Supporting Information: The Facile and Additive-Free Synthesis of a Cell-Friendly Iron(III)-Glutathione Complex

Ziyu Gao,[a,b] Pablo Carames-Mendez,[a] Dong Xia,[a] Christopher M. Pask,[a] Patrick C. McGowan,[a] Paul A. Bingham,[c] Alex Scrimshire,[c] Giuseppe Tronci,*[,b,d] and Paul D. Thornton*[,a]

a. School of Chemistry, University of Leeds, Leeds, UK.
E-mail: p.d.thornton@leeds.ac.uk
b. Biomaterials and Tissue Engineering Research Group, School of Dentistry, St. James's University Hospital, University of Leeds, UK.
E-mail: g.tronci@leeds.ac.uk
c. Materials and Engineering Research Institute, Sheffield Hallam University, City Campus, Howard Street, Sheffield, UK.
d. Clothworkers' Centre for Textile Materials Innovation for Healthcare, School of Design, University of Leeds, UK.

Materials and Methods

Materials

Iron(III) chloride hexahydrate and Iron(II) chloride tetrahydrate were purchased from Sigma-Aldrich, L-glutathione (reduced) was purchased from Alfa Aesar, L-glutathione (oxidised) was purchased from BioSci. Alamar Blue assay kit was purchased from ThermoFisher Scientific.

Fe^{3+}-GSH complex preparation

123 mg (0.4 millimoles) of GSH was added to 4 mL $FeCl_3$ solution (0.1 M), and the mixture was mildly agitated by vortex mixing for 2 min. Then, the complex was precipitated in 40 mL ethanol. The products were collected by centrifugation at 10,000 rpm for 15 min. The Fe^{3+}-GSH complex produced was washed three times with ethanol and dried at 37 °C.

To determine the effect that GSH in its oxidised form (GSSG) has on the oxidation state of Fe, and if it can act as a chelating agent with Fe, 123 mg (0.2 millimoles) of GSSG were added to 4 mL $FeCl_2$ solution (0.1 M). Agitation in ethanol, and product precipitation, collection and drying were performed similar to the previous complex formation with GSH.

Fe^{3+}-GSH complex Analysis

The coordination between Fe^{3+} and GSH was confirmed by UV-Vis optical absorption spectroscopy. Various amounts of GSH solution (0.1 M) were independently added to 600 µL $FeCl_3$ (0.1 M) at different molar ratios. Deionised water was then added to dilute the complex solution to a final concentration of 40 mM $FeCl_3$. Absorbance was measured with baseline correction at 690 nm, at which point no components of the solution absorb significantly. Nine different molar ratios of Fe:GSH between 6:1 and 1:1.5 were assessed (see Figure S1).

The structure of the Fe^{3+}-GSH complex was assessed by fluorescence spectroscopy (FS). 1.0 mg/mL Fe^{3+}-GSH suspension was prepared via ultrasonic agitation for 10 min to achieve a homogeneous H_2O dispersion. 2 mL of each dispersion was used for FS analysis at room temperature, using an excitation wavelength of 408 nm. The blank and control spectra were carried out using 0.1 M $FeCl_3$, 0.1 M GSH and a $FeCl_3$-GSH mixture (1.2:1, 1:1 and 1:1.2 molar ratio) with consistent $FeCl_3$ concentration.

^{57}Fe Mössbauer spectroscopy was applied to confirm the Fe^{3+}-GSH configuration and iron valence. Measurements were carried out using acrylic absorber discs (area: 1.8 cm^2) loaded with sample to achieve a Mössbauer thickness of 1. The 14.4 keV γ-rays were supplied by the cascade decay of 25 mCi ^{57}Co in Rh matrix source, oscillated at constant acceleration by a SeeCo W304 drive unit, detected using a SeeCo 45431 Kr proportional counter operating with 1.745 kV bias voltage applied to the cathode. All measurements were carried out at room temperature (293 K) over a velocity range of ±4 $mm \cdot s^{-1}$, and were calibrated relative to α-Fe foil. Spectral data were fitted using the Recoil software package, using Lorentzian line shapes.

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR, Bruker AV400, 400 MHz) was carried out on Fe^{3+}-GSH solution (D_2O) to investigate the coordination between Fe and GSH. For further structural information, attenuated total reflectance Fourier transform infrared (ATR-FTIR, Bruker) spectroscopy was used to analyse dried Fe^{3+}-GSH powder between 400-4000 cm^{-1}. Circular dichroism (CD) spectroscopy was applied to investigate the chirality change from GSH to Fe^{3+}-GSH complex. CD spectra was carried with a bandwidth of 2.0 nm between 250-750 nm in 10 mm cuvette with 5 ml/mL concentration.

X-ray photoelectron spectroscopy (XPS) was carried out on Fe^{3+}-GSH and GSH powder at room temperature using a Thermo Scientific 250Xi instrument. 1.000eV/step was used for a complete scan and 0.050 eV/step used to scan a particular element.

Electrospray ionisation mass spectrometry (ESI-MS, Bruker maXis impact) was conducted to further elucidate the complex structure. The complex was dispersed in deuterated water and, due to the limited solubility of the Fe^{3+}-GSH complex in deuterated water, deuterated water that contained 20 µL HCl. Positive ion polarity was used in this measurement with scan from 50 m/z to 1500 m/z.

The Fe content of the Fe^{3+}-GSH complex was quantified by thermogravimetric analysis (TGA), with a measurement taken from 10 °C to 710 °C via a heat increase rate of 10 °C/min. The sample was maintained at 710 °C for a further 60 minutes to ensure the complete decomposition of organic matter.

The stability of the Fe^{3+}-GSH complex in H_2O was evaluated by dynamic light scattering (DLS) (Malvern, Nano ZS) at 25 °C with a concentration of 0.5 $mg \cdot mL^{-1}$ in H_2O. Ultrasonic agitation of the solution was conducted prior to analysis. Further analysis of the same samples was carried out after 24 hours at room temperature without any further sample agitation.

