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Solidification mechanisms of chitosan–glycerol phosphate/blood implant for articular cartilage repair

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Summary

Objective: Chitosan–glycerol phosphate (chitosan-GP) is a unique polymer solution that is mixed with whole blood and solidified over microfractured or drilled articular cartilage defects in order to elicit a more hyaline repair cartilage. For clinical ease-of-use, a faster *in situ* solidification is preferred. Therefore, we investigated the mechanisms underlying chitosan–GP/blood implant solidification.

Methods: *In vitro* solidification of chitosan–GP/blood mixtures, with or without added clotting factors, was evaluated by thromboelastography. Serum was analyzed for the onset of thrombin, platelet, and FXIII activation. *In vivo* solidification of chitosan–GP/blood mixtures, with and without clotting factors, was evaluated in microdrilled cartilage defects of adult rabbits ($N = 41$ defects).

Results: Chitosan–GP/blood clots solidified in an atypical biphasic manner, with higher initial viscosity and minor platelet activation followed by the development of clot tensile strength concomitant with thrombin generation, burst platelet and FXIII activation. Whole blood and chitosan–GP/blood clots developed a similar final clot tensile strength, while polymer–blood clots showed a unique, sustained platelet factor release and greater resistance to lysis by tissue plasminogen activator. Thrombin, tissue factor (TF), and recombinant human activated factor VII (rhFVIIa) accelerated chitosan–GP/blood solidification *in vitro* ($P < 0.05$). Pre-application of thrombin or rhFVIIa + TF to the surface of drilled cartilage defects accelerated implant solidification *in vivo* ($P < 0.05$).

Conclusions: Chitosan–GP/blood implants solidify through coagulation mechanisms involving thrombin generation, platelet activation and fibrin polymerization, leading to a dual fibrin–polysaccharide clot scaffold that resists lysis and is physically more stable than normal blood clots. Clotting factors have the potential to enhance the practical use, the residency, and therapeutic activity of polymer–blood implants.

Key words: Chitosan, Articular cartilage repair, Microfracture, Coagulation, Blood, Platelets, Platelet factor 4, Factor XIII, Factor VIIa, Thrombin, Tissue factor, Thrombin–antithrombin (TAT).

Introduction

A current aim and major challenge in orthopedic practice is to regenerate durable cartilage in focal articular lesions[1–3]. Cartilage is avascular and when damaged fails to bleed and therefore lacks an efficient wound repair response. To stimulate a natural wound response, surgeons have developed techniques like bone marrow stimulation involving drilling or microfracture in the base of the débrided cartilage lesion to induce subchondral bleeding and generate conduits to permit bone marrow stem cell migration into the cartilage lesion[3–6]. However in skeletally mature human patients and animal models, these surgical techniques principally result in the formation of a repair tissue predominantly composed of fibrous tissue or fibrocartilage, tissue types with weak biomechanical properties compared to hyaline articular cartilage[2,5,7–13]. Treatments that improve the volume, integration, and hyaline quality of marrow-derived repair tissue are therefore the focus of intense research.

Bleeding and the formation of a blood clot are initiating events of microfracture therapy[6], however the clot is best retained in the bone defect and not on the cartilage surface[14–16]. To improve clot retention in the cartilage lesion following microfracture and drilling, we previously developed a scaffold-stabilized blood clot using chitosan, a cationic, adhesive and biocompatible polysaccharide. Homogenous mixtures of autologous whole blood and solutions of chitosan–glycerol phosphate (chitosan–GP) solidify to form a clot with morphologically normal erythrocytes and a structurally stable fibrous network with chitosan and fibrin fibers[17,18]. Hybrid chitosan clots resist platelet-mediated clot retraction, and remain voluminous, firm and elastic[17]. The formation of a chitosan–GP/blood clot in marrow-stimulated cartilage defects in sheep and rabbit improved the quantity, hyaline quality and integration of repair cartilage with porous subchondral bone compared to repair tissue formed by microfracture or drilling alone[17,19]. Hybrid clots were shown to promote hyaline repair by attracting neutrophils, marrow-derived repair cells, and by stimulating transient angiogenesis and bone remodeling[15].

