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ORIGINAL CONTRIBUTION

Synthesis of multifunctional nanogels using a protected macromonomer approach

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Abstract Nanoparticles possessing multiple functionalities provide synthetic handles for varied surface chemistries, making them useful for a range of applications such as biotargeting and drug delivery. However, the combination of interfering functionalities on the same particle is often challenging. We have employed a synthetic scheme involving chemical protection/deprotection to combine interfering functional groups on the same hydrogel nanoparticle. The synthesis of amine-containing poly(*N*-isopropylacrylamide) nanogels was carried out via free radical precipitation polymerization by incorporating a Fmoc-protected amine poly(ethylene glycol) (PEG) macromonomer. The Fmoc group was then removed to obtain free amines, which were shown to be available for conjugation. We further explored pNIPAm-co-acrylic acid nanogels with a protected amine-PEG, yielding zwitterionic particles. With careful attention to the order of the chemoligation and deprotection steps, these interfering functional groups can be forced to behave in a pseudo-orthogonal fashion, allowing for multiple chemoligation steps that employ both the amine and carboxylic acid groups.

Keywords Nanogels · Chemoligation · Orthogonal functionality · Bioconjugation

Abbreviations

NIPAm	*N*-isopropylacrylamide
pNIPAm	poly(*N*-isopropylacrylamide)
LCST	lower critical solution temperature
AAc	acrylic acid
APS	ammonium persulfate
SDS	sodium dodecyl sulfate
BIS	*N*,*N*′-methylenebis(acrylamide)
PEG	poly(ethylene glycol)
APMA	*N*-(3-aminopropyl)methacrylamide hydrochloride
EDC	1-ethyl-3-methyl-(3-dimethylaminopropyl) carbodiimide
TAMRA-SE	5-carboxytetramethylrhodamine succinimidyl ester
DMSO	dimethyl sulfoxide
DI	deionized
PCS	photon correlation spectroscopy
UV	ultraviolet spectroscopy
VIS	visible spectroscopy
MWCO	molecular weight cut off
Fmoc	fluorenyl-methoxy-carbonyl
NHS	*N*-hydroxysuccinimide
PEO	poly(ethylene oxide)
AFM	atomic force microscopy

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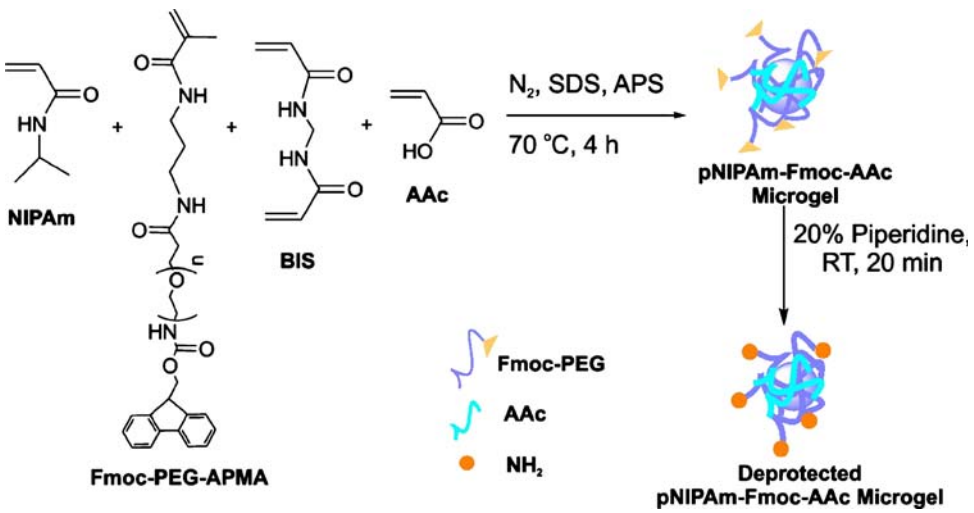
Introduction

Nanoparticles have emerged as versatile building blocks in the development of materials for biomedicine and drug delivery [1, 2], sensors [3–5], nanophotonics [6–8], catalysis [9], and separations [10–12]. One important class of nanoparticles is stimuli-responsive hydrogel nanoparticles or nanogels. There has been a great deal of attention toward increasing the chemical diversity and the complexity of nanogels to design and tailor them for specific applications [5–7, 13–16]. In particular, the specific chemical functionalization of nanogels provides an excellent way to manipulate their responsiveness

to external stimuli such as pH, ionic strength, or light [15–18]. Alternatively, one can impart the nanogel surface with chemical handles for covalent attachment of functionalities in the post-polymerization stage [19–23]. To design complex and versatile nanogels, it is often desired that the particle have multiple different chemical handles. However, in many cases, chemoligations that can be carried out on nanoparticles are complicated and even prohibited by the presence of interfering functional groups. For example, the presence of carboxyl and amine groups on the same particle surface can limit the extensively used carbodiimide-based amide bond-forming chemistry for chemoligation, as it could result in cross-reaction of the two functional groups [24, 25]. Thus, a challenge in the design and synthesis of multifunctional nanoparticles is the selective incorporation of multiple functionalities that behave as orthogonally reactive groups. It is also desirable that such an incorporation scheme be simple and straightforward, as in the approach we describe below.

