SUPPLEMENTAL INFORMATION

Design of thermoresponsive polyamine cross-linked perfluoropolyether hydrogels for imaging and delivery applications

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Supplementary information includes materials, methods and additional characterization data.

Materials and Methods

General Methods

All reagents and materials were purchased from commercial vendors and were used as supplied unless otherwise noted. Pluronic® F127 was purchased from Sigma Aldrich (St. Louis, MO, USA). Polyethylenimine branched (PEI) were obtained from Sigma Aldrich (St. Louis, MO, USA). Glycerol was from Acros Organics (Thermo Fisher Scientific, New Jersey, USA). IR-780 perchlorate (NIR dye) from Sigma Aldrich (St. Louis, MO, USA). Perfluoro(polyethylene glycol dimethyl ether), referred to as PFPE ester, ($CH_3OOCF_2O(CF_2CF_2O)_nCF_2OOCH_3$), where n = 8–13) and PFPE oxide, ($CF_3O(CF_2CF_2O)_nCF_3$, where n = 8–13) were obtained from Exfluor Research Corporation (Round Rock, TX, USA).

Hydrogel Syntheses

Two solutions of Pluronic® F127 surfactant (25 % w/v) were prepared for all formulations. All solutions were prepared in 100 mL volumetric flasks and by stirring with a magnetic stir bar at 4 °C. i) 25 g of Pluronic® F127 were dissolved in deionized water (DI-H_2O). ii) 25 g of Pluronic® F127 were dissolved in potassium buffer of a pH 7.4. The potassium buffer of pH 7.4 was prepared as follows: 250 mL of potassium dihydrogen phosphate 0.2 M and 393.4 mL of sodium hydroxide 0.1 M added in a 1000 mL volumetric flask and dilute with DI-H_2O. The PEI solution (19 mg/mL) was also prepared: 1.9 g of PEI was dissolved in 100 mL of DI-H_2O. The synthesis of all PFPE-PEI crosslink in Pluronic® F127 hydrogels was performed in a three-step process that was carried out in an ice bath to assure a temperature lower than 4°C. First, F127 solution (18 mL) and glycerol (5 mL) were mixed for 30 sec. Second, PEI 19 mg/mL (0.945 mL) was added and mixed for 30 sec. Third, PFPE ester or PFPE oxide (0.9mL) were added and mixed for 90 sec. A THINKY mixer ARE-310 was used for each step of the formulation at 2,000 rpm. Final step was to incubate the hydrogel for 2 hours at 37 °C.

Fluorine Nuclear Magnetic Resonance (^{19}F NMR)

^{19}F NMR spectra were collected on hydrogels with trifluoroacetic acid (TFA) as the internal standard in borosilicate NMR tubes (5 mm diameter). To collect the spectra, a Bruker 300MHz was used. Briefly, the hydrogels, DI-H2O and 0.02% v/v TFA in water solution were mixed in

1:1:2 v/v ratio (400 µL each). ^{19}F NMR peak around −91.5 ppm corresponding to 40 fluorine nuclei was integrated with TFA (set at -76.0 ppm) as a reference.

Fluorine-19 Magnetic Resonance Imaging (^{19}F MRI)

The ^{19}F MRI images were collected by preparing gel A phantoms where gel was loaded in 15mL polypropylene conical tubes. The phantoms were imaged using 7 Tesla 20-cm Bruker Biospec AVANCE III scanner with a 12-cm gradient insert and a 72-mm volume resonator with a tuning range that spanned 1H and ^{19}F resonance frequencies. Following 1H MRI pilot images and 19F pulse calibration, axial ^{19}F images were collected with a RARE (rapid acquisition with relaxation enhancement) sequence with TR/TE= 4000/7ms, RARE factor=8, NEX=32, 0.4mm pixel resolution, and 4mm slice thickness. The ^{19}F MR images are collected and presented in hotiron pseudo color and represent two consecutive slices through hydrogel samples.

