TISSUE ENGINEERING
Volume 11, Number 5/6, 2005

Macrophage Adhesion on Gelatin-Based Interpenetrating Networks Grafted with PEGylated RGD

JEFFREY M. PHILLIPS, M.S.,[1] and WEIYUAN JOHN KAO, Ph.D.[1,2]

ABSTRACT

Human blood-derived macrophage adhesion on interpenetrating networks (IPNs) composed of PEGylated RGD-modified gelatin and poly(ethylene glycol) diacrylate was studied. The interaction between biomaterial immobilized with biofunctional peptides such as RGD and macrophages is central in the design of tissue-engineering scaffolds. PEGylated RGD-modified gelatin was synthesized via several steps involving PEG derivations and characterized by high-performance liquid chromatography, mass spectroscopy, gel permeation chromatography, and the trinitrobenzenesulfonic acid method. IPNs containing modified or unmodified gelatin were cultured with human macrophages and monitored at 2, 24, 96, and 168 h. At each time point, IPNs containing gelatin modified with PEGylated RGD showed a comparable adherent macrophage density as tissue culture polystyrene and a significantly higher cell density than other IPN formulations containing unmodified gelatin or gelatin modified with PEGylated triglycine. Although surface-immobilized RGD can serve to mediate the adhesion of different cell types on the biomaterial surface, the interaction of RGD with immune/inflammatory cells such as macrophages should also be considered when assessing the potential host response of tissue-engineering scaffolds.

INTRODUCTION

THE ARGININE-GLYCINE-ASPARTIC ACID (RGD) SEQUENCE, a ubiquitous cell adhesion motif, has received ongoing interest in biomaterial and tissue engineering research because of the proven ability of this peptide to promote cell adhesion.[1–4] The RGD motif is found in a variety of extracellular matrix (ECM) proteins including fibronectin, vitronectin, fibrinogen, collagens, and laminins.[5,6] Various cell types such as fibroblasts, macrophages, platelets, endothelial cells, and lymphocytes express extracellular membrane integrin receptors that recognize the RGD sequence of these proteins (Table 1).[5–8] Hence, RGD has been used extensively in a wide range of biomedical and tissue-engineering applications including the modification of biomaterials and tissue grafts,[9] biodegradable tissue scaffolds for cartilage regeneration,[10] transplantation matrices for hepatocytes,[11] and the promotion of cellular ingrowth in vascularized tissue matrices.[12] Macrophages, a key cell type in the host inflammatory and immune processes, express a large number of extracellular membrane receptors that recognize a myriad of surface-associated ligands such as ECM proteins.[13] For example, macrophage $\alpha_5\beta_1$ integrins complex with RGD-containing protein, resulting in cellular activation events that include phagocytosis, secretion, migration, and antigen presentation.[4] While biomaterials immobilized with RGD are designed to enhance the adhesion of selected desirable therapeutic cell types such as endothelial cells and keratinocytes in tissue-engineer-

[1]Department of Biomedical Engineering, College of Engineering, University of Wisconsin-Madison, Madison, Wisconsin.
[2]School of Pharmacy, University of Wisconsin-Madison, Madison, Wisconsin.

Table 1. Various Proteins That Contain the RGD Integrin-Binding Sequence, the Integrins That Bind the RGD Sequence, and the Various Cells That Express These Integrins[a]

Protein containing RGD sequence	*Integrins recognizing protein containing RGD sequence*	*Cells expressing integrin that recognize RGD-containing protein*
Fibronectin	$\alpha_5\beta_1$	Macrophages, fibroblasts, platelets, keratinocytes, memory T cells
Fibronectin, tenascin	$\alpha_8\beta_1$	Fibroblasts, neural cells, smooth muscle cells
Fibronectin, vitronectin	$\alpha_v\beta_1$	Endothelial cells
Fibronectin, vitronectin thrombospondin, von Willebrand factor	$\alpha_v\beta_3$	Macrophages, endothelial cells, platelets, B lymphocytes
Vitronectin	$\alpha_v\beta_5$	Fibroblasts, hepatoma cells
Fibronectin, vitronectin tenascin	$\alpha_v\beta_6$	Carcinoma cells
Collagens, laminins, fibronectin	$\alpha_v\beta_8$	Renal tubular epithelial cells
Collagens, fibronectin, vitronectin, fibrinogen, thrombospondin, von Willebrand factor	$\alpha_{III}\beta_3$	Platelets
Collagens, laminins	$\alpha_2\beta_1$[b]	Fibroblasts, platelets, endothelial cells, B and T lymphocytes
Fibronectin, laminins, thrombospondin	$\alpha_3\beta_1$[b]	B lymphocytes, kidney glomerular cells
Fibronectin, VCAM	$\alpha_4\beta_1$[b]	Macrophages, lymphocytes, NK cells, lymphocytes, eosinophils, thymocytes
Laminins	$\alpha_7\beta_1$[b]	Developing skeletal and cardiac muscle, melanoma cells

[a]Adapted from Refs. 1–4.
[b]Denotes weak RGD–integrin binding or binding only under special conditions.

ing applications, the potential recognition of these ligands by macrophages should also be considered.

Several interpenetrating network (IPN) systems have been designed for a variety of tissue-engineering applications including tissue scaffolding, bioadhesives, implant coatings, and the delivery of pharmaceutical or bioactive reagents.[14–17] Polyethylene glycol diacrylate (PEGdA) has been combined with collagens, alginate, gelatin, and hyaluronic acid to form IPNs that have proven successful in delivering bioactive substances and sustaining cell populations both *in vivo* and *in vitro*.[18–20] Previously, we have synthesized multifunctional IPNs composed of PEGdA and chemically modified gelatin as tissue scaffolds and drug delivery matrices.[21,22] These IPNs have demonstrated an increased tensile strength and elasticity when compared with conventional hydrogels and an enhanced drug delivery efficacy *in vivo*.[21,22] The presence of PEGdA in the IPN formulation is designed to provide photoactive moieties and to minimize non-specific cell interaction. A gelatin backbone in these IPNs provides a potential platform for the immobilization of bioactive molecules. The immobilized factor on the gelatin backbone can function in mediating a desirable cellular behavior. In this study, we have modified gelatin with bioactive peptides (i.e., RGD), using heterodifunctionalized polyethylene glycol (PEG) as a linker. The resulting modified gelatin is utilized in the formation of IPNs and the adhesion of macrophages on these substrates is analyzed.