Poly(*N*-isopropylacrylamide) (pNIPAm) nanoparticles are perhaps the most commonly studied nanogels. These nanoparticles undergo a volume phase transition from a hydrophilic (water-swollen) to a hydrophobic (deswollen) state at a lower critical solution temperature (LCST) of about 31 °C in water [26]. The phase transition of these nanogels at a temperature relevant to biological systems makes them interesting for bio-applications [27, 28]. A number of synthetic strategies have been developed for the production of pNIPAm nanogels [22, 23, 29–35]. Interesting architectures such as core/shell nanoparticles have been synthesized via two-stage precipitation polymerization, which offers the ability to spatially localize chemical functionalities to a defined position, to render thermoresponsivity to non-responsive particles, or to modify specific physical properties of the nanoparticles [14, 29, 36]. As argued above, the integration of multiple functional groups onto these thermosensitive nanoparticles is required to explore a range of applications. We therefore present in this paper an improvement over our past methodologies for synthesizing multi-functional nanogels by using a well-established, protection/deprotection organic synthesis strategy.

As diagrammed in Scheme 1, combining the simple and well-established precipitation polymerization technique of nanogel synthesis with traditional organic synthesis techniques for the protection and deprotection of reactive functional groups can yield a straightforward method for synthesizing multifunctional nanogels. This method provides a unique synthetic strategy for combining multiple orthogonal functionalities, which expands the types of chemistry that can be performed on nanogels. In the strategy used here, the nanogels are synthesized using a macromonomer that has a protected amine group. The protecting group can be removed at a later stage to yield the free primary amine for doing further chemistries. The incorporation of carboxylic acid groups along with the amine functionality is also easily obtained with this scheme. The synthesis of zwitterionic (having both positively and negatively charged functional groups) nanogels via traditional precipitation polymerization is limited due to colloidal instability during synthesis; the presence of both cationic and anionic co-monomers, plus charged surfactants (e.g., sodium dodecylsulfate) and initiators (e.g., ammonium persulfate), causes aggregation and the formation of coagulum. A one pot, synthetic route based on precipitation polymerization for synthesizing such zwitterionic nanogels also does not result in efficient incorporation of high concentration of the charged comonomers (especially amines) [37]. However, with the described scheme, a one-pot synthesis of zwitterionic nanogels with high degrees of comonomer incorporation can be efficiently carried out. The protected amine comonomer allows the use of anionic surfactant and initiator in the synthesis. It also provides a route for synthesizing monodisperse, colloidally stable multifunctional nanogels with otherwise interfering functional



Scheme 1 Synthetic scheme based on precipitation polymerization for the synthesis of pNIPAm-based nanogels containing multiple functional groups

groups on the same nanoparticles. These multiple functionalities can be further utilized orthogonally with good control over the access of the functional group at any stage in the post-polymerization modification steps.

Experimental section

Materials All materials were obtained from Sigma Aldrich unless otherwise specified. All chemicals were used as received except NIPAm, which was recrystallized from hexane (J. T. Baker) before use. A bifunctional polyethylene glycol (Fmoc–PEG–NHS, MW 5000) was obtained from Nektar Therapeutics. An amine monomer, *N*-(3-aminopropyl) methacrylamide (APMA) was obtained from PolySciences. Biotin hydrazide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Pierce. Fluorescent dyes, 5-aminofluorescein and 5-carboxytetramethylrhodamine succinimidyl ester (TAMRA-SE) were purchased from Molecular Probes. Water was distilled and then purified using a Barnstead E-Pure system operating at a resistance of 18M Ω and, finally, filtered through a 0.2-μm membrane filter (Pall Gelman Metricel) before use.

Synthesis of the Fmoc-protected PEG methacrylamide macromonomer The Fmoc-protected PEG–NHS (0.20 g, 0.039 mmol, 1 equiv) was reacted with APMA (0.014 g, 0.078 mmol, 2 equiv) at room temperature for 2 h in pH 7.5 phosphate-buffered saline (PBS) buffer. The product was purified by dialysis for a week by changing water three times a day, using Spectra-Por 1,000 MW cut-off dialysis tubing (VWR). The removal of unreacted APMA was confirmed by a ninhydrin (2 wt% ethanol solution) test on a thin-layer chromatography plate. The final product was characterized by ^{1}H NMR for the incorporation of double bonds from the APMA.

