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Protection of Human Genomic DNA from Mechanical Stress by Reversible Folding Transition

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Manipulating intact, long genomic DNA material is of crucial importance for a number of applications in cytogenetics, genomics and biotechnology. The preservation of the genetic information contiguity is a requirement for faster, high-throughput physical DNA mapping and sequencing.[1] Moreover, recent cytogenetic techniques have revealed a higher than suspected frequency of pathology-related large-scale chromosomal rearrangements (up to megabase scale), which can be solely detected on perfectly preserved DNA molecules.[2] Finally, the new perspective of single-molecule-based DNA damage profiling (new generation of genotoxicity tests, radiotherapy follow-up) critically depends on the discrimination between physiologically and experimentally induced DNA damage. For all these applications, the main limitation is the fragmentation of genomic DNA molecules during manipulations. Indeed, basic manipulations (mixing, injection, centrifugation, etc.) induce shearing stress at a scale in the order of a few μm. Human genomic (HG) DNA molecules have the particularity to be very long, with a number of base pairs (bp) in the order of 10^6 and a contour length in the mm range. As a consequence, they experience intense molecular tension when manipulated in solution and are prone to fragmentation.[3] On the other hand, long genomic DNA has the property to undergo a reversible phase transition into very condensed states (of the order of 100 nm) upon addition of appropriate condensing agents.[4] In this work, we exploited this unique property as a way to reversibly reduce DNA size in solution under the typical size at which shearing induces breakage. We established the conditions for fully reversible folding transition of HG DNA. We used molecular combing (MC) to quantitatively analyze the size distribution of ten thousand individual HG DNA molecules with a 1 kbp resolution.[5] Using this strategy, we could demonstrate for the first time perfect preservation of size distribution of HG DNA samples submitted to strong mechanical stress.

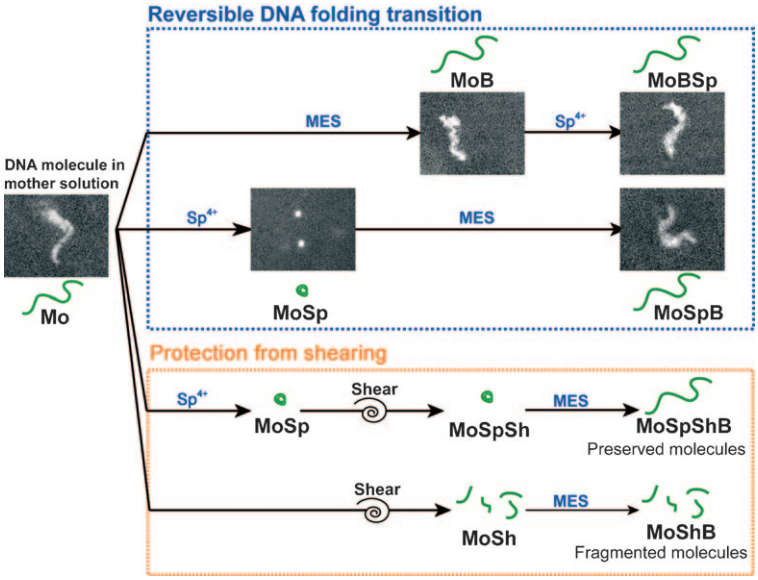

Figure 1. Concept and experimental assay used to obtain DNA reversible phase transition and attest its protection capability. The blue panel depicts the reversible folding transition of single human genomic (HG) DNA molecules in solution. The FM pictures show that DNA folding can be induced when the condensing agent spermine (Sp^{4+}) is added to a low salt solution (Mo) but not to a high salt one (MoB). Unfolding is obtained by increasing the salt concentration upon addition of concentrated MES buffer for molecular combing (MC). The orange panel represents the concept of "protection from shearing": when mechanical shearing is applied to DNA molecules that have been previously compacted, fragmentation is prevented and the initial molecule size distribution is preserved. Experimental conditions are given in the Supporting Information.

