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Thermogelling bioadhesive scaffolds for intervertebral disk tissue engineering: Preliminary *in vitro* comparison of aldehyde-based versus alginate microparticle-mediated adhesion

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ABSTRACT

Tissue engineering of certain load-bearing parts of the body can be dependent on scaffold adhesion or integration with the surrounding tissue to prevent dislocation. One such area is the regeneration of the intervertebral disk (IVD). In this work, poly(N-isopropylacrylamide) (PNIPAAm) was grafted with chondroitin sulfate (CS) (PNIPAAm-g-CS) and blended with aldehyde-modified CS to generate an injectable polymer that can form covalent bonds with tissue upon contact. However, the presence of the reactive aldehyde groups can compromise the viability of encapsulated cells. Thus, liposomes were encapsulated in the blend, designed to deliver the ECM derivative, gelatin, after the polymer has adhered to tissue and reached physiological temperature. This work is based on the hypothesis that the discharge of gelatin will enhance the biocompatibility of the material by covalently reacting with, or "end-capping", the aldehyde functionalities within the gel that did not participate in bonding with tissue upon contact. As a comparison, formulations were also created without CS aldehyde and with an alternative adhesion mediator, mucoadhesive calcium alginate particles. Gels formed from blends of PNIPAAm-g-CS and CS aldehyde exhibited increased adhesive strength compared to PNIPAAm-g-CS alone ($p < 0.05$). However, the addition of gelatin-loaded liposomes to the blend significantly decreased the adhesive strength ($p < 0.05$). The encapsulation of alginate microparticles within PNIPAAm-g-CS gels caused the tensile strength to increase twofold over that of PNIPAAm-g-CS blends with CS aldehyde ($p < 0.05$). Cytocompatibility studies indicate that formulations containing alginate particles exhibit reduced cytotoxicity over those containing CS aldehyde. Overall, the results indicated that the adhesives composed of alginate microparticles encapsulated in PNIPAAm-g-CS have the potential to serve as a scaffold for IVD regeneration.

1. Introduction

Lower back pain (LBP) is a problem world-wide, affecting 80% of adults at some point in their lifetime [1] and results in approximately $100 billion in costs to society annually [2]. In short, disk degeneration results from a decline in the viable cell content of the central nucleus pulposus (NP) of the intervertebral disk (IVD), causing a reduced rate of matrix synthesis, dehydration, and an inability to bear compressive loads [3–6]. The compressive loads are then placed on the outer annulus fibrosus (AF), which can tear and allow the migration of the NP through the AF. Eventually, the NP can impinge on nerve routes causing LBP [7].

In early to mid-stages of degeneration, when extracellular matrix (ECM) repair by NP cells starts to slow, yet the annulus is still competent, there exists a window of time when NP replacement combined with a tissue engineering strategy has the potential to be effective [8]. Tissue engineering is a multidisciplinary field that aims to repair or regenerate lost or damaged tissues and organs in the body [9]. Fundamental strategies in tissue engineering generally combine cellular and scaffold-based approaches [10–13]. While the scaffold provides structural support, cells such

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as bone-marrow or adipose tissue-derived mesenchymal stem cells have the ability to differentiate and form new tissues when exposed to growth factors and cytokines [14].

Ideally, the scaffolds used for NP tissue engineering would be injectable, or *in situ* forming, to minimize damage to the AF upon implantation. Poly (N-isopropylacrylamide) (PNIPAAm) is one such polymer, with a lower critical solution temperature (LCST) behavior at around 32 °C [15]. Below this LCST, PNIPAAm is a miscible, flowable solution in water, forming hydrogen bonds between water molecules and the acrylamide groups. Above the LCST, these bonds are broken in favor of more hydrophobic interactions between the isopropyl group and the carbon backbone. These hydrophobic interactions allow PNIPAAm to form a compact hydrogel at physiological temperature [16]. Previous *in vitro* [17] and *in vivo* [18] work has indicated that PNIPAAm-based materials are biocompatible. Furthermore, polymerization of NIPAAm in the presence of other macromers, such as functionalized poly(ethylene glycol) [18–20] or chondroitin sulfate [21], allows for tailoring of the swelling and mechanical properties of the *in situ* formed gel, an advantage over unmodified natural biopolymers such as alginate [22], chitosan [23], or collagen [24] that have been studied for NP tissue engineering.

We recently investigated a family of *in situ* forming hydrogels based on PNIPAAm grafted with chondroitin sulfate (PNIPAAm-g-CS) [21]. Several of the hydrogel formulations exhibited unconfined compressive modulus values similar to what have been reported for the native NP, 5–6.7 kPa and the graft copolymer was found to be non-cytotoxic in the presence of human embryonic kidney (HEK) 293 cells [25]. However, research has shown that there is a significant risk of expulsion of IVD implants [24,26]. In fact, current NP regeneration strategies are not clinically feasible without significant adhesion to surrounding tissue, since implant expulsion through the damaged annulus can occur during loading and movement [27,28]. This interface is also necessary for the adequate transmission of force across the interface between the implant and the tissue [25]. Thus, there is a need for an adhesive tissue engineering scaffold to repair and regenerate the damaged NP tissue.

