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Dedicated to Professor Bogdan C. Simionescu
on the occasion of his 70th anniversary

DYNAMIC SELF-ORGANIZING SYSTEMS FOR DNA DELIVERY

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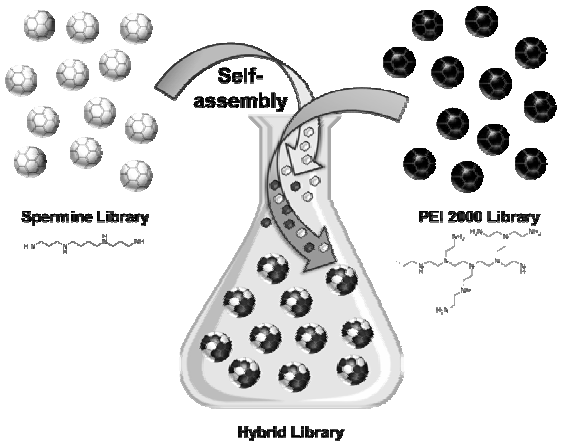
The development of dynamic vectors using the Dynamic Constitutional Chemistry is a well-known strategy and is based on designing systems undergoing self-organization. Thus, prepared Dynamic Combinatorial Frameworks (DCF) are capable of spontaneously generating well-defined organized supramolecular architectures by self-assembly. The design and synthesis of dynamic vectors involved the formation of a combinatorial library of DCFs. While designing the library, we used 1,3,5-benzenetrialdehyde as a trifunctional center, Pegylated squalene derivative for its self-assembly behavior, polyethylene glycol moiety for its biocompatibility properties, branched polyethylene imine as cationic polymer able to complexate DNA and spermine due to its role in cell physiology. By mixing various DCFs between them within the library, we created complex DCFs as adaptive, reorganized vectors for DNA transfection formed by reassociation and rearrangement of components. Obtained hybrid DCFs effectively bind plasmid DNA (pCS2+MT-Luc), are not cytotoxic and, furthermore, prove to be effective in transfection of plasmid DNA on HeLa cell line.



INTRODUCTION

Constitutional Dynamic Chemistry (CDC) became a new evolutional approach,[1-3] to produce chemical diversity, especially important in time of high demand of new "easy to make" materials for drug delivery.[4] We have recently proposed a Dynamic Constitutional Strategy for the construction of Dynamic Constitutional Frameworks (DCF) for DNA recognition.[5] Moreover, we presented the concept of Dynamic Polymers – Dynamers [6,7] which are polymers capable of spontaneously generating well-defined organized supramolecular architectures by self-assembly from their components with high DNA complexation ability, good transfection efficiency, and well tolerated by mammalian cells. The use of reversible interactions as dynamic interfaces between DCF components allow self-adjustment of the system's tridimensional geometry and functional properties. Herein, we report an efficient and simple constitutional approach to conceive Dynamic Constitutional Frameworks as adaptive reorganized vectors for DNA transfection formed by reassociation and rearrangement of components by mixing various DCFs.

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RESULTS AND DISCUSSION

Design and preparation of vectors

The proposed design and synthesis of dynamic vectors involve the formation of a combinatorial library consisting of nine DCFs (Table 1). While designing the library, we have chosen to use: (*i*) a core of 1,3,5-benzenetrialdehyde (TA), serving as a trifunctional center capable of covalent linking of their functionalities *via* the amino-carbonyl/imine reversible chemistry; (*ii*) Pegylated squalene derivative[6] for its self-assembly behavior and for introducing PEG moiety in order to increase membrane penetration and biocompatibility;[8-10] (*iii*) branched polyethylene imine of 2000 Da (PEI 2000) due to its advantageous properties as cationic polymer able to complexate DNA.[11-13] We also included spermine due to its role in cell physiology, such as effects on the structure of cellular macromolecules, gene expression, protein function, nucleic acid and protein synthesis, regulation of ion channels, and providing protection from oxidative damage,[14, 15] thus expecting to obtain DCFs with superior properties compared to the ones previously described.

