Three-minute synthesis of sp^3 nanocrystalline carbon dots as non-toxic fluorescent platforms for intracellular delivery.

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General

Chemicals were purchased and used without further purification. NH_2-FCD formation was conducted in a domestic microwave (Tesco Homebrand) 700W. Concentration centrifugation tubes were GE Healthcare Life Sciences VIVASPIN 6 or 20 with a 10,000 Da molecular-weight cut off filter. 500 Da dialysis membranes were purchased from Sigma-Aldrich. Kochetkov's amination of unprotected carbohydrates was conducted using Biotage Initiator+ microwave reactor. Extracts were concentrated under reduced pressure using both a Büchi rotary evaporator at a pressure of either 15 mmHg (diaphragm pump) and 0.1 mmHg (oil pump), as appropriate, and a high vacuum line at room temperature. ^{1}H & ^{13}C (HSQC and HMBC) NMR and ^{1}H-^{15}N HMBC NMR spectra were measured in D_2O in 400 or 500 MHz Varian (as stated). ^{1}H & ^{13}C NMR chemical shifts are quoted in parts per million (ppm) and referenced to the residual solvent peak (D_2O: ^{1}H = 4.79 ppm); ^{15}N NMR was referenced to NO_3^- ("Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy," Wiley-Interscience, NY, 1979) and coupling constants (J) given in Hertz. Multiplicities are abbreviated as: br (broad), s (singlet), d (doublet), t (triplet), q (quartet), p (pentet) and m (multiplet) or combinations thereof. Assignments were made, where necessary, with the aid of HSQC and HMBC NMR experiments. FTIR was conducted on a Bruker ATR. Dynamic Light Scattering (DLS) and Zeta analysis are carried out using Malvern Instruments, Nano-S90 Red Laser Model ZEN1690 for DLS and Nano-Z ZEN 2600 for Zeta potential. Zeta potential measurements were conducted in distilled H_2O at a concentration, of the FCD, of 250 μgmL^{-1}. The morphology and structure of CDs were examined by transmission electron microscopy (TEM) on a Jeol 2100F with an accelerating voltage of 200 kV. A drop of the NH_2-FCD methanolic suspension (5 mg/mL) was carefully applied to a 200 mesh carbon-coated copper grid and dried at ambient temperature for TEM characterization. Fluorescence measurements were made and conducted on a Perkin-Elmer LS45 in quartz cuvettes. Absorbance measurements were conducted on Cary UV-Vis 50 spectrophotometer in quartz cuvettes. Quantum yield of fluorescence measurements were conducted between both Perkin-Elmer LS45 and Cary UV-Vis 50 spectrophotometer in quartz cuvettes, relative to quinine sulfate (in 0.1M H_2SO_4). Raman spectra were recorded at room temperature using a Renishaw spectrometer and 514 nm green excitation. Curve fitting and data analysis Fityk software was used to assigned the peak locations and fit all spectra. X-ray photoelectron spectroscopy (XPS) was performed using a Kratos Axis Ultra DLD spectrometer, using monochromatic Al ka radiation operating at 144 W power (12 mA x 12 kV). For analysis, samples (dissolved in methanol) were pipetted on to clean gold wafers and the solvent evaporated under vacuum in the fast-entry lock of the spectrometer. Analysis of all regions was taken at a pass energy of 20 eV with a 0.1 eV step for high resolution scans, and 150 eV and a 1 eV step for survey spectra. All data was subsequent calibrated to the lowest C(1s) line at 285 eV and quantified using sensitivity factors supplied by the manufacturer in CasaXPS v2.3.17PR1.1.

Synthetic Protocols

General Protocol for Microwave-assisted Kotchevkov's Amination of Sugars

The microwave vial was charged with reducing sugar (0.25 g), ammonium carbonate (5 fold excess w/w over sugar, 1.25 g) and anhydrous DMSO (0.8 mL). The mixture was purged with N_2 and the tube was sealed and placed in an automated microwave at 60^0C, 250 psi pressure and 10 watts power for 90 minutes. The reaction mixture was freeze dried overnight to remove excess ammonium carbonate and DMSO to afford β-glycosyl amines as hygroscopic solids. (1-aminolactose is typically an orange solid post-freeze drying). Conversion to aminoglycoside confirmed by Ninhydrin treatment.

Synthesis of amine-coated carbon cots: NH_2-FCDs

Glucosamine hydrochloride (1.00 g, 4.63 mmol) was dissolved in distilled H_2O (20 mL) and stirred to achieve complete dissolution in a 250 mL conical flask. 4,7,10-trioxa-1,13-tridecanediamine (TTDDA) (1.11 mL, 5.09 mmol) was then added to the sugar solution and agitated to ensure homogeneity. The conical flask was then placed in a domestic microwave 700W (in a fume cupboard) and the solution heated (3 mins, 700 Watts, full power). A viscous brown residue was afforded which

was dissolved in distilled H_2O (10 mL) and centrifuged through a GE Healthcare Life Sciences Vivaspin 20 MWCO 10000 spin column (8500 rpm, 30 mins). The bulk solution was then reduced *in vacuo* (or lyophilised) to yield a thick brown oil, NH_2-FCDs (~2.0 g).

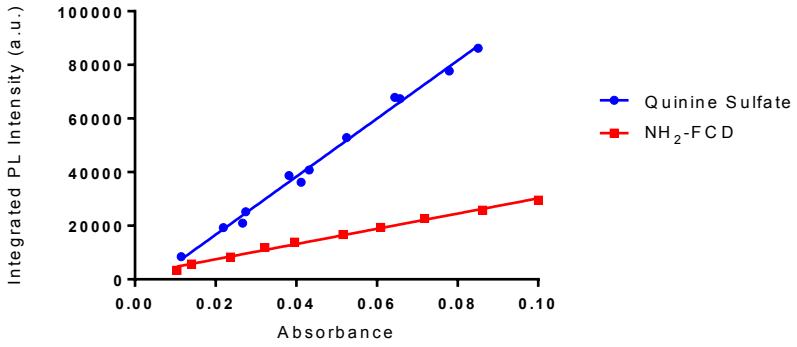
Acid Functionalisation of NH_2-FCDs: COOH-FCDs

250 mg of NH_2-FCDs was dissolved in methanol (5 mL) and succinic anhydride (100 mg) was added. The solution was sonicated for 5 mins before being stirred for 17 hrs. The solution was then reduced *in vacuo* to yield a brown oil. Tetrahydrofuran or diethyl ether (50 mL) was added to the residue and sonicated for 10 mins (repeated at least three times with fresh solvent). Supernatant poured off and centrifuged (8500 rpm, 5 mins) before reconsolidating the solid with the bulk material. The resulting material was dissolved in H_2O (5 mL) and freeze-dried to yield a brown oil-like solid, AmCDs. (350 mg).

Glyco-Functionalisation of COOH-FCDs: Lac-FCD

To COOH-FCDs (10 mg) in distilled H_2O (1 mL) was added CDI (1,1-carbonyldiimidazole) (40 mg) and the solution was sonicated for 15 mins. Glycosylamine (lactose) 2 mL (10mg/mL solution) was added to the solution and this was stirred for 17 hrs in the dark. Dialysed against distilled H_2O (500 MWCO) for 24 hours (H_2O changed regularly). Solution was reduced *in vacuo* to yield a brown product, Lac-FCDs (11 mg).

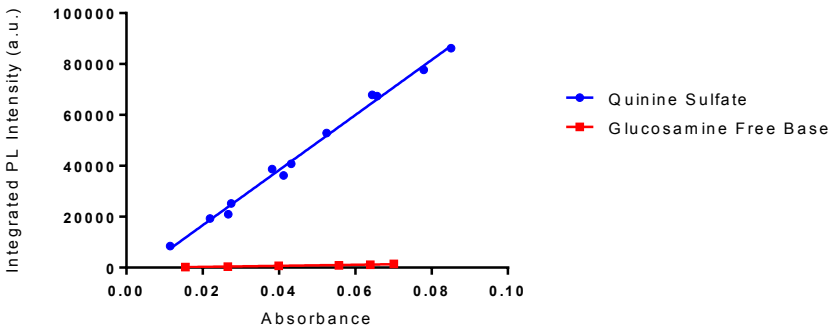
NH_2-FCD Characterisation



The quantum yield of fluorescence (QY or Φ) for NH_2-FCD, in aqueous media (refractive index; η= 1.33) was calculated by measuring the integrated fluorescence intensity (excitation 340 nm) at a range of absorbance values below 0.10. The resulting curve was plotted and compared against quinine sulfate (QS) in 0.1M H_2SO_4 (refractive index; η= 1.33), which is a standard of known QY of 54%. (*I = slope η = refractive index*)

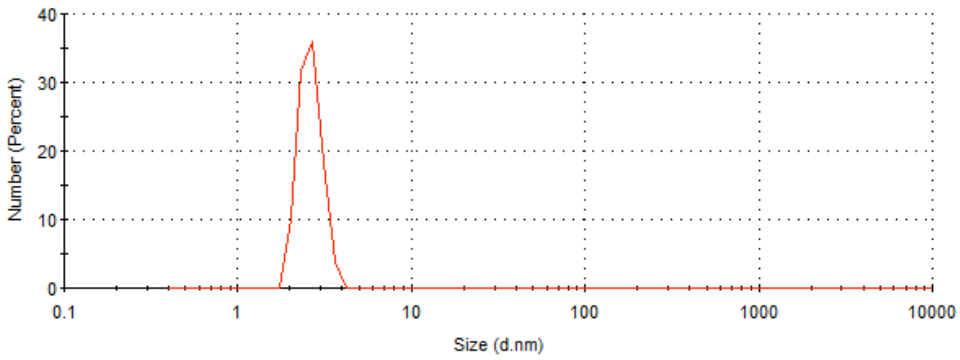
$$\Phi_{FCD} = \Phi_{QS} \times \left(\frac{I_{FCD}}{I_{QS}}\right) \times \left(\frac{{\eta_{FCD}}^2}{{\eta_{QS}}^2}\right) \qquad \Phi_{FCD} = 0.54 \times \left(\frac{330546}{994690}\right) \times \left(\frac{1.33^2}{1.33^2}\right) = \mathbf{0.18}$$

Figure S1A: NH_2-FCD quantum yield of fluorescence determination



$$\Phi_{FCD} = 0.54 \times \left(\frac{18272}{994690}\right) \times \left(\frac{1.33^2}{1.33^2}\right) = \mathbf{0.099}$$

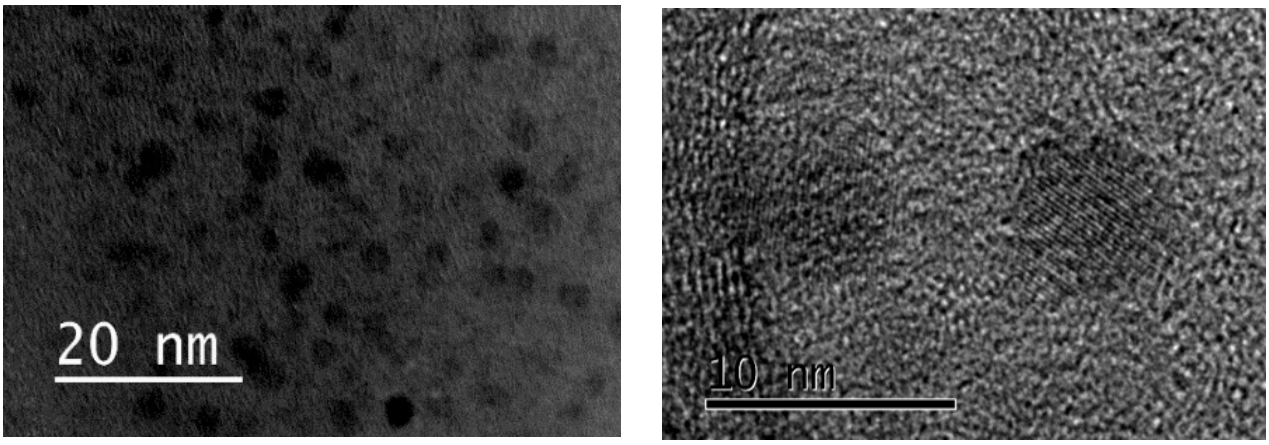
Figure S1B: NH_2-FCD (free base glucosamine) quantum yield of fluorescence determination



Hydrodynamic diameter profile: 2.62 nm ± 0.39

Figure S2: DLS profile of NH_2-FCDs

A



B

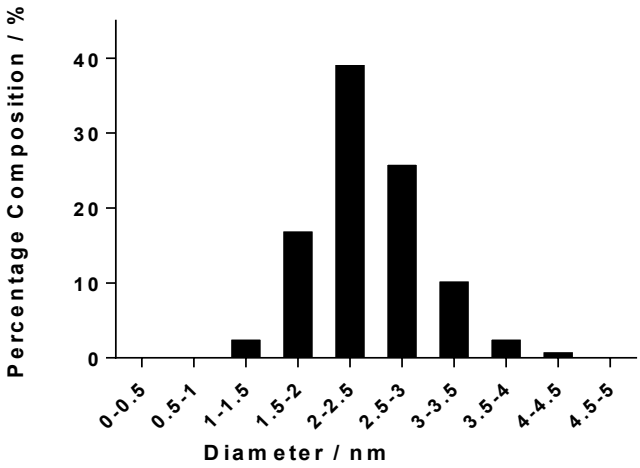


Figure S3: (A) Left: HR-TEM image of NH_2-FCDs Right: HR-TEM image exhibiting lattice fringes of NH_2-FCDs ((a particle of 8 nm in size was chosen to show the lattice fringes, however this size is not representative of the general population).) (B) Histogram of NH_2-FCD diameters, n = 180

