A Novel Thermoresponsive Graft Copolymer Containing Phosphorylated HEMA for Generating Detachable Cell Layers

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ABSTRACT: A novel polymeric formulation based on *N*-isopropylacrylamide (NIPAAm), methylmethacrylate (MMA), and phosphorylated hydroxylethyl methacrylate (Phosp-HEMA) was synthesized and characterized. NIPAAm was copolymerized with a known quantity of MMA to form a poly(NIPAAm–MMA) copolymer and was subsequently grafted with Phosp-HEMA by gamma irradiation to a total dose of 0.5 kGy. The thermoresponsive graft copolymer was characterized by differential scanning calorimetry, Fourier transform infrared spectroscopy, contact angle measurements, and energy dispersive X-ray analysis. The cytotoxicity of the graft copolymer analyzed using L-929 fibroblast cells showed noncytotoxic response. The cell adhesion on the graft copolymer was studied using rabbit corneal cells (SIRC) and human osteoblasts (HOS). The adhered cells were found to spread leading to the formation of cell layers. The cell layers with intact cell–cell and cell–extra cellular matrix contact were detached by lowering temperature below the lower critical solution temperature (29°C) of the graft copolymer. The viability and morphology of the cells in detached cell sheets were assessed by live dead staining and environmental scanning electron microscopy, respectively. This interesting feature of cell adhesion to form cell layers and cell sheet retrieval is implicit to be due to the properties of phosphate moieties on thermoresponsive copolymer. To the authors knowledge there is no previous report on phosphate moiety containing thermo responsive polymeric formulations which can modulate cell adhesion and cell sheet retrieval. J Appl Polym Sci 115: 52–62, 2010

Key words: graft copolymer; Phosp-HEMA; cell culture; cell material interaction; tissue engineering

INTRODUCTION

Tissue engineering is a promising and fast growing approach to restore, maintain, or replace tissue functions destroyed by disease or injury. Primarily, this technique requires a suitable matrix as scaffold to engineer new tissues from isolated cells. The paramount requirement of a suitable polymer to be used as scaffold for such application is its biocompatibility together with the functional requisites, namely the ability to adhere cells, encouraging proliferation and detachment of the cell layer without the usage of proteolytic enzymes as these enzymes can damage cell–cell and cell–ECM (extra cellular matrix) interactions. Despite the wide use of polymers as biomaterials, many of them lack the required functional properties to interface with cells so as to promote cell growth. Therefore, there is an increasing need for the design and development of novel materials capable of modulating the interaction of biological components including cells with their surfaces in a desired fashion. The importance of biomaterials for cell culture as well as for the production of artificial tissues and organs has become increasingly evident in recent years. Regulation of cellular functions is extremely important. For achieving this goal bioactive components are immobilized onto the cell culture substrate. Substrates functionalized with appropriate bio molecules are known to encourage cell adhesion, spreading and proliferation. Synthetic materials possessing the functional features of biological substrates are having interesting applications in the rapidly evolving area of tissue engineering.

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Thermo responsive polymer such as poly(*N*-isopropylacrylamide) [PNIPAAm] has been used as an excellent substrate for cell culture applications.[1,2] In aqueous environment, these materials exhibit a lower critical solution temperature (LCST). PNIPAAm is water soluble and hydrophilic, exhibiting an extended chain conformation at temperature below its LCST, yet undergoing a phase transition to an insoluble and hydrophobic aggregate at temperature above its LCST. The phase transition occurs within a narrow temperature range and is reversible.[3] Because of its unique property of phase transition in response to temperature, this polymer finds diverse application in drug delivery, tissue engineering and regenerative medicine.[4,5] This peculiar ability of the polymer can be utilized to retrieve cells by temperature variation. Cells cultured on thermo responsive polymer detach easily when the temperature is lowered below the LCST avoiding the use of proteolytic enzymes that can damage the cells.[6] Thermo responsive PNIPAAm-grafted surfaces have been used for the effective detachment of hepatocytes and endothelial cells without cell damage.[7] Cell adhesion and growth on PNIPAAm gel has been reported to be influenced by incorporation of biocomponents.[8] A-B type block copolymers consisting of a PNIPAAm segment and a hydrophobic segment can change the LCST. Recently, Zhou et al. synthesized copolymers of NIPAAm and methyl methacrylate (MMA) to produce a gel with desired temperature transitions.[9] Even though poly(2-hydroxyethyl methacrylate) (HEMA) is a widely known biocompatible material, it is less supportive to cell adhesion due to the presence of hydrophilic entities. Different Phosphate moieties are known to be present in many biological components including cell membranes. It is reported that cytocompatibility of pHEMA can be improved by blending with other polymers like poly(caprolactone).[10] We reasoned that graft copolymer based on NIPAAm, MMA and phosphorylated hydroxyl ethyl methacrylate (Phosp-HEMA) would be an interesting material which facilitate cell adhesion and the subsequent growth. To the authors knowledge there is no previous report on phosphate moiety containing thermo responsive polymeric formulations which can modulate cell adhesion and cell sheet retrieval. The present study was carried out with main objectives,[11] i.e., To synthesize a novel polymeric substrate containing NIPAAm, MMA, and Phosp-HEMA which has thermo responsive property to avoid use of proteolytic enzymes, to have a desired LCST suitable for the biological system and to mimic the biological milieu by incorporation of phosphate moiety and[12] to study the efficacy of the novel polymeric material for cell attachment and detachment using different types of cells.

MATERIALS AND METHODS

Materials

N-Isopropylacrylamide (NIPAAm), 2-hydroxyethyl methacrylate (HEMA), Methyl methacrylate (MMA) and 2,2′-azobis (isobutyronitrile) (AIBN) were obtained from Sigma-Aldrich Chemicals, India. Other chromatographic grade solvents were purchased from Spectrochem, India. Minimum essential medium (MEM), fetal calf serum (FCS), 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypsin, penicillin, streptomycin, acridine orange, and ethidium bromide were from Sigma Chemicals, India. Rabbit corneal cells (SIRC), and human osteoblast (HOS) were procured from National Center for Cell Sciences, India and mouse subcutaneous fibroblast L-929 cells were procured from American Type Culture Collection. Cells were maintained in MEM supplemented with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a 95% humidified atmosphere with 5% CO_2.

