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Single-Molecule Studies

Direct Observation of Dynamic Mechanical Regulation of DNA Condensation by Environmental Stimuli**

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Abstract: *Gene delivery is a promising way to treat hereditary diseases and cancer; however, there is little understanding of DNA:carrier complex mechanical properties, which may be critical for the protection and release of nucleic acids. We applied optical tweezers to directly measure single-molecule mechanical properties of DNA condensed using 19-mer poly-L-lysine (PLL) or branched histidine–lysine (HK) peptides. Force–extension profiles indicate that both carriers condense DNA actively, showing force plateaus during stretching and relaxation cycles. As the environment such as carrier concentration, pH, and the presence of zinc ions changes, DNA:HK complexes showed dynamically regulated mechanical properties at multiple force levels. The fundamental knowledge from this study can be applied to design a mechanically tailored complex which may enhance transfection efficiency by controlling the stability of the complex temporally and spatially.*

Gene therapy, the delivery of genetic materials to targeted cells, has great potential for the treatment of hereditary diseases and cancers.[1] Significant efforts have been made to develop effective carriers in viral and non-viral platforms.[1] Non-viral carriers have been extensively investigated because of their lower toxicity and more facile manufacture in comparison to viral vectors. Among non-viral carriers, synthetic cationic peptides have been shown to strongly bind and condense DNA into nanoscale complexes that are effective for delivery.[2] However, the efficiency of delivering genes by synthetic carriers has not yet improved to the point where they are clinically useful. The potential reasons for limited efficiency lie in the set of physiological obstacles that the complexes must navigate: these include the stable transport of packaged DNA in the bloodstream, targeting to specific cells, endocytosis, endosomal escape, unpackaging of DNA, and nuclear transport. Surmounting some of these barriers requires delicate regulation of the forces between nucleic acid and carrier. For example, stable and uniform interaction between carrier and nucleic acid is required to protect DNA in the bloodstream, but later, or in response to changes in the environment, the interaction needs to weaken to release nucleic acids from the complex. However, there is a lack of understanding of the optimal interaction forces required to protect or release DNA and how these forces are regulated under various physiological environments, which prevents rational design of more efficient carriers for gene delivery.

Single-molecule force spectroscopy can provide mechanistic insights into biological processes at a level of detail inaccessible in bulk studies.[3–5] Previously, interaction forces between DNA and a cationic polyaminoamide (PAMAM) dendrimer were measured at the single-molecule level.[6] The interactions are electrostatic, and dendrimers remained bound to DNA after a washing step, exerting circa 10 pN forces during stretching. Recently, the mechanical properties of the complex between DNA and the self-aggregating peptide Kahalalide F were measured using optical tweezers, revealing a two-step kinetic process that forms highly rigid aggregates by electrostatic interaction and hydrophobic collapse.[7]

Herein, we use optical tweezers to directly observe dynamic changes in the interaction forces between DNA and carrier in response to concentration, pH, and metal ions. Custom-built tweezers with high force and spatial resolution are utilized to probe mechanical behaviors of DNA complexes.[8] Two different cationic peptide agents, 19-mer poly-L-lysine (PLL), and histidine–lysine (HK)-based peptides, are examined to compare dynamic behaviors as they interact with DNA.

Histidine–lysine based peptides have been developed as a gene-delivery carrier that contains lysine residues for DNA binding and compaction, and histidine residues to carry out the proton sponge mechanism for endosomal escape.[9] The