Nanogel synthesis The pNIPAm nanogels containing an Fmoc-protected PEG macromonomer were synthesized using the precipitation polymerization method using the Fmoc-protected PEG methacrylamide macromonomer synthesized above as a comonomer. The addition of a pNIPAm–Fmoc shell was performed using a seed and feed method previously described by our group [31]. In this method, a polymer shell with the same or different structure or functionality to that of the core is added onto preformed core particles. In a typical synthesis, preformed pNIPAm core particles are heated to ~70 °C, followed by addition and initiation of the shell monomer solution. As the reaction temperature is well above the volume phase transition temperatures of the core particles, the particles are in a collapsed state. The collapsed particles are hydrophobic and hence tend to capture the growing oligomers, which results in the formation of the cross-linked hydrogel shell. For the synthesis of core/shell nanogels with the Fmoc-protected PEG macromonomer, pNIPAm cores synthesized without the Fmoc macromonomer were used as seeds.

Synthesis of pNIPAm–Fmoc nanogels The synthetic details for each of the nanogels synthesized are presented in Table 1. A total monomer concentration of 70 mM was used for the synthesis of all the core nanogels, while a total monomer concentration of 40 mM was used for the addition of shell onto preformed cores.

Typically, the appropriate amounts of the monomer NIPAm, Fmoc-protected PEG macromonomer, cross-linker *N*,*N*′-methylenebis(acrylamide) (BIS), and the surfactant, sodium dodecyl sulfate (SDS), were dissolved in 9 mL distilled, deionized (DI) water and filtered through a 0.2-μm-membrane filter. The reaction solution was transferred to a three neck flask, stirred, and heated to ~70 °C under a N_2 atmosphere. After 1h, the reaction was initiated by the addition of 1 mL aqueous ammonium persulfate (APS) solution and kept at 70 °C for 4h. The reaction was allowed to cool to room temperature and stirred overnight followed by filtration through Whatman filter paper (no.2). The filtered nanogels were then purified by dialysis against water for ~2 weeks with the water being changed twice per

Table 1 Synthesis of pNIPAm–Fmoc nanogels

Sample name	Total volume (mL)	Wt. NIPAm (g), mol%	Wt. BIS (g), mol%	Wt. Fmoc–PEG (g), mol%	Wt. SDS (g)	Wt. APS (g)	R_h[a] at 25 °C (nm)	R_h[a] at 45 °C (nm)
pNIPAm core (c^0)	200	1.55, 98%	0.0432, 2%	–	0.055	0.0378	136 ± 0.9	56 ± 2.8
pNIPAm–Fmoc core (c^5)	10	0.0737, 93%	0.0022, 2%	0.1830, 5%	0.0043	0.0041	46 ± 3.5	27 ± 2.9
pNIPAm–Fmoc shell[a] (c^0/s^1)	10	0.0439, 97%	0.0120,2%	0.0208, 1%	0.0025	0.0037	184 ± 2.2	134 ± 3.9
pNIPAm–Fmoc shell[a] (c^0/s^5)	10	0.0421, 93%	0.0012, 2%	0.1044, 5%	0.0025	0.0037	153 ± 8.9	103 ± 0.5

The letters in the sample name indicate core (*c*) or shell (*s*), and the superscript indicates the mole percent of Fmoc comonomer in the synthesis.
[a] For the shell synthesis, 0.0156 g of c^0 was used as the core.

day, using Spectra-Por 10,000 MW cut-off dialysis tubing (VWR). The clean nanogels were freeze dried overnight using Labconco lyophilizer. The hydrodynamic radius (R_h) of the synthesized nanogels were measured at 25 °C and 45 °C by photon correlation spectroscopy (PCS).

Synthesis of pNIPAm–Fmoc–AAc shell For synthesizing both carboxyl and amine functional group containing shells, acrylic acid (AAc) was used as comonomer along with the Fmoc-protected PEG methacrylamide macromonomer. The total monomer concentration for the shell was again kept at ~40 mM. The pNIPAm cores (1 mL) were stirred and heated to ~70 °C under a N_2 atmosphere in a three-neck reaction flask. A filtered (using a 0.2-μm-membrane filter), 3 mL aqueous solution of 0.0188 g (83 mol%) NIPAm, 0.0522 g (5 mol%) of the Fmoc-protected PEG methacrylamide, 0.0008 g (2 mol %) of BIS, and 0.0013 g of SDS was added to the reaction flask containing the heated cores. To avoid autopolymerization of AAc at high temperatures, it was added just before the initiation of the reaction. After 1h, 1.5 μL (10 mol %) AAc was added followed by the initiation of the reaction by injection of 0.0019 g APS dissolved in 1 mL DI water. The reaction proceeded at 70 °C for 4 h followed by stirring at room temperature overnight. The reaction was filtered through Whatman filter paper (no.2) and then purified by dialysis against water for ~2 weeks with the water being changed twice per day, using Spectra-Por 10,000 MW cut-off dialysis tubing (VWR). The purified nanogels were freeze dried overnight using Labconco lyophilizer.

