Functionalized Self-Assembling Peptides for Medical Applications

Tzu-Yun Cheng[1], Hsi-Chin Wu[2], Wen-Han Chang[3], Ming-Yuan Huang[3], Chao-Hsiung Lee[3], Tzu-Wei Wang[1]*

[1] *Department of Materials Science and Engineering, National Tsing Hua University, Hsinchu, Taiwan*

[2]*Department of Materials Engineering, Tatung University, Taipei, Taiwan*

[3]*Department of Emergency, Mackay Memorial Hospital, Taipei, Taiwan*

*Corresponding email: twwang@mx.nthu.edu.tw

ABSTRACT

In this study, functionalized self-assembling peptides (SAPs) were designed and developed for the applications of liver bleeding hemostasis and skin wound healing. The physiochemical properties of SAPs were investigated including nanofiber morphology, hydrogel rheology behavior, titration curve and protein secondary structure. The functionalized SAPs have been proven to effectively promote hemostasis efficacy of liver bleeding and skin tissue regeneration process.

Keywords: *Nanomaterial, Peptide, Functionalized, Hydrogel, Hemostasis, Tissue Regeneration*

1 INTRODUCTION

Self-assembling peptides (SAPs) offer several advantages including: 1) high biocompatibility and bio-absorbability, 2) free from animal-derived materials and pathogens, 3) the desired functional extended motifs are easy to incorporate, 4) 3D matrix formation which mimic natural extracellular matrix to support cells proliferation, differentiation and migration, 5) easily conformable to the various shapes of lesion cavities due to liquid-like property before administration to the lesion site. In recent years, several self-assembling peptides have been developed. Among them, $RADA_{16}$ (AcN-RADARADARADARADA-CONH2) is the widely used one. The side-chains of $RADA_{16}$ are one polar arginine (R) or aspartic acid (D) followed by one non-polar alanine (A), which results in forming an amphiphilic oligopeptide and anti-parallel β-sheet structure with one hydrophilic side and the other hydrophobic side. The amphiphilic β-sheet petides form nanofibers in an aqueous solution by hydrogen bonds among the hydroxyl groups and Van Der Waals attraction. Furthermore, the additional functional motifs can be extended from $RADA_{16}$. They can be combined in various ratio and form a SAP with multi-extended motifs carrying various biomedical functions.

In this study, functionalized self-assembling peptides (SAPs) were designed and developed for liver hemostasis and skin wound healing. we specifically linked GRGDS and YIGSR motifs to enrich $RADA_{16}$. GRGDS, located at the tenth type III repeating domain of fibronectin, is an important peptide ligand of fibronectin receptor. The functionalized peptides also incorporated with growth factors of EGF and bFGF for the evaluation of promoting skin burn wound healing process. Our results showed that SAPs were capable of developing into 3D nanofiber matrix which mimics natural extracellular matrix network structure. With the treatment of culture medium or directly injection into wound area, the SAP solution immediately transforms into a 3D hydrogel by the property of sol-gel transition in physiological condition. This emerging biological materials through molecular self-assembly mechanism may serve as a promising controlled release 3D culture system or scaffold to be used in tissue engineering for soft tissue repair and regeneration.

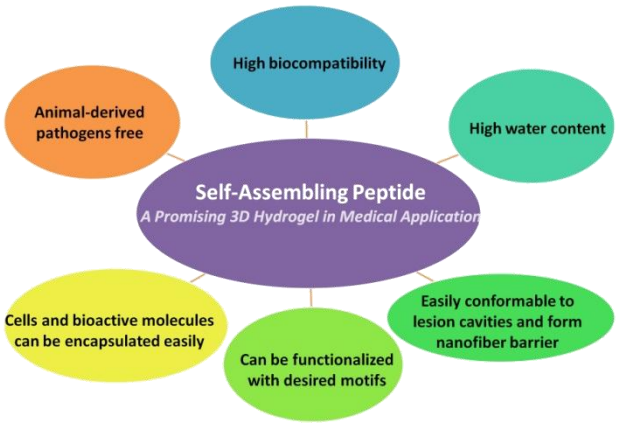

Fig. 1. Advantages of SAPs which offer numerous advantages and may be a promising alternative for 3D scaffold in tissue engineering.

