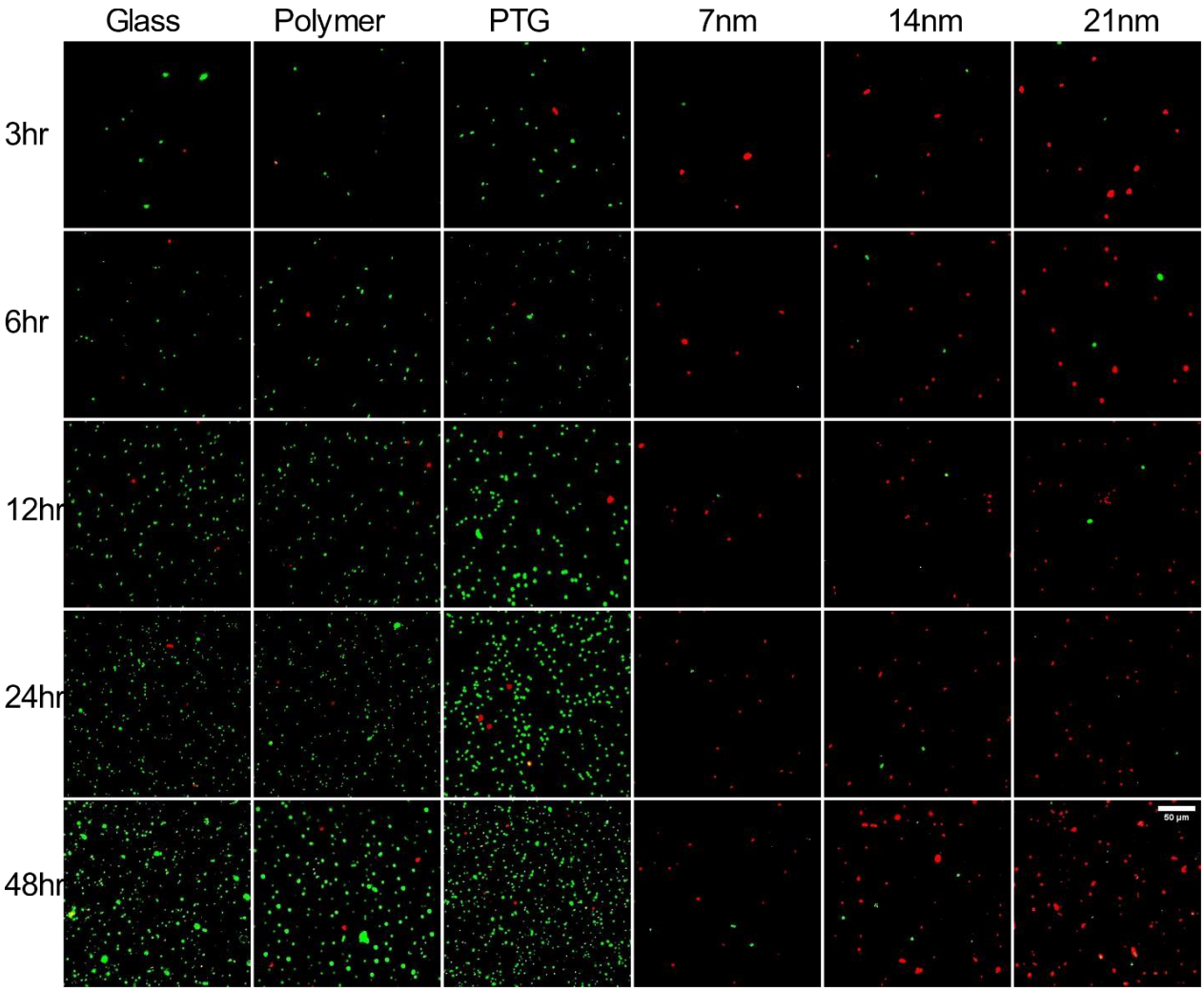

Figure S3. Bactericidal activity of silica nanoparticle-induced topography on *E. coli* K12 at different incubation time. Images were obtained using confocal microscopy and analyzed using Image J software.

