RNA-stabilized protein nanorings: high-precision adapters for biohybrid design

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The equipment of technical devices with biogenic adapter structures enables effective integration of biofunctional units into advanced sensor, filtration or catalytic layouts. Plant virus-derived self-organizing supramolecular complexes are among the most promising soft-matter adapters due to their multivalence on the nanometer scale, precise dimensions, high availability and compatibility with routine conjugation chemistry. Bioengineering can tailor both the templates' shapes and coupling sites and has been applied here to develop nucleoprotein 'nanorings' made of tobacco mosaic virus (TMV) building blocks. Short RNA-scaffolded four-turn helices of ≈ 68 protein subunits and ≈ 9 nm length, with 18 nm outer and 4 nm inner diameters, were generated efficiently in vitro. A structure-directing single-stranded 204-nucleotide ribonucleic acid containing the TMV origin of assembly-yielded colloidal preparations that have structural integrity and are well dispersed in a pH range from about 7 to 9. Two selectively addressable protein types with either an amino or a thiol group accessible were combined in the ring-like objects, allowing dual covalent coupling of a fluorescent dye first and to an isothiocyanate-covered substrate thereafter. Such precisely shaped nanoconstructs with distinct functional groups exposed in high surface densities offer novel opportunities as versatile adapter elements for the fabrication of extended bio/synthetic hybrid materials.

1. Introduction

The combination of biological and inorganic compounds for fabrication of hybrid materials of new functionalities is a growing field of research. Innovative applications tested for biomacromolecular building blocks include light-driven water oxidation,¹ photoelectrochemistry in current collectors,^{2,3} catalysis at bioinorganic interfaces⁴ and marine fouling-resistant coating technology.⁵ In several cases, the use of bioinorganic composites was shown to enhance product transformation rates, quality and/or durability compared to plain inorganic devices. Coating and structuring of inorganic substrates with biological adapter templates such as engineered bacterial S-layer protein lattices,^{6–8} ring-shaped toroidal proteins (tryptophan ribonucleic acid (RNA)-binding attenuation protein⁹ or heme carrier protein 1 (Hcp1) 'donuts'^{10,11}) or

functionalized plant viruses^{12–14} has allowed positioning of nanoparticles as well as installing bioactive molecules with unprecedented efficacies. This has yielded, for example, new types of gas sensors,¹⁵ high-performance proof-of-concept biosensors superior to conventional layouts^{13,14,16–19} and biocatalytic arrays with enzymatic activities increased in comparison to the non-immobilized counterparts.²⁰ Hence, richly available, biologically and chemically tailorable multivalent nano- and mesoscale biotemplates and, namely, plant viruses are regarded as promising interlayers between soft- and hard-matter components, imparting improved functionalities to future detection and biotransformation platforms.^{17,21–26}

Nanoscale building blocks of biological origin provide specific advantages over inorganic materials. Compared to 'top-down'

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processes such as inorganic etching techniques or lithography methods, ‘bottom-up’ biomolecule assembly allows synthesis of highly reproducible structures which can be readily manipulated chemically or genetically.^{24,25,27} On the other hand, inorganic material – for example, of solid-state membranes²⁸ or minerals,^{29,30} – implies durability or high stability even under harsh conditions. A critical point according to the fusion of biological and inorganic components to form hybrid materials is to connect such unequal compounds at their interfaces. This challenge can be tackled by functionalization of the surfaces with reactive groups for chemical coupling, or introducing molecules which interact by van der Waals, ionic or polar interactions.^{2,31,32} A well-established, versatile route of chemical coupling employs silane-based interlayers equipped with reactive groups (e.g. isothiocyanate (ITC)), which can be used, for example, for the functionalization of silica or polymer substrates to immobilize biomolecules.³³ Through a unique combination of top-down patterning of deoxyribonucleic acid (DNA) oligonucleotides on predefined surface patches, their ligation to suitable RNA scaffolds and eventually the bottom-up self-assembly of virus-like particles (VLPs) on the RNA-fashioned substrates, the authors achieved a spatially selective growth of covalently substrate-linked multivalent nucleoprotein ‘nanosticks’ previously.³⁴ The method has exploited the evolutionarily optimized capacity of protein and nucleic acid constituents of the plant-infecting tobacco mosaic virus (TMV) to interact and self-organize into elongated helical complexes and has proven their compatibility with technical environments.

As TMV and its building blocks are available at high yield, are amenable to externally controlled in vitro assembly and exhibit precise dimensions on the nanometer scale, considerable adaptability and stability over a wide range of different conditions,^{24,35} TMV-based nanoobjects have emerged as versatile components in various nanobiotechnology approaches.^{17,21,36} Therefore, the authors intended to advance and modify the RNA-directed fabrication of TMV derivatives to a novel type of colloidal, multiaddressable carrier and adapter templates with superior steric accessibility and advantageous for applications on flat surfaces: bifunctional short RNA–protein helices with an aspect ratio below 1 – that is, a length (respective height if surface-attached) below the diameter of native TMV. This is a tube-shaped, right-handed helical complex of 2130 coat proteins (CPs) and one single-stranded RNA (ssRNA) of 6395 nucleotides (nt) that is sandwiched between the CPs. A single TMV particle is 300 nm long and has an outer diameter of 18 nm and an inner channel of 4 nm width.³⁷ Upon in vitro assembly, the length and also overall (straight or kinked) shape of TMV-like RNA–CP tubes is tunable by altering the numbers of bases^{34,38,39} or origins of assemblies (OAs)⁴⁰ within the ssRNA. The OA is an RNA sequence forming a stem–loop structure, which is necessary for the nucleation reaction starting in vitro the self-organization of RNA and proteins into rod-like particles.⁴¹ Besides the OA-induced assembly, CPs are able to assemble without RNA into VLPs of undefined length.^{42,43} Lower-order intermediate structures of the TMV CP are ‘A-proteins’ of three to six subunits⁴⁴ and 20S aggregates that are a mixture of polar holey double-layered

