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Effect of disulfide bonding and multimerisation on Proteoglycan 4's cartilage boundary lubricating ability and adsorption

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Abstract

Purpose—The objectives of this study were to assess the cartilage boundary lubricating ability of (1) non reduced (NR) disulfide-bonded proteoglycan 4 (PRG4) multimers versus PRG4 monomers, (2) NR versus reduced and alkylated (R/A) PRG4 monomers, and (3) assess the ability of NR PRG4 multimers versus monomers to adsorb to an articular cartilage surface.

Materials and Methods—PRG4 was separated into two preparations, PRG4 multimer enriched (PRG4Multi+) and PRG4 multimer deficient (PRG4Multi−), using size exclusion chromatography (SEC) and characterised by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The cartilage boundary lubricating ability of PRG4Multi+ and PRG4Multi− was compared at a physiological concentration (450 μg/mL) and assessed over a range of concentrations (45, 150 and 450 μg/mL). R/A and NR PRG4Multi− were evaluated at 450 μg/mL. Immunohistochemistry with anti-PRG4 antibody 4D6 was performed to visualise the adsorption of PRG4 preparations to the surface of articular cartilage explants.

Results—Separation into enriched populations of PRG4Multi+ and PRG4Multi− was achieved using SEC and was confirmed by SDS-PAGE. PRG4Multi+ and PRG4Multi− both functioned as

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Author Contributions

All authors contributed to the conception and design of the original study and approved the final submitted manuscript. SA and DP were responsible of the data acquisition and analysis. HH and PM performed purification, assaying, and identification of the anti-PRG4 mAb 4D6. Data analyses and interpretation was performed by SA, DP, JRM and TS. TS, JM & PM obtained funding for the study. The article was first drafted by SA, and critically reviewed by all. All authors take full responsibility for the integrity of the work as a whole.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

effective friction-reducing cartilage boundary lubricants at 450 μg/mL; with PRG4Multi+ being more effective than PRG4Multi−. PRG4Multi+ lubricated in a dose-dependent manner, however PRG4Multi− did not. R/A PRG4Multi− lubricated similar to NR PRG4Multi−. PRG4 containing solutions showed 4D6 immunoreactivity at the articular surface; the immunoreactive intensity of PRG4Multi+ appeared to be similar to SF, whereas PRG4Multi− appeared to have less intensity.

Conclusions—These results demonstrate that the inter-molecular disulfide-bonded multimeric structure of PRG4 is important for its ability to adsorb to a cartilage surface and function as a boundary lubricant. These findings contribute to a greater understanding of the molecular basis of cartilage boundary lubrication of PRG4. Elucidating the PRG4 structure-lubrication function relationship will further contribute to the understanding of PRG4's role in diarthrodial joint homeostasis and disease.

Keywords

proteoglycan 4 (PRG4); PRG4 disulfide-bonded structure; boundary lubrication; cartilage adsorption

2. Introduction

Proteoglycan 4 (PRG4) is a mucin-like glycoprotein synthesised by cells in articular cartilage, meniscus, synovial lining and tendons (1). It is encoded for by the *PRG4 gene* (2), and is analogous to lubricin (3), superficial zone protein (SZP) (4) and megakaryocyte stimulating factor (MSF) (5). PRG4 is present in synovial fluid (SF) and at the surface of articular cartilage where it functions as a critical boundary lubricant necessary for joint health (6), in a dose-dependent manner (7). In addition, PRG4 provides protection by preventing protein deposition and cell adhesion (8). The role played by PRG4 is critical in reducing the friction occurring at the bearing surfaces, which prevents the degradation of cartilage and adhesion of cartilage surfaces when boundary lubrication occurs. Indeed, mutations in the *PRG4 gene* results in an autosomal recessive disorder in humans, camptodactylyarthropathy-coxa vara-pericarditis (CACP), which results in juvenile-onset, non-inflammatory, precocious joint failure (9). Furthermore, alteration in PRG4 concentration within SF due to primary (10-12) and secondary OA (13, 14) in humans and animal models has been shown to affect joint integrity and lubrication.

PRG4 is a mucin-like glycoprotein (6) and shares functionally determinant structural characteristics similar to that of many other mucins (1, 15-17). PRG4 is composed of 12 exons, with exon 6 being the highly glycosylated mucin-like domain that makes up ~50% of the molecule's mass due to the extensive O-linked oligosaccharide substitutions (1, 6, 18, 19). This mucin-like domain is functionally important and determinant as enzymatic removal of the O-linked oligosaccharides, thought to provide repulsive hydration forces, results in diminished lubricating function (1, 17, 19). The cysteine rich N- and C-terminal domains facilitate the formation of functionally determinant intra- and inter-molecular disulfide bonds (17-19) and therefore the formation of PRG4 dimers and multimers (16, 20, 21). Indeed, the cysteine-rich N-terminal has been shown to enable dimerisation, entanglement and self-aggregation (19, 22). The ability to form disulfide-bonded multimers in general is critical to various mucins' functions (16, 20, 21), and this also appears true for

PRG4 (1, 6, 18, 19). There are four different isoforms of PRG4 due to the alternative splicing of exons 2, 4 and 5 in the N-terminal (6). While a recombinant construct representing the N-terminal region of PRG4 (exons 2-5) has been shown to display the capacity to dimerise (22), the potential effect of naturally occurring splice variants on intra- or inter-molecule disulfide bond formation of PRG4 remains to be determined.

