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Solid-Phase ATRP Synthesis of Peptide−Polymer Hybrids

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Abstract: A versatile methodology to prepare hybrid biomaterials by atom transfer radical polymerization from resin-supported peptides has been established. As an example, we have synthesized a GRGDS-functionalized poly(2-hydroxyethyl methacrylate). The peptide−polymer was characterized by solid-state ^{13}C NMR and GPC and found to have a number average molecular weight of 4420 and a polydispersity of 1.47. These values are comparable to those obtained from solution-phase syntheses, suggesting the ATRP reaction is successful from a peptide-conjugated solid support. Solid-state ^{13}C NMR was used to characterize multiple steps in the reaction, and the synthesis was found to be near quantitative. We have performed cell adhesion experiments and observed the GRGDS sequence-promoted cell adhesion, whereas unfunctionalized poly(2-hydroxyethyl methacrylate) did not. By incorporating cell-signaling moieties in materials with defined molecular architecture, it will be possible to control the interactions between polymeric materials and biological systems.

Introduction

The next generation of medical implants will likely incorporate materials designed to have targeted interactions with cells by incorporating ligands for specific cellular receptors.[1−3] In medical implants, cell adhesion to material surfaces is mediated by blood-borne proteins,[4] and an extensive literature exists on materials that resist protein adsorption.[5−7] Since polymeric materials can be engineered to have the necessary mechanical properties, biocompatibility, and protein-adsorption resistance for a variety of biomedical applications,[8] it is of great interest to have flexible synthetic methodologies for synthesizing polymers that incorporate biological signaling moieties such as peptides.[9−12]

A critical requirement for these materials designed to interact with cell receptors is the spatial organization of multiple ligands in order to engage the receptors more effectively. Pierschbacher et al.[13,14] identified the GRGDS peptide sequence of fibronectin, an important adhesion protein found in virtually every tissue in the body, to be important for binding to the membrane receptors, known as integrins. However, it was shown that GRGDS alone has a relatively low affinity for these receptors, approximately 1000 times less than intact fibronectin.[14] This affinity can be greatly amplified by incorporating the GRGDS synergy sequence PHSRN, which is located 30−40 nm from the GRGDS sequence in the parent cell-binding domain.[15,16] Design of materials that are capable of effectively mimicking this environment will rely on controlled-synthesis techniques. Significant progress in developing such techniques has been made in polymer chemistry, and a number of methods are currently available for preparing materials with defined molecular architecture.

Atom transfer radical polymerization (ATRP) is one of the most actively developing areas in polymer chemistry because it allows the synthesis of polymers with well-controlled molecular weight and molecular weight distribution without the stringent requirements on water- and oxygen-removal that are necessary for other types of living polymerizations.[17] Moreover, the chemistry of ATRP is tolerant of many functional groups, thereby permitting the controlled synthesis of a broad range of polymers. The ATRPs of various monomers from different particles were studied extensively for surface modification.[18,19] It has been shown that the ATRP synthesis of poly(methacrylate)s from Wang resin has been reported by Angot et al.[20]

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However, it has not been applied to the peptide-functionalized resin for the synthesis of polymers possessing cell-signaling moieties.

One strategy used to prepare hybrid biomaterials has been the ring-opening polymerization of *N*-carboxyanhydride from a macroinitiator.[21] However, using this method, the polypeptides are limited to one amino acid species. Alternatively, hybrid biomaterials have been prepared by coupling polypeptides and a synthetic polymer. However, this method is often associated with low conversion or strict limitations on the polymer species.[22] Recently, a novel strategy combining solid-phase synthesis and nitroxide-mediated radical polymerization (NMRP) was conducted by Becker et al. in the preparation of a peptide-block copolymer.[23] Detailed characterization of the products was not available because of the different solubilities of the blocks, but this work suggested that it is possible to create peptide−polymer hybrids with controlled architecture.

