Supplementary information

Real-time, non-invasive monitoring of hydrogel degradation using $LiYF_4:Yb^{3+}/Tm^{3+}$ NIR-to-NIR upconverting nanoparticles

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Experimental section

Materials. Medium molecular weight chitosan (CH) (MW 190,000 – 300,000 Da) with a degree of deacetylation 75-85% , hyaluronic acid (HA) sodium salt from *Streptococcus equi* (MW 1500,000–1800,000) and β-glycerophosphate disodium salt hydrate (BGP), lysozyme from chicken egg white, hydrochloric acid, trifluroacetic acid (CF_3COOH), octylphenoxy poly(ethyleneoxy)ethanol (IGEPAL) were purchased from Sigma Aldrich. Oleic acid, n-hexane, cyclohexane, ammonium hydroxide, tetraehyl orthosilicate (TEOS), 1-octadecene, yttrium oxide (Y_2O_3), ytterbium oxide (Yb_2O_3), thulium oxide (Tm_2O_3), and lithium trifluroacetate (CF_3COOLi) were purchased from Alfa Aesar. Genipin was purchased from Challenge Bioproducts co., Limited, Taiwan. Dulbecco's modified Eagle's medium high glucose (DMEM-HG) and glycerol solution were purchased from Gibco Life Technologies Invitrogen. US. Fetal bovine serum albumin (FBS), Trypsin solution (0.25%), LIVE/DEAD® Kit for mammalian cells were purchased from Molecular Probes, Invitrogen US. Cell culture chamber slides were purchased from Thermo Scientific Inc. US.

Synthesis of $LiYF_4$:Yb^{3+}/Tm^{3+} Upconverting Nanoparticles (UCNPs). Yb^{3+}/Tm^{3+} co-doped $LiYF_4$ UCNPs were prepared via thermal decomposition using a previously described method with some modifications.[1, 2] Briefly, trifluoroacetate precursors of Y, Yb and Tm were prepared by dissolving Y_2O_3 (1 mM), Yb_2O_3 (0.27 mM) and Tm_2O_3 (0.005 mM) in a mixture of CF_3COOH and DI water (1:1 volume ratio), in a three neck flask. The mixed solutions were refluxed at 80 ºC for 12 h or until a clear solution was obtained. The precursors were dried at 60 ºC for an additional 12 h. To prepare the UCNPs, the precursor powders were dissolved in a mixture of oleic acid (5 ml) and 1-octadecene (5 ml). Next, CF_3COOLi (2.5 mM) was added, the mixture was heated to 135 ºC and degassed for 30 min using a vacuum pump. In a separate flask,

oleic acid (30 ml) and 1-octadecene (30 ml) were heated to 325 ºC under vacuum. The precursor solution was then injected into a second flask using a syringe pump at a constant rate of 0.5 ml min^{-1} under argon purging. The final mixture was kept at 325 ºC for 60 min under constant stirring. After reaction completion, the temperature was lowered to room temperature. The UCNPs were precipitated using an excess of ethanol, redispersed in n-hexane and centrifuged. The nanoparticles were washed three times and finally dispersed in cyclohexane.

Synthesis of silica-coated UCNPs ($LiYF_4$:Yb^{3+}/Tm^{3+}@SiO_2). Silica-coated UCNPs were prepared using a previously described method with some modifications.[3] Briefly, 40 mg of as-synthesized oleic acid capped UCNPs were dispersed in cyclohexane; 0.1 ml of IGEPAL was added and the mixture was stirred for 10 min. Next, 0.4 ml of IGEPAL and 0.08 ml of NH_4OH were added, and the vial was sonicated for 20 min, followed by the addition of 0.04 ml of TEOS. The final mixture was left at room temperature for 48 h under stirring. Silica-coated UCNPs were then centrifuged, washed and redispersed in DI water.

Synthesis of CH-HA-BGP-GN and CH-HA-BGP-GN/UCNPs@SiO_2 hydrogels. CH-HA hydrogels co-crosslinked with BGP and GN were synthesized using our previously described method. [4] All solutions were cooled to 4 ºC prior to mixing. CH solution (3.1% w/v) was prepared in 0.1M HCl. The pH was adjusted to 7.25 using BGP solution (58% w/v in DI water). Next, HA solution (1% w/v in DMEM-HG) and GN (3%w/v in ethanol) was added to the neutralized CH solution up to final concentrations of 2.5% w/v and 0.01% w/v respectively.

