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Molecular design of dynamically thermoresponsive biomaterials

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Molecular design of dynamically thermoresponsive biomaterials

Dynamically thermoresponsive biomaterials, particularly those utilizing poly(*N*-isopropylacrylamide) (PNIPAAm), have attracted much attention in biomedical applications due to their reversible phase transition (32 °C) near body temperature. These biomaterials provide innovations across drug delivery system, chromatography, and tissue engineering. Molecular designs, such as the incorporation of hydrophilic comonomers or graft copolymers in PNIPAAm hydrogels, enhance rapid kinetics of the gels when jumping the temperature across the phase transition temperature, because of avoiding "skin layer" formation on the surface of the gels. Nanocarriers possessing PNIPAAm coronas facilitate spatial drug delivery and temporally on-demand drug release to targeted cancers in combination with hyperthermic therapy. Downsizing of PNIPAAm hydrogels accelerates the kinetics of shrinkage/swelling, leading to applications as thermoresponsive chromatographic matrices and cell cultureware. PNIPAAm-modified surfaces support thermoresponsive cell culture systems for the non-invasive recovery of intact cell sheets, enabling advanced regenerative therapies and layered 3D tissue formation. Recent developments also integrate growth factor delivery for sustained cell stimulation on culturewares. Newly developed biomaterials, including dynamically thermoresponsive PNIPAAm, are expected to expand the opportunity for novel treatment technologies such as targeted therapies and regenerative medicine.

Keywords: thermoresponsive polymer; poly(*N*-isopropylacrylamide); drug delivery system; chromatography; bioseparation; tissue engineering, cell sheet, regenerative medicine

Subject classification codes: Invited review (commissioned only)

1. Introduction

Thermoresponsive polymers in an aqueous milieu exhibit temperature-dependent changes in the physicochemical properties of the solutions [1-3]. For example, lower critical solution temperature (LCST)-type thermoresponsive polymers are soluble at low temperatures and insoluble at high temperatures [2]. Furthermore, this phase transition phenomenon is reversible. Among various thermoresponsive polymers,

aqueous solutions of poly(*N*-isoprolylacrylamide) (PNIPAAm) and their hydrogels show a sharp phase transition [4,5]. More importantly, the LCST of the PNIPAAm solution is 32 °C [6], which is between room temperature and mammalian body temperature (37 °C); therefore, a phase transition undergoes within a very mild range of temperature change. Due to these characteristics, PNIPAAm has been extensively utilized in thermoresponsive biomaterials and has been the subject of numerous studies [7-10]. In recent years, the utilization of other LCST-type thermoresponsive polymers, including poly(oligo ethylene glycol methacrylates) [11] and poly(2-oxazoline)s [12], have been proposed for thermoresponsive biomaterials as an alternative to PNIPAAm. The thermoresponsive behavior of a copolymer composed of 95% 2-(2-methoxyethoxy)ethyl methacrylate and 5% oligo ethylene glycol methacrylate (M_n = 475 g/mol) [13,14] was comparable to that of PNIPAAm and in some cases even better than that of PNIPAAm. A copolymer of 2-ethyl-2-oxazoline and 2-*n*-propyl-2-oxazoline [15] is also a potential alternative to PNIPAAm. However, to our knowledge, no specific reports have been published showing their superiority over PNIPAAm as biomaterials.

It is a critical point that thermoresponsive biomaterials can respond quickly to temperature changes. For example, chemically crosslinked PNIPAAm hydrogels undergo significant volume changes across the phase transition temperature around 32 °C in response to temperature changes [4]. However, when the temperature is increased rapidly across the phase transition temperature, a skin layer is formed on the surface of the bulk PNIPAAm hydrogel, leading to delayed kinetics of shrinkage [16]. To realize thermoresponsive biomaterials that can dynamically change on demand, molecular design and construction of PNIPAAm-based biomaterials are crucial.

Several strategies have been developed to accelerate the kinetics of PNIPAAm-based biomaterials in response to temperature change. Figure 1 illustrates a concept of dynamically thermoresponsive biomaterials for biomedical applications. Crosslinked PNIPAAm acts as a matrix of water-soluble drugs, allowing the controlled release rate of the drugs in response to the temperature change (Figure 1, middle). When heating above the phase transition temperature, a skin layer on the outermost surface of the gels prevents drug release (Figure 2A). The formation of a skin layer can be avoided by incorporating PNIPAAm graft chains having freely mobile ends [17] or hydrophilic moieties [18] into bulk PNIPAAm hydrogels (Figure 2B). This strategy has been utilized to release drugs rapidly in response to temperature changes. From the viewpoint of polymer physics, downsizing of PNIPAAm hydrogels accelerates the kinetics of shrinkage/swelling (Figure 1, right) because the time needed for a hydrogel phase transition is proportional to the square of the characteristic length of the hydrogel [19]. This strategy has been applied to thermoresponsive chromatographic matrices and cell cultureware for tissue engineering. Meanwhile, an amphiphilic block copolymer having hydrophobic and thermoresponsive PNIPAAm units assembles spontaneously nano-scale micelles in an aqueous milieu (Figure 1, left) [8]. During the self-assembly, the micelles entrap hydrophobic drugs such as anticancer drugs. Also, the PNIPAAm corona of polymeric micelle independently exhibits thermoresponsive dehydration when heating, leading to aggregation and collapse of the micelles.

This review focuses on epoch-making thermoresponsive biomaterials, especially using PNIPAAm, in terms of molecular design to dynamically and rapidly respond to temperature changes. Furthermore, dynamically thermoresponsive biomaterials are categorized into various types of biomedical applications and discussed. Knowledge of

the molecular design of PNIPAAm-based biomaterials in this review can be applied to other thermoresponsive polymers.

2. Molecular design of dynamically thermoresponsive biomaterials for controlled release

2.1. Thermoresponsive hydrogels for on-demand drug release

Hydrogel matrices allow for the sustained release of incorporated drugs from the matrices, and the rate of drug release is governed by diffusion, which is determined by the size of the polymer network in the hydrogel [20]. In contrast, thermoresponsive hydrogels, including crosslinked PNIPAAm hydrogel, exhibit their volume and mesh size changes in response to temperature [21], allowing the control of diffusion of incorporated solutes from the hydrogel. Therefore, the swelling/deswelling behaviors of thermoresponsive hydrogels have systematically been investigated for achieving on-off controlled release of water-soluble drugs by temperature changes. In addition, the applications of thermoresponsive hydrogels often have focused on temperature-controlled drug release because a heating/cooling procedure inside the human body is an accessible approach for temporally modulated or pulsatile drug delivery systems.

Crosslinked PNIPAAm and its copolymer hydrogels are hydrated and swollen below their phase transition temperatures. Once the environmental temperature is raised above their phase transition temperatures, the gels shrink via the dehydration of PNIPAAm. However, the outermost surface region of the gels exposed to warmer temperatures readily shrinks to form a polymer-aggregated surface layer, the “skin layer” (Figure 2A) [16]. Then, this dense skin layer inhibits the outflow of water from the gel interior, preventing the shrinkage of the gels. Based on the unique deswelling behavior, two modes of temperature-controlled drug release from the hydrogels have been designed: “surface regulation” and “bulk matrix squeezing” [22].

