J. Microbiol. Biotechnol. (2001), **11**(6), 922–927

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Certification of Fibroblast Cell Adhesion and Spreading Mediated by Arg-Gly-Asp (RGD) Sequence on Thermo-Reversible Hydrogel

NA, KUN[1], DONG-WOON KIM[2], AND KEUN-HONG PARK[3]*

[1]*College of Pharmacy, Chosun University, 375 Seoseok-Dong, Dong-Ku, Kwangju 501-759, Korea*
[2]*Department of Environmental Science, Kwangyang College, Kwangyang 545-703, Korea*
[3]*College of Dentistry, Seoul National University, Yeongeon-Dong, Jongro-Ku, Seoul 151-100, Korea*

Received: April 21, 2001
Accepted: October 8, 2001

Abstract In an effort to regulate the mammalian cell behavior in entrapment with a gel, we have functionalized hydrogels with the putative cell-binding (-Arg-Gly-Asp-) (RGD) domain. An adhesion molecule of Gly-Arg-Gly-Asp-Ser (GRGDS) peptides, a cell recognition ligand, was induced into thermo-reversible hydrogels, composed of *N*-isopropylacrylamide with small amounts of acrylic acid (typically 2–5 mol% in feed), as a biomimetic extracellular matrix (ECM). The GRGDS containing a p(NiPAAm-co-AAc) copolymer gel was studied *in vitro* for its ability to promote the spreading and viability of cells by introducing a GRGDS sequence. Hydrogel with no adhesion molecule was a poor ECM for adhesion, permiting spreading of only 3% of the seeded cells for 36 h. By immobilizing the peptide linkage into the hydrogel, the conjugation of RGD promoted 50% of proliferation for 36 h. However, the GREDS sequence, nonadhesive peptide linkage, conjugated hydrogel showed only 5% of the seeded cell for the same time period. In addition, with the serum-free medium, only GRGDS peptides conjugated to hydrogel was able to promote cell spreading, while there was no cell proliferation in the hydrogel without GRGDS. Thus, the GRGDS peptide-conjugated thermo-reversible hydrogel specifically mediated the cell spreading. This result suggests that utilization of peptide sequences conjugating with the cell-adhesive motifs can enhance the degree of cell surface interaction and influence the long-term formation of ECM *in vitro*.

Key words: Entrapment, GRGDS, adhesion molecule, hydrogel, proliferation

Mammalian cell culture is a key technique for providing a basic study in biology and for industrial applications as well. The importance of substrata, or biomaterials, such as biological and synthetic extracellular matrices for cell culture, along with the production of artificial tissues and organs, has been increasing. However, it is difficult to regulate cellular functions such as proliferation, secretion, movement, and differentiation with biomaterials alone. For this reason, hydrogel has served as a protective barrier for cells, to mimic at least some of the properties of the ECM, in that it may offer the extracellular environment and it may likely impact cell viability, function, growth, and differentiation in the cell growth substrata [19, 12, 3, 20]. It is effective for surface binding, either on top of the gel films or by using hydrogels. Immobilization by adhesion is generally used to stabilize cells for culture or analytical procedures, providing a structural template for directing cell growth and differentiation, or both.

In order to entrap the cells by adhesion, a wide variety of receptors on cell membranes are used to interact with their ligands, such as peptides [6], proteins, saccharides [9, 11], and other biologically active substances [1]. Cell adhesion to a material, such as a naturally occurring ECM or a synthetic biomaterial, is mediated primarily by the interaction between surface-bound ligands and corresponding receptors on the membrane of the cells [13]. One can expect that materials carrying these ligands not only maintain inherent functions of the cells, but also enhance their unique functions through these interactions. The analysis of the interactions between ligand and receptor and the behavior of cells would help in understanding various cell functions [4]. Typically, a peptide containing the cell-binding domains found in the ECM protein is immobilized on a material in order to promote cell adhesion via ligand-receptor interaction [15, 17-18]. These interactions are mediated by cell surface receptors, such as integrins and transmembrane proteoglycans, which bind to the peptide presenting the specific domain of the ECM protein [21]. Anchorage-dependent cells adhere to and grow on extracellular matrices via ligand-receptor

