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Studying the Effective Concentration of RGDS on RGDS-Poly(DL-Lactic Acid)

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RGDS-PDLLA is a biomimetic material obtained by covalently incorporating RGDS adhesion peptide. RGDS-PDLLA was prepared on basis of BDPLA (butanediamine-grafted polyactic acid). In present work, we prepared RGDS-PDLLA with gradient concentration RGDS and evaluated the cytocompatibility of gradient RGDS-PDLLA and took a novel method for studying the effective concentration of RGDS after grafting on BDPLA. When the concentration of RGDS in the RGDS-grafted-PDLLA (RGDS-PDLLA) varied from 0.142 to 0.302 μmol/g, cell cytocompatibility of osteoblasts was altered depending on the concentration of RGDS. 0.1 μmol/g RGDS on RGDS-PDLLA (RGDS-PDLLA) membrane can effectively promoted cell attachment, cell spreading. The concentration of RGDS increased from 0.142 to 0.302 μmol/g, the adhesion of osteoblasts on RGDS-PDLLA membrane increased slightly. However, we can find that when the RGDS increased to 2.42 μmol/g, the cell on films increased. These results suggest that the cell adhesion is sensitive to the RGDS concentration. By the study, we can confirm the optimal concentration of RGDS on RGDS-PDLLA.

Key Words: RGDS-PDLLA, Effective concentration, Arg-gly-asp-ser (RGDS), Adhesion.

INTRODUCTION

Cell adhesion to extracellular matrix (ECM) is the first stage of histogenesis. In the study of tissue engineering, the interaction between cells and substrate materials must be considered, because it not only determine the adhesion strength but also determine the recovery rate of tissue defect. After suitable adhesion, cells can migrate and proliferate, so it can be said that cell adhesion is the key stage and plays the key role in whole tissue engineering. It is important for designing a novel material to possess nice cell adhesion[1-4]. Polylactic acid (PLA), which is a popular degradable polymer, has a broad range of applications in biomedical engineering. Many researchers prepared bioactive materials based-PLA, such as the PEG-modified-PLA as drug carrier[5], MAH-PLA, BDA-PLA, RGDS-PLA, MGF-PLA, collagen-PLA for bone repair in our laboratory.

RGDS and RGD are important ECM proteins, which are key to the cytocompatibility of cell. We reviewed the Arg-Gly-Asp (RGD) modified biomimetic polymeric materials. We prepared and evaluated the cytocompatibility of a novel bioactive polymer RGDS-PDLLA (RGDS concentration is 5.12 μmol/g), from which we can find that introduction of RGDS can improve the cytocompatibility of PDLLA further. RGD is an ECM protein and can be recognized by cell membrane adhesion receptor, which is important for cells to adhere on substrate materials[1,4,6]. The application of RGD-PDLLA enables us to regulate and control cellular interactions with materials at a molecular level[6]. The receptor binding to the ligand that is presented from the biomimetic material determines the strength of the cell attachment to the surface, the cell migration rate on or through the material and the extent of cytoskeleton organization formation[7,8].

The concentration of active factors has key role in the cytocompatibility of cells. Ohga *et al.*[9] designed multifunctional peptide fibrils using bioactive laminin-derived peptides and evaluated their potential as biomedical materials for tissue engineering, which showed that when the RGD concentration between 1-100 μmol/well (peptide was added into 96 wells plate), the cell showed different phenomenon in different RGD concent. Shin *et al.*[10] also showed that the concentration of peptide has important role in the cytocompatibility of osteoblasts.

In present work, we designed the RGDS-grafted-PDLLA with different concentration of RGDS. How does the concen-

tration of RGDS affect the cytocompatiblity of osteoblast? The cytocompatibility of osteoblasts on RGDS-PDLLA with different concentration of RGDS were also examined.

