Utilisation of plant viruses in bionanotechnology

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Bionanoscience/technology sits at the interface of chemistry, biology, physics, materials science, engineering and medicine and involves the exploitation of biomaterials, devices or methodologies on the nanoscale. One sub-field of bionanoscience/technology is concerned with the exploitation of biomaterials in the fabrication of new nano-materials and/or -devices. In this Perspective we describe examples of how plant viruses, focusing particularly on *cowpea mosaic virus*, a naturally occurring pre-formed sphere-like nanoparticle, are being used as templates and/or building blocks in bionanoscience and indicate their potential for future application.

Introduction

Nanotechnology is a collective term for a wide range of relatively novel technologies; the main unifying theme is that it is concerned with matter on the nanometre scale (Greek nãnos means dwarf). It is a highly multidisciplinary area that describes a field of applied science and technology focused on the design, synthesis, characterisation and application of materials and devices on this scale. Nanosystems may be produced by either microfabrication, making big structures smaller and/or by embedding smaller features into macroscopic materials, or by using the techniques of component assembly and/or supramolecular chemistry to make small molecules bigger; these techniques are also known as top-down and bottom-up design, respectively.

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Bionanotechnology or nanobiotechnology (the terms are synonymous) is a sub-section of nanotechnology. Although, due to its infancy, there is still a lot of bionanoscience to do before bionanotechnology comes to the fore; fundamental research (science) leads on to applications (technology) and exploitation. Bionanoscience/technology involves the exploitation of biomaterials, devices or methodologies on the nanoscale. It also has a multidisciplinary character, as it sits at the interface of chemistry, biology, physics, materials science, engineering and medicine. The subject of bionanotechnology can be divided into two main areas. The first is defined as the use of nanotechnological devices to probe and understand biological systems. The second is concerned with the exploitation of biomaterials in the fabrication of new nano-materials and/or -devices. In this Perspective we describe examples of the latter, especially the bionanoscience of plant viruses and their potential for application, focusing in particular on *cowpea mosaic virus* (CPMV).

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Nicole Steinmetz received her Diploma in Biology with honours from the Rheinisch Westfälische Technische Hochschule Aachen, Germany, 2004, and was awarded the Springorum Denkmünze. Since then she has been a Marie Curie EST Fellow working towards her PhD at the John Innes Centre, Norwich, UK. In 2006 Nicole was awarded the Bryan Harrison Prize, was honoured to participate in the 56th Lindau Meeting of Nobel Laureates and was chosen to be a finalist for the European Young Chemists Award.

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Dave Evans was born in Southampton, England in 1960. He graduated from University College Cardiff, Wales with a 1st Class Honours degree (1980) in chemistry and a PhD (1983). Following post-doctoral research at the University of Arizona and the University of York, he joined the AFRC Unit of Nitrogen Fixation (1986) situated at the University of Sussex. The laboratory was relocated to the John Innes Centre, Norwich (1995) where he is currently a Project Leader in the Department of Biological Chemistry.

Viral nanoparticles as building blocks

During recent years a set of naturally occurring biological nanoparticles such as protein cages (ferritin, heat shock proteins, *etc.*) and a wide range of viral nanoparticles (VNPs) have been utilised as templates for material fabrication. Especially the capsids of viruses have received particular attention. Viruses consist—in their simplest form—of a protein coat (capsid) and nucleic acid (RNA or DNA). The nucleic acid encodes the genetic information allowing reproduction of progeny particles. The function of the capsid is mainly the protection of the nucleic acid, thus viral capsids are extremely robust and rigid and are therefore an excellent tool for bionanotechnological applications. To date many different viruses have been used, including the bacteriophages M13 and MS2, the plant viruses CPMV, *cowpea chlorotic mottle virus* (CCMV), *red clover necrotic mosaic virus* (RCNMV), and *brome mosaic virus* (BMV), and the animal virus *chilo iridescent virus* (CIV).

Viruses have been extensively studied and their biological, genetic, and physical properties are well described. Viral capsids have appealing features for use in bionanotechnology. The most obvious advantage is their size: sphere-like particles with diameters ranging from *circa* 30 nm (*e.g.* CPMV) to 140 nm (*e.g.* CIV), and nanorods with length up to the micrometre scale (*e.g.* M13) are available. The propensity to self-assemble into monodisperse nanoparticles (NPs) of discrete shape and size, with a high degree of symmetry and polyvalency makes them unique bionanoparticles. For many viruses, *in vitro* self-assembly mechanisms of coat protein (CP) monomers, in the presence and absence of the nucleic acid, into intact and stable viral like particles (VLPs) have been observed, for example BMV, CCMV, and *tobacco mosaic virus* (TMV).[1–3]

Mutagenesis of viral capsids is a well established technique and allows alteration of the surface properties of the particles, such as the charge of the particles, amino acids available for selective attachment of molecules, and also allows the display of small peptides on the particle surface. In addition, heterologous expression systems, for example for CPMV and CCMV, have been developed allowing the production of mutant VLPs that would be unlikely to propagate in the natural host.[4,5]

In addition to the property to self-assemble into discrete particles, VNPs show also the propensity for self-organisation. Straightforward crystallisation procedures lead to mesoscale self-organisation, and 2-D and 3-D crystals can be readily obtained;[6,7] also self-supporting crystalline thin films especially of rod-shaped VNPs can be fabricated using the anisotropic nature of the particles.[8,9]

Plant viruses are non-infectious towards other organisms and they do not present a biological hazard. The production of the particles is simple and quick. When produced in the natural host, high expression yields can be achieved, due to their autonomous replication and *in vivo* assembly; for example plant viral particles can be obtained in gram scales from 1 kg of infected leaf material within 2–4 weeks.[10] Heterologous expression systems can also give rise to high yields of VLPs.[5] Last but not least, viral capsids are exceptionally robust. High temperatures can be tolerated, particles remain intact at a wide range of pH values, and also organic solvent–water mixtures can be endured, thus offering conditions for a wide range of chemistry. Therefore, viruses display a number of features that can be exploited for bionanotechnology and so can be regarded as extremely robust, monodisperse building blocks.

Rod-shaped VNP

***Tobacco mosaic virus* (TMV).** TMV is a rod-shaped plant virus. The X-ray structure of the particles has been determined.[11] Each particle consists of 2130 identical protein subunits arranged in a helical motif around a single stranded (ss) RNA molecule to produce a hollow tube of 18 nm by 300 nm with a 4 nm wide central channel.