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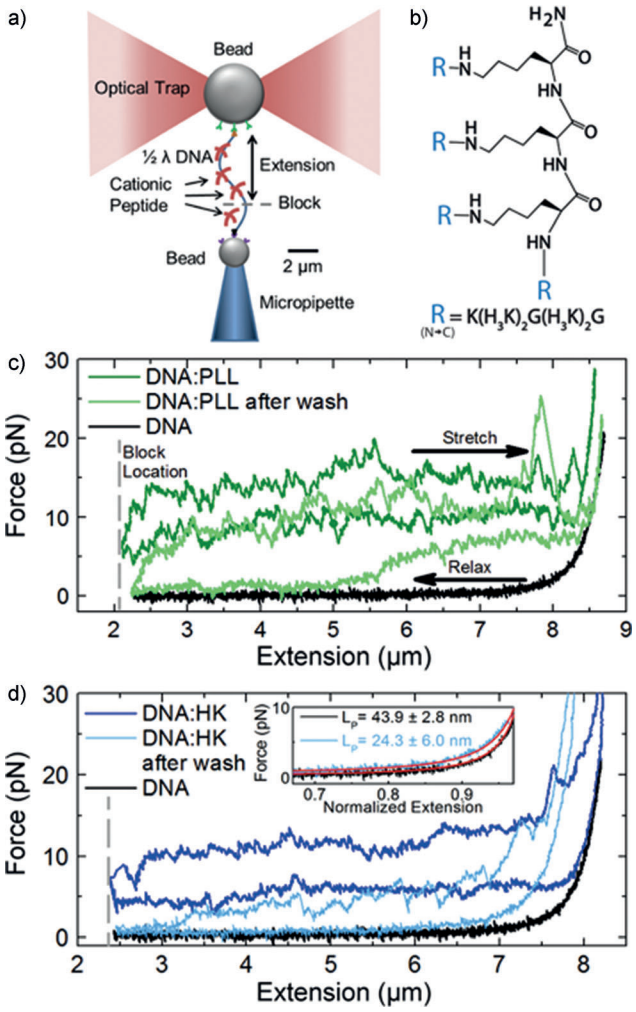

Figure 1. a) The experimental setup. b) Structure of HK peptide, showing a three lysine core and the amino acid sequence of each branch. c), d) Force versus extension profiles of c) 1 μM PLL and d) 1 μM HK solution after peptide injection (dark green and dark blue) and after washing (light green and light blue) with Tris buffer. The force vs extension curve of bare DNA is shown in black. The inset in (d) shows the Worm-like-chain model fits for the relaxation curves of bare DNA and the HK:DNA complex after washing.

circa 80-residue HK peptides used herein have four branches around a three-lysine core (Figure 1b). Each branch has a repeating sequence of HHHK, with the exact sequence of each branch being C-term-GKHHHKHHHGKHHHK-HHHK-*N*-term. Compared to PLL, HK based peptides have been found to be more effective transfection agents in vitro (Supporting Information, Figure S1). Linear PLL is known to condense DNA, and its transfection efficiency from previous work found out to be marginal.[10]

In our experimental setup, half-λ double-stranded DNA (24 kbp) is functionalized with biotin and digoxigenin and tethered between two beads by specific interactions (Figure 1a and Experimental Section).[11] The upper bead was moved up and down in the optical trap to stretch and relax DNA. The extension distance was controlled by setting a block that limits the range of motion of the top bead. During injection of agents, the block location was initially set at about 5 μm from the lower, pipette-fixed, bead. In a typical experiment, 1 μM of condensing agent in a buffer (155 mM NaCl, 0.1 mM Tris pH 7.4, 1 mM EDTA, and 0.05% sodium azide) was injected into the chamber at a rate of 5 $\mu L\,min^{-1}$ while DNA was stretched and relaxed at a rate of 500 $nm\,s^{-1}$. Injection was stopped after 5 μL, when force profiles that clearly deviated from naked DNA features had developed and stabilized. The condensed DNA is identified by the appearance of stretching and relaxation force plateaus and hysteresis. All of the reported force values are based upon at least three experiments unless otherwise specified.

