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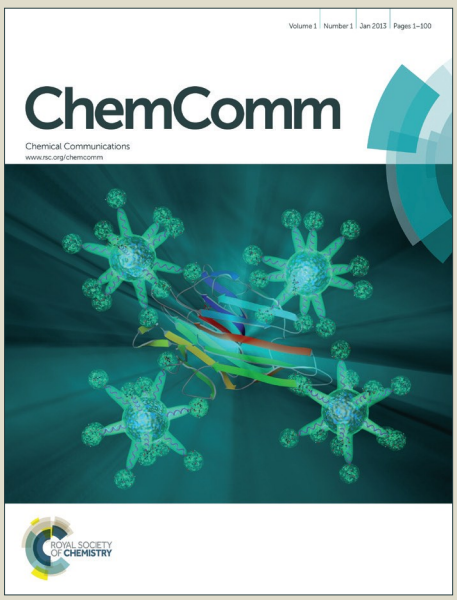

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DyNAvectors: Dynamic constitutional vectors for adaptive DNA transfection

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

Gene therapy is a method used to introduce genetic material into cells to treat disorders. It 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 the application to clinical trials.[3] That is why, the rational design of non-viral vectors has been developed.[4] However due the variety of the DNA targets, the rational design became limited to a low number of components, deliberately positioned and fixed in specific positions on the vector backbone and should be completed by Dynamic Constitutional -DC approaches.[5-7] Extending these concept to material science emerged Dynamic Polymers-Dynamers[8] that are polymers linked through reversible bonds and able to response to internal or external factors by components' exchange. We recently proposed the Dynamic Constitutional Frameworks-DCFs the 3D Dynamers, for DNA recognition.[11] 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 examples with 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.[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 involve the following components: a) 1,3,5-benzenetrialdehyde **1** as a 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 ~100-200 nm in aqueous solution;[10] c) poly-(ethylene-glycol)-bis (3-amino-propyl)-terminated (M_n~1500g/mol^{-1}) PEG$(NH_2)_2$; **3** segments, known to favour solubility in water and to reduce the immunogenicity of the systems;[11] d) low molecular weight branched Polyethyleneimine (*b*PEI, (Mn~800 g/mol^{-1}) **4** as cationic binding sites, able to bind DNA. We know that *b*PEI2500 (25 kDa) are the most effective vectors,[11a] however they present increased cell toxicity.[12] Low molecular weight *b*PEI800 (0.8 KDa), has demonstrated low toxicity and conversely very low transfection activity.[13] Our aim is to We anticipate that *b*PEI800 multivalent presentation on DCFs adaptive backbones might increase its transfection efficiency, keeping the low toxicity levels. Treatment of **1** with different eq. of **2**[14] and **3** (Table 1S) in CH_3CN (rt, 24 h) resulted in a formation of a mixture of linear and cross-linked DCFs (**5** and **6**), supported by ^{1}H-NMR spectral results. The reaction 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 (Figure 1S). By combining **1** and **2** (1:1 molar ratio) in the absence of **3**, the M:D:T ratio is 1:3:1.5. The addition of **3** results in the progressive (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 *b*PEI (Table 1S).

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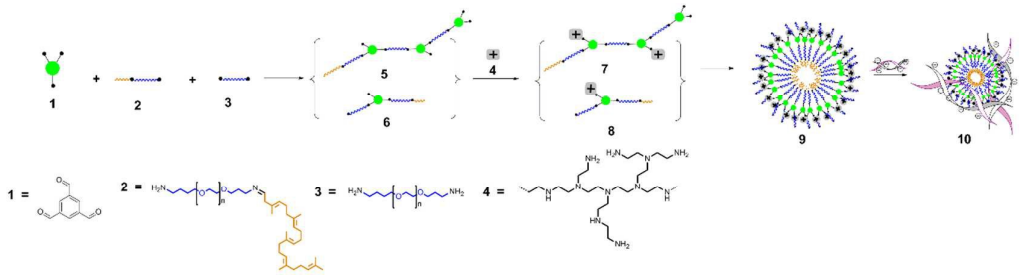

Figure 1. Structure of 1,3,5-benzenetrialdehyde **1** core-connectors, SQ-PEG derivative **2** and poly-(ethyleneglycol)-bis(3-aminopropyl) terminated **3,** segments and DNA binding sites bPEI **4**. The intermediary units (**IU**) **5**, **6** resulted after combination of components **1**-**3** have 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.

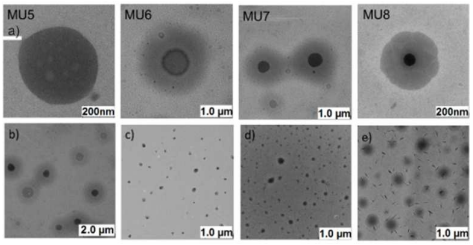

Figure 2. TEM images of core-shell particles of: a) single particles: **MU5**~500 nm; **MU6**~1.4 µm; **MU7**~1.8 µm **MU8**~600nm; b) **MU6**~ 1.4 µm, c) **P6** at N/P=20 (100nm), d) **P6** at N/P=50 (100nm), e) **P6** at N/P=200 (spherical particles 700 nm, tube like particles 250 nm).

