

Research paper

Temperature-sensitive hydrogels composed of chitosan and hyaluronic acid as injectable carriers for drug delivery

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Abstract

Temperature-sensitive hydrogels composed of poly(*N*-isopropylacrylamide) (PNIPAAm) with chitosan (CPN) and chitosan + hyaluronic acid (CPNHA) were grafted in order to examine their physicochemical characteristics, in vitro drug release, and in vivo pharmacodynamics. The sol–gel transition behavior was investigated by UV/visible spectrophotometry, differential scanning calorimetry, and viscometry. A slight difference in the transition temperatures was observed among these polymer systems, with CPN and CPNHA exhibiting higher temperatures compared with PNIPAAm. A zeta potential determination revealed a positive charge for the CPN hydrogel, whereas no or only a negligible charge was observed for PNIPAAm and CPNHA. The entanglement of CPN hydrogels observed using scanning electronic microscopy showed the densest cross-linkage structure, followed by CPNHA and PNIPAAm. Both hydrophilic and lipophilic drugs, including nalbuphine, indomethacin, and the nalbuphine prodrug, were used as model drugs in an in vitro drug release experiment. All 3 hydrogels significantly prolonged drug release. The release rate of hydrophilic nalbuphine increased in the order CPN < CPNHA < PNIPAAm. The drug release of these hydrogels exhibited a trend opposite to that of lipophilic drugs. A cold ethanol tail-flick study was utilized in order to examine the antinociceptive activity of intravenous nalbuphine. CPN and CPNHA prolonged the analgesic duration of nalbuphine with no influence on the onset time. The loading of nalbuphine in the CPNHA hydrogel exhibited the longest analgesic duration (4 h) among the 3 hydrogels tested.

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1. Introduction

Hydrogels, by virtue of their excellent tissue compatibility, easy manipulation, and solute permeability, have played a vital role in the development of drug delivery systems [1]. Considerable interest has been focused on hydrogels that exhibit a phase transition in response to external stimuli such as temperature, pH, ionic strength, and electric potential [2]. Temperature-sensitive polymers exhibit a

lower or upper critical solution temperature in aqueous media. In the case of polymers with a lower critical solution temperature (LCST), the polymers are water soluble below the LCST, but become significantly less water soluble or water insoluble at temperatures above the LCST. The polymer poly(*N*-isopropylacrylamide) (PNIPAAm) has been observed to undergo a temperature-induced sol–gel transition [3]. In the body, PNIPAAm can be degraded to monomers by the action of hepatic glutathione S-transferases, the main metabolite being identified as acrylamide [4]. The clinical applications of PNIPAAm hydrogels are consequently limited due to the carcinogenic or teratogenic toxicity of monomeric acrylamide [2,5].

To date, little work has been published on the development of temperature-reversible hydrogels based on natural

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polymers [6]. Chitosan is a natural copolymer of D-glucosamine and N-acetylglucosamine derived from chitin. Hyaluronic acid (HA) is an abundant non-sulfated glycosaminoglycan component of synovial fluid and extracellular matrices. Both of these can be considered as attractive materials for new biocompatible and biodegradable polymers, having applications in drug delivery, tissue engineering, and viscosupplementation [7,8]. In our previous report, we described how PNIPAAm-grafted chitosan (CPN) was useful as a scaffold for the cultivation of cartilage cells [7]. In the present study, we attempted to develop injectable, temperature-sensitive systems based on PNIPAAm-grafted chitosan and/or HA, which can serve as therapeutic drug delivery systems. This combination of synthetic and natural polymers can help maintain the temperature sensitivity of PNIPAAm, while partly improving its biocompatibility and biomedical properties. This liquid can be administered intravenously into vessels where it is transformed into a gelled drug depot [9].

The drug selected for loading in this study was nalbuphine, a narcotic analgesic used in the treatment of both acute and chronic pain. Due to its short elimination half-life and poor oral bioavailability, frequent injections (e.g., every 3–6 h) of this drug are required [10]. A sustained-release formulation for the parenteral administration of nalbuphine would therefore be highly beneficial. In this work, 2 hydrogels of CPN and PNIPAAm-chitosan-HA (CPNHA) were prepared using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling agents (Fig. 1). To the best of our knowledge, this is the first report to describe the synthesis of CPNHA for the purpose of drug delivery. In this

study, we examined the feasibility of using CPN and CPNHA hydrogels as controlled-release systems for nalbuphine, and evaluated their performance in terms of their physicochemical characteristics, in vitro drug release, and in vivo analgesic activity.

2. Materials and methods

2.1. Materials

Nalbuphine HCl was purchased from Du Pont Merck (North Billerica, MA, USA). Nalbuphine propionate (NAP) was a gift from the School of Pharmacy, National Defense Medical Center, Taipei, Taiwan. Indomethacin, α,α -azobis-isobutyronitrile (AIBN), 2-morpholineethanesulfonic acid (MES), and N-isopropylacrylamide (NIPAM) were from the Sigma Chemical Co. (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Acros Organics (Geel, Belgium). Chitosan [molecular weight (MW) = 150 kDa, deacetylation = 98%] and mercaptoacetic acid (MAA) were purchased from Fluka Chemie GmbH (Buchs, Germany).

2.2. Polymer synthesis

2.2.1. PNIPAAm synthesis

The recrystallized monomer (50 mg) of NIPAM was dissolved in 250 ml of benzene containing 10 mol MAA and 0.5 mol AIBN. After inducing polymerization by stirring in a water bath at 60 °C for 24 h under positive nitrogen pressure, the solvent was evaporated. The crude solid was