Figure 1 depicts the concept and experimental protocol that have been applied to two types of DNA, a prepurified, commercially available lambda phage DNA (λ-DNA, 48.5 kbp, contour length 16.2 μm) and freshly extracted HG DNA (fragments of hundreds of kbp). By fluorescence microscopy (FM) observations of individual molecules stained with YOYO-1 in solution, we established the conditions to get folding/unfolding transition compatible with MC technique (Tables S1 and S2 in the Supporting Information). Figure 1 shows the aspect of individual HG DNA molecules in solution. In the initial mother solution (Mo) all molecules were in the unfolded, elongated coil state: DNA molecules appear very large (8–15 μm) and adopt a random-coil conformation. The addition of a sufficient amount of spermine (Sp^{4+}) resulted in DNA charge neutralization and induced all molecules to fold into the compact state (MoSp).[6] Upon the addition of a concentrated MES buffer solution, progressive unfolding of DNA was observed, which is attributed to the counter-ion exchange between Sp^{4+} and sodium ions coming from MES buffer cations.[7] Control experiments showed that DNA molecules stayed in the unfolded, elongated coil state when MES was added first (MoB), prior to addition of Sp^{4+} (MoBSp). Then, DNA molecules were combed on silanized

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glass substrates[8] and hundreds of FM images were acquired on an automatic platform (see the Supporting Information). Custom image analysis[9] of more than 10000 individual molecules was used to precisely construct the size distribution of combed DNA molecules.

We first studied λ-DNA, which has been widely characterized in MC studies (Figure 2). By FM, we found that λ-DNA (Mo, 100 μM MES buffer, pH 5) can be folded by addition of 2 μM Sp^{4+} (MoSp) and unfolded by final addition of 0.75 M MES, pH 5.75 (MoSpB). Figure 2B shows the size distributions established by MC for the different solutions. First, the size distributions of the λ-DNA control solutions (MoB, MoBSp) have a sharp peak at (25.8 ± 1) μm, which corresponds to the length of an intact λ-DNA genome combed on a surface at a constant stretching factor (1.6 here).[10] Notably, the sample that underwent folding/unfolding transition (MoSpB) shows a size distribution very similar to that of control samples. The position and height of the characteristic peak are preserved, which demonstrates that folding/unfolding does not affect the final conformation of DNA molecules or their response to an elongating force. Once evidenced that our strategy neither damages DNA nor alters the MC stretching factor, we studied its protective capacity to mechanical stress. To this end, DNA was submitted to elongational shearing by syringe-driven flow in a capillary (protocol given in the Supporting Information). When the mother solution was submitted to shear stress (MoShB), the characteristic peak was not observed anymore; this indicates that no more intact molecules were present in the solution. In contrast, when molecules were compacted, sheared, and unfolded (MoSpShB) the distribution appeared very similar to that of the control solution (MoB) and again showed the characteristic peak at (25.8 ± 1) μm. This indicates that folding/unfolding effectively prevents DNA from breakage induced by mechanical stress.

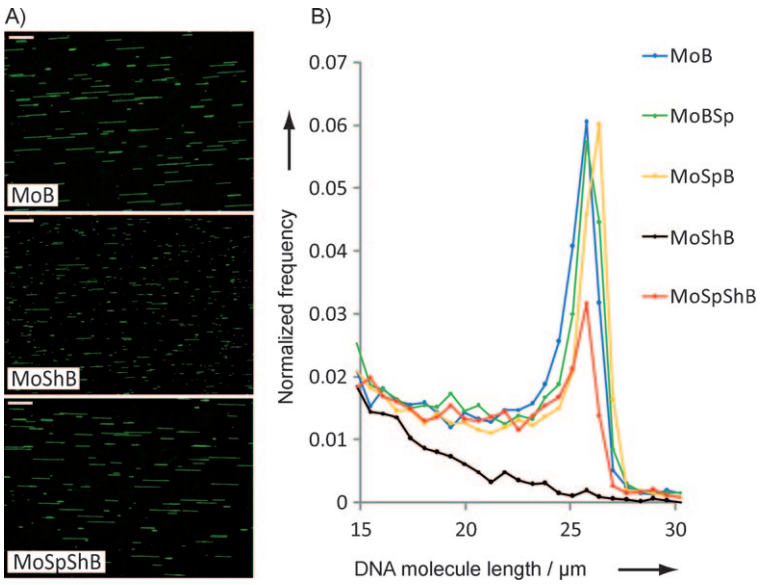

Figure 2. Lambda phage DNA size distribution is perfectly preserved from mechanical stress by reversible folding transition. A) Fluorescence microscopy images (scale bar: 20 μm), and B) size distribution of combed DNA molecules for various conditions. The experimental assay is given in the Supporting Information. MoB: 0.1 μM λ-DNA in 0.75 M MES buffer, pH 5.75; MoBSp: 0.1 μM λ-DNA in 0.75 M MES buffer, pH 5.75 + 2 μM spermine; MoSpB: 0.1 μM λ-DNA + 2 μM spermine in 0.75 M MES buffer, pH 5.75. MoShB: 0.1 μM λ-DNA in 0.75 M MES buffer, pH 5.75 + shearing; MoSpShB: 0.1 μM λ-DNA in 0.75 M MES buffer, pH 5.75 + 2 μM spermine + shearing.

