Electronic Supplementary Information

Biofunctional Metal-Phenolic Films from Dietary Flavonoids

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Materials

Quercetin (Que, ≥95%, Q4951), myricetin (Myr, ≥96%, M6760), fisetin (Fis, F505), iron chloride hexahydrate ($FeCl_3$•$6H_2O$, ACS reagent 97%, 236489), 3-(*N*-morpholino)propanesulfonic acid (MOPS, ≥99.5%, M1254), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich. Luteolin (Lut, 97%, L14186) was purchased from AlfaAesar. Tetrahydrofuran (THF, analytical reagent), dimethylsulfoxide (DMSO, analytical reagent), and ethanol (EtOH, analytical reagent) were purchased from Chem Supply. Polystyrene (PS) particles (D = 3.55 ± 0.07 μm and 3.20 ± 0.13 μm, 10 *w/v%* aq. suspension) were purchased from Microparticles GmbH. Ultrapure water (resistivity of 18.2 MΩ•cm) from an inline Millipore RiOs/Origin water purification system was used for preparing solutions and in all washing/resuspension steps. Aqueous solutions were filtered through a polyethersulfone (PES) membrane with a 0.22 μm pore size (Millex-GP, Merck) and for DMSO solutions a nylon membrane with a 0.45 μm pore size (Labquip Technologies) was used.

Characterization

Differential interference contrast (DIC) images were recorded on an inverted Olympus IX71 microscope (Olympus, Japan). Atomic force microscopy experiments (AFM) were performed on a JPK NanoWizard II BioAFM (JPK Instruments AG, Germany). Typical AFM scans were carried out in tapping mode with BudgetSensors silicon cantilevers (40 N/m, Tap300-G). The AFM film thickness and mean squared roughness values of air-dried capsules were analyzed with the JPK SPM data processing software (version 5.1.11). For height and surface roughness determination, at least 18 capsules were analyzed and values are given as mean ± SD. The single wall thickness was determined to be half of the height of flat, collapsed regions and the roughness is given as values of the root-mean-squared roughness

over 300 × 300 nm^2, fold-free regions of minimum height. Transmission electron microscopy (TEM) images were taken using a FEI Tecnai TF20 instrument (FEI, USA) with an operation voltage of 200 kV. To acquire scanning electron microscopy (SEM) images a Philips XL30 field-emission scanning electron microscope (Philips, Netherlands) was used at an operation voltage of 2.0 kV and a spot size of 2. For AFM, TEM and SEM experiments, aqueous capsule suspensions (5-10 μL) were dropped and air-dried on muscovite mica (grade V-1, G51-09, ProSciTech), formvar-carbon coated copper grids (GSCU200C, ProSciTech) and Piranha-cleaned silicon wafers, respectively. *Caution! Piranha solution is extremely toxic. Extreme care should be taken when handling the solution and only small volumes should be prepared.* SEM samples were gold-coated prior to measurement. To determine the ζ-potentials, capsule suspensions were diluted 1:100 into TRIS buffer (10 mM, pH 7.2) and samples measured on a Zetasizer Nano-ZS (Malvern Instruments, UK). X-ray photoelectron spectroscopy (XPS) data were obtained on a VG ESCALAB220i-XL spectrometer that is equipped with a hemispherical analyzer. Monochromatic Al Kα X-rays (1486.6 eV) at 220 W (22 mA and 10 kV) was used as incident radiation. For survey (wide) and high resolution (narrow) scans analyzer pass energies were set to 100 eV and 50 eV, respectively. Survey scans were performed with 1.0 eV step size and 10 ms dwell time, while high resolution scans were acquired over a 20 eV binding energy range with a 0.05 eV step size and 100 ms dwell time. The base pressure in the analysis chamber was lower than 8.0×10^{-9} mbar and the surface charging effect was compensated by a low energy flood gun. XPS data were processed with CasaXPS software and the C 1s peak was referenced to 285.0 eV for energy calibration. Samples were prepared from a concentrated aqueous capsule suspension that was dropped on a Piranha-cleaned silicon wafer and allowed to air-dry. The amount of Fe per capsule was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Agilent 720-ES). Aqueous suspensions of known capsule concentration were treated

with HCl at a final concentration of 0.66 M, diluted with water and compared to Fe standards of 0.1, 1, 10 and 100 ppm.

