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Ionic liquid-doped and *p*-NIPAAm-based copolymer (*p*-NIBIm): extraordinary drug-entrapping and -releasing behaviors at 38-42 ^{o}C

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Abstract:

Ionic liquid (IL)-doping on the temperature responsive *p*-NIPAAm was achieved by radical copolymerization of N-isopropyl acryl amide (NIPAAm; 90 mol%) and 1-butyl-3-vinylimidazolium bromide ([BVIm]Br; 10 mol%) to give a new temperature responsive copolymer (*p*-NIBIm). The as-prepared *p*-NIBIm copolymer showed the highly increased zeta potential value and the optimal LCST (lower critical solution temperatures) value, respectively, +9.8 mV at pH=7 and 38.2 ^{o}C, compared to those (+0.3 mV at pH=7 and 32.1 ^{o}C) of *p*-NIPAAm. The temperature-dependent size change of the *p*-NIBIm micelles was determined in the range from 25 to 45 ^{o}C by SEM under dry condition and by Zeta Sizer under wet condition, showing a certain size contraction from 253±12.1 to 90.5±7.8 nm in diameter (about 95.4% of volume contraction). The thermo-sensitive behaviors to entrap BSA protein at body temperature (37 ^{o}C) and to release the protein between 38-42 ^{o}C (near the LCST) also were tested by sizing of the complexes of *p*-NIBIm/BSA using Zeta Sizer and also by a colorimetric assay (Bio-Rad DC Protein Assay), resulting in a maximum entrapment of 1.02 mg BSA for 1.0 mg of the polymer at body temperature (37 ^{o}C) and in a maximum release of 0.73 mg BSA for 1.0 mg of the polymer (about 73% releasing of the entrapped amount) at the temperature range of 38-42 ^{o}C. Toxicity of the *p*-NIBIm micelles (in the range

of <0.125 mg/mL) without drug for human embryonic kidney (HEK 293) cells was minimal in vitro. These results revealed that the IL-doped and temperature responsive co-polymeric systems have a very high applicability as a novel delivery system for charged (or polar) molecules as a natural (or synthetic) drug and DNA.

Keywords: thermo-responsive copolymers, ionic liquids, ionic liquid doped copolymers, drug delivery system, ionic copolymers

1 Introduction

Environmentally sensitive materials have recently attracted considerable attention due to their biomedical application owing to the reversible responses to external stimuli, such as temperature,[1] pH,[2] ionic strength,[3] electric field,[4] magnetic field,[5] light,[6] etc..[7-10] Among the external stimuli responsive materials, temperature- or pH-stimuli responsive polymers have been most widely investigated, because these two factors of body tissue could be changed by many diseases and easily regulated by external induction.[7-14] Poly (N-isopropylacrylamide) (*p*-NIPAAm) is one of the most well-known thermoresponsive polymers and has been extensively studied for biomedical applications owing to the reversible thermoresponsive phase transition from a hydrated random coil (or a swelled globule) to a deswelled compact globule at the lower critical solution temperature (LCST = 32).[15–17] However, *p*-NIPAAm has limited applicability as a delivery system due to the too low LCST (below body temperature), a tedious drug-releasing profile in a wide temperature range below body temperature and too low drug-carrying ability (about <0.1 mg drug per 1.0 mg polymer).[8] To resolve these problems, the hydrophilic *p*-NIPAAm segments have been incorporated with relatively hydrophobic polymer segments to prepare thermo-responsive *p*-NIPAAm-based copolymers. The prepared copolymers exhibited some additional advantages for various biomedical applications, for example, a possible tuning of the LCST between 37 (body temperature) and 42 °C (clinical hyperthermic temperature)[18] and creating other property (pH-sensitive),[19,20]

but without a satisfactory improvements in the drug loading level (<36 wt%), the pH-dependant nature of LCST, and the drug-releasing pattern of co-polymeric drug-carriers.[19,21-25]

To achieve more efficient drug delivery exactly to a target site, we hypothesized that co-polymeric drug-carriers (or micelles) have to form stable complexes with drug-molecules for improving drug-carrying ability, to have suitable and constant LCSTs (around 38-40 °C) unaffected by encapsulated drugs and surrounding pH, and to exhibit faster response (for example, within several seconds) for exact drug-releasing at a target temperature range. In this way, the introduction of ionic moieties into a polymer chain will be an effective strategy, because the charged groups within the polymer chain can induce increasing the LCST over body temperature up to clinical hyperthermic temperature and forming stable complexes with negatively (or positively) charged guest molecules via ionic interactions.[26-30] However if the charge density within the temperature-stimulus responsive polymers is easily affected by the external pH values (especially for the cases of polymers with amine functional groups), their LCST values can also undergo a change, leading to a failure to exactly deliver drug molecules to the target site.[31] Therefore the existence of permanently (or pH-independently) ionic moieties within a polymer chain that are not affected by the surrounding pH condition will be essential to transfer drug molecules exactly to the target site with a certain temperature and to reduce response times of drug carriers (or micelles) for drug-releasing.

Here, we prepared a partially positive charged and temperature responsive co-polymer by radical copolymerization of N-Isopropyl acryl amide (NIPAAm) and 1-butyl-3-vinylimidazolium bromide ([BVIm]Br). Imidazolium-based ILs is well known due to their many fascinating properties which have been of special importance in research fields such as surface wettability control, catalyst molecule-supporting, nanostructure construction, and green chemistry.[32-38] The prepared copolymer (*p*-NIBIm) with 10 mol% concentration of the permanently cationic N-butyl-imidazolium unit was appeared to have the LCST (lower

critical solution temperature) of 38.2 ^{o}C and the surface charge of +9.8 mV at pH=7. The temperature-dependent morphology change and the temperature-dependent micelle size change also were determined under dry and wet conditions, respectively, by SEM and Zeta Sizer. The ability to uptake and release a negatively charged protein (BSA), respectively, below and above the LCST (25~45 ^{o}C) were determined with comparing with that of *p*-NIPAAm. The extraordinary protein-entrapping and releasing ability of the IL-doped copolymer will potentially be applied to be used as a novel delivery system for negatively charged molecules such as a natural (or synthetic) drug and DNA.

2 Materials and methods

2.1 Materials

N-isopropylacrylamide (NIPAAm) was purchased from ACROS (USA) and purified by recrystallization from hexanes (HPLC grade, Sigma–Aldrich, USA) prior to use. N-vinylimidazole (NVIm, Sigma-Aldrich, USA), 1-bromobutane (Sigma–Aldrich, USA), ammonium persulfate (APS, Sigma-Aldrich, USA), N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma–Aldrich, USA) were used as received. The Bio-Rad DC Protein Assay II kit was supplied by BMS (Korea). All other reagents and solvents used were analytical grade and used as received.

2.2 Synthesis of 1-butyl-3-vinyl imidazolium bromide, [BVIm]Br, as an IL monomer.

N-vinyl imidazolium-based ionic liquid monomer was prepared via a simple one-step quaternization reaction of N-vinyl imidazole with 1-bromobutane. Reaction mixture of 2.35 g (25 mmol) 1-vinyl imidazole and 4.11 g (30 mmol) 1-bromobutane was heated to 100 ^{o}C for 24 hours by stirring. A dark brown viscous residue was obtained after the complete evaporation of the volatile parts. The organic salt as a monomeric product, 1-butyl-3-vinylimidazolium bromide ([BVIm]Br), was extracted with dichloromethane from the

aqueous solution. 5.92 g of a yellowish viscous liquid was obtained after washing the organic phase with distilled water (DW), evaporation of the organic solvent, and complete vacuum drying. The crude product obtained was purified by flash column chromatography (MC/methanol) to give >90 wt% yield.

