Novel temperature-responsive polymer brushes with carbohydrate residues facilitate selective adhesion and collection of hepatocytes

To cite this article: Naokazu Idota et al 2012 Sci. Technol. Adv. Mater. 13 064206

View the article online for updates and enhancements.

Related content
- Cell membrane-inspired phospholipid polymers for developing medical devices with excellent biointerfaces
  Yasuhiro Iwasaki and Kazuhiro Ishihara
- Fabrication of a thermoresponsive cell culture dish: a key technology for cell sheet tissue engineering
  Jun Kobayashi and Teruo Okano
- Temperature-responsive electrospun nanofibers for ‘on–off’ switchable release of dextran
  Young-Jin Kim, Mitsuhiro Ebara and Takao Aoyagi

Recent citations
- Bio-inspired smart hydrogel with temperature-dependent properties and enhanced cell attachment
  Young-Jin Kim et al
- Synthesis of Thermoresponsive Glycopolymers Combining RAFT Polymerization, Thiol–Ene Reaction, and Subsequent Immobilization onto Solid Supports
  Christian von der Ehe et al
- Temperature-Responsive Poly(-caprolactone) Cell Culture Platform with Dynamically Tunable Nano-Roughness and Elasticity for Control of Myoblast Morphology
  Koichiro Ugo et al
Novel temperature-responsive polymer brushes with carbohydrate residues facilitate selective adhesion and collection of hepatocytes

Naokazu Idota¹, Mitsuhiro Ebura¹, Yohei Kotsuchibashi², Ravin Narain² and Takao Aoyagi¹

¹ Biomaterials Unit, International Center for Materials Nanoarchitectonics (WPI-MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
² Department of Chemical and Materials Engineering and Alberta Ingenuity Center for Carbohydrate Science, University of Alberta, Edmonton, AB T6G2G6, Canada
E-mail: AOYAGI.Takao@nims.go.jp

Received 2 July 2012
Accepted for publication 17 September 2012
Published 23 November 2012
Online at stacks.iop.org/STAM/13/064206

Abstract
Temperature-responsive glycopolymer brushes were designed to investigate the effects of grafting architectures of the copolymers on the selective adhesion and collection of hepatocytes. Homo, random and block sequences of N-isopropylacrylamide and 2-lactobionamidoethyl methacrylate were grafted on glass substrates via surface-initiated atom transfer radical polymerization. The galactose/lactose-specific lectin RCA₁₂₀ and HepG₂ cells were used to test for specific recognition of the polymer brushes containing galactose residues over the lower critical solution temperatures (LCSTs). RCA₁₂₀ showed a specific binding to the brush surfaces at 37°C. These brush surfaces also facilitated the adhesion of HepG₂ cells at 37°C under nonserum conditions, whereas no adhesion was observed for NIH-3T3 fibroblasts. When the temperature was decreased to 25°C, almost all the HepG₂ cells detached from the block copolymer brush, whereas the random copolymer brush did not release the cells. The difference in releasing kinetics of cells from the surfaces with different grafting architectures can be explained by the correlated effects of significant changes in LCST, mobility, hydrophilicity and mechanical properties of the grafted polymer chains. These findings are important for designing ‘on–off’ cell capture/release substrates for various biomedical applications such as selective cell separation.

Keywords: poly(N-isopropylacrylamide), 2-lactobionamidoethyl methacrylate, atom transfer radical polymerization, hepatocytes, selective cell adhesion, cell detachment

1. Introduction
‘Smart’ polymers responding to external stimuli have been actively studied to develop ‘on–off’ switchable materials for applications in biomedical [1, 2], environmental [3, 4] and analytical chemistry fields [5, 6]. Thermoresponsive poly(N-isopropylacrylamide) (PNIPAAm) is one of the most reliable smart polymers that show discontinuous changes in water solubility in the physiological temperature range [7].

