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Evaluation of bioceramic coated materials for orthopaedic applications

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Many surgical metals such as stainless steel, titanium, magnesium and its alloys have been extensively used for the recovery of body structures in human beings. Corrosion is the major reason for failure in metallic implants, when the metal comes in contact with the body fluids it releases metal ions into the surrounding tissues. This may even lead to the second surgery which can be eradicated by the surface modification of the implant with bioceramics using coating techniques. The present work involves the development of coatings on the surface of 316L SS type of stainless steel using a biphasic mixture of bio ceramics HAP/β-TCP in ratio of 7.5:2.5 by electrophoretic deposition (EPD) from a suspension of ethanol. The presence of biphasic coating imparts the property of both bioactivity and bioresorbability to the implant with good adherence of the coatings in body fluids. These coatings provide corrosion resistance and also favour new bone growth. Further, the biocompatibility of these materials can be evaluated by *in-vitro* assay. This includes cytotoxicity tests carried out with normal cell line (Vero cell line) and cancerous cell line (HEP II cell Line). The coated samples have been tested for their biochemical nature using DPPH (2,2 diphenyl 1 picryl hydrazil compound) activity to confirm whether the coated implant is suitable for cancerous patients with its antioxidant property which helps to trap the free radicals.

Keywords: EPD-Electrophoretic deposition, Hydroxyapatite, β-TCP-Tricalcium phosphate, 2 diphenyl 1 picryl hydrazil compound (DPPH)

One of the most rewarding aspects of material science is the use of the biomaterials to alleviate a person suffering from fractures and damages in bone tissues. The advanced development of material science led to the use of surgical grade biomaterials as the materials for implant. Biomaterials are generally synthesized by using several chemical methods in laboratories or extracting them from the natural sources[1]. These synthetic materials can be replaced in the body tissues and organs after they are proven to be biocompatible. Metallic implants such as stainless steels, titanium and magnesium alloys used as orthopedic prostheses provides improved mechanical features that matches with the anatomical structure and functions of the bone. The biocompatibility and corrosion resistance of these implants are primarily determined by their constituent material and surface micro structural properties such as surface roughness, grain size, etc.[2]. Type 316L SS are traditionally used for implantation purposes in orthopaedic surgery owing to their general corrosion resistance, mechanical properties and low cost[3,4].

To overcome the problem of corrosion, the implants can be coated with bio ceramics. Based on the nature of bioceramics the biological properties keep changing. The functions of an implant may be modified to be bioactive with the hydroxyapatite coated implants and bioresorbable with the tri calcium phosphate coated implants[5]. HAP is present naturally in bones, however, it cannot be directly used for making implant devices because of its brittleness and strength limitations, while β-TCP with its bioresorbable property stimulates new bone deposition. Hence, a great deal of research concentrates on the development of HAP/β-TCP as coating and composites[6].

The biocompatibility of implant or matrix for a tissue-engineering product refers to the ability to

perform as a substrate that will support the appropriate cellular activity. After implantation it includes molecular and signalling role inside the system in order to optimize tissue regeneration, without provoking undesirable effects in those cells[7-11]. The selection of HAP and β-TCP bio ceramics is considered to be best as they show similarity with minerals in natural bone and are widely used in hard tissue repair and report supports that they can form a protective layer as both are biocompatible[12,13]. These materials on implantation at osseous site, provides an ideal environment for cellular reaction and leads to osteoconduction which induces bone growth, bonds to implant and promotes a functional interface[14]. Identifying the reliable coating process is another important key factor, since it provides protection to a specific structure exposed to different environment which surrounds the implant inside the human body[15].