The “surface regulation” of PNIPAAm-based gels involves the active use of the skin layer as the barrier of molecular permeation. In particular, PNIPAAm copolymer gels with hydrophobic comonomers such as *n*-butyl methacrylate (BMA) allowed the diffusible release of loaded drugs below their phase transition temperatures, while the dense skin layer on the gels when the deswelling with suddenly increasing the temperature hindered the release of drug and water from the matrix [23,24]. Therefore, the repeated heating/cooling cycles across the phase transition temperatures successfully achieved the pulsatile drug release.

In contrast to the heat-triggered negative drug release mentioned above, heat-triggered positive drug release was achieved by the molecular design for thermoresponsive “bulk matrix squeezing” without forming dense skin layers. The randomly copolymerized gels of PNIPAAm and hydrophilic monomers such as acrylic acid (AAc) were investigated to demonstrate quick and reversible swelling/deswelling behaviors (Figure 2B) [18]. These copolymer gels exhibited a deswelling response without forming a skin layer when raising the temperature above the phase transition temperature, resulting in sustained gel shrinkage. This shrinkage was due to the formation of water channels through the hydrophilic moieties within the skin layer. However, the increased AAc content up to 10 mol% often induced a higher LCST shift and reduced gel shrinkage force, resulting in poor deswelling behavior [25]. This phenomenon may be due to disrupting the repeating *N*-isopropylacrylamide (NIPAAm) moieties by inserting AAc units along the PNIPAAm copolymer.

Hoffman et al. reported that PNIPAAm-g-poly(AAc) graft copolymers show sharp thermal phase transitions independent of total AAc contents, unlike random copolymers [26]. The distinctive phase transition behavior of the graft copolymers was ascribed to the molecularly separated architecture between PNIPAAm and poly(AAc)

segments. These results strongly suggest that multi-functional properties with keeping thermoresponsiveness can be achieved by using molecular design of thermoresponsive polymers such as graft copolymers comprising PNIPAAm and other functional polymers. Using the property of graft copolymer architecture, several approaches were developed for accelerating gel deswelling dynamics rather than molecular diffusion processes. Thermoresponsive comb-type grafted hydrogels, which possessed PNIPAAm-graft chains on a PNIPAAm network, showed rapid deswelling dynamics, even though the chemical composition and crosslinking density were the same as those of regular PNIPAAm gels [17]. Upon heating above the phase transition temperature, before the dehydration of the restrained PNIPAAm network, the freely mobile PNIPAAm-graft chains quickly dehydrated and acted as macromolecular hydrophobes. The hydrophobized graft chains triggered the dehydration of total gels and accelerated the release of water from inside the gels [27]. Another example of thermoresponsive comb-type grafted hydrogels involved hydrophilic poly(ethylene glycol) (PEG)-grafted chains [18]. PEG-grafted chains on the PNIPAAm gel network formed the channels for water penetration across the surface layer above the phase transition temperature, and thus, the gels quickly released internal water and underwent rapid shrinkage.

As an alternative approach for random copolymer gels with rapid shrinkable kinetics, Aoyagi et al. reported PNIPAAm copolymer gels with a functional comonomer, 2-carboxyisopropylacrylamide (CIPAAm) [24]. Poly(NIPAAm-*co*-CIPAAm) chains showed a similar sharp phase transition behavior with pure PNIPAAm, regardless of the additional carboxy groups [28]. This was due to the continuous isopropylamide moieties along the polymer backbone can be maintained after copolymerization. Consequently, the installed carboxy groups assisted the rapidly responsive gel shrinkage above the LCST, even at a high content of 10 mol% hydrophilic comonomers [29].

On the other hand, the introduction of macroscale heterogeneous structures inside thermoresponsive hydrogels was also used to accelerate swelling/deswelling dynamics. For example, macroporous structures inside crosslinked gels increased the surface area of contact between polymer and water, accelerating the diffusion process through polymer networks. Based on this strategy, some researchers have successfully achieved rapid volume changes of hydrogels under the swelling/deswelling process in response to temperature [30].

2.2. Thermoresponsive nanocarriers for on-demand release

In recent decades, nano-scale drug carrier systems, "nanocarriers," have attracted great interest in cancer chemotherapies because they can passively deliver the encapsulated drugs to solid tumors via the enhanced permeability and retention effect [31,32]. However, current nanocarriers face the dilemma of two contradictory functions: one is to encapsulate drugs in nanocarriers stably during the delivery process, and the other is to actively release drugs from nanocarriers at the target site. Therefore, nanocarriers possessing specific stimuli-responsive drug release functions are significantly attractive in terms of achieving higher therapeutic efficacy. In particular, mild heating up to 41–43 ºC hyperthermia has been used as a potential cancer treatment since cancer cells are sensitive to high temperatures [33]. Therefore, the strategy of temperature-triggered release from thermoresponsive nanocarriers can be combined with local hyperthermia for effective synergic cancer therapy.

2.2.1. Thermoresponsive liposomes

Nano-scale liposomes have been intensively explored as cancer-selective drug carriers, especially for tumor-selective drug targeting. In several decades, functional liposomes

were developed by introducing surface functionalization [34], stimuli-responsive functions [35], and polymer-liposome composite technologies [36,37].

In the late 1970s, Yatvin and Weinstein pioneered thermoresponsive liposomal drug carriers, which promoted the release rate of the encapsulated drugs based on the gel-to-liquid crystalline phase transition of phospholipid membranes [38]. However, dipalmitoylphosphatidylcholine-based liposomes have some problems, including small changes in membrane structure and low thermoresponsiveness. To overcome these problems, thermoresponsive lysophospholipid-based liposomal drugs were developed and utilized as the first heat-triggered formulation in human clinical trials [39].

Alternatively, LCST-type polymer-modified liposomes have also been investigated to enhance the release of encapsulated drugs [40,41]. The polymer chains were hydrated and extended below the LCSTs of the modified polymers, whereas the conformation of the dehydrated polymers changed to an aggregated form above the LCSTs. The mechanical distortion derived from the collapse of the modified polymers greatly disturbed the stabilization of liposomal membranes and induced an accelerated release rate of entrapped drugs. The first paper on the temperature-induced distortion of liposomal membrane via the collapse of the modified PNIPAAm chains was reported by Ringsdorf et al. [42]. At the same time, Hoffman et al. proposed a concept of PNIPAAm-modified liposomes as thermoresponsive drug carriers [43].

The modified thermoresponsive polymers' chemical structure and properties significantly influence liposome's thermoresponsive function. The anchoring position of the polymer chain on the liposomal membrane is a significant factor for dynamically thermoresponsive functions (Figure 3A) [40]. Liposomes modified with terminal anchor-possessing PNIPAAm showed a shaper release behavior of loaded drugs within a narrow range of temperature than those fixed via randomly located anchors on

PNIPAAm chains. This possible reason was that PNIPAAm chains anchored at the terminal positions induce their thermal coil-to-globule transitions effectively due to their higher conformational flexibility than those anchored at multiple points of the PNIPAAm chain. Nowadays, another type of thermoresponsive polymer is also available for binding to liposomal membranes to achieve the release of the loaded drugs through the conformational change of the polymer [44].

2.2.2. Thermoresponsive polymeric micelles

Multi-molecular assemblies of amphiphilic copolymers, polymeric micelles, possess high structural stability in aqueous milieu with nano-scale sizes (typically 10–100 nm) for effective drug targeting [45]. Okano and co-workers initially designed polymeric micelles with thermoresponsive PNIPAAm-based coronas (Figure 3B) [46]. Corona-forming PNIPAAm chains acted as a stabilizer of a segmented core-corona structure within the micelle due to the packing of the hydrated and expanded PNIPAAm chains below the LCST. Once the rising temperature was above the LCST, the outer corona switched to be hydrophobic, resulting in the collapse of the micellar corona. Thermoresponsive change in the micelles' structure and properties can trigger the release of loaded drugs from the hydrophobic core.

