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Dynamic Polymers

Degradable Hybrid Materials Based on Cationic Acylhydrazone Dynamic Covalent Polymers Promote DNA Complexation through Multivalent Interactions

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Abstract: The design of smart nonviral vectors for gene delivery is of prime importance for the successful implementation of gene therapies. In particular, degradable analogues of macromolecules represent promising targets as they would combine the multivalent presentation of multiple binding units that is necessary for achieving effective complexation of therapeutic oligonucleotides with the controlled degradation of the vector that would in turn trigger drug release. Toward this end, we have designed and synthesized hybrid polyacylhydrazone-based dynamic materials that combine bis-functionalized cationic monomers with ethylene oxide containing monomers. Polymer formation was characterized by ^{1}H and DOSY NMR spectroscopy and was found to take place at high concentration, whereas macrocycles were predominantly formed at low concentration. HPLC monitoring of solutions of these materials in aqueous buffers at pH values ranging from 5.0 to 7.0 revealed their acid-catalyzed degradation. An ethidium bromide displacement assay and gel electrophoresis clearly demonstrated that, despite being dynamic, these materials are capable of effectively complexing dsDNA in aqueous buffer and biological serum at N/P ratios comparable to polyethyleneimine polymers. The self-assembly of dynamic covalent polymers through the incorporation of a reversible covalent bond within their main chain is therefore a promising strategy for generating degradable materials that are capable of establishing multivalent interactions and effectively complexing dsDNA in biological media.

Introduction

Gene therapies such as antisense[1] and silencing technologies[2] offer promising therapeutic perspectives for various diseases such as cancer. However, synthetic oligonucleotides cannot effectively cross biological membranes due to their negative charges and the resulting electrostatic repulsion with the constituents of cell membranes. Furthermore, they are rapidly degraded by endogenous nucleases, displaying half-lives of minutes in human serum.[3] Although viral vectors are highly effective, their potential inflammatory, immunogenic, and mutagenic effects drastically limit their use and call for the development of nonviral alternatives.[4] Therefore a key challenge in gene therapy is the development of a safe and efficient delivery system that complexes, transports, and releases therapeutic oligonucleotides in cells.[5]

Cationic polymers[6] and dendrimers[7] are well-studied candidates for nonviral vectors in gene delivery. Indeed, they are able to form stable complexes through multiple electrostatic interactions with oligonucleotides. Although artificial vectors may be cheaper to produce and easier to handle, most clinical studies still use viral vectors for transfection.[8] A reason for the weaker efficiency of nonviral vectors may be due to their permanent association with oligonucleotides, which hampers the intracellular delivery of therapeutic oligonucleotides. Indeed, the polyplexes are often trapped in endosomes, which constitutes one of the main obstacles for nonviral gene delivery systems.[9] Therefore smart materials that promote endosome escape through the "proton sponge" effect,[10] or through the chemical[11,12] or enzymatic[11a,13] degradation of the artificial vector represent attractive solutions of great interest.[14] Furthermore, the high molecular weight of polymers and dendrimers prevents their elimination from the body and may lead to toxic effects,[15] and their cationic nature and hydrophilicity lead to association with plasma proteins and results in a short circulation time in blood. Because pegylation is a well-developed strategy for increasing the stability, biocompatibility, and circulation time of biomolecules in vivo,[16] several hybrid polymers[17] combining cationic polyamines and ethylene glycol moieties have been recently prepared and tested as gene carri-

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ers.[18] For example, the pegylation of polyethyleneimine (PEI) leads to a reduction in the surface charge of the polymer as well as a decrease of the interaction with blood components, which therefore reduces cytotoxicity and extends blood circulation time.[19] Thus, degradable hybrid systems represent promising materials for the successful application of artificial gene delivery vectors in the clinic.[12a,g,20]

Dynamic covalent polymers, polymers that are self-assembled from their monomer(s) through multiple reversible covalent bonds,[21] have recently emerged as a class of dynamic materials capable of adaptation through constitutional reorganization.[22] Dynamic covalent polymers may also be considered as smart materials for the multivalent recognition of biomolecules.[23] Indeed, the repeated incorporation of multiple functional monomers into a single structure may lead to the multivalent presentation of several binding units in their polymer states, whereas monomers alone should display a weaker affinity for the target due to their reduced valency.[24] Dynamic covalent polymers may therefore serve as smart multivalent vectors, the degradation of which, upon a physicochemical trigger, leads to the release of the therapeutic agent and generates smaller components that may be more easily eliminated from the body.[12e] Polyacylhydrazone-based dynamic covalent polymers are particularly relevant for the present application as the hydrolysis of acylhydrazones occurs under slightly acidic conditions, at a pH comparable to the pH of late endosomes (5.0–5.5).[14] Furthermore, polyacylhydrazones formed through the condensation of complementary partners, bis-aldehydes and bis-hydrazides, enable the design of alternating hybrid co-polymers. In the context of gene transfection, cationic lipids[12h] and polymer conjugates that incorporate an acylhydrazone bond have recently been reported.[20,25] However, to date, the design of polyacylhydrazone-based dynamic covalent polymers for DNA complexation remains unexplored. It is worth mentioning that, very recently, supramolecular polymers, which are self-assembled and dynamic by nature, have been successfully implemented in transfection assays, thereby highlighting the potential for self-assembled multivalent systems in gene delivery.[26] Therefore there is strong interest in the development of pH-sensitive acylhydrazone-based dynamic covalent polymers for the design of smart gene delivery vectors.

We report herein the preparation of hybrid polyacylhydrazone polymers that alternatively combine within their main chain 1) cationic residues for oligonucleotide binding and 2) neutral polyethylene oxide moieties. We demonstrate that these materials are degradable at acidic pH and effectively complex calf thymus and plasmid DNA.

Results and Discussion

Design

The hybrid polyacylhydrazone-based materials were designed to combine cationic residues and neutral poly(ethylene oxide) moieties. As cationic residues we used diethylenetriamine and guanidinium moieties. Our choice was guided by the observation that monodisperse clusters[11d,12c,13a,27] and polydisperse systems[28a] featuring multiple motifs containing three secondary amines have been shown to effectively complex plasmid DNA. Furthermore, the presence of multiple diaminoethane moieties as side-chains on a polymer has been shown to induce endosomal membrane destabilization similarly to PEI.[28] Guanidinium compounds are well known to form salt bridges with oxyanions, and numerous guanidinium-containing systems, including oligomers, polymers, and dendrimers[11e,12h,29] as well as cationic lipids,[30] have been developed for drug and gene delivery applications. A triethylene glycol was chosen as the neutral moiety, having a similar size to the cationic residue. Both monomers were appended by aldehyde and hydrazide functions at the terminal positions so that self-assembly can occur through a polycondensation process and yield acylhydrazone-based dynamic hybrid polymers consisting of alternating cationic residues and neutral triethylene glycol moieties (Figure 1).

