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Characteristics of platelet gels combined with silk

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ABSTRACT

Platelet gel, a fibrin network containing activated platelets, is widely used in regenerative medicine due the capacity of platelet-derived growth factors to accelerate and direct healing processes. However, limitations to this approach include poor mechanical properties, relatively rapid degradation, and the lack of control of release of growth factors at the site of injection. These issues compromise the ability of platelet gels for sustained function in regenerative medicine. In the present study, a combination of platelet gels with silk fibroin gel was studied to address the above limitations. Mixing sonicated silk gels with platelet gels extended the release of growth factors without inhibiting gel-forming ability. The released growth factors were biologically active and their delivery was modified further by manipulation of the charge of the silk protein. Moreover, the silk gel augmented both the rheological properties and compressive stiffness of the platelet gel, tuned by the silk concentration and/or silk/platelet gel ratio. Silk-platelet gel injections in nude rats supported enhanced cell infiltration and blood vessel formation representing a step towards new platelet gel formulations with enhanced therapeutic impact.

1. Introduction

Platelets play a vital role in normal hemostasis and wound healing. For many years, the scientific community has recognized the role of platelets in tissue repair [1–4]. Recently, commercial entities have investigated implantable or injectable therapies for clinical use that are enriched with supra-physiological levels of platelet growth factors [5,6]. In particular, the benefits of one-time injections of Platelet Rich Plasma (PRP) or platelet gel (PG), in the presence of autologous thrombin and fibrinogen have been explored, with fibrin hydrogel networks containing activated platelets that assemble in 10–20 min. PG can be exogenously applied to wound tissues, conferring benefits due to the released growth factors and the ability to localize the platelet concentrates in the site of injury, a method that was more efficient than the use of recombinant growth factors [7]. The use of PG also provides a microenvironment for the sequential process of tissue regeneration involving migration, proliferation and differentiation of osteogenic and endothelial cells [8,9]. Because of a predictable yet transient liquid (pre-gel) status, PG can be injected alone or in combination with different bone substitutes to avoid the unwanted migration of bone particles [10]. The combination of PG with bone allografts as scaffolds, and with bone marrow stromal cells, can increase the efficacy of PG in animal studies and in clinical applications [11,12].

Although most clinical studies report good outcomes from the use of PRP for the enhancement of healing, many questions remain, particularly with regard to the timing of the therapy and the volume and frequency of treatment [13]. One feature of PRP that can limit efficacy is the rapid and uncontrolled release of growth factors at the site of injection [14,15]. In the absence of long-lasting growth factor (GF) availability, repeated PRP applications are required in order to achieve sustained therapeutic effects. Moreover, while the diversity and quantity of GFs derived from activated platelets are biologically unique, especially compared to factors stored in extracellular matrices (ECM) or those presented by cells at the site of tissue injury, PRP injections cannot supply specific factors in a sequence to fit the needs of true regenerative healing. Since it has been demonstrated that the outcome of healing at a wound site is influenced by fibrin structure, in terms of thickness of fibers, number of branch points, porosity, and permeability of the clot [16], a PG-based method could therefore improve outcomes via mechanical augmentation of the entrapping gel phase.

The objective of the present work was to utilize silk protein gels in combination with platelet gels in injectable formats, to improve

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mechanical properties and growth factor sustained release. The use of silk sonicated silk solutions modulated the release of bioactive platelet growth factors and enhanced the mechanical properties of the system.

2. Material and methods

2.1. Materials

Human peripheral blood was purchased from Research Blood Component, LLC (Boston, MA). Calcium gluconate was purchased from APP Pharmaceuticals (Lake Zurich, IL). *Bombyx mori* silkworm cocoons were supplied by Tajima Shoji Co., LTD (Yokohama, Japan). Histology reagents including ε-poly-L-lysine and Masson's Trichrome were purchased from Sigma Aldrich (St. Louis, MO). Silk filtration and purification were conducted using dialysis tubing from Spectrum Laboratories Inc. (Rancho Dominguez, CA) and centrifugal filter units from Millipore (Billerica, MA). The CellTiter Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI). Cell proliferation assays were conducted using human umbilical vein endothelial cells (HUVECs; Cambrex, East Rutherford, NJ) with control media (endothelial cell basal medium-2) and complete media (endothelial cell basal medium-2, hydrocortisone, human Epidermal Growth Factor (hEGF), Fetal Bovine Serum (FBS), Vascular Endothelial Growth Factor (VEGF), basic Fibroblast Growth Factor-B (bFGF-B), human Recombinant Insulin-like Growth Factor (R3-IGF-1), ascorbic acid, heparin). All cell culture media components were obtained from Lonza (Hopkinton, MA) and used at standard concentrations. The following antibodies were used: mouse anti-phospho ERK (extracellular signal-regulated kinases), mouse anti-actin from Cell Signaling (Danvers, MA), rabbit anti-vascular endothelial (VE) cadherin from LifeSpan BioSciences (Seattle, WA) and rabbit anti-CD31 from Abcam (Cambridge, MA). UO 126 was from Calbiochem (San Diego, CA). Human VEGF, Transforming growth factor β1 (TGF-β1), Platelet-Derived Growth Factor-AB (PDGF-AB) DuoSet and human VEGF affinity purified polyclonal antibody were purchased from R&D system (Minneapolis, MN). Recombinant-VEGF-165 was purchased from Shenandoah Biotechnology Inc. (Warwick, PA).

