Pharm Res (2012) 29:384–396
DOI 10.1007/s11095-011-0560-0

RESEARCH PAPER

Smart DNA Vectors Based on Cyclodextrin Polymers: Compaction and Endosomal Release

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Received: 6 June 2011 / Accepted: 2 August 2011 / Published online: 17 August 2011

ABSTRACT

Purpose Neutral β-cyclodextrin polymers (polyβCD) associated with cationic adamantyl derivatives (Ada) can be used to deliver plasmid DNA into cells. In absence of an endosomolytic agent, transfection efficiency remains low because most complexes are trapped in the endosomal compartment. We asked whether addition of an imidazole-modified Ada can increase efficiency of polyβCD/cationic Ada-based delivery system.

Methods We synthesized two adamantyl derivatives: Ada5, which has a spacer arm between the Ada moiety and a bi-cationic polar head group, and Ada6, which presents an imidazole group. Strength of association between polyβCD and Ada derivatives was evaluated by fluorimetric titration.

Results Gel mobility shift assay, zeta potential, and dark field transmission electron microscopy experiments demonstrated the system allowed for efficient DNA compaction. *In vitro* transfection experiments performed on HepG2 and HEK293 cells revealed the quaternary system polyβCD/Ada5/Ada6/DNA has efficiency comparable to cationic lipid DOTAP.

Conclusion We successfully designed fine-tuned DNA vectors based on cyclodextrin polymers combined with two new adamantyl derivatives, leading to significant transfection associated with low toxicity.

KEY WORDS cyclodextrin · electron microscopy · gene transfer · inclusion chemistry · proton sponge

INTRODUCTION

Cyclodextrins (CD) are cyclic water-soluble molecules of 6 (αCD), 7 (βCD), or 8 (γCD) α-D-glucose units. They present a cone-shape architecture with an apolar cavity that can form an inclusion complex with hydrophobic molecules (1). To generate a cyclodextrin-based DNA delivery system, we made use of a neutral βCD polymer (polyβCD) which was synthesized by polycondensation of βCD and epichlorohydrin (2). In order to enable polyβCD to interact with plasmid DNA, inclusion of amphiphilic cationic connectors is required. This results in the generation of a polycation. The ternary complex, polyβCD/connector/DNA or polyplex, is achieved *via* electrostatic interactions between the polycation and the negatively charged phosphate groups of DNA (Fig. 1a).

A delivery system based on polyβCD presents potentially several interesting characteristics: namely the charge density of the vector can be varied easily by a controlled addition of

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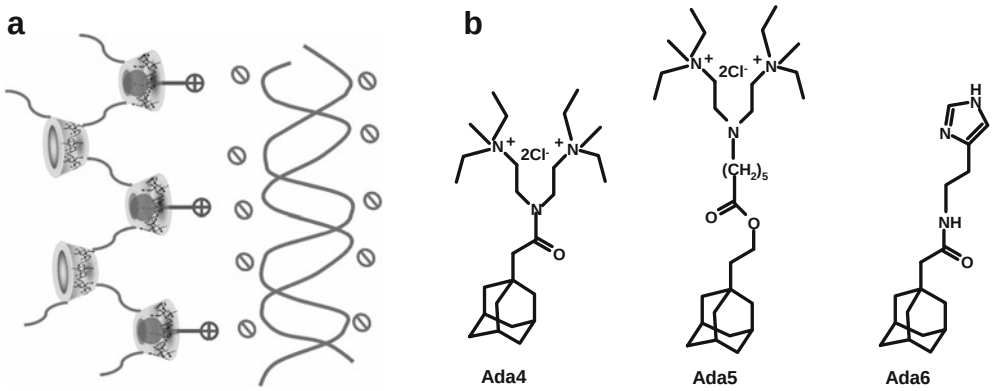

Fig. 1 (**a**) Scheme of polyplex assembly between the βCD polymer, connector, and DNA and (**b**) chemical structure of the Ada connectors.

the connector; it has been shown that CD-bearing vectors can lower the cell toxicity in transfection experiments compared to similar vectors without CD (3) and increase transfection efficiency (4–6); additional amphiphilic connectors containing, for example, cell targeting moieties can be included; and the polyplex characteristics can be modified by changing the nature of the connectors.

In a previous study, we synthesized and evaluated different cationic adamantyl derivatives (Ada) (7). Our results indicated that the transfection efficiency of polyβCD/Ada/DNA polyplexes is highly dependent on the chemical structure of the Ada connector. The nature of the spacer arm between the adamantyl moiety and the charge valency of the connector are two particularly important parameters. The best results until now were obtained with the Ada4 connector (Fig. 1b) bearing a bivalent cationic charge and an amide function along its arm, which limits the dissociation of the polyplexes at physiological ionic strength by combining electrostatic and hydrogen bond interactions between vectors and DNA. The design of an optimal system requires a fine tuning of the interactions in order to ensure both efficient DNA compaction for the cellular uptake and DNA release once the polyplexes reach the nucleus. Further improvements require strengthening of the inclusion complex interactions between polyβCD and connector. This can be provided by the design of a new bicationic Ada derivative, such as Ada5 (Fig. 1b), bearing a longer spacer arm than Ada4. Another required property of the DNA vectors is the ability to perform endosomal release. Previous transfection experiments required the presence of the fusogenic peptide JTS-1 (7). Although efficient in transfection, the system should be optimized by replacing the fusogenic peptide by an adamantane derivative. Polyethylenimines (PEIs) are highly efficient transfection compounds (8,9). Their activity partially relies on their capacity to buffer the capture protons entering the endosome. This induces swelling of the endosomes that leads to membrane disruption (10,11). Midoux *et al.* later showed that coupling histidine residues to polylysine strongly increases the transfection efficiency (12). In fact, the imidazole group of histidine, which has a pKa around 6, allows for mimicking the proton sponge activity of the PEIs. The role of histidine in transfection has been further demonstrated in the context of peptidic delivery vectors (13,14). Moreover, grafting imidazole groups on a cyclodextrin-containing polycation has been shown to increase the transfection efficiency by Davis *et al.* (15)

Based on these results, we developed an adamantane derivative bearing an imidazole group, Ada6 (Fig. 1b). As mentioned before, a new bicationic Ada derivative, Ada5 (Fig. 1b) was also designed in order to improve the decoupling between the electrostatic and inclusion complex interactions. Binary and ternary complexes were then characterized by fluorimetric titrations and zeta potential measurements. Complementary transmission electron microscopy experiments allowed proving the DNA compaction in the DNA vectors. Lastly, the transfection potential of the Ada4 and Ada5-based formulations was evaluated.

MATERIALS AND METHODS

Materials

The β-cyclodextrin (βCD) was a gift from Roquette Company (France), and the βCD polymer (polyβCD) was synthesized by polycondensation of βCD and epichlorohydrin under strong alkaline conditions. The polyβCD synthesis and characterization have already been reported (2). The polyβCD sample used in this study has an average molecular weight of M_w= 160 000 $gmol^{-1}$ with a βCD content of 59% w/w, and its polydispersity index is M_w/M_n=1.9.

