CHAPTER – 3

Materials and Methods

This chapter thoroughly explains the materials selection, synthesis, processing, and characterization methods utilized in the studies presented in chapters 4, 5, 6, 7, and 8. The equipment and variables involved in synthesis and processing, including vacuum arc melting and high-pressure torsion (HPT), are briefly discussed. The structural characterization of the as-cast, annealed, and HPT-deformed CCA specimens was performed using X-ray diffraction and transmission electron microscopy. Optical microscopy and scanning electron microscopy equipped with X-ray energy-dispersive spectroscopy were used to examine the microstructural features and chemical composition of the CCAs. Due to the small size of arc melted specimens, the mechanical response was mostly evaluated using instrumented micro-indentation testing. Corrosion and reciprocating-type wear tests were conducted in a simulated body fluid solution, and in-vitro cytocompatibility was also evaluated. The following sections provide detailed description of the experimental procedures of the aforementioned techniques.

3.1 Materials selection and alloy synthesis

The metal shots of Fe, Cr, Ti, Nb, Mo, and Zr (purity ≥ 99.5%), procured from Alfa Aesar and Sigma Aldrich India Pvt. Ltd., were used to synthesize Ti-Nb-Mo-Fe-Cr and Ti-Zr-Nb-Mo-Fe-Cr-based complex concentrated alloys (CCAs). These elements belong to the 3d transition and refractory transition metal groups, and their fundamental properties are listed in Table 3.1 [5]. The CCAs for the present study were synthesized using the vacuum arc melting (VAM) technique. In this method, metal shots with the nominal compositions of the alloys ($Ti_{10}Nb_{30}Mo_{20}Fe_{20}Cr_{20}$ and $Ti_{35}Zr_{35}Nb_{15}Mo_{5}Fe_{5}Cr_{5}$) were melted under an argon atmosphere. The melting was performed in an arc melting furnace manufactured by VEC SOLUTIONS Pvt. Ltd., India.

Before melting, the mixture of metal shots, with the nominal composition of the alloys, was placed in a water-cooled copper crucible inside the melting chamber of the VAM furnace. The chamber was connected to a high-vacuum system, maintaining a vacuum pressure of approximately 10^{-6} mbar. Melting was performed using a non-consumable tungsten electrode with a diameter of 3 mm, operating at a current range of 260–280 A. Initially, the Ti-getter was melted and held in the liquid state for a sufficient duration to facilitate the absorption of residual oxygen within the melting chamber. Subsequently, the targeted CCA was melted. The resulting CCA button-shaped (20 mm diameter and 10 mm thickness) sample was remelted a minimum of five times, being flipped each time to ensure homogeneous chemical composition.

Table. 3.1: Fundamental properties of the elements selected for the present study [5].

Elements	Crystal Structure (RT)	Atomic Radius (Å)	Melting point (ºC)	Electronegativity	Density (g/cm^3)
Fe	BCC	1.24	1538	1.83	7.88
Cr	BCC	1.25	1907	1.66	7.19
Ti	HCP	1.46	1668	1.54	4.50
Zr	HCP	1.60	1855	1.33	6.51
Nb	BCC	1.43	2477	1.60	8.58
Mo	BCC	1.36	2623	2.16	10.23

3.2 Density measurement

The experimental density of the as-cast CCA specimens was determine using Archimedes' principle, in accordance with ISO 2738 standard. A density measurement kit equipped with an electronic weighing balance (CONTECH Instrument Ltd., India) was utilized. However, the theoretical density of the present CCAs was calculated using the mixture rule [6]:

$$\rho_{mix} = \frac{\sum c_i A_i}{\sum \frac{c_i A_i}{\rho_i}} \text{ .... } (3.1)$$

where, ρ_i, c_i, and A_i are the density, concentration, and atomic weight of the ith element.

