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DUAL STIMULI-RESPONSIVE POLY (N-ISOPROPYLACRYLAMIDE-co-ACRYLIC ACID) COPOLYMERS AND THEIR APPLICATIONS IN IBUPROFEN DELIVERY

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Keywords:

poly(N-isopropylacrylamide-co-acrylic acid), Dual Stimuli-Response, Ibuprofen, Drug Delivery, Salt Concentration

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ABSTRACT: A series of poly(N-isopropylacrylamide-co-acrylic acid) (P(NIPAM-co-AA)) nanoparticles with different contents of acrylic acid were synthesized via free radical polymerization route. Their pH- and thermo-responsive behaviors were investigated using ^{1}H-NMR, FT-IR and SEM, dynamic light scattering, scanning calorimeter and zeta potential measurements. The results elucidated that the P(NIPAM-co-AA) exhibited dual stimuli performances both pH- and temperature- response, showing more sensitive than the pure poly(N-isopropylacrylamide). Their swollen/shrunken behaviors were strongly dependent on the various parameters, including content of acrylic acid monomers, pH value of solution, temperature and salt concentration. Particularly, the electrical repulsive force, surface charge distribution and hydrogen bonding were thought to determine their sensitive properties. Salt effect showed more obvious at high pH than that at low pH, leading to a significant change of particle size distribution and Zeta potential. In addition, the P(NIPAM-co-AA) as matrix loaded-ibuprofen delivery in vitro was also evaluated, exhibiting a faster release rate at pH 7.4 than that at pH 2.0, while the accumulative release amount was larger at 37 ºC than that at 25 ºC in the aqueous ammonia solutions (at pH 7.4). These results demonstrated that the resultant copolymer presented a smart pH and thermo-response in the potential application of controlled drug delivery system.

INTRODUCTION: In the past decades, environmental sensitive polymers have drawn more and more attention in drug delivery systems due to their excellent response to external stimuli changes, especially, pH [1] and temperature [2], in which poly (N-isopropylacrylamide) (PNIPAM) with thermo sensitivity has a low critical solution temperature (LCST) in aqueous solution [3] because the hydrogen bonding between hydrophilic segments of PNIPAM chains and water molecules is dominant. However, the LCST of pure PNIPAM at around 32 °C is below the human physiological temperature, which makes it difficult to use pure PNIPAM as a carrier for drug delivery to have a sharp phase transition at physiological temperature. Additionally, the conventional homopolymer PNIPAM also has a low swelling ratio, slow respond speed [4], poor mechanical strength and rigid network structure, which further limits its applications in many drug delivery fields.

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Therefore, it is very necessary to find an appropriate method to tune the phase transition temperature and swollen-shrunk behaviors of PNIPAM. Although classical carboxylic acids monomers with excellent pH sensitivity such as acrylic acid (AA), allylacetic acid (AAA), methacrylic acid (MAA) and vinylacetic acid

(VAA) can extend and shrink at pH values above and below their *pKa*, these polymers are non-biodegradable and difficult to remove from human body after use.

Therefore, the dual responsive pH- and thermo-sensitive copolymer based on two different stimulus-responsive polymers have attracted more and more attention. For example, Todd and Hofl groups [5, 6] investigated the swelling behaviors of VAA copolymer, and found that VAA-NIPAM microgels were ionized over a narrow pH range with a much larger swelling ratio that of PNIPAM. Karg *et al*., [7] explored the swelling behavior of PNIAM-co-Poly (AAA) at pH 8.0 and pH 10.0 with different incorporated allylacetic acid amounts. Recently, Khan *et al*., [8] and Chen *et al.,* [9] found that the LCST of P(NIPAM-co-AA) became broaden and shifted to higher temperature range after incorporation of a small amount of carboxyl ionizable group into PNIPAM chains. Shibayam *et al.,* [10] also concluded that the influence of the pH values and salt concentrations on their volume phase transition temperature was remarkable. Hu *et al.,* [11] demonstrated their controlled release profiles, showing reversible response to alternating changes in pH and temperature.

All above studies demonstrate that the incorporation of ionic groups into PNIAM networks could modify its LCST, in which the swollen -shrunken ability is also enhanced to some extent. Although a large number of reports on pH-temperature responsive PNIPAM-based polymers have been published, what is important the responsive mechanism of dual stimulus copolymer is still vague. Therefore, how to rational design and precise control of stimulus response performance have become one of the seeking targets for researcher.

Our previous reports demonstrated that the typical polyelectrolyte of poly(methylacrylic acid-covinyltriethoxylsilane)s with charged networks were sensitive not only to the change of pH value, but also to the ionic strength of used medium [12]. The additional electrostatic force and effective charge density derived from various salt ionic concentrations in different solutions played very important roles in the swelling-shrinking of copolymer. Therefore, introduction of ionic monomer into the thermo-response network should modify the critical temperature with a more subtle effect on the mechanism of volume phase transition.

In this study, AA was introduced into the PNIPAM chain via free radical polymerization to synthesize a series of dual sensitive copolymer of P(NIPAM-co-AA), which were characterized systemically with the scanning calorimeter (DSC), Fourier transform infrared spectroscopy (FT-IR), ^{1}H NMR spectra, scanning electron microscopy (SEM). Additionally, influences of AA and salt concentration on the swollen-shrunken behaviors and LCST modification, as well as pH/temperature sensitivity, were investigated with dynamic light scattering (DLS) measurement. Meanwhile, zeta potential analysis was used to explore the essential effect of pH value, monomer content and ionic concentration on surface charge distributions of these copolymers. Furthermore, their loading/ releasing behaviors were evaluated in ibuprofen (IBU) delivery system with different external stimulus.

2. MATERIALS AND METHODS:

2.1. Materials: N-isopropylacrylamide (NIPAM, Aldrich, 97%), N,N-methylenebisacrylamide (BIS, Aldrich, 97%) were obtained from Alfa Aesar company. NIPAM and BIS were respectively re-crystallized from hexane and methanol. Acrylic acid (AA, A. R), potassium persulfate (KPS, A. R), sodium dodecyl sulfate (SDS, A. R), IBU (A. R) were purchased from Sinopharm Chemical Reagent Co., Ltd. Hydrochloric acid (HCl, A. R), ammonia solution ($NH_3 \cdot H_2O$, A. R), and sodium chloride (NaCl, A. R), ethanol (C_2H_5OH), n-hexane (C_6H_6), were purchased from Beijing Chemical Factory.

