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Original Article

Synthesis and characterization of thermoresponsive polymeric nanoparticles

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Abstract We synthesized drug-loaded thermoresponsive poly(N-isopropylacrylamide) with acrylic acid (poly(NIPAM-*co*-AA)) nanoparticles. Dynamic light scattering analysis showed that the size of poly (NIPAM-*co*-AA) nanoparticles was significantly affected by temperatures, indicating that the sizes were changed from 400 nm at 25°C to 100 nm at 37°C. ^{1}H nuclear magnetic resonance (NMR) and fourier transform infrared spectroscopy (FT-IR) analysis demonstrated the synthesis of poly(NIPAM-*co*-AA) nanoparticles, showing that drugs (e.g., doxorubicin, retinoic acid) were conjugated with poly(NIPAM-*co*-AA) nanoparticles. We also analyzed the cumulative release of drugs in a temporal manner, indicating that doxorubicin was highly released from poly(NIPAM-*co*-AA) nanoparticles at 48 hours compared to retinoic acid. Therefore, this thermoresponsive drug-loaded poly (NIPAM-*co*-AA) nanoparticle could be a powerful tool for drug delivery and release applications.

Keywords: Nanoparticle, Polymer, Synthesis, Drug delivery, Controlled release

Introduction

The nanoparticles are of great interest in regulating drug delivery and controlled release[1-4]. Recently, smart thermoresponsive poly(N-isopropylacrylamide) (NIPAM) nanoparticles have previously been used for tissue engineering and biosensor applications[5-9]. For instance, poly(NIPAM-*co*-acrylic acid (AA)) hydrogels have been synthesized and their irreversible recovery of the phase transition has been investigated to understand the effect of the heating and cooling process on the phase transition[10]. The volume phase transition temperature was determined by analyzing the spectra of fourier transform infrared spectroscopy (FT-IR). It was observed that water molecules were ejected from poly (NIPAM-*co*-AA) hydrogels in the heating process, showing that hydrogen bonds of acrylic acid were stable and their size was subsequently decreased. In contrast, NIPAM moieties were swelled by hydrogen bonds of the water in the cooling process, showing thermally irreversible recovery of the phase transition. Poly(NIPAM-*co*-poly(L-lysine)) and poly(NIPAM-*co*-*N*-hydroxymethylacrylamide)-*b*-poly (glutamic acid) has been developed for the thermo- and pH-sensitive polyion complex micelle[11]. The cationic poly(L-lysine) and anionic poly(glutamic acid) was used as a core, whereas hydrophilic poly(NIPAM-*co*-*N*-hydroxymethylacrylamide) was employed as a shell. The temperature enabled the control of the morphology of polyion complex micelles, showing that core-shell polyion complex micelle transformed into core-shell corona structure at high temperature. It investigated the effect of pH on solubility of polyion complex micelles, indicating that uncharged PGA blocks showed low solubility and hydrophobic properties at pH 2. This low solubility and hydrophobic property allowed for self-assembled structures of micelles that could contain insoluble poly(glutamic acid) core and hydrophilic poly(NIPAM-*co*-*N*-hydroxymethylacrylamide) shell. Furthermore, confocal laser scanning images showed endocytosis, indicating that HeLA cells cultured with

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polyion complex micelles remained highly viable. The pH- and thermoresponsive pullulan microspheres have also been developed[12]. Poly(NIPAM-*co*-acrylamide) was grafted on hydrophilic pullulan microspheres. These pullulan microspheres showed internal structures with high density of wracks. The swelling degree of poly(NIPAM-*co*-acrylamide) on pullulan microspheres was increased due to the hydrophilic property of pullulan. The phase transition temperature enabled the control of swelling degree of poly(NIPAM-*co*-acrylamide) on pullulan microspheres, indicating that swelling degree was abruptly decreased around 37˚C. The drug release study demonstrated that the drug release was regulated by cleavage rates of electrostatic bonds and diffusions.

