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Enzymatically Cross-linked Alginic-Hyaluronic acid Composite Hydrogels As Cell Delivery Vehicles

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Abstract

An injectable composite gel was developed from alginic and hyaluronic acid. The enzymatically cross-linked injectable gels were prepared via the oxidative coupling of tyramine modified sodium alginate and sodium hyaluronate in the presence of horse radish peroxidase (HRP) and hydrogen peroxide (H₂O₂). The composite gels were prepared by mixing equal parts of the two tyraminated polymer solutions in 10U HRP and treating with 1.0% H₂O₂. The properties of the alginate gels were significantly affected by the addition of hyaluronic acid. The percentage water absorption and storage modulus of the composite gels were found to be lower than the alginate gels. The alginate and composite gels showed lower protein release compared to hyaluronate gels in the absence of hyaluronidase. Even hyaluronate gels showed only approximately 10% protein release after 14 days incubation in phosphate buffer solution. ATDC-5 cells encapsulated in the injectable gels showed high cell viability. The composite gels showed the presence of enlarged spherical cells with significantly higher metabolic activity compared to cells in hyaluronic and alginic acid gels. The results suggest the potential of the composite approach to develop covalently cross-linked hydrogels with tuneable physical, mechanical, and biological properties.

Keywords

alginic acid; hyaluronic acid; enzymatic cross-linking; composite gels

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1. Introduction

Osteoarthritis, and subsequent cartilage lesions, often resulting from trauma or disease, is one of the leading causes of physical disability worldwide [1]. Articular cartilage is a smooth tissue composed of chondrocytes embedded in a highly organized extracellular matrix (ECM) of collagen and glycosaminoglycans. Unfortunately, articular cartilage has poor healing ability due to the lack of an arterial blood supply, and therefore, superficial cartilage lesions have to depend on slow and inadequate cell mitosis for repair [2-4]. Furthermore, the current treatments for cartilage repair are less than satisfactory in restoring the function of the damaged tissue. Tissue engineering has emerged as an alternative strategy which holds great promise to develop functional cartilage substitutes [5,6]. The regeneration of osteochondral interface is an important area within the field of cartilage tissue engineering, as it allows for functional cartilage to bone integration [7-9]. Osteochondral interfacial tissue has hypertrophic chondrocytes which produce both collagen type II and collagen type X [10,11]. Cartilage in this area is somewhat mineralized, and chondrocytes are organized into vertical columns parallel to vertical collagen fibers [12]. There is a need to develop biomaterials that can maintain chondrocytic phenotype and support cartilage matrix deposition for articular cartilage regeneration as well as chondrocyte hypertrophy and calcified matrix deposition to form cartilage-bone interfaces.

Due to the high water content of cartilaginous tissue, hydrogels are investigated as scaffolds to support cartilage and osteochondral tissue regeneration [13,14]. Hydrogels formed from natural polymers such as alginic acid, and chitosan can maintain chondrocyte phenotype and have been extensively studied as chondrocyte carriers. Alginic acid is a naturally occurring anionic polysaccharide isolated from the cell walls of algae. This linear block copolymer consists of β -(1-4)-linked D-mannuronic acid and α -(1-4)-linked L-guluronic acid [15-20]. Alginic acid gels are known to maintain chondrocyte phenotype in 3D culture, support chondrocyte gene expression, differentiate mesenchymal stem cells, and re-differentiate de-differentiated chondrocytes [21-24]. However, the commonly used calcium ion cross-linked alginate gels present the challenges of long gelation time, release of biologically active calcium ions, and low stability [25]. Due to similarities with natural cartilage matrix, hydrogels made of glycosaminoglycans (GAG) such as hyaluronic acid have been widely studied for cartilage tissue engineering applications [13,26-30]. Hyaluronic acid plays an important role in cellular attachment and function within natural cartilage tissue [31]. Different cross-linking techniques have been investigated to develop hyaluronic acid gels with varying mechanical properties as scaffolds for cartilage tissue engineering [30]. However, the rapid degradation and clearance of the gel may raise limitations in supporting long term tissue regeneration [32]. Clearly, there exists a need to develop improved hydrogel systems that combine the advantages of these natural polymers to serve as cartilage and osteochondral scaffolds.

