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Mini-Review

Understanding the molecular stability of viral capsids from a physics perspective

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ABSTRACT

During its replication cycle, viral capsids undergo various transformations going from protein self-assembly to disassembly. The micro-environment is thought to play a major role in triggering these transformations. In this work, we review the current understanding of the correlation of viral capsid molecular stability with its physical and chemical environment. In particular, by analyzing the forces that act on capsid proteins and the ways in which they are held together, one can gain insight into how capsids maintain their structural integrity under different conditions and how they may be affected by external perturbations such as mutations, drug treatments, or mechanical triggers.

KEYWORDS: viral self-assembly, biophysics, stability, viral disassembly.

INTRODUCTION

The knowledge of the replication cycle of all viruses is relevant in order to decipher the way viruses infect their hosts [1]. From a molecular point of view, the different steps of this cycle represent different "stability" states of viruses. The term "stability" is to be understood here from a physics perspective: a viral capsid is said to be stable if its structural integrity is unchanged during small perturbations of given environmental conditions. Following this line of reasoning, the viral self-assembly corresponds to the emergence of a stable state of the capsid. On the contrary, it is said metastable or unstable if the capsid breaks down during modest perturbations. This last event corresponds indeed to what is called "viral disassembly" in standard virology. Going beyond these simple statements require understanding the molecular origin of replication cycle steps, and in particular the influence of the physical and chemical environment. It is the purpose of this review to present the state of the art of viral capsids' stability investigations from a physics perspective.

Important quantitative investigations in the 1960s of molecular mechanisms underlying self-assembly began with pioneering works of Bancroft [2], and later with works by the group of Zlotnick [3]. In these studies, it was possible to reconstitute *in vitro* virus-like particles (VLP) from purified proteins (with or without the presence of their genome), and to measure kinetics of self-assembly. These *artificial* reconstructions of particles that have similar structures and compositions as real viral capsids allowed to highlight the most relevant features of protein self-assembly mechanisms. This physical approach helped therefore to rationalize most events related to viral self-assembly within cellular environment, but without the full molecular complexity. Later in the 2000s, thanks to the advance of single molecule manipulation techniques, more significant results have been obtained, like the *in vitro* monitoring of active packaging of double stranded DNA inside bacteriophages [4], or the *in vitro* self-assembly of proteins onto a single nucleic acid molecule [5-7]. Overall, this led to the emergence of a new interdisciplinary field named

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"Physical Virology" [8, 9]. Within this approach, new physical tools and theoretical methods are used to measure and quantify the physical properties of viruses. The knowledge of properties, such as morphology, stiffness or stability, is relevant in order to understand how a full replication cycle is achieved.

The outline of this review is the following. First, we will define more precisely the concept of stability down to the single protein scale, and its relation to virus-like particle self-assembly. This will allow us to identify the most important physical and chemical parameters to be considered for the stability. Next, we will provide examples of such parameter tuning that give rise to altered stability. Finally, we will conclude by presenting new research questions to be investigated in this area.

1. Viral capsid structure

The main components of all known viruses are two- or three-fold: a single or multiple genome [10], a self-assembled proteic shell called a *viral capsid* (or viral-like capsid if it does not have the wild-type phenotype), and eventually a lipid envelope surrounding the capsid decorated with envelope proteins [1]. The viral capsid offers therefore a *physical* shield and protection to its embedded genome. We refer the reader to excellent reviews in order to have more information about the structure of viruses [10, 11]. Our focus in this work will be on the proteic shells. These structures are physical realizations of a bidimensional crystal of proteins: in most of the cases, identical proteins are self-assembled thanks to attractive interactions giving rise to a closed, hollow shell structure. Remarkably, the three minimal ingredients in order to perform molecular self-assembly have been highlighted in 2007 by the group of Olson by using *human-scale* building blocks [12]: (i) the molecular building blocks need to have interactions favoring bidimensional sheet-like structures, as opposed to three-dimensional structures; (ii) they need to have a coordination of 5 or 6, such that substructures like pentamers or hexamers are identified within the assembly; (iii) finally thermal motion (or macroscopic agitation within the context of Olson's experiments) helps to nucleate and reconfigure the assembly. As it was shown in reference [12], the concept of self-assembly means here that simple mixing and shaking of the building blocks lead to the emergence of stable ordered complexes, without the need of external or active input. Stability was observed in this case since the same temperature that favored the assembly was not able to dissociate the final structure. We anticipate from a physical perspective that the free energy of the system has reached a deep minimum in this state. In the next section, we will decipher the mechanisms at work during the self-assembly.

