Original Article

Comparison of osteogenic potential of poly-ether-ether-ketone with titanium-coated poly-ether-ether-ketone and titanium-blended poly-ether-ether-ketone: An *in vitro* study

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Abstract

Statement of Problem: Poly-ether-ether-ketone (PEEK), a high-performance semi-crystalline thermoplastic polymer, has been employed to replace the metallic implant components in orthopedics. There were various studies performed in accordance to medical grade PEEK, but the relationship between titanium dioxide (TiO_2)-coated PEEK, TiO_2-blended PEEK, and untreated PEEK still remains complicated, even undefined.

Purpose: The purpose of this study was to compare and quantify the osteogenic potential of untreated PEEK, TiO_2-coated PEEK and TiO_2-blended PEEK.

Materials and Methods: Three groups with ten samples in each group were designed for this study. They were Group 1 - Untreated PEEK, Group 2 - TiO_2-coated PEEK, Group 3 - TiO_2-blended PEEK. The PEEK samples were prepared according to the ISO standard 15309:2013 and milled to size of 15 mm × 2 mm, and the surfaces were finished with grit-blasted alumina of size 20 μm. In this 10 samples were chosen for Group 1. Group 2 samples were prepared by coating TiO_2 nanoparticles by arc ion plating, and Group 3 samples were prepared by blending TiO_2 nanoparticles in HAAKE rheocord with degree of blending analyzed by torque rheometer. These samples were tested for cytotoxicity using human osteosarcoma cells, and alkaline phosphatase (ALP) activity was performed to evaluate and quantify the bone mineralization process. The cross-sectional and the fracture morphology of coatings was observed by a field emission scanning electron microscope (SEM) with the magnification range ×20–×200,000.

Result: Results of cytotoxicity assay and ALP assay of Group 1, Group 2, and Group 3 were statistically analyzed. SEM analysis result clearly showed the difference in the matrix before and after cell adhesion.

Conclusion: The results made it evident that n-TiO_2-coated PEEK was more versatile biomaterial of choice in implant dentistry followed by n-TiO_2-blended PEEK and untreated PEEK.

Key Words: Alkaline phosphatase activity, osteogenic potential, poly-ether-ether-ketone, titanium dioxide-blended poly-ether-ether-ketone, titanium dioxide-coated poly-ether-ether-ketone

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INTRODUCTION

Irrespective of the atrophy, disease or injury of the stomatognathic system, the approach of modern dentistry is to restore the patient with normal function, speech, and health. To support this, over 30 years, dental implant procedures have steadily increased worldwide.[1] The biomaterial discipline has grown ominously over the past decades. Various biomaterials have been introduced in the arena of implant dentistry. For a long time, this was the exclusive domain of titanium or cobalt-chromium.[2] Of recent, titanium and titanium alloys are gaining momentum as biomaterial due to its physical, chemical, and mechanical stability and suitability over other materials. Titanium, the only metal biomaterial to osseointegrate[3] has become the gold standard in implant dentistry due to its frequent usage as implant material in Branemark's studies. Titanium can be alloyed with a wide range of elements to alter its properties, mainly for the purposes of improving strength, high-temperature performance, creep resistance, reaction to aging heat treatments, and formability.[4] However, surface modification of titanium implants disturbs the rate of osseointegration and biomechanical fixation[5,6] which is being overcome through various methods, such as plasma spraying, acid etching.[7] Due to various pitfalls and drawbacks, researchers are now trying to avoid the usage of metals and seek for polymeric materials which can be conveniently used for applications in the field of biomedicine.[8]

Poly-ether-ether-ketone (PEEK), a high-performance semi-crystalline thermoplastic polymer, has been employed to replace the metallic implant components in orthopedics,[9-11] traumatology.[12] Being colorless and having low elastic moduli close to that of bone, it is a viable option for dental implant. In general, PEEK alone is a bio-inert and is not conductive to cell adhesion.[13] Studies revealed that biocompatibility of PEEK was improved on surface modification with titanium.[4] However, newer materials such as PEEK implant materials[5] have low elastic moduli which is very close to that of human cancellous bone, and it has less stress shielding effect when compared to metal implant.[1] In addition, PEEK materials are radiolucent and do not present a medical image shielding problem.[6] Its bio-inertness and hydrophobic surface properties are not suitable for fast bone cell adhesion leaving a longer fusion period between bone and PEEK implant.

