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In vitro cytocompatibility evaluation of a thermoresponsive NIPAAm-MMA copolymeric surface using L929 cells

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Abstract Scaffold free tissue constructs are preferred in tissue engineering as they overcome all the problems associated with scaffolds. Stimuli responsive polymers enable generation of scaffold free multilayered tissue constructs which would in turn reduce the use of biomaterials in vivo. In this study, we investigated cytocompatibility and thermoresponsiveness of a copolymer of *N*-Isopropylacrylamide and Methyl Methacrylate. Thermoresponsive surfaces were prepared by coating tissue culture polystyrene with the copolymer solution in isopropanol. Mammalian fibroblast cells (L929 cells) readily adhered on the copolymer. The viability and cellular activity was ensured through Neutral red staining, MTT assay, Tritiated thymidine uptake assay and Immunofluorescent staining for cytoskeletal organisation. Incubation under lower critical solution temperature of copolymer resulted in intact detachment of cells. To conclude, in-house synthesized cytocompatible smart culture substrate intended for tissue engineering was developed using a cost effective and simple technique. Moreover, presence of methyl methacrylate in the copolymer reduced the lower critical solution temperature facilitating extended in vitro manipulation time. As the copolymer is insoluble in water, the copolymer could be polymerised without additional crosslinkers.

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1 Introduction

Tissue engineering (TE) involves reconstruction of tissues and organs using cells, scaffolds and growth factors/supplements. Although the scaffolds are usually biodegradable, use of scaffold results in the implantation of foreign material into human body. Moreover, some applications of TE namely skin and cornea do not need the use of scaffold. In order to eliminate the use of scaffolds in constructing three dimensional tissue structures and organs, thermoresponsive, smart culture surfaces have been studied [1–3]. These surfaces can be used for detachment of cell layers with intact cell–cell and cell—extra cellular matrix (ECM) [4].

The reversible thermal switching property of thermoresponsive polymers enable it to undergo phase transition between hydrophilic and hydrophobic stages [5]. Above the lower critical solution temperature (LCST) the polymer will be hydrophobic resulting in the attachment and growth of cells, while below the LCST it turns hydrophilic enabling the detachment of cells.

Polymethyl methacrylate (PMMA) is one of the widely used biocompatible polymers in medical applications such as intra ocular lens, bone cement and for dental applications. In this study, largely considering the favourable features of PMMA, a copolymer of *N*-isopropylacrylamide (NIPAAm) and Methyl Methacrylate (MMA) was synthesised and characterised. NIPAAm was incorporated to provide thermoresponsiveness whereby cells can be detached as intact cell sheets, avoiding the use of proteolytic enzymes. Moreover, this can assist in formation of scaffold free tissue constructs. MMA, being considered as an ophthalmic biomaterial in its polymeric form, was incorporated to modulate LCST of the substrate and thereby increasing in vitro manipulation time. Moreover as the copolymer is insoluble in water, use of additional

crosslinkers in the preparation of copolymer can be avoided. Copolymers of Poly(*N*-isopropylacrylamide) (PIPAAm) were already reported for modulation of LCST (32°C) in order to suit different applications like preparation of patterned surfaces and coculture of cells [6, 7]. Besides cell culture applications, copolymers of PIPAAm had been reported as drug delivery systems [8] and embolic agent [9]. The copolymer in this study, being thermoresponsive, could be exploited to generate tissue structures, which do not need scaffolds, like cornea and skin equivalents.

Any material intended for medical application should be safe for the human body. Therefore, all polymeric materials that come in contact with body fluids, tissues in vivo or cells in vitro should be proven cytocompatible before use. The thermoresponsive copolymer coated surface was analysed for its ability in supporting viability, metabolic activity, attachment, spreading and proliferation of fibroblast cells in comparison to tissue culture polystyrene (TCPS). Thermoresponsiveness or temperature induced cell detachment to form detachable cell layers which is required for generation of scaffold free tissue construct was also looked into.

2 Materials and methods

2.1 Materials

N-Isopropylacrylamide, Methyl Methacrylate, azobisisobutyronitrile (AIBN) (Aldrich, USA) Minimal essential medium, Phalloidin-Fluorescein Isothiocyanate (FITC), (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), antibiotics (Sigma chemicals, India) Foetal calf Serum, Trypsin-EDTA (10×) (Gibco BRL, India) Tritiated thymidine (American Radiolabelled Chemicals) and Poly Vinylidene Fluoride Membrane (PVDF) (Millipore, India) were used.

