Fabrication of Thermoresponsive Polymer Gradients for Study of Cell Adhesion and Detachment

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A poly(N-isopropylacrylamide) (PNIPAAm) gradient covalently anchored on a silicon substrate with a linear variation of thickness was fabricated by continuous injection of the reaction mixture (PNIPAAm, CuBr and its ligand, methanol, and water) into a glass chamber containing a silicon wafer, whose surface had been homogeneously immobilized with bromoisobutyryl bromide (BIBB). Because of the good control of the surface-initiated atom transfer radical polymerization (SI-ATRP) technique, the thickness of the PNIPAAm brushes was linearly proportional to the polymerization time. As a result, the gradient length and sharpness could be easily controlled by the experimental parameters such as the polymerization time and the injection rate. The as-prepared PNIPAAm gradients were characterized by ellipsometry, water contact angle, and atom force microscopy to detect their alteration of the thickness, surface wettability, and morphology, confirming the gradient structure. X-ray photoelectron spectroscopy confirmed the surface composition of the PNIPAAm. In vitro culture of HepG2 cells was implemented on the gradient surfaces, revealing that the cells could adhere at 37 °C and could be detached at 24 °C when the gradient thickness was in the range of 20–45 nm. The work thus develops a method to fabricate the stable gradient surface with better quality control, and clarifies in a facile manner the appropriate thickness of the PNIPAAm brushes in terms of cell adhesion and detachment.

1. Introduction

It is known that the surface chemistry of materials has a great impact on their performance in terms of reactivity, catalysis activity, and responsivity to environmental and biological stimuli. Particularly, a gradient surface shows gradual variation of the chemical and/or physical properties along its dimension. There are many advantages to use the gradient surface for various investigations and applications. For instance, a single-gradient material can easily reflect the variation of surface parameters such as molecular weight, chemical compositions, the number of functional groups, grafting density, and the nanostructures, which is of great help to significantly reduce the required sample numbers and improve the working efficiency. More recently, the gradient surface is expected to take more important role in the fields of biomaterials and regenerative medicine, since this kind of surface can provide chemical cues to mediate cell attachment and migration which are key issues for tissue regeneration.

So far, a number of approaches to fabricate the gradients on various substrates have been developed, including gradual immersion, diffusion controlled vapor deposition, corona discharge, scanning tunneling microscopy, photoimmobilization, cross diffusion, electrochemical-potential, and microfluidic techniques, each of which has its own advantages and disadvantages. More recently, surface-initiated atom transfer radical polymerization (ATRP) has attracted great attention in fabricating the surface gradients. This controlled living radical polymerization yields polymers with well-defined molecular weight, low polydispersity, and high functionalities. Therefore,

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higher quality of the surface polymer gradients can be expected by this method.

The thickness of polymer brushes is proportional to the molecular weight, which is one of the most important parameters determining the surface properties. The gradients of polymer thickness on substrates have been prepared by several methods. Typically, the thickness gradients of surface-tethered polymers were first produced by Tomlinson and co-workers. A flat substrate immobilized with initiators was placed vertically into a glass chamber that was filled with ATRP reaction mixture (monomer, catalyst, ligand, and solvent). The level of the polymerization solution was then slowly lowered down using a micropump attached to the bottom of the chamber. However, it is conceivable that residue of the reaction solution inevitably remains on the substrate surface, even though the solution level has been lowered, thereby possibly affecting the quality of the obtained gradients. Later, the same authors modified the method by placing the sample vertically into the polymerization solution using a dippmg apparatus. Alternatively, the reaction solution has also been filled gradually into the chamber to similarly obtain the gradient by Xu et al., which shows better control over the molecular weight of the polymer brushes. In view of the features of the aforementioned methods and for the sake of cell adhesion study, in this work a thermoresponsive polymer gradient is fabricated by using the modified apparatus shown in Figure 1. The procedures include fabrication of a substrate having homogeneously immobilized ATRP initiators and control over the polymerization time by a dynamic injection. Briefly, a silicon wafer immobilized with the ATRP initiators is placed vertically in a glass chamber, into which the polymerization medium is continuously injected using a micropump under ambient nitrogen. Consequently, gradual alteration of the thickness of the polymer brushes is achieved because the length of the polymer chains is directly proportional to the polymerization time. Namely, the chain length is kinetically controlled by the polymerization time, and thereby the sharpness of the gradients can be conveniently controlled by the injection rate.