Fe^{3+}-GSH complex magnetism was measured with a Vibrating-sample magnetometer (Quantum Design, SQUID VSM system) from 0-3 T at room temperature by.

Powder XRD (x-ray diffraction) analysis was scanned from 5°-60° at room temperature, results were fitted by the Lorentz system, before the packing mode was determined using Bragg's law and the Scherrer equation.

$$n\lambda = 2d_{hkl}\sin\theta \qquad \text{(Bragg's law)}$$

where n is a positive integer, λ is the wavelength of the incident wave, θ is scattering angle, d is crystal lattice spacing.

$$\tau = \frac{\mathrm{K}\lambda}{\beta\cos\theta} \qquad \text{(Scherrer equation)}$$

where τ is the mean size of the ordered (crystalline) domains, K is Scherrer constant, λ is X-ray wavelength, β is FWHM (Full Width at Half Maximum) of XRD peak, θ is scattering angle.

Cytotoxicity tests with Fe^{3+}-GSH complex

The Fe^{3+}-GSH complex was dispersed in cell culture medium at an initial concentration of 2.5 mg/mL. L929 murine fibroblasts were cultured (37 °C, 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), and 0.5 % penicillin–streptomycin. The cell suspension (2×10^4 cells·mL^{-1}) was transferred to a 96-well-plate (100 µL per well), followed by the addition of increasing Fe^{3+}-GSH concentrations from 0.125 up to 1.25 mg·mL^{-1} in each well. The cell viability was quantified by Alamar blue assay after 1-7-day culture. Cells cultured on either tissue culture plates (TCPs) or non-treated tissue culture plates (NTCPs) were selected as either positive or negative control, respectively.

Testing of Fe^{3+}-GSH complex with matrix metalloproteinase 13 (MMP-13) *in vitro*

10 mg/mL Fe^{3+}-GSH complex was prepared in 120 mM HCl solution and diluted by deionised water to 2.308 mg/mL. GSH was dissolved in 120 mM HCl solution (1.765 mg/mL, regards TGA data) and set as control group to compare the effect with Fe^{3+}-GSH complex. Both complex and GSH solution were further diluted with deionised H_2O (×4) and each 20 µL diluted samples were added into each well of 96-well- plate, followed by 80 µL H_2O.

Pro-MMP-13 was activated following the commercial protocol. Briefly, 5 µL MMP-13 (10 µg/20 µL) was dissolved in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) working solution (1 mM) to 1 µg/mL and then incubated at 37 °C for 40 minutes. Activated MMP-13 was diluted by AMPA solution (2 mM) to 25 ng/mL and then immediately added to samples in a 100 µL/tube to ensure that the final concentration of enzyme was 12.5 ng/mL and the samples were 25 µL/mL. Deionised water with equal volume of AMPA solution (2 mM) was set as a blank and deionised water with equal volume of activated MMP-13 was set as the none treatment group.

After 12-hour or 24-hour reactions, retained MMP-13 activity was quantified using an MMP activity assay kit (Fluorometric Green, ab112146). Each 50 µL of reacted sample was pipetted into a 96-well-plate, followed by 50 µL MMP Green Substrate solution. The fluorescence of the MMP-13 activity was recorded after a 1-hour reaction in the dark at 37 °C using a microreader (Ex/Em=490/525 nm).

Statistical analysis

All the measurements were carried with at least 3 replicates. Significant difference was calculated through ANOVA with p value of 0.5. $p<0.5$ (*), $p<0.1$(**), $p<0.01$ (***), $p<0.001$ (****), $p<0.0001$ (*****).

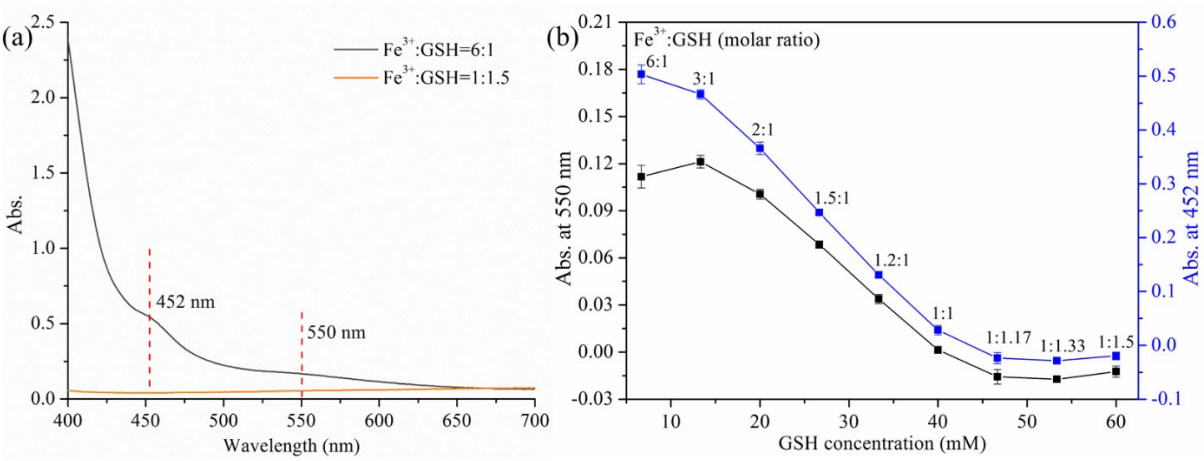

Figure S1 UV-Vis absorption spectroscopy of Fe^{3+}-GSH complex solution formed in H_2O with 40 mM $FeCl_3$. (A): Full wavelength spectrum of solutions containing an $FeCl_3$:GSH molar ratio of either 6:1 (black) or 1:1.5 (orange). (B): Tracking of 452 nm (right-handed y-axis) and 550 nm (left-handed y-axis) absorbances recorded in solutions with an $FeCl_3$:GSH molar ratio in the range of 6:1−1:1.15.