1468-6996/12/064206+09$33.00 © 2012 National Institute for Materials Science Printed in the UK
method can avoid the use of deleterious enzymes, cells are harvested as a single contiguous sheet after they become confluent [12]. These transplantable cell sheets retain cell–cell junctional proteins as well as an intact extracellular matrix underneath the cell sheets, and therefore, they have been stratified in vitro [13] and directly transplanted in vivo [14]. PNIPAAm can also be functionalized by chemical conjugation to biomolecules such as targeting ligands to yield a large and diverse family of polymer-biomolecule hybrid systems [15]. We have developed temperature-responsive polymer-grafted surfaces modified with a series of cell adhesive peptides (Arg-Gly-Asp; RGDs), which is a minimal cell-binding domain in the extracellular matrix (ECM). The RGD-immobilized temperature-responsive surfaces bound cells at 37 °C and spontaneously detached them at 20 °C [16].

The time required for the cell detachment is strongly related to the cell adhesion strength according to the trend: RGD < RGDS < GRGD < GRGDS. These phenomena arise from the specific design of the grafted polymer, which protects peptides from integrin access below the LCST. More precise designs of the grafting architectures have also enabled the investigation of the effect of extensible nanotethers [17] and the synergistic effect of PHSRN sequences [18] on the integrin-mediated cell binding.

Because adhesive interactions between cells and the ECM are governed primarily by integrins, the RGD motif has been shown to enhance the adhesion of various cell types. Although the versatility of the RGD peptide is attractive for surface modification, the use of highly specific sequences with strong affinities toward particular types of cell has gained much attention in the area of cell isolation [19]. An important risk in the clinical application of induced pluripotent stem (iPS) cells, for example, is residual undifferentiated cells that can induce spontaneous tumorigenesis in vivo [20]. To meet safety criteria, the selective separation of targeted cells from residual non-targeted cells is of crucial importance. From these perspectives, we focus on the carbohydrate moiety of glycoproteins, which is one of the most abundant and important biomolecules. The carbohydrates are not only a major source of metabolic energy but are signal biomolecules in a wide range of molecular recognition phenomena, including fertilization, immunological protection, nervous system reactions and viral infection [21]. Many types of glycopolymer with pendent carbohydrate residues have been studied to understand their molecular interactions, mimic biorecognition properties [22, 23], and develop various biomedical applications, such as drug/gene delivery systems for targeting carbohydrate receptors [24], glucose-sensitive biosensors [25] and cell culture substrates using recognition specificity [26–28]. Hepatocyte-specific poly(N-p-vinylbenzyl-D-lactonamide) (PVLA)-coated surfaces, for example, can control an affinity molecular recognition between the asialoglycoprotein receptors (ASGPRs) of hepatocytes and galactose moieties in the polymers [26, 27]. In this system, hepatocytes adhere on cell culture surfaces to form spheroidal shapes and multilayer aggregates in the presence of growth factor, and maintain their differentiated phenotype by regulating liver-specific transcriptions.

Here, we report the construction and characterization of a temperature-responsive ‘on–off’ surface capture and release system for hepatocytes using smart copolymer brushes comprising PNIPAAm and 2-lactobionamidoethyl methacrylate (LAMA), which is known as a sugar-based monomer [29]. Well-defined grafting architectures including a homo, random or hierarchical sequence were achieved by surface-initiated atom transfer radical polymerization (ATRP). The copolymers have been studied in detail by proton nuclear magnetic resonance (^1H-NMR) analysis, gel permeation chromatography (GPC), and turbidity measurements. First, we explored the specific recognition of brush surfaces by the RCA120 lectin above the LCST. The reversible binding was also studied below the LCST. Additionally, HepG2 and NIH-3T3 cells were cultured on the brushes in the absence of serum at 37 °C. The temperature was then decreased to 25 °C to release the adhered cells from the surface (figure 1).

