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Dual-peptide-modified alginate hydrogels for the promotion of angiogenesis

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The ability to supply suitable blood vessel system is a major challenge for artificial thick tissue engineering. Angiogenesis is a key point during the process of microvascular formation. Many bioactive molecules such as extra cellular matrix (ECM) proteins and adhesion peptides derived from the ECM are applied to promote angiogenesis. In this work, two adhesion peptides, YIGSR and REDV, were selected to modify sodium alginate (ALG) to obtain YIGSR- and REDV-alginate conjugates (ALG-YIGSR, and ALG-REDV, respectively). We mixed the two peptide-conjugates together in a series of concentration ratios to prepare bioactive surfaces for *in vitro* studies and hydrogel scaffolds for *in vivo* studies. *In vitro* studies showed that surfaces modified with 1.09 pmol/mm^2 peptide had the best affinity to human umbilical vein endothelial cells (HUVECs) than that with high or low concentrations of peptides. In addition, surfaces modified with dual peptides could significantly promote HUVECs proliferation, where ALG-YIGSR:ALG-REDV at a mole ratio of 5:1 exhibited the best enhancement ability. Furthermore, the *in vivo* angiogenesis results demonstrated that hydrogel scaffolds composed of mixed ALG-YIGSR and ALG-REDV at the 5:1 ratio had angiogenic induction potential by stimulating new blood vessel formation, and showed higher blood vessel density than scaffolds composed of a single peptide. These results demonstrated that a mixed combination of peptide alginate conjugates could be a potential scaffold to stimulate and induce angiogenesis in tissue engineering applications.

angiogenesis, YIGSR, REDV, alginate hydrogel, thick tissue engineering

1 Introduction

A suitable vascular supply is necessary for thick tissue engineering [1,2]. Angiogenesis is a key process during the early stage of new microvascular formation [3]. Endothelial cells' (ECs) adhesion, migration, proliferation, and other behavior are significant factors in the process of angiogenesis [4,5]. Therefore, it is of great importance to give biological scaffolding materials the ability to regulate the growth behavior of ECs, thereby promoting angiogenesis [6,7].

Extracellular matrix (ECM) is a kind of natural material that can support cells and regulate cellular behavior [8]. Many researchers have applied ECM components, including collagen [9,10], laminin [11,12], and fibronectin[13], as scaffolding material for promoting angiogenesis. Cooper and Sefton [13] found that a collagen matrix coated with fibronectin can significantly increase the long-term survival of human umbilical vein endothelial cells (HUVECs) on the matrix, and the cells gradually formed linear vascular structures. However, despite their significant effects in promoting angiogenesis, the application of these ECM components has been limited owing to their relatively high cost and low stability.

It was reported that ECM proteins can interact with cells via some basic units that included only a few amino acid sequences [14], and these adhesive oligopeptides can substitute for ECM proteins in regulating EC behaviors. In addition, these oligopeptides are stable in chemical reactions, and can be artificial and of relatively low cost. The first

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proven peptide was Arg-Gly-Asp (RGD) [15], which is currently the most used oligopeptide in the field of angiogenesis [16]. Some researchers have attempted to mix two peptides derived from the same or different ECM proteins, to investigate whether they have better effects on promoting interactions between ECs and scaffold materials [17,18]. Fittkau *et al*. [19] found that RGD plus the laminin-derived peptide YIGSR enhanced the migration of microvascular endothelial cells (MVECs) by 25% over that of MVECs on RGD alone (p<0.05), whereas RGD plus the fibronectin derived peptide PHSRN had no effect on MVECs migration. This suggested that, in some circumstances, the desired response to scaffolds could be optimized through a combinatory approach to the use of peptides, while in other cases the mixed peptides would have no corresponding effect. Therefore, further studies on cell behaviors in response to a mixture of peptides are necessary.

REDV, derived from the CS-5 part of fibronectin III CS region, can interact with the integrin $\alpha_4\beta_1$ and selectively adsorb ECs but not fibroblasts, smooth muscle cells or platelets [20]. On the other hand, YIGSR, a peptide derived from the laminin B1 chain, represents a class of adhesive peptides that do not interact with the integrin family of cell receptors but with the 67 kDa laminin binding protein (LBP) [21]. This peptide was found to promote the adhesion and spreading of a large number of cell types, including ECs [22]. In previous studies [23,24], we prepared hydrogels made with the peptides YIGSR or REDV conjugated to sodium alginate (ALG) and demonstrated that both the ALG-YIGSR- and ALG-REDV-composited hydrogels can induce angiogenesis significantly compared with the non-modified ALG group. In addition, ALG-YIGSR-modified surfaces showed advantages in ECs adhesion and migration, whereas ALG-REDV-modified surfaces showed superiority in ECs proliferation. Therefore, we think it is reasonable to assume that scaffold materials modified with mixture of YIGSR and REDV can further promote ECs behaviors and angiogenesis than scaffolds modified with either YIGSR or REDV only.