PRG4 exists in both monomeric and multimeric forms, and these may demonstrate differential abilities to adsorb to the surface of cartilage and function as an effective friction-reducing boundary lubricant. Monomers and multimers have been identified in bovine synovial fluid, and their MW reported to range from 1493-867 kDa for multimers and 501-433 and 255-223 kDa for monomers when purified from media conditioned by bovine articular cartilage explants (1, 23, 24). PRG4 preparations purified from such media, and containing both multimers and monomers, have consistently demonstrated cartilage boundary lubricating ability (7). Reduction and alkylation (R/A) of PRG4 has showed diminished binding to the cartilage surface (22) as well as a reduction in lubricating ability at a cartilage-glass interface (25). Therefore, the role of inter-molecular disulfide bonds on PRG4's cartilage boundary lubricating ability, i.e. non-reduced (NR) multimers versus NR monomers, as well as intra-molecular bonds, i.e. NR monomers versus R/A monomers, remains unknown.

Boundary lubricants by definition are able to adsorb to the articulating surfaces on which they reduce friction. Indeed, PRG4 is able to adsorb on the surface of cartilage (22), the cysteine rich C-terminal is thought to enable this attachment (19, 22), and further disulfide-bridging interaction with larger polypeptide chains (17). Consistent with this, R/A inhibits PRG4s ability to bind to cartilage surfaces (22, 25). However, as with cartilage lubricating function, it remains unclear what effect inter-molecular disulfide bonds, i.e. NR multimers versus NR monomers, has on PRG4's ability to adsorb to a cartilage surface.

The objectives of this study were to assess the cartilage boundary lubricating ability of 1) non reduced (NR) disulfide-bonded PRG4 multimers versus PRG4 monomers, and their dose-dependency and (2) NR versus reduced and alkylated (R/A) PRG4 monomers, as well as 3) assess the ability of NR disulfide-bonded PRG4 multimers versus PRG4 monomers to adsorb to a cartilage surface.

3. Materials and Methods

3.1 Lubricant Preparation and Characterisation

3.1.1 PRG4—Cartilage for PRG4 preparation was obtained from fresh skeletally mature bovine stifle joints obtained from a local abattoir (Calgary, AB, Canada), as described previously (7). In brief, bovine stifle joints with intact articular capsules were obtained and discs with the intact articular surface of the cartilage were harvested. The cartilage discs were then cultured in Dulbecco's Modified Eagle's Medium with 0.01% bovine serum albumin for 28 days, with the addition of 25 μg/mL of ascorbic acid and 10 ng/mL of recombinant human transforming growth factor-β to the media (26). Purification of PRG4 from the conditioned media was then performed using diethylaminoethyl anion exchange chromatography (26, 27). The PRG4-rich 0.3 - 0.615 M NaCl eluant was retained,

concentrated with a 100 kDa filter (EMD Millipore, Billerica, MA) and stored at −80°C. The purity of the concentrated and filtered solution was confirmed using 3-8% Tris-Acetate sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by protein stain and western blotting with anti-PRG4 antibody LPN (1, 15) (Life Technologies, Carlsbad, CA). The concentration of this PRG4 preparation was then determined by bicinchoninic acid protein assay (BCA) (Sigma-Aldrich, St. Louis, MO) (28).

3.1.2 PRG4 Separation—Size exclusion chromatography was employed to separate PRG4 multimers and monomers. Sephacryl S-500HR High Resolution gel filtration media packed in a XK16/100 column (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada) was used in conjunction with a fast protein liquid chromatography apparatus (ÄKTA$_{FPLC}$; GE Healthcare Life Sciences). Phosphate buffered saline (PBS) and 0.25% [3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), a zwitterionic detergent (Sigma-Aldrich, St. Louis, MO) was used as the column buffer. The column was equilibrated in the PBS + 0.25% CHAPS buffer a flow rate of 0.2 mL/min, then PRG4 also suspended in PBS + 0.25% CHAPS was injected and 2 mL fractions were subsequently collected. UV absorbance at 280 nm was monitored, and fractions of interest from three predominant peaks were pooled together (**Fig. 1**). Pooled fractions were filtered, concentrated and buffer exchanged using Amicon Ultra-15 Centrifugal 30 kDa cut-off filters (EMD Millipore) using distilled water (dH_2O), centrifuged at 37°C, and re-suspended in PBS.

The recovery of PRG4, based on protein concentration determined by BCA assay, was ~90% following size exclusion chromatography and ~45% following buffer exchange and concentration via filtration.

3.1.3 PRG4 Characterisation—SDS-PAGE and western blotting was used to characterise the apparent MW and size distributions of immunoreactive PRG4 species in each pool, as described previously (1, 15, 23). Materials and equipment for 3-8% SDS-PAGE western blotting and protein staining were obtained from Life Technologies (Carlsbad, CA). Briefly, non-reduced samples were mixed in sample buffer and heated to 70°C for 10 minutes, with or without a reducing agent. These were then loaded onto a 3-8% SDS-PAGE gel followed by protein stain, and/or western blotting using anti-PRG4 antibody (Ab) LPN and monoclonal Ab (mAb) 4D6, as described previously (1, 15, 23, 29).

