

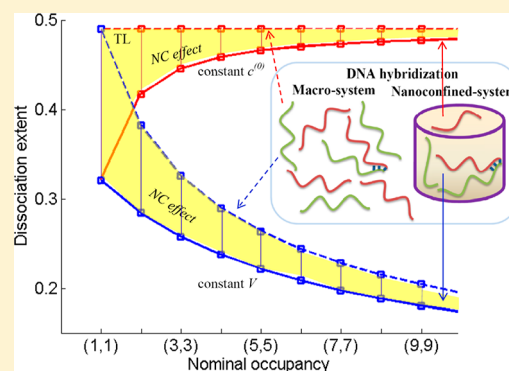
# The Intrinsic Role of Nanoconfinement in Chemical Equilibrium: Evidence from DNA Hybridization

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**ABSTRACT:** Recently we predicted that when a reaction involving a small number of molecules occurs in a nanometric-scale domain entirely segregated from the surrounding media, the nanoconfinement can shift the position of equilibrium toward products via reactant-product reduced mixing. In this Letter, we demonstrate how most-recently reported single-molecule fluorescence measurements of partial hybridization of ssDNA confined within nanofabricated chambers provide the first experimental confirmation of this entropic nanoconfinement effect. Thus, focusing separately on each occupancy-specific equilibrium constant, quantitatively reveals extra stabilization of the product upon decreasing the chamber occupancy or size. Namely, the DNA hybridization under nanoconfined conditions is significantly favored over the identical reaction occurring in bulk media with the same reactant concentrations. This effect, now directly verified for DNA, can be relevant to actual biological processes, as well as to diverse reactions occurring within molecular capsules, nanotubes, and other functional nanospaces.

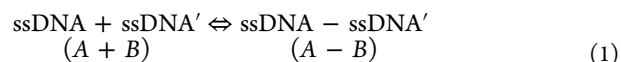
**KEYWORDS:** Nanochemical equilibrium, nanoconfinement, equilibrium constant, mixing entropy, DNA hybridization



Reactions between few molecules confined to a nanospace (molecular capsules, nanotubes, bioreactors, etc.) were found to behave differently compared to macroscopic systems.<sup>1–6</sup> Usually the reactants exhibited enhanced reactivity that was attributed to geometrical constraints preorganizing the molecules, to preferential guest–host interactions, or to increased effective concentrations. On the other hand, remarkable nanoconfinement entropic effects on chemical equilibrium (NCECE) were predicted by us recently using basic statistical mechanics.<sup>1</sup> In particular, extra stabilization of exothermic reaction products is anticipated when a small number of reaction mixture molecules in a nanometric-scale domain is entirely segregated from the surrounding media. The effects are reflected in equilibrium constants and reaction extents larger than the corresponding thermodynamics limit (TL) values. They are related solely to the smallness and confinement of the system, and originate from the inherent reduction in the number of reactant-product mixed microstates.<sup>1</sup> In other words, the entropy of mixing is smaller in such a system and can even vanish in the case of a reaction involving two reactant molecules. In a second paper, it was shown that in addition to this role of the nanosystem size, the magnitude of the product stabilizing NCECE effect can depend on the nanospace size and on deviations from stoichiometry.<sup>7</sup> Furthermore, possible manifestations of the NCECE effect in two widely diverse types of reaction were introduced. Specifically, we demonstrated that the NCECE can stabilize nucleotide dimerization observed within self-assembled molecular cages. Likewise, the effect seems to be pertinent to a longstanding issue in astrochemistry, namely the extra deuteration commonly observed for molecules reacting on

interstellar dust grain surfaces. However, only general trends could be evaluated in that work<sup>7</sup> since quantitative equilibrium data were lacking.