Initially, alkyl-terminated PNIPAAm derivatives were used to form thermoresponsive corona micelles [47]. The contribution of hydrophobic termini to the phase transition temperature of PNIPAAm was significantly notable. The phase transition temperature of hydrophobically terminated PIPAAm derivatives was lower than unmodified PIPAAm because the freely mobile hydrophobic tail promoted dehydration of the proximal NIPAAm units. Interestingly, a long alkyl (e.g., stearoyl) terminated PNIPAAm assembled to form a micelle structure above its critical micelle concentration, and the aqueous micelle solution showed the same LCST as unmodified

PNIPAAm, due to a core-corona structure shielding the hydrophobic alkyl core from interaction with water. Thermoresponsive block copolymer micelles possessing hydrophobic polymeric cores for effective drug loading were prepared using PNIPAAm-based block copolymers [48,49]. An interesting property based on the architectural effect of copolymers was that PNIPAAm-based block copolymer micelles exhibited a unique thermoresponsive behavior compared with randomly copolymerized PNIPAAm derivatives. For example, PNIPAAm-*b*-poly(BMA) (PNIPAAm-*b*-PBMA) block copolymer micelles exhibited an identical thermal phase transition to that of PNIPAAm homopolymer, regardless of hydrophobic PBMA co-introduction [50]. This thermal behavior was attributed to a phase-separated PNIPAAm corona and PBMA core structure. Consequently, the covalently connected hydrophobic PBMA segment does not influence the LCST of the corona-forming PNIPAAm, unlike random copolymers showing lower LCST shifts.

As for the temperature-controlled drug release from thermoresponsive corona polymeric micelles, the consideration of physicochemical properties (e.g., glassy transition temperature: T_g) of core-forming polymers is essential for the dramatically accelerated release of the loaded drugs via the phase transition of the micellar corona. Okano et al. investigated two different types of hydrophobic polymers as inner core modes using polystyrene (PS) and PBMA chosen as glassy-like (rigid: $T_g >$ PNIPAAm's LCST) and rubber-like (flexible: $T_g <$ PNIPAAm's LCST) blocks, respectively [48]. The PNIPAAm-*b*-PS micelles demonstrated limited drug release at temperatures both above and below the PNIPAAm's LCST of 32 °C. In contrast, the PNIPAAm-*b*-PBMA micelles remarkably released loaded drugs over the LCST of the PNIPAAm corona. Characterization of the inner cores of micelles using a hydrophobic fluorescent pyrene revealed that the polarity inside the rubber-like PBMA core significantly increased at

temperature above the LCST of the PNIPAAm corona, unlike that of the glassy-like PS core interior. Therefore, the thermally triggered drug release may be induced by dehydrated outer PNIPAAm coronas aggregation and subsequent structural change of the PBMA core. In addition, for an effective combination of thermoresponsive polymeric micelle carriers with hyperthermic therapy, the thermal transition temperature can be modulated to a desirable temperature of 40 °C (above the normal physiological temperature) by introducing hydrophilic comonomers to the corona-forming PNIPAAm chain [51].

On the other hand, the outermost surface functionalization of thermoresponsive corona micelles was available by mixing various end-functional PNIPAAm-based block copolymers before micelle formation [52,53]. The hydrophobicity and polarity of outermost surface functionalities significantly affected the thermoresponsive behavior of polymeric micelles [54]. Hydrophobic phenyl groups located at the micelle periphery enhanced the dehydration of corona-forming PNIPAAm derivatives. They resulted in a micellar LCST shift to lower temperatures than micelles with surface hydrophilic hydroxy groups [54]. The magnitude of LCST shifts for the polymeric micelles was much larger than linear polymers due to a closely packed thermoresponsive polymer brush structure in micellar corona: the accumulation of hydrophobic groups surrounding polymeric micelles increased the overall hydrophobicity of micelle systems. More Interestingly, thermoresponsive corona micelles formed by mixing phenyl- and hydroxy-terminated block copolymers exhibited only a single sharp phase transition at a temperature within the LCST values of the respective homogeneous surface micelles [55]. These phenomena were probably because respective PNIPAAm-based chains in a close-packed environment may be influenced by surrounding polymer chains and end-functional groups, resulting in cooperative thermal transitions. Uniquely, the thermal

phase transition of the block copolymer-mixed micelles can be readily modulated, dependent on the blended phenyl/hydroxy end-group ratio. These results strongly suggested that micellar LCST values can be controlled via modulating outermost surface chemistry with various stimuli (*e.g.*, pH, light, and specific compounds) to achieve multi-responsive systems [56]. Recently, various advanced polymeric micelles that undergo structural and physicochemical changes in response to stimuli have been explored to achieve both stable drug loading in the micelles during spatial delivery and on-demand release of loaded drugs at the target cancers [8].

3. Molecular design for dynamically thermoresponsive catch-and-release of biomolecules and cells

3.1. Thermoresponsive chromatography

Chromatography is a separation method that utilizes the interaction between a stationary phase and analytes. In 1996, Okano and Kanazawa developed a new concept of thermoresponsive chromatography that utilizes the properties of PNIPAAm [57] (Figure 4). The chromatography system uses the PNIPAAm-modified silica beads as stationary and aqueous mobile phases. Because PNIPAAm hydrophobicity changes with changing temperature, hydrophobic interactions between PNIPAAm and analytes can be modulated by changing the column temperature, leading to dynamic thermoresponsive modulation of analyte retention on the column.

The separation performance of thermoresponsive chromatography is determined mainly by hydrophobic interaction between PNIPAAm and analytes. Thus, the hydrophobicity of PNIPAAm increased with the incorporation of BMA into PNIPAAm by random copolymerization, and poly(NIPAAm-*co*-BMA)-modified silica beads were used as a more hydrophobic stationary phase [58]. Poly(NIPAAm-*co*-BMA)-modified

silica beads packed column can separate relatively hydrophilic analytes such as insulin fragments and amino acid phenylthiohydantoins [58,59].

To effectively separate ionic biomolecules, thermoresponsive ionic copolymers have been utilized to modulate electrostatic interactions with analytes. In 1992, Kim et al. reported that a thermoresponsive ionic copolymer exhibited a temperature-dependent ionic property change attributed to the change in the hydrophobicity of the polymer chains near the ionic functional groups [60]. Utilizing the unique property of thermoresponsive ionic copolymers, thermoresponsive ion-exchange chromatography was developed for effective ionic biomolecules. Poly(NIPAAm-*co*-AAc-*co*-*tert*-butyl acrylamide (tBAAm))-modified silica beads packed column separated basic molecules and peptides by temperature-modulated electrostatic interaction [61,62]. On the contrary, poly(NIPAAm-*co*-*N*,*N*-dimethylaminopropylacrylamide (DMAPAAm)-*co*-BMA) modified silica beads packed column separate adenosine nucleotides by temperature-modulated electrostatic interaction [63].