The versatility of TMV as a biotemplate for fabrication of a range of nanotubular inorganic materials *via* metal deposition has been demonstrated. Different properties of the exterior and interior surface, due to different amino acid compositions, allow spatial and controlled deposition of metals. The use of TMV as a biotemplate for mineralization was first described in 1999, including: co-crystallisation with PbS and CdS, oxidative hydrolysis of iron oxides and sol-gel condensation of SiO_2.[12] Since then a range of materials have been deposited on the viral biotemplate on either the exterior surface or in the central channel: deposition of nickel, cobalt, copper, palladium, platinum, silver, SiO_2, TiO_2, Al_2O_3, on wild type and mutant TMV was achieved.[13–21] Hollow titanium tubes were generated by coating TMV with an ultra thin film of titanium followed by removal of the organic template by oxygen plasma treatment.[22] Also, mesoporous inverse silica replicas have been prepared by sol-gel condensation of SiO_2 on TMV followed by calcination.[23]

Besides their use as templates for mineralization, TMV has been used as a scaffold for the selective attachment of fluorescent dyes and other small molecules. The native virions offer addressable exterior tyrosine residues and interior carboxylates derived from glutamic acid residues.[24] Several mutants displaying reactive cysteine or lysine residues on the solvent exposed exterior surface have been made allowing decoration *via* thiol or amine selective chemistry.[25–27] Fluorescent labelled TMV particles have been used as templates for photo-reduction of metals on the surface of the virion,[24] and recently, the utility of TMV for light harvesting systems has been demonstrated. Such systems may have future uses in photovoltaic or photocatalytic devices.[2]

In addition to the studies in solution, the adsorption properties of TMV on various surfaces such as gold, mica, glass and silicon wafers were studied.[28] A technique for rapid and large scale assembly of thin film coatings and ordered fibres consisting of aligned TMV particles has been reported, metallisation of the virus fibres with silver led to anisotropically conductive arrays of wires of length up to multiple centimetres.[8]

Yi *et al.* [26,27] have used a mild disassembly protocol to partially disassemble the protein coat and to expose the RNA at the 5′ end. Spatially and oriented assembly of TMV on solid supports was achieved in a controlled manner *via* nucleic acid hybridisation using complementary oligonucleotides. The immobilisation of fluorescent labelled TMV onto electrodes was demonstrated.[26] Further, by using differentially labelled TMV particles and a micropatterned substrate, a patterned TMV microarray was constructed (Fig. 1).[27] The assembly of nanopatterned arrays of single TMV particles by the precise immobilisation and positioning of single virions, by using a combination of dip-pen nanolithography and coordination chemistry, was also achieved.[29]

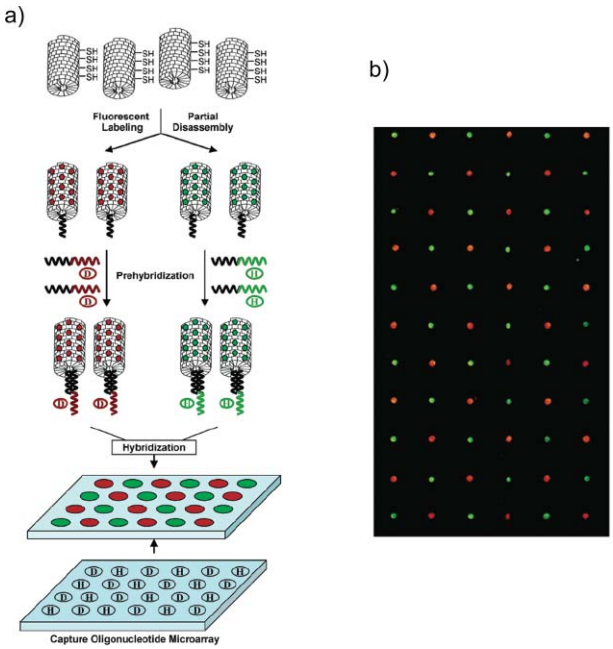

Fig. 1 Microarray consisting of *tobacco mosaic virus* particles. (a) Schematic presentation of the reaction scheme: the patterned assembly of fluorescently labelled, partially disassembled TMV particles onto oligonucleotide microarrays *via* hybridisation; (b) fluorescence microscopy image of the TMV microarray. The spots are *ca.* 20 μm in diameter. Reproduced with permission from reference 27. Copyright 2007 American Chemical Society.

The use of TMV as a template for bionanotechnology is a rapidly growing field, and applications are envisaged in the fabrication of nano-structured electronic devices. The production of a digital memory device based on metallised TMV with incorporated platinum NPs has been reported already.[30]

Sphere-like VNPs

***Cowpea chlorotic mottle virus* (CCMV).** CCMV is a tripartite ssRNA virus from the family *Bromoviridae*. The X-ray structure[31] shows the particles to have an icosahedral symmetry with a diameter of *ca.* 28 nm. The CCMV capsid is composed of 180 identical copies of the CP and the particles display $T = 3$ symmetry.

In the natural host, the plant *Vigna unguiculata*, viral particles accumulate to high titres and typically yields of 1–2 mg of viral particles per gram of infected leaf tissue can be obtained. In heterologous expression systems, comparable high yields can be achieved, for example, expression of CCMV VLPs in *Pichia pastoris* yields up to 0.5 mg per gram of wet cell mass.[5] Heterologous expression in yeast allows large scale production of wild type and genetically modified capsids containing or empty of nucleic acid. The main advantage of heterologous expression is that it allows production of VLPs that would be unlikely to assemble and accumulate in the natural host cells. In general, mutations on the interior surface of the viral capsid, as well as mutations altering the CP subunit interface, eliminate the production of infectious viral particles in plants, but can be produced in large quantities using the yeast expression system.[5] In addition it was found that the CP monomers can self-assemble *in vitro* into intact empty CCMV particles.[3]

CCMV particles undergo reversible pH- and metal ion-dependent structural transitions. These structural transitions are described as a swelling mechanism which results in an approximately 10% increase in virus dimension. The structural transition is a result of an expansion of the pseudo-three-fold axis of the virus particle, the swelling is accompanied by the formation of 60 separate 2 nm sized openings in the protein shell (Fig. 2).[31–33]

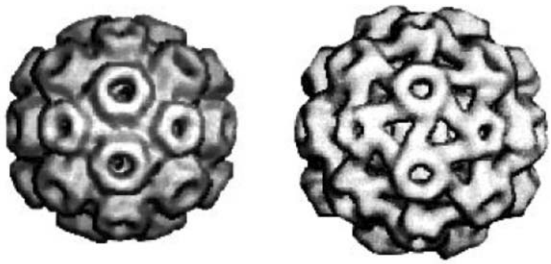

Fig. 2 Cryoelectron microscopy and image reconstruction of the *cowpea chlorotic mottle virus*. In an unswollen condition induced by low pH (on the left), and in a swollen condition induced by high pH (on the right). Swelling at the pseudo-three-fold axis results in the formation of sixty 2 nm pores. Reproduced with permission from reference 35. Copyright 1998 Nature Publishing Group.

