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Plasma Surface Modification of POSS-PCU with Allylamine Enhances the Response and Osteogenic Differentiation of Adipose-Derived Stem Cells

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ABSTRACT

This study present amino functionalization of biocompatible polymer polyhedral oligomeric silsequioxane poly (carbonate-urea) urethane (POSS-PCU) using plasma polymerization process to induce osteogenic differentiation of adipose derived stem cells (ADSCs). Optimization of plasma polymerization process was carried out keeping cell culture application in mind. Thus, samples were rigorously tested for retention of amino groups under both dry and wet conditions. Physio-chemical characterization was carried out using ninhydrin test, X-ray photon spectroscopy, scanning electron microscopy and static water contact analysis.

Results from physio chemical characterization shows that functionalization of amino group are not stable under wet condition and optimization of plasma process is required for stable bonding of amino groups to POSS-PCU polymer. Optimized samples were later tested in vitro in short and long term culture to study differentiation of ADSCs on amino modified samples. Short term cell culture shows that initial cell attachment was significantly ($p<0.001$) improved on amine modified samples (NH_2-POSS-PCU) compared to unmodified POSS-PCU. NH_2-POSS-PCU samples also facilitates osteogenic differentiation of ADSCs as confirmed by immunological staining of cells for extracellular markers such as collagen Type I, and osteopontin. Quantification of total collagen and ALP activity also shows significant ($p<0.001$) increase on NH_2-POSS-PCU samples compared to unmodified POSS-PCU.

A pilot study also confirms that these optimized amino modified POSS-PCU samples can further be functionalized using bone inducing peptide such as KRSR using conventional wet chemistry. This further provides an opportunity for bio-functionalization of polymer for various tissue specific applications.

KEYWORDS: Plasma polymerization, polyhedral oligomeric silsequioxane poly (carbonate-urea) urethane, allylamine, osteogenic differentiation, adipose-derived stem cells

1. INTRODUCTION

Surface modification of polymers is a growing and promising strategy that aims to improve and widen the applications of polymers for biomedical and regenerative purposes. Plasma surface modification is one of such techniques which modify polymers surface without affecting its bulk mechanical properties.[1,2,3,4] This technique provides a solvent-free and thus environmentally friendly alternative to conventional wet chemistry.[5,6,7,8] Number of studies have shown use of plasma modification technique to covalently couple various molecules, and functional groups.[3,4,9,10,11,12] These functional surfaces are very effectively used for covalent coupling of biomolecules,[13,12] growth factors,[14,15] and peptides[4] tailoring the polymeric properties for specific biomedical applications.

Functionalization of polymers surfaces with amine groups is a common method for both studying cellular reponses[11] and further coupling of biological molecules for specific targeted applications.[15] Conventional wet chemistry used silanization as a common method to introduce amino groups on the surface.[16,17] Use of this method is highly dependent on type of substrate and their ability to withstand harsh chemical treatment involved during coupling process. Also, chemical waste generated during process need to be discarded with at most care. Plasma polymerization on other hand, uses only few milliliter of monomers, for example allylamine or acrylic acid, which polymerize on the coated surface during plasma surface modification. Plasma modification process is quick but need to be optimized for individual substrates.

In this study, amino functionalization using plasma polymerization technique of the biocompatible polymer called polyhedral oligomeric silsequioxane poly (carbonate-urea)

urethane (POSS-PCU) was investigated in detail. POSS-PCU is a nanocomposite polymer synthesized and patented by the University College of London (UCL-Nano™; Patent no. US 7820769B2). It is composed of a PCU backbone cross-linked with POSS nanoparticles. Its specific nanostructure enhances the mechanical properties and biocompatibility of this polymer.[18,19,20] POSS-PCU has been used in regenerative medicine to replace and restore tubular structures such as trachea,[21] lachrymal conduits[22] blood vessels ,[23] and its applications are being extended to the treatment of lymphedema, for auricular reconstruction, and for heart valve prosthesis.[24,25,26]

However, its application for restoration of musculoskeletal structures still remains unexplored. POSS-PCU is a non-biodegradable material.[18] It can be molded into porous scaffolds.[3] It is also possible to alter its mechanical properties by simple modifications.[27] This, thus provides an opportunity to use this material for load bearing applications such as bone tissue engineering. However, POSS-PCU is inherently hydrophobic in nature and thus decreasing its wettability and modifying its surface properties are found to be useful for applications where tissue integration and angiogenesis is important.[3]

Adipose-derived stem cells (ADSCs) on other hand show the great clinical potential due to their ability to differentiate into various cell types in the body.[28,29,30,31] They can be are easily harvested in greater number from patient's own fat tissue during surgeries, compared to bone marrow stem cells.[30] Potential of ADSCs in bone regeneration is becoming more obvious with ever new studies conducted in area.[32,33,34,35]

This study thus, aims to functionalize POSS-PCU using plasma polymerization process to alter its wettability and its surface properties for bone tissue engineering application. ADSCs are used to study the effect of this modified POSS-PCU on their osteogenic differentiation.

As a part of this study, plasma polymerization process has been optimized and its stability for biological application has been investigated using X-ray Photon Spectroscopy. The modified samples were later tested in vitro using ADSCs to evaluate potential of these modified samples for bone regeneration in long term. As a proof of concept, we also present use of this method for effective functionalization of short peptide molecules for potential serum-free differentiation of stem cells. This study has implications for developing simple and efficient plasma polymerization process for biomaterial applications of polymers.

