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PERSPECTIVE

Altering the landscape of viruses and bionanoparticles

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In recent years, protein-based nanoparticles or bionanoparticles (BNPs), have been used as primary building blocks to generate ornate nanomaterials for a wide-range of applications. Over the past fifty years, numerous BNPs have been chemically modified or genetically engineered to function as smart drug/gene delivery vehicles, advanced vaccine vehicles, and isolated reaction vessels for inorganic, metallic, and semi-conductive depositions. These studies have contributed invaluable insights to the expansive capabilities of these simple, yet highly robust, nanosized building materials. Here we highlight some of the recent progress in the chemical modifications of BNPs and hopefully inspire the development of many new materials in the near future.

Bionanoparticles as building blocks

Numerous nanostructures have been derived from biological materials as novel biosensors and electronic nanodevices, multifunctional drug/gene delivery agents, advanced vaccine carriers, and multivalent biomaterials for guiding cells.[1–8] The beauty of these particular protein nanosystems lies within their intrinsic genetic programmability, where a specific codon modification translates to the corresponding change in amino acid residue with unsurpassed fidelity, consistency and spatial resolution. Within the context of this article, these nanosized, protein-based systems, which include viruses and virus-like particles (VLPs), ferritins, heat shock proteins (Hsp) and enzyme complexes, and other such biological templates, are defined in a broad category as bionanoparticles (BNPs).[9–14] A key defining characteristic feature of BNP is that several proteins are organized through non-covalent interactions to become highly organized and uniform nanoparticles (< 300 nm in at least one of the dimensions) (Fig. 1).

These particles exhibit distinctive structural symmetries and yet are stable enough to be extensively modified by molecular biology and/or protein chemistry. The distinctive advantage of BNPs stems from this combination of those two techniques (molecular cloning and protein bioconjugation) that allow for the generation of novel BNPs to function as drug/gene delivery vehicles and vaccine carriers with high ligand densities at well-defined nanometer size distributions or as novel nanowires

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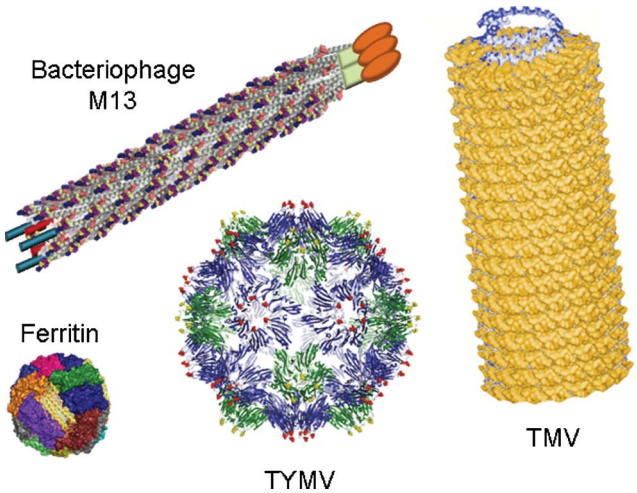


Fig. 1 Representative illustration of BNPs demonstrating the varied shape and sizes with symmetries. Ferritin: an octahedral particle with a diameter of 12 nm; TYMV (Turnip yellow mosaic virus), a 30 nm plant virus with an icosahedral symmetry; TMV (Tobacco mosaic virus), a helical virus, measuring 300 nm long and 18 nm in diameter encasing its genomic RNA; M13 bacteriophage, a filamentous viral particle, 860 nm long and 6.5 nm wide. Models were generated using PyMol (www.pymol.org) with coordinates obtained from RCSB Protein Data Bank (www.pdb.org).