The availability of an efficient heterologous expression system that allows production of empty particles in large quantities on the one hand, and the pH- and metal ion-dependent structural transitions of the CCMV capsid, on the other, made it an obvious target to study host–guest encapsulation. Indeed, the selective encapsulation of an anionic organic polymer, polyanetholesulfonic acid, into the CCMV cage,[34,35] as well as the selective entrapment followed by spatially constrained mineralization of iron oxide[36] and polyoxometalate species such as vanadate, molybdate, and tungstate within the CCMV cage has been reported.[34,35] Internal mineralization of the CCMV cage with polyoxometalate species was achieved by incubating empty particles with precursor ions (WO_4^{2-}, VO^{3-}, and MoO_4^{2-}, respectively) at neutral pH. At pH > 6.5 the particles exist in the open swollen form, allowing the ions to freely diffuse into the interior cavity. By lowering the pH to pH 5.0 two effects are induced: (i) a pH dependent oligomerisation of the inorganic species to form large polyoxometalate species which were readily crystallised as ammonium salts, and (ii) the structural transition of the CCMV cage from the swollen form to the non-swollen form; thus trapping the inorganic material within the viral cage.[34,35] The outer surface of CCMV is not highly charged, therefore the inner and outer surface of the CCMV protein cage provide electrostatically dissimilar environments allowing spatial localisation and control of the mineralization reaction within the protein cage.[34,35]

It was demonstrated that by altering the charge of the interior surface of the CCMV cage from cationic to anionic, the resulting particles favoured the encapsulation of cationic species. A mutant was constructed in which all the basic residues on the amino-terminus of the CP were substituted with acidic glutamic acid. It was found, that due to the electrostatic alterations, the mutant favours strong interaction with ferrous and ferric ions, and that oxidative hydrolysis led to the size-constrained formation of iron oxide NPs encapsulated in the viral cage.[36] In addition to the use of the viral cage structure as a size and shape constrained

reaction vessel for internal mineralization, the use of the particles to nucleate gold NPs on the outer viral surface by the reduction of $AuCl_4^-$ mediated by electron transfer from surface tyrosine residues has been reported.[37]

CCMV displays metal binding sites at the interface of adjacent CP subunits. It has been shown that terbium(III) ions[38] and gadolinium(III) ions[39] can be bound to the inherent metal binding sites. The magnetic properties of the paramagnetic CCMV- Gd^{III} capsids were studied.[39] Although studies on *in vivo* biodistribution, toxicity and immunogenicity have not been reported yet, the paramagnetic CCMV particles are expected to be excellent candidates for medical applications such as magnetic resonance imaging (MRI) contrast agents.

The CCMV capsid offers a large number of selectively addressable amino acids on the exterior surface allowing decoration and multivalent display of molecules. Native CCMV particles display addressable lysines and carboxylates derived from aspartic and glutamic acid. Amine and carboxy-selective chemistry have been used to selectively attach fluorescent dyes; it was found that around 540 dyes can be attached to lysine residues and that up to 560 carboxylates can be decorated.[40] In addition, a genetically engineered cysteine mutant displaying two solvent exposed cysteines per CP was generated and probed with thiol-selective dyes, labelling of around 100 thiol groups (equals a third of the introduced thiols) was achieved.[40] This demonstrates the feasibility of the CCMV virions as a platform for multivalent presentation.

A protocol has been reported that allows controlled, sequential ligand display through mixed self-assembly.[41] Herein, CCMV capsids were independently decorated with two different types of ligand to generate two populations of labelled virions; type I labelled with ligand A and type II labelled with ligand B. The particles were then *in vitro* disassembled and the resulting subunits separately purified. Reassembly was performed using controlled ratios of type I and II subunits, exerting control of the stoichiometry of ligand A and B displayed on the final assembled virions (Fig. 3).

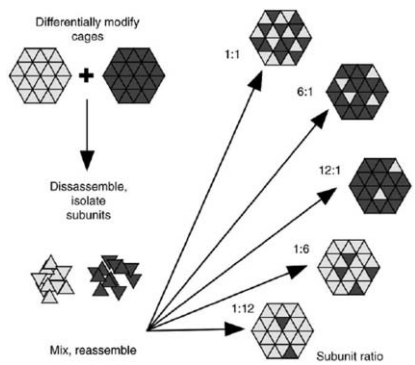

Fig. 3 Scheme for the mixed assembly of multifunctionalised particles. Two populations of particles are labelled, disassembled, and their subunits are purified. The differentially labelled subunits are subsequently mixed together at different ratios during reassembly resulting in multifunctional particles. Reproduced with permission from reference 41. Copyright 2006 Wiley-VCH.

In addition to the studies in solution, CCMV particles have also been used for immobilisation onto surfaces and for the construction of arrays. Immobilisation can be achieved by either chemisorption of cysteine containing CCMV particles onto gold surfaces,[42] or *via* electrostatic interactions of the negatively charged capsids onto positively charged surfaces.[43,44] Furthermore, multilayers consisting of CCMV particles immobilised on a solid support can be constructed *via* electrostatic interactions or complementary biological interactions (biotin–streptavidin(SAv)). Multilayered architectures of alternate polyelectrolytes (polylysine) and CCMV particles and biotinylated CCMV virions and SAv, respectively, were generated by layer-by-layer (LbL) assembly.[43] The ability to construct thin films of CCMV in combination with the possibility of multivalent display of various molecules on the capsid exterior and the possibility to selectively entrap and release materials from the capsids *via* the pH- and metal ion-dependent swelling mechanism, could lead to the development of semi-permeable functionalised membranes or controlled release coatings.

***Brome mosaic virus* (BMV).** BMV is a positive sense ssRNA virus from the family *Bromoviridae*. The structure has been solved at atomic resolution.[45] Capsids of BMV have an icosahedral symmetry with a diameter of *ca.* 28 nm. The capsid is comprised by 180 identical copies of one CP arranged in $T = 3$ symmetry. BMV particles can be produced in plants or in an heterologous expression system in *Saccharomyces cerevisiae*. Further, it has been found that CP monomers assemble *in vitro* into intact VLPs and that BMV, similar to CCMV, has a pH and an ion dependent swelling mechanism.[1,46]

In recent years the incorporation of gold NPs or luminescent quantum dots (QDs) into BMV particles has been studied.[47–50] This has been accomplished by *in vitro* assembly of BMV capsids in the presence of the NPs. It was found that, if sufficiently small (3–5 nm), several gold NPs[48,50] or QDs[49] can be incorporated into the VLP. Particles with sizes up to 16 nm could be incorporated also and this size is close to the inner diameter of the BMV capsids (17–18 nm).[47] The generation of intact, stable VLPs with high yields of incorporated NPs is highly dependent on the surface coating of the NP, polyethyleneglycol (PEG)-coated particles were found to give best yields and result in the formation of structurally intact and stable BMV VNPs.[47,49] The general encapsidation strategy and a transmission electron micrograph showing VLPs with incorporated Au NPs is shown in Fig. 4.[47] Viral cages with incorporated gold NPs and QDs offer new tools for biosensing applications, such as tracking a viral infection process. The utility of VLPs containing NPs for single virus spectroscopy has been demonstrated[50] as has the growth of 2-D and 3-D crystals of these VLPs and it is envisaged that these will lead to new plasmonic metamaterials.[6]