The anticancer drugs (e.g., doxorubicin, paclitaxel)-loaded NIPAM nanoparticles have also been used for cancer therapy applications[13-17]. For instance, doxorubicin-loaded NIPAM nanoparticles have been synthesized for cancer therapy applications[13]. Thermally responsive block copolymers, poly(NIPAM-*co*-*N*,*N*-dimethylacrylamide)-*b*-poly (D,L-lactide-*co*-glycolide), was employed to generate spherical micelle structures. It observed that the size of nanoparticles increased with the chain length of poly(D,L-lactide-*co*-glycolide), because micelles containing longer chain length tended to be aggregated. The drug loading efficiency increased with chain length of poly(D,L-lactide-*co*-glycolide). It also demonstrated that doxorubicin was largely released from micelles at a temperature above lower critical solution temperature (LCST). In contrast, micelle structures were stable at the temperature below LCST, indicating slow diffusion rate of doxorubicin. Dual stimuli-responsive poly(NIPAM-*b*-poly(L-histidine)) micelles have been synthesized for therapy of human hepatocellular carcinoma cells[14]. Doxorubicin was encapsulated inside self-assembled micelles with spherical shapes and intramolecular hydrophobic interactions enabled the increase of drug loading efficiency. It demonstrated that the micelle size and controlled drug release was regulated by temperature and pH conditions, indicating that the size of block copolymer micelles was inversely proportional to pH conditions and doxorubicin was largely released at acidic pH or high temperatures. Thus, it was observed that most human hepatocellular carcinoma cells were dead at acidic pH and high temperatures. Thermoresponsive iron oxide superparamagnetic nanoparticles have previously been synthesized to control the release of doxorubicin in microfluidic devices[15]. The nanobeads were aggregated at high temperatures to become clusters with high magnetic moments. The polyNIPAM was employed as a shell on the surface of nanobeads. FT-IR analysis demonstrated the compositional characterization, indicating that the surface of nanobeads showed different compositions of polymers. Silicon-based microfluidic channels were employed to mimic the circulatory capillaries, showing that magnetic nanobeads aggregated at the corners of zigzag-shaped channels and released doxorubicin in a temporal manner. The gliadin nanoparticles have also been used for apoptosis in breast cancer cells[16]. The gliadin and gliadin-gelatin composite nanoparticles were generated by electrospray deposition methods. X-ray diffraction analysis confirmed that the gliadin-gelatin composite nanoparticles were conjugated and physical structures of proteins were not altered during the electrospray deposition process. The cyclophosphamide, one of anticancer drugs, was released from gliadin and gliadin-gelatin nanoparticles, indicating that gliadin-gelatin composite nanoparticles largely released cyclophosphamide compared to hydrophobic gliadin nanoparticles due to hydrophilic property of gelatin. Furthermore, the western blotting analysis demonstrated that breast cancer cells cultured with cyclophosphamide-loaded gliadin nanoparticles were apoptotic and it was confirmed by down-regulation of Bcl-2 marker. Although doxorubicin-loaded polyNIPAM nanoparticles were developed, retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles were not elucidated. In this paper, we synthesized thermoresponsive retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles and analyzed their controlled release behaviors compared to doxorubicin-loaded poly(NIPAM-*co*-AA) nanoparticles.

Results and Discussion

Synthesis of poly(NIPAM-*co*-AA) nanoparticles

We synthesized thermoresponsive poly(NIPAM-*co*-AA) nanoparticles (Figure 1A). The transmission electron microscope (TEM) image showed that the shape of poly(NIPAM-*co*-AA) nanoparticles was round and homogeneous (Figure 1B). Briefly, NIPAM was conjugated with AA and was subsequently conjugated with potassium persulfate (KPS) initiator at 80˚C to create poly(NIPAM-*co*-AA) nanoparticles (Figure 1C). We observed that the solution of poly(NIPAM-*co*-AA) nanoparticles was significantly affected by temperatures (Figure 2). It represented that the solution of poly (NIPAM-*co*-AA) nanoparticles polymerized at 37.2˚C was opaque, whereas it changed to become transparent at room temperature. Ultraviolet (UV)-visible spectrophotometer analysis demonstrated that the solution of poly(NIPAM-*co*-AA) nanoparticles was absorbed at 350 nm wavelength and their absorbance was abruptly increased over 37.2˚C, whereas the absorbance below 37.2˚C was steadily constant. Thus, we confirmed that