2.2. Synthesis of P (NIPAM-co-AA)-x nanoparticles: 1.8 g of NIPAM, the desired amount of AA, BIS (0.308 g) and SDS (0.05 g) were dissolved in 250 mL deionized water with magnetic stirring under a nitrogen atmosphere. The obtained solution was heated to 70 ºC and kept under a nitrogen atmosphere. After 1h of continuous degassed, KPS (0.135 g) was added into the solution, and polymerization was subsequently carried out at 70 ºC for at least 7 h under a nitrogen atmosphere. After that, the resultant emulsion was centrifuged at a speed of 20,000 rpm, and then the

precipitate was taken out at room temperature for 7 days using a dialysis bag (molecular weight cutoff: 12,000-14,000 Da) and the water was exchanged twice every day. Subsequently, the obtained nanogel of P(NIPAM-co-AA) was lyophilized to collect xerogel for further measurement. According to the different incorporate compositions, the samples were encoded P (NIPAM-co-AA)-x (x denoted monomer AA/NIPAM mass fraction), taking P(NIPAM-co-AA)-1 as example, the mass fraction is 1 %.

2.3. IBU loading by soaking procedure: Incorporation IBU into P(NIPAM-co-AA)-x (in which x is 0, 1) network was performed as following: 300 mg of P(NIPAM-co-AA) powder was dispersed in 40 mL IBU hexane solution (40 mg/mL). Then after 24 h under mild stirring at 25 ºC, the loading IBU of P(NIPAM-co-AA) was filtered and washed repeatedly with hexane, dried in vacuum. High performance liquid chromatography (HPLC) was used to determine the amount of loading IBU.

The drug loading content was calculated by the following equation:

$$\mathrm{LG}\,\% = \frac{m_0 - m_1}{m_2} \times 100\%$$

where m_0 is the weight of IBU thrown into initially, m_1 is the weight of IBU filtrated; m_2 is the weight of IBU loaded polymer P (NIPAM-co-AA)-x. The LG % of pure PNIPAM and P (NIPAM-co-AA)-1 was 0.9 % and 7.9 % respectively.

2.4. *In vitro* release: The samples were pressed into disk and put into the dialysis bag (molecular weight cutoff: 1000 Da), which was immersed into the 20 mL HCl solution (pH=2.0) or 20 mL $NH_3 \cdot H_2O$ solution (pH=7.4) in a shaking water bath at 25 ºC or 37 ºC. In a special interval, 1 mL of the solution release was withdrawn and 1mL fresh solution was supplemented. The concentrations of IBU release were analyzed by UV-visible spectrophotometer at the wavelength of 272 nm. All of measurements were performed in at least twice and standard deviations from the average values were calculated.

The drug releasing content was calculated according to the following formula:

$$C_c = C_t + \frac{V_1}{V_2}\sum_{0}^{t-1} C_i$$

where C_c is the corrected concentration at time t, C_t is the apparent concentration at time t, V_1 is the volume of sample collected, and V_2 is the total volume of release fluids.

2.5. Characterizations: The FT-IR spectrum measurements were preformed on a Nicolet Nexus 470 spectrometer in the wavelength region of 4000-400 cm^{-1} using KBr as the sample holder. ^{1}H NMR spectra of P (NIPAM-co-AA) were recorded in deuterated DMSO by a Bruker AV-3000 spectrometer (Germany) operating at a proton frequency of 400 MHz. The morphology and microstructure of samples were observed using SEM photos by a Hitachi S-4300 electron microscope. The HPLC was used by Agilent Technologies 1200 series, chromatographic column was Extend-C18. UV-vis absorbance spectra were measured using a Shimadzu UV-2600 spectrophotometer.

The series of P (NIPAM-co-AA) samples hydrodynamic diameters and zeta potential values at different pH solution and environmental temperature were determined using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd, Malvern, UK). The LCST behaviors of P(NIPAM-co-AA)-x were tested using DSC measurement (NESCH Germany).

3. RESULTS AND DISCUSSION:

3.1. Structure and morphology of copolymer: The chemical structures of the P(NIPAM-co-AA) and NIPAM were characterized by ^{1}H NMR spectroscopy, as shown in **Fig. 1**. As can be seen in **Fig. 1A**, the typical NIPAM signals at the chemical shift of 5.0-6.2 ppm (a) and 7.8 ppm (b) could be assigned to vinyl protons and acid amides proton, while other signals peaks at 1.2 ppm (d) and 3.9 ppm (c) were due to methyl and methylene proton of isopropyl group [13], respectively. Meanwhile, as compared with that of NIPAM monomer, the spectrum for P (NIPAM-co-AA)-1 exhibited broaden protons signals (a´, c´, f´) because of methyl, methylene and acid amide protons on its main chain of copolymer [14]. Importantly, the disappearance of vinyl protons at 5.0-6.2 ppm in **Fig. 1B** indicated the successful copolymerization

between NIPAM monomer and AA monomer, in agreement with previous reported data [13, 14].

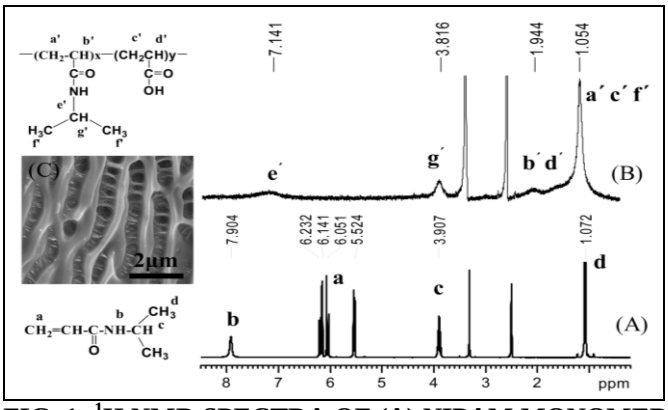
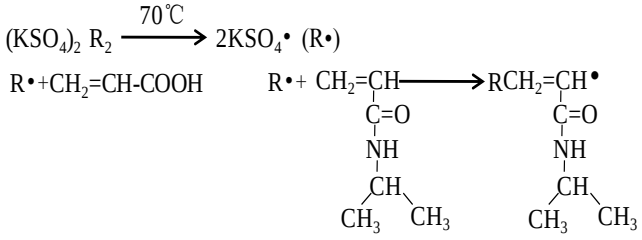


FIG. 1: ^{1}H NMR SPECTRA OF (A) NIPAM MONOMER and (B) P (NIPAM-co-AA)-1. THE TYPICAL SEM IMAGE OF (C) P(NIPAM-co-AA) -1×10.0K (INSET)

The similar spectra were also obtained for other samples. The detailed synthesis procedure was described as shown in **Scheme 1S**. SEM image of freeze-dried P(NIPAM-co-AA) (Taking P(NIPAM-co-AA)-1 as example), as shown in **Fig. 1C** (inset) exhibited that it had a well-regular networks and highly porous structure, and its open pores with continuous boundaries would be very useful in controlled drug delivery carrier. Meanwhile, the FT-IR spectra also confirmed the presence of functional groups of both NIPAM and AA in the obtained P(NIPAM-co-AA) copolymer (shown in **Fig. 2**).

