Measuring cohesion between macromolecular filaments, one pair at a time:
Depletion-induced microtubule binding

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In presence of non-adsorbing polymers, colloidal particles experience ubiquitous attractive interactions induced by the depletion mechanism. We measure the depletion interaction between a pair of microtubule filaments by a method that combines optical trapping, single molecule imaging and umbrella sampling. By quantifying the dependence of filament cohesion on both polymer concentration and solution ionic strength, we demonstrate that the minimal model of depletion based on the Asakura-Oosawa theory fails to describe the experimental data. By measuring the cohesion strength in two- and three- filament bundles we verify pairwise additivity of the depletion interaction for the specific experimental conditions. The described experimental technique can be used to measure pairwise interactions between various biological or synthetic filaments, thus complementing information extracted from bulk osmotic stress experiments.

Ranging from elastic nanopillar arrays [1] to ropes of carbon nanotubes [2] to dense chromatin structures [3], numerous materials of synthetic or biological origin are assembled from filamentous building blocks. The macroscopic properties of such filamentous materials are governed not only by the mechanical properties of the constituents, but also by the interactions between them. These interactions are traditionally measured using bulk osmotic stress experiments in which one applies an external pressure of known magnitude while simultaneously measuring the filament spacing using X-ray scattering [4–6]. Here we describe a complementary single-molecule technique that directly measures pairwise cohesive interactions between a diverse array of filamentous molecules and assemblages. This approach allows us to assemble bundles in a controlled fashion, with predetermined filament number and binding geometry, yielding information that cannot be accessed by bulk methods. It extends microscopy-based methods developed for measurement of interactions between isotropic, colloidal particles [7, 8] to the case of extreme particle anisotropy (e.g. macromolecular filaments).

We study cohesive interactions between microtubules (MTs), cytoskeletal filaments that are assembled from tubulin heterodimers to form rigid tubular structures with an outer diameter of 25 nm, a contour length that can reach tens of microns, and a persistence length of a few millimeters [9, 10]. MTs carry significant negative charge at physiological pH [11]. To assemble bundles, we place MT filaments in a suspension of non-adsorbing polymer, which induces attractive interactions by the depletion mechanism. As two MTs form a bundle, an additional free volume becomes available to polymer coils, thus increasing the overall system entropy and resulting in an effective attraction between the rods (Fig. 1(b)). Its strength and range can be tuned by changing the polymer concentration and size, respectively [12]. Bundle formation requires high ionic strength in order to screen the repulsive electrostatic interactions between the filaments [13].

Tubulin was purified and labeled with biotin and fluorescent dyes (Alexa 568 and Alexa 647) [14]. We used either Poly(ethylene glycol) (PEG, MW=20 kDa) or Dextran (MW=500 kDa) as a depletant. Radius of gyration ($R_g$) of 20k PEG is $\sim$ 7 nm [15]. Dextran 500k has $R_g \approx$ 18 nm [16]. The depleting polymers were in the dilute regime for all the measurements performed. The ionic strength varied between 75 and 650 mM by addition of potassium chloride.

We directly visualized formation of bundled MTs in the presence of the depleting agent (Fig. 1(a)). In the first, rate-limiting step, two diffusing MTs encountered each other and formed a bundle with a random overlap. Subsequently, the bundle maximized the MT overlap. To understand such a process, consider the free energy of a
bundle given by \( G = -\lambda L_0 \), where \( \lambda \) is the cohesive free energy per unit length and \( L_0 \) is the filament overlap length. The magnitude of the force that drives the retraction of filaments is given by \( f = -\partial G/\partial L_0 = \lambda \). Therefore, measuring the retraction force provides information about the attractive depletion interactions as characterized by cohesion energy per unit length, \( \lambda \).

The experimental system used to directly measure \( G \) and determine \( \lambda \) is illustrated in Fig. 2. Briefly, we prepared segmented MTs in which only a short portion (seed) is labeled with biotin. The biotinylated seeds and the biotin-free elongated segments of MTs were labeled with different fluorescent dyes [17]. The entire MTs were stabilized with GMPCPP, a non-hydrolysable analog of GTP, thus ensuring that there is no dynamic instability and monomer turnover. A pair of Neutavidin coated silica beads manipulated with optical traps was manually attached to biotin labeled MT seeds (Fig. 2(a, b)). Subsequently, the two MTs, each attached to a single bead, were brought into close proximity. We then either waited for fluctuations to induce the formation of a filament bundle or sped up the process by applying an external flow [18]. Once the MT bundle formed (Fig. 2(c)), we verified that the MTs overlapped only along the biotin-free elongated segments. This ensured that Neutavidin, which desorbs from beads, did not crosslink biotinylated monomers. Our experimental setup allowed for control of the MT overlap length with nanometer accuracy (Fig. 2(d)).