In the present study, the biopolymer, PEEK was modified with titanium dioxide (TiO_2) particles in two different ways, such as TiO_2-coated PEEK and TiO_2-blended PEEK. The surface modified PEEK was compared and analyzed for osteogenic potential and bone mineralization.

MATERIALS AND METHODS

Sample preparation

Three groups with ten samples in each group were designed for this study. They were

- Group 1 - Untreated PEEK as a control group
- Group 2 - TiO_2-coated PEEK
- Group 3 - TiO_2-blended PEEK.

To standardize the study, 30 disc samples of size 15 mm × 2 mm were milled from PEEK substrate [Figure 1] with ISO 15309:2013 standards. Tungsten carbide cutting tips were used in milling peek into required size. The heat generated is nullified by compressed air which acts as coolant. These discs were polished with 2000 grit SIC abrasive paper and then ultrasonically cleaned 3 min each in acetone, ethanol, and distilled water. From these 30 samples, 10 samples were grouped as control group, Group 1 [Figure 2a].

In Group 2, ten samples were coated with TiO_2 by AIP technique to deposit anatase TiO_2 onto PEEK substrate. Anatase TiO_2 was deposited under oxygen working pressure 0.5 Pa, cathode voltage 20 V, cathode current 90

Figure 1: Poly-ether-ether-ketone ingot before milling

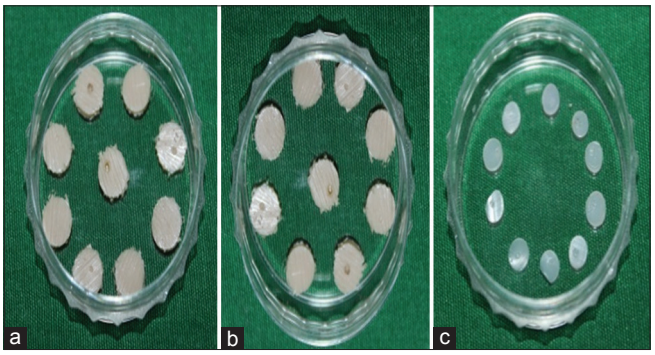

Figure 2: (a) Prepared composite material after milling. Untreated poly-ether-ether-ketone, (b) N-titanium dioxide-coated poly-ether-ether-ketone, (c) N-titanium dioxide-blended poly-ether-ether-ketone

A, and deposition time of 30 min. Crystal structure of the TiO_2 coating was examined using a thin-film X-ray diffractometer. TiO_2 coating adhesion was observed by adhesive tape test which was carried out in harmony with the ASTM D3359-02 standard and followed by microscopic observation to identify any ruptures in the coating [Figure 2b].

Group 3, ten samples [Figure 2c] were prepared by blending TiO_2 nanoparticles with 40 wt%, bending modulus 3.8 GPa and bending strength 93 MPa using an electronic blender HAAKE rheocord in alcohol. The degree of blending was analyzed by the torque rheometer. The samples were then dried in a forced convection oven at 90°C to remove the excess alcohol. The treated samples were preheated to 150°C under a load of 35 MPa, and the temperature was increased to 400°C with a pressure of 15 MPa for 10 min, and after this, the heater was turned off, and after 10 min, the pressure was released. The samples were air cooled to 150°C and removed from the HAAKE rheocord. After treatment, the samples surface morphology was observed using scanning electron microscope (SEM), and the presence of TiO_2 was confirmed.

Evaluation of osteogenic potential

Murine preosteoblastic cell line MC3T3-E1 was plated in all the samples and incubated for 30 min at 37°C to allow adhesion. In this study, alkaline phosphatase (ALP) activity and cytotoxicity evaluation were done to evaluate the osteogenic potential quantitatively.

Alkaline phosphatase activity

ALP increases if there is active bone formation, and it is a byproduct of osteoblastic activity. ALP was measured using ALP assay kit for 48 h. The osteoblast cell compatibility test was conducted four times, and the average was taken. Cells on test specimens were fixed, dehydrated, and critical point dried, cell morphology was observed by SEM.

