Summary of Research Activity
OTKA Grant Final report - NKFIH-ID 109480
Hiererchical mechanobiology of the muscle cytoskeleton
2018. 10. 02.

Summary

In the final year of the proposal much attention has been devoted to wrapping up several projects and publishing our results. Altogether, throughout this grant proposal quite significant discoveries have been made about the muscle cytoskeleton, particularly about the biophysical behavior of the giant protein titin. We discovered domains in the molecule that unfold at very low, physiologically quite relevant forces; a novel method of meniscus-force molecule-stretching allowed us to visualize the location of the domains unfolded by mechanical force; we discovered that titin is able to generate force by the folding of its domains; and we uncovered that titin is not a geometrical template of the thick filament but is likely to regulate thick-filament length by a dynamic mechanism. In additon to our focus strictly on sarcomeric proteins, we also explored addional, related nanoscale systems which allowed us to develop novel methodologies. Accordingly, we explored the behavior of nanoscale lipid systems (liposomes and cochleates), the elasticity and high-resolution structure of filamentous biomolecules (DNA, fibrin, amyloid fibrils), and the nanomechanical behavior of bacteriophages.

The sarcomeric cytoskeleton

The major scope of our research project was the filamentous biomolecular systems of ths striated-muscle sarcomere with an emphasis on the giant protein titin, its various domains, and its interaction with the motor protein myosin-2.

Low-force transitions in titin

Titin, a giant elastomeric muscle protein has been implicated to function as a sensor of sarcomeric stress and strain but with unresolved mechanisms. To gain insight into titin's mechanosensory function here we manipulated single molecules with high-resolution optical tweezers. Discrete, stepwise transitions, with rates faster than canonical Ig-domain unfolding occurred during stretch at forces as low as 5 pN. Multiple mechanisms and molecular regions (PEVK, proximal tandem-Ig, N2A) are likely to be involved. The pattern of transitions is sensitive to the history of contractile events. Monte-Carlo simulations recovered our experimental results and predicted that structural transitions may begin prior to the complete extension of the PEVK domain. High-resolution AFM of titin extended with meniscus forces supported this prediction. Addition of glutamate-rich PEVK-domain fragments competitively inhibited the viscoelastic response in both single titin molecules and muscle fibers, indicating that intra-PEVK-domain interactions contribute significantly to sarcomere mechanics. Thus, under non-equilibrium conditions across the physiological force range, titin extends *via* a complex pattern of history-dependent discrete conformational transitions which, by dynamically exposing ligand-binding sites, may set the stage for the biochemical sensing of the sarcomeric mechanical status.

Globular-domain unfolding visualized with AFM

Although mechanical force unfolds titin's globular domains, the exact structure of the overstretched titin molecule is not known. In this work we analyzed, by using high-resolution atomic force microscopy, the structure of titin molecules overstretched with receding meniscus (**Figure 1.**). The

axial contour of the molecules was interrupted by topographical gaps with a mean width of 27.7 nm that corresponds well to the length of an unfolded globular (immunoglobulin and fibronectin) domain. The wide gap-width distribution suggests, however, that additional mechanisms such as partial domain unfolding and the unfolding of neighboring domain multimers may also be present. In the folded regions we resolved globules with an average spacing of 5.9 nm, which is consistent with a titin chain composed globular domains with extended interdomain linker regions. Topographical analysis allowed us to allocate the most distal unfolded titin region to the kinase domain, suggesting that this domain systematically unfolds when the molecule is exposed to overstretching forces. The observations support the prediction that upon the action of stretching forces the N-terminal ß-sheet of the titin kinase unfolds, thus exposing the enzyme's ATP-binding site and hence contributing to the molecule's mechanosensory function.

