Published in final edited form as:

Soft Matter. 2013 February 7; 9(5): 1589–1599. doi:10.1039/C2SM27496E.

Nanoparticle Formation from Hybrid, Multiblock Copolymers of Poly(Acrylic Acid) and VPGVG Peptide

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Abstract

Elastin-mimetic hybrid copolymers with an alternating molecular architecture were synthesized *via* the step growth polymerization of azide-functionalized, telechelic poly(*tert*-butyl acrylate) (P*t*BA) and an alkyne-terminated, valine and glycine-rich peptide with a sequence of (VPGVG)₂ (VG2). The resultant hybrid copolymer, [P*t*BA-VG2]_n, contains up to six constituent building blocks and has a polydispersity index (PDI) of ~1.9. Trifluoroacetic acid (TFA) treatment of [P*t*BA-VG2]_n gave rise to an alternating copolymer of poly(acrylic acid) (PAA) and VG2 ([PAA-VG2]_n). The modular design permits facile adjustment of the copolymer composition by varying the molecular weight of PAA (22 and 63 repeat units). Characterization by dynamic light scattering indicated that the multiblock copolymers formed discrete nanoparticles at room temperature in aqueous solution at pH 3.8, with an average diameter of 250-270 nm and a particle size distribution of 0.34 for multiblock copolymers containing PAA₂₂ and 0.17 for those containing PAA₆₃. Upon increasing the pH to 7.4, both types of particles were able to swell without being disintegrated, reaching an average diameter of 285-300 nm for [PAA₂₂-VG2]_n and 330-350 nm for [PAA₆₃-VG2]_n, respectively. The nanoparticles were not dissociated upon the addition of urea, further confirming their unusual stability. The nanoparticles were capable of sequestering a hydrophobic fluorescent dye (pyrene), and the critical aggregation concentration (CAC) was determined to be 1.09×10^{-2} or 1.05×10^{-2} mg/mL for [PAA₂₂-VG2]_n and [PAA₆₃-VG2]_n, respectively. We suggest that the multiblock copolymers form through collective H-bonding and hydrophobic interactions between the PAA and VG2 peptide units, and that the unusual stability of the multiblock nanoparticles is conferred by the multiblock architecture. These hybrid multiblock copolymers are potentially useful as pH-responsive drug delivery vehicles, with the possibility of drug loading through concerted H-bonds and hydrophobic interactions.

Introduction

Copolymers composed of blocks that differ in chemical or physical properties have the ability to form a variety of ordered nanostructures in solution that are useful for drug and gene delivery.¹⁻³ In conventional synthetic block copolymer systems, a solubility difference between the blocks usually acts as the driving force for copolymer assembly; the hydrophobic domains have a strong tendency to associate or aggregate in an aqueous solution due to the resulting increase in entropy of the solvent molecules.^{2, 4, 5} The size and shape of the nanostructures are controllable by a variety of factors including the number,

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chemical identity, molecular weight, and hydrophobic-hydrophilic balance of the polymer blocks, or the polarity, ionic strength, temperature, or pH of the solution.⁶ Most block copolymer systems reported to date have a basic diblock or triblock structure. The number of alternating multiblock copolymers ([polyA-polyB]_n) that have been synthesized is much more limited, and far fewer studies have been conducted on their assembly or association behavior. In the few systems that have been investigated, it has been determined that alternating multiblock copolymers can undergo chain looping or folding to form unimolecular or multimolecular micelle structures,⁷ and that the assembly behavior also depends on the number, ratio, and molecular weight of the blocks,^{8, 9} in good agreement with theoretical predictions.^{10, 11}

Many previously reported multiblock copolymers are solely based on synthetic polymers.^{7, 9, 12, 13} When considering the usefulness of self-assembling block copolymer systems for biomedical applications, hybrid synthetic polymer-peptide block copolymers are of interest because the incorporation of the peptides can provide biological functionality or more complex structural properties.¹⁴⁻²⁰ In these polymer-peptide approaches, the majority of hybrid block copolymers are also of diblock or triblock architecture, and there are few studies of [polyA-polyB]_n multiblock hybrid copolymers. We have previously synthesized multiblock elastin-mimetic hybrid polymers (EMHP) with units of the hydrophilic, crosslinking AKA₃KA peptide domains of natural elastin in alternation with poly(ethylene glycol) (PEG).^{21, 22} This structure was designed to mimic tropoelastin, the soluble elastin precursor composed of alternating hydrophilic blocks for crosslinking and hydrophobic blocks that can undergo coascervation as well as provide elasticity to the crosslinked protein.²³

A major advantage of the EMHP synthesis is that its modular nature permits the chemical, structural, biological, and mechanical properties of the hybrid multiblock copolymers to be readily tuned. In this work, we have designed a hybrid multiblock copolymer using poly(acrylic acid) (PAA) and a valine, glycine-rich peptide derived from the hydrophobic domains of natural elastin. Genetically engineered elastin-like polypeptides (ELPs) containing VPGVG repeat units have been proposed to undergo temperature-induced structural transitions caused by hydrophobic dehydration as the temperature is increased,^{24, 25} even when the VPGVG chains are very short (one, three, or five repeats).²⁶ As described herein, the VPGVG-containing multiblock copolymers we have synthesized formed nanoparticles at room temperature by a mechanism that is suggested to involve H-bonds and hydrophobic interactions, and that the formation of nanoparticles of reasonable uniformity is due to the unique, multiblock molecular architecture. The multiblock architecture brings the polymer and peptide units into close proximity, which creates high local concentrations of polymer and peptide and therefore facilitates their interaction and assembly.

Poly(acrylic acid) (PAA) was chosen as the synthetic polymer segment of these EMHPs. Block copolymers of PAA with hydrophobic synthetic polymers such as polystyrene or poly(*n*-butyl acrylate) (P*n*BA) have been used to form pH-responsive micelles or vesicles,^{6, 27-30} hydrogels,^{31, 32} and block copolymer micelle-crosslinked hydrogels.³³ In addition to the pH-responsiveness, the carboxylic acid functional groups of PAA provide a means for future attachment of biologically active molecules. Although multiblock PAA-polystyrene polymers⁷ and hybrid diblock copolymers of PAA/peptide-based materials such as poly(L-valine)^{34, 35} or VPGVG-containing ELPs^{36, 37} have been synthesized, PAA-VPGVG multiblock copolymers have not been reported in the literature. Herein, we have investigated the formation of nanostructures from [PAA-VG2]_n containing two different molecular weights of PAA (22 or 63 repeat units (1,600 or 4,500 g/mol) at pH conditions above and below the pK_a of PAA (~4.5). These novel hybrid polymers could have potential

applications in drug delivery because their modular design allows the chemical, physical, and biological properties to be tuned via interchanging polymer and peptide units of various chemical structure and molecular weight.