A number of adhesives are commercially available; however few possess the necessary characteristics for use in NP tissue engineering. Fibrin adhesives act as a hemostatic plug by mimicking the last stage of blood clotting. The clot is resorbed within days or weeks by macrophages and fibroblasts [29], allowing healing to occur at the site of adhesion. Because they are natural materials, fibrin sealants are completely biocompatible [30]. A number of authors have investigated fibrin for tissue engineering applications and demonstrated successful growth and proliferation of encapsulated MSCs [31–33] and myoblasts [34]. However, the main drawback to this class of adhesives is a low cohesive strength [35,36]. Natural polysaccharides such as alginate [37–39] have been investigated as adhesives, since they form ionic and/or hydrogen bonds with matrix components, such as proteoglycan, in the tissue [40]. As a means of developing stronger adhesives, researchers have also functionalized these biological polymers with aldehyde groups that are capable to bond with amines in the tissue surface via Schiff's base reaction [41–43]. While aldehyde based bioadhesives demonstrate greater adhesive strength, they have been shown to elicit an inflammatory response from cells in contact with the adhesive [44,45].

The objective of this work is to develop an injectable scaffold, based on PNIPAAm-g-CS, which is capable of forming a strong adhesive bond with tissue while maintaining encapsulated cell viability. The design of the adhesive was based on the idea that blending aldehyde-modified CS with PNIPAAm-g-CS would provide a method for easily incorporating a large number of aldehyde functionalities in the polymer, yielding a high adhesive strength due to the formation of covalent bonds with the amines in the tissue matrix. However, the presence of the reactive aldehyde groups can compromise the viability of encapsulated cells [44,46]. To circumvent this problem, we included gelatin-loaded liposomes into the PNIPAAm-g-CS blends with CS aldehyde. The liposomes were formulated to have a lipid bilayer melting point of 37 °C [47], allowing their cargo to be released once the polymer heats to physiological temperature and adhesion has already occurred. It was postulated that the discharge of gelatin would enhance the biocompatibility of polymer by covalently reacting with, or "end-capping", the aldehyde functionalities within the gel that did not participate in bonding with tissue upon contact. As a comparison, formulations were also created without CS aldehyde and with an alternative adhesion mediator, mucoadhesive calcium alginate particles. In this study, we characterized the bioadhesive properties and cytocompatibility of various adhesive formulations to determine the potential of the PNIPAAm-*g*-CS-based systems to serve as a NP tissue engineering scaffold.

2. Materials and methods

2.1. Materials

Chondroitin sulfate A, methacrylic anhydride and NIPAAm monomer were all purchased from Sigma–Aldrich. NIPAAm was purified in excess n-hexane and recrystallized prior to use. Lipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids. Gelatin was purchased from MP Biomedical, with an average molecular weight ranging from 20 to 100 kDa. High-glucose (4.5 g/L) Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS), heat inactivated fetal bovine serum (FBS), trypsin and penicillin-streptomycin (Pen-Strep) for cell cultures were purchased from Life Technologies. DNeasy Blood and Tissue kit for extraction of cellular DNA was purchased from Qiagen. PicoGreen dsDNA Assay kit for cellular DNA quantification was purchased from Life Technologies. *In Vitro* Toxicology Assay kit, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)-based, and Live/Dead Viability/Cytotoxicity kit, for mammalian cells, were purchased from Sigma–Aldrich and Life Technologies, respectively, to study the viability of polymer-encapsulated cells. All solvents were of analytical grade. Fresh porcine cartilage, from pig ears, was obtained from a butcher.

2.2. Poly (N-isopropylacrylamide)-graft-chondroitin sulfate synthesis

Chondroitin sulfate was functionalized with methacrylate groups using methacrylic anhydride (MAA) following a procedure adapted from Bryant et al. [48]. A 25:1 molar ratio of methacrylic anhydride to CS was used resulting in a substitution of 0.1 methacrylate groups per repeat unit of CS, as determined by ^{1}H NMR with D_2O as solvent [49]. Poly (N-isopropylacrylamide) graft chondroitin sulfate (PNIPAAm-g-CS) was synthesized by reacting N-isopropylacrylamide (NIPAAm) monomer with methacrylated chondroitin sulfate mCS in a 1000:1 molar ratio through a procedure previously reported [21].

2.3. Chondroitin sulfate aldehyde synthesis

Chondroitin sulfate was oxidized in the presence of sodium periodate ($NaIO_4$) using a procedure similar to that presented by Reyes et al. [43]. A 5% (w/v) aqueous solution of CS was bubbled with nitrogen gas. Then, $NaIO_4$ and CS were combined in the following weight ratios: 0.5:1, 1:1, and 2:1. The reaction was carried out at

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