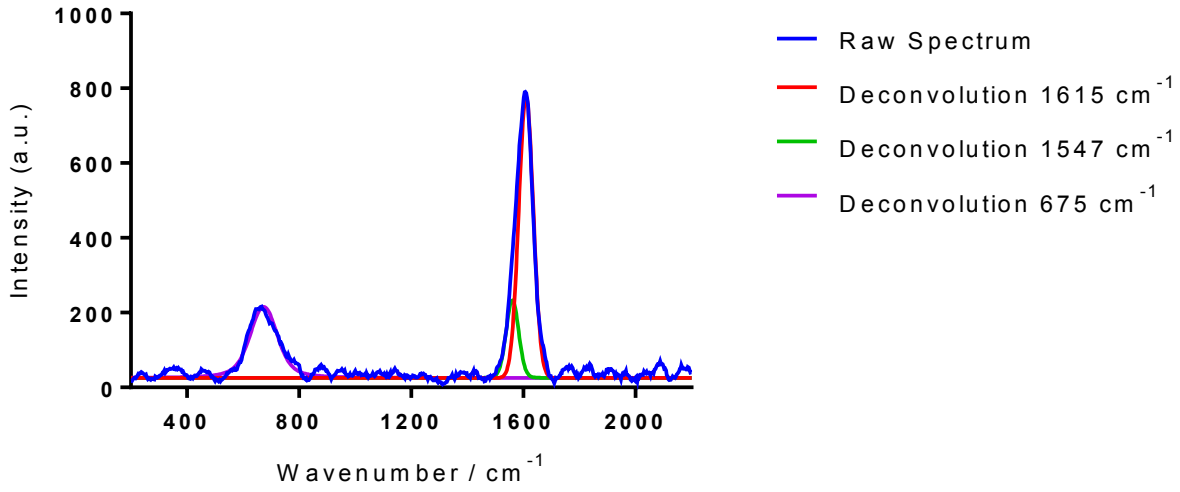


Figure S4A: Raman spectrum of NH_2-FCD

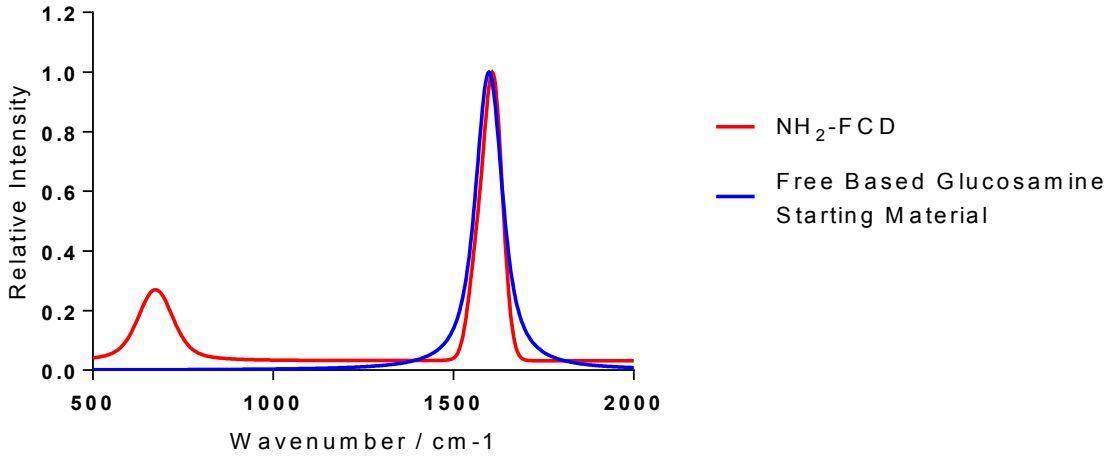
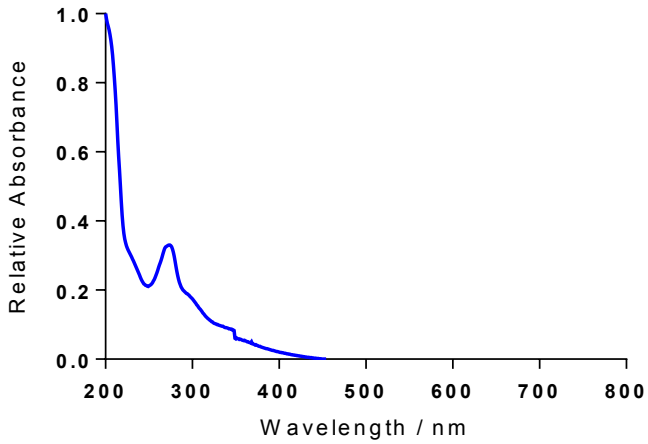


Figure S4B: Relative Raman spectra of NH_2-FCD vs NH_2-FCD from free-based glucosamine



200-450 nm absorbance: typical of CDs, especially with a leading tail into the far-UV region.

270 nm: π-π* transition of aromatic/alkenyl C=C bonds or C=N bonds

Figure S5: Absorbance spectrum of NH_2-FCD

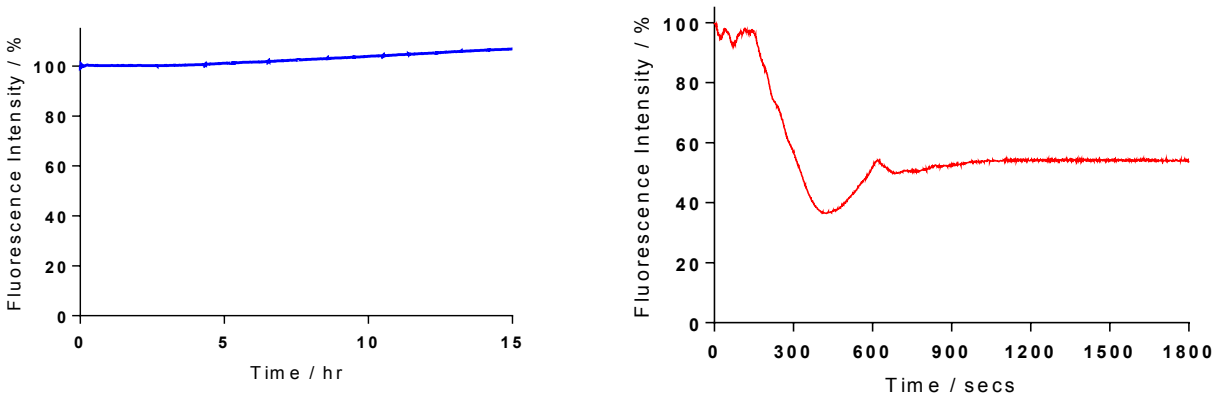


Figure S6: Fluorescence emisison intensity of (left) NH_2-FCD (monitored at 440 nm) with continuous irradiation (excitation at 340 nm) (right) Rhodamine 6G (monitored at 600 nm) with continuous irridation (excitation at 360 nm)

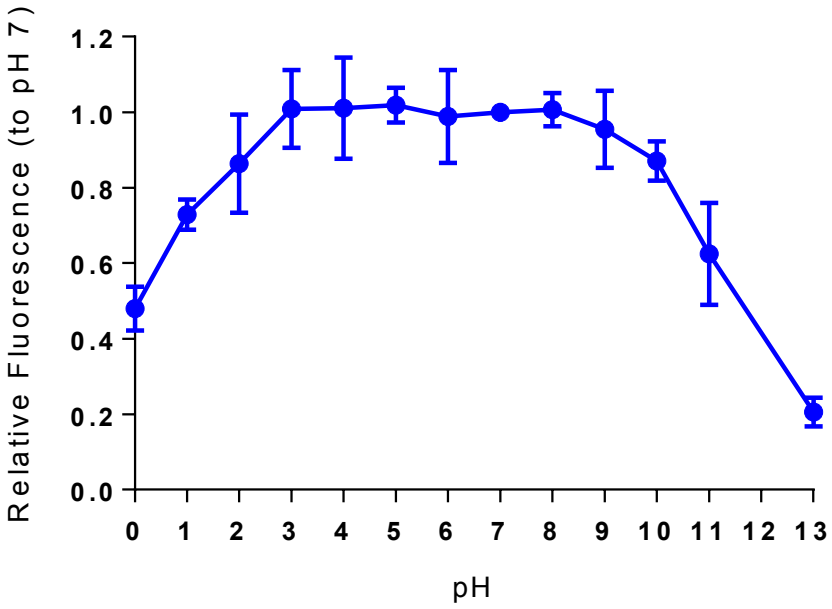
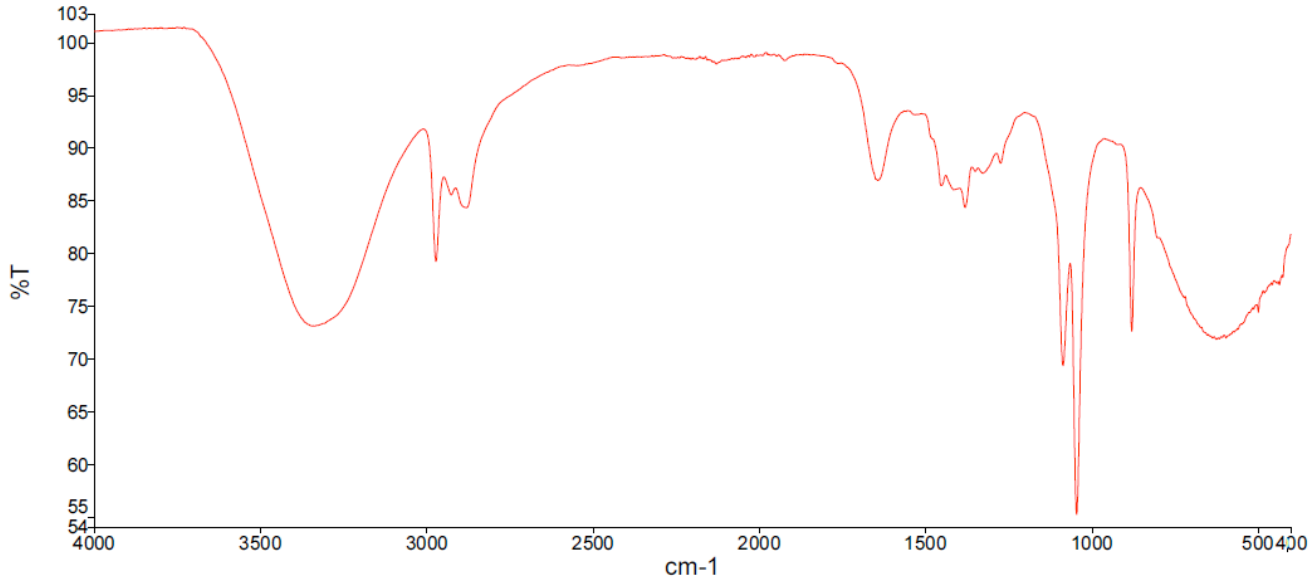


Figure S7: Fluorescence emission intensity of NH_2-FCD (emission 440 nm/excitation 340 nm) over a range of pH values (emission relative to intensity at pH 7)

	Elemental Composition / %				
Batch	**C**	**H**	**N**	**Cl**	**O**
1	44.72	7.63	7.94	7.76	31.96
2	47.59	8.42	7.95	2.12	33.92
3	47.11	8.95	8.08	9.65	26.21
Average	**46.47**	**8.33**	**7.99**	**6.51**	**30.70**

Table S1: Elemental composition of NH_2-FCDs



Key Features: O-H/N-H (3345 cm^{-1}), sp^3 C-H (2974 $cm^{-}1$), amide C=O (NHCO, 1644 cm^{-1}), C-O ether (1045 cm^{-1}), C-Cl (625 cm^{-1})

