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Virus assembly, allostery, and antivirals

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1 **Assembly of virus capsids and surface proteins must be regulated to ensure that the**
2 **resulting complex is an infectious virion. Here we examine assembly of virus capsids,**
3 **focusing on hepatitis B virus and bacteriophage MS2, and formation of glycoproteins in**
4 **the alphaviruses. These systems are structurally and biochemically well-characterized**
5 **and are simplest-case paradigms of self-assembly. Published data suggest that capsid**
6 **and glycoprotein assembly is subject to allosteric regulation, that is, regulation at the**
7 **level of conformational change. The hypothesis that allostery is a common theme in**
8 **viruses suggests that deregulation of capsid and glycoprotein assembly by small**
9 **molecule effectors will be an attractive antiviral strategy, as has been demonstrated with**
10 **hepatitis B virus.**

12 **Is regulation of self-assembly required?**

13 The common structural denominator for a typical small virus is a genome surrounded by a shell
14 composed of dozens of copies of the capsid protein. Many small viruses also have lipid
15 envelopes studded with glycoproteins that can facilitate cell entry. Even a small virus is
16 complex, yet its formation is a canonical example of self-assembly. Given the right conditions,
17 the capsid proteins of many viruses will assemble to capsid-like structures, rapidly, in high yield,
18 and with high fidelity (Table 1). This observation opens up two distinct fields of study: the
19 process of self-assembly and regulation of assembly. The process of self-assembly is best
20 described by physical chemistry; for example, self-assembly can be modeled by a system of
21 differential equations or emulated with small molecules. Regulating assembly, on the other
22 hand, is fundamentally biochemical and ultimately biological in nature. Consider our hypothetical
23 virus where unregulated assembly would yield capsids that did not contain the viral genome and
24 toxic accumulation of fusion protein. These spontaneous assembly reactions must be delayed
25 until the right time and place. Regulation is likely to be at the level of allostery, which is defined
26 as a conformational change in a molecule, usually a protein, that alters its activity, induced by
27 an effector molecule. In addition to allostery, regulation could require viral or host factors.
28 Regulation of the large event of virus assembly by a small reaction, such as inducing
29 conformational change of a single protein, provides the leverage that allows virus replication *in*
30 *vivo*. Disruption of regulation is an ideal target for antiviral therapeutics. Defining the regulation
31 that directly controls the physical chemistry of assembly is a field in its infancy and the focus of
32 this review.

33 In this review, we will separately examine the assembly of virus capsids and the membrane-
34 bound glycoprotein complexes present on the exterior of enveloped viruses. Both the capsid
35 and glycoprotein are protein oligomers; from a physical-chemical perspective, their assembly is
36 similar; from a biological perspective they demonstrably have similar elements of regulation.
37 Though there are numerous experimental systems (Tables 1 and 2), the discussion of capsid
38 assembly will focus on hepatitis B virus (HBV) with significant references to retroviruses and
39 bacteriophage MS2. Furthermore, small molecules that dramatically affect HBV assembly have
40 demonstrable antiviral activity. The discussion on glycoprotein assembly will focus on the
41 formation of the spike complex of alphaviruses as the spike is composed of two proteins which
42 intricately interact and are required for viral entry. Finally, we will discuss potential steps in
43 assembly that could be targeted for designing antiviral therapeutics.

45 **Assembly of virus capsids**

46 The capsids of icosahedral viruses have tens to hundreds of copies of the capsid protein(s).
47 The simplest vision of virus capsid assembly is one where rigid assembly units (AUs) collide by

1 Brownian motion, interact with perfect geometry, and associate irreversibly. Like any utopia, this
2 vision fails examination: assembly simulations are incredibly sensitive to kinetic traps consisting
3 of partial capsids that have a negligible chance of completion due to depletion of subunits
4 (Figure 1).

5 Instead, assembly simulations, from master equations that treat assembly reactions as a well-
6 mixed solution^{2,3} to molecular dynamics that describe stochastic formation of single capsids^{4,5},
7 concur on three generalizations (Box 1). First, weak interactions are necessary to minimize
8 errors and kinetic traps. Weak interactions also contribute to a defined nucleation step. Second,
9 nucleation minimizes the initiation of assembly, decreasing kinetic trapping of intermediates due
10 to depletion of AUs. Third, the initial kinetic phase where there is little capsid formation is due to
11 the time required to build the steady state of intermediates that supports subsequent assembly.
12 More sophisticated mathematical models that incorporate the biological details of individual
13 viruses (e.g. nucleic acid, scaffolding, and allostery) will help to identify opportunities to interfere
14 with assembly.