2. Experimental methods

2.1. Materials

N-Isopropylacrylamide (NIPAAm) was kindly provided by Kohjin (Tokyo) and purified by recrystallization from
n-hexane. LAMA was synthesized and purified according to the previously reported protocol [29]. CuCl was obtained from Aldrich (St Louis, Missouri), and washed with acetic acid to remove any soluble oxidized species. CuCl$_2$ and 2,2′-bipyridyl (bpy) were also purchased from Aldrich. Ethylbenzyl chloride (EBC) was obtained from Kanto Chemical Co. Inc. (Tokyo). Tris(aminomethyl)amine (TREN) was purchased from Sigma Chemical Co. (St Louis, Missouri). Tris(aminoethyl)amine (TREN) was synthesized from TREN as described in reference [30]. (Chloromethylphenyl)ethyltrichlorosilane was obtained from ShinEtsu Chemical Industry (Tokyo). Cover glasses (26 × 76 mm, average thickness 0.8–1.0 mm) were obtained from Matsunami Glass Industry (Tokyo). RCA (26 × 76 mm, average thickness 0.8–1.0 mm) were obtained from ShinEtsu Chemical Industry (Tokyo). Cover glasses were washed with 35% HCl, followed by repeated rinsing with distilled water and drying in vacuo. The clean substrates were immediately put on the bottom of a separable flask with (chloromethylphenyl)ethyltrichlorosilane, an ATRP initiator activated silane, and silanization was carried out for 2 h at 90 °C. After the silanization, the glass substrates were baked at 110 °C for 1 h. P(NIPAAm-co-LAMA) brushes with various feed ratios (molar ratio of NIPAAm/LAMA = 99/1, 97/3, 95/5 and 93/7) were synthesized in mixtures of methanol and water (3/2, v/v) by surface-initiated ATRP. Typically, NIPAAm (1.976 g, 17.5 mmol) and LAMA (0.253 g, 0.5 mmol) were dissolved in 45 ml of the solvent, and the resultant solution was then degassed by freeze-pump-thaw cycles. After being degassed, the monomer solution was poured into another flask containing CuCl$_2$ (8.0 mg, 81 µmol), CuCl$_2$ (1.2 mg, 9 µmol), and Me$_6$TREN (25 µl, 90 µmol) under Ar gas flow. The initiator-immobilized glasses and EBC (13 µl, 90 µmol), which was used as a free ATRP initiator, were added to the solution after stirring for 30 min, and the polymerization proceeded at 20 °C for 24 h. After the polymerization was stopped by exposing the solution to air, the substrates were rinsed with methanol and distilled water, and dried at 25 °C under vacuum. To estimate molecular weights and compositions of polymer chains, the reaction solution was passed through a neutral alumina column to remove the Cu complex, and the polymer was recovered by reprecipitation from diethyl ether.

2.3. Preparation of PNIPAAm-b-PLAMA brushes by surface-initiated ATRP

PNIPAAm-b-PLAMA brushes, where PLAMA stands for poly(LAMA), were prepared by ATRP of LAMA via surfaces pregrafted with PNIPAAm (figure 2(b)) [31]. PNIPAAm monomer (10.184 g, 90 mmol) was dissolved in 45 ml of a mixed solvent (acetonitrile/water = 4/1, v/v), and the solution was degassed by three freeze-thaw cycles. The degassed monomer solution was poured under Ar atmosphere into another flask containing CuCl$_2$ (26.7 mg, 270 µmol), CuCl$_2$ (4.0 mg, 30 µmol), and Me$_6$TREN (85 µl, 300 µmol). The silanized cover glasses and EBC (44 µl, 300 µmol) were added to the solution after stirring for 30 min, and ATRP proceeded at 0 °C for 5 h. CuCl$_2$ and bpy were added to the reaction solution under N$_2$ flow to quench the ATRP, and the polymerization was stopped by exposing the solution to air. The PNIPAAm brush glasses were then repeatedly washed with acetonitrile and water and dried at 25 °C under vacuum. Then, ATRP of LAMA was reinitiated on the quenched PNIPAAm brush surfaces. The reaction solution was passed through a neutral alumina column to remove the Cu complex, and the PNIPAAm, used as a macroinitiator, was recovered by reprecipitation from diethyl ether. Next, degassed LAMA (0.422 g, 9.0 mmol) was prepared in a mixed solvent (methanol/water = 3/2, v/v) as described above, and the ATRP of LAMA proceeded at 20 °C for 12 h. After the reaction, PNIPAAm-b-PLAMA brush glasses were rinsed repeatedly with methanol and water and dried at 25 °C in vacuo. The reaction solution was passed through a neutral alumina column to remove the Cu complex, and the polymer was recovered by reprecipitation from diethyl ether.
2.4. Characterizations