In this report, we described the synthesis of GRGDS-terminated poly(2-hydroxyethyl methacrylate) [poly(HEMA)] using ATRP. Poly(HEMA) is one of most extensively studied biocompatible, hydrogel-forming polymers,[8] which has been used for applications such as contact lenses because it resists protein and cell adhesion. We demonstrate it is possible to incorporate a cell-adhesion peptide in the synthesis of this material on a solid support and that the resulting product has a controlled molecular weight and narrow molecular weight distribution. By incorporating peptides such as GRGDS in materials with defined molecular architecture, it will be possible to control the interactions between polymeric materials and biological systems.

Experimental Section

Certain commercial materials, instruments, and equipment are identified in this article to specify the experimental procedure as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials, instruments, or equipment identified are necessarily the best available for the purpose.

Peptide Synthesis. Peptide synthesis was performed on an Advanced ChemTech Apex396 peptide synthesizer using standard Fmoc chemistry.[24] The Fmoc-protected amino acids were purchased from Advanced ChemTech (Lexington, KY) and used as received. The coupling reactions were facilitated by DIC and HOBt. Peptide synthesis was carried out on a Fmoc-Gly-functionalized Wang resin, which was purchased from Advanced ChemTech (Lexington, KY) and used as received.

Synthesis of Peptide−Initiator. To a 50 mL round-bottom flask was added 0.9 g of GRGDS-Wang resin, (0.54 mmol, 0.6 mmol/g NH_2 functionality). Tetrahydrofuran (20 mL) was added to the flask prior to *N*,*N*-diisopropylethylamine (0.47 mL, 2.7 mmol) and dropwise addition of 2-bromopropionyl bromide (0.28 mL, 2.7 mmol). The mixture was stirred slowly with a magnetic stir bar overnight prior to isolating the supported initiator by filtration. The resin-bound initiator was washed with dichloromethane, acetone, and copious deionized water. The solid was immersed into water and stirred for 4 h. The product (0.85 g) was recovered by vacuum filtration.

Synthesis of GRGDS−Poly(HEMA) via ATRP. The GRGDS-poly(HEMA) bioconjugates were prepared by ATRP. Copper(I) chloride was purchased from Aldrich and purified by stirring in acetic acid overnight, filtering to collect the solids, and washing with ethanol. HEMA monomer was purchased from Polysciences and used as received. The initiator (0.24 g of derivatized peptide-Wang resin, 0.15 mmol), copper(I) chloride (0.015 g, 0.15 mmol), and bipyridyl (bpy) (0.047 g, 0.3 mmol) were placed into a 25 mL pearl-shaped flask. The pearl-shaped flask was degassed with three vacuum/argon fill cycles. Three round-bottom flasks were charged with HEMA, methyl ethyl ketone (MEK), and 1-propyl alcohol, respectively. They were deoxygenated by argon purged through the solution for at least 50 min. The MEK (0.7 mL), 1-propyl alcohol (0.3 mL), and HEMA (0.9 mL, 75 mmol) were added to the pearl-shaped flask in that order, and the flask was heated at 50 °C for 5 h. The beads were then filtered and washed with DMF, then dichloromethane. The beads were allowed to dry in the fume hood overnight before being placed in a vacuum oven to dry (yield: 0.72 g).

Cleavage Reaction. The beads (0.3 g) were placed into a 25 mL flask, and a trifluroacetic acid (TFA, 5.7 mL), triisobutylsilane (TIS, 0.15 mL), water (0.15 mL) solution was added. The solution was stirred for 90 min. The solution was filtered and washed three times with TFA (1 mL). The resulting solution was freeze-dried and redissolved into MEK/1-propanol (70:30, 60 mL) solvent. The GRGDS-poly(HEMA) was obtained by precipitating the solution into hexanes (600 mL) (yield: 0.10 g).