disks of 34 CPs (in two rings each with 17 subunits) and of short protohelices of about two turns with $16\frac{2}{3}$ CP subunits per turn.^{37,45} Bipolar four-layered ‘top-to-top’ stacked disks can also form in CP preparations.^{46,47} Despite the conformational transitions possible between distinct CP disk-like aggregates and short helical tubes, blends of different ring-like structures occurring in CP preparations are easily accessible and well dispersible and might therefore be regarded promising candidate components of hybrid materials. However, their stability depends on pH, temperature, ionic strength and CP concentration^{37,48} – that is, alterations of these conditions inevitably lead to changes in the assemblies’ aggregation state, changing their length or leading to degradation. In particular, pH values even slightly above neutral at low ionic strength will disintegrate wild-type CP disks or protohelices.^{42,49} This does not only impede coupling processes relying on mild alkaline pH; it would also limit the use of successfully established composite materials to a narrow range of conditions.

However, TMV-based ring-shaped nanostructures display repetitive, chemically addressable CP subunits with intermolecular distances most attractive for uses ranging from plasmonics,^{50,51} light harvesting and energy transfer,⁵² enzymatic conversion reactions⁵³ up to intravitral drug delivery.⁵⁴ Hence, they are highly attractive templates for ordering nanoparticles and functional molecules both in suspension and after deposition on solid substrates. Therefore, different strategies to stabilize TMV-derived ring-like assemblies have been pursued in several laboratories. A number of genetically engineered TMV CP variants with enhanced intersubunit attractive forces or covalent binding sites have been established and expressed in *Escherichia coli*, with more robust CP-based rings successfully generated. These included a circularly permuted CP variant with its newly defined N-terminus located in the interior, channel-exposed domain⁵⁵ and a double-arginine (RR-)CP mutant,⁵⁴ both likely to form pairs of inverted single 17-CP disks, CPs with thiol groups enabling disulfide bond formation to adjacent layers⁵⁶ and C-terminally hexahistidine-terminated TMV CP variants from yeast or bacterial cells.^{57–60} They all came out to retain their distinct disk-like conformations up to about pH 8 to a significant proportion, in the case of RR-CP, up to pH 9.⁵⁴ Notwithstanding, electron microscopy revealed for all these distinct mutant CP rings either lateral or longitudinal association to different extents already slightly above pH 7, in various buffers tested, up to the formation of planar disk arrays or rod-like stacks. Thus, prominent fractions of all ring-shaped multimers assembled exclusively from engineered CPs either did not withstand even mildly alkaline conditions at all⁵² or did not remain fully dispersed, respectively, compromising the steric accessibility of individual colloids.

Hence, the authors sought to find out if RNA scaffolds might increase the pH stability and dispersibility of short ring-like, helical TMV CP assemblies to a greater extent than achieved so far with the different CP variants alone. After the authors had initially shown that OA-containing RNAs can direct the length of in vitro-grown TMV-like tubes down to 90 nm efficiently,³⁹ Rego *et al.*³⁸

Figure 1. Exceptionally short VLPs fabricated from TMV CPs and an OA-containing RNA scaffold: ring-shaped four-turn helices ('disks'), well dispersed and almost uniform

used RNAs of 349 nt to assemble TMV CP into almost isometric nucleoprotein cylinders ≈ 16.5 nm long (and 18 nm dia.). These represent the shortest TMV-based ribonucleoprotein complexes generated under controlled conditions so far, with their length still exceeding that of an RNA-free 34-CP disk (4.6 nm) or a stacked disk pair (≈ 10 nm). The authors now aimed at RNA–protein helices with an aspect ratio substantially below 1, resembling two- or four-layered disk-like or protohelical CP aggregates, but less prone to conformational changes – that is, robust, exceptionally short VLPs (Figure 1). To develop efficiently accessible 'flat' helical rings with well-defined numbers of potential coupling sites on their outer rim and end surfaces, a series of three distinct OA-including RNA scaffolds between 102 and 500 nt long was employed, all of them expected to yield ring- to barrel-shaped assemblies. Their capacities to guide the formation of holey nucleoprotein complexes were compared to those of two RNAs lacking an OA. The stability of the resulting short VLPs was evaluated at different pH values. The most reliably forming ring-like structures were then assembled from mixtures of distinct TMV CP types enabling selective covalent coupling to surface-exposed amino or thiol groups, respectively.^{61,62} Finally, the authors examined whether the resulting nanoobjects

could be equipped with fluorescent dyes and immobilized through conjugation to an ITC silane-functionalized substrate thereafter, to serve as proof-of-concept adapter rings installing functional molecules on an accordingly prepared surface area.

2. Materials and methods

2.1 Cloning and in vitro transcription of short RNA constructs

Constructs allowing in vitro transcription of short RNAs included the TMV OA stem-loop complementary DNA (cDNA) sequence, which corresponds to position (pos.) 5447–5515, with its central loop 5'-AAGAAGTCG-3' from pos. 5474–5482^{63,64} in the core OA region spanning pos. 5420–5546^{37,65} of the original TMV National Center for Biotechnology Information reference sequence NC_001367.1.⁶³ Constructs contained either TMV pos. 5442–5521 in pGem-T-Easy-102⁺ or pos. 5350–5531 in pGem-T-Easy-204⁺; see below. OA-cDNA-containing polymerase chain reaction (PCR) products comprising partial TMV and plasmid sequences were amplified with *Pfu* DNA polymerase (EP0571, Thermo Scientific, Darmstadt, Germany) or Q5 high-fidelity DNA polymerase (M0491, New England Biolabs (NEB), Ipswich, Massachusetts, USA) by using a TMV wild-type sequence (p843pe35TMVr.1) as a template.⁵⁸ The amplified PCR products were equipped with an adenosine overhang at their 3'-ends by *Taq* DNA polymerase (NEB). After purification (Qiaquick PCR purification kit, Qiagen, Hilden, Germany), the PCR products and pGem-T Easy vector (Promega, Mannheim, Germany) were cleaved by restriction enzyme *Aat*II (R0117, NEB) and ligated with T4 DNA ligase (NEB). The respective PCR primers provided by Biomers (Ulm, Germany) are listed in Table 1. The 204⁺ nt and 102⁺ nt RNA DNA templates with *Aat*II restriction sites for cloning were amplified by PCR (*Pfu* DNA polymerase; initial denaturation at 95°C, 3 min; three cycles of 95°C, 30 s, 32°C, 30 s, 72°C, 1 min; 27 cycles of 95°C, 30 s, 55°C, 30 s, 72°C, 1 min; final extension at 72°C, 5 min). The ligated vectors with insert (named pGem-T-Easy-102⁺ and pGem-T-Easy-204⁺, with the superscript + indicating presence of an OA sequence) as well as a religated vector (pGem-T-Easy) without insert (yielding RNA devoid of OA, see below) were transformed into chemically competent *E. coli* DH5 α following standard molecular biology procedures,⁶⁶ amplified and extracted by use of a plasmid midi kit (Qiagen). Sequence identity was confirmed by DNA sequencing with a CEQ 8000 genetic analysis system (Beckman Coulter, Krefeld, Germany).