The chemical structure of the different connectors is given in Fig. 1b. The synthesis of Ada4, 2-(1-carbamoylmethyladamantane)-di-(ethyl(diethylmethylammonium) dichloride, has already been described (7). The synthesis scheme of Ada5 and Ada6 is described in Fig. 2. The different solvents and chemicals (Aldrich, Saint-Quentin

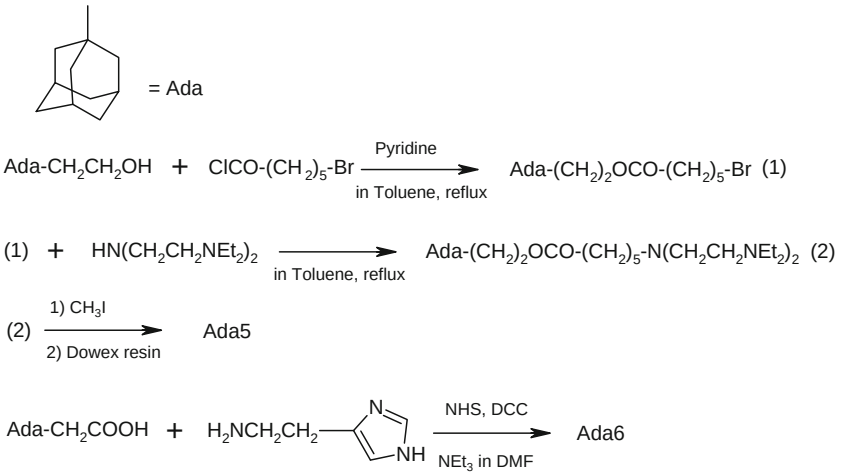

Fig. 2 Synthesis scheme of Ada5 and Ada6 connectors.

Fallavier, France) for these syntheses were used without further purification.

Ada5, (2-(1-adamantyl)ethyl) 6-amino-*N*,*N*-di(ethyl(diethylmethylammonium)chloride)-hexanoate, was prepared following a three-step procedure. 0.6 g of 2-(1-adamantyl) ethanol was dissolved in 100 ml of anhydrous toluene under nitrogen with 2.1 equivalents of anhydrous pyridine. 1.05 equivalents of 6-bromo-hexanoyl chloride were slowly added, and the mixture was left under reflux for 5 h. Then, the mixture was filtered over neutral alumina, the solvent was evaporated, and 1.05 g of (2-(1-adamantyl) ethyl) 6-bromo-hexanoate (1) as a pale yellow oil was obtained (88% yield). Compound 1 was characterized by ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.34$ (t, 2H; AdaCH_2), 1.37 (m, 2H; CH_2), 1.46 (s, 6H; Ada), 1.60 (m, 6H; Ada and 2H CH_2), 1.82 (m, 2H; CH_2), 1.88 (s, 3H; Ada), 2.24 (t, 2H; COCH_2), 3.34 (t, 2H; CH_2Br), 4.06 (t, 2H; CH_2O).

One gram of compound 1 was dissolved in 100 ml of anhydrous toluene with 2.1 equivalents of *N, N, N', N'*-tetraethyldiethylenetriamine. After 24 h of reflux, the mixture was filtered, the solvent was evaporated, and the brown oil was dissolved in diethyl ether, extracted with a saturated solution of $NaHCO_3$ and with water. After drying over $MgSO_4$ and evaporation, 1.2 g of 2-(1-adamantyl) ethyl) 6-amino-*N*,*N*-di(ethyldiethylamino)-hexanoate (2) as a brown oil was obtained. Compound 2 was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH (85/15)). 0.75 g of compound 2 as a pale yellow oil was obtained (35% yield). Compound 2 was characterized by ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.96$ (t, 12H; 4 CH_3), 1.24 (m, 2H; CH_2), 1.35 (t, 2H; AdaCH_2), 1.37 (m, 2H; CH_2), 1.45 (s, 6H; Ada), 1.60 (m, 6H; Ada and 2H CH_2), 1.88 (s, 3H; Ada), 2.22 (t, 2H; COCH_2), 2.39 (m, 2H; CH_2N), 2.48 (m, 16H; 8 NCH_2), 4.05 (t, 2H; CH_2O).

0.75 g of compound 2 was dissolved in 50 ml of diethyl ether with 3 ml of CH_2Cl_2. One millilitre of CH_3I was added, and the mixture was left under reflux for 1 h. The insoluble oil was dissolved in water, extracted with diethyl ether, and freeze dried. 0.85 g of Ada5 as a yellow gum was obtained (72% yield). Ada5 was characterized by ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.31$ (t, 12H; 4 CH_3), 1.25–1.40 (m, 6H; 3 CH_2), 1.45 (s, 6H; Ada), 1.60 (m, 6H; Ada and 2H CH_2), 1.88 (s, 3H; Ada), 2.22 (t, 2H; COCH_2), 2.55 (m, 2H; CH_2N), 3.20 (m, 2H; NCH_2), 3.30 (s, 6H; 2 $^+$NCH_3), 3.55 (m, 8H; 6 $^+$NCH_2), 3.72 (4H; 2 NCH_2) 4.05 (t, 2H; CH_2O).

Ada6, (2-(1-carbamoylmethyladamantane)ethyl-4-imidazole, was prepared following a one-step procedure. 1.18 g de 1-adamantane acetic acid (Aldrich) was dissolved in 100 ml of anhydrous dimethyl formamide and left for 2 h under stirring and nitrogen. 1.5 equivalent of *N*-hydroxysuccinimide, 1 equivalent of *N*,*N*'-dicyclohexylcarbodiimide, 1 equivalent of triethylamine were added, and the mixture was left overnight. 0.75 g histamine was added in the mixture and left for 3 days. The mixture was filtered, the solvent was evaporated, and 30 ml of CH_2Cl_2 were added to the crude sample. After filtration, the organic layer was extracted with a saturated solution of $NaHCO_3$ and with water. After drying over $MgSO_4$ and evaporation, 1.7 g of Ada6 as a yellow compound was obtained. Ada6 was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH (85/15)). 0.48 g of Ada6 as white solid was obtained (25% yield). Ada6 was characterized by ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 1.49$ (s, 6 H; Ada), 1.57 (m, 6H; Ada), 1.87 (s, 5H;3 Ada and AdaCH_2) 2.78 (t, 2H; CH_2), 3.47 (q, 2H; NHCH_2), 6.39 (s, 1H), 6.78 (s, 1H), 7.60 (s, 1H).

Methods

Fluorescence Measurements

The association constants (obtained with a standard deviation of 10%) between the cyclodextrin derivatives and the connectors were measured in ultrapure water using a SLM Aminco 8,100 fluorimeter. A complete description

of the method was previously published in the case of other adamantyl derivatives (16). 4-Amino-*N*-*tert*butylphthalimide, a fluorescent probe able to make inclusion complexes with CDs, was used as a competitor (17) for Ada5 and Ada6. K_{probe} values of 3.1 10^3 and 5.9 10^3 l mol^{-1} have been used for the association constants of the probe with βCD (17) and polyβCD (16), respectively.