2.3 Synthesis of the IL-doped copolymer, poly(NIPAAm-co-BVIm) or *p*-NIBIm

p-NIPAAm-based and partially cationic copolymer with N-butyl imidazolium moieties, poly(NIPAAm-co-BVIm), was synthesized, as shown in Scheme 1. 1.13 g (10 mmol) of NIPAAm and 0.30 g (1 mmol) of 1-butyl-3-vinylimidazolium bromide ([BVIm]Br) were dissolved in 40 mL of DW, and then 10 µL (0.5 mmol) of ammonium persulfate (APS) solution (10%(w/v)) as an initiator and 15 µL (0.1 mmol) of tetramethylethylenediamine (TEMED) as an activator were added to the solution. Prior to polymerization, the reaction solution was purged with nitrogen for at least 30 min to remove oxygen. Polymerizations were performed for 24 hours at 80 ^{o}C. The reaction solution became slightly yellowish and viscous during the reaction. After completion of the reaction, all possible impurities were removed by extraction with MC from the aqueous solution, following dialysis (membrane tubing, molecular weight cutoff 12000-14000 Da, Spectrum Laboratories, Savannah, GA, USA) against DW for 3 days and then freeze drying. To obtain the copolymer product as a pure solid mass, the polymer product clearly dissolved in 20 mL of cold DW was incubated in 60 ^{o}C for one hour and then the precipitated white sold was separated by centrifugation from the aqueous solution. The process was repeated three more times for further purification. Finally the purified polymer product dissolved in 20 mL of cold DW was freeze dried and 1.38 g of the polymer product that looks like white cotton wool was obtained (about 96.5 wt% yield).

2.4 Synthesis of poly(1-butyl-3-vinylimidazolium bromide), *p*-BVIm, and poly(N-isopropylacrylamide), *p*-NIPAAm, as reference polymers

N-vinyl imidazolium-based ionic liquid polymer, *p*-BVIm, and poly(N-isopropylacrylamide), *p*-NIPAAm, were synthesized as follows. 20 mmol of 1-butyl-3-vinylimidazolium bromide ([BVIm]Br) or N-isopropylacrylamide were dissolved in 20 mL of DW, and then 10 µL (0.5 mmol) of ammonium persulfate (APS) solution (10%(w/v)) as an initiator and 15 µL (0.1 mmol) of tetramethylethylenediamine (TEMED) as an activator were added to the solution. Prior to polymerization, the reaction solution was purged with nitrogen for at least 30 min to remove oxygen. Polymerizations were performed for one hour at room temperature. All possible impurities in the aqueous solution were removed by dialysis (membrane tubing, molecular weight cutoff 12000-14000 Da, Spectrum Laboratories, Savannah, GA, USA) against DW for 3 days. The obtained crude product of *p*-BVIm was purified by flash column chromatography (MC/methanol) to give >90-80 wt% yield, while the pure *p*-NIPAAm was obtained by washing with methylene chloride several times.

2.5 Chemical characterization of the polymer

The chemical characterizations of the synthesized monomer and polymer products were performed by FT-IR spectroscopy (Nicolet 380, Thermo Fisher, USA) and ^{1}H-NMR (Bruker, Ultrashield 400 PLUS, USA). FTIR spectra were taken using a KBr window coated with the copolymer solution (5 wt%) in ethanol. ^{1}H-NMR spectra of products were obtained at 400 MHz using d_6-dimethyl sulfoxide as solvent.

2.6 Average molar mass determination of polymers

MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectroscopy (Voyager-DE STR; Negative Polarity) was successfully employed to determine average molar mass of the cationic copolymer. 10 mg of the copolymer sample were dissolved in 1 mL of

water and mixed with α-cyano-4-hydroxycinnamic acid (HCC) matrix solution at a ratio of 1:9 (v/v, polymer : matrix). The MALDI-TOF mass spectra showed the corresponding average molar masses of the copolymer.

2.7 LCST determination of the copolymer

The thermal behaviors of the prepared polymers were studied utilizing DSC (DSC 131 evo, setaram, France). An aqueous solution of the sample polymer was prepared in a concentration of 5.0 wt% in DW. It exhibited endothermic and an exothermic peaks, respectively, in heating and cooling processes with a rate of 2 ^{o}C/min between 25 and 60 ^{o}C.

2.8 Determination of mean size and zeta potential of polymer micelles without and with BSA molecules

Mean sizes of polymer micelles without and with the guest molecules (BSA) under wet condition were measured in a heating process with an interval of 3 ^{o}C between 25 and 45 ^{o}C using Zeta Sizer nano ZS90 (Malvern, France). 1.0 mL of aqueous polymer solutions (0.01 wt%, prepared at 4 ^{o}C) without and with 1.0 mg of BSA were prepared using a mixture of 0.1M acetic acid and 0.2M sodium acetate as buffering. Their size distribution curves were obtained as a Gaussian type curve. The size distribution curves were almost symmetrical to the vertical line passing through the maximum, in all cases. The maximum value is the average size and the distance between the two ends at the base is reflected in the standard deviations. The morphologies of micelles without and with BSA molecules in a dry state were characterized by SEM (S-4300, HITACHI, Japan). For this, the polymer solution (0.1 mg/mL) was dropped onto a cover glass and then dried at 25 ^{o}C and 50 ^{o}C in the dark overnight. The Zeta (ξ) potential values were recorded using a Zetasizer (ZEN 3600, Malvern) at room temperature as a function of pH (3-10) using a mixture of 0.1M acetic acid and 0.2M sodium acetate as buffering in water. The desired pH was adjusted by HCl or NaOH solutions, and pH

values were measured by a pH-meter (Orion 3 star, thermo scientific, Singapore). All the measurments were conducted at least in triplicate for each experimental group and the average values were represented with the standard deviation.

2.9 Determination of colloidal stability of the *p*-NIBIm solution in body temperature

Colloidal stability of the *p*-NIBIm solution was tested by TurbiScane LAb (Leanontech, a pulsed near infrared light source (880 nm)). The copolymer solution (0.5 mg/mL) of 35 mm height was lengthwise scanned every 6h for 72h at 37 ^{o}C. The light backscattered by the sample solution (135^{o}) (or transmission) was measured.

2.10 Determination of the temperature-dependently loaded and released BSA amount of *p*-NIBIm

The amount of BSA molecules that were temperature-dependently loaded in the polymer micelles and released from the micelles was determined by Bio-Rad DC assay. The Bio-Rad DC-Protein assay is a colorimetric assay based on a modified Lowry protein assay method following detergent solubilization. The amount of BSA in solution samples was determined using a calibration curve over the range of 0.20-1.0 mg/mL of the peptide solution. Three sample solutions were prepared by combining 1.0 mL of the aqueous polymer solution (0.1 w/v%) and 1.0 mL of aqueous BSA solution (1.0 w/v%) at 4 ^{o}C. The sample solutions were incubated in a water bath, respectively, of 25, 36, and 42 ^{o}C and then were filtrated through a PTFE membrane filter (0.1 μm pore size, Advantec, Japan) equipped with a syringe at each temperature. 100 μL of the each filtrate solution was put in three new test tubes and 500 μL of reagent A´ (a mixture of 20 μL of reagent S and 1.0 mL of reagent A) and 4mL of reagent B were added into each test tube, following vortexing immediately. After 15 min, the absorbance of the each sample can be read using UV-vis spectrometer (1601PC, Shimadzu) at

750 nm. The experiments were conducted at least in triplicate for each experimental group (at 25, 36, and 42 ^{o}C) and the average values were represented with the standard deviation.

