Advancements in polymer resins for solid-phase peptide synthesis

by

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B.S., Duke University (2014)

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

The use of polymeric supports in solid-phase peptide synthesis has allowed for the facile, rapid synthesis of short peptides in a one bead/one sequence manner. This approach allows for screening peptide libraries for binding and catalytic activities. Once hits are found, the sequence of the active protein can be determined via mass spectrometry. However, problems arise when trying to create large libraries (on the order of 10^9) of peptides. The resins large enough to provide sufficient amounts of peptide sample for sequencing would occupy too great a volume in order to create large quantities. Conversely, attempting to fold a protein on resins with very high loading results in folding errors. In order to overcome these issues, we attempted to develop a novel core-shell polymer support, containing unprotected amine functional groups at low concentration on the resin surface and a large quantity of an amine, protected orthogonally to the conditions of solid-state peptide synthesis, in the core. In principle, this would allow for the synthesis of a sufficient quantity of fully folded proteins on the resin surface to screen for peptide activity while simultaneously synthesizing enough of the variable sequence in the interior of the resin to allow for a high yield of material for sequence determination.

Thesis Supervisor: Jeremiah A. Johnson
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Chapter I. Synthesis of polystyrene microparticles for solid-phase peptide synthesis

Introduction. The use of polymer supports has greatly advanced the artificial synthesis of peptides.[1] This technique, called Solid-Phase Peptide Synthesis (SPPS), was developed by Merrifield and entails growing the desired peptide chain on an insoluble support.[2-5] SPPS allows for the use of a large excess of reagents, because any unreacted chemicals can be facilely removed via filtration.[1] Specifically, an activated carboxylic acid of an N^{α}-protected amino acid reacts with a functional group on the resin or the growing peptide, followed by deprotection of the amino group, after which the cycle is repeated until the desired peptide is grown.[3] The polymer support used by Merrifield was a chloromethylated copolymer of styrene and divinylbenzene (DVB), now referred to as "Merrifield resin."[3] Presently, there are many commercial sources for resin with a variety of handles for peptide synthesis.[6,7]

An application that takes advantage of SPPS is the creation of peptide libraries for drug and affinity agent discovery. Specifically, the "one-bead, one-compound " (OBOC) approach introduced by Lam and colleagues has allowed the synthesis of libraries containing millions of random peptide sequences,[8-10] including libraries containing artificial-,[11] and *D*-[12] amino acids. This method uses a "split-pool synthesis," which entails distributing a batch of beads into different reaction vessels, each with an individual amino acid, allowing the coupling reactions to reach completion, and then recombining the resin. Repetition of this cycle allows for the synthesis of large peptide libraries. For example, using 19 reaction vessels over 5 cycles would result in a library containing 19^5 different peptides (2,476,099 peptides).[9] However, problems arise when trying to create libraries containing billions of peptides: a library on the order of 10^{10} peptides would use 20 kg of standard 100-200 μm resin, which would occupy a prohibitive volume of nearly 200 liters following synthesis.[13] While using 10-20 μm resin would solve this problem, the

commercial available resins in this size range are limited. Thus, we sought to improve on the existing methods to develop a reproducible method of synthesizing 15 μm polystyrene beads.

The traditional way of making polystyrene particles in this size range is the two-stage swelling and polymerization method, developed by Ugelstad and coworkers.[14-28] In this method, a polymer seed particle, dispersed in water, is allowed to absorb a small water-insoluble molecule. Then, the preswelled particle is allowed to swell a second time with an emulsion comprised of the desired monomers and initiators, after which polymerization is allowed to occur. This results in monodisperse particles that are significantly larger than the initial seed (**Figure 1**).[25]

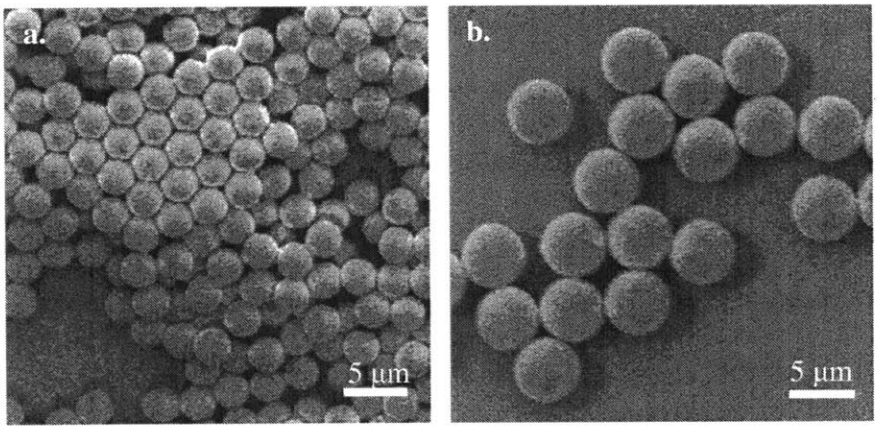

Figure 1. Electron micrographs of **a.** polystyrene seed particles and, **b.** the particles obtained from the seed in A. after two-stage swelling, first with 1-chlorododecane containing benzoyl peroxide, then with styrene, followed by polymerization.

For use in SPPS, the resin must contain a functional handle to which the first amino acid is coupled from its C-terminus. As previously mentioned, the canonical Merrifield Resin is polystyrene crosslinked with divinylbenzene that is functionalized with chloromethyl groups,[3] the latter of which can then be derivatized due to the reactivity of benzyl chlorides as S_N2 electrophiles. Merrifield employed a direct chloromethylation reaction, using chloromethyl methyl ether and a Friedel-Crafts catalyst.[29] However, the toxicity of chloromethyl methyl ether has led to the development of alternate methods for functionalization of resin.[6] One such method is direct

incorporation of functional monomers, such as chloromethyl styrene (CMS) in the two-stage swelling and polymerization process.[30] An alternative method is direct phthalimidomethylation followed by phthalimide cleavage to yield aminomethyl functionalized polystyrene.[6,7] Because the OBOC approach requires that there is a sufficient quantity of peptide for sequencing, we aimed to make high-loading resin in the 10-15 μm size range.

Results and Discussion. Our initial efforts aimed to make crosslinked polystyrene beads in the desired size range in a single step using dispersion polymerization. Dispersion polymerization is a process that starts with stirring a solution consisting of monomer, initiator and a stabilizer. Once polymerization is initiated, the growing polymer precipitates out of solution, leading to sterically stabilized polymer particles.[31,32] While this technique has been used for creating polystyrene particles in the 10-15μm range, there have been difficulties with aggregation and dispersity when trying to make crosslinked particles of this size.[33,34] Winnick and coworkers described a "Two-Stage Dispersion Polymerization," in which a mixture of nonfunctional and functional monomers, including crosslinkers, are added to the mixture after the initial nucleation of nonfunctional polymer.[33-35] They found a linear relationship between the final volume of the particle and the amount of monomer added in this second stage.[33] We attempted to use this method to make appropriately sized particles by heating a solution of styrene, azobisisobutyronitrile (AIBN), and poly(vinylpyrrolidone) (PVP) in ethanol, followed by the dropwise addition of styrene, DVB, and AIBN to the heated mixture. However, all of our attempts to make appropriately sized, crosslinked polystyrene resulted in an unusable aggregate.

