

Chitosan inhibits platelet-mediated clot retraction, increases platelet-derived growth factor release, and increases residence time and bioactivity of platelet-rich plasma *in vivo*

Gabrielle Deprés-Tremblay ¹, Anik Chevrier ², Nicolas Tran-Khanh ², Monica Nelea ² and Michael D Buschmann ^{1,2}

¹ Biomedical Engineering Institute and ² Chemical Engineering Department, Polytechnique Montreal, Montreal, QC, Canada

Running title: Chitosan-PRP implants for tissue repair

Corresponding author: Prof Michael D. Buschmann, Department of Chemical Engineering, Polytechnique Montreal, PO Box 6079 Succ Centre-Ville, Montreal, Quebec, Canada, H3C 3A7, Fax: 514 340 2980 Tel: 514 340 4711 ext. 4931, E-mail: michael.buschmann@polymtl.ca

Abstract

Platelet-rich plasma (PRP) has been used to treat different orthopaedic conditions, however, the clinical benefits of using PRP remain uncertain. Chitosan (CS)-PRP implants have been shown to improve meniscus, rotator cuff and cartilage repair in pre-clinical models. The purpose of this current study was to investigate *in vitro* and *in vivo* mechanisms of action of CS-PRP implants. Freeze-dried formulations containing 1% (w/v) chitosan (80% degree of deacetylation and number average molar mass 38 kDa), 1% (w/v) trehalose as lyoprotectant and 42.2 mM calcium chloride as clot activator were solubilized in PRP. Gravimetric measurements and molecular/cellular imaging studies revealed that clot retraction is inhibited in CS-PRP hybrid clots through physical coating of platelets, blood cells and fibrin strands by chitosan, which interferes with platelet aggregation and platelet-mediated clot retraction. Flow cytometry and ELISA assays revealed that platelets are activated and granules secreted in CS-PRP hybrid clots and that cumulative release of PDGF-AB and EGF is higher from CS-PRP hybrid clots compared to PRP clots *in vitro*. Finally, CS-PRP implants resided for up to 6 weeks in a subcutaneous implantation model and induced cell recruitment and granulation tissue synthesis, confirming greater residency and bioactivity compared to PRP *in vivo*.

Keywords

Chitosan, platelet-rich plasma, clot retraction, platelet activation, implant residency

1. Introduction

Platelets are blood cell components that have recently been implicated in regulation of immune responses, cancer metastasis, vascular development, and angiogenesis, but are primarily responsible for haemostasis in the wound response, while simultaneously playing an essential role in healing by initiating specific cell responses through the release of several growth factors (1). Platelet-derived growth factors have received attention for tissue engineering and regeneration purposes in orthopedics and in other regenerative medicine fields. Unfortunately, growth factors are costly to produce and often inefficient in delivery to specific tissues (2). A more direct approach to produce and deliver growth factors is through injection of platelet-rich plasma (PRP). PRP is an autologous blood-derived product that has an increased concentration of platelets compared to physiological levels. Once the platelets in PRP are activated they release their alpha-granules containing multiple growth factors, among them PDGF, TGF- β , VEGF, EGF and IGF-1. PRP has been used clinically to treat different orthopaedic conditions (3), since it is believed to enhance not only cell proliferation, but also extracellular matrix deposition, remodeling, angiogenesis, and collagen synthesis. However, delivery of PRP to specific sites is problematic since its physical stability is low resulting in rapid dispersion and low residence time (4). In addition, growth factors have a very short half-life and are released rapidly from PRP. Currently, the clinical benefits of using PRP to improve tissue repair and regeneration remain uncertain (3).

Chitosan, a polysaccharide obtained by chitin deacetylation, has been used in several tissue engineering and regenerative medicine applications (5). Chitosan is known to promote wound healing by enhancing migration of inflammatory cells, cell proliferation and matrix formation. This polysaccharide is non toxic, biocompatible and biodegradable, making it suitable for pre-clinical and clinical use. We have worked extensively with chitosan for several years, beginning with the initial discovery that chitosan can be mixed with glycerol phosphate (GP) and still remain soluble in near-neutral conditions of pH and osmolality (6). Chitosan-GP solutions were mixed with whole blood to form voluminous stable clots that are applied to cartilage defects in order to improve repair induced

by marrow stimulation procedures such as microfracture (7-10). We subsequently developed a method to produce lyophilized formulations of chitosan (CS), trehalose (as lyoprotectant) and calcium chloride (as clot activator) that are soluble in PRP and form injectable CS-PRP implants that coagulate rapidly *in situ* (4). We identified some formulation properties that control implant performance and showed that CS-PRP implants have the potential to improve meniscus, rotator cuff and cartilage repair (11-13). Combinations of chitosan and PRP have been used in other pre-clinical injury models as well, however, studies are scarce and report varying levels of success. Chitosan films (DDA 85% and 400 kDa) were used in conjunction with PRP in a rat excision model, and found to improve wound healing when compared to sham control, PRP or chitosan film alone (14). In a rabbit cranial defect model, an 86% DDA chitosan sponge used alone or in combination with PRP failed to improve repair compared to recalcified PRP alone (15). A composite of chitosan (DDA 94% and 680 kDa) and tricalcium phosphate (TP) was mixed with PRP and injected into osseous defects in the goat, where it improved repair compared to chitosan-TP by itself or untreated controls (16).

The purpose of the current study was to (1) investigate possible mechanisms by which chitosan inhibits retraction of CS-PRP hybrid clots *in vitro*, (2) characterize the effect of chitosan, trehalose and a combination of both on platelet activation and granule secretion *in vitro*, (3) characterize the release profile of PDGF-AB and EGF from CS-PRP hybrid clots *in vitro*, and (4) histologically assess the residency, bioactivity and biodegradability of CS-PRP implants *in vivo*. Our starting hypotheses were that (1) chitosan would bind to platelets in a non-specific fashion to inhibit platelet aggregation in hybrid clots and platelet-mediated clot retraction; (2) chitosan would activate platelets and induce granule secretion; (3) the release of growth factors with low isoelectric point (negatively charged at neutral pH), such as EGF, would be more sustained from CS-PRP hybrids than the release of growth factors with high isoelectric points (positively charged at neutral pH), such as PDGF-AB, due to electrostatic interactions with cationic chitosan; (4) CS-PRP implants would reside longer than PRP *in vivo*, where they would induce cell recruitment and angiogenesis, but be degraded within 6 weeks.

2. Materials and methods

2.1 Preparation of freeze-dried chitosan formulations

Raw chitosans were purchased from Marinard, processed in-house and characterized by NMR spectroscopy (17) for degree of deacetylation (DDA) and size-exclusion chromatography/multi-angle laser light scattering (18) for number average molar mass (M_n). Chitosan (80% DDA; M_n 38 kDa) was dissolved in deionized water and 28 mM hydrochloric acid (Sigma-Aldrich) for 16 hours on a rotator at room temperature. Filter-sterilized CaCl_2 (270 mM) and trehalose (15% w/v) solutions (both from Sigma-Aldrich) were then added to reach final concentrations of 1% (w/v) chitosan, 42.2 mM CaCl_2 and 1% (w/v) trehalose prior to filtration through 0.2 μm filters (Millipore). The solution was dispensed in 1-mL aliquots in 3-mL glass vials for freeze-drying with the following cycle: 1) Ramped freezing to -40°C in 1 hour then isothermal 2 hours at -40°C , 2) -40°C for 48 hours at 100 millitorrs and 3) Ramped heating to 30°C in 12 hours then isothermal 6 hours at 30°C , at 100 millitorrs. 10 μL Rhodamine-chitosan tracer (19) of corresponding DDA and M_n was added to the vials that were subsequently used for fluorescent microscopy.

