
RGD peptide-conjugated poly(dimethylsiloxane) promotes adhesion, proliferation, and collagen secretion of human fibroblasts

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Abstract: A novel technique for conjugating Arg-Gly-Asp (RGD) peptides to poly(dimethylsiloxane) (PDMS) surfaces as well as its application to cell culture is presented in this paper. This technique performs RGD conjugation to PDMS through photochemical immobilization of functional NHS groups to PDMS surface followed with linking RGD peptide to the surface via coupling reaction with NHS. A bifunctional photolinker, N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH), was used to conjugate RGD peptide to the surface. Compared to existing methods for peptide conjugation to PDMS, this technique is convenient, efficient, and free of organic contamination to

PDMS surfaces. It can also be used to conjugate other peptides or proteins to most polymeric materials. In addition, cell culture studies showed that the RGD-conjugated PDMS surfaces promoted the adhesion, proliferation, and collagen production of human skin fibroblasts (HSFs). Finally, the RGD-conjugated PDMS surfaces are resistant to autoclaving and UV irradiation, which enables them to be repeatedly used in cell culture studies. © 2006 Wiley Periodicals, Inc. *J Biomed Mater Res* 79A: 989–998, 2006

Key words: RGD peptide; poly(dimethylsiloxane); cell adhesion; proliferation; collagen production

INTRODUCTION

Silicone elastomers, such as poly(dimethylsiloxane) (PDMS), have been widely used in medical applications such as surgical implants and catheters.^{1,2} This family of materials is highly elastic and transparent; it also has superior compatibility with human tissue and body fluids and is biologically inert, for example, it does not support the growth of bacteria. They also resist common sterilization methods, such as alcohol washing, dry heating, steam autoclaving, ethylene oxide, γ -radiation, and electron beam treatments. In addition, they are also important materials that are widely used in microfluidics, microcontact printing, and cell culture studies.^{3,4} Because of the excellent elasticity and transparency, silicone elastomers are especially superior to other materials in cell culture experiments where mechanical loading is applied to cells.^{4–8}

However, like most polymeric materials, silicone polymers are hydrophobic and do not promote cell adhesion, which is critical in a variety of fundamental development and wound healing processes, such as tissue architecture and function regulation, morphogenesis and angiogenesis.^{9,10} Efforts for modifying the surface of silicone polymers have resulted in treating the silicone surfaces with plasma,^{11,12} UV-ozone,^{13,14} or laser.¹⁵ Despite promoting more hydrophilic and cell-adhesive surfaces, the effects of these treatments are short-lived and deteriorate rapidly over time (hydrophobic recovery).^{13,16,17}

Protein coating is another common treatment for modifying the surface of polymers, including silicone elastomers.^{18,19} Cell adhesive proteins, including fibronectin, collagen, and laminin, are often used to promote cell adhesion and work well in many circumstances. However, the use of proteins for surface coating has limitations. For example, after being extracted from other organisms and purified, they may induce undesirable immune responses and increase the risk of infection.²⁰ In addition, proteins are susceptible to proteolysis and may require additional treatments to function properly. Therefore, protein coating may not be effective for long-term biological applications.

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Moreover, the physically adsorbed proteins are susceptible to instability due to desorption over time²¹ or denaturation due to their stochastic orientation, conformational changes, or unfolding on the surface.^{20,22–24}

These problems with protein coating, however, may be overcome by instituting peptide motifs specific to cell recognition on polymer surfaces.^{20,25–27} Peptide conjugation promotes stable, covalent bonds between bioactive molecules and the polymer surface and extends the active life of the surface. Covalent bonding provides an additional degree of freedom: spacer or linker molecules may be introduced to enhance ligand–receptor interactions between the polymer surface and cells if desired. In addition, peptides exhibit higher stability toward cleaning or sterilization conditions, including heating, chemical treatments, and changes in pH. They are also completely stable against enzymatic degradation and should therefore exhibit excellent long-term stability.²⁰

One of the most frequently used peptides for surface coating is peptide that contains Arg-Gly-Asp (RGD) amino acid sequence. RGD sequence is recognized by cell-surface receptors, such as integrins, which are known to mediate cell adhesion. About half of the 24 integrins bind to ECM molecules in a RGD sequence dependent manner.²⁰ As a result, conjugation of RGD molecules to inorganic or polymeric surface has been shown to promote cell adhesion.^{20,28–33} However, there have been few techniques developed for conjugating RGD peptides to PDMS surfaces, all of which consist of multiple reaction steps and result in low conjugation efficiency.^{5,7,34,35} Herein, we report a convenient, photochemically based new approach to conjugate RGD peptides to PDMS surfaces and present the results of adhesion, proliferation, and collagen secretion of human skin fibroblasts on RGD peptide-conjugated PDMS surfaces.

