Harvesting epithelial cell sheet based on thermo- sensitive hydrogel

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ABSTRACT

Thermo-sensitive hydrogels were prepared by graft copolymerization of chitosan and N-Isopropylacrylamide via gamma radiation. Characterization of hydrogels such as ^{13}C-NMR, DSC analysis and swelling test and cell assessments for harvesting living cell sheet were investigated. ^{13}C-NMR and DSC analysis showed chitosan and NIPAAm monomer were grafted via gamma radiation successfully. Swelling ratio and curves results administrated hydrophilicity / hydrophobicity of hydrogel that this property is due to presence of PNIPAAm in different temperatures. The hydrogel was tested for harvesting epithelial cells after carrying out cell culture at 37 °C and incubating the confluent cells at 4°C for spontaneous detachment of cell sheet from hydrogel surface without enzyme treatment. Cell viability assay results and microscopic observations demonstrated that cells could attach to the hydrogel surface and maintain high viability and proliferation ability. Cell detachment efficiency from the hydrogel was high. These unique properties of the hydrogel would make it a promising support for epithelial cell grafting especially cornea regeneration.

Keywords: Thermo-sensitive hydrogel; chitosan; N-Isopropylacrylamide; gamma radiation; epithelial cell sheet

INTRODUCTION

During recent decades several materials and medical devices have been produced for medical purposes. For tissue engineering, it is desirable to recover the monolayer cells in a cell sheet structure at the end of the culture stage without using a biochemical or chemical reagent. Such a cell sheet constructed in vitro could be useful in various clinical situations to regenerate tissues (especially epithelial tissues) such as artificial skin and artificial cornea. 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 thermo responsive culture dishes. Thermo responsive polymers are grafted to dishes covalently, which allow different cell types to attach and proliferate in 37 °C. Cells detach spontaneously when temperature decreases below 32 °C, without using enzymes, and this is due to the natural specification of the intelligent polymers also due to the detachment of the cell metabolic changes made by the polymer resulting from decreasing temperature[5-9]. Environmental sensitive systems or intelligent polymers are those that react to environmental small changes. In fact, 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. Thermo responsive polymers show a balanced and proper hydrophilic-hydrophobic in their structures. They were able to switch on–off the receptor using the transition between extended and coiled form of the molecule[10-12]. PNIPAAm and its copolymers are among those materials which have LCST. PNIPAAm shows LCST in 32 °C. While the temperature is over 32 °C, polymer is solid and also hydrophobic and when it is below 32 °C, it is completely

hydrated and shows hydrophilic properties[13]. Chitosan (CS) is a natural cationic polymer obtained from N-deacetylation of chitin [(1–4)-2-acetamiodo-2-deoxy-d-glucose], which is the second most abundant natural polymer on earth after cellulose. This polysaccharide is considered to be nontoxic, biodegradable; it has been used as an anticoagulant, a wound-healing accelerator, and drug delivery material [14-16]. Graft copolymerization of vinyl monomers onto CS can introduce desirable properties and enlarge the field of the potential applications by choosing various types of side chains [17-20]. In recent years, a number of initiator systems have been developed to initiate grafting copolymerization. Radiation graft copolymerization is a well-established technique for producing polymeric materials that combine the chemical and physical properties of both the base polymer and the grafted monomer[21-23]. In this work, hydrogels based on CS grafted N-isopropylacrylamide were prepared by ^{60}Co gamma radiation, the thermo sensitivity and swelling and structural properties of the polymers were also investigated. In this study, the biocompatible hydrogel surface was used for epithelial cell attachment and proliferation. A continuous and viable cell monolayer would form after cell growth to confluence. When the cells were incubated at 4°C, a cell sheet mimicking natural tissue structure could be obtained with the spontaneous dissolution of the thermoreversible hydrogel and the concomitant cell detachment from the surface.

MATERIALS AND METHODS

Materials

Chitosan was purchased from Fluka company (degree of deacetylation = 98%, molecular weight =1.5 _ 105 g/mol). N-Isopropylacrylamide (NIPAM, Aldrich) were recrystallized from nhexane and methanol freshly before use . Dulbecco's modified eagle's medium (DMEM, Sigma) was used for cell culture.