Then, we applied our method to freshly extracted HG DNA, the length of which can reach up to 10 times the length of intact λ-DNA (up to 500 kbp). This type of sample contains residuals from protein and polysaccharide digestion (see the Supporting Information). To our knowledge, there is no available data regarding in vitro folding transition of HG DNA, and particularly of such high molecular weight. We studied the conditions required to control folding/unfolding of freshly extracted HG DNA by FM observations. At a concentration of 4 μM Sp^{4+}, nearly all molecules in the Mo solution transformed into small, bright and fast-diffusing particles (MoSp); this indicates that folding transition to the condensed state had occurred. Complete unfolding was reached by increasing MES buffer concentration to 0.5 M MES, pH 6.5 (MoSpB).

Figure 3 shows the results obtained with HG DNA samples submitted to folding/unfolding (MoSpB, MoSpShB) and/or mechanical stress (MoShB and MoSpShB). Unlike λ-DNA, the size distribution of freshly extracted HG DNA solutions shows no characteristic peak, but follows an exponential trend of type $y = a e^{-x/\tau}$ (MoB), where x is the size of DNA fragments inside the solution (measured in μm on the FM images of combed DNA), y is the amount of fragments of size x, a is the multiplication factor, and τ is a characteristic decay length. We systematically observed this trend in the solutions obtained using our extraction protocol, which includes various manipulations from chromosomes to deproteinized DNA molecules. For this study, we do not aim at identifying the origin of such profile, but at analyzing the behavior of isolated DNA molecules in solution submitted to mechanical stress. Since this initial distribution was reproducibly obtained for all analyzed samples, we use this curve as the representative distribution of our control sample (MoB solution). Similar to λ-DNA, the size distribution of the sample that underwent folding/unfolding transition (MoSpB) was exactly superposed to the distribution obtained from the control sample (MoB). In Figure 3A, the pictures show HG DNA linearized on MC substrates: when shearing is applied to unfolded DNA (MoShB) a remarkable fragmentation can be directly observed on the images. When folding transition is induced prior to shearing (MoSpShB) the length of the stretched molecules appears similar to the initial one (MoB). This observation is confirmed when we compare the size distributions of the molecules in our samples (Figure 3B). The two samples experiencing folding/unfolding with or without shearing show an exponential trend identical to the mother solution within experimental accuracy (τ = 22.4, 20.7, and 22.6 μm for MoB, MoSpB, and MoSpShB, respectively) while the sample submitted to shearing without folding

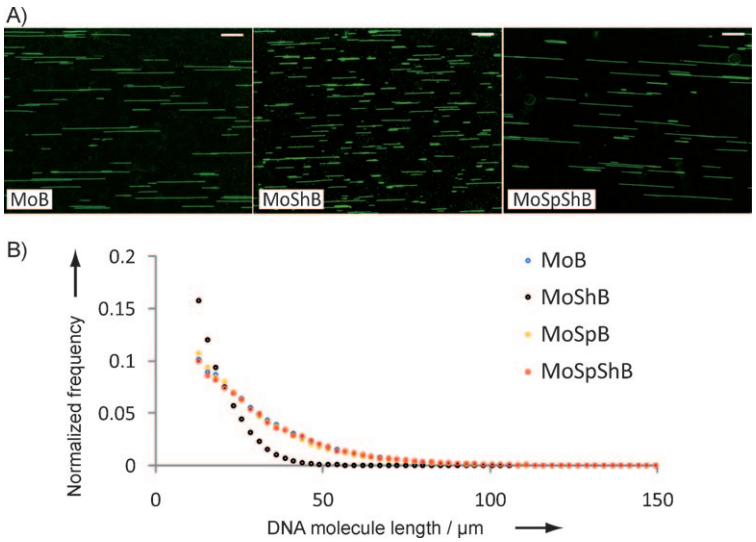

Figure 3. Application to the preservation of human genomic DNA (HG DNA) samples. A) Fluorescence microscopy images (scale bar: 20 μm), and B) size distribution of combed HG DNA molecules for various conditions. Measurement data inferior to 10 μm have not been considered for histogram construction (see the Supporting Information for details). The experimental assay is given in the Supporting Information. MoB: 0.1 μM HG DNA in 0.5 M MES buffer, pH 6.5; MoSpB: 0.1 μM HG DNA + 4 μM spermine in 0.5 M MES buffer, pH 6.5; MoShB: 0.1 μM HG DNA in 0.5 M MES buffer, pH 6.5 + shearing; MoSpShB: 0.1 μM HG DNA in 0.5 M MES buffer, pH 6.5 + 4 μM spermine + shearing.