Initiation:



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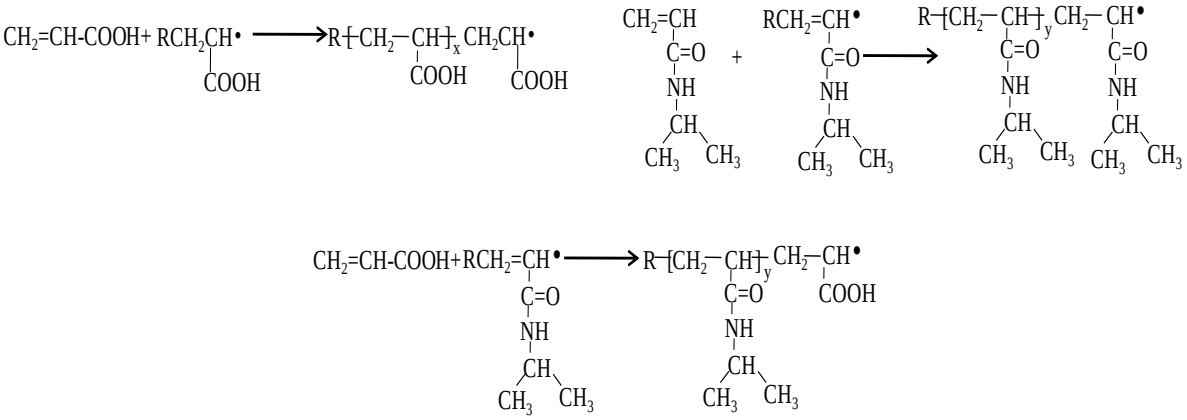
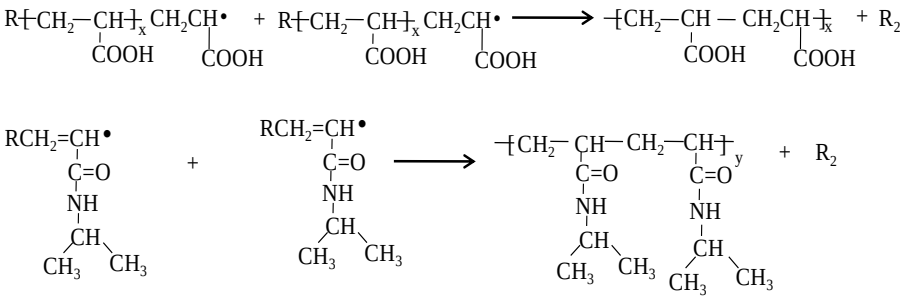


Termination:



SCHEME 1S: SYNTHESIS PROCEDURE PRINCIPLE OF COPOLYMER P(NIPAM-co-AA)

The FT-IR spectra of NIPAM, PNIPAM and P (NIPAM-co-AA) are illustrated in **Fig. 2**. As can be seen in **Fig. 2-a** and **2-b**, the FT-IR spectra of NIPAM monomer and PNIPAM presented the characteristic absorption bands, such as 1652 cm^{-1} and 1545 cm^{-1}, very similar to those of reported results [15]. Meanwhile, **Fig. 2c** revealed the spectrum of P(NIPAM-co-AA), accordingly, its characteristic peak at around 1728 cm^{-1} was attributed to C=O stretching vibration in carboxylic acid group [8], while other three feature bands at 1650 cm^{-1}, 1544 cm^{-1} and 1461 cm^{-1} were ascribed to C=O stretching vibration (amid I band), -NH bending(amid II band) and -C-N stretching vibration of acid amide group, respectively [16-17]. Additionally, the typical band of isopropyl group ($CH\text{-}(CH_3)_2$) at 1363 cm^{-1} and 2969 cm^{-1} corresponded to the -CH and $-CH_3$ stretching vibration [18]. These observations also confirmed the presence of functional groups of both NIPAM and AA in the obtained P(NIPAM-co-AA) copolymer.

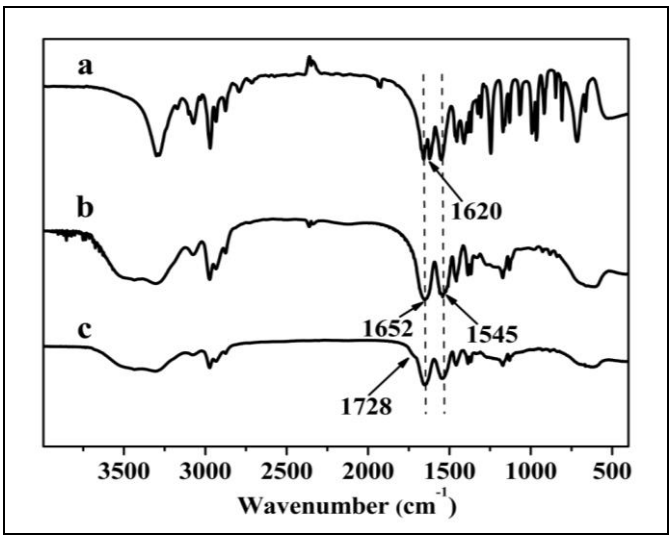


FIG. 2: FT-IR SPECTRA OF (a) NIPAM MONOMER, (b) PNIPAM, AND (c) P (NIPAM-co-AA)