In this research work synthesized hydroxyapatite (HAP)/ β-Tricalcium phosphate (β-TCP) powders in the ratio of 7.5:2.5 (i.e. 0.75mg HAP mixed with 0.25mg β-TCP) have been prepared. HAP/ β-TCP has been chosen because of bioactive character of HAP and bioresorbable character and the phosphate groups of β-TCP which acts as coupling/anchoring agent, it is a good cation absorber and has higher affinity towards the HAP particles and enables new bone formation[16,17]. HAP concentration should be more when compared to β-TCP. These materials have been made into a suspension with ethanol and electrophoretically deposited over 316L SS cathode. *In-vitro* analysis of the coated samples has been carried using MTT assay to assess the biocompatibility with both normal and cancerous cells. Further, these materials have been tested for the DPPH activity to confirm whether the materials can be used as one of the components for the bone tissue scaffolds[18,19]. The extensive study has been carried out using *in-vitro* biocompatibility and the cell proliferation in the synthesized HAP/β-TCP mixture. Figure 1 shows the schematic representation of EPD deposited biphasic HAP/ β-TCP coatings and their biocompatibility evaluation.

Experimental Section

Calcium nitrate tetrahydrate AR (Ca $(NO_3)_2.4H_2O$), diammonium hydrogen phosphate AR $(NH_4)_2HPO_4$, concentrated ammonia (NH_3), cetyl trimethyl ammonium bromide, (CTAB) were purchased from Sisco Research Laboratories (SRL) Ltd., India and all other chemicals were used further without purification. Type 316L SS was used as the substrate

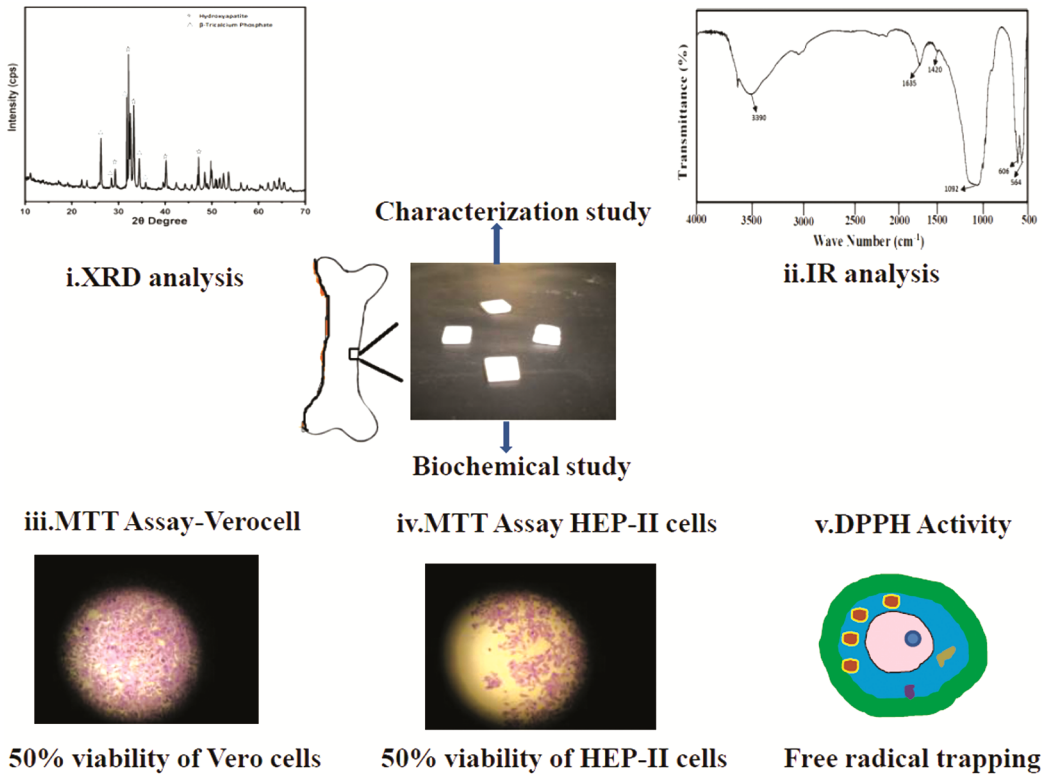

Fig. 1 — Schematic representation of EPD deposited biphasic HAP/ β-TCP coatings and their biocompatibility evaluation.

for coating HAP/β-TCP. The elemental composition of the alloy using direct reading emission spectrometer (Jobin-Yvon, Paris) was given as C- 0.029, Mn-1.8,Cr-17.3, Ni-12.4, Si-.0.7, Mo-2.25, P-0.031, S -0.012.

