

Supporting Information:

Synergistic Induction of Apoptosis in Brain Cancer Cells by Targeted Co-delivery of siRNA and Anti-cancer drugs

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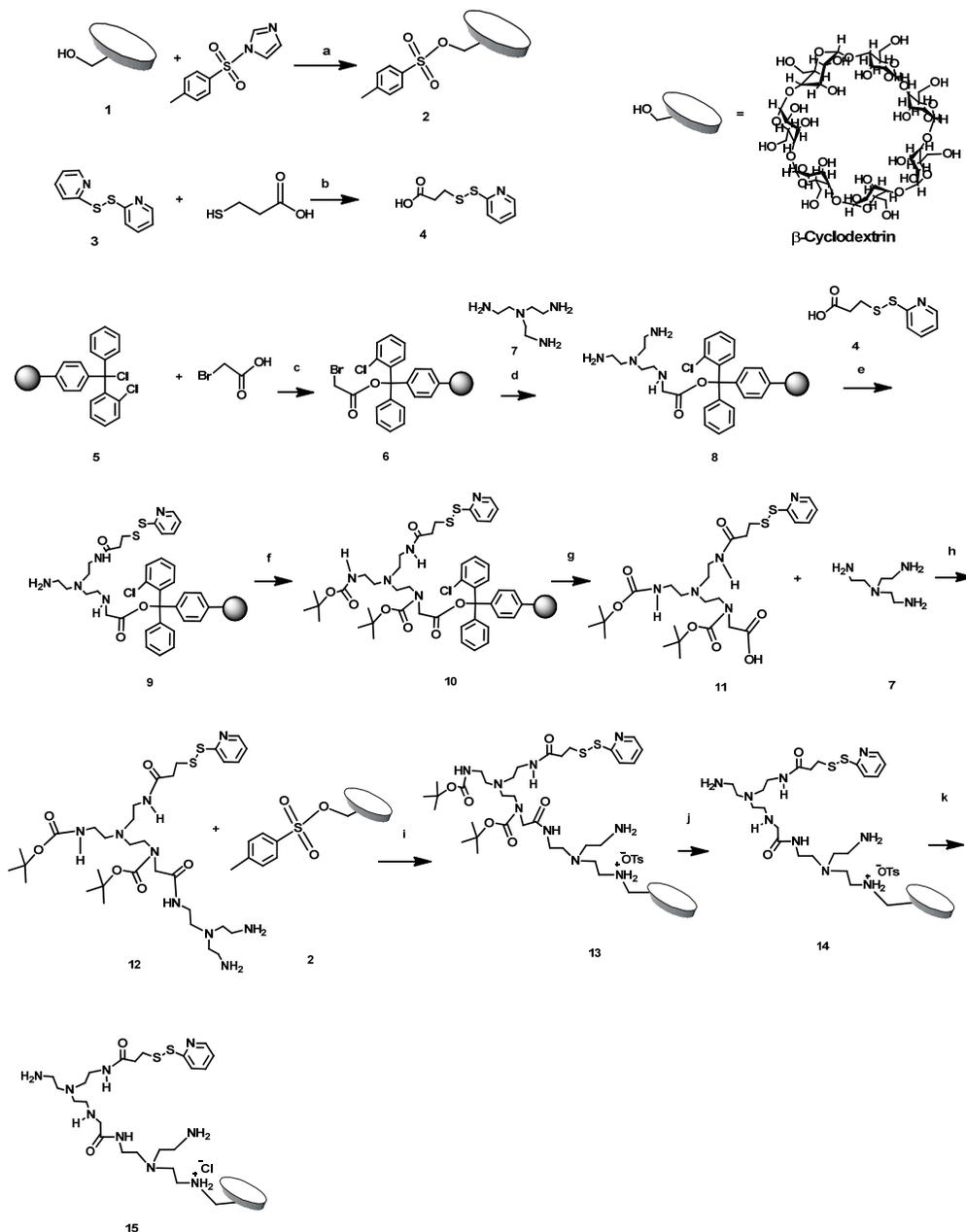
[†] These authors contributed equally to this work.

General:

β -cyclodextrin, tosylimidazole, di-*tert*-butyl dicarbonate, tris(aminoethyl)amine, methyl acrylate, 6, 7-dimethoxyquinazalone, aniline, amberlite IRA 900 were obtained from Sigma-Aldrich and used as received unless otherwise noted. 6-hydrazinonicotinamide and 4-formylbenzamide were from SoluLink. Other chemicals and solvents were of analytical reagent grade. All reactions were conducted in flame-dried glassware with magnetic stirring under an atmosphere of dry nitrogen. Reaction progress was monitored by analytical thin layer chromatography (TLC) using 250 μ m silica gel plates (Dynamic Absorbents F-254). Visualization was accomplished with UV light and potassium permanganate stain, followed by heating. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on either a Varian-300 instrument (300 MHz), Varian-400 instrument (400 MHz) or a Varian-500 instrument (500 MHz). Chemical shifts of the compounds are reported in ppm relative to tetramethylsilane (TMS) as the internal standard. Data are reported as follows: chemical shift, integration, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, br=broad, m=multiplet), and coupling constants (Hz).

Methods

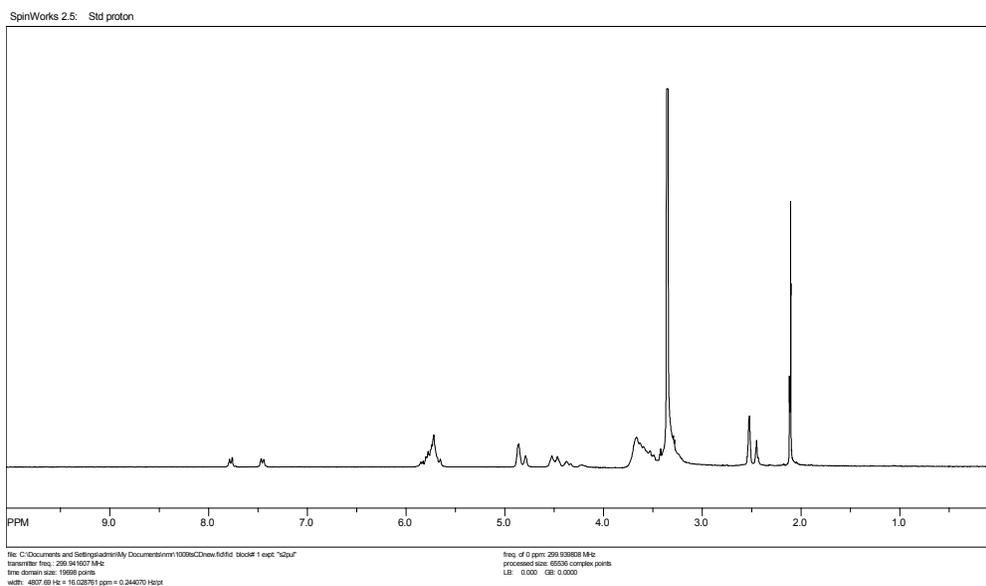
1. Synthesis of DexAM 1



Scheme 1. Synthesis of DeXAM 1. a) ddH₂O, RT, 4h / 1 % NaOH, 10min/NH₄Cl, b) AcOH, EtOH, RT, 12h, c) DIEA, DCM; d) DMF, RT, 2 h, e) DCC, DMAP, DCM, 0°C, 30 min/DMF, RT, 10 h, f) Di-*tert*-butyl dicarbonate, DIEA, DCM, RT, 24 h, g) CF₃CH₂OH, DCM; h) DCC, DMAP, DMSO, 0°C, 30 min/RT, 24h, i) DMF, 90°C, 48 h, j) TFA:DCM (50:50 v/v); k) Amberlite IRA 900

1.1 Synthesis of mono-tosylated cyclodextrin (**2**)

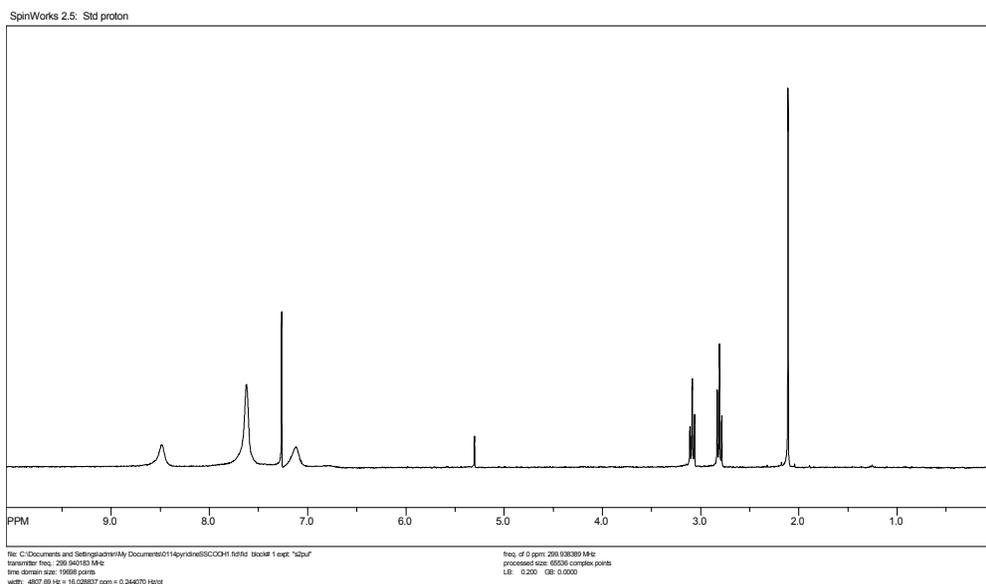
β -cyclodextrin (8.75 g, 7.71 mmol) and tosylimidazole (2.22g, 10.0 mmol) was dissolved in 88 ml deionized water. The solution was vigorously stirred for 4 h at room temperature. Aqueous NaOH solution (1% (w/v), 10.0 ml) was gradually added to the solution and stirred for an additional 10 min. The insoluble solid was filtered off and the filtrate was collected. The filtrate was neutralized to pH 7 using NH_4Cl to induce precipitation. The precipitate was then collected by filtration, washed with cold water (25 ml \times 3) and with acetone (25 ml \times 4). The solid was dried in a drying oven at 60 °C under vacuum (10 mm Hg) overnight to yield **2** as a white solid (4.5 g, 51% yield). ^1H NMR (300 MHz, DMSO- d_6), δ 7.72 (d, $J=8.4$ Hz, 2H), 7.41 (d, $J = 8.4$ Hz, 2H), 5.60–5.89 (m, 14H), 4.75–4.81 (m, 7H), 4.15–4.62 (m, 6H), 3.45–3.72 (m, 28H), 3.15–3.47 (m, 24H), 2.41 (s, 3H). MS (m/z): calculated, 1,288.4 for $\text{C}_{49}\text{H}_{76}\text{O}_{37}\text{S}$; found, 1,311.5 for $[\text{M} + \text{Na}]^+$.



1.2 Synthesis of 2-pyridyl-2-carboxyethyl disulfide (**4**)

2,2'-Bipyridyl disulfide (**3**, 1 g, 4.54 mmol) was dissolved in 15 mL of ethanol (99.5%) followed by addition of 0.4 mL of glacial acetic acid. The solution was vigorously stirred and 0.24 g (2.27 mmol) of 3-mercaptopropionic acid in 5 mL of ethanol was added dropwise. The reaction mixture was stirred at room temperature for 12 h. The excess solvent was then removed under reduced pressure. The resulting oily product mixture was dissolved in 3 mL of hexane/ether (80:20, v/v %). The product was purified by column chromatography using silica gel. The

pyridine-2-thione eluted as a yellow band. The desired product (**4**, 2-pyridyl-2-carboxyethyl disulfide) was collected, and the solvent was removed by evaporation. The residual acetic acid was removed under high vacuum. Isolated yield = 0.4 g (82.2%, based on 0.24 g of the 3-mercaptopropionic acid starting material). $^1\text{H NMR}$ (300 MHz, CDCl_3), δ 2.68–2.73 (t, $J = 7.20$ Hz, 2H), 3.02–3.06 (t, $J = 6.90$ Hz, 2H), 7.20–7.25 (t, $J = 5.0$ Hz, 1H), 7.78–7.87 (t, $J = 7.2$ Hz, 2H), 8.39–8.41 (d, $J = 4.8$ Hz, 1H). MS (m/z): calculated, 215.01 for $\text{C}_8\text{H}_9\text{NO}_2\text{S}_2$; found, 238.28 for $[\text{M} + \text{Na}]^+$.



1.3 Anchorage of the Acidic Function to the Polymeric Support

Briefly, *o*-chlorotrityl chloride resin (**5**, 5 g, 1.2 mmol of Cl/g of resin from Fisher Chemicals) was placed in the solid-phase synthesis vessel (100 mL) and 50 mL of CH_2Cl_2 was added, followed by bromoacetic acid (1.05 g, 7 mmol) and DIEA (0.95 mL, 7.5 mmol). The flask was shaken on a horizontal gyratory shaker for 3 h at room temperature. The solution was filtered and the functionalized resin beads (**6**) were washed three times with CH_2Cl_2 , *i*PrOH and MeOH followed by drying under a stream of nitrogen.

1.4 Reaction of the Polyamine with the Bromoacetyl Resins (**8**)

Tris(2-aminoethyl)amine (**7**, 10-fold molar excess) were dissolved in 50 mL of DMF, added to the vessel containing the functionalized resin beads (**6**) and shaken for 2 h. The reaction was monitored using the ninhydrin test. The product (**8**) was filtered, washed with CH_2Cl_2 and *i*PrOH (20 mL each) and then finally washed with CH_2Cl_2 .

1.5 Attachment of a disulfide linker to the polyamine on the resin (**9**)

To a suspension of pyridyl-2-carboxyethyl disulfide (**4**, 645.87mg, 3.00 mmol) in CH₂Cl₂ (15 mL), DCC (928.485mg, 4.50 mmol, 1.5 eq) and dimethylamino pyridine (36.65 mg, 0.3mmol, 0.1 eq) were added at 0°C and stirred at the same temperature for 30 min. This mixture was added to the vessel containing **8** and shaken for 10 h at room temperature.

