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**Accessory Publication**

# Investigating avidin-biotin interaction on chiral soft structure platform

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**Table of Contents:**

## Material and Methods

**General**- Dichloromethane, N, N-dimethylformamide, methanol, triethylamine and 1, 2-dimethoxy ethane were distilled and dried following standard procedures prior to use. D-Biotin, cholesterol, 4-aminocaproic acid, sodium hydroxide, diethyl ether, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N,N*-dimethyl-4-aminopyridine (DMAP) were purchased from Spectrochem, Mumbai, India, and used without further purification. $^{1}H$ and $^{13}C$ NMR spectra were recorded on JEOL-JNM LAMBDA 400 model operating at 500 and 125 MHz, respectively. Analysis of $^{1}H$ and $^{13}C$ NMR of final product was done by JEOL ECX-500 model operating at 500 MHz and 125MHz respectively. HRMS was recorded at IIT Kanpur, India, on Waters, Q-Tof Premier Micromass HAB 213 mass spectrometer using capillary voltage 2.6-3.2 kV. IR spectra were recorded as KBr pellets on a Bruker Vector 22 FTIR spectrophotometer operating from 400 to 4000 $cm^{-1}$.

**Optical Microscopy (OM):** A 10 μL aliquot of **1** and **2** (3mM solution in 50% MeOH-DCM) at room temperature was placed on a glass slide and allowed to dry by slow evaporation. Optical micrographs were taken by (Leica DM2500M) microscope using 20X lens.

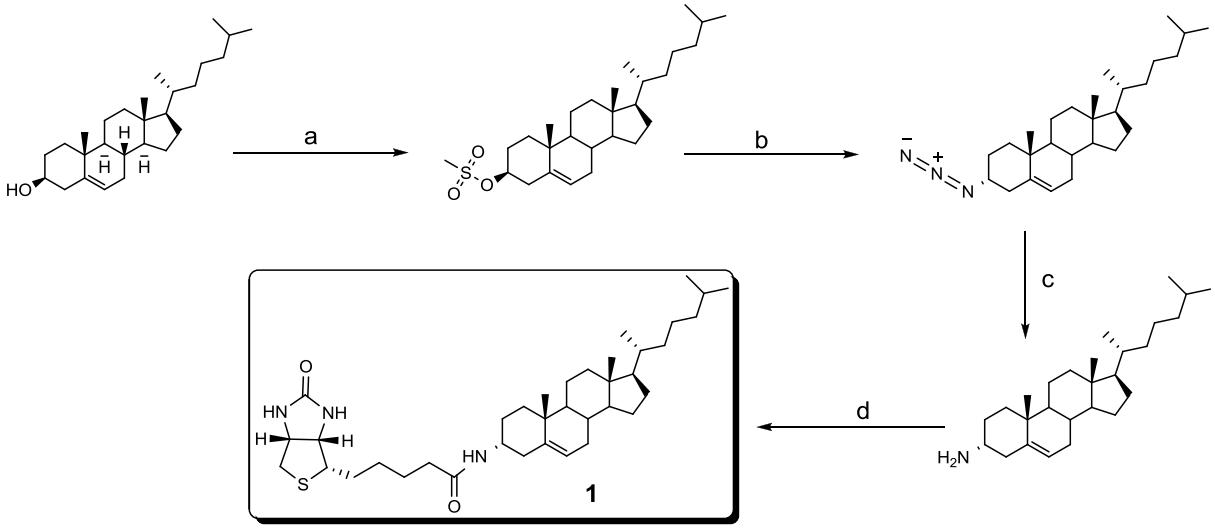
**Fluorescence Microscopy (FM):** Solutions of **1**and **2** (3mM solution in 50% MeOH-DCM) were incubated with Rhodamine B for 6h at room temperature. 10 μL of solutions were loaded on glass slides and dried under lamp. Dye stained structures were examined under a fluorescent microscope (Leica DM2500M), provisioned with a rhodamine filter (absorption 540 nm/emission 625 nm).

**Polarization Optical Microscopy (POM)**: Solutions of **1** and **2** (3mM solutions in 50% MeOH-DCM) were incubated at room temperature for 48 h. 10 μL aliquots of solutions were placed on a glass slide and allowed to dry by slow evaporation and viewed under cross polarizer's.

**Field Emission Scanning Electron Microscopy (FE SEM):** 10 μL aliquots of **1** and **2** (3mM solutions in 50% MeOH-DCM mixture) were dried at room temperature on glass slides further vacuum dried and subsequently coated with gold. FE SEM images were acquired on FEI QUANTA 200 microscope, equipped with a tungsten filament gun, operating at WD 10.6 mm and 20 kV.

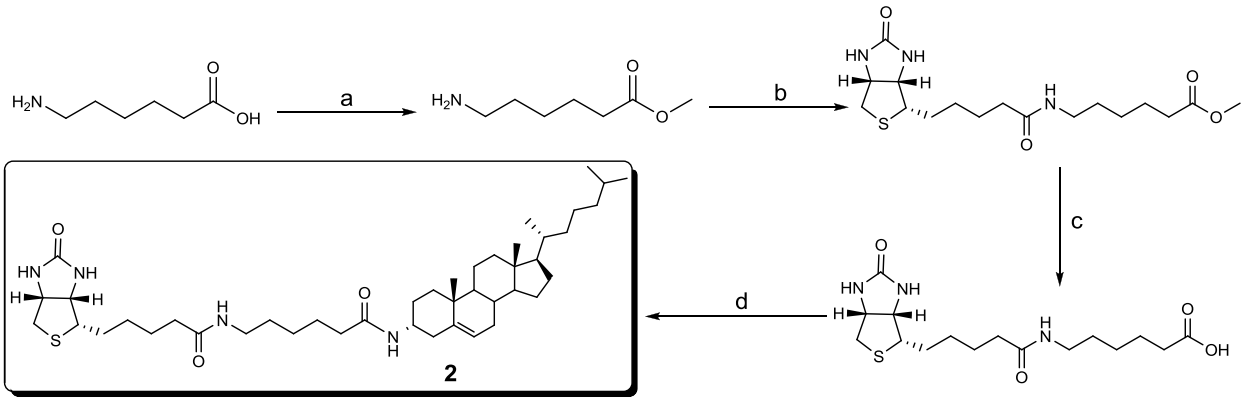
**Atomic Force Microscopy (AFM)**: AFM Samples were imaged with an atomic force microscope (Molecular Imaging, USA) operating under the Acoustic AC mode (AAC), with the aid of a cantilever (NSC 12(c) from MikroMasch). The force constant was 0.6 N/m, while the resonant frequency was 150 kHz. The images were taken in air at room temperature, with the scan speed of 1.5-2.2 lines/sec. The data acquisition was done using PicoView 1.4® software, while the data analysis was done using PicoView. 10 μL aliquots of A and B (3mM solutions in 50% MeOH-DCM mixture) were coated over mica. The sample-coated mica was dried for 30 minutes at room temperature, and finally vacuum was applied for 30 min followed by AFM imaging.

**<u>Synthesis:</u>** The synthesis was carried out by functional group inter conversion of cholesterol to 3α-amino-5-cholestene by reported procedures as shown in scheme 1. Mesylation of cholesterol, synthesis of corresponding azide and its reduction with lithium aluminum hydride to generate 3α-amino-5-cholestene and then direct coupling of carboxylic group of Biotin activated *in situ* with EDC, DMAP to amine in DMF to give conjugate (**1**).



**Scheme 1**: a) $CH_3SO_2Cl$, $Et_3N$, $CH_2Cl_2$, 0 °C; b) $NaN_3$, DMF, reflux at 90 °C for 10h; c) LAH, dry ether, 0 °C, 90 min; d) Biotin, EDC, DMF, DMAP, 12h.

Conjugate **2** was synthesized by incorporating 4-aminocaproic acid linker to biotin by esterification of 4-aminocaproic acid, followed by coupling to Biotin activated *in situ* with EDC and DMAP in DMF. Methyl ester was then deprotected with sodium hydroxide in methanol. The carboxylic acid function was further activated *in situ* with EDC, DMAP and coupled to 3α-amino-5-cholestene in DMF to give conjugate (**2**) as shown in scheme 2.




**Scheme 2**: a) $SOCl_2$, MeOH; b) Biotin, EDC, DMF, DMAP, 12h; c) 1N NaOH, MeOH, 6h; d) 3α-amino-5-cholestene, EDC, DMF, DMAP, 12h.

## Synthesis of 3α-amino-5-cholestene

### Synthesis of cholesterol mesylate:

To 40 mg (0.104 mmol) of cholesterol in 3 mL of dichloromethane was added 157 mg (216 µL, 1.55 mmol) of triethylamine. After cooling to 0 °C, a solution of 29 mg (0.25 mmol) of methanesulfonyl chloride in 10 mL of DCM was added drop wise over a period of 20 min under nitrogen. The reaction mixture was stirred for 3h at 0 °C, then diluted

with ether and washed with 10% aqueous HCl (10 mL) and water (3 x 10 mL). The organic layer was dried over $Na_2S0_4$ and concentrated *in vacuo* to afford 3-α-cholesterol mesylate and the product was used without any further purification. $R_f$ 0.8 in DCM; $^1$H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ (ppm): 0.64(s, 6H), 0.82-0.84(d, 3H), 0.87-0.88(d, 3H), 0.98(s, 6H), 1.03-1.15(m, 5H), 1.19-1.26(d, 2H), 1.30-1.34(m, 3H), 1.41-1.53(m, 9H), 1.75-1.81(m, 1H), 1.85-1.88(dd, 1H), 1.93-2.04(m, 2H), 2.43-2.51(m, 2H), 2.97(s, 3H), 4.48(s, 1H), 5.37(s, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ (ppm): 19.4, 20.7, 22.7, 23.2, 24.6, 27.3, 27.7, 28.1, 29.9, 30.0, 31.9, 35.8, 36.1, 36.76, 37.2, 37.7, 38.6, 39.9, 44.0, 50.8, 55.9, 56.49, 82.00, 123.69, 138.53.

