Bioconjugate Chemistry

Communication

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Top-down Synthesis of Versatile Polyaspartamide Linkers for Single-Step Protein Conjugation to Materials

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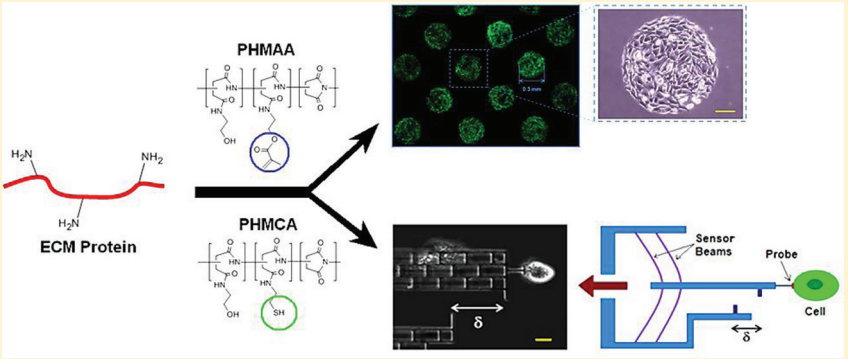
Supporting Information



ABSTRACT: Materials used in various biological applications are often modified with proteins to regulate biomolecular and cellular adhesion. Conventional strategies of protein conjugation accompany monovalent bifunctional protein linkers, which present several limitations in molecular synthesis and protein conjugation. Herein, we present a new strategy of preparing multivalent polyaspartamide linkers in a simple top-down manner, and also demonstrate that the resulting polymer linkers allow us to readily conjugate proteins to both organic and inorganic materials. The top-down synthesis of polyaspartamide linkers was performed by partially opening succinimidyl ring moieties of polysuccinimide (PSI) with the controlled number of nucleophiles reactive to photo-cross-linked hydrogel or gold-coated inorganic materials: (1) Poly(2-hydroxyethyl-*co*-2-methacryloxyethyl aspartamide) (PHMAA) presenting methacrylate was used to micropattern fibronectin or collagen on a hydrogel in order to regulate cell adhesion and growth area on a micrometer scale. (2) Poly(2-hydroxyethyl-*co*-2-mercaptoethyl aspartamide) (PHMCA) presenting thiol functional groups was used to link fibronectin to a gold-coated silicon microelectromechanical probe designed to measure cell traction force. Overall, these multivalent polyaspartamide protein linkers will greatly assist efforts to analyze and regulate the cellular adhesion to and phenotypic activities of a wide array of substrates and devices.

A variety of organic and inorganic materials are widely used in the assembly of in vitro cell culture platforms, biomedical implants, and biomicroelectromechanical systems (bio-MEMS) devices.[1−5] These materials are usually chemically linked with a series of proteins, such as fibronectin, collagen, and antibodies, in order to control cellular adhesion to the materials.[6,7] The number and spacing of these proteins act as insoluble signals to regulate cellular phenotypes.[8−10] Various forms of bifunctional linkers are increasingly used for stable protein conjugation.[11−14] These linkers largely contain two monovalent functional groups: one reactive to a protein and the other reactive to a material. These linkers require multiple modification and purification steps during molecular synthesis and protein conjugation to target substrates, thus necessitating extensive labor. In addition, the number of monovalent linkers should increase in tandem with the amount of protein conjugated to the materials due to their monovalency, which raises concerns over increased cytotoxicity and inadvertent changes in material properties.