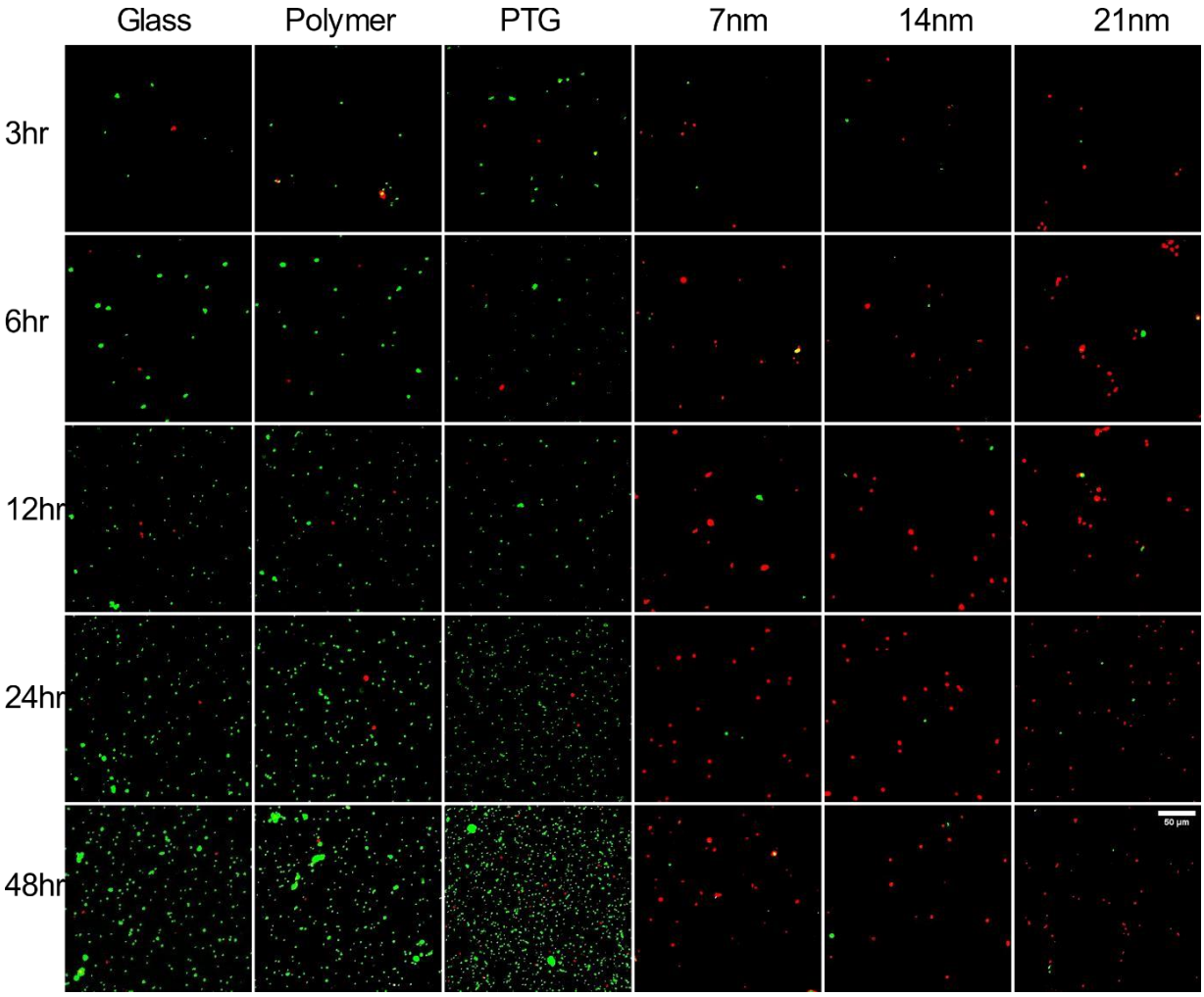

Figure S4. Bactericidal activity of silica nanoparticles-induced topography on *S. epidermidis* at different incubation time. Images were obtained using confocal microscopy and analyzed using Image J software.