2.11 Cytotoxicity assay

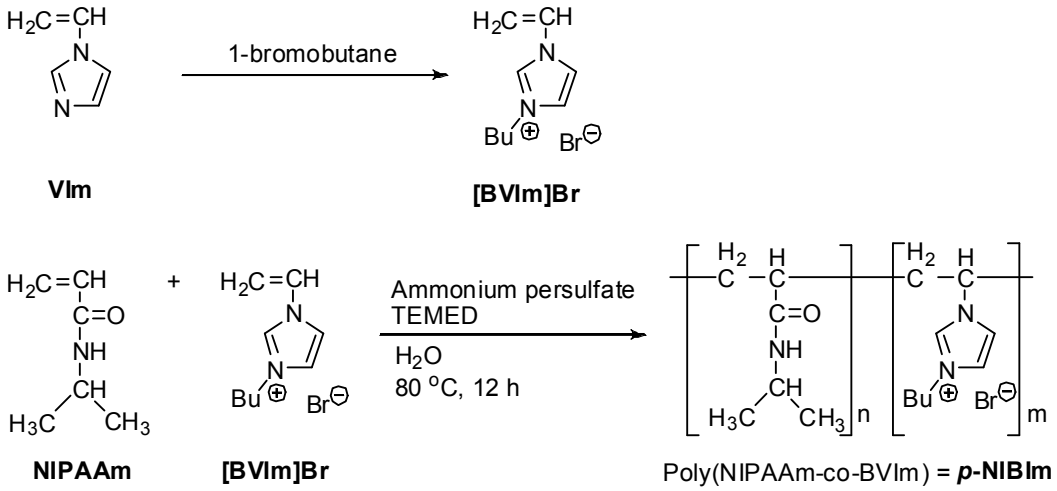
The cytotoxicity on *p*-NIBIm micelles was determined by Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo Laboratories, Japan). The human embryonic kidney cell line, HEK 293, was purchased from American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin and 100 g/mL streptomycin (Gibco, USA) in a humidified incubator with about 90% and 5% CO_2 at 37 °C. To test the cytotoxicity, 100 µL of cell suspensions of HEK 293 cells were seeding at a density of $5x10^3$ cells per well in the 96-well microplates and pre-incubated overnight. Concentration sets of the *p*-NIBIm micelle solutions were prepared by a 2-fold serial dilution with culture media from 8 mg/mL to 0.03125 mg/mL. Each set of 10 µL was added to each well (n = 4) and continued to culture for 1-3 days. At each time point, the plate was further incubated for 2 hours in addition with 10 µL of CCK-8 solution to each well and then the optical density (OD) was measured at absorbance of 450 nm using a microplate reader (PerkinElmer, USA). The rates of the cell viability were calculated by the following equation: Cell viability (%) = ($OD_{p\text{-NIBim}}O/OD_{control}$) x 100%, where $OD_{control}$ was obtained in the absence of polymers and $OD_{p\text{-NIBim}}$ in the presence of polymers.

3 Results and Discussion

3.1 Synthesis and characterization of the IL-doped copolymer, poly(NIPAAm-co-BVIm) or *p*-NIBIm

In this study, a permanently ionic and thermo-sensitive copolymer with the relatively hydrophobic NIPAAm component as a major part and the hydrophilic (or ionic) N-vinyl

imidazolium component (imidazolium-based ionic liquid (IL)) as a minor part was designed and synthesized as a carrier, especially for negatively charged (or ionic or highly polar) guest molecules, including synthetic drug, gene, and protein (Scheme 1). As shown in Scheme 1, the chemical integration of the IL moiety into the *p*-NIPAAm polymer chain was achieved via copolymerization of 1-butyl-3-vinyl imidazolium bromide ([BVIm]Br) with NIPAAm at the molar ratio of 1 to 10. Actually, controlling the concentration of the positively charged (or hydrophilic) imidazolium moiety within the copolymer chain could be expected to be an effective method for tuning the most important physicochemical and morphological properties of thermo-sensitive drug-carriers, such as solubility in water, LCST, surface charge, pH at IEP, micelle size, guest molecule-switching (or -attaching and -detaching) abilities at the suitable temperatures, and the capacity for drug-carrying. Prior to the copolymerization, N-vinyl imidazolium-based ionic liquid monomer ([BVIm]Br; 1-butyl-3-vinylimidazolium bromide) was prepared via a C-N coupling of N-vinylimidazole with 1-bromobutane (see the ^{1}H-NMR spectrum in **Fig. S1** of the supporting information). Then the copolymerization with NIPAAm monomer was accomplished using 10 mol% of [BVIm]Br monomer to prepare poly(NIPAAm-co-BVIm) or *p*-NIBIm. The optimal concentration (10 mol%) of the cationic unit within the prepared copolymer chain to show the most suitable LCST range (38-42 ^{o}C) and an excellent drug-carrying ability was determined by gradual increasing of the IL monomer concentration from 0 up to 10 mol%, because, as mentioned above, the chemical doping of the hydrophilic IL units could be considered to change the most important physicochemical properties of the thermo-sensitive polymer compared to those of the pure *p*-NIPAAm polymer.



Scheme 1. Schematic illustration of the preparation of poly(NIPAAm-co-BVIm) or *p*-NIBIm**.**

We determined the molar mass distribution of the IL-doped copolymer, *p*-NIBIm, using MALDI-TOF mass spectroscopy. The results were compared with those of *p*-NIPAAm and *p*-BVIm that were prepared as references, respectively, from NIPAAm and [BVIm]Br monomers in the same manner. As shown in Fig. 1 and S2, the number- and weight-average molar masses (*Mn* and *Mw*) of the copolymer appeared to be, respectively, around 1353 and 2001 g/mol, showing that the molar mass distribution (*Mw*/*Mn*) is 1.47, while *Mn* and *Mw* of *p*-NIPAAm were, respectively, around 1998 and 2521 g/mol, showing *Mw*/*Mn* = 1.26. For the case of *p*-BVIm, *Mn* and *Mw* were recorded as being around 2102 and 2114 g/mol (*Mw*/*Mn* = 1.0).

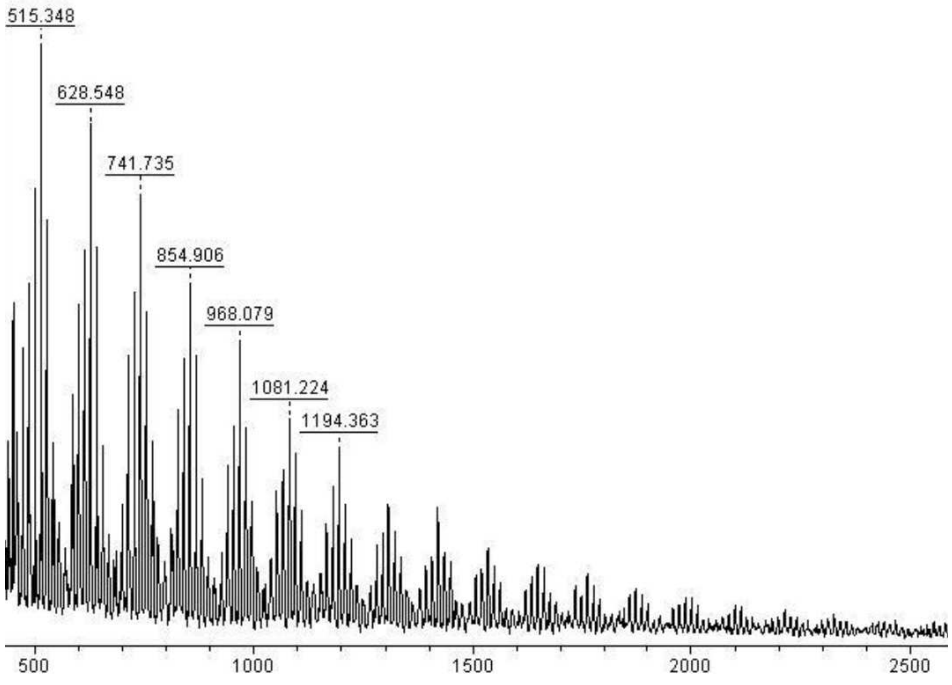


Fig. 1 MALDI-TOF spectrum of the copolymer, *p*-NIBIm.

To assess the presence of the two components, NIPAAm and [BVIm]Br, within the copolymer, *p*-NIBIm, FT-IR (see Fig. S3) and ^{1}H-NMR spectra (see Fig. 2 and S1) were obtained and compared to those of the homopolymers, *p*-NIPAAm and *p*-BVIm. As expected, the characteristic peaks of *p*-NIBIm were observed in the FR-IR spectrum as follows: 3030~2874 cm^{-1} (sp^2 C-H stretching of BVIm units; sp^3 C-H stretching of NIPAAm and BVIm units), 1670~1580 cm^{-1} (C=O stretching of NIPAAm units and C=C and C=N stretching of BVIm units), 1535 cm^{-1} (C(=O)-N-H bending of NIPAAm units), 1458 cm^{-1} (CH_2 bending of NIPAAm and BVIm units), 1366 cm^{-1} (CH_3 bending of NIPAAm and BVIm units), and 1030~1300 cm^{-1} (C-N stretching of NIPAAm and BVIm units). In addition to the characteristic peaks, a broad characteristic band of water appeared at 3200~3600 cm^{-1} for the *p*-NIBIm sample, denoting the hydrophilic character of the copolymeric derivative. ^{1}H-NMR spectrum of the *p*-NIBIm copolymer also is given in Fig. 2 and showed characteristic peaks that belong to the protons of NIPAAm and BVIm monomer units. To check the molar ratio of

both monomer units consisting of the copolymer chain, the integrals of the broad signals (at 3.84 ppm for the isopropyl CH proton of NIPAAm unit and at 4.15 ppm for the N-CH_2 proton of BVIm unit) were compared. In the spectrum, the integrals were, respectively, 10 and 2, and these denote that the copolymer *p*-NIBIm contains about one BVIm unit per 10 NIPAAm units. Consequently, FT-IR and ^{1}H-NMR spectra indicate an effective integration of BVIm monomers in the copolymerization with NIPAAm monomers.