The stringent requirements for quantitative confirmation of the NCECE, namely, monitoring of a very small reaction mixture well-sealed in a nonreactive nanospace, were met in a remarkable single-molecule level experiment reported recently by Shon and Cohen.<sup>8</sup> Thus, a special “Dimple Machine” comprising hundreds of nanofabricated chambers (having 150, 250, 450, and 550 nm radii and ~200 nm depth) was developed, facilitating many chamber trap-measure-refresh cycles in order to obtain good statistics. Two types of 30-mer noncomplementary single-stranded DNA (ssDNA) oligonucleotides labeled with fluorescent dyes were confined together in each chamber and reacted via few hydrogen bonds to form partially hybridized DNA until equilibrium was achieved. The hybridization/dissociation (denaturation, “melting”) reaction



was monitored by colocalization and by fluorescence resonance energy transfer (FRET).<sup>8</sup>

The experiments yielded RT equilibrium probability distributions,  $P_{\text{exp}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B})$ , of finding  $N_{A-B}$  dimers in closed chambers having nominal (low) occupancies ( $N_A^{(0)}, N_B^{(0)}$ ) and volume  $V$ . The data analysis<sup>8</sup> based on the chamber-size

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specific dimer probability distributions, provided similar estimations for the equilibrium constant, and could not unravel the NCECE effect. On the other hand, the present test case uses a chamber-occupancy specific analysis of the experimental measurements, which consistently reflects this effect and provides a quantitative verification of the theoretical predictions regarding the role of nanoconfinement vs the TL macrosystem situation. Thus, starting with  $P_{\text{exp}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B})$  data, the average numbers of product and reactant molecules read

$$\left. \begin{aligned} \overline{N_{A-B}} &= \sum_{N_{A-B}} N_{A-B} P_{\text{exp}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B}) \\ \overline{N_A} &= N_A^{(0)} - \overline{N_{A-B}} \\ \overline{N_B} &= N_B^{(0)} - \overline{N_{A-B}} \end{aligned} \right\} \quad (2)$$

These averages form the basis for obtaining the equilibrium constants and extents for each nanoconfined occupancy separately.

**DNA Dissociation Constants.** For comparison with the experimental TL equilibrium dissociation constant,<sup>8</sup>  $K_d^{\text{TL}}$ , we calculated the corresponding dissociation equilibrium constant for each nominal occupancy ( $N_A^{(0)}, N_B^{(0)}$ ) and chamber volume  $V$

$$K_d = \frac{\overline{N_A} \overline{N_B}}{\overline{N_{A-B}}} \frac{1}{N_{A-V}} \quad (3)$$

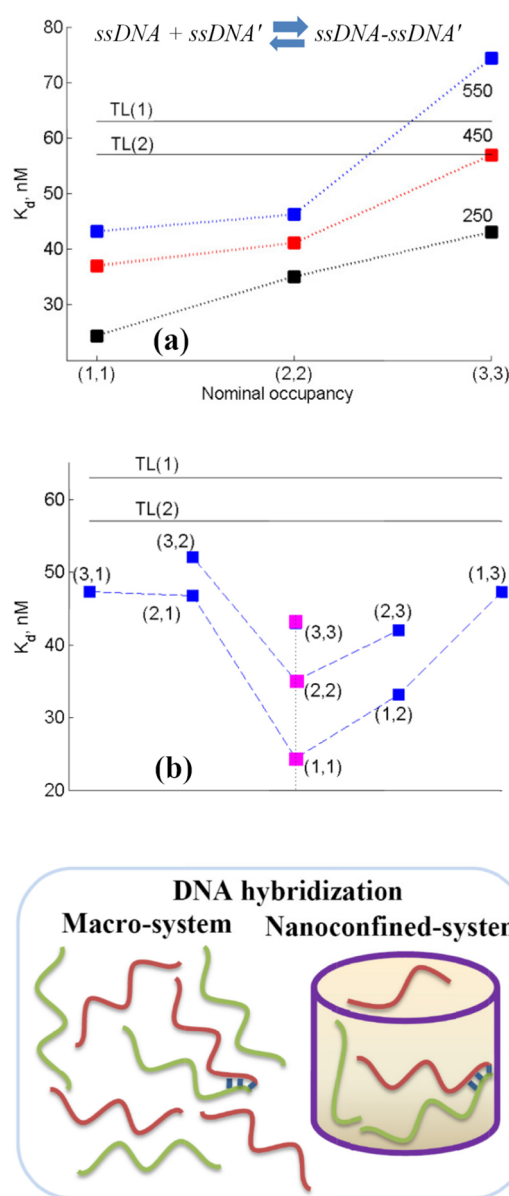
( $N_{A-V}$ , Avogadro number;  $(\overline{N_{A,B,A-B}})/(N_{A-V})$ , molarities at equilibrium;  $K_d = 1/K_{\text{hybrid}}$ ).