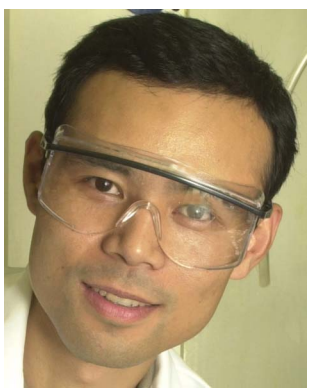
and composite materials.[15–19] In this article, we highlight some of the recent work on the chemical modifications of viruses, ferritin and other BNPs to display multiple motifs and assembly patterns. Then the following section describes recent progress in molecular engineering of reactive sites on BNPs. Since the proteins are organized in a highly repetitive fashion, a single substitution mutation for one protein is identically displayed around the entire particle. This results in a single type of reactive group being placed around the particle at multiple copies in a defined geometry. Only BNPs possess such powerful correlation between modification sites and spatial resolution. In addition, just like phage display, directed molecular evolution can be a powerful tool to achieve selective binding. Still, this article is by no means a comprehensive coverage of the ever-growing theme, but provides a brief summary of the recent work in BNPs and aims to spark new discussions regarding these evolutionarily adept BNPs as useful molecular tools. The audience is encouraged to seek extensive reviews on these protein-based nanosystems for additional details on the development of BNPs as novel materials.[20–26]

Something old, something new, and something used

The harmonious marriage of BNP and chemistry relies on their remarkable stability in organic solvents (up to 20–70% DMF, DMSO and ethanol for some of the viruses we have tested) and heat resistances up to 60–80 °C, which allow for pairing with hydrophobic molecules or dyes at modest reaction conditions. The most common modification of BNPs has been through conventional bioconjugation to target endogenous amino acids by using *N*-hydroxysuccinimide (NHS) esters, maleimides, or isothiocyanates (Fig. 2). Unlike traditional protein modifications, the emphasis for BNPs lies with the high density of modifications within a defined three dimensional space. Such a high degree of modification is often preferred for carbohydrate antigen display in the development of vaccines, the engagement of multiple cell surface receptors, the enhancement of fluorescence signals for imaging or immunoassays, or the generation of light harvesting nanosystems.[27–36]

Chemically labile residues can be identified with these basic conjugation techniques, and the degree of modifications can be adjusted to determine the most stable and/or the maximal loading capacities of each BNP. It is important for future materials development to identify such key residues that will permit anchoring small molecules or large bulky chemical groups on each BNP without losing its quaternary structure. The systematic characterizations on CPMV, CCMV, MS2, Hsp, TMV, Qβ, M13, T4 and TYMV have shed light on the distinct chemical reactivities and physical properties of BNPs. A summarized list of BNPs and their reactivity potentials with references is provided in Table 1. In our experience, each BNP possesses different tolerances to various chemical loadings and preferential modification sites, but many of these limitations have not been clearly defined for many of the particles. A significant portion of the development for BNP conjugations is to determine modification sites, loading densities, and structural retention or ability to reassemble after chemical modifications. And lastly, the modifications should exhibit regioselectivity (interior or exterior surface, specific lysines or cysteines).[37–39]

Despite the fact that these reactions are routine for many proteins, given the large pool of BNPs and their unique chemical compositions, each possesses its own interesting reaction profile. For instance, incubating 50 equivalents of amine reactive dyes for native CPMV[37] provides a highly fluorescent particle, but the similar reaction with TMV or TYMV[47] will result in little or no dye loading. Reactions with NHS ester reagents for CPMV would result in higher loadings at higher concentrations of dye or small molecules; however the particle structure would be less stable and result in lower recovery. In the case of the M13 bacteriophage, NHS ester activated tetramethylrhodamine (TMR) dyes showed selective modification of the major coat protein (pVIII) at two



Qian Wang

Professor Qian Wang received his B.Sc. in Chemistry in 1992 from Tsinghua University. He obtained his Ph.D. in Organic Chemistry from Tsinghua University in 1997 under the supervision of Prof. Yufen Zhao. After his postdoctoral fellowship with Prof. Manfred Schlosser at the University of Lausanne (1997–1999) and Prof. M.G. Finn at The Scripps Research Institute (1999–2003), he started as an Assistant Professor at the University of South Carolina in 2003. He is currently appointed as the Robert L. Sumwalt Professor of Chemistry. His research interest focuses on creating virus-based 3D programmable scaffolds to probe cellular activities.