Figure S8: FTIR spectrum of NH_2-FCD

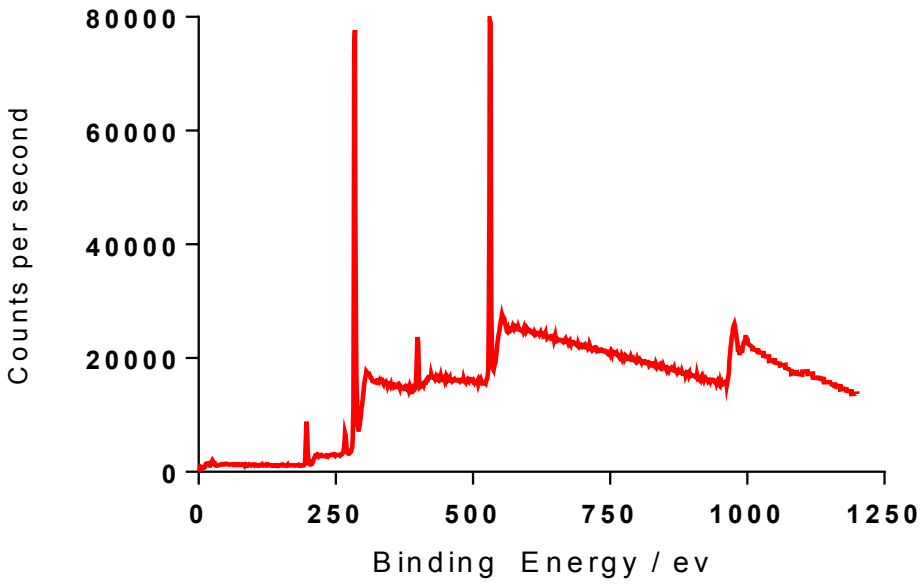


Figure S9: Widefield XPS spectrum of NH_2-FCDs

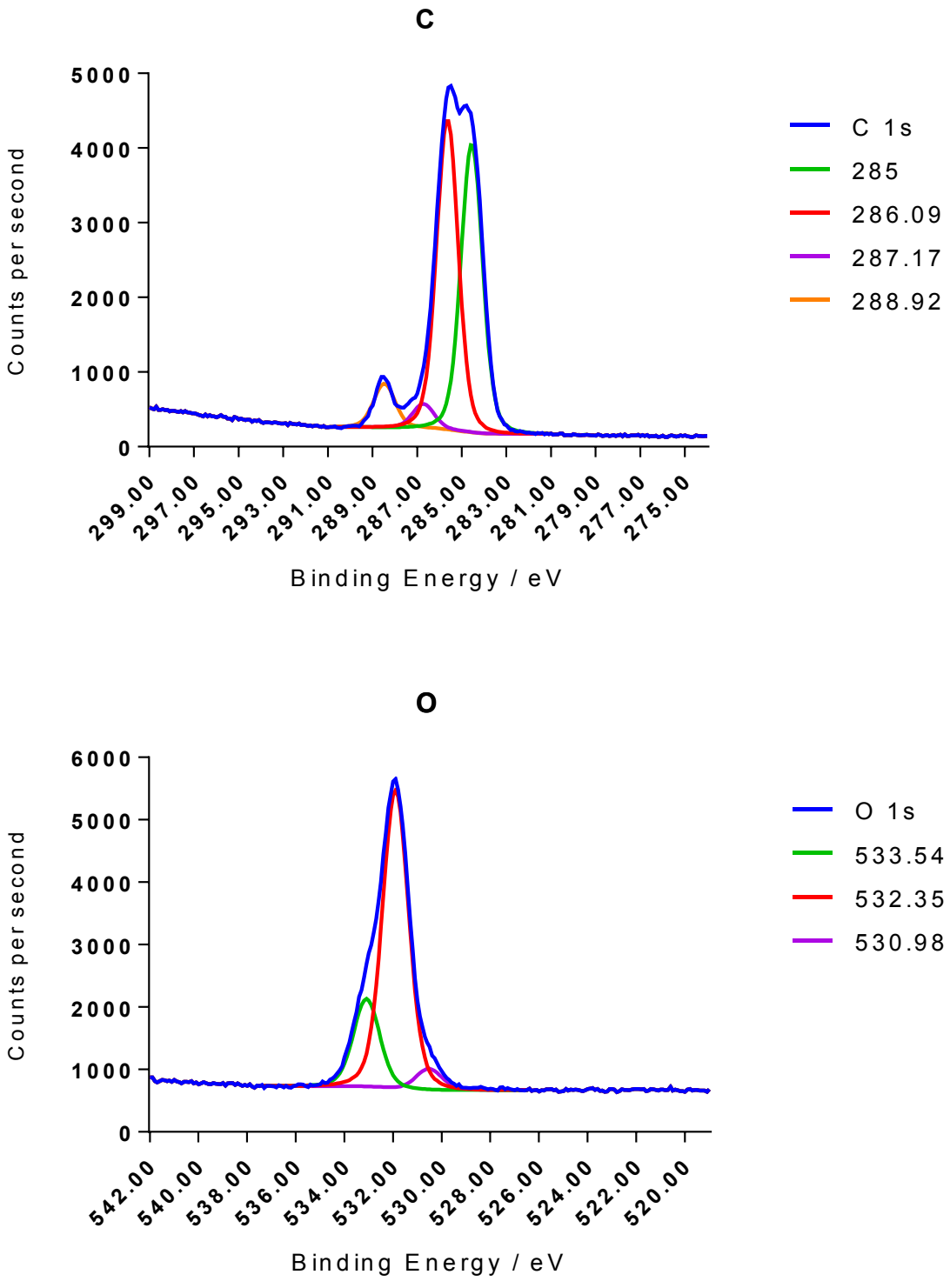


Figure S10: High-resolution XPS for C and O, plus deconvolutions

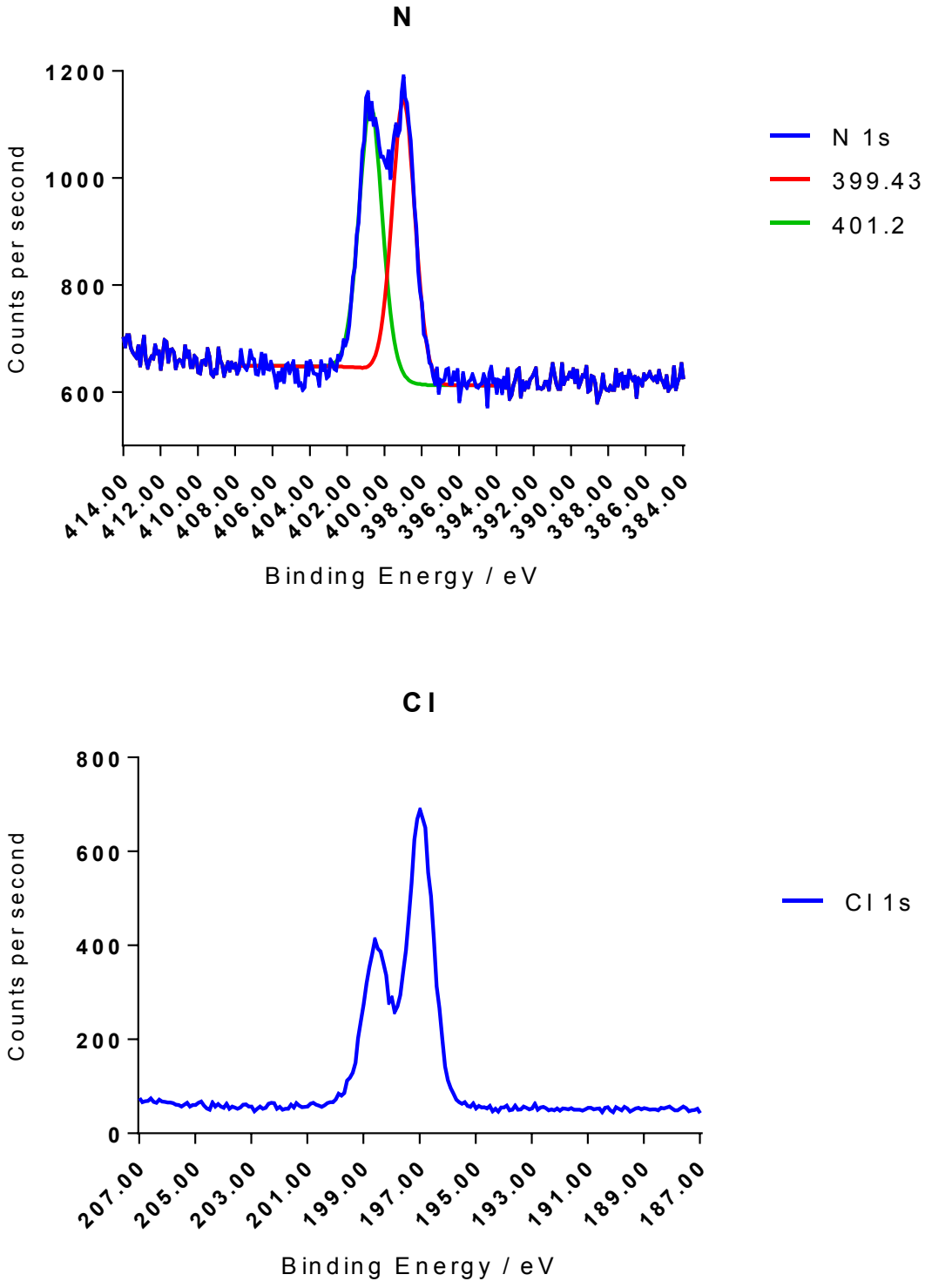
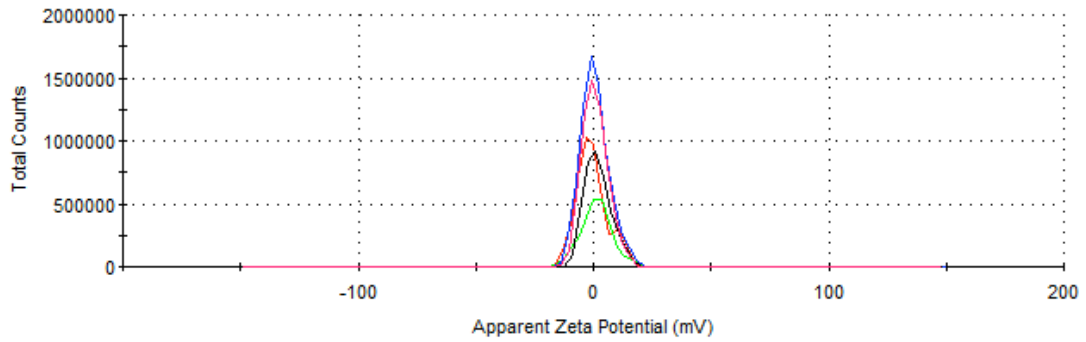


Figure S11: High-resolution XPS for N and Cl, plus deconvolutions

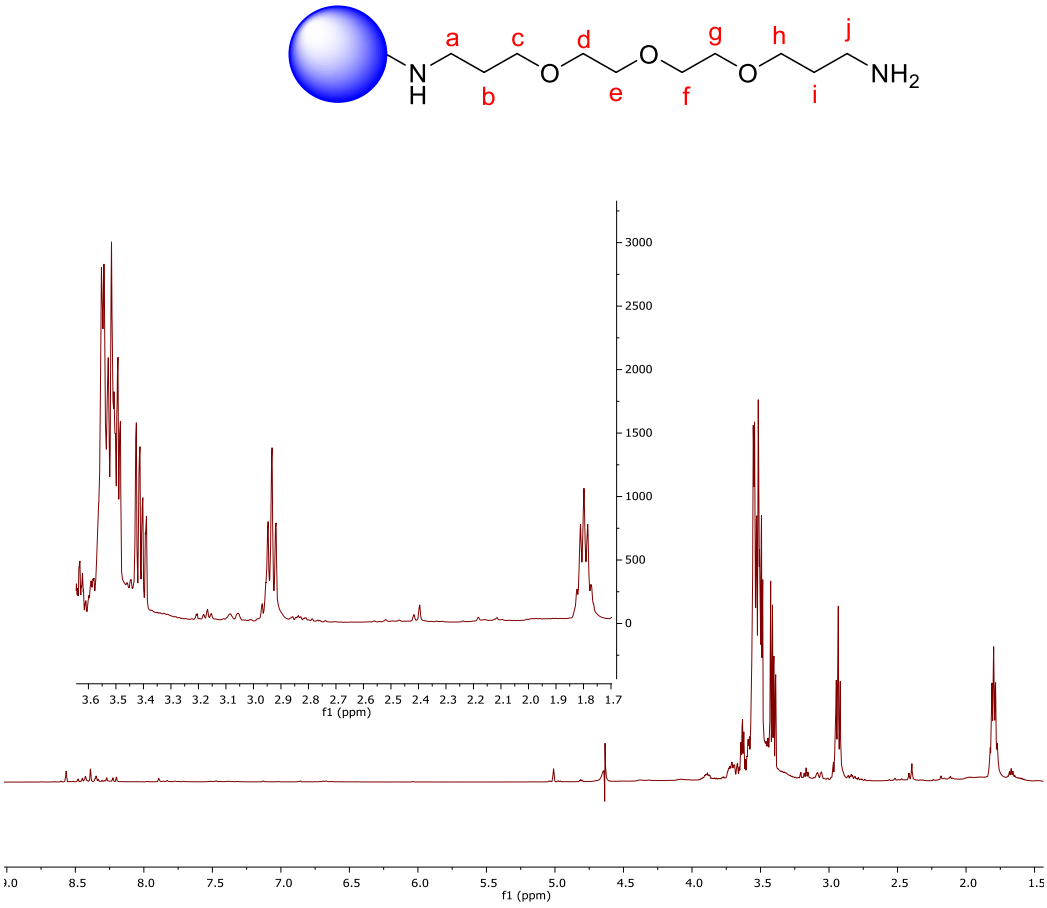
Element	Binding Energy Max / eV	Deconvolution Functionality
C	285	C-C
C	286.09	C-O-C (ether)/C-N (amine)
C	287.17	C=O
C	288.92	O-C=O (ester)
O	530.98	Aromatic C=O
O	532.35	Aliphatic C=O
O	533.54	Aromatic OH
N	399.43	Primary NH_2 (minor amide/pyridine contribution)
N	401.20	Imide

Table S2: XPS deconvolution and functional group assignments



0.78 mV $\pm$ 0.86

Figure S12: Zeta-potential measurement of NH_2-FCDs. Each line represent individual measurements of the same sample, which where used to obtained an average value.



^{1}H Presat NMR: (500 MHz, D_2O) δ: 3.58-3.39 (c-h), 2.94 (t, *J* = 7 Hz, a, j), 1.80 (q, *J* = 6.5 Hz, b, i)