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Mixture of liquid chitosan−GP into whole blood generates a hybrid clot implant that forms within 10−15 min after depositing in large animal or patient microfracture defects[19,20]. A more rapid implant solidification could facilitate treatment of larger defects with curved surfaces and unconfined borders, and also reduce the length of the cartilage repair procedure. Indeed, for clinician ease-of-use, a faster and controlled *in situ* solidification is preferred; however solidification mechanisms are incompletely understood. Many studies previously demonstrated that acidic chitosan solutions and solid chitosan particles are thrombogenic, through mechanisms involving red blood cell (RBC) agglutination, and platelet activation, without direct activation of the clotting cascade[17,21−23]. By comparison, little is understood concerning the hemostatic properties of isotonic and near-neutral pH chitosan−GP solutions.

The first aim of the current study was to elucidate solidification mechanisms of chitosan−GP/blood *in vitro*, using a Thromboelastograph® (TEG), an instrument that measures clotting time and clot tensile strength. We tested the hypothesis that chitosan−GP/blood solidification is thrombin-dependent, and furthermore implicates platelet and Factor XIII activation. The second aim of this study was to develop novel methods to accelerate *in situ* solidification in an *in vivo* animal cartilage repair model. We therefore tested the hypothesis that *in vivo* solidification of chitosan−GP/blood mixtures in microdrilled articular cartilage defects can be accelerated using clotting factors including thrombin (IIa), tissue factor (TF) and recombinant human factor VIIa (rhFVIIa). Tissue factor is a transmembrane receptor constitutively expressed in mainly extravascular tissues. TF binding with trace amounts of activated FVIIa that are present in plasma[24] triggers the extrinsic clotting cascade and thrombin generation. In our *in vivo* experiments, we used a recombinant TF−phospholipid preparation at concentrations previously employed in clinical applications for maxillary bone grafts[25], rhFVIIa at concentrations around threefold greater than the target plasma levels for clinical applications (~2 μg/mL)[26], and thrombin concentrations within the low range used in standard fibrin glue[27]. Our choice of these particular factors was thus based on their known ability to promote repair processes, as well as their current use in other clinical contexts which could help translate their use in cartilage repair strategies involving polymer−blood implants.

Materials and methods

MATERIALS

Medical-grade sterile solutions of chitosan (2.05% w/v, 80% degree of deacetylation, DDA, 1200–2000 mPa s., pH 5.6, <100 EU/mL endotoxins units and <5 ppm heavy metal content) and 500 mM disodium β-glycerol phosphate/50 mM HCl pH 7.1 (GP) were provided by Bio Syntech Inc (Laval, QC, Canada). Chitosan−GP solutions were formed aseptically by combining 1.2 mL chitosan−HCl with 0.3 mL GP. Rhodamine B-isothiocyanate (RITC)−chitosan 0.5% mol/mol RITC/chitosan (80.5% DDA, M_n 144 kDa, polydispersity (M_w/M_n) 1.3) was prepared as 5 mg/mL filter-sterile solutions as previously described[28]. Calibrated viscosity standards (N62000, N1000, S60) were from Canon Instrument Company (State College, PA, USA) and silicon oil (pure silicone fluid) was from Clearco Products (Bensalem, PA, USA). Other reagents included sterile recombinant human Factor VIIa (NovoNordisk, Copenhagen, Denmark), tissue culture-grade purified human thrombin and benzamidine (Sigma−Aldrich, Oakville, ON, Canada), TF (Innovin®) and thrombin−antithrombin (TAT) enzyme-linked immunosorbent assay (ELISA) kits (Dade Behring, Mississauga, ON, Canada), and pooled normal plasma for TAT ELISA sample dilution (Precision Biologic, Dartmouth, Nova Scotia, Canada), affinity-purified goat anti-human platelet factor 4 (PF4) antibody (R&D Systems, Cedarlane, Burlington, ON, Canada), sheep anti-human A and B subunit of Factor XIII (FXIII) antibody and Phe-Pro-Arg- chloromethylketone, a thrombin protease inhibitor (FPR-ck), purified human PF4 and human FXIII (Haematologic Technologies, Essex Junction, VT, USA), donkey anti-goat-horseradish peroxidase (HRP) and donkey anti-sheep-HRP (Jackson Immunology, Montreal, QC, Canada), chemiluminescence substrate kit (Lumi-light, Roche Diagnostics, QC, Canada), and Hyperfilm ECL (Amersham Biosciences, GE Health, ON, Canada). Recombinant tissue plasminogen activator [tPA 1 mg/mL, diluted to 2 μg/mL in phosphate-buffered saline (PBS) with 4% w/v bovine serum albumin (BSA)] was from Hoffmann-La Roche (NJ, USA). Ringer's lactated saline (RLS) buffer (Baxter) was purchased from Lavigne & Dufort (Montreal, QC, Canada).

