3. THE MATERIALS AND METHODS

3.1. SELECTION OF THE SUBSTRATE

The substrate material is selected based on its application as orthopaedic implants. The material must be load bearing in nature. The application of ceramic to the metal surface utilizes the load bearing capability of the metal as well as the biocompatibility of the ceramic [1, 2]. Titanium and its alloys are widely used in load bearing orthopaedic and dental implants [3] due to its superior biocompatibility [4, 5]. Stainless steel is extensively used for load bearing application due to its ease of fabrication and desirable mechanical properties even though it is somewhat corrosive in the body environment [6, 7].

3.1.1. Titanium

Titanium is the working electrode, for the cathodic electrodeposition. Commercially available titanium of grade T-3190 having the dimension 1 x 1 x 0.2 cm$^3$ is used for the present study.

3.1.2. Stainless Steel

Commercially available stainless steel (316L SS) specimens of size 5 x 3 x 0.2 cm$^3$, having the composition Fe, + [Cr: 18.00, Ni: 12.00, Mo: 2.50, Mn: 1.70, P: 0.04, C: 0.02, S: 0.01, Si: 0.15 (in wt. %)] were used as the substrate in the present study.
3.2. STANDARDIZATION OF THE SUBSTRATE

The Ti of grade T-3190 is suitable for further pre-treatment and coating. The 316L SS is very much suitable for implantation purpose. The two substrate materials could be processed easily.

3.3. PRE-TREATMENT OF THE SUBSTRATE

The substrate, both Ti and SS were subjected to pre-treatment. Adhesion of the HA coating to the substrate get improved by pre-treatment. Different pre-treatment methods were applied for Ti and SS. In the case of SS, pre-treatment is different for the two processes adopted in the present study- two interlayer coatings- HA/Ni-P and ZnP coating. Usually metallic substrate had certain demerits [8, 9], which were overcome by the pre-treatment methods. The adhesion of the coating to the metal substrate got improved by the pre-treatments.

3.3.1. Pre-treatment of titanium

The titanium substrate is polished with 600-grit emery paper and cleaned with distilled water. The coatings were immersed in 5% hydrofluoric acid for 1 minute and then rinsed with distilled water and dried in air. The process removes any native surface oxide layer.

Anodic oxidation was carried out for 2 h in 0.5 M NaOH by applying a constant potential of 12 V. In the anodic oxidation process Ti substrate is used as the anode and SS is used as cathode. After the oxidation process the substrate surface is washed with distilled water and dried.
3.3.2. Pre-treatment of stainless steel

The SS substrate exhibited very low adherence without pre-treatment. The pre-treatment method is as follows. The specimens were abraded using 100-grit SiC paper, degreased using 5% NaOH solution at 50 ± 1°C and then etched in a mixed acid solution of HNO₃ (150 g/L) and HF (50g/L) for 5 minutes, at a temperature of 28 ± 1°C to ensure that the surface was free from any superficial oxides [10].

3.3.2.1. Electroless nickel plating

i) Pre-treatment

Acid cleaned substrate is activated by Wood’s nickel strike [ASTM B 656]. Wood’s nickel strike comprises 240 g/L nickel chloride hexahydrate (NiCl₂.6H₂O) and 40 g/L concentrated HCl. The activation was carried out at a temperature of 75°C for 2-5 minute.

ii) The plating process

The electroless bath comprises of 30 g/L nickel sulphate heptahydrate (Spectrochem, 99%), 25 g/L sodiumhypophosphite (Spectrochem, 99%) and 25 g/L succinic acid (NICE, 99%). The temperature of the bath is 80 ± 1°C and the deposition is carried out for 2 h. The pH of the bath is adjusted to 4.5 by the addition of concentrated NH₄OH [11]. Hydroxypatite (HA) particles were added (0 to 100 g/L) during the electroless deposition process with constant stirring.

