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Nanostructured Thermosensitive Polymers with Radical Scavenging Ability

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Abstract: The thermosensitive [60]fullerene end-capped poly(*N*-isopropylacrylamide) was successfully synthesized by the reaction of C_{60} with dithiobenzoate-terminated poly(*N*-isopropylacrylamide), which was prepared by reversible addition-fragmentation chain-transfer (RAFT) polymerization in the presence of azobisisobutyronitrile (AIBN). Its structure was determined by FTIR, UV/Vis, and carbon and proton NMR spectroscopy as well as by size exclusion chromatography (SEC). The novel fullerenated polymer retained the thermosensitivity of poly(*N*-isopropylacrylamide). Moreover, it is soluble in water and most of the common organic solvents. Interestingly, it was able to form nanoparticle clusters in methanol and exhibited significant radical scavenging ability in cell viability and metabolic activity tests with fibroblasts and NOR-3 radicals.

Keywords: fullerenes · nanostructures · polymerization · radical scavenging

Introduction

One of the most promising applications of the fullerenes is based on their biological properties. Very exciting findings pioneered by Wudl,[1] Nakamura,[2] and co-workers have stimulated much interest and generated further successful investigations.[3,4] From a practical point of view, a fundamental issue that must be addressed when dealing with fullerenes is the solubilization of the candidate molecule in polar media that are necessary for biological tests.[3,4] Fullerenes are not soluble in water or in polar organic solvents. Therefore, they have to be chemically modified and a solubilizing appendage covalently attached.[5] Several approaches have been explored for the introduction of fullerenes into an aqueous or polar environment.[3,4] For example, water-soluble fullerene carboxylic acid derivatives, fullerenols, fullerene amino acid and supramolecular derivatives have been prepared and used in studying the biochemical and medicinal activities of fullerenes.[3–7] In particular, the introduction of C_{60} into water-soluble polymers is one of the simple and useful methods for solubilizing the C_{60} in water. Therefore, some C_{60}-containing water-soluble polymers have been prepared for potential biomedical applications.[8]

Poly(*N*-isopropylacrylamide) (PNIPAM) is a representative temperature-responsive polymer that exhibits a rapid and reversible hydration–dehydration change in response to small temperature cycles around its lower critical solution temperature (LCST; 32 °C) in aqueous media.[9–13] In recent years, considerable research attention has thus been focused on PNIPAM-based polymers due to their potential biomedical and pharmaceutical applications, including controlled drug delivery,[9] molecular separation,[10] tissue culture substrates,[11] enzyme activity controlling systems,[12] and materials for improved biocompatibility.[13] However, to the best of our knowledge, there are no reports regarding C_{60}-containing PNIPAM thermosensitive polymers. Therefore, here we report the synthesis, characterization, thermosensitivity, colloidal behavior, and radical scavenging properties of PNIPAM polymers with [60]fullerene end-caps.

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Results and Discussion

Reversible addition-fragmentation chain-transfer (RAFT) polymerization is a radical polymerization technique that controls molecular weight and molecular-weight distribution of polymers. Interestingly, it has been demonstrated that the RAFT-prepared polymers bear functional end groups (-S-C(S)-Ph)[14,15] that are able to further lead to polymerization of monomers in the presence of small amounts of initiators. More recently, Gilbert[14] and Müller[15] independently prepared PNIPAM with different molecular weights by means of RAFT polymerization and confirmed that each PNIPAM chain bears a (-S-C(S)-Ph)-functional endgroup by MALDI-TOF-MS. Similarly, we synthesized PNIPAM homopolymers using benzyl dithiobenzoate as the chain-transfer agent and azobisisobutyronitrile (AIBN) as the initiator by means of RAFT polymerization. Thus, it can be deduced that our prepared PNIPAM polymer also bears an active functional group (-S-C(S)-Ph) at one end of the chains.