Compounds **S1-S4** and **P1** were formed starting from 1,3,5-benzenetrialdehyde (TA), pegylated squalene derivative[6] ($SQPEGNH_2$) and poly(ethylene glycol) bis(amine) of 1500 Da ($PEG(NH_2)_2$), reacted in the first step to form the intermediate units in various equivalent ratios. In the second step, to the mixture PEI 2000 or spermine was added and the completion of reaction was monitored by ^{1}HNMR (Fig. 1), as shown for example of **S2**, *i.e.* disappearance of signals from TA.

Our main interest, however, was to form **PS1-PS4** by combining **S1-S4** with **P1** in 1:1 ratio, thus **S1-S4** and **P1** were obtained as starting DCFs. As resulted **PS** series, each member contains the same amount of PEI (1.5 eq). It should be mentioned that **PS1** and **PS2** have PEG in their composition, while in **PS3** and **PS4** PEG is absent. Through slight variation of squalene moiety we intended to monitor self-assembly properties of obtained DCFs. Essentially, by designing **PS** series (Table 1) we highlighted self-adaptibility and reorganization capability of the vectors. In this case, our intention was to observe and detect differences in transmission electron microscopy (TEM), DNA binding, transfection and cytotoxicity for the **PS1-PS4** series.

Transmission Electron Microscopy investigations

Morphological and dimensional characteristics of **PS** series in water were investigated by TEM and are depicted in Fig. 2.

It was observed that every DCF from **PS** series has different behavior, thus, **PS1** forms "popcorn" like homogeneous particles with an average size of 20 nm, **PS2** – large spherical particles with a size of ~ 1μm, **PS3** forms spherical particles of ~ 500 nm, all together are uniformly distributed and do not have similar size as **P1**, indicating that the ratio of squalene moiety plays an important role. When the ratio of squalene moiety in composition of DCFs was higher, we also have observed a higher tendency for spherical assembly, as in example of **PS2**, **PS3**, **P1.** Moreover, we can conclude that a rearrangement takes place, although self-assembly process seems very complex.

Table 1

Composition of the synthetized library of DCFs

Compound	**$SQPEGNH_2$**	TA	**$PEG(NH_2)_2$**	Spermine	PEI 2000
S1	0.25	1	1.375	0.75	
S2	0.5	1	1.25	0.5	
S3	1	1	-	1	
S4	1	1	-	0.5	
P1	1	1	-	-	3
PS1 (P1+S1) (1:1)	0.6	1	0.7	0.375	1.5
PS2 (P1+S2) (1:1)	0.75	1	0.6	0.25	1.5
PS3 (P1+S3) (1:1)	1	1	-	0.5	1.5
PS4 (P1+S4) (1:1)	1	1	-	0.25	1.5