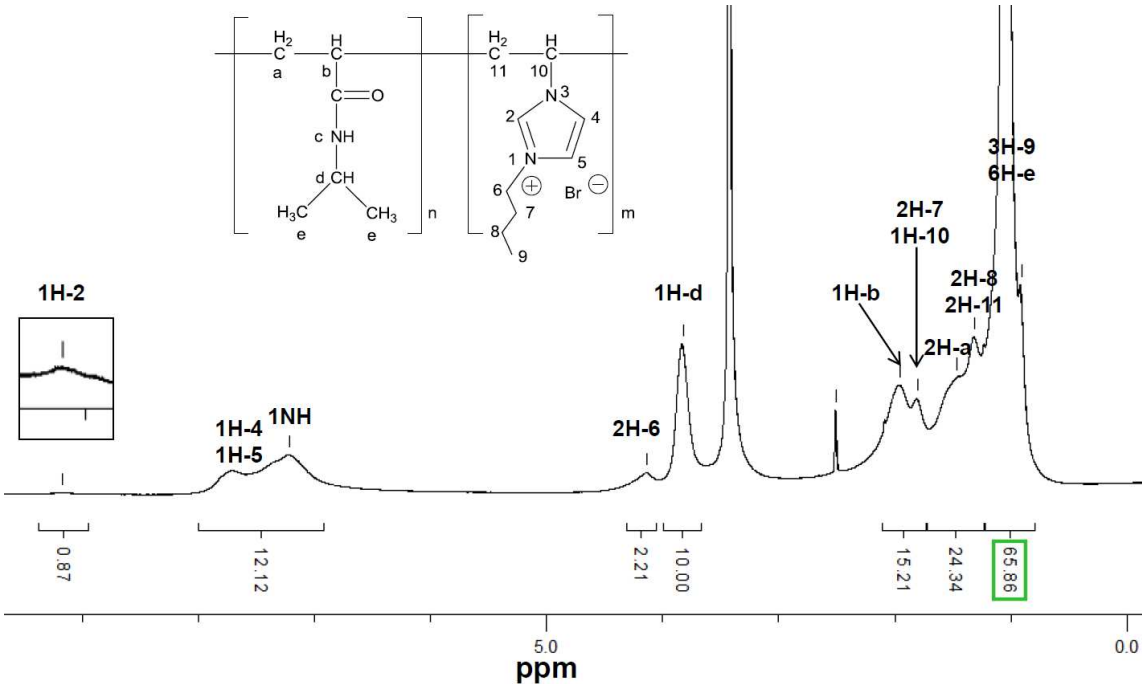


Fig. 2 ^{1}H-NMR spectrum of the *p*-NIBIm copolymer

3.2 Thermal behavior of the IL-doped copolymer, *p*-NIBIm

The thermal behavior of the IL-doped copolymer, *p*-NIBIm, was studied utilizing DSC and compared to those of the homopolymers, *p*-NIPAAm and *p*-BVIm, synthesized as a reference. As well known and expected, an aqueous solution sample (5 wt%) of *p*-NIPAAm exhibited an endothermic peak at 32.1 ^{o}C (the LCST) in the heating process and an exothermic peak at 29.0 ^{o}C in the cooling process (Fig. 3a), while for the case of *p*-BVIm any phase transition at

the temperatures ranging from 5 to 60 °C was not observed. However, the IL-doped copolymer showed a clear phase transition and a good reversibility that was confirmed via several continuous heating and cooling cycles (Fig. 3b and S4a). Fig. 3b shows that an endothermic peak of the copolymer product in the heating process appeared at 38.2 °C (the LCST), whereas an exothermic peak in the cooling process occurred at 36.2 °C. The temperature responsive property of *p*-NIBIm will be caused by the coexistence of relatively hydrophobic (or/and nonionic) and hydrophilic (or/and ionic) parts within the IL-doped copolymer. It is thought that the relatively broad peaks for *p*-NIBIm may be associated with the slightly different concentrations (distribution) of imidazolium units within the copolymer molecules. Moreover, the repulsion between the cationic units (imidazolium units) of the copolymer molecules consisting of a micelle also could be considered to increase the phase transition temperature of the micelle, subsequently resulting in shifting the LCST of the copolymer micelle to the higher temperature than that of *p*-NIPAAm micelle. Theoretically, doping of about 1.0 mol% of BVIm units into *p*-NIPAAm appeared to increase by about 0.6 degree of LCST. The obtained LCST value (38.2 °C) of *p*-NIBIm may be highly meaningful for in vivo applications, because the phase transition temperature is between body temperature (37 °C) and hyperthermia temperature (42 °C). This type of LCST shift also can be found in the previous reports that thermo-sensitive polymers with amine functional groups shifted their LCSTs to higher temperature by protonation under acidic surrounding condition.[21-25] However, unlike these, the *p*-NIBIm copolymer with permanent cationic moieties was not affected by the acidic pH environments (pH=4-6) and their LCST value also cannot be changed (Fig. S4b), demonstrating high applicability as an efficient system to deliver drug exactly to the target site. Consequently it can be considered that the introduction of the permanently cationic moieties within a polymer chain is very effective to increase both the loading efficacy and the transferring stability of drug molecules to the target site.

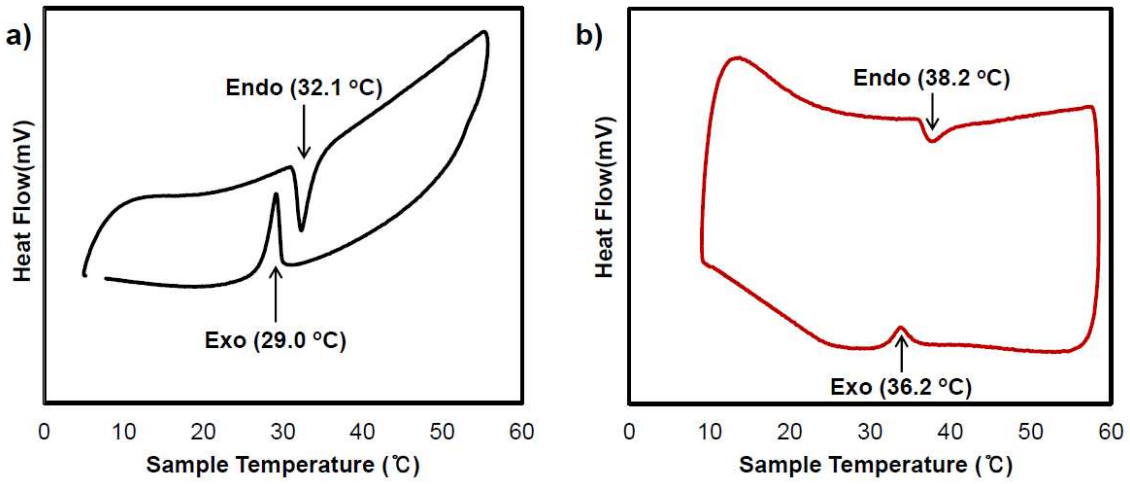


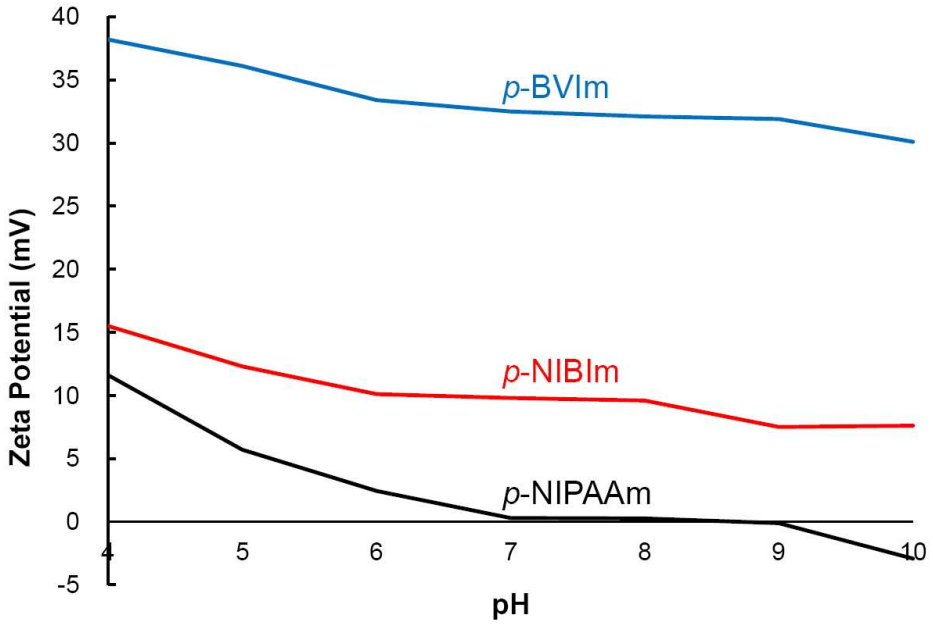
Fig. 3 LCST determination of a) *p*-NIPAAm and b) IL-doped *p*-NIBIm using DSC scan.