Sample preparation

Surface condition of the alloy plays a major role in the development of HAP coating. Type 316L SS alloy is taken in annealed condition was cut into 10 × 10 × 2 mm size pieces for experimental use. The edges of the specimens were polished up to 1000 grit SiC paper to bring it to uniform surface condition. The test specimen was attached to a threaded brass rod using silver paste in between the brass rod and the specimens to get the proper electrical contact. The samples were molded using an epoxy resin (Araldite, Ciba Geigy) in case of the uncoated type 316L SS sample.

HAP/β-TCP was synthesized in the laboratory following established procedures in the lab from literature[17]. The purity was checked by analyzing the chemical composition using titrimetric for calcium and phosphate spectrophotometrically. The crystalline structure was further confirmed by XRD.

The stoichiometric HAP powder synthesized with Ca/P ratio of 1.67 was used as the starting material. The powders were ground finely with a mortar and pestle and sieved with ASTM sieve no.500. The stoichiometric TCP powder was synthesized and the β form of TCP was used for the experiment. The coatings were developed by electrophoretic deposition (EPD) at room temperature from 2% suspension of HAP/β-TCP in the ratio of 7.5:2.5, in ethanol taken in a 100 mL glass beaker, closed with teflon covering. Magnetic stirring was used to keep the particles in suspension. AISI type 316L SS samples were mechanically polished using grit SiC papers from 120 to 600. The electrodes were washed thoroughly with running distilled water, rinsed, degreased with ethanol and dried. The 304 SS sheet was used as anode while the polished sample can be used as a cathode[20]. Both these electrodes were immersed in the suspension. The coatings were obtained at 10V, 15V, 20V, 25V and 30V for various time durations. The substrate, 316L SS was weighed before and after the deposition. Triplicate sample coatings were done for each parameter[21]. The coated samples were sintered for 1 h in furnace at 600°C. The coatings were further characterized to study their properties using XRD and FTIR. MTT assay was used to study the biocompatibility, were cells in the exponential phase of growth are when exposed to toxicity. The crystalline nature of the HAP/TCP powders and coated type 316L SS were studied with (XRD, model: Rich–Seifert, 2000D) using Ni filtered Cu K_d radiation at 30 kV and 20 mA. The 2t angles were swept from 20 to 65· in steps of one degree. The functional characteristics of HAP/TCP powders were characterized with Perkin-Elmer FTIR Paragan series instrument. The spectra of the powders were recorded in the form of KBr (spectroscopic grade) pellet from 4000 - 490 cm^{-1} wave number with a resolution of 4 cm^{-1}. The images were recorded using Canon Power Shot SX530 Digital Camera.

The exposure duration of the cells were determined by the stability of the implant and also by the time required for the maximum damage. The number of surviving cells was then determined by the MTT dye reduction which is a soluble tetrazolium salt that would be converted into insoluble formazan precipitate by viable (mitochondrial reductase) enzymes in the cell. The MTT formazan can be dissolved in a suitable solvent to get a MTT formazon precipitate. The precipitate thus formed can be determined spectrophotometrically. Cell viability studies were carried out on the coated samples by suspending the cells again in growth medium (RPMI 1640 medium with 10% fetal bovine serum) and were observed. The cell suspension was diluted with growth medium to get 2.5-5.08 $\times 10^3$ cells and allowing cell suspension of about 20 mL per micro titration plate. Diluted cell suspensions were transferred to a 9 cm petri dish and 200µL of cell suspensions was transferred into each well of the central 10 columns of a flat bottomed 96 well plate using multichannel micropipette. 200µL of growth medium was added to the eight wells in the column[22] 1&12. The micro titration plate was covered with its lid & incubated in a humidified atmosphere for the biomaterials (HAP/β-TCP mixture). A several fivefold dilutions of the materials (HAP/β-TCP) in growth medium were prepared to give eight concentrations. Cells in eight wells of column 2 and 11 were added with 200 mL of fresh medium and these columns were used as control. 200 µL of samples was transferred to the wells in columns 3 to 10 using micropipette. Plates were covered with its lid and incubated for a definite exposure period. The mixture was removed from all the wells containing cells at the end of its exposure period and the cells were fed with 200 µL of fresh medium. The plates were fed daily for 2-3 PDTs (Population doubling time)[23].