In this study, we prepared different concentrations and combinations of mixed ALG-peptide-modified surfaces and hydrogels using the LBP-binding peptide YIGSR and the integrin-binding peptide REDV. Following this, *in vitro* experiments, including the MTS assay and wound healing assay, were used to investigate the synergistic effect of mixing the peptides. *In vivo* experiments confirmed the angiogenesis promoting ability of the mixed peptides.

2 Experimental

2.1 Chemicals and apparatus

Sodium alginate (ALG; M_w=6.3×10^4, Polydispersity index (PDI)=1.006) was supplied by Qingdao Crystal Rock Biology Development Co. Ltd. (China). 2-Chloro-1-methylpyr (CMPI, purity>98%) and tetrabutylammonium hydroxide (TBA^+OH^-, 40% aqueous solution) were obtained from Xi'ya Chemical Technology Co., Ltd. (China). *D*-glucono-δ-lactone (GDL, purity>99%), phenol (AR), trifluoroacetic acid (TFA, AR), 1,2-ethanedi-thiol (EDT, AR) and thioanisole (MPS, AR), 3-amino-propyltrimethoxysilane (APT MS, >97%) were all purchased from Aladdin Chemical Co., Ltd. (USA). 1-Ethyl-3-(3-dime-thylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and *N*-hydroxysuccinimide (NHS) were obtained from GL Biochem., Ltd. (China). [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was purchased from BestBio (China). 2-(*N*-morpholine) ethanesulfonic acid (MES) was purchased from Dingguo Biotechnology Co., Ltd. (China). The peptides Gly-Tyr(*t*Bu)-Ile-Gly-Ser(*t*Bu)-Arg(Pbf)-Gly-(Prot-GYIGSRG) and Gly-Arg(Pbf)-Glu(O*t*Bu)-Asp(O*t*Bu)-Val(Prot-GREDV) were synthesized by GL Biochem Ltd. (China). All the other chemicals used were of analytical grade. Distilled deionized water was used throughout the experiment. For cell culture, endothelial cell medium (ECM) containing 1% endothelial cell growth supplement (ECGS) and 1% penicillin/streptomyclin solution (P/S), fetal bovine serum (FBS) were supplied by Yuhengfeng biotech CO., Ltd. (China).

The following apparatus were used: FTS-6000 Fourier transform infrared (FT-IR) spectrometer (Bio-Rad, USA), UNITY Plus-400 MHz NMR spectrometer (Varian, USA), TE2000-U Invert Microscope (Nikon, Japan), and AXIS Ultra DLD X-ray photoelectron spectroscopy (XPS; Kratos, UK).

2.2 Cell culture

HUVECs were obtained from the Cell Bank of the Chinese Academy of Sciences (China) and maintained in endothelial cell medium comprising 500 mL of basal medium, 5% FBS, 5 mL of P/S solution, and 5 mL of ECGS, and cultured at 37 °C in a 5% CO_2 humidified environment. HUVECs used in the experiments were at passages 6–9.

2.3 Preparation of ALG-peptides

ALG-peptides were synthesized by two steps according to a previously reported procedure [23]. Briefly, sodium alginate was first converted into its acidified form with HCl and then neutralized to pH 7 with TBA^+OH^-. This was followed by dialysis and freeze drying to obtain tetrabutylammonium-alginate (ALG-TBA). Then, the synthesized ALG-TBA was dissolved in 20 mL of anhydrous dimethyl formamide to form a 1% (*w*/*v*) solution in nitrogen at 0 °C. Then, CMPI was added ([CPMI]:[COOH]=1:2) to activate the ALG-TBA. One hour later, protected peptides YIGSR or REDV ([prot-pep]:[COOH]=1:10) and 0.5 mL of triethylamine were added to react at room temperature for another 48 h. Then, 1 mL of K reagent (TFA:MPS:EDT:phenol:H_2O=

86:5:5:2:2, *v*/*v*) was added to deprotect the peptides. After 1 h, aqueous NaCl (2.5 mol/L, 6 mL) was added to the solution in order to exchange the TBA^+ ions with Na^+ ions. After removal of the TFA under vacuum, the polymers were purified by twice precipitation in ethanol and dissolved in double distilled water, followed by dialysis for 3 d. The final products were obtained after freeze-drying.

2.4 ALG-peptide-modified surface preparation

Glass slides (diameter 15 mm) were consecutively cleaned in acetone, ethanol, and distilled water. They were further incubated in a "piranha" solution (a mixture of 30% hydrogen peroxide and 70% sulfuric acid (*v*/*v*)) for 2 h at room temperature, thoroughly washed with water, and oven dried at 80 °C. Then, the slides were immersed into an APTMS acetone solution (3%, *v*/*v*) for 2 h at room temperature. After consecutively washing with acetone, ethanol, and distilled water, the slides were dried at 80 °C.

ALG and ALG-peptides were mixed with a total mass of 0.25 g, dissolved in 25 mL of MES buffer solution (pH 6.5), and stirred for 4 h. Then, EDC and NHS were added ([COOH]:[EDC]:[NHS]=4:2:1) to each solution and the solution was continuously stirred.

