Effect of the polymer chain length of poly(*N*-isopropylacrylamide) on the temperature-responsive phase transition behavior of its conjugates with [60]fullerene

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In order to develop biomedical materials with specific functionalities, thermoresponsive conjugates [poly(*N*-isopropylacrylamide)-C_{60} (PIPAAm-C_{60})]of [60]fullerene (C_{60}) and PIPAAm with two different polymer chain lengths (4 and 20 kDa) were synthesized by atom transfer radical polymerization. The effects of the polymer chain length on the temperature-responsive phase transition behavior of the synthetic PIPAAm-C_{60} conjugates were probed by means of various physicochemical techniques. The coexistence of unimers and molecular assemblies of PIPAAm-C_{60} was observed by gel permeation chromatography and dynamic light scattering studies in two PIPAAm-C_{60} aqueous solutions below their lower critical solution temperatures (LCSTs). Additionally, below their LCSTs, differences in PIPAAm chain length gave rise to changes in the composition of the unimers and molecular assemblies. In response to temperature, the absorbance of the PIPAAm-C_{60} aqueous solution changed according to a two-step behavior profile. Increasing temperature during the primary stage, where a change in the absorbance of the PIPAAm-C_{60} aqueous solution took place, did not change the transition temperature, regardless of the solution concentration of PIPAAm-C_{60}. This absorbance change was associated with the phase transition of the molecular assemblies of PIPAAm-C_{60}. However, at the second stage, the transition temperature shifted to a higher value with the decrease in the concentration of PIPAAm-C_{60}, in the same manner as free PIPAAm chains. The second change was associated with the phase transition of the unimeric PIPAAm-C_{60}. Differences in PIPAAm chain length gave rise to the change in the phase transition behavior of PIPAAm-C_{60} aqueous solution. Therefore, the chain length of PIPAAm was found to be a predominant factor involved in the solution characteristics of PIPAAm-C_{60}. Consequently, the PIPAAm-C_{60} is expected to be an intelligent biomaterial possessing heat-induced accumulation and bioactivities. © *2010 American Vacuum Society.* [DOI: 10.1116/1.3319348]

I. INTRODUCTION

Fullerene, C_{60}, has recently attracted significant attention because of its unique biological properties, such as antioxidant activity, neuroprotective properties, enzyme inhibition, DNA cleavage, and anticancer activity.[1–6] However, the insolubility of C_{60} in water has hampered many of its biomedical applications, such as photodynamic tumor therapy. One of the strategies to improve its solubility is the chemical modification of C_{60} with hydrophilic compounds, such as alcohols, carboxylic acids, amines, or long-chain hydrophilic polymers.[7–10] To address this issue, we have recently prepared the monoadduct of C_{60} with temperature-responsive poly(*N*-isopropylacrylamide) (PIPAAm) synthesized by means of radical polymerization, and investigated the physicochemical properties of the conjugate (PIPAAm-C_{60}) in water.[11] We have found that PIPAAm-C_{60} exhibits a rapid and reversible temperature-dependent soluble/insoluble transition in response to a narrow range of temperature variation across the lower critical solution temperature (LCST) of the PIPAAm-C_{60}, induced by the phase transition of the PIPAAm chain binding to C_{60}. Turbidity and dynamic light scattering (DLS) measurements implied that below the LCST, the PIPAAm-C_{60} formed a micellelike structure composed of C_{60} molecules as the inner core, whereas above the LCST, the micelles aggregated and the solution turned cloudy. PIPAAm itself is hydrated and expanded in water below its LCST, and changes to a compact form above its LCST by sudden dehydration and inter- and intramolecular hydrophobic interactions.[12–14] The LCST of PIPAAm itself is strongly dependent on its chain length[15] and end groups.[16] PIPAAm with a longer chain or a more hydrophobic end group possesses a lower LCST. In agreement with this, Nakayama and Okano[17] reported that the phase transition of the polymeric micelle comprised of PIPAAm and a hydrophobic polymer was dependent on both the outermost end group of the micelle and the chain length of PIPAAm. Additionally, Chung *et al.*[18] reported that the structural deformations of the micelle's inner core induced by PIPAAm's phase transition were dependent on the hydrophobic component of the inner core.

