

Protein-Polymer Grafts via a Soy Protein Derived Macro-RAFT Chain Transfer Agent

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Abstract Methodology to produce materials derived from renewable resources is of great importance in decreasing both environmental impact as well as dependence on fossil fuels. We report a straightforward method of polymer grafting to soy protein hydrolysates, which are available in surplus. Benzylthiocarbonate moieties were installed on the protein surface via amidation of free amino groups, creating a protein macro chain transfer agent (CTA) for reversible addition fragmentation transfer (RAFT) polymerization. We found that subsection of this soy protein macro-CTA (SP-CTA) to RAFT polymerization conditions with polar acrylate monomers resulted in protein-polymer nanometer-scale particles with solubility properties dictated by monomer polarity. Polymer grafting, particle size and polymerization were characterized by elemental analysis, transmission electron microscopy and gel permeation chromatography. We anticipate that this method of grafting will be of use in generation of new materials based on renewable resources.

Keywords Biomaterials · Grafting · RAFT polymerization · Renewable resource · Soy protein

Introduction

Robust syntheses of renewable resource derived materials are enabling from economic, environmental, industrial

and basic science perspectives [1–3]. Compromise approaches that replace significant quantities of fossil fuel with renewable resources to produce new “hybrid” materials are considered necessary intermediate steps in reducing environmental impact and developing of new uses for bio-derived source material. Soy protein is a starting material of particular interest given its status as a surplus agricultural protein derived from soy bean processing [2]. A large portion of soybean mass is soy protein, and while the soy oil fraction has received much attention as a renewable resource starting material in a number of industrial, food and cosmetic applications, by comparison, less has been done with the protein fraction. Notably, soy protein has been incorporated into plastics [4–6] through injection molding, polymer blends and grafting [7–10] and developed into wood-adhesives [11–13] through chemical modification. Chemical modifications of agricultural protein have primarily focused on reaction of protein amines with active esters, aldehydes [14] and dihydroxyphenyl [12] moieties for crosslinking; there have also been reports of exposure to esterification conditions [11] to address the many free carboxylate sidechains. Protein-polymer grafting has been explored in many different contexts, both for generation of new renewable resource materials as well as for biomedical applications [7, 15–17]. Recently, grafting approaches have been used to produce hybrid materials of soy protein and poly (1,4-dioxanone) (PPDO) [18] as well as wheat protein and polyethyleneoxide diglycidyl ether (PEODGE) [19]. We report herein our chemical strategy for production of acrylate and acrylamide polymer grafts with agriculturally derived soy protein hydrolysate (SP). The lysine residues of SP, comprising 6% of the total amino acid composition, were targeted for acylation using an activated ester containing an S-benzyl

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trithiocarbonate moiety, a known chain transfer agent (CTA) for reversible addition fragmentation transfer (RAFT) polymerization [20, 21]. This method installs multiple polymer capture sites on the SP backbone, facilitating polymer-protein grafting. Furthermore, RAFT provides a living polymerization reaction, permitting the facile synthesis of block co-polymers. RAFT and ATRP polymerization have previously been used to direct polymer growth on a protein scaffold [22–24] through an elegant specific single-site derivatization with a trithiocarbonate CTA [25] that permits retention of the protein bioactivity. For the purpose of synthesizing hybrid materials from soy protein and acrylate monomers, single-site selectivity is neither needed nor desirable, as multiple points of connection should more securely incorporate the protein macro-CTA in the growing polymer network. We set out to examine this process and the effect of varying monomer:protein ratios to manipulate protein solubility. Transmission electron microscopy (TEM) indicated that at mass ratios near unity, protein-polymer nanometer-scale particles were formed with diameter of approximately 300 nm. As the mass fraction of monomer was increased, particle formation decreased, suggesting that increased polymer grafting decreased particle formation. Polar monomers (acrylamide and acrylic acid) were studied to increase protein solubility in water, and indeed graft solubility increased with increasing monomer fraction. Hydrolytic conditions resulted in cleavage of protein and synthetic polymer, as judged by elemental analysis. This synthetic method appears to be an effective route for polymer grafting and allows the preparation of protein-polymer nanometer-scale particles that could be useful in renewable-resource based latex applications (Scheme 1).

