Reactivity Control of Polymer Functional Groups by Altering the Structure of Thermoresponsive Triblock Copolymers

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* Supporting Information

ABSTRACT: A thermoresponsive ABA triblock copolymer bearing an aldehyde group on the thermoresponsive A segments was synthesized. The polymer formed a micellar assembly due to the hydrophobic interactions of the thermoresponsive segment above the lower critical solution temperature (LCST). In contrast, the ABA polymer assembly decomposed upon lowering the temperature below the LCST. Using this structural change, the reactivity of the aldehyde group toward primary amines of albumin and poly(allylamine) was investigated. When the ABA polymer assembly and reactant were mixed above the LCST, Schiff base formation was suppressed because of the aldehyde group being protected by the hydrophobic thermoresponsive core. In contrast, Schiff base formation between the ABA triblock copolymer and the primary amine moiety on the molecules was confirmed below the LCST. The reactivity of the aldehyde functional group can therefore be controlled by altering the structure of the thermoresponsive ABA polymer.

1. INTRODUCTION
The physicochemical properties and structures of thermoresponsive polymers can be altered in response to a heat signal.1,2 For example, thermoresponsive polymers with a lower critical solution temperature (LCST) can form random hydrophilic coils below the LCST, while hydrophobic aggregates are produced above the LCST because of dehydration.3 By applying thermoresponsive polymers to material production, the physicochemical properties of the resulting materials can also be controlled in response to temperature changes across the LCST. As such, thermoresponsive materials have been widely applied to control the interactions with substances, such as cells and proteins, through temperature variations.4–7 In particular, the physicochemical properties of nanoparticles modified with thermoresponsive polymers can be controlled,3–5,8–10 and so such systems are currently being developed as drug carriers for controlling the interactions between the incorporated drugs and the cells.11–13

ABA triblock copolymers possessing LCST-type thermoresponsive properties on the A segments and a hydrophilic polymer on B segment have been used to prepare thermoresponsive nanoparticles.14,15 Because the presence of thermoresponsive segments increases the degree of hydrophobic interactions above the LCST, ABA polymers form a flower-like micelle consisting of a thermoresponsive core. In contrast, above the LCST, the hydrophobic interactions between thermoresponsive segments are relaxed, and the assembled structure can be dissociated.16,17 This structural change occurs reversibly by altering the temperature across the LCST, and so can be employed in nanoparticles prepared from thermoresponsive ABA polymers to control the release of molecules incorporated in the hydrophobic inner core.18 In addition, highly concentrated ABA-type thermoresponsive polymers exhibit sol–gel transitions upon varying the temperature, thereby leading to the development of thermoresponsive injectable gels for biomedical applications.19–21

Furthermore, the abovedescribed structural change of a thermoresponsive ABA polymer can be used to control the interactions between thermoresponsive segments and external molecules. As the ABA polymer assembly possesses a...
hydrophilic polymer on its shell, the interactions between the hydrophobic inner core (composed of the thermoresponsive polymer) and a nanomolecule such as protein can be suppressed because of the exclusion volume effect of the corona-forming hydrophilic polymer. In contrast, an ABA polymer assembly can be dissociated by lowering the temperature below the LCST. Thus, the hydrated thermoresponsive segment is exposed and can come into contact with external molecules. By utilizing the localization change of this thermoresponsive polymer in aqueous media, the interactions between the thermoresponsive segment and the external molecules can be controlled by varying the temperature across the LCST. An ABA polymer possessing a reactive functional group on the thermoresponsive segment can therefore control the reactivity of the introduced functional group through the temperature-dependent structural change of the polymer, and so can be applied in the context of a thermoresponsive molecule reactor.

Thus, we herein report the preparation of thermoresponsive ABA triblock polymers possessing an aldehyde group on their thermoresponsive A segments (Scheme 1). Because aldehyde groups can form Schiff bases by reaction with the primary amines present on molecules, using dynamic light scattering (DLS) and viscoelastic changes, we investigate the formation of a Schiff base by the aldehyde group in a thermoresponsive ABA triblock polymer and primary amine of albumin and poly(allylamine) across the LCST. Finally, we investigate a suitable method for controlling the reactivity of the aldehyde functional group through the temperature-responsive structural change of the thermoresponsive triblock copolymer.

