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Dynamic Constitutional Frameworks (DCF) as nanovectors for cellular delivery of DNA

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We introduce Dynamic Constitutional Frameworks (DCF), macromolecular structures that efficiently bind and transfect double stranded DNA. DCFs are easily synthesizable adaptive 3D networks consisting of core connection centres reversibly linked via labile imine bonds both to linear polyethyleneglycol (PEG, ~1500 Da) and to branched polyethyleneimine (bPEI, ~800 Da). DCFs bind linear and plasmid DNA, forming particulate polyplexes 40-200 nm in diameter. The polyplexes are stable during gel electrophoresis, are well tolerated by cells in culture, and exhibit significant transfection activity. We show that an optimal balance of PEG and b-PEI components is important for building DCFs that are non-toxic and exhibit good cellular transfection activity. Our study demonstrates the versatility and effectiveness of DCFs as promising new vectors for DNA delivery.

Introduction

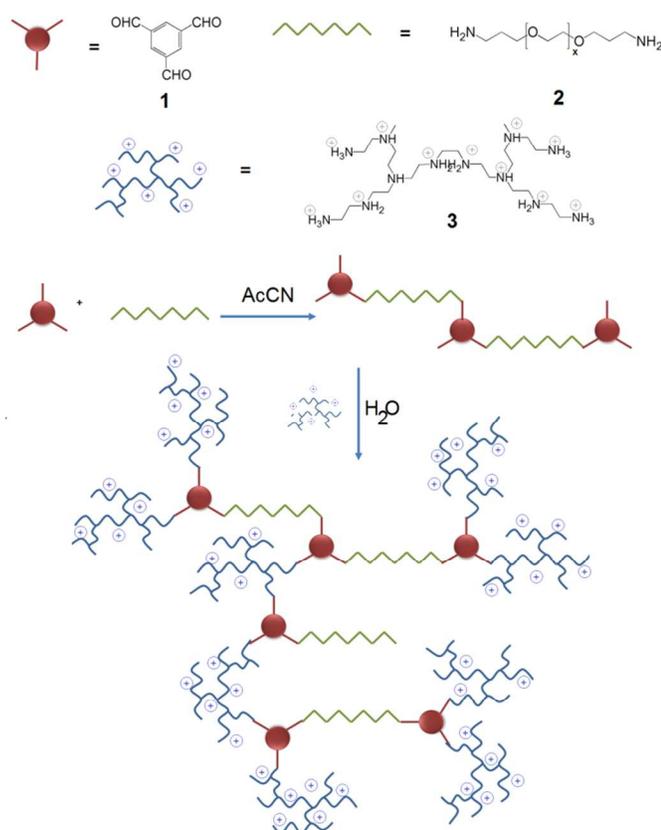
Gene therapy promises to prevent, treat or cure disease by transferring with minimal side effects, therapeutic genetic material to specific cells or tissues, with the aid of either viral and non-viral vectors^{1,2}. Despite their lower transfection efficiency compared to viral vectors, non-viral gene delivery systems have attracted a lot of attention³, due to their unique advantages such as the ability to deliver single genes and lack of infectivity. Convergent strategies have been used for the design of multivalent molecular, supramolecular and nanometric non-viral vectors⁴ mimicking natural delivery functions: membrane penetration, optimal DNA binding and packing, capacity for endosomal escape or nuclear localization, low cytotoxicity and anti-opsonisation functions.⁵ However, due to the enormous variability of both DNA targets and nature of the transfected cells, rational design has been limited to the introduction of a reduced number of components and had to be accomplished by combinatorial approaches. In this context, the Dynamic Combinatorial Strategy proposed by Matile et al.⁶ is one of the most attractive methods for rapid screening, allowing access to active systems from large and complex libraries. By virtue of reversible covalent exchanges between the hydrophilic heads and hydrophobic tails, the fittest *Dynamic molecular transvector* can adapt simultaneously to the DNA and the cell membrane barrier.