Preparation of Complexes

All complexes were prepared in ultrapure water (unless otherwise stated). The binary complexes, polyβCD/Ada4 or polyβCD/Ada5, were generated by mixing a fixed amount of cyclodextrin cavities (20 or 30 equivalents (eq.) depending on the experiments) with various equivalents of Ada4 or Ada5. After 10 min, the ternary polyβCD/Ada4-5/DNA polyplexes formation was achieved by adding DNA plasmid in order to have 1 eq. of the phosphate group. One equivalent corresponds to 4 μg of DNA, or to 12 nmoles of phosphate groups; for polyβCD or Ada, 1 eq. corresponds to 12 nmoles of βCD cavities or Ada, respectively.

For the preparation of the quaternary polyβCD/Ada4-5/Ada6/DNA complexes, we used the following protocol: the polyβCD was mixed with Ada4/5 and Ada6 in ultrapure water. After 10 min, plasmid DNA diluted in ultrapure water was added. After a maturation step of 15 min, the complexes were ready for use. For the preparation of the polyβCD/Ada4-5/DNA/JTS-1 complexes, the following protocol was used: the polyβCD was mixed with Ada4/5 in ultrapure water. After 10 min, plasmid DNA diluted in ultrapure water was added. After a maturation step of 15 min, a given amount of JTS-1 peptide was added, and the complexes were allowed to stay for 10 min at room temperature before they were used.

Zeta Potential Measurements

The zeta potential of the binary and ternary complexes (prepared in ultrapure water) were analyzed using a Zetasizer nano ZS (Malvern Instrument) at 25°C. Measurements were performed in folded capillary cells (DTS 1,060 C, Malvern Instrument) and with a 4 mW He-Ne laser (633 nm). The zeta potential was calculated based on the Hemholtz-Smoluchowski relationship (18) for the polyβCD/Ada5/DNA polyplexes and based on the Hückel approximation for the binary complexes, poly βCD/Ada5 and Ada5/DNA.

Transmission Electron Microscopy (TEM)

TEM sample preparation was performed by positive staining as previously described (19). The polyβCD/Ada/DNA polyplexes were prepared in ultrapure water with various polyβCD/Ada5/DNA and polyβCD/Ada5/Ada6/DNA eq. ratios (20/1/1, 20/1.5/1, 20/2/1, 20/5/1, 20/10/1, 20/20/1 and 20/0.5/0.5/1, 20/1/1/1, 20/1.5/1/1, 20/2/2/1, 20/5/2/1, 30/60/40/1, 30/80/80/1, respectively). They were observed after dilution in water or salt buffer (Tris 10 mM, pH 7.5, NaCl 150 or 300 mM). Five μl of each diluted solution was deposited onto a 600 mesh copper grid coated with a thin carbon film, activated by glow-discharge in the presence of pentylamine (19). After 1 min, grids were washed with aqueous 2% (w/vol) uranyl acetate (Merck, France) and then dried with ashless filter paper (VWR, France). TEM observations were carried out on a Zeiss 912AB transmission electron microscope in filtered cristallographic dark field mode. Electron micrographs were obtained using a ProScan 1,024 HSC digital camera and Soft Imaging Software system.

DNA Retardation Assay

The electrophoretic mobility of the plasmid DNA was evaluated using different Ada/DNA and polyβCD/Ada/DNA ratios. The assay was performed by using a 1% agarose gel (w/v) containing SYBR Safe in a TBE buffer (89 mM Tris/89 mM boric acid/2 mM EDTA, $pH=8.3$). Gels were run for 20 min at 100 V, and DNA was visualized using an UV illuminator.

Cell Culture and Transfection

The transfection experiments were performed using either a CMV-Luc plasmid or an eGFP plasmid encoding either a *luciferase* or GFP gene, respectively, under the control of the human cytomegalovirus (CMV) immediate-early promoter. The human hepatocarcinoma cell line HepG2 (American Type Culture Collection, Rockville, USA) and the human transformed embryonal kidney cells HEK293 (American Type Culture Collection, Rockville, USA) were used for the transfection assays. Briefly, the cells were plated 24 h before transfection in 24-well plates at 37°C in a 5% CO_2/95% air incubator in a complete medium DMEM (Dulbecco's modified Eagle's medium) supplemented with 1% (v/v) of penicillin-streptomycin (Invitrogen) and 10% (v/v) foetal bovine serum (Invitrogen). The cell confluency was typically between 50% and 80% at the beginning of the experiment. All transfection assays have been performed in duplicates. Culture media was removed and replaced by a mixture of 400 μL of serum-free DMEM and 100 μl of polyplexes aqueous solution at the desired polyβCD/Ada/DNA eq. and containing 2 μg of plasmid DNA per well. After 3 h, the transfection medium was removed and replaced with 1 ml of complete medium. Luciferase activity was measured 32 h post-transfection. The luciferase assay was performed as previously described (20). Luciferase background was

subtracted from each value, and the transfection efficiency was expressed as total light units/10 s/well and is the mean of the duplicates. The protein content of the transfected cells was measured using the BioRad protein assay.

The expression level of the green fluorescent protein (GFP) was measured 28 h after transfection using flow cytometry. Briefly, cells were analysed by flow cytometry after they were detached with trypsin-EDTA 0.05% and diluted in PBS. The analysis was carried out on a Facscalibur flow cytometer (Becton Dickinson) using the software CELLQuest (Becton Dickinson). Quantification was achieved using 25,000 morphologically intact cells, and the proportion of eGFP-positive cells is expressed as a percentage.

Where indicated, the transfection experiments were done in the presence of the fusogenic peptide JTS-1 (Genepep) (GLFEALLELLESLWELLLEA; M_W=2,300). Of note, it was previously shown that addition of this amphipathic and pH-responsive peptide to plasmid DNA complexed by a cationic peptide strongly increases the transfection efficiency. JTS-1 (21) was shown to have pH-dependent (i.e. in acidic conditions) membrane destabilizing activity; therefore, it was suggested that this peptide favours escape of DNA from the endosomes once acidification of this compartment occurs. JTS-1, which is negatively charged, associates to the positively charged DNA complexes through electrostatic interactions.

The commercialized cationic lipid DOTAP (22) (*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride) was used at its optimal charge ratio on HEK293 and HepG2 cells (i.e. +/− ratio of 3 and 4, respectively).

Transfection of HepG2 and HEK293 cells in the presence of 10% of serum was performed using the same protocol as the one described for transfection in serum-free condition. Of note, the following polyplexes have been used: for transfection of HepG2 cells, we used polyβCD/Ada5/DNA (30/40/1 eq. ratios and containing 4 µg DNA) mixed either with 15, 30, 40 µg of JTS-1 (which correspond to 6.5, 13 and 17.3 nmoles of JTS-1, respectively, and to 0.54, 1.08 and 1.44 eq.) or 40, 60, 80 eq. of Ada6. For transfection of HEK293 cells, we used polyβCD/Ada5/DNA (30/30/1 eq. ratios and containing 4 µg DNA) mixed either with 15, 30, 40 µg of JTS-1 or 40, 60, 80 eq. of Ada6.

