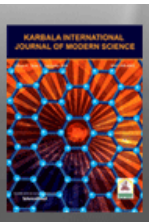

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Biofunctionalization of polyetheretherketone implant material by bone-forming peptide-2 immobilization

Mustafa S. Tukmachi
Department of Prosthodontics, College of Dentistry, University of Baghdad, Baghdad, Iraq,
mustafa.tukmachi@codental.uobaghdad.edu.iq

Hikmat J. Abdul-Baqi
Department of Prosthodontics, College of Dentistry, University of Baghdad, Baghdad, Iraq.

Falah H. Hussein
College of Pharmacy, University of Babylon, Hilla, Iraq

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Abstract

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Keywords

PEEK; Bone-forming peptide-2; Biofunctionalization; ATR-FTIR; AFM; Wettability

RESEARCH PAPER

Biofunctionalization of Polyetheretherketone Implant Material by Bone-forming Peptide-2 Immobilization

Mustafa S. Tukmachi [a,*], Hikmat J. Abdul-Baqi [a], Falah H. Hussein [b]

[a] Department of Prosthodontics, College of Dentistry, University of Baghdad, Baghdad, Iraq
[b] College of Pharmacy, University of Babylon, Hilla, Iraq

Abstract

Polyetheretherketone (PEEK) has favorable biomechanical properties to be used as an implant material. Unfortunately, it is hydrophobic and does not promote cellular adhesion, which could result in poor integration with bone tissue. Biofunctionalization of PEEK surface with osteogenic peptides derived from bone extracellular matrix proteins is an exciting approach to encourage bone formation around the implant. In the current study, bone-forming peptide-2 was immobilized on PEEK surface using two different methods, using dopamine and a diglycidyl ether as conjugate compounds, respectively. Peptide quantification test revealed that the two strategies resulted in the most amount of peptides were attached with 0.5 mM concentration and no further peptides were grafted with a higher peptide concentration. Both methods showed good peptide stability after agitation in aqueous solution. Peptide grafting was confirmed with ATR-FTIR. Surface characterizations with AFM and wettability tests resulted in a significant increase in surface roughness and surface area ratio for the peptide-grafted PEEK compared to unmodified PEEK, which led to a significant enhancement in the wettability of the modified PEEK surface.

Keywords: PEEK, Bone-forming peptide-2, Biofunctionalization, ATR-FTIR, AFM, Wettability

1. Introduction

Implants made of titanium have been the gold standard in dentistry since the 1960s. Because of its excellent biocompatibility, mechanical strength, resistance to corrosion, and ability to integrate with the surrounding bone, titanium has become the preferred material for dental implants [1,2]. However, the elastic modulus of titanium is much higher than that of bone tissue (titanium = 110 GPa and cortical bone = 14 GPa). This distinct mismatch leads to bone resorption and implant failure due to stress shielding of the titanium implant [3,4]. Another disadvantage of titanium is its gray color, which causes a darkening in the periimplant soft tissue around the implant neck with thin gingiva [5].

Polyetheretherketone (PEEK) is one of the most promising replacement materials for titanium in the implant field. It belongs to the family of high-performance thermoplastic polymers. PEEK, as an implant biomaterial, has excellent properties, such as high resistance to chemicals and radiation, bone-like stiffness, light beige color, and ease of machining to the desired shape [6,7]. The modulus of elasticity of unmodified PEEK is 3.6 GPa and that of carbon fiber reinforced PEEK is about 18 GPa, a close match to that of cortical bone [8].

On the other hand, PEEK is bioinert and has poor cellular adhesion properties due to its hydrophobicity. To achieve successful implant treatment, the implant surface has to promote bone growth and attachment, a process called osseointegration [9,10]. The response of osteoblasts (bone-forming cells) to the implanted material depends on the surface properties of that material in terms of topography and surface chemistry [11,12].

PEEK surface modification has the advantage of enhancing its bioactivity while preserving the bulk

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* Corresponding author.
E-mail address: mustafa.tukmachi@codental.uobaghdad.edu.iq (M.S. Tukmachi).

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properties. Biochemical modification of the implant surface, which involves the introduction of chemical cues by covalent immobilization, is one of many surface-modification approaches [13,14].

Promoting the bone-healing process requires bioactive substances that can initiate and guide bone-cell activities. Biofunctionalization is an effective method to mimic the cellular microenvironment by incorporating biomolecules from the bone extracellular matrix (ECM) onto the material surface to initiate the biochemical signaling process necessary for new bone formation and repair [15,16].

