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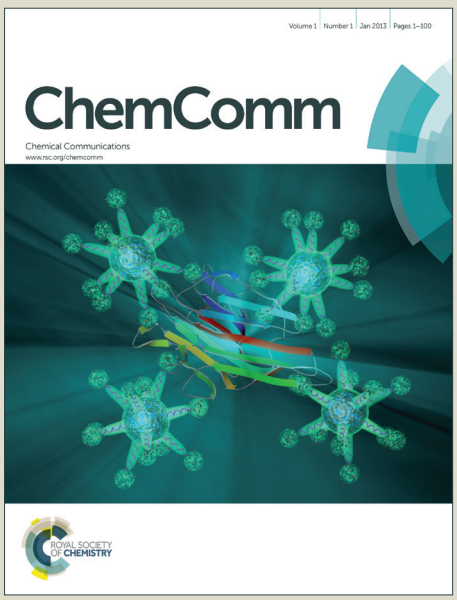

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Dynamic Constitutional Frameworks for DNA Biomimetic Recognition

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Linear and cross-linked Dynamic Constitutional Frameworks generated from reversibly interacting linear PEG/core constituents and cationic sites, shed light on the dominant coiling versus linear DNA binding behaviours, closer to the histone DNA binding wrapping mechanism.

Numerous artificial gene delivery systems utilizing designed molecular or nanocarrier systems have been developed in the last decades.[1-6] Non-exhaustive cell penetrating examples of cationic lipids,[1] peptides,[2] calixarenes,[3] polymeric structures[4] and fullerenes[5] have all been used in this context by using design approaches (Fig 1a). Concurrently, the design of multivalent systems containing DNA coordination, membrane penetration and anti-opsonisation functions has attracted a great deal of interest.[2] Convergent self-assembly strategies have been used for the synthesis of multivalent supramolecular nanodevices, designed to mimic natural delivery functions (Fig. 1b).[1,6] Despite such impressive progress, important application problems, deriving from the enormous variability of both DNA targets and nature of the transfected cells, the rational design became limited to the introduction of a reduced number of components and should be completed by combinatorial approaches. Within this context, the Dynamic Combinatorial Strategy,[7] appeared one of the most attractive screening method for the rapid access to the active systems from large and complex libraries (Fig. 1c, top).