Removal of the Fmoc-protecting group The Fmoc-protected nanogels (~15 mg) were dissolved in 1 mL 20% (*v*/*v*) piperidine in dimethylformamide (DMF) solution and stirred for 10 min at room temperature. Fresh 1 mL 20% (*v*/*v*) piperidine in DMF solution was added after 10 min and allowed to stir for ten more minutes at room temperature. The deprotected nanogels were then purified by dialysis against water for ~2 weeks with the water being changed twice per day, using Spectra-Por 10,000 MW cut-off dialysis tubing (VWR). The successful removal of the Fmoc-protecting group was confirmed by ^{1}H NMR.

Conjugation of 5-carboxytetramethylrhodamine succinimidyl ester to the deprotected nanogels For conjugating the amine reactive fluorophore, 0.2 mg of deprotected nanogels were dispersed in 744 μL of pH 7.4 PBS buffer, and 256 μL of 1 mg/mL solution of 5-carboxytetramethyl rhodamine succinimidyl ester (TAMRA-SE) was added. The solution was stirred at room temperature for 2 h. The conjugated nanogels were then purified by dialysis against water for ~2 weeks with the water being changed twice per day, using Spectra-Por 10,000 MW cut-off dialysis tubing (VWR). The purified nanogels were freeze dried overnight for storage.

5-Aminofluorescein and TAMRA-SE conjugation to pNIPAm–Fmoc–AAc The carboxyl groups on the pNIPAm–Fmoc–AAc were first conjugated with 5-aminofluorescein using carbodiimide coupling. For the reaction, 1.4 mg of pNIPAm–Fmoc–AAc (~0.5 mmol of AAc) nanogels were dispersed in 348 μL of 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer of pH 4.7. To this well-dispersed nanogel solution, 0.173 mg of 5-aminofluorescein and 0.479 mg of EDC were added, and the reaction was allowed to proceed overnight at 4 °C. The nanogels were then purified via dialysis for 2 weeks against 10% aqueous ethanol, with the solvent being changed twice every day. The Fmoc groups on the fluorescein-conjugated pNIPAM–Fmoc–AAc nanogels were then removed, and the nanogels were purified and dried as described above. The free amines thus obtained on the fluorescein-conjugated pNIPAM–Fmoc–AAc nanogels were further used for conjugating TAMRA-SE using a similar protocol as described above.

^{1}H NMR spectroscopy All ^{1}H NMR spectra were recorded on solutions formed by re-dispersing freeze-dried nanogels in either D_2O or DMSO-d_6 at room temperature; a 300-MHz Mercury Varian Unity spectrometer was used for data collection. The water peak arising from residual water inside the nanogels was suppressed to more efficiently observe the proton signals. The amount of Fmoc–PEG macromonomer incorporated was determined by calculating the ratio of the PEG peak (at 3.5 ppm, resulting from the methyl protons in the OCH_2CH_2 repeat units) to the main pNIPAm peak (at 1.1 ppm, resulting from the methyl protons of the isopropyl group) at room temperature. The major source of error in such analysis of the amount of monomer incorporated is from the error in the integration of the individual signals, and it is therefore dependent on the signal-to-noise ratio of the respective signals. Signals with a fair signal-to-noise ratio have moderate errors, whereas poorly resolved signals lead to larger errors. Thus, the errors for the amount of monomer incorporated reported in the paper have been derived from the integration error of the individual signals.

UV-Vis spectroscopy All the absorption spectra were obtained in quartz cuvettes using a Shimadzu UV 1601 spectrophotometer equipped with a temperature controller.

Photon correlation spectroscopy Particle sizes were determined by photon correlation spectroscopy (Protein Solutions) equipped with an integrated Peltier temperature control device, which gives temperature accuracy within ±0.1 °C. The instrument collects scattered light at 90° by a single-mode optical fiber coupled to an avalanche photodiode detector. The

samples were thermally equilibrated at each temperature for 10 min before each measurement. The data presented is an average of at least ten measurements with acquisition times of 10 s. The hydrodynamic radii of the particles were calculated from the diffusion coefficients using the Stokes–Einstein equation. All the data analysis was done using Dynamic Software version 5.25.44 provided by Protein Solutions.

Fluorescence spectroscopy All measurements were done using a Photon Technology International fluorescence spectrophotometer equipped with a model 814 PMT photon-counting detector. The slits were adjusted to achieve a spectral bandwidth of 2 nm, and the spectra were obtained with a 1-nm step size and 1-s integration time. The samples were excited at a wavelength of 490 nm.

Atomic force microscopy All AFM images were obtained in AC mode on an Asylum Research MFP-3D atomic force microscope. Spring constants were calculated using the thermal method. Imaging and analysis was performed using the Asylum Research MFP-3D software (IgorPro, WaveMetrics, Lake Oswego, OR, USA). An Olympus AC160 cantilever with k = 42 N/m, f_0 = 300 kHz was used for imaging. AFM samples were prepared by casting a drop of the sample on micro-cover glass slips (VWR) followed by drying overnight.