Bone morphogenetic proteins (BMPs) are a dominant group of proteins in the bone matrix. Important members of BMPs family are BMP-2 and BMP-7, which play an essential role in signaling osteoblast differentiation and proliferation, the key steps in new bone formation during osseointegration [17–19]. Functionalization of materials with synthetic peptides derived from proteins of the ECM is a new approach to overcome the difficulties of using full-length proteins [16]. In a recent study, a novel bone-forming peptide-2 (BFP-2, with a sequence of 15 amino acids of VEHDKEFFH-PRYHHR) was identified from the immature region of BMP-7. This peptide showed excellent osteogenic activity in vitro and in vivo compared to BMP-7 itself [20].

Peptides can be immobilized on a biomaterial surface through covalent bonding technique, which requires implant surface modification with functional groups such as hydroxyl, amine, or carboxylic groups that react with peptides and attach them to the surface [21,22]. During chemical immobilization, at least one covalent connection is formed between the surface and the target peptide to provide stability and prevent spontaneous peptide uncoupling [23].

This research describes the immobilization of bone-forming peptide-2 on PEEK substrate using two strategies: one based on Michael reaction by using dopamine and the other based on Schiff base formation using a diglycidyl ether. Surface characterizations of PEEK before and after peptide immobilization were performed using ATR-FTIR, AFM, and wettability test. The null hypothesis was that the immobilized peptide does not improve PEEK surface topography and wettability.

2. Materials and methods

2.1. Sample preparation

PEEK disc samples of 10 mm in diameter and 2 mm in thickness were prepared by cutting continuous extruded rods (Energetic Industry Co., Ltd., China). Samples were ground with 500, 800, 1200, 2000, and 2400 grit silicon carbide abrasive papers and were polished with diamond polishing paste to achieve a mirror finish. After that, they were cleaned with ethanol and water successively in an ultrasonic cleaning device.

2.2. Peptide solution preparation

BFP-2 was custom synthesized by ChinaPeptides Co., Ltd. in China. It was supplied as a lyophilized white powder with a purity of 98.22%. Four concentrations of peptide solution (0.25, 0.5, 0.75, and 1 mM) were prepared by mixing the peptide powder with phosphate buffer saline (PBS) with a pH of 7.4. The peptide solutions were aliquoted and kept at −20 °C until used.

2.3. Immobilization methods

2.3.1. First method: immobilization via dopamine

Dopamine was used as an anchor to graft the peptide into the PEEK polymer chains via Michael reaction between the amine group of the peptide and the catechol of the dopamine. PEEK samples were immersed in 2 mg/ml dopamine (DA) (Hangzhou Hyper Chemicals Limited, China) in 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (Hangzhou Hyper Chemicals Limited, China) with pH = 8.5. Samples were shaken in the dopamine solution for 24 h at room temperature. After that, the samples were washed with distilled water three times. DA-PEEK samples were then immersed in 1 ml of each of the prepared peptide solutions and kept at room temperature for 24 h, and subsequently washed with PBS to remove loosely attached peptides. The resulting samples were labelled as P-DA-PEEK. Peptide immersion solutions were kept frozen at −20 °C until used for peptide quantification test. The expected reactions of this method are presented in Fig. 1.

2.3.2. Second method: immobilization via a diglycidyl ether

In this method, a diglycidyl ether was used as a bifunctional crosslinker to conjugate the peptide to PEEK surface. To achieve that, amination of PEEK samples was done first with ethylenediamine at 120 °C for 1, 2, 3, and 4 h. Amine groups on PEEK samples were quantified using ninhydrin assay (Sigma Aldrich, Germany). In brief, an aminated PEEK disc was incubated with 200 µl of KCN, 150 µl of phenol, and 200 µl of ninhydrin reagent in a glass test tube at 98 °C for 10 min in a water bath. When