Cell Viability Assay

Cytotoxicity was evaluated by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay. Briefly, 1 day after transfection of HepG2 cells, the cell culture medium was removed and replaced by serum-free DMEM containing 0.5 mg/ml MTT. After incubation at 37°C for 3 h, the medium was removed, and 200 µl of DMSO was added to each well to dissolve the formazan crystals produced from the reduction of MTT by viable cells. Absorbance was then measured at 570 nm. Untreated cells were used as control.

RESULTS AND DISCUSSION

Chemical Structure of the Connectors

The adamantyl connectors are described in Fig. 1b. Ada5, compared to Ada4, presents a spacer arm formed by an alkyl chain of five carbons between the adamantyl moiety and the bi-cationic polar head group. Presence of the spacer arm is expected to limit the large decrease of the association constant observed from Ada4/βCD to Ada4/polyβCD complexes. In the following parts, the results obtained with Ada5 will be compared with those obtained with Ada4 in order to evaluate the effect of this spacer arm. In addition to an adamantyl group, Ada6 possesses an imidazole moiety that is ionisable in the endocytotic compartments and which thus could be used as a "proton sponge" endosomolytic agent instead of peptides such as JTS-1 in the transfection experiments. The synthesis scheme of Ada5 and Ada6 is given in Fig. 2. Briefly, Ada5 was obtained by a three-step procedure: reaction of 2-(1-adamantyl)ethanol with 6-bromo-hexanoylchloride in order to introduce the spacer arm between the adamantyl moiety and the terminal group; reaction of the obtained bromo derivative with *N*,*N*,*N*',*N*'-tetraethyldiethylenetriamine; quaternization of the terminal amino groups. Ada6 was prepared by a one-step reaction of 1-adamantane acetic acid with histamine *via* a peptidic coupling.

Characterization of the Association Connector-polyβCD

Adamantyl derivatives are known to form stable complexes with βCD as the size of the adamantyl group perfectly fits the βCD cavity. High association constants of the order of 10^4–10^5 l mol^{-1} have been reported in the literature (23). We have also determined high association constant for Ada4 with βCD by fluorimetric titration (7), K~7.8 10^4 l mol^{-1} (Table I). Of note, however, is that different positively charged adamantyl derivatives have shown lower association constants with polyβCD as compared to βCD (7,16). For example, K decreases by a factor 500 for Ada4 with polyβCD compared to βCD (Table I). This has mainly been explained by the closeness of the adamantyl group and the cationic polar head group, leading to strong steric hindrance (7). In this work, the association constants between Ada5 and Ada6 with βCD or polyβCD were also determined by fluorimetric titration. This indirect method

Table I Values of Association Constants K of Different Connectors with βCD and polyβCD Determined by Fluorimetric Titration

Compounds	Ada4 K ($l\ mol^{-1}$)	Ada5	Ada6[a]
βCD	$7.8\ 10^4$	$\sim 4\ 10^5$	$6.5\ 10^4$
polyβCD	$1.5\ 10^2$	$2.6\ 10^3$	$9.2\ 10^2$

[a] In acidic medium

is based on the competition between a fluorescent probe and a non-fluorescent competitor, both forming 1:1 inclusion complexes with the CD cavities. The fluorescence of the probe is largely increased upon βCD complex formation. Therefore, following the fluorescence decrease of the probe/βCD complex by addition of a competitor allows the determination of the competitor association constant with βCD. Table I reports the different data. The association constants obtained for Ada5 and Ada6 with βCD are high, in the order of 10^4–10^5 l mol^{-1}. These values are comparable to the one obtained with Ada4. The binding constants of Ada5 and Ada6 are lowered by around two orders of magnitude between βCD and polyβCD as shown in Table I. Ada5 shows an association constant with polyβCD 17 times higher ($\sim 2.6\ 10^3$ l mol^{-1}) than the one previously determined for Ada4 ($\sim 1.5\ 10^2$ l mol^{-1}). Ada5 is the derivative showing the largest association constant of the Ada compound series (7,16). As expected, the presence of a spacer arm between the adamantyl moiety and the charged head group induces an increase of the association constant. This enhancement of the binding constant should lead to more efficient *in vitro* delivery using Ada5 than Ada4.

Characterization of Interactions Between Vectors and Plasmid DNA

Gel Mobility Shift Assay

We determined, in a first set of experiments, the ability of the different vectors to interact with plasmid DNA by performing a gel shift mobility assay, which allows us to identify the amount of cationic vector required to inhibit the migration of the plasmid DNA during gel electrophoresis. Previously, we reported that Ada4 used in the absence of polyβCD is not able to compact DNA (7), while when associated with polyβCD, it efficiently complexed the plasmid. Similar experiments performed with Ada5 and Ada6 indicated that both compounds were unable to retard DNA migration in the absence of polyβCD (data not shown). As expected due to its charge neutrality at neutral pH, even when associated with the cyclodextrin polymer, Ada6 was unable to complex DNA (Fig. 3). In contrast, polyβCD/Ada5 altered DNA migration, and this was the

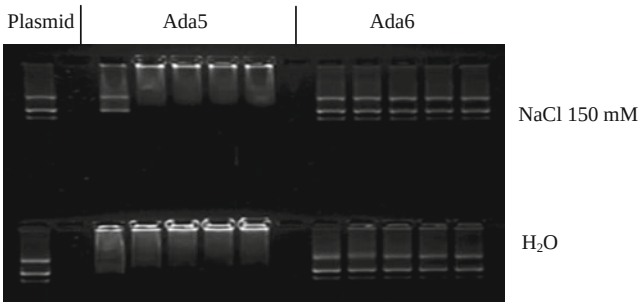

Fig. 3 The DNA retardation assay was performed either in water or 150 mM NaCl using the following conditions. Complexes were generated using constant amounts of cyclodextrin cavities (15 nmoles) and DNA ($1\ \mu g = 3$ nmoles of phosphate groups). For Ada5, we used the following amounts (lanes from the left to the right): 0.5, 1.25, 2.5, 3.75, 5 nmoles. For Ada6, we used 1, 2.5, 5, 7.5 and 10 nmoles.

case at low Ada5/DNA ratio. These results underscore that polyβCD is an essential component of the vector system. Moreover, full DNA retardation is observed for Ada5/DNA ratio higher than 1 in the presence of salt (i.e. 150 mM NaCl; Fig. 3). Under the same conditions, Ada4 provided full DNA retardation only at a ratio above 2. This result underscores the improved properties of the Ada5 vectors.

