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Thermogelling Bioadhesive Scaffolds for Intervertebral Disc Tissue Engineering: Preliminary *In Vitro* Comparison of Aldehyde-Based Versus Alginate Microparticle-Mediated Adhesion

C. Wiltsey¹, T. Christiani¹, J. Williams¹, J. Scaramazza¹, C. Van Sciver¹, K. Toomer², J. Sheehan³, A. Branda⁴, A. Nitzl⁵, E. England⁵, J. Kadlowec⁶, C. Iftode⁵, and J. Vernengo^{1,*}

¹Rowan University, Department of Chemical Engineering, 201 Mullica Hill Rd, Glassboro, NJ 08028, USA

²Medical University of South Carolina, Department of Regenerative Medicine and Cell Biology, 171 Ashley Ave, Charleston, SC 29425

³Rowan University Graduate School of Biomedical Sciences, Department of Cell and Molecular Biology, 42 East Laurel Rd, Stratford, NJ 08084

⁴Saint Joseph College School of Pharmacy, 229 Trumbull St., Hartford, CT 06103

⁵Rowan University, Department of Biological Sciences, 201 Mullica Hill Rd, Glassboro, NJ 08028

⁶Rowan University, Department of Mechanical Engineering, 201 Mullica Hill Rd, Glassboro, NJ 08028

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 disc (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

*Corresponding author: Vernengo, J., vernengo@rowan.edu, Phone: (856)-256-5316, Fax: (856)-256-5242.

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the adhesive strength ($p < 0.05$). The encapsulation of alginate microparticles within PNIPAAm-g-CS gels caused the tensile strength to increase two-fold 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, disc degeneration results from a decline in the viable cell content of the central nucleus pulposus (NP) of the intervertebral disc (IVD), causing a reduced rate of matrix synthesis, dehydration, and an inability to bear compressive loads [3] [4] [5] [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] [11] [12] [13]. While the scaffold provides structural support, cells such 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] [19] [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 has 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 significant risk for expulsion of IVD implants [26] [24]. 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] [32] [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] [38] [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 a Schiff's base reaction [41] [42] [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 [46] [44]. 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 kDa to 100 kDa. High-glucose (4.5g/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 ^1H 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 room temperature in the dark while stirring for six hours. To stop the reaction, 0.55 mL of ethylene glycol was added and the mixture was allowed to stir for an additional hour [50]. The mixture was then placed into 3500 molecular weight cutoff (MWCO) dialysis bag and dialyzed against deionized water (DI) water for a minimum of 24 hours. After the initial dialysis, the CS aldehyde was placed into an acetate buffer bath (pH 4.0, Fisher) for 3 hours before returning the CS aldehyde to dialysis against DI water for an additional 24 hours [51]. After dialysis, oxidized chondroitin sulfate was lyophilized for one week and stored in the fridge prior to use. The degree of substitution (percentage of potential hydroxyl side groups on the CS converted to aldehyde groups) was measured through hydroxylamine hydrochloride titration using the procedure and calculations described by Zhao et al [52].

2.4 Liposome preparation and release studies

Liposomes were synthesized using well established “thin-film” methods [53] [54]. The lipids DPPC and DMPC were combined, dissolved in chloroform and allowed to dry under vacuum overnight. A molar ratio of 90% DPPC and 10% DMPC was used to ensure the liposomes had a melting temperature of 37°C [46]. For each 72.8 mg of DMPC and DPPC, the dried lipid films were hydrated in 3.28 mL of a solution of gelatin in PBS at a concentration of 4 mg/mL (or pure PBS, in the case of unloaded liposomes). The resulting suspensions (22.2 mg lipids/mL) were allowed to hydrate overnight at 60°C with periodic vortexing. Loaded and unloaded liposomes were then extruded with 11 passes through a 0.22 micron filter to break up multilamellar vesicles. Once extruded, liposomes underwent dialysis at 4°C in 50,000 MWCO dialysis bags for one week to separate them from the encapsulated gelatin. Finished liposomes were stored at 4°C prior to use.