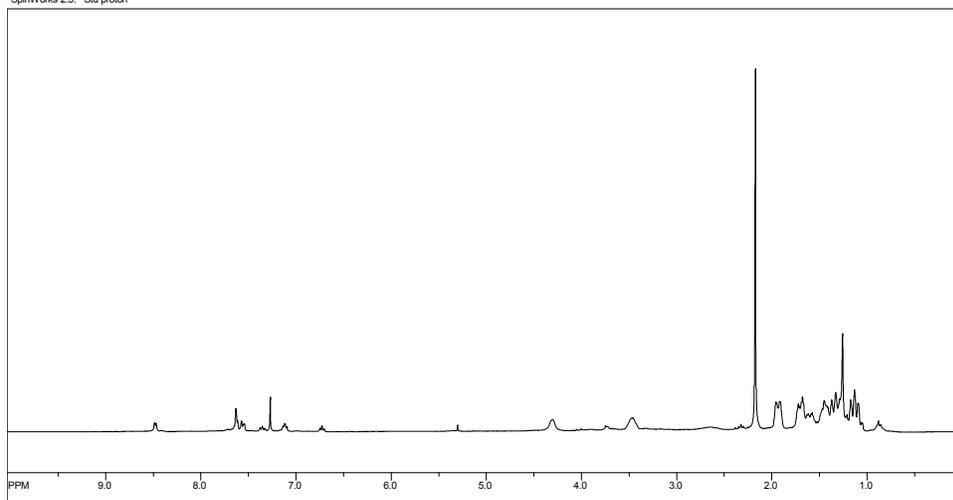
1.6 Protection of the amine groups of the functionalized polyamine on the solid support (**10**)

Di-*tert*-butyl dicarbonate (5.51mL, 24 mmol) and DIEA (4.35mL, 25 mmol) were dissolved in CH₂Cl₂ (15 mL) and added to **9**; the reaction was left overnight at room temperature under shaking. The product (**10**) was filtered, washed with CH₂Cl₂, *i*PrOH and MeOH and finally dried under a stream of nitrogen. The protection of the amine groups was confirmed using the ninhydrin test, which was found to be negative.

1.7 Synthesis of 11-(*tert*-butoxycarbonyl)-2,2-dimethyl-4-oxo-8-(2-(3-(pyridin-2-yl)disulfanyl)propanamido)ethyl)-3-oxa-5,8,11-triazatridecan-13-oic acid (**11**)

The Boc-protected resin (**10**) was placed in a 100 mL solid phase vessel. A solution containing 25 mL of dichloromethane and 25 mL of CF₃COOH was added to it and shaken for 2 h at room temperature. The solution was filtered and the resin washed with 100 mL of CH₂Cl₂. The organic fractions were collected and the solvent evaporated. The crude products were purified by flash chromatography on SiO₂. The fractions containing the products were identified by TLC and characterized using mass spectroscopy and NMR. TLC (CH₂Cl₂:MeOH, 90:10 v/v): *R*_f = 0.65; ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.3 (s, 18H), 2.32 (m, *J* = 6.5 Hz, 8H), 2.66 (t, *J* = 7 Hz, 2H), 3.46 (m, *J* = 7 Hz, 4H), 3.74 (t, 2H), 4.3 (s, 2H), 7.10–7.20 (t, *J* = 5.0 Hz, 1H), 7.26 (t, *J* = 5.0 Hz, 1H), 7.60–7.68 (t, *J* = 7.2 Hz, 2H), 8.44–8.56 (d, *J* = 4.8 Hz, 1H). MS (*m/z*): calculated, 601.26 for C₂₆H₄₃N₅O₇S₂; found, 624.77 for [M + Na]⁺.

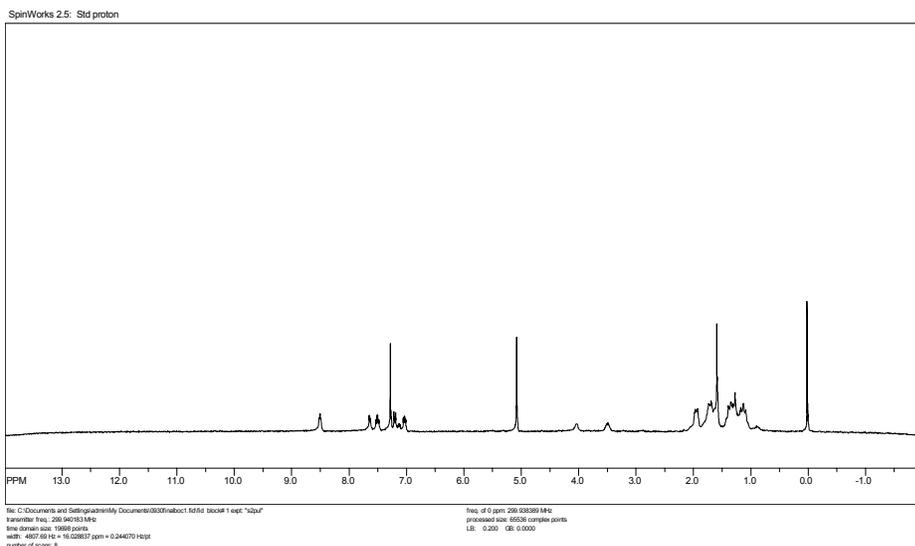
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width: 4807.69 Hz = 16.028837 ppm = 0.244070 Hz/ppm
number of scans: 8
freq. of 0 ppm: 200.938384 MHz
processed size: 65536 complex points
L18: 0.000 000 0.000000

1.8 DCC coupling

To **11** (601.78mg, 1.00 mmol) in DMSO (10 mL), DCC (309.495mg, 1.50 mmol, 1.5 eq) and DMAP (0.1 eq, 12.22 mg) were added at 0°C and stirred for 30 mins. Tris-(aminoethyl)amine (**7**) was added dropwise to the solution and stirred for 30 mins. The mixture was then allowed to stir for 24 h at room temperature. DMSO was then removed under reduced pressure. The product (**12**) was purified by column chromatography using silica gel. Yield: 80%; TLC (CHCl₃:MeOH, 90:10 v/v): *R_f*=0.4; ¹H NMR (400 MHz, DMSO-*d*₆ δ 1.0-1.4 (m, 4H), 1.5 (s, 18H), 1.8 (m, *J* = 6.5 Hz, 14H), 2.00 (m, *J* = 7 Hz, 8H), 3.5 (m, *J* = 7 Hz, 6H), 4.0 (s, 2H), 7.10–7.20 (t, *J* = 5.0 Hz, 1H), 7.26 (t, *J* = 5.0 Hz, 1H), 7.60–7.68 (t, *J* = 7.2 Hz, 2H), 8.44–8.56 (d, *J* = 4.8 Hz, 1H). MS (*m/z*): calculated, 729.40 for C₃₂H₅₉N₉O₆S₂; found, 752.99 for [M + Na]⁺.

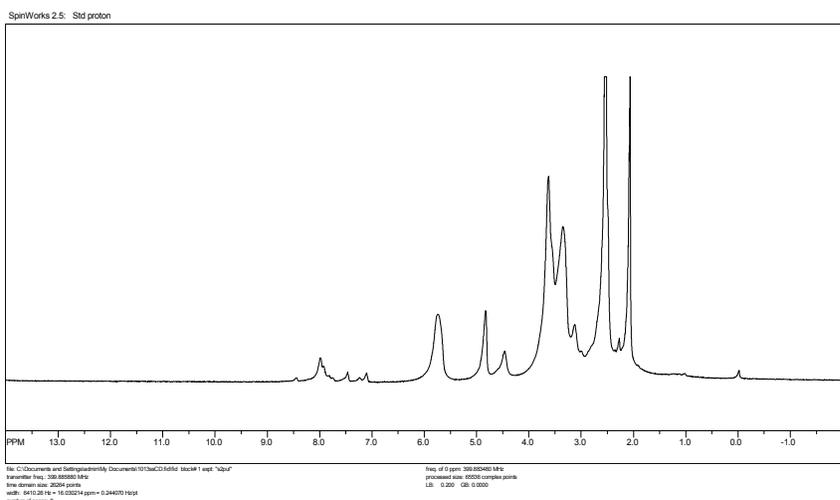


1.9 Synthesis of water-soluble CD polyamine (**13**)

Tosylated CD (**2**, 1.29 g, 1 mmol) and the Boc-protected polyamine (**12**, 730mg, 1 mmol) were dissolved in DMF (10 mL) in a 25-mL one-necked round-bottomed flask, equipped with a Liebig's condenser and a stir bar. The flask was degassed and purged with nitrogen. The mixture was stirred and refluxed at 90 °C for 48 h. The reaction mixture was cooled down to room temperature and the product was precipitated out by the addition of acetone (20 ml). The precipitate was collected by filtration, washed with acetone and dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **13** as a brown solid (1.3g, 91% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.0-1.4 (m, 4H), 1.5 (s, 18H), 1.8 (m, *J* = 6.5 Hz, 14H), 2.00 (m, *J* = 7 Hz, 8H), 3.15–3.47 (m, 24H), 3.45–3.72 (m, 28H), 3.5 (m, *J* = 7 Hz, 6H), 4.0 (s, 2H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.10–7.20 (t, *J* = 5.0 Hz, 1H), 7.26 (t, *J* = 5.0 Hz, 1H), 7.2 (d, *J* = 8.4, 2H), 7.5 (d, *J* = 8.4, 2H), 7.60–7.68 (t, *J* = 7.2 Hz, 2H), 8.44–8.56 (d, *J* = 4.8 Hz, 1H).

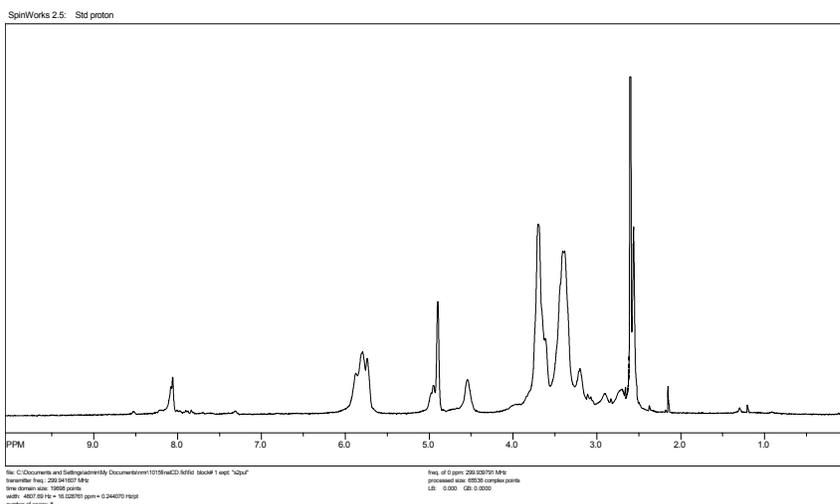
1.10 Deprotection of *N*-Boc amines by TFA (**14**)

The Boc-protected products (**13**) were deprotected using trifluoroacetic acid:DCM (1:1) for 1 h. The solvent was evaporated and the solid washed with acetone. The solid was dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **14** as a brown solid (1.2g, 95% yield). ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.0-1.4 (m, 4H), 1.8 (m, *J* = 6.5 Hz, 14H), 2.00 (m, *J* = 7 Hz, 8H), 3.15–3.47 (m, 24H), 3.45–3.72 (m, 28H), 3.5 (m, *J* = 7 Hz, 6H), 4.0 (s, 2H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.10–7.20 (t, *J* = 5.0 Hz, 1H), 7.26 (t, *J* = 5.0 Hz, 1H), 7.2 (d, *J* = 8.4, 2H), 7.5 (d, *J* = 8.4, 2H), 7.60–7.68 (t, *J* = 7.2 Hz, 2H), 8.44–8.56 (d, *J* = 4.8 Hz, 1H). MH+ 1819.94.

