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Rapid and durable photochemical bonding of cartilage using the porphyrin photosensitizer verteporfin

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SUMMARY

Objective: To evaluate the effectiveness of verteporfin as a photosensitizer to photochemically bond articular cartilage tissues and determine bond durability *in vitro*.
Design: Bond strength induced by verteporfin over a range of concentrations and light exposure conditions was investigated using a disk-annulus model and a pushout test. Exposure was parameterized by varying either irradiance or irradiation time. Bond robustness in a cell-mediated degeneration environment was examined by exposing newly bonded samples to interleukin-1 alpha for the first 4 days of a 7-day culture period, followed by mechanical testing and biochemical and cellular viability assays.
Results: Photochemical bonding using verteporfin produced high bonding shear strengths at relatively low photosensitizer concentrations. Low exposures produced by either low irradiance or short irradiation time were sufficient to produce shear strengths comparable to those previously produced with phthalocyanine photosensitizers with substantially higher light exposure. Photochemically produced bonds were resistant to cell-mediated degeneration *in vitro* with no evident differences in cell viability among treatments.
Conclusions: Verteporfin offers distinct advantages as a photosensitizer for photochemical bonding of articular cartilage due to the production of strong, durable bonds at relatively low light exposures. Further exploration may lead to clinically feasible strategies to augment cartilage repair techniques.

Introduction

Deficiencies in the soft tissue integration of articular cartilage focal repairs have motivated ongoing efforts to enhance the graft–host interface. In osteochondral autograft or allograft transplants, inadequate graft integration can lead to the inability to transfer mechanical loading and nutrients, inferior fibrocartilage formation and further cartilage degeneration[1,2]. Photochemical tissue bonding using a high wavelength light-activated photosensitizer (PS)[3] is an effective technique that produces rapid and overall high-strength initial bonds via the formation of non-native collagen crosslinks[4,5] across the defect interface. Photochemical processes have been used to create collagen crosslinking in various tissues such as skin, blood vessels and cornea[6–8]. Previous work has explored the use of photosensitizers from different chemical families and with different activation light wavelengths in the bonding of articular cartilage. Early studies evaluated a patented 1–8, naphthalamide dye with a peak absorbance wavelength of 430 nm[9]. Subsequent studies examined photosensitizers activated by deep red light (660–690 nm) that penetrates deeper into the tissue[10,11]. In particular, aluminum phthalocyanine chloride (AlPc) was more effective than either a similar phthalocyanine, Al(III) phthalocyanine chloride tetrasulfinic acid (CASPc) or the cationic phenothiazine Methlyene Blue, achieving substantial bond strengths but requiring relatively high light irradiance and irradiation times on the order of 10 min[11]. Reduction of both light irradiance and irradiation time would improve the clinical feasibility of cartilage photochemical bonding, prompting a search for alternative photosensitizer agents.

Verteporfin (or benzoporphyrin derivative, BPD) is an FDA-approved photosensitizer from the porphyrin group, with a similar chemical structure and activation mechanism to the phthalocyanines and a similar activation wavelengths ($\lambda = 689$ nm)[6,12–16]. In particular, the peak absorbance wavelength for verteporfin and AlPc ($\lambda = 675$ nm)[17] are relatively close, supporting direct comparisons of bonding results under comparable conditions. Verteporfin was first

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approved for photodynamic therapy to treat choroidal diseases such as predominantly classic subfoveal choroidal neovascularization[15,16,18]. Activation of verteporfin has been shown to create collagen cross-links that mechanically stiffen corneal tissue, similar to the previous use of riboflavin as a photosensitizer to treat keratoconus[6,19]. These newly formed bonds showed resistance to collagenase A digestion[6], suggesting that photochemical bonds at a cartilage interface may be similarly resistant to enzymatic degradation.

The goal of the studies presented here was to evaluate verteporfin as a candidate photosensitizer for articular cartilage bonding. Benchtop studies presented here examined the interfacial shear strength achieved via photochemical bonding using verteporfin by systematically altering the photosensitizer concentration (Study 1) and light exposure parameters (Study 2). A subsequent study examined the initial stability of the photochemically produced bonds in an artificially challenging environment *in vitro* to simulate factors that are typically present in an injured, diseased or surgically traumatized joint (Study 3). Freshly bonded, viable samples were incubated in the presence of mild to moderate *in vitro* doses of Interleukin-1 alpha (IL-1) to stimulate cell-mediated degeneration and assess whether the newly formed bond is more susceptible than the surrounding tissue to enzymatic degradation.

Method

Materials and equipment

Immature bovine stifles were from Research 87 Inc. (Boylston, MA) and San Jose Valley Veal and Beef (Santa Clara, CA). Protease Inhibitor Cocktail Set I was from Calbiochem (San Diego, CA). Verteporfin, chondroitinase-ABC, low glucose Dulbecco's Modified Eagles Medium (DMEM), Dulbecco's Phosphate Buffered Saline (PBS), gentamicin, L-ascorbate-2-phosphate, glycine, 1,9-dimethylmethylene blue (DMMB) dye, and shark chondroitin sulfate were from Sigma Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was from Fisher Scientific (Pittsburgh, PA). Interleukin-1 alpha was from R&D Systems, Inc. (Minneapolis, MN). Non-essential amino acids (NEAA) and fetal bovine serum (FBS) were from HyClone (Logan, UT). Antibiotic/antimycotic (AB/AM) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution were from Corning (Corning, NY). Sodium chloride was from Acros Organics (Morristown, NJ). The Live/Dead viability/cytotoxicity set was from Invitrogen/Molecular Probes (Eugene, OR).

