Amide-linkage formed between ammonia (NH₃) plasma treated Poly (D, L-lactide acid) scaffolds and bio-peptides: enhancement of cell adhesion and osteogenic differentiation in vitro

Zixing Xu¹, Tao Li¹, Zhaoming Zhong¹, Dingsheng Zha¹, Songhui Wu¹, Fuqiang Liu¹, Wende Xiao¹, Xiaorui Jiang², Xinxin Zhang² and Jianting Chen¹,*

Introduction

Materials and Methods

Fabrication of three-D PDLLA scaffolds.
Preparation of aminated PDLLA (A/PDLLA) and peptides conjugated A/PDLLA (PA/PDLLA) scaffolds.
X-ray Photoelectron Spectroscopy (XPS) analysis.
Appraisal using Confocal Laser Scanning Microscope and High Performance Liquid Chromatography (HPLC).
Cell isolation and culture.
Group settings and Cells seeding.
Analysis of cells adhesion and proliferation on scaffolds.
Analysis of osteogenic differentiation of stem cells on scaffolds.
Statistical analysis.

Results

Effects of NH₃ plasma pre-treatment and FITC-GRGDS conjugation on surface chemistry.
The amounts of FITC-GRGDS anchored to the scaffolds.
Cell adhesion and proliferation in scaffolds.
Expression of osteogenesis-related Genes in BMSCs in scaffolds.

Discussion

Conclusion

Acknowledgements

References

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$^1$Department of Spinal and Orthopedic Surgery, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, People’s Republic of China

$^2$Department of Orthopaedics and Traumatology, Nanfang Hospital, Southern Medical University, 1838 North Guangzhou Avenue, Guangzhou 510515, People’s Republic of China

*To whom correspondence should be addressed.

Tel. +86-20-61641723, Fax +86-20-61641721, E-mail: chenjt99@tom.com

Abstract

The surface characteristics of scaffolds for bone tissue engineering must support cell adhesion, migration, proliferation and osteogenic differentiation. In the study, Poly (D, L-lactide acid) (PDLLA) scaffolds were modified by combing ammonia (NH$_3$) plasma pre-treatment with Gly-Arg-Gly-Asp-Ser (GRGDS)-peptides coupling technologies. The X-ray photoelectron spectroscopy (XPS) survey spectra showed the peak of N 1s at the surface of NH$_3$ plasma pre-treated PDLLA, which was further raised after GRGDS conjugation. Furthermore, N 1s and C 1s in the high-resolution XPS spectra revealed the presence of -C=N (imine), -C-NH- (amine) and -C=O-NH- (amide) groups. The GRGDS conjugation increased amide groups and decreased amine groups in the plasma-treated PDLLA. Confocal microscope and high performance liquid chromatography verified the anchored peptides after the conjugation process. Bone marrow mesenchymal stem cells were co-cultured with scaffolds. Fluorescent microscope and scanning electron microscope photographs revealed the best cell adhesion in NH$_3$ plasma pre-treated and GRGDS conjugated scaffolds, and the least attachment in un-modified scaffolds. Real-time PCR demonstrated that expression of osteogenesis-related genes, such as osteocalcin, alkaline phosphatase, type I collagen, bone morphogenetic protein-2 and osteopontin, was up-regulated in the single NH$_3$ plasma treated and NH$_3$ plasma pre-treated scaffolds following GRGDS conjugation. The results show that NH$_3$ plasma treatment promotes the conjugation of GRGDS peptides to the PDLLA scaffolds via the formation of amide linkage, and combination of NH$_3$ plasma treatment and peptides conjugation may enhance the cell adhesion and osteogenic differentiation in the PDLLA scaffolds.

Keywords: Poly-D,L-lactide acid (PDLLA)  Ammonia (NH$_3$) plasma  Bioactive peptides  Gene expressions  Bone Marrow Mesenchymal Stem Cells (BMSCs)
Introduction

An ideal bone substitute should be compatible with defect surrounding microenvironment, and be recognized by adjacent organs or tissues. So-called ‘third-generation’ biomaterials are being designed to stimulate specific cellular responses at the molecular level\(^1\). Poly (D, L-lactide acid) (PDLLA) is one of the most commonly used biodegradable polymers in the field because of its outstanding properties\(^2\). However, low hydrophilicity/surface energy and lack of bioactive sites have been shown as two negative factors to affect cell adhesion, proliferation and osteogenic differentiation in three-D biomaterial scaffolds\(^3\). To immobilize extracellular matrix (ECM) components to the surface of PDLLA scaffolds, the biomimetic PDLLA may mimic many roles of ECM in vitro. Surface modification is an effective approach for the purpose\(^2,4,5\). Since Arg-Gly-Asp (RGD) tripeptide has been found to be an active sequence of adhesive proteins of ECM, numerous biomaterials have been anchored by RGD for academic studies and clinical applications\(^6,7\). Although various methods have been developed for immobilization of RGD on the surface of the materials, most of them are far from the perfectible goal\(^8-12\).