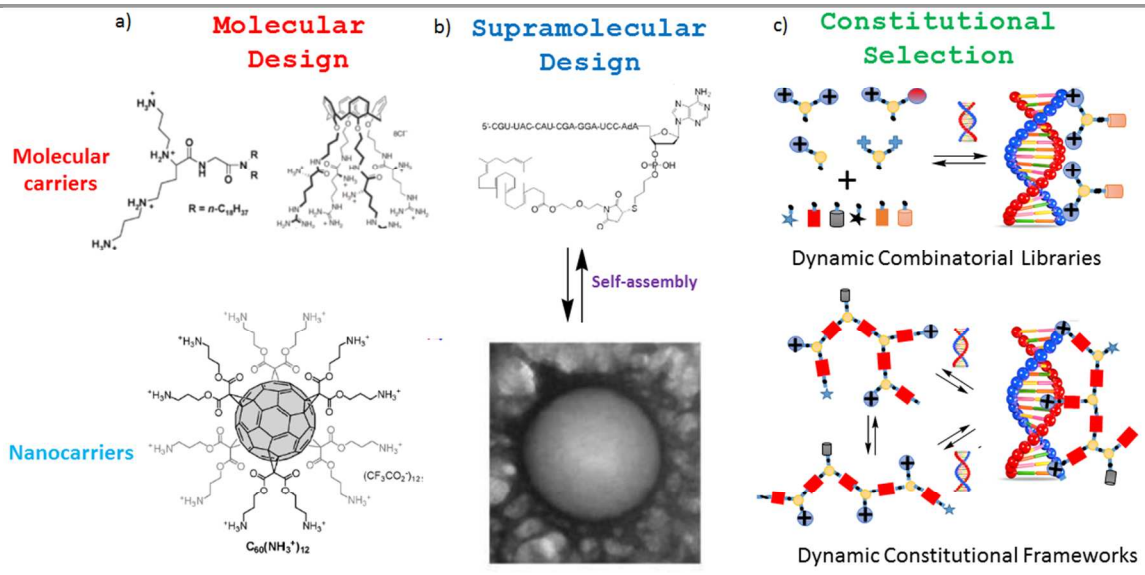

Fig. 1. From molecular DNA carriers to DNA nanocarriers a) Molecular[2,3] and b) Supramolecular Design of active molecules and self-assembled devices[6] and c) Constitutional selection by combinatorial screening of DCL and adaptive selection of nanoscaled Dynamic Constitutional Frameworks for DNA binding. The DCL and DCF contain active (positive charged) or neutral membrane interacting components connected via reversible covalent bonds (black points). Various functionalities can be reversibly added to network components (red) and the core connectors (orange circles) allowing not only the multicomponent variability needed in biological applications, but also a spatially adaptive distribution of active binding sites on target binding/transfection.

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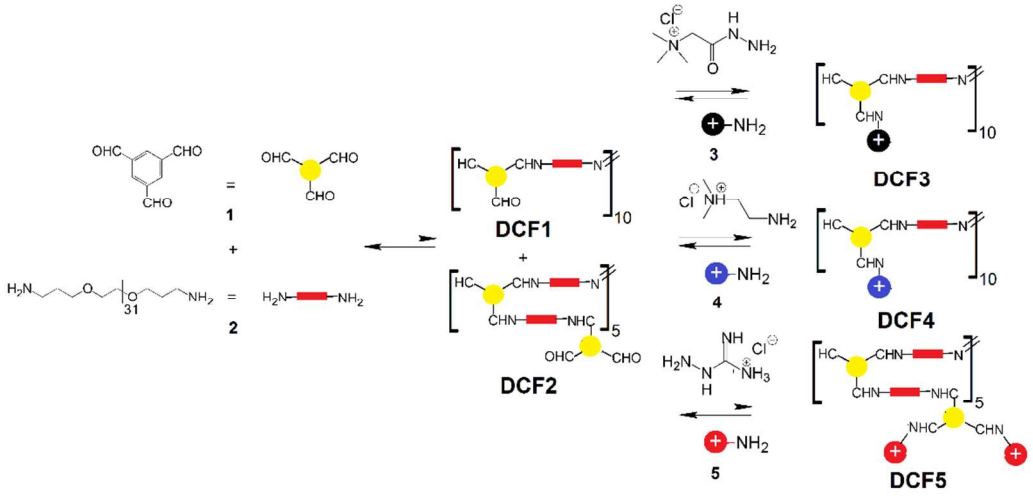

Fig. 2. Schematic representation of the synthesis of obtained **DCFs** combining PEG, **2** (red rectangle) connected via 1,3,5 benzene trialdehyde cores **1**, (yellow circle) resulting in the formation of **DCF1** : **DCF2**, 1:1, mol:mol. Further treatment of this mixture with positively charged heads, **3-5** generates the **DCF3-5** frameworks.

By virtue of the reversible interchanges between the hydrophilic heads and hydrophobic tails, the fittest *Dynamic transfector* can adapt simultaneously to the DNA biotarget and cell membrane barrier.[7] As for the Design approaches, a future alternative Constitutional Selection strategy which may embody the flow of structural information from molecular level to dynamic multivalent devices that bind DNA on their nanosurfaces. This concerns the use of Dynamic Constitutional Frameworks -DCF composed by combinations of linear and/or cross-linked arrays of components reversibly interconnected *via* core connectors and containing functional groups synergistically interacting with DNA and bilayer membrane components (Fig. 1c, bottom). As previously observed[8,9], the DCF may implement adaptive reversible rearrangements of the components toward a high level of correlativity of the its hypersurfaces in interaction with the DNA biotarget[10] and the cell membrane barrier. In this study, linear PEG macromonomers, trialdehyde core connectors and positively charged molecular heads have been used to conceive DCFs for DNA recognition (Fig. 2). 1,3,5-benzenetrialdehyde, **1**, Poly-(ethyleneglycol)-bis(3-amino-propyl)terminated (M_n~1500 g·mol^{-1}), **2** and Girard's reagent T, **3** monoprotonated *N*,*N*-Dimethylethylene amine, **4** or Aminoguanidine hydrochloride, **5** are the building blocks subjected to conceive the **DCFR 1·2·R** R=**3-5**, by using the amino-carbonyl/imine reversible chemistry. Treatment of **1** with 1eq. of **2** in acetonitrile (reflux, 48h) afforded a mixture of linear and cross-linked (Fig. 2) frameworks, whose ^{1}H-NMR spectral properties agree with the formation of 1:1 mixture of **DCF1**:**DCF2** (with M_n~15000-18000 g·mol^{-1}, (Fig.3a). Very interestingly the ^{1}H-NMR spectra of **DCF1**:**DCF2** mixture recorded in CD_3CN and D_2O 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, favoring the imine formation.[9]

