Surface Immobilization of Quaternary Ammonium Compounds for the Enhancement of Antibiotic Efficacy

by

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On a personal note, I would like to express my gratitude to my parents and siblings for their unwavering support and belief in me. I am also thankful to my friends, Helly, Mahejbin, Mansi and Shifa who kept me encouraged throughout the whole research.

Declaration

I, Shikha Tripathi, hereby declare the all the work presented in thesis “Surface Immobilisation of Quaternary Ammonium Compounds for the Enhancement of Antibiotic Efficacy” is my own and it has not been presented in any degree or diploma before. I have also acknowledged assistance and support which I received throughout my work.

This thesis holds a significant milestone in my academic journey, and I hope that key finding of this research will contribute further to research in this field.

Shikha Tripathi

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List of abbreviations

AA: Allylamine

ATCC: American Type Culture Collection

EPS: Extracellular Polymeric Substance

GTAC: Glycidyltrimethylammonium chloride

HAI: Hospital Acquired Infection

IAI: Implant Associated Infection

MBC: Minimum Bactericidal Concentration

MIC: Minimum Inhibitory Concentration

OD: Optical Density ppAA: Plasma

Polymerised Allylamine

QAC: Quaternary Ammonium Compound

TCP: Tissue Culture Plate

WCA: Water Contact Angle

XPS: X-Ray Photoelectron Spectroscopy

ZOI: Zone of Inhibition

ZP: Zeta Potential

Abstract

Presently, bacterial contamination on medical devices has become a major challenge in hospital industry. These medical devices include implants, catheters, and many more. In this research, the issue of bacterial contamination on these implants has been addressed. Previously, various measures have been taken to overcome this issue. One of these measures, is prophylactic antibiotic treatment prior to implant surgery but this could not eliminate this risk up to a desired level. Hence, this issue needs to be resolved because it represents a severe threat to patient health because infections at implant surfaces in human body can also cause death. As per published data from our nanoengineering group, the effect of antibiotics can be influenced by surface chemistry of biomaterials. In the present project, we immobilized glycidyltrimethylammonium chloride (GTAC), a quaternary ammonium compound to a substrate pre-coated with plasma-polymerized allylamine. The aim of this was to enhance the substrate with a strong positive charged compound to investigate our hypothesis. We hypothesized that immobilized GTAC would enhance the substrate surface with an intrinsic antibacterial effect. We also hypothesized that the strong positive charge of the substrate could improve the activity of antibiotics against *Staphylococcus aureus* in the early stages of cell attachment. To test these hypotheses, we used a series of surface characterization techniques (Static Water Contact Angle, XPS, zeta potential) and biological assays (live/dead, zone of inhibition). Further cytocompatibility of these modified surfaces was tested by THP-1 Cytotoxic assay. The GTAC modified surfaces showed antibacterial activity and bacterial viability was found to be 68% for *S. aureus* ATCC 25923 where bacterial viability decreased significantly when antibiotic activity of GTAC modified surfaces was tested in synergy with antibiotics. But cytotoxic tests results showed that cell viability of THP-1 cells dropped on the sixth day of the incubation with these GTAC modified surfaces.

1. Introduction

Pathogenic microorganisms are becoming major challenge for healthcare associated facilities by influencing the functioning of the health-related devices (Jiao et al., 2017). It is well established that health-related devices like joint implants, catheters are used to treat various pathologies, but these also involve a threat of infection (Arciola et al., 2018). Hence, Implant associated infections (IAI) remain a prime concern because reportedly, 70% of hospital acquired infections are found to be associated with an implanted device (Bryers, 2008). In case of joint replacements, IAI occurs in 2% of them (Zimmerli, 2014). Catheters, wound dressing, and ventilators are the some of the short-term medical devices which can be replaced on frequent basis, but it causes inconvenience to the patient and adds extra expenses to the treatment. Unfortunately, the condition becomes severe in case of permanent implants like hip, knee, and heart valve because its replacement requires surgical procedures and this infection may also become threat to patient's life (Cavallaro et al., 2016). IAI occurs when pathogenic microbes encounter an implanted biomaterial, which enables them to colonize and initiate the process of biofilm formation. When bacteria are arranged in a biofilm, they become highly evasive of the host immune system, and their tolerance to antibiotics increases 1000-fold (Olson et al., 2002). Consequently, biofilms cannot be cleared by antibiotic treatment alone, and IAI is usually treated by further invasive surgical procedures and implant replacement (Trampuz and Zimmerli, 2008). Hence, the most attractive intervention is prevention, as opposed to treatment. To achieve this, many research groups have fabricated biomaterial surface modifications that seek to kill bacteria upon attachment. This has been achieved using surface-bound

antimicrobial peptides (Yasir et al., 2020), silver nanoparticle coatings (Riau et al., 2019) and topography modifications (Hayles et al., 2021), among others. However, these strategies have not yet successfully made the transition from laboratory to clinic. Presently the most effective form of prevention in the clinic is the prophylactic administration of antibiotics immediately prior to implant placement surgery (Kim et al., 2020). This usually involves either vancomycin or cefazolin (Kheir et al., 2017)– both of which are antibiotics that target the synthesis of peptidoglycan. While the prophylactic administration of antibiotics certainly helps reduce the rate of IAI, it is presently not sufficient to eliminate the risk entirely, and infections still occur despite this active intervention.

Recent published data by Hayles et al has demonstrated that antibiotic efficacy is influenced by the surface chemistry of biomaterials (Hayles et al., 2024). When the clinically relevant Gram-positive pathogen *Staphylococcus aureus* attaches to a surface harbouring an electrical charge, the charge of the bacterial cell surface is affected. This is believed to occur because *S. aureus* adjusts its surface charge to maximize attachment to the foreign surface. This change in bacterial surface charge is elicited by changes in expression of the *dlt* operon (Peschel et al., 1999), as well as *mprF* (Andrä et al., 2011). The outcome of the upregulation of these genes is the attachment of positively charged compounds to the outer surface of the bacterial cell. Specifically, the *dlt* operon decorates teichoic acids with D-alanine (a positively charged amino acid), while *mprF* synthesizes a positively charged phospholipid called lysylphosphatidylglycerol (LPG), which integrates within the cell membrane.

The present project aims to build on these recent findings, by further examining the interactions between biomaterials, pathogens, and antibiotics. The aim is to evaluate the if the GTAC immobilized substrate can enhance antibacterial activity of antibiotics added externally against

early *S. aureus* biofilm cells. This will be achieved by immobilization of glycidyltrimethylammonium chloride (GTAC) on the surface of a substrate. GTAC is a quaternary ammonium compound (QAC) consisting of a positively charged ammonium head, and a short alkyl chain terminating with an epoxide ring. The epoxide ring is a suitable functional group to be covalently bound to a surface which has been pre-coated with plasmapolymerized allylamine (Cavallaro et al., 2016). This arrangement leaves the positively charged ammonium head to be outwardly facing on the material surface, thereby influencing the net charge of the substrate. Similar QAC-based compounds have been shown to possess strong antibacterial properties by interfering with the integrity of the plasma membrane of bacteria. Further, QACs have previously been immobilized on surfaces and their postimmobilization efficacy has been demonstrated (Isquith et al., 1972).

It is very hard to eradicate biofilm related infection because of its resistance against antibiotics and host immune system due to extracellular polymeric substance (EPS). Directly or indirectly, these notorious microorganisms cause various kinds of infection including implant associated infections. As these implants or biomedical devices are very important part of health care system. Hence, creation of biomedical devices which can kill these microorganisms becomes necessary and it is possible through antibacterial biomaterials which is the construction material for these kind of medical devices (Sun et al., 2015). Moreover, toxicity of QACs is also considered while creation of these biomaterials.

These non-toxic QACs also inhibit the extensive use of antibiotics. These non-toxic QAC surfaces can also be used as replacement for release-based antimicrobial biomaterials because these QAC immobilized contact-killing surfaces do not have to undergo uneven release kinetics and other reservoir exhaustion phenomenon (Jiao et al., 2017).

1.2 Literature

To avoid HAI, it is crucial to break bacterial transmission and to break this chain of infection, medical devices should be protected from bacterial colonisation which can be done by developing antifouling surfaces or antibacterial surfaces which can kill bacteria upon contact. These surfaces are termed as contact-killing surfaces, and these explained in detail given below:

1.2.1 Contact-killing surfaces

These surfaces are created by immobilising bactericides onto them by covalent bonding which makes these surfaces sustainable approach as compared to surfaces equipped with leaching biocides. There are few antibacterial compounds which can be immobilised on these surfaces irreversibly and these do not even leach out and do not cause any harm to the environment and these surfaces do not provide bacterial resistance because these surfaces either act by giving non-specific oxidative stress or killing bacteria by physical damage (Kaur and Liu, 2016).

The most common antibacterial agents causing bactericide upon contact are QACs. Isquith et al (1972) demonstrated the concept of contact-killing surfaces after conducting an experiment to test the preserved antibacterial activity of an organosilicon based QAC after it gets attached to a surface. In this experiment, the coating was done by hydrolytic condensation of 3(trimethoxysilyl)-propyldimethyloctadecyl ammonium chloride (Si-QAC) on glass and cotton. Zone of inhibition test was done to support the non-leaching behaviour of Si-QAC samples and absence of ZOI favoured non-leaching behaviour of these surfaces. Moreover, Si-QAC surfaces also reduced bacterial viability as compared to uncoated samples (Isquith et al., 1972).

1.2.2 QAC-based contact killing surfaces

Menschutkin synthesised QACs for the first time in 1890 by performing a reaction named nucleophilic substitution reaction of tertiary amines with alkyl halide and this reaction was names as 'Menschutkin's reaction'. QACs are positively charged compounds and are generally presented as $N^+ R_1 R_2 R_3 R_4 X^-$ in which Nitrogen atom is covalently attached to four different groups and R may be H-atom, plain alkyl or alkyl group and X represents Halide anion (Jiao et al., 2017) QACs are divided into two groups i.e. short chained and long-chained QACs. When alkyl substitution is longer than 12-C, QACs is long chained (Inácio et al., 2016) whereas in short chained QACs alkyl chain is shorter than 12. But according to Gozzelino et al, a QAC is considered to be long-chained if its alkyl chain is longer than 8 (Gozzelino et al., 2011) whereas Li et al have considered long-chained QACs to have more than 6-carbon in its alkyl chain (Li et al., 2012). The alkyl chain length of QACs influences its antimicrobial activity (Gozzelino et al., 2011).