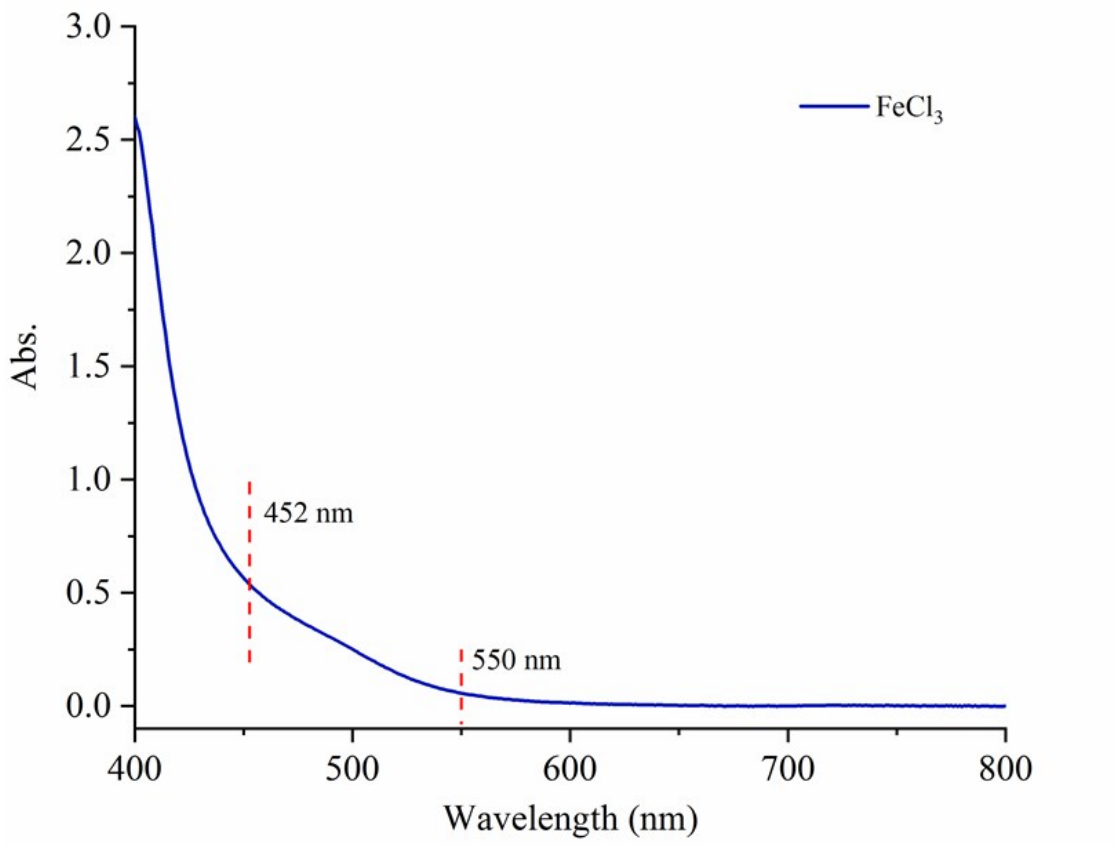

Figure S2. UV-Vis adsorption spectrum of a 40 mM $FeCl_3$ aqueous solution depicting absorption peaks at 452 nm and 550 nm.

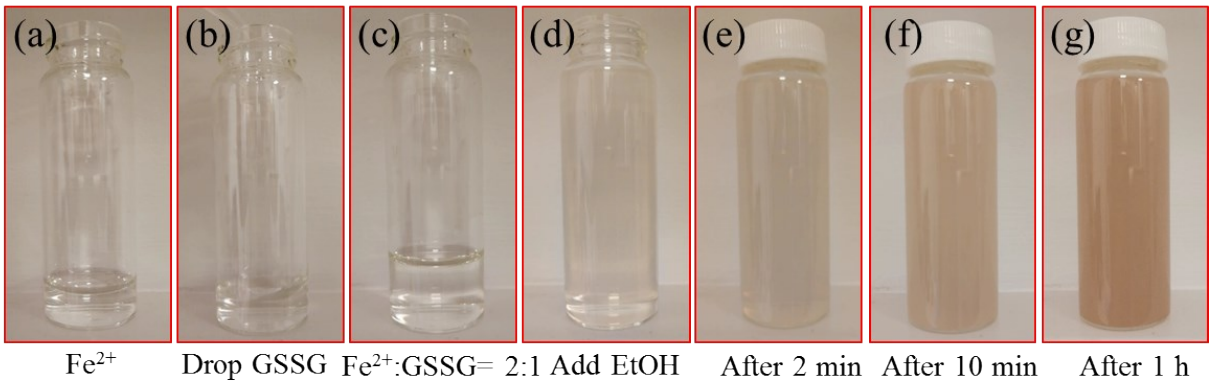

Figure S3. Color change of the ferrous chloride solution following addition of oxidised glutathione, describing the transition from the Fe^{2+} oxidation state to the Fe^{3+}-GSH complex.

