Supporting Information

A Peptide-Based Material for Therapeutic Carbon Monoxide Delivery

John B. Matson,[a] Matthew J. Webber,[b] Vibha K. Tamboli,[c] Benjamin Weber,[a] and Samuel I. Stupp*[ade]

[a] *Institute for BioNanotechnology in Medicine, Northwestern University, Chicago, IL, 60611, USA. Fax: (+312) 503-2482; Tel: (+312) 503-6713; E-mail: s-stupp@northwestern.edu*
[b] *Department of Biomedical Engineering, Northwestern University, Evanston, IL 60208, USA*
[c] *Master of Biotechnology Program, Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL 60208, USA*
[d] *Departments of Chemistry and Materials Science and Engineering, Northwestern University, Evanston, IL 60208, USA*
[e] *Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA.*

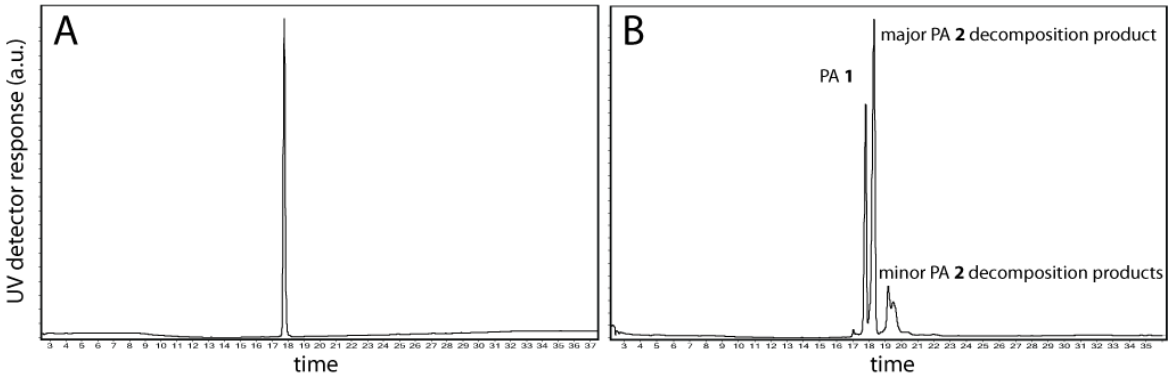

Figure S1. LCMS traces showing UV absorbance at 220 nm of PA **1** (A) and the decomposition products of PA **2** (B). Because complete CO release occurs during the run, only the decomposition products and the PA **1** remaining in the sample are observed.

