Modification and Characterization Poly (N-isopropylacrylamide-co-acrylamide) Grafted Tissue Culture Surface

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Abstract. Poly (N-isopropylacrylamide-co-acrylamide) (PNIAM-co-AM) is known as a temperature-responsive polymer that is used to promote cell adhesion and detachment by using hydrophobic to hydrophilic transition. ATR-FTIR spectra indicate the different wavelengths between ungrafted and grafted PS at around 1650 cm⁻¹ which is secondary amide from PNIAM. AFM topography showed the differences in topography between ungrafted and grafted PS. The contact angles of the grafted and control TCP surfaces show the hydrophilic-to-hydrophobic transition when the temperature is increased. The ability of PNIAM-co-AM surface to support cell detachment was investigated using MC3T3-E1 preosteoblast.

Keywords: Temperature responsive polymer, Poly (N-isopropylacrylamide-co-acrylamide), UV grafting, Temperature grafting, cell sheet engineering

1. Introduction

Poly (N-isopropylacrylamide) (PNAIM) is widely used in tissue engineering because it displays phase change between hydrophobic and hydrophilic at the lower critical solution temperature (LCST) around 32 °C, which is closed to human physiological temperature. [1]. Above the LCST, PNIAM polymer chains show a compact structure due to its hydrophobic behavior, which allow cell adhesion on the surface. At the temperature below 32 °C, PNIAM chain is extended due to its hydrophilic behavior and cell sheet can detach from the surface without any enzyme treatment[2].

Several methods such as electron beam, plasma polymerization and UV polymerization have been used to graft PNIAM on TCPS. Electron beam (EB) polymerization is a successful technique to graft homopolymer PNIAM. However, electron beam requires expensive equipment. Alternatively, plasma polymerization is used to graft PNIAM on surface by one step method in gas phase, but this technique cannot support in large scale production [3]. The last technique, UV polymerization is proposed as a simple method. It can provide a large-scale tissue culture without involving any expensive equipment. [4].

In this study, the UV activated TCPS surfaces were grafted with PNIAM-co-AM by using UV polymerization. The different factors such as amount of solution, concentration and solution removal method were investigated. In addition, a new grafting method of PNIAM-co-AM on TCPS surface using thermal polymerization was studied. The physical properties of all grafted surfaces were characterized. Cell study analysis was also done in order to examine the effect of cell sheet detachment.

2. Materials and Methods

2.1. Materials
NIAM was purchased from Sigma-Aldrich and was re-crystallized in hexane. Sterile commercial tissue cell culture polystyrene (TCPS) dishes (35 mm x 10 mm) and 6-well plates (Corning) were used without any further treatment. Acrylamide (AM) was purchased from MERCK, N, N’-Methylenebisacrylamide (MBAM) and potassium periodate (KIO₄) were purchased from Aldrich. Ammonium persulfate was purchased from UNIVAR. UV lamp (Handheld UVGL-58, 6 W and 254 nm) was used for photopolymerization.

2.2. Preparation of PNIAM-co-AM grafted TCPS

UV polymerization: TCPS was irradiated by UV lamp (6W, 254 nm) for 30 minutes to activate the surface. 500µL of aqueous solution containing 0.0566g monomer PNIAM, 0.0370g monomer AM, 0.00154g crosslinker MBAM, and 0.000575g photoinitator KIO₄ were added to each TCPS dish, covered with aluminium foil and left overnight at room temperature to equilibrate. After 24 hours, the solution was removed under a vacuum condition to evaporate solution. The dishes were exposed to UV light for one hour.

Thermal polymerization: TCPS was irradiated by UV lamp (6W, 254 nm) for 30 minutes to activate the surface. Monomers were dissolved in de-ionized water under nitrogen atmosphere. At 25 minute while purging N₂, 300µl of aqueous solution containing 0.4526g monomer PNIAM, 0.2936g monomer AM and 0.0123g crosslinker MBAM were added. After all monomers are completely dissolved, 0.0046g of ammonium persulfate was added as a thermal initiator. Copolymers of PNIAM-co-Am were prepared by heating the polymer at 60º C in vacuum oven for two hours.