Protein stain (**Fig. 2A**) indicated the peak 2 preparation (PRG4Multi+) contained a distinct high molecular weight (MW) band near the top of the gel (***), a ~1 MDa band (**) (as shown previously (15)), and a weaker ~460 kDa monomeric (15) band (*). The peak 3 preparation (PRG4Multi−) lacked the high putative multimeric MW bands and was enriched in the 460 kDa non-reduced monomer band (*). Peak 3 also contained some faint <460kDa bands. Tandem mass spectrometry (MS/MS) confirmed the bands ~>460kDa (*, **, ***) to be PRG4 (data not shown). Western blotting indicated these preparations contained both LPN (**Fig. 2B**) and 4D6 (**Fig. 2C**) immunoreactive species, with a MW distribution similar to that of the protein stain. Peak 2 preparation was enriched in the high MW species (***, **), whereas peak 3 preparation contained primarily the ~460 kDa monomer (*). Peak 1 was

not resolved on the 3-8% gel as the molecule was too large to enter, though MS/MS indicated PRG4 species were present in those pooled fractions (data not shown).

This characterisation confirms that the two preparations of interest were generated; an enriched population of high MW species of PRG4 (peak 2), which still contained some smaller species, as well as a solution that appeared to lack the high MW PRG4 and was predominantly lower MW species. Henceforth, the terms "*PRG4 multimer enriched preparations*" (PRG4Multi+) for peak 2 and "*PRG4 multimer deficient preparations*" (PRG4Multi−) for peak 3 will be used to describe these preparations.

3.1.4 PRG4 Reduction and Alkylation—Purified PRG4Multi− (1 mg) dissolved in PBS was reduced and alkylated (R/A), essentially as described before (15, 30). The sample was incubated with 100 mM dithiothreitol in PBS for 2 hours at 60°C and 400 mM iodoacetate for 2 hours at room temperature. For protein recovery, the sample was then incubated in three volumes of ice cold ethanol (EtOH) overnight at −20°C. It was then centrifuged at 13000 RPM for 20 minutes (min), with most of the supernatant being removed. The sample was then washed three times with EtOH whilst being centrifuged at 13000 RPM for 20 min. In between each wash the supernatant was removed after each centrifuge. Sample was then centrifuged at 37°C and re-suspended in PBS. The concentration was then determined by BCA protein assay.

3.2 Boundary Lubrication Tests

3.2.1 Sample Preparation—Fresh osteochondral samples were prepared for friction testing from the patellofemoral groove of skeletally mature bovine stifle joints, as described previously (7, 31). Briefly, cores (radius = 6 mm) and annuli (R_{outer} = 3.2 mm and R_{inner} = 1.5 mm) were prepared from harvested osteochondral blocks (31). Samples were then rinsed vigorously overnight in PBS at 4°C to rid the articular surface of residual SF (confirmed by lubrication testing (7, 31)) prior to lubrication testing in PBS. Samples were then bathed in 0.3 mL of the subsequent test lubricants (core bathed in 0.2 mL, annulus bathed in 0.1 mL), completely immersing the cartilage, and left at 4°C overnight prior to the next day's lubrication test. The samples were again rinsed with PBS after each test before incubation in the next test lubricant.

3.2.2 Lubrication Test—The Bose ELF 3200 was used to analyse the cartilage boundary lubrication ability of the PRG4 preparations, using the previously described *in vitro* cartilage-on-cartilage friction test (7, 31). Briefly, all samples were compressed to 18% of the total cartilage thickness. Samples were allowed to stress-relax for 40 minutes to enable fluid depressurisation of the interstitial fluid. The samples were then rotated at an effective velocity of 0.3 mm/s (shown to maintain boundary mode lubrication at a depressurised cartilage-cartilage interface) (31) at +/− 2 revolutions. Samples were then left in a pre-sliding duration (T_{ps}) of 1200, 120, 12 and 1.2 seconds (s). Samples were rotated after each subsequent stationary period in the +/− 2 revolutions. The test sequence was then repeated in the opposite direction of rotation, −/+ 2 revolutions.

3.2.3 Lubricant Test Sequences—In all experiments described below, articulating osteochondral sample pairs were tested 4-5 times in a repeated measures fashion, in PBS (serving as the negative control test lubricant) on the first day and bovine SF (Animal Technologies, Tyler, TX; serving as the positive control test lubricant) on the last day, as done previously (7).

3.2.3.1 Cartilage Boundary Lubricating Ability of PRG4Multi+ vs. PRG4Multi−: The effects of inter-molecular disulfide bonds on the cartilage boundary lubricating ability of PRG4 was determined by comparing NR PRG4Multi+ to PRG4Multi− at a physiological concentration of 450 μg/mL in PBS (32). Furthermore, the potential dose-dependent cartilage boundary lubricating ability of PRG4Multi+ and PRG4Multi− preparations was assessed at concentrations of 45, 150 and 450 μg/mL.

Test Sequence 1 (n = 7): PBS, PRG4Multi−, PRG4Multi+, SF

Test Sequence 2 (n = 4): PBS, PRG4Multi+ @ 45 μg/mL, 150 μg/mL, 450 μg/mL, then SF.

Test Sequence 3 (n = 4): PBS, PRG4Multi− @ 45 μg/mL, 150 μg/mL, 450 μg/mL, then SF.

3.2.3.2 Cartilage Boundary Lubricating Ability of R/A PRG4Multi− and NR PRG4Multi−: To assess the effects of intra-molecular disulfide bonds, the cartilage boundary lubricating ability of R/A PRG4Multi− and NR PRG4Multi− was analysed at a concentration of 450 μg/mL in PBS.

Test Sequence 4 (n = 4): PBS, R/A PRG4Multi−, NR PRG4Multi−, SF

3.3 Immunohistochemistry

3.3.1 Sample preparation—Intact articular cartilage discs (n = 15, diameter = 6 mm) were harvested from bovine stifle joints obtained from a local abattoir. Fresh intact cartilage discs acted as a natural positive control. These were embedded in media (Tissue Tek OCT, Sakura, Torrance, CA) and snap frozen in isopropanol cooled in liquid nitrogen (labelled as “fresh”).

