ADVANCED HEALTHCARE MATERIALS

Supporting Information

for *Adv. Healthcare Mater.*, DOI: 10.1002/adhm.201700162

Multifunctional Polymeric Micelles for Combining Chelation and Detection of Iron in Living Cells

*Zhi Liu, Max Purro, Jing Qiao, and May P. Xiong**

Supporting Information

Multifunctional Polymeric Micelles for Combining Chelation and Detection of Iron in Living Cells

*Zhi Liu, Max Purro, Jing Qiao and May P. Xiong**

Experimental Section

Materials

Pluronic® F127 (F127), sodium hypochlorite solution (NaClO solution, 10-15% available chlorine), sodium bromide (NaBr), 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO), metal chloride salts ($FeCl_3$, $FeCl_2$, NaCl, KCl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $MgCl_2$, $CoCl_2$, $CuCl_2$, $MnCl_2$, $NiCl_2$, $AlCl_3$, $CrCl_3$ and $GaCl_3$), metal nitrate salts ($AgNO_3$), N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and N,N-diisopropylethylamine (DIPEA), were purchased from Sigma-Aldrich. Ferric ammonium citrate (FAC) was purchased from VWR. 1-hydroxybenzotriazole hydrate (HOBt) was purchased from Chem-Impex International. Hydrochloric acid was purchased from Fisher Scientific. Deferoxamine mesylate (DFO) was purchased from the University of Wisconsin Hospital Pharmacy Services (Hospira). Mouse macrophage/monocyte cell line J774A.1 was purchased from American Type Culture Collection (ATCC). Dulbecco's modified eagle medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin solution (100×), the Pierce BCA protein assay kit, and LysoTracker® Red DND-99 were purchased from Thermo Fisher Scientific. Mouse ferritin ELISA kit was purchased from Immunology Consultants Laboratory. All solvents and other reagents were used as supplied without further purification. All NMR spectrums were acquired with a Varian Unity-Inova 400 MHz spectrometer.

Methods

Synthesis of F127-$(COOH)_2$: The terminal hydroxy groups of F127 were oxidized as previously reported by Araki *et al*.[1] Specifically, the equivalent of 0.6 mmol hydroxyl groups (3.78 g F127) was oxidized in water (100 mL) with 100 mg NaBr (0.972 mmol), 100 mg TEMPO (0.640 mmol), and 10 mL NaClO solution at pH 10 for 15 minutes (r.t.). The reaction was quenched by the addition of EtOH (10 mL) followed by acidification to pH 1 with HCl. The oxidized polymer was extracted three times with 100 mL DCM and dried by

rotary evaporation. The polymer was then dissolved in hot EtOH and allowed to recrystallize at -20°C twice to obtain the final purified product. **^{1}H NMR** (400 MHz, DMSO-d_6, 298K): δ = 3.98 (s, OC***H*****$_2$**COOH), 3.20–3.60 (m, CH_2C***H*****$_2$**O of PEG and C***H*****$_2$**CHO of PPG), 1.00 (d, C***H*****$_3$** of PPG).

Synthesis of F127-(DFO)$_2$: The equivalent of 0.25 mmol carboxylic acid groups on the polymer (1.575 g F127-$(COOH)_2$) was reacted with 57.4 mg HOBt (0.375 mmol), 71.9 mg EDC (0.375 mmol), 197 mg DFO (0.3 mmol), and 109 µL DIPEA (0.625 mmol) in 75 mL DMF for 24 hours at room temperature. The reaction mixture was poured into 375 mL EtOH and allowed to recrystallize at -20°C twice to obtain the final purified product. **^{1}H NMR** (400 MHz, DMSO-d_6, 298K): 9.50-9.80 (br, O***H*** of DFO), 7.60-7.80 (br, N***H*** of DFO), 3.81 (s, OC***H*****$_2$**CONH of PEG), 3.20–3.60 (m, CH_2C***H*****$_2$**O of PEG and C***H*****$_2$**CHO of PPG), 2.90-3.10 (m, C***H*****$_2$**-NHCO of DFO), 2.50- 2.60 (m, C***H*****$_2$**-CO of DFO), 2.20-2.30 (m, C***H*****$_2$**-NH of DFO), 1.91 (s,C***H*****$_3$** of DFO), 1.10-1.50 (m, C***H*****$_2$** of DFO), 1.00 (d, C***H*****$_3$** of PPG).