2.2. Platelet gel (PG) preparation

Human platelets were derived from whole blood taken from healthy volunteers under New England Institutional Review Board approval (# 04-144 "The Collection of Whole Blood for Research Purposes") obtained by Research Blood Components (Brighton, MA). The samples were prepared in citric acid/citrate/dextrose solution and maintained sterile over the entire process of PG preparation. Whole blood was centrifuged at 120 × g for 15 min to obtain platelet rich plasma. PRP was subsequently centrifuged at 100 × g for 15 min to eliminate leukocytes in the supernatant. Platelets were recovered by an additional centrifugation at 720 × g for 15 min to obtain a pellet of platelets and a supernatant of plasma poor of platelets (PPP). Platelet count was adjusted to a final concentration of 4×10^6 platelets/μL by resuspending platelets in PPP. Autologous thrombin was prepared by mixing (5:1, v/v) PPP with 0.22 M calcium gluconate [17]. After 15 min incubation at 37 °C and centrifugation at 1000 × g for 15 min, the thrombin-containing supernatant was collected. PGs were obtained by mixing PRP (final concentration 2×10^6 platelets/μL)/autologous thrombin/calcium gluconate 0.22 M (ratio 8:2:1) and incubated in a humidified chamber at 37 °C until use. To evaluate growth factor release from PG, 500 μL of PBS was added to each sample after gelation, which occurred in about 20 min. Separately, plasma enriched in growth factors was formed by removal of the fibrin gel, diluted 1:1 with phosphate buffered saline (PBS; Invitrogen, Grand Island, NY) or 1% w/v silk solution. For growth factor studies, the samples were stored at 37 °C, or room temperature or 4 °C.

2.3. Silk gel preparation

Silk fibroin aqueous solution was obtained from *B. mori* silkworm cocoons using previously described procedures [18]. Briefly, following extraction in boiling ultra pure water containing 0.02 M Na_2CO_3, fibroin was dissolved in 9.3 M LiBr and dialyzed using a 3.5 kDa cut-off dialysis cassette (Pierce Thermo Scientific Inc., Rockford, IL). The boiling time was modified from 30 to 60 min in certain experiments, as indicated, in order to modify the molecular weight of the silk solution as previously described [19]. The resulting 6–8% (w/v) fibroin solution was diluted in ultra pure water to obtain a 4% (w/v) silk solution, or concentrated by placing the solution in the 3.5 kDa dialysis cassettes and letting the excess of water evaporate at RT for periods of time depending on the desired concentration. Solutions were then sterilized by 0.2 μm filtration (Millipore) or by standard 20 min liquid autoclave cycle, as previously reported to sterilize solutions prior to sonication [20]. The resultant solutions were sonicated at different amplitudes and time with a Branson 450 Sonifier (Branson Ultrasonics Co., Danbury, CT) [20]. In order to investigate the impact of the charges in growth factor release, 10% w/v ε-poly-L-lysine or 0.1% w/v silk fibroin ionomers (silk fibroin-poly-L-lysine and silk fibroin-poly-L-glutamic acid ionomers) [21] were added to the 4% silk solution prior to the sonication. The sonicated silk solution, cooled to room temperature in ice for 1 min, was subsequently mixed in a ratio 1:1 with PRP, generating PG-Silk. A volume of 500 μL of the solution was incubated in 24 well plates in an incubator at 37 °C to promote gelation. A volume of 500 μL of PBS was then added to each sample, after gelation was visually confirmed. At different time points (1 h, 1 day, 7 days, 14 days, 21 days) the PBS from each sample was collected and frozen at −80 °C, and completely replaced with fresh PBS.