As for the design approaches, the Dynamic Constitutional Strategy alternative may embody the flow of structural information from molecular to *Dynamic adaptive*

nanotransfactors.⁷ This concerns the use of linear Dynamic Polymers (Dynamers)^{8,9} or of cross-linked Dynamic Constitutional Frameworks (DCF)^{7,10}. These structures are composed of specific components and connector centres, linked together by labile, reversible covalent bonds. Importantly, they undergo exchange, incorporation/decorporation of their subunits, synergistically interacting and adapting the overall nanostructure in the presence of DNA and bilayer membrane components. This might play an important role in the ability to finely mutate and adaptively implement reversible rearrangements of the components, toward a high level of correlativity of their hypersurfaces in interaction with the DNA and the cell membrane barrier. Thus, this strategy leaves the liberty to DNA systems to self-select and self-generate the carrier best adapted for its own transfection.

We have recently showed that linear PEG macromonomers, trialdehyde core connectors and positively charged guanidinium heads can be used to generate DCFs for DNA recognition.⁷ The simplicity and robustness of the synthetic strategy allow rapid screening of conditions for generating systems with optimal DNA presenting/cell membrane synergistic affinities. Among potential components for the DNA recognition/ transfection offered by the available toolbox, large libraries of active compounds may be used for these purposes. Since its first use as gene delivery system³, PEI has been one of the most studied synthetic cationic DNA vector, and branched PEI of high molecular weight is considered to be one of the most efficient gene carrier for plasmid DNA, oligonucleotides and small interfering RNAs (siRNA)s.² The high transfection efficiency

for PEI/DNA polyplexes is attributed to the unique capacity of PEI to buffer endosomes.^{1,11} However, the use of PEI *in vivo* and *in vitro* gene delivery is limited because of its low colloidal stability and its considerable cytotoxicity². To enhance stability and biocompatibility of PEI polyplexes, they can be combined with PEG; however, PEGs shield the positive charges of PEI, which often has the undesired effect of decreasing transfection efficiency.^{2,12}



Scheme 1. Schematic representation of the synthesis of DCFs combining 1,3,5-benzenetrialddehyde cores **1**, (red circle) with PEG **2**, (green) in acetonitrile. Further treatment of this mixture with positively charged PEI **3**, heads (blue) in water generates DCFs. All the reactions have been performed for different molar ratios of components-see text for details.

Results and Discussions

In this study, 1,3,5-benzenetrialddehyde connectors **1**, poly-(ethyleneglycol)-bis(3-aminopropyl) terminated PEG **2**, ($M_n \sim 1500 \text{ g mol}^{-1}$), and branched bPEI **3**, (bPEI, 800 Da) building blocks were used to conceive DCFs for DNA recognition and binding. Treatment of **1** with **2** in different molar ratios (**1** : **2** = 1:1, 3:2) in acetonitrile (r.t., 72h) afforded mixtures of linear and cross-linked non-charged frameworks **A1-5** (Table S1, ESI) assembled via reversible amino-carbonyl/imine chemistry (Scheme 1). Then **A1-5** were reacted with bPEI **3** building blocks in water at varying molar ratios, to generate the poly-charged DCF vectors (Table S2-S6, ESI).