Preparation of TPE-loaded micelles and release studies: Control micelles (C-TFM) encapsulating TPE but without DFO conjugated to F127 terminal ends were prepared by the thin-film hydration method. Briefly, 10 mg of TPE and 300 mg of F127 were dissolved in 20 mL DCM in a round bottom flask. The solvent was removed by rotary evaporation to generate a solid film and residual DCM remaining in the film was further removed under vacuum overnight at room temperature. The resultant thin film was hydrated with 10 mL Tris/HCl buffer (20 mM, pH 7.0) for 30 min to generate micelles and then syringe filtered through a 0.22 µm membrane to remove un-encapsulated TPE. Micelles encapsulating TPE but decorated with DFO moieties (DFO-TFM) were similarly prepared by using a mixture of 200 mg F127 and 100 mg F127-$(DFO)_2$ polymers.

Release of TPE from micelles was investigated both at room temperature and 37°C. Briefly, 10 mL DFO-TFM solution (30 mg/mL) was transferred to a dialysis bag (MWCO 3500 Da) and dialyzed against Tris/HCl (20 mM, pH 7.0) for two days. At predetermined time points, 500 uL of sample was removed from the dialysis bag and freeze-dried. TPE release was analyzed via the measurement of TPE absorption spectrum in DMSO.

Physical characterization of DFO-TFM: The ability of the F127-$(DFO)_2$ polymer to chelate iron(III) was first verified by monitoring the UV/Vis absorption spectra in the presence of iron(III) by scanning between 350–650 nm with a SpectraMax Plus spectrophotometer (Molecular Devices) and confirming the magnitude of the absorbance peak at 430 nm, which is characteristic of the complex concentration in solution when DFO chelates to iron(III) at a 1:1 ratio. Next, because of the proportional relationship that exists between absorbance and complex formation, UV/Vis was used to indirectly determine the amount of DFO in un-encapsulated F127-$(DFO)_2$. Briefly, a series of solutions containing 1.2 mM iron(III) was mixed with increasing concentrations of DFO ranging from 0.1-1 mM was prepared, and the absorption at 430 nm was measured. A linear curve relating DFO concentration to absorption was generated:

$$y = 0.794\,x - 0.0019 \quad R^2 = 0.9999$$

where x is the concentration of DFO (mM) and y is the resulting absorption intensity at 430 nm. The concentration of DFO in DFO-TFM can be calculated based on the fomula.

To investigate the morphology and size of resulting micelles, samples were prepared by freeze-drying a drop of micelle solution on copper grid and transmission electron microscope (TEM) images were taken with a JEM1011 instrument at an acceleration voltage of 100 kV. Hydrodynamic size and polydispersity (PDI) of micelles were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Instruments, UK) and analyzed with Zetasizer software v7.10. The cumulant analysis method was used to calculate

the z-average diameter and PDI. Measurements were conducted on three batches of samples and results are reported as mean ± standard deviation (SD). Prior to measurements, the micelle solution was first clarified by syringe filtration through a 0.22 μm Millipore membrane.

Fluorescence spectra measurement in aqueous media: The fluorescence quenching behavior of TPE encapsulated in the micelles was verified by mixing a solution of DFO-TFM containing 1 mM DFO with different concentration of iron(III) at room temperature and measuring the resulting fluorescence changes at 440 nm. Briefly, iron(III) was dissolved in deionized water to obtain 1.0 mM stock solutions. Before spectroscopic measurements, the working solution was freshly prepared by diluting the high concentration iron(III) stock solution to the corresponding solution and mixed with DFO-TFM solution. The selectivity of DFO-TFM for iron(III) and subsequent competition studies in the presence of other cations was investigated. For the selectivity study, prior to spectrofluorometric measurements, a 1 mM stock solution in deionized water for each metal salt of $FeCl_2$, NaCl, KCl, $CaCl_2$, $ZnCl_2$, $HgCl_2$, $MgCl_2$, $CoCl_2$, $CuCl_2$, $MnCl_2$, $NiCl_2$, $AlCl_3$, $CrCl_3$, $GaCl_3$, and $AgNO_3$ was freshly prepared and mixed with micelle solutions at 1 mM equivalent DFO concentration. For the competition study, iron(III) was added to the micelle solution to pre-chelate it. Next, a 1 or 50-fold excess concentration of other metals was added to a series of these pre-chelated micelle solutions and allowed to incubate for at least 30 min prior to spectrofluorometric measurements. For all studies, an excitation wavelength of 350 nm was used and resulting fluorescence emission spectra was measured at 440 nm with a FluoroMax 4 spectrofluorometer (Horiba Jobin Yvon).