2.2 Preparation of platelet-rich plasma (PRP)

The Polytechnique Montreal institutional ethics committee approved the project and all subjects enrolled in this research (n=3 males and n=3 females, with some donors sampled more than once) responded positively to an Informed Consent Form. For each donor, blood was extracted and anti-coagulated with 12.9 mM sodium citrate. The blood was then centrifuged using an ACE E-Z PRPTM centrifuge at 160 g for 10 minutes at room temperature. The supernatant and first 1-2 mm of erythrocyte sediment was removed and then centrifuged again at 400 g for 10 minutes at room temperature. Only the bottom ~1.5 mL of each tube was retained and resuspended to make PRP. This isolation method yields a leukocyte-rich PRP (L-PRP) which typically contains ~3X more platelets than blood along with a leukocyte fraction (~0.8X that of blood) and a small erythrocyte fraction (~0.2X that of blood). Alexa-647 fibrinogen (Invitrogen) was prepared as a stock solution at 1.5 mg/mL in 0.1 M sodium bicarbonate (pH

8.3), and added to the PRP that was subsequently used for fluorescent microscopy (0.5mL of Alexa-647 fibrinogen added to 4.5mL of PRP).

2.3 Solubilization of freeze-dried chitosan formulations in platelet-rich plasma (PRP)

Each freeze-dried cake (1mL freeze-dried formulation in each vial) was solubilized with 1mL of PRP and mixed vigorously for 10 seconds. The solubilized formulations were dispensed either into glass tubes to assess clot retraction and for imaging or into 48-well culture plates for characterization of release profiles.

2.4 Assessment of clot retraction

Chitosan-PRP formulations (~250 μ L) were dispensed in glass tubes placed on a heat block at 37°C and allowed to clot for 1 hour. Serum was removed and % clot mass lost was quantified by gravimetric measurements. Clots were fixed with 0.5% (v/v) glutaraldehyde (EMS)/0.3% (w/v) paraformaldehyde (Sigma-Aldrich)/0.3% (v/v) Triton X-100 (Sigma-Aldrich). Controls were PRP recalcified with 42.2 mM CaCl_2 . Duplicate clots were prepared for each donor, except for one donor, where one clot was prepared.

2.5 Confocal fluorescent and spinning disk microscopy imaging

Fixed clots were sectioned with a razor blade at ~ 1mm thickness and mounted with Mowiol 4-88 (Fluka)/glycerol (Sigma-Aldrich)/n-propyl gallate (Sigma-Aldrich) mounting medium (prepared in-house) on MatTek glass bottom dishes (Cedarlane). High resolution 2D and 3D images were captured with an Olympus FV1000 spectral confocal laser scanning microscope (Olympus Canada), using a PLAPON Apochromat oil objective (60X, NA 1.42). The excitation/emission wavelengths were 635/644-755 nm for the fibrin network (Alexa-647 fibrinogen tracer), and 543/555-625 nm for the chitosan (rhodamine tracer). Erythrocytes were also imaged for some samples (autofluorescence using 488/500-540 nm). The acquisition parameters were adjusted to avoid signal saturation. 3D images were reconstructed with Imaris software (Bitplane). The confocal microscope is also adapted for a Yokogawa

spinning disk module (Quorum), controlled with the MetaMorph software (Molecular Devices). Given the high dynamic range of the EM-CCD digital camera (Hamamatsu), the spinning disk module was used to capture images with fixed acquisition settings for all the samples without reaching any signal saturation. Only the fibrin network was imaged with this module, using a UPLSAPO Super Apochromat objective (40X, NA 0.95) and excitation/emission wavelengths of 642/662-738 nm.

2.6 Scanning electron microscopy (SEM) imaging

Fixed clots were embedded in paraffin (Fisher), sectioned at 3 μm thickness with a Leica RM2155 microtome and collected on SuperFrost Plus glass slides (Fisher). The sections were then deparaffinized, post-fixed in 2% (v/v) glutaraldehyde (EMS)/0.1 M sodium cacodylate (Sigma) pH 7.2 and washed in water. The post-fixed sections were removed from slides using a super fine point tweezers and placed onto a conductive carbon adhesive tape (EMS). The sections were immobilized by blowing compressed air and then gold sputter coated for 25 seconds using an Agar manual gold sputter-coater (Marivac Inc). SEM images were acquired with a Quanta FEG 200 ESEM (FEI Company) in high vacuum mode with working distance 5.6 to 5.7 mm and accelerating voltage 20kV.

2.7 Transmission electron microscopy (TEM) imaging

Fixed clots were post-fixed in 1% (v/v) osmium tetroxide (Sigma-Aldrich), washed in deionized water, incubated with 2% (v/v) uranyl acetate (EMS) for 1 hour, washed, dehydrated in a graded ethanol series, cleared in xylene and embedded in Embed-812 (EMS) medium at 60°C. 100 nm sections were collected using a diamond blade and an RMC MT-7 ultramicrotome and mounted on copper grids. The images were acquired with a JEM 2000FXII transmission electron microscope (JEOL; Tokyo, Japan) operated at 80 kV.

2.8 Preparation of cell suspension and flow cytometry

Whole blood was anticoagulated with 10.9 mM sodium citrate, centrifuged at 190 g for 15 minutes and the supernatant collected. Supernatant was centrifuged at 2,500 g for 5 minutes to pellet the cells. Cell pellet was resuspended in HEPES/Tyrode's buffer (10 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 5.5 mM glucose) with 1.35% (w/v) BSA (all from Sigma-Aldrich) and left to incubate for 1 hour. The cell suspension was then mixed with, either ADP (Sigma, final concentration 20 μM), solubilized chitosan 80% DDA M_n 38 kDa (final concentration 1% w/v), trehalose (final concentration 1% w/v) or 1% (w/v) chitosan and (1% w/v) trehalose simultaneously. Fluorescein isothiocyanate (FITCs) labeled anti-Pac-1 and anti-CD62P antibodies (Biolegend), which recognize activated platelet GPIIb/IIIa complex and granule membrane protein P-selectin, respectively, were added to the tubes as per manufacturer's instructions and the tubes were incubated at room temperature for 20 minutes. The cells were fixed with 1% (w/v) paraformaldehyde/10 mM HEPES/0.15 mM NaCl pH 7.4 for 15 minutes at room temperature. The samples were then kept on ice until analysis. The fluorescence intensity of 10, 000 events per sample were analyzed using a flow Moflo cytometer (Beckman Coulter Life Sciences).

2.9 Quantification of growth factors released from clots

Freeze-dried chitosan cakes were solubilized in PRP as described above and 250 μL of the solubilized mixture was dispensed into each well of a 48-well plate culture plate and allowed to clot for 1 hour in air atmosphere at 37°C with 5% CO₂. Wells were washed with 500 μL α-MEM cell culture medium (Invitrogen), the culture medium was immediately removed and replaced with fresh α-MEM cell culture medium. The cell culture medium were then removed and replenished at day 1, 3, 5 and 7. Culture medium was centrifuged at 400 g for 10 minutes and frozen at -80°C prior to ELISA quantification using platelet-derived growth factor (PDGF-AB) or epidermal growth factor (EGF) Quantikine kits (Product N° DHD00C and DEG00 from RandD systems). Controls were PRP recalcified with 42.2 mM CaCl₂. Duplicate clots were prepared for each donor, except for one donor, where one clot was prepared.