2. RESULTS AND DISCUSSION

2.1. Preparation of P(NIPAAm-co-NAS)-b-PEG-b-P(NIPAAm-co-NAS). S-1-Dodecyl-S-(3,4′-dimethyl-α′-acetic acid)-trithiocarbonate (DDMAT), the chain transfer agent of reversible addition–fragmentation chain transfer (RAFT) polymerization, was conjugated to the poly(ethylene glycol) (PEG) hydroxyl groups at both termini through a condensation reaction. The resulting terminal conversion efficiency was confirmed by 1H NMR measurements of the proton signals derived from PEG methyne (−CH₂−CH₂O) (4H, 3.4–3.9 ppm) and DDMAT methylene (S(C=S)−CH₃−) (4H, 2.9–3.3 ppm), and was determined to be ~86% (Figure S1).

Thermoresponsive segments bearing succinimide units were connected by the random copolymerization of N-isopropylacrylamide (NIPAAm) and N-acryloylsuccinimide (NAS) using DDMAT−PEG−DDMAT as the macro-RAFT agent. From the resulting 1H NMR spectrum, the proton signals derived from NIPAAm methyne (−CH₂−(CH₃) (1H, 3.9–4.1 ppm), NAS ethylene (−C(═O)−CH₃−CH₂−) (4H, 2.8–2.9 ppm), and PEG methyne (−CH₂−CH₂O) (4H, 3.4–3.9 ppm) moieties were clearly assigned (Figure S2). The conversion of NIPAAm and NAS was 55 and 77.5%, respectively. The numbers of NIPAAm and NAS units were calculated from the proton intensity of each signal and were determined to be 198 and 31, respectively. The polymer termini were then converted to the inert isobutyronitrile group by a radical reaction using Perrier’s method. After the radical treatment, the polymer color changed from yellow to white, and the UV absorbance of the DDMAT unit at 310 nm was no longer observed (data not shown). In addition, gel permeation chromatography (GPC) measurements showed that the radical-treated triblock polymer exhibited a unimodal peak at a similar retention time to the obtained thermoresponsive ABA triblock copolymer prior to the reaction (Figure 1C). The obtained thermoresponsive ABA triblock copolymer, P(NIPAAm-co-NAS)-b-PEG-b-P(NIPAAm-co-NAS), was abbreviated as ABA(NAS).

2.2. Preparation of the Aldehyde-Containing Thermoresponsive ABA Triblock Copolymer. We then converted the succinimide group on the side chain of the NAS unit on ABA(NAS) into an aldehyde group. For this purpose, an acetal group was initially introduced into the thermoresponsive segment by the amino coupling of N,N-diethoxyethylamine (DEEA) to succinimide. From the GPC
chromatogram, it was apparent that the peak shape was comparable to that of ABA(NAS) (Figure 1). In addition, the 1H NMR spectrum showed the complete disappearance of the succinimide (−C(=O)−CH2−CH2−) (4H, 2.8−2.9 ppm) peak, and the appearance of the methyne proton of the acetal unit (−CH−(O−CH2−CH3)2) (1H, 4.5−4.7 ppm) (Figure S3). The acetal-containing triblock copolymer, P(NIPAAm-co-DEEAm)-b-PEG-b-P(NIPAAm-co-DEEAm) (DEEAm: diethoxethylacrylamide) was abbreviated as ABA(acetal).