In order to develop more effective functionalities of PIPAAm-C_{60} as a biomaterial possessing heat-induced accumulation and bioactivities, it is important to probe the effects of the chain length of PIPAAm on the dispersion behavior

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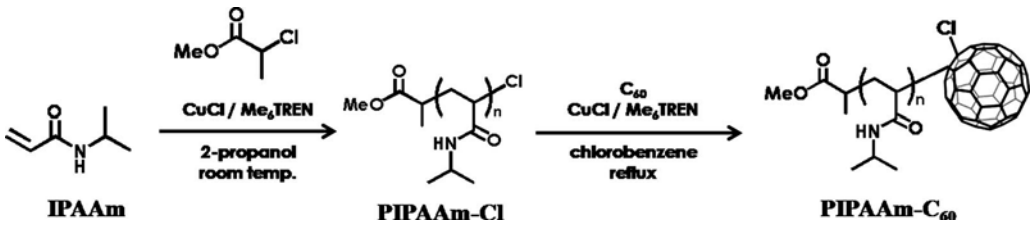

FIG. 1. Reaction scheme for synthesis of PIPAAm-C_{60} by an ATRP technique.

and phase transition of PIPAAm-C_{60} in aqueous solution, in response to temperature. In this study, to eliminate the influence of the polydispersity of PIPAAm to the thermoresponsive solution characteristics of PIPAAm-C_{60}, we have prepared the PIPAAm-C_{60} using the narrow-polydisperse PIPAAm made by atom transfer radical polymerization (ATRP). Then, we have analyzed clearly the thermally sensitive properties of PIPAAm-C_{60} in aqueous solution by turbidity and DLS measurements.

II. EXPERIMENT

A. Materials

C_{60} (99.99%) was obtained from Materials Technologies Research Ltd. (Cleveland, OH, USA). *N*-Isopropylacrylamide (IPAAm) was kindly provided by KOHJIN (Tokyo, Japan) and was purified by recrystallization from hexane and dried at 25 °C *in vacuo*. Tris[(2-dimethylamino)ethyl]amine (Me_6TREN) was prepared according to the method of Queffelec *et al.*[19] Copper (I) chloride (CuCl, 99.995%) and methyl 2-chloropropionate (MCP) (97%) were purchased from Sigma-Aldrich. Chlorobenzene and 2-propanol were obtained from Kanto Chemical Co. (Tokyo, Japan).

B. Preparation of PIPAAm macroinitiator

Well-defined PIPAAm was synthesized by the ATRP technique using CuCl/Me_6TREN as a catalyst (Fig. 1).[15,16] The reactions were carried out at room temperature under argon atmosphere. IPAAm, CuCl and 2-propanol were combined in a Schlenk tube, which was deoxygenated with argon. Me_6TREN was then added and the solution was stirred to allow the formation of the CuCl/Me_6TREN complex. MCP was then added as the initiator. To remove the catalyst and unreacted monomer, the reaction mixture was passed through a neutral alumina column and then the PIPAAm was precipitated in diethyl ether and dried *in vacuo*. The synthesized PIPAAm possessed a chloro group at one end, and hereafter is denoted as PIPAAm-Cl. In this study, two lots of PIPAAm-Cl with different molecular weights were synthesized. The two synthetic PIPAAm-Cls were denoted as P-4 and P-20, and their properties are summarized in Table I.

C. Preparation of PIPAAm-C_{60}

PIPAAm-Cl was used as a macroinitiator for the synthesis of PIPAAm-C_{60} (Fig. 1). PIPAAm-Cl, CuCl, C_{60} (1:1:3 molar ratio), and 100 ml of chlorobenzene were placed in a 300 ml round-bottom flask. After the addition of Me_6TREN (equivalent to macroinitiator), the reaction solution was refluxed for 24 h at 120 °C under argon atmosphere. Next, the solution was evaporated, tetrahydrofuran (THF) was added, and the solution was passed through a neutral alumina column to remove the catalyst and unreacted C_{60}. Finally, the PIPAAm-C_{60} was isolated by precipitation in diethyl ether and dried *in vacuo*. The two PIPAAm-C_{60}'s prepared with P-4 and P-20 were denoted as FI-4 and FI-20, respectively. Their characterization data are summarized in Table I.