**Synthesis of 3 α -azido-5-cholestene:**

To 100 mg (0.216 mmol, 1eq.) of cholesterol mesylate in DMF was added 16.79 mg (0.31 mmol, 1 eq.) of sodium azide and refluxed at 90 °C for 10h. After 10h DMF was evaporated the compound was dissolved in ether and washed with 10% aqueous HCl (10 mL) and water (3 x 10 mL**).** The organic layer was separated, dried over $Na_2S0_4$ and concentrated *in vacuo* to afford 3-α-azido-5-cholestene. $R_f$ 0.95 in DCM; $^1$H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ (ppm): 0.60(s, 6H), 0.78-0.80(d, 3H), 0.83-0.85(d, 3H), 0.93(s, 6H), 0.97-1.04(m, 5H), 1.18(d, 2H), 1.25-1.27(m, 3H), 1.36-1.46(m, 9H), 1.5(s, 1H), 1.76-1.78(dd, 2H), 1.92-1.95(m, 2H), 2.16-2.21(m, 2H), 5.27(s, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ (ppm): 19.4, 20.7, 22.7, 23.2, 24.6, 27.3, 27.7, 28.1, 29.9, 30.0, 31.9, 35.8, 36.1, 36.76, 37.2, 37.7, 39.9, 44.0, 50.8, 55.9, 56.49, 56.77, 121.756, 140.703; HRMS $[M+H]^+$ for $C_{27}H_{46}N_3$ calcd 412.3692, obsd 412.3692

**Synthesis of 3α-amino-5-cholestene:**

To 100 mg (0.242 mmol, 1 eq.) 3 α -azido-5-cholestene in dry diethyl ether (60mL) at 4 °C was added 110.63 mg (2.9 mmol, 12 eq.) Lithium Aluminium Hydride in three equal portions. The reaction was maintained at 4 °C for 30 min, warmed to 23 °C, and stirred for an additional 1.5h. The reaction was quenched by addition of diethyl ether (10 mL) followed by careful drop wise addition of deionized $H_2O$ (12 mL).The organic layer

was washed with saturated aqueous NaCl (2 x 100 mL), dried over anhydrous $Na_2SO_4$, and concentrated *in vacuo* to afford 3α-amino-5-cholestene and the product was used without any further purification. $R_f$ 0.1 in 10% MeOH-DCM; +ve for ninhydrin staining; $^1$H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ (ppm): 0.66(s, 6H), 0.82-0.84(d, 3H), 0.87-0.88(d, 3H), 0.98(s, 6H), 1.03-1.15(m, 5H), 1.19-1.26(d, 2H), 1.30-1.34(m, 3H), 1.41-1.53(m, 9H), 1.75-1.81(m, 1H), 1.85-1.88(dd, 1H), 1.93-2.04(m, 2H), 2.27(s, 1H), 2.43-2.51(m, 2H), 5.37(s, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ (ppm): 19.4, 20.7, 22.7, 23.2, 24.6, 27.3, 27.7, 28.1, 29.9, 30.0, 31.9, 35.8, 36.1, 36.76, 37.2, 37.7, 39.9, 44.0, 47.68, 50.8, 55.9, 56.49, 123.25, 138.16; HRMS $[M+H]^+$ for $C_{27}H_{48}N$ calcd 386.3787, obsd 386.3788.

## Synthesis of D-Biotin 3α-amino-5-cholestene conjugate (1):

### Coupling of Biotin with 3α-amino-5-cholestene (1):

(+) D-Biotin 100 mg (0.41 mmol, 1 eq.), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) 78.46 mg (0.41 mmol, 1 eq.), and *N, N*-dimethyl-4-aminopyridine (DMAP) 64.85 mg (0.49 mmol, 1.2 eq.) were taken in a two-neck round-bottom flask and dissolved in dry DMF (15 mL) under $N_2$ atmosphere. 3α-amino-5-cholestene 194.5 mg (0.50 mmol, 1.2 eq.) was then added and the mixture was stirred for 12h at room temperature. After the reaction was completed solvent was evaporated *in vacuo* and the residue was dissolved in DCM, washed with 1N HCl (2×20 mL) and water. The DCM layer was separated and dried over anhydrous $Na_2SO_4$, and concentrated *in vacuo*. The crude compound was purified through silica gel chromatography with a dichloromethane methanol (95:5) solvent system to give pure Biotin 3α-amino-5-cholestene conjugate [**1**] (175.3 mg, yield 70%). $[\alpha]^{25}_D$ = +9.25(c, 0.004, DCM); $R_f$ 0.3 in 5% MeOH-DCM; mp 134-136 °C; $^1$H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ (ppm), 0.67(s, 3H), 0.70(s, 3H), 0.85-0.86(d, *J=5.5Hz*, 3H), 0.9-0.91(d, *J=4.6Hz*, 3H), 1.01(s, 4H), 1.07-1.14(m, 1H), 1.24(s, 4H), 1.32(m, 1H), 1.40(m, 3H), 1.43(m, 1H), 1.44(m, 2H), 1.47(m, 2H), 1.49(m, 1H), 1.50(m, 1H), 1.51(m, 1H), 1.57(m, 1H), 1.58(m, 1H), 1.66(m, 1H), 1.69(m, 2H), 1.74(s, 2H), 1.82(m, 3H), 1.84(m, 1H), 1.88(s, 1H), 1.91(s, 1H), 1.97-

2.00(t, *J=13.3Hz*, 3H), 2.18(s, 1H), 2.56-2.59(d, *J=13.3Hz*, 1H), 2.73-2.76(d, *J=11.9Hz*, 1H), 2.90-2.92(d, *J=11.9Hz*, 1H), 3.15(s, 1H), 4.12(s, 1H), 4.33(s, 1H), 4.53(s, 1H), 5.37(s, 1H), 5.57(s, 1H), 5.65(s, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ (ppm), 18.69, 18.84, 22.54, 22.66, 22.82, 23.87, 24.26, 24.88, 25.85, 27.99, 28.22. 29.33, 29.48, 29.67, 31.39, 31.60, 31.76, 31.90, 35.76, 36.15, 37.33, 39.47, 39.70, 42.28, 42.65, 42.81, 47.36, 50.43, 56.18, 56.72, 123.77, 151.93, 163.72, 177.05; HRMS $[M+H]^+$ for $C_{37}H_{62}N_3O_2S$ calcd 612.4563, obsd 612.4563

**Synthesis of D-Biotin 4-aminocaproic acid-3α-amino-5-cholestene conjugate (2):**

**Synthesis of Methyl ester of 4-aminocaproic acid:**

4-Aminocaproic acid 100 mg (0.536 mmol, 1 eq.), was taken in a round-bottom flask and dissolved in Methanol (10 mL). Thionyl chloride 63.53 mg (56µL, 1.2 eq.) was then added and the reaction mixture was refluxed for 10h. After completion of reaction solvent, was evaporated *in vacuo*. To the residue was added ice till a white precipitate was formed which was redissolved in DCM and washed with 10% $NaHCO_3$ solution and water. The DCM layer was separated and dried over anhydrous $Na_2SO_4$, and concentrated *in vacuo* to afford a white solid.

**Synthesis of D-Biotin 4-aminocaproic acid methyl ester:**

D-Biotin 100 mg (0.408 mmol, 1eq.), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) 86.37 mg (0.45 mmol, 1eq.), and *N,N*-dimethyl-4-aminopyridine (DMAP) 55 mg (0.45 mmol, 1eq.) were taken in a two-neck round-bottom flask and dissolved in dry DMF (25 mL) and placed under Nitrogen. 4-Aminocaproic acid methyl ester 59.2 mg (0.22 mmol, 1eq.) was then added and the mixture was stirred for 12h at room temperature. After the reaction was completed the solvent was evaporated in high *vacuo* and the residue was dissolved in DCM, washed with 1N HCl (2×20 mL) and water. The DCM layer was separated and dried over anhydrous $Na_2SO_4$, and concentrated *in vacuo* to afford Biotin 4-aminocaproic acid methyl ester. $R_f$ 0.2 in 10% MeOH-DCM;

$^{1}$H NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ (ppm): 1.28-1.36(m, 4H), 1.37-1.39(m, 4H), 1.44-1.46(m, 2H), 1.55-1.59(m, 2H), 2.12-2.14(s, 2H), 2.27-2.30(m, 2H), 2.66-2.70(dd, 1H), 2.8(d, 1H), 2.85-2.88(dd, 2H), 3.13(s, 1H), 3.62(s, 3H), 4.26(s, 1H), 4.47(s, 1H); $^{13}$C NMR (125 MHz, $CDCl_3$) δ (ppm): 24.46, 25.64, 26.30, 28.13, 28.46, 28.85, 33.77, 35.70, 39.11, 40.17, 51.39, 55.69, 60.28, 62.047, 164.517, 174.56 and 174.88.

**Hydrolysis of D-Biotin 4-aminocaproic acid methyl ester:**

D-Biotin aminocaproic acid methyl ester 100 mg (0.23 mmol, 1 eq.) was taken in a 25 mL round-bottom flask and dissolved in MeOH (10 mL).To the reaction mixture 1N NaOH 11.22 mg (0.28 mmol, 1.2 eq.) was added and stirred at room temperature for 6h. After the reaction was completed, the mixture was cooled in an ice bath and acidified with 1N HCl to congo. The hydrolyzed product that precipitated was extracted with ethyl acetate and washed with deionized water. The ethyl acetate layer was then dried over anhydrous $Na_2SO_4$, and concentrated *in vacuo* to afford D-Biotin aminocaproic acid. The acid was used for coupling in the next step without any further purification.

