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Review Article

Viruses and Virus-Like Protein Assemblies—Chemically Programmable Nanoscale Building Blocks

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This paper is dedicated to Professor Yu-Fen Zhao on the occasion of her 60th birthday.

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ABSTRACT

Supramolecular proteins are generated using a limited set of twenty amino acids, but have distinctive functionalities which arise from the sequential arrangement of amino acids configured to exquisite three-dimensional structures. Viruses, virus-like particles, ferritins, enzyme complexes, cellular micro-compartments, and other supramolecular protein assemblies exemplify these systems, with their precise arrangements of tens to hundreds of molecules into highly organized scaffolds for nucleic acid packaging, metal storage, catalysis or sequestering reactions at the nanometer scale. These versatile protein systems, dubbed as bionanoparticles (BNPs), have attracted materials scientists to seek new opportunities with these pre-fabricated templates in a wide range of nanotechnology-related applications. Here, we focus on some of the key modification strategies that have been utilized, ranging from basic protein conjugation techniques to more novel strategies, to expand the functionalities of these multimeric protein assemblies. Ultimately, in combination with molecular cloning and sophisticated chemistries, these BNPs are being incorporated into many applications ranging from functional materials to novel biomedical drug designs.

KEYWORDS

Bionanoparticles, virus, bioconjugation, nanomaterials, bioimaging, drug delivery

Introduction

Biological materials have been extensively utilized as starting precursors to generate ornate nanostructures for materials development [1–7]. For example, the finite number of building blocks and established base-pairing rules in nucleic acids provides a predictable system that has been used to design two- and three-dimensional lattices [8–10], geometrical shapes [11, 12], and other nanopatterns [13, 14]. Nature also employs protein assemblies, such as viruses and virus-like particles (VLPs) [15], ferritins [16, 17], heat shock protein cages [18], and enzyme complexes [19–21] to form robust biosynthetic machineries (Fig. 1). These protein shells, or bionanoparticles (BNPs), are highly organized nanoscale materials with robust chemical and physical properties while still being capable of modification by genetic and chemical methods. A myriad of viruses and VLPs have been genetically and chemically reprogrammed to function as drug/gene delivery vehicles [22–24], vaccines [25–27], and nanomaterials [28–34].

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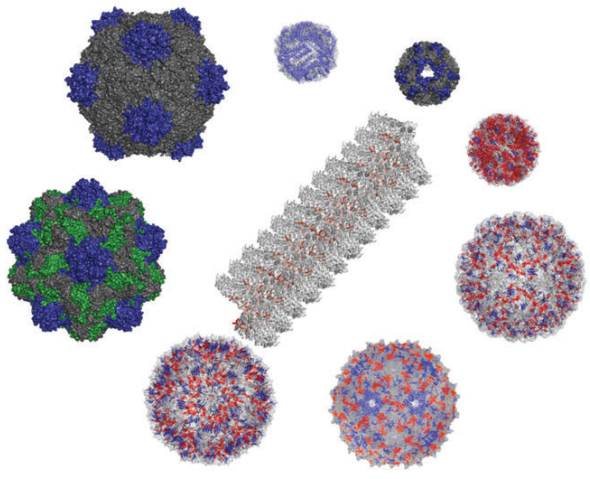

Figure 1 Three-dimensional structures of bionanoparticles: moving clockwise from the top are ferritin, heat shock protein, lumazine synthase, brome mosaic virus, MS2 bacteriophage, cowpea chlorotic mosaic virus, turnip yellow mosaic virus, cowpea mosaic virus and in the center, tobacco mosaic virus. Models were generated using PyMol (www.pymol.org) with coordinates obtained from the RCSB Protein Data Bank (www.pdb.org)

In comparison to conventional synthetic particles, viruses and viral-like protein assemblies are attractive for the development of new materials for the following reasons:

(1) They represent very stable and beautiful self-assembled architectures at the nanometer level with sizes ranging from 10 nm to 200 nm, which are otherwise very difficult to make by standard synthetic methods in the laboratory.

(2) Three-dimensional structures can be characterized at near atomic resolution.

(3) The composition and surface properties of the viruses can be controlled using molecular biology.

(4) They can be purified inexpensively on a large scale, a crucial advantage when considered for materials development.

(5) For each type of virus and virus-like protein assembly, all the particles are identical. We can therefore envision them as truly mono-disperse nanoparticles.

Recently, there have been quite a few detailed review articles which summarize the application of viruses or viral protein cages in biomedicine and materials science [35–39]. In this review, we highlight some of the general approaches utilized to modify these viruses, ferritins, and other protein assemblies in order to impart novel functionalities for nanotechnology applications.

1.1 Chemically addressable bionanoparticles

Many of the basic protein conjugation schemes permeate fundamental BNP chemistry, targeting endogenous amino acids, such as lysines, glutamic or aspartic acids, and cysteines. Less commonly targeted functional groups, such as the phenol ring of tyrosines, have also been incorporated into this approach (Fig. 2). The systematic characterization of cowpea mosaic virus (CPMV) [40–44], along with studies of cowpea chlorotic mottle virus (CCMV) [45], bacteriophage MS2 [46], heat shock protein [18], tobacco mosaic virus (TMV) [47], and turnip yellow mosaic virus (TYMV) [48], have shed light on the unique chemical reactivities and physical properties of these individual BNPs. These initial discoveries have been integred to design BNPs as nano-scaffolds for drug delivery, bioimaging, biomineralization, and even tissue engineering.