*Corresponding author
Phone: 82-2-760-3053; Fax: 82-2-766-4948;
E-mail: phd0410@hanmail.net

interactions [8]. The most common cell-binding domain used extensively as a candidate peptide to enhance cell adhesion onto biomaterial surfaces is recognized as the Arg-Gly-Asp (RGD) sequence [2] or other nonRGD-containing cell binding domains such as tyrosine-isoleucine-glycine-serine-arginine (YIGSR) [7], in addition to leucine-aspartic acid-valine (LDV) [14]. The typical adhesion molecule of the tripeptidyl sequence RGD is a minimum active peptidyl sequence that is quite common with adhesive protein [10], and the mechanism of the cell attachment to the RGD motifs is known as the integrin-mediated specific interaction [22].

The main goal of this study was to certify adhesion molecule-mediated cell adhesion and to spread it into a thermo-reversible p(NiPAAm-co-AAc) copolymer to interact specifically with cell membrane receptors. The thermo-reversible copolymer gel used in this study has been developed as an effective ECM, which contains a key polymer technology for freshly isolated or cultured cell lines [16]. A novel thermo-reversible copolymer gel containing adhesion peptides would lead to cell adhesion and proliferation in response to the RGD sequence-conjugated hydrogels.

MATERIALS AND METHODS

Materials

Gly-Arg-Gly-Asp-Ser (GRGDS) and Gly-Arg-Glu-Asp-Ser (GREDS) peptides were purchased from Ihyo Biochemical Co. Kwangju, Korea. *N*-Isopropylacrylamide (NiPAAm), acrylic acid (AAc), 2,2'-azobisisobutyronitrile (AIBN), 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDC), and Millicell were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). RPMI 1640, fetal bovine serum (FBS), bovine serum albumin (BSA), streptomycin, and penicilin sulfate were all purchased from Gibco.

Preparation of Poly(N-isopropylacrylamide-co-Acrylic acid) (p(NiPAAm-co-AAc)) and Poly(N-isopropylacrylamide-co-Acrylic acid-g-GRGDS) (p(NiPAAm-co-AAc-g-GRGDS)) Hydrogel

Poly(N-isopropylacrylamide-co-Acrylic acid) (p(NiPAAm-co-AAc)) copolymer was synthesized as reported previously [16]. Copolymer of NiPAAm with small amounts of AAc (typically 2–5 mol%) was synthesized by free-radical polymerization in benzene (10 wt% monomer concentration) with AIBN as an initiator (7×10^{-3} mol AIBN/mol of monomer). The monomer solution was bubbled with dried nitrogen gas for 30 min. After adding AIBN, the mixture was degassed under vacuum for 30 min prior to polymerization. The polymerization was conducted at 60°C for 16 h. The polymer precipitated in the benzene as the polymerization reaction proceeded. The precipitated polymer was dissolved in a warm acetone-methanol (90:10, v/v) mixture, precipitated in diethyl ether and then dried *in vacuo* for three days. The polymers were further purified by dialysis (15,000 or 50,000 Molecular Weight Cut Off (MWCO) dialysis tubing; Spectra, Rancho Dominguez, CA, U.S.A.) against distilled water at room temperature (around 25°C) for at least 96 h. The polymers were fractioned by successive solution fraction method at 15°C. Polymers with different conversions were obtained by lowering the polymerization temperature to 50°C and controlling the reaction time. Radical polymerization was also conducted in 1,4-dioxane as a control synthesis.

