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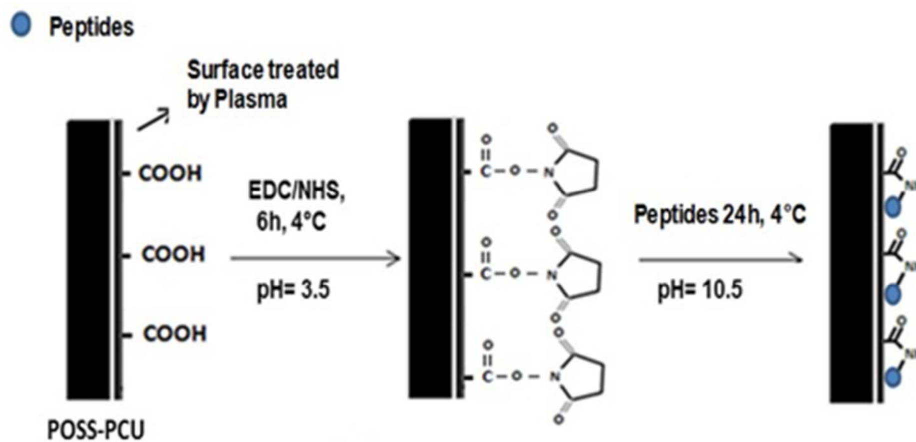

Biofunctionalisation of POSS-PCU for bone tissue engineering by plasma surface treatment
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ARTICLE

Peptide functionalisation of nanocomposite polymer for bone tissue engineering using plasma surface polymerisation

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In this work, we explored modification of new nanocomposite polymer called POSS-PCU for its functionalisation with bone inducting peptides using plasma surface polymerisation method for bone tissue engineering applications. KRSR and FHRRIKA peptides, which are heparin binding domains, have been shown before to induce cells adhesion and bone mineralisation. In this work plasma surface polymerisation process was developed to graft biomimetic peptides on polymeric biomaterial. Plasma polymerisation and peptide coupling was confirmed using various techniques such as Toluidine Blue O assay, IR-Spectroscopy and X-Ray photon Spectroscopy (XPS). Surface chemical analysis confirmed that 10 W power and 20 min of plasma treatment was optimum in order to obtain the highest grafting density of carboxyl groups, which were later used to attach bone biomimetic peptides via carbodiimide chemistry. IR-Spectroscopy and XPS confirmed functionalisation with carboxyl and peptide grafting. Finally, preliminarily *in vitro* study with bone marrow mesenchymal stem cells were performed in order to assess the efficacy of peptide functionalised surfaces to promote differentiation into osteogenic lineage. Cell tests showed increased cell adhesion and osteoblastic differentiation on KRSR and FHRRIKA peptide modified samples. This study has implications for the design and development of new generation of orthopedic implants using plasma modification technique.

Introduction

Implant failure is one of unmet challenges which we face today.[1, 2] Various strategies have been investigated to improve outcome by enhancing implant tissue integration, based on modifying implant interface via chemical or physical modification, in order to introduce functional groups or topographical changes on the surface.[3] In particular, plasma treatment has become popular surface interface modification technique for number of reasons such as: it is solvent free, fast, reproducible and can be applied on different substrates, metallic as well as polymeric materials.[4, 5]
With plasma modification both chemistry and topography can be modified. Recent studies have looked at carboxyl and amino functionalisation of orthopaedic implants to improve tissue integration and shown promising results via improved tissue integration and reduce corrosion on metallic implants.[6, 7] Furthermore, a wide range of biomolecules may be grafted on the surface of the implant to promote osteoinduction. Large proteins or glycosaminoglycans, such as collagen and chondroitin sulfate provide a biomimetic coating on the surface of an implant, which can improve integration once implanted in the body.[8] Growth factors are another type of widely used biomolecules due to their ability to modulate cellular functions, such as decreasing inflammation, enhancing stem cell differentiation, inducing blood vessel formation, or acting as chemoattractants for circulating osteoprogenitors.[9] Furthermore, small peptides derived from protein molecules may also be used to enhance desirable cellular functions, such as adhesion or bone formation from local osteoblasts, therefore allowing potential higher concentration of specific biological cues to be grafted on the surface. [10, 11]
In current study we aimed to investigate peptide functionalisation of a novel non biodegradable nanocomposite polymer called polyhedral oligomeric silsesquioxane (POSS) nanoparticle crosslinked with polycarbonate-based urea-urethane (PCU, UCL-Nano™).[12] This polymer has been extensively tested for various applications for synthetic organs such as the World's First Synthetic Trachea, lachrymal duct replacement and currently undergoing clinical trial as a lower limb by-pass graft. This non-biodegradable material has been shown previously, as anti-thrombogenic,[12] biocompatible[13, 14] and does not cause inflammatory reactions to the surrounding host tissues.[15] The presence of POSS nanocage structures in the polymer enhances the mechanical strength whilst maintaining the radial elasticity, corrosion and oxidation resistance.[13] Therefore, for its intrinsic physico-chemical

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properties POSS-PCU seems a good materials for the development of new generation of orthopaedic implants. Whilst the polymer material has shown good biocompatibility and cell adhesion, the inherent hydrophobicity limited the extent of cell adhesion and potential tissue in growth and vessel formation.

