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Synthesis of poly(*N*-isopropylacrylamide) particles for metal affinity binding of peptides

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Abstract

Temperature-sensitive poly(*N*-isopropylacrylamide) (PNIPAM) microgel particles with metal affinity ligands were prepared for selective binding of peptides containing the His₆-tag (six consecutive histidine residues). The PNIPAM particles were copolymerized with the functional ligand vinylbenzyl iminodiacetic acid (VBIDA) through a two-stage dispersion polymerization using poly(*N*-vinyl pyrrolidone) (PVP) as a steric stabilizer. The resulting particles were monodisperse in size and colloidally stable over a wide range of temperature and ionic strength due to chemically grafted PVP chains. The particle size was also found to be sensitive to ionic strength and pH of the aqueous environment, likely due to the electrostatic repulsion between ionized VBIDA groups. Divalent nickel ions were chelated to the VBIDA groups, allowing selective metal affinity attachment of a His₆-Cys peptide. The peptide was released upon the addition of the competitive ligand imidazole, demonstrating that the peptide attachment to the particles is reversible and selective.

1. Introduction

Colloidal hydrogel particles are attractive carriers for biomolecules into biosensors because they can be synthesized in uniform and controllable size, and the particle diameter can be adjusted through changes in temperature [1, 2], pH [3–5], ionic strength [6, 7], or interactions with metal ions in solution [8]. Crosslinked *N*-isopropylacrylamide (PNIPAM) is a hydrogel with a volume phase transition temperature (VPTT) of around 32–35 °C where the hydrogel collapses upon heating due to thermal disruption of hydrogen bonding and polar interactions [9–11]. The significant change in volume near physiological temperature makes the material attractive for a wide range of potential biomedical applications [12–14]. Dispersion polymerization can be used to produce PNIPAM hydrogels in the form of colloidal microparticles, commonly referred to as “microgels” [1, 15]. Microgel particles have been used in biomedical applications such as bio-separations [16], drug delivery systems [17, 18], and biosensors [19, 20]. Under appropriate conditions, dispersion polymerization results in microgel particles of monodisperse size.

Several studies have demonstrated that the surface of microgel particles can be modified by incorporating functional groups to provide reactive sites for direct coupling of biomolecules such as DNA [20, 21], peptides [22, 23], proteins [24, 25], and biotin for specific binding to

avidin [26]. For the purpose of using PNIPAM microgels as protein or peptide transferring agents, a reversible and site-specific binding mechanism is desired. One common route for reversible and site-specific attachment of proteins is through the strong interaction of transition metal-ligand complexes to a short peptide sequence with six histidine residues in a row called the His₆-tag. Metal affinity purification of proteins is based on the specific binding of the His₆-tag to divalent metal ions, such as Cu, Ni, Co, and Zn, attached to a solid support through chelating groups [27, 28]. The bound His₆-tag can be released upon the addition of imidazole that acts as a competitive ligand to displace the bound His₆-tag [29]. The affinity of the His₆-tag to chelated metal ions has been exploited to attach proteins or peptides to various micro- or nano-particles, including polystyrene particles [30], poly(lactic-co-glycolic acid) particles [31], polyketal particles [32], and magnetic nano-particles [33] but has not been used previously with PNIPAM particles.

One major issue of using PNIPAM microgels as protein carriers is the loss of colloidal stability of the particles in buffer solutions near physiological temperature. It has been reported that PNIPAM micogels aggregate in buffer solutions [34] and during bioconjugation reactions [35]. The PNIPAM particle stability has been shown to depend on the electrolyte concentration and species [36]. At room temperature, the particles in the expanded state are stabilized by a combination of electrostatic repulsion and the steric barrier from extended PNIPAM chains [37, 38]. At physiological temperature, however, the particles are in the collapsed state and are solely stabilized by electrostatic effects. Colloidal stability can be maintained at physiological temperature in high ionic strength buffers by grafting steric stabilizers, such as poly(vinyl alcohol) (PVA) onto the PNIPAM particles [38].