Experimental

Materials

All reagents were used without further purification unless otherwise specified. Dimethyl 2,6-dibromoheptanedioate, copper (I) bromide, copper (I) acetate, *N,N,N',N',N''*-pentamethyldiethylenetriamine (PMDETA), *tert*-butyl acrylate, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium azide, acetic anhydride, *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), toluene, methanol, and dichloromethane (DCM) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Fmoc-L-valine-OH, Fmoc-L-glycine-OH, and Fmoc-L-proline-OH were purchased from Protein Technologies, Inc. (Tucson, AZ, USA), and Fmoc-L-propargylglycine-OH was purchased from AnaSpec, Inc. (Fremont, CA). Water was deionized and filtered through a Barnstead NANOpure Diamond water purification system (Thermo Scientific, Dubuque, IA, USA).

Synthesis of multiblock hybrid copolymers of poly(*tert* butyl acrylate) and (VPGVG)₂ ([P*t*BA-VG2]_n)

Poly(*tert* butyl acrylate) diazide (N₃-P*t*BA-N₃) and X(VPGVG)₂X (XVG2X, (where V = valine, P = proline, G = glycine, and X = propargylglycine) were separately synthesized and characterized (see Supporting Information). Solutions of N₃-P*t*BA-N₃ (0.064 mmol) in 1.7 mL anhydrous DMF, XVG2X (0.064 mmol) in 300 μL anhydrous DMF, and Cu(I) acetate (0.25 equiv. to alkyne) in 200 μL anhydrous DMF were added to a nitrogen-purged, round bottom flask. The mixture was stirred at 80 °C under nitrogen for 24 h followed by precipitation into cold water. The product was collected by vacuum filtration, washed with water, and dried under vacuum to obtain a pale tan solid in 53% yield. ¹H NMR (DMSO-d₆) δ (ppm): 7.10-8.48 (11H, varied, NHCO and triazole C=CH), 5.18 (m, 1H, triazole-CH(NHCOCH₃)CO), 3.95-4.48 (m, 9H, NH-CH-CO), 3.56 (m, 6H, CH₂CH-triazole and proline NHCH₂CH₂CH₂), 2.15 (m, 18H, P*t*BA CH₂CH and proline NHCH₂CH₂CH₂), 1.71-2.0 (m, 20H, P*t*BA CH₂CH, valine CH(CH₃)₂, and proline NHCH₂CH₂CH₂), 1.44 (s, 188H, P*t*BA CH₂CH and C(CH₃)₃), 0.85 (m, 24H, valine CH(CH₃)₂).

Synthesis of [PAA-VG2]_n multiblock hybrid copolymers

[P*t*BA-VG2]_n was added to a mixture of 80/20 DCM/TFA and stirred overnight at room temperature. The solution was diluted with DMF and added to a regenerated cellulose dialysis membrane with a molecular weight cut-off (MWCO) of 1,000 (Spectra/Por, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and dialyzed against water at room temperature for two days in order to remove TFA, changing water 2-3 times per day. The solution was lyophilized to obtain a white solid in 40% yield. To investigate the influence of low molecular weight species on the association of the multiblock copolymers (see below), additional dialysis was carried out for two days using a membrane with a MWCO of 3,500 or 10,000. ¹H NMR (*d*-DMSO, δ) (ppm): 12.5-13.0 (s, COOH), 7.10-8.50 (m, 13H, NHCO and triazole C=CH), 5.31 (m, 1H, triazole-CH(NHCOCH₃)CO), 3.70-4.55 (m, 12H, NH-CH-CO), 3.56 (m, 6H, CH₂CH-triazole and proline NHCH₂CH₂CH₂), 2.20 (m, 20H, PAA CH₂CH and proline NHCH₂CH₂CH₂), 1.25-1.97 (m, 56H, PAA CH₂CH, valine CH(CH₃)₂, and proline NHCH₂CH₂CH₂), 0.85 (m, 24H, valine CH(CH₃)₂).

Association of [PAA-VG2]_n multiblock hybrid copolymers

Lyophilized [PAA-VG2]_n was dissolved in DMF at a concentration of 1 mg/mL and the solution was transferred to a MWCO 1,000 regenerated cellulose dialysis membrane, unless otherwise noted. The solution was dialyzed against water (or 1M urea, when appropriate) at ambient temperature for 3-4 days. Unless otherwise noted, experiments were conducted directly on the dialyzed solution (pH 3.8) or after adjusting the solution pH to 7.4 with 1M NaOH.

Characterization

The successful synthesis of multiblock alternating copolymers was confirmed by ¹H NMR, gel permeation chromatography (GPC) and FT-IR. ¹H NMR spectra were recorded on a Bruker AV600 or AV400 spectrometer in either D₂O (with water suppression by presaturation)^{38, 39} or DMSO-d₆. All spectra were calibrated to the solvent peak and analyzed with Bruker Topspin software. GPC was conducted using a system comprising either two Waters Styragel (HR1 and HR4) columns and a Waters 2695 autosampler pump (Waters Corporation, Milford, MA, USA) with THF as the mobile phase (for PBA-containing samples), or two Waters Ultrahydrogel (Linear and 250) columns and a Waters 515 HPLC pump with phosphate-buffered saline (PBS) as the mobile phase (for PAA-containing samples). All samples were analyzed at a flow rate of 1.0 mL/minute, with detection via a Waters 2996 photodiode array and Waters 2414 refractive index (RI) detector in series. Waters Empower software was used to construct calibration curves of narrow molecular weight polystyrene (PS) or PEG standards for data analysis. FT-IR spectra were collected using a Thermo Nicolet Nexus 670 (Thermo Scientific, Waltham, MA, USA) spectrometer with a DuraSamplIR II ATR accessory (Smiths Detection, Danbury, CT, USA). The polymer or peptide samples were added as solids onto the silicon ATR crystal and gently pressed down during data acquisition (128 scans at 4 cm⁻¹ resolution from 650-4000 cm⁻¹). The data were processed with OMNIC software, and a background of the clean silicon crystal in air was subtracted from all sample spectra.

The assembly properties of [PAA-VG2]_n were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). The samples were filtered (0.22 μm) into a 1.2-cm (outer diameter) glass test tube before measurement. Reported values are an average of at least 10 separate scattering intensity measurements at each temperature. Dynamic light scattering (DLS) analysis was conducted on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom) at a scattering angle of 173° using a low-volume quartz cuvette. The data for each measurement was an average of 12 to 20 runs (the number of runs was determined automatically based on the count rate of the sample), and at least three separate measurements were conducted on each sample. The standard deviation was calculated from an average of results from at least three individual samples. The data were analyzed by Malvern's DTS software (v. 6.01) using a cumulant analysis with a single exponential fit. The size distribution of the particles weighted by intensity and number as well as the particle size distribution were reported based on this analysis. Statistical analysis was conducted on the results of the intensity size distributions for the assembled particles using one-way ANOVA and a p value of < 0.1 was considered statistically significant. Bright field TEM imaging was conducted on a Tecnai 12 (FEI Company, Hillsboro, OR, USA) microscope operating at an accelerating voltage of 120 kV. TEM samples were prepared by depositing a drop particle solution (~5 μL) on a 300 mesh ultrathin carbon-coated copper TEM grid (Electron Microscopy Sciences, Hatfield, PA), blotting with filter paper, and allowing the solvent to evaporate under ambient conditions. Samples were stained by adding a drop (~10 μL) of 2% uranyl acetate aqueous solution (Ted Pella, Inc., Redding, CA), blotting again with filter paper, and allowing the sample to dry before observation.