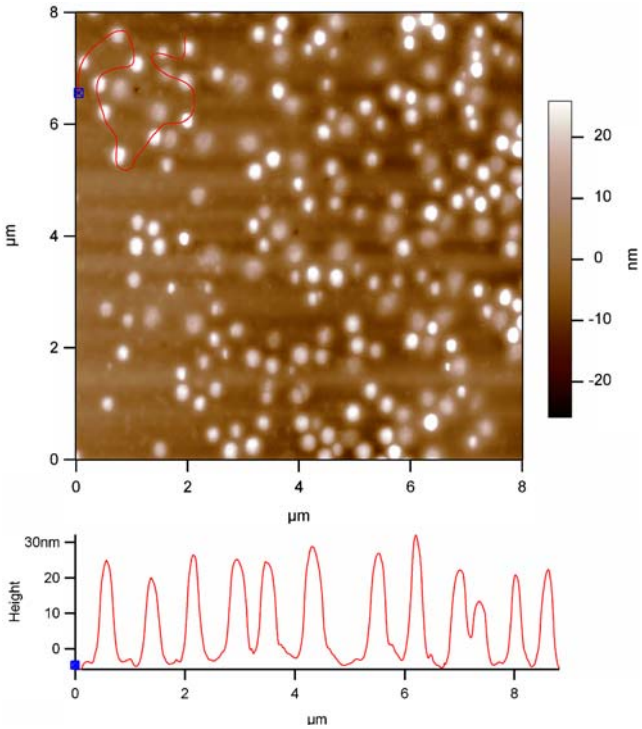


Fig. 1 AFM image (*top*) and the line profile (*bottom*; obtained for the region marked with a *red line* in the AFM image) indicating the morphology and height of protected pNIPAm–Fmoc (c^5) core particles on a glass substrate. Note that small height variations such as those observed in this figure are typically due to deformation of the soft particles on the substrate

Results and discussion

We have synthesized pNIPAm-based nanogels possessing multiple orthogonal functionalities using the scheme illustrated and discussed above (Scheme 1). Fmoc-protected PEG macromonomer was synthesized by simple conjugation of *N*-(3-aminopropyl)methacrylamide with Fmoc–PEG–NHS. Synthesis of pNIPAm nanogels with this macromonomer using precipitation polymerization gave pNIPAm nanogels with Fmoc-protected amine groups. These amine groups can be further deprotected using mild conditions yielding the free amines. Using this synthetic scheme, we have also synthesized nanogels with a multifunctional shell on a pNIPAm core. A core/shell architecture can help increase the surface localization of the functional groups on the nanoparticles, which is often required for effective bioconjugation [21].

The synthesis scheme produced spherical and colloidally stable nanogels as seen from the AFM image of the pNIPAm–Fmoc cores (c^5; Fig. 1) and the regularization histogram (obtained via PCS) of pNIPAm–Fmoc–AAc nanoparticles (Fig. 4). Our core/shell particle naming formalism is laid out in Table 1. The c^5 cores were ~46 nm in radius as measured by PCS before deprotection, whereas they swell to ~57 nm in radius after deprotection (Table 2). This swelling is likely due to the exposure of free amine groups, which makes the polymer chains more hydrophilic. In addition, the removal of the bulky Fmoc groups upon deprotection may also contribute to steric changes (e.g., increase in polymer chain flexibility), resulting in the observed swelling behavior of the nanogels. The nanogels with Fmoc shells (Table 2) showed a similar increase in radius after deprotection (153 to 176 nm at 25 °C), again suggesting the more hydrophilic and flexible nature of the deprotected nanogels.

^{1}H NMR of the synthesized nanogels indicated incorporation of the Fmoc-protected PEG macromonomer. The

Table 2 Hydrodynamic radii (R_h) determined by PCS

Sample	Temperature (°C)	R_h[a] before deprotection (nm)	R_h[a] after deprotection (nm)
5 mol% pNIPAm–Fmoc core (c^5)	25	46 ± 3.5	57 ± 0.9
	45	27 ± 2.9	32 ± 2.4
5 mol% pNIPAm–Fmoc shell[b] (c^0/s^5)	25	153 ± 8.9	176 ± 0.9
	45	103 ± 0.5	105 ± 2.8

[a] Average of three measurements. The errors are standard deviation obtained for the measurements.
[b] Contains a c^0 core with R_h = 136 ± 0.9 nm at 25 °C and R_h = 56 ± 2.8 nm at 45 °C

proton assignments for the pNIPAm polymer are in agreement with its chemical structure and are illustrated in Fig. 2 [38, 39]. The peak at 1.1 ppm can be attributed to the methyl protons of the *N*-isopropyl group (peak a). The resonance for the methylene proton of the isopropyl group is observed at 3.8 ppm (peak b), while the resonances from 1.2 to 2.2 ppm (peaks c and d) are attributed to the protons on the polymer backbone. The methyl protons in the OCH_2CH_2 repeat units from the PEG macromonomer appear at 3.5 ppm (peak e), and the aromatic groups from the Fmoc are seen between 7.2 and 7.8 ppm (peak f). After deprotection, the peak due to the Fmoc moiety disappears, confirming the removal of the protecting group.