Subsequently, ABA(acetal) was dialyzed against sodium acetate buffer (pH 5), followed by treatment with hydrochloric acid (pH 2) to convert the acetal group into an aldehyde group. This transformation was confirmed by the observation of a peak corresponding to the aldehyde group at 9.7−9.9 ppm (Figure 2). Upon increasing the duration of hydrochloric acid treatment, it was found that the conversion to aldehyde groups increased. The aldehyde conversion proceeded by ~25 and 57% after the hydrochloric acid treatment for 5 and 24 h. Analysis by GPC showed that the obtained polymers exhibited a similar peak to ABA(acetal) (Figure 1D). In the experiment, the acetal unit was converted to aldehyde by the acid treatment. Although triblock copolymer possessed ester bonds between PEG and thermoresponsive segments, no possible hydrolysis was observed in the GPC chromatograms. Hydrolysis was observed in the presence of basic molecules during the hydrolysis (Figure S5). In this method, basic molecules such as triethylamine were completely removed before the hydrochloric acid treatment by dialysis using sodium acetate for 3 days. Owing to pretreatment, possible hydrolysis of the ester bond was successfully avoided during the aldehyde conversion.

The obtained polymer, P(NIPAAm-co-DEEAm-co-N-2-formylmethacrylamide)-b-PEG-b-P(NIPAAm-co-DEEAm-co-N-2-formylmethacrylamide) was abbreviated as ABA(aldehyde[x]); where x indicates the number of N-2-formylmethacrylamide units on a single thermoresponsive segment. Characterization of the synthesized polymers is summarized in Table 1.

2.3. Characterization of the ABA Triblock Copolymers. The phase transition behavior of the triblock copolymers was then investigated by measuring the change in turbidity of the polymer solution (Figure 3). The phase transition temperature ($T_p$) values of ABA(acetal), ABA(aldehyde[7]), and ABA(aldehyde[16]) were determined as 32.5, 37.1, and 42.2 °C, respectively. It was found that $T_p$ increased upon the introduction of aldehyde units on the polymer.
thermosensitive unit. As $T_p$ of an NIPAAm copolymer tends to increase upon copolymerization with a hydrophilic co-monomer, the greater hydrophilicity of the aldehyde group compared to that of DEEAm results in the LCST of the thermosensitive chain increasing upon the introduction of an aldehyde unit.

The reactivity of aldehyde toward ABA(aldehyde[16]) was confirmed by the reaction with 2-aminoethanol. After the addition of 2-aminoethanol, the $^1$H NMR signal derived from aldehyde completely disappeared, confirming the presence of imine ($-\text{CH}═\text{N}-$, 1H, 7.7 ppm) (Figure S6). Thus, the aldehyde-modified polymer was able to form the Schiff base by the reaction with primary amine.

Subsequently, DLS measurements were carried out to estimate the structural changes of the polymer across the $T_p$ (Figure 4). Thus, ABA(aldehyde) exhibited a unimodal peak with a volume averaged diameter at 12.2 $±$ 4.2 nm at 37 °C, while the peak became bimodal with volume-averaged diameters of 43.1 $±$ 6.2 and 552 $±$ 114 nm above the LCST (39 °C). Upon decreasing the temperature once again to 37 °C, the aggregate peak disappeared, and a signal was observed at 12.8 $±$ 9.7 nm. This result indicated that the polymer was dispersed in water below the LCST and formed an assembly above the LCST. This assembly possessed a hydrophobic core consisting of dehydrated thermosensitive segments, and a hydrophilic PEG outer shell, which were observed overall as small particles by DLS analysis. However, from the $^1$H NMR analysis, a part of the thermosensitive segment of the hydrophobic core was exposed because the signal from the thermosensitive segment was observed above the LCST (Figure S7). As a result, because of the increased intermicellar hydrophobic interactions, the nanoparticles formed submicron-sized nanoparticle aggregates, which were easily destroyed by lowering the temperature below the LCST (Figure 4C).