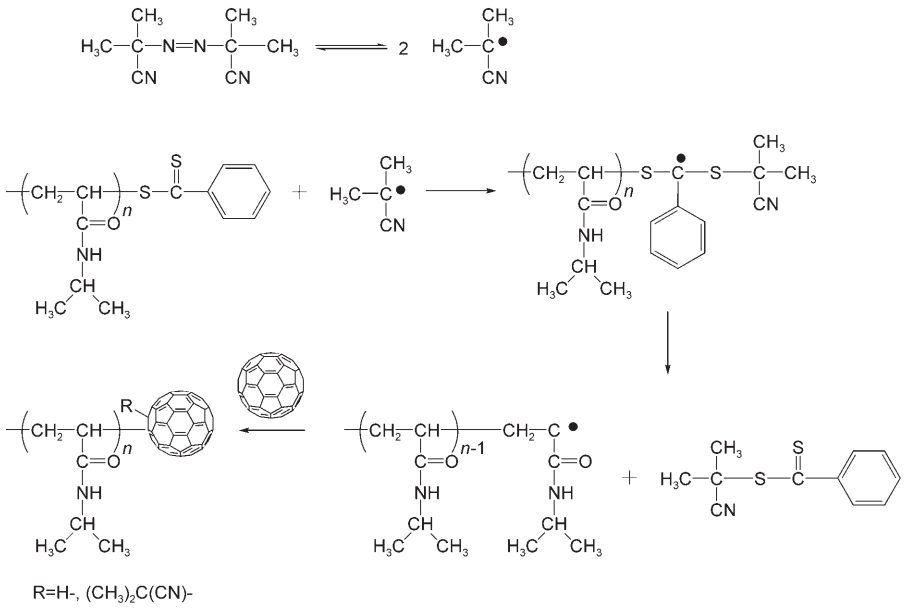
It is known that the addition reaction between C_{60} and free radicals is easily carried out to form fullerenated polymers.[16] According to the RAFT polymerization mechanism elucidated by the MALDI-TOF-MS technique,[14,15] the addition of 2-cyano-2-propyl radicals formed by the decomposition of the initiator AIBN to the C=S part of the endgroup (-S-C(S)-Ph) will result in the formation of macromolecular PNIPAM radicals. The macromolecular radicals easily add to the molecules of C_{60} to give the less active C_{60}-PNIPAM adduct radical. The new adduct radical will be terminated by combination with the other free radicals and/or abstracting a hydrogen atom. The proposed reaction mechanism is shown in Scheme 1.

We found that an *N*,*N*-dimethylformamide–chlorobenzene mixture is a good solvent system for both C_{60} and PNIPAM. Thus, the reaction between C_{60} and the dithiobenzoate-terminated PNIPAM was carried out in such a mixed solvent system at 80 °C in the presence of the initiator AIBN for 48 h. The resulting reaction mixture was separated to give the [60]fullerene end-capped poly(*N*-isopropylacrylamide) polymer (PNIPAM-C_{60}). PNIPAM-C_{60} dissolves in water and methanol, in addition to some organic solvents such as THF.

The UV/Vis absorption spectrum (Figure 1A) of the aqueous solution of PNIPAM-C_{60} is clearly different from that of the starting PNIPAM. A new absorption peak at 260 nm, which is a characteristic absorption for C_{60}, appeared in the spectrum of PNIPAM-C_{60}. This implies that a covalent link has been formed between the PNIPAM and the C_{60}. No other specific band could be detected apart from a new very long tail stretching over several 100 nm. The FTIR spectrum (Figure 1B) of PNIPAM-C_{60} showed the appearance of two new weak peaks at 2240 and 527 cm^{-1}, attributed to the characteristic peaks of the 2-cyano-2-propyl group and the functionalized C_{60} cage, respectively. The remaining peaks are essentially the same as those of the parent PNIPAM. In the ^{13}C NMR spectrum (Figure S1) of PNIPAM-C_{60}, a new broad band at $\delta = 135$–160 ppm (fullerene aromatic carbons) appeared. However, its ^{1}H NMR spectrum (Figure S2) is very similar to that of the parent PNIPAM.

