Dendrimers

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Hormone–PAMAM Dendrimer Conjugates: Polymer Dynamics and Tether Structure Affect Ligand Access to Receptors**

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The development of ligand–polymer conjugates as diagnostic and therapeutic agents raises an interesting question: In what way do the conformational and dynamic features of the macromolecular carrier influence ligand access to its target receptor? Poly(amide)polyamine (PAMAM) dendrimers, in particular, are being widely adapted to biomedical applica-

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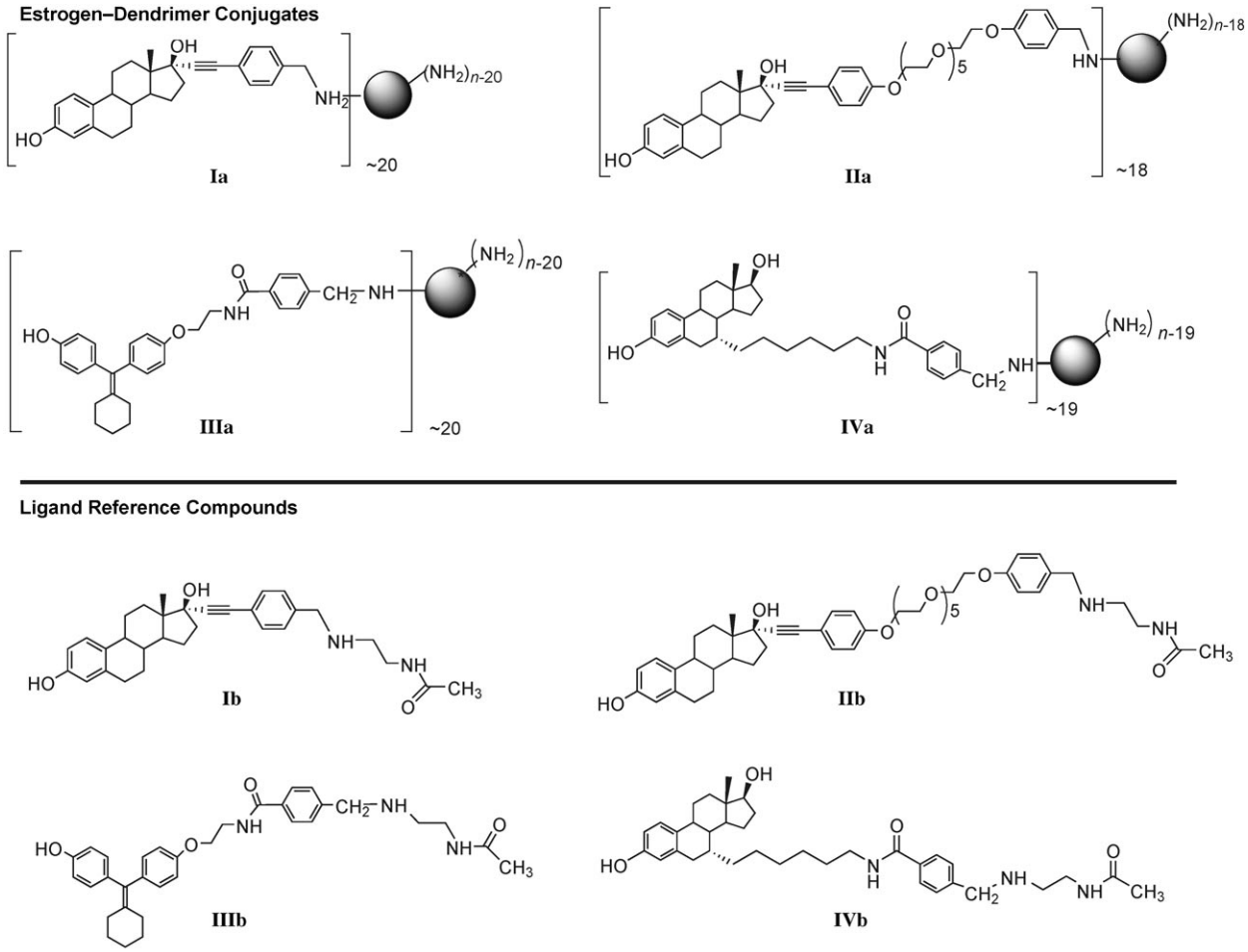
Supporting information for this article (details of the preparation and characterization of estrogen–dendrimer conjugates) is available on the WWW under http://www.angewandte.org or from the author.

