Electronic Supplementary Material (ESI) for Materials Horizons.

Supporting Information

One-pot synthesis of super-bright fluorescent nanogel contrast agents containing a dithiomaleimide fluorophore

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Experimental

General

Ethyleneglycol dimethacrylate (EGDMA), potassium persulfate, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich and used as received. Phloxine B and 5(6)-FAM were purchased from AnaSpec and used as received. Methyl methacrylate (MMA) was purchased from Sigma-Aldrich, and was purified by passing through a short basic alumina column immediately prior to polymerisation. Dithiomaleimide methacrylate (DTMMA) was synthesised as previously reported.[1] All water was purified to a resistivity of 18.2 MΩ·cm using a Millipore Simplicity Ultrapure water system.

All steady state emission, excitation and anisotropy spectra were obtained with a Horiba FluoroMax4 with automatic polarizers, and analyzed in FluoreEssence (Horiba) and Origin 8.6 Pro (Origin Labs). UV-vis spectra were recorded on a Shimadzu UV-2550. Transmission electron microscopy images were obtained on a (JEOL 2000FX). A drop of micelle solution (10 µL, 1 g/L) was placed on a graphene oxide support film on Lacey carbon on 400mesh Cu grid (Agar Scientific). After 1 minute the excess solution was wicked away, and the TEM grid was placed in a desiccator for 30 mins before imaging. Assessment of concentration based emission nanogels against a common, water-soluble, biocompatible dye (Phloxine B) as a reference was performed in DI water at room temperature after dissolving the reference material with stirring, followed by sonication. Afterwards, a serial dilution was generated from a 10% wt/wt solution.

Synthetic protocol

*P(MMA-co-EGDMA-co-DTMMA) nanogel solutions (**NG1-6**)*

In a typical reaction; sodium dodecyl sulfate (0.100 g) was added to water (50 mL, 18.2 MΩ·cm) in a 100 mL RBF. The RBF was sealed with a rubber septum, and the solution deoxygenated by bubbling with N_2. A mixture of MMA (0.500 g, 4.99 mmol), EGDMA, and DTMMA was added to the solution *via* syringe under N_2 flow with constant stirring. Potassium persulfate (10 mg) was added, and the reaction stirred (800 rpm) at 70 °C for 14 h. Polymerisation was terminated by allowing the reaction mixture to cool to room temperature while exposing to air. Excess surfactant was removed by exhaustive dialysis (MWCO 3.5 kDa) against water (18.2 MΩ·cm). Final concentration was determined by weighing the solution recovered after dialysis.

Degree of functionalisation with the DTM fluorophore (*DoF*) and cross-linking density (*CLD*) obtained assuming full conversion of EGDMA were calculated according to equations (1) and (2) respectively.

$$DoF\ (mol\%) = \frac{[DTMMA]}{[MMA + DTMMA]} \times 100 \quad (1)$$

$$CLD\ (wt\%) = \frac{2 \times Mass_{EGDMA}}{Mass_{MMA}} \times 100 \quad (2)$$

The quantities of EGDMA and DTMMA used in the synthesis of **NG1-6** are given below in Table S1.

Table S1. Reagent stoichiometry for the synthesis of **NG1-6**.

	EGDMA (wt%)	DTMMA (mol%)
NG1	1.25 mg (0.25)	6.65 mg (0.3)
NG2	1.25 mg (0.25)	0.665 mg (0.03)
NG3	7.50 mg (1.5)	0.665 mg (0.03)
NG4	25.0 mg (5.0)	0.665 mg (0.03)
NG5	1.25 mg (0.25)	66.5 µg (0.003)
NG6	1.25 mg (0.25)	6.65 µg (0.0003)

Monomer conversion was found to reach >99% by gas chromatography, as expected for an emulsion polymerisation.[2] Consumption of both the methacrylate groups of EGDMA was confirmed by ^{1}H NMR spectroscopy (Fig. S1) for a sample of lyophilised nanogel (*CLD* = 10 wt%, *DoF* = 0 mol%) performed in DMSO-d_6 (a solvent which is expected to swell the nanogel core). No peaks attributable to the vinyl groups of MMA or EGDMA were visible in the ^{1}H NMR spectrum indicating that both the methacrylate groups of EGDMA had reacted during the polymerisation, thereby leading to complete conversion to crosslinking groups.