MATERIALS AND METHODS

Materials

PDMS sheets were prepared by casting the silicone resin, ELASTOSIL[®] RT 601 (Wacker Chemical, Adrian, MI). The silicone resin is composed of components A and B: component A contains platinum catalyst, and component B contains the crosslinker. N-sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH) was purchased from Pierce Biotechnology (Rockford, IL). RGD (H-Arg-Gly-Asp-OH) and GRGDSP (H-Gly-Arg-Gly-Asp-Ser-Pro-OH) peptides were obtained from Bachem Bioscience (King of Prussia, PA). CellTiter 96[®] AQ_{ueous} MTS reagents, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt) (MTS), and phenazine methosulfate (PMS), were obtained from Promega (Madison, WI). The phenol red-free Dulbecco's modified Eagle's me-

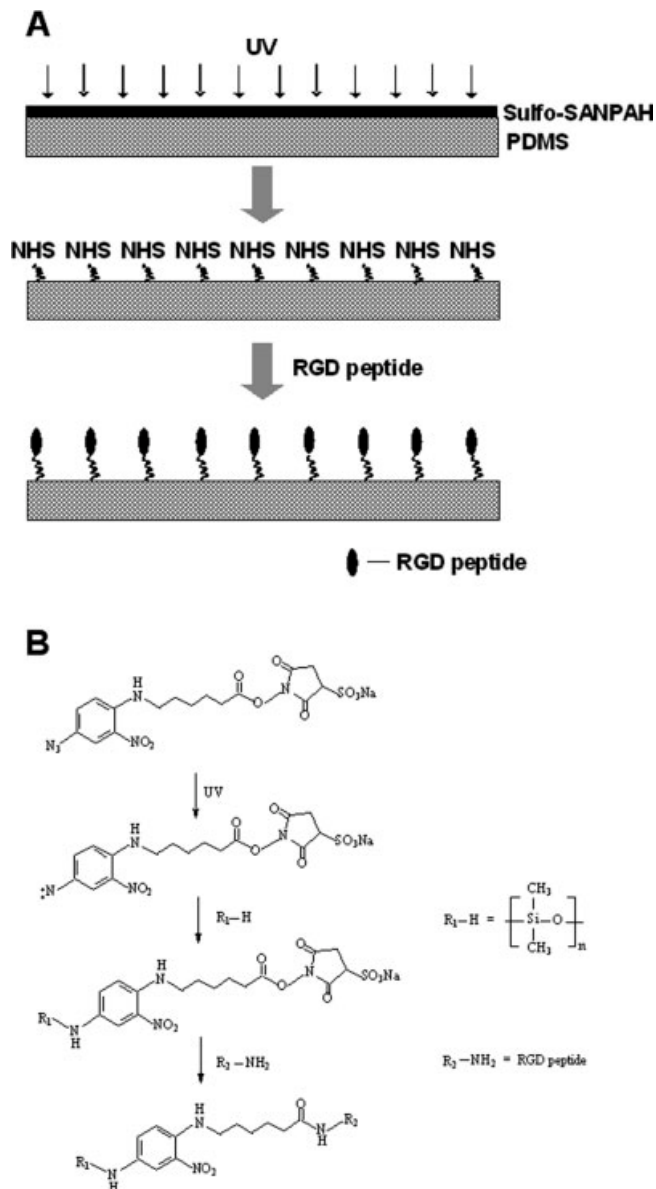


Figure 1. An illustration (A) and the chemical reaction scheme (B) showing the two-step process for conjugating RGD peptide to PDMS surfaces.

dium (DMEM) was purchased from Life Technologies (Gaithersburg, MD). The actin cytoskeleton-focal adhesion staining kit FAK100 was a product of CHEMICON[®] International (Temecula, CA). The Sircol[™] collagen assay kit was purchased from Biocolor Ltd. (Newtownabbey, Northern Ireland). All other chemicals were from Sigma-Aldrich (St. Louis, MO), unless specified otherwise.

Conjugation of RGD peptides to PDMS

The conjugation of RGD peptides included two steps [Fig. 1(A)]. In the first step, a solution of sulfo-SANPAH in deionized water was prepared as follows: sulfo-SANPAH was first dissolved in dimethyl sulfoxide (DMSO) at 0.25 mg/ μ L and then diluted with deionized water to a desired con-

centration. The freshly prepared sulfo-SANPAH solution was placed onto a PDMS sheet, followed by exposure to UV light for 30 min. Then, the PDMS sheet was washed with deionized water and phosphate-buffered saline (PBS) for at least three times. After washing, the membrane was immersed in a RGD peptide solution and incubated at room temperature for 24 h. It was then washed with PBS three more times and kept in PBS for cell culture experiments.

Determination of surface RGD density

The density of RGD peptide on the PDMS surface was measured using a ninhydrin assay.²⁵ A piece of RGD-conjugated PDMS sheet with a surface area of 2 cm² was soaked in 1-mL HCl (5M) in a borosilicate vial and autoclaved at 125°C for 1 h. The solution was then neutralized with NaOH (5M) and mixed with 1 mL of a 2% (w/v) ninhydrin solution containing 3 mg/mL hydrindantin in a mixed solvent of 75% methyl cellosolve and 25% 4M potassium acetate buffer. This solution was incubated at 100°C for 5 min and then immediately placed in an ice-water bath for 3 min. The absorbance of the solution was measured at 570 nm using a microplate reader (Spectra MAX 190, Molecular Devices, CA), and the calibration curve was obtained using RGD solutions of known concentrations.

