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Synthesis and Characterization of Highly Water-Soluble Dendrofulleropyrrolidine Bisadducts with DNA Binding Activity

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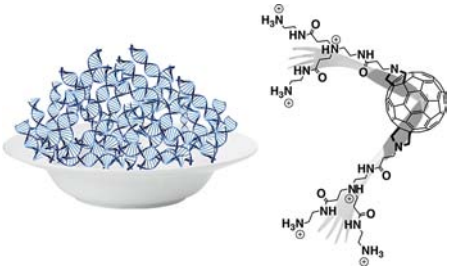
ABSTRACT



The synthesis, characterization and DNA binding studies of a series of polycationic fullerene adducts are reported. These cationic species, exhibiting reasonably high water solubility and a heterogeneous distribution of positive charges, can efficiently complex plasmid DNA. Electrophoresis studies show different DNA binding efficiencies for different adducts, some of which can be considered excellent candidates for DNA binding therapies.

Nonviral approaches for modulating gene expression and for treating genetic diseases rely on the efficient intracellular release of foreign genetic material. This is usually achieved by using polycationic species, such as dendrimers,[1] polymers,[2] nanoparticles,[3] and nanotubes,[4] which can effectively bind oligonucleotides by ionic interactions with the phosphate anions. Cationic fullerene derivatives have shown to be particularly effective gene delivery vectors

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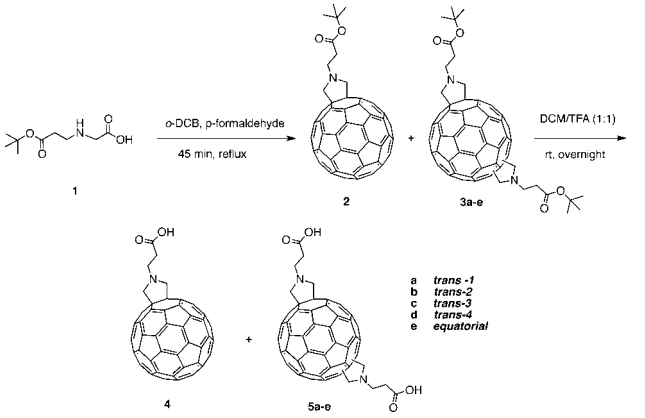
both in vitro[5] and in vivo.[6] Recent studies[5a] have demonstrated that for efficient transfection the size of such fullerene/oligonucleotide complexes must be kept to a submicrometer scale in order to be internalized through endocytosis. The various complexes obtained so far present sizes that span over several orders of magnitude (from several nm to μm), making it difficult to understand the molecular factors that control the transfection mechanism and thus to rationalize the design of more efficient vectors. Moreover, the vectors must be capable of releasing the delivered oligonucleotide in the cytoplasm or in the nucleus. Therefore, it is crucial to design cationic fullerenes that form submicrometer complexes with a good oligonucleotide complexation/decomplexation balance, in order to ensure efficient transfection.[7] C_{60} bisadducts are particularly interesting for this purpose, since the eight different regioisomers can potentially display different oligonucleotide affinities and thus provide a versatile platform for studying, tuning, and improving delivery vectors for specific oligonucleotides.

Herein, we report a synthetic strategy that has allowed us to prepare a library of regioisomerically pure bisadducts that can be further decorated with cationic dendrons. Remarkably such bisadducts exhibit a high solubility in water and DNA binding activity. In fact, electrophoresis studies demonstrate that cationic regioisomerically pure bisadducts exhibit different affinities to DNA and thus that they can be used to control and modulate ionic interactions with oligonucleotides.

To pursue our objectives, we have designed and synthesized a series of fulleropyrrolidine monoadduct and bisadducts equipped with a first generation of polyamidoamine dendrons (PAMAM) that upon protonation provide highly water-soluble polycationic fullerenes. PAMAM dendrons have been selected because of their relatively easy synthesis and also because their dendrimeric analogs are known to interact with DNA.[8] To ensure that the separation of the bisadducts is possible, we have designed a convergent synthetic strategy, in which the different fullerene adducts with a terminal carboxylic group are first synthesized and isolated. Then the individual isolated fullerene adducts are coupled to a first generation PAMAM dendron with a terminal free amine through the formation of an amide.

A 1,3-dipolar cycloaddition was performed on C_{60} using amino acid[9] **1** and *p*-formaldehyde. The mixture was allowed to react for only 45 min, since longer reaction

Scheme 1. Synthesis of Fulleropyrrolidines **4** and **5**



times (2−3 h) result in the formation of tris and higher adducts and their presence renders the recovery of pure isomers difficult and time-consuming. In this way, it was possible to prepare and isolate mono- **2** and bis-fulleropyrrolidines **3a**−**e** (*trans-1*, *trans-2*, *trans-3*, *trans-4*, and *equatorial*). *Cis* isomers were isolated as a mixture and obtained in a low quantity as revealed by HPLC. As a result, five different bisadducts containing *tert*-butyl ester groups were obtained (Scheme 1). Mono- and bis-fulleropyrrolidines (*trans-1*, *trans-2*, *trans-3*, *trans-4*, and *equatorial*) have been characterized by ^{1}H and ^{13}C NMR and UV−vis absorption spectroscopies, which are in perfect agreement with previous work[10] (Supporting Information, Figure S1). The deprotection of the *tert*-butyl group was easily achieved in quantitative yields using a mixture of $CHCl_3$ and trifluoroacetic acid (TFA), obtaining monoadduct **4** and bisadducts **5a**−**e** containing free carboxylic acid residues.