2.10 *Subcutaneous implantation*

The protocol for this study was approved by the University of Montreal ethics committee and was consistent with the Canadian Council on Animal Care guidelines for the care and use of laboratory animals. Five New Zealand White (NZW) male rabbits (> 2.5 kg) were used for the study. Rabbits were anesthetized with ketamine/xylazine cocktail. From each animal, 18mL blood was collected from the ear artery and anticoagulated with sodium citrate (final citrate concentration 12.9 mM). Blood was centrifuged in an ACE EZ-PRP™ centrifuge for 10 min at 160 g and then for 10 min at 400 g to extract PRP, as described above. The back of the rabbit was shaved and the skin disinfected with 3 passages of Baxedin, then with 3 alternating passages of proviodine and isopropanol 70%. Freeze-dried chitosan formulations (300 µL) containing 1% (w/v) chitosan (80%-85% DDA and M_n 36-43 kDa), 1% (w/v) trehalose and 42.2 mM CaCl₂ were solubilised with 300 µL of PRP and shaken vigorously for 10 seconds. A 1-cc syringe equipped with a 26 gauge needle was used to deliver 150 µL of each implant under the skin of the back of the rabbits (n=4 CS-PRP implants injected in each rabbit). Controls consisted of 150 µL PRP recalcified with 42.2 mM CaCl₂ prior to injection (n=2 control PRP implants injected in each rabbit). Animals were sacrificed at 2 weeks (n=2), 4 weeks (n=2) or 6 weeks (n=1) post-implantation. At the time of sacrifice, rabbits were anesthetized with ketamine/xylazine cocktail and euthanized with an overdose of sodium pentobarbital. The skin with attached implants was carefully removed and fixed in 10% neutral buffered formalin (NBF) (Fisher) for 3 days. Each implant was excised using razor blades and a horizontal band was collected from the central area for paraffin embedding. Paraffin sections (5 µm) were collected and stained with Fast Green/Iron Hematoxylin (both from Sigma-Aldrich). The stained sections were then scanned for histological evaluation using a Nanozoomer RS system and images exported using NDPView software (both from Hamamatsu).

2.11 *Statistical analysis*

All statistical analyses were performed with SAS Enterprise Guide 7.1 and SAS 9.4. Data in the text are presented as mean \pm SD. Data in the Figures are presented as median (line); Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. The Mixed model task in SAS Enterprise Guide was used to compare the different groups with post-hoc analysis to look at pairwise differences. Fixed effects were type of sample (CS-PRP vs PRP control) and time, while donor was a random effect, as some donors were sampled more than once.

3. Results

3.1 *Clot retraction and platelet aggregation are inhibited in chitosan-PRP hybrid clots*

Gravimetric measurements showed that PRP clots lost $78\pm 4\%$ of their initial mass after clotting for 1 hour at 37°C (**Fig 1a**). Although there was some variability between donors, % mass loss was significantly less for chitosan-PRP hybrid clots at average $21\pm 18\%$ (**Fig 1a**). Spinning disk microscopy and confocal microscopy images of clots fixed after clotting for 1 hour showed that platelet aggregates were smaller in chitosan-PRP hybrid clots (**Fig 1b, d & f**) compared to PRP clots (**Fig 1c, e & g**).

3.2 *Chitosan coats clot components in chitosan-PRP hybrid clots*

Scanning electron microscopy (SEM) images demonstrated that blood components were coated with chitosan in chitosan-PRP hybrid clots (**Fig 2a & b**). Both cells and fibers were covered by a coating of what is presumed to be chitosan (**Fig 2a & b**). In contrast, the fibrin network was readily visible in PRP clots (**Fig 2c & d**). In transmission electron (TEM) images of chitosan-PRP hybrid clots, chitosan was observed in the space between cellular elements, and also at the surface of fibrin strands, erythrocytes and platelets (**Fig 2e & f**). Erythrocytes were closely packed and platelet aggregates were large in PRP clots (**Fig 2g & h**).

3.3 *Chitosan induces platelet activation and granule secretion in cell suspension*

As expected, incubation with ADP (in green), a known platelet agonist, caused increased Pac-1 and p-selectin expression in a cell suspension (unactivated cells in red) (**Fig 3**). Incubation with 1% (w/v) chitosan (in dark blue) also led to platelet activation (**Fig 3a**) and granule secretion (**Fig 3b**). In contrast, incubation with 1% (w/v) trehalose alone (in yellow) did not induce platelet activation and granule secretion (**Fig 3**). Finally, incubating the cell suspension with 1% (w/v) chitosan simultaneously with 1% (w/v) trehalose (in pale blue) induced platelet activation and granule secretion, albeit less than incubation with 1% (w/v) chitosan by itself (**Fig 3**).

3.4 Chitosan-PRP hybrids provide a greater and sustained release of PDGF-AB and EGF over time

PDGF-AB release into culture medium was ~100 times greater than that of EGF for both chitosan-PRP hybrid clots and PRP clots (**Fig 4**). For both growth factors, a phase of fast release was observed in the first 24 hours, followed by a more controlled release from day 1 to day 7 (**Fig 4**). In addition, for both growth factors, release was still ongoing at day 7 and no plateau had been reached (**Fig 4**). Both time ($p < 0.0001$ and $p < 0.0001$) and sample type ($p < 0.0001$ and $p = 0.0003$) had an effect on the release of PDGF-AB and EGF, respectively (**Fig 4**). The cumulative release of PDGF-AB from chitosan-PRP hybrid clots was significantly greater than the cumulative release from PRP clots from day 1 to day 7 (**Fig 4a**). Similarly, the cumulative release of EGF was significantly greater from chitosan-PRP hybrid clots than from PRP clots from day 3 to day 7 (**Fig 4b**).

3.5 Chitosan-PRP hybrids reside for at least 6 weeks in vivo and induce cell recruitment

No PRP implants could be recovered for histology at any time point tested. In contrast, chitosan-PRP implants were resident for up to 6 weeks post-implantation (**Fig 5**). Host-derived cells were recruited to the implants and were observed invading the implants from 2 weeks on, along with granulation tissue synthesis and new blood vessel formation (**Fig 5**). None of the chitosan implants induced deleterious effects in rabbits. No sign of infection or rejection were noted macroscopically or histologically. Blood work was normal and rabbits did not develop anorexia post-surgery

4. Discussion

One objective of this study was to investigate the mechanisms by which chitosan inhibits platelet-mediated clot retraction and liquid expression in CS-PRP hybrids, as shown in **Figure 1a**. Under static or low shear stress conditions, which is the case here in our *in vitro* assay, platelet aggregation is mainly mediated by the interactions of GPIIb/IIIa on platelet surface with fibrinogen (20). Stimulation of platelets with agonists induces cytoskeletal rearrangement, shape change, protein synthesis, granule secretion and increases the affinity of the GPIIb-IIIa platelet receptor for fibrinogen (21). Platelet aggregation results from binding of multiple platelets to the same fibrinogen molecule (1). Clot retraction, mediated by the platelet actin and myosin contractile system then follows, as long as platelet stimulation, comprising shape change and primary aggregation, are maintained (22, 23). In the absence or non-functioning GPIIb/IIIa, clot retraction does not occur. Confocal, SEM and TEM images (**Figures 1&2**) support our first hypothesis that chitosan physically coats platelets and other components of the blood clot to inhibit platelet aggregation, which is needed for clot retraction. In CS-PRP hybrids, chitosan physically interferes with the ability of the platelets to adhere to each other and the fibrin network and exert mechanical forces.