MATERIALS AND METHODS

Synthesis and characterization of PEGylated-peptide modified gelatin

The reaction scheme for PEGylated-peptide modified gelatin (Fig. 1) has four main stages: (1) the synthesis of

Bis-COOH-PEG* from PEG-diol followed by the addition of a ethyl acetate group to both ends, (2) activation of the terminal COOH groups with *N*-hydroxysuccinimide (*N*-HOSu), (3) peptide conjugation to Bis-NSu-acetate-PEG to form NSu-PEG-peptide, and (4) gelatin modification with NSu-PEG-peptide. All starting materials were received without additional purification and purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

Bis-COOH-PEG synthesis. One equivalent per mole PEG (2000 Da) was dissolved in dry THF followed by the addition of 16 eq/mol sodium hydride (NaH), stirred at room temperature for 1 h in an argon environment. Twenty equivalents per mole of ethyl bromoacetate (EtBrAc) was then added to this solution, stirred under argon at room temperature for 1 h, filtered, precipitated in cold hexane, and dried in a vacuum oven. A mixture of PEG, HO-PEG-EtAc, and Bis-EtAc-PEG was obtained. Although several PEG:NaH:EtBrAc molar ratios were utilized (Table 2A), a respective 1:16:20 ratio resulted in the maximum possible conversion of Bis-COOH-PEG from PEG. The PEG, HO-PEG-EtAc, and Bis-EtAc-PEG dried mixture was dissolved in deionized water followed by the addition of 20 eq/mol of 1 N NaOH, adjusted to pH 12.5, stirred for 1 h, and extracted with methylene chloride. The aqueous phase was removed, adjusted to pH 9.0 with 1 N HCl, and extracted again with methylene chloride. The aqueous phase was removed again, adjusted to pH 3.0, and extracted for a third time. The organic phase solution was evaporated under vacuum in a Rotavapor (BUCCI R-114, Switzerland), and dried in a vacuum oven. The Bis-COOH-PEG (2000 Da) product was characterized with a reversed-phase HPLC system (Gilson, 10 to 100% acetonitrile, 1-mL/min flow rate for 30 min) coupled with UV/Vis and evaporative light-scattering detectors (ELSD).

Bis-NSu-PEG synthesis and peptide conjugation. One equivalent per mole of the mixture containing Bis-COOH-PEG was dissolved in dry THF, followed by the addition of 8 eq/mol N-HOSu. A total of 8 eq/mol of *N,N′*-dicyclohexylcarbodiimide (DCC) was separately dissolved in THF and then added dropwise to this solution (Fig. 1). The solution was stirred at room temperature for 4 h, filtered, precipitated in cold hexane, dried in a vacuum oven, and the resulting Bis-NSu-PEG mixture was characterized by reversed-phase HPLC. A total of 1 eq/mol of the solution containing Bis-NSu-PEG was then dissolved in DMF under argon followed by the addition of 1.5 eq/mol of RGD (98% purity; University of Wisconsin Biotechnology Center) or GGG (99% purity; Bachem, King of Prussia, PA). A total of 1.5 eq/mol of *N,N*-diisopropyl ethylamine (DIPEA) was added to the mixture, and HPLC was used to monitor the peptide conjugation as a function of reaction time at 0, 15, 30, 40, and 60 min. The product NSu-PEG-RGD was further characterized by MS (Fourier transform HiRes MALDI mass spectrometer, 0.1-mg/mL sample dilution in water; IonSpec 7.0T).

Gelatin modification with NSu-PEG-peptide. One equivalent per mole of NSu-PEG-RGD was added to a solution containing 1% gelatin (300 bloom, porcine skin; Sigma Aldrich) in PBS. The mixture was adjusted to pH 8.0 and stirred for 1 h at room temperature. A pressurized ultrafiltration system with a 30-kDa membrane filter was used to separate PEG-peptide modified gelatin from residual Bis-peptide-PEG side products and unreacted NSu-PEG-peptide. MPEG-modifed gelatin was synthesized according to a similar reaction scheme but with MPEG (2000 Da) as the starting material in lieu of PEG-diol. Unmodified gelatin, RGD-PEG-modified gelatin, GGG-PEG-modified gelatin, and MPEG-modified gelatin were characterized with an Ultrahydrogel column in a gel-permeation chromatography (GPC) system (Waters, 80% 0.1 M $NaHO_3$, 20% acetonitrile at a 0.7-mL/min flow rate for 60 min) coupled with a refractive index (RI) detector. The percent modification of gelatin lysyl residues with PEG-peptide was determined by an established method based on trinitrobenzenesulfonic acid (TNBS) and spectrophotometry.[23,24] The number of lysyl residues and percent modification for unmodified and modified gelatins are shown in Table 3.