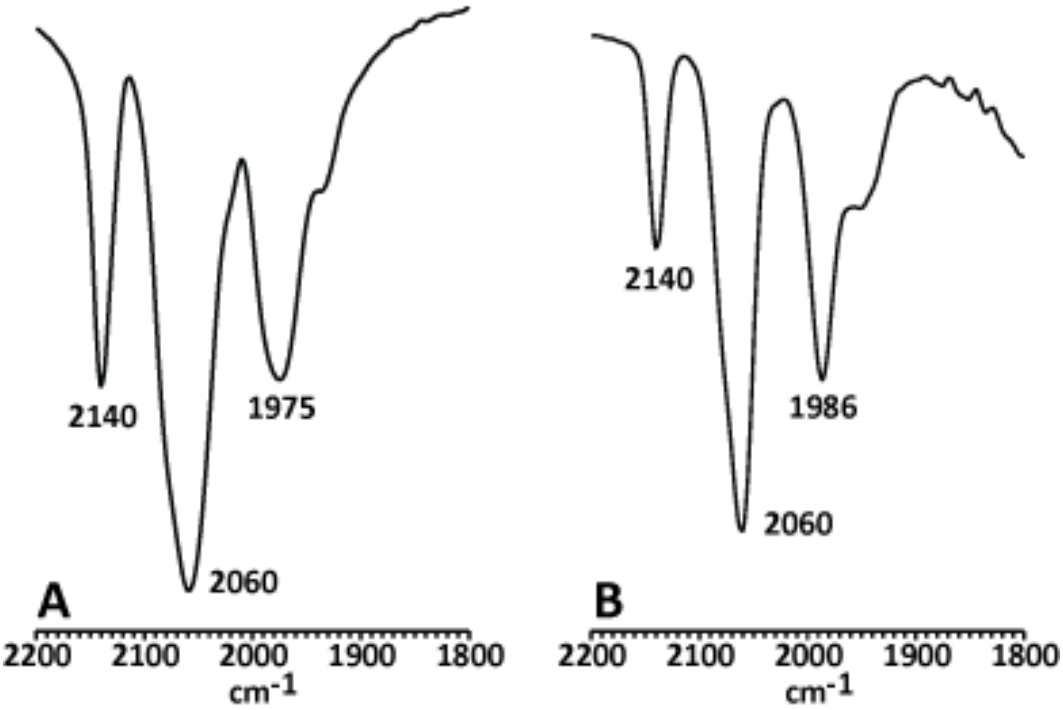

Figure S2. IR spectra of CORM-3 and CO-releasing PA **2**. Both show three ν(CO) bands consistent with a tricarbonyl product. PA **2** was treated with acid to observe all three stretches. Under basic conditions, only two bands were observed at 2023 and 1951 cm^{-1}, consistent with previous literature describing the reversible transformation of one CO ligand to a CO_2 ligand under basic conditions (used in the workup of PA **2**).[3]

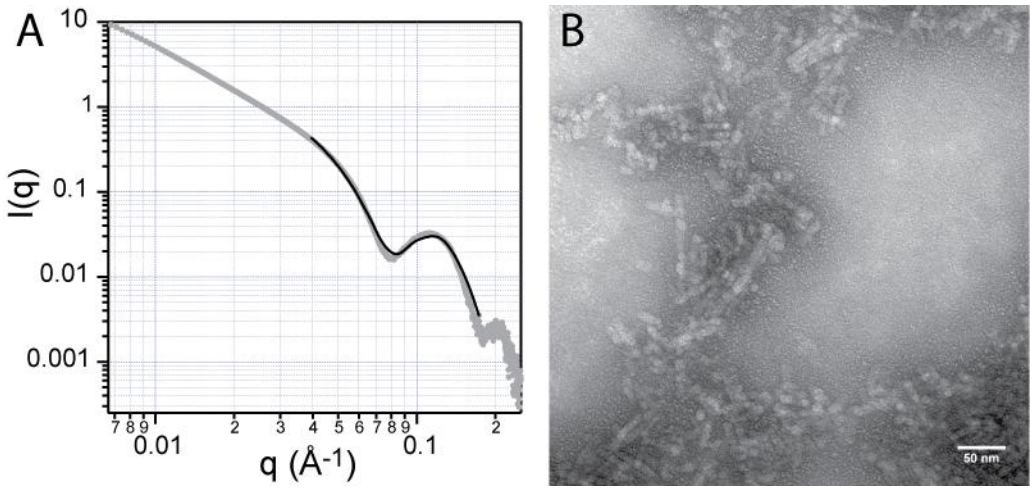

Figure S3. (A) Conventional TEM image of PA **2** cast from PBS at 0.125 wt.% and stained with uranyl acetate showing the presence of short nanofibers. (B) Small angle X-ray scattering (SAXS) of PA **2** in PBS at 0.5 wt. %. The raw data are shown in gray, and the black line shows the fitting to a polydisperse core-shell cylinder model, revealing an average nanofiber diameter of 8.2 nm. Aggregation of the nanofibers limited fitting of the SAXS data in the low *q* region, as has been previously noted.[1]