Synthesis of the bis-hydrazide monomers

4-Formylbenzoic acid was treated with thionyl chloride and *tert*-butyl carbazate to afford aldehyde **1** in a yield of 55% (Scheme 1). Compound **1** was then engaged in a reductive amination reaction with diethylenetriamine[31] or 4,7,10-trioxa-1,13-tridecanediamine to provide *N*-Boc-bishydrazide com-

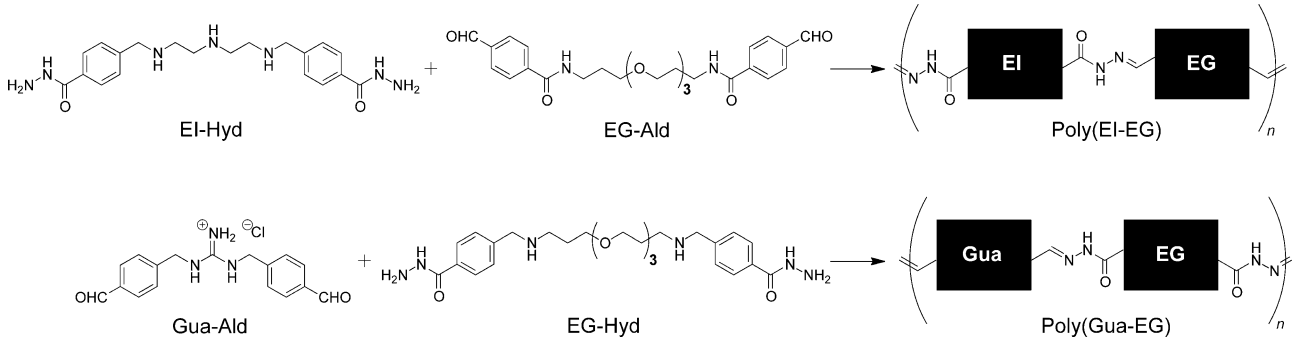
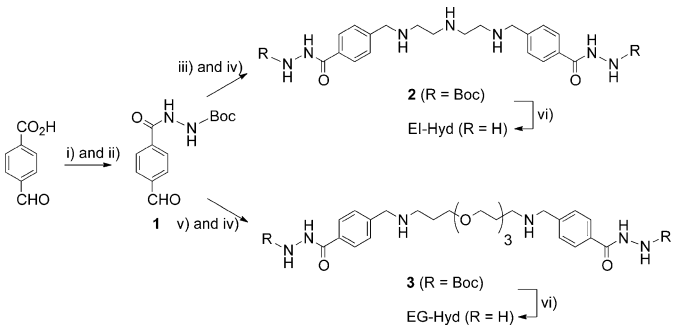


Figure 1. Formation of dynamic hybrid polymers Poly(EI-EG) and Poly(Gua-EG) by the acylhydrazone-based self-assembly of cationic monomers EI-Hyd and Gua-Ald with ethylene glycol containing monomers EG-Ald and EG-Hyd, respectively.

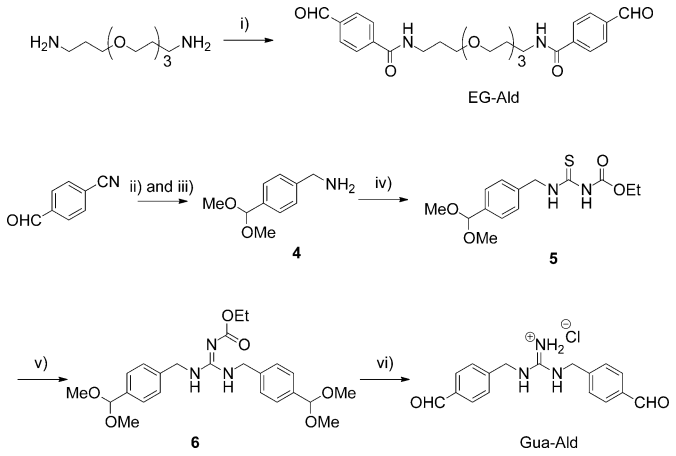


Scheme 1. Synthesis of bis-hydrazide monomers El-Hyd and EG-Hyd. Reagents and conditions: i) $SOCl_2$, toluene, reflux; ii) *tert*-butyl carbazate, Et_3N, CH_2Cl_2, 0 °C to RT; iii) diethylenetriamine, MeOH, 0 °C to RT; iv) $NaBH_4$, MeOH, 0 °C to RT; v) 4,7,10-trioxa-1,13-tridecanediamine, MeOH, 0 °C to RT; vi) TFA/TIS/H_2O (95:2.5:2.5), RT.

pounds **2** and **3**, respectively. Boc deprotection in TFA/TIS/H_2O (95:2.5:2.5) yielded the desired ethyleneimine bis-hydrazide (El-Hyd) and ethylene glycol bis-hydrazide (EG-Hyd) monomers.

Synthesis of the bis-aldehyde monomers

The ethylene glycol bis-aldehyde monomer (EG-Ald) was prepared by the coupling of 4,7,10-trioxa-1,13-tridecanediamine and 4-formylbenzoic acid, mediated by *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC)/1-hydroxybenzotriazole (HOBt), to afford the desired compound as a white solid in a yield of 81% (Scheme 2). The guanidinium bis-aldehyde monomer (Gua-Ald) was prepared in a five-step synthesis starting from commercially available 4-cyanobenzaldehyde: 1) Protection of the aldehyde with trimethyl orthoformate and 2) reduction of the nitrile with $LiAlH_4$ to yield **4** in a yield of 91%, 3) reaction with ethoxycarbonyl isothiocyanate to give protected thiourea **5**, 4) coupling with amine **4** in the presence of EDC to afford the protected guanidine **6**, and 5) acetal and carbamate deprotection with aqueous HCl to afford monomer Gua-Ald as a white solid in an overall yield of 59%.



Scheme 2. Synthesis of bis-aldehyde monomers EG-Ald and Gua-Ald. Reagents and conditions: i) 4-Formylbenzoic acid, EDC, HOBt, Et_3N, CH_2Cl_2, 0 °C to RT; ii) trimethyl orthoformate, HCl_{conc}, MeOH, 45 °C; iii) $LiAlH_4$, THF, 0 °C to RT; iv) ethoxycarbonyl isothiocyanate, Et_3N, CH_2Cl_2, RT; v) **4**, EDC, Et_3N, DMF, 60 °C; vi) 1 M HCl, reflux.