Zeta Potential

In order to study more accurately the interactions with plasmid DNA, we performed zeta potential measurements of the polyplexes. Figure 4 reports the zeta potential as a function of the Ada5 equivalent for the following systems: Ada5/DNA, polyβCD/Ada5 and polyβCD/Ada5/DNA (the concentrations of polyβCD and DNA are fixed to 20 and 1 eq., respectively). In the case of polyβCD/Ada5, the zeta potential is already positive for low Ada5 eq. (the polyβCD polymer alone presents a negative zeta potential value, which is usual for the polysaccharides), and then it progressively increases with Ada5 eq. The efficient complexation of Ada5 with polyβCD leads to a positively charged polyelectrolyte, with a maximum value of zeta potential around + 40 mV. On the contrary, the Ada5/DNA mixture generates a negative zeta potential reflecting the inability of Ada5 alone to mediate DNA complexation. Addition of polyβCD permits obtaining a positive zeta potential value indicating DNA complexation, which is consistent with the gel electrophoresis results. The zeta potential value of the ternary complex polyβCD/Ada5/DNA is always lower (maximum value around + 30 mV) than the one of the binary complex polyβCD/Ada5 at the same Ada5 equivalent (maximum value around + 40 mV). This surface charge decrease, which is expected, is due to the neutralization of cationic charges by the DNA anionic charges. As in the case of Ada4 that also presents a

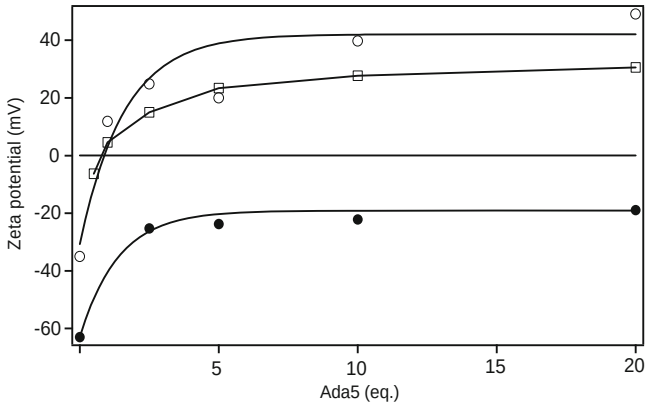

Fig. 4 Zeta potential of the binary complexes (*White circle*) Ada5/polyβCD and (*Black circle*) Ada5/DNA and of the ternary complex (*White square*) polyβCD/Ada5/DNA as a function of Ada5 eq. (the polyβCD and DNA concentrations are fixed at 20 and 1 eq., respectively).

multivalency of the polar head group (7), a neutral surface charge of the polyplexes is obtained at 1 eq. of Ada5, showing the efficiency of Ada5 with polyβCD to make polyplexes with DNA.

Transmission Electron Microscopy (TEM)

A series of experiments was done at different polyβCD/Ada5/DNA ratios in order to characterize the complex formation. In water, free negatively supercoiled DNA plasmids present toroidal shapes corresponding to an apparently open configuration (Fig. 5a) due to the electrostatic repulsion of negative charges along the phosphodiester backbone (24). The addition of polyplexes, at 20/1/1 ratio, restores supercoiled DNA to its canonical plectonemic (or interwound) form, as would the addition of multivalent positive counterions to neutralize the negative charge of DNA (Fig. 5b). The increase of Ada connector concentration in the polyplexes produces a gradual compaction of DNA plasmids and their aggregation (Fig. 5c–e). Typical multimolecular toroidal and rod condensates can be observed (Fig. 5e). DNA-polyplexes' interactions produce the compaction of DNA, and this compaction increases with Ada5 eq. (Fig. 5c–e). At Ada5 eq. higher than 5 (Fig. 5i, l), very large condensates containing many DNA molecules are observed. These preformed condensates in water are reversible: addition of NaCl already at 150 mM destabilizes totally the polyplexes at 20/10/1 ratio (Fig. 5j–k). Even if polyplexes were present onto DNA molecules, such as other cationic counterions, the condensates and the aggregates disappeared. It should be noted, however, that larger NaCl concentration (300 mM) is needed to destabilize totally the polyplexes at 20/20/1 ratio (Fig. 5m–n). This shows the importance of the polyplex composition and particularly the connector concentration to keep relative polyplexes stability in physiological medium.

A series of experiments was also done at different polyβCD/Ada5/Ada6/DNA ratios (Fig. 6a–f). The same general trend was observed; the condensation process is gradual starting at low ratios (20/0.5/0.5/1). It should be noted that the compaction process starts at lower Ada5 eq., since addition of Ada6 in the mixture contributes to the DNA compaction. As observed in the ternary system (without Ada6), the compaction/condensation phenomenon begins by an intramolecular process (Fig. 6b1) and seems to be cooperative, since totally free DNA molecules are observed near complexes on the same grid. At the working pH, part of Ada6 is protonated, which also acts as a connector between polyβCD and DNA. Increasing Ada5 and/or Ada6 leads to the formation of heterogeneous aggregates as illustrated in Fig. 6 d1–e and finally to large and dense aggregates (Fig. 6f1–2).

Very large aggregates formed with ratios 30/60/40/1 and 30/80/80/1 (ratios leading to the best transfection results, see below) are partly destabilized by addition of NaCl (150 mM). On the grid, few compact aggregates together with different and very large structures can then be observed with DNA strands protruding from the aggregates (Fig. 6g–i).

Transfection Efficiency

Next, we evaluated the ability of the polyβCD-based vectors to transfect plasmid DNA into mammalian cells. *In vitro* cell transfection experiments were performed on HepG2 and HEK293 cells using a plasmid DNA encoding a *luciferase* gene. The addition of the JTS-1 peptide to polyβCD/Ada4/DNA complexes has previously been shown (7) to significantly increase the transfection efficiency of Ada4, probably by improving the escape of the complexes from the endosomes.

The transfection studies first revealed that the polyβCD/Ada5/DNA polyplexes have only marginal DNA delivery efficiencies (Figs. 7 and 8). This was expected since we previously reported that polyβCD/Ada4/DNA poorly transfects cells in the absence of JTS-1 (7). In agreement with the data obtained with Ada4, we found that the addition of JTS-1 to polyβCD/Ada5/DNA polyplexes strongly increased the transfection efficiency, reaching levels that are comparable or even higher to those of the cationic lipid DOTAP (Figs. 7 and 8). Then, we generated quaternary complexes by adding increasing amounts of Ada6 to either polyβCD/Ada4/DNA or polyβCD/Ada5/DNA polyplexes. Unexpectedly, the results indicated that addition of Ada6 was unable to improve the transfection efficiency of polyβCD/Ada4-based complexes (Figs. 7 and 8 and data not shown). One possible explanation is that, due to its higher affinity for polyβCD, Ada6 acted as a competitor reducing the