Substituting the measured  $P_{\text{exp}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B})$  in eqs 2 and 3 reveals that the equilibrium “constant” is actually not constant, but depends on the nanosystem (and nanospace) size, and is consistently lower than the TL value ( $K_d^{N_A^{(0)}, N_B^{(0)}, V} < K_d^{\text{TL}}$ , Figure 1). That is, the NCECE effect indeed hinders the dissociation (except for one value within the uncertainties in  $K_d^{N_A^{(0)}, N_B^{(0)}, V}$  and  $K_d^{\text{TL}}$ <sup>8</sup>). In particular, the following distinct NCECE fingerprints verify the predictions:

1. As the nanosystem size gets smaller the DNA hybridization is enhanced;
2. Decreased nanospace size also leads to hybridization enhancement;
3. Increasing deviations from stoichiometry (by extra A or B oligonucleotides) gradually restore the thermodynamic limit value.

The entropic origin of the effect for small numbers of confined oligonucleotides, which will be discussed in more detail below, is related to their reduced molecular mixing with the dimerized product (e.g., in the case of  $N_A^{(0)} = N_B^{(0)} = 1$  either  $A + B$  or  $A - B$  can be present in a chamber, but not concurrently). Since increased entropy of mixing is closely associated with dissociation,  $K_d^{N_A^{(0)}, N_B^{(0)}, V}$  increases with the nanosystem size for each chamber size (Figure 1a). The second fingerprint can be accounted for by more dominating volume-related entropy in larger nanospaces, leading to decreased relative contribution of the NCECE. Similarly, the approach to the TL due to off-stoichiometry (point 3) is related to more possibilities of mixing by the extra A or B molecules.

**Dissociation Extents.** In the case of stoichiometric dissociation reaction this parameter,  $\xi_d \equiv \overline{N_A}/N_A^{(0)}$ , is simply related to the equilibrium constant,

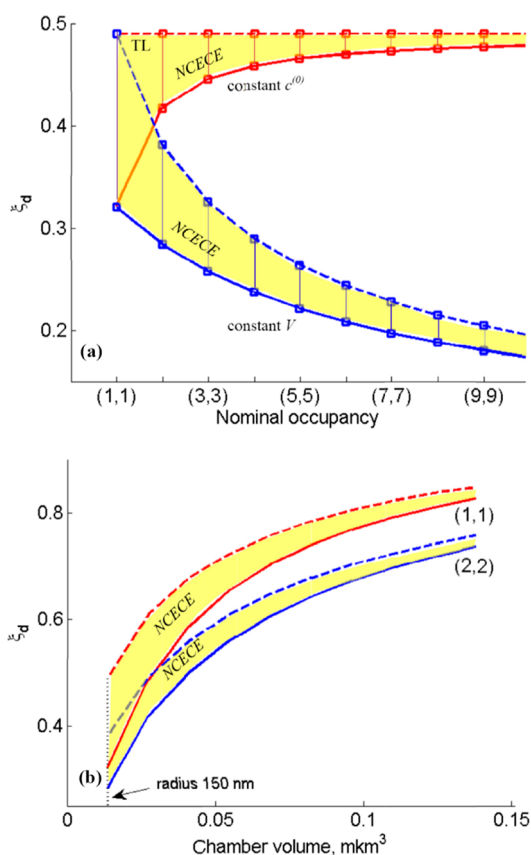


**Figure 1.** Quantitative verification of the NCECE effect: equilibrium dissociation “constants” obtained by a new analysis of measured dimer probability distributions (taken from Figure 4f–i in ref 8). (a) The effects of the nanosystem size occupancy and the nanospace size (radius in nm marked on the right) in the case of stoichiometric reaction mixtures. (b) The NCECE off-stoichiometric effects in 250 nm chambers. Nominal occupancies are indicated. The two experimental values of  $K_d^{\text{TL}}$  are taken from Table 1 in ref 8. The schematics (bottom) illustrate the DNA extra-hybridization under nanoconfinement.

