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# Branched chitosans II: Effects of branching on degradation, protein adsorption and cell growth properties

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**Abstract**

The demand for biodegradable implant materials has fueled interest in chitosan as a biomaterial. In previous work, branched chitosans were synthesized and structurally characterized. In this study the biological properties of branched chitosans were explored. Branched chitosans were synthesized by grafting low molecular weight chitosan chains (1.6, 16 and 80 kDa) to high molecular weight (600 kDa) linear chitosans via reductive amination. Films of the branched materials were evaluated with regard to: lysozyme-mediated degradation; protein adsorption; cell adhesion and proliferation. Branched chitosan with a 1.6 kDa branch length exhibited higher degradation rates than either linear or higher branch length materials. Branched chitosans also exhibited reduced adsorption of bovine serum albumin that was more pronounced with thicker films. Branched chitosans supported proliferation of rat endothelial cells, but growth rates were significantly lower than on linear chitosan. The results of this study demonstrate that control of many aspects of chitosan's physical and biological properties can be achieved by changes in molecular architecture.


*Keywords:* Branched chitosan films; Biodegradation; Protein adsorption; Cell proliferation; Branched polymers

## 1. Introduction

There is a growing need for advanced biomaterials that are biodegradable, can support tissue generation and have mechanical properties comparable to that of native tissue. Chitosan is a promising implantable material that derives potential from its gel-forming properties and cationic nature that allows it to form insoluble ionic complexes with a variety of anionic polymers. Primary amino groups in the chitosan structure can be easily derivatized with useful biological ligands, or modified with other entities to alter mechanical and degradation properties, as well as protein adsorption properties [1–3]. In addition, the material has been shown to promote wound healing, and exhibits a minimal foreign body response with accelerated angiogenesis [4–8]. Chitosan has been used in a variety of biomedical applications including: wound dressings [9–12], drug delivery systems [13,14] and tissue engineered implants [15–19]. In most of these efforts, chitosan has been blended, crosslinked, or grafted with another molecule to bring about changes in properties as required for the specific application. Efforts have been made to enhance chitosan's mechanical properties by incorporating polymers such as poly(ethylene glycol) [20], alginate [21] and silk fibroin [1]. However, addition of another polymer adds an extra level of complexity to the system and may result in adverse changes to other desirable properties.

Given chitosan's linear architecture and its semi-crystalline nature, manipulation of its molecular architecture offers another method of altering the material's physical and biological properties. We previously reported on the synthesis and mechanical characterization of a family of branched chitosan materials [22]. In order to facilitate educated choices of these branched polymers for biomaterial applications, additional characterization is needed.

Literature reports suggest that the low degradation rates of highly deacetylated chitosans are partly due to its semi-

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crystalline nature. Degradation kinetics (as measured by lysozyme-mediated cleavage) is mainly dependent upon two factors, namely molecular weight (MW) and degree of deacetylation (DD) [23–26]. However, changes in molecular architecture introduce material structure changes that can mimic changes in both MW and DD.

Protein adsorption is one of the early events during the interaction of an implant material with a biological system. In addition to monomer chemistry, polymer architecture also can strongly influence chain organization and hence surface characteristics. Thus, inducing branching of linear chitosan may substantially alter some surface characteristics, and in doing so change aspects of protein adsorption properties that could substantially change cell and tissue responses to chitosan implants. Furthermore, the ability to effectively tune interactions between proteins and an implant material's surface can be a useful tool in designing biomaterials specific to particular applications.

In this study, branched chitosans were synthesized as previously reported [22], and various properties relevant to implant performance were evaluated. Specifically, vascular endothelial cell growth kinetics on cast films were characterized along with the adsorption of serum proteins, and lysozyme-mediated degradation kinetics.