DPPH (2,2 diphenyl 1 picryl hydrazil) assay was carried out to study the antioxidant property which is considered as one of the health protecting factor. This method involved the use of free radical 2,2 diphenyl 1 picryl hydrazil (DPPH). The odd or unpaired electron in the DPPH free radical gave a strong adsorption maximum at 517 nm and was purple in colour. This was turned to yellow due to the reduction of DPPH radical to DPPH-H due to the scavenging activity of free radicals.

Results and Discussion

Electrophoretic deposition

EPD of HAP/β-TCP over 316L SS were carried out to optimize the time and voltage of the coatings. The desired time was calculated when the coatings were carried out based on the physical nature of the coatings and this was found to be better at a constant voltage of 20 V. After several assessments the good and adherent coat of HAP/β-TCP over 316L SS was found at 20 min of coating time duration. Similarly, at a constant time of 20 minutes, the different voltages were analyzed to get the maximum coating weight gain. The data presented in Table 1 suggest that the maximum weight gain obtained at 20 min of time when compared to 15, 10, 25 and 30 minutes, respectively. Table 2 indicates that the maximum weight gain obtained at 20V when compared to 15, 10, 25 and 30V, respectively. After optimizing the voltage and time, 20V and 20 minutes were considered as the desired voltage and time to obtain a good adherent coating and the EPD after evaluating the coatings on each substrate with 5 times.

Table 1 — Variation in weight of HAP/ β-TCP with changes in coating time at constant voltage (20 V)

Sample No.	Time (min)	Weight Gain (mg)
1	20 mins	1.3
2	15 mins	0.9
3	10 mins	0.4
4	25 mins	1.1
5	30 mins	0.8

Table 2 — Change in weight of HAP/ β-TCP with change in coating voltage at constant time (20 min)

Sample No.	Voltage applied (V)	Weight gain (mg)
1	20 V	1.5
2	15 V	0.9
3	10 V	0.4
4	25 V	1.0
5	30 V	0.5

Figure 2 shows the images of the coated sample obtained at 20V, 20min on 316L SS after sintering using Canon Power Shot SX530 Digital Camera. The image obtained shows that uniform coatings were formed on the cathode 316L SS which were strongly adhered to the substrate even after sintering. The above image confirms the formation of uniform coatings of the biphasic material HAP/β-TCP on 316L SS after sintering. The images indicate the absence of base metal oxides on the surface of the coatings indicating that the coated metal does not lose its property even after sintering.

Characterization studies

XRD

X-Ray diffraction studies in Fig. 3 (a, b & c) shows the patterns of HAP, β-TCP and HAP/β-TCP composites, respectively which represent that the HAP and β-TCP exists together in the prepared composite. The peaks such as (002), (121), (300), (222), (310), (321), (305), (502), were attributed to the crystalline nature of the HAP and was matched with the JCPDS file no.09-0432. These patterns suggest that there is no structural transformation of HAP after sintering. The peaks obtained for β-TCP are (221), (110), (200), (311), (220), (433) and were matched according to the JCPDS file no 09-1069.

FTIR spectroscopy analysis

Fourier transform infrared spectroscopy is used to determine the different functional groups present in the coated sample. The spectrum was recorded in the range of 4000-490 cm^{-1}. Figure 4 shows the FTIR pattern of (a) HAP, (b) β-TCP and (c) HAP/β-TCP on 316L SS obtained at 20V, 20 minutes after sintering. Theoretically, there are four vibration modes present

Fig. 2 — Images of the coated sample obtained at 20V, 20min on 316 LSS after sintering.