Characterization of RGD-conjugated PDMS surfaces

The attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of the original PDMS, sulfo-SANPAH-treated, and RGD peptide-conjugated PDMS were collected by a BIO-RAD Excalibur FTS-3000MX FTIR system with an ATR attachment. Each sample was scanned 32 times at a resolution of 4 cm⁻¹.

Cell culture

Human skin fibroblasts (HSFs) from the American Type Culture Collection (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C in a 5% CO₂ atmosphere. The cells were seeded on PDMS sheets that had been conjugated with RGD peptide in a cell culture plate. During cell culture experiments, cell morphology, actin filaments, and focal adhesions on RGD-conjugated PDMS surfaces were documented through phase contrast microscopy and fluorescence microscopy. Cell proliferation and total collagen production were also determined in certain experiments (see below).

Cell proliferation assay

Cell proliferation was measured using the CellTiter 96[®] AQ_{ueous} MTS reagents. The cells were briefly cultured in DMEM medium supplemented with 10% FBS and 1% P/S for a predetermined time. The culture medium was then replaced with phenol red-free DMEM containing combined

MTS/PMS solution (DMEM:MTS/PMS = 5:1). The cells were incubated for an additional 1 h at 37°C in a humidified, 5% CO₂ atmosphere. One hundred microliters of the medium was then transferred to a 96-well plate and the absorbance at 490 nm was recorded with a microplate reader.

Immunostaining of actin filaments and focal adhesions

The cultured cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and washed twice with wash buffer (0.05% Tween-20 in 1× PBS). Then the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature and washed twice with wash buffer. Afterward, a blocking solution (4% goat serum in PBS) was applied for 30 min at room temperature. The primary antibody (antivinculin) was diluted to a working concentration in the blocking solution (dilution 1:100) and incubated for 1 h at room temperature. Then it was washed again with wash buffer three times at 15 min each. The goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody was diluted in PBS immediately before use and incubated for 60 min at room temperature. For counterstaining, tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (dilution 1:200) was incubated simultaneously with the secondary antibody for 60 min at room temperature. It was then washed three times (15 min each) with wash buffer. Following this washing step, cells were incubated with 4'-6-diamidino-2-phenylindole (DAPI) solution (dilution 1:1000) at room temperature for 5 min, followed by washing three times, 15 min each, with wash buffer. Fluorescence images of stained cells were then recorded on a fluorescence microscope (Eclipse TE2000-U, Nikon, Japan), with the cells covered with PBS prior to visualization to prevent them from drying out.

Measurement of total collagen in culture medium

Total soluble collagen produced by HSFs in the medium was measured using a Sircol[™] collagen assay kit. The medium was collected after HSFs were cultured in ascorbic acid-supplemented medium for 5 days. For every 100 μL medium in a tube, 1 mL Sircol dye reagent was added and the tube contents gently mixed at room temperature for 30 min. They were then transferred to a microcentrifuge tube and centrifuged at 10,000 rpm for 10 min. The supernatants were drained off and discarded. The collagen-bound dye, to which 1 mL of the alkali reagent was added, remained as a pellet at the bottom of the tube. The collagen-bound dye was then brought back into solution by vortex mixing. An aliquot of the alkali dye solution (200 μL) was taken and the absorbance at 540 nm was measured with a microplate reader. The standard calibration curve was obtained using the soluble collagen standards provided in the kit.

Determination of durability of RGD-conjugated PDMS

The stability of RGD-conjugated PDMS against typical cleaning and sterilization conditions was investigated. Vari-

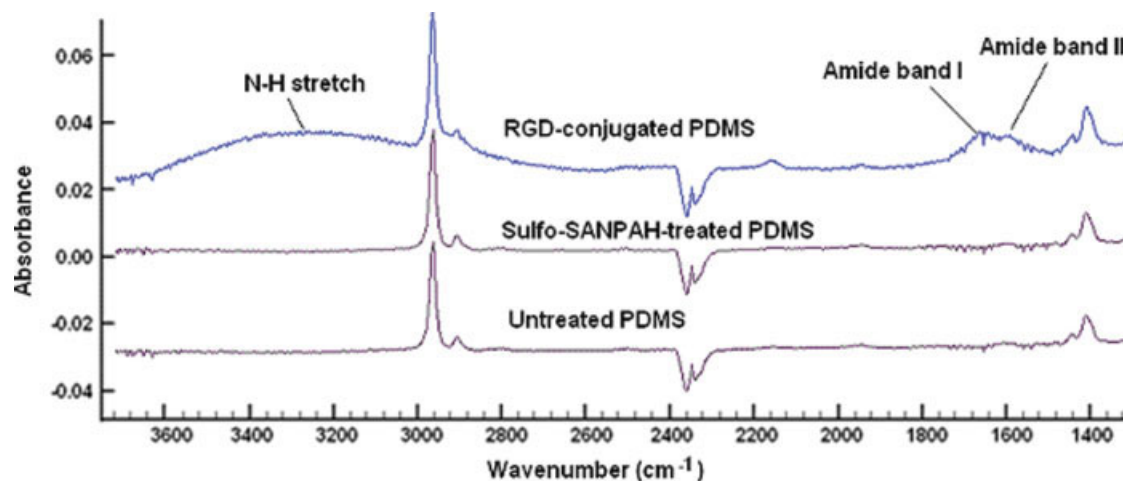


Figure 2. ATR-FTIR spectra of untreated, sulfo-SANPAH-treated, and RGD-conjugated PDMS substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ous treatments, including trypsinization, washing with ethanol, steam autoclaving, and UV irradiation, were applied to RGD-conjugated PDMS sheets. HSFs were cultured on these treated substrates as well as on fresh RGD-conjugated PDMS. MTS assays were performed to quantify the number of cells on these surfaces after 2 days of culture.