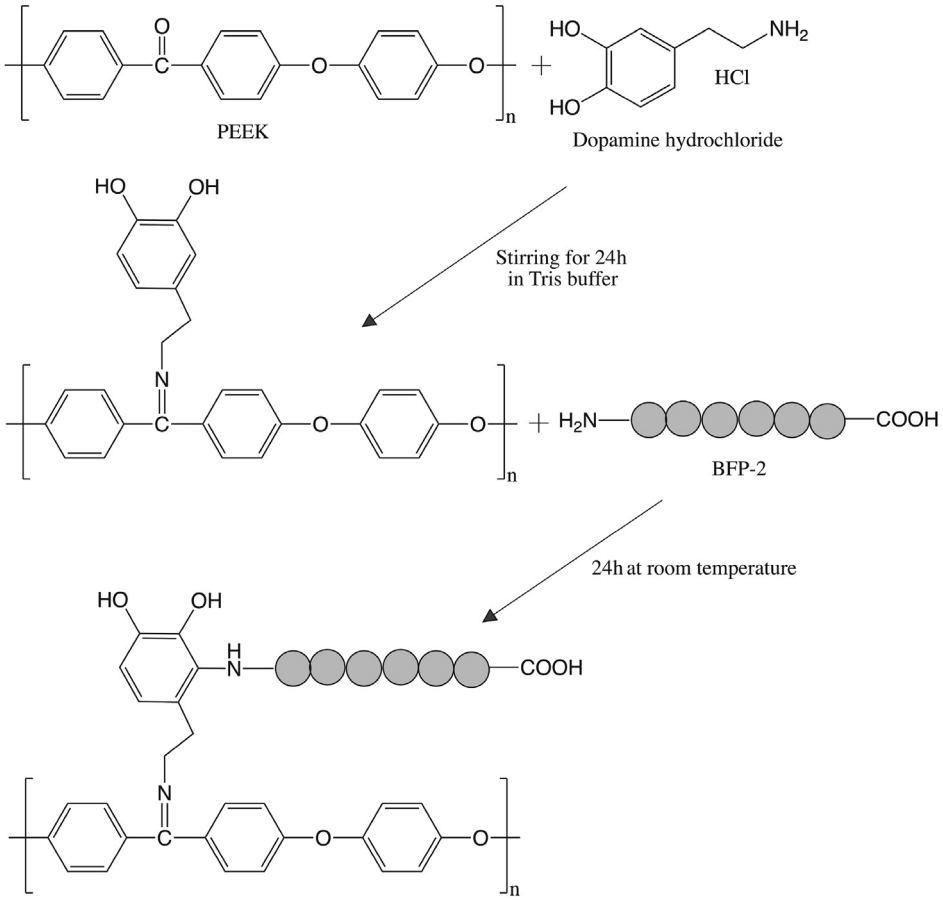

Fig. 1. Reactions of peptide immobilization via dopamine.

reacting with amines, ninhydrin forms Ruhemann's purple dye. After cooling, the solution was diluted with 60% ethanol to a 5 ml volume. An aliquot of 200 μl of the solution was transferred to a new tube and diluted with 60% ethanol to a final volume of 1 ml. The optical density of the final solution was measured by a UV–visible spectrophotometer (Shimadzu, Japan) at 570 nm wavelength. Amination time of 3 h resulted in most amount of amine groups attached on PEEK discs, and there was no increase in amine quantity after 3 h.

Aminated PEEK discs were immersed in 5% (vol/vol) of 1,4-Butanediol diglycidyl ether (Hangzhou Hyper Chemicals Limited, China) in 50 mM carbonate-bicarbonate buffer with a pH of 9 for 2 h. The samples were washed with distilled water and then incubated with 1 ml of each of the prepared peptide solutions for 24 h at room temperature. Subsequently, samples were washed with PBS and the immersion solutions were stored frozen for peptide quantification. The resulting samples were labelled as P-DGE-PEEK. The expected reactions of the diglycidyl ether method are presented in Fig. 2.

2.4. Peptide quantification test

To determine which method and which peptide concentration yielded the most immobilized peptides on PEEK surface, a quantification test was performed. Fluorescamine assay (ChinaPeptides Co., Ltd., China) was used for quantification of primary amines in the reserved peptide solutions (unattached peptides after immobilization) and in the initial peptide solutions (before immobilization). The difference between these two solutions was considered as the amount of attached peptides. Fluorescamine produces a highly fluorescent compound when it reacts with primary amines. The assay was performed by taking a 9 μl aliquot from each immersion solution into a plain tube. A 3 μl of 3 mg/ml fluorescamine in Dimethyl sulfoxide (DMSO) solution was added to the tube and