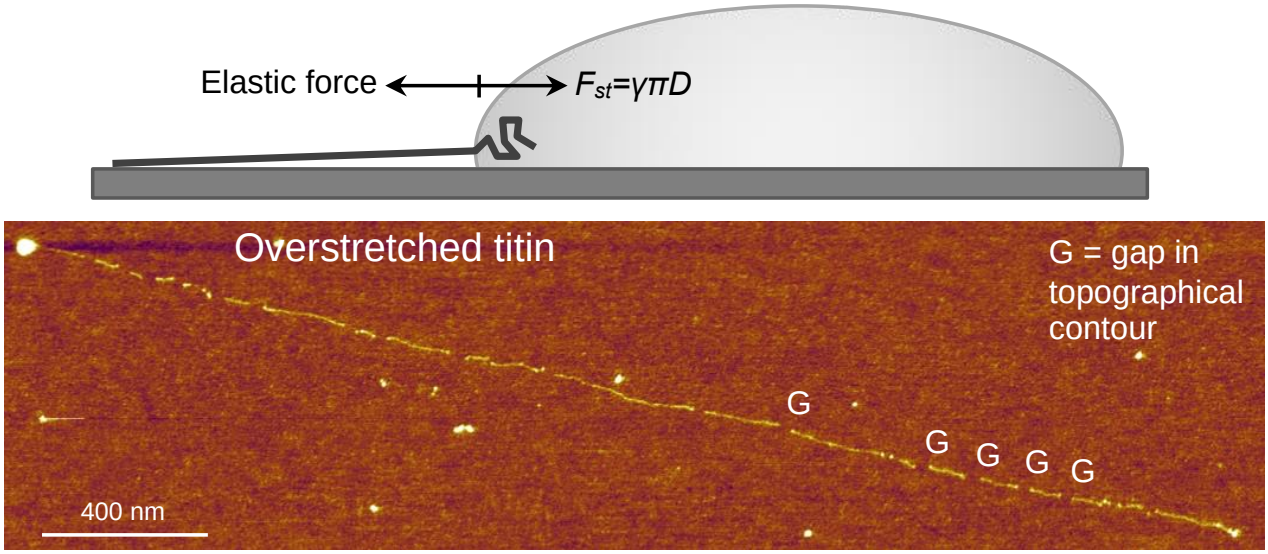

Figure 1. Extension of a single titin molecule with meniscus force, followed by imaging its topographical structure with atomic force microscopy. The unfolded domaine emerge as gaps along the topographical contour of the molecule.

Spatially homogenous distribution of globular domains in mechanically unfolded single titin molecules

Stretch induces the unfolding of titin's globular domains. However, the mechanisms of how domains are progressively selected for unfolding and which domains eventually unfold have for long been elusive. Based on force-clamp optical tweezers experiments we found that, in an paradoxical violation of mechanically-driven activation kinetics, neither the global domain unfolding rate, nor the folded-state lifetime distributions of full-length titin are sensitive to force. This paradox was reconciled by a gradient of mechanical stability so that domains are gradually selected for unfolding as the magnitude of the force field increases. Atomic-force-microscopic screening of extended titin molecules revealed that the unfolded domains are distributed homogenously along the entire length of titin, and this homogeneity is maintained with increasing overstretch (**Figure 2**). While the unfolding of domains with progressively increasing mechanical stability makes titin a variable- viscosity damper, the spatially randomized variation of domain stability ensures that the induced structural changes are not localized but are distributed along the molecule's length. Titin may thereby provide complex safety mechanims for protecting the sarcomere against structural disintegration under excessive mechanical conditions.