3.2. Effect of AA content and pH value on the swelling behaviours: Effects of AA content and pH value on the swelling behavior of P(NIPAM-co-AA) were obtained from the DLS measurement at 25 ºC, in which pH value was adjusted through adding small amounts of HCl solution or $NH_3 \cdot H_2O$ in order to avoid the salt influence. As shown in **Fig. 3** of swelling behavior of the obtained copolymer, with the increase of pH value, the hydrodynamic diameter of P(NIPAM-co-AA) with AA additive presented increases firstly and then decreases tendency, but for PNIPAM without AA additive. Especially, in the range of pH value from 5 to 10, the particle size was increased more significantly than that at low pH range less than 5. For example, as shown in **Fig. 3e**, the hydrodynamic diameter of P(NIPAM-co-AA)-10 was increased from 375 nm at pH 5 to 590 nm at pH 10, which might be due to the features of -COOH stemming from AA chains. When pH value was lower than *pKa* of -COOH group (*pKa* of PAA was around 4.75 [15]), the protonated -COOH group could form hydrogen bonding via intra-molecular and inter-molecular interactions, easily leading to both shrinking of copolymer and deceasing of hydrodynamic diameters. However, when pH value was increased over its pKa, -COOH groups could gradually be deprotonated to $-COO^-$ groups; the ionized carboxylic groups not only could maintain good hydrophilic character but also produce electrostatic repulsive forces among $-COO^-$ groups [16], which directly resulted in the increase of particles size. Thus, the P(NIPAM-co-AA) exhibited a smart pH responsibility. However, the hydrodynamic diameter was decreased sharply at higher pH value (up to 12), mostly because of salt effect of NH_4^+ ions [17].

Meanwhile, it is noticed that the pH effect on swelling behaviors was also depended on the content of -COOH groups existing inside the copolymer chains. As shown in **Fig. 3a**, the smallest particle size of pure PNIPAM was observed, but, the swelling behavior was independent on pH. As shown in **Fig. 3b-e**, P(NIPAM-co-AA-x) (x=1-10) had a larger particle size than pure PNIPAM in both acid or basic medium, which could be explained as the following: When AA was added into PNIPAM framework, the obtained copolymers were more hydrophilic with larger volume due to the dissociate $-COO^-$ groups [18], easily leading to much more water into the nanoporous network and the increased size.

Additionally, the increase of particle size was more remarkable in basic solution than that in acid solution because of the strong hydrophilicities of the -COOH groups dissociated from AA and electrostatic repulsion force among the $-COO^-$ groups [16]. However, at low pH value, the nonionized -COOH groups in AA had less electrostatic repulsion force with a limited increase

of particle size, as shown in **Fig. 3b-e** (pH=2.0-5.0). These results suggested that pH-sensitive properties of these obtained copolymer of NIPAM and AA segment could be successfully tailored through changing AA content. In essence, their features could be affected by variation of the surface charge density. Therefore, it is necessary to explore the relationship of zeta potential with the change of pH value and the content of AA.

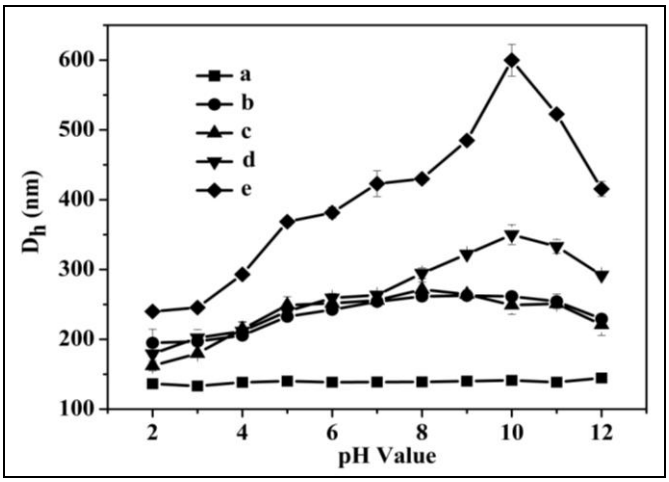


FIG. 3: HYDRODYNAMIC DIAMETERS OF (a) PNIPAM, (b) P(NIPAM-co-AA)-1, (c) P(NIPAM-co-AA)-3, (d) P(NIPAM-co-AA)-7, AND (e) P(NIPAM-co-AA)-10, AS A FUNCTION OF pH VALUE AT 25 °C ON THE BASIS OF PEAK INTESITY OF INTENSITY OF PARTICLE SIZE DISTRIBUTIONS DERIVING FROM DLA MEASUREMENTS

In combination with the DLS characterization, as shown in **Fig. 3**, the variations in the surface charge of copolymer can be used to further confirm the interrelate swelling behaviors with the change of pH value and the content of AA. As shown in **Fig. 4** of the relationship between zeta potential and pH value or various incorporated content of AA, the series of copolymer all had negative potentials that were decreased in the range of pH 2.0-8.0 and then increased at above pH 8.0. With the increase of pH value, -COOH groups were gradually dissociated into $-COO^-$, resulting in the increase of negative charge. At pH of 8.0, -COOH groups were considered to be completely deprotonated, leading to the maximum potential. After that, the zeta potential value was increased to some extent. Therefore, we could speculate that the copolymer reached to ionization equilibrium at around pH of 8.0, and then these negative charges were shielded by NH_4^+ produced from ammonia with the increase of ammonia concentration. As seen in **Fig. 4A-a**, pure PNIPAM had a negative zeta potential derived from $-SO_4^-$ of initiator (KPS) fragments at their chain ends [7]. Noteworthy, **Fig. 4A-b**, **-c**, **-d**, and **-e** showed that the change tendency of zeta potential curves for P(NIPAM-co-AA) (x=1-10) was almost consistent with the hydrodynamic diameter change tendency (seen in **Fig. 3**). Meanwhile, as shown in **Fig. 4B**, the zeta potential was declined with the increase of AA content, such as -24.4 mv for P(NIPAM-co-AA)-1, -32.6 mv for P(NIPAM-co-AA)-3, -28.1 mv for P(NIPAM-co-AA)-7 and -40.8 mv for P(NIPAM-co-AA)-10 at pH 8.0, respectively.

However, in acid solution (pH 3.0), their zeta potential values were very stable. Therefore, DLS analysis and zeta potential measurements suggested that P(NIPAM-co-AA) swelling/shrinking behavior could be effectively adjusted through various pH values and AA content.