To prepare hydrogel/UCNP composites, UCNPs@SiO_2 were dispersed in CH-HA-BGP-GN solutions at a concentration of 0.5 mg/ml followed by incubation at 37 ºC. The ratios of each component in gel/UCNP mixture is given in Table S1.

TEM imaging. Oleic acid-capped and silica-coated UCNPs were dispersed in hexane and DI water respectively. Small aliquots of suspensions were dried on the surface of carbon-coated TEM copper grids (SPI Supplies / Structure Probe, Inc. US). The samples were imaged using a Philips CM200 (Philips, Germany) operated at an accelerating voltage of 200 kV and equipped with Gatan ultrascan imaging system.

Compression testing. To prepare samples for compression testing, CH-HA-BGP, CH-HA-GN, CH-HA-BGP-GN and CH-HA-BGP-GN/UCNPs@SiO_2 gels were cast in cylindrical plastic molds with internal diameter of 9.5 mm. The ratios of CH, HA, BGP, GN and UCNPs@SiO_2 are shown in Table S1. Compression testing was performed using an Electroforce 3200 (Bose Corporation, MN, US) with a 20 N load cell. The heights and diameters of samples were measured using a vernier caliper. All values were averaged by measuring at three different positions on both diameter and length. The compression rate was kept constant at 1.66%/sec for all samples. The gels were compressed to 50% followed by 5s relaxation and recovery at the same displacement rate. Stress-strain curves were plotted to compare the strength of the gels at different strain percentages.

In-vitro hydrogel degradation and UCNPs@SiO_2 release. CH-HA-BGP-GN/UCNPs@SiO_2 were freeze-dried using a lyophilizer (BenchTop K freeze dryer; VirTis, SP Industries, US). The dry scaffolds were cut into small pieces, weighed (*Wi*), immersed in PBS and PBS-Lys at 37 ºC in an incubator. At each time points, the gels were removed from solutions, freeze dried and weighed (*Wf*) again. The PBS and PBS-Lys solutions were saved to determine the amount of released UCNPs@SiO_2. The weight loss was calculated from the following equation:

$$Weight\ loss\ percentage\ (WL\%) = (Wi - Wf) * 100/Wi \qquad \text{(Equation 2)}$$

The amount of released UCNPs@SiO_2 was estimated by collecting emission spectra from the solutions. A calibration curve was first built by dispersing known amounts of UCNPs@SiO_2 in PBS and PBS-Lys. The amount of released UCNPs was then determined from the calibration curve.

Cell viability. UCNPs@SiO_2 suspensions prepared in DI water were autoclaved at 121 ºC for 30 min. Chondrocyte cells were dispersed in DMEM-HG supplemented with concentrations of UCNPs@SiO_2 ranging from 0.05-0.5 mg ml^{-1}. The cells were cultured in 8 well chamber slides (Thermo Scientifc Inc. USA) with a culture density of 10^6 cells ml^{-1} in a standard cell culture incubator.

Live/dead assay was performed after 48 h of culture to evaluate the percentage of live and dead cells. Briefly, glycerol solution (10 ml, 1% v/v) was prepared in PBS followed by the addition of calcein (6 µl) and ethidium bromide (18 µl). Next, culture medium was removed and 300 µl of Live/Dead buffer was added into each chamber. The slides were covered with aluminum foil and kept in the dark for 15 min. Fluorescence images were taken using LSM 5 Carl Zeiss confocal laser scanning microscope using 488 nm and 543 nm excitation wavelengths.

Hydrogel injections into live IVDs. After 72 h of trypsin treatment, two IVDs were injected with approximately 150 µl of CH-HA-BGP-GN mixture containing 0.5 mg ml^{-1} of UCNPs@SiO_2. Two sterile sutures were introduced on the opposite ends of a diameter line of each IVD. These landmarks were used to track gel degradation. The IVDs were then immersed in 30 ml of DMEM-HG in sterile plastic cups and the temperature was raised to 37 ºC to form gels in-situ. The IVDs were then cultured for 3 weeks in a standard cell culture incubator. Cell culture media was replaced every 3 days.