Preparation of Que/Fe^{III} Capsules from Particulate Templates

20 v/v% DMSO: Stock solutions of Que (100 mM in DMSO) and $FeCl_3$•$6H_2O$ (100 mM in water) were prepared freshly prior to use and filtered. The standard protocol used for capsule preparation is as follows: 50 µL of PS particles were washed twice in water (500 µL, 2000 *g*, 1 min) and suspended in 345 µL of water. Subsequently, 95 µL of DMSO, 5 µL of Que and then 5 µL of $FeCl_3$•$6H_2O$ were added to yield the following final concentrations (PS: 10 mg mL^{-1}, Que: 1 mM, $FeCl_3$•$6H_2O$: 1 mM, DMSO: 20 *v/v%* in a total volume of 500 µL). The particle suspension was thoroughly vortexed for 10 s after the individual additions of DMSO, Que and $FeCl_3$•$6H_2O$. The pH was immediately raised by adding 500 µL of MOPS buffer (20 mM, pH 7.3) and the suspension was vortexed for 60 s. Non-coating complexes were removed by centrifugation (2000 *g*, 1 min) and the pellet was washed four times with MOPS buffer (20 mM, pH 7.3). For dissolution of the PS core the pellet was washed four times with 500 µL THF (2000 *g*, 1 min). During the third THF step the suspension was incubated for 1-2 h on a rotator at 22 °C. The resulting hollow capsules were then washed with water (2 × 500 µL, 2000 *g*, 3 min) and resuspended in 300 µL of water for characterization.

For the DPPH assay with coated PS, PS templates were not removed. Instead, particles were washed twice with water after buffer washing steps and resuspended in 500 µL of water.

1 v/v% DMSO: Film formation was performed in the same manner as described for 20 *v/v%* DMSO, except that washed PS templates were resuspended in 440 µL of water and no further DMSO was added. Characterization of capsules obtained from 1 *v/v%* DMSO is shown in Figure S10.

Preparation of Myr/Fe^{III} Capsules from Particulate Templates

Myr/Fe^{III} capsules were prepared in the same manner as described for Que/Fe^{III} capsules in 20 *v/v%* DMSO except that a Myr stock solution of 20 mM in DMSO was used. Therefore, washed PS particles were suspended in 345 µL of water and subsequently 75 µL of DMSO, 25 µL of Myr and then 5 µL of $FeCl_3$•$6H_2O$ were added to yield the final concentrations (PS: 10 mg mL^{-1}, Myr: 1 mM, $FeCl_3$•$6H_2O$: 1 mM, DMSO: 20 *v/v%* in a total volume of 500 µL). For the DPPH assay, PS templates were not removed. Instead, particles were washed twice with water after buffer washing steps and resuspended in 500 µL of water.

Lut/Fe^{III} and Fis/Fe^{III} Film Formation on Particulate Templates

Lut/Fe^{III} films were prepared similarly as described for Que/Fe^{III} capsules in 20 *v/v%* DMSO. Stock solutions of Lut (25 mM in DMSO) and $FeCl_3$•$6H_2O$ (100 mM in water) were prepared freshly prior to use and filtered. 50 µL of washed PS particles were suspended in 340 µL of water) and subsequently, 60 µL of DMSO, 40 µL of Lut and then 10 µL of $FeCl_3$•$6H_2O$ were added to yield the final concentrations (PS: 10 mg mL^{-1}, Lut: 2 mM, $FeCl_3$•$6H_2O$: 2 mM, DMSO: 20 *v/v%* in a total volume of 500 µL). The particle suspension was thoroughly vortexed for 10 s after the individual additions of DMSO and Lut and vortexed for 60 s after the addition of $FeCl_3$•$6H_2O$. No buffer was added. Non-coating complexes were removed by centrifugation (2000 *g*, 1 min) and the pellet washed four times with water.

Fis/Fe^{III} films were prepared in the same manner as described for Lut/Fe^{III} except that a Fis stock solution of 20 mM in DMSO was used. Therefore, washed PS particles were suspended in 340 µL of water and subsequently, 50 µL of DMSO, 50 µL of Fis and then 10 µL of $FeCl_3$•$6H_2O$ were added to yield the final concentrations (PS: 10 mg mL^{-1}, Fis: 2 mM, $FeCl_3$•$6H_2O$: 2 mM, DMSO: 20 *v/v%* in a total volume of 500 µL).