NMR experiments: ¹H-NMR spectral analysis agrees with the formation of mixture imine /aldehyde decorated **A1-5**. The 1:1 and 3:2 mixture are reminiscent with the formation of mostly linear polymers, with $M_n \sim 15000\text{-}18000 \text{ g mol}^{-1}$ as previously observed.⁷ Increasing the amount of **1**, more complex cross-linked frameworks are formed (Fig. S1-S5, ESI). Interestingly, the ¹H-NMR spectra of DCFs mixtures, recorded in CD₃CN and D₂O are similar and remain unchanged for months at neutral pH. As previously observed, the PEG chains may have a protecting effect against the hydrolysis of the imine bonds, favouring the imine formation.⁷ On progressive addition of cationic molecular heads **3**, to **A1-5** mixtures in water, the ¹H-NMR spectra are reminiscent with the formation of completely condensed frameworks (Fig.S1-S5, Table S2-S6). The conversion of the aldehyde groups is almost total on the addition of 0.2 eq. of cationic head **3**. We noticed that, at low concentration of **3** (0.4, 0.6, 0.8 eq.), insoluble aggregates are formed in aqueous solution. When the amount of PEI is increased (1-3 eq.), the hydrophilic behaviours and thus the solubility of colloids in water increase. The ¹H-NMR spectra of all combinations are consistent with the formation of completely condensed imine-networks. This is indicated by the analysis of chemical shifts of the imine and aromatic moieties, which shows for all cases similar and broad patterns of signals, reminiscent of exchanging dynamic networks in solution. Gel electrophoresis DNA binding experiments showed similar binding behaviours for all studied combinations (Table S7 ESI). At this point, we concentrated our next studies to a reduced series of DCF1-6 concerning two series of linear DCF1-3 and cross-linked DCF4-6 networks with different charge contents (Table 1).

Table 1. Different molar ratios of **1**:**2**:**3** used for synthesis of DCF 1-6.

DCF	1	2	3
1	1	1	1.5
2	1	1	2
3	1	1	3
4	3	2	1.5
5	3	2	2
6	3	2	3

X-ray photoelectron spectrometry-XPS experiments: The reactions between 1,3,5-benzenetrialddehyde **1**, PEG **2**, and bPEI **3**, building blocks precursors are also confirmed by high resolution XPS spectra. Figure S6 in ESI, describes the wide scan spectra of **1-3** and DCF1, **3** and **6**, respectively. The high resolution C1s peak between 281.8 - 289.2 eV observed for the 1,3,5-benzenetrialddehyde, **1** sample (Fig. 1a) was deconvoluted in three characteristic peaks at 284.4, 285 and 286.1 eV attributed to C=C, C-C/C-H, C=O bonds, respectively. The C1s peak ranged between 282.8 eV and 287.1 eV observed for the PEG **2**, sample (Fig. 1b) reveals three deconvoluted peaks, corresponding to C-C/C-H at 285 eV, C-N at 286 eV and C-O at 286.5 eV, respectively. Finally, specific C1s types of bindings have been observed for bPEI sample **3**, with specific peaks for C-C/C-H and C-N bonds at 285 and 285.4 eV, respectively (Fig. 1c). The deconvoluted C1s and N 1s spectra

for the **DCF1**, **DCF3** and **DCF6** are represented in Fig. 1d and Fig. 1e respectively. They are fitted with five and four peaks, attributed to C=C, C-C/C-H, C-N, C-O and C=N bonds at 284.4-284.5, 285.0, 285.7-286.0, 286.1-286.4 and 287.1-287.3 eV and to N-C at 398.6-398.9 eV, HN-C at 399-399.4 eV, NH₂-C at 399.5-399.9 eV and N=C at 400.1-400.2 eV, respectively. These data are in agreement with the total conversion of the aldehyde groups in imine groups, in perfect agreement with the results determined by the ¹H-NMR. Elemental compositions calculated based on the wide scan XPS spectra of investigated precursors and DCFs are in agreement with the theoretically calculated ones (Table S8, S9, ESI).

