The targeted behavior of thermally responsive nanohydrogel evaluated by NIR system in mouse model

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Thermally responsive poly(N-isopropylacrylamide-co-Acrylamide) (P(NIPA-co-AAm)) nanohydrogels were synthesized in this study by free-radical precipitation polymerization method. Different lower critical solution temperatures (LCST) of nanohydrogels were obtained by modulating the amount of AAm and characterized by measuring their transmittances of the particle solutions at 500 nm. The diameters within the range of 50–450 nm were achieved by manipulating the amount of sodiumdodecyl sulfate (SDS). Near infrared dye NIRD-12, with excitation and emission wavelengths at 772 nm and 814 nm, was entrapped into the P(NIPA-co-AAm) nanohydrogels for in vivo animal study. The thermally targeted behavior of the nanohydrogels was evaluated by the in vivo fluorescence imaging on different groups of denuded mice, with or without S180 tumors and with or without hyperthermia treatment. Results indicated that this kind of thermally responsive nanohydrogel could only accumulate in the tissue with higher temperature, no matter normal tissue and tumor site. The thermally targeted behavior is passive and non-specific. The targeted location can be selected by hyperthermia treatment and manipulating the suitable LCST of nanohydrogel. Results indicated that the thermally responsive P(NIPA-co-AAm) nanohydrogel could be used as an attracting thermally targeted carrier for drugs, especially for anti-cancer drugs.

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1. Introduction

Incidence of malignant tumors increased rapidly over the past decade, resulting in the extensive utilization of all kinds of cytotoxic drugs. However, conventional administered anti-cancer drugs face shortcomings such as severe side effects and poor therapeutic activity [1]. And the difficulty of accessibility and heterogeneity of the tumor also perplexes the existing chemotherapy. To overcome these disadvantages, a stimuli responsive polymeric device with lower toxicity and targeted effect will be a promising candidate for cancer therapy. Thermally responsive nanohydrogels poly(N-isopropylacrylamide) (PNI PA), including those synthesized by using N-isopropylacrylamide (NIPA) as the main monomer, have been attracting extensive attentions owing to their potential applications in gene delivery [2–8] and novel materials for controlled drug delivery [9–15]. PNI PA undergoes a volume phase transition at lower critical solution temperature (LCST). Its temperature sensitivity lies on the unique amphipathic structure of the main monomer NIPA. Due to the merits of its hydrophilicity and flexibility in aqueous solution when the temperature is below LCST, nanohydrogels can keep in a state of swelling and has the ability to circulate within blood stream, partly like PEGlated stealth particles [16,17]. However, when the temperature goes above LCST, the particles become hydrophobic, aggregate easily and deposit in the heated tissues, resulting in passively thermal accumulation [18]. Synthesis and characterization of PNI PA nanohydrogels, especially those being sensitive to both pH and temperature [19–23], were extensively carried out in recent years, including our previous work [24]. However, most of these studies were focused on the application of PNI PA for controlled drug release and gene delivery. Much less reports cared about their targeted behaviors. In addition, although the dually-responsive nanohydrogels could response to different stimuli, it is difficult to control their LCST in living body because of their property of both pH- and temperature-sensitivity. Further, a remarkable change of their LCST will occur after being covalently labeled with fluorescent probes due to the usage of –NH2 or –COOH for the formation of amide bonds, which was observed in our experiments. Based on these considerations, nanohydrogels responsive to temperature only were synthesized with different size and LCST for the investigation of targeted behavior in mouse model in this study.

To investigate the targeted characteristic of thermally responsive nanohydrogel, we designed the nanoparticles to be systemically soluble after injection and become insoluble and accumulate in the heated tissue. This design was achieved by modulating the LCST of nanohydrogel between normally physiologic temperature (37 °C) and the temperature of hyperthermia treatment (42 °C) [18].

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Imaging techniques possess great advantages in the evaluation of the particles’ targeted behavior. Optical imaging is of particular interest because of its intact property as well as better temporal resolution. Most intrinsic tissue chromophores, including oxyhemoglobin, de-oxyhemoglobin and melanin, have relatively weak absorbance in the near infrared (NIR) spectral range (700–900 nm) [25]. This weak absorption makes the NIR light to have the capability of deep tissue penetration depth. Featured with the ability of deeper tissue penetration as well as non-radioactivity, NIR imaging techniques have increasingly attracted researchers’ interest for non-invasive monitoring of biological signal in living tissue. NIR imaging has been widely used for diagnosis of disease [26], confirmation of therapeutic treatment [27] and evaluation of the rehabilitation [28].