Biopsy punches were from Integra Miltex (York, PA). An AlGaINP FC-671 fiber-coupled diode laser with $\lambda = 686$ nm was from Changchun New Industries Optoelectronics Technology (Changchun, China). A PM 100D power meter and S121 Sensor were from Thorlabs (Newton, NJ). An Instron 5848 Microtester (Norwood, MA) was used for mechanical testing. Live/Dead cell visualization was performed using a Zeiss Axiovert 200M Fluorescence Microscope (Göttingen, Germany). Biochemical analyses were done in a Biotek Synergy HT Microplate reader (Winooski, VT).

Methods

The studies outlined in this paper consisted of two major sets of experiments: benchtop experiments (Study 1 and 2) and *in vitro* experiments (Study 3). *In vitro* experiments followed a similar sample extraction and sample preparation method except under aseptic conditions. In each sub-study described below, the target group size was $n = 6$/condition based on our previous experience and typical practice for similar *in vitro* studies, although joint size and processing issues occasionally resulted in fewer samples for some sub-groups.

Benchtop experiments

Interface strengths were assessed for varied PS concentration (Study 1) and exposures; exposure variations were produced by varying irradiation time while holding the irradiance constant (Study 2A) or varying irradiance while holding irradiation time constant (Study 2B). Study conditions are summarized in Table I. Studies included a negative control group of samples that were digested and press-fitted but were not exposed to photosensitizer or illumination, and a positive control group treated with 5 mM AlPc and irradiated at 1.58 W/cm^2 for 10 min, conditions previously found to produce strong bonding[11]. In each substudy, cores and annuli were randomly allocated to experimental groups.

Verteporfin concentration (study 1). Verteporfin concentrations of 0.01–30mM were used in three separate substudies, with a total of 125 cores and annuli ($n = 5$–17/condition) isolated from 13 joints. Untreated controls were included in each substudy, and light exposure was fixed at 947 J/cm^2 (1.58 W/cm^2 for 10 min).

Exposure study: Study 2 evaluated exposures of 0.1–1925 J/cm^2 with a fixed verteporfin concentration of 1 mM in three separate substudies, with a total of 152 cores and annuli ($n = 5$–12/condition) isolated from 10 joints. Exposure was parameterized by (2A) fixing irradiance (1.58 W/cm^2) and varying irradiation time (0–600sec) or by (2B) fixing irradiation time (600sec) and varying irradiance (0.26–2.29 W/cm^2). A final set of low exposures with different irradiance and irradiation time combinations were explored (Irradiance: 0.05–1.02 W/cm^2; Irradiation Time: 2–75sec, Table I).

Sample preparation. Full thickness cartilage samples (4 mm and 8 mm diameter) were extracted from the femoral condyles and patellofemoral grooves of immature bovine stifles within 24–36 h of slaughter. Plugs were trimmed to 2 mm thickness and a 3.5 mm defect was punched out of the 8 mm diameter discs. Samples were stored at 4°C in 1X PBS with protease inhibitors and tested within 4 days of harvest. Both implants and annuli underwent a 10 min PBS wash followed by a 15 min enzymatic surface digestion at 37°C using 1 U/mL chondroitinase-ABC (CH-ABC) for enhanced bonding[5], followed by a second 10 min PBS wash.

Verteporfin was dissolved in DMSO to an initial concentration of 30mM and subsequently diluted to treatment levels using 1X PBS. Implants were submerged in the photosensitizer for 20 s (as in our previous studies[11]) and press-fitted into annuli. As assessed histologically, verteporfin penetration into cartilage was 7.72 ± 0.66 μm, essentially restricted to the region of chondroitinase digestion (See supplemental information for further details). Bonding between implants and annuli was produced by irradiating photosensitizer-treated samples with an AlGaINP fiber-coupled diode laser with $\lambda = 686$ nm. After light treatment, samples were equilibrated in room temperature PBS for 20 min prior to mechanical testing.

Mechanical bond test. Implants were pushed-out at 0.5 mm/s using a custom hemispherical tip indenter with 3.24 mm diameter, as previously described[11]. Compressive forces were continuously recorded and the peak compressive force was divided by the annulus–implant interface area to calculate shear strength. An overview schematic of the procedure is illustrated in Fig. 1 and representative force-displacement curves are shown in Fig. 3(C).