Polymer formation and characterization

A model reaction was carried out by mixing, in methanol and at room temperature, an ethyleneimine monohydrazide compound with 1.0 equivalent of benzaldehyde to assess the chemoselectivity of the condensation reaction. HPLC analysis after 1 hour showed complete conversion and the formation of a single product, and mass spectrometry revealed an *m*/*z* value corresponding to a condensation product, most likely the acylhydrazone given the known selectivity for hydrazones over imines (see S25 and S26 in the Supporting Information). The polycondensation reactions were then carried out by mixing stoichiometric amounts of bis-aldehyde and bis-hydrazide monomers at concentrations of 200 mM in methanol. Analyses were performed, after evaporation and dilution in [D_6]DMSO, by ^{1}H and DOSY NMR spectroscopy.[32] For both systems, the ^{1}H NMR analysis showed the complete disappearance of the starting aldehyde ($\delta = 10$ ppm, C*H*O) and the appearance of peaks that are characteristic of acylhydrazones ($\delta = 12$ ppm, C(O)N*H*N and $\delta = 8.5$ ppm C(O)NHNC*H*; Figure 2 and S39 in the Supporting Information). At low concentration (10 mM), the ^{1}H NMR spectrum of Poly(EI-EG) shows multiple acylhydrazone signals, which may indicate the formation of different products at intermediate concentrations (see S39 in the Supporting Information).

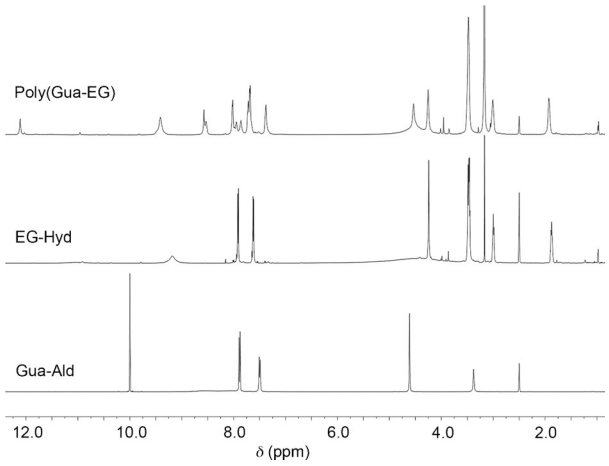


Figure 2.
^{1}H NMR spectra recorded in [D_6]DMSO of EG-Hyd and Gua-Ald and of the condensation reaction between the two reagents at 200 mM after 1 h.

Having demonstrated the formation of acylhydrazones under these conditions, we tested the effect of concentration on the outcome of the polycondensation reactions. Indeed, concentration is known to affect macrocycle/polymer balance in thermodynamically controlled polymerization reactions, polymers being favored at concentrations exceeding the effective molarity of the monomers, with macrocycles prevailing at lower concentrations.[33] The flexible nature of our monomers

should contribute to lowering their effective molarity. The ^{1}H NMR data show a broadening of the peaks as the concentration is raised (Figure 2 and S39 in the Supporting Information). Interestingly, we also noticed a significant increase in the viscosity of the reaction mixtures of both systems, an observation that is in line with polymer formation. DOSY NMR analysis showed the formation, at low concentration, of relatively small species (R_h = 6–17 Å, Table 1, entries 6 and 9) with respect to the monomers (R_h = 5–10 Å, Table 1, entries 1–4), whereas much larger objects (R_h = 134–157 Å, Table 1, entries 5 and 7) are formed at higher concentrations for both systems. Interestingly, two products of different size were observed by DOSY NMR analysis when the polycondensation reaction between EI-Hyd and EG-Ald was carried out at low concentration (10 mM), which confirms the previous observation of two sets of acylhydrazones by ^{1}H NMR spectroscopy. The concentration dependency observed by DOSY NMR analysis is compatible with the formation of macrocycles at low concentration and polymers at higher concentrations.

Table 1. Characterization of the polycondensation reactions by DOSY NMR spectroscopy.

Entry	Compounds	Conc. [mM]	Diffusion coefficient [$m^2 s^{-1}$]	Hydrodynamic radius[a] [Å]
1	Gua-Ald	10	2.32×10^{-10}	5
2	EG-Hyd	10	1.12×10^{-10}	10
3	EG-Ald	10	1.73×10^{-10}	6
4	EI-Hyd	10	1.19×10^{-10}	9
5	Poly(Gua-EG)	200	7×10^{-12}	157
6	Poly(Gua-EG)	10	$(6.4–18.6)\times10^{-11}$	6–17
7	Poly(EI-EG)	200	8.2×10^{-12}	134
8	Poly(EI-EG)	100	$(1.4–3.27)\times10^{-11}$	34–78
9	Poly(EI-EG)	10	$(1.03–1.87)\times10^{-10}$	6–11

[a] The hydrodynamic radii were determined by using the Stokes–Einstein equation.

The LCMS chromatogram of Poly(Gua-EG) shows two populations of products: A sharp peak corresponding to [1 + 1] and/or [2 + 2] macrocycles, and a broad signal that may correspond to oligomers (see S51 and S52 in the Supporting Information). This interpretation is further strengthened by the fact that dilution from 200 to 1 mM leads to the disappearance of the broad signal and an increase in the sharp peak, which is compatible with a chain-to-cycle conversion upon dilution (see S53 and S54 in the Supporting Information). Furthermore, MALDI-TOF mass spectrometric analysis of Poly(EI-EG) and Poly(Gua-EG) confirmed the formation of both open-chain oligomers and macrocycles (see S49 and S50 in the Supporting Information). Taken together, these results indicate that the monomers EI-Hyd and Gua-Ald react with complementary partners EG-Ald and EG-Hyd, respectively, to generate acylhydrazone-based dynamic covalent polymers at high concentrations (typically > 100 mM) whereas macrocycles are the dominant species formed at low concentration. The rearrangement of the polymer chains into smaller cyclic species upon dilution takes place over several hours ($t_{1/2}$ > 50 h, see S53 and S54 in the Supporting Information), which leaves sufficient time for the polymer to associate with its biomolecular target.

