

Cell engineering: nanometric grafting of poly-N-isopropylacrylamide onto polystyrene film by different doses of gamma radiation

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Abstract: Poly-N-isopropylacrylamide was successfully grafted onto a polystyrene cell culture dish and γ -preirradiated in air. In this study, the effect of a γ -pre-irradiation dose of radiation (radiation absorbed dosages of 10, 20, 30, 40 KGy) under appropriate temperature and grafting conditions was investigated. The Fourier transform infrared spectroscopy analysis showed the existence of the graft poly-N-isopropylacrylamide (PNIPAAm) on the substrate. The optimal value of the dose for grafting was 40 KGy at 50°C. The scanning electron microscopy and atomic force microscopy (AFM) images clearly showed that increasing the absorbed dose of radiation would increase the amount of grafting. Surface topography and graft thickness in AFM images of the radiated samples showed that the PNIPAAm at the absorbed dose of radiation was properly grafted. The thickness of these grafts was about 50–100 nm. The drop water contact angles of the best grafted sample at 37°C and 10°C were $55.3 \pm 1.2^\circ$ and $61.2 \pm 0.9^\circ$ respectively, which showed the hydrophilicity and hydrophobicity of the grafted surfaces. Differential scanning calorimetry analysis also revealed the low critical solution temperature of the grafted sample to be 32°C. Thermoresponsive polymers were grafted to dishes covalently which allowed fibroblast cells to attach and proliferate at 37°C; the cells also detached spontaneously without using enzymes when the temperature dropped below 32°C. This characteristic proves that this type of grafted material has potential as a biomaterial for cell sheet engineering.

Keywords: Nanometric grafting, PNIPAAm, polystyrene film, gamma ray, dose, cell engineering

Introduction

During recent decades several materials and medical devices have been produced for medical purposes. Cell sheet engineering has been developed to avoid tissue reconstruction limitations using biodegradable scaffolds or single cell suspension injection.¹⁻⁴ Cell sheets are developed by thermoresponsive culture dishes. Thermoresponsive polymers are grafted to dishes covalently, which allows different cell types to attach and proliferate at 37°C. Cells detach spontaneously without using enzymes when the temperature decreases below 32°C; this is due to the natural specification of the intelligent polymers, and also to the detachment of the cell metabolic changes made by the polymer resulting from decreasing temperature.⁵⁻⁹ Environmentally-sensitive systems or intelligent polymers are those that react to small environmental changes. Those functional polymers that react to their readjustment or physical and chemical changes in the environment are generally known as stimuli-responsive or intelligent polymers. Thermoresponsive polymers

show a balanced and proper hydrophilic-hydrophobic in their structures. They are able to switch on-off the receptor using the transition between the extended and coiled forms of the molecule.^{10–12} Poly-N-isopropylacrylamide (PNIPAAm) and its copolymers are among those materials which have low critical solution temperature. PNIPAAm shows low critical solution temperature at 32°C. While the temperature is over 32°C, the polymer is solid and hydrophobic, but when it is below 32°C it is completely hydrated and shows hydrophilic properties.¹³ Polymer grafting gives considerable thermoresponsive features to surfaces. One of the methods used to create intelligent surfaces is grafting monomers onto polymer surfaces such as polyester (PET) and polypropylene (PP). Chemical and physical polymerization methods such as glow discharge,¹⁴ corona discharge,¹⁵ high energy,¹⁶ ozone,¹⁷ and ultraviolet (UV)^{9,18} have been followed to graft monomers on surfaces. Another important method is γ -radiation diffusion in the material which causes production of secondary electrons, which leads to molecule ionization. Usually on a γ -radiated substrate, free radicals and peroxide groups are produced. During a deoxygenating process using N₂, preirradiated substrates are suspended in a monomer suspension for specific periods of time at the proper temperature.⁸ PNIPAAm and polyacrylic acid are successfully grafted onto different substrates such as polytetrafluoroethylene (PTFE)¹⁹ and polyvinylidene fluoride (PVDF)²⁰ or PET²¹ using γ -radiation. In this study, the monomer of NIPAAm has been grafted onto a polystyrene substrate using different absorbed doses of radiation.