3.3.2.2. Hot-dip galvanization

i) Pre-treatment

Acid cleaned SS is washed in distilled water and dried. The coupons were then dipped in 30% NH₄Cl solution for 30 minutes at 50 ± 1°C to avoid further
surface oxidation and to enhance the adhesion of the molten metal onto the substrate surface during the hot dipping process.

ii) Hot-dip galvanization

The required amount of zinc metal ingot (Binani, India, assay 99.9%) was melted in a graphite crucible kept at 450 ± 5°C in a muffle furnace. The pre-heated SS coupons were then dipped in the bath for about 10 – 15 S. The excess zinc on the surface of the coupons was removed by blowing hot air while withdrawing the strips from the bath. Then the coupons were subjected for conversion coating.

iii) Conversion coating—phosphating

The zinc coated steel coupons were degreased with trichloro ethylene and then pickled in 2.5% trisodium phosphate at 75°C for 10 minutes, followed by rinsing with running water and then distilled water. The surface was etched in 2% H$_2$SO$_4$ for one minute at room temperature and then subjected for phosphating in a bath of composition, ZnO: 5 g/L, H$_3$PO$_4$: 12 mL/L, NaNO$_3$: 6 g/L, and NaF: 0.3 g/L, by immersing the coupons at a temperature of 28 ± 1°C for 30 minutes [12]. After phosphating, the coupons were washed in distilled water and dried at 50-60°C.

3.4. PREPARATION OF HYDROXYAPATITE POWDER

3.4.1. Preparation

The HA powder was prepared by wet-chemical precipitation method. 1.0 M calciumhydroxide is dispersed in distilled water. The solution is vigorously stirred and then 0.6 M orthophosphoric acid is added drop wise into it. The pH of the bath
is kept constant at 10-11 by adding ammonium hydroxide solution. The gelatinous precipitate obtained is stirred vigorously and aged for 20-30 h. The precipitate is separated by filtration. The precipitate is dried at 65-70°C and then calcined at 750-850°C for 2-4 h. The calcined mass is powdered finely and used for incorporation into the electroless nickel plating bath [13].

3.4.2. Characterization of the powder

3.4.2.1. X-ray diffraction analysis

The powder is subjected to X-ray diffraction analysis using X’pert Pro analyzer, The Netherlands. The powder was finely powdered and subjected to XRD analysis using Cu Kα radiation. The composition of the powder was determined based on the standard JCPDS value- 9-432.

3.4.2.2. TEM analysis

The particle size of the prepared HA powder is determined by transmission electron microscopic analysis. TEM analysis was carried out using JEOL TEM instrument after powdering the sample by ultrasonic powdering method.

3.5. Electrodeposition of the coating

3.5.1. Electrodeposition process

Anodically oxidized Ti coupons were the substrate (cathode) for the electrodeposition process. A Pt foil having large surface area was used as the cathode. Ag/AgCl was the reference electrode. The electrolyte was a mixed salt solution of calcium nitrate (Ca(NO₃)₂.4H₂O, 0.084 M) and ammonium dihydrogen phosphate (NH₄H₂PO₄, 0.050 M) having the Ca/P ratio of 1.67. The pH of the bath
was adjusted to 4.5 by adding dilute ammonium hydroxide solution. Temperature of the bath was kept at 65 ± 1°C [14].

3.5.2. Variation of throwing power

The electrodeposition was conducted by varying the deposition parameters. The current density viz: 1, 5 and 10 mA/cm$^2$ is selected. The resultant potential of the working electrode were 1.4, 5.2 and 10.6 V respectively with respect to saturated calomel electrode. The inter electrode distance was kept at three different levels viz: 3, 6 and 12 cm. The potential variation of working electrode during electrodeposition was continuously monitored.

3.5.3. Clay incorporation

Clay particles were incorporated into the electrolytic bath during the electrodeposition process. The electrolyte was stirred vigorously during the electrodeposition process. Montmorillonite (Mont K-10, Sigma Aldrich) was incorporated into the coating in different amounts (0 to 1%).

