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Using Green Emitting pH-Responsive Nanogels to Report Environmental Changes within Hydrogels: A Nanoprobe for Versatile Sensing

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Using green emitting pH-responsive nanogels to report environmental changes within hydrogels: A nanoprobe for versatile sensing

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ABSTRACT

Remotely reporting the local environment within hydrogels using inexpensive laboratory techniques has excellent potential to improve our understanding of the nanometer-scale changes that cause macroscopic swelling or deswelling. Whilst photoluminescence (PL) spectroscopy is a popular method for such studies this approach commonly requires bespoke and time-consuming synthesis to attach fluorophores which may leave toxic residues. A promising and more versatile alternative is to use a pre-formed nanogel probe that contains a donor / acceptor pair and then “dope” that into the gel during gel assembly. Here, we introduce green-emitting methacrylic acid-based nanogel probe particles and use them to report the local environment within four different gels as well as stem cells. As the swelling of the nanogel probe changes within the gels the non-radiative energy transfer efficiency is strongly altered. This efficiency change is sensitively reported using the PL ratiometric intensity from the donor and acceptor. We demonstrate that our new nanoprobe can reversibly report gel swelling changes due to five different environmental stimuli. The latter are divalent cations, gel degradation, pH changes, temperature changes and tensile strain. In the latter case, the nanoprobe rendered a nanocomposite gel mechanochromic. The results not only provide new structural insights for hierarchical natural and synthetic gels, but also demonstrate that our new green-fluorescing nanoprobe provides a viable alternative to custom fluorophore labelling for reporting the internal gel environment and its changes.

1. INTRODUCTION

New approaches for innocuously sensing and reporting the local environment within gels including soft tissue continue to attract much interest¹⁻³. Nanoprobes provide potentially useful detection and remote monitoring of local environments within a range of materials^{4, 5}. Photoluminescence (PL)-based probes are particularly attractive for such monitoring because of the high sensitivity of this technique and relatively low cost equipment⁶⁻¹². Hence, PL has been widely used for studies in life¹³, environmental¹⁴ and biomedical sciences¹⁵. A range of fluorescent dyes, quantum dots, micelles, and nanogel probes have been developed that can be imaged in cells or animals^{16, 17} and used to detect physiological changes involving metal ions¹⁸, sugar¹⁹ pH⁶, and temperature²⁰. Whilst PL-based probes involving fluorophores or quantum dot probes may have high sensitivity, water-dispersity can be problematic and/or high toxicity may limit biological applications^{21, 22}. Molecular fluorophores usually require tailored synthetic strategies for coupling to the test material²³⁻²⁵, which can be time consuming and may involve toxic reactants. Here, a new, versatile, green-emitting fluorescent nanoprobe is “doped” into a range of gels and is shown to report gel swelling changes in response to *five* different local environmental changes.

Non-radiative energy transfer (NRET) is a powerful nanometer-scale spectroscopic ruler for measuring the distance variation between donor and acceptor fluorophores²⁶⁻³⁰. A key criterion for NRET is spectral overlap between donor emission and acceptor absorption^{1, 31}. The distance between the donor and acceptor (R) is typically < 10 nm to facilitate excited-state energy to be transferred from the donor to the acceptor via a dipole-dipole coupling^{32, 33}. NRET has been demonstrated for biosensing³⁴, photodynamic therapy³⁵, nano-actuating devices³⁶ and theranostics³⁷. We recently demonstrated that a nanogel probe could sense the internal pH of hydrogels and report compressive strain³⁸. However, the emission wavelengths were limited to the strongly scattering blue region of the spectrum and the nanoprobes had a

low PL quantum yield (PLQY). For this study green emitting pH-responsive NRET-based nanogel probes (NG_{AM/BDP}) were synthesized that contained methacrylic acid (MAA) as well as (9-anthryl)methacrylate (AM) and BODIPY FL amine (BDP) (see Scheme 1). These new nanogel probes embodied a major NRET design change compared to our earlier system³⁸.

In our previous work AM was used as the *acceptor* in our initial nanogel probe particles³⁸. Here we used a new *acceptor* (BDP), which increased the emission wavelength to the green region. Furthermore, AM is used as the *donor* for the new nanoprobe. This design change had three major benefits: (1) the emission red-shifted by 110 nm to the green part of the spectrum; (2) the PLQY greatly increased; and (3) two well-separated excitation wavelengths (λ_{ex} = 254 or 365 nm) were available which provided enhanced operational versatility for λ_{ex} selection. The NG_{AM/BDP} nanogel probes swell when the pH exceeds the apparent pK_a due to $-COO^-$ formation. In the swollen state the nanogel probe may also be compressed by the surrounding gel matrix which affects NRET and is the subject of this study.

Scheme 1 Depiction of changes of non-radiative energy transfer (NRET) for responsive NG_{AM/BDP} nanoprobe particles. (9-anthryl)methacrylate (AM) and BODIPY FL amine (BDP) were the donor and acceptor, respectively. The nanoprobe was prepared using methyl methacrylate (MMA), methacrylic acid (MAA) and ethyleneglycol dimethacrylate (EGD). NRET increases or decreases when external stimuli cause the nanoprobe particles to de-swell or swell, respectively.

In this study we first characterize the new NG_{AM/BDP} nanoprobes and show they can report divalent cations and sense pH in stem cells. The abilities of the NG_{AM/BDP} probes to detect divalent cations in a polyelectrolyte gel and pH in (non-pH-responsive) gelatin is then investigated. The nanogel probes are also shown to report the triggered degradation of gelatin. We then dope the nanoprobes into a temperature-responsive gel and investigate their response to temperature-triggered gel collapse. Finally, we show that the nanoprobes can also act as tensile stain sensors for a uniaxially stretched nanocomposite gel. This study introduces a new versatile green-emitting nanoprobe that has potential to help resolve some of the

relationships that exist between nanoscale and macroscale swelling changes of hierarchical synthetic and natural gels.