Pyrene encapsulation

The critical aggregation concentration of [PAA-VG2]_n was measured using pyrene as a fluorescent probe to determine the potential of these particles to encapsulate hydrophobic molecules.⁷ A 2.5×10^{-5} M pyrene stock solution was prepared in acetone, 24 μ L of the stock solution was added to empty vials (three repeats for each concentration of polymer solution), and acetone was evaporated under vacuum overnight. The [PAA-VG2]_n stock solutions prepared above were serially diluted with water to a concentration of 4.1×10^{-7} mg/mL. At least 10 different concentrations for both [PAA₂₂-VG2]_n and [PAA₆₃-VG2]_n were prepared, and 1 mL of polymer solution was added to each vial containing pyrene to obtain polymer solutions with a pyrene concentration of 6×10^{-7} M. After stirring in the dark for 4-5 hours, fluorescence measurements were conducted on each sample (excitation = 333 nm, emission = 350-500 nm) using a SPEX Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ, USA) at a 90° angle in a low-volume 1 cm quartz cuvette. The average ratio of the intensity of the vibronic bands at 372 nm (I₁) over 383 nm (I₃) was plotted vs. log of the concentration for each sample. The CAC was determined as the polymer concentration at the intersection point of two regression lines drawn from the linear portions of the graph.

Results and Discussion

Synthesis and characterization of [PAA-VG2]_n

Multiblock EMHPs were previously synthesized with alternating blocks of PEG and a peptide (AKA₃KA)₂ (AK2) derived from the crosslinking regions of natural tropoelastin.²¹ Here, we have utilized (VPGVG)₂ (VG2) peptides derived from the corresponding hydrophobic domains of tropoelastin, in combination with PAA, to form pH-sensitive, associating EMHPs. Few studies have investigated the assembly of hybrid polymer-peptide multiblock copolymers, despite their potential for the development of interesting and novel nanostructures for biological applications. Recently, multiblock poly(acrylic acid)-polystyrene ([PAA-PS]_n) copolymers were synthesized and assembled into well-defined spherical nanoparticles with an average particle diameter of 14 nm in aqueous solution.⁷ In this work, PAA-VG2 hybrid multiblock copolymers were synthesized to create a more biologically relevant system and to determine if the hybrid multiblock copolymers were also capable of self-assembly. To the best of our knowledge, the only reports of conjugates of PAA with ELPs containing VPGVG repeating units are diblock copolymers used in the studies of polyelectrolyte effects on cell attachment.^{36, 37} In other similar investigations, hybrid PAA-poly(L-valine) (PAA-PLV) diblock copolymers assembled into particles via the β -sheet formation of the PLV segments.³⁴ Of the very few hybrid polymer-peptide multiblock copolymers that have been reported, most employ PEG^{21, 40, 41} or polyisoprene⁴² as the synthetic polymer block. The association/assembly properties of PAA-VPGVG or other PAA-peptide multiblock polymers have not been investigated previously.

The elastin-mimetic peptide, X(VPGVG)₂X (XVG2X), was synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) methods. The amino acid X denotes propargylglycine, which was used to impart alkyne functionality to both ends of the peptide for coupling with an azide-terminated synthetic polymer via copper-catalyzed azide-alkyne cycloaddition (CuAAC). The synthesis was straightforward, as analytical HPLC and ESI-MS (Figures S1 and S2) confirmed that the peptide was isolated in high purity without the need for preparative-scale fractionation. ¹H NMR (Figure S3) also confirmed the purity of the peptide, as well as the presence of the alkyne $\text{CH}_2\text{C}\equiv\text{CH}$ and $\text{CH}_2\text{C}\equiv\text{CH}$ peaks at 2.73 and 2.45 ppm, respectively.

Poly(acrylic acid) was chosen as the synthetic polymer component of the EMHPs to impart pH-responsiveness as well as the carboxylic acid functionality for functionalization purposes. The PBA precursor was synthesized via ATRP using CuBr as the catalyst, PMDETA as a copper-stabilizing ligand, and dimethyl 2,6-dibromoheptanedioate as the difunctional initiator to generate bromide groups on either end of the resulting polymer, as shown in Scheme S1. By varying the ratio of monomer to initiator, PBA with $M_n = 2,800$ g/mol (PBA₂₂) and $M_n = 8,000$ g/mol (PBA₆₃) were synthesized for comparison of the effect of PAA length on the particle formation and the pH-responsiveness. A representative ¹H NMR spectrum of Br-PBA₂₂-Br is shown in Figure S4A. The -CHC(O)O(CH₃)₃ protons from the last PBA repeats that are adjacent to the bromide ends of the polymer were observed as a small peak at approximately 4.1 ppm. The molecular weights of both PBA₂₂ and PBA₆₃ were calculated by comparing the ratio of the integrated area of this end group peak with that of the repeating units -CHC(O)O(CH₃)₃ peak at 2.2 ppm. The molecular weights were calculated as $M_n = 2,800$ g/mol (PBA₂₂) and $M_n = 8,000$ g/mol (PBA₆₃) from this NMR end group analysis. In order to generate azide moieties for CuAAC coupling with alkyne-functional VG2 peptides, substitution of the bromide end groups was achieved via a nucleophilic substitution reaction with sodium azide. As shown in Figure S4B, the terminal -CHC(O)O(CH₃)₃ peak was shifted upfield to 3.74 ppm after the reaction, indicating nearly quantitative conversion of bromide to azide groups. The conversion was calculated as 97% based on the integration of the polymer backbone CHCO peak over the area of the azide end group CHCO peak. Similar results were observed from ¹H NMR analysis of the synthesis and azide modification of PBA₆₃ (data not shown). The molecular weights of both PBA precursors were also confirmed via GPC, and the results are summarized in Figure 1 and Table 1. As shown in Table 1, the M_n values of the PBA precursors determined from GPC are in good agreement with those calculated from NMR.