3.3 Surface charge of the partially ionic copolymer, *p*-NIBIm

Zeta (ξ) potential values of *p*-NIPAAm, *p*-NIBIm, and *p*-BVIm were measured as a function of pH (3-10) in a buffering system of 0.1M acetic acid and 0.2M sodium acetate at room temperature with the help of Zeta Sizer to confirm the cationic character of the IL-doped *p*-NIBIm copolymer. As shown in Fig. 4, initially the nonionic polymer, *p*-NIPAAm, showed an isoelectric point (IEP) at pH=8.6 and positive Zeta (ξ) potential values below the IEP (a maximum value of about 11.5 mV at pH=4), indicating that the polymer chain (and it's LCST) consisting of amide functional groups is pH-dependant and weakly positive under neutral condition. However, in the case of the IL-doped copolymer, *p*-NIBIm, no IEP appeared at the range of pH=4~10, indicating that it has permanently positive charges, regardless of the external pH change. The Zeta (ξ) potential change of *p*-NIBIm at the range of pH=4~10, however, appeared to be only about 8 mV, meaning that only 10 mol% of the cationic BVIm moieties are the main factor that affect the charge and the LCST of copolymer micelles. These results will be caused by the coexistence of the pH-independently cationic BVIm moieties and weakly pH-dependent NIPAAm moieties. Only the NIPAAm moieties responded to the protonation or deprotonation process slightly affecting the charge and the

LCST of copolymer micelles. Additionally, Fig. 4 demonstrates that the poly-cationic IL-polymer consisting of 100% BVIm units, *p*-BVIm, showed an extremely high Zeta (ξ) potential value, about 32.5 mV, at pH=7 and the pH-independent behavior of Zeta (ξ) potential values at the range of pH=4~10. The three different polymers with increasing concentration of BVIm unit exhibited increasing Zeta (ξ) potential values at pH=7, for example, +0.3 mV of *p*-NIPAAm, +9.8 mV of *p*-NIBIm, and +32.5 mV of *p*-BVIm.

The colloidal stability of the *p*-NIBIm copolymer solution (0.5 mg/mL) was tested by Terbiscan LAb. For this, the aqueous solution was scanned 12 times at 37 ^{o}C for 72 hours. These were confirmed by measuring the backscattered light (or transmission) of a pulsed near infrared light source of 880 nm wavelength (see Fig. S5). During the entire scanning time, any noticeable change of the light fluxes backscattered by the sample solution was not detected, demonstrating highly stable and uniform dispersion of *p*-NIBIm copolymer micelles in the solution. Here the fluctuations of the light fluxes occurred during the latter scanning periods were caused by air bubbles formed within the closed system of 37 ^{o}C.



Polymer	Zeta potential (mV) at pH=7
p-NIPAAm	+0.3
p-NIBIm	+9.8
p-BVIm	+32.5

Fig. 4 The Zeta (ξ) potential values as a function of pH in 0.1M acetic acid and 0.2M sodium acetate.

3.4 Temperature-dependant morphology change of the IL-doped copolymer micelles, *p*-NIBIm, and their complexes with BSA, *p*-NIBIm/BSA

Temperature-dependant morphology change of *p*-NIBIm micelles in an aqueous solution was tested by scanning electron microscope (SEM). The sample was prepared on a glass by drying a drop of the aqueous sample (0.01 wt%) at 25 (< the LCST) and 50 (> the LCST) ^{o}C. As shown in Fig. 5a and 5b, the SEM images demonstrate that the fibrous bundle-like aggregates (about <3 μm in length) were found for the sample dried at 25 ^{o}C. The copolymer molecules perhaps existed as hydrated random coils or swelled globules at the lower temperature and then the coils or swelled globules might gather or collapse to the fibrous

bundle-like aggregates during the drying process. When the drying temperature increased to 50 ^{o}C, the fibrous bundle-like morphology was dramatically changed to compact globules of about <500 nm in diameter. When looking in depth at the SEM image we can also discover that the compact globule is an aggregate of several smaller globules together of about <200 nm in diameter.

We also tested the morphology change after their complex formation with BSA by SEM. For this, 1.0 mL of aqueous polymer-BSA complex solution (0.011 wt%) containing 0.01 mg of polymer and 0.1 mg of BSA were used. After drying at 25 ^{o}C, a dramatic morphology change from the fibrous bundle-like aggregates (about <3 μm in length) to the circular aggregates (about <1.5 μm in diameter) and aggregates of BSA molecules encapsulated inside the circular aggregates were observed (see, respectively, the red- and blue-colored arrows in Fig. 2c). Here, it should be notable that the circular aggregates have loose spaces, meaning they existed as swelled and hydrated globules encapsulating large amount of BSA molecules under wet conditions at 25 ^{o}C. As shown in Fig. 2d, the compact globule-like aggregates again appeared at the higher drying-temperature of 50 ^{o}C and their mean size also returned to the size of free *p*-NIBIm micelles without BSA (about <200 nm in diameter). Moreover, a large scale of BSA aggregates that were released via phase transition and size contraction during the heating process from the swelled globules encapsulating a large quantity of BSA also was found. Consequently, the SEM results comprehensively illustrate that the morphology and size changes should be clearly related to the temperature-dependant BSA-loading and -releasing behaviors of *p*-NIBIm micelles, which occurred via the thermo-responsive phase transition of *p*-NIBIm micelles from swelled globules to desolvated compact globules in the temperature range (25-50 ^{o}C).

