ABSTRACT
The commitment and differentiation of human mesenchymal stem cells (hMSCs) are guided by bioactive molecules within the extracellular matrix. Among the various approaches to design biomaterials, the functionalization of biomaterial surfaces with peptides from the sequence of proteins from the extracellular matrix is quite common. The purpose of this functionalization is to recruit hMSCs and promote their differentiation into the appropriate lineage. The aim of this work was to investigate the influence of RGD and FHRRIK peptides and peptide sequences taken from bone morphogenic protein (BMP-2) and histone H4 (osteogenic growth peptide; OGP) either tethered alone or as a mixture on the surface of a model material and to also examine the level of hMSC osteogenic commitment without using a differentiation medium. Grafting of the different peptides was assessed by X-ray photoelectron spectroscopy (XPS), while their surface density was quantified by fluorescence microscopy, and their surface properties were assessed by atomic force microscopy (AFM) and contact angle (CA). The osteogenic commitment of hMSCs cultured on the different surfaces was characterized by immunohistochemistry using Runx-2 as an earlier osteogenic marker and OPN, a late osteogenic marker, and by RT-qPCR through the expression of Coll-a1, Runx-2, and ALP. Biological results show that the osteogenic commitment of the hMSCs was increased on surfaces tethered with a mixture of peptides. Results indicate that tethered peptides in the range of pmol mm$^{-2}$ were indeed effective in inducing a cellular response after 2 weeks of cell culture without using an osteogenic media. These findings contribute to the research efforts to design biomimetic materials able to induce a response in human stem cells through tethered bioactive molecules for bone tissue engineering.

KEYWORDS
stem cells, biomimetic materials, bone tissue engineering, mimetic peptides, surface modification
1 INTRODUCTION

Tissue engineering has emerged from the biomaterials field and consists in restoring functional tissues with a combination of cells, scaffolds, and biologically active molecules. The objective of tissue engineering is thus to assemble functional constructs that repair, sustain, heal, or improve damaged tissues or whole organs. One key strategy to achieve this goal is the development of scaffolds to provide not only the early support for cell adhesion, but also a framework to organize the cells into the desired functional tissue. Until now, many natural and synthetic materials have been developed and used in tissue engineering, however, a material suitable for all applications has not yet been identified.

The ideal material for tissue engineering displays perfect biocompatibility for cell seeding, homing, proliferation, and differentiation. The concept of “ideal material” can mainly be achieved by producing a very attractive surface for the cells. To engineer bone tissues, human mesenchymal stem cells (hMSCs) appear to be attractive cell candidates, as hMSCs are pluripotent and are thus able of proliferating and differentiating to various lineages including bone tissue. To produce materials capable of mimicking the stem cell niche, our approach was to functionize a model material surface, namely, polyethylene terephthalate (PET) films, with molecules that would enhance the adhesion or induce the differentiation of hMSCs. As a result, the cell response toward the material would be controlled by intracellular mechanisms due to the interaction between transmembrane proteins and the modified surface.

We investigated the impact of the grafting of adhesion peptides and growth factor mimetic peptides for hMSC differentiation into osteoblasts. For this purpose, we selected two pro-adhesive peptides containing either RGD or FHRRKA (Phe-His-Arg-Arg-Ile-Lys-Ala), a heparin-binding domain peptide; the latter peptide is a more specific peptide toward osteoblast-like cells. Two peptides displaying potential for differentiation were also selected.

The first peptide was taken from a bone morphogenetic protein-2 (BMP-2) sequence. BMPs are cytokines of the transforming growth factor beta family (TGF-β). This protein family is involved in bone formation, development, and remodeling. BMPs interact with their receptors I and II (BMPRI and BMPRII) through noncovalent bonds. Briefly, BMPs bind to BMPRI, after which the BMPRI receptors are phosphorylated. Subsequently, the activated BMPRI phosphorylates a group of Smad proteins (Smad 1/5/8), which interact with a nuclear Smad called Smad4. As shown by Zouani et al., the concomitant grafting of the RGD sequence and a mimetic peptide of a growth factor, such as BMP, can further guide cells to differentiate.