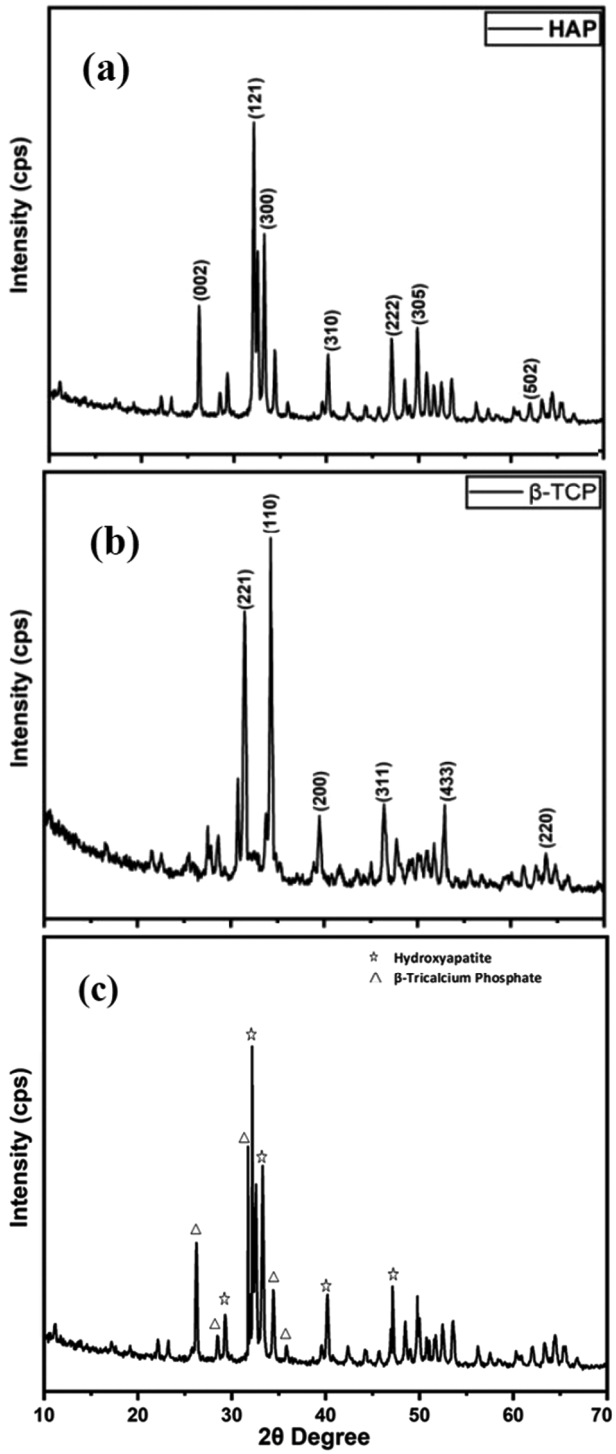

Fig. 3 — XRD pattern of (a) HAP, (b) β-TCP and (c) HAP/β-TCP on 316 LSS obtained at 20V, 20 minutes after sintering.

in phosphate ion q1, q2, q3 and q4. The q1 and q3 phosphate bands in the 900-1200 cm^{-1} and q4 absorption bands in the region 500-700 cm^{-1} are used to characterize the structure of apatite. The strong sharp peaks represent the symmetric and asymmetric