We thus resorted to the two-stage swelling and polymerization method to make our desired particles, as this has been shown to make functionalized, crosslinked particles that are 15μm in diameter.[30] As previously discussed, the two-stage swelling method requires small polymer seed

particles. Because previous reports describe 13μm particles made using a 3μm seed,[30,36] we sought to synthesize such a seed using dispersion polymerization. Initially, we employed a one-stage dispersion polymerization outlined by Winnick consisting of a solution of styrene, AIBN and PVP in ethanol without the addition of any additional monomer,[34] and one described by Suh and coworkers, using styrene as a monomer, PVP and Aerosol-OT as co-stabilizers, ethanol and 2-methoxyethanol as co-solvents, and AIBN as an initiator[30] led to the synthesis of monodisperse ~2μm, uncrosslinked particles, as shown in **Figure 2a**, and **2b**, respectively.

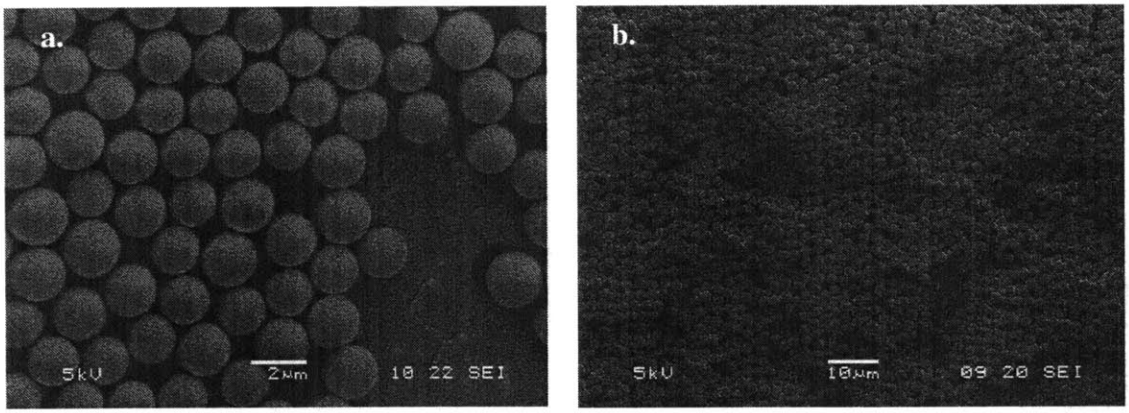

Figure 2. SEM images of 2μm uncrosslinked seed particles made using dispersion polymerization, following methods described by **a.** Winnick[34] and **b.** Suh.[30]

Our next step was to use two-stage swelling and polymerization using CMS to make chloromethylated particles. Following the work of Suh,[30] the polymer seed was first dispersed in water with sodium dodecyl sulfate (SDS) as a surfactant. Then, particles were swollen in an emulsion of 1-chlorododecane in water overnight. Next, the swollen particles were allowed to swell a second time overnight in an emulsion of styrene, CMS, DVB and benzoylperoxide (BPO) as an initiator. After addition of poly(vinyl alcohol) (PVA) as an additional stabilizer, polymerization was allowed to occur overnight. Particles could then either be purified by repeated centrifugation, decantation and redispersion, or by filtration. Moreover, the initial seed could be removed by washing particles with dimethylformamide (DMF).

However, in spite of the fact that the two seed particles described above were similar in size and morphology, they led to different results after treatment with these conditions. The Winnick based seed either gave to a distribution of particle sizes, with the majority of particles being no more than 5μm in diameter (**Figure 3**, below), no swelling at all, or an unusable aggregate.

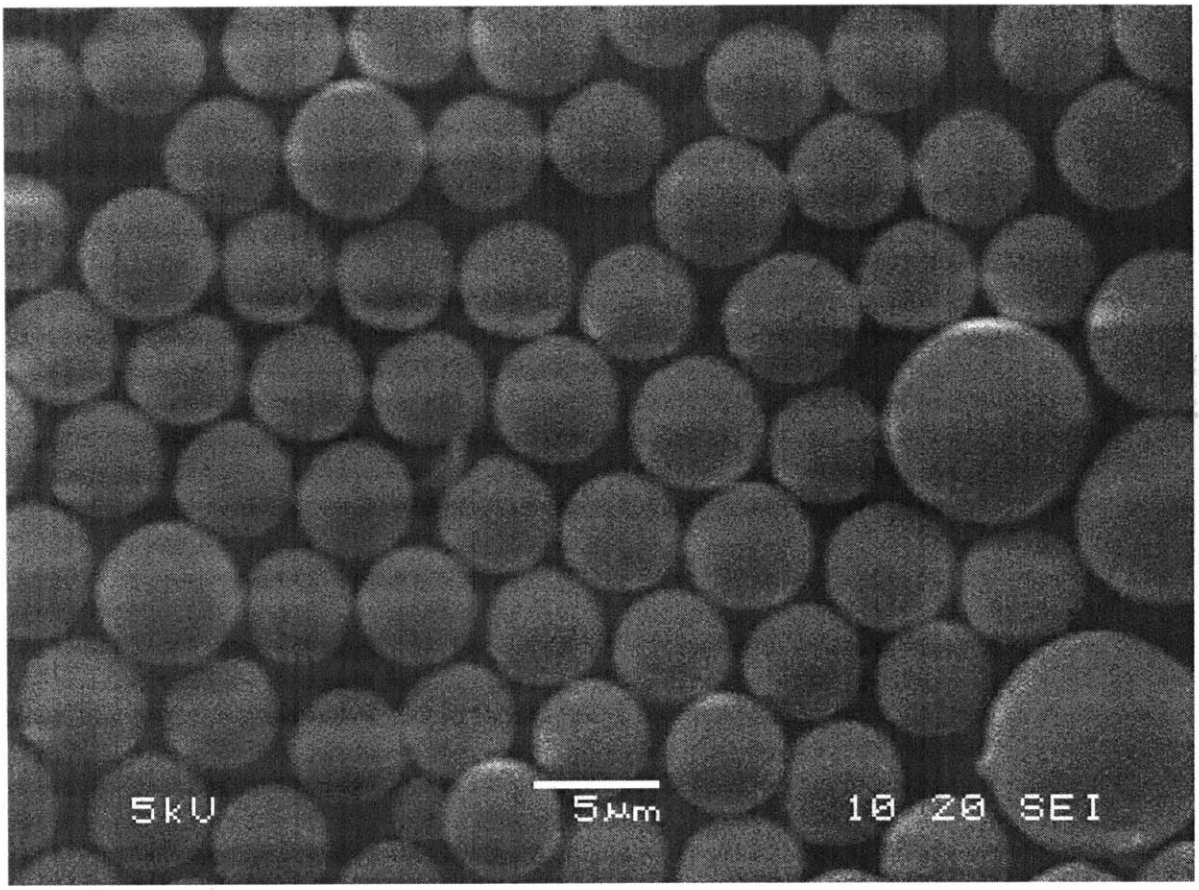

Figure 3. SEM image of particles synthesized via two-stage swelling and polymerization using 2μm seed made by procedures described by Winnick et al.

The seed described by Suh gave more promising results. Swelling this seed with 30% CMS and 1% DVB led to uniform 5μm particles (**Figure 4**). While the desired dispersity was achieved, the size failed to match the reported values for the same procedure.[30] This was likely due to incomplete

uptake of monomers and initiator by the seed, which led to issues with aggregation and low yield of particles.