The release of gelatin from the loaded liposomes was characterized by placing the extruded liposome suspensions into 50,000 MWCO dialysis bags and into a sealed glass jar containing 100 mL of DI water. The jar was stored at 37°C for 7 days and samples were taken from the water surrounding the dialysis bag. A bicinchoninic acid (BCA) assay (Micro BCA™ Protein Assay Kit – Pierce, Thermo Scientific) was used to quantify the amount of gelatin released from the loaded liposomes. Release was characterized at 4°C by the same method and found to be negligible.

2.5 Preparation of calcium alginate particles

Calcium alginate particles were prepared by a method previously described by Won et al [55]. A Fischer Scientific Power Gen 125 homogenizer was set to 15,000 rpm used to emulsify 0.8 mL of Tween 20 and 80 mL of Canola oil. Then, 10 mL of 20 mg/mL aqueous alginate was added drop wise into the Tween and Canola oil and the mixture was homogenized for additional 3 minutes. Next, 4 mL of 2.2 % (w/v) calcium chloride was added dropwise while homogenizing and the mixture was allowed to settle for 15 minutes. The resulting particles were washed and separated using a Forma Scientific, Model 5682 centrifuge with 1400Gs of force for 5 minutes at room temperature. The wash was repeated three times with 2-propanol and DI water, followed by lyophilization. This resulted in microparticles with an average size of 59.7 ± 14.9 microns, determined by visualizing the microparticles under a light microscope and sizing approximately 50 particles.

2.6 Preparation of the adhesives

In all cases, the multi-component adhesives were prepared from 5% (w/v) solutions of PNIPAAm-g-CS in PBS. Formulations contained varying amounts of co-dissolved CS aldehyde and suspended liposomes or alginate particles, all of which were added to the 5% PNIPAAm-g-CS solution at room temperature. Upon heating to physiological temperature, PNIPAAm chains collapse due to hydrophobicity, entrapping the CS-aldehyde and the liposomes or microparticles in the polymer network. In certain control samples, no CS aldehyde, liposomes or alginate particles were added. In the control samples denoted as “free gelatin”, gelatin was co-dissolved in PNIPAAm-g-CS + CS aldehyde solutions at the indicated concentration, rather than encapsulated in liposomes or alginate particles. Table 1 outlines all of the prepared adhesives and their associated concentrations.

2.7 Characterization adhesive properties

The mechanical properties of the adhesives were tested based on the ASTM procedure F2258 for tissue adhesives in tension [56]. The tensile strength of the copolymers at 37°C was tested based on a modified version of ASTM F 2258-05, Strength Properties of Adhesives in Tension [56]. Sections of fresh porcine ear cartilage obtained from a butcher were cut into 1 cm² pieces and affixed to the upper and bottom fixtures of the FGS-200PV E-Force Test Stand using cyanoacrylate adhesive and warmed to 37°C. Cooled hydrogel solution at 15°C (200 µL) was applied to the tissue by dispensing it through a small gauge pipette, the surfaces opposed, and the gel allowed to contact the tissue at 37°C for 5 minutes. The upper fixture was then withdrawn at a rate of 2 mm/min and load-displacement data captured by a computer. Stresses were calculated by subtracting the force exerted by the polymer from the buoyant force of a “blank” (Teflon attachment with no cartilage or adhesive, raised at the same rate) and dividing by the bond area. The maximum stress was simply the highest stress measured during the experiment. All samples were tested in repeats of n = 5. Tisseel® fibrin sealant (Baxter, Deerfield, IL) was tested in parallel as a comparison. It was applied to the porcine cartilage per manufacturer’s instructions and subsequently received the same treatment as the PNIPAAm-based adhesives.

