

# Photo-Crosslinkable Unnatural Amino Acids Enable Facile Synthesis of Thermoresponsive Nano- to Microgels of Intrinsically Disordered Polypeptides

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Hydrogel particles are versatile materials that provide exquisite, tunable control over the sequestration and delivery of materials in pharmaceuticals, tissue engineering, and photonics. The favorable properties of hydrogel particles depend largely on their size, and particles ranging from nanometers to micrometers are used in different applications. Previous studies have only successfully fabricated these particles in one specific size regime and required a variety of materials and fabrication methods. A simple yet powerful system is developed to easily tune the size of polypeptide-based, thermoresponsive hydrogel particles, from the nano- to microscale, using a single starting material. Particle size is controlled by the self-assembly and unique phase transition behavior of elastin-like polypeptides in bulk and within microfluidic-generated droplets. These particles are then stabilized through ultraviolet irradiation of a photo-crosslinkable unnatural amino acid (UAA) cotranslationally incorporated into the parent polypeptide. The thermoresponsive property of these particles provides an active mechanism for actuation and a dynamic response to the environment. This work represents a fundamental advance in the generation of crosslinked biomaterials, especially in the form of soft matter colloids, and is one of the first demonstrations of successful use of UAAs in generating a novel material.

Thermoresponsive hydrogel particles are versatile materials used in a variety of applications, ranging from photonics to tissue engineering and drug delivery.<sup>[1–6]</sup> These particles, made of crosslinked networks of polymers, proteins, or polypeptides,

offer the ability to respond to local changes in temperature, which is manifested as reversible shrinking and swelling in solution due to changes in network solubility. These volumetric changes can be exploited for diverse applications in optics, actuation, cell capture, and drug release.<sup>[7–13]</sup> In addition to their thermal responsiveness, particle size governs many properties of interest (e.g., refractive index, cargo loading capacity, release kinetics, and deformability). Despite the importance of this parameter, an overarching challenge in gel particle engineering is the ability to precisely design and synthesize particles of the same material with uniform size spanning multiple length scales. While techniques such as layer-by-layer deposition, molecular self-assembly, and microfluidic templating provide control over particle size,<sup>[14–20]</sup> these approaches limit size to a specific regime, such as either the nanoscale by self-assembly or the microscale by fluidic templating.<sup>[5,20]</sup>

Here, we present a facile two-step method for fabricating crosslinked gel particles with programmable size across four orders of magnitude (nano-to-meso-to-microscale) that are composed of a thermoresponsive, artificial, repetitive polypeptide designed de novo for this application.

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Elastin-like polypeptides (ELPs), the building blocks of these particles, are stimuli responsive, intrinsically disordered polypeptides that exhibit lower critical solution temperature (LCST) phase behavior in aqueous solution.<sup>[21–28]</sup> At low temperatures, ELPs exist as soluble, disordered chains while at high temperatures above their concentration-dependent transition temperature ( $T_t$ )—also known as the cloud point—the chains desolvate to form polypeptide-rich globules known as coacervates.<sup>[29,30]</sup> The consensus amino acid sequence of ELPs is (Val-Pro-Gly-Xaa-Gly)<sub>N</sub>, where *N* is the number of repeat units and Xaa is any amino acid except proline.<sup>[29]</sup> The influence of both these variables—*N* and Xaa—on the temperature-dependent phase behavior of ELPs is well characterized<sup>[23,28,31,32]</sup> and can be genetically encoded and independently controlled at the gene level. ELPs are an ideal starting material to fabricate thermoresponsive gel particles because they can be synthesized with perfect sequence control, have precisely specified molecular weight, are monodisperse, and can be recombinantly produced in high yield.<sup>[33,34]</sup>

A major challenge in producing crosslinked, stimuli-responsive gel particles lies in the introduction of the crosslinking sites. ELPs can be crosslinked via lysine, cysteine, or tyrosine residues through the addition of an extrinsic crosslinker.<sup>[35–37]</sup> However, these methods are only moderately efficient and often cumbersome, requiring multiple additional processing steps and the use of cytotoxic crosslinking agents.<sup>[38–40]</sup> Further, because a useful application of these particles is as carriers of biological drugs for controlled release, this approach also limits the incorporation of biological cargo via attachment to these chemically reactive amino acids. To develop a bioorthogonal crosslinking approach, and to eliminate the need for an extrinsic chemical crosslinker, we genetically encoded a photo-reactive unnatural amino acid (UAA), *para*-azidophenylalanine (pAzF),<sup>[41]</sup> into the ELP backbone. The bioorthogonal aryl azide group on this residue is activated by irradiation with ultraviolet (UV) light to form a reactive nitrene, which readily inserts into nearby N–H or C–H bonds.<sup>[42,43]</sup> Due to recent advancements in genomically recoded organisms<sup>[44–46]</sup> and multisite UAA<sup>[47]</sup> incorporation by Isaacs and co-workers, multiple copies of a UAA can be site-specifically incorporated into proteins and polypeptides with high yield and >95% accuracy.<sup>[47]</sup> This renders the monodisperse biopolymers crosslinkable immediately after purification. Photo-crosslinkable UAAs have previously been used successfully for probing protein interactions,<sup>[39,48]</sup> photopatterning,<sup>[49]</sup> and immobilization of proteins onto surfaces.<sup>[50,51]</sup> This work is the first demonstration of incorporating multiple copies of a photo-crosslinkable UAA into a polypeptide to create thermally responsive hydrogel particles whose size can be tuned across multiple orders of magnitude, ranging from tens of nanometers to several micrometers.