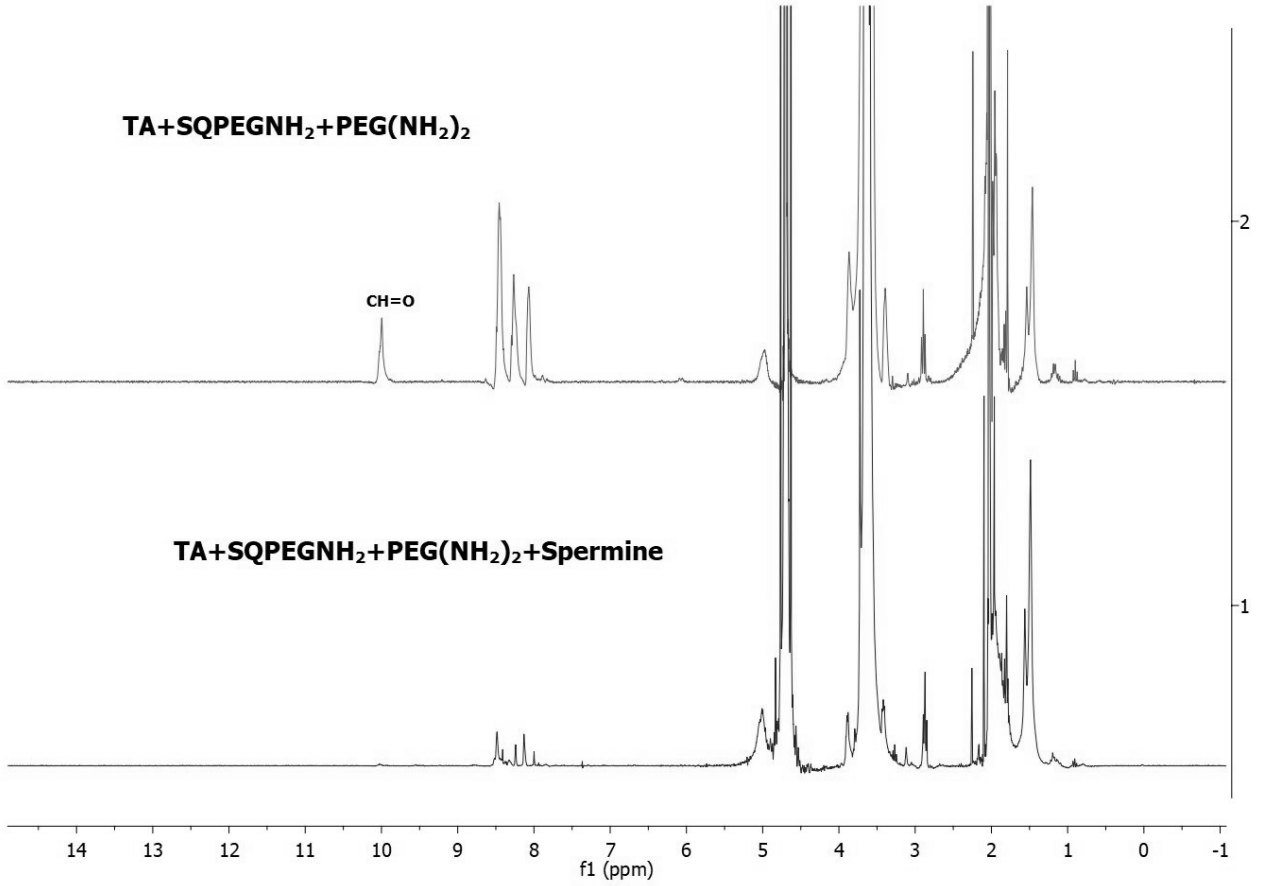

Fig. 1 – ^{1}H NMR monitoring of the formation of **S2.** Up: first step reaction of TA, $SQPEGNH_2$ and $(PEG(NH_2)_2$, bottom: second step after adding spermine.