EXPERIMENTAL

Synthesis and characterization of RGDS-PDLLA: MPLA (MAH grafted to -CH- of PDLLA by free radical reaction) and BDPLA (BDA initiate opening loop of MAH on MPLA) were synthesized and characterized. And then the RGDS-PDLLA was synthesized by introduction of RGDS to terminal -NH_2 of BDPLA[11,12].

MPLA was prepared according to the literature[12]. However, the key difference is to prepare gradient concentration MPLA. The content of MAH in MPLA was 1, 2 and 3 %, respectively. 10 g MPLA (containing 1.11, 2.03 and 3.10 % maleic anhydride in weight) was dissolved in 50 mL tetrahydrofuran (THF), while 0.133 g BDA(mol ratio to MAH is 1.2) was dissolved in 5 mL tetrahydrofuran (THF) and then dropped MPLA solution into BDA solution with stirring below 20 ºC. The reaction lasted for 10 min below 20 ºC and 0.5 h at room temperature. After reaction, the mixture was precipitated with excessive distilled water and yielded BDA modified MPLA (BDPLA). The resulting copolymer was freezed dried and characterized via FTIR and ^{13}C NMR. BDPLA was used for further modification. 0.1, 0.2, 0.3 mg (slow concentration for RGDS-PDLLA-11, RGDS-PDLLA-12, RGDS-PDLLA-13) or10 mg (high concentration for RGDS-PDLLA-1, RGDS-PDLLA-2, RGDS-PDLLA-3) RGDS were dissolved in 50 mL THF and then added 1 g BDPLA (containing 1.01, 1.95 and 3.02 % BDA in weight) into this peptide solution. The detail reaction is taken as described by Niu *et al.*[11] and Luo *et al.*[12].

Characterization of structure and cytocompatibility of RGDS-PDLLA: The confirmation of the gradient concentration of RGDS on RGDS-PDLLA is important for follow-up study. The peptide concentration was determined from amino acid analyzer (AAA). In this study, osteoblasts from newborn Wistar rat calvarias were used. The adhesive force, spreading area and skeletal images were used to characterized the cell adhesion and spread, when osteoblasts were seeded on different films at a density of 1×10^3 cells/cm^2. The MTT assay and ALP activity of osteoblasts, seeded on different films at a density of 2×10^4 cells/cm^2 [11,12]. To contrast the difference among films with gradient RGDS and confirm the effective concentration of RGDS after grafting BDPLA, which is favourable to neutralize the acidity materials during degradation of PDLLA and it has been proved that BDPLA possessed highly improved hydrophilicity.

RESULTS AND DISCUSSION

Concentration of RGDS on RGDS-PDLLA: Gradient concentration of RGDS was immobilized in the BDPLA and was detected by AAA, before which the whole samples must be acid hydrolyzed to produce the free amino acids.

The results of bulk peptide examination by AAA to determine coupling concentration and efficiency were shown in Table-1. These data demonstrated that RGDS was coupled to the BDPLA at a concentration of 0.142, 0.225 and 0.302 μmol/g at low concentration and 2.46, 5.08 and 7.36 μmol/g at high concentration. Considering that the dosage of RGDS was 1 wt % (on BDPLA weight basis) and its molar mass is 433 g/mol, the coupling efficiency was calculated to be 67, 54.5 and 50 % at low concentration and 10.65, 22.00 and 31.87 % at high concentration, respectively. From the results, we can find that the coupling efficiency of the reaction is high to 50-67 % at low concentration, while the coupling efficiency only is 10-30 % at high concentration. It is attributed to the competition mechanism among different RGDS molecule and the limited active reaction sits and BDPLAs. At the low RGDS addition, the active reaction in BDPLA is enough for all RGDS molecule, while at the high RGDS addition, show the competitive response among RGDS.

Result of inverted microscope: From literature[11], it can be find that, comparing with the cells on the PDLLA, MPLA, BDPLA, the cells on the RGDS-PDLLA have nice cytocompatibility (containing nice proliferation, differentiation). Then, how the RGDS affects the cytocompatibility of osteoblasts on RGDS-PDLLA?