Hydrophilic-hydrophobic balance of QACs varies with when alkyl chain length increases which results in variation of killing ability of QACs. As per a study between length of alkyl chain and antibacterial activity of QACs, it has been concluded that long chain QACs (12-14) are most efficient against Gram-positive bacteria and QACs having alkyl chain of 14-16 carbon are more efficient for Gram-negative bacteria. Contrarily, few studies also support that short alkyl chain QACs can also eliminate bacteria efficiently (Kaur and Liu, 2016).

QACs are used in coating of antibacterial surfaces because they show contact killing properties against infectious bacteria for longer period (Sun et al., 2015). These compounds generally kill bacteria by disrupting its cell membrane (Jennings et al., 2015). QACs have previously been immobilized on surfaces and their post-immobilization efficacy has been demonstrated (Isquith et al., 1972). Few examples of QACs used as bactericide are quaternized

poly(2(dimethylamino)ethyl methacrylate), alkyl pyridiniums, N-chloramines and quaternary phosphoniums (QPs) and GTAC.

Functionalised SiO2 beads were prepared by grafting quaternized poly(vinylpyridine) (PVP) chains on SiO2 beads by Kugler et al and the antibacterial activity was tested in two sets where in first test several hundred beads of 1.5 mm diameter were tested against 2 mL of bacterial culture and second test was done with 4 mL of bacterial culture with same number of beads (Kugler et al., 2005). The results showed 98% reduction in bacterial population in former case whereas in later case bacterial reduction was nearly 0% and authors supported former case as result of contact killing by positively charged SiO2 beads (Charged Density= 5 x 1015 N^+/cm2) and former case for no antibacterial activity was explained as: the bacterial cells did not get a chance to encounter a bead in Brownian motion in solution of higher volume (Kugler et al., 2005).

Correia et al conducted a study on contact killing by chitosan scaffold coated with N,Ndimethyldodecylamine quaternized oligo (2-methyl-2-oxazoline) (CHT-OMetOx-DDA) on two strains of bacteria i.e. S. aureus and E. coli (Correia et al., 2015). This experiment on contact-based killing was done by inserting filter equipped transwell inserts in 24-well plate which separates upper part and lower part where upper part contains CHT-OMetOx-DDA and lower part contains bacteria. the pore size of filter was 0.4 μm which is less than the size of bacteria and this separation prevented bacterial killing and bacterial colonies were only killed in absence of that transwell inserts (Correia et al., 2015).

1.2.2 Glycidyltrimethylammonium chloride (GTAC)

GTAC is positively charged, short alkyl chain QAC which is used as a disinfectant. It consists of an ammonium head attached to short alkyl chain which terminates with epoxide ring. This epoxide ring aids in covalent binding of GTAC to plasma polymerised allylamine surfaces

(Cavallaro et al., 2016). The mechanism by which GTACs kill bacteria is not full known (Cavallaro et al., 2016) but it is speculated that strong positively charged ammonium head of GTAC when immobilised on ppAA facing outwards, interferes with membrane potential of bacteria and causes its death because it is generally accepted that QACs kill bacteria by penetrating its NR_4^+ into bacterial membrane, interferes with integrity of bacterial membrane (McCubbin et al., 2006, Ioannou et al., 2007, Ferreira et al., 2011).

Cavallaro et al conducted a study on using concentration gradients of immobilised GTAC on ppAA surfaces and result showed that these modified surfaces shown antibacterial activity against *E. coli* (ATCC 25922) and *S. epidermidis* (ATCC 35984) (Cavallaro et al., 2016).

1.3 Plasma Polymerisation

It is a technique which produces thin polymer films called as 'plasma polymers' which often have novel properties (Vasilev et al., 2009). Organic volatile monomers are used for producing such films with unusual and novel properties. These films can be coated on wide range of substrates and are even suitable for biomedical devices (Vasilev et al., 2009). The thickness of such coatings can be controlled by making adjustment in duration of radiofrequency application or microwave power generator which controls plasma discharge (Vasilev et al., 2009). A vacuum chamber plasma system has been utilised in our research for such coating. Various organic monomers like allylamine, acrylic acid, polyoxazoline can be polymerised in this plasma system. These monomers have different power supply requirements.

1.4 Research question, hypothesis and aims:

Research question:

Can immobilized GTAC on plasma polymerised allylamine surfaces be used to enhance antibiotic efficacy in an in-vitro antibiotic prophylaxis model?

Hypothesis:

To prove this research, the hypothesis was designed as "The immobilization of GTAC on an amine-rich plasma-polymerized substrate will enable enhanced activity of externally added antibiotics against *Staphylococcus aureus*."

Aims:

Following aims were set-up to prove this hypothesis.

Aim 1 –To optimize fabrication conditions for immobilization of GTAC.

Aim 2 – To characterise GTAC surfaces prepared under different conditions.
Aim 3 – To evaluate the intrinsic antibacterial activity of immobilized GTAC.

Aim 4 – To evaluate the influence of immobilized GTAC on the activity of externally added antibiotics.

Aim 5 - Evaluate the cytocompatibility of immobilized GTAC on THP-1 cells.

2. Materials and Methods

2.1 Materials

The materials included 13 mm microscope glass coverslips purchased from Paul Marlienfeld GmbH &Co.KG (Germany), 98% grade Allylamine from Sigma-Aldrich (China), glycidyltrimethylammonium chloride (technical grade ≥ 90%) was purchased from SigmaAldrich (USA). A LIVE/DEAD Baclight Viability kit was purchased from Thermo-Fisher Scientific's Invitrogen.

2.2 Plasma polymerisation of allylamine on substrate

13 mm glass coverslips were used as substrates. Firstly, the substrates were cleaned before allylamine deposition. For cleaning, glass coverslips were first immersed in 100% Acetone and

sonicated for 5 minutes at 50˚C, substrate was again immersed and sonicated at 50˚C for 5 minutes in 100% Ethanol followed by MilliQ water. All coverslips were dried using vacuum drying.

Cleaned coverslips were placed on plasma plate by using clean forceps. A custom-made cold plasma system was used for allylamine deposition (Vasilev et al., 2011). An AA precursor was used to deposit thin AA rich films on glass coverslips. The coverslips were coated on both sides. For surfaces characterisation (zeta potential and ellipsometry), Silicon wafers were also cleaned and coated with AA. The conditions for AA deposition are depicted in the table 1 given below:

Table 1. Depicts parameters for chamber cleaning and allylamine deposition in custombuilt plasma system.

	Precursor	Pressure	Power	Time
Air cleaning of chamber		1.3×10^{-1} mbar	50 W	20 minutes
Allylamine deposition	AA	1×10^{-1}mbar	40 W	2 Minutes

Firstly, plasma chamber and cold trap were wiped with 100% acetone followed by 100% ethanol. Cold trap was filled with liquid Nitrogen. After ensuring all valves are fully closed, speedy valve was slowly opened to create vacuum and to reach at base pressure (2.3×10^{-2} mbar). For air cleaning of the chamber, power was set to 50 W (100%) by rotating output screw and timer was set for 20 minutes by giving prompts in control box. Monomer inlet valve was slowly opened and when a stable pressure of 1.3×10^{-1} mbar reached, RF on button was turned on.

After air cleaning is done, monomer was prepared for deposition. For monomer preparation, appropriate volume of AA (at least 1 to 2 mL) was transferred in monomer flask and monomer flask was fitted in monomer inlet. Afterwards, freezing and thawing of monomer was done three times. For freezing liquid nitrogen was used and thawing was done by using water (room temperature). After its preparation, when it reached at base pressure then chamber was vented by opening venting valve slowly. Substrate was placed on the lower electrode. Chamber was closed again and, power was set to 40 W (80%) and when base pressure reached, monomer flow valve was used to set the stable pressure of 1.0×10^{-1} mbar.

At this pressure, plasma was ignited and time for AA deposition was only 2 minutes shown in figure 1. AA deposition was done on both sides of substrate (coverslip). After AA deposition, samples were carefully taken out of the chamber and placed in 24-well plate and plate was vacuum sealed. Plasma chamber was again cleaned at 1.3×10^{-1} mbar for 20 minutes. Plasma chamber was again wiped with Acetone and Ethanol.

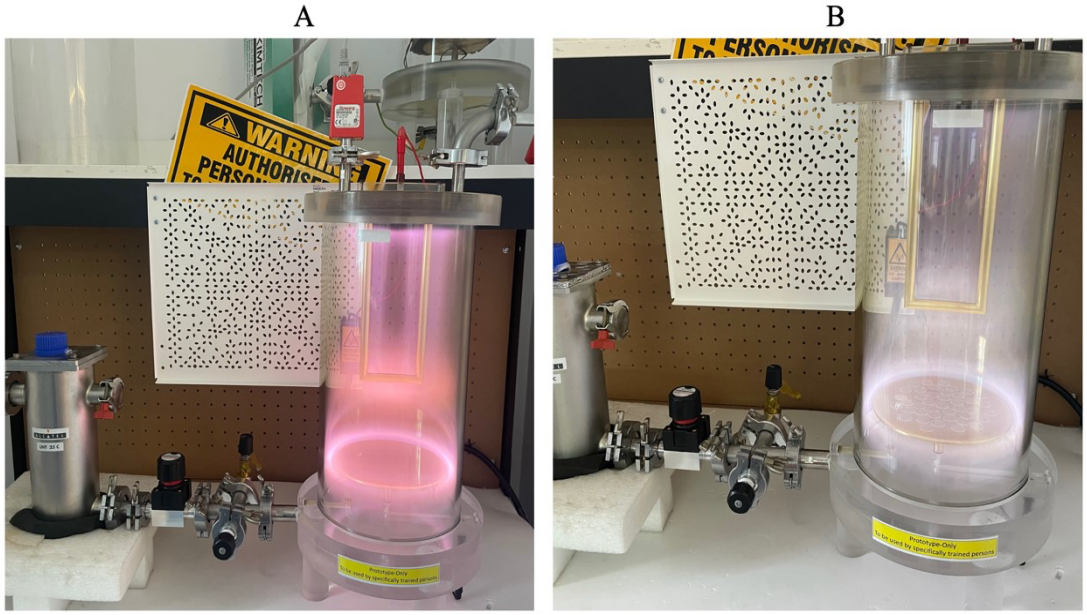


Figure 1: Illustrates custom-built plasma system where A shows air cleaning of the chamber whereas B shows allylamine deposition at substrate.