The amido-activated slides were then immersed into the solution for 10 h at room temperature. After washing 5 times in water to remove the physically adsorbed ALG-peptide molecules, the slides were dried at 80 °C. Figure 1 presents the synthetic route of ALG-peptide and modified surface.

The ALG-peptide modified slides were ultraviolet sterilized and put into 24-well cell culture plates with further UV sterilization overnight, and were then used immediately.

2.5 Surface characterization

The chemical compositions of the surfaces were detected by XPS. Data were analyzed with XPS peak software. The binding energy was corrected by setting the lowest binding energy of the C 1s peak at 284.6 eV.

2.6 Cell behavior investigation on modified surface

2.6.1 Cell adhesion

After reaching about 80% confluence, the cells were detached and seeded onto 24-well cell culture plates with different ALG-peptide modified slides in each well at a density of 3×10^4 cell/well. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. After 4 h, the dead cells were rinsed away with phosphate-buffered saline (PBS). Then, 200 μL of EC medium containing 40 μL of MTS was added to each well, and the plates were incubated for 2 h. The absorbance at 490 nm was measured by a microplate reader. The results of each material were averaged from three parallel samples.

2.6.2 Cell migration

Migration of the cells was investigated via the wound healing assay. The cells were seeded onto 24-well cell culture plates with different ALG-peptide-modified slides in each well at a density of 5×10^4 cell/well and cultured in EC medium. When confluent, the cell monolayers were scratched horizontally with a pipette tip to obtain a monolayer culture with a space without cells. The slides were then rinsed with PBS to remove dislodged cells. EC medium with 2% FBS was then added to the wells in order to maintain growth of

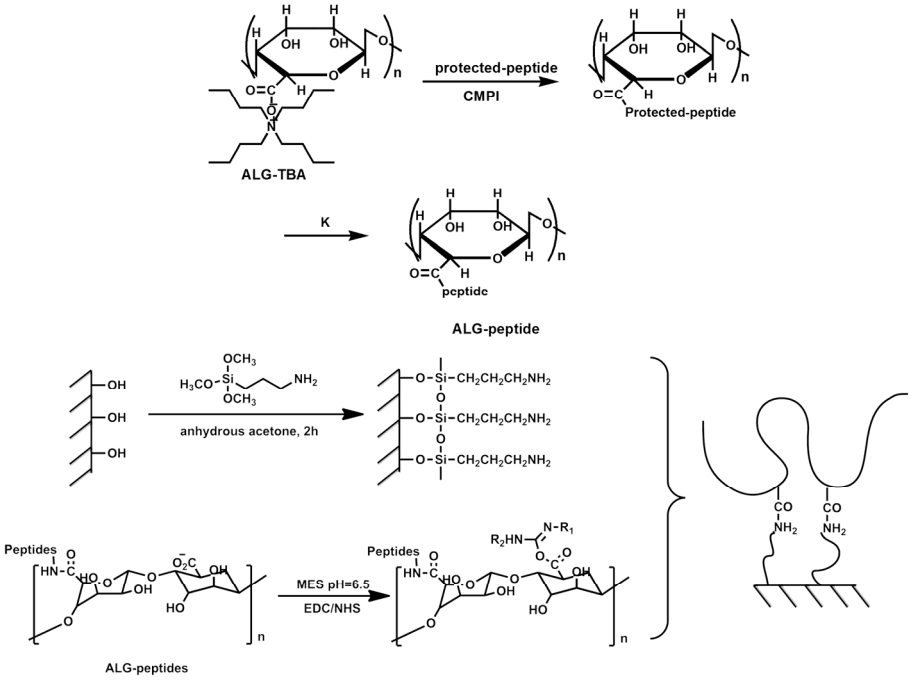

Figure 1 Schematic illustration of ALG-peptide preparation and modification of the activated glass surface.

the cells and avoid any influence of cell proliferation with high-level FBS. Fields along the scraped line were photographed on each well using an inverted fluorescence microscope. After incubation for 8 h, images were taken by the same method and analyzed using Image J software, to obtain a vertical distance H between the scratch boundaries. The migration rate was measured according to the following equation:

$$r_M=(H_0-H_t)/t \quad (1)$$

where r_M is the migration rate, H_0 is the initial distance, H_t is the final distance, and t is the duration of the experiment. Three random fields were chosen for each sample.

2.6.3 Cell proliferation

After reaching about 80% confluence, the cells were detached and seeded onto 24-well cell culture plates with different ALG-peptide-modified slides in each well at a density of 1×10^4 cell/well and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The culture solution was changed every other day. The proliferation of the cells was detected by MTS assay (performed in the same way as for cell adhesion above) on Days 1, 3, 5, and 7. The results of each material were averaged from three parallel samples.

