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Long-term stable fibrin gels for cartilage engineering ☆

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Abstract

It is essential that hydrogel scaffold systems maintain long-term shape stability and mechanical integrity for applications in cartilage tissue engineering. Within this study, we aimed at the improvement of a commercially available fibrin gel in order to develop a long-term stable fibrin gel and, subsequently, investigated the suitability of the optimized gel for in vitro cartilage engineering. Only fibrin gels with a final fibrinogen concentration of 25 mg/ml or higher, a Ca^{2+} concentration of 20 mM and a pH between 6.8 and 9 were transparent and stable for three weeks, the duration of the experiment. In contrast, when preparing fibrin gels with concentrations out of these ranges, turbid gels were obtained that shrank and completely dissolved within a few weeks. In rheological characterization experiments, the optimized gels showed a broad linear viscoelastic region and withstood mechanical loadings of up to 10,000 Pa. Bovine chondrocytes suspended in the optimized fibrin gels proliferated well and produced the extracellular matrix (ECM) components glycosaminoglycans and collagen type II. When initially seeding 3 million cells or more per construct (5 mm diameter, 2 mm thick), after 5 weeks of culture, a coherent cartilaginous ECM was obtained that was homogenously distributed throughout the whole construct. The developed fibrin gels are suggested also for other tissue engineering applications in which long-term stable hydrogels appear desirable.

Keywords: Fibrin; Cartilage tissue engineering; Chondrocyte; Cell culture; Viscoelasticity

1. Introduction

In recent years, fibrin glue has been utilized for different applications in the field of tissue engineering, as it fulfills specific physical and biological requirements [1]. An important fibrin characteristic is an increasing instability and solubility over time in vitro and in vivo, due to fibrinolysis. Rapid degradation could be an advantage in wound sealing or other surgical applications as well as for cell and growth factor delivery. However, this can represent a problem for use as a shape-specific scaffold in tissue engineering [2–5]. Long-term stability and mechanical integrity may be essential for cells that require sufficient time and stiffness to produce their tissue-specific matrix. In addition, mechanically durable gels appear more suited for earlier implantation after cell-fibrin construct preparation, i.e., to shorten the in vitro culture period, and at the same time providing sufficient load-bearing capacity for mechanical loading in vivo. Therefore, optimizing fibrin composition would be a fundamental approach to obtain a scaffold system providing optimal shape stability and integrity for specific applications in tissue engineering. It is known that the variation of fibrin parameters, such as fibrinogen concentration, thrombin concentration, and ionic strength, can generate gels with different appearance, mechanical properties, and stability [6–8]. Ferry et al. [9,10] first reported two different types of fibrin gels: coarse and fine clots. Fine gels consist of a high number of thin and branched fibrin fibers built of protofibrils, resulting in transparent and rigid gels with small pores. Transparency is an additional favourable characteristic as it allows

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observation of the single cells during the early culture period. In contrast, coarse gels are made of thicker fibers due to lateral aggregation of protofibrils, which result in turbid and loose gels with larger pore size. This lateral aggregation can be influenced by several parameters including enzyme and substrate concentration as well as the pH and ionic strength of the solutions [11,12].

Fibrin is widely used in the tissue engineering of cartilage [1]. One major goal of the field is the development of a coherent cartilaginous extracellular matrix (ECM). Using a mechanically strong fibrin gel, however, may immure the single chondrocyte, inhibiting cell proliferation and migration, and subsequently preventing ECM development [13,14]. Therefore, a fibrin system optimized for its mechanical stability has to be tested for the specific application in cartilage engineering. In particular, cell density has to be adjusted to obtain an adequate newly developed tissue [15–22].

In this study, we developed long-term stable fibrin gels and investigated their suitability for cartilage engineering. First, we investigated specific fibrin gel parameters that determine gel stability. To this end, we modified the concentration of fibrinogen and Ca^{2+} as well as the pH and tested the appearance and the stability of the resultant fibrin gels in chondrocyte culture medium over 3 weeks. Additionally, the mechanical properties of the resulting fibrin gels were determined and related to the gel preparation parameters. The optimized fibrin gels were tested as three-dimensional carriers for freshly isolated bovine chondrocytes. In order to obtain an adequate coherent ECM within the newly developed fibrin gels, we investigated the effect of different initial cell densities on ECM production and distribution.

2. Materials and methods

2.1. Materials

Aprotinin solution (Trasylol®) was bought from Bayer (Leverkusen, Germany). Thrombin (as a part of Tissucol®), thrombin dilution buffer, and the commercially available fibrin glue Tissucol® was kindly provided by Baxter (Unterschleißheim, Germany). Bovine fibrinogen and calcium chloride were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Knee joints from 3-month-old bovine calves were obtained from a local abattoir within 12–18 h of slaughter. Type II collagenase and papainase were purchased from Worthington (CellSystem, St. Katharinen, Germany). Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose, fetal bovine serum (FBS), MEM non-essential amino acid solution, penicillin, streptomycin, HEPES buffer, and phosphate buffer solution (PBS) were obtained from Gibco (Karlsruhe, Germany). 149 μm pore size polypropylene filters were purchased from Spectrum (Rancho Dominguez, CA, USA). All cell culture plastics were purchased from Corning Costar (Bodenheim, Germany).