To investigate the effect of temperature on the reactivity of the aldehyde group, ABA(aldehyde[7]) was mixed with the amino-containing albumin (volume average diameter: 7.0 nm at 37 or 39 °C, Figure S8A,B). First, ABA(aldehyde[7]) and albumin were mixed at 25 °C. This mixture was almost similar to ABA(aldehyde[7]) except for the formation of aggregates with 143 nm diameter, and no peak change was observed below 43 °C (Table S1). After phase transition, the mixture

Figure 4. Volume average diameter of the ABA(aldehyde[7]) solution under a range of conditions: (A) at 37 °C, (B) at 39 °C, and (C) upon recooling to 37 °C from 39 °C. (D–H) As above but in the presence of albumin: polymer and albumin were mixed (D) at 25 °C and (E–H) at 50 °C. Measurement was performed (D) at 37 °C, (E) at 39 °C, (F,H) upon cooling to 37 °C from 39 °C (F) at pH 7.4 and (H) at pH 6, and (G) upon heating to 39 °C from 37 °C at pH 7.4.

Figure 5. Photographic images of the (A,B) ABA(acetal) and (C–F) ABA(aldehyde[7]) solutions. The ABA triblock copolymers and PAA were mixed at 50 °C. (A,C) At 50 °C, (B,D) upon cooling to 25 °C from 50 °C, (E) upon reheating to 50 °C from 25 °C, and (F) upon recooling to 25 °C from 50 °C.
formed submicron aggregation and the aggregate was dissociated on cooling at 37 °C (Figure S8E–F). In contrast, after mixing at 50 °C, signals corresponding to the ABA-aldehyde[7]) and albumin mixture were independently observed at 39 °C (Figure 4E). Upon lowering the temperature of this solution to 37 °C, the aggregate and albumin peaks were again observed separately. When this solution was returned to 39 °C again, albumin peak was disappeared and the large aggregation was confirmed. In contrast, upon lowering the pH of the mixed solution, the peak corresponding to the nanoparticle aggregates gradually disappeared (Figure 4H).

These results indicated that the reactivity of the aldehyde unit in ABA(aldehyde[7]) toward albumin was suppressed when ABA(aldehyde[7]) formed an assembled structure above the LCST. In contrast, below the LCST, this structure was disassembled and the aldehyde groups became accessible; therefore, they may react rapidly with the primary amine groups of albumin to form a Schiff base similar to 2-ethanol amine. Upon conjugation with the albumin, the structure of the ABA(aldehyde[7]) assembly became fixed. However, this Schiff base gradually dissociates at low pH values, resulting in collapse of the polymer assembly (Figure 4H). The result indicates that Schiff base formation can be controlled by the temperature-controlled structural changes of ABA(aldehyde).

**2.4. Formation and Characterization of the Hydrogel.**

Solutions of the ABA triblock copolymer (2.0 wt %, 200 μL) and poly(allyl amine) (PAA) (4 wt %, 50 μL) were mixed at 50 °C and the changes in viscoelasticity of the solution at 50 and 25 °C were observed (Figure 5). Although the ABA(acetal)/PAA solution showed no change in viscosity, a mixture of the ABA(aldehyde) and PAA solutions formed a cloudy sol at 50 °C, and the solution viscosity increased upon lowering the temperature. Upon increasing the temperature once again, although the gel became slightly opaque, the solution maintained its gel state, and the strength of the gel in the second cooling cycle was greater than that of the first cooling cycle (Figure 5F). Indeed, the gel structure was maintained beyond one day.

Subsequently, the variation in viscoelasticity of the ABA-(acetal) and PAA solutions was observed using a rheometer (Figure 6). Because this solution formed a precipitate at 50 °C, measurement was performed at 40 and 25 °C. At 40 °C, the storage modulus \(G'(\text{Pa})\) and loss modulus \(G''(\text{Pa})\) of the ABA(acetal)/PAA solution increased gradually with time, and the solution showed a slightly higher storage modulus compared to the loss modulus. However, at 25 °C, the \(G'\) value of the solution became greater than \(G''\). In contrast, the solutions of ABA(aldehyde)/PAA increased in viscoelasticity upon lowering the temperature, and the gel viscoelasticity increased with greater numbers of aldehyde units on the polymer. In addition, the strength of the hydrogel gradually increased at 30 °C. Overall, we found that the \(G'\) values of ABA-aldehyde[7]) and ABA-aldehyde[16]) were 42.5 and 130 Pa, respectively. The experiment was also performed using the ABA(aldehyde[16])/PAA solutions at pH 6.0. In this case, the changes in viscoelasticity were negligible upon altering the temperature, and the \(G''\) values were higher than the \(G'\) values regardless of temperature.