In the present study, we investigated modifying PNIPAM particles by copolymerizing with *N*-(4-vinyl)-benzyl iminodiacetic acid (VBIDA) and poly(*N*-vinylpyrrolidone) (PVP) during a two-stage dispersion polymerization. The VBIDA introduces the metal chelating group iminodiacetic acid that can be used for site-specific attachment of peptides or proteins. The PVP was added as a steric stabilizer and covalently grafted to the particles to prevent the particles from aggregating at physiological temperature in buffers used to maintain protein stability. The novel sterically stabilized PNIPAM particles with iminodiacetic acid groups were investigated for their ability to chelate nickel ions, and to selectively bind and release a model peptide containing the His₆-tag.

2. Experimental Section

2.1 Materials

N-isopropylacrylamide (NIPAM, 97%), *N*, *N*'-methylenebisacrylamide (BIS, 99%), poly(*N*-vinylpyrrolidone) (PVP, average Mw ~55,000 g/mol), potassium persulfate (KPS, 99%), sodium hydroxide (NaOH, 97%), vinylbenzyl chloride (mixture of 3- and 4-isomers, 97%), nickel(II) sulfate (NiSO₄anhydrous, 99.99%) were all purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS, 99%), ethylenediaminetetraacetic acid disodium salt (EDTA, 90–100%), and imidazole (90–100%) were purchased from J.T. Baker. Iminodiacetic acid (IDA, >98%) was purchased from Fluka. DPBS (Dulbecco's phosphate-buffer saline solution) was purchased from Mediatech, Inc. The buffer consists of 2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137.93 mM sodium chloride, and 8.06 mM sodium phosphate dibasic. All reagents were used as received, and deionized water was used in all experiments.

2.2 Synthesis of VBIDA functional comonomer

N-(4-vinyl)-benzyl iminodiacetic acid (VBIDA) was synthesized according to a method described by Kitoh [39]. A solution containing 6.65 g of iminodiacetic acid and 3.3 g of sodium hydroxide was formed in 100 mL of 50% (v/v) methanol/water. The solution was maintained at 60 °C as 7.65 g of vinylbenzyl chloride were added dropwise under continuous stirring. After one-half of the vinylbenzyl chloride had been added in 30 minutes, another 3.3 g of sodium hydroxide was added. The remainder of the vinylbenzyl chloride was then added over 30 minutes. The reaction was maintained at 60 °C and stirred for another 30 minutes after the vinylbenzyl chloride addition was completed. The solution was distilled to reduce the volume to two-thirds of the original volume, and then extracted with ether. Hydrochloric acid was added to acidify the aqueous phase to precipitate out the VBIDA. The precipitated VBIDA was then collected by filtration and dried under vacuum.

2.3 Microgel synthesis

A range of PVP concentrations were investigated to determine synthetic conditions that produce uniform, submicron sized PVP-grafted PNIPAM particles that are sterically stable. A synthesis solution consisting of 0.76 g of NIPAM, 0.04 g of BIS and 0.76 g of PVP dissolved in 80 mL of deionized water was chosen for detailed study. The solution was maintained at a temperature of 60 °C in a 250 mL round bottom flask under continuous stirring while purging with argon for 40 minutes. Polymerization was initiated by adding a 5 mL aqueous solution containing 0.05 g of KPS. The reaction was carried out at 60 °C for 5 hours. VBIDA was incorporated into microgel particles through a two-stage polymerization. The first stage of the reaction was the same as described above for PVP-grafted PNIPAM, except the reaction time was reduced to 17 minutes. The second stage of the reaction was then started by injecting a mixture of 0.05 g KPS, 0.04 g NIPAM and VBIDA in 10 mL of deionized water into the reaction by a syringe. After investigating a range of VBIDA concentrations that yield colloiddally stable particles, VBIDA amounts of 0.2 g, 0.095 g, and 0.01 g were chosen to produce uniform sized particles having different levels of the iminodiacetic acid functional group. The reaction was carried out at 60 °C for a total of 5 hours from initiation of the first stage. The particles are named VBHigh, VBMed, VBLow and PN-PVP to denote the 0.2 g, 0.095 g, 0.01 g, and 0 g of VBIDA used for particle synthesis, respectively. Electrostatically stabilized PNIPAM particles were synthesized following a procedure similar to that first reported by Pelton [1]. A solution of 0.76 g of NIPAM, 0.013 g of BIS and 0.0032 g of SDS was formed in 50 mL of deionized water. After purging with argon, the reaction was initiated by adding a 0.5 mL aqueous solution containing 0.0166 g of KPS. The reaction was carried out at 60 °C for 5 hours. The resulting particles were referred to as PN-SDS. All types of particles were cleaned by repeated centrifugation (13,000 rpm, 3 hours, 15 °C) and redispersion in deionized water four times.

