The Macroarchitecture of Biomimetic Proteoglycans is Responsible for the Micromolecular Engineering of Cartilage Pericellular Matrix

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INTRODUCTION. In articular cartilage, chondrocytes are surrounded by a 3-5 µm thick, structurally distinctive microdomain, the pericellular matrix (PCM). The PCM is pivotal in mediating the biophysical and biochemical cross-talk between chondrocytes and the extracellular matrix (ECM) [1]. In post-traumatic osteoarthritis (OA), degeneration of the PCM is a precursory event, portending histological or biomechanical changes of the ECM partially driven by perturbed cell mechanotransduction, contributing to irreversible cartilage breakdown and proteoglycan degeneration [2]. Recently, our group has synthesized a suite of biomimetic proteoglycans (BPGs), composed of natural chondroitin sulfate bristles (CS) and a poly(acrylic acid) (PAA) backbone, that mimic the macro-architecture of native proteoglycans [3]. We have demonstrated that BPG10, a ~180 kDa mimic with 7-8 CS bristles on a 10 kDa PAA core (Fig. 1a), can passively diffuse through all zones of cartilage *in vivo* and *ex vivo*. Notably, BPG10 preferentially localizes to the PCM (Fig. 1a), and thus, has the potential to molecularly engineer this crucial tissue microdomain [4,5]. In this study, we have demonstrated molecular engineering of bovine cartilage *ex vivo* using BPG10 to modulate PCM biomechanical properties by facilitating enhanced adhesive molecular interactions between aggrecan molecules (the major proteoglycan in cartilage PCM) to a degree that free CS bristles alone could not achieve.

METHODS. *Immunofluorescence-guided AFM (IF-AFM).* BPG10 was synthesized and fluorescently labeled as previously described [3]. Ten-mm lateral femoral osteo-chondral plugs were harvested from adult bovine knees and incubated with either 10 mg/mL BPG10 in 1×PBS or control 1×PBS alone for 24 hrs. Following incubation and resulting diffusion of BPG10 into cartilage, plugs were embedded in OCT without fixation, and 8-µm-thick sagittal sections were obtained using Kawamoto's film-assisted cryosectioning [6]. Using collagen VI-guided IF-AFM, we quantified the indentation modulus, E_{ind}, of the PCM and ECM via a microspherical tip ($R \approx$ 2.25µm, $k \approx$ 0.6 N/m, µMasch) and MFP-3D at 10 µm/s rate [7]. Significance between the matched pairs of $n = 5$ adult bovine specimens was tested via Wilcoxon's signed rank test at $\alpha = 0.05$. *Molecular Force Spectroscopy (MFS).* Microspherical colloidal tips ($R \approx 2.25$ µm, $k \approx 0.03$ N/m, µMasch) were chemically functionalized with thiolated aggrecan via incubation for 48 hrs [8]. Freshly cleaned gold substrates were also functionalized with aggrecan through the same methods. To test if BPG10 or CS increase the adhesion of aggrecan in colloidal force spectroscopy [8], aggrecan-coated tips were programmed to compress aggrecan-coated substrates at ≈ 50% molecular strain for 0 or 20 seconds dwell time in 1× PBS, followed by retraction from the surface with or without the addition of 20 nM free BPG10 or the molar equivalent of CS, 150 nM ($n \geq 180$ measurements). The maximum adhesion force, F_{ad}, and total adhesion energy, E_{ad}, were extracted from each approach-retract force-distance curve (Fig. 1d). Significance was tested by two-sample t-test at $\alpha = 0.05$.

RESULTS. Molecular engineering of cartilage with BPG10 resulted in a significant increase in the elastic modulus, E_{ind}, of the PCM compared to the control by ≈ 44% ($p = 0.0038$). Meanwhile, BPG10 also increased the ECM modulus, albeit to a more moderate extent (≈ 37%) with only a marginal statistical trend ($p = 0.077$, Fig. 1c). The more pronounced impact of BPG10 on PCM micromechanics demonstrates the functional effects of BPG10 molecularly engineering the cartilage and corroborates our observations that BPG10 is present in the ECM and PCM, but preferentially localized to the PCM (Fig. 1a) [5]. Further, the increase in the PCM micromodulus by BPG10 could be explained by molecular interactions of BPG10 with native matrix molecules. Using molecular force spectroscopy, we showed that the presence of free BPG10 significantly increased the adhesion of aggrecan-aggrecan molecules, as demonstrated by the significant increase in both F_{ad} and E_{ad} (Fig. 1e). When opposing aggrecan layers were exposed to a molar equivalent of CS as that on BPG10 (with 7-8 CS chains per BPG10 molecule), there was no detectable increase in F_{ad} at either dwell time. Additionally, the E_{ad} remained unchanged at 0 seconds of dwell time and was reduced at a prolonged dwell time upon addition of free CS (Fig. 1e).