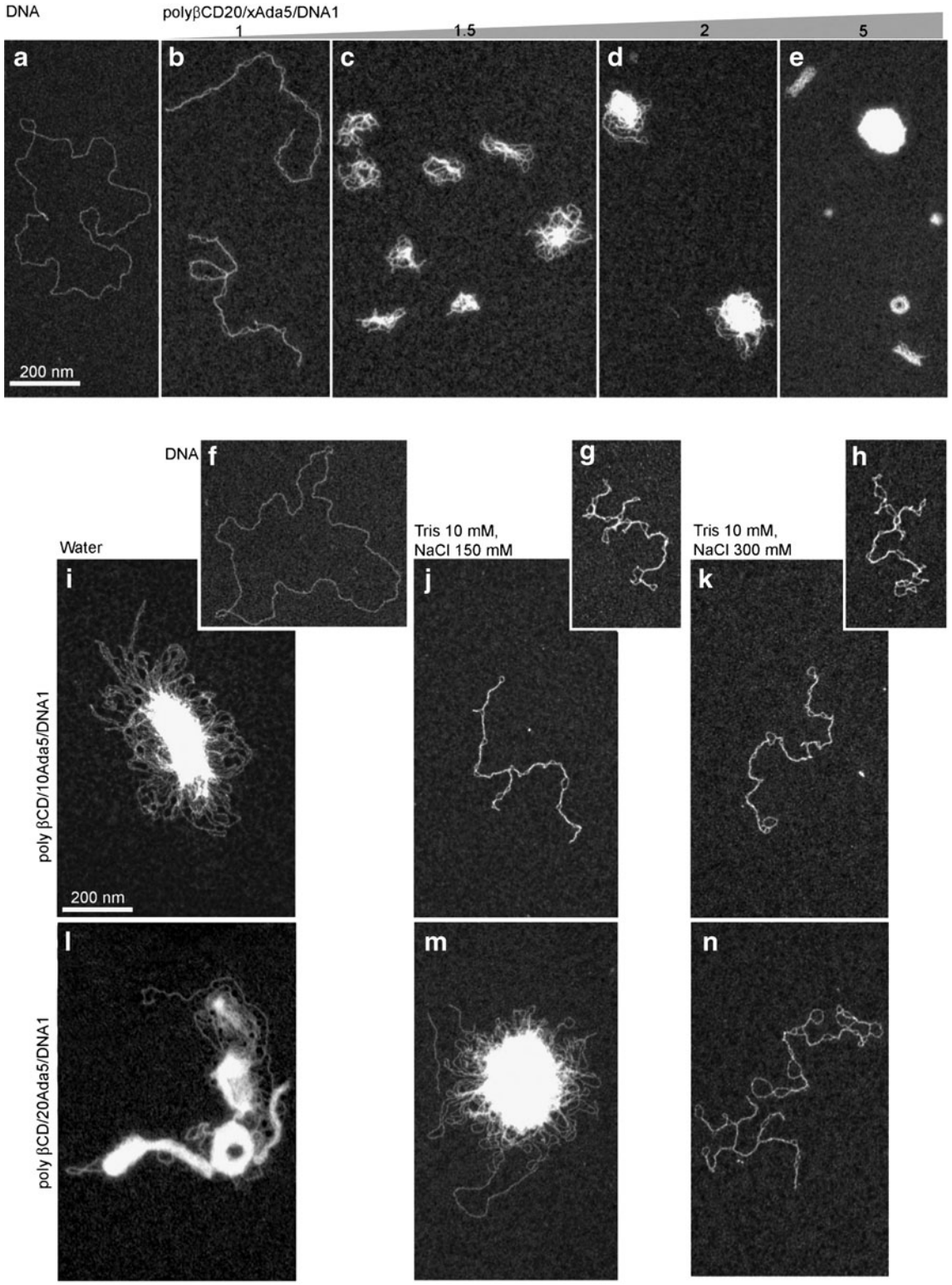

Fig. 5 TEM images of control DNA plasmid in water and its toroïdal shape (**a**) and polyplexes with polyβCD/Ada5/DNA ratios of 20/1/1 (**b**), 20/1.5/1 (**c**), 20/2/1 (**d**), and 20/5/1 (**e**). The fixation of polyplexes to DNA restores the canonical plectonemic (or interwound) forms of supercoiled DNA (**b**). The increase of Ada eq. produces gradual compaction of DNA plasmids and their aggregation (**c**–**e**). Typical multimolecular toroidal and rod condensates can be observed near aggregates (**e**). Large aggregates obtained for (20/10/1) and (20/20/1) ratios (**i**, **l**) are partially or totally destabilized by the addition of monovalent cationic salt (**j**, **m**: NaCl 150 mM; **k**, **n**: NaCl 300 mM). The DNA plasmid control in water, in NaCl 150 mM and 300 mM are reported in (**f**, **g**, **h**).

