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MS-Monitored Conjugation of Poly(ethylene glycol) Monomethacrylate to RGD Peptides

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ABSTRACT: Development of biologically active polymers is an active area of research due to their applications in varied and diverse fields of biomedical research: cell adhesion, tissue proliferation, and drug delivery. Recent advances in chemical modification allow fine-tuning of the properties of biomedical polymers to improve their applications: blood circulation half-life, stimuli-responsive degradation, site-specific targeting, drug loading, etc. In this article, convergent synthesis of polymerizable macromonomers bearing a site-specific ligand (RGD peptide) using a low molecular weight MA-poly(ethylene glycols) (PEGs) is presented. The method affords macromonomers useful as the starting materials to produce biomedical polymers. We found matrix assisted laser desorption/ionization mass spectromerty convenient in monitoring the conjugation process via step-by-step following of PEG modification. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40385.

KEYWORDS: drug delivery systems; biomaterials; nanostructured polymers; molecular recognition; copolymers

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INTRODUCTION

RGD-bearing peptides are known for specific affinity to integrins—transmembrane receptors that mediate the attachment between a cell and its surroundings. Such bioresponsive ligands are routinely used in bioengineering as they facilitate cell adhesion,[1] migration, stratification,[2,3] and active targeting in drug delivery systems.[4] Biocompatibility of RGD-peptides as well as their availability have attracted much attention in the development of various biological scaffolds: tissue regeneration hydrogels,[3,5–7] inorganic implants,[8,9] and site-selective delivery of nanoformulations.[10–18] Anchoring of ligands to scaffolds is crucial to promote cell adhesion because formation of focal adhesions only occurs if ligands withstand the cells contractile forces.[19–21] These forces are able to redistribute weakly adsorbed ligands on a surface, which leads only to weak fibrillar adhesions later on.[19,22–24] Furthermore, cells can remove mobile integrin ligands by internalization usually via receptor mediated endocytosis.[25,26] Hersel *et al.*[27] reviewed two major steps for stable linking of RGD peptide to various polymers: (a) Polymer derivatization: introduction of functional groups, blending, copolymerization or cross-linking with functionalized polymers, and chemical or physical treatment; (b) peptide immobilization via coupling methodologies or chemospecific attachment. Inorganic surfaces are much different from polymeric surfaces and require polymeric coating for metal surfaces, silanol derivatization for silicates[8,9] or specific inorganic reactivity.[16–18]

Polymer conjugation is a well-known and widely exploited technique useful to improve therapeutic properties of peptides.[28] Further, polymer-conjugated drugs or peptides generally exhibit prolonged half-life, higher stability, water solubility, lower immunogenicity, and antigenicity and specific targeting to tissues or cells.[29] Poly(ethylene glycol) (PEG) has been approved by the FDA for several medical applications because of its biocompatibility and low toxicity. It has been extensively studied for various uses including preparation of biologically useful conjugates,[30] surface modification of biomaterials,[31] and induction of cell membrane fusion.[32] There is evidence that introduction of PEG spacer to RGD-bearing hydrogels significantly increases fibroblast spreading[5] or cancer cell adhesion[33,34] versus non-spacer hydrogels. Further, literature is replete with the use of PEGs to tether ligands to the surface of nanoparticles. From this perspective, synthesis of PEGylated RGD-bearing conjugates is of considerable interest.

The fabrication of polymeric nanoparticles can proceed in one of two major ways: dispersion of preformed polymers and in situ polymerization of monomers. Recently, in our group, interest has been shown in two processes of fabricating polymeric

