Advanced Review

Controlling forces and pathways in self-assembly using viruses and DNA

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The ability of both viruses and DNA to self-assemble in solution has continues to enable numerous applications at the nanoscale. Here we review the relevant interactions dictating the assembly of these structures, as well as discussing how they can be exploited experimentally. Because self-assembly is a *process*, we discuss various strategies for achieving spatial and temporal control. Finally, we highlight a few examples of recent advances that exploit the features of these nanostructures.
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INTRODUCTION

Over the last years, great progress has been made in tuning the physical and chemical properties of biopolymers. These properties form the basis for self-assembly behavior, which in turn, has enabled numerous applications beyond the biological realm.[1,2] This 'bottom-up' approach to nanostructure fabrication is extremely attractive, if it can be properly executed. In many of these contexts, proteins and DNA are not only biologically active, but also serve as structural and responsive materials.

The well-defined nanoscale features from either virus or DNA scaffolds open the door to numerous applications. In the biomedical arena, drug delivery, sensing and imaging all rely on size control and the selective presentation of functional groups. The use of natural materials eliminates concerns with respect to biodegradation and reduces issues related to biocompatibility. Furthermore, self-assembly potentially allows for a relatively simple route to the integration of multiple features, making it an attractive fabrication strategy. In addition to uses in biomedical contexts, self-assembled scaffolds potentially address key challenges in the field of opto-electronics. Excellent control over the formation of inorganic nanoparticles and nanowires can be achieved by using biopolymer scaffolds as sacrificial templates. The resulting objects are of great interest in device fabrication due to their dimensions and unique optical and conductive properties. One particularly interesting example, discussed later, is the use of these nanoscale scaffolds for light-harvesting applications.

From a materials science perspective, mechanical considerations of the assembly building blocks are necessary. In addition to given structural features (e.g., helicity), the persistence length p is a key parameter. This quantity p is defined from the exponential decay of the orientational correlation between tangent vectors.[3] Roughly speaking, p is the length between significant changes in direction. Thus objects with lengths much less than p tend to be rigid, whereas those with lengths much greater than p tend to be flexible. For single-stranded DNA (ssDNA), $p <$ 5 nm, whereas for double-stranded DNA (dsDNA), $p \approx 50$ nm.[3,4] Polypeptides are more chemically diverse than DNA and thus p depends strongly on the secondary structure: disordered chains have $p \approx 2$ nm, whereas alpha-helices have $p \approx 200$ nm.[3] It is apparent that a combination of both rigid and flexible segments is required to achieve functional and well-defined structures.[5–9] For more complex structures that can actuate, an understanding of the transitions between different states is also needed.[10]

The large difference in persistence lengths between dsDNA and ssDNA translates to distinct mechanisms for virus assembly. Most dsDNA viruses pre-assemble the protein capsid and load the dsDNA by means of an energy-driven motor. In contrast, ssDNA viruses typically use the ssDNA as a nucleation site, around which the capsid is formed. As a result of these assembly differences, the density of nucleic

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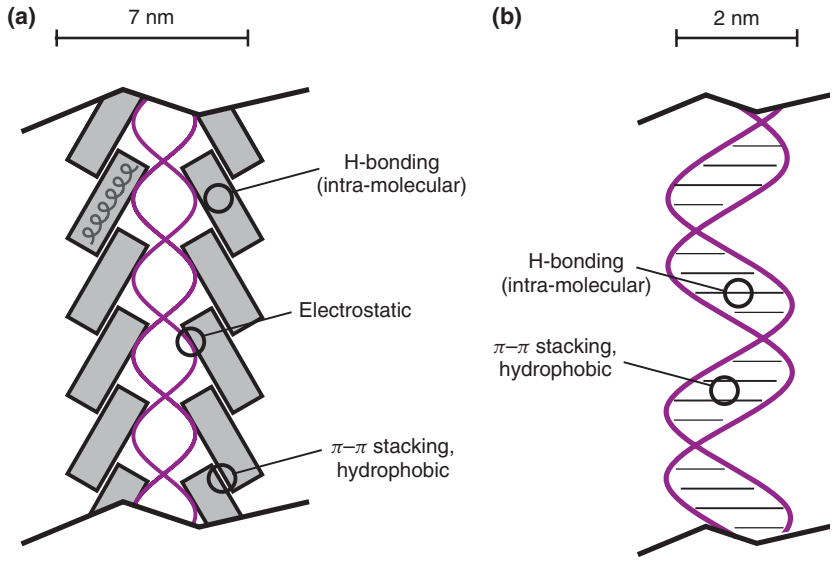

FIGURE 1 | Schematic of relevant interactions within two prototypical structures. (a) Rod-like virus (e.g., fd) and (b) double-stranded DNA.

acid is much higher in dsDNA viruses than in ssDNA viruses. This is a clear example of the correspondence between 'subunit' properties and self-assembly mechanisms. The above concept also holds in other contexts, such as DNA self-assemblies.[11] In that case, rigidity becomes a design variable, which can be altered by changes in the effective diameter d; noting that flexural rigidity scales as d^4 for both hollow and solid cylinders.[12] Such an approach can indeed be exploited to build significantly larger and more rigid DNA objects.[13–15] In addition, so-called crossover motifs in DNA, created by strand exchange throughout multiple helices,[16–19] have also been used to enhance rigidity. Double-crossover motifs increase rigidity by about a factor of two,[20] and it is expected that triple-crossover motifs will have a much greater effect.

While assembly through (mainly) a single type of interaction is conceptually simple, it is likely to be fundamentally limited in terms of structural diversity and robustness. By contrast, properly balanced multiple interactions will lead to a wider range of structures and more forgiving conditions for assembly. Indeed, the control of multiple interactions during assembly remains the ultimate goal of assembly 'by design.' The remainder of this review focuses on (1) chemical and electrostatic interactions between constituents, (2) factors affecting the *process* of self-assembly, (3) and selected examples that illustrate exciting future directions.