The LCST of the synthesized pNIPAm–Fmoc nanogels (~37 °C) is elevated relative to that of the pNIPAm nanogels (32 °C). This is attributed to the fact that, with the incorporation of high molecular weight PEG, the phase transition shifts considerably to a higher temperature, which is due to an increased hydrophilic balance of the particles and, therefore, a reduction in the tendency for the hydrophobic collapse of pNIPAm. Increased LCST values with incorporation of hydrophilic moieties have been observed previously [40]. Virtanen et al. have also observed increased solubilization and increased LCST of pNIPAm by incorporation of poly(ethylene oxide) (PEO) chains [41, 42]. Another interesting effect on the LCST of the nanogels is observed after deprotection. As can be seen from Figure 3, the LCST of the nanogels in water is further elevated to ~40 °C from ~37 °C upon removal of the Fmoc group. The increased LCST after deprotection is again attributed to the increased hydrophilicity of the polymer network due to the exposure of free amine groups following Fmoc deprotection.

To produce multifunctional nanogels containing different functionalities, we incorporated both the Fmoc–PEG methacrylamide macromonomer and AAc as comonomers. The idea was to see if we could localize both amine and carboxyl groups on the surface of the same nanoparticles in high concentrations and further utilize them both for bioconjugation. This has been a challenge in the past, as amine and carboxyl groups have interfering functionalization

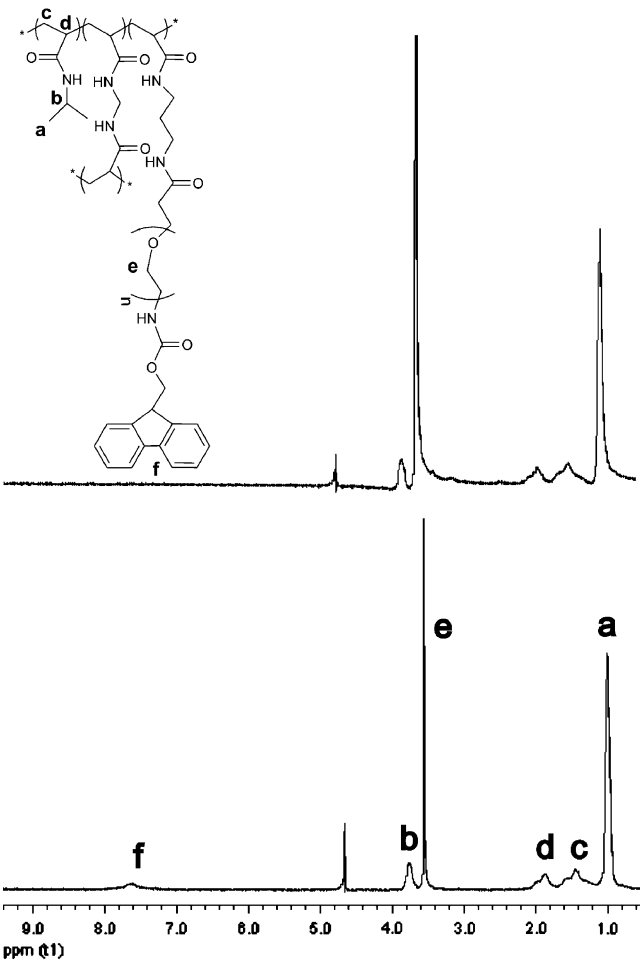


Fig. 2 ^{1}H NMR in D_2O of the pNIPAm–Fmoc (c^5) core nanogels before (*bottom*) and after (*top*) Fmoc group removal

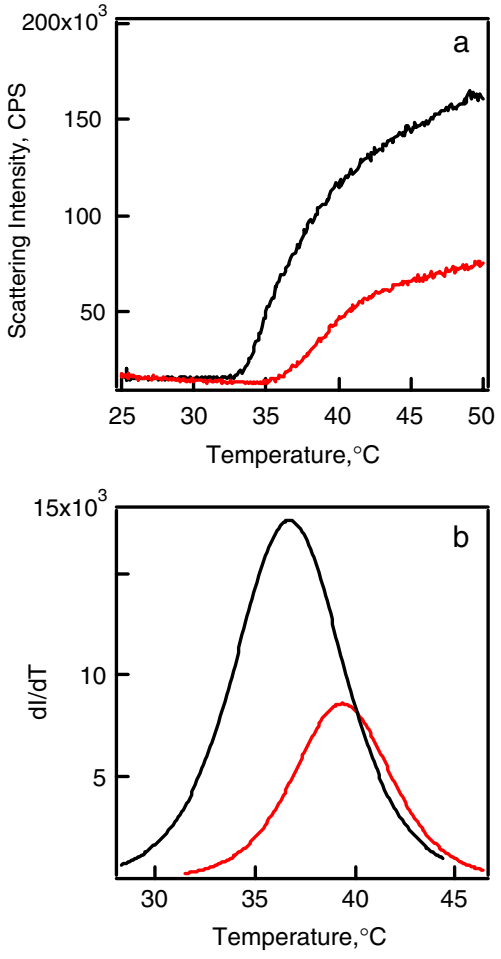


Fig. 3 LCST changes measured by turbidity (**a**) and also depicted as the first derivative of the fit for the turbidity curves (**b**) for protected c^5 (*black*) and deprotected c^5 (*red*)