**Coupling of Biotin 4-aminocaproic acid with 3α-amino-5-cholestene (2):**

(+) D-Biotin 4-aminocaproic acid 150 mg (0.36 mmol, 1eq.), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) 69.53 mg (0.36 mmol, 1eq.), and *N, N*-dimethyl-4-aminopyridine (DMAP) 44.3 mg (0.23 mmol, 1eq.) were taken in a two-neck round-bottom flask under nitrogen and dissolved in dry DCM/DMF (25 mL). 3α-amino-5-cholestene 139.88 mg (0.36 mmol, 1eq.) was then added and the mixture was stirred for 12h at room temperature. After the reaction was completed the solvent was evaporated in high *vacuo* and the residue was dissolved in DCM, washed with 1N HCl (2×20 mL) and water. The DCM layer was separated and dried over anhydrous $Na_2SO_4$ and concentrated *in vacuo*. The crude compound was purified through silica gel chromatography with a dichloromethane-methanol (93:7) solvent system to give pure D-Biotin 4-aminocaproic acid- 3α-amino-5-cholestene conjugate [**2**] (192.8 mg, yield 65%). $[\alpha]^{25}_{D}$ = +6.8(c 0.005, DCM); $R_f$ 0.4 in 10% MeOH-DCM; mp 152-154 °C; $^{1}$H

NMR (500 MHz, $CDCl_3$, 25 °C, TMS) δ (ppm), 0.67(s, 3H), 0.7(s, 3H), 0.84-0.86(d, *J=5.95Hz,* 3H), 0.9-0.91(d, *J=7.35Hz,* 3H), 1.01(s, 4H), 1.07-1.15(m, 1H), 1.24(s, 4H), 1.32-1.34(m, 5H), 1.40(s, 3H), 1.41(m, 1H), 1.43(m, 2H), 1.44(m, 1H), 1.45(m, 1H), 1.49(m, 1H), 1.50(m, 1H), 1.52(m, 1H), 1.54(m, 2H), 1.58-1.60(t, *J=6.9Hz,* 2H), 1.66(s, 1H), 1.68(s, 2H), 1.71-1.73(m, 2H), 1.81-1.84(m, 4H), 1.88(s, 1H), 1.91(s, 1H), 1.97-2.00(t, *J=14.2Hz,* 3H), 2.15-2.19(m, 1H), 2.28-2.31(t, *J=7.8Hz,* 2H), 2.56-2.59(d, *J=15Hz,* 1H), 2.72-2.75(d, *J=12Hz,* 1H), 2.90-2.92(d, *J=11Hz,* 1H), 3.15(s, 2H), 3.22(s, 1H), 4.12(s, 1H), 4.33(s, 1H), 4.53(s, 1H), 5.37(s, 1H), 5.57(s, 1H), 5.65(s, 1H); $^{13}C$ NMR (125 MHz, $CDCl_3$) δ (ppm), 18.69, 18.83, 20.72, 22.55, 22.67, 22.82, 23.87, 24.25, 24.91, 25.27, 25.70, 25.88, 26.03, 26.36, 28.00, 28.23, 28.56, 28.86, 29.12, 29.33, 29.48, 30.15, 31.42, 31.76, 31.93, 34.06, 35.79, 36.16, 37.36, 39.46, 39.70, 42.28, 50.43, 56.17, 56.72, 123.67, 138.72, 163.12, 177.05; HRMS $[M+H]^+$ for $C_{43}H_{73}N_4O_3S$ calcd 725.5403, obsd 725.5403.

**Sample Preparation:**

**For Optical Microscope**: 3mM solutions of **1** and **2** were prepared in 50% MeOH-DCM. 10 μL aliquots of **1** and **2** were then placed on glass slide at room temperature and allowed to dry before viewing under the microscope

**For Fluorescence Optical Microscope**: 3mM solutions of **1** and **2** in 50% MeOH-DCM were stained with rhodamine B. 10 μL aliquots of **1** and **2** were then placed on glass slide at room temperature and allowed to dry before viewing under the microscope.

**For Polarization Optical Microscope:** 3mM solutions of **1** and **2** were prepared in 50% MeOH-DCM and incubated. 10 μL aliquots of **1** and **2** were then placed on glass slide at room temperature and allowed to dry before viewing under cross polarizers.

**For Field Emission Scanning Electron Microscopy (FE SEM):** 10 μL aliquots of **1** and **2** (3mM solutions in 50% MeOH-DCM mixture) were dried at room temperature on

glass slides, further vacuum dried and subsequently coated with gold followed by imaging with FE SEM.

**For Atomic Force Microscopy (AFM)**: 10 μL aliquots of A and B (3mM solutions in 50% MeOH-DCM mixture) were coated over mica. The sample-coated mica was dried for 30 minutes at room temperature, and finally vacuum was applied for 30 min followed by AFM imaging.

**For Avidin studies:** 10 μL aliquots of solutions from **Method B** were then placed on glass slide at room temperature and allowed to dry before viewing under cross polarizers and imaging by **FESEM**.

**The experimental protocol related to aggregation studies of Avidin with self-assembled structures 1 and 2 are presented in greater detail.**

- **Choice of sample concentration**: 1.5 mM solutions of **1** and **2** were prepared in 50% dichloromethane-methanol. This concentration was chosen as no aggregation was observed for **1** and **2** prior to the addition of avidin. The birefringence of the sample was unaffected by decrease in the concentration from 3mM to 1.5mM.

- **Aqueous solution of avidin:** Avidin solutions in water over a range of concentrations (0.75mM – 0.075nM) were prepared.

- **Sample preparation for biotin-avidin interaction studies:**

    - **Method A:** 50 μL of **1** or **2** (1.5mM in 50% dichloromethane-methanol) was added to 50 μL aqueous solution of avidin of a given concentration to make a 100 μL stock solution.

    - **Method B:** 99 μL of **1** or **2** (1.5 mM in 50% dichloromethane-methanol) was added to 1 μL aqueous solution of avidin of a given concentration to make a 100 μL stock solution.

- **Note:** 10 μL aliquots of these stock solutions were then placed on glass slide at room temperature and allowed to dry before viewing under cross polarizers.

- **Observations:**

    - *Samples prepared using Method A:*

- Samples showed a definite trend for aggregation
- Due to large volume of water added to the system, issues like solvent miscibility and solubility of conjugates were inevitable and observed
- Control experiments (without avidin) also showed similar aggregation behavior
- **Sample preparation for control experiments**: 50 µL of **1** or **2** (1.5mM in 50% dichloromethane-methanol) was added to 50µL water to make a 100 µL stock solution.

  We found that,

- The aggregation behavior observed may not be solely due to avidin
- Solvent system may lead to precipitation or aggregation problems
- These experiments were done, but the experimental details and results were not reported in the publication or supporting information

- *Samples Prepared using Method B:*

  - Clear solution was observed without any precipitation
  - Samples showed a definite trend for aggregation (SI Fig 3, 4)
  - Due to small volume of water added a homogeneous system was achieved
  - Control experiment (without avidin) showed no aggregation due to addition of water (SI Fig 5)
  - **Sample preparation for control experiments**: 99 µL of **1** or **2** (1.5 mM in 50% dichloromethane-methanol) was added to 1µL water to make a 100 µL stock solution for control.

    We found that,

  - The aggregation behavior attributed solely due to avidin
  - Issues related to solubility of conjugates in the final solvent system were addressed
  - Denaturing of avidin was not likely as rapid formation of biotin-avidin complex will compete with stability and aggregation of avidin in an organic solvent[1]
  - This protocol was followed and these results were reported in the manuscript

**For BSA studies:** For these controlled experiments, protocols used for biotin-avidin interaction studies were followed replacing avidin with BSA. 10 µL aliquots of solutions from **Method B** were then placed on glass slide at room temperature and allowed to dry before viewing under cross polarizer's and imaging by **FESEM**.

**Control Experiment with Biotin:**

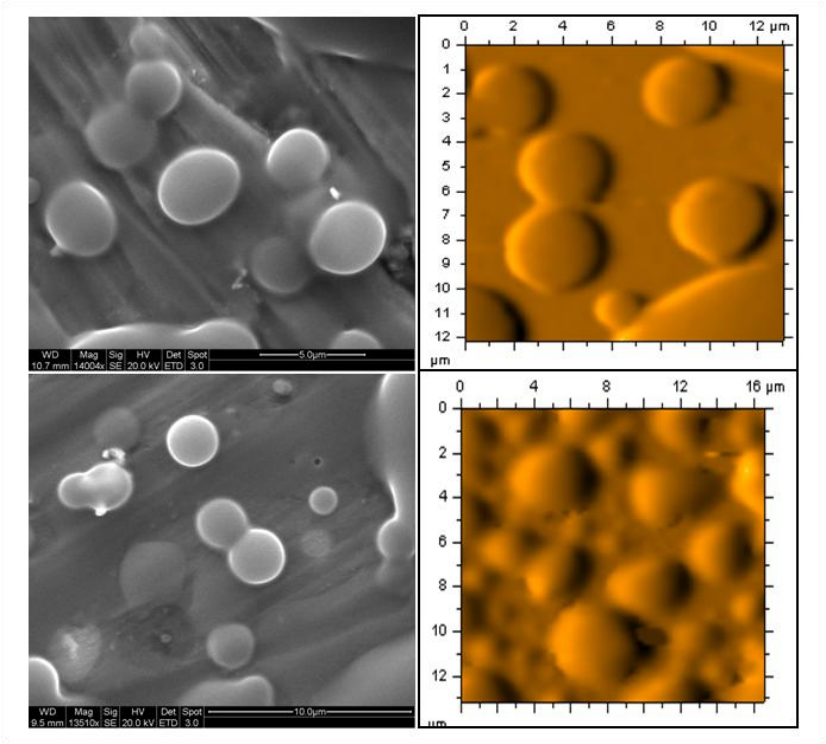
**Conception:** Verify if binding of avidin to biotin cholesterol conjugates is responsible for their loss of birefringence on aggregation. If an avidin solution is added to the solution of conjugates with no free biotin binding site then aggregation and loss of birefringence should not be observed.