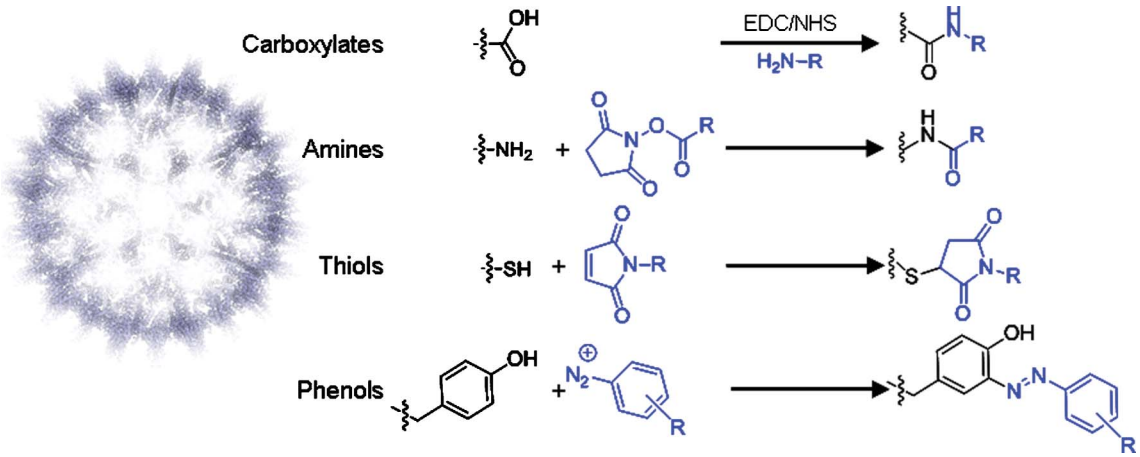


Fig. 2 Conventional conjugations for chemically introducing functional ligands on BNPs. Fluorescent tags, imaging agents, carbohydrates and drugs can be tethered to BNPs *via* the amino group of lysines, the thiol of cysteines, the carboxyl group of aspartic/glutamic acids, as well as on the phenolic group of tyrosine residues with diazonium salts. (EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS, *N*-hydroxysuccimide).

Table 1 Various BNPs with identified modification sites

BNP	Residue	Modification Sites
TMV	Carboxylate[40]	E97 and E106
	Phenol[40,41]	Y139
CPMV	Amine[37,42]	K138, 182, 234, 299 and 2199
	Phenol[43]	Y52, Y103
	Carboxylate[44]	Not assigned
MS2	Amine[45]	K106, K113, and N-terminus
	Phenol[45,46]	Y85
TYMV	Amine[47]	K32
	Carboxylate[48]	Not assigned
M13	Amine[49]	A1 (N-terminus), K8
	Carboxylate[49]	Not assigned
	Phenol[49]	Y21, Y24
Ferritin	Amine[50,51]	K67, K83, K97, K104, K143
	Carboxylate[52,53]	Not assigned
AdV	Amine	Not assigned
	Carboxylate[54]	Not assigned
CCMV	Amine[55]	Not assigned
	Carboxylate[55]	Not assigned
HCRSV	Amine[56]	Not assigned
SIRV2	Amine[57]	Not assigned
	Carboxylate[57]	Not assigned
T4 phage	Amine[29]	Not assigned

sites (amino terminus and lysine 8), with highest fluorescence intensity observed with ~400 dyes per particle.[49] Increasing the number of dyes per phage particle was permissible, however the fluorescence intensity dramatically decreased as the number of dyes increased beyond 400 units per particle likely due to fluorescence quenching.[49] Similar fluorescence enhancement and reduction of fluorophores have also been observed for the T4 phage where the maximum fluorescence intensity was observed for particles labeled with ~350 Cy3 dyes with an inverse correlation of fluorescence intensity and increasing dye per virus particle.[29] This labeling capacity is only a small fraction of the maximal amount of dyes (~19 000) that could be successfully conjugated to T4 particles,[29] which reiterates the importance of identifying loading capacities with functional improvements.