Figure 1. Schematic illustration to show the apparatus for fabricating surface-grafted polymer brushes with a gradient in thickness.

2. Experimental Section

Materials. N-isopropylacrylamide (NIPAAm) was obtained from Shanghai Wing Chemical Company, Ltd. and used after being recrystallized twice in a mixture of toluene and n-hexane (v/v ∼ 1:1). Bromoisobutyryl bromide (BIBB) and 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA,99+%)) were purchased from Aldrich and used as received. 3-(Aminopropyl)triethoxysilane (APS, 99%) was purchased from Hanzhou Guibao Chemical Co., Ltd. and purified by distillation. Copper(I) bromide (CuBr, 98%) was purchased from Sinopharm Chemical Reagent Co., Ltd. and purified by washing with water 5 times, sulfuric acid 10 times, glacial acetic acid 6 times, ethanol 4 times and ether 4 times. It was then dried in a high vacuum oven at 110 °C for about 24 h and stored in high pure nitrogen before use.

Preparation of APS Monolayer on Silicon Wafer. Silicon wafers (P-doped, (111)-oriented, 8–12 Ω cm resistivity, 0.525 mm thickness) were purchased from Ningbo QL Electronics Co., Ltd. and cut into square chips of about 1 cm × 3 cm in size. To remove the organic contaminants, the silicon wafers (chips) were cleaned in a series of ultrasonically agitated solvents (petroleum ether, toluene, acetone, ethanol, and water), each for 5 min. The wafers were etched for 30 s in stirred hydrofluoric acid to remove the native oxide films, followed by rinsing with copious amounts of doubly distilled water and drying under a nitrogen flow. The wafers were further treated with freshly prepared “Pirana” solution (a mixture of 70 vol % concentrated sulfuric acid and 30 vol % of hydrogen peroxide. Caution: pirana solution reacts violently with organic materials and should be handled carefully) at the boiling temperature until no air bubbles evolved (about 20 min). The wafers were finally rinsed with a large amount of doubly distilled water and dried at 90 °C in a vacuum oven for 2 h.

Amination (Si–NH₂) of the above treated wafers was carried out according to the literature. The wafers were contacted only with the condensing vapor of a refluxing mixture of xylene and APS (v/v ∼ 1:12) for 10 h at 170 °C. They were then washed with toluene, ethanol, and water, and dried under a nitrogen flow.

Immobilization of BIBB on the Aminated Silicon Wafers. Immobilization of BIBB on the Si–NH₂ surface was achieved by incubating the Si–NH₂ chips in 20 mL of dry dichloromethane containing 2% w/v pyridine, followed by dropwise addition of 1 mL of BIBB at 0 °C. The mixture was gently stirred at this temperature for 2 h and then left at room temperature for another 12 h. The BIBB-immobilized silicon wafers (Si–Br) were cleaned with acetone and toluene, and dried under a nitrogen flow.

Surface-Initiated ATRP of NIPAAm. Figure 1 shows the schematic illustration of the apparatus for fabricating the surface-grafted polymer gradient. The surface-initiated ATRP of NIPAAm on the BIBB-immobilized surface was carried out using a reaction mixture of NIPAAm (5 g, 44.24 mmol), PMDETA (0.56 mL, 2.68 mmol), and CuBr (0.128 g, 0.92 mmol) in a 1:1 mixture of water and MeOH (20 mL). This reaction mixture was sonicated for 2 min, then stirred and degassed with high pure nitrogen for 30 min. The silicon substrate was fixed with a clamber and vertically put into the glass vial. The vial was then thoroughly purged with nitrogen to get rid of oxygen. The reaction solution was injected into the tube by a microinfusion pump, in which the velocity of flow could be easily tuned. The polymerization was terminated when the liquid level reached the top of the silicon wafer. The silicon substrate was then taken out, rinsed with copious amount of doubly distilled water, incubated in water for 1 day, and then dried in air and stored under nitrogen atmosphere.