2.3 Surface Characterisation of AA coated surfaces

Surfaces characterization of AA coated surfaces was done by measuring water contact angle and Ellipsometry.

2.3.1 Static Water Contact Angle (WCA)

Static Water Contact Angle measurements were done by sessile drop method. A drop of water was placed on AA coated and GTAC immobilised surfaces and horizontal digital microscope was used for taking pictures of the droplet and a tangent was drawn on the edge of each side of droplet by using drop analysis in ImageJ software plus DropSnake Plugin. WCA was measured in triplicates, average of triplicates was calculated and compared.

2.3.2 Measurement of thickness of ppAA coating (ellipsometry)

To measure thickness of deposited coating of AA, a variable angle ellipsometer (VASE, J.A. Woolam Co. USA). WVASE32 (J.A. Woolam) software was used for data analysis (Ruvini L Dabare et al., 2022). Silicon wafers were utilised as the substrate for coating and analysis of coating thickness. All measurements were done in triplicates and average was calculated.

2.4 GTAC Immobilization on ppAA surfaces

The two concentrations of GTAC were 1% (v/v) and 10% (v/v), prepared in milliQ water and its pH was adjusted to 10 by using 0.5 M KOH solution plus there were 4-time intervals (1, 2, 4, 6 hours) for its immobilization on ppAA surfaces. For GTAC immobilisation, ppAA coated substrates were placed in GTAC solution in 24-well plate the schematic of GTAC immobilisation is given in Figure *2*.

After immersing substrates for required time, those were rinsed with 100% ethanol and dried with Nitrogen gas and were placed in 24 well-plates vacuum sealed afterwards.

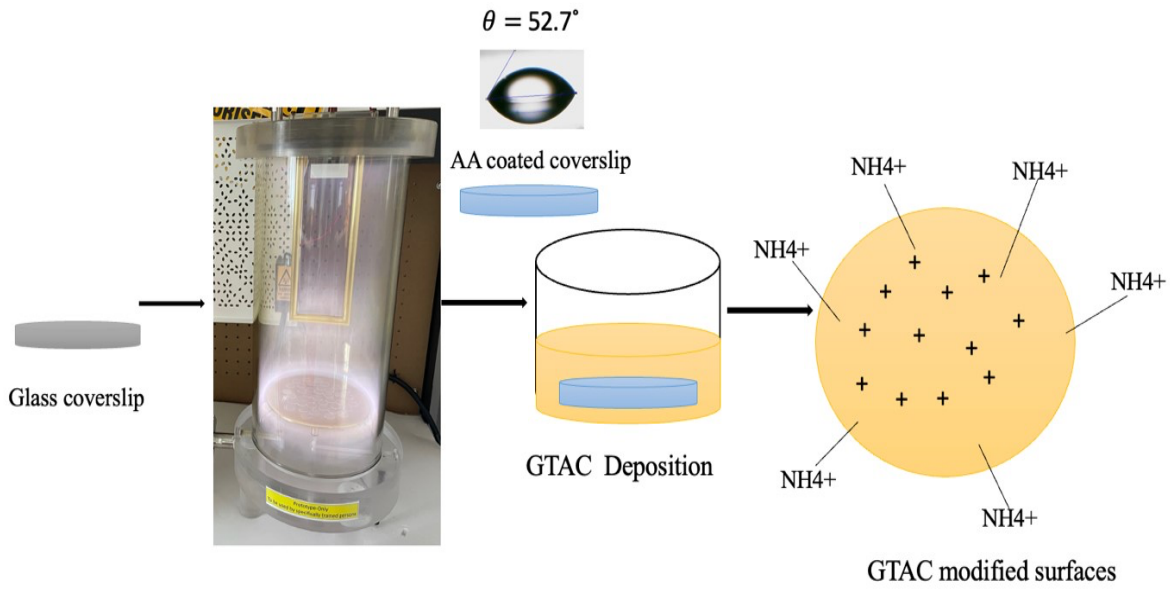


Figure 2: Schematic of plasma polymerization of allylamine on glass coverslip and GTAC fabrication on ppAA in which GTAC modified surface is positively charged due coating of positively charged GTAC and in this figure water contact angle of ppAA is also shown.

2.5. X-Ray Photoelectric Spectroscopy (XPS)

XPS was used to analyse elemental and chemical characterisation of ppAA surfaces and GTAC immobilised ppAA surfaces. Kratos Axis Ultra Spectrometer (Kratos Analytical Ltd, UK) was used to measure spectra and this spectrometer was equipped with a monochromatic Aluminium source which operated at 15 keV and 15mA to record spectra from 0 eV to 1100 eV. Casa XPS software was used for data analysis. The analysis was performed in duplicates.

2.6 Zeta Potential (ZP)

In this research, for Zeta Potential measurements, a ZPA 2.0 (Dataphysics, Germany) was deployed. The pH range was from 5.5-9 with an oscillating flow of KCl(aq) (10^{-3} M) and a narrow gap of nearly 130µm between two GTAC coated surfaces. An automated dosing unit

was adjusting pH between each reading and the stock solution were 1 M HCl (aqueous) and 1M KOH (aqueous).

For Zeta Potential analysis, ppAA Silicon wafers coated with 1% GTAC (1 h) and 10% GTAC (6 h) were used in duplicates.

2.7 Maintenance of Bacterial culture

The bacterial strains included *Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus,* a clinical isolate from a patient suffering from osteomyelitis. Just for reference, later bacterial strain was referred as *Staphylococcus aureus* Bone 20 (a). Tryptone Soy Agar (Oxoid) was used as a nutrient medium to streak bacterial strains. One colony was selected from the streak plate and was inoculated in 10 ml Tryptone Soy Broth (Oxoid) and was incubated for 24 hours in shaker incubator at 37˚C. The optical density of bacterial culture was measured at 600 nm and for incubating bacterial culture with GTAC immobilised surfaces, bacterial culture was diluted to OD 0.01 which is 10^7 CFU/mL.

2.8 Antibacterial testing of GTAC immobilised ppAA surfaces

A LIVE/DEAD BacLight viability Kit was used to assess bacterial viability on modified surfaces. Both bacterial strains were diluted to Optical density of 0.01 (10^7 CFU/mL) in TSB and were incubated with sterile GTAC modified surfaces in 12 well-plates. The GTAC immobilised samples were sterilised by keeping in UV light for 30 minutes. The samples which were incubated with 1 ml of Bacterial culture included coverslips which were uncoated, AA coated and 8 GTAC coated surfaces i.e. 1% GTAC (1 h, 2 h, 4 h and 6 h) and 10% GTAC (1 h, 2 h, 4 h and 6 h). After 24-hours incubation at 37˚C, bacterial culture was removed from well plate and surfaces were gently washed with PBS to remove any unattached bacteria. The

biofilm formed on coverslips was stained with LIVE/DEAD BacLight Viability kit. The stain was prepared by adding 15 µl of each Syto9 and Propidium Iodide (1.5 µL per mL of PBS).

After 15 minutes incubation in dark, sample were immediately imaged under Zeiss LSM880 (Carl Zeiss AG, Oberkochen, Germany) confocal laser scanning microscope. The excitation and emission spectra were 480/500nm and 490/600nm respectively for Syto9 and PI. Micrographs were taken in triplicates at 3 different areas of sample. The green and red cells were quantified with ImageJ software by using the function "split channels". The red and green bacteria were counted by using function "find maxima". Viability was calculated by using the formula, **Bacterial viability: Live cells/total cells x 100.**

2.10 Determination of spatial range of antibacterial effect of GTAC immobilised ppAA surfaces

To confirm that GTAC surfaces are non-leaching, Zone of Inhibition test was done. Eight TSA plates were prepared and 50µl of bacteria was spread on the TSA petri-plate as Zone of Inhibition Test was done for all GTAC fabricated samples in triplicates by placing 3 GTAC coated coverslips on the surfaces of 1 TSA petriplate. The plates were incubated at 37˚C for 24 hours.

2.11 Minimum Inhibitory Concentration of Vancomycin and Cefazolin calculations for *S. aureus* ATCC 25923 and *S. aureus* Bone 20 (a)

To determine MIC, antibiotic solution of 320 µg/mL was prepared. In a 96 well-plate, aliquotes of 95µL of TSB were transferred to each column except for column 1. Column 1 had 170 µL TSB and 20 µL of antibiotic (320 µg/mL). Half dilution was done by doing proper mixing (by sucking up and down by pipette) of solution in previous well before transferring it to the next well and the half dilution was performed from 1 to 2, from 2 to 3 and all the way down to

column 12, leaving only 95µL in all wells. Positive and media controls were also included on each well plate where positive media comprised of TSB and bacteria whereas media control was only TSB. Bacterial concentration of OD_{600} =0.01 (CFU= 10^7 CFU/mL) was prepared and

5 µL of bacterial culture (OD_{600} = 0.01) was transferred in each well. Plate was incubated at 37 ˚C overnight in incubator. MIC was analysed in triplicates. The absorbance of MIC plates was measured in Agilent BioTek Synergy HTX multimode reader.

2.9 Antibacterial testing of GTAC modified surfaces in synergy with antibiotics

The LIVE/DEAD BacLight Viability kit was used for analysing live and dead cells. But for assessing antibacterial activity of GTAC surfaces in synergy with antibiotics, bacterial cultures were only diluted to OD 0.1 (10^6 CFU/mL) and 1 mL of bacterial culture was incubated only for 3-hours and immediately after incubation, triplicate samples were incubated with TSB that has been dosed with different concentrations of antibiotics. Only uncoated, AA coated, and sample coated 10% GTAC for 6 hours were used and for both antibiotics (vancomycin and cefazolin). The concentration of antibiotics was equivalent to 16X, 4X and 1X of its MBC. After incubating those samples for 24-hours at 37˚C, antibiotics were removed, and samples washed with PBS and immediately stained with LIVE/DEAD BacLight Viability kit as done before for testing antibacterial testing of only GTAC fabricated samples. The stained samples were analysed further under laser scanning microscope (Figure *3*).