3.3.2 Specimen processing—Cartilage discs were placed in PBS and shaken vigorously overnight at 4°C to rid the articular surface of residual PRG4, and subsequently frozen at −80°C to prevent further production of PRG4 from viable chondrocytes. Discs were then thawed, and again shaken overnight at 4°C, before incubation with lubricants of interest. Discs were incubated over night at room temperature in test solutions of interest (32); PBS (negative control), PRG4Multimer+ @ 450 μg/mL, PRG4Multi− @ 450 μg/mL, and SF (positive control). Discs were then fixed in OCT and stored at −80°C.

Sections (5 μm thick) were cut using a cryostat microtome (Microm HM550, Thermo Scientific, Waltham, MA) and placed on positively charged glass slides (Superfrost Plus Adhesion Slides, Thermo Scientific). Sections were fixed in 4% paraformaldehyde in PBS and washed in PBS to remove OCT. Samples were blocked with 10% hydrogen peroxide in methanol, followed by 10% goat serum with 1% BSA in PBS in a humidity chamber. Samples were then incubated overnight in anti-PRG4 mAb 4D6 (33); in 1.5% normal goat

serum at a ratio of 1:100. Slides were washed again with PBS and incubated with secondary antibody Alexa Fluor-594 rhodamine-conjugated goat-anti mouse IgG (Life Technologies, Carlsbad, CA) in 1.5% normal goat serum at a ratio of 1:1000. Finally, samples were washed with PBS, mounted with mounting medium containing the nuclear counterstain DAPI (Vectashield, Vector Laboratories, Inc., Burlingame, CA), and sealed with microscope cover slips (VWR Scientific Products, PA). Slides were imaged using Zeiss LSM 780 microscope (Carl Zeiss, Oberkochen, Germany) at a magnification of 20× objective (dry, 0.8 NA). Fluorescence images were obtained for both red (Alexa Fluor-594 rhodamine detected PRG4; excitation/emission of 590/617 nm) and blue (DAPI detected cell staining; excitation/emission of 358/461 nm) fluorescence.

3.4 Statistical Analysis

Two coefficients of friction; static ($\mu_{static,\ Neq}$; resistance of start-up motion from static condition) and kinetic ($<\mu_{kinetic,\ Neq}>$; resistance of steady sliding motion) were calculated for each lubricant, and were averaged between both the + and − revolutions (7). The $<\mu_{kinetic,\ Neq}>$ values increased only slightly with T_{ps}, as observed previously (7), with values at T_{ps} = 1.2 s being on average 20.2 ± 1.9% compared to those at T_{ps} = 1200 s across the four test sequences. Therefore, for brevity and clarity, average $<\mu_{kinetic,Neq}>$ values across T_{ps} for each lubricant are presented, as done previously (34). Unless otherwise indicated, data is presented as mean ± SEM. Statistical analysis was implemented with SPSS 22.0 (IBM SPSS software, New York, NY).

Test Sequence 1—ANOVA was used to assess the effect of lubricant and T_{ps} as a repeated factor on $\mu_{static,Neq}$. ANOVA was also used to assess the effect of lubricant on $<\mu_{kinetic,Neq}>$ values followed by a Least Significant Differences (LSD) post-hoc test to compare PBS, PRG4Multi− and PRG4Multi+.

Test Sequence 2 & 3—$<\mu_{kinetic,Neq}>$ results from test sequence 2 and 3 were pooled together for clarity and ease of comparison. Friedman test was used to assess the effect of lubricant and T_{ps} on $\mu_{static,Neq}$. Repeated measures ANOVA was also used to assess the effect of lubricant on $<\mu_{kinetic,Neq}>$ values followed by a LSD post-hoc test to compare the varying PRG4 concentrations at 45, 150 and 450 μg/mL.

Test Sequence 4—ANOVA was used to assess the effect of lubricant and T_{ps} as a repeated factor on $\mu_{static,Neq}$. Friedman test was used to assess the effect of lubricant on $<\mu_{kinetic,Neq}>$ values, with Wilcoxon signed rank test to compare R/A PRG4Multi− and NR PRG4Multi−.

4. Results

4.1 Boundary Lubrication Tests

4.1.1 Cartilage Boundary Lubricating Ability of PRG4Multi+ vs. PRG4Multi−—

PRG4Multi+ and PRG4Multi− both functioned as effective friction reducing cartilage boundary lubricants at a concentration of 450 μg/mL, with PRG4Multi+ being more effective than PRG4Multi−. Static coefficient of friction ($\mu_{static,Neq}$, resistance of start-up

motion from static condition) varied with pre-sliding duration (T_{ps}) and test lubricant (both $p < 0.001$), with no interaction ($p = 0.427$). Values increased with T_{ps} and were consistently highest in phosphate buffered saline (PBS) and lowest in synovial fluid (SF). PRG4Multi+ and PRG4Multi− were intermediate (**Fig. 3A**). Kinetic coefficient of friction ($<\mu_{kinetic,\ Neq}>$; resistance of steady sliding motion) values exhibited similar trends, varying with lubricant ($p < 0.001$). $<\mu_{kinetic,Neq}>$ values were greatest in PBS and lowest in SF. Values for PRG4Multi + were significantly lower than PRG4Multi− ($p < 0.05$) and both were lower than PBS (both $p < 0.01$) (**Fig. 3B**).