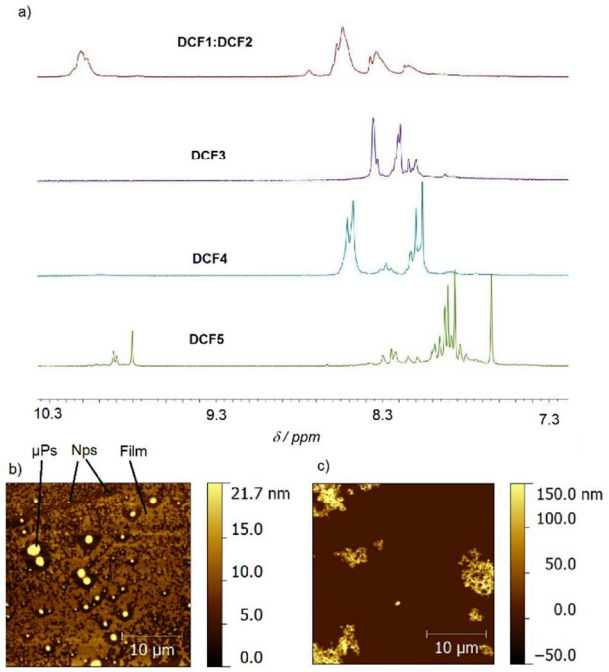

Fig. 3. Aromatic region of ^{1}H-NMR spectra (D_2O) of **DCF1:DCF2** mixture, **DCF3**, **DCF4**, **DCF5.** AFM images of b) **DCF5** and c) **DCF5** with DNA at NP=5 in air, on mica surface.

On progressive addition of cationic molecular heads **3-5** to **DCF1:DCF2** mixture, the ^{1}H-NMR spectra are reminiscent with the formation of linear frameworks **DCF3** and **DCF4** and a more complex cross-linked framework DCF5. The conversion of the aldehyde groups is almost total on the addition of 1-1.5 eq. of cationic head **3-5**. This is proven the analysis of chemical shifts of the imino bonds, showing a very simple pattern of signals for **DCF3** and **DCF4** reminiscent with the presence of the two linear forms presented in Fig. 3a, while the **DCF5** network present a complicated pattern of imino-proton signals, reminiscent to the formation of a complex, cross-linked network. The strong H-bonding between Guanidinium cationic heads and Cl^- anions generate compact and condensed cross-linked architectures, DCF5 while the hydrophobic nature of Ammonium head in weak interactions with Cl^- anions in DCF3 and DCF4 favor the hydrated linear ones.

These assumption have been confirmed by Atomic Force Microscopy (AFM) experiments.[†] The solutions of **DCF1-5** and a mixture of **DCF5** and salmon sperm double-stranded DNA (dsDNA) at an N/P ratio of 5 were imaged by AFM in air on mica surface. As anticipated, uniform film like depositions were found for **DCF1-5** while nano- or μparticles formation were found for **DCF5** (Fig. 3b). After binding with the dsDNA, the **DCF5** nanostructures aggregate all over the surface (Fig. 3c) indicating strong interactions between the **DCF5** and negative charged backbone of the dsDNA.