***Red clover necrotic mosaic virus* (RCNMV).** RCNMV is a bipartite ssRNA-virus from the family *Tombusviridae*; it forms particles with icosahedral symmetry and a diameter of approximately 36 nm. The self-assembly process of the RCNMV capsid is well understood. The assembly of the CP is stabilised by an internal protein/RNA cage and is initiated with the recognition of the origin of assembly site on the viral RNA by the CP and results in the formation of virions with encapsidated RNA.[51] By creating an artificial origin of assembly site on gold NPs the *in vitro* assembly and the formation of VNPs around the gold NPs was achieved. Using this strategy RCNMV CP is able to encapsidate a

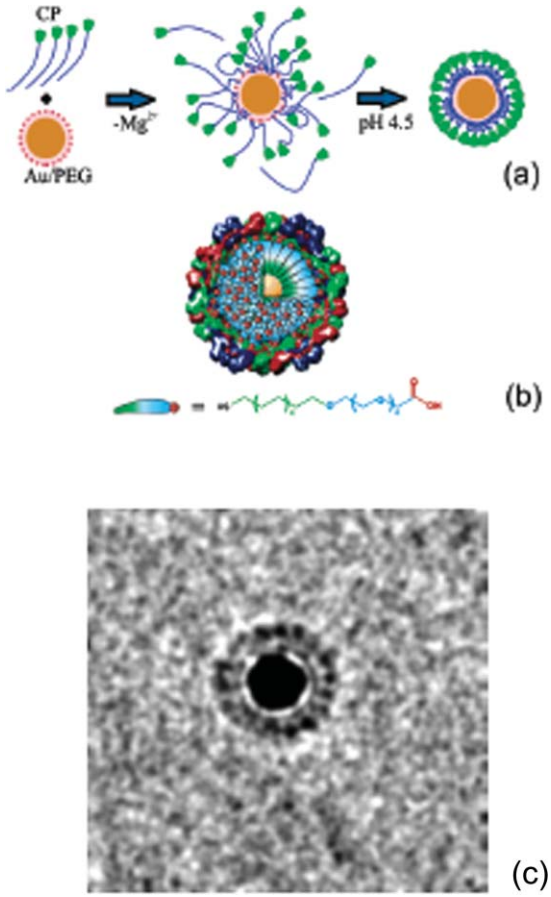

Fig. 4 Gold nanoparticle (NP) incorporation in *brome mosaic virus* (BMV) like particles. (a) Proposed mechanism of viral like particle (VLP) capsid protein (CP) assembly: first, electrostatic interaction leads to the formation of disordered protein–Au NP complexes. The second step is a crystallisation phase in which the protein–protein interactions lead to the formation of a regular capsid; (b) schematic depiction of the encapsidated NP functionalised with carboxyl-terminated PEG chains; (c) cryoelectron micrograph of a single VLP. The regular character of the protein structure coating the 12 nm diameter Au nanoparticle (black disk) is evident. Reproduced with permission from reference 47. Copyright 2006 American Chemical Society.

range of gold core sizes, stable VNPs with encapsidated gold NPs of sizes ranging from five to 15 nm were generated.[52] As suggested for BMV, the hybrid metal containing VNPs may be used as tools for biosensing purposes or as building blocks for the construction of new nanostructured materials.

***Cowpea mosaic virus* (CPMV).** CPMV is the type member of the comovirus genera of the family *Comoviridae*; also known as plant picorna-like viruses as they share similarities in structure, genome organisation and replication strategy with animal picornaviruses.[53] The structure of CPMV is known to near atomic resolution.[54] CPMV virions exhibit an icosahedral symmetry and show protrusions at the icosahedral five-fold and also at the three-fold symmetry axis and a valley at the icosahedral two-fold axis (Fig. 5). The particles are 28 nm in diameter. The virions are formed by 60 copies of two different types of coat proteins, the small (S) subunit and the large (L) subunit. The S subunit has

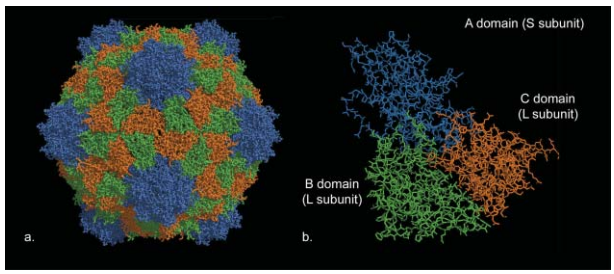

Fig. 5 The structure of the *cowpea mosaic virus* (CPMV) capsid. CPMV capsid (a) and the asymmetric unit (b). The capsid of CPMV is comprised of the small (S) and large (L) subunit. The A domain is shown in blue, the B domain in green and the C domain in orange.

one domain, the A domain, and the L subunit two, the B and C domains. The three domains form the asymmetric unit.

CPMV as an addressable nanobuilding block

In 2002 CPMV for the first time was regarded as an addressable nanobuilding block.[7] Experiments were initially conducted to test selective chemical derivatisation of wild type virions and later, several mutant particles were made, such as cysteine mutants[55,56] and histidine mutants.[57] Different bioconjugation strategies have been applied and a range of biological and organic and inorganic chemical molecules now have been attached to CPMV.

Structural data of the CPMV capsid indicate five exterior lysines to be solvent exposed (Fig. 6). Probing wild type CPMV[7] and lysine-minus mutants[58] with lysine-selective fluorescent dyes and metals confirmed that all lysines are addressable but to a different degree. It was found that a maximum labelling of 240 dyes per wild type CPMV particle can be achieved. In our laboratory we have demonstrated that CPMV displays addressable carboxylates