In vitro study

An *in vitro* study was performed to test initial bond stability in a pro-inflammatory environment (Study 3). Interleukin-1 alpha (IL-1) is a pro-inflammatory factor found at elevated levels in injured or degenerate joints[20] that has been used extensively *in vitro* to study mechanisms of cell-mediated tissue degeneration and to evaluate

Table I
Benchtop study parameters: verteporfin concentration variation study, light exposure study, and low-exposure (0.1–76.4 J/cm^2) study

Study	Photosensitizer	Concentration [mM]	Irradiance [W/cm^2]	Irradiation Time [sec]	Exposure [J/cm^2]
Verteporfin Concentration Study	Verteporfin	0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 30	1.58	600	947
	AlPc (+Control)	5	1.58	600	947
	Control	0	0	0	0
Light Exposure Study	Verteporfin	1	1.58	0, 10, 19, 37, 75, 150, 300, 600	0, 15.8, 30, 58, 118, 237, 474, 947
	Verteporfin	1	0.255, 0.509, 1.02, 1.58, 2.29, 2.68	600	153, 305, 612, 948, 1,374, 1,608
	AlPc (+Control)	5	1.58	600	947
	Control	0	0	0	0
Low-exposure Study	Verteporfin	1	0.05	2	0.1
	Verteporfin	1	0.127	7, 15	0.89, 1.91
	Verteporfin	1	0.255	4	1.02
	Verteporfin	1	0.509	2, 15	1.02, 7.64
	Verteporfin	1	1.02	75	76.4
	AlPc (+Control)	5	1.58	75	118
	Control	0	0	0	0

different repair strategies[21–24]. This study was divided into two sub-studies with mild (1 ng/mL) and moderate (10 ng/mL) IL-1 doses. We note that we use the descriptors mild and moderate in the context of typical *in vitro* doses, as both are hyper-physiologic doses.

Sample preparation. Full thickness cartilage plugs (4 mm and 8 mm diameter) were harvested aseptically from the femoral condyles and patellofemoral grooves of immature bovine stifles and trimmed to 2 mm thick. Prior to treatment, samples were cultured for 1 day in serum-supplemented culture media (DMEM, 10% FBS, 1% NEAA, 10mM HEPES, 1% AB/AM and 50 mg/mL L-ascorbate-2-phosphate). A 3.5 mm defect was created in the 8 mm disc via a biopsy punch. Samples underwent a 10 min PBS wash followed by a 15 min submersion in 1 U/mL CH-ABC at 37°C to mildly digest Sulfated glycosaminoglycan (sGAG) at the tissue surface for enhanced bonding. A final 10 min PBS wash was performed before randomly separating samples into a Control (Ctrl) group and Verteporfin (BPD) group. Treated implants were submerged in 1mM verteporfin for 20 s before being press-fitted, while negative control samples lacked PS treatment. Treated samples were immediately irradiated at 1.58 W/cm^2 for 75 s.

Culture groups. Both Verteporfin and Control groups were randomly sub-divided into subgroups cultured with or without recombinant human IL-1α for the first 4 days of a 7-day culture period, resulting in four treatment groups: Control (Ctrl), Control + IL-1 (I-Ctrl), Verteporfin (BPD), and Verteporfin + IL-1 (I-BPD). Samples were cultured in four mL of culture media with media collected and replaced every day. The experiment was performed twice, using a mild dose of IL-1 (1 ng/mL, 38 cores and annuli from four legs) and a moderate dose of IL-1 (10 ng/mL, 35 cores and annuli from four legs). Shortly after bonding (Day 0; $n = 9–10$/group) or following 7 days of culture (Day 7; $n = 5–6$/group), the interface shear strength was determined as described above.

Cell viability and biochemical analysis. Viability was qualitatively assessed by examining an additional 2 samples per group using a LIVE/DEAD assay. Samples were sliced and placed in 1 mL of 1X PBS wash for 45 min on a plate shaker inside the incubator. The PBS was replaced by 1 mL of dye (2mM ethidium homodimer-1 and 4mM calcein AM) for 45 min. A final 1 mL, 45 min, 1X PBS wash was followed before imaging under a fluorescence microscope. The width of the cell death zone was measured using Fiji (version 2.0.0-rc-66/1.52b; see Supplemental information). sGAG release to culture media was determined via the DMMB assay[25] with pH of 3.0 and the absorption difference between 525 nm and 590 nm wavelengths. An overview schematic of the *in vitro* procedure is shown in Fig. 1.