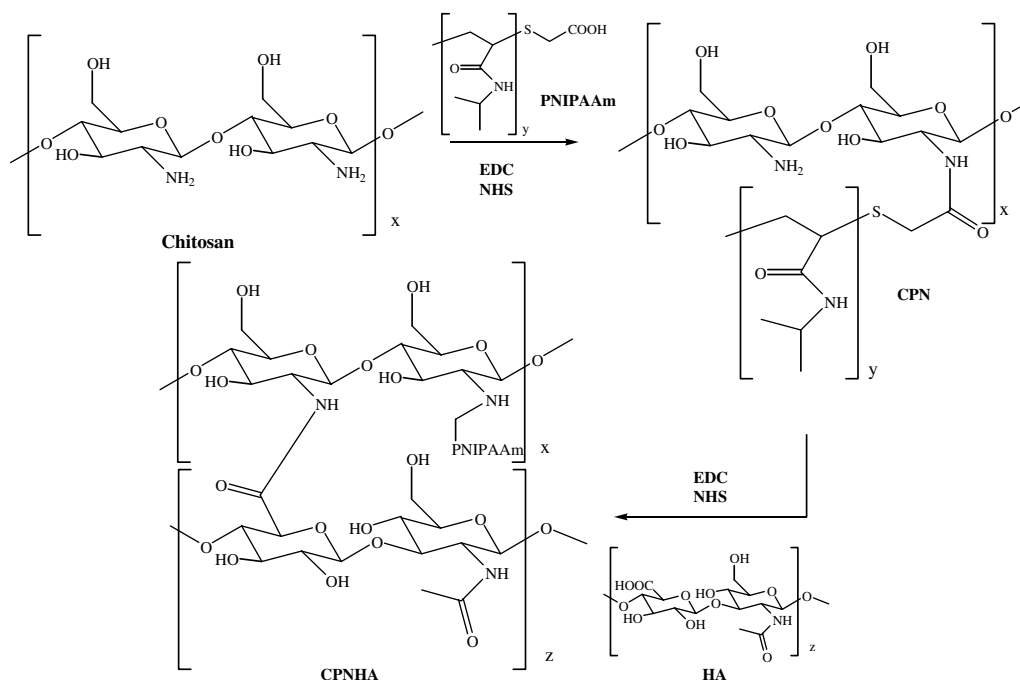


Fig. 1. Chemical reaction scheme for synthesizing CPN and CPNHA.

vacuum-dried, dissolved in acetone, and precipitated by dropwise addition into an ether solution. After being filtered and dried, the polymer was dialyzed against double-distilled water for 7 d (Sepectra/Por3, MW cut-off 3500 Da) and then freeze-dried in order to obtain the polymer.

2.2.2. CPN synthesis

PNIPAAm (10 g) and chitosan (1 g) were dissolved in 100 ml of 0.1 M MES (pH 5.0). EDC and NHS were added to the above solution. The stoichiometric ratio of $-\text{COOH}$ in PNIPAAm- COOH /EDC/NHS was 1:10:50. The conjugation reaction was carried out for 12 h. A 3 M NaCl (30 ml) solution was then added to the solution in a water bath at 50 °C and the mixture incubated for 30 min. Following incubation, the solution was centrifuged at 13,500 rpm and 40 °C for 20 min, diluted, and then dissolved in MES buffer (100 ml). The above steps were repeated 3 times. The resulting product was dialyzed for 4 d (Cellu Sep H1, MW cut-off 50 kDa) and was then freeze-dried in order to obtain the polymer. The yield of CPN was ~40%.

2.2.3. CPNHA synthesis

CPN (2 g) and HA (0.25 g) were dissolved in 100 ml of 0.1 M MES (pH 5.0). HA was extracted from *Streptococcus zooepidemicus* using a method described by Armstrong and Johns [11]. The MW of HA was 1780 kDa (polydispersity = 1.68) determined by size exclusion chromatography [12]. EDC (0.46 g) and NHS (1.38 g) were added to the above solution. The stoichiometric ratio of $-\text{COOH}$ in HA- COOH /EDC/NHS was 1:10:50. The subsequent procedures were the same as those described for the synthesis of CPN. The yield of CPNHA was ~92%.

2.2.4. Measurements of molecular weight, efficiency of grafting, and grafting ratio

The average MW of PNIPAAm was determined by end-group titration. For this purpose, 0.5 g of the polymer was dissolved in 10 ml of water and then titrated with 0.01 N NaOH in order to determine the concentration of the carboxyl end group. The average MW was calculated by using the following equation: $\text{MW}_{\text{PNIPAAm}} = \text{weight of PNIPAAm titrated} / \text{moles of PNIPAAm carboxyl end groups}$.

The efficiency of grafting (%) of CPN was calculated from the relationship $(W_{\text{CPN}} - W_{\text{C}}) / W_{\text{PNIPAAm}} \times 100$, where W_{CPN} is the weight of freeze-dried graft copolymer, and W_{C} and W_{PNIPAAm} are the weights of chitosan and PNIPAAm in the feed, respectively. The grafting ratio was calculated as $(W_{\text{CPN}} - W_{\text{C}}) / \text{MW}_{\text{PNIPAAm}}$ divided by $W_{\text{C}} / \text{MW}_{\text{C}}$. The efficiency of grafting and grafting ratio of CPNHA was calculated in a manner similar to that for CPN.

2.3. Hydrogel preparation

Polymers were mixed with double-distilled water to a concentration of 15% (w/w). The resulting homogeneous,

clear solution (total 10 g) was stored overnight in a refrigerator at 4 °C. Prior to using in the experiments, the mixtures were allowed to reach room temperature (~25 °C).

2.4. Zeta potential determination

The zeta potential of the polymer solutions (15%, w/w) was measured by a Nano ZS[®] 90 Zetasizer (Malvern Instruments, Malvern, Worcestershire, UK) at room temperature. The determination was repeated 3 times per sample for 3 samples.

2.5. LCST determination

The LCST was defined as the temperature at which the solution started to become opaque. The thermoresponsive phase transitions of the aqueous solutions of PNIPAAm, CPN, and CPNHA (15%, w/w) were measured with a UV/visible spectrometer (S2000; Walden Precision Apparatus, Cambridge, UK) by monitoring the absorbance of a 600-nm light beam through the aqueous solutions. The samples were heated at a rate of 0.5 °C/min from 25 to 40 °C. The temperature at which the samples exhibited an absorbance of >2.0 was taken as the LCST.

2.6. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was carried out using a Q10 DSC calorimeter (TA Instruments, New Castle, DE, USA). The thermal analysis profiles of the aqueous solutions of polymers were obtained as the temperature was increased from 25 to 45 °C at a rate of 2 °C/min under nitrogen. The determination was repeated 3 times for the polymers from 3 different batches.

2.7. Viscosity measurement

The temperature effect on hydrogel gelation was studied by measuring the viscosity as a function of temperature between 25 and 32 °C. The viscosities of these systems were determined using a Carri-Med CSL² 100 rheometer (TA Instruments). The diameters of both the cone and plate spindle were 60 mm. The determination mode was set to flow-step measurement with shear rates from 0 to 1000 1/s. The shear stress was set at 2 Pa. The cone angle used for measurements was 2°.