Coating the surface with RGD-containing peptides is one of the simplest methods providing the binding sites for the materials\(^8\). However, it is time-consuming and expensive. Moreover, simple adhesion or embedment of peptides might easy to detach from scaffolds under mechanical vibration or shear stress. An improved method for promotion of the anchorage is the covalently conjugating the peptides to biomaterials\(^11,13\). Surfaces of organic polymers, such as PDLLA, customarily are short of bioactive groups. An aminated surface of poly (caprolactone) (PCL) was reported using 1,6-hexanedimine to incorporate amine groups, and RGD, Tyr-Ile-Gly-Ser-Arg (YIGSR) and Ile-Lys-Val-Ala-Val (IKVAV) peptides consequently were covalently conjugated to the aminated PCL\(^14\). The covalent couple promotes effective and stable conjugation and has been recommended by several researchers\(^15-18\). However, excess use of chemical reagents might lead to complicated reaction, over side reaction, and difficulty to dispose excess reagents.

Low-temperature plasma technique was found to be an efficient method for modifying the surface of biomaterials without changing the bulk properties. It improves the hydrophilicity/surface energy and roughness of the polymer\(^19,20\). Some researchers revealed that cell response of MC3T3-E1 on the poly (L-lactide) (PLLA) treated with plasma in air or in the CO\(_2\) gas were significantly superior to the control\(^20\). In another study, argon (Ar) plasma was introduced to enhance immobilization of Arg-Gly-Asp-Ser (RGDS) tetrapeptide instead of adhesive proteins (e.g., collagen) in PLLA scaffolds\(^21\). Additionally, ammonia (NH\(_3\)) plasma can provide polymer surfaces with N-containing functional groups, which mainly are amine (-NH\(_2\)) groups\(^22\). The functional groups can covalently couple biomolecules, such as RGD tripeptide\(^23\). It increases immobilization of peptides and cell adhesion. The surface of the NH\(_3\) plasma pre-treatment was reported to be easier to anchor collagen\(^24,25\). Culture of mouse 3T3 fibroblasts demonstrated that the method can effectively
facilitate cell migration and that the cultured cells can be distributed evenly throughout the scaffold. These studies disclosed that plasma treatment was helpful in promotion of bioactive macromolecules to the surface of synthesized polymers. However, it was still lack of the direct evidence of successful anchorage and rationale interpretation of immobilization process.

In order to promote peptides-anchorage to the surface of the PDLLA scaffolds, we designed a method of combing NH₃ plasma pre-treatment with Gly-Arg-Gly-Asp-Ser (GRGDS)-peptides coupling technologies. In the study, we explored the variable quantities of N-containing groups conjugated to PDLLA scaffolds in different treatment parameters of NH₃ plasma, and looked for reaction between –NH₂ and carboxyl (–COOH) end group of GRGDS peptides with the aid of cross-linking agents of 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC.HCl) and N-Hydroxysuccinimide (NHS). Since it is a prerequisite for applying the bioactive peptides conjugated PDLLA scaffolds, we investigate the adhesion and osteogenic differentiation of Bone Marrow Mesenchymal Stem Cells (BMSCs) in the scaffolds.

Materials and Methods

Fabrication of three-D PDLLA scaffolds.

Three grams of PDLLA (Mw/Mn=1.85, Foryou Co., Ltd., Huizhou, China) were dissolved into 15mL of dioxane, and then, 12 g of the sieved porogen sodium chloride (salt size 300 μ m-450 μ m) were added into the polylactone solution. The mixture was molded at temperature of 55 ºC - 65 ºC and pressure over 10MPa. After cooling down to −5 ºC for 12 hours, cylindrical composites were extracted, placed in liquid nitrogen for 8-12h, and cryodesiccated for 48h at −45 ºC. The porogen in the composites was washed out by changing water every 4-6 h for a total of 72 h. After vacuum dehydration at 45 ºC for 24h, the cylindrical sponge-like scaffolds (8mm × 8mm × 10mm) were obtained. The scaffolds were sliced into uniform wafers with 1 mm of thickness, and then, sterilized using epoxyethane at 45 ºC for 12h, dehydrated with vacuum for 24h, and ventilated for 5-7d prior to use.

Preparation of aminated PDLLA (A/PDLLA) and peptides conjugated A/PDLLA (PA/PDLLA) scaffolds.