D. Measurements

By ^{1}H NMR spectrometry (500 MHz, JNM-LA500, JEOL, Japan) using D_2O (Kanto Chemical, Japan), number-averaged molecular weights (M_n) of the PIPAAm-Cls were estimated from the ratio of the integral areas of the terminal methoxy protons (3.7 ppm) to that of the methine protons (3.9 ppm) of the polymer side chain (see Fig. S1 in supporting information).[20]

TABLE I. Characterization of PIPAAm-Cl and PIPAAm-C_{60}.

PIPAAm-Cl				PIPAAm-C_{60}			
Code	10^3 M_n [a]	10^3 M_n [b]	M_w/M_n [b]	Code	10^3 M_n [b]	M_w/M_n [b]	C_{60} content (wt %)[c]
P-4	4.65	8.7	1.12	FI-4	10.3	1.13	11.3(13.4)
P-20	20.3	32.8	1.19	FI-20	33.7	1.19	2.8(3.4)

[a]Determined by ^{1}H NMR spectra in D_2O.
[b]Determined using polystyrene standards by GPC analysis in THF.
[c]Measured by DTA on the PIPAAm-Cl and the PIPAAm-C_{60}; the data in parentheses are the theoretical values based on M_n determined by ^{1}H NMR data and the C_{60}-monoadduct structure.

Additionally, M_n and polydispersity indices of the PIPAAm-Cls and PIPAAm-C_{60}'s were determined by gel permeation chromatography (GPC) (CCP-8020, Tosoh, Japan) using standard polystyrenes for calibration. THF was used as the mobile phase (at a flow rate of 1.0 ml/min at 40 °C). GPC elution profiles were obtained with a refractive index (RI) detector system (RI-8022, Tosoh) and a UV detector system (UV-8020, Tosoh, monitoring at 330 nm). To confirm the molecular assemblies of the PIPAAm-C_{60}s in water, the elution profiles for P-4, FI-4, P-20, and FI-20 were obtained from GPC measurements (Model 300 TDA, Viscotek, Texas, USA) with a RI detector (Triple Detector Array, Viscotek). Ultrapure water was used as the mobile phase (at a flow rate of 1.0 ml/min at 25 °C).

The weight percentage of C_{60} incorporated in PIPAAm-C_{60} was determined with thermogravimetric analysis (TGA) measurements (EXSTAR 6000 TG/DTA, Seiko Instruments Inc., Japan) at a heating rate of 10 °C/min under nitrogen. These measurements showed that C_{60} was thermally stable below 600 °C, whereas PIPAAm completely decomposed above 400 °C and only the C_{60} contents remained.

DLS measurements of PIPAAm-C_{60} aqueous solution were carried out on a DLS-7000 instrument (Otsuka Electronics Co., Japan) equipped with an argon ion (Ar^+) laser (488 nm) at a scattering angle of 90° at 20 °C. The concentrations of the two PIPAAm-*b*-C_{60} aqueous solutions were 5.0 mg/ml in ultrapure water. From the time correlation function of the scattering intensity, the decay time distribution function was obtained with an inverse Laplace transform program (CONTIN) provided by ALV (Germany), where the hydrodynamic radius (R_h) was determined from the Stokes–Einstein equation.

Absorbances of 5.0 mg/ml PIPAAm-Cl and 5.0 mg/ml PIPAAm-C_{60} in ultrapure water were measured at 600 nm with a UV-visible-near infrared spectrometer (V-570, Jasco, Japan) equipped with a Peltier-type thermostatic cell holder (ETC-505T, Jasco), at a heating rate of 0.2 °C/min in the temperature range from 25 to 55 °C.

III. RESULTS AND DISCUSSION

A. Identification of the synthetic PIPAAm-C_{60}

To synthesize merely a monoadduct of PIPAAm chains onto C_{60} and avoid the formation of multiple adducts, a higher ratio of C_{60} to PIPAAm-Cl (3:1) was adjusted as the experimental condition. Unreacted C_{60} was removed by dissolving the products in THF in view of the characteristics that the solubility of C_{60} is very small in THF. The GPC elution profiles for P-4 and FI-4 with both RI and UV detectors monitoring C_{60} at 330 nm are shown in Fig. 2. For P-4, there was one peak observed in the GPC curve with a RI detector but no peaks with a UV detector monitoring C_{60} at 330 nm. On the other hand, for FI-4, there was one peak in each of the GPC curves with both RI and UV detectors monitoring C_{60} at 330 nm. The two GPC elution profiles for FI-4 were nearly identical. Therefore, these results indicated

