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Supporting Information

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Hormone-PAMAM Dendrimer Conjugates:

Polymer Dynamics and Tether Structure Affect Ligand Access to Receptors

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Description of Synthesis, Purification and Characterization of Estrogen Dendrimer Conjugates (EDCs)

1. Synthesis of precursor aldehydes and their model compounds (I-b, II-b, III-b, and IV-b)

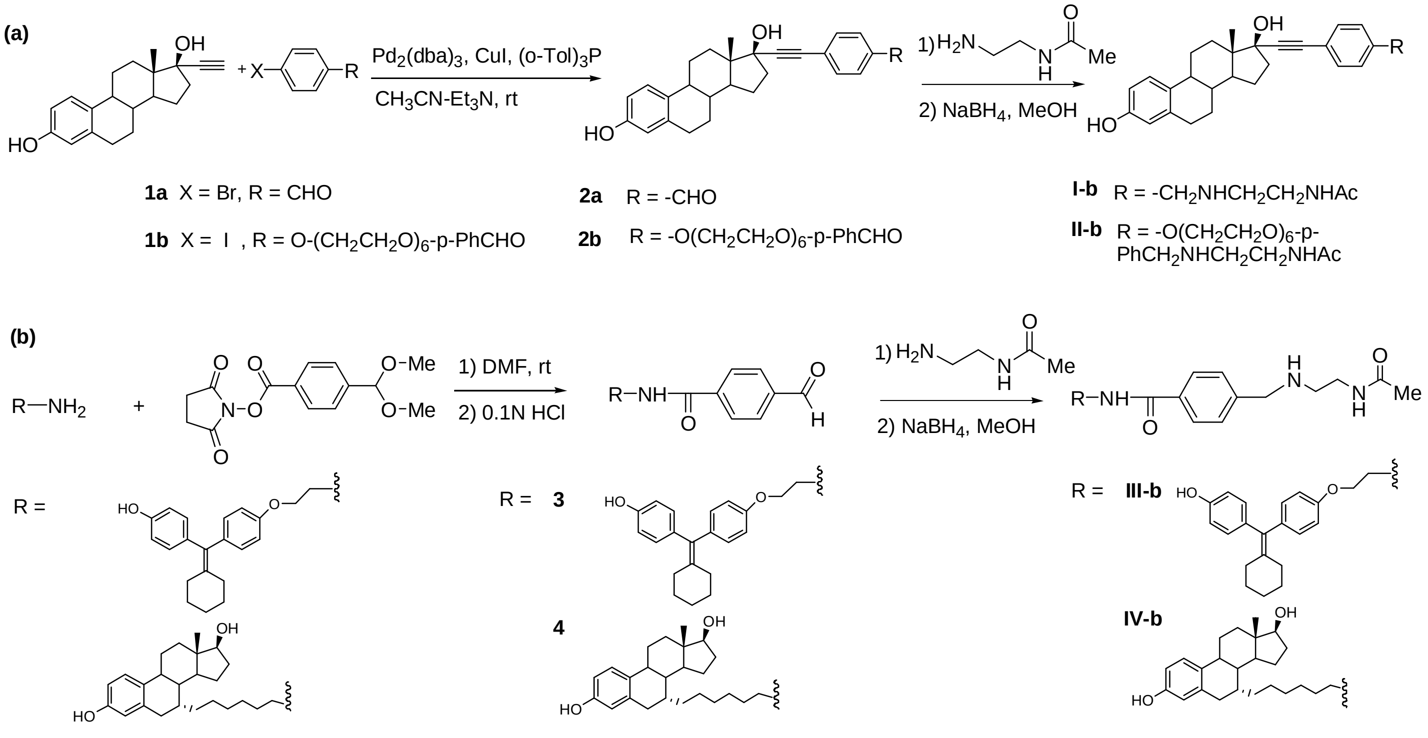

Figure S1. (**a**) Synthetic scheme to prepare for 17α-ethynylestradiol derivatives (**2a** and **2b**) and their EDC monomer compounds (**II-a** and **II-b**). (**b**) Synthetic scheme to prepare for cyclofenil and 7α-hexyl-estradiol derivatives (**3** and **4**) and their EDC monomer compounds (**III-b** and **IV-b**).

As shown in **Figure S1**, Sonogashira coupling reaction of 17α-ethynylestradiol with *p*-bromobenzaldehyde or ω-(*p*-formylphenyl)-hexa(ethyleneglycolyl)phenyliodide afforded corresponding products **2a** and **2b** in good yield. Subsequent reductive amination of **2a** and **2b** with mono acetylated ethylenediamine produced **I-b** and **II-b**. The aldehyde compounds of cyclofenil (**3**) and 7a-hexylestradiol (**4**) were prepared by coupling reaction of mono 2-aminoethyl-cyclofenil or 7a-(ω–aminohexyl)estradiol with p-(dimethoxymethyl)-benzoic acid *N*-hydroxysuccinicimide ester and subsequent hydrolysis. Their EDC monomer reference compounds (**III-b** and **IV-b**) were prepared by reductive amination with mono acetylated ethylenediamine.

2. Preparation of EDC.

As an example of the preparation of an EDC, we show the sequence by which EDC **I-a** is prepared (**Figure S1**). 17a-Ethynylestradiol (EE2), a well known high affinity ligand for ER,[1] is substituted with a benzaldehyde unit at the end of the ethynyl group, as shown in **Figure S1** (compound **2**). Attachment of the aldehyde function to the surface primary amine functionalities of the PAMAM dendrimer is then effected by reductive amination. The other EDCs are prepared in a similar fashion from the corresponding arylaldehyde precursors. The complete synthesis of all of these components is given in the Detailed Experimental Section.

As exemplified in the synthesis of EDC **I-a** (**text Figure 1**), we were able to monitor the course of attachment of the estrogen aldehyde analog to the primary amine functions of the PAMAM dendrimer by ^{1}H NMR (**Figure S2**). When the steroid analog is mixed with the G-6 PAMAM in methanol, spontaneous conversion of the aldehyde signal at d 10.1 (**Fig. S2**, bottom) to the spectroscopically distinct signal for the imine proton at d 8.3 can be observed (**Fig. S2**, middle). The latter signal disappears soon after the addition of sodium borohydride (**Fig. S2**, top). The proton signals from the phenyl ring attached to terminal amine of dendrimer also shifted progressively upfield as the aldehyde was converted to the imine and then the amine. Because they are macromolecules, line broadening effects were observed with all of the EDCs. These and other features of the ^{1}H NMR spectra of these EDCs are discussed below.