Cytocompatibility *in vitro*

Evaluating the cytocompatibility is mandatory to detect the progress of the osteoblastic activity of the test specimens. The cell attachment, cytotoxicity, morphology of cells, and flow cytometric analysis were evaluated by means of MG-63 osteoblast cells which were acquired from the American Type Culture Collection. MG-63 was cultured at 37°C in a humidified, 5% CO_2/95% air incubator, in improved Eagle's medium with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Before *in vitro* testing, all the samples were sterilized by gamma radiation at a total measure of 25 KGγ. The MG-63 cells were seeded by a density of 1 × 105 cells in each of well plates for cell attachment. Total of three culture periods 3, 7, and 14 days for cytotoxicity, cell morphology, and flow cytometric analysis.

Cell attachment

After culture, the culture medium was removed, and the specimen was rinsed with phosphate-buffered solution (phosphate-buffered saline [PBS]) to remove the unattached cells. Adherent cells were incubated on samples at 37°C for another 4 h, and then, 100 μL of the culture medium was transferred into each well. Ultraviolet absorbance was measured using an enzyme-linked immunosorbent assay reader by a wavelength of 450 nm with the reference wavelength of 630 nm.[14]

After different culture periods of 3, 7, and 14 days, the relative growth rate was calculated and evaluated using the water-soluble tetrazolium salt (WST-1) test on the rough and smooth surfaces of all samples.

To observe the cell morphology, the samples were washed with PBS at fixed experimental times 3, 7, and 14 days, and cells were fixed with 4% glutaraldehyde in PBS (pH 7.3) for 30 min. Cell number was measured using a cell counting kit-8, and ALP activities were measured with a p-nitrophenyl phosphate solution. For cell counting, 100 μl of WST-8 was added for each well containing 1 mL of fresh medium followed by incubation for 1 h, and absorbance was measured at 450 nm. After this, each well was washed twice with PBS and 800 μL of p-nitrophenyl phosphate solution was added to each well. After 10 min of incubation at 37°C, the conversion to p-nitrophenol was stopped with 800 μl of 3N NaOH, and the absorbance of p-nitrophenol was measured at 405 nm. ALP-specific activity is expressed as p-nitrophenol absorbance (OD; 405 nm)/WST-8 absorbance (OD; 450 nm). Then, the cells were fixed with 7% ethanol for 1 h, washed, and stained for 10 min using 40 mm alizarin red S solution (pH: 4.2). After this, washing was done with PBS, and the plates were incubated with 10% cetylpyridinium chloride for about 15 min. Then, the samples were collected from respective well, and the absorption of individual supernatant was restrained at 405 nm to determine the amount of calcium deposition.

Data were analyzed by independent variances, and normal distribution of errors was verified for the response variables evaluated. Data were expressed as mean ± standard deviation. Mean difference between the groups were analyzed by one-way ANOVA and followed by Tukey's multiple comparison as *post hoc* test. The $P \leq 0.05$ was considered statistically significant. Statistical analysis was performed using MS Excel.

RESULTS

There was a significant change ($P < 0.01$) in metabolic activity of cells on scaffolds among the groups. On day 3, [Table 1] there was a significant difference in Group 2 ($P < 0.001$) and Group 3 ($P < 0.01$) samples when compared to Group 1 samples. However, no statistical significance ($P > 0.01$) was noted between the Group 2 and Group 3 samples. On day 5, [Table 2] a drastic change in absorbance values were noted in Group 2 when compared to other two groups. Group 2 samples were statistically significant ($P < 0.001$) when compared to other groups. In addition, a significant difference ($P < 0.01$) was noted in Group 3 samples on comparison with Group 1. The results of the study on day 7 [Table 3] showed that the Group 2 samples yield the maximum absorbance as compared with others. Significant difference ($P < 0.001$) was observed in all type of materials on day 7. The result from the graph [Figure 3] makes it evident that the Group 2 samples showed maximum cell proliferation as a result possesses least cytotoxicity against the biomaterial followed by Group 3 samples, whereas the Group 1 samples showed the least adhesion ability.