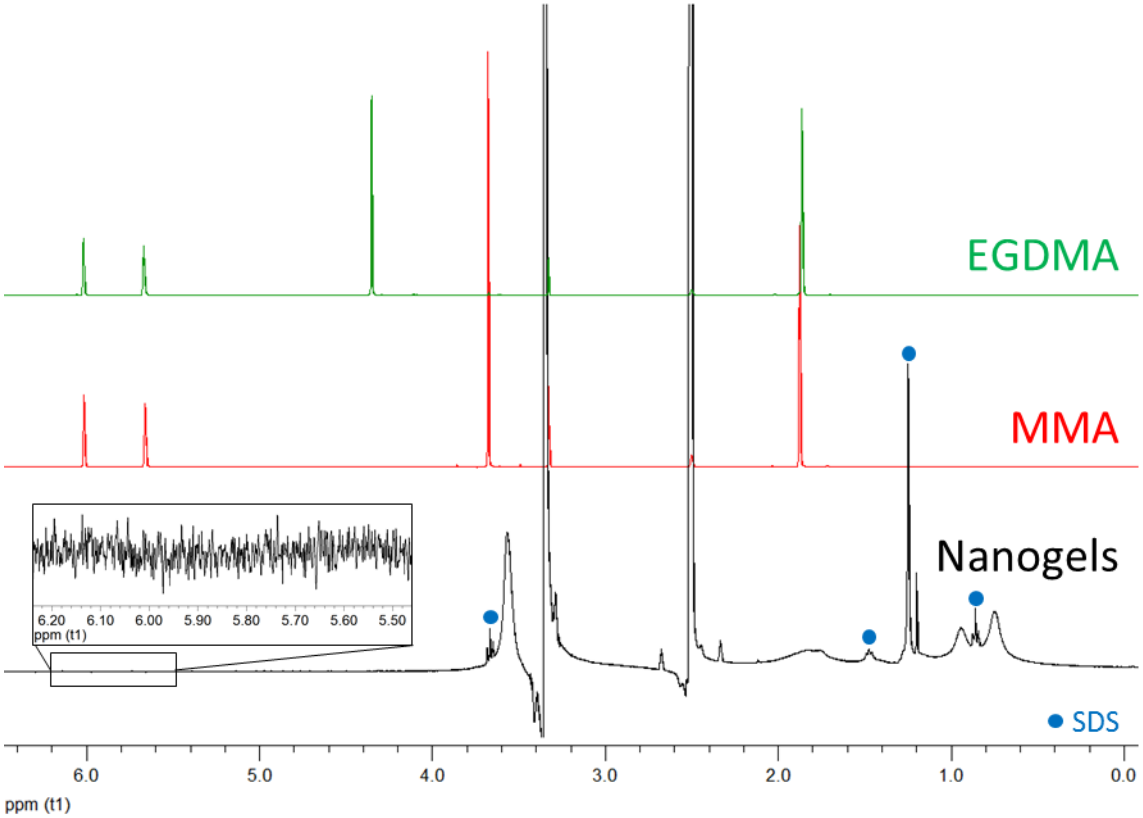

Fig. S1. ^{1}H NMR spectra (400 MHz, DMSO-d_6) of lyophilised nanogels (*CLD* = 10 wt%, *DoF* = 0 mol%), MMA and EGDMA.