2.8. Scanning electronic microscopic (SEM) examination

Hydrogels were frozen at -80 °C and then lyophilized by freeze-drying. Samples were fractured in liquid nitrogen and sputter-coated with gold. The resulting dried samples were examined using a Hitachi S-2400 scanning electron microscope (Tokyo, Japan). All analyses were performed in a blinded fashion; that is, the analyst did not know the identity of the samples when the materials were examined.

2.9. Hydration of hydrogels

A 15% (w/w) solution of polymer in water was gelled in an oven at 37 °C for 1 h. One milliliter of double-distilled water was then added to the samples. Twenty-four hours later, the excess water was removed using a pipette. The remaining solid matrices were then weighed. The weight was recorded, and the hydration ratio of the test samples was calculated from the following expression:

$$\text{Hydration ratio} = (W_H - W_0)/W_0;$$

where W_H is the weight of the hydrated gel and W_0 is the weight of the dried test sample.

2.10. In vitro drug release

The drug release was measured using a Franz diffusion assembly. A cellulose membrane (Cellu-Sep® T2; Membrane Filtration Products, Seguin, USA) with a MW cutoff of 6000–8000 was mounted between the donor and receptor compartments. The donor medium consisted of 0.5 ml of vehicle containing nalbuphine, indomethacin, or NAP (2.8 mM). The receptor medium (5.5 ml) for nalbuphine was pH 7.4 citrate–phosphate buffer. For indomethacin and NAP, a solution of ethanol–pH 7.4 citrate–phosphate buffer (3:7, v/v) was used as a receptor medium. The available diffusion area between the cells was 1.13 cm². The stirring rate and temperature were maintained at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh buffer in order to maintain the sink condition. The drug amounts were determined using a previously reported high-performance liquid chromatography (HPLC) method [13,14]. In another experimental design used for testing nalbuphine release from polymer matrices, the excess water phase excluded from aggregates above the LCST was drained from the hydrogel. Accordingly, only the polymer aggregates were used as the donor phase.

2.11. Polarity of polymers

The hydrophobic fluorescent marker, Nile red ($2.5 \times 10^{-5}\%$, w/w), was used as the model solute, and the molecular environment (polarity) was elucidated by fluorometric spectroscopy based on the solvatochromism of Nile red. Emission fluorescence spectra were determined with a Hitachi F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The spectra of the aqueous solutions of polymers loaded with Nile red were recorded at room temperature with both slit widths set at 10 nm. The excitation wavelength was fixed at 546 nm, and the emission spectra were recorded from 550 to 700 nm at a scanning speed of 300 nm/min.

2.12. In vivo cold ethanol tail-flick test

Male Sprague–Dawley rats weighing between 175 and 225 g were used in this study. All handling and use of

experimental animals for the tests were in accordance with the recommendations and policies of the International Association for the Study of Pain. The apparatus used was a custom-designed temperature circulation system (RTE-140D; Neslab, Newington, NH, USA) with a bath solution of 95% ethanol. A temperature of –30 °C was selected for testing. Vehicles containing 15% polymer and 2.8 mM nalbuphine were intravenously injected into the tail vein at a dose of 7 μmol/kg. Normal saline was used as a control. Animals were firmly held over the opening of the bath with their tails submerged approximately half-way into the bath. The nociceptive threshold was taken as the latency until the rat flicked its tail as a reaction to the bath. The time from immersion to the tail flick was measured to the nearest tenth of a second with a laboratory timer. In order to prevent tissue damage, a 15-s cutoff time was used. With this time cutoff, no frostbite or skin color change was observed on any of the tails throughout the experiment.

2.13. Statistical analysis

Statistical analysis of the differences between different treatments was performed using an unpaired Student's *t* test. A 0.05 level of probability was taken as the level of significance. An analysis of variance (ANOVA) test was also used.

3. Results

3.1. Polymer synthesis

The structure of the novel thermosensitive polymers was identified in our previous study using Fourier transform infrared (FTIR) spectroscopy [6]. In order to conjugate PNIPAAm with chitosan, the –COOH group provided by MAA was added to the structure of NIPAM during its polymerization. There is only a single carboxyl group in every PNIPAAm molecule; therefore, the MW of PNIPAAm can be estimated by titration. The MW of PNIPAAm was estimated to be ~10.9 kDa as determined by the end-group analysis. The –COOH group of PNIPAAm and the –NH₂ group of chitosan were conjugated to form an amide group in the synthesis of CPN. Each chitosan macromolecule can bind to several PNIPAAm molecules. The efficiency of CPN grafting was 33.8%. Based on the grafting ratio of immobilized PNIPAAm to the initial chitosan, the MW of CPN was estimated to be 667 kDa [15]. The carboxyl groups of HA were able to graft to the remaining amine groups of chitosan that had not reacted with PNIPAAm (Fig. 1). The efficiency of grafting and the estimated MW of CPNHA were 24.0% and 684 kDa, respectively.

The zeta potential of a solution of these polymers in double-distilled water (15%, w/w) was determined in order to examine the electric charge before and after conjugation. PNIPAAm itself exhibited no or only a negligible charge

(Table 1), indicating its neutral condition. In acidic pH ranges, the ionizable amino groups in chitosan are protonated (+54.93 mV). Therefore, the formation of CPN gives rise to a net positive charge of +21.40 mV. The anionic characteristics of HA (−83.63 mV) led to the neutralization of the charge after the grafting of CPN and HA. This suggests that most of the amino groups in chitosan were conjugated with HA.

3.2. Sol–gel transition of the hydrogels

A general LCST was first characterized through changes in the UV/visible absorbance at 600 nm. All polymers synthesized in this study were soluble in water (15%, w/w) at room temperature. The LCST determined in the process of ascending temperatures from 25 to 40 °C is referred to as the gel formation temperature, whereas the LCST determined in the process of descending temperatures is the gel melting temperature. The approximate gel formation temperatures of PNIPAAm, CPN, and CPNHA were 32.0 ± 0.2, 32.8 ± 0.3, and 32.7 ± 0.3 °C, respectively (Table 2). The corresponding gel melting temperatures of these hydrogels were 29.0 ± 0.4, 27.0 ± 0.2, and 27.1 ± 0.4 °C. In all cases, the gel–sol transition temperatures were lower than the human body temperature, indicating the feasibility of clinical application. All of the polymer systems exhibited a reversible transition with a change in temperature. Upon cooling, the hydrogels became mobile at temperatures lower than the gelation temperature, indicating some hysteresis between the gelation and gel flowing temperatures. The presence of chitosan and HA in the hydrogels further increased this hysteresis. This may have been due to the significant entanglement among the polymer chains after incorporation of chitosan and HA into PNIPAAm, producing a delayed response to temperature.