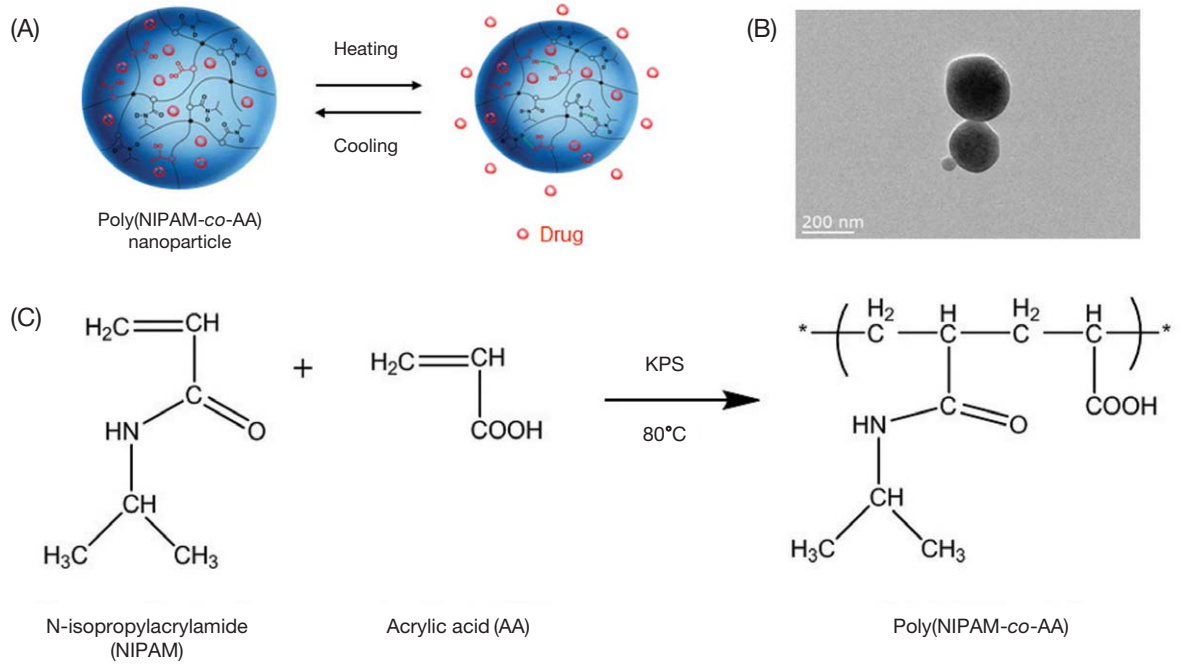

Figure 1. Synthesis of poly(NIPAM-*co*-AA) nanoparticles. (A) Schematic illustration of volume phase transition of poly (NIPAM-*co*-AA) nanoparticles in aqueous solution. (B) TEM image of poly(NIPAM-*co*-AA) nanoparticles. (C) Formation of thermoresponsive poly(NIPAM-*co*-AA) nanoparticles using KPS initiator at 80°C.