The second osteogenic peptide selected for this study was osteogenic growth peptide (OGP), an endogenous peptide found in the human blood system at concentrations in the micromolar level. Discovered in the early 90s, the peptide sequence (Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly) is taken from the C-terminal region of histone H4. However, it has been shown that the active sequence of this peptide is located between the 10−14 amino acid (OGP10−14) sequence. The signaling pathway of OGP is, however, not known with certainty. Moore et al. used this peptide tethered on polymer surfaces to evidence its activity on cell membranes. It appears that the specific orientation of the peptide has an impact, as the study by Panseri et al. showed that the best response was obtained when OGP10-14 was tethered by the N-terminal to the material. Furthermore, the effect of OGP10−14 has always been assessed individually. Given the biological synergy already observed between co-conjugated peptides on surfaces, in terms of differentiation, it is suggested that this synergistic behavior could also be observed with OGP as one of the conjugated peptides.
The goal of this study was therefore to develop surfaces with different peptides tethered onto the surface of PET material with a well-controlled density. PET material was chosen for its easy surface modification and as a model material. To assess the peptide grafting and their surface density, peptide-conjugated surfaces were characterized by X-ray photoelectron spectroscopy, fluorescence microscopy, atomic force microscopy, contact angle, and the Toluidine Blue O test. The biological impact of the tethered surfaces on the differentiation of human mesenchymal stem cells (hMSCs) into the osteoblastic lineage was assessed using RT-qPCR and immunochemistry.

2 MATERIALS AND METHODS

2.1 Materials.

PET samples were taken from a commercial crystalline biaxially oriented film obtained from Goodfellow (Lille, France). The bioriented film had a thickness of 75 μm. Inorganic reagents (NaOH, KMnO₄, H₂SO₄, HCl, glacial acetic acid), acetone, acetonitrile, dimethylaminopropyl-3-ethylcarbodiimideethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and 2-(N-morpholino)-ethanesulfonic acid (MES), and Toluidine Blue-O (TBO) were purchased from Sigma-Aldrich (Lyon, France). GRGDSPC (RGD), GFHRRIKA (FHRRIKA), GYGFGG (OGP), RKIPKASSVPNTELSAISMLYL, which is a BMP-2 mimetic peptide previously identified by our group (BMP), GRGDSPCTAMRA, and RKIPKASSVPNTELSAISMLYL-FITC fluorescent peptides were synthesized by GeneCust, (Ellange, Luxembourg).

2.2 Methods.

2.2.1 Surface Preparation of PET and Covalent Grafting of Different Peptides.

PET surfaces were functionalized according to the protocol described by Choll et al. with some adjustments. Briefly, the PET surfaces were hydrolyzed and oxidized to generate carboxylic acid groups on the surface (referred to as PETCOOH). Oxidized surfaces were used as a control for the biological experiments. The surfaces were then immersed in a solution of ethylcarbodiimide hydrochloride (EDC) (0.2 M) + N-hydroxysuccinimide (NHS) (0.1 M) + 4-(morpholino)ethanesulfonic acid (MES) (0.1 M) in Milli-Q water to convert the surface carboxylic acid groups into activated esters. Because of the presence of MES during the activation step, the previously generated COOH group on the surface of the PET were not dissociated, as the pH of the solution remained acidic. Peptide immobilization onto the activated PET surfaces was subsequently performed by immersing the previous surfaces into the peptide solution in PBS, at 10⁻⁵ M (of one peptide or as 1:1 molar ratio of two peptides), for 16 h at room temperature. Following covalent immobilization, the surfaces were sonicated with Milli-Q water for 15 min to remove the physically attached peptides. Figure 1 illustrates the different surface modification steps. Surfaces with a single peptide and double grafted through the N-terminal to the surface were prepared.

2.2.2 Surface Characterization.

2.2.2.1 Toluidine Blue O.

Following the oxidation step described previously, the concentration of carboxyl groups onto the film was assessed by means of the toluidine blue-O (TBO) protocol, in which the dye stains the negatively charged 5.10⁻⁴ M of TBO was prepared by dissolving TBO powder in a NaOH solution (pH=10). The functionalized PET surfaces were individually placed in 15 mL tubes with 10 mL of TBO solution on a shaking plate for 6 h. Following TBO binding, the supernatant was removed from the tubes and the stained PET surfaces were successively washed twice with a NaOH solution (pH
10) and Milli-Q water. A solution of 1:1 v/v H₂O:CH₃COOH (5 mL) was added on the PET surfaces and left to react during 10 min to detach the TBO dye from the carboxyl groups. Thereafter, 200 μL of the washing solution from each sample was placed into a 96-well plate and the absorbance at 633 nm was measured using a UV−vis spectrometer. Non-specific adsorption was assessed after 1 day of contact between the surface and the 1:1 v/v H₂O/CH₃COOH solution. A calibration curve obtained with different dilutions of TBO in 1:1 v/v H₂O/CH₃COOH was used to determine the concentration of the carboxyl groups (see Figure S1). Calculation of the carboxylic acid surface concentration was based on the assumption of a 1:1 stoichiometric reaction between TBO and the carboxylic acid groups. The TBO assay was performed on spared surfaces and not on those used for further modification and biological assessment.