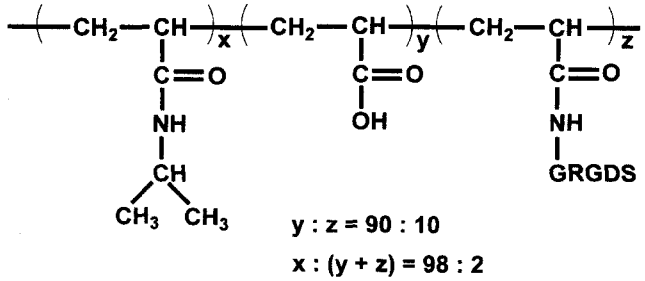

Fig. 1. Chemical structure of RGD-conjugated thermo-reversible hydrogel.

The GRGDS was coupled to poly(NiPAAm-co-AAc) by 1-ethyl-3-dimethylaminopropyl carbodiimide hydrochloride (EDC)-mediated amide bond. In order to immobilize the GRGDS peptide linkage into poly(NiPAAm-co-AAc), the carboxylic group of synthesized poly(NiPAAm-co-AAc) copolymer was activated with EDC in 50 mM of TEMED buffer at 4°C for 24 h, and then the solution was dialyzed against 10 l of distilled water. After the activation of carboxylic groups, GRGDS in TEMED buffer solution was added to the activated carboxylic group of hydrogel. The reaction mixture was kept in a cold room for 1 day. It was then dialyzed against 10 l of distilled water, and P(NiPAAm-co-AAc-g-GRGDS) was isolated by freeze-drying. The chemical structure is shown in Fig. 1. The content of the GRGDS peptide onto p(NiPAAm-co-AAc) copolymer was determined by NMR.

The gelation process was examined by applying the vial inversion method at various polymer concentrations. The temperature at which the solution no longer flowed in response to the vial inversion was caused by the gelation temperature. The temperature was kept constant for 5 h prior to inverting the vial in a water bath. This experiment was carried out at 1°C intervals.

Cell Culture

3T3-L1 fibroblast cells were precultured in RPMI 1640 in 5% CO_2 and 95% air at 37°C. The medium was supplemented with 10% fetal calf serum, 50 mg/l streptomycin, and 75 mg/l penicillin sulfate. Aqueous polymer solutions (5, 6, 8, and 10 w%, 300 μl) of each

copolymer were mixed with cells (1×10^5 cells). The cell suspensions mixed with copolymer were placed in a 12-mm diameter Millicell, having a 12 μm pore size, and then cultured in RPMI 1640 combined in 5% CO_2 and 95% air at 37°C. A competitive inhibition was studied by incubating cells in a serum-free media with 1.0 mg/ml of GRGDS or GREDS for 20 min prior to seeding. Cell growth and viability were monitored as a function of time for up to 10 days. The cell morphology entrapped in the copolymer gel was observed by using a phase-contrast microscope. The grown cell number entrapped in the copolymer gel was counted by using a hemacytometer.

RESULTS AND DISCUSSION

Gelation Processing Test

A high molecular weight (MW) of N-isopropylacrylamide copolymers with small amounts of acrylic acid (AAc) (typically 2–5 mole% in feed) were synthesized by a free-radical polymerization method in benzene. The molecular weights and compositions of the polymers in this study, determined by conventional characterization techniques, were reported [16]. As the typical gelation process in an aqueous polymer solution (5 wt%) in a phosphate buffered saline (PBS; pH 7.4, ionic strength (I)=0.2 M) with increasing temperature, the clear solution clouds at 29°C (cloud temperature) subsquently become immobile due to gel formation, without any significant gel induction time at 35°C (gelation temperature). Accordingly, the initially formed gel is translucent and becomes more opaque with further increasing temperature when it is above 43°C (gel shrinking temperature), when the gel starts to shrink (data not shown).