Our second and third aims were to investigate whether platelets are activated in CS-PRP hybrid clots and, if so, how platelet-derived growth factors are released from CS-PRP hybrid clots. Chitosan (DDA > 90% and 50 kDa) was previously shown to be a platelet agonist and stimulate platelet activation and GPIIb/IIIa expression *in vitro* (24). In another study, stimulation of platelet suspensions with chitosan (DDA 84%) induced p-selectin and GPIIb/IIIa expression, in a process that was shown to be modulated by plasma and extracellular matrix proteins (25). Consistent with these previously published data and our third hypothesis, we found that chitosan induces platelet activation and granule secretion in cell suspensions (**Figure 3**), even more so than ADP (20 μ m), a known platelet agonist. Interestingly, incubation of cell suspension with trehalose along with chitosan slightly decreased expression of Pac-1

and p-selectin compared to incubation with chitosan alone. This is consistent with published reports that lyoprotectants impede haemostatic mechanisms (26, 27).

Even though test conditions in the flow cytometry assay are different than in the hybrid clot system, we expected platelets within the CS-PRP hybrid clots to be activated and release their granule content, and this was ascertained by ELISA assays. In the case of physical adsorption of growth factors to chitosan, release is believed to be controlled by the electrostatic interactions that exist between the growth factors and the chitosan (28). Therefore, our third starting hypothesis was that the isoelectric point of platelet-derived growth factors would determine how growth factors would be released from our CS-PRP hybrids. The isoelectric point of PDGF is 9.8 (29) and, under physiological conditions, we expected ionic repulsion between positively charged PDGF-AB and cationic chitosan to result in burst release. Meanwhile, EGF, which has an isoelectric point of 4.6 (30) would be expected to bind to chitosan under physiological conditions and be released in a more sustained manner. In contrast to this, we found that CS-PRP hybrid clots sustained and increased release of both PDGF-AB and EGF for 1 week *in vitro*, which suggests that additional factors are controlling their release in this system (**Figure 4**).

We found that the cumulative levels of PDGF-AB and EGF released in the culture medium were higher in the case of CS-PRP clots compared to PRP clots (**Figure 4**). These results were not completely unexpected. Kutlu et al (31) previously prepared CS-PRP scaffolds by either adding PRP to a chitosan gel before freeze-drying or by delivering PRP to a lyophilized chitosan sponge. Sustained release of PDGF-BB was achieved in the first group, similarly to what we observed here, while a sharp burst release was observed in the second group. Interestingly, both of their CS-PRP scaffolds secreted higher cumulative levels of PDGF-BB when compared to unactivated PRP or PRP activated with type I collagen, similarly to what was found here for PDGF-AB. Hattori et al (32) showed that platelets in whole blood are activated when mixed with solutions of chitosan with different DDA and M_w . Of particular relevance to our study, the amount of PDGF-AB released was the highest when chitosans of DDA 75-85% and M_w 50-190 kDa were used in conjunction with calcium chloride than when calcium chloride was used by itself.

Shen et al (33) showed that stimulation with chitosan of DDA > 90% and 450 kDa induced release of PDGF-AB and EGF from PRP for up to 60 minutes. Shimojo et al (34) prepared lyophilized scaffolds containing different concentrations of chitosan (DDA 83% and M_w 400 kDa), loaded the scaffolds with PRP activated with autologous serum and calcium chloride and showed that PDGF-AB cumulative release was higher from the scaffolds compared to activated PRP alone, provided that the scaffolds be lyophilized at low temperatures. In a subsequent study, Shimojo et al (35) showed that stabilizing the chitosan scaffolds by treating them with NaOH prior to loading them with PRP was another way to increase cumulative PDGF-AB release from scaffolds lyophilized at -20°C .

With regard to growth factor release, it is important to consider the contribution of each cell type present in the PRP preparation. Platelets are the main contributors to growth factor release from PRP and positive correlations were previously found between platelet doses and the amount of released growth factors including PDGF-AB, TGF- β 1, VEGF and EGF (36, 37). While it appears that the inclusion of leukocytes in PRP increases the content of some pro-inflammatory cytokines (38-41), the effect of leukocytes on growth factor content and release is still not fully understood. Previous studies found that leukocyte-rich PRP contained higher concentrations of growth factors compared to leukocyte-poor PRP, but that may be due to the fact that systems that include the buffy coat layer are usually more efficient at capturing platelets (39, 42-46). However, positive correlations and close associations were also found between PRP leukocyte counts and levels of PDGF-AB, VEGF and EGF (36, 37), which suggests that leukocytes contribute to the release of growth factors from PRP. Here, we found increased cumulative PDGF-AB and EGF release from CS-PRP clots compared to PRP clots (**Figure 4**). One possible reason for this is that chitosan used in conjunction with calcium chloride stimulates platelet activation and granule secretion more than calcium chloride by itself. Another possibility would be that leukocytes, especially monocytes, present in CS-PRP hybrids are secreting higher amounts of growth factors than in PRP without chitosan. While it has been reported that M0 and polarized M2a macrophages secrete PDGF-BB (47, 48), and that biodegradable chitosan particles (DDA 81.5% and M_n 132 kDa) enhance release of

PDGF-BB from M0 and M2a macrophages (49), we believe that the main contributors to growth factor release in the CS-PRP hybrid system are platelets and not monocytes, for the following reasons: 1) Monocytes typically require specific stimulatory signals to become macrophages, 2) There is a low number of monocytes present in each CS-PRP clot, compared to the number of platelets (on average ~ 2500X more platelets than monocytes); 3) In light of previous reports on the amount of growth factors released by M0 and M2a macrophages, it seems unlikely that such a limited number of monocytes could secrete the amount of PDGF-AB and EGF that was measured here in the culture medium.

Our fourth aim was to investigate the implants *in vivo*, and, as previously shown (4), CS-PRP hybrids exhibited longer residency and higher bioactivity than PRP (**Figure 5**). In a subcutaneous implantation model in the mouse, porous chitosan scaffolds were found to elicit neutrophil migration into the implantation area along with angiogenic activity, as was shown here as well (50). It is of interest to mention that the site of implantation along with implant volume influences biodegradability. We have previously shown that CS-PRP implants are degraded within 3 weeks in meniscus tears in the sheep (13), and between 2-8 weeks in rotator cuff tears (11) and cartilage lesions in the rabbit (12) compared to more than 6 weeks in the current study. One limitation of this study is the low number of animals used for the *in vivo* subcutaneous implants study. Another limitation is the limited number of growth factors studied for the release profiles. We chose both EGF and PDGF-AB, since they are frequently assessed in the literature, and they have widely different isoelectric points. Nevertheless, future studies should involve a greater number of platelet-derived growth factors. It is also important to mention that *in vitro* release profiles cannot be extrapolated to the *in vivo* situation; however, these data improve our understanding of the CS-PRP technology.

5. Summary

Chitosan physically coats platelets, blood cells and fibrin strands in CS-PRP hybrid clots, thus inhibiting platelet aggregation, which is required for clot retraction. Platelets are activated, granules secreted and higher levels of PDGF-AB and EGF are released from CS-PRP hybrid clots compared to PRP clots *in vitro*. Finally, CS-PRP implants reside for at least 6 weeks post-implantation subcutaneously and induce cell recruitment and granulation tissue synthesis, confirming a longer residency and higher bioactivity compared to PRP *in vivo*.

Acknowledgments

We acknowledge the technical contributions of Geneviève Picard and the funding sources (Canadian Institutes of Health Research, Canada Foundation for Innovation, Groupe de Recherche en Sciences et Technologies Biomédicales, Natural Sciences and Engineering Research Council of Canada, Ortho Regenerative Technologies Inc).

Competing interests

AC and MDB hold shares and MDB is a Director of Ortho Regenerative Technologies Inc.