tions,[1–5] yet little is known about how their complex, pH-dependent conformation and flexibility[6–8] affects the accessibility of covalently attached ligands. We have used spectroscopic and physical methods to characterize ligand dynamics and access to the receptor in four estrogen–dendrimer conjugates (EDCs) that were prepared to study pathways of estrogen signaling.[9] Curiously, EDCs having long or hydrophobic tethers engender considerable ligand shielding that results in poor access to the receptor, whereas those with short tethers expose the ligand so it binds to the receptor with little impediment from the dendrimer carrier.

The four EDCs (Scheme 1) are based on a generation six (G6) PAMAM dendrimer, with a molecular weight of approximately 58000,[1] comparable to that of bovine serum albumin (67000) used to construct other estrogen–protein conjugates.[10,11] Three are linked to estradiol, either through 17α-ethynyl (**Ia** and **IIa**) or 7α sites (**IVa**), which are known to tolerate substitution,[12] while the fourth is based on the nonsteroidal ligand cyclofenil (**IIIa**).[13] The linking tethers are short (**Ia** and **IIIa**), moderate (**IVa**), or long (**IIa**, hexaethylene glycol). Four compounds (**Ib**–**IVb**) in which the tether chain was terminated by an (acetamido)ethylamine function, which mimicks the first dendrimer attachment site, were prepared for reference.

The EDCs were prepared by reductive amination of the appropriate estrogen aryl carboxaldehyde with the primary amine termini of the G6 PAMAM. Imine formation proceeded spontaneously, and reduction with borohydride was quantitative; these steps could be followed by ^{1}H NMR spectroscopy. The final ligand:dendrimer ratio (18:1–20:1, Scheme 1), determined by MALDI MS, simply reflected the reaction stoichiometry. The resulting EDCs were purified by ultrafiltration.[9]

Although conformationally mobile, G6 PAMAMs are believed to be roughly spherical up to the generation five layer.[6,7] Conformation and flexibility are predicted to be pH-dependent,[6,8] with low pH values favoring rigid, extended forms (because of Coulombic repulsion) and high pH values producing more dense compact forms. At neutral pH, the peripheral amine groups are partially protonated, with the outer layers constituting a relatively flexible framework having pockets,[6–8] a characteristic through which PAMAMs can be used as drug and gene delivery systems.[14,15] In our EDCs we have added a peripheral hydrophobic group (estrogen) to a macromolecule that has a relatively hydrophilic surface, but which is also porous with a more hydrophobic interior. Thus, the disposition of the ligand with respect to the surface—Is it extended outward and thus



Scheme 1. Estrogen–dendrimer conjugates (EDCs, **Ia**–**IVa**) and reference compounds (**Ib**–**IVb**).

available to a receptor, or is it buried within the G6 layer and inaccessible to other proteins?—becomes an important issue.

NOESY spectra show interactions between the ligand and the dendrimer backbone (and sometimes tether). In both the short- and long-tether EDCs the aromatic signals (C-1, 2, 4; a–d) show strong cross-peaks in D_2O with 4/5 resonances that can be assigned to the dendrimer backbone (f–k); these cross-peaks are very weak in methanol (Figures 1 and 2, top versus bottom spectrum). Significantly, the long-tether EDC in D_2O (**IIa**, Figure 2, bottom) shows there are strong interactions between the resonances of the ethylene glycol spacer (eg) and the aromatic resonances of the ligand (C-1, 2, 4), even though