Finally, hydrogel stability was investigated by immersing the hydrogel in the phosphate buffer solution at different pH (Table 2). After one day, \(G'\) of the hydrogel in pH 4, 6, and 7 became 53, 93, and 96 Pa, respectively. Although pH was important for the formation of the Schiff base, the hydrogel maintained its stability at pH 6. In contrast, \(G'\) of hydrogel decreased in the pH 3 solution. The results indicated the Schiff base in the hydrogel was not rapidly decomposed during the low pH treatment.

It was also found that ABA(aldehyde) and the primary amine-bearing polymer formed an irreversible gel upon altering the temperature (Figure 7). Because gel formation was suppressed by lowering the pH, it was apparent that a cross-linking point was formed through Schiff base formation between the aldehyde group on ABA(aldehyde) and the primary amine moiety of PAA, as shown in the DLS results. ABA(aldehyde) and PAA immediately formed a hydrogel upon reducing the temperature, and the strength of this gel gradually increased, thereby indicating that the reaction between the aldehyde and the amino group proceeded gradually with time as the temperature decreased. Interestingly, upon increasing the temperature above \(T_g\) once again, the gel strength further increased. Upon increasing the temperature across the LCST, the thermoresponsive segment became hydrophobic, and as a result, hydrophobic interactions formed to give a new cross-linking point in the hydrogel. This ultimately resulted in the formation of a dense gel network and a stiffer hydrogel.

From the abovementioned results, it was apparent that the reactivity of the aldehyde functional group in the thermores-
responsive ABA triblock copolymer could be controlled by temperature-dependent structural changes across the LCST.

3. CONCLUSIONS

We herein reported the preparation of thermoresponsive ABA triblock copolymers bearing aldehyde groups on the thermoresponsive segments at both termini. The polymer reversibly formed assembled structures through altering the temperature across the LCST of the thermoresponsive segment. Owing to this structural change, the reactivity of the aldehyde moiety on the thermoresponsive segment toward a primary amine group of albumin and poly(allylamine) was successfully controlled. In response to the dissociation of this assembly upon lowering the temperature, the aldehyde group reacted rapidly with the primary amine to form a Schiff base. This Schiff base was stable in the experimental temperature range employed herein, thereby resulting in the formation of a stable irreversible hydrogel from a mixture of ABA(aldheyde) and the amine-based polymer. Using this mechanism, the thermoresponsive ABA block copolymer could be employed as a molecular reactor for medical applications, such as an injectable hydrogel that controls the sol–gel transition through the application of an external heat signal.

4. MATERIALS AND METHODS

4.1. Materials. NIPAAm was purchased from KJ Chemicals (Tokyo, Japan) and was purified by recrystallization from toluene and n-hexane. PEG (M<sub>n</sub>: 10 kDa), oxalyl chloride, and chloroform were purchased from Sigma-Aldrich (St Louis, MO) and were used without further purification. DDMAT was purchased from Trylead Chemical Technology (Hangzhou, China). NAS, 2,2′-azobis(2,4-dimethylvaleronitrile) (V-65), 2,2′-azobis(isobutyronitrile) (AIBN), dichloromethane (super dehydrated), acetone, toluene, n-hexane, ethanol, 1,4-dioxane, diethyl ether, DEEA, triethylamine, N,N-dimethylformamidine, hydrochloric acid, sodium carbonate, acetic acid, and sodium acetate trihydrate were purchased from Wako Pure Chemical (Osaka, Japan) and were used without purification. PAA (M<sub>n</sub>: 150 kDa, 40 wt % solution) was kindly provided by Nittobo Medical (Tokyo, Japan).