15 Experimental observation of assembly of empty HBV capsids agrees well with the predictions of
16 theoretical models⁶ (Box 1). HBV has a T=4 icosahedral capsid composed of 120 assembly
17 units, the homodimeric core protein (Cp)⁷. Cp can be assembled *in vitro* in response to ionic
18 strength⁶ and the kinetics are sigmoidal⁶. The average association energy between two subunits
19 is -3 to -4 kcal/mol, corresponding to a millimolar dissociation constant. Because subunits are
20 tetravalent, the weak association energy corresponds to a micromolar pseudo-critical
21 concentration⁸. *In vitro* HBV assembly is resistant to, but not altogether immune to, kinetic
22 traps^{9,10}.

23 HBV is a simplest case system, a homopolymer of dimeric AUs. In cowpea chlorotic mottle virus
24 and bacteriophage HK97, different complexes of AUs participate in assembly^{11,12}. In many
25 viruses, scaffold proteins support assembly. Bacteriophage P22 has a scaffold protein that
26 thermodynamically and kinetically contributes to assembly^{13,14}. Excess P22 scaffold can
27 actually block assembly by trapping numerous intermediates¹⁴. Scaffolds can play roles in
28 switching morphologies, as in bacteriophages P2 and P4¹⁵, and complex roles in subsequent
29 maturation¹⁶. Thus, scaffold proteins could direct geometry, stability, and a measure of
30 regulation by imposing stepwise assembly.

31 Viral nucleic acid can also serve as a molecular scaffold. Viral RNA has been considered as an
32 'antenna' to attract free AUs¹⁷ and as a solid that attracts and organizes AUs on its surface¹⁸.
33 Assembly driven by RNA can be thought of in terms of the McGhee-von Hippel model of non-
34 specific protein binding to a surface¹⁹, which considers that the association for nucleic acid (NA),
35 K_{NA} , is modified by a cooperativity coefficient, ω , based on the protein-protein association
36 constant. An ω value of 1 indicates no cooperativity; an ω value greater than 1000 results in
37 steep cooperativity and reactions that appear to be two-state. Cowpea chlorotic mottle virus,
38 which does not assemble under RNA-binding conditions, binds RNA with low cooperativity,
39 displaying gradual assembly and partial capsids²⁰. HBV, where the reciprocal of the pseudo-
40 critical concentration is $\sim 10^5$ (equivalent to ω) under physiological conditions, binds RNA
41 tenaciously and with high cooperativity, resulting in quantitative assembly under mild
42 conditions²¹. A nucleic acid scaffold can thus concentrate the capsid protein and provide
43 additional association energy.

44

45 **Assembly and nucleic acids as allosteric effectors**

46 Simple theoretical models fail to describe experimental results where induced conformational
47 changes activate assembly. This behavior fits the definition of allostery. Nucleic acid-regulated

1 allostery is observed with bacteriophage MS2 and retroviruses, e.g. human immunodeficiency
2 virus (HIV) and Rous sarcoma virus (RSV).

3 Retroviral Gag, the 'capsid protein' of immature retroviruses, is a multidomain protein. The
4 elements of Gag that are most important for this discussion are the two-domain capsid (CA)
5 segment (the primary mediator of Gag-Gag interactions), a spacer peptide that follows CA, and
6 the RNA-binding nucleocapsid (NC) segment. The earliest suggestion of allostery in retrovirus
7 assembly came from *in vitro* assembly studies using DNA oligomers and Gag^{22, 23}. Gag
8 dimerization was critical for assembly -- HIV Gag would not assemble with very short oligos nor
9 if the oligo had high affinity sequences separated by a low affinity sequence, suggesting that
10 protein-protein interaction was required to activate assembly²⁴. Replacing NC with a leucine
11 zipper promoted dimerization of Gag and was sufficient to drive assembly in cells²⁵, though
12 other factors were required *in vitro*²⁶. What transitions are driven by protein oligomerization and
13 DNA-binding? Small angle neutron scattering (SANS) indicated that HIV Gag had a propensity
14 to fold on itself¹⁷, thus, straightening might be a critical assembly transition. NMR studies have
15 indicated that the spacer sequence C-terminal to Gag (in HIV and RSV) can refold^{27, 28}. This
16 refolding transition, driven by binding nucleic acid, might expose surfaces on CA to allow
17 interaction²⁸ and generate a new, potentially helix-rich interaction between the spacer
18 peptides^{29, 30}. Recent tomographic studies of immature HIV demonstrate CA-CA interactions
19 and spacer peptide-spacer peptide interactions^{30, 31}. Thus, it appears that Gag undergoes one or
20 more conformational transitions to assemble into an immature lattice. These conformational
21 transitions are involved in Gag assembly and are consistent with a regulatory effect, i.e. they
22 prevent assembly by obstructing interactions²⁸ and this obstruction can be removed by binding a
23 co-factor²⁶.