The size exclusion chromatographic (SEC) trace (Figure 1C) of PNIPAM-C_{60} recorded by refractive index (RI) is almost the same as that of the parent PNIPAM, the only difference being that the elution time of PNIPAM-C_{60} is a little shorter than that of PNIPAM. This also indirectly confirmed that the C_{60} moiety was incorporated into one end of the polymer chains. The number-average molar mass and polydispersity index of PNIPAM-C_{60} resulted in $M_{n,SEC} = 12\,400\ g\,mol^{-1}$ and $M_w/M_n = 1.22$. However, as observed in a previous work,[17] there is a marked deviation between the SEC-determined and theoretical molecular weights ($M_{n,theory} = 23\,590\ g\,mol^{-1}$). The main cause for this discrepancy is that polystyrene standards were used for calibration.

The temperature-dependent transmittance changes of aqueous solutions of PNIPAM-C_{60} and PNIPAM are shown in Figure 2 (left). The aqueous solution of PNIPAM-C_{60} was transparent at lower temperature, while the transmittance of the solution changed drastically from transparent to turbid above characteristic temperatures. The PNIPAM-C_{60} solution exhibited the temperature-induced phase transition between the hydrated and dehy-



Scheme 1. Proposed mechanism for the reaction of dithiobenzaote-terminated poly(*N*-isopropylacrylamide) homopolymer with [60]fullerene.

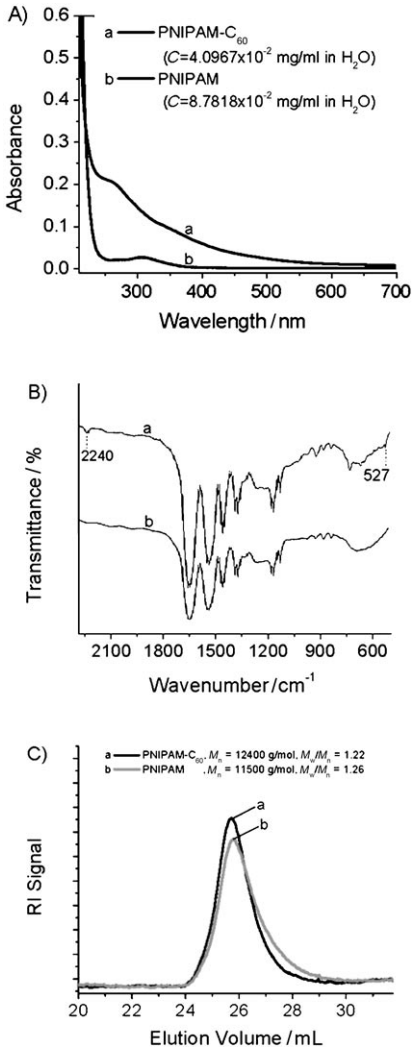


Figure 1. A) UV/Vis spectra, B) FTIR spectra, and C) SEC curves of the [60]fullerene end-capped PNIPAM (a), and the dithiobenzoate-terminated PNIPAM (b).

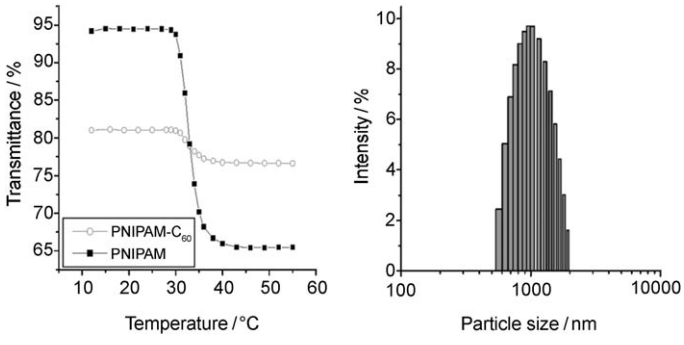


Figure 2. Temperature dependence of optical transmittance changes for PNIPAM-C_{60} and dithiobenzaote-terminated PNIPAM polymers (all sample solutions contained 10^{-2} wt% polymers in water) (left), and size distribution of the particles formed by [60]fullerene end-capped PNIPAM in water at room temperature (the diameter is plotted on a logarithmic scale) (right).

drated states of the polymer. As can be seen in Figure 2, the lower critical solution temperature (LCST) of PNIPAM-C_{60} is the same as that of the parent PNIPAM.