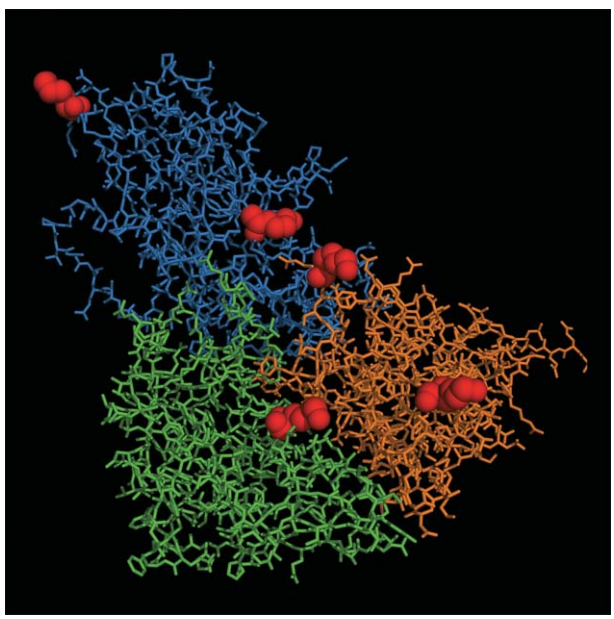

Fig. 6 Addressable lysine residues on the exterior surface of the *cowpea mosaic virus* (CPMV) capsid asymmetric unit. The A domain is shown in blue, the B domain in green and the C domain in orange; addressable lysine residues are highlighted as red spheres.

on its solvent exposed surface (Fig. 7).[59] The structural data from CPMV suggest eight to nine carboxylate groups, derived from aspartic and glutamic acids, to be on the solvent exposed exterior surface.[60] We probed the reactivity of the carboxylates using a fluorescent carboxylate-selective chemical dye, *N*-cyclohexyl-*N*′-(4-(dimethylamino)naphthyl)carbodiimide (NCD4). Using UV–visible spectroscopy, native gel electrophoresis and denaturing gel electrophoresis, covalent modification with the dye was confirmed. The latter showed that the dye was attached to both the S and the L subunit.

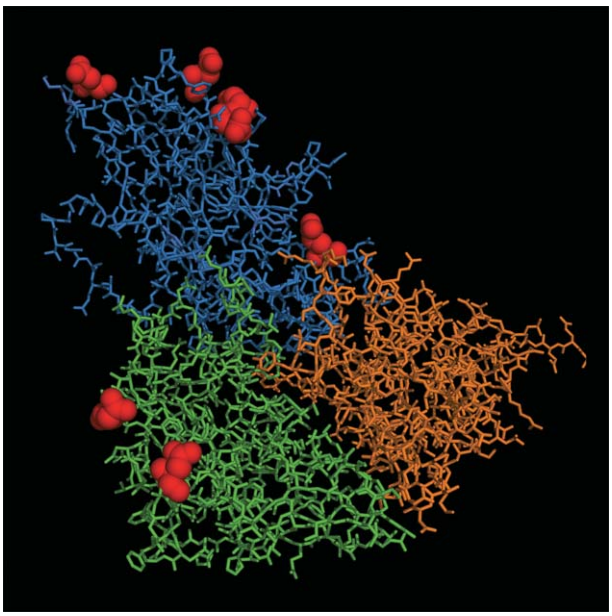

Fig. 7 Surface exposed carboxylates on the asymmetric unit of the capsid of the *cowpea mosaic virus*. The A domain is shown in blue, the B domain in green and the C domain in orange. Addressable surface exposed carboxylates derived from aspartic and glutamic acids are highlighted as red spheres.

Further, native CPMV particles display addressable tyrosine residues. Structural data and the study of tyrosine minus mutants demonstrated that two tyrosines located in the S subunit (Fig. 8) are available for chemical conjugation.[61] Besides the reactive exterior, addressable groups have been found on the interior of the capsid as well. CPMV has at least two reactive interior cysteine residues,[7] but no cysteines can be found on the solvent-exposed exterior.[54] It was demonstrated that chemical derivatisation of the internal cysteines can be achieved using small thiol reactive compounds.[7,62] Thus small molecules apparently can diffuse through the capsid, perhaps through the small hole that appears in the crystal structure at each five-fold axis.[54] At its narrowest point the opening of the hole is 0.75 nm, this explains why internal labelling with nanogold, with a diameter of 1.4 nm, could not be achieved[7,62] whilst smaller molecules can diffuse into the internal cavity and then react with internal cysteines.

Mutant CPMV nanobuilding blocks

The availability of the cDNA clones of RNA1 and RNA2 and chimaeric virus technology allow modification of the capsid

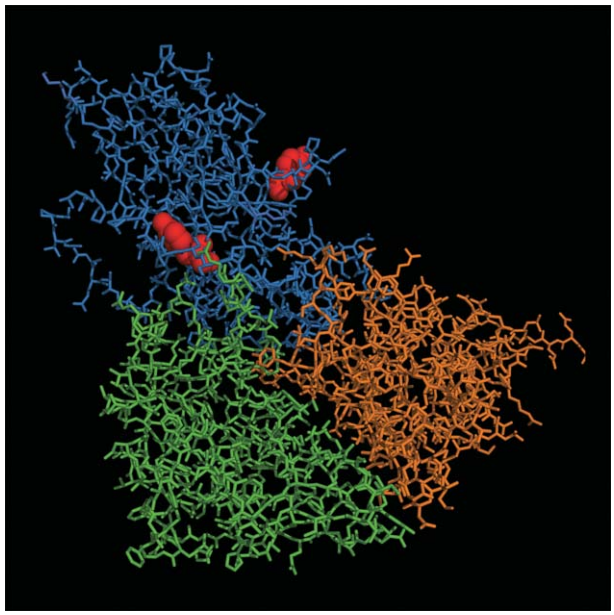

Fig. 8 Addressable tyrosine residues on the exterior surface of the *cowpea mosaic virus* (CPMV) capsid. The A domain is shown in blue, the B domain in green and the C domain in orange. Addressable tyrosine residues are shown as red spheres.

surface *via* site-directed and insertional mutagenesis with relative ease.[63–65] Previous work designed to employ CPMV as a scaffold for the presentation of antigenic peptide sequences[66–69] established that additional amino acids can be inserted into the highly surface exposed βB–βC loop and βC′–βC″ on the S subunit and the less solvent exposed βE–βF loop on the L subunit, without compromising the ability of the resulting mutant virus to propagate in the host plant. Provided the inserted peptide is less than 40 amino acids and has a pI below 9.0, the yields of modified particles are in general similar to those obtained for wild type CPMV.[67] On the basis of this knowledge a set of minus mutants and amino acid added mutants has been generated.

Thiols are among the most useful functional groups found in proteins; they can react with a large number of organic and inorganic reagents. Since native CPMV particles do not display any cysteines on the exterior solvent-exposed surface, genetic protein modification reactions can be used to introduce cysteines at defined locations. A series of CPMV cysteine added mutants ($CPMV_{CYS}$) have been made.[55,56] Mutants displaying cysteine residues on the surface exposed βB–βC loop and βE–βF loop, as well as double mutants, were generated, and their addressability was demonstrated using a variety of molecules such as nanogold or thiol-reactive fluorescent dyes.[7,56]

A major problem with $CPMV_{CYS}$ mutant particles is their propensity for interparticle aggregation *via* the formation of disulfide bonds. We have very recently reported a protocol for the chemical introduction of protected, addressable thiols to wild type CPMV particles (rather than by genetic modification).[70] Using the versatile labelling reagent *N*-succinimidyl-*S*-acetylthiopropionate, exposed lysines were modified to give CPMV particles decorated with thioacetate (SAc) groups. The advantage of the chemically engineered CPMV-SAc_n particles is that the introduced thiol is protected, this prevents the formation of disulfide bonds and

subsequent aggregation during storage. The protecting group can be easily removed with hydroxylamine hydrochloride. Usefully, the deprotected CPMV-SH_n particles also do not aggregate over three weeks in solution, a consequence of the position of the thiol groups on the capsid surface.