Statistical analyses

All data are expressed as mean \pm SD. One-way analysis of variance (ANOVA) was used for statistical analysis, followed by Fisher's PLSD test for multiple comparisons. An unpaired student's *t*-test was also used where appropriate. A *p*-value of less than 0.05 was considered to be statistically significant.

RESULTS

RGD peptides were conjugated to PDMS

The surfaces of PDMS before and after sulfo-SANPAH and RGD treatments were characterized through ATR-FTIR analysis. There was no absorption above 3000 cm^{-1} on the ATR-FTIR spectra of both untreated PDMS and PDMS with sulfo-SANPAH treatment (Fig. 2). After treated with sulfo-SANPAH and incubated with RGD peptide solution, however, a broad absorption peak appeared in the range of $3000\text{--}3600\text{ cm}^{-1}$, which was centered around 3300 cm^{-1} and corresponded to the N—H stretching vibration. In addition, a group of two peaks appeared at 1640 and 1590 cm^{-1} , respectively, which corresponded to the specific absorption of amide bands I and II. These absorption peaks are a result of the conjugation of RGD peptide to PDMS, because untreated PDMS and sulfo-SANPAH do not contain amide groups.

By comparing the ATR-FTIR spectra of untreated PDMS with PDMS that was incubated with RGD solu-

tion for 24 h and then thoroughly washed with deionized water, we did not find any signal of RGD peptide that was possibly absorbed in/onto the PDMS surfaces (data not shown). This indicated that the RGD peptide could not be simply coated on PDMS surfaces without sulfo-SANPAH treatment for covalent binding.

Using a ninhydrin assay, we found that the surface density of the two types of RGD peptides (RGD and GRGDSP) was significantly dependent on the concentration of peptide solution in the second step of RGD peptide conjugation, that is, coupling of the peptide to PDMS surface by reacting with NHS groups. However, there was no apparent difference between the surface density of RGD and GRGDSP if reacted at the same peptide concentration (Fig. 3). Additionally, the density of RGD peptides on PDMS surface was also dependent on the concentration of sulfo-SANPAH solution during the first step of RGD peptide conjugation, that is, sulfo-SANPAH immobilization through UV activation (data

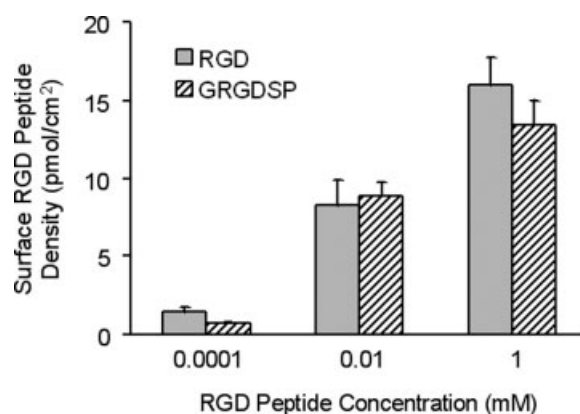


Figure 3. Relationship between the RGD peptide density on PDMS surface and the concentration of peptide solution in the RGD conjugation reaction. The surface density of both RGD and GRGDSP increased with the concentration of peptide solutions ($p < 0.001$).

