CHAPTER 2
MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter elaborately describes the materials used, experimental procedure involved for the preparation of thin films of AgNPs and thin films of CuNPs on the primary heart valve material Pyrolytic Carbon (PyC). This chapter also elucidates the techniques used for cell culture studies and cytotoxicity assessment of AgNPs and CuNPs coated PyCs on animal and human cell lines. The thin film preparation method namely PLD technique and preliminary characterization methods such as Scanning Electron microscopy (SEM), Energy Dispersive X-ray analysis (EDXA), Atomic Force microscopy (AFM) and Surface profilometry (SP) were the techniques employed to characterize the prepared silver and copper thin films coated PyC samples. Blood samples for in vitro studies were obtained from K.J. Hospital Research and Post Graduate centre (Chennai, India). This study was approved ethical clearance from the Tamil Nadu government (Ref.No. KJ/188/2003). The volunteers were briefed on the study and an informed consent was obtained from every volunteer for the collection of blood samples based on inclusion and exclusion criteria. The volunteers were in the age group of 22 to 40 years devoid of any blood thinning medications. The blood samples were analyzed for in vitro assessment within 3 to 4 hours of sample collection. The chemicals and reagents used for this study were of AR grade purchased from ERBA and Sigma-Aldrich. The culture media Muller Hinton (HA) agar was procured from Golecchha Diagnostics, Chennai, India. All other reagents used were of analytical grade.
2.1.1 Substrate preparation for thin film deposition

Pyrolytic Carbon was purchased from Goodfellow Cambridge Ltd. Ermine Business Park, Huntingdon, England PE29 6WR. The carbon plate was cut into multiple pieces of 10 mm x 10 mm x 2 mm size. All the PyC pieces were cleaned and polished by mechanical means. Initially the substrates were kept in 100 ml of pure de-mineralized water and ultrasonicated for 5min. Mechanical polishing is required to attain a surface finish of 1 µm. This is carried out by using abrasive paper of mesh size 220, 320, 400, 600 and 1200. The sheets of different grade are placed over a wheel and the PyC substrates are pressed over the sheets to start the mechanical cleaning process. Using high mesh size polishing paper the surface roughness will reduce gradually. This was done till the substrate becomes flat, smooth and mirror finish reflection quality. Finally the substrate was polished using 0.5 micron Diamond paste for further ultra smooth surface. Then the substrates polished were placed in acetone and ultrasonically cleaned for 15 minutes to remove impurities. The prepared substrates were finally placed in methanol for 15 minutes prior to deposition process.

Figure 2.1 Image of Pyrolytic Carbon purchased from Goodfellow Cambridge, U.K.
2.2 THIN FILM PREPARATION AND CHARACTERIZATION TECHNIQUES

2.2.1 Thin film Deposition

2.2.1.1 Pulsed Laser Deposition of silver thin films and copper thin films on PyC

PLD is one of the well known methods among the physical vapor deposition process, carried out in a vacuum system and shares some process characteristics common with molecular beam epitaxy and sputter deposition. The schematic of the process is shown in Fig. 2.2. In this process, a pulsed laser is focused onto a target of the material to be deposited. The laser pulse ablates or vaporizes small amount of the material creating plasma called plume with sufficiently high laser power density. At the early stage of the laser pulse, a dense layer of vapour is formed in front of the target. Energy absorption during the process of laser pulse causes both the pressure and temperature of the vapour to increase and forms plasma plume. The ablation plume, consisting of ablated material is of highly forward directional in nature irrespective of the angle of incidence of the laser beam and provides the material flux for film growth. Figure 2.2 shows the photograph of the Pulsed Laser Deposition (PLD) facility used for the preparation of Ag and Cu thin films on PyC substrates. Important components of this system are (i) Nd:YAG laser, (ii) UHV Chamber with associated vacuum pumping, target carrousel and substrate heating system and (iii) gas feeding system.
2.2.1.2 Nd:YAG laser

The Nd:YAG laser is used for the preparation of AgNPs and CuNPs thin films. It is an optically pumped solid-state laser which is capable of operating on four levels energy system denoted by E1 to E4 as shown in Figure 2.3. The neodymium (Nd) ions are doped in the Yttrium-Alumina-Garnet (YAG) matrix having two absorption bands (E3 & E4) which are excited using flash pumps by optical pumping. From these excited energy levels, by a non-radiative transition the neodymium ions will move into the upper laser level (E3). Then stimulated emission occurs from the laser level of upper to the lower laser level (E3 to E2). The wavelengths of the emitted
photons are being around 1064 nm in the IR region. A non-radiative transition occurs from the lower laser level to the ground level which is denoted by E1.