The aim of this study was to improve the tissue integration of the POSS-PCU polymer for orthopaedic implants by plasma treatment by introducing carboxyl functional groups, followed by grafting bone inducing peptide sequences on the surface. Two recently identified peptides sequences, able to interact with transmembrane proteoglycans, KRSR (lysine-arginine-serine-arginine) and FHRRIKA (phenylalanine-histidine-arginine-arginine-isoleucine-lysine-alanine), were grafted on the membrane surface. KRSR represents a sequence found in five different bone related adhesive proteins: fibronectin, vitronectin, bone sialoprotein, thrombospondin, and osteopontin,[16] whereas FHRRIKA is derived from bone sialoprotein.[17] It has been reported in various studies that the KRSR motif is selective for osteoblast attachment to surfaces, but less so for either endothelial cells or fibroblasts.[18] Dee et al. have also demonstrated osteoblast attachment on KRSR-modified surfaces with similar levels as typically found on RGD modified ones.[16] Furthermore, it has been reported in literature that FHRRIKA motif enhanced the matrix mineralisation. [19] Therefore, as demonstrated by Schuler et al. the combination of two different peptide sequences, RGD and FHRRIKA or RGD and KRSR, could potentially result in enhanced cell behaviour.[20]

To confirm grafting of peptides, the functionalised membranes were extensively characterised using a range of techniques, which include, water contact angle, attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and X-ray Photon Spectroscopy (XPS) to evaluate the effect of the plasma treatment and peptide functionalisation on the physico-chemical characteristics. Furthermore, the peptide functionalised POSS-PCU membranes were further investigated *in vitro* culture with bone marrow derived mesenchymal stem cell to access their differentiation into bone. This study has important implications for developing new implantable materials, which promote bone tissue integration to overcome implant failure, caused by poor integration.

Experimental

Materials

N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, purum ≥98%; CAS No: 25952-53-8), N-hydroxysuccinimide (NHS, purum ≥98%; CAS No:6066-82-6), Toluidine Blue O (TBO; CAS No: 92-31-9), acrylic acid (CAS No: 79-10-7), N,N-Dimethylacetamide (≥99%; CAS No: 127-19-5) were purchased from Sigma Aldrich (Sigma- Aldrich®, UK); FHRRIKA, Fluorescein isothiocyanate (FITC)-FHRRIKA, KRSR and FITC-KRSR peptides were synthesised and purchased from Biomatik. The purity of the peptides was more than 95 %, analysed by analytical HPLC. All materials and chemicals were used as received without any additional purification.

Production of dense POSS-PCU membranes

The polymer was synthesised, as described previously.[12] In brief, polycarbonate polyol (Mw= 2000 Da) was mixed with trans-cyclohexanechloroydrinisobutyl-silses-106 quioxane (Hybrid Plastics Inc) using a 500 ml flask containing a mechanical stirrer and nitrogen inlet. The POSS cage dissolves into the polyol solution at 70 °C. Under nitrogen at a temperature of 75-85 °C flake 4,4'-methylenebis (phenyl 109 isocyanate) (MDI; Sigma-Aldrich) was then added to the mixture for a total of 90 minutes, forming a pre-polymer. To create a polymer solution, DMAC was then added to the pre-polymer. Following cooling the 40 °C, chain extension was then carried out by the addition of ethylenediamine and diethylamine in DMAC in a drop wise manner. One-butanol chain stopper is used to impede further unwanted polymerization. Non-porous POSS-PCU samples used were fabricated via casting of 14 g of 18 % POSS-PCU solution onto a large steel plate with a diameter of 14 cm. The scaffold-containing plates were then left overnight in the oven at 65 °C before removing the polymer gently using tweezers and cutting them into circular scaffolds of 1.55 cm diameter.

Surface functionalisation

A) Plasma treatment

For plasma polymerisation, a glass cylindrical tube of internal diameter 10 cm and length 40 cm was used (QVF, UK). A copper coil was wound around the tube and earthed aluminium/brass flanges ended the tube connecting it with a radio frequency (RF) signal generator (Coaxial Power Systems Ltd., UK). The base pressure achieved was lower than 0.1×10^{-3} mBar and an operating pressure of 6.0×10^{-3} mBar was employed for acrylic acid. The monomer for polymerisation was placed in round-bottomed flasks, connected to the reactor vessel via needle valves through one of the flanges. We used flow rate 2 cm^3 $stpmin^{-1}$ and different times of exposure (10 min and 20 min) for POSS-PCU samples polymerisation as described in Table 1.

The plasma was initiated and sustained by a radiofrequency power source of frequency 13.56 MHz. Different discharge powers of 10 and 20 W were used (Table 1).

B) Peptide grafting

The plasma treated membranes and the synthetic peptide sequences (KRSR: FHRRIKA= 1:1 molar ratio) were reacted using a solution of EDC and NHS as coupling reagents (EDC:NHS= 4:1 molar ratio) in phosphate buffered saline (PBS) at pH 3.5, for 6 h at 4 °C (Scheme 1). Then, the activated

Table 1. Process parameters used for the plasma treatment.

Sample code	Beam power (W)	Exposure times (min)
P1	10	10
P2	10	20
P3	20	10
P4	20	20

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carboxylic groups of the plasma treated samples were reacted with a solution containing the soluble peptides (0.05% w/v) in PBS at pH 10.5, for 24 h at 4 °C. The solution containing unreacted peptides was withdrawn and the membranes were rinsed three times with distilled water. Then the aqueous solution was removed and the membranes were dried in oven at 37 °C.