The LCST value estimated by UV/visible absorbance is, however, not a sufficient descriptor of the phase transition behavior of these polymeric materials. The sol–gel transition of the polymers was also observed by the thermal properties derived from the DSC measurements. Fig. 2 shows DSC thermograms of the hydrogels. Significant endothermic peaks were observed for the 3 thermosensitive

Table 1
The zeta potential of various hydrogels

Polymer	Zeta potential (mV)
PNIPAAm ^a	0.02 ± 0.21
CPN ^b	21.40 ± 1.80
CPNHA ^c	1.23 ± 0.06
Chitosan	54.93 ± 0.87
HA ^d	−83.63 ± 1.05

Each value represents the mean ± SD (n = 3).
^a PNIPAAm, poly(*N*-isopropylacrylamide).
^b CPN, chitosan-*graft*-PNIPAAm.
^c CPNHA, chitosan-*graft*-PNIPAAm and HA.
^d HA, hyaluronic acid.

Table 2
The sol–gel transition temperature (°C) of hydrogels (15% w/w polymer) by various examination methods

Method	PNIPAAm ^a	CPN ^b	CPNHA ^c
UV/visible	32.0	32.8	32.7
DSC ^a	32.0	32.1	32.1
Viscosity	29.9	31.1	30.5

^a PNIPAAm, poly(*N*-isopropylacrylamide).
^b CPN, chitosan-*graft*-PNIPAAm.
^c CPNHA, chitosan-*graft*-PNIPAAm and HA.

hydrogels at ~32 °C. This indicates that the hydrogels exhibit a single heat capacity peak in the temperature range of the phase transition. It was found that the temperatures at the onset of the DSC endotherms were 2 °C lower than those of the maximum endotherms. The temperature of the endothermic maxima is referred to as the LCST [16]. The DSC profiles show that after chitosan and HA were conjugated into the PNIPAAm system (CPN and CPNHA), the maxima of the endothermic peaks shifted slightly from 31.99 ± 0.04 to 32.11 ± 0.05 °C (Table 2). This difference is statistically significant (*p* < 0.05).

The sol–gel transition behaviors were further illustrated by a viscosity analysis. Fig. 2 shows the viscosity of the hydrogels as a function of temperature, where an abrupt increase in viscosity at ~30 °C marks the onset of the gelation process. The gelation was dependent on the type of polymer used. The gelling temperature increased in the order of CPN > CPNHA > PNIPAAm Fig. 3.

3.3. Morphology of freeze-dried hydrogels determined by SEM

The structures of the hydrogels (shown in Fig. 4) were determined by examining cross sections under an SEM. The microscopic structure of PNIPAAm revealed that the spherical pores were interconnected throughout the scaffold matrix. As shown in Fig. 4a, the PNIPAAm system

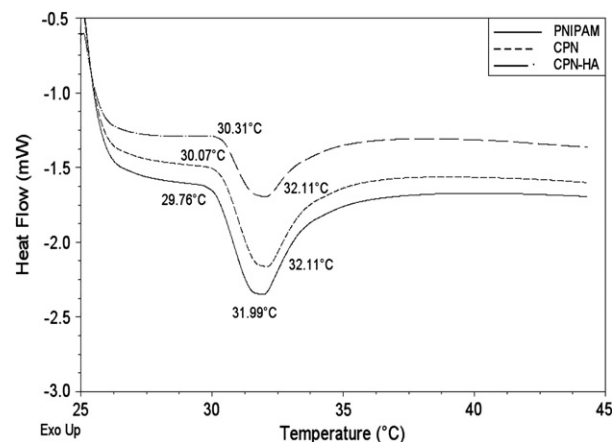


Fig. 2. DSC heating curves for PNIPAAm, CPN, and CPNHA hydrogels (15%, w/w). The curves are displaced along the ordinate for better visualization.

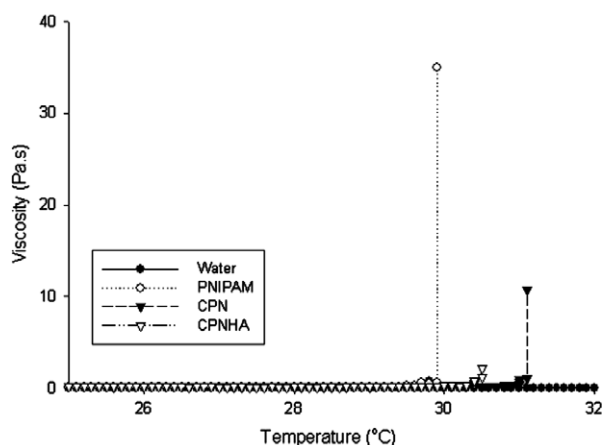


Fig. 3. Effect of temperature between 25 and 32 °C on the viscosities of the PNIPAAm, CPN, and CPNHA hydrogels (15%, w/w) determined by a Carri-Med CSL² 100 rheometer. The diameter of both the cone and plate spindle was 60 mm. The shear stress was set at 2 Pa.

had a relatively flat appearance and large pores ($\sim 10 \mu\text{m}$). Fig. 4b illustrates the structure of CPN. The left side of the micrograph depicts the surface of the CPN hydrogel. One remarkable characteristic of CPN was the deep pores and smooth surface. There were also fewer pores in the surface as compared to those in the cross section. The network structure of CPN was more condensed than that of PNIPAAm. The pore sizes of CPN and CPNHA (Fig. 4c) were ~ 1 and $\sim 5 \mu\text{m}$, respectively. The hydrogels of CPNHA exhibited a porous structure composed of sheets with inter-connecting channels.

3.4. Hydration capacity of the hydrogels

The water contents held by the hydrogel matrices are shown in Table 3. Those matrices that are able to hold more water may increase the possibility of encapsulating drugs within the system. CPN and CPNHA were observed to retain twice the amount of water compared to PNIPAAm. There was, however, no significant difference ($p > 0.05$) between the hydration capacities of CPN and CPNHA.