24 In the RNA bacteriophage MS2, the role of allostery mediated by nucleic acid is unambiguous
25 (Figure 2). These phages are organized with T=3 quasi-symmetry; their capsids are comprised
26 of 90 chemically identical dimers that fit into AB and CC environments³². B half-dimers cluster
27 around icosahedral fivefold vertices, A and C half-dimers alternate around icosahedral
28 threefolds (quasi-sixfolds). The A and C half-dimers are very similar, while a loop on the B
29 subunits has a different conformation, suggesting a structural switch. Uhlenbeck and co-workers
30 showed that a specific RNA stem-loop from the genome, TR, was necessary and by itself
31 sufficient to induce capsid assembly^{33, 34}. By NMR, TR-free dimer was shown to be symmetrical
32 in solution, suggestive of a CC dimer, but TR-bound dimer was asymmetric³⁵. Pure RNA-free
33 dimers or pure TR-saturated dimers do not assemble rapidly, i.e. they appeared kinetically
34 trapped³⁵. Addition of the other form of coat protein to trapped species resulted in rapid
35 assembly³⁶. These results showed that both forms of dimer, symmetric and RNA-bound
36 asymmetric, are needed for efficient assembly and that RNA was an allosteric effector. Non-TR
37 RNA stem-loops also trigger these effects, although they are much weaker binders, implying
38 that multiple RNA-coat protein interactions within the capsids also contribute to conformer
39 switching^{36, 37}. Normal mode analysis has suggested that the RNA-induced conformational
40 change might be one of dynamics, rather than static dimer conformations³⁸, and is consistent
41 with the lack of sequence-specificity. Analyzing mixed assembly reactions by mass
42 spectrometry has provided a clear example of assembly by one dimer at a time rather than via
43 coalescence of oligomers³⁵. Examination of assembly reactions with TR incorporated into a
44 longer RNA have lead to the surprising conclusion that assembly might be directed by an RNA
45 scaffold³⁶, funneling the system along only a few of the very many possible assembly paths³⁶.
46 Thus, initiation by an allosteric event and limitation by allosteric responses to protein-RNA and
47 protein-protein interaction gives MS2 assembly the appearance of following a deterministic path.
48 Interestingly, recent theoretical studies suggest that only a relatively small number of

1 intermediates will ever be used during assembly as a consequence of kinetic availability and
2 thermodynamic stability³⁹.

3

4 **Allostery in capsid construction**

5 HBV Cp assembly is also an example of allosterically controlled assembly, even though its
6 assembly superficially resembles association of rigid bodies. For example, HBV Cp binds
7 cooperatively to Zn⁺⁺, resulting in increased intrinsic fluorescence (indicating conformational
8 change), and faster assembly kinetics¹⁰. Conformational differences between free Cp dimer and
9 the same protein in the context of capsid are evident in the crystal structure of an assembly
10 defective mutant (Figure 3b)⁴⁰. Dimers from capsid are symmetrical about the dimer interface⁴¹,
11 while free dimer is asymmetric. Comparison of the structures suggests a mechanical linkage of
12 sub-domains connected to a central chassis; these sub-domains displace one another like so
13 many molecular dominos, leading to movements of up to 9 Å and a conformation that is
14 geometrically incompatible with an icosahedral structure. In comparison to the crystal structure,
15 an NMR study under non-assembly conditions showed a symmetric dimer that was readily
16 asymmetricized by interaction with a Cp-binding peptide⁴². Thus, the dimer is able to adopt
17 multiple conformations, or families of conformations, tentatively categorized as assembly-active
18 and assembly-inactive.

19

20 **Allostery and antiviral strategies based on assembly**

21 For allostery, there must be a conformational change and an allosteric effector. Synthetic HBV
22 assembly effectors have now been identified, the heteroaryldihydropyrimidines (HAPs) and
23 phenylpropanamides. These show promise as antiviral agents, though the identity of the natural
24 allosteric effector still remains unknown.