A series of CS-g-NIPA hydrogels were prepared in the following procedures: pure CS dissolved in 5% aqueous acetic acid (25 ml) in a glass reaction bottle, the monomer was added to the CS solution (W% CS/monomer =1/2. Mohr's salt (ammonium ferrous sulphate) was added to the mixture to minimize homopolymerization during irradiation. The solution was deoxygenated by purging with nitrogen for 30 min. The sealed reaction bottles were irradiated at a dose 20 kGy. After irradiation, the product was extracted with methanol in a Soxhlet extractor for 48 h, in order to remove the unreacted monomer, homopolymer and other impurities. The hydrogel was dried at 40 °C in a vacuum oven overnight.For cell culture; SW742 (NCBI C146) colon carcinoma and adenocarcinoma cells (obtained from National Cell Bank of Iran, NCBI) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. They were incubated at 37°C in a humidified CO2-incubator with 5% CO2 and 95% air. For Cytotoxicity assay, The effect of diethylenetriaminepentacetic acid (DTPA) on these cell lines, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), colorimetric assay was applied [9]. Briefly, asyn-chronously growing cells (1.5 × 104 cells/ml) were transferred into 96-well culture plates containing 200 μl of medium and incubated for 24 h. Various concentrations of DTPA (0-100 μM) were added and incubated for different time intervals followed by MTT assay. The percent of cell viability was calculated as the (mean OD of treated cells/mean OD of control cells.

13C CP/MAS NMR analysis

13C CP/MAS NMR spectra were recorded on a Varian UNITY plus 400 NMR spectrometer in solid state at a frequency of 100.6MHz for 13C nuclei. The sample was placed in a zirconia rotor (5 mm), the spinning rate was set at 4.5 kHz at the magic angle, and 13C chemical shifts were referenced to external hexamethylbenzene.

Swelling measurement

Swelling of the hydrogel was determined gravimetrically after immersing the grafted product in water for an hour, the surface of the hydrogel was wiped with filter paper in order to remove the free water and weighed.The swelling ratio was determined as,

Swelling ratio: Ws/Wd

Where Ws and Wd are the weights of swollen and dry hydrogels, respectively.

Differential scanning calorimetry (DSC)

The samples were investigated by thermal analysis using the DSC device (Netzschdsc200F3) with the heating rate of 5 degree per minute from 0°C to 60°C in a nitrogen gas atmosphere.

Cell culture on hydrogels

Aliquots of cell suspension in RPMI medium containing 300,000 SW742 epithelial cells were seeded on a 6-multiwell cell culture plate (orang), which was pre-coated with hydrogels. The plate was incubated in a incubator (37 °C, CO2) for 3 h for cell attachment, followed by rinsing off the loosely attached cells with phosphate buffer solution, and adding 2 ml of fresh medium for cell culture in a incubator (37 °C, CO2) for 7 days. The proliferation of cells was determined by MTT assay for measurement of viable cell number. The MTT tetrazolium compound is reduced by living cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product is directly proportional to the number of viable cells in the culture. The assays were performed by adding 1 ml of MTT solution (Sigma) and 9 ml fresh medium to each well after aspirating the spent medium, and incubating at 37 °C for 4 h with protection from light. Colorimetric measurement of formazan dye was performed at a wavelength of 570 nm using a microplate reader (RAYTO).

Detachment of cells cultured on hydrogels

For cell detachment, SW742 cells were seeded onto the hydrogels at a density of 1,000,000 cells and cultured at 37 °C under a humidified atmosphere of 5% CO2. Cell detachment was evaluated by incubating the cultures at 4 °C for up to 60 min to dissolve the hydrogels with concomitant cell detachment. Culture medium containing the detached cells and the dissolved was transferred to a new well. Number of detached cells and cells attached to the original well was determined by MTT assay.