2 EXPERIMENT

Preparation of SAP solution

$RADA_{16}$ (AcN-RADARADARADARADA-$CONH_2$), $RADA_{16}$-GRGDS (AcN-RADARADARADARADAGRGDS-$CONH_2$), $RADA_{16}$-YIGSR (AcN-RADARADARADARADAYIGSR-$CONH_2$), with purity >85%, were purchased from Biopharmaceutical firms (Chinese Peptide Company, Hangzhou, China) and used without further purification. Purity of the peptides was confirmed by analytical high performance liquid chromatography. Identities of the peptides were confirmed

by mass spectrometry analysis. All aqueous peptide solutions were prepared by using Milli-Q water (18.2 MΩ), stored at 4 °C and sonicated for 30 min before use.

Measurement of apparent pK1, pK2, and isoelectric point (pI) of SAPs

The pH of the peptide solutions was measured by a glass microelectrode (InLab Micro, Mettler Toledo Inc, Switzerland), which was connected to the pH meter (SP-701, Suntex, Taiwan). Before use, the pH meter was calibrated by a 2-point calibration (pH7 and pH4, or pH7 and pH10). 100 µl of the 1.0% (w/v) peptide solution was applied for measurement at room temperature and 2µl of NaOH (0.1N) was added each time with a pipette, followed by vortex for about 5 secs. The apparent *pK1*, *pK2*, and *pI* of each peptide were determined by the pH titration curve.

CD for secondary structure

The secondary structure of peptides was studied using circular dichroism (CD) measurements of 0.01% (w/v) peptide solutions. The data were collected on an Aviv model 202 spectrophotometer (Aviv Instrument Inc., US) with a 1mm path length at room temperature.

AFM for morphology

The morphology of peptide scaffolds was analyzed using atomic force microscopy (AFM). In brief, 10 µl of 1.0% (w/v) peptide solution was placed on the surface of clean cut silicon wafer. Each sample was set on the mica for 30min, washed with 100 µl water three times and then dried by blowing anhydrous nitrogen. AFM images were collected with a Scanning Probe Microscope (DI Dimension 3100, Veeco Instruments Inc, US) using the dynamic force mode in ambient air. Mickromasch NSC15 micro-cantilevers were chosen with tip radius of 10 nm, spring constant of 40 N/m, and frequency of 325 kHz.

Rheology for gelation behavior *analysis*

RheoStress 600 (Thermo Scientific Instrument Inc., US) equipped with a cone and plate geometry system (cone with diameter: 35mm, angle: 2°) was used in rheology properties analysis. 200 µl of 1% (w/v) peptide solution was loaded on the plate for measurement. For the gelation behavior study, additional 100 µl of medium or normal saline (0.9% NaCl) was slowly added around the peptide sample to trigger the peptide solutions assembling into hydrogel. During all measurements, frequency sweeps ranging from 100 rad/s to 0.1 rad/s were performed at a gap height of 1.05 mm and a constant shear stress of 1 Pa at room temperature.