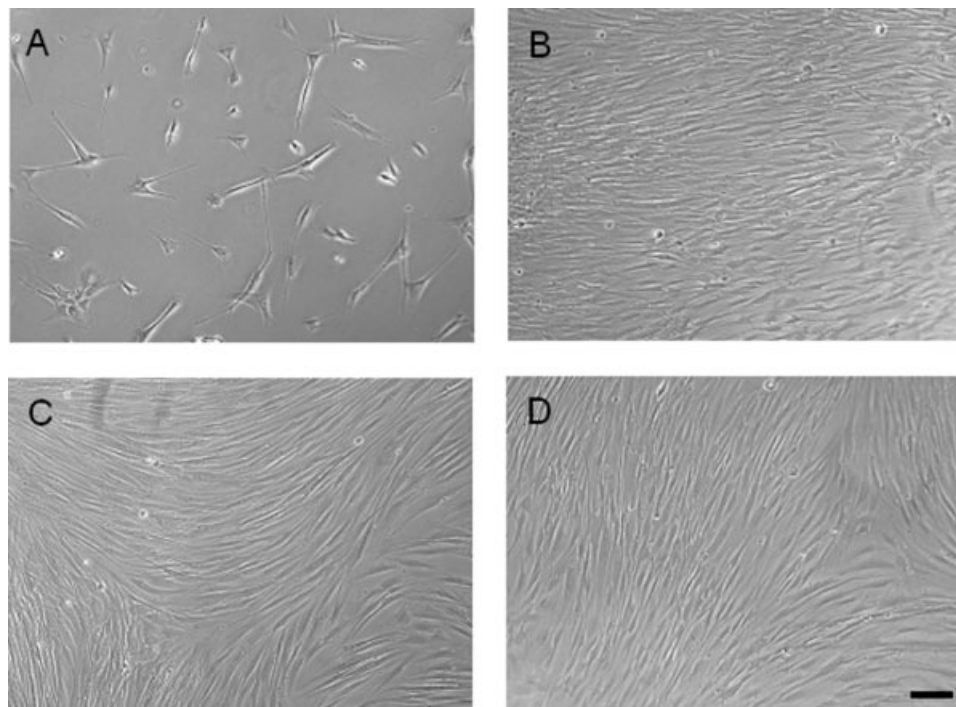


Figure 4. Phase contrast microscopy images of HSFs on (A) untreated PDMS; (B) sulfo-SANPAH-treated PDMS; (C) sulfo-SANPAH-treated and RGD-conjugated PDMS; and (D) TCPS. Images were taken at 2 days of culture; Scale bar, 100 μm .

not shown). On PDMS that was not treated with sulfo-SANPAH, but was incubated with RGD solution, no RGD peptide was detected by ninhydrin assay. This again signifies that RGD peptide was not physically adsorbed on or trapped in PDMS substrate.

Human skin fibroblasts adhered on RGD-conjugated PDMS

When HSFs were seeded on untreated PDMS surfaces, few cells attached [Fig. 4(A)]. On PDMS treated with sulfo-SANPAH but not incubated with RGD peptide, the cells adhered well and proliferated rapidly [Fig. 4(B)]. When HSFs were plated to PDMS treated with both sulfo-SANPAH and RGD peptide, cells spread well and appeared more elongated in shape, compared with the PDMS surface treated with sulfo-SANPAH only. The morphology of HSFs on RGD-conjugated PDMS [Fig. 4(C)] did not show apparent difference from that of HSFs on standard tissue culture polystyrene (TCPS) dishes [Fig. 4(D)]. Furthermore, when HSFs were cultured on RGD-conjugated PDMS surfaces, bundles of actin microfilaments (stress fibers) and focal adhesions were formed (Fig. 5), implying the strong interaction between the cells and the substrate.

As the concentration of RGD peptides in the conjugation reaction increased and therefore the surface density of RGD on PDMS increased, the number of cells grown on sulfo-SANPAH-treated PDMS increased ($p < 0.01$, Fig. 6). However, there was no significant differ-

ence between the numbers of HSFs on RGD-conjugated surfaces and those on GRGDSP surfaces.

HSFs proliferated on RGD-conjugated PDMS

A comparison was made between the numbers of HSFs on sulfo-SANPAH-treated PDMS surfaces with

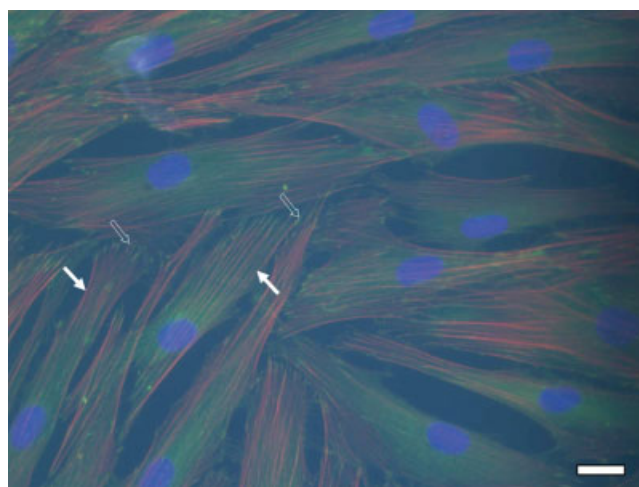


Figure 5. Fluorescence microscopy images of HSFs cultured on a RGD-conjugated PDMS substrate. The cells contain bundles of stress fibers (solid arrows), and their focal adhesions are also clearly seen (blank arrows). The cells were fixed and stained after 2 days in culture; Scale bar, 20 μm . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

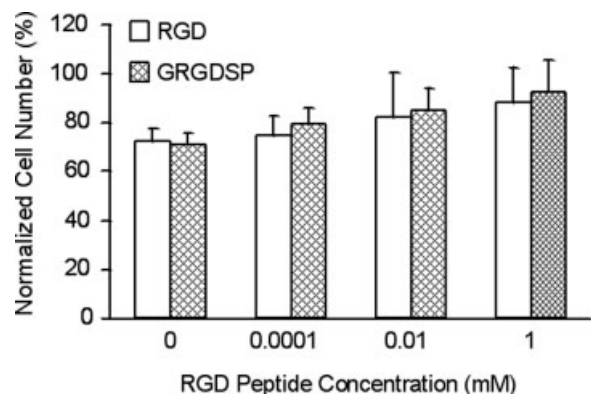


Figure 6. The number of HSFs grown on sulfo-SANPAH-treated PDMS substrates with various RGD peptide concentrations during conjugation reaction. The numbers of cells were normalized with respect to the number of HSFs on TCPS. The cells were in culture for 2 days.