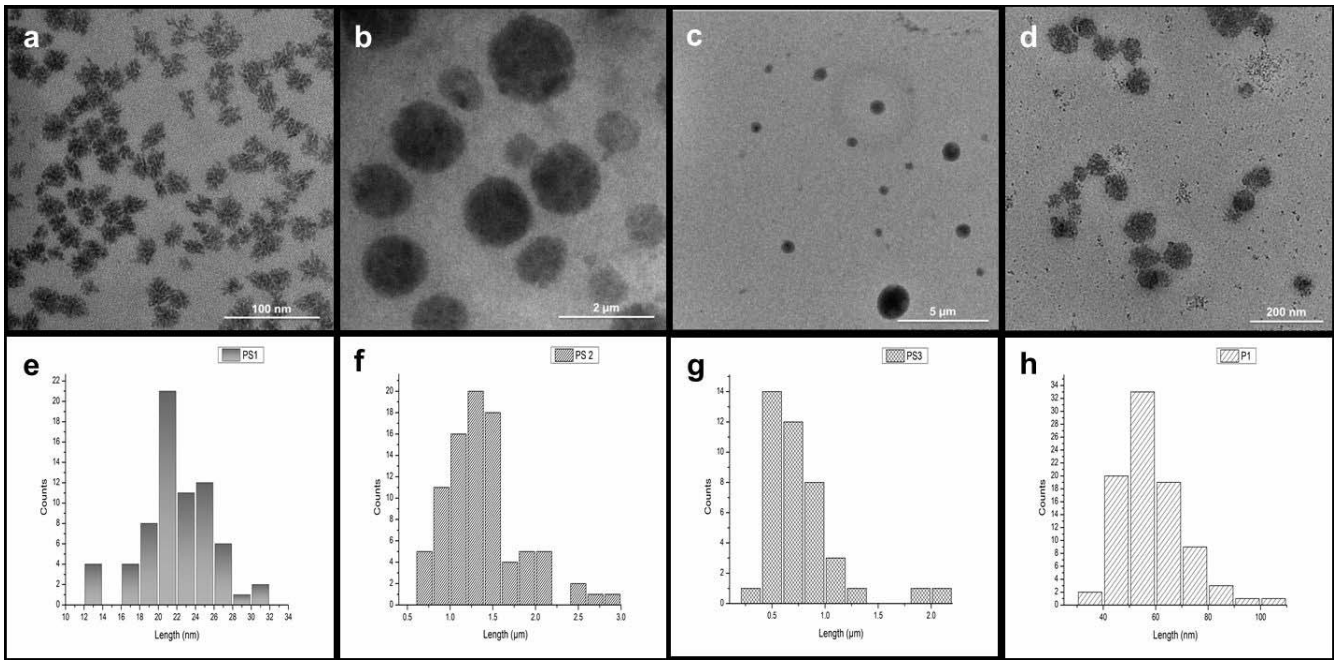

Fig. 2 – TEM images of: a) **PS1**; b) **PS2**; c) **PS3**; d) **P1** and the corresponding size distribution (e-h).