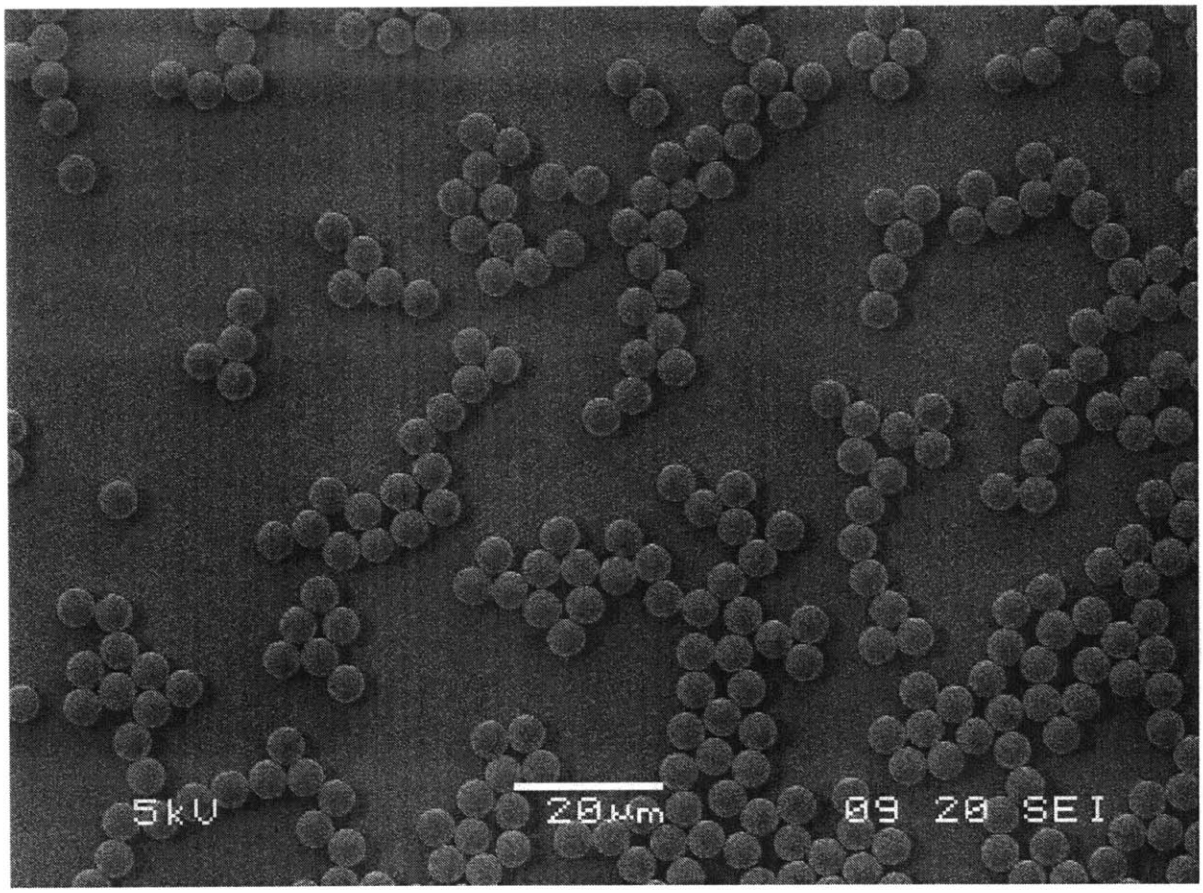

Figure 4. SEM image of uniform 5µm particles synthesized via two-stage swelling and polymerization using 3µm seed made by procedures described by Suh et al.

We employed various strategies to try and overcome these size and aggregation issues. Some unsuccessful attempts are summarized below:

- In an attempt to avoid solvation of the initiator in water, we dissolved BPO in 1-chlorododecane prior to the first stage of swelling. This resulted in no improvement compared to our previous efforts.
- Employment of water miscible co-solvents, such as acetone and 1,2-dichloroethane, during the first stage of swelling has been shown to improve uptake of the water

insoluble oil by the seed particle.[21,25,37] However, our attempts at using co-solvents failed to drastically improve uptake of oil. Moreover, because the co-solvent would decrease the amount of monomer absorbed by the seed by increasing monomer solubility in the water phase, it had to be removed *in vacuo* prior to the second stage of swelling. This proved to be unnecessarily difficult and time-consuming as the presence of a surfactant forced slow evaporation due to uncontrollable bubbling.

One strategy that was somewhat successful involved removing excess monomer after the second stage of swelling but before polymerization. This was accomplished by momentarily stopping stirring of the mixture, allowing the lower density monomer to float above the higher density swelled seed, and physically removing this unabsorbed monomer. However, this only decreased the amount of aggregation following polymerization and did not improve the size of the final particles.

In an attempt to grow larger particles, we decided to study how varying the initiator and amount of DVB would influence the final size of the particles. When comparing different initiators, we found that BPO was better absorbed by the seed than AIBN, resulting in less aggregation and particles with greater mechanical stability. Moreover, decreasing the weight percentage of BPO from 1% to 0.1% increased the particle size. However, this change resulted in a greater size dispersity. We also found that decreasing the amount of DVB greatly improved both size and dispersity, with the lowest dispersity achieved by completely omitting DVB. However, decreasing the crosslinking would also lower the mechanical strength of the resin, which would limit its usefulness in SPPS.[1] Thus, we reasoned that, in spite of the prior reports of using a 3μm polystyrene seed to grow 15μm, 1% crosslinked polystryrene particles,[21,22,25,30] we needed to use a larger seed in order to grow particles in the desired size range.

Thus, we first attempted to use dispersion polymerization to make a larger seed. Ober and colleagues reported making 7μm polystyrene particles via dispersion polymerization by using ethanol and 2-methoxyethanol as co-solvents, BPO as an initiator, and hydroxypropyl (HPC) as the initiator.[32] While we were able to successfully make 7μm particles with a reasonably low dispersity (**Figure 5**), these particles failed to incorporate any monomer added during the two-stage swelling and polymerization and we sought other means of making larger seed particles that could be used to grow larger resin.