$$K_d = \frac{\xi_d^2}{(1 - \xi_d)} c^{(0)} \quad (4)$$

with  $c^{(0)} = N_{A,B}^{(0)}/N_{A-V}$  denoting the nominal molarity of each of the two oligonucleotides. The reaction extent is more transparent compared to the equilibrium constant with regard to the progress of the equilibrated reaction, including the  $A - B$  melting-temperature (at  $\xi_d = 0.5$ ). However, its explicit, direct dependence on  $c^{(0)}$  (eq 4) should be separated in small systems from possible NCECE effects (via  $K_d(c^{(0)})$ ), in contrast to the TL case. The extent dependence on the chamber occupancy

(or  $c^{(0)}$ ) and size was computed using a theoretical expression for the dimer probability distribution,  $P_{\text{theor}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B})$ , introduced before,<sup>8</sup> thus enabling us to go beyond the limited experimental data. As can be seen from Figure 2a, decreasing



**Figure 2.** (a) The dependence of the dissociation extent on the nanospace nominal occupancy: solid blue line, constant  $V$ , chamber radius 150 nm; solid red line, the chamber volume is set to increase proportionally to the occupancy (constant  $c^{(0)}$ ); dashed lines correspond to the TL with the same molarities  $c^{(0)}$  as in the nanosystems. (b) The dependence of the dissociation extent on the chamber volume (solid lines; TL, dashed lines). Note: in small volumes toward 150 nm radius,  $\xi_d(1,1) < \xi_d^{\text{TL}}(2,2)$  due to dominance of the NCECE effect. In all cases, the gap between dashed and solid lines quantifies pure NCECE effects on the extent, namely, hybridization under nanoconfined conditions is significantly favored over the identical reaction occurring in bulk media with the same reactant concentrations.

the size of a stoichiometric nanosystem in a fixed-size chamber (constant  $V$  line) results in increase of the dissociation extent both for the nanosystem and the TL, in accordance with the expected direct concentration effect. However, the remarkable gaps between the two lines, especially for low occupancies, signify the role of the NCECE in diminishing the dissociation extent (hybridization enhancement), namely acting in opposite direction to the concentration effect. The pure NCECE-induced reduction of  $\xi_d$  is quantified also by the gaps between the constant  $c^{(0)}$  lines as is shown in Figure 2a. On the other hand, if the nominal occupancy is fixed, the reduction in dissociation for smaller nanospaces (due to increased  $c^{(0)}$ ) is amplified by the NCECE effect (Figure 2b), that is, the NCECE and the concentration effects act in the same direction. Thus, our main conclusion regarding all above cases is that the

