Alternate Method for Grafting Thermoresponsive Polymer for Transferring *In Vitro* Cell Sheet Structures

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ABSTRACT: Retrieval of cells for various applications involves enzymatic or mechanical methods that hamper the cell–cell and cell–extracellular matrix (ECM) binding. Poly(*N*-isopropylacrylamide) (PIPAAm) is a known temperature-sensitive polymer that exhibits a lower critical solution temperature (LCST) around 32°C and is hydrophobic over LCST and hydrophilic below LCST. PIPAAm-grafted culture surface can be used for detaching adhered cells by lowering the temperature below LCST. In this study, polymerization and grafting of PIPPAm was done by gamma (γ) ray irradiation instead of the conventional method of electron beam irradiation. The efficacy of the grafted surface was confirmed by the successful growth of different cell lines such as L-929, NRK-49F, HOS, and SIRC. The cell sheet structures with intact cell–cell and cell–ECM contact was detached by lowering incubation temperature below 20°C. Live–dead staining of cells before and after transfer showed that cell sheet structures maintained viability. This approach of synthesizing thermoresponsive surface by γ-ray irradiation method can be used to culture many other cell types and could be utilized to prepare *in vitro* tissue constructs for bioengineering. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 2245–2251, 2007

Key words: biomaterials; bioengineering; FTIR; irradiation; polystyrene

INTRODUCTION

Different types of scaffolds are available for tissue engineering and regeneration. However, most of them do not allow sufficient cell migration to establish adequate cell–extracellular matrix (ECM) and cell–cell interaction. Anchorage-dependent cells require either mechanical or enzyme method for cell retrieval.[1] The disruption of cell–cell and cell–ECM interactions during retrieval of cells by enzymatic or mechanical methods can be avoided by intact recovery as cell sheets. Studies on polymeric substances responding to pH, salt, and temperature have been investigated for their kinetic and thermodynamic aspects of phase transition behavior.[2,3]

Stimuli responsive polymers have been used to culture and detach cells by changing incubation temperature.[4,5] The well-known polymer, poly(*N*-isopropylacrylamide) (PIPAAm), with a lower critical solution temperature (LCST) of about 32°C, shows temperature-dependent hydrophilicity and hydrophobicity. Owing to its unique property of phase transition in response to temperature, this polymer finds diverse application in drug delivery,[6] tissue engineering,[7,8] and regenerative medicine.[9] Usually polymerization and grafting of PIPAAm (homopolymer) is done by irradiation with electron beam,[10] using sophisticated equipments like electron beam accelerator. Chemical or photopolymerization methods have been used to synthesize copolymers with *N*-isopropylacrylamide (NIPAAm).[11] Thermoresponsive gel without PIPAAm has been synthesized by γ-irradiation polymerization and studied using mammalian cell cultures.[12] In this study, an attempt has been made to make a thermoresponsive surface for cell culture by grafting homopolymer of PIPAAm on tissue-culture dishes by γ-ray irradiation. The grafted surface was used to culture and transfer cell sheets of various cell types such as L-929 (mouse subcutaneous connective tissue fibroblast), NRK-49F (normal rat kidney), SIRC (rabbit corneal epithelium), and HOS (human osteosarcoma) by lowering the temperature.

MATERIALS AND METHODS

N-Isopropylacrylamide (NIPAAm) (Polyscience); Iscoves modified Dulbecco's medium (IMDM), fetal calf serum (FCS), trypsin, penicillin and streptomycin, and FITC-conjugated phalloidin (Sigma, India); and Durapore filter membrane [0.45 μm, hydrophilic polyvinylidene fluoride (PVDF)] (Millipore, India) were used.

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Preparation of PIPAAm-grafted cell culture surfaces

The NIPAAm solution at concentrations of 45% (w/w) in isopropyl alcohol was used to graft tissue culture-grade polystyrene (TCPS) dishes (Nunc). A thin film of monomer was added uniformly and spread evenly. The monomer was polymerized and grafted on the dishes by γ-ray irradiation, using Panoramic Batch Irradiator (Bhaba Atomic Research Center, India). The plates were washed thoroughly with cold, sterile deionized water to remove unreacted monomer and unbound polymer followed by immediate drying in nitrogen atmosphere. The grafted culture dishes were sterilized either by γ-ray irradiation or ethylene oxide and stored at room temperature until use. The ungrafted TCPS dishes were taken as control.