Physico-chemical characterisation of the functionalised POSS-PCU surface

A) Water contact angle. Contact angle analysis in static conditions was carried out using a CAM 200 KSV instrument, using Drop Shape Analysis System DSA 10 software. For all analyses, bidistillate water drops (6 µL) were used. At least 9 measurements for samples were averaged.

B) Toluidine blue O (TBO) assay. Toluidine blue O was used to determinate the amount of carboxyl groups on the samples before and after plasma treatment as reported by Fazley Elahi et al. [21] The samples were placed in wells containing 1 ml each of a 0.5 mM TBO aqueous solution (pH 10) at 37 °C for 4 h. The treated samples were then rinsed with 1 ml of 0.1 mM NaOH solution (pH 10) for 2 min in order to remove the unbound TBO. The bonded TBO on the surface of the samples was desorbed with 1 ml of 50% (v/v) acetic acid solution for 10 min. The absorbance of the solution at 620 nm was measured using a UV-Vis spectrophotomer (Lambda 2S Perkin Elmer). The amount of carboxyl groups on the surface of the samples is directly proportional to the adsorbed TBO amount. A calibration curve of different concentration of TBO against its respective absorbance value was made and with reference to this curve, the grafting density at different process parameters of plasma treatment was determined.

C) Grafted peptides quantification. The solution containing unreacted FITC-KRSR and FITC-FHRRIKA and the three solutions used for rinsing the membranes were analysed by UV-Vis Spectroscopy at room temperature in the spectral range from 550 to 400 nm using a fluorescence plate reader (Leica DM2500) in order to measure indirectly the amount of unreacted peptide, as:

Amount of grafted peptides = $[Pep_{initial} - (Pep_{residual} + Pep_{rinsing})]$,

where $Pep_{initial}$ is the initial concentration of FITC-peptides solution, $Pep_{residual}$ is the FITC-peptides concentration after incubation with the membranes and $Pep_{rinsing}$ is the FITC-peptides concentration in the rinsing solutions. Three measurements were performed and data are reported as average value and standard deviation.

Furthermore, FITC-KRSR and FITC-FHRRIKA grafted on the surface of the POSS-PCU membrane were investigated by a fluorescence microscope (Leica DM2500), as a complementary and quick assessment of peptide functionalisation.

D) Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Chemical analysis of functionalised coated films was performed by attenuated total reflectance Fourier transform infrared spectroscopy over a wavelength of 4000–550 cm^{-1} using a Nicolet iS 10 spectrometer (resolution 4 cm^{-1}; 32 scans).

E) X-ray Photon Spectroscopy (XPS). Surface composition was determined by X-Ray Photoelectron Spectroscopy on Theta Probe (Thermo Scientific, East Grinstead, UK), which uses a micro- focused AlKa X-ray source (1486.6 eV), operated with a 400 µm spot size (100 W power). Survey spectra were collected at a pass energy of 200 eV, a step size of 1 eV and a dwell time of 50 ms, with the spectrometer operated in standard (not angle-resolved) lens mode. Three points were analysed on each sample surface as received. Charge neutralisation was used throughout the analysis. High resolution regional spectra were collected using a pass energy of 40 eV, a step size of 0.1 eV and a dwell time of 200 ms. High resolution spectra envelopes were obtained by curve fitting synthetic peak components using the software CasaXPS.

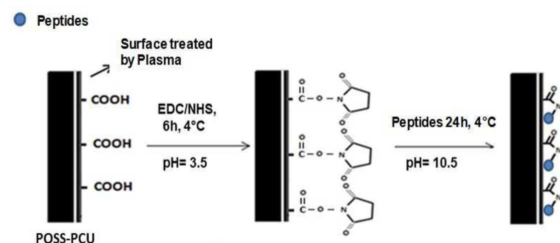
Cell tests

A) Bone Marrow mesenchymal cells isolation. Bone marrow mesenchymal stromal cells (BM-MSCs) were isolated from 4–5 week-old male Wistar rats, following the method described by Santocildes-Romero et al.[22] The femora of five individuals were dissected under aseptic conditions, cleaned of soft tissues and immersed in 10 ml Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), supplemented with 100 U/ml penicillin (Sigma-Aldrich) and 1 mg/ml streptomycin (Sigma-Aldrich). The ends of the femora were removed and the bone marrows were flushed into 5 ml DMEM supplemented with 10 U/ml penicillin, 0.1 mg/ml streptomycin, 20 M ml-glutamine (Sigma-Aldrich) and 10% v/v fetal calf serum (FCS; Biosera). The suspended cells were seeded into 75 cm^2 culture flasks containing 10 ml cell culture medium and incubated at 37 °C and 5% CO_2 for 24 h. Afterwards, the non-adherent cell populations and debris were washed away with fresh cell culture medium. The cell cultures were inspected daily and the medium was changed every 48–72 h. Once the cultures achieved near-confluence, the adherent cells were detached using a solution of 0.05 % trypsin/ 0.02 % ethylenediaminetetra-acetic acid (Sigma-Aldrich), pooled into a single population and seeded for experiments.