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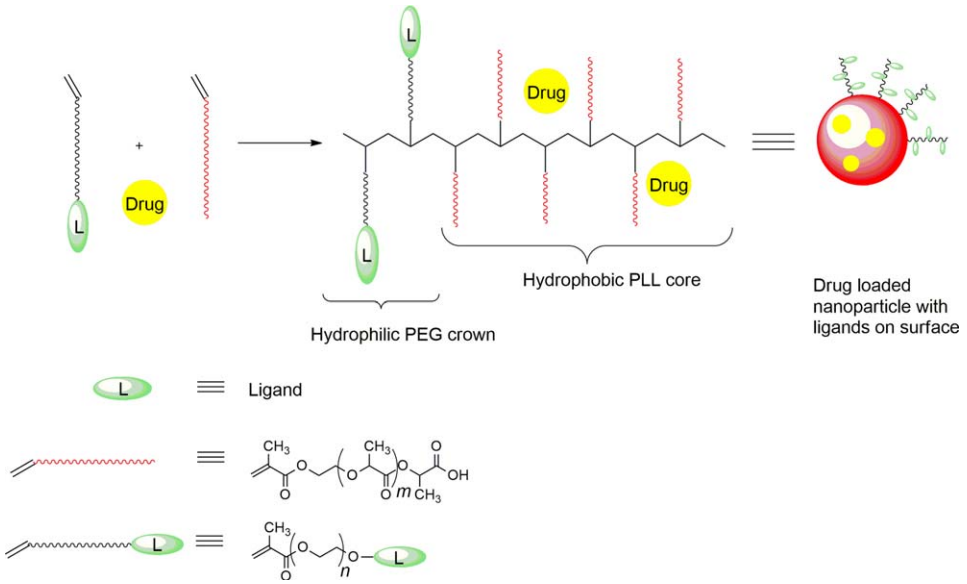


Scheme 1. General scheme for synthesis of drug-loaded PLL-nanoparticles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

nanoparticles by *in situ* polymerization: free radical dispersion polymerization and emulsion polymerization.[35–37] Nanoparticles with hydrophobic core form simultaneously with hydrophilic crown, which is exposed to the aqueous medium. The method also allows simultaneous drug loading (Scheme 1). The surface of the nanoparticle is predetermined by hydrophilic macromonomer and can be tuned *via* various modifications. Polymerizable RGD-bearing macromonomers hold great potential for preparation of drug-loaded nanoparticle formulations for site specific delivery by in situ polymerization technique.

Solid supported synthesis of polymerizable macromonomer-bearing RGD-ligand was reported earlier by Maynard et al.[38] and later on by Ayres et al.,[39] and Perlin et al.[40] Solid phase synthesis allows efficient control of sequence specificity; however, it has limitations on the scale and it restricts application of high molecular weight PEG due to the probability of surface intermolecular aggregation.

Polydisperse-PEG macromonomers of $M_{av} > 3000$ bearing RGD peptide are prepared by liquid phase methodology via convergent coupling of the methacrylated PEG *N*-hydroxy succinimidyl ester (MA-PEG-CO-SI) and free RGD peptide. Completion of reaction is monitored by terminal amino group analysis[5] or by the disappearance of proton peak of *N*-hydroxy succinimidyl ester.[3] Other reports simply rely on excess of activated PEG.[6,7,41] One should bear in mind that excessive amount of activated substrate often results in PEGylation of Arginine residue, which may significantly decrease RGD-affinity.

In order to couple MA-PEG with RGD-peptide selectively, a highly specific "click" coupling procedure was employed by Shokeen et al.[42] Gaining selectivity, however, requires additional modification of both peptide and MA-PEG. Unfortunately, the authors did not provide any structural evidence of the macromonomer aside from proton NMR. We concluded that significant lack of spectroscopic evidence in synthesized macromonomers may lead to uncertainty in biological activity assessment. NMR alone cannot show complete conjugate characterization. Gel permeation chromatography, which is widely used in evaluating average MW of polymers and macromonomers, has limited precision, especially for low MW polymers. Besides, GPC is a relative method, requiring calibration with a standard of known MW.