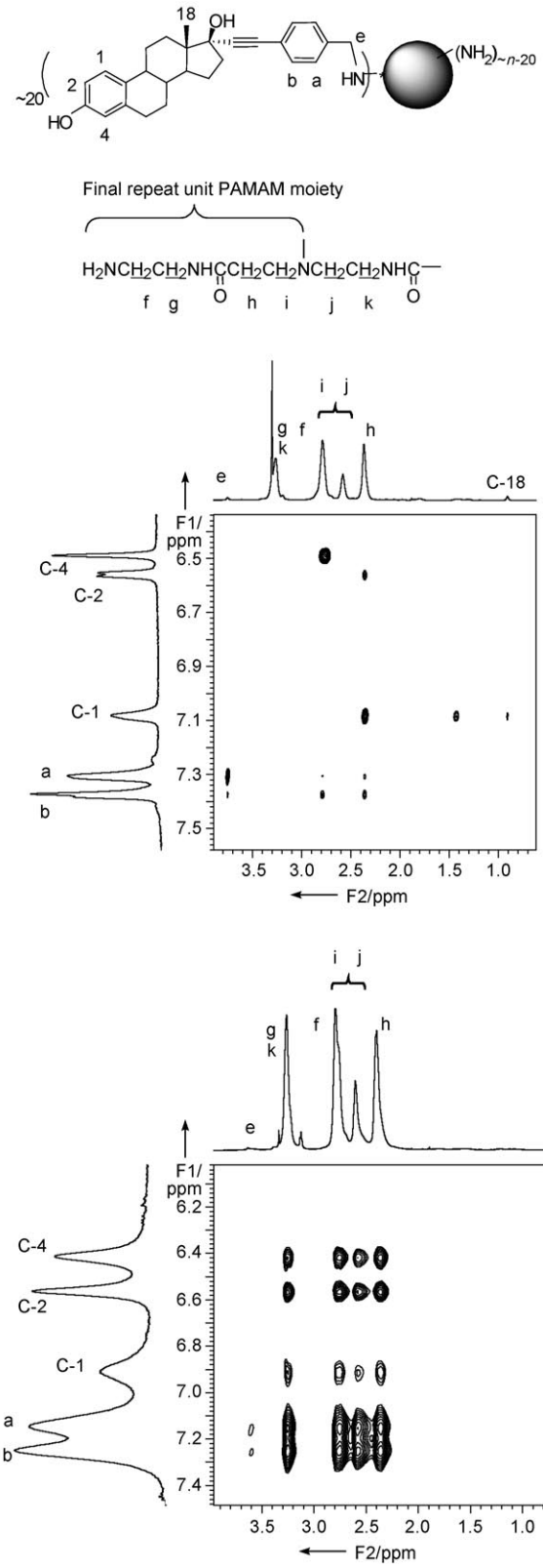


Figure 1. Aliphatic and aromatic region of the ^{1}H NMR NOESY spectra of short-tether EDC **Ia** in CD_3OD (top) and in D_2O (bottom).