2.8 *In vitro* cytocompatibility studies

2.8.1 Cell culture and seeding of the polymer scaffolds—Human embryonic kidney 293 cells (HEK-293) were grown to 80% confluency in DMEM supplemented with 10% fetal bovine serum, and 100 U/mL penicillin-streptomycin, in a humidified incubator at 37°C with 5% CO₂. The medium was changed every 2 days. For cytocompatibility assays, cells were harvested from 100 mm dishes by trypsinization, counted with the hemacytometer and resuspended in 300 µl growth medium (monolayer) or polymer solution and seeded onto 24 well dishes at a density of 1×10⁶ cells/mL. Dishes were subsequently incubated for 10 minutes at 37°C for polymer gelation. After the addition of 600 µl warm growth medium to each well, while maintaining the dish on a heat pad set at 37°C (to prevent polymer transition to liquid state), cells were incubated for proliferation at 37°C for 5 days prior to either PicoGreen, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) or Live/Dead assay. Cells grown in a monolayer, in the absence of polymer, were used as the positive control. For XTT only, 400 µl/ml sodium citrate was added to the polymer + alginate in order to dissolve the calcium alginate microparticles [57], allowing for easier separation of the cells from the polymer. The same concentration of sodium citrate was added to the monolayer growth medium in order to replicate the growth conditions used for cells encapsulated in polymer + alginate. Killed cells in PNIPAAm-g-CS (negative control) were treated with 70% methanol for 30 minutes prior to exposure to the viability detection reagents. All samples and controls were prepared in replicates (five for Live/Dead and six for PicoGreen and XTT) in independent experiments (n=3, XTT and Live/Dead; n = 5, PicoGreen).

2.8.2 Cell viability and proliferation—To quantitatively determine cell viability and proliferation within the scaffold, the PicoGreen DNA quantification assay was used. After 5 days in culture, HEK-293 cells were subjected to enzymatic lysis and total DNA purification according to the DNeasy Blood & Tissue Kit protocol from Qiagen. The resulting DNA

samples were further processed as per the Quanti-iT PicoGreen protocol (Life Technologies). In brief, 1:300 dilutions of the DNA samples were prepared with Tris-EDTA and mixed with 1 mL aqueous solution of the Quanti-iT PicoGreen reagent. A portion of each sample (300 μ L) was transferred to 96 wells and incubated for 5 min in the dark before fluorescence reading at 485 nm excitation and 520 nm emission in a SpectraMax® M5 multimode plate reader (Molecular Devices). After correcting for blank fluorescence values, the DNA concentration of each sample was determined from a high-range DNA standard curve generated with lambda DNA.

The XTT in vitro toxicology assay rather PicoGreen was chosen to quantify cell survival in the alginate-containing polymer because the polymer's enhanced bioadhesive properties interfered with the purification of cellular DNA by spin columns. In this study, the tetrazolium salt XTT mixed with 1% N-methyl dibenzopyrazine methyl sulfate (PMS) was used in an assay adapted from the manufacturer's instructions (Sigma-Aldrich). At the end of the 5 day growth period, the medium was removed from cells and replaced with 300 μ L 20% reconstituted XTT in DMEM without phenol red or serum. Metabolically active cells reduce the XTT to an orange colored formazan derivative detectable colorimetrically. For color development, dishes were covered with foil and incubated at room temperature for 24 hrs with gentle rocking to ensure even distribution of the XTT reagent. The XTT supernatant was read in a Multiscan Ex microtiter plate reader (Thermo Electron Corporation) at both 450 nm (specific absorbance) and 690 nm (non-specific absorbance) and averaged absorbance values ($A_{450\text{nm}} - A_{690\text{nm}}$) were computed for each assayed condition. Relative cell viability was then calculated by normalization to averaged readings for no cell wells.

At 5 day incubation, for the Live/Dead assay, the surrounding growth medium was removed from the cells and replaced with 300 μ L mix/well of 6 μ M ethidium bromide and 2 μ M calcein in PBS for 40 minutes at room temperature. Cells were examined under fluorescence microscopy after staining to qualitatively determine live (green) and dead (red) cells.

2.9 Statistical Analysis

For each experiment, sample means ($n = 5$ replicates) were compared using a two-tailed Student T's test (Microsoft Excel, $\alpha = 0.05$), unless otherwise stated. Samples with a p-value of less than 0.05 ($p < 0.05$) were determined to be statistically significant. In each of the plots, error bars represent the 95% confidence interval.

3. RESULTS

3.1 Gelatin release from the liposomes

The release of gelatin from the liposomes was quantified over time. After 30 minutes elapsed, representing the time period immediately after gelation, the average cumulative release from the lipid vessels was $2.6 \times 10^{-2} \pm 0.01$ mg gelatin per mg of dry particles ($n=3$ replicates). We also determined from the release study that, after 7 days, 0.62 ± 0.03 mg of gelatin per mL of liposome solution was cumulatively released. This concentration was used when the effect of free gelatin on the adhesive properties and cytocompatibility of the scaffold were studied (sections 3.3 and 3.5, respectively).