References

1. Jurk K, Kehrel BE. Platelets: physiology and biochemistry. *Seminars in thrombosis and hemostasis*. 2005;31(4):381-92.
2. Lee K, Silva EA, Mooney DJ. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. *J R Soc Interface*. 2011;8(55):153-70.
3. Sheth U, Simunovic N, Klein G, Fu F, Einhorn TA, Schemitsch E, et al. Efficacy of autologous platelet-rich plasma use for orthopaedic indications: a meta-analysis. *The Journal of bone and joint surgery American volume*. 2012;94(4):298-307.

4. Chevrier A, Darras V, Picard G, Nelea M, Veilleux D, Lavertu M, et al. Injectable chitosan-platelet-rich plasma (PRP) implants to promote tissue regeneration: In vitro properties, in vivo residence, degradation, cell recruitment and vascularization. *Journal of tissue engineering and regenerative medicine*. 2017. In press.
5. Muzzarelli RAA. Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. *Carbohydrate Polymers*. 2009;76(2):167-82.
6. Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, et al. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. *Biomaterials*. 2000;21(21):2155-61.
7. Hoemann CD, Hurtig M, Rossomacha E, Sun J, Chevrier A, Shive MS, et al. Chitosan-glycerol phosphate/blood implants improve hyaline cartilage repair in ovine microfracture defects. *Journal of Bone and Joint Surgery-American Volume*. 2005;87A(12):2671-86.
8. Stanish WD, McCormack RG, Forriol F, Mohtadi N, Pelet S, Desnoyers J, et al. Novel Scaffold-Based BST-CarGel Treatment Results in Superior Cartilage Repair Compared with Microfracture in a Randomized Controlled Trial. *Journal of Bone and Joint Surgery-American Volume*. 2013;95A(18):1640-50.
9. Shive MS, Stanish WD, McCormack RG, Forriol F, Mohtadi N, Pelet S, et al. BST-CarGel® Treatment Maintains Cartilage Repair Superiority over Microfracture at 5 Years in a Multicenter Randomized Controlled Trial. *Cartilage*. 2015 6(2):62-72.
10. Méthot S, Changoor A, Tran-Khanh N, Hoemann CD, Stanish WD, Restrepo A, et al. Osteochondral Biopsy Analysis Demonstrates That BST-CarGel Treatment Improves Structural and Cellular Characteristics of Cartilage Repair Tissue Compared With Microfracture. *Cartilage*. 2015.

11. Deprés-Tremblay G, Chevrier A, Buschmann MD, editors. Freeze-dried chitosan-PRP in a rabbit model of rotator cuff repair. Transactions Orthopaedic Research Society; 2017 San Diego, CA, USA.
12. Dwivedi G, Chevrier A, Hoemann CD, Buschmann MD. Freeze dried chitosan/platelet-rich-plasma implants improve marrow stimulated cartilage repair in rabbit chronic defect model. Transactions Orthopaedic Research Society; 2017 San Diego, CA, USA.
13. Ghazi zadeh L, Chevrier A, Hurtig MB, Farr J, Rodeo S, Hoemann CD, et al., editors. Freeze-dried chitosan-PRP injectable surgical implants for meniscus repair: results from pilot ovine studies. Transactions Orthopaedic Research Society; 2017 San Diego, CA, USA.
14. Mohammadi R, Mehrtash M, Hassani N, Hassanpour A. Effect of Platelet Rich Plasma Combined with Chitosan Biodegradable Film on Full-Thickness Wound Healing in Rat Model. Bulletin of emergency and trauma. 2016;4(1):29-37.
15. Oktay EO, Demiralp B, Senel S, Cevdet Akman A, Eratalay K, Akincibay H. Effects of platelet-rich plasma and chitosan combination on bone regeneration in experimental rabbit cranial defects. The Journal of oral implantology. 2010;36(3):175-84.
16. Bi L, Cheng W, Fan H, Pei G. Reconstruction of goat tibial defects using an injectable tricalcium phosphate/chitosan in combination with autologous platelet-rich plasma. Biomaterials. 2010;31(12):3201-11.
17. Lavertu M, Xia Z, Serreqi AN, Berrada M, Rodrigues A, Wang D, et al. A validated ¹H NMR method for the determination of the degree of deacetylation of chitosan. J Pharm Biomed Anal. 2003;32(6):1149-58.
18. Nguyen S, Winnik FM, Buschmann MD. Improved reproducibility in the determination of the molecular weight of chitosan by analytical size exclusion chromatography. Carbohydrate Polymers. 2009;75(3):528-33.

19. Ma O, Lavertu M, Sun J, Nguyen S, Buschmann MD, Winnik FM, et al. Precise derivatization of structurally distinct chitosans with rhodamine B isothiocyanate. *Carbohydrate Polymers*. 2008;72(4):616-24.
20. Jackson SP. The growing complexity of platelet aggregation. *Blood*. 2007;109(12):5087-95.
21. Platelet function : assessment, diagnosis, and treatment Quinn M, Fitzgerald D, editors. Totowa, NJ, USA: Humana Press; 2005.
22. Cohen I, Gerrard JM, White JG. Ultrastructure of clots during isometric contraction. *The Journal of cell biology*. 1982;93(3):775-87.
23. Cohen I. The mechanism of clot retraction. Platelet membrane glycoproteins. New York, NY, USA: Springer US; 1985. p. 299-323.
24. Chou TC, Fu E, Wu CJ, Yeh JH. Chitosan enhances platelet adhesion and aggregation. *Biochemical and biophysical research communications*. 2003;302(3):480-3.
25. Lord MS, Cheng B, McCarthy SJ, Jung M, Whitelock JM. The modulation of platelet adhesion and activation by chitosan through plasma and extracellular matrix proteins. *Biomaterials*. 2011;32(28):6655-62.
26. Bakaltcheva I, Reid T. Effects of blood product storage protectants on blood coagulation. *Transfusion medicine reviews*. 2003;17(4):263-71.
27. Luostarinen T, Niiya T, Schramko A, Rosenberg P, Niemi T. Comparison of Hypertonic Saline and Mannitol on Whole Blood Coagulation In Vitro Assessed by Thromboelastometry. *Neurocritical care*. 2011;14(2):238-43.
28. Yao K, Li J, Yao F, Yin Y. Chitosan-Based Hydrogels: Functions and Applications. Boca Raton, FL, USA: Taylor and Francis Group LLC CRC Press; 2012.

29. Antoniades HN, Scher CD, Stiles CD. Purification of human platelet-derived growth factor. *Proceedings of the National Academy of Sciences of the United States of America*. 1979;76(4):1809-13.
30. Taylor JM, Mitchell WM, Cohen S. Epidermal growth factor. Physical and chemical properties. *The Journal of biological chemistry*. 1972;247(18):5928-34.
31. Kutlu B, Aydin RST, Akman AC, Gumusderelioglu M, Nohutcu RM. Platelet-rich plasma-loaded chitosan scaffolds: Preparation and growth factor release kinetics. *Journal of Biomedical Materials Research Part B-Applied Biomaterials*. 2013;101B(1):28-35.
32. Hattori H, Ishihara M. Feasibility of improving platelet-rich plasma therapy by using chitosan with high platelet activation ability. *Experimental and Therapeutic Medicine*. 2016;DOI: 10.3892/etm.2017.4041.
33. Shen EC, Chou TC, Gau CH, Tu HP, Chen YT, Fu E. Releasing growth factors from activated human platelets after chitosan stimulation: a possible bio-material for platelet-rich plasma preparation. *Clin Oral Impl Res*. 2006;17():572-8.
34. Shimojo AA, Perez AG, Galdames SE, Brissac IC, Santana MH. Performance of PRP associated with porous chitosan as a composite scaffold for regenerative medicine. *TheScientificWorldJournal*. 2015;2015:396131.
35. Shimojo AA, Perez AG, Galdames SE, Brissac IC, Santana MH. Stabilization of porous chitosan improves the performance of its association with platelet-rich plasma as a composite scaffold. *Materials science & engineering C, Materials for biological applications*. 2016;60:538-46.
36. Magalon J, Bausset O, Serratrice N, Giraudou L, Aboudou H, Veran J, et al. Characterization and comparison of 5 platelet-rich plasma preparations in a single-donor model. *Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association*. 2014;30(5):629-38.