$^1$H-NMR spectra of the free polymers were acquired using a JNM-GSX300 spectrometer (300 MHz, JEOL, Tokyo) to confirm the successful synthesis and determine the chemical composition. The molecular weight and polydispersity of the polymers were determined at 40 °C using a GPC system (TOSOH TSK-GEL α-2500; Tosho, Tokyo) equipped with a refractive index detector (RI-2031; JASCO International Co, Ltd, Tokyo). The transmittance of the polymer aqueous solution at 500 nm was continuously monitored at a heating rate of 1.0 °C min$^{-1}$ using a UV-visible spectrometer (V-550; JASCO International Co, Ltd). The LCST was determined as the temperature at which transparency decreased to 50%. Temperature-dependent aqueous wettabilities of the polymer brush surfaces were analyzed by static contact angle measurements using a digital camera. The sample temperature was regulated with a temperature-controlled plate within a deviation of ±0.1 °C. Contact angles were determined from the shapes of water droplets, and the results were expressed as a mean of three measurements with standard deviation (SD). The thicknesses of dry grafted copolymer brushes on the surfaces were determined by ellipsometry (M-2000U, J A Woollam Co, Lincoln, Nebraska). The measurements were performed at three different angles of incidence (55°, 60° and 65°), in the wavelength range from 300 to 1000 nm. The average dry thickness of a polymer brush was estimated by fitting the experimental data with the Cauchy model, taking refractive indexes $n = 1.50, 1.52$ and $1.46$ for the glass substrate, (chloromethylphenyl)ethylsilane [32], and PNIPAam [33], respectively. The grafting density, $\sigma$ (chains nm$^{-2}$), of the polymer brushes on the surfaces was calculated using

$$\sigma = \frac{h \rho N_A}{M_n},$$

where $h$ is the polymer layer thickness (nm), $\rho$ is the density of dry PNIPAam film [34], $N_A$ is Avogadro’s number and $M_n$ is the number-average molecular weight of the free polymer chains.

2.5. Adsorption of RCA$^{120}$ onto NIPAAm and LAMA copolymer brush surfaces

RCA$^{120}$ is a protein that specifically binds to glucose/lactose structures. The adsorption of fluorescein isothiocyanate (FITC)-conjugated RCA$^{120}$ onto NIPAAm–LAMA copolymer brush surfaces was examined at different temperatures. A stock solution of FITC-conjugated RCA$^{120}$ was diluted to 2 $\mu$g ml$^{-1}$ using 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) buffer. The NIPAAm-LAMA copolymer brush glasses were immersed in the solution and incubated for 30 min at 37 °C. After the incubation, the glass substrates were rinsed with a HEPES buffer and dried by $\text{N}_2$ gas flow. The fluorescence images of the glass surfaces were photographed using a fluorescence microscope (IX71 N-23Fi/DIC, Olympus, Tokyo). The reversible binding of RCA$^{120}$ was also studied below the LCST. The surfaces were allowed to bind RCA$^{120}$ above the LCST; according to the above protocol, the temperature was decreased from 37 to 25 °C for 1 h, and the fluorescence images of the surfaces were acquired. To estimate the adsorption of RCA$^{120}$ on the polymer brush surfaces, the images were analyzed using ImageJ software [35].

2.6. Cell culture

HepG2 cells were suspended in serum-free DMEM with penicillin and streptomycin at a concentration of $1.0 \times 10^5$ cells ml$^{-1}$. The cells were then seeded on the polymer brush glass substrates and cultured at 37 °C. For the cell detachment assay, after 24 h of incubation at 37 °C, the cells were transferred to an incubator maintained at 25 °C. The remaining cell numbers on the surfaces were monitored and imaged using a phase-contrast microscope (IX71 N-23Fi/DIC, Olympus, Tokyo). NIH-3T3 mouse fibroblast cells were also used as a non-LAMA specific control.