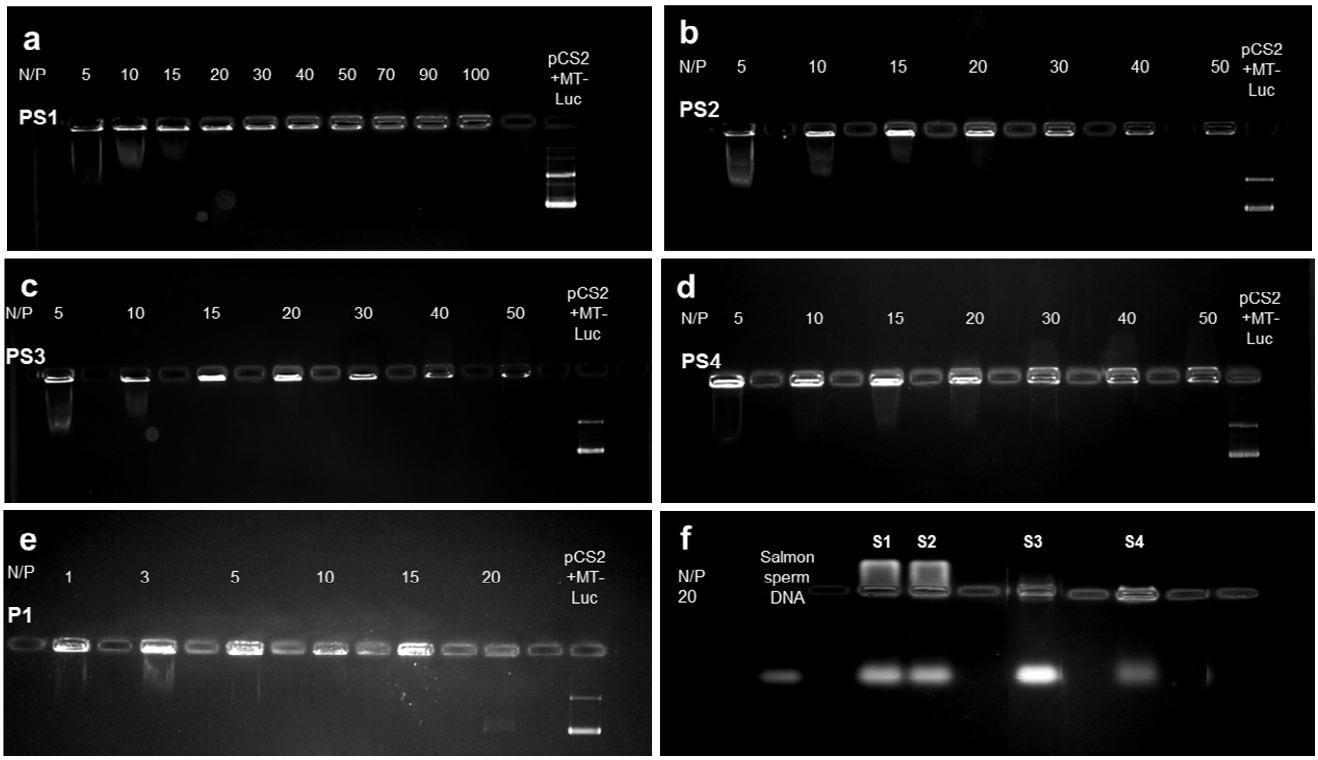

Fig. 3 – Agarose gel electrophoresis assays for compounds of **PS**, **S** series and **P1**. Agarose gel (1% w/v) in TAE (Tris-Acetate-EDTA) buffer at pH 7.4. The amount of plasmid DNA (pCS2-MT-Luc) was kept constant in all the experiments and used as reference in line pCS2-MT-Luc. For compounds of **S** series, salmon sperm dsDNA was used.

DNA binding properties

Obtained library of DCFs contains spermine and/or PEI in its composition and being polyamine or cationic polymer within interaction with DNA, it formis polyplexes. It is known that complexation and condensation behavior of polyplexes is dependent on several polymer characteristics such as molecular weight, number and the density of charges and the ratio of polymer to DNA (N/P). Therefore, low molecular weight cationic polymers require higher N/P ratios for a complete binding of DNA compared to highly-branched or higher weight derivatives. The binding ability of polyplexes formed from **S1-S4, P1, PS1-PS4** and DNA was investigated by agarose gel electrophoresis. Fig. 3 shows electrophoretic lanes of free DNA (pCS2-MT-Luc (Fig. 3 a-e) or salmon sperm dsDNA (Fig. 3 f)) and those of polyplexes formed from **S1-S4, P1, PS1-PS4** and pCS2-MT-Luc / salmon sperm ds DNA at different N/P ratios. The reduction of DNA electrophoretic mobility is the result of condensation between positive charges of the compounds and the negative charged phosphate groups of nucleic acid.

Unpredictably polyplexes formed from **S1-S4** (Fig. 3f) presented extremely weak interactions, while polyplexes formed from **P1, PS1-PS4** (Fig. 3a-e) showed a strong interaction of cationic moiety and fully bind the DNA starting with N/P ratio of 10 (Fig 3a-e), concluding that spermine moiety presents a very weak ability to bind DNA, compared to PEI 2000 moiety. However, the presence of spermine seems to not disturb the binding ability of hybrid **PS** series.

Transfection efficiency

Next, we investigated the transfection efficiency of DCFs by luciferase assay at two N/P ratios and by fluorescence imaging. From Fig. 4 we observed that transfection efficiency for **S1-S4** was poor at both N/P (50, 100) ratios and, on the other hand, the efficiency of **P1** was much higher. It reveals that in transfection, a crucial role is played by the cationic moiety,[16] along with the presence of PEG [6] thus, hybrid **PS** series present the best transfection for **PS2**, that contains only half of the amount of PEI in its composition compared to **P1** (Table 1) and PEG moiety. When **PS2** was compared to **PS4,** both containing the same ratio of PEI (1.5 eq) but **PS4** do not contain PEG moiety, showing a weaker transfection ability, thus proving the important role of PEG in composition of DCFs.