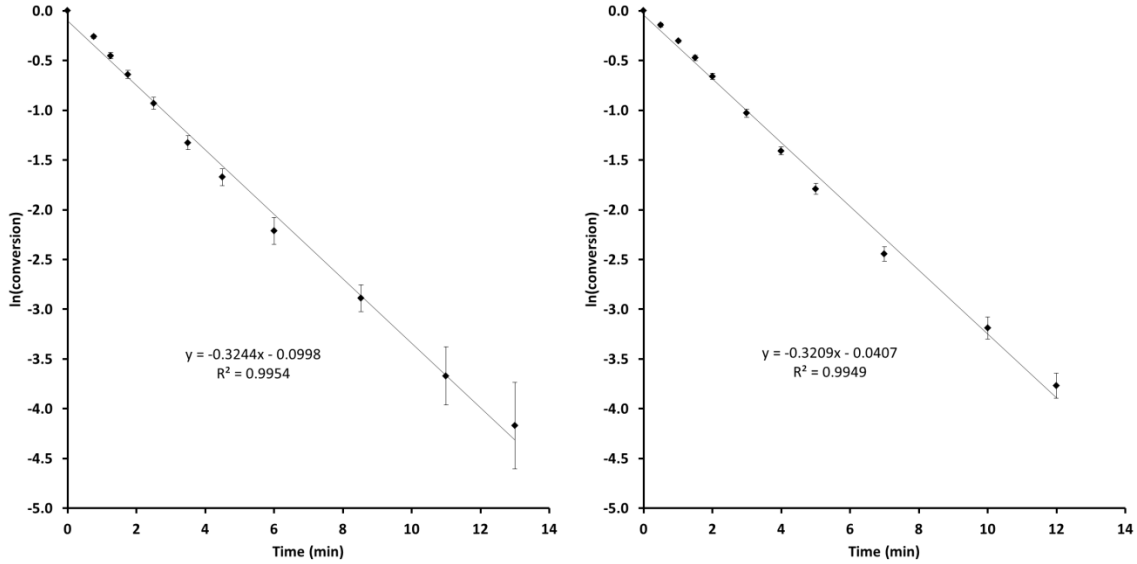

Figure S4. First order kinetics plots of CORM-3 (left) and CO-releasing PA **2** (right).

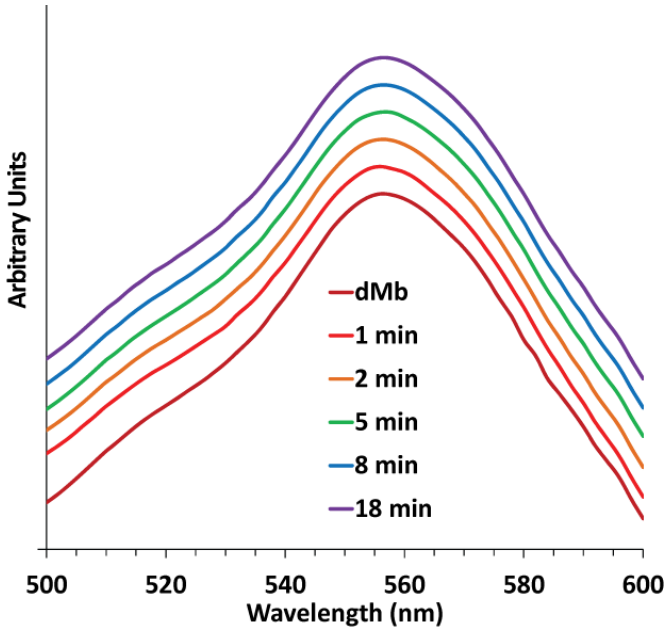

Figure S5. Absorbance spectra showing dMb over time upon addition of control PA **1**. The heights of the spectra were adjusted for clarity. No conversion to COMb is observed.

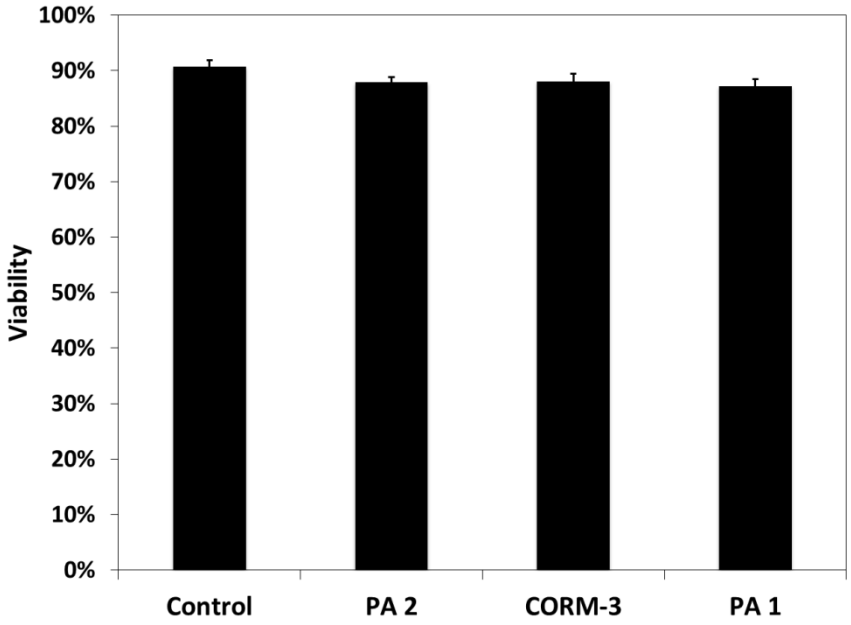

Figure S6. Viability of H9c2 cardiomyocytes after 24 hours without H_2O_2 treatment. No statistically significant differences between any of the treatment groups are observed, indicating that none of the compounds are cytotoxic at the concentrations tested.