Using our previous work on controlling the size of ELP coacervates within aqueous microdroplets as a guideline,<sup>[52]</sup> we designed two ELPs containing photo-crosslinkable pAzF residues<sup>[53]</sup> to serve as the constituent polypeptides for the gel particles (**Figure 1A**; Table S1, Supporting Information). One polypeptide, termed photo-crosslinkable ELP (PCE), is composed of 80 pentameric repeats of the VPGVG motif with four regularly spaced pAzF residues, a sufficient number to form a crosslinked hydrogel network.<sup>[36]</sup> The second ELP in our system

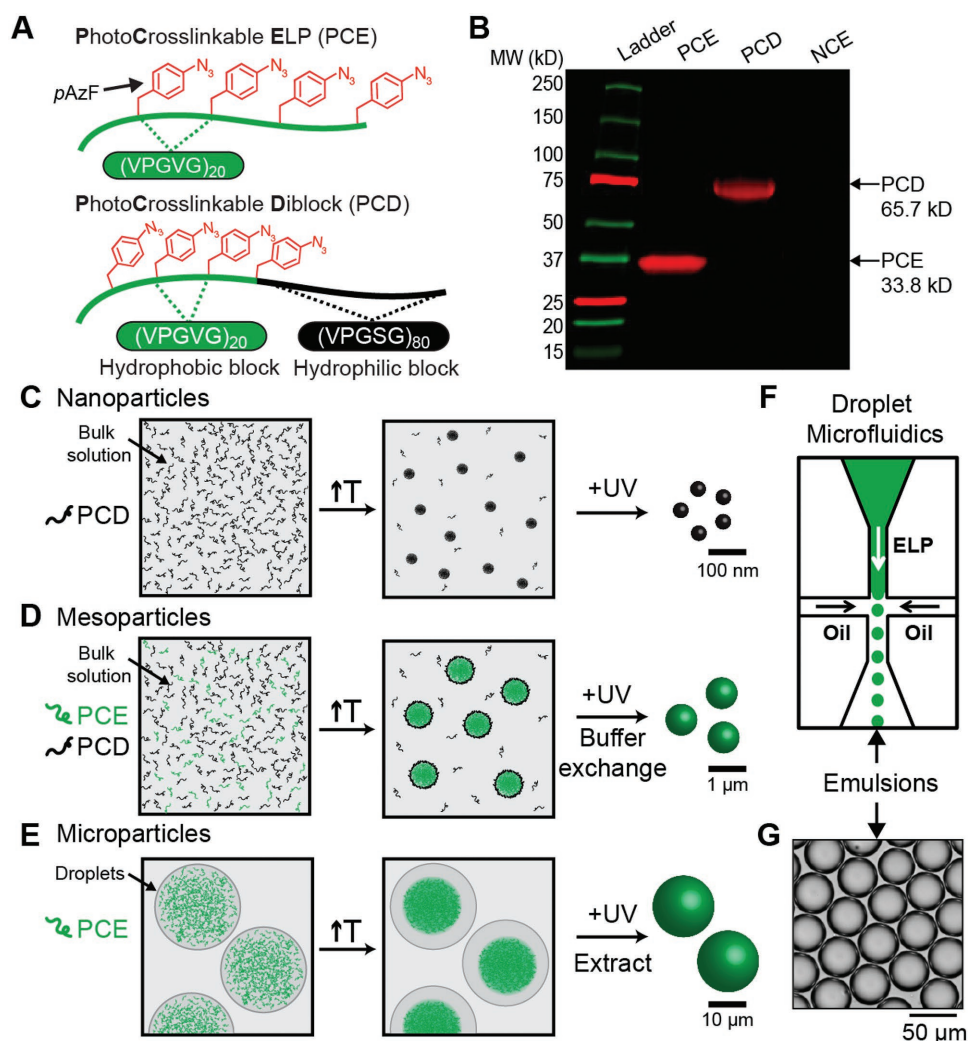
is a self-assembling photo-crosslinkable diblock (PCD) comprised of two distinct repetitive blocks—an N-terminal hydrophobic PCE block and a C-terminal hydrophilic ELP block. We successfully expressed these polypeptides in a genomically recoded *Escherichia coli* strain<sup>[44–46]</sup> and purified them in high quantities (>50 mg L<sup>−1</sup> culture; see Figure S1 in the Supporting Information) using inverse transition cycling, a non-chromatographic method for purifying ELPs.<sup>[29]</sup> To confirm the successful incorporation of functional pAzF, we labeled PCE and PCD with a fluorophore by a “click” reaction<sup>[54]</sup>—a strain-promoted alkyne-azide cycloaddition with a dibenzocyclooctyne (DBCO)-conjugated Cy5 fluorophore—and visualized the labeled peptide polymers by fluorescent imaging of a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (**Figure 1B**). We further confirmed >99% incorporation efficiency of pAzF by electron spray ionization liquid chromatography/mass spectrometry (LC/MS) (**Figure S2**, Supporting Information), which is consistent with previous reports.<sup>[47]</sup>

After synthesizing pAzF-containing ELPs, we employed a two-step strategy to fabricate thermoresponsive gel particles of uniform size spanning multiple length scales (**Figure 1C–E**). We first tuned the temperature of solutions containing PCD, PCE, or a mixture of both to form particles, and then stabilized the resulting particles by crosslinking with UV light. For the smallest, nanoscale size regime we use only PCD, a thermally responsive amphiphile. Raising the temperature above its critical micellization temperature (CMT) in bulk solution triggers its self-assembly into spherical micelles (**Figure 1C**; **Figure S3**, Supporting Information) and subsequent UV irradiation crosslinks the micelle cores, leading to the formation of nanogels.

To create hydrogel particles that span the mesoscale, we combine diblock PCD and monoblock PCE in solution (**Figure 1D**). By tuning the temperature above both the CMT of the PCD and the  $T_t$  of PCE, but below the  $T_t$  of PCD, we can generate mesoscopic coacervates of PCE whose size is limited by the PCD that serves as a surfactant and limits coarsening of the PCE coacervates. By tuning the ratio of PCE to PCD, we can generate particles of different sizes spanning the mesoscale. UV irradiation then stabilizes these hydrogel particles, which can be subsequently buffer exchanged to remove excess PCD for downstream applications.

At the largest, microscale size regime, we utilized microfluidic droplet-generating chips to create monodisperse water-in-oil emulsion droplets containing PCE (**Figure 1E–G**). Tuning the temperature either above or below the  $T_t$  of PCE and subsequent UV irradiation crosslinks the PCE coacervates into microscopic particles. Gel particle size can easily be controlled by modulating the starting concentration of PCE and the temperature at which crosslinking occurs.