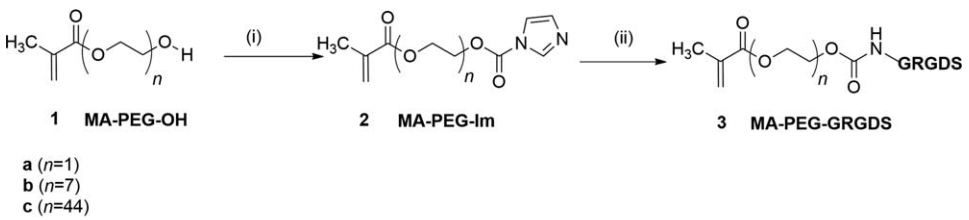
Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been widely accepted as a useful method to study various types of biomolecules due to its soft ionization properties.[43,44] Unlike electrospray ionization (ESI) that produces multiple charged ions, the MALDI process almost exclusively generates singly charged ions,[45] which can greatly help reduce complexity of mass spectra for easier and more confident data interpretation.[46] Enjalbal and group disclosed convenient MALDI-TOF MS analysis of soluble PEGs.[47] The choice of MALDI-MS was governed by the fact that simpler mass spectra were generated, thus allowing easier and more rapid data interpretation. Such automated high throughput process, including data acquisition and interpretation, is applicable to the monitoring of multistep modification of low MW PEGs.

Here, we propose the two step synthesis of MA-PEG-GRGDS macromonomer **3** according to Scheme 2, with the use of MS to monitor each step in order to assure completion.

EXPERIMENTAL

Chemicals and Reagents

1,1'-Carbodiimidazole (CDI), *N,N*-diisopropylethylamine (DIPEA), 2-Hydroxyethyl methacrylate (HEMA), solvents for reactions and HPLC analysis were purchased from Aldrich. PEG Monomethacrylates: $M_{Av} = 400$ and 2000 were supplied by Monomer-Polymer & Dajac Laboratories and Polysciences, respectively,



Scheme 2. General scheme for synthesis of RGD-bearing macromonomers.

and used as received without purification. GRGDS-peptide trifluoroacetate was purchased from American Peptide Company.

Instrumentation

HPLC preparative separation was performed on Hewlett-Packard liquid chromatography system Series 1100 equipped with UV-detector using Zorbax SB300SB-C18 reversed-phase semi-preparative column (250 × 9.6 mm, 5 min). The flow rate was 4.0 mL/min and the effluent was monitored at 210 nm. The gradient started with 15% of Acetonitrile versus 85% water (0.1% Trifluoroacetic acid) and it was changed linearly to 85% over a period of 15 min.

MALDI-ToF measurements were performed on a Voyager DE-STR instrument (Applied Biosystems) equipped with a pulsed nitrogen laser (20 Hz, 337 nm). For the Voyager DE-STR, the following conditions were used: acceleration voltage 20 kV, grid voltage 64% (reflectron) and delay time 350 ns. The spectrum was recorded in the reflectron mode with DHB (dihydrobenzoic acid) as matrix. A total of 300 laser shots were summed for each spectrum.

For the measurements on the Voyager DE-STR, 10 μL of sample (1mg/mL in DCM) was mixed with 30 μL of DHB (10 mg/mL in Methanol) matrix solution and vortexed for 30 s. Resulting solution was then spiked (1 µL) on a stainless steel Bruker Anchor100 sample plate. The preparation was allowed to dry at room temperature and subjected to MS. ESI-MS was recorded on Thermo LTQ Orbitrap Mass Spectrometer (MS), using Nano Electro Spay (NSI). MS was operated in FT positive ion mode with resolution of 30,000. Samples were diluted up to concentration of 10^{-6} M in MS grade solvent mixture H_2O-acetonitrile (MeCN) (50/50) prior injection. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer using the residual proton/carbon resonance of the solvent as the internal standard.

General Procedure for Synthesis of Carbamates 2 (Scheme 2)

PEG monomethacrylates **1** (1 mmol) and CDI (1.2 mmol) were dissolved in 5 mL of dichloromethane (DCM) and left to stir at room temperature for 48 h. Reaction mixture was then washed with saturated sodium carbonate, dried over anhydrous sodium sulfate and evaporated to give colorless oil (**a–b**) or white solid (**c**) with quantitative yield.