In this study, thermally responsive poly(N-isopropylacrylamide-co-Acrylamide) (P(NIPA-co-AAm)) with LCST of 37 °C to 42 °C and size of about 50 nm was synthesized by free-radical precipitation polymerization method. And a near infrared organic dye 12 (NIRD-12) was physically labeled to the P(NIPA-co-AAm) hydrogel nanoparticles by hydrophobic association effect. The dye-loaded nanohydrogel was then systematically administrated into experimental mice with or without bearing s180 tumors. NIR imaging system was designed to monitor the dynamic behavior of the nanohydrogel in the denuded mice, with or without local hyperthermia treatment.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPA, ≥99% in purity) and N,N'-methylene-bis-acrylamide (BIS) were purchased from Aldrich-Chemie (Steinheim, Germany) and Tianjing Chemical Research Institute (Tianjing, China) respectively, and used without further purification. Potassium persulfate (KPS, the initiator of the polymerization process) and sodiumdodecyl sulfate (SDS) were supplied by Huakang Technology Company (Jiangsu, China). Acrylamide (AAm) was purchased from Sinopharm Chemical Regent co., Ltd (Shanghai, China). Near infrared dye 12 (NIRD-12) was obtained from Huahai Lanfan Chemical Technology Company (Liaoning, China). Water for all reactions, solution preparation and sample purification was double distilled.

2.2. Preparation and characterization of P(NIPA-co-AAm) nanohydrogel

2.2.1. Preparation of P(NIPA-co-AAm) nanohydrogel

A series of P(NIPA-co-AAm) nanohydrogels with different feed compositions (Table 1) was synthesized by free-radical precipitation polymerization method reported previously [9]. Their diameters and LCST could be controlled by modulating the amounts of SDS and AAm, respectively. In detail, monomer NIPA (1000 mg), AAm (25–200 mg), cross-linker BIS (27 mg) and surfactant SDS (50–200 mg) were added to 100 mL of double distilled water and with a magnetic stirrer. The solution was nitrogen-purged for 40 min at room temperature (RT). And then the polymerization was initiated by adding 75 mg of KPS and lasted for 4 h under nitrogen atmosphere at a temperature of 70 ± 1 °C. The resultant nanohydrogels were cooled down to RT and then dialyzed (molecular weight cutoff 10 kDa) against double distilled water for 5 days. The diazoyed aqueous solution of nanohydrogel was then lyophilized to obtain dried powder for subsequent research.

2.2.2. Characterization of P(NIPA-co-AAm) nanohydrogel

2.2.2.1. Determination of LCST in different conditions. Optical transmittance of aqueous nanohydrogel solutions (NHG1–5, 5 mg/mL) at various temperatures was measured at 500 nm with a Lambda 35 UV–visible spectrophotometer (Perkin Elmer, America). Samples and reference were thermostated by putting the cells in a water bath with temperature of 30 °C to 50 °C. And at least 10 min was allowed for each of the samples to reach equilibrium temperature. Due to the fact that the transparent P(NIPA-co-AAm) solutions will become opaque when the temperature increases above the transition point, the lower critical solution temperature (LCST) of nanohydrogel was defined as the temperature producing 50% decrease in optical transmittance. The measurements were repeated three times for each sample.

To investigate the effect of ionic strength (IS) on the LCST of hydrogel particles, LCST of NHG4 were determined under different solvents with particular IS, including Hank solution (a simulated body fluid, its composition was listed in Table 2, IS=0.1605), 0.9% NaCl solution (w/w, IS=0.1513), aqueous solution (pH 4.0, IS=0.0001), aqueous solution (pH 10.0, IS=0.0001). NHG4 concentration in each solution was 5 mg/mL.

2.2.2.2. Diameter and morphology measurement of nanohydrogel. The average diameters and their distribution of P(NIPA-co-AAm) nanohydrogels (NHG4, 6–9, 5 mg/mL) were measured by Mastersizer 2000 Laser Particle Size Analyzer (LPSA, Malvern, British) with a helium–neon laser (10 mW max, Wavelength of 633 nm) as the light source at the scattering angle of 90°. The available detecting size interval is 2 nm–3 μm.

