DNA Recognition

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Dynamic Expression of DNA Complexation with Self-assembled Biomolecular Clusters**

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Abstract: *We report herein the implementation of a dynamic covalent chemistry approach to the generation of multivalent clusters for DNA recognition. We show that biomolecular clusters can be expressed in situ by a programmed self-assembly process using chemoselective ligations. The cationic clusters are shown, by fluorescence displacement assay, gel electrophoresis and isothermal titration calorimetry, to effectively complex DNA through multivalent interactions. The reversibility of the ligation was exploited to demonstrate that template effects occur, whereby DNA imposes component selection in order to favor the most active DNA-binding clusters. Furthermore, we show that a chemical effector can be used to trigger DNA release through component exchange reactions.*

Self-assembly processes are extensively used in biological systems for generating functional nanostructures that display emergent properties and functions.[1] A central goal of supramolecular chemistry has been to exploit self-assembly processes in order to generate synthetic bioactive systems.[2] Harnessing multivalency for achieving bioactivity is of great interest. Indeed, multivalency is often desired to compensate for the weakness of non-covalent interactions in aqueous media.[3] Thus, self-assembly approaches based on host–guest chemistry,[4] metal coordination,[5] supramolecular polymer formation,[6] peptide assembly,[7] and DNA/PNA hybridization[8] have been recently developed to achieve the multimerization of bioactive moieties.[9] Compared to covalent nanoconstructs, supramolecular self-assemblies are adaptive and feature constitutional dynamics.[10] These unique properties may be exploited for a) identifying, from dynamic combinatorial libraries, bioactive assemblies generated through target-induced component selection, or b) for functional control through component exchange.

The recent advent of dynamic covalent chemistry (DCC)[11] has opened new opportunities for the generation of bioconjugates by covalent self-assembly.[12] In particular, the (acyl)hydrazone ligation is particularly attractive since it is a chemoselective ligation that takes place in aqueous media under mild acidic conditions. It is compatible with the presence of many biological targets, which is of interest for in situ bioorthogonal chemistry.[13] Furthermore, the acylhydrazone ligation is a reversible reaction often used in DCC. It is therefore ideally suited for the generation of dynamic bioactive self-assemblies.

The multivalency approach has been implemented to the recognition of nucleic acids with low-molecular-weight cationic clusters. These systems are typically prepared by tethering multiple ligands onto a single scaffold through irreversible click-type reactions. Cationic clusters based on cyclodextrine,[14] dendrimer,[15] calixarene,[16] fullerene,[17] and pillar[5]arene[18] scaffolds decorated with ammonium or guanidinium ligands have thus been reported. However, few examples exploit a self-assembly approach based on DCC. Disulfide-bond formation has been used for generating multivalent DNA-binding peptides by oxidative cross-links.[19] More recently, imines were used for DNA recognition by dynamic constitutional frameworks[20] and functionalized nanoparticles.[21] We report herein the implementation of a self-assembly approach using DCC for the in situ generation of biomolecular clusters that effectively complex dsDNA through multivalent interactions. Such a dynamic self-assembly approach, using a reversible reaction compatible with the presence of the DNA target, should enable real-time monitoring of DNA complexation as the self-assembly proceeds and leads to an increase of valency.

Our biomolecular clusters are made of a cyclic peptide scaffold functionalized with modified amino acids through multiple acylhydrazone ligations. Thus, we prepared peptide **1** and used **ArgHyd**—a modified arginine bearing a hydrazide group at the C-terminus—and **AcHyd** for generating, respectively, cationic or neutral biomolecular clusters (Figure 1 and Supporting Information (SI), Sections 1–3). Unlike many organic scaffolds such as calixarenes and fullerenes, these peptides have the advantage of being water-soluble—which is an essential prerequisite for carrying out the self-assembly process in the presence of a biological target. This type of cyclic peptide scaffold has been previously shown to adopt a highly constrained conformation in which the four side chains point toward the same direction.[22] The glyoxylic aldehyde moiety of **1** can be engaged in oxime and

