Orientation of DNA minicircles balances density and topological complexity in kinetoplast DNA

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Abstract

Kinetoplast DNA (kDNA), an essential mitochondrial structure common to trypanosomes and *Leishmania*, contains thousands of DNA minicircles that are densely packed and topologically linked into a chain mail-like network. Experimental data indicate that every minicircle in the network is, on average, singly linked to three other minicircles (i.e., has mean valence 3) before replication and to six minicircles in the late stages

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of replication. The biophysical factors that determine the topology of the network and its changes during the cell cycle remain unknown. Using a mathematical modeling approach, we previously showed that volume confinement alone can drive the formation of the network and that it induces a linear relationship between mean valence and minicircle density. Our modeling also predicted a minicircle valence two orders of magnitude greater than that observed in kDNA. To determine the factors that contribute to this discrepancy we systematically analyzed the relationship between the topological properties of the network (i.e., minicircle density and mean valence) and its biophysical properties such as DNA bending, electrostatic repulsion, and minicircle relative position and orientation. Significantly, our results showed that most of the discrepancy between the theoretical and experimental observations can be accounted for by the orientation of the minicircles with volume exclusion due to electrostatic interactions and DNA bending playing smaller roles. Our results are in agreement with the three dimensional kDNA organization model, initially proposed by Delain and Riou, in which minicircles are oriented almost perpendicular to the horizontal plane of the kDNA disk. We suggest that while minicircle confinement drives the formation of kDNA networks, it is minicircle orientation that regulates the topological complexity of the network.

Keywords: Kinetoplast DNA, DNA topology, minicircle, Mathematical Modeling

1   Introduction

Kinetoplastids are single cell flagellated protozoa some of which are parasites that cause the human diseases African and American trypanosomiasis and Leishmaniasis [1, 2]. The mitochondrial DNA of these organisms, called kinetoplast DNA (kDNA), is organized into a catenated network composed of few dozen maxicircles and thousands of minicircles. Maxicircles do not encode tRNAs however they do contain ribosomal genes and other mitochondrial genes; their size ranges from 20 to 40 kb. Minicircles on the other hand range in size from 1kb to 2.5 kb and encode for guide RNAs that edit maxicircles transcripts [3, 4, 5]. The formation and maintenance of this network is still a major puzzle in spite of significant advances through numerous biochemical and molecular studies (for a review see [6]).

In kinetoplastid parasites, kDNA is confined within a small cylindrical structure, called the kinetoplast disk, in which DNA concentration is comparable to that of the bacterial nucleoid [7]. Minicircles are relaxed (instead of supercoiled) and topologically linked into a planar network [8]. During the early phase of the kDNA replication cycle in *Crithidia fasciculata*, an insect parasite whose kDNA network structure has been studied in the most detail and closely resembles that of kDNA networks from human parasites, minicircles are singly linked to three other minicircles (i.e. they have mean valence equal to three) ([8], Figure 1A). The mean minicircle valence increases from three to six in kDNA

S-phase returning to three prior to segregation of daughter networks [9, 10].

In our earlier work we hypothesized that volume confinement is the main contributing factor to the formation of the kDNA network. To test this hypothesis, we introduced a mathematical model in which kDNA minicircles were modeled as randomly oriented geometric circles whose centers were placed on a planar square lattice grid (Figure 1B). Our modeling showed, in agreement with experimental observations, that a network containing nearly all minicircles is the most likely conformation at high minicircle density and that the mean minicircle valence increases linearly with minicircle density [11, 12].

In this work, we first show how the predicted mean valence at the observed *in vivo* minicircle density is two orders of magnitude greater than that experimentally observed in kDNA. This surprisingly large discrepancy between the predicted and observed mean valences suggests that biophysical factors other than confinement contribute to the topological properties of the network. Type II topoisomerases (Topo II) actively simplify the topology of DNA [13] even under crowded cellular conditions and could maintain a lower mean valence. However, this biological factor seems unlikely since genetic depletion of the mitochondrial Topo II in *T. brucei* results in networks with simplified topologies [14, 15]. We therefore hypothesize that other biophysical factors, inherent to the minicircle network, may play an important role in creating a network topology simpler than expected. To test this hypothesis we provide a complete quantitative characterization of the effects induced by minicircle position, bending, electrostatic repulsion and orientation on the topological properties of the kDNA network. These factors are of immediate interest for understanding the formation of the kDNA network and have been qualitatively discussed in the literature [6, 7, 9, 10, 16, 17] but never quantitatively studied. Our results show that, while all these factors are consistent with the observed experimental data, only minicircle orientation contributes significantly to the reduction of the predicted mean valence. Our findings are consistent with Delain and Riou's experimental observations and proposed three dimensional kDNA model [16]. In their model, minicircles are arranged almost perpendicular to the circular base of the kinetoplast disk (also known as the horizontal plane of the condensed kDNA disk). We propose that this arrangement of minicircles is the evolutionary solution in Kinetoplastids to the reduction of topological complexity induced by DNA condensation.

2 Data, Assumptions and Modeling Methods

2.1 Physical characteristics of *C. fasciculata* kDNA networks

The mathematical models in this study are based on previously published data of the *C. fasciculata* kDNA network (see [6, 7] for reviews). In *C. fasciculata* about 5,000 2.5 kb relaxed minicircles are confined into the kinetoplast

disk, a cylindrical structure .4 μm in height and 1 μm in diameter. Minicircles are organized into a planar topological network in which every pair of neighboring minicircles is singly linked by a Hopf link (see Figure 1A, [8]). In a non-replicating network, each minicircle is linked to an average of three other minicircles. The area of the horizontal plane of the condensed *in vivo* kDNA disk can be expressed in terms of the minicircle radius, and its value is about $47.94r^2$, where $r = .128\mu$m is the radius of a minicircle. Thus the density of minicircles in the kDNA network of *C. fasciculata* is about 104.3 minicircles per squared minicircle radius.
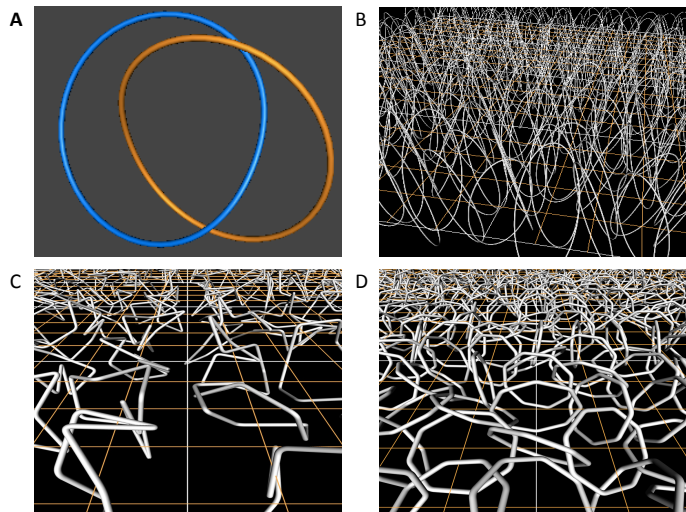

Figure 1: Hopf Links and Models proposed to analyze the topological properties of the minicircle network. A: A Hopf link; B: A grid of minicircles whose orientation has been biased. Volume effects are not considered and minicircle thickness is shown to help trace the trajectory of each minicircle; C: A grid of minicircles represented by freely jointed closed chains to study the effects of DNA bending; D: A grid of minicircles, represented by octagonal polygons, to study the effects of volume exclusion due to electrostatic interactions.

2.2   Biological and Mathematical Assumptions

The models described below are based on the following assumptions:

[**Biological assumption**] The centers of the kDNA minicircles are distributed in the horizontal plane of the kDNA disk. This assumption is based on two experimental observations. First, the height of the kinetoplast disk

is about half the length of the minicircles [7, 16, 18] and second, kDNA networks extracted from *C. fasciculata* are planar instead of three dimensional [8].

[**Mathematical assumption 1**] Minicircles have their centers at the vertices of the regular square lattice. This simplification is supported by the authors' previous work [11, 19] that shows that the topological properties of the network do not change significantly when minicircles are placed on triangular or hexagonal lattices or even when they are randomly displaced from these lattices. We refer to such a lattice region with the minicircles placed in it as *minicircle grid* (See Figures 1B, 1C and 1D).

[**Mathematical assumption 2**] Minicircles are represented by geometric flat circles (Figure1B) or by flexible minicircles (Figure 1C). The use of rigid flat circles is justified by three observations: first kDNA minicircles are relaxed and not supercoiled [8], second they are linked by a single Hopf link (Figure 1A), the only possible link between two geometrical minicircles and third the linking probability of two flexible chains with a given radius of gyration can be seen as the linking probability of two rigid circles whose radius is the same value as the radius of gyration of the flexible chains with a noise component. We used freely jointed minicircles, instead of the more commonly used wormlike chain model [32], because the statistical properties of these protein-bound molecules may significantly deviate from those of the wormlike chain (Figure 1D) and because the bending of the wormlike chain [33] falls in between the freely jointed minicircles and the rigid minicircles even if localized sequence induced bending were to be introduced.

2.3 Quantitative description of minicircle networks

The *density of minicircles* is defined as the number of minicircles per squared radius of the minicircle (namely its length divided by 2π) and the density of minicircles is simply the reciprocal of this distance squared. A minicircle grid in which most minicircles are linked forming a topological network is called a *saturation network*, and the average density at which the saturation network forms is called *mean saturation density*. In our studies we measured saturation as a percentage to avoid artificially high saturation densities caused by minicircles along the grid boundary that have a smaller probability of linking than do minicircles within the grid. In this a network becomes saturated when 99% of the minicircles are linked together. The *mean valence of a minicircle* is the average valence value of a minicircle over the entire ensemble of possible minicircle grids. In all the calculations described below, unless stated otherwise, the mean saturation density was estimated by sampling $10,000$ networks, and valence calculations for each density were performed by estimating the average number of minicircles linked to a non-boundary, randomly selected minicircle over $1,000$ samples of 7×7 minicircle grids.

2.4 Modeling minicircle orientation

Methods for modeling orientation have been previously described [20]. The orientation of any minicircle is given by the orientation of the vector perpendicular to the plane containing the minicircle, which in turn is given by the the tilting and the azymuthal angles. In [20] we showed, through computer simulations, that restrictions on the tilting angle (1) can increase the density of minicircles while keeping the topology of the network simple and (2) preserve the linear relationship between density and mean valence. The azymuthal angle, on the other hand, showed highly nonlinear responses and was not considered biologically relevant. In this paper, we used the methods in [20] to estimate the effects of tilting angles that are consistent with experimental data.

2.5 Modeling the effects of DNA bending

We used freely-jointed minicircles to study the role of DNA bending (Figure 1C). This minicircle model reflects the effects of DNA sequence and/or histone-like proteins that condense DNA molecules hence reducing their overall radius of gyration and linking probability (Figure 1C). Methods have been reported elsewhere and will only be briefly reviewed here [21]. To generate grids of freely-jointed minicircles, we sampled minicircle conformations using the generalized hedgehog algorithm [22, 23], positioned them at the vertices of the lattice, and computed the linking number between pairs of neighboring minicircles following the Gaussian integral formulation [24]. In this study minicircles were represented by freely-jointed fragments. When modeling naked double stranded DNA in free solution one uses the value of $300bp$ per fragment (i.e. one Kuhn length). In this study we overemphasized the effects of chain bending by representing minicircles with twice the number of Kuhn lengths of the minicircle. Saturation densities were estimated on grids of size 100×100.

2.6 Modeling the effects of volume exclusion due to electrostatic repulsion of DNA chains

Minicircles with electrostatic volume exclusion were modeled by segmented polygons that traced the contour of the rigid minicircle (Figure 1D) or by freely jointed circular chains (Figure 1C); volume exclusion was modeled by an impenetrable cylinder around each segment of the polygon [25]. Two parameters defined these models: (1) the number of segments used to represent the DNA minicircle; and (2) the radius of the cylinder that defines the excluded volume (denoted by τ). These values were normalized by the length of the minicircle in order to compare them with experimental data.

We developed new algorithms to generate grids of minicircles with volume exclusion due to two important factors. First, the linking probability of two minicircles is not independent of whether other neighboring minicircles are topologically linked or not. This dependency is negligible in systems with no volume

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exclusion but it plays a key role otherwise. Second, previously defined algorithms mostly produce non-admissible conformations, due to the overlapping of minicircles. This algorithm, whose details are reported in a separate paper [26], had two stages. In the first stage we estimated the linking probability between two minicircles in small (10×10) grids and in the second stage we used the estimated linking probabilities and a 'coin-tossing' argument to generate large (1000×1000) grids.

Large samples of small minicircle grids were generated using a simulated annealing algorithm, since even for this small grids, methods used in our previous studies [11, 20] produced mostly unacceptable conformations. This algorithm starts with a minicircle grid, which may or may not be acceptable. The grid is assigned an energy penalty, given by the number of intersecting minicircles. If the initial energy is positive, a fixed number of minicircles are randomly selected and their orientations are randomized uniformly over all possible directions. The new configuration is accepted or rejected according to the standard Monte-Carlo criterion. This process terminates when a zero energy state is reached (meaning that an acceptable minicircle grid has been found), or when a pre-determined number of steps, between 300 and 1,000 (a value dependent on the density of minicircles), had occurred before reaching a lower energy state. The output of this algorithm is a large set of tables that contain the linking probability of two neighboring minicircles in the grid together with the topological state of other neighboring minicircles.

Next, we generated large grids that did not explicitly represent the minicircles but just their positions on the grid. To decide whether two neighboring minicircles in the large grid were topologically linked or not we first determined the topology of the nearby minicircles and looked for the table (produced in the first stage of the algorithm) that had the same combination of linked/unlinked neighboring minicircles. Once the table was identified the linking status of the two minicircles under consideration was decided using a 'biased coin tossing' algorithm with the linking probability given by the probability estimated in the first stage of the algorithm and given by the table.

3   Numerical Results

3.1   Mathematical modeling revealed a discrepancy between predicted and observed minicircle density and mean minicircle valences

In our previous work we rigorously showed that, for any fixed minicircle grid size, a saturation network will form with a probability rapidly approaching 1 as the minicircle density increases [12]. Numerical results from these studies estimated that the mean saturation density is $D_{Sat} \approx 1.37$ and its corresponding mean valence ≈ 4.84. The estimated D_{Sat} is therefore much smaller than the density of the kDNA network (≈ 104.3) and we can safely conclude, in agree-

ment with experimental observations, that the kDNA network in *C. fasciculata* is saturated. There is however a significant discrepancy between the experimentally observed and the expected mean valences: at the density of ≈ 104.3, we predict a mean valence close to 400, while the experimentally determined valence in kDNA is only at 3 [10]. The observed differences are robust with respect to the relative position of minicircles in the grid [11, 19] thus validating our hypothesis and our use of the square lattice in this and other studies (Mathematical Assumption 1).

3.2 Neither DNA bending nor volume exclusion due to electrostatic interactions can account for the discrepancy between observed and predicted mean valences

As discussed in the methods section, the model that evaluates the effects of volume exclusion due to DNA electrostatics, has two parameters: (1) the number of segments representing the minicircle; and (2) the radius of the cylinder that defines the excluded volume. In [26] we concluded that (1) had negligible effects on the topology of the network and in this study we used a fixed value of 22 segments. The effects of (2) were estimated and revealed that saturation density increases linearly with the value of τ according to $D_{sat}^{\tau} = 1.3511 + 8.2905\tau$, a value that is consistent with the calculated value for rigid minicircles for $\tau = 0$ ($D_{sat}^{0} \approx 1.354$) [26]. Two values of the parameter τ were considered of biological relevance: $\tau \approx .008$ and $\tau \approx .02$. The value $\tau \approx .008$ corresponds to 2nm and it is the geometric diameter of the DNA double helix. The value $\tau \approx .02$ corresponds to 5nm and it is the effective diameter of the DNA double helix under physiological conditions (*i.e.* NaCl concentration of 0.15M) [25].
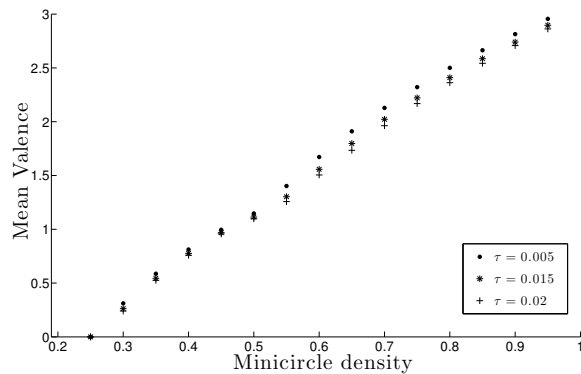

Figure 2: Estimation of the mean minicircle valence as a function of the minicircle density for biologically significant radii. The sample size for each data point is 10^5 and the sizes of the error bars are less than the sizes of the plotted data points.

Figure 2 shows the relationship between minicircle density and mean valence for different excluded volumes. The Figure clearly shows two important phenomena: first, there is a linear relationship between mean valence and minicircle density for each fixed τ value; second increasing minicircle volume exclusion reduces the value of the mean valence. The linear relationship between mean valence and minicircle density result is in good agreement with our previous studies [11, 12, 20] and therefore help validate the results presented here. The inverse relation between volume exclusion and mean valence supports the hypothesis that volume exclusion has a simplifying effect on the topology of the network [9]; interestingly both phenomena are conserved for moderate increases of the parameter τ [26] hence we conclude that our results do not change even if the salt conditions are somewhat different from those discussed above. Based on these results we argue that the topological simplification is very small and conclude that volume exclusion does not have leading role on determining the topology of the network.

We found significant differences when we took DNA bending into account. It is well known that factors such as stretches of specific DNA sequences [27] and DNA-protein interactions [28, 29, 30] can alter the overall shape of minicircles. To study DNA bending we used the freely-jointed model [31]. As described in the methods section we represented each minicircle by a freely jointed polygonal chain with 16 edges. Using this model we estimated a mean saturation density of $3.04\pm.06$ [21], a value not much larger than that observed for rigid mincircles. The mean valence at this density was ≈ 80 a significant decrease from 104.3 but not large enough to reach the experimentally observed values.

To test whether a combination of DNA bending and volume exclusion could have a significant effect on the mean valence of the network we added volume to the freely jointed minicircles. Instead of generating an entire network of minicircles, as in the previous studies, we compared the linking probabilities of two rigid minicircles and of two flexible minicircles with excluded volume. The radius of the rigid minicircles was equal to the radius of gyration of the flexile minicircles. We argue that if the linking probabilities between the two are similar then the induced network structure will not be too different. Results of these calculations are shown in Figure 3. The column on the left shows the comparison between the linking probabilities of the two models for different excluded volumes ($\tau = 0.01, 0.03$ and 0.05) and different bending rigidities ($seg = 6, ..., 20$); the column on the right shows a magnification of the graph on the left for those linking probabilities for which the network forms. As expected the geometric minicircle (x-axis) reaches a linking probability equal to one faster than flexible minicircles, however there is a strong linear relationship for most of the compared values (left column) which is robust with respect to the bending rigidity of the minicircle. These results therefore suggest that adding volume exclusion to freely jointed minicircles simplifies the topology of the network but, as argued above, the effects are not strong enough to significantly decrease the value of the mean valence from 80 to near 3.
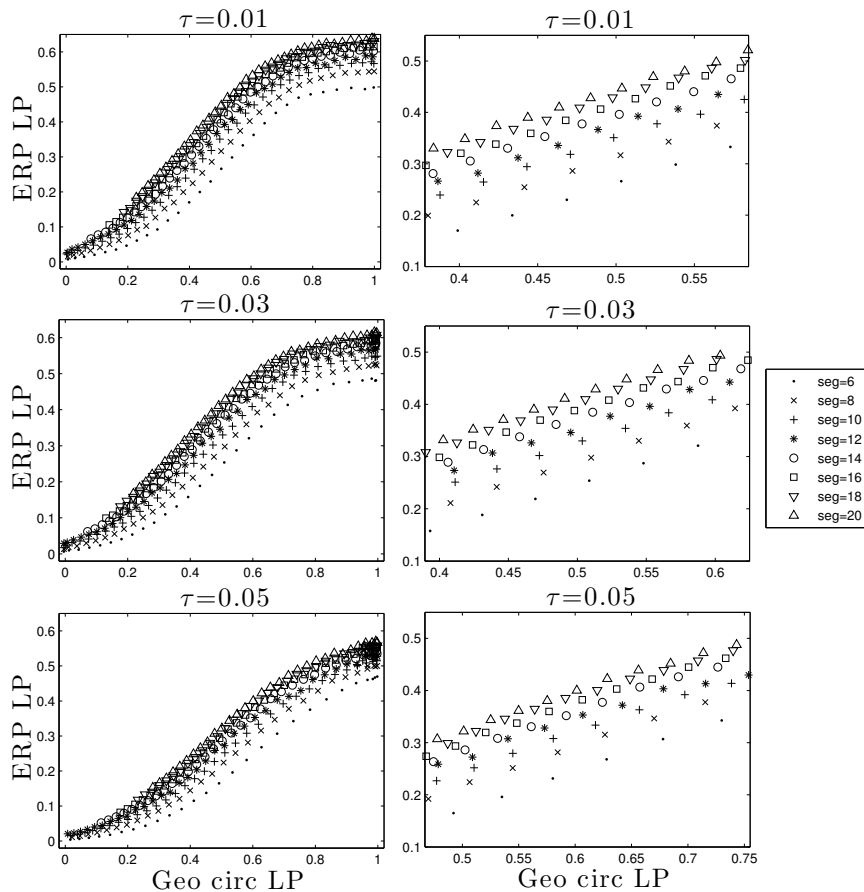
Figure 3: Relationship between the linking probability (LP) of rigid (Geo Circ) and freely jointed minicircles (ERP) with volume exclusion.

3.3 The relative orientation of minicircles significantly decreases mean valence and explains most of the discrepancy between observations and predictions

Experimental studies show that the height of the kinetoplast disk is roughly half of the length of the minicircle length and that minicircles are aligned almost perpendicular to the horizontal plane of the kDNA disk [16, 17]. In a previous study we characterized the effect of minicircle orientation on the topological properties of minicircle grids [20]. We therefore extended our previous study by sampling tilting values near 90 degrees. Clearly the tilting angle cannot be exactly 90 degrees because minicircles cannot be linked in this situation even if the azimuthal angle has no restrictions. Numerical estimations of the

mean saturation densities, as well as the corresponding mean valences at these densities, are plotted in Figure 4. The angle that best represents the observed experimental data was 88 degrees. At this angle, the mean saturation density is 94.74 with a mean valence of 11.17. These values are within the same order of magnitude of the minicircle density and mean valence observed experimentally in a kDNA network and less than two fold of the replicated network.
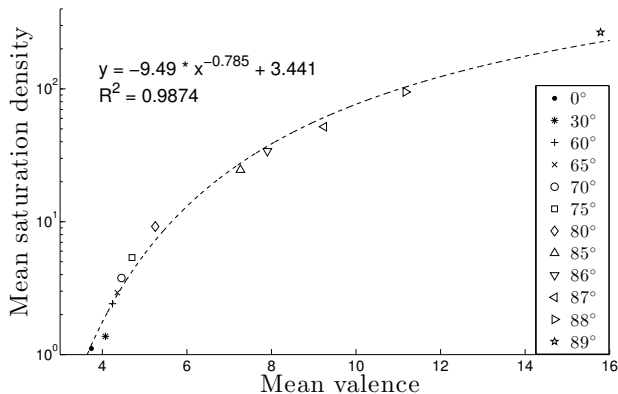

Figure 4: Estimated average saturation density for various restriction angles. Each data point in the figures is based on samples of sample size 1000 and minicircle grids of dimension 1000×1000. The 95% standard error bars are less than .0005 in all cases.

4 Conclusion

Mitochondrial DNA from most organisms in the group of Kinetoplastida is composed of numerous minicircles and maxicircles. Interestingly, only the parasites in the group contain a large network of topologically linked molecules [34]. The establishment of the kDNA network has been a topic of interest and various hypotheses have been proposed with confinement being the prevailing one of biophysical nature (see [6, 7, 34] for reviews). Although this hypothesis is supported by indirect experimental results that show that the topological complexity of single and multiple circular chains increases in the presence of condensing agents [35, 36, 37, 38, 39, 40, 41] it remains to be determined how to directly test this hypothesis *in vivo* since disruption of the kDNA structure has been shown to be severely detrimental for the cell (reviewed in [6]).

In our previous work [12] we used mathematical modeling of the *C. fasciculata* kDNA network to test for confinement. Our results, in agreement with experimental data, supported the confinement hypothesis and showed that a network is the most likely conformation when minicircles are in a confined

volume and that in confined conditions the mean minicircle valence increases linearly with density. This second observation is particularly relevant since it is in close agreement with the observed changes in the topology of the kDNA network during the cell cycle [9]. Our mathematical modeling approach also estimated, for the first time, the variance of the mean valence and predicted a critical density threshold in the evolution of kDNA in which the network first formed (i.e. a critical percolation density) and the topology became an essential component of the kinetoplast DNA .

One would intuitively predict that the topology of the network is very complex since 5,000 minicircles of 0.5 μm in diameter are condensed into a horizontal disk that is just 1 μm in diameter. This intuitive argument is at odds with experimental results that show that the mean minicircle valence in kDNA is only three. How can the topology of the network remain so simple given the high density of minicircles? In this study we tested the most plausible biophysical factors that may contribute to such phenomenon. We first tested those factors that have been previously proposed in the most recent literature [10]: DNA bending induced by DNA sequence or DNA binding proteins and volume exclusion induced by electrostatics. Our results show that neither factor can account for the observed mean valence *in vivo*. On the other hand we found that the proposed 3D kDNA model by Delain and Riou [16], in which DNA filaments appear to be preferentially oriented to the height of the disk, can bring the value of the mean valence closer the value observed experimentally. We therefore propose that the topological simplicity of minicircle networks is mostly controlled by the stacking of minicircles and modulated to a lesser extent by DNA bending and volume exclusion among other factors.

One may wonder what are the mechanisms keeping minicircles in such position. Experimental evidence suggest that the Tripartite Attachment Complex, a set of filaments that connect the flagellar basal body to the kDNA, may have an important role on this positioning and in preserving a simplified topology. In fact, mutations on members of these complex [43, 44] show enlarged and potentially more complicated networks. Other proteins, including histone-like or DNA binding proteins [45, 46, 47, 48], may also be involved in the positioning of minicircles since their mutations disrupt the topological features of the network, driving it into a more complicated topological state. Finally it is also possible that the position of some minicircles is not directly determined by a protein complex but instead by some neighboring minicircles, since it is well known that at high concentrations DNA molecules align parallel to each other (e.g., [49, 50]).

Our proposed arrangement of minicircles, with a tilting angle of about 88 degrees with respect to the horizontal plane of the condensed kDNA disk, produces a network that is consistent with the available experimental data. We however believe that there is still room for improvement, for instance our results above show a mean valence that is very close to that observed in nature (in particular it is less that 2 fold the value of the replicated network) but still a little

larger than the value observed in the pre-replicated network. This suggests that further experimental and computational studies need to be performed to accurately quantify the relative contribution of DNA bending and volume exclusion in the reduction of topological complexity in kDNA since multiple combinations of the two could reach the observed mean valence.

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