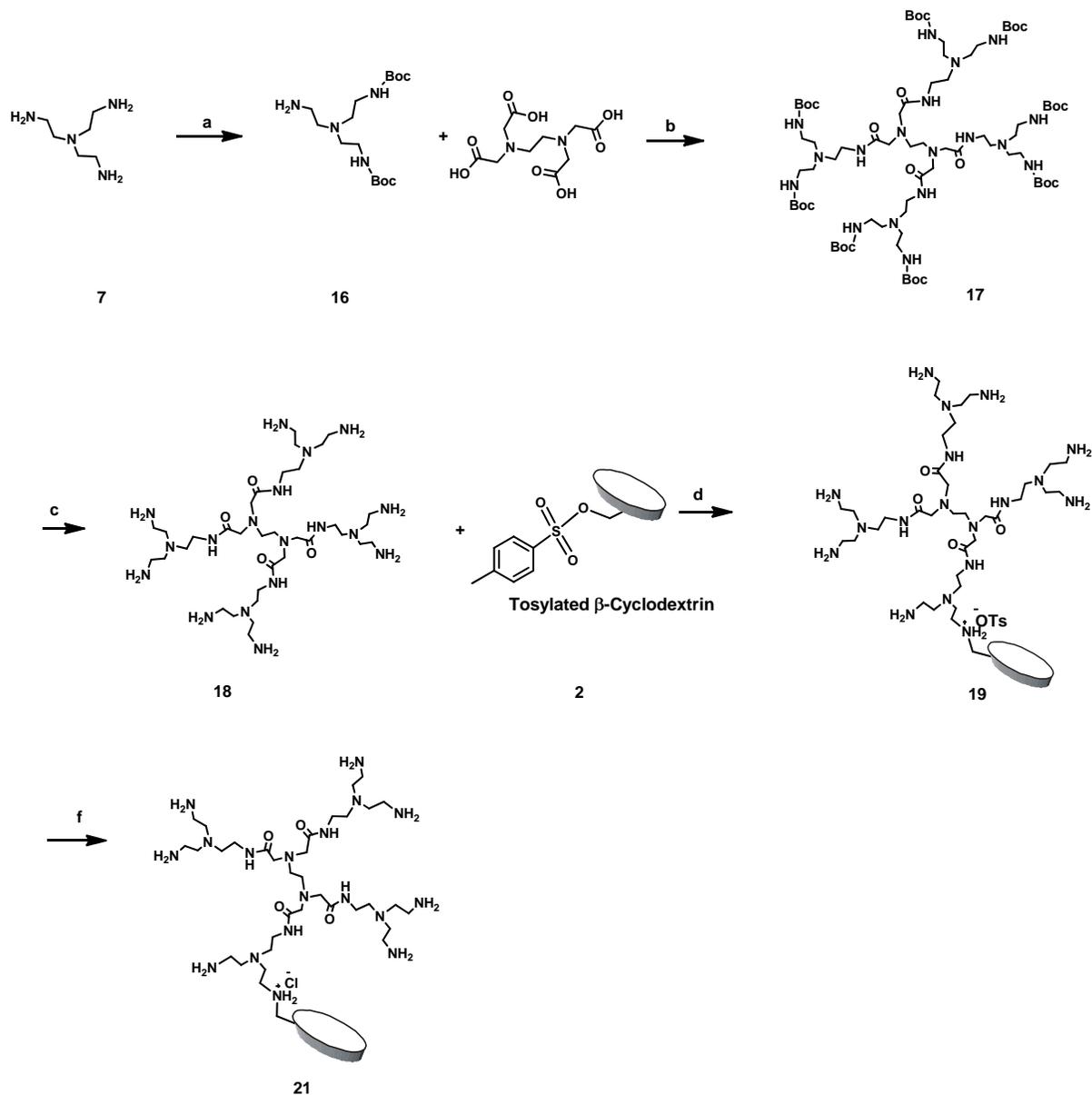


1.11 Anion exchange reaction (**15**)

CD Polyamine tosylate (**14**, 1.4 g, 1 mmol) was dissolved in 40 mL deionized water. A 50 mL solid-phase synthesis vessel was packed with Amberlite IRA-900 ion-exchange resin to about half the vessel volume. The solution was transferred into the solid phase vessel. After 1 h, the eluent was collected and the water distilled off under reduced pressure using a vacuum pump. The solid residue was dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **15** as a brown solid. Yield: (1.15g, 95% yield); ¹H NMR (400 MHz, DMSO-*d*₆) δ 1.0-1.4 (m, 4H), 1.8 (m, *J* = 6.5 Hz, 14H), 2.00 (m, *J* = 7 Hz, 8H), 3.15–3.47 (m, 24H), 3.45–3.72 (m, 28H), 3.5 (m, *J* = 7 Hz, 6H), 4.0 (s, 2H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.10–7.20 (t, *J* = 5.0 Hz, 1H), 7.26(t, *J* = 5.0 Hz, 1H), 7.60–7.68 (t, *J* = 7.2 Hz, 2H), 8.44–8.56 (d, *J* = 4.8 Hz, 1H). MH+ 1684.20.



2. Synthesis of DexAM 2

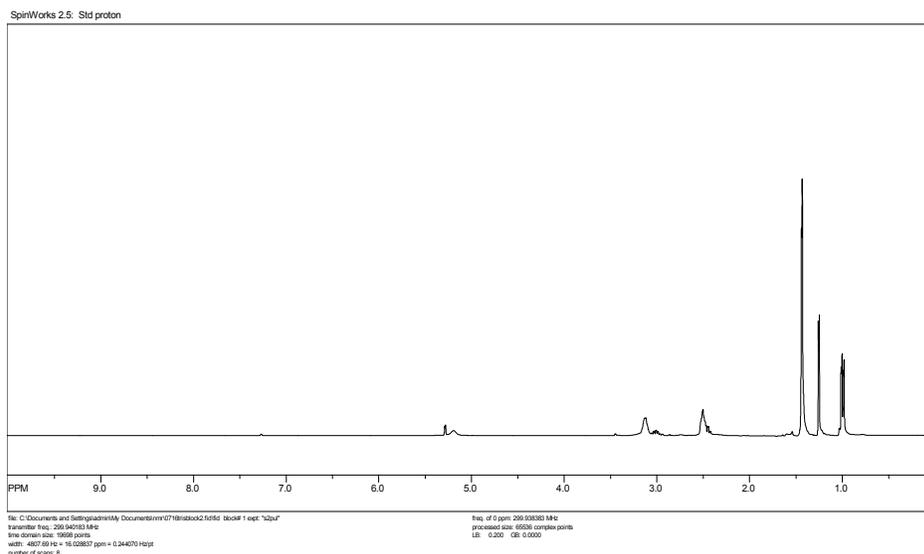


Scheme 2: Synthesis of DexAM 2. a) DCC, DMAP, DCM, MeOH, b) DCC, DMAP, DCM, MeOH, c) TFA:DCM (50:50 v/v), d) DMF, 90°C, 48h, e) DMSO, RT, 24 h, f) Amberlite IRA900

2.1 Bis-[2-(tert-butoxycarbonylamino)ethyl]-(2-aminoethyl)amine (**16**)

Tris(2-aminoethyl)amine (**7**, 14.6g, 100mmol) was dissolved in 40 mL of dry CH_2Cl_2 and cooled to 0 °C. A solution of di-*tert*-butyl dicarbonate (44.11mL, 200 mmol) in 50 mL dry CH_2Cl_2 was added dropwise over 1h. The mixture was stirred at room temperature for 24h. After removal of the solvent under reduced pressure, the remaining yellow oil was dissolved in ethyl acetate and

washed twice with 0.5 N NaOH. The aqueous phase was diluted with brine and re-extracted with ethyl acetate. The combined organic phases were dried over MgSO₄ and the solvent removed under reduced pressure. The crude products were purified by flash chromatography on SiO₂. The fractions containing the desired product were identified by TLC and characterized by mass spectroscopy and NMR. The product (**16**) was isolated as a pale-yellow oil. Yield: (18.02 g, 52%); TLC (CH₂Cl₂:MeOH, 90:10 v/v): *R_f* = 0.65; ¹H-NMR (DMSO-*d*₆) δ 1.37 (s, 18 H), 2.38-2.44 (m, 6 H), 2.49-2.54 (m, 2 H), 2.95 (d, 4 H), 5.30 (s, 2 H). MS (m/z): calculated, 346.26 for C₁₆H₃₄N₄O₄; found, 369.46 for [M + Na]⁺.



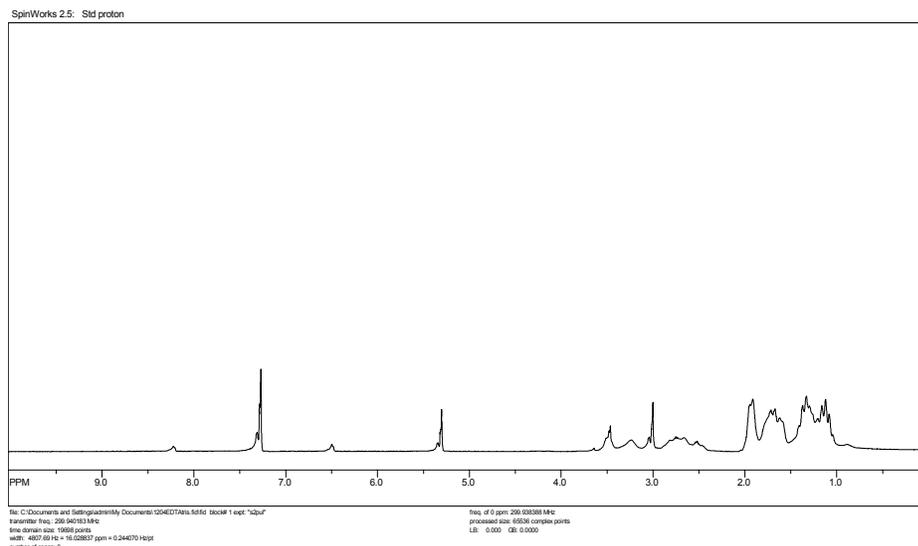
2.2 Synthesis of 4-armed Boc-protected amines

To EDTA (2.93g, 10.0 mmol) in CH₂Cl₂ (45 ml), DCC (9.9g, 48 mmol, 4.8 eq) and DMAP (0.4 eq, 489.0 mg, 4mmol) were added at 0°C and allowed to stir for 1 h. This solution was then added to **16** in MeOH (15ml) and stirred for 24 h at room temperature. Yield: (12.85g, 60%); TLC (CH₂Cl₂/MeOH, 90:10 v/v): *R_f* = 0.4; ¹H NMR (DMSO-*d*₆, 400 MHz): δ 1.37 (s, 18 H), 1.00-1.49 (m, 16H), 1.8 (s, 4H), 2.40-2.85 (m, 40H), 3.00 (s, 8H), 3.45-3.55 (m, 8H), 8.20 (s, 4H). MS (m/z): calculated, 1605.08 for C₇₄H₁₄₄N₁₈O₂₀; found, 1629.03 for [M + Na]⁺.

2.3 Deprotection of *N*-Boc amines by TFA to obtain polyamine of DexAM 2 (**18**)

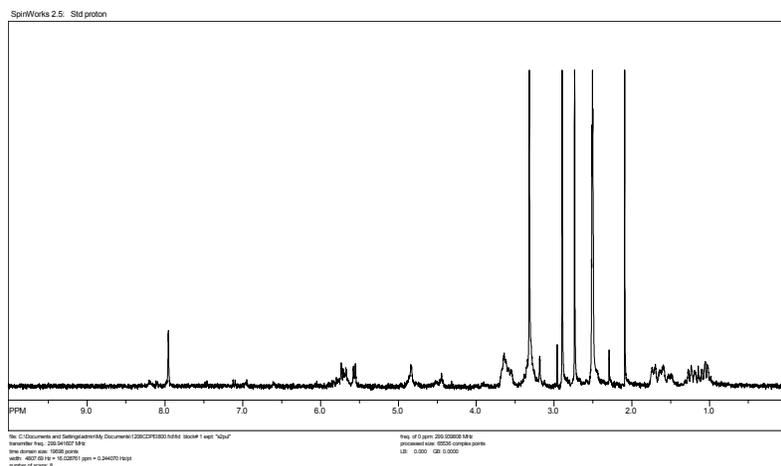
To 4 arm-boc-protected amines (**17**, 1.6g, 1mmol) was added 40% TFA in dichloromethane (10 ml). After 3 h, the reaction mixture was concentrated to give **18** as clear oil. The solvent was evaporated and the crude products were purified by flash chromatography on SiO₂ to obtain **18**. Yield: (772.92mg, 96%); TLC (CH₂Cl₂/MeOH, 9:1): *R_f* = 0.1; ¹H NMR (DMSO-*d*₆, 400 MHz): δ 1.00-1.49 (m, 16H), 1.8 (s, 4H), 2.40-2.85 (m, 40H), 3.00 (s, 8H), 3.45-3.55 (m, 8H), 8.20 (s, 4H).

MS (m/z): calculated, 804.66 for C₃₄H₈₀N₁₈O₄; found, 828.11 for [M + Na]⁺.



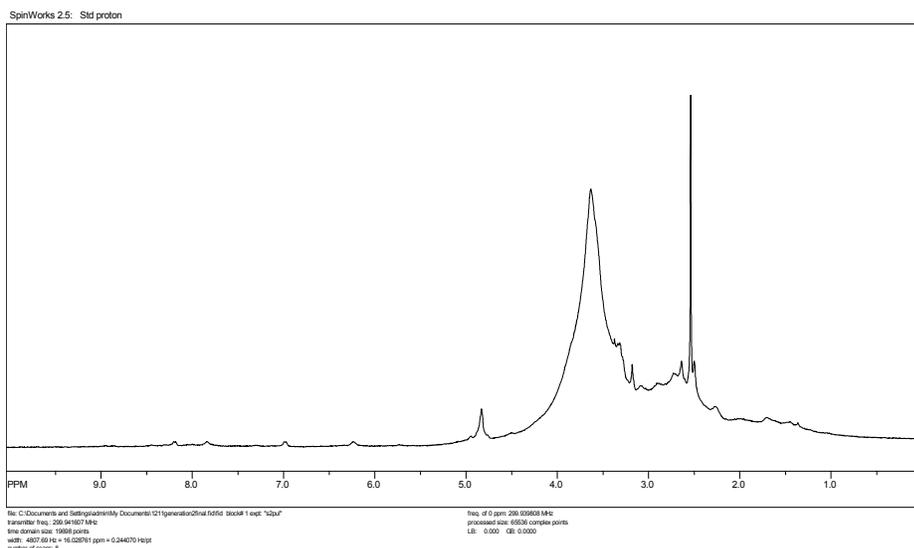
2.4 Synthesis of water-soluble CD-polyamine tosylate (19)

Tosylated cyclodextrin (**2**, 2.58 g, 2 mmol) and amine (**18**, 1.61g, 2 mmol) was dissolved in DMF (10 mL) in a 25-ml one-necked round-bottomed flask equipped with Liebig's condenser and a stir bar. The flask was degassed and purged with nitrogen. The mixture was stirred and refluxed at 90 °C for 48 h. The reaction mixture was cooled down to room temperature and the product precipitated out by the addition of acetone (20 ml). The precipitate was collected by filtration, washed with acetone and dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **19** as a brown solid. Yield: (3.77g, 90%); ¹H NMR (DMSO-*d*₆, 400 MHz): δ 1.00-1.49 (m, 16H), 1.8 (s, 4H), 2.40-2.85 (m, 40H), 2.41 (s, 3H), 3.00 (s, 8H), 3.15–3.47 (m, 24H), 3.45-3.55 (m, 8H), 3.45–3.72 (m, 28H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.2 (d, J=8.4, 2H), 7.5 (d, J=8.4, 2H), 8.0 (s, 4H). MH⁺ 2095.29.