3.3 High pressure torsion

The HPT deformation was performed at room temperature using a Bridgman-type anvil. During the process, the upper part of the anvil remained stationary, while the lower part rotated continuously at a speed of 1 revolution per minute. A 10 mm diameter and 1 mm thick disc of the as-cast $Ti_{35}Zr_{35}Nb_{15}Mo_5Fe_5Cr_5$ CCA was deformed under a pressure of 4 GPa, with varying numbers of rotations, including 1, 3, 5, and 7 turns. The equivalent von Mises strain (ε_{EV}) in the alloy during HPT processing was estimated using the following equation [148]:

$$\varepsilon_{EV} = \frac{2\pi N r}{\sqrt{3}.t} \text{ .... } (3.2)$$

where, N, r, and t are the number of rotations, radially outward distance, and thickness of the disc, respectively.

3.4 Annealing treatment

The annealing treatment of the as-cast CCAs was carried out in a muffle furnace. First, the as-cast samples were sealed in a vacuum quartz tube backfilled with argon gas.

These sealed samples were then placed in the furnace at various temperatures for a fixed holding time of 20 hours. Annealing experiments of the $Ti_{10}Nb_{30}Mo_{20}Fe_{20}Cr_{20}$ and $Ti_{35}Zr_{35}Nb_{15}Mo_5Fe_5Cr_5$ CCAs were performed over temperature ranges of 600°C to 1200°C and 600°C to 1100°C, respectively, wherein the annealing temperature was increased in steps of 100°C. For example, $Ti_{10}Nb_{30}Mo_{20}Fe_{20}Cr_{20}$ CCA specimens were annealed at 600, 700, 800, 900, 1000, 1100 and 1200°C and out of these seven samples, only those showing some distinct change in phase evolution were selected for further characterization. The maximum annealing temperature for each composition was selected corresponding to approximately 0.55 T_m, where T_m is the melting temperature calculated using the mixture rule, to assess the thermal stability of the alloys. For the $Ti_{10}Nb_{30}Mo_{20}Fe_{20}Cr_{20}$ CCA, no significant changes in phase fractions were observed up to 700°C. Similarly, for the $Ti_{35}Zr_{35}Nb_{15}Mo_5Fe_5Cr_5$ CCA, no significant change in phase fraction was detected at 600°C. However, significant change in phase fractions occurred at 800°C, 1000°C, and 1200°C for $Ti_{10}Nb_{30}Mo_{20}Fe_{20}Cr_{20}$ CCA, and at 700 °C, 900 °C, and 1100 °C for $Ti_{35}Zr_{35}Nb_{15}Mo_5Fe_5Cr_5$ CCA. Therefore, the specimens annealed at these temperatures were characterized further to study the microstructure, mechanical response, corrosion behaviour and other properties. In all cases, the samples were annealed for a fixed holding time of 20 hours, followed by furnace cooling.

3.5 Microstructural characterization

In order to study the microstructural features of the as-cast, annealed, and HPT-deformed CCA specimens, the following techniques were used.

3.5.1 X-ray diffraction

The XRD analysis in this study was conducted using two instruments: Rigaku Miniflex-600 (operating at 40 kV and 15 mA) equipped with Cu-Kα radiation (λ = 1.54 Å) and the PANalytical EMPYREAN (operating at 40 kV and 40 mA) equipped with Co-Kα radiation (λ = 1.79 Å). The as-cast and annealed $Ti_{10}Nb_{30}Mo_{20}Fe_{20}Cr_{20}$ (Cu-Kα radiation) and $Ti_{35}Zr_{35}Nb_{15}Mo_{5}Fe_{5}Cr_{5}$ (Co-Kα radiation) specimens were scanned over an angular range (2θ) of 20° to 90° and 20° to 110°, respectively, using a scanning speed of 5°/min with a step size of 0.02°. In addition, XRD analysis of the HPT-deformed $Ti_{35}Zr_{35}Nb_{15}Mo_{5}Fe_{5}Cr_{5}$ CCA was performed using a Rigaku SmartLab system operating at 45 kV and 100 mA with Cu-Kα radiation, scanning over a 2θ range of 20° to 100°. After performing Pseudo-Voigt fitting of the XRD peak profiles, phase analysis of the as-cast, annealed, and HPT-processed CCAs was carried out with the help of the "International Centre for Diffraction Data (ICDD)" PDF2 database.