2. Viral self-assembly

In order to understand what could alter viral capsid stability, it is necessary to know more about the way protein self-assemble into a viral capsid. We will provide here qualitative arguments that can be understood without a precise mathematical framework. For the sake of illustration, we use a simple model that captures the most important physical ingredients in order to mimic viral capsid structures [13]. Within this model, several proteins are grouped into an effective building block with elastic properties. The use of a simple geometric shape, like a triangle as a building block, allows to reproduce most of the viral shapes observed.

2.1. Energetics of a single molecular bond

Obviously, proteins will self-assemble if they have attractive interactions. The first step in deciphering self-assembly process is therefore to characterize such interactions. Typical building blocks interactions result from the combination of multiple factors associated to the chemical nature of the amino acids within the protein: Van der Waals, electrostatic, hydrophobic and/or hydrophilic interactions, or multiple hydrogen bonding. Without loss of generality, it can be argued that the net protein-protein potential $E(r)$ has the following generic features: a strong small-distance repulsion, the presence of a minimum at intermediate distance, and an asymptotic zero value at large distance. Such a potential is represented for example in Figure 1b, taking the example of pure Lennard-Jones potential. It represents the energetics of a single bond between the building blocks or the protein. The relevant features of this potential are associated with two characteristic

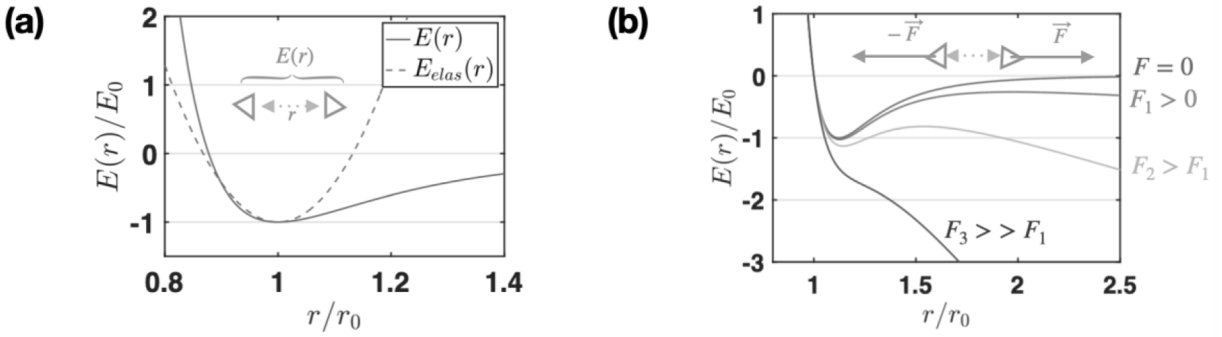

Figure 1. Typical protein interaction potential. (a) The typical potential involved in protein self-assembly and its elastic approximation are shown. Lennard-Jones potential has been chosen for the sake of illustration. The dashed line represents the harmonic approximation of the potential close to its minimum at $r = r_0$ for which $E\ (r = r_0) = -E_0$. (b) Effect of a stretching force F on the interaction potential. When the force is weak, the bond remains, while for larger force, it is broken (no energy minimum at finite distance).

parameters: the energetic depth of the potential well E_0, and the position of the minimum r_0, which is the average distance between neighboring protein's center of mass within the self-assembled structure. At finite temperature, there will be fluctuations in their relative positions. It is legitimate to ask how stable such a bond can be in relation to these fluctuations. The mean energy brought by thermal fluctuations is proportional to the temperature. Its value at room temperature is roughly $kT = 0.5\ kcal.mol^{-1}$ when evaluated for one mole of molecules, or equivalently its value is $kT = 4pN.nm$. The comparison of the adhesion energy E_0 with thermal energy kT provides a way to address the stability of the molecular bond. This can be quantified more precisely by evaluating the rate of bond dissociation, which is a standard result from statistical physics. This is done by using the classical Kramers formula $k_{dissoc} \cong k_0 e^{-E_0/(kT)}$ where k_0 is the typical rate of fluctuations, which depends on the shape of the potential well. One concludes from this formula that this is the ratio between E_0 and kT that governs the dynamics of bond disruption. The larger is this ratio, the smaller is the dissociation rate, and therefore the more stable is the bond. Note that the inverse of this dissociation rate gives the average lifetime of the molecular bond.

