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Biocompatible poli(amidoamine) (PAMAM) dendrimer octa-substituted with α-cyclodextrin towards controlled release of doxorubicin hydrochloride from its ferrocenyl prodrug

Electronic Supplementary Information (ESI)

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Table of contents

S1. Experimental section

S1.1 Materials and methods

Chemical reagents and solvents were commercially purchased and purified according to the standard methods, if necessary. Air- and moisture-sensitive reactions were carried out using commercially available anhydrous solvents under an inert atmosphere of nitrogen. The NMR experiments were carried out using a Varian VNMRS 500 MHz spectrometer (^{1}H NMR at 500 MHz or ^{13}C NMR at 125 MHz) equipped with a multinuclear z-gradient inverse probe head. Unless otherwise stated, the spectra were recorded at 25 °C. Standard 5 mm NMR tubes were used. ^{1}H and ^{13}C chemical shifts (δ) were reported in parts per million (ppm) relative to the solvent signal: DMSO-d_6, δ_H (residual DMSO) 2.50, δ_C 39.5. No internal standard was added in order to exclude any interactions with α-cyclodextrin (αCD), if any. NMR spectra were analyzed with the MestReNova v12.0 software (Mestrelab Research S.L). ^{1}H DOSY (Diffusion Ordered SpectroscopY) NMR experiments were performed using a stimulated echo sequence incorporating bipolar gradient pulses[1] and with convection compensation[2]. The gradient strength was logarithmically incremented in 15 steps from 25% up to 95% of the maximum gradient strength. The DOSY Toolbox software was used for DOSY NMR spectra processing (*The DOSY Toolbox – version 2.5, 2014, Mathias Nilsson, School of Chemistry, University of Manchester, UK*). Diffusion coefficient value (*D*) was given in 10^{-10} m^2 s^{-1}. The concentration of the sample for ^{1}H DOSY NMR measurement was $0.5 \cdot 10^{-3}$ M. Fourier-transform infrared (FT-IR) spectra were recorded in the Attenuated Total Reflectance (ATR) mode with the Thermo Nicolet Avatar 370 spectrometer with spectral resolution of 2 cm^{-1} (150 scans). The wavenumbers for the absorption bands *v* were reported in cm^{-1}. UV-Vis measurements in doxorubicin hydrochloride (DOX*HCl) release trials were performed with a VWR UV-3100 PC spectrometer with the spectral resolution of 1 nm. TOF-MS (ESI) measurements were performed with a Q-Exactive ThermoScientific spectrometer. Elemental analyses were performed using CHNS Elementar Vario EL III apparatus. Each elemental composition was reported as an average of two analyses. TLC analysis was performed using Merck Silica gel 60 F254 plates.

Monoaldehyde of α-cyclodextrin (αCD-CHO; **2**) was obtained based on the literature method.[3]

S1.2 Synthesis of octa-αCD-PAMAM (3)

To a stirred solution of poli(amidoamine) (PAMAM) dendrimer G1.0 (50.0 mg, 0.035 mmol, 1 eq) in dry methanol (3 mL) a solution of αCD-CHO (272.0 mg, 0.280 mmol, 8 eq) in dimethylsulfoxide (6 mL) was added. The reaction mixture was stirred under argon atmosphere at room temperature for 72 hours. Solid sodium triacetoxyborohydride (356.1 mg, 1.68 mmol, 48 eq) was added and the reaction mixture was further stirred under argon atmosphere at room temperature for 4 hours. The content of the reaction flask was diluted with distilled water (20 mL) and was dialyzed (MWCO 3500 Da) against distilled water for 72 hours. A resultant solution was lyophilized to obtain octa-αCD-PAMAM (**3**; 257.6 mg; 80 %) as white solid.

NMR spectra were acquired in DMSO-d_6 : D_2O=1:1 *v*/*v* solvent system to obtain proper, significant concentration of octa-αCD-PAMAM for the measurement of these spectra.

^{1}H NMR (500 MHz, DMSO-d_6 : D_2O = 1:1 *v*/*v*, ppm), δ_H 4.84-4.82 (bm), 3.78-3.74 (bm), 3.68-3.61 (bm), 3.42-3.35 (bm), 3.13-3.10 (bm), 2.61-2.58 (bm), 2.43-2.38 (bm), 2.24-2.18 (bm)

^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6 : D_2O = 1:1 *v*/*v*, ppm), δ_C 175.7, 175.6, 102.1, 100.5, 100.4, 84.5, 82.0, 77.7, 77.4, 74.0, 73.9, 73.5, 72.8, 72.4, 71.9, 71.8, 71.7, 71.3, 70.1, 66.4, 64.5, 64.1, 61.2, 58.4, 52.0, 50.0, 49.9, 49.7, 48.5, 40.4, 39.4, 38.1, 37.6, 37.5, 33.5

^{1}H DOSY NMR (500 MHz, DMSO-d_6 : D_2O = 1:1 *v*/*v*, $m^2 \cdot s^{-1}$), D $0.746 \cdot 10^{-10}$

FT-IR (ATR, cm^{-1}), *v* 3375, 3080, 2945, 2845, 1645, 1605, 1555, 1505, 1340, 1290, 1135, 1075, 1030, 780, 670

Elemental analysis: calculated for: C, 46.42; H, 6.61; N, 4.18%; found: C, 46.36; H, 6.58; N, 4.02%

ESI-MS (TOF): calcd. for $C_{350}H_{593}N_{26}O_{244}$ $[M+H]^+$ = 9066.49, found: m/z 9066.52

S1.3 Synthesis of Fc-COO-DOX*HCl (5)

Ferrocenecarboxylic acid (**4**; 30 mg, 0.131 mmol, 1 eq) was dissolved in DMSO (2 mL). Solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCl; 51.0 mg, 0.262 mmol, 2 eq) was added and the reaction mixture was stirred under argon atmosphere at room temperature for 30 minutes. A solution of DOX*HCl (76.0 mg, 0.131 mg, 1 eq) and 4-dimethylaminopyridine (DMAP; 8.0 mg, 0.066 mmol, 0.5 eq) in DMSO (3 mL) was added and the reaction mixture was further stirred under argon atmosphere at room temperature for 48 hours. A reaction mixture was diluted with CH_2Cl_2 (40 mL) and it was washed with distilled water (3x20 mL) and brine (2x10 mL), dried with anhydrous $MgSO_4$, filtered, and the solvent was removed in vacuum. The resultant residue was purified by column chromatography (10% CH_3OH/CH_2Cl_2) to give Fc-COO-DOX*HCl (**5**; 78.8 mg, 76% yield) as red-orange solid.