As PLL or HK binds to DNA, the force profile of bare DNA (black trace in Figure 1c and d) is gradually changed to show plateaus during a stretching and relaxation cycle (dark green in Figure 1c, dark blue in Figure 1d). The appearance of these force plateaus shows that both PLL and HK actively condense DNA. Plateau forces remained at relatively similar levels; no distinctive sawtooth pattern was seen in the profiles, indicating that the binding between DNA and both agents is rather uniform, without apparent large loops or bridged structures. The stretching plateau is at 11.2 ± 0.7 pN for the DNA:HK complex and 12.6 ± 1.6 pN for DNA:PLL (Supporting Information, Figure S2). Upon slowing the pulling rate down to 50 $nm\,s^{-1}$, a decrease in the stretch plateau and an increase in the relaxation force are observed, indicating that the force profile becomes quasi-equilibrium behavior at the slower rate (Supporting Information, Figure S3). These force plateau levels are similar to those of PAMAM dendrimers, which showed stretching plateau at about 10 pN,[4] but they are well below the range of rupture forces of nucleosomes, which are observed between 20 and 40 pN.[12]

For HK, the relaxation plateau occurs 6.6 ± 0.4 pN, and similarly PLL is at 8.1 ± 1.4 pN. These relaxation plateau values are similar to the critical condensation force at which an active condensation starts for protamine (ca. 6 pN) and spermidine (ca. 7 pN).[13] The relaxation plateaus for HK and PLL are higher than that of PAMAM dendrimer (ca. 4 pN), and the critical condensation forces of other multivalent cations such as spermine (ca. 3 pN), cobalt hexamine (1.5 pN), and cobalt sepulchrate (3.5 pN).[6,13] The plateau forces decreased as ionic strength increased, demonstrating that the driving force of this active condensation is mainly due to electrostatic interactions between the phosphate backbone and the cationic peptides (Supporting Information, Figure S4).

To mimic the process of diluting prepared complexes into the bloodstream or a cellular environment lacking free cationic peptides, we washed away free condensing agent by injecting the Tris buffer into the chamber. Upon washing the PLL:DNA complex (Figure 1c, light green), double relaxation plateaus (at 0.8 ± 1.2 pN and 5.1 ± 3.6 pN) were observed in two out of three experiments, suggesting formation of heterogeneous structure; single plateau behavior was observed once (Supporting Information, Figure S5). The stretching plateau decreased slightly (10.5 ± 3.6 pN), indicating that most of PLL molecules still remain bound to DNA after buffer injection.

In sharp contrast, HK showed a significant decrease in both stretching and relaxation plateaus after washing (Fig-

ure 1 d, light blue). The relaxation plateau disappeared, showing a bare-DNA-like behavior, while the stretching plateau value decreased to 4.8 ± 5.3 pN. The decreases in both plateau forces is attributed to detachment of a significant number of HK molecules from the DNA, thus leading to a net negatively charged complex that exerts lower condensing force after washing. This result is similar to the concentration dependence of condensation force of multivalent ions, for which a decrease in condensation force is observed at lower concentration.[13]

The relaxation curve of DNA:HK complex returned to worm-like chain behavior, with a reduced persistence length (L_p) of 24.3 ± 6.0 nm ($n = 5$) compared to a naked DNA (L_p = 43.9 ± 2.8 nm, $n = 6$) (Figure 1 d, inset). The decrease in L_p is presumably due to charge screening by the HK that still remains bound to DNA after washing,[14] as observed for the condensation of DNA by other condensing agents.[7,15] The observations that the tensile resistance during stretching deceases significantly and the relaxation profile resembles naked DNA behavior indicate that HK:DNA complex may release HK readily, allowing more efficient unpackaging when the concentration of cationic agents in the surroundings is low.

