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Relationships between surface roughness/stiffness of chitosan coatings and fabrication of corneal keratocyte spheroids: Effect of degree of deacetylation

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ABSTRACT

Fabrication of the cell spheroids from corneal keratocytes has important implications to the advance in tissue engineering while stimulation from the interface of a biopolymer coating has the ability to modulate this event. This study aims to investigate the dependence of keratocyte migration, proliferation, and differentiation on the surface roughness/stiffness of the chitosan coatings through modifications by degree of deacetylation (DD). After a series of deacetylation process, chitosan coatings with increasing DD exhibited significantly decreased surface roughness and increased surface stiffness. Relationships between the behaviors of rabbit corneal keratocytes (RCKs) and biopolymer coatings with varying DDs (between 75% and 96%) were also found during in vitro cultivation. Both the surface roughness increase and stiffness decrease could lead to enhanced cell migration, which is the main driving force for the early stage spheroid formation on chitosan substrates (e.g., within 8 h). With these stimulations from the substrate interfaces, the size and morphology of RCK spheroids were greatly affected by the DD of chitosan. When fabricated on a lowered DD of chitosan material, the spheroids had a larger size with abundant extracellular matrix produced around the cells. At a later stage of spheroid cultivation (e.g., 5 days), significantly higher amount of RCKs on chitosan coatings was noted with increasing DD, indicating the substrate interface effects on cell proliferation. The keratocan expression of RCK spheroids grown on a lowered DD of chitosan was up-regulated, suggesting that both the surface roughness increase and stiffness decrease may facilitate the microenvironment for preservation of cellular phenotype. Overall, our work contributes to the scientific understanding of the keratocyte behaviors and spheroid fabrications in response to DD-mediated surface roughness/stiffness of chitosan coatings.

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1. Introduction

Cell culture is a fundamental and important topic in tissue engineering since it simulates cell behaviors outside the native tissues with the possibility of engineering cells exhibiting desirable structure-function relationships. The underlying challenges in cell culture are to maintain the characteristic structures, behaviors, and metabolisms of the cells outside their complex tissues and organs [1]. Typically, formations of three-dimensional (3-D) spheroids and 2-D monolayers are the two most common cell configurations during cultivation while the environmental conditions may determine the corresponding configurations in either 2-D or 3-D. Studies in fabrication of 3-D spheroids received many interests over the 2-D monolayers given that the cells grown in a 3-D culture manner closely mimicked in vivo conditions and exhibited a higher similarity to the real tissues in many aspects. The unique 3-D structures are often accompanied with a high rate of proliferation and differentiation from cells, and their structure-function relationships have been reviewed by Lin and Chang [2]. It is generally believed that the most important aspect of spheroid cultivation is perhaps bridging in vitro cell assays and in vivo animal models. In addition, a 3-D spheroid culture technique has been used for purification and elimination of the undifferentiated cells as demonstrated by Hattori et al. [3]. Other biomedical applications that have been investigated by 3-D spheroids include the providing of ideal conditions for

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bioprinting and tissue regenerations [1,2]. Owing to these advantages, we seek for cultivation of keratocytes into 3-D spheroids using a biopolymer substrate while addressing the interfacial effects on the self-adjustment of spheroid configuration.

Keratocytes, located in corneal stroma, are the major cellular components residing between the collagen lamellae. One of the important functions of keratocytes involves in the synthesis of extracellular matrix (ECM) that is essential to the strength and transparency of cornea. Upon injury or age-related damage to cornea, quiescent keratocytes initiate their cell cycles in proliferation, differentiation, and migration to the injury sites. However, depending on the types of injury and specific environmental conditions of the cornea, keratocytes may proliferate and differentiate into other cell types such as fibroblasts or myofibroblasts that may not function properly in native eyes and can sometimes reduce the vision [4]. Due to this issue, there is an unmet challenge in tissue engineering to allow the cultivation of corneal keratocytes in vitro while preserving their phenotype. To achieve this, cultivation of keratocytes into 3-D spheroids becomes an alternative route. In addition, several studies have suggested the formation of keratocyte spheroids while preserving the phenotypic expression. For example, Scott et al. showed the formation of keratocyte spheroids with specific phenotypic markers in a serum-free medium [5]. In another study, Li et al. demonstrated the formation of keratocyte spheroids in suspension with the addition of methylcellulose to promote spheroid formation [6]. Furthermore, Funderburgh et al. compared spheroid formation from the effect of matrix during cultivation [7]. Their results suggested that the expression of keratocan, a proteoglycan component found uniquely from keratocytes, was higher in unattached spheroids than those attached to the matrix during cultivation. In general, these researchers showed the possibility in the formation of keratocyte spheroid especially from an aqueous solution/suspension.