2.2 Preparation of copolymer

NIPAAm and MMA were taken in 9:1 ratios and dissolved in benzene. Temperature was raised to 60°C. AIBN was added as initiator and nitrogen was bubbled through the whole reaction mixture for 10 min. It was kept stirring at 60°C until the polymerization (as shown in the Scheme 1) was completed. The copolymer was then dissolved in acetone and again precipitated in water. Process was repeated thrice to remove unreacted monomers. The final cleaned copolymer was dissolved in isopropanol.

2.3 Characterisation of copolymer

2.3.1 Fourier transform infrared spectroscopy (FTIR)

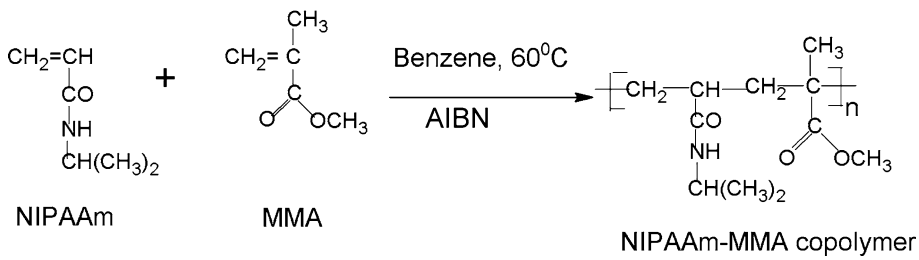
Powdered copolymer was mixed with optical grade Potassium Bromide (KBr) mixture. Spectra were measured for the mixture as well as pure KBr which was kept as blank in the range of 400–4000 cm^{-1} on a Nicolet 5700 spectrometer. Spectra were recorded at a resolution of 4 cm^{-1} and number of scans were 32.

2.3.2 Differential scanning calorimetry

Lower critical solution temperatures of the copolymers was determined using Differential Scanning Calorimetry (DSC) (DSC 2920 TA Instruments, Delaware, USA) with TA 4000 controller as reported earlier [10]. Prior to measurements the samples were immersed in deionised water at room temperature and allowed to swell to equilibrium. About 10 mg of equilibrium swollen sample was sealed hermetically and thermal analysis was performed from 25°C to 55°C at a heating rate of 3°C in nitrogen atmosphere.

2.4 Preparation of copolymeric surfaces (NIPAAm-MMA)

The copolymer solution was coated on TCPS by adding the solution to the surface for a few seconds and removing the excess. The coated plates were then kept in hot air oven at 65°C overnight. The excess unbound polymer was washed with cold distilled water and left in oven for drying. Before cell culture experiments, the plates were sterilised by ethylene oxide.



Scheme 1 Synthesis of NIPAAm-MMA copolymer

2.5 Characterisation of copolymeric surface

2.5.1 Attenuated total reflectance Spectra

The surface chemistry of NIPAAm-MMA dish was assessed using Attenuated Total reflectance spectra (ATR-FTIR). A Thermo Nicolet 5700 model Fourier Transform infrared spectrophotometer (FTIR) fitted with a horizontal attenuated total reflection (ATR) accessory with diamond crystal was used to obtain spectrum. Spectra were taken in the wavelength region 400 cm^{-1} to 2000 cm^{-1} from 32 scans.

2.5.2 Profilometry

The coating thickness as well as the surface profile of the NIPAAm-MMA dishes was assessed using Talysurf CLI 1000 (Taylor Hobson UK) with the software Talymap Gold (version 4.1). For analysing coating thickness, samples were prepared by coating on one half of the dish and the other half was left bare, in order to form a dip. A surface of 10 mm × 2 mm, covering this dip was measured at a speed of 500 μm/s using high resolution non contact Confocal point Gauge (CLA) with a range of 300 μm. The profile was extracted from the measured region and the step height measurement ($n = 6$, where n is the number of replicates) was taken manually.

For analysing surface roughness, 1 mm × 1 mm of the surface area of coated and bare dishes were measured. At least 10 profile lines were extracted from the levelled surface measurement, from which surface roughness was calculated by the software.

2.6 Cell culture

L929 mouse fibroblast cell line, used in this study, was procured from American type culture collection (ATCC). The cells were maintained in Minimum Essential Medium with 5% serum, under humidified conditions at 37°C.

2.7 Cytocompatibility studies

The cells were trypsinised and seeded onto polystyrene dishes as well as NIPAAm-MMA plates at a concentration of 2×10^4 cells/cm^2 for all the experiments. Cell viability on the copolymer was tested using a vital stain, neutral red [11]. Neutral red solution (0.5 mg/ml) in normal saline was added to the cells after 24 h of culture on the copolymer and incubated for 10 min at 37°C.