^{1}H NMR (500 MHz, DMSO-d_6, ppm), δ_H 14.03 (s, 1H), 13.25 (s, 1H), 7.88 (d, *J* = 5.0 Hz, 2H), 7.62-7.61 (m, 1 H), 7.14 (d, *J* = 8.2 Hz, 1H), 5.47 (s, 1H), 5.28 (d, *J* = 3.7 Hz, 1H), 4.96 (t, *J* = 4.4 Hz, 1H), 4.87-4.85 (m, 2 H), 4.78-4.76 (m, 2H), 4.59 (d, *J* = 5.9 Hz, 2H), 4.30 (t, *J* = 1.6 Hz, 2H), 4.22 (q, *J* = 6.6 Hz, 1H), 4.13 (s, 5H), 3.96 (s, 3H), 3.51 (dd, *J* = 6.3, 2.6 Hz, 1H), 2.97 (q, *J* = 18.3 Hz, 2H), 2.26-2.22 (m, 1H), 2.13 (dd, *J* = 14.2, 5.6 Hz, 1H), 2.02 (td, *J* = 13.0, 4.0 Hz, 1H), 1.55 (dd, *J* = 12.5, 4.5 Hz, 1H), 1.17 (d, *J* = 6.4 Hz, 3H)

^{13}C{^{1}H} NMR (125 MHz, DMSO-d_6, ppm), δ_C 213.8 (2H), 186.5, 186.4, 172.9, 168.2, 160.7, 156.1, 136.2, 135.5, 134.6, 134.1, 112.0, 119.7, 119.0, 110.8, 110.6, 100.4, 76.5, 75.0, 69.9, 69.8 (2H), 69.3 (5H), 68.3 (2H), 68.1, 66.8, 63.7, 45.2, 36.6, 32.1, 29.8, 17.0

^{1}H DOSY NMR (500 MHz, DMSO-d_6 : D_2O = 1:1 *v*/*v*, $m^2 \cdot s^{-1}$), *D* $3.699 \cdot 10^{-10}$

FT-IR (ATR, cm^{-1}), *v* 3470, 3310, 2925, 2820, 1750, 1615, 1550, 1485, 1375, 1330, 1215, 1155, 1020, 990, 835, 750, 665

ESI-HRMS (TOF): calcd. for $C_{38}H_{38}ClFeNO_{12}$ $[M]^+$ = 791.1432, found: m/z 791.1434

R_f (10% CH_3OH/CH_2Cl_2) = 0.72

S1.4 Synthesis of nanoconjugate {Fc-COO-DOX*HCl}@{octa-αCD-PAMAM} (6)

A solution of Fc-COO-DOX*HCl (**5**; 28.0 mg, 0.0352 mmol, 8 eq) in C_2H_5OH (3.5 mL) was added to a stirred solution of octa-αCD-PAMAM (**3**; 40.0 mg, 0.0044 mmol, 1 eq) in distilled water (8 mL). The resultant solution was stirred at 65°C for 48 hours. The reaction mixture was placed in a laboratory refrigerator (-20°C) and then it was lyophilized for 48 hours to give nanoconjugate {Fc-COO-DOX*HCl}@{octa-αCD-PAMAM} (**6**; 68.0 mg, quantitative yield) as light-orange solid.

^{1}H DOSY NMR (500 MHz, DMSO-d_6 : D_2O = 1:1 *v*/*v*, $m^2 \cdot s^{-1}$), *D* $0.358 \cdot 10^{-10}$

FT-IR (ATR, cm^{-1}), *v* 3375, 3080, 2945, 2825, 1750, 1645, 1605, 1550, 1505, 1340, 1220, 1135, 1075, 1030, 780, 670

Elemental analysis: calculated for: C, 50.99; H, 5.86; N, 3.09%; found: C, 50.93; H, 5.82; N, 3.06%

ESI-MS (TOF): calcd. for $C_{654}H_{899}Cl_8Fe_8N_{34}O_{340}$ $[M+H]^+$ = 15404.53, found: m/z 15404.57

S1.5 Controlled drug release trials with nanoconjugate {Fc-COO-DOX*HCl}@{octa-αCD-PAMAM} (6)

For the controlled drug release trials, the nanoconjugate {Fc-COO-DOX*HCl}@{octa-αCD-PAMAM} (**6**) was dissolved in a buffer solution of pH 4.7 or buffer solution of pH 7.4. This solution was dialyzed (MWCO 1000 Da) against buffer solution of pH 4.7 or buffer solution of pH 7.4 for three days. Supernatants were collected in the following time intervals (2h, 6 h, 8 h, 12 h, 18 h, 24 h, 30 h, 48 h, 60 h, 72 h). The amount of released DOX*HCl was estimated based on the UV-Vis analyses (absorbance at λ_{max} = 500 nm) and the calculation was based on the calibration curve for the native DOX*HCl. The plot was constructed based on the cumulative release of DOX*HCl.

S1.6 Biological assays

The microfluidic device was fabricated in poly(dimethylsiloxane) (PDMS, Sylgard 184, DC), using a double casting technique.[4] The microsystem was dedicated for three-dimensional (3D) spheroid culture. The microsystem construction contained concentration gradient generator structure (CGG) which allows to obtain control and two different concentrations of the tested substance in a single microsystem/experiment. Moreover, the devise was designed as a direct measuring tool for spectroscopic analysis.[4]

The breast cancer cell line (MCF-7) was used in this research. The MCF-7 cells were cultured in DMEM medium (Biowest) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% of 25 mM L-glutamine and 1% of 100 mM penicillin/streptomycin (Sigma-Aldrich). The cell suspension (about $4 \cdot 10^6$ cells·mL^{-1}), prepared using standard protocols was introduced into the microsystem using a peristaltic pump (Ismatec Reglo-Digital MS-4/12). The introduced cells were incubated for 24h (37°C, 5% CO_2) to form spheroids.