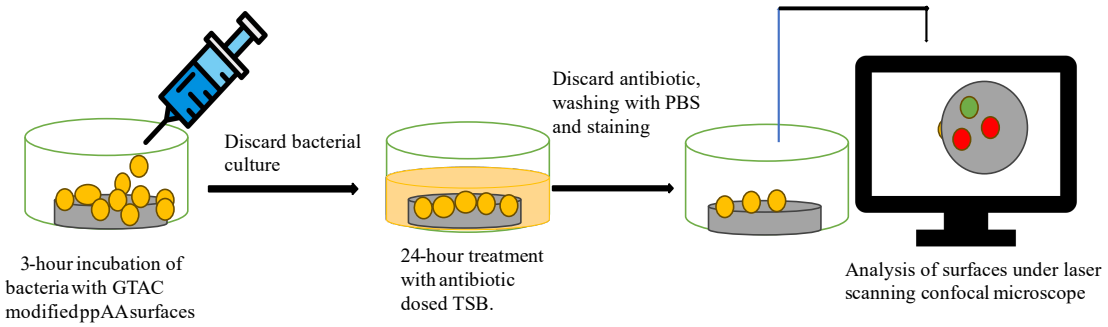


Figure 3: Schematic of testing of antibacterial activity of GTAC fabricated surfaces in synergy with antibiotics and its analysis by confocal laser scanning microscope.

2.12 THP-1 Cytotoxicity Assay for Cytocompatibility testing of modified surfaces

THP-1 monocytes were differentiated by using Phorbol 12-myristate 13-acetate (PMA) to produce macrophage like THP-1 cells. Those cells at density of 5 X 10^4 were seeded per sample. Cytocompatibility tests were done on 4 samples (uncoated, allylamine, 1% GTAC coated for 1 hour and 10% GTAC coated for 6 hour) in triplicates. After 24 hours, cells were fixed with 4% paraformaldehyde for 45 minutes followed by treatment with 0.1% Triton-X (Sigma) for 10 minutes. Subsequently, cells were incubated for 1 hour in Alexa 488-phalloidin (Thermo Scientific). Afterwards, 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Scientific) was incubated for 45 minutes. After these treatments, these coatings were washed with 2 mL PBS and, phalloidin and DAPI were used to stain macrophage like THP-1 cells and were visualised under confocal laser scanning microscope.

3. Results and Discussion

3.1 Surface Characterization

3.1.1 Water Contact Angle Measurements and Ellipsometry analysis

Water contact angle measurements were taken from left and right side of the water droplets for ppAA c, 1 % GTAC coated (1 hour and 6 hour) and 10% GTAC coated (1 hour and 6 hour) and WCA was obtained after calculating average of triplicate values. The lowest angle in the sample coated 10% GTAC for 6 h was 36.3° and for ppAA surfaces, WCA was 52.7° and it was highest of all WCA (Figure *4*). As Static Water Contact Angle represents wettability of the surface, lower WCA suggests, that this GTAC modified surface is hydrophilic (Fedorova, 2020) and this is the reason why water spreads out more on this surface as compared to other surfaces. The thickness of ppAA film was analysed at three different samples and average thickness was 26.7 nm when measured by ellipsometry (Figure *5*).

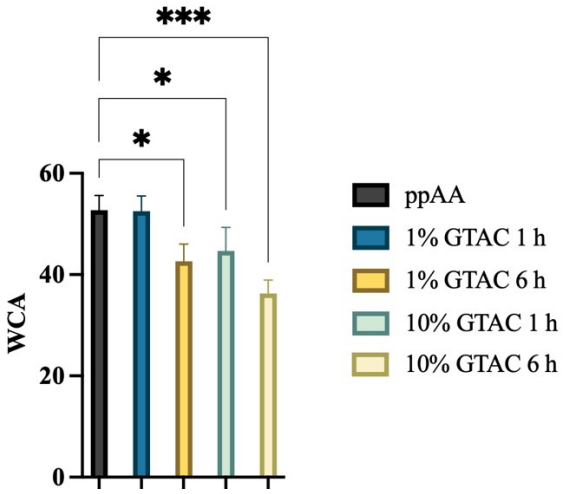


*Figure 4:Graphical representation of water contact angle versus ppAA coated, 1% GTAC, *p<0.5, ***p<0.001, n=3*

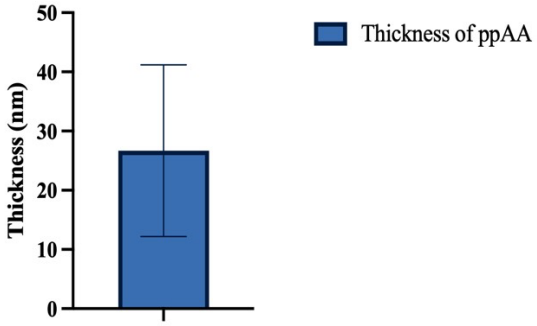


Figure 5: Thickness of ppAA coating (ellipsometry) n=3.

3.2 X-Ray Photoelectron Spectroscopy (XPS)

XPS was used to analyze the nitrogen content and confirm binding of GTAC on ppAA surfaces by scanning bonding energy region of N1s region. The given Figure *7* shows that peak of ppAA surfaces is lying between 399-402 eV and another peak of GTAC 1 h 1% samples and 10% GTAC 6 h at 400eV. A new peak can be seen at peak at 400 eV in 10% GTAC 6-hour sample indicates presence of quaternary ammonium cation (Kistamah et

al., 2009). The percentage NR_4^+ in ppAA samples was zero and in 1% GTAC (1 h) sample, the average percentage of NR_4^+ was 0.13% and in 10% GTAC (6 h), it was 1.6% shown in graph of Figure *7*.

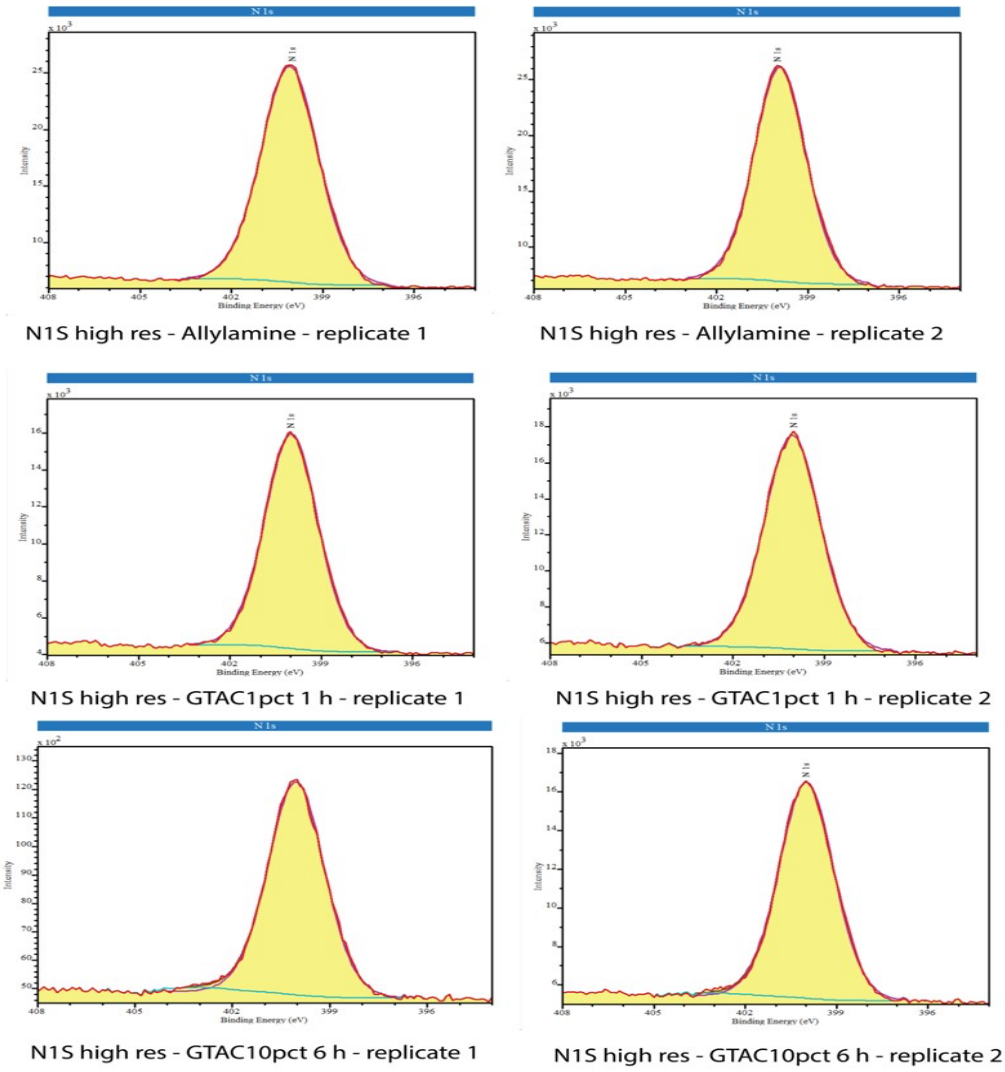


Figure 6: N1s High resolution spectra images obtained by XPS analysis of ppAA, 1%GTAC 1 h and 10% GTAC 6 h samples in duplicates.

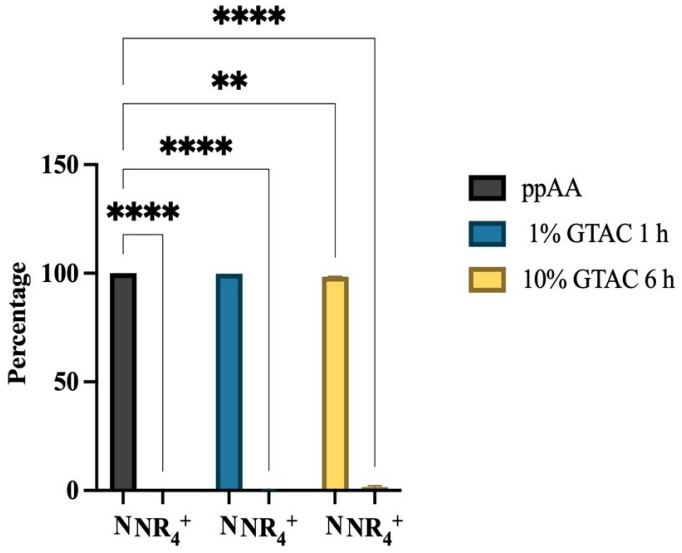


*Figure 7:Shows percentage of N and NR4+ in ppAA, 1% GTAC 1 h and 10% GTAC 6 h samples, ****p<0.0001, **p<0.01, n=2.*

3.3 Zeta Potential (ZP)

The given graph (Figure *8*) shows ZP values versus pH and the ZP lies between -50 eV to 50 eV, pH ranging from 5.5 to 10. The comparison was of ZP values was performed between the samples coated with 10% GTAC (for 6 hour) and 1 % GTAC (for 1 hour). The ZP value for the 10% GTAC (6 hour) sample changes but remains positive from pH 5.5 to 9 whereas it fluctuates from positive to negative and again becomes positive ZP value for 1% GTAC (1 hour). It is possible due to contaminations in the samples (Mierczynska-Vasilev, 2019). At pH 7, the ZP of 10% GTAC (for 6 hour) is 21.7 eV and ZP was -0.2 eV at pH 7.2 for 1% GTAC (1 hour).