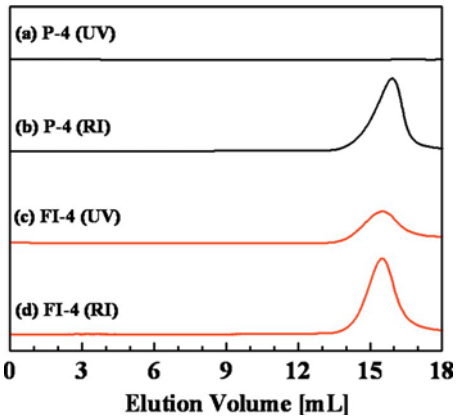

FIG. 2. (Color online) GPC curves of P-4 and FI-4 by [(a) and (c)] UV detection and [(b) and (d)] RI detection, in THF at 40 °C at a flow rate of 1.0 ml/min.

that the PIPAAm chain of FI-4 was covalently bound to a C_{60} molecule. Moreover, the GPC curve for FI-4 by RI showed the absence of multiple or broadened peaks. The M_w/M_n of FI-4 is almost the same value as P-4, as shown in Table I. These findings implied neither further ATRP-induced chain extension of PIPAAm bound onto C_{60} nor product of PIPAAms multiple adducts with a C_{60} molecule. Thus, FI-4 was confirmed to be a PIPAAm monoadduct with a C_{60} molecule. Likewise, FI-20 was also identified to be a PIPAAm monoadduct with a C_{60} molecule. The TGA profiles of P-4 and P-20 showed that the weight of the sample was negligible after 500 °C. On the other hand, the C_{60} was still stable below 600 °C. Thus, based on the weight loss of the samples at 500 °C, the C_{60} content of FI-4 and FI-20 was calculated. The experimental values for C_{60} content in both FI-4 and FI-20 from TGA roughly agreed with theoretical values estimated from the molecular weight of the PIPAAm monoadduct with C_{60}, as shown in Table I.

B. Characterization of dispersed PIPAAm-C_{60} in water below its LCST

The GPC elution profiles for P-4, P-20, FI-4, and FI-20 with a RI detector using ultrapure water as the mobile phase at 25 °C below the LCST of all samples are shown in Fig. 3. The GPC curve for P-4 showed the presence of only one

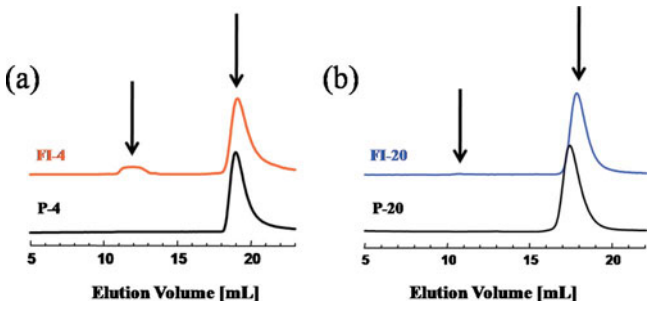

FIG. 3. (Color online) GPC curves of (a) P-4 and FI-4 and (b) P-20 and FI-20 in water. These measurements were performed at a concentration of 5.0 mg/ml at 25 °C with a flow rate of 1.0 ml/min.

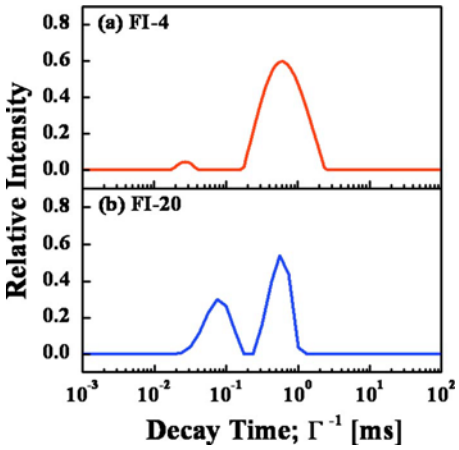

FIG. 4. (Color online) Decay time distribution functions of (a) FI-4 and (b) FI-20 at 25 °C.