CLOTTING FACTOR PREPARATION FOR *IN VITRO* AND *IN VIVO* EXPERIMENTS

Factor rhFVIIa was reconstituted with sterile water for injection from Abbott (QC, Canada) at 500 μg/mL. Purified human IIa lyophilized powder containing 0.15 M sodium chloride and 0.05 M sodium citrate buffer at pH 6.5 was reconstituted with sterile water for injection at 100 U/mL. Innovin® or recombinant TF containing phospholipids was freshly reconstituted with 2 mL sterile water for injection, to give 5 nM TF according to a recombinant TF standard curve (S. Butenas, personal communication)[29]. For *in vitro* TEG experiments, clotting factors were diluted with RLS before loading in the TEG cups.

COAGULATION ANALYSES BY THROMBOELASTOGRAPHY, ELISA AND WESTERN BLOT

Clot tensile strength was evaluated for up to 180 min with four Thromboelastograph® (TEG) (5000 series TEG analyzer Software Version 3, Haemoscope, Niles, IL, USA) instruments in tandem, a set-up permitting the simultaneous analysis of eight samples[30]. Venous peripheral whole blood was drawn from healthy non-fasting consented donors (three males and eight females, 29−47 years old, with one male and two females studied twice), according to institutional ethics approved protocols. A first blood draw was used to generate chitosan−GP/blood mixtures, and a second blood draw from the same donor was performed within 3 h to analyze unmodified whole blood. Blood was mixed with chitosan−GP at a 3:1 v/v ratio by vigorous shaking for 10 s in glass mixing vials containing six sterile surgical stainless steel mixing beads (0.39 g each, Salem Specialty Ball Co., Canton, CT) as described[17]. Unmodified whole blood was transferred to sterile borosilicate mixing vials to control for exposure to glass prior to transferring into TEG reaction cups. TEG plastic reaction cups (made of Cyrolite G20) were pre-loaded with 40 μL of either tPA (2 μg/mL in PBS with 4% w/v BSA), or clotting factor diluted in RLS to give the following target concentrations after adding 320 μL sample: 0.08−10 U/mL IIa; 5 μg/mL rhFVIIa; 0.7−278 pM TF; and rhFVIIa + TF (5 μg/mL rhFVIIa + 0.7 pM TF). Clot tensile strength was recorded manually as the amplitude (the distance in mm on the *y*-axis between the two traces). At set time intervals, the whole reaction cup was transferred to ice cold quench buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 10 mM benzamidine, 33 μM FPR-ck, pH 7.4) at a 1:9 ratio of blood:quench buffer, vortexed for 10 s, cleared by a double centrifugation (2500*g*, 15 min, 4°C) and kept as aliquots at −80°C until analyzed for thrombin generation by TAT ELISA[30], or by Western blot for platelet activation by appearance of serum PF4[31] and for activation of FXIII as determined by cleavage of FXIII A subunit[32]. The equivalent of 4 μL serum, also adjusted for dilution of blood by chitosan−GP, was loaded into each well of a 17.5% acrylamide non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for PF4 and 2 μL serum on a 10% acrylamide SDS-PAGE reducing gel for the A and B subunits of FXIII. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane, blocked for 1 h with 5% non-fat milk powder in 50 mM Tris, 150 mM NaCl, 0.1% Tween-20 pH 7.4 (TBST) at 50°C, and probed with goat anti-PF4 (0.2 μg/mL) then 1:10,000 diluted affinity-purified donkey anti-goat-HRP (0.08 μg/mL) or Sheep anti-human A and B subunits of FXIII antibody (9.8 μg/mL) and donkey anti-sheep-HRP (0.08 μg/mL), followed by chemiluminescence (Lumi-Light). Purified proteins were used as positive controls for PF4 (20 ng/well) and FXIII (0.22 μg/well).

IN VIVO ARTICULAR CARTILAGE REPAIR MODEL

All animal experimentation was carried out with protocols approved by the University of Montreal Animal Division, using skeletally mature New Zealand White rabbits ($N = 33$ rabbits, 16 males, 17 females, >8 months randomly assigned to different groups; 8 out of these 33 rabbits received bilateral knee implants for a total of $N = 41$ unilateral or bilateral defects treated with implant, see Table I). Rabbits were anesthetized with an intramuscular injection of ketamine−xylazine−buprenorphine cocktail then placed under 3% isoflurane/8% oxygen gas anesthesia. They were subjected to small knee arthrotomies in order to create bilateral 3.5 × 4 mm