The 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 *N*-hydroxysulfosuccinimide (Sulfo-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.[52,53] Francis and co-authors reported efficient coupling of the interior glutamic acid residues *via* EDC/HOBt with a variety of primary and secondary amines.[40] Their study indicated the activation of carboxylates on TMV had been restricted to the glutamic acid residues exposed to the surface of the inner channel. Studies with TYMV,[47] CPMV,[44] and CCMV[55] had demonstrated that dyes and small molecules can be linked to the carboxylic groups using the similar EDC/NHS reactions without affecting the structural integrity of the viral particles.

Beyond the traditional amines and carboxylates, it is worth noting that tyrosines have become routine modification sites, with the incorporation of alkynyl derivatized diazonium salt orthogonal reactions can be accomplished on BNPs and proteins, leaving the amines, thiols and carboxylates for additional reactions.[41] When considering tyrosine residues for bioconjugation, the electron donating effect of the hydroxyl group makes the phenol group of tyrosine susceptible to an electrophilic attack at the *ortho*-position of the OH group. Using diazonium salts (Ar-N≡N$^+$), tyrosine can be selectively functionalized with relative ease. This reaction has been applied to selectively modify the surface tyrosines on TMV and MS2.[40,45,46] However, the reactivity is diminished when the diazonium reagents are used without an electron withdrawing group on the aromatic ring. Francis and co-workers improved the reactivity with sequential derivatizations to render the tyrosine more susceptible to sophisticated modifications.[28,45] The nitro substituted diazonium salt afforded an excellent modification of the tyrosine residue located in the interior surface of bacteriophage MS2 to hold drugs and MRI contrast agents.[28,45] The method was 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 tyrosine residues.

A major thrust in biopharmaceuticals has been to design multifunctional nanoscaffolds carrying imaging agents, cell targeting, and 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 would eventually lead to such multi-functional constructs. A CPMV mutant, which expresses a reactive cysteine residue inserted between residues G98 and K99 of the large subunit, still possesses all of the natural chemical reactivity of the exposed lysine, K38.[38] Therefore, one can sequentially modify the lysines with a NHS-ester (or isothiocyanate) functionalized group and cysteines with a maleimide group.[38] For example, a cysteine CPMV mutant tagged with fluorescent dyes and immunoglobulins (chicken or mouse IgGs) have been used to test its potential use to enhance the detection limits of immunoassays.[30] Wang and coworkers had dual modified TYMV particles with a terbium complex for time-resolved immunoassays.[48] Douglas, Young, and coworkers generated cysteines within the interior shell of MjHsp and linked cell targeting moieties to the exterior surface to direct the modified nanoparticles to melanoma cells.[58] Wang and coworkers had generated an M13 phages modified with folic acid,[49] which were then co-assembled with poly-caprolactone and poly-2-vinylpyridine block co-polymers to form core-shell nanoparticles for selective delivery of doxorubicin to breast cancer cells.[59]

Site-directed mutagenesis for selective modification

A disadvantage for targeting endogenous amino acids is that multiple reactive sites can be present. One process has been to systematically substitute the reactive sites with non-reactive substitutes, for instance lysine residues on CPMV with arginines.[42] The replacement of amines with guanidine groups allowed the measuring of the reactivities of the different lysines on the surface of CPMV and the generation of a particle with a uniquely reactive lysine on the exterior surface.[42] Similar engineering feats can also be fashioned for many of the BNPs to introduce or to reduce reactive groups on the surface (Table 2).

