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Binomial distribution for quantification of protein subunits in biological Nanoassemblies and functional nanomachines

Huaming Fang, PhD[a], Peng Zhang, PhD[b], Lisa P. Huang, PhD[c], Zhengyi Zhao, BS[a], Fengmei Pi, MS[a], Carlo Montemagno, PhD[d], Peixuan Guo, PhD[a,*]

[a]*Nanobiotechnology Center, Department of Pharmaceutical Sciences, and Markey Cancer Center, University of Kentucky, Lexington, KY, USA*
[b]*Department of Surgery, University of Michigan, Ann Arbor, MI, USA*
[c]*Oncoveda, Tumor Biology Center, Medical Diagnostic Laboratories, L.L.C., Hamilton, NJ, USA*
[d]*National Institute for Nanobiotechnology, Department of Chemical and Materials Engineering, University of Alberta, Edmonton, Canada*

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Abstract

Living systems produce ordered structures and nanomachines that inspire the development of biomimetic nanodevices such as chips, MEMS, actuators, sensors, sorters, and apparatuses for single-pore DNA sequencing, disease diagnosis, drug or therapeutic RNA delivery. Determination of the copy numbers of subunits that build these machines is challenging due to small size. Here we report a simple mathematical method to determine the stoichiometry, using phi29 DNA-packaging nanomotor as a model to elucidate the application of a formula $\sum_{M=0}^{Z}\binom{Z}{M}p^{Z-M}q^{M}$, where p and q are the percentage of wild-type and inactive mutant in the empirical assay; M is the copy numbers of mutant and Z is the stoichiometry in question. Variable ratios of mutants and wild-type were mixed to inhibit motor function. Empirical data were plotted over the theoretical curves to determine the stoichiometry and the value of K, which is the number of mutant needed in each machine to block the function, all based on the condition that wild-type and mutant are equal in binding affinity. Both Z and K from 1-12 were investigated. The data precisely confirmed that phi29 motor contains six copies (Z) of the motor ATPase gp16, and K = 1.

Key words: phi29; Viral DNA packaging; ATPase; Nanobiotechnology; Copy number quantification

Background

Living beings produce a wide variety of nanomachines, ordered structures, and patterned arrays at the nanometer scale, i.e. biomotors,[1-3] exosomes,[4] pumps,[5] arrays,[6,7] valves,[8-10] or membrane pores.[11-13] The complexity and intricacies of these natural nanoparticles have inspired the development of biomimetic nanodevices.[14,15] These nanomachines or patterned structures can be manipulated to build sophisticated nanodevices or parts for arrays, chips, MEMS,[16] actuators,[17] molecular sensors,[18-20] molecular sorters,[21] single pore DNA sequencing apparatus[11-13] or other revolutionary electronic and optical devices[22,23] *ex vivo*. Medical applications of these or their derivatives could be used for pathogen detection, disease diagnosis, and drug and therapeutic RNA delivery.[19,20,24,25] Also common to living system is the transportation of dsDNA. In the ASCE (additional strand catalytic E) family including the AAA+ (ATPases Associated with diverse cellular Activities) and the FtsK-HerA superfamily, there are nanomotors that facilitate a wide range of functions involved in dsDNA riding, tracking, packaging, and translocation.[26,27] These functions are critical to DNA replication, DNA repair, replication, recombination, chromosome segregation, DNA/RNA transportation, membrane sorting, cellular reorganization, cell division, and bacterial binary fission.[28]

Conflict of interests: PG is a co-founder of Kylin Therapeutics, Inc., and Biomotor and RNA Nanotechnology Development Corp. Ltd.

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*Corresponding author. Peixuan Guo-Department of Pharmaceutical Sciences, Lexington, KY, USA.
E-mail address: peixuan.guo@uky.edu (P. Guo).

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The members of ASCE family usually assemble as multimeric, often hexameric,[26,29] ring-shaped particles and act in coordination to perform cellular functions that involve multiple components in multi-step reactions. The hexameric geometrical shape facilitates bottom-up assembly in nanomachine manufacturing and can produce stable patterned arrays as parts for nanodevices. Quantitative analysis of copy number in these systems is difficult since the biological reactions that they catalyze are rapid and the intermediates are difficult to isolate and characterize.

Viral DNA packaging motors are intriguing molecular motors since they can package the lengthy genomic DNA to near-crystalline density into a pre-formed protein shell known as the procapsid.[3] The pivotal component of the DNA packaging motor is the ATPase, which forms a ring structure and converts chemical energy from ATP hydrolysis into mechanical force to physically move the genomic DNA into the procapsid. The bacteriophage phi29 is an excellent model system for studying viral DNA packaging motors.