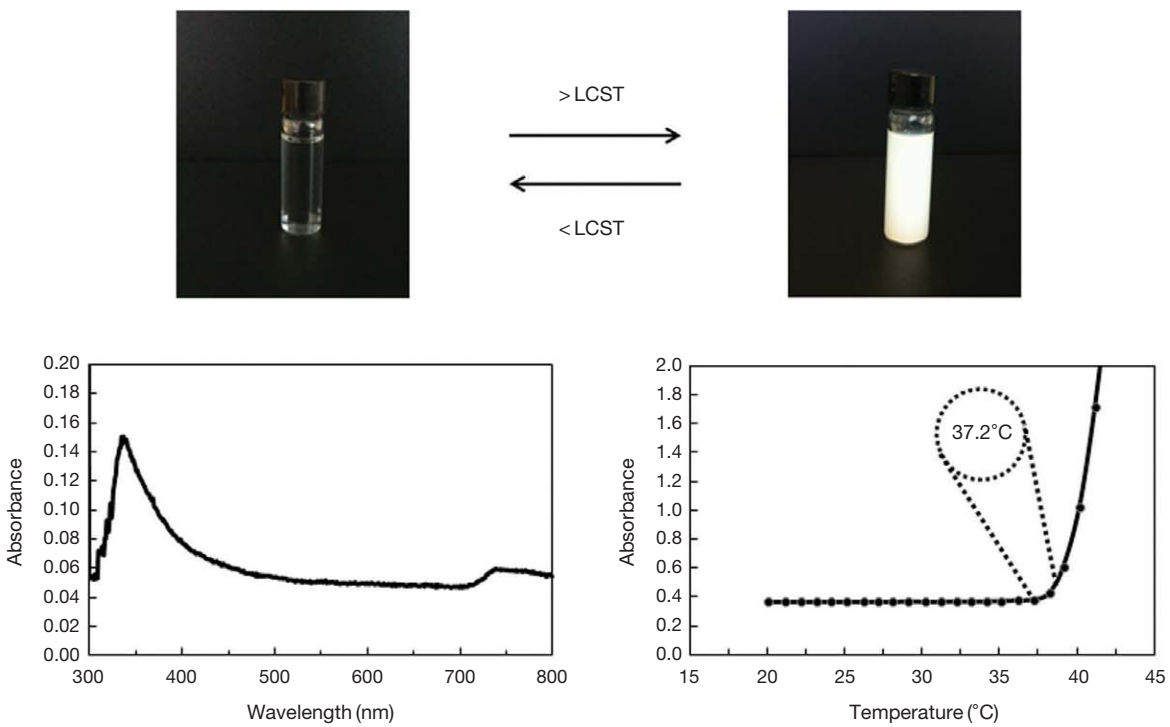

Figure 2. Thermoresponsive property of poly(NIPAM-*co*-AA) nanoparticles. UV-visible spectrophotometer analysis of poly (NIPAM-*co*-AA) nanoparticles (LCST, 37.2°C).