2. MATERIALS AND METHODS

2.1. Polymer synthesis

Polymer synthesis of POSS-PCU was carried on as described before.[36] Briefly, 72 g of polycarbonate polyol and 2 grams of *trans*-cyclohexanediol isobutyl-POSS were placed in a 500 ml reaction flask equipped with stirrer and nitrogen inlet. The mixture was heated to 125°C and then cooled to 60°C. Then 18.8 g of flake 4, 4'-diphenylmethane diisocyanate were added to the mixture under nitrogen at 70-80°C for 90 min to form a pre-polymer and 156 g of dry dimethylacetamide (DMAC) were added to the pre-polymer to form a solution. After cooling the solution at 40°C, the chain extension was done by adding a mixture of 2 g of ethylenediamine and 0.05 g of diethylamine in 80 g of DMAC. Once the chain extension was completed, a mixture of 4 g 1-butanol and 80 g DMAC were added slowly to the polymer to form a 20% solution of DMAC in POSS-PCU.

2.2. Scaffold production

The obtained polymer solution was poured into square metallic mold of 12×16.5 cm to obtain films of 0.02 mm thickness. After being cured at 65°C for around 16 hrs, solvent was evaporated and the films were cut in circles of 1.6 cm diameter to fit in 24-well plates. All samples were then washed once with deionized water, sterilized with 70% (v/v) ethanol followed by further 3 washes with deionized water. Samples were dried and kept in desiccator until use.

2.3. Plasma surface polymerization

POSS-PCU films were treated in a low-temperature pulsed DC discharge plasma at 40 kHz ("FEMTO" Low pressure plasma system, Diener electronic GmbH+Co, Germany). Every time, before machine was used, plasma chamber was cleaned using oxygen gas for 30 min at a pressure of 0.4 mbar. After chamber was cleaned, polymeric samples were introduced in to the plasma machine. Plasma polymerization of POSS-PCU was carried out in 2 stages. First stage involves activation of polymeric samples with oxygen plasma for 5 min. In the second stage, samples were exposed to monomer of allylamine ($C_3H_5NH_2$) along with carrier gas oxygen (0.4 mbar) which was polymerized using plasma process (Fig. 1). Different polymerization times were investigated to generate primary amine groups at the surface of POSS-PCU. Samples were stored under vacuum using a desiccator immediately after plasma modification.

2.4. Ninhydrin Assay

Ninhydrin is used as a chemical assay for the colorimetric detection of α-amino acids by its reaction with primary amines that produces a purple colored chromophore. In our study a ninhydrin spray (Sigma-Aldrich, UK) was used to evaluate the presence of amine groups on

POSS-PCU samples after plasma polymerization process. The amine-modified samples were sprayed with ninhydrin in a fumed hood and immediately placed in an oven at 110° for 3 min. Samples with amino groups, turned purple in color following reaction with ninhydrin. Unmodified samples were used as a control.

2.5. Water Contact Angle Measurement

The static water contact angle of the POSS-PCU samples were analyzed using sessile drop method using the DSA 100 instrument (KRUSS, Germany). 3 µl volume of deionized water was dispensed by a syringe pump through a flat tip needle of 0.5 mm diameter. The average contact angle was calculated using KRUSS drop shape software (version 1.90.0.14). Measurement of a single drop was performed on six independent samples (n=6).

2.6. X-ray Photoelectron Spectroscopy (XPS)

Grafting of primary amines and chemical modification of POSS-PCU was evaluated by an XPS analysis on a K-Alpha™ X-ray photoelectron spectrometer (Thermo scientific, UK) using a monochromatic Al Kα X-ray (hν =1486.6 eV) with an incident angle of 45° and a photoelectron-collection take-off angle of 90° under a high-vacuum of $<5\times10^{-7}$ mbar. Three points of 400 µm were analyzed twice for each sample. The XPS analysis determined the survey and high resolution spectra in the regions of C1s, N1s, O1s, and Si for the elemental and chemical state identification. Data was acquired using Thermo Advantage v.5941 and analyzed using CASAXPS software.

2.7. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used for the topographical evaluation of the amine-modified surfaces. The samples were attached to aluminum stubs with double sided sticky tabs. Samples were sputtered coated with gold using a Q150R rotary-pumped coater (Quorum Technologies, UK). Images were acquired and processed using EVO® LS15HD Scanning Electron Microscope (Carl-Zeiss, Germany).

2.8. Peptide coupling

Covalent binding of bone inducing peptide KRSR was achieved by a carbodiimide reaction. Amine functionalized POSS-PCU samples were incubated with a mixture of 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 0.2 M N- hydroxysuccinimide (NHS) (Sigma-Aldrich, UK) and 100µg/ml of KRSR (Genscript, USA) in a ratio of 1:1:1. 200 µl of the solution was poured in a 24-well plate containing the amine-modified POSS-PCU films after activation with phosphate-buffered saline (PBS, pH 7.4) for 30 min (Fig. S2). This reaction was carried out overnight at room temperature. When fluorescently labeled peptides were used, all samples were kept protected from exposure to light.