Figure S13: ^{1}H Presat NMR of NH_2-FCDs

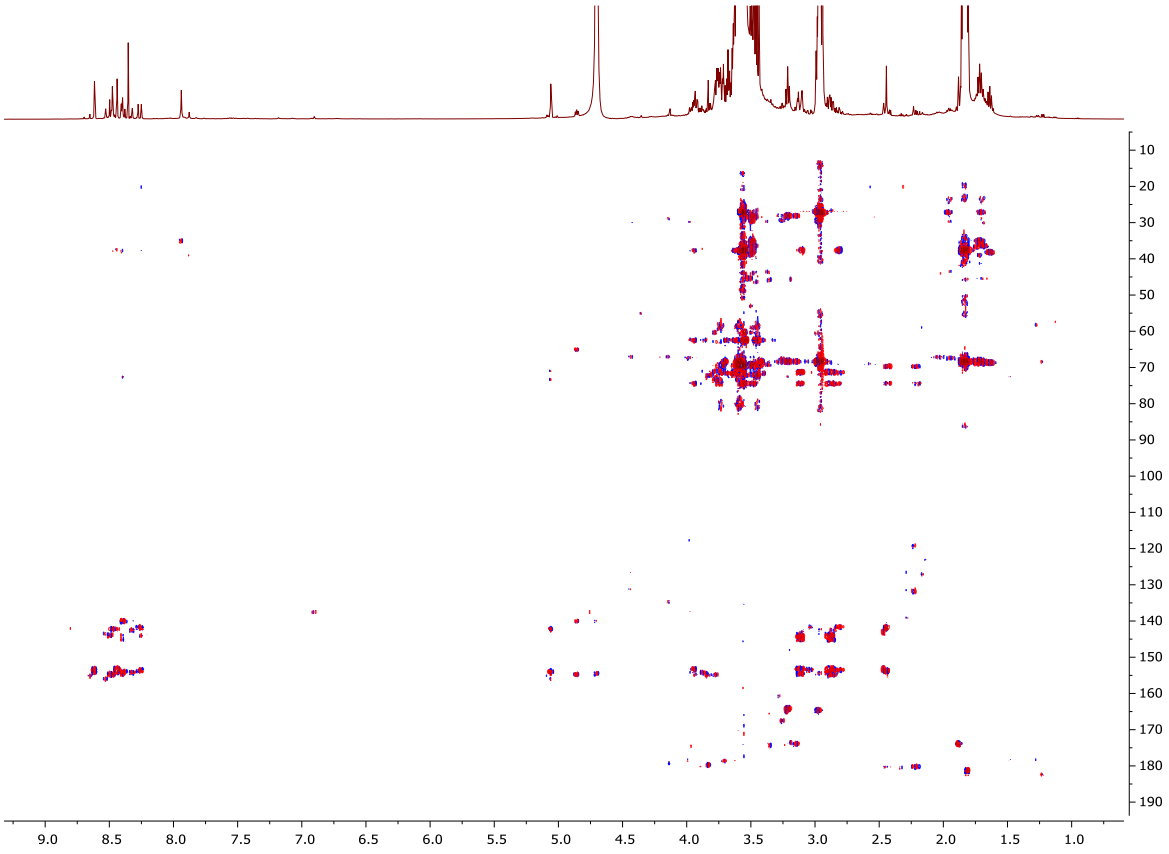
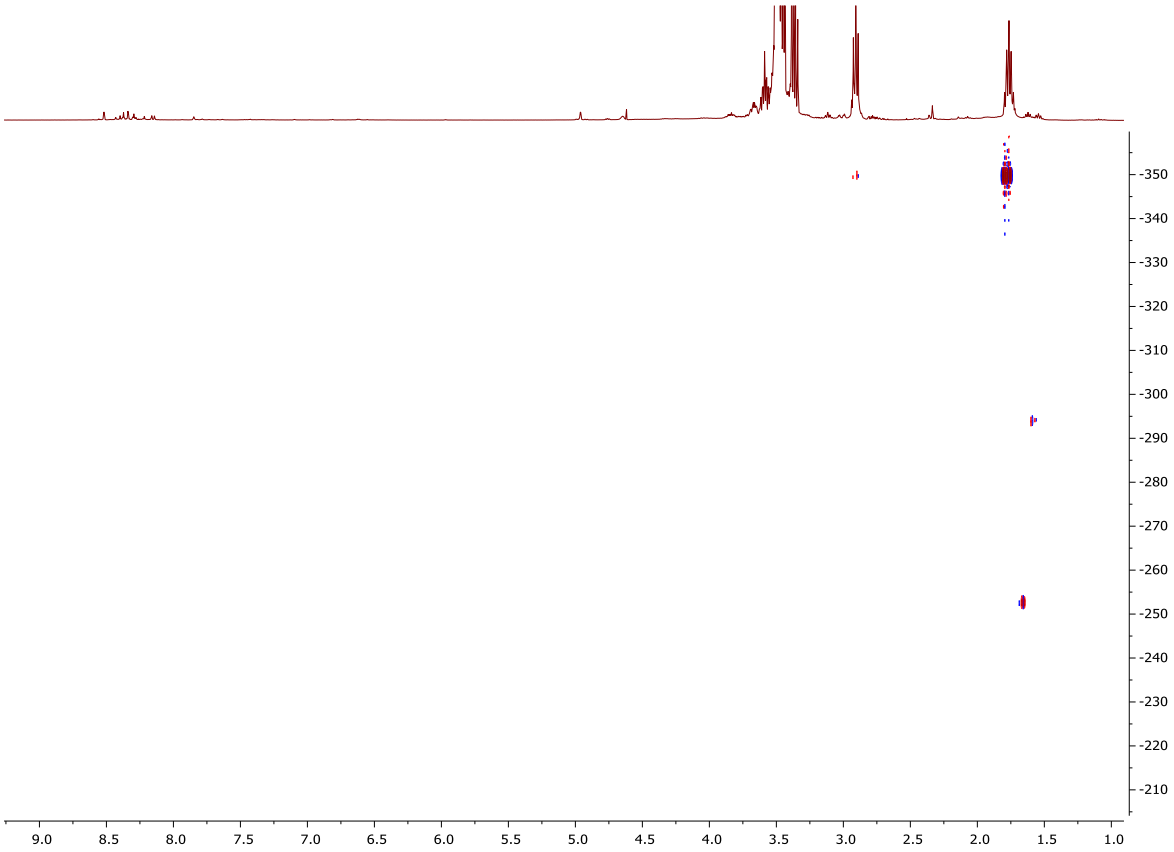
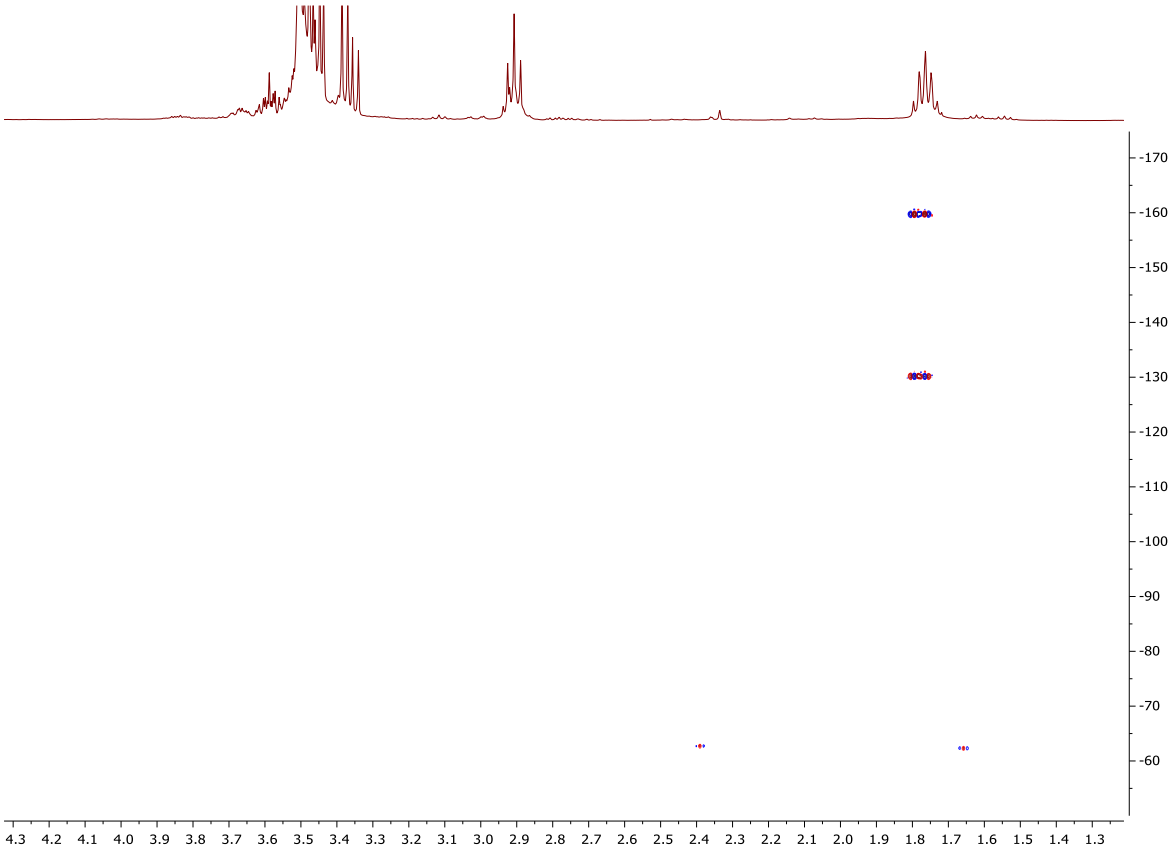


Figure S14: ^{1}H-^{13}C HMBC of NH_2-FCDs



^{1}H-^{15}N HMBC NMR (500 MHz, D_2O) RNH_3^+ distal amine (1.77 ppm, -350 ppm), amide NHCO (1.65 ppm, -253 ppm), (1.55 ppm, -293 ppm)

Figure S15: ^{1}H-^{15}N HMBC of NH_2-FCDs, ^{15}N spectral range (-350 to -200 ppm)

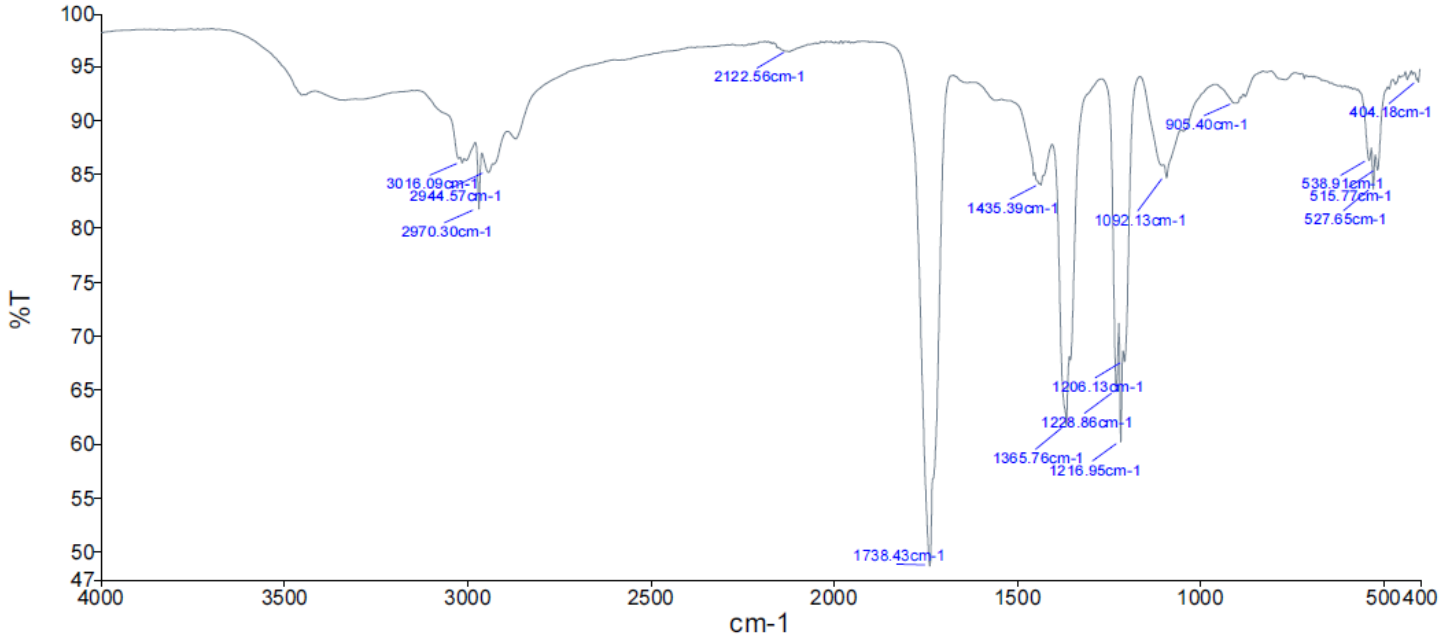


^{1}H-^{15}N HMBC NMR (500 MHz, D_2O) N-containing heteroaromatics e.g. pyridine, imidazole, pyrazine (1.77 ppm, -160 and -130 ppm), imine (1.66 and 2.38 ppm, -62 ppm)

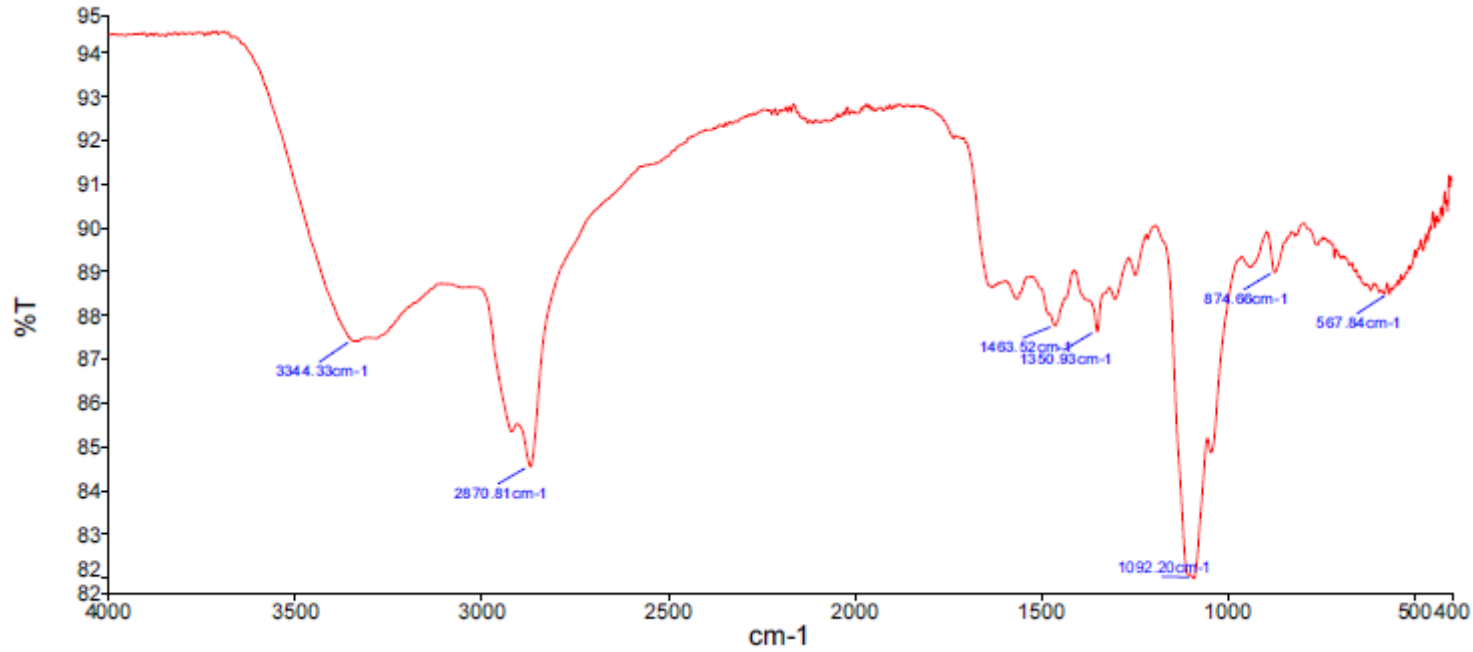
Figure S16: ^{1}H-^{15}N HMBC of NH_2-FCDs, ^{15}N spectral range (-170 to -50 ppm)