Both steps of the conjugation sequence (**Fig. S2**) appear to go quantitatively, with the stoichiometry of estrogen per dendrimer being determined simply by the molar ratio of estrogen to dendrimer (range 18-20). The EDCs were purified by using ~30,000 MW cutoff membrane filter in MeOH solution, and in cell-based activity experiments described elsewhere, these conjugates were shown to be free from non-attached ligand (<0.1% free ligand contamination).[2]

As indicated in **text Figure 1**, the EDCs were prepared at a ~20:1 ratio of steroid to dendrimer. Conjugates prepared at higher ratios (e.g., 40:1) were insoluble in polar aprotic solvents or acidic or basic buffers, even at 70-80 °C. By contrast, those prepared at this ~20:1 ratio gave material that retained excellent aqueous solubility, some (**I-a**) being soluble up to 30 mM (based on attached ligand concentration) at neutral pH. (This is remarkable, considering that the water solubility of the free amine monomeric analogs **Ib-IVb** (Cf. text **Fig. 1**) is less than 1 µM).

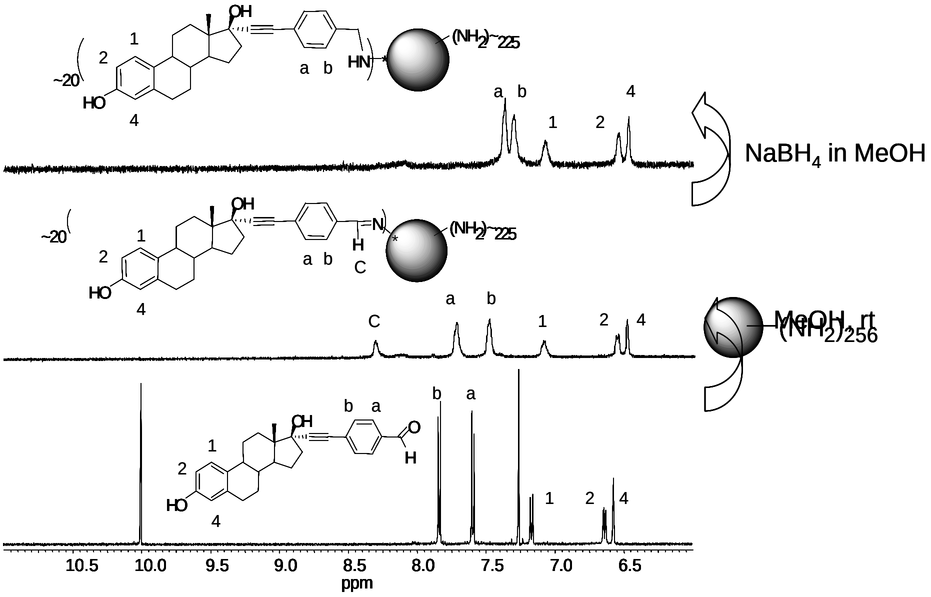

Figure S2. Aromatic region of ^{1}H NMR (CD_3OD) of free EE_2 (bottom), imine intermediate, and final EDC **I-a** after reductive amination.

1D ^{1}H NMR Spectra of EDC I-a and II-a, and Calculation of T_1 and T_2 and τ_c Values

In deuterated methanol, peaks in the aromatic region of both the short- and the long-tether EDCs are relatively sharp (**Fig. S3a**), showing a splitting pattern that is similar, though somewhat broader than those of the free ligands (compare with **Fig. S2**). A much more pronounced broadening effect in the aromatic region, however, was found in the ^{1}H NMR spectra in deuterated water (**Fig. S3b**), particularly with the long-tether EDC **II-a**, where peaks resolved in methanol merge in D_2O. Spectroscopic assignments in D_2O were facilitated by raising the temperature (**Fig. S3c**) and by reference to the NOSEY experiments, discussed below. In addition, the proton chemical shifts of the A ring hydrogens, the C-18 methyl group, and the linker aromatic protons were assigned by comparison with the chemical shifts of the corresponding model compounds **I-b** and **II-b** (see **Fig. S1**).

In the case of the short-tether EDC (**Fig. S3b**, left), all of the aromatic protons as well as the C-18 methyl resonances broadened and shifted upfield in D_2O compared to CD_3OD, consistent with a movement to an environment of greater shielding. The greatest upfield shift was experienced by the C-18 and C-1 protons of the steroid, with the aromatic protons of the linker phenyl group showing a somewhat smaller shift, and the C2 and 4 protons shifting to only a small extent. Significantly, all of the resonances of the short-tether EDC remain resolved, though broadened.

By contrast, in the case of the long-tether EDC (**Fig. S3b**, right), the aromatic protons showed a greater line broadening effect with the change of solvent from CD_3OD to D_2O, suggesting a even more pronounced change in environment than experienced by the short-tether EDC. At room temperature (**Fig. S3b**, right), the signals for the all three aromatic protons of the steroid A ring seemed to merge, and they appeared quite upfield, at around 6.5 ppm. The ^{1}H NMR spectrum of the long-tether EDC in D_2O at 90 °C is somewhat sharper, so it is possible to distinguish each aromatic proton (**Fig. S3c**, right). The C-1 proton of the A ring has shifted the most, 0.3 ppm upfield (its resonance at this much higher field position confirmed by interpretation of a 2D COSY ^1H NMR spectrum, and the other aromatic protons of the A ring appeared at ~0.1 ppm upfield compared to their position in deuterated methanol. In addition, the aromatic protons on the terminal and ligand-attached linker benzene rings appeared at 0.2 ppm upfield and 0.3 ppm upfield, respectively.