3.5.4. Alkaline post treatment

The calcium phosphate coating deposited during the electrodeposition process contains less stable phases which are less biocompatible than hydroxyapatite. In order to make the coating biocompatible the calcium phosphate coating was treated in 0.1 M NaOH solution at 60°C for two days [15].
3.6. CHARACTERIZATION OF THE COATING

The composition, surface morphology, stability, corrosion resistance, bioactivity and behaviour in aggressive physiological solution of the electrodeposited coating were evaluated in detail.

3.6.1. Physicochemical characterization

3.6.1.1. Porosity of the inter layers and coating

a) Porosity of electroless nickel-phosphorous interlayer was evaluated by ferroxyl reagent test. Ferroxyl reagent is a solution of potassium ferricyanide, sodium chloride and agar agar in hot water. Reagent is applied on the surface of the coating and looked for prussian blue colouration. The prussian blue colouration indicates the exposure of the steel surface to the environment.

b) Porosity of the hot-dip galvanized layer: Normal ferroxyl test is not suitable for zinc coating, hence modified ferroxyl reagent test is selected for the determination of porosity [16]. An external anodic potential of 0.400 V was impressed on the coupons. Ferroxyl reagent was applied on the surface of the coupons for 30 minutes. The appearance of any prussian blue colouration indicates the exposure of steel coupons towards the environment.

c) Porosity of calcium phosphate coatings

The porosity of the electrodeposited coating was evaluated from the surface morphology of the coating.

3.6.1.2. Evaluation of the adherence of the coating

a) Hydroxyapatite coated coupons were scratched with a nylon brush and it was immersed in distilled water for 1 hour. The variation in open circuit potential
was then monitored and from this any exposure of the substrate towards the environment was determined.

The HA coated coupons were scratched with graphite pencils (H, HB and 2B) and then immersed in distilled water for 1 hour. Then the surface potential variation was analyzed by immersing the coatings in 0.9 % sodium chloride solution. The potential was determined with respect to saturated calomel electrode.

The HA coating was subjected to very small anodic current, since the coating after implantation was exposed to the aggressive body condition and stray currents in the body. The coatings were subjected to 0.5 mA/cm$^2$ anodic current for 1 h and then the potential variation was evaluated in physiological solution with reference to the saturated calomel electrode (S. C. E).

3.6.1.3. Evaluation of the thickness of the coating

a) The thickness of the coating formed on the inter layer was evaluated by scanning electron microscope.

b) The thickness of the coatings were evaluated based on the equation

$$T = \frac{\Delta w}{A \times \rho}$$

$T =$ thickness, $\Delta w =$ weight of the deposited plate (calculated based on the weight difference of the coating before and after deposition), $A =$ area of the plate and $\rho =$ density of the coating.
3.6.2. Surface morphology of the coating

The surface morphology determines the bio activity of the developed coating [17]. The surface morphology was evaluated by optical micrograph as well as SEM.

3.6.2.1. Optical micrograph

The electrodeposited coatings were washed in distilled water, acetone and then dried in air. The surface morphology of the coating was analyzed by optical micrograph (Olympus SZ 61, Taiwan).

3.6.2.2. Scanning electron micrograph

Strips having the surface area of 1 cm$^2$ were cut from the coated coupons. The coatings were washed in distilled water, dried in air and then the surface was gold coated using Polaron SC 7620 sputter coater. The gold coating was carried out in order to make the surface conductive during the analysis and to avoid any surface charging effect. Then the surface morphology was analyzed by scanning electron microscope. Both the plane surface and the cross sectional surface were evaluated.

3.6.2.3. Atomic force micrograph

The topographic analysis of the samples was performed using a commercial atomic force microscope (AFM, molecular imaging, USA, Picoscan 2100) instrument in contact mode. A phosphate (n) doped silicone coated cantilever (force constant- 0.05-3 N/m) was used for imaging. The height mode was used to scan the surface. The system automatically keeps the force and hence the distance between the tip and the surface remain constant. The AFM images were recorded over a scan area of 1×1 µm$^2$ and 5×5 µm$^2$ respectively. The AFM images were
displayed in the way, lower and highest regions are recorded in variations of colour intensity. The coating surface was cleaned with acetone and then dried before the analysis.