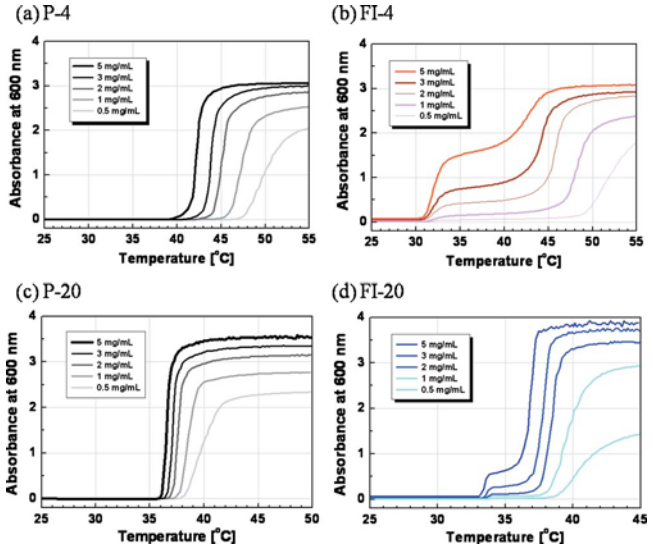

FIG. 5. (Color online) Temperature dependence of optical transmittance at 600 nm of (a) P-4, (b) FI-4, (c) P-20, and (d) FI-20, in aqueous solutions at a concentration of 5.0 mg/ml and heating rate of 0.2 °C/min.

peak, whereas the GPC curve for FI-4 showed the presence of two peaks. As shown in Fig. 2, from the GPC measurement for FI-4 with RI using THF as the mobile phase, only one peak was observed, similar to that of P-4 using ultrapure water as the mobile phase. The FI-4 peak that eluted at approximately the same time as that of P-4 was assigned to the unimer, namely, a PIPAAm-C_{60} (FI-4) molecule. The primary peak of FI-4, which eluted at an earlier time than that of P-4, indicated the existence of a species with a higher molecular weight than a single FI-4 molecule and would correspond to a molecular assembly of FI-4. It was suggested that the molecular assembly of FI-4 formed a core-shell micelle structure composed of an inner core of C_{60} molecules and an outer shell layer of PIPAAm chains, below the LCST of FI-4, resulting from intermolecular hydrophobic interactions between C_{60} molecules in water. Therefore, for FI-4, the unimer and the molecular assembly coexisted in water at 25 °C. Similar to P-4, the GPC curve for P-20 showed the presence of only one peak and the GPC curve for FI-20 showed the presence of two peaks. However, the primary peak of FI-20, which eluted earlier than that of P-20, was slight. This indicated that the amount of the molecular assembly tended to decrease with increasing polymer chain length in PIPAAm-C_{60}. A longer polymer chain would give rise to a decrease in intermolecular hydrophobic interactions among C_{60} molecules due to the decrease in the C_{60} contents, and increases the polymer wrapping effects to C_{60} molecules through a charge-transfer complexation with polymers containing a carbonyl group.[21–23]

From DLS measurements on both FI-4 and FI-20 at 20 °C (below each LCST), two decay modes were observed in the decay time distribution, as shown in Fig. 4. The first and slow modes of FI-4 correspond to hydrodynamic radius (R_h) values of 4.7 and 87.3 nm, respectively, whereas those of FI-20 correspond to 9.5 and 83.0 nm, respectively. In view of the GPC results, it is inferred that the first mode is ascribed to the unimeric FI-4 and FI-20, whereas the slow mode is associated with the molecular assembly of FI-4 and FI-20. Consequently, from the DLS measurements, it was also confirmed that the unimer and molecular assembly of PIPAAm-C_{60} coexist in water below their LCSTs.

C. Effect of the chain length of PIPAAm on the thermal transitions of PIPAAm-C_{60}

The absorbance changes in FI-4 and FI-20 at various concentrations of the PIPAAm-C_{60} aqueous solutions in response to temperature, compared to that of P-4 and P-20 are shown in Fig. 5. For P-4 and P-20, the one-step absorbance change of the aqueous solution was observed in response to increasing temperature at a rate of 0.2 °C/min from 25 to 55 °C, regardless of the concentration. However, the temperature at the onset of the absorbance change shifted lower with an increase in the concentration of P-4 and P-20, on the basis of the changes in the polymer-solvent interaction.[24] On the other hand, for both FI-4 and FI-20, two-step absorbance changes were observed in response to temperature. Interestingly, the temperature at the primary onset of the rapid absorbance change was approximately constant, independent of the PIPAAm-C_{60} concentration, whereas the temperature at the second onset was dependent on the PIPAAm-C_{60} concentration in the same manner as with P-4 and P-20. In view of these observed concentration effects, the first and second phase transitions of these PIPAAm-C_{60} solutions are suggested to be associated with aggregation of the molecular assembly and unimer of PIPAAm-C_{60}, respectively.