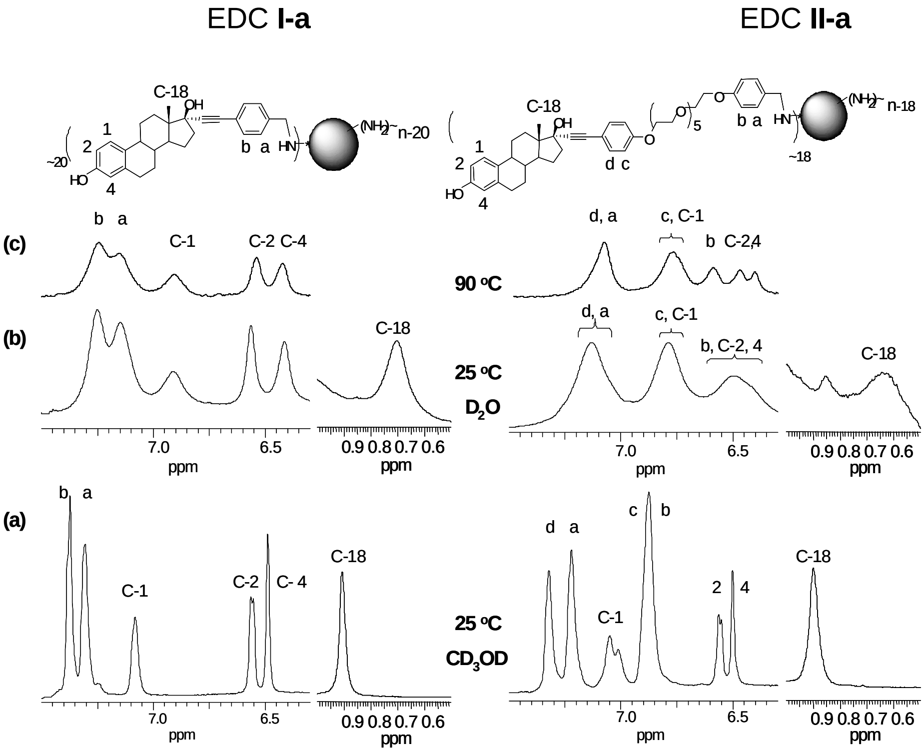

Figure S3. ^1H NMR of CH_3 at 18 position and A ring of EE_2 and linker aromatic region of ([G-6]-Short-Tether-EE2 **I-a** (left) and [G-6]-$(eg)_6$-EE2 **II-a** (right). (a) ^1H NMR in CD_3OD (**I-a,** left and **II-a** right) at 25 °C, (b) ^1H NMR in D_2O (**I-a,** left and **II-a** right) at 25 °C, and (c) ^1H NMR in D_2O (**I-a,** left and **II-a** right) at 90 °C.

The upfield shifts and pronounced broadening of the signals of both EDCs upon solvent change from CD_3OD to D_2O indicates that there is a change in environment to one that is more shielded and more restricted in D_2O than that experienced by the ligand (and tether) in CD_3OD. This is particularly true with the long-tether EDC **II-a**, suggesting that the longer tether of this EDC gives the hydrophobic estrogen ligand great flexibility to explore more environments, more so than in the short-tether EDC. This issue is explored further in the text of the paper.

The formulas below[3] describe T_1 and T_2 in terms of the magnetogyric ratio, angular momentum, magnetic field strength, and correlation times, as follows:

$$1/T_1 = 2/3\ \gamma^2\langle\Delta H^2\rangle[\tau_\chi/\{1+(\omega\tau_\chi)^2\} + 4\tau_\chi/\{1+(2\omega\tau_\chi)^2\}]$$

$$1/T_2 = 1/3\ \gamma^2\langle\Delta H^2\rangle[3\tau_\chi + \tau_\chi/\{1+(\omega\tau_\chi)^2\} + 4\tau_\chi/\{1+(2\omega\tau_\chi)^2\}]$$

where γ is the magnetiogyric ratio of the proton, ω is the proton resonance frequency. When $\omega\tau_c >> 1$ (i.e. slow motion), the ratio of $1/T_1$ to $1/T_2$ can be represented as a $T_2/T_1 \approx 8 / \{6(\omega\tau_c)^2 + 11\}$.

This formula can be rearranged to describe the rotation correlation time, τ_c.

$$\tau_c = (1/\omega)\ [\{8\ (T_1/T_2) - 11\}/6]^{1/2}$$

^{1}H COSY NMR Spectrum of the Long Tether EDC (II-a)

The ^{1}H COSY spectra of the long tether EDC (**II-a**) were in D_2O and methanol. The spectrum in water was taken at 80 °C; in D_2O at room temperature in this solvent the aromatic region could not be resolved because of extensive broadening of that spectral region.

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STANDARD PROTON PARAMETERS

Pulse Sequence: relayh
 Solvent: D2O
 Temp. 80.0 C / 353.1 K
File: COSY_KSH8-14-long_80oc_D2O_6-25-05
INOVA-500  "sundsl"

 Relax. delay 1.300 sec
 COSY 90-90
 Acq. time 0.102 sec
 Width    4996.3 Hz
 2D Width 4996.3 Hz
 16 repetitions
 128 increments
OBSERVE   H1, 499.6979344 MHz
DATA PROCESSING
 Sine bell 0.051 sec
F1 DATA PROCESSING
 Sine bell 0.013 sec
FT size 1024 x 1024
Total time 48 min, 42 sec
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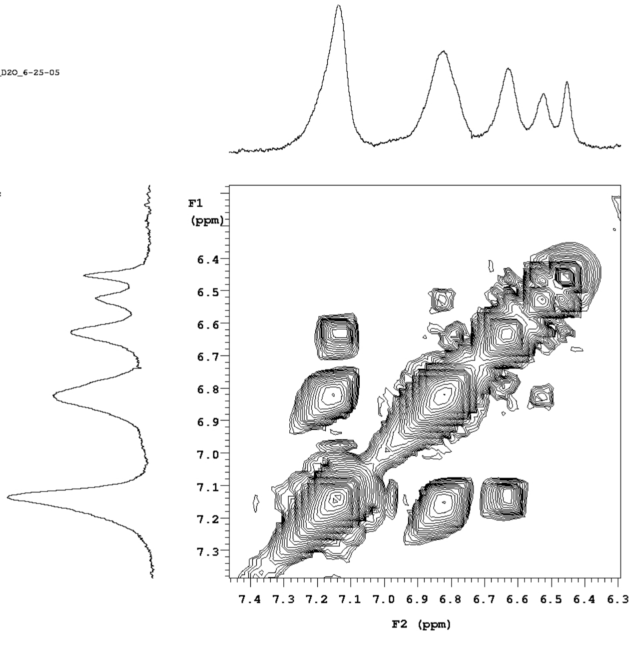
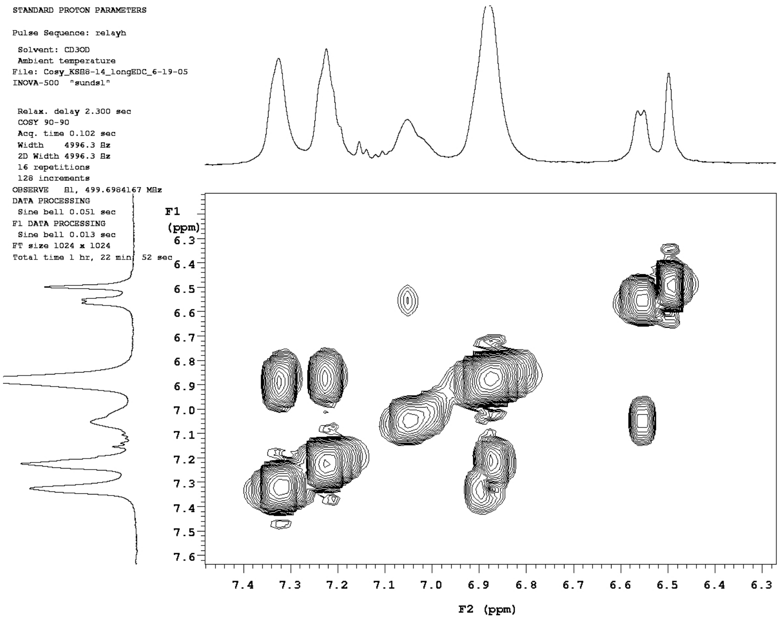