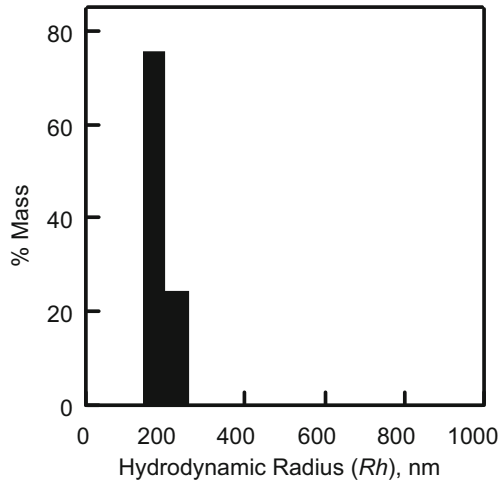


Fig. 4 A representative regularization histogram obtained from PCS indicating the colloidal stability (lack of aggregation) of the multifunctional nanogels

chemistries. The protected amine comonomer allowed the use of anionic surfactant and initiator, and the synthesis proceeded without any aggregation, even with high concentrations of the two comonomers (5 mol% amine and 10 mol% carboxylic acid). The nanogels obtained were fairly monodisperse (~13–15% according to PCS) and colloidally stable as can be seen from the regularization histogram obtained for the pNIPAm–Fmoc–AAc nanogels at pH 5.0 at 25 °C (Fig. 4). The successful incorporation of the AAc comonomer is indicated by the pH responsivity of the nanogels, as seen in the PCS studies (Table 3); pNIPAm–Fmoc–AAc nanogels at 25 °C showed a decrease in radius from 167 nm at pH 5.0 to 145 nm at pH 3.0. This change in size in response to pH for AAc-containing pNIPAm nanogels is well established and is due to changes in the interaction between the polymer chains and the solvent, as well as a decrease in the internal osmotic pressure upon protonation of the AAc moieties [31, 43]. At a pH above the pK_a of AAc (pK_a = 4.2), there is Coulombic repulsion between the polymer chains due to the negatively charged carboxyl groups. This repulsion is reduced at a pH below the pK_a because the carboxyl groups are mostly protonated, thereby causing the nanogels to deswell. Also, infrared spectroscopy spectra of the nanogels (data not shown) showed appearance of the carbonyl stretching peak

Table 3 Hydrodynamic radii (R_h) as a function of temperature and pH for pNIPAm–Fmoc–AAc nanogels as determined by PCS

Temperature (°C)	R_h[a] at pH 5.0 (nm)	R_h[a] at pH 3.0 (nm)
25	167 ± 1.6	145 ± 4.1
45	102 ± 1.4	84 ± 8.2

[a] Average of three measurements. The errors are standard deviations obtained for the measurements.

Table 4 Fmoc-protected macromonomer incorporation

Sample	Mole percent of monomer added	Mole percent of monomer detected by ^{1}H NMR[a]
pNIPAm–Fmoc core (c^5)	5.0	3.9 ± 0.2
pNIPAm–Fmoc shell (c^0/s^1)	1.0	1.3 ± 0.1
pNIPAm–Fmoc shell (c^0/s^5)	5.0	3.5 ± 0.3
pNIPAm–Fmoc–AAc shell	5.0	5.3 ± 0.2

[a] In some cases, the value obtained is slightly higher than expected possibly due to slight overestimation of the integrated peak area.