Solution of tested compound (100 µgmL-1) was made by dilution of primary stock solution (1 mg·mL^{-1}) in culture medium. Such prepared solution and fresh cell culture medium were introduced into the microsystem via inlet D2 (**Fig. S1**) and inlet 2E (**Fig. S1**) respectively. In middle row of gradient generating structure (CGG) compound solution and culture medium were mixed at 1:1 (v/v) ratio. Due to the CGG, we tested three different concentration of these substances in single microsystem/experiment (0, 50 and 100 µg·mL^{-1}). Thus, cells in microwells in upper, middle and lower row were incubated with examined compound concentration of 100 µg·mL^{-1}, 50 µg·mL^{-1} and 0 µg·mL^{-1}, respectively. Cells were incubated with tested substances for 24h (37°, 5% CO_2). Next, we evaluated cel viability using alamarBlue® (AbD Serotec) assay. Fluorescence measurements of MCF-7 cells viability was performed daily using Varian Cary Eclipse Fluorescence Spectrophotometer equipped with a Microplate Reader (Agilent). This measurement was based on the introduction of 10% alamarBlue® solution (4.5 µL·min^{-1}, 15 min) to the microsystem and incubated for 15 min (37°C, 5% CO_2). After this time, the fluorescence intensity was measured (Ex: 552 nm, Em:583 nm) using a microplate reader and chip holder. Chip holder was a plastic plate with hole for microsystem. The fluorescence intensity was possible due to microchambers arrangement. [4] After the fluorescence analysis, culture medium was exchanged (4.5 µL·min^{-1}, 15 min) and the microsystem was incubated for the next day (37°C, 5% CO_2).

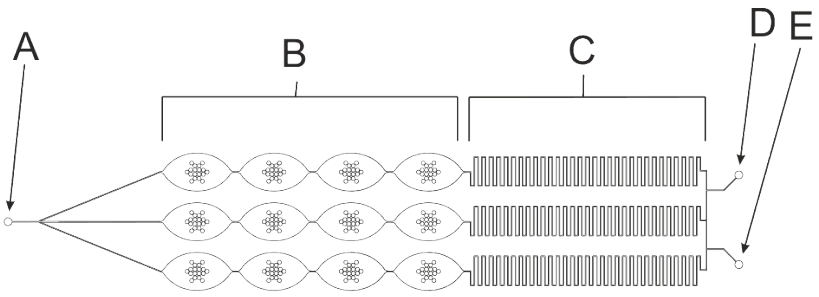

Fig. S1. Scheme of microfluidic device: (A) inlet/outlet , (B) microchambers with microwells for spheroid culture, (C) concentration gradient generator (CGG), (D) inlet/outlet , (E) inlet outlet

S2. NMR spectra

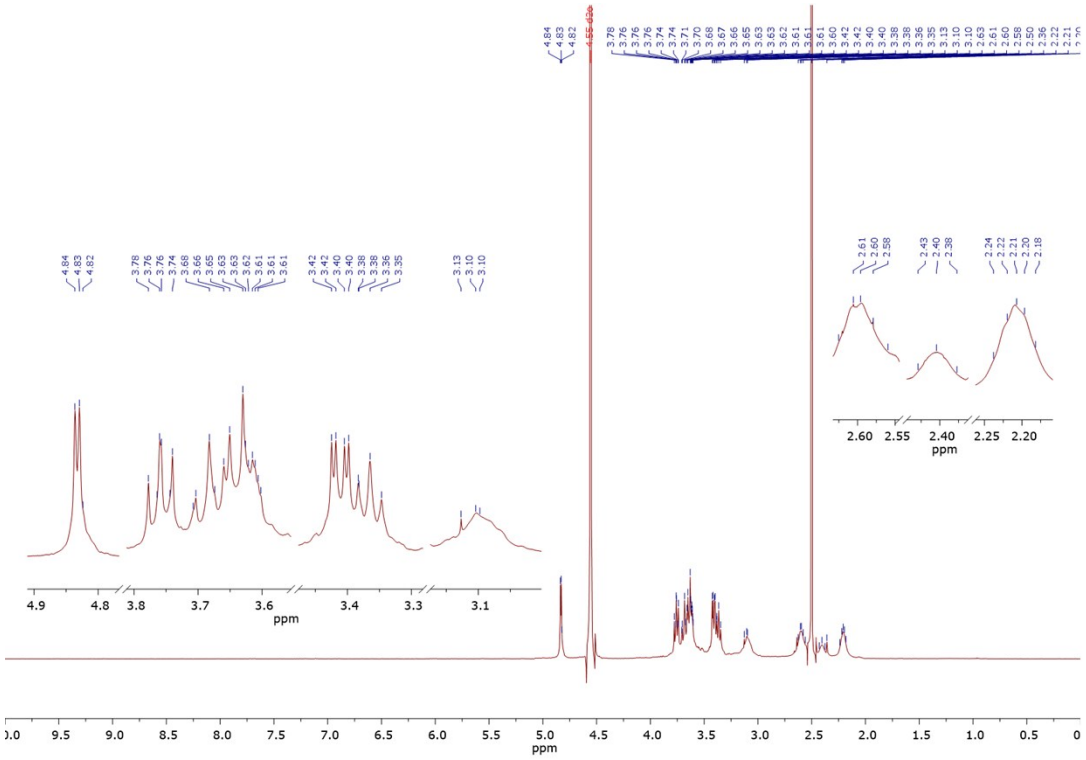

Fig. S2. ^{1}H NMR (DMSO-d_6 : D_2O = 1:1 *v*/*v*, 500 MHz) spectrum of octa-αCD-PAMAM (**3**)