To observe the morphology of the nanohydrogel and further confirm the size measurement from LPSA, transmittance electron microscope (TEM) pictures of nanohydrogel (NHG4) were taken by H-7000 TEM (Hitachi, Japan) with accelerated voltage of 75 kV. A small amount of aqueous solution of lyophilized powder (5 mg/mL) was placed on a polyvinyl formal coated grid surface with a filter paper. A drop of 1% phosphotungstic acid was immediately added to the surface of the grid. After 1 min excess, fluid was removed and the grid surface was air dried at RT before loaded in the microscope for observation.

2.3. Physically labeling of P(NIPA-co-AAm) with NIRD-12 and optical characterization of free and entrapped NIRD-12

2.3.1. Physically labeling of P(NIPA-co-AAm) with NIRD-12

NIRD-12, a kind of hydrophobic dye with maximum absorption at 772 nm and maximum emission at 814 nm, was dissolved in a small amount of dimethyl sulfoxide (DMSO). After that, nanohydrogel solution (NHG4, 5 mg/mL) was dropwisely added to the solution and the mixture was put into dark environment and incubated at RT with constant agitation. The mixture solution was sampled every 3 h and centrifuged. The supernatant solution with nanohydrogel was with freshly double distilled water for 4 days. The resultant NIRD-12-loaded nanohydrogel solution was then lyophilized to obtain dried powder for subsequent research.

Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>NIPA (g)</th>
<th>AAm (g)</th>
<th>BIS (g)</th>
<th>SDS (g)</th>
<th>KPS (g)</th>
<th>H₂O (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHG1</td>
<td>1.000</td>
<td>0.025</td>
<td>0.027</td>
<td>0.200</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG2</td>
<td>1.000</td>
<td>0.050</td>
<td>0.027</td>
<td>0.200</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG3</td>
<td>1.000</td>
<td>0.100</td>
<td>0.027</td>
<td>0.200</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG4</td>
<td>1.000</td>
<td>0.150</td>
<td>0.027</td>
<td>0.200</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG5</td>
<td>1.000</td>
<td>0.200</td>
<td>0.027</td>
<td>0.200</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG6</td>
<td>1.000</td>
<td>0.150</td>
<td>0.027</td>
<td>0.150</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG7</td>
<td>1.000</td>
<td>0.150</td>
<td>0.027</td>
<td>0.100</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG8</td>
<td>1.000</td>
<td>0.150</td>
<td>0.027</td>
<td>0.075</td>
<td>0.075</td>
<td>100</td>
</tr>
<tr>
<td>NHG9</td>
<td>1.000</td>
<td>0.150</td>
<td>0.027</td>
<td>0.050</td>
<td>0.075</td>
<td>100</td>
</tr>
</tbody>
</table>

W% stands for W_AAm/W_total×100%.
where \( W_{\text{Entrapped-NIRD-12}} \) was obtained by the calibration curve of NIRD-12. The diameter and LCST of NIRD-12-loaded NHG4 were also determined.

2.3.2. Optical characterization of free and entrapped NIRD-12

Absorbance and fluorescence spectra of free and entrapped NIRD-12 were measured by using Lambda 35 UV–visible spectrophotometer (Perkin Elmer, America) and S2000 spectrometer (Ocean Optics, USA), respectively. A NL-FC-2.0-763 semiconductor laser (\( \lambda = 765.9 \text{ nm} \), Enlight, China) was used to excite fluorescence. The photo stability of free and entrapped NIRD-12 was evaluated by measuring their relative fluorescence intensity under continuous exposure to the laser for 2 h, with 5 min interval for data acquisition.