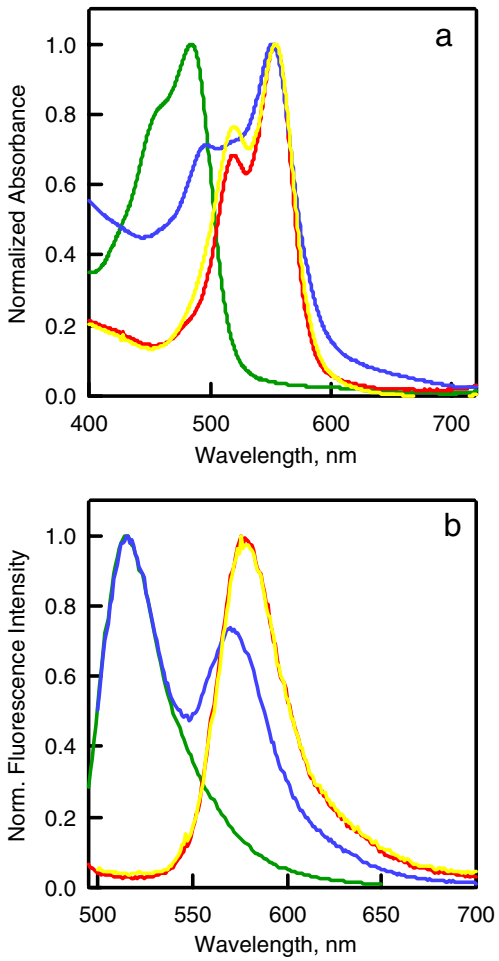


Fig. 5 Absorption (**a**) and emission (**b**) spectra for the multifunctional nanogels conjugated with fluorescent dyes. *Green* 5-Aminofluorescein conjugated to the carboxylic acid groups on the Fmoc-protected pNIPAm–Fmoc–AAc nanogels. *Yellow* TAMRA-SE conjugated with the free amines on the deprotected pNIPAm–Fmoc–AAc nanogels and *red* pNIPAm-Fmoc nanogels. *Blue* 5-Aminofluorescein and TAMRA-SE conjugated to the carboxylic acid and free amine groups, respectively, on the deprotected pNIPAm–Fmoc–AAc nanogels

at 1,715 cm^{-1} due to the carboxyl groups of AAc. ^1H NMR also confirmed the incorporation and deprotection of the Fmoc–PEG macromonomer.

We investigated the amount of the Fmoc–PEG macromonomer incorporated in the nanogels by ^1H NMR after the synthesis and purification. The amount of Fmoc–PEG macromonomer incorporated was determined by the ratio of the PEG peak (at 3.5 ppm, resulting from the methyl protons in the OCH_2CH_2 repeat units) to the main pNIPAm peak (at 1.1 ppm, resulting from the methyl protons of the isopropyl group) calculated at room temperature. All of the ratios are shown in Table 4. From the table, it can be seen that incorporating the very hydrophilic PEG macromonomer in the synthesis is difficult at high mole percent. This is because the synthesis is based on the hydrophobic collapse of growing pNIPAm chains, and this typically makes the incorporation of hydrophilic monomers difficult [17, 34]. However, our results suggest that if the growing chain is relatively hydrophilic (as obtained from incorporation of AAc), then the targeted concentration of PEG can be effectively incorporated. This phenomenon is currently under further investigation.

To further explore whether the amine and carboxylic acid groups are available for chemoligation, we conjugated 5-aminofluorescein to the acid groups and TAMRA-SE to the amine groups. Figure 5a and b shows the absorption and the fluorescence spectra of the conjugated nanogels, respectively. The carboxylic acid group can be easily conjugated to 5-aminofluorescein using carbodiimide chemistry if the amine group is kept protected, as seen by an emission maximum at 520 nm due to the conjugated fluorescein. After the conjugation of carboxyl group, the deprotection of the amines yielded free amine groups, which were further used for conjugation with TAMRA-SE. The pNIPAm–Fmoc and pNIPAm–Fmoc–AAc nanogels both showed emission maxima at 578 nm due to conjugated TAMRA. From the spectrum, it can be seen that for pNIPAm–Fmoc–AAc nanogels conjugated with both 5-aminofluorescein and TAMRA-SE, two distinct absorption and emission peaks were observed. There is a shift in the emission wavelength of TAMRA-SE from 578 to 570 nm for the pNIPAM–Fmoc–AAc nanogels conjugated with both 5-aminofluorescein and TAMRA-SE. Note that this shift is not observed for a simple aqueous solution containing the two dissolved dyes (data not shown). Although the precise reason for this is not known, we tentatively attribute the spectral shift to the difference in the local environment of the TAMRA fluorophores due to the presence of fluorescein molecules on the same nanoparticles. Regardless of the origins of these photophysical effects, these studies clearly suggest that the exposed functional groups on the nanogels are easily available for standard chemoligation reactions without functional group interference.

Conclusions

We have reported a very simple synthetic procedure for incorporating traditionally interfering functional groups such as amines and carboxylic acids in hydrogel nanoparticles. Well-established synthetic methodologies for protection and deprotection borrowed from organic chemistry were used in synthesizing colloidally stable nanogels equipped with these interfering groups. One advantage of using this technique is that it gives control over the access and availability of the functional groups at any stage in a post-polymerization conjugation strategy. The multiple functional groups present on the nanogels can be readily conjugated to multiple ligands. In summary, even though the functional groups are interfering, the protection of one of the groups allows the use of an otherwise interfering chemoligation step. The two functional groups on the nanogel can thus be functionalized in a pseudo-orthogonal fashion. This scheme for nanoparticle synthesis and subsequent functionalization provides a facile route for the future attachment of biologically important molecules such as targeting ligands, reporter molecules, and therapeutic agents onto the same nanoparticle, thus broadening their utility. Also, the synthetic route described provides very good control over the size, composition, and architecture of the nanoparticles, making it an attractive way to synthesize materials with complex topology.

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