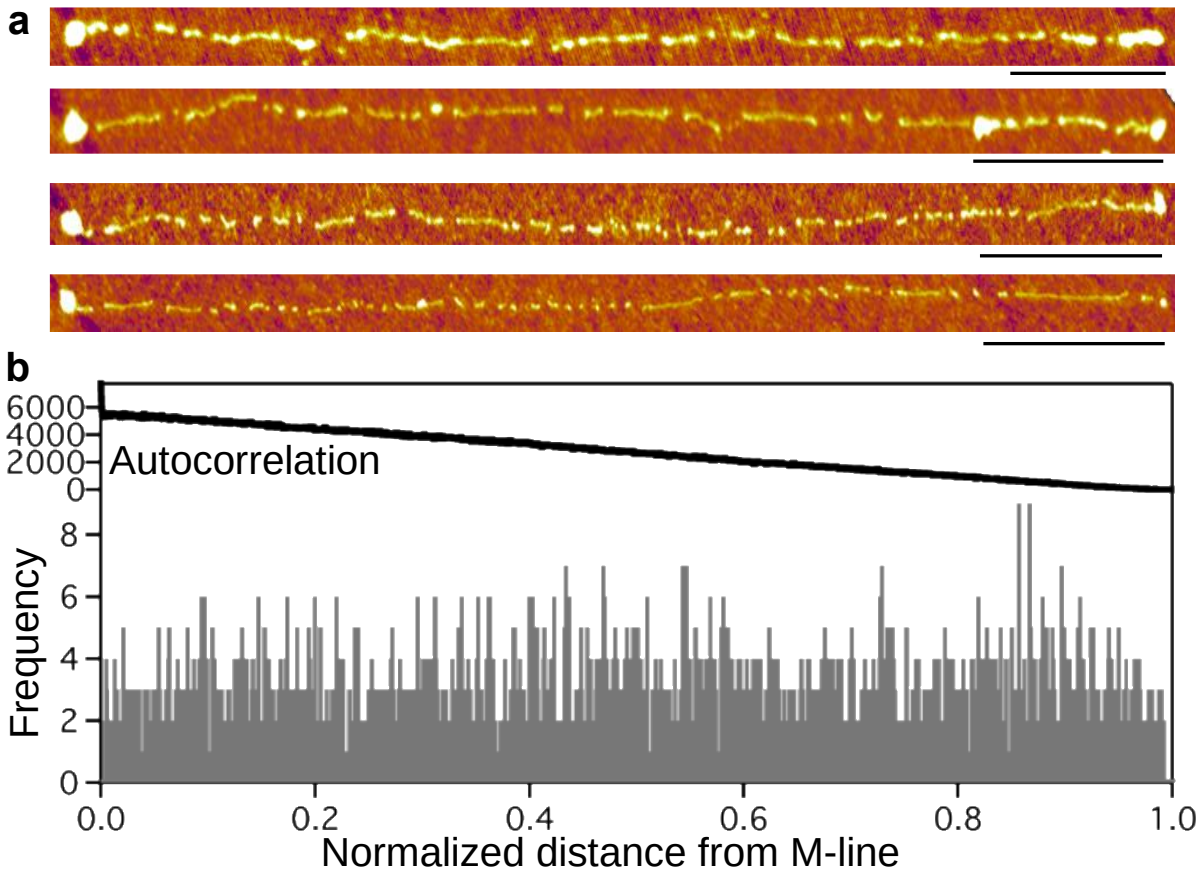

Figure 2. a. AFM images of single titin molecules stretched with receding meniscus. The images from top to bottom display progressively longer molecules. The M-line end of titin is identified by the large globular head. **b.** Spatial frequency of unfolded domains along the normalized contour of titin.

Generation of force by domain folding in titin.

In this work we demonstrated that the giant titin molecule is able to generate a few piconewtons of force through the folding of its globular (pre-unfolded) domains. As the sarcomere is stretched, titin extends hierarchically according to the mechanics of its segments. Whether titin's globular domains unfold during this process and how such unfolded domains might contribute to muscle contractility are strongly debated. To explore the force-dependent folding mechanisms, here we manipulated skeletal-muscle titin molecules with high-resolution optical tweezers. In force-clamp mode, after quenching the force (<10 pN), extension fluctuated without resolvable discrete events. In position-clamp experiments the time-dependent force trace contained rapid fluctuations and a gradual increase of average force, indicating that titin can develop force *via* dynamic transitions between its structural states *en route* to the native conformation. In 4 M urea, which destabilizes H-bonds hence the consolidated native domain structure, the net force increase disappeared but the fluctuations persisted. Thus, whereas net force generation is caused by the ensemble folding of the elastically-coupled domains, force fluctuations arise due to a dynamic equilibrium between unfolded and molten-globule states. Monte-Carlo simulations incorporating a compact molten-globule intermediate in the folding landscape recovered all features of our nanomechanics results. The ensemble molten-globule dynamics delivers significant added contractility that may assist sarcomere mechanics, and it may reduce the dissipative energy loss associated with titin unfolding/refolding during muscle contraction/relaxation cycles.