In the viruses we have tested so far, no reactive cysteine residues are found on the exterior surface of BNPs, a logical argument that evolution had disfavored particles forming inter-particle cross-linkages *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 thiol 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 to no attachments to the virus.[38,39,60] Johnson and collaborators had tactfully engineered the cysteine residue on surface-exposed loops based on several design criteria.[61] The mutants had expressed the cysteine residue as part of an added small loop or as a point mutation, resulting in 60 or 120 copies of the inserted thiol being displayed symmetrically around the 30-nm-diameter particle (Fig. 3A).[38,39] 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 concentrations of the maleimide electrophiles at neutral pH (Fig. 3B).[38,39]

Biologically-relevant proteins (T4 lysozyme, Her2 and LRR domain of internalin) could be anchored *via* the use of bi-functional linkers tethered to the engineered cysteine residue on the icosahedral virus, while still retaining the structural and

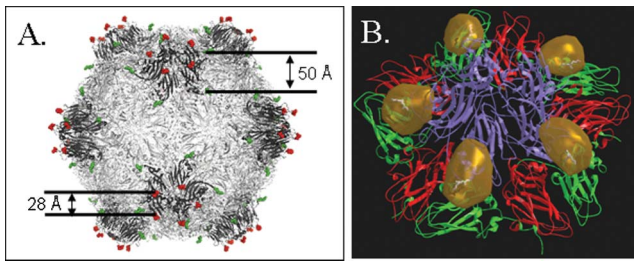


Fig. 3 Ribbon model of CPMV (**A**) with genetically engineered cysteines on the protruding loop (βB-βC) shown here in red, which had tendency to aggregate without the presence of reducing agents. The particles with cysteine inserted in a recessed loop (βE-βF) shown in green, are more resistant to aggregation. The distance between each cysteine residue, based on crystal structure, can be varied with nanometer resolution. (**B**) The electron density difference after modification with gold nanoparticles shown in gold around pentameric geometry.[39] Copyright Wiley-VCH Verlag GmbH & Co. KGaA.

biological functionalities of the virus and its conjugates.[62] The innate structural feature of the virus had also been exploited to pattern the gold nanoparticles around the three-dimensional space.[63] In another system, Hsp from *Methanococcus jannaschii* (MjHsp) had 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 a decrease in pH.[64]

Native TMV particles are nearly unreactive towards amine or thiol-selective reagents, making TMV a prime candidate for inserting reactive sites around the virus particles. Both types of mutants have been successfully expressed in tobacco plants and purified in large quantities to utilize the virus platform as vaccine carriers[65] or DNA microarrays.[66,67] We have tested the loading densities of both lysine and cysteine mutants of TMV with fluorescein molecules and small alkynyl derivatives (Fig. 4). Both reactive sites follow a typical reaction profile with an increasing number of dyes attached to the viral particles with an increasing molar ratio of dye until a plateau phase is reached. Pushing the reaction beyond this plateau phase would provide higher modifications per particle, but resulted in decreased yields of intact viral particles and even decreased the fluorescence intensities.

Culver *et al.* designed and functionalized the cysteine-substituted TMV particles with fluorescent dyes, and the modified TMV particles were then partially disassembled to expose the single-stranded viral RNA. The exposed ssRNA was hybridized to complementary DNA sequences patterned on surfaces.[66,67] Francis and co-workers expressed TMV coat protein in a bacterial system to generate cysteine substituted recombinant TMV coat proteins, which were conjugated with fluorescent chromophores for the purpose of generating a light-harvesting system.[35] 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.[87] These studies highlight a key feature of BNPs, which is that chemically reactive groups can be genetically engineered to selectively position the drug molecules, imaging agents and biologically relevant molecules on the three-dimensional template with spatial precision. Francis and

Table 2 Genetic modifications in BNPs

BNP	Type of insert	Expression host
TMV	Antigenic peptides,[17,68] RGD peptides[69,70] cysteine,[66] lysine,[65] His_6 tag[71]	Tobacco plants and bacterial expression
CPMV	Peptide antigens,[72–74] cysteine,[38] lysine,[42] His_6 tag[75]	Cowpea plants
CCMV	subE mutant,[76] cysteine[77]	Cowpea plants and bacterial expression
Ferritin	AG4,[11] Co2 peptide,[78]	Native host
P22	Cysteine[79]	Bacteria host
FHV	Antigenic insert[79]	Native (insect cells) and recombinant (baculovirus)
MjHsp	Metal binding peptides,[80] cysteine,[81] RGD peptide[10]	Bacteria host
MS2	Cysteine,[58] unnatural amino acid[82,83]	Bacteria host
HBVLP	Unnatural amino acid[84] His_6 peptide[85]	Recombinant
M13	Metal binding peptides,[12] proteins[86]	Bacteria host
Qβ	Unnatural amino acids[83,84]	Bacteria host and cell free