Cytotoxicity studies: J774A.1 macrophage cells were seeded in 96-well plates at a density of 3,000 cells/well, cultured at 37°C, 5% CO_2 and 100% humidity with DMEM complete

medium (supplemented with 10% (v/v) heat-inactivated FBS, 100 I.U./mL penicillin and 100 µg/mL streptomycin), and allowed to settle for 24 h. Cells were then treated with 1 mM free DFO or DFO-TFM at equivalent DFO concentrations (prepared by 1:3 serial dilutions) for 48 h; C-TFM were also similarly prepared. Cell viability was measured with the metabolism-based resazurin assay. Briefly, the substrate resazurin was dissolved in cell culture medium at a concentration of 44 µM, added to each well (100 µl) and incubated at 37°C for 4 h. The fluorescence was measured with excitation at 560 nm and emission at 590 nm, on a SpectraMax Gemini EM microplate reader. Readings from the wells without cells were used as E_{blank}, and the readings from control cells without treatment ($E_{control}$) represented 100% cell viability. The viability of cells treated at different concentrations can be calculated from the following equation:

can be calculated from the following equation:

$$Cell\ viability\ \% = \frac{E_{sample} - E_{blank}}{E_{control} - E_{blank}} \times 100$$

Similarly, cytotoxicity was also evaluated in J774A.1 cells supplemented with excess iron. For this study, cells were iron-overloaded for 24 h by incubation with culture medium containing 100 µM ferric ammonium citrate (FAC) as previously reported prior to addition of the various preparations.

Chelation properties in iron-overloaded cells: J774A.1 macrophage cells were seeded in 6-well plates at a density of 30,000 cell/well and allowed to settle for 24 h at 37 °C, 5% CO_2 and 100% humidity with DMEM complete medium. Cells were then treated with 100 µM FAC for 24 h, washed with PBS and treated with free DFO or DFO-TFM micelles at 10 µM or 50 µM equivalent DFO concentrations for 48 h. At the end of the incubation period, cells were lysed with lysis buffer (150 mM NaCl, 10 mM Tris, 1% Triton X-100 and protease inhibitor cocktail, pH 7.4) and total protein concentration was measured with the BCA protein

assay kit. Cellular ferritin concentration was measured with a mouse ferritin ELISA kit. The results are plotted as the ratio of ng of ferritin per μg total protein concentration.

Fluorescence imaging and monitoring iron chelation in live cells: J774A.1 macrophage cells were seeded in glass-bottom cell culture dishes (NEST Biotechnology Co., LTD.) at a density of 30,000 cells/well and allowed to settle for 24 h at 37°C. The next day, cells were treated with 0, 50 or 100 μM FAC in DMEM complete medium for another 24 h and subsequently washed with PBS prior to returning to the incubator for an additional 48 h. Afterwards, cells were allowed to co-incubate with 1 mg/mL of DFO-TFM in culture medium at 37 °C for 1 h prior to adding LysoTracker® Red DND-99 at a final concentration of 1 μg/mL and incubating for another 0.5 h. At the end of incubation period with LysoTracker, the cells were washed three times with PBS, fresh growth medium was added and live cells were directly imaged with a Zeiss LSM 710 Confocal Microscope. Fluorescence images were captured by monitoring blue and red channels and exciting cells with the laser at 405 nm for TPE and 633 nm for LysoTracker® Red DND-99, respectively.

To monitor the turn-off iron chelation process in live cells over time, J774A.1 macrophage cells were iron overloaded for 24 h by incubation with 50 μM FAC, washed with PBS and incubated for another 48 h. As before, cells were then co-incubated with 1 mg/mL DFO-TFM or C-TFM at 37°C for 1 h. After washing with PBS and replacing with fresh culture medium, cells were returned to the incubator for either 1 or 10 h respectively. LysoTracker was added for 0.5 h, cells were washed three times with PBS and fresh growth medium was added prior to taking images with the Zeiss LSM 710 Confocal Microscope. All fluorescence images were captured via blue and red channels but to more easily visualize the different fluorescence quenching effects when the images are merged, all CLSM pictures corresponding to the blue channel were digitally switched to green.