PL spectroscopy and NIR imaging. The IVDs were placed in clear sterile plastic zipper bags and filled with 20 ml of DMEM-HG. The IVDs were irradiated using an unfocused fiber-coupled 975 nm diode laser (Thorlabs Inc., NJ, US) with a maximum power of 330 mW. The laser current was set at 300 mA (~120 mW) during all measurements. Laser power density was approximately 2.5 W/cm^2. The irradiation time varied from 5 to 8 sec. Such low power density and short irradiation time is unlikely to cause any cell damage or heating of the tissue during image capturing. All NIR images were captured using an infrared converted Nikon D80 digital camera equipped with a 135 mm *f* 2.8 objective (Nikon corp., Japan). An 800 nm Hard Coated Broadband Bandpass Interference Filter: 50 nm FWHM (Edmund Optics Inc. US) was placed in between the IVD and the camera. Upconversion PL spectra were recorded using an Avaspec-2048L-USB2 spectrophotometer (Avantes, Netherlands). The movement of IVDs was controlled using a graduated stage.

Images analysis. Image J software was used to analyze all NIR images. A previously reported method was used to measure the PL intensity of images.[5] Briefly, the area and integrated density of each image and each image background was measured. The corrected total image intensity (CTII) was then calculated using following equation:

$$Corrected\ total\ image\ intensity\ (CTII) = Integrated\ density\ of\ image - (Area\ of\ image * Mean\ background\ intensity)$$

(Equation 3)