B) Cell culture and seeding. Before cell seeding, material samples (1.5×1.5×1 cm) were sterilised in a 70% ethyl alcohol solution (EtOH, Sigma-Aldrich) for 30 min, washed in PBS and incubated with an appropriate medium for 3 h. The medium was then discarded. BM-MSCs cells were detached using 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid and seeded on samples and in 24-well polystyrene tissue culture plates (TCPs) as controls at a density of 30,000 cells/cm^2.

C) Cell metabolic activity. After culturing cells for 6 hours (to evaluate the cell adhesion), 1, 3 and 6 days, the medium was removed and the sample were transferred to new multiwell plates; 10 % PrestoBlue solution (5 mg/mL in DMEM; Fisher

Scheme 1. Schematic representation of the functionalisation strategy based on EDC/NHS complex.



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Scientific) was added to the cell monolayers. The multiwell plates were incubated at 37 °C for an additional 1 h. Then, the dark blue solution was removed (0.2 mL) and quantified spectrophotometrically at 560 nm (Leica DM2500). The results are reported as fluorescent unit. The mean and the standard deviations were obtained from five different experiments.

D) Cell Morphological analysis For the morphological evaluation, after 6 days of culturing period, samples were washed with PBS and fixed with 4 % formalin solution (0.5 mL) for 15 min at room temperature (RT). The cells were washed with PBS, containing 0.2 % Triton X, for 2 min. After the fixation and permeation steps, cells were washed again and stained with 4,6-Diamidino-2-phenyindole dilactate (1:1000 DAPI, Sigma-Aldrich) for 2 min at RT, and Phalloidin-Tetramethylrhodamine B isothiocyanate (10 µM phalloidin Sigma-Aldrich) for 1 h at RT. Finally, cells were washed and observed with the help of Axioplan 2 imaging fluorescent microscope with a digital camera QIC AM 12-bit (Zeiss).

E) Alkaline Phosphatase (ALP) assay

The BM-MSCs differentiation was studied after 21 days of cell seeding in standard and osteogenic cell culture medium (added after 7 days of cell culture with the same composition of the standard medium plus 50 µg/ml ascorbic acid (Sigma-Aldrich), 10 mM β-glycerophosphate (Fluka Biochemika) and 10^{-8} M dexamethasone (Sigma-Aldrich)). ALP activity was measured by incubating 100 µL of each samples with 0.5 mL alkaline buffer solution (Sigma-Aldrich) and 0.5 mL of stock substrate solution (40 mg p-nitrophenyl phosphate disodium, Sigma-Aldrich), diluted in 10 mL of distilled water, at 37 °C for 1 h. Production of p-nitrophenol in the presence of ALP was measured by monitoring light absorbance of the solution at 410 nm using a Perkin Elmer Lambda 25 UV/Vis Spectrometer. The mean and the standard deviation were obtained from three different experiments. Alkaline phosphatase absorbance values were normalised for cell number calculated using the PicoGreen® dsDNA reagent (Invitrogen, USA).

Statistical analysis

Experiments were run at least in triplicate for each sample. All data were expressed as mean ± SD. Statistical analysis was determined by using Analyse-it v2.22 software. The statistical differences between groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared as significant at (*) at $p < 0.05$ and very significant (**) at $p < 0.0001$.

Results and discussion

In this study, the potential application of plasma-treated POSS-PCU and, then, grafted with bone peptide sequences was explored in order to impart biomimetic properties to be used as new generation of orthopaedic implants. The POSS-PCU membranes were prepared by solvent casting and functionalised by plasma treatment, after an appropriate optimisation of the process parameters, to obtain a high density of -COOH reactive groups able to react via carbodiimide with heparin-biding domain peptides. Modification strategy represents a suitable trend in fabricating proactive implant surfaces, which are able to mimic proteins found within the extracellular matrix. Such bioligands have the ability to bind to the cell via specific cell surface receptors. The RGD sequence is the most prominent ligand addressing integrins, a family of receptors present in most cell types. Other sequences, used in this work, such as -KRSR- and –FHRRIKA- bind transmembrane proteoglycans (e.g. heparin sulphate). These peptide patterns are found in the ECM of osteoblasts, and are therefore similarly interesting for bone-contacting implant modification applications.[16, 23]

A) Plasma treatment

The POSS-PCU films were treated by means of radiofrequency plasma under acrylic acid (AAc) flow. Different treatment times (10 and 20 min) and power (10 and 20 W), as reported in Table 1, were selected in order to evaluate the effect of the plasma treatment on the sample surfaces. In particular, the plasma polymerisation is able to induce modifications into surface morphology, roughness, chemistry, and wettability strongly related to the beam power and treatment times.[24] To select the optimal plasma treatment parameters, the membranes were characterised by different techniques to evaluate the physico-chemical properties before and after the treatment.

A colorimetric TBO assay was used to determinate the amount of carboxyl groups formed after the plasma modification. The amount of bound dye was quantified by measuring the solution optical density at 534 nm. The amount of the carboxyl groups was calculated by referring to a calibration curve from 0.005% w/v to 0.0035% w/v of a TBO/50% acetic acid solution recorded at the same conditions (not shown). The amount of carboxylic groups on control (unmodified) POSS-PCU samples was also calculated.