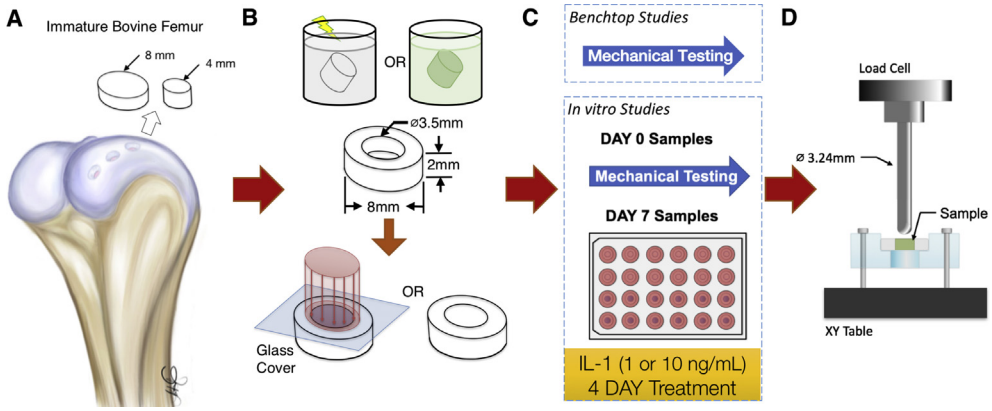

Fig. 1. Procedure followed in benchtop and *in vitro* studies. *In vitro* studies followed aseptic procedure. A) Full thickness four and 8 mm chondral grafts were extracted from bovine stifles and trimmed to 2 mm thickness. A 3.5 mm defect was created in the 8 mm diameter disc. B) Grafts (8 and 4 mm diameter) were exposed to chondroitinase ABC for 15 min and 4 mm grafts in bonded group were treated with photosensitizers at set conditions. 4 mm grafts were press-fitted into annuli and irradiated at set conditions (negative control group was not irradiated). C. In benchtop studies, samples were mechanically tested. In *in vitro* studies, samples were either mechanically tested (Day 0 samples) or cultured (Day 7 samples) with or without an altered environment (1 or 10 ng/mL of IL-1) for first 4 days of 7-day culture period. D) Mechanical testing of samples to determine bond shear strength.

Data analysis

Statistical analyses were performed using Minitab v17 (Minitab, Inc., State College, PA). Shear strengths in the verteporfin concentration study and light exposure studies were analyzed via single factor general linear models. Significance was set at $P < 0.05$ and Bonferroni's test was used for pairwise comparisons when the factor was significant. A power law function was determined via nonlinear regression analysis of combined strength vs exposure data from the varied irradiance and varied irradiation time studies. In the *in vitro* study, shear strength and sGAG release were analyzed via multifactor general linear models with significance set at $P < 0.05$ and Bonferroni's test for pairwise comparisons on significant interactions or non-interacting factors. For shear strength, group treatment (Control and Verteporfin), day of mechanical test (day 0 or day 7) and IL-1 treatment (with or without IL-1 treatment) were set as factors with day of testing nested within treatment group. In the sGAG release analysis, media collection day and treatment group (with or without IL-1) were set as factors with treatment group nested within media collection day. Values are reported as mean ± 95% confidence intervals (CI).

Results

Benchtop studies

Verteporfin produced a substantial shear strength (Fig. 2) at even the lowest concentration tested (0.01mM, 178 ± 42 kPa, $P < 0.001$). All verteporfin concentrations (0.01–30mM) produced shear strengths significantly greater than negative controls, with a maximum mean of 269 ± 20 kPa for 1mM ($P < 0.001$ vs controls). As in studies with other photosensitizers[11], bond strength dropped off at higher concentrations, and the highest concentration tested (30mM) produced shear strengths of 142 ± 35 kPa, significantly lower than those at all lower concentrations ($P < 0.048$).

In the constant irradiance study, all irradiation times (10–600 s, Fig. 3(A)) produced shear strengths that were significantly greater than controls ($P < 0.001$). Samples in Study 2A that were treated with verteporfin and had a 0 s irradiation time did not significantly differ from controls (106 ± 28 kPa, $P > 0.999$). The low exposures show that substantial shear strengths could be achieved within very short time periods as shear strength significantly increased with only 10 s exposure at 1.58 W/cm^2. Verteporfin achieved shear strengths that are equivalent or higher to those produced by the optimal parameters of AlPc (205 ± 33 kPa). Similarly, when the irradiances were varied and the irradiation time was held constant (0.255–2.292 W/cm^2, Fig. 3(B)), the shear strengths produced were significantly greater than controls ($P < 0.001$) with mean shear strength values higher than that produced with optimal parameters of AlPc (183 ± 34 kPa). Maximum mean shear strength (285 ± 77 kPa) was achieved for an irradiance of 1.02 W/cm^2.

In the exposure variation study (Study 2), even the lowest exposure tested (0.1 J/cm^2: 0.05 W/cm^2 irradiance for 2 s irradiation time) produced a significantly greater shear strength than controls (165 ± 31 kPa vs 47 ± 13 kPa, $P < 0.001$). A plot of shear strength vs exposure for all samples in Study 2 (Fig. 4) reveals an apparent power law relationship between shear strength and exposure, with a power law function of $y = 186.55x^{0.0486}$ ($R^2 = 0.29$). This relationship is consistent with the power law relationship found for shear strength vs exposure for AlPc[11], indicating that bond strength is largely determined by the total power delivered to the cartilage interface rather that the rate of delivery or light irradiance. It is interesting to note that shear strengths achieved with verteporfin at lower exposures (0.1 J/cm^2, 165 ± 31 kPa) were substantially greater to those previously achieved at the lowest exposure used with aluminum phthalocyanine chloride (AlPc) (15.8 J/cm^2, 125 ± 27), a phthalocyanine photosensitizer previously tested under similar conditions[10,11]. Shear strengths for verteporfin at low exposures (1.02 J/cm^2: 220 ± 56 kPa) were comparable to those achieved by AlPc at higher exposures (1,600 J/cm^2: 219 ± 39 kPa).