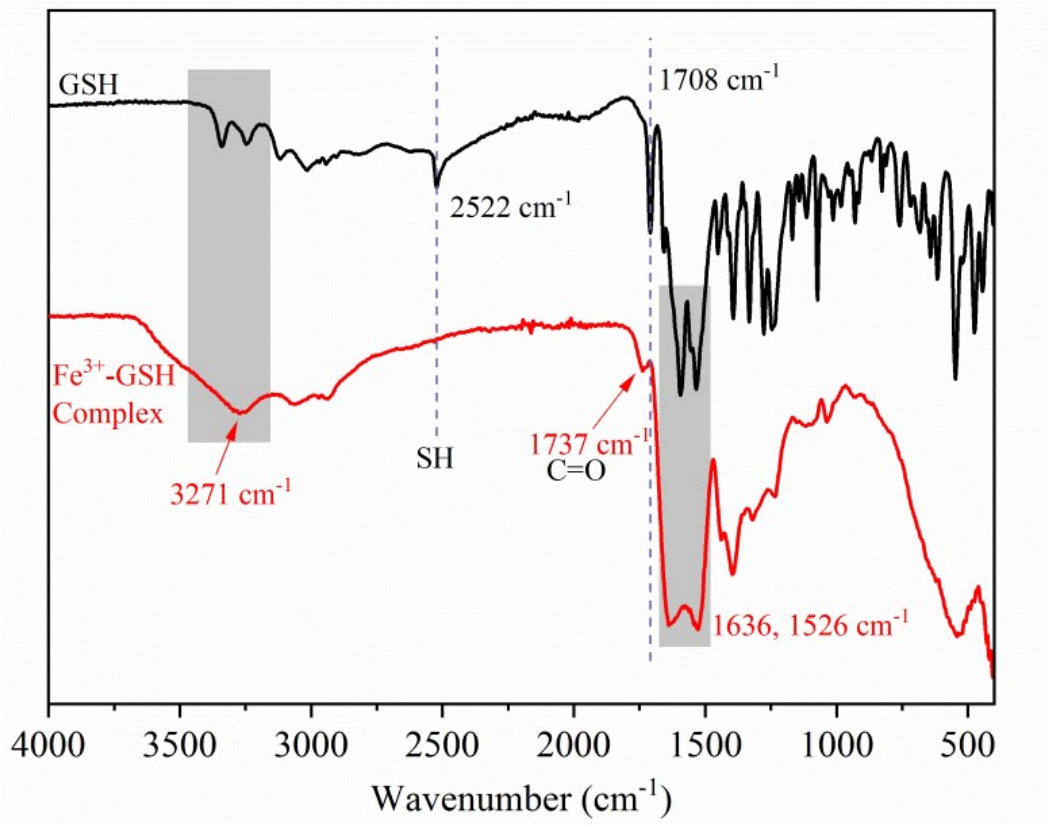

Figure S4 IR spectra of GSH (black) and the Fe^{3+}-GSH complex (red).

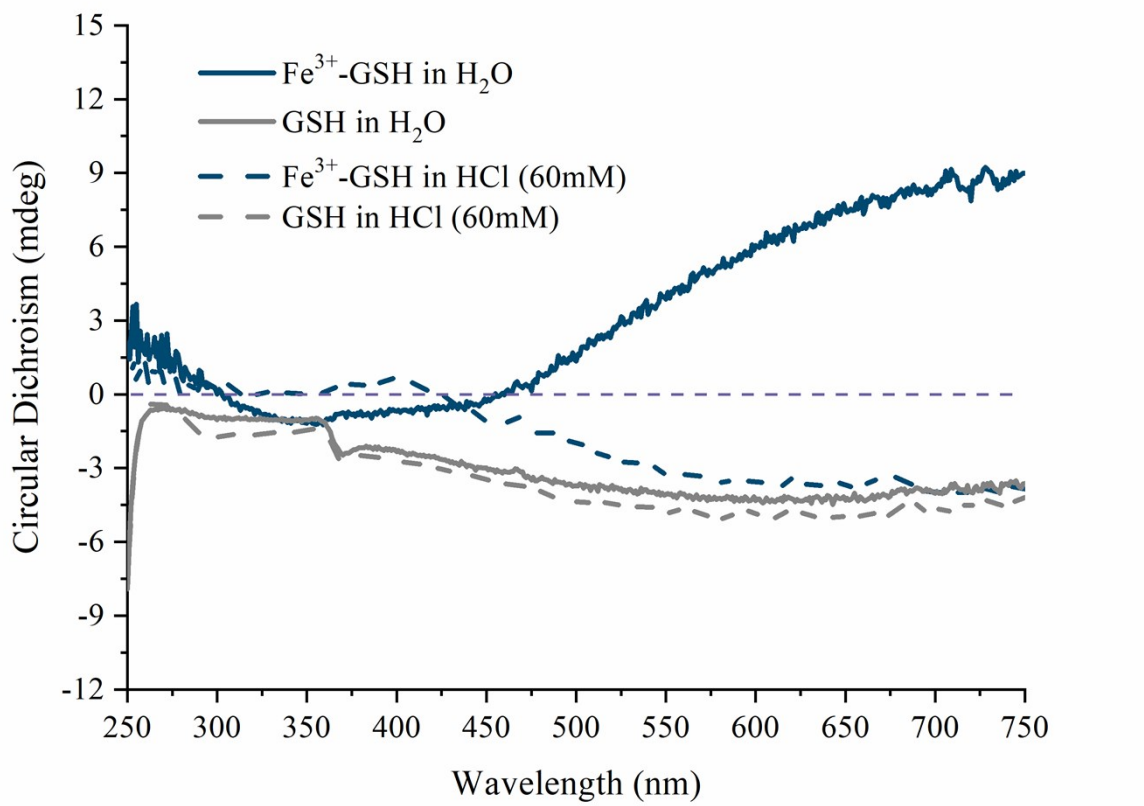

Figure S5 Circular dichroism spectra of GSH (grey solid) and Fe^{3+}-GSH complex (blue solid) in deionised water and GSH (grey dash) and Fe^{3+}-GSH complex (blue dash) in 60 mM HCl.