Light scattering

Static light scattering (SLS) and dynamic light scattering (DLS) measurements were performed simultaneously on an ALV CGS3 goniometer with a HeNe laser operating at $\lambda = 632.8$ nm. The temperature of the toluene bath was regulated using a Julabo F32-ME refrigerated and heating circulator set to 20 °C. Refractive index increment (dn/dc) was measured by injecting samples of a known concentration into a Shodex RI-101 refractive index detector. The response was calibrated using solutions of poly(styrene) in toluene. All samples were prepared in a Karstulan Mikrofil Laminar Flow Cabinet and filtered prior to analysis using a 0.45 µm Nylon syringe filter.

DLS

For each sample intensity autocorrelation functions ($g_2(q,t)$) were recorded for all concentrations (c = 5, 4, 3, 2 & 1 g/L) and angles (θ = 40°-150°, 10° step) corresponding to a scattering vector (q) range of $8.19\times10^{13}\ m^{-2} \leq q^2 \leq 6.53\times10^{14}\ m^{-2}$. Intensity autocorrelation functions were fitted with the REPES routine using GENDIST software,[3, 4] which performs an Inverse Laplace transformation to produce a distribution of relaxation times $A(\tau)$. The apparent diffusion coefficient (D_{app}) at each concentration was calculated as the average value of $\tau^{-1}\cdot q^{-2}$, and the diffusion coefficient (D) calculated by extrapolation of D_{app} *vs*. c to $c = 0$ (Fig. S2). Hydrodynamic radius (R_h) was then calculated from the Stokes-Einstein equation (3).

$$R_h = \frac{k_B T}{6\pi\eta D} \tag{3}$$

The volume of the nanogels (V_{NG}) was calculated from R_h, as the volume of a sphere with radius R_h, as shown in equation (4).