2.4 Microgel characterization

Microgel particle size was measured using a Brookhaven Instruments model 90 Plus dynamic light scattering (DLS) instrument on samples dispersed in deionized water or DPBS. For the temperature dependent particle size measurements, the data were taken after the temperature had stabilized. All data reported are averages of three repeats, and the error bars represent the standard deviation of the mean of three measurements. Proton nuclear magnetic resonance (¹H NMR) spectra of the microgels were recorded in D₂O with a Bruker Avance 400 MHz NMR spectrometer at 295K. The NMR samples were prepared by freeze-drying and redispersing the washed particles in D₂O.

2.4 Examination of colloidal stability with adsorbed PVP

PN-SDS particles were washed and then incubated with PVP at the same PVP concentration, time and temperature used in the polymerization process. A dispersion containing 0.08 g of washed PN-SDS particles and 0.076 g of PVP was prepared in 8.5 ml of deionized water, and heated to 60°C for 5 hours. The resulting particles were cleaned by repeated centrifugation (13,000 rpm, 3 hours, 15°C) and redispersion in deionized water four times. The sizes of unwashed and washed particles were measured by DLS in DPBS buffer.

2.5 Peptide binding

Nickel was chelated to VBIDA-containing microgel particles by dispersing the particles in 50 mM NiSO₄ under constant stirring overnight at room temperature. Unbound nickel was removed by dialysis (CE membrane, Spectra/por, MWCO=300kDa) against 1 L of deionized water three repeated times overnight at room temperature. To estimate the amount of nickel bound to the particles, a known amount of latex particles were treated with 100 mM EDTA under continuous stirring overnight at room temperature to remove nickel bound to the particles, as suggested by the methodology for nickel-based affinity chromatography [40]. The particles were then removed by centrifugation and the nickel content of the supernatant was determined by atomic adsorption spectroscopy (AAnalyst 600, Perkin-Elmer). For peptide binding, approximately 5.5 mg of particles (based on dry weight of particle suspension) having chelated nickel were incubated at 4 °C with continuous end over end rotation in 500 µL of binding buffer (50 mM phosphate buffer, pH 8, 0.3 M NaCl) containing 0.1 mg/mL of His₆-Cys peptide (synthesized by GenScript USA, Inc., purity 93%). The microgel particles were then separated by centrifugation (13000 rpm, 4 hr, 15 °C), and aliquots of the supernatant were collected for peptide concentration measurement. The microgel particles were then washed three times by centrifugation/redispersion in 2 ml of wash buffer (50 mM phosphate buffer, pH 8, 0.3 M NaCl, 20 mM imidazole). The wash buffer contained a low concentration of imidazole to minimize non-specific binding, as suggested by His₆-tagged protein purification protocols [40, 41]. The washed microgel particles with bound peptide were resuspended in an elution buffer (50 mM phosphate buffer, pH 8, 0.3 M NaCl, 250 mM imidazole) and incubated at 4 °C overnight with continuous end-over-end rotation. Microgel particles were separated from the elution buffer by centrifugation (13000 rpm, 4 hr, 15 °C) and the peptide concentration in the supernatant was measured by a Pierce 660 nm protein assay (Pierce Biotechnology). Selected samples were left in the elution buffer for a much longer time (3 days) to examine the effect on the particle dispersion and peptide elution. The amount of peptide adsorbed was obtained by subtracting the amount left in the supernatant from the initial concentration. All reported values were obtained by taking the average of at least three measurements.