Cell Culture on Peptide-Conjugated Hydrogel

In order to define a method for peptide conjugation into otherwise, poorly adhesive hydrogels, we needed to engineer a biospecific cell adhesion to this material such as an adhesion peptide. The peptide employed in this study was an RGD-containing sequence, chosen to serve as a model peptide for the investigation of the method of peptide conjugation. Figure 2 illustrates the growth of fibroblast cells as a function of time for p(NiPAAm-co-AAc) copolymer gel with the GRGDS and GREDS sequences, or without peptides. The active peptide GRGDS promoted cell adhesion and proliferation in a time-dependent manner. After 36 h in culture, GRGDS in the hydrogel promoted approximately 50% spreading. However, when the copolymer gel was conjugated with the inactive GREDS peptide, the hydrogel did not promote the cell proliferation. Cell adhesion and proliferation to the copolymeric networks containing GRGDS conjugation were biospecific, i.e. these were mediated by cell membrane receptor interaction with the conjugated peptide, while the inactive peptide GREDS

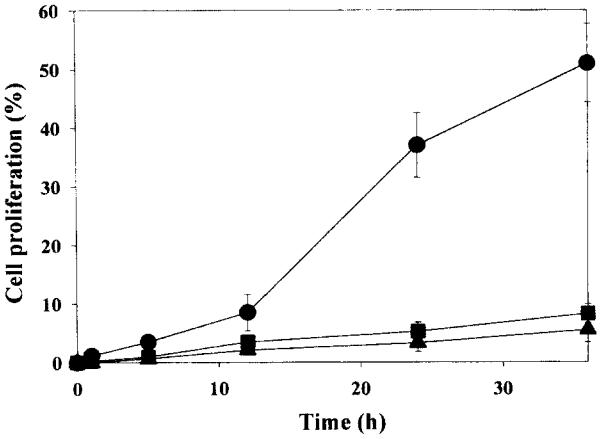

Fig. 2. Fibroblast cell spreading on RGD peptide immobilized into thermo-reversible hydrogel.
●: GRGDS-conjugated hydrogel, ■: GREDS-conjugated hydrogel, and ▲: without peptides conjugated hydrogel. The error bars represent mean±SD (n=5).

did not support cell spreading at similar copolymeric networks when it was similarly immobilized. The results support that GREDS exhibited very little nonspecific cellular adhesion on this copolymer gel. It is true that cell spreading on the GRGDS-conjugated gel was essentially identical to that on the biologically inactive peptide GREDS. The results also indicate that the growth of fibroblast cells cultured in reconstituted RGD sequence conjugated p(NiPAAm-co-AAc) is significantly different from that grown in the gel without it.

Also, cell growth as a function of time for a synthetic or natural matrix was investigated. The synthetic matrix of p(NiPAAm-co-AAc-g-GRGDS) showed similar behavior of cells on collagen-coated polystyrene. This result indicates

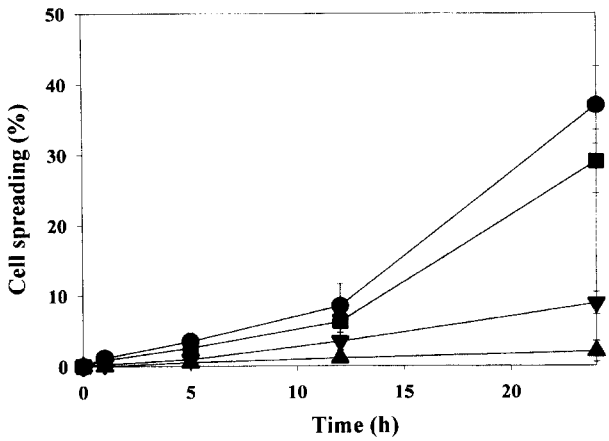

Fig. 3. Inhibition of fibroblast cell spreading on RGD peptide immobilized hydrogel by the presence of serum.
●: serum-containing and ■: serum-free medium GRGDS-conjugated hydrogel; ▲: serum-containing and ▼: serum-free medium GEGDS-conjugated hydrogel. The error bars represent mean±SD (n=5).

Table 1. Effect of addition of soluble peptides, RGD or RED, on inhibition of cell spreading.