4.1.2 Concentration Dependent Cartilage Boundary Lubricating Ability of PRG4Multi+ and PRG4Multi−—

PRG4Multi+'s effective friction-reducing cartilage boundary lubricating was dose-dependent over the concentrations tested (45, 150, 450 μg/mL), while that of PRG4Multi− was not. Friedman test on the PRG4Multi+ $\mu_{static,Neq}$ data varied across test lubricant and T_{ps} (both $p < 0.001$). Friedman test on the PRG4Multi− $\mu_{static,Neq}$ data varied across lubricant ($p < 0.001$), but at the T_{ps} level only varied at 1200 s and 120 s ($p < 0.05$) with no variation at 12 s and 1.2 s ($p = 0.06$). Values for both increased with T_{ps} and were consistently highest in PBS and lowest in SF.

$<\mu_{kinetic,Neq}>$ values exhibited similar trends. PRG4Multi+ varied with lubricant ($p < 0.01$). PRG4Multi− did not vary with lubricant ($p = 0.95$). $<\mu_{kinetic,Neq}>$ values were greatest in PBS and lowest in SF, with all PRG4 preparations being intermediate. Values for PRG4Multi+ at 450 μg/mL were significantly lower than PRG4Multi+ at 45 μg/mL ($p < 0.05$) and 150 μg/mL ($p < 0.01$), there was no difference between PRG4Multi+ at 45 μg/mL and 150 μg/mL ($p = 0.19$). No significant difference was found between PRG4Multi− samples across all three concentrations of interest ($p > 0.05$) (**Fig. 4**).

4.1.3 Cartilage Boundary Lubricating Ability of R/A PRG4Multi− and NR PRG4Multi−—

R/A PRG4Multi− and NR PRG4Multi− both functioned as effective friction-reducing cartilage boundary lubricants, with no significant differences between the two lubricants. $\mu_{static,Neq}$ varied with T_{ps} and test lubricant (both $p < 0.01$), with no interaction ($p = 0.156$). Values increased with T_{ps} and were consistently highest in PBS and lowest in SF, with R/A PRG4Multi− and NR PRG4Multi− being intermediate (**Fig. 5A**). $<\mu_{kinetic,Neq}>$ values exhibited similar trends, varying with lubricant (both $p < 0.01$). $<\mu_{kinetic,Neq}>$ values were greatest in PBS and lowest in SF. Values for R/A PRG4Multi− and NR PRG4Multi− were not significantly different from each other ($p = 0.375$) (**Fig. 5B**).

4.2 Immunohistochemistry

Immunohistochemistry (IHC) (**Fig. 6**) of fresh cartilage discs indicated a 4D6-immunoreactive layer of PRG4 localised at the articular surface but not at the cut surface. Vigorous shaking in PBS overnight appeared to remove the majority of PRG4. There was relatively little to no immunoreactivity observed for the non-immune controls, as indicated by the extremely faint or complete lack of rhodamine staining at the articular surface in the mAb (−) images, respectively. Shaken samples that were incubated in PRG4 containing solutions showed 4D6 immunoreactivity (red) at the articular surface and cut edge. The immunoreactive intensity of PRG4Multi+ appeared to be similar to that of SF, whereas

PRG4Multi− appeared to have less intensity. The 4D6 immunoreactive layer at the articular surface appeared to have a greater intensity to that observed at the cut edge of the explant for PRG4 containing solutions. There was no immunoreactivity observed for the non-immune control. Cartilage discs incubated in PBS alone showed some immunoreactivity, though not to the same intensity as those incubated in PRG4 containing solutions.

5. Discussion

The objectives of this study were to assess the cartilage boundary lubricating ability and the cartilage adsorption of NR disulfide-bonded PRG4 multimers versus NR PRG4 monomers. The results demonstrate inter-molecular disulfide-bonded multimeric structure of PRG4 is important for its ability to adsorb to a cartilage surface and function as a boundary lubricant. PRG4 multimer enriched preparations (PRG4Multi+) reduced friction in a dose-dependent manner, and appeared to adsorb to the articular cartilage surface to a greater extent than multimer deficient preparations (PRG4Multi−). PRG4Multi− still reduced friction and adsorbed to the articular surface, though not to the same extent as PRG4Multi+. These findings contribute to a greater understanding of the molecular basis of articular cartilage boundary lubrication of PRG4.

The PRG4Multi+ and PRG4Multi− preparations used in this study were generated using size exclusion chromatography. Chromatogram results indicated pooled peaks were not distinctly separated. As such, pooling of the chosen fractions could have resulted in cross-contamination in each of the PRG4Multi+ and PRG4Multi− preparations. SDS-PAGE results indicated predominant preparations of PRG4Multi+ and PRG4Multi− with some overlap between pooled samples. Subsequent evaluation of more select and fewer fractions, selected closer to the peak in each pool in an attempt to avoid cross-contamination, were performed. However, subsequent SDS-PAGE results showed no apparent improvements compared to the original pooling approach. Also, fractions collected from the peak shoulders found in peaks 2 and 3 were examined via SDS-PAGE and were again similar to those from the overall pool. Furthermore, losses were significant when trying to avoid cross-contamination and analyse the shoulders within the peaks, therefore for practical reasons fractions were pooled as described. Additional separation and characterisation method development would allow for further functional examination of different putative monomeric PRG4 species (24), as well as higher order (e.g. dimers, multimers) PRG4. Nevertheless, the methods developed and employed here resulted in the generation of enriched populations for PRG4 multimers/monomers that were appropriate for this study.