At this stage, dendron **12** was synthesized with a free amine, as anchoring points suitable for coupling to the fullerene building blocks **4** and **5a**−**e**, and two *tert*-butyloxycarbonyl (Boc)-protected amines. Five steps were needed to afford the desired compound **12** as outlined in Scheme 2. Ethylenediamine was protected with a carbobenzyloxy group (CBz) by treatment with *N*-(benzyloxycarbonyloxy)-succinimide. Thus, a large excess of the ethylenediamine was used in order to avoid bisprotection, obtaining monoprotected ethylendiamine **8**. Then, a double aza-Michael reaction was carried out on **9** in the presence of an excess of methyl acrylate, resulting in bis-methyl ester derivative **10**. Attempts to react compound **9** with *N*-Boc-ethylenediamine to afford directly **12** result in very low yields despite the use of various conditions. To overcome this, an excess of ethylenediamine was added to a solution of **9** and stirred for 7 days, yielding **10**. Then, the terminal amines were protected by treatment with di-*tert*-butyl dicarbonate obtaining a mixture of unreacted, mono-, and bis-protected

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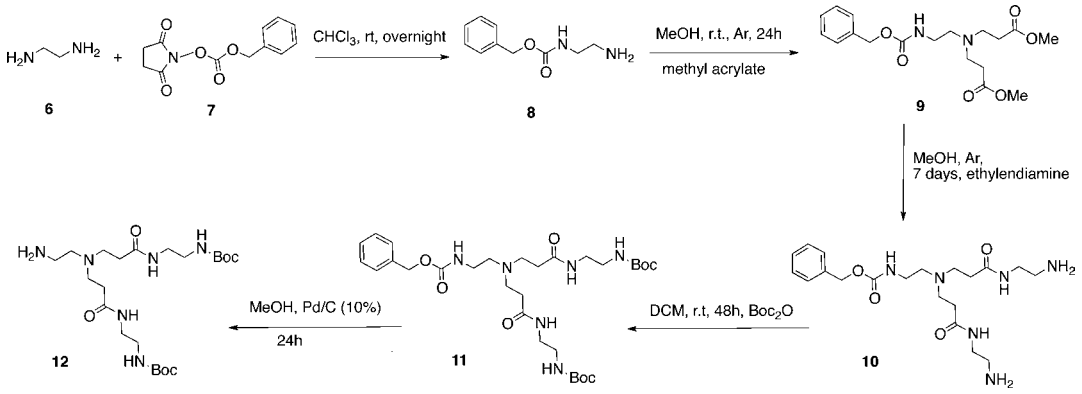
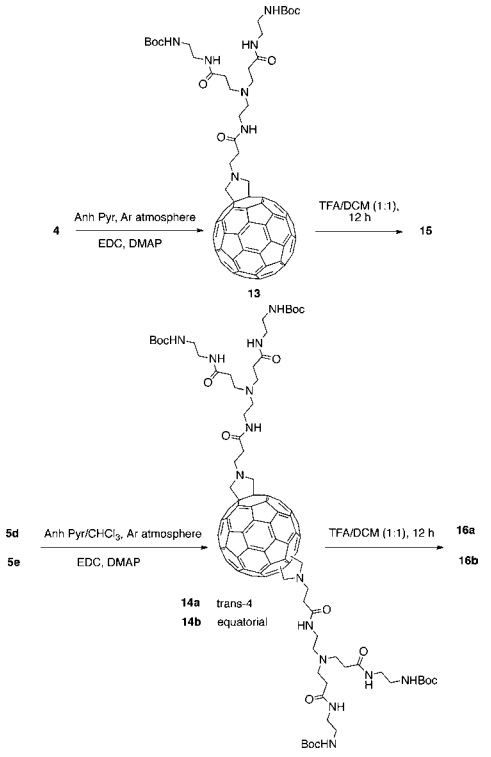
Scheme 2. Synthesis of First-Generation PAMAM Dendron **12**



Scheme 3. Coupling and Deprotection Reactions



dendron, which was easily separated by chromatography to afford **11**. The CBz group was easily removed by hydrogenation using Pd/C as catalyst giving **12** in quantitative yields. To optimize the coupling between the fullerene derivatives and the dendron moieties, several conditions were tested using as model monoadduct **4**. The activation of its carboxylic residue with 10 equiv of EDC and DMAP in pyridine was the most convenient procedure to obtain **13**, leading to the remarkable yield of 70% (Scheme 3). Finally, TFA was added to a solution of **13** in chloroform to achieve full deprotection of the Boc-group in a few hours, as revealed by TLC and MS spectra, obtaining **15**.