NH_2-FCDs Formation Characterisation

(A) 30 secs



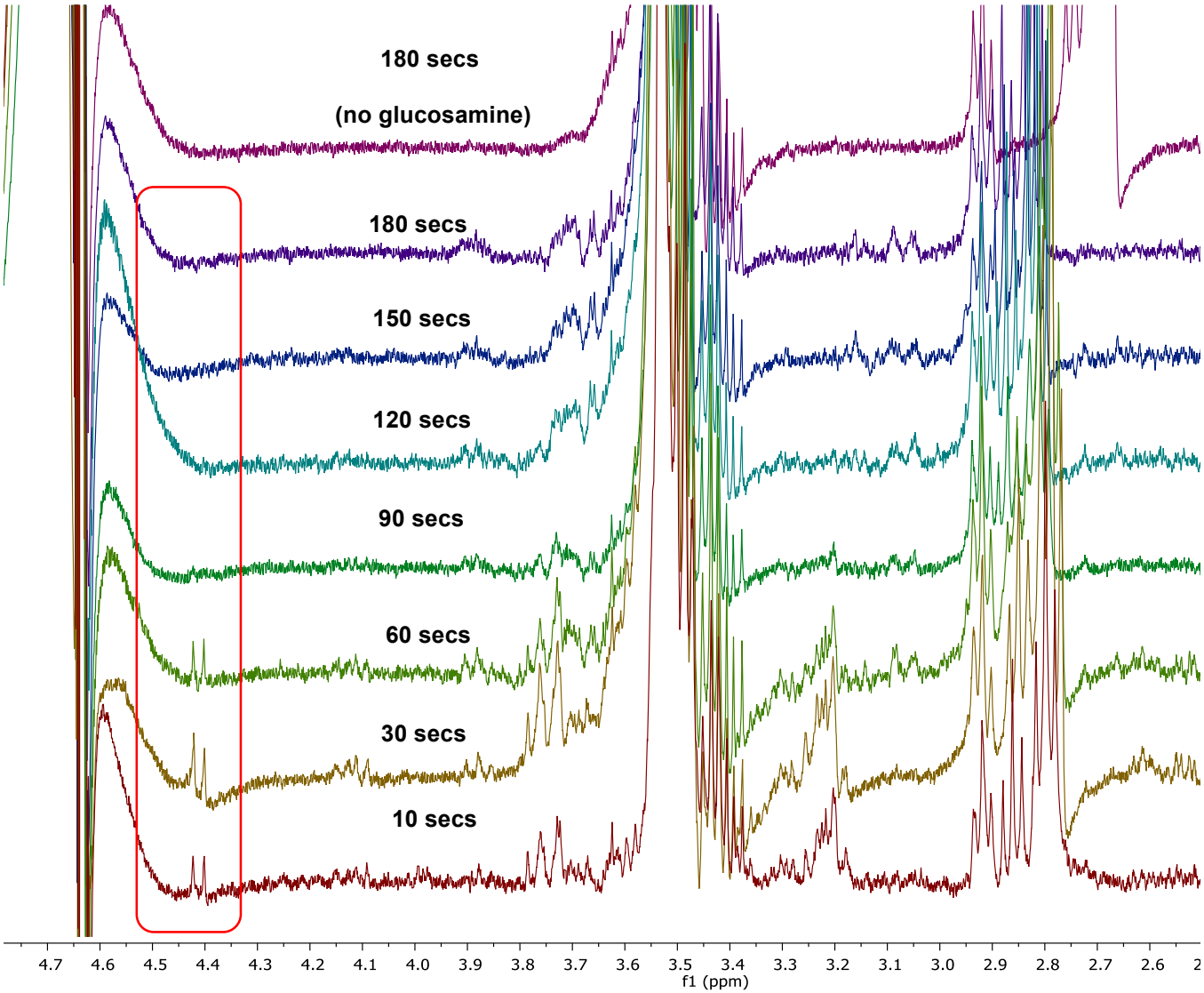
(B) 90 secs



Key features: (A) aldehyde C=O (1738 cm^{-1})

(B) aldehyde C=O not present, O-H/N-H (3344 cm^{-1}), sp^3 C-H (2870 cm^{-1}), C-O ether (1092 cm^{-1}), C-Cl (567 cm^{-1})

Figure S17: FTIR spectrum of NH_2-FCDs reaction after (A) 30 secs and (B) 90 secs



Anomeric proton at 4.4 ppm diminishes over the reaction time course. Indicative of loss of carbohydrate architecture, probably via ring-opening to subsequent aldehyde formation (Figure S15 (A))

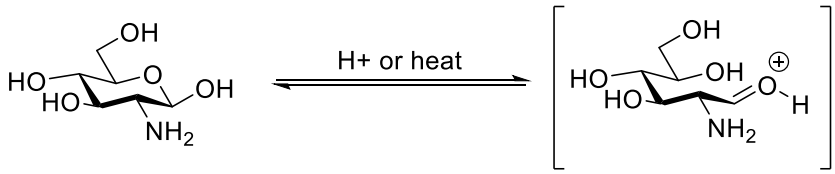


Figure S18: ^{1}H NMR reaction monitoring of NH_2-FCD at different points (10-180 secs)

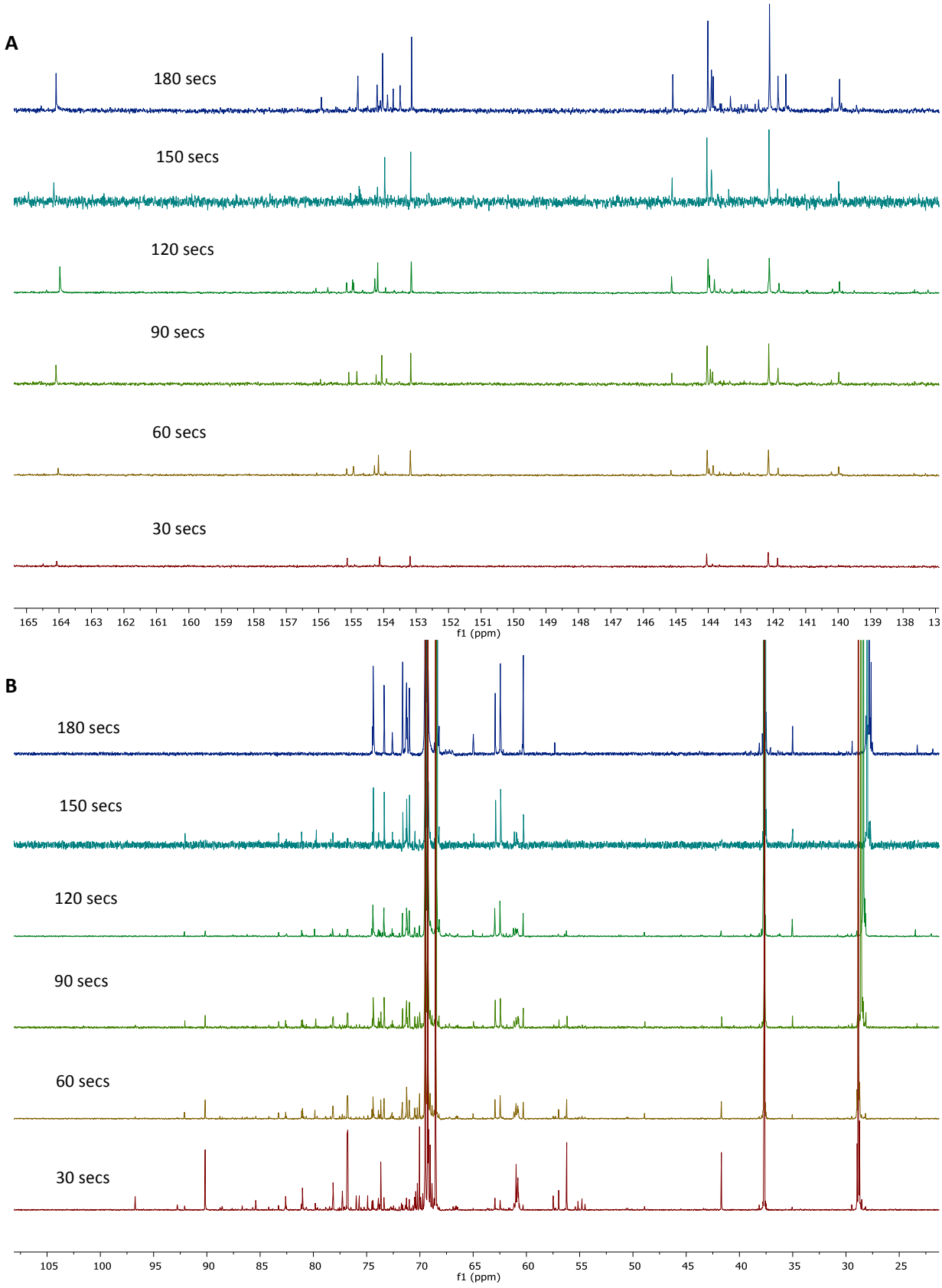


Figure S19: ^{13}C NMR reaction monitoring of NH_2-FCD at different time points (30-180 secs) (A) 137-165 ppm (B) 20-110 ppm

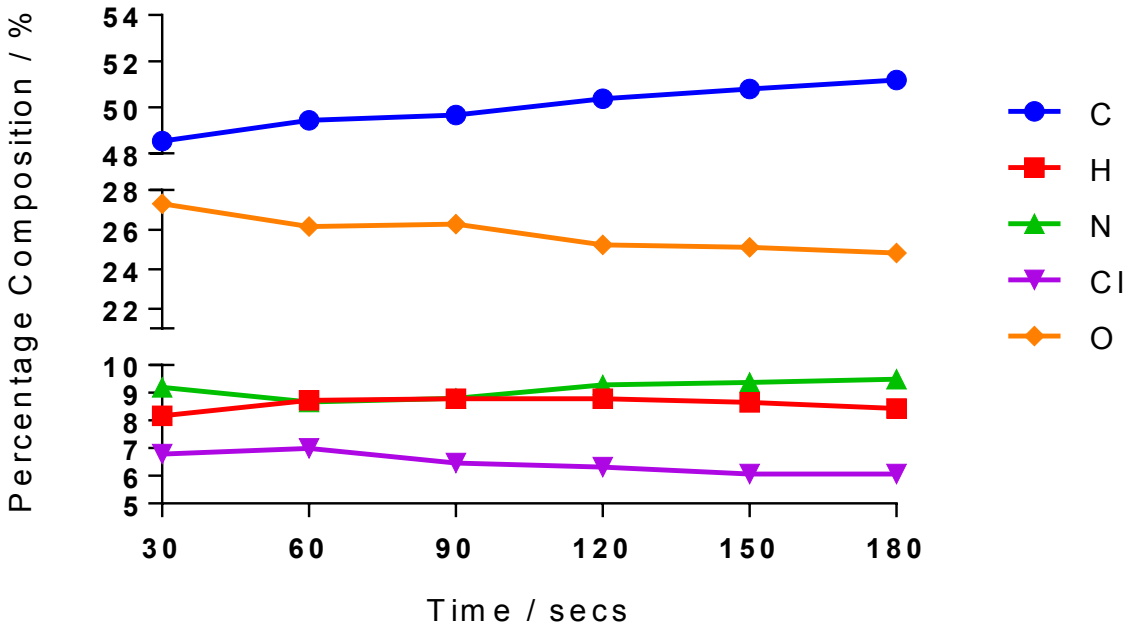
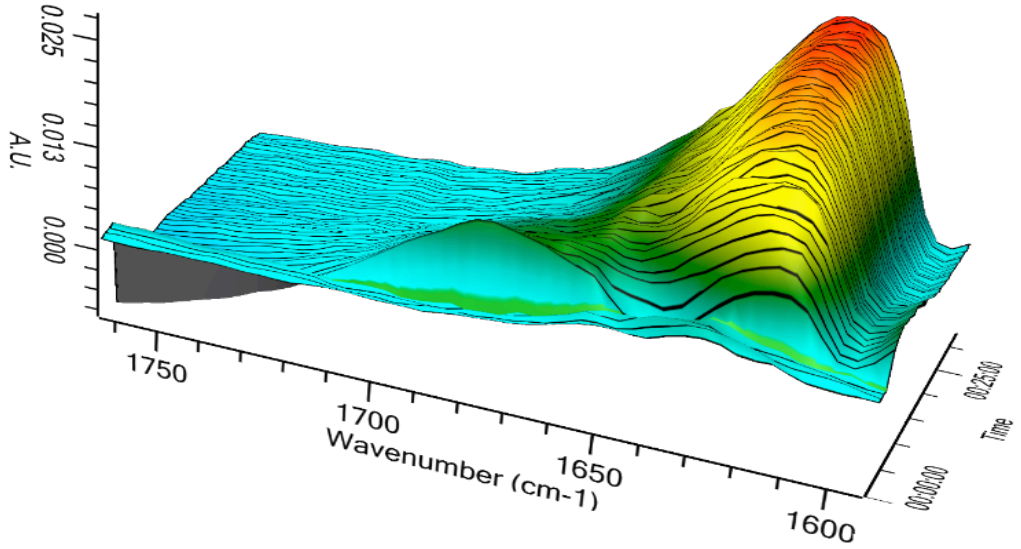


Figure S20: Elemental analysis of different aliquots during the reaction monitoring of NH_2-FCD at different time points (30-180 secs)