3.6.3. Composition of the coating

3.6.3.1. X-ray diffraction analysis

The composition of the coating was evaluated using X ray diffraction analysis using Cu Kα radiation (X’pert pro analyzer, The Netherlands). The coatings were rinsed with distilled water, acetone and then dried in air before the XRD analysis.

3.6.3.2. EDX analysis

The composition of the coating had a significant role in the bio activity of the coating [18]. The elemental composition of the coating was determined by EDX analysis (EDAX, Oxford make).

3.6.4. Study of the corrosion behaviour and stability of the coating

3.6.4.1. Electrolytes used for the evaluation

a) Physiological saline solution

The corrosion behaviour of the biologically active coatings was normally determined in 0.9% sodium chloride solution, known as physiological saline solution [4]. It was prepared by dissolving 0.9 g sodium chloride in 100 mL distilled water.

b) Ringer’s physiological solution

The corrosion behaviour of the biological specimens was also evaluated in
Ringer's physiological saline solution having the composition: sodium chloride-8.6 g/L, calcium chloride dihydrate- 0.66 g/L and potassium chloride- 0.6 g/L [19].

3.6.4.2. Open circuit potential evaluation

The corrosion resistance tendency was evaluated by the measurement of the open circuit potential (O. C. P.) i.e., the equilibrium potential of the anode when it is in contact with the electrolyte. It signifies the tendency of the metal to corrode and change its potential with time without the application of an external current and can relate to the important phenomena occurring at the metal surface.

The corrosion resistance characteristics of the HA coated ZnP coatings were evaluated in 0.9 % NaCl solution. The trend of the variation in O. C. P. was recorded for a period of two weeks at a temperature of 28 ± 1°C. A saturated calomel electrode was used as the reference electrode.

3.6.4.3. Anodic polarization

The potential variation of the coated substrate on the application of an anodic current was evaluated in stagnant Ringer's solution (50 mL) using a potentiostat of BAS, U. S. A. The temperature of the electrolyte was kept at 37 ± 1°C. 1 cm² exposed area of the coated substrate was the working electrode, a platinum mesh was the counter electrode and SCE was the reference electrode. The potential variation was noted for a current density from 0 to 2.5 mA/cm².

3.6.4.4. Cyclic voltammetric analysis

The current- voltage characteristics at the different location of the coatings were evaluated in 0.9 % sodium chloride solution at 37 ± 1°C using a potentiostat of BAS, U. S. A. 1 cm² exposed area of the coupons were washed in acetone, dried and used as the working electrode. A Pt mesh was the counter electrode and
Ag/AgCl was the reference electrode. The electrode was kept in 0.9% sodium chloride solution for 55 minutes to reach the constant O. C. P. value. The I-V characteristics evaluation was carried out at a scan rate of $0.1 \times 10^{-3}$ mA/S. Anodic current density varied from 0 to $5 \times 10^{-3}$ mA/cm$^2$ for 2$^{nd}$ and 10$^{th}$ consecutive cycles. The electrolyte was not renewed for the overall analysis period of each sample. No visible breakdown of the coating was observed during the initial scan period. On repeating the scan cycles some visible mass loss was observed.

3.6.4.5. Electrochemical impedance spectroscopic analysis

Electrochemical impedance spectroscopic analysis was widely used to evaluate the corrosion resistance of biomaterials [20, 21]. EIS study was carried out using an AUTOLAB PGSTAT 30 with FRA2 software of FRA version 4.9. The equivalent circuit is a two layer model for the surface film [22]. The equivalent circuit used for the present study is shown below.

![Equivalent circuit](image)

**Fig. 3.1.** The equivalent circuits used for the EIS analysis two layer model for a) porous surface film and b) sealed porous surface film.
Where $R_e$ is the solution resistance of the test electrolyte and electric leads. $C_p$ is the capacitance of the coating, $R_p$ is the charge transfer resistance associated with the penetration of the electrolyte through the pores or pinholes in the coating, $R_b$ is the polarization resistance, $C_b$ is the capacitance at the substrate/electrolyte interface, $C_{ho}$ & $R_{ho}$ were the capacitance/resistance of the hydrates/precipitates on the surface. The values obtained based on this equivalent circuit closely fit to the experimental values. The electrolyte used for this EIS analysis was Ringer’s physiological solution. 1 cm$^2$ exposed area of the coating, Pt, Ag/AgCl were used as the working, counter and reference electrode respectively. Frequency ranged from 1 MHz to 10 Hz. The analysis was carried out after attaining a constant O.C. P.