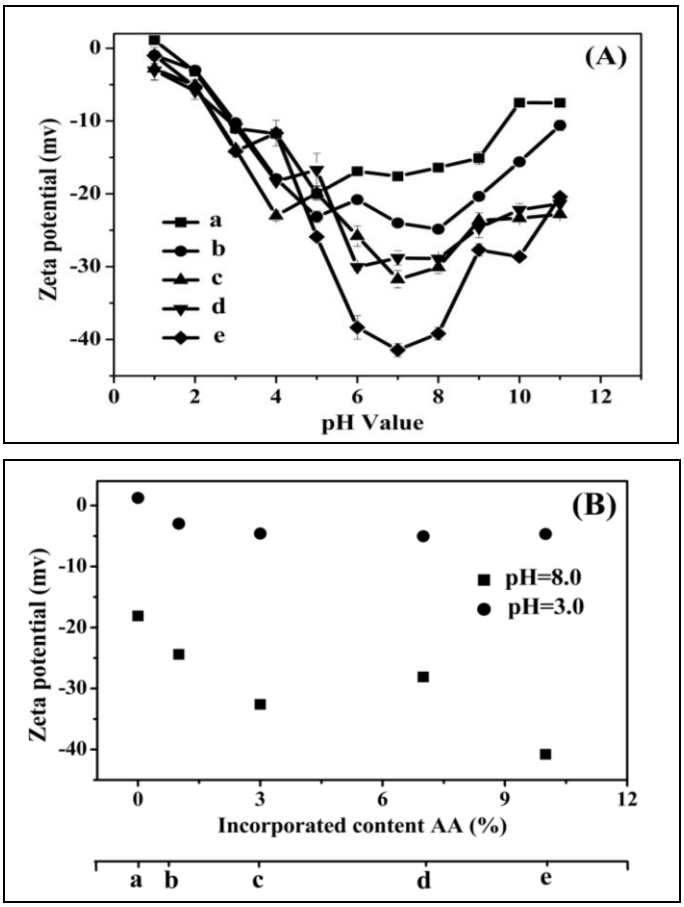


FIG. 4: ZETA POTENTIAL-DEPENDENT PROFILES ON pH VALUE WITH (A) VARIOUS INCORPORATED CONTENT OF AA AT 25 °C AND (B) AT pH=3.0, pH=8.0 FOR (a) PNIPAM, (b) P(NIPAM-co-AA)-1, (c) P(NIPAM-co-AA)-3, (d) P(NIPAM-co-AA)-7, AND (e) P(NIPAM-co-AA)-10.

3.3. Effect of AA content and temperature on the swelling behaviours: The temperature-response behaviors of copolymer were investigated by observation of the variation of hydrodynamic diameters in ultrapure water at pH 6.8 with different temperature from 20 ºC to 60 ºC. Overall, as presented in **Fig. 5**, the hydrodynamic diameter of P(NIPAM-co-AA) was continuously decreased with the increase of temperature, as described in Introduction section, the main reason is that the P(NIPAM-co-AA) with thermal sensitive segments had volume shrinkage above its LCST. However, compared with that of pure PNIPAM (shown in **Fig. 5a**), the transition temperature range of P(NIPAM-co-AA) had a broad distribution towards higher temperature with the increase of AA content (**Fig. 5b**, **4c**, **4d** and **4e**). It was known that the LCST behavior resulted from a balance of hydrophobic and hydrophilic groups in the obtained polymer [17, 18, 19]. The introduction of hydrophilic AA into the polymer strengthened the strong interaction between copolymer and water molecule while weakened the hydrophobic interaction of isopropylacrylamide groups. The hydrophobic interaction was also enhanced with the increased temperature, thus, higher temperature was needed to compensate the hydrophilic interaction of the AA chain with water molecule.

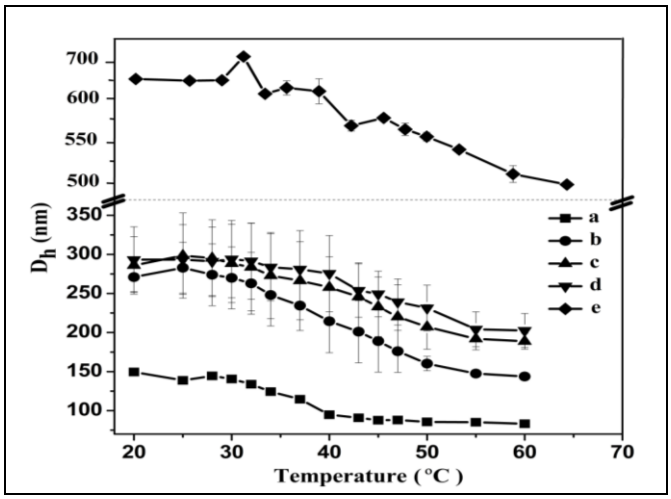


FIG. 5: INFLUENCE OF TEMPERATURE ON HYDRODYNAMIC DIAMETERS (BASED ON PEAK INTENSITY OF DLS PROFILES) WITH DIFFERENT AA CONTENTS IN AQUEOUS SOLUTION, (a) PNIPAM, (b) P(NIPAM-co-AA)-1, (c) P(NIPAM-co-AA)-3, (d) P(NIPAM-co-AA)-7, AND (e) P(NIPAM-co-AA)-10.

Secondly, as shown in **Fig. 3** and **4**, at near pH 7.0, the electrostatic repulsive force among depronated -COO^- could partly offset the chain aggregation effectively because of hydrophobic interaction among isopropyl groups [19, 20]. Therefore, with a higher content of AA, the electrostatic repulsive force had the more strength, leading to the transition temperature towards higher temperature range.

In order to further confirm that AA content had strong impact on the thermo-responded behavior, the DSC measurements of the swollen P(NIPAM-co-AA) in ultrapure water at pH 6.8 were conducted. As shown in **Fig. 6**, when the occurrences of the phase transition for swollen samples were accompanied by thermal effect with increased temperature, an endothermic peak could be observed on DSC curves in **Fig. 6(a-e)**. The onset given temperature was determined from the DSC data at the baseline below 30 ºC and the other passing through the rising slope of transition. The temperature at which the two lines intersected was defined as the onset.

Overall, as shown in **Fig. 6(a-e)**, the phase transition onset temperature rose from 31.6 ºC for PNIPAM to 33.2 ºC for P(NIPAM-co-AA)-1, 33.6 ºC for P(NIPAM-co-AA)-3, 33.7 ºC for P(NIPAM-co-AA)-7, 33.9 ºC for P(NIPAM-co-AA)-10, respectively. These results clearly showed an endothermic peak shift to a higher temperature region with the increase of AA content, due to the comprehensive actions between hydrophobic interaction and electrostatic repulsive force [21].