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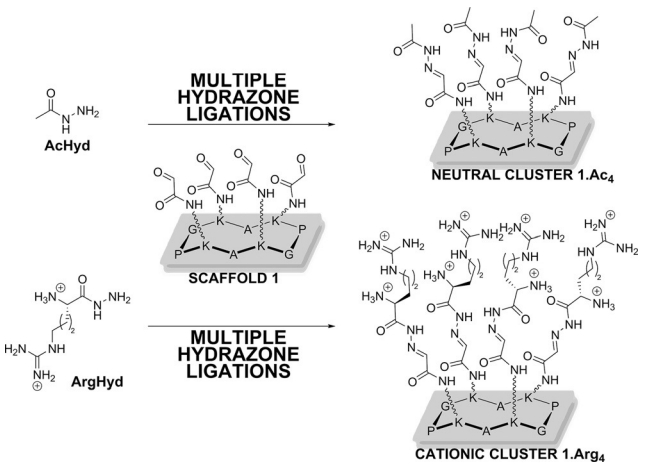


Figure 1. Multimerization of modified amino acids by acylhydrazone ligation. Structures of peptide scaffold **1**, hydrazide building blocks **AcHyd**, **ArgHyd**, and tetravalent clusters **1.Ac$_4$** and **1.Arg$_4$**.

(acyl)hydrazone ligations.[23] For instance, tetravalent nanoconstructs that show bioactivity in vitro and in vivo were previously prepared from scaffold **1** using oxime ligation.[24]

Upon addition of 8 equiv of **AcHyd** (2 equiv per aldehyde) onto an aqueous solution of **1** (1 mM)[25] in acetate buffer (pH 5.0), we observed, by HPLC, the complete conversion, within a few hours, into a single product identified by MALDI-ToF mass spectrometry as the tetravalent bioconjugate **1.Ac$_4$**. Likewise, the reaction between **1** and **ArgHyd** leads to the formation of **1.Arg$_4$** (SI, Section 4).

The ability of the tetravalent clusters to complex double-stranded DNA was assessed by a fluorescence displacement assay with Ethidium Bromide (EthBr) and calf thymus DNA (ctDNA). In this EthBr assay, a DNA complexation is detected by a fluorescence emission decrease. The results indicate that neither the peptide scaffold **1**, nor the hydrazide building blocks **AcHyd** and **ArgHyd** effectively complex ctDNA (Table 1, entries 1–3). While the neutral cluster **1.Ac$_4$** was also found unable to complex ctDNA, the cationic cluster **1.Arg$_4$** was pleasingly found to effectively complex ctDNA at N/P = 80 within 1–2 h (Table 1, entries 4 and 5). DNA complexation was also observed at N/P = 2 in low- and high-salt concentration (Table 1, entries 6 and 7), as well as at pH 7.2 (fluorescence emission = 2.6).

Table 1: DNA complexation assessed by fluorescence displacement assay.

Entry	Compound	N/P[a]	[NaCl] [mM]	Fluorescence emission [%][b]
1	**1**	n.a.[c]	9.4	98.0
2	**AcHyd**	n.a.[d]	9.4	96.8
3	**ArgHyd**	240[d]	9.4	87.9
4	**1.Ac$_4$**	n.a.[c]	9.4	94.2
5	**1.Arg$_4$**	80[c]	9.4	0.0
6	**1.Arg$_4$**	2[c]	9.4	3.9
7	**1.Arg$_4$**	2[c]	150	0.0

[a] N/P represents the ratio of positive charges (N) per phosphodiester (P); **ArgHyd** and **1.Arg$_4$** were considered to bear, respectively, 3 and 8 positive charges. [b] Measured after 2 h. [c] Experiments carried out with 1 mM of compounds. [d] Experiments carried out with 8 mM of compounds.

Gel retardation assays confirm that the cluster **1.Arg$_4$** effectively complexes plasmid DNA (pDNA) at N/P > 10—a threshold value comparable to other cationic clusters[15b,c,16,17]—and that hydrazide **ArgHyd** does not complex pDNA in the range of N/P = 20–500 (Figure 2 A,B). Isothermal titration calorimetry (ITC) confirms the binding of **1.Arg$_4$** to ctDNA at pH 7.2 and low salinity (9.4 mM NaCl), by showing a favorable exothermic binding ($\Delta H = -4.3 \pm 0.13$ kcal mol^{-1}) accompanied with an entropic gain ($T\Delta S = 3.4 \pm 0.06$ kcal mol^{-1}) that may be due to DNA desolvation (Figure 2 C). The integrated ITC data were fit to a one set of sites binding model, indicating a binding constant for the interaction of 4.4×10^5 M^{-1} and a *N*-value of 0.5. This would suggest that the binding involves one cluster **1.Arg$_4$** for two DNA base pairs. At 150 mM NaCl, ITC shows a much weaker interaction with a binding constant of 1.4×10^3 M^{-1} (SI, Section 5). Overall, these results reveal the importance of multivalency and electrostatic interactions in the complexation of DNA by cationic cluster **1.Arg$_4$**.