To resolve these challenges encountered with the use of monovalent linkers, this study presents a simple top-down synthetic strategy to prepare a polyaspartamide linker with controlled multivalencies of reactive groups to both proteins and materials. In addition, this study demonstrates that the polymeric linkers allow us to conjugate cell adhesion proteins with a single step. Polysuccinimide (PSI) with the average

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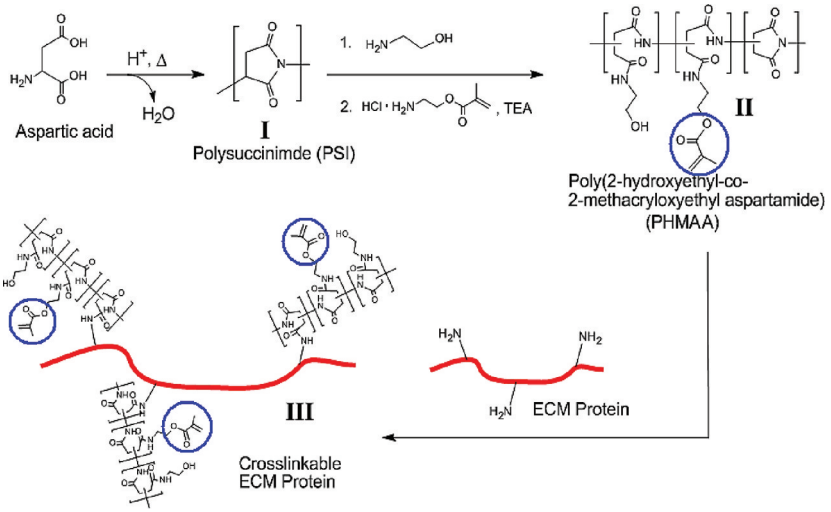
Scheme 1. Synthesis of PHMAA and Its Reaction with Fibronetin (Fn) to form Fn-PHMAA[a]



[a]Chemically reactive methacrylic groups are marked with blue circles.