4.2. Instrumentation. 1H NMR (400 MHz) spectroscopy was carried out using an NMR ECS-400 instrument (JOEL, Tokyo, Japan). GPC was performed using a GPC LC-2000Plus series system (JASCO, Tokyo, Japan) equipped with two Shodex columns (KD-804 and KD-802, Showa Denko, Tokyo, Japan). The eluent was composed of N,N-dimethylformamidine containing 3 mmol/L lithium bromide and 0.5% triethylamine, and a flow rate of 1.0 mL/min was employed. The hydrodynamic diameters were measured by DLS using an ELSZ-2PL instrument (Otsuka Electronics, Osaka, Japan). The optical transmittance of each polymer solution (10 mg/mL) at a range of temperatures (heating rate = 0.5 °C/min) was measured by UV/vis spectrophotometry (UV−2500PC) (Shimadzu, Kyoto, Japan) (λ: 550 nm). The phase transition temperature (T<sub>p</sub>) was defined as the temperature at which the light transmittance of a solution reached 50%. The viscoelasticity was recorded using an AR-G2 rheometer (TA instruments, New Castle, DE, USA).

4.3. Synthesis of the DDMAT-Conjugated PEG. The DDMAT-conjugated PEG was prepared using Lodge’s method with slight modifications.3,15 More specifically, DDMAT (3.65 g, 10.0 mmol) was dried under reduced pressure for 24 h to remove any moisture. Subsequently, dichloromethane (super dehydrated) (20 mL) and oxalyl chloride (8.6 mL, 12.7 mmol) were added and the mixture was allowed to react at 25 °C for 3 h under an Ar atmosphere. After this time, dichloromethane and the hydrogen chloride byproducts were removed by drying under reduced pressure. Subsequently, PEG (1.0 g, 0.5 mmol) was dried under reduced pressure for 24 h to remove any moisture from the polymer. After this time, PEG and the activated DDMAT were dissolved in dehydrated dichloromethane (80 mL) and stirred at 25 °C for 24 h under an Ar atmosphere. After the reaction, the solution was filtered and dichloromethane was removed under reduced pressure. The obtained solid was dissolved in toluene and purified by repeated precipitation with dropwise addition to hexane. Finally, the obtained solid was dried under reduced pressure.

4.4. Synthesis of P(NIPAAm-co-NAS)-b-PEG-b-P(NIPAAm-co-NAS). DDMAT−PEG−DDMAT (0.186 mmol), NIPAAm (134 mmol), NAS (14.9 mmol), and V-65 (3.72 μmol) were dissolved in 1,4-dioxane (100 mL), and the resulting solution was allowed to react at 60 °C for 3 h under an Ar atmosphere. After this time, the produced polymers were recovered by repeated precipitation in an excess of diethyl ether. Subsequently, the obtained polymer and AIBN (60 times excess against DDMAT) were dissolved in ethanol and stirred at 30 °C for 24 h under an Ar atmosphere. Purification of the polymer was by repeated precipitation in diethyl ether.

4.5. Introduction of the Aldehyde Group into the Thermoresponsive Segments. P(NIPAAm-co-NAS)-b-PEG-b-P(NIPAAm-co-NAS), DEEA (10 times molar equivalent of NAS), and triethylamine (10 times molar equivalent of NAS) were dissolved in dichloromethane (100 mL) and the solution was stirred at 25 °C for 24 h. After this time, the solvent was removed by evaporation and replaced by acetone. The resulting solution was dialyzed against 0.5 mol/L sodium acetate buffer (pH 5) for 3 d, and then dialyzed continuously.
for 1 d against pure water. Thereafter, hydrochloric acid was added dropwise to the dialysis solution to adjust the pH to 2. After 5 or 24 h dialysis, the solution was dialyzed against pure water for 1 d. The obtained polymer solution was maintained at 4 °C in the absence of light prior to further use.