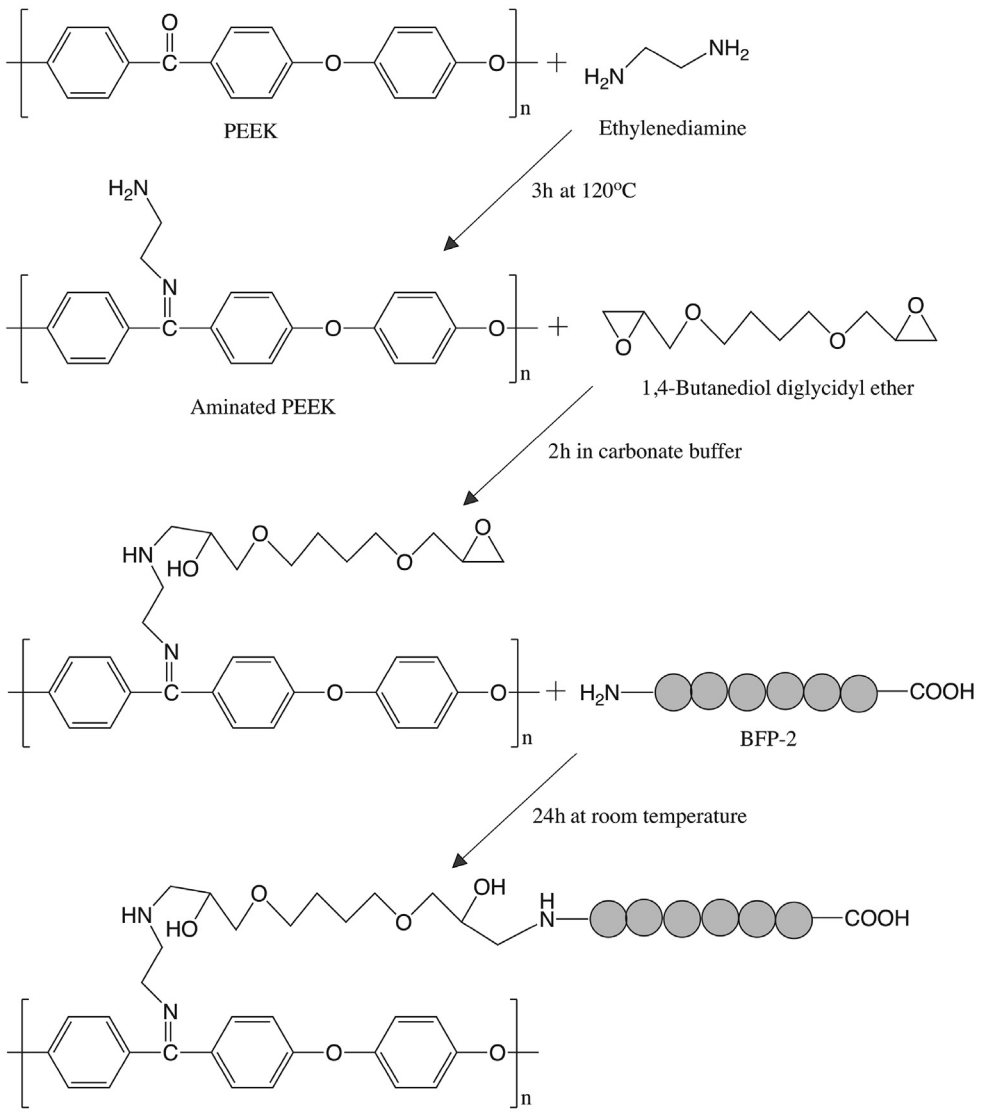

Fig. 2. Reactions of peptide immobilization via a diglycidyl ether.

incubated in the dark at room temperature for 15 min. Then the solution was diluted with DMSO to a final volume of 3 ml. The relative fluorescence units (RFU) of the final solution were measured by a Spectrofluorophotometer (RF-5301PC, Shimadzu, Japan).

2.5. Peptide stability test

The strength of peptide attachment can be tested by a stability test. Each of the P-DA-PEEK and P-DGE-PEEK samples were immersed in 5 ml PBS and challenged against agitation by a tube roller device (Spiramix 5, Denley, England) for 24 h at room temperature. The amount of detached peptides in the 1 ml of the immersion liquid was quantified by fluorescamine assay as described above. To calculate the percentage of detached peptides, the amount of attached peptide in 9 μl was proportionated to that in 1 ml, and the percentage was calculated by dividing the detached peptides in 1 ml by the attached in 1 ml then multiplied by 100.

2.6. Surface characterization

Surfaces of unmodified PEEK (control), P-DA-PEEK, and P-DGE-PEEK were characterized by:

1. Attenuated total reflection Fourier transform infrared (ATR-FTIR). The transmittance spectrum recorded from 4000 to 600 cm^{-1} was obtained by ATR-FTIR device (IRAffinity-1,

Shimadzu, Japan) to determine the formation of new bonds with PEEK polymer chains.
2. Atomic force microscopy (AFM). A 3D topographical image along with the average surface roughness (S_a) in nanometers and the surface area ratio (S_{dr}) were obtained for each sample by contact AFM (BenYuan CSPM-5500, Being Nano-Instruments Ltd. Beijing, China) operating in tapping mode.
3. Wettability test. Wettability of PEEK samples was assessed by a contact angle goniometer (Cam110, Creating Nano Technologies Inc., Taiwan) with a sessile drop of deionized water.

2.7. Statistical analysis

All data were analyzed using Prism 8 (GraphPad Software, USA). Results were presented in bar charts with the mean values (n = 10) written inside the bars and the standard deviation written above the bars. One-way ANOVA and Tukey's HSD (honestly significant difference) post-hoc tests were performed to determine the significance among groups and for multiple comparisons, respectively. A P-value >0.05 is statistically non-significant (NS), <0.05 is significant (S), and <0.01 is highly significant (HS).