Experiment

Materials

Polystyrene dishes with the dimensions of 1 × 1 cm² and 1 mm thickness, ethanol and methanol (Merck, Whitehouse Station, NJ), N-isopropylacrylamide (NIPAAm) (Sigma-Aldrich, St. Louis, MO), n-hexane (Merck), distilled water, polystyrene, and fibroblast cells (L929) were used in this study. Polystyrene dishes were put in a solution of ethanol-methanol with a 50/50 ratio for 24 hours to dissolve impurities and oils existing on the surface of the dishes. After removal from the solution, the dishes were washed in distilled water. For recrystallization of NIPAAm, 10.3 g of NIPAAm (Sigma-Aldrich) was dissolved in 125 mL n-hexane and the solution was then placed in a refrigerator to make the NIPAAm ready for grafting.

Irradiation

A 60Co- γ -radiation source, supplied by Karaj Atomic Research Centre, Iran, was used for the irradiation of the

samples. The dose rate was 1 KGy/h. Irradiations were carried out in the air under ambient conditions.

Graft polymerization

In this study, 60Co- γ -radiation with a radiating absorbed dose of 1 KGy/h was used. Pre-irradiated polystyrene samples (for 10, 20, 30, 40 hours) were put in a preprepared solution including recrystallized NIPAAm with distilled water which was degassed by nitrogen gas (2 bar mass flow rate) for 20 minutes. This process was done to increase the efficiency of the free radical polymerization (deoxygenation). The samples in this solution were put in a water bath at 50°C for 2 hours; then the samples were brought out, washed in distilled water and put into distilled water for 72 hours, after which they were taken out for analysis. The effect of the γ -radiation dose on the grafting degree was calculated using the following formula: Grafting (%) = $(w - w^0) 100 / w^0$, where w and w^0 indicate the weights of the grafted and ungrafted samples, respectively.

Fourier transmission infrared spectroscopy

The samples were examined by Fourier transmission infrared spectroscopy (FTIR; Bruker-Equinox 55; Bruker Optics, Billerica, MA) before and after adjustment. The samples were scratched into powder and were produced as capsule using KBr, and then, were put to investigation.

Scanning electron microscopy and atomic force microscopy

The surface characteristics of various modified and unmodified films were studied with the help of scanning electron microscopy (SEM; Cambridge Stereoscan, model S-360; Cambridge Instruments, Wetzlar, Germany) to analyze the changes in the surface morphology. The films were first coated with a gold layer (Joel fine coat, ion sputter for 2 hours) to provide surface conduction before their scanning. The surface topology characteristics and the thickness of various modified films as well as the unmodified films were studied with the help of atomic force microscopy (AFM; TMX 2010; Motorola, Schaumburg, IL) to analyze the changes in the surface topology.

Contact angle analysis

The sample surfaces' static contact angles were investigated by a contact angle-measuring device (Krüss G10; Krüss, Matthews, NC) following the sessile drop method.

The formed contact angle was defined as the angle between solid/liquid and liquid/vapor join surface. In order to review the sample surfaces' hydrophilic/hydrophobic behavior at high and low temperatures, the samples were examined at two different temperatures of 10°C and 37°C and the contact angles were measured at these temperatures.

Differential scanning calorimetry

The samples were investigated by thermal analysis using a differential scanning calorimetry (DSC) device (Netzsch DSC 200 F3 Maia®; Netzsch Instruments, Exton, PA) with a heating rate of 5°C per minute from 0°C to 60°C in a nitrogen gas atmosphere.