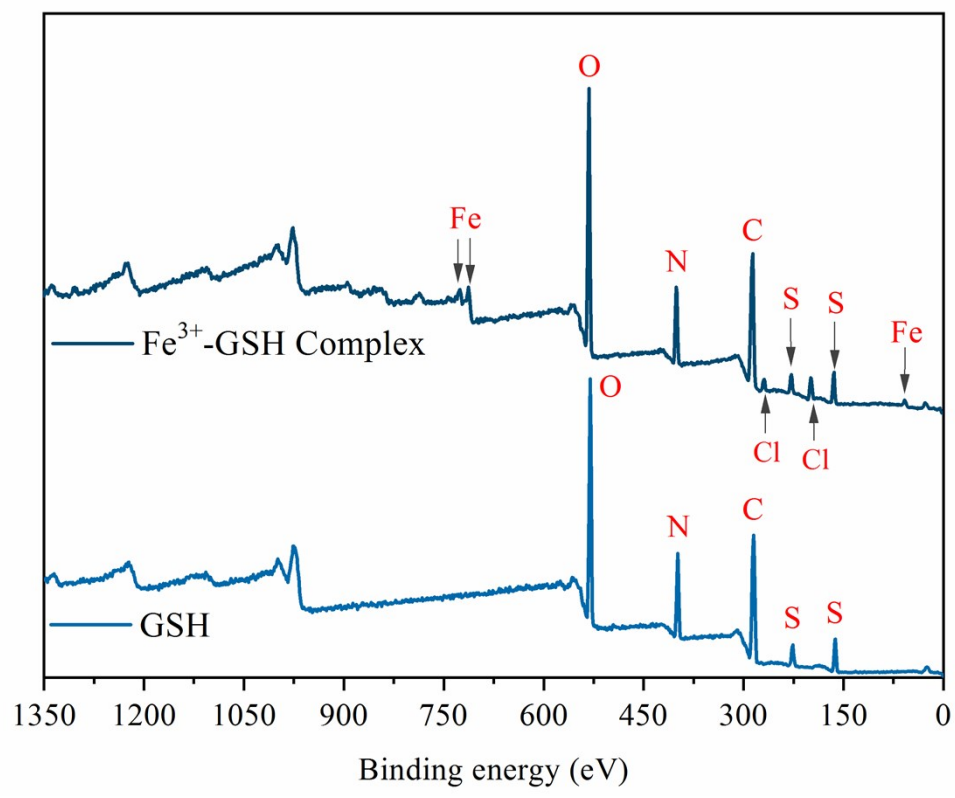

Figure S6 X-ray photoelectron spectroscopy (full spectrum) of Fe^{3+}-GSH complex and GSH.

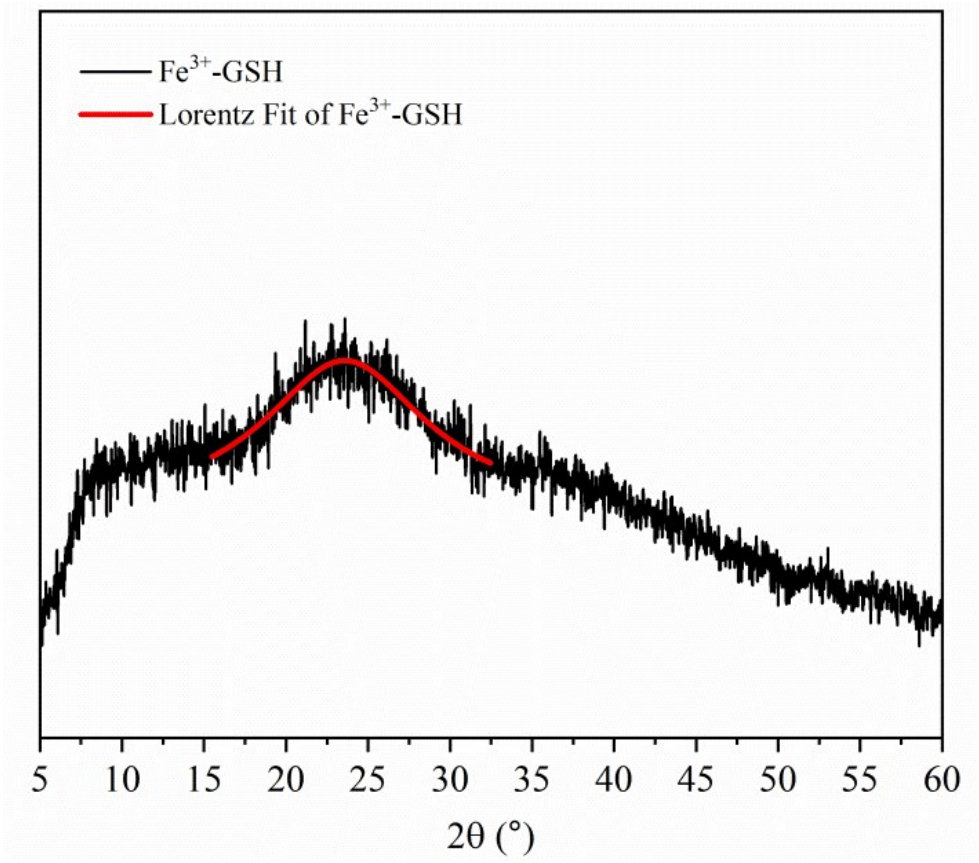

Figure S7 X-ray powder diffraction spectrum of Fe^{3+}-GSH.