The degree of polymerization (DP) of a dynamic covalent polymer is determined by the equilibrium constant of the reversible reaction used for the assembly of the main chain, in this case the formation of acylhydrazone. Although the results described above clearly indicate the formation of open chains at high concentrations, the DOSY NMR and MALDI-TOF MS data tend to indicate the formation of oligomers or short polymers (DP < 100) rather than very long polymer chains. Nevertheless, it is worth mentioning that cationic clusters containing a limited and rather small number of binding units have recently been shown to effectively complex DNA, thereby highlighting the potential of dynamic oligomers for transfection applications.[27,34]

pH-sensitive polymer degradation

Acylhydrazones are known to undergo exchange with oxyamines to form oximes.[35] Indeed, we verified with a model compound containing a single acylhydrazone that the reaction with an excess of methoxyamine in aqueous solution leads to the formation of the oxime compound at the expense of the initial acylhydrazone with a pH-dependent rate (see S27–S31 in the Supporting Information). Thus, to assess the dynamics of the cationic dynamic covalent polymers, the stability of Poly(Gua-EG) was monitored by reversed-phase HPLC at different pH in the presence of 5 equivalents of methoxyamine per acylhydrazone, which may act as a chain terminator. The results showed the formation of the bis-oxime GuaOx and the bis-hydrazide EG-Hyd with pseudo-first-order kinetics, which confirms that Poly(Gua-EG) is able to undergo monomer exchange in the presence of a competing nucleophile. Poly(Gua-EG) was found to be relatively stable at pH 7.0, whereas it readily undergoes exchange at acidic pH, the half-lives ($t_{1/2}$) of formation of GuaOx showing a strong pH dependency ($t_{1/2}$ > 200 h at pH 7.0, $t_{1/2}$ = 54 h at pH 5.0; Figure 3 and S55–S59 in the Supporting Information).

The direct hydrolysis of a model compound containing a single acylhydrazone was also tested at different pH and monitored by reversed-phase HPLC (see S32–S38 in the Supporting Information). The hydrolysis took place faster at pH 5.0 ($t_{1/2}$ = 50 h) than at pH 7.0 ($t_{1/2}$ = 200 h), which is of strong interest for the design of smart delivery systems that may promote endosome escape through a degradation of the delivery vehicle that is triggered by the acidic pH of late endosome compartments. The hydrolysis of Poly(Gua-EG) in aqueous buffers at different pH was also monitored by HPLC but only the polymer-to-macrocycle conversion was observed (see S60–S63 in the Supporting Information). Overall, the results indicate that these dynamic polymers rearrange into smaller species at acidic pH by component exchange and hydrolysis.

Ethidium bromide displacement assay

The ability of the dynamic materials to bind dsDNA was assessed by a fluorescence displacement assay with calf thymus

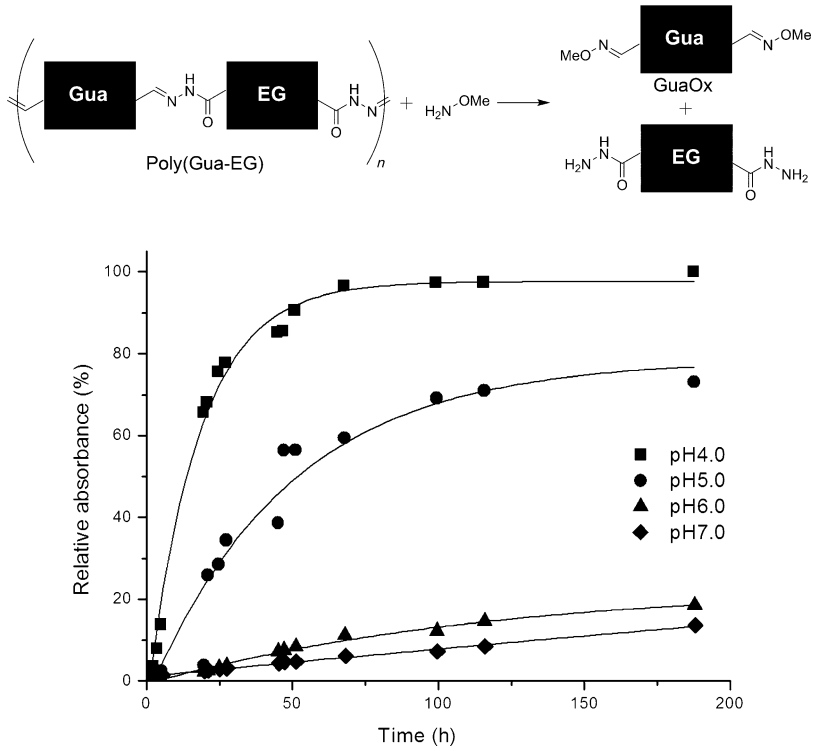


Figure 3. Kinetics for the formation of GuaOx from Poly(Gua-EG) in the presence of 5.0 equivalents of methoxyamine at different pH.

DNA (ctDNA) and ethidium bromide (EthBr) in aqueous buffer at pH 7.2. The fluorescence of EthBr increases upon intercalation with dsDNA and the competitive complexation of a cationic polymer may result in structural changes of the dsDNA that decrease its binding affinity for EthBr. As a consequence, the fluorescence signal decreases upon polyplex formation. We therefore titrated a solution of ctDNA and EthBr against Poly(EI-EG) and Poly(Gua-EG) and monitored the fluorescence emission at 590 nm (Figure 4). The results were quantified by determining the charge excess (CE) of the cationic polymers relative to ctDNA. The charge excess is defined as the nominal number of positive charges of the polymers divided by the number of negative charges present on the dsDNA. We assumed all the amines to be protonated and all the phosphodiesters to carry one negative charge. As such, the CE is equivalent to the N/P ratio. CE_{50} values correspond to the charge excess required for a 50% decrease in the fluorescence (Table 2).

Spermine was used as a control as it is a well-known biogenic ligand of dsDNA involved in cell growth.[36] Spermine displays no significant binding under high-salt conditions and moderate binding under low-salt conditions ($CE_{50}=14.4$). This result is in line with literature data.[27c] Similarly, Poly(EI-EG) also displayed a salt-dependency that is typical of polyamines. However, the magnitude of the changes in fluorescence is much greater than for spermine, reaching $CE_{50}=6$ under low-salt conditions, thereby confirming the polymeric nature of Poly(EI-EG) and the advantage provided by the presence of multiple diethylenetriamines within the main chain. It is worth mentioning that PEIs generally achieve complete complexation at $N/P>4$.[37] Monomer Gua-Ald showed almost no fluorescence change under low- and high-salt conditions. However, we observed a sharp decrease in fluorescence upon addition of Poly(Gua-EG) ($CE_{50}=1.2$). Interestingly, no salt-dependency was observed in this case. Again, this result confirms that the presence of multiple repetitions of a guanidinium group within the main chain of a dynamic covalent polymer is sufficient to achieve effective ctDNA complexation.[38] Compared with Poly(EI-EG), Poly(Gua-EG) shows a lower CE_{50}, thereby indicating that guanidiniums are more effective than amines at promoting interactions with dsDNA in these hybrid materials.