2.6 Kinetics of microgel polymerization

The NIPAM monomer consumption was monitored by high-performance liquid chromatography (HPLC) analysis of the reaction mixture. Samples were collected at regular time points, transferred to small tubes by syringe, and frozen immediately with liquid nitrogen. The frozen samples were stored at 0 °C and thawed before analysis. All HPLC data were taken on a Shimadzu LC-2010A Liquid Chromatograph using a Shim-pack CLC-ODS-(M) C18 column. HPLC analysis was performed using a gradient flow from 0 to 30% acetonitrile (0.1 % trifluoroacetic acid) in distilled water for 15 minutes, followed by a linear concentration to 100 % acetonitrile (0.1 % trifluoroacetic acid) at 30 minutes, with a flow rate of 0.5 ml/min. Analytes were detected at 220 nm. The NIPAM, BIS and VBIDA peaks were identified by comparing to pure standard solutions, and the peak areas were compared to the peak areas obtained from the initial mixture.

3. Results and Discussion

3.1 PVP-grafted PNIPAM particles

Microgel particles display temperature dependent colloidal stability due to the swelling and collapse of the polymer network [37, 38]. When the particles are in the expanded state, the high water content inside the particles causes the Hamaker constant to be similar to the surrounding water so that the attraction between particles is weak [37]. In addition, dangling PNIPAM chain ends can extend from the particles in the swollen state to provide steric repulsion between particles [38]. In the collapsed state, the Hamaker attraction between particles is stronger and the steric repulsion from PNIPAM chains is lost. As a result, PNIPAM particles can easily aggregate when in the collapsed state, particularly when the ionic strength is high. It has been demonstrated that grafting a steric stabilizer to the surface of PNIPAM microgel particles can prevent aggregation even in high ionic strength buffers at physiological temperature and pH [38]. For the present study, PVP was chosen as a steric stabilizer based on its successful use as a steric stabilizer in dispersion polymerization of styrene [42]. PVP was shown to easily graft to the particle surface during dispersion polymerization of styrene, although it has never been employed in the dispersion polymerization of PNIPAM.

In preliminary experiments, PVP was found to be a suitable polymeric stabilizer for PNIPAM, based on the uniform size and the low polydispersity of the resulting particles. Particles were washed by repeated centrifugation and resuspension in deionized water to remove free PVP. The particles were then dispersed in DPBS buffer and the particle diameter was measured by dynamic light scattering as a function of temperature. As shown in Figure 1A, the PN-PVP particles display a temperature dependent diameter of ~390 nm at 25 °C and decreased to ~250 nm at 40 °C. The data is well represented by a Boltzmann sigmoid equation, and a transition temperature of ~31 °C is determined from the inflection point of the curve fit. The PN-PVP particles are stable in the buffer over the entire temperature range of 25–40 °C, since no increase in particle size or polydispersity due to aggregation was measured by dynamic light scattering. In contrast, the measured diameter of electrostatically stabilized microgel particles (PN-SDS) was found to increase sharply above 31 °C due to particle aggregation in the buffer solution. The sharp increase in the size of the PN-SDS is accompanied by an increase in measured polydispersity, confirming particle aggregation. Electrostatic repulsion from the adsorbed SDS is insufficient to prevent PNIPAM particles from aggregating in the buffer solution at physiological temperature. The improved colloidal stability of PN-PVP is attributed to the steric repulsion from the PVP chains that were either physically adsorbed or chemically grafted onto the PNIPAM microgel particles during the dispersion polymerization process.

In the dispersion polymerization of styrene in alcoholic media, PVP was found to be an effective steric stabilizer due to the chemical grafting of PVP segments to the polystyrene chains [42–44]. The most plausible mechanism for this chemical grafting is the formation of PVP macroradicals during the free radical polymerization process [45, 46]. In the presence of free radicals, reactive sites are formed on PVP when hydrogen atoms at the methine and methylene positions are abstracted, leading to subsequent grafting to the polymer produced by the reaction [47]. We found that the PN-PVP particles remained stable in DPBS buffer at physiological temperatures after repeated washing steps to remove physically adsorbed PVP. Therefore, it is likely that PVP is also grafted to the PNIPAM particles, forming a robust stabilizing layer that is not removed by desorption.