*Abbreviations used: Bis-COOH-PEG, bis-carboxylate-PEG; Bis-EtAc-PEG, bis-ethylacetate-PEG; Bis-NSu-PEG, bis-*N*-succinimidyl-acetate-PEG; DCC, 1,3-dicyclohexylcarbodiimide; DIPEA, *N,N*-diisopropyl ethylamine; DMF, *N,N*-dimethyl formamide; DMPA, 2,2-dimethoxy-2-phenyl-acetophenone; EDTA, ethylenediaminetetraacetic dianhydride; ELSD, evaporative light scattering; ECM, extracellular matrix; EtBrAc, ethyl bromoacetate; FN, fibronectin; GPC, gel-permeation chromatography; HO-PEG-COOH, α-hydroxy-ω-carboxy-PEG; HO-PEG-EtAc, α-hydroxy-ω-ethylacetate-PEG; HO-PEG-NSu, α-hydroxy-ω-*N*-succinimidyl-acetate-PEG; HO-PEG-GGG, α-hydroxy-ω-glycine-glycine-glycine-acetate-PEG; HO-PEG-RGD, α-hydroxy-ω-arginine-glycine-aspartic acid-acetate-PEG; HPLC, high-performance liquid chromatography; IPN, interpenetrating network; Mp, peak molecular weight; MPEG, polyethylene glycol monomethyl ether; MPEG-COOH, α-methoxy-ω-carboxyl-PEG; MPEG-EtAc, α-methoxy-ω-ethyl-acetate-PEG; MPEG-IPN, MPEG-modified gelatin IPN; MPEG-NSu, α-methoxy-ω-*N*-succinimidyl-acetate-PEG; MS, mass spectroscopy; N-HOSu, *N*-hydroxysuccinimide; NSu-PEG-GGG, α-*N*-succinimidylacetate-ω-glycine-glycine-glycine-PEG; NSu-PEG-RGD, α-*N*-succinimidylacetate-ω-arginine-glycine-aspartic-PEG; OD, optical density; PBSE, PBS with 5 mM EDTA; PEG, polyethylene glycol (diol) 2K; PEGdA, polyethylene glycol diacrylate; TCPS, tissue culture polystyrene; TNBS, trinitrobenzenesulfonic acid.

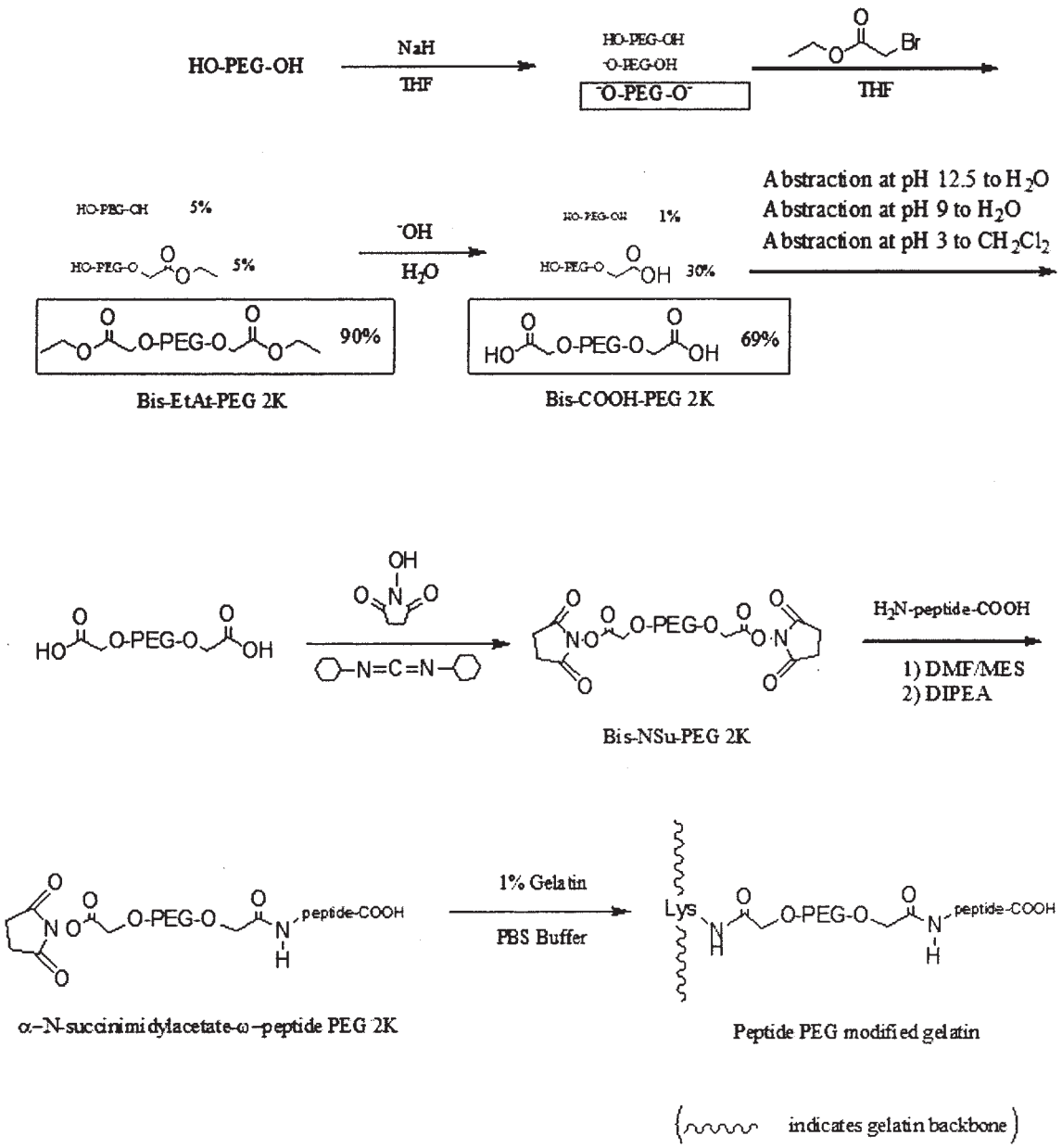

FIG. 1. Synthesis scheme of peptide-PEG-modified gelatin.

IPN synthesis

Modified or unmodified gelatin was dissolved in deionized water at 37°C. This solution was combined with PEGdA (600 Da) in a 4:6 gelatin:PEGdA weight ratio to form a 10 wt% solution according to previously established procedures.[25] Briefly, 20 μL of 2,2-dimethoxy-2-phenyl-acetophenone (DMPA) initiator solution (0.02 g of DMPA in 0.5 g of PEGdA) was added to the gelatin–PEGdA mixture, vortexed, injected into a PTFE mold (diameter, 7 mm; thickness, 0.75 mm), and secured between two glass slides. IPN solution within the PTFE molds was irradiated with UV light (maximal intensity, 365 nm; 21,700 μW/cm^2 at a distance of 2.5 cm from mold surface, UVP, model B 100 AP) for 3 min from both sides. The resulting IPNs were allowed to cool for several minutes and then removed from the PTFE molds.