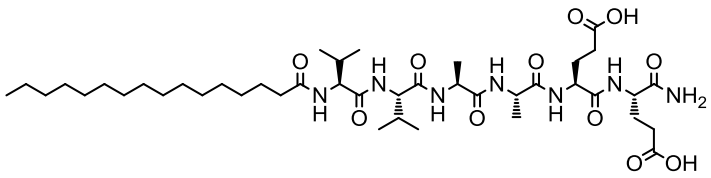

Figure S7. Chemical structure of gelling PA $C_{16}V_2A_2E_2$ (PA **3**).

Materials and Methods

Rink Amide MBHA resin and Fmoc-protected amino acids were purchased from Novabiochem Corporation or Anaspec Inc. $[Ru(CO)_3Cl_2]_2$ was purchased from Strem Chemicals. All other reagents and solvents were purchased from Aldrich, Fischer, or Novabiochem and used as received. CORM-3 was synthesized according to previously published methods.[2]

PA synthesis and purification. PA **1** was synthesized at the Peptide Synthesis Core at the Institute for BioNanotechnology in Medicine on a CEM Liberty microwave-assisted peptide synthesizer from Rink amide MBHA resin. The first residue was a Lys(Mtt) residue. For each coupling, 5 equiv of Fmoc-protected amino acid in DMF was added with 5 equiv HBTU in DMF and 10 equiv DIEA in NMP (HBTU = O-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate; DIEA = N,N-diisopropylethylamine). Fmoc removal was accomplished using a solution of 20% piperidine in DMF and 0.1 M HoBt. Default settings for microwave

power and duration were used. The palmitic acid tail was added using the same coupling conditions. After addition of the palmitic acid tail, removal of the Mtt group from the ε-amine of the Lys(Mtt) was accomplished by manual addition of 4% TFA and 4% TIPS in CH_2Cl_2 (TIPS = triisopropylsilane). Lastly, Boc-Asp-OtBu (4 equiv) was added to the lysine ε-amine with HBTU (3.95 equiv) and DIEA (6 equiv). The PA was manually cleaved from the resin using a peptide cleavage solution of 95% TFA, 2.5% TIPS and 2.5% H_2O. Concentration of the cleavage solution in vacuo and precipitation of the residue into cold Et_2O afforded the crude product. Purification of PA **1** by preparative-scale HPLC was carried out on a Varian Prostar 210 HPLC system, eluting with a gradient of 2% ACN to 100% ACN in water on a Phenomenex C18 Gemini NX column (150 x 30 mm) with 5 μm pore size and 110Å particle size. 0.1% NH_4OH was added to both mobile phases to aid PA solubility. Product-containing fractions were confirmed by ESI mass spectrometry (Agilent 6510 Q-TOF LC/MS), combined, and lyophilized after removing ACN by rotary evaporation. MS: (M+): calc'd: 1395.82; found: 1395.82.

PA **2** was synthesized by reaction of purified PA **1** with $[Ru(CO)_3Cl_2]_2$ and NaOMe. Briefly, PA **1** (10.9 mg) was dissolved in hexafluoroisopropanol (HFIP), following which HFIP was removed by rotary evaporation and high vacuum. The residue was taken up in dry MeOH (1 mL) and NaOMe (61 μL of a 0.5 M solution). To this suspension was added a solution of $[Ru(CO)_3Cl_2]_2$ (2.8 mg) in 250 μL dry MeOH. The reaction mixture was shaken for 36 h while protected from light. The product was recovered by centrifuging the mixture (1.7 rpm for 2 min) and removing the supernatant. The pellet was washed three times with MeOH and then taken up in a solution of 0.05% NH_4OH (10 mL). The solution was then lyophilized to yield 6.7 mg PA **2** as a fluffy pale yellow powder. MS: (PA-$Ru(CO)_2(CO_2)$ + Na): calc'd: 1618.68; found: 1618.74. IR: 2032, 1951. Based on the MS and the IR, the observed product is a mixed CO/CO_2 structure, which forms reversibly under basic conditions from the tricarbonyl species, as reported by Motterlini for CORM-3.[3]

Analytical LCMS of PA **1** and the product from synthesis of PA **2** was performed using an Agilent 1200 system with an Agilent 6250 quadrupole-time-of-flight mass spectrometer using a Phenomenex Gemini C18 column (5 μm particle size, 150 x 1.0 mm) eluting with a gradient of 5% ACN to 95% ACN in water, with each solvent containing 0.1% NH_4OH. UV absorbance was monitored at 220 nm.