Figure S4. ***Aromatic expansion of COSY NMR for [G6]-(eg)$_6$-EE$_2$ (long-tether EDC, II-a) in D$_2$O at 80 °C.***



*COSY in methanol-d$_4$ was taken at room temperature.

Figure S5. ***Aromatic expansion of COSY* NMR for the Long Tether EDC II-a in Methanol-d$_4$***

Detailed Experimental Section

Materials and Methods

Compounds and materials were supplied from the sources indicated: radiolabeled estradiol ([^{3}H]E$_2$) ([6,7-^{3}H]estra-1,3,5(10)-triene-3,17β–diol, 52 Ci/mmol) (Amersham Biosciences, Piscataway, NJ), 17α-Ethynylestradiol (Steraloids, Newport, RI), PAMAM generation-6 dendrimer, sodium borohydride, *N*-Acetylethylenediamine, 4-bromobenzaldehyde, dibenzylidene acetone palladium (Pd$_2$(dba)$_3$), tri(*o*-toluyl)phosphine, and copper(I) iodide (Aldrich, Milwaukee WI), Amicon Centriplus YM-30 filter (Milipore, Bedford, MA), 17β-estradiol (Sigma, St. Louis, MO). 17α-[4-(ω-Methansulfonyl-hexa(ethylene glycolyl)phenyl] ethynyl-estra-1,3,5(10)-triene-3,17β-diol, 7a-(6-aminohexyl) estra-1,3,5(10)-triene-3,17β-diol, and 4-hydroxyphenyl-4'-aminoethoxyphenyl-methylenecyclohexane was prepared according to a literature procedure.[4, 5]

Proton ^{1}H NMR spectra including measuring spin-spin and spin-lattice relaxation time were recorded on a Varian Inova-500 at 500 MHz with methanol-d_4 or D_2O. Carbon ^{13}C NMR spectra were obtained on a Varian Inova-500 at 126 MHz with methanol-d_4 or D_2O. High- and low-resolution electron-ionization or matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were obtained on a Q-TOF Ultima API (Waters Co. Ltd.) or Voyager (Applied Biosystems Co. Ltd.) using DHB as a matrix.

Description of the Synthesis of the Aromatic Aldehyde Precursors for EDC Synthesis

17a-[(4-Formylphenyl)]ethynyl-estra-1,3,5(10)-triene-3,17b-diol [EE$_2$-Ph-CHO] 2a

To a mixture of 17α-ethynylestradiol **1a** (590 mg, 2 mmol), *p*-bromobenzaldehyde (740 mg, 4 mmol), $Pd_2(dba)_3$ (92 mg, 0.1 mmol), and P(*o*-Tol)$_3$ (243 mg, 0.8 mmol) in TEA (40 ml) was added CuI (112 mg, 0.6 mmol) under an argon atmosphere at rt. This reaction mixture was stirred for 4 h at rt. EtOAc (40 ml) was added to the concentrated reaction mixture, which was then washed with water (50 ml x 3). The organic phase was dried under vacuum and loaded on a silica gel column. Elution with a mixture of n-hexane and EtOAc (2:1) gave an off-white solid, EE$_2$-Ph-CHO (590 mg, 74%, R_f 0.45): mp 138-140 ^{o}C (dec.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.93 (s, 3H), 1.25-2.81 (m, 15H), 6.47 (d, 1H, J = 2.4 Hz), 6.53 (dd, 1H, J = 2.4 Hz, J = 8.0 Hz), 7.09 (d, 1H, J = 8.0 Hz), 7.60 (d, 2H, J = 10.5 Hz), 7.87 (d, 2H, J = 10.5Hz), 9.97 (s, 1H, aldehyde); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.09, 23.15, 26.64, 27.40, 29.82, 33.33, 39.26, 39.65, 43.85, 47.98, 50.15, 66.10, 85.48, 97.21, 110.00, 112.94, 115.48, 126.80, 129.79, 132.43, 132.63, 135.68, 138.47, 191.78; HRMS (EI) m/z calcd for $C_{27}H_{28}O_3$ (M^+) 400.203845, found 400.203872; Elemental analysis: Calcd for $C_{27}H_{28}O_3$ 1.0H_2O C 77.48, H 6.74, found C 77.80, H 6.74.

17a-[4-(? -(p-Formylphenyl)-hexa(ethyleneglycolyl)phenyl]ethynylestra-1,3,5(10)-triene-3,17ß-diol 2b

To the mixed solution of 17α-[4-(ω-methansulfonyl-hexa(ethyleneglycolyl)phenyl]ethynylestra-1,3,5(10)-triene-3,17β-diol (146.0 mg, 0.2 mmol) and K_2CO_3 (200.0 mg) in DMF (10 ml) was added *p*-hydroxybenzaldehyde (122.0 mg, 1.0 mmol) at rt. This solution was continued to stir until sulfonate was not detected on TLC. After typical work-up procedure, the title compound (129.0 mg, 85 %) was collected through silica gel column chromatography using ethyl acetate as a very viscous liquid.^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.90 (s, 3H), 2.80-2.86 (m, 2H), 3.64-3.71 (m, 18H), 3.85-3.87 (m, 2H), 4.09-4.12 (m, 2H), 4.18-4.20 (m, 2H), 6.06 (s, 1H), 6.56 (d, J = 2.5 Hz, C3), 6.62 (dd, J = 8.5, 2.5 Hz, 1H, C2), 6.83 (d, J = 10 Hz, 2H), 7.00 (d, J = 10 Hz, 2H), 7.12 (d, J = 8.5 Hz, 1H, C1), 7.35 (d, J = 10 Hz, 2H), 7.81 (d, J = 10 Hz, 2H), 9.86 (s, 1H); ^{13}C ($CDCl_3$, 126 MHz) δ 16.57, 26.71, 27.93, 29.73, 32.12, 34.85, 37.75, 44.53, 48.62, 51.10, 55.62, 67.61, 67.94, 69.66, 69.86, 70.76, 70.82, 71.03, 71.09, 83.95, 93.02, 112.89, 114.77, 115.11, 115.52, 116.18, 126.46, 130.23, 133.20, 135.17, 137.76, 138.36, 153.83, 158.79, 164.09, 191.18; MS (ESI) m/z Calcd for $C_{45}H_{56}O_{10}$ (M^++1) 757.4, found 757.6.