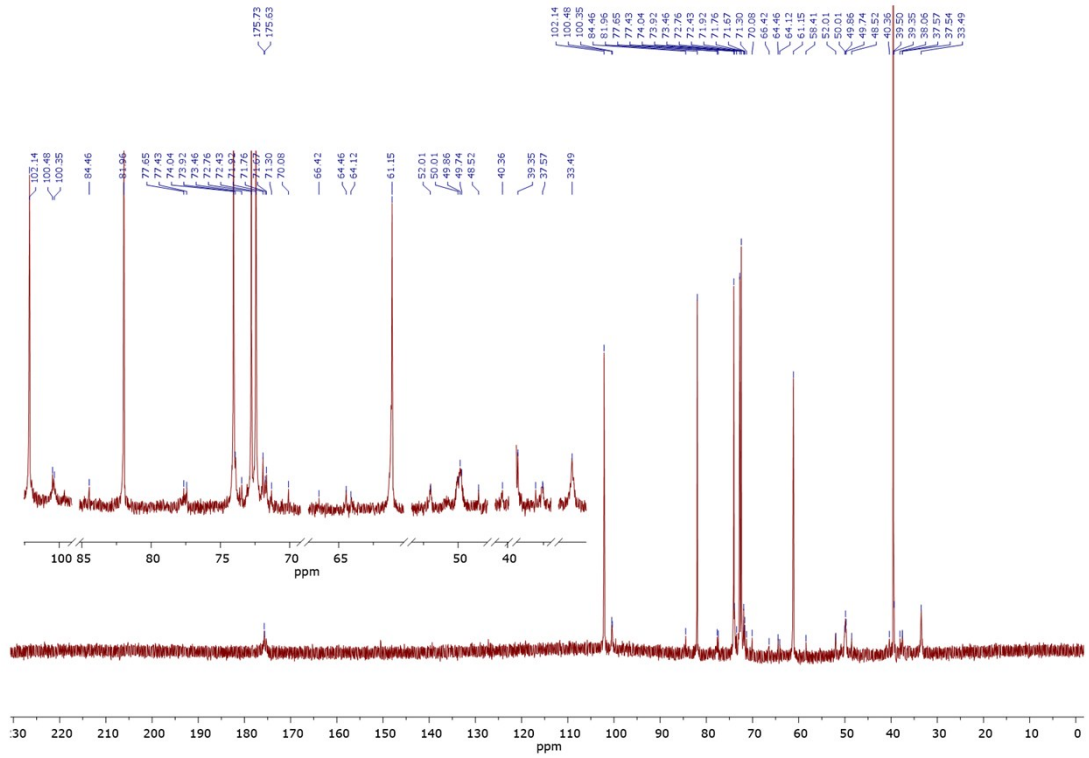

Fig. S3. ^{13}C NMR (DMSO-d_6 : D_2O = 1:1 *v*/*v*, 125 MHz) spectrum of octa-αCD-PAMAM (**3**)

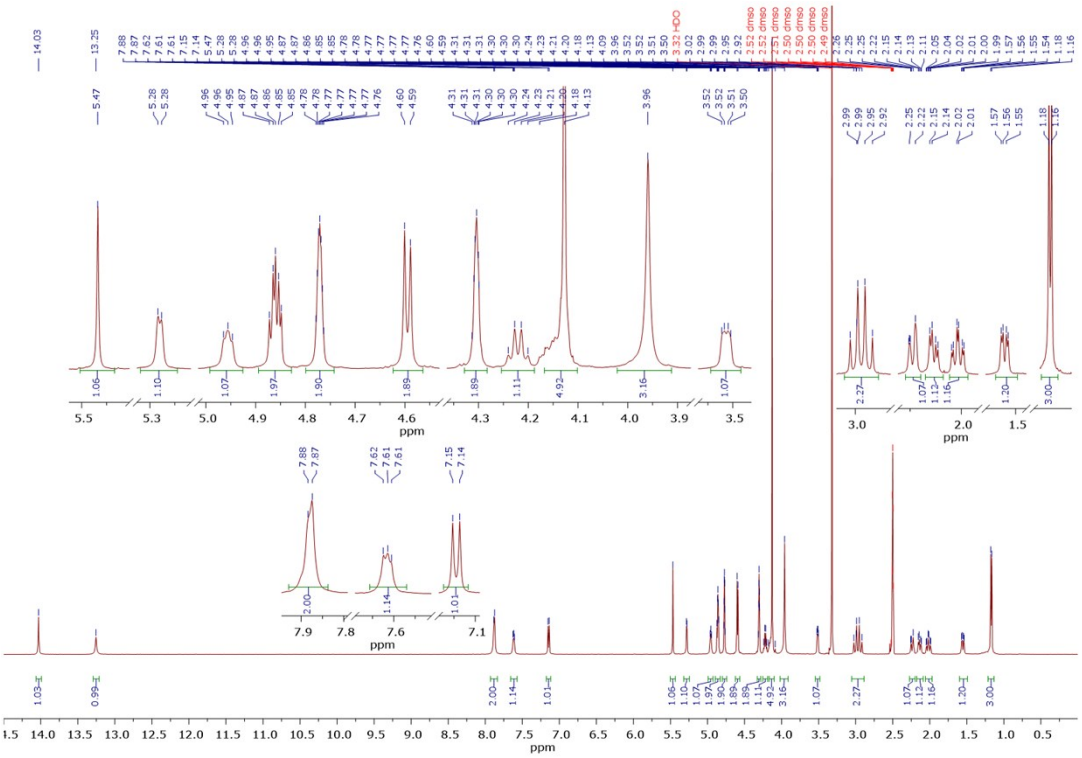

Fig. S4. ^{1}H NMR (DMSO-d_6, 500 MHz) spectrum of Fc-COO-DOX*HCl (**5**)