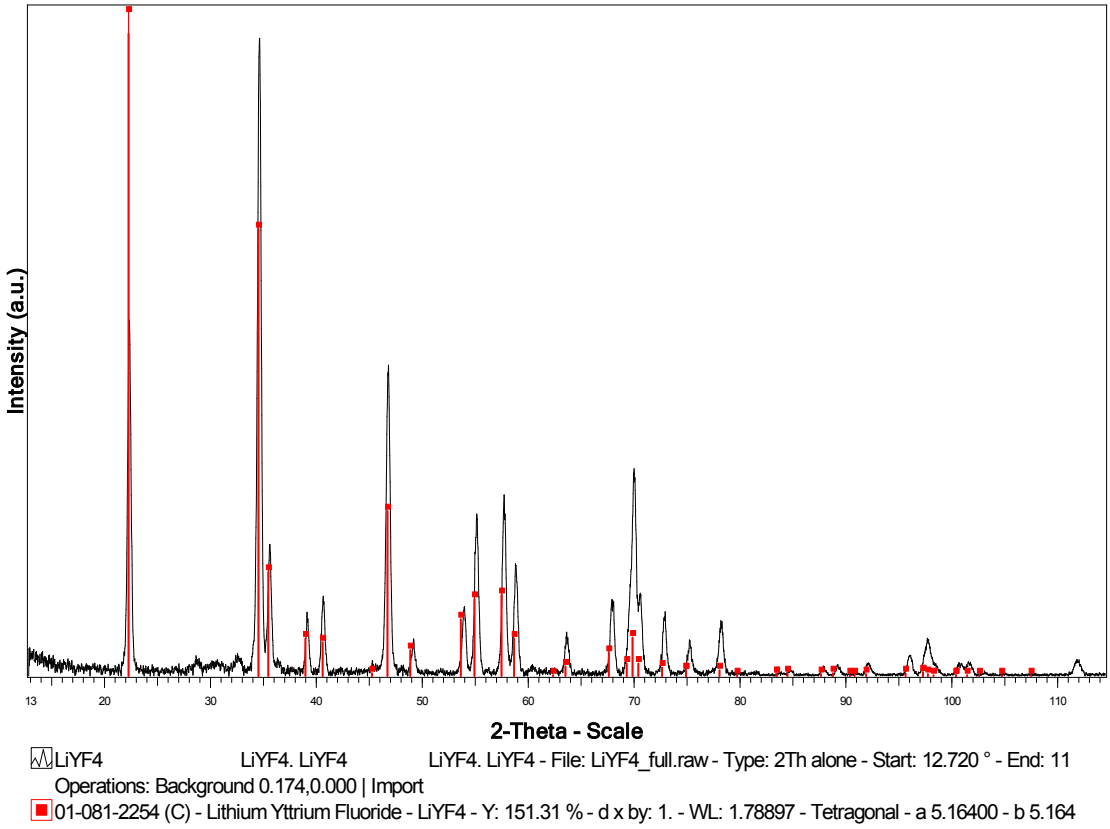

Figure S1. X-ray diffraction patterns of UCNPs (black) and reference spectrum of $LiYF_4$ (red).

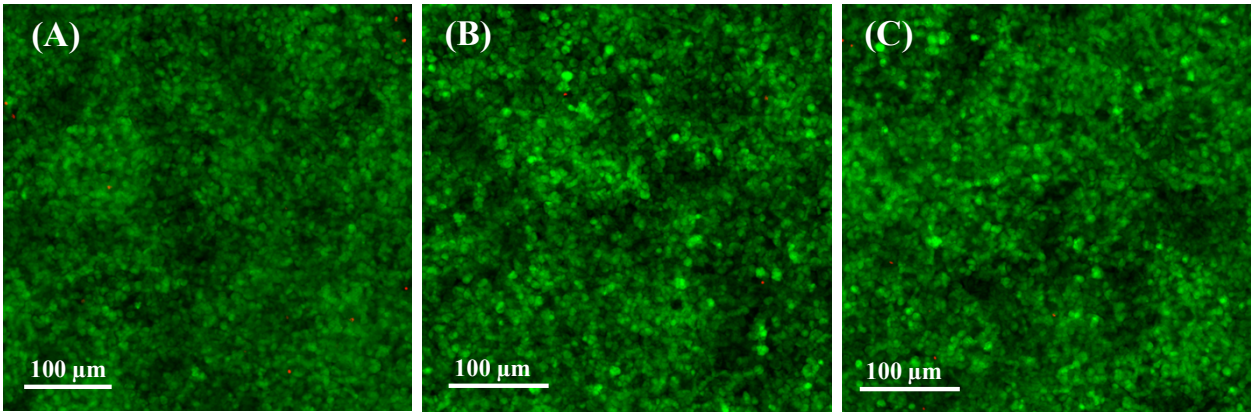

Figure S2. Combined live/dead images of chondrocyte cells cultured in DMEM-HG supplemented with (A) 0.1, (B) 0.3 and (C) 0.4 mg/ml of UCNPs@SiO_2.