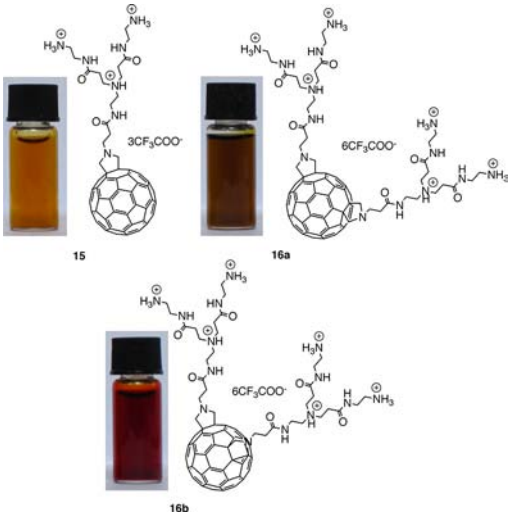


Figure 1. Water solutions (pH = 7.0, 10^{-3} M) and molecular structure of **15**, **16a**, and **16b**.

The same procedure was adopted in the coupling with the bisadducts **5a**–**e**, but in this case a much lower yield was obtained (20%), most probably due to the limited solubility of the bisadducts in pyridine. The change of the solvent to a $CHCl_3$/pyridine mixture allowed to obtain homogeneous solutions of the fullerene derivatives and yields as much as 70% for bisadducts **5d** and **5e**. Because of the high yields obtained for **14a** and **14b**, only these were deprotected for performing the DNA binding experiments. Bisadducts **16a** and **16b** were obtained quantitatively from **14a** and **14b** after 48 and 72 h of reaction respectively, in the presence of TFA.

Monoadduct **15** and bisadducts **16a** and **16b** exhibit a high solubility in water and their characteristic brown, greenish and redish color as depicted in Figure 1. In addition, the aggregation profiles determined by dynamic light scattering (DLS) measurements confirm that no aggregation is observed up to 10^{-3} M (see the Supporting Information for

details). The solubility of monoadducts and bisadducts varies drastically as expected from the different number of solubilizing dendrons. Monoadduct **15** presents a maximum solubility of 1.02 mg/mL, while isomer **16a** (*trans-4*) presents a solubility 10 times higher (10.02 mg/mL). Interestingly, the *equatorial* isomer **16b** is much more soluble than its *trans-4* analogue with a remarkable solubility value of 23.90 mg/mL. The solutions of **15**, **16a**, and **16b** were perfectly stable after several weeks, and neither visual precipitation nor changes on their absorption characteristics was observed.

Different concentrations of fullerene adducts **15**, **16a**, and **16b** were incubated with increasing amounts of plasmid DNA (pDNA), and gel electrophoresis was used to estimate the presence of unbound pDNA, both supercoiled pDNA (form I) and free nicked circular pDNA (form II). Studies on monoadduct **15** showed mostly free pDNA and some binding to form II at high concentrations (316.8 μM) corresponding to an amine/phosphate ratio (N/P) of 15 (Figure 2, left panel). Remarkably, in the case of bisadducts **16a** and **16b**, a concentration 21 times lower (14.9 μM, N/P ratio = 1) was enough to bind completely both forms of pDNA. A shorter range concentration experiment was carried out in order to study the difference between isomers **16a** and **16b** (Figure 2, right panel), which shows clear differences in their DNA binding activity. The *equatorial* isomer **16b** exhibits a remarkable higher performance with respect to *trans-4* isomer **16a**. In fact, in the case of **16b** only traces of unbound form II pDNA were observed at concentrations as low as 9.9 μM. Instead, in the case of **16a** both traces of unbound forms I and II can be appreciated.

In conclusion, we have synthesized, isolated, and fully characterized a fulleropyrrolidine monoadduct and five bisadducts with terminal carboxylic acids, three of them (monoadduct and bisadducts *trans-4* and *equatorial*) further decorated with a first generation PAMAM dendron to obtain highly water-soluble cationic derivatives. The terminal positive charges contained in their structure lead to a very efficient pDNA complexation as

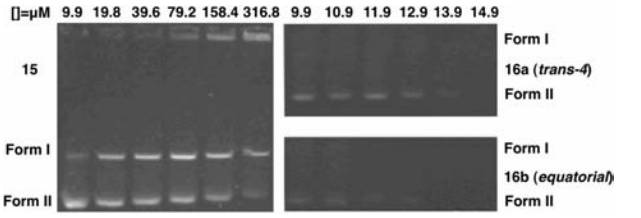


Figure 2. Images of electrophoretic gels of pDNA with monoadduct **15** (left panel) and bisadducts **16a** (right panel, top) and **16b** (right panel, bottom).

demonstrated by gel electrophoresis. In addition, remarkable differences between bisadduct isomers have been found concerning solubility, aggregation profile, and DNA complexation ability, opening the door to a new family of tunable fulleropyrrolidine-based gene transfection vectors. The study of the activity of all different bisadducts with higher dendron generations is currently underway, and the results will be reported in due course.

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Supporting Information Available. Synthetic procedures of all compounds and ^{1}H NMR, ^{13}C NMR, and HPLC spectra of key compounds. DLS characterization graphics of **15**, **16a**, and **16b**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.