Multifunctionalised cationic fullerene adducts for gene transfer: design, synthesis and DNA complexation†

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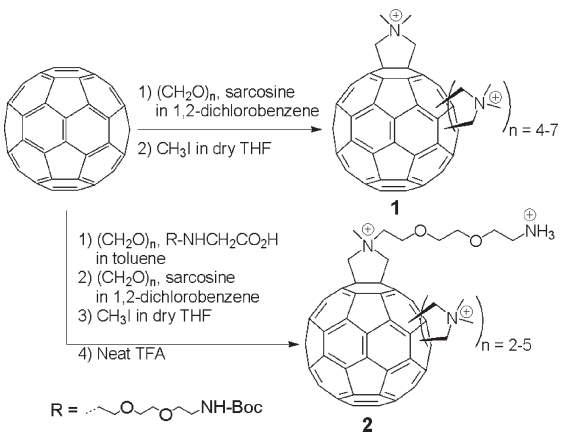
Cationic poly-*N*,*N*-dimethylfulleropyrrolidinium derivatives have been designed and synthesised to complex plasmid DNA for gene delivery.

The biological applications of fullerenes continue to attract increasing attention.[1] Fullerenes can be considered useful systems for drug delivery because they can be multifunctionalised, they can act as drug adsorbents and form particles in the nanoscale range.[2] Very few examples of delivery of therapeutic agents using fullerenes as the active carrier have been reported in the literature. [60]Fullerene has been conjugated to paclitaxel or complexed to a melanoma targeting antibody for chemo- and immunotherapy, respectively.[3,4] Pristine fullerene has been used as solid adsorbent for erythropoietin to engineer a new drug vector with improved bioavailability and uptake.[5] Alternatively, fullerene derivatives were used as counter-ions to modulate the cellular uptake and the anion carrier activity of cell-penetrating peptides.[6,7]

In the field of nucleic acid delivery, Nakamura *et al.* pioneered the potential use of fullerene derivatives as carriers for gene delivery.[8] A cationic fullerene derivative has been designed and used to condense plasmid DNA (pDNA). Transfection of COS-1 cells was then achieved by optimising various parameters including the fullerene/base pair ratio, the transfection time and the amount of pDNA. The cationic fullerene was able to preserve DNA in the cytoplasm and presented a higher efficiency in transfection than lipofectin, a commonly used, commercially available lipid-based transfecting agent. More recently, a library of twenty-two fullerene derivatives were prepared and a new efficient transfecting compound was isolated.[9] According to this SAR study, the size of the fullerene/DNA aggregates is a key point for the development of efficient fullerene-based gene vectors. The complexes should be as small as possible to improve gene expression. However, it has been observed that the fullerene/pDNA complexes can attain dimensions in the micrometer scale in the cell culture and that the expression is strongly influenced by the cell culture medium used to form the complexes.[8–10] In the search for new gene delivery vectors, we have conceived and designed a new class of cationic fullerene. In the field of carbon-based nanomaterials, we have already developed an efficient method to render biocompatible both fullerenes and carbon nanotubes (CNT).[11,12] As functionalised CNT were previously described as new potential vectors for therapeutic molecules (including peptides, nucleic acids and small drugs), the same approach of polyfunctionalisation was extended to C_{60}.[13,14] We demonstrated that it is possible to multifunctionalise C_{60} using the 1,3-dipolar cycloaddition reaction of azomethine ylides.[15] When a large excess of reagents is used, up to nine pyrrolidine rings around the fullerene sphere can be introduced.[15] Based on such experimental observations, two types of multifunctionalised C_{60} were prepared. C_{60} was refluxed in 1,2-dichlorobenzene for 1 h in the presence of a large excess of sarcosine and paraformaldehyde (Scheme 1). During multiple additions the colour of the reaction mixture changed from purple to dark red. The poly-*N*-methylfulleropyrrolidines were isolated by column chromatography to recover a dark red solid. The mass analysis of the polyadducts revealed the presence of a regioisomer mixture with a number of *N*-methylpyrrolidine moieties between 5 and 8, as previously reported (see ESI,† Fig. S2).[15] The integration of the peaks showed that the most abundant compounds with six and seven addends were present at 30 and 35%, respectively. The NMR spectrum in $CDCl_3$ displayed two broad bands at 2.5 and 3.4 ppm corresponding to the protons of the methyl group and the pyrrolidine ring, respectively (see ESI,† Fig. S4). The mixture was highly soluble in organic solvents and in water under sonication. The separation of the different isomers was beyond the scope of this work,[16,17] since we wanted to use the mixture of the



Scheme 1 Multiple 1,3-dipolar cycloadditions of azomethine ylides to C_{60}.