For instance, based on the crystal structure of CPMV (Fig. 3(a)) [49], only a few lysine residues per asymmetric unit appear exposed to the solvent (Fig. 3(b)), whereas the majority of the lysine groups are found buried or interacting with neighboring residues (Fig. 3(c)). Studies indicated that reactions with *N*-hydroxysuccinimide (NHS) ester-functionalized fluorescein dyes preferentially targeted these surface-exposed lysines [41, 50].

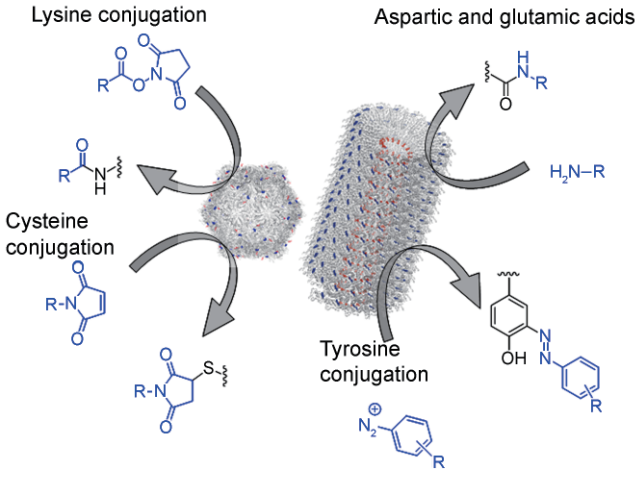

Figure 2 Conventional protein conjugation strategies targeting the endogenous amino acids (lysine, cysteine, aspartic or glutamic acids, and tyrosine) on bionanoparticles

Johnson and colleagues characterized the relative reactivity of the five lysines by sequentially replacing each residue with arginines, and found that each lysine possessed varying reactivities and no single lysine residue accounted for all of the reactivity of the virus [50]. The authors suggest that each lysine being housed in different microenvironments would account for the varied reactivity of the different residues. A chemical reactivity study of TYMV showed that lysine at position 32 had significant reactivity towards fluorescein NHS esters, whereas other lysines in various locations were less reactive. According to its structural data, TYMV protrudes a flexible *N*-terminal region housing the reactive lysine, K32, thus presenting that particular amino group in a more solvent-accessible state than the other lysines [51]. In one particular insect virus, *Nudaurelia capensis ω* virus, the particles undergo a pH-induced structural shift, shrinking in size with decreasing pH. The virus, as predicted, exhibited varied chemical reactivity based on its different conformations, with the larger, more porous state having more chemical conjugates than the smaller, less permeable configuration [52].

Similar variations in reactivity are also observed with other endogenous residues. The exposed carboxylic groups of BNPs have been demonstrated to undergo selective modification upon activation by using a co-catalysis system, 1-(3-dimethylaminopropyl-3-ethylcarbodiimide) hydrochloride (EDC) and NHS. These reagents have been previously used in the derivatization of carboxylic groups of ferritins with long chain aliphatic amines, resulting in stable, hydrophobic macromolecules [53, 54]. In recent years, systematic characterizations implemented with EDC coupling reactions have been conducted on various plant viruses (CPMV, TYMV, CCMV, and TMV) [45, 47, 48, 55]. Schlick and co-authors reported efficient coupling via EDC in the presence of 1-hydroxybenzotriazole (HOBT) [47]. Their study indicated that the activation of carboxylate groups on TMV was restricted to the glutamic acid residues exposed to the inner channel surface with no detectable attachments to the exterior surface. Studies with TYMV [51], CPMV [55], and CCMV [45] also revealed several carboxylate groups, which were solvent-accessible, and capable of being modified with small molecules.

In the case of tyrosine, the electron donating effect of the hydroxyl group makes the phenol group susceptible towards an electrophilic attack at the ortho-position to the OH group. Using diazonium salts ($Ar{-}N{\equiv}N^{+}$), tyrosine can be selectively functionalized with relative ease [56]. This reaction has been applied to selectively modify the surface tyrosines on TMV and MS2 [46, 47]. However, the reactivity is diminished when diazonium reagents without an electron withdrawing group on the aromatic rings are used. To circumvent this shortcoming, Francis and co-workers further improved the reaction by sequential derivations in order to render the tyrosine more susceptible to sophisticated modifications [57, 58]. A nitro-substituted diazonium salt afforded

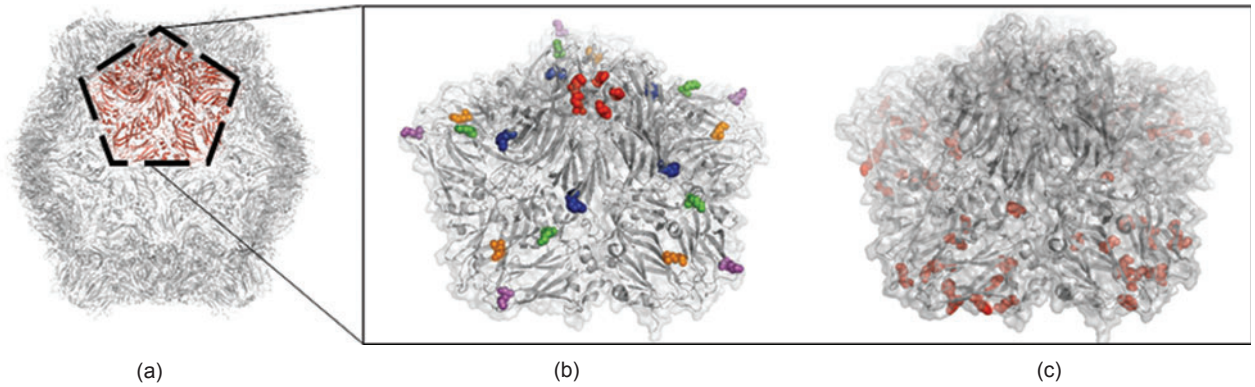

Figure 3 (a) Ribbon model diagram of cowpea mosaic virus with the pentameric unit highlighted in red; (b) surface exposed lysines along the five-fold axis highlighted in color (K182 in red, K138 in blue, K299 in green, K2199 in orange, and K234 in purple); (c) according to crystallographic data, the red spheres are lysines found to be inaccessible to small molecule reactions. Images are rendered with PyMol with coordinates obtained from the RCSB Protein Data Bank