Figure 2.3 Energy level representation of a Nd:YAG laser

Nd:YAG lasers are useful for several applications ranging from medical, materials processing to military due to their capability producing very high-power emissions. The advantage of Nd:YAG lasers lies in the fact that they can operate in both pulsed and continuous wave modes. Nd:YAG lasers operating at pulsed mode are typically operated in the Q-switching mode. The Q-switching is an optical switch which is inserted in the laser cavity, before it opens waiting for a maximum population inversion in the neodymium ions. In this Q-switched mode, pulse duration of less than 10 ns and output powers of 20 MW can be achieved. In this study, an Nd:YAG laser (M/s Quanta Systems, Italy) operating at 1064 nm wavelength with a maximum pulse energy of 800 mJ, pulse width of 5 ns and repetition rate of 10 Hz was used. The laser beam, having 8 mm diameter at the output port, is steered into the PLD chamber using anti-reflection coated BK7 mirrors using optical mounts.
and focused using a 3-mirrored telescope having focal length of approximately 50 cm. The beam is focused to a circular spot having 1.0 mm diameter.

2.2.1.3 UHV Deposition chamber

The ultra high vacuum deposition chamber is an bakeable stainless steel spherical chamber (M/s Excel Instruments, India) pumped by a turbo molecular pumping unit (550 l/sec, M/s Leybold, Germany) which is backed by a rotary vane pump. An oil free vacuum environment inside the chamber is ensured by the deployment of the turbo molecular pumping system. This pumping system is also very efficient in removing inert carrier gases in a low turnaround times with high pumping speed. This operation takes place at a base pressure of \( \sim 2 \times 10^{-7} \) mbar. The low background pressure, minimal contamination and better reproducibility are the main advantages of maintaining ultra high vacuum environment. The multi target carrousel and substrate heating assemblies are the components of the chamber. Substrates are positioned just in front of the target. Apart from gadgets for vacuum measurement like Pirani and Penning gauges, the laser port, target and substrate assembly are also configured in such a way that the laser at an oblique incidence of 45° falls on the surface to provide maximum ablation yield.

2.2.1.3 Target holder and automated target carousel controller (ATCC)

The target holder assembly fixed inside the ultra high vacuum chamber consists of a six-target turret with DC motor and 0- 360° graduated rotatable positioner which are mounted on a DN 150 CF flange. The DC motor will aid in the process of rotating the targets about their own axis. The targets can be fixed in a target assembly which can hold up to a maximum of six numbers are indexed through the calibrated rotatable positioner. Any
desired targets fixed to the target assembly can be brought to the focus of laser beam by rotating them in the central axis. During laser ablation process, development of deep craters on the target surface can be prevented by rotating the target on its own axis and exposing a fresh target surface for ablation. The target contamination shield is also housed by a target assembly to prevent other targets contamination due to plume generated during the ablation process of one target. The ATCC is a microprocessor based controller that controls the motion of DC motor. This aids in growth of thin films of Ag and Cu thin films on prosthetic heart valve material PyC.

Figure 2.4 Multi target holder and automated target carousel controller (ATCC)

2. 2.1.4 Thin film deposition process

The Nd:YAG (Q-switched laser) capable of operating at 1064 nm was introduced through a quartz window into ultra high vacuum (UHV) compatible reaction chamber. This was focused to approximately 2 mm diameter circular spot at an oblique incidence of 45° at the target surface. The laser pulse duration was 5 ns were maintained during the deposition process. A high purity (>99.9%) silver and copper target of 24.5 mm diameter and 10
mm thickness was used as a targets for coating the heart valve material PyC. The silver and copper targets rotated at 3 rpm to prevent crater formation and to ensure fresh material is exposed for laser ablation. Prior to deposition, the PyC substrates were de-greased, cleaned, polished and vacuum dried. The ablating plume produced was made to deposit on the substrates which are positioned on a substrate holder, located just in front of the target. A target-substrate distance of 40 mm was maintained during the deposition. The ultra high vacuum chamber was evacuated to a base pressure of \(\sim 5.0 \times 10^{-9}\) mbar. The vacuum was created using a turbo-molecular pumping system which is backed by a rotary pump.