We also tested ctDNA complexation in fetal calf serum as it is well known that plasma proteins may interact with cationic polymers and destabilize the DNA–polymer complex. In comparison with the experiments carried out in aqueous buffer, we observed a significant decrease in the binding efficiency of Poly(Gua-EG) ($CE_{50}=5.8$) whereas Poly(EI-EG) ($CE_{50}=17.1$) essentially retained a comparable activity (Figure 4B). This observation may be accounted for by the fact that, unlike Poly(EI-EG), which is made of ethyleneimines of low basicity, Poly(Gua-EG) is probably more protonated at physiological pH (pK_a guanidinium ≈ 12.5) and therefore more susceptible to the presence of anionic proteins.

Table 2. ctDNA binding properties for spermine, Poly(EI-EG), Gua-Ald, and Poly(Gua-EG) as measured by the ethidium bromide displacement assay.[a]

Compound	[NaCl] [mM]	Nominal charge	CE_{50}
spermine	9.4	4+	14.4
Poly(Gua-EG)	9.4	3+	1.2
Poly(EI-EG)	9.4	3+	6
Gua-Ald	9.4	1+	>30[b]
spermine	150	4+	>30[b]
Poly(Gua-EG)	150	3+	1.2
Poly(EI-EG)	150	3+	>20[b]
Gua-Ald	150	1+	>30[b]

[a] The experiments were carried out in HEPES buffer (100 mM HEPES, 10 μM EDTA, pH 7.2) with 9.4 or 150 mM NaCl. The amount of calf thymus DNA (0.1 mg) and the final concentration of ethidium bromide (42 μM) were kept constant. [b] Estimated value.

Gel retardation assay

Gel electrophoresis was used to confirm the ability of these materials to complex dsDNA. In this case, we used a pET-15b

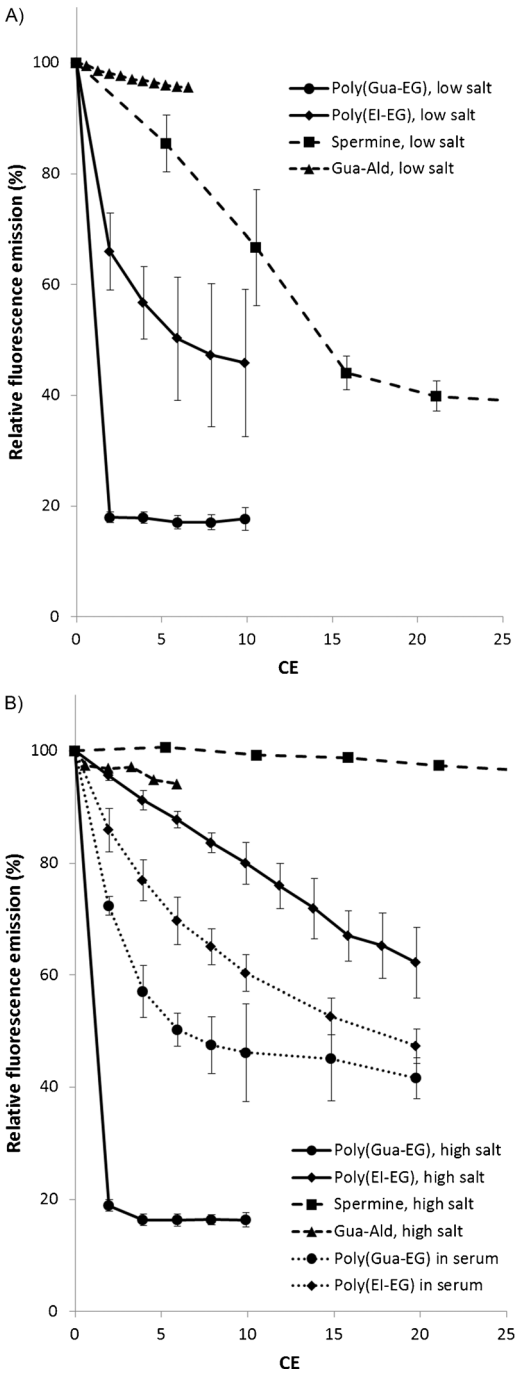


Figure 4. Fluorescence titration of a solution of calf thymus DNA and ethidium bromide by spermine, Poly(EI-EG), Gua-Ald, and Poly(Gua-EG) in the presence of: A) 9.4 mM NaCl (low-salt concentration) in buffered water (pH 7.2) and B) 150 mM NaCl (high-salt concentration) in buffered water (pH 7.2) or fetal calf serum (serum). The experiments were conducted in duplicate or triplicate. Error bars represent the standard deviation.

plasmid DNA and tested different N/P ratios (0.1–10). The results showed no shift of the plasmid band with either spermine, Gua-Ald, or, surprisingly, with Poly(EI-EG) (see S64 in the Supporting Information). However, complexation was detected by the disappearance of the native plasmid band with Poly(Gua-EG) at $N/P > 2$ (Figure 5). Thus, this gel retardation assay confirmed that the guanidinium-based dynamic covalent polymer is more effective at complexing dsDNA than the polyamine-based polymer. The fact that Poly(EI-EG) does not seem to complex the plasmid in this experiment may be due to the higher pH of the Tris-acetate-EDTA (TAE) buffer (pH 8.2), which reduces the degree of protonation of Poly(EI-EG) compared with in the fluorescence displacement experiment, which was carried out at pH 7.2.

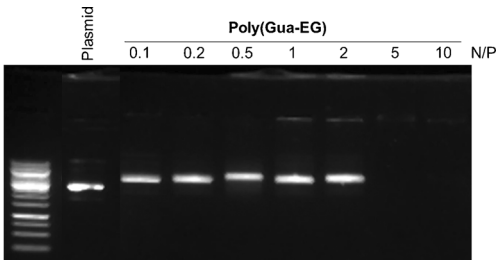


Figure 5. Gel electrophoresis of plasmid DNA with Poly(Gua-EG).

Taken together, these results demonstrate that the cationic polyacylhydrazone-based materials described herein are capable of effectively complexing dsDNA in aqueous buffer and biological serum as a result of the multivalent presentation of multiple binding units within their main chain.

Conclusion

We have reported the design and synthesis of two hybrid dynamic covalent polymers that combine cationic moieties and short ethylene oxide groups within their main chain. These materials self-assemble through a polycondensation reaction. This process yields mainly macrocycles at low concentration and open-chain oligomers at high concentration, as evidenced by DOSY NMR spectroscopy. We have demonstrated the dynamic nature of these materials through exchange reactions with methoxyamine and direct hydrolysis. Interestingly, exchange and hydrolysis take place much faster at pH 5.0 than at pH 7.0, which is of prime importance for promoting endosome escape through the pH-controlled degradation of the delivery vehicle. Furthermore, we have demonstrated, by an ethidium bromide displacement assay and gel electrophoresis, that effective complexation of dsDNA takes place, in aqueous buffer and in biological serum, at N/P ratios comparable to PEI polymers. In summary, these self-assembled hybrid materials combine two attractive features for application as smart vectors for gene delivery: 1) The effective complexation of dsDNA in biological media and 2) acid-catalyzed degradation that should promote endosome escape.