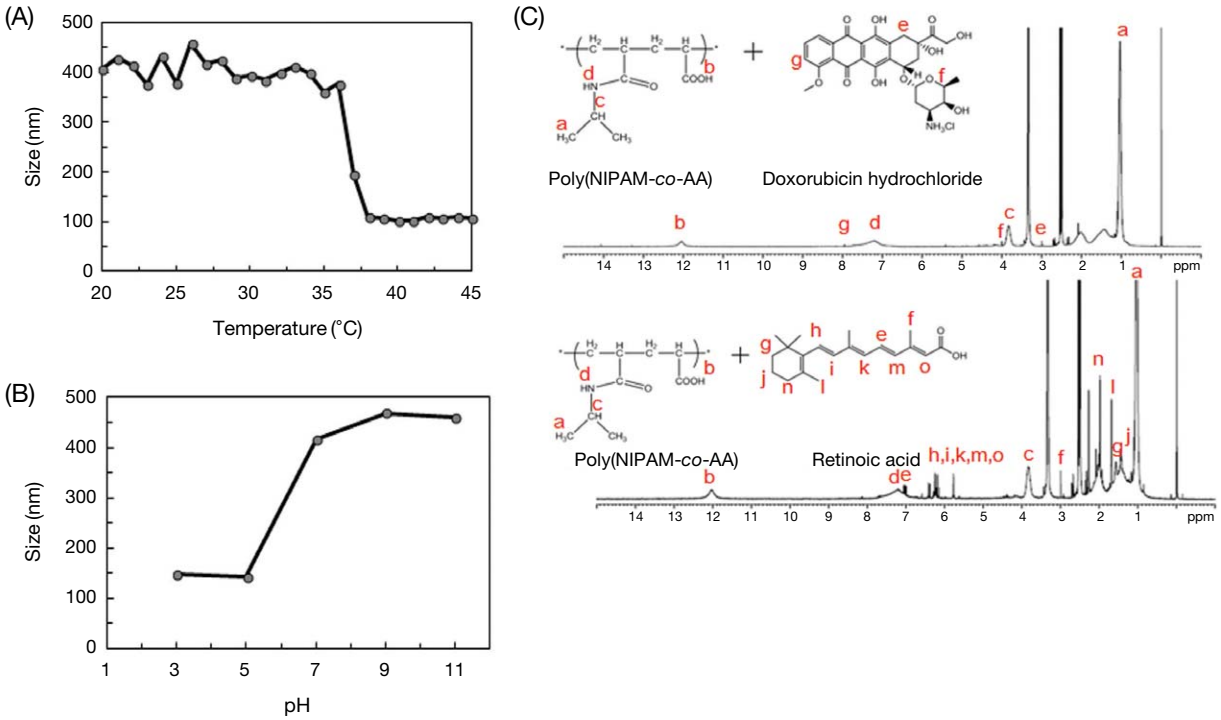

Figure 3. Characterization of poly(NIPAM-*co*-AA) nanoparticles. (A-B) The particle size analysis of poly(NIPAM-*co*-AA) nanoparticles in response to temperature and pH. (C) ^{1}H NMR spectra of doxorubicin-loaded poly(NIPAM-*co*-AA) and retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles.

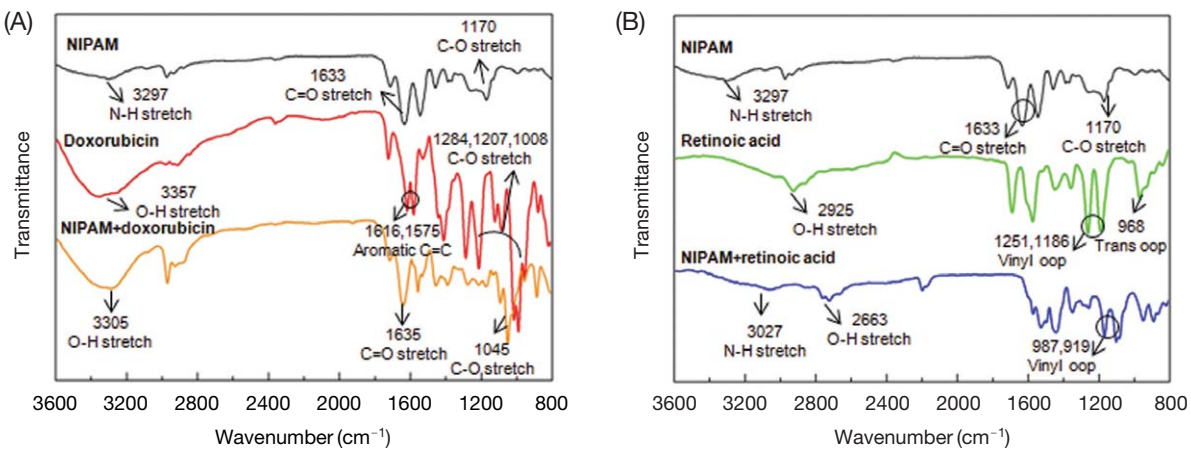

Figure 4. Analysis of FT-IR spectra. (A) FT-IR spectra of poly(NIPAM-*co*-AA), doxorubicin, and doxorubicin-loaded poly (NIPAM-*co*-AA) nanoparticles. (B) FT-IR spectra of poly(NIPAM-*co*-AA), retinoic acid, and retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles.

LCST for the phase transfer was 37.2°C.

Characterization of poly(NIPAM-*co*-AA) nanoparticles

We characterized poly(NIPAM-*co*-AA) nanoparticles using dynamic light scattering and ^{1}H nuclear magnetic resonance (NMR) analysis. Dynamic light scattering analysis showed that temperatures enabled the control of the sizes of poly(NIPAM-*co*-AA) nanoparticles, indicating that their sizes were changed from 400 nm at 25°C to 100 nm at 37°C (Figure 3A). In addition to temperatures, pH played an important role in regulat-