At < 0.5 Eq of *b*PEI800, insoluble aggregates **MU2** and **MU4** are formed in aqueous media. Further increasing of the *b*PEI800 amount led to colloidal solutions. Interestingly, in the ^{1}H-NMR spectra recorded in D_2O, the aromatic and the imide signals of **7** and **8** mixtures are highly broadened (Figure 2S), showing the total consumption of aldehydes. The formation of colloidal species **9** in solution is responsible for this protecting effect against the hydrolysis of the imine bonds. The formation of imine -N=C- bonds is confirmed by X-ray photoelectron spectroscopy (XPS) (Fig. 3S). The Energy-dispersive X-ray spectroscopy (EDX) (Fig. 4,5S) allow to conclude that elemental compositions of C, N and O elements in the samples are in agreement with the theoretical ones (Table S2). The formation of discrete assemblies of **MU** and their DNA plasmid pEYFP polyplexes **P** were confirmed by Transmission electron microscopy (TEM) (Fig. 2a). It was found that the **MU** form µm spherical particles, with the hydrophobic squalene core and the PEG/PEI hydrophilic corona (Fig. 2). Moreover, it is obvious that **P** present the more compact structures in comparison to the non-complexed **MU**. The size of nanoparticles of **P** is strongly dependent of N/P values varying between 20-100 nm for N/P ratios lower than 50 (Figure 2b-d). Surprisingly, at N/P 200 cylindrically-shaped particles with size about 250 nm and spherical particles of about 700 nm were observed (Figure 2 e).

DNA binding ability: The ability of DyNAvectors to condense negatively charged DNA plasmid pEYFP was investigated by agarose gel electrophoresis (Figure 6S). Retardation assay performed for **MU**: pEYFP polyplexes having different N/P ratios exhibits a complex behavior, revealed by the migrating 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 (Figure 3a). The use of PEG$(NH_2)_2$ **3** and *b*PEI800 **4** cationic binding sites in **P3**, **P5**-**P7**, (Figure 3b-f) or increasing amounts of *b*PEI800 **4** cationic binding sites in **P8** (Figure 3g) induce a strong increase of interactions with pEYFP starting with N/P ratio of 5, in accordance with disappearance of the smear under the loading pocket. Remarkably, all **P** show higher complexation ability when compared with *b*PEI800, used as 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 µg of plasmid pCS2+NLS-eGFP; plates were inspected under microscope 48 h post-transfection (Fig. 3a) and transfected cells were quantified by flow cytometry (Fig. 3b). The cytotoxicity of DyNAvectors (Fig. 4c) was determined by Propidium iodide (PI) fluorescence assay, known to present high affinity for double stranded dsDNA.[15,16] The transfection results, in line with previous reported values for the non-viral vectors[17] show that the multivalent presentation of *b*PEI800 increase the efficiency of both **P6** (12 %) and **P8** (2%) polyplexes at N/P ratio of 50, than "monomeric" positive control *b*PEI800 (<1%).

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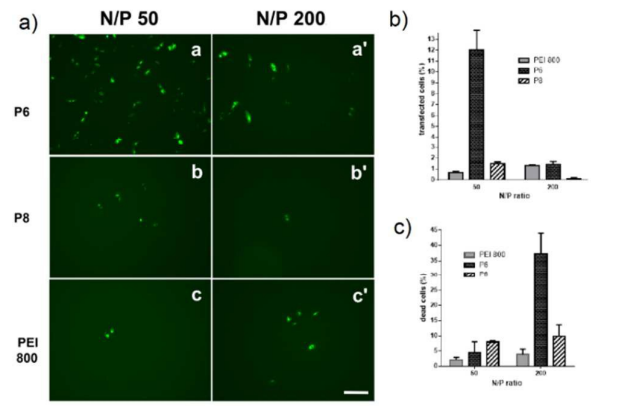

Figure 3. a) Morphology of HeLa cells 48 hours post transfection with polyplexes carrying pCS2+NLS-eGFP plasmid; polymer/plasmid (N/P) ratio=50 (a, b, c) 200 (a', b', c'). Scale bar, 200 µm. b) eGFP gene transfection efficiency determined by flow cytometry; c) Cytotoxicity profiles of polyplexes based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

P6 demonstrated six time higher transfection than **P8**, having PEG not only in SQ-moiety, but also PEG as external constitutive component, stabilizing the **P6** in a serum-rich environment (Fig. 3a). The size of the **P6** at N/P 50 was found to be around 100 nm (Fig. 2d) which dimensionally agree with requirements for nanoparticle for gene delivery.[10-17] Also, **P6** proved to be nontoxic at this N/P ratio and it is being lower than 10% (Fig. 3c), compared with other non-viral vectors, previously considered in literature.[1,2] At N/P ratio 200 an important decrease in transfection for **P6** and **P8** was observed (Fig. 3b). Transfection of **P6** was slightly higher than *b*PEI800 and **P8** exhibiting 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 in no surprise since, it is known < 150 nm sizes correlates with better transfection than their bigger counterparts.[8] Higher cytotoxicity of **P6** is associated with the increase of bPEI800 concentration and with 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 *b*PEI800 cationic DNA binding groups with the core centres. They adaptively generate multivalent polyplexes with variable sizes that transfect HeLa cells and proved low cytotoxic levels. This very simple screening strategy, let us easily to 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 in 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 of easily identify 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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