Table 1. Primers designed for cloning

RNA product	Primer name	Primer sequence	Annealing temperature: °C
102 ⁺	102_cl_AatII_fwd	5'-GAATTCGACGTCCGTGAGAGACGGAG-3'	32, ^a 55 ^b
	102_cl_rev	5'-GCCTGATCGACATAGGGAC-3'	
204 ⁺	204_cl_AatII_fwd	5'-GAATTCGACGTCCGCGGGTTTCTGTCC-3'	45, ^a 51 ^b
	204_cl_rev	5'-AATTTGCAAGCCTGATCGACA-3'	

^a Initial annealing temperature (three cycles) to ensure hybridization in early PCR stages
^b Subsequent annealing temperature (27 cycles) for specific hybridization during PCR product amplification
*Aat*II restriction sites underlined; cl, cloning primers

Table 2. Primers yielding DNA amplicons as templates for RNA in vitro transcription

RNA product	Primer name	Primer sequence	Annealing temperature: °C
102 ⁺	102_iv_rev	5'-TGCCTGATCGACATAGGGACATC-3'	60
204 ⁺	204_iv_rev	5'-TAACTTTGCAAGCCTGATCGACAAGG-3'	60
500 ⁺	500_iv_rev	5'-GATTCATTAATGCAGCTGGC-3'	48
90 ⁻	102_iv_rev	5'-CCATATGGTCGACCTGCAGGC-3'	60
308 ⁻	308_iv_fwd	5'-CGCGCGTTGGCCGATTCAAT-3'	69
	308_iv_rev	5'-ATCACTAGTGAATTCGCGGCC-3'	
	308_iv_T7_fwd	5'-taatacgactcactatagggCGCGGTTGG-3'	40, ^a 61 ^b
	308_iv_rev	5'-ATCACTAGTGAATTCGCGGCCGCTG-3'	62
All except 308 ⁻	pGem_iv_fwd	5'-CGCCAGGGTTTCCAGTCAC-3'	60

^a Initial annealing temperature (three cycles) to ensure hybridization in early PCR stages

^b Subsequent annealing temperature (27 cycles) for specific hybridization during PCR product amplification
Small letters indicate part of the T7 RNA polymerase promoter; iv, in vitro transcription template primers

The DNA templates for in vitro transcription of the different RNAs were synthesized by PCR by using the isolated plasmids as templates and the primers listed in Table 2. The PCR products were separated by gel electrophoreses, and the required bands were isolated with a Qiaquick gel extraction kit (Qiagen) and reamplified by PCR by using the same primer combinations and purification protocol mentioned earlier. Elution from the Qiaquick PCR purification kit (Qiagen) made use of dimethyl dicarbonate (DMDC)-treated deionized (dd) water (H₂O) (18.3 MΩ cm; purified by a membraPure system, Aquintus, Bodenheim, Germany). The PCR DNA templates for 102⁺ nt RNA (template: pGem-T-Easy-102⁺), 204⁺ nt RNA (template: pGem-T-Easy-204⁺) and an OA-free 90⁻ nt RNA (template: pGem-T-Easy) were amplified by *Pfu* DNA polymerase (initial denaturation at 95°C, 3 min; 30 cycles of 95°C, 30 s, 60°C, 30 s, 72°C, 1 min; final extension at 72°C, 5 min). The DNA template for the third OA-containing 500⁺ nt RNA (PCR template: pGem-T-Easy-204⁺) was amplified the same way mentioned earlier but using an annealing temperature of 48°C, whereas an in vitro transcription template for a further OA-free 308⁻ nt RNA (PCR template: pGem-T-Easy-204⁺) was synthesized in three steps: The first PCR product was amplified without the region of the T7 RNA polymerase promoter (Q5 DNA polymerase; initial denaturation at 98°C, 30 s; 30 cycles of 98°C, 10 s, 69°C, 10 s, 72°C, 10 s; final extension at 72°C, 1 min). Second, this PCR product – extracted from an agarose gel – was fused with the T7 RNA polymerase promoter (*Pfu* DNA polymerase; initial denaturation at 95°C, 3 min; three cycles of 95°C, 30 s, 40°C, 30 s, 72°C, 30 s; 27 cycles of 95°C, 30 s, 61°C, 30 s, 72°C; final extension at 72°C, 5 min). Finally, the gel-extracted product was reamplified (*Pfu* DNA polymerase; initial denaturation at 95°C, 2 min; 30 cycles of 95°C, 30 s, 62°C, 30 s, 72°C, 30 s; final extension at 72°C, 5 min) and purified the same way mentioned earlier. All steps were monitored by standard electrophoresis methods,⁶⁶ using the NEB DNA loading dye (B7021). The samples were heated for 10 min at 60°C prior to separation in up to 2% agarose in 1× Tris–borate–ethylenediaminetetraacetic acid (EDTA) (TBE) and staining with ethidium bromide. The molecular weight marker used was NEB N3231.