Hemostasis Efficacy of SAPs

The Sprague-Dawley rats were obtained from the Center for Laboratory Animals of the Mackay Memorial Hospital. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Department of Medical Research in Mackay Memorial Hospital. The following surgical procedure for liver injury was performed. Briefly, rats (380–450 g) were anesthetized with Zoletil 50 (55mg/kg body weight) and placed on supine position. Then the abdomens were opened and the livers were exposed. On the liver lobes, 2.2mm size diameter punctures were created with glass micropipette as experimental design. For the three test groups, the wound was immediately treated with hemostatic agent (1% peptide solution of $RADA_{16}$, $RADA_{16}$-GRGDS, or $RADA_{16}$ -YIGSR). For the control groups, the thermal cautery (positive control) and without hemostatic agent treatment (only with normal saline, negative control) were conducted. The time to hemostasis of each treatment (N=15) were evaluated and recorded by the judgment of stopping oozing in the wound. All animals received gentamicin sulfate (3mg/kg body weight) and the abdominal incision was closed in the end of the surgery. After various predetermined time span (1day, 3days, 1week, 2weeks, 3weeks), the rats (n=3) were sacrificed to harvest the liver tissue for histological analysis.

Histological and immunohistochemical analysis

The harvested liver tissues were fixed in 4% paraformaldehyde, dehydrated in graded alcohols, and then embedded in paraffin. Sections of 4-6 µm in thickness were sliced, and then stained with hematoxylin-eosin (H/E) and immunohistochemical analysis. For immunohistochemical analysis, primary antibodies including mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (Millipore), mouse monoclonal directed against CD68 (Millipore) and rabbit polyclonal anti-liver mitochondria carbamoyl phosphate synthetase 1 (CPS1) (Abcam) were applied. Secondary detection system was performed by IHC Select® HRP/DAB kit (Millipore), and biotinylated goat anti-mouse IgG and goat anti-rabbit IgG were used. Analysis was performed under an optical microscope (Axiostar scope A1, Carl Zeiss Inc., Germany) with a CCD system.

3 RESULTS

Table 1. The summary of pK1, pK2, and isoelectric point *(pI)* of different functionalized SAPs