Surface Characterization. The chemical compositions of the NIPAAm brushes were characterized by an ESCALAB 220i-XL X-ray photoelectron spectrometer (XPS). The monochromatic Mg KR line (1253.6 eV) was used as the excitation source. After peak fitting of the C1s spectra, all the binding energies were calibrated against the C1s peak at 284.6 eV of the saturated carbon. The pressure in the analysis chamber was maintained at 10⁻⁸ Torr or lower during the measurement. Survey spectra from 0 to 1200 eV binding energy (BE) were recorded at 100 eV pass energy with an energy step of 1.0 eV, with a dwell time of 100 ms for one scan. All data achieved by XPS were analyzed using the software of XPS Peak 4.1.

The static water contact angles of the neat and polymer functionalized silicon surfaces were measured at saturated humidity by a sessile-drop method on a DSA 100 water contact measuring system (Kruess, Germany). The apparatus was equipped with a microscope and illumination system to visualize the water droplets sitting on the substrates. Before each measurement, the samples were immersed in water at a given temperature for 1 h and dried.
in vacuum at the same temperature. Then the samples were placed into a chamber mounted to the apparatus, whose temperature was controlled by a superthermostat (Haake, Germany) and equilibrated to the corresponding temperature for 15 min. Water droplets at the same temperature were put onto the samples. The water volume was continuously increased, and the advancing contact angle was obtained. Each value was averaged from five parallel measurements and reported as mean \((\text{standard deviation})\).

The AFM topographic images were obtained in a dynamic force mode in triple distilled water using V-shaped silicon cantilevers (Nanoprobe, Veeco; spring constant 0.12 N/m; tip radius 20–60 nm) with a SPI3800N atomic force microscope, Seiko Instruments, Inc. Before the AFM measurement, the samples were incubated in triple distilled water for 12 h. The scanning frequency was set at 0.5–1 Hz. The applied normal force between the tip and the sample was kept as low as possible (~1 nN).

The thickness of the polymer brushes grafted on the silicon substrates was measured by ellipsometry. The measurement was carried on a variable-angle spectroscopic ellipsometer (model VASE; J. A. Woollam, Inc., Lincoln, NE) at incident angles of 65°, 70°, and 75° within a wavelength range of 400–2000 nm. The thickness was calculated from the ellipsometric parameters \(\Delta\) and \(\Psi\), by assuming a refractive index of the polymer layer of 1.46. A Cauchy layer model provided with the instrument was used for all the organic films. Each value was averaged from nine parallel measurements at the same \(x\)-direction position but different \(y\)-direction position and reported as the mean ± standard deviation.

Figure 5. (a) Advancing water contact angles on the gradient film as a function of position at 24 and 40 °C, respectively. (b) Advancing water contact angle as a function of temperature. In (b), a uniform PNIPAAm film grafted on a silicon wafer with a thickness of 25nm was used.

Figure 6. SEM images of HepG2 at positions of 0, 6, 12, 18, and 24 mm from (a) to (e), respectively. The cells were cultured at 37 °C for 8 h.