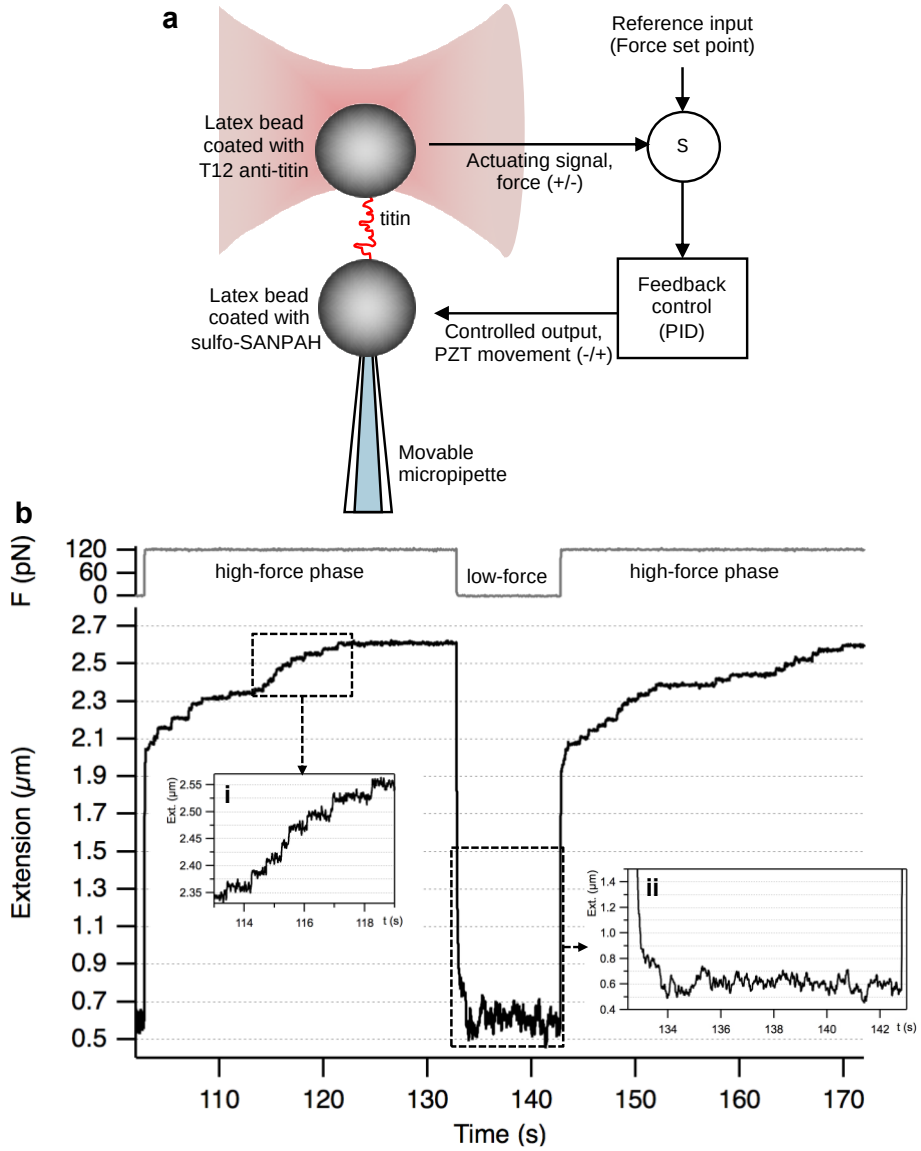

Figure 3. Manipulation of single titin molecules with optical tweezers. **a.** Schematics of the force-clamp optical tweezers experiment. **b.** Typical unfolding, refolding and unfolding experimental cycle at constant high, low and high forces.