[4-Hydroxyphenyl-4'-[{(4-formyl)phenylcarbonyl}aminoethoxy]phenyl]methylenecylohexane (Cyclofenil-Et-Ph-CHO) (3)

To the solution of (4-hydroxyphenyl-4'-aminoethoxyphenyl)methylenecylohexane (323 mg, 1 mmol) in DMF was added 4-(dimethoxymethyl)benzoic acid *N*-hydroxysuccinicimide ester (350 mg, 1.19 mmol) and triethylamine (100 μl) at rt. The reaction was stirred until no more amine was detected on tlc. Ethanolamine (100 mg) was added to destroy the remaining ester. Dilution of reaction mixture with water (10 ml), extraction with ethyl acetate (10 ml x3), drying over $MgSO_4$, passed through short silicagel pad to remove polar compound, and evaporation gave [4-hydroxyphenyl-4'-[{(4-dimethoxymethyl)phenylcarbonyl}-aminoethoxy]phenyl]methylenecylohexane (Cyclofenil-Et-ph-CH(OMe)$_2$) (403 mg, 80%); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.51-1.58 (m, 6H), 2.20-2.24 (m, 4H), 3.32 (s, 6H), 3.86 (q, J = 6.5 Hz, 2H), 4.13 (t, J = 6.0 Hz, 2H), 5.43 (s, 1H), 6.12 (t, J = 6.5 Hz, 1H, NH), 6.73 (d, J = 8.5 Hz, 2H), 6.81 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 8.5 Hz, 2H), 7.02 (d, J = 8.5 Hz, 2H), 7.51 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 8.5 Hz, 2H); ^{13}C ($CDCl_3$, 126 MHz) δ 27.06, 28.88, 32.68, 39.85, 52.84, 66.93, (1 or 3 carbon of aliphatic region is lack), 102.51, 114.01, 114.98, 127.16, 127.29, 131.26, 133.45, 134.52, 135.96, 136.82, 138.74, 141.80, 154.21, 156.88, 167.67; MS (ESI) m/z Calcd for $C_{31}H_{36}NO_5$ (M^++1) 502.3, found 502.3.

To this aldehyde (50 mg, 0.1 mmol) in THF (1 ml) and acetone (0.1 ml) was added 1 drop of 1N HCl. The reaction was monitored by tlc. When the reactant was disappeared on tlc, sat. $NaHCO_3$ was added to the solution. Product was extracted with ethyl acetate (5 ml x 3), followed by drying and evaporation to afford 35 mg (77%) of white solid: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.51-1.58 (m, 6H), 2.19-2.24 (m, 4H), 3.85-3.89 (m, 2H), 4.14 (t, J = 4.5 Hz, 2H), 6.74 (d, J = 8.5 Hz, 2H), 6.81 (d, J = 8.5 Hz, 2H), 6.93 (d, J = 8.5 Hz, 2H), 7.14 (d, J = 8.5 Hz, 2H), 7.92 (s, 4H), 10.05 (s, 1H); ^{13}C ($CDCl_3$, 126 MHz) ? 27.01, 28.85, 28.87, 32.65, 32.68, 40.06, 66.68, 114.00, 115.02, 127.99, 130.16, 131.23, 131.29, 133.45, 135.78, 136.99, 138.49, 138.77, 139.59, 154.44, 156.76, 166.98, 191.94; mp 80.5-82; HRMS (ESI) m/z Calcd for $C_{29}H_{30}NO_4$ (M^++1) 456.2175, found 456.2181.

7α-[(4-formylphenyl)carbonylaminohexyl]-1,3,5(10)-triene-3,17ß-diol (7α-hexylPh-CHO, 4)

As described in preparation of **3**, (7α-[(4-dimethoxymethylphenyl)carbonylaminohexyl]-1,3,5(10)-triene-3,17ß-diol was obtained from the reaction of 7α-(ω-aminohexyl)-1,3,5(10)-triene-3,17ß-diol (50 mg, 0.13 mmol) with 4-(dimethoxymethyl)benzoic acid N-hydroxysuccinicimide ester (100 mg, 0.34 mmol) as a sticky liquid: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.78 (s, 3H), 2.67 (d, J = 16.5 Hz, 1H), 2.84 (dd, J = 17 Hz, 5.5 Hz, 1H), 3.35 (s, 6H), 3.38-3.44 (m, 2H), 3.74 (t, J = 6.5Hz, 1H), 5.63 (brs, 1H), 6.18 (t, J = , 1H, NH), 6.57 (d, J = 2.5 Hz), 6.63 (dd, J = 8.5, 2.5 Hz, 1H), 7.13 (d, J = 8.5 Hz, 1H), 7.52 (d, J = 8.0 Hz, 2H), 7.78 (d, J = 8.0 Hz, 2H); ^{13}C ($CDCl_3$, 126 MHz) δ (127 Hz) 11.28, 22.85, 25.48, 27.06, 27.45, 28.09, 29.71, 29.79, 30.72, 33.47, 34.85, 37.07, 38.38, 40.36, 42.22, 43.60, 46.67, 52.91, 52.93, 82.21, 102.63, 113.21, 116.45, 127.03, 127.26, 131.85, 135.00, 137.20, 141.62, 153.92. 167.60; HRMS (ESI) m/z calcd for $C_{34}H_{48}NO_5$ (M^++1) 550.3519, found 550.3516.