Alkyne-functional VG2 peptide and azide-terminated PBA were coupled to form PBA-VG2 alternating multiblock copolymers via CuAAC in the presence of copper(I) acetate, as shown in Scheme 1. The GPC traces in Figures 1A and 1B show a molecular weight increase from the PBA building block to the respective multiblock copolymers, corresponding to an average of 3 to 6 blocks per chain for both [PBA₂₂-VG2]_n ($M_n = 7,830$ g/mol, $M_w = 15,030$ g/mol) and [PBA₆₃-VG2]_n ($M_n = 15,600$ g/mol, $M_w = 28,650$ g/mol). GPC was conducted on the crude polymer after precipitation, and a portion of low molecular weight PBA remained present in both samples, possibly as a result of the incomplete azide functionalization of PBA or the presence of cyclized reaction products.⁴³ The presence of unreacted or cyclized polymers, however, is not a concern because, as we will show below, PAA alone is not capable of assembly, and if cyclized products are present, they do not seem to affect the ability of the multiblock copolymers to assemble into stable, uniform particles. In the ¹H NMR spectrum of [PBA₂₂-VG2]_n (Figure S5A), peaks from both the PBA and peptide segments are present, and the integrations of the peaks correspond to molar ratios of approximately 1 to 1 polymer to peptide units. The triazole CH peak, which should appear at about 7.9 ppm, cannot be distinguished because the peptide NH peaks (7.8-8.5 ppm) in DMSO overlap with the triazole peak.

After the successful synthesis of [PBA-VG2]_n, the *tert*-butyl groups were hydrolyzed with TFA to generate [PAA-VG2]_n. The ¹H NMR spectra indicate that for multiblock copolymers with different PAA block lengths (Figure S5B and S5C for multiblock copolymers containing PAA₂₂ and PAA₆₃, respectively), the *t*-butyl units at 1.44 ppm completely disappeared and a broad COOH peak appeared at 12.5-13.0 ppm. The small peak remaining at approximately 1.28-1.38 ppm is likely from the methylene groups of the initiator and not residual *t*-butyl protons, since the area of this peak corresponded to the expected ratio of approximately six initiator CH₂ protons per PAA chain. To ensure that the multiblock copolymer backbone was not degraded by the hydrolysis procedure, [PAA₂₂-

VG2]_n along with the PAA₂₂ starting material were analyzed by aqueous GPC, as shown in Figure 2. The [PAA₂₂-VG2]_n peak molecular weight was calculated by comparison with PEG standards as $M_n = 7,580$ g/mol and $M_w = 11,200$ g/mol. These molecular weight values are similar to those calculated for the [P β BA₂₂-VG2]_n mentioned above (7,830 g/mol), indicating that there was no significant degradation of the peptide domains of the multiblock copolymers as a result of the deprotection procedure.

The successful synthesis of [PAA-VG2]_n multiblock copolymers was further confirmed by FTIR, as shown in Figure 3 (representative spectra for polymers containing P β BA₂₂ or PAA₂₂). Upon modification of Br-P β BA₂₂-Br to N₃-P β BA₂₂-N₃, the antisymmetric N=N=N stretch at $2,100\text{ cm}^{-1}$ was clearly observed. The spectrum for the VG2 peptide shows the characteristic Amide I band at $1,630\text{ cm}^{-1}$ and Amide II band at $1,552\text{ cm}^{-1}$. After coupling of N₃-P β BA-N₃ and VG2 to form [P β BA₂₂-VG2]_n multiblock copolymers, the presence of both the Amide I and Amide II bands indicated the incorporation of peptide into the multiblock, and the disappearance of most of the azide band due to triazole formation was apparent. The broad band around $3,400\text{ cm}^{-1}$ in the [P β BA₂₂-VG2]_n spectrum may originate from the residual water due to incomplete drying. Following the hydrolysis of the *t*-butyl groups to carboxylic acid groups, the C=O stretch was broadened and shifted to lower wavenumbers ($1,710\text{ cm}^{-1}$ as compared to $1,730\text{ cm}^{-1}$ in P β BA) and a very broad band from carboxylic acid OH stretching centered around $3,400\text{ cm}^{-1}$ appeared, indicating the successful hydrolysis to [PAA₂₂-VG2]_n. The presence of the azide band in the [P β BA₂₂-VG2]_n and [PAA₂₂-VG2]_n multiblock copolymers are expected as azide groups are retained as some fraction of the end groups of the multiblock polymers. The presence of unreacted PAA, as suggested by the GPC results in Figures 1 and 2, may also contribute to the presence of residual azide groups.

Formation of [PAA-VG2]_n nanoparticles

A solvent exchange process,^{4, 7, 33} involving dissolution in DMF followed by dialysis against water at an ambient temperature, was employed to investigate the capability of the multiblock polymers to form nanoparticles. When physical mixtures of PAA₂₂ (or PAA₆₃) and VG2 were dialyzed from DMF into water at similar weight concentrations as in the multiblock copolymers, large, polydisperse particles were detected. For the PAA₂₂/VG2 physical mixture, the particles were approximately 550 nm in size, and the PAA₆₃/VG2 mixture showed two distributions of 600-900 nm and 165 nm. Control experiments were also conducted by dialyzing the PAA or peptide alone in DMF against water by the same procedure, and neither PAA nor the VG2 peptide alone was capable of forming nanoparticles (data not shown). For PAA₆₃ alone, species of 1-1.5 nm were detected in the number distribution by DLS, and large aggregates of up to 1 μm were observed only in very small amounts. For the VG2 peptide alone, mostly isolated chains (species of less than 1 nm) were observed, along with very small amount of ill-defined aggregates of up to 1 μm in size. Since the PAA and peptide were not able to assemble alone, and the PAA/VG2 mixtures formed nanoparticles, physical interactions between the PAA and peptide domains may provide a driving force for assembly at temperatures below the inverse transition temperature of the VG2 peptide.

The PAA-VG2 multiblock copolymers were thus subjected to the same solvent exchange process described above for the PAA/VG2 physical mixtures; the resulting solutions were analyzed via dynamic light scattering (DLS) methods to determine particle sizes (Figure 4). Our results indicate that this method yielded particles with average diameters (size distribution based on intensity) of approximately 250-270 nm for both [PAA₂₂-VG2]_n and [PAA₆₃-VG2]_n in water at pH 3.8 (dashed lines in Figure 4A and 4B, respectively), with slightly larger particles formed at pH 7.4 (see below). The number-average size distributions showed similar results with no indication of the presence of smaller particles (dashed lines

in Figures S6A and S6B). The size distribution for the multiblock copolymers was unimodal in all cases, and size distribution of the $[PAA_{22}\text{-VG2}]_n$ particles was 0.34 at 25 °C. The $[PAA_{63}\text{-VG2}]_n$ particles had a lower average particle size distribution of 0.17, which may be due to the formation of more stable particles by the longer PAA chains (see below). These values are similar to that of 0.35 measured for PS-PAA multiblock particles with 9 PS-PAA repeat units and polydispersity index of 0.16 for particles assembled from PS-PAA multiblock copolymers with 4 repeat units,⁷ which is notable considering that the PAA-VG2 multiblock copolymers do not possess definitive hydrophobic segments (such as PS) to drive the assembly.