2.7.1 Cell attachment and doubling time

The experiment was performed based on Prasad et.al [12] with minor modifications. In brief, L929 cells in log phase were labelled with tritiated thymidine by maintaining in a medium containing 5 μCi/ml of tritiated thymidine until they reached confluency. Then the cells were trypsinised and seeded to 35 mm TCPS and NIPAAm-MMA culture dishes in triplicates. To assess initial attachment of cells, the medium from culture dishes were removed and unattached cells were pelleted after 4 h of cell seeding. DNA from the unattached cells were extracted for ^3H counting according to Shivkumar et al. [13]. Aliquots of 4×10^4 cells were used to determine the radioactive count of known number of cells. ^3H count was measured in a liquid scintillation counter (TRIATHLER Multilabel-tester, Hidex, Finland). The attached cells were calculated based on the count of unattached cells and count of known number of cells.

For the quantification of doubling time, the cells were maintained in fresh medium containing 2.5 μCi/ml for 72 h. The cells were harvested afterwards and ^3H count was measured as described above. Doubling time was calculated from the count obtained at 4 h and 72 h using the formula:

$$\text{Doubling time} = \log(2)/K \quad \text{where}$$
$$K = (2.3 \times \log(N1/N0))/T1\text{-}T0$$

where T0 is the initial time point; T1 is final time point; N0 is number of cells at T0 and N1 is number of cells at T1

2.7.2 MTT assay

After growing the cells for 48 and 96 h, MTT (0.5 mg/ml in serum free MEM) was added and incubated for 4 h at 37°C [14]. The cells were then lysed and the converted dye was solubilised using isopropanol for 20 min with mild shaking. The released purple formazan product was measured at 570 nm (BioTek plate reader, USA). The assay was done in triplicates and medium alone was considered as reagent blank.

2.7.3 Cytoskeleton staining

The cells were fixed with 4% paraformaldehyde and permeabilised with PBS containing 0.1% Triton X 100. The cells were then stained by phalloidin conjugated with FITC for 30 min. After thorough washing with PBS, the cells were observed under inverted fluorescent microscope (Leica DMI 6000, Leitz, Germany).

2.8 Cell detachment

L929 cells were cultured on NIPAAm-MMA plates until almost confluency. After removal of medium a PVDF membrane was placed on top of the cells and incubated below LCST of copolymer for 30 min. The membrane was then slowly peeled off the culture surface after incubation.

2.9 Statistical analysis

The results were expressed as mean ± standard deviation. Significance was determined using one way analysis of variance (ANOVA) and $P < 0.005$ was considered as significant.

3 Results

3.1 Characterisation of copolymer

3.1.1 Fourier transform infrared spectroscopy (FTIR)

The infrared spectra of NIPAAm-MMA copolymer is shown in Fig. 1.The major peaks at 1627 cm^{-1} and 1538 cm^{-1} characteristic of –NH–CO stretching and bending modes of NIPAAm, confirmed the presence of NIPAAm in the copolymer. The carbon–oxygen single bond (–CO–O–CH_3) stretching of unconjugated ester around 1130 cm^{-1} confirmed the presence of MMA in copolymer. The carbon–oxygen stretch of methyl ester around 979 cm^{-1} further substantiated the result. The carbon–oxygen stretching vibration of ester group around 1170 cm^{-1} could also be seen in the spectra which gave a further evidence for the presence of MMA in the copolymer.

3.1.2 LCST of NIPAAm-MMA copolymer

Thermoresponsive smart polymer undergoes phase transition from soluble to insoluble state when temperature is raised. LCST is the point where the hydrophobic interaction of isopropyl group of NIPAAm outweighs the hydrophilic nature of the amide group on the pendant groups, forcing water out of the polymer. Figure 2 shows the DSC scan of the copolymer. The LCST of the copolymer was centered around 29°C while that of PIPAAm was centered around 32°C.

3.2 Characterisation of thermoresponsive surface

3.2.1 ATR

ATR spectrum of both coated and uncoated surfaces are given in Fig. 3. The spectrum of the coated surface showed the characteristic peak of amide carbonyl group of NIPAAm around 1650 cm^{-1} while it was completely absent in bare TCPS dishes. Meanwhile, the strong peak due to monosubstituted aromatic ring was present in TCPS at 1600 cm^{-1}.