2.7 Preparation of alginate composite hydrogels

Alginate composite hydrogels were synthesized according to the previously reported procedure. Briefly, 2.5 wt% ALG or ALG-peptide solution and $CaCO_3$ powder were mixed completely ([$CaCO_3$]:[COOH]=0.8). Under vigorous stirring, GDL was added ([GDL]:[$CaCO_3$]=2) to the mixture. Then, the mixture was injected into molds and allowed to gel at room temperature for 24 h. The final products were washed with double-distilled H_2O until the filtered water was neutral in pH. The hydrogels were finally cut into a shape with a diameter of 8 mm and thickness of 2 mm for later use.

2.8 Evaluation of angiogenesis *in vivo*

The prepared composite hydrogels were immersed in 75% ethanol solution for about 40 min for sterilization and then rinsed several times with sterile saline to remove alcohol components. The composite hydrogels were implanted into the subcutaneous pockets of SD female mice (180–200 g) on symmetrical sides of the back. Each kind of hydrogel was implanted with 4 parallels. The incision was subsequently sutured to close. (This part of the experiment was entrusted to the Experimental Animal Center of Tianjin.)

The implants were harvested at 21 d after implantation. First, the mice were anesthetized and perfused with India ink by tail vein injection. Blood circulation was allowed for 5 min in order to ensure that all the blood vessels were marked with the ink. Finally, the implants were retrieved from the mice and fixed in 10% formalin for 24 h, paraffin embedded, sectioned, and stained with hematoxylin and eosin. The histological sections were observed using a microscope and photographed.

Five photographs were taken on each tissue section at ×100 magnification. The number of mature, ink-stained blood vessels was counted around the hydrogel material in three stochastic circles (diameter=100 μm). The density of newly formed blood vessels was recorded as numbers/mm^2.

All applicable institutional and/or national guidelines for the care and use of animals were followed.

2.9 Statistical analysis

All data are presented as mean±SD. Statistical analysis was performed using Origin 8.5 software. A two-tailed paired Student t test was used to compare differences. Significance difference was accepted at $p<0.05$.

3 Results and discussion

3.1 Characterization of ALG-peptide surface

The composition and structure of the peptide-modified polymers (ALG-peptides) were characterized by ^{1}H NMR and FT-IR (Figures S1 and S2, see Supporting Information online). The grafting ratio of the peptide in alginate was determined using element analysis, and was 4.8% and 4.9% for ALG-YIGSR and ALG-REDV, respectively. The surface chemical compositions were analyzed by XPS after each reaction. In the case of the Glass-NH_2 surface, XPS detection found that after treatment with ATMPS, the atomic ratio of nitrogen to carbon (N/C ratio) of amido-activated slides, which is proportional to the relative content of N element around the surface, was increased compared with the original glass slide (Table 1). After reacting with ALG-REDV, the N/C ratio decreased, which can be attributed to the lower N/C ratio in ALG-REDV than in ATPMS. The peptide amount modified on surface was calculated according to the surface element content, and found to be 1.09 pmol/mm^2. Further evidence was provided by analysis of the C 1s peaks (at 284.6 eV) after each reaction (Figure 2). The curve-fitted C 1s core level spectrum revealed the presence of C–NH_2 and O=C–NH bonds (Figure 2(b)), a C–N_3H_4 bond (Figure 2(c)), and the peak area increase of the O=C–NH bond (Figure 2(c)), which confirmed

Table 1 N/C ratios of the surface after each reaction, detected by XPS

Samples	Atomic Conc. (%)			N/C (%)
	C	O	N	
Glass	65.81	33.47	0.73	1.11
Glass-NH_2	25.08	72.49	2.44	9.13
Glass-ALG-REDV	55.54	40.47	3.99	7.18