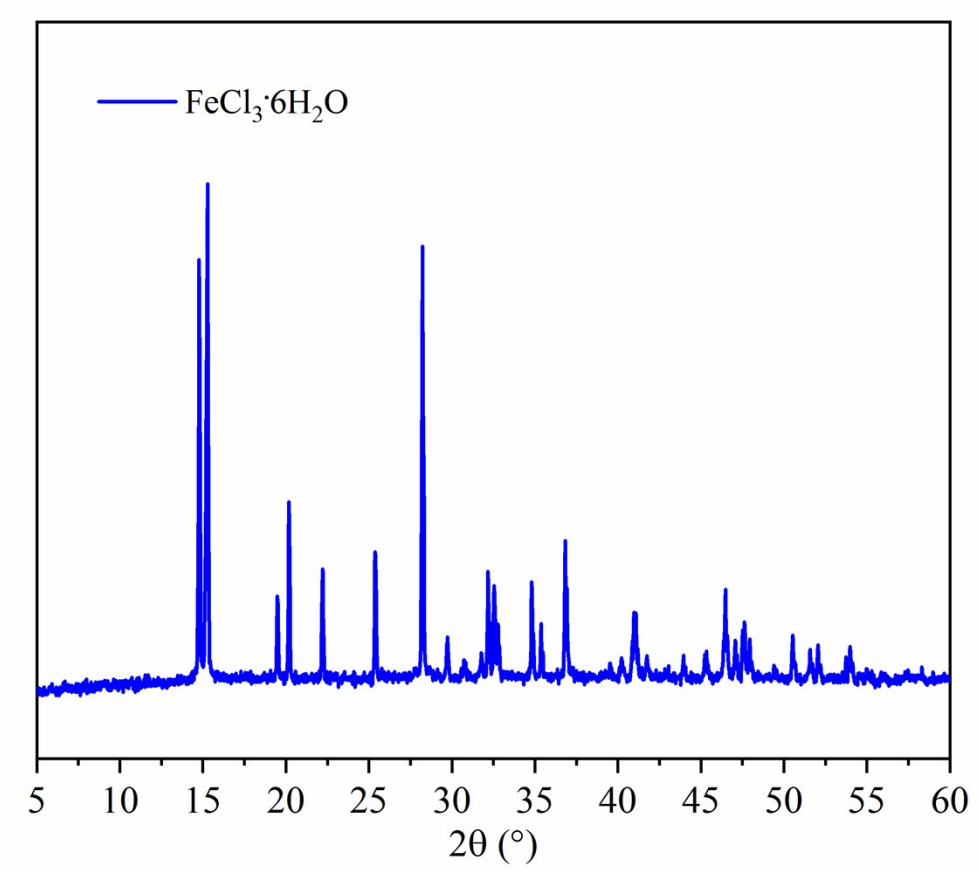

Figure S8 X-ray powder diffraction of Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$)

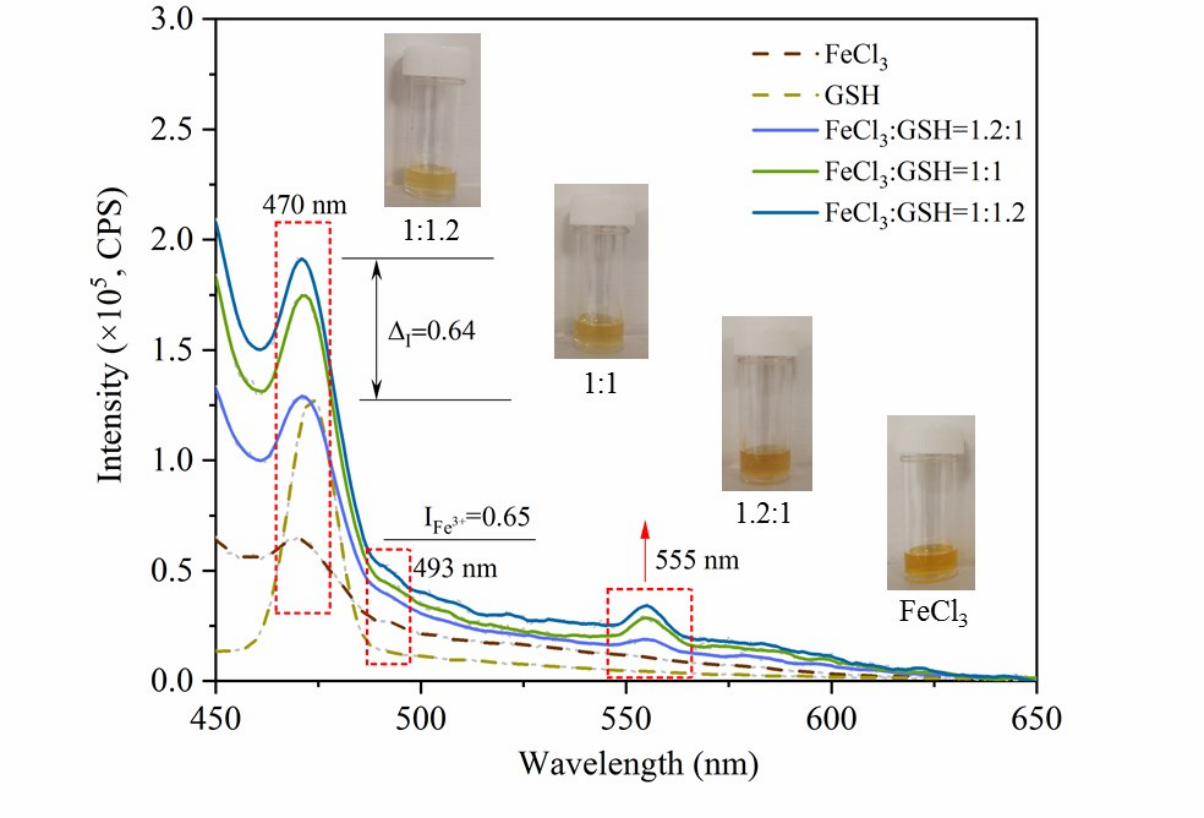

Figure S9 UV-Vis fluorescence spectra of $FeCl_3$ (--), GSH (--) and $FeCl_3$-GSH mixture with molar ratios of 1.2:1 (—), 1:1 (—) and 1:1.2 (—). Spectra were recorded with an excitation wavelength of 408 nm.