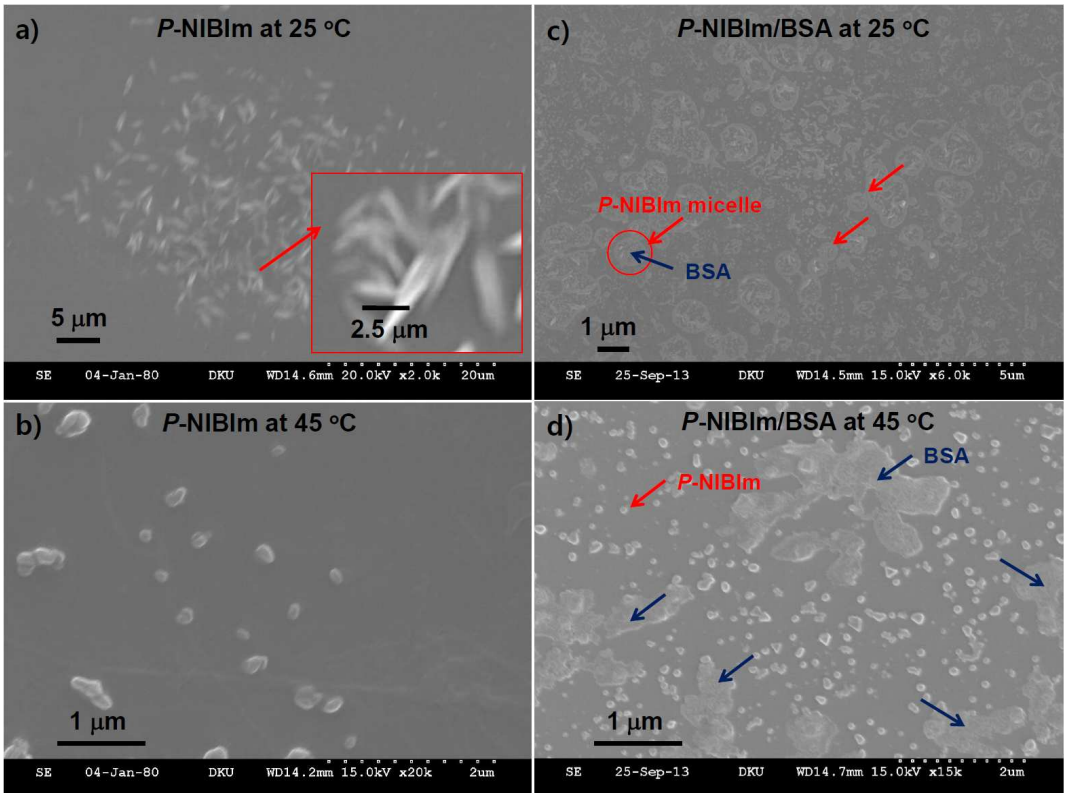


Fig. 5 SEM microscopic images of the *p*-NIBIm copolymer and the *p*-NIBIm/BSA complex micelles: a) and b) *p*-NIBIm samples prepared by drying, respectively, at 25 and 50 ^{o}C; c) and d) *p*-NIBIm/BSA complex samples prepared by drying, respectively, at 25 and 50 ^{o}C (red circle and arrow: swelled and deswelled *p*-NIBIm micelle, blue arrow: BSA aggregate).

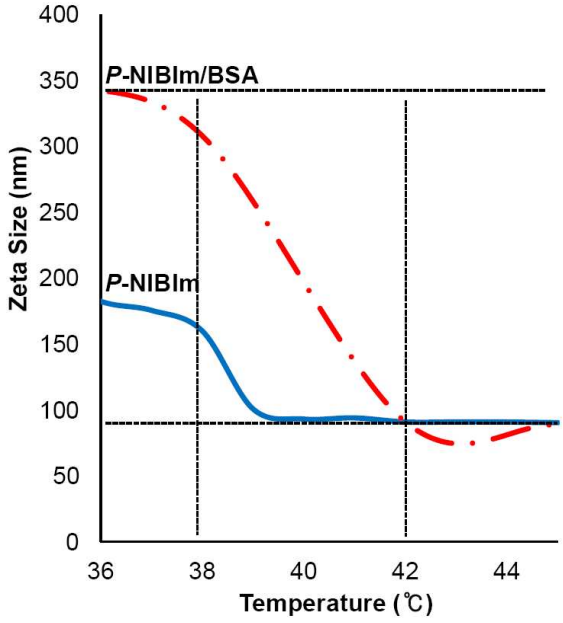
3.5 Temperature-dependant size change of the IL-doped copolymer micelles, *p*-NIBIm, and their complexes with BSA, *p*-NIBIm/BSA

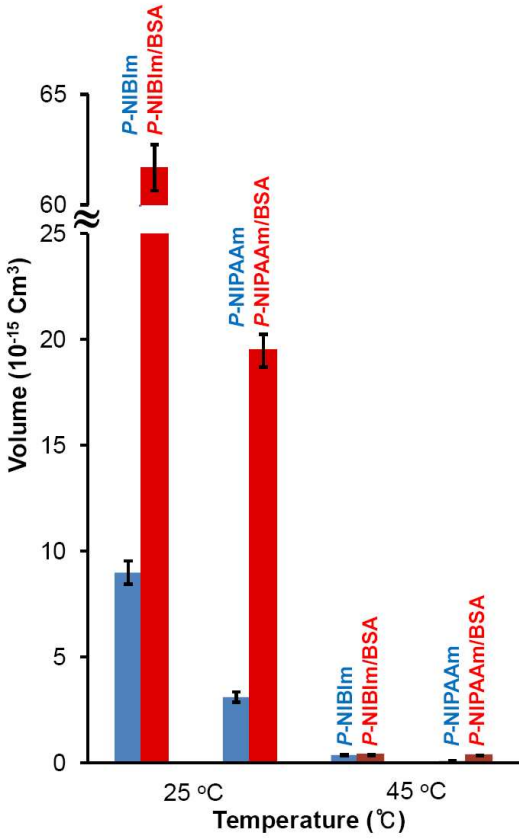
The size (or volume) change of *p*-NIBIm micelle and *p*-NIBIm/BSA complex micelle, depending on the temperature change ranging from 25 to 45 ^{o}C, was tested under wet conditions by Zeta Sizer. For this, aqueous samples (0.1 wt%) of the IL-doped copolymer, *p*-NIBIm, without and with BSA (1.0 wt%) were subjected to the instrument. As summarized in Fig. 6, the micelle volume of the pure *p*-NIBIm sample was reduced by about 8.09×10^{-15} cm^3 (decrease in diameter from 253±12.1 to 90.5±7.8 nm) with increasing temperature from 25 to

45 ^{o}C, showing a slow reduction between 25-37 ^{o}C and a rapid contraction around the LCST (38-39 ^{o}C). The size decrease obviously is caused by the phase transition of swelled globules and their volume contraction to compact globules. In contrast, the nonionic polymer, *p*-NIPAAm, showed the volume contraction of only 3.05×10^{-15} cm^3 (decrease in diameter from 180.6±5.1 to 39.8±3.6 nm) under the same temperature condition and a rapid deswelling around the LCST (32-34 ^{o}C) with a little initial reduction below 31 ^{o}C (see Fig. S6). The copolymer *p*-NIBIm with permanent positive charge clearly revealed the following interesting properties compared to the *p*-NIPAAm: the about 6 degree higher LCST, the 1.4-2.3 fold larger micelle size in diameter in the entire region of the tested temperatures, and the about 2.7 fold larger volume contraction in cm^3. As mentioned above, the molar masses (*Mn*, *Mw*) of *p*-NIBIm were smaller than those of *p*-NIPAAm. Nonetheless, there is no doubt that the increase of the micelle diameter can be caused by the strong repulsive force between positive charged imidazolium rings inside the copolymer micelles. Moreover, it also should be noted that the level of the micelle contraction for the IL-doped copolymer (*p*-NIBIm) at the tested temperature range (25-45 ^{o}C) is much higher than the level of the nonionic polymer (*p*-NIPAAm). All these results may indicate that the *p*-NIBIm copolymer, which may exist as swelled micelles below the LCST, can be expected to show a high adsorption efficacy of negatively charged molecules including drugs, genes and proteins owing to the charge-charge interaction between the positively charged host molecules and negatively charged guest molecules. Moreover, the high level of the micelle contraction at the LCST also may indicate an efficient releasing of the entrapped guest molecules via the thermo-responsive phase transition from a swelled globule to a desolvated compact globule.

The next step in this research is to test whether the IL-doped *p*-NIBIm micelles will function as planned, for example, to encapsulate and to release the protein BSA as a negatively charged model molecule, respectively, below and above the LCST. For this, the temperature dependent micelle size change of the aqueous *p*-NIBIm sample containing the

protein BSA was tested in the temperature range of 25-45 ^{o}C using Zeta Sizer (see Fig. 6) and compared to that of *p*-NIPAAm (see Fig. S6). 1.0 mL of the aqueous complex solution containing 0.1 mg of the polymer, *p*-NIBIm or *p*-NIPAAm, and 1.0 mg of BSA were subjected to the instrument. As shown in the Figures, the sizes of *p*-NIBIm/BSA and *p*-NIPAAm/BSA complex micelles at 25 ^{o}C appeared to be, respectively, 491.0±7.2 nm and 332.0±9.8 nm in diameter, meaning the volumes of, respectively, about 62.0×10^{-15} cm^3 and 19.1×10^{-15} cm^3. When the temperature further increases over the respective LCSTs, *p*-NIBIm/BSA complex micelles were reduced by about 61.9×10^{-15} cm^3 in volume (from 491.0±7.2 nm in diameter at 25 ^{o}C to 55±6.5 nm in diameter at 45 ^{o}C), showing a slow reduction between 25-37 ^{o}C and a rapid contraction around the LCST (37-42 ^{o}C), whereas *p*-NIPAAm/BSA complex micelles exhibited a reduction of about 18.7×10^{-15} cm^3 in volume (from 332±9.8 nm in diameter at 25 ^{o}C to 89±5.7 nm in diameter at 45 ^{o}C), showing a slow and consistent reduction between 25-39 ^{o}C. Here it is very impressive that *p*-NIBIm/BSA complex micelles were intensively contracted in a certain range (38-42 ^{o}C) around the LCST, while *p*-NIPAAm/BSA complex micelles were continually contracted over the entire range of 25-45 ^{o}C. The about 3.3 times larger volume and volume contraction of *p*-NIBIm/BSA complex micelles than *p*-NIPAAm/BSA complex micelles may reflect the higher capacity to load and release drugs.