The plasma treatment was done in a Model DL-01 Plasma Chamber (Omega Co., Ltd., Suzhou, China) under anhydrous ammonia gas. The wafers of PDLLA scaffolds were placed in the plasma reactor chamber. The chamber was evacuated to less than 10 Pa before filling with the NH₃. After the pressure of the chamber was stabilized at 30Pa, plasma treatment was initiated for 2, 5, 10, 20 and 30 min using a power of 50 W and pulsed frequency of 13.56 MHz. The plasma-treated scaffolds (aminated PDLLA, A/PDLLA) were exposed to the NH₃ for 10 min before removing from the chamber. Three-D scaffolds (treated with or without plasma) were pre-wetted with ethanol 70% for 3 h. After the pre-wetted, phosphate buffered saline (PBS, Sigma, USA) was used to replace ethanol. Next, the scaffolds were immersed into the sterile GRGDS solution labelled with or without FITC (Shanghai Bootech Bioscience &
Technology Co., Ltd, China) which contained EDC.HCl and NHS (Sigma, USA) with the molar ratio of peptides, EDC.HCl and NHS was 1:1.5:1.5. The solution was brachytely sloshed in room temperature for 24hs. The consequent scaffolds were marked ‘peptides conjugated A/PDLLA (PA/PDLLA)’.

**X-ray Photoelectron Spectroscopy (XPS) analysis.**
XPS was performed on polymer scaffolds before and after plasma treatment and following conjugation of FITC-GRGDS in order to determine the changes of surface chemistry using a Ultra DLD spectrometer (Kratos Analytical Ltd, UK) with a monochromatized Al Kα x-ray source \( (hν = 1486.6 \text{ eV}) \). The anode voltage and current were 15 kV and 10 mA. Survey spectra were collected using a pass energy of 160 eV with 1 eV/step, while region scans were collected with a pass energy of 40 eV under the rate of 0.1 eV/step. The pressure in the analysis chamber was maintained at \( 5 \times 10^{-9} \text{ Torr} \) or lower during each measurement. Binding energy was referenced to the C 1s neutral carbon peak at 284.6 eV. The XPS analysis of the plasma treated sample was done in 48 h after the treatment.

**Appraisal using Confocal Laser Scanning Microscope and High Performance Liquid Chromatography (HPLC).**
Amounts of FITC-GRGDS conjugated to scaffolds were appraised by confocal laser scanning microscope and HPLC. Various intensities of green fluorescence ignited by scaffolds indirectly reflect the amounts of peptides anchored to the scaffolds. Prior to observe, the scaffolds were flushed with deionized water 3 times, dried by a filter paper, and then sliced to less than 10 μm of the thickness for Leica TCS SP2 AOBS Laser Spectral Confocal Microscope (Leica, Germany). The scaffolds were treated with 10% of aqueous ammonia and standing several hours to complete degradation before analysis of HPLC. Agilent 1100 HPLC system (Agilent, USA) was utilized for detection. HPLC conditions were set up as follow: ① VYDAC C18 column (4.6 mm × 250.0 mm, 5 μm, USA) was used as stationary phase; ② 5% of methanol (containing 0.1% trifluoroacetic acid, TFA) and 95% of methanol (containing 0.1% TFA) were used as mobile phase A and B respectively, by linear gradient elution, at the flow rate of 1.0ml/min and detection wavelength of 220 nm. Aqueous solution of 0.001 mg/mL of FITC-GRGDS was prepared as standard solution. Five different sample injection volumes (5, 10, 15, 25 and 30 μL) of standard solution were selected for HPLC analysis. Three replicates were done in the each sample volume. A standard curve was made by mean of peak areas and amounts of peptides. Peak areas from chromatogram linearly correlate to corresponding sample injection volume.

**Cell isolation and culture.**
Primary BMSCs were harvested from the long bones of young adult male Sprague-Dawley (SD) rats (Experimental Animal Center of Southern Medical University). Four SD rats were executed by excess anesthesia of 3% pentobarbital sodium and then immersed into 75% aqueous ethanol for sterilization. The femurs and tibias were aseptically excised from the hind limbs, cleaned of soft tissue, and rinsed
with Dulbecco’s Modified Eagle Medium, DMEM, 4.5 g/L glucose with L-glutamine (Gibco, USA) plus 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin-streptomycin, and 0.3 μg/ml amphotericin B (Sigma, USA). The ends of the bones were removed and the marrow cavity was flushed with 5 mL PBS. The isolated marrow was centrifuged, re-suspended in the growth medium, and seeded in 25 ml culture flask. The flasks were incubated in a humidified 5% CO\textsubscript{2} incubator at 37°C. The medium was changed every 3 days to remove haematopoetic and other unattached cells. The cells were subcultured when they were 90% confluent. For tracing the cytobiological behaviour of BMSCs in scaffolds, the lentivirus-based Green Fluorescent Protein-transfected BMSCs (GFP-BMSCs) were purchased from Cyagen Biosciences Inc. Reagents and culture methods were in accordance with primary BMSCs culture after resuscitation of frozen GFP-BMSCs (Cyagen, USA). The growth medium was changed to osteogenic medium containing DMEM 4.5 g/L glucose (10% FBS), 10 nmol/L dexamethasone, 10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate (Sigma, USA) to induce osteogenic differentiation of primary BMSCs and GFP-BMSCs, when they were 70% confluent at 5-6 passages. Then, it was replaced with fresh osteogenic medium every 3 days.