ALP, being the by-product of osteogenic activity, was read calorimetrically. After 7 days of incubation, the Group 2 samples were found to possess a significant difference when compared to Group 3 ($P < 0.01$) and Group 1 ($P < 0.001$) samples. In addition, a significant difference ($P < 0.01$) was observed between Group 3 and

Table 1: Tukey Honestly Significant Difference *post hoc* tests for multiple comparisons of MTT absorbance at 570 nm at 2 days standpoint

Dependent variable	Group	Mean difference	*P*
MTT - Absorbance at 570 nm at 2 days standpoint	Group-A		
	Group-B	−0.0280	<0.001
	Group-C	−0.0204	0.003
	Group-B		
	Group-C	0.0076	0.285

Table 2: Tukey Honestly Significant Difference *post hoc* tests for multiple comparisons of MTT absorbance at 570 nm at 5 days standpoint

Dependent variable	Group	Mean difference	*P*
MTT - Absorbance at 570 nm at 5 days standpoint	Group-A		
	Group-B	−0.0718	<0.001
	Group-C	−0.02360	0.003
	Group-B		
	Group-C	0.0482	<0.001

Table 3: Tukey Honestly Significant Difference *post hoc* tests for multiple comparisons of MTT absorbance at 570 nm at 7 days standpoint

Dependent variable	Group	Mean difference	*P*
MTT - Absorbance at 570 nm at 168 h	Group-A		
	Group-B	−0.0738	<0.001
	Group-C	−0.0288	<0.001
	Group-B		
	Group-C	0.0450	<0.001

Table 4: Tukey Honestly Significant Difference *post hoc* tests for multiple comparisons of alkaline phosphatase activity at day 7

Dependent variable	Group	Mean difference	*P*
ALP activity (IU/L) at day 7	Group-A		
	Group-B	−37.8	<0.001
	Group-C	−20.4	0.002
	Group-B		
	Group-C	17.4	0.006

ALP: Alkaline phosphatase

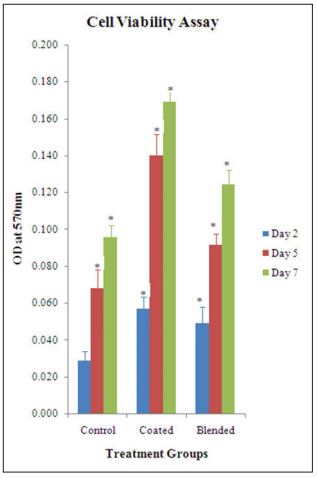

Figure 3: Graph depicts the cytotoxicity effect of osteoblast cells against the specimen samples with a period of 2, 5, and 7 days. Values are expressed in mean ± standard deviation; $n = 5$; significance with Turkey's multiple comparison followed by one-way ANOVA. *$P < 0.01$ when compared to control group

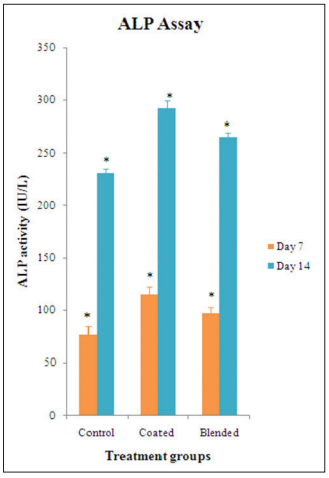

Figure 4: Levels of alkaline phosphatase produced (IU/L) by coated, blended, and control groups with an incubation period of 7 and 14 days. Values are expressed in mean ± standard deviation; $n = 5$; significance with Turkey's multiple comparison followed by one-way ANOVA. *$P < 0.01$ when compared to control group

Group 1 samples [Table 4]. The reduced cytotoxicity of the biomaterial aids maximum adhesion of cells resulting in the production of ALP in higher levels. On further incubation of cells to 14 days, the ALP produced was found to be statistically significant ($P < 0.001$) between all types of biomaterials. Graph [Figure 4] depicts that the amount of ALP produced after 14 days of seeding was comparatively higher than the other period. However, the Group 2 samples possessed maximum levels of ALP activity followed by Group 3 samples [Table 5].

Scanning electron microscope analysis

To understand the effect of implant surface morphology on cell culture and biologic responses of Group 1, Group 2, and Group 3 samples, SEM analysis was carried out. The surface topographies observed by SEM for osteoblast cells, seeded onto the Group 1, Group 2, and Group 3 samples, after an incubation period of 14 days [Figures 5-7]. The images clearly depict that the efficacy of cell adhesion and proliferation for osteoblast cells. From the figure, it was noted that the surface of Group 2 [Figure 6a] and Group 3 [Figure 7a] samples was smoother than Group 1 samples [Figure 5a]. In addition, it was seen that the cell proliferation seems to be rapid leading to bone formation in Group 2 [Figure 6b] followed by Group 3 [Figure 7b] and Group 1 [Figure 5b].

