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HUVEC cell affinity evaluation and integrin-mediated mechanism study on PHSRN-modified polymer

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ABSTRACT

To investigate the role of the peptide Pro–His–Ser–Arg–Asn (PHSRN) in cell adhesion and growth, PHSRN- and Gly–Arg–Gly–Asp–Ser (GRGDS)-containing polymers (P-PN5 and P-GS5, respectively) were synthesized by modification of poly(D,L-lactide-*co*-*beta*-malic acid) (PLMA) with the corresponding peptides. The cell affinities of the modified polymers were evaluated by adhesion and proliferation experiments with human umbilical vein endothelial cells (HUVECs). The results showed that P-PN5 had comparable ability to that of P-GS5 in supporting HUVEC adhesion and growth. Furthermore, the integrin-mediated mechanism of cell-substrate interaction was investigated. The results showed that P-PN5 had similar binding affinity and binding strength towards $\alpha_5\beta_1$ compared to those of P-GS5. The findings suggest that PHSRN is capable of mediating the adhesion and growth of HUVECs independently and that PHSRN-modified polymers might be used as biologically compatible materials.

1. Introduction

In recent years, the use of synthetic polymeric materials has increased rapidly in tissue engineering. However, conventional synthetic polymers usually have significant drawbacks because of inadequate interaction with cells [1]. Research has revealed that small active sequences in extracellular matrix proteins exert a critical influence on cell–matrix interactions. Thus, modification with small active sequences could be an effective strategy for promoting the cell affinity of biomaterials [2].

Arg–Gly–Asp (RGD), the minimal protein alternative characterized from fibronectin, is able to trigger cell adhesion effectively [3–5]. A great number of different RGD peptides have been combined freely with different polymers and cell lines for academic studies or medical applications. Walluscheck et al. [6] assessed endothelial cell attachment on RGD-containing peptide-coated polytetrafluoroethylene vascular grafts, and found that a graft coated with GRGDSPL showed higher endothelial cell attachment (30.6%) and retention (62.9%) after shear stress than one coated with fibronectin (26.0%/45.5%).

Besides RGD-type peptides, many other small fragments have been identified as exerting significant influence on cell behavior, such as YIGSR (from laminin) [7], DGEA (from collagen) [8] and KRSR (from heparin) [9]. To develop synthetic matrices with different properties, one or more types of fragments could be selectively immobilized on polymers according to the target cell types and specific cell responses.

Pro–His–Ser–Arg–Asn (PHSRN), in the 9th type III domain of fibronectin, is known to act synergistically with RGD in the adjacent 10th domain for cell adhesion and spreading [10]. However, in many reports it has been considered that PHSRN itself has no biological activity [11–15] and a consensus on the function of PHSRN on polymers has not been reached yet. Fittkau et al. [16] reported that polyethylene glycol (PEG) hydrogels containing PHSRN showed little adhesion and no spreading of endothelial cells. Although many groups have reported that PHSRN cannot mediate cell attachment independently [16–19], Feng and Mrksich [20] showed that BHK cells and 3T3 Swiss fibroblasts attached efficiently to self-assembled monolayers presenting either GRGDS or PHSRN peptide. Therefore, the role of the peptide PHSRN in mediating cell adhesion needs to be further investigated.

In this work, experiments were designed to study the HUVEC cell affinity of the peptide PHSRN. To this end, PHSRN- and GRGDS-containing polymers (P-PN5 and P-GS5, respectively) were synthesized by modification of PLMA with the sequences PHSRN and GRGDS derived from fibronectin (Scheme 1), and the attachment and growth of HUVECs on these polymers were evaluated. Then, the integrin-mediated mechanism of cell–substrate interaction was investigated by measuring the interaction of peptide-containing polymers with integrin $\alpha_5\beta_1$.

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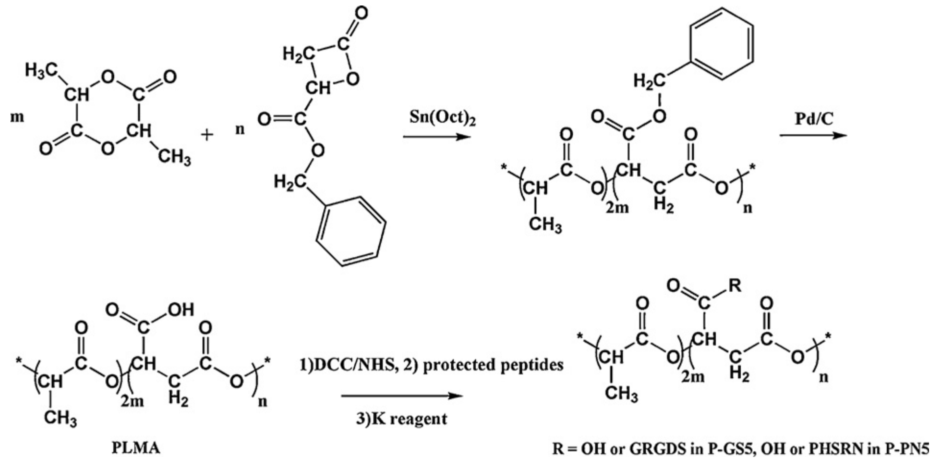

Scheme 1. The synthetic route to the modified polymers, m:n = 9:1.