In vitro transcription involved application of a MEGAscript® T7 High-Yield Transcription Kit (Ambion, Austin, Texas, USA) following the manual instructions. An amount of 100 ng DNA template was used in a reaction mix of 20 µl total volume and incubated for 6 h at 37°C. The transcripts were precipitated in (final concentrations (FCs) 1.3 M lithium chloride, 8.6 mM EDTA and 71% (v/v) ethanol overnight at –20°C to optimize the precipitation of short RNA products. The pellet was dissolved in DMDC-treated dd water. Transcript length and quality were analyzed by gel electrophoresis⁶⁶ as follows: Aliquots were supplemented with denaturing formamide-containing RNA gel loading dye according to the manufacturer's instructions (R0641, Thermo Scientific, Darmstadt, Germany), heated for 10 min at 95°C, separated in 3% agarose in TBE and stained with ethidium bromide. The molecular weight marker used was SM1831 (Thermo Scientific, Darmstadt, Germany).

2.2 Assembly of RNA-stabilized disks

Wild-type TMVwt⁵⁸ and genetically modified TMVCys and TMVLys⁶¹ were purified according to Gooding and Hebert.⁶⁷ RNA-free CP was prepared by disintegration of intact TMV particles in 66.7% (v/v) acetic acid for 20 min on ice.⁶⁸ The viral RNA was removed by centrifugation for 20 min at 20 000g and 4°C. The supernatant containing the free CP was dialyzed in tubing (Spectra/Por 7 dialysis membrane, 8 kDa molecular weight cutoff, Spectrum Laboratories, Rancho Dominguez, California, USA) against dd water at 4°C with water changes every 8 h until the isoelectric point was reached and the CP precipitated (24–48 h). The flocculating CP was collected by centrifugation the same way mentioned earlier, and the pellet resuspended in 75 mM sodium potassium phosphate (SPP) pH 7.5. This CP solution was centrifuged for 10 min at 10 000g, and the supernatant transferred into a fresh reaction tube. The concentration of the CP was determined by ultraviolet (UV) spectroscopy with a NanoDrop D-1000 spectrophotometer (PqLab, Erlangen, Germany) at a wavelength of 280 nm, using the extinction coefficient of TMV CP (1.3 ml/(mg cm)).⁶⁹ For a typical assembly reaction, 10 mg/ml CP was incubated in 75 mM SPP (pH 7.2) for at least 48 h at 20°C according to Butler.⁷⁰ RNA transcripts were mixed with CP in a 1:15 weight-to-weight ratio at an FC of

6 mg/ml CP and 0.4 µg/µl RNA and incubated overnight at 30°C. To investigate the initiation efficiency of nucleoprotein particle formation, RNA-containing or RNA-free CP solutions were incubated for 24 h at 30°C, 3 h at 30°C followed by 21 h at 10°C and 24 h at 10°C. Nucleoprotein particle formation was analyzed by native gel electrophoresis as in the following section.

2.3 Native gel electrophoresis

The samples that were expected to contain nucleoprotein particles and the corresponding controls were analyzed by native gel electrophoresis. Aliquots corresponding to 12 µg CP were supplemented with the loading buffer (10 mM SPP pH 7.2, 0.1% (w/v) bromophenol blue, 10% glycerol) and separated in 2.5–3.0% agarose gels in TBE (98 mM Tris pH 8.0, 89 mM boric acid, 2 mM EDTA). Protein bands were stained with Coomassie Brilliant Blue R250 (Serva Electrophoresis, Heidelberg, Germany). In later stages of the experiments, VLPs equipped with fluorescent dyes were also analyzed by native gel electrophoresis. Such samples were first analyzed under UV illumination and subsequently stained by Coomassie Brilliant Blue R250.

2.4 Transmission electron microscopy

The length of the nucleoprotein particles was assessed by transmission electron microscopy (TEM). Samples (15 µl) containing 50 µg/ml CP were placed on Parafilm M (American National Can, Menasha, Wisconsin, USA), and 400-mesh Formvar carbon-coated copper grids (Science Service, Munich, Germany) hydrophilized by brief ethanol treatment were placed for 5 min on the droplets. Excess sample solution was removed with filter paper; the grids were rinsed with five droplets of ultrapure water and stained with 2% uranyl acetate solution for 90 s. TEM analysis was carried out using a Tecnai G2 Sphera electron microscope (FEI, Hillsboro, Oregon, USA) with a Tietz F214 camera (TVIPS, Munich, Germany).

2.5 Stability tests at different pH values

The stability and structural integrity of the nucleoprotein particles at different pH values were examined by native gel electrophoresis and TEM analysis. A buffered solution (75 mM SPP pH 7.2) containing 4.3 mg/ml wild-type CP was incubated with 0.3 µg/µl 204⁺ nt RNA overnight at 30°C to yield four-turn helices. A control experiment with RNA-free wild-type CP was carried out under the same conditions. The pH was then adjusted to 8.0, 9.0, 10.0 or 11.0 by the addition of suitable amounts of a 100 mM trisodium phosphate (Na₃PO₄) stock solution. To yield acidic conditions, the pH was adjusted to 6.0 by the addition of suitable amounts of a 5% acetic acid stock solution. After 1 h at room temperature, the samples were analyzed on 3% (w/v) agarose gel the same way mentioned earlier, and for TEM analysis, the grids were prepared as described earlier.