DISCUSSION. Our data clearly illustrates the capability of BPG10 to functionally molecularly engineer the micromechanics of the PCM and to a lesser degree the ECM. The preferential association of BPG10 to the PCM is surprising in that BPGs are made with a synthetic polymer core, and there are no protein motifs to endow specific interaction with native biomolecules. We attribute this localization to the preferential molecular interactions between BPG10 and naturally existing PCM molecules, such as aggrecan. We have shown that free BPG10 can provide intermolecular interactions to significantly increase aggrecan-aggrecan adhesion (Fig. 1e). Aggrecan, the major proteoglycan in cartilage, is more concentrated [9], and undergoes much faster turnover [10] in the PCM compared to the ECM. Due to the similarity in molecular composition and brush-like macro-architecture between BPG10 and aggrecan, it is possible that BPG10 interacts with aggrecan to form supramolecular networks through non-specific interactions such as hydrogen bonding, charge-charge interactions, and physical entanglement. Since aggrecan is also present in the ECM at a lower concentration, this also explains the presence of BPG10 in the ECM, albeit with a lower concentration. Further, we have demonstrated the importance of the molecular architecture of our biomimetic proteoglycans. When an equivalent amount of CS is added to the aggrecan environment, it does not enhance adhesive events between aggrecan molecules like we have previously observed upon the addition of BPG10. At longer dwell times, when more interactions are typically able to occur, CS inhibits aggrecan-aggrecan binding potential to a significant extent. The 3-D brush-like architecture of BPG10, mimicking natural proteoglycan structure, is a driving factor in its ability to molecularly engineering cartilage tissue. Our ongoing studies are examining the impact of BPG10 on PCM micromechanics in human OA cartilage, its interactions with other PCM predominant molecules, and its effects on chondrocyte mechanotransduction in both normal and OA tissues.

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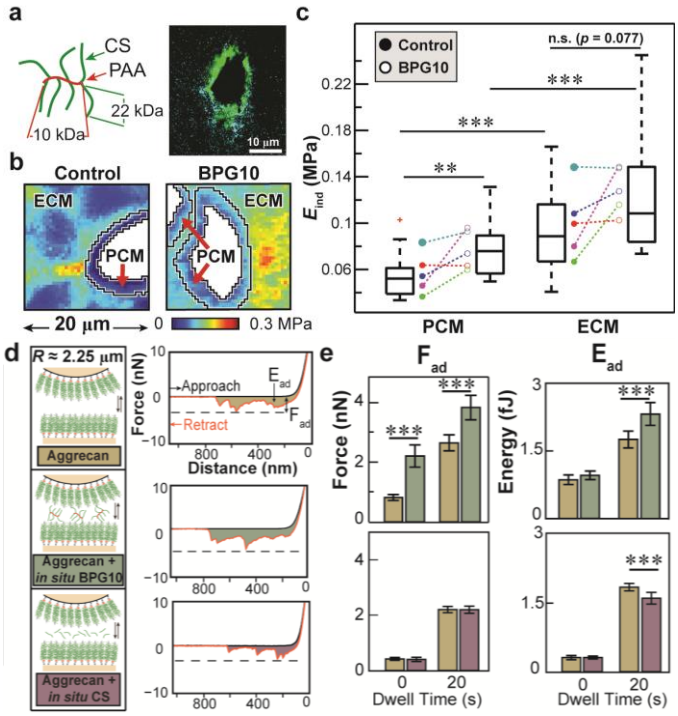

Figure 1. a) Schematic of BPG10 with IF image illustrating BPG10 (blue) localized to the PCM (green). **b)** Representative indentation modulus maps of PBS (Control) or BPG10 diffused tissue. **c)** BPG10 diffused cartilage demonstrated an increased E_{ind} in the PCM. Box-and-whisker plots of PCM and T/IT-ECM micromodulus for the Control and BPG10 tissue with each matched pair of circles representing the average modulus from one of $n = 5$ animals. **d)** Schematics and representative force-distance curves of MFS demonstrating opposing aggrecan compression alone, with 150 nM free CS, or 20 nM free BPG10. **e)** Adhesion force (F_{ad}) and energy (E_{ad}) of the corresponding MFS experiments with mean ± 95% CI, from n = 3 experimental repeats (**: $p < 0.01$, ***: $p < 0.001$).