Cell investigations

The fibroblast cell suspension (L929) from a mouse tail was prepared according to the ISO10993 standard. The fibroblast cell suspension was transferred to a flask (25 cc) containing 5 mL Dulbecco's modified Eagle's medium (DMEM; 2Mm L-glutamine, penicillin [100 lu/mL], streptavidin [100 µL/mL]) and fetal bovine serum (FBS) 10%. The suspension was then placed in an incubator (5% CO₂, 37°C). The fibroblast cells were proliferated in the flask and were washed using FBS/ethylenediaminetetraacetic acid (EDTA). Then the trypsin enzyme/EDTA was added to the flask (4°C), and the flask was incubated for 2 min. The culture media (FBS/DMEM) was added to the flask, and the cells were gently pipetted. The cell suspension was transferred to a falcon tube (15 mL) and centrifuged (1410 rpm) for 5 min. The solution was removed and the precipitation was transferred to a new flask (75 cc) for reculturing. The surface of the samples was well cleaned using cotton and alcohol. Pieces of cell culture (0.5 × 0.5 cm) from the petri dish (control) and the main sample were cut and placed individually in one of the Petri dish wells by using a sterilized pincer. 50,000 cells/well were seeded into a 12-well culture plate, removed by pipette and were poured onto the control and the main samples. Then all of the samples were placed in a Memmert incubator at 37°C for 48 hours. The samples were removed from the incubator and refrigerated at 10°C for 2 hours before being studied through a Nikon Eclipse Ts-100 photonic microscope (Nikon, Tokyo, Japan).

Results and discussion

The pre-irradiated polystyrene samples dosed at 10, 20, 30 and 40 KGy were weighed at a constant temperature.

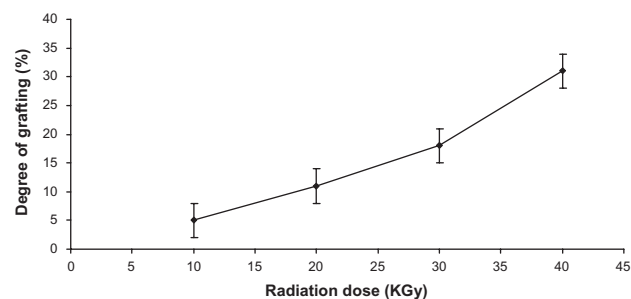


Figure 1 Grafting as a function of dose at a reaction temperature of 50°C.

Figure 1 shows the grafting increase rate versus the increase in radiation dosage. The maximum rate of graft growth on the polystyrene substrate (average 31%) was achieved under the highest dosage of radiation (40 KGy)

Fourier transmission infrared spectroscopy

FTIR spectra results of the regular unadjusted and the γ -radiation-adjusted polystyrene samples are shown in Figure 2. The FTIR spectra of the NIPAAm grafted by the γ -radiated polystyrene are shown in the lower part of Figure 2. The PNIPAAm characteristic points include 1601 cm⁻¹ which indicate -NH groups, 1730–1830 cm⁻¹ which indicate C=O groups, 3025 cm⁻¹ which indicate CH₃ groups, and 3443 cm⁻¹ which indicate NH groups, in

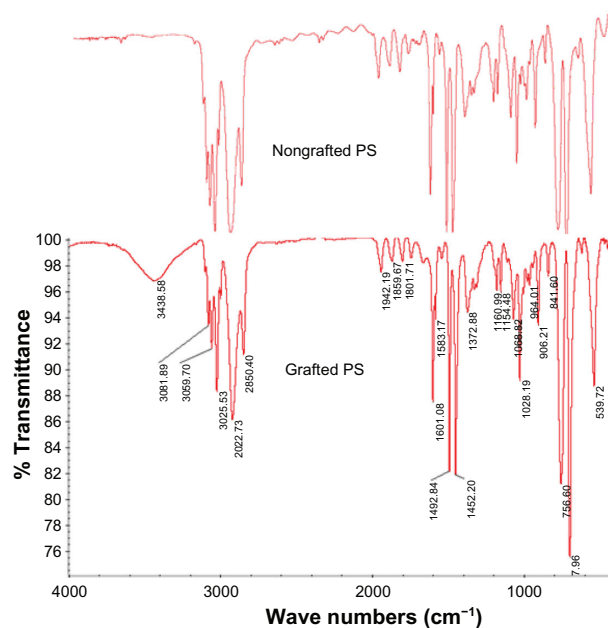


Figure 2 FTIR spectra of the nongrafted polystyrene (upper) and grafted polystyrene treated with γ -rays (radiation dose: 40 KGy) (lower).