excellent modification of tyrosine residues located inside the bacteriophage MS2, enhancing its use for storage of drugs and as an MRI contrast agent [57–59]. This protocol has been expanded with a larger pool of reagents and reaction conditions for TMV, demonstrating this method to be a flexible and orthogonal procedure for BNP modification at tyrosines (Fig. 4).

1.2 Chemical modification in combination with genetic mutation

Surprisingly, no reactive cysteine residues are exposed on the exterior surface of most BNPs, a reasonable assumption being that evolution has disfavored particles forming inter-particle cross-links via disulfide bonds. This presents the unique opportunity to genetically position the cysteine residue on strategic locations of viruses and protein shells, after which the sulfhydryl group can be selectively targeted with thiol-selective reagents. For instance, the virus coat protein of CPMV expresses 14 cysteine residues (3 in the small subunit and 11 in the large subunit); however, treatment of wild-type CPMV with thiol-selective reagents yielded little or no attachments to the virus [42, 44]. Lin and collaborators have engineered the cysteine residue on surface-exposed loops based on several design criteria [60]. The mutants expressed the cysteine residue as part of an added small loop or as a point mutation (Fig. 5(a)), resulting in 60 copies of the inserted thiol being displayed symmetrically around the 30-nm-diameter particle (Fig. 5(b)). Compared to the native virus, the new inserted cysteines demonstrated higher reactivity, with nearly all of the inserted thiol groups being chemically modified at very low concentration of a maleimide electrophile at neutral pH (Fig. 5(c)) [40]. Biologically-relevant proteins (T4 lysozyme, Her2, and LRR domain of internalin) could be anchored through the use of a bifunctional linker to the surface of an icosahedral virus via the engineered cysteine residue, meanwhile retaining the structural and biological functionalities of the virus and its conjugates [61]. The innate structural features of the virus have also been exploited to pattern gold nanoparticles around the three-dimensional space [62]. In another system, the heat shock protein from Methanococcus jannaschii (MjHsp) has been engineered with a cysteine residue housed within the interior and by coupling the reactivity of the cysteine with a pH-sensitive maleimide derivative, an antitumor drug was linked to the interior surface and selectively released upon decrease in pH [63]. Culver et al. designed and functionalized cysteine substituted-TMV particles with fluorescent dyes, and the modified TMV particles were then partially disassembled to expose the single-stranded viral

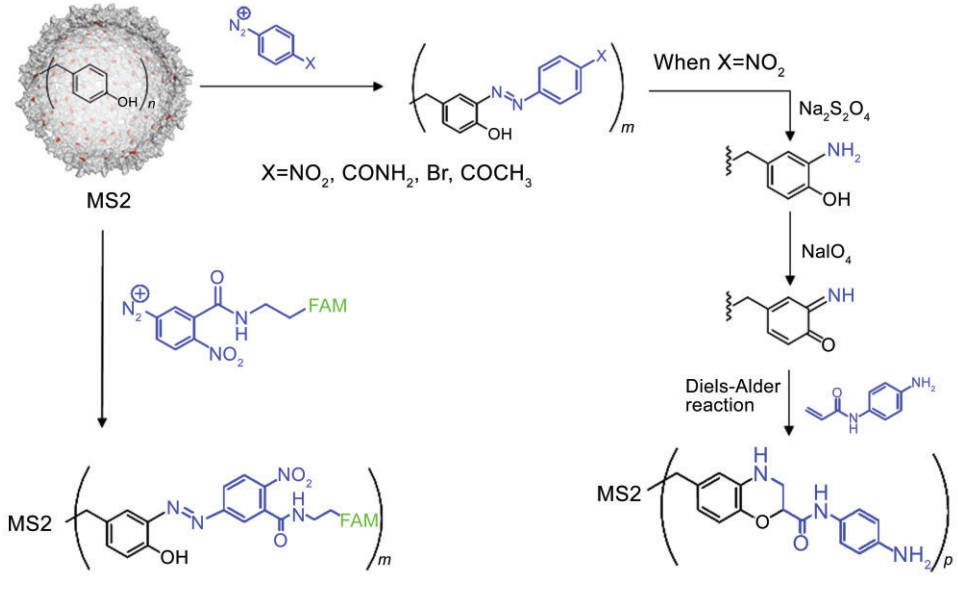

Figure 4 Scheme of tyrosine modification in bacteriophage MS2 using various diazonium salts