Monocyte adhesion assay

Human monocytes were isolated from citrated whole blood of healthy adult volunteers according to an established density gradient, nonadhesion method.[26] All IPN samples were sterilized via γ irradiation (1 Mrad) and placed in individual wells of 48-well TCPS plates and cultured with monocytes at a concentration of 10^6

Table 2. Molar Ratios of Reactants Used in the Synthesis of Peptide-Conjugated PEG

A. Bis-COOH-PEG synthesis

PEG 2K in THF (mmol)	*NaH (mmol)*	*EtBrAc (mmol)*
1	1.5	1.5
1	1.7	1.7
1	2	2
1	4	4
1	8	8
1	16	16
1	16	20
1	16	40

B. Bis-Nsu-PEG synthesis and peptide conjugation

Bis-NSu-PEG 2K in DMF (mmol)	*Peptide (RGD, GGG) (mmol)*	*DIPEA (mmol)*
1	1.5	1
1	1.5	1.5
1	2	1.5
1	2	2

cells/mL (i.e., 5×10^5 cells/cm^2 surface area) in RPMI culture medium containing 10% autologous serum at 37°C under 5% CO_2.[1,27] At 2, 24, and 96 h, samples were washed with RPMI medium to remove nonadherent cells and fresh culture medium was added. At 2, 24, 96, and 168 h, samples were washed and stained with Wright's stain, and adherent macrophages were quantified using a computer-assisted video analysis system (MetaMorph version 4.1; Universal Imaging, Downingtown, PA) coupled to an inverted light microscope (Eclipse TE300; Nikon, Tokyo, Japan). Adherent cell density is expressed as number of cells per square millimeter of surface area.

Statistical analysis

All experimental results were expressed as means ± standard error of the mean. HPLC data ($n = 3$), GPC data ($n = 2$), and TNBS data ($n = 3$) were averaged. Monocyte adhesion data (three independent samples of each substrate in triplicate for each of the four culture time points) were analyzed by two-way analysis of variance

Table 3. Total Number of Modified Gelatin Lysyl Residues and Percent Modification as Determined by TNBS Method[a]

Modified gelatin	*No. of lysyl residues*	*Percent modification*
Unmodified gelatin	6.653 ± 0.070	0
MPEG-modified gelatin	0.829 ± 0.035	87.4 ± 0.5
GGG-PEG-modified gelatin	1.170 ± 0.027	81.1 ± 0.4
RGD-PEG-modified gelatin	1.235 ± 0.061	81.6 ± 0.9

[a]All values are expressed as means ± SEM; $n = 3$.

and Tukey post hoc testing at $p < 0.01$ using SigmaStat version 2.03 software (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Synthesis of gelatin modified with PEGylated peptide

Molar ratios of reagents versus PEG derivative, abstraction conditions, and reaction time are all critical in the synthesis of gelatin modified with PEGylated peptide. The conversion of PEG to Bis-COOH-PEG relied heavily on the molar ratios of the ionizing reagent, NaH, and EtBrAc, to PEG. Several PEG:NaH:EtBrAc ratios were utilized (Table 2A); however, a ratio of 1:16:20 resulted in the maximal conversion of PEG to Bis-COOH-PEG while minimizing the side product intermediate HO-PEG-COOH. High NaH concentration ensured the ionization of both terminal hydroxyl moieties. Similarly, EtBrAc, in a proportionally large concentration allowed the ethyl acetate group to add to both ends of the ionized PEG species. The percent yield of each species at all stages of the synthesis is quantified on the basis of normalized peak areas of HPLC chromatograms (Table 4). After the addition of ethyl bromoacetate, the Bis-EtAc-PEG species resulted in ~90% yield with the HO-EtAc-PEG and PEG species at 5% each. Subsequently, abstractions at several pH levels were performed to optimize the condition for separating each of these three species. At pH 12.5, PEG abstracted to methylene chloride because of its high solubility in the organic phase, while the COOH groups of Bis-COOH-PEG and HO-PEG-COOH ionized, leaving the two species in the aqueous phase. A second abstraction at pH 9.0 resulted in the separation of Bis-COOH-PEG from most of the HO-COOH-PEG, leaving the compounds in the aqueous and organic phases, respectively. A third abstraction of Bis-COOH-PEG to methylene chloride for solvent removal occurred at pH 3.0. After abstraction, Bis-COOH-PEG was characterized by HPLC with a peak resulting at an elution time range between 6.3 and 7.3 min (Fig. 2). The peak to the right of the Bis-COOH-PEG peak indicates the

Table 4. HPLC Elution Time, Normalized Peak Area, and Percent Yield for PEG Derivatives and Intermediates Synthesized from PEG 2K[a]

Compound[b]	*Elution time (min)*	*Normalized peak area* (N_i)	*Yield (%)*	*UV signal*
PEG	10.6	1	100	No
PEG	10.6	1	5	No
HO-PEG-EtAc	11.6	19.0	90	Weak
Bis-EtAc-PEG	12.8	1.1	5	Weak
Bis-COOH-PEG	6.5	64.6	69	No
HO-PEG-COOH	7.8	27.5	30	Weak
PEG	10.6	1	1	Weak
COOH-PEG-NSu	9.2	2.9	3	No
PEG	10.6	1	1	No
HO-PEG-NSu	12.0	15.6	17	Weak
Bis-NSu-PEG	13.2	69.9	78	Medium
Bis-RGD-PEG	7.3	131.2	56	No
NSu-PEG-RGD	8.6	77.3	33	No
PEG	10.6	1	0	No
Bis-NSu-PEG	13.1	25.2	11	Medium
Bis-GGG-PEG	7.4	45.6	34	No
NSu-PEG-GGG	8.8	64.7	48	No
PEG	10.6	1	1	No
Bis-NSu-PEG	13.2	23.1	17	Medium

[a]All values are expressed as a mean of peak data from three independent syntheses ($n = 3$). Peaks were normalized with the ELSD signal of the internal PEG 2K to calculate the percent yield ($N_i/\Sigma N$), where N_i is the normalized peak area for each peak and ΣN is the sum of N_i.