Fourier Transform Infrared Spectroscopy (FT-IR)

The hydrogels were lyophilized and analyzed using a Nicolet Nexus 470 (Thermo Fisher) with a Diffuse Reflectance attachment FT-IR.

Microscopy

For initial characterization, hydrogels were observed and images were obtained at room temperature using an Olympus BX-51 optical microscope with dual polarizing filters. For 90-day stability studies, images were obtained at room temperature using an EVOS Cell Imaging System (Thermo Fisher Scientific). Scanning electron microscopy (SEM) image was obtained using a Hitachi scanning microscope on a lyophilized sample of hydrogel A (PFPE ester/PEI/F127, no buffer).

Image Processing

All images were converted from RGB color to an 8-bit image with a linear grayscale range from 0 to 255. Since there is a significant difference in the gray value between the dark outline of droplets and the light background, the image was converted, without further manipulation, to a binary (black and white) using the auto-threshold command. The droplets were filled by using the *Fill Holes* command, where the program uses the 4-connected background elements to fill the

background of the object. All droplets were counted and measured using the analyze particles command. The results of this algorithm include the size of each individual droplet in μm^2 that was further analyzed using R3.6.3. Droplet surface area average and median were calculating using built in functions in R. P-values were calculated using a two-sided Welch t-test.

Rheology

The rheological properties of hydrogels were characterized using a stress-controlled rheometer (AR-2000, TA Instruments) equipped with 40 mm diameter parallel plate geometry. Each test was carried out at a gap of 0.5 mm and the sample temperature was controlled under 0.1 °C deviation using a Peltier plate. In order to prevent sample evaporation, the solvent trap of the upper-tool was wetted using deionized water. For each sample, the linear viscoelastic domain was determined by performing strain sweeps at an angular frequency of ω= 1 rad s^{-1} at 25 °C. Frequency sweeps were then carried out from 100 to 0.1 rad s^{-1} on freshly loaded samples at a low-strain amplitude of γ_0 = 0.3%.

pH Measurements

The pH was measured at ambient temperature using an Orion Ross Sure-Flow electrode (ThermoFisher), attached to an Orion Star A111 pH meter. Before taking any measurements, the pH meter was calibrated using three standard buffers from Spectrum at pH 4.01 ($\pm$ 0.01 at 25°C), 7.00($\pm$ 0.01 at 25°C), and 10.01 ($\pm$ 0.01 at 25°C).

Resveratrol Micelle Preparation

Resveratrol micelle solution was prepared by adapting previously reported methods.[1-2] Resveratrol in acetone (8mL, 25mg/mL) and F127 in acetone (20mL, 100mg/mL) were added to a 250mL round bottomed flask. After rotating on a rotary evaporator at 150rpm for 30 minutes, acetone was evaporated at 35°C, 556mbar. The resulting product was rehydrated with 40mL distilled water, resulting in a resveratrol-loaded F127 micelle with a final concentration of 5mg/mL resveratrol and 50mg/mL F127. The micelle was filtered through a pore size of 0.45µm.

Resveratrol-Loaded Hydrogel Preparation

Resveratrol-loaded hydrogels were prepared using the same procedure as the resveratrol-free hydrogels, with one modification. Prior to addition of the other hydrogel components, 30% w/v F127 in distilled water (15mL) and resveratrol-loaded F127 micelle (3mL) were combined and mixed at 2000rpm for 30 seconds using a THINKY Mixer ARE-310. This enabled incorporation of the resveratrol micelle without impacting the final F127 concentration of the hydrogel, since resveratrol-free hydrogels were prepared using 25% w/v F127 solution (18mL).