3.2.2 Profilometry

Profile extraction and step height measurement showed that the coating thickness of NIPAAm-MMA was

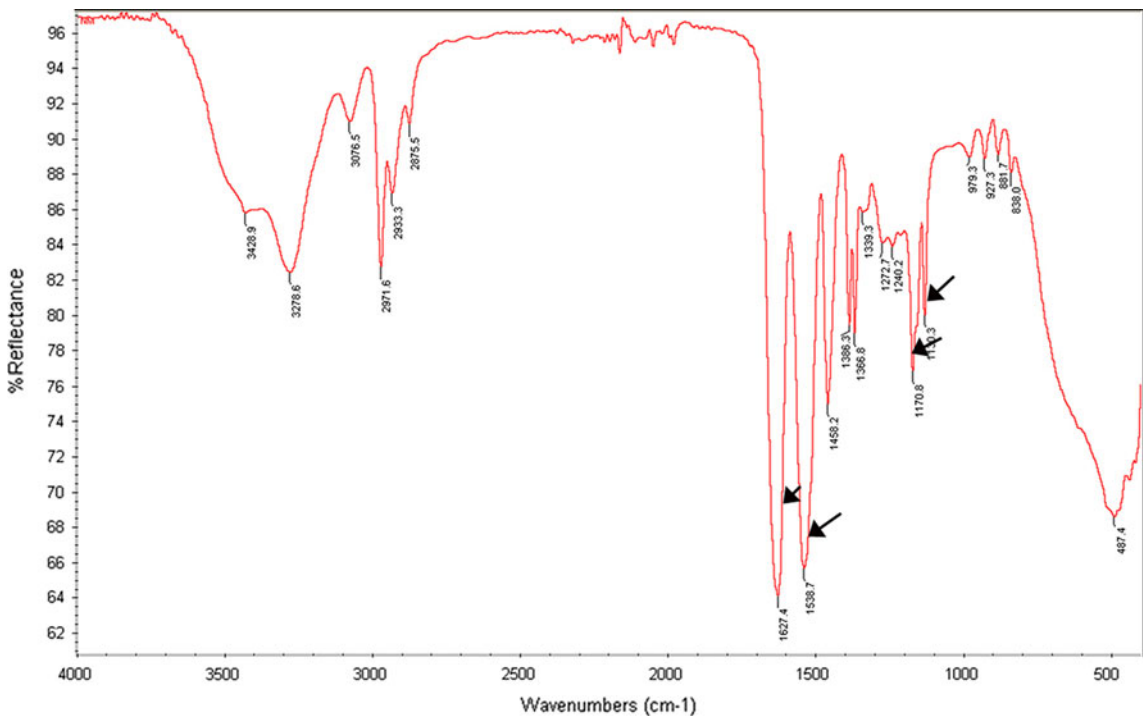


Fig. 1 FTIR spectrum of NIPAAm-MMA copolymer. Arrows indicate peaks confirming presence of both monomers in the copolymer

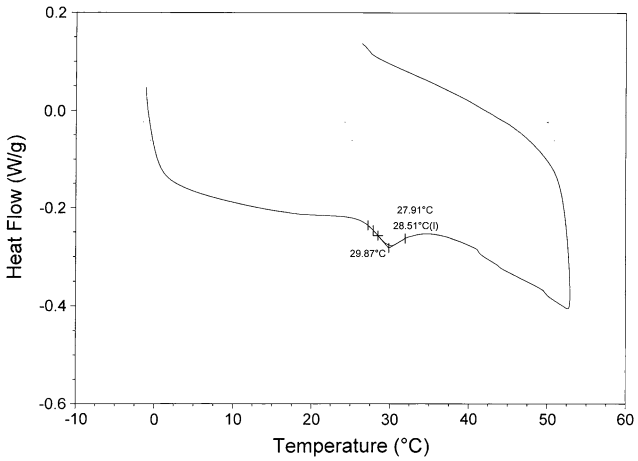


Fig. 2 DSC scan of the copolymer. The LCST was found to be centered around 29°C

1.12 ± 0.016 μm. A 1 × 1 mm coated and uncoated surfaces were scanned for surface topography. According to the 3D images, the uncoated surface had rougher topography compared to coated surfaces (Fig. 4). Roughness calculation using software confirmed this observation. The roughness parameters of both coated and uncoated surfaces are given in Table 1. This indicated that coating with NIPAAm-MMA covered and smoothened the TCPS surfaces.

3.3 Cytocompatibility studies

3.3.1 Cell morphology

The L929 cells cultured on NIPAAm-MMA showed its characteristic spindle shaped morphology under phase contrast microscope throughout the culture period (Fig. 5a). The cells on copolymer were viable and healthy as shown by neutral red staining after 24 h (Fig. 5b).