In addition, dynamically thermoresponsive biomaterials to modulate affinity interactions with specific biomolecules have a potential for non-invasive separating systems. Hoffman et al. pioneeringly reported that thermoresponsive expansion and shrinkage of PNIPAAm molecule bound near the biotin-binding site in recombinant streptavidin controls the binding between biotin and streptavidin [64]. They also clarified that the degree of shielding by thermoresponsive poly(*N*,*N*-diethylacrylamide) (PDEAAm) depends on both the size of the biotinylated protein and the size of the PDEAAm [65]. These papers revealed that the size of a thermoresponsive polymer and a target protein and the distance between a tethered thermoresponsive polymer and a ligand are dominant for regulating thermoresponsive affinity. However, the binding

process was irreversible because the affinity binding between streptavidin and biotin is typically strong (dissociation constant, $K_D = 4 \times 10^{-14}$ mol/L [66]).

For practical applications such as affinity chromatography, an effective method for the purification of proteins, thermoresponsive affinity control needs to be reversible: capturing a target protein and then releasing it on demand. Therefore, utilizing an optimal combination of ligand and protein, a new type of affinity chromatography was developed through thermoresponsive extension and shrinkage of PNIPAAm [67,68]. Both PNIPAAm and Cibacron Blue F3G-A as ligands for albumin were immobilized on polymethacrylate beads, and the prepared beads were used as affinity chromatography matrices [67]. At 37 °C, PNIPAAm on the beads shrank, and Cibacron Blue F3G-A was exposed, leading to the capture of albumin. By reducing the temperature to 20 °C, the shrunken PNIPAAm on the beads was extended, reducing the interaction between Cibacron Blue F3G-A and albumin and the subsequent elution of albumin from the column. Utilizing this property, the temperature-modulated separation of albumin from human serum was conducted.

Another approach to thermoresponsive affinity chromatography was developed using temperature-modulated competitive inhibition-induced shrinkage of PNIPAAm [68]. *Ricinus communis* agglutinin (RCA_{120}) and lactose were conjugated to PNIPAAm, and RCA_{120} and lactose-conjugated PNIPAAm were modified to agarose-based Sepharose beads. At low temperatures, the beads capture asialotransferrin through the affinity between asialotransferrin and RCA_{120}. By increasing the temperature, PNIPAAm shrank, and conjugated RCA_{120} and lactose were close, leading to the displacement of asialotransferrin with lactose from the immobilized RCA_{120}. Based on this property, asialotransferrin was separated by temperature modulation.

Thermoresponsive ionic copolymer brush-modified beads have been investigated as thermoresponsive protein adsorption materials. Atom transfer radical polymerization (ATRP) can graft PNIPAAm brushes onto silica beads, and the prepared beads exhibit strong hydrophobic interactions with analytes because of the large amount of PNIPAAm on the beads [69-71]. Thermoresponsive ionic copolymer brushes were prepared by introducing ionic monomers into thermoresponsive polymer brushes. The prepared thermoresponsive ionic copolymer brush exhibited temperature-modulated strong electrostatic and hydrophobic interactions with proteins, leading to the purification of proteins [72-77]. Poly(NIPAAm-*co*-DMAPAAm-*co*-tBAAm) brush-modified silica beads exhibited temperature-modulated adsorption of albumin [72]. At low temperatures, the hydrophobic interaction between the copolymer and albumin was weak because the copolymer was hydrated. In contrast, at 40 °C, the copolymer dehydrated and became hydrophobic, leading to the adsorption of albumin on the copolymer brush through combined electrostatic and hydrophobic interactions. Similarly, poly(IPAAm-*co*-2-acrylamido-2-methylpropanesulfonic acid (AMPS)-*co*-BMA) brush exhibited temperature-modulated adsorption and release of antibody drug rituximab [76].

Block copolymer brushes with bottom ionic segments exhibited effective temperature-modulated protein adsorption and desorption [78,79]. Poly(3-acrylamidopropyl trimethylammonium chloride-*b*-PNIPAAm brush-grafted silica beads exhibited temperature-modulated adsorption and desorption of milk serum proteins [78]. At high temperatures, the upper PNIPAAm segment shrunk, and proteins tended to access the basal cationic segment, leading to the adsorption of proteins through combined electrostatic and hydrophobic interactions. Poly(AMPS)-*b*-PNIPAAm

brushes also exhibit effective temperature-modulated adsorption of basic proteins, α-chymotrypsinogen A, and lysozyme [79].

3.2. Cell separation using thermoresponsive polymer

In tissue engineering and regenerative medicine, cell separation methods that maintain cell activity are in high demand. Thus, the PNIPAAm-modified interface has been investigated as a cell separation material that maintains cell activity (Figure 5).

The PNIPAAm brush-modified glass substrate exhibited temperature-modulated adhesion and detachment of vascular cells and myoblast at 37 °C and 20 °C, respectively [80]. During cell detachment from the PNIPAAm brush, endothelial cells promptly detached, whereas myoblasts gradually detached. By utilizing the difference in the detachment rate from the PNIPAAm brush, endothelial cells and myoblasts were separated.

As cells have intrinsic electrostatic properties, thermoresponsive ionic polymer brushes have been investigated to improve cell separation performance [81-84]. One example is mesenchymal stem cell (MSC) separation using a thermoresponsive cationic copolymer brush [81] (Figure 5A). MSC exhibits relatively strong anionic properties compared to other cells. Thus, MSC selectively adhered to the thermoresponsive cationic polymer poly(NIPAAm-*co*-DMAPAAm-*co*-tBAAm) brush-grafted glass substrate, while other types of cells did not. By reducing the temperature to 20 °C, the adhered MSCs on the copolymer brush were successfully recovered.

Thermoresponsive block copolymer brushes with affinity ligands have been developed for effective capture of target cells [85-87] (Figure 5B). A block copolymer brush with bottom poly(2-hydroxyethyl methacrylate (HEMA)-*co*-propargyl acrylate) and top poly(NIPAAm-*co*-HEMA) segments were prepared through two-step ATRP [85]. Then, the Arg-Glu-Asp-Val (REDV) peptide, which has an affinity for endothelial

cells, was introduced into the basal layer of the block copolymer brush. At 37 °C, the upper segment poly(NIPAAm-*co*-HEMA) shrunk, and the REDV peptide was exposed on the outer surface of the copolymer brush, leading to the capture of endothelial cells. By reducing the temperature to 20 °C, the upper PNIPAAm segment was extended, leading to shielding of the affinity between the REDV peptide and endothelial cells, and the endothelial cells were recovered from the copolymer brush.

A block copolymer brush with a poly(*N*-*p*-vinylbenzyl-*O*-*β*-D-galactopyranosyl-(1→4)-D-gluconamide) (PVLA) segment was utilized for the effective capture of hepatocytes [86]. PVLA-*b*-PNIPAAm was grafted onto a glass substrate through two steps of ATRP. At 37 °C, the upper PNIPAAm shrank and the galactose moiety of the bottom PVLA was exposed to the outer surface of the copolymer brush, leading to selective adhesion of hepatocytes on the copolymer brush through the affinity between galactose and asialoglycoprotein receptor (ASGPR) of hepatocytes. By reducing the temperature to 20 °C, the PNIPAAm segment was extended, leading to a decrease in the affinity between PVLA and hepatocytes, and the hepatocytes were recovered from the copolymer brush.

To increase the throughput of cell separation, thermoresponsive polymer-modified microfibers have been investigated because they have larger surface areas than thermoresponsive polymer-modified glass substrates [88,89] (Figure 5C). At 37 °C, adipose tissue-derived MSC selectively adhered to dehydrated PNIPAAm brushes on the microfibers, whereas adipocytes and endothelial cells did not. By reducing the temperature to 20 °C, the PNIPAAm became hydrophilic, leading to the detachment of adhered MSC from the microfibers. Thus, PNIPAAm brush-modified microfibers can separate MSC from contaminant cells by simply changing the temperature.