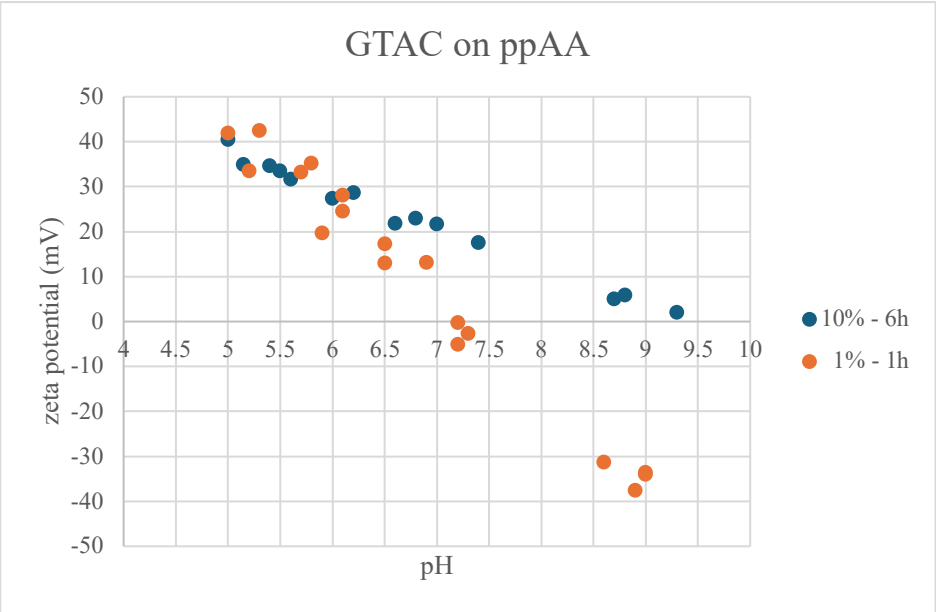


Figure 8: Zeta potential versus pH analysis of 1% GTAC 1 h and 10% GTAC 6 h samples where former sample has negative zeta potential at pH 7.

3.4 Antibacterial testing of GTAC immobilised surfaces

3.4.1 *Staphylococcus aureus* ATCC 25923

Bacterial Viability was assessed to analyse the effect of GTAC coated surfaces on *S. aureus* ATCC 25923. The two stains used in LIVE/DEAD viability kit work on the basis nature of cell envelope which means if a cell envelop is perforated, it will absorb propidium iodide and will appear red under confocal laser scanning microscope and the healthy cells get stained from SYTO9 on the cell envelop and appear green (Hayles et al., 2024). Figure 9 shows highest number of live cells in uncoated substrate and lowest green cells on 20% GTAC immobilised ppAA substrate.

For *Staphylococcus aureus* ATCC 25923, the highest viability was confirmed in uncoated samples and its average viability was 95.40% ± 0.5% whereas it was almost similar in ppAA surfaces also with average viability of 95.38%±2.2% as shown in Figure *10*. In contrast, the lowest survival of bacterial colonies was on the sample coated with 10% GTAC for 6 hours and its average viability was 60.85%±3.1%. From this result, it is

understood that coating of 10% GTAC for 6 hours is the most effective concentration and time of coating.

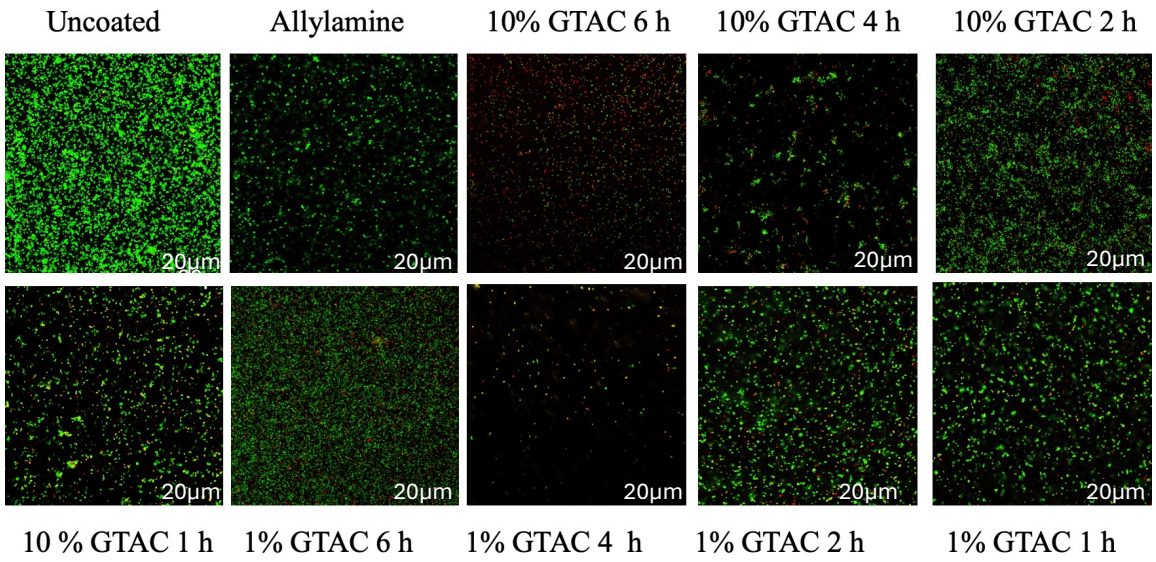


Figure 9: Shows images obtained from analysis of LIVE/DEAD assay of S. aureus ATCC 25923 under confocal laser scanning microscope where in uncoated sample highest number of green cells can be visualised whereas in 10% GTAC 6 h sample, least number of green cells are seen.

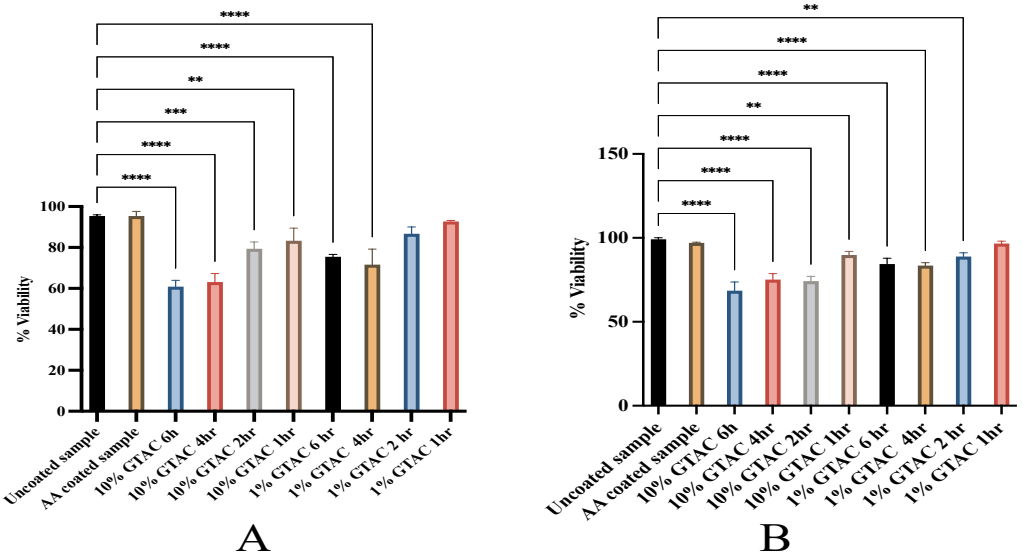


Figure 10: Graphical representation of percentage viability versus various fabricated samples where A shows percentage viability of S. aureus ATCC 25923 on 10 different surfaces where highest viability is on uncoated sample whereas lowest viability is on sample

*coated with 10% GTAC (6 h) and B shows percentage viability of S. aureus Bone 20 (a), **p<0.01, ***p<0.001,****p<0.0001, n=3.*

3.4.2 *Staphylococcus aureus* Bone 20 (a)

For *S. aureus* Bone 20 (a), lowest viability was found in the surface which was coated with 10% GTAC solution for 6 hours and highest bacterial population survived in uncoated samples as shown in Figure *12*. It can also be seen from the above Figure *11* that the bacterial viability is quite similar in uncoated as well as in Allylamine coated samples but its little bit higher in uncoated. It may be due to presence of N-atom in allylamine. From this experiment, it was confirmed that the ppAA sample coated with 10% GTAC for 6 hours had highest ability to reduce bacterial population on its surface and the average viability was 68.55%±5%.

The positively charged ammonium head of GTAC disrupts bacterial cell wall followed by leakage from cell wall (Jiao et al., 2017), because when the microorganism encounter the antimicrobial compound having concentration more than its minimum inhibitory concentration, it causes more interaction between microorganism and the QACs and it even reduces the chance of bacterial survival by breaking its cell wall (Jiao et al., 2017).

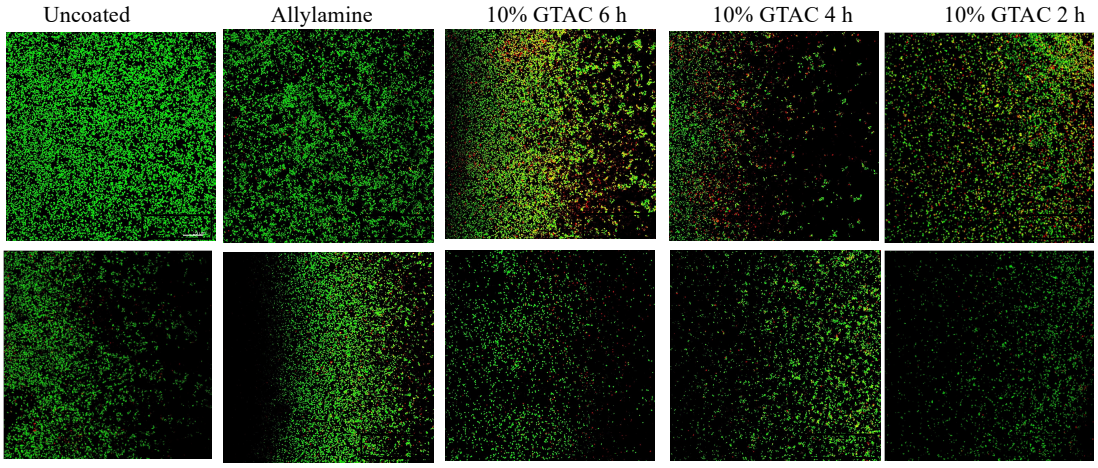


Figure 11:Shows LIVE/DEAD Assay images of S. aureus Bone 20 (a) obtained by laser scanning confocal microscope where highest number of viable cells is found on uncoated sample followed by allylamine (ppAA) and lowest number of green cells on sample coated with 10% GTAC for 6 h.