Solid-State NMR and Solution NMR. ^{1}H NMR was recorded on a JEOL 270 MHz spectrometer. Solid-state ^{13}C NMR spectra were obtained on a noncommercial spectrometer operating at 2.35 T corresponding to a ^{13}C frequency of 25.19 MHz. Cross polarization and magic angle spinning were utilized in the usual way.[25] The sample spinning frequency was fixed at 4.0 kHz so that there would be no overlap between center bands and sidebands. The proton and ^{13}C rf levels used in CP and decoupling corresponded to nutation frequencies of 65 and 69 kHz, respectively. The CP time was 1.0 ms, and the time between scans was 4 s. Total scans varied from 20 000 to 48 000. Chemical shifts are given with respect to liquid tetramethylsilane, using the methine resonance of adamantane as a secondary external standard at 29.5 ppm.

Gel Permeation Chromatography. The GPC measurements were performed on a Waters HPLC system (Waters Corporation, Milford, MA) equipped with a 510 pump, a 717 plus injector, and a 410 differential refractometer. DMF with 10 mM LiBr was used as mobile phase at a flow rate of 1.0 mL/min. Sample concentration of 0.5−1 w/v and injection volume of 100 μL were used. Calibration was accomplished with 10 polystryene standards (from Polymer Laboratories LTD) and molecular weight ranged from 325 000 to 580. A column set used for chromatography consists of a Waters Styragel HR4 7.8 × 300 mm column and a Waters Styragel HR2 7.8 × 300 mm column. Empower software (Waters Corporation) was used for calibration and calculation.

Hydrogel Preparation. Hydrogels of poly(HEMA) and GRGDS−poly(HEMA) were prepared by coating the bottom of a six-well tissue culture poly(styrene) plate with 0.05 g of lyophilized polymer. Deionized water (18 MΩ) was added dropwise until a gel was observed at the bottom of the dish.

Cell Attachment. Mouse NIH-3T3 fibroblasts were a gift from Dr. Kenneth Yamada at NIDCR/NIH. The cells were cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with calf serum (volume fraction of 10%) and penicillin/streptomycin (volume fraction of 1%). All cell culture reagents were obtained from Gibco, UK. Cells were incubated at 37 °C under 10% CO_2. Cells grown to 80% confluence were passaged by trypsinization for 1 min twice, diluted, and inoculated into a fresh tissue culture flask.

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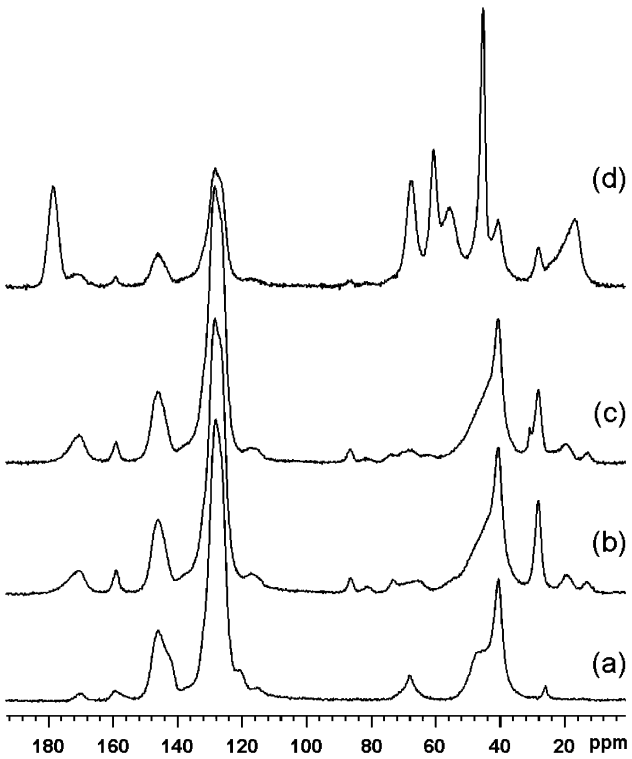


Figure 1. Solid-state ^{13}C NMR spectra of Fmoc-glycine Wang resin (a), GR(Pbf)GD(Obut)S(But) Wang resin (b), GR(Pbf)GD(Obut)S(But)-initiator Wang resin (c), and GR(Pbf)GD(Obut)S(But)-poly(HEMA) Wang resin (d).