2. Materials and methods

2.1. Materials

N,*N*′-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) were purchased from Acros. Protected peptides H–Gly–Arg(Pbf)–Gly–Asp(O*t*Bu)–Ser(*t*Bu)–OH (M_w = 854.98, Purity = 87.90%) and H–Pro–His(Trt)–Ser(*t*Bu)–Arg(Pbf)–Asn(Trt)–OH (M_w = 1402.65, Purity = 88.80%) were synthesized by GL Biochem Ltd. Dimethylformamide (DMF) and dichloromethane (DCM) were dried with CaH_2 and distilled. Other reagents were of analytical reagent grade.

2.2. Synthesis of peptide-containing polymers

PLMA (ratio between lactic acid and malic acid = 9:1, $M_w = 4.5 \times 10^4$) was prepared according to the literature procedure [21,22]. To graft peptides to the PLMA, 0.2 g polymer was dissolved in 5 mL anhydrous DCM and activated with DCC and NHS in a 1:1 molar ratio under nitrogen atmosphere at room temperature for 24 h. After filtering off the *N,N*′-dicyclohexylurea formed, the activated polymer was obtained by precipitation from diethyl ether. The product was redissolved in 5 mL anhydrous DMF and modified with equal molar amounts of the requisite protected peptides at room temperature for 4 days. After deprotection in 2 mL reagent K for 2 h at room temperature, the polymers P-GS5 and P-PN5 were obtained by precipitation from diethyl ether and thoroughly washed with diethyl ether and ethanol, then dried under vacuum at 30 °C for 2 days.

2.3. Preparation of the polymer substrates

The polymers were dissolved in chloroform at a concentration of 10 mg/mL and the solutions were filtered through a 0.22 μm dialyzer to remove any insoluble impurities and bacteria. Films (diameter = 15 mm) were fabricated by spin-coating the solutions onto cover slips rotated at a speed of approximately 2000 rpm for 60 s and then dried under vacuum. The dried films were placed in a 24-well TC plate (Falcon, USA) and sterilized by ultraviolet irradiation for 30 min before cell seeding.

2.4. Characterization

^{1}H NMR spectra were recorded on a Varian Mercury VX300 spectrometer (400 MHz) with tetramethylsilane as the internal standard and $CDCl_3/CF_3COOD$ as the solvent. FT-IR spectra were recorded on a Nicolet Magna-560 spectrometer. The surface peptide contents were measured by X-ray photoelectron spectroscopy (XPS, Kratos AXIS Ultra DLD). The molecular areas were calculated by Chem 3D Ultra 8.0. The surface morphologies of the polymer films were observed with an atomic force microscopy (AFM) at room temperature with a NanoIII(R)A system (Veeco, New York, NY), and the images were analyzed by the V5.30r3.sr3 Program Software (Veeco).

2.5. Cell cultures

HUVECs were harvested from fresh human umbilical cord and cultured in M199 medium (Sigma–Aldrich, St. Louis, MO) containing 2.2 mg/mL sodium bicarbonate (Fisher, Fairlawn, NJ), 90 μg/mL sodium heparin (Sigma–Aldrich, St. Louis, MO), 100 IU penicillin, 100 μg/mL streptomycin (Invitrogen Corporation, Grand Island, NY), 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO), 1 mM L-glutamine (Invitrogen Corporation, Grand Island, NY), and 50 mg/mL ECGS (Technoclone, Vienna, Austria) [23]. Cells were split at a ratio of 1:2 after growing to 80–90% confluence. HUVECs used in experiments were at passages 3–5.

2.6. Assay for cell adhesion

HUVECs were trypsinized and resuspended in culture medium at a density of 8×10^4 cells/mL. Aliquots of 1 mL of cell suspension were placed in the wells containing films of PLMA, P-GS5 and P-PN5 (n = 3). After culturing at 37 °C in a 5% CO_2 atmosphere for 24 h, the culture medium was removed from the wells, and the polymer films were washed with phosphate buffered saline (PBS). Then, the cells were trypsinized and counted using a hemocytometer.

2.7. Assay for inhibition of cell adhesion

Inhibition by soluble peptides: besides aliquots of cell suspension, 0.2 mg portions of corresponding soluble peptides were added to the wells of P-GS5 and P-PN5 (n = 3). Soluble GRGDS was added to the wells containing films of P-GS5, and soluble PHSRN was added to the wells containing films of P-PN5. After culturing at 37 °C in a 5% CO_2 atmosphere for 24 h, the number of cells was counted.

Inhibition by anti-integrin antibodies: samples of 1 mL of cell suspension were incubated with 15 μg of antibodies (anti-integrin α_5, β_1, $\alpha_\nu\beta_3$ antibodies, Shanghai Hushang Biotechnology) at room