Concentration range of Avidin solutions at which aggregation was observed: **0.075µM-0.0075µM**

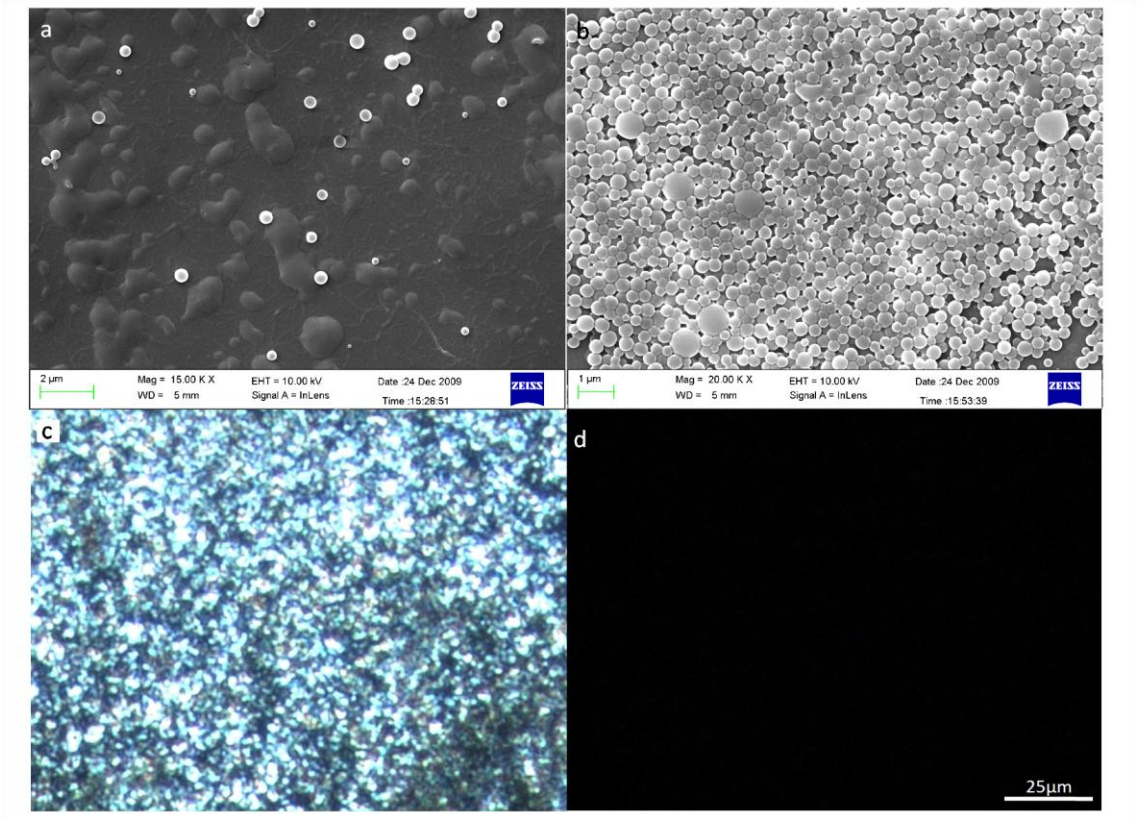
**Solutions Prepared:** 0.0075mM and 0.75µM solutions of avidin were prepared in water and 1.5mM solutions of **1** and **2** were prepared in 50% DCM-MeOH.

**Experiment:** According to the sample preparation protocol 1µL of avidin solution was added to 99µL of **1** and **2** to observe aggregation. However in this control experiment all the biotin binding sites of avidin were preoccupied with biotin before the addition. Initially 0.03mM and 0.003mM solutions of biotin were first prepared in water and then in eppendorf equal volumes of avidin and biotin solutions were mixed resulting in an avidin solution with its biotin binding sites now blocked. This solution was then added to solutions of **1** and **2**. As expected no aggregation or loss of birefringence was observed (**SI Fig 6**). The Maltese cross patterns of light extinction observed before addition were also observed after the addition of avidin solutions saturated with biotin. Therefore experiment conclusively proves that avidin binding to biotin cholesterol conjugates is responsible for aggregation and for loss of birefringence.

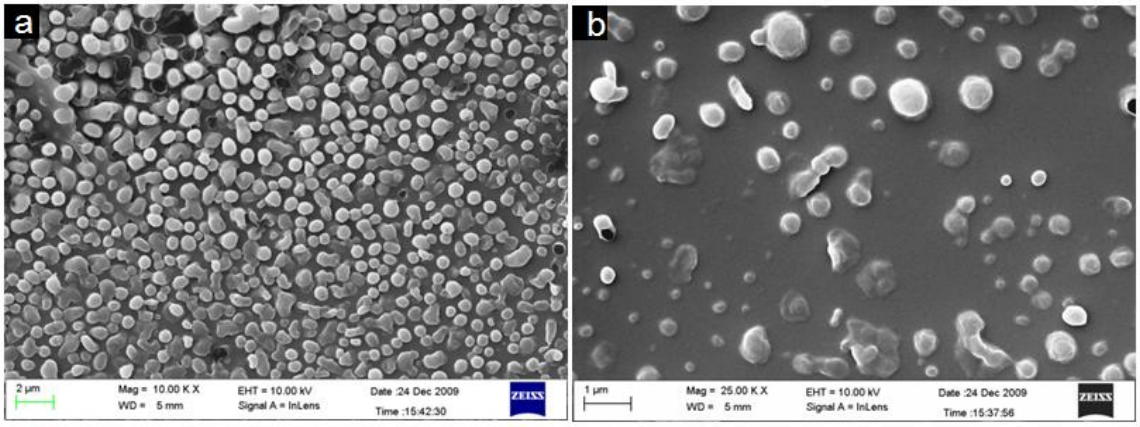
## Figures



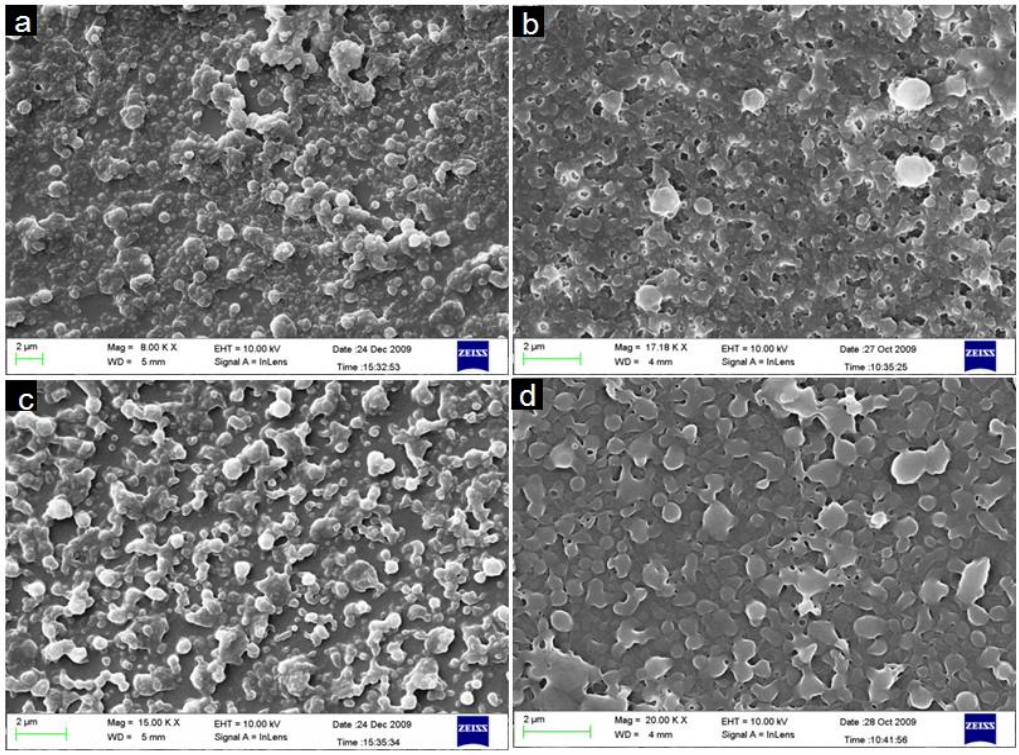
**SI Fig 1**: SEM and AFM images of 1.5mM solutions in 50% DCM-MeOH: of **1** (Top row) and **2** (Bottom row).