37. Weibrich G, Kleis WK, Streckbein P, Moergel M, Hitzler WE, Hafner G. Comparison of point-of-care methods for preparation of platelet concentrate (platelet-rich plasma). *Int J Oral Maxillofac Implants*. 2012;27(4):762-9.
38. Anitua E, Zalduendo MM, Prado R, Alkhraisat MH, Orive G. Morphogen and proinflammatory cytokine release kinetics from PRGF-Endoret fibrin scaffolds: Evaluation of the effect of leukocyte inclusion. *Journal of biomedical materials research Part A*. 2014.
39. Cavallo C, Filardo G, Mariani E, Kon E, Marcacci M, Pereira Ruiz MT, et al. Comparison of platelet-rich plasma formulations for cartilage healing: an in vitro study. *The Journal of bone and joint surgery American volume*. 2014;96(5):423-9.
40. Sundman EA, Cole BJ, Fortier LA. Growth Factor and Catabolic Cytokine Concentrations Are Influenced by the Cellular Composition of Platelet-Rich Plasma. *American Journal of Sports Medicine*. 2011;39(10):2135-40.
41. Pochini AC, Antonioli E, Bucci DZ, Sardinha LR, Andreoli CV, Ferretti M, et al. Analysis of cytokine profile and growth factors in platelet-rich plasma obtained by open systems and commercial columns. *Einstein (Sao Paulo, Brazil)*. 2016;14(3):391-7.
42. Castillo TN, Pouliot MA, Kim HJ, Dragoo JL. Comparison of Growth Factor and Platelet Concentration From Commercial Platelet-Rich Plasma Separation Systems. *American Journal of Sports Medicine*. 2011;39(2):266-71.
43. Leitner GC, Gruber R, Neumuller J, Wagner A, Kloimstein P, Hocker P, et al. Platelet content and growth factor release in platelet-rich plasma: a comparison of four different systems. *Vox Sanguinis*. 2006;91(2):135-9.

44. Masuki H, Okudera T, Watanebe T, Suzuki M, Nishiyama K, Okudera H, et al. Growth factor and pro-inflammatory cytokine contents in platelet-rich plasma (PRP), plasma rich in growth factors (PRGF), advanced platelet-rich fibrin (A-PRF), and concentrated growth factors (CGF). 2016;2(1):19.
45. Fitzpatrick J, Bulsara MK, McCrory PR, Richardson MD, Zheng MH. Analysis of Platelet-Rich Plasma Extraction. Variations in Platelet and Blood Components Between 4 Common Commercial Kits. *The Orthopaedic Journal of Sports Medicine*. 2017;5(1):2325967116675272.
46. Parrish WR, Roides B, Hwang J, Mafilios M, Story B, Bhattacharyya S. Normal platelet function in platelet concentrates requires non-platelet cells: a comparative in vitro evaluation of leucocyte-rich (type 1a) and leucocyte-poor (type 3b) platelet concentrates. *BMJ Open Sport & Exercise Medicine*. 2016;2(1).
47. Spiller KL, Anfang RR, Spiller KJ, Ng J, Nakazawa KR, Daulton JW, et al. The role of macrophage phenotype in vascularization of tissue engineering scaffolds. *Biomaterials*. 2014;35(15):4477-88.
48. Spiller KL, Nassiri S, Witherel CE, Anfang RR, Ng J, Nakazawa KR, et al. Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. *Biomaterials*. 2015;37:194-207.
49. Fong D, Ariganello MB, Girard-Lauziere J, Hoemann CD. Biodegradable chitosan microparticles induce delayed STAT-1 activation and lead to distinct cytokine responses in differentially polarized human macrophages in vitro. *Acta biomaterialia*. 2015;12:183-94.
50. VandeVord PJ, Matthew HWT, DeSilva SP, Mayton L, Wu B, Wooley PH. Evaluation of the biocompatibility of a chitosan scaffold in mice. *Journal of biomedical materials research*. 2002;59(3):585-90.

Figure Legends

Figure 1. After clotting for 1 hour at 37°C, clot retraction and serum expression (expressed as % clot mass lost) was greater from PRP clots compared to CS-PRP hybrid clots (a). Data are presented as mean (diamond) and median (line) of n=7 clots from 3 different donors; Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. The Mixed model task in SAS Enterprise Guide 7.1 and SAS 9.4 was used to compare the different groups using sample (CS-PRP vs PRP) as a fixed effect and donor as a random effect. * p < 0.05 compared to PRP. Platelet aggregates were smaller in CS-PRP hybrid clots (b, d & f) compared to PRP clots (c, e & g), as shown by spinning disk microscopy images (b & c), confocal microscopy images (d & e) and 3-D stacks of confocal microscopy images (f & g). An Alexa-647 fibrinogen tracer was added to allow imaging of fibrin-covered platelets and fibrin in white. A Rhodamine-chitosan tracer was added to allow imaging of chitosan in red.

Figure 2. Chitosan (CS) appeared to be coating cellular and fibrous elements in scanning electron microscopy (SEM) images of CS-PRP clots (a & b) while the fibrin (F) network was readily visible in PRP clots (c & d). Chitosan (CS) was found in the space between cellular elements and also at the surface of erythrocytes (E), platelets aggregates (P) and fibrin (F) in transmission electron microscopy (TEM) images of CS-PRP clots (e & f). Erythrocytes (E) were tightly packed and platelet aggregates (P) were large in TEM images of PRP clots (g & h).

Figure 3. a) Flow cytometric analysis of PAC-1 (a) and p-selectin (b) staining of cell suspension (unactivated in red). As expected, incubation with ADP (20 μm in green) and chitosan (in dark blue), both known platelet agonists, led to platelet activation (a) and granule secretion (b). In contrast, incubation with trehalose alone (in yellow) had no effect on platelet activation (a) or granule secretion (b). Interestingly, incubation with chitosan and trehalose simultaneously (in pale blue) decreased the intensity of both fluorescent signals compared to incubation with chitosan alone (in dark blue).

Figure 4. PDGF-AB (a) and EGF (b) cumulative release in culture medium was greater in the case CS-PRP clots compared to PRP clots. Data are presented as mean (circle) and median (line) of n=13 clots from 6 different donors; Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. The Mixed model task in SAS Enterprise Guide 7.1 and SAS 9.4 was used to compare the different groups with post-hoc analysis to look at pair-wise differences using sample and time as fixed effects and donor as a random effect. * $p < 0.05$ compared to PRP. Insets show total amount of growth factors released.

Figure 5. Formulations containing 1% (w/v) chitosan (CS M_n 38-43kDa and DDA 80-85%) with 1% (w/v) trehalose and 42.2 mM CaCl_2 were solubilized in autologous PRP and injected subcutaneously in rabbits, where they were found to be resident and induce cell recruitment at two weeks (a-c), four weeks (d-f), and six weeks (g-i) post-implantation. Invasion of the CS-PRP implants by host cells was accompanied by granulation tissue (GT) synthesis and formation of new blood vessels (BV). In contrast, recalcified PRP implants were completely degraded within a few days. Outlines in a, d & g show where higher magnification images b, c, e, f, h & i were acquired.