Four decades of extensive study have provided many details about the phi29 DNA packaging motor,[8,30-32] which is composed of three essential co-axial rings: a dodecameric connector ring located at the vertex of the procapsid, a hexameric pRNA ring bound[31] to the N-terminus of the connector,[33] and a hexameric ring of ATPase gp16 that surrounds the helical region of pRNA[26,30] (for review, see[9]). Sequence alignment has revealed that phi29 ATPase gp16 belongs to the FtsK/HerA family of dsDNA translocases that is a member of the FtsK/Her superfamily.[34] The proteins of this family usually form hexameric rings and function in coordination. Native gel shift assays, capillary electrophoresis (CE), Hill constant determination, and titration of mutant subunits using computational binomial distribution have determined gp16 to be hexameric following a monomer → dimer → tetramer → hexamer pathway.[26] The hexameric motor uses a revolution mechanism without rotation.[9,26,35-37]

A variety of methods have been attempted to quantify the reaction in viral DNA packaging motors.[38,39] The stoichiometry of pRNA has been determined utilizing binomial distribution to predict the number of pRNA bound on the procapsid, taking advantage of the highly sensitive *in vitro* phi29 phage assembly system with 10^8 magnitude of sensitivity.[40,41] This system can produce 10^8 virions (PFU, plaque form unit) per ml without any background. The goal of this study is to develop a simple mathematical method for determining the copy number of protein subunit in a biological complex. A Walker B mutant ATPase gp16 (gp16/ED) was constructed that dramatically inhibited the virion assembly activity of wild type gp16. The Walker B mutant gp16 could assemble into a ring on the motor together with wild type gp16. The probability of various combinations of mutant and wild type gp16 on the motor was predicted with binomial distribution. The production of the virion could be predicted under the assumption that Z gp16 copies are needed to drive the packaging motor and K copies of inhibitive mutant gp16 are required to block the motor. The pre-requisition of this method in the stoichiometry determination is that the mutant and the wild-type ATPase have an equal affinity in substrate binding. Since we can construct different mutant gp16 ATPase with only single mutation and inactive the entire system with only one amino acid modification. This trivial change will not significantly change the system, making it a feasible approach for the usage of the binomial distribution equation. This method could serve as a way to study the stoichiometry and mechanism of many other biological complexes.

Methods

Construction of mCherry-gp16 and mutant eGFP-gp16 plasmids

The strategy for construction of mCherry-gp16 was the same as that used for the construction of the eGFP-gp16 clone, as previously described.[42] The mCherry gene was amplified by PCR (Polymerase Chain Reaction) with appropriate primers and digested with XbaI and KpnI. The mCherry-gp16 gene was then inserted into the previously re-engineered His-gp16 on pET32 Xa/Lic vector[43] to replace the thioredoxin gene. The Walker B mutant, eGFP-gp16/ED, was cloned with the Stratagene Quick Change site-directed mutagenesis kit. The amino acid residues D255 and E256 of gp16 were mutated to E and D, respectively, with the appropriate primers. For Walker A mutant, the G27 amino acid residue was mutated to D.

Expression and purification of eGFP-gp16, mutant eGFP-gp16 and mCherry-gp16.

The expression and purification of eGFP-gp16 were described previously.[42] The walker B mutant eGFP-gp16/ED, walker A mutant eGFP-gp16/G27D and wildtype mCherry-gp16 protein were expressed and purified by the same protocol. Briefly, the expression of the proteins was induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in BL21 (DE3) *Escherichia coli*. The harvested cells were resuspended in buffer A (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 15% glycerol, (2-carboxyethyl) phosphine (TCEP) and 0.1% Tween-20) containing 5 mM imidazole. The cells were then passed through a French press and cell debris was removed by centrifugation. 0.1% Polyethylenimine (PEI) was added to remove the nucleic acids and other proteins. Lysate was loaded onto a Ni-resin column for further purification.

In vitro virion assembly assay

The components used in the *in vitro* phage phi29 assembly assay, including the procapsid, pRNA, DNA-gp3, gp9, and gp11-12-13-14 were prepared, as previously described.[41,44] The *in vitro* phi29 assembly assay was also carried out as previously described.[43,45] Briefly, 10 μl of purified procapsid (1 mg/ml) was mixed with 1 μl of 100 ng/μl pRNA and 3 μl of reaction buffer (10 mM ATP, 6 mM 2-mercaptoethanol, and 3 mM spermidine in TMS) at ambient temperature for 30 min. Then, DNA-gp3 and re-engineered gp16 diluted in PEG buffer (15% PEG 8000, 5% glycerin, 100 mM NaCl, 20 mM Tris–HCl, pH 7.8) were added at room temperature for 1 h to initiate the DNA packaging.

Finally, 10 μl of gp8.5-9 extract from *E. coli HMS174* containing plasmid pARgp8.5-9 and 20 μl of gp11-12-13-14 extract from *E. coli HMS174* containing plasmid pARgp11-12-13-14 were incubated with the DNA packaging mixture for 2 h at room temperature. The virions produced (PFU/ml) were quantified by