3.2 Tuning of aldehyde content in the adhesive

We aimed to understand the relationship between the aldehyde content in the scaffold and its adhesive tensile strength. There are two ways to vary the aldehyde content in the scaffold: first, by the degree of substitution of the CS; second, by the overall concentration of CS aldehyde in the polymer solution at room temperature. To begin, adhesive testing was carried out on the hydrogel solutions containing 5% (w/v) PNIPAAm-g-CS + 3% (w/v) chondroitin sulfate (CS) aldehyde. The CS aldehyde was synthesized using three mass ratios of NaIO₄: to CS, 0.5:1, 1:1 and 2:1, resulting in an average degree of substitution of the CS of 34, 42 and 72 percent. The data in Figure 1 indicate that varying the degree of substitution produced no statistically significant ($p > 0.05$) differences in the adhesive tensile strength of the copolymer. However, compared to the 5% PNIPAAm-g-CS alone, it can be seen that the incorporation of any degree of substitution of CS aldehyde in the range tested produces a statistically significant ($p < 0.05$) increase in tensile strength.

The effects of varying the concentration of CS aldehyde within the polymer matrix were also investigated. For this and all subsequent tests, the degree of substitution was held constant at 42% while the overall concentration of CS aldehyde in the polymer solution at room temperature was varied between 0 and 5% (w/v). Tensile test results in Figure 2 show that 3% CS aldehyde had a significantly ($p < 0.05$) higher tensile strength than the other samples. At concentrations above 3% CS aldehyde, the adhesive strength begins to decrease. Concentrations above 5% were found to be too viscous to characterize. Thus, 3% CS aldehyde was used in all subsequent studies.

3.3 Adhesive properties of the biomimetic scaffolds containing liposomes

To analyze the effect of increasing liposome concentration on adhesive strength, samples containing 5% (w/v) PNIPAAm-g-CS and 3% (w/v) CS aldehyde were combined with liposomes to obtain suspensions containing 0, 2.78, 5.55, 11.1 and 22.2 mg gelatin-loaded lipids per mL of polymer solution. The DS of the CS aldehyde was held constant at 42%. The results in Figure 3 indicate that incorporation of liposomes into the polymer solution at any of the concentrations tested resulted in a statistically significant decrease in tensile strength compared to 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde ($p < 0.05$). The data suggest that liposomes interfere with the ability of the scaffold to adhere to the substrate.

To confirm that the liposomes were interfering with adhesion, further tensile tests were performed to determine the effects of individual liposome components (free gelatin and unloaded liposomes) on the adhesive properties of the system in comparison with 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde. The results, shown in Figure 4, indicate that the addition of free gelatin (0.62 mg/mL polymer solution) slightly reduced adhesive strength to a level that was statistically similar to that seen with PNIPAAm-g-CS alone, confirming the need for encapsulation of the gelatin. However, the addition of unloaded liposomes (22.2 mg lipids/mL) produced another significant ($p < 0.05$) decrease in maximum stress compared to PNIPAAm-g-CS, but the magnitude of the drop was much greater than what was seen with free gelatin. These data confirm that the liposomes have an unfavorable effect on adhesion.

3.4 Adhesive properties biomimetic scaffolds containing alginate particles

Because alginate has been known to have mucoadhesive properties [58], we investigated alginate particles as an alternative adhesion mediator to liposomes. Calcium alginate microparticles were combined with the 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde. The particles were suspended in this polymer solution at concentrations of 25, 50 and 75 mg/mL. These concentrations were chosen because they are one-half, equal to, and twice the concentration of PNIPAAm-g-CS in aqueous solution. No concentrations above 75 mg/mL were used because of the excessive viscosity of the suspension. The formulations containing 50 and 75 mg/mL of suspended alginate particles in 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde (Table 2) exhibited tensile strength that is not significantly different ($p < 0.05$) than 5% PNIPAAm-g-CS + 3% CS aldehyde. Thus, in contrast to the liposomes, adding these particles to the adhesive system does not necessarily have to result in a decrease in adhesive stress.