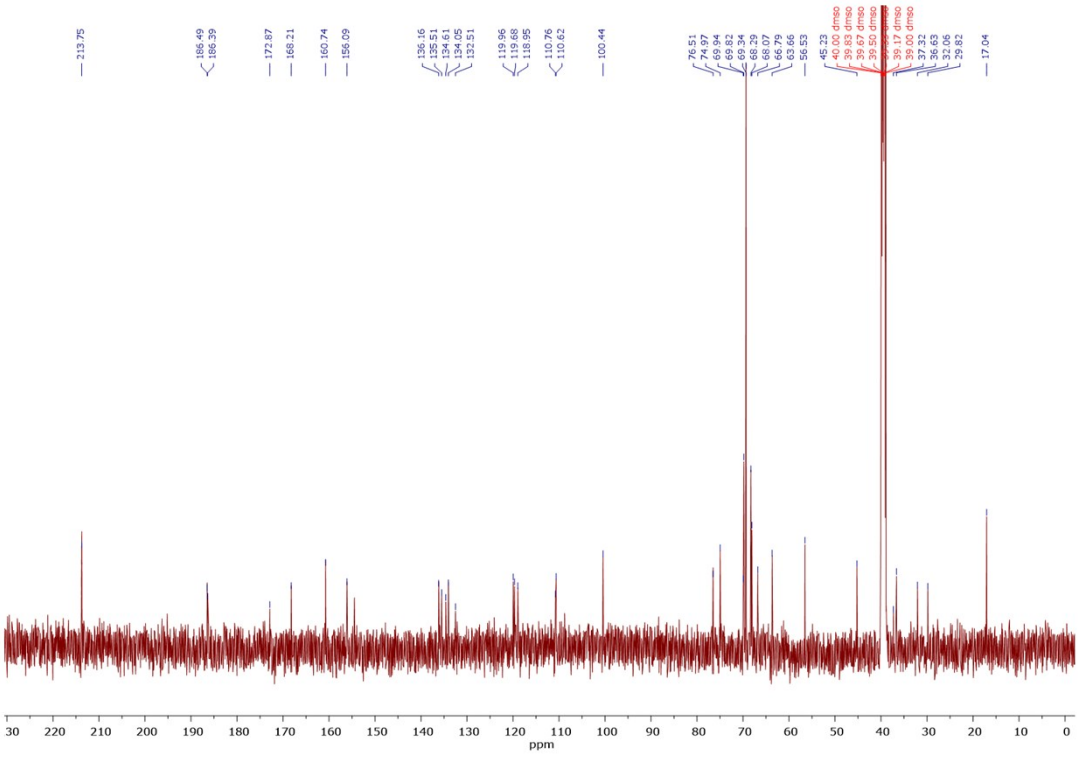

Fig. S5. ^{13}C NMR (DMSO-d_6, 125 MHz) spectrum of Fc-COO-DOX*HCl (**5**)

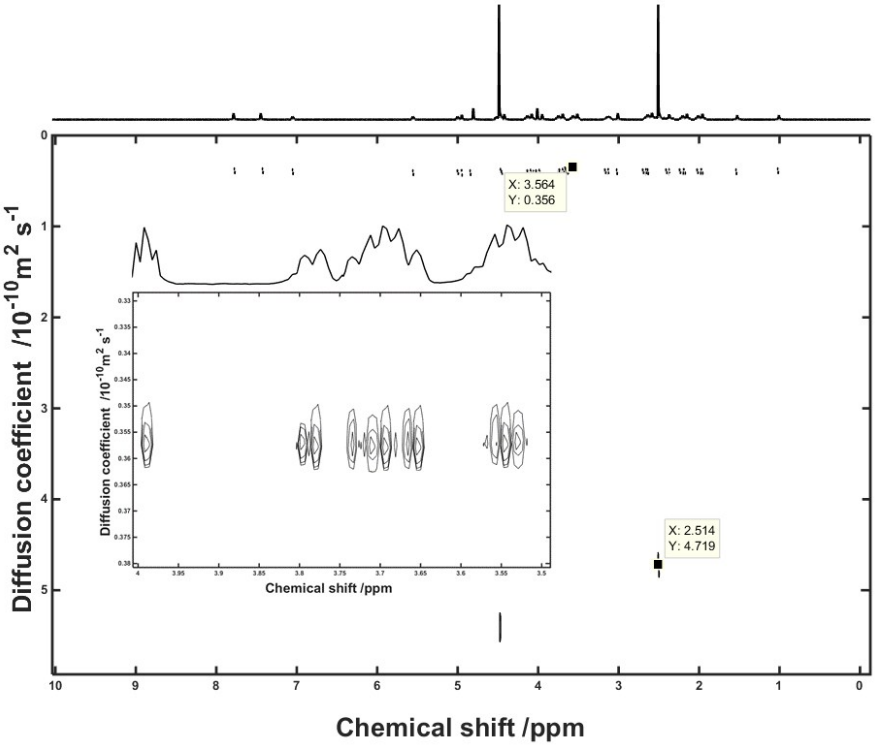

Fig. S6. ^{1}H DOSY NMR (DMSO-d_6 : D_2O = 1:1 *v*/*v*, 500 MHz) spectrum of nanoconjugate {Fc-COO-DOX*HCl}@{octa-αCD-PAMAM} (**6**). The 4.00-3.50 ppm inset of the spectrum is also presented.

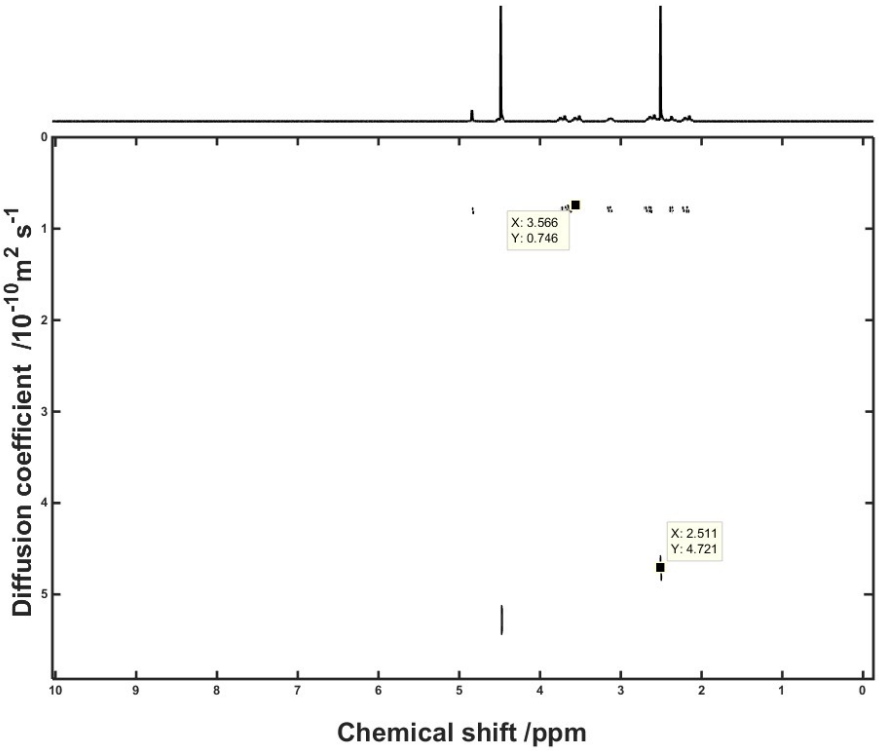

Fig. S7. ^{1}H DOSY NMR (DMSO-d_6 : D_2O = 1:1 *v*/*v*, 500 MHz) spectrum of octa-αCD-PAMAM (**3**)