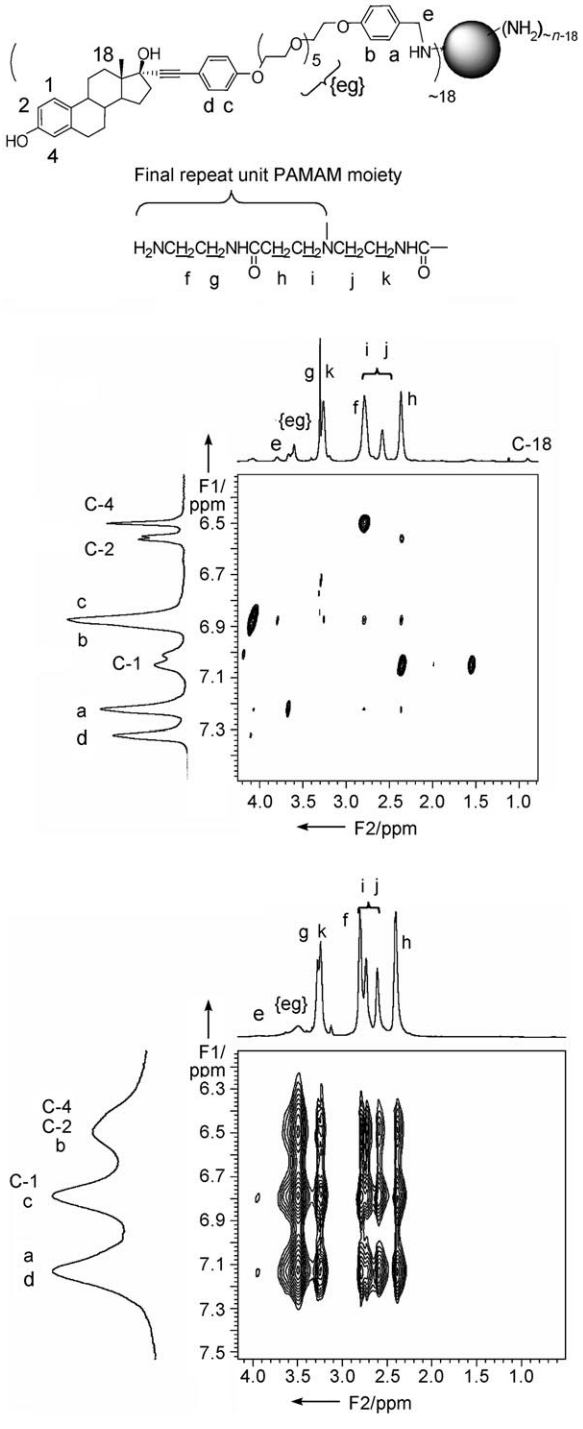


Figure 2. Aliphatic and aromatic region of the ^{1}H NMR NOESY spectra of long-tether EDC **IIa** in CD_3OD (top) and in D_2O (bottom).

they are at opposite ends of the ligand. Thus, in an aqueous environment, but not in methanol, the ligand portion of the long-tether EDC becomes enwrapped by the ethylene glycol tether and buried within the dendrimer with sufficient stability to generate large NOE signals. Although oligoethylene glycol derivatives are water compatible and flexible, they have a coiled structure, engendered by the gauche preference of the glycol unit,[16–19] which probably contributes to this ligand-tether-dendrimer interaction.

Spin–lattice and spin–spin relaxation times T_1 and T_2 for the C-4 proton of the EDCs **Ia** and **IIa**, as well as their reference compounds **Ib** and **IIb**, are given in Figure 3. The