Synthesis of Phosp-HEMA

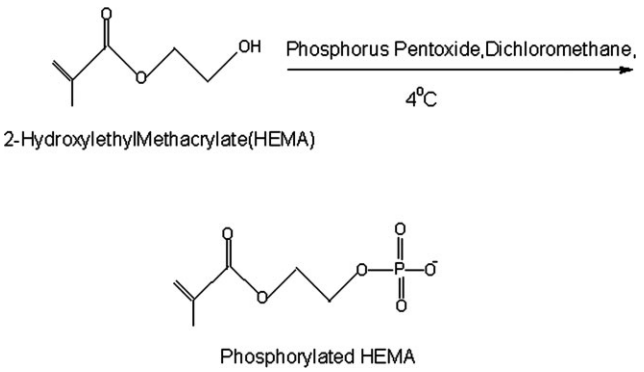
Phosphorylated HEMA was prepared using the method described by Swart et al.[13] Briefly, 9.17 g HEMA was added drop wise over 90 min to a stirred suspension of 10 g phosphorus pentoxide in 100 mL dichloromethane while maintaining the reaction temperature between 0 and 5°C (Scheme 1). Stirring was continued for further 90 min until the reaction mixture reached room temperature. The Phosp-HEMA was obtained as viscous yellow oil and the solvent was removed in a rotary evaporator. The Phosp-HEMA was stored at 4°C until use.

Synthesis of copolymer of NIPAAm and MMA

NIPAAm (2 g) and MMA (0.1 g) were copolymerized in presence of AIBN in benzene at 60°C as depicted in Scheme 2. After complete polymerization, the polymer was dissolved in iso-propanol and subsequently precipitated in *n*-hexane. The process was repeated 3 or 4 times to remove the unreacted monomers. The precipitated copolymer was dried under vacuum at room temperature for 24 h. Then it was dissolved in iso-propanol and was cast in tissue culture grade petri dishes (35 mm diameter) and glass cover slips (10 mm diameter). The coated dishes and coverslips were washed with cold sterile deionized water 3–5 times.

Synthesis of graft copolymer of Phosp-HEMA and NIPAAm-MMA copolymer

The copolymer coated on tissue culture dishes and glass cover slips were dried in a hot air oven at a temperature range of 65–75°C and cooled to room



Scheme 1 Schematic diagram of synthesis of Phosphorylated HEMA.

temperature. Sufficient quantity of Phosp-HEMA was added to the dried copolymer and allowed to stand at room temperature for 4 h. This allowed the copolymer to swell and excess Phosp-HEMA was removed. The samples were then exposed to γ-ray irradiation from a Panoramic Batch irradiator (BARC, India) to a total dose of 0.5 kGy at a rate of 0.2 Mrad/h. The polymer-grafted surface was rinsed many times with ice cold deionized water to remove traces of unreacted monomer and unbound polymer. The extent of grafting on NIPAAm-MMA coated sample was calculated by measuring the weight before and after grafting with Phosp-HEMA. The modified culture dishes were then sterilized by either γ-ray irradiation or ethylene oxide and stored at room temperature until use.

Fourier transform infrared spectra

The infrared (IR) spectra were recorded on a Nicolet (Madison, WI) model impact 410 Fourier transform IR (FTIR) spectrophotometer. The polymer was placed on a potassium bromide window and FTIR spectrum was obtained in the range 400 cm^{-1} to 4000 cm^{-1} from 50 scans.

Energy dispersive x-ray analysis

Energy dispersive X-ray analysis (EDS) was performed using an EDX model 6051 SP (Oxford Instruments, UK) attached to the Scanning electron microscope. Before the measurement, a thin layer of gold was coated on the surface of the polymers.

Lower critical solution temperature measurement

Lower critical solution temperature of the copolymers was determined using Differential Scanning Calorimetry (DSC) (DSC 2920 TA Instruments, Delaware) with TA 4000 controller as reported earlier.[14] Before the measurement, the samples were immersed in deionized water at room temperature and allowed to swell to equilibrium. About 10 mg of equilibrium swollen sample was sealed hermitically and thermal analysis was performed from 25°C to 55°C at a heating rate of 10°C/min in nitrogen atmosphere and LCST was recorded. The samples were cooled to 25°C and reheated to 55°C to understand any change in the thermal history.

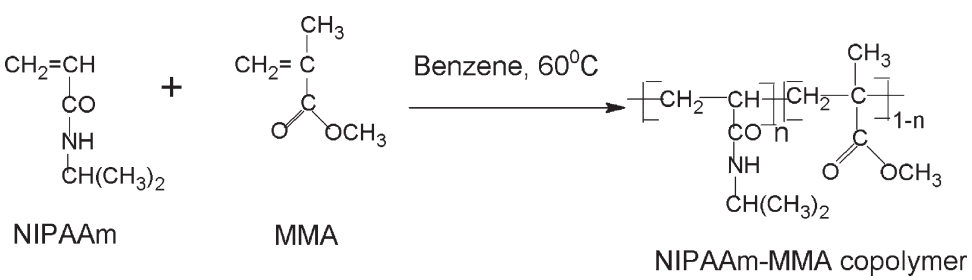
Copolymers containing different compositions of NIPAAm and MMA were synthesized. The polymer with NIPAAm and MMA in 20 : 80 ratio was used for this study considering its lower critical solution temperature (LCST).

Water contact angle measurement

Water contact angle (WCA) was determined using OCA15 plus Video-based contact angle measuring device (Data physics, Germany) to characterize the relative hydrophilicity or hydrophobicity of the polymeric surface. After dispensing a water drop on polymer surface, measurements were carried out under constant temperature: (23°C). The contact angles on the left and right side of the drop was recorded to calculate the mean. Multiple samples were used and a minimum of three different fields from each sample were analyzed.