3. Results and discussion

3.1. Characterization of NIPAAm and LAMA copolymers and the grafted surfaces

To construct an ‘on–off’ switchable platform for the selection of hepatocytes, temperature-responsive copolymer brushes comprising NIPAAm and LAMA were prepared by surface-initiated ATRP. This method has been successfully used to synthesize polymer brushes with well-defined molecular weights and narrow molecular weight distributions. The sensitivity of polymer brushes to external stimuli heavily depends on their grafting architectures [31], and therefore, we prepared both random and block architectures by one-step and sequential polymerizations, respectively. A sacrificial initiator EBC was added to the polymerization reaction to generate free copolymers. This allowed to the study of the composition and molecular weight by $^1$H-NMR and GPC of the grafted copolymer brushes, respectively. It has been reported that the number-average and weight-average molecular weight (M$\bar{n}$ and M$\bar{w}$), and thus, the polydispersity index (M$\bar{w}$/M$\bar{n}$) of the polymer generated in solution from the sacrificial initiator agreed well with those of the polymer chains [36]. The addition of a free initiator also allowed to the adjustment of the ratio of monomer and initiator concentrations, which is an important factor for controlling the chain growth in the ATRP.

Random copolymer brushes with various compositions of NIPAAm and LAMA were synthesized using Me$_6$TREN as a ligand, which provides good control of the molecular weight of acrylamide-type monomers [37]. Block copolymer brushes were prepared by sequentially growing the blocks under identical reaction conditions. The LAMA blocks were prepared using bpy owing to its excellent ability to control the molecular weight of methacrylate-type monomers. The copolymer parameters are summarized in table 1. All the copolymers possess relatively narrow molecular weight distributions (M$\bar{n}$/M$\bar{w}$ = 1.15–1.43) as determined by GPC. The $^1$H-NMR spectra of the copolymers reveal the presence of characteristic signals of NIPAAm and LAMA; the proton signals at 3.5–4.5 ppm in figure 3 were assigned to LAMA.
Table 1. Properties of free NIPAAm and LAMA copolymers and the brush surfaces

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence</th>
<th>LAMA in feed [mol%]</th>
<th>(M_n \times 10^{-4})</th>
<th>(M_w / M_n)</th>
<th>LAMA content [mol%]</th>
<th>Thickness [nm]</th>
<th>Graft density [chains/nm^2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNIPAAm homopolymer</td>
<td>0.0</td>
<td>1.84</td>
<td>1.18</td>
<td>0.0</td>
<td>7.6</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Random-1</td>
<td>1.0</td>
<td>1.32</td>
<td>1.43</td>
<td>1.1</td>
<td>6.8</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Random-5</td>
<td>P(NIPAAm-co-LAMA)</td>
<td>3.0</td>
<td>1.55</td>
<td>1.25</td>
<td>5.2</td>
<td>7.3</td>
<td>0.36</td>
</tr>
<tr>
<td>Random-9</td>
<td>5.0</td>
<td>1.70</td>
<td>1.15</td>
<td>9.1</td>
<td>7.6</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Block-3</td>
<td>PNIPAAm-b-PLAMA</td>
<td>0/100(^d)</td>
<td>1.91</td>
<td>1.29</td>
<td>3.2</td>
<td>8.2</td>
<td>0.33</td>
</tr>
</tbody>
</table>

\(^a\) Determined by GPC (standard: PEG; eluent: DMF with 10 mmol/L LiBr; flow rate: 0.6 ml min\(^{-1}\); temperature: 40°C).
\(^b\) Estimated from \(^1\)H-NMR spectra (figure 2).
\(^c\) Determined by ellipsometry under dry condition (incidence angles: 55°–65°, wavelength range: 300–1000 nm).
\(^d\) First/second step of sequential ATRP processes.

The LAMA contents of the random copolymers determined by \(^1\)H-NMR were 1.1, 5.2 and 9.1 mol%, and the LAMA content of the block copolymer was estimated to be 3.2 mol%. The LAMA contents in random copolymers were slightly higher than those in the feed because of the different reactivity ratios for NIPAAm and LAMA. On the other hand, a smaller molecular weight of the PLAMA segment in the block copolymer was obtained from GPC. It is plausible that the PLAMA formed a compact conformation in the GPC eluent owing to the poor solubility in dimethylformamide (DMF).