A



B

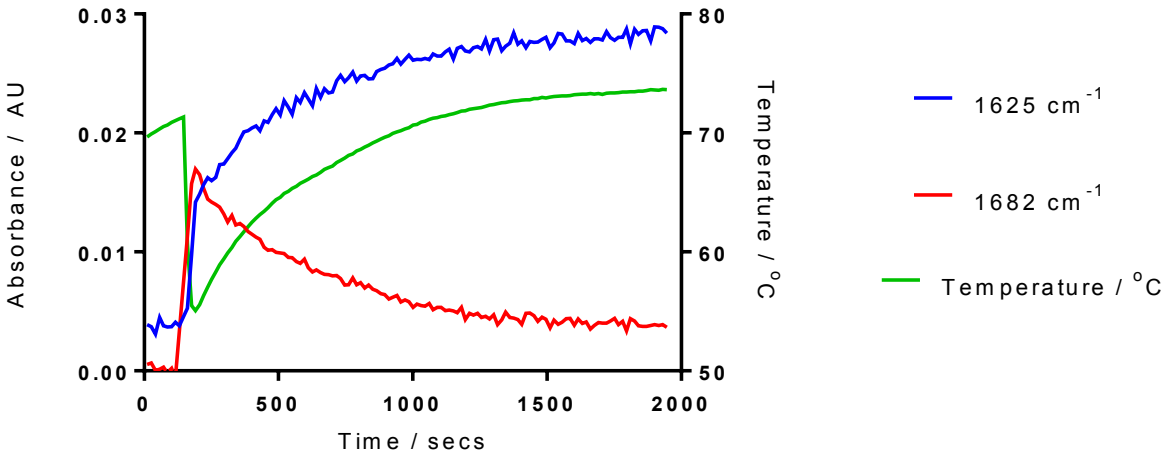
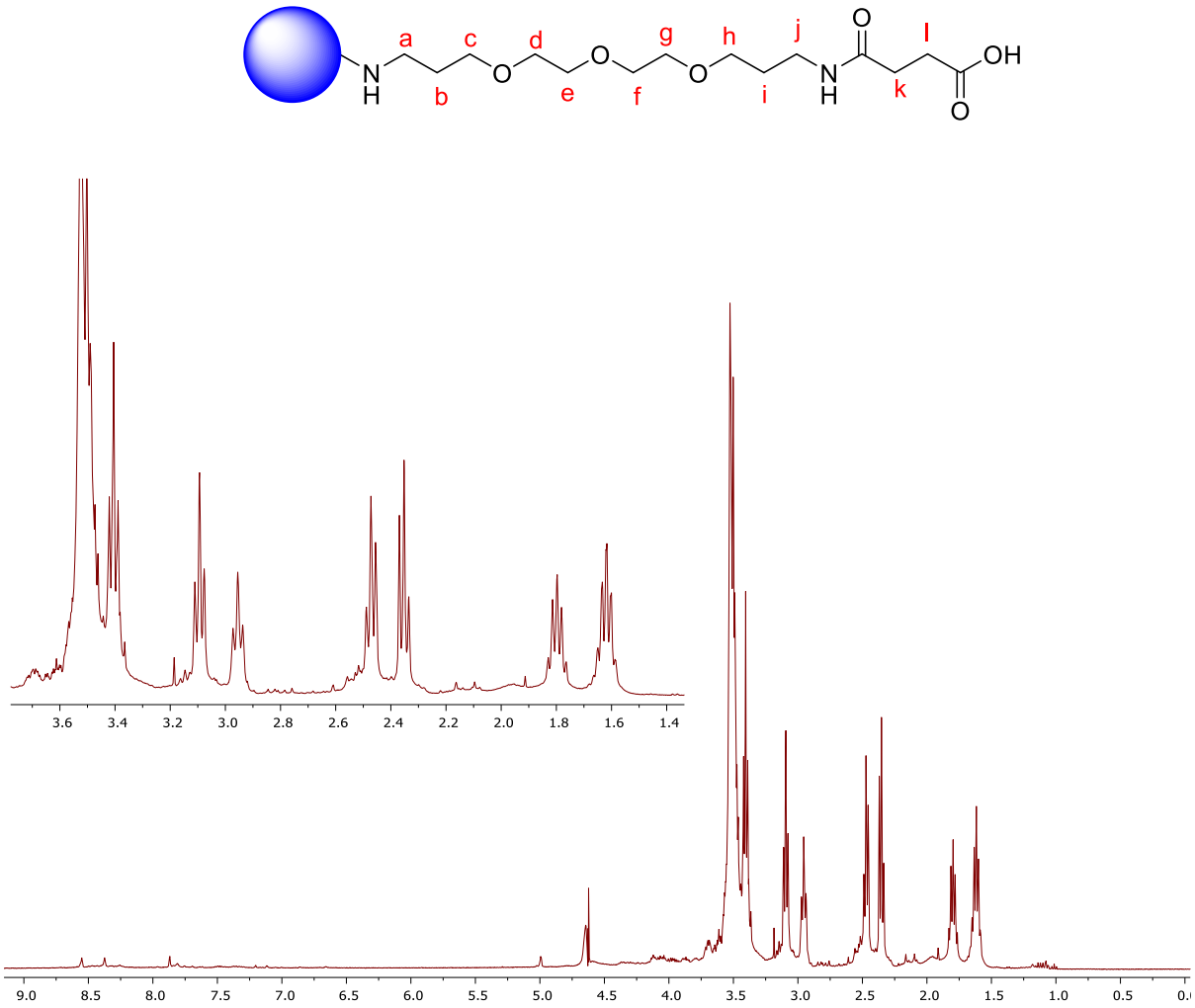


Figure S21: (A) 3D-ReactIR plot of NH_2-FCDs formation under hydrothermal conditions at 70°C (B) Graph of ReactIR peak intensities monitored over time

COOH-FCDs and Lac-FCDs Characterisation



^{1}H Presat NMR (500 MHz, D_2O) δ: 3.57-3.45 (d-g), 3.41 (t, *J* = 8 Hz, c, h), 3.10 (t, *J* = 8 Hz, j), 2.95 (t, *J* = 8 Hz, a), 2.47 (t, *J* = 8 Hz, l), 2.35 (t, *J* = 8 Hz, k), 1.80 (p, *J* =8 Hz, b), 1.62 (p, *J* =8 Hz, i)

Figure S22: ^{1}H NMR of COOH-FCDs

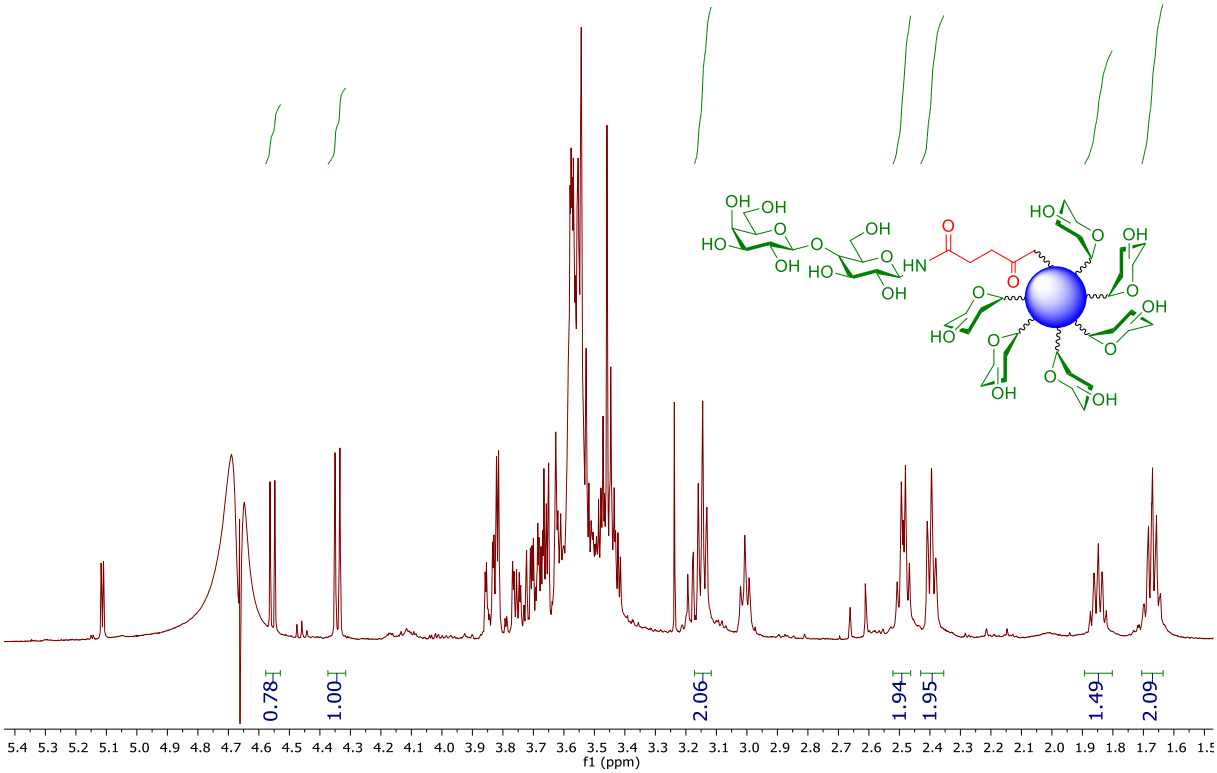
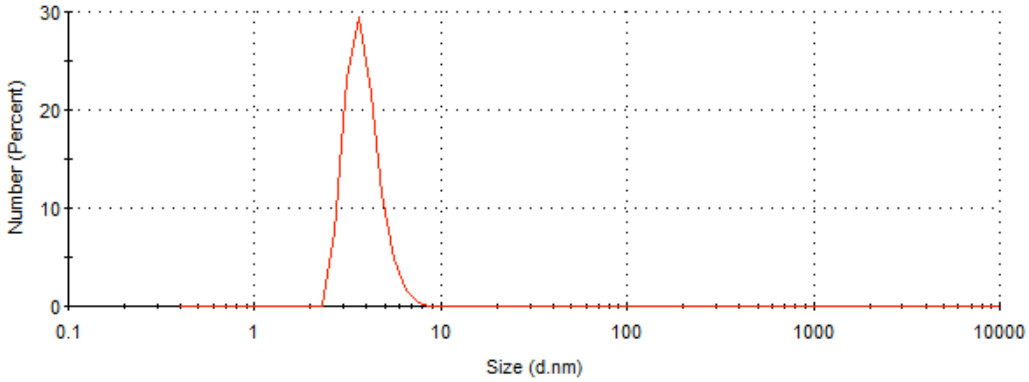
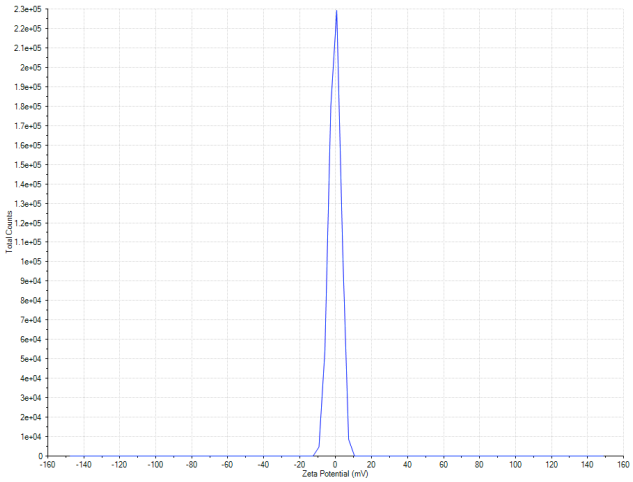


Figure S23: ^{1}H Presat NMR of Lac-FCDs



Hydrodynamic diameter profile: 3.85 nm $\pm$ 0.89

Figure S24: DLS profile of COOH-FCDs



-0.44 mV $\pm$ 3.12

Figure S25: Zeta-potential measurement of COOH-FCDs

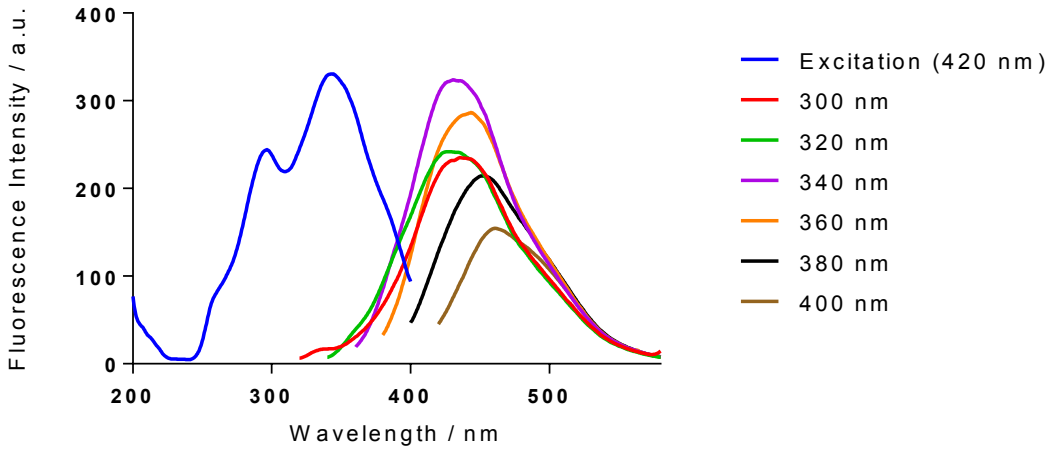
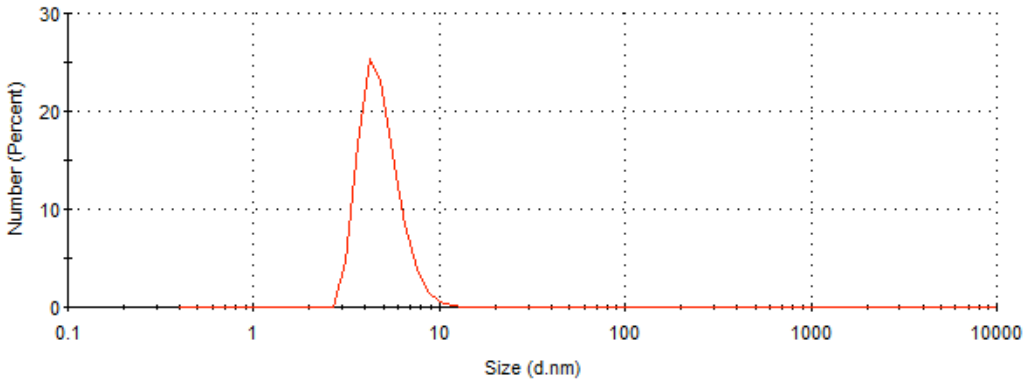
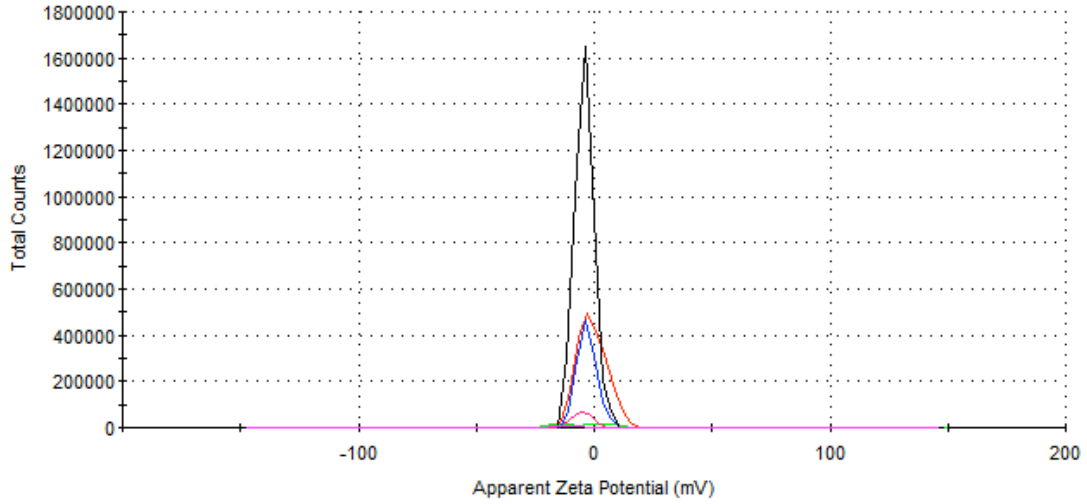


Figure S26: Excitation (420 nm emission) and emission spectrum of COOH-FCDs at various excitation wavelengths



Hydrodynamic diameter profile: 3.65 nm ± 1.23

Figure S27: DLS profile of Lac-FCDs



-0.807 mV ± 3.84

Figure S28: Zeta-potential measurement of Lac-FCDs. Each line represent individual measurements of the same sample, which where used to obtained an average value.