INTER- AND INTRAMOLECULAR FORCES DRIVING SELF-ASSEMBLY

The interactions underlying self-assembly are numerous,[1,2,21] but we focus only on those pertinent to *aqueous solutions*. Figure 1 highlights dominant interactions within two archetypal self-assemblies: viruses and DNA structures.

Hydrogen-Bonding Effects

Hydrogen-bonding in particular is responsible for direct interactions between both backbones and side-chains of biopolymers. In proteins, these interactions can be of either short- or long-range nature, leading to secondary structures such as alpha-helices and beta-type structures. However, long range intramolecular H-bonding in proteins remains a difficult challenge to predict and control. In DNA structures, hydrogen-bonding is mainly responsible for highly specific molecular recognition (i.e., base-pairing). However, because sequences (or parts thereof) can repeat, off-target interactions are possible and must be minimized. Therefore sequences underlying a DNA nanostructure must be designed to strike an optimum between stability and specificity. This optimization can be achieved by restriction of similar and symmetric sequences, repetitive sequences, and GC content. Because of the increasing number of interactions as assemblies grow in size, computational tools have been developed to facilitate sequence design.[22,23] Other hydrogen-bonding interactions among DNA bases can lead to non-Watson–Crick structures. For example, guanine-rich strands can form four-stranded 'G-quadruplexes', and cytosine-rich strands can form intramolecular 'i-motifs' (especially at low pH).[24] Importantly, the strength of a single hydrogen-bond is $\approx k_BT$.[25] Therefore the resulting interactions are labile, and readily reversed, near room temperature. Interactions strengths near k_BT also allow thermal annealing to be used as a means to minimize defects.

Hydrophobic Effects

In water, hydrogen-bonding plays another crucial role, being responsible for the so-called 'hydrophobic effect'. At the molecular scale this effect is manifested as a competition between water molecules forming a network and their individual degrees of freedom.[26,27] Although the crossover between these two regimes is broad, at length-scales less than about 1 nm, the energy penalty scales with volume. At length-scales above roughly 1 nm, an interface is formed and the energy penalty scales with area. Such crossover behavior suggests implications for self-assembly: volume effects are likely to dominate at initial stages (e.g., precursors or subunits), whereas area effects are more likely to be important for larger intermediate structures and nearly complete assemblies. The analogy of this crossover behavior with nucleation phenomena is evident. In the context of assembly subunits, hydrophobic patches or domains generally form the basis for weakly directional interactions. These domains are not buried internally, but rather, they are surface-exposed. In a manner similar to protein subunits, the hydrophobic bases of DNA side-chains are driven away from solution, toward the interior of the double-helical chain. We note that the penalty for breaking substantial numbers of hydrogen bonds, together with entropic effects, is also responsible for the hydrophobic cores found in micelles and vesicles.[21,28–30]

Electrostatic Effects

Another key player in aqueous self-assembly is electrostatics. While direct attractive or repulsive effects are fairly well-understood (e.g., the formation of salt bridges), the situation is typically far more complex. Charged groups, including the phosphate backbone of nucleic acids and appropriate protein side-chains, are surrounded by counterions and co-ions. This cloud of ions is responsible for a concentration-dependent 'screening' that progressively reduces the interaction strength.[31] In addition, ion valency and size lead to measurable and relevant higher-order effects. As a result, the interplay of numerous charged species can give rise to counter-intuitive results, such as the net attraction between like-charged objects.[32]

For DNA self-assembly, electrostatic effects are relatively straightforward. It is now understood that DNA–DNA pairing (repulsion) is strengthened (weakened) with increasing ionic strength, due to charge screening. An example of the direct effect of ion size and valence comes from DNA self-assembly, where Mg^{2+} ions play a key role in stabilizing dsDNA.[33] In fact, attraction between DNA strands is substantially stronger in the presence of higher valence ions,[34,35] and can lead to the formation of toroidal structures.[36,37] Beyond molar monovalent and millimolar divalent ion concentrations (i.e., nonphysiological), the transition from B-DNA (right-handed) to Z-DNA (left-handed) can be induced.[38]

Muthukumar et al. have modeled electrostatic effects in both dsDNA virus and ssDNA virus assembly. The role of the virus coat protein during assembly is, in part, to neutralize the charge of the nucleic acid. For dsDNA viruses, the effect of electrostatics is minor (due to counterion condensation) and repulsive in nature. The assembly process is instead dominated by a combination of bending energy, excluded volume, and confinement.[39] For ssDNA viruses, electrostatics play a far more significant role. The negative charge of the DNA is mainly neutralized by the presence of highly basic 'arms' on the coat protein subunits. As a result, the ssDNA exists as a loosely packed shell, detached from the interior capsid surface. Using a self-consistent approach, it was shown that genome length does not correlate with capsid geometry, but rather, with the net charge of the subunit peptide arms.[40]

Another ionic effect comes from the Hofmeister series,[41,42] which divides anions into two classes. The so-called chaotropes are weakly hydrated, and tend to increase the interfacial tension between dissolved macromolecules and the aqueous environment, favoring reduced solubility. Chaotropes are also capable of directly binding to macromolecules, causing polarization, which tends to increase solubility. This competition leads to nonlinear behavior with chaotrope anion concentration. On the other hand, the so-called kosmotropes are strongly hydrated, and in addition to the interfacial tension effect, can polarize the first hydration shell of macromolecules. The above two effects work together to reduce solubility in a concentration-dependent fashion. For both types of anions, it has now been shown that the bulk properties of water (i.e., its hydrogen-bonding network) are *not* altered.[43] The relative position of a particular anion within the Hofmeister series thus has important consequences for macromolecular solubility. In contrast to nonspecific charge-screening effects, chaotropes were found to reduce the stability of dsDNA (i.e., depress the T_m).[44] The Hofmeister trend was observed for this destabilizing effect, with thiocyanate SCN- being the most potent anion. A more recent study examining kosmotrope effects (e.g., F^- and SO_4^{2-} on dsDNA found no stabilizing role.[45] This apparent violation of the Hofmeister series was interpreted as being due to anion-specific interactions with nitrogen and oxygen atoms in DNA. We note that although Hofmeister effects are generally observed at ionic strengths greater than 0.1 M,[26,45] this is well within the range of

physiological relevance. To our knowledge, however, there has been little exploration of Hofmeister effects on virus or capsid assembly.