HEMA-Im (2a). *Pale colorless oil.* HEMA-Im was verified by ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.91 (s, 3H, CH_3), 4.47 (t, $J = 3.6$ Hz, 2H, CH_2–O), 4.63 (t, $J = 3.6$ Hz, 2H, CH_2–O), 5.59 (m, 1H, vinyl C–H), 6.11 (m, 1H, vinyl C–H), 7.05 (s, 1H, C–H), 7.40 (s, 1H, C–H), 8.11 (s, 1H, C–H). In good agreement with previously reported data (Supplementary information 2).[48]

MA-PEG-Im (2b). *Pale colorless oil.* ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.86 (s, 3H, CH_3), 3.50–3.80 (m, 32H, O–CH_2–CH_2–O), 4.19–4.22 (m, 2H, CH_2–O), 4.47–4.50 (m, 2H, CH_2–O), 5.49 (m, 1H, vinyl C–H), 6.04 (m, 1H, vinyl C–H), 6.98 (s, 1H, C–H), 7.37 (s, 1H, C–H), 8.07 (s, 1H, C–H). ^{13}C NMR (400 MHz, $CDCl_3$, δ, ppm): 18.2 (CH_3), 63.8–70.6 (–OCH_2CH_2O–), 117.1 (CH), 125.6 (vinyl C), 130.6 (CH),

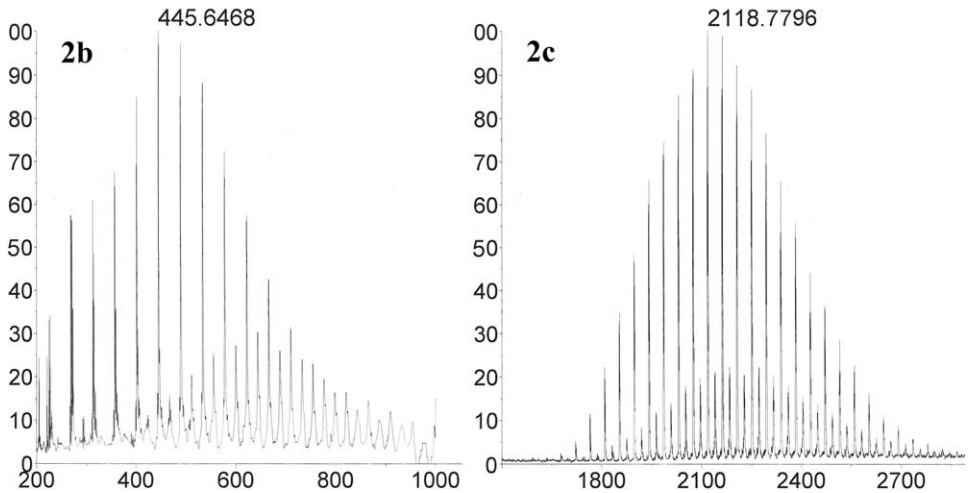


Figure 1. MALDI spectra of MA-PEG-CO-Im **2**. Lower domes correspond to sodiated ions.