The properties of PNIPAAm-g-CS formulations containing alginate particles without the addition of CS aldehyde were also investigated. The addition of 50 or 75 mg/mL of alginate particles suspended in 5% (w/v) PNIPAAm-g-CS caused the tensile strength to increase two-fold over that of 5% PNIPAAm-g-CS + 3% CS aldehyde ($p < 0.05$), as shown in Table 2. Thus, the adhesive strength of the system was maximized with the addition of alginate particles to PNIPAAm-g-CS, without the inclusion of the CS aldehyde. Importantly, the PNIPAAm-g-CS + alginate particles (50 or 75 mg/mL) exhibited an over four-fold increase in adhesive tensile strength compared to Tisseel® fibrin sealant, the current standard for biocompatible adhesives.

3.5 *In vitro* cytocompatibility of the biomimetic scaffolds

The cytocompatibility of the adhesives was examined using 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde, as well as the same solution with gelatin-loaded liposomes (22.2 mg lipids/mL). The level of cell survival and proliferation allowed by these polymers was determined with the PicoGreen assay. The DNA amount isolated from 5 day cultures of HEK-293 cells seeded at 10^6 /ml with the various controls and growth medium is presented in Figure 5. With the incorporation of CS aldehyde, there was a significant decrease ($p < 0.05$) in DNA content compared to PNIPAAm-g-CS, which is expected since aldehydes are known to induce cytotoxic response in cells [44] [45]. The addition of free gelatin (0.62 mg/mL polymer solution) to PNIPAAm-g-CS + CS aldehyde did not produce a significant change ($p > 0.05$) in DNA content compared to PNIPAAm-g-CS + CS aldehyde, indicating that the use of gelatin to “end cap” the aldehyde groups within the polymer may not have the desired effect of reducing the cytotoxicity. Furthermore, the inclusion of liposomes in the scaffold, whether they were loaded or unloaded, caused a significant decrease ($p < 0.05$) in DNA content compared the PNIPAAm-g-CS + CS aldehyde. Confirming this, Live/Dead results at 5 days encapsulation (Figure 6) show poor viability for the samples containing CS aldehyde, loaded or unloaded liposomes, and free gelatin. An estimated 65–86% dead cells were observed under these conditions compared to PNIPAAm-g-CS alone. Taken together, the results indicate that both the CS aldehyde and the liposomes induce a cytotoxic response in the cells.

Formulations containing 50 mg/mL of alginate particles combined with PNIPAAm-g-CS (and no aldehyde) were seeded with HEK-293 cells as described in section 2.8.1 and were characterized after 5 days of growth at 37°C by XTT cytotoxicity (Figure 7) and Live/Dead (Figure 8) assays. Both tests indicate good viability for the adhesive containing PNIPAAm-g-CS and alginate particles. The relative cell viability with PNIPAAm-g-CS + alginate was not found to be significantly different ($p>0.05$) from that with the PNIPAAm-g-CS polymer alone, decreasing only 1.3 fold (Figure 7). This extent of viability is confirmed by images obtained with the Live/Dead assay (Figure 8), which show mostly living (green) cells in cultures with the alginate-containing polymer. We have also found that HEK-293 cells continue to proliferate over 21 days in culture when seeded within the PNIPAAm-g-CS + alginate particle polymer (data not shown). At the same time, the cell survivability in the PNIPAAm-g-CS polymer is almost as good ($p>0.05$) as that in the monolayer (no polymer). Overall, these results showed that the polymer with the highest adhesive strength of all our samples tested, PNIPAAm-g-CS + alginate particles, also displayed good biocompatibility.

4. DISCUSSION

In this work, we describe the development and characterization of an adhesive system that supports tissue repair, which is an important contribution to tissue engineering strategies for the treatment of lower back pain. By combining an injectable polymer, PNIPAAm-g-CS, with alginate microparticles, we developed a formulation that has a significantly higher adhesive tensile strength than fibrin glue, the current standard for biocompatible adhesives. This property was imparted without significantly affecting the scaffold's ability to support HEK-293 cell survival and proliferation, as demonstrated by both quantitative and qualitative viability assays over 5 days in culture (Figures 6–9). Moreover, the adhesive scaffold creates an environment that is also compatible with long-term (21 days) cell proliferative activity (data not shown). Such a permissive milieu could be conducive to differentiation of encapsulated stem cells with regenerative potential.