Figure 7. (a) and (b) Morphology of HepG2 on a silicon wafer surface homogeneously grated with a 25nm PNIPAAm film. (a) After being cultured for 8 h and (b) for 1 day on the same position. (c) The cells were cultured for 8 h on a 60 nm PNIPAAm film.
Using the apparatus shown in Figure 1, the gradient PNIPAAm brushes were obtained on the BIBB-immobilized silicon wafer. A mixture of NIPAAm, CuBr, PMDETA, and MeOH/H2O was continuously injected into the chamber under nitrogen atmosphere at ambient temperature, in which the BIBB-immobilized silicon wafer with a total length of 30 mm was previously placed. From the bottom to the solution level (defined as the 0 position of the as-prepared gradient), the polymerization time was gradually decreased, leading to a steady decrease of the chain length. As a result, the PNIPAAm gradient was prepared as illustrated in Figure 2a. The thickness of the grafted PNIPAAm brushes was measured by ellipsometry from nine equally spaced positions along the 30 mm long gradient. Figure 2a shows that the thicknesses of the PNIPAAm brushes were increased approximately linearly on the silicon substrates. To demonstrate the versatility, here three surface gradients with variable sharpness were prepared by using injection rates of 1, 2, and 5 mL/h, respectively. Since a total of 5 mL of solution is needed to submerge the 30 mm long substrate, the injection time, i.e., the reaction time, was set at 300, 150, and 60 min, respectively. It shows that, with a slower injection rate, a sharper gradient was made on the same length of a silicon substrate. Here, a reaction mixture of NIPAAm/CuBr/PMDETA/MeOH/H2O was employed in the polymerization of NIPAAm, as similarly reported by others.49,50 PMDETA/Cu(I)Br is a quite efficient catalyst for ATRP of NIPAAm and acrylates. The excess PMDETA can induce chain transfer reaction.49,51 Therefore, Cu(II) is not necessarily added. Indeed, our reaction system remained homogeneous throughout the polymerization.

Compared to the monotonous growth of the gradients obtained at 2 mL/h and 5 mL/h, a transition appeared on the profile of the sample obtained at 1 mL/h, illustrating a slower growth of the film thickness at longer position. To clarify the reason and to elucidate the surface grafting polymerization of the PNIPAAm, the PNIPAAm was homogeneously polymerized on the silicon substrates for different times by surface-initiated ATRP (Figure 2b). It shows that the film thickness was increased linearly before 250 min, after which a transition was similarly observed. This result indicates that the process of the surface-initiated ATRP of NIPAAm is controllable, although the growth rate was decreased at longer polymerization time. The slower growth rate at longer reaction time might be a result of loss of the active chain ends. In such a case, the active end groups may be buried in the film and thus become inaccessible to the monomers, followed by bimolecular coupling and disproportional reactions that consume the active chains.49,52,53 Another possible explanation is the deactivation of the catalyst by forming a competitive complex with the growing PNIPAAm, which has been reported for the polymerization of (meth)acrylamides using linear amines.54 It is worth mentioning that the homogeneously polymerized film has a thinner thickness than that of the gradient film at the same polymerization time, which should be attributed to the different use of nitrogen during the polymerization. High pure nitrogen was used throughout the polymerization during preparation of the surface gradients, whereas only initial nitrogen purge was applied before the polymerization of the nongradient film. The

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### Cell Culture

For cell culture, the substrates having the PNIPAAm films were immersed in 75% ethanol for 1 h, washed three times with phosphate buffered saline (PBS, pH 4.7), sterilized for about 1 h by UV irradiation. The substrates were then placed into the wells at a density of 5 × 10^4 cells/well and incubated in Dulbecco’s modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37 °C for a predetermined time under a humidified 5% CO2 atmosphere. For study of the cell adhesion on the PNIPAAm gradient surfaces, the surfaces were washed gently twice in PBS at 37 °C after cultivation for 8 h to remove the unattached cells, fixed with 2.5% glutaraldehyde for 8 h at 37 °C, dehydrated in a series of ethanol solutions (50–100%), each for 15 min, and immersed in acetic isopentadiester solution for another 30 min. The mechanism of the fixative action of glutaraldehyde is due to the formation of cross-links between the protein molecules in the cell membrane.48 The samples were dried by a critical point drying method and observed under a scanning electron microscope (SEM, StereoScan 260, Cambridge) after coating a thin gold layer.