2. EXPERIMENTAL DETAILS

2.1 Materials. Methyl methacrylate (MMA, 99%), methacrylic acid (MAA, 99%), ethyleneglycol dimethacrylate (EGD, 98%), and glycidyl methacrylate (GMA, 97%), sodium dodecyl sulfate (SDS, 98.5%), ammonium persulfate (APS, 98%), di(ethylene glycol) methyl ether methacrylate (MEO₂MA, 95%), poly(ethylene glycol) methyl ether methacrylate (OEGMA, $M_n = 500$, 95%), potassium persulfate (KPS, 99%), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 96%), phosphate buffered saline (PBS), potassium dihydrogen phosphate (PDP, 98%), NaOH (97%), CHCl₃ (99%), hexane (95%), *N,N*-dimethylformamide (DMF, 99%), acrylamide (AAM, 99%), *N,N*-methylenebis(acrylamide) (MBAAM, 99%), gelatin (type A), glutaraldehyde (GTA, 25%) and AlamarBlue™ cell viability reagent were all purchased from Sigma-Aldrich and used as received. AM was obtained from Carbosynth Limited. BDP (BODIPY FL amine which is a borondipyrromethene dye) was obtained from Lumiprobe Ltd. Laponite XLS (Clay) was obtained from Rockwood Ltd (UK). 2,2'-Azobis[*N*-(2-carboxyethyl)-2-methylpropionamidine]tetrahydrate (V57, 95%) was obtained from Wako Chemicals. Alexa Fluor 594 phalloidin and MesenPRO RS™ Basal Medium was obtained from Invitrogen Thermo Fisher Scientific. All chemicals were used as received. The water used was multiply filtered and deionized.

2.2. Nanogel synthesis

The materials used to prepare all the nanogels are given in Table S1 and characterization data are shown in Table S2 (ESI†).

2.2.1. Synthesis of NG-*MAA* and NG-*MAA*_{GMA} nanogel particles

NG-*MAA*_{GMA} particles were used to prepare the gels studied in Fig. 2. The synthesis of vinyl-functionalized NG-*MAA*_{GMA} consisted of two steps. The first step was the synthesis of NG-*MAA* particles. These particles were used to prepare NG-*MAA*_{GMA} (below) and also the NG_{BDP} control particles (later).

NG-*MAA*: This nanogel was prepared by semi-continuous emulsion polymerization (see Scheme S1A, ESI†). SDS (1.2 g) in water (240 mL) was added to a three-neck round flask equipped with a mechanical stirrer and reflux condenser. The contents were de-oxygenated and purged with nitrogen. The co-monomer solution (53.1 g) used to prepare the NG contained MMA (42.1 g, 419 mmol), MAA (10.1 g, 116 mmol), EGD (1.1 g, 5.0 mmol). The temperature of the aqueous phase was increased to 80 °C and then aqueous APS (2.2 g of solution with a concentration of 8.0 wt. %) was quickly added. The feed was then commenced and continued for 4 h. The feed rate was 0.32 ml/min. All NG-*MAA* dispersions were purified via extensive dialysis.

NG-*MAA*_{GMA}: NG-*MAA* particles were vinyl-functionalized using GMA via an epoxide ring-opening reaction following earlier work³⁹. The precursor NG-*MAA* dispersion (120 g, 5.0 wt.%) was reacted with GMA (4.5 g, 31.5 mmol). The pH was adjusted to 5.1 and the dispersion heated at 40 °C for 8 h. The NG-*MAA*_{GMA} particles were washed extensively with CHCl₃ and residual solvent removed by rotary evaporation.

2.2.2. Synthesis of NG_{AM} nanoparticles

Blue-emitting NG_{AM} control nanogels were also prepared (see Scheme S1B, ESI†). This system was the precursor used to prepare NG_{AM/BDP} (see below). The initiator was added to begin emulsion polymerization and the feed rate and other details were the same as used for

NG-*MAA* (see above). In this case AM (0.20 g, 0.76 mM) was dissolved in the co-monomer solution (53.0 g) and then together fed into the water (240 mL) containing 1.2 g SDS in three-neck flask which was stirred magnetically. Before the growth commenced the contents were deoxygenated using nitrogen bubbling. The reaction was allowed to proceed for 4 h. To remove free fluorophore the dispersion was dialyzed against water/DMF (volume ratio: 80/20) for 3 days and then pure water for an additional 7 days.

2.2.3. Synthesis of NG_{AM/BDP} nanoprobe particles

The method used to prepare NG_{AM/BDP} involved post-reacting BDP with pre-formed NG_{AM} nanogels (see Scheme S1B, ESI†). Briefly, aqueous NaOH solution (1.0 mL, 4.0 M) was added dropwise to NG_{AM} dispersion (132.6 mL, 0.5 wt.%), and then activated by adding DMTMM (0.59 g, 2.1 mM) for 5 min. Then BDP solution (0.71 mL, 5.86 mM) was added and the reaction continued for 16 h at 25 °C. The pH was 8.9. The final nanoprobe dispersion was purified extensively by dialysis against water for an additional 7 days.