3.3.2 Cell attachment and doubling time

The results of tritiated thymidine uptake assay on both substrates indicated that there was no significant difference in cell attachment and doubling time. The initial attachment rate of L929 cells on both TCPS and the copolymer is given in Fig. 6a. Similar numbers of cells were seemed to adhere on both substrates. Likewise, the similar doubling time of the cells on the substrates (Fig. 6b) indicated that the copolymer supports good cell proliferation.

3.3.3 Cell metabolism

MTT assay proved that cells cultured on copolymer were equally active and healthy compared to that on TCPS

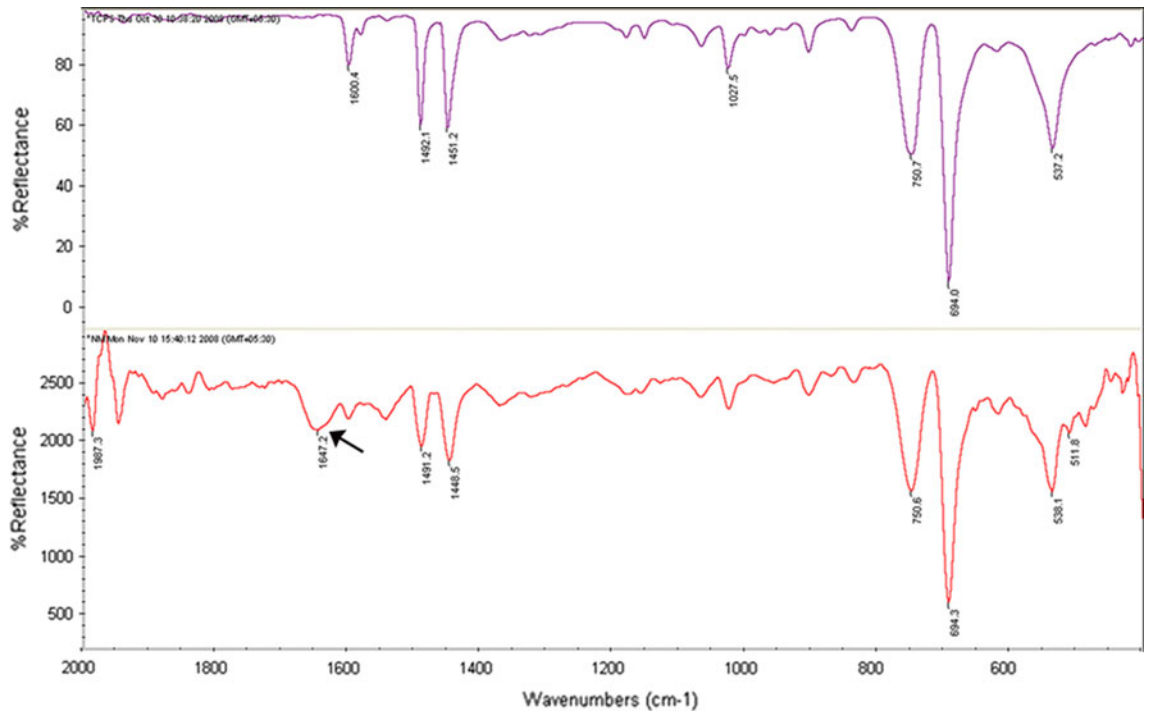


Fig. 3 ATR spectrum of the copolymer. The peak around 1650 cm^{-1} indicated presence of NIPAAm (*arrow*), thereby confirming coating of the copolymer on TCPS surface

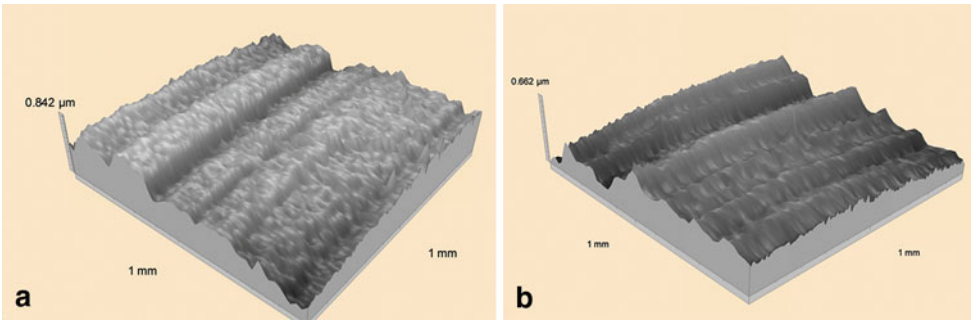


Fig. 4 Three dimensional images for surfaces of **a** TCPS and **b** NIPAAm-MMA obtained by profilomery. The coating of the copolymer appeared to smoothen TCPS surfaces