25 HAPs were discovered in a search for non-nucleoside inhibitors of HBV replication and only
26 later shown to affect HBV production by a core protein-dependent mechanism⁴³. *In vitro*
27 experiments showed that HAPs could misdirect assembly and, more critically, HAPs strengthen
28 dimer-dimer association and accelerate assembly kinetics, sometimes by orders of magnitude⁹.
29 ⁴⁴. A crystal structure of a HAP-capsid complex (Figure 3a) shows that HAPs lead to a change
30 in capsid quaternary structure (but not Cp tertiary structure), bowing out icosahedral fivefolds
31 while flattening hexagonal arrangements of dimers⁴¹. These two effects explain the basis of
32 assembly misdirection by destabilizing fivefolds while favoring sheets of Cp dimers⁹. The HAP
33 molecule fills a hydrophobic pocket at the dimer interface, increasing buried surface area and
34 presumably the association constant while slightly distorting the geometry of Cp-Cp
35 interaction⁴¹.

36 Using a series of HAPs, a strong correlation was observed between antiviral effect and the rate
37 of capsid formation, and a negligible correlation between antiviral effect and HAP stabilization of
38 Cp-Cp interaction⁴⁴. The low concentrations of the most effective HAPs required to suppress
39 HBV replication suggest that misdirection (which requires stoichiometric concentrations of HAP)
40 is not central to antiviral activity⁹. Similarly, phenylpropanamides, which were recently shown to
41 accelerate assembly with little capsid stabilization and no assembly misdirection⁴⁵, inhibit virus
42 production in culture to yield empty capsids⁴⁶.

43 We propose that the kinetic effects of HAPs and phenylpropanamides are the critical predictor
44 of antiviral activity^{44, 45}. This hypothesis leads to predictions about the nature of assembly and its
45 allosteric activation. First, as per assembly models, a kinetic effect will be most evident in the
46 nucleation step. Starting capsid assembly at an inappropriate time (e.g. without the viral genetic

1 material) is likely to be bad for the virus. Second, faster kinetics indicate that effectors decrease
2 the energy barrier to assembly, which can be best explained by invoking an effector-induced
3 conformational change. The graphic of the HAP site in the context of free and the capsid
4 conformations (Figure 3c) supports this hypothesis. The key point is that only the nucleation
5 step of an assembly reaction needs be affected to gain an overall enhancement of assembly
6 kinetics (Figure 3d). Thus, the few effectors needed for nucleation can be leveraged to consume
7 many copies of Cp for the production of failed virus.

8 As a point of comparison to assembly effectors, there are molecules that inhibit other allosteric
9 transitions in viruses, such as stabilization of picornaviruses to uncoating and inhibition of HIV
10 maturation. The WIN compounds (e.g. pleconaril) bind to a hydrophobic pocket in rhinoviruses
11 and polioviruses and entropically inhibit the structural transition that enables release of the viral
12 genome^{47, 48}. A peptide inhibitor of HIV, 'CAI', inhibits the structural transitions between the N-
13 terminal and C-terminal domains of CA that are associated with maturation, possibly favoring
14 inappropriate geometries⁴⁹. CAI also blocks assembly of immature Gag. However, consideration
15 of assembly models suggests that simply inhibiting assembly could be losing strategy as it is
16 easily overcome by overproduction of the capsid protein(s)⁵⁰.

18 **Viral glycoprotein spikes**

19 While capsid and envelope glycoproteins have different functions, they share common
20 principles that regulate their assembly. Viral glycoproteins, or spikes, are multimers that are
21 assembled sequentially before becoming competent for catalyzing cell entry. The protein-
22 protein interactions within a spike and between spikes are analogous to the interactions
23 between AUs that drive capsid assembly. The processing events of the spikes parallel the
24 nucleation events that initiate capsid assembly. Furthermore, factors such as environmental
25 pH and peptides digested from the spikes as they are folded and refolded function as allosteric
26 regulators and sensors during assembly.

27 The assembly of viral glycoprotein spikes requires a coordinated, highly regulated, processive
28 mechanism to be successful. The alphavirus system, which is discussed below, demonstrates
29 that even with less than a half-dozen proteins, the virus has developed an assembly mechanism
30 that relies on cellular signals and the environment to initiate conformational or allosteric changes
31 in the assembly process. Stabilizing any of the discrete conformations along the spike
32 assembly path by binding of a small molecule would have tremendous potential for disrupting
33 the timing and compartmentalization of this complex series of reactions.