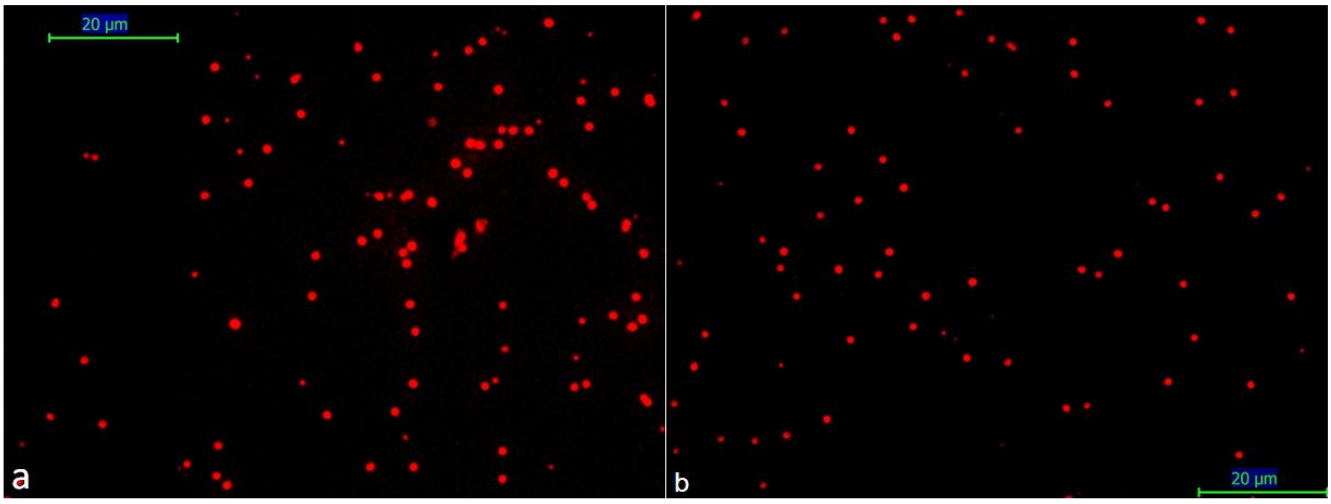

**SI Fig 2**: FE-SEM micrographs: a) **2** alone; b) Clustering of **2** on avidin addition; c) Optical microscopy images of aggregation on avidin addition; d) polarization optical microscopy images showing complete loss of birefringence on avidin addition between cross polarizer's



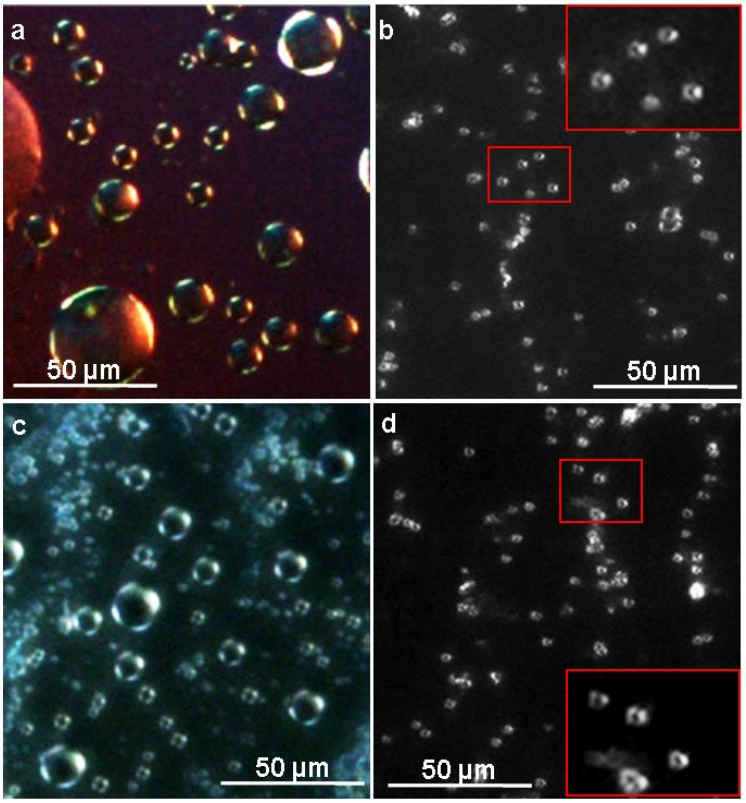

**SI Fig 3**: SEM images of aggregation at lower concentrations of avidin showing no definite aggregation a) at 0.75 nM avidin with **1**; b) at 0.75 nM avidin with **2**




**SI Fig 4**: SEM images of aggregation at higher concentrations of avidin showing pre formed aggregation a) at 0.0075mM avidin with **1**, b) at 0.0075mM avidin with **2**, c) at 0.75µM avidin with **1,** d) at 0.75µM avidin with **2**



**SI Fig 5:** Florescence optical microscope images with rhodamine B in control experiment with water: a) for **1** (1.5mM) in 50% DCM-MeOH on addition of water alone; b) for **2** (1.5mM) in 50% DCM-MeOH on addition of water alone.




**SI Fig 6:** (a) Maltese cross patterns of 1.5 mM solution of **1** in 50% MeOH-DCM between cross-polarizers; (b) Addition of avidin (with its biotin binding sites preoccupied with native biotin) to 1.5 mM solution of **1** in 50% MeOH-DCM between cross-polarizers; no change in the light extinction pattern is observed (inset shows the zoomed image of the Maltese cross patterns); (c) Maltese cross patterns of 1.5 mM solution of **2** in 50% MeOH-DCM between cross-polarizers; (d) Addition of avidin (with its biotin binding sites preoccupied with native biotin) to 1.5 mM solution of **2** in 50% MeOH-DCM between crossed polarizer's showing no change in the light extinction pattern, inset shows the zoomed image of the Maltese cross patterns.

# Spectra

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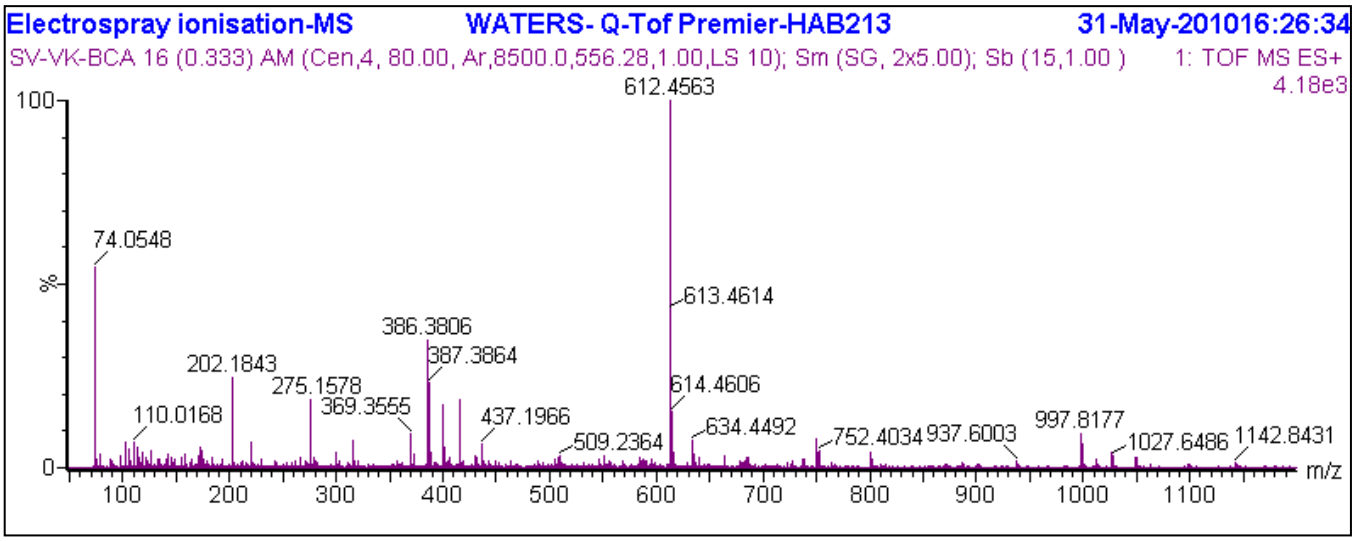
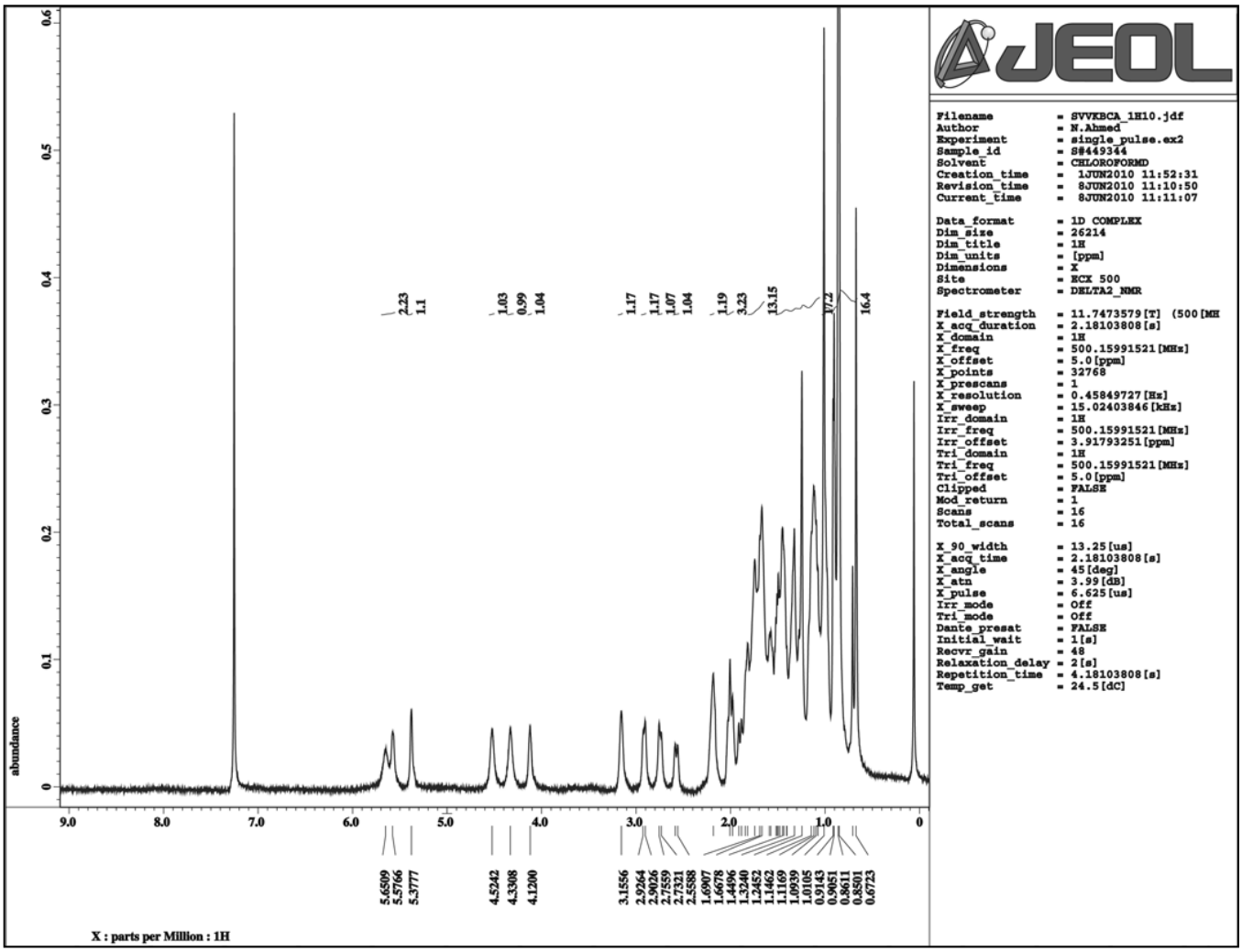