2.6 Fluorescence labeling of mixed assembled disks by Atto 488–maleimide conjugation

Bifunctional nucleoprotein particles were generated by a mixed assembly approach.⁶⁰ CP_{Cys} and CP_{Lys} were combined in 75 mM

SPP at pH 7.2 to an FC of 3 mg/ml each and incubated for 24 h at 4°C to allow the disassembly of 20S and higher aggregates into A-protein and smaller aggregates, to achieve a homogeneous distribution of the different genetically modified CPs in the intended disk-like structures later. Afterward, the CP_{Cys}–CP_{Lys} blended solution was exposed for 24 h to room temperature, to obtain precursor aggregates for the nucleation process of VLPs. A 0.6 µg/µl FC of 204⁺ nt RNA was added to the CP_{Cys}–CP_{Lys} (FC 2.4 mg/ml each) solution and incubated overnight at 30°C. For chemical modification of the resulting VLPs, the products (0.028 µmol CP_{Cys}–CP_{Lys}) were mixed with 0.023 µmol Atto 488–maleimide, which corresponds to a 1.7-fold molar excess of Atto 488–maleimide per CP_{Cys} in a volume of 103 µl and incubated for 2.5 h under agitation (horizontal shaking at 300 revolutions per minute (rpm)) at 30°C. After the conjugation reaction, the chemically modified bifunctional nucleoprotein assemblies were purified by gel filtration by using PD SpinTrap G-25 columns (GE Healthcare, Freiburg, Germany).

2.7 Fabrication of gold-patterned ITC-functionalized glass substrates

To obtain solid supports with areas functionalized with ITC and areas without reactive groups, glass slides were spin-coated with photoresist AR-P 5350 for 40 s at 4000 rpm and prebaked on a hot plate for 4 min at 105°C. For patterning, the photoresist was exposed to UV light by using a lithography mask and subsequently developed in AR300-35 and immersed in a bath of ultrapure water. Substrates were then metalized with a layer of 2 nm thermally evaporated chromium and afterward with 30 nm gold. In a liftoff process, the photoresist was removed by acetone and ultrasonication.

For 3-isothiocyanatopropyl-triethoxysilane (ITC-silane) deposition, these gold-patterned substrates were treated in oxygen plasma to activate the surface and subsequently placed together with fluid ITC-silane (ABCR, Karlsruhe, Germany) inside a desiccator and evacuated at 100 mbar for 6 h to evaporate the fluidic ITC-silane. Gold-patterned substrates with site-specific ITC functionalization on the glass areas were removed from the desiccator and, after storage for 12 h at room temperature, rinsed with methanol to remove physisorbed ITC-silane molecules.

2.8 Disk and control dye immobilization on ITC-functionalized substrates

Fluorescence-labeled bifunctional nucleoprotein particles were immobilized on ITC-silane-covered glass substrates patterned with sputtered gold. A volume of 40 µl fluorescence-labeled bifunctional nucleoprotein structures (of in total 4.8 mg/ml CP) was mixed with 10 µl 0.2 M disodium phosphate (Na₂HPO₄) and 20 µl 0.1 M disodium phosphate. For the conjugation reaction between lysine moieties of the CP_{Lys} and the ITC groups of ITC-silane, the pH was adjusted to 9 by the addition of 7–8 µl 0.1 M trisodium phosphate. A control sample was prepared containing Atto 488–maleimide without nucleoprotein particles: 40 µl 77 µM Atto 488–maleimide in 75 mM SPP was mixed with 10 µl 0.2 M disodium phosphate and 20 µl 0.1 M disodium phosphate. Fluorescence-labeled bifunctional

nucleoprotein particles (30 μ l) or fluorescent dye, respectively, adjusted to pH 9 were dropped onto an ITC-covered glass substrate and spread by a coverslip that was placed onto the droplet and incubated overnight at room temperature in a humid chamber in the dark. The substrates were then cleaned three times with 10 ml ultrapure water and air-dried. Fluorescence signals were analyzed by fluorescence microscopy with an Axiovert 200M microscope (Zeiss, Oberkochen, Germany) using a fluorescein isothiocyanate (FITC) filter set.

2.9 Atomic force microscopy analysis of chemically immobilized disks on ITC-functionalized substrates

For imaging particle distributions on different surface modifications, atomic force microscopy (AFM) analysis was performed by a NanoScope IIIa-controlled MultiMode 2 atomic force microscope (Bruker, Ettlingen, Germany) equipped with a type 'E' scanner. A silicon nitrate cantilever with a typical resonance frequency of 75 kHz and a force constant of approximately 0.2 N/m (MikroMasch, HQ:NSC18/AU BS) was used for imaging 5 μ m $\times$ 5 μ m scan regions in tapping mode at a scan speed of 0.5 Hz, with a resolution of 512 $\times$ 512 points and a scan angle of 0°. Image data were processed with the NanoScope Analysis 1.40 software (Bruker).

3. Results and discussion

3.1 TMV-based nucleoprotein tubes and rings by way of OA-containing RNA scaffolds

Different RNAs with (+) and without (−) the TMV OAs, named after their length and potential OA presence (nt)^{+/−} in the following, were designed to find out whether they would give rise to RNA-scaffolded short VLPs in combination with TMV CP in vitro (see the scheme in Figure 1). Their efficacies and reliabilities upon the fabrication of tube- and ring-like assemblies with low aspect ratios were evaluated, seeking for efficiently generated products with increased stability compared to RNA-free CP holey disks, but without pronounced tendency for interparticle aggregation. Three RNAs containing the complete TMV OA core sequence⁶⁵ were expected to assemble into VLPs of different lengths (from 4.6 to 23 nm, *d*: 18 nm) and helix turn numbers (Table 3): 102⁺ nt RNA, 204⁺ nt RNA and 500⁺ nt RNA. A 90[−] nt RNA and a 308[−] nt RNA both without OA served as controls as they should not be able to induce VLP formation,

according to the generally accepted model of OA-dependent nucleation of TMV assembly in vitro.³⁷ Additionally, the length of the 90[−] nt RNA falls below that of an $\approx$ 102 nt RNA stretch incorporated between a CP helix generated from a two-layered 34-CP 20S intermediate that is postulated to initiate nucleoprotein tube formation through interaction with the OA stem-loop.⁷¹