Figure 1. Peptide grafting procedure on PET surfaces.

2.2.2.2 X-ray Photoelectron Spectroscopy (XPS).

Surface chemical compositions were determined after each step of the peptide grafting by XPS using a PHI 5600-ci spectrometer (Physical Electronics, Eden Prairie, MN, USA). Survey spectra were recorded using a standard aluminum X-ray source (1486.6 eV) with charge neutralization, while high-resolution C 1s XPS spectra were recorded using a standard magnesium X-ray source (1253.6 eV) without charge neutralization. The detection angle was set at 45° with respect to the sample surface. The analyzed area was 0.5 mm². The curve fitting procedures for C 1s were performed by means of a least-squares Gaussian−Lorentzian peak fitting procedure following Shirley background subtraction. The C−C and C−H contributions to the C 1s high-resolution spectra were referenced at 285 eV. Three measurements per sample on three different samples were carried out to ascertain the reproducibility of the surface chemistry.

2.2.2.3 Atomic Force Microscopy (AFM).

AFM was used to characterize surface roughness and morphology (Dimension 3100, Digital Instruments, Veeco, Woodbury, NY, USA). It was performed in tapping mode with an etched silicon tip (OTESPA, tip radius <10 nm) at a rate of 1Hz at room temperature. The roughness average parameter (Rₐ) obtained from 20 × 20 μm² topographical images was used to determine surface roughness. The images were then analyzed using WSxM software. Three measurements per sample on three different samples were carried out to ascertain the homogeneity of the surface modification.

2.2.2.4 Contact Angle (CA).
The contact angles of water microdrops on the different surfaces were measured using the VCA-2500XE video contact angle system (AST Products Inc., Billerica, MA, USA). Static water contact angles were measured by deposition on the different PET surfaces of a droplet of 1 μL of deionized water. Six measurements were performed on each PET sample (at least two materials for each condition).

2.2.2.5 Evaluation of Peptide Density by Fluorescence Microscopy.

To ascertain peptide density on the PET surfaces, the surfaces were tethered with fluorescent peptides. The fluorescence intensity of these surfaces was then assessed by fluorescence microscopy according to previous works carried out by our group. briefly, FITC or TAMRA fluorochromes were covalently conjugated to the end of each peptide through a lysine amino acid residue (K). The fluorescent peptides were then immobilized onto the PET surfaces as aforementioned. Quantification of fluorescence intensity on the surfaces with either FITC-labeled or TAMRA-labeled peptides was performed using a fluorescence microscope (Leica DM5500B, Wetzlar, Germany) equipped with Leica MMAF software. A calibration curve was first established by measuring the fluorescence intensity of a series of FITC and TAMRA droplets (from 1.1 to 18.4 nmol) deposited on virgin PET using fluorescence microscopy at a magnification of 2.5. The surfaces grafted with FITC-labeled or TAMRA-labeled peptides were then imaged under identical conditions. Finally, the surface density of each fluorescent peptide grafted was measured using the calibration curve (see Figure S2).

2.2.3 Cell Culture.

Human MSCs from bone marrow (one donor) purchased from PromoCell (Heidelberg, Germany) were grown in mesenchymal stem cell basal media (MSCBM2) (PromoCell) in a humidified atmosphere containing 5% (v/v) CO₂ at 37 °C. For each experiment, hMSCs between passages 4 and 5 were seeded on PET materials at a density of 5000 cells/cm² in serum-free α-MEM during the first 6 h. The medium was then changed to α-MEM supplemented with 10% (v/v) fetal bovine serum FBS (Gibco) with no additional growth factors and was changed every 72 h. hMSC differentiation on the different PET substrates was evaluated after 14 days of cell culture. Because of the large number of materials required to perform the biological analyses, the decision was made to perform cell cultures at one specific time point. To define the optimal time to assess gene expression, exploratory experiments at 1, 2, and 4 weeks, using fluorescent microscopy, were performed with a smaller amount of materials. Oxidized PET was used as the control surface in each biological experiment.

