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Processing chitosan for tissue regeneration

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ABSTRACT

Chitosan is a linear polysaccharide derived by deacetylation of very abundant chitin. Chitosan is metabolized into non-toxic D-glucosamines by lysozymes present in the body and hence is biodegradable. Hence, chitosan has attracted significant interest in tissue regeneration applications. Various processes have been developed to form porous structures, needed for guiding cell growth. The processes include freeze-drying, thermo-gelation and electrospinning. Recent advances show that the porous structures formed from these processes are useful in cell colonization, however, show different cellular responses and mechanical characteristics. Chitosan has also been blended with various macromolecules to alter the biological and mechanical characteristics. This article summarizes the recent advances in processing chitosan-based scaffolds and their biomechanical alterations.

KEYWORDS: chitosan, lysozyme, scaffolds, freeze drying, electrospinning, hydrogels, cell growth, mechanical properties

1. INTRODUCTION

There has been a significant investigation into biodegradable polymers for use in regenerative and drug delivery applications. Biodegradable scaffolds are used to support and guide the in-growth of cells, which is essential for successful tissue regeneration. Further, tissue regeneration is a dynamic and complex process involving soluble mediators, extracellular matrix elements, and cells[1]. One can regenerate tissues by creating conducive microenvironment. One of the primary steps is forming matrices with pore characteristics and biomechanical properties similar to extracellular components. Chitosan has been the subject of many investigations in these applications. Chitosan is a biodegradable, biocompatible linear polysaccharide. Chitosan is formed by the alkaline deacetylation of chitin, the second most abundant polysaccharide occurring in nature[2]. In the commercial production of chitosan, Chitin is harvested as a waste-product from the structural components of crustaceans typically used in food-processing industry. Hence, chitosan has advantages of low cost and easy availability compared to many biodegradable polymers. Chitosan with different physicochemical characteristics i.e., molecular weight, crystallinity, and degree of deacetylation can be obtained by controlling the alkaline treatment. Chitosan is soluble in weak acids and interacts with negatively charged molecules, such as glycosaminoglycans, proteins and nucleic acids in solution to form complexes that can be processed into beads, gels, fibers, or films. Chitosan has been an active contender in the development of biodegradable scaffolds. Significant advances have been made in generating porous scaffolds using chitosan and understanding the behavior of cells on these scaffolds. Some of these advances are summarized below.

2. Generating porous structures

Chitosan dissolves in mild acidic water (below pH 6.3), unlike many other polymers which require high acidity or organic solvents that raise

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toxicity concerns. This has made easy processing into various forms[3]. One could freeze the chitosan solution at various temperatures and sublimate (also called lyophilization or freeze-drying) to obtain porous structures of different shapes. Since the process is done at low temperatures, other bioactive molecules can be incorporated during the fabrication process. Variety of symmetrical and non-symmetrical scaffolds can be formed based on the mold shape used while freezing the solution. The initial freezing temperature controls the crystal size of water and hence the porous structure present in the scaffold. Varying the concentration and the direction of cooling affects the pore size and orientation. An optimum pore size in the range of 50 -150 μm is the target while developing these scaffolds for supporting cell ingrowth for majority of the mature cell types (except osteoblasts and osteocytes present in the bone). When scaffolds with the pore sizes >300 μm are formed, many mammalian cell types are unable to completely colonize due to the difficulty in crossing large bridging distances.

Formed porous structures possess open pores that are conducive for cell infiltration. However, the freezing process forms a skinny layer (Figure 1) at the top, which is less porous than the interior of the scaffold and would limit the infiltration of cells. Surface analysis images show these images rather than the interior. Hence one has to be cautious about the freezing process and corrective methods could be implemented to remove the skinny layer. One way to remove is to put a wet filter paper on top of the frozen scaffold and refreeze the assembly. Frozen filter paper attaches to the surface of the frozen chitosan due to water crystals crisscrossing the interface. Peeling off that paper prior to lyophilization removes the skinny layer (Figure 1).