At the level of a single molecular bond, there are therefore two major ways to alter the stability of the bond: either decreasing the value of the typical energy E_0, which represents the height of the energetic barrier to be crossed in order to rupture the bond, or increasing the temperature. The latter possibility is not realistic in a biological system, in which temperature is roughly constant. We will come back to this feature later in the argumentation, as this still constitutes a way of challenging the stability. The former possibility is more realistic, as it can be achieved by changes in the micro-environment. For example, changes in the ionic environment might trigger variations in the value of the potential well E_0, and this might happen in different regions of a cell, where ionic conditions are regulated by ionic channels for example.

Yet, there is a third way to alter single bond stability: to use a mechanical force on the bond. This route can be reproduced using single molecule manipulation techniques, and it offers a natural way to probe the interactions between proteins [14]. These techniques rely on the application of a mechanical force on two proteins held by molecular handles [15]. The most current set-up uses either optical or magnetic tweezers, for which the external mechanical forces are provided respectively by lasers or magnetic fields [6, 7]. When an external force F is applied to the interaction potential, the resulting net potential is modified by taking into account the mechanical work of the applied force $E_F(r) = E(r) - F.r$. Such a modified potential is shown in Figure 1b for different values of the force. It is observed that the main effect of this force is to lower the energetic barrier to be crossed in order to break

the bond, or even to suppress this barrier. As a consequence, the lifetime of the bond is reduced by the presence of the force, and this is what explains the breaking of the bond. Useful quantitative information can also be obtained by measuring the average distance $< r >$ of the protein-protein bond as a function of applied force F without reaching the rupture. For most of the molecular potentials, there is a linear relationship between the force applied and the equilibrium distance, $F = k_{elas}(< r > - r_0)$. This formula reminds the reader of the classical mechanical response of a macroscopic spring. It shows that, even down at a molecular scale, an elastic response of a molecular bond is observed. This is not that surprising, given that most of the spectroscopic techniques like NMR or infrared spectroscopy rely on the detection of such harmonic vibrations of molecular or chemical bonds [16].

The elastic constant k_{elas} contains information on the curvature of potential with respect to distance, as it is proportional to the potential well $k_{elas} \sim E_0/r_0^2$. Indeed for small perturbation around the potential minimum, the true potential can be approximated by a harmonic function of the distance, as shown in Figure 1a [17].

2.2. Energetics of protein self-assembly

So far, we only discussed the stability at the scale of a single protein-protein bond, and it is not clear whether these results might apply to an extended structure like a viral capsid. Let us consider the process of self-assembly in a solution with disperse building blocks. In order to start a new assembly, the building blocks need to find themselves in the volume of solution under consideration. The probability of successful contacts between building blocks and the nucleation of a new assembly increases with the concentration of the solution. After a concentration threshold has been reached, the blocks will grow into a curved surface. The growth of the surface induces an energetic cost associated to its rim [18]: indeed, a building block far from the rim has more neighboring blocks than the one sitting on the rim. This energetic cost is called a line tension and it represents a penalty for the growth. In the case of a spherical or icosahedral VLP, the length of the line first increases, and then decreases. Therefore there is a *global* energetic barrier for both assembly and disassembly of the building blocks, as shown in Figure 2. In this configuration, each building block, which are triangles in our model representing the assembly of three proteins, can bind three other triangles. That is, if one wants to remove a given building block from the assembled structure, one has to provide three times the energetic cost of a single bond. As a consequence, the structure *per se* of the assembly increases its stability with respect to rupture. Note that the presence of the genome further stabilizes the assembly [18].

3. Challenging the stability

In the previous sections, by exploring the energetics of viral self-assembly, we have suggested several origins for capsid stability. This knowledge can now be used in order to experimentally challenge the stability. We present below a list of non-exhaustive scenarios.