The cartilage boundary lubricating ability of the PRG4Multi+ preparation used here appeared similar in its magnitude and dose-dependency to that previously reported for unseparated NR PRG4 (7). These results are consistent with previous studies that have shown lack of disulfide-bonds reduces both PRG4s boundary lubricating ability (30) and attachment to the cartilage surface (22). PRG4Multi− did lubricate at cartilage-cartilage interface compared to PBS, however not to the same extent as PRG4Multi+ and not in a dose-dependent manner. Furthermore, NR PRG4Multi− with their intra-molecular bonds intact functioned in a similar manner to R/A PRG4Multi− that lacked those bonds.

Given the repeated measures method used here for friction testing, used previously in other studies to examine lubricants in increasing concentration (7), there was the potential for a carryover effect in test sequences 1 and 4 where distinct test lubricants were evaluated. As such additional testing was conducted where the order of test lubricants was reversed: Test Sequence 1a (n = 4): PBS, PRG4Multi+, PRG4Multi−, SF; Test Sequence 4a (n = 4): PBS, NR PRG4Multi−, R/A PRG4Multi−, SF. For Test Sequence 1a, values of $<\mu_{kinetic,Neq}>$ for PRG4Multi+ (0.143 ± 0 .024) were not statistically different than PRG4Multi− (0.111 ± 0.012) ($p = 0.077$) and both were lower than PBS (0.237 ± 0.030) (both $p < 0.05$). However, the trend towards a difference was apparent, suggesting there could be carryover effect if from testing in PRG4Multi+ first, therefore validating the initial test sequence 1 employed. Furthermore, the values of values of $<\mu_{kinetic,Neq}>$ obtained for PRG4Multi+ and Multi− at 450 μg/mL in Test Sequence 1 where similar to those in Test Sequence 2 and 3, respectively, also suggesting data from Test Sequence 1 was not significantly affected by carry over. For Test 4a, values of $<\mu_{kinetic,Neq}>$ for NR PRG4Multi− (0.168 ± 0.045) were similar to R/A PRG4Multi− (0.122 ± 0.023) ($p = 0.149$) and both were lower than PBS (0.350 ± 0.021) (both $p < 0.05$), consistent with the observations from the initial test sequence 4. Collectively, these results along with those in the initial test sequence 1 and 4, support the findings summarised above.

As indicated above, a repeated measures approach was employed for the lubricant testing in this study, as done previously. While repeated measures approach reduces variability, and therefore increases power within subject comparisons, it was employed here based on previous work (7). Furthermore, this experimental design enabled a significant number of measurements on a reasonable number of biological sample pairs. In this study, 19 osteochondral sample pairs were prepared and used in the four main test sequences, in which 84 lubricating tests were conducted (each taking ~3 hr, for a total of 252 hr of testing). Although 84 individual osteochondral sample pairs could have been prepared and used, resulting in significantly more experimental time and cost, the experiment approach and design employed here enabled the relevant objectives to be completed and conclusions to be drawn. Future studies may require the one-time use of osteochondral sample pairs.

To assess the potential effects of the separation process on PRG4's cartilage boundary lubricating ability, PRG4 was subjected to SEC, reconstituted and buffer exchanged via filtration into PBS. The cartilage boundary lubricating ability of the reconstituted sample was then tested via friction test and compared to unprocessed PRG4 (n = 4, all methods as previously). $<\mu_{kinetic,Neq}>$ for reconstituted-PRG4 (0.147 ± 0.033) was slightly higher but not statistically different to that of unprocessed PRG4 (0.098 ± 0.016) ($p = 0.126$). This suggests the process did not significantly or irreversibly affect PRG4's lubricating ability alone and therefore was appropriate for this study. Future studies could examine potential alternative dissociative agents for the use in purification/separation of lubricants of interest (e.g. PRG4) from conditioned media and/or SF.

It is still unknown how these disulfide-bonds interact to form dimers through either the C- and/or N-termini interactions (1, 20, 21). In addition, it is yet to be elucidated how these molecules interact with each other to form larger putative multimers and entanglements with other forms of PRG4, be it multimers or monomers, or even with other SF molecules such as

hyaluronan (7, 28, 35). Collectively, these data indicate inter-molecular disulfide-bonds and therefore PRG4's multimeric structure plays a greater role in PRG4's dose-dependent cartilage lubricating ability compared to its monomeric structure. One possible explanation for these observed differences is PRG4Multi+'s ability to adsorb and accumulate at the articular cartilage surface.

PRG4Multi+ demonstrated an increased localisation at the articular cartilage surface. IHC provided qualitative information on PRG4Multi+ and PRG4Multi−'s ability to adsorb to an articular cartilage surface; however quantity cannot be determined but these results do provide visual evidence for the extent of spatial adsorption at the cartilage surface. Shaken samples soaked in PBS as negative controls appeared to have minimal amounts of PRG4 at the cartilage surface. However, the immunoreactivity at the cartilage surface of PBS soaked samples was substantially less intensity compared to fresh or repleted samples. The effective removal of the PRG4 at the surface by this method is further supported by the high friction coefficient values measured for these samples tested in PBS, compared to those of fresh samples tested in PBS (7, 31). This would also remove any such residual PRG4 prior to testing in the first test lubricant where it could have potentially confounding effects; especially for a lower concentration or altered test lubricant(s) (overnight vigorous shaking between test lubricants was not possible as it would have resulted in a test duration of 8-10 days during which the osteochondral samples would likely have started to degrade). The approach of vigorous shaking in PBS to remove residual PRG4 was employed here to avoid potential alterations to the articular cartilage surface resulting from the use of other enzymatic, ionic, or mechanical methods (22, 32, 33).

