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Screening of functionalized self-assembling peptide nanofiber scaffolds with angiogenic activity for endothelial cell growth

Xiu-mei WANG[1], Lin QIAO[1], Akihiro HORII[2]

1. Key Laboratory of Advanced Materials, Department of Materials Science and Engineering, Tsinghua University, Beijing 100084, China;
2. Olympus America Inc., 3500 Corporate Parkway, Center Valley, PA 18034, USA

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Abstract: Promotion of angiogenesis in tissue engineering is of vital significance to the survival of transplants, which leads the way of tissue regeneration. In this study, we screened six short peptides with potentially angiogenic activities to functionalize self-assembling peptide nanofiber scaffolds RADA16-I (Ac-$(RADA)_4$-$CONH_2$). Fluorescence microscopy images of endothelial cells morphology on peptide scaffolds exhibited good cell attachments and survivals on these six functionalized peptide scaffolds, especially on peptide scaffolds RAD/KLT and RAD/PRG. Cell proliferation examination also confirmed the excellent cell growth on these two functionalized peptide scaffolds. Functional peptide motifs KLT (KLTWQELYQLKYKGI) and PRG (PRGDSGYRGDS) had positive effects on endothelial cell survival and growth, which implied that these two peptides might have great promise for promoting angiogenesis *in vitro* and *in vivo*.

Key words: self-assembling peptide; angiogenesis; tissue engineering; endothelial cell

1 Introduction

The self-assembling peptide scaffolds have been demonstrated as unique biologically inspired materials for various applications including tissue engineering and regenerative medicine, 3-D tissue cell culture, drug release and biomineralization [1−3]. Among them, RADA16-I (Ac-$(RADA)_4$-$CONH_2$) has been widely used for tissue engineering as an ideal 3-D cell culture system and analog of extracellular matrix (ECM) with excellent biological compatibility, degradation, and bioactivity. According to different functional requirements, specific bioactive short peptide motifs can be easily incorporated with pure RADA16-I so as to endow the functionalized peptide scaffolds "biological intelligence".

Angiogenesis is of vital significance in tissue engineering and regenerative medicine. An adequate blood vessel supply to the newly formed tissue and within the transplanted scaffold is essential in determining the success of new tissue regeneration. Long-term survival and function of constructed tissue substitutes require new blood vessels to provide enough nutrients and oxygen to the cells within the transplants and new tissues [4−7]. Therefore, promotion of angiogenesis in tissue engineered organs determines its clinical applications and has been one of the major topics of tissue regeneration.

To achieve the goal of tissue reconstruction, an ideal tissue engineering scaffold should meet many specific requirements, such as biocompatibility, non-immunogenicity, biodegradation, and 3D cell culture, especially promoting vascular invasion [8−10]. In consideration of the importance of vascularization of tissue engineered scaffolds, new class of angiogenic self-assembling peptide nanofiber scaffolds was designed and synthesized.

In this work, we screened six short peptides with potentially angiogenic activities and good water-solubility, which were used to functionalize self-assembling peptide nanofiber scaffolds RADA16-I (Ac-$(RADA)_4$-$CONH_2$) through directly coupling pure RADA16-I with short biologically angiogenic peptide motifs. The process of their synthesis, self-assembly and gelation were under research. In particular, their ability to support endothelial cell growth was also evaluated for screening ideal functional motifs.

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Corresponding author: Xiu-mei WANG; Tel: +86-10-62772977; Fax: +86-10-62781160; E-mail: wxm@tsinghua.edu.cn

2 Materials and methods

2.1 Peptide solution preparation and gel formation

2.1.1 Peptide solution preparation

All the lyophilized designer peptides used in this work were custom-synthesized by CPC Scientific (Purity >85%, San Jose, CA). These peptides were dissolved in MilliQ water at a final concentration of 1% (w/v, 10 mg/mL) and then sonicated for 30 min (Aquasonic, model 50T, VWR, NJ). After sonication, they were filter-sterilized (Acrodisc Syringe Filter, 0.2 mm HT Tuffrun membrane, Pall Corp., Ann Arbor, MI) for succeeding uses. The functionalized peptide solutions were mixed in a volume ratio of 1:1 with 1% pure RADA16-I solution to get 1% functionalized peptide mixtures.

2.1.2 Gel formation for two-dimensional (2-D) cell culture

Desired numbers of sterilized culture plate inserts (10 mm in diameter, 0.4 mm Millicell-CM, Millipore, MA) were placed in a 24-well culture plate with 400 μL culture medium in each well. 100 μL of peptide solution (functionalized peptide mixtures or pure RADA16-I) was loaded directly into each of the inserts and then incubated for at least 1 h at 37 °C for gelation. 400 μL of culture medium was very carefully added onto the gel and then incubated on the plate overnight at 37 °C. Once the gel was formed, the medium was carefully removed and changed twice more to equilibrate the gel to physiological pH prior to plating the cells. A certain number of cells in 400 μL of medium were seeded on the top of the gel and then the insert was moved to a new 12-well culture plate with 800 μL of medium in each well for 2-D cell culture.

2.2 Cell culture of human umbilical vein endothelial cells (HUVECs)

Primarily isolated HUVECs were commercially obtained from Lonza Inc. (Walkersville, MD) and routinely grown in endothelial growth media EGM-2 (Lonza Inc., Walkersville, MD) on regular tissue-culture plates. All the experiments were conducted with cells between passage 5 and passage 8. Sub-confluent (~6×10^4 cells per insert) of HUVECs was seeded on the top of the scaffolds for 2-D cell culture.

2.3 Circular dichroism (CD)

Peptide samples were prepared by diluting 1% peptides in water to a working concentration of 25 mmol/L. Samples were analyzed at room temperature in a quartz curette with a path length of 0.5 cm and in a wavelength range of 195−250 nm, and the CD spectra were collected.

2.4 AFM examination

1 μL of 1% peptide solution was diluted with 19 μL of Milli-Q water. 1 μL of dilution sample was dropped onto a freshly cleaved mica surface for 5 s and then rinsed with 100 μL of Milli-Q water. The peptide sample on the mica surface was then air-dried. The images were obtained by AFM (Nanoscope IIIa, Digital Instruments, Santa Barbara, CA) operating in tapping mode. The functionalized peptides were examined by AFM before and after mixing with RADA16-I, respectively.

2.5 Fluorescence microscopy

Cells on the peptide hydrogels were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Fluorescent Rhodaminphalloidin and SYTOX Green (MolecularProbes, Eugene, OR) were used for labeling F-actin and nuclei, respectively. Images were taken using a fluorescence microscope (Axiovert 25, ZEISS) or laser confocal scanning microscope (Olympus FV300).

2.6 Cell proliferation assay

The number of cells on the scaffolds was determined by the fluorometric quantification of amount of cellular DNA. The scaffolds with cells were collected for DNA purification (QIAamp DNA Mini Kit, QIAGEN). 100 mL of purified DNA sample was mixed with 100 mL DNA binding fluorescent dye solution (0.5 mL Picogreen reagent in 100 mL TE buffer, Quant-iT PicoGreen dsDNA Reagent and kits, Invitrogen). The fluorescent intensity of the mixed solution was measured with a fluorescence spectrometer (Wallace Victor2, 1420 Multi-label counter, excitation at 485 nm and emission at 510 nm, Perkin-Elmer, MA). All the data were statistically analyzed to express in the standard deviation of the mean. The *t*-test was performed and $p<0.01$ was commonly accepted to be statistically significant.

3 Results and discussion

In order to design and screen functional peptide motifs with angiogenic activities, we mainly considered from the important angiogenic factors during the physiological process of angiogenesis. It has been reported that angiogenesis is dependent strongly on endothelial cells (ECs) survival and the suppression of ECs apoptosis, which depend on the growth factors, such as VEGF, bFGF, Angiopoietin-1, cell attachment to ECM, and cell-cell interactions[11−14]. And the related matrix proteins mainly contain intergrin, vitronectin, fibronection and so on. In this study, we searched several biological short peptide motifs from ECM proteins and

relative growth factors according to currently scientific publications[15−19]. The preliminarily selected peptides sequences and descriptions are listed in Table 1.

Table 1 Functional peptides used in this work

No.	Code	Sequence	Description
1	SVV	SVVYGLR	Osteopontin/equivalent to VEGF in migration effect
2	LKK	LKKTETQ	Actin binding site on thymosin $\beta 4$
3	VGV	VGVAPG	Elastin/endothelial cell migration and tubulogenesis
4	RED	REDV	Fibronectin/endothelial cells adhesion
5	KLT	KLTWQELY-QLKYKGI	VEGF mimicking peptide/bind to VEGF recepters
6	PRG	PRGDSGYRGDS	Repetitive RGD

These functional peptide motifs were used to functionalize self-assembling peptide nanofiber scaffolds RADA16-I through directly coupling with pure RADA16-I with several Glycine residues as a space linker for keeping the flexibility of functional peptides. Although self-assembling peptide RADA16-I scaffold has excellent biocompatibility and has been widely used in different kinds of tissue engineering fields, it has no tissue specificity and lack of unique interactions with specific tissue cells[15]. It has been demonstrated that the functional peptide motifs could significantly improve the biological function of the peptide materials. We therefore extended the self-assembling peptide RADA16-I through solid phase synthesis at the C-terminal with these six functional peptide motifs to increase the potentially angiogenic activities of the peptide scaffolds. The functionalized self-assembling peptides were mixed with pure RADA16-I in a volume ratio of 1:1 to facilitate their self-assembly and gelation. The CD spectra of peptide mixture solutions (as shown in Fig.1) showed typical beta-sheet structures, which is very important and necessary for peptides self-assembly and nanofiber formation. Pure functionalized peptide didn't show typical beta-sheet structure mainly because of the interference and dilution of functional motif. While the functionalized self-assembling peptide could interact with RADA16-I and underwent spontaneous self-assembly through the self-assembling motif $(RADA)_4$ forming long nanofibers with protruded functional motifs. A schematic illustration is shown in Fig.2.

The self-assemblies of functionalized peptides before and after mixing with RADA16-I were examined

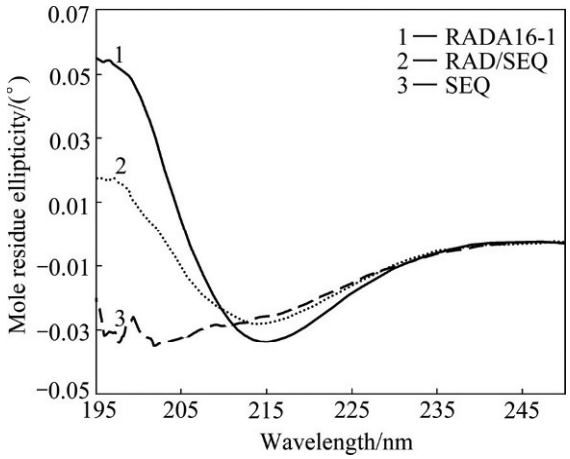

Fig.1 Typical CD spectra of self-assembling peptides: 1—RADA16-I peptide with high beta-sheet content; 2—Peptide mixtures of RADA16-I with functionalized peptides showing considerably less beta-sheet contents; 3—Pure functionalized peptide without typical beta-sheet CD spectrum

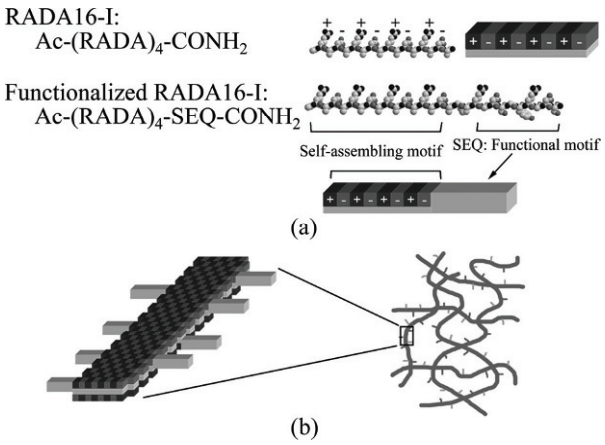

Fig.2 Molecular models of designer peptides and schematic illustrations of self-assembling peptide nanofiber scaffolds: (a) Molecular modes of designer peptides RADA16-I and functionalized peptide; (b) Schematic illustrations of self-assembling peptide nanofibers formation after mixing RADA16-I with functionalized peptide

by AFM, as shown in Fig.3. The AFM images confirmed the self-assembling long nanofiber formation of the peptide mixtures (Figs.3(a2−f2)), which demonstrated that the addition of functional peptide motif sequences has no distinct impediment on the self-assembly of the functionalized peptides. We also noted that the functionalized self-assembling peptide itself only formed short fibers or conglomeration structures (Figs.3(a1−f1)), which implied that the additional functional amino acid sequences impacted the self-assembly probably because of steric hindrance. However, once the functionalized peptide mixed with self-assembling peptide RADA16-I, it could well interact with RADA16-I to form typical uniform nanofiber assembling structure.

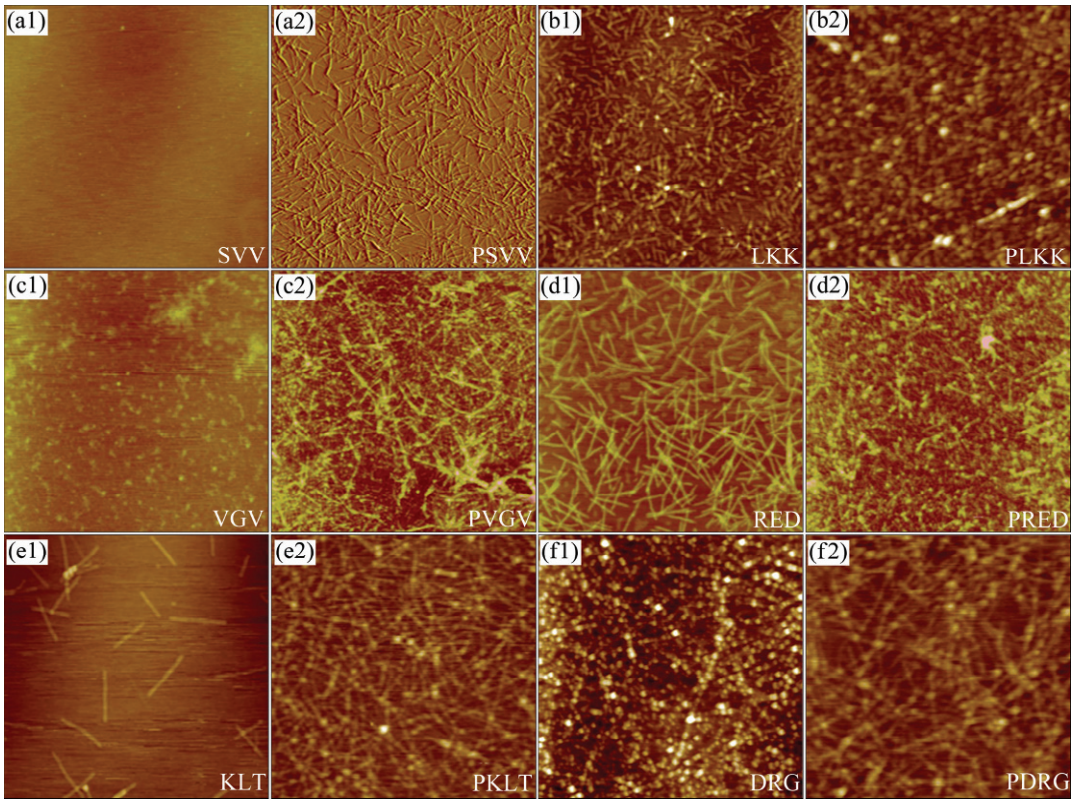

Fig.3 AFM morphologies of self-assembling peptides: (a1)−(f1) Functionalized self-assembling peptide; (a2)−(f2): Functionalized self-assembling peptide mixtures with RADA16-I

In order to screen fine functionalized peptide scaffolds for 2-D endothelial cells growth, ECs attachment, survival, and proliferation properties were examined in this work. Same numbers of HUVECs were seeded on the top of different peptide scaffolds for 2-D cell culture up to one week. Type I collagen and Martrigel were used as positive controls. Figure 4 shows the typical morphologies of HUVECs on different scaffolds after two-day cell culture. Compared with the unmodified self-assembling peptide RADA16-I, functionalized peptide KLT and PRG significantly increased the HUVECs attachment and survival. While endothelial cells growth on the scaffolds of SVV, LKK, VGV, RED was not improved significantly. This may be resulted from different motif presentations and self-assembling processes of these peptides. In this study, the functionalized self-assembling peptides were synthesized by direct extension from the C-terminal of the self-assembling peptide RADA16-I using solid phase synthesis with different functional peptide motifs. Therefore, the biological functions of the functionalized self-assembling peptide scaffolds depended on not only the bioactivities of functional motifs, but also the presentations, such as mobility, conformation and orientation. These four functional peptide motifs all contained a certain number of hydrophobic amino acid, such as Valine. Valine inhibited the water solubility of the functionalized peptides and also impacted the spatial configurations of these peptides, which had a negative effect on peptide self-assembly. These hydrophobic amino acids in the functional motifs probably extruded into the self-assembling nanofiber backbones. Therefore, there were no enough functional motifs exposed to the environments and interacted with cells. One-week cell cultures on these peptide scaffolds showed same morphologies with two-day cell cultures.

From the cell morphology observation, HUVECs on the PPRG scaffold exhibited good attachment and cell survivals and uniformly attached with the scaffold, which was quite similar with collagen scaffold. HUVECs on the PKLT scaffold also exhibited good attachment but not uniformly or isotropically distributed on the surface of scaffold. Endothelial cells on PKLT scaffold formed capillary-like structure. This result indicated that functionalized peptide KLT had the ability to induce the morphology differentiation of endothelial cells. Detailed investigations on the angiogenesis properties *in vivo* and *in vitro* induced by PKLT and PPRG peptide scaffolds will be introduced in other papers.

We also estimated cell proliferation on these peptide scaffolds by examining DNA contents extracted from the scaffolds after three-day cell culture (Fig.5). The cell

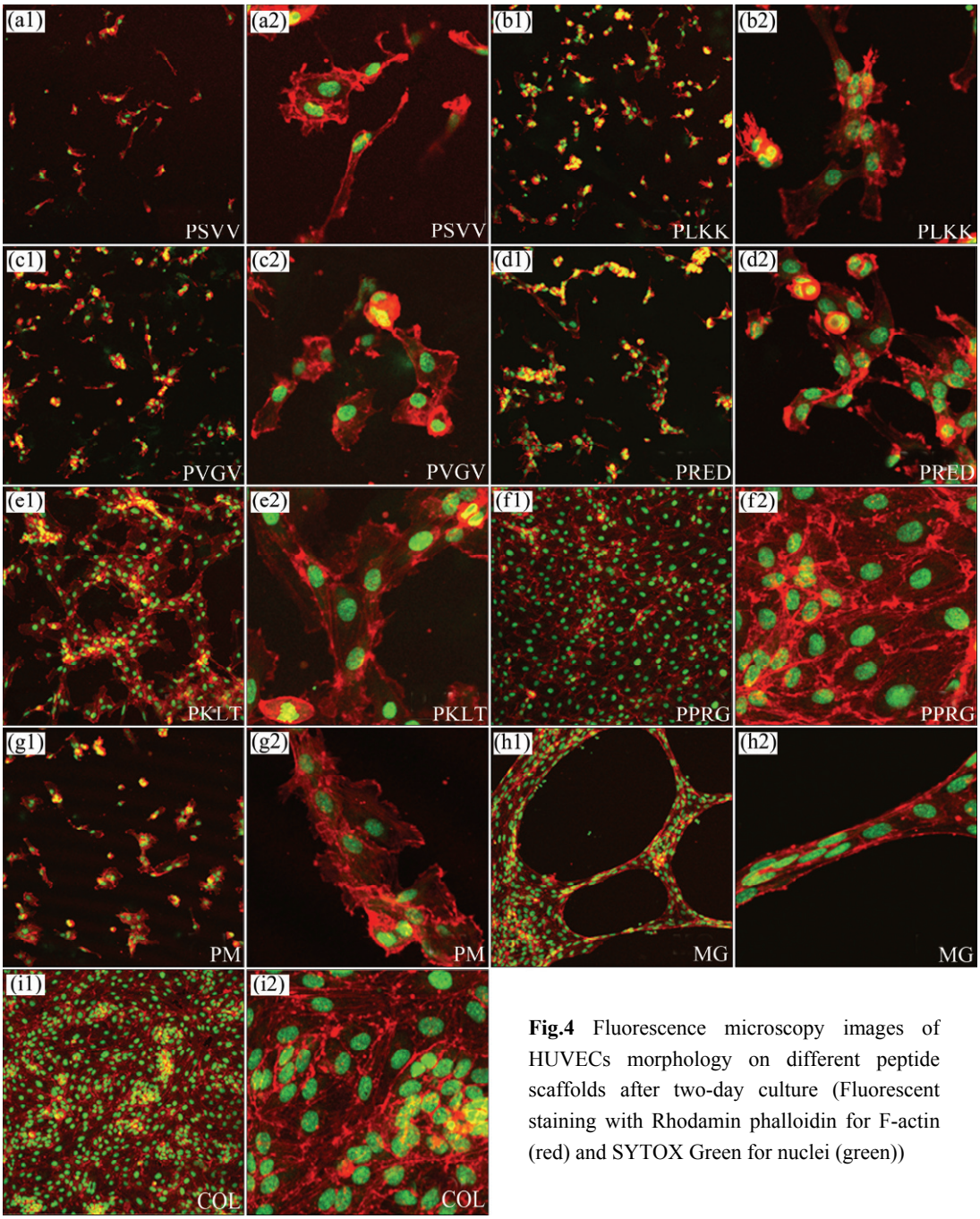

Fig.4 Fluorescence microscopy images of HUVECs morphology on different peptide scaffolds after two-day culture (Fluorescent staining with Rhodamin phalloidin for F-actin (red) and SYTOX Green for nuclei (green))

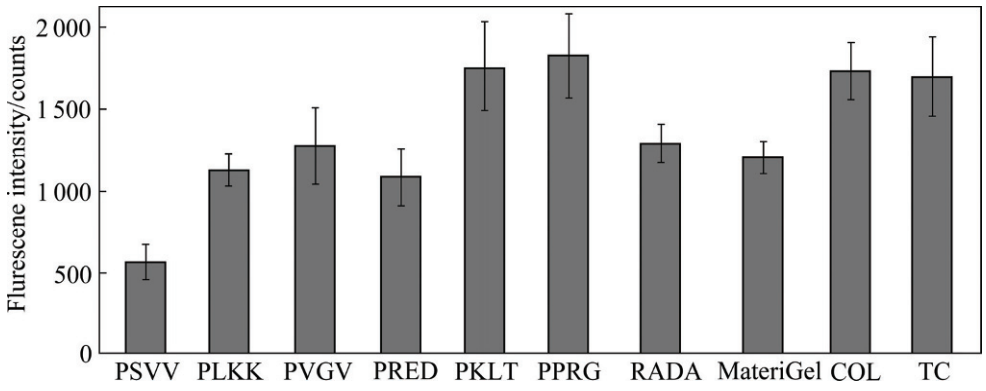

Fig.5 HUVEC numbers on different functionalized peptide nanofiber scaffolds after three-day cell culture with collagen, Matrigel and tissue culture plate (TC) as control

number on scaffold PKLT and PPRG were significantly more than other scaffolds including collagen-I. The result was consistent with the cell attachment examinations from fluorescence microscopy observations.

The functionalized peptide nanofiber scaffolds have unique advantages over many other synthetic biomaterials. The terminal of self-assembling peptide RADA16-I can be modified with different functional peptide motifs to meet various requirements of tissue engineering and regenerative medicine[20]. We could design and screen ideal tissue specificity peptide motifs to increase the tissue specificity and biological activities of the peptide hydrogels fulfilling different clinical application requirements in neural regeneration, cardiovascular tissue repair, and so on so forth.

4 Conclusions

In this study, we screened six short peptides with potentially angiogenic activities to functionalize self-assembling peptide nanofiber scaffolds RADA16-I (Ac-$(RADA)_4$-$CONH_2$). The CD spectra of peptide solutions showed typical beta-sheet structures, which is very important and necessary for peptides self-assembly and nanofiber formation. AFM examinations confirmed the self-assembling nanofiber formation of the peptide mixtures, which demonstrated that the addition of functional peptide motif sequences has no distinct impediment on the self-assembly of the functionalized peptides. Fluorescence microscopy images of endothelial cells morphology on peptide scaffolds exhibited good cell attachment and high viability especially on functionalized peptide scaffolds PKLT and PPRG. Cell proliferation examination also confirmed the excellent cell growth on these two functionalized peptide scaffolds. Functional peptide motifs KLT (KLTWQELYQLKYKGI) and PRG (PRGDSGYRGDS) had positive effects on endothelial cell survival and growth, which implied that these two peptides might have great promise for promoting angiogenesis *in vitro* and *in vivo*.

References

[1] ZHANG S G. Fabrication of novel biomaterials through molecular self-assembly [J]. Nature Biotechnology, 2003, 21: 1171−1178.