3.1. Chemical trigger

Metastability is an inherent part of viral life cycle as the entry and exit of viruses into host cell correspond respectively to the loss or gain of stability of the capsid. Within our physics-based analysis, the genome release in a cell is associated to cellular chemical triggers. Several examples of such mechanisms are available in the literature. We illustrate this on a well-documented case. For SV40 virus, it has been shown in 2009 for example that a specific mutation can alter the ability of SV40 mutant to disassemble [21]. The rationale was that the wild type SV40 has binding sites for calcium between pentamers which are responsible for capsid metastability: in the presence of calcium, the capsid is stable, while in a calcium-free medium the capsid disassembles, unlike mutant capsids. The calcium plays a similar crucial role in the case of cowpea chlorotic mottle virus (CCMV) stability [22, 23]: this small plant viral capsid exhibits two different configurations upon local pH change: in the absence of calcium, the capsid swells its radius by 10% when the pH is increased from 5 to 7, while in the presence of calcium, no swelling is observed. These two

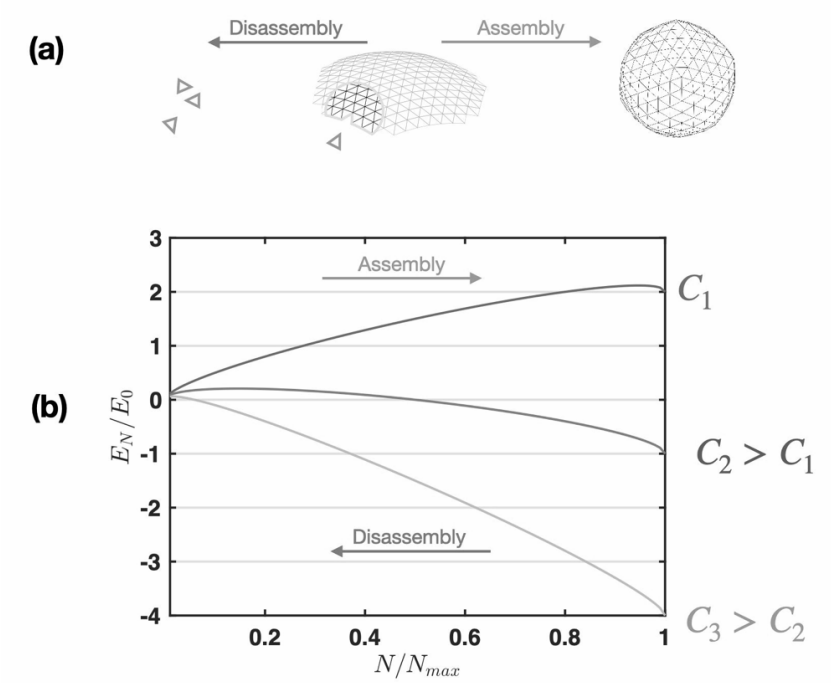

Figure 2. Energetics of self-assembly. (a) Cartoon of self-assembly scheme. (b) Energy of intermediate assembly as a function of the number of incorporated building blocks. The formula used to plot these curves can be found in different works from the literature [18-20]. The three curves correspond to the three different solution concentrations of the building blocks. Once the concentration is larger than the threshold concentration (here for C2 and C3), the assembly becomes favorable.

examples show that calcium is an effective trigger of metastability. This trigger is very relevant for viruses, as calcium levels are tightly regulated within cell compartments and are therefore subjected to concentration change. From a physical point of view, calcium ions effectively affect the height of energetic barrier to be crossed in order to destabilize a viral capsid.

Recently, it has been shown explicitly that it is possible to reversibly modulate the structure of virus-like particles by changing the pH [24]. These authors used coat proteins of CCMV functionalized with a hydrophobic buoy whose properties are tunable with pH or temperature. As a result, they could form T=3 icosahedral capsids at pH=5, and T=1 capsids at pH=7.5. The T-number is associated to a structural classification of icosahedral capsids. The change from one form to the other was reversible, but most importantly, the only way to go from one form to the other is to disassemble and reassemble. In this case, the pH plays the role of affecting the stability. This is another quantitative example of the influence of micro-environment on the stability/metastability of viral capsids.