2.2.4. Synthesis of NG_{BDP} nanoparticles

To prepare green-emitting NG_{BDP} control particles (Scheme S1A, ESI†) purified NaOH (1.0 mL, 4.0 M) was added to NG-*MAA* (132.6 mL, 0.5 wt.%) dispersion and then DMTMM (0.59 g, 2.1 mM) added. After 5 min, BDP solution (0.71 mL, 5.86 mM) was added to the activated NG-*MAA* and allowed to react for a further 16 h at 25 °C. The final pH was ~ 8.9. The reaction mixture was purified extensively by dialysis against water for an additional 7 days.

2.2.5. Synthesis of NG-*OEG*_{GMA} nanogel particles

NG-*OEG*_{GMA} particles were used to prepare the temperature-responsive gels studied in Fig. 4. The synthesis of NG-*MAA*_{GMA} consisted of two steps. The first was the synthesis of NG-*OEG* particles. These were used to prepare NG-*OEG*_{GMA} (below).

NG-OEG: This nanogel was synthesized using aqueous precipitation copolymerisation. Briefly, SDS (60 mg) was dissolved in water (240 mL) and the solution was purged with nitrogen gas in a three-neck reactor for 60 min. A comonomer solution (2.77 mL) of MEO₂MA (2270 mg, 12.05 mmol), OEGMA (267 mg, 0.53 mmol), MAA (29.8 mg, 0.35 mmol) and EGD (5.8 mg, 0.03 mmol) was prepared. An aqueous solution of KPS (60 mg in 12 mL water) was subsequently added after the temperature reached 70 °C. The polymerization was continued for 6 h under a nitrogen atmosphere and cooled to room temperature. The dispersion was extensively dialysed.

NG-OEG_{GMA}: The pH of the NG-OEG dispersion (1.0 wt.%, 120 mL) was adjusted to 5.0 and then GMA (0.65 mL) was added while stirring. The dispersion was left to stir for 8 h at 40 °C. The dispersion was then washed with hexane twice and residual hexane removed.

2.3. Hydrogel synthesis

All of the gels discussed below contained 0.10 wt.% NG_{AM/BDP} nanoprobe.

2.3.1. Synthesis of DX NG-MAA(NG_{AM/BDP}) hydrogel

DX NG-MAA_{GMA} (NG_{AM/BDP}) gels were prepared using two mixtures. Mixture A comprised 101 mg of a stock solution prepared using NaOH (120 µL, 4.0 M) and V57 (56 µL, 87 mM). Mixture B contained water (391 µL), NG-MAA_{GMA} (935 µL, 23.1 wt.%) and NG_{AM/BDP} (373 µL, 0.48 wt.%). Addition of Mixture A to Mixture B caused a viscosity increase. The pH was increased to 7.4 (which was greater than the pK_a) by rapidly vortex mixing the two mixtures for at least 2 min, which ensured that a physical gel formed. A covalently interlinked gel was formed by holding the temperature at 58 °C for 5 h.

2.3.2. Synthesis of DX NG-*OEG*(NG_{AM/BDP}) hydrogel

DX NG-*OEG*_{GMA} (NG_{AM/BDP}) gels were prepared following previous work⁴⁰. Mixture A contained NaOH (40 μ L, 4.0 M) and V57 (50 μ L, 87 mM). Mixture B contained NG-*OEG*_{GMA} (1.2 mL, 17.5 wt.%) and NG_{AM/BDP} (0.31 mL, 0.48 wt.%). After blending the mixture became viscous when the pH was adjusted to 7.8 by rapidly vortex mixing the two mixtures for at least 2 min. The viscous dispersion was subsequently cured at 58 °C for 14 h.

2.3.3. Synthesis of Gelatin(NG_{AM/BDP}) hydrogel

To prepare glutaraldehyde (GTA) crosslinked Gelatin(NG_{AM/BDP}) gels, gelatin dispersion (20 wt.%) was prepared by dispersing gelatin particles thoroughly in water (1.0 mL) under magnetic stirring for at least 1 h at 60 °C. NG_{AM/BDP} (414 μ L, 0.48 wt.%), water (486 μ L), and GTA (0.1 mL, 1.0 w/v%) were added and mixed thoroughly. The final hydrogel pH was 7.0 and curing was conducted at room temperature for 2 h.

2.3.4. Synthesis of PAAm-Clay(NG_{AM/BDP}) hydrogel

AAm (399 mg, 4.77 mM), MBAAm (0.40 mg, 2.59 μ M), NG_{AM/BDP} (414 μ L, 0.48 wt.%), Laponite XLS (Clay) (320 μ L, 6.25 w/v%), NaOH (23 μ L, 0.10 M) and V57 (200 μ L, 24 mM) were added to water (1.04 mL) and mixed thoroughly. The final pH was about 8.6 and curing was conducted at 58 °C for 3 h.

2.4. Physical measurements

Titration measurements were conducted in the presence of NaCl (0.050 M) at room temperature with standard 0.10 M NaOH solution and a Mettler Toledo DL15 instrument. The z-average particle size (d_z) was determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS90. All measurements were conducted at 25 °C unless otherwise

stated. Electrophoretic mobility measurements were obtained using a Malvern Zetasizer Nano-2S instrument. TEM images were obtained using a FEI Tecnai 12 BioTwin instrument operating at an accelerating voltage of 110 kV and were stained using uranyl acetate solution. The nanogel dispersions were deposited onto 200 mesh copper TEM carbon grids and then allowed to dry at room temperature. Number-average diameters from TEM images were obtained using Image-J software. Confocal laser scanning microscopy images (CLSM) images were obtained using a broadband confocal Leica TCS SP5 microscope. UV-visible absorption spectra were recorded with a Hitachi U-1800 UV spectrophotometer with a scan rate of 240 nm / min. All the measurements were carried out at 25 °C Uniaxial tensile stress-strain data were conducted using an Instron series 5569 load frame equipped with a 10 N tensile testing head at a rate of 20 mm s⁻¹ using cuboid shaped molds with a central rectangular region of length 20 mm. The modulus values were calculated using the stress-strain data obtained in the strain range of 0 and 10 %. The swelling behaviour of each gel was measured using a gravimetric method. The volume swelling ratio Q value for the gels was calculated using:⁴¹