Invitro study

In the first pro-inflammatory study with a mild (1 ng/mL) IL-1 dose, the Verteporfin group (BPD) had a substantial greater Day 0 shear strength than the negative controls (Fig. 5(A), BPD vs Ctrl: 257 ± 26 kPa vs 75 ± 15 kPa, $P < 0.001$). Shear strength in Day 7 Verteporfin groups (BPD and I-BPD) decreased significantly relative to the Day 0 Verteporfin group ($P < 0.001$) yet remained significantly greater than Day 7 Control counterparts (Ctrl vs BPD: 49 ± 17 kPa vs 173 ± 33 kPa and I-Ctrl vs I-BPD: 49 ± 10 kPa vs 165 ± 19 kPa, $P < 0.001$). There were no significant differences between Control groups on Day 0 and 7 ($P > 0.999$). IL-1 supplementation did not significantly affect bond strengths in either Verteporfin group for Day 7 (BPD vs I-BPD: 173 ± 33 kPa vs 165 ± 19 kPa, $P > 0.999$). Neither bonding nor Il-1 treatment significantly affected sGAG release to the media (Fig. 5(B), $P > 0.999$).

In the moderate (10 ng/mL) IL-1 dose study, the shear strength significantly differed between the Control and Verteporfin groups on Day 0 (Fig. 6(A), Ctrl vs BPD: 83 ± 35 kPa vs 241 ± 22 kPa, $P < 0.001$) and remained significantly different on Day 7 for groups not treated with IL-1 (Ctrl vs BPD: 43 ± 26 kPa vs 211 ± 53 kPa, $P < 0.001$) and those treated with IL-1 (I-Ctrl vs I-BPD: 83 ± 32 kPa vs 246 ± 86 kPa, $P < 0.001$). Neither the Control nor Verteporfin groups exhibited significant differences in shear strength across different days and treatment groups ($P > 0.999$). sGAG release into the media was significantly elevated in IL-1 treated samples for

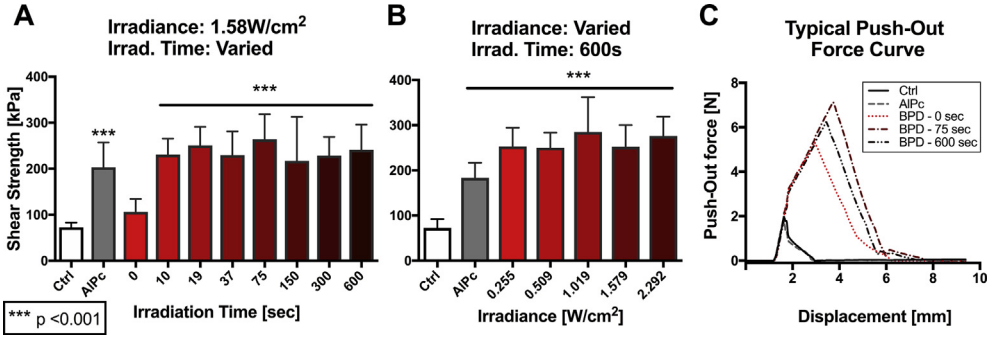

Fig. 2. Shear strengths produced with varied verteporfin concentrations (0.01–30mM, exposure 947 J/cm^2). The Control (Ctrl) group was not treated with PS or irradiated. The AlPc group was treated with 5mM AlPc and irradiated at 1.58 W/cm^2 for 600 s. All concentrations of verteporfin produced shear strengths that were significantly greater than the control group ($P < 0.01$). Verteporfin was very consistent in achieving high bond shear strengths.

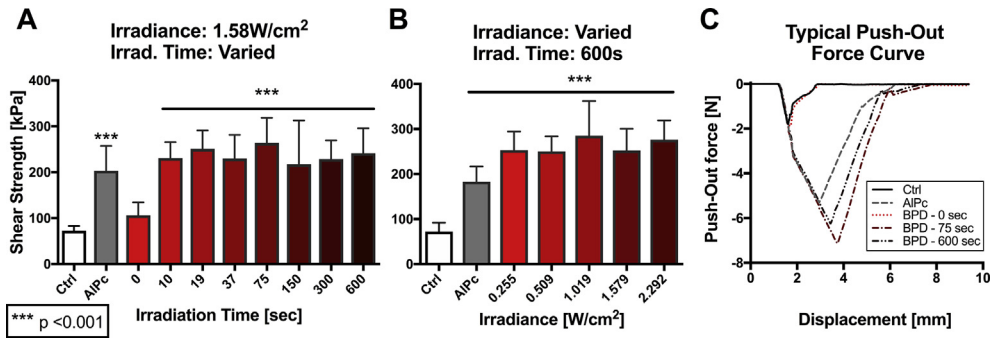

Fig. 3. Shear Strengths for the exposure variation study. A) Exposure was first explored by holding irradiance constant (1.58 W/cm^2) and varying irradiation time from 0 to 600 s (1mM verteporfin). B) Conversely, the irradiance was varied between 0.255 W/cm^2 and 2.292 W/cm^2 while the irradiation time was held constant (600 s, 1mM verteporfin). Control (Ctrl) groups were not treated with PS or irradiated. AlPc groups were treated with 5mM AlPc and irradiated at 1.58 W/cm^2 for 600 s. C) Representative force-displacement push–out curves for Ctrl, AlPc, and BPD samples. BPD samples displayed in this plot had irradiation times of 0, 75, and 600 s at an irradiance of 1.58 W/cm^2.