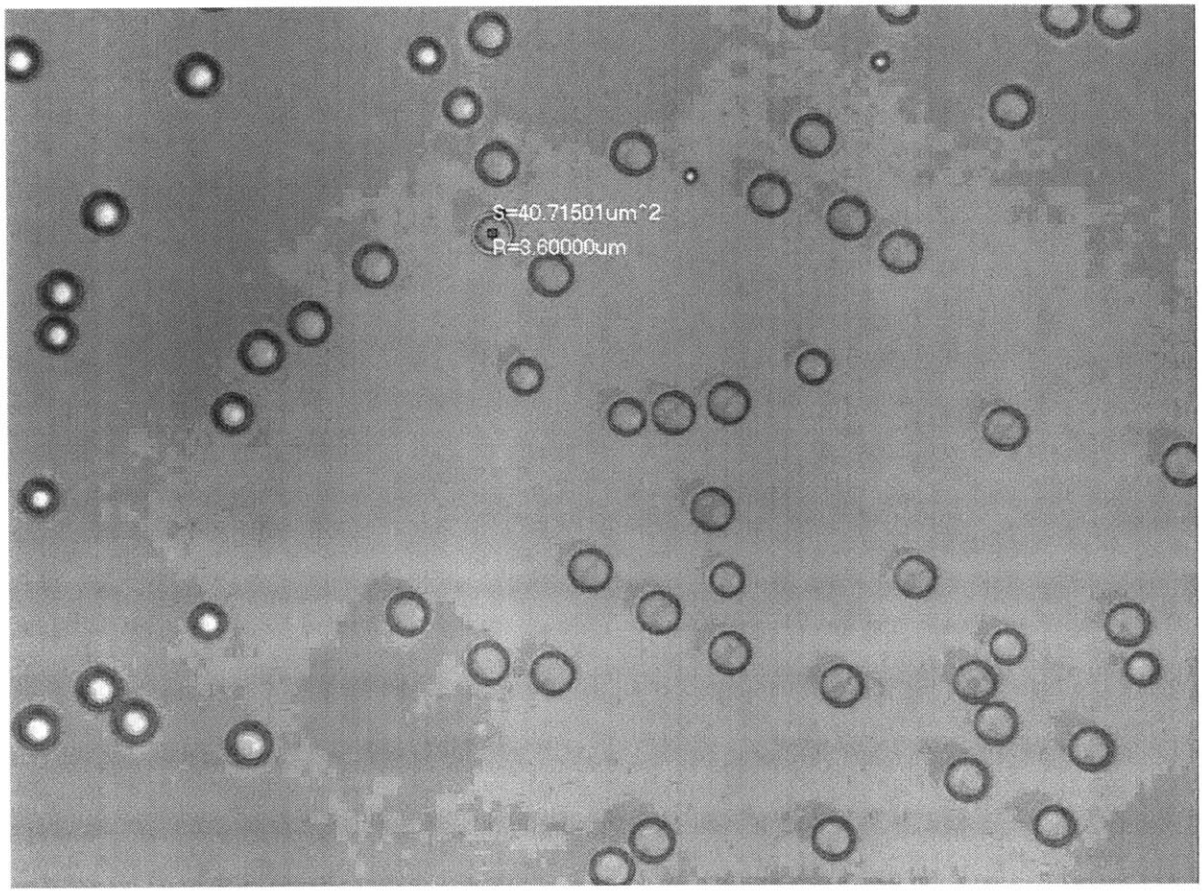

Figure 5. Optical image of 7.2μm seed particles made using dispersion polymerization.

Intrigued by the low dispersity of the non-crosslinked particles we made using two-stage swelling and polymerization, we decided to subject these particles to a second round of growth.

Much to our delight, we were able to make 10μm particles using a 5μm seed that contained 5% CMS (**Figure 6**). While 15μm particles would allow for a lower loading per individual bead, these 10μm particles were sufficient for further use.

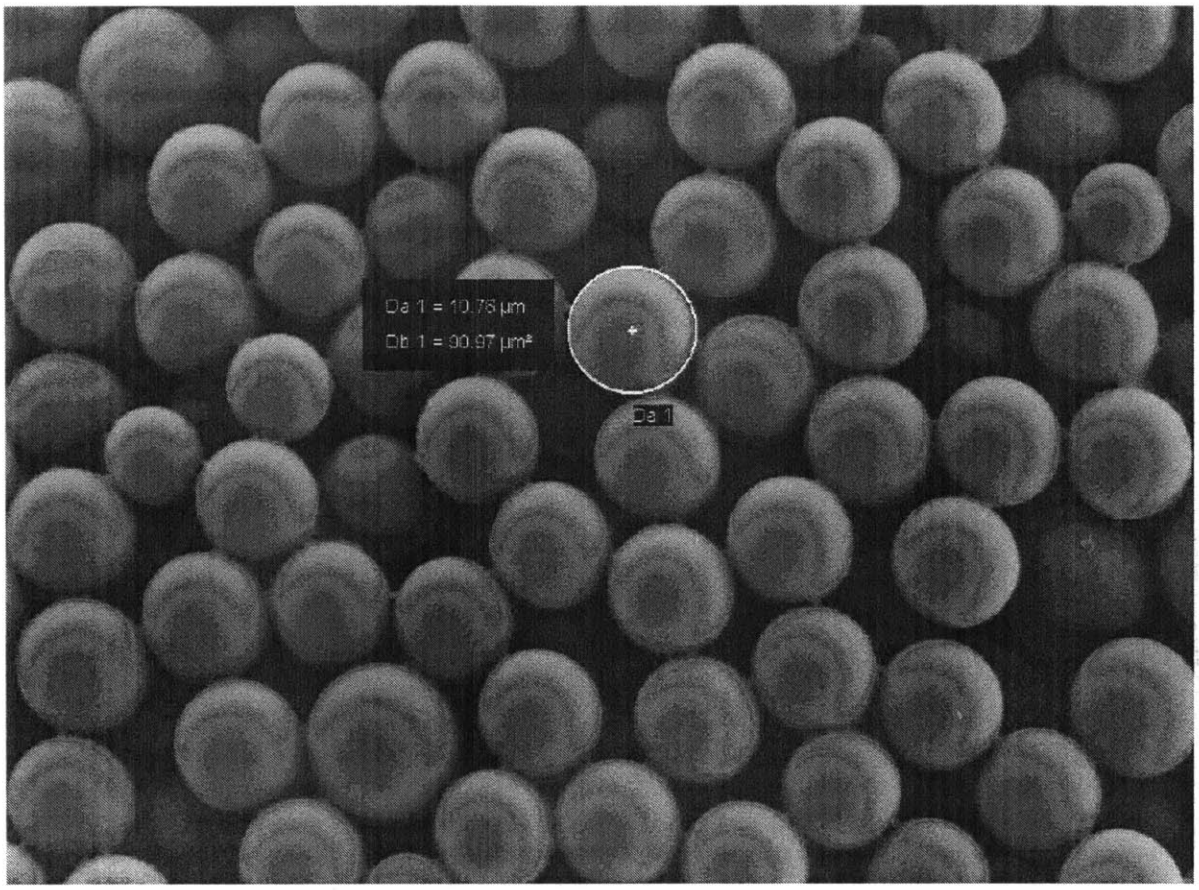

Figure 6. SEM image of 10μm polystyrene particles made after successive iterations of 2-stage swelling and polymerization.

Our initial efforts towards making functional resin involved varying the CMS to monomer ratio during the second stage of swelling. Increasing the amount of CMS led to a decrease in the amount of monomer incorporated. For example, 50% CMS led to half-sphere particles following a DMF wash, while 100% CMS failed to grow the particles and incorporate chloromethyl groups at all. Nevertheless, we continued with these imperfect particles in order to test amino functionalization.

Due to the electrophilicity of benzyl chloride functional groups, we sought to incorporate amines or amine precursors via S_N2 reactions. Initially, we focused on using ethylene diamine as an amine source, due to its ease of use, relative nontoxicity, and the potential for incorporating two amine groups per functional group, thus doubling the loading. However, after determining the loading via coupling of an Fmoc-Gly-OH residue using HATU and DBU and subsequent Fmoc deprotection and UV-Vis absorption of the DBU-dibenzofulvene (DBU-DBF) adduct to determine the amount of Fmoc-Gly-OH successfully bound to the resin,[38] we found much lower loadings than expected given the amount of CMS added in the polymerization; much less than the double loading that we had hoped for (**Figure 7**).

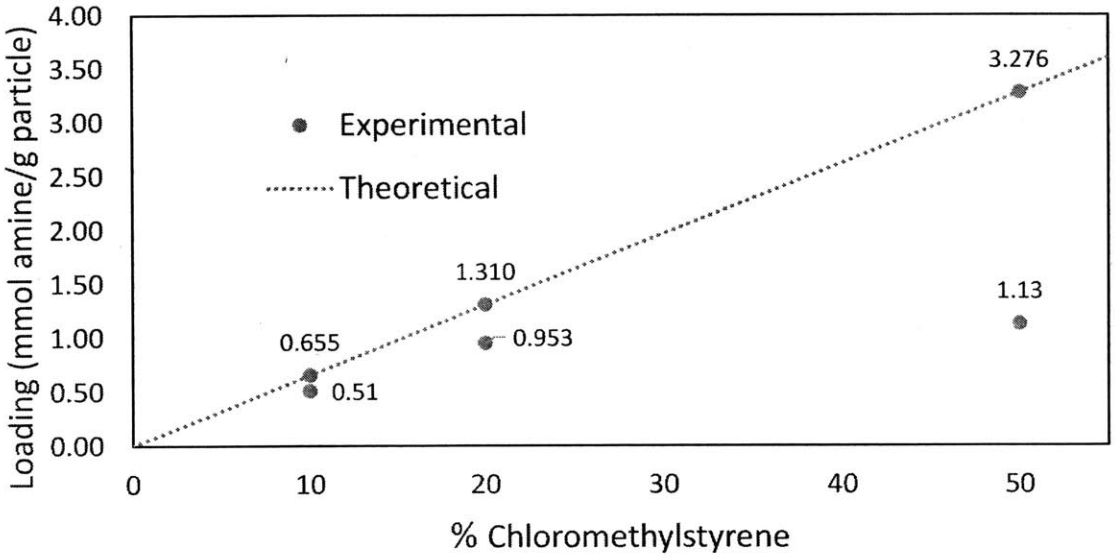

Figure 7. Plot comparing the amount of CMS added to polymerization and the resin loading following functionalization with ethylene diamine.