or without RGD conjugation at various days of culture (Fig. 7), with HSFs grown on untreated PDMS and TCPS as control. At the first 2 days, the HSFs seeded on sulfo-SANPAH-treated PDMS substrates (either with or without RGD conjugation) adhered and grew well but at a rate slower than those on TCPS. During the following 2 days, the number of HSFs on sulfo-SANPAH-treated PDMS substrates continued to increase, whereas the number of HSFs on TCPS remained constant. As a result, the number of HSFs on RGD-conjugated PDMS exceeded that of the cells on TCPS after 4 days. After an additional 2 days, more cells were also found on sulfo-SANPAH-treated PDMS surfaces than on TCPS. Compared with PDMS that was immobilized with sulfo-SANPAH, but without RGD conjugation, RGD-conjugated PDMS surfaces promoted faster cell proliferation at 6 days ($p < 0.05$),

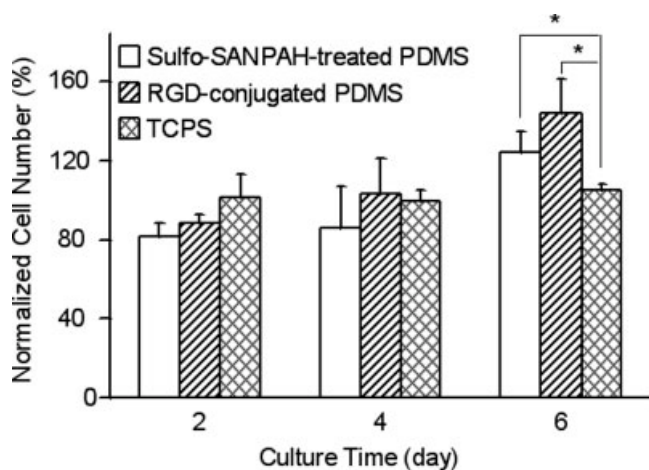


Figure 7. The proliferation of HSFs cultured on sulfo-SANPAH-treated PDMS, RGD-conjugated PDMS and TCPS substrates at different days of culture. The numbers of cells were normalized with respect to the number of HSFs on TCPS at 2 days of culture; * $p < 0.01$.

although both substrates appeared to stimulate similar increases in cell number during the first few days of culture. However, few cells were seen on untreated PDMS substrates (data not shown).

RGD-conjugated PDMS promoted collagen production in HSFs

To promote collagen secretion without cell proliferation, HSFs were seeded on treated PDMS substrates at a high density so that they were confluent at the beginning of culture. In addition, ascorbic acid (AA) was supplemented at a concentration of 25 $\mu\text{g}/\text{mL}$ to the medium after 16 h of culture to facilitate collagen synthesis in the fibroblasts. After another 5 days of culture, the medium was collected to determine the concentration of total soluble collagen produced by the cells. Meanwhile, the number of cells was quantified with MTS assay. The collagen secretion of HSFs was therefore normalized with respect to the number of cells to eliminate the effect of cell number difference when making comparison between two groups. The results are summarized in Figure 8.

Compared with HSFs cultured on TCPS, those on either RGD- or GRGDSP-conjugated PDMS substrates produced about 20% more collagen ($p < 0.01$). No statistically significant difference in collagen production was found between HSFs cultured on RGD- and GRGDSP-conjugated PDMS. HSFs on RGD- or GRGDSP-conjugated PDMS were also found to produce more collagen than those on PDMS surfaces that were immobilized with sulfo-SANPAH, but not conjugated with RGD peptide ($p < 0.05$). The collagen production of HSFs on sulfo-SANPAH-treated PDMS did not show a significant difference from those on TCPS. Collagen assay was not performed for HSFs on untreated PDMS substrates

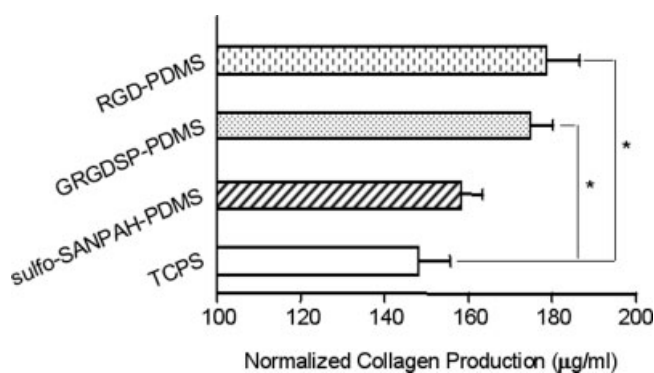


Figure 8. Total soluble collagen production by HSFs cultured on various substrates. The concentrations of collagen in culture medium were measured using Sircol assays and normalized with respect to the cell numbers that were represented by the O.D. values in MTS assays. The cells were in culture for 5 days in ascorbic acid-supplemented medium; * $p < 0.01$.