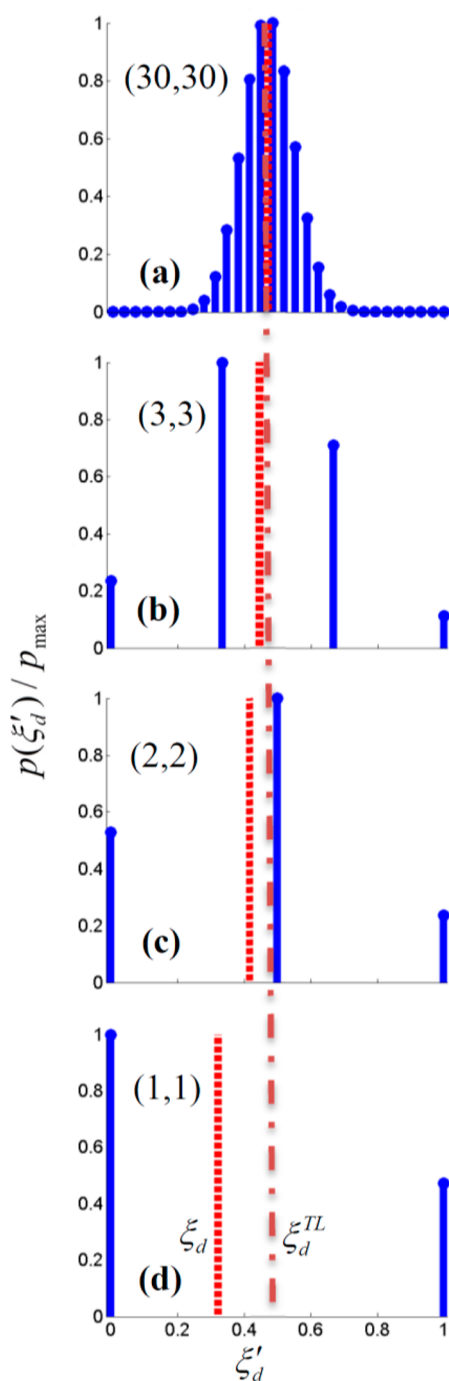
shift of equilibrium in favor of hybridization cannot be explained merely by the fact that the occupied chambers correspond to higher concentrations than the bulk value (this pure concentration effect alone was invoked before<sup>8</sup> to explain the shift). To illustrate further this point, extents are computed for equimolar macroscopic mixture of the two types of ssDNA oligonucleotides prepared at the nominal concentrations of 30 nM used in the experiments and for (1,1) occupancy 150 nm chambers (120 nM) with and without the NCECE effect. Thus, using in eq 4  $K_d^{\text{TL}} = 57$  nM (obtained as a fitted parameter<sup>8</sup>)  $\xi_d^{\text{TL}}$  is 0.72, while for the nanosystem  $\xi_d$  is reduced to 0.49 due to the concentration effect alone. The actual, NCECE-affected, reaction extent turns out to be significantly lower,  $\xi_d = 0.32$ , so that the melting temperature should be significantly higher than RT. This NCECE-induced DNA stabilization can play a role in biological processes like replication and transcription<sup>9,10</sup> if under proper equilibrium (or near equilibrium) nanoconfinement conditions.

The origin of the NCECE effect can be elucidated by inspecting the probability distribution of discrete levels,  $\xi'_d$ , of the stoichiometric hybridization reaction extent (Figure 3) obtained using  $P_{\text{theor}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B})$ . As the number of molecules in the constant- $c^{(0)}$  system decreases, the TL-like bell-shaped  $\xi'_d$  probability distribution of relatively large systems ((30,30) in Figure 3a) first broadens and becomes asymmetric together with increased fluctuations (Figure 3b–d). Moreover, the concomitant reduction in the number of reactant-product mixed  $\xi'_d$  levels for lower occupancy ( $N_A^{(0)}, N_B^{(0)}$ ) reveals the entropic origin of the NCECE effect. Thus, it promotes the shift of the reaction equilibrium toward hybridization (lower average extent,  $\xi_d$ , Figure 3b,c), until for (1,1), the reaction smallest system, reactant-product mixed levels are entirely absent and the dissociation is the smallest among the four cases (Figure 3d). Nevertheless, some dissociation persists ( $\xi_d \neq 0$ ) due to thermal fluctuations of the reaction extent, which are significant in nanosystems. On the other hand, if mixing entropy is not taken into account in a macroscopic system, the reaction would go to completion. Another distinction between nano and macro systems concerns the variations in  $\xi_d$  (Figure 3a–d) despite the constant concentration, apparently reflecting the nonextensivity of small systems<sup>11</sup> (see also the constant  $c^{(0)}$  lines in Figure 2a).

Beyond the above computations,  $P_{\text{theor}}^{N_A^{(0)}, N_B^{(0)}, V}(N_{A-B})$  can be used to explore the temperature dependence of NCECE both by deriving analytical expressions to gain more insight into nanoconfined phenomena, and numerically. First, substitution of  $P_{\text{theor}}^{1,1,V}(N_{A-B})$  in eqs 2 and 3 for  $N_A^{(0)} = N_B^{(0)} = 1$  gives