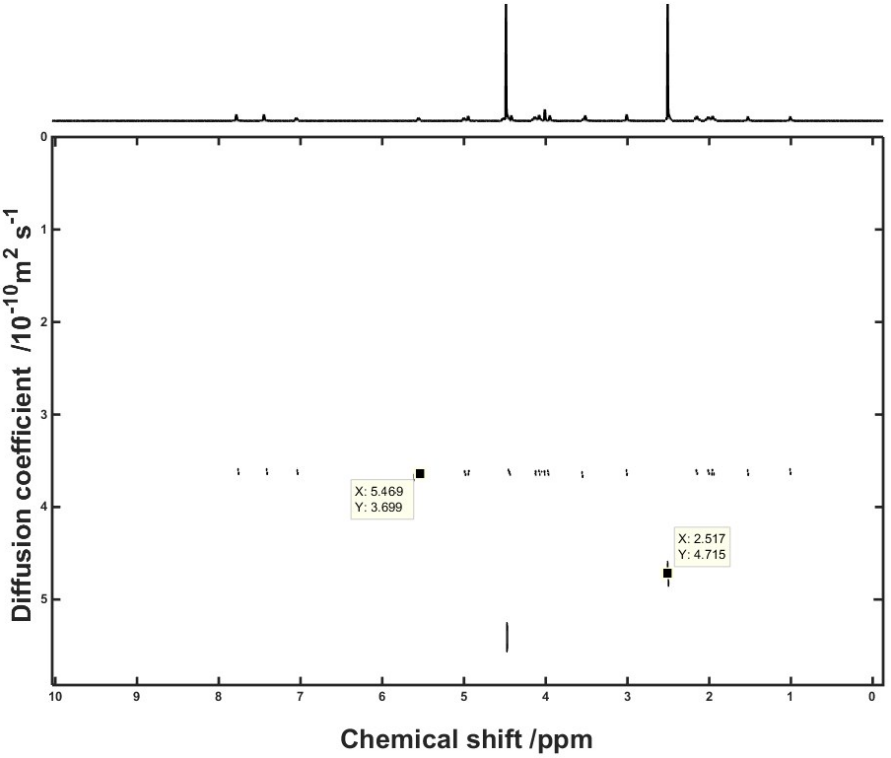

Fig. S8. ^{1}H DOSY NMR (DMSO-d_6 : D_2O = 1:1 *v*/*v*, 500 MHz) spectrum of Fc-COO-DOX*HCl (**5**)

S3. FT-IR spectra

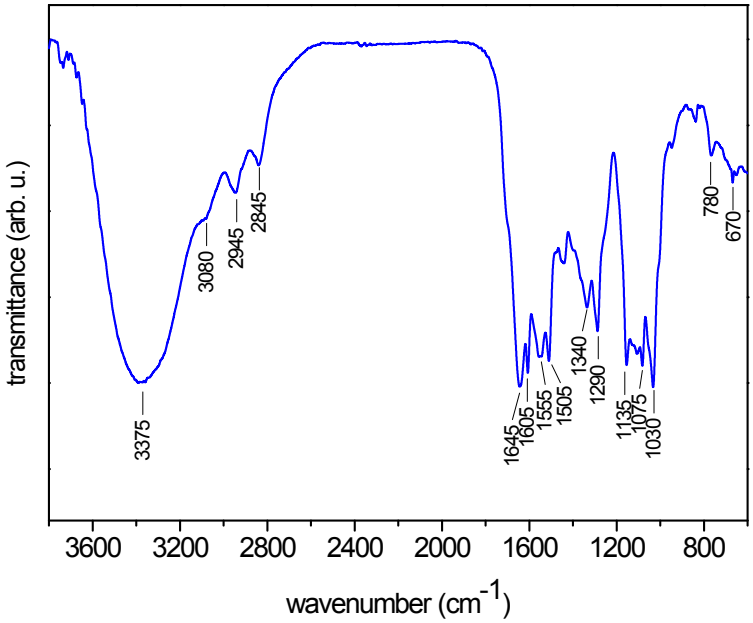

Fig. S9. FT-IR (ATR) spectrum octa-αCD-PAMAM (**3**)

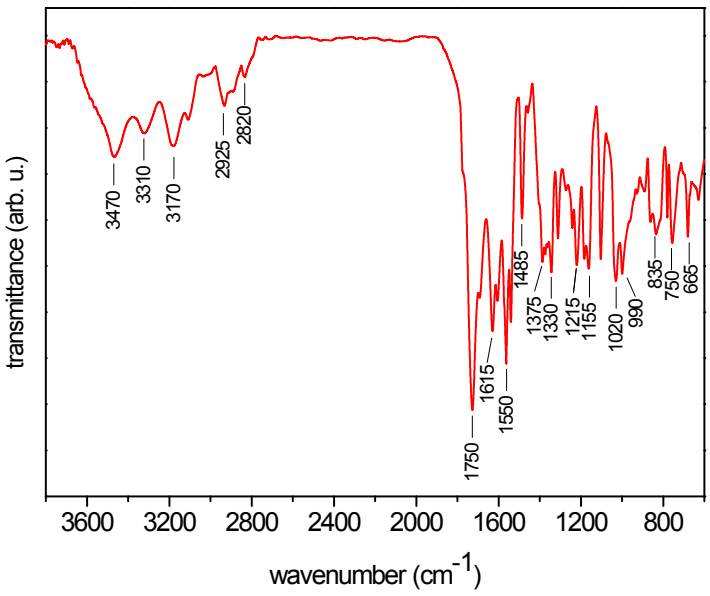

Fig. S10. FT-IR (ATR) spectrum Fc-COO-DOX*HCl (**5**)

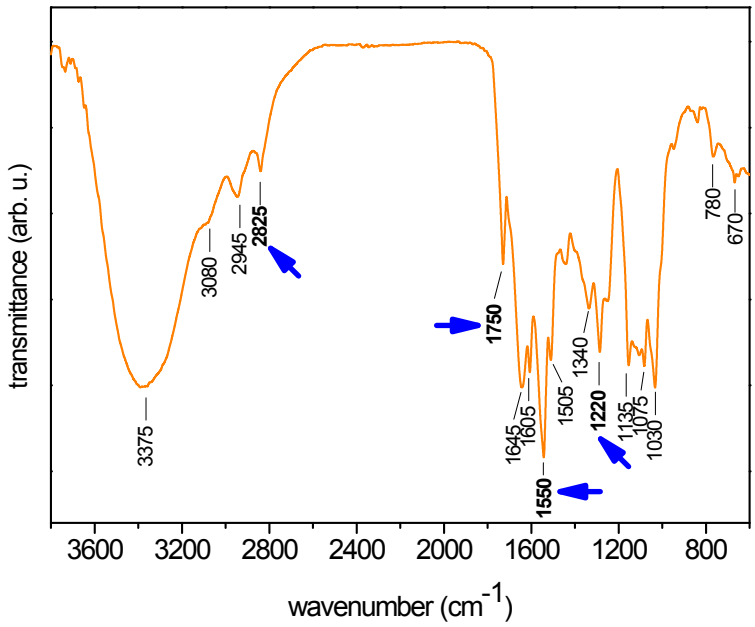

Fig. S11. FT-IR (ATR) spectrum {Fc-COO-DOX*HCl}@{octa-αCD-PAMAM} (**6**). The most significant absorption bands that were ascribed to the DOX*HCl moiety of guest **5** were bold and marked with blue arrows