In the present study, the phase fraction of the constituent phases was determined using the following equations [149-151].

$$f(BCC1) = \frac{\Sigma A(BCC1)}{\sum A(BCC1)+\Sigma A(BCC2)+\Sigma A(TiCr_2)} \text{ ………….. } (3.3)$$

$$f(BCC2) = \frac{\Sigma A(BCC2)}{\sum A(BCC1)+\Sigma A(BCC2)+\Sigma A(TiCr_2)} \text{ …………... } (3.4)$$

$$f(TiCr_2) = 1- f(BCC1) - f(BCC2) \text{ …………….. } (3.5)$$

where f(BCC1), f(BCC2), and $f(TiCr_2)$ correspond to the phase fractions of the BCC1, BCC2, and $TiCr_2$ phases, respectively. A(BCC1) refers to the area beneath the XRD peak associated with the BCC1 phase; A(BCC2) indicates the area beneath the peak for BCC2; and $A(TiCr_2)$ denotes the area beneath the XRD peak related to the $TiCr_2$ phase.

In addition, the crystallite size and lattice strain were calculated using the Williamson–Hall method [151-153]. The following equation was used to determine these parameters:

$$\beta \cos\theta = \frac{0.9\lambda}{t} + 4\varepsilon \cdot \sin\theta \; \ldots\ldots\ldots\ldots\ldots \; (3.6)$$

where, β represents the total broadening, λ is the wavelength, θ is the Braggs angle, ε is the lattice strain, and t is the crystallite size. The total broadening was determined by subtracting the broadening of a standard Si sample to eliminate the effects of instrumental broadening.

The macro-texture measurements of the as-cast and annealed CCA were performed using a Rigaku Ultima X-ray diffractometer (XRD) equipped with Cu Kα radiation (λ = 1.5403 Å). A four-circle diffractometer goniometer with Schulz back-reflection geometry was employed for the precise measurement of the alloy's bulk texture. The pole figure measurement using XRD is limited to a maximum tilt angle of 70° due to increased defocusing errors at higher tilt angles [154]. Therefore, multiple incomplete pole figures were analyzed to gather information on the various orientations present in the polycrystalline sample. In this study, incomplete pole figures, such as (110), (200), and (211), were utilized to obtain the orientation distribution function (ODF). The ATEX software was used to generate the texture plots (IPF and ODF) from the X-ray texture data [155].

3.5.2 Optical microscopy

The samples for optical microscopy were prepared using standard metallographic techniques. First, the samples were flattened using a belt grinder with 60 µm grit size silicon carbide paper. Following this, they were polished with emery paper of varying grit sizes, ranging from 400 to 2500. Final polishing was performed on a ChemoMet cloth. The polished samples were then etched with Kroll’s reagent for 8–10 seconds. The microstructure of the etched samples was examined and captured at various magnifications using a Leica DFC295 optical microscope.

3.5.3 Scanning electron microscopy

The morphological features of the as-cast, annealed, and HPT-processed samples were investigated using the Carl Zeiss EVO 18 SEM, as well as the high-resolution Quanta 200F and NOVA NanoSEM 450 FESEM, operated at appropriate accelerating voltages. Both secondary electron (SE) and backscattered electron (BSE) imaging modes were utilized in the present study. SE mode was utilized for topographical analysis, while BSE mode was applied to detect contrast changes associated with variations in chemical composition. Additionally, SEM equipped with X-ray energy dispersive spectroscopy (EDS) was used for elemental analysis. The sample preparation for SEM followed the same procedure as for optical microscopy, as detailed in Section 3.5.2.