We then investigated, using the EthBr assay, whether the self-assembly of the clusters can be performed in the presence of ctDNA and lead to DNA complexation. We found that, when mixing **1** with **ArgHyd** (8 equiv) in low salinity acetate buffer (pH 5.0), a fluorescence emission decrease is observed, which indicates that DNA complexation takes place (Figure 3 A). Since both **1** and **ArgHyd** are ineffective to complex DNA under these conditions, this result demonstrates that in situ self-assembly occurs through acylhydrazone ligation and leads to the programmed expression of the bioactive cationic cluster **1.Arg$_4$**. In contrast, no fluorescence decrease was observed upon mixing **1** with **AcHyd** (Figure 3 A). Since it was previously demonstrated that the ligation reaction does proceed under these conditions, this result confirms that the expressed neutral cluster **1.Ac$_4$** does not complex DNA. When using sub-stoichiometric amounts of either **AcHyd** or **ArgHyd**, HPLC and MALDI-ToF mass spectrometry show the formation of clusters of lower valency ($n < 4$), respectively **1.Ac$_n$** and **1.Arg$_n$** (SI, Sections 6 and 7). In situ DNA complexation shows that at least 2 equiv of **ArgHyd** are necessary to achieve maximal DNA complexation in low-salt condition whereas more than 4 equiv of **ArgHyd** are required in high-salt condition (SI, Section 8). These results show that the DNA-complexing properties of the cationic clusters **1.Arg$_n$** increases with their valency and that the tetravalent cluster **1.Arg$_4$** is responsible for ctDNA complexation at high salinity.

The reversibility of the acylhydrazone enables exchange reactions to take place and may lead to interesting adaptive behaviors. HPLC analyses showed the formation of a library of several products upon mixing, at pH 5.0, the peptide scaffold **1** with **AcHyd** (8 equiv) and **ArgHyd** (8 equiv) (SI, Section 9). However, the constitution of this dynamic system was found to be strongly biased toward the formation of the neutral cluster **1.Ac$_4$**. Exchange reactions (i.e. addition of **ArgHyd** onto **1.Ac$_4$** or addition of **AcHyd** onto **1.Arg$_4$**) confirm that the library is under thermodynamic control, with equilibrium being reached after 6–12 days (SI, Section 9).