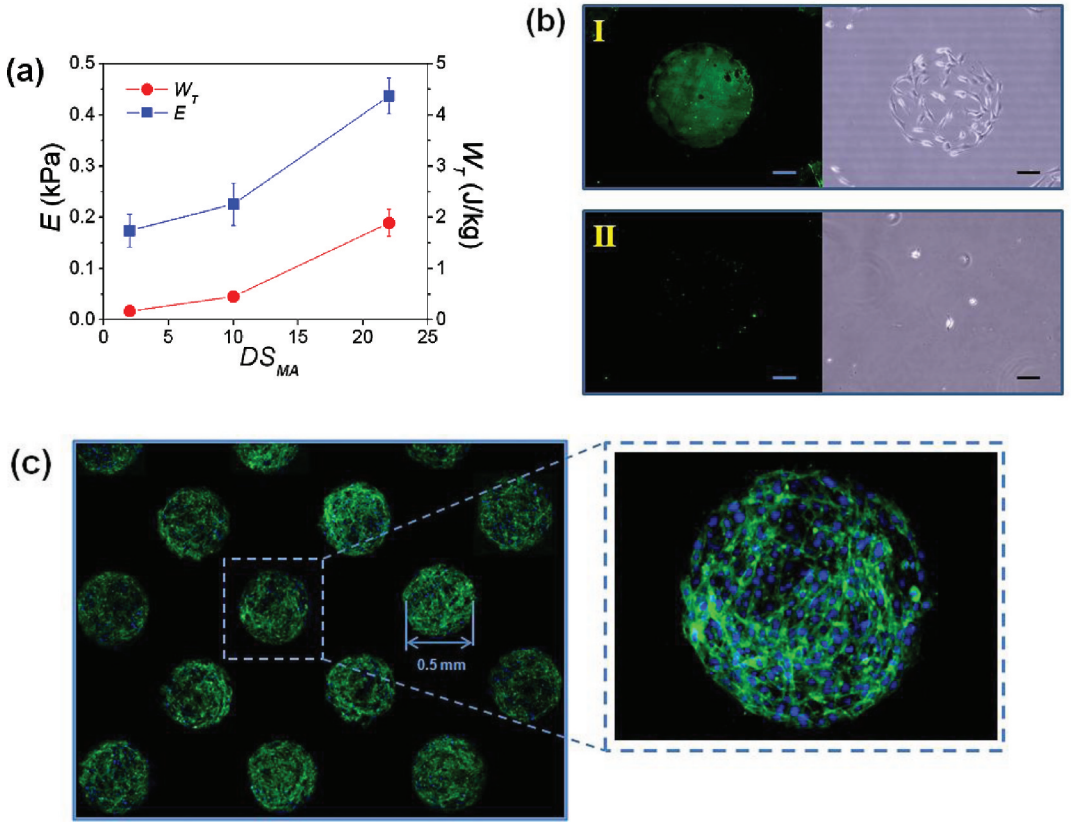


Figure 1. (a) Elastic modulus (E) and tensile energy (W_T) of polyacrylamide hydrogel cross-linked with PHMAA with varying degree of methacrlyate substitution (DS_{MA}). (b) Circular pattern of Fn-PHMAA (**I**, left) or pure Fn (**II**, left) on polyacrylamide hydrogel visualized with immunoflurescent labeling. Fibroblast adhesion was more active on the Fn-PHMAA pattern (**I**, right), compared with pure Fn pattern (**II**, right) (Scale bar: 100 μm.) (c) Proliferation of fibroblasts within the circular pattern of Fn-PHMAA visualized with fluorescein-phalloidin (green) and DAPI (blue), respectively (a magnified image of a circular pattern shown on the right).

molecular weight of 22 000 g mol^{-1} was synthesized by acid-catalyzed polycondensation of aspartic acid and used as a starting molecule (Scheme 1 (**I**) and Figure S1a in Supporting Information).[15] PSI consists of a series of succinimidyl units which allow ring-opening nucleophilic addition.[16,17] In this study, we partially opened the succinimidyl rings of PSI with a controlled amount of amine-based nucleophiles that are reactive to a target material, in order to create a polyaspartamide linker in a top-down manner. The protein of interest, which also contains amine groups, would be conjugated to the intact succinimidyl groups of polyaspartamide in the same fashion as the amine-based nucleophilic substituents. This synthetic strategy allows the linker to present a variety of functional groups needed for conjugation to specific

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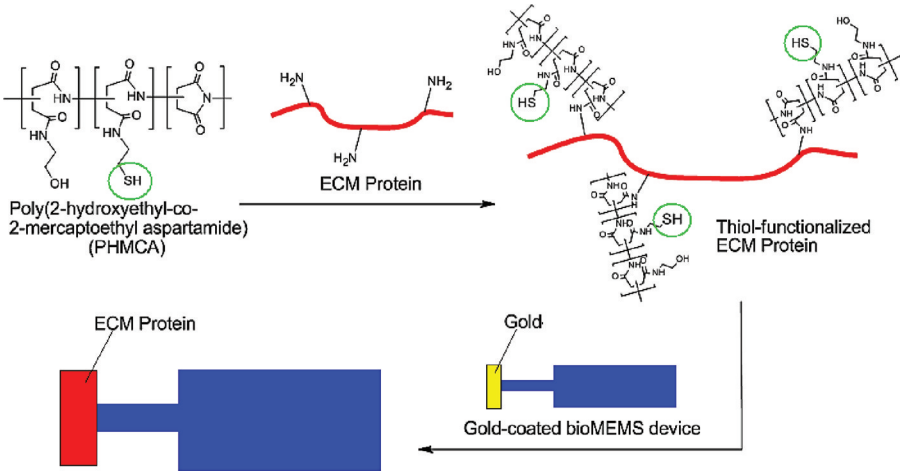
Scheme 2. Synthesis of PHMCA and Its Reaction with Fibronetin (Fn) to Form Fn-PHMCA[a]



[a]Chemically reactive thiol groups are marked with green circles. Fn-PHMCA was used to conjugate Fn to gold-coated bio-MEMS device.

materials simply by using nucleophiles containing different functional groups. Here, we demonstrate the function and versatility of this polyaspartaminde linker with two applications: (1) protein conjugation to a hydrogel using a polyaspartamide linker containing methacrylic groups, and (2) protein conjugation to a gold-coated bio-MEMS device using a polyaspartamide linker containing thiol groups.