Table 5: Tukey Honestly Significant Difference *post hoc* tests for multiple comparisons of alkaline phosphatase activity at day 14

Dependent variable	Group	Mean difference	*P*
ALP activity (IU/L) at day 14	Group-A		
	Group-B	−61.2	<0.001
	Group-C	−34.0	<0.001
	Group-B		
	Group-C	27.2	<0.001

ALP: Alkaline phosphatase

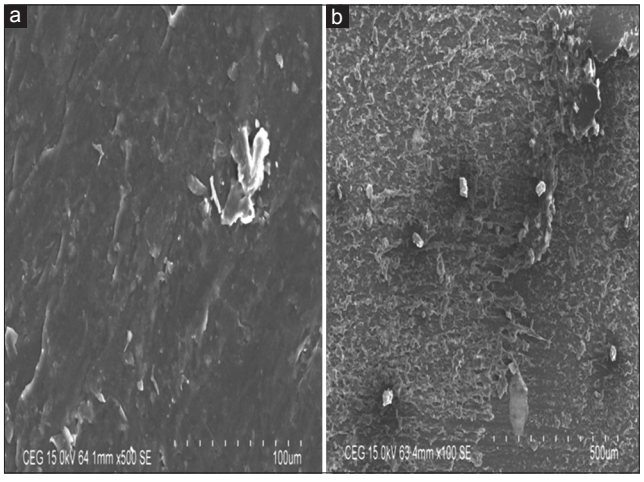

Figure 5: (a) Scanning electron microscope images of untreated poly-ether-ether-ketone before bone response, (b) scanning electron microscope images of untreated poly-ether-ether-ketone after bone response

DISCUSSION

PEEK is a synthetic and tooth-colored polymer and has melting point around 335°C. Studies have shown that PEEK can be easily modified by incorporation of other materials such as carbon fibers, hydroxylapatite, and titanium.[15] The mechanical and physical properties of PEEK is similar to bone and dentin, so increasing the bioactivity of PEEK without disturbing their mechanical properties is a foremost challenge.[16,17] Kurtz and Devine studied the mechanical properties of peek and its clinical impact in the field of spine implants, dental implants, total joint replacements, and fracture fixation implants. He involved the process of evaluating the osteogenic potential of peek and concluded that these composite biomaterials are effectively inert biologically speaking radiolucent alternatives to metallic biomaterials.[18] Hence, in this study, PEEK was modified by coating and blending with TiO2, and the osteogenic potential was compared. The first generation machined smooth surface implants were replaced by textured implant surfaces which comprise mechanical blasting with acid etching, bioactive coatings, anodized, and more recently, laser-modified surfaces.[19,20] Nano TiO_2 coating produces profound osteogenic potential *in vitro* cell culture.[21,22] Long-term performance of surgical implants is often constrained by their surface properties. Low wear resistance of titanium primes to the problem of reduced life of the implants which can be overcome to greater extent using suitable surface coatings. The four primary factors that affect the biocompatibility between biomaterial and cell contact are composition of the surface, surface energy, roughness of the surface, and surface topography.[23] Knowing that PEEK is a hydrophobic material, having

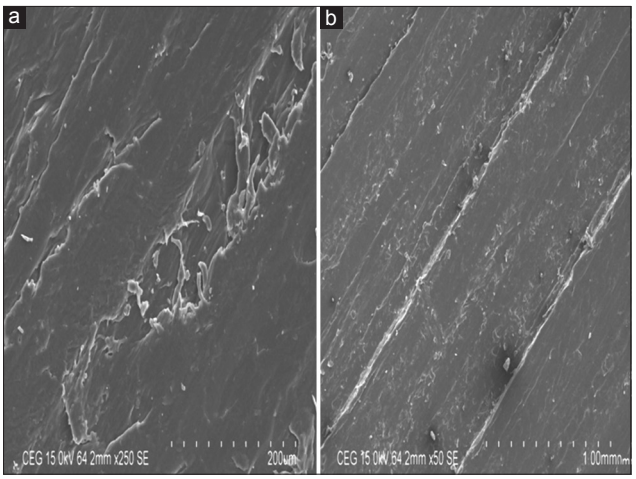

Figure 6: (a) Surface topography of titanium dioxide-coated poly-ether-ether-ketone before bone response by scanning electron microscope analysis, (b) surface topography of titanium dioxide-coated poly-ether-ether-ketone after bone response by scanning electron microscope analysis