Abbreviations: FTIR, Fourier transform infrared spectroscopy.

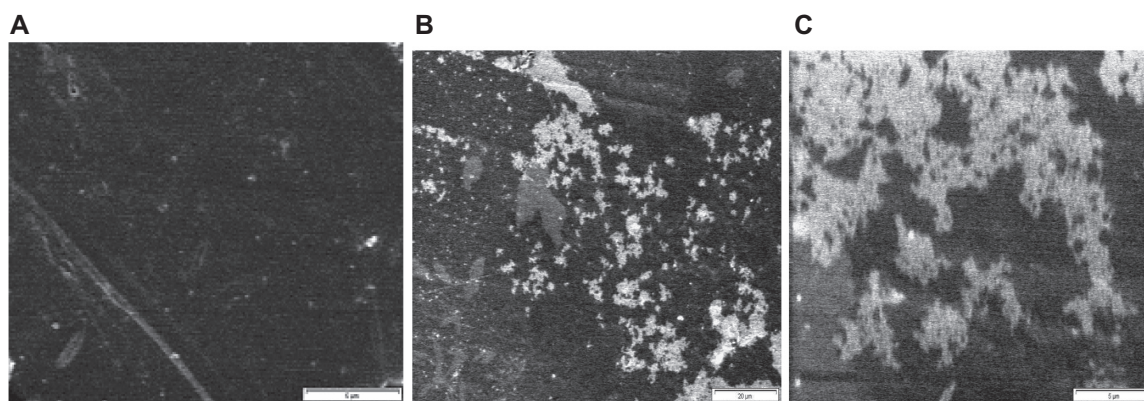


Figure 3 **A)** Scanning electron microscopy of nongrafted polystyrene. Magnification 5000 $\times$. **B)** Scanning electron microscopy of grafted polystyrene under 10 KGy. Magnification 1000 $\times$ (scale: 20 μ m). **C)** Magnification 5000 $\times$ (scale: 5 μ m).

PNIPAAm. All these points are found in the PNIPAAm-grafted polystyrene samples. This demonstrates grafting between PNIPAAm and the polystyrene surface through γ -radiation coating activation.

Scanning electron microscopy and atomic force microscopy

The images for investigating the adjusted samples through γ -radiation are shown in Figures 3–11, which shows the PNIPAAm graft at different radiation dosages in polystyrene. Figure 3a is the SEM image obtained from a normal polystyrene substrate sample; the visible lines indicate slight superficial scratches which are clearly seen in a 5000 $\times$ magnification. The SEM images of the PNIPAAm-grafted samples show the existence of the grafted PNIPAAm on the

polystyrene surfaces. The quantity of the radiated-absorbed dose of 10 KGy is very low, as is clearly shown in Figures 3b and 3c by the number of white spots in the 1000 $\times$ and 5000 $\times$ magnifications of the SEM images of the grafted surfaces. The surfaces' topography and the created graft thickness on the surface as shown in the AFM images (Figure 4) also confirms this statement. The average graft thickness was about 100 nm and the white spots indicate the roughness created during radiation.

Figures 5 and 6 show the AFM and the SEM images of the PNIPAAm-grafted samples under a radiation-absorbed dose of 20 KGy. These images demonstrate that the graft quantity was increased, as is clearly visible in the 1000 $\times$ and 5000 $\times$ magnifications. The topography of the surfaces shown in the AFM images indicates roughness created as a result of PNIPAAm grafting on the polystyrene surfaces. The observed graft thickness in these AFM images is about 45 nm.

Figures 7 and 8 show images of AFM and SEM, PNIPAAm-grafted samples under a radiation absorbed dose of 30 KGy. According to the images, the quantity of the graft has been increased. The surface topography and the created thickness on the surface is shown in the AFM images. These also indicate more rough surfaces which could be the result of a complete PNIPAAm graft on the polystyrene surface. The observed graft thickness in the AFM images is about 90 nm.