Stacking Effects (π–π)

Another direct, but noncovalent, interaction between building blocks is π–π stacking.[46] Aromatic side-chains in proteins provide ample opportunity for these interactions. In addition to contributions to protein folding (i.e., intramolecular stability), π–π stacking can mediate interactions between distinct capsid subunits (i.e., intermolecular stability). Arnold et al. have shown that π–π stacking between tryptophan and phenylalanine on adjacent coat proteins is largely responsible for the unique circular dichroism spectrum of the rod-like filamentous phage (fd) virus.[47] Using the quasispherical cowpea mosaic virus (CPMV) capsid, an elegant chemical approach was used to demonstrate tyrosine–tyrosine interactions, and subsequently stabilize them by covalent cross-linking.[48] A more recent example is in the adeno-associated virus, where it was shown that a conserved phenylalanine in the capsid subunit is essential for assembly and genome packaging.[49] In this case, the stabilizing interaction is believed to be intermolecular π–π stacking between phenylalanine and a proline in an underlying subunit. Similarly, DNA bases contribute to overall assembly stability, in part, through π–π stacking along the helical axis. As expected, alteration of stacking through the use of 7-deazapurine analogs slightly destabilized the dsDNA.[50] In addition, DNA charge transport properties,[51] and the fluorescence of incorporated 2-aminopurine,[52,53] rely on the π–π stacking interaction. Thus stacking provides a sensitive measure of DNA stability and conformational changes.

FACTORS AFFECTING SELF-ASSEMBLY

Concentration

From a mass-action perspective, simply increasing the (protein or DNA) subunit concentration will drive assembly of larger structures. However, for most virus systems, the reverse path due to dilution has not been demonstrated, suggesting that virus assembly may not be a true equilibrium situation. Bearing this point in mind, relatively simple viruses have provided the most experimental results to date. Tobacco mosaic virus (TMV) is perhaps the best known, as it was the first virus to be reconstituted *in vitro* (see Butler's work[54] for a review). For rod-like and most quasispherical viruses, the stoichiometry [coat protein]:[nucleic acid] $\gg$1. Therefore, it is not a surprise that protein–protein interactions dominate the initial stages of virus assembly. Under appropriate initial conditions, TMV coat proteins exist as a distribution of small aggregates,[55] which in this case are collectively referred to as subunits. When the pH is lowered, the subunits evolve into two-layer disks, in a concentration-dependant manner. In particular, above a critical concentration, subunits will preferentially assemble into disks–behavior that is reminiscent of amphiphile micellization.[21,56] The transition to final rod-like particles can be accelerated by up to one order of magnitude by the addition of disks, suggestive of cooperative assembly. Also accelerating the rate of assembly is the TMV nucleic acid (i.e., RNA) which is believed to nucleate the formation of rods by threading through the central disk cavity.[55] These protein–nucleic acid interactions strengthen the relatively weak and nonspecific (i.e., hydrophobic) protein–protein interactions.

Quasispherical plant viruses such as cowpea chlorotic mottle virus (CCMV) have provided an additional perspective. Capsid assembly (i.e., without nucleic acid) may be particularly useful to probe early stages of assembly, due to the exclusive focus on protein–protein interactions. As expected, the rate of CCMV capsid assembly increases with the concentration of subunit, although there is a corresponding increase in the fraction of misformed products.[57] These misformed products are interpreted from the postulated mechanism of capsid assembly: short-time kinetic data indicate association from subunits into pentamers, and capsid assembly then proceeds by the cooperative addition of subunits to pentamers (i.e., the intermediate nucleating species). As the subunit concentration increases (>20 μM), however, pentamers accumulate and thereby deplete the free subunits needed for proper capsid growth. This high pentamer concentration therefore favors misformed products, presumably brought about by pentamer self-association. Mechanisms to minimize improper assembly include a slow nucleation step and 'autostery'. The latter concept proposes that subunit flexibility allows for assembly-incompetent intermediates, keeping free subunits available through dissociation steps.[58] Note that such flexibility requirements highlight the need to bear in mind the mechanical properties of subunits, as alluded to earlier. In the presence of RNA, CCMV assembly is thought to follow a different pathway, primarily through the initial formation of planar hexamers, which are induced to curve by contact with RNA.[59] Irrespective of the actual intermediate type (pentamer vs hexamer), protein–nucleic acid interactions stabilize the resulting virus particles, as compared to protein capsids, which can form various unusual structures (e.g., tubes, double-shells).[60]

Brownian dynamics simulations provide a useful counterpoint to experimental approaches. For example, Hagan and Chandler[61] have verified the so-called 'kinetic trap' (i.e., improper assembly at high subunit concentration). More importantly, they have also found evidence for autostery (i.e., assembly-incompetent intermediates). One of the virtues of simulations is the monitoring of all possible species, revealing different modes of assembly. Indeed, the above simulations reveal that although individual subunit addition is predominant, a significant amount (>30%) of cooperative addition can occur.[61] These findings challenge the general applicability of sequential subunit addition, revealing that the balance of entropy loss/energy gain in some cases favors multimer addition. Furthermore, cooperative addition during assembly mitigates the dependence on the availability of free subunits, increasing robustness.