As shown in **Fig. 6a,** the process of phase transition for pure PNIPAM was mainly dominated by hydrophobic interaction. However, the phase transition of P(NIPAM-co-AA) needed to overcome the electrostatic repulsive force from -COO^- groups. Therefore, higher content of AA and larger electrostatic repulsive force made their phase transition temperature shift to the higher temperature region, which was in well agreement with that temperature influence on their hydrodynamic diameters, as shown in **Fig. 5**.

Furthermore, it is noted that the DSC curves shape in the peak height and the peak area of these copolymers were significantly changed with different AA contents, in which the enthalpy value during phase transition procedure was calculated according to the software from the calorimeter

manufacturer. Interestingly, the obtained results showed that the enthalpy values were decreased with the increase of AA content from 2.3 J/g for pure PNIPAM, to 1.9 J/g for P(NIPAM-co-AA)-1, 0.92 J/g for P(NIPAM-co-AA)-3, 0.88 J/g for P(NIPAM-co-AA)-7 and 0.49 J/g for P(NIPAM-co-AA)-10, respectively, particularly, the enthalpy value of pure PNIPAM is in a good agreement with Zhang's report [22]. These enthalpy values were mainly related to the disrupted or weakened hydrophilic interaction and hydrogen bonding between polymer and molecule water (defined as structure water) [23]. Since the isopropylacrylamide groups in copolymer skeleton contained a smaller amount of structure water at high temperature than that at low temperature, it only needed very limited energy to weak these interactions, finally leading to the high transition temperature with low enthalpy value.

Therefore, these demonstrations concluded that P(NIPAM-co-AA) could maintain the temperature response property, and AA content had a significant impact on the phase transition temperature.

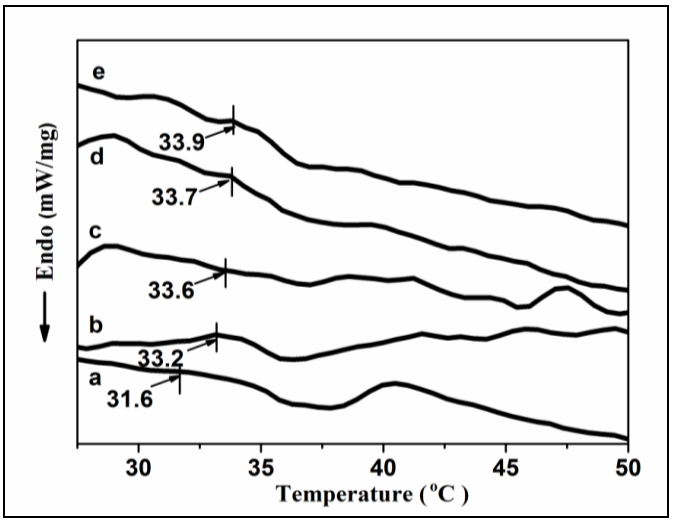


FIG. 6: DSC PROFILES OF THE SWELLEN SAMPLES AT 25 °C (SIGNAL MAGNIFIED THREE TIMES), (a) PNIPAM, (b) P(NIPAM-co-AA)-1, (c) P(NIPAM-co-AA)-3, (d) P(NIPAM-co-AA)-7, AND (e) P(NIPAM-co-AA)-10

3.4. Effect of NaCl concentration on swelling behaviors with different AA contents: As above discussion, the incorporation of different AA contents into the copolymer network not only affected the sensitivity of pH and temperature but as-expected the swelling behavior, which could be controlled by the different ionic concentrations as well as ionic strength [7]. In this case, we investigated the influence of NaCl concentration on zeta potential and hydrodynamic diameters at pH 3.0 and 8.0, as shown in **Fig. 7**. Firstly, as seen in **Fig. 7A**, hydrodynamic diameter of polymer was increased with the increase of incorporated AA amount. In detail, the pure PNIPAM had a small hydrodynamic radius (135 nm at pH 3.0 and 138 nm at pH 8.0). After AA was incorporated into PNIPAM network, its hydrodynamic radius was gradually increased at both pH 3.0 and 8.0, as shown in **Fig. 3** and **Fig. 4**.

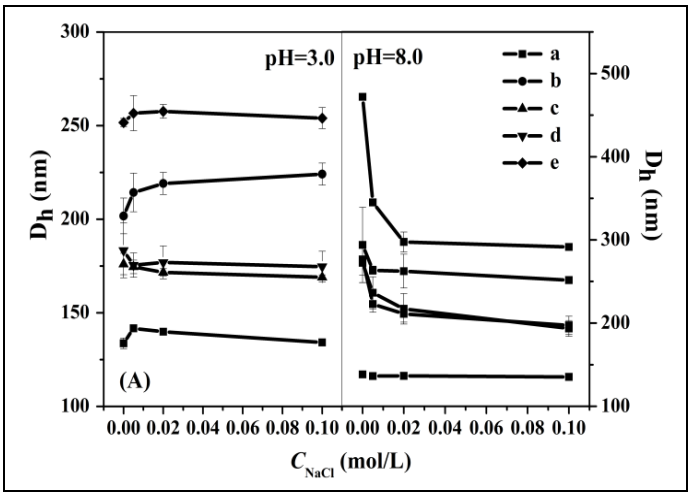
In addition, at pH 3.0 hydrodynamic diameter of copolymers was only slightly depended on salt concentration from 0.0 mol/L to 0.1mol/L. Conversely, at pH 8.0, the hydrodynamic diameter was decreased sharply at an initial stage of NaCl concentration of 0.0-0.02 mol/L, and then stayed unchanged. Taking P(NIPAM-co-AA)-10 as an example, as shown in **Fig. 7A** at pH 8.0 its hydrodynamic diameter was around 472 nm without sodium ion, and then sharply decreased to 297 nm when NaCl concentration was around 0.02 mol/L. After that NaCl concentration even up to 0.1 mol/L, it remained almost the same at about 290 nm.