We first synthesized crosslinked nanogels by thermally triggering the self-assembly of PCD in bulk solution into micelles and then crosslinking with UV light.<sup>[19,55,56]</sup> We use the terms “nxPCD” to denote the non-crosslinked micelles, and “xPCD” for these micelles after they are crosslinked into nanogels by UV irradiation. We characterized the nanoscale assemblies of PCD before and after crosslinking with UV light in bulk solution. Thermal ramp dynamic light scattering (DLS) of dilute xPCD in solution confirmed the stabilization

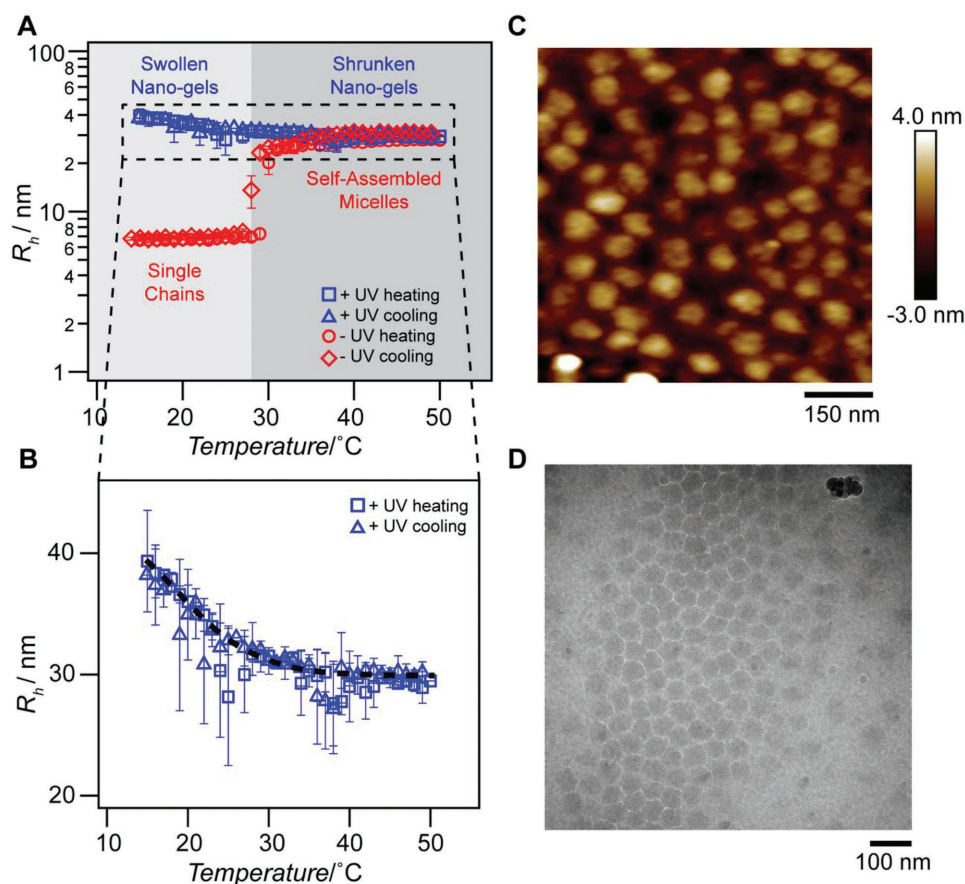


**Figure 1.** Design of size-controlled, thermoresponsive gel particles composed of disordered polypeptides containing photo-crosslinkable unnatural amino acids. A) Schematic of the two disordered polypeptides used in this study—PCE and PCD. B) Fluorescent SDS-PAGE gel of PCE, PCD, and NCE (non-crosslinkable ELP, (VPGVG)<sub>80</sub>) labeled with DBCO-Cy5. PCE and PCD contain pAzF residues that react with DBCO-Cy5, while the negative control NCE is not fluorescently labeled via click. C) Strategy for generating nanoparticles by heating and crosslinking PCD in bulk solution. D) Strategy for generating mesoparticles by heating and crosslinking mixtures of PCE and PCD in bulk solution. The PCD acts as a surfactant to stabilize PCE particles after heating, and buffer exchanging removes free PCD following crosslinking. Mesoscale particles can also be generated in microfluidic droplets for fluorescent spatial tracking. E) Strategy for generating microparticles by heating and crosslinking solutions of PCE in microfluidic droplets, followed by particle extraction from droplets. F) Schematic of a chip-based microfluidic droplet generator for generating monodisperse ELP-containing water droplet templates. G) Bright-field images of monodisperse water droplets containing photo-crosslinkable ELPs.

of self-assembled nanosized gel particles (Figure 2A; Figure S4, Supporting Information). These xPCD particles demonstrate reversible thermal responsiveness by shrinking upon heating as the hydrophobic core desolvates and swelling upon cooling (Figure 2B). In contrast, the nxPCD particles assemble upon heating into nanoscale micelles but disassemble completely into single chains upon cooling (Figure 2A). Static light scattering (SLS), performed on both nxPCD and xPCD, performed above the CMT corroborated the particle sizes measured by DLS (Table 1; Figures S5 and S6, Supporting Information).

We visualized the xPCD particles by tapping mode atomic force microscopy (AFM) performed below the CMT, which showed highly monodisperse nanogels and confirmed their

spherical shape (Figure 2C; Figures S7 and S8, Supporting Information). At the same temperature, nxPCD does not form any visible nanoscale assemblies in the absence of crosslinks to stabilize the chains against dissociation (Figure S9, Supporting Information). We further confirmed, using cryogenic transmission electron microscopy (cryo-TEM), that xPCD exists as monodisperse particles above the CMT (Figure 2D; Figure S10, Supporting Information). These uniform, spherical xPCD nanogels are ideally suited as delivery vehicles as they can be generated in solution and exist as stable micelles across a range of temperatures. Their size and solvation, both of which decrease with increasing temperature, are useful tunable attributes of these thermally responsive nanogels as they may provide an “active” mechanism to release encapsulated cargo



**Figure 2.** PCD self-assembles into micelles upon heating and can be photo-crosslinked to form thermoresponsive nanogels. A) Hydrodynamic radius ( $R_h$ ) of nxPCD (red; non-crosslinked) and xPCD (blue; crosslinked) as a function of temperature. nxPCD self-assembles from single chains into monodisperse micelles above the critical micellization temperature (CMT  $\approx 27$  °C at 100  $\mu$ M), whereas xPCD does not disassemble into single chains when the temperature is lowered below the CMT. B) Hydrodynamic radius ( $R_h$ ) of xPCD as a function of temperature demonstrates that xPCD nanogels exhibit thermal responsiveness as seen by the decrease in  $R_h$  with increasing temperature. Black dashed line represents the best sigmoidal curve fit for visualization. Error bars represent the polydispersity percentages ( $n = 5$ ). C) Tapping mode AFM images of xPCD below the CMT ( $T = 22$  °C) indicate highly monodisperse, crosslinked nanogels with a diameter of  $60.4 \pm 11.7$  nm. D) Cryogenic transmission electron microscopy (TEM) images of xPCD above the CMT ( $T = 37$  °C).