$$V_{NG} = \frac{4\pi R_h^3}{3} \tag{4}$$

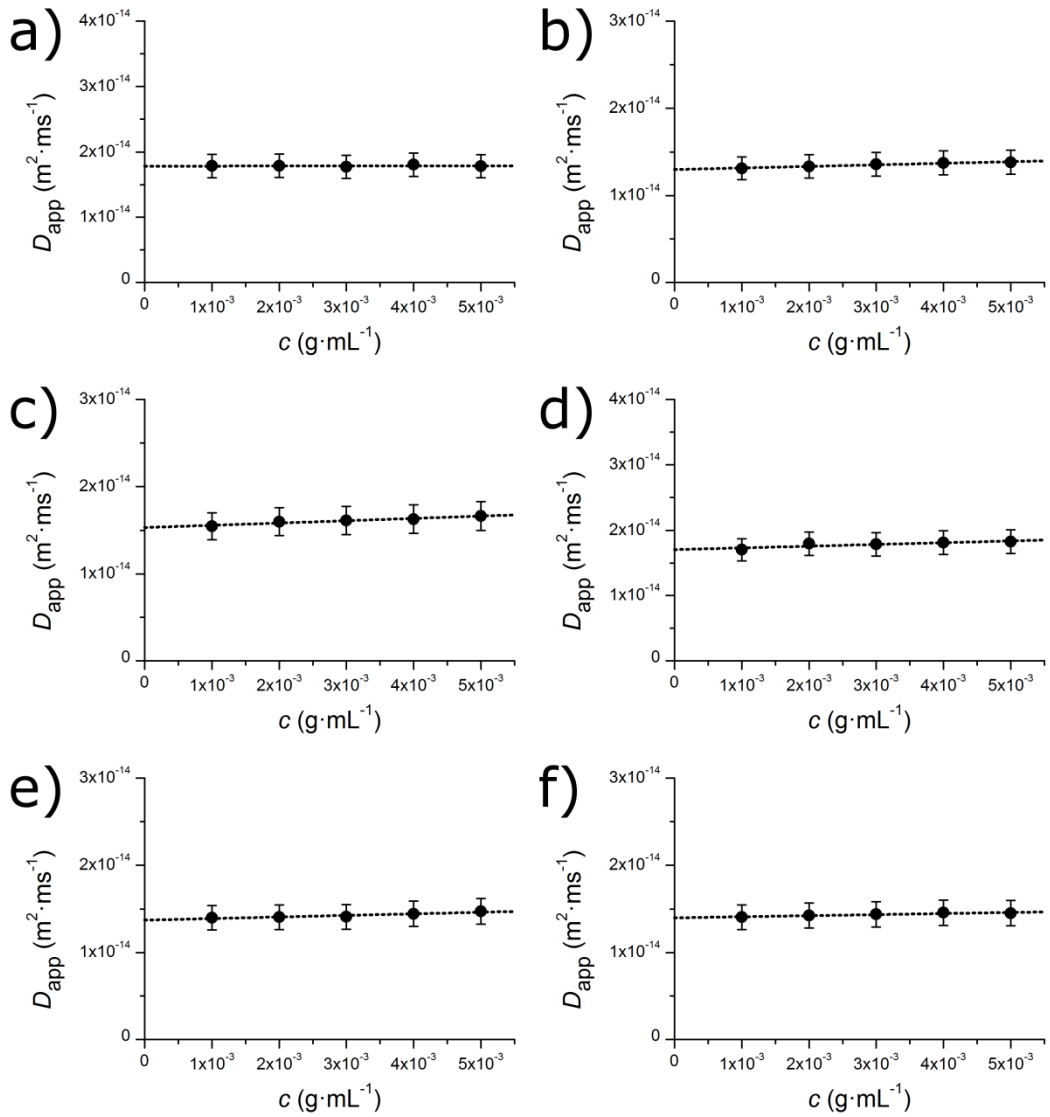

Fig. S2. Plots to determine the diffusion coefficient (*D*) for nanogel solutions; a) **NG1**, b) **NG2**, c) **NG3**, d) **NG4**, e) **NG5**, f) **NG6**.

SLS

For each sample the average intensity of scattered light (I_{sample}) was measured over 60 s for all concentrations (c = 5, 4, 3, 2 & 1 g/L) and angles (θ = 40°-150°, 10° step) corresponding to a scattering vector (q) range of 8.19×10^{13} m^{-2} ≤ q^2 ≤ 6.53×10^{14} m^{-2}. The Rayleigh ratio (R_θ) was calculated according to equation (5), where $I_{solvent}$ and $I_{standard}$ are the average intensity of light scattered by the solvent (water) and standard (toluene) respectively, and $R_{\theta,standard}$ is the known Rayleigh ratio of the standard (toluene).

$$R_\theta = \frac{I_{sample} - I_{solvent}}{I_{standard}} R_{\theta,standard} \quad (5)$$

The Rayleigh ratio was related to particle size using the Zimm equation (6), where R_g is the radius of gyration, M_w is the weight average molecular weight, and A_2 is the second virial coefficient.

$$\frac{Kc}{R_\theta} = \frac{q^2 R_g^2}{3M_w} + \frac{1}{M_w} + 2A_2 c \quad (6)$$

The Zimm equation is valid in the regime $R_g \cdot q < 1$, which for a HeNe laser (λ = 632.8 nm) and maximum angle of observation θ = 150° corresponds to R_g < 40 nm. K is a collection of constants given by equation (7), where $n_{standard}$ is the refractive index of the standard (toluene), dn/dc is the refractive index increment of the sample solution (measured using a differential refractometer), and N_A is Avogadro's number.

$$K = \frac{4\pi^2 n_{standard}^2 \left(\frac{dn}{dc}\right)^2}{N_A \lambda^4} \quad (7)$$

For each concentration, plotting Kc/R_θ *vs.* q^2 gave a straight line. Because the nanogel particles were smaller than $\lambda/20$, there was a negligible phase difference between light emitted from the various scattering centres within each particle, so the gradient of Kc/R_θ *vs.* q^2 was zero.[5] The result is that it was not possible to calculate R_g. The intercept (or average value) of Kc/R_θ *vs.* q^2 corresponds to a zero angle Rayleigh ratio ($Kc/R_{\theta,0}$). Measurements were made at a range of concentrations, so that concentration could be extrapolated to zero to remove the effect of non-ideal interactions between particle and solvent (Fig. S3). This plot of $Kc/R_{\theta,0}$ *vs.* c gave a straight line with intercept $1/M_w$.