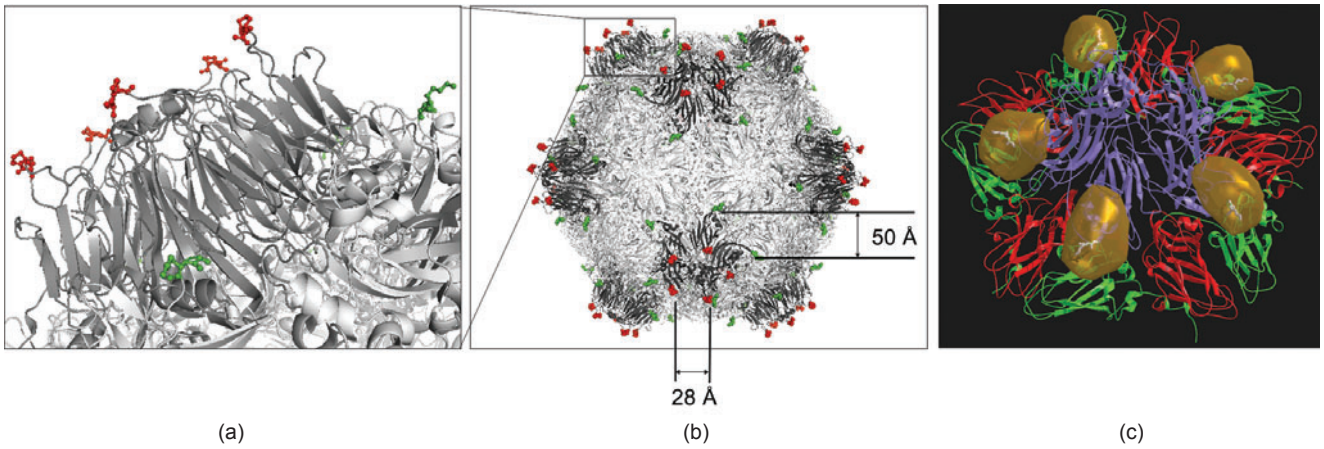

Figure 5 (a) Cysteine residues engineered on CPMV on the protruding loop (βB−βC), shown here in red, which have a tendency to aggregate without the presence of reducing agents. The lesser exposed loop (βE−βF), shown in green, is more resistant to aggregation. (b) Each mutation resulted in 60 reactive thiol groups positioned around a predictable 3-D space, with a 28 Å spacing between each thiol group on the βB−βC (red) loop versus a 50 Å spacing on the βE−βF (green) loop. (c) The electron density difference after modification with gold nanoparticles, shown in gold, clearly shows the attachment site is at the cysteine. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reprinted with permission

RNA. The exposed ssRNA strand was then utilized to hybridize to complementary DNA sequences patterned on surfaces [64, 65]. Francis and co-workers expressed TMV coat protein in a bacterial system to generate cysteine substituted-TMV coat proteins, which were modified with fluorescent chromophores for the purpose of generating a light-harvesting system. By controlling the pH and ionic strength, the proteins self-assembled into long fibrous structures which were capable of positioning the chromophores for efficient energy transfer [66].

These studies highlight an important feature of BNPs, namely that chemically reactive groups can be genetically engineered to selectively position drug molecules, imaging agents, and biologically relevant molecules on the three-dimensional template, which is extremely difficult to realize using synthetic nanoparticles. In particular, the regio-precision of surface functionalization can be crucial for many biomedical applications. For example, in their recent endeavor, the Finn group exploited the architectural features of the virus to explore how spatial distribution and polyvalent display of antigenic carbohydrates would modulate the mammalian immune response [67]. Moreover, a new way to enhance carbohydrate immunogenicity has been reported by means of ordered display on the surface of the CPMV capsid [68, 69]. In another study, murine polyoma VLPs genetically engineered with eight glutamic acids and one cysteine residue in one of its exterior loops tethered the anti-tumor antibody B3 via electrostatic interactions [70]. The antibody fragment was modified at the *C*-terminus with a peptide sequence ($Arg_8CysPro$), which associates with the mutant VLP, and the complex is further stabilized by the formation of a disulfide linkage. This coupling reaction yielded polyoma VLPs with 30–40 antibody fragments bound to the surface, allowing the modified VLPs to bind to breast carcinoma cells with high efficiencies [70].

1.3 Novel bioconjugation techniques

Not all proteins are created equal, ergo all BNP scaffolds do not exhibit similar reactivities or selectivity. The need for selective reactions in complex biomolecules such as BNPs creates a new challenge to design alternative schemes to covalently modify proteins in aqueous or physiological solutions, while retaining their original structural integrity and functionality. Reactions such as Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) [71] and other examples of transition metal catalysis [72] have addressed this need by successfully demonstrating cell-surface labeling and virus modifications.

The CuAAC reaction, a member of the family of click reactions, has emerged as an excellent tool to

covalently fuse two different biological molecules together with relative ease [71, 73]. The exclusive reactivity and selectivity of both azido-and alkyne groups have placed this reaction among the favorites for protein coupling reactions due to its mild reaction conditions and high reaction rates. Although the presence of Cu(I) as a catalyst often interferes with proteins, the addition of a co-catalyst can dramatically enhance the reaction and quantitative amounts of modified proteins are recovered after the reaction. In the presence of bipyridine derivatives, one class of new co-catalysts [74], the efficiency of the reaction has been significantly improved so that micromolar concentrations of virus can be covalently attached with mid-micromolar concentrations of small molecules, polymers, and proteins, all with excellent purity and yield [75]. Over past few years, this reaction has been expanded to conjugate various small molecules and cell-targeting moieties onto viruses. The azide or alkyne moiety, after being attached to lysine, cysteine or tyrosine residues using conventional conjugation chemistry, can be rapidly reacted with high efficiency when only milli- to micromolar concentrations of valuable reagents are present [76]. The sequentially derivatized BNPs were then conjugated with fluorogenic dyes, peptides, proteins, polyethylene glycol (PEG) polymers and complex carbohydrates [67, 75–80] (Fig. 6). The Finn group has further broadened this modification strategy by directly incorporating an azido-containing non-natural amino acid at the interior surface of the bacteriophage Qβ, positioning a chemically unique site within the virus [79].