DNA fragments of accordingly combined TMV- and pGem-T Easy nucleic acid sequences cloned downstream the T7 RNA polymerase promoter were amplified by PCR, to serve as templates for in vitro transcription of the five RNA scaffolds. Out of these, the 102⁺ nt RNA was produced by way of the plasmid construct pGem-T-Easy-102⁺, and both the 204⁺ nt RNA and 500⁺ nt RNA by way of pGem-T-Easy-204⁺ as the PCR template. This construct also yielded the DNA amplicon underlying the 308[−] nt RNA (transcribed from a pGem-T-Easy sequence portion in minus orientation), whereas for the 90[−] nt RNA, an empty pGem-T-Easy vector fragment (of plus orientation) was used (refer to Supplementary Material Table S1 for the experimentally verified sequences of the DNA templates). The lengths of all PCR-generated DNA templates were judged by agarose gel electrophoresis (Figure 2(a)) and were in agreement with the expectations (Table 4). Since successful CP encapsidation of RNAs with non-viral portions fused to a TMV-derived OA-containing segment had been demonstrated earlier,^{62,71} assembly competence was expected for all RNA sequences given that a productive interaction with appropriately prepared CPs took place.

The distinct RNA molecules transcribed in vitro by T7 RNA polymerase exhibited the predicted lengths as deduced from their electrophoretic migration velocities in comparison to RNA molecular weight standards (Figure 2(b) and Table 4), within the limits of experimental accuracy. All transcripts with and without OAs were then incubated with wild-type CP for different periods of time at variable temperatures, and the products were subjected to TEM and native gel electrophoresis (Figure 3). In order to allow comparing scaffolding efficacies, a moderate stoichiometric excess of RNA over CP was applied, which would lead to full consumption of free CPs in the case of optimum scaffold performance. Indeed, all OA-containing RNAs induced and guided the formation of VLPs of the expected sizes at 30°C with good efficiencies (although not 100%, in agreement with the authors' recent studies⁶²), as detected after a period of 3 h: The

Table 3. RNA scaffolds with (+) and without (−) TMV OA designed to generate short VLPs of different lengths and helix turn numbers or to serve as controls

Name	Expected length: nm	Turn number	VLP formation
102 ⁺ nt RNA	4.6	2	Yes
204 ⁺ nt RNA	9.2	4	Yes
500 ⁺ nt RNA	23.0	10	Yes
90 [−] nt RNA	—	—	No
308 [−] nt RNA ^a	−(13.8) ^a	−(6) ^a	(With low efficiency) ^a

^a Although for RNAs without TMV OA, VLP formation is not expected, an OA-related secondary structure is predicted for RNA 308[−], potentially resulting in the products indicated in parentheses. See text for details

Figure 2. Electrophoretic analysis of DNA templates for in vitro transcription and the corresponding RNA products. (a) Purified DNA templates for the preparation of five RNA types: 204⁺ nt RNA, 102⁺ nt RNA, 90⁻ nt RNA, 308⁻ nt RNA and 500⁺ nt RNA constructs with (+) or without OA (-) as indicated above the lanes (2% agarose in 1× TBE). M, 100 base pair (bp) DNA ladder (NEB). (b) RNA transcripts (3% agarose in 1× TBE; denaturing sample buffer). M, RiboRuler low-range RNA ladder (Life Technologies). Ethidium bromide stain.

Table 4. DNA templates including the T7 RNA polymerase promoter and RNAs in vitro transcribed thereof: expected and apparent lengths measured through gel electrophoresis

RNA	DNA length: bp		Deviation: %	RNA length: nt		Deviation: %
	Exp.	Meas.		Exp.	Meas.	
204 ⁺	271	259	4.8	204	208	1.7
102 ⁺	169	169	0.1	102	94	8.4
90 ⁻	157	161	2.7	90	88	2.0
500 ⁺	327	331	1.3	308	306	0.6
308 ⁻	567	587	3.5	500	501	0.3

Exp., expected length; Meas., apparent measured length

cloudy signals of free CP in the gels were reduced substantially in the three lanes with OA-RNAs, and newly occurring accelerated protein band species indicated the presence of compact CP complexes of increased negative charge, as expected for small VLPs (Figure 3(a), data not shown). Electron microscopy revealed particles with the typical TMV diameter in these samples, of which laterally attached ones were chosen to verify the RNA-controlled length (Figure 3(b) and Table 3). By contrast, the reaction mixture of 90⁻ nt RNA (lacking an OA) with CP did not produce comparable novel bands upon native electrophoresis (Figure 3(a)), thus excluding the generation of non-specific RNA-CP adducts other than VLPs and hinting at a specific OA-triggered assembly with the respective RNAs. Some disk-shaped structures found in the 90⁻ nt RNA sample by TEM (Figure 3(b)) had been expected, due to the RNA-independent presence of 20S CP disk-like aggregates in the CP preparation. Such disks were also detected microscopically in control experiments with RNA-free CP exposed to the same conditions, which in native agarose gels revealed no bands other than the cloudy signals typical of pure TMV CP preparations (Figure 4, data not shown).

Interestingly, however, the second control 308⁻ nt RNA without TMV OA sequence nevertheless led to the formation of weak VLP-indicative bands upon electrophoresis, and the detection of correspondingly sized tubes by TEM, after incubation with CP at 30°C. In silico structure analysis calculated by mfold⁷² predicted a potential secondary structure of an OA-resembling stem-loop in this RNA molecule (Supplementary Material Figure S1), consisting of a nonanucleotide loop like in the genuine TMV OAs but with a deviant sequence, and a stem of 14 bp with several analogies to the original as well (see Supplementary Material Figure S1 and compare Turner *et al.*'s⁷³ paper). Previous investigations on the TMV OA loop sequence have extracted essential features of necessary base positions inside,^{73,74} which are also realized in the 308⁻ nt RNA (Supplementary Material Figure S1). Therefore, a pseudo-OA initiation activity exerted by the non-homologous but structurally similar stem-loop derived from pGem-T Easy may exist, in agreement with the previous results of Turner *et al.*⁷³ and earlier findings on pseudo-OA motifs by Taliansky *et al.*⁷⁵ and Kaplan *et al.*⁷⁶ The free energy for the putative pseudo-OAs in the 308⁻ nt RNA sequence is predicted to