First, we synthesized a polyaspartamide linker that can conjugate cell adhesion proteins to a hydrogel that is conventionally used for in vitro cell culture. For this purpose, designated amounts of ethanolamine and 2-aminoethyl methacrylate were added sequentially to PSI to prepare poly(2-hydroxyethyl-co-2-methacryloxyethyl aspartamide) (PHMAA) (Scheme 1 (**II**) and Figure S1b in Supporting Information). The addition of 2-aminoethyl methacrylate to PSI presented the linker with methacrylic groups needed for radical polymerization. The ethanolamine allowed us to control the number of unreacted succinimidyl groups independent of the number of methacrylic groups, and also to render the linker water-soluble by presenting hydroxyl groups.

These hydroxyl groups were not reactive toward unreacted succinimidyl groups of the PHMAA, as confirmed with minimal changes in the peak integrals corresponding to unreacted succinimidyl groups in the ^{1}H NMR spectrum (Figure S1b in Supporting Information). Separately, PSI was mixed with ethylene glycol to further determine whether hydroxyl groups are reactive toward succinimidyl groups. The ^{1}H NMR spectrum of the resulting product was identical to that of original PSI (Figure S1d in Supporting Information), without showing any changes in peaks corresponding to unreacted succnimidyl groups. On the other hand, PSI mixed with ethylenediamine resulted in immediate polymeric precipitation.

The ability of PHMAA to copolymerize with gel-forming monomers was evaluated by examining whether the PHMAA can cross-link acrylamide monomers to form a hydrogel and to further control hydrogel properties. PHMAA was able to cross-link acrylamide to form a hydrogel via in situ free radical polymerization (Figure S2 in Supporting Information). Furthermore, the PHMAA could increase the elastic modulus and resistance to fracture of the hydrogel with the degree of methacrylic substitution (DS_{MA}), the percentage of succinimidyl groups conjugated with methacrylic groups (Figure 1a). We suggest that mechanical properties of the hydrogel should be further tuned by increasing concentrations of acrylamide and PHMAA. In addition, mechanical properties of hydrogels were not significantly changed during incubation in neutral media, which addresses the lack of significant hydrolysis of unreacted succinimidyl units of PHMAA.

PHMAA was then reacted with fibronectin (Fn), a cell adhesion protein, to prepare Fn reactive to a hydrogel (Fn-PHMAA) (Scheme 1, **III**). The DS_{MA} of PHMAA used to conjugate Fn was kept constant at 10%. The reaction between PHMAA and Fn was confirmed by monitoring the decrease of free amine groups quantified with TNBS (trinitrobenzenesulfonic acid) assay (Table S1 and Figure S3 in Supporting Information).[18] Increasing the amount of Fn while keeping PHMAA constant led to a more rapid decrease of amine groups in the TNBS assay. Furthermore, ^{1}H NMR analysis of PHMAA reacted with varying amounts of glycine, as a model amino acid, showed that the degree of substitution of glycine (DS_{Gly}), analyzed with peak integral ratio of the reacted glycine to the total succinimidyl groups, could be varied from 3.6% to 11.9%. These results show that the multivalency of unreacted succinimidyl groups in PHMAA facilitated reactions with multiple amine groups in protein.

The resulting Fn-PHMAA was used to control the size and spacing of cell adhesion domains on a polyacrylamide hydrogel, combined with a microcontact printing technique (Figure S4 in Supporting Information).[19] Briefly, a circular array of Fn-PHMAA was placed on a glass surface using a microcontact stamp onto which the Fn-PHMAA was physically adsorbed. Both the diameter and spacing of the circles were kept constant at 500 μm. The subsequent in situ cross-linking reaction between acrylamide and *N*,*N*,*N*′,*N*′-methylene bisacrylamide on top of the micropatterened Fn-PHMAA resulted in a polyacrylamide hydrogel which presented circular arrays of fibronectin with regular size and spacing on its surface, as confirmed with immunofluorescent labeling (Figure 1b). The fibronectin mass per circular pattern, determined from a colorimetric immunoassay, was kept constant at 5 μg cm^{-2}. In contrast, the same polyacrylamide hydrogel fabrication on top of pure Fn without PHMAA, a negative control, resulted in limited conjugation of Fn to the surface.