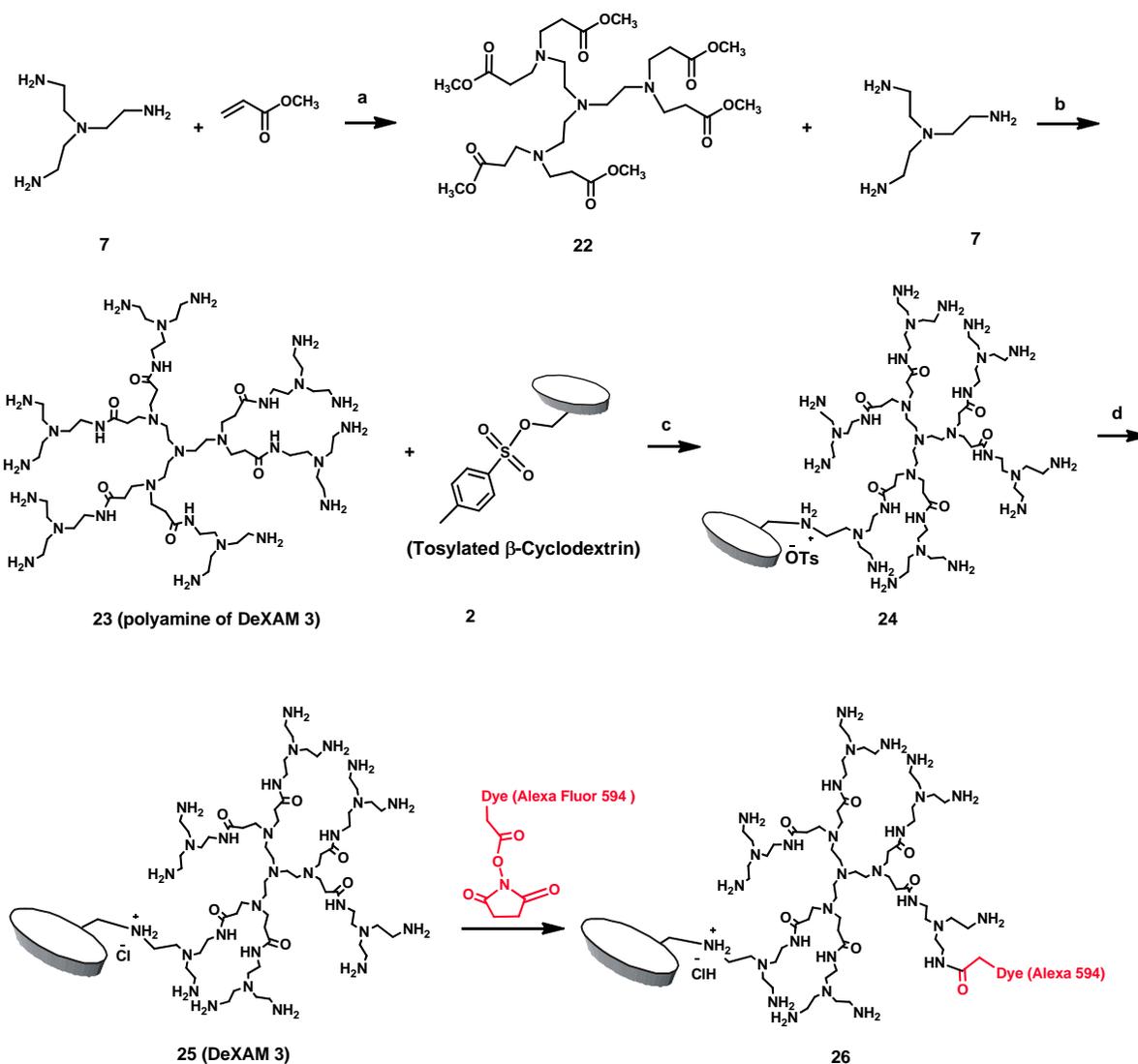


2.5 Anion exchange reaction for obtaining DexAM 2 (20)

CD-polyamine tosylate (**19**, 2.2g, 1 mmol) was dissolved in 40 mL deionized water. A 50 mL solid-phase synthesis vessel was packed with Amberlite IRA-900 ion-exchange resin to about half the vessel volume. The solution was transferred into the solid phase vessel. After 1 h, the eluent was collected and the water was distilled off under reduced pressure using a vacuum pump. The solid residue was dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **20** as a brown solid. Yield: (2.05g, 95%); ^1H NMR (DMSO- d_6 , 400 MHz): δ 1.00-1.49 (m, 16H), 1.8 (s, 4H), 2.40-2.85 (m, 40H), 2.41 (s, 3H), 3.00 (s, 8H), 3.15-3.47 (m, 24H), 3.45-3.55 (m, 8H), 3.45-3.72 (m, 28H), 4.15-4.62 (m, 6H), 4.75-4.81 (m, 7H), 5.60-5.89 (m, 14H), 7.10-7.20 (t, J = 5.0 Hz, 1H), 7.26 (t, J = 5.0 Hz, 1H), 7.60-7.68 (t, J = 7.2 Hz, 2H), 8.2 (s, 4H), 8.44-8.56 (d, J = 4.8 Hz, 1H). MH $^+$ 2156.82.



3. Synthesis of DexAM 3 and its conjugation to Alexa-594 dye

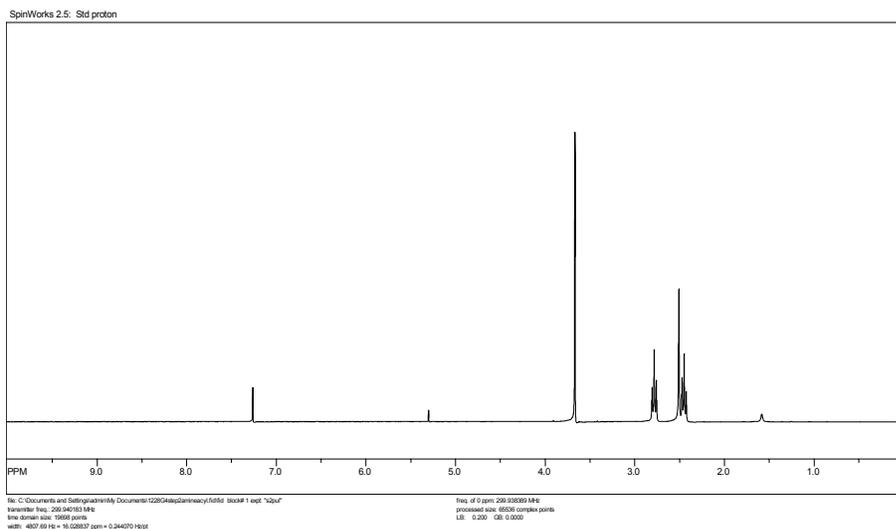


Scheme 3. Synthesis of DeXAM 3. a) MeOH, 0°C, 1h/ RT, 48h, b) MeOH, 0°C, 1 h, RT, 7 days, c) DMF, 90°C, 48 h. d) Amberlite IRA900

3.1 Synthesis of hexamethyl-3,3',3'',3''',3''''-((2,2',2''-nitriлотris(ethane-2,1-diyl)tris(azanetriyl)) hexapropanoate (22)

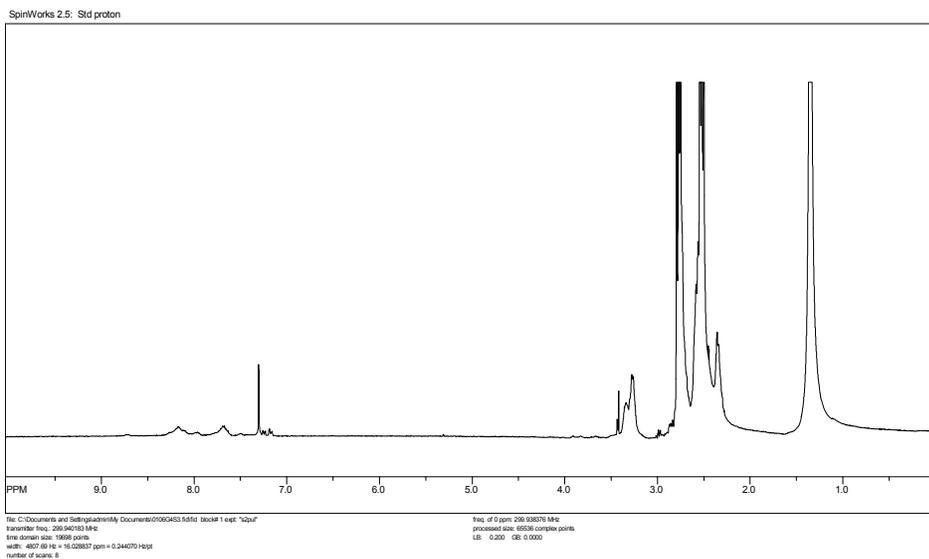
A solution of tris(aminoethyl)amine (7, 4.3872g, 30 mmol) in methanol (25 mL) was added dropwise to a stirred solution of methyl acrylate (19.37 g, 225 mmol) in methanol (25 mL) for 1 h in an ice-water bath. The resulting solution was stirred for 1 h in an ice-water bath and then allowed to warm to room temperature and stirred for further 48 h. The solvent and excess acrylate were removed under reduced pressure using a rotary evaporator. The residue was

purified by column chromatography to afford the product (**22**) as a colorless oil. Yield: 16.88 g, 85%); NMR (300 MHz, CDCl₃): δ 2.44 (t, J=6.9 Hz, 12H), 2.49 (s, J=6 Hz, 12H), 2.74 (t, J=6.9 Hz, 12H), 3.67 (s, 18H). MS (m/z): calculated, 662.37 for C₃₀H₅₄N₄O₁₂; found, 685.76 for [M + Na]⁺.



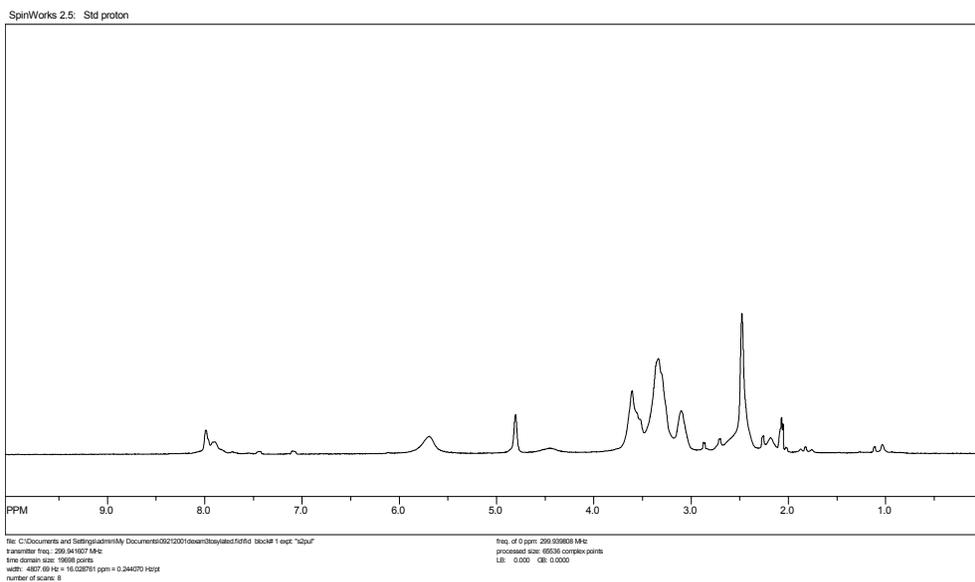
3.2 3,3',3'',3''',3''',3''''-(2,2',2''-nitriлотris(ethane-2,1-diyl)tris(azanetriyl))hexakis(N-(2-(bis(2-aminoethyl)amino)ethyl)propanamide) (**23**)

A solution of **22** (2.17 g, 3.3 mmol) in methanol (20 mL) was added dropwise to solution of tris(aminoethyl)amine (**7**, 5.8 g, 39.6 mol) in methanol (20 mL) and stirred over a period of 1 h in an ice bath. The resulting solution was allowed to warm to room temperature and stirred for 7 days at room temperature at which time no methyl ester was detectable by NMR spectroscopy. The solvent was removed under reduced pressure using a rotary evaporator and then the excess tris(aminoethyl)amine was removed using an azeotropic mixture of toluene and methanol (90:10 v/v). The remaining toluene was removed by azeotropic distillation using methanol. Finally, the remaining methanol was removed under vacuum. The residue was purified by dialysis and centrifugal filtration to afford the desired product. Finally the product was kept under vacuum to obtain the amino-terminated product (**23**, 4.4 g, 99%) as colorless oil. NMR (300 MHz, CDCl₃): δ 1.25 (s, J=6.0 Hz, 24H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, J=8 Hz, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.25 (t, 12H), 8.0 (s, 6H). MS (m/z): calculated, 1347.14 for C₆₀H₁₃₈N₂₈O₆; found, 1370.0391 for [M + Na]⁺.