35 **Assembly of alphavirus spike complexes**

36 Alphaviruses have 80 trimeric spikes, each ~70 Å in height. Each spike is a trimer of an E2-E1
37 heterodimer, with the E2 protein at the top of the spike and the E1 protein lying underneath,
38 close to the lipid membrane (Figure 4)⁵¹. In addition to interactions within each heterodimer,
39 there are interactions between heterodimers. An E1 molecule from one heterodimer contacts
40 the stalk of an adjacent E2 molecule, which is part of another heterodimer⁵¹. Each spike
41 interacts with the nucleocapsid core via the cytoplasmic region of E2 and the E2 and E1
42 proteins interact uniquely with each other via their transmembrane regions⁵¹.

43 The E2 protein binds to host-cell receptor and the E1 protein mediates fusion between the viral
44 membrane and the host cell membrane. The receptor binding domain in E2 is positioned above
45 the fusion peptide of E1⁵¹. One could speculate that upon receptor-binding, unzipping of the
46 E2-E1 dimer must be initiated to ultimately expose the fusion protein to the host membrane.

1 What might control this association and subsequent disassociation? Viral fusion occurs in the
2 endosome and is pH dependent⁵². pH sensors or amino acid residues that destabilize the E2-
3 E1 dimer or that stabilize the post-fusion E1 trimer conformation have been identified⁵³.
4 Interfering with the assembly of the E2-E1 dimer, either through stabilization or destabilization,
5 would severely affect the allosteric regulation by the sensor residues during fusion and, as a
6 result, cell entry would be blocked.

7 The association of the alphavirus E2-E1 dimer is a regulated process that occurs early in
8 assembly. The alphavirus structural proteins are translated from a sub-genomic RNA as a
9 single polyprotein of capsid-E3-E2-6K-E1. The capsid protein autoproteolytically cleaves itself
10 from the rest of the polyprotein in the cytoplasm⁵⁴. The remaining structural polyprotein is
11 targeted to the endoplasmic reticulum and cleaved to pE2 (or p62, corresponding to E3+E2),
12 6K, and E1 proteins by cellular proteases⁵⁵. In the ER, E1 undergoes several conformations
13 (α , β , γ) each differing in the number of disulfide bond rearrangements. pE2 associates only
14 with the E1 β intermediate. As a pE2-E1 dimer and during trimerization, E1 continues to
15 undergo conformational changes until a stable E1 ϵ conformation is reached. Late in the
16 secretory pathway E1 ϵ becomes metastable, possibly in response to cleavage of pE2 during
17 virus maturation (see below)⁵⁶.

18 One could speculate that pE2 undergoes similar disulfide bond rearrangement as E1 during
19 assembly. Both the E1 and E2 proteins have 12 cysteine residues in their ectodomain, and
20 both interact with resident disulfide isomerases⁵⁷. pE2 has several CXXC motifs, characteristic
21 of disulfide isomerase substrates⁵⁸. It has been suggested that E3 might function as a disulfide
22 isomerase during alphavirus assembly⁵⁹. In the absence of pE2, E1 is not transported to the
23 plasma membrane; in the absence of E1, low amounts of pE2 are transported to the plasma
24 membrane⁵⁶. These results emphasize the necessity of forming a specific pE2-E1 dimer early in
25 assembly.

26 Recently, a translational frameshift was identified in the 6K protein coding region, producing a
27 TF (for transframe) protein⁶⁰. The synthesis of TF presents a conundrum for maintaining a
28 balance of pE2 and E1 protein during assembly. When the 6K protein is translated, equal
29 amounts of pE2 and E1 are translated. However, when TF is translated, E1 translation is
30 abrogated, presumably drastically impacting the assembly of the pE2-E1 heterodimer.

31

32 **Activation of viral fusion proteins**

33 Viral glycoproteins fill many roles; the most common is mediating fusion between the viral and
34 host membrane via the fusion protein. The atomic structures of viral fusion proteins in the pre-
35 and post-fusion conformations have been determined and led to their classification based on
36 structural similarities: Class I (orthomyxo-, paramyxo-, retro-, filo-, arena-, and coronaviruses),
37 Class II (alpha- and flaviviruses), and Class III (rhabdo-, baculo- and herpesvirus)^{61, 62}. The
38 three classes are trimers in their fusion-active conformation. For most viruses, the final step of
39 glycoprotein spike assembly is the activation of the fusion protein. This step brings the fusion
40 protein into a metastable state, priming it for rapid fusion with the host cell upon proper
41 activation.