	Addition of peptide	
	RED	RGD
GRGDS-conjugated gel	33.4±3.16 (%)	13.4±5.16 (%)
GRGDS-free gel	6.1±2.94 (%)	6.7±3.17 (%)

that the synthetic RGD sequence can mimic the function of adhesion molecules of collagens when it is covalently conjugated to modify the poorly adhesive hydrogel (data not shown).

Spreading of cells seeded in a serum-free medium was tested to confirm further evidence for peptide-mediated cell proliferation (Fig. 3). In a serum-free medium, cell spreading to GRGDS-conjugated gel was only slightly less than cell spreading in a serum-containing medium. However, cell spreading to GREDS-conjugated gel was significant in a serum-containing medium, while there was no cell spreading in a serum-free medium. This result indicates that adhesion and spreading are principally mediated by the adhesion peptide in the hydrogel since the serum contained numerous factors, in addition to adhesion proteins which may indirectly influence cell spreading.

Inhibition of Cell Spreading by Soluble Peptide

Table 1 summarizes the cell spreading to GRGDS-conjugated gel with RGD or RGE soluble peptides for 24 h. The fibroblast cell spreading to GRGDS-conjugated gel was obviously reduced by pretreatment of the soluble GRGDS, while the inclusion of pretreatment of soluble GREDS had no significant impact on cell spreading, as evidenced by the morphology and the degree of spreading of the attached cells. Pretreatment of fibroblast cells with soluble GRGDS peptide strongly blocked the spreading of cells. This suggests that the soluble form of GRGDS peptides interacted in advance with receptors of fibroblast cells expressed on the cell membrane. Therefore, the fibroblast cells did not recognize the sites of the GRGDS peptide-conjugated copolymer gel. These observations support the specificity of the RGD signal in mediating fibroblast cell attachment and spreading on GRGDS-conjugated gels.

Long-Term Cultivation of 3T3-L1 Fibroblast Cells

Cell density as a function of time was used as an index of cell proliferation. Figure 4 illustrates that the number of cells grown on the adhesion molecule of RGD peptide into p(NiPAAm-co-AAc) copolymer is approximately 2.5 times larger than the initially seeded cells after 5 days. The growth of fibroblast cells on the reconstituted RGD peptide-conjugated p(NiPAAm-co-AAc) increased 4 times as much in 10 days, while there was no significant increase in growth in the absence of the RGD sequence or a different type of peptide sequence. Thus, it is likely that fibroblast cells recognize the binding sites of RGD ligand of substrata and then spread. In a cell proliferation, it may be expected that cell-cell or cell-matrix interactions occur through a ligand-receptor specific interaction.

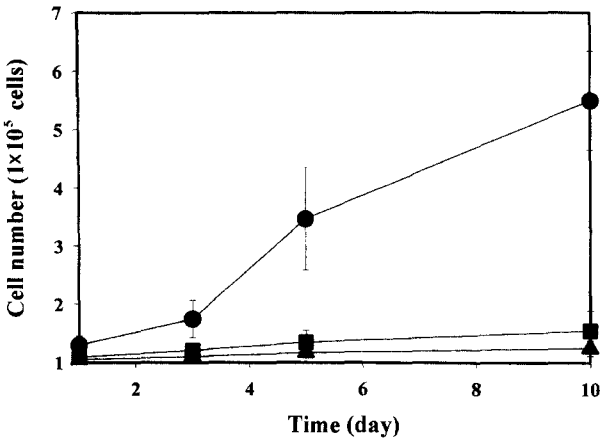

Fig. 4. Cell growth of fibroblast cells entrapped in gel with GRGDS or GREDS peptide and without peptides for a long-term culture.
●: GRGDS-conjugated gel, ■: GREDS-conjugated gel, and ▲: without peptides conjugated gel. The error bars represent mean±SD (n=5).