Table 1 Roughness parameters of both coated and uncoated surfaces

Roughness parameters	Uncoated	Coated with NIPAAm-MMA
R_a	6.06 ± 0.679 nm	3.37 ± 0.188 nm
R_q	7.39 ± 0.876 nm	4.04 ± 0.262 nm
R_p	12.1 ± 1.62 nm	5.54 ± 0.325 nm

R_a—arithmetic average of absolute values, R_q—root mean squared, R_p—maximum peak height

(Fig. 7). The cells grown on copolymer maintained their metabolic function even after 96 h. The copolymer did not have any negative influence on the metabolic functions of the cells.

3.3.4 Cytoskeletal organisation

Actin cytoskeleton organisation play important role in morphological stability, adhesion and proliferation of cells. Therefore a comparative analysis was done on both substrates. Even distribution of actin filaments showed good cell spreading on copolymer (Fig. 8).

3.4 Cell detachment

L929 cells, cultured on NIPAAm-MMA, were incubated at 20°C after keeping a PVDF membrane on it. On removal of the membrane, it was observed that cells which came in contact with the membrane had been completely

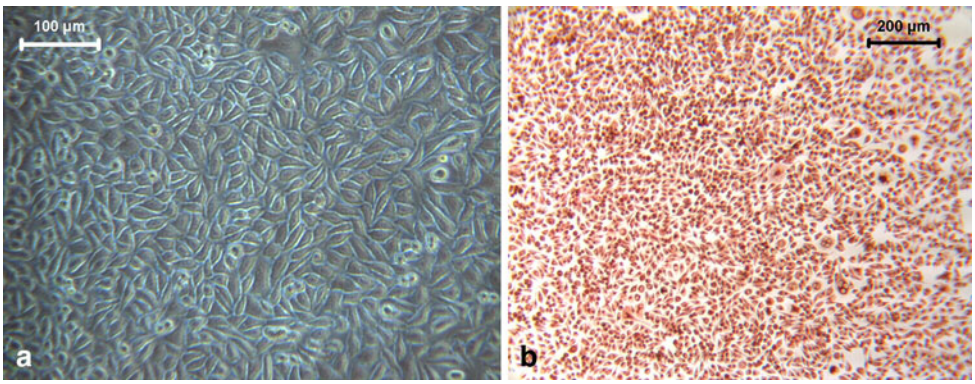


Fig. 5 L929 cell culture on NIPAAm-MMA copolymer. **a** Phase contrast micrograph after 72 h and **b** Neutral red stained cells indicating their viability on copolymer

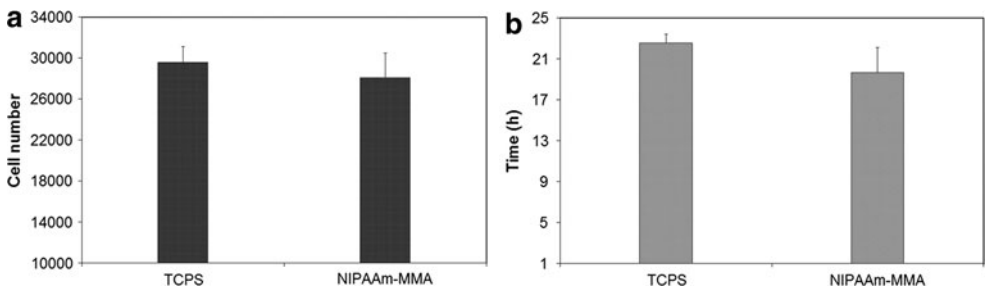


Fig. 6 Results of tritiated thymidine uptake assay. **a** Graph showing cell attachment rate on both substrates and **b** doubling time of L929 on both substrates. There was no significant difference in the attachment rate or doubling time of L929 cells between the substrates ($n = 3$)

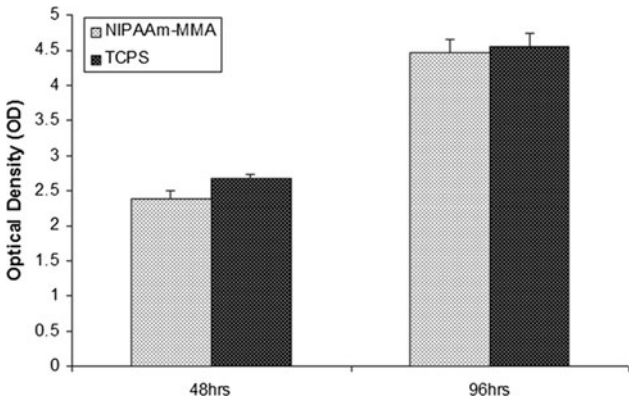


Fig. 7 MTT assay revealed that the cells were metabolically active even after 4 days of subculture and the copolymer supported growth of metabolically active cells ($n = 3$)

transferred from the culture dish to the PVDF support (Fig. 9), demonstrating its thermoresponsive property. This was achieved by the phase transition of copolymer around its LCST leaving the culture surface hydrophilic at low temperatures.