42 The requirement of a chaperone protein and its cleavage during assembly is a property of the
43 Class II fusion proteins. The cleavage of E3 from pE2 occurs in the trans-Golgi⁶³ and marks the
44 transition from an immature to mature or fusogenic alphavirus particle. Immature and mature
45 alphaviruses are structurally similar, but immature particles have reduced infectivity levels
46 compared to mature particles because of the presence of E3 and/or because E1 is not in a
47 metastable state able to facilitate fusion⁶³. Retaining the E3 protein until the trans-Golgi might

1 serve to prevent premature fusion of the assembled spikes with membranes in the host cell
2 secretory pathway. Thus, E3 is an effector of spike activity. At the plasma membrane the spikes
3 are arranged in large hexagonal arrays⁶⁴, creating a platform for virus budding. Host proteins
4 have not been identified within the alphavirus glycoprotein array, suggesting lateral interactions
5 between the spikes are important, in contrast to vesicular stomatitis virus⁶⁵, HIV⁶⁶, and influenza
6 virus⁶⁷ which also bud from the plasma membrane. Though not usually described as such, pE2
7 acts as an effector of E1 activation at two different times, as a chaperone and by releasing E3
8 for activation.

9 Similar to pE2, the flavivirus prM protein chaperones folding of the flavivirus E protein, and
10 cleavage of the pr peptide is required to transition from the immature to the mature flavivirus. In
11 contrast to alphaviruses, immature and mature flaviviruses have radically different structures⁶⁸.

12 Class I fusion proteins do not have a distinct chaperone. Activation of the fusion proteins occurs
13 by cleaving the fusion protein itself. Multiple extracellular and cellular proteases have been
14 proposed to cleave influenza HA0 both intra- and extracellularly, and we suggest that additional
15 proteases for other viruses will also be identified⁶⁹. The class III fusion proteins do not have a
16 chaperone protein nor do they require a cleavage step to activate the protein into a fusion-active
17 form. The vesicular stomatitis virus G protein is found in three reversible conformations in a pH-
18 dependent equilibrium. The reversible conformations are a unique property among Class III
19 fusion proteins, suggestive of allosteric conformational changes, which also prevents premature
20 fusion during assembly⁷⁰. Thus, the classification of fusion proteins is dependent on the
21 structure of the fusion protein as well as the regulatory mechanism for its activation. Both
22 processes are likely controlled through allosteric mechanisms.

23 We suggest that most viral families rely on a series of 'sensing' events that control protein
24 disulfide bond linkages, glycosylation, acylation, phosphorylation, oligomerization, and transport
25 to the appropriate cellular membrane for viral budding. These sensors respond to pH, redox,
26 and lipid environment as the proteins are assembled and transported through the host secretory
27 pathway⁵³ to allosterically enhance assembly and ensure productive spike formation.

28

29 **Conclusions and future directions: interfering with conformational change and control** 30 **during viral assembly**

31 While targeting specific conformations is an attractive antiviral strategy, we further propose that
32 one should consider the dynamic nature of virus assembly and that interfering with the
33 environmental sensors and their targets could be equally beneficial. Both capsid and
34 glycoprotein assembly are carefully orchestrated events requiring activation, by known and
35 unidentified effectors. For example, we have learned from the HAP molecules that interfering
36 with assembly is not equivalent to inhibiting assembly. In addition, weak interactions between
37 protein subunits or glycoprotein complexes minimize production of misassembled spikes. Thus,
38 altering the kinetics, misdirecting the intermediates, stabilizing an interaction to prevent
39 disassembly are all viable options that disrupt a highly evolved set of reactions to achieve the
40 same end goal: reduced virus propagation.

41

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47

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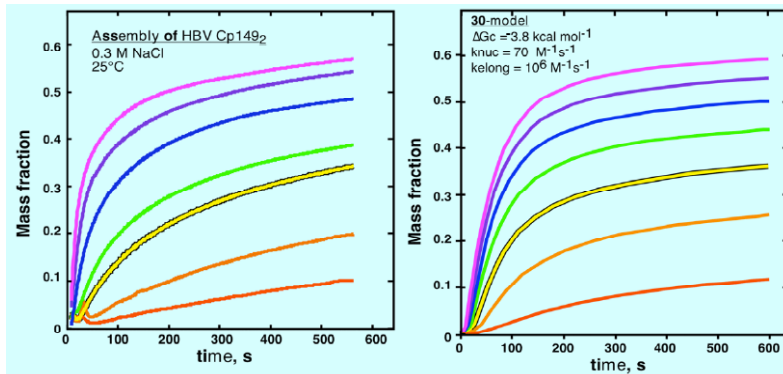
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 17
 18
 19

