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DyNAvectors: dynamic constitutional vectors for adaptive DNA transfection

Dynamic Constitutional Frameworks are prepared and tested as modular DyNAvectors for DNA transfection. Depending on their tunable structure, they constitutionally self-adapt to the DNA targets, allowing a rapid identification of most effective vectors with high complexation ability, good transfection efficiency, and well tolerated by mammalian cells.

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DyNAvectors: dynamic constitutional vectors for adaptive DNA transfection[†]

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Dynamic constitutional frameworks, based on squalene, PEG and PEI components, reversibly connected to core centers, allow the efficient identification of adaptive vectors for good DNA transfection efficiency and are well tolerated by mammalian cells.

Gene therapy is a method used to introduce genetic material into cells to treat disorders. It is well known that viral vectors have superior transfection capacity but their use is limited by their induction of immune responses and virus-pathogenicity.^{1,2} Alternatively, the non-viral vectors present lower transfection but their cytotoxicity limits their application to clinical trials.³ That is why, the rational design of non-viral vectors has been developed.⁴ However, the rational design has become limited to a low number of synthetic components fixed in specific positions on the vector backbone and should be completed by Dynamic Constitutional (DC) approaches.^{5–7} Extending the DC concepts to materials science led to the emergence of Dynamic Polymers (Dynamers⁸) that are polymers linked through reversible bonds and are able to respond to internal or external factors by exchange of components. We recently proposed the Dynamic Constitutional Frameworks (DCFs), the 3D Dynamers, for DNA recognition.⁷ The ability to adaptively implement spatial rearrangements of such reversible materials may induce a high level of correlativity of their 3D architectures and external surfaces in interaction, for example, the DNA and the cell membrane barrier. In other words, this leaves the DNA to self-generate the fittest material, for its own transfection. The DNA target itself is used to self-select an active DyNAvector from a virtual mixture of architectures, resulting in

a highly useful simplified screening process. Within this context, the use of dynameric materials for DNA transfection is an emerging field.^{7,9} Herein, after the DNA recognition studies we further report an efficient and simple constitutional approach to conceive DCFs as multivalent DyNAvectors for DNA transfection (Fig. 1). They simultaneously exhibit optimal DNA binding, transfection yield to standard agents and preserve high HEK 293T cell viability.

DyNAvector synthesis. The synthesis involves the following components: (a) 1,3,5-benzenetrialdehyde 1 as the core centre, able to cross-link the network components and DNA-binding sites via the amino-carbonyl/imine reversible chemistry; (b) PEG-ylated squalene (SQ-PEG) 2 hydrophobic component, known to form stable particles with diameters \sim 100–200 nm in aqueous solution;¹⁰ (c) poly-(ethylene-glycol)-bis (3-amino-propyl)terminated ($M_n \sim 1500 \text{ g mol}^{-1}$) PEG(NH₂)₂ 3 segments, known to favour solubility in water and to reduce the immunogenicity of the systems;¹¹ and (d) low molecular weight branched polyethyleneimine (bPEI), $(M_n \sim 800 \text{ g mol}^{-1})$ 4 as cationic binding sites, able to bind DNA. We know that bPEI2500 (25 kDa) is the most effective vector;^{11a} however, they present increased cell toxicity.12 On contrary, bPEI800 (0.8 kDa) has demonstrated low toxicity and conversely very low transfection activity.¹³ We anticipate that the multivalent presentation of bPEI800 on DCF adaptive backbones might increase its transfection efficiency, keeping the toxicity levels low. Treatment of 1 with different equiv. of 2¹⁴ and 3 (Table S1, ESI[†]) in CH₃CN (rt, 24 h) resulted in the formation of a mixture of linear and cross-linked DCFs (5 and 6), supported by ¹H-NMR spectral results. The reactions have been monitored by following the aldehyde chemical shifts corresponding to mono-, M di- D and trialdehyde T-type compounds for which the corresponding imine chemical shifts can be observed in the spectra (Fig. S1, ESI⁺). By combining 1 and 2 (1:1 molar ratio) in the absence of 3, the M:D:T ratio is found to be 1:3:1.5. The addition of 3 results in the progression (M:D:T = 1:3:0.5 at 1:2:3 molar ratio of 1:1:0.5) to the complete consumption of T (M:D = 1:3 at 1:1:1 ratio). By decreasing the ratio of component 2 to 1:0.5:1 the ratio M:D remains 1:3. Then, the mixture of 5 and 6 was treated with various amounts of bPEI (Table S1, ESI⁺).

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Fig. 1 Structure of 1,3,5-benzenetrialdehyde 1 core-connectors, SQ-PEG derivative 2 and poly-(ethyleneglycol)-bis(3-aminopropyl) terminated 3, segments and DNA binding sites bPEI 4. The intermediary units (IU) 5, 6 resulted after combination of components 1–3 have been combined with 4 to give multivalent charged unit (MU), 7,8; SQ-based self-assembly 9 results in the formation of DyNAvector nanoparticles binding DNA to form polyplex (P) 10 used as gene delivery system.

