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Applications of chitosan-based thermo-sensitive copolymers for harvesting living cell sheet

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ABSTRACT

A thermo-sensitive chitosan-based copolymer hydrogel was used for harvesting living cell sheets. The hydrogel was tested for harvesting 3T3 cells after carrying out cell culture at 37 °C and incubating the confluent cells at 20 °C for spontaneous detachment of cell sheets from hydrogel surface without enzyme treatment. Results from cell viability assay and microscopy observations demonstrated that cells could attach to the hydrogel surface and maintain high viability and proliferation ability. Cell detachment efficiency from the hydrogel was about 80%. The detached cell sheet retained high viability and could proliferate again after transferred to a new culture surface.

1. Introduction

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 [1,2]. Using poly(*N*-isopropylacrylamide) (PNIPAAm), it is possible to rapidly recover intact cell sheets from culture surfaces using temperature drop as the mechanism for cell detachment. PNIPAAm is well known for its unique reversible solubility transition at the lower critical solution temperatures (LCST) of about 32 °C in an aqueous solution [3,4]. Cells will attach and proliferate at 37 °C (above the LCST) on tissue culture polystyrene (TCPS) surface grafted with PNIPAAm. By lowering the temperature to a value below the LCST, a cell sheet layer could be obtained by detaching cells from a well-designed culture surface as a result of hydration and swelling of grafted PNIPAAm chains [5]. PNIPAAm has been immobilized on TCPS by photolithography and vapor-phase deposition of plasma polymerization [6–8]. Recently, Okano's group has developed a novel technique of cell sheet engineering by grafting PNIPAAm to TCPS with electron beam irradiation. However, the whole grafting process is relatively complicated and time-consuming [9,10].

In this study, a simple and inexpensive method was proposed by simply pouring a thermo-sensitive chitosan-*g*-poly(*N*-isopropylacrylamide) (CPN) polymer solution into a dish at room temperature and incubating 37 °C for hydrogel formation. The biocompatible hydrogel surface was used for cell attachment and proliferation. A continuous and viable cell monolayer would form after cell growth to confluence. When the cells were incubated at 20 °C, a cell sheet mimicking natural tissue structure could be obtained with the spontaneous dissolution of the thermo-reversible hydrogel and the concomitant cell detachment from the surface. The feasibility of our method was examined for the CPN copolymer hydrogel using 3T3 fibroblast cells as a model cell line.

2. Experiment

2.1. Materials

N-Isopropylacrylamide (NIPAM, Aldrich) and azobisisobutyronitrile (AIBN, Sigma) were recrystallized respectively from *n*-hexane and methanol freshly before use. Mercaptoacetic acid (MAA, Fluka) was used as a chain transfer agent to synthesize

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carboxyl-ended poly(*N*-isopropylacrylamide) (PNIPAM-COOH). Chitosan (degree of deacetylation = 98%, molecular weight = 1.5×10^5 g/mol) was obtained from Fluka. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Acros. Dulbecco's modified eagle's medium (DMEM, Sigma) was used for cell culture.

2.2. Synthesis of chitosan-g-PNIPAM (CPN) copolymer

CPN was synthesized by following the method reported previously by our group [11]. In brief, PNIPAM-COOH was first prepared by free radical polymerization of NIPAAm monomer and MAA in benzene using AIBN as an initiator. The molar ratio of NIPAAm:MAA:AIBN is 100:10:1. CPN was synthesized by conjugating the carboxylic acid group of PNIPAM-COOH to the amine group of chitosan, with the weight ratio of PNIPAM-COOH to chitosan equal to 50, in the presence of EDC and NHS in 2-morpholinoethane sulphonic acid (MES) buffer (0.1 M, pH 5.0). The copolymer was purified by thermo-induced precipitation and re-dissolution cycles and dialysis, and lyophilized for storage in a desiccator at room temperature.

2.3. Cell culture on CPN

Aliquots of cell suspension in DMEM medium containing 1×10^5 3T3 fibroblast cells were seeded on a 24-multiwell cell culture plate (Nunc), which was pre-coated with autoclaved 10% (w/v) CPN hydrogel. The plate was incubated in a 37 °C CO_2 incubator 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 37 °C CO_2 incubator for up to 7 days. Proliferation of cells was determined by MTS assay for measurement of viable cell number. The MTS 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 40 μl of MTS solution (Promega) and 200 μl fresh medium to each well after aspirating the spent medium, and incubating at 37 °C for 3 h with protection from light. Colorimetric measurement of formazan dye was performed at a wavelength of 490 nm using an ELISA plate reader (Tecan Sunrise).