**Group settings and Cells seeding.**

PDLLA scaffolds were prepared in a diameter of 8mm-circle with 1mm-thickness and divided into 3 groups, Group P (untreated) as control group, Group A (PDLLA pre-treated with 20 min of NH\textsubscript{3} plasma) and Group PA (PDLLA pre-treated with 20 min of NH\textsubscript{3} plasma and conjugated GRGDS peptides). Cell qualitative experiments of adhesion and proliferation were done with undifferentiated GFP-BMSCs which were cultured in the growth medium. Primary BMSCs were used to study osteogenic differentiation of stem cells in three-D scaffolds, which were cultured in osteogenic medium for 3 days. Before seeding, cells were trypsinized and resuspended in the medium. Then the suspension was added drop-wise on the top of the scaffold at a density of nominal cells/scaffold in 50 μL of the culture medium. After 3 hours of the initial cell attachment, 1.5 ml of medium was added to each scaffold to continue culture. The medium was replaced by fresh one every 3 days.

**Analysis of cells adhesion and proliferation on scaffolds.**

After seeding at an initial density of 5.0×10\textsuperscript{4} GFP-BMSCs/scaffold and culture of the composites for 6 and 12 days, adhesion and proliferation of cells on scaffolds were observed qualitatively using a IX71 fluorescence microscope (Olympus, Japan) and a S-3000 Series Scanning electron microscope (SEM, Hitachi, Japan) at 15 kV. At each time point, three composites in each group were flushed, fixed with 2.5% glutaraldehyde (GA), air dried, and coated with gold prior to microstructural observation.

**Analysis of osteogenic differentiation of stem cells on scaffolds.**

After seeding at an initial density of 5.0×10\textsuperscript{5} BMSCs/scaffold and culture of the composites for 3, 7 and 14 days, two composites in each group were rinsed. Total
RNA isolation was performed. Reverse of transcription and real-time quantitative polymerase chain reaction (RT-qPCR) is described below. A RNAiso Reagent (Takara, Japan) was used to isolate total RNA from composites. After rinsed in PBS, each composite was placed in an EP tube and clipped into 1 mm³ pieces. One ml Trizol was added to each tube and the performance was done in accordance to the method described previously. The OD$_{260/280}$ of extracted total-RNA was measured in a ND-1000 ultraviolet spectrophotometer (Nano Drop, USA) to determine RNA concentration. One μg of total RNA was used in a reverse transcription reaction with PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan). Complementary DNA products were stored at −20 °C. Effects of osteogenic differentiation of BMSCs in scaffolds were further assessed by RT-qPCR to measure the mRNA expression of osteocalcin (OCN), alkaline phosphatase (ALP), type I collagen (COL I), bone morphogenetic protein-2 (BMP-2) and osteopontin (OPN) in all three groups. The reaction with SYBR® Premix Ex Taq™ (Takara, Japan) was carried out using Applied Biosystems 7500 Sequence Detection System (ABI, USA). Two-Step PCR amplification followed 1 cycle of 30 seconds at 95 °C, 40 cycles of 5 seconds at 95°C and 1 cycle of 34 seconds at 60°C. Settings of melting curve were maintained system default and analysis was performed to affirm specific amplification without genomic DNA contamination. All samples were performed with average results in triplicates and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene. Primer pairs (Invitrogen, USA) used in real-time PCR were listed below: OCN, forward 5’-GCAGGAGGGCGATAGGTG-3’ and reverse 5’-AAGCCAATGTGTTGCAGCTA-3’; ALP, forward 5’-GACTGACCCCTTCCCTCTCG-3’ and reverse 5’-GTGGTGCAATCCTCGCCTCCT-3’; Col I, forward 5’-CTGGAACCTCAAGAAGTCC-3’ and reverse 5’-CAAGTTCCGGTGTACCTG-3’; BMP-2, forward 5’-CGGACTGGTGCTTCTCTAA-3’ and reverse 5’-GGGGAAGCAGCAGACACTAGA-3’; OPN, forward 5’-GAGTTTGGCAGCTAGGAGA-3’ and reverse 5’-TCTGGTTCTCTGATGATGGTCA-3’; GAPDH, forward 5’-GGACCAGGTGGTCTCTTG-3’ and reverse 5’-TGTTGGCAGCTAGGAGC-3’. Relative expression levels of the each gene were normalized against the Ct value of GAPDH and determined using the 2$^{-ΔΔCt}$ method. Expression levels of genes in Group P on Day 3 were designed as ‘one’ fold as control. The relative expression of each gene in the each group at different time points was comparable with control.