Figures 9 and 10 show the AFM and SEM images of PNIPAAm-grafted samples under a radiation absorbed dose of 40 KGy. According to the images the quantity of the graft has been increased. The surface topography and the created thickness on the surface is shown in the AFM images. These also indicate more rough surfaces which could be the result of a complete PNIPAAm graft on the polystyrene surface. The observed graft thickness in the AFM images is about 60 nm.

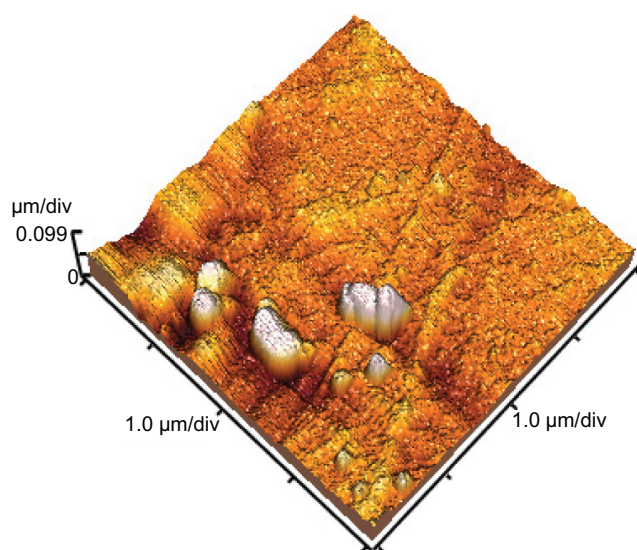


Figure 4 Atomic force microscopy of grafted polystyrene under 10 KGy (scale: 1 $\times$ 1 μ m).

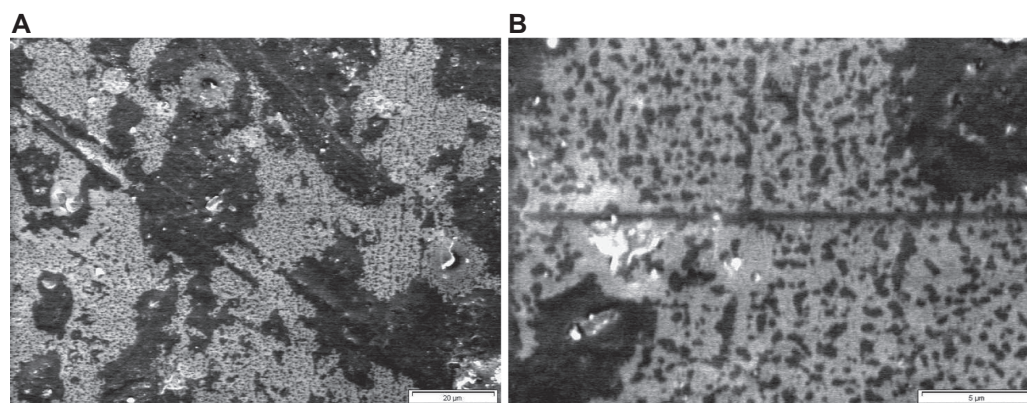


Figure 5 Scanning electron microscopy of grafted polystyrene under 20 KGy. **A)** Magnification 1000 $\times$ (scale: 20 μ m). **B)** Magnification 5000 $\times$ (scale: 5 μ m).

Contact angle analysis

The angles of surface samples of normal polystyrene adjusted by γ -radiated PNIPAAm which were measured at 10°C and 37°C temperatures are shown in Table 1. The data for the best-grafted sample (40 KGy pre-irradiated sample) indicate that, at 10°C and 37°C, the samples show different contact angles, which is also another reason for the existence of PNIPAAm grafting onto polystyrene surfaces.

The contact angle averages of 55.3° and 61.2° were calculated for 10°C and 37°C temperatures. The results indicate a contact angle decrease below 32°C temperature (10°C), and show the hydrophilic surface feature. The contact angle increases above 32°C temperature (37°C), which also shows the hydrophobic surface feature.