S4. ESI-MS spectra of octa-αCD-PAMAM (3) and nanoconjugate 6

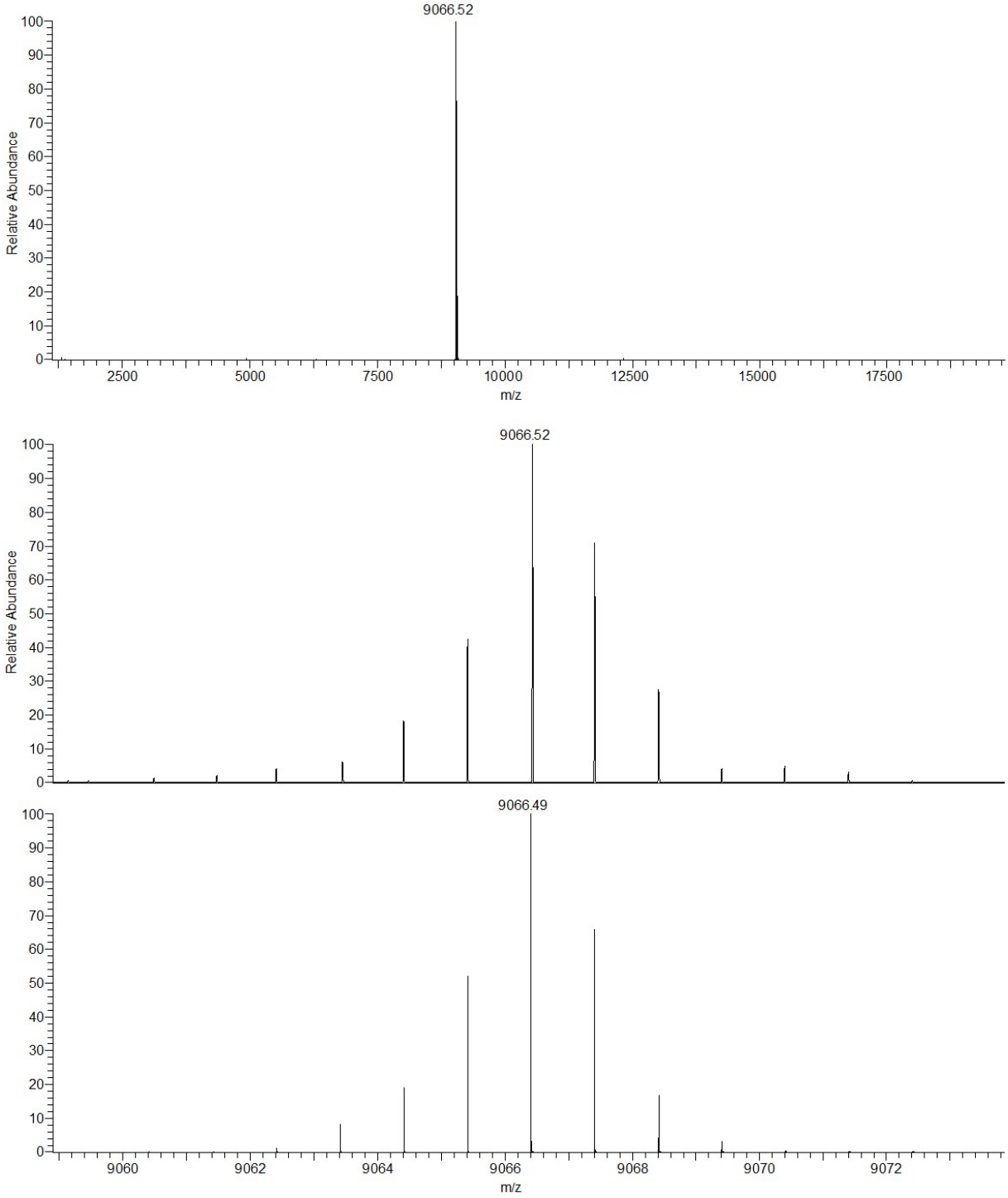

Fig. S12. ESI-MS (TOF) spectrum of octa-αCD-PAMAM (**3**): top – measured (full spectrum) middle – measured (inset), bottom – calculated (inset)

