MATERIALS & METHODS
4. MATERIALS AND METHODS

This section contains the details of the experimental procedures adopted for the synthesis of nanohydroxyapatite powders, passivation of 316L stainless steel (SS) and coatings of HAP over SS surface. The techniques used for characterizing the powders and alloy substrates and their operating conditions are also furnished.

4.1 Synthesis and characterization of nanohydroxyapatite powders

4.1.1 Synthesis of nanohydroxyapatite by conventional and microwave coupled hydrothermal method

In the conventional hydrothermal synthesis, analytical grade calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$.4H$_2$O), Merck, India) and dipotassium hydrogen phosphate (K$_2$HPO$_4$.3H$_2$O, Merck, India) were used for preparing calcium and phosphate precursor solutions respectively. The precursor solutions were prepared in double distilled water and the stoichiometric ratio was maintained at 1.67. The pH of the solution was adjusted to 12 by adding ammonia solution. The resulting suspension was allowed under constant magnetic stirring for an hour and then the contents were sealed in Teflon container heated at 60-100 °C. The white precipitate was washed with double distilled water and ethanol followed by drying in air oven. The resulting powder was then calcined and sintered at different temperatures to obtain pure nano-HAP powders.

In the microwave coupled method the suspension obtained after mixing the precursor solutions were placed in a microwave oven (2.45 GHz, 900 W,
LG, India) and irradiated at different microwave powers (720 & 900 W) for 30 min. After allowing the contents to be cooled the white precipitate formed is washed several times with double distilled water, dried, then calcined and sintered. In the surfactant assisted microwave synthesis, 50 ml solution of 0.024 M $K_2HPO_4 \cdot 3H_2O$ and an appropriate amount of CTAB was added and stirred at room temperature for 30 min to make ensure cooperative interaction and the make self assembling process complete.

![Flow chart for conventional and microwave coupled hydrothermal synthesis.](image)

**Fig. 4.1.** Flow chart for conventional and microwave coupled hydrothermal synthesis.
Then 50 ml of calcium nitrate tetrahydrate solution (0.04 M) was introduced dropwise and the resulting mixture was stirred thoroughly with magnetic stirring. Finally the pH of the solution was adjusted to 12 by adding ammonia solution. A white suspension thus obtained was then pushed in to a microwave oven (2.45 GHz, 900 W, LG, India) for continuous heating for 30 min. The resultant white precipitate was thoroughly washed with distilled water to remove the residual CTAB and then dried at 120 °C for 12 hours followed by calcination at different temperatures. The obtained nano-HAP powders were characterized by adequate sophisticated analytical techniques.

4.1.2 Synthesis of nanohydroxyapatite by conventional and ultrasonic coupled sol-gel method

The procedure adopted for synthesizing nanohydroxyapatite through conventional sol-gel synthesis is furnished as follows:

For the synthesis of hydroxyapatite, calcium nitrate tetrahydrate (Ca(NO₃)₂. 4H₂O), diammonium hydrogen phosphate ((NH₄)₂HPO₄) were used as precursors for calcium and phosphorus respectively. All the chemicals used were of analytical grade and the solutions were prepared using double distilled water.

In a typical synthesis procedure, diammonium hydrogen phosphate was dissolved in 1:1 ethanol/double distilled water mixture and a stoichiometric amount of dissolved calcium nitrate was subsequently added dropwise into the hydrolyzed phosphate sol with continuous stirring for 1 h at 60° C. The two solutions were mixed in a Ca/P molar ratio of 1.67. The as-formed gel was
aged for 16 h at 60 °C, then washed with double distilled water, dried and sintered at 300, 600 and 900 °C in a muffle furnace for 4 h. In ultrasonically coupled synthesis, the gel formed by mixing the precursor solutions was allowed for ultrasonic agitation for 1 h at 60 °C. The resultant white precipitate which was washed, dried and sintered at 300, 600 and 900 °C for 4 h to obtain nano-HAP powders.