For the DPPH assay, coated particles were resuspended in 500 µL water.

Que/Fe^{III} and Myr/Fe^{III} Film Formation on Planar PS

PS pieces of ca. 1 cm × 1 cm were cut from a Corning® petri dish, rinsed with water and isopropanol and air-plasma treated in a Harrick plasma cleaner before coating. Que/Fe^{III} films on PS were formed in a 1.75 ml Eppendorf tube and water (395 µL), DMSO (95 µL), Que (5 µL, 100 mM in DMSO) and $FeCl_3$•$6H_2O$ (5 µL, 100 mM in water) were added successively with intermediate vortexing steps. MOPS buffer (500 µL, 20 mM, pH 7.3) was added to raise the pH. Three washing steps were performed with the same buffer. For films in Fig. 1a, the deposition cycle was repeated 5 times, followed by extensive rinsing with water and drying of the films in a stream of N_2. Myr/Fe^{III} films were prepared as described for Que/Fe^{III} films with the following differences in added volumes and concentration of stock solutions: In a 1.75 ml Eppendorf tube with templating PS, water (395 µL), DMSO (75 µL), Myr (25 µL, 20 mM in DMSO) and $FeCl_3$•$6H_2O$ (5 µL, 100 mM in water) were added successively with intermediate vortexing steps before addition of MOPS buffer (500 µL, 20 mM, pH 7.3).

Stability Measurements by Flow Cytometry

For stability studies Que/Fe^{III} and Myr/Fe^{III} capsules were kept on a rotator in water at 22 °C in the dark with a concentration of ca. 5×10^5 capsules μL^{-1}. At indicated time points 10 µL was diluted with 250 µL of water for flow cytometric analysis to count the number of capsules. Capsule counting was carried out with an Apogee A50-Micro flow cytometer (Apogee Flow Systems, U.K.). Data shown are the mean ± SD from three independent measurements.

UV/Vis Absorption Spectroscopy

Base-line corrected UV/Vis absorption spectra were acquired on a Varian Cary 4000 UV/Vis spectrophotometer (Varian, USA). Free flavonoids were measured at a concentration of 8 µM

in water/EtOH 1:24 (*v/v*). Aqueous capsule suspension (50 µL) was diluted with EtOH (1150 µL) prior to measurement. For disassembly, capsule suspensions were exposed to 0.1 M HCl and incubated for 30 min at 22 °C. To quantify the amount of Que per capsule, a calibration curve was measured including Que concentrations of 3.75, 6.25, 12.5, 25, and 50 µM with the same solvent composition as used for capsule disassembly. Readout was performed with a Tecan infinite M200 Pro plate reader at an absorption wavelength of 374 nm.

Fluorescence Emission Spectroscopy

Fluorescence spectra were recorded on Horiba FluoroLog spectrophotometer with an excitation wavelength of λ_{exc} = 380 nm. Que was measured at a concentration of 10 µM water/EtOH 1:15 with 0.1 M HCl. Sample preparation of capsule suspension and disassembly at acidic pH as described for UV/Vis spectroscopy.

DPPH Assay

To assess the radical scavenging capacity (RSC) of free flavonoids and flavonoid MPN films, a previously published protocol[1] was used with slight modification for use with a plate reader. Readout was performed with a Tecan infinite M200 Pro plate reader. DPPH solutions were prepared freshly prior to use. Here, the RSC of flavonoid MPN films was measured at a DPPH concentration of 60 µM by mixing 780 µL of EtOH, 30 µL of an aqueous suspension of flavonoid/Fe^{III} coated PS (D = 3.2 µm) and 90 µL of DPPH (700 µM in EtOH). The number of particles was the same in all experiments and ca. $6•10^6$ (determined by flow cytometry). Control samples were prepared by replacing flavonoid/Fe^{III} coated PS either with pure water or uncoated PS particles. Samples were incubated in the dark on a rotator at 22 °C. At indicated time points 200 µL of particle suspension was taken, centrifuged (1 min, 2000 *g*)

and 150 µL of supernatant transferred into a 96-well plate for readout of absorption at 517 nm.

For comparison of free Que, Que/Fe^{III} films on PS and Que/Fe^{III} capsules, the concentration of Que was set to 10 µM (determination of Que content in films and capsules, see UV/Vis absorption spectroscopy) and the assay performed as described for flavonoid/Fe^{III} films above.