The circular patterns of Fn on the polyacrylamide hydrogel were recognized by cells plated on the gel. Fibroblasts seeded on the hydrogel surface with circularly patterned Fn-PHMAA

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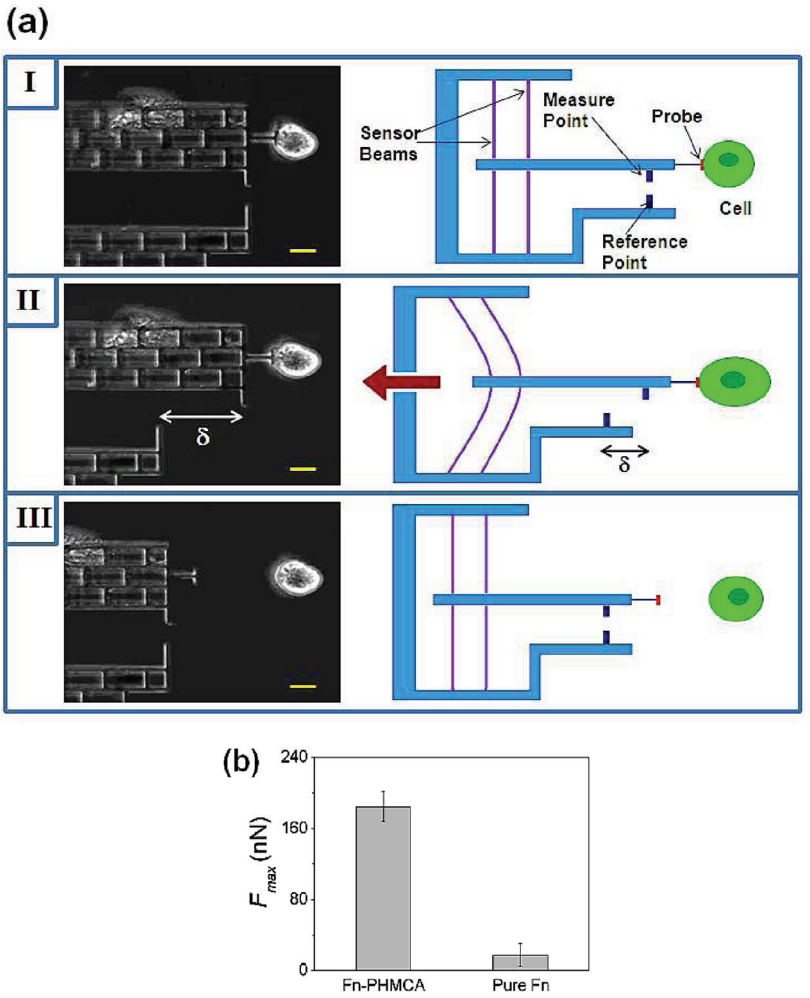

Figure 2. (a) Schematic description of measurement of cell force response using a bio-MEMS device. First, the probe conjugated with Fn is placed in contact with cell (**I**). After incubation, the device is moved away from the cell laterally (red arrow) at a constant rate (**II**). The probe is connected to sensor beams. Due to the cell's adhesion to the probe, it is displaced from the original position, as indicated by the difference between the measurement point and reference point. The probe is continuously pulled until it is finally detached from the cell (**III**). (Scale bar: 15 μm.) (b) The maximum applied force (F_{max}) was significantly larger when the probe was conjugated with Fn-PHMCA than with pure Fn.

adhered exclusively on the patterned area (Figure 1b-**I**). Furthermore, the cells adhered to the circular pattern proliferated over time, while remaining confined to the patterned area (Figure 1c and Figure S5 in Supporting Information). In contrast, the hydrogel patterned with pure Fn displayed limited cell adhesion (Figure 1b-**II**).