Fig. 4.2. Flow chart for conventional and ultrasonic coupled sol-gel synthesis.
4.1.3 Characterization of nanohydroxyapatite powders

4.1.3.1 Fourier transform infra-red spectroscopic (FT-IR) studies

The nanohydroxyapatite powders obtained by both the synthetic routes were characterized by Fourier transform infra-red spectrometer (Nicolet 380 FT-IR spectrophotometer) over the wave number ranging from 4000-400 cm\(^{-1}\) with a number of scans 32 and resolution of 4 cm\(^{-1}\). For this, a small amount of HAP powder was blended with KBr and then pressed into discs for obtaining FT-IR spectrum.

4.1.3.2 X-ray diffraction (XRD) studies

The phase composition and the crystallinity of the calcined and sintered nano-HAP powders were determined by X-ray diffraction (XRD, Bruker D-8 Advance-Germany) with a CuK\(\alpha\) radiation \(\lambda=1.5406\)Å generated at 35 kV and 25 mA. Data were collected over the 2\(\theta\) range 20-60° with a step size of 0.010° and a count time of 0.2 s.

4.1.3.3 Scanning electron microscopic (SEM) studies

The particle size and morphology of the synthesized nano-HAP powders were characterized by scanning electron microscope (SEM, JEOL Japan) operated at 20 kV.

4.1.3.4 Transmission electron microscopic (TEM) studies

The morphology and the microstructure of the obtained nano-HAP powders were characterized by transmission electron microscopic (TEM)
images obtained from Philips CM12 transmission electron microscope operated at 120 kV.

4.1.3.5 Thermogravimetric analysis (TGA)

The thermal behavior and stability of the HAP samples was evaluated by thermogravimetric analysis performed over the temperature range of 0-1200 °C (Mettler TA 3000 thermal analyzer).

4.2 Surface passivation of 316L stainless steel

4.2.1 Passivation of 316L SS in borate buffer solution

The passive layer on the alloy surface was formed in borate buffer solution under potentiostatic conditions at selected potentials such as 200 mV, 640 mV and 800 mV vs SCE. The borate buffer solution (0.4 M) was prepared using sodium tetra borate (Na₂B₄O₇.10H₂O) and the pH of the solution was 9.3. The potentials were selected on the basis of polarization behavior exhibited by the stainless steel specimens in borate buffer solution. After the electrochemical passivation, the specimens were rinsed with deionized water, dried and further evaluations of the passivated surface were performed by electrochemical and surface analytical techniques.

4.3 Development of HAP coatings on 316L stainless steel

4.3.1 Dip-coating method

Hydroxyapatite sol was prepared by the sol-gel method [61] and the specimens were coated by a computer controlled dip coater provided with a dual way electronic switch. The withdrawal rates were varied in order to obtain
crack free, uniform, bioresistive coatings. The coated specimens were air dried for 30 min and then sintered in a microwave oven at a 520 W for duration of 10 min.

4.3.2 Electrodeposition method

Electrodeposition was carried out using a three electrode cell assembly (Fig. 4.3) with the 316L SS specimens serving as the cathode, while platinum electrode was taken as the anode. The saturated calomel electrode (SCE) was taken as the reference electrode. Flat samples of 316L SS with the composition (wt. %) C — 0.0222, Si — 0.551, Mn — 1.67, P — 0.023, S — 0.0045, Cr — 17.05, Ni — 11.65, Mo — 2.53, Co — 0.136, Cu — 0.231, Ti — 0.0052, V — 0.0783, N — 0.0659 and rest Fe were used as substrates. These working electrodes were prepared by embedding them in epoxy resin mounting leaving areas of 1 cm² for exposure to the electrolyte. The surfaces exposed to the electrolyte were prepared by sequential polishing with silicon carbide papers up to 1200 grit. After polishing, the surface was degreased with acetone and then rinsed with deionized water.