PIPAAm-modified bead-packed columns have been investigated as effective cell-separation systems [90-93]. Poly(DMAPAAm)-*b*-PNIPAAm (PDMAPAAm-*b*-PNIPAAm) brush-modified silica beads packed column was developed as a thermoresponsive MSC separation column [92] (Figure 5D). At 37 °C, the upper PNIPAAm segment shrunk, and the cationic property of basal PDMAPAAm was expressed, leading to the adsorption of MSC on the copolymer. In contrast, the contaminant cells did not adsorb onto the copolymer but passed through the packed beads. By reducing the temperature to 20 °C, the PNIPAAm segment was extended, and the adsorbed MSCs were detached from the copolymer. Thus, the copolymer brush modified beads packed column effectively separated MSC from contaminant cells simply by changing temperature.

3.3. Thermoresponsive cell culture surfaces

Cells adhere and grow via cell-binding proteins such as fibronectin that are non-specifically adsorbed to the surface of culture substrates. These processes are typically passive and irreversible. In 1990, Okano et al. invented and reported that the introduction of PNIPAAm molecules onto the surface of the culture substrate, such as a tissue culture polystyrene (TCPS) dish, facilitated dynamic control of the interaction with the adsorbed proteins and cells [94]. Tethered PNIPAAm to the surface of TCPS exhibits reversible hydration/dehydration changes across the phase transition temperature of 32 °C. Eventually, the PNIPAAm-grafted TCPS at 37 °C showed hydrophobic, allowing cells to attach to the surface via adsorbed serum proteins in the culture medium [95,96]. Cells cultured on the hydrophobized PNIPAAm-grafted TCPS grew [97] and reached confluence as well as on TCPS.

A noteworthy feature of the PNIPAAm-grafted TCPS dish is that the interaction between the substrate and the cultured cells can be dynamically regulated only by

reducing temperature. When the temperature was lowered below the LCST of PNIPAAm (e.g., 20 °C), the grafted PNIPAAm became hydrated [94-96], and the confluent cells spontaneously started to detach themselves. Finally, the contracted "cell sheet" was liberated from the surface [98]. In other words, the hydration of PNIPAAm on the surface triggered a weakening of the interaction with the proteins on the bottom of the cultured cells, and the cells were detached as a contiguous sheet by the traction forces of the sheet derived from organized cytoskeleton within spread cells [99-101].

In addition, the sheet was non-invasively detached without proteolytic treatments, such as using trypsin-EDTA. Thus, the extracellular matrix (ECM) was held on the bottom of the sheet [98]. The ECM acts as a glue to adhere to another culture dish or biological tissue. The detached cell sheet is flexible and can fit to the curvature of surfaces. This feature is advantageous when attaching it to curved organs such as eyeballs or hearts, enabling efficient transplantation of cell sheet tissues. To manipulate soft cell sheets, they can be grabbed using a hydrophilic membrane through the surface tension of the medium between the sheet and membrane [10].

Electron beam (EB) polymerization was used to covalently bond crosslinked PNIPAAm to polystyrene surface [94,97]. A uniform NIPAAm monomer solution was spread on the surface of a commercially available TCPS, and then the surface was irradiated with an EB. EB-induced NIPAAm polymerization started from the main polystyrene chain, and crosslinking between PNIPAAm co-occurred. The crosslinked PNIPAAm layer was grafted onto the surface. The most critical factor in allowing cell sheet recovery by temperature change is the controlled thickness of the grafted PNIPAAm. When a grafted PNIPAAm layer of about 30 nm or more was grafted onto TCPS, cells did not adhere or spread even though the temperature was 37 °C above the phase transition temperature [96]. In other words, when the thickness was about 20 nm,

cell adhesion and detachment could be switched by temperature change. Recently, a thermoresponsive culture dish can be easily fabricated by the physisorption of a block copolymer composed of hydrophobic PBMA and thermoresponsive PNIPAAm using the spin-coating method [102,103]. The coated block copolymer layer on the culture dish showed high water stability owing to the hydrophobic PBMA anchor, regardless of temperature.

In recent years, various types of stimuli-responsive cell culture surfaces that respond to physical and chemical stimuli, such as light [104], electricity [105-107], and pH changes [108,109], have been reported for the fabrication of cell sheets [110]. However, light stimulation requires a light source, and high-energy radiation such as UV may damage the DNA of cells. Guillaume-Gentil et al. reported that a cell sheet on RGD peptide-conjugated polyelectrolyte-coated electrically conductive glass was detached by applying a positive potential to the glass substrate [111]. Fukuda et al. reported that cells were cultured on self-assembled monolayers of RGD peptide-conjugated alkanethiols. They reductively desorbed from the gold substrate by applying negative potential, allowing the cultured cell sheets to rapidly detach from the gold surface [105-107]. However, the detachment of cells occurs irreversibly, and voltage is required for electrical stimulation by a device. On the other hand, a useful feature of thermal control is that cell sheets can be collected using commodity equipment such as a temperature-controlled incubator. Another advantage is that a wealth of knowledge has been accumulated regarding recovering cell sheets using thermoresponsive culture dishes.

Noting that biological tissues have a similar structure to laminated sheets, we proposed “cell sheet engineering,” a method of reconstructing three-dimensional (3D) tissue structures by laminating cell sheets [10,112]. Specifically, this method involves

attaching a detached cell sheet to another cell sheet and repeatedly laminating them to construct 3D tissue. To date, the first clinical applications in humans using single-layer or laminated autologous cell sheet transplants were conducted to regenerate corneal epithelium, myocardium, esophagus, periodontal ligament, and cartilage. Recent clinical applications of cell sheet transplantation were summarized in another paper [113].

Furthermore, we designed next-generation thermoresponsive culture surfaces that mimic the extracellular matrix, such as cell adhesion and affinity binding with growth factors. For example, a PNIPAAm-grafted surface modified with heparin can immobilize various heparin-binding proteins, such as basic fibroblast growth factor (bFGF) [114,115] and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [116,117], through affinity interaction. The immobilized heparin molecules and heparin-binding proteins formed complexes that maintain their activity; eventually, the heparin-modified PNIPAAm-grafted culture surface continuously stimulated cultured cells. The surface bound with bFGF accelerated fibroblasts' proliferation and sheet formation [114]. Meanwhile, the surface bound with HB-EGF inhibited the dedifferentiation of hepatocytes and maintained liver-specific functions [116,117]. More importantly, the swelling/shrinkage changes of PNIPAAm chains caused by temperature can be used to dynamically control the affinity interaction between heparin and heparin-binding proteins through multipoint binding. When the temperature was lowered to 20 °C, below the LCST of PNIPAAm, the affinity interaction between heparin and heparin-binding proteins weakened as the PNIPAAm chains swelled and extended, and heparin-binding growth factors were recovered along with the detached cultured cells [114,116]. Thus, the heparin-modified PNIPAAm-grafted culture surface can simultaneously achieve sustained stimulation of cell receptors by heparin-binding proteins and cell sheet recovery by lowering the temperature.