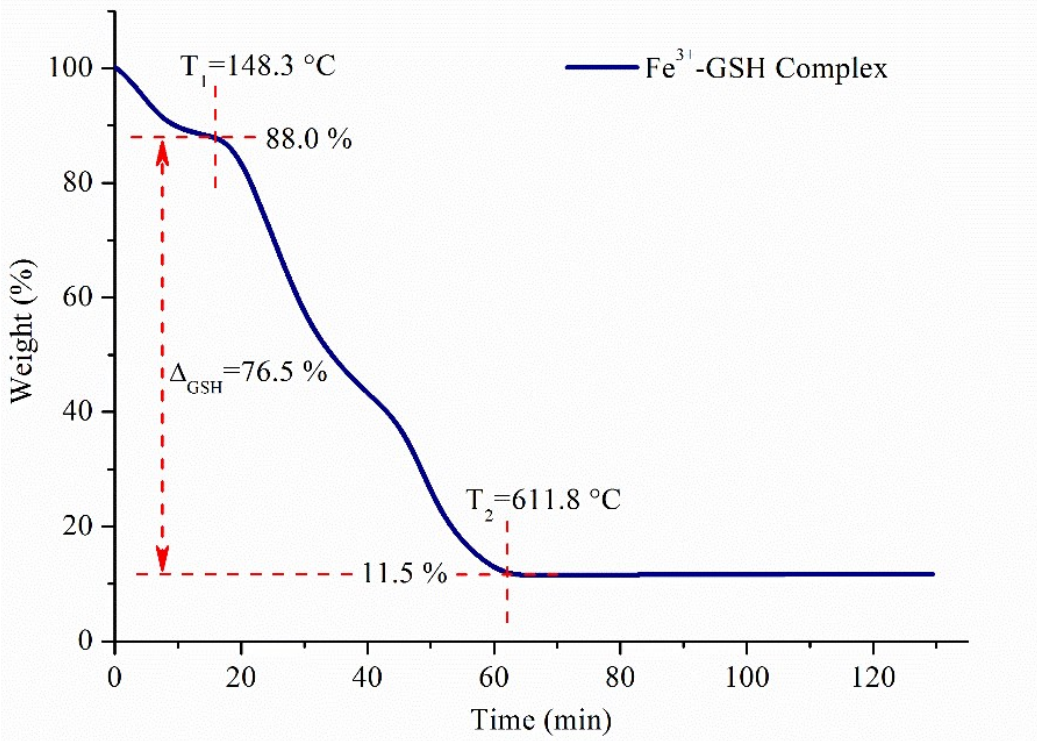

Figure S10 TGA thermogram corresponding to the Fe^{3+}-GSH complex.

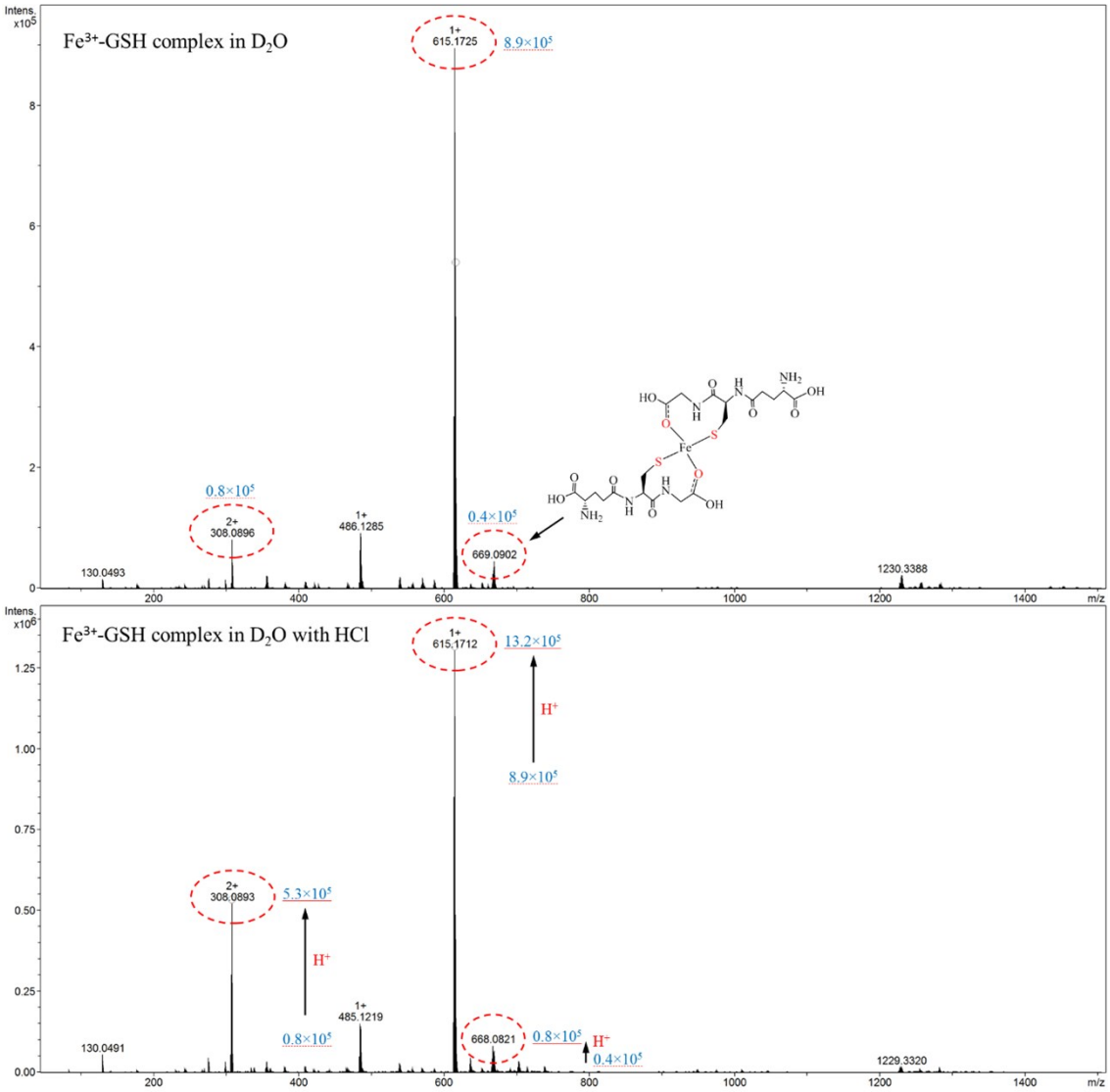

Figure S11 Mass spectra of Fe^{3+}-GSH complex in D_2O without (a) and with HCl (b).