The thicknesses of grafted polymer brushes on the glass substrates depended on their molecular weight. This result is plausible because all the brushes were created by the same method on the same silanized surfaces. The PNIPAAm copolymer brushes had a relatively high graft density of 0.3–0.4 chains nm\(^{-2}\).

Figure 4(a) shows temperature-dependent transmittance changes at 500 nm for the copolymers in water, whereas figure 4(b) reveals the effect of LAMA content on LCST for random and block copolymers of NIPAAm and LAMA. The LCST of the random copolymers rises as the LAMA content increases simply because of the hydrophilic nature of LAMA monomer units along the backbone. In contrast, the block copolymers show a constant LCST, independent of the LAMA moiety. As the temperature is raised, the PNIPAAm blocks independently phase-separate, as is the case for the NIPAAm homopolymer, whereas a random copolymer of similar composition may remain soluble [38].

Aqueous wettabilities on the copolymer brushes were investigated using static contact angle measurements. Figure 5 compares the contact angles between homo, random, and block copolymer brushes at 25 and 37°C, which are the corresponding temperatures in the following RCA\(_{120}\) adsorption and cell culture experiments. A contact angle of ca 77° was measured for the initiator-modified glass substrate. For the LAMA homopolymer brush, the contact angle decreased to ca 36° and was independent of temperature. The PNIPAAm homopolymer brush demonstrated temperature-responsive changes in the contact angles, i.e. the contact angle was smaller than 54.9° at 25°C and larger than 64.9° at 37°C. For random copolymer brushes, on the other hand, no significant change in the contact angle was observed between 25 and 37°C, although they became slightly hydrophobic at 37°C. This is closely related to the observed increase in LCST by temperature-dependent transmittance measurements of free-copolymer solutions. For the block copolymer brush, the contact angles were 53.9° and 63.4° at 25 and 37°C, respectively.

3.2. Adsorption of RCA\(_{120}\) on NIPAAm and LAMA copolymer brushes

Most biomolecules have immense recognition power (specific binding), but they also physically adsorb onto a solid substrate without specific receptor recognition (nonspecific adsorption).
Figure 4. (a) Temperature-dependent transmittance changes for NIPAAm and LAMA copolymers in water (open circles: PNIPAAm, open triangles: Random-1, open squares: Random-5, open diamonds: Random-9 and solid squares: Block-3). (b) Effect of LAMA content in copolymers on their LCST, which was determined as the temperature at which transmittance decreased to 50% (solid square: PNIPAAm, open circles: random copolymer and open triangle: block copolymer).

Therefore, both specific and nonspecific binding properties of the designed copolymer brushes were studied using FITC-conjugated lectins. Lectins are proteins that specifically bind carbohydrate, and the RCA_{120} used in this study, in particular, has a high binding affinity for galactose/lactose moieties [39]. The adsorption of the RCA_{120} on polymer brushes was observed by fluorescence microscopy. The fluorescence images of homo, random, and block copolymer brushes after incubation with RCA_{120} at 37°C are shown in figures 6(a)–(d). To quantify the adsorbed FITC–RCA_{120} on the brushes, the fluorescence images were analyzed using ImageJ software to invert and split the colors into red, green, and blue components. The average green intensities of the images are shown in figure 6(e). The initiator-immobilized sample showed high intensity owing to the hydrophobic nature of its silanized surface, exhibiting a nonspecific adsorption of the lectin. In the case of the PNIPAAm brush, the intensity was much weaker than those of the other brushes when the temperature was decreased to 25°C. In contrast, the highest intensity was observed for the PLAMA brush despite its hydrophilicity, indicating specific binding of RCA_{120} to this brush. Both random and block copolymer brushes also showed higher adsorption of RCA_{120} at 37°C than the NIPAAm homopolymer brush, and the intensity was slightly higher for the block brush than the random brush, even though the LAMA content was lower for the block. This is probably due to the hierarchical structure of block copolymer brushes, where LAMA units are exposed to the solution. Importantly, no significant decrease in the intensity was not observed for the block copolymer brushes when the temperature was decreased from 37 to 25°C, even when the block copolymer had the LCST between 25 and 37°C; this is because of the extremely high affinity constant of RCA_{120}. Yoshizako et al reported reversible capture/release of bovine serum albumin (BSA) using PNIPAAm-grafted silica beads co-immobilized with Cibacron Blue F3G-A (CB), and the association constant of BSA against CB on the surface was \(10^2–10^4\, \text{M}^{-1}\) [40]. The affinity constant value, \(K_A\), for RCA_{120} binding to the PLAMA brush has been measured as \(1.86 \times 10^5\, \text{M}^{-1}\) by surface plasmon resonance spectroscopy [41].