Effect of RGD Peptide Conjugation by Concentration

GRGDS conjugation into thermo-reversible hydrogels varied with the amount of peptide by 1, 5, and 10%. Figure 5 illustrates the percentage of fully spreaded cells on hydrogels containing GRGDS. The GRGDS promoted cell adhesion and spreading in a dose-dependent manner. After 24 h in culture, GRGDS in a 10% conjugated hydrogel promoted approximately 55% spreading. However, the dose-responsiveness of the two hydrogels was also quite

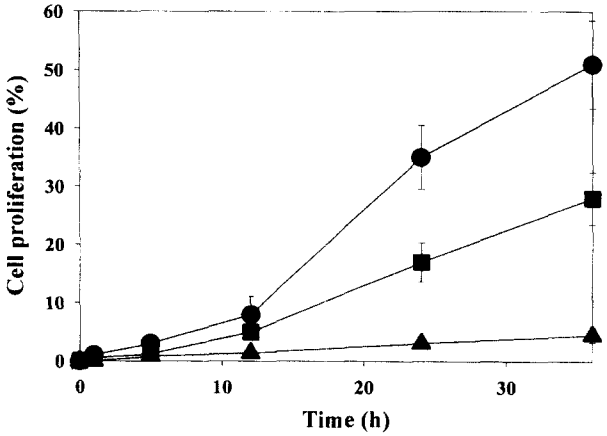

Fig. 5. Enhancement of cell proliferation on RGD peptide-conjugated hydrogel by different GRGDS peptide concentrations.
●: 10%, ■: 5%, and ▲: 1% GRGDS-conjugated gel. The error bars represent mean±SD (n=5).

similar when compared to the similar concentrations of 5% with 5% peptide-conjugation hydrogels promoting about 19%, and 1% peptide-conjugation hydrogels promoting about 7% spreading at 24 h. The lowest concentration was not much greater than the control gel (without the peptide gel).

Observation of Cell Morphology

Phase contrast photographs of fibroblast cells on an RGD sequence-conjugated copolymer gel against time are shown in Fig. 6. In the presence of GRGDS-conjugated gel, fibroblasts started to form the aggregates [Fig. 6(a)]. By the well-characterized interaction of RGD with integrins on the surfaces of adjacent cells, this specific binding leads to cell aggregation. After forming the cell aggregation, proliferated cells were observed significantly for the RGD-conjugated poly(NiPAAm-co-AAc) gel [Figs. 6(b) and 6(c)]. There was no significant difference between size of cell aggregates produced in the presence and absence of soluble GREDS peptides. When fibroblast cells were seeded on GREDS-conjugated hydrogel, only small aggregates were formed, while there was a big difference of cell proliferation. Although GRGDS-conjugated gel promoted cell aggregation, unconjugated gel and GREDS-conjugated hydrogel did not lead to cell aggregation, because they were not adhered onto the hydrogel and they maintained their morphology as a single cell (data not shown). Given the well-characterized interaction of RGD with integrins, GRGDS-conjugated hydrogel enhance aggregation by binding to integrins on surfaces of adjacent cells; this binding leads to cell aggregation [5]. In cell proliferation, it may be expected that cell-cell or cell-matrix interactions occurred through a ligand-receptor specific interaction. Therefore, the cell aggregates produced by peptide-polymer conjugates may have enhanced cell function compared with a single cell.

