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Enhanced Tissue Compatibility of Polyetheretherketone Disks by Dopamine-Mediated Protein Immobilization

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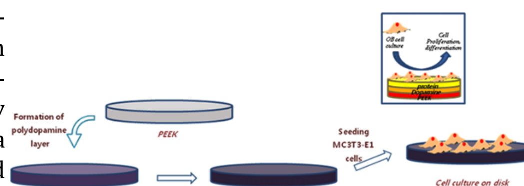
Abstract: Polyetheretherketone (PEEK) disks with hydrophilic and bioactive surface properties were prepared by covalent immobilization of collagen (Col) or insulin (In) on poly-dopamine (D)-coated PEEK samples using WSC (water soluble carbodiimide) as an activating agent. The poly-dopamine on the PEEK disk was coated by carrying out self-assembled polymerization of the dopamine neurotransmitter in a basic medium at pH 8.5. The poly-dopamine coating of the PEEK surface facilitated the deposition of a uniform layer of aligned molecules of collagen, which increased the bioactivity of the PEEK surfaces allowing for adhesion, proliferation, and osteogenic differentiation of MC3T3-E1 cells in a minimum essential medium in the presence of 5% CO_2 at 37 °C. The collagen-modified PEEK surface had higher bioactivity for MC3T3-E1 cells, compared to the self-assembled poly-dopamine coated PEEK surface or pristine PEEK alone. Alkaline phosphatase, von Kossa, and Alizarin red staining of MC3T3-E1 cells cultured on a collagen-modified PEEK surface, were all found to be increased compared to staining of cells cultured on other PEEK surfaces. The self-assembled polymerization of dopamine on a PEEK surface was found to be useful for the immobilization of proteins such as collagen and insulin. Thus, collagen- and insulin-immobilized PEEK provide an opportunity to enhance the bioactivity of the PEEK samples allowing for better cells adhesion and tissue integration for potential use in tissue implants.



Keywords: polyetheretherketone, dopamine, collagen, MC3T3-E1 cells, osteogenic differentiation.

1. Introduction

Polyetheretherketone (PEEK) is biologically inert,[1] semi-crystalline linear polycyclic aromatic thermoplastic material with current medical utility. PEEK is biocompatible, chemically stable, and radiolucent. However, for implantation, its use in clinical practice is limited due to poor integration with adjacent bone tissues. Though elastic modulus of PEEK is much lower (8.3 GPa) in comparison to metallic implants such as, Ti alloy (116 GPa), Co-Cr alloy (210 GPa) but comparable to elastic modulus of human cortical bone (17.7 GPa). Despite its clinical limitations, the high-performance thermoplastic PEEK is becoming a popular component, especially for orthopedic, traumatic, and spinal applications.[2-6] Radiolucent spinal implants are generally made from PEEK as it does not interfere during computer tomography (CT) or magnetic resonance imaging (MRI) scanning. With the emergence of a carbon fiber-reinforced PEEK composite it has been a major feature in fracture fixation and femoral prosthesis in artificial hip joints.[7-10] Over the past few years, PEEK and its composites have attracted a great deal of attention from material scientists and orthopedists. Efforts are being made to improve its bone-implant interface activity utilizing the chemical structure, properties, and the combination of ketone and ether functional groups between aryl rings in polyaryletherketone.[2,9,12] *In vitro* and *in vivo* studies have shown that PEEK is not toxic or mutagenic, and does not cause significant inflammation.[11,13] The US FDA has recently approved the use of PEEK in the construction of custom craniofacial implants to support mandibular dentures.[14]

To utilize the full potential of the PEEK and to improve its bioactivity, two major strategies have been used including surface modification such as plasma coating,[15-17] or formation of a composite with other biocompatible materials. To fabricate scaffolds with improved affinity for the attachment of mesenchymal cells, surface modification has been carried out either chemically, by reacting a bioactive material, or physically by coating a biologically active material on the surface of the scaffolds.[18-20] Recently Tsou *et al.* used titanium dioxide (TiO_2) coatings to develop osteoblast compatible PEEK[21,22] and after prolonged implantation in rabbits, histological observations showed that there was significant bone regeneration. The shear strength of the bone/implant interface increases with an increase in the implantation period and the bone bonding performance of a titanium dioxide coated implant is found to be superior to that of pristine PEEK. The electron beam deposition technique has also been used for

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the deposition of titanium to enhance the biological properties on PEEK.[23] To promote the osseo-integration of PEEK, bioactive hydroxyapatite,[24,25] tri-calcium phosphate[26] and strontium-containing hydroxyapatite have been used as PEEK coatings.[27] However, modification of PEEK by ceramics decreases the mechanical properties of the PEEK and the biomechanical property of the modified PEEK is no longer similar to that of human cancellous bone.[2,5,13]

In order to increase cell adhesion, PEEK has been functionalized by coupling with amino acids and proteins.[28,29] *In vitro* studies have indicated that immobilization of recombinant human bone morphogenetic protein-2 (rhBMP-2) on collagen-coated PEEK was able to increase the rate of osteogenic differentiation significantly when cells were cultured on modified PEEK disks, which suggested the importance of surface modification of PEEK in orthopedic and spinal clinical applications.[30] Recent studies have also indicated that poly-dopamine (PDA) is found to be useful in cell adhesion and differentiation on various substrates,[31-34] including scaffolds fabricated with poly(lactic acid)[35,36] and poly(ε-caprolactone),[37] which indicates that an attempt at a multifunctional surface modification of PEEK scaffolds should be considered especially considering the mussel-inspired coating of organic and inorganic materials as reported by other workers.[38,39] Dopamine undergoes self-polymerization in alkaline media and is able to form a universal coating enriched with catechols that enables biomolecular and cellular immobilization *via* a simple dipping process. Generally, cell adherence is favored to the surfaces, which are hydrophilic and enriched with functional groups such as -NH_2 or -COOH;[40-42] to promote the cellular compatibility of surfaces, various materials such as growth factors and proteins[29,30,43-45] have been used.

In this study, to overcome the prevalent hydrophobicity and poor integration of PEEK to host tissues,[46] an attempt has been made to add multifunctional properties to the surface of PEEK by immobilization of collagen or insulin on self-assembled poly-dopamine coated PEEK in order to enhance cells adhesion and tissue integration so as to explore PEEK's potential applications in bone tissue engineering and implantation. The poly-dopamine coating on a PEEK surfaces was selected because it was likely to ensure a uniform distribution and thickness[47-49] of immobilized collagen that would allow development of surfaces with enhanced cells adhesion, proliferation, and osteogenic differentiation of pre-osteoblasts.