Attenuated total reflection–Fourier transform infra red spectrophotometric analysis of the surface

A Nicolet (Madison, WI) model Impact 410 Fourier transform infra red spectrophotometer (FTIR) and a horizontal attenuated total reflection (ATR) accessory with ZnSe crystal was used to obtain spectrum of TCPS, irradiated TCPS, poly(NIPAAm), and PIPAAm-grafted TCPS. Specific peak representing monosubstituted aromatic ring of TCPS and amide group of PIPAAm was analyzed at 1600 and 1650 cm^{-1}, respectively.

Water contact angle measurements

Water contact angles of grafted and ungrafted surfaces were measured in NRL contact angle goniometer, using deionized water at 37°C and 27°C. A minimum of five different fields were measured from each sample and the mean was calculated.

Surface analysis using scanning electron microscope (SEM)

The grafted and ungrafted surfaces were sputtered with gold using an ion sputter device (Hitachi, E 101). The samples were observed at high magnification using scanning electron microscope (SEM; Hitachi S 2500), and surface morphology was compared.

Cell culture

L-929 cells were procured from American Type Culture Collection (USA) and NRK-49F, SIRC, and HOS were procured from National Center for Cell Sciences (India). Cells were maintained in IMDM supplemented with 5% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C, in a 95% humidified atmosphere with 5% CO_2.

Culture of cells on grafted surface

Cells were seeded on PIPAAm-grafted and untreated culture dishes at a density of 2×10^5 cells/dish. The cultures were incubated at 37°C until subconfluency, with medium change at an interval of 3 days.

Transfer of cells from PIPAAm-grafted surface without enzyme treatment

L-929, NRK-49F, SIRC, and HOS cultures on PIPAAm were transferred by temperature variation without enzyme treatment. After removing excess medium, a PVDF membrane soaked with culture medium was placed over the confluent cell layer. An additional 200-μL culture medium was added over the membrane to avoid drying, and the cultures were incubated at temperatures below 20°C for 15–20 min. During incubation, the cells detached from grafted surface and adhered on to the membrane as cell sheet. The membrane together with the cells were peeled off using forceps and kept on a new culture dish, with the cell side facing down. With an additional 200 μL fresh medium over the membrane, cells were incubated at 37°C for 10–15 min. The cell sheets were transferred from membrane to new surface and were cultured as normal.

Observation under phase contrast microscope, fluorescence microscope, and SEM

Attachment, detachment, and reattachment of cells were monitored under phase contrast microscope (Leica DMIL, Germany).

L-929 cells, on untreated and PIPAAm-grafted surfaces, were fixed with 4% paraformaldehyde and used for staining cytoskeletal proteins using FITC-conjugated phalloidin. Cells were rinsed with phosphate buffered saline (PBS), permeabilized with Triton-X 100, and stained by incubating at room temperature with FITC-conjugated phalloidin for 15 min. After rinsing, cells were mounted on microslides and observed under fluorescence microscope (Nikon, Eclipse E-600) using FITC filter.

The viability characteristics (live–dead staining) of L-929 cells cultured on grafted surface were compared with that of cells on normal surface. Cells were stained with a mixture of acridine orange and ethidium bromide (0.05 μg/mL in PBS) for 1 min. Stained samples were observed under fluorescence microscope (Nikon filter cubes G-2A and FITC). Images captured separately using different filters were superimposed to get live–dead staining. To observe whether cell transfer manipulation affects viability, HOS cells transferred to new surface and cultured for 24 h was also subjected to live–dead staining as described earlier.