2.9. Cell culture

Human ADSCs were purchased from Lonza, UK. Cells were thawed from liquid nitrogen at passage three and cultured in a growth medium composed of Dulbecco's Modified Eagle's Medium (DMEM, low-glucose, pyruvate; Thermofisher Scientific, UK) supplemented with v/v of 10% fetal bovine serum (Thermofisher Scientific, UK), 1% penicillin-streptomycin (Sigma-Aldrich, UK), and 1% L-glutamine (Lonza, UK). Cells were seeded in 75cm² flasks and maintained in a humidified incubator at 37°C and 5% CO_2. Once a confluence of 60-70% was reached, cells were sub cultured following trypsinization. Cell culture media was changed every

2-3 days. For the differentiation experiments, growth medium supplemented with 0.1 mM dexamethasone, 50 μg/ml ascorbic acid and 10 mM β-glycerolphosphate was used.[37,38,39]

2.10. Assessment of cellular attachment and spreading

Cell attachment and spreading was monitored by staining filamentous actin (F-actin) using phalloidin. Cells were seeded at a density of 20,000 cells/well. Samples were washed with PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 20 min and washed gently with 0.1% (v/v) Tween in PBS (Sigma-Aldrich, UK). Permeabilized of samples is achieved with 0.1% (v/v) triton X100 in PBS (Sigma-Aldrich, UK) for 4 min. Samples were then blocked by incubation with 1% (w/v) bovine serum albumin (BSA) in PBS during 30 min followed by incubation with rhodamine phalloidin (1:50 in PBS) (Thermofisher Scientific, UK) for 30 min. Samples were washed and incubated with 300 nM of 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Thermofisher Scientific, UK) for 5 min excess dye was removed by washing samples with 0.1% (v/v) Tween 20. Samples were mounted on glass slides using a drop of Fluoromount (Sigma-Aldrich, UK), to avoid photo bleaching. Cell images were acquired using fluorescence microscope. Three random images per sample were collected from triplicate samples for quantitative analysis of cell attachment and morphology. Cell density was calculated by counting the number of nuclei in 9 different images per sample and results were expressed in cells/cm². Cell morphology was quantified in terms of mean cell area and circularity index using ImageJ software (n=50).

2.11. Metabolic activity and cell growth

To assess the cytotoxicity and metabolic activity, alamarBlue® assay (Life Technologies, UK) was used. AlamarBlue® is a blue fluorescent dye irreversibly reduced to a pink fluorescent dye

(*Resorufin)* in the mitochondria of metabolically active cells. The amount of fluorescence or pink colour produced indicates the oxidation-reduction potential of the cells and therefore their metabolic activity. During the experiment, samples were incubated with 20,000 cells per well. The metabolic activity of cells was measured using alamarBlue® assay kit as per manufacturer's instructions. Briefly, cell seeded samples were incubated with 10% (v/v) alamarBlue® dye in cell culture media for 4 hrs. 100 µl of reduced dye was then place into 96 well plates and fluorescence was measured at excitation and emission wavelength of 530 and 620 nm using Fluoroskan Ascent FL, (Thermo Labsystems, UK) (n=6).

After analysis of the metabolic activity, samples were washed with PBS and 400 µl of DNA-free water were poured into each 24-well plate. Cell lysis was done by freezing-thawing three times the samples at -80°C and 65°C. Cellular quantification was performed using a DNA Quantitation Kit Fluorescence Assay (Sigma-Aldrich, UK). Assay was performed as per manufacturer's instructions. The fluorescence was measured at excitation and emission wavelength of 360 and 460 nm using Fluoroskan Ascent FL (n =6) (Thermo Labsystems, UK).

To determine the DNA concentration, a standard concentration curve was obtained using standards samples of calf thymus DNA. The fluorescence of the samples was then converted in DNA concentration by using the equation derived from the standard curve.

2.12. **Osteogenic differentiation and mineralization**

2.12.1. Alkaline Phosphatase (ALP) Activity

ALP activity was measured using ALP Assay Kit (Abcam, UK) at 7, 14 days, after cell seeding. Assay is based on hydrolysis of the substrate pNPP by the enzyme produced by the differentiated

cells. This reaction gives a yellow color that can be read in a plate reader at a wavelength of 405 nm. Assay was performed as per manufactures instructions. Briefly, 50 µl of cell lysate from three repeats (n=3) were transferred to a 96-transparent-well-plate and 31.25 µl of 5mM *p*NPP were added and incubated for 60 min protected from light at room temperature. Then, 12.5 µl of stopping solution was added to each well. Colorimetric measurements were performed using an Anthos 2010 microplate reader (Biochrom, UK) at a wavelength of 405 nm. Quantification of ALP was done using standard curve. All results where then normalized to their corresponding DNA concentration to know the ALP activity expressed in µg/ml of DNA.

2.12.2. Collagen production using Sirius Red colorimetric assay

Sirius Red was used to quantify the amount of total collagen production on day 14 and 21. Briefly, cell seeded scaffolds were first fixed in methanol overnight at -20 °C. After washing with PBS they were staining at room temperature for 4 hrs with the 0.1% Picro Sirius Red (PSR) solution (Sigma Aldrich, UK). Excess dye was removed by washing 3 times with 0.1% acetic acid. To quantify amount of collagen produced per sample, PSR stained samples were incubated with 200 µl of 0.1N sodium hydroxide. Samples were placed on a rocker at room temperature for 1 hour before the optical density (OD) was measured at 540 nm with the Anthos 2020 microplate reader (Biochrome Ltd, UK). Standard curve was prepared using various amount of Bovine collagen (1, 5, 10, 20, 30, 40 and 50 µg) as per manufactures instruction (n =6), which was later used to quantify amount of collagen per sample.