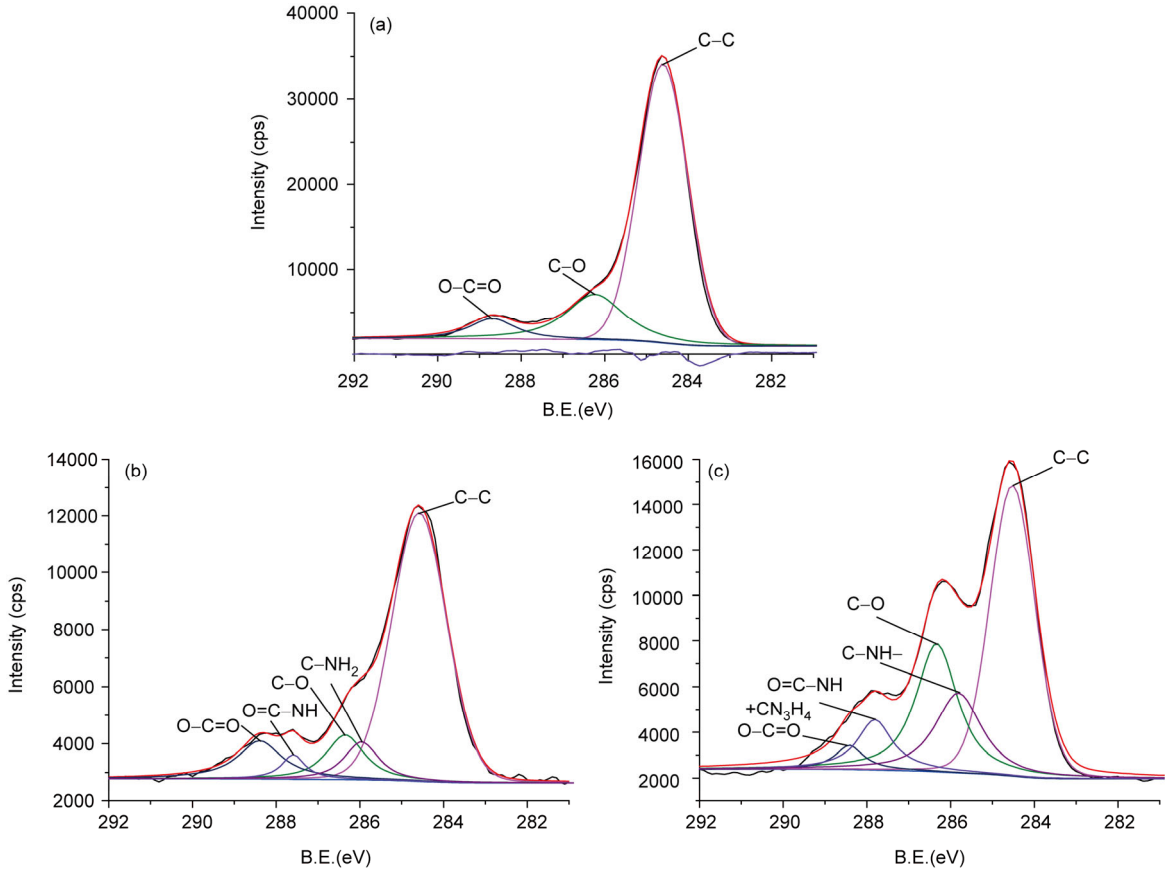

Figure 2 XPS C 1s spectra of different surfaces. (a) Glass; (b) glass-NH_2; (c) glass-ALG-REDV.

the successful immobilization of amino groups and ALG-REDV, respectively.

3.2 Cell behavior on modified surfaces

Angiogenesis is a complicated process. *In vivo* angiogenesis is mainly divided into the following stages [25]. First, in response to angiogenic stimuli, EC-pericyte contacts are disrupted, leading to activated EC and pericyte phenotypes, degradation of the basement membrane and increased vessel permeability. Then, ECs migrate into the surrounding tissue, adhering and proliferating on the matrix, forming new blood vessels that secrete to the ECM to form capillaries. Finally, the microvasculature gradually grows and assembles with pericytes (such as smooth muscle cells) again, forming a mature blood vessel network. Therefore, scaffold materials that can promote ECs migration, adhesion, and proliferation have the potential ability to stimulate angiogenesis.

3.2.1 Optimization of peptide concentration on modified surfaces

To explore the effect of peptide density on cell behavior, surfaces modified with three different peptide amounts (~0.36, ~1.09, and ~4.39 pmol/mm^2) were obtained by physically mixing the ALG-peptides and ALG in different ratios and defined as ALG-peptide-L, ALG-peptide-M, and ALG-peptide-H, respectively. Figure 3 shows the HUVECs adhesion, migration, and proliferation responses to the surfaces with different peptide concentration. Representative images of the scratched cell monolayers are shown in Figure S3.

The cell adhesion rate on all peptide surfaces changed in a peak-shape trend; that is, it first increased and then decreased with the increase of peptide concentration. That is to say, the surface with ALG-peptide-M exhibited better adhesion behavior than that with ALG-peptide-L or ALG-peptide-H. This pattern applied to both YIGSR and REDV (Figure 3(a)). The cell migration and proliferation results showed a similar tendency (Figure 3(b, c)). All of these results showed that the surface with a medium peptide concentration was more effective on HUVECs behavior. This indicates that there may be an optimal peptide concentration for cell behavior. When the surface is modified with a low density of peptides, it cannot provide enough sites for interaction between receptors from the cell surface (integrin) and ligands from the material (peptides). Conversely, surfaces with a high peptide density attract far more cells through specific interaction; these cells crowd around the surface and only a small number can spread well on materials,