Profilometry

Profilometry is the technique used to measure the coating thickness as well as to assess the surface profile. The surface profile was assessed using Talysurf CLI 1000. A surface of 12 × 2 mm^2 was measured at a speed of 50 μm/s using high resolution non contact Confocal point Guage (CLA) with a range of 300 μm.



Scheme 2 Schematic diagram of synthesis of NIPAAm-MMA copolymer.

The thickness of graft copolymer was determined by manual adjustment of the step height measurement in the software with a guassian filter of 25 μm from four different fields from each sample.

Cell culture studies

Cell culture studies were done with cell lines HOS, SIRC, and L-929. Cytotoxicity, cell adhesion, and cell sheet retrieval from graft polymer-coated surface was analyzed. Viability and transfer efficacy of cell sheet to new surface was also studied.

Cytotoxicity evaluation of graft copolymer

Cytotoxicity of the material was assessed by direct contact method based on ISO10993.5 using L-929 fibroblasts.[15] Briefly, 1×10^4 cells were seeded per well to a 24 well plate and incubated in CO_2 incubator at 37°C with 5% CO_2 and 95% humidity, until a monolayer is formed. Triplicate samples of polymer-coated cover slips were kept on cell monolayer such that polymer makes direct contact with the cells. ZdEC containing polyvinyl chloride (PVC) and ultra high molecular weight poly ethylene (UHMWPE) were used as positive and negative controls, respectively. The cultures were incubated as mentioned earlier and the cell response was evaluated at the end of 24-h incubation by observing under an inverted phase contrast microscope (Leica DMIL, Germany).

Cell adhesion on graft copolymer

The suitability of graft copolymer for cell adhesion was studied using SIRC and HOS cells. To graft copolymer coated 35 mm diameter culture dishes, cells were seeded at a density of 2×10^5 cells/dish and incubated for 72 h at 37°C in a 95% humidified atmosphere with 5% CO_2. The cells seeded on normal tissue culture dishes (TCPS) was taken as control. Cell adhesion was evaluated under inverted phase contrast microscope.

Cell proliferation on graft copolymer

NIPAAm-MMA copolymer and NIPAAm-MMA-Phosp-HEMA grafted on 35-mm culture dishes were used for cell proliferation study. Approximately 7×10^3 cells/mL were seeded on modified surfaces to compare the effect of phosphorylation on NIPAAm-MMA Cell activity was estimated at 24 h and 48 h by MTT assay. Briefly, cells on modified surfaces were incubated with serum free medium containing 0.5 mg/mL MTT for a minimum of 4 h at 37°C. After discarding MTT reagent, cells were rinsed with PBS and incubated with isopropanol for 20 min in a shaker incubator at 70 rpm. The contents were transferred to a 96 well plate and quantitatively estimated in a multiwell plate reader at 540 nm. The results were compared and the significance was calculated using *t*-test.

Cell sheet retrieval from graft polymer-coated surface

Approximately 2×10^5 HOS cells were seeded on graft copolymer coated culture dishes and allowed to form monolayer. Equal number of cells were seeded on the monolayer and continued culture to obtain a double layer. To retrieve the double layer, culture medium was removed and cells were kept below 10°C for 15–20 min. During low temperature treatment, the adhesion strength between the cell construct and the polymer was decreased and detached as cell sheet. The detached cell sheet construct was peeled off while observing under a three dimensional (3D) stereo microscope (Leica S8APO, Germany).

Viability staining

To understand the effect of low temperature treatment in cell transfer manipulations, viability of retrieved HOS cell sheet construct, was assessed. Immediately after retrieval, cell constructs were rinsed twice with PBS and incubated for 1 min in a mixture of acridine orange (AcOr) and ethidium bromide (EtBr) (each 0.5 μg/ ml in PBS). Stained samples were observed under fluorescence microscope (Nikon Eclipse E600) using G-2A and FITC filter cubes. Images of the cell sheet observed using the two filters were captured separately and manually superimposed to get a single image of live-dead staining.

Transfer efficacy of cell sheet construct

To analyze the growth potential of retrieved cell construct, cell sheet with approximate size of 2×2 mm^2 was transferred to a normal culture dish (TCPS). The cell sheet was allowed to settle and adhere by incubating in 250 μL culture medium for 4 h. After initial incubation, sufficient culture medium was added and the transferred sheet was incubated for further 72 h. The spreading and growth of cells from the transferred cell sheet was monitored under phase contrast microscope.

Environmental scanning electron microscopy

The HOS double layer construct was fixed in 4% paraformaldehyde and directly observed under environmental scanning electron microscopy (FEI