2.12.3. Osteopontin and Collagen type I Immunocytochemistry

Cell seeded samples were washed with PBS and fixed for 20 min with 4% paraformaldehyde. Samples were washed 3 times with 0.1% tween and permeabilized with 0.1% Triton X-100 for 5

min. Then, samples were blocked 1% BSA for 30 min and incubated with mouse monoclonal anti-collagen I (1:500) or rabbit polyclonal anti-osteopontin (1:600) (both from Abcam, UK) overnight at 4°C. Samples were then washed with 0.1% Tween and incubated with a secondary antibody for 1 hour. A goat polyclonal anti-mouse IgG coupled with FITC (1:500) (Merck KGaA, Germany) and a goat polyclonal anti-rabbit IgG coupled with Alexa Fluor 488 (1:1000, Abcam, UK) were used for collagen and osteopontin staining respectively. Samples were washed thoroughly with 0.1% Tween and incubated with 300 nM DAPI for 5 min to stain cell nuclei. Samples were washed mounted on a glass slide with Fluoromount (Sigma, UK). Samples incubated only with the secondary antibody were used as control, to confirm the absence of unspecific staining.

2.13. Statistical analysis

All error bars on data are expressed as standard errors from the mean. The statistical significance was determined at 95% level using a one-way ANOVA, were * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$. Post-hoc statistical analysis of the means of individual groups was performed using Tukey's Kramer multiple comparison test.

3. RESULTS

3.1. Amine functionalization POSS-PCU using plasma polymerization process

3.1.1. Ninhydrin assessment

The presence of primary amine groups on the surface was confirmed by the ninhydrin test. The intensity of *Ruhemann's purple* was proportional to the treatment time for plasma polymerization

using allylamine. Untreated POSS-PCU presented the lowest intensity while 10 min treatment showed an intense purple colour (see Fig 2A).

3.1.2. Contact angle measurement

Changes in polymer wettability pre and post plasma polymerisation with allylamine was confirmed using static water contact angle measurements. Static water contact angle for untreated POSS-PCU was 104±3°. The hydrophilicity of the samples increases with plasma treatment time, shown by decreasing WCA values (see Fig 2B). After 5 min there was no significant difference in WCA values.

3.1.3. Scanning electron microscopy (SEM)

Surface roughness as a result of polymer deposition was investigated using SEM both before and after washing with di-ionized water. Washing of samples was performed to check stability of polymer coupling. From Fig 3 A, it is clear that as the POSS-PCU samples were treated with plasma polymerization, roughness increased with time. However, when samples were washed excess amount of polymer which remained loosely attached to polymer (not chemically crosslinked) is removed during washing process. Fig 3B shows untreated POSS-PCU sample, which is used as a control.

3.1.4. X-Ray Photoelectron Spectroscopy (XPS) analysis

Surface chemical modification was investigated by XPS analysis, which probes the top 10 nm of the sample surface, and is therefore an effective measure of changes in surface chemistry and composition. The surface elemental composition of POSS-PCU is shown in the supplementary information (Fig. S1). In all samples only C, N, O, and Si were detected on the surface, the

detection limit of XPS for light elements being ~1 atomic%. The surface composition of untreated POSS PCU was consistent with our previous reports.[3] The atomic percentage of N was used as an indicator of amine functionalization post plasma polymerization with allylamine as shown in Fig. 3C. To evaluate the effect of plasma polymerization time on amine functionalization, XPS data was gathered from samples after varying lengths of amine plasma modification, and also after modification with an O_2 plasma. Fig. 3C shows increase in N% as a functional of time from 1 to 5 min. In the untreated POSS PCU 3% atomic N was present, which is broadly consistent with the expected molecular structure. Upon treatment with O_2 plasma the N level dropped slightly. Treatment with amine plasma for only 1 minute resulted in a large increase in surface nitrogen to above 15 atomic%, representing a significant change in the surface chemistry, and suggesting successful attachment of amine functionality to the polymer surface. Amine plasma treatment for longer times did not significantly increase the nitrogen content of the surface: after 5 min. of amine plasma, the surface contained 17 atomic % N, and after 10 minutes of amine plasma the N level dropped slightly to 15 atomic%. Thus extended amine plasma treatment time does not increase the level of surface modification above what is seen after 1 minute.

In order to access stability of amine functionalization, samples were incubated in deionized water overnight, to stimulate wet conditions during cell culture. XPS analysis of samples post washing showed very significant reduction in N% for samples treated with amine plasma up to 5 minutes. For 1, 2 and 5-minute plasma treatment samples, washing with deionized water led to a reduction in N% to the levels seen in untreated POSS PCU – i.e. an almost total removal of the attached amine modification. However, samples polymerized for 10 min showed retention of N (10 atomic %), confirming stability of amino groups post polymerization. This shows that until 5

min, attached amine layer is loosely bond to the polymer surface, however when treated for 10 min, amino functionality becomes more strongly attached.