Before bundle formation, each bead with an attached MT fluctuated around the minimum of the harmonic potential imposed by the optical traps. Upon bundle formation, the average bead separation, \( R \), decreased due to the retraction force (Fig. 3(a)). While measurement of the reduction in average spacing for known trap stiffness directly yields the magnitude of the retraction force \( f = \lambda \), we improved statistics by incorporating the fluctuations around the equilibrium position. Specifically, we performed umbrella sampling to measure the free energy, \( G \), of the bundle as a function of \( R \) [19, 20]. As beads fluctuated in the optical traps [21], we measured their center-to-center separation \( R \) and constructed normalized probability distributions: \( P_{\text{bundle}}(R) \) and \( P_{\text{calibration}}(R) \) for the bundled and unbound configurations, respectively (Fig. 3(b)). Both distributions had Gaussian shapes whose width is determined by the stiffness of the traps. Presence of the bundle reduced the mean bead separation. Since the separation \( R \) and the overlap length \( L_0 \) are simply related [22], the bundle free energy as a function of the overlap length, \( L_0 \), is given, up to an additive constant, by:

\[
G(L_0) = -k_B T \log \left( \frac{P_{\text{bundle}}(L_0)}{P_{\text{calibration}}(L_0)} \right) + \text{const} \tag{1}
\]

We extracted \( G(L_0) \) by using the measured distributions in Eq. 1. The cohesion force per unit length is given by \( \lambda = -\partial G/\partial L_0 \), which was calculated from the slope of the weighted linear fit to the experimental points (Fig. 3(c)). For example, the probability distributions depicted in Fig. 3(b) yielded \( \lambda = 25 \pm 1 \, k_B T/\mu m \), which is equivalent to a retraction force of \( \sim 0.1 \, \text{pN} \). A single experiment samples only a small region of the bundle overlap (\( \approx 100 - 150 \, \text{nm} \)) that is accessible by thermal fluctuations. To extend this range, we manually changed the overlap length and repeated the measurement. We found \( \lambda \) to be independent of the MT bundle overlap, \( L_0 \), in agreement with our initial assumptions (Fig. 3(d)).

We measured how the cohesion strength depends on both the suspension ionic strength and depleting polymer concentration. For a fixed ionic strength, \( \lambda \) increased linearly with increasing polymer concentration. For a fixed polymer concentration, \( \lambda \) decreased with decreasing ionic strength (Fig. 4). These results can be qualitatively understood by considering that the cohesive potential between aligned MTs is governed by a combination of the attractive depletion interaction and electrostatic repulsion (Fig 5(a)). In the Asakura-Oosawa (AO) model, the depletion strength depends linearly on the depletant concentration, in agreement with the experimental findings. Upon decreasing ionic strength, the range and effective strength of the electrostatic repulsion increases, thus
leading to decreased cohesion strength as observed in the experiments. When the ionic strength is sufficiently low, the depletion attraction is insufficient to overcome repulsion between the negatively charged filaments, thus suppressing bundling.