$$Q = \rho_p \left(\frac{Q_{(m)}}{\rho_s} + \frac{1}{\rho_p} \right) - \frac{\rho_p}{\rho_s} \quad (1)$$

where $Q_{(m)}$ is the mass swelling ratio. ρ_s and ρ_p are the densities of the solvent (water) and polymer, respectively. The ρ_s value used for water was 1.0 g/mL. The ρ_p value used for DX NG-*MAA*_{GMA}, DX NG-*OEG*_{GMA} or Gelatin was 1.2 g/mL. We used the linear swelling ratio ($\alpha = Q^{1/3}$) in this study to report swelling. Photoluminescence (PL) spectra were obtained using an Edinburgh Instruments FLS980 spectrometer. Experimental details regarding the PL measurements for PLQY determination, dependence on pH-triggered swelling, added divalent cations, temperature-triggered hydrogel swelling/deswelling, gelatin degradation, tensile strain-dependent study and NRET analysis are all described in the ESI†.

2.5. Cytotoxicity assays

Human adipose-derived stem cells viability / proliferation test for nanogel probe dispersions were measured using an AlamarBlue™ assay at 1 and 3 days. The cells were seeded on 24-well culture plates in Gibco™ MesenPRO RS™ Basal Medium and grown overnight. After removal of the original medium in each well, the cells were seeded in 24-well plates at a density of appropriate 5.0×10^4 cells per well in (about 310 μL) of complete medium pH at 7.4 containing predetermined concentrations of NG_{AM/BDP}. A nanogel-free cell was used as a control. The cell-seeded dispersion was incubated for 1 day at standard conditions (37 °C under 5% CO₂ and 95% humidity). Afterwards, cell culture media was removed and 0.5 mL of AlamarBlue™ assay in PBS solution (0.01 mg/ml) was used to test each well plate after being incubated for 4 h under standard conditions. Finally, 150 μL of each sample was transferred to a 96-well plate. The cell viabilities were determined by using fluorescence at an excitation wavelength of 540 nm with an emission wavelength of 590 nm using a TECAN Infinite 200 plate reader (Tecan, Männedorf, Zurich, Switzerland). Experiments were performed at least three times for each time point and concentration. The cell viability was calculated based on the following equation³:

$$\text{Cell viability (\%)} = \frac{I_{\text{Sample}}}{I_{\text{Control}}} \times 100\% \quad (2)$$

In Equation 2, I_{sample} and I_{control} represented the intensity of sample and control wells, respectively. Experimental details regarding cell imaging are given in the ESI†.

3. RESULTS AND DISCUSSION

3.1. Nanogel probe characterization and environmental reporting properties

The new NRET NG_{AM/BDP} nanogel probes (Scheme 1) were synthesized using emulsion copolymerization with AM followed by DMTMM activated coupling⁴² of BDP (see Scheme S1B, ESI†). They contained 0.24 mol % of AM and 0.11 mol % of BDP as determined by UV-visible spectroscopy and the Beer-Lambert law (see Fig. S1A and S1B, ESI†). The MAA content and apparent pK_a determined from potentiometric titration data (Fig. S4, ESI†) were 34.8 mol% and 7.1, respectively. De-swollen (collapsed) NG_{AM/BDP} had an average diameter of 20 ± 1 nm based on TEM (Fig. S3A, ESI†) and were negatively charged as shown by electrophoretic mobility data (Fig. S5, ESI†). (Other size characterization data for NG_{AM/BDP} are shown in Table S2, ESI†.) The NG_{AM/BDP} probes showed PL emission over the wavelength range of 370 to 600 nm (see Fig 1A). (PL spectra for the parent AM and BDP molecular dyes are shown in Fig. S1C and S1D (ESI†).) The spectral overlap of the AM PL emission and BDP absorption spectra was substantial which enabled NRET (see Fig. S1E, ESI†). The Förster distance (R_0) is the distance between the donor and acceptor at which the energy transfer efficiency is 50%¹ and was calculated as 2.9 nm. (See ESI† for NRET analysis.) Furthermore, the NG_{AM/BDP} particles were very bright with a PLQY of 86%. Compared to the blue-emitting nanogels previously studied³⁸, the PL emission for the present nanoprobe was red-shifted by ~ 110 nm and is also six times brighter.

Figure 1 (A) PL spectra for NG_{AM}/BDP measured with different Mg²⁺ concentrations ($\lambda_{ex} = 254$ nm). (B) Intensity ratio of donor and acceptor peaks (I_D/I_A) and d_z vs. Mg²⁺ concentration at pH 7.4. (C) I_D/I_A for five divalent cations (30 mM) at pH 8.6, or at pH 7.4 for Zn²⁺. (D) Variation of d_z and I_D/I_A with pH. The insets were obtained using i) $\lambda_{ex} = 254$ nm and ii) $\lambda_{ex} = 365$ nm at the pH values shown. Cellular imaging (merged color channels) of the NG_{AM}/BDP particles in stem cells at pH values of (E) 6.0 and (F) 7.4, respectively. The arrows highlight the location of NG_{AM}/BDP in the cells. Scale bars: 25 μm.