DNA nanostructures present an opportunity to rationally design subunits, and presumably exert greater control over assembly. Some of the early structures were constructed from subunits with high degrees of symmetry, for reasons of simplicity, efficiency, and attractiveness toward generating macroscopic objects or surfaces.[62,63] On the other hand, the disadvantages of high symmetry are apparent for creating discrete nanoscale objects, since excessive concentrations will lead to poorly-defined aggregates. To minimize improper assembly, concentrations for the assembly of discrete DNA nanostructures are generally sub-micromolar.[64–66] Under such conditions, the yields of desired structures can reach 90% or even higher. Interestingly, it has been demonstrated that DNA assembly into different structures can be guided by the concentration of subunits.[65] Here the subunit was deliberately designed to be symmetric three-point star motif. By controlling concentration, either tetrahedra dodecahedra, or 'buckyballs' could be generated from essentially identical subunits. However, as noted above, undesired aggregates became increasingly likely with concentration, reducing the overall yield. An alternative approach to creating DNA structures called 'origami' relies on many unique strands interacting with a long single-stranded scaffold, and thus is far less sensitive to the effects of concentration and stoichiometry.[67] DNA assemblies clearly present themselves as models to test assembly and further our understanding.

Solution Conditions

Because proteins are polymeric, their local environment can modulate pK_a values and consequently lead to differential pH sensitivity. The electrostatic nature of protonation/deprotonation indicates that the ionic character of the solution will be of equal importance. In viruses, the underlying mechanism appears to be the protonation state of carboxyl side-chains,[55,59,60] which mainly alters interactions between the subunits and nucleic acid. For example, the swelling of CCMV with increased pH is a result of deprotonation-induced repulsion. The combined effects of pH and ionic strength impact the assembly of both viruses and simpler capsids,[54,55,59,60] and again we refer to TMV and CCMV as examples. Depicted in Figure 2a is a map of the various transitions that can occur

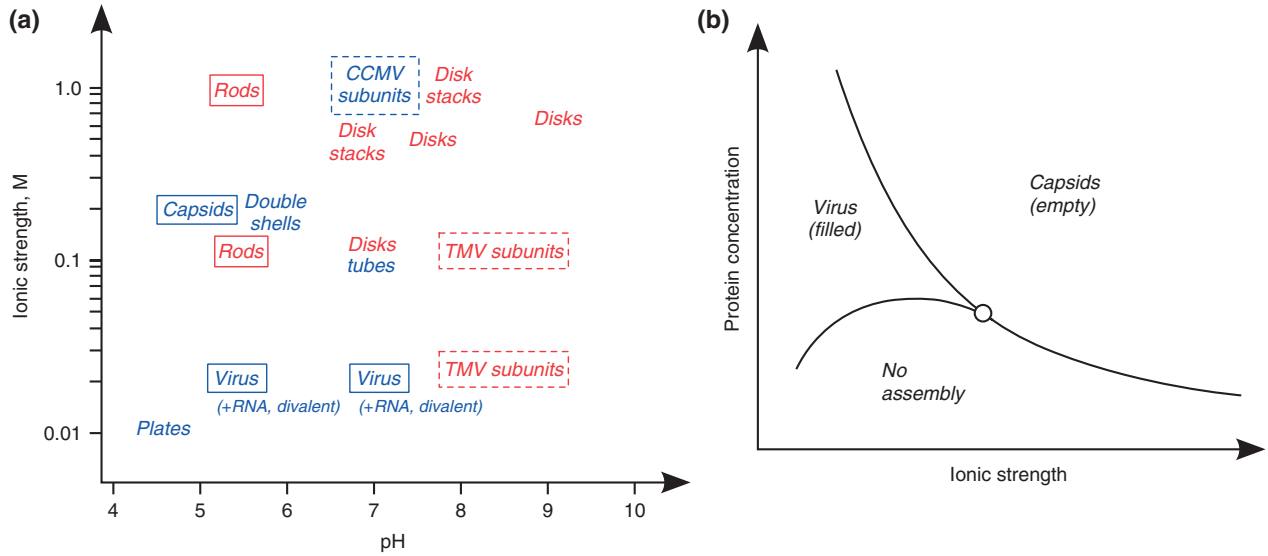

FIGURE 2 | (a) Map of pH and ionic strength effects on TMV (red) and CCMV (blue) coat protein assembly. (Reprinted with permission from Refs 55, 59, 68); (b) Map of protein concentration and ionic strength. (Adapted from Ref 70)

between TMV subunits, intermediates, and assemblies. Also contributing to the stability of both TMV and CCMV are divalent cations such as Mg^{2+}, presumably through salt-bridging effects and/or charge neutralization. However, all divalent cations are not equal, as Ba^{2+} has been shown to have minimal stabilizing effect.[68] Even in the case of capsid assembly (i.e., without nucleic acid), deprotonation is still relevant, as it affects the relatively weaker electrostatic interactions between subunits, allowing hydrophobic contacts to predominate.[57,60] As briefly mentioned earlier, CCMV capsid assembly is especially sensitive to solution conditions. Depending on the pH, ionic strength, and presence of multivalent cations, various morphologies can be induced.[60] These morphologies include quasispherical capsids, double-shell forms, various tubular shapes, and even plate-like structures. As compared to the conditions needed for true virus assembly (protein + nucleic acid), *lower* pH and *higher* ionic strength are required to achieve hollow quasispherical capsids.[60,68] It is worth noting that both capsid assembly and intact virus assembly display hysteresis in their assembly, suggesting that the pathways are not truly equilibrium (i.e., reversible) steps. As an example of this hysteresis, the CCMV behaves normally even at pH 5, although assembly cannot occur at that same pH.[60,68] Although CCMV is quite distinct in character from TMV, the underlying interactions are similar in nature, and thus we tentatively superimpose CCMV assembly states onto the TMV map in Figure 2a. Indeed, to obtain 'filled' capsids *in vitro* (i.e., coat proteins + nucleic acid), theoretical models indicate that a balance of electrostatic interactions is necessary,[69,70] achieved by modulation of the ionic strength (Figure 2b). These results illustrate the complexity of an apparently simple 2-component assembly, again highlighting the need for well-defined and tunable models.