10% GTAC 1 h 1% GTAC 6 h 1% GTAC 4 h 1% GTAC 2 h 1% GTAC 1 h

3.5 Determination of spatial range of antibacterial effect of GTAC immobilised ppAA surfaces

After 24-hour incubation, bacterial growth was found to be inhibited only under the surface of GTAC modified coverslips and there was no zone of inhibition as shown in Figure *13* and figure 12 shows ZOI under AA coated substrates. Hence, it proves that this GTAC modified surface is non-leaching and its antibacterial action is based on contact. QAC modified surfaces are known to be antimicrobial in both in leaching and non-leaching conditions. But leaching may involve uncontrolled release of chemicals from the modified surface, and it may not be effective for long-term (Bruenke et al., 2016).

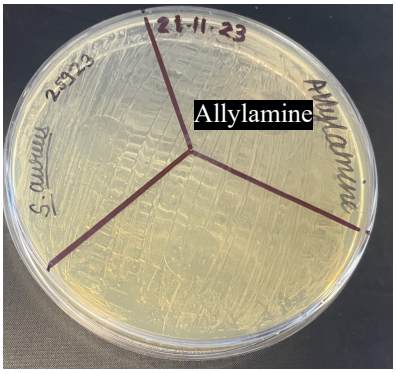


Figure 12: the above given figure does not show any ZOI or antibacterial activity under AA coated cover slips

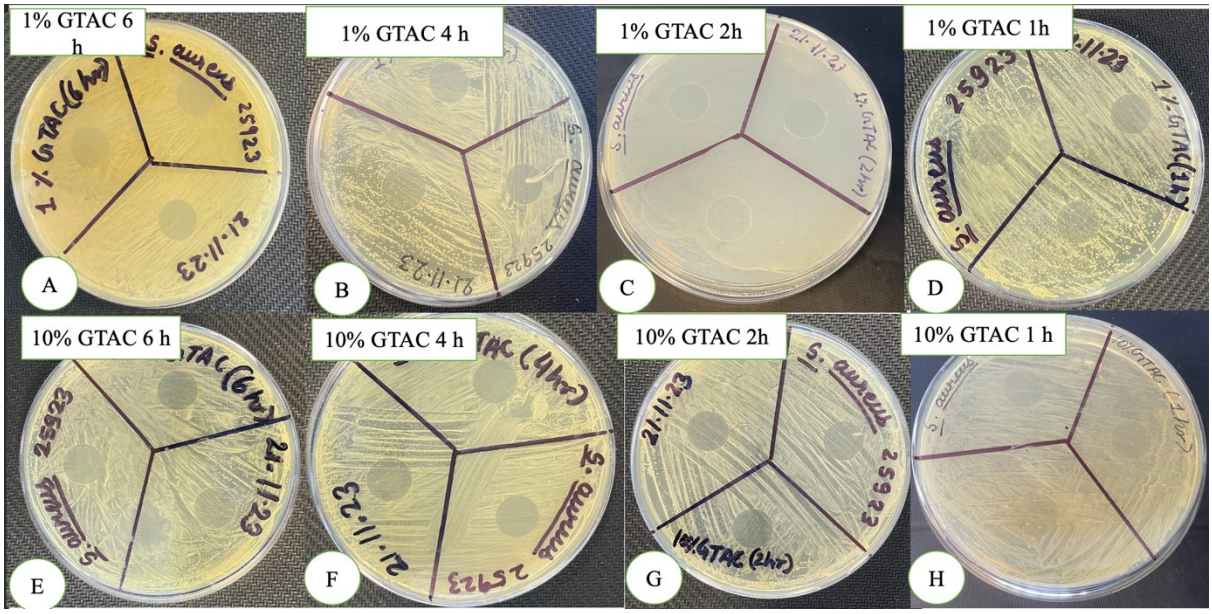


Figure 13:Results of Zone of Inhibition which show that antibacterial effect GTAC coating is contact based not by leaching out as there is no ZOI in any sample petriplate.

3.6 MIC and MBC of antibiotics

3.6.1 *S. aureus* ATCC 25923

For this strain MIC of vancomycin and cefazolin is shown in figure *Figure 14* and Figure *15*. After overnight incubation, absorbance was measured, and least absorbance of bacteria was found at the concentration of 1 µg/mL for vancomycin which is its MIC value and its MBC was 2 µg/mL and for cefazolin least absorbance was at the concentration of 0.25 µg/mL (MIC) and its MBC value was 0.5 µg/mL as given in Table *2*. These results of MIC and MBC are similar with research conducted by Andrew et al (Hayles et al., 2024).

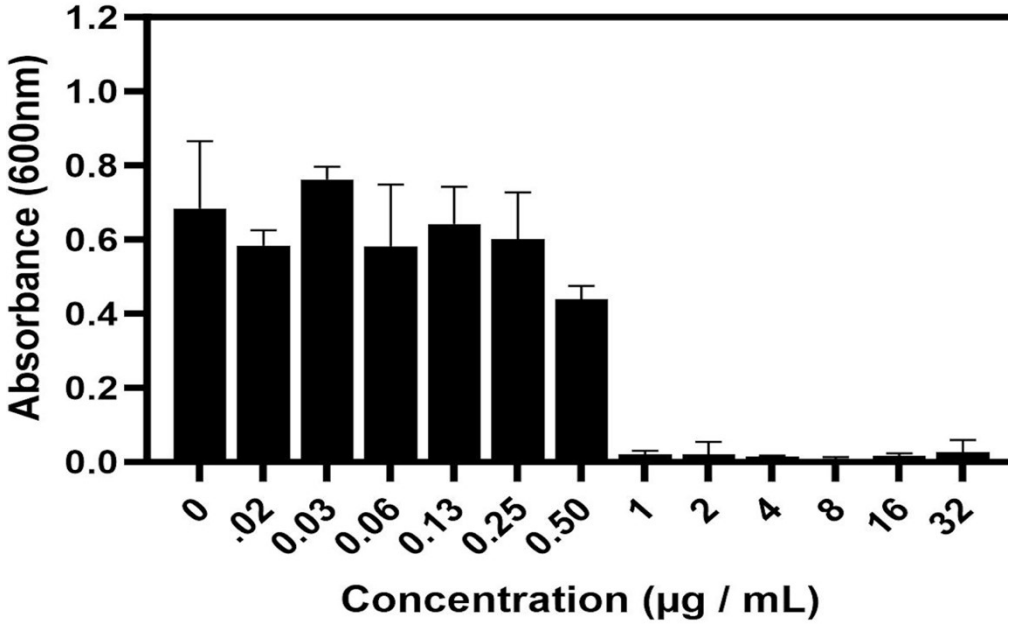


Figure 14:Graphical representation of absorbance versus concentration (µg/mL) of vancomycin against S. aureus ATCC 25923 to analyze its MIC value for killing the bacteria.

Table 2. MIC and MBC values of vancomycin and cefazolin for *S. aureus* ATCC 25923.

Antibiotic	**MIC**	**MBC**
Vancomycin	1 µg/mL	2 µg/mL
Cefazolin	0.25 µg/mL	0.5 µg/mL

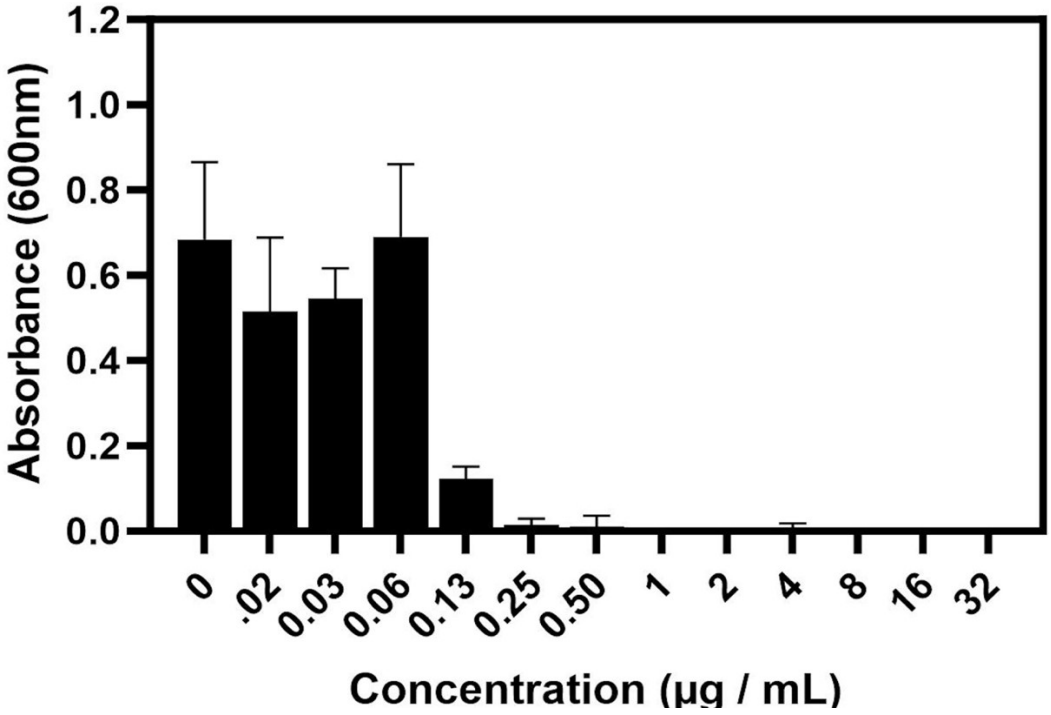


Figure 15:Graphical representation of absorbance (nm) versus Concentration (µg/mL) of vancomycin against S. aureus ATCC 25923 to analyze its MIC and MBC value for killing bacteria.