Many bioconjugation pathways have been recently elaborated, opening new opportunities to selectively modify BNPs. For instance, selective alkylation of phenol groups in tyrosine residues has been achieved using π-allylpalladium complexes [81] in protein concentration levels as low as 5 μmol/L. Chymotrypsin A and bacteriophage MS2 displaying surface-exposed tyrosines were selectively modified, whereas proteins lacking exposed tyrosines, even in the presence of a reactive cysteine residue, were not modified. Finn and colleagues approached tyrosine modification by using the tripeptide (Gly-Gly-His) in the presence of nickel acetate and magnesium monoperoxyphthalate to generate a tyrosyl radical [82]. In both reaction strategies, the authors suggested that the hydrophobic, aromatic ring interactions with the transition metal catalysts enhanced the selectivity towards tyrosine residues.

1.4 Multifunctional bionanoparticles for *in vivo* imaging and drug delivery

A new direction in biopharmaceuticals has been to design multifunctional scaffolds that contain imaging

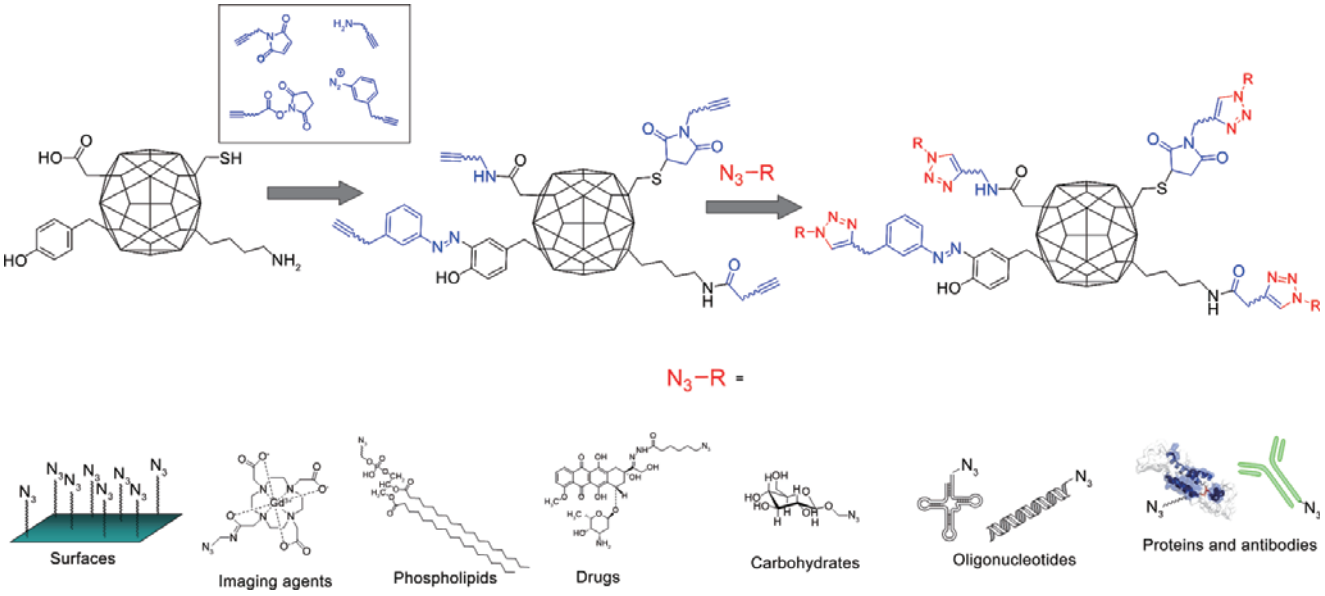

Figure 6 The CuAAC reaction permits sequential modification of bionanoparticles with a universal handle, demonstrated in this figure using an alkyne-derivatized functional group

agents, cell targeting, and therapeutic drugs to reduce non-specific cargo release and side-effects in patients. Combinations of the aforementioned bioconjugation strategies have been utilized to afford additional handles on BNPs, which should eventually lead to such multi-functional constructs. Therefore, both signaling moieties (like fluorescent or magnetic molecules) and biological recognition motifs (such as antibodies, oligonucleotides, ligands, receptors, or chemical sensors) can be simultaneously attached to BNPs, leading to possible nanosized vehicles for biosensing or drug delivery.

For example, a CPMV mutant, expressing the uniquely reactive cysteine residue between residues G98 and K99 of the large subunit, still possesses all of the natural chemical reactivity of the exposed lysine, K38 [43, 50]. Therefore, one can sequentially modify the lysines with an NHS-ester (or isothiocyante) functionalized group and the cysteines with a maleimide group [43]. In one case, the CPMV mutant was functionalized with fluorescent dyes and immunoglobulin (chicken or mouse IgGs) to test its potential use in immunoassays (Fig. 7(a)) [83]. It has also been shown that fluorescent dyes can be anchored on CPMV with controlled separation, which prevented the formation of non-fluorescent dimers and subsequent quenching, and thus afforded highly fluorescent viral nanoparticles [84]. Such kind