Though both preformed and injectable hydrogels have been used for tissue regeneration, injectable hydrogels present several advantages, such as mild and cytocompatible gelation processes, minimally invasive delivery, homogeneous distribution of cells or molecules at the injection site, and ability to fill irregular defects [33]. Among the various cross-linking methods used to create injectable gels, enzyme catalyzed reactions are raising significant interest due to the mild, cell friendly gelation process. Cross-linking phenol derivatives of polymers using hydrogen peroxide in the presence of HRP is a potential enzymatic route to develop injectable gels [33,34]. Lee et al. demonstrated the feasibility of synthesizing tyramine conjugated hyaluronic acid that can be cross-linked in the presence of HRP and hydrogen peroxide [35]. A similar chemical reaction can be used to modify sodium alginate for synergistic ionic and enzymatically cross-linked gels [36].

The focus of the present study is to develop and characterize an enzymatically cross-linked alginic-hyaluronic acid composite injectable hydrogel system as a potential cell delivery vehicle for cartilage and /osteocondral interfacial tissue regeneration. Tyraminated alginic acid and hyaluronic acid were used to develop the enzymatically cross-linked composite injectable gels. The physical, mechanical, and biological properties of the 50:50 alginate/hyaluronic acid composite gels were compared to enzymatically cross-linked alginate and hyaluronic acid gels.

2. Materials and Methods

2.1 Materials

Alginic acid sodium salt, N-3-dimethylaminopropyl-N-ethyl carbodiimide hydrochloride, N-hydroxysuccinimide, tyramine, sodium hydroxide, HRP, bovine serum albumin (BSA), 0.4% Trypan-blue dye solution, and hydrogen peroxide were obtained from Sigma-Aldrich, USA. Phosphate buffered saline (PBS), Ethidium homodimer-1 and calcein AM fluorescent dyes, fetal bovine serum (FBS), penicillin, streptomycin, and 0.05% Trypsin-EDTA were purchased from Invitrogen-GIBCO, USA. Promega cell titer 96 aqueous one solution cell proliferation assay (MTS reagent) was purchased from Promega. BCA Protein Assay Kit was purchased from Thermo Scientific, USA. Complete media was made by adding 1% penicillin, 1% streptomycin, and 10% FBS to minimal essential medium. This media is referred to as cMEM.

2.2 Methods

2.2.1 Synthesis of tyramine substituted sodium alginate—Tyramine substituted alginic acid was prepared according to a reported procedure with some modifications [37]. Alginic acid sodium salt (500 mg) was dissolved in 45 mL of 1.0 M-(N-morpholino)ethanesulfonic acid (MES buffer). Tyraminated alginate was prepared by activating the carboxyl groups of alginate with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (175mg) and N-hydroxysuccinimide (66 mg) followed by reacting with tyramine (180 mg). The solution was stirred for 24 h, dialysed in 1 M sodium hydroxide solution for 3 h followed by milli-Q water for 48h and freeze dried.

Tyramine substituted sodium hyaluronate (2.8%) was procured from Life Core Biomedical USA. Briefly, tyramine was coupled to hyaluronic acid by the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide according to a previous protocol [54].

2.2.2 Determination of Tyramine Substitution in Sodium Alginate—The extent of tyramine substitution in sodium alginate was determined using UV-spectrophotometer. Briefly, the absorbance at 275 nm of the aqueous solutions of tyramine-substituted alginate was determined and the concentration of the phenolic group in the solutions was estimated from a standard curve prepared using different concentrations of tyramine.

2.2.3 Preparation of Enzymatically Cross-linked Gels—The tyraminated hyaluronic acid (HA) and sodium alginate (AA) solutions (1% (w/v)) were prepared in calcium free phosphate buffered saline solution (PBS) containing 10U/ml HRP. The 50:50 composite solution (AA:HA) was prepared by mixing equal volumes of HA and AA solutions. Gelation was initiated by the addition of aqueous hydrogen peroxide solution (1.0% (v/v)).