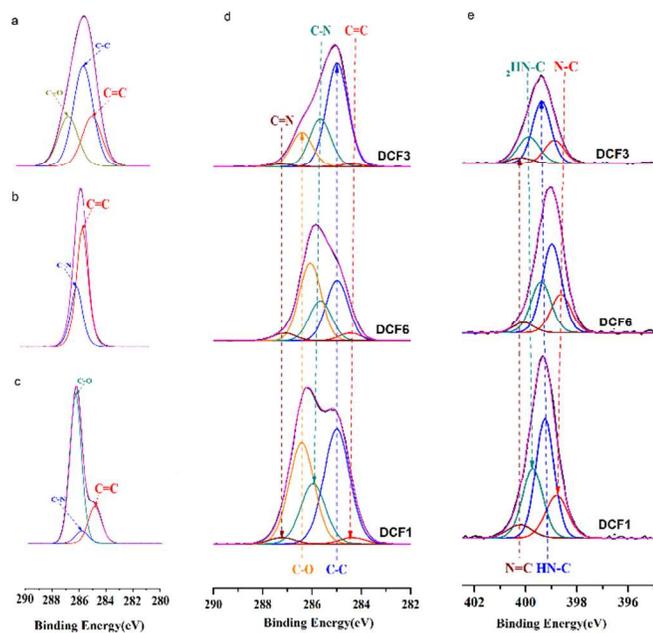


Fig. 1. Deconvoluted high resolution XPS spectra of C 1s peaks of a) 1, b) 2, c) 3, d) **DCF1,3,6** and e) N 1s peaks of **DCF1,3,6**.

DNA polyplexes based on the DCF1, DCF3 and DCF6: Two types of polyplexes of **DCF1**, **DCF3** and **DCF6** were generated by mixing DCFs with linear dsDNA (salmon sperm DNA; ~200-300 base pairs) and a circular dsDNA (plasmid pCS2+MT-Luc; 5991 base pairs). All related studies were performed considering compositions precisely formulated between the carriers and the dsDNA, calculated as ratios between the molar fraction N/P of nitrogen in the DCF conjugates and the molar content of phosphorous in the DNAs.

Morphological and dimensional behaviour of DNA/DCF polyplexes: To ensure prolonged circulation in blood vessels, polyplex particle size should be compacted, roughly between 10 and 200 nm. Entities below 10 nm are quickly cleared through the kidney, while the ones above 200 nm are cleared by the reticulo-endothelial system^{12d}. For these reasons it was important to determine the size of particulate DNA polyplexes, generated by **DCF**-dsDNA association in aqueous solution. First, we investigated their solution behavior by Dynamic Light Scattering (DLS) (Fig. 2a). Our results show that DCFs have an

average diameter of 3 nm, while **DCF**-salmon sperm dsDNA polyplexes have an average diameter of 80 nm. This suggests that salmon sperm dsDNA with sub-nanometric dimensions is well packed by the DCF, leading to formation of bigger aggregates, in which likely several DNA molecules fold and bind around several DCFs (Fig. 2b).