A set of five CPMV histidine mutants ($CPMV_{HIS}$) is also available.[57] Six contiguous histidine residues were genetically inserted at different positions of the capsid, in the βE–βF loop on the L subunit, the βB–βC loop and at the carboxy-terminus of the S subunit. The affinity to nickel(II) cations was verified for all mutants, although the affinity varies from mutant to mutant. The histidine tag provides a novel attachment site for chemical or biological moieties; the addressability was confirmed by labelling the virus with nanogold derivatised with a Ni-nitrilotriacetic acid crosslinker.[57]

It is evident, therefore, that CPMV wild type and mutant particles can be regarded as robust and multi-addressable nanobuilding blocks. During the last five years a large number of different biological as well as organic and inorganic chemical moieties have been attached to the virions for different applications and these are listed in Table 1.

From surface decoration to applications

CPMV wild type and mutant particles display different reactive groups on the exterior and interior surface, a wide range of selective chemistry is available allowing modification. In addition to standard (bio)conjugation techniques, *e.g.* the use of *N*-hydroxysuccinimide (NHS) activated molecules in order to decorate amine containing residues (lysines) or maleimide reactive moieties for selective modification of thiols (cysteines), the feasibility of copper(I)-catalysed azide–alkyne [3+2] cycloaddition (click-chemistry) for bioconjugation of the CPMV platform has been demonstrated, permitting efficient attachment of a range of molecules such as carbohydrates, peptides, polymers and proteins.[72,73,89]

Inorganic particles such as QDs and nanotubes,[80] and nanogold particles have been successfully attached.[7,55–57,79,94] Blum *et al.*[79] used CPMV as a nanoscaffold to build a 3-D conductive molecular network: gold NPs were attached to solvent exposed thiols on $CPMV_{CYS}$ mutant particles and subsequently interconnected by molecular wires, thus creating a 3-D conducting network (Fig. 9). This may lead to the use of the CPMV building block for the construction of nano-electronic circuits.

In addition, we have shown that CPMV particles can be decorated with redox-active compounds yielding electroactive VNPs.[59,81] Ferrocenes are well characterised molecules, noted for their stability and their favourable electrochemical properties. The availability of a large variety of derivatives makes them a popular choice for biological applications such as labels or sensors for electrochemical detection.[95] Ferrocenecarboxylate was used for facile, covalent decoration of amine groups on the CPMV particle surface using standard coupling procedures (Fig. 10).[81] Electrochemical studies confirmed the presence of redox active nanoparticles. Cyclic voltammetry showed that the complex possesses an electrochemically reversible ferrocene/ferrocenium couple. The oxidation process was diffusion controlled, and using the Randles–Sevcik equation,[96] the number of ferrocene molecules was calculated to be around 240 per

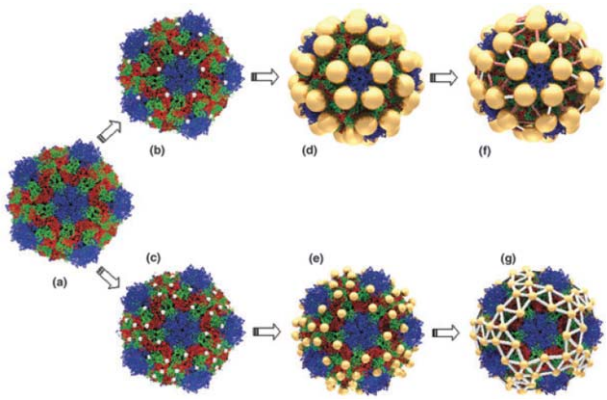

Fig. 9 Schematic of the procedure used to create molecular networks on the surface of the *cowpea mosaic virus* (CPMV). (a) CPMV capsid structure from crystallographic data; (b) $CPMV_{CYS}$ EF mutant with one cysteine (white dots) per subunit in the so-called EF loop; (c) $CPMV_{CYS}$ double mutant (DM) mutant with two cysteines per subunit; (d) EF with 5 nm gold NPs bound to the inserted cysteines; (e) DM with 2 nm gold NPs bound to the inserted cysteines; (f) EF mutant with the 5 nm gold particles interconnected using different wiring molecules (in red and white); (g) DM mutant with the 2 nm gold particles interconnected with wiring molecules. Reproduced with permission from reference 79. Copyright 2005 Wiley-VCH.

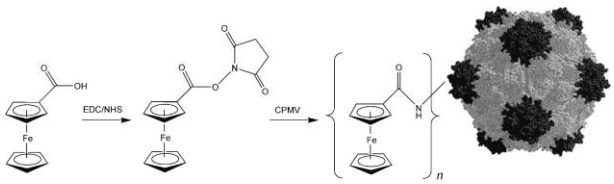

Fig. 10 Decoration of *cowpea mosaic virus* (CPMV) particles with ferrocene. Coupling of ferrocenecarboxylic acid to CPMV particles by activation with *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS). Reproduced with permission from reference 81. Copyright 2006 Wiley-VCH.

CPMV particle. The appearance of a unique reversible process in the cyclic voltammogram indicates that the multiple ferrocenyl centres behave as independent, electronically isolated units; therefore the CPMV-Fc_n conjugates are similar to metallodendrimers and could find applications as multielectron transfer mediators in electrocatalytic processes of biological and industrial importance.

In a second study, addressable carboxylate groups on wild type CPMV particles were utilised as anchor groups for an organic, redox-active viologen derivative, methyl(aminopropyl)viologen (MAV).[59] Cyclic voltammetric studies on viologen decorated CPMV-MAV_n nanoparticles showed the characteristic two successive, one electron, reversible steps of the methyl viologen moieties (Fig. 11). The reduction processes were diffusion controlled. Evidently the attached moieties behave as independent, electronically isolated units. The number of viologen molecules attached to each CPMV virion was estimated by use of the Randles–Sevcik equation, and it was found that around 180 viologens decorated each viral particle.