3. Results and discussion

Wet chemistry was used in this study for surface modification of PEEK biomaterial to enhance its bioactivity. Wet surface treatment is considered more applicable and feasible than other surface treatment strategies since it does not involve elevated temperatures, which might affect bulk properties [21]. The chemical modification of PEEK usually involves the use of ketone groups (C=O) to anchor functional groups or compounds, or the conversion of ketone groups into hydroxyl or amine groups, which in turn are used to further graft bifunctional spacers and biomolecules.

In this study, two strategies were applied to graft BFP-2 into PEEK polymer surface using dopamine and 1,4-Butanediol diglycidyl ether as an anchor and a bifunctional spacer, respectively. Dopamine is an organic compound of the catecholamine family. It can be grafted onto many polymer surfaces by self-polymerization in an alkaline medium with no need for surface activation or pretreatment. The peptide was grafted to the dopamine through Michael addition reaction between the primary amine of the peptide and the catechol of the dopamine. The 1,4-Butanediol diglycidyl ether is an organic chemical that has two glycidyl (epoxide) groups at both ends. The glycidyl group is highly reactive with primary amines at room temperature. This chemical was used in this study to conjugate the amine group of the peptide at one end and the amine on the PEEK surface (created by amination) at the other end.

Bone-forming peptide-2 is an osteogenic peptide derived from bone morphogenetic protein-7. A smart approach is to utilize peptides derived from the active domains of bone extracellular matrix (ECM) proteins to initiate the cascade of osteogenesis, angiogenesis, and ossification of new bone during bone healing around dental implants [20,24].

3.1. Peptide quantification and stability

Mean values (n = 10) of RFU for both quantification and stability tests are presented in Table 1.

In both immobilization methods, the amount of attached peptides did not significantly increase when increasing the concentration of peptide solution above 0.5 mM, possibly because of saturation of all binding sites for the peptides. The P-DGE-PEEK showed a slightly higher amount of attached peptide (than the P-DA-PEEK. This could be due the high affinity of the diglycidyl to react with amine groups of the aminated PEEK and the peptide.

All groups showed comparable peptide stability with a percentage of detached peptides of around 1%. The P-DGE-PEEK samples resulted in a slightly higher percentage of detached peptides than the P-DA-PEEK samples, but still not significant. This proves that the peptide was strongly bonded to the polymer chains and no dissolution of covalent bonds happened in aqueous solution with agitation.

Table 1. Results of peptide quantification and stability tests.

Immobilization method		P-DA-PEEK				P-DGE-PEEK			
Peptide concentration (mM)		0.25	0.5	0.75	1	0.25	0.5	0.75	1
RFU mean	Attached in 9 μl	248	313	319	320	242	326	330	325
	Attached in 1 ml	27556	34778	35444	35556	26889	36222	36667	36111
	Detached in 1 ml	261	332	341	342	274	367	382	353
Percentage of detached/attached		0.95%	0.95%	0.96%	0.96%	1.01%	1.01%	1.04%	0.98%

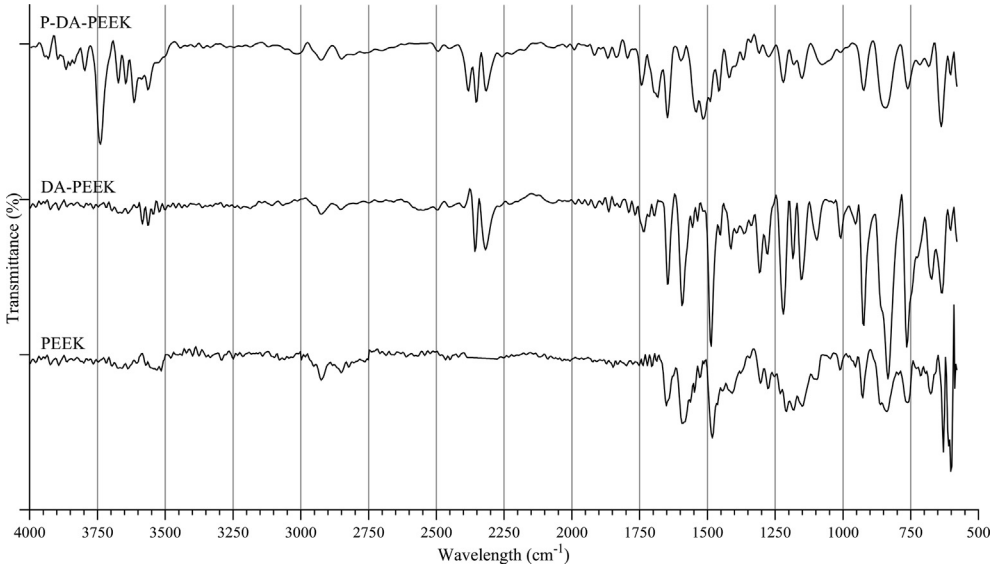

Fig. 3. ATR-FTIR spectra of PEEK samples of dopamine immobilization method.