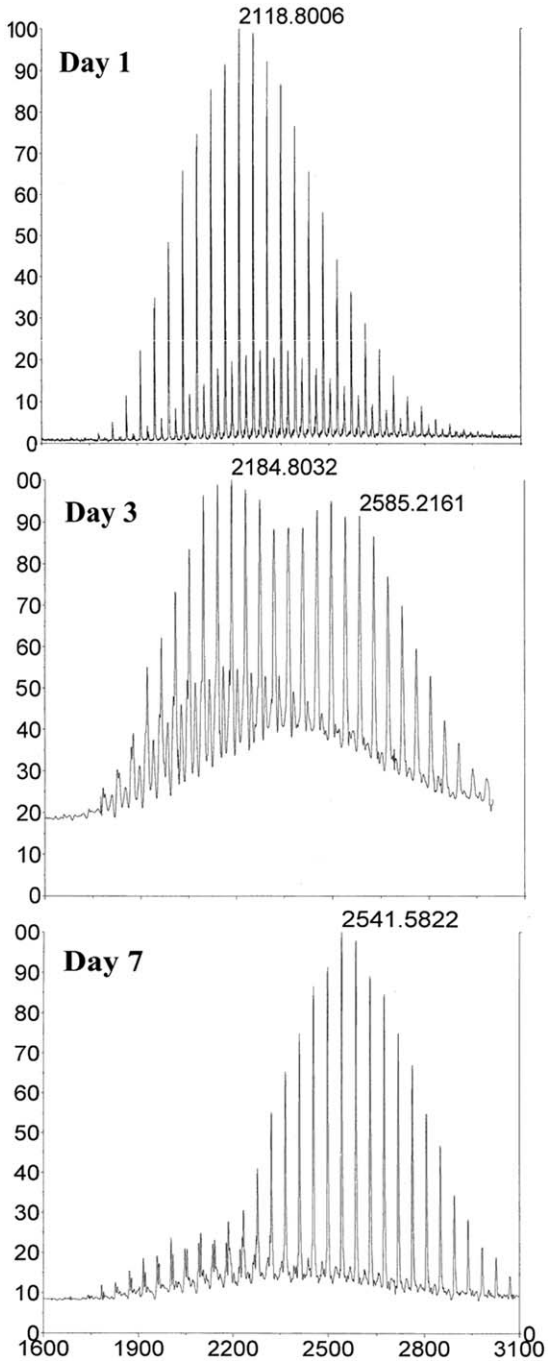


Figure 2. Daily progress on synthesis of **3c**.

136.1 (vinyl C), 137.1 (CH),148.6 (CO), 167.2 (CO). MALDI-ToF-MS: calcd. 445.2186 for n=6 $[(M + H)]^+$, found 445.6642.

MA-PEG-Im (2c). White solid, mp = 47–51 °C. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 1.84 (s, 3H, CH_3), 3.30–3.38 (m, 212H, O–CH_2CH_2–O), 4.17–4.21 (m, 2H, CH_2–O), 4.45–4.47 (m, 2H, CH_2–O), 5.47 (m, 1H, vinyl C–H), 6.02 (m, 1H, vinyl C–H), 6.96 (s, 1H, C–H), 7.35 (s, 1H, C–H), 8.05 (s, 1H, C–H). ^{13}C NMR (400 MHz, $CDCl_3$, δ, ppm): 18.0 (CH_3), 63.6–70.4 (–OCH_2CH_2O–), 116.9 (CH), 125.4 (vinyl C), 130.4 (CH), 135.9 (CH), 136.9 (vinyl C),148.4 (CO), 167.0 (CO). MALDI-ToF-MS: calcd. 2118.215 for n=44 $[(M + H)]^+$, found 2118.7796 (Supplementary information 3, 4, 5, 6, 7, 8).

General Procedure for Synthesis of Macromonomer 3 (Scheme 2)

Two solutions were prepared: A: 0.1 M solution of DIPEA in anhydrous DMSO and B: 0.1 M solution of Carbamate **2** in anhydrous DMSO. 5 mg of GRGDS peptide trifluoroacetate salt (8.4 μmol) was dissolved in 200 μl of solution A. 84 μl of solution B (8.4 μmol of **2**[1]) was added subsequently. Reaction was monitored by MALDI-ToF-MS for checking when signal of **2** vanishes. Reaction took 18 hours for **3a**, 72 hours for **3b** and a week for **3c**. After the reaction was complete, the mixture was diluted with 200 μL of DCM followed by precipitation in 2 mL of ether. Precipitate was then washed with ether 2–3 times, dissolved in 1 ml of water, filtered through 0.45 μm filter and freeze-dried overnight in a previously weighed vial.

HEMA-CO-GRGDS (3a). *White powder.* Yield, 5.9 mg. ^{1}H NMR (400 MHz, D_2O, δ, ppm): 1.31 (d, J = 4,8 Hz, residual DIPEA), 1.50–1.85 (m, 4H, CH_2–CH_2), 1.89 (s, 3H, CH_3), 2.75–2.93 (m, 2H, Asp CH_2), 3.16–3.21 (m, 2H, Arg CH_2), 3.80–3.95 (m, 6H), 4.30–4.42 (m, 7H), 5.70 (m, 1H, vinyl C–H), 6.11 (m, 1H, vinyl C–H), 7.45 (s, 1H, N–H), 8.67 (s, 1H, N–H). MALDI-ToF-MS: calcd. 647.2637 for $[(M + H)]^+$, found 647.2875 (Supplementary information 9, 10).

