Rescue of Inflammation-Induced Cartilage Degradation via Biomimetic Proteoglycan-Dexamethasone Conjugate

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INTRODUCTION: Articular cartilage chondrocytes are surrounded by a 2-4 μm thick, structurally distinctive pericellular matrix (PCM). In osteoarthritis (OA), degeneration of the PCM is a leading event of disease initiation, contributing to disrupted chondrocyte mechanotransduction and downstream deleterious metabolic changes, resulting in the breakdown of cartilage [1]. Molecularly engineering the PCM holds promising potential for modulating the mechanosensitive activities of chondrocytes to promote regeneration and attenuate disease progression [2]. Our group has synthesized a suite of biomimetic proteoglycans (BPGs), which are composed of natural chondroitin sulfate bristles (CS) and a poly(acrylic acid) (PAA) backbone. These molecules mimic the nano-architecture and water uptake of native proteoglycans [3, 4]. We have demonstrated that BPG10, a ~170 kDa mimic with ~7-8 CS bristles attached onto a 10 kDa PAA core (Fig. 1a), can passively diffuse through all zones of cartilage *in vivo* and *ex vivo*, preferentially localize in the PCM and territorial-ECM (T-ECM). As a result, these BPGs can modulate the local micromechanics of the PCM and in turn, the mechanosensitive activities of residing chondrocytes [5-7]. Building upon this material, our group attached dexamethasone (Dex), a widely used anti-inflammatory glucocorticoid for OA treatment, to BPG via a stable, pH-mediated amide bond to facilitate controlled release of Dex and amelioration of cartilage degradation (Fig. 1a). In this study, we synthesized and characterized BPG-Dex, quantified controlled release *in vitro*, and demonstrated the effect of BPG and BPG-Dex to attenuate the inflammatory response induced by IL-1β using an adult bovine cartilage explant model.

METHODS: *Materials preparation*. BPG was synthesized as previously described, resulting in a poly(acrylic acid, PAA) core and CS bristles [8]. BPG-Dex was synthesized, purified, and chemically characterized by FTIR and HPLC to determine reaction efficiency, drug release profiles, and drug loading capacity, as well as drug release at pH 7.4 and 6.5 to determine sensitivity of release with pH levels relevant to normal and OA cartilage. *Bovine cartilage sample preparation*. Cylindrical cartilage plugs (4 mm diam) were harvested from $n = 3$ fresh adult bovine knee joints, washed with PBS, and incubated undisturbed in chemically defined chondrogenic DMEM (1% ITS+Premix, 50 μg/mL L-proline, 0.9 mM sodium pyruvate, 50 μg/mL ascorbate 2-phosphate) for 24 hours prior to stimulation with a low dosage of 1 ng/mL recombinant bovine IL-1β for 3 days in 1.5 mL DMEM at 37ºC. Fresh IL-1β was administered every 24 hours for the 3-day duration. BPG, BPG-Dex, and Dex were dissolved in complete DMEM at the following concentrations: BPG (10 mg/mL), BPG-Dex (10 mg/mL), and Dex (25 μM). Each treatment well (n = 3 plugs) was incubated with 1.5 mL of drug solution for 48 hours and then fresh media was replenished every 48 hours for the remainder of the explant study. *Histology*. Plugs were processed for histology by fixing in 4% PFA in 4ºC overnight and then sequentially dehydrated and paraffin embedded [9]. Samples were sectioned at 6-μm thick and stained with Safranin-O/Fast Green (Saf-O/FG) to assess gross-level proteoglycan and collagen changes among groups. *Statistics.* Samples were assessed via measurements of sGAG void ($thickness_{sGAG\ void}$) obtained using ImageJ (n = 87 measurements from n = 9 plugs per condition across n = 3 biological repeats). One-way ANOVA with Tukey's post-hoc test for multiple comparisons was performed on sGAG thickness measurements for all groups at a significance level of $\alpha = 0.05$.

RESULTS: FTIR analysis of the BPG-Dex starting materials and final product showed an addition of aromatic alkene bonds (C-H: 2938 cm^{-1}) from dexamethasone and carbonyl bonds (C=O: 1764 cm^{-1}) and ester bonds (C-O: 1335 cm^{-1}) from the ester linkage between glycine and dexamethasone (Fig. 1b), indicating successful covalent attachment of Dex to BPG. Release of Dex was faster in pH 7.4 release media due to rapid cleavage of the ester bonds compared to pH 6.5 conditions (Fig. 1c). Cartilage viability was maintained during the length of explant culture as demonstrated by live/dead assay (Fig. 2d) as well as sGAG staining between day 1 control (D1C) and day 16 control (D16C), which showed no significant differences (Fig. 2a). IL-1β induced an inflammatory and catabolic response within cartilage, resulting in a salient increase in the thickness of sGAG-depleted regions (Fig. 2c). Free Dex (Dex) had the greatest sGAG loss among treatments administered and was not statistically different to that of the IL-1β treated cartilage (Fig. 2b), indicating no clear signs of rescue from cartilage degradation as measured by sGAG (Fig. 2a-c). In contrast, BPG demonstrated attenuation of degeneration with a significant increase in sGAG retention compared to both IL-1β and Dex conditions ($p<0.01$, Fig. 2b,c). Furthermore, BPG-Dex (BPG-Dex) had the greatest rescue of inflammatory conditions with sGAG loss statistically comparable to that of both the day 1 and 16 control groups.