Samples prepared by both methods were dried at room temperature under vacuum condition for 24 hours and washed with ethanol three times to remove unreacted monomers. Finally, the dishes were dried in vacuum oven for 24 hours.

2.3. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR technique is used to identify the secondary amide group in PNIAM-co-AM grafted sample. Fourier Transform Infrared Spectroscopy (FTIR) Thermo Nicolet 6700 was combined with the accessory of ZnSe (450ºC) Attenuated Total Reflection crystal (ATR). The surface of sample was placed over the ATR crystal in which maximum pressure was pressed on the sample. The FT-IR spectrometer was equipped with KBr beamsplitter. The spectra were recorded in the range of 680-4000 cm⁻¹ with a spectral resolution of 2 cm⁻¹.

2.4. Contact angle Measurement

The dynamic contact angle measurement was used to examine the thermo-responsive nature of the PNIAM-co-AM grafted surfaces by observing the change in surface wettability as a function of temperature. An ungrafted polystyrene surface was used as a control. The sample was kept on an aluminium plate to control the temperature at 40ºC and 10ºC. The samples were allowed to equilibrate 15 minutes before each measurement. The image of water droplet on each sample was captured at three different positions and the contact angles were measured by using ImageJ program.

2.5. Atomic Force Microscopy (AFM)

Atomic Force Microscopy (AFM) machine was used to examine the roughness and the topography of PNIAM-co-AM grafted samples. Atomic Force Seiko Instrument SPA 400 microscope was used to measure the samples in air condition. A long cantilever with a spring constant of 0.06 Nm⁻¹ and a resonant frequency of 10 kHz was used to attach with silicon nitride AFM probe. The surface images were taken by using the tapping mode with a scan rate of 0.5 Hz.

2.6. Cell Study Analysis

Mouse preosteoblast MC3T3-E1 cells (passage 10–20 for all experiments) were provided by Faculty of Medicine, Chulalongkorn University (Thailand). The MC3T3-E1 cell-lines (1 x 10⁶ cells/ml) were seeded onto the sterilized PNIAM-co-AM grafted 35mm culture dish. And they were cultured for 2weeks in alpha-MEM medium. These plates were incubated at 37ºC under a CO₂ (5%) atmosphere to promote cell attachment, spreading and proliferation. Culture media were changed every three days. For cell detachment, non-adherent cells were removed by washing with 2 ml PBS and 3 ml fresh media were added into culture plate. The culture well plates were incubated at 10ºC for increasing the PNIAM hydration. After that, the temperature was increased to 20ºC in order to allow cellular metabolism and accompanying morphological
changes. The cell layers were harvested by gently agitated the surface with culture medium. Cell morphology in each well plate was observed by inverted microscope (Sundrew MCXI600, Vienna, Austria).

3. Result

3.1. Preparation of PNIAM-co-AM grafted TCPS

UV and thermal polymerization of PNIAM-co-AM grafted TCPS was synthesized because they required simple method and inexpensive equipment. Both techniques were prepared with different conditions showed in Table I in order to optimize the uniformity of substrate and cell detachment.

Table I: Outlines the parameters used in synthesis of each PNIAM-co-AM samples.

<table>
<thead>
<tr>
<th>Codea</th>
<th>NIAM/AM (mmol/mL)</th>
<th>Evaporation time (hour)</th>
<th>Amount of solution (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV polymerization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNIAM-AM1-3hr</td>
<td>1:1</td>
<td>3</td>
<td>500</td>
</tr>
<tr>
<td>PNIAM-AM1-5hr</td>
<td>1:1</td>
<td>5</td>
<td>500</td>
</tr>
<tr>
<td>PNIAM-AM1-7hr</td>
<td>1:1</td>
<td>7</td>
<td>500</td>
</tr>
<tr>
<td>Thermal Polymerization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNIAM-AM1-300</td>
<td>1:1</td>
<td>-</td>
<td>300</td>
</tr>
<tr>
<td>PNIAM-AM1-400</td>
<td>1:1</td>
<td>-</td>
<td>400</td>
</tr>
<tr>
<td>PNIAM-AM1-500</td>
<td>1:1</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>PNIAM-AM0.5-500</td>
<td>0.5:0.5</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>PNIAM-AM0.25-500</td>
<td>0.25:0.25</td>
<td>-</td>
<td>500</td>
</tr>
</tbody>
</table>

a Sample code PNIAM-AMX-YZ denotes PNIAM-co-AM grafted dishes with concentration mole ratio (X is NIAM/AM in mmol/mL), evaporation time (Y in hours) and amount of solution (Z in µL).