2. Experimental

2.1. Materials and methods

PEEK, as a brownish color rod, (crystallinity 32%, T_g 143 °C, T_m 343 °C) was purchased from Evonik Company, Germany. The circular PEEK disks of 1.0 cm diameter and 1.5 mm in thickness were cut using a die and were sterilized before surface modification with dopamine and collagen. A 0.5 wt% PEEK solution in 4-chlorophenol was prepared and used to cast thin, pale-yellow, films for recording of their FT-IR spectra, as well as to record optical micrographs of MC3T3-E1 cells (5×10^4 cells/mL) seeded onto collagen covalently immobilized on poly-dopamine modified PEEK film surfaces. Dopamine hydrochloride (DA) (MW, 89.64 g mol^{-1}), rat tail collagen (α-1 (I) chain) and insulin were obtained from Sigma-Aldrich (Castle Hill, Australia). Hydroxyproline and leucine were used as standards to determine the amount of collagen loaded onto the PEEK samples. Tris (hydroxymethyl) aminomethane (Tris) buffer solution (pH 8.5), 1-ethyl-3-(3-dimethylaminopropyl dicarbodiimide hydrochloride) (EDC), *N*-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Company, (USA), and used without further purification. Alizarin red staining kits were purchased from Millipore (Billerica, MA, USA), Type 1 collagen was purchased from Bioland Company (Korea). The cells were cultured in α-minimum essential medium (α-MEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1.0% penicillin G-streptomycin at 37 °C under a 5% CO_2 atmosphere. The culture medium was changed every other day. Mouse pre-osteoblast cells (MC3T3-E1) were purchased from Korea cells bank (Seoul, South Korea) and stored in liquid nitrogen before carrying out cells seeding experiments. A 10×10^{-3} mmol phosphate buffer saline (PBS) solution (pH 7.4) containing 87×10^{-3} mmol Na_2HPO_4, 14×10^{-3} mmol KH_2PO_4, 131×10^{-3} mmol NaCl and 27×10^{-3} mmol KCl was purchased from Sigma-Aldrich. All other chemicals and solvents used in experimental work were high purity reagents and were purchased from Sigma-Aldrich.

2.2. Instrumental analysis

The surface morphologies of PEEK disks were determined by recording FE-SEM (FE-SEM, 400 Hitachi, Tokyo, Japan) micrographs. To record the FE-SEM micrographs, the PEEK disks were fixed on the holder using carbon tape and then sputter-coated with platinum. The platinum-coated PEEK disks were then examined by FE-SEM under a high vacuum. The presence of dopamine, collagen and insulin on the PEEK surface was confirmed by recording the FT-IR spectra (FTIR Mattason, Galaxy 7020A) of dopamine and collagen on KBr pallets and comparing the spectra to the FT-IR spectra of dopamine- and collagen-modified PEEK films. The X-ray photoelectron spectra (ESCA, ESCA LAB VIG microtech Mt 500/1, Etc, East Grinstead, UK) of dopamine, collagen, and covalently immobilized collagen or insulin on dopamine-coated PEEK disks were recorded using monochromatized Mg Kα radiation at 1253.6eV operating at an anodic power of 150W. The spectrometer energy scale was calibrated using Au $4f_{7/2}$ photoelectron peak at a binding energy of 83.98 eV. An X-ray photoelectron survey spectrum of PEEK samples was used for qualitative and quantitative chemical analyses of modified surfaces of PEEK samples using the binding energies and peak intensities of the elements in the survey spectra.

To analyze the surface properties of dopamine and covalently immobilized collagen or insulin on dopamine-coated PEEK samples, the contact angle (θ) measurements were carried out using a sessile drop method (Kruss contact angle equipment model DS10) using deionized water. The average contact angle value reported is the mean of three measurements. Fluorescent micrographs of covalently immobilized proteins on poly-dopamine-coated PEEK samples were recorded to confirm the uniform

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immobilization of collagen and insulin on poly-dopamine-modified circular PEEK disks. Fluorescent collagen (Rho-col) was prepared by dissolving collagen at 1 mg/mL in 0.1 M carbonate–bicarbonate buffer (pH 9.5) and then incubated at 4 °C for 24 h in a solution of 5(6)-carboxyrhodamine 6G (Rhodamine) solution (0.02 mg/mL). After dialysis against deionized water (MWCO 1000 membrane), the fluorescent collagen was dried. To immobilize the fluorescent collagen on dopamine-coated PEEK, the rhodamine-collagen (100 μg/mL) was activated by adding EDC (10 mmol/L) and NHS (25 mmol/L) in deionized water and then poly-dopamine-coated PEEK (PEEK-P) disks were immersed in the activated collagen solution and allowed to react at 5 °C for 48 h. Finally, the PEEK disks were washed with de-ionized water and fluorescence micrographs were obtained using a fluorescence microscope to visualize the presence of collagen using a TRITC filter.

2.3. Covalent immobilization of collagen or insulin on polydopamine-coated PEEK samples

Compared to physically immobilized collagen, covalently immobilized collagen is preferred by MC3T3-E1 cells; hence, for this reason collagen was covalently immobilized on the PEEK disks. To achieve this, the surface of the circular PEEK disks (1.0 cm diameter) was modified by self-assembled polymerization of dopamine in a Tris buffer at alkaline medium (pH 8.5) to create a supporting substrate for the covalent immobilization of collagen. The mussel inspired polydopamine coating technique is able to provide strong adhesive interactions with various materials and biomolecules containing amine and thiol functional groups.[50,51] Five circular PEEK disks were immersed in 100 mL of 10 mmol/L Tris buffer solution (pH 8.5) containing 2.0 mg/mL dopamine and the solution was stirred for 24 h at room temperature. The resultant poly-dopamine-coated disks (hereafter referred to as PEEK-D) were carefully removed and dried at room temperature, after rinsing three times with deionized water. To covalently immobilize proteins on the PEEK-D disks, a solution of activated proteins was prepared by adding 2.5 μg of EDC (10 mM) and 0.25 μg NHS (25 mM) to a 5 mL solution containing 100 μg/mL proteins and the poly-dopamine coated disks (PEEK-D) were immersed in this solution and incubated for 48 h at 5 °C. Finally, after washing with deionized water, the collagen- or insulin-immobilized disks (hereafter referred to as PEEK-D-Col) were stored in a PBS solution (pH 7.4) at 5 °C. Collagen was also physically coated on pristine PEEK disks to compare the extent of collagen immobilization and cellular activities. The amount of collagen immobilized on the disks was determined by recording liquid chromatographs for different amount of hydroxyproline and by recording their areas at different amount of hydroxyproline.