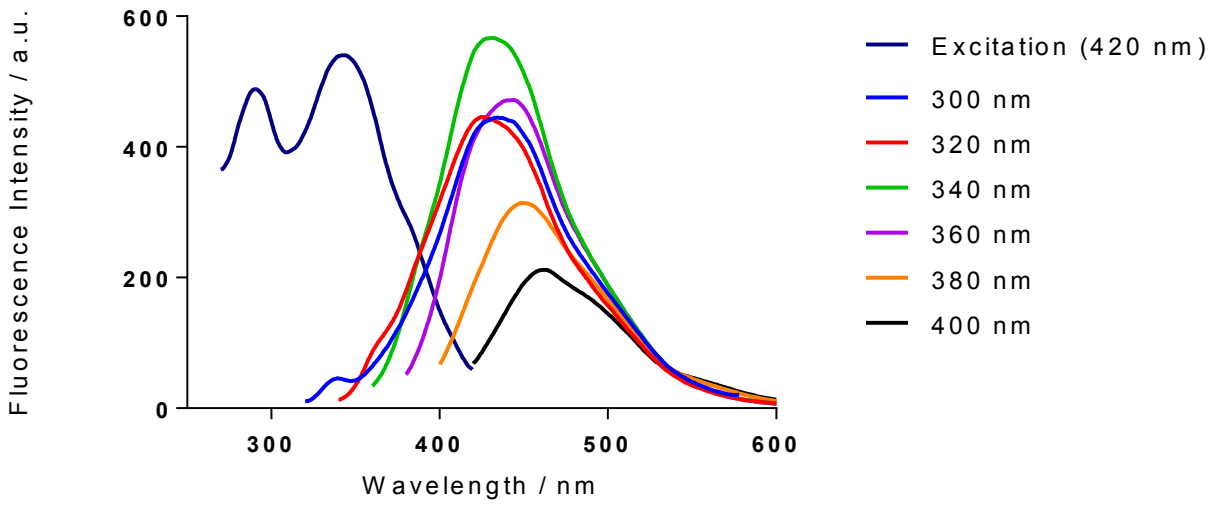


Figure S29: Excitation (420 nm emission) and emission spectrum of Lac-FCDs at various excitation wavelengths

Cell Cultures, Toxicity Assays and Confocal Microscopy Protocols

Cell culture

HeLa cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) with 1g glucose/L, GlutaMAX™ and 10% fetal bovine serum (FBS); MDA cells were grown in DMEM with 4.5 g glucose/L, GlutaMAX™and 10 % FBS. Media were supplemented with antibiotic-antimycotic (Anti-Anti). Confluent cultures were detached from the surface using trypsin (Tryp LE Express) and plated at 10^4 cells/well in either petri dishes (Mat-Tek 35 mm, with 14mm glass microwell) for imaging, or 96-well plates for toxicity tests (8 replicates per condition, i.e. toxicant concentration and time point) for 1 hour, 1 day and 3 days. For 7 days incubation 24-well plates were used, maintaining seeding density and number of replicates as above.

For toxicity assays, the chosen FCDs (at concentrations of 2000, 1000, 500, 250, 100, 50, 10 μg mL^{-1} followed by 10 times dilutions down to 10^{-6} μg mL^{-1} (1 hour, 1 day and 3 days) and 10^{-3} μg mL^{-1} (7 days)) were added 24 h after plating, in medium with reduced FBS (5%) to avoid possible protection from high serum. Tests were carried out after the required incubation time, as specified below. All cell culture media and additives were purchased from Invitrogen, Life Technologies (Thermo-Fisher).

Toxicity assays

The influence of NH_2/COOH/Lac-FCDs on cell metabolism was assessed using 5% v/v AlamarBlue in medium without any FBS. Alamar blue is cytosolic substrate for reductive metabolism (resazurin to resorufin) whose fluorescence spectrum changes on reduction. The total number of live cells was evaluated using Calcein AM, which is transformed into fluorescent calcein the cytoplasm of live cells. After exposure, cultures were washed with PBS, and incubated with both dyes simultaneously for 1h.

Confocal Microscopy

All images were acquired on a Leica SP5 confocal system equipped with a Leica DMI 6000 inverted microscope. For the excitation of the FCDs 405 nm excitation was used. Cells were live and imaged in Living Cell Imaging Solution (ThermoFisher Scientific). The images were analysed using Volocity software (PerkinElmer). See full list of images at the end of ESI.

Toxicity Assay Results

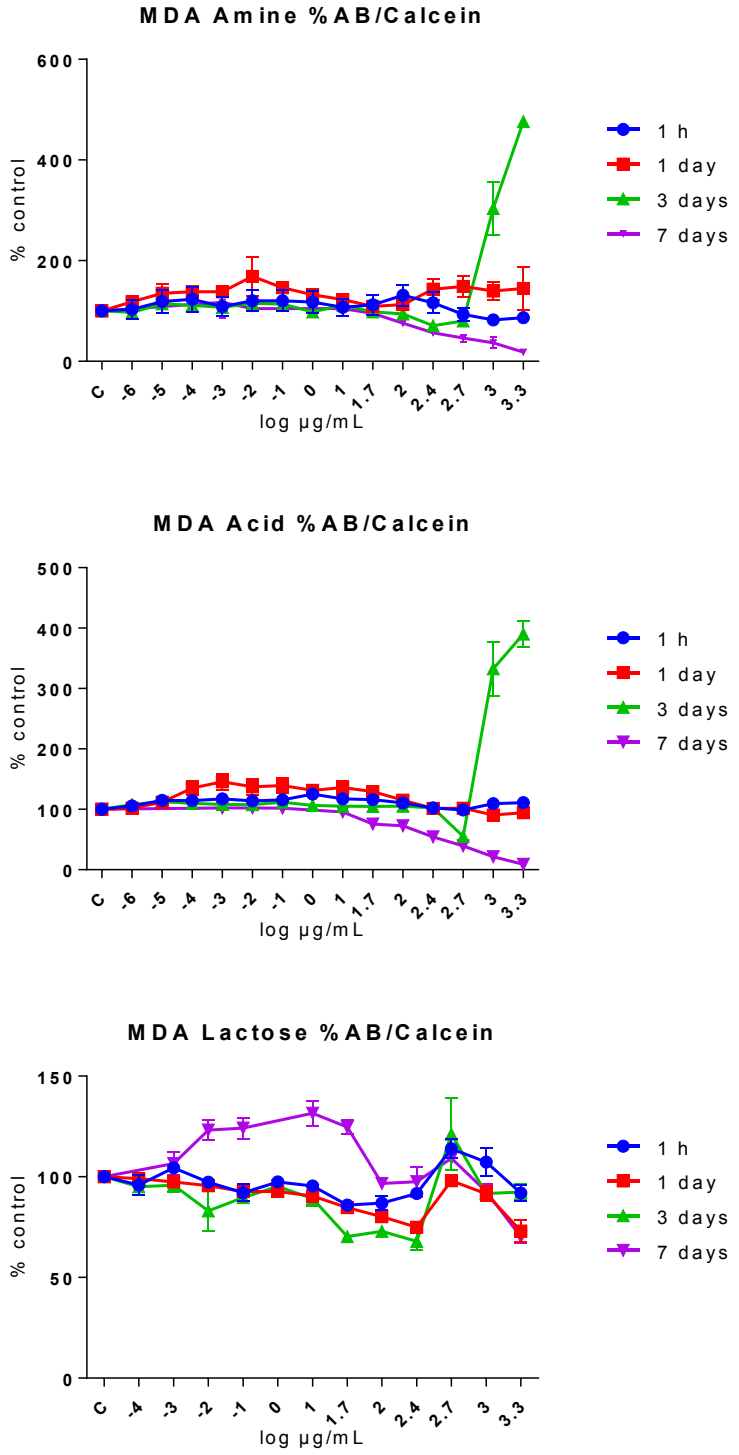


Figure S30: MDA - AB/Calcein measurements giving a measure of reductive metabolism per cell (RMPC) relative to control (C)

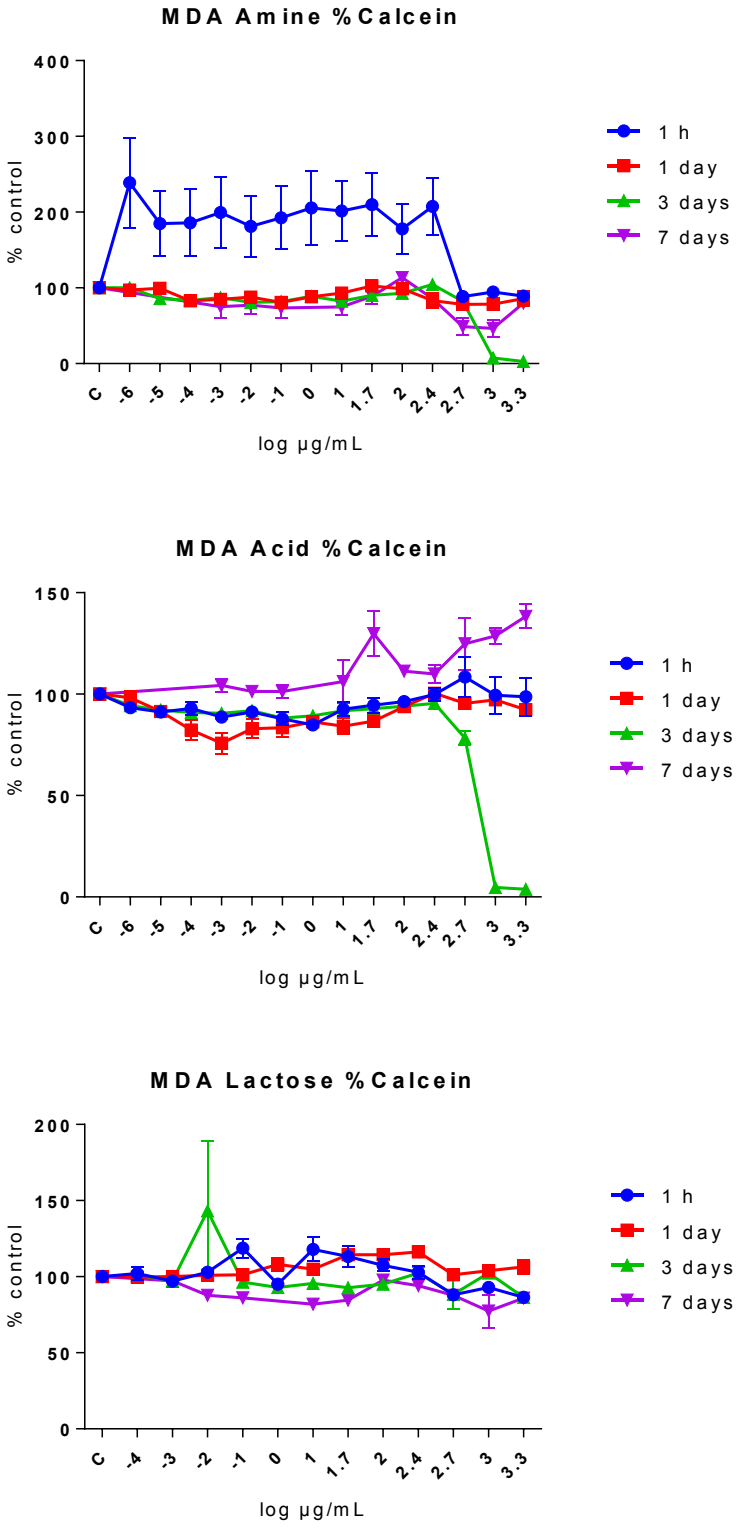


Figure S31: MDA - Calcein measurements giving a measure of live cells relative to control (C)