Measuring the topology of interactions between titin molecules and myosin thick filaments.

In this work we explored the hypothesis whether titin mifght serve as a geometric ruler for the formation of the myosin thick filament. In the A-band titin is associated with the myosin thick filament. It has been speculated that titin may serve as a ruler for thick-filament formation due to the super-repeat structure of its A-band domains. Accordingly, titin might provide a template that determines the length and structural periodicity of the thick filament. However, the accessibility of A-band anti-titin antibody epitopes and structural reconstructions from electron-microscopic images suggest that titin runs on the thick filament surface, raising important questions about how the two filaments determine or affect each other's structural arrangement. Here we tested the titin ruler hypothesis by mixing titin and myosin at in situ stoichiometric ratios (approximately 300 myosins per 12 titins) and dialyzing the sample against buffers of different filament-forming ionic strength (KCl concentration range 50-150 mM). The topology of the filamentous complexes were investigated with atomic force microscopy. We found that the samples contained distinct, separate populations of titin molecules and myosin thick filaments. We were unable to identify complexes in which myosin

molecules were regularly associated to either mono- or oligomeric titin in either conformationally relaxed or stretched states of the titin filaments. Occasionally we observed myosin thick filaments with titin oligomers attached to their surface. Thus, the electrostatically driven self-association is stronger in either myosin and titin than their binding to each other, and it is unlikely that titin functions as a geometrical template for thick-filament formation. Conceivably, associated proteins, such as myosin-binding protein C, and additional mechanisms are required to modulate and regulate the in situ interactions between titin and the myosin thick filament.

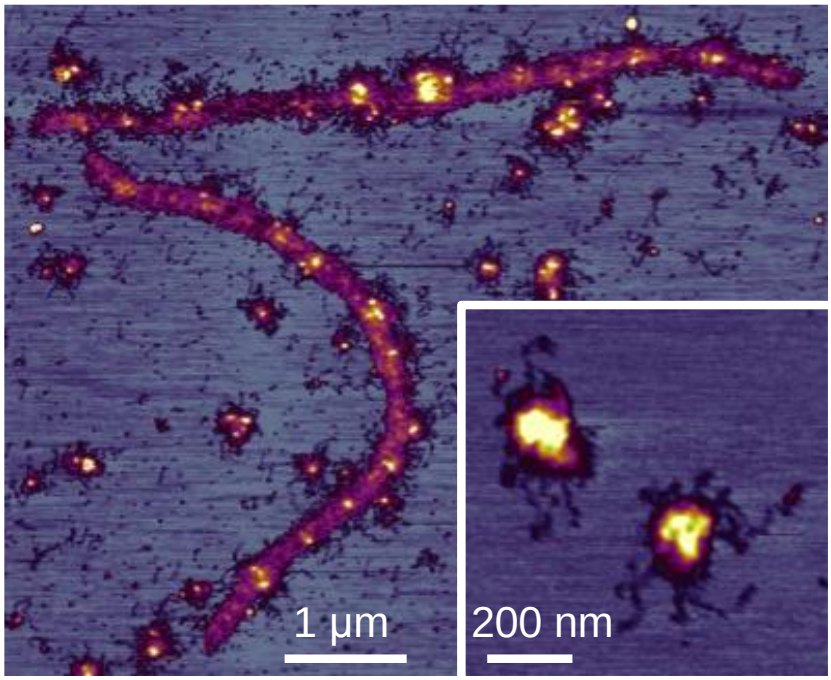

Figure 4. AFM image of thick-filament—titin oligomer mixture. The titn oligomers cover the surface of the thick filament. **Inset** shows a magnified view of titin oligomers.

Extrasarcomeric cytoskeleton

Pobably the most important component of the striated-muscle cytokeleton outside of the myofibrils is the desmin intermediate filament system. We focused on the nanomechanics of desmin filaments exposed to hydrodynamic (meniscus) forces.