2.2.4 hMSC Differentiation along Osteoblastic Lineage.

2.2.4.1 RT Quantitative Real-Time PCR.

hMSCs were lysed in TRIZOL reagent (Invitrogen) to isolate the total RNA, and a TurboDNA free kit (Ambion, Illkirch-Graffenstaden, France) was used to remove contaminating DNA from the RNA preparations. Two micrograms of purified total RNA was used to synthesize cDNA using Thermo Scientific Maxima Reverse Transcriptase (Thermo Scientific, Illkirch-Graffenstaden, France) and random primers (Thermo Scientific). cDNA aliquots (4 ng) were then amplified in a 10-μL reaction volume containing 500 nM primers and the SsoAdvancedTM Universal SYBR Green Supermix (BioRad, Marnes-la-Coquette, France) in a CFX96TM Real-Time PCR Detection System (BioRad). The PCR cycling parameters were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of PCR reactions (95 °C for 5 s and 60 °C for 10 s). C₀ values for the gene of interest were normalized against RPC53 and PPIA. Bestkeeper software was used to determine the normalization effectiveness of each reference gene among all of the samples under study. The relative expression
levels were calculated using the comparative method ($2^{−ΔΔCt}$), with the controls arbitrarily set at 1. Table 1 lists the primers used for amplification.

<table>
<thead>
<tr>
<th>genes</th>
<th>primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx-2</td>
<td>5′-AAGTGCAGGTGAACATTTCT-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TCTCGGGGGCTCTGAGTGA-3′ (reverse)</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>5′-ATGCCCCGGAGCTTCACAAG-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TGGTGGAGCTGACCTTGTGAG-3′ (reverse)</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>5′-GACGTGAGCTGATAGCTGA-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TTGGGAGGACAGAATG-3′ (reverse)</td>
</tr>
<tr>
<td>collagen α1</td>
<td>5′-ACATGGTGCTGCTGACC-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-TGATTGGGATGGATCTCCTG-3′ (reverse)</td>
</tr>
<tr>
<td>PPIA</td>
<td>5′-CCGGGTCTGGCATCTTGTG-3′ (reverse)</td>
</tr>
<tr>
<td>RPC53</td>
<td>5′-ACCTCGGTGACACTGACAG-3′ (forward)</td>
</tr>
<tr>
<td></td>
<td>5′-AGGAGTTGACACCTTCCAGA-3′ (reverse)</td>
</tr>
</tbody>
</table>

2.2.4.2  Immunofluorescence Staining.

Immunofluorescent staining was performed 2 weeks after cell seeding on three surfaces per condition. The cells were fixed in 4% paraformaldehyde (PFA) solution, permeabilized with 0.5% Triton-X 100, and subsequently blocked with 1% BSA/PBS solution (BSA: bovine serum albumin). Samples were then incubated with rabbit anti-Runx2 primary antibody (Abcam, Cambridge, United Kingdom) and mouse antiosteopontin (Sigma-Aldrich, Lyon, France) at 37 °C for 1 h, and coupled with Alexa Fluor 568 goat antirabbit IgG secondary antibody (Invitrogen, Illkirch-Graffenstaden, France) and Alexa Fluor 488 rabbit antimouse IgG secondary antibody (Invitrogen) for 30 min at room temperature. The samples were then mounted with Fluoroshield using DAPI from Sigma-Aldrich (Lyon, France) and were observed under a fluorescence microscope (10×).

2.2.5  Statistical Analysis.

All of the data were presented as the mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) and Tukey’s test for numerous comparisons using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Significant differences were determined for p values of at least ≤ 0.05. * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001.

3  RESULTS

3.1  Single Peptide Grafting.

3.1.1  Physico-chemical Characterization.

3.1.1.1  Carboxylic Group Density Assessment by Toluidine Blue O.

The amount of functional carboxylic group on the different surfaces was assessed by means of the toluidine blue-O TBO test. Densities obtained for the native and oxidized PET surfaces were 18 (equivalent to $1.12 \times 10^{16}$ COOH group per mm$^2$) and 134 pmol mm$^{-2}$ (equivalent to $8.05 \times 10^{16}$ COOH group per mm$^2$).
COOH group per mm²), respectively. These results show the effectiveness of the hydrolysis and oxidation steps to generate more functional COOH groups on the PET surface.