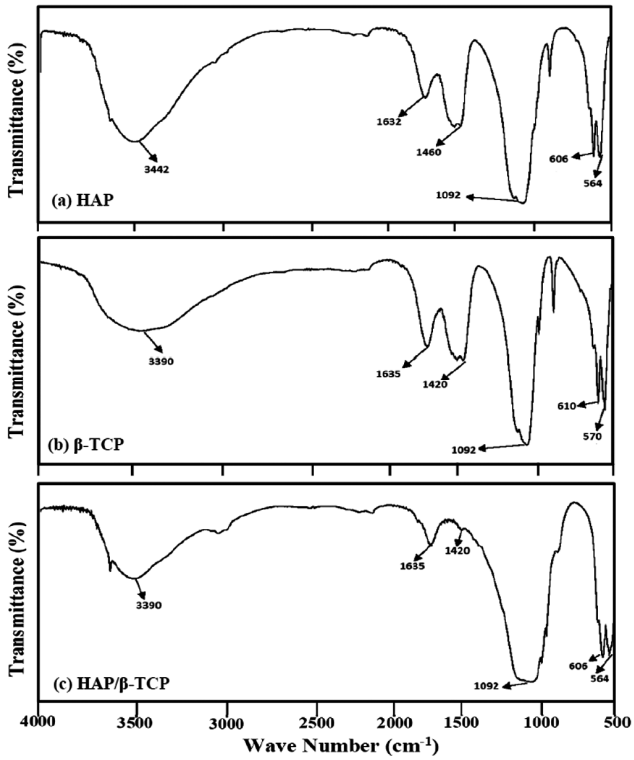

Fig. 4 — FTIR pattern of (a) HAP, (b) β-TCP and (c) HAP/β-TCP on 316 LSS obtained at 20V, 20 minutes after sintering.

stretching mode of vibration for PO_4^{3-} group. Specifically, the peak obtained between the range of 510-610 cm^{-1} and 1010-1094 cm^{-1} corresponds to the q4 and q3 modes of phosphates ions, respectively. The peak at 1420cm^{-1} corresponds to the presence of carbonate group (CO_3^{2-}). The peak at 3390 cm^{-1} represents the presence of O-H stretching vibrations.

Biocompatibility assay

A cell line of normal cell (Vero cells) and the cancerous cell (HEP II cells) obtained from NCCS, Pune, India was used for the MTT assay.

Cell viability and biocompatibility studies using vero cells

The percentage of cell viability in normal vero cell is given in the Table 3. These results show that 30% cells are viable at the concentration of 5 µg and EPD coatings coated over the sample alone can interact with the cells. This indicates good compatibility of the coatings. Figure 5 shows the cell cytotoxicity dose for HAP/β-TCP on 316L SS at 20V, 20 minutes after sintering- Normal cells (CCD: CCD 50 =1.6µg). Hence, the study suggests that more than 50% viability can be seen while using the coated material as an implant device in normal cells.

Figure 6 shows cell viability in vero cells (A) normal vero cell line (B) 50% cell viability with

Table 3 — Changes in % of cell viability of Vero-Cells with changes in biphasic HAP/ β-TCP coatings

Concentration of HAP/ β-TCP (µg)	Dilution used	Optical Density (nm)	% of Cell Viability
5	1:1	0.12	30
2.5	1:2	0.12	33
1.25	1:4	0.21	56
0.625	1:8	0.22	59
0.3125	1:16	0.25	68
0.156	1:32	0.29	77
0.078	1:64	0.32	87
0.039	1:12	0.33	90
0	1:64	0.37	100

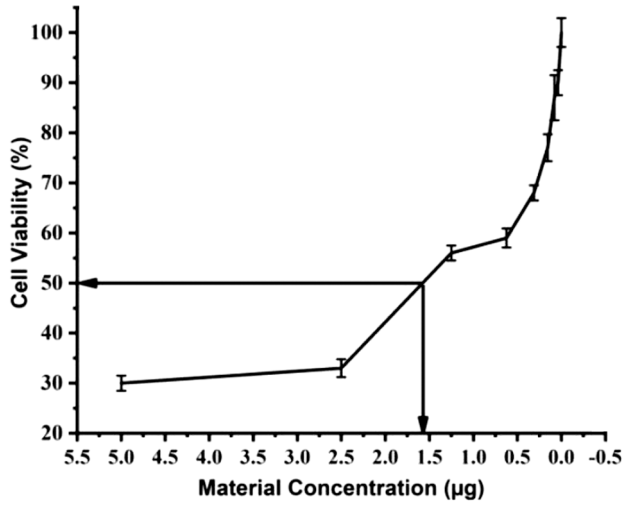

Fig. 5 — Cell cytotoxicity dose for HAP/β-TCP on 316 LSS at 20V, 20 minutes after sintering- normal cells (CCD: CCD 50 =1.6µg).

