Synthesis and Characterization of HA-grafted Thermo-reversible Chitosan Hydrogel for Cartilage and Meniscus Tissue Engineering

Tai-Hong Cheng,[1] Jyh-Ping Chen[1,2]
[1]Department of Chemical and Materials Engineering, [2]Graduate Institute of Biochemical and Biomedical Engineering, Chang Gung University, Kwei-San, Taoyuan 333, Taiwan, Republic of China

Statement of Purpose: Cartilage and meniscus have very low cell-to-matrix ratio, which led to limited ability of self-repair. Hence, to successfully achieve articular cartilage and meniscus tissue regeneration, a scaffold as the cell carrier for chondrocytes and meniscus cells should closely mimic the *in vivo* environment of the knee joint, and contain 3-D inter-connected pores with adequate mechanical strength. Chitosan has a very similar structure to glycosaminoglycans (GAG) present in cartilage and meniscus. Hyaluronic acid (HA), which is abundant in the synovial fluid, is known to have a high capacity for water adsorption and retention. In this study, an injectable thermo-responsive ((chitosan-*g*-poly(*N*-isopropylacrylamide))-*g*-HA) (CPN-HA) hydrogel was synthesized. The hydrogel was characterized in details and its potential use in cartilage and meniscus tissue engineering was also evaluated.

Methods: The CPN-HA hydrogel was synthesized by grafting carboxyl acid-ended poly(*N*-isopropylacrylamide) (PNIPAM-COOH) and HA onto chitosan in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The LCST of the polymer solution was measured by determining solution turbidity at different temperature and is defined as the temperature where solution turbidity is half the difference between the maximum and the minimum values. The volume shrinkage ratio was defined as the percentage of water volume loss from the polymer solution after gelling at 37□. Chondrocytes and meniscus cells were obtained from knee cartilage of New Zealand rabbits. Cell viability was determined by an *in situ* fluorescence assay using FDA. To evaluate cell growth, cell numbers were determined by DNA content. Cell functions were determined by measuring GAG concentrations with 1,9-dimethyl-methylene blue (DMB) and total collagen concentrations from hydroxyproline content after hydrolysis. Secretions of tissue-specific sulfated GAG and type II collagen were demonstrated by histological examinations and immunohistochemical stainings.

Results/Discussion: The molecular weights of PNIPAM-COOH, CPN, and CPN-HA are 2.1×10^4, 1.12×10^6, and 1.19×10^6, respectively. The dependence of absorbance of polymer solution on temperature shows a hysteresis loop with higher liquid-to-solid phase transition temperature. However, regardless of the chemical composition, LCST of all thermo-sensitive polymers is around 30□. The complex shear modulus (G*) of the polymer rises abruptly around the LCST and increases with temperature thereafter, corresponding to a sharp phase change and a more rigid and solid-like hydrogel in appearance. From the results of water content and volume shrinkage ratio, the CPN-HA hydrogel (5% (w/v)) has the highest water content (14.25 g water/g polymer) and the lowest volume shrinkage (19.84%), implying that the network structure of CPN-HA can accommodate more water and stabilize structural integrity. From SEM micrographs of chondrocytes and meniscus cells cultured in CPN-HA, the hydrogel contains interconnected pores in the range of 10 to 30 μm with isolated cell aggregates surrounded by thick extracellular matrix (ECM) found within the polymer network. For cell proliferation in the hydrogel, cell numbers increased steadily with time and the cell number was more than twice that cultured on TCPS after 6 weeks. Similarly, the 3-D hydrogel culture system gave 2 to 3-fold increase in collagen synthesis and GAG production when compared with 2-D dish culture. FDA viability staining strongly supports the cytocompatibility of CPN-HA as chondrocytes and meniscus cells are still of high viability after cultured for a extended period of time (114 days) in the hydrogel. Hematoxylin-Eosin (HE) stain also showed well differentiated chondrocyte morphology with round cells surrounded by dense ECMs. Alcian blue staining identified the abundant accumulation of sulfated GAG around cells. From immunostaining of cell construct after cultured for 20 weeks, type II collagen could still be positively stained and identified for chondrocytes. Furthermore, the mechanical strength of the cell/scaffold construct was substantially enhanced after long-term cell culture. From a tissue engineering perspective, the increase in mechanical strength represents structure stabilization of the cell/scaffold construct and a possible advantage in reconstruction of the damaged tissues. From those results, CPN-HA can be shown to be a useful injectable hydrogel for *in vitro* culture of chondrocytes and meniscus cells to maintain cell viability, and promote cell proliferation and differentiation.

Conclusions: CPN-HA, a HA-grafted thermo-reversible chitosan hydrogel synthesized by conjugating PNIPAM-COOH and HA to chitosan backbone using EDC, was synthesized and characterized in this study. This injectable thermo-reversible hydrogel can be used for tissue engineering of cartilage and meniscus. Results from SEM observations, viability staining, cell proliferation, and collagen and GAG synthesis, suggest that CPN-HA hydrogel could provide a suitable *in vitro* environment for maintaining cellular functions, promoting cell proliferation and differentiation, and will find potential applications in inducing cartilage and meniscus tissue growth.

References:

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