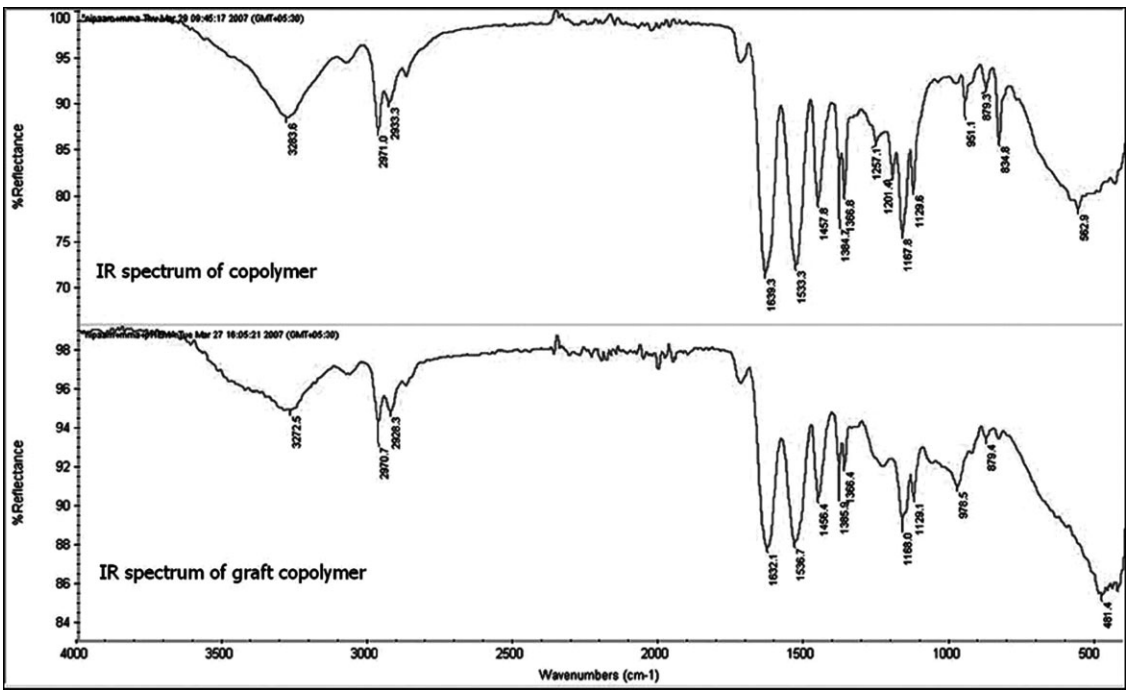


Figure 1 FTIR spectrum of (a) NIPAAm-MMA copolymer (b) Graft copolymer; peak at 1638 cm^{-1} and 1533 cm^{-1} characteristic of —NHCO, showing the presence of NIPAAm in the copolymer. Peak around 978 cm^{-1}, characteristic stretching mode of PO_4 showing the presence of phosphate moiety in the copolymer.

QUANTA 200, Japan) using high vacuum secondary electron detector.

Cytoskeletal staining

The HOS cell sheet fixed in 4% paraformaldehyde was rinsed with phosphate buffered saline (PBS) and permeabilized with Triton-X 100 (0.1% in PBS) for 10 min followed by incubation with FITC- conjugated Phalloidin (Sigma) for 15 min. After rinsing with PBS, cell sheet was mounted on micro slide and observed under Laser Scanning Confocal Microscope (Carl Zeiss 510 Meta, Germany) at an excitation of 488 nm.

RESULTS

Characterization of NIPAAm–MMA–Phosp-HEMA graft copolymer

Fourier transform infrared spectra

The infrared spectra of NIPAAm-MMA copolymer and graft copolymer of NIPAAm–MMA–Phosp-HEMA are shown in Figure 1. A major peak at 1638 cm^{-1} and 1533 cm^{-1} characteristic of —NHCO, shows the presence of NIPAAm in the copolymer. The graft copolymer showed an additional peak around 978 cm^{-1} which represents a characteristic stretching mode of PO_4 confirming the presence of phosphate moiety in the copolymer. The FTIR spectrum of the copolymer showed no additional peaks indicating the absence of unreacted monomers in the copolymer. From the weight measurement of NIPAAm-MMA copolymer before and after grafting, it was found that there was only 1% change in the net weight due to grafting. Since the percentage of grafting is very low considerable alterations in the spectrum cannot be expected.

Energy dispersive X-ray spectroscopy analysis

The EDS spectrum of graft copolymer depicted in Figure 2 apparently shows the peak associated with Phosphorus, reflecting the presence of Phosp-HEMA in the graft copolymer. Peaks representing the elements Au and Pd can also be seen which might have arisen from the gold coating done before sample analysis. The ungrafted copolymer surface did not show characteristic peak of phosphorus (Figure not shown).

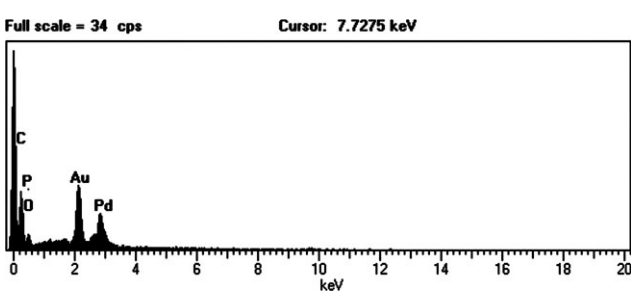


Figure 2 EDS trace of copolymer containing Phosp-HEMA (graft copolymer); showing the peak associated with phosphorus. This indicates the presence of Phosp-HEMA in the graft copolymer.

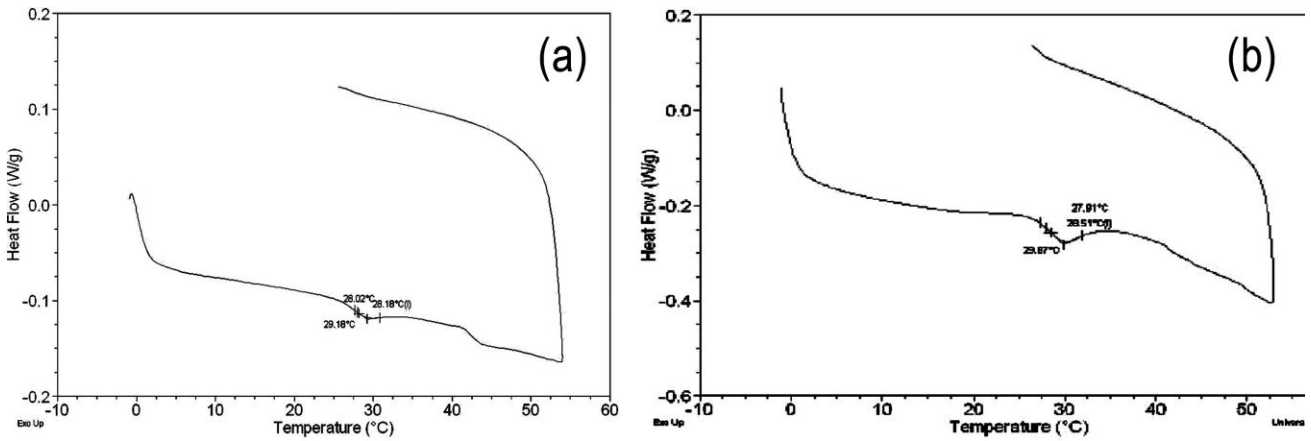


Figure 3 DSC trace of (a) graft copolymer showing the LCST around 29.18°C. (b) DSC trace of copolymer showing the LCST around 29.87°C.