Fig. 1 shows the carboxyl density calculated for the samples treated

Figure 1. Carboxylic density (nmol/cm^2) of the untreated and treated sample by plasma for different time and power values. P1: 10 min at 10 W; P2: 20 min at 10 W; P3: 10 min at 20W; P4: 20 min at 20W. n= 3.

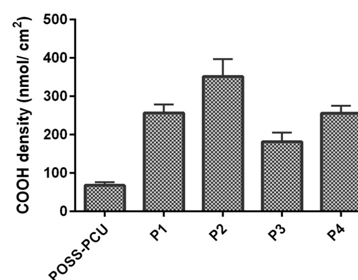
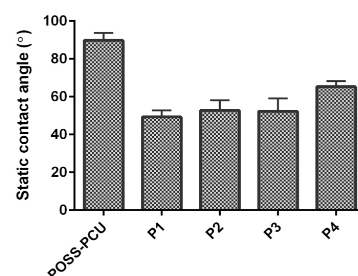


Figure 2. Static contact angle values for the untreated and treated sample by plasma for different time and power values. P1: 10 min at 10 W; P2: 20 min at 10 W; P3: 10 min at 20W; P4: 20 min at 20W. n= 9.



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by plasma for different period of time and power. It is possible to observe that there is a noticeable differences for the different process parameters, which range from 182 ± 23 nmol/cm^2 for 10 minutes at 20 W (P3 sample) to 352 ± 45 nmol/cm^2 for 20 min at 10W (P2 sample). Furthermore, the plasma treatment was successfully obtained as showed by the significant differences of the values found for the treated samples, compared with the untreated POSS-PCU sample (68 ± 8 nmol/cm^2).

Surface hydrophilicity of the polymeric films was measured using water contact angle, in order to investigate POSS-PCU films wettability before and after plasma treatment. Fig. 2 shows the values of contact angle obtained with the different parameters.

The water contact angle of POSS-PCU film decreased after plasma treatment. The untreated film surface was hydrophobic, as expected, having contacts angle of 90 ± 4°. The plasma treatment, increased the wettability of all the samples, and, in particular, the water drop contact angle decreased to 53 ± 5° with a reduction of about 42 % after flow exposure for 10 minutes at 20 W (P2 sample).

The reduction in contact angle values clearly indicates increased surface hydrophilicity, which may have been caused by the introduction of hydrophilic functional groups (-C-O, and C=O or –COOH) on the surface.[25, 26] The achievement of a moderate hydrophilicity is important to improve cell adhesion, spreading and proliferation.[27, 28]

To evaluate the presence of carboxylic groups on the POSS-PCU surface a chemical analysis of the films before and after plasma treatment was determined by ATR-FTIR and XPS. ATR-FTIR spectra of treated POSS-PCU sample are compared with the vibration modes of individual functional groups of the nanocomposite polymer backbone (Fig. 3). The peak at 3321 cm^{-1} could be identified as -NH vibrations, presumably from urea and urethane components of the polymer. Larger peak at 2953 cm^{-1} corresponds to -CH vibrations from the methylene segments within the polymer chain, and the side peak at 2868 cm^{-1} to -CH vibrations from -NH groups. Peaks at 1738 cm^{-1} was identified as -C=O vibration arising from carbonate and urethane segments. The -C=C bonds of MDI (hard crystalline segment) were evident at 1529 cm^{-1}. Peaks at 1244 cm^{-1} were assigned to O-CO-O vibrations from the long chain polymer backbone. The peak at 1110 cm^{-1} could be assigned to POSS containing siloxane bonding (Si-O-Si).[29, 30] Comparison between spectra in Fig. 3 shows that the ATR-FTIR spectrum of poly acrylic acid (PAAc)-grafted film containing carboxyl group is not significantly different from untreated film. As it is noted in literature, a typical carbonyl absorption band of PAAc in grafted copolymers is usually found between 1710 and 1730 cm^{-1}.[31, 32] Therefore, there is a possibility that the intense carbonyl group signal of the polyurethane group in the backbone of POSS-PCU nanocomposite overlaps with the much lower intensity peak of the correspondent PAAc group.[31] Hence, the carbonyl peak at 1730 cm^{-1} from PAA is masked with inherent vibration of carbonyl functional groups in POSS-PCU.

To confirm the obtained graft polymerisation by plasma, X-ray photon spectroscopy was performed. Fig. 4a shows the XPS survey spectrum of untreated POSS-PCU and Fig. 4b-e show treated POSS-PCU by plasma following different process parameters. Five elements were detected before and after plasma treatment, namely carbon (C), oxygen (O), nitrogen (N), silicon (Si) and chlorine (Cl) due to the polymeric chemical structure. The C_{1s} in peaks were fitted to three components (Fig. 4f) at (1) 284.8 eV, (2) 285.8 eV, (3) 289.1 eV, which were attributed to aliphatic carbon bonds or carbon-hydrogen bonds (-C-H- or –C-C-), to C-O-, and to ester groups (>C=O or -COOH), respectively.

Table 2 shows the percentage of various carbon environments on the surface of the samples and the atomic O/C fractions were calculated from the peak intensity ratios.

Figure 3. ATR-FTIR spectra of untreated and treated sample by plasma for different time and power values. P1: 10 min at 10 W; P2: 20 min at 10 W; P3: 10 min at 20W; P4: 20 min at 20W. n= 3.

