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ScienceDirect

Procedia Engineering 110 (2015) 143 – 150

Procedia Engineering

www.elsevier.com/locate/procedia

4th International Conference on Tissue Engineering, ICTE2015

# Investigation of chitosan-glycol/glyoxal as an injectable biomaterial for vocal fold tissue engineering

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**Abstract**

Injectable hydrogels offer promising tissue engineering approaches for vocal fold (VF) tissue repair. Research on VF tissue engineering scaffolds has largely been focused on derivatives of hyaluronan and collagen. Although chitosan hydrogels have been extensively investigated for various soft tissues, their potential use for VF tissue engineering has been overlooked. The aim of the present study was to investigate cross-linked Chitosan-glycol (GCs)/glyoxal (Gy) hydrogels for VFLP tissue engineering. The effects of Gy concentration on cell viability, viscoelastic properties, enzymatic degradation, and cell migration were studied. Six different groups of cell-seeded hydrogels, consisting of immortalized human vocal fold fibroblasts encapsulated in GCs/Gy hydrogels, were prepared to obtain target concentrations of $2\times10^6$ cells/ml, GCs 2% and Gy 0.02% (Group#1), 0.015% (Group#2), 0.01% (Group#3), 0.0075% (Group#4), 0.005% (Group#5), or 0.0025% (Group#6). The storage and loss moduli were 629±35Pa and 9±1 Pa, 560±28 Pa and 9±1Pa, 489±41 Pa and 8±1 Pa, 307±25 Pa and 4±1 Pa, 149±31 Pa and 3±1 Pa, 55±17 Pa and 3±1 Pa for groups 1, 2, 3, 4, 5, and 6, respectively. The viability rates were above 90% for all groups, 3 hours after encapsulation. The viability rates were 60.0±2.2%, 80.3±2.2%, 83.5±0.5%, 83.1±1.3%, 88.2±0.1%, and 88.0±1.1% for groups 1,2,3,4,5, and 6, respectively, one week after encapsulation inside GCs/Gy hydrogels. The average cell motility speed was 0.09±0.03 µm/minute, 0.07±0.043 µm/minute, and 0.09±0.02 µm/minute for groups 4, 5, and 6, respectively. Following four weeks enzymatic degradation study, the mass loss was 10%, 21%, and 100% for groups 4, 5, and 6, respectively. Our results indicated that GCs/Gy hydrogels could be potential candidates for use in human VF tissue repair and regeneration.


Peer-review under responsibility of IDMEC-IST

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Peer-review under responsibility of IDMEC-IST
doi:10.1016/j.proeng.2015.07.022

*Keywords:* Chitosan; Human fibroblasts; Viability; Migration; Degradation;Vocal fold; *In vitro*.

## 1. Introduction

The human vocal folds (VFs), located within the larynx, include soft connective tissues, such as the epithelium, the basement membrane zone, and the lamina propria (LP). These tissues are attached to the vocalis muscle and anchored to the thyroid and arytenoid cartilages [1]. In normal phonation, the VF mucosa and ligament undergo vibrations at frequencies ranging from 20 Hz to 3 kHz and amplitudes of a few millimeters. Voice disorders can impose occupational risks especially for teachers, signers, and public speakers, and may also have emotional consequences.

Scarring is a common voice disorder. Vocal fold scarring affects the mucosa and the LP. It may result from the surgical removal of benign or malignant VF lesions, phonotrauma or intubation over an extended period of time. Early scarring appears one to three months following VF injury, and may take up to twelve months for the remodeling phase completion and scarred VFLP maturation. Scarred VF tissue is fibrotic with diminished elastin but excessive disorganized collagen deposition [2]. As a result, the viscoelastic properties of the LP are significantly altered. Using a rabbit model, it was found that the stiffness and dynamic viscosity of the LP are one order of magnitude larger in scarred LP than in normal tissue [3]. Since the mechanical properties of the LP must be within a specific range to ensure proper phonation, viscoelastic biomaterials are often injected to treat scarring by compensating for the excessive stiffness of the scarred tissue. But, these materials degrade over time, and periodic re-injection is required. This shortcoming may be avoided through the use of tissue engineering approaches, which are intended to effectively modulate wound healing and regenerate functional VF tissue. The main motivation for the current investigation is to design, fabricate, characterize, and examine a new injectable biomaterial for VF tissue engineering.

Injectable hydrogels offer promising tissue engineering approaches for VF tissue healing. Biomaterials based on natural hydrogels such as hyaluronic acid (HA), gelatin (Ge), chitosan, alginate and fibrin have been used to promote soft tissue regeneration [4-6]. Vocal fold tissue engineering investigations have been largely focused on derivatives of hyaluronic acid and collagen. These macromolecules are the main components of the VFLP extracellular matrix. Hyaluronic acid is a non-sulfated glycosaminoglycan and a major contributor to maintain VF tissue viscosity. The HA-Ge hydrogels cross-linked by disulfide bond formation are biocompatible [7] and has viscoelastic properties similar to VFLP [8]. The injection of HA-Ge hydrogels in rabbits after unilateral injury resulted in less fibrotic tissue. Favorable biomechanical properties were achieved compared to those of the controls injected with saline. One major problem with bulk HA-based hydrogels is their fast degradation *in vivo*. Alternatively, an injectable biomaterial based on densely cross-linked micro-gels of HA and Ge was developed [9], and was shown to be biocompatible in a preliminary animal study [10].

Chitosan is a linear poly-saccharide containing heteropolymer of randomly distributed β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine units [11]. Chitosan, derived from chitin by partial deacetylation, is the second most abundant natural biopolymer [12]. Similarities to glycosaminoglycans, abundance in nature, biocompatibility, low production cost, and low immune-stimulatory activities have made this polymer very appealing for drug delivery, wound healing, and tissue engineering applications [13]. Chitosan is biodegradable, and can be metabolized by certain human enzymes, especially lysozyme. Chitosan hydrogels have extensively been investigated for variety of soft tissues such as skin [14], cartilage [15], blood vessels [16], and brain [17]. However, they have not been investigated for their potential use in VF tissue engineering.

Ionic and covalent cross-linking are the most common methods for the fabrication of chitosan hydrogels. Covalent cross-linking forms a permanent network, and therefore the hydrogels may reside longer *in vivo*, thereby better for tissue engineering applications. Ionic cross-linking, in contrast, is more prone to break down with the body fluids, and is then more appropriate for drug delivery applications [18].

The aim of the present study was to investigate cross-linked Glycol Chitosan (GCs)/glyoxal (Gy) hydrogels for VFLP tissue engineering applications. The effects of cross-linker (Gy) concentration on cell viability, viscoelastic properties, enzymatic degradation, and cell migration kinematics were studied.