Experimental

Materials and Characterization

Carbon disulfide ($\geq 99.0\%$), benzyl bromide ($\geq 98.0\%$), triethyl amine, monomers and solvents were purchased from Aldrich and used as received with the exception of: acrylic acid ($\geq 99.0\%$), which was distilled under reduced pressure prior to use, and butyl acrylate ($\geq 99.0\%$), which was washed with 5% NaOH solution prior to use. 2,2'-Azobis(2-methylpropionitrile) (AIBN) was purchased from Aldrich ($\geq 98.0\%$) and re-crystallized twice from ethanol. Mercaptopropionic acid ($\geq 99.0\%$) was purchased from Acros. Phosphotungstic acid was purchased from Malinckrodt. Soy protein hydrolysate (SP) was obtained from Archer Daniels Midland. Synthetic yields refer to chromatographically and spectroscopically (NMR and MS) pure compounds. Size exclusion chromatography was carried out on a Ultrahydrogel™ Linear 7.8×300 mm column using 0.1 M sodium nitrate solution with refractive index detection (Shodex RI-71). TEM imaging was performed on Tecnai G2 Spirit microscope using an accelerating voltage of 80 kV.

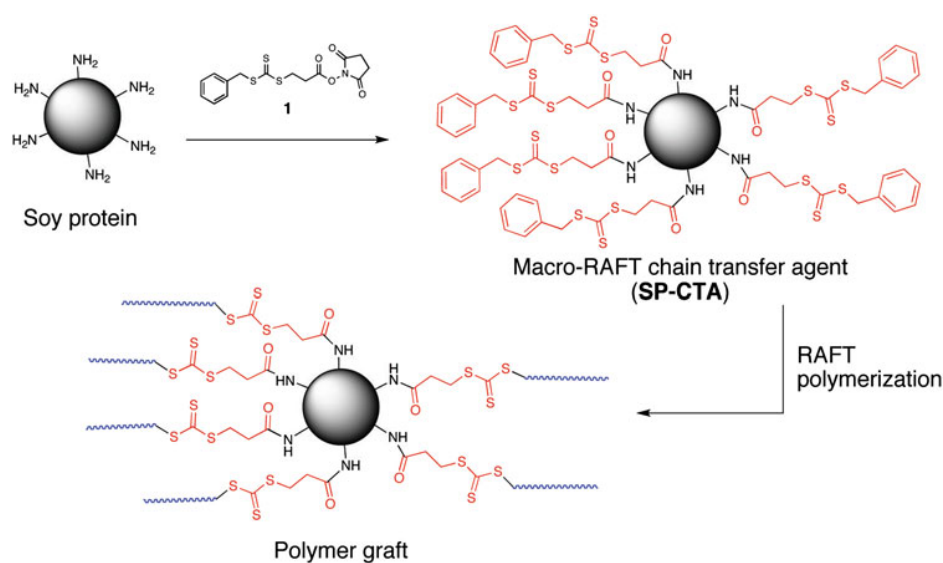
Synthesis

Compounds **2** and **3** were prepared according to literature procedures, [25] briefly described below.

3-Benzylsulfanylthiocarbonylsulfanyl-Propionic Acid (**3**)

3-Mercaptopropionic acid (5 mL, 5.75 mmol) was added to a potassium hydroxide solution (6.5 g, 11.5 mmol) in water (60 mL). Carbon disulfide (7.5 mL) was added

Scheme 1 Transformation of soy protein isolates into macro-RAFT chain transfer agents through acylation with trithiocarbonate chain transfer agent



dropwise to the above solution and the orange solution was allowed to stir for 5 h. The mixture was then heated with benzylbromide (9.9 g, 5.75 mmol) for 12 h at 80 °C. After cooling, chloroform (75 mL) was added and the reaction mixture was acidified with 6(N) hydrochloric acid until the organic layer became yellow. The water phase was extracted with chloroform (3 × 25 mL). The combined organic layers were dried over anhydrous sodium sulfate and solvent was removed under reduced pressure. The product was purified by flash column chromatography with a 3:1 hexane/ethyl acetate mixture as an eluent to yield a yellow powder 14.0 g, 90%). ¹H NMR (CDCl₃): 2.84 (t, 2H, J = 7.15 Hz), 3.62 (t, 2H, J = 7.15 Hz), 4.61 (s, 2H), 7.27 (m, 5H), 10.1 (b, 1H, OH). ¹³C NMR (CDCl₃): 30.7, 32.8, 41.4, 127.7, 128.6, 129.1, 134.6, 177.9, 222.5. ESI-MS (m/z) [M + Na]⁺ calcd for C₁₁H₁₂O₂S₃Na: 295.0, Found: 295.0.