For assessing the radical scavenging activity of repeated cycles, 760 µL of EtOH, 50 µL of an aqueous suspension of Que/Fe^{III} coated PS (D = 3.2 µm) and 90 µL of DPPH (600 µM in EtOH) were mixed and incubated in the dark on a rotator at 22 °C. Note that the particle concentration was higher in this experiment, corresponding to a Que concentration of 18 µM. After 30 min the particle suspension was centrifuged (1 min, 2000 *g*) and 150 µL of supernatant was transferred into a 96-well plate for readout of absorption at 517 nm. The remaining supernatant was collected for UV/Vis spectroscopy and the pellet washed twice with EtOH. The pellet was redispersed in 740 µL of EtOH, 50 µL of water and 90 µL of DPPH (600 µM in EtOH) to arrive at the starting conditions. This process was repeated for three cycles.

All DPPH experiments were performed in triplicates and data shown are the mean ± SD.

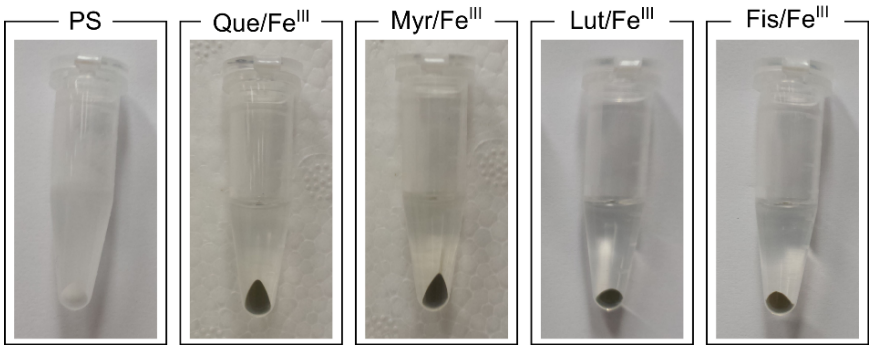

Fig. S1 Photographs showing colored pellets after film formation on PS particles and washing.

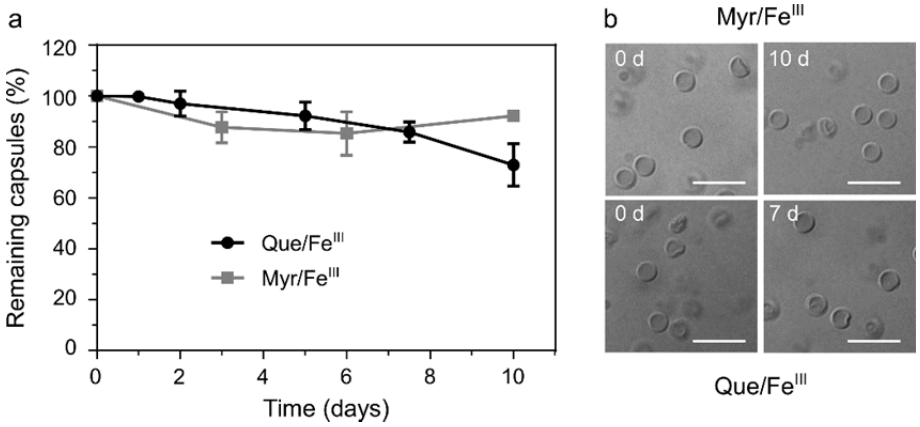

Fig. S2 Stability of Que/Fe^{III} and Myr/Fe^{III} capsules in water at 22 °C was assessed over 10 days by flow cytometry (a) and optical microscopy (b). Scale bars are 10 μm.