[b]Each subgroup of compounds corresponds to the products resulting at the end of each reaction stage shown in Fig. 1.

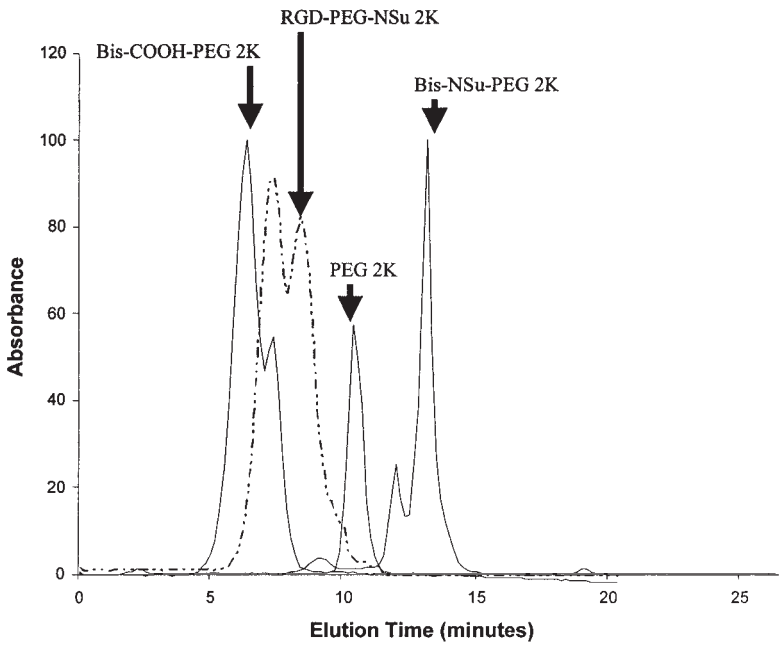

FIG. 2. ELSD signals of HPLC chromatograms of PEG 2K, bis-COOH-PEG 2K, bis-NSu-PEG 2K, and RGD-PEG-NSu 2K. Products were characterized with a reversed-phase HPLC system (Gilson, 10 to 100% acetonitrile at at 1-mL/min flow rate for 30 min, coupled with a UV/Vis detector and an evaporative light-scattering detector [ELSD]). For each material, a representative chromatogram from three independent syntheses is shown.

presence of a small amount of HO-PEG-COOH remaining in the final product. Bis-COOH-PEG resulted in a final yield of 69% (Table 4).

The terminal COOH groups of Bis-COOH-PEG subsequently were activated with N-HOSu to facilitate further peptide conjugation.[28,29] The Bis-NSu-PEG product was characterized by HPLC and resulted in a peak at a higher elution time range of 12.9 to 13.2 min (Fig. 2), when compared with Bis-COOH-PEG, because of the increased hydrophobicity of the activating *N*-succidimidyl groups. Two smaller peaks near elution times of 9.2 and 12.0 min indicate the presence of COOH-PEG-NSu and HO-PEG NSu, respectively (Fig. 2). The final yield of the Bis-NSu-PEG product was 78% (Table 4).

During the conjugation of Bis-NSu-PEG with peptides, both Bis-NSu-PEG:peptide:DIPEA molar ratios and reaction time were important in obtaining the highest possible yield of NSu-PEG-peptide. Several Bis-NSu-PEG:RGD:DIPEA molar ratios were utilized (Table 2B). A respective ratio of 1:1.5:1.5 resulted in

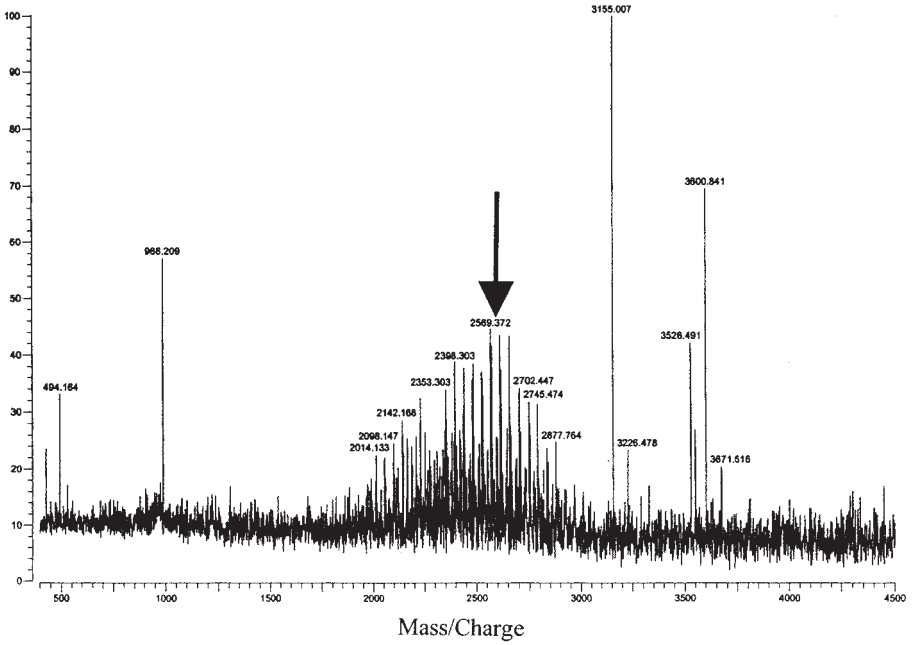

FIG. 3. MS spectroscopy of RGD–PEG conjugate. Peak molecular weight is identified by the arrow at 2569.372 g/mol.