Although keratocytes are anchorage-dependent cells, the formation of cell spheroids may become feasible with proper stimulations from the interface through cell-matrix crosstalks. Among all potential biopolymer substrates, chitosan coating has gained an increasing interest due to its excellent biocompatibility for cell culture and outstanding ability to promote spheroid formation. For example, Cheng et al. demonstrated the formation of spheroids from human adipose-derived stem cells (ASC) on chitosan coatings [8]. Their results suggested that chitosan materials enhanced spheroid formation of ASCs over 7 days of culture as compared to those on TCPS control. In another study, Lin et al. reported the formation of melanocyte spheroids on chitosan coatings and compared the characteristics of the spheroids with different cell seeding densities [9]. These two examples clearly demonstrated the possibility of using chitosan coatings as substrate materials to fabricate spheroids during cell cultivation. However, research involving in the use of chitosan coatings for cell culture rarely considered the degree of deacetylation (DD) of chitosan as a crucial factor in spheroid formation, especially in keratocytes. DD is one of the most important physicochemical parameters affecting the surface properties of chitosan coatings as reported by Foster et al. [10]. In addition, the cell behaviors such as migration, aggregation, proliferation, and differentiation are known to be modulated by tuning the interface properties of the substrate materials. These considerations motivate us to address the gap in keratocyte spheroid fabrication on chitosan materials due to the effect of DD. As a promising ophthalmic biomaterial, chitosan is a polysaccharide consisting of randomly distributed units of β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine [11]. It is usually obtained by alkaline *N*-deacetylation process of chitin, which may simultaneously lead to chemical depolymerization of biopolymer. Therefore, in this study, the deacetylated chitosan samples with varying DDs were prepared by heat-alkaline treatment under a nitrogen atmosphere to avoid the molecular weight modification due to oxidative mechanisms [12]. The molecular weight of biopolymer was controlled at the same level to ensure the clarification of the role of DD of chitosan in the bioengineering of corneal keratocyte spheroids.

In the present study, we hypothesized that the DD-mediated surface roughness/stiffness of chitosan coatings plays important roles on the migration, proliferation, and differentiation of keratocytes. In addition, the cultivation of keratocyte spheroids and their corresponding morphology might be modulated by DD of the chitosan coatings. In a study by Chen et al., bovine corneal keratocyte spheroids were formed on a chitosan (DD value of 85%) coating [13]. Although it is promising to see that the keratocyte spheroids maintained phenotypes under serum-containing culture conditions, the effects of DD of chitosan on the fabrication of keratocyte spheroids and their corresponding cell behaviors remained unclear. Therefore, this work aims to investigate keratocyte spheroid fabrication on chitosan coatings of varying DD values. We found that chitosan deacetylation significantly affected the surface characteristics such as surface roughness and stiffness of the coating biomaterials. Furthermore, cell behaviors and spheroid fabrication were mediated through these stimulations from the substrate interface. For the first time, here, we demonstrated that the surface roughness/stiffness of chitosan coatings, as a result of DD,

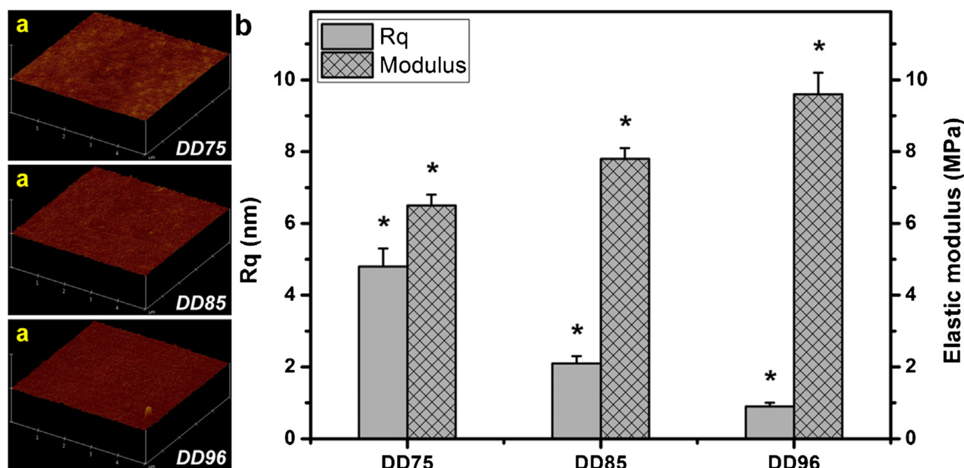

Fig. 1. AFM measurements on surfaces of TCPS and samples coated with chitosan of varying DDs. (a) 3-D height images; (b) Rq (i.e., mean square roughness) and elastic modulus. Values are mean ± SD (n = 3). *P < 0.05 vs all groups.

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