**Table 2.1 The Deposition Parameters of (A) silver thin films coated on PyC & (B) copper thin films coated on PyC**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base pressure</td>
<td>(9 \times 10^{-6}) mbar</td>
</tr>
<tr>
<td>Deposition Pressure</td>
<td>(9.2 \times 10^{-6}) mbar</td>
</tr>
<tr>
<td>Energy (Laser)</td>
<td>180mJ</td>
</tr>
<tr>
<td>Repetition rate &amp; Pulse Width</td>
<td>10Hz, 5ns</td>
</tr>
<tr>
<td>No. of laser pulses</td>
<td>5,000, 7,500, 10,000, 12,500, 15,000</td>
</tr>
<tr>
<td>Substrate temperature (PyC)</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>Target</td>
<td>Silver, Copper</td>
</tr>
<tr>
<td>Laser Wavelength ((\lambda))</td>
<td>1064 nm</td>
</tr>
<tr>
<td>Target to substrate distance</td>
<td>4 cm</td>
</tr>
</tbody>
</table>
2.3 CHARACTERIZATION TECHNIQUES

Materials characterization at the microscopic level is an important area of fundamental and technological interest and this field is continuously evolving towards improvements in the limits of existing techniques and the development of novel characterization tools with superior capabilities. The characterization techniques used in this study were carefully chosen so as to follow the effect of process parameters on the structure, morphology and composition of the coatings and their properties.

2.3.1 Surface Profilometry

Surface profiler is used to measure the film thickness of the surface. In the present study DEKTAK 6M stylus profiler (Veeco, India) was used to measure the AgNPs thin films and CuNPs thin films coated PyCs at various ablation pulses. The measurements are taken electromechanically by moving the sample beneath the diamond tipped stylus. The high precision stage moves the sample placed area beneath the stylus according to user programmed scan length, speed and stylus force. The Linear Variable Differential Transformer (LVDT) is coupled to the stylus. As the stage moves the sample the stylus rides over the surface of the sample and variations on the surface cause the stylus to be translated vertically. Electrical signals are produced due to position of the LVDT changes corresponding to the movement of the stylus. The signal produced is proportional to the change in the position, which is then converted to a digital format. The digital format is obtained through an analog-to-digital converter. The digitized signals are stored and available for the display, measurement and printing.
2.3.2 Scanning Electron Microscopy (SEM)

SEM is a versatile tool applied for surface characterization of materials. This technique is capable of producing resolution of about few nm and can operate at wide range of magnifications (10 to 300,000 X). The mechanism of SEM includes a tungsten filament which forms the source for high energy electrons (primary electrons) which is incident into a fine probe using electromagnetic lenses to scan the surface of the sample. These primary electrons are subjected to either elastic or inelastic scattering after interacting with atoms of the specimen. This result in secondary electrons (SE), X-ray photons, visible light, heat and the diffracted back scattered electrons.

The technique is considered to be non destructive except for the fact that often electron beams can bring about radiation induced surface damage. During inelastic scattering, a part of the incident electron energy is donated to the target atom. In that case, when the energy transferred is greater than the work function of the specimen results in SE emission, the energy is $< 50$ eV and are emitted within few nm of the specimen. A fraction of the primary electrons which have undergone elastic scattering with the atomic
nucleus (without loss of KE) are back scattered. These backscattered electrons (BSEs) have energies comparable to the primary electron beam. When the energy of the emitted electron is > 50 eV, is termed as BSE (ref).