IR Spectroscopy. IR spectra were taken as KBr pellets on a Thermo Nicolet, Nexus 870 FT-IR spectrometer. To observe all three ν(CO) stretches, PA **2** was taken up in dilute NaOH solution and treated with HCl to pH 3 then immediately lyophilized.

TEM. TEM samples were cast from 0.125 wt. % solutions in PBS onto carbon film on copper 300 mesh TEM grids (Electron Microscopy Sciences). Samples were then stained with a 2% solution of uranyl acetate in water. Images were taken on an FEI Tecnai Spirit G2 with an accelerating voltage of 120 kV.

SAXS collection. SAXS measurements were performed using beam line 5ID-D, in the DuPont-Northwestern-Dow Collaborative Access team (DND-CAT) Synchrotron Research Center at the Advanced Photon Source, Argonne National Laboratory. An energy of 15 keV corresponding to a wavelength λ=0.83 $Å^{-1}$ was selected using a double-crystal monochromator. The data were

collected using a CCD detector (MAR) positioned 245 cm behind the sample. The scattering intensity was recorded in the interval $0.006 < q < 0.27$ A-1. The wave vector defined as $q = (4\pi/\lambda)\sin(\theta/2)$, where θ is the scattering angle. Samples were analyzed in 1.5 mm quartz capillaries at 0.5% by weight in PBS. The 2-dimensional SAXS images were azimuthally averaged to produce one-dimensional profiles of intensity (I) vs *q*, using the two-dimensional data reduction program FIT2D. Scattering of a capillary containing only solvent was also collected and subtracted from the corresponding data. No attempt was made to convert the data to an absolute scale.

SAXS modeling. Data analysis was based on fitting the scattering curve to an appropriate model by a least-squares method using software provided by NIST (NIST SANS analysis version 7.0 on IGOR).[4] The scattering intensity of a monodisperse system of particles of identical shape can be described as:

$$I(q) = NP(q)S(q)$$

where N is the number of particles per unit volume, P(q) is the form factor revealing the specific size and shape of the scatterers and S(q) is the structure factor that accounts for the interparticle interactions.[5] In dilute solutions, where the interactions between the objects can be neglected, S(q) equals one. In a polydisperse system of particles having identical shape, the total intensity scattered from a can be described by:

$$I(q) = N\int_0^\infty D_n(R)P(q,R)dR$$

where $D_n(R)$ is a distribution function and $D_n(R)dR$ is the number of particles, the size of which is between R and R + dR, per unit volume of sample.

A form factor for a simple polydisperse core-shell cylinder, where the core and the shell have a uniform electron density, is given by:

$$P(q) = \int_0^{\pi/2} \sin\theta \cdot d\theta \cdot \left[V_l(\rho_l - \rho_{solv}) \frac{\sin\left(\frac{qH_l\cos\theta}{2}\right)}{\frac{qH_l\cos\theta}{2}} \frac{2J_1(qR_l\sin\theta)}{qR_l\sin\theta} + V_p(\rho_p - \rho_l) \frac{\sin\left(\frac{qH_p\cos\theta}{2}\right)}{\frac{qH_p\cos\theta}{2}} \frac{2J_1(qR_p\sin\theta)}{qR_p\sin\theta} \right]^2$$

$$V_x = \pi R_x^2 H_x$$

where $J_1(x)$ is the first order Bessel function. Theta is defined as the angle between the cylinder axis and the scattering vector, q. R_p and R_l are the core and shell radii respectively. H_p and H_l are the core and shell lengths and ρ is electron density.

The polydispersity of the core radius is modeled using a log-normal distribution

$$D_n(R_p) = \frac{\exp\left(-\frac{1}{2}\left[\frac{\ln\left((R_p/R_0\right)}{\sigma_p}\right]^2\right)}{\sqrt{(2\pi)}\sigma_p R_p}$$

where R_o is the mean core radius and σ is equivalent to the standard deviation of the log-normal distribution.

Analysis of PA **2** using the polydisperse core-shell cylinder form factor gave a mean core radius of 2.6 Å and a mean shell radius of 38.2 Å for a mean diameter of 8.2 nm. The polydispersity (σ) was 0.56.