Figure 3. Comparison of RNA-induced VLP formation efficiencies under variable temperature regimes and corresponding nanostructures. (a) Native gel electrophoresis indicates the assembly efficiencies with the five different RNAs with (+) or without OA (–), as denoted above the images (see also Figure 2). Top line: temperature conditions (30°C for 24 h; 30°C for 3 h and subsequently 10°C for 21 h; 10° for 24 h; 2.5% agarose in 1× TBE. Protein stain: Coomassie Brilliant Blue R25). OAs +/-: presence of TMV OA sequence. The presence of a TMV OA correlates with substantially increased assembly efficiencies. (b) TEM analysis displays CP assemblies occurring in the reaction products. Brackets indicate laterally attached ring-like structures of the dimensions expected for RNA-scaffolded products or, in the case of RNA 90–, plain CP disks. These are indistinguishable from putative nucleoprotein particles formed with an RNA of this size, but occurred at ≈15 times lower frequencies compared to the assembly with 102+ nt RNA. For details, refer to the text

be $\Delta G = -21.00$ kcal/mol, in comparison to -18.30 kcal/mol for the genuine TMV OAs. Hence, despite a deviant primary sequence, the OA-resembling hairpin loop might promote an assembly of virus-like structures to a limited extent. Since, however, simple poly(A) molecules may also undergo 20S disk-dependent encapsidation into VLPs⁷⁷ and since details of the in vitro TMV-like particle assembly process and putative variable modes thereof remain under ongoing debate,^{37,78} alternative mechanistic explanations cannot be excluded.

As the primary goal of this study lay in a reliable fabrication of short ring-like VLPs, the three RNAs containing the TMV OA were then subjected to suboptimal assembly conditions with CP at 10°C, where they turned out to guide nanotube formation with substantially different efficiencies. While both 204+ nt RNA and 500+ nt RNA retained convincing assembly performance, that of the 102+ nt RNA was extremely reduced – a trend also detectable for conventional reactions at 30°C at a closer look (compare Figure 3(a), from left to right for VLP and free CP bands). This could be due to unfavorable secondary structures of this RNA, most pronounced at lower temperatures, thus limiting nucleation

and tube growth progression rates. An additional, tempting explanation might be derived from the length of the 102+ nt RNA: Despite its exact fit to the RNA portion incorporated between a two-turn helical CP segment corresponding to a 20S CP intermediate postulated to initiate assembly at the OA,³⁷ experimental evidence suggests an instability of the resulting RNA-scaffolded two-turn helices. Representing the first nascent state of nanotubes, they seem to undergo stabilization only through addition of a further disk.⁷⁷ This was shown to demand for a continuous RNA stretch of at minimum ≈150 nt upon use of poly(A),⁷⁷ for which reason four-turn helices with the 204+ nt RNA might form with significantly improved efficiency.

Taken together, both the native electrophoretic and the TEM analysis indicated consistently that the authors were able to generate three types of short VLPs stabilized by TMV OA-containing RNAs. The capacities of the 204+ nt RNA and the 500+ nt RNA to direct nucleoprotein particle formation seemed to be tolerant toward reduced temperatures, whereas the 102+ nt RNA came out to be less efficient and thus less fail-safe under non-optimized reaction conditions than its longer counterparts

Figure 4. Stability test of 204⁺ nt RNA-scaffolded disks (short VLPs) and RNA-free CP aggregates exposed to different pH values for 1 h. (a) Native gel electrophoretic analysis of in vitro-assembled disks after parallel incubation at the pH indicated above the lanes (2.5% agarose in 1× TBE; protein stain: Coomassie Brilliant Blue R250; 204⁺: RNA 204⁺ containing OA). (b) TEM analysis of RNA-stabilized disks (left) and CP aggregates without RNA (right). Uranyl acetate negative stain

were. Since short VLPs of low aspect ratio and thus ring-like shape were the focus of this study, in order to explore their suitability for technical applications as adapter structures on flat surfaces, the following experiments employed the 204⁺ nt RNA scaffold only: because of its dimensions advantageous for molecular display and combination purposes (length about 9.2 nm corresponding to four-turn helices), its multivalence of ≈68 CP subunits and its reliable assembly with TMV CP. In the following, the authors will call the resulting ring-shaped nucleoprotein construct ‘disk’ for simplicity, as its dimensions are related to those of the commonly so-called four-layer disks which, however, usually contain two disk domains in opposite (bipolar head-to-head) orientation⁴⁶ as also assumed for certain recombinant CP disk variants.^{54,55}

3.2 Stability test of RNA-scaffolded disks under variable pH conditions

As outlined in Section 1, the limited pH range up to maximally pH 8.0 applicable to all but one⁵⁴ type of engineered RNA-free

holey TMV CP disks, and their inherent tendencies to aggregate by longitudinal multimerization or stacking and/or lateral array formation, prevents uses relying on colloidal particle suspensions and chemical modifications even under mildly alkaline conditions. To find out if the 204⁺ nt RNA-scaffolded helical disk-like structures might clear a way out of these restrictions, the VLP assemblies were studied in detail regarding their pH stability. The short VLPs and control samples of RNA-free CP preparations containing disk aggregates were exposed to different pH values in parallel for 1 h and analyzed by native gel electrophoresis and TEM thereafter (Figure 4). Electrophoretic separation revealed the presence of VLP-specific nucleoprotein bands after incubation in a pH range from 6.0 to 9.0. Upon exposure to pH values of 10.0 and above, the respective bands disappeared (Figure 4(a)). No such bands were detected for control samples of RNA-free CP disk-containing preparations. TEM analysis disclosed details of the interparticle aggregation state of the underlying structures not possible to discriminate by native electrophoretic separation (Figure 4(b)). Nucleoprotein disks exposed to pH 7.2–9.0 had