as compared to the typical “passive” diffusive mechanism of release from most hydrogels.<sup>[57–60]</sup>

We next developed crosslinked particles with sizes spanning the mesoscopic range from 100 nm to 1  $\mu$ m in both bulk solution and microdroplets utilizing insights from our previous study. In that work, we showed that mesoscopic coacervates of uniform size could be obtained by emulsifying mixtures of ELP diblocks and monoblocks, and thermally triggering the phase separation of both species.<sup>[52]</sup> Upon heating these mixtures to a temperature,  $T$ , such that the  $\text{CMT}_{\text{diblock}} < T < T_{\text{t-diblock}}$  and  $T > T_{\text{t-monoblock}}$  we observed two concurrent events: (i) the assembly of diblocks

into nanoscale micelles and (ii) the assembly of monoblocks into stable mesoscopic coacervate droplets where the diblock micelles prevent coalescence of the coacervate droplets. Importantly, we showed that the size of the mesoscale coacervates can easily be tuned by adjusting the relative ratio of mono-to-diblock ELP.

To determine the appropriate temperature for mesoscale particle formation, we first investigated the bulk LCST phase separation of PCE (Figure S11, Supporting Information). To visualize the phase separation within microdroplets, we fluorescently labeled the N-termini of the polypeptides for spatial tracking with fluorescence microscopy. When emulsified and heated, PCE undergoes the canonical liquid–liquid phase separation of monoblock ELPs and coarsening to form uniform liquid spheres (Figure S12 and Movie S1, Supporting Information), indicating that the pAzF residues do not have an adverse effect on PCE phase behavior. Next, we carried out light scattering of PCD to determine the CMT of PCD and its  $T_t$ —the temperature at which micelles of PCD undergo phase separation into a coacervate (Figures S3A, Supporting Information). These experiments allowed us to identify a range of

**Table 1.** Static light scattering characterization of nxPCD and xPCD at 37 °C.

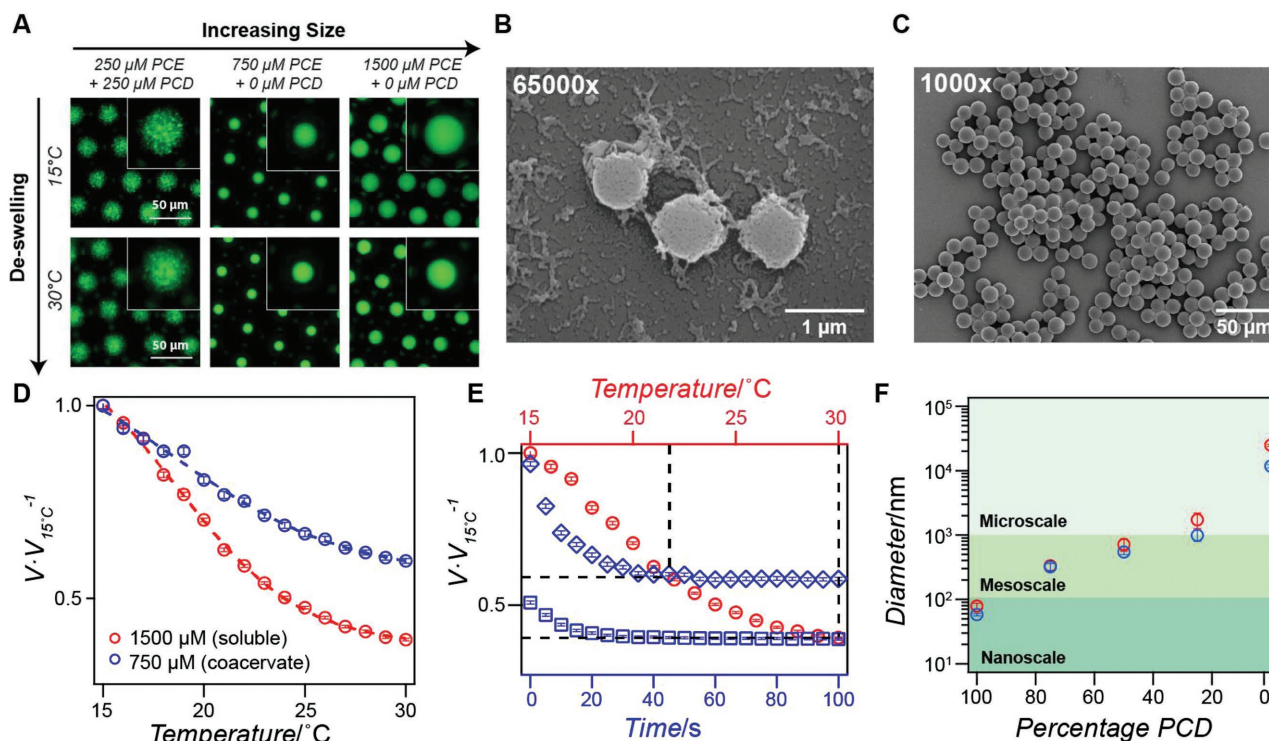
	$R_g$ [nm]	$R_h$ [nm]	$\rho$	$\text{MW}_{\text{micelle}}$ [g mol <sup>-1</sup> ]	$N_{\text{agg}}$
nxPCD	16.4	27.4	0.60	$1.1 \times 10^7$	162
xPCD	19.2	29.1	0.66	$1.3 \times 10^7$	200