Myoglobin kinetics assay. For the soluble assays all solutions were prepared in 100 mM phosphate buffer at pH 7.4. A myoglobin solution (2 mg/ml) was degassed by bubbling with nitrogen for at least 15 min. To this degassed solution was added a freshly prepared solution of sodium dithionite (24 mg/ml) at 1:10 dithionite/dMb (v/v), which gave a 108 μM solution of dMb. The dMb solution was added to an appropriate amount of either CORM-3 or PA **2** to yield a ~80 μM solution of CO-releasing compound. This solution was quickly transferred to a cuvette and visible spectra were taken at room temperature at predetermined time points using a Hewlett Packard 8452A diode array spectrophotometer, measuring between 500 nm and 600 nm with a step of 2 nm. Quantification of CO release was calculated from the obtained spectra according to the equation below as previously reported2:

$$\frac{[COMb]}{[Mb]+[COMb]} = \left(\frac{\varepsilon_{d542}}{\varepsilon_{iso}} - \frac{A_{542}}{A_{iso}}\right) \cdot \left(\frac{\varepsilon_{iso}}{\varepsilon_{d542} - \varepsilon_{CO542}}\right)$$

Parameters are defined as follows: ε_{d542}, ε_{CO542} and ε_{iso} are the extinction coefficients of dMb at 542 nm, COMb at 542 nm and of the isosbestic point at 550 nm, respectively; A_{542} and A_{iso} are the measured absorbances at each time point at 542 and 550 nm, respectively. Experiments were run in triplicate.

For the gel release assays, PA **2** (200 μg) was dissolved in a solution of PA **3** (45 μL; 1.1 wt. % in 10 mM NaOH). This solution was quickly gelled by addition of $CaCl_2$ (5 μL; 1 M), then 1 mL dMb solution (prepared as above) was added on top. Lastly, a layer of mineral oil was added on top of the dMb solution to prevent conversion to oxy-Mb. Conversion of dMb to COMb was observed and quantified as above.

SEM. SEM samples were prepared by adding a solution of PA **3** (45 μL, 1.1% in 10 mM NaOH) to a lyophilized sample of PA **2** (200 μg) followed by gelation with 5 μL $CaCl_2$ (1 M). After 10 minutes, gels were fixed using 4% glutaraldehyde and 3% sucrose in PBS by layering fixative on top of the gel for 90 min. Gel samples were then dehydrated using a graded ethanol series, followed by drying at the critical point using a Tousimis Samdri-795 critical point dryer. Dry samples were mounted on SEM stubs with carbon conductive cement (Electron Microscopy Sciences) and coated with 8 nm OsO_4 using an osmium plasma coater (Structure Probe, Inc.). Images were taken using a Hitachi S-4800-II SEM.

H_2O_2 assay. H9c2 cardiomyocytes were cultured in high glucose DMEM containing 10% heat-inactivated FBS in an incubator at 37 °C with 5% CO_2. Cells were plated at 4,500/well in a 96-well plate and cultured overnight. The following day, media was exchanged for growth media containing 500 μM H_2O_2 and cells were cultured for two hours. Control cells were treated with growth media without H_2O_2. Following 2 hour H_2O_2 exposure, the media was removed and replaced with growth media for the controls or growth media supplemented with 25 μM CORM-3, PA **1**, or PA **2** and cells were incubated with these treatments for 24 hours. Following this time, viability was determined using a two-color Live/Dead stain (Invitrogen) to stain live cells with calcein AM (green) and dead cells with ethidium homodimer (red). Images of the center of each well (n = 5-8) were collected using a standard inverted fluorescent microscope and the total number of live and dead cells in each image was manually counted by an observer blinded to

experimental treatment in order to determine the viability, which was defined as the percent of live cells relative to the total number of cells.

Statistical Analysis. All error bars for cell experiments indicate the standard error of the mean. Differences between groups were determined using a one-way analysis of variance (ANOVA) with a Student-Newman-Keuls multiple comparisons *post-hoc* test using GraphPad InStat v3.0b.

[1] Matson, J. B.; Stupp, S. I. *Chem. Commun*. **2011**, **47**, 7962-7964.
[2] U. Hasegawa, A. J. van der Vlies, E. Simeoni, C. Wandrey, J. A. Hubbell, *J. Am. Chem. Soc.* **2010**, *132*, 18273-18280.
[3] T. R. Johnson, B. E. Mann, I. P. Teasdale, H. Adams, R. Foresti, C. J. Green, R. Motterlini, *Dalton Trans.* **2007**, 1500-1508.
[4] S. R. Kline, *Journal of Applied Crystallography*, 2006, **39**, 895-900.
[5] O. Glatter and O. Kratky, *Small angle x-ray scattering*, Academic Press, London ; New York, 1982.