Statistical Analysis: Statistical analysis was performed with GraphPad Prism 5.0 software. Statistical significance between groups was assessed with one way ANOVA; $p < 0.05$ was considered statistically significant.

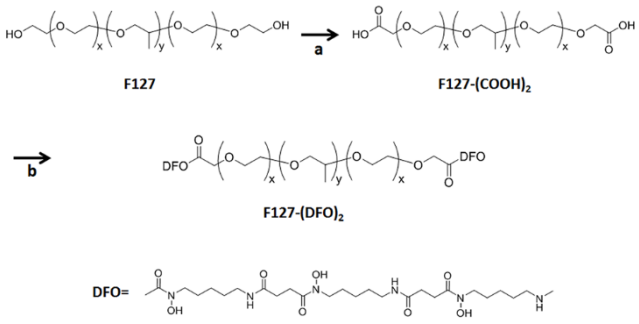


Figure S1. Schematic illustration of the synthetic steps for F127-$(DFO)_2$. a) NaBr, TEMPO, NaClO, water, b) HOBt, EDC, DIPEA, DMF.

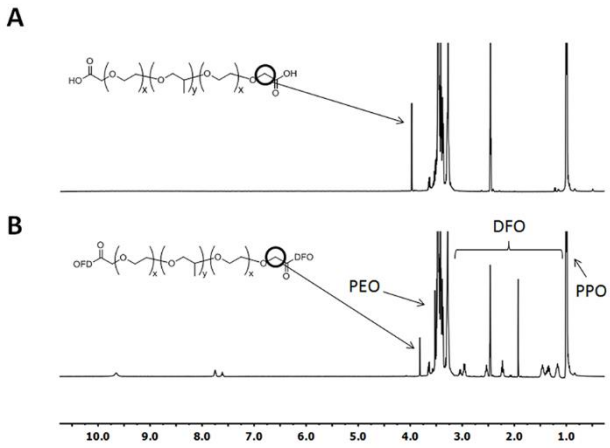


Figure S2. ^{1}H NMR spectra of F127-$(COOH)_2$ (**A**) and F127-$(DFO)_2$ (**B**).

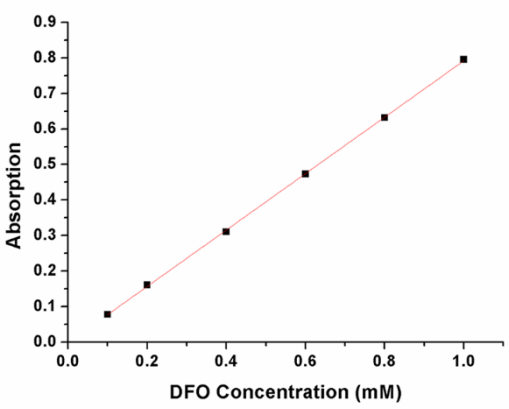


Figure S3. Determination of DFO content in DFO-TFM by UV-Vis spectroscopy method. As the concentration of DFO increases, the absorption at 430 nm also increases linearly and is proportional to the DFO concentration.

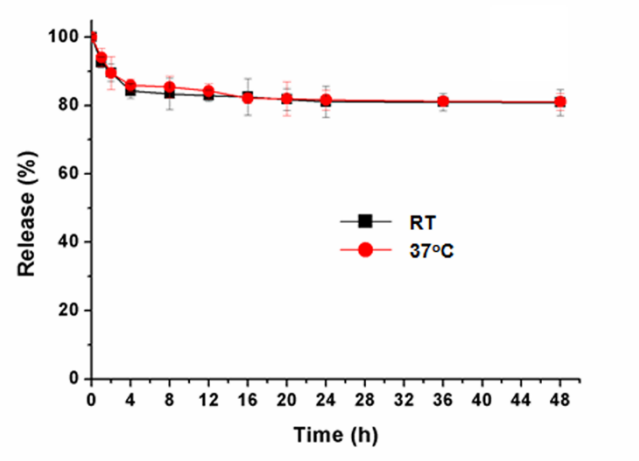


Figure S4. Release profiles for DFO-TFM in Tris/HCl (pH 7.0) at room temperature and 37 °C. All of the measurements were conducted in triplicate.