To simulate the low pH environment that the DNA complex would experience upon endocytosis, acetate buffer at pH 5 (10 mM acetate, 155 mM NaCl, 1 mM EDTA, and 0.05 % azide) was introduced into the chamber at a rate of 10 μL min^{-1} after washing with the Tris buffer. The pH drop did not elicit any changes in mechanical behavior of the DNA:PLL complex (Supporting Information, Figure S5), whereas for the DNA:HK complex, stretching and relaxation plateaus reappeared at 14.0 ± 1.8 pN and 10.0 ± 1.0 pN, respectively (Figure 2). The return of both plateaus at pH 5 is most likely due to the protonation of the 48 histidine residues ($pK_a \approx 6$) in each HK molecule that remains bound during washing. The increased positive charge density of HK allows stronger electrostatic interactions between HK and DNA. The stretching plateau and relaxation plateau levels decreased as salt concentration increased from 150 mM to 1M, confirming that electrostatic interactions are the major driving force for active condensation at low pH (Supporting Information, Figure S4).

The dynamic response of the DNA:HK complex was further examined in the presence of divalent cations. When 1 mM Zn^{2+} in the acetate buffer (pH 5) was injected into the chamber, the DNA:PLL force profile did not change (Supporting Information, Figure S6), whereas the DNA:HK complex became mechanically very stiff and its contour length significantly decreased, contracting nearly to the block location at about 5.5 μm (Figure 3 a; Supporting Information, Figure S7). The maximum force available in the optical trap (ca. 100 pN) was not large enough to disrupt the complex formed in the presence of Zn^{2+}. Subsequent stepwise movement of the block location in 500 nm increments (dotted gray line) caused corresponding decreases in the contour length of the complex (arrows in Figure 3 a and b). This indicates that when the complex is allowed to contract, it interacts with zinc ion to form highly mechanically resistant structures (Figure 3 b). The average final contour length was 2.87 ± 0.26 μm when the final block location was about 2 μm (Figure 3 b). The compaction does not always continue indefinitely (2 out of 4 experiments), and there is a slack region where the complex extends at relatively low force (< 2 pN) with no apparent hysteresis (Figure 3 a). Worm-like chain fitting of

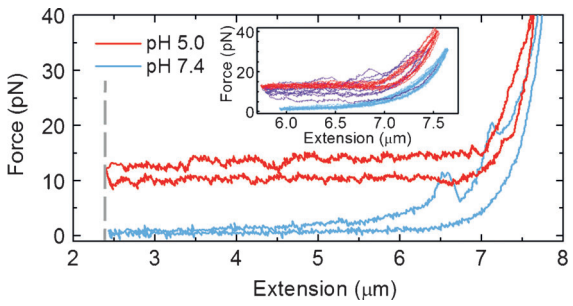

Figure 2. The effect of pH on force vs extension profile of DNA:HK complex. Force curves at pH 7.4 after washing and at pH 5 are shown in light blue and red, respectively. Inset: dynamic transitional behavior (purple) of force profiles while the pH was changing from 7.4 to 5.