Resveratrol-Loaded Micelle and Hydrogel Quantification

Resveratrol-loaded micelles and hydrogels were quantified 48h after production using HPLC (DIONEX Ultimate 3000, Thermo Scientific™). Micelle or hydrogel was diluted in methanol to a theoretical concentration of 10µg/mL and shaken for 15 minutes to ensure complete dissolution. Samples were then further diluted 1:1 in water and quantified using an isocratic method of 50% methanol, 50% water and a flow rate of 0.5mL/minute. C18 Hypersil Gold 150x4.6mm (Thermo Scientific) was used. A Resveratrol peak was observed at approximately 6.7 minutes, 305nm.

Resveratrol Hydrogel Release Study

Resveratrol release was assessed using Slide-A-Lyzer™ dialysis cassettes (Thermo Fisher) with volume range 0.5-3mL and molecular cut off 20K. A solution of 1% w/v Tween 80 in phosphate buffered saline at pH 7.4 was used as the dialysate. After soaking the dialysis membrane in the dialysate for 5 minutes, 3mL of hydrogel or micelle solution was injected into the dialysis membrane and excess air was removed. Dialysis cassettes were placed in a 250mL beaker containing 215mL of dialysate. The dialysate was stirred continuously at 125rpm throughout the study. The study was conducted at ambient temperature. At scheduled time points, 0.5mL samples were removed and replaced with 0.5mL of dialysate. All samples were taken in triplicate. Time points were taken at the following hours: 1, 2, 3, 4, 6, 22, 28, 34, 46, 55, 71, 83, 95, 107, 118, 144, 168, 192, 216, 244, 271, 291.

Release Study Sample Preparation and Analysis

Resveratrol release samples were analyzed using the same HPLC method used to quantify the resveratrol-loaded micelles and hydrogels. To prepare release samples, 0.5mL of methanol was added to the 0.5mL of collected release sample. If necessary, samples were further diluted using a

1:1 solution of methanol and water. Cumulative percent release was calculated using the following equation:

$$Q = \frac{C_n V_t + \sum_{i=1}^{n-1} C_i V_s}{C_o V_b} * 100$$

Where Q is the cumulative percentage of resveratrol released, C_n is the resveratrol sample concentration at the nth time point, V_t is the total volume of dialysate (215mL), C_i is the resveratrol sample concentration at the n-1th sampling interval, V_s is the sample volume (0.5mL), C_o is the resveratrol concentration of the hydrogel or micelle added to the dialysis cassette, and V_b is the volume of hydrogel or micelle added to the dialysis cassette at the 0h time point (3mL).

Cellular Viability

Hydrogels were evaluated for cell viability in a phagocytic cell line, RAW 264.7 murine macrophages. Cells were plated in a 96-well plate at 3,000 cells/well and cultured at 37°C, 5% CO_2 for 48h prior to exposure. Hydrogels were dissolved in warm cell culture media at a concentration of 50mg/mL. The solutions were shaken for 30 minutes to ensure complete gel dissolution. Hydrogel solutions were then used to perform seven serial dilutions such that hydrogel concentrations were 25mg/mL, 12.5mg/mL, etc. Cells were exposed to hydrogel solutions in media for 24h. After 24h, the plate was washed twice with warm media, then Cell Titer Glo® assay was performed following kit instructions. Data were plotted using GraphPad Prism 8.4.

FIGURES:

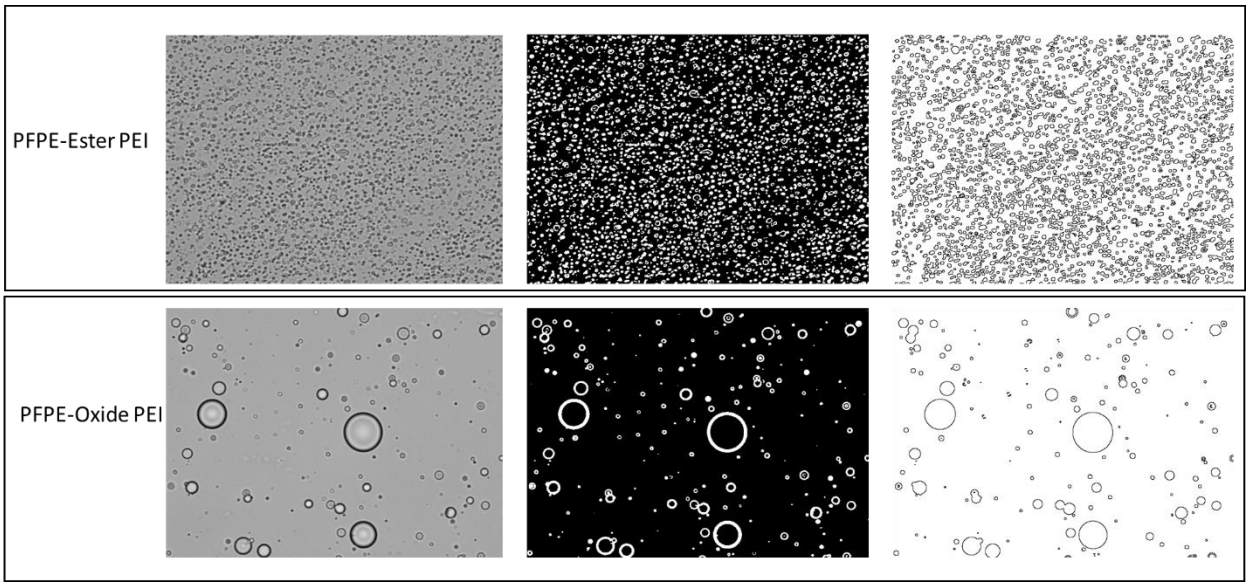

Figure S1. Example of image analysis using the macros created in ImageJ. From right to left, the first image shows the 8-bit greyscale images followed by the binarized thresholder images and, finally, the outlines for every identified droplet in the individual hydrogel.

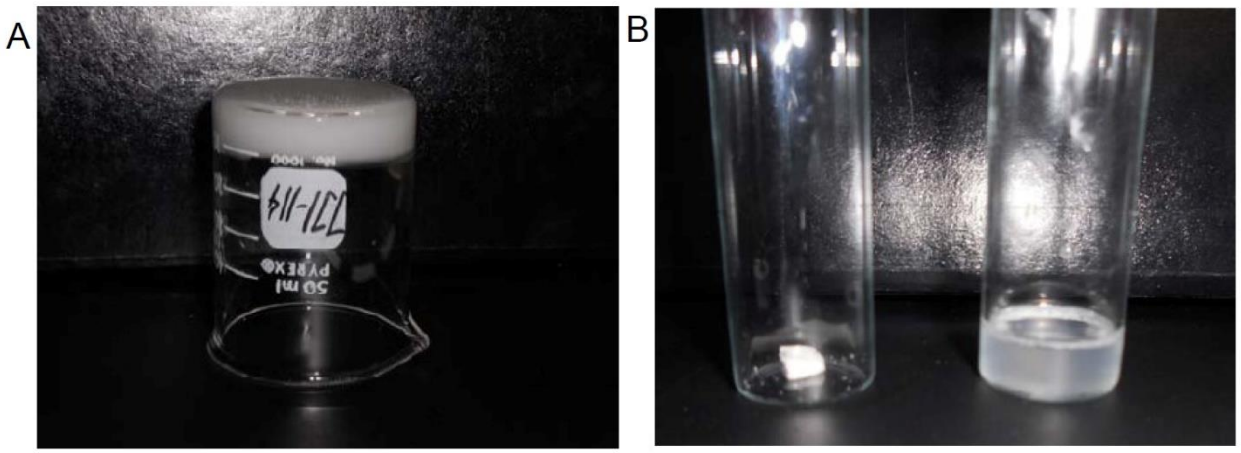

Figure S2. Hydrogel A visual observations at room temperature: A) Hydrogel A 24 after storage at RT. B) Lyophilized hydrogel A into solid material (left) and dissolution of lyophilized hydrogel A in deionized water at room temperature (right). Dissolution of lyophilized hydrogel A creates a homogenous dispersion of PFPE droplets (by visual inspection only).