For study of the cell detachment from the gradient surfaces, the 8 h cultured samples were subjected to low-temperature treatments as follows. The 37 °C medium was replaced by a 24 °C medium and maintained at this temperature for 30 min. After the medium was replaced with a 24 °C medium, the samples were maintained at 37 °C for 20–30 min. The same washing and fixation procedures were then applied to fix and observe the attached cells as described above.

## 3. Results and Discussion

### Fabrication and Characterization of the PNIPAAm Gradients

To obtain the PNIPAAm gradients using the grafting-from ATRP approach, BIBB (one of the ATRP initiators) was homogeneously immobilized onto the silicon surface. For this purpose, the silicon wafer was functionalized first by treatment at ambient temperature, in which the BIBB-immobilized silicon wafer with a total length of 30 mm was previously placed. From the bottom to the solution level (defined as the 0 position of the as-prepared gradient), the polymerization time was gradually decreased, leading to a steady decrease of the chain length. As a result, the PNIPAAm gradient was prepared as illustrated in Figure 2a. The thickness of the grafted PNIPAAm brushes was measured by ellipsometry from nine equally spaced positions along the 30 mm long gradient. Figure 2a shows that the thicknesses of the PNIPAAm brushes were increased approximately linearly on the silicon substrates. To demonstrate the versatility, here three surface gradients with variable sharpness were prepared by using injection rates of 1, 2, and 5 mL/h, respectively. Since a total of 5 mL of solution is needed to submerge the 30 mm long substrate, the injection time, i.e., the reaction time, was set at 300, 150, and 60 min, respectively. It shows that, with a slower injection rate, a sharper gradient was made on the same length of a silicon substrate. Here, a reaction mixture of NIPAAm/CuBr/PMDETA/MeOH/H2O was employed in the polymerization of NIPAAm, as similarly reported by others.49,50 PMDETA/Cu(I)Br is a quite efficient catalyst for ATRP of NIPAAm and acrylates. The excess PMDETA can induce chain transfer reaction.49,51 Therefore, Cu(II) is not necessarily added. Indeed, our reaction system remained homogeneous throughout the polymerization.

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Figure 8. HepG2 numbers on the PNIPAAm gradients at 37 °C (■) and 20 °C (○). Data were averaged from three parallel experiments.
residue oxygen in the latter may partially deactivate the active chain ends, leading to a slower polymerization rate.

The chemical compositions of the PNIPAAm gradient were confirmed by XPS. Figure 3a shows a survey spectrum of the Si-g-PNIPAAm surface, obtained at a position where the thickness of the PNIPAAm layer is 45 nm (measured by ellipsometry). The C/N/O molar ratio was measured to be 74.8:11.8:13.9, which is close to the theoretical ratio of 75.0:12.5:12.5 for PNIPAAm. The C1s core-level spectrum (Figure 3b) of Si-g-PNIPAAm surface can be curve-fitted into three peaks with binding energies of 284.6, 286, and 287.6 eV, respectively. Peak 1 at 284.6 eV corresponds to the two CH$_3$- groups in the isopropyl and the –CH$_2$- and –CH- in the PNIPAAm backbone. Peak 2 at 286 eV is attributed to the –CH- unit adjacent to the –NH- group, and peak 3 at 287.6 eV corresponds to the –CO- group. Along a 30 mm gradient obtained at an injection rate of 1 mL/h, the C/N ratio was decreased sharply from 7:1 to about 6:1 and then kept constant (Figure 3c). This alteration tendency again confirms the successful grafting of PNIPAAm, which is consistent with the fact that the detection depth of XPS is <10 nm too.