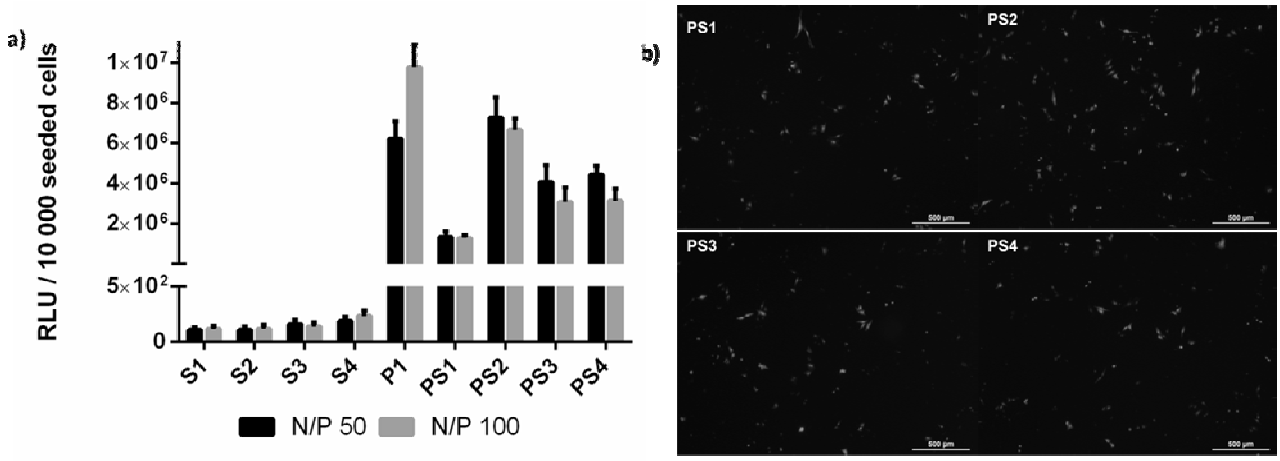

Fig. 4 – a) Transfection efficiency determined by luciferase assay at 50 and 100 N/P ratios; b) Fluorescence microscopy images showing the expression of GFP in HeLa cells transfected with PS/pCS2+NLS-eGFP polyplexes at 50 N/P ratio.

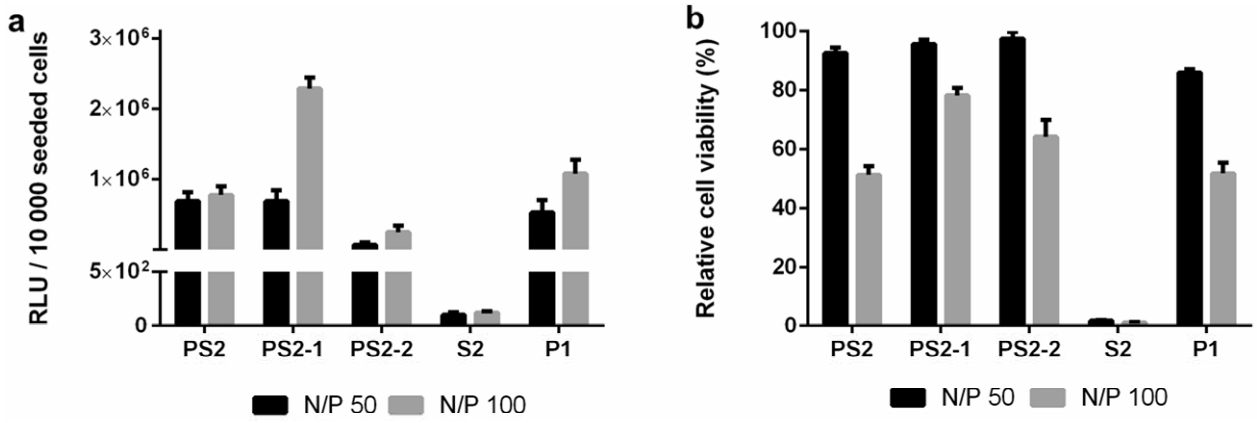

Fig. 5 – a) Transfection efficiency determined by luciferase assay; b) *In vitro* viability assay using MTS method.