Ascorbic acid, deoxyribonucleic acid, diaminobenzidine, dimethylmethylene blue, glutaraldehyde, glycine, hematoxylin, proline, and safranin-O were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chloramin-T, formalin 37%, and *p*-dimethylaminobenzaldehyde (*p*-DAB) were from Merck (Darmstadt, Germany).

Hoechst 33258 dye was obtained from Polysciences (Warrington, PA, USA) and L-hydroxyproline from Fluka (Neu-Ulm, Germany). Chondroitin sulfate was from ICN (Aurora, Ohio, USA) and Tissue Tek was from Sakura Finetek (Torrane, CA, USA). Biotinylated secondary anti-mouse/rabbit IgG, Vectastain ABC-kit and DAB-kit as well as normal horse serum were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). Type II collagen monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (USA). Type I collagen antibody (monoclonal anti-collagen type I col-1) mouse ascites fluid was obtained from Sigma-Aldrich (Saint Louis, Missouri, USA).

2.2. Preparation of fibrin gels

For improvement of fibrin gel appearance and stability, the concentration of fibrinogen and Ca^{2+} and the pH were modified. To study the influence of fibrinogen concentration, a range of 12.5–100 mg/ml purified fibrinogen containing approximately 60% protein was dissolved in 10,000 KIE/ml aprotinin solution. Fibrinogen solutions were mixed with the same volume of thrombin at a concentration of 5 U/ml in 40 mM $CaCl_2$ (500 U/ml thrombin diluted 1:100 in Baxter dilution buffer containing 40 mM $CaCl_2$) and allowed to gel in a silanized glass ring with an inner diameter of 5 mm for 45 min at 37 °C. This preparation procedure resulted in fibrin gels with a final fibrinogen concentration ranging from 6.25 to 50 mg/ml, and final $CaCl_2$ and thrombin concentrations of 20 mM and 2.5 U/ml, respectively; the pH of the resultant gels was 7.0. For modification of the Ca^{2+} concentration, fibrinogen solutions with a fixed concentration of 100 mg/ml were mixed with equal volumes of thrombin at 5 U/ml in a dilution buffer containing 5–40 mM $CaCl_2$ and allowed to gel as described above (2.5–20 mM final concentration of $CaCl_2$). For variation of the pH, fibrinogen solutions with a fixed concentration of 100 mg/ml were mixed with equal volumes of thrombin at 5 U/ml at a pH between 6 and 9.5 in dilution buffer containing 40 mM $CaCl_2$. The pH in the thrombin solutions was adjusted using 0.01 M HCl and 0.01 M NaOH; the pH of the final fibrin gel was equal to the pH in the thrombin solution. After removing the glass rings, all fibrin gels were covered with 4 ml of chondrocyte culture medium without aprotinin and incubated at 37 °C for 3 weeks. Tissucol® was prepared according to the protocol provided by the supplier. For all fibrin gels, aprotinin was only used for gel preparation and not added to the medium during the experimental period. All gels were visually examined to assess turbidity and shrinkage, i.e., contraction directly after preparation as well as degradation and/or dissolution over time.

2.3. Rheological characterization of fibrin gels

For rheological experiments, an AR 2000 rheometer (TA Instruments, Alzenau, Germany) with 20 mm steel plate geometry was used. For fibrin gel preparation, the two liquid components were mixed and then transferred to the lower plate of the rheometer. Solutions were allowed to gel in the measuring gap (1000 μm) at 37 °C for 45 min. To prevent dehydration, the measuring gap was covered with a solvent trap filled with distilled water. For dynamic measurements, an increasing oscillatory stress ranging from 0.1 to 10,000 Pa at a fixed oscillatory frequency of 1 Hz at 37 °C was applied, and the storage (G') and loss (G'') moduli were recorded. For static measurements, creep tests were performed applying a constant stress of 100 Pa for 5 min and recording the resulting deformation over time, followed by creep recovery.

2.4. Cell culture

Primary chondrocytes were isolated from the surface of the femoral patellar groove. The cartilage was enzymatically digested overnight in DMEM containing 4.5 g/l glucose, 10% FBS, 584 mg/l glutamine, 0.1 mM MEM non-essential amino acids, 10 mM HEPES, 0.4 mM proline, 50 μg/ml ascorbic acid, 50 U/ml penicillin, 50 μg/ml streptomcin, and 470 U/ml of type II collagenase. The digest was repipetted, filtered through a 149 μm filter, and washed three times with PBS. The cell number was determined using a hemocytometer. Based on the results from gel development, fibrin