Dynamically, thermoresponsive hydrogels can fabricate 3D structures to form cell tissues of various shapes. Healy et al. proposed that shrunken PNIPAAm hydrogels at body temperature (37 °C) were utilized for efficient cell transplantation, while loosely crosslinked PNIPAAm hydrogels were fluidic as an injectable support at room temperature [118]. Many types of thermoresponsive injectable materials have recently been proposed to apply stem cell transplantation therapies [119]. Matsumoto et al. proposed using a PNIPAAm hydrogel template with various shapes to fabricate large-size aggregates of bone marrow-derived MSCs [120]. The aggregates were detached by lowering the temperature due to the volume change of the PNIPAAm hydrogel template. At the same time, the hydrophilicity of the gel surface was maintained over a wide temperature range. Shin et al. reported the preparation of thermoresponsive Tetronic®-tyramine hydrogel immobilized with cell-adhesive fibronectin via polydopamine coating for imparting cell adhesiveness [121]. A cell sheet cultured on the gel was mechanically detached due to the contraction of the gel on lowering the temperature, allowing the cell sheet to be readily manipulated and transplanted [122,123]. Together, dynamically thermoresponsive biomaterials enable various tissue engineering applications, including 3D organization and easy manipulation, expanding the feasibility of tissue engineering for the application of regenerative therapy.

4. Conclusions

Dynamically thermoresponsive biomaterials have opened up the possibility of various biomedical applications. Furthermore, it is expected that they will be combined with multiple modalities, such as medical devices, diagnostic devices, and regenerative therapies. Thermoresponsive nanocarriers are expected to achieve more precise and location-specific drug delivery in combination with hyperthermia or high-intensity focused ultrasound. In addition, separation technology using thermoresponsive

chromatography facilitates the accurate separation of biomolecule-based drugs such as antibodies, cell sources for cell therapy, and tissue engineering. Combining cell sheet engineering with automated robotics for cell sheet manipulation will be promising for the large-scale production of clinical-grade tissues [124,125]. In the future, newly developed biomaterials, including dynamically thermoresponsive PNIPAAm, are highly expected to expand the opportunity for novel treatment technologies.

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Disclosure statement

The authors declare no conflicts of interest associated with this manuscript.

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J.K. designed the framework of this review article. M.N. wrote the manuscript on thermoresponsive biomaterials for controlled release. K.N. wrote the manuscript on thermoresponsive chromatography and cell separation. J.K. wrote the other parts. J.K.,

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Figure captions

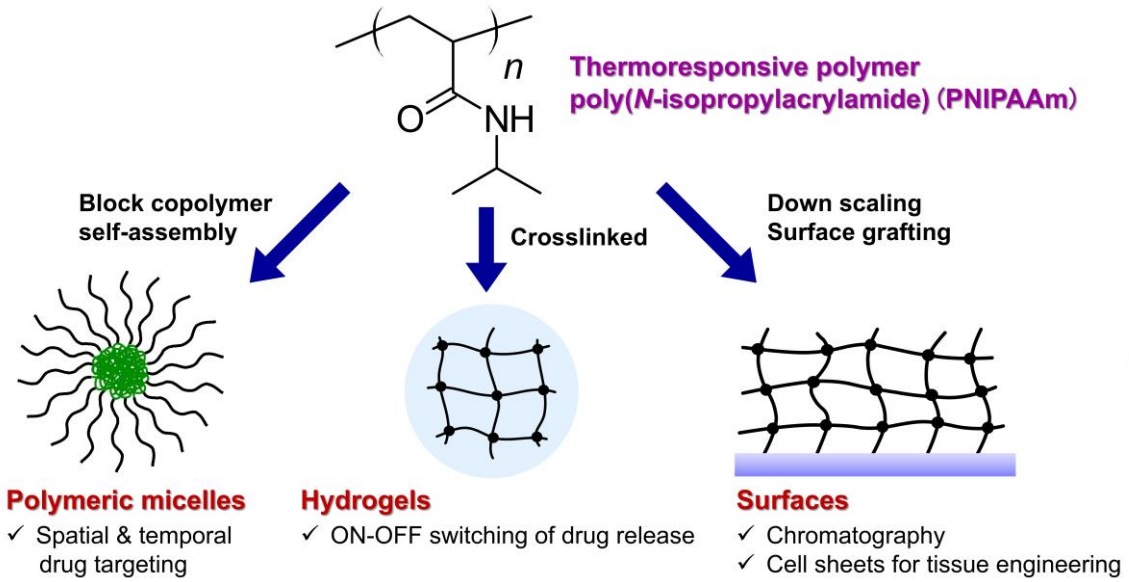


Figure 1 Scheme of molecular design of thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAAm) for biomedical applications.

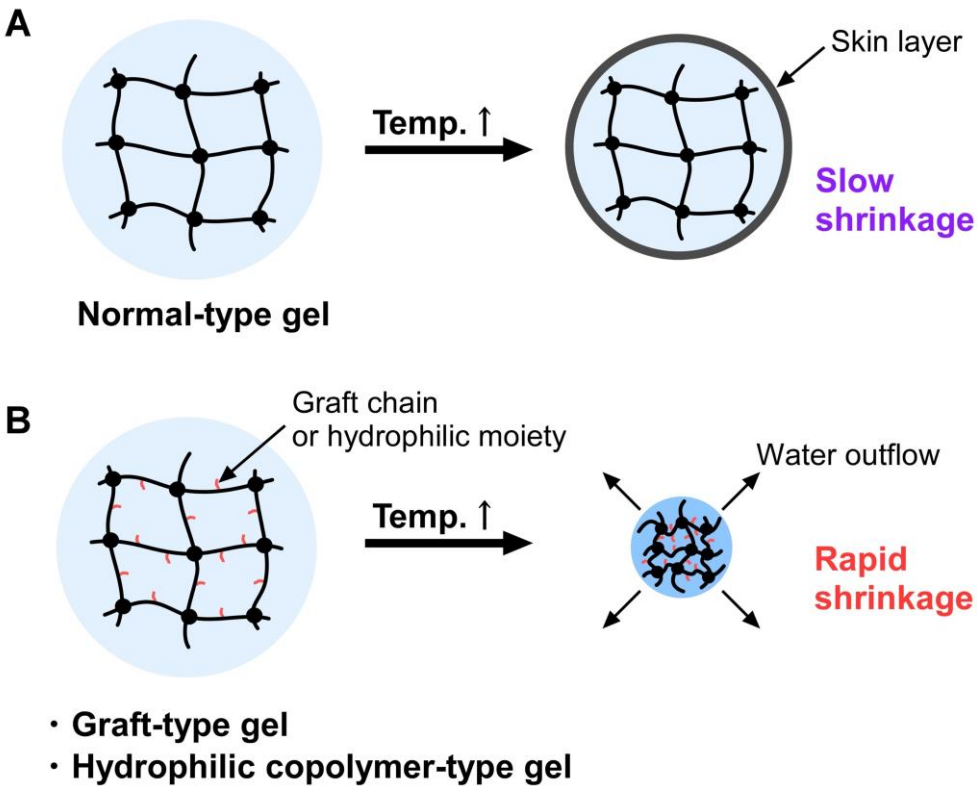


Figure 2 Schematic illustration of deswelling process for (A) normal-type PNIPAAm gel and (B) its graft-type or hydrophilic copolymer-type gel.