20 **Box 1. An analytical description of capsid assembly**



21
 22 Figure I. Comparison of HBV capsid assembly with simulations of capsid assembly. Assembly
 23 of a concentration series of HBV core protein (left) compared to assembly simulations of a 30-
 24 mer model (right), where subunits have an HBV-like geometry and the nucleus is trimeric¹. The
 25 protein concentration increases from 4 μM (red) to 10 μM (purple) in 1 μM increments. The
 26 calculation is parametrized to resemble HBV assembly. For the simulation, the bimolecular rates
 27 for nucleation (k_{nuc}) and elongation (K_{elong}) were $70 \text{ M}^{-1}\text{s}^{-1}$ and a diffusion-limited $10^5 \text{ M}^{-1}\text{s}^{-1}$,
 28 respectively; the pairwise association energy was -3.8 kcal/mol .
 29

30
 31
 32
 33 Probably the simplest way to describe assembly of a capsid of N assembly units (AUs) is as a
 34 progressive series of intermediates¹⁰⁰. To quantify this description, one can assume that all
 35 contacts between assembly units have the same energy, energies are additive, microscopic
 36 forward rates are identical (subject to statistical considerations), and that backward rates are the
 37 product of forward rates, dissociation constants and statistical terms. The result is a series of

1 rate equation each with terms for assembly and disassembly of n -mer and $(n+1)$ mer,
2 respectively. Two equations in the series are unique: the monomer equation references all
3 intermediates and the final equation is a dead-end with only one build-up and one build-down
4 term.

$$5 \quad d[n\text{-mer}]/dt = k_{\text{forward},n}[(n-1)\text{mer}][\text{monomer}] - k_{\text{forward},n+1}[n\text{-mer}][\text{monomer}] \\ 6 \quad \quad \quad - k_{\text{backward},n}[n\text{-mer}] + k_{\text{backward},n+1}[(n+1)\text{mer}]$$

7 Incorporating weak interactions between AUs and a slow nucleation step to limit the initiation of
8 assembly results in robust reactions with minimal kinetics traps where errors and (meta)stable
9 intermediates accumulate^{4, 39}. This model necessarily leads to sigmoidal kinetics and, at
10 equilibrium, a pseudo-critical concentration of free AU^{5, 100}. The resulting model recapitulates
11 most of the features observed *in vitro* (Figure I).

12 Assembly can be described in much greater detail as stochastic reactions using discrete event
13 simulators or coarse-grained molecular dynamics⁴. These molecular simulations provide detail,
14 showing conditions where association of intermediates could be an important path, where
15 reversibility is critical⁵, and how subunit geometry can critically affect the size and shape of
16 assembly products⁴. This statistical mechanical view of assembly leads to a view of assembly
17 that is fundamentally similar to the thermodynamic-kinetic view of the previous paragraph⁴.
18 However, the detail provides additional insights into the reactions and the behaviors that can be
19 anticipated by biological molecules.

20

21

22 Figure legends

23 **Figure 1. Successful assembly and kinetic traps.** Assembly of a dodecahedron from
24 pentagonal assembly units (faded colors) is more robust if regulated by nucleation. In nucleated
25 assembly (top path), the initial association reactions are relatively slow, resulting in rare
26 nucleation centers (brightly colored oligomer) and plenty of assembly units to allow completion
27 of capsids. If assembly is unregulated (lower path), many partial capsids will form and very few
28 assembly units will remain, kinetically trapping these intermediates. Of course, this trap might
29 resolve if stochastic dissociation of some intermediates allows others to go to completion;
30 alternatively, partial capsids can associate incorrectly, resulting in aberrant structures.