The average number of DTM units per nanogel (DoF_{NG}) was calculated from the molar degree of functionalisation (DoF) and the particle M_w according to equation (8), where M_{MMA} is the molar mass of methyl methacrylate, and the final contribution of SDS to the particle mass was assumed to be negligible.

$$DoF_{NG} = DoF \times \frac{M_w}{M_{MMA}} \quad (8)$$

Using DoF_{NG} and V_{NG}, the concentration of DTM confined in the nanogels ([DTM_{NG}]) could also be calculated, according to equation (9), where N_A is the Avogadro constant.

$$[DTM] = \frac{DoF_{NG}}{V_{NG} \times N_A} \quad (9)$$

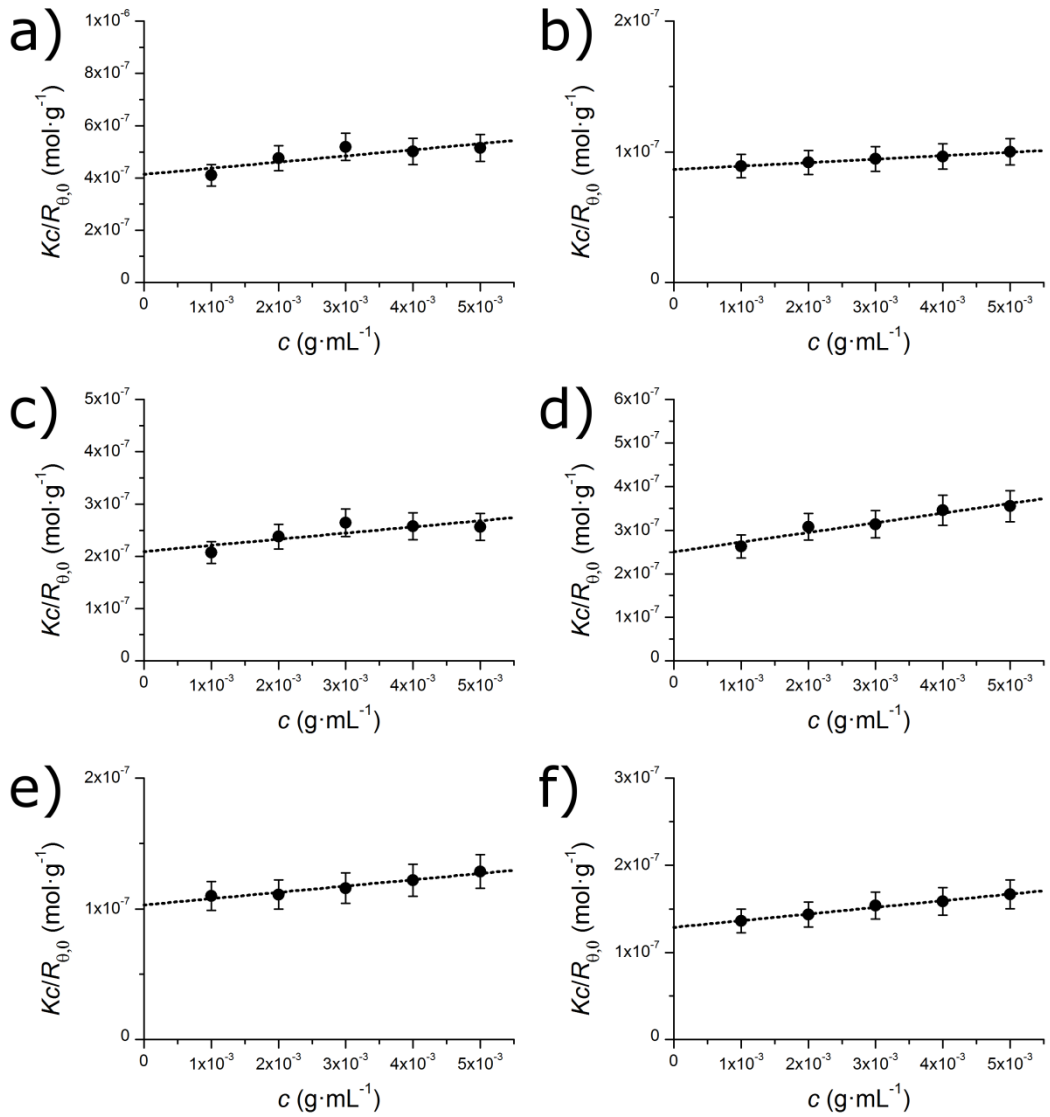

Fig. S3. Plots to determine the nanoparticle molecular weight (M_w) for nanogel solutions; a) **NG1**, b) **NG2**, c) **NG3**, d) **NG4**, e) **NG5**, f) **NG6**.

Quantum Yield and Brightness