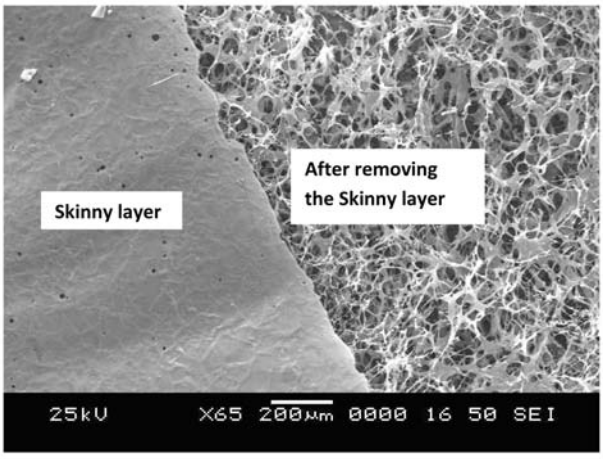

Figure 1. Scanning electron micrograph of chitosan scaffold formed by freeze-drying.

Another problem with freeze-drying is large distribution and random orientation of pores. For obtaining more uniform porous structure, maintaining a constant temperature gradient is necessary. Freezing the solution by placing the molds on top of a pre-cooled aluminum block is one such simple approach.

Once the porous structure is formed, it is stabilized by removing the acid used to reduce the pH of water for dissolution. Different approaches are used to remove the acid such as neutralization with a base such as NaOH or washing with an alcohol such as ethanol. Alkaline neutralization may shrink the scaffold while alcohol treatment does not alter the shape. Since ethanol is also used as a disinfectant, using this approach serves both the purposes of disinfection and elimination of the acid. However, it has to be cautioned that these processes do show significant differences in cell-material interactions. The freeze-drying concept has been exploited to make chitosan for use in regenerating various tissues[4].

Alternative processing techniques to form porous structures have been developed such as thermosensitive hydrogels using polyoles to adjust the pH, phase inversion technique, and electrospinning[5]. Developing thermosensitive injectable hydrogels is the primary target as they can conform to the shape present during the gelation point. For example, injecting the solution into the damage cartilage area offers less invasive surgical approach than using a pre-formed scaffold. When chitosan solution is adjusted to pH 7.4 using β-glycerophosphate (GP), the solution converts to a hydrogel at body temperature[6]. Essentially the solution remains in liquid phase at room temperature and gels irreversibly upon increasing to body temperature. Advances into the mechanical characteristics show major weakness of these hydrogels relative to the required

target tissues. Hence, injectable hydrogels have been formed with increased concentration. Increasing the concentration of chitosan increases the rate of gelation and the mechanical strength of the hydrogel while maintaining the porous structure conducive for nutrient transport[7,8]. Further, the porous architecture of the hydrogel shows spongy open pores, which is suitable for the diffusion of nutrients to the entrapped cells[9,10]. Long-term implications of these new formulations of hydrogels on cell growth and regeneration of tissues need further evaluation.

Electrospinning is a widely established polymer processing technology which allows generation of fibers (in nanometer to micrometer size) that can be collected to form non-woven structures[11]. A jet of the solution is drawn toward a collector dries to form fibers. By choosing a suitable polymer concentration and appropriate solvent system, fiber size can be controlled (40 nm to 10 μm). Since the technology allows the possibility of presenting the polymers in the form similar to the extracellular matrix elements present in the body, there has been a significant effort to adapt the technology in tissue regeneration, and drug delivery. A significant challenge in electrospinning chitosan is finding solvents with high volatility in addition to being environmentally friendly[12]. When low volatile solvents such as acetic acid are used in generating scaffolds, the rate of formation is very slow in the order of one milliliter per hour due to increased packing of fibers. Further the formed porous structures have decreased pore size due to multiple layers of fibers deposited to obtain thicker structures that withstand mechanical handling in other steps. For example, fibers have to be peeled-off from the collector plate and transferred to the tissue culture condition which could mechanically damage the structure. The reduced pore size restricts the cellular interactions to surface only. Recent advances have shown that controlling fiber sizes and pore sizes is possible using appropriate deposition volume and collector plate design[11]. Fiber sizes and alignment significantly influence cellular activity. Using these new techniques one could generate scaffolds with larger pore sizes and utilize the concept of layer-by-layer assembly to generate thicker structures. Recent advances in forming alcohol gels of chitosan could potential be adapted in electrospinning[13]. Nevertheless, determining an optimum solvent system and processing conditions would advance the utilization of chitosan in tissue regeneration.