The block diagram of field emission secondary electron microscope (FE-SEM) equipment is shown in Figure 2.5; the electrons coming out from the specimen surface (SEs and BSEs) are collected by appropriate detectors. The detector output controls the brightness of a cathode ray tube (CRT) where each point on the specimen surface corresponds to each point on the screen. The secondary electrons are the most common signal used in the SEM and have multiple applications e.g. analysis of surface morphology and its particles size, fracture analysis, microstructural homogeneity and determination of coating thickness. In addition to secondary electrons imaging, energy dispersive X-ray (EDX) analysis is also a useful tool which is widely used for chemical analysis. The collision of primary electrons with electrons of the atom can cause ejection of core electrons. The excited atom relaxes to ground level by either emitting a characteristic X-ray photon or an Auger electron. The X-rays signals emitted can be sorted by either energy or by wavelength using an EDX detector or by a wavelength spectrometer electron probe micro analyser (EPMA). The distributions reveal about the type of element and also give the information about its spatial distribution in the sample. The output signal from the detectors corresponds to the amount of X-ray photons from the area subjected to electron bombardment. In EDX equipment the output data is in the form of a histogram of counts versus X-ray energy. The characteristic X-rays are considered as the fingerprints which provide elemental composition of the samples. The essential components of an electron microscope are electron emission gun, electron lenses, and sample stage, detectors for all signals of interest, display and data acquisition devices.
A Field Emission Source (FES), also known as a cold cathode field emitter, does not need heating of the emitter material. The emission is achieved by placing the emitter in a huge electrical potential gradient (0.5 to 30 kV). The FES is usually a tungsten wire fashioned into a sharp point. The significance of the small tip radius (~ 100 to 10 nm) is that an electric field can be concentrated to an extreme level, becoming so big that the work function of the material is lowered, enabling easy generation of electrons. The electron beam produced by the field emission is 1000 times narrower than that produced in a conventional SEM and produces cleaner images with much less electrostatic distortions and high spatial resolution (< 2 nm). This high spatial resolution permits one to image nanoscale topography and ultra small scale phenomena. In the present study FE-SEM was used to study the surface morphology and measurement of particle size of the AgNPs thin films and CuNPs thin films in secondary electron mode using Carl Zeiss SUPRA55,
Germany. Semi-quantitative elemental analyses were carried out using the EDX facilities attached to the FE-SEM.

2.3.3 Atomic Force Microscopy (AFM)

The Atomic Force Microscope (AFM) is one of the most recently invented characterization technique and is an imaging tool with vast dynamic range spanning the realms of optical and electron microscopes. The AFM probes the sample surface with a sharp tip, a few micrometers long and less than 10 nm of tip diameter located at the free end of a cantilever that is 100 to 200 µm long. When this tip is brought close to the sample surface, cantilever will bend or deflect due to the forces between the tip and the sample surface. This bending is detected using the reflected laser beam from the cantilever by a position sensitive photo detector. With the advantages of using suitable tips it is possible to measure the physical properties such as static charge distribution, localized friction and elastic moduli and surface conductivity. There are many forces which may contribute to the bending or deflection of cantilever used for AFM. The important force that is associated with the technique of AFM is an inter-atomic force called the van der Waals force. This force is dependent upon the distance between the tip and the sample surface. The AFM can be operated in typically three modes viz., contact, non-contact and intermittent contact modes (ref). In this study, we have mainly used the intermittent-contact mode which is described briefly in the following paragraph.

Intermittent-contact atomic force microscopy (IC-AFM) or Tapping mode or Semicontact (SC-AFM) is one of the three modes of AFM. The SC-AFM is similar to non-contact-AFM (NC-AFM). In IC-AFM the vibrating cantilever tip is brought near to the sample surface, so while probing the surface it just barely hits, or “taps,” the sample. In this mode the cantilever is driven at a frequency just below the resonant frequency. The amplitude of the vibration will increase when bringing the tip closer to the sample. This induces a decrease of the vibration amplitude, which is used to control the
tip-sample distance. By monitoring the aforementioned changes images representing surface topography is obtained. The main advantage of using IC-AFM instead of contact or non-contact AFM is that they less likely to cause damage to the sample than contact AFM because it does not involve the friction or drag mechanism thereby eliminating lateral forces between the tip and the sample. IC-AFM is more effective than the other two modes of AFM for imaging scan sizes of larger area which results in greater variation in sample topography. Since IC-AFM has overcome the limitations of the other two modes of AFM, it has become an important AFM technique. A multi mode scanning probe microscope (Solver Pro, M/s NT-MDT, Netherlands) was used in this study to measure the surface roughness of uncoated PyCs and AgNPs thin films and CuNPs coated PyCs at various ablations pulses ranging from 5,000 to 15,000. The cantilever used is a Au coated Si with force constant ~11 N/m and having a tip radius ~ 10 nm. Prior to acquiring surface roughness measurements, the system and scanner were calibrated using standard grids with periodic structure.