Similarly, mainly due to the increase of ionic concentration in the pH 8.0 medium [24], the repulsion force among $-COO^-$ groups was decreased with the shrinkage of copolymer volume and the decrease in particle size. At pH 3.0, the -COOH groups were not protonated with weakly internal repulsion force and the impact of sodium ion on the copolymer structure was very weak, which led to the constant hydrodynamic diameter of P(NIPAM-co-AA)-10 even at the highest concentration of added NaCl (up to 0.1ml/L, as shown in **Fig. 7A** at pH 3.0).

In fact, the influence of salt concentration on the surface charge distributions of copolymer is remarkable [21], which could be confirmed by zeta potential measurements. As shown in **Fig. 7B**, zeta potential of copolymer exhibited a relatively smaller change at low pH (3.0), but significantly increased with the increase of NaCl concentration at pH 8.0 within the range from 0.0 to 0.02 mol/L. For example, the zeta potential of P(NIPAM-co-AA)-10 was steeply increased from -40.0 mv to -

15.2 mv when Na^+ concentration was increased from 0 to 0.02 mol/L, and then slowly increased from -15.2 mv to -10.0 mv when Na^+ concentration was from 0.02 to 0.1 mol/L. As mentioned in **Fig. 4B**, at low pH value, the -COOH group was protonated, resulting in seldom surface charge of copolymer, therefore the Na^+ existence had no effect on their zeta potential and hydrodynamic diameters. Conversely, in the basic medium, the copolymer had a bulk surface negative charge, which was the primary reason for the continued shrinkage and improved zeta potential. These observations implied that Na^+ played a significant role in electrostatic screening of these surface negative charges and diminished the charge density accompanied with charges shielded [25].

Moreover, at pH 8.0, **Fig. 7A** indicated that hydrodynamic diameter of pure PNIPAM was not changed significantly. As shown in **Fig. 7B**, zeta potential of pure PNIPAM had the similar trend with other copolymers. These results illustrated that the seldom surface negative charge of pure PNIPAM could be shielded by Na^+ due to formation of the $-CONH_2$ groups. Therefore, P(NIPAM-co-AA) was thermo- and pH-responsive with hydrodynamic diameter changes. The surface charge of copolymer could also be adjusted through the incorporation of different AA contents into the copolymer chains, which could systematically control their swelling-shrinking behaviors. Meanwhile, effects of NaCl concentration on the surface charge of the copolymer and occurrences of electrostatic screening would be very important to their stimuli behaviors in the drug delivery applications.



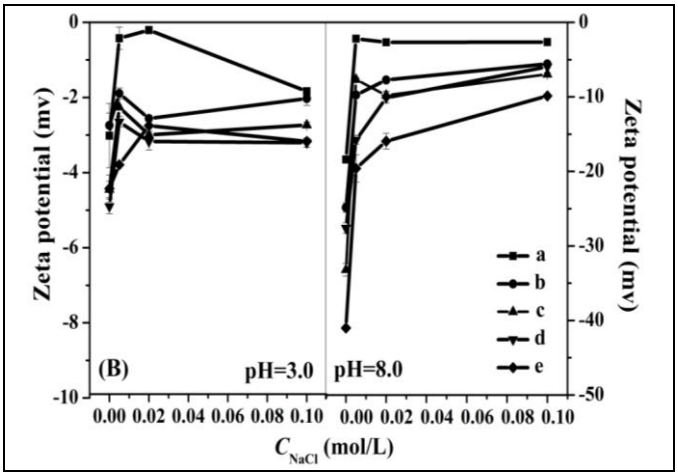


FIG. 7: EFFECTS OF NaCl CONCENTRATION ON (A) HYDRODYNAMIC DIAMETERS AND (B) ZETA POTENTIAL OF COPOLYMER WITH DIFFERENT AA CONTENTD AT 25 °C AT pH=3.0 AND pH=8.0, (a) PNIPAM, (b) P(NIPAM-co-AA)-1, (c) P(NIPAM-co-AA)-3, (d) P(NIPAM-co-AA)-7, AND (e) P(NIPAM-co-AA)-10

3.5. Effect of pH value and temperature on IBU-released performance of resultant copolymer: It is widely known that the drug delivery system is complex and the release behaviors of loaded drug have a close relationship with the swelling performance of copolymer matrix under different conditions. Thus IBU was used as a model drug to carry out the drug releasing measurement and further confirmed dual-response property of the copolymers.

Fig. 8 shows the IBU-release profiles from drug-loaded copolymer at different temperature in HCl (pH 2.0) or $NH_3 \cdot H_2O$ aqueous solution (pH 7.4). As profiled in **Fig. 8a** and **b**, it is observed that the release behaviors of pure PNIAM at pH 2.0 and 7.4 were very similar with IBU equilibrium release percentages of about 78.8 % and 80.7 %, respectively, clearly showing that the release of IBU from pure PNIPAM had poor pH-sensitivity at both pH 2.0 and 7.4 due to the nature of PNIPAM structure to determine the swelling feature as mentioned-above characterizations as illustrated in **Fig. 3, 4** and **5**. However, as shown in **Fig. 8c** and **d**, the IBU-release from P(NIPAM-co-AA)-1 at pH 7.4 was much faster than that at pH 2.0. The cumulative release reached up to about 58.7 % before 5 h at pH 7.4 while the release amount was only about 23.5 % in the pH 2.0 solution. The highest cumulative release after 24 h was 80.7 % at pH 7.4 and 48.7 % at pH 2.0, respectively.

On the other hand, as compared with that in **Fig. 8a** and **d** the release rate of IBU from P(NIPAM-co-AA)-1 at low pH value was evidently slower and the release amount was 48.7 % at 24 h, less than that from pure PNIAM in the same medium. Oppositely, as shown in **Fig. 8b** and **c**, the release rate of IBU for P(NIPAM-co-AA)-1 and pure PNIPAM at high pH value was similar and their release amount was around 80.7% at 24 h.

Evidently, these results indicated that IBU-release from P(NIPAM-co-AA)-1 was closely related to the pH conditions. According to the above discussion, since the copolymer of PNIPAM chains exhibited volume shrinkage as 37 ºC above its LCST, the different release behaviors between pH 2.0 and 7.4 were mainly attributed to the AA additives, which had an impact on "switch on-off " dependence via pH value to control drug release.