The micro-texture characterization of both the as-cast and annealed CCA specimens was performed using EBSD on a JEOL field emission gun scanning electron microscope (FEG-SEM) of model JSM-7100F (JEOL, Japan). The specimens for EBSD were metallographically prepared using emery paper and then cloth polishing with alumina. To obtain a high-quality polished surface, VibroMet polishing with colloidal silica was performed at a frequency of 30 Hz for 8 h. After polishing, the specimens were properly cleaned to remove contaminants. Ultrasonic cleaning in a methanol medium was performed for 30 minutes to eliminate silica particles. The collected EBSD data was analyzed using TSL OIM software.

3.5.4 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to investigate the fine microstructural characteristics and also to confirm the structure obtained from XRD analysis. In this study, TEM was performed using a TECNAI G^2 T20 microscope operated at 200 kV. For TEM sample preparation, the as-cast sample was crushed into fine particles. These fine powder particles were dispersed in ethanol and subjected to 15–20 minutes of ultrasonication to prevent particle agglomeration. Subsequently, 1–2 drops of the solution were drop-cast onto 3 mm carbon-coated Cu grids with a 200-mesh size. This was dried using an infrared lamp for 30 min.

3.6 Mechanical properties

3.6.1 Hardness and elastic modulus

Vickers hardness tester and instrumented microindentation tester were used to measure the hardness and elastic modulus of the CCAs. For this, mirror polished samples were prepared using the same procedure as describe in Section 3.5.2. The hardness was measured using Leco, LV 700 Vickers hardness tester at 2 and 3 kgf load and 15 s dwell time. Additionally, the hardness and elastic modulus of the alloys were calculated using Anton-Paar, MHT^3 instrumented microhardness tester with a Vickers diamond indenter tip. The Oliver-Pharr technique was utilized for the aforementioned measurements by analyzing loading and unloading curves [99]. Following equations were used to calculate the elastic modulus of the present CCAs.

$$\frac{1}{E^*} = \frac{1-\nu_i^2}{E_i} + \frac{1-\nu_s^2}{E_s} \quad \ldots\ldots\ldots\ldots\ldots (3.7)$$

$$S = \frac{dP}{dh} = \frac{2}{\sqrt{\pi}} E^* \sqrt{A} \ldots\ldots\ldots\ldots.. (3.8)$$

where, E^* denotes the reduced modulus of the indenter/sample system; E_i and ν_i represent the elastic modulus and Poisson ratio of the indenter, respectively; E_s and ν_s represent the elastic modulus and Poisson's ratio of the sample, respectively; S the slope of the unloading curve at the maximum displacement; and A refers to the projected area. The elastic modulus and Poisson's ratio of the indenter were 1141 GPa and 0.07, respectively [99]. The hardness and elastic modulus, along with their corresponding standard deviations, were calculated by averaging the results from 15 successful indentations. These indentations were performed with a spacing of 150 μm between consecutive indents.

3.6.2 Estimation of elastoplastic response

In this study, tensile testing of the CCA could not be performed due to the limited size of the samples produced by VAM. As a result, evaluating the strength-ductility index was not possible. To overcome this limitation, the instrumented micro-indentation data was used to estimate the alloy's elastoplastic response (stress-strain curve), enabling the analysis of its plasticity.

The method proposed by Dao et al. [156] was used to convert the instrumented micro-indentation response (P–h curve) into a corresponding stress-strain curve. This approach utilizes key parameters extracted from the P–h curve, including C (loading curvature), h_m (maximum indentation depth), h_r (residual depth), $\left(\frac{dP_u}{dh}\right)_{h=h_m}$ (unloading slope at maximum depth), E^* (reduced modulus), and $\frac{h_r}{h_m}$ to estimate the parameters strain hardening exponent (n), and yield strength (σ_y). The dimensionless functions Π_1 and Π_2 are utilized to calculate the values of the stress at 3.3% plastic strain ($\sigma_{0.033}$) and n [156].