The PL spectra for NG_{AM}/BDP (Fig. 1A) showed a maximum at 513 nm due to BDP (labelled *I_A*) and also at 413 nm due to AM (labelled *I_D*) which were both sensitive to Mg²⁺ as well as Zn²⁺, Ba²⁺, Sr²⁺ and Ca²⁺ (see also Fig. S6 and S7, ESI†). As the Mg²⁺ concentration increased the NG_{AM}/BDP particles de-swelled and NRET became more efficient. The ratio of the donor to acceptor PL intensities (I_D/I_A) was very sensitive to cation-triggered nanoprobe deswelling. As shown in figures 1B and 1C the I_D/I_A values could be measured using excitation wavelengths (λ_{ex}) of 254 or 365 nm. The I_D/I_A ratio closely followed the decrease

in d_z as the Mg^{2+} concentration increased using either λ_{ex} . Indeed, Fig. 1B shows that $\text{NG}_{\text{AM/BDP}}$ can act as an Mg^{2+} ion probe for concentrations of 3.0 mM or higher. The I_D/I_A ratio decreased by nearly half when 30 mM of Ca^{2+} , Mg^{2+} , Ba^{2+} , Sr^{2+} or Zn^{2+} was present (Fig. 1C). In contrast, K^+ had no effect on I_D/I_A . This difference is because divalent cations such as Ca^{2+} form an ionic crosslinks with carboxylate groups⁴³. These ionic crosslinks not only caused the whole nanogel particle to de-swell but simultaneously decreased the average separation of the donor and acceptor (i.e., R) at the sub-10 nm scale. A decrease of R increased energy transfer as evidenced by the decreased I_D/I_A ratios.

$\text{NG}_{\text{AM/BDP}}$ is also pH-responsive and increasing the pH caused I_D/I_A to increase (see Fig. 1D). This trend is due to decreased energy transfer between AM and BDP as a consequence of increased donor-acceptor separation. The data show that the I_D/I_A values closely followed the d_z values. These pH-triggered changes in the PL spectra (shown in Fig. S8B and S8C, ESI†) caused pronounced changes of the emission that were visible to the eye. The $\text{NG}_{\text{AM/BDP}}$ dispersion became more blue at high pH (see insets of Fig 1D). These changes were not due to UV-visible absorption changes as those spectra were pH independent (see Fig. S8A, ESI†). Furthermore, I_D/I_A showed reversibility between pH 6.0 and 8.0. The d_z and I_D/I_A values also closely followed each other (see Fig S9, ESI†). There was, however, a slight upward drift of the data after three pH-switching cycles which may be due to an increase in ionic strength. Nevertheless, these data confirm the strong link between NRET and nanoprobe swelling. The strong correlation between pH-triggered changes in d_z (which measure the whole nanoparticle size) and I_D/I_A (which is sensitive to sub-10 nm distance) shows that (a) both of the fluorophores were well connected to the nanogel particle network and (b) affine swelling occurred.

The stability of both d_z and I_D/I_A as a function of time with excitation wavelengths of 254 nm and 365 nm for the NG_{AM/BDP} nanoprobe was investigated in the presence of aqueous Mg²⁺, Ba²⁺, and Sr²⁺ (30 mM) at pH 8.6. (See Fig S10, ESI†). The d_z and I_D/I_A values changed by an average of less than 5% over a 6 day period and demonstrated good stability.

Biocompatibility is essential for potential imaging and theranostic applications of nanoprobe^{44, 45}. The cytotoxicities of the NG_{AM/BDP} nanoprobe after 1 and 3 days of incubation with human adipose-derived stem cells were evaluated. The cell viabilities were greater than ~90% after incubation for 3 days (see Fig. S11A, ESI†) using concentrations where strong PL signals were recorded. The latter is supported by Fig. S11B (ESI†) where PL spectra are shown at the nanoprobe concentrations used for the cytotoxicity studies. The NG_{AM/BDP} probes can also report the local pH within stem cells. As shown in Fig. S12 (ESI†) intense cytoplasmic red, blue, and green fluorescence signals from the actin-stained Alexa Fluor 594 were observed using CLSM. We note that there is significant auto-fluorescence in the image under the green filter. The merged color channel images are shown in figures 1E and F. NG_{AM/BDP} can be seen concentrated mainly in the cytoplasm of these cells (arrows). Moreover, the concentrated nanoprobe were green and blue when the pH of the cells was 6.0 (Fig. 1E) and 7.4 (Fig. 1F), respectively. This color difference is due to differences in the swelling of the pH-responsive nanoprobe, which were more swollen at pH 7.4 (See Fig. 1D).

An interesting question concerns whether it might be possible to detect cations or the pH in cancer cells? We believe that NG_{AM/BDP} nanoprobe should be able to detect the pH of cancer cells using ratiometric PL intensity⁴⁶. This is because the pH inside the cancer cells is about 7.0 - 7.2⁴⁷⁻⁴⁹ and is lower than the pH in normal cells (7.4). If NG_{AM/BDP} were endocytosed into such cancer cells, the I_D/I_A ratio signal of NG_{AM/BDP} should decrease. As for the effect of nanoprobe on divalent cations in cancer cells, it should, in principle, be also able to detect

such ions⁵⁰⁻⁵² including Mg^{2+} . However, the sensitivity of the probe is limited to greater than ~ 3 mM. From the literatures⁵³⁻⁵⁶, the body's Mg^{2+} ions are mainly concentrated in bones, muscles and soft tissue cells. The concentration of Mg^{2+} ions in the cells is about 5-20 mmol. Our nanoprobe ($\sim 3 - 30$ mmol) should, in principle, be suitable for detection in this range.