3.2. Cell attachment and morphology

Cell attachment and morphology on plasma polymerized samples was assessed using fluorescence microscopy after 4 hrs of cell seeding (as shown in Fig. 4A). Significantly higher ($P<0.01$) number of cells attached on amine functionalised compared to unmodified POSS-PCU. Similarly, increase in cell spreading was observed on amino modified samples, where cells remained predominately spread compared to unmodified POSS-PCU, where cells maintained rounded morphology. Glass coverslip were used as a positive control, where cell remained spread.

Quantitative assessment of cell morphology was carried out by measuring average cell area and circularity (as shown in Fig. 4B and C), where cell grown on amino modified POSS-PCU showed significantly higher average cell area ($p<0.001$) compared to unmodified samples. Circularity which is used as a measure of cellular elongation, decreases on amino modified samples compared to unmodified ones, further confirming changes in morphology.

3.3. Production ECM proteins and osteogenic markers

Collagen produced by cells on test samples was assessed both qualitatively and quantitatively. Immunostaining of collagen type I confirms its expression on all test samples (as shown in Fig.5 A). However, higher level of expression was observed on NH_2-POSS-PCU compared to POSS-PCU. Quantification of total collagen production by Sirius Red Assay (Fig 5B), shows

significantly higher level of collagen production on NH_2-POSS-PCU compared to pristine POSS-PCU on day 14 ($p<0.001$) and 21 ($p<0.01$).

Along with collagen type I, differentiation of ADSCs to osteogenic cells was confirmed by immuno-staining of osteopontin. Osteopontin is a bone ECM protein, which was expressed on all test samples (as shown in Fig. 6A) suggesting POSS-PCU both with and without amine modification was capable of inducing osteogenic differentiation of ADSCs.

ALP activity of the cells was measured to evaluate their ability to transcript and express this enzyme which is an early marker of osteogenic differentiation (days 5 to 14).[40] Quantification of ALP activity (Fig 6B) showed significantly higher value on NH_2-POSS-PCU compared to unmodified POSS-PCU and control (glass coverslip) samples ($p<0.001$) at day 14.

3.4. Peptide coupling

Following confirmation of NH_2 functionalization of POSS-PCU, pilot study was carried out to explore coupling of bone inducing peptide on these surfaces. Fluorescently labelled bone inducing peptide KRSR was coupled on to amino functionalized sample of POSS-PCU (10 min) to confirm presence and stability of peptide by fluoresce microscopy (Fig. 7A). Quantification of fluorescent intensity (Fig 7B) confirms significant increase fluorescence intensity ($p<0.001$) on covalently attached sample compared to physically adsorb one ($p<0.05$), confirming higher level of peptide present on plasma modified sample. NH_2-POSS samples was used as a background control. Fluorescence intensity following physio-adsorption suggest nonspecific background from unbound peptide.

3. DISCUSSION

Plasma polymerization is well known surface modification technique, which has been widely adopted and used for biomaterials modification due to its simplicity and reproducibility. In current study, we investigated use of this technology to optimize functionalization of POSS-PCU with amino groups using plasma polymerization process. Later, optimized samples were evaluated for their potential to enhance osteogenic differentiation of ADSCs cells for bone regenerative applications.

Firstly, various surface analysis methods are employed to confirm amino functionalization. POSS-PCU in its native state is hydrophobic in nature (WCA~100-120°).[41] Following plasma polymerization a drop in WCA was observed for all the samples, suggesting improved wettability of the polymer. Wettability plays pivotal role in cell-materials interactions. Protein adsorption and their conformation is strongly linked to wettability of the polymers.[42] Improved wettability of POSS-PCU is seen as favorable for essential protein adsorption and subsequent cell attachment.

Ninhydrin test was used as qualitative test to confirm presence of primary amine post plasma polymerization process. Intensity of purple color increases with treatment time confirming higher density of amino groups on the surface.

However, major question which remains unanswered is stability of these amino groups on POSS-PCU samples. This is important consideration which is not looked into previously by researchers. Leaching of unreacted monomer or polymer can affect cellular behavior. Since testing the stability of surface chemistry of the samples is difficult after cell culture, samples were rigorously washed with di-ionized water over night to remove any unattached, unreacted

monomer/polymer. XPS analysis of polymerized samples prior to washing shows increase in N%, which is used as an indicator for amine functionalization (as shown in Fig. 3C). However, when washed samples were evaluated, N% drops to similar values presented by unmodified POSS-PCU until 5 min of polymerization (as shown in Fig. 3C). This suggest that polymerization process did not lead to chemical coupling of amino functionalities on POSS-PCU within 5 min and require at least 10 min. of polymerization to form stable bonding. It is possible that activation process of 5 min with O_2 gas prior to plasma polymerization was not enough to initiate polymer coupling on the material, and longer activation time may be required to reduce polymerization time. By providing 10 min of polymerization, post 5 min activation provides enough surface activation to initiate irreversible coupling of amino functional groups on POSS-PCU samples. SEM analysis of samples before and after washing confirms finding from XPS, where deposition and retention of polymer was only visible on samples polymerized for 10 min (as shown in Fig. 3A).