At <0.5 equiv. of bPEI800, insoluble aggregates MU2 and MU4 are formed in aqueous media. Further increasing the bPEI800 amount led to colloidal solutions. Interestingly, in the ¹H-NMR spectra recorded in D₂O, the aromatic and the imide signals of 7 and 8 mixtures are highly broadened (Fig. S2, ESI[†]), showing the total consumption of aldehydes. The formation of colloidal species 9 in solution is responsible for this protection effect against the hydrolysis of the imine bonds. The formation of imine -N=C- bonds is confirmed by X-ray photoelectron spectroscopy (XPS) (Fig. S3, ESI⁺). Energy-dispersive X-ray spectroscopy (EDX) (Fig. S4 and S5, ESI†) allows us to conclude that the elemental compositions of C, N and O in the samples are in agreement with the theoretical ones (Table S2, ESI[†]). The formation of discrete assemblies of MU and their DNA plasmid pEYFP polyplexes P were confirmed by transmission electron microscopy (TEM) (Fig. 2a). MU were found to form µm spherical particles, with the hydrophobic squalene core and the PEG/PEI hydrophilic corona (Fig. 2). Moreover, it is obvious that P present more compact structures in comparison to the noncomplexed MU. The size of nanoparticles of P is strongly dependent on N/P values varying between 20 and 100 nm for N/P ratios lower than 50 (Fig. 2b-d). Surprisingly, at the N/P ratio of 200 cylindrically-shaped particles with a size of about 250 nm and spherical particles of about 700 nm were observed (Fig. 2e).

DNA binding ability. The ability of DyNAvectors to condense negatively charged DNA plasmid pEYFP was investigated by



Fig. 2 TEM images of core-shell particles of: (a) single particles: MU5 \sim 500 nm; MU6 \sim 1.4 μ m; MU7 \sim 1.8 μ m MU8 \sim 600 nm; (b) MU6 \sim 1.4 μ m, (c) P6 at N/P = 20 (100 nm), (d) P6 at N/P = 50 (100 nm), (e) P6 at N/P = 200 (spherical particles 700 nm, tube like particles 250 nm).

agarose gel electrophoresis (Fig. S6, ESI⁺). Retardation assay performed for MU: pEYFP polyplexes having different N/P ratios exhibits a complex behavior, revealed by the migration spot splitting of supercoiled and nicked circle topologically-distinct forms or pEYFP. The reduction of DNA electrophoretic mobility is the result of condensation between positive charges of the vectors and the negative charged phosphate groups of DNA. Free PEG, polyplex P1 present weaker interactions with DNA showing the importance of $PEG(NH_2)_2$ 3 which does not interfere with DNA complexation (Fig. S6a). The use of $PEG(NH_2)_2$ 3 and bPEI800 4 cationic binding sites in P3, P5-P7, (Fig. S6b-f) or increasing amounts of bPEI800 4 cationic binding sites in P8 (Fig. S6g) induces a strong increase of interactions with pEYFP starting with an N/P ratio of 5, in accordance with the disappearance of the smear under the loading pocket. Remarkably, all P's show higher complexation ability when compared with bPEI800, used as the reference.

In vitro transfection efficiency-TE and cytotoxicity. In order to evaluate *in vitro* TE, HeLa cells in 24 well plates were treated with **P** comprising 1.5 μ g of plasmid pCS2 + NLS-eGFP; plates were inspected under a microscope 48 h post-transfection (Fig. 3a) and transfected cells were quantified by flow cytometry (Fig. 3b). The cytotoxicity of DyNAvectors (Fig. 3c) was determined by propidium iodide (PI) fluorescence assay, known to present a high affinity for double stranded dsDNA.^{15,16}

The transfection results, in line with previously reported values for the non-viral vectors,¹⁷ show that the multivalent presentation of bPEI800 increases the efficiency of both **P6** (12%) and **P8** (2%) polyplexes at the N/P ratio of 50, compared to "monomeric" positive control bPEI800 (<1%).

P6 demonstrated six times higher transfection than **P8**, having PEG not only in the SQ-moiety, but also as an external constitutive component, stabilizing **P6** in a serum-rich environment (Fig. 3a). The size of **P6** at N/P 50 was found to be around 100 nm (Fig. 2d) which dimensionally agrees with requirements for nanoparticles for gene delivery.^{10–17} **P6** proved to be nontoxic at this N/P ratio (dead cells <10%) (Fig. 3c), similar with other non-viral vectors, previously reported in literature.^{1,2} At N/P ratio 200 a significant decrease in transfection for **P6** and **P8** was observed (Fig. 3b). Transfection of **P6** was slightly higher



Fig. 3 (a) Morphology of HeLa cells 48 hours post transfection with polyplexes carrying pCS2 + NLS-eGFP plasmid; polymer/plasmid (N/P) ratio = 50 (a–c) 200 (a'–c'). Scale bar, 200 μ m. (b) eGFP gene transfection efficiency determined by flow cytometry; (c) cytotoxicity profiles of polyplexes based on PI assay.

than that of bPEI800 and **P8** exhibited the lowest efficiency. TEM images showed the formation of large spherical particles of **MU6** (700 nm) (Fig. 2e) and tube shaped particles of **P6** (250 nm). This came as no surprise since, it is known that particles with <150 nm sizes correlate with better transfection than their bigger counterparts.⁸ The higher cytotoxicity of **P6** is associated with the increase of bPEI800 concentration and the increase in particle size observed in TEM images (Fig. 2).

In conclusion, adaptive DyNAvectors have been synthesized via constitutional self-assembly of PEG and squalene with bPEI800 cationic DNA binding groups with core centres. They adaptively generate multivalent polyplexes with variable sizes that transfect HeLa cells and have been proved to have low cytotoxicity levels. This very simple screening strategy, lets us easily conclude at this stage that among all studied compositions P6 proves to be an interesting transfection agent with an efficiency of 12% at N/P = 50, while presenting very importantly a minimal toxicity (<10%), reminiscent of the most known examples of non-viral vectors.^{16,17} These findings provide insights into the identification, via self-fabrication, of the multivalent adaptive DyNAvectors for optimal DNA binding, membrane penetration and transfection functions. We believe that the novel Dynamic Constitutional Approach presented here has the potential for the easy identification of potential highly active vectors for DNA delivery. Work is currently in progress to further develop more efficient systems for targeted DNA transfection on different cell lines, which may be used in therapy.

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