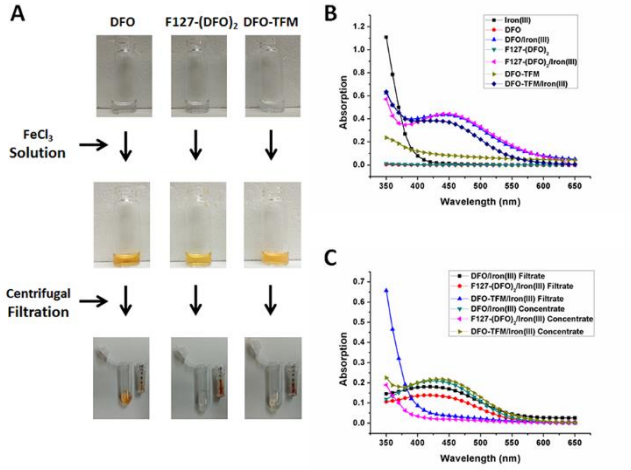


Figure S5. (**A**) Optical images of DFO, F127-$(DFO)_2$ and DFO-TFM before (first row of vials) and after (second row of vials) addition of iron reveal clear and yellow solutions respectively. After centrifugal filtration with a microcentrifuge filter tube (mwco 10,000 g/mol), the yellow colored suspension in F127-$(DFO)_2$ and DFO-TFM containing chelates remained in the filter unit and the clear solution containing excess iron passed through; in contrast, the yellow colored suspension in DFO passed through the filter into the filtrate (third row of images). (**B**) UV-Vis absorption spectrum of iron(III) and DFO, F127-$(DFO)_2$, DFO-TFM in the absence and presence of iron(III). (**C**) UV-Vis absorption spectrum of the concentrate of F127-$(DFO)_2$ and DFO-TFM displays strong absorption at ca. 430 nm and no absorption for the filtrate after extensive washing with the centrifugal filtration unit. Both filtrate and concentrate of DFO display strong absorption at ca. 430 nm.

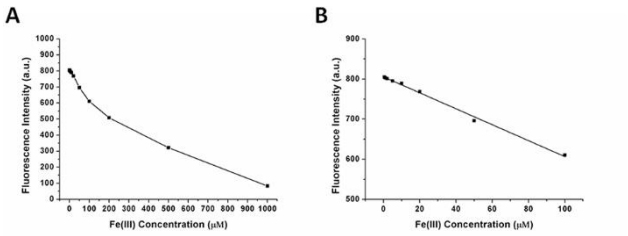


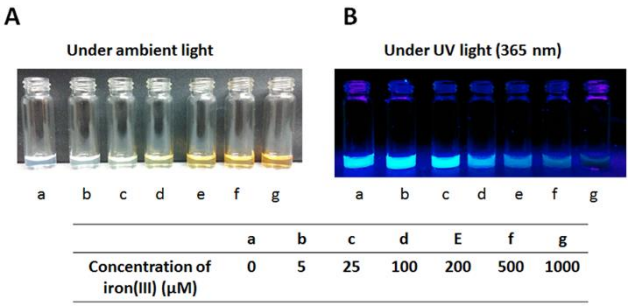
Figure S6. The fluorescence intensity ratio of DFO-TFM at different iron(III) concentrations in pH 7.0 Tris/HCl solution (λ_{exc}=350 nm).

The detection limit (DL) was calculated as following:

$$F = 805.48691 - 1.98933 \times C(\mu M) \qquad R^2 = 0.9954$$

$$DL = \frac{3 \times S.D.}{k} = 0.83(\mu M)$$

Where *F* is the fluorescence intensity, *k* is the slope of the curve equation, and S.D. represents the standard deviation for the DFO-TFM solution's fluorescence intensity ratio in the absence of iron(III).



	a	b	c	d	E	f	g
Concentration of iron(III) (μM)	0	5	25	100	200	500	1000

Figure S7. Visible color (**A**) and fluorescence (**B**) images of DFO-TFM after addition of various concentrations of iron(III) in Tris/HCl solution pH = 7.0.

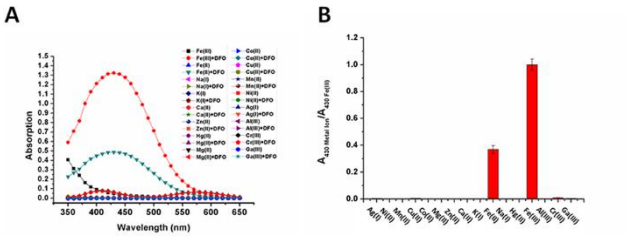


Figure S8. Absorption intensity changes ([$A_{430\ Metal\ ion}/A_{430\ Fe\ (III)}$]) for free DFO (**A**) and DFO-TFM (**B**) upon addition of Ag(I), Ni(II), Mn(II), Cu(II), Co(II), Mg(II), Zn(II), Ca II), K(I), Na(I), Fe(II), Hg(II), Fe(III), Al(III), Cr(III) and Ga(III) respectively (λ_{exc} = 350 nm). Results are presented as mean ± SD (n = 3).