temperatures,  $T$ , such that  $\text{CMT}_{\text{PCD}} < T < T_{\text{t-PCD}}$  and  $T > T_{\text{t-PCE}}$  for a given concentration in order to take advantage of these distinct phase behaviors and form mesoscale particles. For example, at 100  $\mu\text{M}$ ,  $\text{CMT}_{\text{PCD}}$  is  $\approx 27^\circ\text{C}$ ,  $T_{\text{t-PCD}}$  is  $\approx 65^\circ\text{C}$ , and  $T_{\text{t-PCE}}$  is  $\approx 23^\circ\text{C}$ ; therefore, heating to any temperature in the range of  $27^\circ\text{C} < T < 65^\circ\text{C}$  will result in mesoscale particle formation. Having verified that PCE and PCD satisfied these conditions, we next created microdroplets containing equimolar concentrations of PCE and PCD and heated to  $T = 30^\circ\text{C}$ , to induce the phase separation of PCE and self-assembly of PCD (Movie S2, Supporting Information). Heating PCE in the presence of PCD results in visible PCE coacervates that do not coarsen over time but rather exhibit Brownian motion due to the surfactant-like PCD chains at the interface between the PCE-rich and PCE-poor phases (Movie S2, Supporting Information). Exposing the mesoscopic PCE–PCD coacervates to UV light efficiently crosslinks them into particles that do not resolubilize upon cooling below the cloud point temperature of PCE (Figure 3A; Movie S3, Supporting Information). By varying the ratio of PCE:PCD, it is possible to create mesogels

whose size spans the entire mesoscale regime; increasing the concentration of PCE relative to PCD creates coacervate droplets with increasing size which then can be photo-crosslinked into hydrogel particles (Figure S13, Supporting Information). Scanning electron microscopy (SEM) imaging of these particles confirms the ability to easily tune their size within the mesoscale range (Figure 3B; Figures S14 and S15, Supporting Information).

To extend the size of the particles further into the microscale, and expand their utility for drug delivery, optics, cosmetics, and other applications, we created microdroplets containing only PCE. Without PCD chains present in solution, PCE phase separates to form spherical, microscale coacervates with dimensions dictated by the template droplet size (Movie S1, Supporting Information). We can tune the size of these particles by modulating the initial concentration of PCE and then crosslinking at a temperature either above or below its  $T_{\text{t}}$ . We used this approach to generate microparticles of 12 and 25  $\mu\text{m}$  in diameter, respectively. To generate 25  $\mu\text{m}$  microgels, we crosslinked droplets containing soluble PCE ( $T < T_{\text{cloud point PCE}}$ )



**Figure 3.** Thermoresponsive meso- and microgels with tunable swelling and deswelling behavior. A) Fluorescence microscopy images of left) AlexaFluor 488-labeled mesogels, middle) 12  $\mu\text{m}$  xPCE microgels, and right) 25  $\mu\text{m}$  xPCE microgels within water-in-oil emulsion droplets. The concentration of PCE and PCD is shown above each panel. Top) At  $T < T_{\text{cloud point PCE}}$  the gels are swollen and bottom) at  $T > T_{\text{cloud point PCE}}$  the gels are shrunk. B) SEM images of mesoscale particles with a mean particle diameter of  $865 \text{ nm} \pm 199 \text{ nm}$  ( $n \geq 200$ ). C) SEM images of microscale particles with a mean particle diameter of  $12.1 \mu\text{m} \pm 0.8 \mu\text{m}$  ( $n \geq 200$ ). D) Gel volume (normalized to the volume at  $15^\circ\text{C}$ ) as a function of temperature for 750  $\mu\text{M}$  xPCE particles (blue points) and 1500  $\mu\text{M}$  xPCE particles (red points). The dashed lines represent the best sigmoidal fit and the error bars represent the SEM ( $n = 150$  droplets). E) Normalized gel volume as a function of time for xPCE microgels (1500  $\mu\text{M}$ ) quenched heated from 15 to  $22^\circ\text{C}$  (blue diamonds) or 22 to  $30^\circ\text{C}$  (blue boxes). The red circles indicate the equilibrium volume of xPCE gels as a function of temperature; the black dashed lines are visual aids that intersect at the equilibrium volume at 22 and  $30^\circ\text{C}$ . The error bars represent the standard error of the mean ( $n = 150$ ). F) Gel particle diameter as a function of PCD content (PCE percentage =  $100 - \text{PCD}$ ) demonstrating the ability to fabricate uniform gel particles spanning multiple length scales. The two data points shown represent the largest (red) and smallest (blue) particles observed via DLS (100–25% PCD) illustrating the size range that can be achieved by modulating temperature, with error bars representing the polydispersity percentage ( $n \geq 5$ ). The red and blue points at 0% PCD illustrate the particle size obtained with different starting concentrations of PCE, and error bars represent the standard deviation ( $n \geq 100$ ).

at a concentration above chain overlap to enable network formation. After crosslinking, these microgels rapidly reach their equilibrium concentration and upon heating expel water and shrink due to enhanced chain–chain interactions and desolvation (Figure 3A). To create the 12  $\mu\text{m}$  microparticles, we heated PCE droplets ( $T > T_{\text{cloud point PCE}}$ ) to form uniform microcoacervates (one coacervate per droplet) and then irradiated the droplets with UV light to crosslink the coacervates into microgels. These microgel particles were extracted from their template droplets and imaged by SEM, revealing highly monodisperse, spherical particles (Figure 3C; Figures S14 and S15, Supporting Information). These particles are also highly stable; they can be lyophilized and reconstituted in solution, and stored at temperatures ranging from  $-80$  to  $37$   $^{\circ}\text{C}$  for at least one week without loss of structural integrity or change in size (data not shown).

We next investigated the thermal responsivity of xPCE microgels by monitoring their size as a function of temperature using temperature-controlled fluorescence microscopy. We observed that the 12  $\mu\text{m}$  and 25  $\mu\text{m}$  xPCE microgels exhibit well-controlled deswelling upon slow heating from  $15$  to  $30$   $^{\circ}\text{C}$  (Figure 3D; Movies S4 and S5, Supporting Information). In both microgels, the temperature-dependent deswelling follows a sigmoidal relationship similar to that observed for xPCD nanogels, though this effect is more prominent for the 25  $\mu\text{m}$  xPCE microgels due to a less densely packed network. This steady decrease in microgel size with increasing temperature is due to the decreased excluded volume of each monomer comprising the network, which in turn directly affects the swelling behavior of the gel.<sup>[61]</sup> These results demonstrate that by simply changing the temperature at which crosslinking is performed (i.e., upon solutions of soluble or insoluble PCE), it is possible to generate microgels that controllably shrink down to 40% of their original volume (Figure 3D).