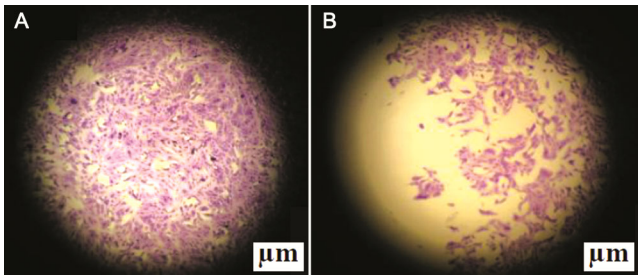

Fig. 6 — Cell viability in vero cells (A) normal Vero cell line (B) 50% cell viability with biphasic HAP/ β-TCP coated samples.

biphasic HAP/ β-TCP coated samples under Light microscopy. 30% of the coating weight was calculated at different potentials and it was standardized to a coat value of 0.01 µg and this indicates that these biphasic materials are not toxic to the cells. The material biocompatibility data are presented in Table 3. Normally, 50% of cells are viable, which indicates that the materials show a good

Table 4 — Changes in % of cell viability of HEP II-Cells with changes in biphasic HAP/ β-TCP coatings

Concentration of HAP/ β-TCP (µg)	Dilution	Optical Density (nm)	% of Cell Viability
5	1:1	0.18	27
2.5	1:2	0.19	29
1.25	1:4	0.21	32
0.625	1:8	0.26	39
0.3125	1:16	0.38	58
0.156	1:32	0.45	68
0.078	1:64	0.55	83
0.039	1:12	0.56	85
0	1:64	0.65	100

biocompatibility with the particular cell line. Hence, from the standardization procedure one can confirm whether a particular material is not toxic to the particular cell line.

The biphasic coatings obtained by electrophoretic deposition; the maximum amount of the material distribution occurs over the metal was found to be 0.454 µg. IC 50 value represents when the viability is 50% which indicates the concentration corresponding to the 50% viability. It is an accepted concentration used as an implant. In the present work the 50% viability corresponds to the 1.6µg /mL and this is very much high when compared to the concentration of material coated over the substrate. For the samples with maximum coating weight of about 0.454 µg shows more than 90% viability and this confirms that the materials after coating and sintering shows good biocompatibility.

Cell viability of HEP II cells

The changes in percentage (%) of cell viability of HEP II-Cells with changes in biphasic HAP/ β-TCP coatings are given in Table 4. The analysis shows 27% cells were viable at the concentration of 5 µg. It was suggested that the weight corresponding to 50% viability shows good compatibility for that particular size. Figure 7 shows the cell cytotoxicity dose for HAP/β-TCP on 316L SS at 20V, 20 min after sintering- Cancerous cells (CCD: CCD 50 =0.4µg). When the substrate is subjected to EPD for 20 minutes duration at 20V maximum weight gain of 0.454 µg is obtained, which is more than 0.4 µg. Figure 8 shows the cell viability in HEP II CELLS (A) HEP II cells in addition of HAP/ β-TCP under light microscopy (B) HEP II cells after addition of HAP/ β-TCP with 50% cell viability under light microscopy. The stained structure in the micrographs