Figure 2.7 Scheme showing the main components of Atomic Force Microscope(http://www.nanoparticles.pacificnanotech.com)
Most AFMs currently in use will detect the position of the cantilever with optical techniques. In the most common scheme a laser beam bounces back of the cantilever onto a position-sensitive photo detector (generally a four segment PSPD). When the cantilever starts to bend the position of the laser beam on the detector also shifts. The PSPD itself can measure displacements light as small as even upto 10 Å. The ratio of the path length between the cantilever and the detector to length of the cantilever will produces a mechanical amplification. As a result, the system can detect sub-angstrom vertical movement of the cantilever tip. The basic schematic showing the primary components of the light lever atomic force microscope is shown in Fig. 2.5. A multi mode scanning probe microscope (Solver Pro, M/s NT-MDT, Netherlands) was used in this study. The cantilever used is Au coated Si with force constant ~11 N/m and having a tip radius ~ 10 nm. Prior to acquiring specimen topography, the system and scanner were calibrated using standard grids with periodic structure.

2.4 ANTIMICROBIAL STUDIES

Antimicrobial activity of silver and copper nanoparticles synthesized by pulsed laser deposition was evaluated by standard Zone of Inhibition (ZOI) microbiology assay.

2.4.1 Culturing of Bacteria

The bacterial organisms chosen for the present study included the gram positive bacteria namely Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, (Methicillin resistant Staphylococcus aureus (MRSA) and gram negative Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa and Proteus vulgaris. The inoculation of the culture was made with the inoculation loop by back and forth streaking on nutrient agar surface. Individual colonies formed from the biomass upon incubation were isolated aseptically and transferred into different nutrient agar slant tubes. Then the tubes were incubated at 37°C overnight and the pure cultures of organisms were obtained.
2.4.2 Culturing of fungi

The fungal organisms chosen for the present study included *Candida albicans* and *Aspergillus niger*. The inoculation of the culture is made with an inoculation loop by back and forth streaking on solid rose bengal agar/potato dextrose agar surface. Individual colonies formed from the biomass upon incubation were isolated aseptically and transferred into different nutrient agar slant tubes. Then the tubes were incubated at 37°C overnight and the pure cultures of organisms were obtained.

2.4.3 Zone of inhibition studies for bacteria

Antibacterial activities of silver and copper nanoparticles synthesized PLD were evaluated by standard Zone of Inhibition (ZOI) microbiology assay. The culture medium used for antibacterial tests was Muller Hinton (HA) agar. Mueller-Hinton agar contains beef extract powder, an acid digest of casein, soluble starch and agar. For inoculation of culture plates each bacterium was swabbed uniformly using a sterile cotton swab. The test samples consisted of PyC samples coated with AgNPs and PyC samples coated with CuNPs. Uncoated PyC served as controls. All samples were placed in the plates containing the test bacteria. The culture plates were then incubated at 37°C for 24 hrs and observed for the ZOI formation.

2.4.4 Zone of inhibition studies for fungi

Antifungal activities of silver and copper nanoparticles synthesized by PLD were evaluated by standard ZOI microbiology assay. The culture medium used for antifungal tests was Rose Bengal/ Potato Dextrose agar. For inoculation of culture plates each fungus was swabbed uniformly using a sterile cotton swab on the culture plates. Test samples which consisted of PyC samples coated with AgNPs and PyC samples coated with CuNPs and control samples which consisted of uncoated PyCs were placed in the plates containing the test fungus. The culture plates were then incubated at 37°C for 24 hrs and observed for the ZOI formation.
2.5 HAEMOCOMPATIBILITY STUDIES

This study was cleared by Institutional ethical committee and informed consent was obtained from the participants in the study. Human healthy male and female volunteers in the age between 22 to 40 years were enrolled in this study. The hemocompatibility of thin films of AgNPs and CuNPs coated PyCs was tested by measuring the Clotting time (CT), thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (aPTT). The haemocompatibility was also assessed by the anti–coagulation assay and platelet adhesion studies. The results obtained for the test samples were compared with that of uncoated controls.