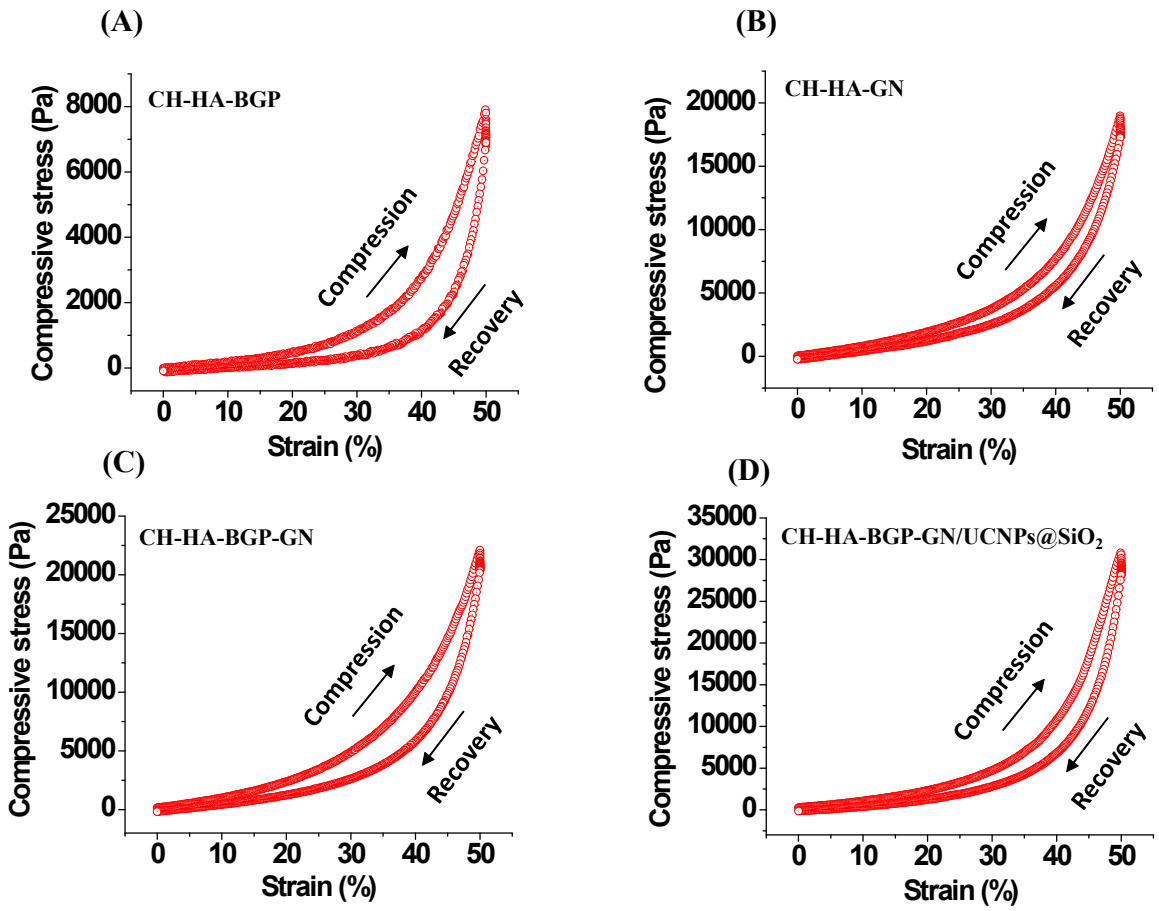

Figure S3. Compression and recovery curves as a function of time measured on CH-HA gels crosslinked with (A) BGP, (B) GN, (C) BGP-GN and (D) BGP-GN with the addition of UCNPs@SiO_2 at a concentration of 0.5 mg/ml. All samples are compressed to up to 50% of their original height followed by recovery with 5 sec relaxation at the top. All gels show complete structure restoration after being relaxed from 50% compression.

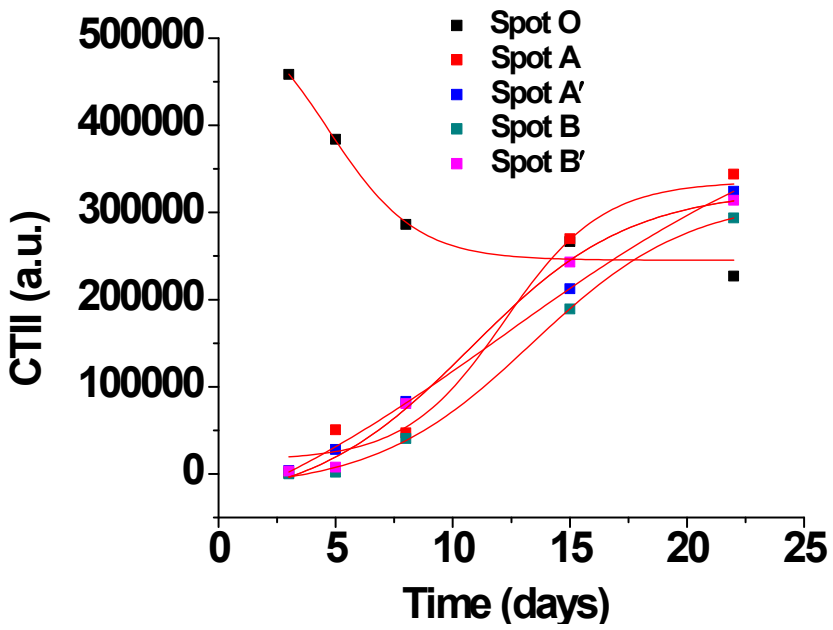

Figure S4. Plots of corrected total image intensity (CTII) at spot O, A, A′, B and B′ of live IVD as a function of time.