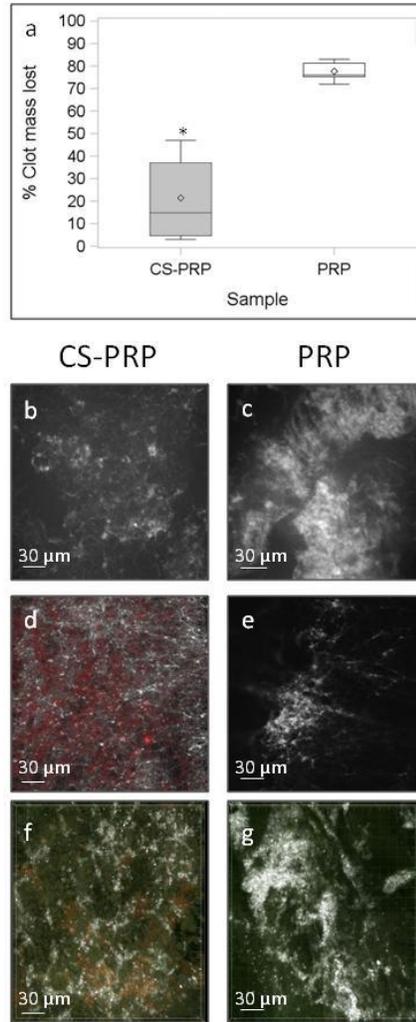


Figure 1. After clotting for 1 hour at 37°C, clot retraction and serum expression (expressed as % clot mass lost) was greater from PRP clots compared to CS-PRP hybrid clots (a). Data are presented as mean (diamond) and median (line) of n=7 clots from 3 different donors; Box: 25th and 75th percentile; Whisker Box to the most extreme point within 1.5 interquartile range. The Mixed model task in SAS Enterprise Guide 7.1 and SAS 9.4 was used to compare the different groups using sample (CS-PRP vs PRP) as a fixed effect and donor as a random effect. * p < 0.05 compared to PRP. Platelet aggregates were smaller in CS-PRP hybrid clots (b, d & f) compared to PRP clots (c, e & g), as shown by spinning disk microscopy images (b & c), confocal microscopy images (d & e) and 3-D stacks of confocal microscopy images (f & g). An Alexa-647 fibrinogen tracer was added to allow imaging of fibrin-covered platelets

and fibrin in white. A Rhodamine-chitosan tracer was added to allow imaging of chitosan in red in d) and orange in f).

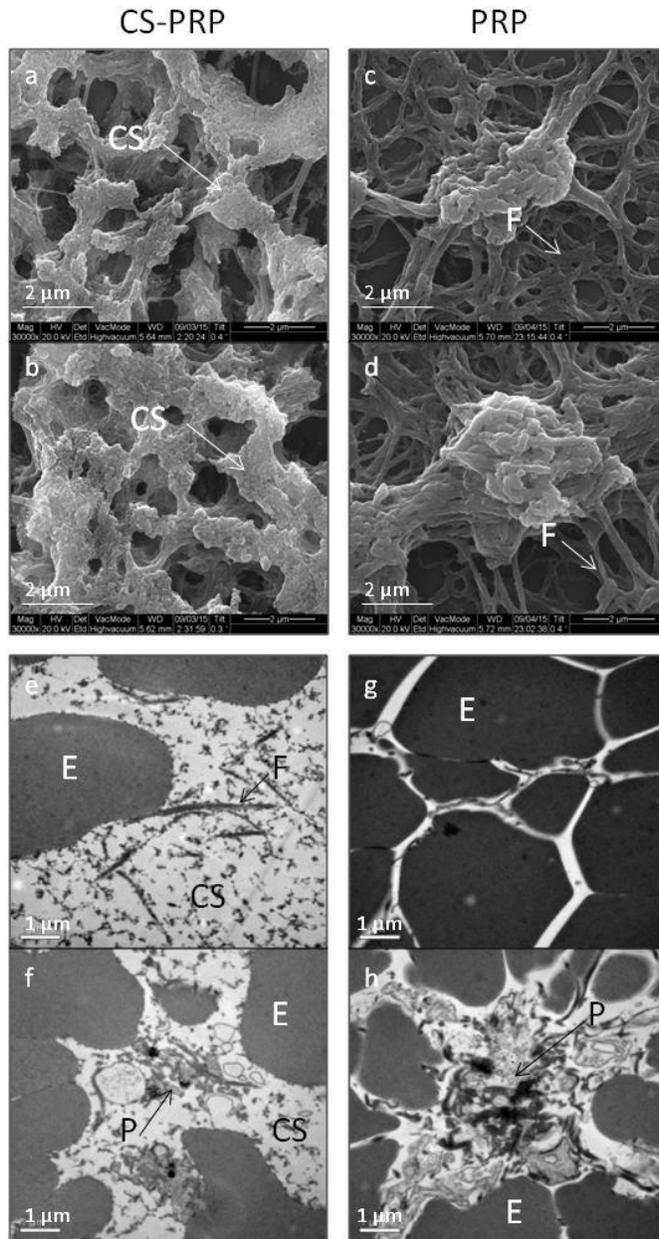


Figure 2. Chitosan (CS) appeared to be coating cellular and fibrous elements in scanning electron microscopy (SEM) images of CS-PRP clots (a & b) while the fibrin (F) network was readily visible in PRP clots (c & d). Chitosan (CS) was found in the space between cellular elements and also at the surface of erythrocytes (E), platelet aggregates (P) and fibrin (F) in transmission electron microscopy (TEM)

images of CS-PRP clots (e & f). Erythrocytes (E) were tightly packed and platelet aggregates (P) were large in TEM images of PRP clots (g & h).