ing sizes of poly(NIPAM-*co*-AA) nanoparticles, showing that the size of poly(NIPAM-*co*-AA) nanoparticles was approximately 400 nm at pH 7.4 (Figure 3B). In contrast, the sizes were decreased to 150 nm at acidic pH. To confirm the synthesis of poly(NIPAM-*co*-AA) nanoparticles, we employed ^{1}H NMR analysis, showing that drugs (e.g., doxorubicin, retinoic acid) were conjugated with poly(NIPAM-*co*-AA) nanoparticles (Figure 3C). We observed that poly(NIPAM-*co*-AA) nanoparticles and doxorubicin were crosslinked by charges, whereas the hydrophobic bonding induced conjugation of poly(NIPAM-*co*-AA) nanoparticles and retinoic acid. To further confirm whether drugs (e.g., doxorubicin, retinoic acid) conjugated with poly(NIPAM-*co*-AA) nanoparticles, we performed FT-IR spectra analysis (Figure 4). We observed FT-IR spectra of poly (NIPAM-*co*-AA), doxorubicin, and doxorubicin-loaded poly(NIPAM-*co*-AA) nanoparticles, showing the presence of aromatic C=C stretch (1575 cm^{-1}, 1616 cm^{-1}) in doxorubicin (Figure 4A). The transmittance pattern of doxorubicin-loaded poly(NIPAM-*co*-AA) nanoparticles at 1600-800 cm^{-1} was similar compared to bare doxorubicin. It also revealed that O-H stretch was observed in doxorubicin (3357 cm^{-1}) and doxorubicin-loaded poly(NIPAM-*co*-AA) nanoparticles (3305 cm^{-1}). These results confirmed that poly(NIPAM-*co*-AA) nanoparticles were conjugated with doxorubicin. Furthermore, we investigated FT-IR spectra of poly (NIPAM-*co*-AA), retinoic acid, and retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles (Figure 4B). We observed vinyl oop stretch in retinoic acid and retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles. In contrast, there was no vinyl oop stretch in bare poly(NIPAM-*co*-AA) nanoparticles, suggesting that poly(NIPAM-*co*-AA) nanoparticles would be conjugated with retinoic acid.

Cumulative release analysis of drugs from poly(NIPAM-*co*-AA) nanoparticles

We observed the cumulative release of drugs (e.g., doxorubicin, retinoic acid) from poly(NIPAM-*co*-AA) nanoparticles (Figure 5). It demonstrated that doxorubicin was highly released (85%) from poly(NIPAM-*co*-AA) nanoparticles at 48 hours compared to retinoic acid (70%). Although the percentage of cumulative release of doxorubicin and retinoic acid was similar at 4 hour (~40%), the percentage of doxorubicin released from poly(NIPAM-*co*-AA) nanoparticles was steadily increased for 48 hours. Although the electrostatic bonding between doxorubicin and poly(NIPAM-*co*-AA) nanoparticles is stronger than hydrophobic bonding between retinoic acid and poly(NIPAM-*co*-AA) nanoparticles[18], doxorubicin was highly released from poly (NIPAM-*co*-AA) nanoparticles at 48 hours compared to retinoic acid, because retinoic acid was more hydrophobic than doxorubicin. The drug release from poly (NIPAM-*co*-AA) nanoparticles was affected by the hydrogen bond and solubility. The hydrogen bond between poly(NIPAM-*co*-AA) and water was weak and the solubility of poly(NIPAM-*co*-AA) was decreased when the temperature increased above LCST. As a result, the size of poly(NIPAM-*co*-AA) nanoparticles was decreased and the diffusion rate of drugs in nanoparticles was increased, as previously described[19]. Thus, drugs could be released from poly(NIPAM-*co*-AA) nanoparticles at 37°C.

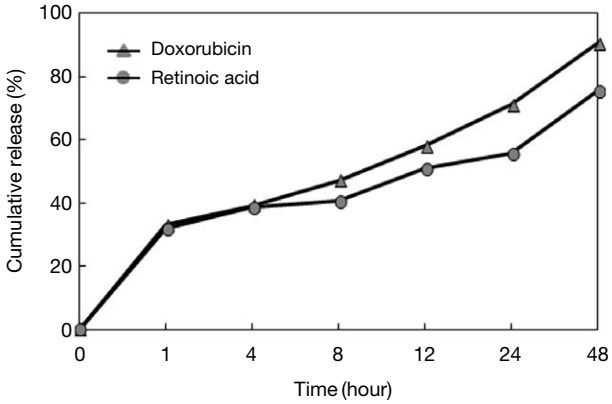

Figure 5. Analysis of controlled release of drugs (e.g., doxorubicin, retinoic acid) from poly(NIPAM-*co*-AA) nanoparticles at pH 7.4 and 37°C.