The temperature-dependent amphiphilic PNIPAM-C_{60} polymer can undergo self-association by a hydrophobic C_{60} cage, leading to the formation of a micellar structure in an aqueous phase at temperatures lower than its LCST. The characteristics of the PNIPAM-C_{60} polymer micelles in the aqueous phase were investigated by using dynamic light-scattering measurements. The particle-size distribution is presented in Figure 2 (right). Interestingly, the PNIPAM-C_{60} polymer is able to form nanoparticle clusters in methanol. Figure 3 shows the TEM image of particles formed in methanol.

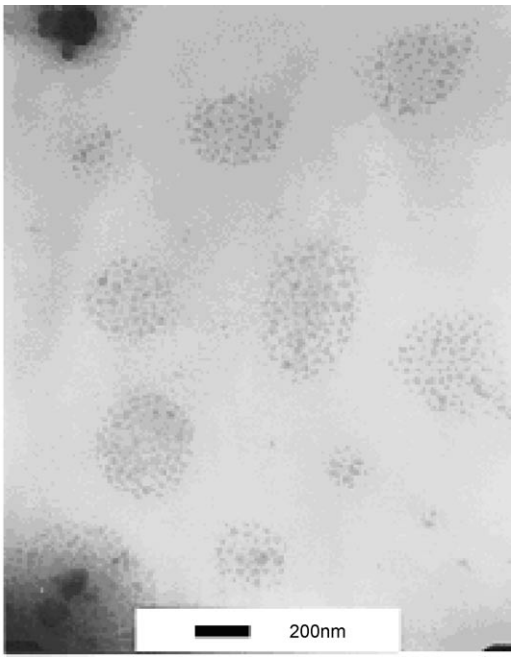


Figure 3. Transmission electron micrograph of the particles formed by PNIPAM-C_{60} in methanol.

PNIPAM-C_{60} could have potential biomedical applications including the treatment of acute or chronic inflammation. Different cells are known to produce radicals, such as $NO^{\bullet-}$ and $O_2^{\bullet-}$, which harm biomolecules and affect the viability, replication, and the regulatory and biosynthetic functions of cells. However, fullerenes and their derivatives are known to have a radical scavenging ability.[7] The effects of radicals on the cellular metabolism and viability were mimicked by the addition of NOR-3, a potent $NO^{\bullet-}$ radical donor, to NIH3T3 murine fibroblasts according to previous experimental procedures.[18] As observed in Figure 4, the NOR-3 radicals significantly reduced the cell viability and metabolic activity ($p = 0.001$). The addition of PNIPAM-C_{60} ameliorated the $NO^{\bullet-}$ effects and significantly increased the cell viability and metabolic activity ($p < 0.02$). Also, the addition of PNIPAM-C_{60} did not reduce the cell viability or metabolic

