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Thermo-responsive poly(N-isopropylacrylamide)-grafted hollow fiber membranes for osteoblasts culture and non-invasive harvest

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ABSTRACT

Hollow fiber membrane (HFM) culture system is one of the most important bioreactors for the large-scale culture and expansion of therapeutic cells. However, enzymatic and mechanical treatments are traditionally applied to harvest the expanded cells from HFMs, which inevitably causes harm to the cells. In this study, thermo-responsive cellulose acetate HFMs for cell culture and non-invasive harvest were prepared for the first time via free radical polymerization in the presence of cerium (IV). ATR-FTIR and elemental analysis results indicated that the poly(N-isopropylacrylamide) (PNIPAAm) was covalently grafted on HFMs successfully. Dynamic contact angle measurements at different temperatures revealed that the magnitude of volume phase transition was decreased with increasing grafted amount of PNIPAAm. And the amount of serum protein adsorbed on HFMs surface also displayed the same pattern. Meanwhile osteoblasts adhered and spread well on the surface of PNIPAAm-grafted HFMs at 37 °C. And Calcein-AM/PI staining, AB assay, ALP activity and OCN protein expression level all showed that PNIPAAm-grafted HFMs had good cell compatibility. After incubation at 20 °C for 120 min, the adhering cells on PNIPAAm-grafted HFMs turned to be round and detached after being gently pipetted. These results suggest that thermo-responsive HFMs are attractive cell culture substrates which enable cell culture, expansion and the recovery without proteolytic enzyme treatment for the application in tissue engineering and regenerative medicine.

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1. Introduction

It's important for regenerative medicine including tissue engineering and cell transplantation to harvest a large number of therapeutic cells, usually in the order of 10^{10} cells for each patient [1,2]. Owing to the large scale area-to-volume ratio involved, hollow fiber membranes (HFMs) provide a tremendous amount of surface area for high cell number in relatively small volume of media. Additionally, HFMs have the ability for the efficient exchange of nutrients and waste products across the fiber wall without any cellular damage. Therefore, cell large-scale harvest can be easily achieved with three-dimensional HFM culture system [3]. On the other hand, repeated trypsinization is required in the process of passaging culture to harvest adhered cells from the surface of HFMs. However, this proteolytic enzyme treatment not only invasively destroys cell-to-cell junctions but also irreversibly damages cell membrane-associated matrix proteins, leading to the reduction of cell viability, the loss of cell function or cell death [4–6]. Therefore, alternative HFMs enabling non-invasive cell harvest without proteolytic enzymes and providing intact cells would be an advantageous candidate for the application of HFMs to large-scale culture of therapeutic cells.

Currently, the novel cell harvest strategies have been focused on stimuli-responsive polymer mediated detachment. For example, thermo-responsive polymers, pH-responsive polymers [7] and alginate hydrogel [8], these polymers can reversibly change their microstructures from collapse to expansion in response to the external stimuli (changes of temperature or pH etc.). Poly(N-isopropylacrylamide) (PNIPAAm), a thermo-responsive polymer, is the most widely studied in cell culture and non-invasive recovery because its physiologically proximal lower critical solution temperature (LCST) is around 32 °C [9]. In 1990, Okano group [10] firstly prepared a thermo-responsive surface using PNIPAAm for cell culture and harvest. At 37 °C, the thermo-responsive surface exhibits weak hydrophobic property, which is suitable for cell adhesion and growth. When the temperature is below LCST, the surface becomes hydrophilic and its volume expands, the combined effects force the cells to detach from the substrate. This cell harvest method makes cell membranes remain intact [6]. Consequently, cells retain better growth activities and biological functions compared with those harvested by enzymes or cell scrapers.

Since the physical structures and properties of PNIPAAm polymers can be easily controlled by simply shifting the temperature, a few types of PNIPAAm modified surfaces and hydrogels have been developed in recent years for cell culture and harvest [11–14]. Among these thermo-responsive microcarriers have been constructed via atom transfer radical polymerization (ATRP) for the control of cell adhesion and cell detachment without proteolytic enzyme treatment [11,12]. And a variety of

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thermo-responsive hydrogels consisting of PNIPAAm and natural polymer materials (chitosan, hyaluronic acid, collagen etc.) have been prepared. It was found that the incorporation of natural polymer materials could enhance cell attachment [13,14]. On the other hand, several PNIPAAm modified HFMs have been reported to control membrane flux thermo-responsively [15,16]. Wang et al. [15] prepared PNIPAAm-modified poly(vinylidene fluoride) HFMs by free radical polymerization using potassium persulfate as the initiator. The changes of flux of pure water and the rejection of ovalbumin with temperature demonstrated that the obtained PNIPAAm-modified PVDF HFMs have thermo-sensitivity. Li et al. [16] prepared dual stimuli-sensitive (pH- and thermo-sensitive) polyethersulfone HFMs modified with a terpolymer of poly(N-isopropylacrylamide-co-methacrylic acid-co-methyl methacrylate). The significant change of the pore sizes with temperature indicated that the P(NIPAAm-MAA-MMA)-modified PES HFMs had thermo-sensitivity. However, to date no work has been done regarding applying thermo-responsive HFMs for the purpose of cell culture and non-invasive harvest. Therefore this study aims to develop the thermo-responsive HFMs for large scale expansion and non-invasive harvest of cells.

To this end, the thermo-responsive PNIPAAm-grafted HFMs were prepared via free radical polymerization using cerium ammonium nitrate (CAN) as a chemical initiator. Then the physical, structural and thermal properties of the obtained PNIPAAm-grafted HFMs were investigated using different techniques. Moreover, the cellular compatibility of thermo-responsive HFMs was analyzed by means of Alamar Blue (AB) assay, alkaline phosphates (ALP) activity and Osteocalcain (OCN) protein expression. Finally, the ability of cell detachment induced by temperature reduction was assessed.