Following standardization of plasma polymerization process with amino groups, we evaluated effect of amino modified POSS-PCU on ADSCs to study biocompatibility of modified material and its ability to differentiate cells in osteoblastic lineage for bone tissue engineering applications. Similar study, looking at allylamine modification of glass coverslip (model surfaces) was piloted with ADSCs, which shows enhance osteogenic differentiation into osteogenic lineage on amino modified glass surfaces.[11] However, translation of these amine modified model surfaces into real life application is not anticipated. Thus, relevance of current study become more obvious from clinical translation point of view, where POSS-PCU polymer has been recognized as successful biomaterial and used for various clinical applications.

Short term in vitro study with ADSCs shows enhance cell attachment and spreading on NH_2-POSS-PCU, compared to unmodified polymer (as shown in Fig. 4A). NH_2-functionalized polymers have previously been shown to promote cell attachment and spreading, which is thought to be due to protonation of amino groups providing positively charged surface.[43,44,45] Amine modified surfaces provide high pH which enhance proliferation and osteogenic activity of osteoblastic cells.[43,44] Song et al. studied the osteoblastic behavior of MC3T3-E1 cells on amine-modified titanium and showed that amine-plasma polymerized surfaces enhance adhesiveness and spreading of cells and showed elevated expression of paxillin and actin cytoskeleton on the modified surfaces.[46,47] Our results are in line with previous findings, where enhanced cell attachment and spreading was observed on NH_2- POSS-PCU samples.

When ADSCs were differentiated on these samples using osteo-inductive media, expression of various osteogenic markers become visible on all samples, however significantly elevated level of total collagen, osteopontin and ALP activity was only observed on NH_2-POSS-PCU samples (Fig 5 and 6). This confirms that even though unmodified POSS-PCU supported differentiation of ADSCs to osteogenic lineage, presence of amino functionality enhances this effect significantly. This provides a greater opportunity for bone regenerative applications where quicker bone integration and bone formation of implant is essential.[4,3]

We have previously showed use of carboxyl functionalized POSS-PCU for coupling of short bone inducing peptides via plasma polymerization using EDC-NHS chemistry.[4] However this for the first time, we also provide alternative method where amino functionalized POSS-PCU was also used to couple short peptide sequences successfully (as shown in Fig. 7). This, thus confirms versatility in modification of POSS-PCU polymer with known functional groups (NH_2 or

COOH) and their exploitation for successful functionalization with short bio active peptide sequences.

Even though we used allylamine as a common monomer which was reported in number of previous studies, optimization of amino functionalization and retention of coupled functional groups for POSS-PCU polymer was reported for first time for biological applications. It is likely that plasma polymerization process and stability of coupled functional groups is substrate dependent. The care must be taken while using any new material for plasma polymerization, in terms of evaluating stability of functional groups for intended applications.

4. CONCLUSION

This study helps to optimize amino functionalization of polymer using plasma polymerization technique. Also, provides necessity of confirming the stability of plasma polymerization samples for biological applications, where unreached monomer/polymer can leach out from the surface. Amino modified polymer shows ability of enhance osteogenic differentiation of adipose derived stem cells on clinically applied material, paving the ways for new bone regenerative applications.

5. ASSOCIATED CONTENT

Supporting information

XPS survey spectrum of elemental composition of POSS-PCU and schematic diagram of plasma polymerization using allylamine, followed by peptide coupling on POSS-PCU polymer using conventional EDC-NHS chemistry.

6. ACKNOWLEGMENT

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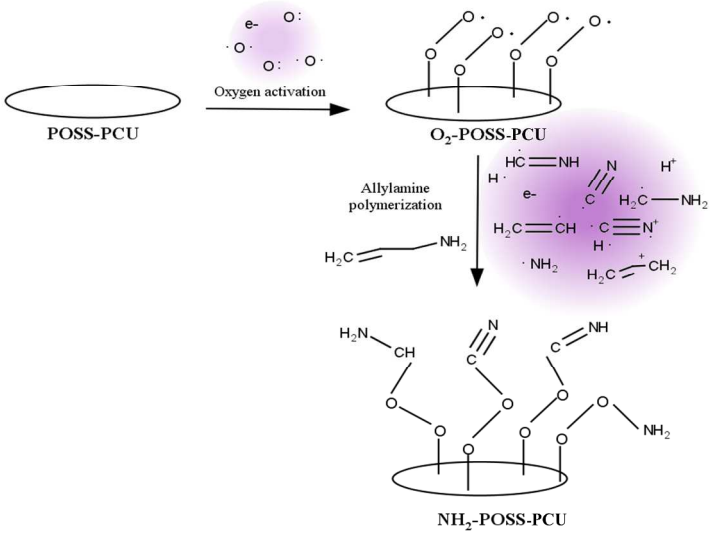

Figure 1. Schematic diagram of plasma polymerization of POSS-PCU using Allylamine hydrochloride.