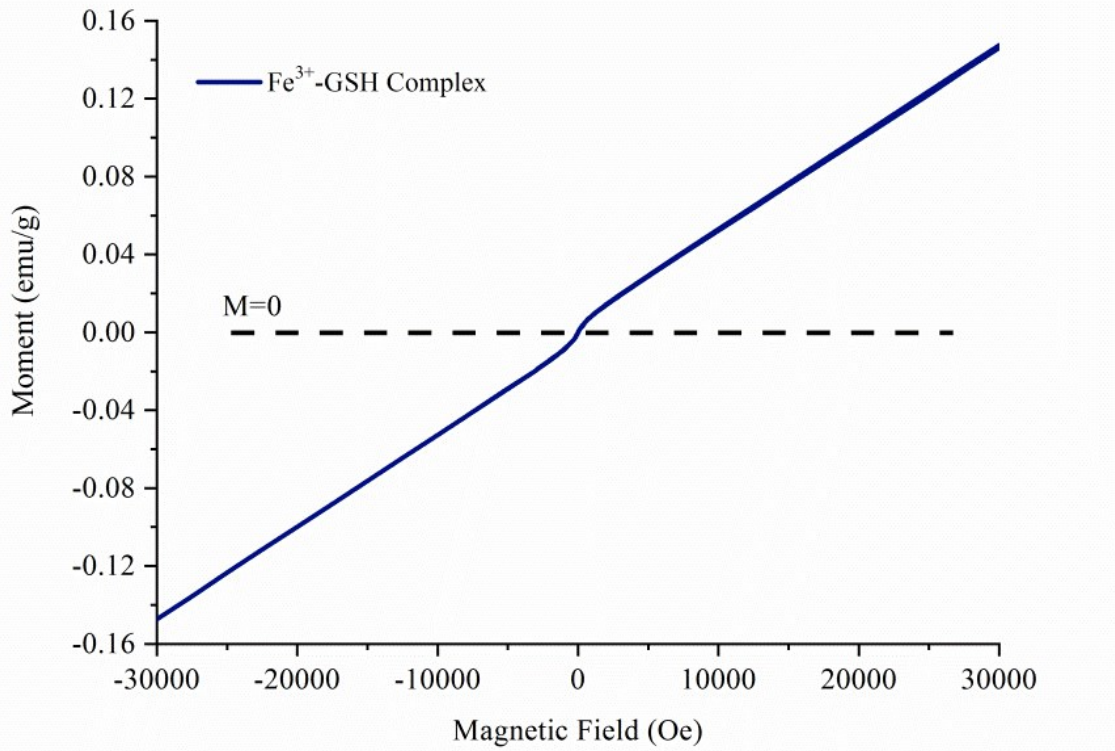

Figure S12 Vibrating-sample magnetometer measurement of Fe^{3+}-GSH from -3 T to 3 T magnetic field at room temperature.

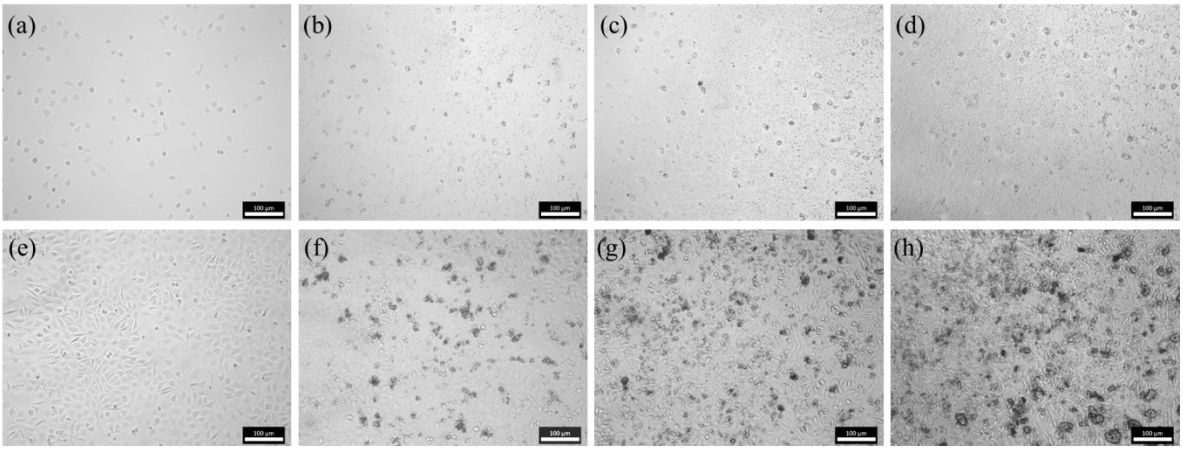

Figure S13 (a-d) Optical images captured following 1-day (a-d) and 7-day (e-h) culture of L929 murine fibroblasts on either TCPs (a, e), or cells culture medium supplemented with Fe^{3+}-GSH complex at a concentration of 0.125 mg·mL^{-1} (b, f), 0.25 mg·mL^{-1} (c, g) or 0.5 mg·mL^{-1} (d,h). Scale bar = 100 µm

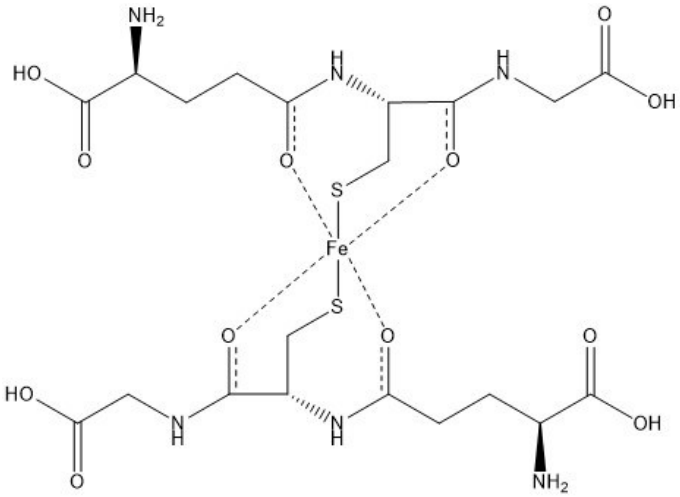

Figure S14 A proposed structure of the octahedral complex formed by Fe(III) and GSH in aqueous solution.

Table S1. Statistical analysis of L929 cells viability following 7-day cell culture on either TCPs or cell culture medium supplemented with varied concentrations of Fe^{3+}-GSH complex. p<0.5 (*), p<0.1(**), p<0.01 (***), p<0.001 (****)

	1 day	2 days	3 days	5 days	7 days
TCPs	***	***	****	****	
0.125 mg/ml	****	----	****	****	
0.25 mg/ml	****	***	****	***	
0.5 mg/ml	****	***	****	****	
0.75 mg/ml	****	****	****	****	
1 mg/ml	****	****	****	***	
1.25 mg/ml	**	****	****	***	