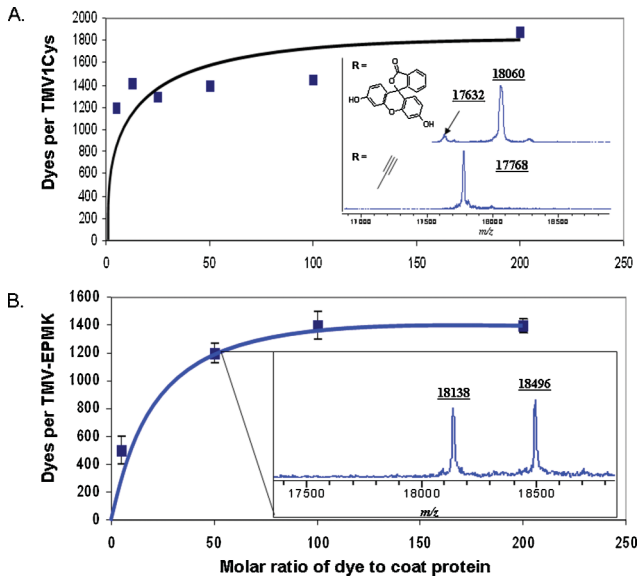


Fig. 4 A chemical modification profile of BNP. (**A**) TMV1Cys mutants were modified with varying ratios of maleimide–fluorescein at which 200 equivalents of dye per subunit permitted maximal loading density. The corresponding MALDI-TOF MS (inset) of fluorescein modified TMV1Cys indicate a small group of unmodified coat proteins (m/z 17 632) with a mass shift ($\Delta m/z$ 428) corresponding to maleimide dye per subunit. Smaller molecules such as the propargyl maleimide permitted near complete modification of the virus as indicated by MALDI-TOF MS (inset, m/z 17 768). (**B**) NHS-ester–fluorescein loading on lysine mutants of TMV (TMV-EPMK) showed lower reactivity than cysteine mutants, achieving approximately 50–60% modification of the coat protein at 200 molar equivalents. Reaction with higher ratios of dyes would typically decrease the yield of intact virus particles. Error bars denote mean ± s.d. of three separate experiments performed in triplicates.

collaborators have also generated dual-labelled TMV and MS2 by utilizing intrinsic amino acids to functionalize the exterior and the interior with small molecules, dyes and polymers.[40,45] Notably, all of the dually modified virus scaffolds had consistently retained their structural integrity throughout the reactions, which often consist of mixture of aqueous and organic solvents. Ongoing studies for these BNPs will involve many more *in vitro* and *in vivo* experiments of cell targeting, uptake, delivery and cytotoxicity, but the anticipated immunogenic response will still be a major obstacle to overcome for all protein-based scaffolds.

Alternative modification methods for BNPs

There is still a major need for selective reactions in complex biomolecules such as BNPs. One practical approach has been to rely on reactions, such as Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) and transition metal catalysis, which have addressed this need by successfully demonstrating cell-surface labeling and virus modifications. CuAAC reaction has emerged as an excellent tool to covalently fuse two different biological molecules together with relative ease.[88,89] The exclusive reactivity and selectivity of both azido- and alkynyl 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 has dramatically enhanced the reaction and quantitative amounts of modified proteins are recovered after the reaction. Under one of the new co-catalysts, 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.[90] Over past few years, Finn and coworkers expanded this reaction to conjugate small molecules, peptides, proteins, complex sugars and polymers onto the viruses.[1,19,27,85,90–92] The group had broadened this chemical modification strategy by directly incorporating an azide containing non-natural amino acid to the interior surface of bacteriophage Qβ, positioning a chemically unique site within the virus.[84] In their recent endeavour, the group exploited the architectural features of the virus to explore how the spatial distribution and polyvalent display of antigenic carbohydrates would modulate the mammalian immune response.[1,27] This study underscores the advantages of BNPs with their geometrical symmetries and genetically encoded building blocks can become important tools to probe biological events. Francis and coworkers reported in their studies the incorporation of a non-natural amino acid, *para*-amino-L-phenylalanine, on MS2 by using amber suppression technology. Such display technology on MS2 permitted cell-targeting peptides and DNA aptamers to be decorated on the bioscaffold.[18]