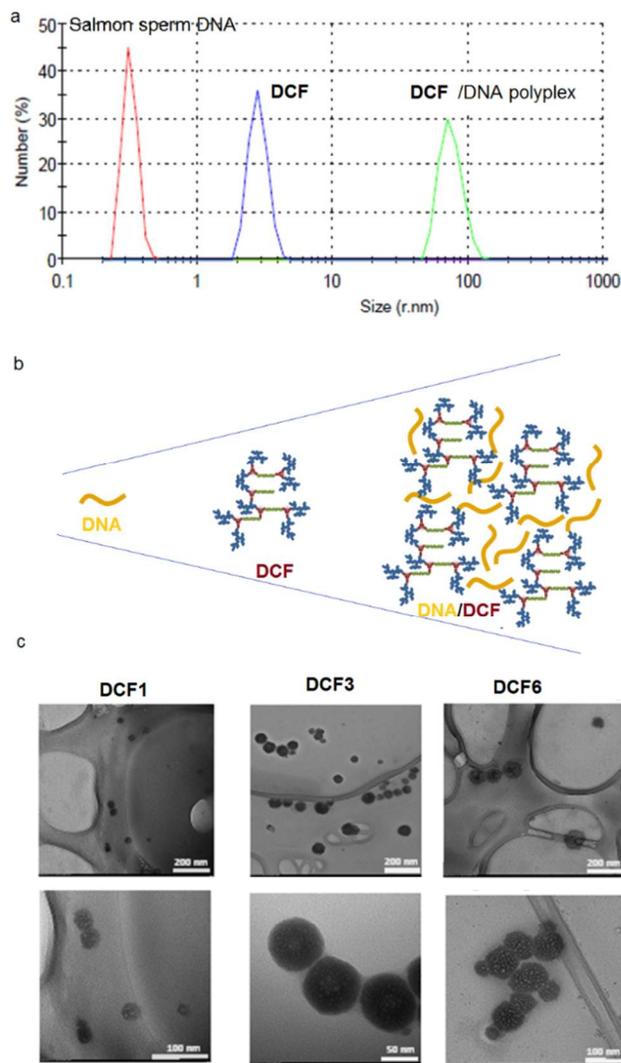


Fig. 2. a) DLS measurements for dsDNA, DCF and dsDNA:DCF polyplex; b) Size of dsDNA, DCF and dsDNA-DCF polyplex and their proposed self-aggregation mechanism; c) TEM micrographs for **DCF1**, **3**, **6** · pCS2+MT-Luc polyplexes at N/P=10.

TEM imaging (Fig. 2c) showed formation of spherical aggregates, whose sizes varies between 40-125 nm, depending on the 1 : 2 : 3 molar ratio; the aggregates display a narrow size distribution within each sample. The inside areas of the aggregates present different densities, perhaps due to a different packaging capacity of conjugates for DNA, determined by the availability of the positive charges of bPEI conjugate toward negative charges of DNA.

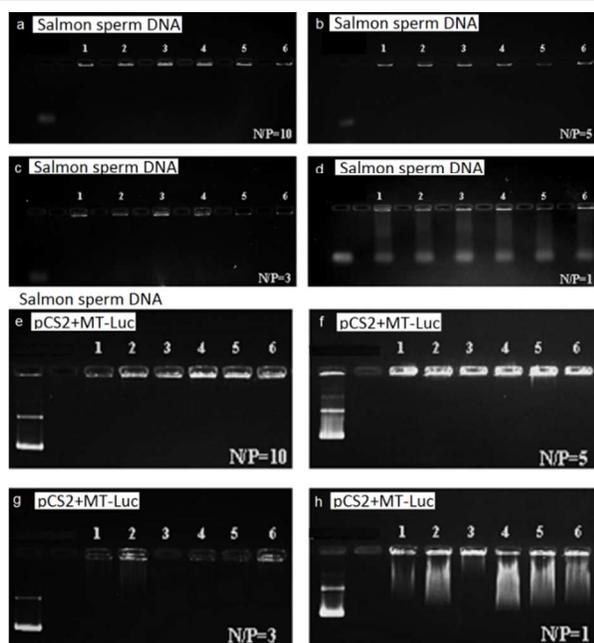


Fig. 3. Agarose gel retardation assays in the case of **DCF1-6** · salmon sperm dsDNA at a) N/P=10, b) N/P=5, c) N/P=3, d) N/P=1 ratios and **DCF1-6** · pCS2+MT-Luc polyplexes at e) N/P=10, f) N/P=5, g) N/P=3, h) N/P=1 ratios.

DNA binding ability of DCF1-6 polyplexes was investigated by agarose gel electrophoresis (Fig. 3). Salmon sperm dsDNA or plasmid pCS2+MT-Luc were complexed with **DCF1-6**, at different N/P ratios. In the case of the linear salmon sperm dsDNA, polyplex electrophoretic migration is completely blocked at N/P ratio=3. At this value, DLS and TEM analysis shows that packing between DNA and **DCF1-6** takes place with formation of aggregates in solution and in solid state. These results also agree with previous reports, showing that nearly all DNA is packed by bPEI of 25 kDa for N/P ratio=3 and that transfection efficiency can be enhanced for higher N/P ratios.^{12c} We observed a significant difference between the ability of DCFs to condense salmon sperm dsDNA versus the stiffer, higher molecular weight plasmid DNA. Electrophoretic migration of plasmid DNA is completely blocked for N/P ratios above 5 in the case of **DCF1-6**. In this respect, a levelling effect is obvious in the case of N/P =1 or 3 where **DCF1-6** are less effective in binding plasmid DNA. Comparatively, for these N/P ratios, the best binding is observed for **DCF3**, suggesting a good balance between the linear geometry of the PEG backbone and the high content of bPEI, which is perhaps optimal for packaging and protection of plasmid DNA.