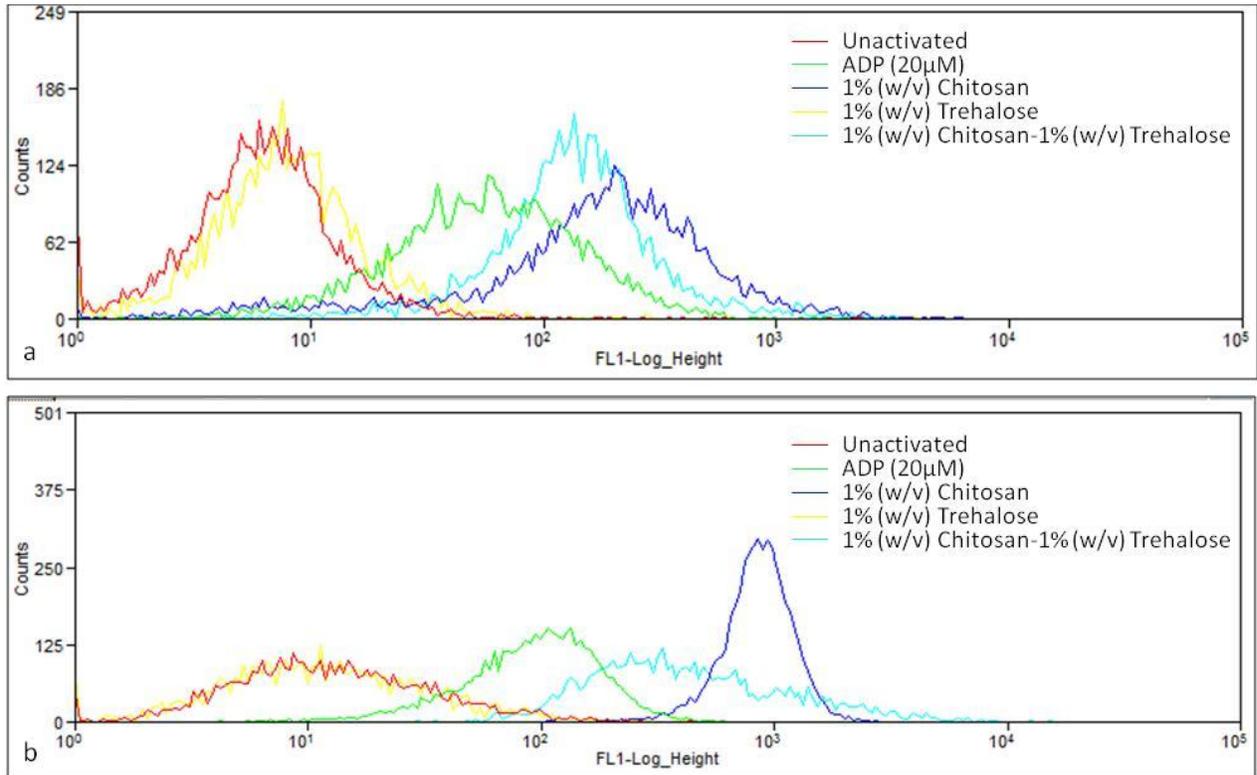


Figure 3. a) Flow cytometric analysis of PAC-1 (a) and p-selectin (b) staining of cell suspension (unactivated in red). As expected, incubation with ADP (20 μm in green) and chitosan (in dark blue), both known platelet agonists, led to platelet activation (a) and granule secretion (b). In contrast, incubation with trehalose alone (in yellow) had no effect on platelet activation (a) or granule secretion (b). Interestingly, incubation with chitosan and trehalose simultaneously (in pale blue) decreased the intensity of both fluorescent signals compared to incubation with chitosan alone (in dark blue).

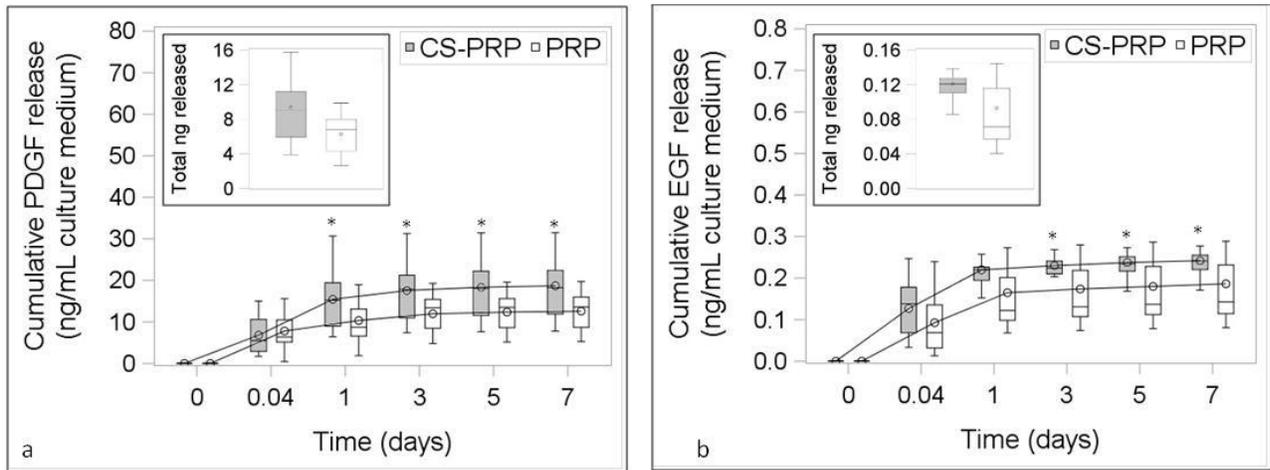


Figure 4. PDGF-AB (a) and EGF (b) cumulative release in culture medium was greater for CS-PRP clots compared to PRP clots. Data are presented as mean (circle) and median (line) of n=13 clots from 6 different donors; Box: 25th and 75th percentile; Whisker: Box to the most extreme point within 1.5 interquartile range. The Mixed model task in SAS Enterprise Guide 7.1 and SAS 9.4 was used to compare the different groups with post-hoc analysis to look at pair-wise differences using sample and time as fixed effects and donor as a random effect. * p < 0.05 compared to PRP. Insets show total amount of growth factors released.

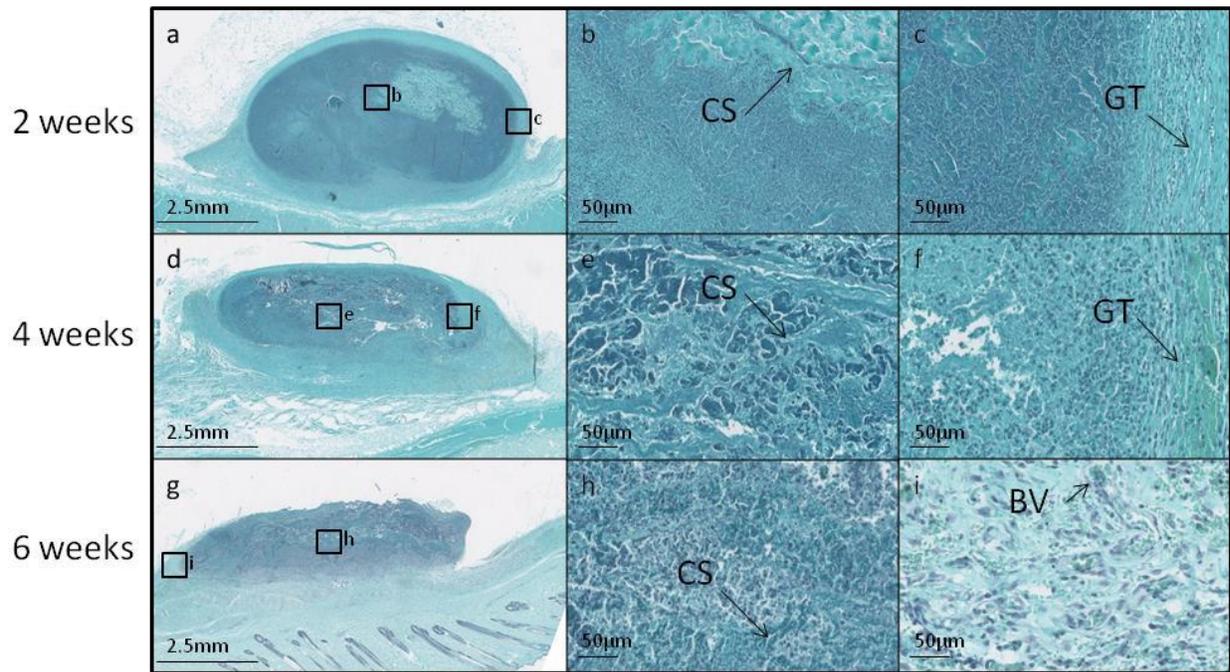


Figure 5. Formulations containing 1% (w/v) chitosan (CS M_n 38-43kDa and DDA 80-85%) with 1% (w/v) trehalose and 42.2 mM CaCl_2 were solubilized in autologous PRP and injected subcutaneously in rabbits, where they were found to be resident and induce cell recruitment at two weeks (a-c), four weeks (d-f), and six weeks (g-i) post-implantation. Invasion of the CS-PRP implants by host cells was accompanied by granulation tissue (GT) synthesis and formation of new blood vessels (BV). In contrast, recalcified PRP implants were completely degraded within a few days (not shown since the implant was absent). Outlines in a, d & g show where higher magnification images b, c, e, f, h & i were acquired.