**Statistical analysis.**

Statistical analysis was performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation. The Welch’s test was used as an alternative analysis of variance when samples variances were unequal. To evaluate differences between or among groups, analysis of variance (ANOVA) was performed with post hoc pairwise testing, if necessary, using Dunnett’s T3 test. Significant difference was considered when P<0.05.

**Results**

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Effects of $\text{NH}_3$ plasma pre-treatment and FITC-GRGDS conjugation on surface chemistry.

XPS survey spectra shows the presence of nitrogen at 399.8 eV (binding energy, BE) and sulfur at 164.0 eV in addition to carbon and oxygen after plasma treatment and conjugation (Figure 1). The peak of N 1s in the spectra increased with increasing the treatment time except at the 30 min. During each treatment, N 1s peak has accordingly further increased after conjugation of peptides. Although S 2p peak was slightly higher than the background noise level at 0, 2 and 5 min modification, it became obvious after 10 min pre-treatment peptides-conjugation. The percentage of the atomic composition and the relative O/C, N/C ratios were listed in Table 1. Before peptides conjugation, percentage of oxygen atom and the ratio of corresponding O/C were slightly increased after 0, 2 and 5 min of plasma treatment and sharply decreased after 10 min. The percentage and the ratio declined after the conjugation. C 1s in the spectra from pristine samples was deconvoluted into four peaks with binding energies of 284.6, 286.6, 287.0 and 289.1 eV, which were attributed to carbons in -C-C- or -C-H, -C-O-, >C=O and -COO- groups, respectively (Figure 2A). $\text{NH}_3$ plasma treatment and FITC-GRGDS conjugation produced two new peaks with binding energies of 285.7 and 288.3 eV, which were attributed to –C-NH- (amine) and -C=O-NH- (amide) groups, respectively (Figure 2B-D). N 1s in the spectra from plasma treated scaffolds was deconvoluted to three components corresponding to nitrogen in 398.8 (-C= N, imine group), 399.8 (amine group) and 401.2 eV (amide group). The relative compositions changed after FITC-GRGDS conjugation (Figure 3). Table 2 shows the fractions of various carbon functional groups or nitrogen functional groups on the surface of the pristine, $\text{NH}_3$ plasma-treated (20 min) and further peptides modified scaffolds. $\text{NH}_3$ plasma treatment brings about N-containing radicals, which are primarily amine groups. Further conjugation results in the decline of amine groups and raise of amide groups.

Amounts of FITC-GRGDS anchored to the scaffolds.

Qualitative conjugation of the peptides to the scaffolds was visually exhibited by the fluorescent confocal images (Figure 4). The fluorescent intensities have obviously strengthened with the time of plasma treatment except the 30 min of plasma pre-treatment and peptides conjugation. Qualitative results of peptides anchored to the scaffolds were determined by HPLC and listed in Table 3 (Figure 5). Conjugation was undetectable in the scaffolds with 0 (untreated), 2 and 5 min of $\text{NH}_3$ plasma pre-treatment and FITC-GRGDS conjugation. The peak of the anchored peptides appeared at 20 min of the $\text{NH}_3$ plasma pre-treatment. It is significantly elevated from 10 min and reduced at 30 min ($P<0.05$). Changes of conjugated peptides accompany to the fluorescent intensities observed by confocal microscope.

Cell adhesion and proliferation in scaffolds.

Adhesions and proliferations of GFP-BMSCs in all scaffolds were observed (Figure...
6). After 6 days of culture, the number of cells adhered to the scaffolds in Group PA is the highest among the three groups. The intensities of green fluorescences exhibited GFP-BMSCs were the lowest in pristine scaffolds (Group P). The density of fluorescence in the scaffolds in Group A and Group PA is more homogeneous than the density in Group P. After being cultured for 12 days, it was difficult to discern the difference of cell numbers between Group A and Group PA. It could be seen from SEM photographs that most cells adhered to the aperture walls or pore spaces of scaffolds by filopodia. Cells were polygen or spindle-shaped and contacted each other. In the same range of vision, less cells attached to the scaffolds were seen in Group P. After 12 days of culture, cells attached to the scaffolds were exhibited the best adhesion in Group PA. It was obvious that cells were spread adequately and densely and coated in the pore spaces. During the same period, cells laid sparsely in group P and partial cells became pyknotic in Group A.