3.5. In vitro drug release

The cumulative amounts of nalbuphine released from the hydrogels as a function of time are shown in Fig. 5a. Double-distilled water was used as the control. The release of nalbuphine was virtually complete at 10 h. Compared to the free control, entrapment in the hydrogels resulted in a prolongation of nalbuphine release. Fig. 5a shows that the initial phase of nalbuphine release was faster than the terminal phase. This was consistent for all hydrogels. The retardation of nalbuphine release was highest in CPN, followed by CPNHA and PNIPAAm.

Nalbuphine and indomethacin have the same MW (357 Da); however, a large discrepancy was observed in

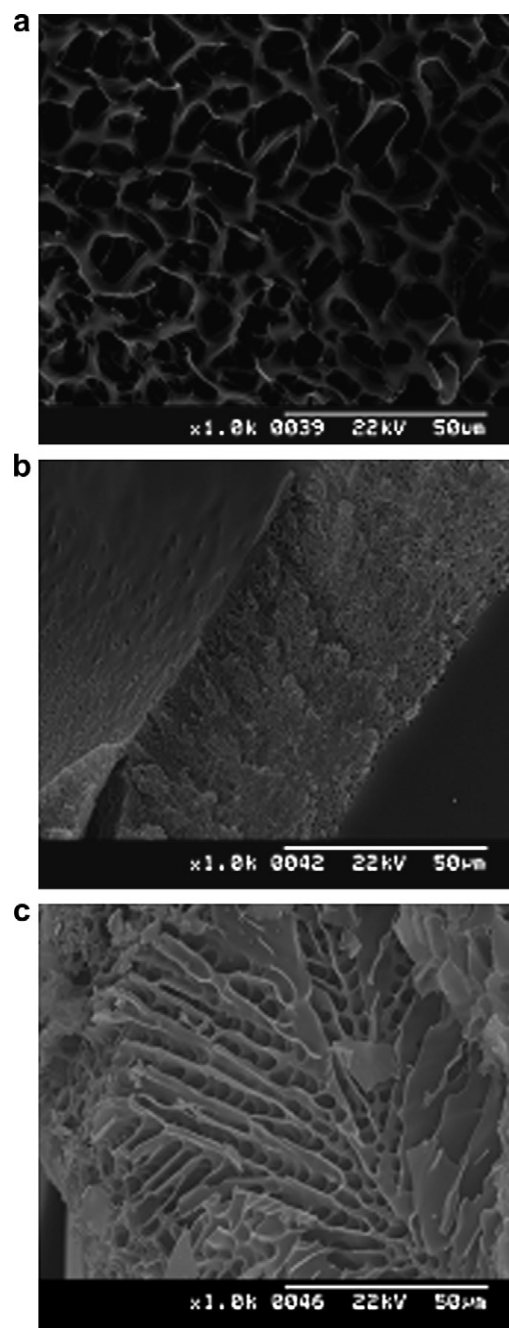


Fig. 4. SEM images (magnification, 1000 \times) of hydrogels after the freeze-drying process composed of PNIPAAm (a), CPN (b), and CPNHA (c) at a concentration of 15% (w/w).

Table 3

The water content of various polymers at 37 °C

Polymer	Water content (water weight/polymer weight)
PNIPAAm ^a	0.63 ± 0.30
CPN ^b	1.25 ± 0.18
CPNHA ^c	1.22 ± 0.27

Each value represents the mean \pm SD ($n = 3$).

^a PNIPAAm, poly(*N*-isopropylacrylamide).

^b CPN, chitosan-graft-PNIPAAm.

^c CPNHA, chitosan-graft-PNIPAAm and HA.

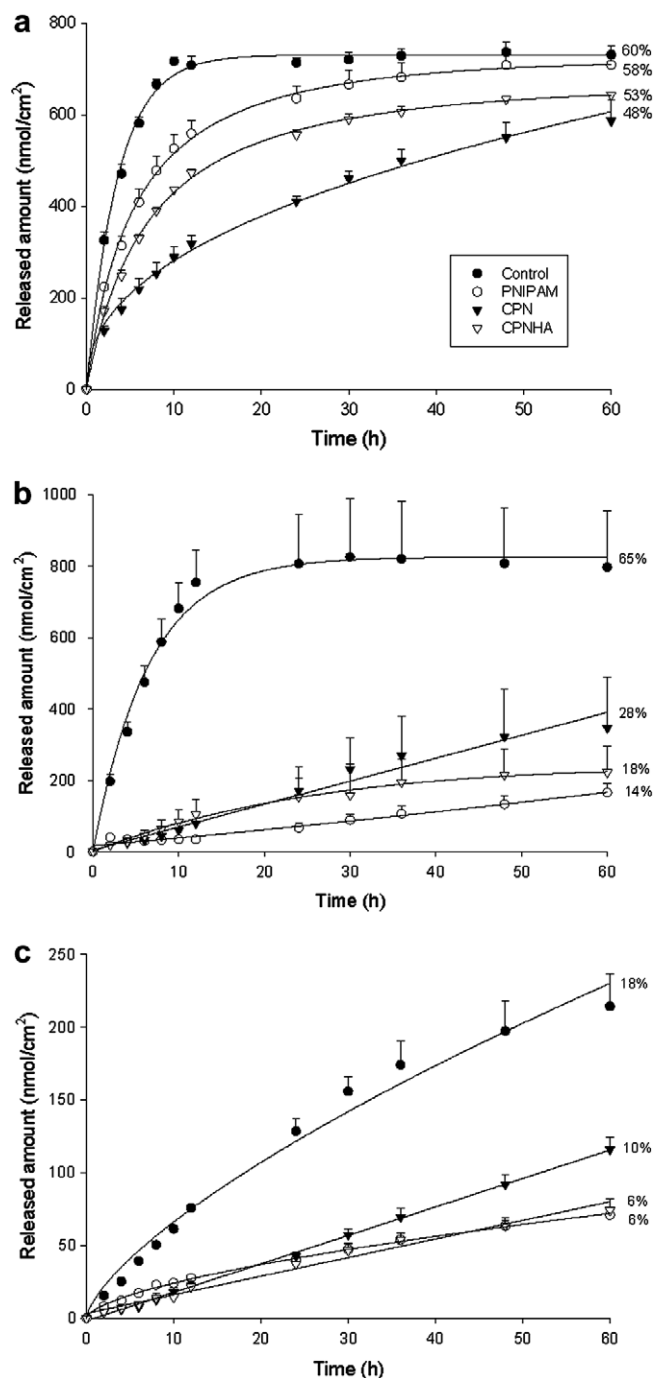


Fig. 5. Release amount-time profiles of naltuphine (a), indomethacin (b), and NAP (c) across a cellulose membrane from PNIPAAm, CPN, and CPNHA hydrogels (15%, w/w). Release amount values (nmol/cm²) represent the amount of drug in the receptor per diffusion area of a Franz cell. Each value represents the mean \pm SD. ($n = 5$). The amount of drug released as a percentage of the total drug dose is shown behind the release curves.