Our observations further suggest that each particle comprises many multiblock copolymer chains, given the significant increase in the particle dimensions over the expected dimensions of the multiblock polymers (5 nm, see radius of gyration calculations for PAA and $[PAA_{22}\text{-VG2}]_n$ multiblock copolymers in the Supporting Information). It should be noted that particles were not formed in DMF solutions of the $[PAA\text{-VG2}]_n$ multiblock copolymers before the solvent exchange; diameters of 5-6 nm were measured by DLS (data not shown), indicating that the multiblock copolymers are molecularly dispersed in DMF. The multiblock $[PAA\text{-VG2}]_n$ copolymers formed smaller and more uniform particles (ca. 250 nm) than the PAA/VG2 physical mixtures (ca. 550 nm) mentioned above, suggesting that the high local concentrations of the PAA and peptide segments, combined with the close proximity of these segments in the multiblock architecture, leads to the formation of particles of a smaller diameter. The $[PAA\text{-VG2}]_n$ particles also demonstrated long-term stability, as their diameters and particle size distributions did not change significantly when measured after up to five months of storage at 4 °C in aqueous solution.

As discussed above, the GPC analysis of the $[PAA\text{-VG2}]_n$ multiblock polymers suggests that the product may contain a small amount of residual homopolymers and/or cyclic species that could potentially affect the assembly properties of the multiblock copolymers. A control experiment was thus performed in which the $[P\beta A_{63}\text{-VG2}]_n$ precursor was subjected to additional dialysis (in DMF) using membranes with a MWCO larger than 1,000 (3,500 and 10,000), intended to provide conditions in which the removal of any residual small polymers would be facilitated. The molar mass of the XVG2X peptide and the $P\beta A$ homopolymer (1,090 and 8,000 g/mol, respectively) are smaller than the pore size of the membranes (3,000 and 10,000) employed for purification purposes. In addition, cyclic species generated during step growth polymerization are of a smaller hydrodynamic volume as compared to the corresponding homopolymers.⁴⁴⁻⁴⁶ The purified samples were subjected to the same conditions described above for particle formation. No significant changes in the particle size, determined by DLS, was observed after the additional purification procedure. Therefore, the potential presence of any unreacted homopolymers and the cyclic byproduct, in samples dialyzed against the smaller MWCO membranes, is not suggested to influence the overall assembly properties of the multiblock copolymers.

The particle stability and size change upon an increase in solution pH were assessed by DLS, and the representative data are presented as the solid lines in Figure 4 and Figure S6. The pH of the above $[PAA\text{-VG2}]_n$ particle solutions was measured as 3.8 after dialysis in water, well below the pK_a of PAA (~4.5), thus most of the carboxylic acid groups should be protonated and uncharged. When the pH of solutions of pre-formed $[PAA\text{-VG2}]_n$ particles was raised to 7.4 (by addition of NaOH), the particles did not dissociate, but instead swelled to a larger size (285-300 nm for multiblock copolymers containing PAA_{22} , and 330-350 nm for multiblock copolymers containing PAA_{63}) as shown in Figure 4. The repulsion between the negatively charged carboxylates of PAA caused greater swelling in the PAA_{63} multiblock copolymer particles due to their longer length and greater molar fraction (calculated as $\sim 2.10 \times 10^{-4}$), in comparison with the PAA_{22} multiblock copolymers (molar

fraction $\sim 1.82 \times 10^{-4}$). The average diameters of these nanoparticles did not change over a month in solution during storage, indicating the colloidal stability of the hybrid particles.

The structure of the $[\text{PAA-VG2}]_n$ nanoparticles was investigated by TEM (Figure 5); images were captured on films cast from solution at room temperature and stained with uranyl acetate. The observed particles had diameters of 265 ± 59 nm and 335 ± 104 nm for $[\text{PAA}_{22}\text{-VG2}]_n$ at pH 3.8 and 7.4, respectively, and 368 ± 101 nm and 340 ± 67 nm for $[\text{PAA}_{63}\text{-VG2}]_n$ at pH 3.8 and 7.4, respectively. These values were similar to those measured by DLS at 25 °C, (250-270 nm at pH 3.8 for both multiblock copolymers, and 285-300 nm and 330-350 nm for $[\text{PAA}_{22}\text{-VG2}]_n$ and $[\text{PAA}_{63}\text{-VG2}]_n$, respectively, at pH 7.4). Intriguingly, many of the nanoparticles contained irregularly-shaped regions that were stained darker than the exterior areas of the particles (Figure 5C, 5D). Such structures could arise from the compartmentalization of the PAA chains in the particle interior during the dialysis process or the local collapsing of vesicle-like, membrane structures induced during the drying process. In-depth mechanistic studies are currently underway to understand the origin of the internal structures.

It is notable that at pH 3.8, the particles are irregularly shaped and had a diffuse and rugged outer edge, while at pH 7.4, the particles were more uniformly spherical with smoother edges. This effect was more evident in the $[\text{PAA}_{63}\text{-VG2}]_n$ particles (Figures 5C and 5D), possibly due to the negative charges of the PAA at higher pH values, which would improve the overall hydrophilicity of PAA and increase the charge repulsion within and between the PAA segments. Minimization of charge repulsion would likely drive more of the PAA chains to the outside of the particles, causing the particles to have a more defined spherical shape. In similar work involving the assembly of PAA-polyvaline diblock copolymers, the increase in surface charge and hydrophilicity of PAA at pH 7.4 was also predicted to result in the formation of more stable and spherical particles.³⁵ The DLS data in Figure 6, of particles (formed by dialysis at room temperature, as shown in Figures 4 and 5) in aqueous solution at pH 3.8 and 7.4 and at temperatures ranging from 25 to 80 °C, show that there is no statistically significant difference in the diameters of the particles under these conditions ($p > 0.05$). The size increase with pH as observed by DLS was not apparent in the TEM images, likely owing to the dehydrated condition for image acquisition. Thus, the assembled particles are pH-sensitive, but not temperature-responsive.

The NMR spectra of the peptide alone in DMF, $[\text{PAA}_{22}\text{-VG2}]_n$ in DMF, and $[\text{PAA}_{22}\text{-VG2}]_n$ in D_2O at pH 3.8 and pH 7.4 after particle formation by solvent exchange are shown in Figure S7. Upon comparison of the spectra, the peptide peaks are still present after assembly and do not appear to decrease in intensity relative to the PAA peaks, as quantified by the ratios of the PAA backbone CH_2CHCO peak at 2.4 ppm to the peptide backbone CH peaks at 3.9-4.6 ppm, which were calculated to be approximately 2.5 in both DMF and D_2O . Similar results were observed for $[\text{PAA}_{63}\text{-VG2}]_n$, for which the ratios of the same peaks were 5.7 in DMF and 5.9 in D_2O (after solvent exchange to form particles). Given the lack of any soluble multiblock polymers in the solutions of the nanoparticles, it is possible that the peptide may be aggregated in smaller domains throughout the particle rather than in a single inner core.^{47, 48} Alternatively, the peptide segments may aggregate in the interior of the nanoparticles, but are readily solvated by water. The solvated peptide chains have high mobility, and can be detected by ^1H NMR, similar to interpolyelectrolyte complex micelles.^{49, 50}