3.6.4.6. Evaluation of the deposition potential and rate of deposition

i) The deposition potential was evaluated during the electrodeposition process. The working electrode was taken as the cathode and saturated calomel electrode as the anode and the potential variation during the electrodeposition process was monitored.

ii) Rate of deposition

The deposition rate was evaluated by monitoring the weight difference of the coating after various periods of deposition. Then a graph was plotted with the deposit weight against time. From this the rate of deposition of calcium phosphate coating was obtained.

3.6.5. In vitro characterization

Bioactivity of the developed coatings was evaluated by soaking the coatings in simulated body fluid.
3.6.5.1. Simulated Body Fluid (S. B. F)

Simulated body fluid is a solution having the composition similar to human blood plasma. The composition of the body fluid is indicated in the Table 2.1. The reagents used for preparing S. B. F. are shown in Table 2.2.

Table 2.1. The composition of the simulated body fluid in comparison to human blood plasma

<table>
<thead>
<tr>
<th>Ion</th>
<th>S. B. F.</th>
<th>Human blood plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>142.0</td>
<td>142.0</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>148.8</td>
<td>103.0</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>4.2</td>
<td>27.0</td>
</tr>
<tr>
<td>HPO₄²⁻</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2.2. Chemicals used for preparing simulated body fluid

<table>
<thead>
<tr>
<th>Sl. No:</th>
<th>Reagent</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>7.996</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>0.350</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.224</td>
</tr>
<tr>
<td>4</td>
<td>K₂HPO₄·3H₂O</td>
<td>0.228</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂·6H₂O</td>
<td>0.305</td>
</tr>
</tbody>
</table>
3.6.5.2. Biomimetic evaluation

S. B. F was prepared by the following method

1. The bottles were washed with 1 N HCl, ion exchanged distilled water and dried.

2. 500 mL distilled water is put in a covered polyethylene bottle.

3. The reagents were added one by one while stirring with a magnetic stirrer.

4. Temperature of the bath is kept at 37 ± 1°C.

5. The pH is adjusted to 7.40 by adding HCl.

6. The solution is then made up to the required volume.

7. The solution is kept at 5-10°C in a refrigerator.

The coatings were soaked in S. B. F. for 14 days. The S. B. F. is refreshed in every day and sealed to remain sterile [23, 24]. The biomimetic growth was evaluated by the surface morphological analysis. The HA growth was evaluated by optical microscope and SEM. The variation of surface potential during the biomimetic growth was evaluated with reference to S. C. E.

3.6.6. Characterization under special conditions

3.6.6.1. In aggressive physiological media

The pH of the Ringer’s solution was changed to acidic one. Initial pH 6.74 of the Ringer’s solution was changed to 4.5 by adding a drop of 0.1 M HCl. A pH of 5 mimics the acidic body environment during the early inflammation period.
The coatings were immersed in the acidic Ringer's solution for 2, 4, 6 & 8 days. The variation in O. C. P. was noted with respect to S. C. E. Surface morphology was also evaluated.

3.6.6.2. Re growth in simulated body fluid

The destructed coatings were analyzed for re growth characteristics in S. B. F. for 14 days at 37 ± 1°C. The variation in O. C. P. was analyzed during the re growth process. After re growth the surface morphology of the coating was evaluated.

3.6.6.3. Evaluation of the release of harmful metal ions

The release of Zinc ions into the S. B. F. solution was evaluated by Atomic absorption spectroscopic analysis (AAS, GBC Aventa Absorption Spectrophotometer). 1 mL of the solution is put in the corresponding flame. Absorbance value and the concentration of zinc ions were recorded.

3.7. REFERENCES