Differential scanning calorimetry

The samples were investigated by thermal analysis using a DSC device (Netzsch DSC 200 F3 Maia®; Netzsch Instruments) with a heating rate of 5°C per minute from

0°C to 60°C in a nitrogen gas atmosphere. A review of DSC analysis of the grafted samples indicates the critical temperature for the grafted PNIPAAm. Figure 11 shows the DSC thermogram from which the curve slope at 36°C and 30°C and the critical temperature point at 32°C are obtained. This shows no significant change in the smart polymer critical temperature during the radiation and grafting process.

Cell culture results

The cell attachment and detachment in the grafted polystyrene Petri dish were studied with the fibroblast cells (1929) of mouse tail. Figure 12a shows the growth of the fibroblast cells on the grafted Petri dish. In the images, the grafted sample shows good cell adhesion at 37°C. In Figure 12b the cells have detached spontaneously (cell sheet) from the grafted sample after the temperature dropped below 10°C.

Conclusion

The effect of a γ -radiation dose on polymer grafting in a polystyrene cell culture dish sample was studied in this article. The FTIR spectrum showed the existence of the grafted polymer on the polystyrene surfaces. The imaging and gravimetric analysis of the amount of grafting under radiation dosages of 10, 20, 30, and 40 KGy indicated that an increase in the grafting quantity is achieved by increasing the dose or lengthening the duration of the radiation. Therefore, in this study, optimal grafting was obtained for preirradiated samples under a radiation dose of 40 KGy. Increasing the radiation dosage increased the number of sites and free radicals on the polystyrene surface, which led to an increase in the graft. The SEM images showed the morphology of the grafted surfaces at different absorbed dosages; we could clearly observe and

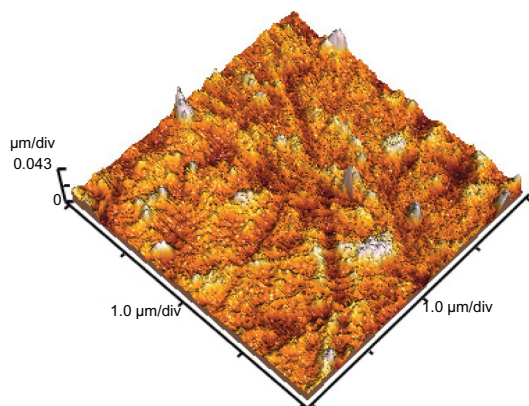


Figure 6 Atomic force microscopy of grafted polystyrene under 20 KGy (scale: 1 $\times$ 1 μ m).

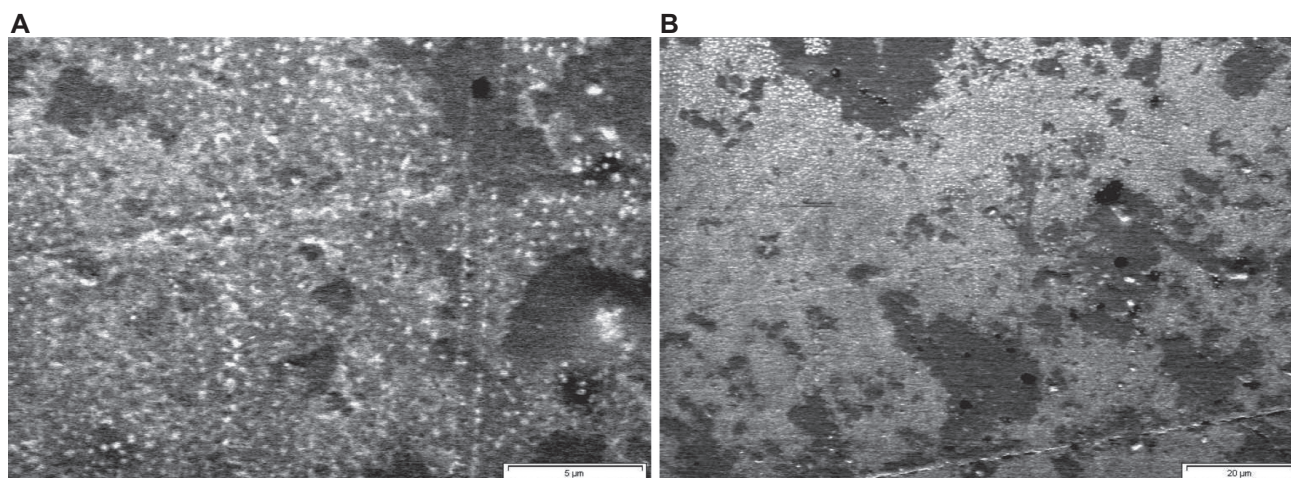


Figure 7 Scanning electron microscopy of grafted polystyrene under 30 KGy. **A)** Magnification 1000 $\times$ (scale: 20 μ m). **B)** Magnification 5000 $\times$ (scale: 5 μ m).