RESULTS

^{13}C CP/MAS NMR spectroscopy analysis

^{13}C CP/MAS NMR spectra of CS and grafted CS were shown in Fig. 1. The peak assignments of ^{13}C chemical shifts of CS were 53.4 (C2), 56.8(C6), 71.4 (C3,5), 79.2 (C4), and 101.6 (C1) ppm respectively. Except for the signals of CS denoted as ‘c’ in ‘Figure 1’ several new peaks denoted as ‘d’ in ‘Figure 1’ were found at a range from 10–45 ppm in grafted CS, which should be assigned to aliphatic signals of grafted PNIPA, this further confirms the grafting of PNIPA onto CS. On the other hand, it is known that CS molecule contains two reactive groups at C2 and C6 positions. The grafted CS showed downfield shifts for both, which implies that the grafting reaction may have taken placed on C2 and C6 positions. In addition, we also noticed that all peaks in the grafted CS were broadened as the result of the paramagnetic effect of Fe^{3+} of ammonium ferrous sulphate on the ^{13}C NMR signals.

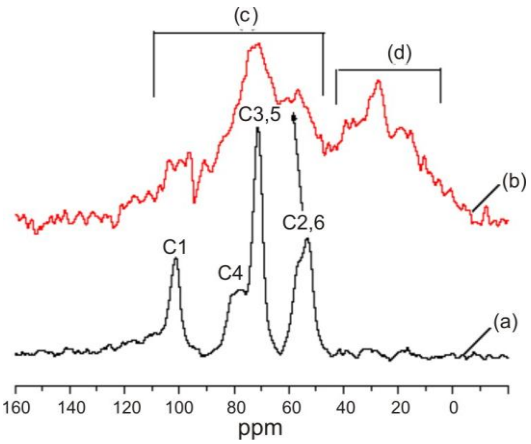


Figure 1. ^{13}C-NMR spectra of (a) CS and (b) CS-g-NIPA

Swelling measurement

Swelling ratio of the hydrogels in 4,10,25,37 and 40 °C in the distilled water was investigated. ‘Table 1’ indicated swelling ratio of the hydrogels in different temperatures. Swelling ratio for 37 and 40 °C was calculated 1 which demonstrated hydrophobicity of samples and for 4, 10 and 25 °C was about 4 which showed hydrophilicity of the samples. ‘Figure 2’ showed swelling / temperature ratio of hydrogels. The curve slop related to critical temperature of the gels at 32 °C and this demonstrated presence of PNIPAAm in the hydrogels and no significant change in polymer LCST during radiation and graft process.

Table 1. Swelling ratio of hydrogel in different temperatures

T°C	Ws/Wd
4	4.25
10	4.15
25	4.11
37	1.10
40	1

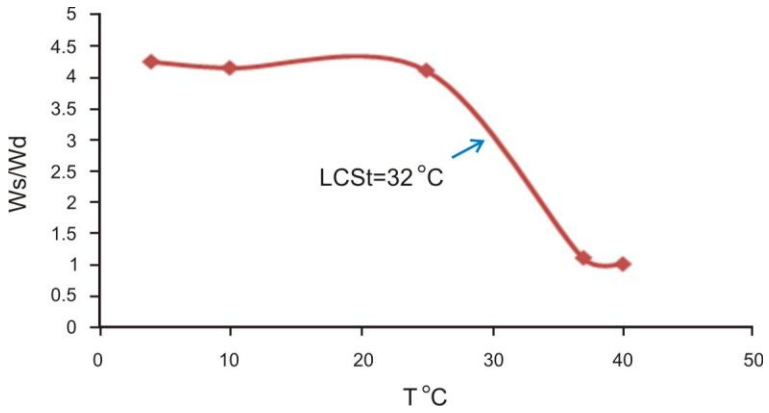


Figure 2. Ws/Wd ratios to T°C, the curve slop in 32°C showed presence of PNIPAAm in hydrogel

Differential scanning calorimetry (DSC)

The samples were investigated by thermal analysis using the DSC device (NETZSCHDSC200F3), with the heating rate of 5 degree per minute from 0°C to 60°C in a nitrogen gas atmosphere. The grafted samples' DSC analysis review showed a critical temperature of the grafted PNIPAAm. 'Figure 3' shows the DSC thermogram in which the curve slope in 26°C and pick in 32°C are obtained. This shows no significant change in the smart polymer critical temperature during radiation and graft process.