Cell adhesion to substrate materials has two stages: non-specific adhesion and specific adhesion. In order to reflect two kinds of adhesive characteristics, the adhesive force and spreading area of osteoblasts were studied after culturing for 0.5 and 4.0 h, respectively and the results were shown in Figs. 1 and 2. In the experiments, there are 6 kinds of BPLA (stand for RGDS-PDLLA), cells adhesive force and spreading area on each BPLA was obviously enhanced compared to the PDLLA, BDPLA ($p < 0.01$). Introduction of adhesion peptide

TABLE-1
CONTENT OF RGDS IN RGDS-PDLLA

Amino acid	Contents (mg/g)			Molar mass (g/mol)	Mole number (μmol/g)		
	1 %	2 %	3 %		1 %	2 %	3 %
Arg	0.411	0.817	1.203	174	2.36	4.70	6.91
Gly	0.201	0.399	0.583	75	2.68	5.32	7.77
Asp	0.323	0.678	0.965	133	2.42	5.10	7.26
Ser	0.248	0.547	0.785	105	2.36	5.21	7.48
Mean value	–	–	–	–	2.46	5.08	7.36
Amino acid	Contents (mg/g)			Molar mass (g/mol)	Mole number (μmol/g)		
	1 %				1 %		
Arg	0.020	0.038	0.057	174	0.115	0.218	0.328
Gly	0.011	0.017	0.020	75	0.147	0.227	0.267
Asp	0.019	0.029	0.041	133	0.143	0.218	0.308
Ser	0.017	0.025	0.032	105	0.162	0.238	0.305
Mean value	–	–	–	–	0.142	0.225	0.302

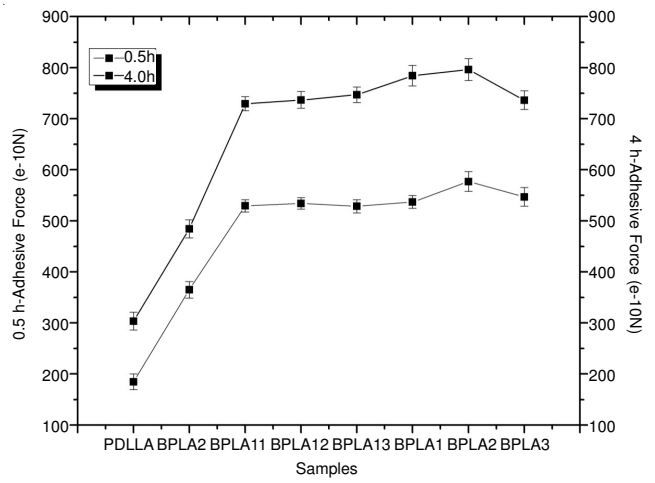


Fig. 1. Adhesive force of osteoblasts cultured on different substrate membrane (n = 25, $p < 0.01$)

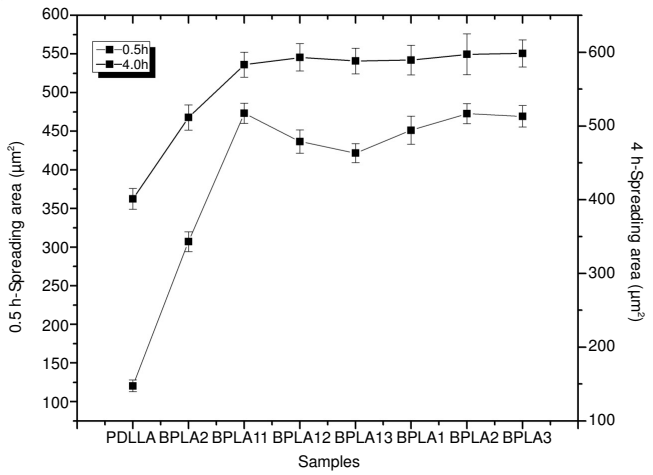
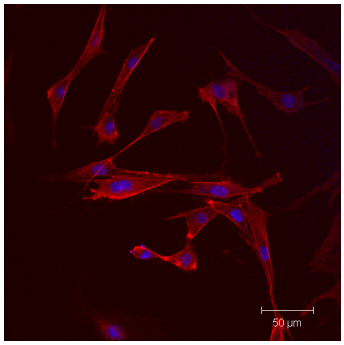