We attempted to use alternative nucleophiles, specifically the phthalimide and the azide anions. While we were unable to successfully functionalize the particles using potassium phthalimide, we had more promising results using sodium azide. After installing azides, the Staudinger reaction to yield amines proved successful as monitored by the disappearance of the

characteristic azide stretch in the FTIR spectrum at 2100 cm^{-1} (**Figure 8**). Nevertheless, due to the inability to make particles with sufficiently high loading, found to be 0.5 mmol/g as determined by the colorimetric protocol described above (**Figure 9**), using this strategy, we were forced to explore other strategies for functionalization.

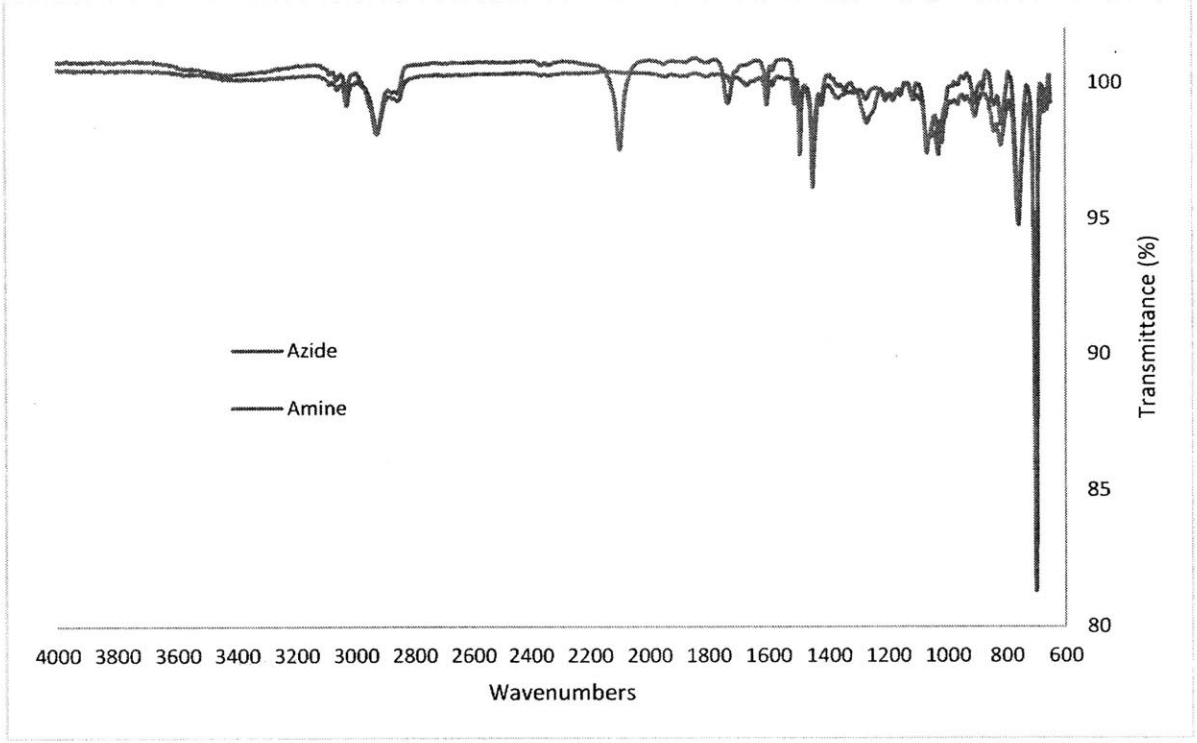

Figure 8. FTIR spectra of azide resin before and after being subjected to the conditions of the Staudinger reduction.

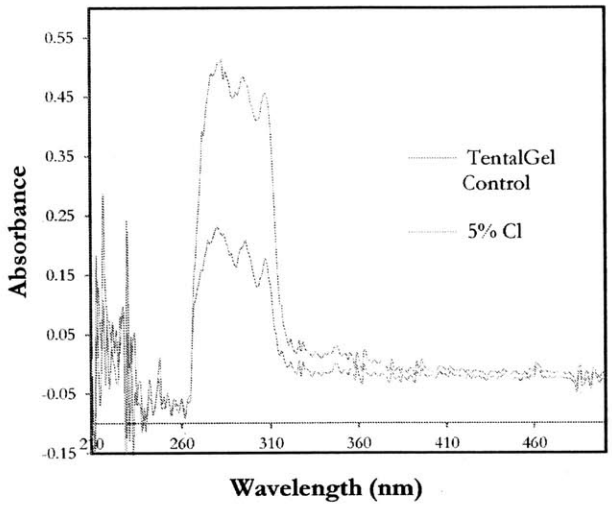

Figure 9. Absorption spectra of DBU-DBF adduct collected from resin functionalized using 5% CMS, reacted with NaN_3, and treated to the conditions of the Staudinger reaction. Loading is determined by comparison to absorption spectra of DBU-DBF adduct from TentaGel® control.

First, we attempted to synthesize and use functional monomers for the two-stage swelling and polymerization. However, neither azidomethyl styrene, nor phthalimidomethyl styrene resulted in successful polymerizations. Next, we looked to directly incorporate phthalimidomethyl groups to polystyrene resin via electrophilic aromatic substitution, specifically the Tscherniac-Einhorn reaction.[39,40] Not only would this reaction functionalize the resin, but it would afford us the opportunity to synthesize resin with two orthogonally protected amino groups, one installed as a phthalimide via the Tscherniac-Einhorn reaction, the other as an azide. Moreover, the azide could prove useful as either an amine precursor as initially intended, or as the azide partner in the strain-promoted azide-alkyne cycloaddition for further derivatization. We were inspired to explore conditions described by Brimble in coworkers, which describe a faster phthalimidomethylation reaction and phthalimide cleavage using the less toxic ethanolamine instead of hydrazine.[6] By magnetically stirring 10μm particles with *N*-(hydroxymethyl)phthalimide and methanesulfonic acid (MsOH) as a catalyst in dichloromethane, we were able to make resin with 3.0 mmol/g loading

after cleavage of the phthalimide group via reflux in a mixture of ethanol and ethanolamine for 15 h. However, we found that this resin had poor structural integrity, with many of the particles being broken following this functionalization. To avoid this issue, we chose to agitate the mixture in the first reaction using a shaker instead of a magnetic stir bar. Much to our delight, this resulted in structurally sound particles with loading varying from 2.5 mmol/g to 3.5 mmol/g, as shown by the representative absorbance spectra below (**Figure 10**).