Experimental Section

General procedures and materials

All solvents, including anhydrous solvents, and reagents were purchased from Acros Organics, Alfa Aesar, Fluka, or Sigma–Aldrich and used without further purification. Reactions under dry conditions were performed under argon in flame-dried glassware. ^{1}H NMR, ^{13}C NMR, and DOSY NMR spectra were recorded at 250 MHz

for ^{1}H and 63 MHz for ^{13}C (Bruker Avance 250), 400 MHz for ^{1}H and 100 MHz for ^{13}C (Bruker Avance 400), and 600 MHz for ^{1}H and 150 MHz for ^{13}C (Bruker Avance III) in deuteriated chloroform, methanol, or dimethyl sulfoxide. Chemical shifts are reported in ppm relative to the residual solvent peak. Data are reported as follows: Chemical shift (δ), multiplicity (s for singlet, d for doublet, t for triplet, q for quartet, quint for quintuplet, and m for multiplet), coupling constant (J in Hertz), and integration. DOSY NMR spectroscopy was carried out at the Laboratoire de Mesures Physiques, IBMM-Université Montpellier 2. TLC analyses were performed on Merck silica gel 60F_{254} with detection under UV light (254 nm) or by staining with either a ninhydrin solution in ethanol or a 2,4-dinitrophenylhydrazine (DNPH) solution, followed by heating. Flash chromatography was performed on silica gel (40–63 μm) purchased from Merck. LC/ESI-MS analyses were performed on a Waters HPLC 2695 instrument (EC Nucleosil 300-5 C_{18}, 125× 3 mm column, Macherey-Nagel) equipped with a Waters 996 DAD detector and a Waters Micromass ZQ mass spectrometer (positive mode detection) with the following linear gradients of solvent B (90% acetonitrile, 9.9% water, and 0.1% TFA) and solvent A (99.9% water and 0.1% TFA): Method A: 5 to 100% of solvent B in 5 min; flow: 1 mL/min; Method B: 5 to 100% of solvent B in 20 min; flow: 1 mL/min. Retention times (t_R) are given in minutes. MALDI-TOF and high-resolution mass spectrometry (HRMS, positive mode) were carried out at the Laboratoire de Mesures Physiques, IBMM-Université Montpellier 2 by using Ultraflex III and Micromass Q-Tof instruments, respectively. Fluorescence analyses were carried out on an A F-2500 HITACHI fluorescence spectrophotometer.

Synthetic procedures and characterizations

***tert*-butyl 2-(4-formylbenzoyl)hydrazine-1-carboxylate (1)**: Thionyl chloride (145 μL, 2.01 mmol) was added to a solution of 4-formylbenzoic acid (100 mg, 0.67 mmol) in dry toluene (3 mL) under argon . The reaction solution was stirred at reflux overnight under argon. The solvent was removed under reduced pressure under dry conditions to afford the crude 4-formylbenzoyl chloride as a yellow solid. Then *tert*-butyl carbazate (88 mg, 0.67 mmol) and triethylamine (139 μL, 1.00 mmol) were added to a stirred solution of the crude acyl chloride in dry CH_2Cl_2 (3 mL) at 0 °C. The reaction solution was then warmed to room temperature and stirred for 48 h. After removal of the solvent, the residue was diluted with EtOAc (15 mL), washed with saturated $NaHCO_3$ solution (15 mL) and brine (15 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The resulting crude material was purified by flash chromatography (petroleum ether/EtOAc gradient 7:3 to 5:5, v/v) to provide **1** (yield 55%) as a white solid. R_f = 0.31 (petroleum ether/EtOAc 6:4, v/v); t_R = 3.16 min (Method A); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 10.04 (s, 1H; *H*aldehyde), 8.93 (br s, 1H; *NH*), 7.94 (d, J = 8.0 Hz, 2H; *CH*=*CH*), 7.87 (d, J = 8.0 Hz, 2H; *CH*=*CH*), 6.96 (br s, 1H; *NH*), 1.48 ppm (s, 9H; *tBu*); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 191.5, 165.8, 156.2, 138.6, 136.8, 129.9, 128.2, 82.6, 28.3 ppm; MS (ESI): *m/z* calcd for $C_{13}H_{17}N_2O_4^+$ $[M+H]^+$ 265.12; found: 265.24; HRMS (ESI): *m/z* calcd for $C_8H_9N_2O_2^+$ $[M-Boc+H]^+$ 165.0664; found: 165.0665.

***tert*-Butyl 2-[4-({[2-({2-[({4-[2-(*tert*-butoxycarbonyl)hydrazinylcarbonyl]phenyl}methyl)amino]ethyl}amino)ethyl]amino}methyl)-benzoyl]hydrazine-1-carboxylate (2)**: Compound **1** (200 mg, 0.76 mmol) was added to a solution of diethylenetriamine (41 μL, 0.38 mmol) in dry methanol (2 mL) under argon at 0 °C. The reaction mixture was stirred at room temperature for 48 h. Then sodium borohydride (43 mg, 1.14 mmol) was added to the solution cooled to 0 °C. The reaction was warmed to room temperature and stirred for 24 h. After removal of the solvent, the residue was purified by flash chromatography (*i*PrOH/H_2O/NH_4OH gradient 7:0.1:0.1 to 7:0.25:0.25, v/v/v) to provide **2** (yield 26%) as an oil. R_f = 0.39 (*i*PrOH/H_2O/NH_4OH 7:0.5:0.5, v/v/v); t_R 3.02 min (Method A); ^{1}H NMR (400 MHz, CD_3OD): δ = 7.82 (d, J = 8.0 Hz, 4H; *CH*=*CH*), 7.46 (d, J = 8.0 Hz, 4H; *CH*=*CH*), 3.82 (s, 4H; Ph*CH*$_2$N), 2.72 (s, 8H; NH*CH*$_2$*CH*$_2$), 1.50 ppm (s, 18H; *tBu*); MS (ESI): *m/z* calcd for $C_{30}H_{46}N_7O_6^+$ $[M+H]^+$ 600.35; found: 600.65.