The relative quantum yield of NG1 in aqueous solution was calculated according to literature,[6] using as a standard 5-(6)-carboxyfluorescein (5(6)-FAM) which has $Q = 0.92$. Emission spectra for 5(6)-FAM and **NG1** were recorded at $\lambda_{ex} = 445$ nm, the $\lambda_{ex,max}$ for 5(6)-FAM. Emission spectra were integrated using OriginPro 8.5, and integrated emission *vs. Abs* plotted for 5(6)-FAM and **NG1** (Fig. S4).

Using the ratio of the gradients of these plots it was calculated that Q(**NG1**) = 0.54.

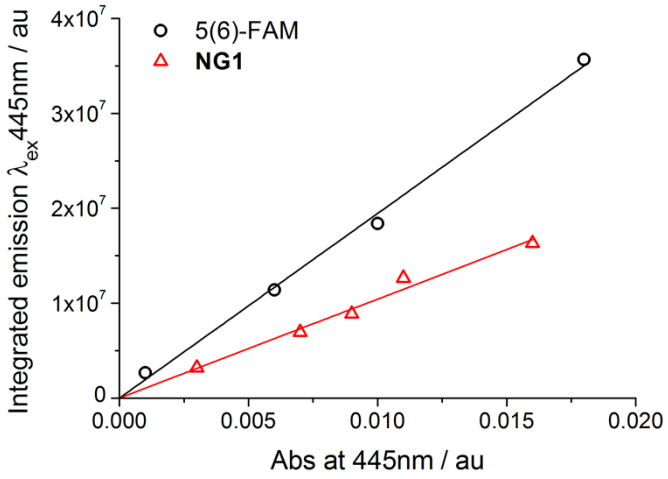

Fig. S4. Plot of integrated emission *vs. Abs* to determine Q(**NG1**).

The molar extinction coefficient (ε) for the aqueous solution of **NG1** at $\lambda_{em,max} = 405$ nm was determined using a BMG LABTECH FLUOstar OPTIMA microplate reader. Absorption was measured for **NG1** solutions at five different concentrations (in triplicate), and ε calculated from the resultant standard curve (Fig. S5).