In alkaline solution, -COOH groups of P(NIPAM-co-AA) were deprotonated, and the electrostatic repulsion among -COO^- groups caused the volume swelling of polymer network. These conditions could provide an "open channel" to promote the gradual diffusion of drug into solution [26], in this case, the IBU *pKa* (*pKa*=4.91) was an important factor governing its release. Therefore, its ionization was increased at pH 7.4 and the electrostatic repulsion force was obviously generated between IBU molecules and polymer chains. Oppositely, in acidic solution (pH 2.0), -COOH groups of P(NIPAM-co-AA) were protonated and also formed into hydrogen bond among polymer chains, resulting in volume shrinking of the polymer network. Thus, the loaded IBU inside the polymer network was confined in the "closed channel" and the diffusion rate was slowed down. Apparently, these results also illustrated that the particle size of P(NIPAM-co-AA) had a close relationship with the IBU-releasing performances. As the above discussion in **Fig. 3**, at the same temperature in alkaline solution the P(NIPAM-co-AA) presented the larger particle size than that in acidic medium. Comparably, at the same temperature the large particle size of P(NIPAM-co-AA) with the swollen structure is more favorable to the IBU diffusion from copolymer network than the small particle size of that with the shrinkable structure.

Additionally, **Fig. 8** presents the temperature responsive effect on IBU release behaviors at 25 ºC and 37 ºC. As shown in **Fig. 8b** and **e**, there was a ''burst" release profile during the initial stage for pure PNIPAM. IBU was completely released in 11 h at 25 ºC, and the release amount of IBU only reached to 70.0 % at 37 ºC. Meanwhile, IBU release rate from pure PNIPAM at 25 ºC was much faster than that at 37 ºC due to the complete collapse of polymer network at 37 ºC (above LCST) that blocked the diffusive release of IBU into solution [27]. On the other hand, the hydrophilic interactions between PNIPAM and water molecules at 25 ºC (below the LCST) led to polymer swelling with open network and therefore the easier IBU releasing [28].

In contrast, as shown in **Fig. 8c** and **f**, the IBU release amount of P(NIPAM-co-AA)-1 at 37 °C reached up to 80.7% at equilibrium that was much higher than that at 25 °C, indicating that the releasing behavior of IBU from P(NIPAM-co-AA)-1 was strongly depended on the temperature. As shown in **Fig. 5**, the phase transition temperature of P(NIPAM-co-AA)-1 was broaden from 35 °C to 40°C due to the electronic repulsive force of -COO^- groups on the AA chain of copolymer. When the temperature was around 37 °C, the occurrence of copolymer configuration caused a segmental collapse with open pores and the drug releasing was accelerated through the open pore. Therefore, the shrinkage of PNIPAM chains played a critical role in squeezing out the entrapped drug at 37 °C [29]. Additionally, the mobility of IBU through the network was enhanced as the temperature was increased. Thus, the release rate of IBU from the copolymer at 37 °C was faster than that at 25 °C.

This result is significant because of high release amount near the human body temperature, which can make the drug efficiently used. Also, **Fig. 8e** and **f** present that the release of pure PNIPAM was clearly faster than that of P(NIPAM-co-AA)-1 at 25 ºC because the presence of AA chains in the copolymer network overcame the "burst release" effect of IBU from used copolymer, which was mentioned in influence of temperature on swelling behavior though at below LCST temperature. On the whole for the pure PNIAM, IBU release behaviors exhibited the temperature-response but independent pH-sensitivity.

However, the P(NIPAM-co-AA)-1 clearly indicated both pH- and temperature dual-response, in which their network structure had greatly effects on the release behaviors.

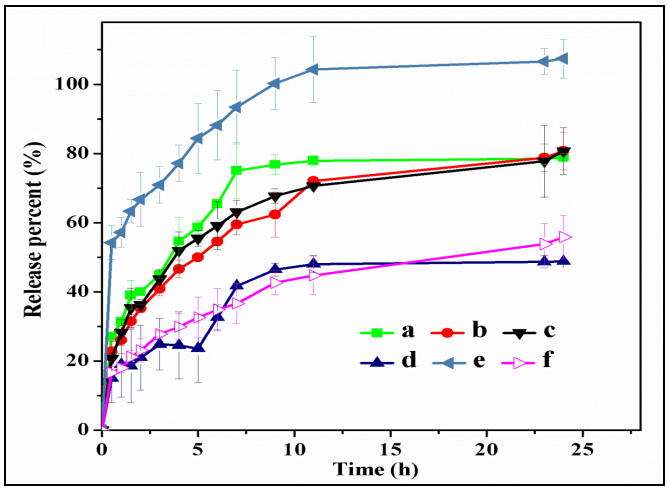


FIG. 8: *IN VITRO* RELEASE BEHAVIORS OF IBU-LOADED COPOLYMER AT DIFFERENT TEMPERATURE IN VARIOUS AQEOUS SOLUTIONS, (a) AT 37 °C IN CONTAINED PNIPAM HCl SOLUTION (pH 2.0), (b) AT 37 °C IN CONTAINED PNIPAM $NH_3 \cdot H_2O$ SOLUTION (pH 7.4), (c) AT 37 °C IN CONTAINED P(NIPAM-co-AA)-1 $NH_3 \cdot H_2O$ SOLUTION (pH 7.4), (d) AT 37 °C IN CONTAINED P(NIPAM-co-AA)-1 HCl SOLUTION (pH 2.0), (e) AT 25 °C IN CONTAINED PNIPAM $NH_3 \cdot H_2O$ SOLUTION (pH 7.4), (f) AT 25 °C IN CONTAINED P(NIPAM-co-AA)-1 $NH_3 \cdot H_2O$ SOLUTION (pH 7.4).

CONCLUSIONS: The dual stimuli-responsive P(NIPAM-co-AA) copolymers have been prepared successfully by copolymerization of NIPAM and acrylic acid with free radical polymerization method. Their swelling behaviors could be effectively controlled through tuning pH and temperature, in which, the influences of AA amount and ionic concentration on the particle size and zeta potential were obvious under alkaline solution. The phase transition temperature of copolymer with the increasing AA content shifted toward higher values. Meanwhile, *in vitro* IBU-releasing profiles of P(NIPAM-co-AA) indicated that release amount was much greater at pH 7.4 than at pH 2.0 at 37 ºC but faster at 37 ºC than at 25 ºC at pH 7.4, showing much sensitive to pH value and temperature Therefore, the incooperation AA chains into PNIPAM could overcome burst effect of PNIPAM-based network to some extent, which might be potentially used to prepare the smart functional hybrid materials for applications in drug delivery.

CONFLICT OF INTEREST: The authors declared that there are no any conflicts of interest.

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