Importantly, this multicomponent system is highly versatile in that various other natural polysaccharides, such as methyl cellulose, alginates, and hyaluronic acid may be incorporated into the PNIPAAm network. Furthermore, incorporated particles can be designed to release any combination of native long chain of extracellular matrix (ECM) proteins as well as short peptide sequences that can cause specific interactions with cell receptors. As such, the system can be tailored to the needs of any tissue engineering application that requires the formation of a stable, bonded interface with host tissue.

In these experiments, the adhesive properties of the scaffold were studied as a function of its composition. First, the degree of aldehyde substitution of the CS and overall CS aldehyde concentration in the gel were varied. While varying the degree of substitution of the CS between 24 and 72% did not produce a significant ($p>0.05$) effect on adhesive stress, increasing the amount of CS aldehyde (with a constant degree of substitution of 42%) from 1 to 3 % (w/v) produced a significant increase in adhesive strength ($p<0.05$), which was expected due to the aldehyde-mediated reaction with tissue. However, at 5 % (w/v) CS aldehyde, the maximum adhesive stress began to decrease. This can be attributed to the higher viscosity of solutions containing high amounts of CS aldehyde. The adhesive

behavior of our system is at least in part attributable to mechanical interlocking with the texture of the porcine skin. Solutions with a high viscosity have a limited ability to penetrate the texture of the tissue surface, lowering the adhesive strength of the polymer. This phenomenon was also observed in our prior work with PNIPAAm-g-CS [21]. PNIPAAm-g-CS solutions with suspended alginate particles at concentrations higher than 75 mg/mL also exhibited increased viscosity and decreased adhesive strength (data not shown).

Lipid vesicles, encapsulating the protein derivative gelatin were also incorporated into the scaffold. Liposomes were chosen for this application because the melting of the lipid bilayer at 37°C [54] could be exploited in order to achieve a burst release of gelatin, once the polymer heats to body temperature, shortly after adhesion to the surrounding tissue. However, the inclusion of liposomes significantly decreased ($p < 0.05$) maximum stress (Figures 3 and 4). As was previously mentioned, adhesion partially relies on mechanical interlocking with the tissues. It is postulated here that the liposomes, composed of fatty acids, formed a “slippery layer” which prohibited the CS aldehyde from effectively contacting the tissue surface and forming covalent or physical bonds. Furthermore, the liposomes, both loaded and unloaded, caused appreciable decreases in DNA content of the scaffolds (Figure 5), indicative of a cytotoxic response. Liposomes have been widely investigated for intracellular drug delivery, which is generally believed to be mediated by the adsorption of liposomes onto cell membrane surfaces and subsequent endocytosis [59]. Yet, prior work by other investigators has found that cellular uptake of neutral phospholipids, such as of DPPC and DMPC, is minimal [60] [61]. Contrary to our results, neutral liposomes have been reported as cytocompatible [62]. However, it is important to note that the present study was conducted at liposome concentrations two orders of magnitude higher than what has been studied previously [62]. It is possible that in our system this caused a higher than expected degree of cellular uptake, causing excess disturbances to the cell membranes, resulting in cytotoxicity.

The incorporation of alginate particles into PNIPAAm-g-CS enhanced the adhesive strength of the scaffold. In fact, 5 w/v (%) PNIPAAm-g-CS containing 50 or 75 mg/mL of alginate microparticles had a significantly higher max adhesive strength ($p < 0.05$) than 5 w/v (%) PNIPAAm-g-CS + 3 w/v (%) CS aldehyde (Table 2). This was an unanticipated result, as we expected that covalent bonds formed between the CS aldehyde and the tissue would be stronger than the hydrogen bonding interactions between the alginate particles and glycoproteins in the tissue. However, one should consider the strength of the network that is bonding with the tissue. The alginate particles are composed of a cohesive crosslinked network, as opposed to solubilized and loosely associated CS aldehyde chains. Thus, the PNIPAAm-g-CS samples with alginate particles required a higher force for detachment from the substrates compared to PNIPAAm-g-CS + CS aldehyde.