Subsequent hydrolysis of this aldehyde dimethyl acetal as described in prepararion of **3** gave off-white solid (25 mg, 38%): ^{1}H NMR (MeOH-d_4, 500 MHz) δ 0.77 (s, 3H), 2.68 (d, J = 16.5 Hz, 1H), 2.86 (dd, J = 17 Hz, 5.5 Hz, 1H), 3.39-3.4 (m, 2H), 3.73 (t, J = 6.5Hz, 1H), 6.32 (s, 1H, NH), 6.56 (d, J = 2.5 Hz), 6.64 (dd, J = 8.5, 2.5 Hz, 1H), 7.12 (d, J = 8.5 Hz, 1H), 7.92 (d, J = 8.0 Hz, 2H), 7.97 (d, J = 8.0 Hz, 2H), 10.07 (s, 1H); ^{13}C (MeOH-d_4, 126 MHz) δ 11.30, 22.85, 25.57, 27.13, 27.45, 28.16, 29.78, 30.50, 30.71, 33.47, 34.83, 37.08, 38.35, 40.58, 42.196, 43.60, 46.67, 82.21, 113.21, 116.44, 127.27, 127.86, 130.12, 131.72, 137.16, 138.35, 140.12, 154.06, 166.78, 191.90; mp 114-115.5; HRMS (ESI) m/z calcd for $C_{32}H_{42}NO_4$ (M^++1)

504.3114, found 504.3096.

Synthesis of Control Compounds (I-IVb)

17a-[4-(p-Acetylaminoethylamino)phenyl]ethynylestra-1,3,5(10)-triene-3,17b-diol (EE$_2$-Ph-NHAc) (I-b)

To the solution of 17α-[(4-formylphenyl)]ethynyl-estra-1,3,5(10)-triene-3,17β-diol [EE$_2$-Ph-CHO] (100.0 mg, 0.25 mmol) in methanol (5 ml) was added N-acetyl ethylenediamine (10.0 mg) at rt. When aldehyde disappeared on TLC, NaBH$_4$ was added. The solution was concentrated under vacuum and loaded on silica gel column. Elution with 20% MeOH-CH$_2$Cl$_2$ gave off white solid (80 mg, 66%). mp 100-101.5 ^{o}C; ^{1}H NMR (MeOH-d$_4$, 500 MHz) d 0.92 (s, 3H), 1.92 (s, 3H), 2.73 (t, J = 6.5, 2H), 2.75-2.79 (m, 2H), 3.33 (t, J = 6.5, 2H), 3.80 (s, 2H), 6.47 (d, J = 2.5, 1H, C3), 6.53 (dd, J = 9.0, 2.5, 1H, C2), 7.09, (d, J = 9.0 Hz, 1H), 7.32 (d, J = 8.0 Hz, 2H), 7.39 (d, J = 8.0, 2H); ^{13}C NMR (MeOH-d$_4$, 126 MHz) d 12.29, 21.33, 22.63, 26.57, 27.45, 29.54, 33.22, 33.54, 38.31, 38.70, 39.99, 44.03, 49.96, 52.32, 53.88, 66.69, 79.64, 84.88, 110.00, 112.56, 114.84, 122.66, 126.10, 128.61, 131.26, 131.43, 137.63, 154.79, 172.56. mp 100-101.5 ^{o}C. HRMS (ESI) m/z calcd for $C_{31}H_{39}N_2O_3$ (M$^+$+1) 484.2961, found 487.2958

17a-[4-{w-(p-Acetylaminoethylamino)phenyl-(hexaethylene glycolyl)}phenyl] ethynyl-estra-1,3,5(10)-triene-3,17ß-diol (EE2-(eg)$_6$-Ph-NHAc) (II-b)

Model Compound ***II-b*** (35 mg, 58%) was obtained from N-acetyl ethylenediamine (100 mg) and 17a-[4-(? -(p-formylphenyl)-hexa(ethylene glycolyl)phenyl]ethynylestra-1,3,5(10)-triene-3,17ß-diol (55 mg, 0.072 mmol) according to the procedure preparing **I-b**. ^{1}H NMR (MeOH-d$_4$, 500 MHz) δ 0.91 (s, 3H), 1.97 (s, 3H), 2.76 (t, J = 6.0 Hz, 2H), 2.81-2.88 (m, 2H), 3.34 (q, J = 6.0 Hz, 2H), 3.62-3.75 (m, 16H), 3.82-3.88 (m, 4H), 4.07-4.14 (m, 4H), 6.09 (m, 1H), 6.10 (s, 1H), 6.56 (s, 1H), 6.57 (d, J = 8.5 Hz, 1H), 6.84 (d, J = 8.5 Hz, 1H), 6.86 (d, J = 8.5 Hz, 1H), 7.11 (d, J = 8.5 Hz, 1H), 7.17 (d, J = 8.5 Hz, 1H), 7.36 (d, J = 8.5 Hz, 1H); ^{13}C NMR (MeOH-d$_4$, 126 MHz) δ 16.58, 21.66, 23.51, 26.73, 27.95, 29.75, 32.13, 34.88, 37.56, 37.77, 39.32, 44.53, 48.09, 53.12, 55.63, 67.63, 69.86, 69.95, 70.77, 70.83, 71.01, 71.07, 79.66, 84.98, 110.01, 112.96, 114.82, 114.85, 115.61, 126.38, 129.59, 132.98, 133.11, 133.20, 135.11, 137.78, 138.33, 154.03, 170.95. HRMS (ESI) m/z calcd for $C_{49}H_{67}N_2O_{10}$ (M$^+$+1) 843.4796, found 843.4813.

Cyclofenil-Ph-NHAc (III-b)

As described in ***I-b***, ***III-b*** (15 mg, 54%) was obtained from the reaction of ***3*** (25 mg, 0.05 mmol) with 2-aminoethyl aetamide as a white solid: ^{1}H (MeOH-d$_4$, 500) δ 1.51-1.62 (m, 6H), 1.98 (s, 3H), 2.18-2.24 (m, 4H), 3.19 (t, J = 6.5 Hz, 2H), 3.51 (t, J = 6.5 Hz, 2H), 3.75 (t, J = 6.0 Hz, 2H), 4.15 (t, J = 6.5 Hz, 2H), 4.29 (s, 2H), 6.66 (d, J = 8.5 Hz, 2H), 6.85 (d, J = 8.5 Hz, 2H), 6,86 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 8.5 Hz, 2H), 7.57 (d, J = 8.5 Hz, 2H), 7.90 (d, J = 8.5 Hz, 2H); ^{13}C (126 MHz, CD_3OD) δ 21.22, 26.62, 28.30, 32.15, 33.43, 35.70, 39.54, 41.05, 50.67, 56.20, 66.54, 110.06, 113.97, 114.24, 127.89, 129.47, 130.35, 130.43, 134.67, 134.93, 135.74, 136.41, 137.30, 155.63, 157.96, 168.09, 173.98; Mp 134-135.5 ^{o}C; HRMS (ESI) m/z calcd for $C_{33}H_{40}N_3O_4$ (M^{+}+1) 542.3021, found 542.3019

7a-[4-{w-(p-Acetylaminoethylamino)phenyl}carbolyl}aminohexyl]-estra-1,3,5(10)-triene-3,17ß-diol (IV-b)