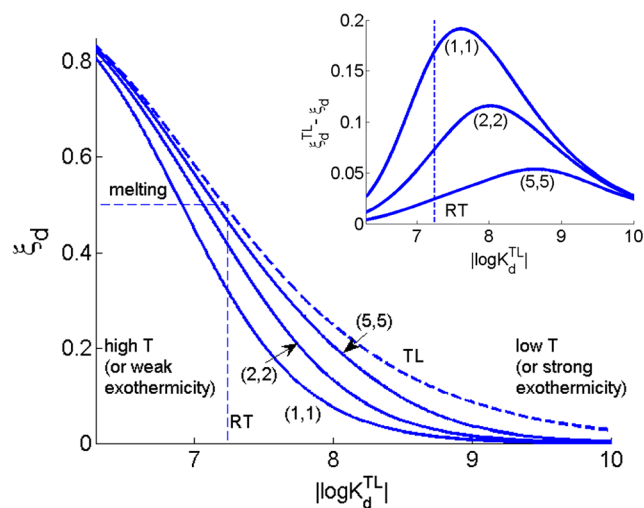
$$K_d^{1,1,V} = \frac{N_{\text{Av}} V}{N_{\text{Av}} V K_d^{\text{TL}} + 1} (K_d^{\text{TL}})^2 \quad (5)$$

Since at low temperatures the dissociation is nearly absent ( $K_d^{\text{TL}} \ll 1$ ), for small chambers  $K_d^{1,1,V} \sim (K_d^{\text{TL}})^2$ . Hence, a plot of  $\log K_d^{1,1,V}$  versus inverse temperature has a slope that is twice larger than the  $\log K_d^{\text{TL}}$  linear slope, in full accordance with our previous prediction made in the lattice-gas model framework.<sup>1</sup> In addition to this low-temperature-limit behavior, Figure 4 presents the variations in the average reaction extent with  $\log K_d^{\text{TL}}$ . As seen, the NCECE effect on  $\xi_d$  is significant for intermediate values, while in the case of weak dissociation (low  $T$  or strong  $A-B$  exothermicity) and especially upon nearly complete dissociation (high  $T$  or weak exothermicity) the effect is milder (smaller deviations from the TL line). In the RT



**Figure 3.** Elucidation of the NCECE origin in the case of DNA hybridization: the probability distribution function (at RT, scaled to the maximal value) of the reaction extent discrete levels ( $\xi'_d$ ) and its average value ( $\xi_d$ ) computed for decreasing nanosystem sizes,  $(N_A^{(0)}, N_B^{(0)})$ , corresponding to a chosen constant  $c^{(0)}$  (2 ssDNA per 150 nm chamber). Note: in the  $N_A^{(0)} = N_B^{(0)} = 1$  limiting case (d), the shift in  $\xi_d$  is maximal since mixed reactant–product extent levels are entirely absent.

experiment, the intermediate  $\xi_d$  values are due to the low reactant nominal concentrations used, and the computed maximal effect occurs at lower temperatures depending on the nominal occupancy (Figure 4, inset). It can be finally noted that in the case of reactions preserving the total number of molecules (treated by us in ref 1, e.g.,  $2AB \rightleftharpoons A_2 + B_2$ ) the



**Figure 4.** The dependence of the dissociation extent on  $\log K_d^{TL}$  ( $\sim 1/T$ ) computed for three nominal ssDNA occupancies and the thermodynamic limit. In the nanosystems, melting occurs at progressively higher temperatures compared to the macroscopic TL system, signifying the NCECE stabilizing effect. Inset: The corresponding reductions in the dissociation extents.

NCECE effect exhibits a similar temperature dependence, but is concentration and volume independent.

To conclude, the nanoconfinement entropic effect on chemical equilibrium predicted previously is verified for the first time by a new direct analysis of recently reported measurements concerning DNA hybridization inside confined nanofabricated chambers. The NCECE effect is found to consistently diminish the DNA dissociation constant and extent, that is, to induce its extra stabilization beyond common thermodynamic-limit concentration effects. This can have important biological implications, and in principal similar results are expected for other confined DNA as well as RNA molecules. The remarkable agreement between theoretically predicted tendencies and those measured experimentally confirms the universal nature of the NCECE effects, whose entropic origin is elucidated, and which should also play a role in nanometric-scale chemistry of diverse nonbiological confined reactions.

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### Notes

The authors declare no competing financial interest.

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