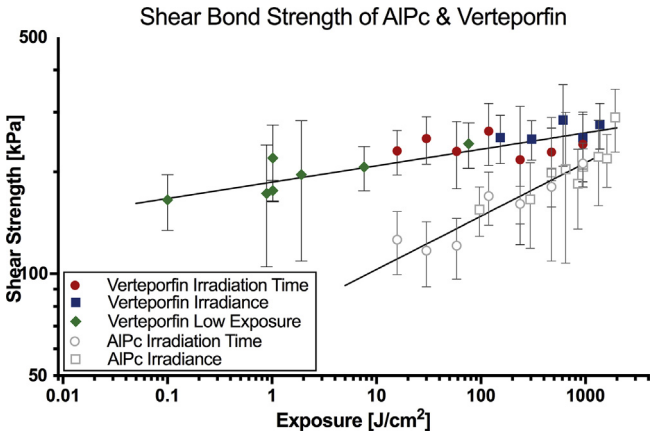

Fig. 4. Shear strength vs total light exposure for verteporfin reveals a power law relationship between bond shear strength and exposure, $y = 186.55x^{0.0486}$ ($R^2 = 0.29$). Data points in red and blue are from the varied irradiation time and varied irradiance studies presented in Fig. 3, and data points in green are from additional tests performed at very low exposures. Data points in white are shear strength values obtained using AlPc from a previous study[11]. The power law relationship between bond shear strength and exposure for AlPc is: $y = 71.57x^{0.1577}$ ($R^2 = 0.395$)[11].

both Control and Verteporfin groups on Day four and for the Verteporfin group on day 5 [Fig. 6(B)].

LIVE/DEAD cell staining for all subgroups in the IL-1 study, (control, mild and moderate IL-1 dose), showed an expected zone of cell death near the cut surfaces with viable cells throughout the tissue (Fig. 7). The cell death zone was approximately 100 μm wide in control and treated samples on day 0 and in control samples on day 7, but at approximately 200 μm was significantly wider in day 7 bonded samples than in all other groups ($P < 0.001$; see supplemental information for additional details).

Discussion

Previous studies have shown the feasibility of using photochemical treatment to integrate cartilage grafts to native tissue, but the light exposure conditions required for substantial bonding were at the limit of clinical feasibility (long exposure at high intensity to bond small implants)[9–11]. In the studies presented here, verteporfin, an FDA approved photosensitizer not previously used for articular cartilage bonding, produced substantial bond strengths (Fig. 4) at light exposures well below the lower limit for effective bonding with AlPc, the most effective agent in our previous comparison study[11]. This raises the prospect of achieving effective implant-host integration with only a few seconds of illumination at

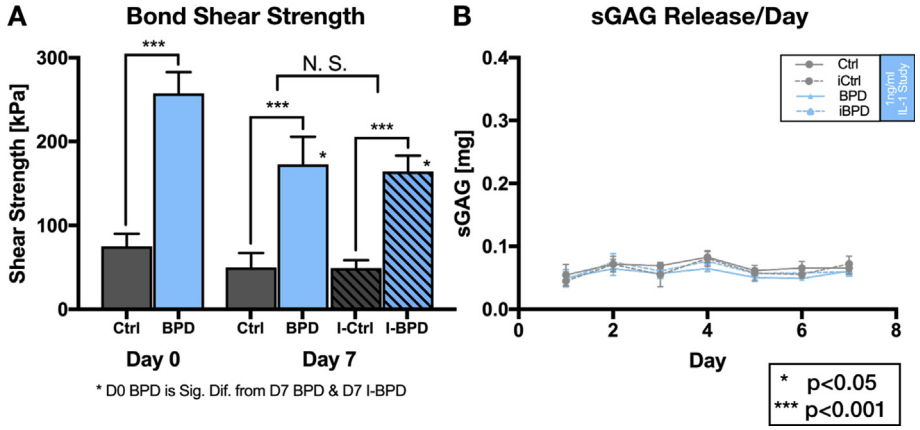

Fig. 5. Shear strengths for Days 0 and 7 of the mild IL-1 dose Study (1 ng/mL IL-1 dose, BPD group bonding conditions: PS concentration—1 mM BPD; irradiance—1.58 W/cm^2; irradiation time—75 s). No significant difference was observed between Control (CTRL) groups on either Day 0 or 7 ($P > 0.999$). Verteporfin (BPD) groups were significantly different from Control groups for Day 0 and for both groups on Day 7 ($P < 0.001$). Importantly, no significant difference was observed for BPD groups (IL-1 treated and non-treated) on Day 7 ($P > 0.999$). Shear strengths between BPD groups decreased from Day 0 to Day 7 ($P < 0.05$). Sulfated glycosaminoglycan (sGAG) release into the media did not significantly differ among groups on all days.