Immunoreactivity was observed at the non-articular surface of samples soaked in PRG4 containing solutions. This demonstrates PRG4's ability to attach to the articular cartilage surface, as well as cut surfaces. Future studies could consider incubation of osteochondral samples instead of cut cartilage explants to prevent adsorption to lower cut surfaces. Future work is required to quantify the amount of PRG4Multi+ and PRG4Multi− at the articular cartilage surface. One potential approach, previously employed, is the radiolabeling of PRG4 (25) and quantifying the amount of PRG4 accumulated at the cartilage surfaces. Such studies would provide further insight into a possible relationship between an ability of PRG4Multi+ to accumulate at a cartilage surface and function as a dose-dependent boundary lubricant.

The occurrence of altered PRG4 structural composition in OA SF, in terms of relative abundance of PRG4 multimers and monomers, and potential functional consequences in terms of cartilage boundary lubricating ability remains to be determined. Previous studies demonstrated that some OA SF patients lack normal levels of PRG4, have a HA MW distribution shifted lower, and demonstrated diminished cartilage lubricating function that can be restored with PRG4 (13, 14). Perhaps some of the OA patients that do have normal overall PRG4 levels could lack normal PRG4 multimer/monomer distribution (i.e. diminished multimeric content) and therefore normal cartilage lubricating function. Future work could involve method development for the analysis of PRG4 structural composition in SF, as well as examination of the potential effect of PRG4 multimer/monomer distribution on PRG4's synergistic friction reducing interaction with hyaluronan (7, 28). Elucidating the

PRG4 structure-lubrication function relationship in SF, will further contribute to the understanding of PRG4's role in diarthrodial joint homeostasis and disease.

Acknowledgements

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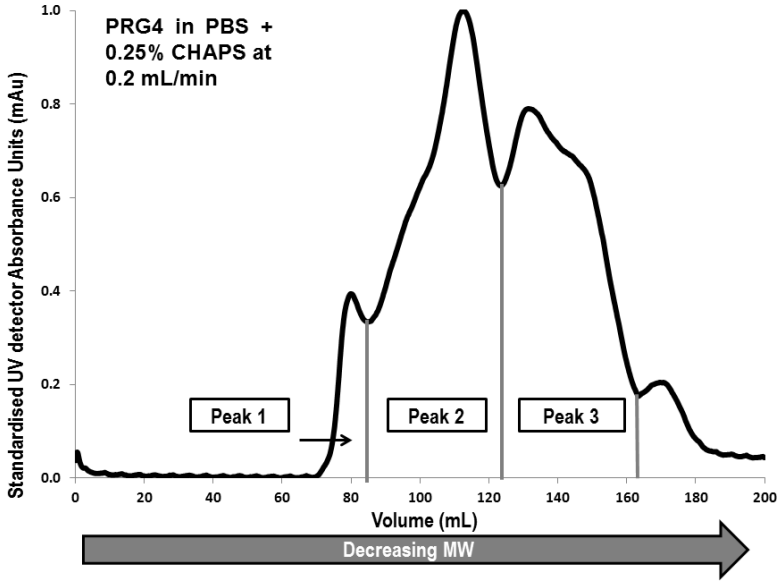

Figure 1.

Size exclusion chromatography chromatogram showing three peaks of interest. Peak 2 is shown to be PRG4Multi+ and peak 3 is shown to be PRG4Multi−.

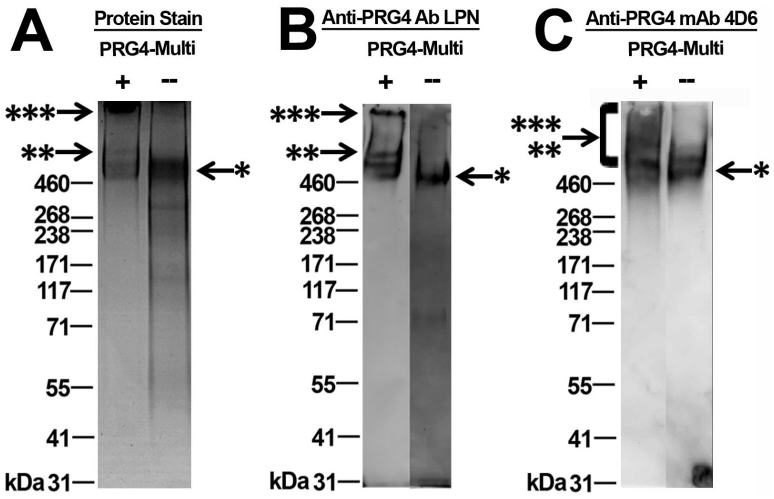

Figure 2.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of PRG4Multi− and PRG4Multi+ preparations; protein stain (A) and western blotting with Ab LPN (B) and mAb 4D6 (C).

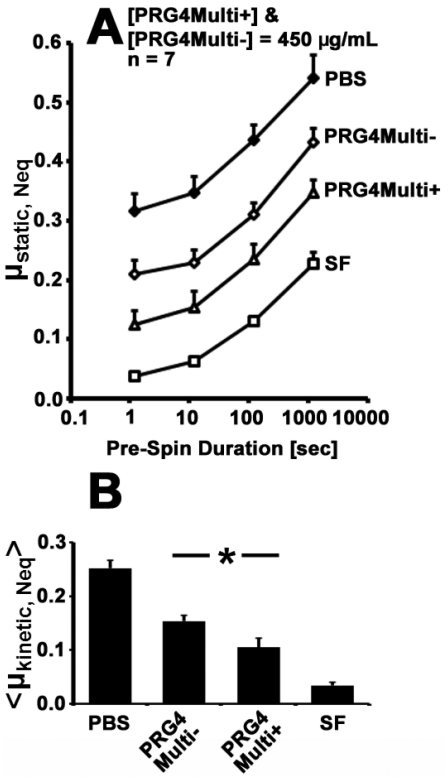

Figure 3.