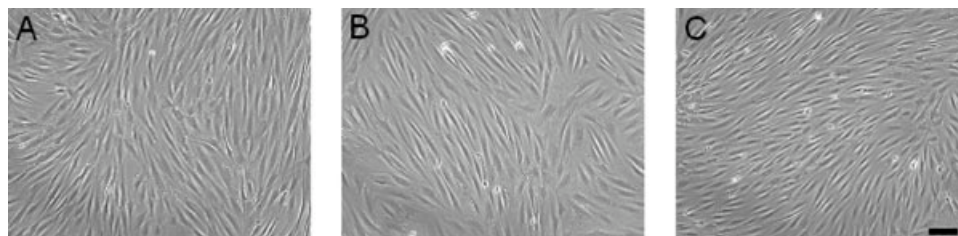


Figure 9. The effect of various treatments of RGD-conjugated PDMS substrate on the proliferation of HSFs. (A) RGD-conjugated PDMS subjected multiple treatments, including trypsinization, alcohol washing, and steam autoclaving; (B) fresh RGD-conjugated PDMS; and (C) TCPS. Phase contrast microscopy images were taken at 2 days of culture; Scale bar, 100 μm .

because few cells remained on these surfaces after the same culture time.

RGD-conjugated PDMS resisted harsh treatments

The possibility of RGD-conjugated PDMS to withstand typical cleaning and sterilization conditions was tested by culturing HSFs on the RGD-conjugated PDMS substrates that were subjected to different treatments: trypsinization, washing with ethanol, steam autoclaving, or UV irradiation. A RGD-conjugated PDMS substrate was subjected to repeated cycles of cell culture followed with different treatments. Specifically, the substrate was seeded with HSFs and cultured for 1 day; after that the cells were removed by trypsinization. The substrate was then washed with ethanol before a second 1-day cell culture was performed on it. Again, the cells were trypsinized, and then the substrate was sterilized by autoclaving. After the third batch of HSFs was cultured for 1 day, the same treatments, that is, trypsinization followed with autoclaving, were repeated. After these treatments, the substrate was still able to sustain good cell adhesion and promote proliferation (Fig. 9). There was little difference between the morphology of HSFs on treated and fresh RGD-conjugated PDMS, as well as on TCPS. Quantitative measurements of cell numbers with MTS assays showed that trypsinization and washing with ethanol virtually had no effect on RGD-conjugated PDMS substrates, while the harsh treatments with autoclaving or UV exposure only slightly affected cell proliferation (Table I).

DISCUSSION

We present a novel technique for RGD conjugation to PDMS through photochemical immobilization of functional NHS groups on PDMS surface, which is followed by coupling of RGD peptide. Sulfo-SANPAH is used as a bifunctional photolinker. The two functional groups (azido group and NHS ester group) of the sulfo-SANPAH molecule are the keys for RGD

conjugation. The photosensitive azido group links the sulfo-SANPAH molecule to the PDMS surface under UV irradiation. The azido group decomposes into nitrene upon exposure to UV light [Fig. 1(B)].³⁶ Nitrene is an extremely reactive intermediate, which reacts with the PDMS surface by extracting a hydrogen atom from the polymer backbone and forming a link between the sulfo-SANPAH molecule and PDMS. The amino-reactive NHS group reacts with the amino end of the RGD molecule, thus linking RGD to the PDMS surface. This results in permanent covalent bonding between RGD peptides and PDMS surface. The whole conjugation procedure can be completed in two steps of reactions, which are accomplished in an aqueous environment without significant use of organic solvents (only a very small volume of DMSO is used to dissolve sulfo-SANPAH). Therefore, this technique for RGD peptide conjugation is convenient, efficient, and free of organic contamination. The surface density of RGD peptides conjugated on PDMS, which was in the magnitude of tens pmol/cm^2 , was comparable to those on other inorganic or polymeric substrates.^{32,33} Note that the reactivity of nitrene is nonspecific to virtually all hydrocarbon backbones of polymers, and the reaction between NHS esters and amine groups is very efficient. Hence, it is possible that our technique can also be used to conjugate other peptides, proteins or aminated biomolecules such as DNA and polysaccharides to most polymeric materials.

Few studies concerning the RGD conjugation to PDMS have been performed. Current methods are

TABLE 1
Normalized Numbers of HSFs on RGD-Conjugated PDMS Substrates Subjected to Various Treatments

Treatment	Normalized Cell Number (%)
None (fresh RGD-conjugated PDMS)	100
Trypsinization	100 \pm 8
Washing with ethanol	98 \pm 6
Autoclaving once	92 \pm 4
Autoclaving twice	84 \pm 5
UV irradiation	88 \pm 6

Cell numbers were normalized with respect to that on fresh RGD-conjugated PDMS substrates.

developed either by the surface coupling of functional groups through silanization reaction or by the surface grafting of functional polymers, followed by a conjugation reaction between the surface functional groups and RGD molecules.^{5,7,34,35} These methods involve multiple steps; therefore, the whole conjugation procedure is relatively long and conjugation efficiency might be low. In addition, the organic solvents that are frequently used in these reactions might swell the PDMS, thus causing damage to its physical and chemical properties. The technique used in this study, however, is free of these problems. An additional advantage of the presented technique is that the density of the RGD peptide on PDMS can be easily controlled by simply varying the concentration of either the sulfo-SANPAH solution in the first step of reaction or the RGD peptide solution in the second step.