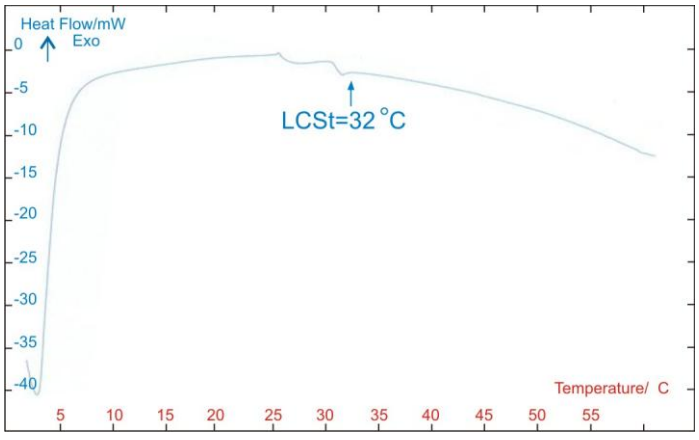


Figure 3. DSC spectra of the hydrogel grafted by gamma radiation (radiation dose : 20 KGy)

Cell results

At physiological temperature (37°C), the hydrogel (25°C) turns into a rigid gel within 10 min and the phase transition is reversible. Biocompatibility data demonstrated that the hydrogel supported epithelial cell adhesion and proliferation and the cells also maintained high viability 'Figure 4'. After cultured for 7 days on gel, all cells are alive, suggesting that hydrogel is suitable for cell attachment and proliferation 'Figure 4a' and viability is 90% , When cells were placed outside the incubator and the medium cooled from 37 to 4 °C, almost all cells are alive 'Figure 4b' and viability is up 80% .

'Figure 5a' shown cells grown on control surface (TCPS) that grown completely (95%). 'Figure 5b'shown good cells grown on gel surface (90%) at physiological temperature (37°C), 'Figure 5c and 5d' shown cells grown detached from the hydrogel surface spontaneously, in the absence of enzymes (trypsin/EDTA). Cell detachment efficiency from the hydrogel was high. In contrast, cells grown on TCPS dishes did not show such temperature-dependent cell sheet detachment, implying that cell sheet detachment from the hydrogel was not due to side effects such as CO2 concentration or pH, but was due to the thermo-responsiveness of the hydrogel.

After a longer period of cell cultivation for 7 days, confluent cells formed a continuous monolayer cell sheet on the surface of the hydrogel. The cell sheet spontaneously detached from the surface of the thermo-reversible hydrogel when cooled to 4 °C without treating with any enzymes. As shown from 'Figure 5c and 5d' detachment of the cell monolayer started from the edge of the cell monolayer.After 60 min incubation at 4 °C, a monolayer cell sheet could be lifted up from the edge upon mild perturbation of the medium. A living cell sheet completely detached from the culture surface could be obtained within 60 min 'Figure 5c and 5d'. Although the cell sheet was folded into an irregular shape by contractile forces between cells, cell–cell connections were well preserved in the rolled- up sheet. These results demonstrate that cold treatment effectively released the cell sheet from the plate without considerable damage of the cell–cell connections.