Solution conditions used to generate DNA nanostructures do not vary substantially.[13,62–67,71–73] The pH of the solution is typically between 7.4 and 8.3, presumably for physiological relevance and stability. The total ionic strength is generally kept <50 mM, as might be expected, so as to favor hybridization without undue loss of specificity. Lastly, because of its ability to stabilize dsDNA, even at low concentrations, Mg^{2+} is typically used at concentrations ≈15 mM. It becomes apparent that the exquisite specificity (not the strength) of DNA–DNA interactions overrides many of the subtleties found in virus and capsid assembly.

Temperature

For obvious biological reasons, the role of temperature has not been extensively studied in the context of virus assembly. However, the rate of TMV assembly can be increased with temperature,[55] suggesting a thermally-activated process that is consistent with nucleation phenomena. The formation of larger, and misformed, aggregates is also favored at higher temperatures, likely due to increased (hydrophobic) protein–protein interaction strength.[55] Experimentally, increased temperature favors the association of CCMV protein subunits, although specificity does not substantially change.[60] A possible exception, and an interesting avenue to explore further, would be viruses that are harbored in extremophilic (e.g., high *T*/low pH/high salt) bacteria.[74]

In contrast to virus assembly, temperature provides an extremely useful means for regulating DNA self-assembly. Because temperature is orthogonal with respect to concentration, it can aid to deconvolute effects on assembly. DNA–DNA recognition is a collective and reversible process, where the 'melting' temperature T_m of DNA is defined as the temperature at which there are equal populations of single-stranded and double-stranded forms, and provides a useful reference point. The initial state that precedes assembly is often well above T_m, generally only a few degrees below the boiling point of water, so as to minimize any unforseen intrastrand structures (e.g., hairpins, loops). The designed architectures then ideally form as cooling takes place. As might be expected, the assembly time increases with the final size of the object, and may be considered a separate effect.[23] Ignoring such size effects for the moment, most DNA assembly procedures employ a gradual cooling to room temperature over a period of several hours to several days.[62,65,71,75–77] For substantially denser ('origami') structures, the time intervals of cooling are surprisingly short, only a few hours or less.[67,73,78] The shortened interval for assembly may be a benefit of unique strand pairings (i.e., high specificity), as opposed to symmetric subunit self-interactions (i.e., low tolerance for errors). The rapid assembly of small DNA tetrahedra (<5 min) from four unique DNA strands is consistent with the above picture of increased specificity during assembly. Together, these results point to the *rate* of temperature change as a key variable. Indeed, exploiting the rate of cooling is the well-known process of annealing, and in principle allows for error correction during assembly. To date there has been little exploration of annealing routes to improved assembly, although a few reports hint at future possibilities.[79,80] An interesting use of temperature control has been recently demonstrated with ssDNA-coated colloidal particles, where the ssDNA forms hairpin structures that either cause particle aggregation or remain inert, depending on the thermal history.[81]

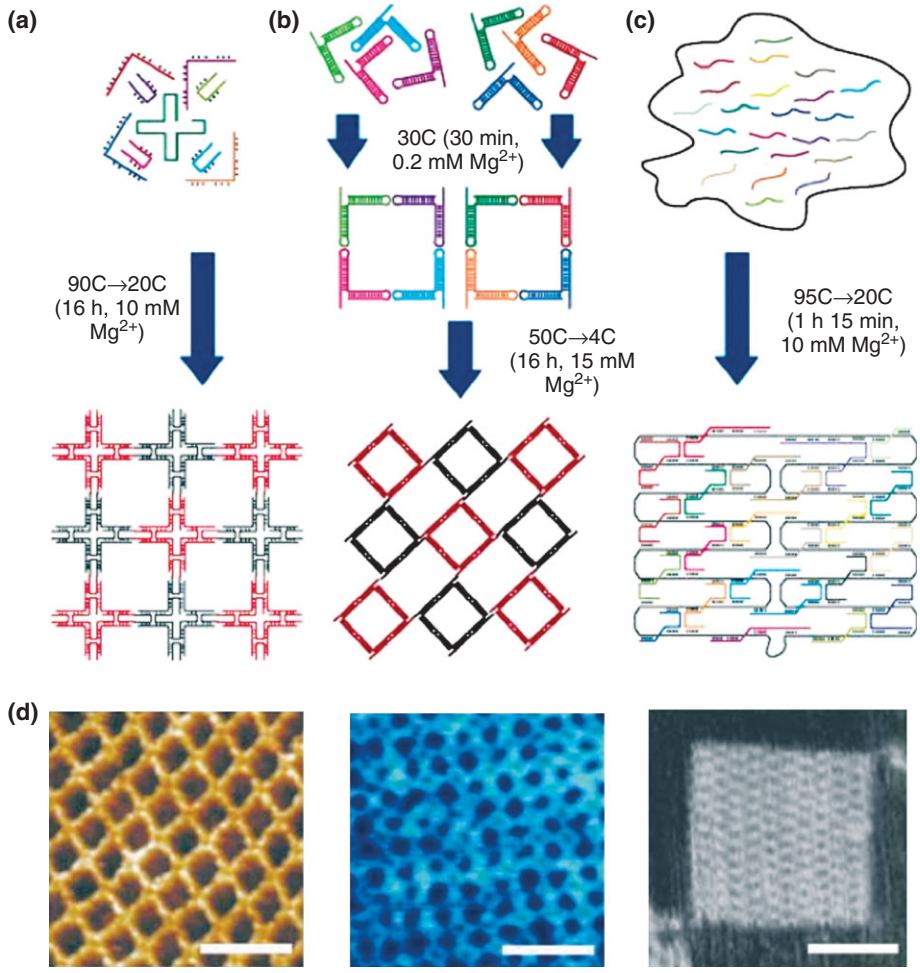

FIGURE 3 | Schematic and examples of strategies for nucleic acid self-assembly. (Reprinted with permission from Ref 82. Copyright 2006 Elsevier) (a) One-pot self-assembly: all the components are mixed together, followed by a gradual cooling.[62] (b) Step-wise self-assembly: subsets of components are separately assembled into intermediate structures, then mixed in a step-wise fashion to yield the desired final architecture.[83] (c) Scaffolded self-assembly (e.g., DNA origami): a long ssDNA is folded into an arbitrary shape with short strands acting as 'staples'.[67] (d) Atomic force microscopy (AFM) confirms the formation of the designed structures.