4-[({2-[(2-{[4-(hydrazinylcarbonyl)benzyl]amino}ethyl)amino]ethyl}amino)methyl]benzhydrazide (EI-Hyd): Compound **2** (25 mg, 0.42 mmol) was dissolved in a solution of TFA/H_2O/TIS (95:2.5:2.5, v/v/v; 10 mL) and stirred for 48 h at room temperature. After removal of 90% of the solvent, diethyl ether was added to the residual solution. The precipitate residue was triturated with Et_2O and filtered. The material was then lyophilized twice to afford the trifluoroacetate salt EI-Hyd as a sticky solid in 80% yield. The yield was calculated by a ^{1}H NMR titration method using *tert*-butyl alcohol as an internal reference. ^{1}H NMR (600 MHz, d_6-DMSO): δ = 7.92 (d, J = 8.3 Hz, 4H; *CH*=*CH*), 7.64 (d, J = 8.3 Hz, 4H; *CH*=*CH*), 4.29 (s, 4H; Ph*CH*$_2$N), 3.29-3.27 ppm (m, 8H; NH*CH*$_2$*CH*$_2$); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 165.3, 158.8 (TFA), 135.9, 132.1, 130.0, 127.7, 116.0 (TFA), 49.8, 43.2 (2C); HRMS (ESI): *m/z* calcd for $C_{20}H_{30}N_7O_2^+$ 400.2461; found: 400.2456.

***tert*-Butyl 2-{4-[({13-[({4-[2-(*tert*-butoxycarbonyl)hydrazinylcarbonyl]phenyl}methyl)amino]-4,7,10-trioxatridecyl}amino)methyl]-benzoyl}hydrazine-1-carboxylate (3)**: Compound **1** (480.10 mg, 1.82 mmol) was added to a solution of 4,7,10-trioxa-1,13-tridecanediamine (200 mg, 0.91 mmol) in dry methanol (5 mL) under argon at 0 °C. The reaction solution was stirred at room temperature for 48 h. Then sodium borohydride (103.30 mg, 2.73 mmol) was added to the solution cooled to 0 °C. The reaction was warmed to room temperature and stirred for 24 h. After removal of the solvent, the residue was purified by flash chromatography (*i*PrOH/H_2O/NH_4OH gradient 7:0.1:0.1 to 7:0.2:0.2, v/v/v) to provide **3** as an oil in 22% yield. R_f = 0.27 (*i*PrOH/H_2O/NH_4OH 7:0.2:0.2, v/v/v); t_R 2.64 min (Method A); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65 (d, J = 8.0 Hz, 4H; *CH*=*CH*), 7.24 (d, J = 8.0 Hz, 4H; *CH*=*CH*), 3.74 (s, 4H; Ph*CH*$_2$N), 3.58–3.56 (m, 4H; NHCH$_2$CH$_2$*CH*$_2$), 3.54–3.51 (m, 8H; O*CH*$_2$*CH*$_2$*O*), 2.71 (t, J = 6.0 Hz, 4H; NH*CH*$_2$), 1.79 (quint, J = 6.0 Hz, 4H; NHCH$_2$*CH*$_2$CH$_2$), 1.46 ppm (s, 18H; *tBu*); MS (ESI): *m/z* calcd for $C_{36}H_{57}N_6O_9^+$ $[M+H]^+$ 717.42; found: 717.40.

4-{[(13-{4-[(Hydrazinylcarbonyl)phenyl]amino}-4,7,10-trioxatridecyl)amino]methyl}benzhydrazide (EG-Hyd): Compound **3** (145.6 mg, 0.20 mmol) was dissolved in a solution of TFA/H_2O/TIS (95:2.5:2.5, v/v/v; 8 mL) and stirred for 24 h at room temperature. After removal of 90% of the solvent, diethyl ether was added to the residue. The precipitate was triturated with Et_2O and filtered. The crude material was then lyophilized twice to afford the trifluoroacetate salt EG-Hyd as a sticky solid in 55% yield. The yield was calculated by a ^{1}H NMR titration method using *tert*-butyl alcohol as an internal reference. t_R 2.93 min (Method B); ^{1}H NMR (600 MHz, [D_6]DMSO): δ = 7.92 (d, J = 8.1 Hz, 4H; *CH*=*CH*), 7.62 (d, J = 8.1 Hz, 4H; *CH*=*CH*), 4.24 (s, 4H; Ph*CH*$_2$N), 3.48–3.45 (m, 12H; NHCH$_2$CH$_2$*CH*$_2$O*CH*$_2$*CH*$_2$O), 3.00 (t, J = 6.0 Hz, 4H; NH*CH*$_2$), 1.88 ppm (quint, J = 6.0 Hz, 4H; NHCH$_2$*CH*$_2$CH$_2$); ^{13}C NMR (150 MHz, [D_6]DMSO): δ = 165.3, 158.7 (TFA), 136.2, 131.9, 130.0, 127.7, 116.0 (TFA), 69.7, 69.5, 67.3, 49.6, 44.6, 25.8 ppm; HRMS (ESI): *m/z* calcd for $C_{26}H_{41}N_6O_5^+$ 517.3138; found: 517.3135.

4-(Dimethoxymethyl)benzylamine (4): The procedure was adapted from a previous report.[39] Trimethyl orthoformate (19 mL, 173.4 mmol) and concentrated aqueous HCl (260 μL, 8.67 mmol) were added to a solution of 4-cyanobenzaldehyde (3.79 g, 28.9 mmol) in dry MeOH (50 mL) under argon. The reaction solution was heated at 45 °C for 3 h 30 min. The solvent was then removed under reduced pressure and the residue was diluted with

EtOAc (50 mL), washed with a saturated $NaHCO_3$ solution (2× 50 mL) and brine (2×50 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The resulting colorless oil (yield 98%) was used in the next step without further purification. Characterization data are in agreement with published data for 4-(dimethoxymethyl)benzonitrile.[39] R_f = 0.50 (petroleum ether/EtOAc 8:2, v/v); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (d, J = 8.1 Hz, 2H; *CH*=*CH*), 7.48 (d, J = 8.1 Hz, 2H; *CH*=*CH*), 5.34 (s, 1H; *CH*(OMe)$_2$), 3.22 ppm (s, 6H, *CH*$_3$). The following procedure was adapted from a previous report.[40] A solution of the previously obtained 4-(dimethoxymethyl)benzonitrile (505 mg, 2.8 mmol) in anhydrous THF (3.5 mL) was then added dropwise to a stirred suspension of lithium aluminium hydride (215 mg, 5.6 mmol) in anhydrous THF (3.4 mL) under argon at 0 °C. The reaction mixture was stirred at room temperature overnight. The mixture was then cooled to 0 °C and basified by the addition of a 1 M NaOH solution (2 mL). The mixture was filtered through Celite and rinsed with EtOAc (30 mL). The organic filtrate was washed with a saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain **4** as a yellow oil (93% yield), which was used directly in the next step without further purification. Characterization data are in agreement with published data for compound 4.[40] ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.41 (d, J = 8.1 Hz, 2H; *CH*=*CH*), 7.31 (d, J = 8.1 Hz, 2H; *CH*=*CH*), 5.38 (s, 1H; *CH*(OMe)$_2$), 3.87 (s, 2H; PhCH$_2$N), 3.32 ppm (s, 6H; *CH*$_3$).