Conclusions

We synthesized drug-loaded thermoresponsive poly (NIPAM-*co*-AA) nanoparticles. We observed that the size of poly(NIPAM-*co*-AA) nanoparticles was affected by temperatures (400 nm at 25°C, 100 nm at 37°C). ^{1}H NMR and FT-IR analysis showed that drugs (e.g., doxorubicin, retinoic acid) were conjugated with poly (NIPAM-*co*-AA) nanoparticles. We also analyzed the cumulative release of drugs, showing that doxorubicin was largely released from poly(NIPAM-*co*-AA) nanoparticles at 48 hours compared to retinoic acid. Therefore, this poly(NIPAM-*co*-AA) nanoparticle could be a potentially powerful carrier for controlled release applications of various drugs.

Materials and Methods

Materials

NIPAM, N,N-methylenebisacrylamide (MBA), AA, KPS, doxorubicin, retinoic acid were purchased from

Sigma-Aldrich. The cellulose membrane tubing with molecular weight cut-off (6,000-8,000 g mol^{-1}) was used for dialysis.

Preparation of poly(NIPAM-*co*-AA) nanoparticles

Polymerization was carried out in a three-necked glass flask for 4 hours at 70˚C in nitrogen atmosphere. NIPAM (0.4 g, 0.026 mmol) and MBA (0.004 g, 0.026 mmol) crosslinker was dissolved in distilled water and were stirred with 400 rpm at room temperature for 20 minutes. The temperature increased to 70˚C and 0.05 g KPS added for polymerization. The polymerization solution was dialyzed for 4 days using a dialysis membrane.

Characterization of poly(NIPAM-*co*-AA) nanoparticles

The sizes of poly(NIPAM-*co*-AA) nanoparticles were investigated using a dynamic light scattering (Zetasizer Marvern instruments, France). For the size distribution analysis, nanoparticles were dispersed in a deionized water and were sonicated using a sonicator (Sonics: Vibracell) at 30% amplitude for 2 minutes. The sample was analyzed at various temperatures (20-50˚C) and a fixed scattering angle of 90˚. The particle size and surface morphology of poly(NIPAM-*co*-AA) nanoparticles was characterized by TEM (JEM-2100F). An aliquot of lyophilized of poly(NIPAM-*co*-AA) nanoparticles was resuspended in ethanol. A drop of nanoparticles was suspended on a carbon film coated on a 400 mesh copper grid and was subsequently dried overnight.

Thermal response of poly(NIPAM-*co*-AA) nanoparticles

The thermal response of nanoparticles was measured by UV-visible spectrophotometer. The transmission of nanoparticles was measured by UV-visible spectrophotometer in the range of 200-800 nm and 20-50˚C.

Analysis of nuclear magnetic resonance spectra

^{1}H NMR spectra of doxorubicin-loaded poly(NIPAM-*co*-AA) and retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles were analyzed by a Bruker spectrometer (400 MHz). ^{1}H NMR spectra were measured by using dimethyl sulfoxide (DMSO-d_6) and deuterium oxide (D_2O).

Fourier-transform infrared spectra analysis

FT-IR spectroscopy of doxorubicin, retinoic acid, poly (NIPAM-*co*-AA), doxorubicin-loaded poly(NIPAM-*co*-AA), and retinoic acid-loaded poly(NIPAM-*co*-AA) nanoparticles was analyzed by using a NICOLET AVATAR 330 FT-IR spectrometer. FT-IR measurements were performed at 25˚C. FT-IR spectra were performed between 3,600 cm^{-1} and 800 cm^{-1}.

Drug release profile of poly(NIPAM-*co*-AA) nanoparticles

50 mg poly(NIPAM-*co*-AA) nanoparticles with drugs (e.g., retinoic acid, doxorubicin) were placed in phosphate buffered saline (PBS, 0.1 M, pH 7.4, 5 mL) and were incubated at 37˚C with stirring (100 rpm). The nanoparticle suspension was centrifuged and 1 mL of the release medium was exchanged with fresh medium to maintain the total volume of 5 mL. Drugs released from nanoparticles were measured by UV-spectrophotometer (retinoic acid: 350 nm, doxorubicin: 496 nm wavelength) for 48 hours. Drug concentrations were calculated from the calibration curve.

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