The ability of **DCF1-5** to bind negative dsDNA was evaluated using the agarose gel retardation assay (the reduction of DNA electrophoretic mobility as a consequence of condensation between positive charges of the compounds and the negative charged phosphate groups of nucleic acid).[‡] Aqueous samples with different N/P ratios (N/P 1, 3, 5, 10, 15, 20) were obtained by mixing dsDNA (200 base pairs, (bp)) with appropriate quantities of **DCF1:DCF2**, (negative control), **DCF3**, **DCF4**, **DCF5** in buffer solution and then loaded on the gel (Fig.4). As anticipated, the experiment with neutral **DCF1:DCF2** as negative control, resulted in no dsDNA retention caused by the frameworks interaction, the same amount of dsDNA (lanes 2-7) migrating similarly and having comparable intensity with the reference dsDNA (Fig.4a, lane 1). Similar results were obtained in case of linear **DCF3** and **DCF4** were at different values of N/P ratios, no dsDNA retention was observed (Fig. 4b,c) indicating no interactions between the charged **DCF3** and **DCF4** and dsDNA. The cross-linked **DCF5** has shown clear retention of dsDNA starting with N/P ratio of 3 (Fig. 4d, lane 3) demonstrating strong interaction between the Guanidinium moieties on the surface of aggregates with the negatively charged dsDNA backbone. The observed difference of DNA-binding affinity may also be due to the greater capacity of the guanidinium *vs*. ammonium group to interact with phosphodiesters as previously reported.[11] Increasing the N/P ratio led to the better DNA binding, reminiscent with the disappearance of the smear (partial interaction between DNA and **DCF5**) under the loading pocket (Fig.4d, lanes 4-7). Thanks to this positive result, the binding of a longer DNA plasmid (pEYFP,[†] 4500 bp) by **DCF5** has been tested. No significant difference can be observed between the **DCF5** ability to condense flexible, lower weight dsDNA (200 bp), and the stiffer, higher weight plasmid DNA (4500 bp),[†] for which starting N/P ratio of 1 are effective (Fig. 4e, lane 8).