The ability of $[\text{PAA-VG2}]_n$ multiblock copolymers to sequester small, hydrophobic molecules was investigated using pyrene, an aromatic hydrocarbon that shows considerable fine structure in the fluorescence spectrum of its monomer.⁵¹ Pyrene is often used as a fluorescent probe in micellar systems because the ratio of the vibronic bands in the

fluorescence spectrum of the monomer is sensitive to the polarity of the solvent environment.^{7, 52, 53} The third vibronic band (I_3 , 383 nm) has been established as varying most in intensity relative to the first vibronic band (I_1 , 372 nm).⁵¹ Consequently, the ratio I_1/I_3 is plotted for pyrene in the presence of varying concentrations of $[PAA_{22}\text{-VG2}]_n$ and $[PAA_{63}\text{-VG2}]_n$, as shown in Figure 7. At polymer concentrations below the CAC, there are no aggregates present and I_1/I_3 is high, indicating that pyrene is in a polar environment. As the concentration increases to a value at which particle formation is possible, I_1/I_3 decreases as pyrene is partitioned into the hydrophobic regions of the particles. The CAC was calculated as the concentration at the intersection point of the two linear regions of the plot, and did not differ significantly between $[PAA_{22}\text{-VG2}]_n$ and $[PAA_{63}\text{-VG2}]_n$, which had CAC values of 1.09×10^{-2} mg/mL and 1.05×10^{-2} mg/mL, respectively. These values are much lower than those obtained for most other peptide-containing amphiphilic copolymers, further indicating the higher stability of the multiblock copolymers here. For example, PEG-valine triblock copolymers with short (five amino acid) valine segments on either end of PEG had CAC values of 0.032-0.299 mg/mL depending on the nature of the polyvaline end group.⁵⁴ The ability of the particles to encapsulate pyrene demonstrates their potential usefulness for future encapsulation of hydrophobic drugs, and the swelling behavior reported above should allow drugs to be released readily.

Given the fact that the multiblock copolymers are composed of PAA and peptide blocks, concerted H-bonding interactions may contribute to the particle assembly. H-bonds between the peptide segments have been exploited for the assembly of hybrid polymers into particles or fibrils.^{41, 55, 56} In PAA-containing hybrid polymers such as PAA-polyvaline diblock copolymers, micellar assembly was proposed to take place via H-bonds between the peptide chains, as well as those between the PAA and peptide units.³⁴ In addition, H-bonding of PAA with poly(ethylene oxide) (PEO) has been employed for the assembly of synthetic polymer systems that do not contain any peptides.⁵⁷⁻⁵⁹ In our studies, urea, a common denaturation reagent used to disrupt H-bonding in proteins, was used to probe the contribution of H-bonding to particle formation. Specifically, the effect of urea addition on particle assembly was studied by adding urea to solutions of the unassembled $[PAA\text{-VG2}]_n$ or PAA/VG2 physical mixtures in DMF followed by dialysis against 1M urea in water, at concentrations of urea greater than the anticipated concentration of associating units in the polymers. Particles formed from the $PAA_{22}/VG2$ physical mixture in 1M urea were larger in size compared to the particles formed in pure water, according to the DLS size distribution by intensity (Figure 8A). However, the intensity distribution is strongly influenced by the presence of large particles, and in the number-average distribution, the particle diameter of the physical mixture of $PAA_{22}/VG2$ (Figure 8C) decreased, indicating the disruption of a significant number of the particles. In the DLS intensity-average distribution of the $PAA_{63}/VG2$ physical mixture, the main peak at about 650 nm was maintained, and a second, smaller peak at ~140 nm appeared (Figure S8A). The number-average distribution (Figure S8C) showed that the $PAA_{63}/VG2$ particle diameter also decreased, as was the case for the $PAA_{22}/VG2$ physical mixtures.

In contrast, the diameter of the $[PAA_{22}\text{-VG2}]_n$ multiblock nanoparticles was slightly increased in the presence of urea in the intensity-average as well as the number-average DLS size distributions (Figures 8B and 8D, respectively), in contrast to the physical mixtures, in which a reduction in size was observed in the number-average size distribution (Figure 8C). This illustrates the increased stability of the multiblock nanoparticles against disintegration. The diameter of $[PAA_{63}\text{-VG2}]_n$ multiblock particles also increased in the presence of urea in both intensity- and number-average size distributions (Figure S8B and S8D, respectively). At a sufficiently high concentration, urea may cause intermolecular H-bonding between polymer chains, resulting in larger particles instead of breaking existing H-bonds.⁶⁰ For $[PAA\text{-VG2}]_n$, this effect became more obvious at higher concentrations of urea

(2M or 5M), which caused a larger increase in particle size, while 10M urea caused the solution to become very viscous. This is most likely because a crosslinked network was being formed, as observed for PNIPAM at high urea concentrations.⁶⁰ Therefore, the urea concentration was not increased above 1M for the dissociation experiments.

The urea experiments suggest that the particles formed from the multiblock copolymers are more stable against disruption in comparison to particles formed by a simple physical mixture of PAA and VG2 peptide. Similar diblock PAA-polyvaline copolymers (PAA-PLV) were proposed to assemble through β -sheet formation of the polyvaline blocks as well as PAA-PLV and PAA-PAA H-bonding,³⁴ but the copolymers were able to form only small, random aggregates with polyvaline of 60 repeat units.³⁵ The [PAA-VG2]_n multiblock nanoparticles presented here are able to form discrete nanoparticles even though the peptides are of much shorter length. In the PAA-PLV particles, it was suggested that the structure was most likely composed of smaller collapsed micelles connected by intermolecular H-bonds,³⁵ and the [PAA-VG2]_n multiblock copolymers described here may also be stabilized as a result of a combination of H-bonds and hydrophobic interactions between PAA and the VG2 peptide. The control experiments showed that physical mixtures of PAA and VG2 formed particles, which did not occur for PAA or VG2 alone, suggesting that the nanoparticle formation is at least partially driven by H-bonding of PAA with the VG2 peptide (at pH 3.8). H-bonding can also occur between the PAA side chains or between amide linkages in the peptide backbone. Hydrophobic interactions can also occur between the peptide side chains or the PAA backbone. The potential contribution of the hydrophobic interactions is indicated by the lack of full dissociation of the particles with the addition of urea, as well as by the ability of the particles to encapsulate pyrene as a hydrophobic probe. The multiblock nanoparticles are smaller and suggested to be more stable against disruption (than the particles formed by physical mixtures) because of their unique molecular architecture, which would facilitate the interaction of the PAA and peptide units by bringing them into close proximity, thus enforcing intramolecular association.