3-Benzylsulfanylthiocarbonylsulfanyl-Propionic Acid Chloride (2)

Compound **3** (8 g, 29.4 mmol) was dissolved in chloroform (40 mL). Thionyl chloride (10 mL) was added slowly to the mixture under nitrogen and allowed to stir for 1 h. The solvent and excess thionyl chloride was removed under reduced pressure to afford yellow oil (8.5 g, 99%) which was used for the next step. ¹H NMR (CDCl₃): 3.34 (t, 2H, J = 7.10 Hz), 3.61 (t, 2H, J = 7.10 Hz), 4.60 (s, 2H), 7.30 (m, 5H). ¹³C NMR (CDCl₃): 30.5, 41.5, 45.5, 127.8, 128.6, 128.7, 134.4, 172.1, 221.9.

Synthesis of RAFT NHS Ester (1)

Compound **2** (8.5 g, 29.4 mmol) was added to a mixture of *N*-hydroxysuccinimide (3.39 g, 29.4 mmol) and triethylamine (2.98 g, 29.4 mmole) in chloroform (75 mL) and allowed to stir for 12 h at room temperature. After evaporation of the solvent under reduced pressure, the crude product was obtained as a yellow solid. The crude product was purified by gel column chromatography with a 3:1 hexane/ethyl acetate mixture as an eluent (4.5 g, 42%). ¹H NMR (CDCl₃): 2.82 (s, 4H), 3.08 (t, 2H, J = 7.20 Hz), 3.67 (t, 2H J = 7.20 Hz), 4.59 (s, 2H), 7.28 (m, 5H). ¹³C NMR (CDCl₃): 25.5, 30.3, 41.5, 127.8, 128.6, 129.2, 134.5, 166.8, 168.8, 222.2. ESI-MS (m/z) [M + Na]⁺ C₁₅H₁₅NO₄S₃Na: calculated: 392.0, found: 392.0.

Acylation of Soy Protein with CTA-NHS (SP-CTA) SP (1.0 g) was dissolved in 25 mL of PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.0). SP solution was then added to RAFT-Ester (1.0 g) solution in DMSO (225 mL). The mixture was allowed to run for 2 h at room temperature. Product (SP-CTA) was precipitated by acidification

2 N HCl as a yellow solid, which was washed with methanol (6 × 30 mL) and dried under reduced pressure to afford 0.85 g SP-CTA.

RAFT Polymerization In a typical graft polymerization, 50 mg of SP-CTA was mixed with 4 mg AIBN and monomer, with monomer to protein weight ratios varying from 1 to 10, in 1 mL DMSO. The reaction mixture was freeze-pump-thaw (3×) degassed and reacted in a sealed tube at 65 °C for 24–48 h to complete consumption of monomers (>95%). The product was precipitated out of DMSO by acetone (acrylamide and acrylic acid grafts), followed by drying under reduced pressure. Triethylamine was added in 1:1 ratio with respect to acrylic acid for SP-CTA and acrylic acid RAFT polymerization reaction. Product was dissolved in PBS (20 mg/mL) and filtered using a centricon 30 kD molecular weight cutoff filtered. Filtrant was washed with water and freeze dried.

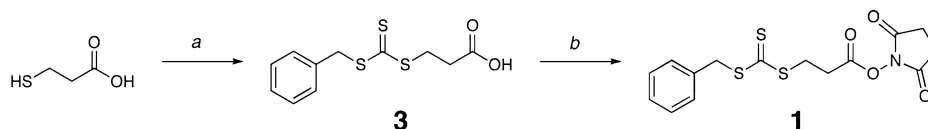
Protein-Polymer Graft Hydrolysis SP-CTA-PAA (polyacrylic acid) (15–40 mg) was dissolved in PBS (20 mg/mL), pH was adjusted to 12 using ammonium hydroxide and allowed to stir for 2 h. Protein was isolated by acid precipitation with 2 N HCl followed by drying under reduced pressure at room temperature. The supernatant was centricon filtered (3kD cutoff) and filtrant washed with water three times and freeze dried.