Finally, we evaluated the deswelling kinetics of the xPCE microgels to better understand their response rate to changes in temperature. We performed two step-wise heating ramps (from  $15$  to  $22$   $^{\circ}\text{C}$  and  $22$  to  $30$   $^{\circ}\text{C}$ ) and monitored the size of the 25  $\mu\text{m}$  xPCE microgels as a function of time after reaching the target temperature (Figure 3E; Movies S6 and S7, Supporting Information). In both instances, the microgels respond similarly and reach their equilibrium size in  $\approx 40$  s, indicating a rapid response to the temperature change of the environment, which is ideal for applications requiring on-demand feedback. The deswelling dynamics we observed are consistent with those reported for poly(*N*-isopropylacrylamide) microgels of similar size.<sup>[62]</sup> In thermoresponsive particles, the swelling/deswelling response rate is proportional to the rate of diffusion of water into/out of the particle, which is governed by the overall size and density of the polymer network. Given ELPs' finely tuned and well-characterized response to temperature, these particles are well-positioned to serve as ideal carrier vehicles, actuators, and sensors.

In conclusion, we have developed a simple and convenient method to fabricate thermoresponsive gel particles from genetically encoded polypeptides that are tunable from the nanoscale to microscale in size (Figure 3F). Particle size can easily be adjusted by tuning the relative concentrations of the pAzF-containing ELP diblock and monoblock. Particle size is increased from the nano- to mesoscale by increasing the ratio

of monoblock (PCE) to diblock ELP (PCD). To extend into the microscale regime, microfluidic droplets provide a necessary level of control for templating PCE coacervates, with droplet dimensions directly dictating particle size. The reversible swelling/deswelling behavior of the particles is rapid (i.e., on the order of seconds) and easily tuned by specifying the phase—soluble versus coacervate—of the polypeptides prior to crosslinking. Future work with this material system will allow further fine-tuning of gel network density at any given temperature. For example, changing the ELP sequence (i.e., guest residue, length) will modulate the functional dependence of monomer-excluded volume on temperature,<sup>[61]</sup> and altering the concentration of pAzF residues can also easily tune the swelling properties. The simplicity and flexibility of this new method for creating thermoresponsive nano-, meso-, and microgels enables the fabrication of particles with potential utility in a wide variety of applications in biomaterials, optics, and pharmaceuticals.

## Experimental Section

**Materials:** pET24+ vectors were purchased from Novagen (Madison, WI). Oligonucleotides and gBlocks encoding sequences of interest were purchased from Integrated DNA Technologies (Coralville, IA). Ligation enzymes, restriction enzymes, and calf intestinal alkaline phosphatase were purchased from New England Biolabs (Ipswich, MA). EB5 $\alpha$  chemically competent *E. coli* cells were purchased from Bioneer (Taunton, MA). Genomically recoded *E. coli* cells were generously provided by Prof. Farren Isaacs (Yale University). All *E. coli* cultures were grown in 2xYT media comprised of sodium chloride ( $5$  g  $\text{L}^{-1}$ ; Alfa Aesar, Ward Hill, MA), tryptone ( $16$  g  $\text{L}^{-1}$ , Becton, Dickinson and Co., Franklin Lakes, NJ), and yeast extract ( $10$  g  $\text{L}^{-1}$ , Becton, Dickinson and Co., Franklin Lakes, NJ). *p*-Azidophenylalanine hydrochloride was purchased from Synchem, Inc. (Elk Grove Village, IL). Kanamycin sulfate was purchased from EMD Millipore (Billerica, MA) and chloramphenicol was purchased from Sigma-Aldrich (St. Louis, MO). Protein expression was induced with isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) from Gold Biotechnology (St. Louis, MO) and L-(+)-arabinose from Sigma-Aldrich (St. Louis, MO). All salts were purchased from Alfa Aesar (Ward Hill, MA).  $1\times$  phosphate buffered saline (PBS) tablets ( $10\times 10^{-3}$  M phosphate buffer,  $140\times 10^{-3}$  M NaCl, and  $3\times 10^{-3}$  M KCl, pH 7.4 at  $25$   $^{\circ}\text{C}$ ) were purchased from EMD Millipore (Billerica, MA). Molecular organic fluorophores (NHS-Alexa Fluor 488, dibenzocyclooctyne-Cy5) were purchased from Life Technologies (Grand Island, NY) and Sigma-Aldrich (St. Louis, MO), respectively. DNA extraction kits, DNA gel purification kits, and PCR purification kits were purchased from Qiagen Inc. (Germantown, MD). Mineral oil was purchased from Sigma-Aldrich (St. Louis, MO). ABIL EM 90 and TEGOSOFT DEC surfactants were purchased from Evonik Industries (Essen, Germany). A glass single emulsion droplet-generating chip was purchased from Dolomite Microfluidics (Royston, UK). Syringe pumps were purchased from Chemex Inc. (Stafford, TX). Isobutanol was purchased from IBI Scientific (Peosta, IA). All solvents for LC/MS analysis were LC/MS grade, purchased from Thermo Fisher Scientific (Waltham, MA). SEM samples were deposited on silicon wafers on aluminum specimen mounts, purchased from Electron Microscopy Sciences (Hatfield, PA). Quantifoil grids (Quantifoil Micro Tools GmbH, Großlobichau, Germany) or Lacey carbon grids (Ted Pella, Redding, CA) were used for TEM imaging.