Fibroblasts were seeded at a density of 50 000 cells/well in a six-well plate onto polymer films. After incubating for 24 h, the cells were washed with phosphate-buffered saline (PBS) and fixed with glutaraldehyde (Sigma) in PBS (volume fraction of 1.5%) for 1 h at room temperature.

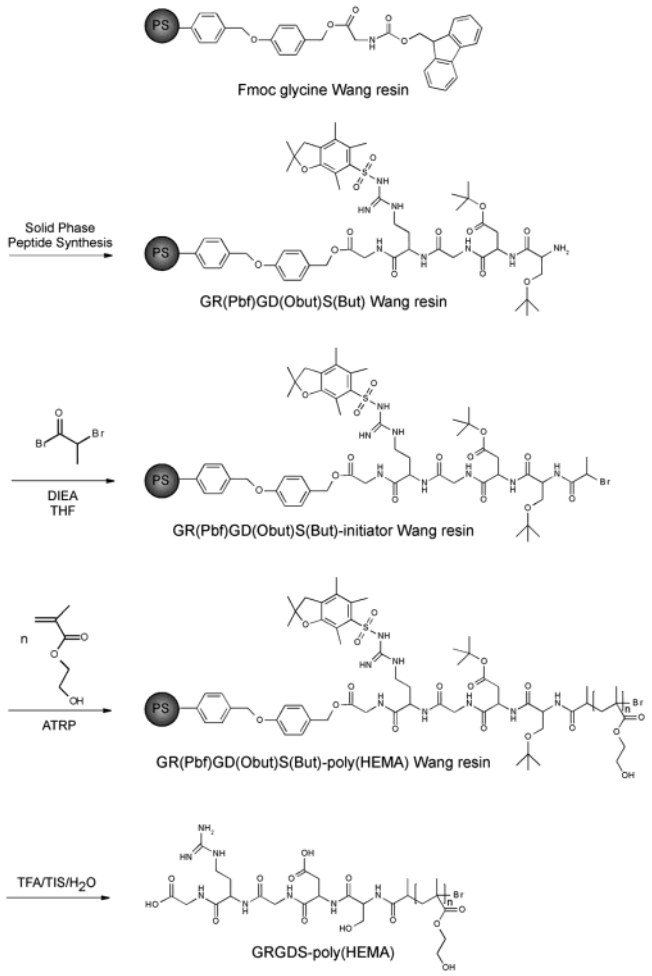
Results and Discussion

We employed solid-state ^{13}C NMR to follow changes in the spectrum of the Wang resin after successive steps in the synthesis of the peptide−polymer hybrids. We were able to assign resonances in the spectrum to follow the addition of peptide to the resin, the coupling of the ATRP initiator to the peptide, and the polymerization of the HEMA monomer. These reactions were confirmed by MALDI-TOF MS, ^1H NMR, and GPC analyses of the products cleaved from the resin. Nearly quantitative agreement was observed between the results of the solid-state NMR spectra and these other characterization techniques.

A. Synthesis and Characterization of Polypeptide GR-(Pbf)GD(OBut)S(But)-Wang Resin. The solid-state ^{13}C NMR spectrum of Fmoc-glycine Wang resin is shown in Figure 1a. Extensive resonance assignments were noted in the Supporting Information. The resonance near 170 ppm represents a single carbonyl ester carbon associated with the Fmoc-Gly terminus. Amide carbonyls are also expected to appear in this region; however, the carbonyl attached to the glycine nitrogen is shifted upfield, due to the oxygen substitute.