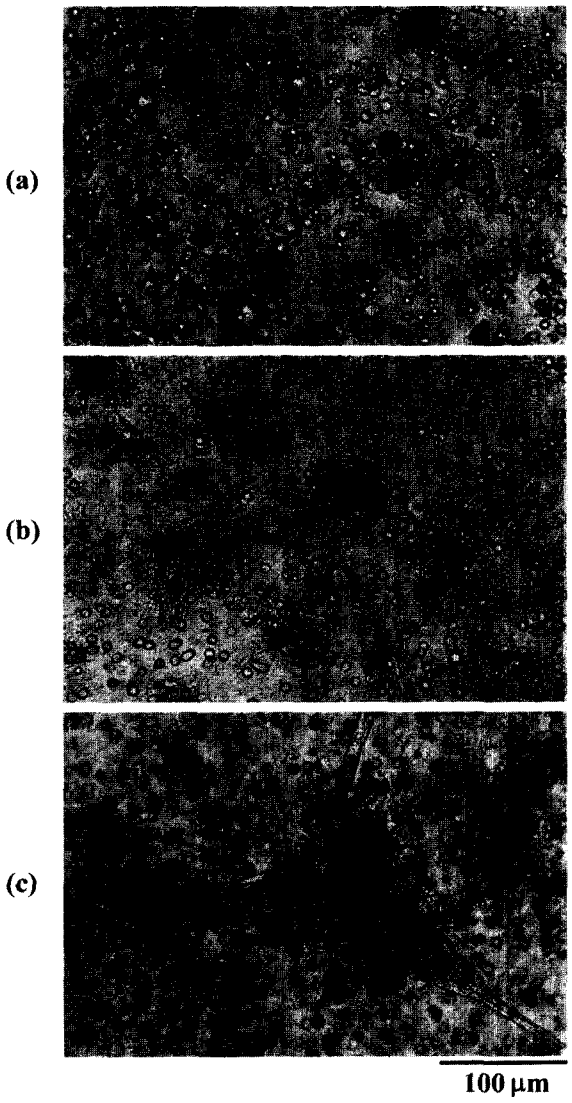

Fig. 6. Photomicrographs of fibroblasts cultured against time on hydrogels containing RGD peptides.
(a): 1 day later, (b): 3 days later, and (c): 10 days later.

From the above results, the behavior of fibroblast cells in RGD-containing hydrogel was found to be similar to the behavior of cells in a natural extracellular matrix of collagen. This indicates that the synthetic RGD sequence can mimic the function of natural ECM when covalently conjugated to hydrogel. Furthermore, cell aggregation by polymer-RGD conjugates is an interesting model system for the study of receptor-ligand interactions. For this reason, RGD-conjugated hydrogel might be useful in the field of tissue engineering that interacts through chemical signaling with cells, in addition to providing some mechanical support.

Acknowledgment

This work was supported by Korea Research Foundation Grant (99-041-00257F).

References

1. Andrade, J. D. and V. Hlady. 1986. Protein adsorption and materials biocompatibility: A tutorial review and suggested hypotheses. *Adv. Polym. Sci.* **79:** 1–63.
2. Aota, S. I., T. Nagai, and K. Yamada. 1991. Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. *J. Biol. Chem.* **266:** 15938–15943.
3. Brandley, B. K. and R. L. Schnaar. 1998. Covalent attachment of an Arg-Gly-Asp sequence peptide to derivatizable polyacrylamide surfaces: Support of fibroblast adhesion and long-term growth. *Anal. Biochem.* **172:** 270–278.