**Gene Synthesis:** Genes encoding PCE and PCD were first synthesized using recursive directional ligation by plasmid reconstruction, as described elsewhere.<sup>[34]</sup> In brief, we modified a pET-24+ cloning vector (m-pET-24+) to contain endonuclease recognition sites for *AclI*, *BseRI*, and *BglI*. We digested the modified vector with *BseRI* and ligated the desired ELP repeat sequence into the vector. The ELP sequence was created by annealing together complementary ssDNA strands that encode

for the desired amino acid sequence along with “sticky end” overhangs. An amber stop codon (DNA sequence “TAG”) was introduced as an oligonucleotide into a second population of m-pET-24+ between the start and stop codons by digesting with BseRI. The vector containing the ELP sequence was then digested with AclI and BglI to create an “A” population and the TAG-containing vector was digested with BseRI and BglI to create a “B” population. The two populations of cut vectors are complementary to one another such that when ligated together the ELP sequence and TAG codon are seamlessly encoded between the start and stop codons of m-pET-24+ vector. This process was repeated to form populations of an “A” cut of ELP-TAG and a “B” cut of ELP-TAG that were subsequently ligated together to double the length of the base repeat sequences. This method was continued until the desired length of the gene was obtained. The genes encoding the final sequences of PCE and PCD (see Tables S1 and S2, Supporting Information) were confirmed by DNA sequencing. The gene encoding NCE (non-crosslinkable ELP) was constructed with the same protocol and confirmed by DNA sequencing (Tables S1 and S2, Supporting Information).

We replaced the T7 promoter and terminator sequences of m-pET-24+ with a pTac promoter and rrnB terminator, with vector reconstruction performed by GenScript USA Inc. (Piscataway, NJ). This vector, m-pTac, is compatible with expression in the genomically recoded *E. coli*. We transferred the genes of interest by digesting the PCE- and PCD-containing m-pET-24+ vectors with BseRI/BamHI, extracting the insert using agarose gel separation and purification, and ligating with similarly digested m-pTac. These vectors were then cotransformed with the modified pEvol tRNA/aaRS vector that contained two copies of the pAcFRS.1.t1 synthetase into the genomically recoded *E. coli* (both generously provided by F. J. Isaacs).

**Protein Expression, Purification, and Characterization:** Liquid cell cultures (50 mL) of strains harboring pEvol and ELP plasmids were inoculated from frozen glycerol stocks and grown to confluence overnight. Cultures were then inoculated at 1:20 dilution in 2xYT media (1 L) supplemented with kanamycin (45  $\mu\text{g mL}^{-1}$ ) and chloramphenicol (25  $\mu\text{g mL}^{-1}$ ). aaRS expression was simultaneously induced by the addition of arabinose (0.2%) and pAzF ( $1 \times 10^{-3}$  M). Cells were grown at 34 °C in a shaking incubator at 200 rpm for 6 h, at which time ELP expression was induced by the addition of IPTG ( $1 \times 10^{-3}$  M), and the cultures incubated at 34 °C for an additional 18 h. Cell pellets were harvested by centrifugation at 3500  $\times g$  and resuspended in 1xPBS (20 mL). Cells were lysed by sonication for a total of 3 min (Misonix; Farmingdale, NY) and DNA was precipitated by addition of polyethylenimine (10%; MP Biomedicals, Santa Ana, CA). Precipitated DNA and cellular debris were removed by centrifugation at 20 000  $\times g$  at 4 °C. Proteins were then purified using four rounds of inverse transition cycling, as described elsewhere.<sup>[63,64]</sup> Briefly, solutions of proteins were heated and salt (NaCl) was added to induce the phase transition of the ELP, centrifuged to collect all insoluble material at 35 °C, 20 000  $\times g$  (“hot spin”), and resuspended in cold 1xPBS. Upon cooling, the ELP resolubilizes, while contaminants remain insoluble and can be removed by centrifugation at 35 °C, 20 000  $\times g$  (“cold spin”). Protein purity was characterized by 4–20% gradient Tris–HCl (Biorad, Hercules, CA) SDS-PAGE and staining with copper chloride (0.5 M; Fisher Scientific, Hampton, NH). Protein yield was determined gravimetrically after dialysis into nanopure water and lyophilization. Incorporation of pAzF was qualitatively confirmed by fluorescent labeling with excess DBCO-Cy5 followed by separation on SDS-PAGE and imaging on a Typhoon 9410 Variable Mode Imager (GE Healthcare, Pittsburgh, PA). All protein samples were kept in the dark to protect pAzF from ambient light.

**Temperature-Controlled Spectrophotometry:** Cloud point transition temperatures ( $T_i$ ) were determined by temperature-controlled spectrophotometry using a Cary 300 (Agilent Technologies). Samples containing various concentrations of the polypeptide in 1xPBS were heated at 1 °C  $\text{min}^{-1}$  and the optical turbidity at 650 nm was recorded every 0.1 °C. The cloud point was determined as the maximum of the first derivative of the turbidity as a function of temperature.

**Droplet Formation:** To create water-in-oil emulsion droplets, two liquid phases—a dispersed, aqueous phase of ELPs in 1xPBS and an organic, continuous phase comprised of TEGOSOFT DEC/ABIL EM 90/mineral oil (75%/5%/20% v/v)—were injected into the microfluidic droplet generator at constant flow rates using precision syringe pumps. The flow rates of the dispersed and continuous fluids were tuned to ensure droplet formation in the dripping regime; in these experiments, the dripping regime was achieved using a constant flow rate of 250  $\mu\text{L h}^{-1}$  for the organic continuous phase and 75–100  $\mu\text{L h}^{-1}$  for the aqueous, dispersed phase. The production of droplets within the microfluidic device was monitored using a 5 $\times$  objective on an inverted microscope (Leica) equipped with a digital microscopy camera (Lumenera Infinity 3-1 CCD).

**Particle Extraction from Droplets:** Microscale particles were extracted from droplets using isobutanol to disrupt the water/oil phase boundary and create two continuous phases. Isobutanol was added at a ratio of 10:1 with droplets in solution and vortexed gently to mix. Droplet rupture and therefore particle extraction was evident by a change in the solution from turbid and yellow-white to clear and colorless. This solution was then centrifuged for 1 min at 20 000  $\times g$  and the supernatant removed. The remaining pellet contained the stable, crosslinked particles and was resuspended in PBS or water for SEM imaging. Particle integrity, shape, and size were confirmed to be intact and unchanged before and after droplet extraction via light microscopy.

**Mass Spectrometry:** Solutions of PCE and PCD ( $20 \times 10^{-6}$  M) were prepared in 5% acetonitrile/0.2% formic acid/water and mass spectra acquired on an Agilent 1100 LC/MSD Trap SL (Agilent Technologies, Santa Clara, CA). Samples were injected into a Phenomenex Luna C18 column (50  $\times$  1 mm, 3  $\mu\text{m}$ ; 0.2% formic acid in water as buffer A, 0.2% formic acid in acetonitrile as buffer B) and then into the mass spectrometer using a fully automated system. Spectra were acquired in positive mode followed by analysis and deconvolution using LC/MSD Trap Data Analysis software (Agilent Technologies, Santa Clara, CA). Mass spectra were acquired at the Mass Spectrometry shared facility at Duke University.