their hydrophilicities. NAP also exhibited a high lipophilicity, with an octanol–water partition coefficient ($\log P$) of 1.05 ($\log P$ of naltuphine = 0.17) [17]. As shown in Fig. 5b, the hydrogels retarded indomethacin release to a high degree. No burst release of indomethacin was observed. Indomethacin release increased in the order

CPN > CPNHA > PNIPAAm. A similar trend was observed in the release profiles of NAP (Fig. 5c). However, there was no significant difference ($p > 0.05$) between the profiles of CPNHA and PNIPAAm for NAP. Only $\sim 20\%$ of the NAP dose was released from the donor.

Since thermosensitive polymers can undergo dehydration and phase separation above the LCST [15], naltuphine release was also examined by separating the polymer aggregates from the aqueous phase at 37 °C. The unencapsulated drug in the polymer systems was thus excluded in this study. The release of naltuphine from the aggregates of hydrogels is depicted in Fig. 6. The release percentage of the drug was relatively low as compared to the complete hydrogel systems. This may be due to the loss of the drug in the extracted water. Moreover, some drug molecules may be strongly held by the solid matrices, and are therefore difficult to release. PNIPAAm and CPNHA exhibited drug release in the period of 0–4 h with no apparent release thereafter. Naltuphine was continuously released from the matrix of CPN over a 24-h period. The total amount of drug released increased in the order CPN > CPNHA > PNIPAAm.

3.6. Polarity of the hydrogels

Nile red is a dye whose absorption bands vary in shape, position, and intensity with the nature of the environment. The emission spectra of Nile red in the hydrogels are shown in Fig. 7. The emission maximum moved to a longer wavelength when chitosan was incorporated into PNIPAAm (CPN, 604 to 608 nm). Nile red emission shifts to longer wavelengths indicate an increase in environmental polarity [18]. CPNHA not only exhibited a wavelength shift (604 to 608 nm) but also a reduction in the fluorescence intensity (3532 to 2602 au). This is because the fluorescence is quenched in a more hydrophilic environment [18].

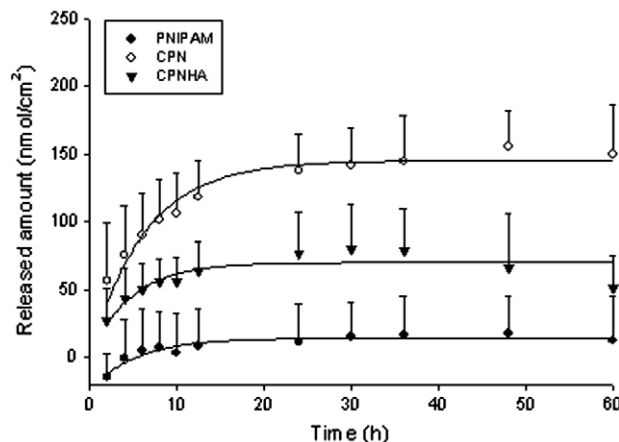


Fig. 6. Release amount-time profiles of naltuphine across a cellulose membrane from PNIPAAm, CPN, and CPNHA hydrogel matrices after draining the excess water. Each value represents the mean \pm SD. ($n = 5$).

3.7. In vivo cold ethanol tail-flick test

The PNIPAAm, CPN, and CPNHA hydrogels were intravenously injected into rats in order to examine their pharmacological effects. The antinociceptive activity of nalbuphine was determined using a cold ethanol tail-flick test. No animals died after the intravenous injection of these polymer systems. As shown in Fig. 8, nalbuphine in an aqueous solution without polymers (control) produced a quick and significant effect on the latency of the tail-flick response. Free nalbuphine could extend the latency to 12-s after a 20-min administration. After 1.5 h, the analgesic effect of nalbuphine had vanished. The loading of nalbuphine into PNIPAAm did not affect the antinociceptive profiles ($p > 0.05$). Nalbuphine entrapped in the CPN hydrogel resulted in a longer (up to 3 h) duration of activity. Although there was some fluctuation of the analgesic profiles at the late stage (3–6 h) for CPNHA, the analgesic duration of the CPNHA hydrogel may be extended to 4 h since there was a significant increase of analgesic latency ($p < 0.05$) as compared to the control.

4. Discussion

Thermosensitive polymers undergo abrupt changes in solubility in response to increases in the environmental temperature (i.e., the LCST). The temperature dependence of certain molecular interactions, such as hydrogen bonds and hydrophobic effects, contributes to phase separation [19]. Above the LCST, interactions between hydrophobic groups become dominant, leading to an entropy-driven polymer collapse and phase separation [16]. Due to dehydration of the hydrated amide group above the LCST, PNIPAAm graft chains are precipitated forming multimolecular aggregates [20]. Supporting evidence for this model comes from the DSC experiments on solutions of the polymers, which demonstrated endothermic phase separation.

It was found that the maxima of the DSC endotherms of these hydrogels corresponded closely to their LCST as

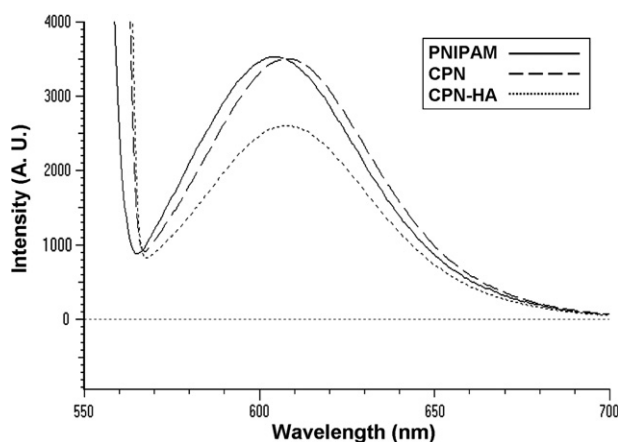


Fig. 7. Fluorescence emission spectra of Nile red ($2.5 \times 10^{-5}\%$, w/w) in PNIPAAm, CPN, and CPNHA hydrogels (15%, w/w).