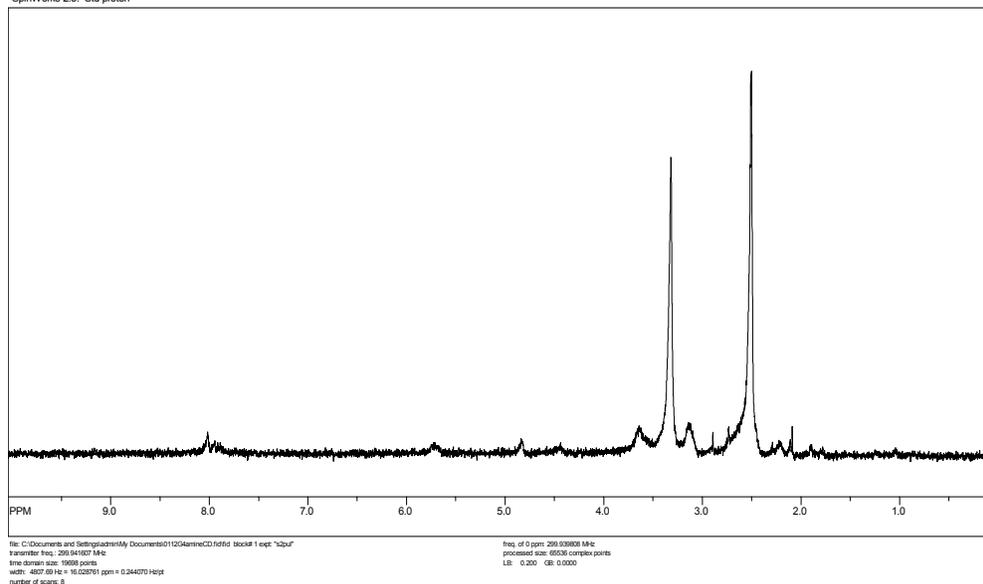
3.3 Synthesis of water-soluble CD-polyamine tosylate (**24**)

Tosylated cyclodextrin (**2**, 2.58 g, 2 mmol) and the polyamine (**23**, 2.694g, 2 mmol) were dissolved in DMF (10 mL) in a 25 mL one-necked round-bottomed flask equipped with Liebig's condenser and a stir bar. The flask was degassed and purged with nitrogen. The mixture was stirred and refluxed at 90 °C for 48 h. The reaction mixture was cooled down to room temperature and product precipitated out by the addition of acetone (20 mL). The precipitate was collected by filtration, washed with acetone and dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **24** as a brown solid (4.75g, 90% yield). ¹H NMR (300 MHz, DMSO-d₆), δ 1.25 (s, J=6.0 Hz, 24H), 2.41 (s, 3H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, J=8 Hz, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.15–3.47 (m, 24H), 3.25 (t, 12H), 3.45–3.72 (m, 28H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.21 (d, J = 8.4 Hz, 2H), 7.52 (d, J=8.4 Hz, 2H), 8.0 (s, 6H). MS (m/z): MH+ 2638.09.



3.4 Anion exchange reaction for obtaining DexAM3 or D3 (25)

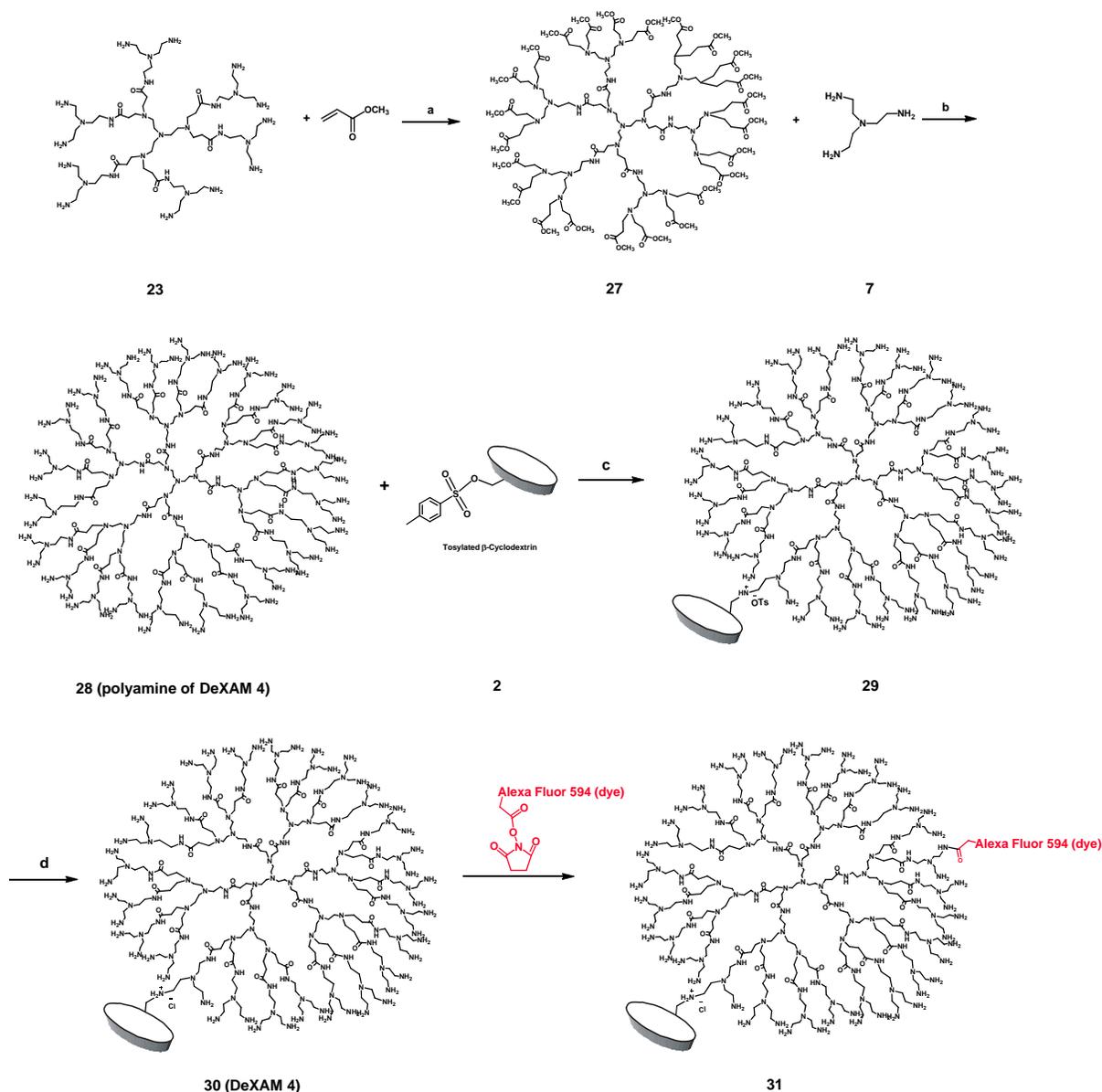
CD-polyamine tosylate (**24**, 2.637g, 1 mmol) was dissolved in 40 mL deionized water. A 50 mL solid-phase synthesis vessel was packed with Amberlite IRA-900 ion-exchange resin to about half the vessel volume. The solution was transferred into the solid phase vessel. After 1 h, the eluent was collected and the water was distilled off under reduced pressure using a vacuum pump. The solid residue was dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **25** as a brown solid. Yield: (2.376g, 95% yield). ¹H NMR (300 MHz, DMSO-d₆), δ 1.25 (s, J=6.0 Hz, 24H), 2.41 (s, 3H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, J=8 Hz, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.15–3.47 (m, 24H), 3.25 (t, 12H), 3.45–3.72 (m, 28H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 8.0 (s, 6H). MH⁺ 2502.35.



3.5 Conjugation of Alexa-Fluor 594 dye to the CD-Polyamine (26)

Alexa Fluor-594 dye-succinidimyl ester (100 nM, Molecular Probes, Invitrogen) and DexAM 3 (25, 100 nM) were dissolved in PBS buffer solution (0.5 mL). The reaction mixture was allowed to vortex for 5 minutes. After being vortexed, the mixture was shaken at room temperature for 2 h.

4. Synthesis of DeXAM 4 and conjugation to Alexa 594 dye



Scheme 4. Synthesis of DexAM 4, a) MeOH, 0°C, 1 h, RT, 60 h, b) MeOH, 0°C, 1 h, RT, 7 days c) DMF, 90°C, 48h, d) Amberlite IRA900

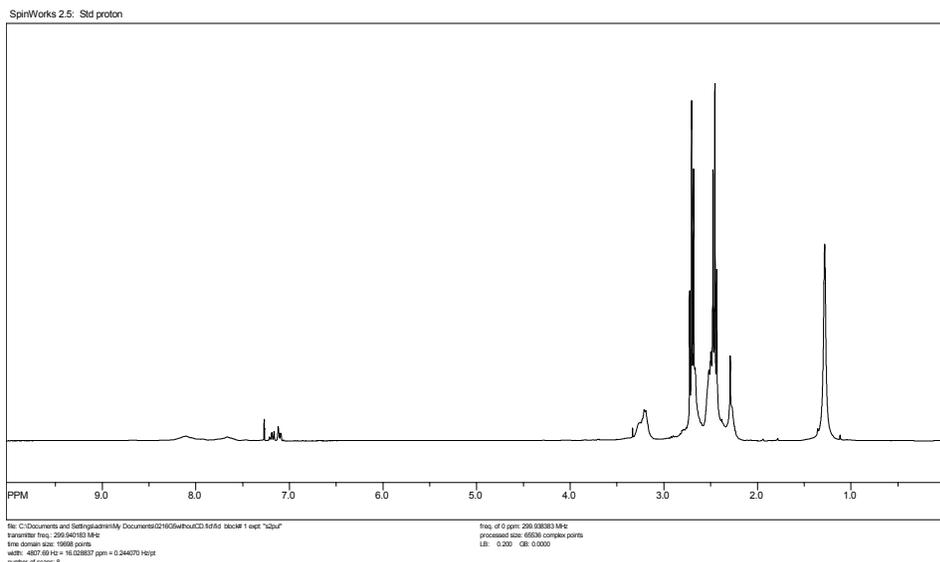
4.1 Synthesis of methyl ester of **23** (**27**)

A solution of **23** (1.48g, 1.1 mmol) in methanol (5 mL) was added dropwise to a stirred solution of methyl acrylate (2.84 g, 33.0 mmol) in methanol (5 mL) for 1 h in an ice bath. The resulting solution was stirred for 30 min in an ice bath and then for 60 h at room temperature. The

volatiles were removed under reduced pressure. The residue was purified by column chromatography using DCM:MeOH (10:1 v/v) to afford the desired product (**27**) as a yellow oil. Yield: (3.41 g, 91%); NMR (300 MHz, CDCl₃): δ 2.44 (t, J=6.9 Hz, 12H), 2.49 (s, J=6 Hz, 12H), 2.74 (t, J=6.9 Hz, 12H), 3.67 (s, 18H). MS (m/z): calculated, 3410.03 for C₁₅₈H₂₈₄N₂₆O₅₄; found, 3435.08 for [M + Na]⁺.

4.2 Synthesis of the polyamine of DexAM 4 (**28**)

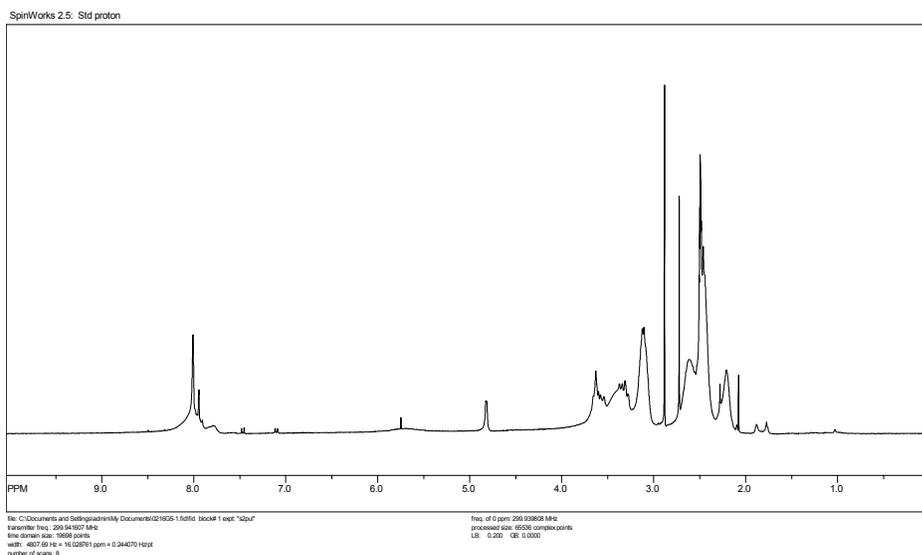
A solution of ester **27** (3.41g, 1 mmol) in methanol (20 mL) was added dropwise to a stirred solution of tris(aminoethyl)amine (**7**, 7.02 g, 48 mmol) in methanol (20 mL) over a period of 1 h in an ice bath. The resulting solution was allowed to warm to room temperature and stirred for 7 days at room temperature at which time no methyl ester was detectable by NMR spectroscopy. The solvent was removed under reduced pressure the excess tris(aminoethyl)amine was removed using an azeotropic mixture of toluene:MeOH (90:10 v/v). The product was further purified by washing with anhydrous ether twice, yielding a highly viscous liquid. Finally the product was kept under vacuum to provide the amino-terminated final product (**28**) as a light yellow liquid. Yield (6.1 g, 99%); NMR (300 MHz, CDCl₃): δ 1.25 (s, J=6.0 Hz, 24H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, J=8 Hz, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.25 (t, 12H), 8.0 (s, 6H). MS (m/z): calculated, 6151.06 for C₂₇₆H₆₁₈N₁₂₄O₃₀; found, 6177.66 for [M + Na]⁺.