Transfection ability: We measured the transfection efficiency of **DCF1, 3** and **6** polyplexes by assaying the uptake by HeLa cells of pCS2+MT-Luc plasmid, which drives expression of firefly luciferase. HeLa cells were treated with polyplexes formed by mixing a fixed quantity of pCS2+MT-Luc DNA plasmid (400 ng per well of a 96-well plate) with varying amounts of DCFs, followed by luciferase assays 48 hours later.

As shown in Fig.4, all tested polyplexes transfect HeLa cells to a level comparable with bPEI, or slightly better at N/P ≥ 100 .¹³

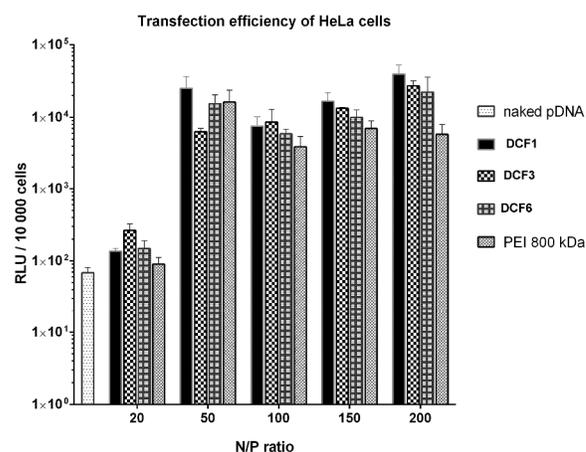


Fig. 4. Transfection efficiency at different N/P ratios measured at 48 hours. HeLa cells were treated with polyplexes made by combining pCS2+MT-Luc plasmid with polymers: **DCF1**, **DCF3**, **DCF6** and PEI 800. Naked pDNA is presented as reference. Results are given as relative light units/ 10 000 cells.

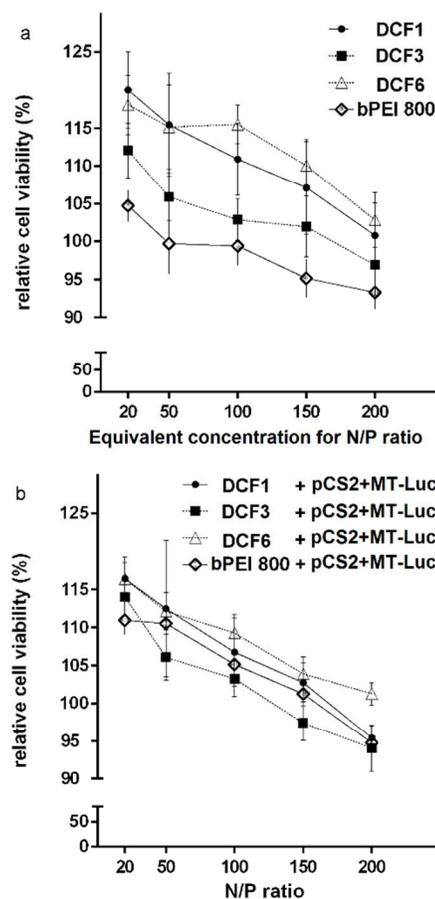


Fig. 5. Cytotoxicity profiles of a) **DCF1, 3, 6** and PEI and b) their respective **DCF1, 3, 6** (used at the same concentration with that employed to calculate N/P) and PEI-pCS2+MT-Luc polyplexes based on MTS assay. The viability of cells grown in medium alone (control cells) was considered 100%.