3.3. Control of cell adhesion on NIPAAm and LAMA copolymer brushes

Cell adhesion to synthetic materials can be guided via specific interactions with cell surface receptors by modifying the substrate surface with short peptide sequences derived from ECM proteins. Most commonly, these cell adhesion peptides...
are based on the RGD sequence, which is derived from the cell attachment domain of fibronectin and specifically binds to integrin receptors that are present on the cell surface [42]. Carbohydrate-mediated cell recognition has also been used to enhance selective interactions between materials and cells. In particular, the binding of multivalent galactose residues to ASGPRs on the hepatocyte membrane is the most extensively studied example [43]. In this study, newly designed temperature-responsive glycopolymer brushes were used to demonstrate the selective adhesion of hepatocytes above the LCST and their collection below the LCST. First, the specificity of the sugar moiety in LAMA in the copolymer brushes for cell adhesion was investigated using NIH-3T3 fibroblasts and HepG2 cells. To confirm the biospecificity between LAMA and hepatocyte, a cell adhesion assay was carried out under serum-free conditions, as the serum-containing medium commonly includes various cell adhesion proteins. Figure 7 shows phase contrast images of NIH-3T3 fibroblasts (a–d) and HepG2 cells (e–h) on the polymer brush surfaces at 37 °C after one-day culture. NIH-3T3 fibroblasts barely adhered to the surfaces in the absence of serum proteins, because they do not express ASGPR and could not interact with LAMA sequences in the polymer brushes [44]. By contrast, adhesion was observed for HepG2 cells on the LAMA-incorporated polymer brush surfaces, whereas the PNIPAAm brush did not demonstrate cell adhesion. The attached HepG2 cells on the LAMA-incorporated brush surfaces mainly showed spherical morphology. Akaike and colleagues reported that hepatocytes selectively adhere to the surfaces coated with poly(N-p-vinylbenzyl D-lactose lactonamide) (PVLA) by interaction between the sugar residues and ASGPRs. The morphologies of the attached cells depended on the amount of coated PVLA on the surfaces (spreading and spheroidal shapes for 1 and 100 μg ml⁻¹ of PVLA coatings, respectively) [26, 27]. The round morphologies of the attached HepG2 cells observed in this study imply that the condensed sugar density on the surface was achieved by surface-initiated ATRP. Therefore, these results strongly indicate that HepG2 cells recognized the LAMA moiety in the polymer brush and maintained the adhesion state.

Figure 8 compares the densities of attached HepG2 cells on the polymer brush surfaces after 1 day of culture at 37 °C and subsequent culture at 25 °C for another 1 h. HepG2 cells adhered to the LAMA-incorporated brush surfaces very well at 37 °C, although the initiator-immobilized surface is the most hydrophobic studied here. This result signifies that HepG2 adhesion to LAMA is not affected by the hydrophobic interaction. The density of attached cell number increased with the density of galactose residues in the random copolymer brushes. Interestingly, the density of HepG2 cells was higher on the Block-3 brush Random-5 brush even though the latter contained larger amounts of LAMA. This can be explained by the shielding effect of the random copolymer brush on ligand recognition. Namely, the steric hindrance of a random copolymer brush could prevent the interaction between HepG2 cells and receptors with the galactose residues, whereas the LAMA segments of the block copolymer brushes are exposed to the HepG2 cells. These observations agree well with the results of RCA₁₂₀ adsorption assays. A significant difference was, however, observed when the cell culture temperature of the cell culture was suddenly reduced to 25 °C. The cells started to spontaneously detach from the Block-3, but not from the random copolymer brushes. This is closely related to the observed increase in LCST and surface wettability of random copolymers. Because Block-3 has LCST between 25 and 37 °C, the temperature decrease induced a drastic hydration and extension of the