$$\Pi_1 = \frac{C}{\sigma_{0.033}} = -1 \cdot 131 \left[\ln\left(\frac{E^*}{\sigma_{0.033}}\right)\right]^3 + 13.635 \left[\ln\left(\frac{E^*}{\sigma_{0.033}}\right)\right]^2 - 30.594 \left[\ln\left(\frac{E^*}{\sigma_{0.033}}\right)\right] + 29.267$$

………… (3.9)

$$\Pi_2\left(\frac{E^*}{\sigma_r}, n\right) = \frac{1}{E^* h_m}\left(\frac{dP_u}{dh}\right)_{h_u = h_m} = (-\ 1.40557n^3 + 0.77526n^2 + 0.15830n - 0.06831)\left[\ln\left(\frac{E^*}{\sigma_{0.033}}\right)\right]^3$$

$$+ (17.93006n^3 - 9.22091n^2 - 2.37733n + 0.86295)\left[\ln\left(\frac{E^*}{\sigma_{0.033}}\right)\right]^2$$

$$+ (-\ 79.99715n^3 + 40.55620n^2 + 9.00157n - 2.54543)\left[\ln\left(\frac{E^*}{\sigma_{0.033}}\right)\right]$$

$$+ (122.65069n^3 - 63.88418n^2 - 9.58936n + 6.20045) \text{ …………………… (3.10)}$$

In the sharp indentation test, the representative strain approach is essential for correlating material properties, such as representative stress and strain, with loading parameters. The representative strain is determined based on the semi-angle of the sharp indenter [157]. Equations 3.11 and 3.12 were used to calculate the representative strain and stress [157-158].

$$\varepsilon_r = 0.2 \cot\theta \text{ …………… (3.11)}$$

$$\sigma_r \approx \frac{H}{2.8} \text{ …………………... (3.12)}$$

where, ε_r and σ_r are the representative strain and stress, respectively, θ is the semi-angle of the sharp indenter, and H is the hardness in MPa. The yield strength of the materials was also calculated using the representative strain method, as expressed in equation 3.13 [159].

$$\sigma_{0.033} = \sigma_y \cdot \left(1 + \frac{E^*}{\sigma_y} \cdot 0.033\right)^n \text{ ………… (3.13)}$$

The ultimate tensile strength (σ_{UTS}) of the materials is estimated using the equation 3.14 [157].

$$\sigma_{UTS} = E^n {\sigma_y}^{1-n} \left(\frac{n}{e}\right)^n \text{ …………………. (3.14)}$$

where, e is the natural base.

3.7 Electrochemical corrosion testing

Electrochemical tests were performed using a three-electrode system connected to a computer-controlled potentiostat (CorrTest CS350). In this experiment, the test samples act as the working electrode, while a platinum wire mesh and a saturated Ag/AgCl electrode were used as the counter electrode and reference electrode, respectively. These experiments were carried out in 3.5 wt.% NaCl and simulated body fluid (SBF) solutions. The SBF solution (pH = 7.31 ± 0.04) was prepared according to the methodology of Kokubo and Takadama, with its chemical composition detailed in Table 3.2 [160]. Additionally, to achieve a quasi-stationary value of the open-circuit potential (OCP), the samples were allowed to corrode freely in the solution for 60 minutes.

Table 3.2 Chemical composition of the SBF solution (For 1000 ml solution) [160].

Sl. No.	**Reagents**	**Amount**
1	NaCl	8.035 g
2	$NaHCO_3$	0.355 g
3	KCl	0.225 g
4	$K_2HPO_4.3H_2O$	0.231 g
5	$MgCl_2.6H_2O$	0.311 g
6	1.0 M HCl	39.0 ml
7	$CaCl_2$	0.292 g
8	Na_2SO_4	0.072 g
9	$((HOCH_2)_3CNH_2)$	6.118 g

3.7.1 Potentiodynamic polarization tests

The potentiodynamic polarization experiments were conducted at a scan rate of 0.5 mV/s, with a scanning range from -0.5 V to 1.5 V relative to the OCP. To ensure reproducibility, each experiment was conducted in triplicate using a freshly prepared electrolyte solution, in accordance with ASTM G5-95 standard. The corrosion rate (CR) is determined from the potentiodynamic polarization data using the following equation [161].

$$CR(mm/y) = \frac{3{\cdot}27\times10^{-3}\times EW\times I_{Corr}}{\rho} \quad \ldots\ldots\ldots\ldots\ldots.. (3.15)$$

where, EW is the equivalent weight of the alloy, $\mathrm{I_{Corr}}$ is the corrosion current density (μA/cm^2), and ρ is the density of the alloy (g/cm^3).