Conclusions

Hybrid multiblock copolymers of PAA and the (VPGVG)₂ peptide were synthesized via CuAAC. The PAA-VG2 multiblock copolymers formed nanoparticles with monomodal distribution in water at room temperature as indicated by DLS and TEM. The particles swelled while maintaining their stability at increased pH values. Upon addition of pyrene as a hydrophobic probe, the PAA-VG2 multiblock particles in solution exhibited a CAC similar to or lower than that of most other reported values for hybrid polymer-peptide particles. Physical mixtures of PAA and VG2 form larger and less uniform particles, suggesting that nanoparticle formation may be driven by H-bonding between the PAA and VG2 segments as well as by hydrophobic interactions. This possibility was corroborated by the observed disruption of the particles, formed from the physical mixtures, by the addition of urea; the multiblock particles, however, remained stable in the presence of urea likely owing to their unique molecular architecture. These results thus suggest that the multiblock architecture is critical for driving the formation of uniform, stable nanoparticles owing to the localization of the PAA and peptide segments, and that although H-bonding contributes to particle formation, the particles could not be fully dissociated due to the additional contribution of hydrophobic interactions. We are currently investigating the utility of the multiblock copolymer nanoparticles in drug delivery applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Yingchao Chen, Darrin Pochan, Chaoying Ni, and Frank Kriss for TEM instrument assistance and data interpretation, and Xiaoqian Ma and Bruce Chase for help with FTIR. NMR spectra were obtained with instrumentation supported by NSF CRIF: MU, CHE 0840401. Funding was provided by the NSF/DMR Biomaterials Program (Jia, Career: DMR0643226; Kiick: DMR0907478), the NSF Integrative Graduate Education & Research Traineeship (IGERT), the Center for Neutron Science at the University of Delaware (US Department of Commerce, #70NANB7H6178), and the National Institutes of Health (P20RR017716).

References

1. Kwon GS, Okano T. *Pharm. Res.* 1999; 16:597–600. [PubMed: 10349998]
2. Gaucher, G. v.; Dufresne, M.-H. l. n.; Sant, VP.; Kang, N.; Maysinger, D.; Leroux, J-C. *J. Control. Release.* 2005; 109:169–188. [PubMed: 16289422]
3. Blanz A, Armes SP, Ryan AJ. *Macromol. Rapid Commun.* 2009; 30:267–277. [PubMed: 21706604]
4. Riess G. *Prog. Polym. Sci.* 2003; 28:1107–1170.
5. Alexandridis P, Yang L. *Macromolecules.* 2000; 33:3382–3391.
6. Choucair A, Lavigneur C, Eisenberg A. *Langmuir.* 2004; 20:3894–3900. [PubMed: 15969376]
7. Greene AC, Zhu J, Pochan DJ, Jia X, Kiick KL. *Macromolecules.* 2011; 44:1942–1951. [PubMed: 21552373]
8. Hadjiantoniou NA, Triftaridou AI, Kafouris D, Gradzielski M, Patrickios CS. *Macromolecules.* 2009; 42:5492–5498.
9. Popescu MT, Athanasoulis I, Tsitsilianis C, Hadjiantoniou NA, Patrickios CS. *Soft Matter.* 2010; 6:5417–5424.
10. Halperin A. *Macromolecules.* 1991; 24:1418–1419.
11. Gindy ME, Prud'homme RK, Panagiotopoulos AZ. *J. Chem. Phys.* 2008;128.
12. Hadjiantoniou NA, Krasia-Christoforou T, Loizou E, Porcar L, Patrickios CS. *Macromolecules.* 2010; 43:2713–2720.
13. Popescu M-T, Tsitsilianis C, Papadakis CM, Adelsberger J, Balog S, Busch P, Hadjiantoniou NA, Patrickios CS. *Macromolecules.* 2012; 45:3523–3530.
14. Fuks G, Talom RM, Gauffre F. *Chem. Soc. Rev.* 2011; 40:2475–2493. [PubMed: 21229168]
15. Krishna OD, Kiick KL. *Biopolymers.* 2010; 94:32–48. [PubMed: 20091878]
16. Rabotyagova OS, Cebe P, Kaplan DL. *Biomacromolecules.* 2011; 12:269–289. [PubMed: 21235251]
17. Klok H-A. *J. Polym. Sci. Part A: Polym. Chem.* 2005; 43:1–17.
18. Borner HG, Schlaad H. *Soft Matter.* 2007; 3:394–408.
19. Pan H, Yang J, Kopeckova P, Kopecek J. *Biomacromolecules.* 2011; 12:247–252. [PubMed: 21158387]
20. Yang J, Luo K, Pan H, Kopeckova P, Kopecek J. *React. Funct. Polym.* 2011; 71:294–302. [PubMed: 21499527]
21. Grieshaber SE, Farran AJE, Lin-Gibson S, Kiick KL, Jia X. *Macromolecules.* 2009; 42:2532–2541. [PubMed: 19763157]
22. Grieshaber SE, Farran AJE, Bai S, Kiick KL, Jia X. *Biomacromolecules.* 2012 online, DOI: 10.1021/bm3002705.
23. Vrhovski B, Weiss AS. *Eur. J. Biochem.* 1998; 258:1–18. [PubMed: 9851686]
24. Li B, Alonso DOV, Bennion BJ, Daggett V. *J. Am. Chem. Soc.* 2001; 123:11991–11998. [PubMed: 11724607]
25. Urry DW, Hugel T, Seitz M, Gaub HE, Sheiba L, Dea J, Xu J, Parker T. *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.* 2002; 357:169–184. [PubMed: 11911774]
26. Reiersen H, Clarke AR, Rees AR. *J. Mol. Biol.* 1998; 283:255–264. [PubMed: 9761688]
27. Gao L, Shi L, Zhang W, An Y, Jiang X. *Macromol. Chem. Phys.* 2006; 207:521–527.
28. Zhang L, Eisenberg A. *J. Am. Chem. Soc.* 1996; 118:3168–3181.