$^{1}$H NMR of **1**: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X : parts per Million : 1H
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1.1
1.03
0.99
1.04
1.17
1.17
1.07
1.04
1.19
3.23
13.15
17.2
16.4
Filename = SVVKBCA_1H10.jdf
Author = N.Ahmed
Experiment = single_pulse.ex2
Sample_id = S#449344
Solvent = CHLOROFORMD
Creation_time = 1JUN2010 11:52:31
Revision_time = 8JUN2010 11:10:50
Current_time = 8JUN2010 11:11:07
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Dim_units = [ppm]
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X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 32768
X_prescans = 1
X_resolution = 0.45849727[Hz]
X_sweep = 15.02403846[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 3.91793251[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 13.25[us]
X_acq_time = 2.18103808[s]
X_angle = 45[deg]
X_atn = 3.99[dB]
X_pulse = 6.625[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
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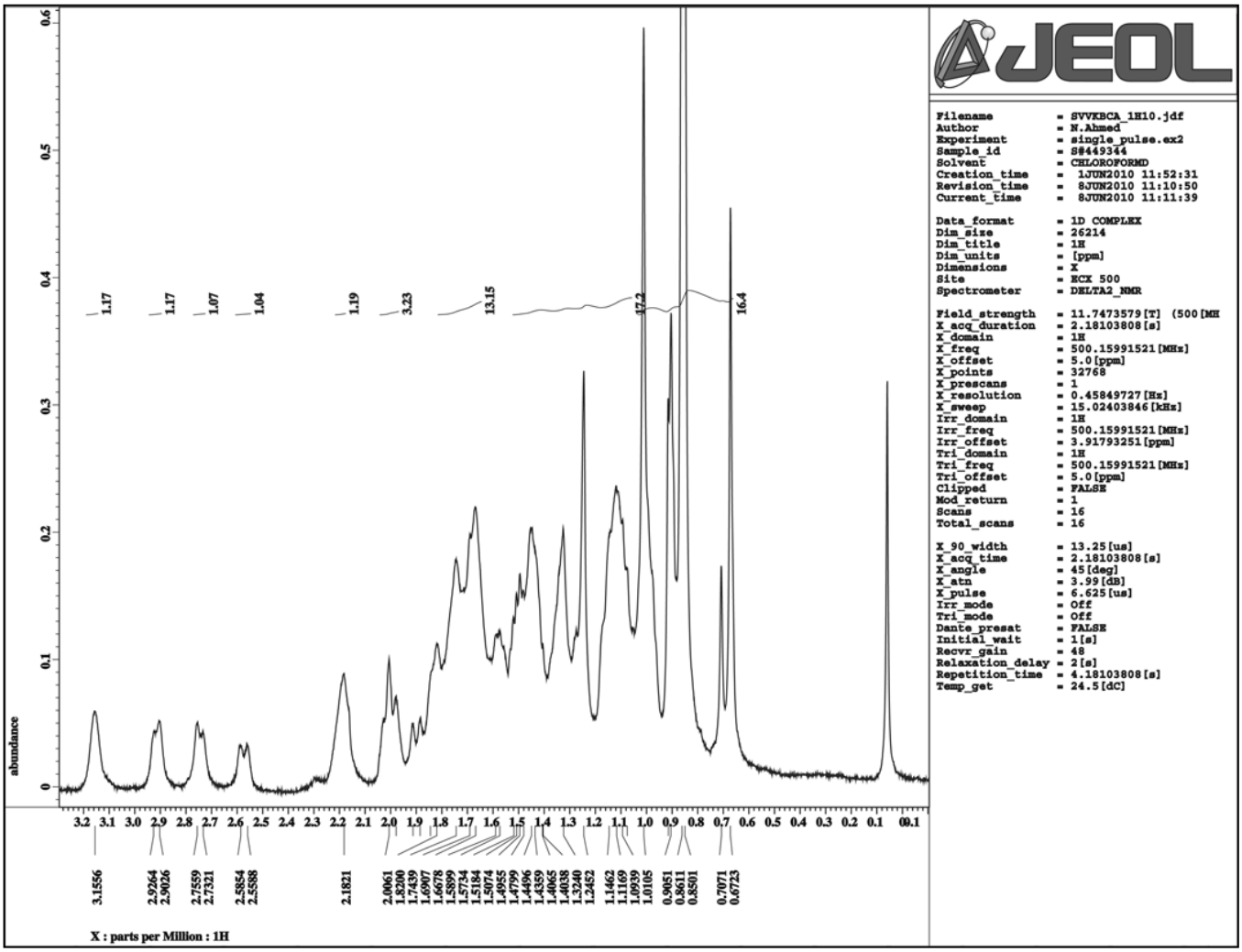
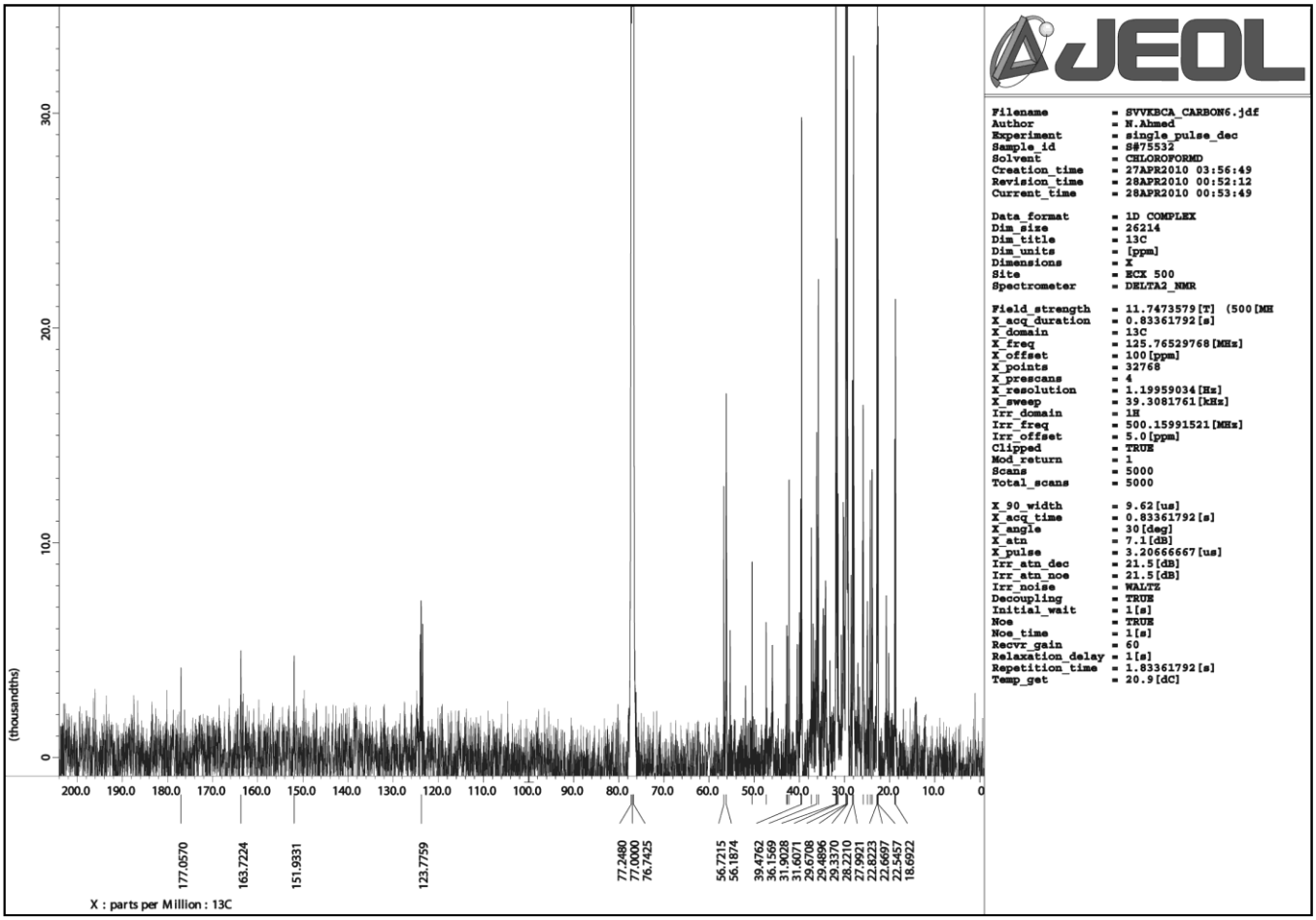