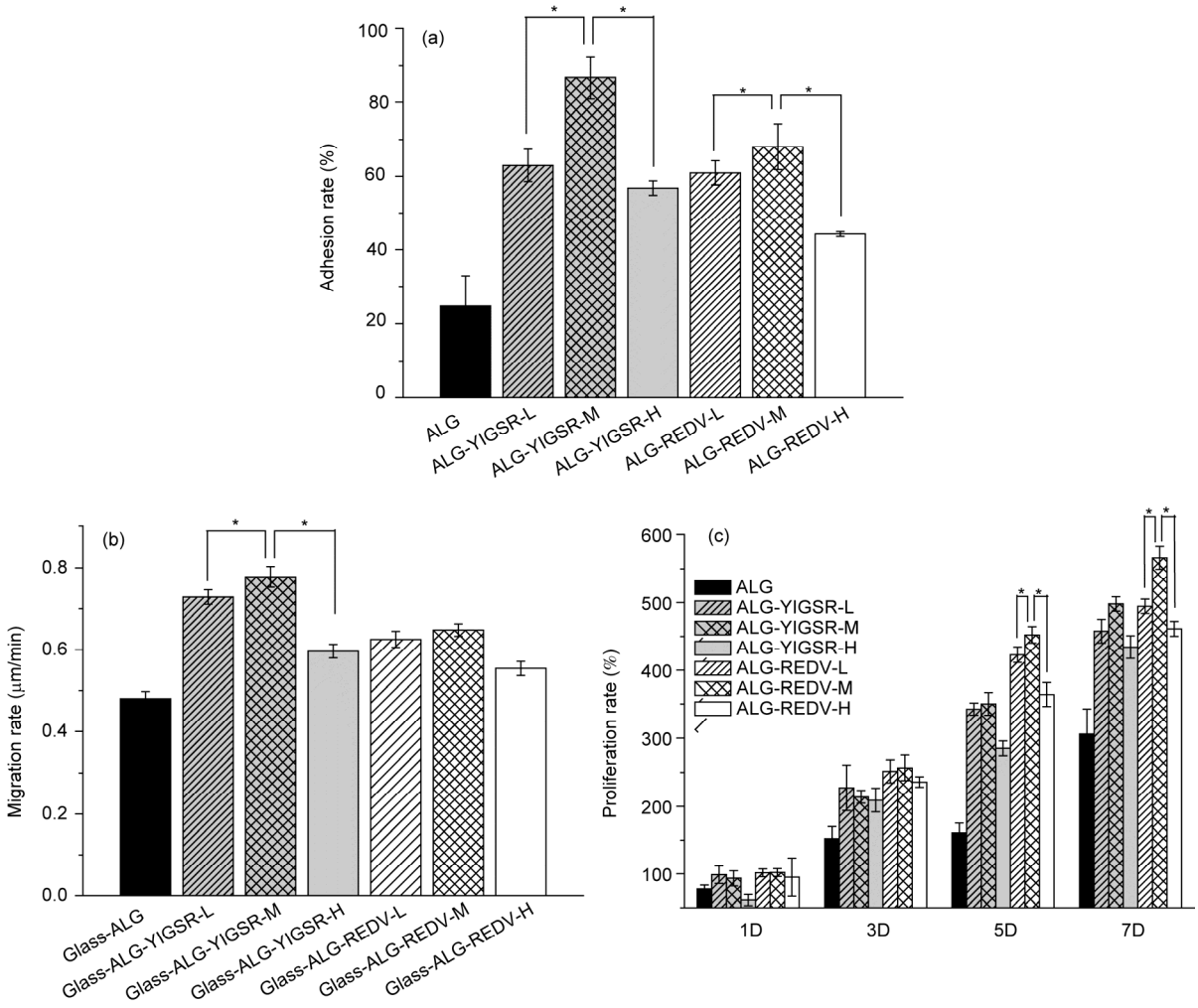

Figure 3 HUVECs adhesion (a), migration (b), and proliferation (c) in response to surfaces with different peptide densities. The proliferation rate was calculated versus the numbers of cells seeded at the first day. The results are given as the mean±SD (n=3). * Indicates a significant difference between groups (p<0.05).

resulting in reduced focal adhesion. Cell adhesion is usually the basis and premise of other cell behaviors such as migration, and the impeding effect of spread can affect cell proliferation to a certain extent. These results were similar to the data from other researchers. Mann and West [26] found that an optimal concentration of cell adhesive ligand was needed to achieve appropriate adhesion proliferation and migration for smooth muscle cells.

3.2.2 Surfaces modified with dual peptides

Based on the results above, ALG-YIGSR and ALG-REDV were mixed together and covalently conjugated on the slices (at a constant total concentration as 0.5 mmol/L) for studying the synergetic effect of dual-peptide-modified surfaces on HUVEC behavior. Figure 4 shows the HUVECs adhesion, migration, and proliferation responses to the dual-peptide-modified surfaces with different mixing ratios. Figure 4(a) shows the cell adhesion result. The cell adhesion on dual-peptide surfaces was significant lower than that with a single peptide. The reason for this phenomenon is not very clear at present, but we attributed it to interaction between the two peptides themselves, thus obstructing the binding between each peptide and its cell receptor. Since the two peptides have an opposite charge, they were likely to bind with each other during the mixing stage before modulating the slide surface. Moreover, the test time for cell adhesion was 4 h long, which was not long enough for the receptor to completely compete with peptides and, in turn, to form stable binding with them. Figure 4(b) shows that the HUVECs migration rate increased with the increase of YIGSR proportion on the surface. This result conformed to our previous research that the YIGSR peptide has a positive effect on HUVECs migration [23].