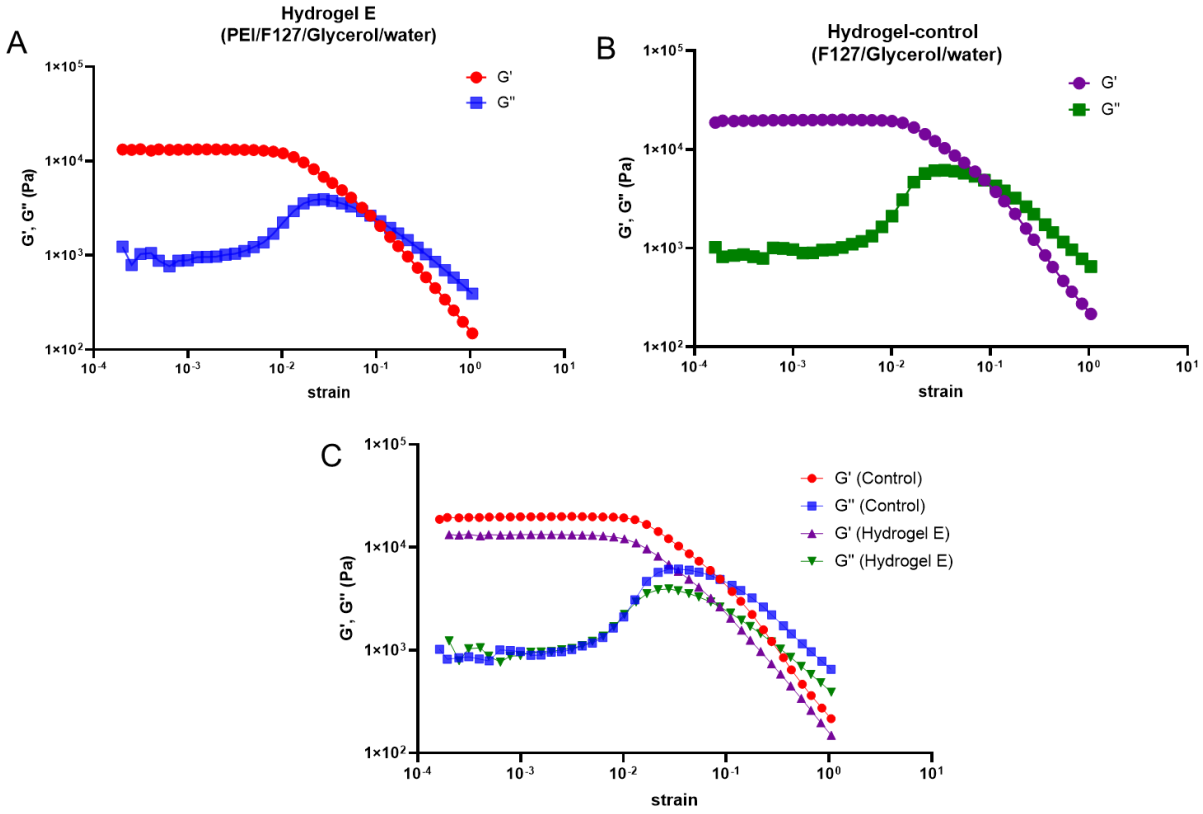

Figure S3. Rheological behavior comparisons between control hydrogels represented as elastic modulus G′ and viscous modulus G″ during sweeps and strain amplitude sweeps: A) control hydrogel without PFPE, Hydrogel E (PEI/F127/Glycerol/water); B) control hydrogel without PFPE and without PEI (F127/Glycerol/water); C) Same data plotted together for hydrogel E and control hydrogel. Concentration of F127 is matched between these formulations.