From **PS** series we spotlighted **PS2** as being the optimal DNA transfector from the developed library due to its high transfection efficiency and lower ratio of PEI. In order to understand more deeply the rearrangement, self-adaptive, self-assembly processes we prepared **PS2** in two different ways, *i.e.* **PS2-1** was formed from TA, $SQPEHNH_2$, $PEG(NH_2)_2$), PEI 2000 and spermine by mixing all together and respecting the ratios of components from Table 1, and **PS2-2** formed from TA, $SQPEHNH_2$, $PEG(NH_2)_2$, PEI 2000, spermine mixed in the presence of DNA (pCS2-MT-Luc). Fig. 2 represents the transfection efficiency and cytotoxicity of **PS2**, **PS2-1** and **PS2-2** (Fig. 5).

The luciferase assay (Fig. 5a) revealed that *de novo* formed **PS2-1** had similar transfection efficiency at N/P 50, but it showed a significant improvement at N/P 100 when compared to **PS2**, **PS2-2** and **P1**. On the other hand, **PS2-2,** which is formed in the presence of DNA, showed weaker transfection efficiency at both tested N/P ratios (50 and 100). Thus, we can conclude that reorganization of components in DCFs occurs indeed, due to dynamicity of the obtained systems, even though *de novo* formation of **PS2-1** proved to give a better transfection at N/P 100.

The *in vitro* cell viability assay (Fig. 5b) revealed that all three compounds (**PS2**, **PS2-1**, **PS2-2**) show no significant toxicity at N/P 50, while at N/P 100 the vector becomes more cytotoxic. Remarkably, the presence of spermine does not influence the cytotoxicity of **PS2**, **PS2-1**, **PS2-2,** even though its own toxicity is very high (**S2**, Fig. 5b).

EXPERIMENTAL

Materials. Squalene (purchased from Sigma, ≥ 98%), 1,3,5-benzenetrialdehyde (purchased from Manchester Organics, 98%), poly-(ethyleneglycol)-bis(3-aminopropyl) terminated (Mn~1500 g/mol) (purchased from Aldrich), branched polyethylenimine (2kDa, 50 wt. % in H_2O) (purchased from Aldrich), Spermine (purchased from Aldrich), Salmon Sperm ds DNA low molecular weight (purchased from Fluka).

Synthesis of S2. A mixture of 1,3,5-benzenetrialdehyde (10 mg, 1 eq), SQ-PEG-NH_2 (57 mg, 0.5 eq) and PEG$(NH_2)_2$ (115 mg, 1.25 eq) were dissolved in acetonitrile and stirred overnight at room temperature. Solvent was evaporated and the residue redissolved in water and spermine (6.22 mg. 0.5 eq) was added and stirred for another 48 h at room temperature. Solution was kept as stock solution for further experiments. S1, S3, S4 were obtained in a similar manner, respecting ratios from Table 1.
Synthesis of P1. In the first step, 1,3,5-benzenetrialdehyde (5 mg)**,** SQ-PEG-NH2 (57 mg) were dissolved in 1500 µL acetonitrile. Reaction mixture was stirred for two days at room temperature. In the second step, the solvent was evaporated and the residue was redissolved in 100 µL water and PEI2000 (184 mg) in 200 µL water were added and stirred for another 48 h at room temperature. Solution was kept as stock and used for further experiments.
Synthesis of PS series. S series (S1,S2,S3,S4) and P1 compounds were simply combined in a 1:1 equivalent ratio of 1,3,5-benzenetrialdehyde to form a mixture of polycationic micelles that were stirred for one week in order to allow their components to reassemble and form new assemblies.