2.4. Cellular response of collagen-immobilized polydopamine-coated PEEK Disks - *In-vitro* MC3T3-E1 cells adhesion

To evaluate the effect of surface properties of the different PEEK disks on the bone forming activity of MC3T3-E1 cells, the circular surface-modified PEEK disks were cut and placed in the wells of a 4-well cell culture plate after sterilization with UV irradiation for 2 h. Non-osteogenic α-minimum essential medium (500 μL, α-MEM: Gibco, Tokyo, Japan) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was added to each well and MC3T3-E1 cells were then seeded at a cell density of 5×10^4 cells/mL. The MC3T3-E1 cells cultures were incubated in a humidified atmosphere at 37 °C in the presence of 5% CO_2 for three days to evaluate the cells adherence properties to the different PEEK disks (pristine PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In). If cells were seeded for more than one day, the medium was changed every second day. After incubation, the supernatant medium was carefully removed and the PEEK disks incubated with cells were fixed for 10 min using 2.5% solution of glutaraldehyde after washing twice with phosphate buffered saline solution. Finally, the scaffolds were dehydrated with a critical point drier (EMS 850 Critical Point Dryer, Hatfield, PA, USA) and used to record FE-SEM micrographs (400-Hitachi, Tokyo, Japan) after drying and sputter gold coating.

2.5. MTT assay

To evaluate the bioactivity of the different PEEK disks (PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In) on MC3T3-E1 cell proliferation, a MTT assay was performed by seeding MC3T3-E1 cells on the PEEK disks for one or three days. To perform the MTT assay, sterilized circular PEEK disks were placed in the wells of a 4-well cell culture plate and 500 μL of non-osteogenic a-minimum essential medium was added to each well before seeding MC3T3-E1 cells at a cell density of 5×10^4 cells/mL on each PEEK disk. After incubation of MC3T3-E1 cells for three days, the culture medium was carefully removed and the scaffolds were washed twice with phosphate buffered saline solution. To determine the extent of proliferation of MC3T3-E1 cells, the cells seeded on the PEEK disks were incubated for approximately 4 h at 37 °C with 500 μL of MTT solution containing 500 μg/mL of MTT. After removal of the MTT solution, the formazan purple crystals, produced by the MC3T3-E1 cells seeded on the PEEK disks, were dissolved by adding 250 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) to each well for about 10 min. The absorbance of the extracted solution was recorded at 570 nm using a Symergy HT multi-detection microplate reader (Symergy HT, BioTek, USA). The absorbances obtained from negative controls were subtracted from the measured values.

2.6. ALP activity

To evaluate the osteogenic differentiation of MC3T3-E1 cells cultured on different surface modified PEEK disks, the alkaline phosphatase (ALP) activity of MC3T3-E1 cells was determined. To achieve this, sterilized, surface-modified, PEEK disks were placed into 4-well cell culture plates and MC3T3-E1 cells were plated on top at a cell density of 3×10^4 cells/mL. After incubation in a humidified atmosphere at 37 °C in the presence of 5% CO_2, the cells were cultured for fifteen days in the presence of 500 μL of α-MEM. ALP staining of the MC3T3-E1 cells was carried out using protocol prescribed by the supplier of the ALP staining kit (Sigma-Aldrich). PEEK disks were washed with deion-

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ized water and fixed for about 30 sec using a citrate-acetone-formaldehyde fixative solution (citrate solution 25 mL, acetone 65 mL, and 8 mL 37% formaldehyde solution). The MC3T3-E1 cells on the PEEK disks were stained for approximately 30 min at room temperature using an alkaline-dye mixture containing 48 mL of Fast Blue RR salt solution and 2 mL of a naphthol AS-MX phosphate alkaline solution. After staining, the PEEK disks were rinsed with deionized water for approximately 2 min to remove any residual dye. After this rinsing, the PEEK disks were placed in Mayer's Hematoxylin solution for approximately 10 min and then optical microscopic images (Nikon E 4500, Japan) of the ALP-stained cells were obtained.

2.7. Alizarin red staining

Alizarin red staining of MC3T3-E1 cells seeded on the different surface-modified PEEK disks was carried out to evaluate the osteogenic response of MC3T3-E1 cells cultured on PEEK, PEEK-D, PEEK-D-Col and PEEK-D-In disks. Briefly, MC3T3-E1 cells were cultured on circular PEEK disks placed in the wells of a 4-well cell culture plate at a cell density of 5×10^4 cell/mL in a humidified atmosphere of 5% CO_2 at 37 °C in α-MEM. The medium was then carefully aspirated and the disks were washed twice with PBS solution before attached cells were fixed for approximately 15 min at room temperature, using a 10% solution of formaldehyde. After fixation, the cells were rinsed three times (10 min each) with distilled water to remove any residual formaldehyde. After removal of the excess water, Alizarin red staining of the seeded cells was performed by adding 1 mL of Alizarin red solution/well and incubating the samples for approximately 30 min. After staining, the excess Alizarin red was removed by gentle washing with distilled water. To measure the Alizarin red staining of the cells on the different PEEK disks, the digital images of the stained cells were captured using optical microscope equipped with high end camera (Axioplan 2, Carl Zeiss camera).

2.8. Von kossa assay

Von Kossa staining was carried out in order to evaluate the calcium deposition response of MC3T3-E1 cells cultured on PEEK disks with different surface properties. MC3T3-E1 cells were seeded onto circular PEEK disks, which were fitted into the wells of a 4-well cell culture plate. Seeding of MC3T3-E1 cells was carried out at a cell density of 5×10^4 cells/mL and cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C in α-MEM for approximately fifteen days. The PEEK disks containing the attached MC3T3-E1 cells were gently washed three times with PBS for 5 min each time, and were then fixed for 30 min in the presence of 10% formaldehyde. After fixing, the cell-seeded disks were again washed three times with distilled water for 10 min and then exposed to UV irradiation for 5 min after treatment with a 5% solution of $AgNO_3$. The UV-irradiated PEEK disks were then washed twice with PBS to remove residual $AgNO_3$ and then immersed in a 5% solution of $Na_2S_2O_3$ for 5 min. Finally, the PEEK disks were washed twice with distilled water and digital images of the stained cells were captured by optical microscope equipped with a camera (Nikon E 4500, Japan).

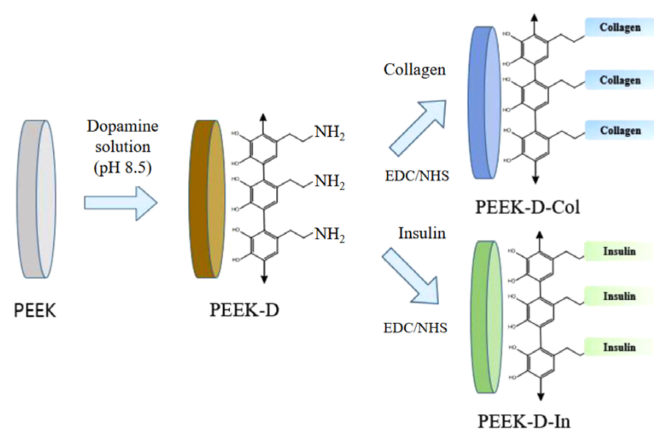
2.9. Statistical analysis

All experiments were performed in triplicate and data are presented as means ± standard deviations. Statistically significant differences were determined using a one way ANOVA considering $P<0.05$ and $P<0.01$ as statistically significant and very significant, respectively, whereas $P>0.05$ was considered as being statistically insignificant.