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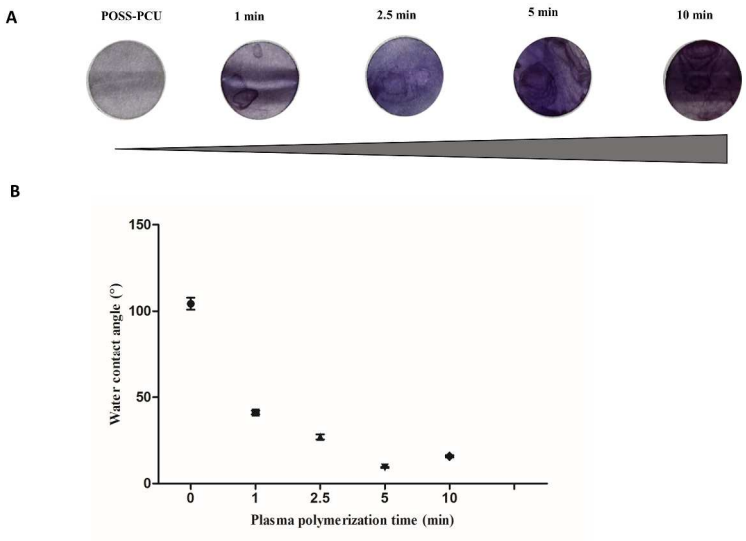

Figure 2. [A] Ninhydrin test to confirm presence of primary amines on plasma polymerized POSS-PCU samples at different time period. Intensity of purple colour is proportional to polymerization time. [B] The effect of plasma polymerization time on wettability of POSS-PCU surface, measured by static water contact angle (n=6).

338x190mm (300 x 300 DPI)

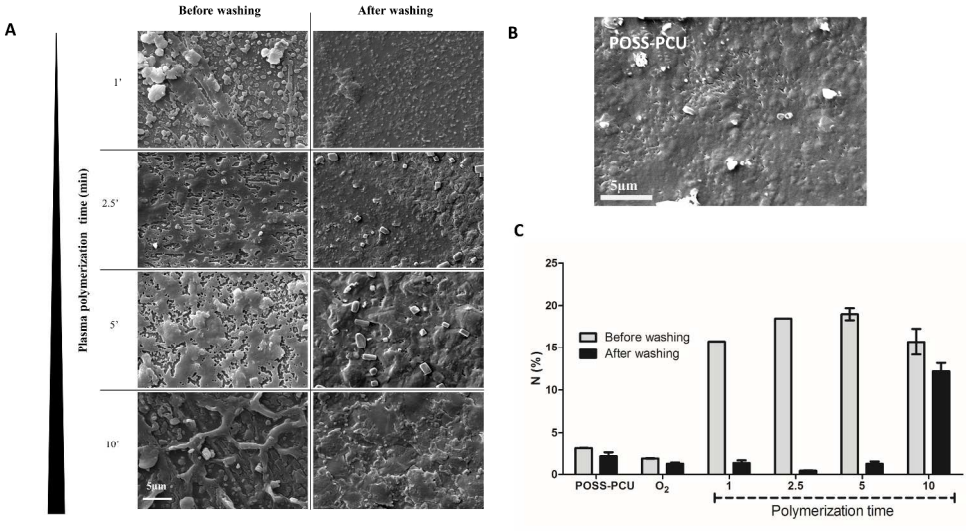

Figure 3. [A] Scanning electron micrographs of plasma polymerization POSS-PCU samples before and after washing with di-ionised water for 24 hrs. [B] POSS-PCU was used as control. [C] XPS analysis of allylamine polymerized samples for various duration of time. Elemental composition of N% before and after washing overnight using di-ionised water.

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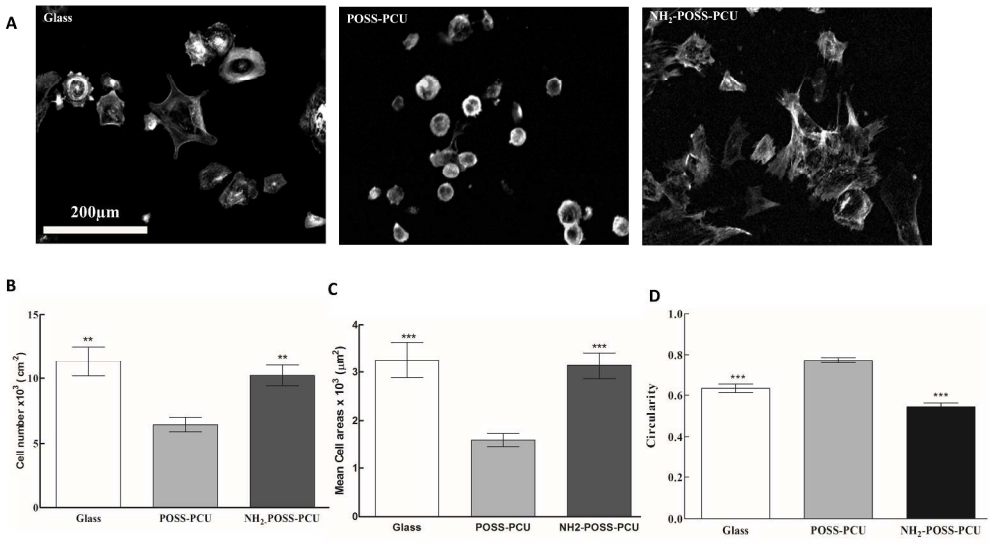

Figure 4. [A] Typical ADSCs morphology on glass, POSS-PCU and NH2-POSS-PCU samples as investigated by F-actin staining, quantification of cell attachment is reported in [B] as cell density (n=50), morphology of cells was quantified using mean cell area [C] and circularity [D] , where n=50.