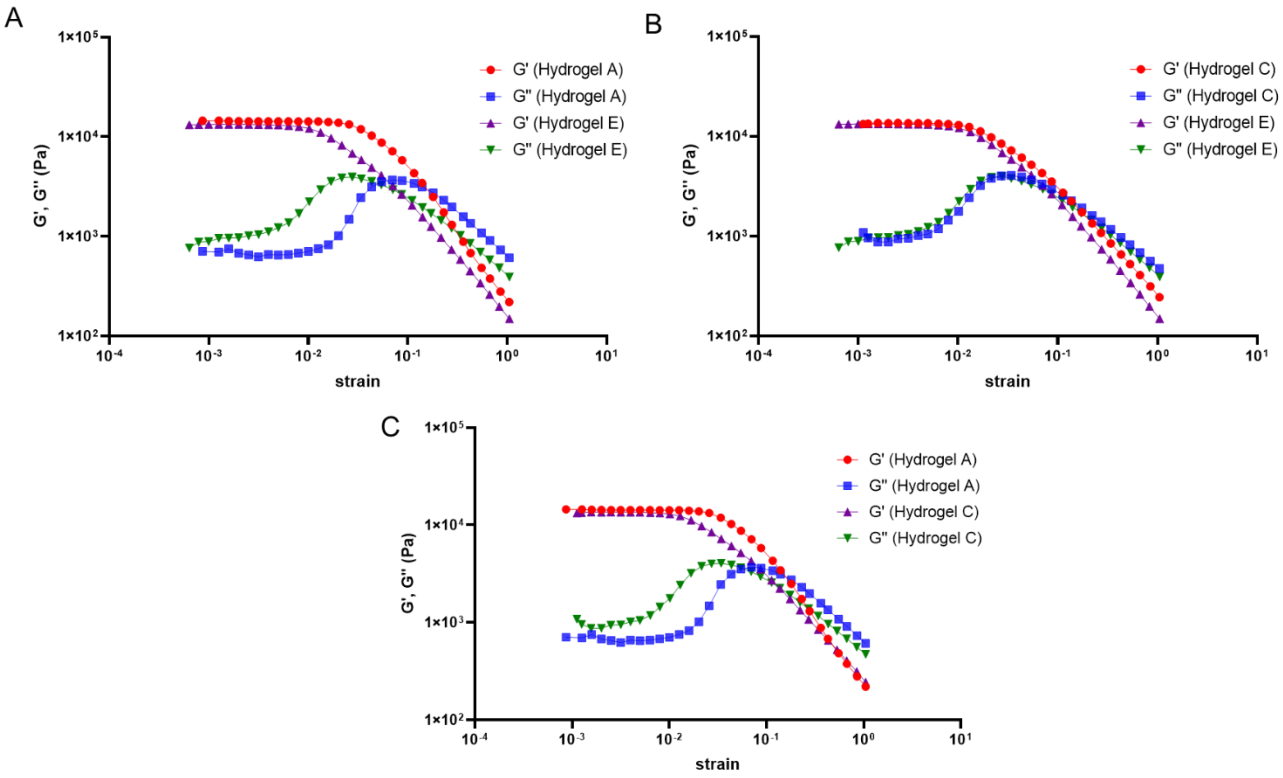

Figure S4. Rheological behavior comparisons between hydrogels represented as elastic modulus G′ and viscous modulus G″ during sweeps and strain amplitude sweeps: A) PFPE ester/PEI/F127/glycerol cross-linked (Hydrogel A) compared to control hydrogel without PFPE, Hydrogel E (PEI/F127/Glycerol/water); B) PFPE oxide/PEI/F127/glycerol non-crosslinked (Hydrogel C) compared to control hydrogel without PFPE, Hydrogel E (PEI/F127/Glycerol/water); C) Same data plotted together for hydrogels A and C. Concentration of F127 is matched between these formulations.