To support the hypothesis of PVP being chemically grafted onto the PNIPAM particles, a control experiment was carried out with pre-synthesized, electrostatically stabilized PNIPAM particles. The electrostatically stabilized PN-SDS particles were washed, then

incubated with the same concentration of PVP used in the PN-PVP polymerization and heated to 60 °C for 5 hr. If the PVP is associated to the particles by physical adsorption, the resulting particles should have the same colloidal stability as the PN-PVP particles. As shown in Figure 1B, after incubating with PVP, the particles remained stable in the DPBS solution up to the temperature of 40 °C. After washing, however, the particles aggregated above 31 °C. This behavior is significantly different from the PN-PVP particles that remain colloidally stable after multiple washing steps. The results indicate that physical adsorption of PVP is sufficient to provide colloidal stability, but the physically adsorbed PVP is easily removed by washing. Therefore, we conclude that chemically grafted PVP is mainly responsible for the enhanced colloidal stability of the PN-PVP particles in high ionic strength environment.

3.2 Copolymerization of VBIDA

The dispersion polymerization procedure was modified with the addition of VBIDA comonomer to introduce metal-chelating functionality to PNIPAM microgels. Sharply lower yield was obtained when the VBIDA comonomer was added at the start of the dispersion polymerization. It is likely that the yield is low because of the formation of water soluble VBIDA-rich oligomers and polymers that disrupt the particle nucleation [48]. To overcome the disruption of microgel particle formation caused by VBIDA, we employed a two-stage dispersion polymerization [49]. In the first stage, the reaction was initiated without VBIDA to nucleate PNIPAM particles. The second stage was then started by adding additional monomer, initiator, and VBIDA comonomer. In that way, VBIDA can be incorporated during the particle growth stage after particle nucleation is complete. Three samples were synthesized, using VBIDA:NIPAM ratios of 2:8, 1:8, and 0.1:8, respectively for the samples labeled VBHigh, VBMed, and VBLow.

Figure 2 shows the particle diameter versus temperature for VBIDA containing particles synthesized by two-stage dispersion polymerization. Particle size results are shown for samples dispersed in deionized water and also in DPBS buffer. The resulting particles were uniform in size, and the diameters were temperature dependent as expected. The VPTT was found to be ~33 °C in water, and was reduced in the DPBS buffer to ~30 °C for VBIDA containing particles and ~31 °C for PN-PVP. The lower VPTT in buffer is likely due to electrostatic screening of charges in the interior of the particles. The particle size increases with increasing amount of VBIDA used in the synthesis when the particles are dispersed in water, but there is an opposite trend when the particles are dispersed in the buffer. The particles without VBIDA have similar size in water and buffer, in both the expanded and collapsed state. The particle size results suggest that VBIDA was incorporated in the interior of the particles. Electrostatic repulsion between the negatively charged VBIDA groups causes the particle size in water to increase with increasing amount of VBIDA. Electrostatic repulsion is suppressed by the high ionic strength of the buffer, so there is a smaller size difference with varying VBIDA content. Moreover, the difference between particle sizes in buffer and in deionized water due to the loss of electrostatic repulsion is more significant when the VBIDA content is higher. The particles all remained stable in buffer up to 37 °C, due to the steric stabilization provided by grafted PVP.

Since VBIDA introduces weak acid groups, it is expected that particles containing VBIDA will respond to changes in pH as well as ionic strength. The negative charge on VBIDA will be lost as pH is reduced due to protonation of the acid groups. Figure 3 shows the particle size at 25 °C in deionized water compared to the size in dilute HCl solution of pH 2. The particle size is not significantly changed by the pH for the sample without VBIDA. However, all samples containing VBIDA show a smaller particle size at the lower pH. The difference in particle size at the low pH compared to the size in deionized water is greatest for the sample synthesized with the highest amount of VBIDA and is least for the sample

synthesized with the lowest amount of VBIDA. The results in Figure 3 provide additional evidence in support of VBIDA being incorporated into the particles, and indicate that the amount of VBIDA incorporated is proportional to the amount of VBIDA monomer added during particle synthesis.