3. Results and discussion

PEEK is biologically inert but possesses useful mechanical properties that are suitable for use in bone implants. To enhance the surface bioactivity of PEEK, and therefore its tissue integration, modification with collagen has been found to be a useful method of changing its surface properties. The self-assembled polymerization of dopamine in an alkaline medium is able to produce an adhesive layer of poly-dopamine on PEEK disks which aids in the strong immobilization of collagen[52] that occurs through chemical bonding between the amino groups of collagen and the hydroxyl groups of the polydopamine[53] as has been shown for the surface immobilization of enzymes and stem cells by other workers.[54,55] The covalent binding of collagen to the PEEK disks is particularly useful since it creates a uniform layer of collagen that provides a better control on the thickness, density, and molecular orientation of the collagen molecules[56,57] on PEEK disk surfaces (Scheme 1). Collagen is a major component of bone extracellular matrix and helps in improving cell adhesion, proliferation, and differentiation on various biomaterial surfaces.[58-62] Compared to other methods, collagen immobilization has been found to be non-toxic and provides a suitable method to improve tissue integration of implants;[13,31,32] other methods have generally involved the introduction of a toxin to the implant surfaces.[63]

The PEEK disks prepared with covalently immobilized collagen or insulin were characterized for the presence of dopamine and protein such as collagen and insulin and were then evaluated for bioactivity by seeding MC3T3-E1 cells onto their surfaces and analyzing cell proliferation and differentiation.



Scheme 1. Self-assembled polymerization of dopamine (D) and covalent immobilization of collagen (Col) or insulin (In) on PEEK sample surfaces.

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3.1. Characterization of collagen-immobilized polydopamine-coated PEEK samples

To immobilize collagen onto the PEEK disk surface, dopamine was polymerized on five circular PEEK disks using a dopamine self-polymerization reaction. To achieve this, dopamine (2.0 mg/mL) in a 10 mM Tris buffer (pH 8.5) was used. The polymerization of dopamine on the PEEK disks produces quinine functional groups, which help in the chemical anchoring of collagen. The change in color of the PEEK disks to a dark brown or black color was considered a primary indication of the successful self-assembled polymerization of dopamine occurring *via* catechol oxidation. The process took almost 1 h to complete. The poly-dopamine-coated PEEK disks (PEEK-D) were then immersed in a solution containing EDC/NHS-activated collagen or insulin to react chemically with the poly-dopamine-coated PEEK disk surface. Spectrophotometric analysis at 350 nm of the remaining dopamine solution, as well as washings of the dopamine-modified disks, indicated that more than 88% of the dopamine was successfully coated onto the PEEK disk surface. The self-assembled polymerization and deposition of dopamine on the surfaces of biomaterials has been shown to be dependent on solution pH.[64,65] The amount of collagen immobilized on the dopamine-coated PEEK disks was determined using measurement of hydroxyproline. A calibration curve was constructed from the peak areas of chromatograms of liquid chromatography measurements of solutions of hydroxyproline standards (1,000-5,000 nmol/L) in 0.1 M formic acid. Following this, the amount of immobilized collagen was estimated by determining the amount of hydroxyproline left in solution after chemical immobilization onto the dopamine-modified PEEK disk surface. This analysis indicated that more than 95% of the collagen was successfully immobilized onto the poly-dopamine-coated PEEK disks. This high rate of immobilization can be attributed to activation of collagen by the EDC/NHS reagents and to collagen cross-linking reactions that occur during chemical immobilization.

3.2. Scanning electron microscopy for surface morphology

The SEM micrographs of PEEK disks recorded after sputter coating with platinum provided an insight into variations in the surface morphology of PEEK disks after coating with dopamine or chemical immobilization of collagen on the dopamine-coated PEEK disk surfaces (Figure 1). The SEM micrograph of a pristine PEEK disk (Figure 1(a)) clearly showed that the surface of the pristine PEEK disk was smooth and lacking surface porosity. In contrast, after the self-assembled polymerization of dopamine, the PEEK-D surfaces were found to be rough with folded structures consisting of the self-polymerized dopamine (Figure 1(b)). After chemical immobilization of collagen on the dopamine-coated PEEK disks the rough surface seen in Figure 1(b) was evident but with the evenly distributed fine structure of the collagen fibers also being evident (PEEK-D-Col, Figure 1(c)). These data confirm that coating of dopamine onto the PEEK disk was able to produce a uniform layer of collagen following its chemical immobilization.[56,57,66]

3.3. Contact angle measurements

The coating of dopamine on the PEEK disks was confirmed by comparing the surface wettability of PEEK-D disks with that of pristine PEEK disks (Figure 2). These measurements were made using contact angle measurements. The contact angle of the PEEK-D disks was decreased from 84° to 64° (Figure 2(a),(b)). This decrease in contact angle that occurred on coating of dopamine onto the PEEK disk has been used previously to confirm successful coating of dopamine onto a PEEK disk surface.[67] Contact angle measurements were also used to confirm the chemical immobilization of collagen onto the PEEK-D-Col surface.

The contact angle for PEEK-D-Col disks (Figure 2(c), 39±0.75°) and PEEK-D-In (Figure 2(d), 29±1.12°) was found to be significantly lower than the contact angle observed with pristine PEEK disks (Figure 2(a), 84±1.06°) or with PEEK-D disks (Figure 2(b), 64±1.10°). These contact angle measurements clearly confirmed

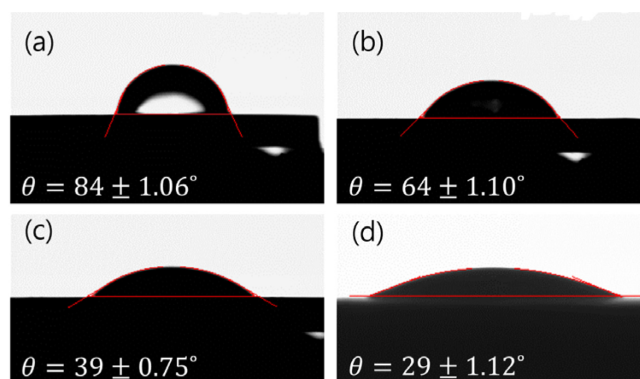

Figure 2. Contact angle measurements of pristine PEEK surface (a), PEEK-D surface (b), PEEK-D-Col surface (c), and PEEK-D-In surface (d).