Polymer	Micelle size in diameter (nm)		
	36 ºC	38 ºC	42 ºC
p-NIBIm	182.5±7.4	159.9±8.7	92.0± 6.7
p-NIBIm/BSA	340.2±6.2	308.0±9.8	92.0±10.3

Fig. 6 The temperature-dependent micelle size changes of *p*-NIBIm copolymer under wet conditions.

3.6 Temperature-dependant BSA loading and releasing behaviors of the IL-doped copolymer, *p*-NIBIm

As the final step for this research, the quantitative determinations of the loaded and released BSA concentrations were accomplished by Bio-Rad DC-Protein assay. For this, three aqueous samples (2 mL) containing 1.0 mg of the thermo-sensitive polymer (*p*-NIBIm or *p*-NIPAAm) and 10 mg of BSA molecule were prepared at 4 ^{o}C and then the temperature of the three samples was slowly increased, respectively, to 25 (room temperature), 37 (body temperature), and 42 ^{o}C (clinical hyperthermic temperature). Each sample solution was rapidly filtered through a syringe filter equipped with PTFE membrane filter (0.1 μm pore size, Advantec,

Japan) at each temperature, following adding the DC protein assay reagent to each filtrate and measuring the UV/Vis absorbance of the filtrate at λ_{max}=750 nm. The BSA concentrations in the filtrate and encapsulated in polymer micelles were calculated via a standard curve, created by plotting the known BSA concentration on x axis and the absorbance of BSA/DC complexes at λ_{max}=750 nm on y axis (Fig. S7). Fig. 7 shows different BSA-loading capacities of the *p*-NIBIm and the *p*-NIPAAm samples at 25 ^{o}C. The BSA amount encapsulated in 1.0 mg of *p*-NIBIm polymer appeared to be about 5 times larger than that of *p*-NIPAAm, for example, 3.28 mg for *p*-NIBIm and 0.64 mg for *p*-NIPAAm. These quantitative results interestingly are consistent with the results of micelle volume increase obtained by Zeta Sizer at 25 ^{o}C (see Fig. 6). The higher BSA adsorption ability of *p*-NIBIm micelles is definitely associated with the charge-charge interaction between negatively charged BSA molecules and positively charged imidazolium rings within the swelled micelles of the copolymer chains.

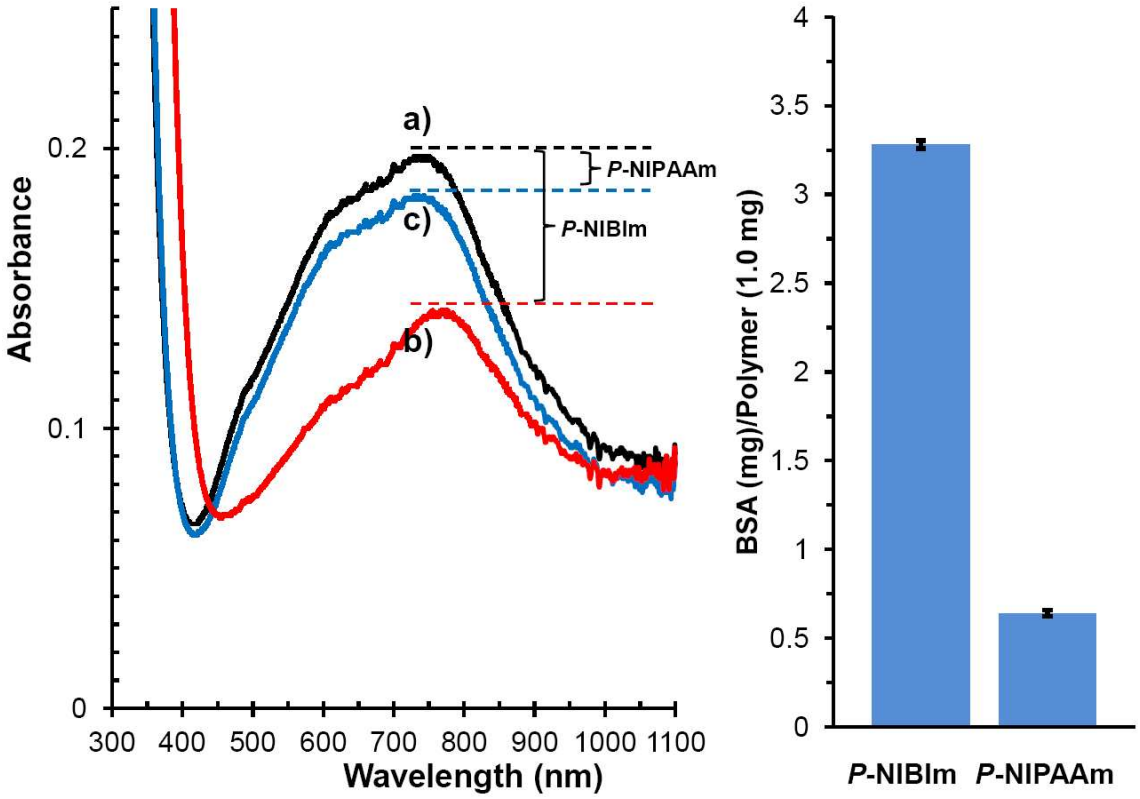


Fig. 7 The different BSA-loading capacities of *p*-NIBIm and *p*-NIPAAm at 25 ^{o}C: a) the initial BSA concentration, b) the filtrate obtained from the *p*-NIBIm/BSA solution at 25 ^{o}C and c) the filtrate obtained from the *p*-NIPAAm/BSA solution at 25 ^{o}C.

The BSA concentrations remained in micelles of 37 ^{o}C and further released from the micelles between 38~42 ^{o}C were taken, respectively, as the loaded and released BSA amounts especially for the *p*-NIBIm polymer sample. Loading and releasing tests of the BSA molecules for *p*-NIBIm, therefore, were accomplished through elevating the temperature of the sample solution prepared at 4 ^{o}C to body temperature (37 ^{o}C) and clinical hyperthermic temperature (42 ^{o}C). Fig. 8 shows BSA-loading capacity at 37 ^{o}C and BSA-releasing capacity at 38-42 ^{o}C for the *p*-NIBIm micelles. When the temperature of two *p*-NIBIm/BSA solution samples increased, respectively, to 37 and 42 ^{o}C, as shown in Fig. 8, respectively, 1.4 mg and 0.38 mg of BSA were left in the copolymer micelles. This means that 1.0 mg of *p*-NIBIm can load 1.4 mg of BSA at body temperature and extrude 1.02 mg (72.9%) of the in the micelle of 37 ^{o}C remaining BSA amount via a deswelling process of the swelled copolymer micelles between 38~42 ^{o}C, while *p*-NIPAAm micelles can encapsulate only 0.64 mg at 25 ^{o}C and extrude almost all the BSA molecules below body temperature. In conclusion, the IL-doped copolymer *p*-NIBIm, unlike the *p*-NIPAAm, are considered to be potentially useful as a smart delivery system of negatively charged molecules, such as BSA protein, because *p*-NIBIm can carry up to about 1.0 mg drug per 1.0 mg of the polymer to the target site between body temperature and hyperthermic temperature.