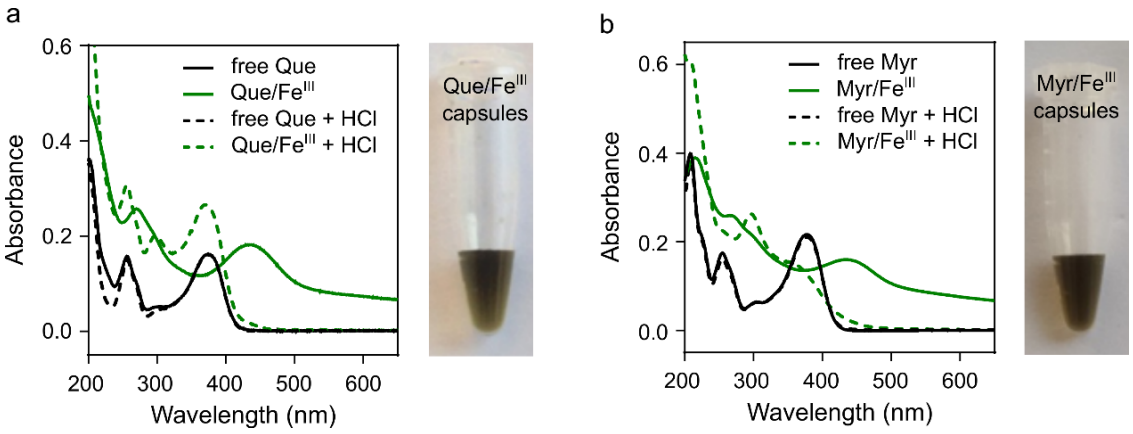

Fig. S3 UV/Vis spectra of Que (a) and Myr (b) samples. Graphs show spectra of the free flavonoids, capsule suspension and disassembled capsules upon HCl treatment. Photographs on the right of the spectra show aqueous capsule suspensions.

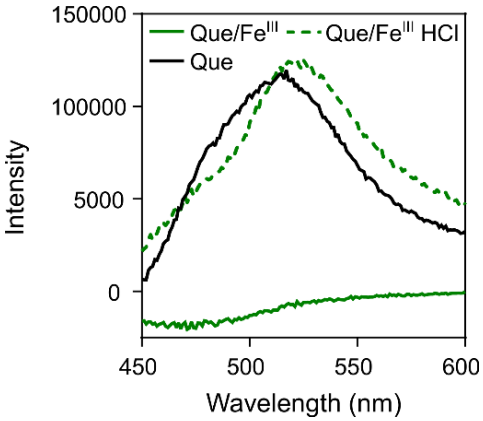

Fig. S4 Fluorescence emission spectra of free Que, Que/Fe^{III} capsule suspensions and a Que/Fe^{III} capsule suspension treated with HCl. Excitation wavelength is λ_{exc} = 380 nm.