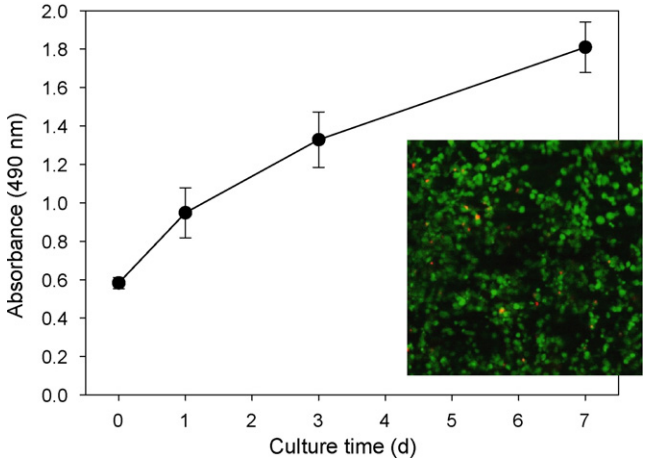

Fig. 1. Attachment and proliferation of 3T3 cells seeded on chitosan-g-poly(*N*-isopropylacrylamide) hydrogel. The insert shows the viability of attached cells at day 7 by Live/Dead assay where live cells are in fluorescence green and dead cells are in fluorescence red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

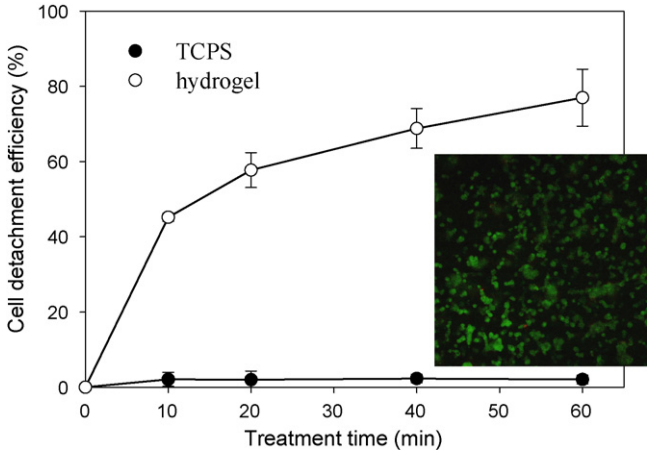

Fig. 2. Detachment efficiency of 3T3 cells from chitosan-g-poly(*N*-isopropylacrylamide) hydrogel and TCPS (control) after cultured at 37 °C for 3 h and incubated at 20 °C for different times. The insert shows the viability of detached cells from the hydrogel by Live/Dead assay after treatment for 60 min. Live cells are in fluorescence green and dead cells are in fluorescence red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Cells were assayed for viability by the Live/Dead Viability/Cytotoxicity Assay Kit (Invitrogen). The kit provides two molecular probes, calcein AM and ethidium homodimer-1 (EthD-1), to simultaneously differentiate live and dead cells. Cells were stained with a mixture of 2 mM EthD-1 and 4 mM calcein AM and the fluorescence-stained cells were imaged using an inverted fluorescence microscope (Leica DMIL).

2.4. Detachment of cells cultured on CPN

For cell detachment, 3T3 cells were seeded onto the hydrogel or a TCPS surface at a density of 1×10^6 cells and cultured at 37 °C under a humidified atmosphere of 5% CO_2. Cell detachment was evaluated by incubating the cultures at 20 °C for up to 60 min to dissolve the copolymer hydrogel with concomitant cell detachment. Culture medium containing the detached cells and the dissolved copolymer was transferred to a new well. Number of detached cells and cells attached to the original well was determined by MTS assay. The percentage of detached cells (%) was defined as the absorbance of detached cells divided by that of total cells (detached cells plus cells left in the original well). The viability of detached cells was assessed by the Live/Dead assay.

For cell re-attachment and re-growth, the content of each well after the cold treatment was aspirated and transferred to a fresh 24-well TCPS culture plate. The plate was then returned to the 37 °C CO_2 incubator to allow cell attachment and growth. Proliferation of the transferred cells in each well was assessed using MTS assay.

3. Results and discussion

The number average molecular weight and degree of polymerization of PNIPAM-COOH are 2×10^4 g/mol and 180, respectively. From the molecular weight and degree of deacetylation of chitosan, 1 g of chitosan will contain 6.2 mmol amino groups for conjugation with the carboxylic acid groups of PNPAAm-COOH. With NH_2/COOH molar ratio equal to 2.59 during the conjugation reaction, 32% of the available NH_2 groups in chitosan have been conjugated with PNIPAAm and the molecular weight of CPN is 5.99×10^6 g/mol.