**Expression of osteogenesis-related Genes in BMSCs in scaffolds.**

The expression levels of osteogenesis-related genes in the each group were increased (P<0.001) at each time point, more obvious in Group A and PA (Table 4). The statistical significances from multiple comparisons are indicated on the histogram (Figure 7). The results from qPCR show that osteogenesis-related gene expression in Group PA was higher than in Group P on Day 3. Also, it was higher than in Group A except OCN mRNA on the same day. The gene expression levels of OCN (13.13±1.28), Col- I (23.71±6.51) and OPN (27.4±7.17) in Group A were the highest among the three groups on Day 7, and it was the second place in Group PA. Most of the gene expressions were elevated in all groups on Day 14. Gene expression levels in Group A and Group PA were higher than the control. OCN (49.21±7.03), ALP (24.26±3.41) and BMP-2 (11.82±2.38) mRNA in Group PA were significantly higher than those in Group A (P<0.05).

**Discussion**

In the study, we found that the surface of pristine PDLLA scaffolds showed carbon and oxygen in the form of -C-C- or -C-H, -C-O-, >C=O and -COO- bonds characteristic for the polymers. It is in accordance with previous reports 20,31,32. After NH$_3$ plasma treatment, N 1s in high-resolution XPS spectra indicated that the amine group (-NH$_2$) was at the most prominent composition. Meanwhile, nitrogen content increased obviously up to 6.85 % at 20 min of the treatment. By further reacted with FITC-GRGDS solution, the content further increased and S 2p peak (derived from FITC) appeared, which suggest the ‘anchorage’ of the FITC-GRGDS on scaffolds surface. Confocal microscope and HPLC verified the increase of anchored peptides after the ‘anchorage’ process with increase of plasma pre-treatment time until 20 min. Therefore, with increase of reactive -NH$_2$ groups, quantities of FITC-GRGDS anchored on PDLLA scaffolds increased accordingly. Moreover, the decrease of amine groups and the increase of amide groups were obtained after ‘anchorage’ process as expected. In briefly, NH$_3$ plasma treatment promotes the conjugation of
GRGDS peptides to the PDLLA scaffolds via the formation of amide linkage (-C=O-NH-) between surface reactive –NH₂ and -COOH end group of GRGDS peptides.

Surface characteristics of scaffolds for bone tissue engineering must support cell adhesion, migration, proliferation and osteogenic differentiation 33-36. Numerous methods of surface modification have been developed 13,14,19,37-39. In the study, we combine NH₃ plasma treatment with peptides coupling technologies to affect the aforementioned cytobiological behaviors of BMSCs in PDLLA scaffolds. The application of NH₃ plasma treatment enhances the surface energy and hydrophilicity by increasing the roughness and introducing nitrogen and oxygen functionalities to the surface 2,22,40,41. The N-containing groups attached to the surface are primarily amine, amide, and/or imine. It has been proved that the polymers with the amine and amide radicals promote attachment of cells in the culture 26,31,34,42-45. The enriched polar groups on polymer surface can provide many sites to obtain the ECM proteins by polar interaction and hydrogen bonding 24, or directly induce the interaction of cells with the reactive groups 45. However, unsuitable prolonging of treatment time may result in loss of new generated polar groups 46. Our results show that 30 min of NH₃ plasma pre-treatment lead to the decline of nitrogen content and conjugation of FITC-GRGDS peptides. The following two reasons may explain decrease of nitrogen in the 30-min group: 1. Oxygen content on PDLLA surface was increased with the prolonged treatment time of 20 min to 30 min. Therefore, nitrogen content was relatively decreased; 2. The cleavage of the chemical bonds may have been caused due to the excessive treatment 46.

RGD tripeptide as an integrin recognition site is the most effective and wide employed peptide sequence for stimulating cell adhesion on biomaterial surfaces 47,48. Numerous studies have demonstrated that RGD peptides promote increase of adhesion of osteogenic cells and MSCs to many types of biomaterials 47,49-51. Once RGD sequence is present on the polymers surface and is recognized by and binded to integrins at focal points of cell adhesion, it will initiate an integrin-mediated cell adhesion process and activate signal transduction between the cell and RGD sequence. RGD sequence influents cell behaviors including adhesion, proliferation, and osteogenic differentiation in the synthetic scaffolds.