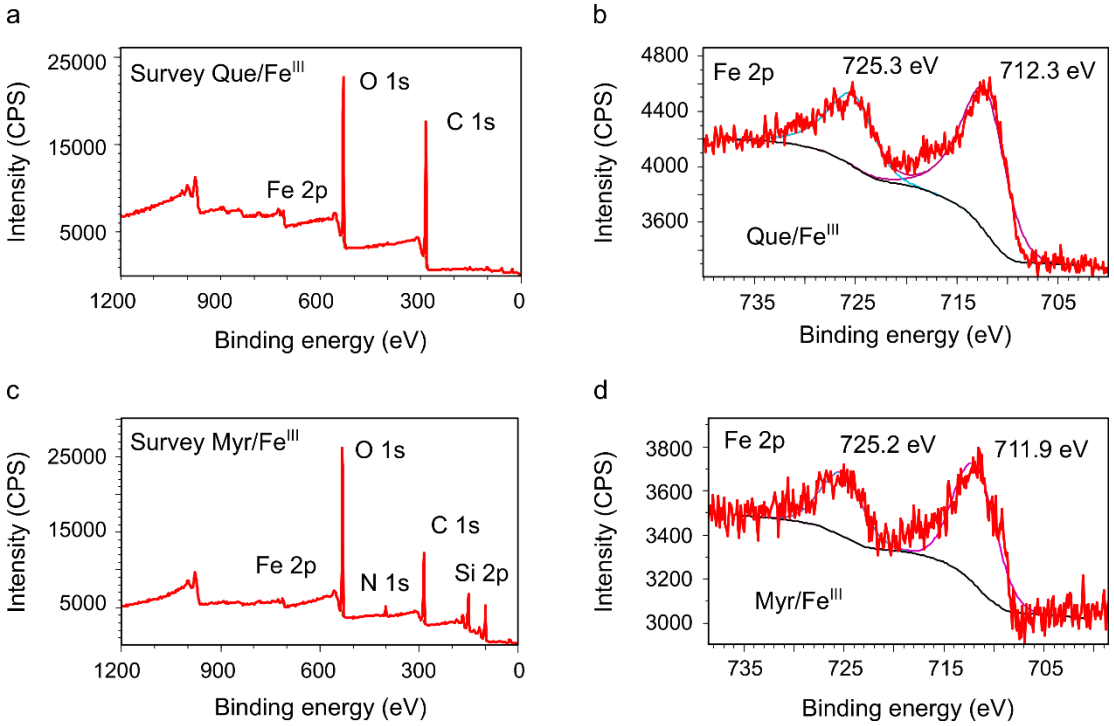

Fig. S5 XPS spectra of Que/Fe^{III} and Myr/Fe^{III}. Survey spectra (a, c) give the elemental composition of air-dried Que/Fe^{III} and Myr/Fe^{III} capsules, respectively. Fe 2p core-level spectra (b, d), indicating the oxidation state of Fe in both systems.

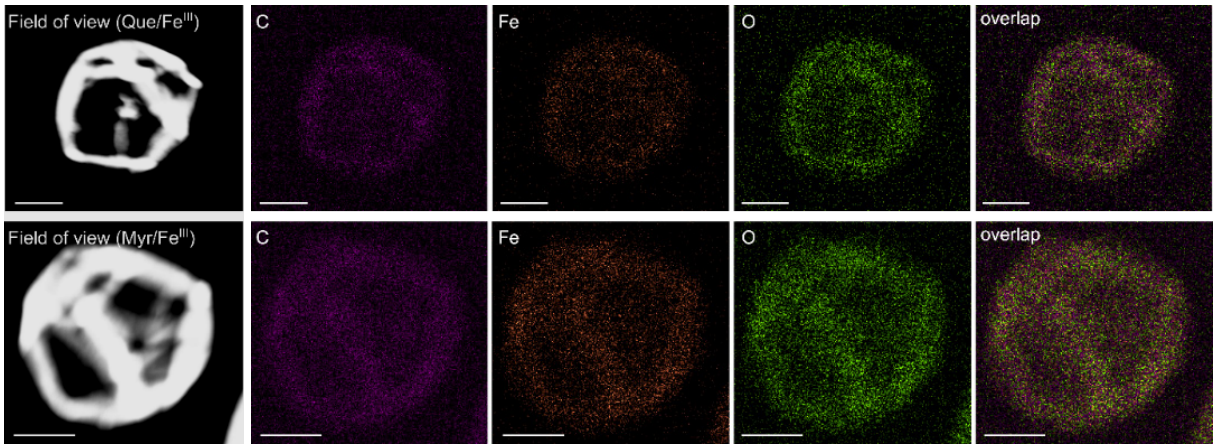

Fig. S6 EDX elemental mapping (C, Fe, O) of Que/Fe^{III} (top row) and Myr/Fe^{III} (bottom row) capsules. Scale bars: 1 μm.

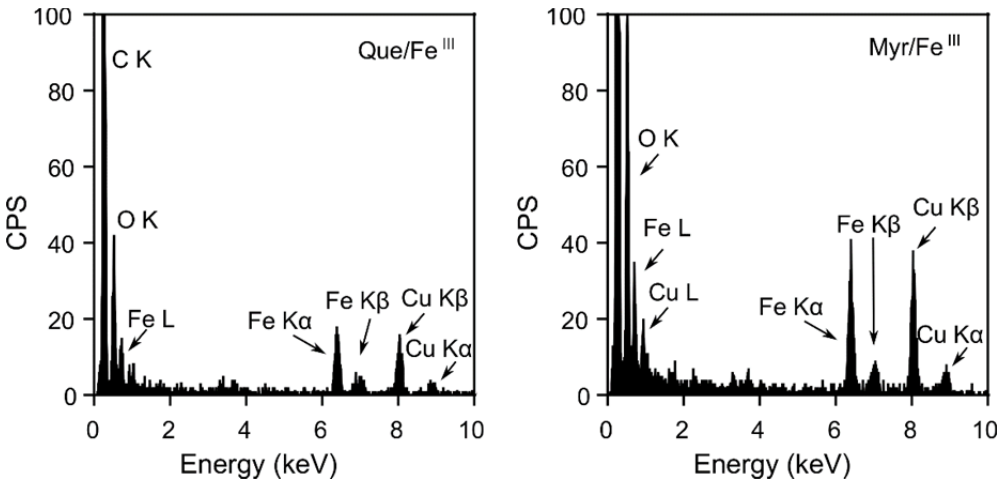

Fig. S7 EDX spectrum of Que/Fe^{III} (left) and Myr/Fe^{III} (right) capsules.