2.5.1 Lee and White tube inversion method for clotting time estimation

The clotting time was determined by Lee and White tube inversion method. For this five different test tubes (pre warmed) containing AgNPs or CuNPs coated PyC test samples prepared at five different ablation pulses ranging from 5,000 to 15,000 pulses were taken. Then 1ml of blood was collected by vein puncture under aseptic conditions into each glass tube containing the coated test sample. A pre warmed glass tube into which 1 ml of blood was collected in a similar manner and contained the uncoated PyC sample served as control for comparison. Another test tube into which plain blood was collected in the similar manner but didn’t contain any PyC samples served as another control. Then all the tubes were heated at 37°C in a water bath and tilted once in every 30 seconds till blood clots were formed.

2.5.2 Thrombin time (TT)

For TT measurement, plasma was separated from 0.5 ml of blood by centrifuging the blood sample for 15mins at 1500 rpm. Then 200 μ L of plasma was transferred into each test tube. Five different test tubes (pre
warmed) containing AgNPs or CuNPs coated PyC test samples prepared at five different ablation pulses ranging from 5,000 to 15,000 pulses served as test samples. Test tube containing 200 μL of plasma and uncoated PyC sample served as control for comparison. Another test tube into which plasma was collected in a similar manner without any sample served as another control. Then, to all the test tubes 200 μL of the thrombin reagent was added and the tubes were tilted back and forth till the clot was formed. The time taken for the formation of fibrin clots were formed in all the test tubes was noted.

2.5.3 Prothrombin time (PT)

For PT measurement, plasma was separated from 0.5 ml of blood by centrifuging the blood sample for 15mins at 1500 rpm. Then 200 μL of plasma was transferred into each test tube. Five different test tubes (pre warmed) containing AgNPs or CuNPs coated PyC test samples prepared at five different ablation pulses ranging from 5,000 to 15,000 pulses served as test samples. Test tube containing 200 μL of plasma and uncoated PyC sample served as control for comparison. Another test tube into which plasma was collected in a similar manner without any sample served as another control. All tubes were incubated for 120s at 37°C. Then 200 μL of PT reagent was added to each test tube and incubated for 120s at 37°C. The time taken for the formation of the fibrin clot in all the test tubes was noted.

2.5.4 Activated partial thromboplastin time (aPTT)

For aPTT measurement, plasma was separated from 0.5 ml of blood by centrifuging the blood sample for 15mins at 1500 rpm. Then 200 μL of plasma was transferred into each test tube. Five different test tubes (pre warmed) containing AgNPs or CuNPs coated PyC test samples prepared at five different ablation pulses ranging from 5,000 to 15,000 pulses served as test samples. Test tube containing 200 μL of plasma and uncoated PyC
sample served as control for comparison. Another test tube into which plasma was collected in a similar manner without any sample served as another control. All tubes were incubated for 120s at 37°C. Then 200 μL of aPTT reagent was added to each test tube and incubated for 200s at 37°C. This was followed by the addition of 200μL of CaCl₂ to each tube for the initiation for intrinsic clotting cascade. The time taken for the formation of the fibrin clot in all the test tubes was noted.

2.5.5 Anti – coagulation assay

In this method, 3 ml of blood was collected by vein puncture under aseptic conditions into a pre warmed glass tube without any anticoagulant. Five different test tubes containing AgNPs or CuNPs coated PyC test samples prepared at five different ablation pulses ranging from 5,000 to 15,000 pulses served as test samples. A pre warmed glass tube into which 3 ml of blood was collected in a similar manner and contained the uncoated PyC sample served as control for comparison. Another test tube into which blood was collected in a similar manner without any sample served as another control. The anti – coagulant effect of AgNPs or CuNPs coated PyCs were evaluated by noting the time taken for the clot formation.