3.2. ATR-FTIR

The FTIR spectrum of pristine PEEK showed a carbonyl stretching vibration at ≈1650 cm^{-1} and ring vibrations at 1590, 1500, 1485, and 1410 cm^{-1}. The diphenyl ether group (C−O−C) appeared at 1270 and 1190 cm^{-1}. The bending motion of C−C and (-C=O) groups appeared around 1300 cm^{-1}. By comparing the FTIR spectra of PEEK with PEEK-DA in Fig. 3, it was clear that the dopamine was bonded to the PEEK polymer chains. Thus, the intensity of carbonyl groups was sufficiently decreased with the appearance of a new band stretching frequency vibration of the azomethine group (C=N) of the Schiff base observed at ≈1700−1750 cm^{-1}. The spectrum displayed strong bands at 3550 cm^{-1} corresponding to the stretching frequency vibrations of phenolic (OH).

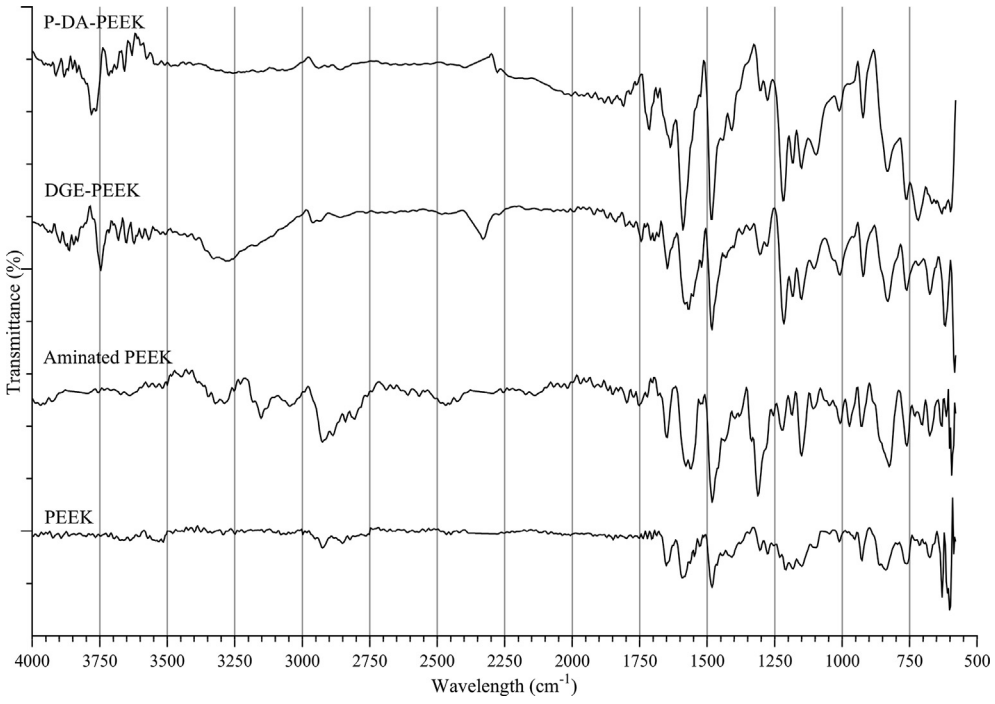

Fig. 4. ATR-FTIR spectra of PEEK samples of diglycidyl ether immobilization method.

The FTIR spectrum of P-DA-PEEK showed a sharp singlet peak above 3500 cm^{-1} which was definitely assigned to the phenolic OH. The appearance of additional peaks around 1710 cm^{-1} and 3500-4000 cm^{-1} which belong to the C=O and −COOH groups, respectively, confirmed the loading of peptide-dopamine onto the PEEK surface. Furthermore, the band intensity at 1100 cm^{-1} was for C−N (peptide bond with the dopamine).

The peaks around 2350 cm^{-1} belonged to atmospheric CO_2.

In Fig. 4, the amination of PEEK was confirmed by the appearance of new band stretching frequency vibration of the azomethine group (C=N) of the Schiff base at ≈1700 cm^{-1}. The spectrum displayed new bands at 3300 cm^{-1} corresponding to the stretching frequency vibrations of NH_2, and at 2750−2900 cm^{-1} corresponding to the stretching frequency vibrations of aliphatic C−H.