2.2.4 Percentage Water Uptake—The extent of water absorption by the enzymatically cross-linked gels was evaluated by incubating the gels in PBS at 37°C. Briefly, AA, HA and AA:HA mixture solutions were cross-linked in the presence of 10U HRP solution with

H₂O₂ (1.0%) to form the respective gels. The initial weights (W_{initial}) of the wet gels were determined before incubation in PBS at 37°C. After 7 days, the weights of the wet gels (W_{fin}) were determined. The extent of water absorption was calculated by:

$$\% \text{Water uptake} = (W_{\text{fin}} - W_{\text{initial}}) / (W_{\text{initial}}) * 100$$

2.2.5 Rheology—Solutions of HA, AA, and AA:HA (1% w/v) were prepared in HRP solution in PBS (10U/mL). Gelation was initiated by adding 1.0% H₂O₂ solution. The oscillatory shear measurements of the elastic modulus (G') and the viscous modulus (G'') were measured at room temperature using a constant stress rheometer (TA Instruments Discovery Hybrid Rheometer 3 DHR-3). The value of G'' was obtained for each gel type to determine the limit of the linear viscoelastic regime using an amplitude sweep from 0.001 to 10% strain. The solution was allowed to gel on the plate, and dynamic frequency sweeps were then performed in the linear viscoelastic region to determine values of G and G'' to compare the mechanical performance of the different gels. Rheological testing conditions were as follows: angular frequency: 1 rad/s; % strain: 0.005%; gap width: 700 μ m; volume of solution loaded: 220 μ L.

2.2.6 Protein Release—The HA, AA, and AA:HA solutions (1% w/v) were prepared in 10U HRP solution as described before. The BSA loaded gels were prepared by adding BSA (22 mg/mL) and H₂O₂ (1.0% v/v) to respective polymer solutions. The protein loaded gels were then incubated in PBS at 37°C on a shaker table. 100 μ L samples of supernatant PBS were removed and replaced with 100 μ L of fresh PBS at time points of 1, 3, 7, 10 and 14 days. The extent of BSA release was determined by BCA protein assay kit using calibration curves obtained from standard solution of BSA.

2.2.7 Cell Culture—The ATDC-5 cell line was selected to evaluate the ability of the gels to sustain cell viability and support proliferation. ATDC-5 is an established murine chondrocytic cell line widely used for studying chondrocyte functions. In culture, ATDC-5 is known to follow well characterized stages of chondrogenesis, showing morphology, gene and protein expression profiles characteristic of primary cells [38]. ATDC-5 cells were cultured in cMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

2.2.8 Cell Encapsulation in the Gels—ATDC-5 cells were encapsulated in HA, AA and AA:HA gels as follows. Tyraminated HA, AA and AA:HA solutions (1% w/v) were prepared in 10U HRP solution as discussed before. The cells were suspended in the polymer solutions (80,000 cells/150 μ L) followed by the addition of H₂O₂ solution (1.0%). The encapsulated cells were then cultured in cMEM at 37°C for predetermined time points.

2.2.9 Viability of the Encapsulated cells—The viability of the cells in the gels was determined using Live/Dead Viability/Cytotoxicity kit (Molecular Probes, Carlsbad, CA). Live/Dead stain is a vital fluorescence double-stain that utilizes calceinAM and ethidium homodimer-1 probes to measure membrane integrity and cellular esterase activity to assess cell viability. The ATDC-5 cells encapsulated in HA, AA, and AA:HA gels were cultured at 37°C in cMEM and imaged at predetermined time points. At these time points, the gels were washed and incubated at 37°C for 15 min in PBS containing 2mM calcein AM and 2mM ethidium homodimer-1. Cells stained green (live cells) and red (dead cells) were imaged using a Zeiss LSM 510 confocal microscope.