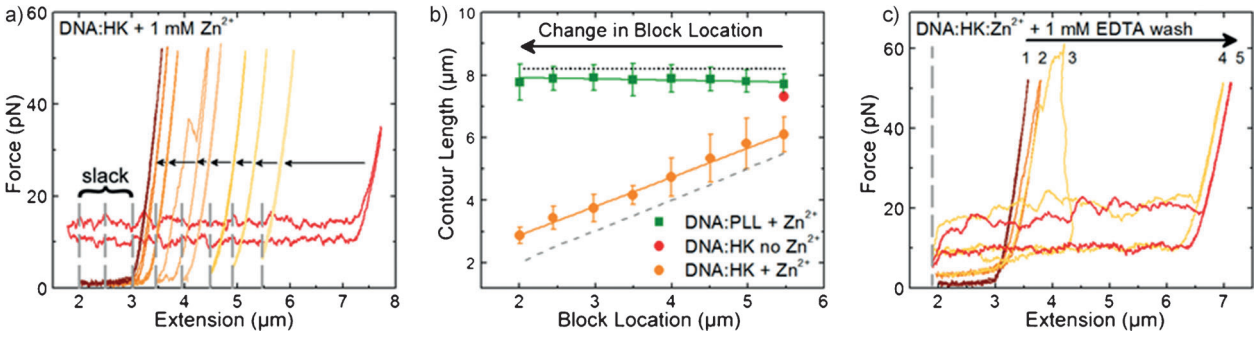

Figure 3. The effect of Zn^{2+} on the mechanical behavior of the DNA:HK complex. a) The red force profile for the DNA:HK complex was obtained after washing at pH 7.4 and then decreasing the pH to 5. As 1 mM Zn^{2+} is added, the maximum extension decreases markedly, indicating the formation of a mechanically rigid complex. Sequential movements of the block location by 500 nm (yellow to brown) resulted in a further decrease of extension as indicated by arrows. b) A plot of contour length (from fits to the worm-like chain model) versus block location for the DNA:PLL complex and the DNA:HK complex. The dotted black line represents the original DNA contour length, and the dashed gray line shows the 1:1 line between block location and contour length as a guide to the eye. c) Recovery of extension upon washing with 1 mM EDTA. Numbers indicate sequential stretching and relaxation cycles.

the slack region reveals that the persistence length is much longer than that of naked DNA, up to an average of 187.5 ± 23.0 nm ($n = 3$; Supporting Information, Figure S8). The shape of the force profile along with the increased persistence length indicates that a very stiff complex is formed as DNA:HK complex interacts with Zn^{2+}. A similar behavior is observed when DNA is collapsed by a hydrophobic peptide, although in that case the rigid complex is constrained by hydrophobic forces.[7,16]

The phenomenon of Zn^{2+} inducing the formation of a stiff DNA:HK complex is reversible upon injection of 1 mM EDTA in the acetate buffer at pH 5. Within a few stretching/relaxation cycles, the initial contour length is recovered, with reappearance of force plateaus, showing that removal of Zn^{2+} allows the rigid complex to recover its original force characteristics (Figure 3c). The dissociation constants of Zn^{2+}/EDTA and Zn^{2+}/Histidine are 1×10^{-16} M[17] and 8.8×10^{-13} M[18] respectively, so EDTA should readily chelate Zn^{2+} ions away from histidines in the rigid complex. Other cations such as Mg^{2+} and Ca^{2+} had no effect on force profiles and did not form a mechanically resistant complex (Supporting Information, Figure S9).

The highly mechanically resistant complex induced by Zn^{2+} is proposed to originate from chelation by multiple imidazole groups in histidine residues.[19] Incorporation of a bi-histidine metal chelation site into a protein, GB1, has demonstrated an increase in mechanical stability in the presence of Ni^{2+}.[20] For DNA:HK, the fact that the relaxation by moving the block location is required to form the stiff complex suggests that participation of multiple histidines in HK polymers that are apart from each other along the DNA is essential for polydentate coordinated complex formation. The rupture force of Ni^{2+}/histidine coordination is a few hundred pN based on force spectroscopy.[19] The dissociation constant of Zn^{2+}/histidine (8.8×10^{-13} M) is lower than that of Ni^{2+}/histidine (5×10^{-7} M), and the corresponding $\Delta G_{\text{dissociation}}$ is calculated to be 16.5 kcal mol^{-1} and 8.6 kcal mol^{-1}, respectively.[17,21] Although mechanical stability may not be directly correlated with thermodynamic stability,[22] we expect that a similar or higher force would be needed to disrupt the DNA:HK in the presence of zinc ions considering the nature of the interaction is the same.