3.1.1.2 Characterization of Surface Chemical Composition by XPS.

XPS characterization of the native PET exhibited the expected elements, namely, carbon and oxygen (Table 2). The C1s spectrum exhibited three components present in the PET polymer: O−C=O at 288.9 eV, C−O at 286.5 eV, and C−C at 285.0 eV. The C/O experimental ratio was 2.8, which was close to the expected value of 2.5. However, the differences between the theoretical and experimental values were likely due to organic surface contamination. XPS analysis of the PETCOOH oxidized materials revealed the expected increase of the oxygen amount on the surface and a lower C/O ratio: C/OPET-Native = 2.8 and C/OPET-COOH = 2.0. The differences between the theoretical and experimental percentages were due to contamination. As expected, nitrogen was observed following the NHS grafting (Table 2). However, it should be noted that the %N detected as well as the C/N ratio was dependent on the peptide structure (number of amino acids and composition). For example, the smallest peptides, namely, OGP (6 amino acid residues) and RGD (7 amino acid residues), exhibited the lowest nitrogen composition, 2.2 and 2.1%, respectively, and the highest C/N ratio values (Table 2). In contrast, the surface grafted with BMP, which was the longest peptide under study (22 amino acid residues), displayed the highest nitrogen content of 3.1 and the lowest C/N ratio of 23.

Table 2. Experimental Composition (%) Obtained by XPS Analysis of Different Modification Steps of the PET Surface

<table>
<thead>
<tr>
<th>atomic %</th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>C/O</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>native PET</td>
<td>73.1 ± 0.3</td>
<td>26.6 ± 0.2</td>
<td>2.7 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PET-COOH</td>
<td>69.9 ± 0.2</td>
<td>30.2 ± 0.1</td>
<td>2.3 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>activated</td>
<td>68.1 ± 0.9</td>
<td>29.2 ± 0.9</td>
<td>2.8 ± 0.4</td>
<td>2 ± 1</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>RGD</td>
<td>70.0 ± 0.6</td>
<td>27.9 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>2 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>FTIRRAKA</td>
<td>71.6 ± 0.4</td>
<td>25.7 ± 0.7</td>
<td>2.7 ± 0.3</td>
<td>2 ± 1</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>BMP</td>
<td>71.5 ± 0.6</td>
<td>25.4 ± 0.6</td>
<td>3.1 ± 0.1</td>
<td>2 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>OGP</td>
<td>72 ± 1</td>
<td>26 ± 1</td>
<td>2.2 ± 0.1</td>
<td>2 ± 1</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>

To support the survey data and the grafting efficiencies, high-resolution (HR) spectra of C 1s were performed and are shown in Figure 2. First, as expected, the C 1s spectrum of the native surface exhibited three components associated with the PET structure: O−C=O at 288.9 eV, C−O at 286.5 eV, and C−C/ C−H at 285.0 eV (Figure 2). Following the hydrolysis/oxidation, a slight change in HR C 1s was observed, mainly due to the chain breakage (less C−O detected) leading to carboxylic group formation (higher contribution of O−C=O). Regarding the carboxylic group activation, PET-NHS sample in Figure 2, the new band at 287.5 eV assigned to the N−C=O bonds clearly shows the modification efficiency. Following the peptide grafting, the C 1s signals provided evidence confirming the presence of the different peptides: an increase in the alkyl band (C−C/C−H) at 285 eV and a slight increase of the characteristic peptide bond (N−C=O at 287.5 eV). Furthermore, the slight decrease of the band at 288.9 eV, associated with the O−C=O bond of the PET, concluded the PET surface coverage due to the peptide grafting. The survey and HR C 1s results thus confirm the expected surface modifications. Nitrogen, of course, remained present after peptide grafting, displaying, however, a lower relative atomic percentage (Table 2) because all of the peptides had higher C/N and O/N ratios in their structure.
Figure 2. C 1s XPS spectrum after the different surface modification steps leading to peptide conjugation. Light blue curves: C−C at 285 eV; light green curves: C−O at 286.5 eV; orange curves: N−C=O at 287.5 eV; dark green curves: C(−O)=O bonds at 288.9 eV.