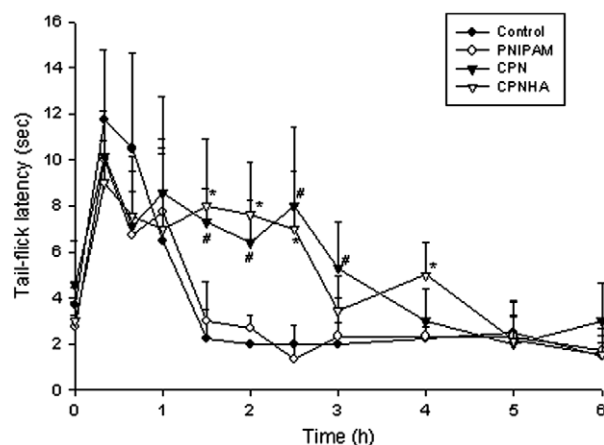


Fig. 8. Antinociceptive activity of nalbuphine ($7 \mu\text{mol/kg}$) in an aqueous solution, and in PNIPAAm, CPN, and CPNHA hydrogels (15%, w/w) after intravenous administration in rats using the cold ethanol tail-flick test. Each value represents the mean \pm SD. ($n = 4$). #, the value for the CPN hydrogel was significantly higher ($p < 0.05$) than that of the control. *The value for the CPNHA hydrogel was significantly higher ($p < 0.05$) than that of the control.

determined by UV/visible spectrophotometry. PNIPAAm exists as individual chains with a coil conformation at temperatures lower than $\sim 32^\circ\text{C}$, but undergoes a sharp coil-to-globule transition at higher temperatures to form inter- and intrachain associations resulting in precipitation [21]. A small but significant shift in the maximum endothermic peak to a higher temperature was observed for CPN and CPNHA. A delicate hydrophilicity/hydrophobicity balance within the polymer structure is responsible for the LCST phenomenon [22]. The incorporation of hydrophilic polymers reduces the number of hydrophobic groups and increases the hydrophilicity of the system. This leads to an increase in the LCST, since the hydrophobic interactions, which increase with temperature, are compensated for at the higher temperatures by increased polymer-water interactions [16]. This result suggests that the hydrophilic properties of chitosan and HA interfere with the hydrophobic interactions of PNIPAAm. Another possible explanation for the increase in the LCST is that increased physical cross-linking in the polymer structure induces a higher sol-gel transition temperature [23]. The slower response of PNIPAAm in aqueous solution compared with CPN and CPNHA is probably due to its grafted structure, which sterically hinders self-aggregation. Although some discrepancies exist among the transition temperatures of PNIPAAm, CPN, and CPNHA, the sol-gel transitions of these hydrogels were very close to each other. This indicates that PNIPAAm continued to dictate the transition behavior of the hydrogels. The hydrophobic structures therefore had a significant effect on controlling the overall phase transition [3].

The viscosity of the polymer systems increased sharply with an increase in temperature and the hydrogels eventually became a non-flowing mass at approximately 30°C . The gelling temperature determined by viscometry was

lower than the LCST as determined by UV/visible spectrophotometry and the 2 °C incremental measurements using DSC. This suggests that the polymers were first converted to a gel form, followed by the occurrence of endothermic heat of phase separation. Since CPN exhibited a significant positive charge, the charged group in the CPN structure may have led to a strong interaction with water [16]. Accordingly, among the hydrogels tested in the viscosity examination, CPN was observed to exhibit the highest gelling temperature. The discrepancy in the LCST of the 3 polymer systems determined by viscosity was larger than that determined by DSC (Table 2). This may indicate that the gel-forming property is a preferable technique for differentiating between the various hydrogels. Although the transition temperatures determined by various methods were approximate for these hydrogels, CPN, and CPNHA always exhibited a slower sol–gel transition than did PNIPAAm.

CPN and CPNHA exhibited a cross-linked structure with smaller pores compared to PNIPAAm. It is probable that chain entanglement further enhanced the formation of the hydrogel networks [24]. It is notable that HA conjugation could expand the pores inside the hydrogel structure. This may have been due to the very highly swollen gel-like characteristic of HA [18]. HA may unfold the condensed entanglement of the hydrogels.

Generally, with an increasing extent of cross-linking, there is an appreciable decrease in the hydration ratio of a hydrogel [7,25]. However, this was not the case in the present study. In the process of hydration of a polymer material, water binds to the most hydrophilic groups. The addition of a hydrophilic polymer can therefore enhance water uptake and swelling [1]. Moreover, the amide group, which is rich in CPN and CPNHA, is regarded as highly hydrated [16]. Both mechanisms contributed to the greater hydration capacity of CPN and CPNHA in contrast to PNIPAAm. HA did not further increase the water content of the hydrogels.

One of the key applications of thermoreversible hydrogels is in controlled drug delivery. In the present study, approximately 60% of nalbuphine in the aqueous solution (control) was released to the receptor by the end of the experiment (60 h). The limited amount released may have been due to the use of the Franz diffusion assembly. Since drugs are released into a definitive volume of the receptor (5.5 ml in this study), the drug loading in the receptor medium is limited [26]. Thus, there may no longer exist a concentration gradient between the donor and receptor. Although the Franz cell permitted only a limited loading in the receptor, this method is still useful for differentiating the release capabilities of various formulations.

The hydrogels exhibited an initial burst release of nalbuphine, which could be attributed to untrapped drug residing on the exterior of the polymer matrices. Since the hydrogel matrix of PNIPAAm contained less water than the other 2 polymers, a greater number of drug molecules were observed on the outside of the matrices of this

hydrogel. This may therefore have contributed to the higher burst release observed with the PNIPAAm hydrogel. The structure, pore size, and polymer composition of a gel are all taken into account in the context of drug release. The solute diffusion coefficient (D_{123}) inside the hydrogel can be described by the general equation for diffusion [1]:

$$D_{123} = D_{13}f(r_1, \Phi, \xi);$$

where D_{13} is the diffusion coefficient of the solute in the pure solvent phase, f signifies that D_{123} is the function of three variables, r_1 , Φ , and ξ . r_1 is the size of the solute molecule, Φ is the polymer volume fraction of the gel, and ξ is the mesh size of the gel. Nalbuphine occupied the water channels of the hydrogel from which it was released by diffusion along a concentration gradient. The slowest nalbuphine release among the hydrogels was observed for CPN, and this may be linked to the material density as revealed by SEM. With a denser matrix, hydrogels may entrap nalbuphine within the matrix for a longer period.