MA-PEG-GRGDS (3b). *White powder.* Yield, 5.0 mg. ^{1}H NMR (400 MHz, D_2O, δ, ppm): 1.31 (d, J = 4,8 Hz, residual DIPEA), 1.50–1.85 (m, 7H, CH_2–CH_2), 1.89 (s, 3H, CH_3), 2.60 (residual DMSO), 2.70–2.92 (m, 4H, Asp CH_2), 3.16–3.21 (m, 4H, Arg CH_2), 3.50–3.99 (m, 40H), 4.22 (s, 2H), 4.30–4.40 (m, 6H), 5.70 (m, 1H, vinyl C–H), 6.11 (m, 1H, vinyl C–H), 7.45 (s, 2H, N–H), 8.66 (s, 1H, N–H). MALDI-ToF-MS: calcd. 911.4210 for n=7 $[(M + H)]^+$, found 911.3727 (Supplementary information 11, 12).

MA-PEG-GRGDS (3c). *White powder.* Yield, 13.3 mg. ^{1}H NMR (400 MHz, D_2O, δ, ppm): 1.31 (d, J = 4,8 Hz, residual DIPEA), 1.50–1.85 (m, 4H, CH_2–CH_2), 1.92 (s, 3H, CH_3), 2.70–2.92 (m, 2H, Asp CH_2), 3.16–3.21 (m, 2H, Arg CH_2), 3.50–3.99 (m, 231H), 4.21–4.24 (m, 2H), 4.30–4.40 (m, 4H), 5.73 (m, 1H, vinyl C–H), 6.15 (m, 1H, vinyl C–H), 7.47 (s, 2H, N–H), 8.69 (s, 1H, N–H). MALDI-ToF-MS: calcd. 2585.4210 for n=45 $[(M + H)]^+$, found 2585.2410 (Supplementary information 13, 14).

RESULTS AND DISCUSSION

Activation of MA-PEG-OH 1

Synthesis of the macromonomer reported in this work involves two steps. First is the activation of terminal hydroxyl group of PEG-derivative **1** with CDI giving carbamate **2**. Slight CDI excess of 0.2 equivalent was taken in order to assure that derivatization was complete. Carbamates **2** are stable at room temperature. They can be isolated in pure form and stored for a prolonged time in a freezer without degradation. All three carbamates **2** were characterized by MALDI-MS and NMR. MALDI spectra of the intermediates **2** were calibrated versus starting materials **1**, that were considered pure. Spectra resolution

[1]Molecular weights of carbamates **2** were calculated from ^{1}H NMR data.

Table I. Accuracy Estimation for MALDI-MS Measurements of 3

Macromonomer **3**	**a**	**b**			**c**		
Calculated for $[M+H]^+$	647.2637	867.3948	911.4210	955.4472	2541.394	2585.421	2629.447
Found	647.2875	867.3589	911.3737	955.3794	2541.1947	2585.245	2629.247
Accuracy (ppm)	37	41	52	71	78	68	76

Table II. Synthesis of Macromonomers 3 Details

Quantity (mg)	Activated carbamate **2** (mmol)	**GRGDS** peptide (mmol)	Isolated macromonomer **3** (mmol/Yield, %)
a	2.3 mg (0.01)	5 mg (0.0083)	5.4 mg (0.0083/100)
b	5.6 mg (0.01)	5 mg (0.0083)	3.2 mg (0.0032/38)
c	20.0 mg (0.01)	5 mg (0.0083)	13.3 mg (0.0046/55)

obtained did not allow getting clear isotopic pattern. Main peaks corresponding to each fraction were taken as one signal and used for the characterization. Proton and carbon spectra of HEMA derivative are in good agreement with what had been reported earlier.[48] NMR spectra for MA-PEG-Im derivatives are similar differing in integrals for ethylene oxide chain. Both **2b** and **2c** MALDI spectra represent nice distribution of signals referring to monocharged molecules of **2** but not to starting material or side products (Figure 1).