---

**Figure 6.** Adsorption of FITC-conjugated RCA₁₂₀ on NIPAAm and LAMA copolymer brushes. Fluorescence images taken at 37 °C for (a) PNIPAAm brush, (b) PLAMA brush, (c) Random-5, and (d) Block-3. (e) Average green intensity for RCA₁₂₀-adsorbed surfaces at 25 and 37 °C.
Figure 7. Phase contrast images of NIH-3T3 fibroblasts (a–d) and HepG2 cells (e–h) on PNIPAAm (a, e), PLAMA (b, f), Random-5 (c, g), and Block-3 (d, h) after one-day culture at 37 °C under serum-free conditions. Bar: 100 µm.

Figure 8. Density of HepG2 cells remaining on NIPAAm and LAMA copolymer brush surfaces after the temperature was decreased from 37 to 25 °C.

In addition, cells tend to detach from the surface if they lose intracellular tension. Recent studies have revealed that mechanical and structural factors are critical for controlling cell functions [46]. Therefore, the obtained results are attributed to the correlated effects of significant changes in mobility, hydrophilicity, and mechanical properties of the grafted polymer chains. Those factors can be controlled by not only the composition and sequence of comonomers but also the grafting density and chain length. Further studies are needed to investigate the correlations between the architectures of grafted polymers and surface properties.

Given the current public focus on stem cell biology, cancer diagnostics, and regenerative medicine, the development of highly effective and noninvasive cell separation techniques has been strongly desired [47]. Conventional fluorescence-activated and magnetically activated cell sorting techniques have been commonly used to separate target cells from a heterogeneous cell mixture [48]. They are increasingly applied to microfluidic systems because of the intrinsic compatibility of cell size with microchannels [49]. These methods, however, require labeling of the cells with either fluorescent or magnetic nanoparticles prior to the separation, and the sorting throughput is limited for microfluidic systems owing to their small sample volume. Our strategy of selective adhesion/detachment enables label-free cell separation using only variations in temperature. The simplicity, ease of use, sensitivity and noninvasive nature of this technique make it a particularly attractive alternative to more complex methods based on high throughput cell separation.

4. Conclusions

LAMA-incorporated temperature-responsive polymer brush surfaces with different grafting architectures were prepared via surface-initiated ATRP. The LCST increases with the
LAMA content in random polymers, whereas it is independent of the LAMA content and type in block copolymers. The copolymer surfaces containing LAMA selectively attached HepG2 cells via the biospecific affinity between galactose residues and ASGPR, whereas NIH-3T3 cells barely adhered. The attached HepG2 cells were collected from the block copolymer surfaces only by cooling them below the phase transition temperature, whereas almost all the attached HepG2 cells remained on the random copolymer brush surfaces owing to the increased surface hydrophilicity. This methodology can be extended to the preparation of smart bioactive interfaces with diverse architectures and to cell screening. The versatility and noninvasive protocol of our temperature-responsive system may enable the realization of label-free high-throughput cell separation for stem cell biology, cancer diagnostics, and regenerative medicine.

Acknowledgments

The first author was financially supported by the Research Fellowships of the Japan Society for Promotion of Science (JSPS) for Young Scientists. This research was partially supported by the Japan Society for the Promotion of Science (JSPS) through the ‘Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program),’ initiated by the Council for Science and Technology Policy (CSTP).

References

[29] Nara I and Armes S P 2003 Biomacromolecules 4 1746
[34] Ishida N and Biggs S 2007 Langmuir 23 11083
[38] Chen G H and Hoffman A S 1995 Nature 373 49