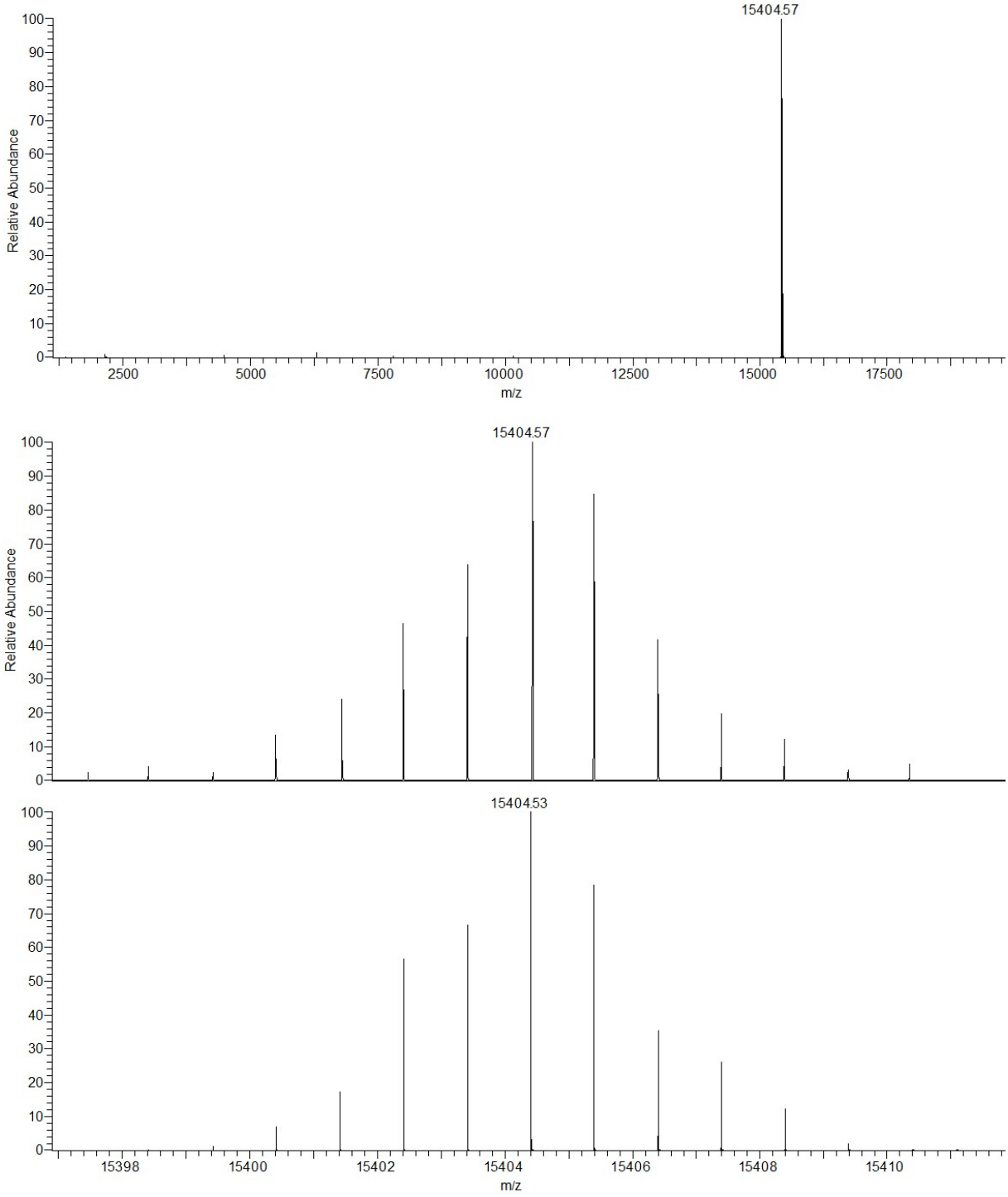

Fig. S13. ESI-MS (TOF) spectrum of nanoconjugate **6**: top – measured (full spectrum) middle – measured (inset), bottom – calculated (inset)

S5. UV-Vis analyses on the complexation phenomenon

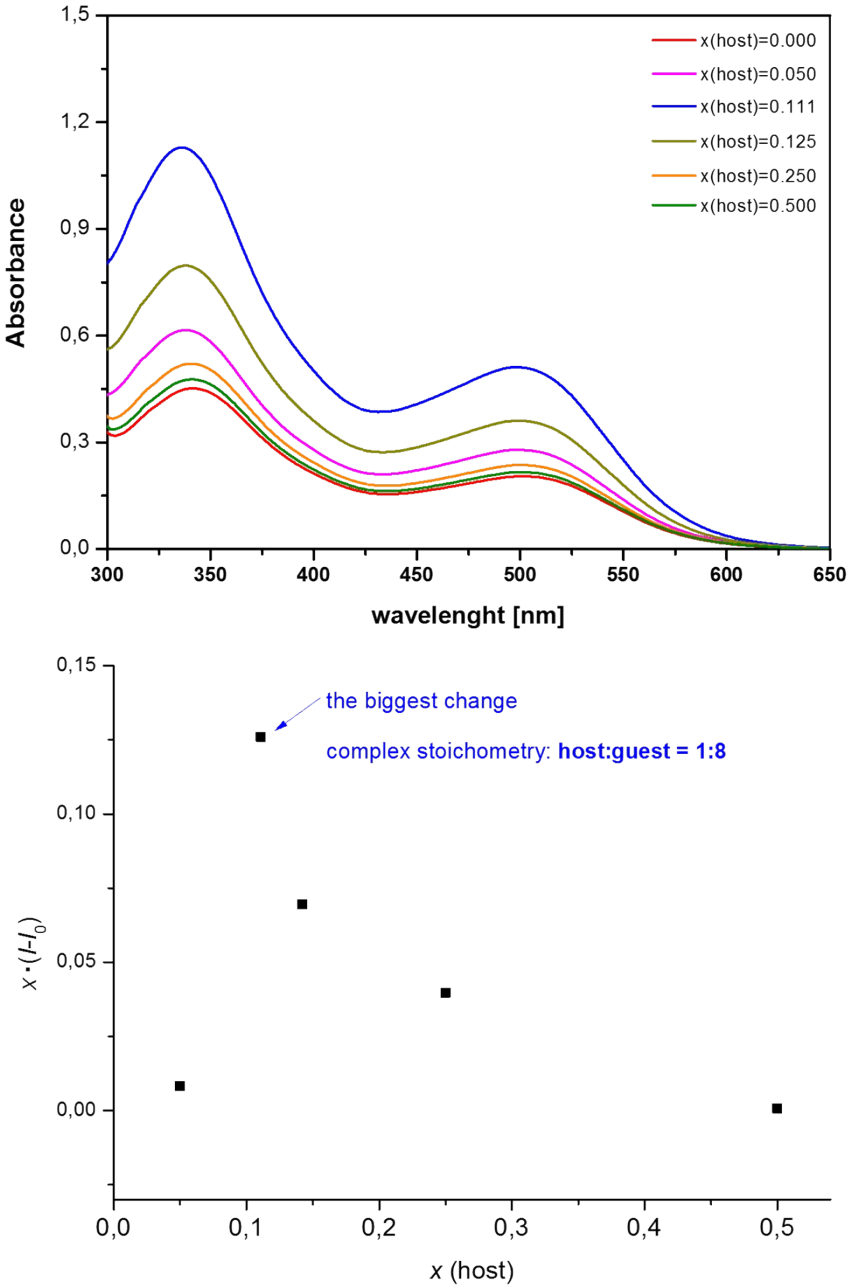

Fig. S14. (**Top**) UV-Vis titration spectra for the interactions between guest **3** and host **5** in the DMSO : H_2O = 1:1 *v*/*v* solvent system (*x*(host) stands for the molar fraction of host **3** in the mixture); (**Bottom**) Job's plot constructed on the basis of UV-Vis analyses (absorbance at *ca.* 342 nm) .

S6. References

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