Unlike protein coating or conjugation, where the protein is either easily desorbed from the surface or denatured, RGD peptide conjugation is a permanent and stable polymer surface modification. This study showed that RGD peptide-conjugated PDMS can be subjected to various cleaning and sterilization conditions without significant loss of its activity in promoting cell adhesion and proliferation. Therefore, it is reusable, meaning that in addition to convenience and efficiency, this technique is also cost-effective.

As shown in this study, both RGD and GRGDSP that were conjugated to PDMS promoted adhesion and proliferation of HSFs. Interestingly, a mere sulfo-SANPAH treatment without RGD conjugation also modified PDMS surface in a way that facilitated cell adhesion and proliferation. Since all our cell culture studies were performed in serum-supplemented medium, one might suspect that certain serum proteins could be conjugated to the sulfo-SANPAH-treated PDMS through the NHS ester groups during cell culture, thereby promoting cell attachment. However, we believe this was not the case. The NHS esters are water-sensitive and subjected to rapid hydrolysis in aqueous environments free of primary amines. The half-life of hydrolysis, for example, is 4–5 h at pH 7.0 and 0°C for a homobifunctional NHS ester. Hydrolysis rate also increases with increasing pH and occurs even more readily in dilute solutions. Given the fact that in our study the NHS-substrates were also incubated in PBS (pH 7.4) at room temperature for 24 h, a typical incubation time used for all RGD conjugation reactions, there should be a very small number of reactive NHS groups left. Therefore, it is unlikely that during cell culture, the serum proteins in culture medium could be conjugated to PDMS surfaces. It is possible, however, that the modified PDMS surface favors adsorption of serum protein as a result of hydrophilicity changes due to immobilization of sulfo-SANPAH and consequent hydrolysis of NHS esters. In addition, this enhanced cell adhesion effect of

sulfo-SANPAH treatment might also be caused by the presence of surface hydroxyl groups, which have been proved to be effective in enhancing cell adhesion on polymeric materials.^{37–40}

With RGD conjugation, however, cell adhesion and proliferation were further enhanced in long-term cell cultures (e.g., 6 days, see Fig. 7). We also found that at the first 1 or 2 days, HSFs in culture attached and grew better on TCPS than on sulfo-SANPAH-treated PDMS either with or without RGD peptide conjugation. After 2 days of culture, however, the number of HSFs on sulfo-SANPAH-treated PDMS substrates began to exceed that of those on TCPS. In addition, compared with HSFs cultured on PDMS immobilized with sulfo-SANPAH, but without RGD conjugation, cells on RGD-conjugated PDMS proliferated faster.

Two peptides, RGD and GRGDSP, were used for surface modification of PDMS. We found that both of them appeared to function similarly in promoting adhesion and proliferation of HSFs on PDMS. Previous studies showed that longer RGD sequence-containing peptides, such as GRGDSP, GRGDSPC, and CGRGDSY, improved cell attachment more than short RGD peptide.^{20,41,42} A better conformation adjustability of these long-chain peptides might account for the better performance of these peptides. However, we did not observe a significant difference between RGD and GRGDSP peptides in our studies. This could be resulted from the flexibility of spacer arm of the sulfo-SANPAH molecule, which is a pentylene group with a length of 18.2 Å. This spacer allows the RGD sequence to adopt a preferential conformation to signal cell membrane receptors.

Besides cell adhesion and proliferation, collagen production of HSFs cultured on various PDMS substrates was measured. It was found that conjugation of RGD peptides enhanced collagen production of HSFs on PDMS substrates. Similar results from other researchers with RGD-modified substrates also support our findings.⁴³ Compared with the commonly used TCPS, RGD peptides-conjugated PDMS showed even better performance in promoting collagen production of HSFs. The introduction of additional cell binding sites through RGD modification might induce subsequent intracellular signaling events to promote the production of matrix proteins, including collagen.⁴⁴ Again, we did not observe a difference between the collagen production of HSFs on RGD- and GRGDSP-conjugated PDMS substrates.

Finally, it should be noted that while this study, along with many other studies, showed RGD peptides effectively promote cell adhesion, proliferation, ECM protein secretion and other cellular functions, these short peptide sequences cannot replace adhesive proteins such as fibronectin. In other words, the peptides can recapitulate certain key functions of native proteins, but not all functions. For example, it has been

shown that fibronectin and RGD behaved similarly in terms of promoting cell spreading; however, the traction forces of fibroblasts were markedly different on the substrates that were coated with fibronectin and RGD peptides, respectively.⁴⁵

In summary, a convenient, efficient and cost-effective technique to conjugate RGD peptides has been developed in this study. Two kinds of RGD peptides, RGD and GRGDSP, were covalently immobilized to PDMS surface using sulfo-SANPAH as the photolinker under UV irradiation. Both sulfo-SANPAH immobilization and RGD peptide conjugation promoted HSF adhesion and proliferation on PDMS; however, HSFs cultured on RGD peptide-conjugated surfaces adhered better and proliferated faster than those on sulfo-SANPAH surfaces. The adhesion of HSFs was dependent on the RGD peptide density on PDMS surface. Both RGD and GRGDSP conjugation equally well promoted adhesion, proliferation, and collagen production in HSFs. Finally, being able to resist harsh cleaning and sterilization conditions, RGD peptide-conjugated PDMS substrates are reusable for cell culture experiments.

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