29. Liu F, Eisenberg A. *J. Am. Chem. Soc.* 2003; 125:15059–15064. [PubMed: 14653740]
30. He W-D, Sun X-L, Wan W-M, Pan C-Y. *Macromolecules.* 2011; 44:3358–3365.
31. Elliott JE, Macdonald M, Nie J, Bowman CN. *Polymer.* 2004; 45:1503–1510.
32. Philippova OE, Hourdet D, Audebert R, Khokhlov AR. *Macromolecules.* 1997; 30:8278–8285.
33. Xiao LX, Liu C, Zhu JH, Pochan DJ, Jia XQ. *Soft Matter.* 2010; 6:5293–5297. [PubMed: 21278815]
34. Sinaga A, Ravi P, Hatton TA, Tam KC. *J. Polym. Sci. Part A: Polym. Chem.* 2007; 45:2646–2656.
35. Sinaga A, Hatton TA, Tam KC. *Macromolecules.* 2007; 40:9064–9073.
36. Janorkar AV, Rajagopalan P, Yarmush ML, Megeed Z. *Biomaterials.* 2008; 29:625–632. [PubMed: 18006054]
37. Swierczewska M, Hajicharalambous CS, Janorkar AV, Megeed Z, Yarmush ML, Rajagopalan P. *Acta Biomater.* 2008; 4:827–837. [PubMed: 18178532]
38. Cavanagh, JF.; W. J.; Palmer, AG.; Rance, M.; Skelton, NJ. *Protein NMR spectroscopy: principles and practice.* Elsevier Inc.; Burlington, MA: 2007.
39. Hoult DI. *J. Magn. Reson.* 1976; 21:337–347.
40. Sahin E, Kiick KL. *Biomacromolecules.* 2009; 10:2740–2749. [PubMed: 19743840]
41. Rathore O, Sogah DY. *J. Am. Chem. Soc.* 2001; 123:5231–5239. [PubMed: 11457385]
42. Zhou CC, Leng BX, Yao JR, Qian J, Chen X, Zhou P, Knight DP, Shao ZZ. *Biomacromolecules.* 2006; 7:2415–2419. [PubMed: 16903690]
43. Golas PL, Tsarevsky NV, Sumerlin BS, Matyjaszewski K. *Macromolecules.* 2006; 39:6451–6457.
44. Tsarevsky NV, Sumerlin BS, Matyjaszewski K. *Macromolecules.* 2005; 38:3558–3561.
45. Eugene DM, Grayson SM. *Macromolecules.* 2008; 41:5082–5084.
46. Nicolay R, Matyjaszewski K. *Macromolecules.* 2011; 44:240–247.
47. Grieshaber SE, Nie T, Yan C, Zhong S, Teller SS, Clifton RJ, Pochan DJ, Kiick KL, Jia X. *Macromol. Chem. Phys.* 2011; 212:229–239. [PubMed: 21359141]
48. Wanka G, Hoffmann H, Ulbricht W. *Macromolecules.* 1994; 27:4145–4159.
49. Maggi F, Ciccarelli S, Diociaiuti M, Casciardi S, Masci G. *Biomacromolecules.* 2011; 12:3499–3507. [PubMed: 21866922]
50. De Santis S, Diana Ladogana R, Diociaiuti M, Masci G. *Macromolecules.* 2010; 43:1992–2001.
51. Kalyanasundaram K, Thomas JK. *J. Am. Chem. Soc.* 1977; 99:2039–2044.
52. Astafieva I, Zhong XF, Eisenberg A. *Macromolecules.* 1993; 26:7339–7352.
53. Colombani O, Ruppel M, Schubert F, Zettl H, Pergushov DV, Muller AHE. *Macromolecules.* 2007; 40:4338–4350.
54. Goh SL, Platt AP, Rutledge KE, Lee I. *J. Polym. Sci. Part A: Polym. Chem.* 2008; 46:5381–5389.
55. Smeenk JM, Schon P, Otten MJB, Speller S, Stunnenberg HG, van Hest JCM. *Macromolecules.* 2006; 39:2989–2997.
56. Top A, Roberts CJ, Kiick KL. *Biomacromolecules.* 2011; 12:2184–2192. [PubMed: 21553871]
57. Pradip, Maltesh C, Somasundaran P, Kulkarni RA, Gundiah S. *Langmuir.* 1991; 7:2108–2111.
58. Hao JK, Yuan GC, He WD, Cheng H, Han CC, Wu C. *Macromolecules.* 2010; 43:2002–2008.
59. Gao W-P, Bai Y, Chen E-Q, Li Z-C, Han B-Y, Yang W-T, Zhou Q-F. *Macromolecules.* 2006; 39:4894–4898.
60. Sagle LB, Zhang Y, Litosh VA, Chen X, Cho Y, Cremer PS. *J. Am. Chem. Soc.* 2009; 131:9304–9310. [PubMed: 19527028]

Scheme 1.
Synthesis of $[\text{PAA-VG2}]_n$ multiblock polymers.

Figure 1.

Characterization of *PtBA* and multiblock polymers by GPC. A: GPC traces of *PtBA*₂₂ dibromide, diazide, and [*PtBA*₂₂-VG2]_n multiblock polymer; B: GPC traces of *PtBA*₆₃ dibromide, diazide, and [*PtBA*₆₃-VG2]_n multiblock polymer. All samples were analyzed in THF with a refractive index detector.

Figure 2. Aqueous GPC traces (PBS, pH 7.4) of PAA_{22} and $[\text{PAA}_{22}\text{-VG2}]_n$. Data were acquired with the refractive index detector. Molecular weights calculated from comparison with the PEG standards.

Figure 3.
ATR-FTIR spectra of PtBA₂₂ and VG2 peptide starting materials and PBA₂₂-VG2 and PAA₂₂-VG2 multiblock polymers.

Figure 4. DLS size distribution by intensity for $[PAA_{22}-VG2]_n$ (A) and $[PAA_{63}-VG2]_n$ (B) at pH 3.8 (dashed line) and pH 7.4 (solid line).

Figure 5. TEM images of assembled [PAA-VG2]_n nanoparticles (dried from solutions of 1 mg/mL in water and stained with uranyl acetate). A: [PAA₂₂-VG2]_n at pH 3.8, B: [PAA₂₂-VG2]_n at pH 7.4, C: [PAA₆₃-VG2]_n at pH 3.8, D: [PAA₆₃-VG2]_n at pH 7.4.

Figure 6. Summary of DLS analysis of [PAA-VG2]_n multiblock copolymer particles (average diameter based on size distribution by intensity) at pH 3.8 and 7.4 in water at temperatures from 25 to 80 °C.

Figure 7. Ratio of pyrene I_1/I_3 band fluorescence at various concentrations of [PAA₂₂-VG2]_n (A) and [PAA₆₃-VG2]_n (B) in water. Critical aggregation concentration was calculated as the intersection point of the two linear regions of the plots.

Figure 8.

DLS size distributions of PAA₂₂/VG2 physical mixtures (A: intensity, C: number) and [PAA₂₂-VG2]_n multiblock copolymers (B: intensity, D: number) in water without urea (dashed lines) and with urea during particle formation (solid lines).

Molecular weight analysis by GPC and NMR (GPC molecular weight values were calculated from polystyrene standards; NMR analysis was conducted in CDCl_3).

Table 1

	M_n (NMR) (g/mol)	M_n (GPC) (g/mol)	M_w (GPC) (g/mol)	M_p (GPC) (g/mol)	PDI
Br-PBA ₂₂ -Br	2,800	2,570	3,090	2,850	1.2
N ₃ -PBA ₂₂ -N ₃		2,600	3,130	2,890	1.2
[PBA ₂₂ -VG2] _n		7,830	15,030	18,130	1.9
Br-PBA ₆₃ -Br	8,000	8,290	10,740	9,660	1.3
N ₃ -PBA ₆₃ -N ₃		8,390	10,880	9,790	1.3
[PBA ₆₃ -VG2] _n		15,600	28,650	50,150	1.8