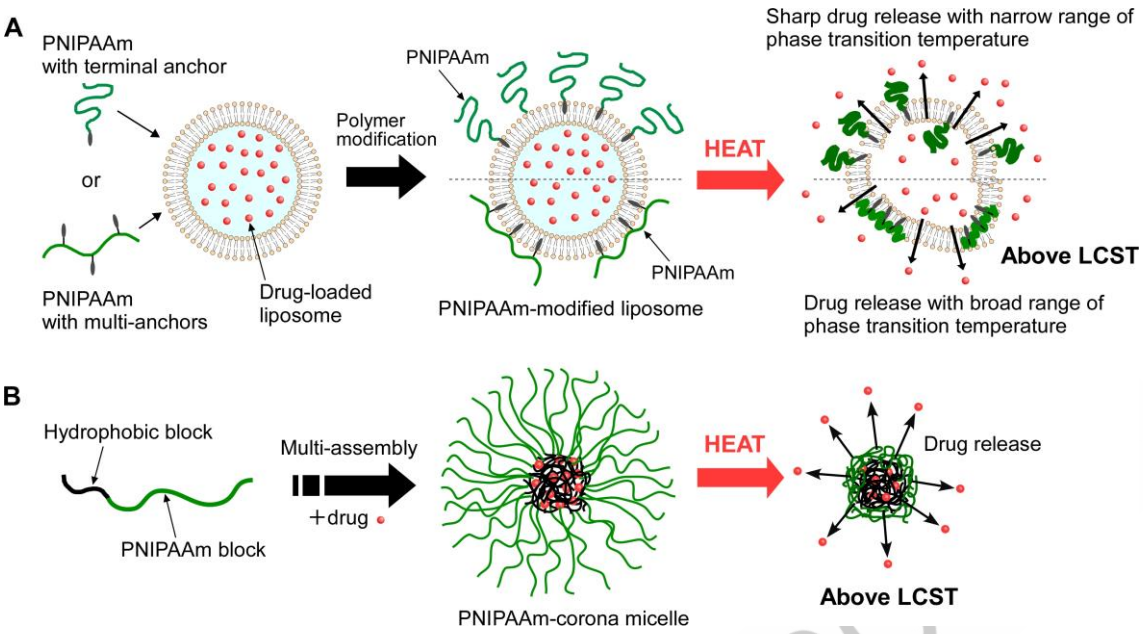


Figure 3 Schematic illustration of thermoresponsive nanocarriers with PNIPAAm segments. (A) PNIPAAm-modified liposome and (B) PNIPAAm-based block copolymer micelles.

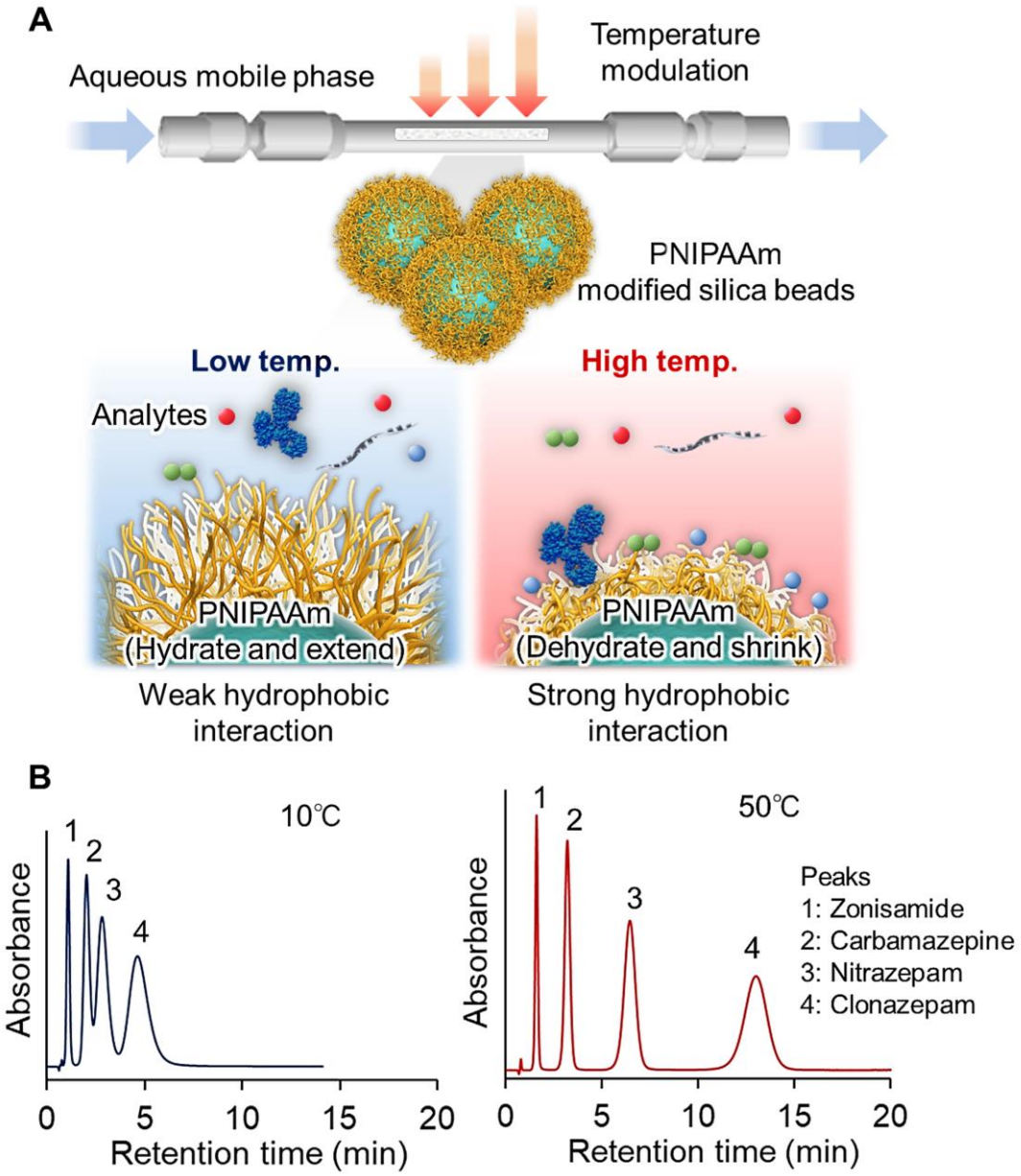


Figure 4 Schematic illustration of thermoresponsive chromatography. (A) Concept of thermoresponsive chromatography and (B) chromatograms at 10 °C and 50 °C.