## 2. Materials and methods

### 2.1. Synthesis of branched chitosan

Branched chitosan materials were synthesized as previously described [22] using a two-step procedure. The first step involved synthesis of low MW chitosan polymers by nitrous acid depolymerization [27,28] and the second step involved grafting the low MW chains (i.e. the branches) to high MW chitosan backbones using reductive amination [29]. Chitosan (90% deacetylated, 600 kDa, Fluka) was dissolved in 1% acetic acid to form a 1.5 wt.% solution. Portions of this solution were depolymerized by addition of $NaNO_2$ solution [27,28] to give solutions with average MW of 1.6, 16 and 80 kDa. The nitrous acid depolymerization procedure generates a reactive aldehyde group at the reducing end of each new low MW chitosan molecule. To prepare branched chitosans, 50 ml volumes of high MW chitosan solution and methanol were mixed. To this solution was added an appropriate volume of a low MW (depolymerized) chitosan solution. The volume of low MW solution added was determined by the stoichiometry of the desired branch density. Finally, 0.1 g of $NaCNBH_4$ was added and the mixture was stirred at room temperature for 4–5 h. The branched chitosan product was purified by precipitation with 30% ammonia solution, followed by water washing, then redissolved in 1% acetic acid. Using this procedure a range of branched products varying in branch density and branch length were synthesized [22]. In this work, branch density is defined as the moles of branch molecule (i.e. low MW chitosan) added per mole of primary amine in the chitosan backbone polymer, assuming 100% reaction.

### 2.2. Biodegradation studies

Degradation rates of cast chitosan films were evaluated using the dry weight loss method. Films of branched or linear chitosan were cast from solution by air-drying 25 ml of 1.5 wt.% solutions in 10 cm, non-adhesive, polystyrene petri dishes in a fume hood. Residual acetic acid was extracted from the dried membranes by washing with absolute ethanol 4–5 times. The membranes were then rehydrated through an ethanol series (80%, 60% and 40%) and finally equilibrated with water. Rectangular film specimens (1 × 2 cm) were cut and their initial wet weights were noted. Specimens were then immersed in PBS containing 30 mg ml$^{-1}$ lysozyme and incubated at 37 °C in a 5% $CO_2$ atmosphere. Degraded samples were collected at time zero and every 4 days up to day 20. Collected samples were washed thoroughly with water, blotted on filter paper and their wet weights were documented. Dry weight and water content were determined by drying membranes in an oven at 105 °C overnight. Degradation was calculated as reduction in dry weight as a function of time. Statistical comparisons were done using Student *t*-test (paired two samples for means).

### 2.3. Evaluation of protein adsorption

#### 2.3.1. Quantification of total adsorbed protein

Protein adsorption studies were conducted using cast films of linear and branched chitosans, with a branch density of 0.1 and branch lengths 1.6, 16 and 80 kDa. Three different film thicknesses were employed (denoted as 1, 2 and 3 in Fig. 3). Films were prepared by casting and air-drying 1.5 wt.% chitosan solutions using 0.4, 0.8 and 1.2 ml/well in standard 24-well tissue culture plates. The dried films were neutralized using 10% ammonia solution, washed extensively with water and equilibrated with PBS for 6 h at 4 °C. After aspiration of the PBS, the films were overlayed with 1.2 ml/well of a protein solutions for 36 h at 4 °C. The protein solutions used were bovine serum albumin (BSA, 2 mg ml$^{-1}$ in PBS) and fetal bovine serum (5% in PBS). After incubation, wells with adsorbed protein were washed with PBS four times, for 1 h each with constant rotary shaking in order to remove loosely bound proteins and proteins passively entrapped within the hydrogel films. Adsorbed proteins on the films were quantified in situ using a modified Lowry assay (DC Protein Assay kit from Bio-Rad Laboratories).

#### 2.3.2. Quantification of adsorbed fibronectin and vitronectin

The relative adsorption of fibronectin and vitronectin to chitosan films was evaluated after film exposure to 10% FBS in PBS. Films of intermediate thickness were prepared as described above, with the exception that 48-well plates were employed with 0.4 ml of chitosan

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