4.3 Synthesis of water-soluble CD-Polyamine tosylate (**29**)

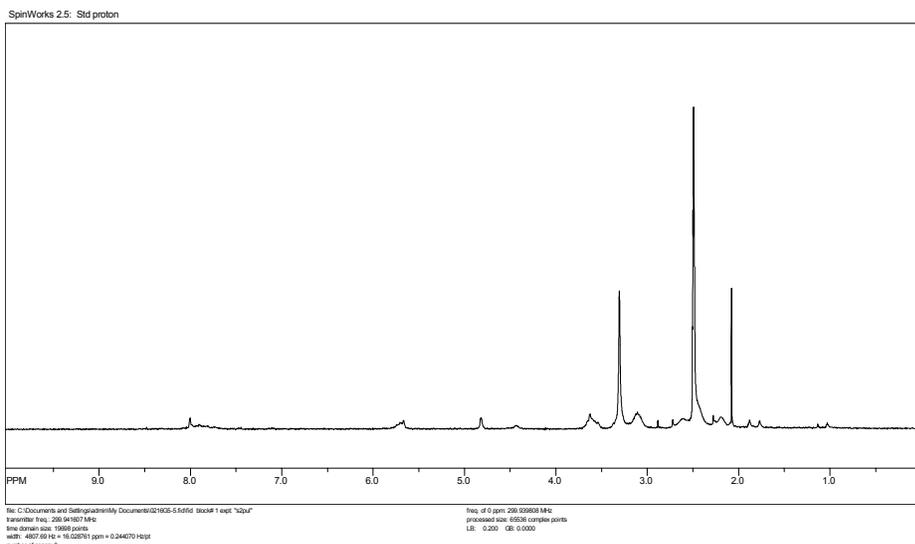
Tosylated CD (**2**, 1.29g, 1mmol) and polyamine of DexAM 4 (**28**, 6.1g, 1 mmol) were dissolved

in DMF (10 mL) in a 25 mL one-necked round-bottomed flask equipped with Liebig's condenser and a stir bar. The flask was degassed and purged with nitrogen. The mixture was stirred and refluxed at 90 °C for 48 h. The reaction mixture was cooled down to room temperature and product precipitated out by the addition of acetone (20 mL). The precipitate was collected by filtration, washed with acetone and dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **29** as a brown solid. Yield: (6.6g, 89%); ¹H NMR (300 MHz, DMSO-d₆), δ 1.25 (s, J=6.0 Hz, 24H), 2.41 (s, 3H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, J=8 Hz, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.15–3.47 (m, 24H), 3.25 (t, 12H), 3.45–3.72 (m, 28H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 7.21 (d, J = 8.4 Hz, 2H), 7.52 (d, J=8.4 Hz, 2H), 8.0 (s, 6H). MH+7444.84 or 8733.01



4.4 Anion exchange reaction for obtaining DexAM 4 or D4 (30)

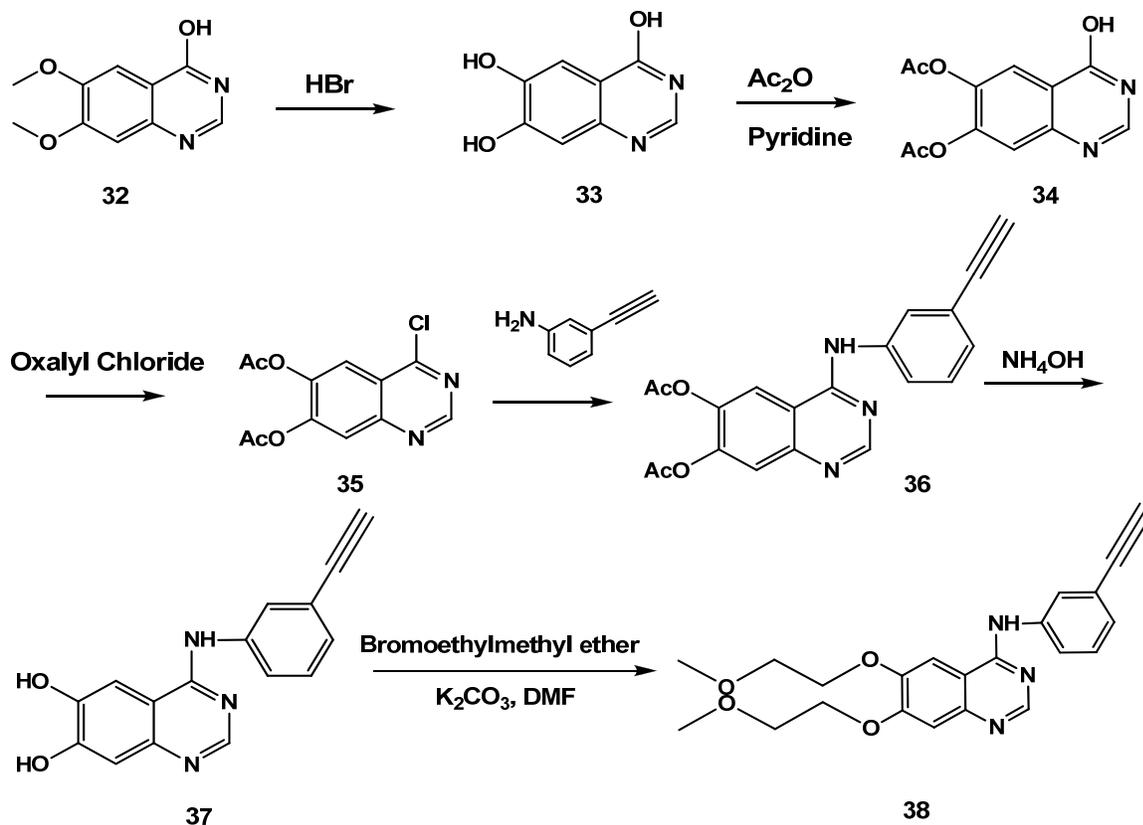
CD-polyamine tosylate (**29**, 3.72g, 0.5 mmol) was dissolved in 40 mL deionized water. A 50 mL solid-phase synthesis vessel was packed with Amberlite IRA-900 ion-exchange resin to about half the vessel volume. The solution was transferred into the solid phase vessel. After 1 h, the eluent was collected and the water was distilled off under reduced pressure using a vacuum pump. The solid residue was dried overnight at 60 °C in a vacuum oven (10 mm Hg) to yield **30** as a brown solid. Yield: (3.47g, 95%); ¹H NMR (300 MHz, DMSO-d₆), δ 1.25 (s, J=6.0 Hz, 24H), 2.41 (s, 3H), 2.44 (t, J=6.9 Hz, 12H), 2.48 (m, J=8 Hz, 72H), 2.74 (t, J=6.9 Hz, 12H), 3.15–3.47 (m, 24H), 3.25 (t, 12H), 3.45–3.72 (m, 28H), 4.15–4.62 (m, 6H), 4.75–4.81 (m, 7H), 5.60–5.89 (m, 14H), 8.0 (s, 6H). MH+7309.10 or 8462.53.



4.5 Conjugation of Alexa-Fluor 594 dye to DexAM 4 (31)

Alexa Fluor-594 dye (100 nM, Molecular Probes) and DeXAM 4 (**30**, 100 nM) were dissolved in PBS buffer solution (0.5 mL). The reaction mixture was allowed to vortex for 5 minutes. After being vortexed, the mixture was shaken at room temperature for 3 h.

5. Synthesis of Erlotinib (38)

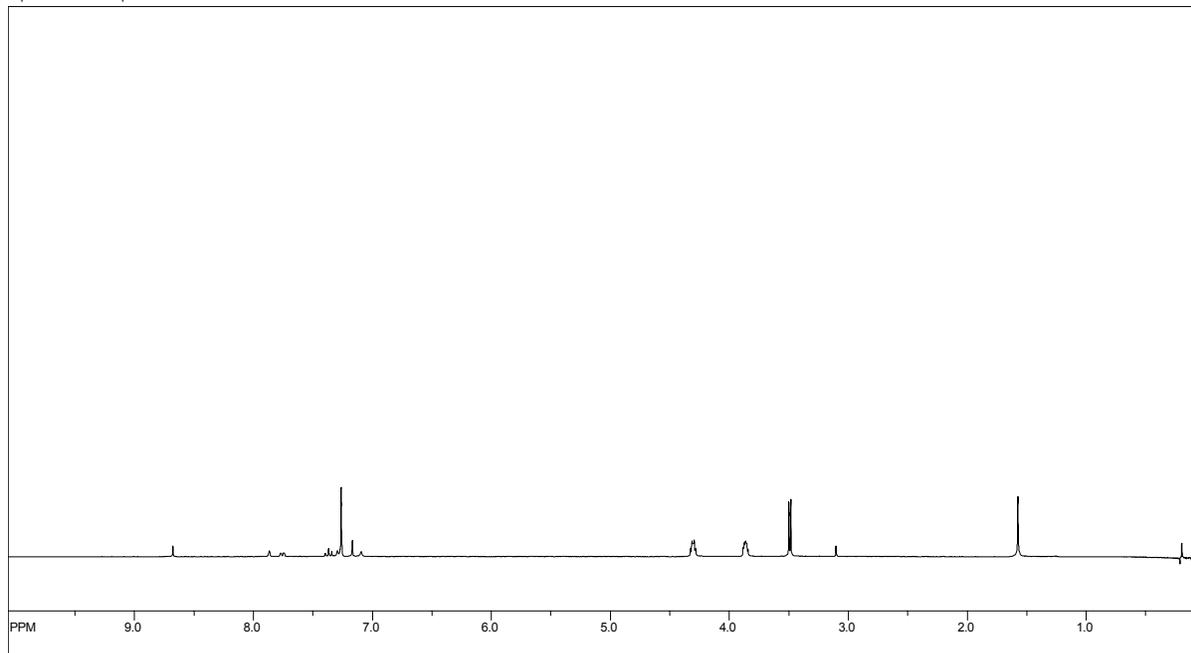


Scheme 5. Synthesis of Erlotinib

The EGFR tyrosine kinase inhibitor N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine (commonly known as Erlotinib) was synthesized using previously reported protocols albeit with slight modifications^[3] (**Scheme 5**). Briefly, 5.0 g of the 6, 7-dimethoxyquinazolinone (**32**) was suspended in concentrated HBr and refluxed under nitrogen to obtain **33** as a white solid. This was then suspended in 23 mL of acetic anhydride in presence of 200 μ L of pyridine. The reaction mixture was refluxed under nitrogen for 3 h and the acetic anhydride/acetic acid was removed *in vacuo* to yield the diacetate (**34**) in 91% yield. The diacetate was converted to the chloro derivative (**35**) by reacting it with oxalyl chloride which was then reacted with 3-ethynylaniline to yield **36** as an off-white solid. The product was converted to the dihydroxy derivative (**37**) by hydrolysis using conc. NH₄OH. **37** was dissolved in 10 mL of dry DMF followed by the addition of 11.4 g of K₂CO₃. Potassium Iodide (2 g) and bromomethylethyl ether (3.5 g) were added to the above solution and stirred overnight at 45 °C. The solvent was

removed *in vacuo*, dissolved in DCM, washed with water and dried over MgSO₄. The solvent was evaporated to obtain the product (**38**) as a brown solid. Yield: (3.56g, 95%); ¹H NMR (CDCl₃) δ 8.60 (s, 1H), 7.96 (w, 1H), 7.85 (s, 1H), 7.70-7.76 (m, 1H), 7.42-7.36 (m, 3H), 7.12 (s, 1H), 4.13-4.21 (m, 4H), 3.73-3.78 (m, 4H), 3.40 (s, 3H), 3.08 (s, 1H)

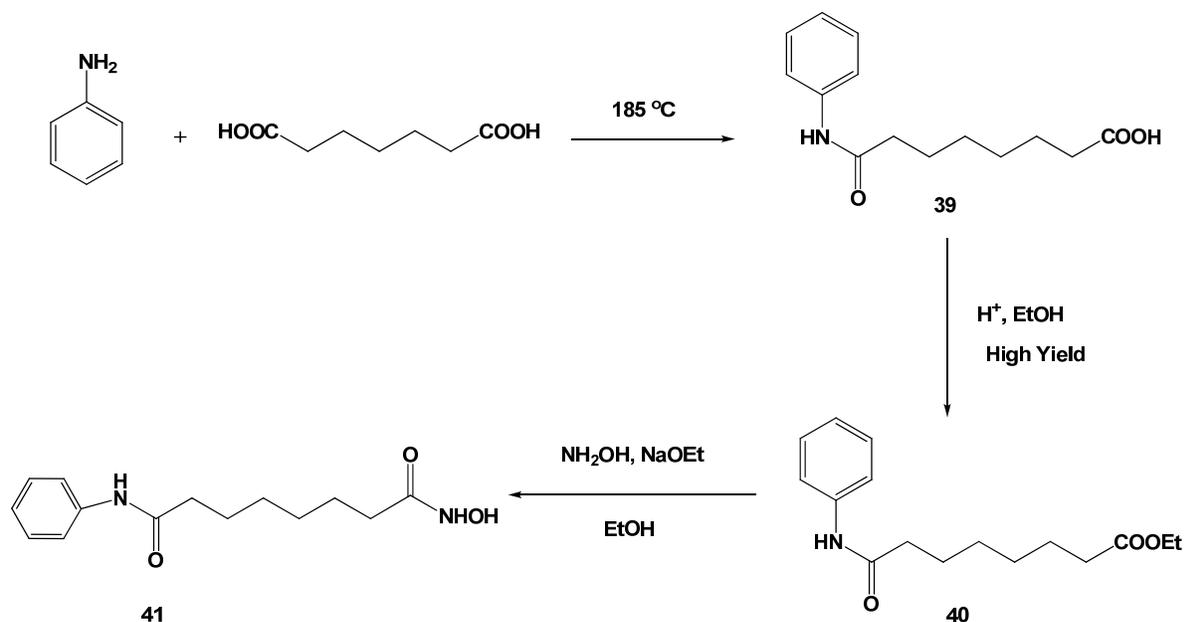
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time domain size: 10550 points
width: 4907.69 Hz = 16.028837 ppm = 0.244070 Hz/pt
number of scans: 8

freq. of 0 ppm: 299.938388 MHz
processed size: 65536 complex points
LB: 0.200 GB: 0.0000

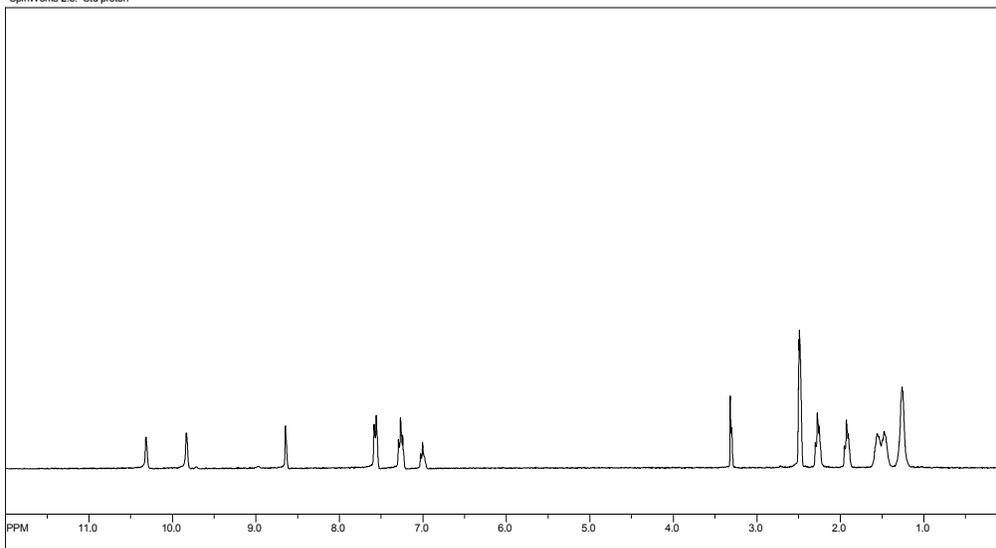
6. Synthesis of SAHA (41)