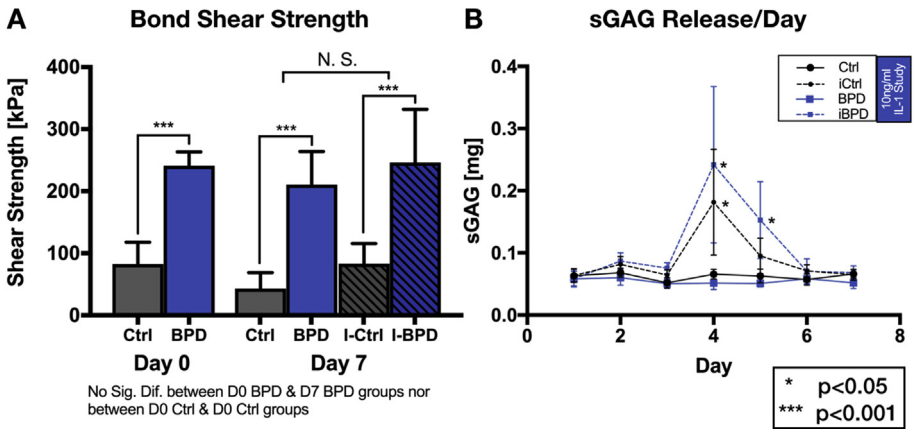

Fig. 6. Shear strengths for Days 0 and 7 and sGAG release to media for a moderate dose of IL-1 (10 ng/mL IL-1 dose, BPD group bonding conditions: PS concentration—1 mM BPD; irradiance—1.579 W/cm^2; irradiation time—75 s). Shear strengths between Control groups and between BPD groups did not significantly differ between Day 0 and 7 and between groups treated with and without IL-1 on Day 7 ($P > 0.999$). Bonded groups were significantly different from Control (CTRL) group for assigned day and treatment group ($P < 0.001$). Most importantly, bonded groups treated with and without IL-1 had no significant differences ($P > 0.001$). sGAG release into the media was significantly different for both Control and BPD groups treated with IL-1 on Day four and BPD group treated with IL-1 on Day 5 ($P < 0.001$).

low light exposure, substantially enhancing the clinical feasibility of this strategy[1].

While the increased cell death adjacent to the interface 7 days after treatment is concerning, delayed cell death up to ~100 μm from the interface is unlikely to be a direct consequence of the bonding procedure itself. The laser light was applied to the entire sample, the verteporfin was restricted to within 10 μm of the interface, and the photochemical reaction products decay rapidly (μs-ms) and would thus not affect cells away from the interface during or after laser illumination. We suspect that the delayed cell death could have been induced by ambient light activation of verteporfin as it diffused into the tissue, as samples were kept in the tissue culture hood for up to ~2 h after bonding for practical reasons Indeed, a recent study suggested that ambient light exposure could explain many purported light-independent effects of verteporfin, including cytotoxicity[42]. While sustained ambient light exposure after bonding would not be encountered in a surgical setting, verification of the specific mechanism and identification of treatment parameters and surgical procedures that do not generate this delayed cell death will be important. As live/dead staining is a relatively simple approach to assessing cellular effects, long-term viability and phenotypic stability will be important considerations in moving this approach towards the clinic.

The disc-annulus model used in quantifying bond shear strength in the benchtop and *in vitro* studies is representative of a chondral/osteochondral defect model where the interface between the graft and native tissue is perpendicular to the articulating surface[10,26–28]. Advantages of integrating cartilage via photochemical bonding over other integrating techniques include the rapid and durable production of high bond strengths—in particular for verteporfin, bond strengths of 219 kPa can be produced within 2 s of being exposed to an irradiance of 0.1 W/cm^2. In comparison, other integration techniques such fibrin glues or tissue transglutaminase produced bond strengths of 40–50 kPa under ideal conditions, and 2 weeks of *in vitro* tissue apposition created bond strengths of 20–100 kPa[29,30] Photochemical bonding using verteporfin can create high and durable bond strengths that do not cause negative effects to surrounding tissue produced by cyanoacrylates, photo-thermal welding or suturing[5,10,31].

Since photochemical bonding is a zero-length bonding mechanism[10,32], a mild enzymatic surface digestion is needed to release sGAG and create a collagen rich substrate to covalently crosslink proteins/