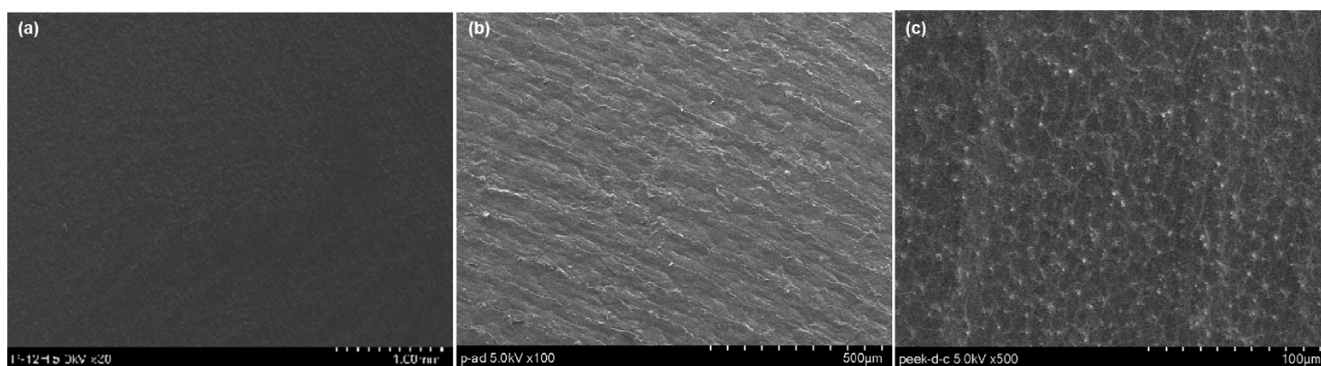

Figure 1. SEM micrographs of a pristine PEEK disk (a), PEEK-D disk (b), and PEEK-D-Col disk (c).

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the loading of dopamine, collagen and insulin onto the PEEK disk surfaces, and also indicated that modification of the PEEK disk surface with either dopamine or protein (collagen and insulin) increased the surface hydrophilicity significantly. This would be expected to assist in cell adhesion to the PEEK surface and subsequent bone implant integration with adjoining bone tissues.

3.4. FT-IR spectra

To further confirm both dopamine coating and protein immobilization on the PEEK disk surface, the FT-IR spectra of PEEK-D, PEEK-D-Col and PEEK-D-In disks were recorded and compared to the FT-IR spectrum of pristine PEEK disk (Figure 3). The FT-IR spectrum of pristine PEEK (Figure 3(a)) showed a carbonyl stretching vibration at 1653 cm^{-1} and skeletal ring vibrations at 1593, 1500, 1485, and 1410 cm^{-1}. The bending motion of C-C and (=O)-C groups appeared around 1305 cm^{-1}. The asymmetric stretching vibration bands of the diphenyl ether group appeared at 1277 and 1190 cm^{-1}. By comparing the FT-IR spectra of pristine PEEK with PEEK-D (Figure 3(a),(b)), it was clear that there was no significant variation in the PEEK FT-IR characteristic peaks between the two, indicating that poly-dopamine formed a self-assembled layer on the PEEK surface without undergoing chemical bond formation.

The appearance of an additional peak around 1630 cm^{-1} in PEEK-D sample confirmed the loading of poly-dopamine onto the PEEK surface (Figure 3(b)). In addition, the band intensity was increased by further increasing the polymerization of the dopamine on PEEK sample surfaces.

On comparing the FT-IR spectrum of PEEK-D-Col (Figure 3(c)) and PEEK-D-In (Figure 3(d)) film with the FT-IR spectrum of pristine PEEK (Figure 3(a)) and PEEK-D (Figure 3(b)) films, new peaks, characteristic of collagen, appeared at 1660 cm^{-1} and 1550 cm^{-1} corresponding to the stretching vibration of the carbonyl group of amide-I (-CONH-) and the stretching vibration for amide II (-CONH-), which were formed due to the chemical reaction of collagen with poly-dopamine coated PEEK. These data support the presence of collagen on the PEEK disk surface and also confirmed the fact that the collagen was chemically bonded to the poly-dopamine-coated PEEK disk.

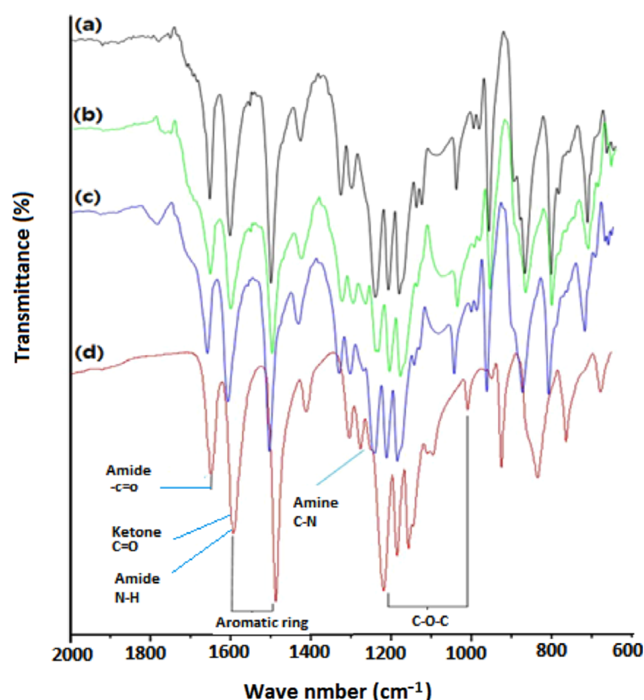

Figure 3. FT-IR spectra of pristine PEEK (a), PEEK-D (b), PEEK-D-Col (c), and PEEK-D-In (d).

3.5. X-Ray photoelectron analysis

In addition to FT-IR characterization, pristine PEEK, PEEK-D, PEEK-D-Col and PEEK-D-In were also analyzed using X-ray photoelectron spectroscopy to confirm the presence of dopamine and proteins on the PEEK disk surfaces (Figure 4 and Table 1). The X-ray photoelectron spectra of pristine PEEK (Figure 4(a)) and PEEK-D (Figure 4(b)), PEEK-D-Col (Figure 4(c)) confirmed the modification of the PEEK disk surfaces with dopamine and collagen. The appearance of a peak at 399 eV, corresponding to N1s in the PEEK-D sample (Figure 4(b)) and a shift in its binding energy to 399.5 eV in the PEEK-D-Col Sample (Figure 4(c)) confirmed the presence of dopamine and collagen, respectively.

In pristine PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In, the binding energies of C1s and O1s remained almost constant at 284 eV and 531 eV, respectively, suggesting that the interactions of these elements were not influenced by dopamine coating and/or protein immobilization. The variation in atomic percentage of elements detected by the X-ray photoelectron survey analysis (Table 1) therefore provided sufficient proof to support the conclusions of successful dopamine coating and collagen or insulin immobilization on the PEEK disks. In particular, the presence of 4% nitrogen in the PEEK-D sample (Table 1) and its increase to 10.7% in the PEEK-D-Col and 11.63% in the PEEK-D-In sample confirmed dopamine coating and protein immobilization. A fluorescence analysis of thin film (0.06 mm) PEEK disks coated with poly-dopamine and immobilized collagen also confirmed the deposition of collagen on the dopamine-coated PEEK disk surface.