Lower critical solution temperature

The LCST of the graft copolymer from the DSC scans was found to be around 29.18°C [Fig. 3(a)] and that of copolymer was 29.87°C [Fig. 3(b)]. The result shows that grafting process has not much changed the LCST of the copolymer.

Water contact angle measurement

Water contact angle of NIPAAm-MMA copolymer and graft copolymer were 43.6 ± 1° and 35.7 ± 0.4°, respectively (Table I). The graft copolymer exhibited more hydrophilicity when compared to copolymer. The contact angle of graft copolymer is less than the contact angle of copolymer reflecting that graft copolymer acquired additional hydrophilicity due to the presence of Phosp-HEMA chains.

Profilometry

Profilometry was used to measure the thickness of the coating over bare TCPS. The coating thickness was found to be 0.966 ± 0.006 μm and these results were found suitable for cell culture application.

Cell culture studies on graft copolymer

Cytotoxicity evaluation

The cytotoxicity of the graft copolymer assessed by direct contact method using L-929 cells confirmed the non cytotoxic nature. Figure 4 shows the normal morphology of cells around the graft copolymer similar to the negative control. This shows the suitability of modified surface for cell culture application.

TABLE I
Water Contact Angle Measurements of Copolymer and Graft Copolymer at 23°C

NIPAAm-MMA copolymer (degree)	Graft copolymer (degree)
42.4	35.3
44.3	35.9
44.1	36.1

Cell adhesion on graft copolymer

When SIRC and HOS cells were seeded on the modified surface, cells adhered and formed monolayer similar to cells cultured on normal TCPS. Both cell type exhibited characteristic morphology on graft copolymer similar to the control (Fig. 5).

Cell proliferation

The cell proliferation on NIPAAm-MMA copolymer and Phosp-HEMA and NIPAAm-MMA copolymer-grafted plate was estimated at different time points by MTT assay. Results showed enhanced cell proliferation on graft copolymer of NIPAAm-MMA-Phosp-HEMA compared to NIPAAm-MMA (Fig. 6) This increase was found to be statistically significant at 24 h ($P < 0.0001$) and 48 h ($P < 0.0026$).

Cell sheet construct

Osteoblast cells cultured on the temperature sensitive graft copolymer coated culture dish was retrieved as cell sheet construct by nonenzymatic method. As depicted in Figure 5(d), cells seeded on graft copolymer adhered and spread to form monolayer. Additional supply of cells and further culture resulted in a double layered cell sheet structure. Incubation below 10°C for 20 min was sufficient to detach the HOS double layer structure from the thermo responsive polymer. The loosely bound cell sheet peeled off physically, had an appropriate diameter of 15–20 mm and maintained the intact double layered sheet structure (Fig. 7).

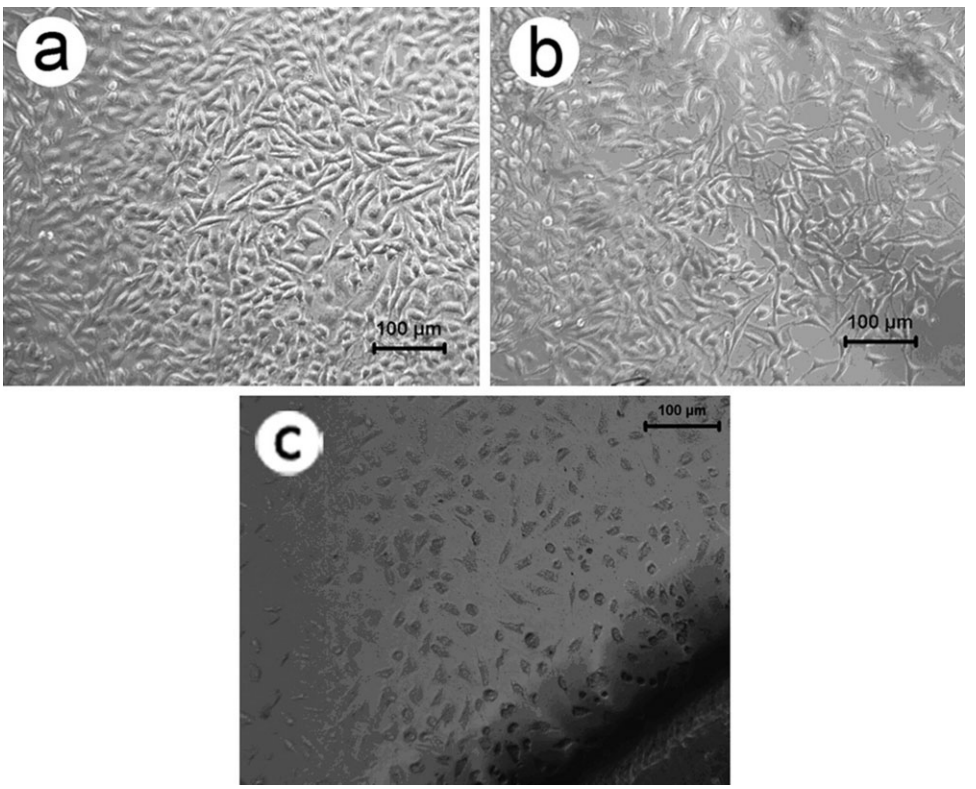


Figure 4 Cytotoxicity test of the polymeric material (a) L 929 cells showing normal morphology shown by negative control. (b) L 929 cells showing normal morphology similar to negative control after contact with material. (c) L-929 cells showing cytotoxic response shown by positive control.