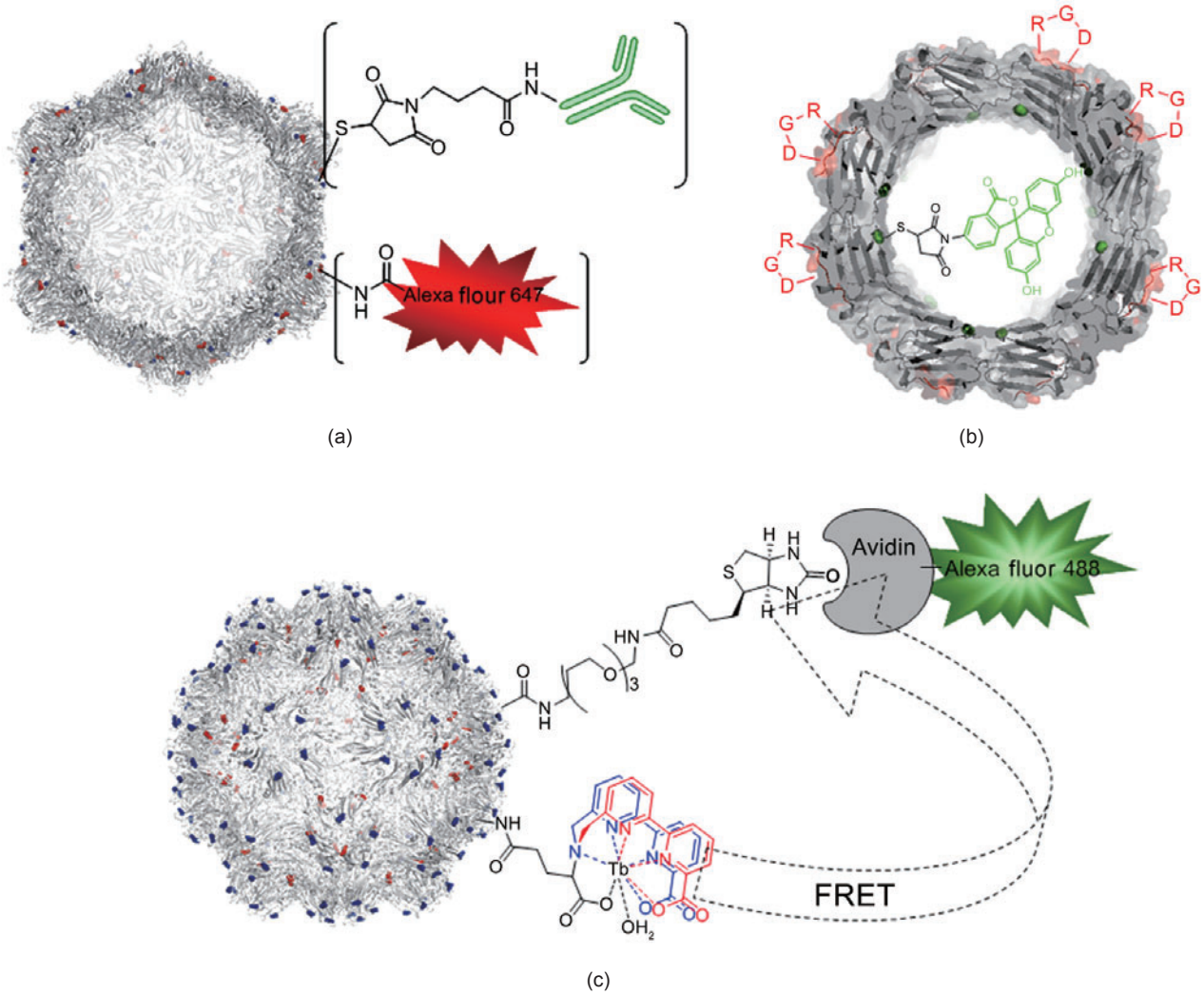

Figure 7 (a) Bifunctional CPMV displaying fluorescent dye and immunoglobulin for immunoassays; (b) integrin targeting peptide sequence (Arg-Gly-Asp) engineered on a heat shock protein as a potential drug delivery agent to target melanomas; (c) terbium ligand complex conjugated to TYMV to generate time-resolved fluoroimmuno assays

of engineered viral particles can be used as probes in microarray-based genotyping assays and sandwich immunoassays with improved sensitivities [83, 84].

Francis and co-workers generated doubly labeled TMV and MS2 by utilizing intrinsic amino acids to functionalize the exterior and the interior with small molecules, dyes, and polymers [47, 57]. Douglas, Young, and co-workers generated MjHsp with cysteines engineered in the interior of the shell and the cell targeting moieties on the exterior to direct the protein shell to melanomas (Fig. 7(b)) [85]. Wang and group recently generated TYMV labeled with a terbium complex and biotin as a prototype BNP for time-resolved fluoroimmuno assays (Fig. 7(c)) [48], whilst Culver and co-workers have recently developed an elegant approach to assemble multifunctional TMV onto a solid substrate [65]. In addition to genetic modification of the surface protein to afford a reactive cysteine residue that was labeled with fluorescent markers, the particle was treated with an alkaline solution in order to remove ~20 coat protein subunits from the virus 5′-end, exposing ~60 nucleotides. Such kind of particles can be further annealed with complementary DNA printed on a patterned microarray platform [64]. Using this strategy, Yi and co-workers prepared encoded microparticles via nucleic acid hybridization with mutant TMVs, which were suggested to have broad applications in multiplexed target sensing [86].

Notably, all of the doubly-modified BNP scaffolds consistently retained their structural integrity throughout the reactions, which often involved a mixture of aqueous and organic solvents. Further studies of these BNPs are likely to involve *in vitro* and *in vivo* models of cellular targeting, uptake and drug delivery, but the anticipated immunogenic response will be a major obstacle to overcome for all protein-based scaffolds. To address this potential problem, Manchester and co-workers employed the CuAAC reaction to conjugate the folic acid-PEG ligand to a mutant CPMV. While pegylation of CPMV completely eliminated background binding of the virus to tumor cells, the folate moiety allowed CPMV-specific recognition of tumor cells bearing the folate receptor [87].