Overall, the adhesive composed of alginate microparticles suspended in PNIPAAm-g-CS is more promising than any formulation containing CS aldehyde, due to the higher adhesive properties and better cytocompatibility. The current material studies are groundwork for future studies, where the compressive behavior of the material will be characterized. Furthermore, the structure-property relationships developed with this system can be applied to improving the adhesive strength further. For instance, particles composed of other

polysaccharides that are known to be mucoadhesive, such as chitosan [58] can be studied. The bioadhesive will also be tested in shear mode in contact with isolated porcine annulus fibrosis tissue. It is important to test in shear mode because it is akin to the likely mode of failure in an intervertebral disc if the hydrogel were to be extruded through the annulus due to insufficient adhesion. To our knowledge, there are no existing studies on the requisite adhesive characteristics to prevent expulsion of a hydrogel nucleus replacement. We plan to demonstrate the efficacy of the adhesive in resisting expulsion and restoring original mechanical properties to a spinal motion segment exposed to repetitive cycles of flexion and extension.

Furthermore, the differentiation of encapsulated adipose derived stem cells (ASCs) toward nucleus pulposus phenotype will be studied in order to determine the feasibility of the adhesive as a three-dimensional culture system for IVD regeneration. ASCs have been recognized to have tremendous potential in IVD tissue engineering [63] [64]. In preliminary 3 day-tests with the bioadhesive polymer, we found that ASCs display viability profiles similar to those obtained with the HEK-293 cells (data not shown).

5. Conclusions

The objective of this work is to develop an injectable scaffold, based on PNIPAAm-g-CS that is capable of forming a strong adhesive bond with tissue while maintaining encapsulated cell viability. Results of our biomaterial characterization studies indicate adhesive strength of PNIPAAm-g-CS was maximized with the addition of alginate particles to PNIPAAm-g-CS, without the inclusion of the CS aldehyde or gelatin-loaded liposomes. Qualitative and quantitative cytotoxicity results indicate that both the CS aldehyde and the liposomes induce a cytotoxic response in the cells. On the contrary, formulations containing 50 mg/mL of alginate particles combined with PNIPAAm-g-CS (and no aldehyde) showed good cytocompatibility. Overall, we present a novel system with potential to serve as an adhesive tissue engineering scaffold for the IVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Adhesive strength of hydrogels formed from solutions containing 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde as a function of aldehyde substitution. The incorporation of any degree of substitution in the range studied significantly increased ($p < 0.05$) the adhesive strength over control samples with no CS aldehyde. No differences ($p > 0.05$) were seen between the various degrees of substitution of CS aldehyde.

Figure 2.

The adhesive strength of hydrogels formed from 5% (w/v) PNIPAAm-g-CS and varying % (w/v) of CS aldehyde. Samples containing 3 w/v% CS aldehyde have a statistically higher ($p < 0.05$) adhesive stress than samples containing 0, 1 or 5 w/v% CS aldehyde. Samples of 0, 1 and 5% CS aldehyde were determined to be statistically similar ($p > 0.05$).

Figure 3.

The effect of increasing liposome concentration on the adhesive strength of hydrogels formed from solutions containing 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde. Increasing the liposome concentration yielded a decreasing trend in the adhesive strength, indicating that the lipids interfered with adhesion.

Figure 4.

The effect of liposomes and gelatin on the adhesive strength of 5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde. Incorporation of loaded or unloaded liposomes produces a statistically significant decrease the adhesive strength compared to PNIPAAm-g-CS.

Figure 5. Biocompatibility by PicoGreen assay of PNIPAAm-gCS + aldehyde polymer formulations. Five day cultures of HEK-293 cells encapsulated in PNIPAAm-gCS + aldehyde with and without liposomes or with free gelatin were compared with the PNIPAAm-gCS polymer. * Significant decrease of DNA content ($p < 0.05$) compared to PNIPAAm-g-CS; ** DNA content does not differ significantly ($p > 0.05$) compared to PNIPAAm-g-CS + aldehyde. Error bars represent 95% confidence intervals for $n = 5$ (with 4 replicates per experiment).