Fig. 2. Spreading area (b) of osteoblasts cultured on different substrate membrane (n = 25, $p < 0.01$)

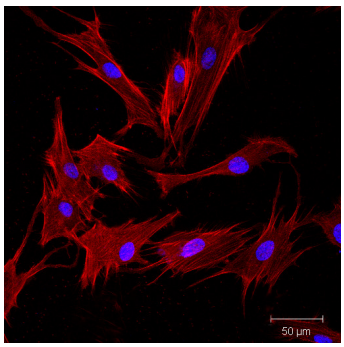
RGDS could increase the adhesion of osteoblasts on the PDLLA films, which was accordant in the two kinds of adhesive characteristics[11].

Cell adhesion is the first phase of cell/substrate interactions, which will have an important role in the cell's capacity to proliferate and to differentiate it on substrate films. Numerous polymers have been functionalized with RGD peptide for tissue engineering application, since RGD has been found to promote cell adhesion[13]. Massia and Hubbell[14] showed that a surface density of as low as 1 fmol/cm^2 of RGD was sufficient for cell adhesion to an otherwise no adherent surface and as low as 10 fmol/cm^2 was sufficient for formation of focal contacts and stress fibers.

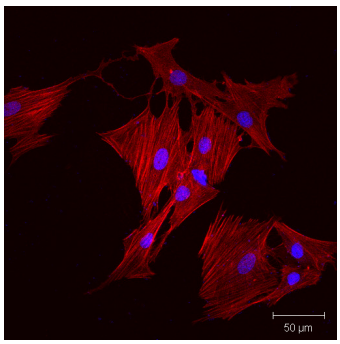
Moreover, to confirm the cell surface receptor-spectic of RGDS on the PDLLA, one can find that, when the molar number is 0.142 μmol/g, meaning 14.2 fmol/cm^2 (based on the results of AAA and assuming a density of 1 g/cm^3 and 1 nm access layer) at low RGDS addition, the adhesive force and spreading area was sufficient for cell adhesion to the modified PDLLA. And then, the molar number increased to 0.302 μmol/g, even to 7.36 μmol/g at the high RGDS addition, however, the cell adhesion did not show the obvious change. Fig. 3 shows the skeletal images of osteoblasts on different materials. The RGDS-PDLLA11 and RGDS-PDLLA1 showed the more spreading area than PDLLA. In addition, the spreading area of osteoblasts on RGDS-PDLLA1 is higher than osteoblasts on RGDS-PDLLA11. It is important that the osteoblasts on RGDS-PDLLA11 and RGDS-PDLLA1 showed the more fibers, especially, the osteoblasts on the RGDS-PDLLA1, which is coincident to the literature[14].



(a) PDLLA



(b) RGDS-PDLLA11



(c) RGDS-PDLLA1

Fig. 3. Skeletal images of osteoblasts on different materials

Result of proliferation and differentiation measurement: Osteoblasts viability and proliferation were affected by adhesion of cells on substrate. From the results of MTT (Fig. 4), we can find that the number of osteoblasts increased with increasing of RGDS concentration at the low concentration after cultivating 2days, in addition, the number of osteoblasts increased tinily with increasing of RGDS concentration. However, we can find that when the RGDS concentration increased from 0.302-2.46 μmol/g, the number of osteoblasts increased 50 %. Compared with the control PDLLA,