2.4. MTT assay of P(NIPA-co-AAm)

MTT assay was conducted following the standard protocol reported previously [21,29]. Human Vein Endothelial Cell Line (ECV304) was chosen as the target cells. In detail, 200 \( \mu \)L of ECV304 in RPMI 1640 (2.0×10^5 cells/mL) was added into each well in a 96-well plate and incubated for 24 h in humidified atmosphere containing 5% \( \text{CO}_2 \) at 37.0 °C. The culture medium in each well was replaced by 200 \( \mu \)L of RPMI 1640 containing P(NIPA-co-AAm) nanohydrogel (NHG4) with particular concentrations (0 mg/mL, 0.02 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL). Medium with zero concentration of NHG4 was used here as control. And the mixture was further incubated for 48 h. RPMI 1640 with NHG4 was replaced by 180 \( \mu \)L of fresh RPMI 1640 and 20 \( \mu \)L of MTT solution (5 mg/mL). After incubation for another 4 h, the medium containing MTT was removed from each well and 200 \( \mu \)L of DMSO was added and shaken at RT. The optical density (OD) was measured at 490 nm with a Microplate Reader (Biorad, USA). The viable rate could be calculated by the following equation: viable rate = \( \frac{\text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \times 100\% \), where \( \text{OD}_{\text{treated}} \) was obtained in the presence of nanohydrogel and \( \text{OD}_{\text{control}} \) was obtained in the absence of nanohydrogel.

2.5. Animal experiment

The Kunming mice (half male and half female) used in this study were purchased from Laboratory Animal Resources of China Pharmaceutical University, which were 6–8 weeks old and weighed at about 18–22 g. All experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (document no. 55, 2001) and the guidelines for the Care and Use of Laboratory Animals of China Pharmaceutical University.

2.5.1. In vivo acute toxicity evaluation

NHG4 aqueous solutions with different concentrations (2.5 mg/mL, 5 mg/mL, 10 mg/mL, 20 mg/mL, 40 mg/mL) were prepared by dissolving different amounts of lyophilized powder of nanohydrogel into 5 mL of 0.9% NaCl solution (w/w). The mice were divided into six groups and marked as groups A, B, C, D, E and F. Each group contained 10 mice. Each mouse of groups A to E was injected by tail vein with 0.2 mL of nanohydrogel solution with concentration of 2.5 mg/mL, 5 mg/mL, 10 mg/mL, 20 mg/mL and 40 mg/mL, respectively. Group F mice were used as control with the injection of 0.2 mL of 0.9% NaCl solution (w/w). After injection, mice of all groups were sent to a room with constant temperature of 26 °C with enough supply of food and water. The survival rates in each group were counted after 72 h post-injection.

2.5.2. In vivo thermally targeted test

The mice were denuded with its hair snipped. Each mouse was anesthetized with an intraperitoneal injection of 1 mg/g of ethyl carbamate and immobilized in a Lucite jig. 15 healthy mice were evenly divided into three groups. For group A mice, one leg of each mouse was heated by a temperature-controlled water sack and maintained at 42 °C. The local temperature of the heated leg was monitored by a needle-like temperature sensor (Hengcheng Technology, Guangzhou, China). As soon as the local temperature of the heated leg reached 42 °C, 200 \( \mu \)L of NIRD-12-loaded NHG4 nanohydrogel solution (containing 100 \( \mu \)g/mL of NIRD-12) was injected through tail vein into the physiologically normal mouse. The dynamic behaviors of nanohydrogel were monitored by NIR imaging system, which was introduced in our previous work [30]. The similar process was carried out for injection of 200 \( \mu \)L of NIRD-12 solution (containing 100 \( \mu \)g/mL of NIRD-12) into the group B mice for control. The group C mice without hyperthermia treatment were injected with the same amount of NHG4 nanohydrogel as in group A for another control. Fluorescence images of all the experimental mice were taken continuously for 12 h and the typical images at 1 h post-injection.

To confirm the thermally targeted ability of the nanohydrogel on tumor tissue, similar protocol was carried out on s180 tumor-bearing mice (n=15), with the hyperthermia treatment site changing from leg to tumor tissue in subxiliary (Fig. 5B).

3. Results and discussion

3.1. Preparation of P(NIPA-co-AAm) nanohydrogels

A series of P(NIPA-co-AAm) nanohydrogels with different LCST and diameters was successfully synthesized. In the process of synthesis, KPS was used as an initiator for the polymerization. When the reaction temperature increased to 70 °C, one molecule of KPS would change into two molecules of free radicals because of the breakage of the peroxide bond of KPS. The resultant free radicals initiated the polymerization reaction and formed the nanohydrogels.