Figure 6.

Representative Live/Dead fluorescence images of 5 day cultures of HEK 293 cells encapsulated in: (A) PNIPAAm-g-CS, (B) PNIPAAm-g-CS + CS aldehyde (68% dead cells out of 638 cells counted), (C) PNIPAAm-g-CS + CS aldehyde + unloaded loaded liposomes (86% dead cells out of 563 cells counted), (D) PNIPAAm-g-CS + CS aldehyde + loaded liposomes (85% dead cells out of 566 cells counted), and (E) PNIPAAm-g-CS + CS aldehyde + 0.6 mg/mL free gelatin (65% dead cells out of 559 cells counted). Positive (cell monolayer) and negative (methanol killed cells in PNIPAAm-g-CS) controls are shown in F and G, respectively. Calcein AM- based intracellular green fluorescence is observed in live cells, while dead cells display red fluorescence due to the uptake of ethidium homodimer-1. The indicated central area in panels B–D was used to estimate the number of dead cells of the total counted. Experiment was repeated three times (with three replicates per experiment). Scale bars are 100 μ m.

Figure 7.

Short term viability of HEK-293 cells in PNIPAAm-g-CS + alginate particles. At 5 day encapsulation in PNIPAAm-g-CS with or without alginate particles, survival of HEK-293 cells was compared by XTT. Methanol killed and polymer free cells were used as negative and positive controls, respectively. * Not a significant reduction ($p > 0.05$) of cell viability compared to PNIPAAm-g-CS ** Viability is not significantly different ($p > 0.05$) compared to cell monolayer. Error bars represent 95% confidence intervals for $n = 3$ (with six replicates per experiment).

Figure 8.

Representative Live/Dead fluorescence images of 5 day cultures of HEK 293 cells encapsulated in: (A) PNIPAAm-g-CS and (B) PNIPAAm-g-CS + alginate particles (7.3% dead cells out of 738 cells counted). Positive (cell monolayer) and negative (methanol killed cells in PNIPAAm-g-CS) controls are shown in C and D, respectively. Calcein AM- based intracellular green fluorescence is observed in live cells, while dead cells display red fluorescence due to the uptake of ethidium homodimer-1. The indicated central area in panel B was used to estimate the number of dead cells of the total counted. Experiment was repeated three times (with three replicates per experiment). Scale bars are 100 μm .

Table 1

Control samples and additional blended formulations used for bioadhesive testing and characterization.

Samples	Concentration at Room Temperature
5% (w/v) PNIPAAm-g-CS with:	Control
CS aldehyde	1%, 3%, 5% (w/v)
Unloaded alginate particles	25, 50, 75 mg/mL solution
5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde with:	Control
Degrees of aldehyde substitution	34%, 42%, 72%
Unloaded alginate particles	25, 50, 75 mg/mL solution
Loaded liposomes	2.78, 5.55, 11.1, 22.2 mg lipids/mL solution
Unloaded liposomes	22.2 mg lipids/mL solution
Free gelatin	0.62 mg/mL solution

Table 2

The effects of incorporating varying concentrations of alginate microparticles into PNIPAAm-g-CS and PNIPAAm-g-CS + CS aldehyde. The addition of 50 or 75 mg/mL of alginate particles suspended in 5% (w/v) PNIPAAm-g-CS caused the tensile strength to increase two-fold over that of 5% PNIPAAm-g-CS + 3% CS aldehyde ($p < 0.05$) and four-fold over Tisseel® fibrin sealant ($p < 0.05$).

Samples	Tensile Strength (kPa)
Tisseel® Fibrin Sealant	0.413 ± 0.153
5% (w/v) PNIPAAm-g-CS	1.108 ± 0.182
25 mg/mL alginate particles	2.060 ± 1.066
50 mg/mL alginate particles	3.898 ± 1.608
75 mg/mL alginate particles	4.160 ± 0.794
5% (w/v) PNIPAAm-g-CS + 3% (w/v) CS aldehyde	1.960 ± 0.189
25 mg/mL alginate particles	1.301 ± 0.241
50 mg/mL alginate particles	1.991 ± 0.344
75 mg/mL alginate particles	1.940 ± 0.307