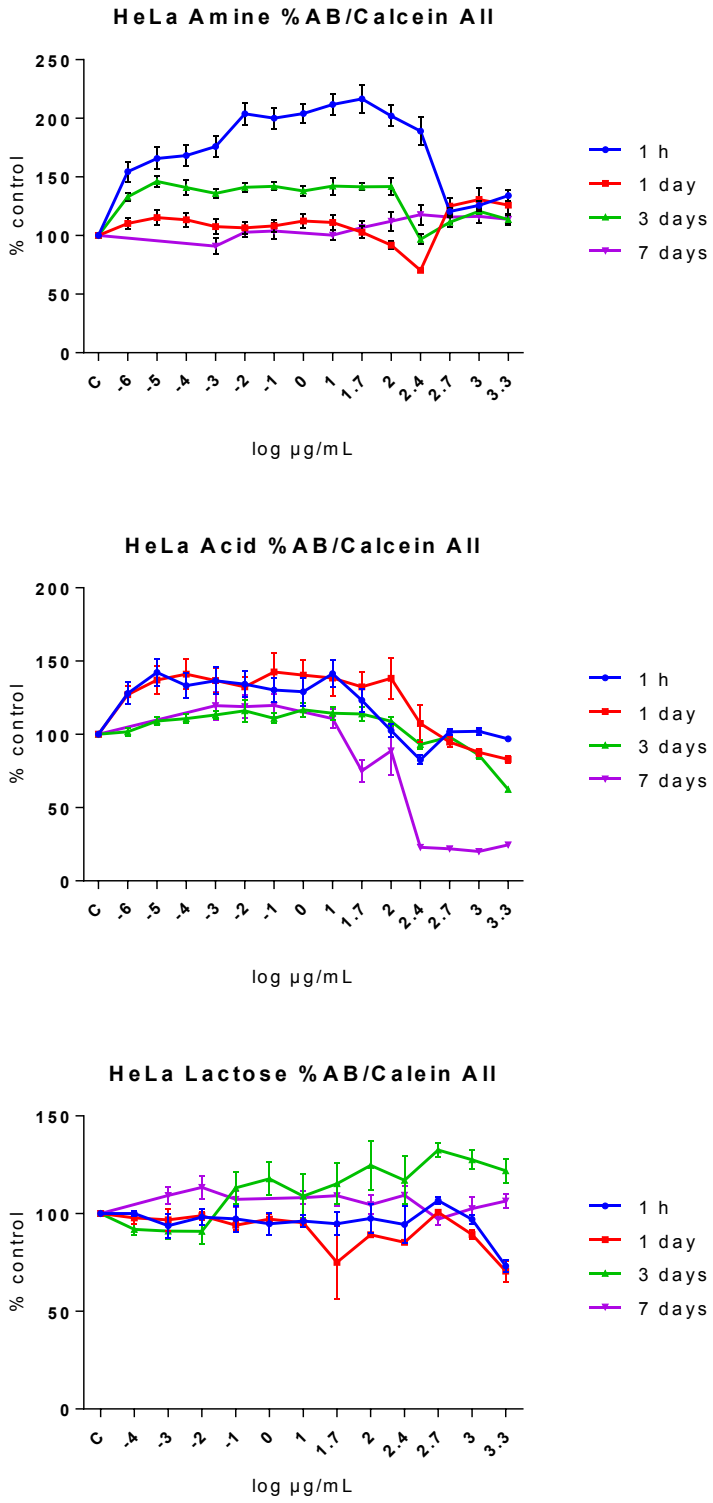


Figure S32: HeLa - AB/Calcein measurements giving a measure of reductive metabolism per cell (RMPC) relative to control (C)

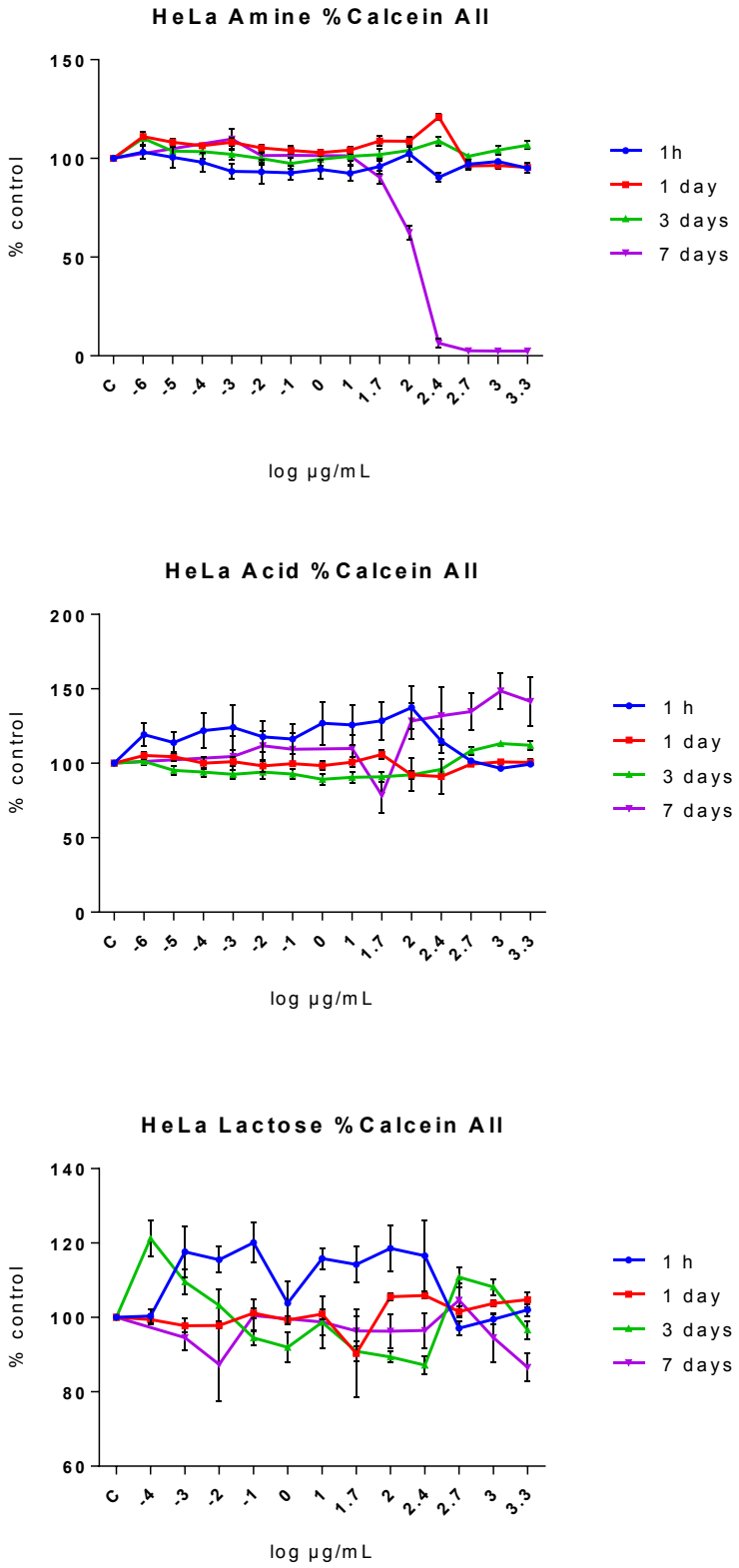
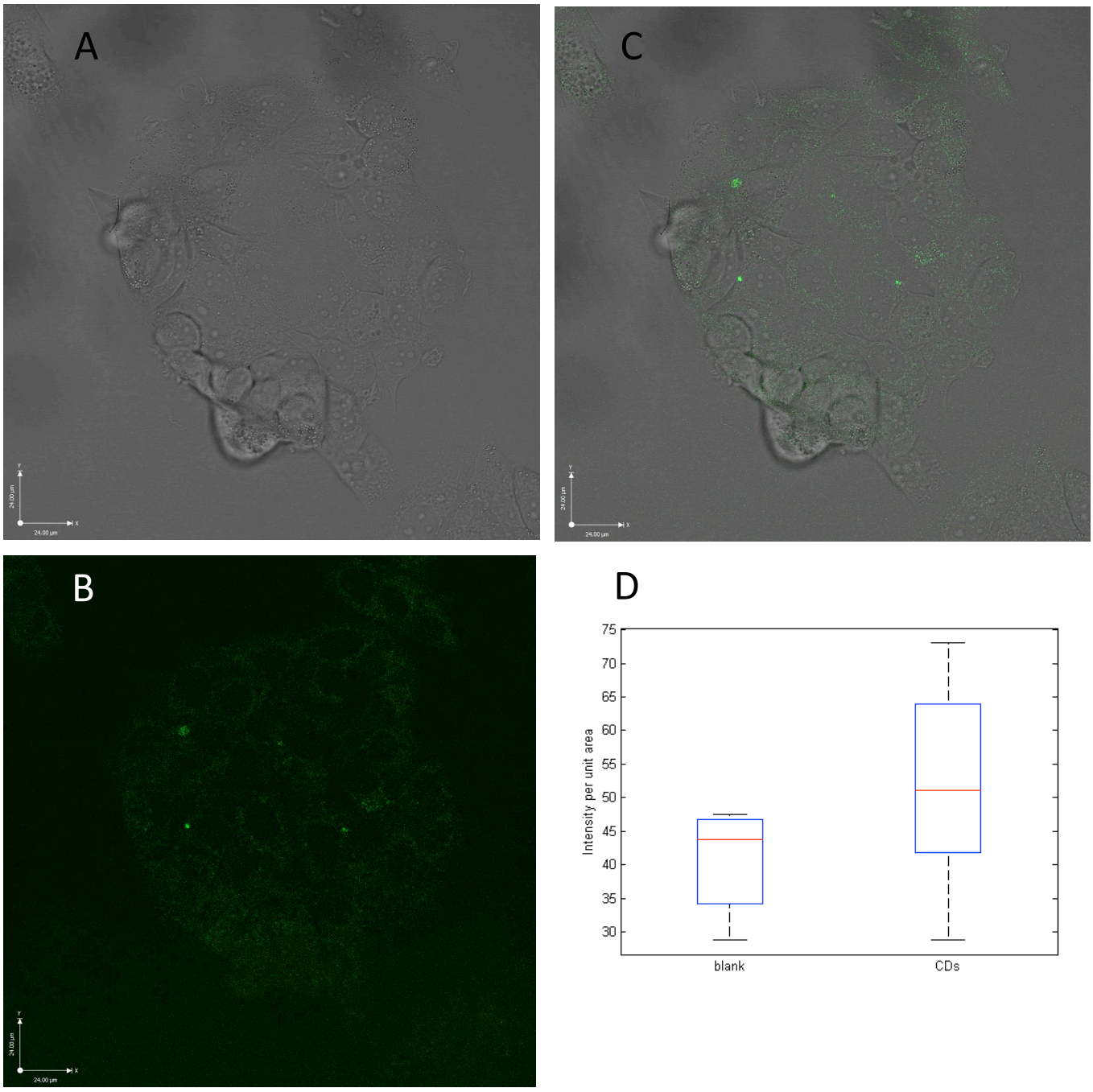


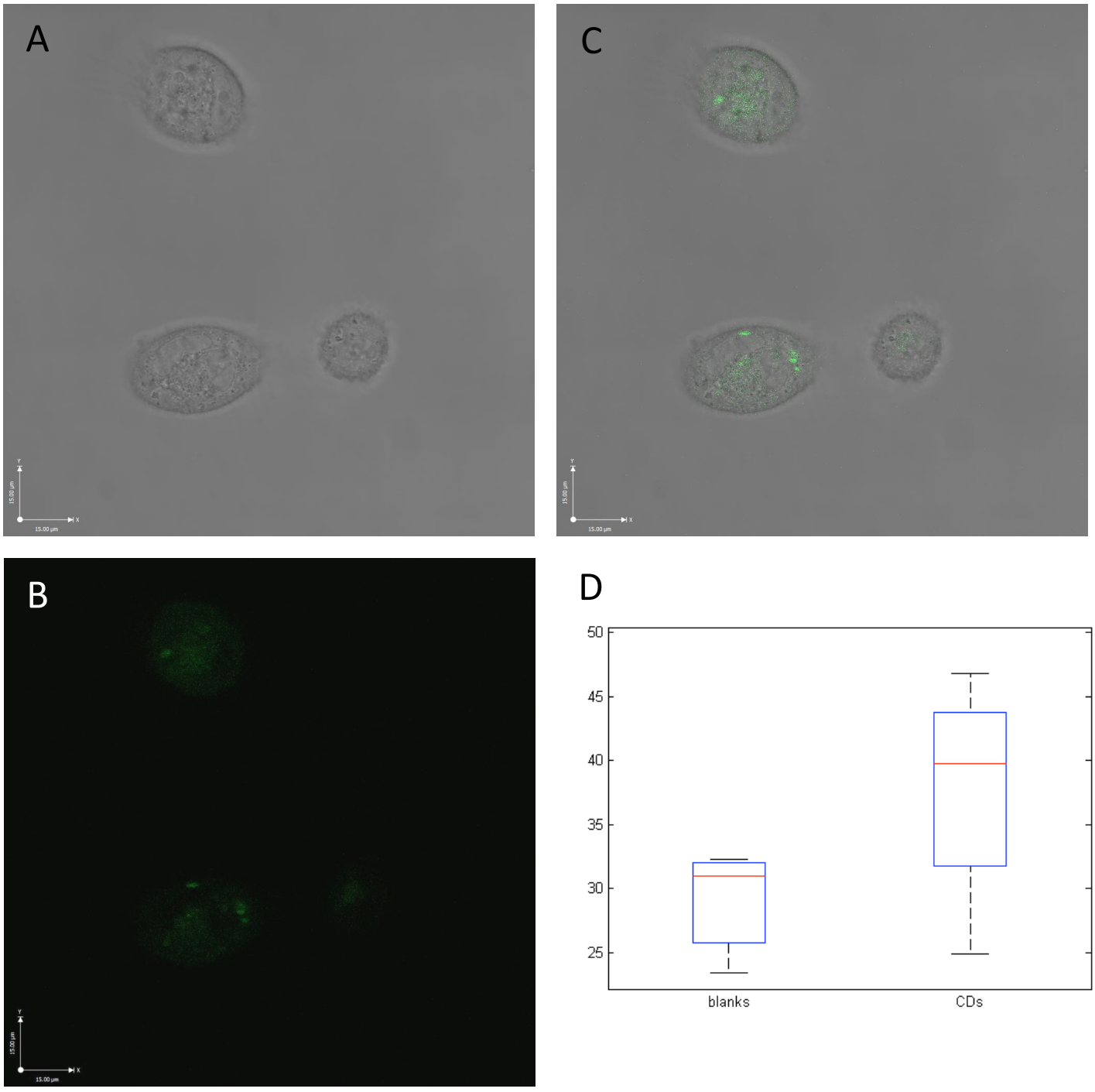
Figure S33: HeLa - Calcein measurements giving a measure of live cells relative to control (C) over a range of concentrations

Confocal Microscopy Images



(A) Bright field
(B) Fluorescence channel (excitation: 405 nm)
(C) Mixed channel
(D) Graph showing average cell fluorescence intensity per unit area for untreated vs treated cells

Figure S34: Confocal images of HeLa cells: exposed to Lac-FCDs for 2 hours at 1 mg/mL



(A) Bright field
(B) Fluorescence channel (excitation: 405 nm)
(C) Mixed channel
(D) Graph showing average cell fluorescence intensity per unit area for untreated vs treated cells

Figure S35: Confocal images of MDA cells: exposed to Lac-FCDs for 2 hours at 1 mg/mL