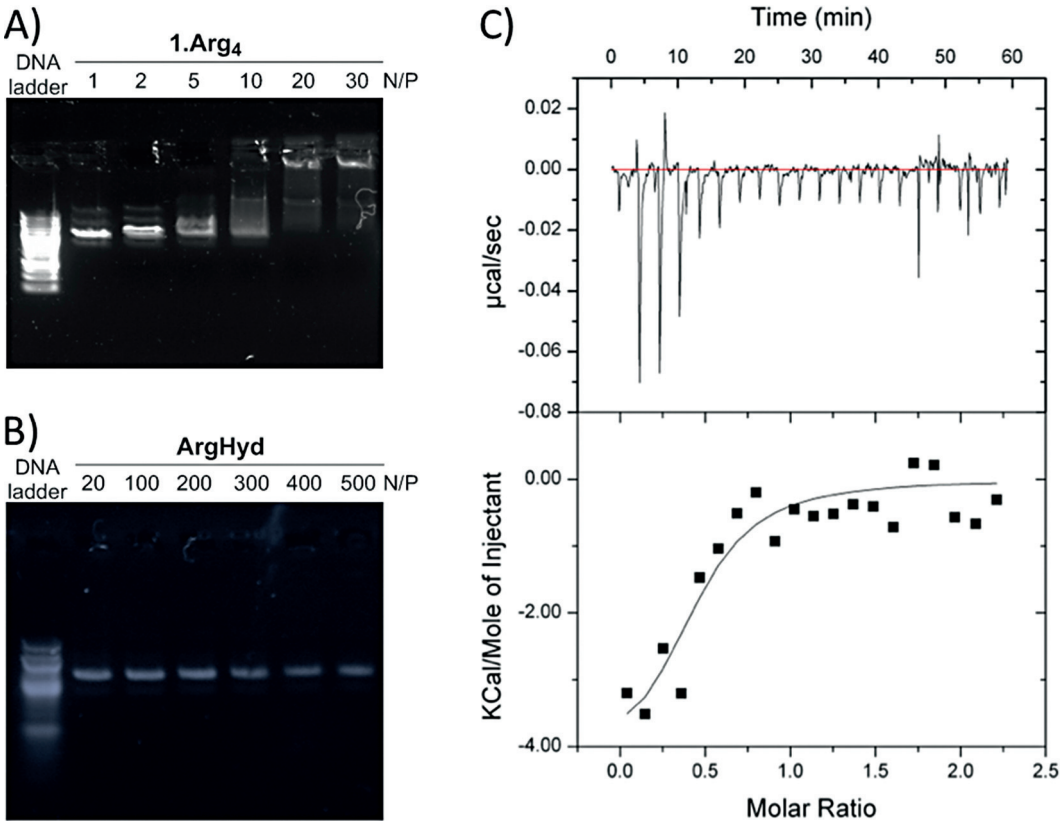


Figure 2. Complexation of DNA. Gel electrophoresis at different N/P ratio of: A) **1.Arg$_4$**, B) **ArgHyd**; C) ITC binding isotherms. Top: raw data; down: integrated heat flow signals as function of the ligand/DNA base pair molar ratio.

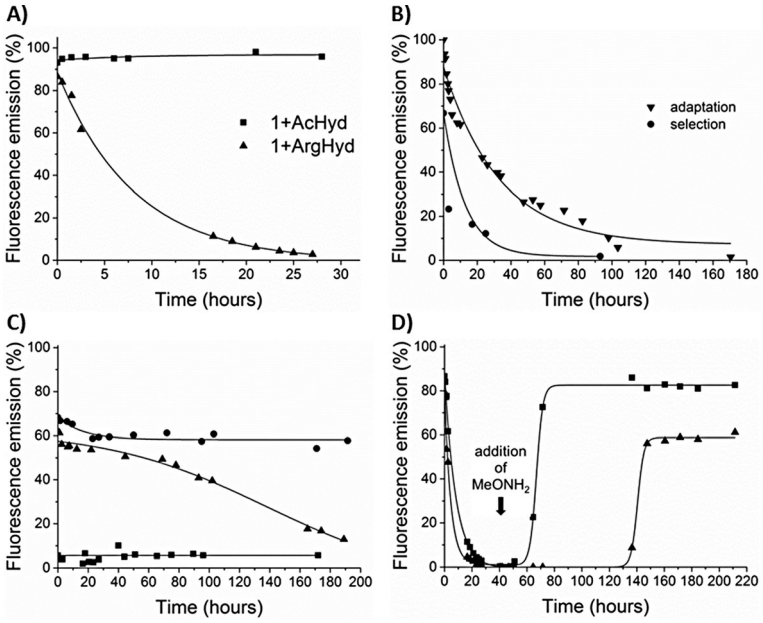
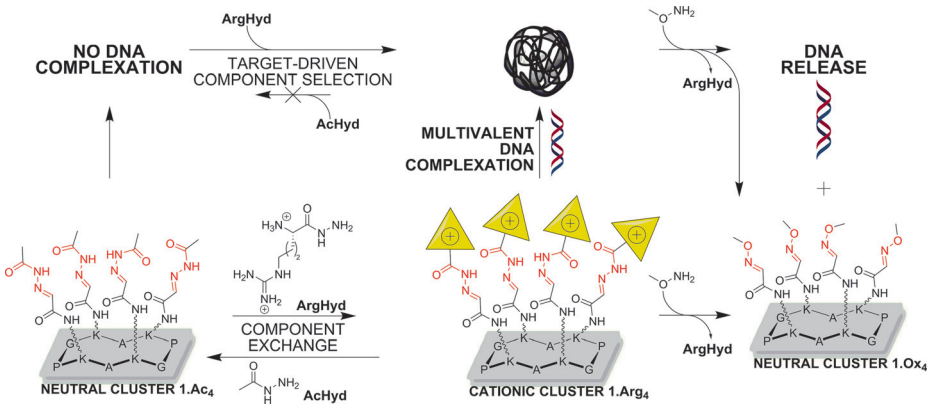