$^{13}$C NMR of **1**: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10.0
0
200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0 0
177.0570
163.7224
151.9331
123.7759
77.2480
77.0000
76.7425
56.7215
56.1874
39.4762
36.1569
31.9028
31.6071
29.6708
29.4896
29.3370
28.2210
27.9921
22.6623
22.6607
22.5457
18.6922
X : parts per Million : 13C
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Author = N.Ahmed
Experiment = single_pulse_dec
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Solvent = CHLOROFORMD
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Revision_time = 28APR2010 00:52:12
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X_offset = 100[ppm]
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X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
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Irr_offset = 5.0[ppm]
Clipped = TRUE
Mod_return = 1
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X_acq_time = 0.83361792[s]
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X_pulse = 3.20666667[us]
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Irr_atn_noe = 21.5[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 1[s]
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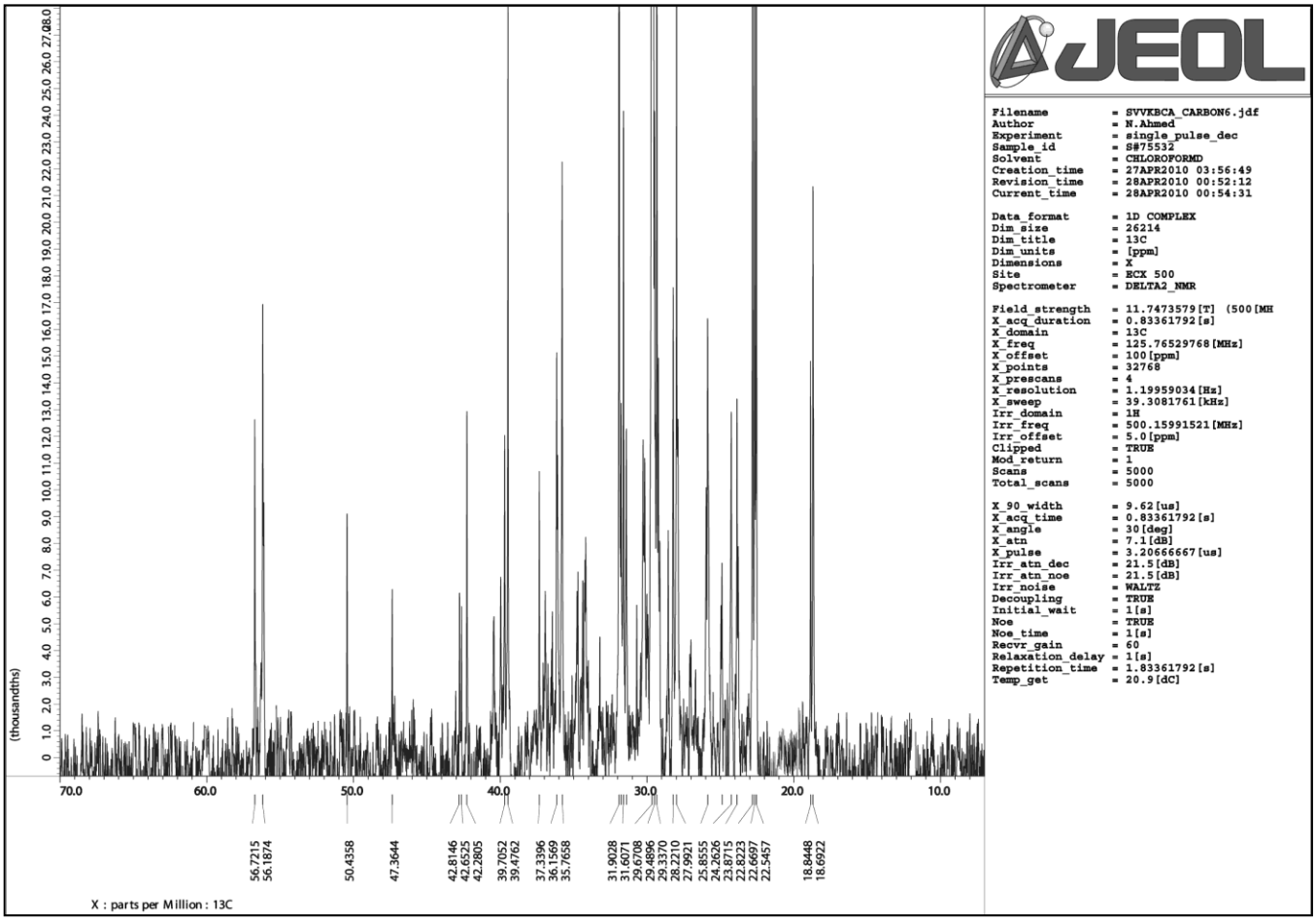
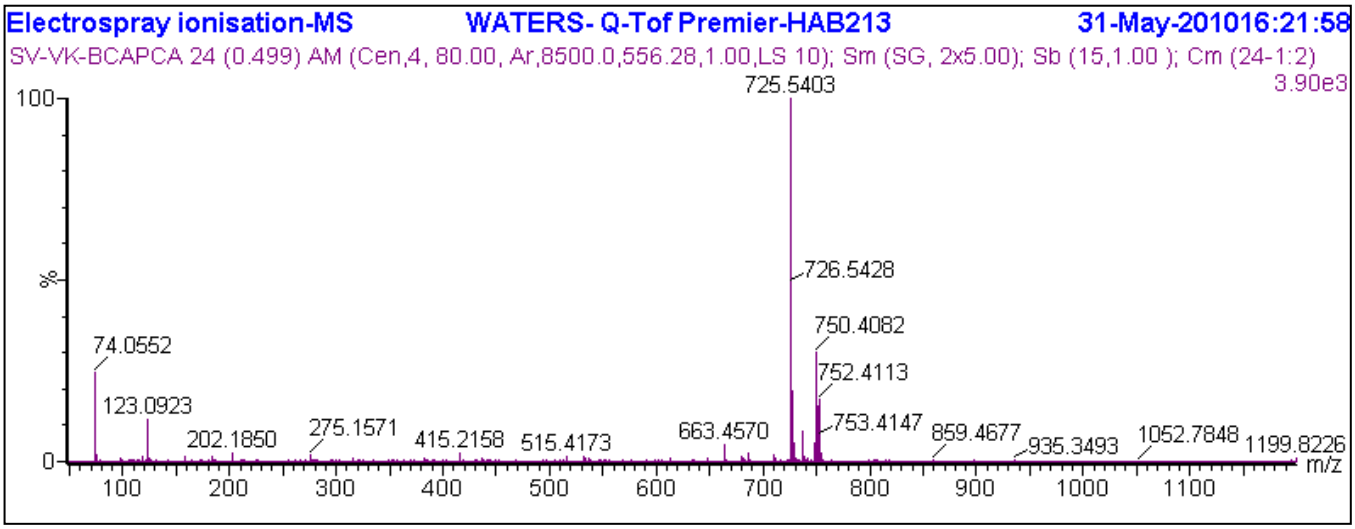
$^{13}$C NMR of **1 (Expanded)**:



HRMS of **2**: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31-May-201016:21:58
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100
%
0
725.5403
726.5428
750.4082
752.4113
753.4147
74.0552
123.0923
202.1850
275.1571
415.2158
515.4173
663.4570
859.4677
935.3493
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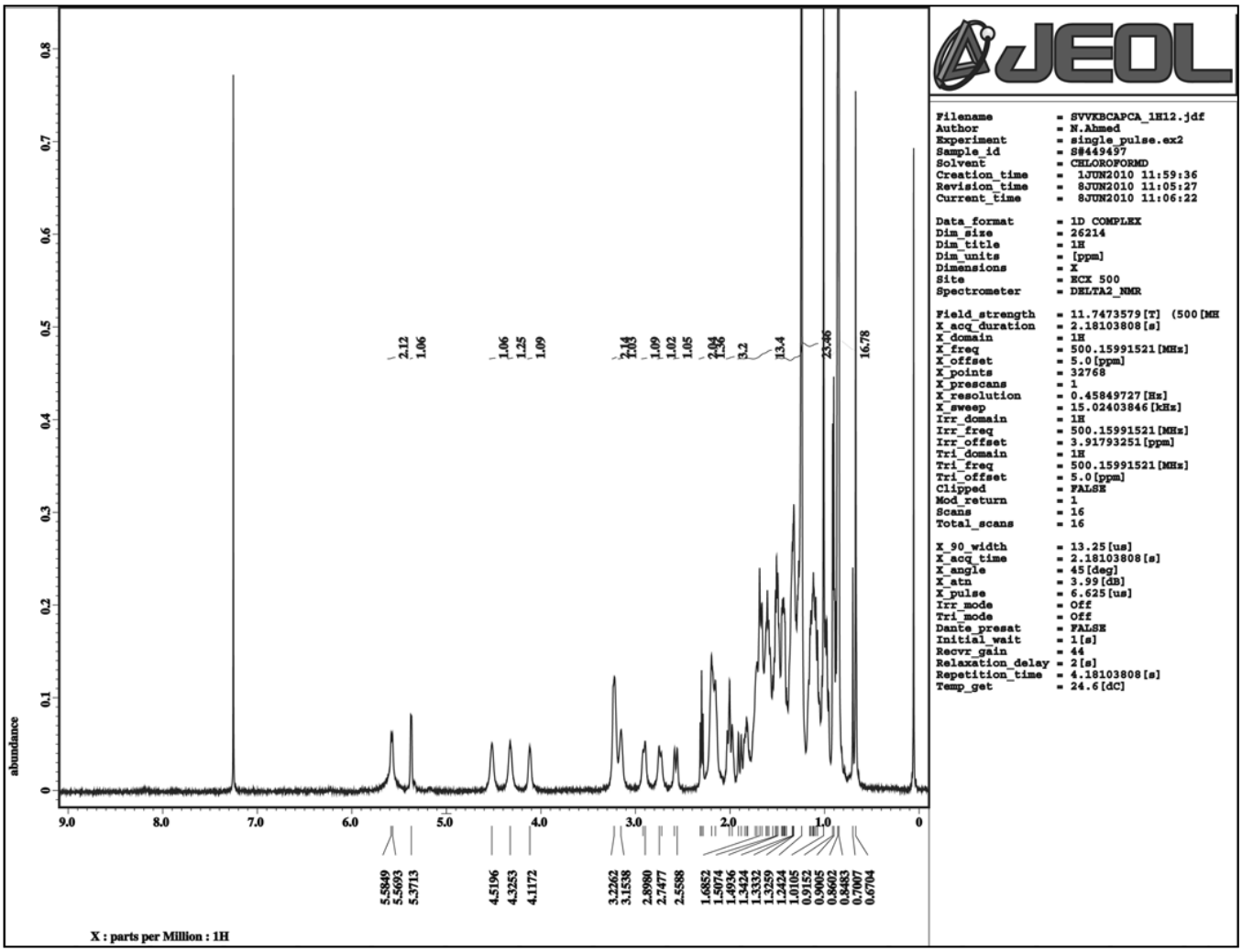
m/z
100
200
300
400
500
600
700
800
900
1000
1100