It indicated that the solution was spread uniformly at 3 and 5 hours and gone at 7 hours left. Thermal polymerization, the uniformity of solution spread on substrate was varied from 300µl, 400µl and 500µl. The results showed that 500µl solution could uniformly spread over the surface.

3.2. Fourier Transform Infrared Spectroscopy (FTIR)

Figure 1 shows the ATR-FITR spectra of un-grafted polystyrene and PNIAM-co-AM grafted on PS samples. The wavenumber of 1652 cm⁻¹ which refers to secondary amide group (-CONH-) [5]. This peak is clearly seen in every sample when compared with the ungrafted PS spectrum.

Fig. 1: ATR-FTIR spectra of PNIAM-co-AM grafted on TCPS by both UV polymerization and thermal polymerization which varying condition at wavelength 1400-2000 cm⁻¹.

3.3. Contact Angle Measurement
The hydrophobic-hydrophilic transition was studied at two different temperatures. Table II showed the ungrafted polystyrene surface exhibits hydrophobic characteristic at both 10°C and 40°C. From UV polymerization, the different evaporation time samples showed the different droplet angles between hydrophobic to hydrophilic transitions. From thermal polymerization, it showed that only mole ratio 1:1 was implied to more hydrophobic to hydrophilic change. Therefore, decreasing the concentration of PNIAM dropped the ability of thermo-responsive property of the grafted surface.

Table II: Contact angles measurement of polystyrene surfaces and PNIAM-co-AM grafted PS surfaces by UV polymerization at different temperature

<table>
<thead>
<tr>
<th>UV polymerization</th>
<th>Thermal polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface</td>
<td>Temp.=40°C&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PNIAM-AM1-3hr</td>
<td>64.74±0.84°</td>
</tr>
<tr>
<td>PNIAM-AM1-5hr</td>
<td>61.26±1.61°</td>
</tr>
<tr>
<td>PNIAM-AM1-7hr</td>
<td>63.46±0.52°</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>68.27± 4.34°</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are expressed as mean±standard deviation; n =3.

3.4. Atomic Force Microscopy (AFM)

AFM images of the ungrafted and PNIAM-co-AM grafted TCP surfaces at different conditions are shown in Figure 2 (a-f). The surface topography of PNIAM-co-AM grafted on TCP surfaces demonstrate the existence of copolymers which are confirmed by the white spots on the grafted TCP surface compared to the image of ungrafted TCP.

![AFM Images](image)

Fig. 2: AFM images of (a) ungrafted TCP (b) PNIAM-co-AM grafted without evaporation, (c) PNIAM-AM1-3hr, (d) PNIAM-AM1-5hr (e) PNIAM-AM1-300 and (f) PNIAM-AM1-500.

3.5. Cell study analysis

Mouse pre-osteoblast MC3T3-E1 cells were culture to achieve strong cell-cell junctions at 37°C. The cell detachment was performed using 30 minutes incubation at 10°C followed by additional incubation at 20°C for 60 minutes. After temperature reduction, MC3T3 cell are detached from PNIAM-AM1-5hr sample by flushing the surface with culture media in figure 3. This detachment was not seen in the control surface. In other samples, cell sheets were not detached because samples were not yielded with the uniform of surface and thickness of copolymer [6, 7].
Fig. 3: PNIAM-AM1-5hr of (A) cell attachment (B) cell detachment by flushing the surface with culture media.

4. Conclusion

In this study, Poly (N-isopropylacrylamide-co-acrylamide) was successfully grafted on PS culture surface from both polymerization techniques which can be confirmed by ATR-FTIR, contact angle and AFM techniques. From cell detachment, MC3T3-E1 cells were detached only on PNIAM-AM1-5hr from UV polymerization technique without detached in thermal polymerization. The uniformity and thickness will be further studied to improve the percentage of cell detachment from both UV polymerization and thermal polymerization of PNIAM-co-AM.

5. Acknowledgement

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6. Reference