3.7.2 Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) spectra were recorded after stabilization of the OCP in the corrosive medium. Measurements were performed over a frequency range of 10 mHz to 100 kHz, with a sinusoidal potential signal of 10 mV amplitude applied relative to the OCP. The resulting data were subsequently analyzed and fitted using the CS Studio5 software (ZView® tool).

3.8 Wear and friction studies

The reciprocating wear and friction experiments were conducted under wet conditions (SBF solution) using a micrometric linear tribometer, in accordance with the ASTM G133 standard. Disc-shaped test samples with a diameter of 12 mm and a thickness of 5 mm were used for this study, while a tungsten carbide (WC) ball served as the counter material. Throughout the experiments, a fixed stroke length of 5 mm and an oscillation frequency of 2 Hz were maintained. However, the applied load varied between 10 N and 25 N, and the sliding time ranged from 30 minutes to 120 minutes. To measure the wear weight loss, a precise electronic balance with an accuracy of 0.1 mg was used to record the weight both before and after each experiment. Additionally, the specific wear rate was calculated using the following equation [162-163].

$$\mathrm{SWR}\ (\mathrm{mm}^3/\mathrm{N\,.\,mm}) = \frac{\Delta \mathrm{w}}{\mathrm{P}\times\rho\times\mathrm{D}} \ \ldots\ldots\ldots\ldots\ldots\ (3.16)$$

$$\mathrm{D} = 2.\mathrm{f.t.S_L} \ \ldots\ldots\ldots\ldots\ldots\ (3.17)$$

where, Δw represents the weight loss (g), P is the applied load (N), D is the sliding distance (mm), f denotes the oscillation frequency, t is the sliding time, S_L is the stroke length, and ρ is the density of alloy (g/mm^3).

In addition, after the wear experiments, the surface of the wear track was analyzed using atomic force microscopy (AFM). In this study, the NTEGRA Prima AFM was used for topographical examination. A scanning rate of 0.5 Hz and a scanning area of 60 µm × 60 µm were selected for the analysis.

3.9 Biocompatibility tests

3.9.1 Wettability tests

The wettability of the present CCAs was measured using a contact angle measuring device (Drop Shape Analyzer-DSA 25, Kruss) equipped with a computerized digital camera. Prior to the experiments, cylindrical samples with a diameter of 10 mm and a length of 5 mm were prepared according to the procedure described in section 3.5.2. They were then washed adequately with acetone and subsequently dried. Forceps and gloves were used during sample handling to prevent surface contamination from oils, dirt, or dust. A 2 µL drop of distilled water was dispensed onto the CCA sample using a syringe, and photographs were taken 30 seconds after the drop was applied.

3.9.2 In-vitro cell viability, adhesion, and proliferation tests

The in-vitro cytocompatibility experiments were conducted using human bone osteosarcoma cells (MG-63) and Human Embryonic Kidney cells (HEK-293). Both cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. MG-63 cells were cultured in Minimum Essential Medium (MEM), while HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), both supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. These cells were kept in a humidified atmosphere with 5% CO_2 at 37°C in a CO_2 incubator. For further experiments, 1×10^6 cells were seeded in a culture flask with an area of 25 cm^2. When the confluency of these cells reached at 80-90% then they were collected and plated for subsequent experiments.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate the cell viability and proliferation behavior. Cell viability was assessed using this assay, which is based on the ability of cells to convert the soluble tetrazolium salt MTT into insoluble formazan crystals. [69,164]. In brief, all samples underwent autoclaving at 121°C, followed by a 10-minute exposure to UV light prior to the experiment. A 98-well plate was then prepared, with each well containing a distinct alloy, including CCA, 316L SS, and cp-Ti.

