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# Bacteriophage T4 Capsid: A Unique Platform for Efficient Surface Assembly of Macromolecular Complexes

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We report the first description of a macromolecular complex display system using bacteriophage T4. Decorated with two dispensable outer capsid proteins, Hoc (155 copies) and Soc (810 copies), the 120 nm × 86 nm T4 capsid particle offers a unique binding site-rich platform for surface assembly of hetero-oligomeric complexes. To display the 710 kDa anthrax toxin complex, two bipartite functional fusion proteins, LF-Hoc and LFn-Soc, were constructed. Using a defined *in vitro* binding system, sequential assembly was performed by first attaching LF-Hoc and/or LFn-Soc to *hoc*$^{-}$*soc*$^{-}$ phage, saturating the Hoc and Soc binding sites. Trypsin-nicked PA63 was then assembled into heptamers through specific interaction with the capsid-exposed LFn domain. EF was then attached to the unoccupied sites of PA63 heptamers, completing the assembly of the tripartite anthrax toxin. Negative electron microscopy showed decoration of each capsid with a layer of heptameric PA63 rings. Up to 229 anthrax toxin complexes, equivalent to a total of 2400 protein molecules and a mass of about 133 MDa (2.7 times the mass of capsid shell), were anchored on a single particle, making it the highest density display reported on any virus. The phage T4 capsid lattice provides a stable biological platform allowing maximum display of large hetero-oligomeric complexes *in vitro* and offers insights for developing novel vaccines, analysis of protein–protein interactions, and structure determination of complexes.



*Keywords:* phage T4; macromolecular assembly; Hoc; Soc; phage display

## Introduction

Since the first description of peptide display on phage fd,[1] numerous display systems using phages,[2–6] animal and plant viruses,[7,8] and bacterial surfaces,[9,10] have emerged for a variety of biological applications. All these systems are *in vivo*-based, generally limited to displaying a single component, a peptide, domain, or protein, fused to an essential, or conditionally essential, outer structural protein. Display of multiple components, or macromolecular complexes, is very limited, or not feasible, using the classic *in vivo* phage display approaches.

One of the limitations of *in vivo* display is that no control can be exerted on intracellular expression, structure, and assembly of foreign protein(s). In the few examples where attempts were made to display complexes, for instance tetramers of *Escherichia coli* β-galactosidase (116 kDa) on phages λ[4,11] or T7,[12] the copy numbers obtained were quite low; only ≤ one copy of β-galactosidase tetramer per phage particle was displayed using gpV (major tail protein of phage λ; 192 copies per particle) or gp10 (major capsid protein of phage T7; 415 copies per particle). Higher copy numbers, up to 34 per particle, were achieved when it was displayed through the phage λ outer capsid protein, gpD (420 copies per particle),[4] but no additional studies have been reported to adapt this system for general display of hetero-oligomeric complexes.

Abbreviations used: aa, amino acid(s); EF, edema factor; gp, gene product(s); LF, lethal factor; LFn, N-terminal domain of lethal factor; LTx, lethal toxin; PA, protective antigen; SOE, splicing by overlap extension.

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Efficient assembly of macromolecular complexes on phage particle will enable novel applications. Where neutralization epitopes are conformationally constrained in a complex (e.g. trimeric HIV gp120/gp41 envelope glycoprotein),[13–15] a system able to display complexes would allow presentation of epitopes in native conformations. Particles displaying complexes can be used as baits to identify novel interactions to hitherto unknown ligands. Cryo-EM and image reconstruction of displayed phage can generate structures of bound complexes that are refractory to crystallization.[16]