2. Development of functional materials based on non-covalent interactions

The intrinsic chemistry of BNPs extends their applications far beyond the basic covalent annealing of different functional groups on the surface. Non-covalent interactions (i.e., metal–ligand, lectin–sugar, charge–charge, π–π stacking, and many other interactions) have emerged as excellent approaches for introducing novel functionalities to into BNPs. BNP-based inorganic materials, greatly advanced over the past years by Mann, Douglas, Young, and Belcher, have made important contributions to the synthesis of inorganic nanoparticles [32, 88–91]. Highly uniform, water-soluble protein shells provide the added benefits of preventing aggregation and sequestering mineralization reactions with various metallic ions [92–94].

TMV, a rod-like BNP with a length of 300 nm and 18 nm in diameter, which can be purified from infected tobacco plants in large quantities, has been exploited as template to grow metal or metal oxide nanoparticles such as iron oxyhydroxides, CdS, PbS, gold, nickel, cobalt, silver, copper, iron oxides, CoPt, $FePt_3$, and silica [95–102]. From electrophoretic measurements, the isoelectric point of TMV is around 3.4 and so at neutral pH, the TMV surface has net negative charge. In order to achieve successful coating based on the electrostatic interactions, the deposition conditions should be varied in order to match the interaction between the virion surface and the deposition precursor. In the case of silica coating, carrying out the reaction at pH<3 results in a positively charged TMV surface that will have strong interactions with the anionic silicate sols formed by hydrolysis of tetraethyl orthosilicate (TEOS). In contrast, CdS, PbS, and iron oxides can be successfully coated on the outer surface at near neutral pH by specific metal ion binding with the glutamate and aspartate residues [96]. As for metal deposition, in some cases, a suitable activation agent is needed in order to realize successful coating [101]. Pd(II) and Pt(II) are two typical activation agents. The metal deposition can occur either inside the inner channel or at the outer surface of TMV [101]. Genetically engineered TMV can show enhanced

deposition of metal onto its surface [24, 103]. In this case, native TMV was genetically altered to display multiple metal binding sites through the insertion of two cysteine residues within the amino-terminus of the virus coat protein. *In situ* chemical reductions successfully deposited silver, gold, and palladium clusters coating onto the genetically modified TMV without any activation agent [103]. Furthermore, mixing TMV with aniline and a mild oxidant resulted in highly uniform, micron length nanofibers measuring approximately 22 nm in diameter, likely due to the non-covalent interactions between aniline and TMV [30]. These nanofibers were readily dispersed in water and addition of dopants increased the conductivity of the composite nanofibers [31].

Another good example is bacteriophage M13, a filamentous bacteriophage that measures 800–900 nm in length and 6–8 nm in diameter. In 1992, Brown pioneered the use of phage display for the screening and binding of inorganic materials [104]. Belcher and co-workers expanded this phage display technique to decorate M13 bacteriophage with different metal binding peptides specific to inorganic materials, such as semiconductor materials like GaAs, InP, ZnS, CdS, and magnetic materials like FePt, CoPt, Co, and Au, or composites [24, 105–107]. Nanowires, nanorings [108], nanofibers [109], films [106, 110], and other nanostructures [107, 111, 112] have also been engineered by displaying specific receptors at the pⅢ of the phage networks and other assemblies with predictable structures. Furthermore, nanostructures that are more complex can be produced by incorporating two or more fusion proteins in the same mutant M13 particles. For example, both pⅧ and pⅢ of M13 have been engineered as templates to assemble Au and CdSe nanocrystal/hetero-nanocrystal arrays and Au nanowires [113, 114]. These studies demonstrate that various substrate-specific motifs can be independently selected from type 8 or type 3 libraries (where the genes encoded pⅧ and pⅢ are mutated) and then genetically incorporated into M13 structures to produce versatile hybrid materials with hetero-functionalities. Such bio-inorganic hybridized materials have been used to generate thin, flexible lithium batteries [115].

Douglas and Young used CCMV, a spherical virus composed of 180 identical 20-kDa protein subunits, as a nano-sized reactor to develop hybrid inorganic materials. Since the inner surface of each protein subunit of CCMV presents nine basic residues (arginine and lysine) and creates a highly positively charged interior, a wide variety of negatively charged polyoxometalate species (vanadate, molybdate, and tungstate) were selectively mineralized at pH=5 within empty CCMV particles devoid of the nucleic acid; the size of the capsid cavity defines the size of mineralized particles (Fig. 8(a)) [88]. Similarly, a genetically engineered mutant CCMV particle with a negatively charged interior (the nine basic residues at the *N*-terminus of CCMV were replaced with glutamic acid) was used to sequester a nanoparticle of iron oxide through spatially constrained oxidative