2.2.10 Cell Proliferation—The proliferation/metabolic activity of the cells encapsulated in the gels was determined using MTS assay. Briefly, the ATDC-5 cells encapsulated in HA,

AA and AA:HA gels were cultured at 37°C in cMEM for 7, and 14 days. At predetermined time points, the gels were washed and incubated at 37°C in media containing the MTS reagent (CellTiter Aqueous one solution, Promega) for 2 h. The hydrogel-cell constructs were then homogenized and the absorbance of the solution was then determined at 490 nm using a plate reader (BioTek Synergy HT).

Statistical data analysis: All data is presented as mean $\pm$ S.D. (standard deviation) for n=3, unless stated otherwise. Statistical analyses were performed by t-test; $p < 0.05$ was considered statistically significant.

3. Results and Discussion

Synthesis of tyraminated alginic acid

Standard carbodiimide mediated coupling of amino groups of tyramine with carboxylic groups of sodium alginate was used to develop tyraminated alginic acid. Figure 1 shows the phenolic content of tyramine modified alginic acid (1% solutions) as a function of reaction time under the described reaction conditions. The phenolic content of the alginic acid increased with time of reaction. The 24 h reacted sample showed approximately 4 times the phenolic content when compared to the 6 h reacted sample.

Preparation of enzymatically cross-linked gels

Chemically cross-linked gels are considered superior to physically cross-linked gels due to the controllable mechanical and degradation properties. Several cross-linking methods have been investigated to develop covalently cross-linked alginate and hyaluronic acid gels by functionalizing the polymers. Photocross-linked alginate and hyaluronic acid systems were developed from acrylated polymers as potential cell compatible matrices for tissue engineering [39, 40]. Recently enzyme mediated covalent cross-linking of polymers using HRP, hematin or transglutaminase was developed as a versatile system of significant biomedical interest due to the mild gelation process and the ability to modulate the gel physical and mechanical properties by varying the reaction parameters [33-35,41,42]. In the HRP mediated reaction, the concentrations of the reagents (tyramine modified polymers, HRP and H₂O₂) play important roles in determining the gelation time. In the present study, irrespective of the HRP and H₂O₂ concentrations studied, the mixture of 1% solution of tyraminated alginic and hyaluronic acid formed uniform composite gels with gelation time of less than 10s.

Percentage water uptake

In the present study, the water uptake ability of the gels were evaluated in the wet state to understand the gel behavior upon injection. The enzymatically cross-linked sodium alginate hydrogel showed significant increase in size after incubation in phosphate buffer solution. This is consistent with the behavior of photo cross-linked alginate hydrogels wherein the extent of gel swelling is dependent on the degree of cross-linking [39]. Enzymatically cross-linked hyaluronic acid on the other hand showed a decrease in mass of the gel with incubation time in PBS (in the absence of hyaluronidase) suggesting possibility of some gel shrinkage (Figure 2). Similar to the sodium alginate gels, the composite alginate-hyaluronic acid gels showed an increase in gel water uptake upon incubation in PBS. However, the extent of water uptake by the composite gel was significantly lower than that of the sodium alginate gels (Figure 2). Injectable biomaterials are designed to fill irregular spaces, and therefore ideally, gelation should not cause significant swelling or shrinkage as this might compromise the gel's intended functions. The data demonstrates the advantage in using a composite approach to modulate the extent of water uptake by injectable alginate and hyaluronic acid gels.