In the these two studies, the feasibility of CPMV as a nanobuilding block for chemical conjugation with redox-active compounds

Table 1

Area of research/purpose of study/potential application	Chemical/biological moiety conjugated to CPMV	Reference
Structural studies: Fluorescent and metal decorated CPMV wild type and mutant (cysteine added and histidine added, as well as lysine and tyrosine minus mutants) particles for structural analysis, *i.e.* testing the addressability of the particles and obtaining information about the conformation of attached moieties on the capsid surface. Also, testing the feasibility of various bioconjugation techniques.	Fluorescein Nanogold particles *N*-Cyclohexyl-*N′*-(4-(dimethylamino)naphthyl)carbodiimide Treatment with the nickel(II) complex of the tripeptide glycine-glycine-histidine in the presence of magnesium monoperoxyphthalate. Visible light irradiation in the presence of tris(bipyridyl)ruthenium(II) dication and ammonium persulfate. Ethylmercury phosphate Fluorescein and rhodamine (double labelling) Stilbene Metallic gold particles (0.9 μm in diameter) Stilbene and polyethyleneglycol (double labelling) *N*-Succinimidyl-*S*-acetylthiopropionate Azides Alkynes Fluorescein-azide and fluorescein-alkyne derivatives Transferrin-alkyne conjugate Arginine-glycine-aspartate (RGD) peptide Polyethyleneglycol Carbohydrates	58,71 59 61 57 7,56 62 70 72 73
CPMV as a reporter particle for various studies: - study of self-assembly of NPs at liquid–liquid interfaces - standard microtubule gliding assay - DNA microarray protocols - direct and indirect sandwich immunoassays - use of nanoprobes in order to gain information about the structure of hyaluronan.	Oregon green Tetramethylrhodamine NeutrAvidin and AlexaFluor 648 (double labelling) Chicken IgG and AlexaFluor 546 (double labelling) NeutrAvidin and Cy5 (double labelling) Antibodies and AlexaFluor 647 (double labelling) Chicken and/or mouse IgG and AlexaFluor647 and biotin (double labelling)	74 75 76 77 78
Nano/microelectronics: Generation of electro-active CPMV particles and construction of three dimensional conducting circuits at the nanometre scale. Also covalent network formation of CPMV with quantum dots or carbon nanotubes for the design of new nanostructured materials.	Nanogold Nanogold followed by interconnection of the gold NPs *via* 1,4-C_6H_4[*trans*-4-$AcSC_6H_4C{\equiv}CPt(PBu_3)_2C{\equiv}C]_2$ and/or oligophenylenevinylene Ferrocene Methylaminoviologen Quantum dots Carbon nanotubes	55,79 80 59,81
Biomedical applications: Labelled CPMV particles as reporter particles for non-invasive *in vivo* imaging *via* fluorescence microscopy, diffuse optical tomography, and magnetic resonance imaging. PEGylation as a strategy for reduced immunogenicity of CPMV virions for diagnostic and therapeutic applications. Glycosylated CPMV as a tool for cellular recognition and signalling events (glucose assay and hemagglutination). CPMV as a platform for covalent clustering of carbohydrates as a tool in the study of carbohydrate-based cellular processes. CPMV as a platform for the presentation of biologically active proteins with potential for vaccine development, drug delivery, and therapeutic applications.	Oregon green Fluorescent red NIR-782 Gd^{III} complexes Tb^{III} complexes AlexaFluor 555 AlexaFluor 488 Fluorescein Polyethyleneglycol Fluorescein and polyethyleneglycol (double labelling) Mannose Mannose and fluorescein (double labelling) Side chain neoglycopolymer T4 lysozyme LRR internalin B Intron 8 of herstatin Transferrin-alkyne conjugate Arginine-glycine-aspartate (RGD) peptide Polyethyleneglycol Carbohydrates	82 83 84,85 86 87 88 89 90 73
Material fabrication: Controlled formation of VNP arrays in solution and on solid supports achieved *via* biospecific recognition.	Oligonucleotides Mannose Biotin Biotin and AlexaFluor 488 (double labelling) Biotin and AlexaFluor 568 (double labelling) AlexaFluor 488	91 88 56,71 92 93

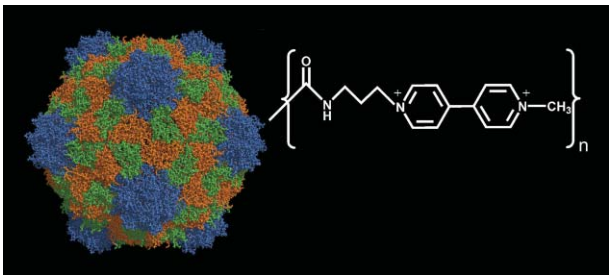

Fig. 11 Methyl(aminopropyl)viologen decorated *cowpea mosaic virus* particles.

was demonstrated. The resulting robust, and monodisperse particles could serve as a multielectron reservoir that may lead to the development of nanoscale electron transfer mediators in redox catalysis, molecular recognition and amperometric biosensors and to nanoelectronic devices such as molecular batteries or capacitors.

Fluorescent CPMV derived VNPs find potential applications in: (i) biomedicine, (ii) as reporter molecules in sensors or assays,[77,76] (iii) as fluorescent tags for understanding biological processes on the nanoscale.[74,75] The main advantage of the viral nanoscaffold, besides biocompatibility, is the multivalent display of reporter molecules, and that high local dye concentrations without fluorescent quenching can be achieved, this increases the signal-to-noise ratio and therefore the detection sensitivity.[83] The utility of the VNPs as a tool for *in vivo* non-invasive intravital vascular imaging has been explored[82,86] and in a recent study, the bio-distribution, toxicity, and pathology of the particles has been studied in detail.[85] It has previously been reported that CPMV particles induce antibody responses.[87] PEGylation is known as an effective strategy in order to minimise molecular interactions, and the study of PEGylated CPMV particles showed that this strategy can be used to effectively reduce the immunogenicity of the VNPs.[87] Besides the use of fluorescent labelled CPMV particles, the potential of gadolinium modified CPMV as a MRI contrast reagent has also been shown.[84] CPMV provides a platform for biomedical applications; the VNPs can provide new routes for targeted imaging and also targeted drug delivery.