Cytotoxicity of DCF1-6 and DCF1-6 polyplexes: To assess toxicity of DCF1,3,6 polyplexes, we used an assay that measures mitochondrial reductase activity (MTS)¹⁴. Only cells with uncompromised mitochondrial function reduce tetrazolium salt to formazan. Using this assay, we observed an increase in cell viability with decreasing N/P ratio; interestingly, a slight increase in cell proliferation relative to untreated controls was observed for N/P ratios of less than 150. We determined that cells treated with DCF1 and DCF6 and their polyplexes show the highest viability, while DCF3 and bPEI 800 show higher toxicity. (Fig.5). DCF1 and DCF6 with higher PEG content lead to increased cellular proliferation compared to DCF3 that incorporates a higher fraction of bPEI. DCF3 showing high DNA binding and transfection ability, and having the highest bPEI concentration is associated with higher cytotoxicity. Our findings are in accord with the effect of PEG on cytotoxicity that has been described in previous studies¹⁵.

Conclusions

The present study describes the synthesis and characterization of a class of DNA nanovectors based on specific frameworks of components and connector centres, linked by reversible covalent bonds. The dynamic self-assembly of PEG components with bPEI cationic binding groups around the aromatic core connectors lead to adaptive spatial distributions in the presence of interacting DNA biotargets.

The DCF polyplexes reported here are able to act as gene nanovectors, by forming stable polyplexes with dsDNA. Depending on the type and amount of associated DNA and on the molar ratio of bPEI / PEG, polyplexes have dimensions ranging between 40 and 125 nm. All tested vectors were capable of transfecting DNA into HeLa cells and demonstrated low cytotoxic levels; even at an N/P=200 cell viability is over 90% relative to untreated control cells. We can conclude that the presence of the PEG component and a moderate amount of b-PEI in DCFs are both important in the construction of highly transfecting and cytofriendly polyplexes. Perhaps an optimal balance between the linear geometry of the PEG backbone and high content of bPEI favours optimal packaging and protection of DNA. Furthermore, cell viability was always above 90 %, demonstrating that our vectors are well tolerated by cells.

Our findings provide novel insight in the development, *via* a simple synthetic strategy, of multivalent adaptive nanovectors carrying out several functionalities for optimal DNA binding, membrane penetration, DNA delivery and anti-opsonisation functions. We believe that the approach presented here has the potential of solving critical problems in DNA delivery to cells that stem from the enormous variability of both DNA targets and cell types to be transfected. Work is currently in progress to further develop such dynamic adaptive systems.

Experimental Section

Materials: 1,3,5-benzenetricarboxaldehyde (97%), **1** was purchased from Manchester Organics. Poly(ethyleneglycol) bis (3-aminopropyl)terminated (PEG, $M_n \sim 1,500$ g/mol), **2** branched polyethylenimine, (bPEI, 800 Da), **3** were purchased from Sigma-Aldrich, and were used without further purification. Low molecular weight salmon sperm DNA was purchased from Fluka (St. Louis, MO, USA).

Synthesis of A1: 1,3,5-benzene-tricarboxaldehyde (0.3014 g, 1.8582 mmol) was dissolved in acetonitrile (40 ml) under magnetic stirring. To this mix was added PEG (2.7884 g, 1.8582 mmol) dissolved in acetonitrile (10 ml), and the reaction was stirred for 72 hours, at room temperature. The solvent was removed by rotary evaporation (40°C, under vacuum), affording the product (2.8 g).

Synthesis of DCF1: A1 (0.030 g, containing 0.0202 mmol of **1**) was dissolved in water (1 ml). To this solution was added bPEI (0.024 g, 0.0303 mmol) dissolved in water (0.284 ml). The reaction was stirred for 12 hours on a vortex and then incubated at room temperature for 72 h.

NMR: Spectra were recorded on a Bruker instrument operated at 300 MHz. All samples were dissolved in D₂O and analyzed at room temperature. Chemical shifts were recorded in ppm.

X-ray photoelectron spectroscopy (XPS) were collected with a KRATOS Axis Nova instrument (Kratos Analytical, Manchester, UK), using AlK α radiation, with 20 mA current and 15 kV voltage (300W). XPS survey spectra were collected in the range of -10÷1200 eV with a resolution of 1 eV and a pass energy of 160 eV. For all elements identified from survey spectra, high-resolution spectra were collected using pass energy of 20 eV and step size of 0.1 eV. Data were analysed using Vision Processing software (Vision2 software, Version 2.2.10). The binding energy of the C1s peak was normalized to 285 eV.