Gel Mechanical properties

The mechanical properties of hydrogels play a significant role in supporting tissue regeneration by 1. Transiently supporting the mechanical function of the tissue to be regenerated and 2. Presenting a tissue mimic mechanical microenvironment to modulate cell functions. Both ionic and covalent cross-linking methods have been investigated to develop hydrogels with a wide range of mechanical properties to match the properties of various soft tissues. Similar to the extent of gel water uptake, the polymer composition significantly affected the elastic modulus of the hydrogels (Figure 3). The 1% sodium alginate gel showed an elastic modulus of ~ 11 kPa (Figure 3). By varying the concentration of sodium alginate and calcium chloride, Banerjee et al demonstrated the feasibility of developing ionically cross-linked sodium alginate with elastic modulus ranging from 1-10 kPa[43]. Similarly, Rouillard et al., developed photo cross-linked alginate hydrogels with elastic modulus ranging from 10-20 kPa by varying the extent of cross-linking [44]. The elastic modulus of enzymatically cross-linked hyaluronic acid gels also has shown to depend on the extent of cross-linking [35]. Lee et al developed covalently cross-linked gels with elastic modulus ranging from 0.8-3 kPa using methacrylated hyaluronic acid with different concentrations of methacrylic groups [35]. Hyaluronic acid gels with compressive strength in the range of ~5-11kPa were developed by Toh et al using 2% (wt/v) tyraminated hyaluronic acid by varying the concentration of H₂O₂ [45]. Under the present reaction conditions, we observed an elastic modulus of ~2kPa for 1% (wt/v) tyraminated hyaluronic acid. The composite gel on the other hand showed an elastic modulus of ~6 kPa, showing the possibility of increasing the elastic modulus of injectable hyaluronic acid gels by the addition of alginic acid. Similarly, using interpenetrating polymer network scaffolds of sodium hyaluronate and sodium alginate, Chung et al showed the feasibility of increasing the strength of hyaluronic acid scaffold by the addition of sodium alginate [32]. The incorporation of hyaluronic acid has been demonstrated to change the structure of ionically cross-linked alginate gels, thereby modifying the composite gel properties [46]. The enzymatically cross-linked composite gels may therefore serve as an alternative approach to develop potential soft tissue scaffolds with altered mechanical properties.

Release of BSA from the Gels

The open, water rich environment of hydrogels usually leads to an initial burst release of the encapsulated molecules [47,48]. Hyaluronic acid is enzymatically degradable *in vivo*, and hyaluronic acid gels have been shown to release encapsulated macromolecules by degradation as well as diffusion in a controlled manner [35]. Figure 4 shows the release of a model protein, BSA from alginic acid, hyaluronic acid and alginate/hyaluronate composite gels in PBS in the absence of hyaluronidase. As shown in the figure, hyaluronic acid gel showed a significantly high protein release compared to the other gels upon incubation in PBS. Approximately 50-80% protein release (lysozyme and alpha-amylase) was reported from enzymatically cross-linked hyaluronic acid gels in the presence of 2.5U/ml hyaluronidase within 25 h [35]. The absence of burst release and significantly low release of BSA over time from hyaluronic acid gel in the present study (~10% in 14 days) can be attributed to the significant intra-molecular interaction between BSA and hyaluronic acid polymer, thereby limiting the diffusion controlled release of the protein [49]. The sodium alginate gel as well as the composite alginate/hyaluronic acid gel showed significantly lower protein release compared to the hyaluronic acid gel. This has been attributed to the significant electrostatic interactions between BSA and the anionic polysaccharide [50]. In summary, the incorporation of hyaluronic acid in the composite gel did not significantly affect the albumin release profile compared to the alginic acid gel, however, incorporation of hyaluronic acid may significantly change the gel degradation and corresponding release of protein *in vivo*, since hyaluronic acid is known to be degraded by the enzyme hyaluronidase present in the body.