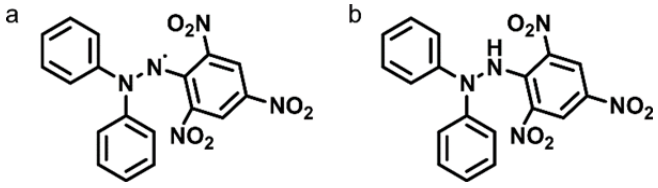

Fig. S8 Molecular structures of a) the DPPH radical and b) the reduced DPPHH.

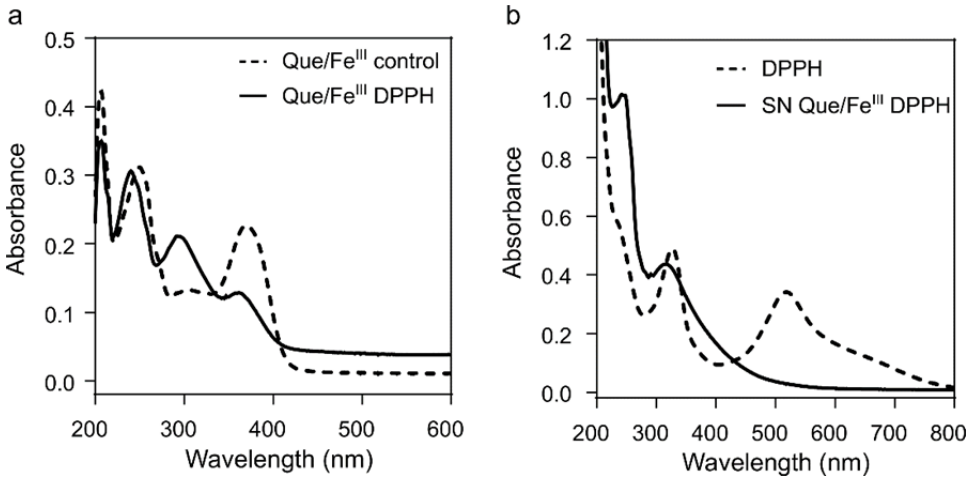

Fig. S9 UV/Vis spectra of DPPH-treated Que/Fe^{III} films. After 30 min exposure to DPPH films were recovered, washed and disassembled at 0.1 M HCl (a). UV/Vis spectrum of DPPH (30 µM in ethanol) and of the supernatant (SN) collected from films after 30 min exposure to DPPH (b).

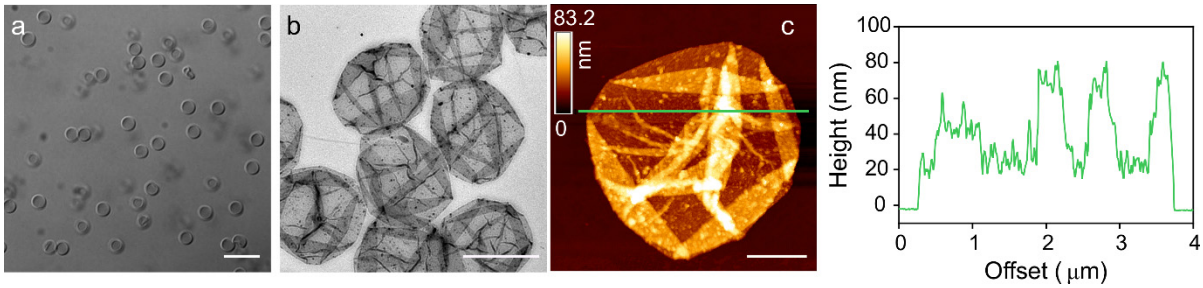

Fig. S10 Characterization of Que/Fe^{III} capsules formed in the presence of 1 *v/v%* DMSO by DIC microscopy (a) (scale bar 10 μm), TEM (b) (scale bar 3 μm) and AFM (c) with height profile of indicated cross section in green (scale bar 1 μm).

References

1 Y. Deligiannakis, G. A. Sotiriou and S. E. Pratsinis, *ACS Appl. Mater. Interfaces*, 2012, **4**, 6609–6617.