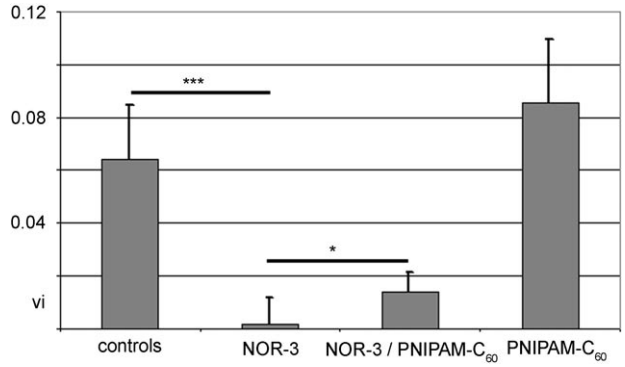


Figure 4. Viability index (vi, *y* axis) and metabolic activity of fibroblasts in the presence of PNIPAM-C_{60} and/or radical NOR-3 (controls represent the addition of neither PNIPAM-C_{60} nor NOR-3). The data show the mean value ± standard deviations ($n=8$ individual samples) of one representative experiment. For statistical analysis a two-sided T-test was performed (MSExcel®) and p values <0.05, <0.01, or <0.001 were considered to be significantly different and marked in the artwork accordingly (*, **, ***).

activity; the changes in comparison to nontreated controls were statistically not significant. Although PNIPAM-C_{60} shows the capability to enhance significantly the cell viability and metabolic activity, its full pharmacological potentials as well as the general toxic effects are under investigation.

Conclusion

The thermosensitive [60]fullerene end-capped poly(*N*-isopropylacrylamide) polymer was successfully prepared by means of reversible addition-fragmentation chain-transfer (RAFT) polymerization and further characterized by FTIR, UV/Vis, and NMR spectroscopy, and by size exclusion chromatography. The new fullerenated polymer retains the thermosensitivity of the poly(*N*-isopropylacrylamide). Moreover, it dissolves in common solvents such as water and methanol. It is able to form nanoparticle clusters in methanol and also exhibits a significant radical scavenging ability as demonstrated by cell viability and metabolic activity tests with fibroblasts and NOR-3 radicals.

Experimental Section

Materials: 2,2′-Azobis(isobutyronitrile) (AIBN; 97%, Aldrich) was purified by recrystallization from methanol. *N*-Isopropylacrylamide (NIPAM; 97%, Aldrich), was purified by multiple recrystallization from a mixture (60/40, v/v%) of toluene and hexane. The chain transfer agent, benzyl dithiobenzoate (BTB), was prepared by the ester interchange reaction between carboxymethyl dithiobenzoate [s-(thiobenzoyl)thioglycolic acid] and benzyl mercaptane.[19]

Synthesis of dithiobenzoate-terminated homopoly(*N*-isopropylacrylamide) by RAFT polymerization: NIPAM (14.847 g, 131.2 mmol), AIBN (20.0 mg, 0.122 mmol), and BTB (65.9 mg, 0.270 mmol) were dissolved in 1,4-dioxane (32 mL) to give a clear solution. The solution was transferred into an ampoule and degassed through five freeze–thaw–evacuate cycles, sealed under vacuum, and heated at 60°C for 24 h. The polymerization mixture was poured into a large excess of diethyl ether to precipitate the resulting polymer. The polymer was purified by reprecipitation from 1,4-dioxane into a large excess of diethyl ether three times, and then dried under vacuum at room temperature to yield 6.368 g of an orange polymer. The conversion of the monomer styrene was determined to be 42.45% by gravimetry. The molecular weight of the dithiobenzoate-terminated homopoly(*N*-isopropylacrylamide) polymer was determined by SEC. Also, its theoretical molecular weight was calculated according to the equation: $M_{n,theory}=([M]_i/[CTA]_i)fM_0+M_{CTA}$, in which $[M]_i$ and $[CTA]_i$ are the initial concentrations of the monomer NIPAM and the transfer agent BTB, respectively, f is the fractional conversion, and M_0 and M_{CTA} are the molecular weights of the monomer NIPAM and the used RAFT agent BTB. SEC: $M_{n,SEC}=11\,500$, $M_w=14\,500$, $M_w/M_n=1.26$; $M_{n,theory}=23\,590$.