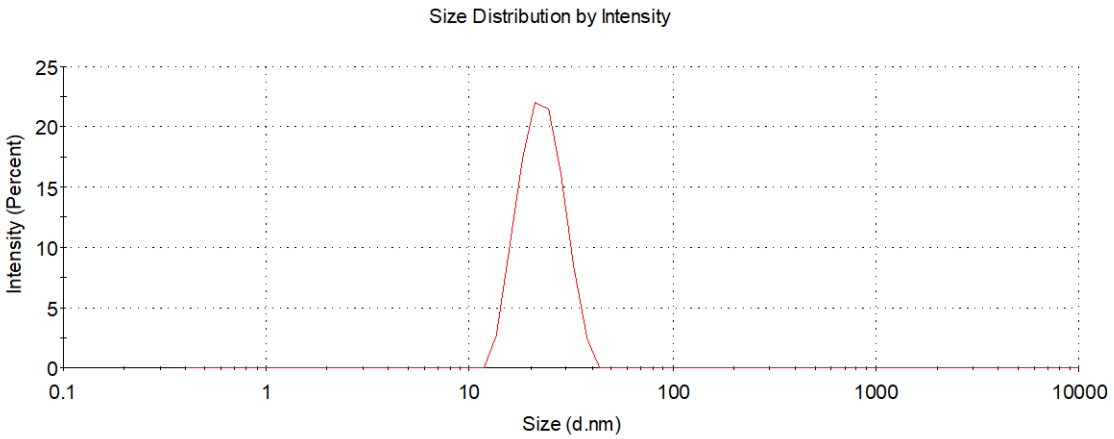

Figure S5. Size distribution by intensity of resveratrol-loaded F127 micelles, measured by dynamic light scattering (DLS). Micelles had an average diameter of 22.05±0.22nm and an average polydispersity index (PDI) of 0.022±0.004.

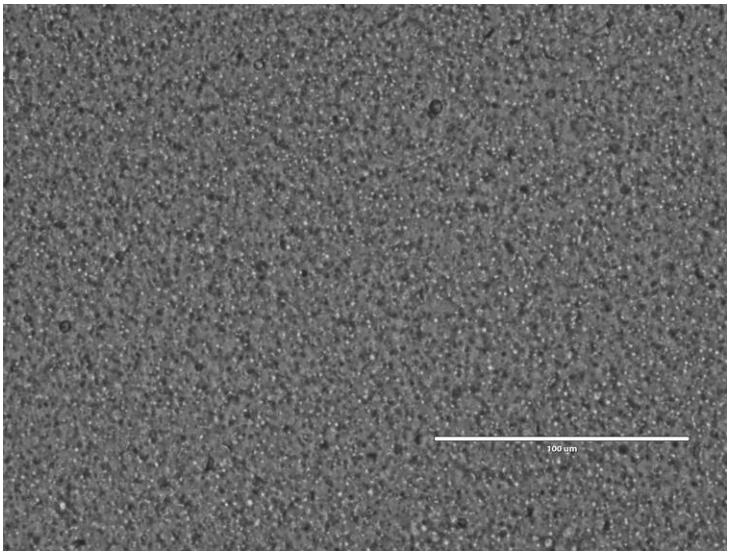

Figure S6. White light optical microscopy of resveratrol-loaded Hydrogel F (Evos Microscope), 40X magnification.

References

1. Carlson, L. J.; Cote, B.; Alani, A. W.; Rao, D. A., Polymeric micellar co-delivery of resveratrol and curcumin to mitigate in vitro doxorubicin-induced cardiotoxicity. *J Pharm Sci* **2014,** *103* (8), 2315-22.
2. Rao, D. A.; Cote, B.; Stammet, M.; Fatease, A. M. A.; Alani, A. W., Evaluation of the Stability of Resveratrol Pluronic Micelles Prepared by Solvent Casting and Simple Equilibrium Methods. *Pharm. Nanotechnol.* **2016,** *4*, 120-125.