As described in ***I-b***, ***IV-b*** (13 mg, 44%) was obtained from the reaction of ***3*** (26 mg, 0.05 mmol) with 2-aminoethyl aetamide as a white solid: ^{1}H (MeOH-d$_4$, 500) δ 0.77 (s, 3H), 1.93 (s, 3H), 2.22-2.35 (m, 2H), 2.70 (t, J = 6.0 Hz, 2H), 2.75-2.84 (m, 1H), 3.35 (t, J = 6.0 Hz, 2H), 3.64 (t, J = 8.5 Hz, 1H), 3.81 (s, 2H), 6.45 (d, J = 2.0 Hz, 1H), 6.54 (dd, J = 8.0, 2.0 Hz, 1H), 7.07 (d, J = 8.0 Hz, 1H), 7.43 (d, J = 8,0 Hz, 2H), 7.77 (d, J = 8,0 Hz, 2H); ^{13}C (126 MHz, CD_3OD) δ 21.37, 22.41, 25.24, 25.61, 26.84, 27.40, 27.87, 29.22, 29.47, 33.40, 34.52, 36.41, 37.04, 38.42, 38.67, 39.74, 42.47, 43.30, 46.56, 52.45, 68.13, 81.35, 112.75, 115.74, 126.70, 127.25, 128.38, 130.63, 133.50, 136.44. 143.26, 154.83, 168.74, 172.40; HRMS (ESI) m/z calcd for $C_{36}H_{52}N_3O_4$ (M^{+}+1) 590.3958, found 590.3947

General Procedure for the Preparation of EDCs (I-IVa)

Short-Tether EDC ([G6]-EE$_2$, I-a)

To the solution of PAMAM G6 (200 mg, 0.17 μmol, 5% methanol solution) in methanol (2.0 ml) was added EE$_2$-Ph-CHO (1.7 mg, 3.4 μmol) in methanol (1.0 ml) at room temperature. This solution was stirred continuously and monitored until no aldehyde could be detected by thin layer chromatographic analysis. ^{1}H

NMR (500 MHz, methanol-d_4; all signals are broad) δ 8.31 (s, imine), 7.72 (s, ArH), 7.48 (s, ArH), 7.09 (s, 1-CH of A ring), 6.56 (brd, 2-CH of A ring), 6.49 (s, 4-CH of A ring), 3.26 (s, dendrimer), 2.79 (s, dendrimer), 2.57 (s, dendrimer), 2.36 (s, dendrimer), 1.89 (s), 1.42 (s), 1.28(s), 0.92 (s, 19-CH_3).

To the reaction mixture in methanol was added a freshly prepared 1 M $NaBH_4$ methanol solution (10 µl). This solution was stirred for 30 min at rt. The reaction mixture was transferred to an Amicon filter (Centriplus YM-30, cut-off MW 30,000); the volume was adjusted to be about 10 ml of methanol, and the filter was centrifuged at ~3000 x g until the entire solution had passed through the filter (~ 5 hrs). This wash-centrifugation procedure was repeated for a total of five times. The concentrated residue was dried under vacuum to afford a yellowish solid, **EDC *I-a*** (12 mg), which was immediately dissolved into DI water. Upon long-term storage as a solid, in the refrigerator, this material changed its physical properties so that it became insoluble in water. ^1H NMR (500 MHz, methanol-d_4; all signals are broad) δ 0.92 (s, 19-CH_3), 1.29 (s, ligand), 1.42 (s, ligand), 1.80 (s, ligand), 2.36 (s, dendrimer), 2.57 (s, dendrimer), 2.79 (s, dendrimer), 3.26 (s, dendrimer), 3.75 (s, ligand-$BnC\underline{H}_2$-NH-G6), 6.49 (s, 4-CH of A ring), 6.55 (s, 2-CH), 7.09 (s, 1-CH), 7.32 (s, ArH), 7.40 (s, ArH); ^{13}C NMR (126 MHz, methanol-d_4) δ (12.61), 22.79, 26.69, 27.52, 29.60, 33.62 (G6), 37.44 (G6), 38.70, 38.91, 40.01, 40.84 (G6), 41.65 (G6), 44.06, 48.42, 48.64 (G6), 49.96 (G6), 52.34 (G6), 52.71, 53.80, 63.21, 72.68, 79.58, 85.00, 112.85, 115.09, 122.27, 126.30, 128.54, 131.20, 131.50, 137.71, 155.05, 164.62, 173.47 (G6), 173.95 (G6). See **Table 1** for MALDI-TOF.

Long-Tether EDC ([G6]-(eg)$_6$-EE$_2$, II-a)

Long-tether EDC ***II-a*** (12 mg) was obtained from the reaction of PAMAM G6 (200 mg, 0.17 µmol, 5% methanol solution) and (2.6 mg, 3.4 µmol) 17a-[4-(? -(*p*-formylphenyl)-hexa(ethyleneglycolyl)phenyl]-ethynylestra-1,3,5(10)-triene-3,17ß-diol following the same procedure as was used for preparing the short-tether EDC (***I-a***).^1H NMR (CD_3OD, 500 MHz) δ 0.90 (s, 19-CH_3), 2.36 (s, $COCH_2$, G6), 2.58 (s, NCH_2 or CH_2CO, G6), 2.79 (s, CH_2NH_2, $(CH_2)_2N$-, G6), 3.26 (s, $CONHCH_2$, G6), 3.60 (s, eg), 3.66 (s, eg), 3.79 (s, ligand-$BnC\underline{H}_2$-NH-G6), 4.08, 6.01, 6.50 (s, 4-CH of A ring), (d, J = 5 Hz, 2-CH of A ring), 6.88 (s, terminal ArH), 7.01, 7.05 (s, s, 1-CH of A ring), 7.22, 7.32 (s, s, ligand attached ArH); ^{13}C NMR (CD_3OD, 126 MHz) δ

15.95, 26.66, 27.92, 29.47, 31.67, 33.03 (G6), 34.86, 37.45 (G6), 38.03, 38.69, 40.87 (G6), 41.70 (G6), 44.56, 45.07, 48.70 (G6), 49.98 (G6), 52.36 (G6), 53.81, 55.68, 67.46, 67.57, 69.56, 69.68, 70.41, 70.58, 83.64, 92.87, 112.08, 114.45, 115.13, 116.03, 125.95, 129.73, 131.40, 132.91, 134.64, 137.62, 137.95, 155.06, 173.45 (G6), 173.99 (G6). See **Table 1** for MALDI-TOF.