2.5.6 Platelet adhesion studies

Platelet adhesion study was performed to investigate the adhesion of platelets on control PyC and PyCs coated with thin film of AgNPs and CuNPs prepared at 7,500 and 10,000 ablation pulses. For this blood was drawn from a healthy adult volunteer and was treated with sodium citrate to prevent coagulation. It was then centrifuged at 1000 rpm for 5 min and the platelet rich plasma was separated from red blood cells and incubated at 37 °C for 10 min. To the 20 μl of the platelet rich plasma, 20 μl of thrombin reagent was added and placed at 37°C for 10min. The samples were then rinsed with 0.9% saline solution to remove any weakly adhered platelets from the
samples. Washed fibrin clots were fixed in 2.5% glutaraldehyde in PBS maintained at a pH of 7.4 and further rinsed with PBS for the removal of loosely adhering platelets. The platelet rich plasma treated films were then examined for platelet adhesion and thrombus formation using a field emission scanning electron microscope.

2.6 CYTOTOXIC STUDIES

2.6.1 Cell Culture preparation

Reagents such as, Minimal Essential Media (MEM), Fetal bovine serum (FBS), Trypsin, Roswell Park Memorial Institute medium (RPMI), methylthiazolyl diphenyl- tetrazolium bromide (MTT) were used for culturing of fibroblast and PBM cells.

2.6.2 Culturing of Fibroblast cells

Ten day old embryonated eggs were selected after candling and the shell was cleaned with tincture of iodine or 70% alcohol. The shell over the air sac area was ruptured to cut open the shell membrane and extra embryonic membranes. Curved forceps was then inserted below the neck of the embryo and the embryo was lifted gently. The lifted embryo was placed in a Petri dish containing Minimal essential medium (MEM) and the embryo was washed 2 to 3 times. The head and limbs were carefully cut and removed. The internal organs were eviscerated and removed, and then the embryo was washed again in MEM. The embryo was transferred to a 100ml beaker which was then chopped to small pieces. Again the minced embryo was washed with MEM. Minced embryo was transferred to a trypsinization flask and 0.25% trypsin (5% trypsin stock diluted in PBS) was added at the rate of 5 ml per embryo which was then incubated for 10-15 min with intermittent shaking. The supernatant was carefully removed and discarded. Trypsin was added again and incubated for 20-30mins with intermittent shaking. Supernatant was
removed and kept at 4°C after adding calf serum to a final concentration of 5%. This procedure was repeated and the supernatant was collected. The collected supernatant was pooled and kept at 4°C for 10mins. The cell suspension was then passed through a stainless steel mesh filter with a mesh size of 80-150μm and further centrifuged at 800-1000 rpm for 10min at 4°C. The cells were suspended in a growth medium and the cell concentration was adjusted to 1x 10⁶ cells /ml. The cell suspension was passed into muslin cloth covered beaker to remove cell clumps and they were seeded into culture vessels. The culture vessels were then incubated at 37°C and examine at every 24 hrs for the formation of monolayer.

2.6.3 Culturing of PBM cells

8 ml of blood was collected from a healthy volunteer. 3 ml of alcevors solution was added to 8ml of blood and mixed well which was overlayered with 4 ml HiSep. The mixture was centrifuged at 1600 rpm for 20 minutes. Buffy coat (PBMc) was carefully aspirated and added to a fresh tube containing 5 ml RPMI medium. This was further centrifuged at 1600 rpm for 10 minutes. Supernatant was removed and the pellet was resuspended in 4 ml RPMI medium + 1ml serum + antibiotic solution. 1.5 ml was added to each well of six well plates.