3.6.2 *S. aureus* Bone 20 (a)

For this strain also, MIC and MBC values of vancomycin and cefazolin (Figure *16*, Figure *17* and Table *3*) are same as obtained for *S. aureus* ATCC 25923 which means MIC and MBC for vancomycin is 1 µg/mL and 2 µg/mL for vancomycin and 0.25 µg/mL and 0.5 µg/mL respectively.

Table 3. MIC and MBC values of vancomycin and cefazolin for *S. aureus* Bone 20 (a).

Antibiotic	**MIC**	**MBC**
Vancomycin	1 µg/mL	2 µg/mL
Cefazolin	0.25 µg/mL	0.5 µg/mL

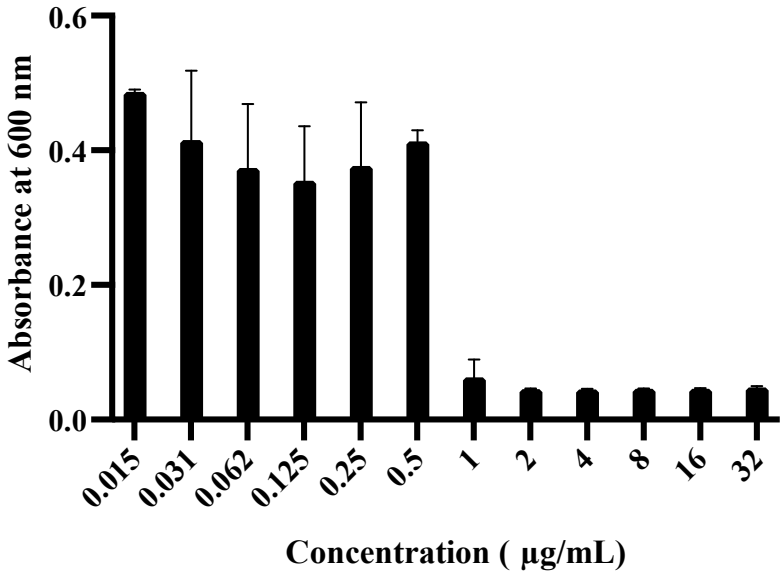


Figure 16:Graphical representation of absorbance (nm) versus Concentration (µg/mL) of vancomycin against S. aureus Bone (20a) to analyse its MIC and MBC value for killing bacteria.

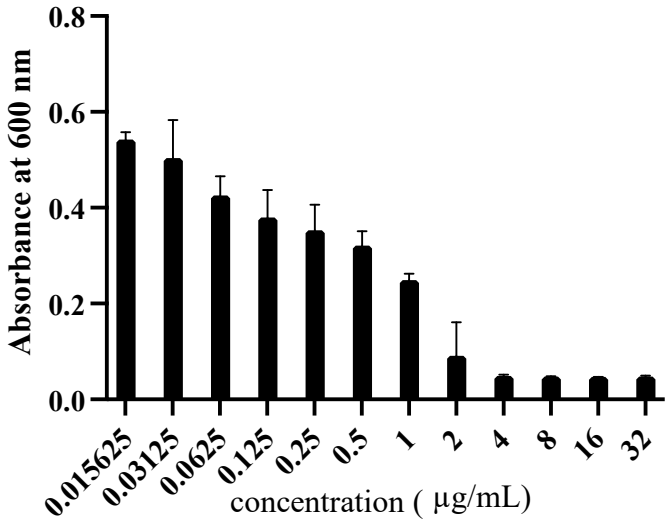


Figure 17:Graphical representation of absorbance (nm) versus Concentration (µg/mL) of vancomycin against S. aureus Bone 20(a) to analyze its MIC and MBC value for killing bacteria.

3.7 Antibacterial testing of GTAC coated surfaces in synergy with antibiotics

To confirm that GTAC immobilized surfaces can enhance the action of antibiotics, firstly, GTAC surfaces were incubated with bacterial culture for 3 hours and then with antibiotic for 24-hour and were imaged under confocal laser scanning Microscope. The images of

S. aureus ATCC 25923 and *S. aureus* bone 20 (a) visualized under microscope are given in Figure *18* and Figure *19*. Two antibiotics i.e., vancomycin and cefazolin were selected to be tested. Vancomycin is positively charged whereas cefazolin is negatively charged antibiotic. Firstly, MIC and MBC of these antibiotics were determined. The MBC for vancomycin and cefazolin was 2 µg/ml and 0.5 µg/ml respectively. Both antibiotics were tested with 1X, 4X and 16X concentrations (of MBC). In 1X vancomycin, percentage viability of *S. aureus* ATCC 25923 in 10% GTAC (6 h) was found to be 65.99±4.28 whereas in *S. aureus* Bone 20 (a), it was 20.19%±1.80%. In 4x concentration of vancomycin percentage viability of *S. aureus* ATCC 25923 and *S. aureus* Bone 20 (a), with 10% GTAC samples was 40.97%±4.99% and 25.46%±6.7% respectively. For 16x vancomycin, the percentage viability of S. aureus ATCC 25923 and for *S. aureus* Bone 20 (a) was 36.15%±3.09% and 13.63%±2.09% respectively.

For 1x cefazolin, the percentage viability of *S. aureus* ATCC 25923 and for *S. aureus* Bone 20 (a) in 10% GTAC (6 h) was 65.69±4.2 and 72.32%±1.5% whereas 4x cefazoline, the percentage viability of *S. aureus* ATCC 25923 and for *S. aureus* Bone 20 (a) in 10% GTAC (6 h) was 16.35%±9.8% and 38.50±11.63. The percentage viability of *S. aureus* ATCC 25923 and for *S. aureus* Bone 20 (a) in 10 % GTAC (6 h) with 16X cefazolin was 33.42%±2.7% and 24.82%±5.8% respectively shown in Figure *20 and Figure 21*.

The viability of *S. aureus* ATCC 25923 was lower in all concentrations of cefazolin as compared with vancomycin as shown in Figure *21* whereas figure 22 shows opposite results where bacterial viability is high in cefazolin as compared to vancomycin.

But it notable that bacterial viability decreased significantly when bacteria was incubated with antibiotics followed by incubation on GTAC immobilized ppAA surfaces.

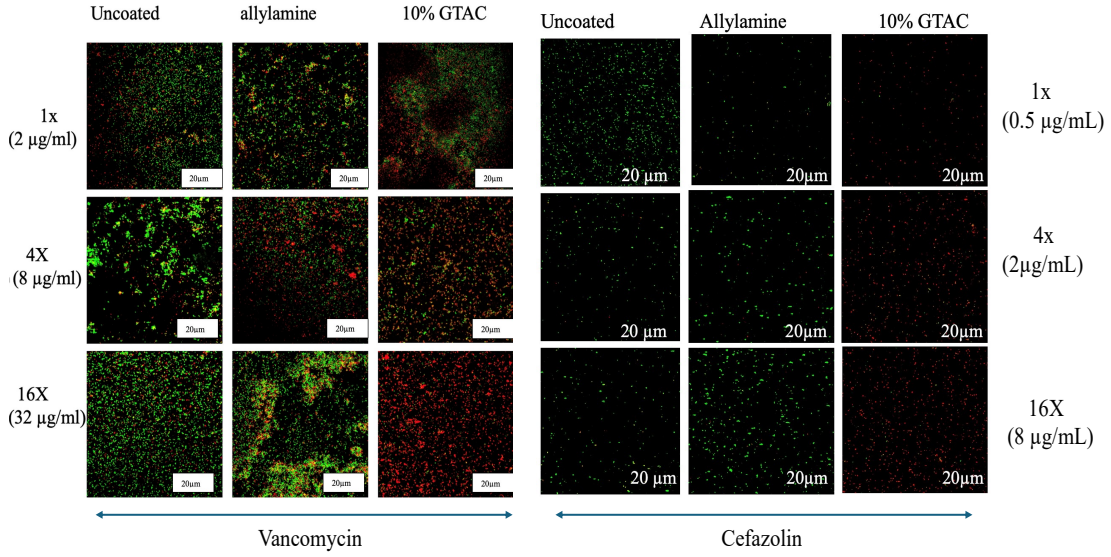


Figure 18: Shows images obtained by analysis of LIVE/DEAD assay samples under laser scanning microscope where the antibacterial activity of samples (uncoated, allylamine coated and 10% GTAC 6 h sample) in synergy with antibiotics was tested against S. aureus ATCC 25923,