After their formation, the nanohydrogels should be purified by dialysis before their practical usage. The complete purification of the nanohydrogels after 5 day’s dialization was determined by measuring the absorbance of the dialysate at the wavelength range of 200–300 nm. The unreacted components and lower polymerized by-product, dialized from the dialysis bag, have absorption within the wavelength range of 200–300 nm. After 5 days of dialysis, the dialysate has no absorbance at that wavelength range, which indicated the complete clearing of the nanohydrogel.

3.2. Characterization of LCST of P(NIPA-co-AAm) nanohydrogel

3.2.1. LCST of P(NIPA-co-AAm) nanohydrogel

LCST of nanohydrogels (NHG1–5, with Wt % (Wt % stands for \( W_{\text{Aam}}/W_{\text{NHG}} \times 100\% \)) of 2.5%, 5.0%, 10.0%, 15.0% and 20%, Table 1) were measured in our research. Fig. 1(A) displayed the profiles of the transmittances of different P(NIPA-co-AAm) with the change of temperature (n=3). The LCST for NHG1–5 were 35.5 °C, 36.5 °C, 39 °C, 43 °C and 48 °C, respectively. The result indicated that LCST increased with the increase of Wt %, as shown in Fig. 1(B) (n=3).

The hydrophilicity of AAm in nanohydrogels, the stronger it produces hydrogen bond interaction in aqueous solutions. This stronger bond requires more energy to destroy it and results in the increase of LCST. Thus, LCST of nanohydrogels could be adjusted by modulating Wt %.

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**Table 2**

Chemical composition of Hank solution (n mol/L)

<table>
<thead>
<tr>
<th>Ion</th>
<th>Na^+</th>
<th>K^+</th>
<th>Ca^{2+}</th>
<th>Mg^{2+}</th>
<th>Cl^-</th>
<th>HCO_3^-</th>
<th>HPO_4^{2-}</th>
<th>SO_4^{2-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>142</td>
<td>5</td>
<td>2.5</td>
<td>1.5</td>
<td>147.8</td>
<td>4.2</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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Nanohydrogel undergoes volumetric phase transition when the temperature exceeds its LCST. This phase transition may be explained by the thermal breakage of the nanohydrogel–water hydrogen bonds and the enhanced inter- and intra-molecular hydrophobic interactions. At this moment, water of hydration is propelled out of the hydrogel due to the collapsing of their structure. LCST is a key parameter of the thermally responsive nanohydrogel. It determines the potential application of nanohydrogel in targeted drug delivery [31].

Except for the parameter of Wt %, the ionic strength (IS) of the solution is also an important factor affecting the LCST of nanohydrogel [32]. Generally, the addition of electrolytes provokes flocculation of the aqueous colloidal dispersions because of the salting out effect. The similar phenomenon should be observed in our thermally responsive nanohydrogel dissolved in those solvents. Compared to pure water, ionic solvents did decrease the LCST of the nanohydrogels. More decreases were observed in the solutions with higher IS such as Hank solution and 0.9% NaCl solution. Less shifts were obtained in the solutions with lower IS, including aqueous solution of pH 10.0 and 4.0. This phenomenon could be explained as the sudden removal of the protective hydration layer of nanohydrogel caused by the reinforcement of IS, resulting in the exposure of the hydrophobic residues of nanohydrogel. The decrease of LCST of nanohydrogel in 0.9% NaCl solution (w/w) was nearly same with that of Hank solution because of their similar IS. Similar explanation could be applied to the other two solutions. The similar LCST of nanohydrogel in aqueous solutions with pH 4.0 and 10.0 further confirmed the independent of the P(NIPA-co-AAm) on pH conditions.

### 3.2.2. Diameter and morphology of nanohydrogel

Fig. 2(A) \( n=3 \) showed the diameters of nanohydrogels (NHG4, 6–9) as a function of the concentrations of SDS. When the concentration of SDS was 0.5 mg/mL (NHG9), nanohydrogel with a diameter of about 440 nm was obtained. And when the concentration of SDS increased to 2 mg/mL (NHG4), the diameter of resulted nanohydrogel decreased to about 50 nm (51 ± 8, mean and standard deviation). The narrow distribution of the diameters for the nanohydrogels determined by Laser Scattering Analysis (LSA) (Fig. 2(B)) indicated the uniformly particle sizes. The morphology of NHG4 was exhibited by TEM photos (Fig. 2(C)), which further confirmed the particle size from LSA. TEM pictures showed that the nanohydrogels were well dispersed as individual particles with spherical shape. The result in our study was consistent with reports that SDS could be used to regulate the size of nanohydrogel [9]. Electrostatic repulsion force of the NIPA monomers in the process of polymerization was strengthened accordingly with the increase of SDS, which resulted in the shorter chain of the polymer, and thus, the smaller particle size.