CONTROLLING SELF-ASSEMBLY PATHWAYS

Out of the many possible self-assembly strategies, the most common approach is a 'one-pot' assembly, where all components are mixed together at the same time. External triggers are subsequently used to initiate assembly; for capsid coat proteins these are often a sudden change in (1) solution pH or (2) ionic strength.[55,57,60,84] Slower changes in solution conditions are also possible, but obviously less desirable for practical reasons. DNA self-assembly also makes wide use of the one-pot approach,[5,62–64,85–87] and here the component DNA strands are slowly cooled from an initial high temperature to yield the desired structures (Figure 3a). This one-pot method is clearly facile, yet offers little control over the spatial and temporal interaction of subunits.

In step-wise self-assembly, various subunits are kept separate (or intermediates are assembled separately) and then combined in a particular order (Figure 3b). Viruses appear to increasingly use step-wise and hierarchical assembly pathways, as they become more structurally and compositionally diverse. *In vivo*, these pathways are certainly necessary to stabilize transient structures and/or provide metastable intermediates, and the end result is an impressive spatio-temporal control. These tasks are mainly carried out by additional proteins that act as provisional scaffolds, connector units, ATP-driven motors, etc.[88,89] An in-depth discussion of the particulars is beyond the scope of this article, but we direct the reader to an excellent introduction.[90] *In vitro*, a variation of the step-wise strategy was used by Gillitzer et al., with differentially modified versions of CCMV coat protein.[91] The

step-wise assembly of DNA nanostructures is currently less common, but has been successfully executed in a few cases.[71,72,75] Notably, the first polyhedral DNA object, a cube, was constructed using such an approach.[75] One particular advantage of step-wise assembly is that reduced numbers of connecting interfaces are needed, since the formation of intermediates is separated from the assembly of larger structures. As a result, particular subunits can (in principle) be re-used at different stages of the assembly.[72] In the case of DNA, the T_m of the intermediates and final structures must be kept well separated, a requirement which is not strictly needed in one-pot approaches.

Scaffolded self-assembly is yet another alternative, and is sometimes referred to as nucleated or templated self-assembly.[82] This approach utilizes a scaffold or template DNA to direct subsequent assembly of other strands (Figure 3c). Scaffolded self-assembly is unique in that it is a one-pot method yet also exerts spatial control over the order of assembly. Discrete structures such as octahedra have been constructed with this approach, using a long ssDNA strand and a few short linker strands.[92] The concept has been extended and named 'origami', where the long ssDNA template is folded by use of many unique linker strands, generating arbitrary shapes. In its first demonstration, two-dimensional shapes were raster-filled by the template strand, generating structures with high yields and little sensitivity to both purity and stoichiometry.[67] The DNA 'origami' method has more recently been used to produce both simple[73,78] and complex[13,14] three-dimensional structures.

APPLICATIONS AND FUTURE DIRECTIONS

The well-defined sizes of viruses and DNA nanostructures make them attractive candidates for further manipulation.[93–98] In biomedical contexts, roles for these structures in drug delivery, sensing, and imaging are readily apparent.[99–109] In optical and electronics applications, benefits could be gained from nanoscale mineralization and metallization.[62,110–118] Due to space limitations, we briefly present only a few illustrative examples.

A prerequisite to any such applications is the ability to selectively introduce chemical reactivity or other responsive features.[119] The amino acid side-chains of protein subunits naturally provide such an opportunity. The most heavily exploited moiety for conjugation is the ϵ-amino group of lysine. Using NHS esters and isothiocyanates as linkers, a wide variety of attachments have been demonstrated, including: redox-active molecules,[120–122] PEG,[123] carbohydrates,[124] quantum dots,[125,126] and even carbon nanotubes.[126] Strable and Finn have recently given an overview of conjugation strategies,[95] mainly in the context of CPMV. Nevertheless, their elegant discussion is broadly applicable to other viruses and nanoscale particles. We adapt their summary, including a few other viruses of interest, in Table 2.

TABLE 1 | Nanoscale Features of Self-Assembling Viruses and DNA Nanostructures

Structure	Characteristic dimensions (nm)	Core
CPMV	$R = 28$	ssRNA
CCMV	$R = 26$	ssRNA
TMV[1]	$d_o = 18$, $d_i = 4$, $L = 300$	ssRNA
fd, M13[1]	$d_o = 7$, $d_i = 2$, $L \approx 900$	ssDNA
DNA structures	$R = 7 - 50$	dsDNA

[1] Symbols d_o and d_i refer to outer and inner diameters, respectively.

A key objective of the above strategies is *selective* functionalization. In a few cases, the native virus capsid structure provides solutions: (1) size-selective features,[106,139] or (2) surface-accessibility.[127,135] Additional control can be obtained through the unique reactivity of cysteines, and more recently,[140] tyrosine and tryptophan (see Table 2). Building upon the above approaches, copper-catalyzed azide–alkyne cycloaddition 'click' reactions have been increasing in popularity, due to their attractive features.[95,128,130,138] In a clever integration of multiple techniques, Strable et al. genetically incorporated azide- and alkyne-containing methionine analogs into viral particles, enabling direct 'click' reactions with dyes and proteins.[141] By contrast with viruses, numerous DNA modifications have been established,[142] and for the most part are now readily available from commercial vendors.