The protected polypeptide GR(Pbf)GD(Obut)S(But) was prepared by standard Fmoc chemistry. The solid-state ^{13}C NMR spectrum of GR(Pbf)GD(Obut)S(But)-Wang resin is shown in Figure 1b. The 170 ppm resonance should represent six carbons, after the synthesis of GR(Pbf)GD(OBut)S(But); these include

Scheme 1 Solid-Supported Peptide and Polymer Syntheses



the four peptide amide carbonyls and the two ester carbonyls, one from the original glycine and the other associated with the D(Obut) side chain. Compared with the glycine Wang resin, which has one amide bond, the intensity at 170 ppm of GRGDS-Wang resin increased by a factor of 5.9 ± 0.4. This indicates nearly quantitative conversion from Fmoc-Gly Wang resin to GR(Pbf)GD(OBut)S(But)-Wang resin. The predominant appearance of a proton-cationized molecule peak corresponding to GRGDS sequence at 491 *m*/*z* in a MALDI-TOF MS spectrum (see Supporting Information) further confirmed the successful synthesis of GRGDS-Wang resin.

B. Synthesis and Characterization of GR(Pbf)GD(OBut)S-(But)-Initiator Wang Resin. The peptide-initiator was prepared from the condensation reaction of the amino group of the serine and 2-bromopropionyl bromide according to the procedure proposed by Angot and co-workers,[20] shown in Scheme 1. The resulting secondary bromide has been widely used as initiator for various monomers in atom transfer radical polymerization.[17]

The difference solid-state ^{13}C NMR spectrum of GRGDS-initiator Wang resin and GRGDS-Wang resin is shown in Figure 2. Given that there are no nonoverlapping resonances originating from the initiator in the ^{13}C NMR spectrum of the GRGDS-initiator Wang resin, we used a difference spectrum (Figure 2) to estimate the extent of reaction with the initiator. This difference spectrum (2a) is generated from the spectra of the GRGDS-initiator Wang resin (2b) and the GRGDS-Wang resin

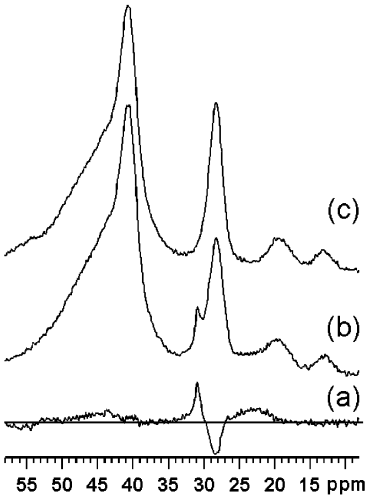


Figure 2. Expanded region of solid-state ^{13}C NMR spectra of GR(Pbf)-GD(Obut)S(But) Wang resin (c) and GR(Pbf)GD(Obut)S(But)-initiator Wang resin (b). The difference spectrum of the above (a) and a baseline was drawn for clarity in part a.

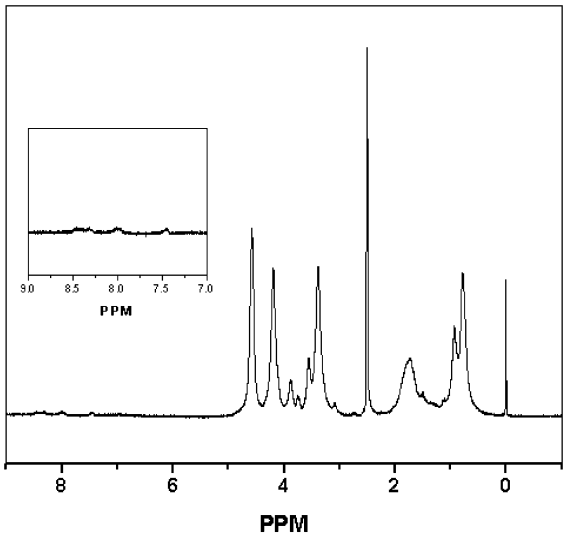


Figure 3. ^1H NMR spectrum of GRGDS-poly(HEMA) in DMSO-d_6.