**Surface Morphology.** Wet mode tapping-mode AFM was employed to investigate the surface morphology of the PNIPAAm brushes at different regions, as typically shown in Figure 4 (samples obtained at an injection rate of 2 mL/h). The representative root-mean-squared (rms) roughness was steadily increased as a function of positions along the PNIPAAm gradient. The underlying layer, i.e., the initiators-immobilized surface, is exceptionally uniform and smooth with an rms roughness of 0.35 nm. The rms roughness at the end part of the gradient surface is lower than 7 nm, a value not large for such a thick film. The representative topographical images at positions of 7, 15, and 23 mm of this gradient are shown in the insets of Figure 4. They show a typical protuberant domain structure of polymer films on the surface. The number of these domains was increased, but their lateral size (about hundreds nanometers) was decreased along with the increase of the film thickness. The surfaces measured at a dry state were more uniform (data not shown), but no significant difference on the rms and morphology had been measured.

**Water Contact Angle.** PNIPAAm has a LCST at about 32 °C in pure water. It exhibits a random coil structure below the LCST and a collapsed globular structure above the LCST. Figure 5a shows the advancing water contact angle of the PNIPAAm gradient as a function of thickness measured at 24 and 40 °C, respectively. The water contact angle (80°) at the position of 0 was recorded on a uniform Si–Br substrate. The variation of the contact angle in Figure 5a suggests that the wettability of the PNIPAAm-functionalized surfaces is thickness dependent and thermally switchable. The water contact angles of the PNIPAAm films measured at 24 °C are always smaller than that measured at 40 °C, regardless of the film thickness. Figure 5b shows a typical profile of the water contact angle as a function of temperature. On this 26 nm thick PNIPAAm film, variation of the water contact angle followed a sigmoidal regime. The sharpest change occurred between 30–34 °C with a jump of the water contact angle from 65° to 93°. The LCST is estimated at about 32 °C, which is consistent with the results observed by Sun et al. and He et al. on the surface-bound PNIPAAm brushes. This alteration profile is very similar with those recorded by using other parameters such as absorbance and dynamic radius of this thermoresponsive PNIPAAm, illustrating the thermal responsivity of the grafted PNIPAAm brushes. The intrinsic mechanism of the transition has been well-known, i.e., the competition between the intermolecular and intramolecular hydrogen bonding of PNIPAAm and water molecules. At a

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**Figure 9.** SEM images of HepG2 at positions of (a) 0, (b) 2, (c) 4, and (d) 8 mm after the cells were cultured at 37 °C for 8 h, followed at 24 °C for 30 min.

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temperature below the LCST, the intermolecular hydrogen bonding between the PNIPAAm and water molecules is dominant, leading to an extending molecular conformation and hydrophilic surface. At a temperature above the LCST, the formation of intramolecular hydrogen bonding between C=O and N–H groups results in a compact and collapsed conformation of PNIPAAm, and thereby a more hydrophobic surface.

Figure 5a shows also that at 24 °C the contact angle was monotonously decreased from 80°, the Si–Br substrate, to 39°, the PNIPAAm film with a thickness of 90 nm. However, at 40 °C, it was first increased to a maximum value of 93.5° on a PNIPAAm film with a thickness of 15–20 nm, and was then decreased gradually with an eventual value of 57°. This alteration tendency is attributed to the interplay between the substrate and the PNIPAAm brushes. When the PNIPAAm thickness is thinner than 10 nm, the surface wettability is inevitably affected by the “relatively hydrophilic” Si–Br surface. When the thickness of the PNIPAAm reaches ~15–20 nm, influence of the substrate can be neglected, and thereby the highest water contact angle (~93–95°) appears. At this stage, the PNIPAAm brushes growing from the Si–Br surface are highly dense. Consequently, the molecular mobility and hydration is significantly weakened. The subsequent decrease of the contact angle implies the progressive increase of hydrophilicity, which is a result of the more freedom of the uneven grown chains at the longer polymerization time. Indeed, the fact that thicker PNIPAAm brushes are more hydrophilic regardless of the temperature has been reported by Okano and co-workers. With the increase of the PNIPAAm thickness, the density of the polymer chains from the inside layer to the outmost layer is decreased because the active end groups gradually lose their activity and/or are buried within the film. As a result, the outmost layer of the PNIPAAm brushes has a higher degree of freedom and mobility, thus it is easily hydrated and behaves more hydrophilic. In fact, we have observed that, on a grafted PNIPAAm film with a thickness of >200 nm, a water droplet spread very quickly, with a final static water contact angle of <10°. It has to note that the surface wettability is influenced significantly by the surface hierarchical structure too, in particular with a combination of nano- and microstructures. Figure 4 shows that the roughness of the PNIPAAm gradient was increased slightly along with the increase of film thickness. However, we believe the influence of the surface roughness on the surface wettability of the gradient films should be rather trivial since the absolute value is within a small range and the overall surface morphology does not change significantly.