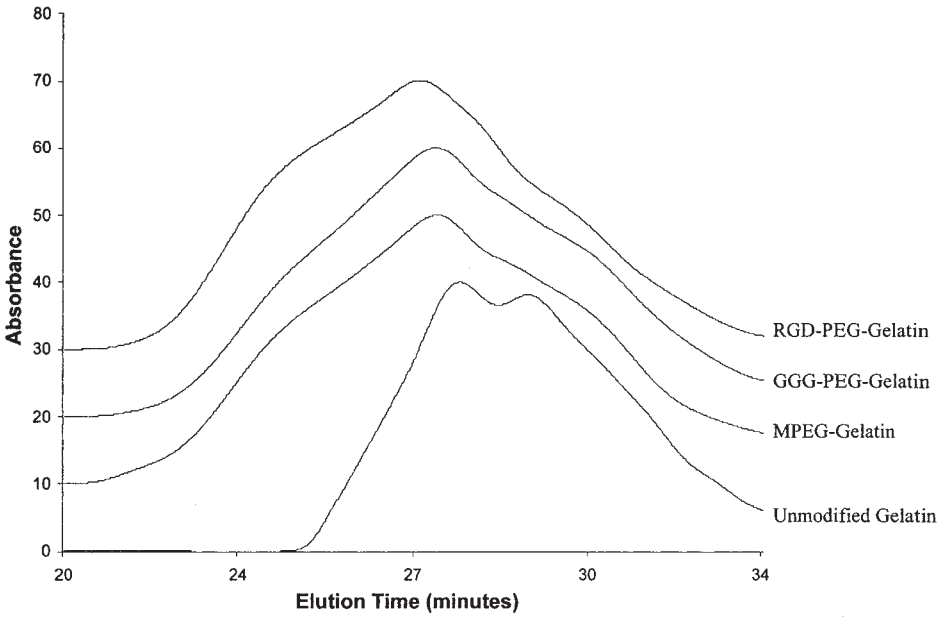

FIG. 4. Gel-permeation chromatography (GPC) chromatograms. Gelatin and various forms of modified gelatin were characterized with an Ultrahydrogel column in a GPC system (Waters, 80% 0.1 M $NaNO_3$ with 20% acetonitrile at a 0.7-mL/min flow rate for 60 min coupled with an refractive index [RI] detector). For each material, a representative chromatogram of two independent syntheses is shown.

the highest conversion to NSu-PEG-peptide. HPLC was used to monitor the conjugation reaction as a function of reaction times of 0, 15, 30, 40, and 60 min. A reaction time of 40 min resulted in the optimal formation of NSu-PEG-RGD. NSu-PEG-RGD was characterized by HPLC and resulted in a peak at an elution time range of 8.3 to 8.7 min (Fig. 2). The larger peak at elution time 7.3 min indicates Bis-RGD-PEG species that formed in greater yield. The final yield of NSu-PEG-RGD was 33% (Table 4). Conjugation of Bis-NSu-PEG with GGG was also performed according to similar procedures and resulted in a yield of 48% (Table 4). The RGD-PEG conjugation was verified by MS, showing a peak molecular weight (Mp) of 2569.372 g/mol (Fig. 3), whereas the theoretical calculated molecular weight of RGD-PEG is 2545 g/mol.

Subsequently, the mixture containing NSu-PEG-peptide and Bis-RGD-PEG was utilized to modify the lysine residues of gelatin (Fig. 1). Bis-peptide-PEG side products were not expected to conjugate to gelatin lysyl groups because of the absence of the activating NSu moiety. To separate PEG-peptide-modified gelatin from Bis-peptide-PEG and residual unreacted NSu-PEG-peptides, the final reaction mixture was passed through a pressurized ultrafiltration (Millipore, Bedford, MA) with a 30-kDa filter membrane. Unmodified gelatin, RGD-PEG-modified gelatin, GGG-PEG-modified gelatin, and MPEG-modified gelatin were further characterized by GPC. GPC peak elution time for each of the modified gelatins decreased, indicating an increase in molecular weight in comparison with unmodified gelatins (Fig. 4). The largest species, RGD-PEG-modified gelatin, eluted first with GGG-PEG-modified gelatin and MPEG-modified gelatin eluting later. TNBS confirmed gelatin lysyl group modification for these three species with 81.6, 81.1, and 87.4% modification, respectively (Table 3).

TABLE 5. ADHERENT MONOCYTE DENSITY ON TCPS AND VARIOUS IPNs CONTAINING MODIFIED AND UNMODIFIED GELATIN, DETERMINED AT 2, 24, 96, AND 168 h OF CULTURE[a]

	Culture time			
	2 h	*24 h*	*96 h*	*168 h*
TCPS	587 ± 157[c]	502 ± 236[c]	260 ± 152[c]	262 ± 160[c]
Unmodified gelatin IPN	177 ± 70[b]	71 ± 23[b]	28 ± 20[b]	12 ± 6[b]
MPEG-modified gelatin IPN	109 ± 26[b]	106 ± 31[b]	23 ± 9[b]	12 ± 6[b]
GGG-PEG-modified gelatin IPN	116 ± 60[b]	128 ± 57[b]	28 ± 25[b]	15 ± 10[b]
RGD-PEG-modified gelatin IPN	437 ± 166[c]	417 ± 238[c]	233 ± 142[c]	191 ± 121[c]

[a]All values are expressed as number of cells per mm^2 surface area, mean ± SEM; $n = 3$, in triplicate.
[b]Significantly different from TCPS positive control at $p < 0.01$, ANOVA.
[c]Significantly different from unmodified gelatin IPN negative control at $p < 0.01$, ANOVA.