3.2. Temperature trigger

As it was mentioned earlier, thermal motion might help to cross an energetic barrier if its amplitude is large enough. Focusing on the energetic barrier of protein bond inside a self-assembled capsid, it is possible to disassemble a capsid by raising temperature. Additional information about the protein bond strength can also be obtained by changing the temperature. This was illustrated recently by our team and by others [25-27]. Destabilization of adeno associated virus (AAV) vector particles induced by increasing controlled temperature was investigated at the single capsid level by imaging the population of particles using atomic force microscopy (AFM). Depending on the temperature, it was observed that genome release can occur *in vitro via* two alternative pathways: either the capsid remains intact and

linearly ejects the genome, or the capsid is ruptured, leaving genomic ssDNA in a compact entangled conformation. In both cases, one or several proteins are lost from the original structure, and therefore an energetic barrier has been crossed. From a quantitative analysis of the different populations, the authors concluded that partial or full destabilization of the capsids is initially associated to an energetic barrier whose height is roughly 20 kT, which is a large value considering that spontaneous barrier crossing occurs for energies of the order of kT. Thus, by dramatically challenging the stability up to the breakpoint, we were able to access an equilibrium property, namely the height of the energy barrier that holds the protein inside the capsid. Overall, these results may explain why AAV is one of the most stable capsids in the viral era, as the price of disassembling is high.

3.3. Direct mechanical trigger

Atomic force microscopy is a technique allowing to both image and exert forces on molecular structures deposited on surfaces, as depicted in Figure 3a [28]. In 2004, it has been used, for example, to measure the elastic response of bacteriophages [29]. This technique is so precise that it can distinguish a wild-type bacteriophage from a mutant without genome, based on their difference in measured stiffness [30].

The stiffness is defined indeed as the ratio between the applied force and the deformation of the capsid. In other words, for two different stiffness, the required force to obtain the same deformation in both cases is larger for the larger stiffness. Since the deformation is made possible thanks to the relative motion of proteins, it is clear that the measure of the stiffness also provides information on the bond strength potential. The stiffness is directly proportional to the strength of the protein-protein bond, the precise coefficient of proportionality depending mainly on geometrical features of the capsid, like its radius and its thickness.

Within the context of continuum elasticity, the strength of protein-protein bond is characterized by the so-called Young modulus Y, which has the physical dimensions of pressure. Knowing the depth of the bond's potential E_0, and the position of its minimum r_0, the order of magnitude of Young Modulus can be estimated by the formula $Y \sim E_0/r_0^3$. Speaking about orders of magnitude, Young modulus ranges from $10^{-3} GPa$ for polystyrene foam to $10^3 GPa$ for graphene or diamond, which are among the toughest material. Typical values for viruses range from $0.2 GPa$ for small RNA viruses like CCMV to $1.8 GPa$ for $\phi 29$ bacteriophages, which are thought to be among the toughest capsids as they need to sustain the mechanical pressure of their high density genomes (double-stranded DNA) [29].

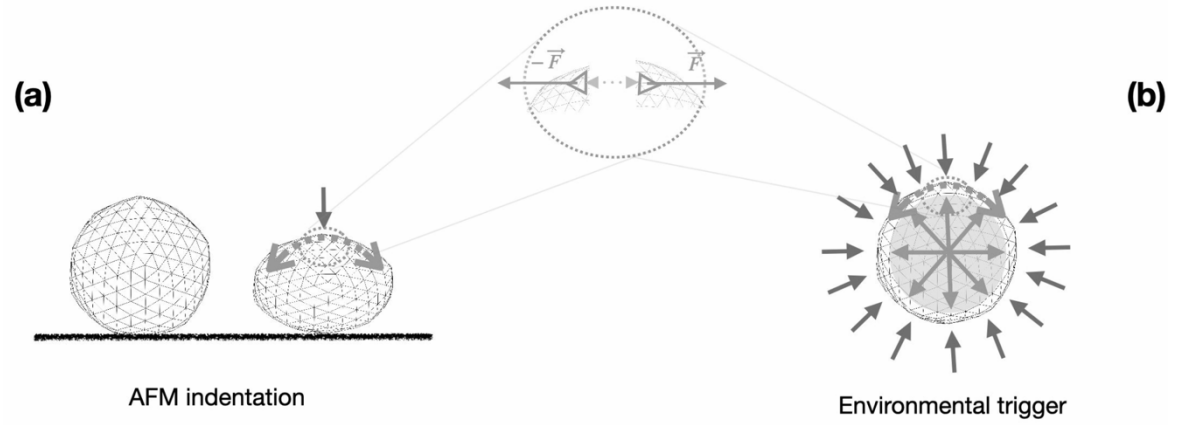

Figure 3. Mechanical trigger of stability/instability. (a) AFM tip exerts an external force (plain vertical arrow) and induces a deformation of the capsid, which is associated to lateral stress (dashed double arrow). (b) Osmotic pressure difference (plain inward and outward arrows) induces mechanical stress within the viral capsid (dashed double arrow). In both cases, protein bonds are put under tension, and are susceptible to breaking.