For chain length effects, the magnitude of the first-step change in the absorbency of FI-4 was larger than that of FI-20, but the magnitude of the second-step change was smaller. However, the temperature at the second onset of FI-4 was higher than that of FI-20. In correlation with this behavior, Xia *et al.*[15] reported that the LCST of a PIPAAm chain synthesized by ATRP shifted lower with increasing

molecular weight of PIPAAm. These findings indicate that the second-step change in absorbance is brought about by aggregation of the unimeric PIPAAm-C_{60}.

In regard to the reversibility, the transmittance changes in the PIPAAm-C_{60} solution were measured in response to reversible temperature changes across the LCST. Increasing the temperature, the PIPAAm-C_{60} solution was turbid, and then, the transmittance was restored by cooling (see Fig S2 in supporting information).[20] The transmittance profile in response to reversible temperature changes possessed the hysteresis property. Interestingly, the PIPAAm-C_{60} with shorter polymer chain showed the transmittance profile with the larger hysteresis in response to reversible temperature changes (data not shown). The detail of the hysteresis will be described elsewhere.

Here, it should be noted that although unreacted PIPAAm-Cl might exist in scarce amounts in the PIPAAm-C_{60} system, it is difficult to completely remove this component because of the same characteristics of both PIPAAm-Cl and PIPAAm-C_{60}. However, we believe that the unreacted PIPAAm-Cl, namely, free polymer chain, would not trigger the two-step phase transition behavior of the PIPAAm-C_{60}. As a verification experiment for this, the transmittance changes in the mixture solution of micelle comprised of poly[styrene-*block*-(*N*-isopropylacrylamide-*co*-*N*, *N*′-dimethylacrylamide)] [PSt-*b*-P(IPAAm-*co*-DMAAm)] and free PIPAAm chain were measured in response to temperature changes (see Fig S3 in supporting information).[20] Consequently, the mixture showed not two separated phase transition but one sharp transition. The LCST of the mixture lied between that of pure micelle and pure free polymer, and was dependent on the weight ratio of polymer/micelle (p:m). The phase transition of the mixture containing free PIPAAm in a low dose was nearly identical with that of the pure micelle. The above result confirms our suggestion that free PIPAAm would not induce to the two-step phase transition in this PIPAAm-C_{60} system.

The foregoing results led us to conclude that the first and second phase transitions of these PIPAAm-C_{60} solutions are associated with the aggregation of the molecular assembly and unimer of PIPAAm-C_{60}, respectively. Moreover, this work demonstrates that the phase transition behavior of PIPAAm-C_{60} in water is controllable by varying the chain length of PIPAAm. Preliminary experiments have shown that the PIPAAm-C_{60} conjugates have effective bioactivities, such as superoxide generation by photoirradiation. Further studies are continuing in our group and will be published in due course.

IV. CONCLUSIONS

Thermoresponsive conjugates PIPAAm-C_{60} comprising a C_{60} molecule and a PIPAAm chain as a thermoresponsive segment with varying chain lengths were synthesized by ATRP polymerization. Unimeric PIPAAm-C_{60} and a micellelike molecular assembly of PIPAAm-C_{60} coexisted in the PIPAAm-C_{60} aqueous solution below their LCSTs. The existence ratio of the molecular assembly to the unimer in the PIPAAm-C_{60} aqueous solution decreased with an increase in the chain length of PIPAAm. The PIPAAm-C_{60} aqueous solution showed two-step phase transitions associated with aggregation of the molecular assembly and the unimer in response to temperature. The chain length of PIPAAm was found to be the predominant factor involved in the solution characteristics of PIPAAm-C_{60}. Consequently, the PIPAAm-C_{60} is expected to be an intelligent biomaterial possessing heat-induced accumulation and bioactivities.

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