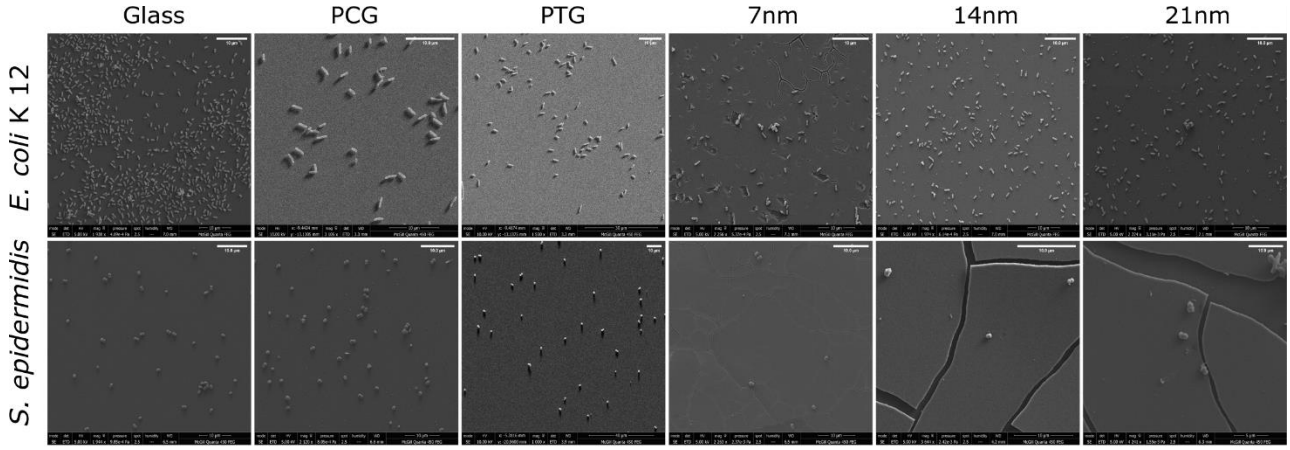

Figure S5. SEM images of bacterial cells at 48-hour incubation. Fewer bacterial cells observed on surfaces modified with silica nanoparticles.

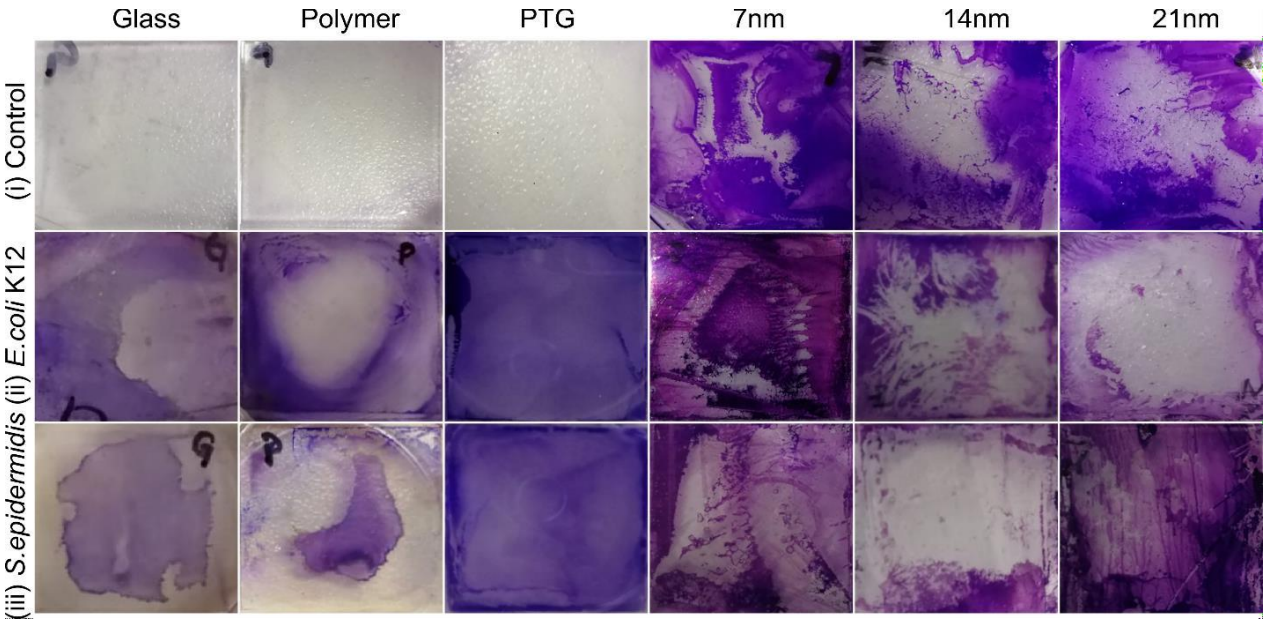

Figure S6. Qualitative image of coverslip treated with 1% crystal violet after 48 hours incubation. (i) Control – clean glass, polymer deposited, and silica nanoparticles deposited coverslips immersed in LB media without any bacteria for 48 hours. (ii) Clean glass, polymer posited, and silica nanoparticles deposited coverslips seeded with bacterial culture (ii) *E. coli* K12 and (iii) *S. epidermidis* after 48-hour incubation.