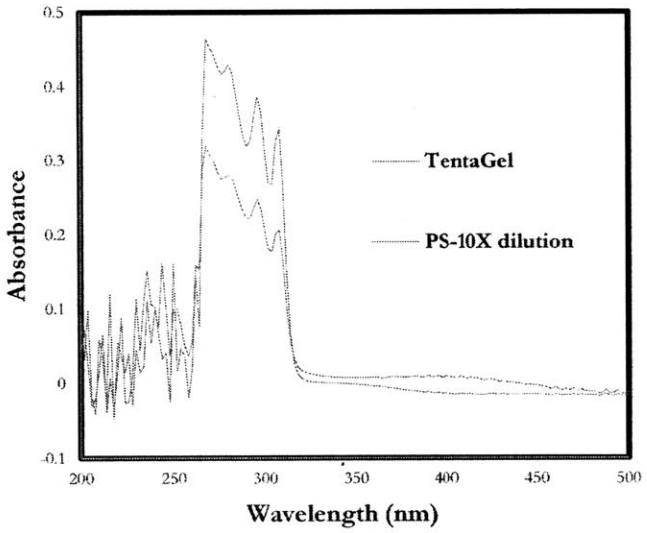

Figure 10. Figure 11. Absorption spectra of DBU-DBF adduct collected from resin functionalized by direct phthalimidomethylation and diluted tenfold compared to adduct collected from the TentaGel® control. Loading for this particular sample was determined to be 3 mmol/g.

Next, we sought to create core-shell particles. This resin would be particularly useful for making a library of peptides where only a specific region of the peptide is grown at a given time. This variable region, which would ideally provide the peptide/protein with desired binding or catalytic activity, would be needed in a greater amount than the full peptide for sequencing purposes. To achieve this goal, we aimed to have orthogonally protected amino groups: one in small quantity on the surface of the resin for the full-length peptide/protein, the other in a large quantity for the variable region. Moreover, the amino group intended to be used to grow the

variable region would have to be protected orthogonally to the Fmoc chemistry used for SPPS. This goal was achieved by coupling amino functionalized particles with NHS-PEG4-N_3 in an aqueous buffer solution. Because the resin showed poor swelling properties in this solution, only the exterior was functionalized. To prove that only the exterior was functionalized, we reacted particles with DBCO-PEG-Cy5 for confocal imaging (**Figure 11**). Particles synthesized only showed fluorescence on their exterior, showing that they were, in fact, core-shell particles.

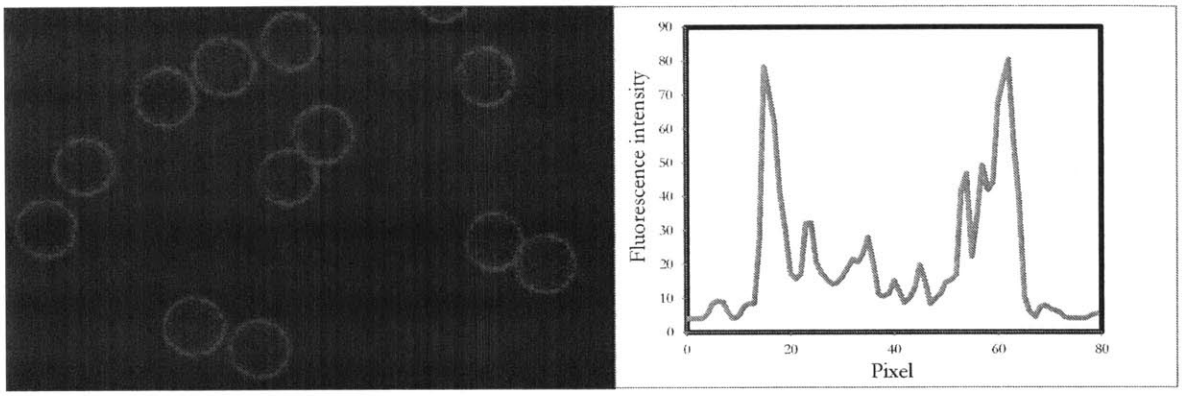

Figure 12. a. Confocal fluorescence microscopy image (λ_{ex}/ λ_{em} = 633 nm / 670 nm) of resin particles functionalized with PEG-Cy5 and **b.** graph showing fluorescence intensity as a function of depth.

To make a synthetically useful core-shell resin, we first reacted the amino groups of the resin with azide groups on the shell with a Rink amide linker, followed by protection of the new amino group with the alloc protecting group, as it is orthogonal to the standard conditions of Fmoc SPPS. Next, we reacted the unreacted azide groups with DBCO-PEG5K-NH_2 for core-shell particles. Unfortunately, efforts to make both the full protein sequence of the trypsin inhibitor EETI-II (sequence = GCPRILMRCKQDSDCLAGCVCGPNGFCGSP) and the active site of the native peptide (GCPRILMR), which has no influence on the protein's folding, on this high-loading core-shell resin led to impure peptides, as evidenced by shoulder peaks on the LC-MS spectrum (**Figure 12**). Instead, we put effort into optimizing small, high loading resin with hopes of being able to produce a significant amount of peptide with only a small quantity of resin.

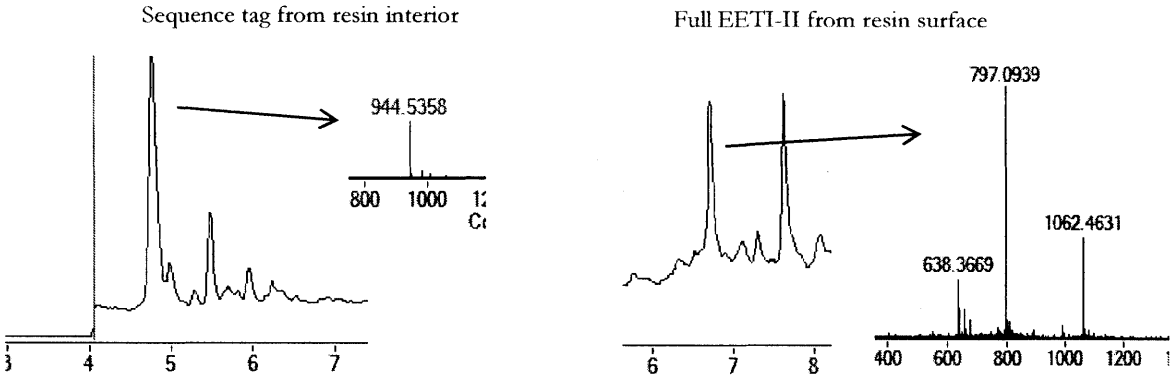

Figure 13. LC-MS total ion current vs. time for the synthesis of EETI-II and the sequence tag on small, high loading, core-shell resin. Inset of charge state series of desired product at the peak apex (calc. monoisotopic mass, sequence tag = 943.510, obs. = 944.5358).

For this purpose, we chose to use 10μm resin with various functional groups and attempt to make two test peptides, the relatively simple sequence ALFALFA and the challenging sequence VQAAIDYING, using our collaborators' (the Pentelute laboratory) rapid-flow based peptide synthesis system.[41] Our initial efforts using resin with a high amine loading were problematic, even with the easier sequence, with deletions occurring on later residues. Because we attributed this issue to swelling problems, we aimed to improve the swelling properties of our resin without greatly sacrificing the loading. Our first attempt was to add a small triple glycine (GGG) spacer before attaching the Rink linker, but while this improved swelling, we encountered deletions when making the test peptides. We next coupled a small amount of PEG-amine to the resin prior to functionalizing it with linker. Much to our delight, we were able to successfully synthesize 35 mg of ALFALFA from 50 mg of our resin compared to just 8 mg of peptide from 180 mg of the commercial resin, TentaGel®, which served as a point of comparison (**Figure 13**). Moreover, the purity of this synthesized peptide, determined by LC-MS, was sufficiently high (**Figure 14**). However, the more challenging sequence VQAAIDYING was found to have 10% of the characteristic deletions of the ninth and tenth residues,[42] emphasizing the limited viability of small, high-loading resin.