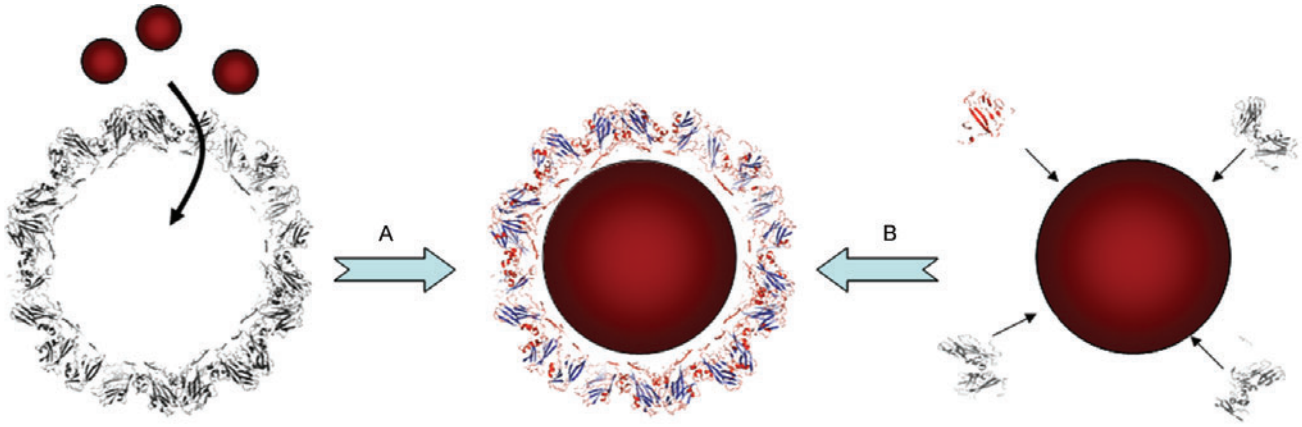

Figure 8 Schematic illustration of two different strategies to prepare hybrid inorganic/viral core-shell particles: (A) using a void viral capsid as reaction vector to synthesize an inorganic core; (B) using different inorganic particles to nucleate the assembly of coat proteins

hydrolysis of Fe (Ⅱ) [91]. This strategy has also been employed to encapsulate horseradish peroxidase enzymes, where a single enzyme reactor was constructed and the activities were studied at the single-molecule level [116, 117].

To synthesize hybrid inorganic/viral core-shell particles, it was demonstrated that inorganic particles could serve as a core to nucleate the assembly of viral capsids (Fig. 8(b)). For example, Dragnea and co-workers used gold nanoparticles, quantum dots, and magnetic nanoparticles to induce the organization of the coat proteins of brome mosaic virus (BMV) [118–121]. Therefore, nanoparticles can be encapsulated by self-assembly in viral capsids, yielding VLPs in size similar to the native virus. Furthermore, manipulation of the diameter of the packaged material can be used to vary the degree of packaging. Another strategy of packaging nanoparticles in viral capsids was reported by Franzen, where the origin of assembly sequences was used as a trigger to package nanoparticles [122]. Association of these sequences on nanoparticles provides recognition to the coat protein of wild-type red clover necrotic mosaic virus (RCNMV), and self-assembly of coat protein then begins until a VLP is formed with the nanoparticles fully packaged within the coat protein. The specific non-covalent interaction between the RCNMV coat protein and the sequences tethered on the nanoparticles offers great stability and specificity in encapsidation of nanoparticles that can be further explored for packaging other cargos [123].

In another study, virus-modified particles were fabricated by the layer-by-layer approach and fused with rubella virus-like particles (RVLPs) [124, 125]. Hence, it may be possible to fabricate particles with virus functions at the surface of colloidal particles along with other biological properties on the nano/micro meter scale. It can be concluded that non-covalent interactions, including layer-by-layer techniques, may be a general approach for the transfer of biological functionalities of various kinds of viruses onto colloids, capsules, and flat surfaces [126–129].

3. Discussion

The major features of BNPs that have been exploited to generate novel biotemplates in the nanometer range have been highlighted throughout the paper. In short, their superb symmetry and size uniformity, and system modularity in combination with genetic engineering place BNPs as a material distinct from polymeric materials. Viruses, ferritins, enzyme complexes, chaperonines, and carboxysomes can range widely in shape and size (spherical, rod-like, from tens to hundreds of nanometers) with diverse chemical and physical properties, thereby presenting an extensive selection of primary building blocks. Since these characterized BNPs only represent a small fraction of the entire biodiversity, investigation of other BNPs and their unique properties would be of particular interest. For instance, viruses isolated from extreme environments (e.g., high temperature) would likely possess unparalleled thermal stabilities which could allow for additional chemical reactions at temperatures that would normally destroy other BNP templates. Comparative analysis amongst other BNPs may also elucidate which structural features sequences would tolerate chemical and genetic modifications, large peptide insertions, and structural integrity.

Numerous studies have demonstrated the potential of BNPs in biomedical applications made possible by attaching ligands and small molecules to redirect the protein carriers to different cell types for drug/gene delivery and cell imaging. The nanosized probes can further be modified with chemical compounds, such as bio-imaging agents (near infrared fluorescent dyes, magnetic contrast imaging agents) and drugs at high local concentrations to increase detection sensitivity and efficacy in therapeutic applications. Furthermore, the BNPs can be modified with polymers to boost their half-life in the host, either by shielding the protein structures from enzymatic degradation or the host's immune response.

Despite the advantages the BNPs possess, a protein-based system exhibits similar limitations to any other biological system. Unlike their inorganic nanoparticle counterparts, the biological system exhibits much lower stabilities at high temperatures, in various organic solvents, and during long-term storage. In addition, while these protein systems

possess the potential to generate libraries, only one type of virus (bacteriophage M13) has a tested virus-display and screening procedure. The possibility of generating large arrays of mutant viruses, ferritins, and BNPs and screening these systems against specific targets is essential in order to realize the full potential of these BNPs. Ultimately, through the combination of these various functionalization schemes, BNPs could be designed as versatile templates with nanosized features for material synthesis that would not be feasible via the traditional top-down fabrication techniques.

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