For transition metal catalyzed reactions, the less commonly targeted amino acid residues (tyrosine and tryptophan) can be selectively modified to expand the chemical repertoire beyond the conventional lysines and cysteines. The unique structural feature of tyrosine has been shown to support multi-faceted reactions other than simple nucleophilic or electrophilic substitution reactions

with amino, thiol or carboxylic groups. For instance, selective alkylation of phenol group in tyrosine residues has been achieved using π-allylpalladium complexes in protein concentration levels as low as 5 μM.[93] Bacteriophage MS2 displaying surface exposed tyrosines had been selectively modified, whereas proteins lacking exposed tyrosines, even in the presence of a reactive cysteine residue, had not been modified. Another strategy involves the use of rhodium carbenoids to selectively target the indole group of tryptophan residues. This reaction has been reported as a chemoselective method to anchor small molecules onto the tryptophan residues.[93,94] Finn and colleagues approached the tyrosine modification by using tripeptide (Gly–Gly–His) in the presence of nickel acetate and magnesium monoperoxyphthalate to generate a tyrosyl radical.[43] The addition of a nucleophilic group, such as azido-functionalized cysteine, provided a new chemical address on the virus particle beyond the conventional bioconjugation schemes. In both reaction strategies, the authors suggest that the hydrophobic, aromatic ring interactions with the transition metal catalysis enhanced the selectivity towards tryptophan and tyrosine residues.

Conclusions

Nature has designed many biogenic systems with exquisite symmetries and complexities at nanometer scales. Viruses, ferritins, enzyme complexes, chaperonin proteins and carboxyomes can be all classified within this category of BNPs, and each with their own distinctive features that can be exploited and tailored to suit each specific application. Each type of particle differs widely in shape and size with diverse chemical and physical properties. These physical differences provide researchers with an extensive selection of possible building blocks for the appropriate application. The thermal and pH stabilities, unique higher order assembly patterns, spatial resolution (including the overall shape of the particle and the distance between each reactive site/functional group) and genetic mutation limitations are all possible variations of these expansive platforms.

In summary, BNPs provide a number of distinct advantages. Nature provides a variety of BNPs with different sizes, shapes and biological features. Secondly, these systems can be designed with various functionalities for special application through genetic or chemical modifications. In addition, the controlled assembly of these uniform nanoparticles can result in unique hierarchical structures for a variety of applications. And finally, industrial scale production in a small laboratory space with relatively low maintenance is attractive for many academic researchers. On the other hand, in comparison to polymers or other nanomaterials, these BNPs are not as physically robust. As seen from many of the listed examples, BNPs are evolutionarily "designed" to fit a biological function within moderate parameters. Despite this current limitation, many of the future designs could lead to more robust BNPs that are more permissive towards wide range of chemical modifications and physical stresses.

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 for therapeutic applications. Furthermore, BNPs can be modified with polymers to boost the half-life of the biological system by shielding the protein structures from either enzymatic degradation or immune response. BNP chemistries also extend beyond the covalent annealing of two different functional groups. 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 the BNPs. Typically the modification approach on a nanoparticle will depend on the final application. Whether it is specific cell-targeting for drug/gene delivery, with simultaneous *in vivo* imaging for biomedical purposes or as tools for inorganic metal depositions, intravital imaging agents or biomaterials for guiding cell growth, adjusting the total number of molecules displayed on the BNP and generating consistent, uniform function will be a significant part of biotemplated nanomaterials.

Acknowledgements

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