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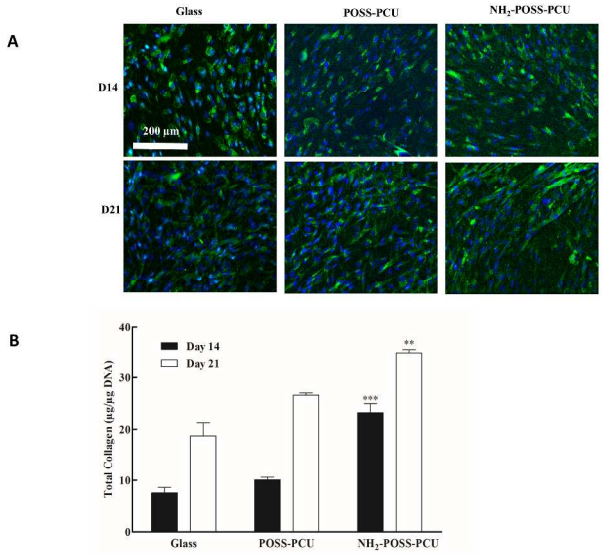

Figure 5. Immunological staining for Collagen type 1 at day 14 and 21 [A]. Quantification of total collagen production on day 14 and 21 [B]. Statistical significance was reported with respect to POSS-PCU, where n= 6.

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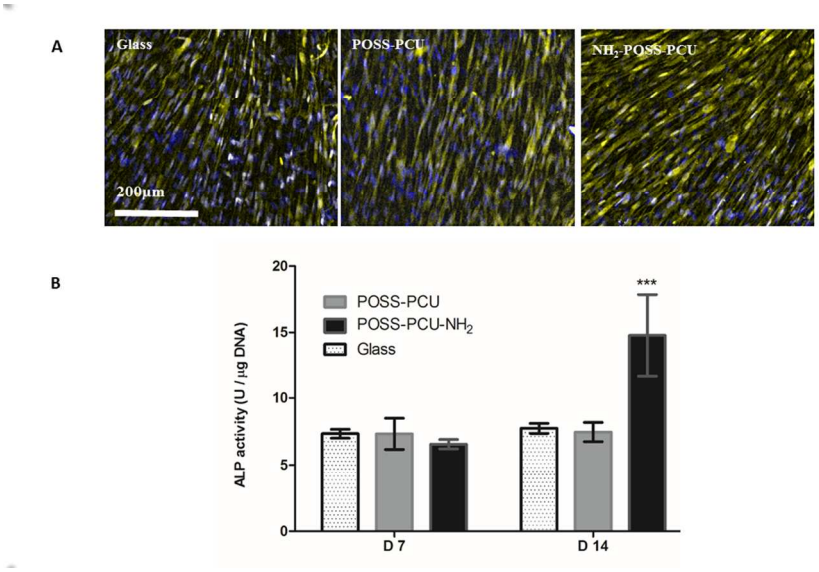

Figure 6. Immunological staining for Osteopontin on day 21, where glass coverslip is used as positive control [A]. ALP quantification after day 7 and 14 is shown in [B], Statistical significance was reported with respect to control (glass coverslip), where n= 6.

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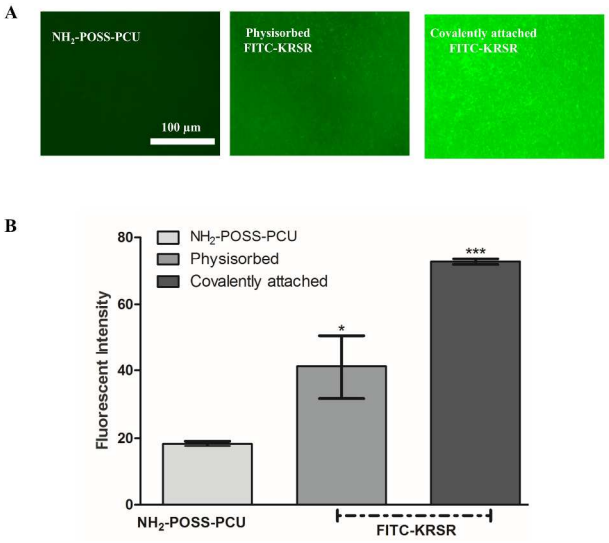

Figure 7. Fluoresce images of FITC-labelled KRSR peptide followed by amino polymerisation of POSS-PCU samples using conventional EDC-NHS chemistry where [A] NH2-POSS-PCU was used as a control, showing background intensity along with physio adsorbed peptide. Covalently attached KRSR shows highest level of fluorescent intensity. Quantification of fluoresce intensity using microscope is shown in [B], which confirms intensity of attached peptide is highest for covalently attached FITC-KRSR (n=3).

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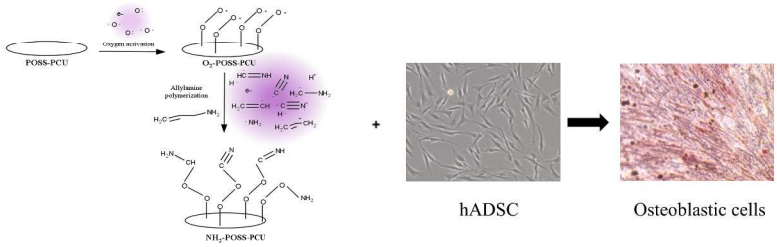

Abstract Image

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