Static ($\mu_{static,Neq}$) (**A**) and kinetic $<\mu_{kinetic,Neq}>$ (**B**) friction coefficients of PRG4Multi− and PRG4Multi+ at 450 μg/mL. Where * is $p < 0.05$.

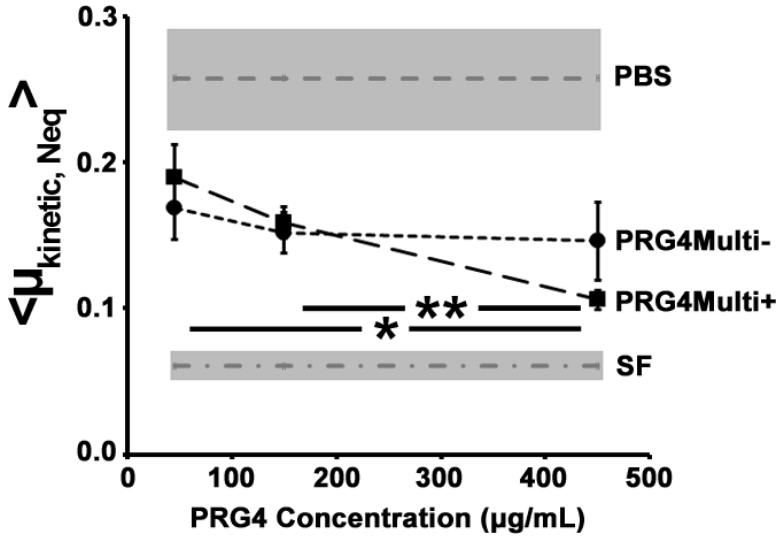

Figure 4.

Kinetic $<\mu_{kinetic,Neq}>$ dose-response friction coefficients of PRG4Multi− and PRG4Multi+ at 45, 150 and 450 μg/mL.

Where * is $p < 0.05$ and ** is $p < 0.01$ for PRG4Multi+. There are no significant differences across the different concentrations of PRG4Multi−. Sample sizes, PBS and SF; n = 8, PRG4Multi− and PRG4Multi+; n = 4.

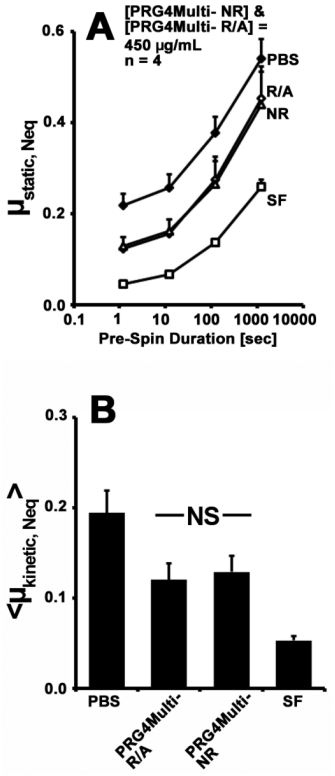

Figure 5.

Static ($\mu_{static,Neq}$) (**A**) and kinetic $<\mu_{kinetic,Neq}>$ (**B**) friction coefficients NR PRG4Multi− and R/A PRG4Multi− at 450 μg/mL.

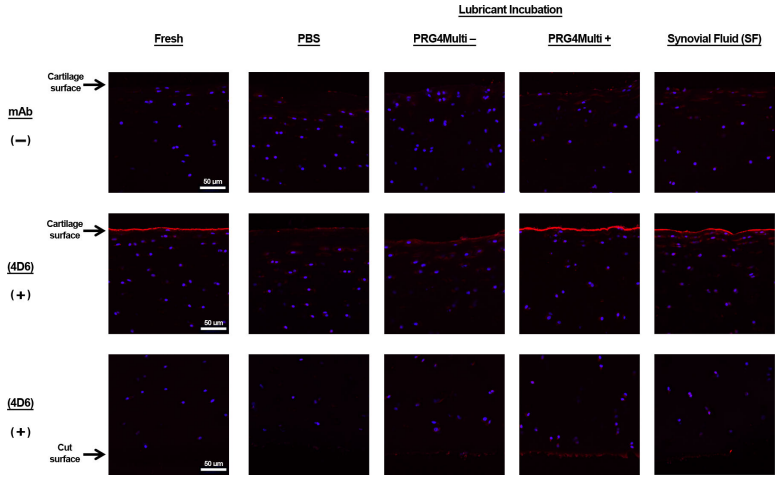

Figure 6.

Immunolocalisation of PRG4 at an articular cartilage surface. Key: Fresh – samples taken directly from joint and snap-frozen (control). All other samples were shaken overnight in PBS at 4°C, frozen over night at −80°C, shaken again in fresh PBS at 4°C and soaked in solutions of interest overnight at room temperature. Solutions: PBS (negative control), SF (positive control), PRG4Multi− and PRG4Multi+, with PRG4 at physiological concentrations of 450 μg/mL. (−) signifies negative samples that lacked primary antibody (Ab) 4D6 but contained secondary gt-anti mouse Ab, (+) signifies positive samples that contained both primary and secondary Abs. Blue indicates DAPI staining of chondrocyte cells and red indicates 4D6-immunoreactivity.