3. Cell interactions

Chitosan has been blended with various molecules in an attempt to improve i) biological properties and ii) mechanical properties. Cells predominantly adhere to surfaces through integrins, a family of transmembrane receptors, which require peptide sequences in the polymeric structure for cell adhesion[14]. Chitosan being a polysaccharide contains no such peptides in its sequence. It used to be a popular belief that degree of deacetylation (DD) of chitosan controlled the spreading of cells via electrostatic interactions. Reduced cell spreading of chitosan membranes was attributed to strong electrostatic interactions. However, recent advances have shown that chitosan films do not allow cell spreading in the absence of serum proteins[15]. Most common method of culturing cells outside the body is using serum harvested from animal sources. Sera contain a number of proteins essential for cell growth including transferrin to facilitate oxygen transport. Proteins such as fibronectin and vitronectin found in the serum are important for cell attachment. Chitosan binds to many serum proteins due to the positive charge and then modulate the adhesive interactions. However, it is widely recognized that there are significant variations in serum composition from preparation to preparation (in addition to clinical safety concerns).

When chitosan is used in any form, cellular interactions are mediated via the initial interactions of the serum proteins with the surface. These interactions are unpredictable as the content of serum proteins binding to chitosan may vary due to the concentration of serum proteins and changes in DD as positive charge is controlled by DD of chitosan. Hence, one could observe changes in cell adhesive interactions from preparations to preparations via indirect mechanisms. Another variability is the form in which chitosan is presented to cells, which could also affect the cellular interactions[11,16]. Changes in physical properties of the porous scaffold such as hydrophilicity, stiffness, porosity, fiber alignment

and pore size affect cell morphology, attachment and function. For example, pore size of the scaffold affects cell binding, migration, depth of cellular in-growth, cell morphology and phenotypic expression. Surface features such as edges, grooves, steps, roughness and pores of substratum significantly influence cell behavior.

Lack of cell adhesive domains has necessitated blending various proteins such as collagen (or gelatin), fibronectin, laminin and fibrin, that contain peptides for cell adhesion[17]. These blends are achieved by exploiting the positive charge of chitosan or by cross-linking with bifunctional compounds such as glutaraldehyde. Many studies show the toxicity caused by the bifunctional compounds and hence their usage is restricted. Blending by electrostatic interactions seems to produce stable structures under physiological conditions. Gelatin-chitosan complexes can be formed without cross-linkers and extensive analyses show stability and functionality[15]. Presence of collagen or gelatin along with chitosan restores cell spreading. However, when other polysaccharides are blended, stability of those interactions has to be tested. For example, chitosan scaffolds can be immobilized with glycosaminoglycans or their analogues without significantly altering the structure of the scaffold. However, the ionic interaction after the formation of the scaffold is unstable, and the initial high content of glycosaminoglycans could dissociate rapidly[18]. If these scaffolds are used in long-term cultures, only a partial amount may be retained. Alternatively, immobilizing the glycosaminoglycans or their analogues in solution and then forming scaffolds may be much more stable[19]. There have been many studies showing significant promise in tissue regeneration application[20]. One could use calcium hydroxide to remove the acid instead of NaOH or ethanol. If calcium based alkaline agents are used to neutralize chitosan, the calcium molecules could mediate cell adhesion through cadherins. Influence of these interactions on cell function has to be tested.