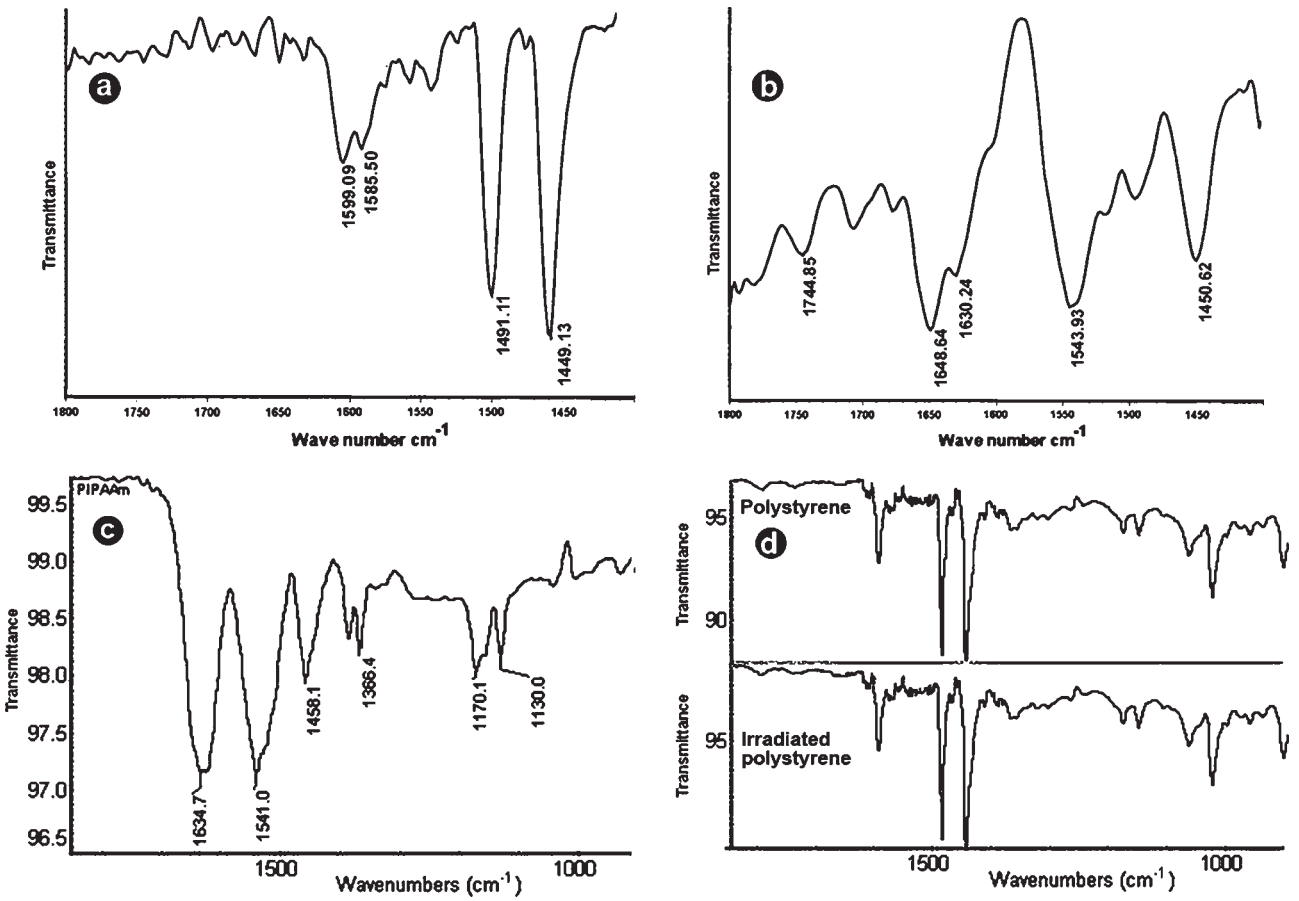

Figure 1 ATR FTIR spectrum of TCPS (a) depicting the peak of monosubstituted aromatic ring at 1600 cm^{-1} and PIPAAm-grafted TCPS (b) showing the amide peak around 1650 cm^{-1}. The FTIR spectrum of (c) polymerized NIPAAm showing amide peak at 1650 cm^{-1}. Comparison of (d) FTIR spectrum of polystyrene before and after γ irradiation. There is no degradation of polystyrene due to irradiation.

For SEM analysis, cells cultured on TCPS and PIPAAm-grafted TCPS, and fixed in glutaraldehyde (3% in 0.1*M* phosphate buffer) were dehydrated in graded alcohol followed by critical point drying (Hitachi, HCP-2). The samples were gold-coated and observed under SEM.

RESULTS AND DISCUSSION

Thermoresponsive PIPAAm, having LCST around 32°C in aqueous medium, is being used for various biomedical research and applications. Poly(*N*-isopropylacrilamide) finds application as attached to solid surface, crosslinked hydrogels, and biomolecules to create new grafted surfaces, comb-type grafted hydrogels, and modified thermoresponsive bioconjugates.[13] The polymer expresses temperature-dependent wettability changes in the form of hydrated extended chain conformation below LCST and dehydrated compact form above LCST. PIPAAm crosslinked with copolymers has been developed and used as thermal on–off switching surface for drug loading and releasing.[14] Poly(*N*-isopropylacrilamide) exhibits hydrophilic and hydrophobic swelling changes in response to small temperature variation. When grafted on solid surfaces, the polymer exhibits changes in surface properties.[15]

Generally, surface modification with addition and blending processes has problems like leaching out of additives.[16] Hence grafting methods are preferred due to simplicity of procedure and good surface modification. Different polymerization and grafting method has been studied to synthesize thermoresponsive culture surface. Chemical methods and UV irradiation have been used for synthesizing/modifying thermally reversible PIPAAm copolymers for cell culture applications.[11,17,18] Such procedures require additional entities like polymerization initiators. Only method reported to graft PIPAAm homopolymer for cell culture application is by using high-energy electron beam irradiation.[10]

In this study, PIPAAm was grafted on TCPS by γ irradiation to obtain thermoresponsive surface for culture of different cell lines and their retrieval. This method has the advantage of being the most commonly used sterilization method instead of previously

reported method with electron beam, thereby eliminating the need for sophisticated equipment like electron beam accelerator.[19] This method also avoids the necessity of other copolymers as in the case of photopolymerization and chemical synthesis.

The thermoresponsive surface enables to acquire cells from their culture environment with intact cellular arrangements and organizations as designed *in vitro*. Zammaretti and Jaconi[20] reviewed the different approaches for cardiac tissue engineering, revealing significance of thermoresponsive surface for such purpose. During detachment as a confluent layer from temperature-sensitive surface, the cells maintain interactions with cells and ECM, whereas ECM interaction with grafted surface decreases.

The grafting was characterized by ATR-FTIR, and thermoresponsiveness was assessed by water contact angle and cell retrieval. The ATR-FTIR spectrum of surface confirmed the grafting of PIPAAm on culture plate by the presence of amide peak at 1650 cm^{-1}, instead of the monosubstituted aromatic ring peak of TCPS at 1600 cm^{-1} [Fig. 1(a,b)]. The amide peak at 1650 cm^{-1} on grafted TCPS was also compared with the FTIR spectrum of polymerized NIPAAm [Fig. 1(b,c)]. The presence of amide peak at 1650 cm^{-1} on the grafted TCPS by γ-ray method conforms to earlier reports.[21] To analyze possible degradation of TCPS by γ irradiation, FTIR spectrum of culture dishes before and after irradiation were compared [Fig. 1(d,e)]. Both spectra were identical, indicating that there was no degradation of polystyrene by γ-irradiation. SEM is used as an important tool for analyzing the surface characteristics of polymeric biomaterials.[22] The surface morphology of the grafted and unmodified TCPS, evidenced by SEM, did not show any alteration on the surface (Fig. 2). Contact angle measurements on the normal and grafted culture surfaces showed the hydrophilic–hydrophobic changes with variation in temperature. At 37°C, water contact angle measurement of polymer-grafted surface showed relatively hydrophobic contact angle ($\theta = 35.8^\circ \pm 1.61^\circ$) when compared with contact angles at lower temperature of 27°C ($\theta = 32.65^\circ \pm 1.02^\circ$). The contact angles of untreated culture dishes were almost same ($\theta = 40.6^\circ \pm 2.5^\circ$) when compared with that at lower temperature of 27°C ($\theta = 39.55^\circ \pm 2.99^\circ$).

The difference in the contact angle is due to the temperature-influenced hydration of PIPAAm-grafted surface. The grafted surface was more hydrophilic below room temperature when compared with ungrafted surface. The results of water contact angle from different areas of grafted surface confirm the even grafting of polymer using γ-ray irradiation method.

L-929, NRK-49F, SIRC, and HOS cell lines have been cultured on temperature-responsive PIPAAm homopolymer for nonenzymatic cell-sheet retrieval. Cells seeded on grafted surfaces adhered, spread, and proliferated to form monolayer (Fig. 3). The surface was found to be noncytotoxic to cells during culture time, and the cells showed similar morphology as on control TCPS.

The cell adhesion is an important factor for cell morphology, spreading, and proliferation that greatly depend upon the surface properties of biomaterial.[23] To study the cell material interaction, SEM has been used for assessing cellular responses to biomaterials.[24] Under SEM, L-929 cells revealed intact morphology and similar adhesion pattern of cells on PIPAAm surface in comparison with cells on ungrafted TCPS [Fig. 4(a,b)].

Cytoskeletal organization of L929 cells on PIPAAm-grafted TCPS under fluorescence microscope showed normal pattern [Fig. 4(c,d)]. The morphological feature of cell lines in this study on grafted surfaces were comparable to cells cultured on TCPS, as demonstrated by actin filament distribution.

Retrieved L929 cell sheets structures from PIPAAm surface were also stained for cytoskeletal organization. Cells in the folded cell sheet demonstrated cortical staining [Fig. 4(e)]. Cortical staining of cells is

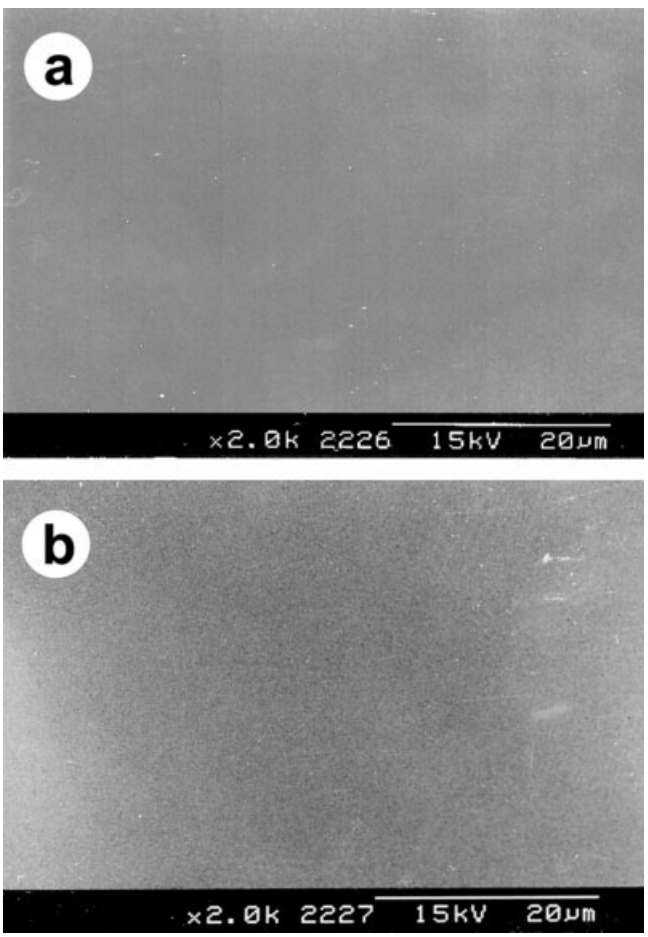

Figure 2 SEM of (a) ungrafted and (b) grafted culture dish showing no alteration in surface morphology.

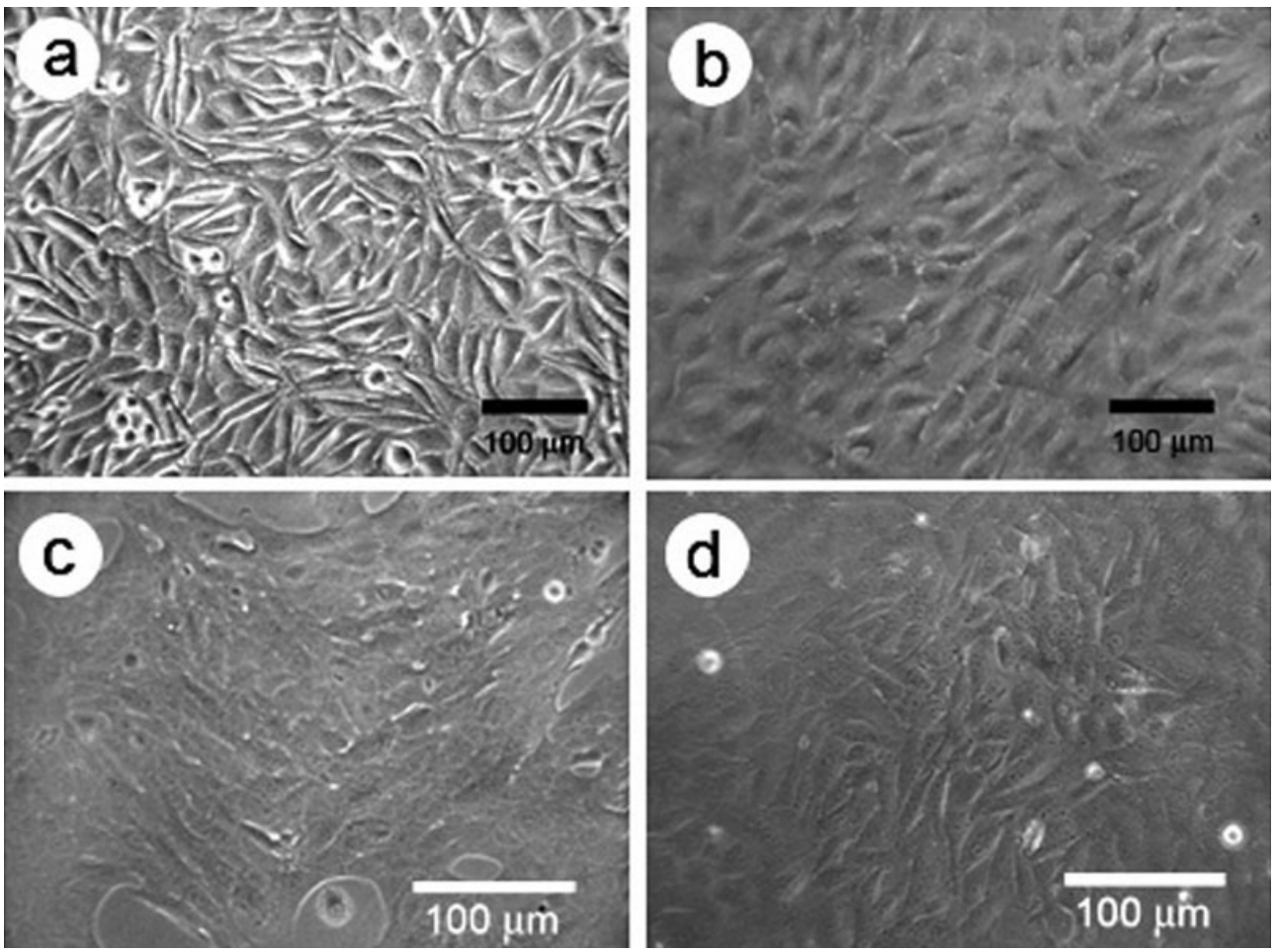

Figure 3 Morphology of (a) L-929, (b) NRK-49F, (c) SIRC, and (d) HOS cells on PIPAAm-grafted surface. The morphology is similar to that of cells cultured on ungrafted TCPS.

noticed during low adhesion to substrate.[25] Hence it can be deduced that the cortical pattern observed is due to the nonadhered stage of individual cells in sheet, which confirms the maintenance of cell–cell and cell–ECM binding of cell sheets. It has been reported that the adhesive strength of monolayer on

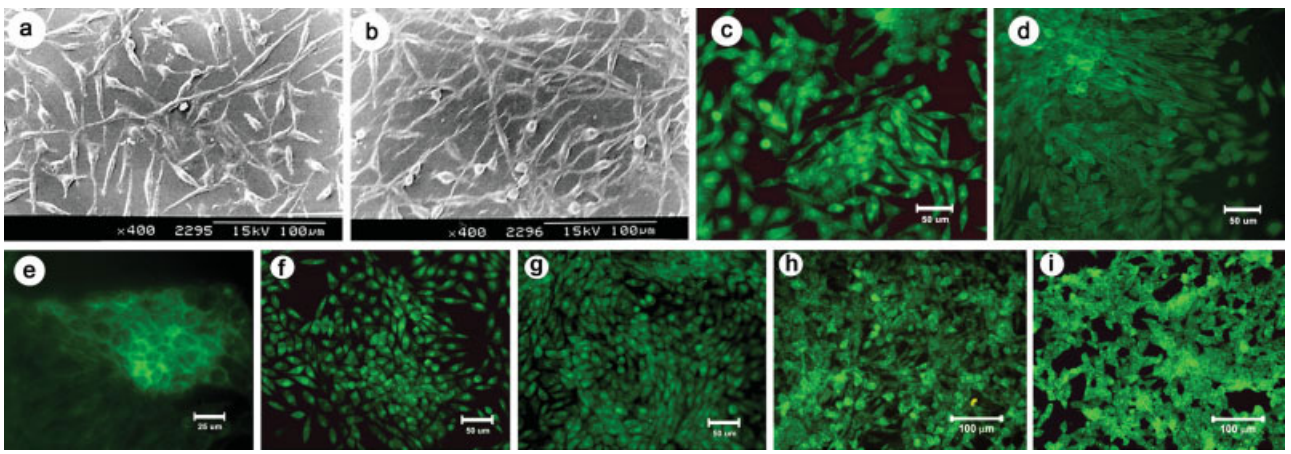

Figure 4 Images (a–g) L-929 cells and (h,i) HOS cells. SEM showing (a) ungrafted surface and (b) grafted surface. Actin cytoskeletal staining of cells on (c) TCPS and (d) grafted TCPS and (e) cortical staining pattern of cell sheet. Viability staining of cells on (f) TCPS and (g) PIPAAm-grafted surface showing live cells (green) and dead cells (red). Live–dead staining of (h) HOS cells on PIPAAm and (i) transferred cell sheet grown for 24 h showing 100% viability. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

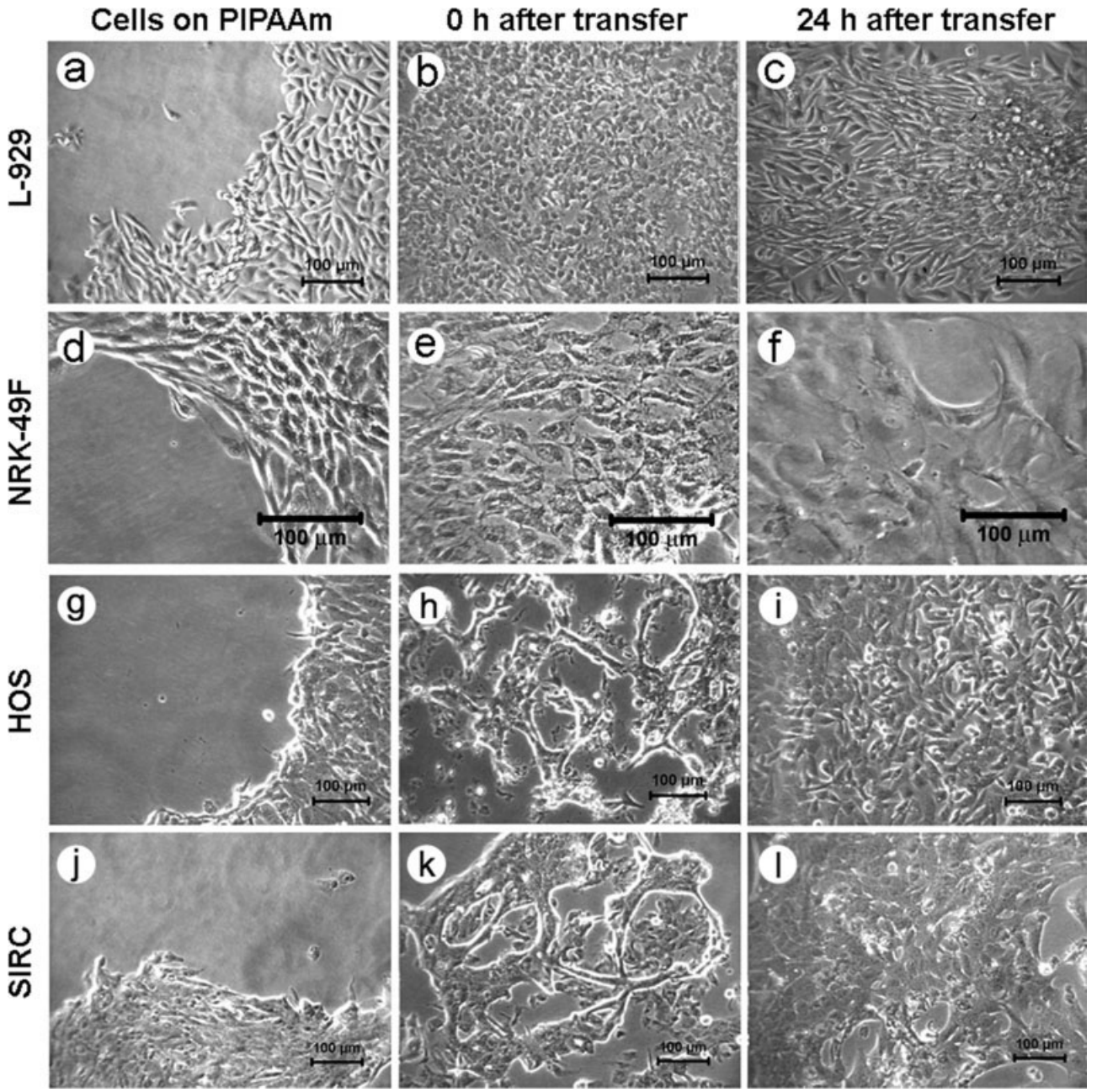

Figure 5 Cell sheet transfer with the help of membrane support. Images in each row show the removed cell monolayer, transferred cell sheet at 0 h, and transferred cell sheet after 24 h, respectively. Cell lines used were L-929 (a, b, and c), NRK-49-F (d, e, and f), HOS (g, h, and i), and SIRC (j, k, and l).

copolymer form of PIPAAm-grafted culture dishes reduces considerably below LCST.[26]

Live–dead staining of L929 cells on PIPAAm surface established that all cells were viable similar to cells on ungrafted surface [Fig. 4(f,g)]. To detect whether the transferred cell sheet maintained viablility, transferred HOS cells were assessed for viability after 24 h. Live–dead staining of transferred HOS cell sheets maintained 100% viability [Fig. 4(h,i)].

Transfer of L-929, NRK-49F, SIRC, and HOS cell monolayer was done from grafted surface (Fig. 5) by incubating at low temperature below 20°C, with the hydrophilic PVDF membrane on it. Low-temperature initiated hydration of the surface and thereby cell detachment, and confluent cells adhered to the membrane, retaining cell–cell contacts. The cells at confluent stage was removed along with membrane [Fig. 5(a,d,g,j)]. Upon reincubation at 37°C at the new surface, the cells from the membrane got adhered as intact cell sheet construct [Fig. 5(b,e,h,k)]. Observation of cells at 0 h showed the transfer of intact cell patch to new surface. The transferred cells on new dishes grew with normal morphology [Fig. 5(c,f,i,l)] within 24 h. Cell attachment on hydrophobic surface

involves tight binding of various proteins like albumin, myoglobin, and fibronectin on which the cells attach.[27] Culture of cells on PIPAAm-grafted surface above LCST is similar to that of cultured cells in normal condition. During low-temperature incubation below 20°C, all cell types like L-929, NRK-49F, SIRC, and HOS cultured on PIPAAm detached without any enzyme treatment. Low temperature helps in detachment of intact cell sheet structures due to increase in hydration of surface, as evidenced by the water contact angle of PIPAAm. Kim et al. showed the osteoblast cell sheet manipulation with PIPAAm-based copolymer-grafted culture surface.[28] Here we studied osteoblast cell sheet transfer from γ-irradiated homopolymer of NIPAAm-grafted culture surface. Maintenance of intact cell sheet structures with cell–cell and cell–ECM contact has been proved to be useful in enhanced cellularization of porous scaffolds for tissue engineering applications.[8]

CONCLUSIONS

Cell culture on PIPAAm-grafted surface avoids the routine cell detachment procedures like mechanical or enzyme treatments, which can alter the cell architecture. By using PIPAAm-grafted temperature-responsive surface synthesized by γ irradiation, cultured cells can be harvested without deprivation of differentiated functions. From the earlier results, it is clear that PIPAAm-grafted surface on TCPS synthesized by alternate γ-ray irradiation method is comparable to the surfaces synthesized by electron beam irradiation. Moreover, γ-ray irradiation facilitates batch processing for polymerization and grafting of PIPAAm. Acquiring cells as monolayer make it possible to manipulate to form three-dimensional tissue lattices. The use of such surface can further help in creating multilayered tissue construct using different cell types. This might be useful in tissue engineering applications as a cell transplant tool for construction of multilayered cell sheets with different cell types.

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