Figure 4(c) shows the cell proliferation results on the dual-peptide-modified surface. The proliferation rate was calculated versus cell culture plate. At Day 5, cells on the

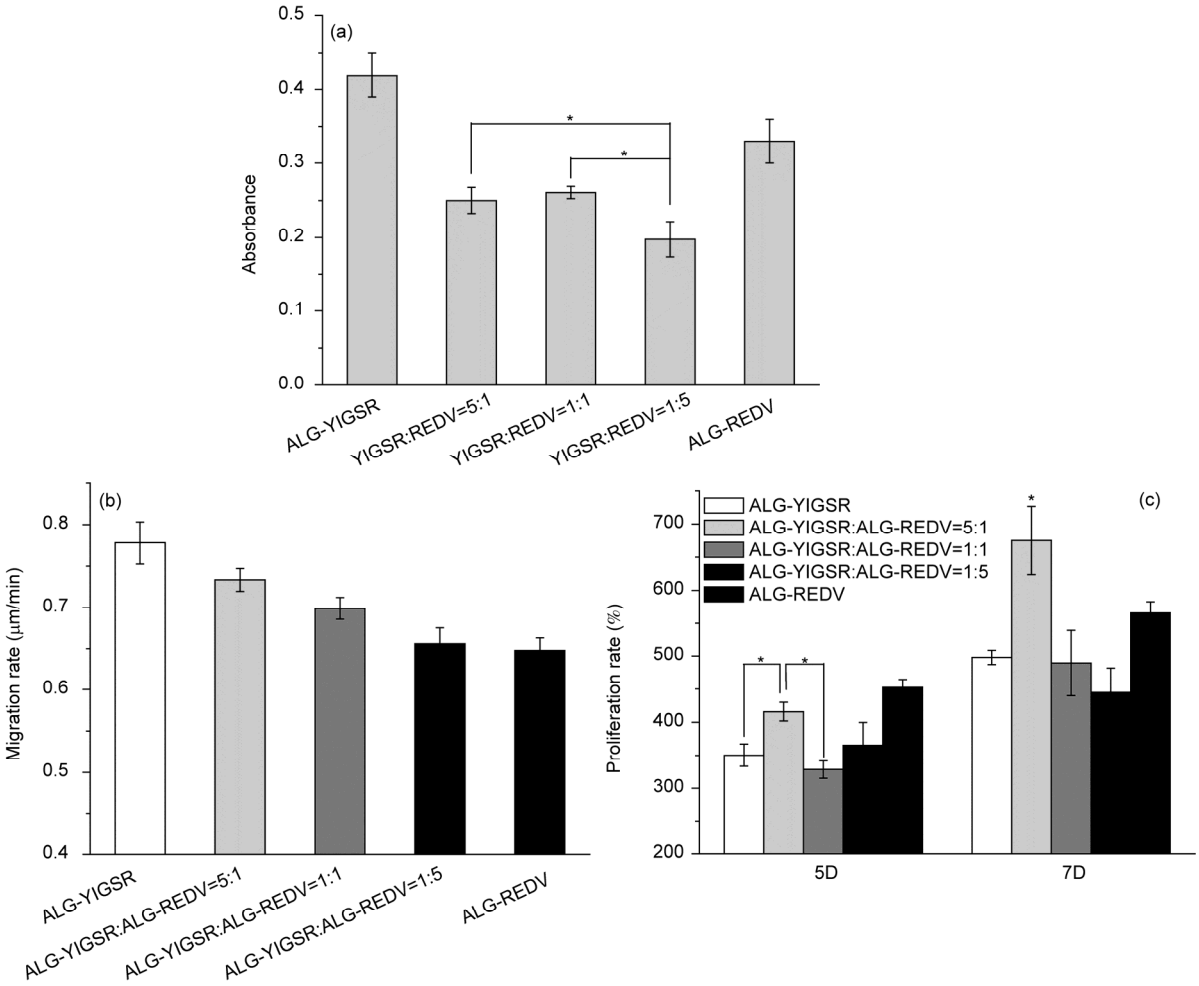

Figure 4 HUVECs adhesion (a), migration (b), and proliferation (c) in response to surfaces modified with different mixing ratios of two peptides. The proliferation rate was calculated versus the number of cells seeded at the first day. The results are given as the mean±SD (n=3). * Indicates a significant difference (p<0.05).

surface modified by YIGSR:REDV at a ratio of 5:1 proliferated significantly more than the other groups. On Day 7, the proliferation superiority was more obvious, being even higher than the proliferation on the single peptide REDV. The 5 or 7 d test time for cell proliferations enabled the dual peptides to bind with their receptors rather than interact with each other. Based on the above results, the surface modified with 5:1 YIGSR:REDV was concluded to have the greatest superiority in promoting cell proliferation and does not have inferiority in cell adhesion and migration, meaning that this mixing ratio of 5:1 has a certain advantage on a variety of behaviors of ECs. In a previous study, we found that the proliferation rate of ECs may play the most important role in angiogenesis. Therefore, we chose materials with a mixing ratio of 5:1 for the subsequent *in vivo* study.