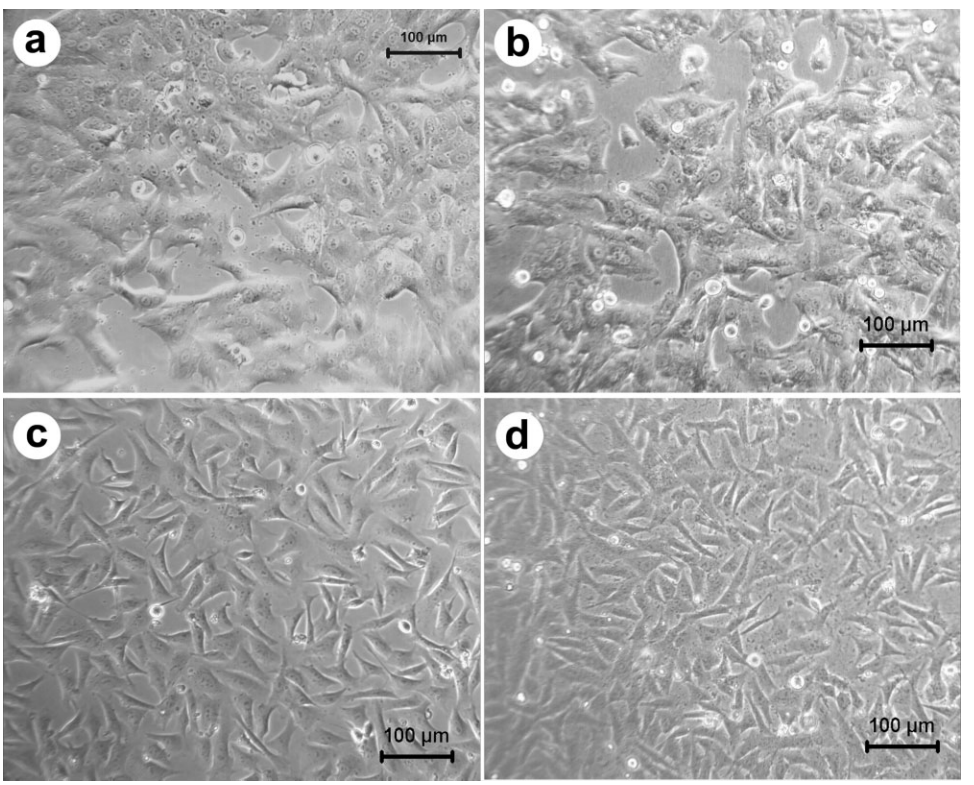


Figure 5 Cell adhesion on polymer. (a) SIRC on TCPS; (b) SIRC on graft polymer; (c) HOS on TCPS; (d) HOS on graft polymer. The morphology of SIRC and HOS on graft copolymer was similar to TCPS.

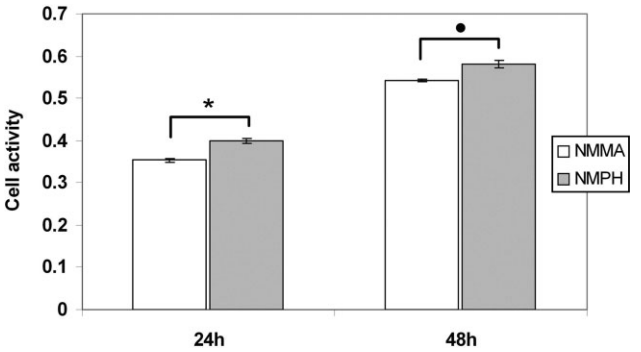


Figure 6 Cell proliferation on copolymer of NIPAAm-MMA copolymer (NMMA) and NIPAAm-MMA and Phosp-HEMA-(NMPH) grafted plates. Significance of cell proliferation was calculated. *$P < 0.0001$, **$P < 0.0026$.

Viability of cells

The live-dead staining of retrieved cell sheet using AcOr and EtBr showed that cells in the sheet construct maintained 100% viability (Fig. 8). It can be noticed that either low temperature treatment or the peeling method has not affected the viability of the cells.

Transfer efficacy of cell sheet

The growth potential of retrieved cell construct was examined by transferring the cell sheet to a new surface. At the end of 72-h incubation, cells from the sheet construct spread and grew on transferred surface (Fig. 9). This proves that the cell sheet construct retained the normal structure and functions of cells to a greater extend even after the cell sheet manipulation process.

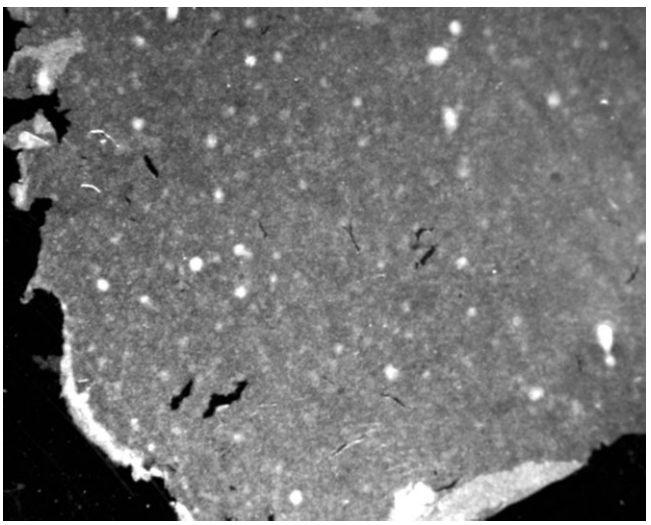


Figure 7 HOS cell sheet retrieved by temperature variation and viewed under 3D stereo microscope. The dual layered cell sheet showed tissue architecture.

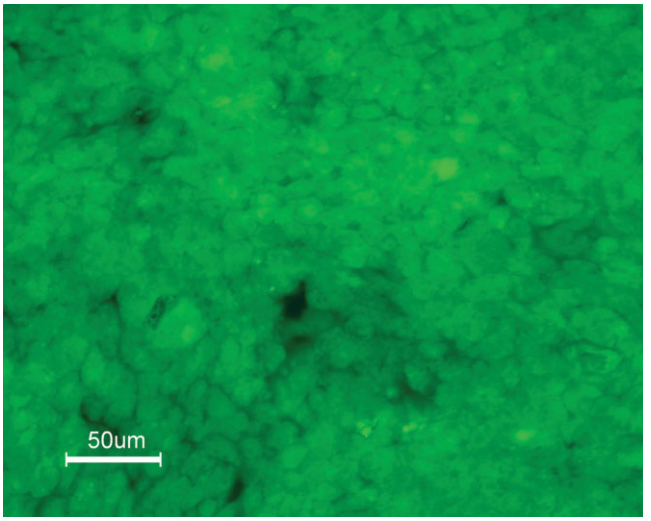


Figure 8 Live dead staining of HOS sheet after retrieval from graft copolymer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Tissue architecture of cell sheet

ESEM enables the observation of biological samples and cells in its native form. The cell sheet construct was examined under ESEM to get a magnified view of the tissue like architecture. The ESEM analysis showed the double layered structure and intact tissue like architecture of HOS cell sheet (Fig. 10).

Cytoskeletal distribution of cells in sheet construct

The cytoskeletal distribution of HOS cell sheet construct observed under confocal microscope by visualizing fluorescent labeled actin cytoskeletal structures revealed a cortical staining pattern.

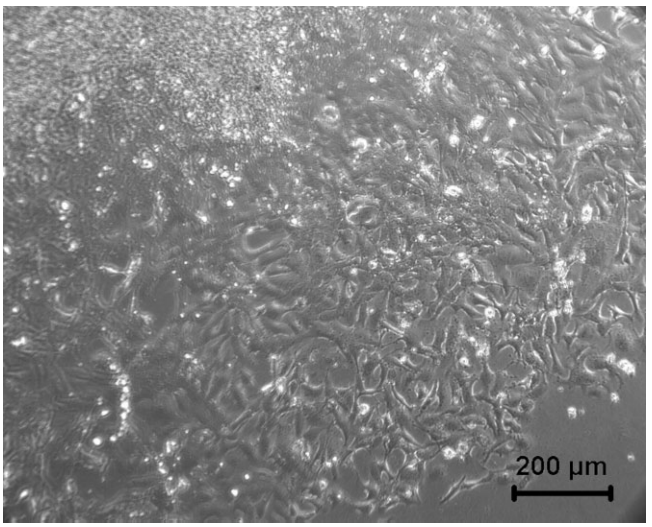


Figure 9 Morphology of the HOS cells migrating from transferred cell sheet to culture dish observed after 72 h.

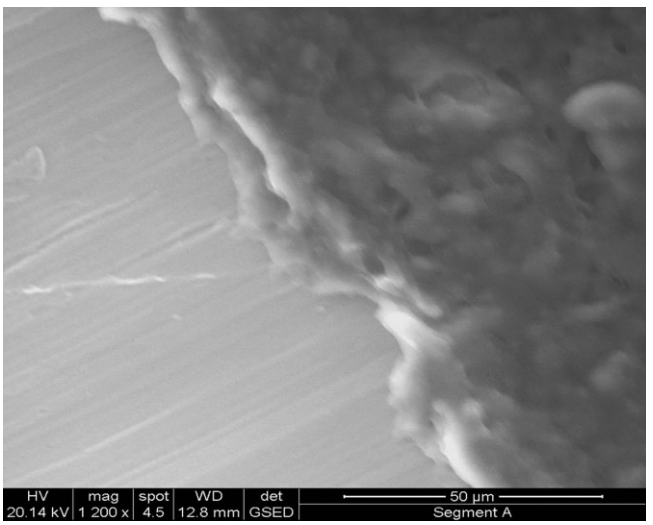


Figure 10 ESEM of HOS cell sheet showing intact double layer tissue structure.

Because of cytoskeletal-driven contractile forces, cell sheet was sometimes wrinkled or folded (Fig. 11).

DISCUSSION

In recent years, many new classes of materials have been figured in tissue engineering application for the recovery of cells and cell sheets. Among these materials, PNIPAAm is unique, since it undergoes a sharp coil-globule transition resulting into a phase separation in water at its LCST around 32°C.[16] The LCST of PNIPAAm can be tuned to specific applications by copolymerization with other comonomer having either higher hydrophilic or hydrophobic nature. It is widely known that LCST is lowered by copolymerizing with hydrophilic co-monomer.[17,18] In the present study graft copolymer of NIPPAm MMA and Phosp-HEMA showed LCST around 29°C which is lower than 32°C of PNIPAAm. Interestingly, LCST of the copolymer is not affected by the incorporation of Phosp-HEMA chains. It is reasoned that grafted chains are confined more to the surface of the copolymer which in turn affect the bulk features to lesser extent. The presence of peak representing phosphorus in the EDS trace further manifest the presence of Phosp-HEMA chains in the bulk of the polymer. From the contact angle measurement carried out at room temperature (23°C) difference in hydrophilicity was noted. The grafted copolymer surface exhibited a lower contact angle than copolymer, further indicating that the surface is enriched with hydrophilic entities such as PO_4 groups.

Cell adhesion is influenced by the physical and chemical properties of the surface through a nonreceptor mediated or receptor mediated attachment mechanism. Nonreceptor mediated cell adhesion is governed by nonspecific cell–material interaction mainly through hydrogen bonding, electro static, polar, and ionic interactions between molecules on cell membranes and functional chemical groups on the material surface. Cells can adhere to the hydrophobic surface, while they are unable to adhere to the highly hydrated hydrophilic surface.[19] In this study, cell adhesion might have been mediated by nonreceptor type interaction as the surface did not contain any of the molecules such as collagen or cell specific amino acid sequences as reported earlier. Phosphorus is a component of DNA, RNA and also cell membranes, where it is found in association with lipids (phospholipids). It is thus an essential element for all living systems especially in the form of phosphates which plays a major role in structural frame work of biological molecules such as DNA and RNA. Calcium phosphate or apitites is known to stiffen bones and are used as scaffolds for tissue engineering. Phosphate moieties along with calcium have been known to enhance osteoblast cell adhesion.[20,21] Copolymers of Phosphorylated HEMA and MMA are reported to provide stability to cell cultures and favor collagen production in corneal keratocyte cultures.[22] The interesting observation of cell adhesion and proliferation on the graft copolymer illustrate the advantageous role of PO_4 groups in favoring cell–polymer interactions.

Cell–cell and cell–ECM interactions are mediated by cell membrane proteins. Commercially available tissue culture dishes are modified to favor

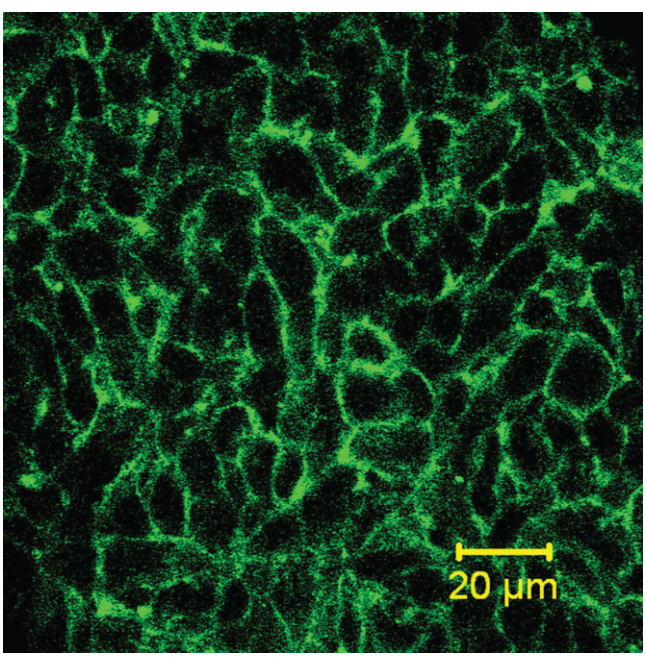


Figure 11 Cortical staining pattern of actin cytoskeletal structure of HOS cell sheet. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

adsorption of ECM proteins and thereby cell attachment and proliferation. Harvesting cells using conventional trypsinisation method disrupts both ECM proteins as well as membrane proteins to release cells separately and requires extended time to form a monolayer again. The thermoresponsive polymer-coated surface used in this study was hydrophobic at 37°C and cells could adhere and grow. Low temperature incubation resulted in a reversible hydrophilic surface due to hydration and swelling of the polymer[11,12] and cells were detached as sheet with intact cell–cell and cell –ECM contact. Kim et al. showed osteoblast cell sheet manipulation using PNIPAAm-based terpolymeric-grafted culture surface.[16] In this study, phosphorylated HEMA was grafted on a copolymer of NIPAAm and MMA to culture osteoblasts and cell sheet manipulation showed similar efficiency. Here, the adherence and spreading of the transferred cell sheet on TCPS showed intact cell–cell and cell–ECM contact due to the noninvasive procedure. Cell sheet structures maintaining intact cell–cell and cell–ECM contact have been proved to be useful in enhanced cellularization of porous scaffolds for tissue engineering applications.[23] Similarly, HOS cell sheet structure generated using graft copolymer can be used for various tissue engineering applications.

The cytocompatibility studies demonstrated that the new graft copolymer was non cytotoxic favoring cell adhesion and spreading similar to cells cultured on TCPS. The cell material interaction depends on the surface energy/charge of the material and is mediated by secondary factors including van der Waals forces and cell surface proteins.[23] Cell adhesion can be enhanced by adding bio-functional molecules on the material surface. Thermoresponsive culture surfaces have been modified by immobilizing peptides for enhanced cell adhesion.[24] This study shows that similar effect could be achieved by incorporating synthetic entity, poly(phosphorylated HEMA). The material depicted in this study is predominantly hydrophilic in nature as reflected by the contact angle measurement. Above LCST, the PNIPAAm component assumes conformation which is predominantly more hydrophobic. The cells seeded could stabilize on the hydrophobic patches at the surface of the copolymer and the polar PO4 groups present on the copolymer surface execute electrostatic interaction. We assume that these interactions may presumably affect the charge distribution on the cell surface, leading to conformational alterations in the cell membrane molecules favoring further cell activities.

In recent years, substantial efforts have been directed to the design and fabrication of structured materials with functional properties. In particular, much attention has been focused for the preparation of functionalized surfaces exhibiting specific features in terms of control of cell adhesion, activation and proliferation. This study, shows that addition of phosphate molecules could improve the function of synthetic polymers towards better cell interaction.

CONCLUSIONS

Cell sheet manipulation using a novel temperature responsive culture surface coated with the noncytotoxic, cytocompatible, and functional group-based polymer was shown to be very useful in generating tissue structures. This unique temperature responsive polymer switches from hydrophilic surface below its transition temperature (29.18°C) to hydrophobic surface above this temperature. Cells such as SIRC, L-929 and HOS adhered and spread on surface similar to cells on uncoated TCPS. By reducing the temperature below 10°C, the cell sheet could be detached from the surface using forceps. The lifted cell sheet could be transferred onto another surface, where it adhered and spread. Incorporation of phosphate moiety to graft copolymer retains duel characteristics, enhancement of biofunctionality and stimuli responsiveness for intact cell sheet retrieval. These findings suggest that this novel nontoxic, cytocompatible polymer-coated surface will be useful for generating cell sheet constructs. Since the graft copolymer supported more than one cell line, it is concluded that this novel polymer could have wide applicability in tissue engineering.

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