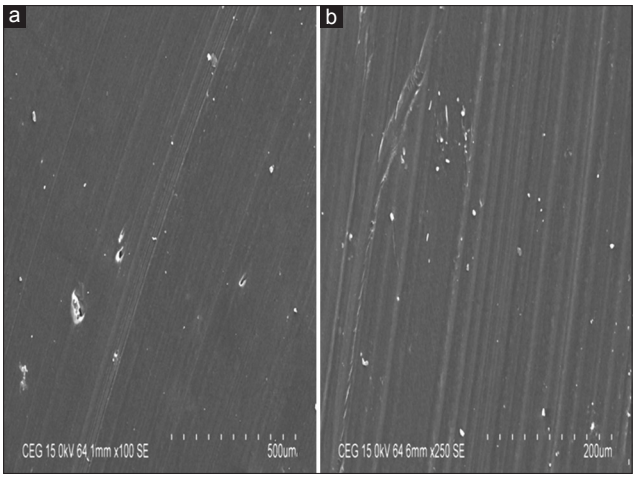

Figure 7: (a) Scanning electron microscope analysis of titanium dioxide-blended poly-ether-ether-ketone before bone response, (b) scanning electron microscope analysis of titanium dioxide-blended poly-ether-ether-ketone after bone response

exceptional biomechanical properties, there is a further need to improve its bioactivity for application in dental and orthopedic fields. Najeeb *et al*. studied the effects of TiO_2 nanoparticles on human neutrophils. They determined the effects of TiO_2 on two neutrophil functions requiring longer exposure periods between nanoparticles and cells and concluded that TiO_2 has neutrophil agonistic properties.[15] The present study was aimed in evaluating the *in vitro* bioactivity of surface-modified PEEK and PEEK ingot itself. Hence, because of this, property titanium in oxide form was used to modify the PEEK surface in this study. The developed biomaterials were found to be nontoxic resulting in good biocompatibility when assayed. According to ISO standards, a material is considered biocompatible only when it is nontoxic to cells during *in vitro* testing. The fabricated biomaterial did not show any toxic effect against the osteoblast cells. It was found that the TiO_2 nanoparticles incorporated PEEK significantly improved the cell adhesion than untreated PEEK.

Gaggl *et al*. studied topographically modified surfaces of general implants without introducing chemical treatment. They concluded titanium plasma method and alumina oxide blasted implant surfaces did not produced optimal surface purity. However, laser processing was the new method of treating implant surfaces to produce high degree of purity while coating pure metals on biomaterials.[24] In this study, titanium dioxide-coated samples showed better cell adhesion and osteoblastic activity similar to mentioned above. Gutwein and Webster studied proliferation of osteoblast and chondrocytes exposed to different sizes of alumina as well as titanium particles at various concentrations was investigated in a *in vivo* study. The study concluded the wear debris at the implant-bone interface was lower in nanoparticle-treated implant than in the conventional implant material.[25] Hence, in this study, PEEK surface was modified by roughing the surface and which was coated and blended by TiO_2, and the osteogenic potential was compared. Germanier *et al*.[26] studied early bone opposition on a modified sandblasted large grit acid-etched (SLA) surface coated with peptide-modified polymer in the maxilla of miniature pigs and compared to standard SLA surface. At 2 weeks, the modified SLA surface-coated implants showed significantly higher percentage of bone-implant contact (BIC) as compared to the controls and concluded that the coatings may promote enhanced bone opposition during early stages of bone regeneration. Hence, in this study, PEEK surfaces were modified with TiO_2 and the osteogenic potential was examined. Zhao *et al*.[27] evaluated that surface roughness and surface-free energy were important factors that regulate cell response to biomaterials and concluded that there is increased cell response to increase in surface energy. Hence, in this study, PEEK surface was modified, and the cell response was examined.