Biomedical Applications

Because of their multivalent capacity, quasispherical viral capsids have been explored to improve magnetic resonance imaging through increased binding and altered relaxivity of contrast agents.[103–106] Recent strides in this area have demonstrated the attachment of greater than 100 ligands per particle.[105,106] The rod-like phage (fd) virus has been widely exploited for surface display of peptides and proteins, primarily for identifying receptor–ligand interactions.[143–150] The extension of viruses or capsids into drug or gene carriers is an active area,[99–102] and will ultimately interface with the immune system.[151–153] The ability to selectively functionalize virus scaffolds allows the creation of multifunctional structures, for example, capable of both targeting and imaging[107,108] (Figure 4a).

TABLE 2 | Attachment Strategies and Linkers for Surface Modification (Reprinted with permission from Ref 95)

Scaffold	Moieties	Linker	Ref.
CPMV	Lys	NHS ester	122, 123, 125, 127–131
	Lys	Isothiocyanate	124, 127
	Lys	carbodiimide/carboxyl	126
	Cys	Maleimide	120, 121, 129
	Cys	Bromoacetamide	121, 124, 128
	Tyr	Azidoalkyl cystine	48
	Asp, Glu	Carbodiimide/amine	132
CCMV	Lys	NHS ester	104, 133
	Cys	Maleimide	133, 134
	Asp, Glu	Carbodiimide/amine	133
TMV	Lys	Carbodiimide/triazole	135
	Cys	Maleimide	136, 137
	Tyr	Diazonium	135, 138

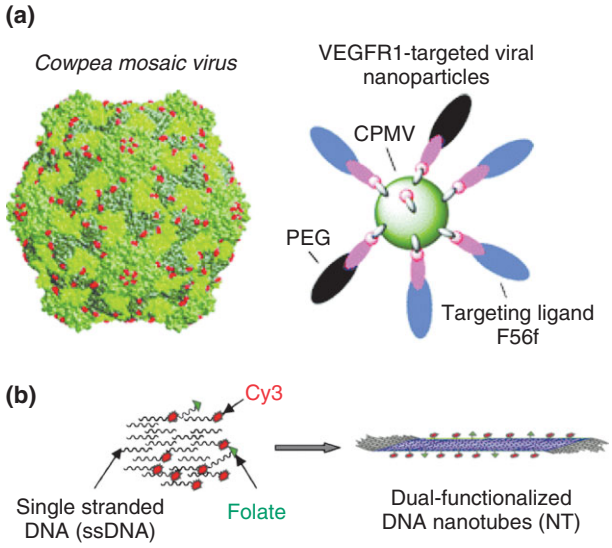

FIGURE 4 | Self-assembled nanoscale scaffolds provide multifunctional capabilities in biomedical applications. (a) CPMV scaffolds with individually tunable levels of VEGFR-1 peptide ligand (for targeting) and PEGylated fluorescein (for imaging). (Reprinted with permission from Ref 108. Copyright 2010 American Chemical Society) (b) DNA nanotubes with individually tunable levels of folate (for targeting) and Cy3 dye (for imaging). (Reprinted with permission from Ref 154. Copyright 2008 American Chemical Society)

DNA nanostructures have been used as scaffolds for templating biomolecules, potentially useful for sensing. For example, by selectively modifying component DNAs with biotin, two-dimensional arrays (with nanoscale spacing) were created that could sequester streptavidin.[76] A related approach has incorporated aptamer sequences into the DNA scaffold, allowing recruitment of specific proteins.[155,156] Due to their well-defined properties, DNA nanostructures can also be potentially used as delivery vehicles.[109] Early demonstrations of this concept include encapsulation of a single cytochrome c protein in a DNA cage,[157] and encapsulation of gold nanoparticles.[158] Similar to viral scaffolds, the modular nature of DNA assembly has allowed the creation of DNA scaffolds with both targeting and imaging moieties (Figure 4b).[154,159] We further note that the size and mechanical properties of nanoscale DNA structures may offer novel opportunities to interface with cells.[160] Toward therapeutically-relevant action, the several groups have created DNA-hybrid[161] and DNA hydrogels.[162] The Luo group further demonstrated cell-free protein synthesis within DNA hydrogels, achieving *in situ* production of target proteins with high yield.[163]

Opto-electronic Applications

TMV has been explored as a scaffold for nanowire synthesis of CdS, PbS, nickel, and cobalt, using the native reactivities of surface-exposed carboxyls.[113,114] The more versatile fd virus has been genetically modified to display specific peptides that promote either cobalt or gold binding[117] (Figure 5a). Although the mechanism is not fully understood, hybrid gold–-cobalt wires demonstrated improved electrochemical performance over cobalt-only wires. Nevertheless, achieving end-to-end orientations over long distances, as well as establishing contacts in devices, remain open challenges.

A recent approach to achieve light harvesting uses the interior channel of TMV, decorated with chromophores.[136] Cysteine-displaying subunits were selectively functionalized with either donor