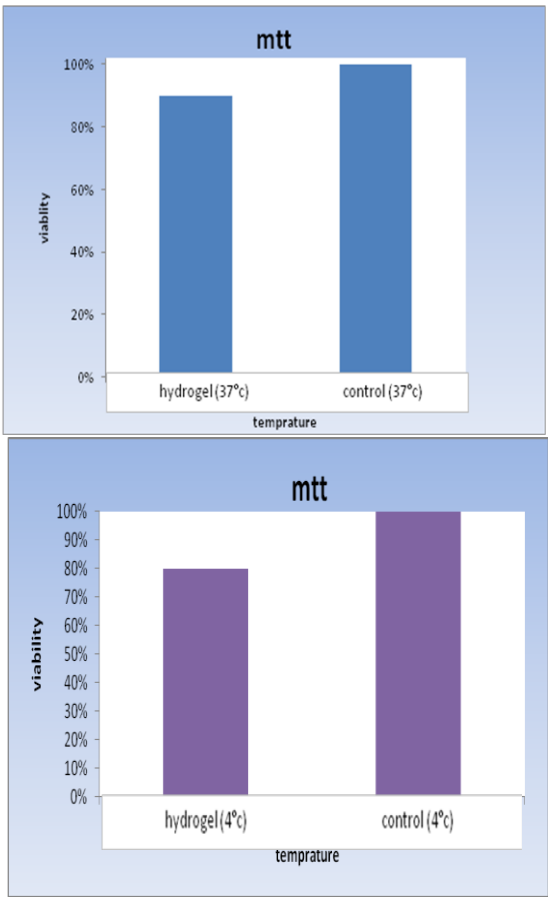
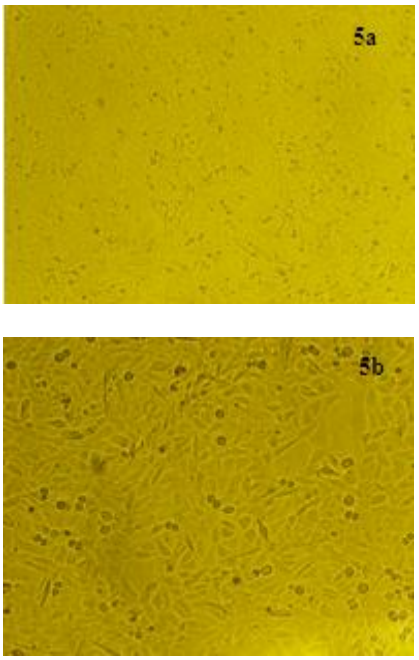


Figure 4. Good cells viability on gel surface at physiological temperature (37°C) (Fig.4a), and after 60 min incubation at 4 °C (Fig. 4b) and control surface (TCPS)



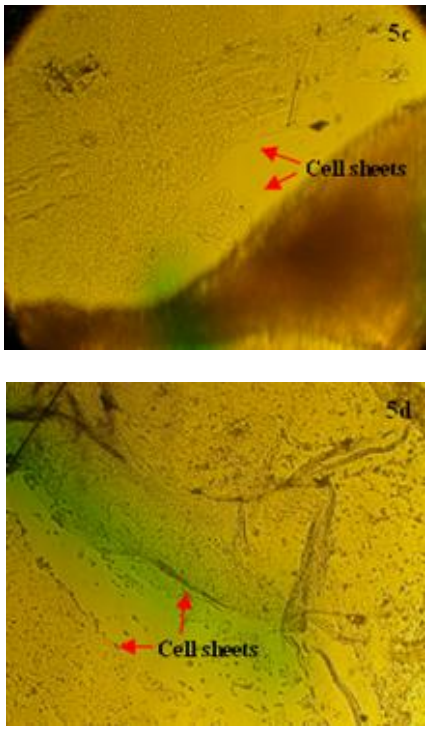


Figure 5. Cells growth on control surface (TCPS) 'Figure 5a'. Good cells grown on gel surface at physiological temperature (37°C) 'Figure 5b'. Detachment of the cell monolayer after 60 min incubation at 4 °C 'Figure 5c and 5d'

DISCUSSION

In this work, hydrogels based on CS grafted N-isopropylacrylamide were prepared by 60Co gamma radiation, the thermo sensitivity and swelling and structural properties of the polymers were also investigated. The CS-g-NIPA hydrogels showed good safety and thermosensitivity and swelling property. Epithelial cells grown well on the hydrogel surface at 37°C and shown high viability that this administrated biocompatibility and non toxicity of our hydrogel. MTT analysis showed good viability of hydrogel at 37°C. Cells also (cell sheet) detached spontaneously when temperature decreased at 4°C, without using enzymes. MTT analysis showed good viability of the hydrogel at 4°C that this administrated no significant change in cell viability. This unique property of the hydrogel would make it a promising support for cell grafting and especially for cornea regeneration.

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