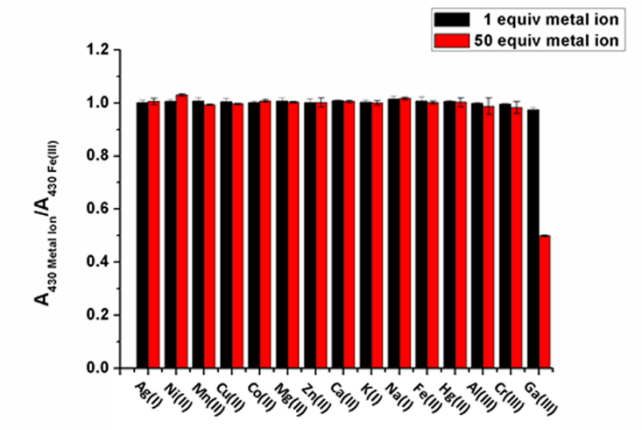


Figure S9. Absorption intensity changes ([$A_{430\ Metal\ ion}/A_{430\ Fe\ (III)}$]) of DFO-TFM pre-chelated to Fe(III) upon addition of 1x or 50x equivalence Ag(I), Ni(II), Mn(II), Cu(II), Co(II), Mg(II), Zn(II), Ca(II), K(I), Na(I), Fe(II), Hg(II), Al(III), Cr(III) and Ga(III) respectively. Results are presented as mean ± SD (n = 3).

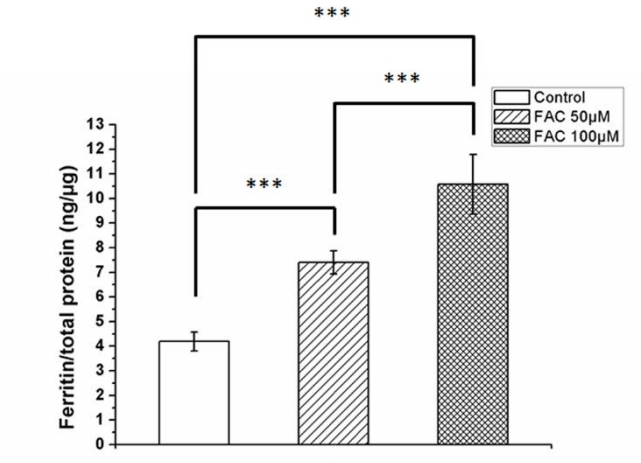


Figure S10. Ferritin expression levels in J774A.1 cells after 24 h incubation with 0, 50, or 100 μM FAC. Results are presented as mean ± SD (n = 3). *** $p < 0.001$.

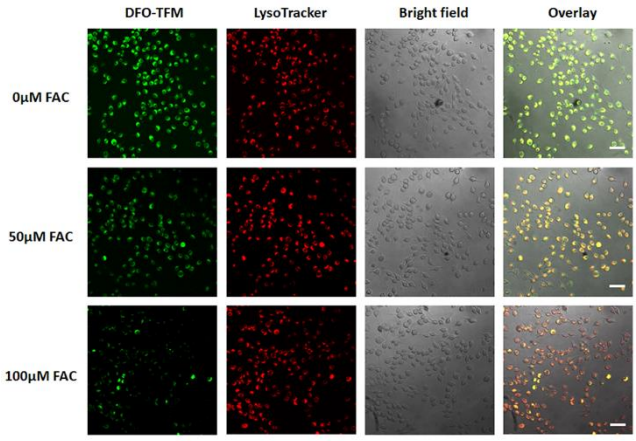


Figure S11. Fluorescence images of J774A.1 cells incubated with DFO-TFM for 1 h after iron-overloading cells with 0, 50 or 100 μM FAC for 24 h. Images from left to right correspond to DFO-TFM fluorescence, LysoTracker fluorescence, bright field, and overlaid images (scale bars: 50 μm).

References:

[1] J. Araki, C. M. Zhao, I. Kohzo, *Macromolecules* **2005**, *38*, 7524.