An increase in the rate of drug release is attributable of the repulsive forces between the drug and the polymer [2]. Nalbuphine was positively charged in the hydrogels and the CPN backbone also has a high positive charge density. Thus, electrostatic repulsion can be ruled out as the mechanism for the controlled release of nalbuphine. CPN and CPNHA are hydrophilic polymers that may exhibit an affinity for hydrophilic nalbuphine, forming solute-hydrogel chemical interactions. The nalbuphine released from CPN and CPNHA may thus have been hindered by this dipole-dipole interaction. In order to explore the mechanisms of chemical interactions, indomethacin and NAP – drugs with higher lipophilicities – were introduced to examine their effect on the release from hydrogels.

In general, a direct comparison of the release of different drugs from hydrogels should be avoided owing to the different solubilities of the same formulations of different drugs. However, the trend of drug release from different formulations can be discussed. The trend of indomethacin release from various hydrogels was opposite to that of nalbuphine release (Fig. 5b). A larger amount of unreleased indomethacin in PNIPAAm as compared to CPN and CPNHA may be ascribed to the strong hydrophobic interaction. This possibly confirms the importance of chemical interactions between the solute and hydrogel. A similar result was obtained for NAP, a lipophilic prodrug of nalbuphine. A further observation was the low release of NAP from the donor. A previous study demonstrated that the release of the parent drug and its prodrug was a function of lipophilicity, with slower release of more lipophilic drugs [13]. Since the polarity of the hydrogels may greatly influence drug release, the molecular environment of the polymers was elucidated. The Nile red emission results (Fig. 7) confirmed that CPN and CPNHA exhibited a higher hydrophilicity as compared to PNIPAAm.

The reduction in the release by the hydrogels was more significant for indomethacin and NAP than for nalbu-

phine. Higuchi [27] developed an equation describing the release of a drug dispersed in matrix dosage systems. One form of the equation [$Q = (2ADC_s t)^{1/2}$] states that the amount of drug released at time t per unit area of exposure (Q) is proportional to the square root of the total concentration (dissolved and undissolved) of drug in the matrix (A), the diffusion coefficient of the drug in the matrix (D), the solubility of the drug in the matrix (C_s), and the time (t). Since indomethacin and NAP could not be completely dissolved in the hydrogels, drug release was significantly reduced from the polymer matrix.

The formulation factor (various hydrogels) influencing indomethacin and NAP release was not as pronounced as compared to nalbuphine. Based on the theory cited by Topp and colleagues [28,29], lipophilic solutes may diffuse through the hydrophobic as well as hydrophilic pathways of the hydrated polymer environment; therefore, the various polymers had no significant effect on the total release rate of indomethacin and NAP through the hydrogels. However, it has been suggested that the major permeation pathway of solutes with a hydrophilic nature is the hydrated portion of the hydrogel. Accordingly, the entanglement of the polymer backbone may influence the permeation process.

CPN exhibited a steeper release slope at the late stage of the experiment (12–60 h) for all the drugs examined. This possibly suggests continual erosion of the CPN hydrogel by excess water. This phenomenon was particularly significant for indomethacin and NAP since there was almost no difference ($p > 0.05$) among the amounts of drug released from all hydrogels at the initial stage of the experiment (0–12 h). Previous studies also indicate the possibility that chitosan is eroded within the water phase [30,31]. The nalbuphine encapsulated in the CPN matrices after draining the aqueous phase exhibited a higher release as compared to PNIPAAm and CPNHA. Gradual disintegration of the polymer chain network leads to an enlarged mesh size, thus enabling the entrapped drug to diffuse out more readily [21]. HA incorporation (CPNHA) could inhibit this erosion. Clearly, after a determined period, the remaining nalbuphine became trapped in the matrix and could not be completely released unless the matrix was dissolved in a medium.

The cold ethanol tail-flick method in rats has been reported to sensitively and reproducibly determine the antinociception of opioid agonists and mixed agonist-antagonists [32]. Although nalbuphine in aqueous solution (control) exhibited a rapid onset of analgesia with a maximal effect at 20 min, the analgesic duration of ~1.5 h was too short because of the short elimination half-life. The onset of antinociceptive activity was quickly attained after a 20-min duration by all hydrogels tested. This onset was the same as that observed for the control, which may have been due to the release of unencapsulated nalbuphine from the hydrogels. The loading of nalbuphine in CPN and CPNHA prolonged the analgesic duration. This can be assumed to have been a result of the protection and

retarded release of free nalbuphine from the hydrogels. Although CPN slowed drug release more significantly than did CPNHA, the analgesic duration of CPN was shorter than that of CPNHA. This may have been due to the disintegration of CPN under in vivo conditions. Further studies are required in order to confirm this speculation.

5. Conclusions

Injectable, thermoreversible hydrogels were developed by chemically grafting PNIPAAm onto chitosan and HA chains. The incorporation of chitosan and HA into PNIPAAm did not greatly affect the transition temperature as determined by UV/visible spectrophotometry, DSC, and viscometry. The hydrogels produced all had gelation temperatures well below body temperature; thus, they readily became gels, making them ideally suited to function as injectable drug depots. The hysteresis of the reversed LCST was significantly lowered in CPN and CPNHA hydrogels as compared to PNIPAAm by a 2 °C reduction in temperature. Based on SEM images, CPN exhibited the densest entanglement of its cross-linked structure, followed by CPNHA and PNIPAAm. Each polymer system had distinct characteristics as a carrier for drugs. The pore size of the hydrogel structure and the affinity between the drug and hydrogel may have contributed to the main mechanisms determining drug release. CPN might have disintegrated in the in vitro and in vivo environments. The presence of HA in the systems (CPNHA) helped to prevent this disintegration. The analgesic duration and activity for nalbuphine in the hydrogels were well correlated with the in vitro release profiles. Based on the current in vitro and in vivo results, it can be concluded that such biocompatible delivery systems can facilitate the controlled release of nalbuphine and improve the duration of action after intravenous administration in rats, particularly in the case of the CPNHA hydrogel. The use of either CPN or CPNHA can reduce the amount of PNIPAAm utilized in the thermosensitive systems, thus reducing toxicity. In order to enhance the clinical applicability of these hydrogels, the safety and sterility of the polymer materials should be elucidated in the further studies.

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