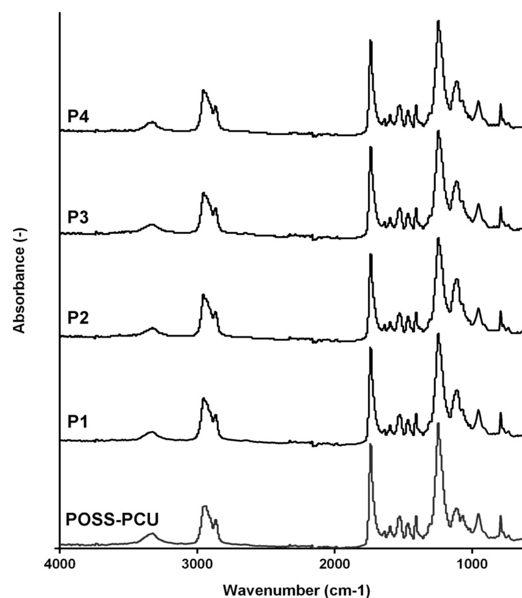

Figure 4. XPS surveys of untreated (a) and plasma treated POSS-PCU film (P1, b; P2, c; P3, d; P4, e) and high resolution C_{1s} spectrum for P1 sample (f).

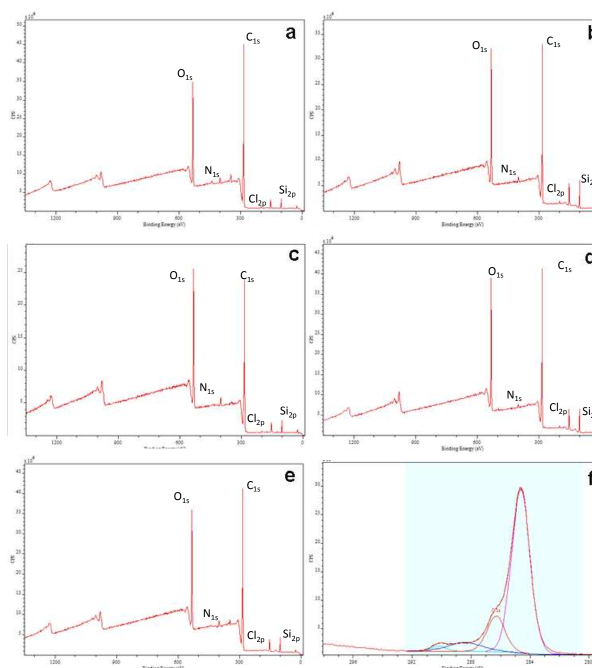

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Table 2. Binding Energy (eV) of core-Levels and atomic O/C fractions of POSS-PCU samples before and after plasma treatment.

Sample	289.1 eV >C=O or –COOH (%)	286.9 eV -C-O- (%)	284.8 eV –C-H-or –C-C- (%)	O/C
POSS-PCU	6.17	9.13	84.7	0.215
P1	14.1	16.2	69.7	0.322
P2	14.5	22.7	62.6	0.372
P3	12.4	23.0	64.8	0.308
P4	12.9	17.5	69.6	0.321

From these results, it was possible to observe the substantial increase of oxygen after plasma treatment. From Table 2 the percentage of >C=O or –COOH- and –C-O- increased after plasma treatment, but the percentage of –C-H- or -C-C- decreased after polymerisation. from results it is clear that after the treatment, a part of –C-H- or –C-C- bonds were oxidised to –C-O- and >C=O or -COOH bonds by generated active species, which led to an increase in the fraction of –C-O- and >C=O or –COOH and a decrease in –C-H or –C-C- bonds.

Moreover, the value of O/C ratio allowed selection of the best parameters of treatment time and operating power for the plasma treatment. For plasma treated POSS-PCU the value of the ratio was the highest, the untreated sample (from 0.215) to P2 sample treated for 10 min at 20 W showed highest ratio (0.372) among other modifications. Thus, based on above results, 10 minutes and 20 W were selected as parameters for plasma treatment to introduce highest density of carboxyl groups, which were later used for peptide grafting.

B) Peptide grafting

Peptide grating following plasma modification was confirmed by various techniques, namely fluorescence microscopy, ATR-FTIR and XPS. Fluorescently labelled peptides were used to quickly access presence of peptides. To confirm the incorporation of the bone peptide sequences, FITC-KRSR and FITC-FHRRIKA were grafted on the POSS-PCU surface and were imaged by fluorescence microscopy as shown in Fig. 5. It is clear from the images that peptides were available on the surface. However to confirm if chemical coupling of peptide occurred, ATR-FTIR and XPS were used.

ATR-FTIR spectra of plasma treated POSS-PCU membrane for 10 min at 20 W (Fig. 6a) showed the typical characteristics absorption bands of the amide groups, which confirm that the binding membrane-synthetic peptides occurred. The bands due to the vibration of amide A, amide I and amide II can be seen at 3280 cm^{-1}, 1626 cm^{-1} and 1530 cm^{-1} respectively. Furthermore, XPS spectrum (Fig. 6b) complimented these results. Fig. 6b showed an increase in the intensity of the N_{1s} peak at 399.5 eV (from 0.9 ± 0.2 % for untreated membrane to 3.8 ± 0.4 % for the functionalised membrane), indicating that the POSS-PCU samples have been successfully functionalised with the grafting of the bone peptide sequences.