TEM picture of NHG4 (Fig. 2(C)) indicated some aggregations of the nanoparticles. That is because the nanohydrogel particles can only disperse well in solution. In the process of sample preparation for TEM, water was evaporated and the nanohydrogel particles then gradually aggregated together. In addition, the surface of the frame is hydrophobic carbon membrane, which facilitated the aggregation of particles dissolved in the aqueous solution.

### 3.3. Physically labeling of nanohydrogel with NIRD-12 and optical characterization of free and entrapped NIRD-12

#### 3.3.1. Physically labeling of nanohydrogel with NIRD-12

NIRD-12 is an extremely hydrophobic dye with maximum absorbance at 772 nm and maximum emission at 814 nm. It could be easily entrapped into nanohydrogel NHG4 by hydrophobic association effect. Because of the poor solubility of NIRD-12, it was firstly dissolved in a small amount of DMSO before incubated with nanohydrogel solution. 24 h incubation of nanohydrogels with NIRD-12 was proven to be the optimal duration with highest entrapment. The loading efficiency of nanohydrogel for NIRD-12 was calculated with the calibration curve of NIRD-12 \( y=0.2535x-0.0008, R^2=0.9997 \) at about 4% (w/w). The LCST and diameter of the dye-loaded nanohydrogel have no apparent change before and after physically NIRD-12 labeling (data not shown here).

#### 3.3.2. Optical characterization of free and entrapped NIRD-12

NIRD-12 is easy to form dimmer or multimer in aqueous solution because of its poor solubility. After being entrapped into nanohydrogel
by the hydrophobic association effect, its stability will be strongly enhanced. Red shifts of both absorbance and fluorescence spectra of NIRD-12 were observed ([Fig. 3(A, B)]) after the process of physically labeling. This could be explained as the stabilization of nanohydrogel for entrapped dye, which can decrease the transition energy of the chromophores of NIRD-12, so a red shift of maximum absorption happened. The red shift of maximum absorption can result in the red shift of maximum emission. And the interior hydrophobic micro-environment of the nanohydrogel can also affect the maximum absorption and emission. The photo stability of NIRD-12 was significantly enhanced after being entrapped by nanohydrogel ([Fig. 3(C)]) because of this kind of stabilization. A similar explanation could be found elsewhere [33]. The enhancement of the dye’s photo stability will enable the real-time monitoring of the dynamic process of dye-loaded nanohydrogel in mouse model for a relatively longer time.

3.4. Cytotoxicity of P(NIPA-co-AAm)

MTT assay was carried out to evaluate the cytotoxicity of P(NIPA-co-AAm) on the ECV304 cells. The inhibition of nanohydrogel (NHG4) on the proliferation of ECV304 was studied. The nanohydrogel exhibited no apparent cytotoxicity ([Fig. 4, n=6]). The proofs of little or non-cytotoxicity of NIPA-based nanohydrogels were reported by many groups [2,4,21,29,34]. Incorporation of NIPA in the NIPA-based

Fig. 2. (A) Diameter of P(NIPA-co-AAm) as a function of SDS concentration (n=3); (B) Size distribution of P(NIPA-co-AAm) (NHG4) at 25 °C; (C) Transmittance electron microscope (TEM) pictures of P(NIPA-co-AAm) (NHG4).
3.5. In vivo acute toxicity on mouse model

The in vivo acute toxicity test described in Section 2.5 demonstrated the low toxicity of P(NIPA-co-AAm) (NHG4). Even group E with the highest dosage (400 mg/kg) had a death rate of zero (data not shown here).

3.6. In vivo thermally targeted test

For application in drug delivery, the particles usually should maintain their diameters at less than 100 nm to lower the possibility of being scavenged by the reticuloendothelial system (RES) [35]. In order to avoid this effect, nanohydrogel with diameter of about 50 nm at RT temperature was used for thermally targeted test. In addition, LCST of the nanohydrogels was adjusted between 37 °C and 42 °C (NHG4), which allowed the nanohydrogel to circulate systemically within the blood stream in the state of swelling and accumulate in heated tissue over 42 °C.