Control over the Cell Adhesion and Detachment. Various types of cells can adhere and proliferate on the collapsed PNIPAAm surface above the LCST, whereas they can detach spontaneously by only lowering the culture temperature below the LCST. Although this fact is generally true for the PNIPAAm film, controversy still exists in terms of cell response to the thickness of the PNIPAAm brushes. Moreover, different fabrication methods may yield PNIPAAm brushes with different performance, in particular the cell response. The PNIPAAm gradient provides a powerful sample to investigate the influence of brush thickness on cell adhesion and detachment. For this context, HepG2 cells were cultured on the gradient surfaces for 8 h at 37 °C before being fixed with glutaraldehyde. As shown in Figure 6, on the Si–Br (Figure 6a, 0 position) and PNIPAAm surfaces with a thinner thickness (Figure 6b, 6 mm position), most cells showed an elongated morphology. Along with the extension of the position, the number of cells was decreased accompanied with a progressive decrease of the elongated cells but increase of the round cells (Figure 6c,d). At the position of 24 mm, only a few round cells were observed (Figure 6e). To clearly view the morphology, magnified images of the cells are presented in Figure 7. The initially elongated HepG2 cells (Figure 7a) became well-spread after being cultured for 1 day (Figure 7b) on the 25 nm thick PNIPAAm brushes, confirming that this surface is appropriate for cell growth too. In contrast, on the thicker PNIPAAm surface, 60 nm thick as a typical example (Figure 7c), clear filopodia could be visualized from the few attached cells. Many of the round cells died after 1 day of culture, and the remaining cells on the surface had no change in their morphology (data not shown).

Figure 8 summarizes the statistical numbers of the cells at different positions, namely the PNIPAAm thickness. When the PNIPAAm thickness was smaller than 50 nm, the cells could attach very well on the substrates with no significant difference between all the positions. Then along with the increase of the PNIPAAm thickness, the number of adhered HepG2 cells was gradually decreased. At the position of 14 mm (with a PNIPAAm thickness of about 45 nm), the number of attached cells was decreased significantly (p < 0.01). At the position of 16 mm (film thickness 55 nm), the number of adhered cells were only 1/4 of those on the thinner PNIPAAm films (<30 nm). When the thickness of the PNIPAAm film reached 65 nm, most of the HepG2 cells could not attach.

Next, the cell detachment from the gradient PNIPAAm surface was determined. After the cells were cultured for 8 h, they were further incubated at 24 °C for 30 min, followed by fixation with glutaraldehyde. As shown in Figure 9a, no apparent detachment of the cells from the surface at the 0 position was observed. Most cells at the position of 2 mm (Figure 9b) corresponding to a PNIPAAm thickness of 8.3 nm became contracted, but did not detach from the surface. By contrast, at the position of 4 mm (PNIPAAm thickness 15.6 nm, Figure 9c), some of the cells became globular and many cells detached. When the thickness of PNIPAAm reached 28.4 nm at the position of 8 mm, no cells could be found on the surface (Figure 9d).