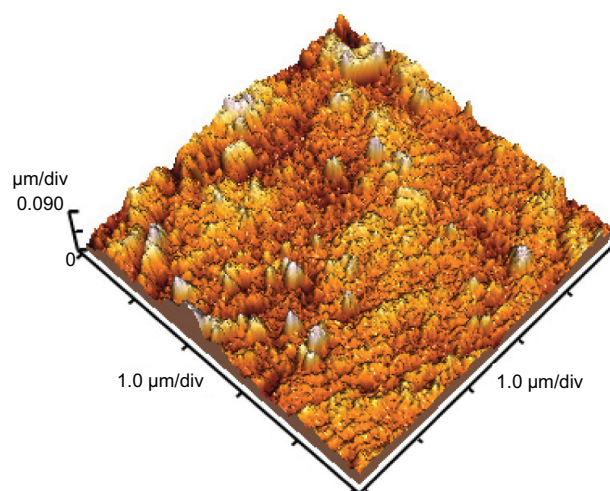


Figure 8 Atomic force microscopy of grafted polystyrene under 30 KGy (scale: 1 $\times$ 1 μ m).

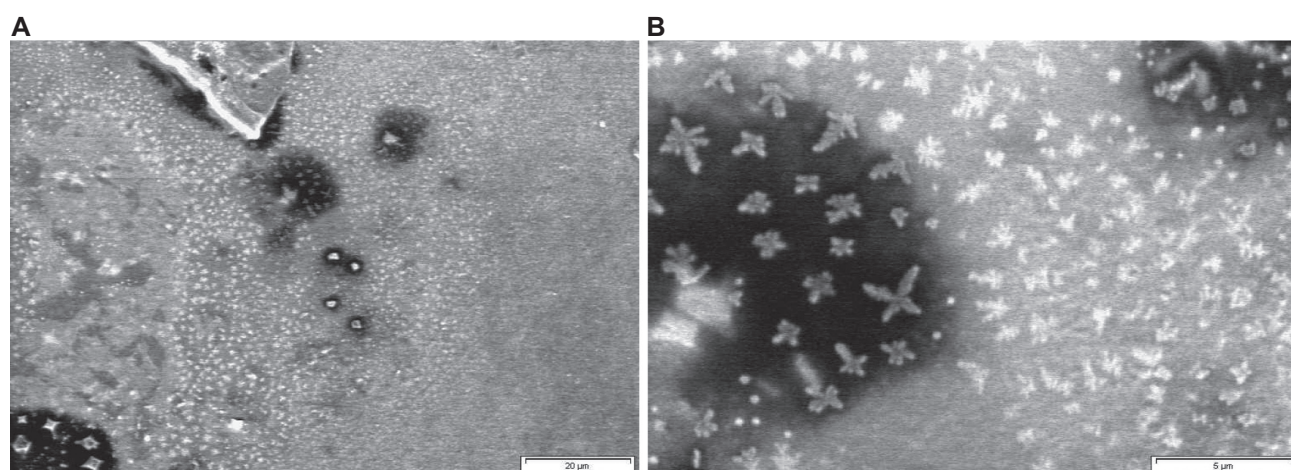


Figure 9 Scanning electron microscopy of grafted polystyrene under 40 KGy. **A)** Magnification 1000 $\times$ (scale: 20 μ m). **B)** Magnification 5000 $\times$ (scale: 5 μ m).