Our mechanical measurements using a single DNA molecule complexed with peptides demonstrated that environmental changes can cause significant differences in the mechanical properties of a DNA:peptide complex. Specifically, the DNA:HK complex showed dynamic changes in mechanical properties at multiple force levels, which could be exploited during the gene delivery process. When a DNA:peptide complex is injected into the bloodstream, it may undergo shear or elongational forces which may disrupt the complex, increasing chances of degradation of DNA.[23] Based upon our data, addition of Zn^{2+} to histidine-containing peptides resulted in a highly mechanically stiff complex, which could be advantageous in maintaining the stability of the complex when it is in contact with bodily fluids. Furthermore, coordinated Zn^{2+} is expected to increase the zeta potential of the complex, which should enhance its interactions with a cellular membrane.[10] Both pathways will contribute to enhanced transfection efficiency. Zn^{2+} is considered relatively non-toxic and is maintained at μM concentrations in the blood plasma. Within the cytosol, femtomolar concentrations of Zn^{2+} are maintained by metalloregulatory proteins.[24–26] These Zn-binding proteins may compete with HK for Zn^{2+} ions and facilitate release of the DNA:HK complex. Additionally, if the protonation occurs to the extent that the HK:DNA complex becomes overcharged, some HK molecules may shed, accelerating unpackaging process.

Recently, single molecule techniques have provided direct evidence about how mechanical force may influence gene regulation.[27] For instance, individual nucleosomes are disrupted at 20–40 pN.[12] The procession of RNA polymerase is halted at around 25 pN[3] and DNA helicase is known to exert 10–15 pN to separate DNA.[28] In our washing experiments, DNA:HK showed lower stretching and relaxation plateau forces than DNA:PLL, which suggests that HK is more easily removed from DNA than PLL. The fact that the stretching plateau force is about 5 pN suggests that HK is likely to be a more effective carrier in terms of allowing the unpackaging of DNA by endogenous nucleic acid processing systems. Previously, it was shown that multivalent cations such as spermine are completely washed away from DNA, as indicated by disappearance of stretching and relaxation force plateaus.[29] In contrast, the DNA complex with PAMAM dendrimer does not show changes in force plateaus after washing, indicating that dendrimers are irreversibly bound.[6] One key parameter that may govern the mechanical response to washing is the number of charged groups per molecule. Spermine has a +4 charge, and PAMAM (5th generation) has +128 charges, whereas HK and PLL contain +17 (at pH > 7) and +20 charges, respectively. Despite small difference in total charges per molecule, the observed large variation in washing effect between HK and PLL may be attributed to a linear charge density along its peptide backbone; at pH 7.4, PLL has a charged group on every residue, whereas only about 25% of total residues along each HK branch are charged. Therefore, both the total charge of the molecule and its charge density need to be considered to optimize interactions between DNA and condensing agents. It is also likely that secondary interactions such as hydrogen bonding between the HK peptide and nucleic acid may play a role in stabilizing the complex.[30]

Transfection studies indicate the importance of size and charge regulation of cationic polymers in controlling transfection efficiency.[31,32] However, there have been few studies probing how charge regulation affects mechanical properties of the complex directly under various environments. Poly-L-lysine, one of the first polymers studied for non-viral gene therapy, is a poor carrier unless coupled with endosomolytic agents.[2,33] Polymers consisting of lysine and histidine or other imidazole-containing groups are able to efficiently transfect an array of cell lines, up to four orders of magnitude more effectively than PLL, without cytotoxic effects.[34,35]

Our observations at the single molecule level reveal that there are multiple levels of interaction forces between DNA and HK peptide depending on the environment. After washing, the minimal level of interaction forces (< 5 pN) was observed for HK, which is an ideal behavior for a tran-

scriptional process to work inside a nucleus without significant mechanical barrier. At pH 5, the stretching plateau of DNA:HK complex increased to 14 pN. The increased stretching force indicates strong association of DNA with HK and it may keep DNA from degrading under an acidic environment inside the endosome. Moreover, very rigid Zn^{2+} bound complex may be suited to protect DNA in the blood stream where shear forces, competing agents, and serum nucleases are present. Finally, these adaptive force responses to environmental changes can be further adjusted to a delicate level by varying peptide sequences, linear charge density, and branching patterns, with the end goal being higher transfection efficiencies. We also suggest that single molecule techniques may serve as a platform for screening condensing agents for desirable mechanical properties prior to in vitro and in vivo experiments.

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