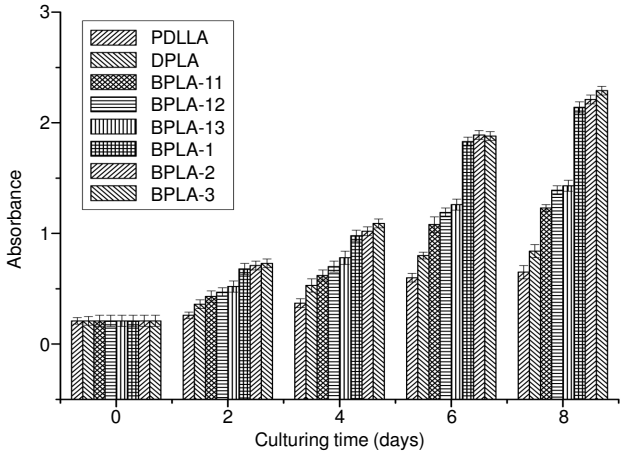


Fig. 4. MTT assay of osteoblasts cultured on different substrate films

BDPLA films, cell viability of osteoblasts cultured on RGDS-PDLLA was significantly higher ($p < 0.01$), which can be attributed to the introduction of RGDS for enhancing the adhesion and spread. However, to explain the remarkable increase, the RGDS concentration increased from 0.302 to 2.46 μmol/g. Whether or not the influence of RGDS concentration on the differentiated function is similar to the MTT results.

Fig. 5 indicated the total ALP activity of osteoblasts cultured on different polymer surface after culturing 4, 7, 10 days, which can assess the differentiated function of rat osteoblasts. In general, the ALP activity increased from 4-10 days of culture on all substrates. Moreover, we can find that, the ALP activity of osteoblasts seeded on RGDS-PDLLA-1, 2, 3 is distinctly higher than that of osteoblasts seeded on RGDS-PDLLA-11, 12, 13.

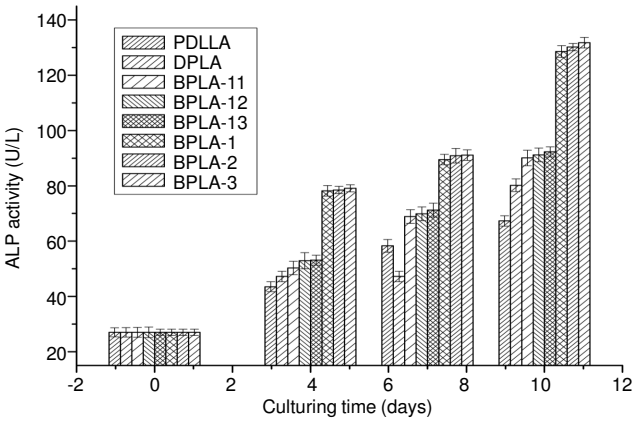


Fig. 5. ALP activity of osteoblasts cultured on different substrate films

From the results of adhesion, spread, proliferation and differentiation research, it is concluded that introduction of RGDS on PDLLA can obviously promote cell attachment and viability. The available RGDS concentration of accommodating the adhesive force and spreading area of osteoblasts on the RGDS-PDLLAs is 10 fmol/cm^2 (0.1 μmol/g). When the RGDS concentration is high to 246 fmol/cm^2 (2.46 μmol/g), it is ineffective for increasing of adhesive force and spreading area, however, it is necessary for increasing of the osteoblasts number on RGDS-PDLLA, which can be attributed to the specific adhesion. In addition, the results is can be explained by the literature[15], which showed that the cell has different dependent on varied concentration of bioactive factor at different phase.

Conclusion

In present work, RGDS-PDLLAs with different concentration RGDS were successfully introduced into PDLLA to prepare a bioactive material determining the influence of RGDS concentration on cytocompatibility of osteoblasts. The modified substrates were verified qualitatively and quantitatively with AAA and classical chemical reaction. The osteoblasts behavior on different RGDS-PDLLA revealed that the introduction of RGDS has positive effect on the attachment and viability of osteoblasts at low RGDS concentration, whereas has effect on more apparent effect on the functional behavior of osteoblasts at high RGDS concentration. The involvement of adhesion peptide can further improve its cytocompatibility.

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