The FTIR spectrum of DGE-PEEK showed new peaks at 3400 cm^{-1} and 3270 cm^{-1} which are definitely assigned to the OH and NH respectively. Also, the appearance new band at 1200-1280 cm^{-1} assigned to the C−O of ether. While, the stretching and contracting of the bonds in epoxide ring can be seen shifted from 1280 to 1230, 950−810, and 880−750 cm^{-1}.

The FTIR spectrum of P-DGE-PEEK showed appearance of additional peaks around 1700 cm^{-1} and 3500-4000 cm^{-1} which belong to the C=O and −COOH groups, respectively confirmed the loading of peptide onto the modified PEEK surface. Also, the band intensity at 1100 cm^{-1} was for C−N (peptide bond with the ether).

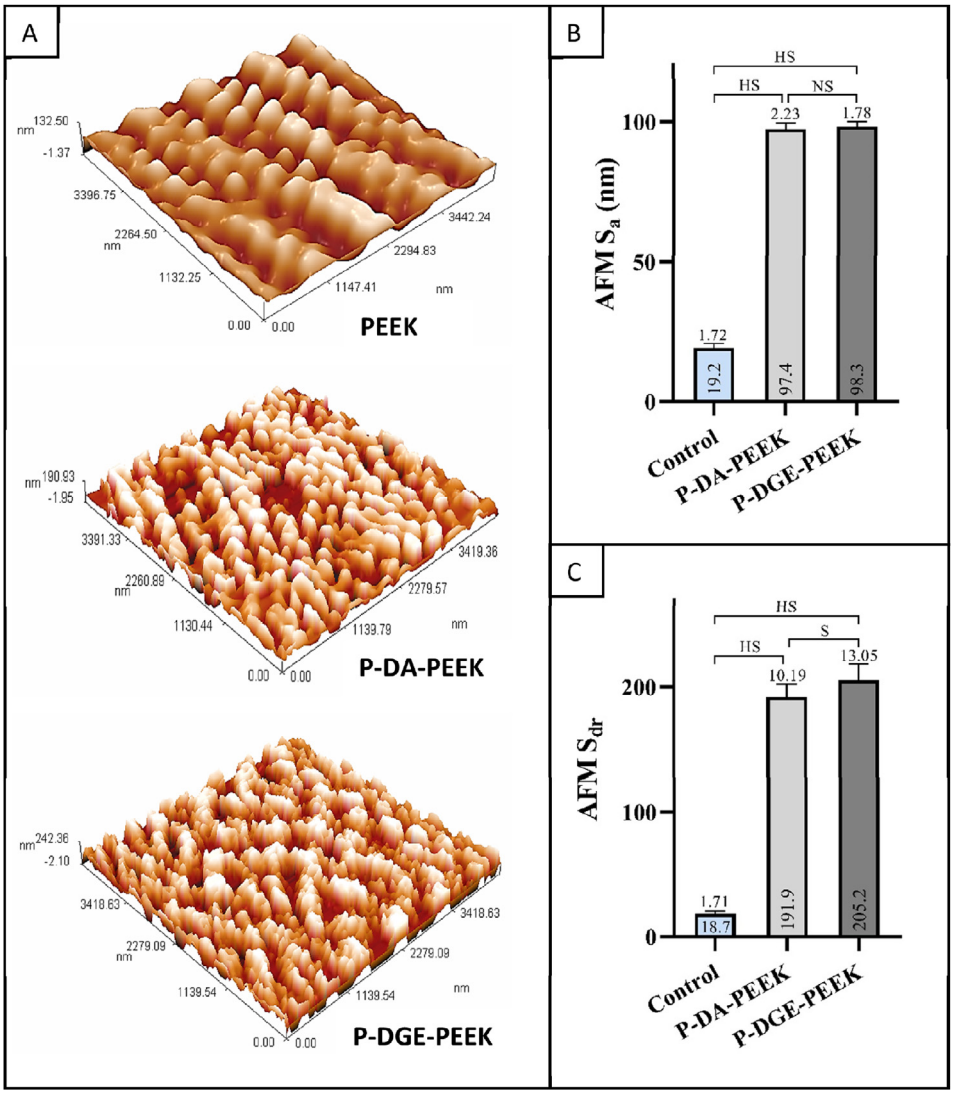

Fig. 5. AFM results, A: 3D topographical images, B: Mean values of S_a with Tukey's HSD significance, C: Mean values of S_{dr} with Tukey's HSD significance.