4 Discussion

Thermoresponsive polymers are widely studied and utilised for many biomedical applications including tissue engineering. The capability of these polymers to undergo phase transitions with response to temperature changes makes them an attractive culture substrate in tissue engineering. Due to their thermoresponsive property, these polymers result in the detachment of cultured cells along with an intact extracellular matrix which would enable a firm and rapid integration of the retrieved cell sheets with the host tissue after transplantation. These scaffold free cell constructs could be used in tissue and organ reconstruction, which would eventually reduce implantation of foreign materials like biomaterials/polymers inside the body. Even though thermoresponsive polymers are not implanted in vivo during most of its tissue engineering applications, cytocompatibility evaluation could not be avoided as it comes in contact with cells in vitro. Here, a thermoresponsive copolymer was prepared, characterised and evaluated for its cytocompatibility.

A material is said to be cytocompatible when it induces no toxic response, support cell adhesion, spreading, migration, proliferation and preserve cell functionality. Extensive studies for the different aspects of cytocompatibility of PIPAAm, a traditional thermoresponsive substrate, or its copolymers were not reported. Non cytotoxicity of PIPAAm as a culture substrate was demonstrated by studying cell morphology [5]. Okano et al. studied cell growth on PIPAAm by determining amount of DNA in the cultured cells [2]. They observed that PIPAAm coated surfaces could be compared to standard tissue culture dishes. Potential cytotoxicity of a copolymer of PIPAAm, MMA and Hydroxy Ethyl Methacrylate on L929 cells was evaluated using MTT assay [15]. The assay was also employed to study proliferation rate of human dermal fibroblasts on the copolymer. Quan et al. reported a better cytocompatibility of P(NIPAAm-co-Propyl acrylic acid) nanogel than PIPAAm nanogel [8].

In this study, different methods were adopted to evaluate the various aspects of cytocompatibility of the copolymer. Toxic response could be ruled out as the cells were viable and healthy on copolymer, as evident by the neutral red staining. Initial cell attachment has a major role in cell material interaction as all the following events are dependent on this factor. In our study, tritiated thymidine uptake assay was used to analyse cell adhesion and doubling time. Radio active labelled thymidine would be incorporated into DNA during replication. Hence, the resultant radioactive count would be proportional to the population comprising of actively proliferating cells and recently proliferated cells. As the seeded L929 cells were already labelled, radioactive count of the unattached cells after 4 h was indirectly used to assess attached cells. Results suggested that the NIPAAm-MMA copolymer encouraged cell attachment and proliferation. The doubling time of cells were also not affected when grown on copolymer. Good cell spreading demonstrated by actin staining further confirmed proper cell attachment. In MTT assay, a yellow

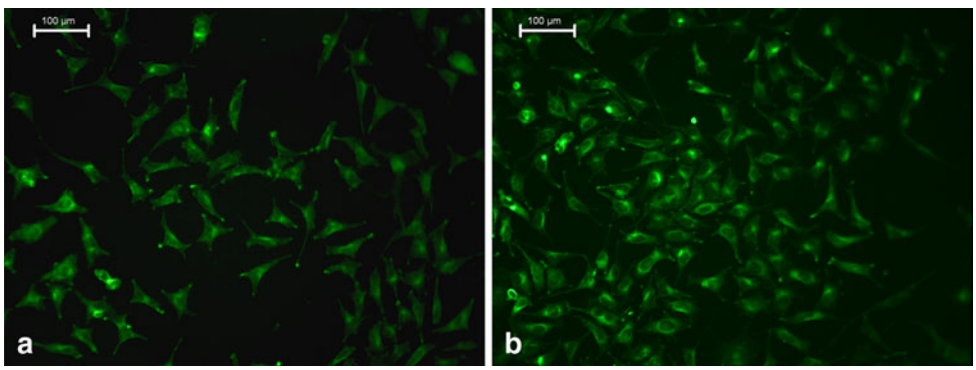


Fig. 8 Actin cytoskeletal organisation of L929 cells on both substrates. The cells were fixed and stained with Phalloidin FITC for 30 min. **a** TCPS and **b** NIPAAm-MMA copolymer