transition shows a much stronger decay (MoShB, $\tau = 9.6$ μm). These results demonstrate that reversible folding transition perfectly prevents the fragmentation of very long HG DNA molecules submitted to mechanical stress, and this even in unpurified solutions.

The "protection" capability of spermine on DNA has already been observed in some biochemical protocols.[11] Here, we performed single-molecule measurements on very large sample population and showed that the size distribution of genomic DNA molecules (λ-DNA, HG DNA) are perfectly preserved from mechanical stress by using reversible folding transition. To interpret the mechanism of protection, it is interesting to consider the characteristic scales of scission that molecules manipulated in solution undergo. It is well established that DNA molecule breakage occurs when the chain is forced into a highly extended configuration.[8,11] A recent theory for polymer chain scission suggested that this breakage-favorable condition is reached when the polymer extended length L_E, which can be assumed close to its contour length L, reaches the Kolmogorov scale η, which scales as $\eta \approx d\,Re^{-3/4}$, where d is a characteristic dimension of the flow, and Re is the Reynolds number.[12] In our system, d corresponds to the inner diameter of the needle used for the shearing experiments (500 μm), and Re ranges between 1000 and 2000 (see the Supporting Information). The Kolmogorov scale η is then between 1.7 and 2.8 μm. According to this hypothesis, molecules with contour length $L \gg 1$ μm experience a molecular tension that leads to covalent bond breakage. Both λ-DNA and HG DNA used in our experiments are much larger than the calculated Kolmogorov scale, and as predicted by the theory, both undergo intense fragmentation. On the contrary, when phase transition is used, λ-DNA folds into toroidal structures of approximately 100 nm in diameter,[4a,c,13] which are much smaller than η and are thus not affected by the applied shearing. After unfolding, the initial size distribution of DNA molecules is perfectly recovered. To our knowledge, the aspect and size of globular forms generated by long HG DNA molecules in heterogeneous solutions has never been reported in the literature. We calculated the DNA packaging limit of a typical 100 nm toroid, assuming hexagonal packing of the DNA chains in the cross section of the toroid.[13] The maximal DNA length that can be folded in these structures is $L_{max} \approx 50$ μm, which corresponds to approximately 145 kbp (see the Supporting Information for calculation details). Thus, the majority of the DNA molecules present in our solutions should form unimolecular toroidal structures. Molecules exceeding 150 kbp should form more complex condensates or aggregated toroidal or rod-like structures. These larger condensates do not probably exceed the hydrodynamic size of 1 μm and, as observed in the experiments, they do not significantly break down in our system. Complementary to size effect, the folding of DNA into well-defined structures with ordered morphology (toroids, rods, etc., with hexagonal packing)[13] can also contribute to increase DNA mechanical resistance.

We have demonstrated that our method is suitable for manipulating freshly extracted human DNA without further uncontrolled fragmentation. The ability to manipulate and eventually extract DNA in the condensed phase opens new perspectives for biological applications focused on the recovery of the genetic information coded into individual, preserved DNA molecules. Commonly used methods for genome sequencing are based on disruption of DNA and subsequent reconstruction of the original order. Preserving the intactness of the genetic material isolated from cells is equal to the possibility of directly reading the information incorporated in chromosomes, and opens new ways for sequencing and mapping based on single-molecule analysis. Moreover, direct detection of large genomic rearrangements (for instance, the number of repeats of a specific sequence) or localization of a viral genome in the host DNA require manipulating hundreds of kbp of DNA without loss of their contiguity.

In this communication, we quantitatively characterized the "protection" capability of reversible folding transition on human genomic DNA. Our results are derived from tens of thousands of measurements of individual molecules, which confer great statistical relevance on our study. Our findings show that our method can be used to prevent very long DNA from breakage in a fully reversible manner, even in complex unpurified solutions. These two aspects are requirements for future integration in biological protocols.

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