(2c). We have added a baseline for clarity in 2a. In the difference spectrum, the relatively broad peak at 22 ppm is attributed to the methyl group of the initiator moiety. This resonance appears as an isolated resonance (not shown) at the same position and with the same line width when the initiator is reacted directly with a Wang resin, i.e., a resin without the GRGDS sequence. The fact that this methyl resonance occurs in a region where the overlapping resonances are relatively weak greatly enhances the reliability of the difference intensity relative to, say, a region such as 35−55 ppm, where intensities in the parent spectra are strong. Integration of the 22 ppm resonance is slightly complicated by a lack of good baseline in the 26−36 ppm region. Attachment of the initiator has apparently modified the structure or conformation of the carbons in this latter region so as to cause some chemical shifts. Thus, relatively strong difference features are observed in this latter shift region; however, the near-offsetting positive and negative areas of these features support the notion that little new intensity is associated with this region. Thus we approximate the methyl integral by integrating the positive-going intensity shown above the sketched baseline, between 18 and 26 ppm in Figure 2a. The integration ratio of this methyl resonance to the resonance at 170 ppm of Figure 1b, which corresponds to six carbonyl groups, indicates that the ratio of initiator sites to the number of GRGDS sites originally available is 1.1 ± 0.2. This value includes a correction for stronger spinning sidebands associated with the carbonyl carbons. Hence, the suggestion is strong that most GRGDS sites have reacted.

In the MALDI-TOF spectrum of this product, the disappearance of the GRGDS proton-cationized molecule peak at 491 *m*/*z* and the emergence of a dominant peak at 625/627 *m*/*z* (corresponding to the proton-cationized molecule peak of GRGDS-initiator) in the MALDI-TOF (see Supporting Information) also confirmed the quantitative conversion from the GRGDS sequence to GRGDS-initiator.

C. Characterization of ATRP Polymerization of HEMA from Solid Support. The ATRP polymerization of HEMA was first reported by Beers et al. using MEK/propanol (70:30) as solvent and Robinson et al. using methanol/water (50:50) as solvent.[26,27] The MEK/propanol system was chosen for this study because the hydrophobic Wang resin composed of polystyrene did not swell well in the methanol/water system. New ^{13}C solid-state resonances associated with this product appeared at 20, 43, 53, 64, 67, and 178 ppm. This confirmed the successful polymerization of HEMA. On the basis of the integration ratio of the resonance at 178 ppm (corresponding to the ester carbonyl group of poly(HEMA)) to that at 170 ppm (corresponding to the six carbonyl groups of the two ester and four amide bonds in the initiated polypeptide), the degree of polymerization (DP) was 34 ± 3.

The product was cleaved from the resin at the benzylic ester linkage by adding excess TFA/TIS/H_2O (95:2.5:2.5) solution. The TIS functioned as scavenger to quench the reactive cationic species generated from the cleavage of the protecting group. The ^1H NMR spectrum of the detached polymer is shown in Figure 3.

Ester hydrolysis in TFA deprotection has been reported previously;[28] however, no significant hydrolysis of the backbone ester group of the poly(HEMA) was found under these conditions. This is evidenced in Figure 3 by the integration ratio of 2:2.5 for the poly(HEMA) resonance at 4.2 and 4.8 ppm, which correspond to the two pairs of methylene protons of the hydroxyethyl moiety, to the broad region from 0.8 to 1.7 ppm, the two backbone methylene protons and the three methyl side chain protons. The GPC profile of GRGDS-poly(HEMA) is shown in Figure 4.