The electrolyte for deposition was prepared by dissolving analar grade 0.42 M of Ca (NO₃)₂•4H₂O and 0.25 M of K₂HPO₄ (Merck, India) in deionized water with gentle magnetic stirring. The temperature of the electrolyte was maintained at 50 °C and the deposition was carried out at the cathodic potentials of -1200 mV, -1400 mV and -1600 mV for duration of 1 h. After the deposition was completed, the electrodes were immersed in 1 M NaOH for 24 h then rinsed with deionized water and oven dried at 80 °C for 1 h.
4.3.3 Electrochemical evaluation of 316L stainless steel

4.3.3.1 Specimen preparation

The electrochemical experiments were performed with the 316L stainless steel working electrodes which were cut into an overall apparent size of $1.0 \times 1.0 \times 0.3$ cm and used. The electrodes were then embedded in epoxy resin with an electrical connection and exposed area of $1\text{cm}^2$. Before each experiment the specimens were mechanically abraded with silicon carbide papers (from grits 120 to 1200), washed with distilled water, degreased with acetone and dried at room temperature.

4.3.3.2 Cyclic potentiodynamic polarization studies

The surface resistivity and coating efficiency of 316L SS specimens were assessed by cyclic potentiodynamic polarization studies. A three-electrode cell assembly was used for all the electrochemical measurements. The saturated calomel electrode was used as the reference while platinum
served as the counter electrode. The 316L SS specimens were employed as the working electrode. All the potentials mentioned in the text are with reference to saturated calomel electrode (SCE). The electrolyte used to simulate the physiological medium was Ringer’s solution, whose composition is given as NaCl: 8.6 g/L, CaCl₂·2H₂O: 0.66 g/L and KCl: 0.6 g/L. Potentiodynamic polarization studies were carried out using the electrochemical workstation CH 760 C (CH instruments, USA) using the experimental set-up shown in Fig. 4.4. The investigated potential range was from -1200 mV to 1000 mV (SCE) with a scan rate of 10 mV/s.

Fig. 4.4 Experimental set-up employed for electrochemical evaluations using electrochemical workstation CH 760 C.

4.3.3.3 Electrochemical impedance spectroscopic (EIS) studies

Electrochemical impedance is the complex combination of solution resistance, interface capacitance, charge transfer resistance and mass transfer resistance. At high frequencies the solution resistance predominates whereas at
low frequencies charge transfer and mass transfer become the main contributors to impedance. This can normally be analyzed using Nyquist diagrams, where the imaginary part of impedance is plotted as a function of real part. Similarly Bode plots obtained by plotting the frequency and impedance can be used for further interpretation. All the experiments in the present study were conducted at the open circuit potential.

The EIS measurements were performed with the frequency ranging from 1 Hz to 100 KHz and the data obtained was fitted using the internally available software using the equivalent circuits shown in Fig. 4.5. Typical Nyquist and Bode plots were obtained from the impedance studies and the results were further assessed.

Fig. 4.5. Equivalent circuit used for (a) uncoated and (b) coated 316L SS.
4.3.3.4 Cyclic voltammetric (CV) studies

The CV studies were performed in borate buffer solution by sweeping the potential from -1000 mV to 1000 mV at different scan rates such as 20, 50 and 100 mV s\(^{-1}\). The characteristic oxidation and reduction peaks were indentified from the cyclic voltammogram and explained.

4.3.4 Surface evaluations of 316L SS

4.3.4.1 Scanning electron microscopic (SEM) studies

The surface morphology of the passivated and HAP coated 316L SS specimens was studied by scanning electron microscope (JSM 840A Scanning microscope, JEOL-Japan).

4.3.4.2 Atomic force microscopic (AFM) studies

The 3D topography of the coatings was assessed by atomic force microscope (SPA 300, Seiko instruments, Japan).