Osteogenic differentiation of BMSCs in NH₃ plasma treated and peptides conjugated PDLLA scaffolds has not been well studied. To apply the new type of modified bone substitute in vivo, it is necessary to develop osteogenic differentiation of BMSCs in the scaffolds. The results from qPCR indicate that the both modified methods, single NH₃ plasma treatment and plasma pre-treatment following GRGDS conjugation, promote osteogenic differentiation of BMSCs in the scaffolds. After 3 days of culture, expressions of osteogenesis-related genes in Group PA were higher than Group A except OCN. After 7 days of culture, the extents of up-regulated expression of OCN, COL I and OPN mRNA in Group A were the most obvious, even surpassed the combined modification in Group PA. Above results indicate that single NH₃ plasma treatment may promote earlier osteogenic differentiation of BMSCs in the scaffolds. Since the expression of osteogenesis-related genes were higher in Group
PA on Day 3, it might be resulted a better initial adhesion of BMSCs in the scaffolds with NH$_3$ plasma treatment and peptides conjugation than with single NH$_3$ plasma treatment. With extending culture time to the 14$^{th}$ day, expressions of all related genes in Group PA and OCN, ALP mRNA in Group A were further up-regulated in great extents. Meanwhile, changes of expression in COL I, BMP-2 and OPN mRNA in Group A were not obvious. After 14 days of culture, mRNA expressions in OCN, ALP and BMP-2 in Group PA were the highest among the 3 groups. It reflects that combination of NH$_3$ plasma treatment and peptides conjugation may enhance the osteogenesis in vitro.

**Conclusion**

In conclusion, NH$_3$ plasma treatment promotes the conjugation of GRGDS peptides to the PDLLA scaffolds via the formation of amide linkage (-C=O-NH-) between surface reactive –NH$_2$ and -COOH end group of GRGDS peptides. Furthermore, combination of NH$_3$ plasma treatment and peptides conjugation may enhance the osteogenesis in vitro.

**Acknowledgements**

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**References**

Figure 1. XPS survey spectra was done on the surface of scaffolds before (0 min) plasma pre-treatment and 2, 5, 10, 20 and 30 min after the pre-treatment and accordingly, conjugation of FITC-GRGDS. (A) Before FITC-GRGDS conjugation, N1s peak increases obviously with plasma treatment time except 30 min-treatment. (B) After FITC-GRGDS conjugation, N1s peak further increases accordingly.

399x700mm (300 x 300 DPI)
Figure 2. C1s in high-resolution XPS spectra in scaffolds: (A) Control. (B) Modified by NH3 plasma treatment for 20 min. (C) Modified by FITC-GRGDS conjugation without NH3 plasma pre-treatment. (D) Modified by NH3 plasma pre-treatment for 20 min and FITC-GRGDS conjugation.
Figure 3. N1s in high-resolution XPS spectra in scaffolds: (A) Modified by NH3 plasma treatment for 20 min. (B) Modified by FITC-GRGDS conjugation without NH3 plasma pre-treatment. (C) Modified by NH3 plasma pre-treatment for 20 min and FITC-GRGDS conjugation.
Figure 4. Intensities of green fluorescence observed by confocal microscope in scaffolds: (A) Control. (B) Modified by FITC-GRGDS conjugation without NH3 plasma pre-treatment. (C) Modified by NH3 plasma treatment for 2 min and FITC-GRGDS conjugation. (D) Modified by NH3 plasma treatment for 5 min and FITC-GRGDS conjugation. (E) Modified by NH3 plasma treatment for 10 min and FITC-GRGDS conjugation. (F) Modified by NH3 plasma treatment for 20 min and FITC-GRGDS conjugation. (G) Modified by NH3 plasma treatment for 30 min and FITC-GRGDS conjugation.
Figure 5. Quantification of FITC-GRGDS conjugated to various times of NH3 plasma pre-treatment for 10, 20 and 30 min and peptides conjugation in scaffolds.

* Comparison between 10 min and 20 min of NH3 plasma pre-treatment, P<0.001; Comparison between other two pairs of groups, P<0.01.
Figure 6. Cells adhesions and proliferations were observed using fluorescent microscope (A - F) and SEM (G - O) in various scaffolds in Group P, A and PA, A - C and G - I observed on Day 6, D – F and J - L observed on Day 12, M - O without cells seeding (control).
Figure 7. Fold changes of osteogenesis-related genes with prolonging time for: (A) OCN mRNA. (B) Col 1a1 mRNA. (C) ALP mRNA. (D) BMP 2 mRNA. (E) OPN mRNA.