3.2. Nanoprobe reporting of ionic crosslinking in a polyelectrolyte hydrogel

Recently, a new family of polyelectrolyte hydrogels have been reported which are assembled by covalent interlinking of MAA-containing nanogel particles³⁹. Those gels are termed doubly crosslinked nanogels (DX NGs) and are optically transparent³⁹. We prepared such a gel by covalently interlinking NG- MAA_{GMA} as depicted in Fig. 2A. The NG- MAA_{GMA} particles had a collapsed diameter of 18 ± 4 nm as determined by TEM (Fig. S3B and Table S2, ESI†). The apparent $\text{p}K_a$ was 6.9. (Other characterization data are shown in Fig. S4 and Table S2, ESI†). A low concentration of NG $_{\text{AM/BDP}}$ (0.10 %) was included during gel preparation to form nanoprobe doped DX NG- $\text{MAA}(\text{NG}_{\text{AM/BDP}})$ gels. The PL spectra for DX NG- $\text{MAA}(\text{NG}_{\text{AM/BDP}})$ gels (Fig. S13A-B, ESI†) showed a maximum at 513 nm (I_A) due to BDP and also at 413 nm (I_D) due to AM. The I_D/I_A ratio and the gel's linear swelling ratio (α) decreased with increasing concentration of Mg^{2+} (Fig. 2B) and these parameters closely followed each other. (Note that $\alpha = Q^{1/3}$, where Q is given by equation 1.) Major decreases of I_D/I_A occurred in the presence of divalent cation solutions (30 mM) due to ionic cross-linking of the COO^- groups (see Fig. 2C and Fig. S13C to S13E, ESI†). Hence, the nanoprobe reported macroscopic gel swelling changes due to ionic crosslinking within polyelectrolyte DX NGs.

Figure 2. (A) Depiction of DX NG-MAA(NG_{AM}/BDP) gel formation. GMA is glycidyl methacrylate. (B) Linear swelling ratio (α) and I_D/I_A as a function of Mg^{2+} concentration. The pH was 7.4. (C) I_D/I_A ratio for the gels in the presence of four cations (30 mM) at pH 7.4.

The reporter NG_{AM}/BDP particles were not covalently attached to the DX NG-MAA network. If the cation-triggered swelling change of the macroscopic gel network was different to the internal gel swelling changes covalent linking of NG_{AM}/BDP to the network may have been required for accurate reporting of the internal gel swelling. Our data show that such a connection was not required. This suggests that the swelling changes at the macroscopic and nanogel size scales were similar, i.e., affine swelling occurred.

3.3. Nanoprobe reporting of degradation and internal pH within a natural gel

The abilities to remotely report the onset of polymer degradation or internal pH in gels are important for biomaterial applications⁵⁷⁻⁵⁹. Gelatin is a natural biocompatible hydrogel^{60, 61}

that is used as a biomaterial and as a food ingredient⁶²⁻⁶⁴. Here, crosslinked Gelatin was prepared by reacting the amine groups with glutaraldehyde (GTA)⁶⁵ (see Fig. 3A). The crosslinked gels did *not* show pH-triggered changes in α at 25 °C over time periods less than 24 h. However, the gel degraded at pH 7.4 and 32 °C, which is proposed to be due to imine hydrolysis⁶⁶. In the first 4 h of degradation 30% of the mass was lost at 32 °C. Samples were taken from the supernatant and UV-visible and PL spectra recorded (see Fig. S14, ESI†). We found that scattering from Gelatin fragments strongly affected I_D . Therefore, the concentration of released nanoprobes, and hence gel degradation was followed using I_A (see Fig. 3B). Hence, the increase in nanoprobe concentration in the supernatant due to nanoprobe release from the gel enabled monitoring of the early stages of gel degradation.

Figure 3. (A) Depiction of crosslinked Gelatin(NG_{AM}/BDP) gel formation. GTA is glutaraldehyde. Measurable gel degradation occurred slowly at 32 °C and pH 7.4. In contrast

no significant disintegration was detectable at 25 °C. **(B)** Degradation of Gelatin(NG_{AM}/BDP) gel studied using PL ($\lambda_{ex} = 254$ nm) at 32 °C. The inset shows a tube containing the original gel (time = 0) at pH 7.4 as well as the gel after 2 h (yellow arrow). The PL data were obtained using the dispersed phase. **(C)** Variation of α and I_D/I_A with pH at 25 °C for Gelatin(NG_{AM}/BDP) gel. The inset pictures were obtained using $\lambda_{ex} = 254$ nm at the pH values shown. The scale bars are 10 mm.

Whilst the Gelatin gel degraded at 32 °C over a period of hours (above), it showed negligible degradation at 25 °C and was effectively non-pH-responsive in the first 24 h. The PL spectra for Gelatin(NG_{AM}/BDP) gel in various pH buffers was measured (Fig. S15, ESI†). Interestingly, the PL spectra for the gels were pH-dependent and the I_D/I_A ratio increased strongly even though the α values hardly changed (see Fig. 3C). Gels constructed using pH indicators confirmed the internal gel pH over the range of 5.6 to 9.0 (Fig S17, ESI†). Hence, the nanoprobes reported the internal pH of a gel in the absence of gel swelling changes. This result shows that the nanogels were able to swell and deswell within the gelatin network. The data suggest that the nanogel particles were not connected to the gelatin network. Rather, they were only loosely caged within spaces that were comparable to their swollen volume. This enabled them to swell and de-swell freely within the Gelatin in response to local pH changes. A high resolution SEM images of a Gelatin(NG_{AM}/BDP) gel freeze-dried at pH 5.6 is shown in Fig. S16 (ESI†). The undulating morphology is consistent with a soft gel.