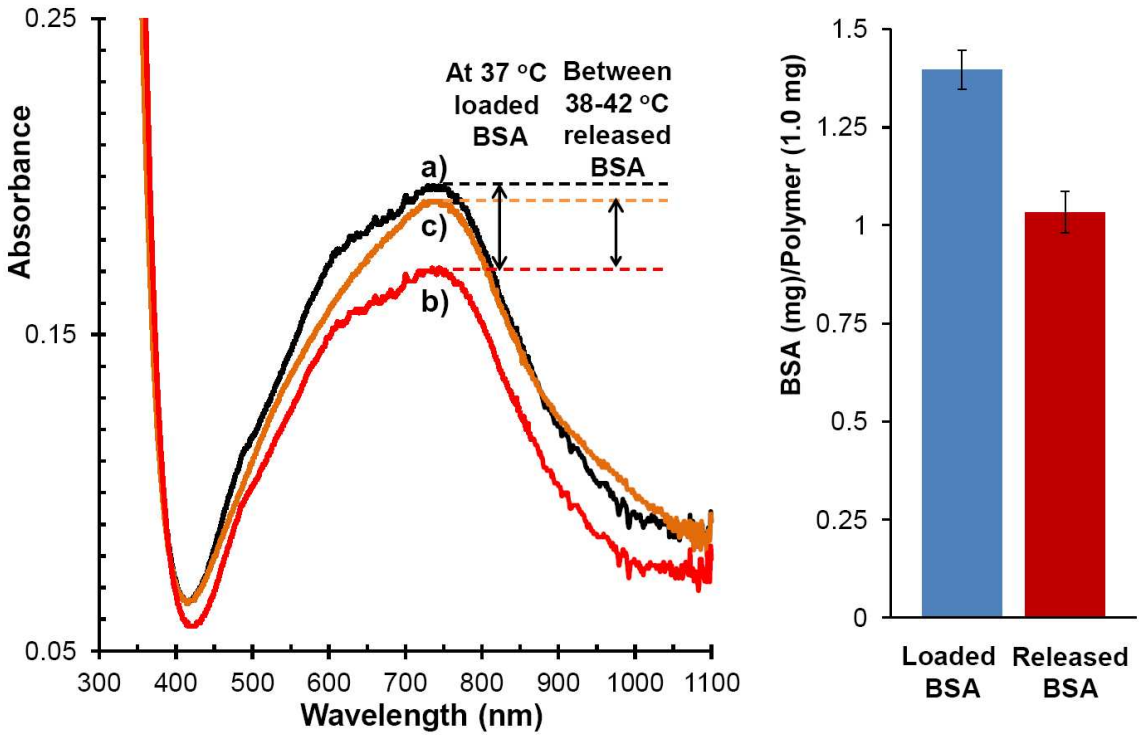


Fig. 8 The BSA-loading and -releasing capacities of the *p*-NIBIm copolymer, respectively, at 37 °C and 38-42 °C: a) the initial BSA concentration (5mg/mL), b) filtrate obtained at body temperature (37 °C) and c) filtrate obtained at 42 °C.

3.7 Cytotoxicity assay

The *in vitro* cytotoxicity of *p*-NIBIm was assessed by the CCK-8 assay with HEK 293 cells. Fig. 9 well shows the concentration-dependent effects of *p*-NIBIm on cell viability. After 1 day exposure to the copolymer up to 0.5 mg/mL, the *p*-NIBIm micelles of up to 0.5 mg/mL had very low toxicity for HEK 293 cells as at least 90% cells remained alive up, while an obvious increase of the cytotoxicity was observed in the *p*-NIBIm micelle concentration range of above 1 mg/mL. After incubation for 3 days, the HEK 293 cells showed still high viability of around 90% in the *p*-NIBIm concentration up to 0.125 mg/mL, but the cell viability slowly was reduced to less than 75% from the micelle concentration of 0.5 mg/mL. These results suggest that the *p*-NIBIm micelles have no obvious cytotoxicity to the HEK 293 cells in the

range of <0.125 mg/mL and are highly applicable as a thermoresponsive drug delivery system in the human body.

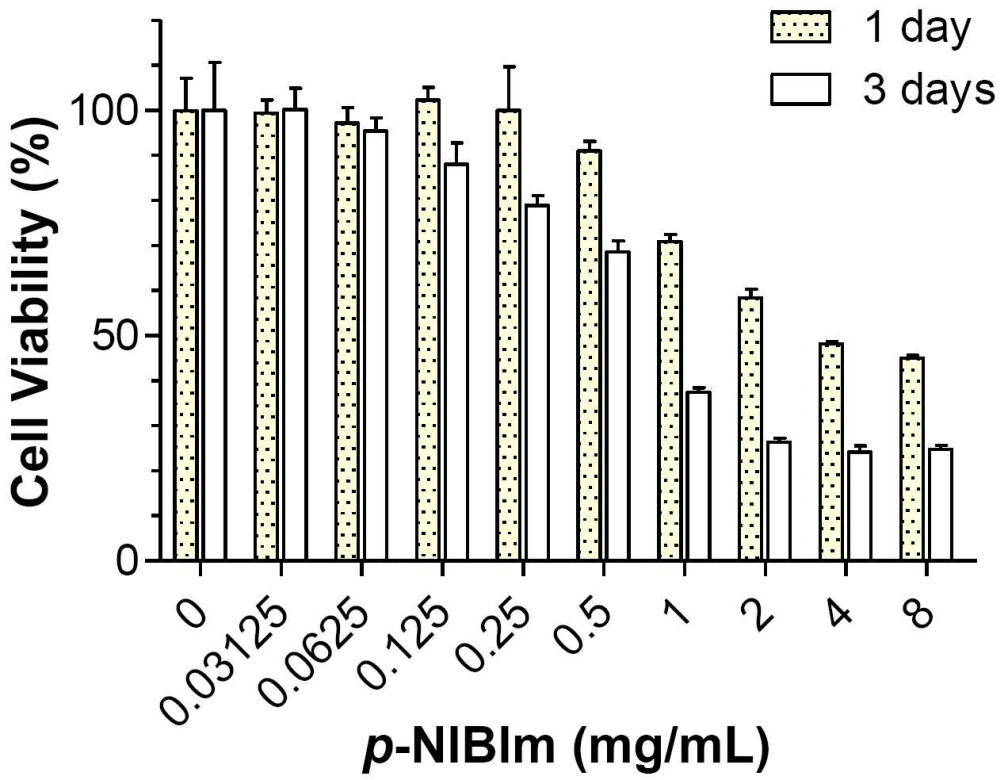


Fig. 9 Viability of HEK 293 cells incubated with *p*-NIBIm micelles in the concentration range of 0~8 mg/mL for 1 day and 3 days at 37◦C. Data were shown as mean ± S.D. (n = 4).

4. Conclusions

To summarize, ionic liquid (IL)-doping on temperature responsive *p*-NIPAAm was achieved by radical copolymerization of N-isopropyl acryl amide (NIPAAm; 90 mol%) and 1-butyl-3-vinylimidazolium bromide ([BVIm]Br; 10 mol%) to give a new temperature responsive copolymer (*p*-NIBIm). The as-prepared *p*-NIBIm copolymer exhibited a highly increased Zeta potential value and optimal LCST (lower critical solution temperatures) value, respectively, +9.8 mV at pH=7 and 38.2 ^{o}C, compared to those (+0.3 mV at pH=7 and 32.1 ^{o}C) of *p*-NIPAAm without the ionic moiety. The temperature-dependent size change of the *p*-NIBIm micelles was determined in the range from 25 to 45 ^{o}C by SEM under dry conditions

and by Zeta Sizer under wet conditions, showing a certain size contraction from 253±12.1 to 90.5±7.8 nm in diameter (about 95.4% volume contraction). The thermo-sensitive behaviors to entrap BSA protein at body temperature (37 ^{o}C) and to release the protein between 38-42 ^{o}C (near the LCST) also were tested by sizing the complexes of *p*-NIBIm/BSA using Zeta Sizer, by SEM, and also by colorimetric assay (Bio-Rad DC Protein Assay), resulting in a maximum entrapment of 1.4 mg BSA (about 140% loading) for 1.0 mg of the polymer at body temperature (37 ^{o}C) and in a maximum release of 1.02 mg BSA for 1.0 mg of the polymer (about 73% release of the entrapped amount) at the temperature range of 38-42 ^{o}C. The in vitro toxicity of the *p*-NIBIm micelles without drug for human embryonic kidney (HEK 293) cells was minimal in the range of <0.125 mg/mL. These results revealed IL-doped and temperature responsive co-polymeric systems have high applicability as a novel delivery system for negatively charged molecules as a natural (or synthetic) drug and DNA.

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Ionic liquid-doped and *p*-NIPAAm-based copolymer (*p*-NIBIm): extraordinary drug-entrapping and -releasing behaviors at 38-42 °C

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Temperature-dependent size changes of *p*-NIBIm and extraordinary BSA-entrapping and -releasing behaviors of *p*-NIBIm/BSA complexes at 38-42 °C