Finally, a quantification of the bonded KRSR+FHRRIKA to the membranes was performed using FITC-KRSR and FITC-FHRRIKA. The measured amount of grafted peptides was 80.4% ± 3.8 % (reported as a percentage of the initial amount of peptides).

***In vitro* cell tests**

The adhesion and proliferation of bone marrow mesenchymal stromal cells on the untreated and functionalised POSS-PCU membranes were evaluated, with particular attention to the effect of the bone peptide sequences on the cell response.

Following the initial adhesion of cells on bone substitutive materials, a series of processes take place, affecting osteointegration and osteoconduction.[33] In this work PrestoBlue assay was used to evaluate BM-MSCs viability, after 6 hours (to study cell adhesion after a relatively short period after cell seeding)

Figure 5. Fluorescence microscopy images of (a) untreated and (b) functionalised POSS-PCU with the grafting of FITC-KRSR and FITC-FHRRIKA (ratio 1:1). Bar= 100 µm.

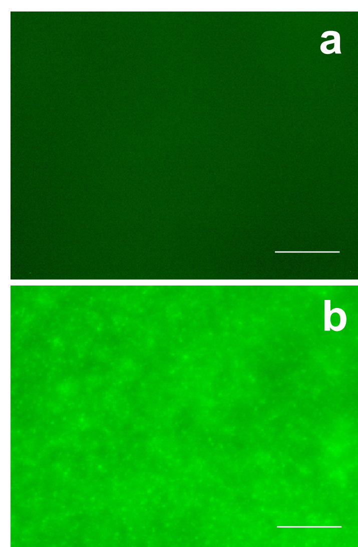

Figure 6. (a) ATR-FTIR spectrum of untreated POSS-PCU (dashed line) and peptide functionalised POSS-PCU membrane (continuous line); (b) XPS survey of the peptide functionalised POSS-PCU membrane.

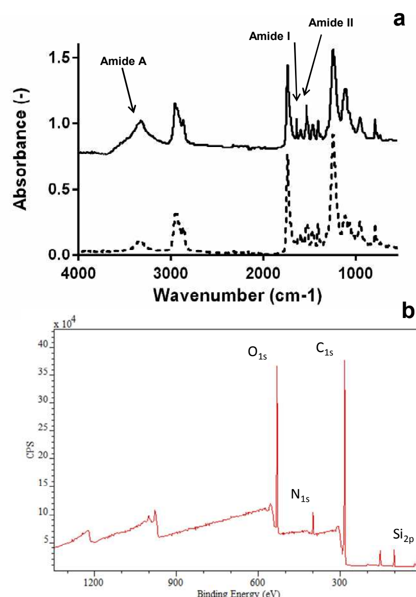

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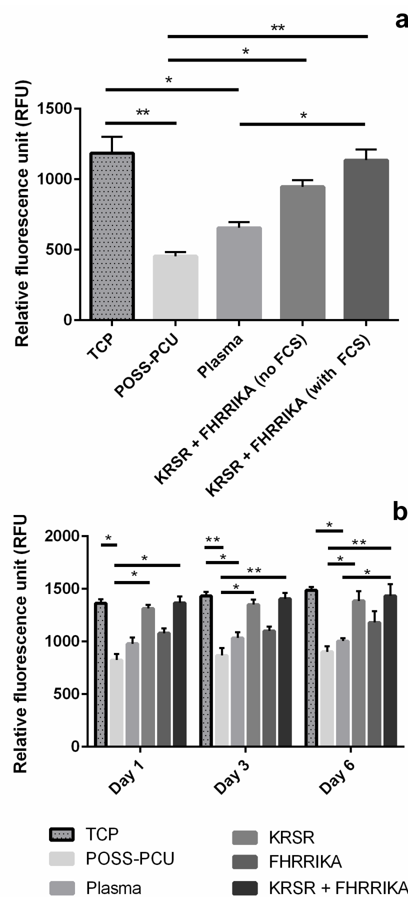
Figure 7. BM-MSCs attachment after 6 hours (a) and proliferation after 1, 3 and 6 days (b) assessed using a PrestoBlue method on the tissue culture plates (TCP as control), untreated, plasma treated and peptide functionalised POSS-PCU membranes. Data are represented as the mean ± SD for n= 5, and statistical comparison by Kruskal-Wallis One Way Analysis of Variance on Ranks showed significant difference at $p < 0.05$ (*) and very significant different $p < 0.0001$ (**)



and after 1, 3 and 6 days. The results are shown in Fig. 7a and b, respectively. At 6 hours after BM-MSCs seeding, PrestoBlue assay showed that the samples with the peptide grafting demonstrated a good adhesion (1134 ± 75) , comparable respect to the tissue culture plates (TCP; 1185 ± 115) and higher than the pure POSS-PCU and the plasma treated membranes (453 ± 29 and 654 ± 42 respectively). Furthermore, we tested also the adhesion without the FCS addition in the culture medium, in which we found that the absence of serum influenced slightly the cell attachment (945 ± 47). Serum proteins are responsible for initial attachment of cell on implant surfaces. No significant difference for cell attachment between serum and without serum containing media on peptide modified surfaces suggest that grafted peptides are either not masked by serum proteins or they are in right conformation and by themselves capable of initialising cell attachment. Long-term viability study using PrestoBlue test displayed an increase of cell viability along the cell incubation period. In particular, the grafting of the bone peptide sequences on the membranes enhanced significantly the cell viability compared to their un-functionalised counterparts (1462 ± 104 for KRSR+FHRRIKA and 954 ± 51 for POSS-PCU respectively), as observed after 3 and 6 days of incubation, showing value comparable with the TCP control (1487 ± 29).