2.6.4 Counting of the isolated cells and primary culturing

To determine the cell number during the growth curve experiments cells were plated at an initial density of 5,000 cells per well and counted using haemocytometer at the specific time point up to eight days. Each grid square of the haemocytometer slide represents a volume of 10⁷ µl and the cells were counted in 10 squares in 1 ml of the cell suspension. Each trial had triplicates and was conducted three times. Cell suspensions were dilute enough so that the cells did not aggregate.
The primary culture was taken out from CO\textsubscript{2} incubator. The cell plate was immediately placed in the biosafety hood. The cells were cleaved of the plate from trypsin. After 80% confluence, the cells had to be cleaved. The cell plates were put in the incubator for about 5mins. The cell dislodgement was checked under microscope to make sure that all of the cells have come off the plates by observing the cells floating and moving in the solution. 5 ml of the MEM with FBS was then added to the plates and the cell suspension was collected into 15 ml centrifuge tubes. The cells were then centrifuged at room temperature for 5mins. The cell pellet was then resuspended in MEM medium containing 5% FBS. This was then transferred to six well culture plates. The amount of cells present was checked under a microscope with 40x objectives. The plates were then incubated maintained at 5% CO\textsubscript{2} at 37 \textdegree C for the cells to grow. The live cells grow within 24-48 h. After that period the plates were taken out from the incubator. The cells were then used for the study.

2.6.5 \textit{In vitro} cytotoxic assessment of silver and copper thin films coated PyC and control PyC by MTT Assay

Human peripheral mononuclear cells (PBMc) and chick fibroblast cells were seeded separately into 96 well plates at a density of $1 \times 10^6$ cells/well in 200 µl culture medium (MEM medium for fibroblast cells and RPMI medium for PBM cells) and allowed to form a monolayer/attach to the plates for 12 hours at 5% CO\textsubscript{2} incubator before treatment. After the incubation period, the culture medium was replaced by Finally, 20µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added to each wells in order to determine the viable cells for the photo toxicity assessment. After 4hrs of incubation, viable cells were determined by reading the absorbance at 540nm. The effect of the samples on % cell viability is calculated using the following formula:

$$\text{% cell viability } = \left( \frac{\text{treated cells}}{\text{control cells}} \right) \times 100\%$$
2.6.6 Optical microscopy studies of silver and copper thin films coated PyC and control PyC treated with cell lines

The interactions of AgNPs coated PyCs and CuNPs coated PyCs with chick fibroblast and human PBM cells was studied using an inverted optical microscopy. Both cell lines were seeded at a density of 1 x 10^6 cells/ml in six well plates. The plates seeded with cells were kept at 37°C in a 5% CO₂ incubator for a time period of 24 hours. After 24 hours of cell attachment the plates containing chick fibroblast cells as well as the plates containing PBM cells were washed with PBS twice and exposed to PyC samples coated with AgNPs at 7,500 and 10,000 ablation pulses. Similarly another set of plates were exposed to PyC samples coated with CuNPs thin films prepared at 7,500 and 10,000 ablation pulses. Uncoated PyC samples served as controls. After the exposure period the test as well as the control plates was removed from the incubator and the cells were washed twice with PBS. Then the cells were imaged using a Nikon Eclipse TS100 inverted microscope. All images were recorded using a Nikon ELWD 0.3/OD75 camera.

2.7.7 Live and dead cell staining of cells treated with silver and copper thin films coated PyC and uncoated PyC samples

The live and dead cell staining was performed by carrying out epifluorescence microscopy study, a simple technique to quantify the amount of dead and viable cells using acridine orange-ethidium bromide staining. Acridine orange will be taken up by viable as well as dead cells giving green fluorescence. The green fluorescence is due to the fact they bound to double stranded DNA in living cells whereas, red fluorescence is the result of binding to single stranded DNA which is dominant in dead cells. 1x10^6 cells of fibroblast and PBM were separately seeded into six well plates and incubated for 24h maintained at 37°C with 5% CO₂ atmosphere. The thin films of AgNPs and CuNPs coated PyCs prepared at two different ablation pulses
namely 7,500 and 10,000 were treated with chick fibroblast and human PBM cells and incubated for 10h. After incubation period, the samples treated with cells are detached with 0.25% trypsin-EDTA and washed with phosphate buffer saline. Fifteen microlitres of both the cells for two different ablation pulses were placed separately on a pre warmed clean glass slide. The cells were then mixed with 10 ml of acridine orange (100 mg/ml) and ethidium bromide (100 mg/ml). The cells were viewed under a Nikon Eclipse E600 epifluorescence microscope with 200x magnifications.