2. Materials and methods

2.1. Materials

Cellulose acetate HFMs with an outer diameter and wall thickness of 175 μm and 13 μm, respectively, were obtained from KAWASUMI. In order to eliminate the possible impurities (such as dust), the fibers were first washed in ethanol at 25 °C for 120 min. Then the fibers were rinsed with deionized water and dried in an air oven at 25 °C for 2 days. N-isopropylacrylamide (NIPAAm), supplied by J&K Chemical Reagent Co., Ltd, was used as a graft monomer and recrystallized from hexane. CAN was purchased from Sinopharm Chemical Reagent Co., Ltd. as an initiator. Hexane and nitric acid were supplied by Tianjin Kemiou Chemical Reagent Co.,Ltd. All chemicals were analytic grades and were used as received without further purification. Deionized water was used in all procedures.

2.2. Preparation of PNIPAAm-grafted HFMs by free radical polymerization

Before grafting polymerization, cellulose acetate HFMs were immersed in 0.05 M NaOH solution at 25 °C for 24 h, then washed with deionized water to remove the alkali solution. The purpose of alkaline treatment is to achieve the deacetylation of cellulose acetate [17,18], and to form the hydroxyl, which can be oxidized by CAN and produce free radicals. As an initiator, CAN leads to formation of free radicals during oxidation of C2 and C3 hydroxyl groups of anhydro-D-glucose of cellulose, which then initiates the graft polymerization of NIPAAm vinyl monomer (Fig. 1) [19–22].

PNIPAAm-grafted HFMs were prepared by free radical polymerization according to the reported protocols with some modifications [19,20]. A typical procedure (e.g., HFMs-0.2, 0.2 represents that the feed concentration of NIPAAm is 0.2 M) was as follows: alkaline treated HFMs (abbreviated as HFMs-0, single length: 4 cm, diameter: 175 μm, total number: 500) were loaded into a round bottom flask equipped with a three-way stopcock, in which 25 mL nitric acid solution ($[HNO_3]$ = 0.1 M) was added, followed by bubbling with nitrogen for 5 min to deoxygenate the reaction mixture. The CAN (0.137 g, 0.25 mmol) was introduced into the reactor to carry out the initiation step. The CAN initiator concentration, initiation time and temperature were 10 mM, 30 min and 60 °C, respectively. After the initiation step, the recrystallized NIPAAm (0.565 g, 5 mmol) was added into the reactor and graft copolymerization of NIPAAm onto the HFMs was carried out under nitrogen at 25 °C for 6 h. After grafting copolymerization of NIPAAm, the sample was rinsed and washed with deionized water to remove the initiator, ungrafted PNIPAAm homopolymer and unreacted monomers. The washed HFMs were dried in oven at 25 °C for at least 24 h.

2.3. Surface characterization of PNIPAAm-grafted HFMs

A Perkin Elmer Fourier Transform Infrared (FTIR) Spectrometer (Spectrum 2000) was used to characterize the changes of surface chemical structure. The FTIR spectrometer was purged with N_2 atmosphere for 30 min before and after sample loading. FTIR measurements in the attenuated total reflectance (ATR) mode were performed over 64 scans in the range of 500–4000 cm^{-1} with 4 cm^{-1} resolution. The data was analyzed using OriginLab 8.

The grafted amount of PNIPAAm on HFMs surface was calculated by an increment in the nitrogen composition derived from the amide bonds of PNIPAAm. The nitrogen content of the HFMs was measured by an Elemental analyzer (VarioELIII, Elementar, Germany). The grafted amount of PNIPAAm on the HFMs surfaces was calculated as follows [23]:

$$m_p(\mu g/cm^2) = \frac{N_p\% - N_0\%}{(N_{P,theor}\% - N_0\%)S} \times 10^6$$

where N_p % and N_0 % represent the nitrogen content in PNIPAAm modified and unmodified HFMs, respectively, $N_{P,theor}$ % represents the theoretical nitrogen content of PNIPAAm, and S represents the specific surface area of HFMs, 1116 cm^2/g.

Morphology of the samples was observed using a NOVA NanoSEM 450 Scanning Electron Microscope (SEM). Dry samples were coated with a thin layer of gold using a coating unit, in order to increase electrical conductivity of the samples.

2.4. Thermo-responsive behavior characterization

1) Dynamic contact angle measurement

The dynamic contact angles between the deionized water (20 °C and 37 °C) and the external surface of the HFMs were measured based on Wilhelmy plate method using a DCA-322 dynamic contact angle analyzer manufactured by American ThermoCahn Company. The testing conditions were wetting speed 0.03 mm/s, immersion depth 3 mm and detection threshold 0.1 mg. Five HFMs parallel to each other were measured at the certain wetting speed; the dynamic contact angle could be calculated from the determined wetting power of the HFMs. Every test was repeated three times to eliminate the errors.

2) Protein adsorption determination

Adsorption of serum protein on the surface of PNIPAAm-grafted HFMs was demonstrated at 37 °C and 20 °C to evaluate the temperature responsive property of the surface. 1 mL, 2% serum protein in DMEM was incubated with PNIPAAm-grafted HFMs in every well of a 24-well plate at 37 °C and 20 °C for 24 h, and the total weight of PNIPAAm-grafted HFMs in one well was adjusted to 3 mg. The 10 μL supernatant of the incubated serum protein solution was collected in a 96-well plate, then 200 μL BCA working liquid was added and incubated for 30 min at 37 °C. The optical density (OD) at the emission wavelengths of 562 nm and 620 nm was measured by an Enzyme-linked immune detector (Thermo scientific, USA). The optical density of serum protein adhered to the surface of HFMs was estimated based

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