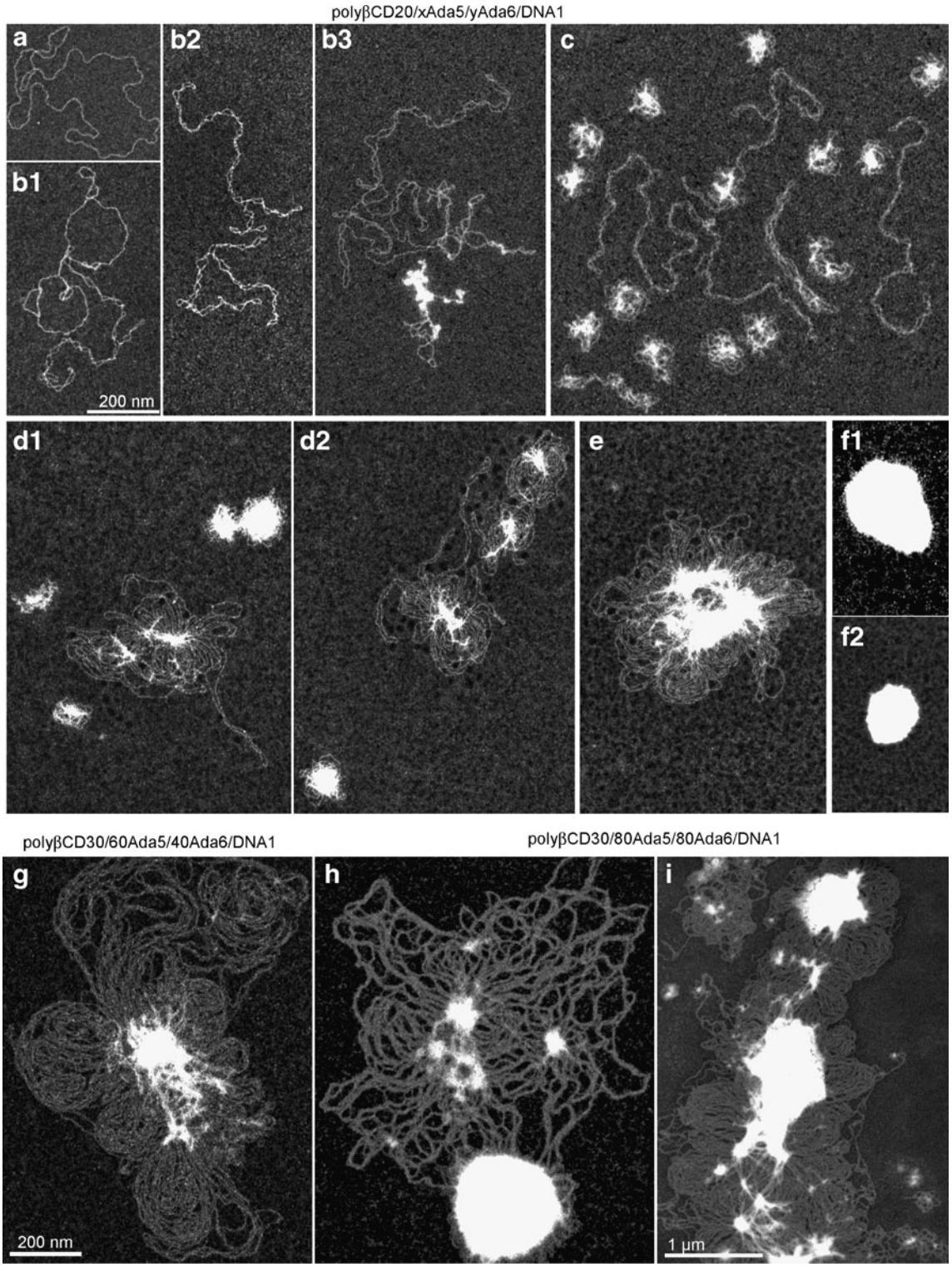

Fig. 6 TEM images of control DNA plasmid in water (**a**) and polyplexes with polyβCD/Ada5/Ada6/DNA ratios of 20/0.5/0.5/1 (**b1**–**b3**), 20/1/1/1 (**c**), 20/1.5/1/1 (**d1**–**d2**), 20/2/2/1 (**e**), 20/5/2/1 (**f1**–**f2**). The condensation process is gradual starting at low ratios 20/0.5/0.5/1 (**b1–b3**). The compaction/condensation phenomena begin by an intramolecular and cooperative process (**c**). The increase of Ada5 and/or Ada6 leads to the formation of heterogeneous aggregates (**d1–2, e**) and finally to large and dense aggregates (**f1–2**). Very large aggregates formed with ratios 30/60/40/1 and 30/80/80/1 are partly destabilized with increasing ionic strength (NaCl 150 mM). Different and very large structures can then be observed with DNA molecules protruding from the aggregates (**g–i**).

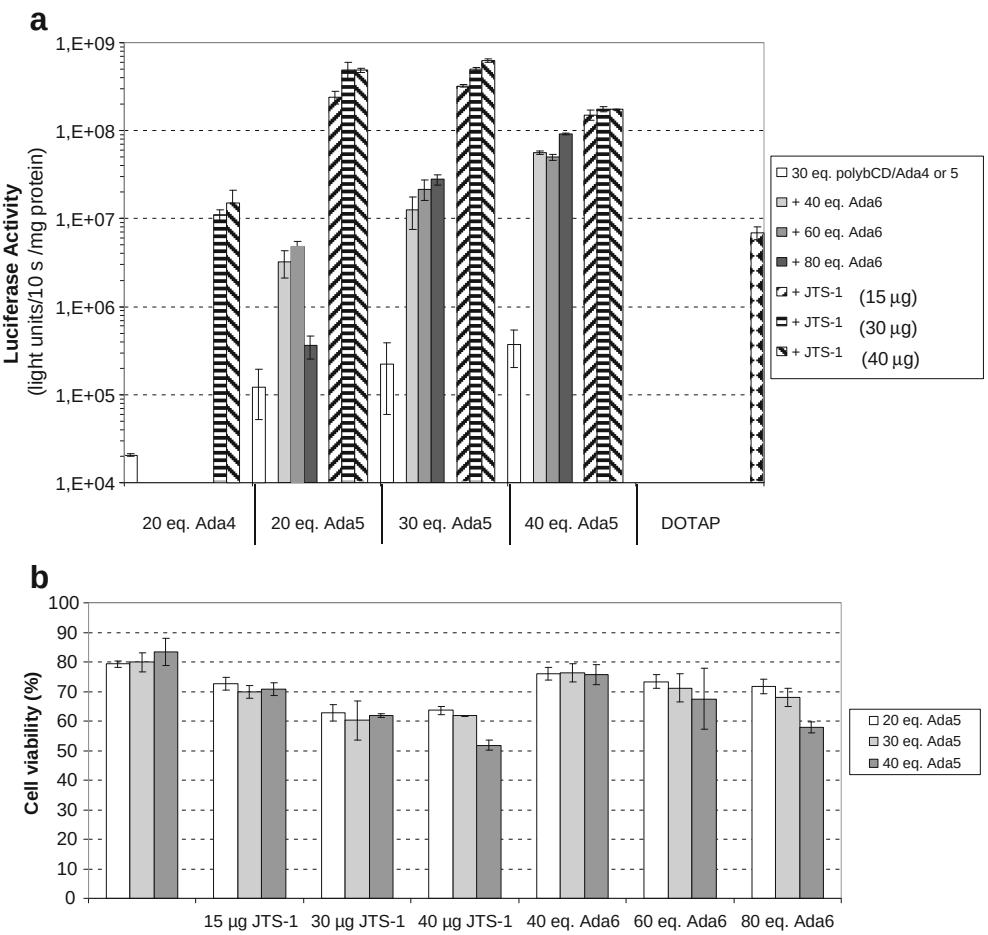

Fig. 7 Evaluation on HepG2 cells of the transfection efficiency and cytotoxicity of the polyβCD polyplexes (the polyβCD and DNA concentrations are fixed at 30 and 1 eq., respectively; 1 eq. corresponding to 4 μg of DNA (12 nmoles of phosphate groups)). (**a**) The complexes were generated in ultrapure water, as described in detail in the experimental section. For each amount of Ada4 (20 eq.) and Ada5 (20, 30 and 40 eq.) three different concentrations of Ada6 (40, 60 and 80 eq.) were tested. For comparison, we tested quaternary polyplexes containing different amounts of JTS-1. The transfection efficiency, measured at $t = 32$ h, is expressed as total light units/10 s/mg protein and is the mean of the duplicates. (**b**) The MTT assay was performed in duplicates 24 h after transfection of HepG2 cells. Absorbance was measured at 570 nm and untreated cells were used as control (100%).

incorporation of Ada4 into the polymer. In contrast, when Ada6 is mixed with polyβCD/Ada5, the efficiency was significantly increased. In fact, the optimal formulation was almost as effective as the best JTS-1 formulation on both cell lines (Figs. 7 and 8). The toxicity of the polyβCD/Ada5/Ada6/DNA complexes on HepG2 cells following transfection was estimated by performing the MTT colorimetric assay. The results indicate that the

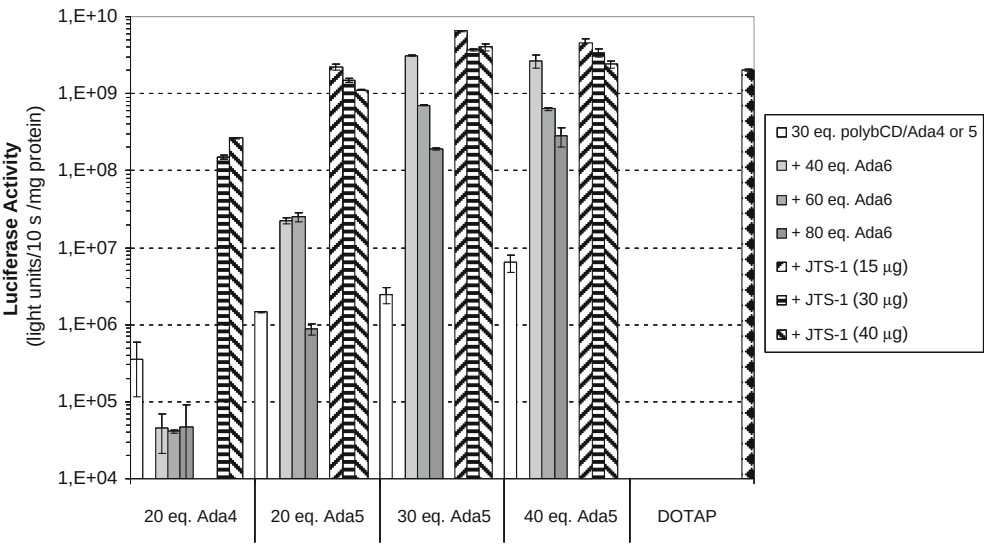

Fig. 8 Evaluation of the transfection efficiency on HEK293 cells. The complexes were generated as described in the legend of Fig. 7. The *luciferase* levels were measured 32 h after transfection; efficiency is expressed as total light units/10 s/mg protein and is the mean of the duplicates.