Synthesis of Polymerizable Macromonomer 3

The next step involves nucleophilic substitution of imidazole with terminal glycine amino-group of GRGDS peptide in DMSO in the presence of Hunig Base (DIPEA). It is hard to control such reactions due to low load and decreased reaction rates in case of higher molecular weight PEGs. Excessive amounts of Imidazole derivatives **2**, however, could be applied, but double acylation of other nucleophilic centers (–OH of Serine and $–NH_2$ of Arginine) of peptide might occur giving a product of reduced biological activity. We did add 1 equivalent of activated PEG **2** and tracked the reaction using ESI-MS for **3a** and used MALDI mass-spectrometry for **3b** and **3c**. Even though ESI-MS spectrum for **3b** looks clear with well distributed ions, switching to a higher molecular weight PEG gives a very complex spectrum; while the use of MALDI gives a clear distribution of singly charged ions (Figure 2). Reaction of **2c** with peptide took at least 7 days to accomplish according to MALDI. We were able to see gradual vanishing of **2c** dome and appearance and rise of dome corresponding to **3c** (Figure 2).

Three most abundant signals were chosen in order to assess overall accuracy of MALDI-MS (See spectra in SI) measurements. Data are given in Table I.

Resulting mixtures (**3a–c**) were diluted with ether in order to precipitate the product. Imidazole was supposed to be gone with the solvent leaving the product alone. Since the product is a dicarboxylic acid we observe precipitation of its DIPEA salt. The quantities isolated are given in Table II.

All three samples were diluted in 600 μL of D_2O and subjected to ^1H NMR. Proton spectra showed the methacrylate residue represented by vinyl singlets at 6.1 and 5.7 ppm (Figure 3) and methyl singlet at 1.9 ppm common for all three macromonomers. Ethylene glycol protons for macromonomers of different molecular weights show up at 4.3 and 3.8 ppm. One also can observe characteristic Arginine methylene multiplets at 1.6, 1.8, and 3.1 ppm. In all cases **3a,c** we were able to isolate the product with accurate integral ratio of vinyl and arginine methylene protons as 1:2 (Figure 3). As for **3b,** we assume the reaction resulted in the mixture of macromonomer and peptide 1:1. Peptide was successfully separated using preparative HPLC.

CONCLUSIONS

Precise monitoring of polymeric species during modifications has always been a challenge. We have shown in this article the

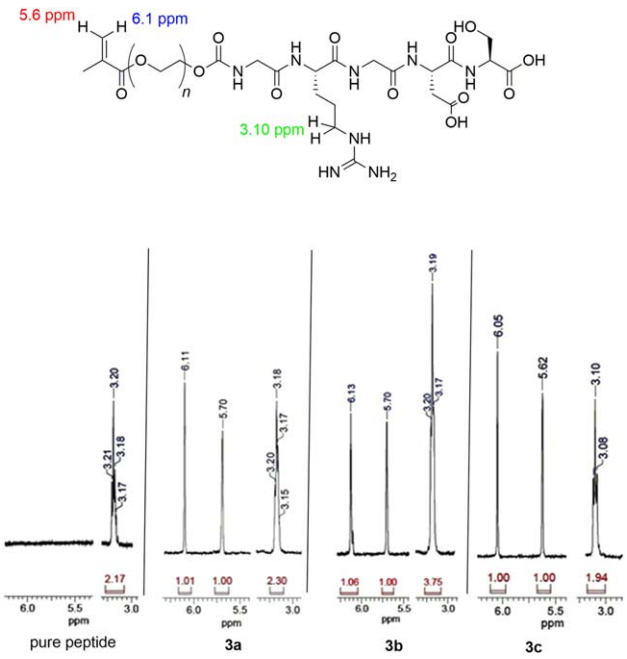


Figure 3. Characteristic vinyl and methylene protons of **3a–c**. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

use of MALDI for immediate data acquisition of mixture contents and monitoring progress on polymer modification *en route* the synthesis of RGD-bearing PEG monomethacrylate macromonomers with clear spectra of high resolution not obtainable with other methods such as GPC, NMR, and ESI-MS.

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