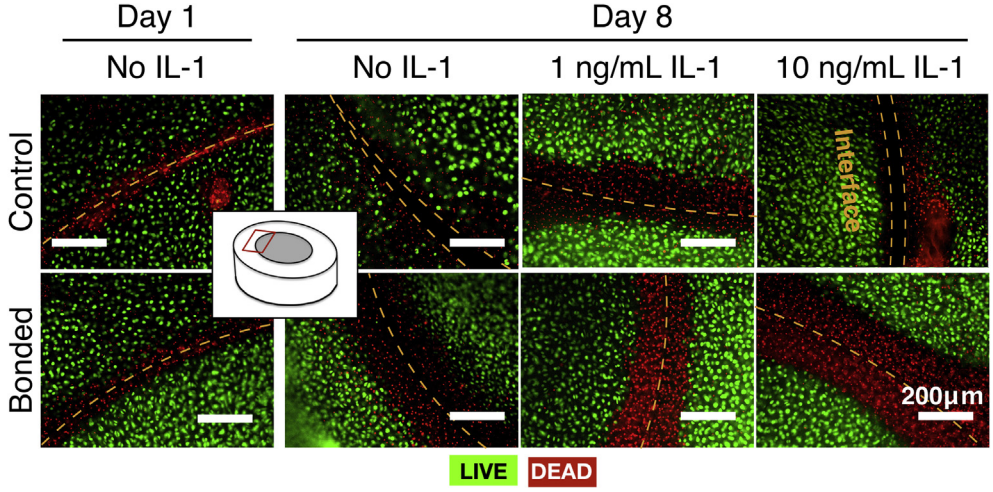

Fig. 7. Cell viability for Days 1 and 8 of IL-1 studies. Baseline studies represent control groups, for day 1 and 8. Control row of images indicated samples that did not undergo photosensitizer treatment or laser irradiation, while Bonded row of images indicate samples that underwent both photosensitizer and laser irradiation.

microfibrils across the interface[28,33–35]. Enzymatic cartilage surface digestions have previously been shown to promote integration due to the higher collagen content and cell density at the compressed interface[26,30]. A highly crosslinked collagen interface would not affect nutrient transfer to graft as nutrients are diffused primarily through the cartilage's surface with the aid of joint loading[36,37].

Similar to CASPc and AlPc, photosensitizers previously explored[10,11], verteporfin acts through both type I and type II mechanisms with a tendency to favor the type II mechanism (driven by singlet-oxygen generation) as its primary form of creating crosslinks within and across the interface[38,39]. This is apparent in the exposure study 2A and 2B that show that crosslinking occurs immediately after irradiation with negligible increase of bond strength over time. One possible explanation for the negligible increase in shear strength is rapid consumption of oxygen at the interface and minimal replenishment during continued light exposure[5,40]. The type II mechanism has been previously found to be important for bonding collagen rich tissues as the singlet oxygen reacts with selected amino acids, primarily histidine, to create bonds between neighboring proteins and amino acids[31,41].

Bond shear strength using verteporfin is affected by overall light exposure and photosensitizer concentration. Consistent with previous findings with AlPc[11], the bond strength achieved at a fixed verteporfin concentration is predominantly dictated by the overall light exposure rather than individually by the intensity or duration of irradiation. Also similar to patterns observed with AlPc and CASPc[10,11] the bond strength for a fixed exposure increased with concentration at intermediate concentrations (0.05–5mM) but decreased at higher concentrations. A possible explanation for the decrease in shear strengths at higher concentrations is oversaturation of photosensitizer at and adjacent to the interface that depletes oxygen rapidly upon activation and reduces the degree of type II crosslinking across the interface. Although these overall patterns are similar between verteporfin and AlPc, verteporfin was able to produce shear strengths with very low total exposure comparable to the maximal values produced with AlPc at substantially higher exposure. As a practical matter, the ability to produce effective bonding in seconds at relatively low exposure (as opposed to minutes at moderate laser power) offers substantial advantages for clinical translation.

Although the rapid production of high shear strengths is important for cartilage repair in the clinical setting, bond stability and robustness in traumatic or injured joints is needed for successful overall repair. In culture, shear strengths of bonded samples remained higher than Controls for both IL-1 doses tested. Importantly, IL-1 supplementation did not detrimentally affect the bonded interface during culture as neither shear strengths nor cell viability staining were affected, suggesting that the newly created interface is not preferentially susceptible to cell-mediated catabolism, even at the moderate dose of IL-1. The persistent bond strength over time in culture may be attributed to the non-native collagen crosslinking that is less susceptible to degeneration[34], to protection conferred by the high collagen density at the press–fit interface, or to minimal expression of collagen-degrading enzymes at early time points. Nevertheless, the initial insensitivity to cell-mediated catabolism suggests that the bonds would be relatively insensitive to the initial inflammatory environment in a patient's joint.

Verteporfin is a promising photosensitizer agent for integrating AC implants due to the high shear strengths achieved at relatively low light exposures, and the bond effectiveness was not reduced by mild to moderate degenerative environments induced by IL-1 exposure. Verteporfin is an FDA approved photosensitizer for photodynamic therapy, which would facilitate either experimental off-label use or eventual approval for a different application as in these studies. These studies have shown the feasibility for using verteporfin for photochemical bonding of articular cartilage, motivating further exploration of verteporfin as a strategy to augment clinical cartilage repair. A preclinical animal model is an essential next step towards determining the efficacy of verteporfin and photochemical bonding in the enhancement of cartilage integration *in vivo*.

Contributions

All authors contributed to study conception and design. Studies were performed by ALA and AIM and analyzed by MEL (levenston@stanford.edu) and ALA. MEL and ALA participated in data interpretation and writing the manuscript. All authors reviewed the final manuscript.

Competing interests

The authors have no conflicts of interest to disclose.

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Supplementary data

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