Wong *et al*. evaluated that polyether ether ketone containing material such as strontium, titanium have enhanced properties. This *in vitro* study represents the mechanical properties and human osteoblast-like cell response to composite material. Strontium, titanium tends to increase the bioactivity of PEEK composites.[28] Hence, the present study also showed more cell growth when the surface of PEEK was modified with TiO_2. Han *et al*. made a study with titanium (Ti) layer using an electron beam deposition. The result indicated Ti-coated implant showed better BIC than pure PEEK, ALP assay was used to evaluate bone formation.[29] Based on this, in the present study, PEEK was coated with TiO_2 using arc ionic plating technique, and blending was done by electronic blender (HAAKE rheocord).

In this study, the TiO_2 nanoparticles and PEEK composite were fabricated successfully. Two different fabrications were done to make a comparative report between the coated and the blended PEEK as researches have been carried out only with TiO_2 coatings as the biomaterial chosen for the study.[30,31] The results of MTT assay suggest that the cells are nontoxic to the biomaterial and enhance better cell adhesion as the period increases. It was evident from the results that the increase in absorbance was directly proportional to the number of viable cells. The TiO_2-coated PEEK showed an increased level of absorbance than others revealing that it aids maximum cell adhesion ability followed by TiO_2-blended PEEK. Cell proliferation was enhanced up to 7 days in the coated groups.

On the other hand, the results of ALP activity, a direct measure of osteogenic potential, exhibit increased levels of ALP (IU/L) on day 14 in all the groups than day 7. However, the TiO_2-coated groups possess the maximum ALP activity than other groups on both time periods. In a humid environment, crystalline TiO_2 coatings will form negatively charged – OH^- groups at their surface. Since – OH^- groups absorb Ca^{2+} and PO_4^{3-} to form bone-like apatite, cell adhesion and growth[32,33] were greatly facilitated. Moreover, an AIP-prepared TiO_2 coating surface contains microparticles that were activated by cathode titanium to provide better cell adhesion. The bone-like apatite can be made possible on a long-term implant restoration which can be analyzed through ALP activity.

In vitro studies showed that TiO_2 nanoparticles do not cause any severe cytotoxicity or interfere in cell cycle progression but improved bioactivity of surface-modified PEEK. Further, it was justified with the results of SEM analysis that the TiO_2-coated PEEK showed an obvious bone-like formation than untreated PEEK. The blended PEEK samples also showed an promising growth but were not distinct than coated sample. Although there are several biomaterials available for dental implants, due to number of advantages, PEEK was chosen for this research. Since PEEK is inert in nature, a composite along with TiO_2 in a surface-modified fashion was enabled, and the TiO_2-coated PEEK showed least cytotoxicity and maximum ALP activity indicating that it could be the ideal biocomposite material which can be used in the field of dentistry and orthopedics.

Limitations of the study

In this study, only the ALP activity and the cytotoxicity had been used to prove the osteogenic potential of the samples. Different ways of coating n-TiO_2 on PEEK are there, but only arc ion plating was used for coating PEEK in this study. Coating of only the oxide form of titanium was used in this study even though coating of pure titanium is possible through other surface coating mechanisms.

Further scope of study

The study revolves around evaluating the osteogenic potential using ALP activity and the cytotoxicity. Osteogenic potential can also be evaluated through various other methods such as calcium assay, evaluating bone markers, cell morphology, and flow cytometric analysis. In the present study, PEEK was modified by TiO_2, but other surface treatments such as deposition of noble metals or hydroxyapatite plasma spraying can also be done and evaluated for bone response in future.

CONCLUSION

In this study, untreated PEEK, TiO_2-coated PEEK, and TiO_2-blended PEEK samples were compared and evaluated for osteogenic potential and concluded as follows:

1. TiO_2-coated PEEK exhibited maximum ALP activity followed by TiO_2-blended PEEK and untreated PEEK
2. TiO_2-coated PEEK exhibits least cytotoxicity followed by TiO_2-blended PEEK and maximum toxicity attributed to the untreated PEEK
3. The results make it evident that TiO_2-coated PEEK was more versatile biomaterial of choice in implant dentistry followed by TiO_2-blended PEEK.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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