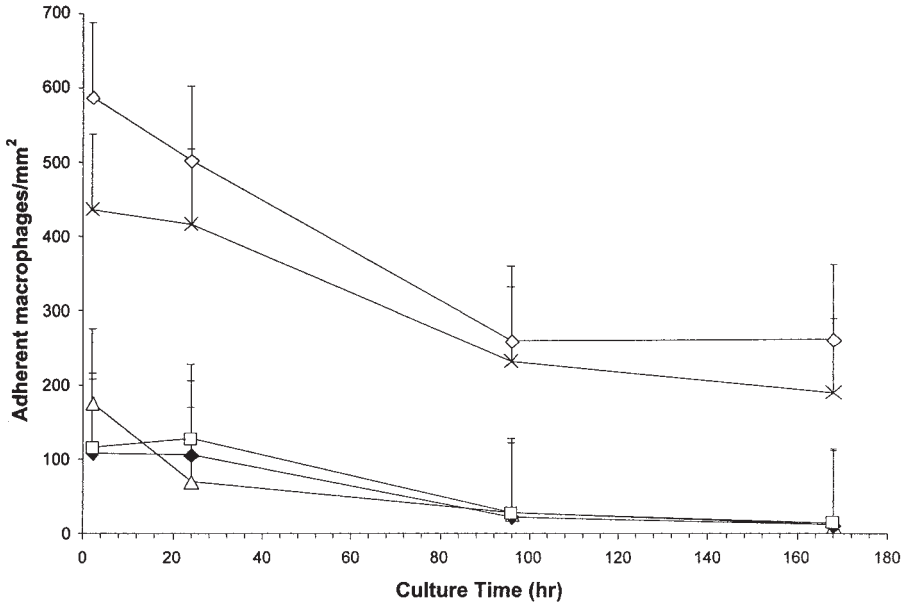

FIG. 5. Adherent monocyte density on TCPS (◇), RGD-PEG-modified IPN (×), unmodified gelatin IPN (△), GGG-PEG-modified IPN (□), and MPEG-modified IPN (◆) at 2, 24, 96, and 168 h.

Monocyte adhesion assay

Adherent monocyte densities at 2, 24, 96, and 168 h on TCPS or IPNs containing unmodified gelatin or gelatin modified with MPEG, GGG-PEG, or RGD-PEG were quantified and expressed as number of cells per square millimeter of surface area (Table 5 and Fig. 5). At each time point, adherent monocyte densities on IPNs with unmodified gelatin, or gelatin modified with MPEG or GGG-PEG, were significantly lower than that on TCPS controls, but not different from each other. At each time point, RGD-PEG-modified gelatin IPNs showed a comparable macrophage density with TCPS and a significantly higher adherent monocyte density than each of the other IPN formulations. Adherent macrophage density on all substrates decreased gradually with increasing culture time. This is expected from nonproliferative primary macrophages. On the basis of these results, the presence of PEGdA in the IPN formulation played a significant role in minimizing cell adhesion (i.e., IPN containing unmodified gelatin). This allows for the modification of IPNs with immobilized ligands such as peptides and soluble factors (i.e., drugs, growth factors) to specifically affect the behavior of a selected cell type. The RGD peptide sequence has been incorporated extensively into biomaterials to promote the interaction of different types of cells, such as fibroblasts and keratinocytes. Because immune/inflammatory cells such as macrophages also recognize surface-immobilized RGD sequences, a more detailed study of ligand–receptor complexation, subsequent intracellular signaling, and phenotypic behavior is needed. For example, the PHSRNG_6RGD peptide, but not RGDG_6PHSRN, RGD, or PHSRN, has been shown to play a unique and necessary role in mediating foreign body giant cell formation from the fusion of macrophages.[2,13] Giant cells are standard histological markers of end-stage inflammation; however, the causal relationship between materials biocompatibility and the presence of giant cells is highly debated. The judicial selection of bioactive ligands in promoting the interaction of a specific cell type, but not others, requires a mechanistic understanding of ligand–receptor association and subsequent cellular events.

CONCLUSION

Molar ratios of PEG derivatives versus other reactants, abstraction conditions, and reaction time were all critical in the synthesis of gelatin modified with PEGylated-peptides as a component in IPN formulation. IPNs containing RGD-PEG-modified gelatin showed a significantly higher macrophage density when compared with IPNs containing unmodified gelatin and peptide controls. While RGD promotes the adhesion of a variety of cell types, the role of this peptide on immobilization in mediating macrophage interaction should also be addressed in tissue-engineering design.

ACKNOWLEDGMENTS

This work was supported by NIH grants EB-00290 and HL-77825. The authors thank Jing Li for guidance in PEG derivative synthesis and characterization, Rick Witte for assistance in IPN polymerization, and Gary Girdaukas for use of mass spectroscopy equipment and software.