3.4. Nanoprobe reporting of temperature-triggered gel de-swelling

Remotely reporting the swelling of temperature-responsive gels is potentially important in applications for subcutaneous release or capture of therapeutics⁶⁷. We studied the ability of NG_{AM}/BDP to act as a probe within an injectable temperature-responsive gel⁴⁰. The gel (DX NG-OEG(NG_{AM}/BDP)) was constructed using covalent interlinking of NG-OEG_{GMA} nanogels

in the presence of a small amount of $\text{NG}_{\text{AM/BDP}}$ (see Fig. 4A). Linear poly(OEGMA-MEO₂MA) copolymers have tunable lower critical solution temperatures⁶⁸. The collapsed particle size for NG- OEG_{GMA} was 58 nm based on TEM (Fig. S3C, ESI†). The d_z values for these gel building blocks (pH 5.6) decreased from 137 nm at 4 °C to 55 nm at 40 °C (see Fig. S18, ESI†). Additional characterization data for NG- OEG_{GMA} are given in Table S2 (ESI†).

Figure 4. (A) Depiction of the formation and temperature-responsive behavior of covalently interlinked DX NG- $\text{OEG}(\text{NG}_{\text{AM/BDP}})$ gel. (B) Variation of α and I_D/I_A with temperature for DX NG- $\text{OEG}(\text{NG}_{\text{AM/BDP}})$ gels. (C) Reversible α and I_D/I_A changes for DX NG- $\text{OEG}(\text{NG}_{\text{AM/BDP}})$ with temperature ($\lambda_{\text{ex}} = 365$ nm). The inset shows photographs of a DX NG- $\text{OEG}(\text{NG}_{\text{AM/BDP}})$ gel in an upturned cuvette at 5 and 40 °C ($\lambda_{\text{ex}} = 365$ nm). Scale bar: 10 mm.

The PL spectra for the DX NG- $\text{OEG}_{\text{GMA}}(\text{NG}_{\text{AM/BDP}})$ gels were temperature-dependent (Fig S19, ESI†). The gels scattered light when they de-swelled which prevented the I_D/I_A ratios being used when $\lambda_{\text{ex}} = 254$ nm. However, using the higher λ_{ex} of 365 nm overcame that

problem since there was negligible light scattering at that wavelength. The I_D/I_A ratio and α decreased with increasing temperature (see Fig. 4B). This experiment demonstrated the versatility of the NG_{AM/BDP} probes because two λ_{ex} were available. Here, the pH-responsive nanoprobe reports the contraction or expansion of the nanoprobe network in response to temperature-triggered swelling or deswelling of the macroscopic DX NG-*OEG* network. Variable temperature PL data for the NG_{AM/BDP} nanoprobe confirmed that temperature did not affect I_D/I_A (see Fig S20, ESI†). Fig. 4C shows an upturned cuvettes containing DX NG-*OEG*_{GMA}(NG_{AM/BDP}) gel. The color changed from light blue at 4 °C (gel swollen state) to green at 40 °C (gel de-swollen state) under 365 nm UV light. (Gel deswelling is also evident.) In addition, cyclic α and I_D/I_A data for DX NG-*OEG*(NG_{AM/BDP}) gel showed excellent reversibility (see Fig. 4C). These data demonstrate that NG_{AM/BDP} was able to report temperature-responsive gel swelling / deswelling.

In the DX NG-*OEG*(NG_{AM/BDP}) gel the pH-responsive NG_{AM/BDP} probe particles were dispersed within an interlinked network of NG-*OEG* particles. The swelling extents for this type of gel are altered by temperature-triggered changes in hydrophobicity⁴⁰. The nanoprobe particles were compressed at 40 °C when NG-*OEG* particles de-swelled (Fig. S18, ESI†). Interestingly, the minimum value for I_D/I_A of ~ 1.16 (Fig. 4B) was relatively high compared to the other gels discussed above (Fig. 2B and 3C). This is likely due to the compressive force from DX NG-*OEG* matrix (due to hydrophobic association) being relatively weak compared to the swelling force within NG_{AM/BDP} which originates from electrostatic repulsion of COO⁻ groups.

3.5. Nanoprobe reporting of uniaxial stretching of a nanocomposite gel

The ability to remotely report changes in gel stretching is desirable for applications of high toughness gels in tissue engineering and soft robotics⁶⁹⁻⁷¹. Nanocomposite gels have high toughness⁷² and were prepared by free-radical polymerization of AAm in the presence of

Laponite clay nanoparticles and small quantity of $\text{NG}_{\text{AM/BDP}}$ (see Fig. 5A). The nanoprobe doped gels are denoted as $\text{PAAm-Clay}(\text{NG}_{\text{AM/BDP}})$. Cyclic tensile stress–strain data for the gel (Fig. 5B) showed little hysteresis, in agreement with earlier reports⁷³. The tensile breaking strain, breaking stress and Young's modulus values (Fig. S21A, ESI†) were 435%, 54 kPa and 26 kPa, respectively, and are comparable to those for cartilage⁷⁴. The gel changed from blue to green when stretched (see inset of Fig. 5B) and was therefore mechanochromic. The I_D/I_A ratios obtained from the PL spectra (Fig. S21B, ESI†) decreased from 1.91 to 0.74 ($\lambda_{\text{ex}} = 254$ nm) and 1.13 to 0.62 ($\lambda_{\text{ex}} = 365$ nm) as the strain increased from 0% to 200% (Fig. 5C). The reversibility of tensile strain was investigated (Fig. 5D) and was excellent. This is the first time the ability of nanoprobe particles have been used to monitor tensile strain within a gel. Taking inspiration from the behaviour of micrometer-sized microgels within stretched microgel-reinforced hydrogels⁷⁵ we propose that stretching of the nanocomposite gel caused the nanogel probes to stretch in the direction of strain (z) and de-swell in the perpendicular directions. Hence, the R increased along the z axis but decreased along the x and y axes. Whilst we do not have any direct experimental to support this conjecture, the decrease of I_D/I_A upon stretching is indirect support for the dominance of strain-induced $\text{NG}_{\text{AM/BDP}}$ de-swelling on the overall value for R . A technique such as small-angle neutron scattering would be required to explore this proposed mechanism, which is beyond the scope of the present work which emphasizes inexpensive equipment for gel swelling studies.