3.1.1.3 Characterization of Surface Topography.

The roughness after each PET modification step and that of the functionalized materials was measured by AFM (Figure 3A). The AFM images exhibited no peptide aggregates on the peptide-grafted PET surfaces, regardless of the conjugated peptide. Roughness was shown to increase following the different modification steps, from 3.4 ± 0.7 nm for the native PET to 11.4 ± 0.4 nm for the PET-NHS. The results obtained for the tethered surfaces varied from 7.3 ± 0.6 nm in the case of the PET-RGD to 12 ± 2 nm for the PET-FHRIKA. However, these differences were not found to
be statistically significant. The contact angles were measured after each step of the surface modification (Figure 3B). The native PET surfaces were the most hydrophobic, with a contact angle of 67 ± 3°. The water contact angle significantly decreased on the PET-COOH (57 ± 3°) and PET-NHS (58 ± 2°) surfaces. Following the peptide grafting on the surfaces, the wettability of the different tethered surfaces was found to be comparable, regardless of the conjugated peptide involved. The contact angle of the different tethered surfaces was not statistically different.

Figure 3. (A) Surface roughness and (B) contact angle measured after the different surface modification steps leading to peptide conjugation (n = 6).

3.1.2 Peptide Surface Concentration.

3.1.2.1 Peptide Density Assessment by Fluorescence Microscopy.

As previously reported,25 peptide density was estimated by conjugating either TAMRA or FITC-coupled peptides by fluorescence microscopy. The measured peptide surface density varied between 1.1 ± 0.1 and 1.41 ± 0.05 pmol mm⁻², depending on the tethered peptide (Table 3). Moreover, the fluorescent microscopy images clearly evidence the homogeneity of the grafting.

Table 3. Peptide Density Measurements for Different Tethered Surfaces with Peptides (Scale Bar: 100 μm)

<table>
<thead>
<tr>
<th>Tethered surfaces</th>
<th>RGD</th>
<th>FHRRIKI</th>
<th>BMP</th>
<th>OGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide density (pmol.mm⁻²)</td>
<td>1.1 ± 0.2</td>
<td>1.41 ± 0.05</td>
<td>1.3 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

3.1.2.2 Extent of hMSC Osteogenic Differentiation in Response to Single Peptides.

To discriminate between different hMSC phenotypes on the different tethered surfaces, immunofluorescence staining was performed to assess the protein expression of Runx-2, an early
regulator of osteoblastic differentiation, and osteopontin (OPN), a late marker expressed in preosteoblasts. Human MSCs seeded on oxidized PET in identical cell culture conditions were used as negative controls. Surfaces with a single tethered peptide were first investigated. Cell nuclei were stained with DAPI (Figure 4). The cells on the control surfaces showed no expression of Runx-2 or OPN after 2 weeks of cell culture. The FHRRKA surfaces showed an expression of Runx-2 and a weak expression of OPN, whereas the BMP and OGP tethered surfaces exhibited a strong expression of OPN and Runx-2.

![Figure 4](image)

Figure 4. Immunofluorescence images of Runx-2 (red) and osteopontin (OPN) (green) after 2 weeks of cell culture on the single peptide-grafted surfaces. Nuclei are stained with DAPI (n = 3; scale bar: 40 μm).

To complement the immunofluorescence analysis, RT-qPCR was performed to screen for potential changes in hMSC phenotype on the different tethered surfaces (Figure 5). The gene expression of various osteogenic markers was assessed after 2 weeks of cell culture. The results clearly indicate that the tethered surfaces significantly enhanced the expression of early osteogenic markers (Runx-2, Coll-a1 and ALP), compared to that observed with the control surfaces. An overexpression of Runx-2 on the FHRRKA-tethered surfaces was clearly evidenced.

To better understand the induction process, the relative expression of alkaline phosphatase (ALP) and collagen-I α-1 (Coll-a1) was also evaluated. The secretion of these two proteins is characteristic of the extracellular matrix of native osteoblasts. A significant increase of Coll-a1 and ALP was observed in the bioactive functionalized materials, regardless of the peptide (Figure 5). Specifically, the highest levels of the underlying osteogenic markers were observed on the FHRRKA-tethered surfaces compared to what was observed on the control surfaces. We also evaluated the expression of OCN, known as a late osteogenic marker, which indicated an osteoinductive potential, compared that in the control surfaces. However, no significant difference in OCN expression was observed between the different conditions.