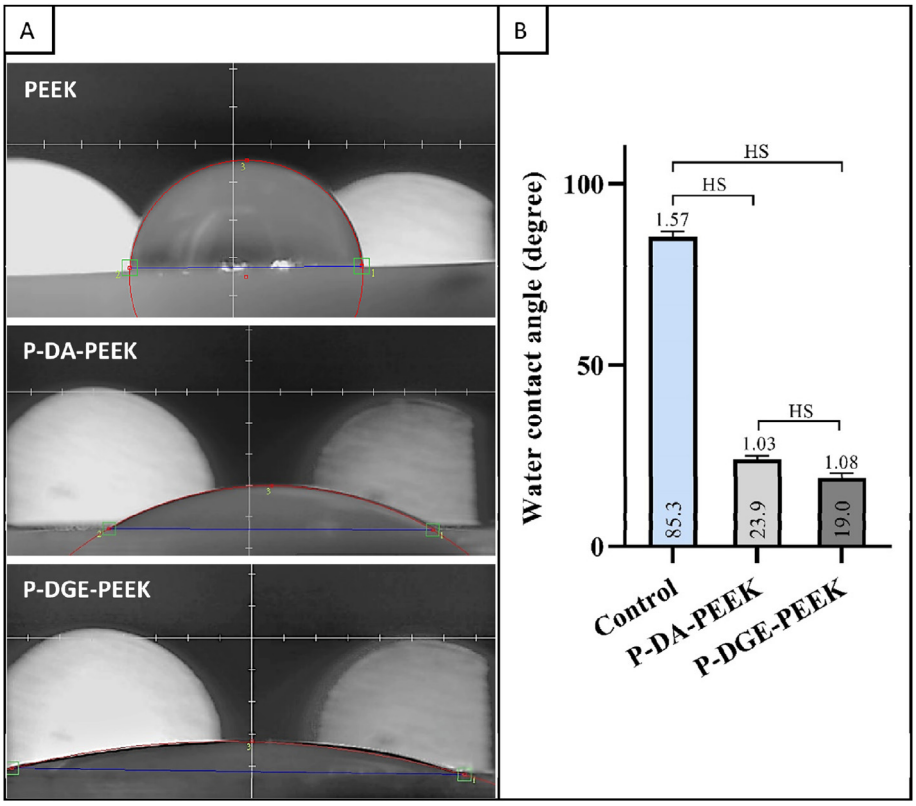

Fig. 6. A: Water contact angle measurements, B: Mean values of water contact angle with Tukey's HSD significance.

3.3. AFM

The 3D images of AFM showed changes in the surface topography with an increase in the number of valleys of P-DA-PEEK and P-DGE-PEEK compared to the control group. This resulted in highly significant increases in average surface roughness (S_a) and surface area ratio (S_{dr}) in both peptide groups compared to the control group (Fig. 5). The increase in roughness could be attributed to the grafting of peptide molecules on the surface along with the conjugate compounds [25]. The P-DGE-PEEK samples showed slightly higher surface roughness values (98.3 nm) than P-DA-PEEK samples (97.4 nm). This could be explained by the fact that the 1,4-Butanediol diglycidyl ether ($C_{10}H_{18}O_4$) has a longer carbon atom chain, which can undergo folding phenomena, compared to dopamine ($C_8H_{11}NO_2$) which is shorter and has a stiffer aromatic backbone. The folding of the diglycidyl ether was projected as roughness in nanoscale on the PEEK surface.

3.4. Wettability

Results showed that there was a highly significant decrease in water contact angle measurement for both peptide groups in comparison to the unmodified PEEK. The P-DGE-PEEK had the lowest value (19°), while the P-DA-PEEK and PEEK had values of 23.9° and 85.3°, respectively (Fig. 6). This decrease in the contact angle indicates a better wettability for the modified PEEK. The enhancement in the wettability of peptide-grafted PEEK is due to the hydrophilicity of the peptide, which is attributed to its polar groups (OH, NH_2, and COOH). Polar molecules are very good at attracting water molecules, mostly through the formation of hydrogen bonds. Water can generate electrostatic interactions (charge-based attractions) with other polar molecules due to its polarity.

The increase in wettability is also related to the surface topography and the increase in the surface area ratio according to Wenzel's theory [26] which stated that adding surface roughness and increasing the surface area leads to an enhancement in the wettability. This could explain the higher wettability for P-DGE-PEEK than P-DA-PEEK, since it showed higher surface roughness and surface area values.

4. Conclusion

Bone-forming peptide-2 was successfully immobilized on PEEK surface using the two described

methods. The null hypothesis was rejected since the grafted peptide improved PEEK surface properties in terms of surface chemistry, topography, and wettability. This approach of PEEK functionalization might have the potential to be used as an implant material with further investigation both in vitro and in vivo.

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