Figure 3. In situ monitoring of DNA complexation: A) self-assembly of peptide scaffold **1** with **AcHyd** (■) or **ArgHyd** (▲); B) libraries of **1**, **AcHyd** (8 equiv), **ArgHyd** (8 equiv), ctDNA (selection, ●), and **1.Ac$_4$**, **1.Arg$_4$**, ctDNA (adaptation, ▼); C) after addition of i) **AcHyd** onto **1.Arg$_4$** (9.4 mM NaCl, ■), ii) **ArgHyd** onto **1.Ac$_4$** (9.4 mM NaCl, ▲; 150 mM NaCl, ●). D) DNA release triggered by component exchange (addition of 8 equiv of methoxyamine 42 h after in situ DNA complexation by cluster **1.Arg$_4$** at pH 5.0 (■) and 7.2 (▲).

Using the EthBr assay, we found that this library displays a high fluorescence emission signal which indicates weak DNA complexation, most probably because it is mainly made of inactive cluster **1.Ac$_4$**. However, when carried out at N/P = 2, the fluorescence signal slowly decreases over a period of 100 h, thereby showing that an active cluster has been generated by the dynamic system (Figure 3B). A similar behavior was observed in competition experiments that consist in adding the peptide scaffold **1** to a mixture of **AcHyd** (8 equiv), **ArgHyd** (8 equiv) and ctDNA (Figure 3B). In contrast, no such fluorescence decrease was observed at N/P = 80 (SI, Section 10). We interpret these results by DNA acting as a template and shifting the composition of the dynamic system—through component selection or exchange reactions—in order to favor the formation of the best binder (**1.Arg$_4$**). Such template effects are indeed expected to occur when most components of the system may be involved in DNA complexation, that is, at low N/P. The UV/Vis monitoring of cluster formation from scaffold **1** shows that ctDNA does not affect the observed initial rate with **AcHyd** but induces a two-fold rate enhancement with **ArgHyd** (SI, Section 11). Further evidences of this template effect were obtained by exchange reactions: the

addition of **ArgHyd** onto a mixture of ctDNA and **1.Ac$_4$** at low salinity causes a slow decrease in the fluorescence emission signal (Figure 3C). In contrast, no changes were observed at high salinity where DNA complexation is two order of magnitude weaker, or following the addition of **AcHyd** onto a mixture of ctDNA and **1.Arg$_4$** (Figure 3C).



Scheme 1. Dynamic processes involving biomolecular cluster interconversion through component exchange, and DNA binding.

Taken together, these results indicate that DNA acts as a template and leads to the preferential incorporation of **ArgHyd** to produce the best DNA-binding clusters, while impeding the incorporation of **AcHyd** (Scheme 1).

Acylhydrazones may undergo exchange with oxyamines, leading to oximes.[24a] Indeed, HPLC analyses show that, upon addition of methoxyamine (8 equiv) into a solution of **1.Arg$_4$** at pH 5.0, the latter undergo a complete conversion into tetraoxime cluster **1.Ox$_4$**, identified by MALDI-ToF mass spectrometry (SI, Section 12). We then added methoxyamine into a solution of **1.Arg$_4$** complexed to ctDNA and observed a complete recovery of the fluorescence emission signal. This fluorescence recovery was found to be pH dependent—taking place faster at pH 5.0 than at pH 7.2—which is consistent with the observed pH-dependency of the hydrazone-to-oxime conversion (Figure 3D and SI, Section 12). Overall, the effect of methoxyamine may be explained by the production of neutral cluster **1.Ox$_4$** and monovalent **ArgHyd** which are both weaker DNA-binding agents than tetravalent cluster **1.Arg$_4$**. DNA release can thus be triggered by using a simple chemical effector that operates through component exchange (Scheme 1). Thus far, controlled DNA release has been reported using optical switches[26] or degradable vectors.[15b,27]

In summary, we reported herein a novel approach based on DCC for generating biomolecular clusters that effectively bind DNA through multivalent interactions. Template effects were evidenced, whereby DNA imposes component selection and adaptation of the dynamic system in order to favor the formation of its best binder. This target-assisted approach may be of interest for the development of specific nucleic acid binders. Component exchange reactions were exploited to trigger DNA release upon addition of a chemical effector, thereby opening new avenues for the design of chemically-responsive systems.

Keywords: bioconjugates · DNA recognition · dynamic covalent chemistry · multivalency · self-assembly

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