Additionally, 1×10^5 viable cells were seeded to each well of a 96-well flat-bottom ELISA plate containing 100 µL of complete culture media. The plates were then incubated in a CO_2 incubator for different durations, with the media changed every other day to ensure normal culture conditions. After media removal, 15 µL of freshly prepared MTT solution (5 mg/mL) was added to each well, and the plates were incubated at 37°C for 3 hours. The solution was carefully removed to prevent disturbing the formazan crystals, after which 100 µL of Dimethyl Sulphoxide (DMSO) was added to each well to dissolve the crystals. The plate was then incubated in the dark at room temperature (RT) for one hour. The cell viability was determined by measuring the optical density (OD) at 595 nm using a microplate reader (Bio-Rad, USA) [165].

The MTT experiment results are expressed as the mean ± standard deviation. Additionally, statistical evaluation was performed using one-way analysis of variance (ANOVA), with significance considered at $P < 0.05$.

To qualitatively assess apoptosis in MG-63 cells, the dual fluorescent staining method using Acridine Orange (AO) and Ethidium Bromide (EtBr) was used. AO, a green fluorescent dye, is capable of straining both live and dead cell membranes, while EtBr selectively stains the DNA of dead cells. Early signs of apoptosis include nuclear condensation, cell shrinkage, and membrane blebbing [166]. In a 6-well culture plate, 1×10^5 cells were seeded into each well and incubated overnight in a CO_2 incubator. On the subsequent day, the cells were treated with the test samples and subsequently incubated for three days at 37°C in a humidified environment containing 5% CO_2. After incubation, the cells were rinsed with PBS and stained with EtBr and AO, each at a concentration of 100 µg/ml. The staining was carried out in the dark for 30 minutes at 37°C. Subsequently, the cells were washed again with PBS and examined under a confocal microscope.

The influence of the test samples on cellular nuclear morphology was examined using 4,6-diamidino-2-phenylindole (DAPI) staining, supplied by Sigma Chemical Co. (St. Louis, MO, USA). A total of 1×10^5 MG-63 cells were seeded into a 6-well culture plate and incubated overnight in a CO_2 incubator. The cells were treated with the samples and then cultured for another three days at 37°C in a CO_2 incubator with 5% CO_2. Afterward, they were washed and stained with DAPI (5 μg/ml) and incubated in the dark at RT for 30 minutes. Morphological changes in the cells and their nuclear membranes were then examined using confocal microscopy.

The mitochondrial membrane potential ($\Delta\Psi_m$) was evaluated using the Rhodamine-123 (Rh-123) probe. A total of 1×10^5 cells were seeded onto 6-well culture plates and allowed to adhere overnight. Afterward, the cells were exposed to CCA samples, as well as control samples (reference materials: 316L SS and cp-Ti). The cells were then maintained in a CO_2 incubator for three days. Then they were washed with PBS, and then treated with Rh-123 at a concentration of 5 μg/ml, and incubated in the dark at RT for 30 minutes. Subsequently, the cells were analyzed under a confocal microscope to observe any morphological changes.

The intracellular production of reactive oxygen species (ROS) was measured using DCFH-DA (2,7-dichloro-dihydrofluorescein diacetate) staining, a fluorescent probe that detects the presence of peroxyl, hydroxyl, and other ROS. Following the seeding of 1×10^5 cells into 6-well culture plates, the cells were incubated overnight. Afterward, the cells along with all the samples were placed in a CO_2 incubator for three days. Following a 30-minute staining with DCFH-DA dye (5 mM/ml), the cells were rinsed with PBS. Subsequently, images were acquired using confocal microscopy in both the green fluorescence channel and phase contrast mode.