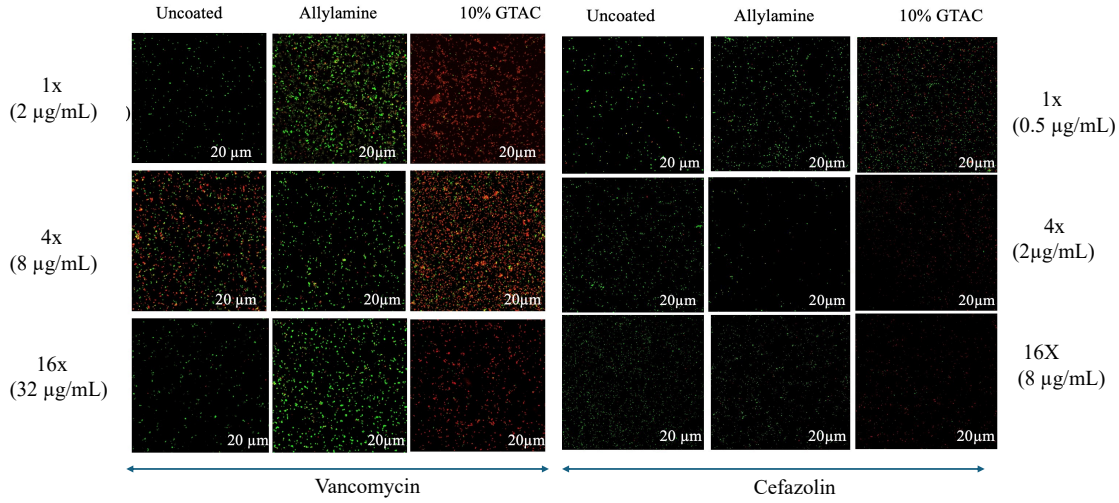
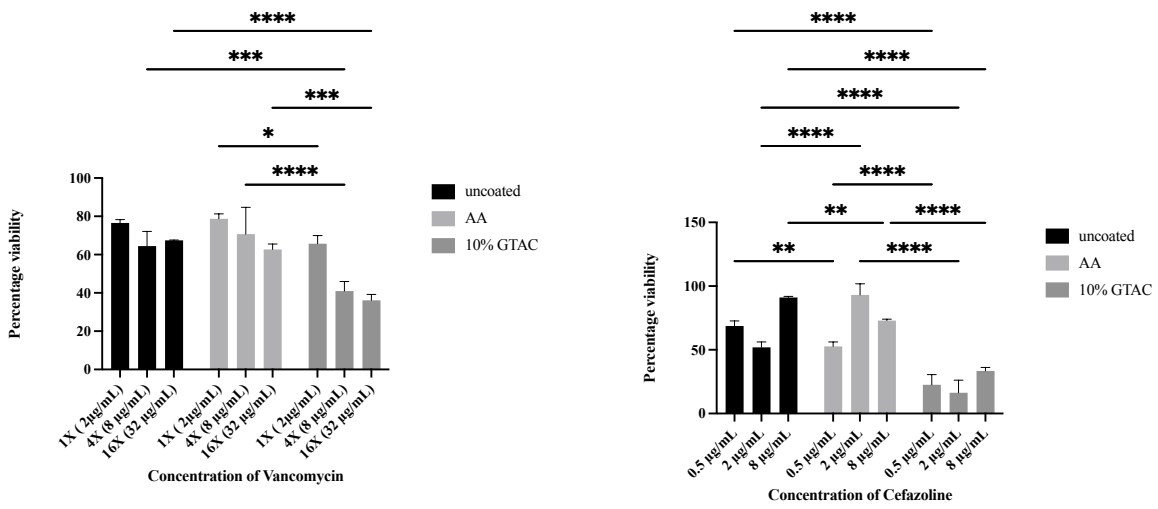


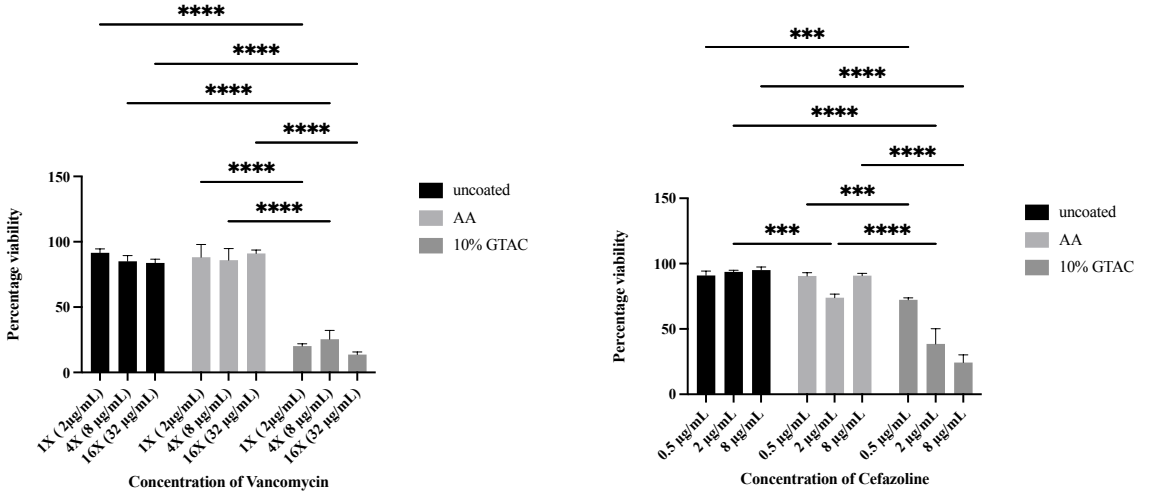
Figure 19: Shows images obtained by analysis of LIVE/DEAD assay samples under laser scanning microscope where the antibacterial activity of samples (uncoated, allylamine coated and 10% GTAC 6 h sample) in synergy with antibiotics was tested against S. aureus Bone (20a).



A B

Staphylococcus aureus ATCC 25923

*Figure 20: Shows percentage viability of S. aureus ATCC 25923 under the three MBC (1X, 4X, 16X) of vancomycin and cefazolin, *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001, n=3*



A B

Staphylococcus aureus Bone 20 (a)

*Figure 21: Shows percentage viability of S. aureus Bone (a)under the three MBC (1X, 4X, 16X) of vancomycin and cefazolin, *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001, n=3*

3.8 THP-1 Cytotoxicity assay

Cytocompatibility was done one 4 different samples (uncoated, ppAA samples, 1% GTAC 1 h and 10% GTAC 6 h). For each group of samples, there were two biological replicates and each biological replicate had 3 technical replicates, hence, there were total 6 replicates per sample. The cytotoxic effect of fabricates sample was tested on day 1 and day 6. The images obtained of THP-1 cells incubated with all sample were analyzed under confocal laser scanning microscope shown in Figure *21* and the percentage viability is shown in graph given in Figure *22*.

The percentage viability of uncoated samples, ppAA samples, 1% GTAC 1 h and 10% GTAC 6 h (all normalized to TCP) on day 1 was 103.07%±3.6%, 106.57%±8.20%, 109.21%±7.39% and 109.16%±4.94% respectively.

On day 6, percentage viability for uncoated samples, ppAA samples, 1% GTAC 1 h and 10% GTAC 6 h (all normalized to TCP) was 89.75%±2.38%, 74.70%±13.11%, 89.04%±4.33% and 70.29%±9.13% respectively.

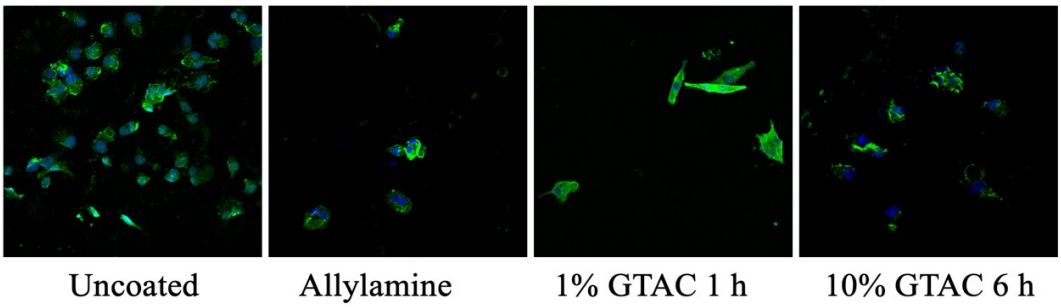


Figure 22: Represents results for cytocompatibility test of the Uncoated, ppAA (allylamine), 1% GTAC 1 h and 10% GTAC 6 h samples with THP-1 cell obtained by sample analysis under confocal laser scanning microscope.

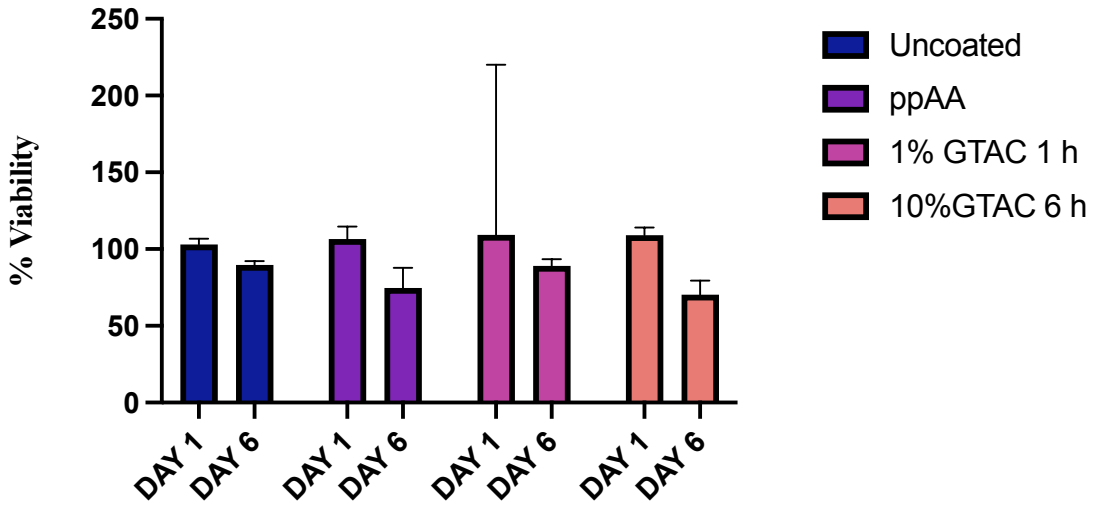


Figure 23: Shows percentage viability of THP-1 cells with four samples (uncoated, ppAA, 1% GTAC 1 h and 10% GTAC 6). on day 1 and day 6 of incubation where there is no loss of viability on day first but on day 6, there is some loss of viability in cells incubated with ppAA and 10% GTAC (6 h) which is 74.70%±13.11% , n=6 and 70.29%±9.13%, n=6.

From the data presented above, there was no loss of viability on day 1 but the percentage viability of macrophage like THP-1 decreased on day 6 as the ppAA coated sample and ppAA sample coated with 10% GTAC (6 h) caused some toxicity to the cells. The cytotoxic effects of ppAA surfaces and 10% GTAC (6 h) are nearly similar at day 6. It suggests that optimization of concentration and density of GTAC is necessary to balance the antibacterial and cytotoxic effect of these modified surfaces.

4. Conclusion

Overall, fabrication parameters for GTAC immobilization were optimised followed by the surface characterisation of GTAC immobilised surfaces. Intrinsic antibacterial activity of GTAC immobilised surfaces was tested where the ppAA surfaces coated with 10% GTAC for 6 h showed highest antibacterial activity for both bacterial strains and these surfaces

caused significant loss in viability of bacterial cells when tested in synergy with antibiotics and among both antibiotics, cefazolin was more effective as compared to vancomycin in case of *S. aureus* ATCC 25923. As per the hypothesis, GTAC immobilised ppAA surfaces enhanced the antimicrobial properties of externally added antibiotics against *S. aureus*. As the 10% GTAC immobilised ppAA surface was toxic for THP-1 cells, the fabrication concentration and time needs to be optimised in future to balance out the antibacterial and cytotoxic effects of GTAC coated compounds on human cells.

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