Immobilisation of CPMV on solid supports and the construction of arrays

Immobilisation of biomolecules and the assembly of biomolecules in defined arrays is a desired requirement of (bio)nanotechnology. Thin films of immobilised proteins on solid supports are of growing interest and find applications in biosensors, information processing, optics and biomedicine.[97,98] In contrast to the large amount of studies with CPMV in solution, there are only a few reports where CPMV particles have been used as building blocks for the construction of arrays on solid supports. CPMV particles have been bound onto surfaces using different strategies. For example, $CPMV_{HIS}$ mutants have been immobilised on NeutrAvidin surfaces bridged with biotin-X-NTA molecules followed by decoration of the viral particles with QDs.[92] CPMV cysteine mutants have been successfully immobilised on maleimido-functionalised patterned templates; these templates were prepared by either microcontact patterning or scanning- and dip-pen nanolithography. Using the latter the controlled fabrication of a nanopatterned array consisting of single VNPs was achieved.[99,100]

To further extend the utility of CPMV virions as tools in bionanotechnology, we have shown that CPMV particles can be utilised as building blocks for the construction of mono-, bi- and multilayer arrays on surfaces in a controlled manner.[93] CPMV virions were labelled with two different ligands: fluorescent dyes that enabled differential detection and biotin molecules that allowed the construction of arrays from the bottom-up *via* a layer-by-layer approach. The construction of the layers was achieved using the high molecular recognition between SAv and biotinylated virions (CPMV-biotin). Immobilisation of CPMV particles on solid supports was achieved by either direct binding of CPMV cysteine mutant particles onto gold by a gold–thiol interaction, or by indirect immobilisation of CPMV-biotin mediated by a thiol-modified SAv. In detail, one set of viral wild type particles were labelled with the fluorescent dye AlexaFluor (AF) 488 and biotin, both groups were attached to surface available lysines. Another batch was labelled with AF568 and biotin, also at addressable lysines. Both types of building block, CPMV-biotin-AF488 and CPMV-biotin-AF568, displayed around 40 biotin moieties and around 200 dyes (Fig. 12, building blocks). Unspecific adsorption on gold surfaces of any of the building blocks was ruled out, indicating that binding of the building blocks occurred in a controlled manner based on a sulfur–gold interaction (SAv-thiol) and on specific interactions between biotin-bound-CPMV and SAv.

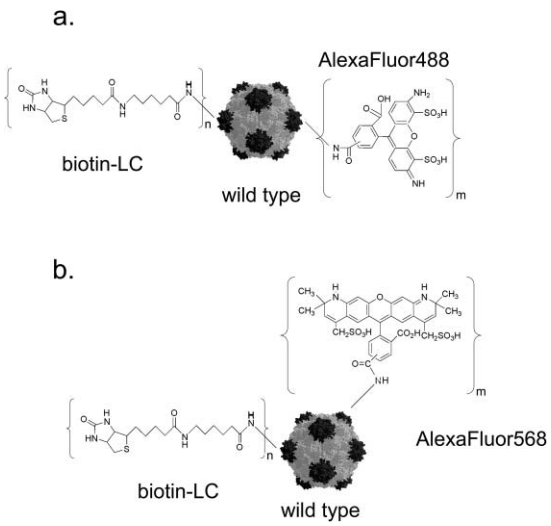

Fig. 12 Representation of chemically modified *cowpea mosaic virus* (CPMV) particles. (a) CPMV-biotin-AF488; (b) CPMV-biotin-AF568. Reproduced with permission from reference 93. Copyright 2006 American Chemical Society.

CPMV bilayers comprised of [CPMV-biotin-AF488–streptavidin–CPMV-biotin-AF568], and *vice versa*, on SAv functionalised gold surfaces were fabricated and analysed (Fig. 13). Fluorescence microscopy imaging of the CPMV arrays was consistent with successful binding of both viral building blocks. The fluorescent viral particles were spread evenly over the

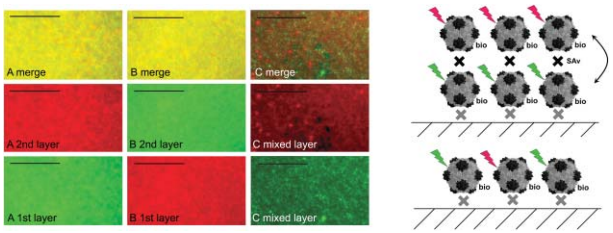

Fig. 13 Bilayers and a mixed monolayer of biotinylated (bio) and fluorescent labelled *cowpea mosaic virus* (CPMV) particles on Au-slides imaged *via* fluorescence microscopy (left) and diagrammatic representation of layer structures (right). The green flag and the red flag show the AlexaFluor dyes AF488 and AF568, respectively. The black cross depicts streptavidin (SAv), the grey cross shows a thiol-modified SAv. The scale bar is 10 μm. (A) Bilayer of CPMV-biotin-AF488 and CPMV-biotin-AF568, CPMV-biotin-AF488 in the 1st and CPMV-biotin-AF568 in the 2nd layer, merge shows the overlaid images from the 1st and 2nd layer. (B) Bilayer of CPMV-biotin-AF568 and CPMV-biotin-AF488. (C) Mixed monolayer of CPMV-biotin-AF488 and CPMV-biotin-AF568. Reproduced with permission from reference 93. Copyright 2006 American Chemical Society.

whole surface and a dense coverage was achieved. The overlaid image demonstrated that the individual images line up well, indicating that the virions are sitting atop each other. To further support these observations, a mixed layer was immobilised on the gold surface and analysed in the same way (Fig. 13). The merged images do not line up, consistent with the particles occupying the same layer and competing for the same binding sites. The construction of a trilayered array, by incorporation of CPMV cysteine mutants in the first layer, has also been shown (Fig. 14).[93] The feasibility of CPMV particles as nanobuilding blocks for the controlled fabrication of arrays on solid supports has now been demonstrated and different immobilisation strategies were found to be applicable. The introduction of functional molecules in the one-, two-, or three-dimensional arrays of uniform nanoparticles may provide potential for the development of novel functional devices on the nanoscale.

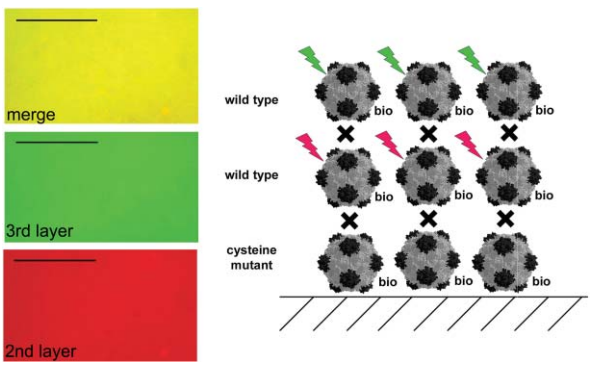

Fig. 14 Triple layer of CPMV particles on gold slides. Imaged by fluorescence microscopy (left) and diagrammatic representation of layer structures (right). The green and red flags show the AlexaFluor dyes AF488 and AF568, respectively. The black cross depicts streptavidin. The scale bar is 10 μm. Reproduced with permission from reference 93. Copyright 2006 American Chemical Society.

Outlook

In recent years it has been recognised that biological structures and biomolecules are promising tools for bionanotechnological applications. Plant viruses are a valuable addition to the bionanotechnology toolbox for the construction of new materials, sensors and devices on the nanoscale and for use in medical applications, from imaging to drug targeting and delivery. This new area of bionanoscience/technology is rapidly developing, a highly interdisciplinary area that involves collaborations between virologists, chemists, physicists, materials scientists and medics: it is exciting to be working at the virus–chemistry interface!

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