Scheme 6. Synthesis of SAHA

The histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) was synthesized according to previously reported methods^[4] (**Scheme 6**). Briefly, freshly distilled aniline (4.09 g, 0.044 mol) and suberic acid (6.96 g, 0.040 mol) were heated at 185-190 °C for 10 min to yield suberanilic acid (**39**) as a white solid in 41% yield. This was then converted to the methyl ester (**40**) by refluxing with methanol for 22 h. Hydroxylamine hydrochloride (2.17 g, 0.0312 mol) was dissolved in 15 mL of ethanol in a 50 mL flask equipped with magnetic stirring and an addition funnel. Solid methyl suberanilate (4.10 g, 0.0156 mol) was added, which dissolved readily, followed by the addition of sodium methoxide solution. The reaction mixture was stirred for 26 h at room temperature and then rinsed with 100 mL of water where most of it dissolved. Glacial acetic acid (4.0 g) was added with stirring. The resulting heavy precipitate was filtered, rinsed with water, then slurried with another 75 mL of water, filtered, and rinsed again. The solid was dried at room temperature, affording the product (**41**) as a white solid. Yield: (1.82 g, 47%); MP = 159-160.5 °C; ¹H NMR (DMSO-d₆) δ 10.33 (s, 1H), 9.84 (s, 1H), 8.66 (s, 1H), 7.57 (d, J = 7.61, 2H), 7.27 (t, J = 7.2, 2H), 7.00 (t, J = 7.4, 1H), 2.27 (t, J = 7.61, 2H), 1.92 (t, J = 7.4, 2H), 1.56 (p, J = 6.7, 2H), 1.47 (p, J = 6.21, 2H), 1.26 (m, 4H)

SpinWorks 2.5: Std proton

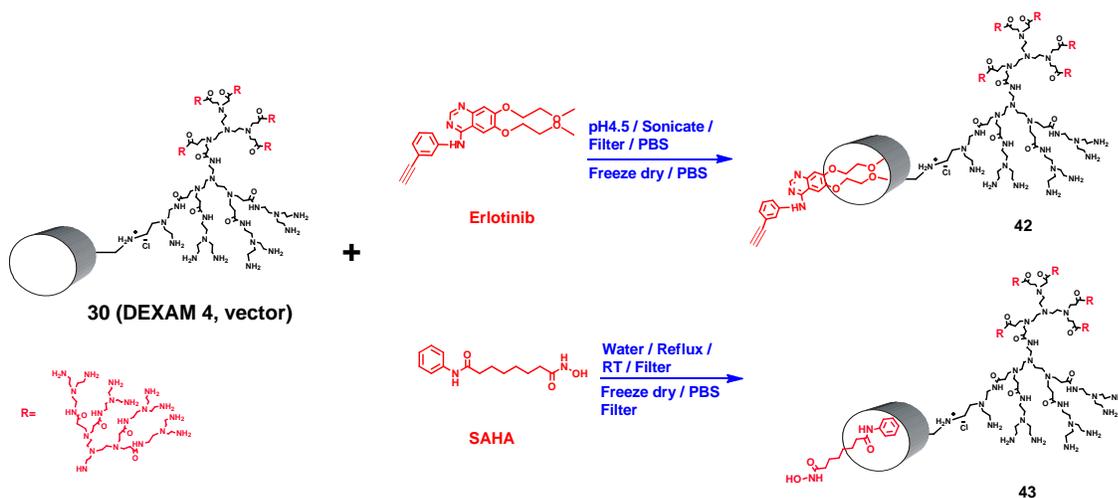


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processed size: 65036 complex points
SI: 0.200 CFS 0.000

transmitter freq.: 299.941507 MHz
time domain size: 15669 points
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number of scans: 8

7. Inclusion of Drugs into DexAM complexes



Scheme 7. Inclusion of anticancer drugs into DexAM 4

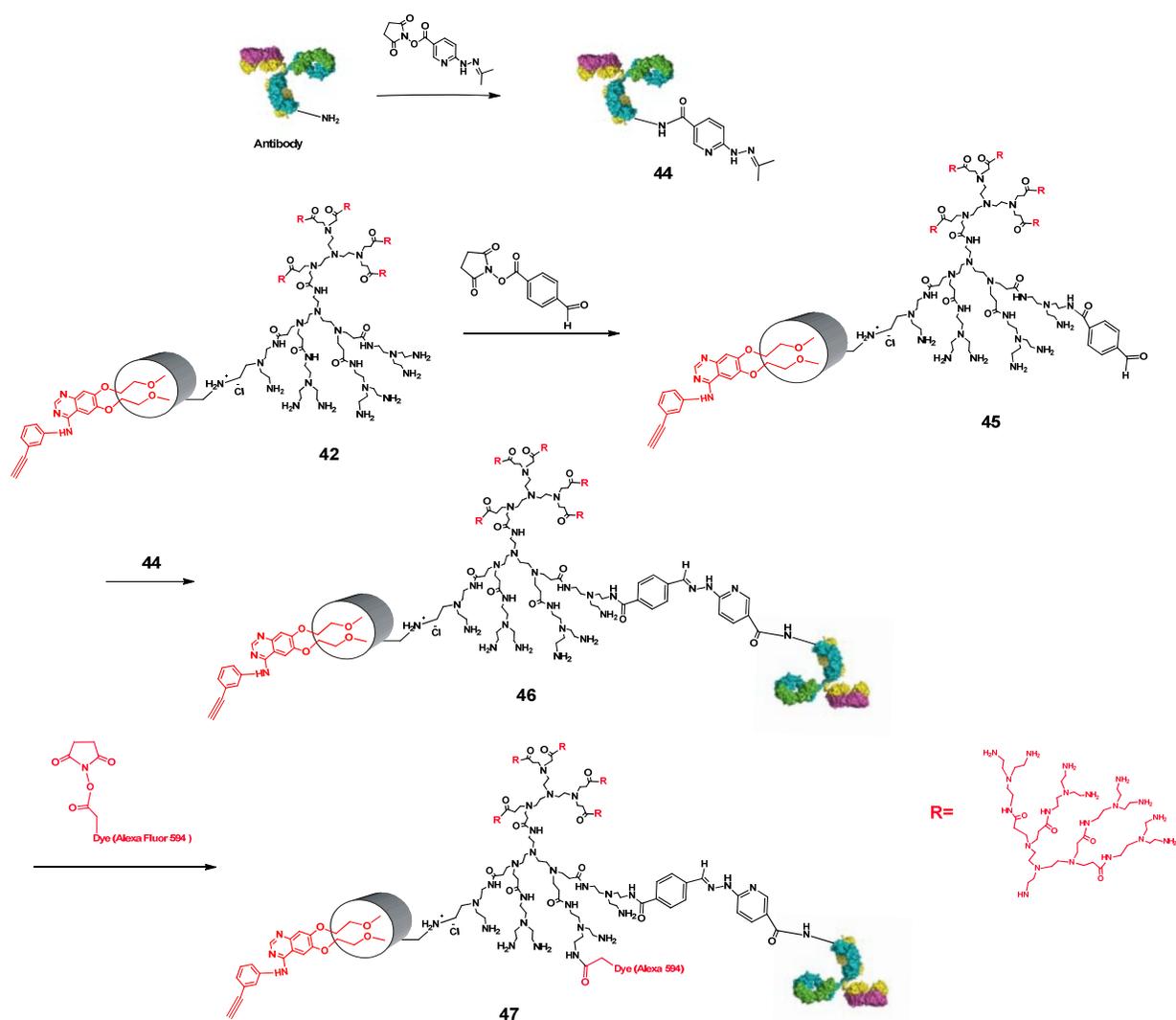
7.1 Formation of Erlotinib-DexAM 4 inclusion complex (42)

Erlotinib (5mg, 13 μmol) was dissolved in 10 mL of acetate buffer (pH=4.5). The erlotinib solution was added to vials containing pure DexAM 4 (26 μmol). The contents of the vials were vortexed for 10 min, sonicated for 30 min and stirred vigorously for 8 h. The resulting complex was freeze-dried. Thereafter, 200 μL of PBS was added to the DexAM complex (10 μmol) and allowed to stand at room temperature to dissolve for a few minutes. The solution was then filtered and purified using an appropriate molecular weight cut-off (MWCO) centrifugal filter (Millipore, Billerica, MA, USA).

7.2 Formation of SAHA-DexAM 4 inclusion complex (43)

Suberoylanilide hydroxamic acid (SAHA) was solubilized in 2 molar equivalents of DexAMs in distilled water. Briefly, 8 mg of SAHA was added to a solution of 370mg of DexAM 4 in 3 mL of water, heated until fully dissolved, stirred for 8 h, and then rapidly cooled on ice to room temperature. This solution was filtered and freeze-dried. Thereafter, 200 μL PBS was added to DexAM complex (10 μmol) and allowed to stand at room temperature to dissolve for a few minutes. Finally, the complex was purified through by centrifugal filtration using an appropriate MWCO membrane. DexAM-SAHA solutions of various concentrations were prepared by maintaining the molar ratio between SAHA and DexAMs.

8. Antibody conjugation to DexAM 4 complexes



Scheme 8. Conjugation of EGFR antibody to DexAM 4 complexes

8.1 Functionalization of antibody with 6-hydrazinonicotinamide (44)

6-hydrazinonicotinamide (HyNic), an aromatic hydrazine was attached to the amine group of antibody. Briefly, 6-hydrazinonicotinamide (11 nM, HyNic, SoluLinK) and EGFR Antibody (10 nM) were mixed in 2 mL of buffer (pH = 6.0) and vortexed for 5 minutes. After being vortexed, the mixture was shaken at room temperature for 2 h.

8.2 Functionalization of DexAM with 4-formylbenzamide (45)

4-formylbenzamide (4FB), an aromatic aldehyde was linked to the amine group of DeXAMs. Briefly, 4-FB (11 nM, SoluLinK) and DeXAM 4 (10 nM) were dissolved in 2 mL buffer (pH = 6.0) and the reaction mixture vortexed for 5 minutes. After being vortexed, the mixture was shaken at room temperature for 2 h.

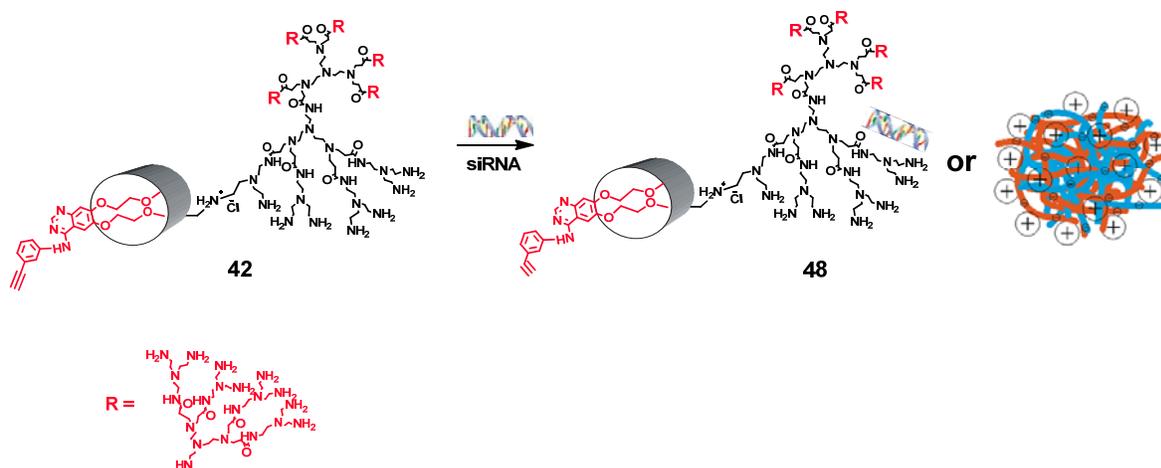
8.3 Antibody conjugation to DexAMs (46)

44 and **45** were vortexed together for a few minutes at room temperature thereby leading to the formation of a stable bis-aryl hydrazone bond between the antibody and DexAM 4 (**46**). A similar conjugation strategy was used for attaching the antibody to DexAM 4-SAHA complex (**43**)

8.4 Conjugation of Alexa Fluor-594 dye to antibody-modified DexAMs (47)

Alexa Fluor-594 (100 nM, Molecular Probes, Invitrogen) and EGFR antibody-conjugated DexAM 4 (**46**, 100 nM) were dissolved in PBS buffer solution (0.5mL) and vortexed for 5 minutes. After being vortexed, the mixture was shaken for 2 h at room temperature.

9. Formation of polyplexes between siRNA and DexAMs



Scheme 9. Polyplex formation using antibody-modified DexAMs and siRNA.

siRNA complexation: DexAM/siRNA complexes were prepared by adding equal volumes of DexAM solution (DexAM dissolved in dH_2O) and siRNA (dissolved in water) at different concentrations of siRNA and DexAMs. The resultant solution was then incubated for 30 min at room temperature before transfection.