Synthesis of PS2 library. PS2 was synthetized by mixing P1 and S2 in 1:1 equivalent ratio of 1,3,5-benzenetrialdehyde.
PS2-1 In the first step, 1,3,5-benzenetrialdehyde (10 mg), SQ-PEG-NH2 (86.3 mg) and PEG(NH2)2 (57.8 mg) were dissolved in 2500 µL acetonitrile. Reaction mixture was stirred for two days at room temperature. In the second step, the solvent was evaporated and the residue was redissolved in 1000 µL water and spermine (3.11 mg) and PEI2000 (185 mg) in 398 and 925 µL water, respectively, were added. The mixture was stirred for 48 h at room temperature.
PS2-2 was synthesised in the same manner with the difference that plasmid DNA (pCS2+MT-Luc) was added in one step before adding the spermine and PEI2000, in order to obtain a structure like polyplex .

Cell cultures. HeLa cells (from CLS-Cell-Lines-Services-GmbH, Germany) were cultivated in tissue culture flasks with alpha-MEM medium (Lonza) supplemented with 10% fetal bovine serum (FBS, Biochrom GmbH, Germany) and 1% Penicillin-Streptomycin-Amphotericin B mixture (10K/10K/ 25 µg in 100 mL, Lonza). Medium was changed with fresh once every 3 or 4 days. Once confluency was reached, cells were detached with 1x Trypsin-Versene (EDTA) mixture (Lonza), washed with phosphate buffered saline (PBS, Invitrogen), centrifuged at 200 x g for 3 minutes and subcultured into new tissue culture flasks.

Preparation of polyplexes. The polyplexes with the plasmid DNA (pCS2+MT-Luc) were prepared at different molar ratios (N/P), considering the content of nitrogen from PEI in vectors, and the content of phosphate groups of plasmid DNA. Plasmid DNA (500 ng/µL) was mixed with the appropriate amounts of PEI and vector solution at N/P ratios of 50 and 100, and incubated at room temperature for 30-60 minutes to generate vector/DNA polyplexes.

***In vitro* gene transfection study.** Cells (HeLa) were seeded 24 hours prior to transfection into a 96-well culture plate at a density of $1x10^4$ cells per well in 100 µL culture medium (alpha-MEM medium (Lonza) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B mixture (10K/10K/25 µg in 100 mL, Lonza)). At the time of transfection the medium in each well was replaced with 100 µL of mixture containing fresh medium and polyplexes. After 48 hours in the humidified incubator at 37ºC and 5% CO_2, 100 µl Bright Glo Luciferase Assay Reagent (Promega) was added to each well, and the luminescence is measured within a 4 minutes interval. At least six biological replicates were performed for each compound.
For **fluorescence imaging studies**, the polyplexes were prepared with plasmid DNA (pCS2+NLS-eGFP) which encodes enhanced green fluorescent protein. The same protocol as the one described above is used for fluorescence imaging studies, specifying that the transfection period was 48 hours and the plates were inspected using an inverted microscope Leica DMI 3000 B and images were acquired with fluorescence GFP filter.
***In vitro* cytotoxicity study (MTS assay).** Cytotoxicity of transfected cells was performed using the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) at 48 hours post-transfection, following the manufacturer's protocol. Briefly, cells were transfected with pCS2+NLS-eGFP plasmid as described earlier.[17, 18] At 44 hours post-transfection, 20% MTS reagent (v/v) was added. Cells were incubated at 37 °C for 4 hours, and absorbance was measured at 490 nm, using a plate reader (EnSight, PerkinElmer). Cell viability was expressed as a relative percentage compared with untreated cells.[1]

CONCLUSIONS

Here we applied dynamic chemistry constitutional approach to conceive Dynamic Constitutional Frameworks (DCFs), as adaptive and reorganized vectors for DNA transfection, which are formed by reassociation and rearrangement of components when various DCFs are mixed between them. We showed that the amount of squalene moiety presented in DCFs affects its self-assembly structure, proved by TEM analysis. We identified that the presence of spermine moiety in DCF structure exhibits very weak ability in binding DNA, however, its presence in hybrid **PS** series seems not to disturb their binding property. We spotted **PS2** as being the optimal DNA transfector from the developed library, which gave us an insight into the importance of the PEG and PEI presence in the composition of DCF. Furthermore, we established that the reorganization of components in DCFs occurs indeed due to the dynamicity of the obtained systems.

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