3.3 Copolymerization kinetics

The kinetics of the two-stage polymerization of VBIDA-containing particles was studied by monitoring the monomer concentrations using HPLC. Two types of particles, PN-PVP and VBHigh, were compared, and the conversion of NIPAM, BIS and VBIDA was plotted as shown in Figure 4. Due to the occurrence of both physical and the chemical association of PVP under the polymerization conditions, the decrease of PVP detected by HPLC was not considered as a real conversion here. In both cases, the initial conversion of BIS was faster than NIPAM, which agrees with the previous observation by Wu [50]. Due to the higher BIS conversion in the early stage of the polymerization, the crosslink density is higher in the interior of the particles, giving the outer layer a less dense network structure. For PN-PVP particles, NIPAM and BIS conversions increased rapidly in the first 60 min and reached 65% and 89%, respectively. The reaction slowed down afterward, and at the end of the reaction BIS was 99% converted and NIPAM was 95% converted.

For VBHigh particles, the conversions of NIPAM and BIS were similar to the PN-PVP in the first 17 min; but after the VBIDA addition, NIPAM and BIS conversions were impacted. The consumption of BIS was slightly hindered within the first 8 min and then resumed, while NIPAM conversion was delayed for at least 43 min. During this time period of 43 min, about 56% VBIDA was consumed, suggesting that the VBIDA is more reactive than NIPAM and therefore preferentially polymerized once it is present in the reaction. The results implied the formation of a VBIDA-rich layer as VBIDA was introduced. After this rapid VBIDA conversion, a PNIPAM-VBIDA copolymer layer was then formed outside the VBIDA-rich layer. It is worth to mention that the VBIDA conversion reached 99% at the end of the polymerization, suggesting that almost all VBIDA comonomers were incorporated into the VBHigh particles. Thus we assume that for VBMed and VBLow particles the VBIDA added was also completely incorporated.

From the kinetics data we assumed that the VBIDA comonomer was completely incorporated to the particles, as well as the BIS crosslinker. To understand the amount of PVP grafted to the particles, the molar ratio between PVP and NIPAM was determined by ¹H NMR. The particles were washed, freeze-dried, and then redispersed in D₂O for NMR analysis. By comparing the signals from the hydrogens from PNIPAM and PVP (specifically on the tertiary carbon of the isopropyl group and on the γ -carbon of the pyrrolidone ring), the molar ratio and thus the weight percentage of PVP relative to PNIPAM can be determined (Table 1). Although the initial PVP amount in the feed was fixed for the synthesis of the four types of particles, the final amount of grafted PVP varied and followed the trend of PN-PVP>VBLow>VBMed>VBHigh. Since the PVP grafting occurs when PVP molecules are attacked by free radicals to form PVP macroradicals through hydrogen abstraction, it is competing with the incorporation of other components. As demonstrated by the kinetics data, VBIDA has relatively higher reactivity than NIPAM. Therefore, it is likely the PVP grafting was hindered when the more reactive VBIDA was present in the polymerization reaction.

3.4 Ni(II) chelation

The particles were incubated overnight in Ni(II) solution to examine the ability of the VBIDA groups to chelate nickel. Figure 5 shows the amount of chelated nickel per unit mass of particles. As expected, the amount of chelated nickel is proportional to the amount

of VBIDA used in the synthesis of the particles. To obtain the data in Figure 5, chelated nickel was removed from the particles by EDTA solution. It was assumed that, since EDTA is a much stronger chelating agent than VBIDA, all Ni(II) bound to the particles would be removed by the EDTA. The concentration data shown in Figure 5 are from atomic adsorption spectroscopy of the EDTA solution used to extract chelated nickel from the particles, not directly from the particles. While this method of measuring nickel content may lead to some error, the nature of any error would be to underestimate nickel chelated to the particles if there is incomplete removal of nickel by EDTA. The results in Figure 5 do confirm that nickel is bound to the particles, and suggest that the bound nickel is chelated to VBIDA groups as expected. It should be noted that the steric stabilizer PVP grafted to the surface of the particles also has the ability to complex metal ions [51]. The amide groups in the pyrrolidone rings contain nitrogen and oxygen electron donors that can coordinate to metal ions. Complexation of nickel to PVP may be responsible for the small amount of chelated nickel measured in Figure 5 for the PN-PVP sample. However, the amount nickel chelated to PN-PVP is much lower than for particles containing VBIDA. The effect of metal chelated to PVP should be considered when examining metal affinity binding of proteins or peptides.

3.4 His₆-Cys peptide binding

The particles were charged with Ni(II) ions, and equilibrated with His₆-Cys peptide in binding buffer to test for metal affinity binding. Total peptide binding was evaluated by taking the difference in peptide concentration in the supernatant before and after peptide capture, as measured via Pierce 660 nm protein assay. Next, reversible binding was evaluated by recording the amount of peptide that could be displaced from the particles by imidazole. We found that particle size is unaffected by imidazole, opening up the possibility to tune size by temperature (Figure 2) and deliver on demand from a targeted particle size. The quantity of peptide was normalized by the weight of particles used in the reaction, as shown in Figure 6. The amount of peptides bound on particles was found to be 4.0 nmol/mg and 1.6 nmol/mg for VBHigh and VBMed particles, respectively. Very low to zero peptide binding was observed for VBLow and PN-PVP particles, and for all types of particles without Ni(II) ions. The results show that peptide binding depends on both the incorporation of VBIDA into the particles and the presence of Ni(II) ions, indicating that the peptide was bound through specific interaction with the chelated nickel. Compared to the amount of Ni(II) ions bound on particles (7.7 nmol/mg and 5.7 nmol/mg for VBHigh and VBMed respectively), the binding sites were not completely occupied by peptides, possibly due to a fraction of the VBIDA groups being inaccessible to the peptide. The copolymerization kinetics results discussed in section 3.3 indicate that the VBIDA preferentially reacts over NIPAM immediately after addition of the VBIDA monomer. As a result, some of the VBIDA in the core of the particles may be less accessible for peptide binding.

Ideally, all the peptide bound to the particles should be able to be released by imidazole. However, the data show that the recovery of peptide by imidazole elution was ~47% for both VBHigh and VBMed particles. The peptide that was not released was possibly trapped in aggregates of particles that were observed after centrifugation. For both VBHigh and VBMed particles, visible aggregates were observed after the first centrifugation step with the particles containing chelated nickel. After transferring the particles to the elution buffer overnight, the particles redispersed and aggregates were no longer visible. However, the average diameter measured by DLS remained larger than before centrifugation, indicating that some aggregates remained that were too small to be seen by the naked eye. Therefore, we speculate that the incomplete elution of bound peptide was due to a fraction of the peptide remaining entrapped in the particle aggregates.

A separate set of experiments was conducted to determine the factors leading to the formation of particle aggregates upon centrifugation. The VBHigh and PN-PVP particles with or without nickel chelation were centrifuged after incubation with peptide alone, imidazole alone, or peptide and imidazole together. The particle size was measured after overnight incubation. The particles were then centrifuged and resuspended in the elution buffer. Particle size was measured immediately after resuspension and again after 3 days in the elution buffer. The matrix of experiments revealed that the PN-PVP particles did not form aggregates under any of the conditions investigated. VBHigh particles only formed aggregates when centrifuged with chelated nickel. Aggregates were observed after centrifugation of the VBHigh particles containing chelated nickel whether they were incubated with peptide or not. The aggregates disappeared completely after 3 days in the elution buffer. The results show that particle aggregation can be avoided completely by avoiding centrifugation. For example, dialysis can be used to remove unbound peptide without aggregating the particles, but quantification of the amount of peptide removed is difficult from the large volumes of solution used for dialysis.

One possible explanation of the aggregation phenomenon might be the formation of Ni(II)-(VBIDA)₂ complex. Ideally, three of the six coordination sites of nickel ion are occupied by VBIDA, while the other three are coordinated to histidine residues on the peptide. However, the sites for peptide binding could also be occupied by another VBIDA group, similar to the case of IDA functionalized polystyrene resin reported by Waki [52]. During centrifugation, the distance between particles is greatly reduced, thereby increasing the local concentration and promoting the formation of Ni(II)-(VBIDA)₂ complex, in some cases between two VBIDA groups on different particles. This new linkage serves as a bridge to hold particles together to form aggregates after centrifugation. This hypothesis is also supported by the observation that the aggregates could be broken up completely by incubation in the presence of imidazole for several days, because imidazole competes for the coordination sites and therefore breaks the coordination complex formed between particles. The reversible aggregation observed indicates that the particles may be potentially assembled into dissociable hydrogels. Bulk hydrogels could possibly be formed by assembling nanoparticles through Ni(II)-(VBIDA)₂ complexes bridging multiple particles. The bulk hydrogel could then be induced to dissociate into the constituent nanoparticles upon addition of a ligand, such as imidazole, that competes with VBIDA for binding to nickel.

4. Conclusions

Our results show that VBIDA was successfully incorporated into PVP stabilized PNIPAM microgel particles. When nickel ions are chelated to the VBIDA groups, a metal affinity matrix results that targets binding of histidine residues. The data show that the particles selectively bind to His₆-Cys peptide, a model His₆-tagged biomolecule. Since the peptide binding depends on the presence of metal ions and peptides are released upon addition of imidazole, the binding must occur specifically through the VBIDA-Ni(II)-His₆ complex formation. Steric stabilization imparted by PVP allows the particles to remain stable in a wide range of temperature, pH, and ionic strength. Under centrifugation the concentrated particles tend to aggregate in the presence of nickel ion, possibly due to the formation of Ni(II)-(VBIDA)₂ complex that can be removed by adding imidazole. These temperature responsive particles have the potential to act as reversible carriers of proteins and peptides, and potentially assembled into dissociable hydrogels.

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Highlights

Thermo-responsive PNIPAM particles were synthesized as peptide carriers.

Good colloidal stability under physiological conditions was achieved.

Reversible Ni-mediated peptide binding was demonstrated.

Figure 1.

Particle diameter in DPBS buffer versus temperature for (A) PN-PVP and PN-SDS particles and (B) unwashed and washed PN-SDS particles after incubating with PVP under the polymerization condition. The curves represent the Boltzmann sigmoid equation fits of the data, and the straight line represents the increase in diameter due to the flocculation of the PN-SDS particles.

Figure 2.

Temperature dependence of particle size in (A) deionized water and (B) DPBS buffer. The curves are Boltzmann sigmoid equation fits of the data.

Figure 3. Comparison of particle sizes measured in water and dilute HCl (pH = 2) at 25 °C. The error bars represent the standard deviation of the mean of three size measurements.

Figure 4. Conversion of monomers versus polymerization time for the synthesis of (A) PN-PVP particles and (B) VBHigh particles. The conversion was determined by normalizing the consumed amount of the monomer by the initial amount of the monomer; both amounts were obtained from HPLC analysis.

Figure 5. Nickel ion chelation to the particles as measured by atomic absorption spectroscopy. The bound Ni(II) ions were released from the particles by EDTA treatment. The error bars represent the standard deviation of the mean of three measurements.

Figure 6.

Analysis of amount of His₆-Cys peptide bound to and eluted from the particles. The particles without Ni(II) ions were used as control experiments. Sample names ending in "+Ni" indicate the particles contained chelated nickel. The error bars represent the standard deviation of the mean of three repeated experiments.

Table 1

Composition of PN-PVP, VBHigh, VBMed and VBLow particles. PVP and VBIDA amounts are represented as the weight percentages relative to NIPAM.

	PVP (wt%)^a	VBIDA (wt%)^b
PN-PVP	7.86	0
VBLow	6.09	1.25
VBMed	4.08	11.88
VBHigh	2.41	25.00

^a weight percentage relative to NIPAM monomer in the feed; estimated by peak areas in ¹H NMR spectrum of the samples.

^b weight percentage relative to NIPAM monomer in the feed; calculated from the amount of VBIDA in the feed, assuming VBIDA is completely converted.