Cell Viability and Proliferation

Hydrogels are known to provide a conducive microenvironment to support cell viability by enhancing nutrient and gaseous diffusion. Figure 5 shows the viability of ATDC-5 cells encapsulated in alginic, hyaluronic and composite alginic/hyaluronic acid hydrogels. At 2, 7 and 14 days, >95% of the cells encapsulated in all three gels remained viable (green fluorescence), indicating the excellent cytocompatibility of the gels. Moreover, all the gels retained the round cellular phenotype. The cells encapsulated in alginate and composite gels also showed an increase in size with more spherical morphology that might indicate the possibility of cellular hypertrophy, which needs to be further investigated [51]. Figure 6 shows the MTS results showing the metabolic activity of the cells in the gels. As shown in the figure, there is a significant increase in metabolic activity of cells encapsulated in the composite gel compared to alginate and hyaluronic acid gels at 14 days. Moreover, the metabolic activity of the cells encapsulated in the gels significantly increased from day 7 to day 14. Chondrocytes are known to have good proliferative ability when cultured in monolayers. Eslaminejad et al has shown that chondrocytes show significantly lower proliferative ability when cultured in calcium alginate gels with a doubling time of ~10 days [52]. By 14 days, the metabolic activity of the cells in the composite gels was found to be significantly higher than that in the hyaluronic and alginic acid gels. The increase in cellular activity in the composite gel is presumably due to the increased permissive environment presented by the alginic acid and the stimulatory environment presented by the hyaluronic acid [53]. Several factors are known to affect cell functions upon encapsulation in hydrogels. Banerjee et al have shown using alginate gels that the proliferation of encapsulated neural stem cells decreases with increases in gel modulus [43]. Similarly, Toh et al using hyaluronic acid gels showed that increase in gel stiffness can adversely affect mesenchymal stem cell differentiation towards chondrogenic lineage [45]. In the present study, even though the composite gel showed higher initial modulus than the hyaluronic acid, the increase in cell proliferation in the gel is presumably also due to the increase in water absorption of the composite gel compared to the hyaluronic acid gel, which might decrease the gel mechanical properties with time in culture.

Conclusions

The enzyme mediated cross-linking of polysaccharides is a potentially mild *in situ* gelation method to form composite hydrogels. The addition of hyaluronic acid was found to decrease the percentage water uptake by the gels. Similarly, the composite approach can be used to modulate the mechanical properties of the hydrogel. The hyaluronic and alginic acid gels showed significant interaction with the cationic macromolecule BSA. Since hyaluronic acid gels are degradable *in vivo*, the composite gels could be engineered to control the release profile of similar macromolecules via diffusion and degradation mediated mechanisms. The gels were found to be cytocompatible and the composite gel was able to support higher cellular metabolic activity compared to alginic acid and hyaluronic acid gels.

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Highlights

Composite injectable hydrogels were developed from alginic and hyaluronic acid using enzymatic crosslinking

The composite approach helped to modulate the physical, and mechanical and properties of the gels

The gels supported cell viability

Figure 1.
Tyramine content of modified alginic acid determined by UV-spectrophotometer

Figure 2. Percentage water uptake by the gels after 7 days in PBS. **AA percentage water uptake was significant to both AA:HA and HA. *AA:HA composite gel's percent water uptake was significant to HA gels. Significance computed using a 2 tailed t-test with $p < 0.05$.

Figure 3.

Storage modulus of 1% HA, AA and AA:HA composite gels. **AA modulus was significant to both AA:HA and HA. *AA:HA composite gel's modulus was significant to HA gels. Significance computed using a 2 tailed t-test with $p < 0.05$.

Figure 4. BSA release from AA, HA, and AA:HA composite gels. *BSA release was significantly higher for HA gels at all time points when compared with AA and AA:HA composite gels. Significance computed using a 2 tailed t-test with $p < 0.05$.

Figure 5.

Live/Dead assay showing the morphology and viability of ATDC5 cells encapsulated in AA, HA and AA:HA composite gels. A, D, and G display cells in AA gels at time points 2, 7, and 14 days respectfully. B, E, and H display cells in AA:HA composite gels at time points 2, 7, and 14 respectfully. C, F, and I display cells in HA gels at time points 2, 7, and 14 respectfully. All images were captured with a Zeiss LSM 510 confocal microscope at 20x magnification.

Figure 6.

Metabolic activity of ATDC-5 cells encapsulated in AA, HA, and AA:HA composite gels after 7 and 14 days. **Cellular metabolic function was significantly higher for ATDC-5 cells encapsulated in AA:HA composite gels when compared to both HA and AA gels alone after 14 days. Significance computed using a 2 tailed t-test with $p < 0.05$.