31

32 **Figure 2. Structure and assembly of bacteriophage MS2.** (a) The T=3 capsid of MS2 has 60
33 AB dimers (blue and green, respectively) and 30 CC dimers (red), so that A and C subunits
34 alternate around the quasi-sixfold axes. Icosahedral fivefold and quasi-sixfold (icosahedral
35 threefold) vertices are shown in this representation. (b) The CC dimers are necessarily
36 symmetric with an extended loop (highlighted in the open triangles) connecting the F and G β
37 strands at either end. Bound RNA, the TR stem-loop (in yellow), induces asymmetry in the AB
38 dimers so that the FG loop of the B subunit (in the pentagon) adopts a unique conformation that
39 allows it to pack around the fivefold vertices. (c) Pure CC dimers (red) or pure AB dimers (green
40 and blue), with bound RNA, assemble rapidly when mixed, leading to the hypothesis that
41 assembly proceeds in a stepwise reaction. The hexameric threefold cluster is one of the major
42 complexes identified by mass spectrometry, suggesting a highly deterministic assembly path.
43 This hypothesis has been strongly supported by further examination of RNA-driven assembly⁹⁸.
44 This figure was adapted from Ref. ³⁵.

45

1 **Figure 3. Structure an assembly of hepatitis B virus core. (a)** The HBV capsid is
2 constructed from 120 dimers arrayed with T=4 icosahedral symmetry. One of the 60 icosahedral
3 asymmetric units, an AB and CD dimer, are highlighted against on the 20 triangular facets that
4 surface an icosahedron. Shown are two overlaid crystal structures of HBV (blue, PDB 2G33)
5 and HBV with bound HAP1 assembly effector (red, PDB 2G34). More red is visible as HAPs
6 cause the capsid to expand. Sixty HAPs are bound with high occupancy to a pocket in the C
7 subunits (two are shown as white squares)⁴¹. **(b)** Dimers from the capsid (yellow) are
8 symmetrical and identical. Asymmetric free dimers (blue, PDB 3KXS) are presumably in an
9 assembly-inactive conformation. **(c)** A free dimer is color coded to show the chassis (blue) that
10 is identical to the dimer from capsid and the three mobile subdomains (red, green, and orange).
11 The subdomains are connected to the chassis by flexible joints, either Gly or Pro. Movement of
12 one subdomain will force the others to shift their positions. A HAP molecule (magenta), modeled
13 into its binding site, coordinates mobile subdomains with the chassis, facilitating the transition to
14 an assembly-active state⁴⁰. **(d)** HAPs accelerate assembly, even without misdirecting the
15 reaction. Assembly of 10 μM Cp initiated by ionic strength in the presence of 0 to 15 μM HAP1⁹.
16 The inset micrographs show that 2 μM HAP1 yields spherical virus-like particles very fast,
17 whereas higher HAP concentrations yield aberrant structures that scatter much more light.

18

19 **Figure 4. Structure of Sindbis virus. (a)** Surface view, looking down the icosahedral 2-fold
20 axis, of Sindbis virus at 20 Å resolution. The triangular spikes are trimers of the E2-E1
21 heterodimer. **(b)** Cross-section through an 11 Å resolution structure of Sindbis virus. The
22 glycoprotein spikes are on the surface of the virus and their transmembrane region is seen
23 traversing the lipid bilayer (transmem. helices). The nucleocapsid core is internal to the lipid
24 bilayer with the RNA appearing disordered. The capsid and glycoproteins of alphaviruses both
25 have icosahedral symmetry and their symmetry axes are aligned. This is in contrast to most
26 enveloped viruses. **(c)** A surface view of the glycoprotein spikes showing the arrangement of
27 the E2 and E1 proteins. Four monomers of E1 have been fit into the cryo-electron microscopy
28 density of the 9 Å resolution Sindbis map and are shown in yellow, blue, red, and green. The
29 yellow monomers surround the icosahedral three-fold axis while the blue, red, and green
30 monomers surround the quasi-three-fold axis. The density corresponding to the E1 proteins has
31 been subtracted from the density of the entire spike. The remaining density, which corresponds
32 to the E2 protein, is shown in gray. The E2 protein covers the E1 protein and makes up the top
33 portion of the spike. Interactions between E2-E1 heterodimers, along with those within the
34 heterodimer, are thought to stabilize the spike complex. **(d)** A model of the allosteric changes
35 that occur during assembly of the viral glycoproteins. The polyprotein is cleaved by cellular
36 proteases to the individual proteins (green, E3; blue, E2; red, E1; 6K is not shown for clarity).
37 Dimerization of pE2 and E1 to (pE2-E1) is followed by trimerization of the heterodimer, (pE2-
38 E1)₃. Last, cleavage of E3 from pE2 leaves a fusion competent trimer, (E2-E1)₃. Changes in
39 color indicate conformational changes in addition to changes in aggregation state. Panels (a),
40 (b), and (c) were adapted from Ref.^{51, 99}

41

42