It is interesting to note that the polydispersity of GRGDS-poly(HEMA) prepared by ATRP from solid support (1.47) was found similar to the polydispersity (1.3−1.5) of poly(HEMA) prepared by ATRP in solution under similar reaction conditions.[26] This suggests that ATRP synthesis of poly(HEMA) from a solid support was successful, even following solid support peptide synthesis.

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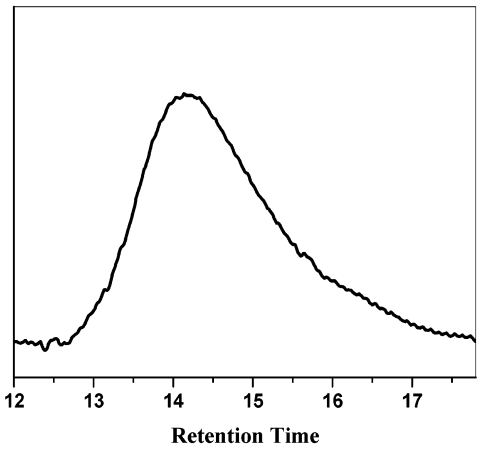

Figure 4. GPC profile of the GRGDS-poly(HEMA).

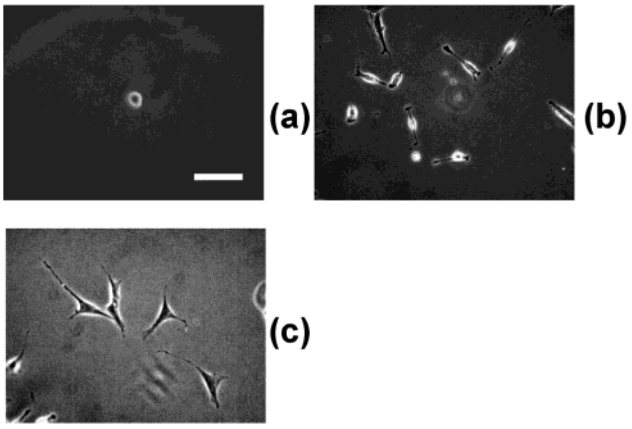

Figure 5. Microscopic images of fibroblast on poly(HEMA) (a), GRGDS-poly(HEMA) (b), and tissue culture polystyrene (c). The bar in the lower right of panel (a) represents 50 μm.

D. Cell Attachment. In Figure 5 are shown the results of cell-adhesion studies to poly(HEMA), GRGDS-poly(HEMA), and tissue culture polystyrene.

Negligible cell attachment was observed on the poly(HEMA) film after 24 h cell culture, consistent with previous reports that cell adhesion is suppressed on the poly(HEMA).[29] This is thought to be due to the hydrophilic character of poly(HEMA), which resists adsorption of serum proteins necessary for cell adhesion. In contrast, cell attachment and spreading was found on the GRGDS-poly(HEMA) film after 24 h cell culture, albeit more weakly than on tissue culture polystyrene surface. Moreover, the viability of spread cells on the GRGDS-poly-(HEMA) film after 24 h cell culture also suggests that the copper catalyst could be removed simply by washing the beads with copious solvent and that the final product retains its biocompatible character.

Conclusions

A versatile methodology to prepare peptide−synthetic polymer hybrid materials has been demonstrated and GRGDS-poly-(HEMA) was synthesized as a model compound. The synthesis was monitored by solid-state ^{13}C NMR, and the resultant GRGDS-poly(HEMA) was characterized by ^{1}H NMR and GPC. The relative low polydispersity indicated the ATRP reaction was successful, even from a peptide-loaded solid support. The cell-adhesion and cell-spreading experiments suggested the GRGDS sequence was active in promoting the cell adhesion and spreading. This methodology provides many opportunities to prepare the well-defined hybrid peptide−synthetic materials through solid-phase peptide and ATRP syntheses.

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Supporting Information Available: The methodology and spectra of MALDI-TOF MS as well as the extensive resonance assignment in ^{13}C solid-state NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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