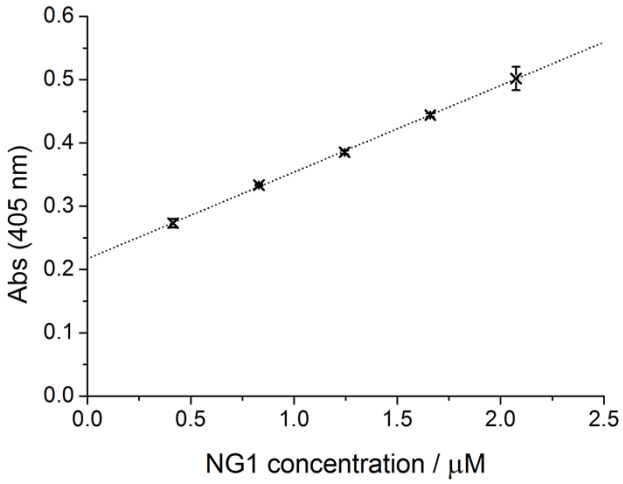

Fig. S5. Standard curve for the determination of molar extinction coefficient (ε) for **NG1**.

With ε known at 405 nm, the molar extinction spectrum ($\varepsilon(\lambda)$) for **NG1** could then be plotted (Fig. S6). Brightness as a function of excitation wavelength ($B(\lambda_{ex})$) was then calculated according to

equation (10), where $Ex(\lambda)$ is the excitation spectrum ($\lambda_{em} = \lambda_{em,max}$ = 510 nm) with Ex(445 nm) the value of excitation at 445 nm, Q(445 nm) is the quantum yield calculated at λ_{ex} = 445 nm, $Abs(\lambda)$ is the absorption spectrum and Abs(445 nm) is the absorption at λ = 445 nm.

$$B(\lambda) = \varepsilon(\lambda) \cdot \frac{Em(\lambda) \cdot Q(445\ nm) \cdot Abs(445\ nm)}{Em(445\ nm) \cdot Abs(\lambda)} \quad (10)$$

The obtained spectrum for $B(\lambda)$ is shown in Fig. S6. At $\lambda_{ex,max}$ = 420 nm, brightness is 4.61×10^4 $M^{-1}\cdot cm^{-1}$ for **NG1**.

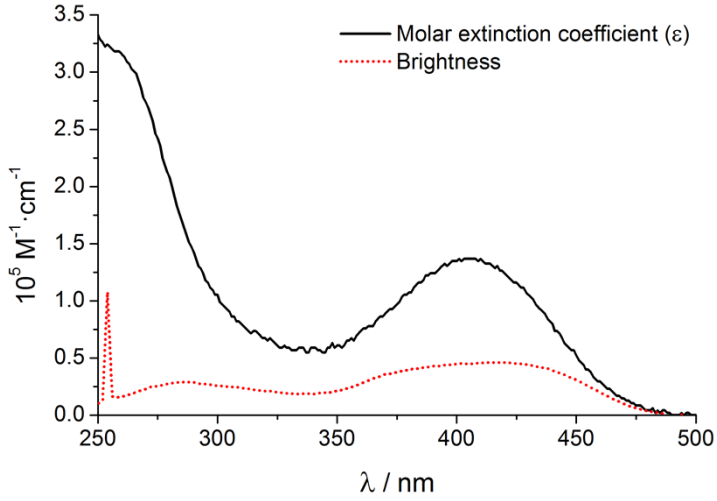

Fig. S6. Molar extinction coefficient ($\varepsilon(\lambda)$) and brightness ($B(\lambda)$) spectra for **NG1**.

Time-correlated single photon counting (TCSPC)

Solution state

Time correlated single photon counting (TCSPC) was employed to obtain all fluorescence lifetime spectra. This was done with a Fluorotime 100 fluorometer and 405 nm solid state ps diode laser source (PicoQuant) in matched quartz 0.7 mL cells (Starna Cell). Instrument response functions (IRF) were determined from scatter signal solution of Ludox HS-40 colloidal silica (1% particles in water *w*/*w*). Analysis was performed on Fluorofit (PicoQuant). Full width half maximum (FWHM) for the 405 nm laser head was 59 ps, pulse frequency was 2.5 MHz, and maximum power was 0.21 mW (attenuated by variable neutral density filters to prevent count pile up and maintain counting rates below 25 kcps). Bin sizes of 64 ps were used, collecting to a maximum bin occupancy of 10^4 counts. All IRF deconvolved exponential fits were performed with the 3 or 4 exponents selected for completeness of fit as determined by boot-strap chi-squared analysis in Fluorofit.