**Ultraviolet-Initiated Crosslinking:** PCE and PCD were UV crosslinked using an Omnicure Series 1000 lamp (Ontario, Canada) equipped with a liquid light guide and a 250–450 nm filter set. PCD micelles were crosslinked to form nanogels by heating a solution of PCD in 1xPBS to 40 °C for 5 min to ensure micellization and then exposed to 50% intensity UV for 10 s. Similarly, emulsions containing PCE or mixtures of PCE + PCD were brought to a desired temperature ( $T = 30$  °C for photo-crosslinking PCD + PCE mixtures,  $T = 15$  °C for photo-crosslinking soluble PCE, and  $T = 30$  °C for photo-crosslinking PCE coacervates) on the Linkam LTS120 precision Peltier heating and cooling microscope stage and subsequently exposed to 50% intensity UV for 10 s.

**Light Scattering:** DLS measurements were performed over a temperature range of 15–50 °C using a Wyatt DynaPro temperature-controlled instrument (Wyatt Technology, Santa Barbara, CA). Samples for the DLS system were prepared in 1xPBS and filtered through Whatman Anotop sterile syringe filters (0.2  $\mu\text{m}$ ; GE Healthcare Life Sciences, Pittsburgh, PA) into a quartz crystal cuvette (12  $\mu\text{L}$ ; Wyatt Technology, Santa Barbara, CA). Five acquisitions were taken at each temperature, and the results presented represent the mean  $R_h$  or  $D_h$  of the sample at each temperature. The error bars represent the polydispersity percentage determined from the five data points taken at each temperature. It should be noted that the sizing measurements on all PCD + PCE combination mixtures demonstrated a small percentage of PCD nanoscale micelles and, at some temperatures, larger populations that accounted for small percentages of the overall composition. These data points are not presented in Figure 3D.

SLS measurements were performed using an ALV/CGS-3 goniometer system (Langen, Germany). Samples for the ALV/CGS-3 goniometer system were prepared in 1xPBS and filtered through Whatman Anotop sterile syringe filters (0.2  $\mu\text{m}$ ) into a disposable borosilicate glass tube (10 mm; Fischer Scientific, Pittsburgh, PA). Simultaneous SLS measurements were obtained at angles between 30° and 150° at 5° increments, with each angle consisting of three runs for 15 s, at



37 °C. The differential refractive index ( $dn\ dc^{-1}$ ) was determined by measuring refractive index at five different concentrations at 37 °C using an Abbemat 500 refractometer (Anton Paar, Graz, Austria). Static light scattering data were analyzed by partial Zimm plots using the ALVSTAT software to determine the radius of gyration ( $R_g$ ) and molecular weight.  $N_{agg}$  was determined by dividing the particle molecular weight by molecular weight of an individual PCD chain, as calculated from the amino acid sequence.

**AFM:** Samples for AFM imaging were prepared by placing a drop ( $\approx 20\ \mu\text{L}$ ) of sample solution ( $6\ \text{mg mL}^{-1}$  in water) onto a freshly cleaved mica surface. The samples were then incubated for 15 min and subsequently gently rinsed with Milli-Q  $\text{H}_2\text{O}$  and dried with  $\text{N}_2$  gas. AFM images were acquired in Tapping Mode under ambient conditions using a Nanoscope MultiMode AFM (Bruker). TappingMode silicon cantilevers were used for all the AFM images ( $k_F = 40\ \text{N mol}^{-1}$ ,  $f_{res} = 300\ \text{kHz}$ ). The particle sizes were determined using ImageJ open source software.

**Cryo-TEM:** Cryo-EM imaging was performed on a Tecnai G<sup>2</sup> Spirit BioTWIN (FEI-Company, Eindhoven, the Netherlands, and Hillsboro, OR) operated at 120 kV. xPCD samples were adsorbed onto holey carbon grids. Either 2.0  $\mu\text{m}$  holes, 2.0  $\mu\text{m}$  spacing, Quantifoil grids (Quantifoil Micro Tools GmbH, Großbichau, Germany), or Lacey carbon grids (Ted Pella, Redding, CA) covered with a thin continuous carbon film were used. Samples were vitrified using an FEI Vitrobot Mark IV device. xPCD samples were loaded onto the grid in the Vitrobot chamber at room temperature (22 °C) with the relative humidity set to 100%. Samples were blotted for 2 s with a force set to 3, plunged directly into liquid ethane and transferred into liquid nitrogen. Grids were then transferred into a Gatan 626 cryoholder (GATAN Inc., Pleasanton, CA) and inserted into the TEM. 2D microscopic images were acquired using an FEI Eagle 4k  $\times$  4K CCD camera in low-dose condition at varying magnifications with a dose not exceeding  $15\ \text{e}^-\ \text{\AA}^{-2}\ \text{s}^{-1}$ .

**SEM:** Microparticles were extracted from droplets and resuspended in water. An aliquot of sample (5  $\mu\text{L}$ ) was drop cast onto a silicon wafer attached to an aluminum stub with double-sided adhesive carbon tape and allowed to air dry for at least 4 h. The dried samples were sputter coated with gold for 250 s (Denton Desk IV, Moorestown, NJ) and then imaged with an FEI XL30 SEM-FEG at 7 kV. Mesoparticles were crosslinked in bulk and diluted 1:100 in water prior to sample preparation.

**Heating and Optical Imaging:** The PCE or PCD + PCE emulsion samples were collected on a glass microscope slide and heated using a precision Peltier heating and cooling stage (Linkam LTS120) equipped with a Linkam PE95 digital temperature control unit. The spatial distribution of Alexa Fluor 488-labeled (25% molar fraction N-terminal labeled) PCE was characterized via fluorescence microscopy using an upright Zeiss Axio Imager A2 microscope with a 20 $\times$  objective and the appropriate filter set (ex 470/40, em 525/50). Particle sizing of visible microscale particles was done using MATLAB.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

bioinspired materials, hydrogel particles, recombinant proteins, unnatural amino acids, unstructured polypeptides

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