The same results were also observed when type I collagen and human airway smooth muscle cells (haSMCs) were used instead of Fn and fibroblasts (Figure S6 in Supporting Information). For this study, collagen linked with PHMAA (DS_{MA} = 10%) was used to pattern the polyacrylamide hydrogel surface with a circular array of collagen at a density of 10 μg cm^{-2}, following the same procedure as described in SI Figure S4. haSMCs exclusively adhered and proliferated within this circularly patterned hydrogel area. This result verifies that PHMAA can chemically link a variety of cell adhesion proteins to the hydrogel and control spatial organization of a wide array of anchorage-dependent cells.

Next, we synthesized a polyaspartamide linker modified with a controlled number of thiol groups for its use in protein conjugation to gold-coated inorganic substrates (Scheme 2). Gold has been extensively used in biosensors such as bio-MEMS and surface plasmon resonance (SPR), due to its inertness, robust structural integrity, and excellent conductivity.[20,21] These gold-coated devices are commonly modified with cell adhesion proteins or antibodies using a thiol-substituted bifunctional monovalent linker, which is typically synthesized via multiple substitution and purification steps. Here, we synthesized poly(2-hydroxyethyl-2-mercaptoethyl aspartamide) (PHMCA) with the controlled number of thiol groups. The same synthetic procedure to create PHMAA in Scheme 1 was used, except that cysteamine was used as a nucleophile to present thiol groups (Scheme 2 and Figure S1c in Supporting Information). The number of thiol groups in PHMCA, which are available to react with gold, was determined to be 10 per molecules, according to a colorimetric assay using Ellman's reagent.[22] The percentage of cysteamine that side-reacted to form thioester linkages was 24% (see Supporting Information). PHMCA was then mixed with Fn to prepare the Fn-PHMCA, and the reaction between PHMCA and Fn was confirmed with TNBS assay, as conducted for Fn-PHMAA (data not shown).

The resulting Fn-PHMCA was used to chemically modify the surface of the gold-coated probe of a bio-MEMS device which is designed to measure force exerted by cells under local uniaxial stretching.[23,24] The bio-MEMS device is made from a single crystal silicon wafer, and the surface of its probe was

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coated with gold to prevent nonspecific interaction with cells (Figure S7 in Supporting Information). Then, the probe was immersed in a solution of Fn-PHMCA to chemically link Fn to the probe (Scheme 2). Separately, another device was also immersed in a solution of pure Fn as a negative control.

The probe modified with Fn-PHMCA or pure Fn was allowed to contact a fibroblast to form specific bonds between Fn and cellular integrins. The device was then pulled from the cell at a rate of 1 μm s^{-1}, and its maximum stretching force (F_{max}) before the cell detachment from the probe was calculated by the equation $F_{max} = k\delta$, where k is the spring constant of the sensor beams to which the probe is attached, and δ is the displacement of the probe from its original position (Figure 2a). The probe coated by the pure Fn was readily detached from the cell with only a small displacement (average $\delta \approx 6$ μm, $F_{max} \approx 20$ nN), indicating poor linkage between Fn and the probe. In contrast, the cell contacted by a Fn-PHMCA modified probe exerted a significant force resulting in an average δ of 55 μm ($F_{max} \approx 185$ nN) (Figure 2b). Furthermore, the F_{max} values were minimally changed during repeated use of the same probe, demonstrating that the chemical linkage of Fn-PHMCA to the gold-coated probe was well retained.

In summary, this study presents a novel strategy of synthesizing versatile polyaspartamide linkers that allow convenient conjugation of proteins to a variety of materials. The synthetic strategy of introducing functional groups by nucleophilic addition to PSI in a top-down manner allowed us to independently control the number of units reactive to proteins and those to materials in a highly efficient manner. In addition, these linkers allow a convenient one-step conjugation of various proteins to organic and inorganic materials. However, the chemical reaction between the linker and protein may cause a small portion of proteins to be denatured, the extent of which should be more carefully examined in future studies. These mutivalent linkers therefore present several attributes superior to conventional monovalent linkers. Furthermore, we expect that the versatility of the polyaspatamide linkers, due to their synthetic strategy of presenting functional groups in a simple top-down manner, will make it possible to further utilize a wide array of conjugation chemistries necessary for tailormade materials.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental methods and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

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