Table S2. Kinetic data for solution state fluorescence emission decay spectra.

	τ_1	A_1	τ_2	A_2	τ_3	A_3	τ_4	A_4	$\tau_{Av,I}$	$\tau_{Av,A}$
NG1	5.8±0.4	0.24	23.2±0.1	0.73	65.5±1.5	0.03	-	-	26.0±0.3	20.3±0.5
NG2	5.9±0.4	0.26	21.5±0.1	0.71	58.7±1.2	0.04	-	-	24.4±0.3	18.8±0.5
NG3	5.8±0.4	0.27	22.2±0.2	0.69	57.6±1.2	0.04	-	-	25.1±0.3	19.2±0.5
NG4	5.6±0.4	0.27	22.7±0.2	0.68	57.6±1.1	0.04	-	-	25.8±0.3	19.5±0.5
NG5	5.1±0.4	0.27	22.7±0.2	0.69	58.6±1.2	0.04	-	-	26.1±0.3	19.6±0.6
NG6	5.9±0.3	0.27	23.9±0.2	0.42	65.0±1.7	0.02	1.02±0.2	0.28	25.5±0.4	13.4±1.1

Solid state

Solid state samples were prepared by applying a drop of nanogel solution to a glass cover slide, and leaving to air dry to form a film. Fluorescent decay spectra for solid samples were obtained by performing fluorescence lifetime imaging microscopy (FLIM) using a FLIM LSM upgrade kit for the FV1000 (PicoQuant) mounted on a FV1000 (Olympus) confocal microscope on a IX-81 inverted base (Olympus). A PlanApo N 60x oil lens (NA 1.42, Olympus) was used for all imaging. The FV1000 system was driven with the FV10-ASW v3.1a software platform (Olympus) with scan rates of 4 µs/pixel at 256×256 pixels. FLIM images and spectra were collected using bins of 128 ps with a 405 nm laser (LDH-P-C-405B, PicoQuant) driven at 2.5 MHz. FWHM for the 405 nm laser head was 59 ps and maximum power was 0.21 mW (attenuated by variable neutral density filters to prevent count pile up and maintain counting rates below 25 kcps). SymphoTime 64 (Picoquant) software was used for collection and analysis of FLIM images and spectra. All IRF deconvolved exponential fits were performed with the 3 or 4 exponents selected for completeness of fit as determined by boot-strap chi-squared analysis in Fluorofit.

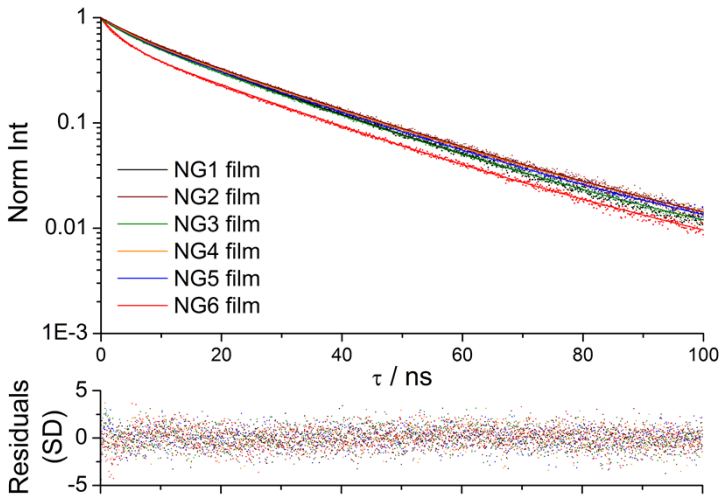

Fig. S7. Fluorescence lifetime decay spectra (points) with fitting (line) and residuals (bottom) for dehydrated films of **NG1-6**.

References

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