Synthesis of [60]fullerene end-capped poly(*N*-isopropylacrylamide) (PNIPAM-C_{60}): Dithiobenzoate-terminated PNIPAM (500.0 mg, 2.12×10^{-2} mnmol based on the molecular weight of $M_{n,theory}=23\,590\,g\,mol^{-1}$, $M_w/M_n=1.26$), C_{60} (150.0 mg, 20.81×10^{-2} mmol), and AIBN (30.0 mg, 18.27×10^{-2} mmol) were dissolved in the mixed solvent (90/10, v/v%) of chlorobenzene and *N*,*N*-dimethylformamide (50 mL). The reaction mixture was degassed through four freeze–thaw–evacuate cycles, sealed under vacuum, and then heated at 80°C for 48 h. The resulting mixture was precipitated into a large excess of diethyl ether to give a crude product (0.590 g). The crude product was added into an Erlenmeyer flask with methanol (1000 mL). The methanolic mixture was stirred at room temperature for 2 h and was left to stand overnight. The insoluble solid was filtered off (the unreacted C_{60} and adduct of C_{60} with 2-cyano-2-propyl radicals). The filtrate was evaporated under reduced pressure to dryness to give a methanol-soluble crude product (0.500 g). The methanol-soluble crude product was dissolved in of THF (125 mL) to give an unclear solution, and then the solution was centrifuged to remove trace insoluble residue, and a clear solution was obtained. After removing THF under reduced pressure, the methanol-soluble crude product (0.474 g) was obtained and further purified according to the following typical procedure. A specific weight ratio $\chi_w=1.226$ in a methanol–water mixture at room temperature was first determined under which the precursor PNIPAM homopolymer is just soluble, then the crude product was dissolved in MeOH in advance to give a solution, then a suitable amount of water was added dropwise into the polymer solution (the amount of water added can be calculated using χ_w and the amount of MeOH used, in this case, 2.00 g of MeOH vs. 1.600 g of H_2O). The methanol–water mixture solution was vigorously shaken, and centrifuged to give two phases. The upper phase, which contained unreacted PNIPAM, was removed, and then the same amounts of MeOH and water were added in turn into the brown lower phase, and shaken and centrifuged again. The procedure was repeated three times. The lower brown phase was dried to give 0.275 g of pure product. Yield: 53%.

Characterization: ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Inova instrument (500 MHz) in $CDCl_3$, with tetramethylsilane as the internal reference. FTIR spectra were recorded on KBr slide in transmission mode on a Nicolet Impact 400 spectrometer. UV/Vis spectra and optical density measurements were conducted on a Cary UV/Vis spectrophotometer (Cary Varian Optical Spectroscopy Instruments). Size exclusion chromatography (SEC) was carried out on a Viscotek SEC assembly consisting of a model P1000 pump, a model T60 dual detector, a model LR40 laser refractometer, and three mixed-bed columns (10 cm) by using THF as eluent with a flow rate of 0.70 mL min^{-1}. Polymer concentrations for SEC experiments were prepared in a concentration of about 3 mg mL^{-1}. The SEC system was calibrated by using narrow standards prior to use. The polystyrene standards were purchased from American Polymer Standards Corp. The particle size and distribution were determined using laser scattering technique (DynaPro 99 Molecular Sizing Instrument). TEM measurements were performed on a Philips CM12 transmission electron microscope operated at 100 kV. Samples for TEM were deposited on Cu-grid and allowed to air dry.

Cell viability and metabolic activity tests: The NIH3T3 fibroblasts were inoculated in 96-well plates at a starting density of 3000 cells per well in Dulbecco's modified Eagle medium (DMEM) enriched with 10% bovine

serum and antibiotics.[18] Then the PNIPAM-C_{60} solution (ad 1.25 mg mL^{-1}), NOR-3 solution (Calbiochem, in DMSO ad 250 μM) or both were added to the cells and mixed. Nontreated cells served as controls. The cells were incubated in a cell incubator (humidified controlled atmosphere, 10% CO_2) overnight and the metabolic activity was measured by the MTT assay as described.[18] The data show the mean value ± standard deviations ($n=8$ individual samples) of one representative experiment. For statistical analysis a two-sided T-test was performed (MSExcel®) and p values <0.05, <0.01, or <0.001 were considered to be significantly different and marked in the artwork accordingly (*, **, *** in Figure 4).

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