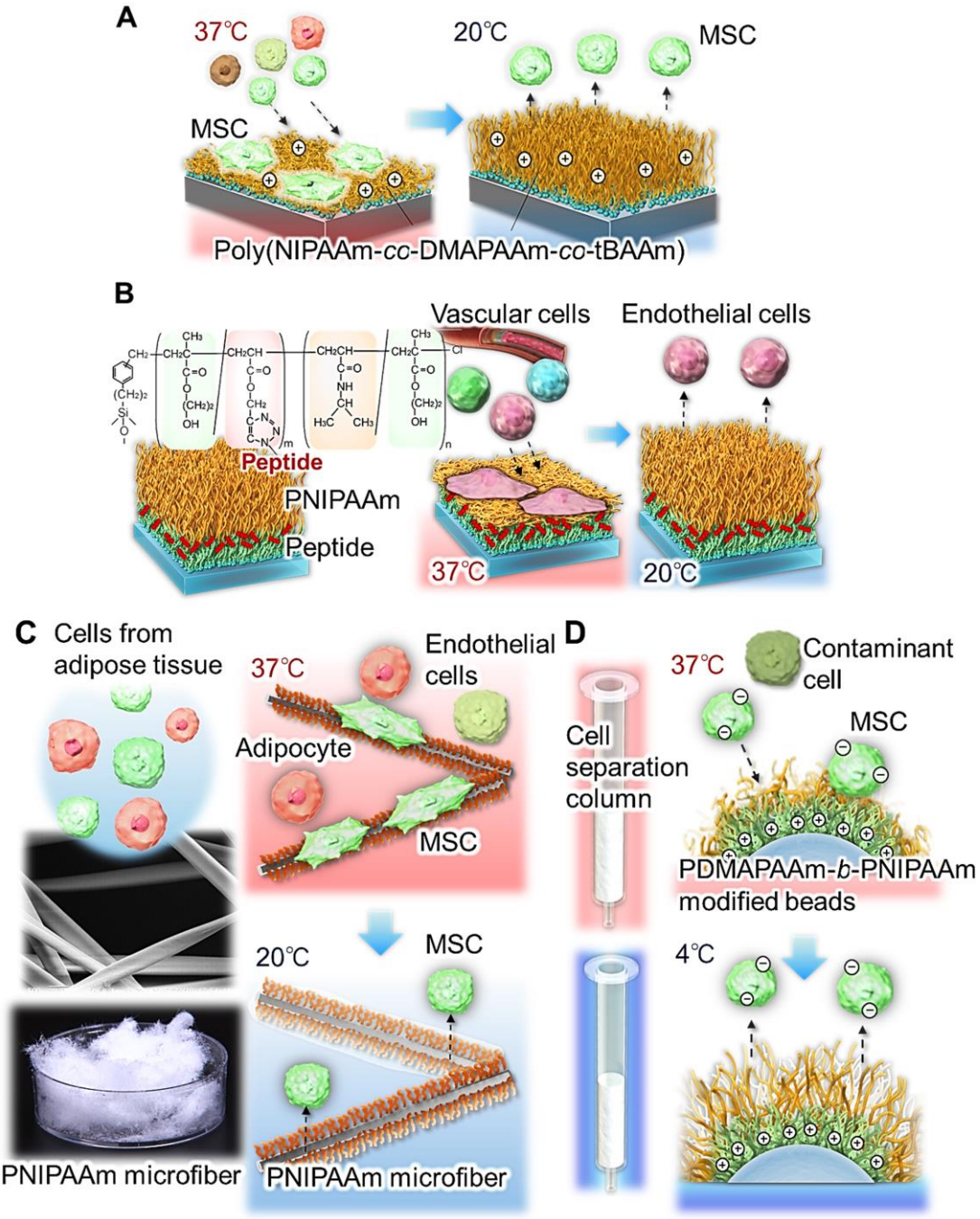


Figure 5 Schematic illustration of thermoresponsive cell separation materials. (A) Thermoresponsive cationic copolymer brush, (B) thermoresponsive polymer brush with affinity peptide, (C) thermoresponsive microfiber, and (D) thermoresponsive cell separation column.

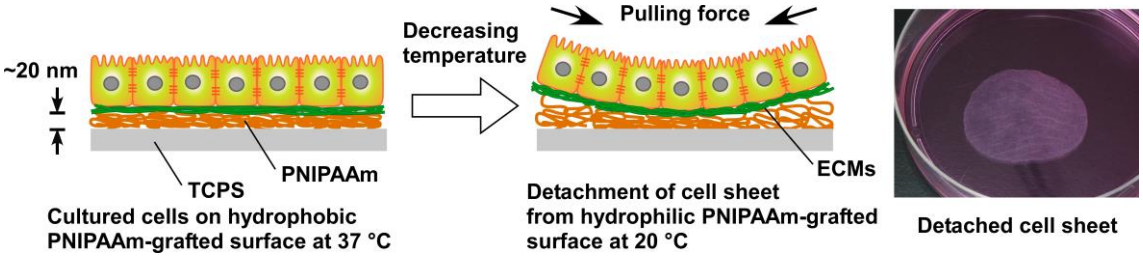
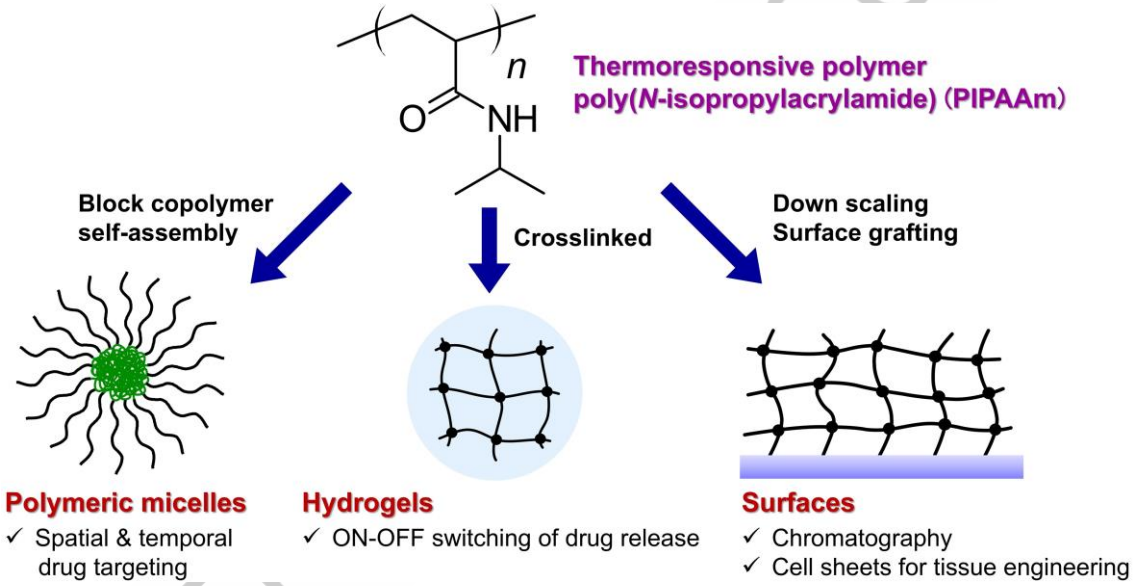


Figure 6 Cell sheet preparation using a temperature-responsive cell culture surface. PNIPAAm-grafted surface exhibits hydrophobicity and is cell adhesive at 37 °C, and changes to hydrophilic and cell non-adhesive at 20 °C. The PNIPAAm-grafted surface enables the cultured cells to detach themselves as a contiguous sheet only upon reducing temperature. The detached cell sheet holds ECM beneath the sheet. Reprinted from Kobayashi, J. (2019) [10] with permission from Wiley.



Graphical Abstract

Statement of Novelty: This paper focuses on designing molecular architectures, such as polymer grafting structures, monomer design, and the size effects of molecules, to achieve dynamically thermoresponsive biomaterials.

Biographical note

Dr. Jun Kobayashi obtained his B.S. and M.S. degrees at Waseda University, Tokyo, Japan. He received his Ph.D. degree in Chemical Engineering from Waseda University in 2003. In 2003, he carried out his postdoctoral research at the Institute of Advanced Biomedical Engineering and Science at Tokyo Women's Medical University (TWMU), Tokyo, Japan. Since 2004, he joined TWMU as Research Assistant Professor, and as Assistant Professor in 2011. Present research topics are intelligent thermoresponsive surfaces for tissue engineering, especially in hepatic tissue engineering.

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