Particle size measurements were performed on a Nano Zetasizer (Malvern Instruments Ltd., UK) operating at 633 nm and recording the back scattered light at an angle of 173°. Measurements were done in a cuvette with a 10 mm path length. Sample temperature was allowed to equilibrate for 3 min before each measurement. The light scattering was recorded for 200 s with 10 replicate measurements.

TEM microscopy images were obtained on a HT7700 Hitachi Transmission Electron Microscope. Samples were prepared by placing a drop of aqueous suspension of polyplexes on a carbon-coated copper grid, then allowing the solvent to evaporate at room temperature. The grids were imaged in high-resolution mode, under an operating potential of 100 kV. For each sample, the size of 500 aggregates was measured from TEM micrographs, using Image J software.

Preparation of plasmid DNA. Plasmid pCS2+MT-Luc which encodes for firefly luciferase (Harvard University, Boston) was propagated by molecular cloning in E.Coli DH5 α , extracted and purified with EZNA ENDO-FREE PLASMID MINI II kit (Omega Bio-Tek, Inc.).

Preparation of polyplexes. The polymer was dissolved in pure water (Millipore), after which the appropriate amount of DNA was added and the mix was vortexed for 10s, followed by incubation for 30 minutes before use.

Gel retardation assay. Polyplexes were prepared with varying N/P ratios: 10, 5, 3 and 1. The polyplex solution (5 μ L polymer and 5 μ L salmon sperm DNA / 1 μ L plasmid solutions) was mixed with 3 μ L TAE buffer pH=7.4 and 10 μ L sucrose solution. The samples were then loaded on a 1% agarose gel. Electrophoresis was carried out at 90 V for 60 minutes for salmon sperm DNA and 120 minutes for plasmid. The gel was stained with ethidium bromide and was then imaged on a UV trans-illuminator.

Cell culture. Transfection of DNA by polyplexes was assayed in HeLa cells. HeLa cells (from CLS-Cell-Lines-Services-GmbH, Germany) were grown in tissue culture flasks with alpha-MEM medium (Lonza) supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin-streptomycin-amphotericin B mixture (Lonza).

Measuring in vitro transfection efficiency. HeLa cells were harvested by trypsinization, counted with a Countess Automated Cell Counter (Invitrogen), and seeded at a density of 10⁴ cells/well in 96 well white opaque culture microplates (PerkinElmer) with 100 μ L/well. Cells were transfected 24 hours later with 400 ng of pDNA per well, mixed with DCFs or bPEI 800 Da at various N/P ratios. Both pDNA and polymer were separately diluted in 10 μ L alpha-MEM without FBS and antibiotic. These 2 solutions were then mixed, vortexed briefly and then incubated at room temperature. After one hour, the transfection mixture was added to cells without removing the medium. After 48 hours, transfection efficiency was evaluated with Bright-Glo(TM) Luciferase Assay System kit (Promega) on EnSight plate reader (PerkinElmer). Each experiment was performed in triplicate.

Cytotoxicity assay. Cytotoxicity was measured using the CellTiter 96@AQueousOne Solution Cell Proliferation Assay (Promega). HeLa cells were seeded at a density of 10⁴ cells/well in 96 well plates, in 100 μ L DMEM without phenol red (Lonza) supplemented with 10% FBS. The next day, cells were transfected with polyplexes as described above, after which the cells were grown for another 44 hours. At least 3 biological replicates were performed for each polyplex type and N/P ratio, and each experiment was repeated 3 times. After 44 hours, 20 μ L of CellTiter 96@AQueousOne Solution reagent were added to each well, and the plates were incubated for

another 4 hours before reading the result. Absorbance at 490 nm was recorded with an EnSight plate reader (PerkinElmer).

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Notes and references

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