4.3.4.3 Inductively coupled plasma-atomic emission spectroscopic (ICP-AES) analysis

The ICP-AES analysis was performed to estimate the amount of metal ions leached out from the alloy specimen during its immersion in Ringer’s solution. The samples were impressed at a potential of 460 mV for 1 h to study extent of dissolution of various metal ions from the alloy surface. Then the amount of ions leached out from the metal surface was assessed quantitatively through ICP-AES (Perkin Elmer optima 5300 DV) technique.
4.3.5 Mechanical Studies

4.3.5.1 Measurement of shear strength

The shear strength of the HAP coated and passivated-HAP coated 316L SS samples were assessed by universal testing machine for which the samples were taken in cylindrical shape and tested.

4.4 *In vitro* studies

4.4.1 Testing in simulated body fluids

The bioactivity of an implant material is assessed in terms of its ability to form bone-like apatite layer over the surface when immersed in simulated body fluids (SBF). SBF was used as an incubation solution for apatite formation on the surface treated 316L SS samples. The ion concentrations of SBF are nearly equal to those of the human blood plasma, as shown in Table 1. The SBF was prepared using Kokubo's formulation [252].

<table>
<thead>
<tr>
<th>Table 4.1 Ionic composition of Human blood plasma and SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ions</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Human Blood Plasma (mM)</td>
</tr>
<tr>
<td>SBF (mM)</td>
</tr>
</tbody>
</table>

Table 4.2 lists the reagents that are to be used for preparing 1000 mL of SBF solution. The SBF was prepared in double distilled water by dissolving the reagents one by one from 1ˢᵗ to 8ʰ in the order given in Table 4.2 and the
temperature was maintained at 36.5±1.5 °C. Finally, the pH of the solution was adjusted to 7.40 with HCl and tris-hydroxymethyl aminomethane (Tris) and the finally the temperature was set to 36.5 °C. All the samples were kept in a vertical position inside polypropylene tubes (one per tube). After soaking in SBF for different periods, the samples were gently washed with double distilled water and dried in desiccators and analyzed further.

Table 4.2 Reagents and their amounts to be used for preparing SBF

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagents</th>
<th>Amount (g/L)</th>
<th>Formula weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>8.035</td>
<td>58.4430</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO₃</td>
<td>0.355</td>
<td>84.0068</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.225</td>
<td>74.5515</td>
</tr>
<tr>
<td>4</td>
<td>K₂HPO₄.3H₂O</td>
<td>0.231</td>
<td>228.2220</td>
</tr>
<tr>
<td>5</td>
<td>MgCl₂.6H₂O</td>
<td>0.311</td>
<td>203.3034</td>
</tr>
<tr>
<td>6</td>
<td>1.0 M HCl</td>
<td>39 ml</td>
<td>36.5</td>
</tr>
<tr>
<td>7</td>
<td>CaCl₂</td>
<td>0.292</td>
<td>110.9848</td>
</tr>
<tr>
<td>8</td>
<td>Na₂SO₄</td>
<td>0.072</td>
<td>142.0428</td>
</tr>
<tr>
<td>9</td>
<td>Tris</td>
<td>6.118</td>
<td>121.14</td>
</tr>
<tr>
<td>10</td>
<td>1.0 M HCl</td>
<td>0–5 ml</td>
<td>36.5</td>
</tr>
</tbody>
</table>

4.4.2 Cell count

Osteoblasts-like cells were extracted from adult rats. The cells were washed twice in a phosphate buffer solution (PBS) then collected by centrifugation. Under standard incubation conditions (5% CO₂, 37.1 °C), the cells were incubated for one week in different cultural media containing Dulbecco’s modified Eagle’s medium (DMEM), dexamethasone, vitamin C and β-sodium glycerol-phosphate. The surface treated and HAP coated samples
were then placed in six-well plates to allow the growth of the cells. Each well contained cell density of $5 \times 10^4$ cells/ml. The cell attachment was characterized by removing the specimens every day for digestion in a 0.25% trypsin-EDTA solution and the number of cells is counted using a hematocytometer.

4.4.3 Cell viability

The cell viability was assessed by staining in Tryptan blue dye and then subsequent viewing in optical microscope. The number of viable cells was counted per unit square using the optical microscope (Carl Ziess, Germany).