258x205mm (300 x 300 DPI)
Table 1. Changes of the atomic composition and the relative ratios on the surface of the scaffolds before and after peptides conjugation

<table>
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<tr>
<th>Treatment times</th>
<th>O(%)</th>
<th>C(%)</th>
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<td></td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>0min</td>
<td>31.55</td>
<td>26.95</td>
<td>68.45</td>
<td>71.81</td>
<td>UD*</td>
<td>1.02</td>
</tr>
<tr>
<td>2min</td>
<td>32.33</td>
<td>26.93</td>
<td>66.22</td>
<td>70.23</td>
<td>1.45</td>
<td>2.56</td>
</tr>
<tr>
<td>5min</td>
<td>32.82</td>
<td>26.57</td>
<td>64.94</td>
<td>69.17</td>
<td>2.24</td>
<td>3.94</td>
</tr>
<tr>
<td>10min</td>
<td>25.40</td>
<td>23.15</td>
<td>69.92</td>
<td>69.97</td>
<td>4.68</td>
<td>6.34</td>
</tr>
<tr>
<td>20min</td>
<td>23.58</td>
<td>21.82</td>
<td>69.57</td>
<td>68.91</td>
<td>6.85</td>
<td>8.58</td>
</tr>
<tr>
<td>30min</td>
<td>24.09</td>
<td>23.05</td>
<td>70.32</td>
<td>69.44</td>
<td>5.59</td>
<td>7.03</td>
</tr>
</tbody>
</table>

I: before peptides conjugation; II: after peptides conjugation
UD: Undetectable
Table 2. Fractions of various carbon functional groups or nitrogen functional groups from the deconvoluted C 1s or N 1s XPS spectra

<table>
<thead>
<tr>
<th>Samp</th>
<th>C 1s (%)</th>
<th>N 1s (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>-C-C- or -C-H</td>
<td>-C=O-N</td>
</tr>
<tr>
<td>I</td>
<td>48.0</td>
<td>26.3</td>
</tr>
<tr>
<td>II</td>
<td>35.8</td>
<td>14.8</td>
</tr>
<tr>
<td>III</td>
<td>36.1</td>
<td>36.4</td>
</tr>
<tr>
<td>IV</td>
<td>24.4</td>
<td>30.1</td>
</tr>
</tbody>
</table>

I: control; II: modified by NH₃ plasma treatment for 20 min; III: modified by FITC-GRGDS conjugation without NH₃ plasma pre-treatment; IV: modified by NH₃ plasma pre-treatment for 20 min and FITC-GRGDS conjugation.
Table 3. Quantification of conjugated peptides in the various scaffolds ($\bar{X} \pm S$, n=5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conjugated peptides ($\times 10^{-6}$ mg per mg of scaffold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-III</td>
<td>UD</td>
</tr>
<tr>
<td>IV</td>
<td>6.86±0.90</td>
</tr>
<tr>
<td>V</td>
<td>23.03±2.92</td>
</tr>
<tr>
<td>VI</td>
<td>11.97±1.80</td>
</tr>
</tbody>
</table>

I : Modified by FITC-GRGDS conjugation without NH$_3$ plasma pre-treatment. II - VI: Modified by FITC-GRGDS conjugation with NH$_3$ plasma pre-treatment for 2, 5, 10, 20 and 30 min, respectively.

UD: Undetectable
Table 4: Relative expression levels of osteogenesis-related genes ($2^{\Delta\Delta CT}$) and differences among three groups (n=6)

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th></th>
<th></th>
<th></th>
<th>Day 7</th>
<th></th>
<th></th>
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<th></th>
<th>Day 14</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>OCN</td>
<td>G1-1</td>
<td>ALP</td>
<td>BMP2</td>
<td>OPN</td>
<td>OCN</td>
<td>G1-1</td>
<td>ALP</td>
<td>BMP2</td>
<td>OPN</td>
<td>OCN</td>
<td>G1-1</td>
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<td>G1-1</td>
<td>ALP</td>
<td>BMP2</td>
<td>OPN</td>
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<td></td>
<td></td>
<td>1.64 ± 0.07</td>
<td>2.75 ± 0.39</td>
<td>1.57 ± 0.41</td>
<td>0.76 ± 0.09</td>
<td>0.53 ± 0.06</td>
<td>13.13 ± 1.28</td>
<td>2371 ± 4.51</td>
<td>3.42 ± 0.62</td>
<td>5.96 ± 0.68</td>
<td>27.4 ± 7.17</td>
<td>41.26 ± 2.99</td>
<td>24.05 ± 2.39</td>
<td>18.25 ± 4.85</td>
<td>5.57 ± 0.5</td>
<td>25.75 ± 4.85</td>
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<td>245.004</td>
<td>451.997</td>
<td>32.208</td>
<td>60.635</td>
<td>189.065</td>
<td>207.489</td>
<td>62.302</td>
<td>45.318</td>
<td>118.186</td>
<td>12.077</td>
<td>93.658</td>
<td>31.362</td>
<td>18.622</td>
<td>30.578</td>
<td>39.411</td>
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