4. Cook, A. D., J. S. Hrkach, N. Cao, I. M. Johnson, U. B. Pajvani, S. M. Cannizaro, and R. Langer. 1997. Characterization and development of RGD-peptide-modified poly(lactic acid-co-lysine) as an interactive, resorbable biomaterial. *J. Biomed. Mater. Res.* **35:** 513–523.
5. Dai, W., J. Belt, and W. M. Saltzman. 1996. Fibroblast aggregation by suspension with conjugates of poly(ethylene glycol) and RGD. *Biotechnol. Bioeng.* **50:** 349–356.
6. Drumheller, P. D., D. L. Elbert, and J. A. Hubbell. 1994. Multifunctional poly(ethylene glycol) semi-interpenetrating networks as highly selective substrates for bioadhesive peptide grafting. *Biotechnol. Bioeng.* **43:** 772–780.
7. Grzesik, W. J. and P. G. Robey. 1994. Bone matrix RGD glycoproteins: Immunolocalization and interaction with human primary osteoblastic bone cells *in vitro*. *J. Bone Miner. Res.* **9:** 487–496.
8. Kim, S.-K., S.-H. Yu, J.-Y. Lee, A. Rademacher, D.-H. Lee, and J.-K. Park. 2001. Effect of collagen concentration on the viability and metabolic function of encapsulated hepatocytes. *J. Microbiol. Biotechnol.* **11:** 423–427.
9. Kobayashi, K. and H. Sumitomo. 1988. Oligosaccharide-carrying styrene-type macromers. Polymerization and specific interactions between the polymers and liver cells. *J. Macromol. Sci. Chem.* **A25:** 655–667.
10. Kugo, K., M. Okuno, K. Masuda, J. Nishino, H. Masuda, and M. Iwatsuki. 1994. Fibroblast attachment to Arg-Gly-Asp peptide-immobilized poly(gamma-methyl L-glutamate). *J. Biomater. Sci. Polym. Ed.* **5:** 325–327.
11. Lee, J. H., J. W. Jung, I.-K. Kang, and H. B. Lee. 1994. Cell behavior on polymer surfaces with different functional groups. *Biomaterials* **15:** 705–711.
12. Lin, H. B., Z. C. Zhao, C. Garcia-Exheverria, D. H. Rich, and S. L. Cooper. 1991. Synthesis of novel polyurethane copolymer containing covalently attached RGD peptide. *J. Biomater. Sci.* **3:** 217–227.
13. Maeda, M., Y. Izuno, K. Kawasaki, Y. Kaneda, Y. Mu, Y. Yasuo, S. Nakagawa, and T. Mayumi. 1997. Amino acids and peptides. Preparation of Arg-Gly-Asp (RGD) hybrids with poly(ethylene glycol) analogs and their antimetastatic effects. *Chem. Pharm. Bull.* **45:** 1788–1792.
14. Massia, S. P. and J. A. Hubbell. 1990. Covalent surface immobilization of Arg-Gly-Asp- and Try-Ile-Gly-Ser-Arg-containing peptides to obtain well-defined cell adhesive substrates. *Anal. Biochem.* **187:** 292–301.
15. Massia, S. P. and J. A. Hubbell. 1992. Vascular endothelial cell adhesion and spreading promoted by the peptide REDV of the IICS region of plasma fibronectin is mediated by integrin $\alpha 4\beta 1$. *J. Biol. Chem.* **267:** 14019–14026.
16. Na, K. and K.-H. Park. 2000. Conjugation of Arg-Gly-Asp (RGD) sequence into thermo-reversible gel as an extracellular matrix for 3T3-L1 fibroblast cell culture. *Biotechnology Letters* **22:** 1553–1556.
17. Rezania, A. and K. E. Healy. 1999. Biomimetic peptide surface that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteoblast-like cells. *Biotechnol. Prog.* **15:** 19–32.
18. Saiki, I. 1997. Cell adhesion molecules and cancer metastasis. *Jpn. J. Pharmacol.* **75:** 215–242.
19. Shin, J. S., H. I. Chang, H. C. Sung, and C. W. Kim 1999. The effects of fetal bovine serum, epidermal growth factor, and retinoic acid on adult rat islets embedded in collagen gels. *J. Microbiol. Biotechnol.* **9:** 150–156.
20. Tatarkiewicz, K., E. Sitarek, M. Sabat, and T. Orlowski. 1996. Long-term culture of non-purified rat islets embedded in hydrogel matrix. *Transplant Proc.* **28:** 831–832.
21. Vukicevic, S., F. P. Luyten, H. K. Kleinman, and A. H. Reddi. 1990. Differentiation of canalicular cell processes in bone cells by basement membrane matrix components: Regulation by discrete domains of lamine. *Cell* **63:** 437–445.
22. Weigel, P. H., R. L. Schnaar, M. S. Kuhlenschmidt, E. Schmell, R. T. Lee, Y. C. Lee, and S. Roseman. 1979. Adhesion of hepatocytes to immobilized sugars: A threshold phenomenon. *J. Biol. Chem.* **254:** 10830–10838.