The statistical data shown in Figure 8 reveal quantitatively that almost no cells were detached from the Si–Br substrate and the PNIPAAm brushes with a thickness of 8.3 nm. When the thickness of the PNIPAAm brushes reached 15.6 nm, half of the attached cells were detached. In contrast, on the thicker PNIPAAm films almost all the cells were detached. Taking into account attachment and detachment performance, one can get the following conclusions: (1) when the thickness is smaller than 20 nm, the HepG2 cells can attach well at 37 °C but can not be effectively detached from the surface by simply lowering the temperature; (2) above 45 nm, the cells can not attach well but can easily detached; (3) between 20 and 45 nm, the cells have satisfactory response in terms of attachment and detachment by the temperature switching.

The reasons for the observed phenomena should be attributed to the structure of the PNIPAAm covered substrates. At the initial polymerization stage, the brushes are very uniform to form a dense film structure. Consequently, the ability of water infiltration is largely restricted. Moreover, PNIPAAm defects might exist to expose the substrate, which is more favorable for cell attachment. Therefore, the cells can adhere more tightly,
even at a temperature below the LCST. In contrast, in the very thick PNIPAAm films, the molecular chains are more likely to be inhomogeneously distributed. Those longer chains have larger freedom and mobility, which make the films more hydrophilic. Consequently, the thick PNIPAAm films can repel the cells. When the film thickness is 20–45 nm, the PNIPAAm brushes that are close to the silicon surface are tethered on the substrates in a manner similar to that of the thinner PNIPAAm brushes, i.e., <20 nm. However, the outer layer of the brushes should have an appropriate extent of freedom, and can respond to the temperature switching. Therefore, the brushes with this length and structure have satisfactory performance in terms of cell adhesion and repulsion. In this sense, the multidistributed length of the PNIPAAm brushes is important for the temperature controlled cell adhesion and detachment. It is worth mentioning that Okano and co-workers\(^62\) have reported a narrower range of thickness (20–30 nm) of the PNIPAAm film for the cell adhesion and detachment. In their work, electron beam grafting polymerization was used, resulting in a cross-linked film structure. Moreover, instead of silicon wafer they used tissue culture polystyrene as the substrate, whose surface is rougher, and thus the grafting density is lower. This difference illustrates that the cell adhesion and detachment on the thermal responsive PNIPAAm brushes is also dependent on the fabricating methods. Nevertheless, our approach can produce the thermoresponsive surface with a broader range of thickness for manipulating the cell adhesion and detachment.

4. Conclusions

We have demonstrated in this work that combination of ATRP and a microinjection can produce thermoresponsive surfaces with a gradual variation of the PNIPAAm thickness. Grafting kinetics study reveals that the thickness of the PNIPAAm films increases linearly with the polymerization time, and the sharpness of the gradient can be easily manipulated by experimental parameters such as the injection rate. At room temperature, i.e., below the LCST of PNIPAAm, the surface wettability of the PNIPAAm gradients increases monotonously with increase of the film thickness. However, at 37 °C, i.e., above the LCST, the gradient surface becomes more hydrophobic when the film thickness is smaller than 20 nm, and then turns out to be more hydrophilic along with the increase of the PNIPAAm thickness. When the film thickness reaches 200 nm, the water contact angle can be as low as <10°. Moreover, surface roughness of the gradient film increases slightly along with the increase of the film thickness. At 37 °C, cells can adhere and proliferate on the surfaces with a PNIPAAm thickness of ≤45 nm, but can not adhere on the surfaces with a still higher PNIPAAm thickness (>45 nm). In vitro culture of HepG2 cells obtains the following results: (1) below 20 nm, the HepG2 cells can well attach at 37 °C, but can not be effectively detached by simply lowering the temperature; (2) above 45 nm, the cells can not attach well but can be easily detached; (3) between 20 and 45 nm, the cells can satisfactorily attach and be detached by the temperature switching. Therefore, our approach can produce the thermoresponsive surface with a broader range (20–45 nm) of PNIPAAm thickness for manipulating the cell attachment and detachment.

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