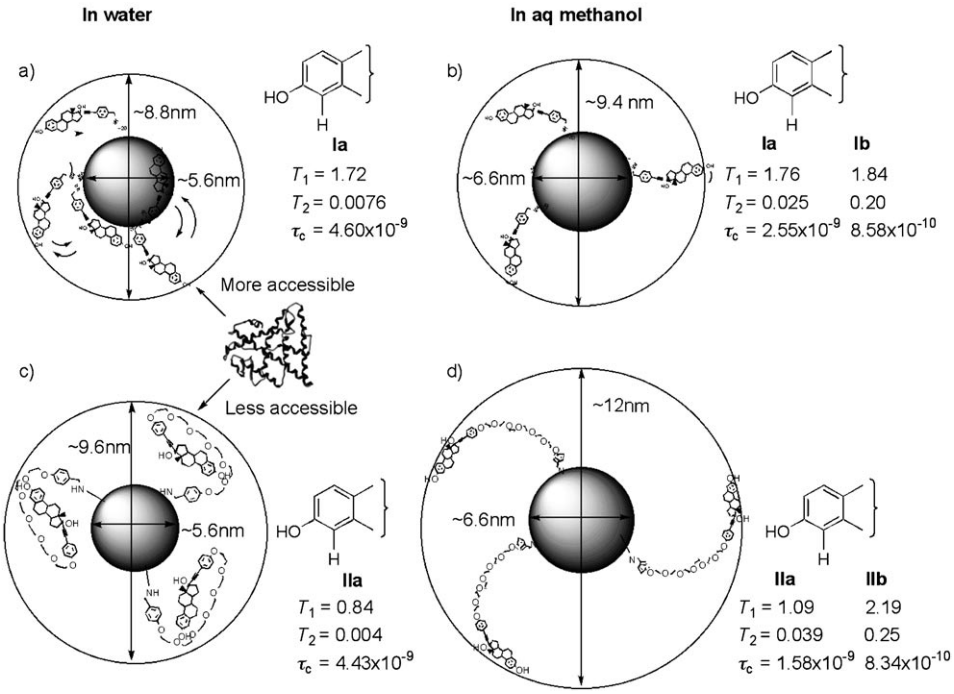


Figure 3. ^{1}H NMR relaxation and rotational correlation times (in seconds) and hydrodynamic diameter measurements for the short-tether (**Ia**) and long-tether (**IIa**) EDCs: a) EDC **Ia** in water, b) EDC **Ib** in 20% MeOH in water, c) EDC **IIa** in water, d) EDC **IIb** in 20% MeOH in water. The diameters, measured by dynamic light scattering studies, are shown schematically; the inner circle represents the PAMAM and the outer circle the EDC diameter. The spin–lattice and spin–spin relaxation times (T_1 and T_2) and the rotational correlation time (τ_c) are given for the C-4 proton of the 17α-ethynylestradiol in the EDCs and control compounds. The abbreviated structure of the ligand is meant to represent the degree to which the ligand is extended or enveloped relative to the PAMAM.

T_1 value for the C-4 ligand proton in the long-tether EDC (**IIa**) in D_2O is considerably less than that in CD_3OD; in the latter solvent, the T_1 value is very similar to that of the monomeric ligand analogue **IIb**. In contrast, there was little difference in the T_1 value for the short-tether EDC (**Ia**) in the two solvent systems, both values being similar to those of the monomeric ligand analogue **Ib**. This finding suggests that in hydrophilic solvents, only the ligand in the long-tether EDC (**IIa**) undergoes a change of environment, which causes its motion to become more restricted (increasing spin–lattice relaxation; smaller T_1 values). The T_2 values of both the short- and long-tether EDCs decrease upon shifting from CD_3OD to D_2O, but the decrease was more pronounced for the long-tether EDC (**IIa**). The T_2 values for the monomeric ligand analogues (**Ib** and **IIb**) are, as expected, much larger.

The rotational correlation times of the C-4 proton on the A ring of the steroid, calculated from the T_1 and T_2 values,[20] show that the tumbling motion of two monomeric ligands (**Ib** and **IIb**) in CD_3OD is very similar, whereas the tumbling motion of the two EDCs showed a twofold difference in methanol, but only a 1.3-fold difference in D_2O. We interpret the effect of solvent on the rotational correlation times to mean the following: in CD_3OD, the size difference between the short- and long-tether EDCs is pronounced because the long-tether EDC (**IIa**) has an extended structure; by contrast, in water, the ligand of the long-tether EDC becomes encapsulated in the dendrimer interior, so its molecular size is less different from that of the short-tether EDC.

To further evaluate the apparent size of the EDCs, we determined the hydrodynamic diameter of the G6 PAMAM and the EDCs in water and 20% aqueous methanol solution by dynamic light scattering (DLS; Figure 3). In all cases, the diameters are smaller in water than in aqueous methanol, but the two EDCs show very different solvent effects. The radius of the short-tether EDC (**Ia**) in both solvents is nearly the same as the sum of the PAMAM radius and the length of the ligand moiety (ca. 1.5 nm, determined by molecular modeling studies). By contrast, the radius of the long-tether EDC (**IIa**) in aqueous MeOH is much larger, consistent with a stretched-out conformation, whereas in water its radius is much smaller, similar to that of the short-tether EDC. Thus, the short-tether EDC (**Ia**) appears to have a more rigid structure, with the ligand projecting outward from the peripheral region of the dendrimer in a relatively solvent-insensitive manner. By contrast, the long linker in EDC **IIa** allows the hydrophobic ligand to reach back into the interior region of the PAMAM in aqueous media.

The hydrodynamic diameters of the other two EDCs (**IIIa** and **IVa**) were approximately 8.6 nm and 45 nm in water, respectively. The cyclofenil-functionalized EDC (**IIIa**), with a short linker, has a radius close to that of the dendrimer plus the length of the cyclofenil moiety (1.4 nm), as was the case with **Ia**. The unexpectedly large diameter measured for the

Table 1: Relative binding affinity (RBA) values for the EDCs and reference compounds for ERα and ERβ.