Bacteriophage T4 capsid provides a unique binding site-rich platform for display of macromolecular complexes. It is a prolate icosahedron (length, 120 nm; width 86 nm) composed of the major capsid protein, gp23*† (49 kDa, 930 copies), which forms the hexagonal lattice of the capsid shell; the vertex protein, gp24* (47 kDa, 55 copies), which occupies 11 of the 12 5-fold vertices; and the portal protein, gp20 (61 kDa, 12 copies), a dodecamer that forms the unique head-tail connector vertex through which DNA enters and exits the phage head (see Figure 6).[17] Early biochemical studies by Ishii and Yanagida,[18] and recent cryo-electron microscopy reconstructions,[17,19,20] revealed the presence of two dispensable proteins, Hoc (highly antigenic outer capsid protein, 39 kDa) and Soc (small outer capsid protein, 10 kDa), on the outer surface of the particle. These decorate the capsid at high density with a combined total of 965 copies per particle. Hoc, a monomer, is present up to 155 copies per capsid in the center of the gp23* hexon, whereas 810 copies of Soc, visualized as trimers on capsid shell, bridge the adjacent hexons.[17–20] Foreign peptides or full-length proteins have been fused to the N and C termini of either protein and displayed on the capsid surface.[5,21–23]

Recently, we have developed an *in vitro* approach that distinguishes itself from the classic *in vivo* display in that it allows efficient and controlled display of large full-length proteins on the capsid surface through specific interactions between Hoc and capsid.[24] A variety of proteins, anthrax toxins, phage packaging proteins, and HIV antigens of various size (up to 90 kDa), structure, and biological function, have been fused to Hoc, over-expressed in *E. coli*, purified and assembled on the phage particles in a native functional state, under defined binding conditions.[24,25]

Here we report a novel approach to assemble‡ macromolecular complexes on bacteriophage T4. The native anthrax toxin complex consisting of three components, protective antigen (PA, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (EF, 89 kDa), having a size of about 710 kDa, was used as a model to develop the system. In the anthrax toxin pathway, PA interacts with the host cell receptor, ATR or CMG2 receptor, through the C-terminal domain-4 of PA[26] following which it is cleaved at amino acid (aa) 167 by the membrane-bound protease, furin. The activated form, PA63 (63 kDa), forms a ring-shaped heptamer,[27] which interacts with the N-terminal domain of LF (LFn) and/or EF (EFn) to form the biologically active anthrax lethal toxin (LTx) and edema toxin (ETx). LF and EF are then translocated into the cytosol through the heptamer channel where they exert toxic effects.[28]

Our strategy involves the attachment of LF§ or LFn to phage T4 through Hoc/Soc-capsid interactions so that the LFn domain is exposed on the outer surface. Soluble PA was nicked to PA63 by trypsin to expose the LFn-interacting PA domain-1′. The PA63 heptamerizes and assembles on phage T4 through interaction with the T4-exposed LFn domain. Assembly was further extended to EF by its binding to the unoccupied sites of PA63 heptamer, thus generating the complete anthrax toxin consisting of "three layers" of bound proteins on T4 surface.

The data showed that a massive number of pure, functionally intact protein molecules, about 2400 molecules of anthrax toxin components with a total mass of 133 MDa can be assembled into complexes on the T4 capsid through Hoc and Soc attachment sites. To our knowledge, this system represents the first report of a stable, efficient, phage platform for high density display of large hetero-oligomeric complexes. The data provide insights on the stoichiometry of bound complexes as well as potentially novel applications in vaccine development, structure determination, and analysis of protein–protein interactions.

## Results

### Bipartite functional LF-Hoc and LFn-Soc fusions

The biochemical pathway for LTx formation involves interaction of the N-terminal LFn domain (aa 1–263) with the PA domain-1′ of two adjacent subunits of the PA63 heptamer.[31] Based on the solved structures of PA and LF,[32,33] we hypothesized that fusion of LF or LFn to the N terminus of Hoc or Soc would create a bipartite functional protein with the N-terminal LFn domain exposed for interaction with PA63 heptamer, whereas the C-terminal Hoc/Soc domain would be available for

†*Represents the cleaved mature capsid protein following T4 capsid assembly-dependent maturation cleavages.

‡Assembly refers to the binding of Hoc/Soc fusion proteins to the symmetrically arranged binding sites on the T4 capsid surface. The terms assembly, binding, display and attachment are interchangeably used to refer this process.

§In this study, null mutants of LF and EF, $LF_{E687C}$ and $EF_{K346R}$, which exhibit no detectable MAPKK protease and adenyl cyclase activities, respectively, were used to eliminate the toxicity of anthrax toxin complexes.[29,30] The mutants however retained their binding functions and immunogenicity.