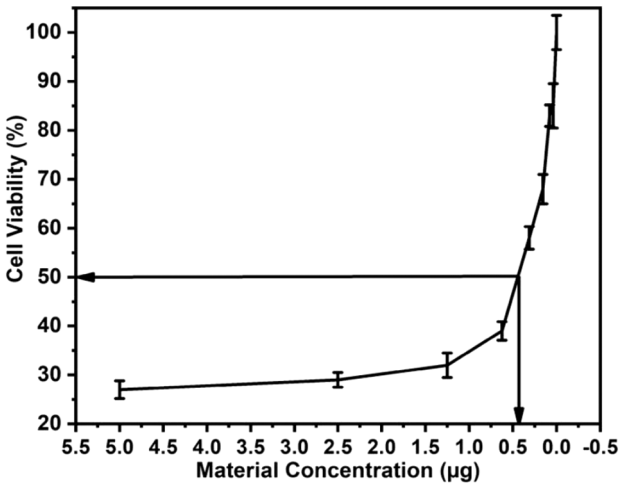

Fig. 7 — Cell cytotoxicity dose for HAP/β-TCP on 316 LSS at 20V,20 minutes after sintering- cancerous cells (CCD: CCD 50 =0.4µg).

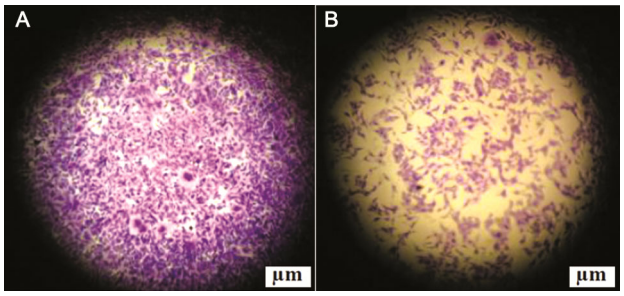

Fig. 8 — Cell viability in HEP II cells (A) HEP II cells before addition of HAP/ β-TCP under light microscopy and (B) HEP II cells after addition of HAP/ β-TCP with 50% cell viability under light microscopy.

indicates that the viable cells which are very less. Hence, the study suggests that more than 50% viability cannot be obtained while using the coated material as an implant device in cancerous cells. This study concludes that the implant device coated with HAP/β-TCP over 316L SS with 20V and 20 min duration of the substrate with a maximum of 5 g should show the biocompatibility with vero cells and not to the HEP II cells.

DPPH assay

Figure 9 shows DPPH activity of HAP/β-TCP on 316L SS at 20V, 20 min after sintering. DPPH assay proves that the coated sample (at 20V, 20 min) has the antioxidant activity above 50% which is required for implants to be placed inside the biological medium. All values obtained are acquired from UV spectrophotometer for assays.

% Anti-oxidant activity = {(absorbance at blank) – (absorbance at test) / (absorbance at blank)} ×100 …(1)

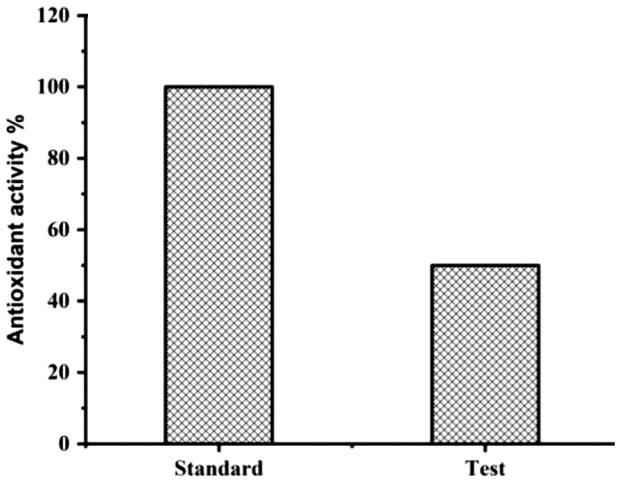

Fig. 9 — DPPH activity of HAP/β-TCP on 316 LSS at 20V & 20 minutes, after sintering.

From the above test it has been inferred that the sample traps the free radicals.

Conclusion

Biphasic coatings of HAP and β-TCP have been successfully coated on medical grade 316L SS electrophoretic deposition. No changes in crystalline structure and vibration properties have been observed before deposition and after sintering of the biphasic coatings. This biphasic coating are nontoxic which has been proved by the cytotoxicity test. The DPPH assay shows the antioxidant activity of the sample which proves that the sample traps the free radicals in the biological media. The study indicates that biphasic coatings are biocompatible and can be used for orthopaedic applications.

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