Figure 5. (A) Depiction of PAAm-Clay(NG_{AM}/BDP) gel formation and stretching. (B) Cyclic tensile stress-strain data for the gel. Images for the gel (i) before, (ii) during application of strain and (iii) after being allowed to relax are also shown ($\lambda_{ex} = 254$ nm). Scale bar is 10 mm. (C) I_D/I_A vs. tensile strain for PAAm-Clay(NG_{AM}/BDP) gel. (D) Reversible tests from tensile strain 0 % and 200 % for PAAm-Clay (NG_{AM}/BDP) gel. The legend in (C) also applies to (D).

3.6. Comparison of nanoprobe swelling in different gels

Figure 6A shows all of the ranges of I_D/I_A values reported for NG_{AM}/BDP in this study using $\lambda_{ex} = 365$ nm. The minimum and maximum I_D/I_A values represent, respectively, the maximum and minimum extents of deswelling for NG_{AM}/BDP in each environment. Comparing the range observed for dispersed NG_{AM}/BDP (System (1)) with the doped gels the minimum and maximum probe swelling achieved within Gelatin (System 4) and the nanocomposite gel (System 5) was similar to that in the dispersed state. The probe sensitivity increases with increasing breadth of the I_D/I_A values. Hence, the nanoprobe's swelling and de-swelling was

most sensitive to those environments. It is noted that the I_D/I_A ranges measured for NG_{AM/BDP} using $\lambda_{ex} = 254$ nm show similar trends to those in Fig. 6A (see Fig. S22, ESI†).

Figure 6. I_D/I_A ranges ($\lambda_{ex} = 365$ nm) for all of the systems studied.

In the case of the polyelectrolyte DX NG-MAA(NG_{AM/BDP}) gel (System 2) the breadth of I_D/I_A values (and the decrease in I_D/I_A) was less compared to the dispersed NG_{AM/BDP} nanoprobe (System 1). This result implies restricted probe swelling and less de-swelling. Consequently, the nanogel building blocks that comprise these gels were less able to swell and de-swell, which is likely due to the inter-nanogel linkages. Whilst the observation of less nanoprobe swelling confirms an earlier report³⁸, the identification of impeded deswelling is new and is probably only now detectable due to less interference due to light scattering for NG_{AM/BDP}. The I_D/I_A value range for the temperature-responsive DX NG-OEG gels (System 3) were relatively small and shifted vertically compared to the other systems. The decreased I_D/I_A range is because the internal swelling pressure of these polyelectrolyte nanoprobe was relatively strong compared to weaker temperature-triggered changes of the surrounding network swelling (due to hydrophobic association) which sought to deform the nanoprobe.

Nanoprobes that report environmental responses have been widely reported in the literature. However, it is a rarity for one nanoprobe to be as versatile as NG_{AM/BDP} in terms of reporting

gel responses to a multitude of stimuli. We compare the number of responses detected by NG_{AM/BDP} to related systems in Fig. S23 and Table S3 (ESI†). This analysis shows that NG_{AM/BDP} is the most multi-responsive in the context of reporting environmental stimuli from gel hosts. The multi-responsive nature of NG_{AM/BDP} originates from the MAA-based nanogel network that holds the NRET fluorophores. That network is dependent on electrostatic repulsion between fixed COO⁻ groups which is, in turn, sensitive to pH and complexing metal ions. Furthermore, the extent of network swelling is also sensitive to external compression from a surrounding network. Consequently, the success of NG_{AM/BDP} is due to the combination of a sensitive COO⁻-based network and NRET.

4. CONCLUSIONS

This study has introduced a new versatile green emitting nanogel probe with the ability to detect a wide range of environmental responses when included in synthetic and natural gels at very low concentrations. The NG_{AM/BDP} particles show reversible NRET responses and are cytocompatible. The nanoprobe can not only sense the pH and ions in solution, but can also report pH when included into host gels and stem cells. The nanoprobe enabled fluorescent sensing of internal pH, temperature, ions, gel degradation, and tensile strain. In addition, the nanoprobe provided new information about that internal environment of complex gels such as the pH- and temperature-responsive DX NGs. The sensitivity of the probes was highest for the gelatin and nanocomposite gels. However, all four different types of gels were able to have their internal network responses to changes in environmental stimuli reported and remotely detected via PL. This approach obviates the need to use bespoke chemistry to attach NRET dye pairs to specific hydrogels. In terms of applications, NG_{AM/BDP} nanogel probes loaded into injectable gels, may lead to load-supporting gels with built-in fluorescent strain reporting for next-generation biomaterials. The two DX NG systems studied here have been

previously shown to be injectable^{39, 40}. NG_{AM/BDP} reported five different stimuli when dispersed within four different hydrogels: pH, cations, network degradation, temperature and stretching. This nanogel probe can be considered as a highly versatile probe for sensing hydrogel environment changes.

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Using green emitting pH-responsive nanogels to report environmental changes within hydrogels: A nanoprobe for versatile sensing

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Low concentrations of new stimuli-responsive blue-green-fluorescent nanogel probes within *four* different gels and stem cells reversibly report *five* different stimuli: Cations, degradation, pH, temperature and tensile strain.