Fig. 5A exhibited the typical fluorescence images of different healthy group mice at 1 h post-injection. Fig. 5A(a) represented the image in group A mice, with hyperthermia treatment on the right leg and dye-loaded NHG4 injection. Fig. 5A(b) was a typical image in group B mice, with same hyperthermia treatment and pure dye injection. Fig. 5A(c) was an image in group C mice, with same dye-loaded NHG4 injection and removing of hyperthermia treatment. Bright fluorescence signals were observed in livers of all the mice immediately after the injection, indicated a quick accumulation of the particles and dyes in livers. However, obvious fluorescence signal appeared in the heated legs of group A mice at 1 h post-injection (Fig. 5A(a)). No other bright signals were observed in control groups B and C mice except for the liver area (Fig. 5A(b) and (c)). The fluorescence imaging was still obviously observed at 12 h post-injection for group A mice (data not shown here). The in vivo fluorescence images demonstrated the thermal targeted characterization of the temperature-responsive nanohydrogel.

The thermal targeted effects were also carried out in s180 tumor mouse model (Fig. 5B(d, e and f)). Fig. 5B(d) represented the image in group D mice, with hyperthermia treatment on tumor site and injection of dye-loaded NHG4. Fig. 5B(e) and (f) were images for group mice with and without hyperthermia, and with pure dye or dye-loaded NHG4 injection, respectively. Results indicated that the nanohydrogel NHG4 can accumulate in the tumor site with higher temperature (Fig. 5B(d)). The fluorescence imaging was still obviously observed at 12 h post-injection for group D mice (data not shown here).

Fig. 5. A. Thermally targeted test using physiologically normal mice: (a) Systemically injection of dye-loaded nanohydrogel with hyperthermia treatment (42 °C) of the right leg (n=5); (b) Systemically injection of pure dye solution with hyperthermia treatment (42 °C) of the right leg (n=5); (c) Systemically injection of dye-loaded nanohydrogel without hyperthermia treatment (n=5). B. Thermally targeted test using s180 tumor-bearing mice: (d) Systemically injection of dye-loaded nanohydrogel with hyperthermia treatment (42 °C) of s180 tumor tissue (n=5); (e) Systemically injection of pure dye solution with hyperthermia treatment (42 °C) of s180 tumor tissue (n=5); (f) Systemically injection of dye-loaded nanohydrogel without hyperthermia treatment (n=5).
here). No fluorescence was observed in the control group mice, implying that free NIRD-12 had no tumor targeting effect even with the help of hyperthermia treatment (Fig. 5B(e)), and the nanohydrogel could not accumulate in non-heated tumor tissue (Fig. 5B(f)).

For mice of each group, fluorescent imaging could also be observed except for liver and hyperthermia tissues. This does not mean a systemic distribution of the NIRD-12-loaded nanohydrogel. Fluorescent imaging in abdominal region was caused by those nanohydrogel metabolized by liver and excreted to intestine. And weak fluorescent imaging of the whole body was caused by the residual hair.

In vivo animal studies demonstrated the thermally targeted behavior of the nanohydrogels. These thermally responsive nanohydrogels with suitable LCST and size could accumulate in both normal and pathological tissues with the help of hyperthermia treatment. It provided a promising way in the medical application by incorporating with hyperthermia treatment. This thermally targeted effect of the nanohydrogel is passive and non-specific to any particular cell or tissue. The modification of specific ligands or antibodies to the hydrogel would greatly enhance its wide application in tumor diagnosis and treatment.

4. Conclusion

Thermally responsive nanohydrogels were synthesized and characterized in this study. The thermal targeted behavior was proved by the in vivo animal study. The various thermally targeted tests indicated that the thermally responsive nanohydrogel can only accumulate in the higher temperature tissue, no matter normal tissue or tumor. The targeted behavior of this kind of thermally responsive nanohydrogel is passive and non-specific. The targeted locations can be selected by manipulating the suitable LCST of nanohydrogel and tissue heating process. Results indicated that the thermal responsive nanohydrogel in combination with local hyperthermia treatment provided a promising way for tumor therapy. Entrapment of anti-cancer drugs into this kind of nanohydrogels for tumor therapy will be our future work.

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References