Supporting Figures

- Figure S1 siRNA complexation ability of DexAMs using Picogreen assay
- Figure S2 Comparison of particle diameters and zeta potentials of polyamines with and without cyclodextrins
- Figure S3 Polymer mediated cytotoxicity in glioblastoma (U87-EGFP) cells
- Figure S4 Quantification of siRNA-mediated EGFP gene silencing
- Figure S5 Phase contrast and fluorescent images of siRNA-mediated EGFP gene silencing using Xtremegene
- Figure S6 Dose response curves for Erlotinib and SAHA in U87-EGFRvIII cells

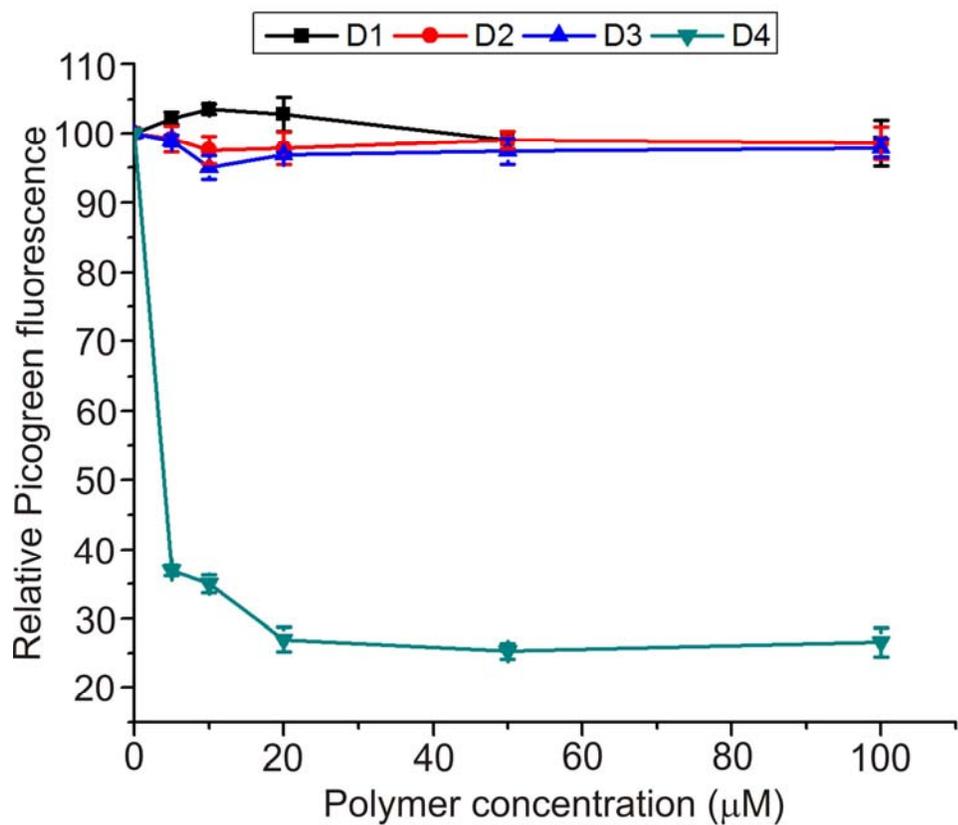


Figure S1. siRNA complexation efficiency of different DexAMs measured by Picogreen dye exclusion assay (Absorption = 480 nm, Emission = 520 nm)

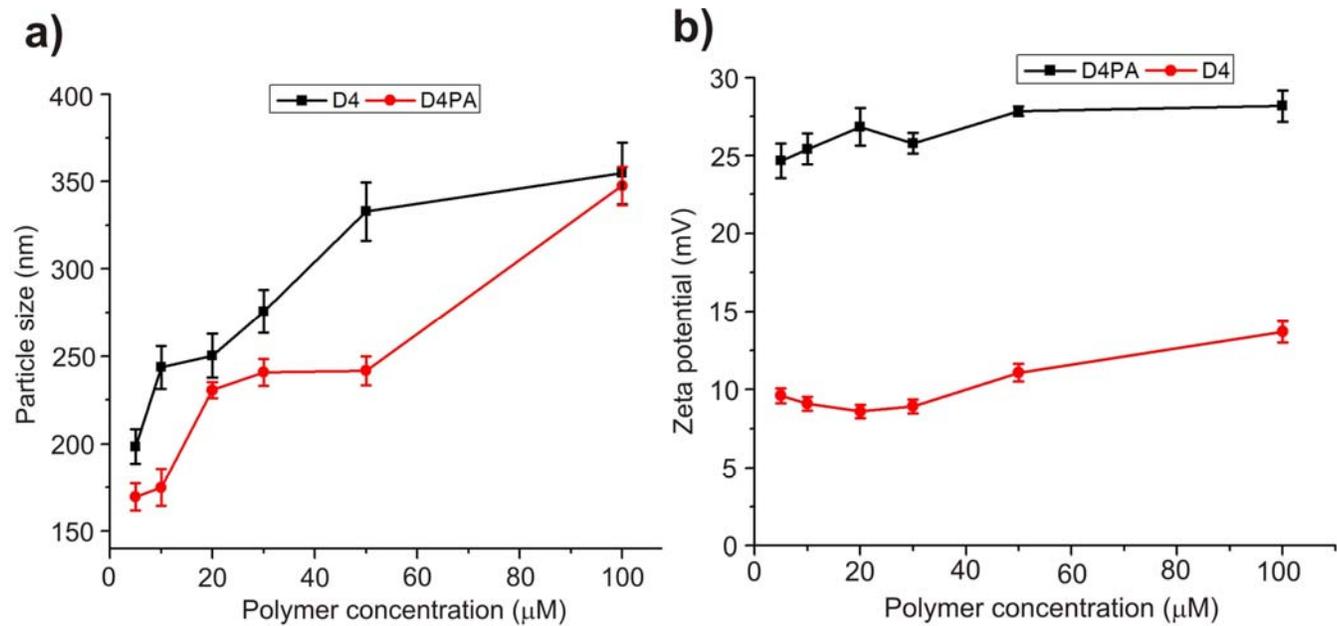


Figure S2: Comparison of particle diameters (a) and zeta potentials (b) of polyamines with and without cyclodextrins. The results are an average of three independent experiments and their respective standard errors.

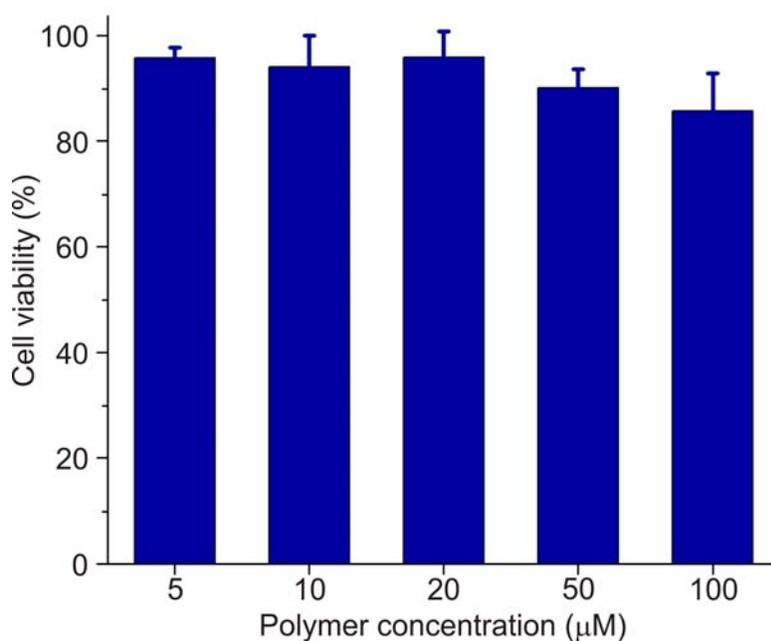


Figure S3: Polymer mediated cytotoxicity in glioblastoma (U87-EGFP) cells. The percentage of viable cells was estimated using MTS assay following incubation of cells with the DexAM-4 for 96 h. The data was obtained as absorbance of water-soluble formazan at 490 nm, following incubation with only DexAMs for 96 h. The fluorescence for treated samples was normalized to that of untreated controls.

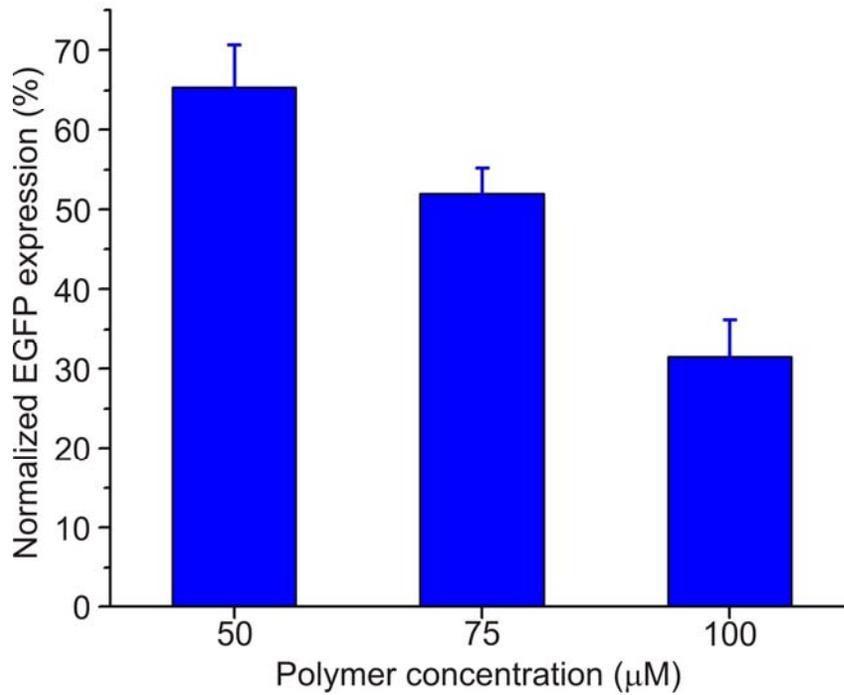


Figure S4: Quantification of siRNA-mediated EGFP gene silencing. U87-EGFP cells were incubated with DexAM-siRNA polyplexes for 12h and analyzed 72h post transfection using fluorescence microscopy. The knockdown efficiency of DexAM-siRNA polyplexes at different concentrations was quantified using Image J software after background subtraction. The results are expressed as mean of three independent experiments (n=3).

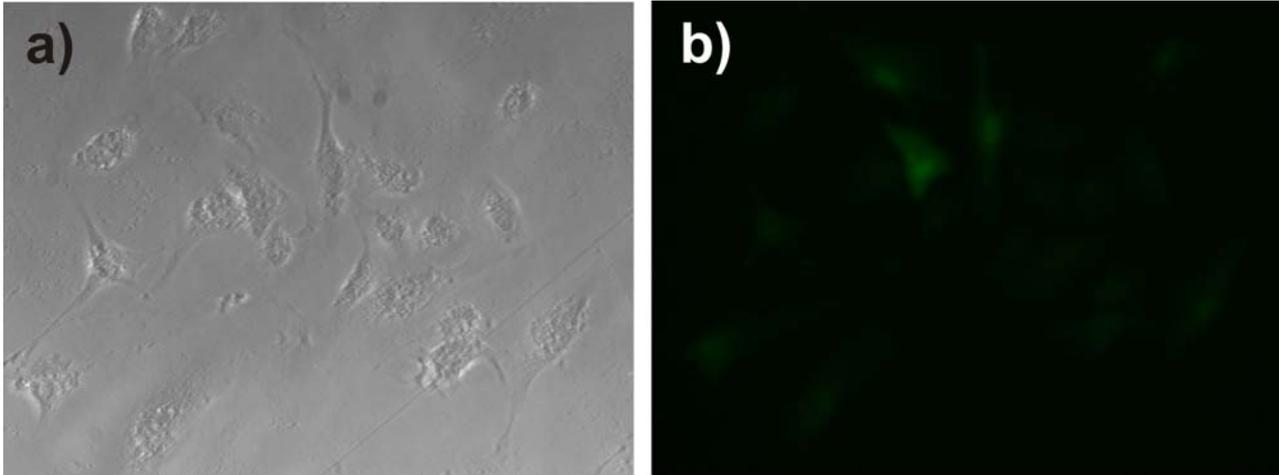


Figure S5: Phase contrast **(a)** and fluorescent **(b)** images of siRNA-mediated EGFP gene silencing using Xtremegene. U87-EGFP cells were incubated with Xtremegene-siRNA polyplexes for 12h and analyzed 96h post transfection using fluorescence microscopy. The polyplexes were formed at a ratio of 1:2 of siRNA to Xtremegene in serum-free media.

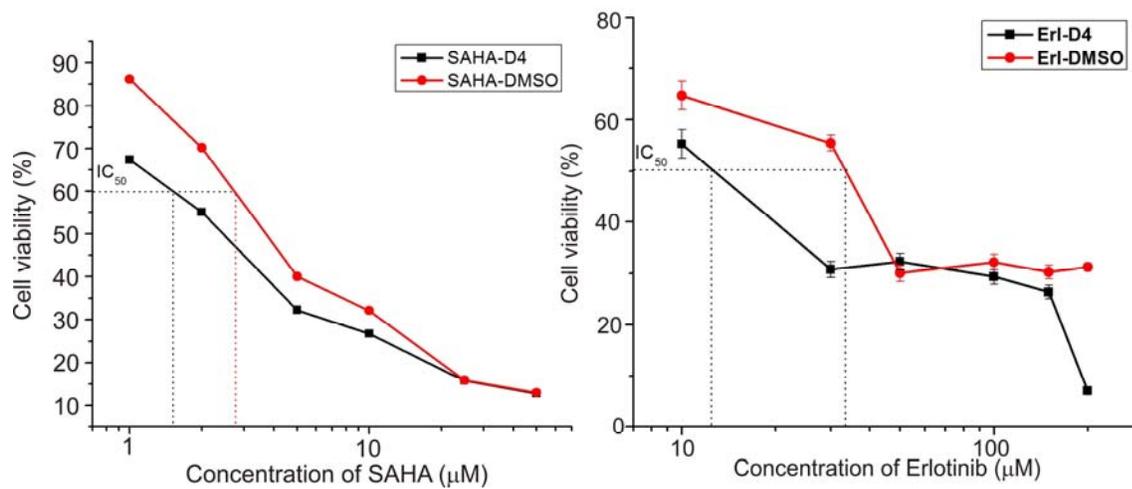


Figure S6: Dose response curves for Erlotinib and SAHA in U87-EGFRvIII cells. For cell viability studies, SAHA and Erlotinib were dissolved in DMSO as well as complexed within cyclodextrin moiety of DexAM-4. Cell viability is represented as the absorbance of the formazan product formed, with that of control (untreated) cells considered as 100%. The data represents mean of three independent experiments.

References

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