	pKa1	pI	pKa2
$RADA_{16}$	2.96	7.13	10.61
$RADA_{16}$-GRGDS	2.70	6.90	11.30
$RADA_{16}$-YIGSR	2.32	8.5	10.08

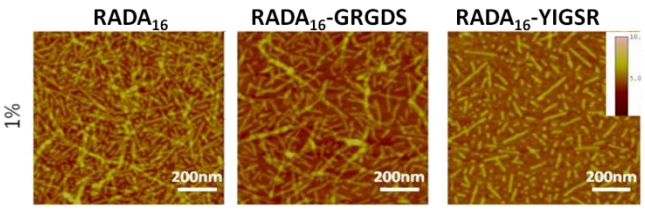

Fig. 2. Interwoven nanofibrous network structure of SAPs by AFM

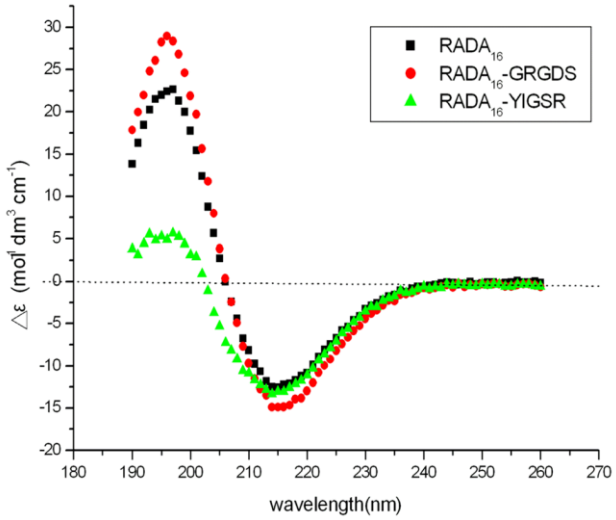

Fig. 3. Typical β-Sheet Structure of SAPs by CD Spectrum

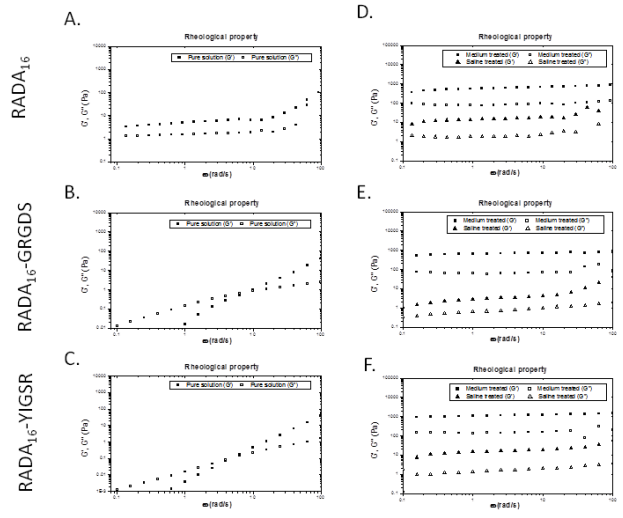

Fig. 4. Rheology property of 1 % (w/v) $RADA_{16}$, $RADA_{16}$-GRGDS, and $RADA_{16}$-YIGSR in pure water (A, B, C) and with medium or saline treatment (D, E, F). (G' is the elastic (storage) modulus representing the elasticity of material and the ability of the material to store energy. G" is the viscous (loss) modulus representing the ability of the material to dissipate energy.) (n=3)

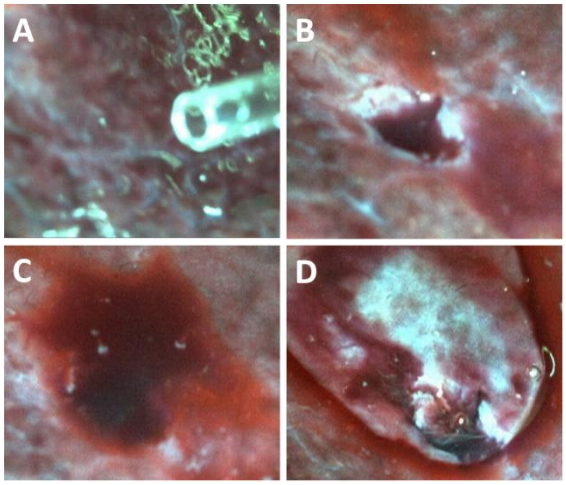

Fig. 5. Punctures were created in median lobe (for SAP groups) and left lateral lobe (for PBS group). (A, B) The lobes of rat liver were exposed and stabbing wound defect was created with glass micropippette, leading to extensive bleeding (C). Various treatments were then conducted in the puncture cavities for the evaluation of hemostasis efficacy. (D) With injection of SAP into the damaged site, SAPs underwent self-assembling immediately in the presence of blood and gelationed into hydrogel to achieve complete hemostasis.

Hemostasis Efficacy

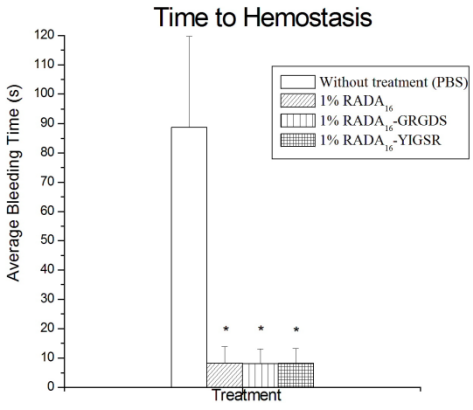

Fig. 6. All of the peptide solutions have excellent hemostasis efficacy. (*Student T test P< 0.01). On the other hand, it required more than 90s for the PBS controls.