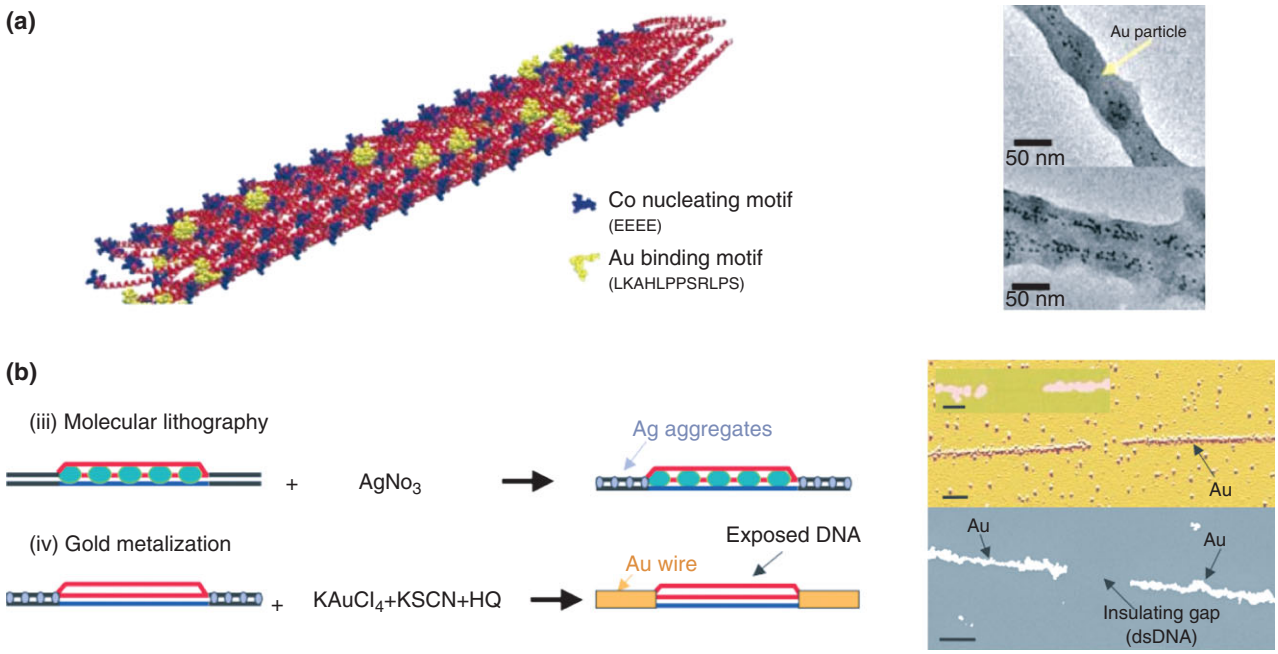

FIGURE 5 | Self-assembled nanoscale scaffolds as sacrificial templates for inorganic metallization. (a) Selective templating of gold or cobalt on the rod-like fd virus. (Reprinted with permission from Ref 117. Copyright 2006 American Association for the Advancement of Science) (b) Molecular lithography approach to creates patterned nanowires from DNA templates. (Reprinted with permission from Ref 164. Copyright 2002 American Association for the Advancement of Science) In both cases, microscopy techniques directly confirm the desired structures.

or acceptor chromophores, in separate steps, and then assembled. An impressive 'antenna effect' was observed for the 2-chromophore system, with the large improvements over donor-free and single-donor systems being attributed to the redundant pathways made possible by polyvalency.[136] The spectral overlap was improved by the addition of a second donor, leading to an overall efficiency of ≈90%. It was also noted that TMV rods were significantly more efficient as compared to disks.[136,137] Further work revealed that rod systems exhibit a linear dependence on defects, supporting the notion that energy transfer in rods occurs mainly via redundant axial transfer whereas disks are limited to lateral transfer.[137] In a preliminary study, fd virus has also been used as a template for light harvesting.[165] Zinc-porphyrins were conjugated to surface-exposed amines via carbodiimide coupling. Effects included a decrease of intrinsic tryptophan fluorescence, suggesting exposure of native residues to the aqueous environment and possible π–π interactions with the porphyrin rings. Transfer of photons was hypothesized to occur via long-range dipole–dipole interactions and quenching occurred due to electron coupling of the pigments.[165]

DNA-based nanostructures have also been explored as an alternative to 'top-down' fabrication. Even with recent innovations in photolithography, achieving feature sizes below 20 nm is a challenge due to the wave properties of light. In a 'bottom-up' approach, DNA nanostructures can be used as sacrificial templates to fabricate metallic patterns, similar to the DNA-templated creation of highly conductive silver nanowires.[62] Patterning can even be performed on the DNA molecules themselves, called 'molecular lithography', where interactions between proteins and DNA are utilized to eventually give sequence-specific patterning.[118,164] In an early demonstration of this approach, a DNA binding protein was allowed to bind a ssDNA template and then this complex subsequently recognized an adsorbed target dsDNA strand, thus serving as a resist (Figure 5b). As the metallization proceeded, only exposed regions of the DNA were coated, achieving nanoscale patterning on single molecules. Despite such promising results, it is likely that top-down and bottom-up efforts will need to be integrated, due to practical issues of cost and efficiency. Indeed, Kershner et al.[166] have recently combined lithography (top-down) and DNA origami (bottom-up) to achieve *controlled* placement of nanoscale objects over μm^2 areas.

CONCLUSIONS

As we complete this journey, we may ask: are there any rules that emerge? We have seen that the nature of the interactions depend exquisitely on the chemical and

physical properties of the building blocks. The nature of the environment (e.g., pH, ionic strength, *T*) also plays a central role in modulating the 'bare' interactions. In spite of the complexity, some rough guidelines for effective self-assembly do appear to take shape. In particular, one should aim to maintain: (1) a modest subunit concentration, (2) fairly low ionic strength (<0.5 M), and (3) neutral or slightly acidic pH. Under such conditions, no single interaction type will dominate, allowing the interplay of multiple effects to favor 'proper' self-assembly instead of 'improper' aggregation. That the chemical interactions require a balance brings us back where we began, if we recall the role of mechanical rigidity. Indeed, during the self-assembly process, the degree of registry between interacting groups is thought to be more important than the actual interaction strengths.[167]

Just as with any recipe, the use of one-pot mixing is seductive in its simplicity. However, predicting the optimal pathways for a given assembly remains far from clear, and will be key to realizing efficient nanoscale self-assembly for any intended application. Furthermore, it is apparent that many biological self-assemblies (i.e., the living ones) do not exist in a state of equilibrium. Uncovering the roles of metastable states, and rates of change during assembly, are exciting avenues for future exploration.

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