Sample Preparation for TEM SP-polymer products were dissolved in water or methanol and 1 drop of this solution was spotted on carbon-coated copper grids and air-dried. Samples were stained with 2% phosphotungstic acid, pH 7 (NaHCO₃) or imaged without stain; the same structural features were observed with and without stain.

Results and Discussion

Synthesis of Soy Protein Macro-RAFT Agent

The *N*-hydroxysuccinimide ester of the known RAFT chain transfer agent (trithiobenzylcarbonate) was readily prepared using standard procedures. The stable NHS activated-ester was used to provide a convenient method for acylation of the free amino groups of soy protein isolate, while generating an innocuous *N*-hydroxysuccinimide byproduct that is easily removed by product precipitation (Scheme 2). This active ester, which has a bright yellow color characteristic of the trithiocarbonates that is common to RAFT CTAs, was reacted with soy protein isolate in DMSO solution to yield a yellow-colored protein derivative following isolation. The acylation reaction was followed by ninhydrin analysis for free amines. Though the



Scheme 2 Preparation of NHS-ester of trithiocarbonate CTA. *a* Carbon disulfide, benzyl bromide, $\text{KOH}_{(\text{aq})}$, 80 °C; *b* (1) SOCl_2 , (2) NHS, Et_3N

acylation never achieved a completely negative signal for free amino groups, greatly diminished signal and a steady state was reached. Thus, it appears that acylation can react with most of the amino groups of soy protein, some are not accessible to the NHS-ester, but are accessible to ninhydrin test conditions (Scheme 2).

Nanoparticle Formation

Upon exposure of SP-CTA and monomers to RAFT polymerization conditions at protein-monomer, protein-polymer nanometer-scale particles were formed with solubility properties and elemental composition consistent with a grafted species. Polymerization in the presence of control soy protein (SP-Ac), acylated with acetic anhydride instead of CTA NHS ester (**1**), yielded products that rapidly separated into polymer and protein components upon protein precipitation in aqueous acidic media. When polymerizations were carried out with acrylamide monomer, the product protein-polymer was increasingly water soluble as the monomer:protein weight ratio was increased from 1

to 10, with protein-polymer solubility ranging from 1.2 to 47 weight percent in water, though at high monomer ratios, the product is essentially polyacrylamide. Analysis of the water-soluble product by dynamic light scattering (DLS) indicated a nanoparticle suspension (Table 1), which coalesced into larger particles when dried and analyzed further by transmission electron microscopy (TEM). Electron microscopy analysis indicated the formation discrete particles, with particle density higher with lower monomer ratios. Comparison of protein:monomer ratios of 1:2 and 1:10 indicate many more particles at a 1:2 ratio, with particles roughly ~400–500 nm diameter while at the 1:10 ratio, there were far fewer particles which were of smaller size (Fig. 1). This supports the notion that particle formation is driven by protein aggregation while aqueous solubility is dominated by the grafted polar polyacrylamide. As the protein mass fraction decreases, the aggregating fraction decreases relative to the solubilizing fraction, resulting in fewer particles and smaller overall size. Polyacrylamide itself does not yield nanoparticle structures without SP-CTA. Identical experiments with SP-Ac in place of

Table 1 Particle and elemental analysis of SP-CTA/polymer grafts

Weight ratio SP-CTA/monomer	Monomer	ζ -Potential (mV)	Diameter (nm)	C/N graft	C/N precipitate (ammonolyzed)	C/N soluble polymer (ammonolyzed)
1:2	Acrylamide	-5.41	129	3.43	-	-
1:10	Acrylamide	-1.66	66	-	-	-
1:2	Acrylic acid	-16.95	125	7.23	4.05	18.87
1:10	Acrylic acid	-22.35	162	14.41	3.96	16.00