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polyadducts to complex pDNA (*vide infra*). In addition, the direct use of the polyadducts without tedious chromatographic separations would offer a great advantage in terms of practical applications. Next, these poly-*N*-methylfulleropyrrolidines were alkylated with an excess of iodomethane in dry THF leading to the highly water-soluble poly-*N*,*N*-dimethylfulleropyrrolidinium derivative **1** (Scheme 1).[11,16,18] Although it was difficult to determine the amount of positive charges, we assumed a complete methylation of all pyrrolidine rings affording an average number of ~6.05 charges per fullerene.[11,16,18] We performed then a diffusion-ordered spectroscopy (DOSY) NMR analysis in D_2O to determine the hydrodynamic radius of the different species of **1** present in the aqueous solution. This type of experiment gives a direct measure of the size of the self-assembling molecules in solution and allows detecting the presence of possible aggregates. An average hydrodynamic radius of 9.8 Å was obtained. This supports the fact that the population of the aggregated molecules is very low and not observable (less than 10% if present) when **1** is dissolved in pure water.

To be considered a good transfection vector, a molecule has to bind tightly to DNA. The cationic poly-*N*,*N*-dimethylfulleropyrrolidinium salt **1** was therefore used to evaluate its capacity to condense pDNA based on positive/negative charge interaction. The pDNA used in this study was the highly purified 7.2 kbp pCMV-βgal expressing β-galactosidase. We studied first the migration of **1**–pDNA complexes by agarose gel electrophoresis and the degree of pDNA condensation by ethidium bromide (EtBr) exclusion (Fig. 1). Using increasing amounts of **1**, pDNA was condensed in pure water and in serum-free DMEM cell culture media. The complexation of pDNA by **1** excluded EtBr intercalation, quenching the fluorescence signal, and affected the shift in the migration rate of free pDNA inside the electrophoretic field. The poly-*N*,*N*-dimethylfulleropyrrolidinium **1** strongly condenses pDNA already at 1 : 1 positive/negative charge ratio when dissolved in water (Fig. 1, left panel). Only in the DMEM buffer fluorescent bands towards the cathode, derived from free, not fully complexed DNA are visible (Fig. 1, right panel). The large smears above the wells likely correspond to the excess of free cationic **1** migrating towards the anode. The analysis of the gel suggests that there is a strong interaction between the poly-*N*,*N*-dimethylfulleropyrrolidinium **1** and the pDNA.

The capacity of **1** to complex pDNA was then confirmed by transmission electron microscopy (TEM). Initially, pDNA and compound **1** were separately observed on the TEM grid. Although the electron density of pDNA is very weak, it looked like a film made of filaments organised randomly (see ESI,† Fig. S7). The poly-*N*,*N*-dimethylfulleropyrrolidinium **1** was instead very difficult to detect. This might be explained by the presence of the multiple positive charges around the C_{60} core resulting in a strong repulsion among the fullerene spheres thus preventing the formation of clusters in the nanoscale range. Only very few small aggregates of **1** were visible (see ESI,† Fig. S8), in agreement with the DOSY measurement. The poly-*N*,*N*-dimethylfulleropyrrolidinium **1** was mixed in water with pDNA at 5 : 1 positive/negative charge ratio and deposited after complexation on the TEM grid. The **1**–pDNA complexes are clearly visible confirming the capacity of the cationic fullerene mixture to heavily condense nucleic acids (Fig. 2). The pDNA appears globular, coiled and very compact, forming a network around the cationic fullerenes similar to a chain of pearls with an average diameter of ~30 nm (Fig. 2, right). Moreover, at the background of the images, no uncomplexed, free pDNA forming fibre-like motifs was visible confirming the complete complexation of pDNA onto the fullerene derivatives.

The spatial arrangement of these complexes is quite different in terms of organisation and dimensions from that found by Nakamura and co-workers using a different cationic fullerene.[19,20] These authors observed several types of large aggregates between the aminofullerene and pDNA. The cationic fullerene initially formed disks up to 150 nm in diameter with a densely packed pDNA, which evolved to more complex objects as the amount of fullerene increased.[19,20] A direct correlation between the structure of the complexes and the pDNA expression is not really possible because transfection occurred only at very high positive/negative charge ratio – a condition that resulted in the formation of large macroscale clusters of pDNA/fullerenes in the cell culture.[8,10]

Next, we quantified the binding between **1** and pCMV-βgal, by measuring their interaction using surface plasmon resonance (SPR). The SPR technique allows the determination of the affinity between macromolecules forming supramolecular assemblies. In particular, the binding affinity is an important parameter for the design of gene delivery systems based on electrostatic interaction.[21] For this study, we have prepared a second type of poly-*N*,*N*-dimethylfulleropyrrolidinium salt. Indeed, compound **1** cannot be immobilised on the sensor chip because it lacks a suitable functional group. We have introduced an additional amino group on the fullerene core. C_{60} was first monofunctionalised using a Boc-N-protected triethylene glycol derivative of glycine in the presence of paraformaldehyde as previously reported (Scheme 1).[22] Then, the monoadduct underwent a multiple addition using a large excess of sarcosine and paraformaldehyde in 1,2-dichlorobenzene at 120 °C for 2 h to avoid the risks of removing the Boc protecting

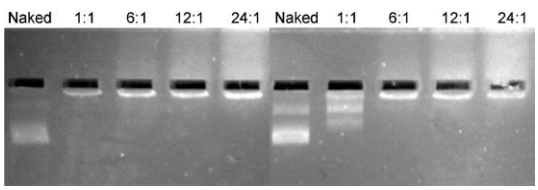

Fig. 1 Electrophoretic motility of **1**–pDNA complexes. In the left and right panels, lane (1) (Naked) represents 0.2 μg free pDNA. All other lanes contain fullerene derivative **1** complexed to 0.2 μg DNA at various (±) charge ratios: lane (2), 1 : 1; lane (3), 6 : 1; lane (4), 12 : 1; line (5), 24 : 1. The left panel shows the complexes formed in pure water. The right panel shows the complexes formed in serum-free DMEM cell culture media.