According to the RT-qPCR and immunocytochemistry results on the single-tethered surfaces, the surfaces inducing the most osteoblastic differentiation of hMSCs were the FHRRKA, BMP, and OGP-grafted surfaces. Given the immunofluorescence results, the protein expression was greater in the BMP and OGP-tethered surfaces. Therefore, the BMP and OGP surfaces provided the most effective differentiation of hMSCs into osteoblasts on a modified PET surface.

3.2 Extent of hMSC Osteogenic Differentiation in Response to Combined Peptides.
The synergistic effect of dually grafted peptides at a ratio of 1:1 on PET material surfaces was also investigated. The peptide combinations selected for this study were as follows: RB, RGD+BMP; RO, RGD+OGP; FB, FHRRRIKA+BMP; and FO, FHRRRIKA+OGP. Regarding the immunofluorescence results, the surfaces grafted with a blend of FHRRRIKA and an osteoinductive peptide (FB, FHRRRIKA+BMP; FO, FHRRRIKA+OGP) exhibited no improved osteoinductive activity compared to that observed in the control surfaces (Figure 6). On the other hand, the surfaces with a blend of RGD and BMP or OGP (referred to as RB and RO in the Figure 6) displayed promising osteogenic potential for the differentiation of hMSCs on the PET model surfaces, particularly in the case of RGD + OGP.

![Gene expression dynamics](image1.png)

**Figure 5.** Gene expression dynamics after 2 weeks of Runx-2, Coll-α1, alkaline phosphatase (ALP), and osteocalcin (OCN) on the single peptide-tethered surfaces (n = 5).

![Immunofluorescence images](image2.png)

**Figure 6.** Immunofluorescence images of Runx-2 (red) and OPN (green) after 2 weeks of cell culture on the double peptide-tethered surfaces (RB, RGD+BMP; RO, RGD+OGP; FB, FHRRRIKA+BMP; FO, FHRRRIKA+OGP) (n = 3; scale bar: 40 μm).
To complete the immunofluorescence analysis, RT-qPCR was performed. Cells on the FB and FO surfaces overexpressed Runx-2 after 2 weeks compared to what was observed on the control surfaces (Figure 7). Coll-α1 was overexpressed for all of the cells cultured on the double-tethered surfaces. However, Coll-α1 expression failed to exhibit differences between all of the different surfaces tethered with a blend of peptides. Furthermore, the expression of alkaline phosphatase was greater on the surfaces tethered with FHRRRIKA and BMP or OGP peptides. OCN expression was not found to significantly differ from that on the control surface.

In the double-grafted surfaces, the protein expression measured by immunochemistry was more significant in the RGD coupled with an osteoinductive peptide, while the gene expression determined by RT-qPCR was more important on the surfaces tethered with FHRRRIKA peptide.

Figure 7. Gene expression dynamics after 2 weeks of Runx-2, Coll-α1, alkaline phosphatase (ALP), and osteocalcin (OCN) on the double peptide- tethered surfaces (n = 5).

4 DISCUSSION

Clinicians currently supply growth factors directly to the regeneration site. This method is ineffective, however, due to the short biological life and rapid diffusion of injected growth factors to the surrounding tissue. Moreover, biological effects are often unpredictable due to systemic administration. The use of peptides and their immobilization on biologically compatible biomaterial surfaces appears to be a promising solution to overcome issues associated with targeted delivery. Furthermore, it is known that BMP activity is upheld when the peptide is grafted onto surfaces through a different pathway. There are various advantages to using grafted BMP. Indeed, it makes it possible to control the local density of the growth factor and the orientation of the molecule and prevents the growth factor from moving into the blood flow when the materials are implanted into patients. Today, there are numerous studies on the impact of single peptides grafted onto biomaterial surfaces, but only a few examine tethered peptide blends. Ma et al. suggested that there was an optimal codensity of BMP-2 mimetic peptide and RGD to obtain optimal hMSC differentiation. Furthermore, Moore et al. showed that the minimum density of BMP-2 peptide
required to induce an upregulation of Runx-2 was 0.8 pmol/mm². In the present study, peptide density was assessed by fluorescence microscopy with a monolayer of peptides on the surface of the material, which was confirmed by XPS results that clearly show that the variation in the atomic surface composition followed the expected surface concentration variation considering the various peptide chemical structures. In addition, despite the anticipated greater steric hindrance for BMP-2 surface grafting, the surface density of all four investigated peptides was shown to be almost identical. These data also suggest that the PET surfaces were effectively tethered with a thin homogeneous layer of peptides. Furthermore, the measured peptide surface densities are in agreement with the findings of Chollet et al., who estimated the RGD density on PET to be 1.7 pmol mm⁻². Other physicochemical characterizations were performed such as roughness evaluation and hydrophilicity. Although roughness increased through the modification steps, no significant difference was observed between the investigated tethered surfaces, therefore ruling out the possibility that cell behavior was driven by differences in surface roughness. Similarly, the contact angle measurements indicate that surface hydrophilicity variations could not explain cell fate differences when cultured on the different peptide-grafted materials. Identifying specific biochemical cues is of singular importance when assessing the osteogenic ability of biomaterials used as a bone tissue engineering scaffold. In previous studies by our group, RGD peptide was shown to enhance the osteoinductive potential of a BMP-2 mimetic peptide on mouse calvaria-derived preosteoblast-like cells on PET surfaces. Similar results were obtained on bioglass surfaces with the same RGD/BMP-2 mimetic peptide blend and hMSCs. The present study focused on comparing the response of hMSCs with these peptides and others previously identified in the literature.