Chapter 3 – Conclusion

The antibacterial effects of silica nanoparticles achieved by the modification of surface topography at a nanoscale level has been experimentally shown in this study. Several studies have employed silica nanoparticles to modulate the adhesion of cells on surfaces and in suspension. For instance, adherent mammalian fibroblast cells exhibited two to three-fold reduction in proliferation, altered cellular orientation, and decreased adhesion on surfaces modified with silica nanoparticle [40], while reduced cellular attachment, spreading, and proliferation of human endothelial cells was also observed [41]. It was shown, that the incubation of bacteria with silica nanoparticles resulted in aggregates of silica nanoparticles on the surface of bacterial membrane, suggested to be the result of electrostatic interactions between the bacterial cell wall and silica nanoparticles [57]. Surface topography variations in the order of nanometers is proved here as a single contributing factor for modifying glass and plastic (polypropylene) surface to fabricate an antibacterial, smart surface. We have used abundantly available, silica nanoparticles as the antibacterial material to coat any substrate (in this study with glass and plastic). The effectiveness of nanoscale surface modifications was proved using bacterial colonization studies and live and dead cell assay using specific fluorescent dyes on gram-positive and gram-negative bacterial model species (*Staphylococcus epidermis* and *Escherichia coli*). Bacterial cells were grown on modified surfaces up to 48-hours of incubation at 30°C, the results showed topographic-induced bactericidal effects by silica nanoparticle on both the model bacterial strains used in this study. The impact of bactericidal effects was observed on bacterial cells as morphological changes like cellular disfiguration, cell membrane punctures and random cell debris on the surface with longer incubation times under the influence of the nanoparticle surface coatings.

The size of the silica nanoparticles used in this study namely, 7 nm, 14 nm, 21 nm are very specific to this study, which have shown a strong, size-specific bactericidal effects. This study

proved that the 7 nm nanoparticle were the most effective in reducing bacterial attachment and subsequent biofilm formation typically for gram negative bacterial species than gram positive bacteria. However, on the whole, bacterial cells, including gram positive *S. epidermidis* on 14 nm nanoparticle exhibited the second most-effective in delivering impaired cellular proliferation and cell damage. All the results prove that such surface modifications can be an effective alternative surface coating material to prevent bacterial attachment and subsequent biofilm formation.

3.1 Future work

3.1.1 Additional parameters

Based on these preliminary experimental outcomes, the following objectives can be explored in the future both at nano-and micro levels. Although we have evidence of the proof of concept in the size dependent toxicity, variations can be made by including many other different sized silica nanoparticles to determine the critical size for membrane damage leading to cell lysis. Specifically, our study shows 14 nm silica nanoparticles has the highly pronounced cellular damage in both gram-positive and gram-negative test species while 7 nm has highest bactericidal effects in terms of proliferation on gram negative species. We also show that on surface with 21 nm nanoparticle gram-negative bacteria exhibit an adaptive strategy (VBNC state) by changing its phenotypic morphology from rod to circular. By including several more sized silica nano particles a critical diameter can be acclaimed. This would provide an evidence of bacterial response or adaptive strategy to topographical dimensions. Additionally, atomic force microscopy studies can be performed to probe the mechanical property of bacterial cell membrane and understand the mode of action of silica nano particles on bacteria before and after seeding on the test surface.

3.1.2 Combinatorial studies with Microfluidics

Motility mediated virulence characterizes bacteria's efficiency to find nutrients rich niches, and to colonize these habitats effectively in short period of time. However, any recent study on bacterial motility in confined spaces studied their responses in the scale of hundreds or to the least over tens of micron. In certain cases of bacteremia infections, bacteria survive in the blood vessels and in intra tissue spaces that are sub ten-micron habitats. The preliminary results can be progressed by studying the bacterial motility in confined geometries and spaces on a microfluidic device. The outcomes will be essential to categorize specific type of geometries that would prevent

effectively the bacterial colonization as a feature of physical barrier and knacks to trap bacteria in these geometries for applications in diagnostics. *E. coli* assimilates a spatio-temporal statistical signaling network during chemotaxis movements towards the nutrient receptors. These signaling networks and correlated activity of transcription factors in a single cell organism provide evidence to higher level of regulatory hierarchy. The versatility of microfluidics allows the development of various systems with different levels of complexity curating it on experimental need basis. Additionally, parallelly arranged channels can increase the throughput of the experiment by increasing the range of samples tested simultaneously. Analyzing the power of behavioral characteristics in primitive single organism like *E.coli* to explore, proliferate, colonize, quorum sense and assimilating its correlation with its ability to adapt and modify itself according to the surrounding and nutrient flow or availability can be a stepping stone to strategically build a system to eradicate biofilm mediated bacterial infections. The outcomes of these objectives will be used to demonstrate the nano-modified surfaces with real-time biomedical applications particularly in medical facilities including catheter, prosthetics, medical devices.

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