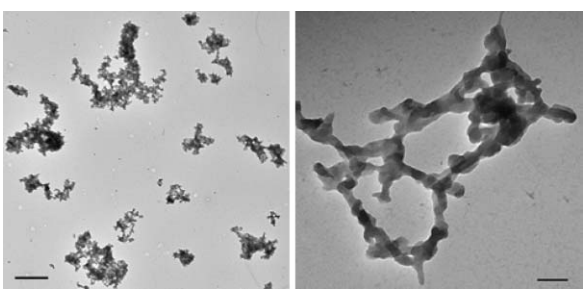

Fig. 2 TEM images of **1** complexed to pDNA in 5 : 1 (±) ratio. Scale bars correspond to 2 μm (left) and 100 nm (right), respectively.

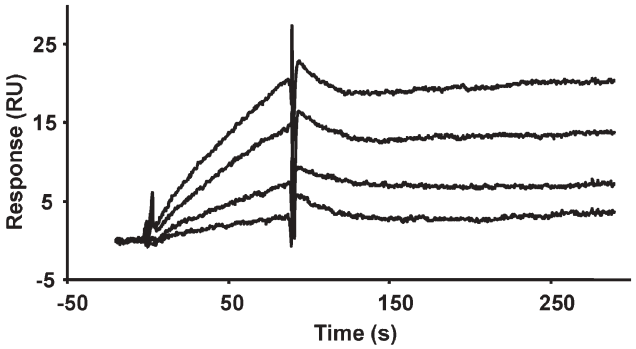

Fig. 3 Sensorgrams of the interaction of the plasmid DNA with **2** obtained by increasing the concentration of the pDNA at each run: 2.62 nM, 5.25 nM, 10.5 nM, and 21 nM from the bottom to the top curve.

group at the reflux temperature of the solvent. The poly-*N*-methylfulleropyrrolidines were again isolated by column chromatography to recover a dark red solid. The mass spectrum shows a mixture of the polyadducts with a number of pyrrolidine rings between three and six (see ESI,† Fig. S6). The most abundant is the isomer with five rings which is present in ~45%. The pyrrolidine rings were subsequently methylated using an excess of iodomethane in dry THF affording the cationic fullerene polyadducts **2** after the final acid cleavage of Boc (Scheme 1). The compound **2** is structurally very similar to **1**. Both compounds interact with the pDNA in a similar manner as they possess approximately the same amount of positive charges. The poly-*N*,*N*-dimethylfulleropyrrolidinium **2** was immobilised on the sensor chip *via* a stable amide bond between its amino group at the end of the triethylene glycol chain and the dextran carboxylic functions on the chip gold surface, activated in turn with carbodiimide and *N*-hydroxysuccinimide. After this coupling, increasing concentrations of pDNA from 2.62 to 21 nM were injected to evaluate the capacity of **2** to interact with the nucleic acid. Compound **2** gradually complexed the pDNA, evidenced by an increase of the signal during the association phase (Fig. 3). During the dissociation phase, the pDNA was slowly released. The (apparent) association rate constant (k_a) and the (apparent) dissociation rate constant (k_d) corresponded to 8.05×10^5 M^{-1} s^{-1} and 1.04×10^{-3} s^{-1}, respectively. The analysis of the fitting parameters enabled us to calculate an apparent equilibrium association constant of 7.74×10^8 M^{-1}, which represents the affinity between pDNA and the cationic fullerene **2**. Sub-nanomolar equilibrium constants between biomolecules are generally rare. The value found for the complex between **2** and pCMV-βgal is however indicative of a strong interaction, similar to that obtained for complexes formed between the same pDNA and functionalised cationic CNT.[21] This SPR study using compound **2** supports the data obtained with fullerene derivative **1** which was very efficient to complex pDNA as demonstrated by gel electrophoresis and TEM. On the basis of these results, the poly-*N*,*N*-dimethylfulleropyrrolidinium derivatives we have developed are promising vectors for gene transfer. In comparison to the molecules developed by Nakamura *et al.*, which require a certain percentage of potentially toxic organic co-solvent (DMF or DMSO) for complete solubilisation,[8,10] they have the advantage of being totally water soluble. The preliminary results on cell uptake and gene transfer (see ESI,† Fig. S9–12) show the potential of this new class of vectors, as it is also supported by their lack of toxicity and their ability to penetrate into the cells.

In conclusion, we have designed a novel class of cationic multifunctionalised fullerenes able to tightly complex pDNA. We are currently improving the efficiency of these interesting cationic polyadducts fullerene-based vectors to obtain higher levels of gene expression.

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