EDC or reference compound	I		II		III		IV	
	a	**b**	**a**	**b**	**a**	**b**	**a**	**b**
RBA[a] (ERα)	3.8 ± 0.8	3.6 ± 0.2	0.9 ± 0.1	3.3 ± 0.5	2.9 ± 0.2	2.8 ± 0.3	2.4 ± 0.3	10.3 ± 0.4
RBA[a] (ERβ)	1.9 ± 0.3	2.3 ± 0.4	0.2 ± 0.1	1.2 ± 0.4	5.8 ± 0.1	10.2 ± 0.4	2.7 ± 0.3	14.4 ± 3.8
ligand–receptor access ratio[b]	83–106 %		17–27 %		60–104 %		19–23 %	
nature of tether explanation	**short**		**long**		**short**		**medium**; **hydrophobic**	
	Good ligand–receptor access		ligand access restricted by encapsulation by PAMAM and tether		good ligand–receptor access		ligand access restricted by EDC aggregation	

[a] RBA = relative binding affinity determined in competitive radiometric binding assays.[21,22] RBA = {IC_{50}[estradiol]/IC_{50}[compound]} × 100. Values are the mean ± range or SD of 2 or more independent experiments. The RBA for estradiol is 100; K_d value for estradiol is 0.2 nM for ERα and 0.5 nM for ERβ. [b] Ligand–receptor access ratio is defined as {RBA[EDC]/RBA[reference compound]} × 100.

EDC **IVa**, with a moderate length but hydrophobic pentamethylene group linker, is most likely the result of aggregation.

We determined the ligand access to the receptor in all four EDCs by comparing their binding affinities for the estrogen receptors with those of their reference compounds, which were designed to mimic the EDC ligand in all respects except not having the dendrimer attached (Scheme 1). Binding affinities, determined by a known method,[21,22] are expressed in Table 1 as relative binding affinity (RBA) values, where the affinity of estradiol is set at 100. The values for the EDCs are based on the ligand-equivalent concentration, which corrects for the fact that multiple ligands are attached to each dendrimer.

All of the compounds have RBA values in the range 0.2–14.4. Most notably, the short-tether EDCs (**Ia**, **IIIa**) have RBA values for the estrogen receptors (ERs) that are very close to those of the corresponding model compounds (**Ib** and **IIIb**). In contrast, both the long and moderate tether length EDCs (**IIa** and **IVa**) have much lower affinities than the reference compounds. The ratio of EDC to reference compound affinity, expressed in percent, can be termed the "ligand–receptor access index" and provides a direct measurement of ligand access to the receptor. The two short-tether EDCs **Ia** and **IIIa** have ligand–receptor access indices of 83–106 % and 60–104 %, respectively, whereas the EDCs with either long- or medium-length tethers (**IIa** and **IVa**) have indices of only 17–27 % and 19–23 %, respectively. Thus, as expected from the spectroscopic and hydrodynamic measurement studies, the short-tether EDCs (**Ia** and **IIIa**) provide much better ligand access to the receptor than do the longer tether EDCs (**IIa** and **IVa**), for the reasons summarized in the Table 1.

We have shown that tethering ligands to polymeric macromolecules can significantly affect ligand access to a receptor. In our EDCs, where the ligand is hydrophobic (for example, an estrogen) and is attached to a macromolecule having a flexible structure with surface invaginations (for example, PAMAM), ligand access becomes a function of the tether: Short-tether EDCs are able to maintain ligand exposure, thus providing essentially unimpeded access to the ERs; in contrast, ligands attached through long or hydrophobic tethers experience masking by burrowing into the PAMAM or by aggregation and thus result in poor access of the ligand to the receptor. Our findings hold important implications for the future design of drug– or hormone–polymer conjugates where receptor interaction by the polymer-bound ligand is the goal, and our investigation provides approaches that can be used to evaluate the behavior of new ligand–polymer conjugates. We believe that these principles will help ensure success in the future development of such novel polymer-based biological reagents.

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