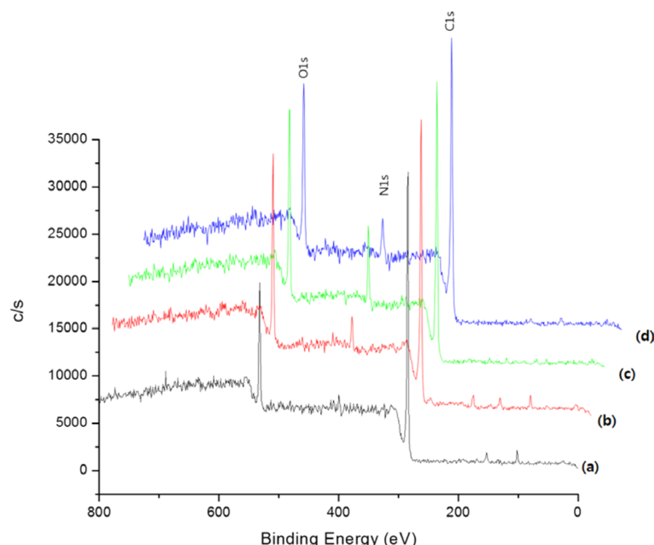

Figure 4. X-Ray photoelectron spectra of pristine PEEK (a), PEEK-D (b), PEEK-D-Col (c), and PEEK-D-In (d).

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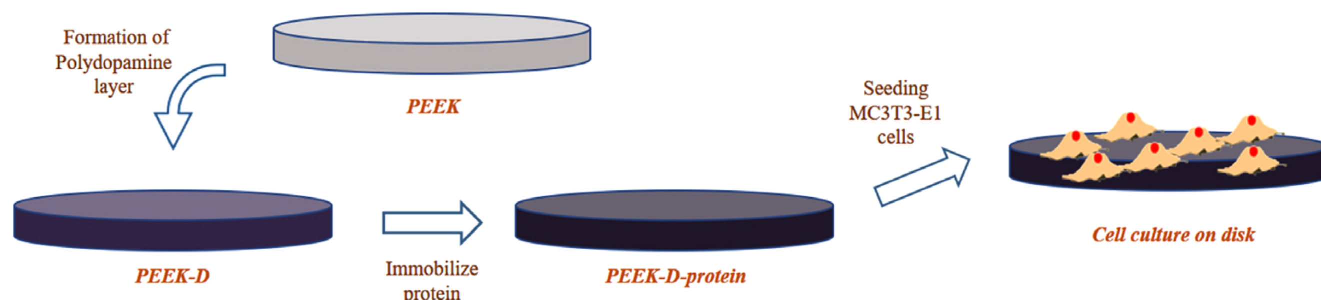
Table 1. Atomic percent of PEEK disks calculated from survey scan spectra of X-ray photoelectron spectroscopy

Substrates	Atomic percent of elements (%)			
	C	O	N	S
PEEK	85.6	13.6	0.8	0
PEEK-D	81.2	14.8	4.0	0
PEEK-D-Col	70.1	18.9	10.7	0.3
PEEK-D-In	68.36	19.04	11.63	0.97



Scheme 2. Collagen immobilized on polydopamine coated PEEK disk surface and its bioactivity for MC3T3E1 cells adhesion and proliferation.

3.6. Cellular response of MC3T3-E1 cells on pristine and modified PEEK disks

The chemical immobilization of collagen on dopamine-coated PEEK disk surfaces was expected to significantly improve the culture of MC3T3-E1 cells (Scheme 2) because the collagen produced a surface similar to natural extra-cellular matrices.[58-60]

The cellular response to modified PEEK surfaces has previously been shown to depend on incubation time, as well as the orientation and distribution of collagen on the PEEK surface.[56,57] In this study, the immobilization of collagen in presence of activating agents (EDC/NHS) not only helped in chemical immobilization of collagen but also produced cross-linked collagen with interconnected pores which is desirable for bone tissue formation and tissue growth. The SEM and contact angle measurements clearly indicated that modification of the PEEK disk with collagen increased hydrophilicity due to the presence of amino acid groups[31,34-35] and numerous collagen amino acid motifs, such as the RGD sequence;[59] these factors are essential for good cell bio-compatibility in sharp contrast with hydrophobic surfaces.

3.7. *In vitro* MC3T3-E1 cells adhesion

To evaluate MC3T3-E1 cell adhesion on modified PEEK disks, the pristine PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In were used for seeding MC3T3-E1 cells. After three days of culture the extent of cell adhesion was assessed using an analysis of SEM micrographs of pristine PEEK (Figure 5(a)), PEEK-D (Figure 5(b)), PEEK-D-Col (Figure 5(c)), and PEEK-D-In (Figure 5(d)).

The adhesion of MC3T3-E1 cells on pristine PEEK was found to be relatively low; this result was not unexpected given that the surface of pristine PEEK was very smooth and inherently hydrophobic, and so would not be expected to provide a good surface for cell adherence (Figure 5(a)). In contrast, PEEK-D-Col, and PEEK-D-In surfaces, both of which adopted a rough appear-

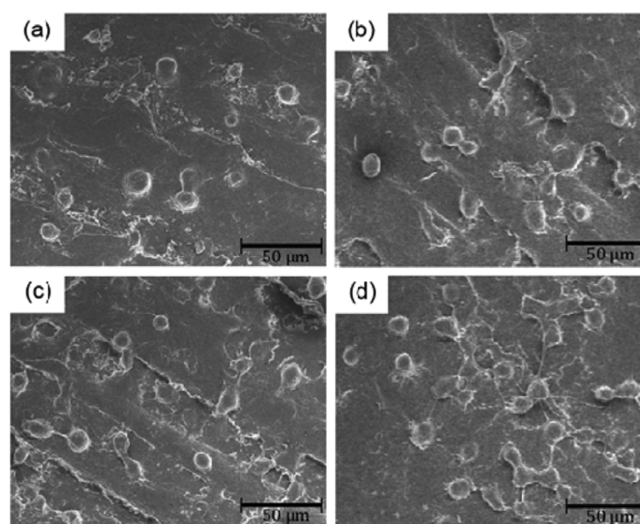


Figure 5. MC3T3-E1 cell adhesion after 24 h culture on pristine PEEK (a), PEEK-D (b), PEEK-D-Col (c), and PEEK-D-In (d).

ance after modification,[68] and also provided chemical functional groups that assist in the adherence of the seeded cells, did indeed show increased cell adherence (Figure 5(c), (d)). Thus the smooth surface of unmodified PEEK is one of the factors that contribute to its bio-inert character. Coating with poly-dopamine alone increased surface roughness and also enhanced the surface hydrophilicity, as confirmed by contact angle measurements (Figure 2(b)). However, further modification with collagen provided various additional surface attributes ranging from physical to chemical interactions suitable for enhancing cells adhesion. Figure 6 indicated that the extent of cell adhesion increased significantly at 48 h on collagen-modified PEEK films (Figure 6(c)). This is assumed to be due to the presence of the cross-linked three dimensional structure of the collagen that continues to increase cell adhesion up until 48 h (Figure 6(c)). The MC3T23E1

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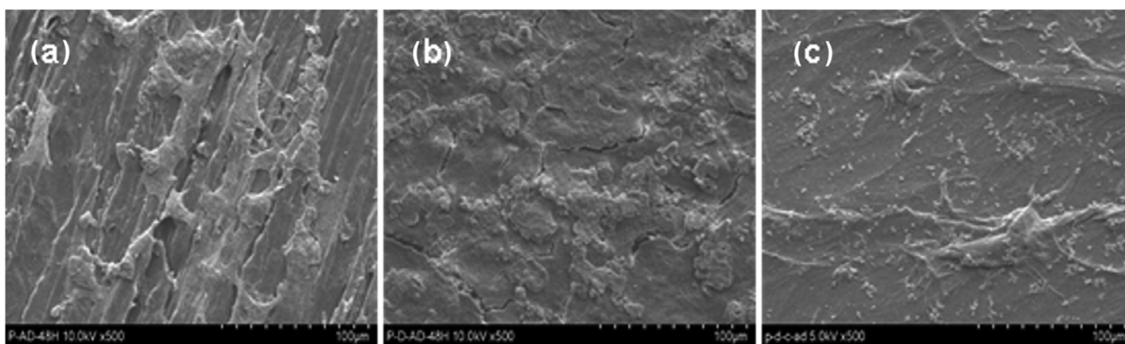

Figure 6. Optical micrographs showing MC3T3-E1 cell adhesion on films of pure PEEK (a), PEEK-D (b), and PEEK-D-Col (c). All cells were incubated for 48 h in α-MEM.

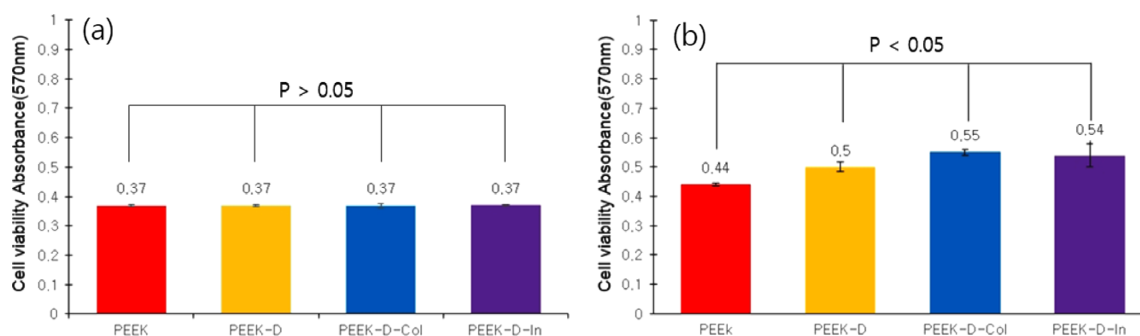

Figure 7. MTT assay of MC3T3-E1 cell cultured in α-MEM for one (a) and three (b) days on pristine PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In.

cells seeded for 48 h on collagen-modified PEEK also showed well developed lamellipodia (Figure 6(c)) in comparison to the cells seeded on the pristine PEEK and polydopamine-coated PEEK matrix (Figure 6(a),(b)).

Taken together these observations clearly indicate that modification of PEEK with collagen increases desirable PEEK surface properties more significantly than modification by simple polydopamine, and this is a major reason for the increases in cell adhesion observed.

3.8. MTT assay

MC3T3-E1 cell proliferation on pristine and modified PEEK surfaces was evaluated using the MTT assay. MC3T3-E1 cells were seeded onto pristine PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In circular disks, for one and three days, and proliferation was assessed by quantifying the amount of purple formazan produced by mitochondrial reduction of the thiazolyl blue tetrazolium bromide. The results of the MTT assay indicated that the rate of proliferation of MC3T3-E1 cells cultured on the pristine PEEK sample for 3 days was lowest and increased very poorly on increasing the cell seeding time from one day (Figure 7(a)) to three days (Figure 7(b)).

However, in contrast, the rate of proliferation of cells seeded on both PEEK-D and PEEK-D-Col increased from day 1 (Figure 7(a)) to day 3 (Figure 7(b)). This clearly indicates that the modification of the PEEK surface with collagen provides better opportunities for cell proliferation, beyond just increasing adhesion, presumably because the collagen can act as an authentic extracellular matrix.

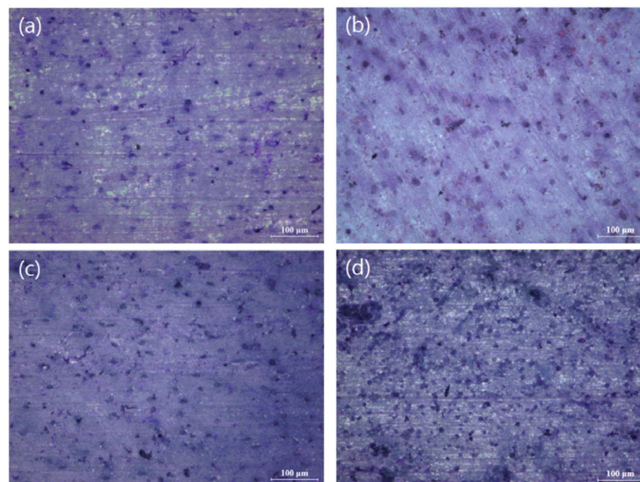

Figure 8. ALP activity of MC3T3-E1 cells on pristine PEEK (a), PEEK-D (b), PEEK-D-Col (c), and PEEK-D-In (d).

3.9. ALP activity

Osteogenic surfaces generally enhance the formation of osteoblast-associated genes[69] and markers including collagen Type I (Coll-I), alkaline phosphatase (ALP),[70] osteopontin (OPN), osteocalcin (OCN), and bone sialoprotein (BSP) in a well-established temporal sequence during the development of osteoblast or bone formation. Collagen Type I (Coll-I), and ALP are considered as early markers of osteogenic differentiation,[37,71] whereas OCN and OPN are considered to be late markers. In this study, we elected to evaluate MC3T3-E1 cell differentiation by evaluating alkaline phosphatase activity (ALP) in cells cultured on different PEEK surfaces (Figure 8). The data showed that the ALP response of

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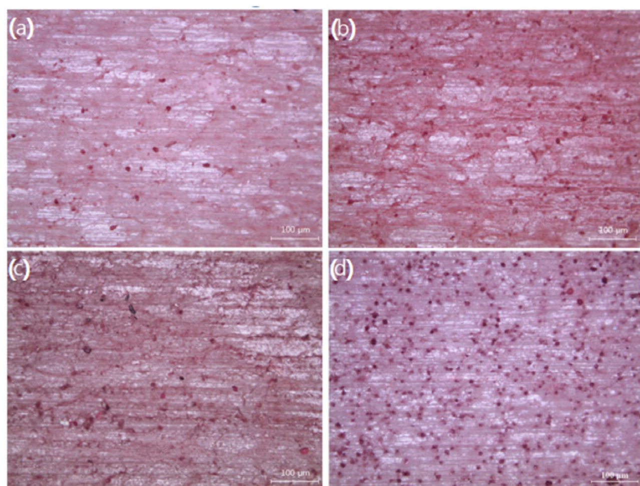

Figure 9. Alizarin red staining of MC3T3-E1 cells cultured for fifteen days on pristine PEEK (a), PEEK-D (b), PEEK-D-Col (c), and PEEK-D-In (d).

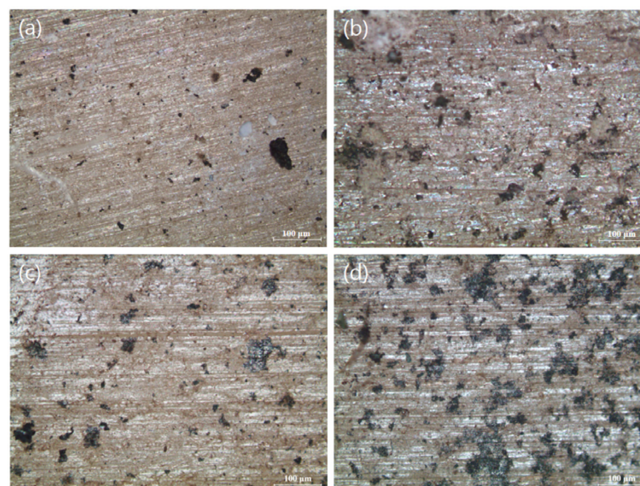

Figure 10. von Kossa assay for MC3T3-E1 cells cultured for fifteen days on pristine PEEK (a), PEEK-D (b), PEEK-D-Col (c), and PEEK-D-In (d).

MC3T3-E1 cells varied significantly depending on if the cells were seeded on PEEK (Figure 8(a)), PEEK-D (Figure 8(b)), PEEK-D-Col (Figure 8(c)), and PEEK-D-In (Figure 8(d)).

This variation in ALP activity of MC3T3-E1 cells clearly indicated that modification of PEEK by collagen and insulin improved the expression of alkaline phosphatase, which is important for osteogenic differentiation and bone formation.

3.10. Alizarin red staining

Alizarin red staining indicates the process of transformation of undifferentiated MC3T3-E1 cells to osteoblasts and their subsequent mineralization leading to bone formation.[69] *In vitro* Alizarin red stain is able to visualize the process of osteogenesis by producing a red color on calcium deposition. In this study, we examined the osteogenic properties of the various PEEK surfaces on MC3T3-E1 cells using Alizarin red staining (Figure 9). Comparing the intensity of Alizarin red staining for MC3T3-E1 cells seeded on pristine PEEK (Figure 9(a)), PEEK-D (Figure 9(b)), PEEK-D-Col (Figure 9(c)), and PEEK-D-In (Figure 9(d)), it was clear that the osteogenic level of MC3T3-E1 cells differentiated on the pristine PEEK surface was very low (Figure 9(a)) compared to the osteogenic level of MC3T3-E1 cells cultured on PEEK-D-Col and PEEK-D-In (Figure 9(c),(d)) with the maximal osteogenic effect being observed for culture on PEEK-D-In (Figure 9(d)). The effects on Alizarin red staining are therefore in close agreement with the effects shown by ALP staining.

3.11. Von kossa assay

The post-proliferative mineralization stage of MC3T3-E1 cells cultured on the various PEEK surfaces could be evaluated by analyzing the expression of non-collagenous osteogenic matrix proteins such as osteonectin (OSN), osteopontin, and osteocalcin. However, the expression of these proteins in the early stages of osteogenesis is very low. The regulation and expression of osteocalcin protein is controlled by osteocalcin mRNA, which can be detected at early stages of osteogenesis but detection of mRNA markers for the expression of osteonectin, osteopontin, and osteocalcin is time consuming and costly in comparison to using a simplified von Kossa protocol for detecting the osteocalcin protein marker by mineralization. The von Kossa protocol is able to assay the effect of surface structure and properties of the materials in causing cells differentiation and mineralization. MC3T3-E1 cells were cultured on pristine PEEK, PEEK-D, PEEK-D-Col, and PEEK-D-In for 15 days and calcium deposited within the cells were stained using the von Kossa protocol (Figure 10). This stain produces a deposit of silver ions wherever a calcium deposit had previously existed.

On comparing the response of the MC3T3-E1 cells cultured on the various PEEK surfaces by examining the deposition of the silver ion black spots, it was apparent that cells cultured on the insulin-immobilized PEEK surface was found to have well developed black spots, which confirmed that modification of the PEEK surface with covalently immobilized insulin was highly osteogenic (Figure 10(d)) compared to the pristine PEEK surface (Figure 10(a)), the PEEK-D surface (Figure 10(b)), and the PEEK-D-Col (Figure 10(c)).

4. Conclusions

The self-assembled polymerization of dopamine on PEEK was found to be useful in allowing for deposition of a uniform layer of collagen or insulin that could enhance the bioactivity of the PEEK surface. Specifically, the self-assembled layer of poly-dopamine on the PEEK surface provided an opportunity for covalent immobilization of collagen in the presence of EDC and NHS as activating agents. Using this approach, the bioactivity of PEEK increased significantly as indicated by increases in MC3T3-E1 cell adhesion, proliferation, and differentiation. The covalent immobilization of proteins such as collagen and insulin provides cell binding sites on the PEEK surface, which should be capable of triggering binding to cells and allow for integration with adjoining tissues so that PEEK can be used in bone implantation. The coating of poly-dopamine on the PEEK surface also provides an opportunity to tailor the PEEK surface with colla-

gen layers of different thickness and orientations to allow control of the cellular response, and ultimately allow for tissue integration of PEEK implants. The collagen- and insulin-modified PEEK surface show high osteogenic properties, especially as compared to the unmodified pristine PEEK surface, which clearly indicates that collagen- and insulin-modified PEEK could be a useful material for bone tissue engineering and implants applications.

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