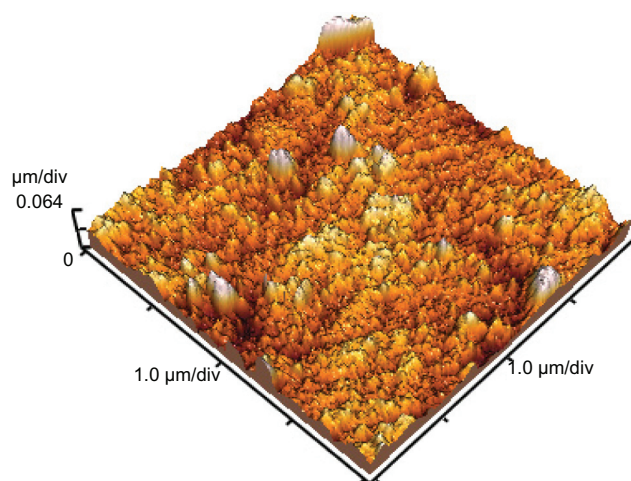


Figure 10 Atomic force microscopy of grafted polystyrene under 30 KGy (scale: $1 \times 1 \mu\text{m}$).

compare our graft increases at higher doses. The topology of the surfaces shown in the AFM images also confirmed this claim. The graft thickness of the study samples was about 50–100 nm, which can be attributed equal to the radiation dose intensity to all samples. The contact angles

Table I Contact angle for normal and grafted samples

T(°C)	$\theta_{\text{H}_2\text{O}}$ of Normal samples	$\theta_{\text{H}_2\text{O}}$ of grafted samples
37	94	$61.2 \pm 0.9^\circ$
10	91	$55.3 \pm 1.2^\circ$

55° and 61° obtained at 10°C and 37°C temperatures, as well as the polymer critical temperature constancy (32°C) measured by the DSC method confirmed that the grafting caused no change in the PNIPAAm operation and function. Thermoresponsive polymers were grafted to dishes covalently, which allowed the fibroblast cells to attach and proliferate at 37°C . Also cells (cell sheet) detached spontaneously when the temperature decreased below 32°C , without using enzymes. This characteristic proved that such a type of grafted material has potential as a biomaterial for cell sheet engineering.

Disclosure

The authors report no conflicts of interest in this work. This project was supported by a formal research grant from the Islamic Azad University, Tonekabon Branch.



Figure 11 Differential scanning calorimetry spectra of the grafted polystyrene by γ -ray (radiation dose: 40 KGy).

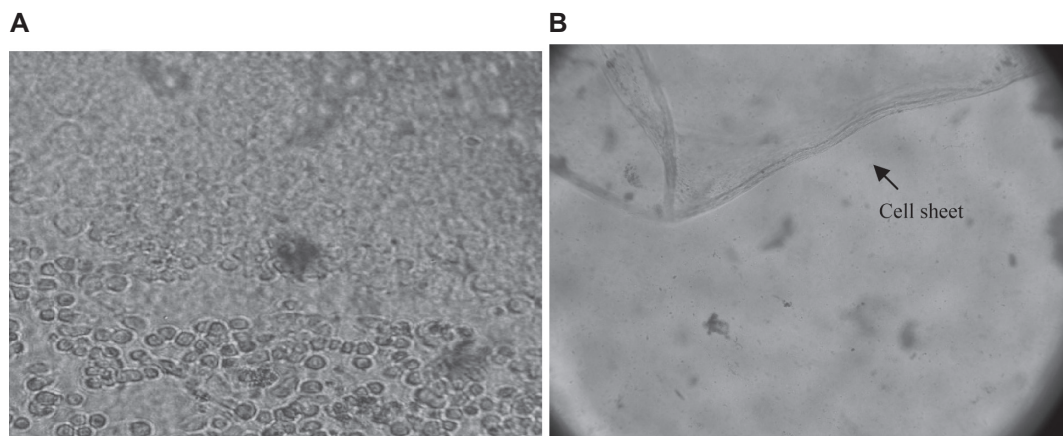


Figure 12 **A)** The growth of fibroblast cells on the grafted Petri dish at 37°C. **B)** the cells detached spontaneously from the grafted sample when the temperature dropped below 10°C.

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