At the last time point of incubation, the silk/platelet gel samples (500 μL/well) were solubilized in 500 μL urea 8 M at 37 °C, a process which required 30 min for completion based on visual confirmation. Residual salt was removed by dialysis against deionized water (by using dialysis tubing with MWCO 2 kDa) for 16 h. The solution obtained was concentrated to a final volume of 500 μL by using centrifugal filter units (3 kDa cut-off).

In some experiments, PG-Silk samples mixed with silk fibroin ionomers were analyzed for their ability to swell. The weight of the samples was recorded 1 h after preparation. After adding 500 μL of PBS, samples were allowed to swell for 24 h and measured again, and the percentage weight gain was calculated.

2.4. Growth factor evaluation

PG-Silk was evaluated for growth factor release at 1 h, 1 day, 7 days, 14 days and 21 days incubation. The platelet gels were removed from 37 °C storage, centrifuged at 2000 × g for 15 min at RT in order to separate the gel and the PBS supernatant rich in growth factors, which was immediately stored at −80 °C. At the final 21-day collection point, the PG-Silk samples were solubilized in 500 μL urea 8 M for 30 min at 37 °C, as described above. The collected growth factor solutions were quantified through enzyme-linked immunosorbent assay (ELISA) kits for platelet-derived growth factor-AB (PDGF-AB), transforming growth factor-beta1 (TGF-β1) and vascular endothelial growth factor (VEGF) according to manufacturer's instructions.

The theoretical net charges at neutral pH of the growth factors analyzed, including VEGF, PDGF-AB and TGF-β1, were calculated from the primary sequences, obtained from the UniProt Knowledgebase (http://uniprot.org), by using the Innovagen Peptide property calculator software (http://pepcalc.com).

2.5. Rheometer testing

In order to monitor the gelation kinetics and capture the rheological shear stiffness of the various PG-Silk formulations, several designated samples of silk solution diluted in water, or PPP, or PG (ratio 1:1) were prepared for rheology analysis and compared to platelet gel alone. Dynamic oscillatory time sweeps were performed using an ARES strain-controlled rheometer (TA Instruments, New Castle, DE) with 25-mm-diameter stainless steel cone-and-plate geometry (1° cone angle) at 0.051-mm measuring gap distance. In a typical experiment, the silk solution was applied slowly via pipette on the rheometer plate to prevent shearing of the sample immediately after sonication. The normal force applied on the sample during lowering of the top plate was limited to 20 g. A low viscosity mineral oil was used to prevent sample evaporation from the sides of the plate, as previously described [22]. An air-driven oven was used to control temperature at 37 °C for the duration of the test. Dynamic oscillatory time sweeps were collected at a low strain amplitude ($\gamma = 1\%$, $\omega = 3.14$ rad/s) to prevent possible sample damage due to applied shear during measurements. This testing protocol was applied continuously over 1 h for PG alone, which decayed quickly after reaching its maximum, and 16 h for all silk containing groups, which asymptotically approached a maximum threshold over that time span.

2.6. Compression testing

For unconfined compression testing, silk solutions were generated from cocoons boiled from 30- to 60-min and all solutions with concentration ranging from 4 w/v% to 12 w/v%. Where possible, sterilization via filtration or autoclave cycle was performed in advance of sonication. For all groups, a larger volume of sonicated silk (2 mL–3 mL) was mixed with either PPP or PG in a 1:1 ratio and cast into 30 cm petri dishes [20,23]. Plugs were prepared after gelation using a 6 mm inner diameter biopsy punch. The gel plugs were left in PBS prior to testing. A strain-to-failure test was used to extract an elastic modulus. A minimum of $N = 4$ samples were evaluated and were tested on a 3366 Instron machine (Norwood, MA) equipped with unconfined compression platens and a 100 Newton (N) load transducer and sample data exported using Bluehill Software Version 2.0. Each sample was compressed at a strain rate of 0.1%/s, beginning after nominal tare loads (0.005 N) were reached and sample heights recorded. The compressive stress and strain were determined by normalizing against sample geometries and the elastic modulus was calculated as the best fit linear regression established at a 5% strain range of each stress/strain curve [20,23].

2.7. Cell proliferation assay

HUVECs were seeded in 96 well plates at a density of 5000 cells/well, supplemented with control medium. One day later, media was fully replaced with complete medium in positive control groups, control medium containing 25% (v/v) PG-Silk supernatant in experimental groups, or control medium alone in negative control groups. During this step the cells that did not initially attached to the plates were removed. After 3 days, the MTS assay was performed by following the manufacturer's instructions. The absorbance was measured at 490 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). In order to evaluate molecular pathway activation, cells were prepared as described above and, one day after seeding,