REFERENCES

1. Kao, W.J., and Hubbell, J.A. Murine macrophage behavior on peptide-grafted polyethylene glycol-containing networks. Biotechnol. Bioeng. **59,** 2, 1998.
2. Kao, W.J., Liu, Y., Gundloori, R., Li, J., Lee, D., Einerson, N., Burmania, J., and Stevens, K. Engineering endogenous inflammatory cells as delivery vehicles. J. Control. Release **78,** 219, 2002.
3. Kao, W.J. Evaluation of protein-modulated macrophage behavior on biomaterials: Designing biomimetic materials for cellular engineering. Biomaterials **23/24,** 2213, 1999.
4. Liu, Y., and Kao, W.J. Human macrophage adhesion on fibronectin: The role of substratum and intracellular signaling kinases. Cell Signal. **14,** 145, 2002.
5. Ruoslahti, E. RGD and other recognition sequences for integrins. Annu. Rev. Cell Dev. Biol. **12,** 697, 1996.
6. Koster, J. Integrin Subunits and Combinations. Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands, 2002. Available at URL http://integrins.hypermart.net/combinations (accessed March 2005).
7. Muller, U., Bossy, B., Venstrom, K., and Reichardt, L.F. Integrin $\alpha_8\beta_1$ promotes attachment, cell spreading, and neurite outgrowth on fibronectin. Mol. Biol. Cell **4,** 433, 1995.
8. Marshall, J.F., Rutherford, D.C., McCartney, A.C.E., Mitjans, F., Goodman, S.L., and Hart, I.R. $\alpha_v\beta_1$ is a receptor for vitronectin and fibrinogen, and acts with $\alpha_5\beta_1$ to mediate spreading on fibronectin. J. Cell Sci. **108,** 1227, 1995.
9. Hersel, U., Dahmen, C., and Kessler, H. RGD modified polymers: Biomaterials for stimulated cell adhesion and beyond. Biomaterials **24,** 4385, 2003.
10. Hsu, S.H., Whu, S.W., Hsieh, S.C., Tsai, C.L., Chen, D.C., and Tan, T.S. Evaluation of chitosan–alginate–hyaluronate complexes modified by an RGD-containing protein as tissue-engineering scaffolds for cartilage regeneration. Artif. Organs **28,** 693, 2004.
11. Pinkse, G.G., Voorhoeve, M.P., Noteborn, M., Terpstra, O.T., Bruijn, J.A., and De Heer, E. Hepatocyte survival depends on β_1-integrin-mediated attachment of hepatocytes to hepatic extracellular matrix. Liver Int. **3,** 218, 2004.
12. Zisch, A.H., Lutolf, M.P., Ehrbar, M., Raeber, G.P., Rizzi, S.C., Davies, N., Schmokel, H., Bezuidenhout, D., Djonov, V., Zilla, P., and Hubbell, J.A. Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth. FASEB J. **17,** 2260, 2003.
13. Kao, W.J., Lee, D., Schense, J.C., and Hubbell, J.A. Fibronectin modulates macrophage adhesion and FBGC formation: The role of RGD, PHSRN, and PRRARV domains. J. Biomed. Mater. Res. **55,** 79, 2001.
14. Nakayama, Y., and Matsuda, Y. Photocurable surgical tissue adhesive glues composed of photoreactive gelatin and poly(ethylene glycol) diacrylate. J. Biomed. Mater. Res. **48,** 511, 1999.
15. Barber, T.A., Golledge, S.L., Castner, D.G., and Healy, K.E. Peptide-modified p(AAm-co-EG/AAc) IPNs grafted to bulk titanium modulate osteoblast behavior *in vitro*. J. Biomed. Mater. Res. **64A,** 38, 2003.
16. Okino, H., Nakayama, Y., Tanaka, M., and Matsuda, T. *In situ* hydrogelation of photocurable gelatin and drug release. J. Biomed. Mater. Res. **59,** 233, 2002.
17. Elisseef, J., McIntosh, W., Anseth, K., Riley, S., Ragan, P., and Langer, R. Photoencapsulation of chondrocytes in poly(ethylene oxide)-based semi-interpenetrating networks. J. Biomed. Mater. Res. **2,** 164, 2000.
18. Park, Y.D.,Tirelli, N., and Hubbell, J.A. Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks. Biomaterials **24,** 893, 2003.
19. Desai, N.P., Sojomihardjo, A., Yao, Z., Ron, N., and Soon-Shiong, P. Interpenetrating polymer networks of alginate and polyethylene glycol for encapsulation of islets of Langerhans. J. Microencapsul. **17,** 677, 2000.
20. Cho, C.S., Han, S.Y., Ha, J.H., Kim, S.H., and Lim, D.Y. Clonazepam release from bioerodible hydrogels based on semi-interpenetrating polymer networks composed of poly(ϵ-caprolactone) and poly(ethylene glycol) macromer. Int. J. Pharm. **181,** 235, 1999.
21. Zilinski, J., and Kao, W.J. Tissue adhesiveness and host response of *in situ* photopolymerizable interpenetrating networks containing methylprednisolone acetate. J. Biomed. Mater. Res. **68A,** 392, 2004.
22. Burmania, J., Martinez-Diaz, G., and Kao, W.J. Synthesis and physiochemical analysis of interpenetrating networks containing modified gelatin and poly(ethylene glycol) diacrylate. J. Biomed. Mater. Res. **67A,** 224, 2003.
23. Ofner, C.M., and Bubnis, W.A. Chemical and swelling evaluations of amino group crosslinking in gelatin and modified gelatin matrices. Pharm. Res. **13,** 1821, 1996.
24. Einerson, N.J., Stevens, K.R., and Kao, W.J. Synthesis and physiochemical analysis of gelatin-based hydrogels for drug carrier matrices. Biomaterials **24,** 509, 2002.
25. Witte, R.P., Blake, A.J., Palmer, C., and Kao, W.J. Analysis of PEG–diacrylate macromer polymerization within a multicomponent semi-interpenetrating polymer network (semi-IPN) system. J. Biomed. Mater. Res. **71A,** 508, 2004.
26. McNally, A.K., and Anderson, J.M. Complement C3 participation in monocyte adhesion to different surfaces. Proc. Natl. Acad. Sci. U.S.A. **91,** 10119, 1994.
27. Kao, W.J., and Liu, Y. Intracellular signaling involved in macrophage adhesion and FBGC formation as mediated by ligand–substrate interaction. J. Biomed. Mater. Res. **62,** 478, 2002.
28. Martinek, K., Klibanov, A.M., Goldmacher, V.S., and Berezin, I.V. The principles of enzyme stabilization. I. Increase in thermostability of enzymes covalently bound to a complementary surface of a polymer support in a multipoint fashion. Biochim. Biophys. Acta **485,** 1, 1977.
29. Hurwitz, E., Klapper, L.N., Wilchek, M., Uarden, U., and Sela, M. Inhibition of tumor growth by poly(ethylene glycol) derivatives of anti-ErbB2 antibodies. Cancer Immunol. Immunother. **49,** 226, 2000.

Address reprint requests to:
Weiyuan John Kao, Ph.D.
777 Highland Ave.
School of Pharmacy
University of Wisconsin-Madison
Madison, WI 53705

E-mail: wjkao@pharmacy.wisc.edu