3.3 *In vivo* study

Based on the discussion above, the surface modified with 5:1 YIGSR:REDV was chosen for the subsequent *in vivo* study. A rodent subcutaneous model was used to evaluate the tissue response *in vivo*. Figure 5 shows the histological micrographs of the control and 5:1 YIGSR:REDV hydrogels explanted after 21 d. Blood vessels were visualized based on perfusion with India ink. In all the tested groups, tissue invaded the hydrogels, with newly formed blood vessels surrounding the implant (red arrows). The blood vessel density in the dual-peptide group was evidently higher than that in the control groups. Quantitative analysis was performed for the neovascular density on the basis of histologically stained samples (Figure 6). The blood vessel density of the dual-peptide group was significantly higher than that of all the other single peptide groups, especially the REDV group (p<0.05).

In our previous work [24], *in vitro* experiments showed that REDV-modified materials can significantly promote ECs proliferation, while *in vivo* experiments showed that a REDV-modified ALG hydrogel can significantly promote angiogenesis. These positively correlated results indicated that the proliferation rate of ECs may play an important role

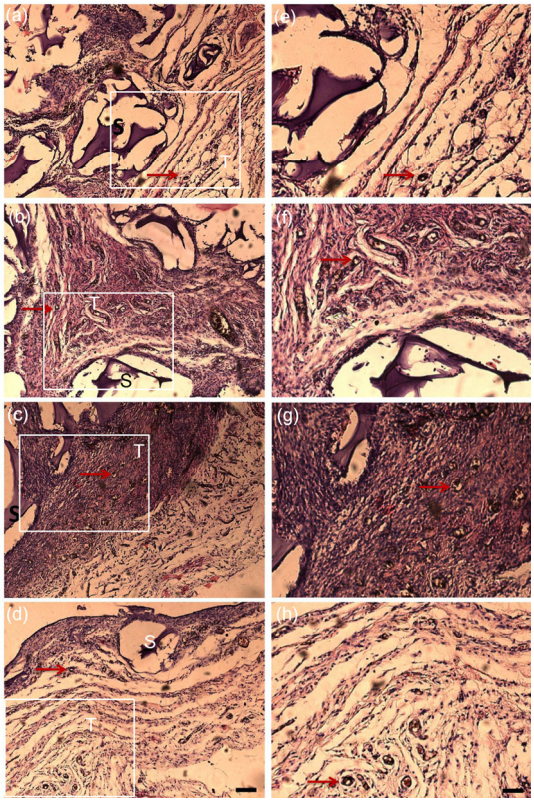

Figure 5 (a–d) Histological micrographs of various scaffolds implanted after 21 d with ALG (a), ALG-YIGSR (b), ALG-REDV (c), or ALG-YIGSR:ALG-REDV at 5:1 ratio (d); (e–h) amplifications of (a–d), respectively. "S" remaining scaffold; "T" tissue. The red arrow shows the newly formed blood vessels (color online). Original magnification: ×100 ((a–d), bar=100 μm), ×200 ((e–h), bar=200 μm).

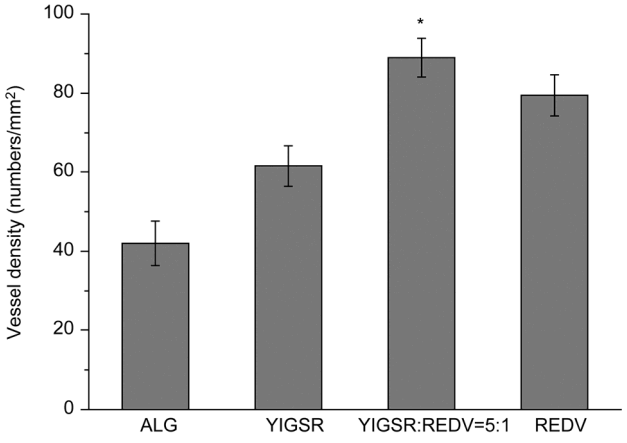

Figure 6 The blood vessel density of scaffolds with various peptides implanted after 21 d. The results are given as the mean±SD (n=15). * Indicates a significant difference between the mixed group and other groups (p<0.05).

in angiogenesis. Similarly, in this study, the blood vessel formation was consistent with the trend of ECs proliferation. Therefore, the ability of the dual-peptide group in promoting cell proliferation and inducing angiogenesis was quite excellent and greater than that of REDV in the single-peptide-modified group.

4 Conclusions

This study has demonstrated that a surface modified with a medium peptide concentration was more effective on HUVECs behaviors than surfaces modified with low or high peptide concentrations. In addition, surfaces modified with a mixture of peptides could significantly promote HUVECs proliferation, with the YIGSR:REDV mixing ratio of 5:1 exhibiting the best ability for such enhancement. More importantly, in the *in vivo* study, the blood vessel density in hydrogels composed of 5:1 ALG-YIGSR:ALG-REDV showed significant advantage over the other single-peptide groups, demonstrating the potential ability of scaffolds with ALG-YIGSR:ALG-REDV at a 5:1 ratio to promote angiogenesis in tissue engineering.

Supporting information

The supporting information is available online at chem.scichina.com and link.springer.com/journal/11426. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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