4. Mechanical characteristics

The microscale mechanical properties experienced by the cells and the bulk material properties that provide physical support for both the cells and the surrounding tissue become important during tissue regeneration. Since chitosan is derived from the exoskeletal components, it is always thought that the mechanical strength could be similar to that. In dry state, chitosan is less ductile and needs to improve the tensile properties. However, many restrictions on the usable concentration of chitosan are from the degradability issues. Since chitosan degradation is mediated by enzymes, thicker structures would restrict the diffusivity of the enzyme and limit the degradation. Chitosan is metabolized into non-toxic D-glucosamines by lysozymes in the body[21]. Lysozyme is an innate non-immunologic antibacterial enzyme and is one of the most structurally well-characterized carbohydrate hydrolases[22]. The three-dimensional structures of both the uncomplexed state and the complexed state, the substrate binding site are known and also hydrolysis is through the acid catalytic reaction with a peak reaction rate occurring around pH 4.5 to 5.5. Lysozyme-dependent degradation kinetics of chitosan is sensitive to DD[21] due to substrate specificity and columbic interactions. At very high DD, in fact, adsorption of lysozyme does not result in the depolymerization of chitosan with 100% DD not conducive to degradation by the enzymatic action[23]. Majority of the studies utilize 70- 90% DD chitosan. Slow degradation rate of chitosan (> 1 year) limits its quantity in scaffolds needed for faster tissue regeneration. Many scaffolds are formed with reduced concentration of chitosan. Some of the preparations of porous scaffolds formed by freeze-drying show that the porous scaffold of chitosan has a bulk modulus in kilopascals, much below that of many tissues in the body[15]. Modifications to chitosan structure, for example, adding branches and other molecules to the primary structure have been considered. Although initial results show increased degradation with the addition of few branches[24], the effect of altering the chitosan chain itself on the degradation of the polymer has not been well understood. Nevertheless, majority of the approach for changing mechanical properties utilize physical blending of other synthetic polymers which can be tailored to required mechanical properties[4]. Degradation studies show an improvement in some cases, probably due to

the local influence of lysozymal activity due to pH changes[25].

Different preparations of chitosan scaffolds show different stress-strain characteristics in hydrated conditions at 37°C, most relevant to tissue regeneration applications. The elastic moduli of chitosan films are in Mega-Pascals where as that of porous structures formed by freeze-drying are in kilo-Pascals[15]. Some preparations of hydrogels show an elastic modulus in Pascals[6]. Since cellular activity is also influenced by scaffold stiffness of the substrate[26], cell colonization behavior on these forms could be altered. Further, the rigidity of the scaffolds may affect the formation of extracellular matrix elements which can affect cellular activity. Further investigation into these alterations would help understand the utility of chitosan[16]. Further, many studies assess the elastic (also referred as storage) modulus and viscous (also referred as loss) assuming linear viscoelasticity[27], which show that these values depend on the testing frequency. However, biological tissues display a complex non-linear viscoelastic behavior which is time-dependent and load-history-dependent[28-30]. Knowing how tissues in the body function is crucial to estimate tissue performance during stress and strain. Some studies have extended the analysis to three Maxwell elements in series with an elastic element to model one cycle of stress relaxation of based scaffolds[31]. Stress relaxes in time under constant strain, and the tissue shape progressively creeps under constant load. Chitosan-based scaffolds prepared by freeze-drying show nearly 90% relaxation of stress after each stage[32]. The relaxation behavior is independent of the concentration of chitosan. This is unlike some of the popular synthetic polymers poly-lactic-co-glycolic acid[33] and poly-caprolactone[34] which show 30 to 40% relaxation. However the stress and strain limits used for chitosan scaffold analyses are much smaller than those used for synthetic polymers. Further comparative analysis would help to understand the advantages of chitosan relative to other existing polymers.

5. FUTURE OUTLOOK

Utilization of chitosan for tissue regeneration applications has seen different phases from the beginning of its discovery. Recent focus has been on evaluating new methodologies to form scaffolds with various physico-chemical characteristics, particularly those biologically inspired nanoscale scaffolds mimicking the *in vivo* environment. Further, many functional groups available in chitosan polymeric chain also help in blending other molecules. With better understanding of the viscoelastic behavior of chitosan and its blends in relation to various tissue parts, chitosan is poised for success in tissue regeneration. With further evaluation of how cells behave on different chitosan scaffolds, one could see various uses of these scaffolds in not only tissue engineering, but also for advancing biotechnology, drug discovery and toxicology.

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