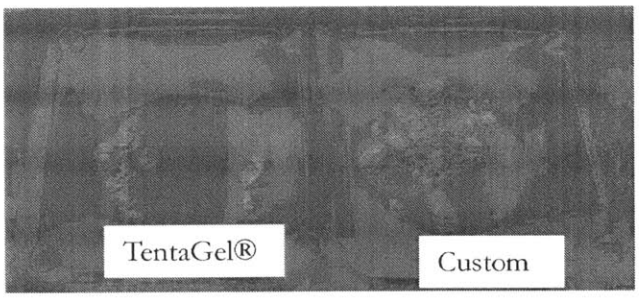

Figure 14. Lyophilized ALFALFA made from 180 mg of TentaGel® compared to 50 mg of custom resin.

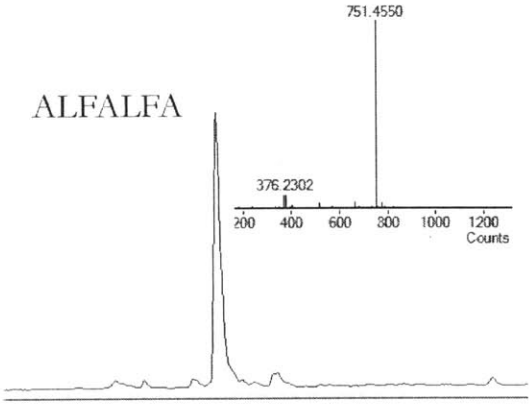

Figure 15. LC-MS total ion current vs. time for the synthesis of ALFALFA on small, high loading resin. Inset of charge state series of desired product at the peak apex (calc. monoisotopic mass = 751.432, obs. = 751.4550)

Conclusions. While we initially sought to synthesize 10-15 μm, high-loading core-shell resin, we encountered many difficulties along the way. The traditional 2-stage swelling and polymerization method used to make particles in the desired size range resulted in particles that were too small for use, so we were forced to repeat this process to achieve the requisite size. While we were successful in making high-loading core-shell particles, when this resin was used for a functional test of a full peptide and a sequence tag, the results were subpar. We had some success in using the small, high loading resin to make greater quantities of simple sequences than commercially available resin, highlighting the potential of this resin. However, due to purity issues

with more difficult sequence, more work is needed to optimize the particle properties. Future work would entail fully optimizing the resin, with or without the core-shell functionality.

Experimental Methods

General Considerations. Styrene, chloromethyl styrene, and divinyl benzene were purchased from Aldrich and passed through a column of basic alumina to remove inhibitor. Azide-PEG4-NHS ester was purchased from KeraFAST. DBCO-PEG2K-NH_2 and DBCO-PEG2K-Cy5 were purchased from Nanocs. N^{α}-Fmoc amino acids, Rink amide linker, and 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HATU) were from Chem-Impex International. TentaGel M-NH_2 polyethylene glycol-polystyrene hybrid resin (20μm, 0.25 mmol/g) was purchased from Rapp Polymere. N,N-Dimethylformamide (DMF), dichloromethane (DCN), methanol (MeOH), diethyl ether, and HPLC-grade acetonitrile used for peptide synthesis and analysis were from EMD Millipore. 1,2-Ethanedithiol (EDT) was from Alfa Aesar. Methane sulfonic acid was from Fluka. Solvents for LC-MS were purchased from J.T. Baker and Fluka. All other reagents and solvents were purchased from Aldrich or VWR and used as received. Elemental analysis was performed by Atlantic Microlab, Inc.

Instrument information. Optical images were taken using an OMAX MD82ES10 biological compound microscope with a built-in 1.3MP digital camera. Images were taken at 800X magnification.

Scanning Electron Microscope images were taken at the MIT Center for Materials Science and Engineering's (CMSE) Electron Microscope Facility using either a JEOL 5910 General Purpose SEM or a Zeiss Merlin High-resolution SEM. Samples were prepared by placing a drop

of a dilute aqueous particle suspension on a silicon wafer followed by evaporation of the water under ambient conditions. Moreover, samples for the JEOL were sputter-coated with gold by Dr. Yong Zhang at the MIT CMSE.

Fluorescence images were taken using a Leica SP2 confocal fluorescence microscope. All images were digitally post-processed to improve brightness and contrast.

Fourier-Transform Infrared (FTIR) Spectroscopy was performed using a Nexus Model 470/670/870 Spectrophotometer using the Omnic software package.

Absorbance measurements were collected using a Hewlett Packard 8452a diode array spectrophotometer and a quartz cuvette with a path length of 1 cm.

Peptides were analyzed on an Agilent 6520 Accurate Mass Q-TOF LC-MS with an Aglient C3 Zorbax SB column (2.1 x 150 mm, 5 μm packing) with a flow rate of 0.4mL/min o the following gradient: 0.1% (v/v) formic acid in water (A) with 1% 0.1% (v/v) formic acid in acetonitrile (B) for 3 minutes, 1-61% B ramping linearly over 15 min, and 61% B for 4 min.

2 μm polystyrene seed (adapted from Winnick and coworkers).[34] Styrene (12 g, 115 mmol) was added to a three-neck round bottom flask containing AIBN (600 mg, 3.65 mmol), polyvinylpyrrolidone (PVP) (MW=55K, 4 g), and Triton X-305 (0.72 g) dissolved in ethanol (100 mL). The solution was purged with N_2 for 30 minutes before being heated to 80 °C while stirring magnetically at 200 rpm for 12.5 hours. The reaction was allowed to cool to room temperature and was exposed to air. Particles were purified by repeated centrifugation and redispersion in EtOH. Particle diameter was determined to be 2 μm by SEM.

2 μm polystyrene seed (adapted from Suh and coworkers).[30] Styrene (10 g, 96 mmol) was added to a three-neck round bottom flask containing AIBN (100 mg, 0.61 mmol), PVP-K30 (1.8 g), and Aerosol-OT (0.40 g) dissolved in a mixture containing ethanol (55.6 mL) and 2-methoxyethanol (45.5 mL). The solution was purged with N_2 for 30 minutes before being heated to 70 °C while stirred magnetically at 200 rpm for 24 hours. The reaction was allowed to cool to room temperature and was exposed to air. Particles were purified by repeated centrifugation and redispersion in EtOH (3x), followed by centrifugation and redispersion in 0.25% aqueous SDS solution (5x). Particle diameter determined to be 2μm by SEM.

Two-stage swelling and polymerization. In a typical procedure, a colloidal dispersion of polystyrene seed particle in 0.25% aqueous SDS was diluted to 42.5 mL at a concentration of 0.35% w/w (150 mg seed) in a three-neck round bottom flask. 1-chlorododecane (0.15 g) emulsified by ultrasonic homogenization in 0.25% aqueous SDS (7.2 mL) was added to the dispersion, which was stirred magnetically at 450 rpm for 10 hours at 30°C, after which the dispersion was diluted with an additional 70 mL of 0.25% aqueous SDS. Then, a mixture of monomer totaling 9 g containing 1% BPO (90 mg) was added to the dispersion, which continued to stir for an additional 10 hours. After this step, the mixture was carefully transferred to a separatory funnel and excess monomer was removed. After transfer back to the three-neck round bottom flask, 125 mL of an aqueous solution containing 2% PVA and 0.25% SDS was added, and the mixture was purged with N_2 for 30 minutes. The mixture was then heated at 70 °C for 18 h. The reaction was allowed to cool to room temperature and was exposed to air. Particles were purified by repeated centrifugation and redispersion in 0.25% aqueous SDS solution until the

supernatant was clear (3x). Typically 150-200 mg resin was isolated. Particle size was characterized by optical microscopy. Specific recipes are tabulated below:

Seed	Monomers	Comments
2 μm polystyrene seed (Suh)	30% CMS, 1% DVB	5 micron particles
2 μm polystyrene seed (Suh)	10% CMS, 1% DVB	5 micron particles
2 μm polystyrene seed (Suh)	10% CMS	5 micron particles
10% CMS particles	5% CMS, 1% DVB	12 micron particles

Colorimetric loading determination. Resin and TentaGel as a positive control were dispersed in 1 mL of DMF in a 15 mL centrifuge tube. 1.1 mmol Fmoc-Gly-OH (327 mg) or 1.1 mmol Rink Amide linker (593.5 mg) was dissolved in 2.5 mL of 0.4 *M* HATU (1.0 mmol), activated for 30 s with 0.5 mL DIEA. 700 μL of the resulting solution was added to each resin aliquot. After agitation for 10 min, resin aliquots were diluted to 15 mL with DMF. The aliquots were then washed by repeated centrifugation and redispersion in DMF (3x). 2.5 mL of a 2% DBU in DMF solution was added to the decanted resin. After 30 min of agitation, the reactions were diluted to 10 mL with DMF. Following centrifugation, 100 μL of the supernatant was diluted to 5 mL with DMF and the UV-Vis absorption of DBU-dibenzofulvene adduct was recorded and loading was determined by comparison of absorbance to positive control.

Resin functionalization via S_N2. In a typical reaction, chloromethyl functionalized resin dispersed in DMF was added to a 40 mL vial. To this vial, an excess of nucleophile and 1 crystal of potassium iodide as a catalyst were added. The mixture was stirred at 70 °C for 16 h. Once the reaction was

allowed to cool to room temperature, the mixture was centrifuged and washed with DMF (3x). Specific nucleophiles used are tabulated below:

Nucleophile	Comments
Ethylene diamine	Low loading achieved
Sodium Azide	Presence of azide stretch monitored by FTIR
Potassium Phthalimide	No reaction by FTIR

Staudinger reaction on resin. Azide particles containing 0.6 mmol N_3 was transferred to 5 mL THF in a vial. Triphenyl phosphine (315 mg, 1.2 mmol) and a few drops of water were added to the mixture. This dispersion was then allowed to stir at room temperature overnight. Loss of azide was characterized by FTIR.

Direct phthalimidomethylation. Resin (~250 mg) was dispersed in DCM (12 mL). *N*-(hydroxymethyl)phthalimide (750 mg, 4.22 mmol) and methanesulfonic acid (1000 μL) were added to the mixture. The mixture was then agitated in a shaker for 20 h. The mixture was filtered and washed with DCM, DMF, and EtOH before drying under high vacuum. 280 mg resin was isolated. Resin was brought to next step before further characterization.

Phthalimide Cleavage. Phthalimide functionalized resin (280 mg) was dispersed in 20 mL EtOH with 4 mL ethanolamine. The mixture was stirred at 85 °C at 150 rpm for 24 h. Resin was filtered and washed with EtOH, DMF, and DCM. Resin loading was determined to be 2.5 mmol/g colorimetrically, which was confirmed by elemental analysis (%N = 3.86%).

Surface PEG4-azide functionalization. 55.6 mg of 6 μm $-NH_2$ functionalized particles (loading = 3.5 mmol/g) were dispersed in 5 mL 1X PBS buffer solution containing 0.1% Triton X-100. Excess N_3-PEG4-NHS ester is added and the mixture was agitated for 15 h. Resin was centrifuged and washed with DMF. Resin was brought to next steps before further characterization.

PEGylation via strain-promoted azide-alkyne cycloaddition. Surface coated PEG4-N_3 particles were dispersed in 1 mL 1X PBS buffer solution containing 1% Triton X-100. 100 μL of the particle mixture was transferred to two 1 mL vials. 100 μL of a solution containing 1.0 mg DBCO-PEG5K-NH_2 in 400 μL ddH_2O was added to one vial, while 100 μL of a solution containing 1.0 mg DBCO-PEG2K-Cy5 in 400 μL ddH_2O was added to the other vial (for fluorescence imaging). The vials were wrapped in foil and mixed at 55 °C for 24 h. Fluorescently labeled particles were characterized using a confocal fluorescence microscope.

Core-shell resin. Surface coated PEG4-N_3 particles were treated to the conditions of colorimetric loading determination using the Rink amide linker (loading of remaining amino groups determined to be 3.157 mmol/g). 60 mg of this resin was dispersed in 5 mL DMF. 500 mg alloc-succinimate (10 equiv) and 300 μL Et_3N (>10 equiv) were added to this mixture, which was agitated in a shaker for 24 h, when a Kaiser test was found to be negative. Resin was then washed with DMF. Particles were then PEGylated with DBCO-PEG2K-NH_2 as described above.

Rapid-flow based peptide synthesis. All peptides were synthesized on the flow-based platform reported by Pentelute and coworkers.[41] All reagents (coupling, wash, deprotection) were preheated

to 60 °C immediately before reaching the synthesis vessel. The standard 3-minute cycle was as follows:

1. Amide bond formation (coupling) – 30 s at 6 mL/min
2. Removal of coupling reagent (wash) – 60 s at 20 mL/min
3. N^{α}-Fmoc removal (deprotection) – 20 s at 20mL/min
4. Removal of deprotection reagent and products (wash) – 60 seconds at 20 mL/min
5. Manual manipulations, not included above – 10 s

Coupling. The coupling solution consisted of 1 mmol of N^{α}-Fmoc and side chain protected amino acid dissolved in 2.5 mL of 0.4 M HATU in DMF and 0.5 mL of DIEA. In order to minimize racemization of Cys residues, only 190 μL DIA were added for activating this amino acid. Coupling solution contained at least four equivalences of activated amino acid with respect to the resin. Amino acids were dissolved in activating agent solution up to several hours before use, and DIEA was added within 1 min of use.

Side chain protecting groups were as follows: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Gln(OtBu), Lys(Boc), Ser(tBu), Tyr(tBu).

Wash. Excess reagent and reaction by-products were washed out from the synthesis vessel with 20 mL of DMF delivered at 20 mL/min over 1 minute.

Deprotection. N^{α}-Fmoc protecting groups were removed with 6.6 mL of 20-50% (v/v) piperidine in DMF delivered at 20 mL/min over 20 seconds.

Alloc deprotection. Resin was washed for 3 min, removed from the reactor and mixed in a solution containing 90 mg tetrakis(triphenylphosphine)palladium(0) and 1.2 mL phenylsilane in 6 mL

DCM for 20 min at room temperature. Following removal, resin was re-added to the reactor and washed for an additional 3 min.

Standard cleavage of peptide from resin. All peptides were cleaved from the resin and side chains were deprotected with a standard cleavage cocktail of 2.5% (v/v) EDT, 2.5% (v/v) H_2O, and 1% (v/v) TIPS in TFA for 7 min at 60 °C. Cold diethyl ether was added and the precipitate was washed three times with additional cold diethyl ether. The solid was then dissolved in a solution comprised of 0.1% TFA, 49.95% acetonitrile, and 49.95% water. Peptides were analyzed by LC-MS.

Peptide sequences synthesized.

Sequence	Resin	Results
EETI-II & Sequence tag	50 mg core-shell (2.5 mmol/g)	Impure peptide, insignificant recovery
ALFALFA	50 mg PEGylated resin (2.5 mmol/g)	35 mg peptide recovered
ALFALFA	180 mg TentaGel® (0.25 mmol/g)	8 mg peptide recovered
VQAAIDYING	51 mg PEGylated resin (2.5 mmol/g)	63.4 mg recovered, ~10% deletion
VQAAIDYING	180 mg TentaGel® (0.25 mmol/g)	40 mg recovered, pure

Chapter II. References

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