[2] HARTGERINK J D, BENIASH E, STUPP S I. Self-assembly and mineralization of peptide-amphiphile nanofibers [J]. Science, 2001, 294: 1684−1688.

[3] MICKLITSCH C M, PAJAGOPAL K, YUCEL T, et al. Hydrophobicity affects beta-hairpin hydrogelation [J]. Biopolymers, 2005, 80: 515.

[4] FEDER J, MARASA J C, OLANDER J V. The fromation of capillary-like tubes by calf aoric endothelial cells grown in vitro [J]. Journal of Cellular Physiology, 1983, 116: 1−6.

[5] VERNON R B, LARA S L, DRAKE C J, et al. Organized type I collagen influences endothelial patterns during “spontaneous angiogenesis in vitro”: planar cultures as models of vascular development [J]. In Vitro Cell Dev Biol, 1995, 31: 120−131.

[6] CHALUPOWICZ D G, CHOWDHURY Z A, BACH T L, et al. Fibrin II induces endothelial cell capillary tube formation [J]. The Journal of Cell Biology, 1995, 130(1): 207−215.

[7] BROWN K J, MAYNES S F, BEZOS A, et al. A novel in vitro assay for human angiogenesis [J]. Laboratory Investigation, 1996, 75(4): 539−555.

[8] GOLDBERG M, LANGER R, JIA X Q. Nanostructured materials for applications in drug delivery and tissue engineering [J]. Journal of Biomaterials Science-Polymer Edition, 2007, 18(3): 241−268.

[9] BETTINGER C J, BRUGGEMAN P P, MISRA A, et al. Biocompatibility of biodegradable semiconducting melanin film for nerve tissue engineering [J]. Biomaterials, 2009, 30(17): 3050−3057.

[10] PATEL Z S, MIKOS A G. Angiogenesis with biomaterial-based drug- and cell-delivery systems [J]. Journal of Biomaterials Science-Polymer Edition, 2004, 15(6): 701−726.

[11] D’ANDREA L D, GATTO A D, PEDONE C, et al. Peptide-based molecules in angiogenesis [J]. Chem Biol Drug Des, 2006, 67: 115−126.

[12] KOUVROUKOGLOU S, DEE K C, BIZIOS R, et al. Endothelial cell migration on surfaces modified with immobilized adhesive peptides [J]. Biomaterials, 2000, 21: 1725−1733.

[13] AGUZZI M S, GIAMPIETRI C, DE MARCHIS F, et al. RGDS peptide induces caspase 8 and caspase 9 activation in human endothelial cells [J]. Blood, 2004, 103: 4180−4187.

[14] SUN G M, SHEN Y I, KUSUMA S, et al. Functional neovascularization of biodegradable dextran hydrogels with multiple angiogenic growth factors [J]. Biomaterials, 2011, 32(1): 95−106.

[15] HORII A, WANG X M, FABRIZIO G, et al. Biological designer self-assembling peptide nanofiber scaffolds significantly enhance osteoblast proliferation, differentiation and 3-D migration [J]. PLoS ONE, 2007, 2(2): e190−198.

[16] GENOVE E, SHEN C, ZHANG S G, et al. The effect of functionalized self-assembling peptide scaffolds on human aortic endothelial cell function [J]. Biomaterials, 2005, 26: 3341−3351.

[17] DANDREA L D, LACCARINO G, FATTORUSSO R, et al. Targeting angiogenesis: Structural characterization and biological properties of a de novo engineered VEGF mimicking peptide [J]. Proc Natl Acad Sci. USA, 2005, 102: 14215−14220.

[18] NOWAKOWSKI G S, DOONER M S, VALINSKI H M, et al. A specific heptapeptide from a phage display peptide library homes to bone marrow and binds to primitive hematopoietic stem cells [J]. Stem Cells, 2004, 22: 1030−1038.

[19] PALOU E, PIROTTO F, SOLE J, et al. Genomic characterization of CD84 reveals the existence of five isoforms differing in their cytoplasmic domains [J]. Tissue Antigens, 2000, 55: 118−127.

[20] YOKOI H, KINOSHITA T, ZHANG S G. Dynamic reassembly of peptide RADA16 nanofiber scaffold [J]. Proc Natl Acad Sci. USA, 2005, 102: 8414−8419.