Stretching desmin filaments with receding meniscus reveals large axial tensile strength

Desmin forms the intermediate filament system of muscle cells where it plays important role in maintaining mechanical integrity and elasticity. Although the importance of intermediate-filament elasticity in cellular mechanics is being increasingly recognized, the molecular basis of desmin's elasticity is not fully understood. We explored desmin elasticity by molecular combing filaments with forces calculated to be as large as 4 nN. Average filament contour length increased 1.55-fold axial on average. Molecular combing together with EGTA-treatment caused the fragmentation of the filament into short, 60-120-nm-long and 4-nm-wide structures. The fragments display a surface periodicity of 38 nm, suggesting that they are composed of laterally attached desmin dimers. The axis of the fragments may deviate significantly from that of the overstretched filament, indicating that they have a large orientational freedom in spite of being axially interonnected. The emergence of protofibril fragments thus suggests that the interconnecting head or tail domains of coiled-coil desmin dimers are load-bearing elements during axial stretch. A paper was published n the Journal of Structural Biology.

Mechanics of nanoscale biomolecular systems

In addition to our main focus of exploring the behavior of the muscle cytoskeleton, we carried out experiments on alternate nanoscale biomolecular systems as well, wit the intention of developing and fine-tuning our single-molecule biophysics toolkit.

Extreme resilience of cochleate nanoparticles

Cochleates, which are prospective nanoscale vehicles for the delivery of proteins, pharmaceuticals and numerous other molecular species, are rolls of negatively-charged phospholipid membrane layers. The membrane layers are held together by calcium ions; however, neither the magnitude of membrane-interaction forces, nor the overall mechanical properties of cochleates have been known. In our work we manipulated individual cochleate nanoparticles with atomic force microscopy to characterize their nanomechanical behavior. We found that their stiffness (4.2-12.5 N/m) and membrane-rupture forces (45.3-278 nN) are orders magnitude greater than those of the tough viral nanoshells. Even though the fundamental building material of cochleates is a fluid membrane, the combination of supramolecular geometry, the cross-linking action of calcium and the tight packing of the ions apparently lead to extreme mechanical resilience. The supramolecular design of cochleates may provide efficient protection for encapsulated materials and give clues to understanding biomolecular structures of similar design, such as the myelinated axon.

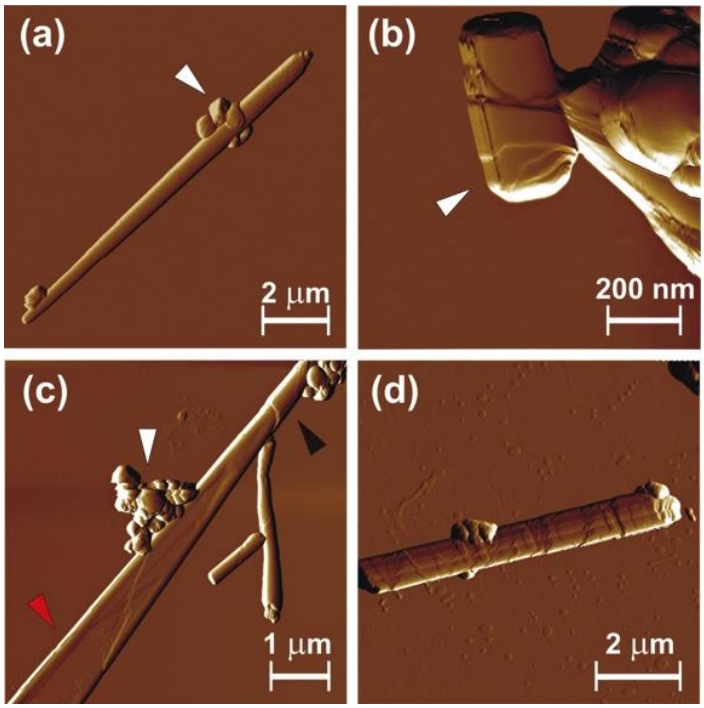

Figure 5. AFM morphology of cochleate nanoparticles.