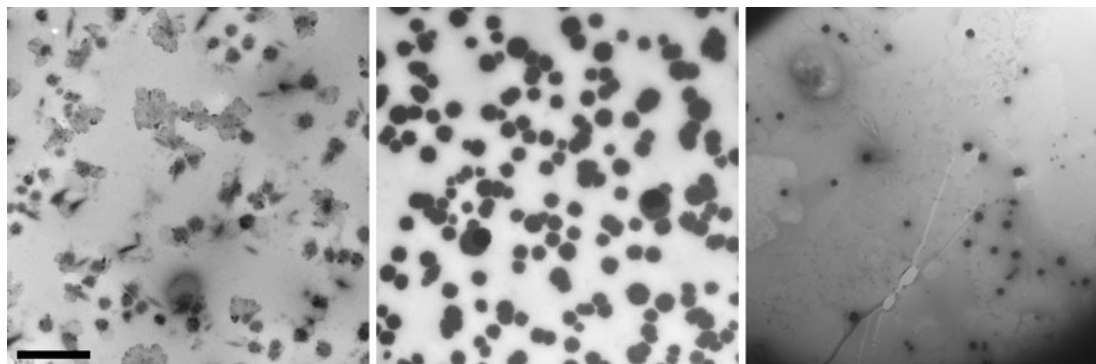


Fig. 1 Transmission electron micrographs of soy protein co-polymerizations with acrylamide monomer. Scale bar = 2 μm . (Left) SP-Ac, weight ratio to acrylamide = 1:2; (Middle) SP-CTA, weight ratio to acrylamide = 1:2; (Right) SP-CTA, weight ratio to acrylamide = 1:10

SP-CTA also yielded micrographs with nanoparticle structures of similar size, but these particles were loosely distributed among amorphous film-type structures, possibly representing non-protein associated polyacrylamide sheets. Interestingly, SP-CTA itself is insufficiently soluble in water for TEM analysis, and partial grafting of polyacrylamide is required for aqueous solubility and TEM sample preparation. The SP-CTA macro-RAFT agent is only soluble in DMF or DMSO, which resulted in featureless micrographs that are not directly comparable to the polymer graft series due to the different sample preparation conditions (Fig. 1).

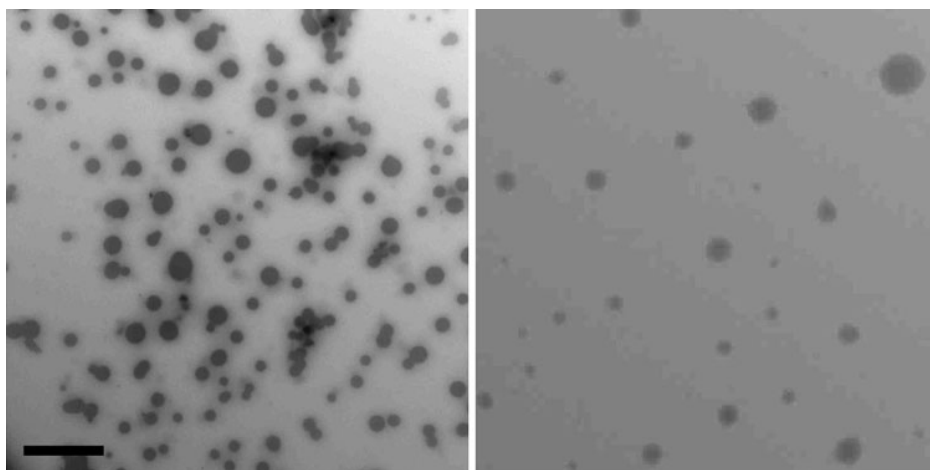
The grafted materials were most conveniently analyzed by elemental analysis. As polyacrylamide has similar C/N ratio as unmodified soy protein (C/N \sim 3.3), the grafting procedure was repeated with acrylic acid monomer, which should markedly increase the carbon content without increasing nitrogen content, resulting in a more marked change in C/N ratio. These RAFT copolymerizations yielded similar nanoparticle formation behavior as judged by TEM (Fig. 2). Again, as the weight percent of protein was decreased, particle size and frequency also decreased. Elemental analysis of the grafted material indicated a clear increase in carbon:nitrogen as the monomer weight fraction was increased, consistent with grafting. As before, when control co-polymerizations with SP-Ac were run, the product (Fig. 2) material was unstable with regard to separation into protein and polymer components while acrylic acid co-polymerization with SP-CTA resulted in stable product solutions. Interestingly, DLS and zeta potential analysis of aqueous solutions of these protein-polymer hybrids indicated small particles of 66–129 nm and a zeta potential trend consistent with the monomer used; we speculate that these small particles form size-limited aggregates upon drying, resulting in the approximately half micron protein-polymer hybrid particles observed by TEM. Soy protein itself has a high negative charge deriving from