$^{1}$H NMR of **2**: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X : parts per Million : 1H
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1.06
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5.5693
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4.3253
4.1172
3.2262
3.1538
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2.5588
1.6852
1.5074
1.4936
1.3424
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Author = N.Ahmed
Experiment = single_pulse.ex2
Sample_id = S#449497
Solvent = CHLOROFORMD
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Revision_time = 8JUN2010 11:05:27
Current_time = 8JUN2010 11:06:22
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X_sweep = 15.02403846[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 3.91793251[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
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X_acq_time = 2.18103808[s]
X_angle = 45[deg]
X_atn = 3.99[dB]
X_pulse = 6.625[us]
Irr_mode = Off
Tri_mode = Off
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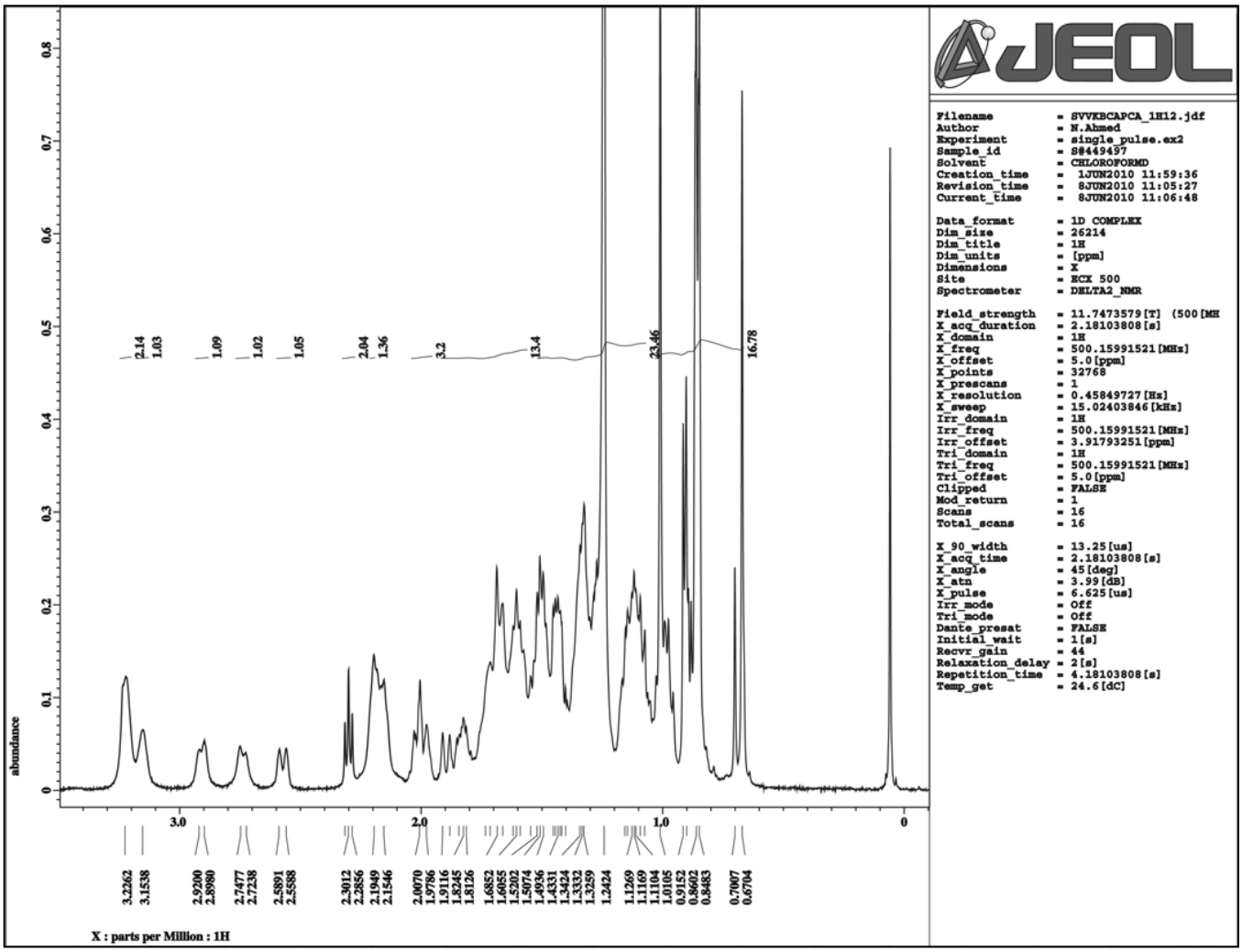
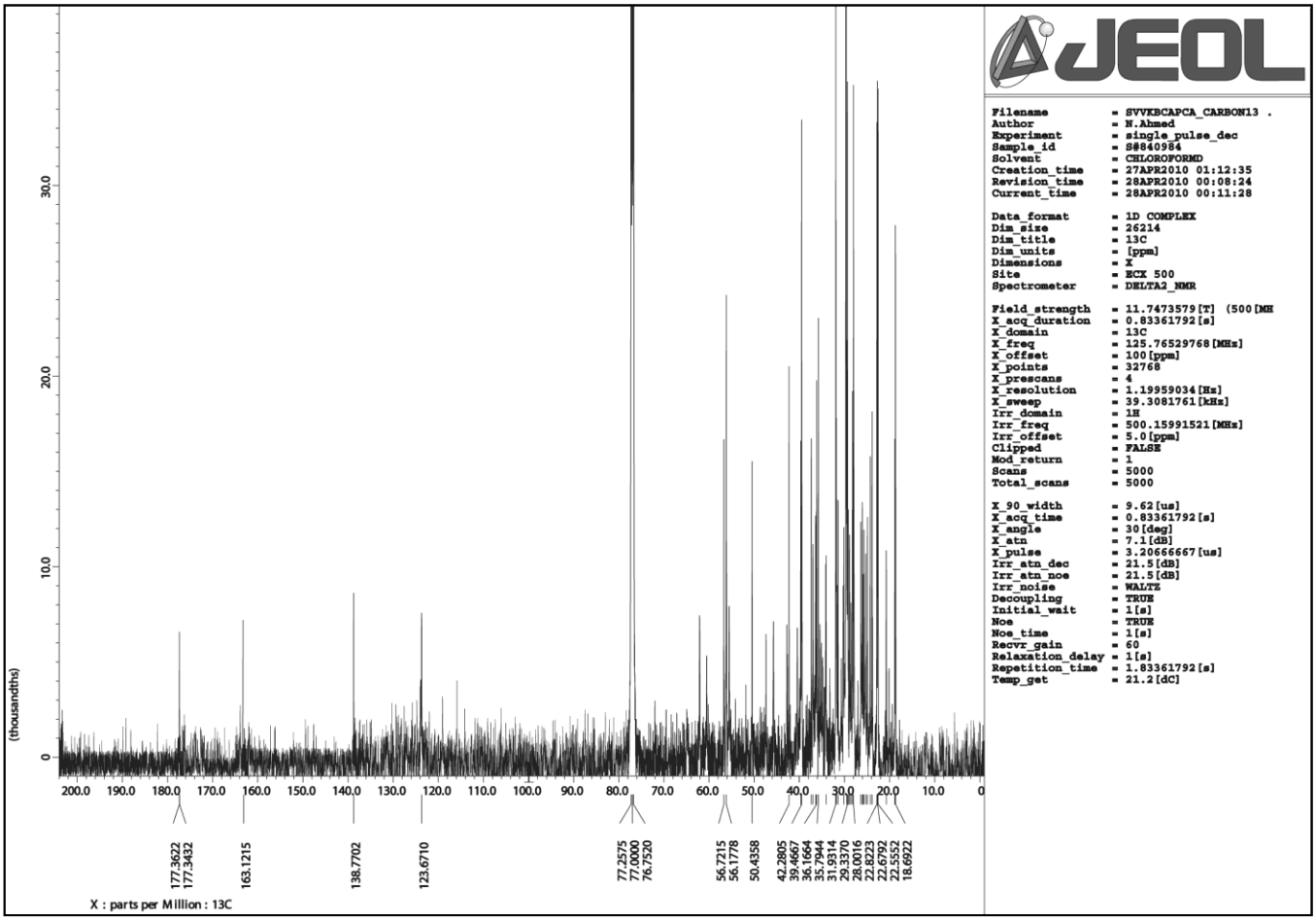
$^{1}$H NMR of **2(Expanded)**:



$^{13}$C NMR of **2**:


(thousandths)
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20.0
10.0
0
200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0 0
177.3622
177.3432
163.1215
138.7702
123.6710
77.2575
77.0000
76.7520
56.7215
56.1778
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42.2805
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X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 5000
Total_scans = 5000
X_90_width = 9.62[us]
X_acq_time = 0.83361792[s]
X_angle = 30[deg]
X_atn = 7.1[dB]
X_pulse = 3.20666667[us]
Irr_atn_dec = 21.5[dB]
Irr_atn_noe = 21.5[dB]
Irr_noise = WALTZ
Decoupling = TRUE
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Noe = TRUE
Noe_time = 1[s]
Recvr_gain = 60
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Temp_get = 21.2[dC]

$^{13}$ NMR of **2 (Expanded)**: