On the Unusual Stability of Succinimidyl Esters in pNIPAm-AAc Microgels

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In this contribution, we describe the effects of amide coupling reactions on the physical properties of thermoresponsive hydrogel microparticles (microgels). These microgels, when treated *via* aqueous carbodiimide/sulfo-succinimide coupling protocols, displayed a dramatic modulation of the microgel phase transition thermodynamics. UV spectrophotometry was used to determine that this modulation was due to remarkably stable hydrogel conjugates of sulfo-NHS that resisted degradation under standard hydrolysis protocols. These intermediates result in a shift of the phase transition, along with a large increase in equilibrium microgel swelling degree, due to an increase in chain−chain Coulombic repulsion. Only aggressive hydrolysis protocols resulted in the recovery of the native microgel phase transition, suggesting that an unusually stable succinimidyl ester is formed in the microgel during coupling.

INTRODUCTION

Protein−polymer hybrid materials have been of great interest for some time, especially in the areas of drug delivery (*1*), the modulation of protein:ligand binding (*2*), biosensing (*3*, *4*), and chromatography (*5*). Awareness has also been increasing in the use of thermoresponsive polymers for responsive biomaterials (*6*). Our group has worked extensively on bioconjugates of hydrogel microparticles and nanoparticles (microgels) (*3*, *4*, *6*−*8*) and through the course of these studies has observed unusual phenomena when "standard" amide bond-forming conjugation protocols are used. Therefore, a series of investigations was initiated to more completely study these phenomena. In the course of this investigation it was discovered that the use of *N*-hydroxysulfosuccinimide (sulfo-NHS) in a carbodiimide coupling scheme (*9*) resulted in a dramatic modulation of the microgel phase transition thermodynamics. We report that these effects are due to unusually stable polymer-conjugated succinimidyl ester moieties that persist for many months in aqueous media and are resistant to standard methods of NHS ester hydrolysis. These results may have significant implications for how bioconjugation strategies are developed for complex nanomaterials such as porous, amphiphilic hydrogel particles.

Thermoresponsive microgels such as those composed of poly(*N*-isopropylacrylamide) (pNIPAm) and acrylic acid (AAc) are discussed extensively elsewhere (*10*−*12*). The presence of acrylic acid monomers in the microgels provides a means to form an amide bond with an amine (*9*). Specifically, the water-soluble carbodiimide reagent 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC) is useful in that it allows for the formation of amide linkages between amine substituents on a molecule (e.g., protein, ligand, fluorescent tag, etc.) and the carboxylic acid incorporated via the acrylic acid in the microgel (*3*, *13*, *14*).

This coupling procedure is essentially the aqueous phase version of the standard amide coupling reaction used for peptide synthesis in organic media. The aqueous phase reaction suffers from significant drawbacks relative to its organic solvent counterpart. Most importantly, the isoacylurea formed by the reaction of EDC and carboxylic acid is quite labile, as water attacks it to regenerate the carboxylic acid (*15*). In order to increase the conjugation efficiency, sulfo-NHS is frequently used, as shown in Scheme 1. This reagent replaces the isoacylurea to form a succinimidyl ester that hydrolyzes at a slower rate (*9*, *16*). The hydrolysis of the NHS ester is shown in Scheme 2. Conjugations such as these are essentially complete after several hours. It is generally accepted that the pH can be raised to quickly hydrolyze any remaining active esters, thereby quenching the reaction (*13*). Alternatively, the conjugated materials can be incubated overnight in PBS buffer (0.01 M, pH 7.4), which will result in hydrolysis of the ester bond.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were obtained from Aldrich unless otherwise noted. The coupling reagents 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide HCl (EDC) and *N*-hydroxysulfo-succinimide (sulfo-NHS), as well as phosphate-buffered saline (PBS) were used as received. The buffer component 2-[*N*-morpholino]ethanesulfonic acid (MES) was purchased from Sigma and used as received. Water used in all syntheses was distilled and then purified using a Barnstead E-Pure system. A 0.2 μm filter incorporated into this system removed particulate matter.

Conjugate Preparation. Aqueous carbodiimide coupling is a well-known cross-linking technique (*9*, *13*). Here, 100-μL of pNIPAm-AAc microgel solution (15 g/L) was mixed with 500 μL of MES buffer (0.1 M, pH 5.5). The coupling reagents EDC (4.4 mM final concentration) and sulfo-NHS (7 mM final concentration) are added to the microgel solution. and the prepared microgels are allowed to mix on a shaker table for at least 48−72 h. The microgels are then cleaned of excess reagents and spent reactants by successive centrifugation (5× at 14000*g*) and resuspension in PBS buffer (0.01 M, pH 7.4). Efforts to hydrolyze the active esters included addition of hydroxylamine (0.1 M, 25 μL), as well as various concentrations of NH_4OH. None of these resulted in significant loss of sulfo-NHS over the incubation period. Cleaned microgel conjugate solutions were then stored at 5 °C.

Photon Correlation Spectroscopy (PCS). The radii of the native and EDC/sNHS-treated pNIPAM-AAc microgels were determined in aqueous solution by PCS (Protein Solutions, Inc.). The temperature of the microgel samples was controlled via a Peltier device (±0.1 °C) integrated into the sample holder.

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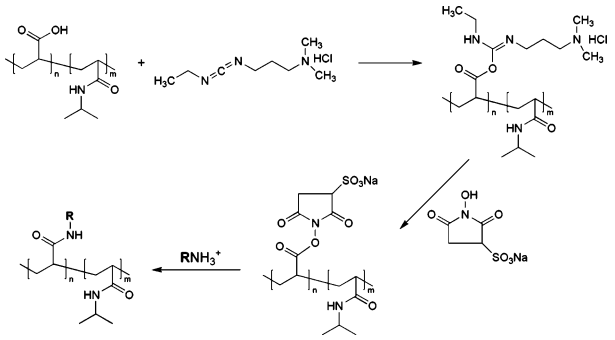
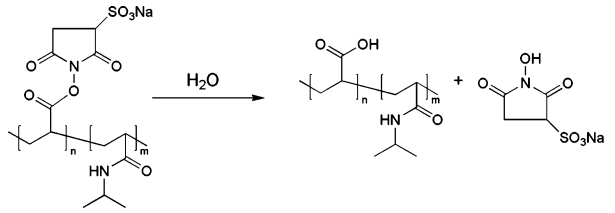
Scheme 1. EDC-NHS Coupling of a Primary Amine to the pNIPAm-AAc Copolymer



Scheme 2. Hydrolysis of the NHS-ester



Microgel samples were thermally equilibrated for 10 min before measurements were taken. The hydrodynamic radii of the particles were calculated from diffusion coefficients using the Stokes−Einstein equation. All correlogram analyses were performed with manufacturer-supplied software (Dynamics v.5.25.44, Protein Solutions, Inc.). The data presented are the averaged values of 20 measurements, with a 10 s integration time for each.

Phase Transition (Cloud-Point) Experiments. Phase transition curves of scattering intensity versus temperature were constructed from experiments performed on a steady-state fluorescence spectrophotometer (Photon Technology International) equipped with a Model 814 PMT photon-counting detector. The instrument was controlled by manufacturer-supplied software (Felix v.1.41, Photon Technology International). In the experimental setup, the λ_{ex} and λ_{em} were set to the same value and the band-pass was adjusted to 2 nm. Light from the instrument impinged on the experimental sample, and the scattered light was collected at a 90° angle. The programmable temperature controller (Linkam Scientific Instruments model PE 60) provides real-time solution temperature data via a temperature probe with accuracy of ±0.1 °C.

UV Analysis. UV data was collected on a Shimadzu UV1601 spectrophotometer. Samples were analyzed by pipetting 100 μL of the centrifuged microgel supernatant into 1 mL of PBS (0.01 M, pH 7.4)

RESULTS AND DISCUSSION

In this study, we treated pNIPAm-co-AAc microgels with EDC and sulfo-NHS under standard conditions (*13*), and the resultant behavior of the treated microgels was investigated. Microgels treated under these conditions were analyzed via light scattering in order to ascertain any change in the phase transition behavior of the microgels. The light scattering data in Figure 1a show a typical phase transition curve for pNIPAm-AAc microgels at pH 3.5 (open circles). The solution scattering intensity increases at the phase transition temperature due to water expulsion from the microgels and concomitant polymer phase separation. The adjacent curve (filled circles) was obtained from microgels that had been treated with EDC and sulfo-NHS, followed by rigorous cleaning by repeated centrifugation/resuspension steps. This sample does not display any evidence of a normal phase transition. Rather, the scattering stays at a

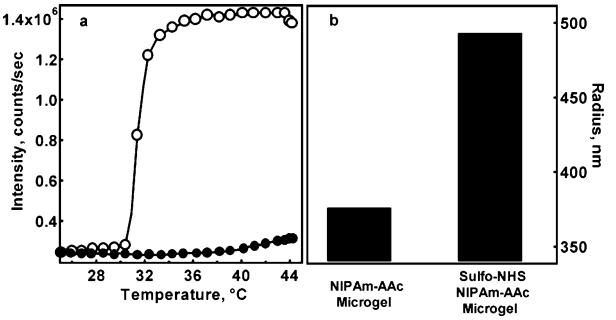


Figure 1. (a) Scattering intensity as a function of temperature for 0.11 mg/mL solutions of native pNIPAm-AAc microgels (open circles) and EDC/sulfo-NHS-treated pNIPAm-AAc microgels (filled circles) in water at pH 3.5. (b) Radii of native and EDC/sulfo-NHS-treated microgels as determined by photon correlation spectroscopy at 25 °C.

relatively low level until ~38 °C at which point the scattering intensity begins to increase gradually. Samples of microgels that were reacted with only EDC or only sulfo-NHS did not show a modulation of the phase transition temperature (see Supporting Information). It has been shown that hydrogels synthesized with anionic monomers exhibit phase transitions at higher temperatures due to Coulombic repulsion between polymer chains (*10*−*12*). The shifted phase transition in Figure 1a may similarly be the result of charge repulsion between sulfo-succinimidyl moieties attached to the microgel via the active ester. Hence, this implies that these active esters conjugated to the microgels are less susceptible to hydrolysis than what is typically observed, where the half-life for the NHS-ester is typically on the order of a few hours (*13*). The photon correlation spectroscopy (PCS) data shown in Figure 1b support the conjecture that sulfo-NHS is still bound to the microgels. At pH 3.5, the microgels treated with EDC and sulfo-NHS show a much larger radius than that of the native microgels. The treated microgels show a size monodispersity similar to the native microgels, ruling out aggregation as a factor in the increased radius. On the other hand, Coulombic repulsion of neighboring charged sulfonate groups would account for this effect, as an expanded polymer network results in larger microgel radii (*17*).

To confirm the presence of sulfo-NHS on the treated microgels, more extreme hydrolysis conditions than that found in simple aqueous media were employed. Such a hydrolysis condition can be achieved via a solution of 95% ethanol−5% PBS (0.01 M, pH 7.4). A small amount of freeze-dried polymer previously treated with EDC/sNHS was added to the solution and heated to 40 °C. The polymer solution was incubated for several hours, cooled to room temperature, and allowed to equilibrate on a shaker table for 72 h. To serve as a control, a microgel sample was mixed with an aliquot of sulfo-NHS (no EDC), cleaned by centrifugation, and carried through the same hydrolysis protocols. Both sets of microgels were then separated from solution via centrifugation. Supernatant was collected before and after hydrolysis treatment and analyzed via UV spectroscopy. Sulfo-NHS ($\epsilon \sim 8500\ M^{-1}\ cm^{-1}$) has a peak in the UV at approximately 268 nm in PBS (0.01 M, pH 7.4). Supernatant from treated microgels preceding the EtOH-PBS treatment resulted in no detectable loss of sulfo-NHS (Figure 2, open circles). However, supernatant from treated microgels incubated in EtOH−PBS showed a small release of sulfo-NHS. No release of sulfo-NHS was detectable from the supernatants of the control sample (data not shown). These data serve to confirm the presence of sulfo-NHS esters on the microgels and also reveal that the ester is hydrolyzing, but apparently at a very slow rate. Following the procedure outlined above, the treated microgels exhibited modestly shifted phase transition temperatures. This shift to a lower phase transition temperature is most likely due to the hydrolysis of a small amount of sulfo-NHS

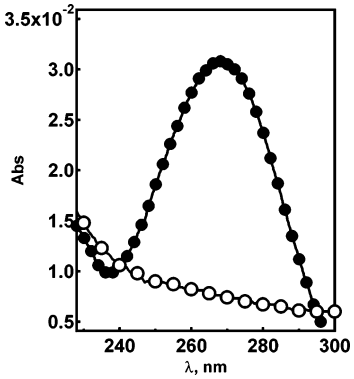


Figure 2. UV spectra of supernatant solutions recovered from EDC-sulfoNHS-modified microgels before EtOH−PBS incubation (open circles) and after 72 h incubation in EtOH−PBS (filled circles). λ_{max} = 268 nm.

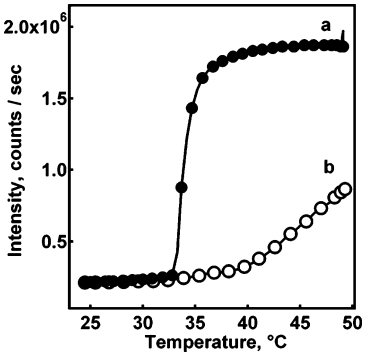


Figure 3. Scattering intensity as a function of temperature for 0.11 mg/mL solutions of (a, filled circles) ethanol−aq NaOH-incubated EDC and sulfo-NHS NIPAm-AAc microgels and (b, open circles) EDC and sulfo-NHS-treated pNIPAm-AAc microgels in water at pH 3.5.

and a resultant decrease in Coulombic repulsion between chains (Supporting Information).

In an effort to completely hydrolyze the succinimidyl ester and recover the native phase transition behavior of the microgels, EDC/sulfo-NHS-treated microgels were dispersed in a more basic solution of EtOH and aq NaOH. The polymer dispersion was heated to 50 °C for 15 min, cooled to room temperature and heated again to 50 °C for 15 min before being incubated for 24−48 h at room temperature. The microgels were separated from the reaction medium by centrifugation and redispersed in water. The results from light scattering experiments on these microgels (Figure 3) reveal that the phase transition temperature changed dramatically. Before incubation (open circles), the microgels exhibited phase transitions around 45−50 °C. The incubated microgels (filled circles) show a phase transition at ∼34 °C. The highly basic ethanolic solution allows for greater hydrolysis of the sulfo-NHS ester moieties and regenerates the original acid sites, resulting in the regeneration of a phase transition typical for native pNIPAm-AAc microgels at pH 3.5. Note that under these conditions, sulfo-NHS itself degrades and is not detectable *via* UV spectroscopy. The above data suggest that the environment of the microgel interior is such that much harsher conditions are necessary for the hydrolysis of sulfo-NHS esters than would be typically expected. The consequence is the long-lived stable esters that affect the solution properties of the microgels themselves.

In addition to the changes in physical properties, these long-lived esters appear to be responsible for decreasing the efficiency of amine conjugation. Experiments with protein, ligand, and fluorophore coupling have resulted in peculiar results, namely, *higher* coupling yields when sulfo-NHS is omitted from the EDC coupling protocols. The data in Figure 4 show an example

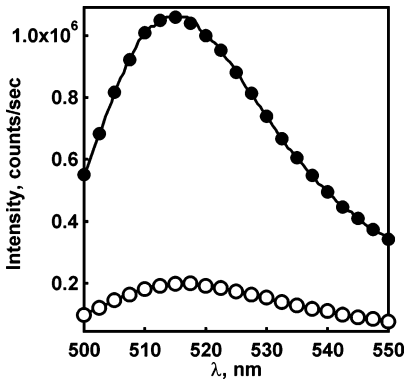


Figure 4. Fluorescence intensity of from a dispersion of microgels conjugated with 1 μL of fluoresceinamine (5.0 mg/mL) *via* EDC (4.4 mM) coupling (filled circles) and microgels conjugated with 1 μL fluoresceinamine (5.0 mg/mL) *via* EDC (4.4 mM) and sulfo-NHS (0.77 mM) coupling (open circles). All microgel solutions conjugated with sulfo-NHS result in lower fluorescence intensity proportional to the amount of sulfo-NHS added, suggesting that the sulfo-NHS competes for sites with the fluorophore by forming a stable ester.

of this effect. Here, the fluorophore fluoresceinamine was EDC coupled to pNIPAM-AAc microgels both with and without sulfo-NHS. When sulfo-NHS is included in the reaction, a dramatically lower yield of fluorophore coupling is observed relative to the EDC-only reaction. Effects such as this have been observed in our lab for proteins (avidin, streptavidin, and various IgGs), ligands (biotin, peptides), and other amine-containing fluorophores. We interpret these results as being due to competition for the chemoligation sites. That is, acid groups that react with sulfo-NHS are not as available for amide coupling as those that do not react with sulfo-NHS.

CONCLUSIONS

In summary, we have shown that treatment of pNIPAm-AAc microgels with the carbodiimide coupling reagents EDC and sulfo-NHS results in microgels containing 'long-lived' sulfo-NHS intermediates, despite the use of standard hydrolysis protocols. These intermediates result in a shift of the phase transition of the microgels to higher temperatures, along with a large increase in equilibrium microgel swelling degree, due to an increase in chain−chain Coulombic repulsion. Despite the typically labile nature of the ester bond in aqueous media, no hydrolysis of the sulfo-NHS ester is observed in standard aqueous solutions, while water/ethanol mixtures allow for hydrolysis rates that are detectable by UV spectroscopy. Extremely aggressive hydrolysis in basic ethanol affords almost complete removal of the sulfo-NHS moieties, thus regenerating the normal microgel phase transition behavior.

While the mechanistic origins of these unusual reactivities are not clear from these studies, it is perhaps not surprising that hydrolysis rates would be perturbed in hydrogel phases, given previous observations of differences in water mobility in microgel networks (*18, 19*). However, the susceptibility of the NHS ester to nucleophilic attack should not necessarily be sensitive to the behavior of water in the network, as water does not directly participate in that reaction. To address this issue, we are currently undertaking more detailed mechanistic studies on model polymers. Regardless of the mechanisms involved in the present study, these results point to the dramatically different reactivity that may be present in complex macromolecular materials such as hydrogels and other novel nanostructures, a point that should be acknowledged when applying "standard" chemoligation strategies to nonstandard materials. In light of the enhanced stabilities of the NHS esters, our group now performs acid−amine chemoligation reactions using EDC only,

as the NHS step obviously decreases the overall yield of the desired coupling reaction.

ACKNOWLEDGMENT

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Supporting Information Available: Phase transition curves of microgels exposed to various EDC, EDC/sulfo-NHS, and hydrolysis treatments. This material is available free of charge *via* the Internet at http://pubs.acs.org/BC.

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