Preparation and analysis of dispersed cochleate nanoparticles.

In this work we managed to devise a method of disaggregating cochleates, which are membraneous nanoscale structures that can potentially be used to encapsulate chemicals. Cochleates, calcium-stabilized membrane rolls of nanoscale diameter, promise a unique and efficient way of delivering lipid-soluble drugs, proteins or nucleic acids into biological systems because they protect the encapsulated material against enzymatic or chemical degradation. Self-aggregation, which typically arises during production and storage is a major obstacle that has so far precluded the development of

an efficient cochleate-based drug-delivery system. Here we show that citric acid, added transiently in a narrow concentration range, effectively disperses cochleate aggregates, stabilizes the disperse state for long-term storage and preserves the canonical ultrastructure and topological characteristics of cochleate nanoparticles.

Aggregation of PEGylated liposomes driven by hydrophobic forces.

In this work we investigated the stability of liposomes, which we intend to utilize as microcompartments. Polyethylene glycol (PEG) is widely used to sterically stabilize liposomes and improve the pharmacokinetic profile of drugs, peptides and nanoparticles. Here we report that ammonium sulfate (AS) can evoke the aggregation of PEGylated vesicles in a concentration-dependent manner. Liposomes with 5 mol% PEG were colloidally stable at 0.7 mM AS, but above this concentration they precipitated to form micron sized aggregates with irregular shape. While aggregation was reversible up to 0.9 M AS, above 1 M fusion occurred, which irreversibly distorted the size distribution of liposomes. Zeta potential of liposomes markedly increased from -71±2.53 mV to 2.02±0.45 mV upon raising the AS concentration from 0 to 0.1 M, but no considerable increase was seen during further AS addition, showing that the aggregation is independent of surface charge. There was no aggregation in the absence of the PEG chains, and increasing PEG molar % shifted the aggregation threshold to lower concentrations. Modifications of infrared spectrum of PEGylated vesicles suggests that AS dehydrates PEG chains. Other kosmotropic salts also led to aggregation, while chaotropic salts did not, which indicates a general kosmotropic phenomenon. The driving force behind aggregation is likely to be the hydrophobic effect due to salting out the polymer similarly to what happens during protein purification or Hydrophobic Interaction Chromatography. Since liposome aggregation and fusion may result in difficulties during formulation and adverse reaction upon application, the phenomena detailed in this paper may have both technological and therapeutical consequences.

Nanomanipulation and nanomechanical characterization of T7 bacteriophages.

In this work we manipulated individual T7 naoparticles and discoveres reversible, stepwise structural transitions in them. These particles may be user for encapsulating biomolecules. Viruses are nanoscale infectious agents constructed of a proteinaceous capsid that protects the packaged genomic material. Nanoindentation experiments using atomic force microscopy have, in recent years, provided unprecedented insight into the elastic properties, structural stability and maturation-dependent mechanical changes of viruses. However, the dynamics of intercapsomeric interactions are still unresolved. Here we used high-resolution nanoindentation experiments on mature, DNA-filled T7 bacteriophage particles. The elastic regime of the nanoindentation force trace contained discrete, stepwise transitions that cause buckling of the T7 capsid with magnitudes that are integer multiples of ~0.6 nm. Remarkably, the transitions are reversible and contribute to the rapid consolidation of capsid structure against force during cantilever retraction. The stepwise transitions were present even following the removal of the genomic DNA by heat treatment, indicating that they are related to the structure and dynamics of the capsomeric proteins. Dynamic force spectroscopy experiments revealed that the thermally activated consolidation step is ~10^4 times faster than spontaneous buckling, suggesting that capsid stability is under strong dynamic control. Capsid structural dynamics may play an important role in protecting the genomic material under harsh environmental impact. The nanomechanics approach employed here may be used to investigate the structural dynamics of other viruses and nanoscale containers as well.

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