Cyclofenil EDC (III-a)

Cyclofenil EDC ***III-a*** (11 mg) was obtained from the reaction of PAMAM G6 (200 mg, 0.17 μmol, 5% methanol solution) with [4-Hydroxyphenyl-4'-[{(4-formyl)phenylcarbonyl}aminoethoxy]phenyl]-methylene-cylohexane (Cyclofenil-Et-ph-CHO) ***(3)*** (1.55 mg, 3.4 μmol) following the same procedure as was used for preparing the short-tether EDC (***I-a***). ^{1}H NMR (CD_3OD, 500 MHz) δ 1.54 (s, Cyclofenil -$CH_2CH_2CH_2$-), 2.20 (s, =$(CH_2)_2$), 2.37 (s, $COCH_2$, G6), 2.57 (s, NCH_2 or CH_2CO, G6), 2.79 (s, CH_2NH_2, $(CH_2)_2$N-, G6), 3.26 (s, $CONHCH_2$, G6), 3.75 (s, ligand-BnC$\underline{H}_2$-NH-G6), 3.82 (s, Ph-CONH-C$\underline{H}_2$-CH_2-Cyclofenil), 4.15 (s, Ph-CONH-CH_2-C$\underline{H}_2$-Cyclofenil), 6.69 (s, ArH), 6.85 (s, ArH), 6.96 (s, ArH), 7.43 (s, tether ArH), 7.82 (s, tether ArH); ^{13}C (MeOH-d_4, 126 MHz) δ 26.70, 28.62, 32.32, 33.58 (G6), 37.43 (G6), 38.67, 39.55, 40.56 (G6), 40.90 (G6), 48.60 (G6), 49.94 (G6), 52.29 (G6), 63.20, 66.24, 109.94, 113.93, 114.65, 125.64, 127.50, 128.44, 130.76, 133.50, 134.29, 136.40, 137.60, 157.35, 160.30, 164.56, 173.87 (G6), 174.11 (G6). See **Table 1** for MALDI-TOF.

7α-E_2-Hexyl-EDC (IV-a)

Cyclofenil EDC ***III-a*** (12 mg) was obtained from the reaction of PAMAM G6 (200 mg, 0.17 μmol, 5% methanol solution) with *7α-**[(4-formylphenyl)carbonylaminohexyl]-1,3,5(10)-triene-3,17ß-diol (7α-hexylPh-CHO, 4)*** (1.71 mg, 3.4 μmol) following the same procedure as was used for preparing the short-tether EDC (***I-a***). ^{1}H NMR (CD_3OD, 500 MHz) δ 0.75 (s, 19-CH_3), 1.35 (s, ligand), 1.58 (s, ligand), 1.72 (s, ligand), 1.79 (s, ligand), 1.89 (s, ligand), 2.37 (s, dendrimer), 2.58 (s, dendrimer), 2.79 (s, dendrimer), 3.26 (s, dendrimer), 3.64 (s, ligand), 3.82 (s, ligand-BnC$\underline{H}_2$-NH-G6), 6.47 (s, 4-CH of A ring), 6.55 (s, 2-CH 4-CH of A ring), 7.06 (s, 1-CH 4-CH of A ring), 7.42 (s, ArH), 7.78 (s, ArH); ^{13}C (MeOH-d_4, 126 MHz) δ 10.86, 22.59, 25.38.26.96, 27.96, 29.48, 30.66, 33.62 (G6), 34.70, 35.30, 37.20, 37.47 (G6), 38.45, 38.73, 39.88, 40.66 (G6), 40.93 (G6), 42.51, 43.39, 45.11, 46.61, 48.68 (G6), 49.97 (G6), 63.23, 72.70, 81.33, 112.91, 115.93, 126.90, 127.36, 128.42, 130.61, 133.52, 136.50, 143.55, 154.98, 164.54, 173.49 (G6), 173.81 (G5), 174.07 (G6). See **Table 1** for MALDI-TOF.

The mass and polydispersity of the EDCs were determined by MALDI-MS; results are presented in **Table 1**. It is notable that the G6 PAMAM dendrimer has a measured MW of ca. 44,000, considerably less than the nominal MW of 56,000, indicating that there are some defects occur in the synthesis of this high generation dendrimer. The mass increments between EDCs **I-IVa** and the G6 PAMAM dendrimer itself were used to determine the average number ligands that became attached to the dendrimer. The polydispersity of the EDC is relatively low.

Table 1. MALDI-TOF data of EDCs and G6 PAMAM dendrimer

	G6	***[G6]-EE$_2$, I-a***	***([G6]-(eg)$_6$-EE$_2$, II-a***	**cyclofenil –EDC, *III-a***	**7a-E2-EDC, IV-a**
M_n	43,525	50,934	57,427	52,162	52,792
M_w	44,691	52,716	58,946	53,890	54,077
PI*	1.02	1.03	1.03	1.03	1.02

* PI = Polydispersity index

Other Methods

Estrogen Receptor Binding Affinity Assays

Relative binding affinities were determined by a competitive radiometric binding assay as previously described,[S2,3] using 10 nM [^{3}H]estradiol as tracer (Amersham BioSciences, Piscataway, NJ), and purified full-length human ERα and ERß (PanVera/InVitrogen, Carlsbad, CA). The dendrimer conjugates were assayed from 10^{-4} to 10^{-9} M as equivalents of estradiol (20 fold lower than the molar concentration of the dendrimer itself). Incubations were for 18-24 h at 0 °C, and the receptor-dendrimer complexes were absorbed onto hydroxyapatite (BioRad, Hercules, CA), and the unbound dendrimer was washed away.

The binding affinities are expressed as relative binding affinity (RBA) values, with the RBA of estradiol for both receptors being set at 100. The values given are the average ± range or SD for two or more independent determinations. Estradiol binds to ERα with a K_d of 0.2 nM and to ERß with a K_d of 0.5 nM.

Dynamic Light Scattering

These measurements were performed at 25 °C using a DynaPro-MS/X instrument from Protein Solutions, Inc., (Charlottesville, VA), equipped with He-Ne Laser (10 mW) operating at 632.8 nm. All samples were measured at a 90° scattering angle. Deionized water and HPLC grade methanol were filtered through a 0.2 μm Nylon filter from Millipore before use. The autocorrelation function of intensity was analyzed by the cumulants analysis method to obtain the average diffusion coefficient, D, of the particles and the polydispersity. The hydrodynamic diameter, D_h, was calculated from the Stokes-Einstein equation ($D_h = k_B T/3phD$, where $k_B T$ is the thermal energy and h is the viscosity of the continuous phase).

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