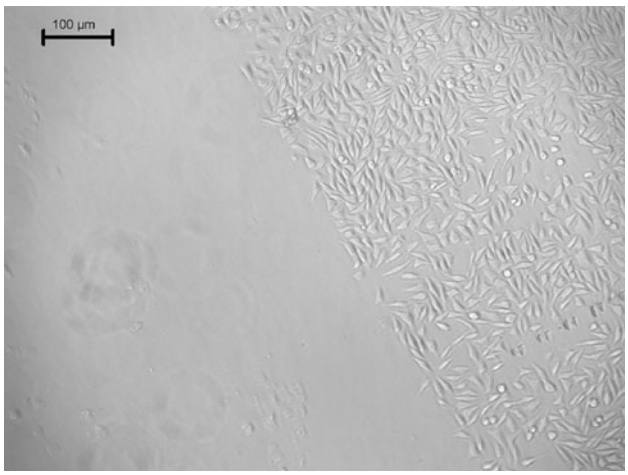


Fig. 9 Cell detachment from the copolymeric substrate due to its thermoresponsive nature. L929 cells were cultured on the copolymer till subconfluency and transferred to PVDF membrane by incubating below LCST. Arrow indicates the region where the membrane was kept

tetrazole is converted to a purple formazan compound by succinic dehydrogenase enzyme present in the mitochondria. Thus, only healthy metabolically active cells would contain formazan crystals which was then solubilised and read. As the experiment was done for two time points, it would indirectly give an idea about cell proliferation also. Results of MTT assay agreed with that of tritiated thymidine assay and ruled out any subcellular toxic effects. No significant difference in cell function even after 4 days ensured the cytocompatibility of the copolymer. Therefore, this study revealed that NIPAAm-MMA is as cytocompatible as the standard tissue culture surface.

LCST is the temperature above which the entropy factor dominates. PIPAAm has a particular hydrophobic-hydrophilic ratio above and below LCST. Introduction of hydrophobic MMA disturbs this balance and changes the LCST. However, the LCST of the copolymer did not deviate much probably due to the low amount of MMA incorporated. It had been already reported that introduction of hydrophobic monomers like MMA decreases the transition temperature of the copolymer [16, 17]. Transfer of L929 cells to PVDF membrane under LCST of the culture surface confirmed the stimuli responsive nature of the substrate. The hydrophilic nature of the copolymer below its LCST resulted in weakened interaction between the surface and cells with ECM compared to that between the PVDF membrane and cells, enabling the transfer [18]. Transferring of cultured L929 cells from PIPAAm to PVDF membrane was reported earlier from this lab [19].

The surface roughness of the copolymer showed noticeable difference compared to TCPS. The decrease in roughness was due to the smoothening of TCPS surfaces during the coating, thus giving indirect evidence for the presence of copolymer on the surface. It had been reported that surface roughness also play an important role in determining the cell material interactions [20, 21]. Our cell culture studies on copolymer suggested that a surface roughness of R_a 3.37 ± 0.188 nm was enough for the fibroblast cell line to attach and grow.

Different fabrication methods like electron beam irradiation [22, 23], plasma polymerisation [24] and gamma ray irradiation [19] were reported for synthesizing thermoresponsive culture surfaces. Most of these techniques might not be available for all researchers because of its expensive nature. In this study, we utilised a cost effective and simple technique to prepare these surfaces. Our technique would enable increased use of thermoresponsive surfaces for tissue reconstruction and other various tissue engineering applications as it could be done in any average laboratory.

Copolymers of NIPAAm and MMA along with other monomers had already been reported for controlled drug release [25, 26]. Reports on the use of NIPAAm-MMA copolymer for cell culture purposes are scanty. To the best of our knowledge, this is the first report about the application of NIPAAm-MMA for cell culture.

5 Conclusions

In this study, a smart, thermoresponsive copolymeric surface using a cost effective and simple technique was prepared and proved its cytocompatibility for tissue engineering applications like skin and cornea. Physicochemical characterisation techniques confirmed presence of NIPAAm and MMA in the copolymer and the coating on TCPS surfaces. The ideal thermoresponsive nature with good cytocompatibility makes it a promising substrate for cell culture and tissue reconstruction. Eventhough few reports had already been available about the use of copolymers consisting NIPAAm and MMA for drug delivery purposes, this study would be the first report depicting its application for cell culture and preparation of scaffold free cell/tissue constructs.

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