Assuming that the capsid has a radius R and thickness h, the relation between the stiffness measured by AFM and the Young modulus is given by $k_{cap} = A\frac{Yh^2}{R}$, where A is the numerical prefactor [31]. As a consequence, by measuring directly the stiffness of viral capsid, it is possible to deduce its Young modulus Y, and therefore the typical energy of the bond E_0. Notice also that under the application of large forces, viral capsid rupture can be observed and characterized [31-33].

3.4. Indirect mechanical trigger

There is another indirect way to mechanically probe the bond between the proteins. Indeed, it is a classical physics result that a difference in osmotic pressure between inside and outside of a shell, which is permeable to the solvent, induces a lateral stress or tension within the shell, as depicted in Figure 3b. Quantitatively, for a capsid of size R and thickness h, a pressure difference Δp induces a mechanical stress σ (with dimension of a pressure) which is written as $\sigma = \frac{2\Delta pR}{h}$. This is the Laplace law. This law helps to explain why osmotic shock can be responsible for capsid rupture [17, 34]: the increase in osmotic pressure difference caused by changing the environment of viral capsid induces large tension within the protein layer, and the process is very similar as the application of pulling forces on each protein bond, up to the rupture. We discuss below a few examples, showing different aspects of Laplace law for viruses.

A first example of this link between the pressure and the strength of the capsid is given by the maturation of some viruses. This is the case, for example, for bacteriophage lambda [35]. The packaging of double stranded DNA into a pre-assembled capsid induces a strong mechanical pressure on the inner wall of the capsid due to the high molecular density and small volume of the capsid. In the case of bacteriophage lambda, this process of DNA packaging is accompanied with 20% increase in radius (from 50 nm to 60 nm), and thickness reduction. This swelling helps partially to reduce the stress within the capsid, but additional gpD proteins are still needed to reinforce the strength of the capsid. Otherwise the capsid breaks down before full wildtype DNA length packaging.

As mentioned before if the osmotic pressure inside a capsid is larger than the outer osmotic pressure, this leads to some *positive tension* within the shell (proteins are pulled apart). In contrast, if the outer osmotic pressure is larger than the inside osmotic pressure, we expect some negative tension within the shell (proteins are pushed against each others). This has been quantitatively measured by atomic force microscopy in a recent work on brome mosaic virus (BMV) subjected to excess osmotic pressure or crowding: the measured stiffness of BMV capsid increases with outer osmotic pressure [36]. However, note that if the pressure is increased beyond a threshold, then the stiffness decreases again, before the capsid breaks down.

Ongoing research works on respiratory viruses further highlight the link between the stability of viral capsids and their local environment [37]. The key parameter in this context is the relative humidity of surrounding air. Indeed, respiratory viruses are found in the droplets and aerosols emitted by a sick host, and the fate of these particles is intimately related to the relative humidity of ambient air, which determines the dynamics of droplet or aerosol evaporation. As a consequence, major changes in osmotic pressure inside viral capsids are expected within droplets due to evaporation. This might explain why viability of viruses depends strongly on the relative humidity and therefore on the seasonality of some epidemic.

CONCLUSION

In this review, we have analyzed the stability of viral capsids from a physical point of view. We have highlighted the important role played by the protein-protein bonds that hold the capsid together.

As a consequence, we could correlate on the one hand perturbations of this protein potential such as changes in the direct environment of the viral capsid to changes of viral global stability, and on the other hand the loss of stability such as the one induced by mechanical constraints on viral capsid to the modification of protein bond potential.

All the mentioned works are part of a two-decade old approach to study viruses: the physical virology. During this period, new technical approaches and new concepts have helped traditional molecular virology to progress further in understanding all the steps of viral replication. We hope that the present synthesis might help virologists to catch these news ideas coming from a complementary field. More discoveries are to come with the progresses in physics and virology.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interests.

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