To quantitatively assess the relationship between measured MT cohesion and physical and structural features of MTs, we developed two theoretical models. In the simplest primitive model, MTs were treated as uniformly charged, hollow cylinders of radius \( a = 12.5 \) nm with surface charge density \( \sigma_s = -0.23 \) e/\( \text{nm}^2 \). The depletion-induced attraction was computed by modeling polymer coils as effective hard spheres with radius \( R_b = 2R_d/\sqrt{\pi} \). The binding free energy predicted by the minimal-energy separation of the primitive model overestimates the measured cohesion strength by a factor of \( ~5 - 7 \), implying an overestimation of depletion attraction and/or an underestimation of repulsions. To explore the possibility that repulsive forces generated by negatively charged, flexible “bristle-like” c-terminal tails of tubulin contribute to this discrepancy, we developed a bristle-stabilized MT model, which includes additional salt-dependent electrostatic forces between c-terminal bristles anchored to MT surfaces. Here, we adopted the electrostatic brush model of Pincus et al.\cite{28} to calculate the dependence of bristle height, \( h \), on the ionic strength. Treating the bristles as 13-segment chains\cite{29} (segment size 0.5 nm) with charge \( -8e \) per bristle as an approximately flat brush of areal density of one bristle per tubulin monomer \( \sigma_B = 1/4^2 \text{nm}^{-2} \), we predicted bristle heights to vary from 2.6 nm to 4.8 nm over the range of salt concentrations measured, scaling roughly as \( h \sim [K^+]^{-1/3} \) in the high-salt limit (Fig. 5(a), inset)). We assumed uniformly stretched bristles with the bristle charge density at radial distance \( r \) from the MT center given by \( \rho_B(r) = Q_d \sigma_B/rh \). Electrostatic repulsion in the bristle-stabilized model was computed from the linearized-PB interaction between superposed bare surface charge of MTs and bristle charge density, \( \rho_f(r) = \sigma_s (r-a) + \rho_B(r) \), while depletion was treated as before, such that polymers were assumed to penetrate the bristles freely. These interactions increase filament separation by roughly twice the bristle height at the expense of decreasing depletion attractions. While calculated binding energies were closer to the experimental values, they still overestimated the measured \( \lambda \) by a factor of \( ~2-3 \) (Fig. 5(c)). Comparing the primitive and bristle-stabilized model predictions (Fig. 5(b)) suggests that the larger surface separation due to charged c-terminus bristles is fundamental to MT-MT interactions. However, in light of apparent overestimation of the polymer-induced depletion by the AO theory, a more detailed representation of polymer interactions with the MT geometry is needed to quantitatively recapitulate measured cohesion strengths.

Depletion interactions between spherical colloids and polymers are pairwise additive as long as the depleting
polymer is significantly smaller than the spherical colloid [30, 31]. To investigate the additivity of the depletion between rod-like particles, we characterized properties of multi-filament bundles. We induced the formation of a three-MT bundle by bringing a third filament near an existing two-MT bundle. Once adsorbed, the third MT always migrated to the existing two-filament overlap region, since such configurations minimize the depletant excluded volume (Fig. 6(a, b)). We found the cohesion strength $\lambda$ for the 3-MT bundles to be consistently twice the value measured for the 2-MT bundles (Fig. 6(c)). These experiments indicate that depletion interactions with a $\sim$7 nm range are still pairwise additive for the filaments with hardcore diameters of $\sim$25 nm. The electrostatic interactions decrease the range of the depletion attraction and thus likely further reduce the importance of many-body overlap configurations.

Polymer-induced depletion interactions with nanometer range have been extensively studied between micron-sized colloidal particles [8, 32, 33]. Here we have measured the depletion interactions in the protein limit [34, 35], in which the size of the non-adsorbing polymer ($R_g$) approaches the size of the colloidal object (MT filament diameter). We found significant discrepancies between our measurements and predictions of a simplified AO model. These were only partially reconciled by a more refined microscopic description of MT structure. To ultimately discern the source of this discrepancy, several other effects, beyond the scope of this manuscript, need to be investigated. For example, the AO model is only accurate when polymer coils are significantly smaller than colloidal particles [34]. Though the accuracy of the AO model has been studied for spherically symmetric particles, there are few equivalent studies for rod-like colloids [36]. Furthermore, bending degrees of freedom are severely constrained in a bundle, corresponding to an additional entropic cost of bundling for finite-persistence length filaments. This entropy loss was important for understanding depletion-induced bundling of actin filaments [37]; since the bending entropy loss scales inversely with the fourth root of the bending stiffness, it could yield significant corrections even for stiff MTs. Finally, we have treated the depletant as ideal non-adsorbing polymers; excluded volume interactions could affect the magnitude of the depletion attraction [38].

In conclusion, we have developed a technique to measure attractive depletion interactions between filamentous structures. These results should be relevant for biological or synthetic systems where depletion forces arising from globular polymers or other non-adsorbing particles play an important role [39, 40]. Furthermore, they are also important in materials science, where the depletion effect is frequently used to engineer interactions between colloids and to drive self-assembly of diverse structures [41–47]. Finally, the methods described here can easily be extended to other filamentous systems as well as alternate interactions such as counterion-induced bundling of polyelectrolyte filaments [48–52].

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