Histological analysis

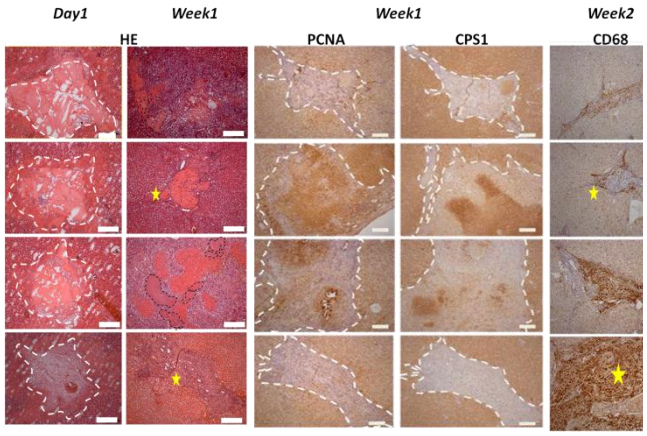

Fig. 7. H/E stain and Immunohistochemistry *of different SAPs on wound healing and regenerative liver tissue. (*anti-CD68 for macrophage, anti-PCNA for proliferating cell, anti-CPS1 for hepatocyte.)

Wound Healing and Histology analysis

No	Treatment agent (500μL)
I	Normal saline (Open wound control)
II	*Pre-form gelation* 1%$RADA_{16}$
III	*Pre-form gelation* 1%$RADA_{16}$ with 20ug/ml bFGF and EGF
IV	1X Matrigel

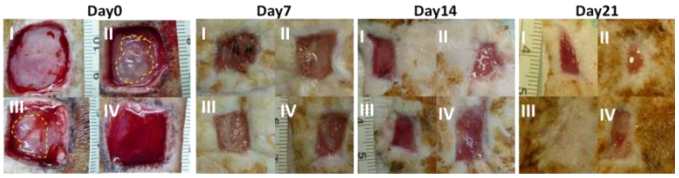

Fig. 8. Skin wound healing process with different time span from day 0 to day 21. Group III ($RADA_{16}$ incorporated with growth factors) demonstrated with better skin tissue re-epithelialization, dermal regeneration and wound area contraction. The results indicated that SAPs hydrogel with GFs can significantly promote the cutting wound healing process in rat animal model.

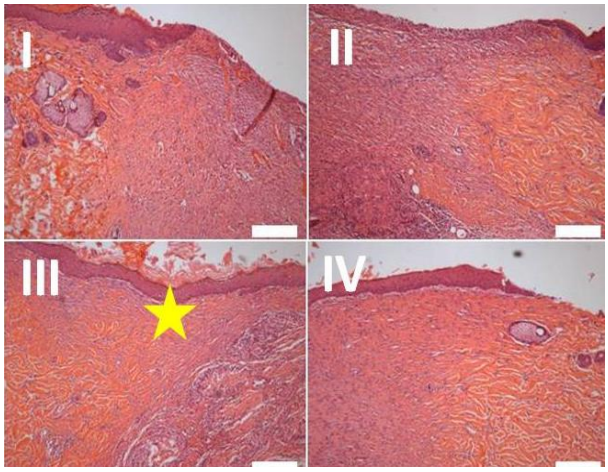

Fig. 9. The histological result of H/E stain after 3 weeks after transplantation of SAPs hydrogel (Scale bar 200 μm)

4 CONCLUSION

In this study, we have successfully developed functionalized SAPs with extended motifs of GRGDS and YIGSR, respectively, and evaluated their physiochemical properties compared to $RADA_{16}$ nanopeptides. The results from animal study showed that immediate hemostasis could be achieved within 10 seconds. In addition, the histological analysis demonstrated that the extended motifs significantly promote liver tissue regeneration. We also designed SAPs with incorporated growth factors to enhance skin wound healing process. The SAPs incorporated with EGF and bFGF growth factors have significant effect on skin epithelialization and dermal regeneration. To sum up, SAPs can be a promising material in medical applications. Future researches may concentrate on the investigation of extending SAPs with various functional motifs and combination SAPs with stem cells or generic materials for more clinical applications in tissue engineering and drug delivery system.

ACKNOWLEDGEMENT

The authors thank for the research funding support from NSC 100-2628-E-007 -022 -MY3 and NSC 99-2320-B-007 -001 -MY3