Each cell culture experiment was performed in serum-free media for the first 6 h post seeding (to avoid protein fouling) and in the absence of soluble osteogenic factors (to avoid interference with culture media) over 2 weeks of cell culture. Human MSC differentiation toward the osteoblastic lineage usually occurs in response to BMP proteins or their mimetic peptides. Differentiation is then achieved through the interaction of BMPs with BMPR-I and BMPR-II, which in turn activates SMAD 1/5/8 signaling pathways leading to the overexpression of Runx-2. The gene expression analysis of the osteogenic markers showed that surfaces containing BMP-2 mimetic peptide alone at a density of 1.3 pmol mm⁻² upregulated ALP gene expression and OPN protein expression levels in hMSCs. Our observations are supported by Moore et al., who investigated the osteogenic differentiation of hMSCs with a tethered BMP-2 surface density in basal medium. These authors noticed an increase in Runx-2 expression, as BMP-2 concentration increased along a peptide gradient ranging from 0 to 1.4 pmol.mm⁻².

In contrast, the surfaces grafted with FHRRIKA peptide exhibited osteogenic potential, as was evidenced in the gene expression profile. These results are in agreement with previous studies showing FHRRIKA peptide promoting matrix mineralization. However, the immunofluorescence results show no proof of protein transcription after 2 weeks of cell culture. Furthermore, the cells cultured on surfaces grafted with FHRRIKA and BMP-2 or OGP exhibited no overexpression of the investigated markers. Even if the protein expression was conditioned by an overexpression of RNA, the expression peaks may not be observable at the same time scale. Consequently, it is possible to observe no RNA overexpression and an overexpression of the corresponding protein.

Furthermore, the RGD+BMP bifunctionalized surfaces exhibited an increase in osteogenic marker expression consistent with previous findings. Although the synergic effect between BMP and RGD peptides has been reported in the literature, contradictory results have been published, as Kim et al. and Koepsel et al. have reported that the effect of RGD and BMP-2 peptides on stem cell responses may in fact be due to differences in peptide density, which can significantly impact cell behavior, such as the way they attach onto biomaterials, as well as the medium conditions used for in vitro experiments. Overall data show that a synergistic effect between RGD and OGP peptides is observed in terms of differentiation with no need for additional osteogenic supplements.
One of the main applications of this work would thus be in the creation of osteoinductive systems for use in regenerative medicine or tissue engineering such as, for example, differentiated cells harvested and loaded into 3D biodegradable matrices for cell-based therapies.

5 CONCLUSION

We demonstrated the feasibility of easy-to-produce 2D model materials to observe the osteogenic differentiation of hMSCs in response to tethered molecules. The effect of different tethered peptides on hMSC differentiation was evaluated by RT-qPCR, immunofluorescence, and alkaline phosphatase activity analyses. Results show that a peptide density in the range of pmol mm$^{-2}$ was effective in inducing a cellular response after 2 weeks of cell culture in basal medium. BMP mimetic peptide was indeed shown to induce hMSC commitment and differentiation toward the osteoblastic lineage. The OGP sequence appeared to be more efficient in the presence of the RGD sequence, thereby suggesting that these peptides synergistically increased the commitment of hMSCs into osteoblast cells in the absence of osteogenic media after 2 weeks. These findings contribute to ongoing research efforts to design biomaterials that induce stem cell responses through bioactive molecules for bone tissue engineering.

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