4.6. DLS Measurement. The triblock copolymer was dissolved in Dulbecco’s modified phosphate buffer saline (1 mg/mL). Before adding the albumin, 1 mL of polymer solution was maintained at 25 or 50 °C, followed by addition of 100 μL of albumin solution (1 mg/mL). For the temperature ramp experiment, the sample cuvette was held in the machine until the polymer solution achieved the target temperature. To lower the pH of the solution, hydrochloric acid was added to the polymer and albumin mixture until the pH became 6.

4.7. Hydrogel Formation. The pH of PAA (4 wt %) solutions was adjusted to 7.0 using sodium hydroxide. The triblock copolymer (2 wt %) and PAA (4 wt %) solutions were warmed in a hot incubator at 50 °C, and then equivalent volumes of the two solutions were mixed on a hot plate at 50 °C. The resulting mixture (200 μL) was used on the rheometer plate, measurement was performed for 10–30 min at 40 or 50 °C (above Tg). Next, the temperature was lowered to 25 °C using the AR-G2 rheometer software. For measuring pH stability, the hydrogel was immersed in pH 7.0 phosphate buffer. After holding the sample on the rheometer plate, measurement was performed for 10–30 min at 40 or 50 °C (above Tg). Next, the temperature was lowered to 25 °C using the AR-G2 rheometer software. For measuring pH stability, the hydrogel was immersed in pH 7.0 phosphate buffer for one day. In addition, the hydrogel was immersed in phosphate buffer solutions of different pH. After removing the solution, the hydrogel was applied on the rheometer plate and the time sweep measurement was performed at 25 °C. All the measurements were performed at a frequency of 1 Hz and a strain of 1%.