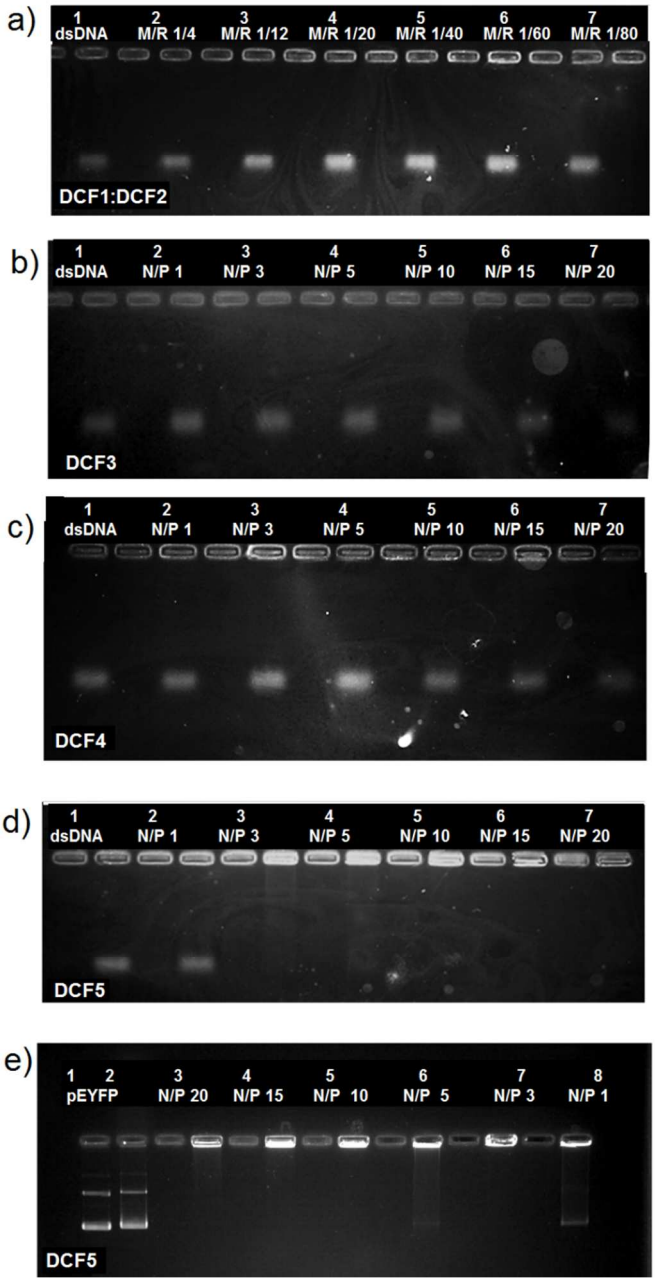

Fig. 4. Agarose gel electrophoresis assays for compounds a) **DCF1**:**DCF2** b) **DCF3**, c) **DCF4**, d) **DCF5/** dsDNA and e) **DCF5**/pEYFP. The amount of dsDNA and pEYFP was kept constant in all the experiments and used as reference in lane 1. For **DCF1**:**DCF2**/DNA : 1/4, 1/12, 1/20, 1/40, 1/60, 1/80 mass ratios were used (lanes 2-7). For **DCF3-5,** N/P ratios of 1, 3, 5, 10, 15, 20 (lanes 2-7) were used.

Conclusions

Our findings show that Dynamic Constitutional Frameworks-DCF (3D adaptive Dynamers)[8,9] story may reserve novel surprises, relevant to the biological and medicinal research, especially when the biotarget families like DNA have a multitude of members. The DCF stability in neutral water, as well as the adaptive spatial distribution of their multivalent biointeracting heads may lead to the discovery of active components self-adapting by itself to the DNA targets and the cellular barriers.

The present study revealed a new paradigm: the DNA binding is effected by cross-linked compact Guanidinium framework **DCF5** (at impressive lower N/P ratios of 1 even for longer DNA strands), while the linear Ammonium frameworks **DCF3, 4** showed no binding properties. This shed light on the dominant coiling versus linear DNA binding behaviours, closer to the histone wrapping DNA binding mechanism.[11] The simplicity of the synthetic strategy presented here can be easily used to self-generate Dynamic Constitutional Networks presenting relative DNA/cell membrane synergistic affinities, toward the systematic rationalization of active delivery systems. In other words, this strategy leaves the liberty to DNA systems to self-select and self-generate the most adapted carrier for its own active and optimal transfection. Work is in progress to pursue such studies.

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Notes and references

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† *Atomic Force Microscopy (AFM)*. The AFM images were obtained using an Ntegra Spectra instrument (NT-MDT, Russia) operated in tapping mode under ambient conditions. Silicon cantilever tips (NSG 10) with a resonance frequency of 140-390 kHz, a force constant of 5.5-22.5 Nm^{-1} and tip curvature radius of 10 nm were used. Typically, to prepare AFM samples, 10 µL aliquotes of sample solutions were deposited on freshly cleaved mica substrate and dried in air at room temperature prior the imaging.

‡*Agarose gel electrophoresis*. **DCL1-5** were mixed with the corresponding amount of dsDNA or pEYFP-plasmid and then incubated for 30 minutes at room temperature. The resulted solutions were loaded in a 1 % agarose gels, and electrophoresis experiments were carried out in TAE buffer solution (40 mM Tris–HCl, 1%, acetic acid, 1 mM EDTA, pH = 7.4) at 90 V for 120 minutes. The migration of dsDNA in free and complexed states was visualized under UV light, after gels staining with ethidium bromide. pEYFP, plasmid vector triggers the expression of the genetically engineered enhanced yellow-green *Aequorea victoria* fluorescent protein (EYFP). pEYFP exhibits a more complex behaviours, due to the plasmid self-assembly particularities, revealed by the migrating spot splitting of topologically-distinct forms (supercoiled form migrate faster comparing with nicked circle form) (Fig.4e lane 1).

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