\sim 30% carboxylate side chains (aspartate and glutamate) side chains and forms \sim 100–300 nm particles with negative zeta potential around -6 to -8 mV under these conditions. Acrylamide grafting does not have a strong effect on zeta potential, though it remains negative, with larger monomer fractions leading to a decrease in zeta potential. These measurements at high monomer fraction also report smaller sized particles, reflecting the solubilizing effect of polymer grafting that is expected to both increase polarity as well as sterically block aggregation via protein–protein interactions. Grafting of acrylic acid, which should be largely ionized at the neutral pH of the measurements, reveals the expected increasingly negative zeta potential (increasing magnitude) with increasing mass fraction of acrylic acid (AA) monomer used (Table 1). Particle size as measured by DLS increases slightly instead of decreasing at 1:10 SP-CTA/AA relative to a 1:2 ratio, though the accuracy of the DLS measurement may not be high enough to discriminate reliably under these high monomer conditions which exhibit lower particle density (Table 1).

Graft Hydrolysis

Subjecting the hybrid graft material to conditions which nucleophilically cleave [26] the trithiocarbonate resulted in fragmentation of the hybrid into polymer and protein components. The hybrid materials were reacted with aqueous ammonium hydroxide, followed by acid quench. In this procedure, the acid precipitated material had an elemental composition consistent with soy protein starting material, and mass loss corresponding to the monomer fraction used; near theoretical mass recovery of protein was obtained after drying. The soluble fraction likewise exhibited a high C/N ratio by elemental analysis, possibly reflecting incomplete hydrolytic cleavage or retention of the ammonium counterion. Notably, treatment of the SP-CTA co-polymerization with acrylic acid under low pH

Fig. 2 Transmission electron micrographs of soy protein co-polymerizations with acrylic acid monomer. Scale bar = 2 μ m. (Left) SP-CTA, weight ratio to acrylic acid = 1:2, (Right) Sp-CTA, weight ratio to acrylic acid = 1:10



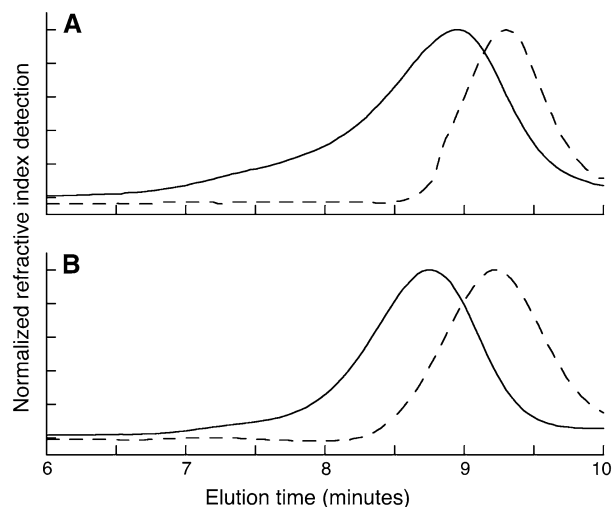


Fig. 3 GPC analysis of soy protein:acrylic acid grafts before ammonolysis (*continuous line*) and after ammonolysis (*broken line*). **a** SP-CTA:acrylic acid = 1:2, **b** SP-CTA:acrylic acid = 1:10

conditions without prior hydrolytic treatment did not result in precipitation. The grafted materials were soluble in NaNO_3 elution buffer for GPC analysis, and in all cases, the aminolyzed material eluted with later retention times than the graft, indicating a decrease in aggregate size following cleavage of the polymer from the soy protein nucleus (Fig. 3).

Conclusions

We have demonstrated the application of RAFT polymerization approaches to protein-polymer grafting of soy protein. We anticipate that this methodology will be useful for generation of renewable resource hybrid materials. Hybrid soy protein and polyacrylate and polyacrylamide materials readily formed nanometer-scale particles upon drying, suggesting possible applications as synthetic latexes [2, 27, 28]. Furthermore, increasing monomer proportion diminished particle formation, suggesting that collapse of protein-polymer grafts is driven by protein-protein aggregation.

Acknowledgments Instrumentation and support for this work was provided in part by the Institute of Materials Research at the Ohio State University, the Ohio Soy Council and the Ohio Bioproducts Innovation Center.

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