DISCUSSION: This study demonstrated that a disease-modifying drug, such as Dex, can be coupled to BPG using an environment-susceptible linker to provide long-term controlled release *in vitro* up to 42 days. Further, under an inflammatory environment *ex vivo*, cartilage degeneration as measured by sGAG staining, can be significantly attenuated via BPG alone. This effect was further amplified by the use of BPG-Dex, surpassing the efficacy of free Dex administration. BPG alone has demonstrated the addition of sGAGs or reduction in sGAG depleted thickness, likely due to mechanobiological effects previously documented by BPG alone [5]. Early treatment regimens with BPG suggest that BPG was able to attenuate IL-1β-induced degeneration (e.g. increased cytokines, increased enzyme production, decreased proteoglycan production) as measured by sGAG depleted thickness measurements. The addition of BPG-Dex, further attenuated sGAG, hallmarking nearly a complete rescue. BPG-Dex delivers Dex at a 200-2000 times lower dose than those used for intra-articular injections, which may reduce unintended adverse effects of free Dex on the joint clinically. These findings provide direct molecular evidence that the BPG-Dex conjugate can molecularly engineer cartilage in early stages of IL-1β induced inflammation and thus, has the potential to serve as a multi-functional, minimally invasive therapeutic to reverse OA progression by protecting chondrocyte PCM microenvironments.

SIGNIFICANCE/CLINICAL RELEVANCE: This study demonstrates that BPG alone and BPG-Dex conjugate can be used to rescue cartilage GAG depletion by mitigating the inflammatory response, and thus, could potentially be used as a therapeutic for OA.

REFERENCES: [1] Chery+ 2020. [2] Guilak+ 2018. [3] Kim+ 2015. [4] Prudnikova+ 2019. [5] Kahle+ 2022. [6] Phillips+ 2019a. [7] Phillips+ 2019b. [8] Prudnikova+ 2018. [9] Han+ 2019.

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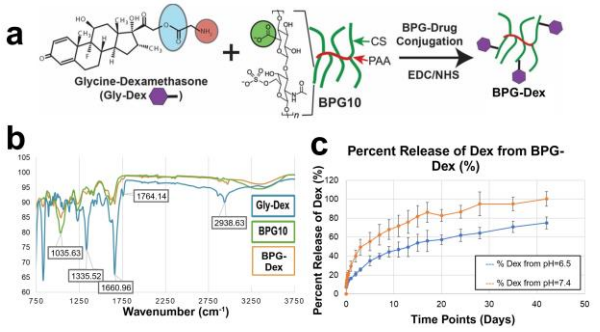

Fig. 1 a) Scheme for BPG-Dex synthesis. Glycine-Dex (Gly-Dex) reacts with BPG through standard EDC/NHS chemistries to yield the desired BPG-Dex drug construct. **b)** FTIR spectra of glycine-dexamethasone (GD), BPG, and BPG-glycine-dexamethasone (BPG-Dex). **c)** Percent of Dex released from BPG-Dex in pH = 7.4 and 6.5 PBS. Cumulative release percentage was tracked over the course of 42 days from $n = 3$ repeats per condition. Half of the total Dex is released in 3 days in basic release conditions and 50% of the total Dex is released in 13 days from acidic release conditions.

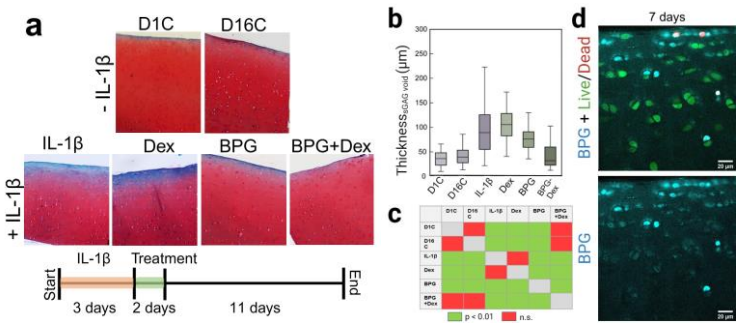

Fig. 2 a) Histological staining of Safranin-O (sGAG) and Fast Green (collagen) of controls and treatment groups. Top row: controls from 24 hours after explantation (D1C) and age-matched (D16C). Bottom row: All groups that received IL-1β including the control condition (IL-1β) and three treatment groups (Dex, BPG, and BPG-Dex). **b)** sGAG void thickness assessment of controls and treatment groups with **c)** table of statistical significance between groups. **d)** Retention of BPG within cartilage explants for 7 days. Top image: live cell (calcein AM, green) and dead cell (ethidium homodimer-1, red), and BPG (cyan). Bottom row: BPG diffused through cartilage and localized in the PCM region.