Table II Transfection of HEK293 Cells Using eGFP as Reporter Gene

DNA complexes	GFP positive cells (%)	GFP positive cells (%)	Average (%)
30 eq. polyβCD/40 eq. Ada5/4μg eGFP	2.5	3.5	3
30 eq. polyβCD/40 eq. Ada5/15 μg JTS-1/4μg eGFP	28.2	22.7	25.45
30 eq. polyβCD/40 eq. Ada5/40 eq. Ada6/4μg eGFP	22.4	28.4	25.4

cytotoxicity of the complexes is lower with the Ada6 containing polyplexes as compared to polyplexes generated with JTS-1 (Fig. 7b).

Next, HEK293 cells were transfected using an eGFP expression cassette which allows determining the percentage of cells that expresses the transgene. As shown in Table II, at 28 h after transfection, 25% of the cells transfected with polyβCD/Ada5/Ada6/DNA were eGFP positive, a percentage similar to that obtained with polyβCD/Ada5/DNA/JTS-1. Notably, in the absence of Ada6 or JTS-1, only a marginal number of HEK293 expressed the transgene.

In vitro transfection experiments reported in the literature are often realized in serum-free culture medium for a few hours before changing the medium for fresh medium supplemented with serum (25–27). In the presence of serum, cationic complexes adsorb onto negatively charged proteins and this in turn usually results in a reduction of the transfection efficiency. We therefore asked whether polyβCD/Ada5/DNA/JTS-1 and polyβCD/Ada5/Ada6/DNA complexes keep their high efficiency in the presence of 10% of serum. The results indicate that JTS-1 containing polyplexes were almost insensitive to the presence of serum (a drop of only 3.3- and 1.14-fold on HepG2 and HEK293 cells; Fig. 9). However, although still significant, the efficiency of the polyβCD/Ada5/Ada6/DNA complexes was reduced in presence of serum: a reduction of 17- and 58-fold of the *luciferase* expression was observed on HEK293 and HepG2 cells, respectively (Fig. 9). It is unclear why the Ada6-containing polyplexes are more sensitive to serum than the JTS-1-containing polyplexes. One explanation could be that the negative charges of JTS-1 reduce the surface charge of the polyplexes, which in turn reduces the interactions with serum proteins.

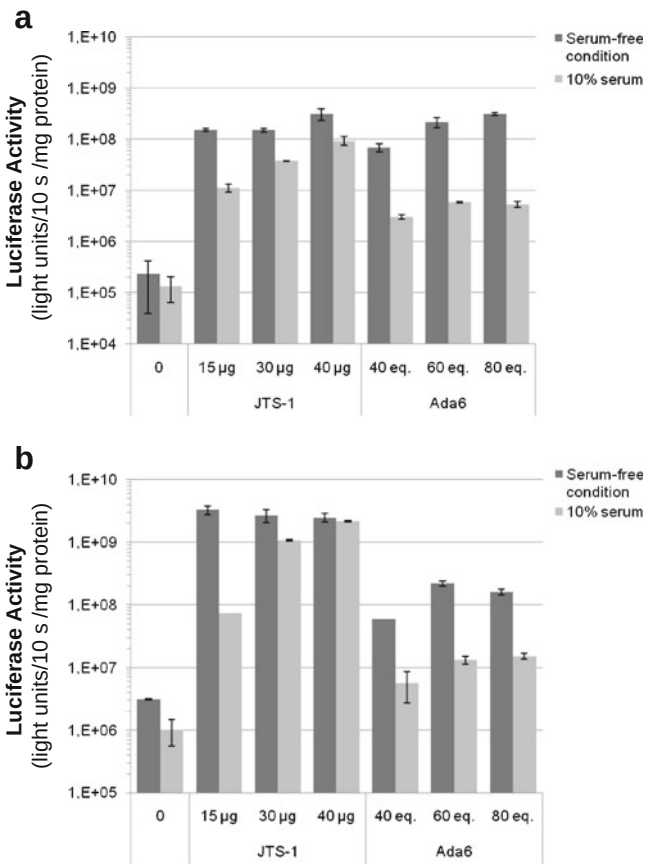

Fig. 9 Evaluation of the transfection efficiency of the polyplexes in the presence of 10% of serum. (**a**) The following polyplexes have been used for transfection of HepG2 cells: polyβCD/Ada5/DNA (30/40/1) mixed either with 15, 30, 40 μg of JTS-1 or 40, 60, 80 eq. of Ada6. (**b**) Transfection of HEK293 cells was done using the following conditions: polyβCD/Ada5/DNA (30/30/1) mixed either with 15, 30, 40 μg of JTS-1 or 40, 60, 80 eq. of Ada6.

CONCLUSION

In the present work, we have shown that it is possible to develop an efficient transfection system by adding to the polyβCD/Ada5/DNA polyplex an adamantane derivative presenting an imidazole group. This latter compound improves the transfection probably by increasing the endosomal escape of DNA. This is supported by the fact that Ada6 successfully replaced the fusogenic peptide JTS-1 in the delivery system. Our results also indicate that the nature of the spacer arm (and ultimately the affinity of the connector for polyβCD) is important since Ada6 associated with the cationic Ada4 did not allow efficient transfections.

In conclusion, we have developed a versatile system based on the use of a cyclodextrin polymer combined with a cationic connector for complexing the DNA, and with an imidazole containing Ada for improving endosomal escape. These systems can be very promising due to their modularity and low cell toxicity.

However, some of their properties such as sensitivity to serum are not yet optimal and future work will focus on the design of the cyclodextrin polymer architecture. On the other hand, the development of procedures which are able to remove neutralizing factors present in the plasma could help to increase the efficiency of delivery systems like the present one (28). Plasmapheresis is in this context a very promising candidate since it is a safe medical procedure which is performed worldwide in adult and it is able to remove various substances from the plasma including antibodies, immune complexes, mediators of inflammation or complement activation, cholesterol-containing lipoproteins, etc. The development of such protocols could be extremely beneficial for the field of gene therapy, since it could open the way to the clinics for delivery systems which are partially inactivated by neutralizing factors present in the blood.

A final step will also consist in the development of an even more sophisticated system allowing cell targeting. This aim could be achieved by including into the polyβCD/Ada5/Ada6/DNA system a connector presenting a ligand recognized by a cell receptor.

ACKNOWLEDGMENTS & DISCLOSURES

We would like to thank the Association Française contre les Myopathies (AFM) for financial support of this work.

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