This results are consistent with previous reported work by Schuler et al.,[20] where KRSR absorption led to higher amount of osteoblast cells attachment on the surface of titanium substrates, as also confirmed by PrestoBlue comparing the viability values between

Figure 8. Fluorescence microscopy of BM-MSCs morphology after 6 days of culture on: (a) POSS-PCU; (b) plasma treated; (c) KRSR grafting; (d) FHRRIKA grafting and (e) KRSR+FHRRIK grafting. DAPI in blue colour and stains nucleus of cells; Phalloidin in green colour and stains the actin filamentous. Bar= 50 µm.

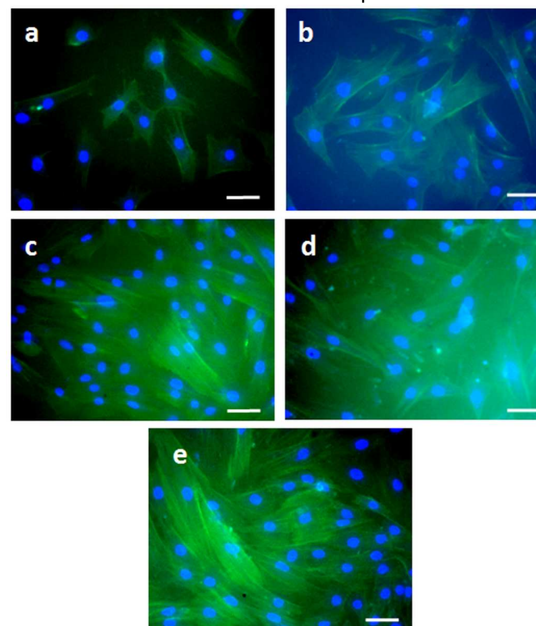
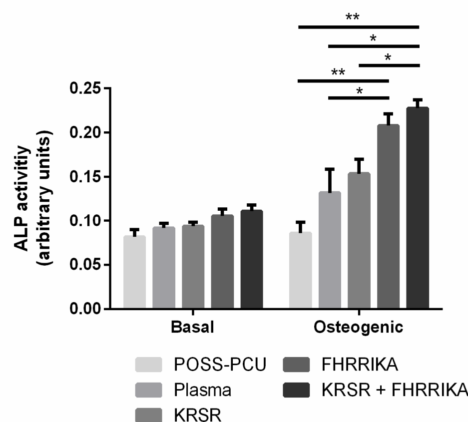


Figure 9. ALP activity of BM-MSCs osteoblast-like cells on untreated, plasma treated and peptide functionalised POSS-PCU membranes after 21 days of culture. Data are represented as the mean ± SD for n= 3, and statistical comparison by Kruskal-Wallis One Way Analysis of Variance on Ranks showed significant difference at $p < 0.05$ (*) and very significant different $p < 0.0001$ (**).



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the POSS-PCU membranes functionalised with only KRSR (1411 ± 21) and with only FHRRIKA (1137 ± 34) at 6 days. These results were further complimented by fluorescent staining of the cells at day 6. After 6 days of culture, BM-MSCs adhered on the surface of the samples grafted with the peptide sequences, showing a spreading behaviour with the formation of actin filaments (in green marked by phalloidin staining) (Fig. 8c-e). In the case of POSS-PCU or plasma treated samples, cells spreading was less pronounced and confirmed by PrestoBlue assay results that showed a lower metabolic activity on untreated and plasma treated POSS-PCU compared to their peptide-modified counterparts (Fig. 8a-b).

Finally, ALP tests was performed to preliminary assess the bone forming potential of the BM-MSCs cells, showing that functionalised POSS-PCU membranes affect dramatically the ALP activity of the BM-MSCs cultured in osteogenic medium (ALP value for POSS-PCU without and with peptides incorporation were 0.085 ± 0.012 and 0.227 ± 0.009, respectively) as shown in Fig. 9. In particular, this behaviour can be attributed to the presence of FHRRIKA that, according also to the literature, is able to enhance the bone mineralisation,[17] as confirmed by the ALP activity for the sample containing only FHRRIKA (0.207 ± 0.013) compared to with only KRSR (0.153 ± 0.016). KRSR on other hand has been shown to act as adhesion peptide for osteoblast cells similar to RGD [16, 19].

Conclusions

This study demonstrated that modelling of variable parameters of AAc grafting through plasma treatment can be predicted to optimise the amount of COOH-grafted onto the chemically inert POSS-PCU nanocomposite film. The following grafting, via carbodiimide chemistry, of specific bone peptide sequences (KRSR and FHRRIKA) induced appropriate results in terms of viability and ALP activity of BM-MSCs. In particular, the combination of the two peptides demonstrated to be suitable for inducing osteoblastic differentiation. This study has implications for development of simple and efficient method for peptide functionalisation of various biomaterials, which can promote tissue specific growth.

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