1-Ethoxycarbonyl-3-[(4-dimethoxymethyl)benzyl]thiourea (5): A solution of **4** (83 mg, 0.46 mmol) in dry CH_2Cl_2 (1.5 mL) was added to a solution of ethoxycarbonyl isothiocyanate (50 μL, 0.42 mmol) and triethylamine (70 μL, 0.50 mmol) in dry CH_2Cl_2 (1 mL) was added under argon. The reaction mixture was stirred at room temperature for 2 h and then concentrated in vacuo. The crude material was purified by flash chromatography (petroleum ether/EtOAc 8:2, v/v) to provide **5** (yield 90%) as a white solid. R_f = 0.36 (petroleum ether/EtOAc 8:2, v/v); ^{1}H NMR (250 MHz, $CDCl_3$ neutralized by K_2CO_3): δ = 9.98 (br s, 1H; *NH*), 8.60 (br s, 1H; *NH*), 7.43 (d, J = 7.5 Hz, 2H; *CH*=*CH*), 7.33 (d, J = 7.5 Hz, 2H; *CH*=*CH*), 5.38 (s, 1H; *CH*(OMe)$_2$), 4.85 (d, J = 5.0 Hz, 2H; PhC*H*$_2$N), 4.18 (q, J = 7.5 Hz, 2H; OC*H*$_2$CH$_3$), 3.30 (s, 6H; OC*H*$_3$), 1.27 ppm (t, J = 7.5 Hz, 3H; OCH$_2$C*H*$_3$); ^{13}C NMR (63 MHz, $CDCl_3$ neutralized by K_2CO_3): δ = 179.5, 152.9, 137.8, 136.6, 127.7, 127.3, 102.7, 62.8, 52.7, 49.2, 14.2 ppm.

1,3-Bis[(4-dimethoxymethyl)benzyl]-2-ethoxycarbonylguanidine (6): Triethylamine (230 μL, 1.6 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (38 mg, 0.19 mmol) were added to a solution of **5** (51 mg, 0.16 mmol) in anhydrous DMF (1 mL) . The reaction mixture was stirred for 30 min at room temperature and then a solution of **4** (30 mg, 0.16 mmol) in anhydrous DMF (900 μL) was added. The reaction mixture was heated at 60 °C overnight. The solvent was then removed in vacuo and the crude material was purified by flash chromatography (petroleum ether/EtOAc 5:5, v/v) to provide **6** (yield 84%) as a colorless oil. R_f = 0.36 (petroleum ether/EtOAc 5:5, v/v); ^{1}H NMR (250 MHz, $CDCl_3$ neutralized by K_2CO_3): δ = 7.37 (d, J = 7.7 Hz, 4H; *CH*=*CH*), 7.18 (br s, 4H; *CH*=*CH*), 5.34 (s, 2H; *CH*(OMe)$_2$), 4.43 (br s, 4H; PhC*H*$_2$NH), 4.11 (q, J = 7.1 Hz, 2H; OC*H*$_2$CH$_3$), 3.29 (s, 12H; OC*H*$_3$), 1.29 ppm (t, J = 7.1 Hz, 3H; OCH$_2$C*H*$_3$); ^{13}C NMR (100 MHz, $CDCl_3$ neutralized by K_2CO_3): δ = 164.6, 160.3, 137.9 (2C), 127.4, 127.1, 102.9, 60.9, 52.8, 45.1, 14.7 ppm; HRMS (ESI): *m*/*z* calcd for $C_{24}H_{34}N_3O_6^+$ 460.2448; found: 460.2450.

1,3-Bis(4-formylbenzyl)guanidinium chloride (Gua-Ald): Protected guanidine **6** (1.12 g, 2.44 mmol) was suspended in a 1 M aqueous HCl solution (12 mL) and the solution was heated at reflux for 13.5 h. The reaction mixture was concentrated in vacuo and the crude material was purified by flash chromatography (MeCN/H_2O gradient 98:2 to 97:3, v/v) to provide, after freeze-drying, Gua-Ald as a white solid (yield 86%). R_f = 0.66 (MeCN/H_2O 8:2, v/v); t_R = 2.84 min (Method A); ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 10.0 (s, 2H; *H*aldehyde), 7.89 (d, J = 12.0 Hz, 4H; *CH*=*CH*), 7.50 (d, J = 12.0 Hz, 4H; *CH*=*CH*), 4.61 ppm (s, 4H; PhC*H*$_2$N); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 192.8, 156.3, 144.4, 135.4, 129.7, 127.6, 43.8 ppm; HRMS (ESI): *m*/*z* calcd for $C_{17}H_{18}N_3O_2^+$ 296.1399; found: 296.1397.

***N*-{13-[2-(4-Formylphenyl)acetamido]-4,7,10-trioxatridecyl}-4-formyl)benzamide (EG-Ald)**: 4-Formylbenzoic acid (681.5 mg, 4.54 mmol) and 1-hydroxybenzotriazole hydrate (HOBt; 920 mg, 6.81 mmol) were added to a solution of 4,7,10-trioxa-1,13-tridecanediamine (500 mg, 2.27 mmol) and triethylamine (315 μL, 2.27 mmol) in dry CH_2Cl_2 (10 mL) under argon. Then the solution was cooled to 0 °C and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (1.3 g, 6.81 mmol) was added. The reaction mixture was warmed to room temperature and stirred for 48 h. After removal of the solvent, the residue was diluted with EtOAc (20 mL), washed with a saturated $NaHCO_3$ solution (20 mL) and brine (15 mL), dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude material was purified by flash chromatography (EtOAc/MeOH 95:5, v/v) to provide EG-Ald (yield 81%) as a white solid. R_f = 0.37 (EtOAc/MeOH 95:5, v/v); t_R = 3.65 min (Method A); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 10.06 (s, 2H; *H*aldehyde), 8.66 (t, J = 4.0 Hz, 2H; *NH*), 8.01–7.96 (m, 8H; *CH*=*CH*), 3.54–3.48 (m, 8H; OC*H*$_2$C*H*$_2$O), 3.45 (t, J = 8.0 Hz, 4H; NHCH$_2$CH$_2$C*H*$_2$), 3.35–3.30 (m, 4H; NHC*H*$_2$), 1.76 ppm (quint, J = 8.0 Hz, 4H; NHCH$_2$C*H*$_2$CH$_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 193.0, 165.4, 139.7, 137.7, 129.5, 127.9, 69.8, 69.6, 68.3, 36.9, 29.3 ppm; HRMS (ESI): *m*/*z* calcd for $C_{26}H_{33}N_2O_7^+$ 485.2288; found: 485.2292.

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