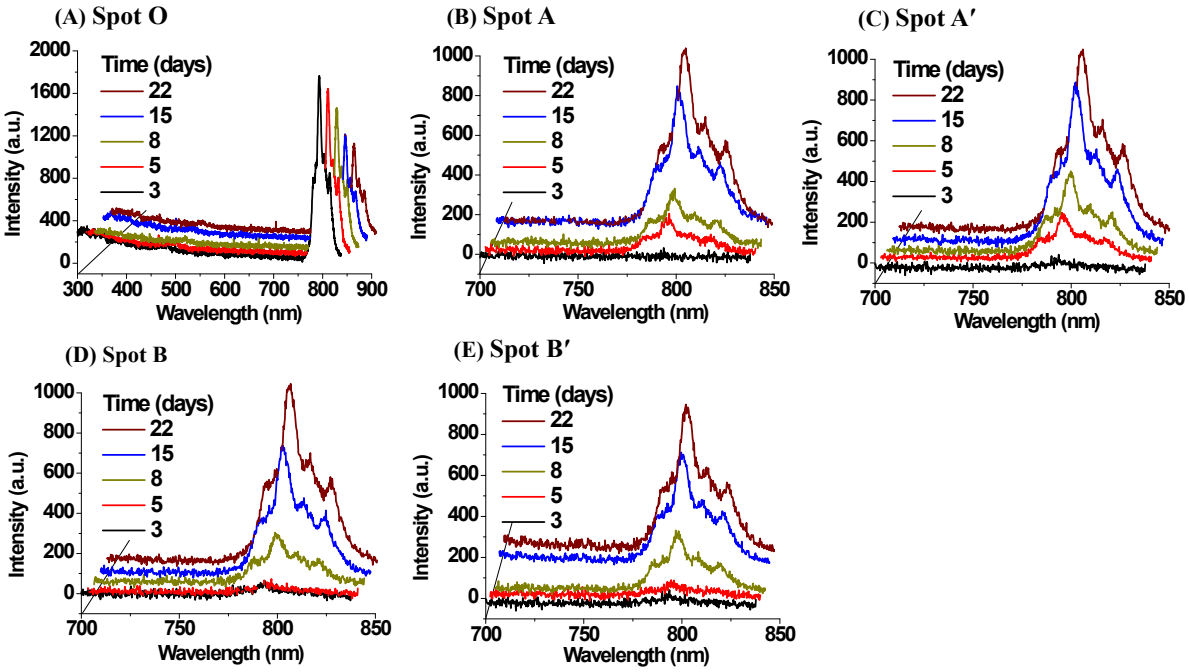

Figure S5. Upconversion PL spectra of UCNPs@SiO_2 recorded at different locations on the IVD as a function of degradation time. The spot location refers to the schematic shown in Figure 4B. The complete spectra are shown in A, whereas in B-E we reported only the region containing peaks (700-850 nm) for clarity.

Table S1. Names and compositions of samples studied in this work

Samples	CH [w/w%]*	HA [w/w%]*	BGP	GN [w/w% of CH]	UCNPs@SiO_2 [w/v%]‡
CH-HA-BGP	97.5	2.5	Yes	No	No
CH-HA-GN	97.5	2.5	No	0.01	No
CH-HA-BGP-GN	97.5	2.5	Yes	0.01	No
CH-HA-BGP-GN/UCNPs@SiO_2	97.5	2.5	Yes	0.01	0.5

(*) The w/w% in CH and HA columns refers to the final weight percentages of CH and HA powders in the gel mixture, not the overall gel weight.
(‡) The w/v% in UCNPs@SiO_2 column refers to the weight (mg) of UCNPs dispersed in the final volume (ml) of gel.

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2. J. C. Boyer, F. Vetrone, L. A. Cuccia and J. A. Capobianco, *J. Am. Chem. Soc.*, 2006, **128**, 7444-7445.
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4. G. Jalani, D. H. Rosenzweig, G. Makhoul, S. Abdalla, R. Cecere, F. Vetrone, L. Haglund and M. Cerruti, *Macromol. Biosci.*, 2015, **15**, 473-480.
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