REFERENCES

1. Hoffman, A. S. Stimuli-responsive polymers: Biomedical applications and challenges for clinical translation. Adv. Drug Delivery Rev. 2013, 65, 10–16.
2. Liu, F.; Urban, M. W. Recent advances and challenges in designing stimuli-responsive polymers. Prog. Polym. Sci. 2010, 35, 3–23.
3. Schild, H. G. Poly(N-isopropylacrylamide): Experimental and application. Prog. Polym. Sci. 1992, 17, 163–249.
4. Kanazawa, H.; Sunamoto, T.; Matsushima, Y.; Ikikchi, A.; Okano, T. Temperature-Responsive Chromatographic Separation of Amino Acid Phenylthiohydantoin Using Aqueous Media as the Mobile Phase. Anal. Chem. 2000, 72, 5961–5966.
5. Stayton, P. S.; Shimoboji, T.; Long, C.; Chilkoti, A.; Ghen, G.; Harris, J. M.; Hoffman, A. S. Control of protein–ligand recognition using a stimuli-responsive polymer. Nature 1995, 378, 472.
6. Yamada, N.; Okano, T.; Sakai, H.; Karikusa, F.; Sawasaki, Y.; Sakurai, Y. Thermo-responsive polymeric surfaces; control of attachment and detachment of cultured cells. Makromol. Chem., Rapid Commun. 1990, 11, 571–576.
7. Winnik, F. M.; Ringsdorf, H.; Venzmer, J. Interactions of surfactants with hydrophobically-modified poly(N-isopropylacrylamides). J. Fluorescence probe studies. Langmuir 1991, 7, 905–911.
8. Cammas, S.; Suzuki, K.; Sone, C.; Sakurai, Y.; Katoaka, K.; Okano, T. Thermo-responsive polymer nanoparticles with a core-shell micelle structure as site-specific drug carriers. J. Controlled Release 1997, 48, 157–164.
9. Neradovic, D.; Van Nostrum, C. F.; Hennink, W. E. Thermoresponsive polymer micelles with controlled instability based on hydrodynamically sensitive N-isopropylacrylamide copolymers. Macromolecules 2001, 34, 7589–7591.
10. Akimoto, J.; Nakayama, M.; Okano, T. Temperature-responsive polymeric micelles for optimizing drug targeting to solid tumors. J. Controlled Release 2014, 193, 2–8.
11. Akimoto, J.; Nakayama, M.; Sakai, K.; Okano, T. Thermally controlled intracellular uptake system of polymeric micelles possessing poly(N-isopropylacrylamide)-based outer coronas. Mol. Pharmaceutics 2010, 7, 926–935.
12. Yatvin, M.; Weinstein, J.; Dennis, W.; Blumenthal, R. Design of liposomes for enhanced local release of drugs by hyperthermia. Science 1978, 202, 1290–1293.
13. Chung, J. E.; Yokoyama, M.; Yamato, M.; Aoyagi, T.; Sakurai, Y.; Okano, T. Thermo-responsive drug delivery from polymeric micelles constructed using block copolymers of poly(N-isopropylacrylamide) and poly(butylmethacrylate). J. Controlled Release 1999, 62, 115–127.
14. Kirkland, S. E.; Hensarling, R. M.; McConaughy, S. D.; Guo, Y.; Jarrett, W. L.; McCormick, C. L. Thermoresponsive hydrogels from RAFT-synthesized BAB triblock copolymers: steps toward biomimetic matrices for tissue regeneration. Biomacromolecules 2008, 9, 481–486.
15. He, Y.; Lodge, T. P. Thermoresversible ion gels with tunable melting temperatures from triblock and pentablock copolymers. Macromolecules 2008, 41, 167–174.
16. Loh, X. J.; Cheong, W. C. D.; Li, J.; Ito, Y. Novel poly(N-isopropylacrylamide)-poly[(R)-3-hydroxybutyrate]-poly(N-isopropylacrylamide) triblock copolymer surface as a culture substrate for human mesenchymal stem cells. Soft Matter 2009, 5, 2937–2946.
17. Ueki, T.; Nakamura, Y.; Usui, R.; Kitazawa, Y.; So, S.; Lodge, T. P.; Watanabe, M. Photoreversible gelation of a triblock copolymer in an ionic liquid. Angew. Chem., Int. Ed. 2015, 54, 3018–3022.
18. Taktak, F. F.; Bütin, V. Synthesis and physical gels of pH-and thermo-responsive tertiary amine methacrylate based ABA triblock copolymers and drug release studies. Polymer 2010, 51, 3618–3626.
19. He, C.; Kim, S. W.; Lee, D. S. In situ gelling stimuli-sensitive block copolymer hydrogels for drug delivery. J. Controlled Release 2008, 127, 189–207.
20. Tisitsilianis, C. Responsive reversible hydrogels from associative “smart” macromolecules. Soft Matter 2010, 6, 2372–2388.
(21) Yoshida, Y.; Kawahara, K.; Inamoto, K.; Mitsumune, S.; Ichikawa, S.; Kizuya, A.; Ohya, Y. Biodegradable injectable polymer systems exhibiting temperature-responsive irreversible sol-to-gel transition by covalent bond formation. ACS Biomater. Sci. Eng. 2016, 3, 56–67.
(22) Kataoka, K.; Kwon, G. S.; Yokoyama, M.; Okano, T.; Sakurai, Y. Block copolymer micelles as vehicles for drug delivery. J. Controlled Release 1993, 24, 119–132.
(23) Murakami, Y.; Yokoyama, M.; Okano, T.; Nishida, H.; Tomizawa, Y.; Endo, M.; Kurosawa, H. A novel synthetic tissue-adhesive hydrogel using a crosslinkable polymeric micelle. J. Biomed. Mater. Res. 2007, 80A, 421–427.
(24) Cordes, E. H.; Jencks, W. P. On the mechanism of Schiff base formation and hydrolysis. J. Am. Chem. Soc. 1962, 84, 832–837.
(25) Perrier, S.; Takolpuckdee, P.; Mars, C. A. Reversible addition–fragmentation chain transfer polymerization: end group modification for functionalized polymers and chain transfer agent recovery. Macromolecules 2005, 38, 2033–2036.
(26) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. Temperature-responsive bioconjugates. 2. Molecular design for temperature-modulated bioseparations. Bioconjugate Chem. 1993, 4, 341–346.