maintained their characteristic organization as well-dispersed four-turn helices, whereas at pH 6.0, oligomers and longer aggregates up to rod-shaped structures were formed. The latter probably represent head-to-tail aggregates of the nucleoprotein disks, which are also described for whole TMV particles at acidic pH values.⁷⁹ At pH 10.0 and 11.0, disaggregated clusters were detected with largely undefined contours. Degradation of whole TMV particles typically occurs at pH values above 9.0;^{80,81} thus, the disintegration of the RNA-scaffolded disks in this pH range coincides with previous studies. TEM of the RNA-free CP disk-containing solutions visualized generally less-defined complexes. Ring-like structures occurred almost exclusively between pH 6.0 and 7.2, between an excess of bulky material. They completely disaggregated at pH 8.0 and above. This comparative study of the distinct disks at different pH values thus unequivocally confirmed a strong, beneficial stabilization exerted by the RNA scaffold enclosed inside the TMV CP helix and an impressive shift of the structures' equilibrium state toward defined and well-dispersed ring-shaped objects. Hence, the novel disks seemed suitable for chemical reactions under moderately alkaline conditions, and for initial tests as carrier particles.

3.3 Bifunctional TMV disks to serve as fluorescence carrier and adapter templates

To prepare TMV-based adapter rings accessible to orthogonal coupling of distinct molecule species, two types of CPs exposing selectively addressable amino acid side-chains on their outer rim were combined in single nucleoprotein particles by mixed simultaneous assembly.⁶⁰ Genetically modified CPs each equipped with an additional amino or thiol group⁶¹ were incorporated at a 1:1 ratio into the four-turn helices to yield statistically blended arrangements of ≈ 68 CPs with about 32 molecules per type. Such heterovalent disks were fabricated in order to interconnect them with two different functionalities, to serve as proof-of-concept adapter colloids between fluorescent labels and a chemically modified substrate in this study, as depicted in Figure 5.

The engineered CP types could be integrated into single particles as efficiently as the wild-type protein used in the previous tests and equipped with maleimide-activated fluorescent dye molecules by way of their reactive thiol groups. Native gel electrophoresis showed the expected deviating migration of the two input CPs, mutated either at amino acid position 3 from serine to a thiol-exposing cysteine (C or

Figure 5. Flowchart of serial functionalization and coupling reactions: fluorescence labeling of heterovalent bifunctional disks, ITC-silane functionalization of a silica substrate and immobilization of the disks equipped with multiple dye molecules. (a) Fluorescence labeling of thiol groups exposed on disks with Atto 488-maleimide. (b) Coupling of ITC-terminated silane to a silica surface in a gas phase reaction. (c) Thiourea bond formation between primary amino groups exposed on the fluorescently labeled bifunctional disk and ITC at pH 9

CP_{Cys}) or at amino acid position 158 from threonine to an amino group-exposing lysine (K or CP_{Lys})⁶¹ Figure 6, lower part). These exchanged amino acids confer selective dual coupling functionality to the outer rim of the ring-shaped VLP assemblies (Figure 5(a)). After combining the two protein varieties without the addition of RNA, both migrate separately (C-K) in clearly distinct bands

Figure 6. Preparation and fluorescence labeling of bifunctional disks. Native agarose gel analysis reveals educts and mixed assembled disks of 204⁺ nt RNA with 50% CP_{Lys} (K) and 50% CP_{Cys} (C) labeled with Atto 488–maleimide, prior to and after removal of unbound Atto 488 by way of gel filtration (G25). Top: UV-illuminated gel reveals fluorescent disks (VLPs) and free residual Atto 488; bottom: Coomassie Brilliant Blue R250 stain detects proteins or nucleoprotein complexes (2.5% agarose in 1× TBE). For details, refer to the text

(Figure 6, C-K alone; lower part). These two bands disappeared as soon as RNA 204⁺ had been added to guide the proteins' mixed assembly into blended VLPs (C-K + 204), giving rise to a novel more diffuse band with a higher electrophoretic velocity (Figure 6, lower part). Conjugation of maleimide-activated Atto 488 to the thiol groups of these hetero-multivalent nanorings (see also coupling scheme in Figure 5(b)) increased the migration velocity of the bifunctional disks due to the additional net charges of –1 per coupled Atto 488 molecule (Figure 6, C-K + 204 + Atto 488, lower part) and enabled fluorescence detection of the functionalized complexes (Figure 6, upper part). Excess free Atto 488–maleimide exhibited the highest electrophoretic mobility and was successfully removed from the reaction mixture by gel filtration, as obvious for the final fluorescent disk preparation analyzed through electrophoretic separation (Figure 6, C-K + 204 + Atto 488 + G25, rightmost lane).

The structural integrity of the heterovalent adapter VLPs, and of the Atto 488-functionalized, purified disks was confirmed by TEM. Uniform ring-like structures were detected before and after dye coupling and gel filtration (Supplementary Material Figure S4). Control samples of the two mixed CP varieties devoid of scaffold RNA did not contain any disk-like assemblies if treated under the same conditions (Supplementary Material Figure S4(a)). The RNA-stabilized TMV-based disks thus were equipped with fluorescent molecules by way of their CP_{Cys} thiol functions efficiently, as demonstrated also by other laboratories for accordingly engineered TMV or TMV-like rods.^{38,82,83} The second type of reactive groups – that is, the amino function of K158 exposed at the structurally disordered and hence probably protruding⁴⁶ C-terminus of every CP_{Lys} subunit – remained accessible to subsequent conjugation steps.

3.4 Immobilization of bifunctional disks on ITC-silane-covered substrates

Such brightly fluorescent nanorings displaying multiple dye molecules in combination with numerous selectively addressable anchoring sites represent attractive functional, biodegradable particles: They may procure signal amplification in biodetection setups (see the paper of Soto and Ratna⁸⁴ and the references therein) or serve as tracers in dilutable coatings to monitor optically the surface distribution of coapplied or interlinked effector compounds. The authors sought to find out whether the TMV-based assemblies would be appropriate for respective uses. Atto 488-labeled, purified bifunctional disks were thus spread convectively on lithographically gold-patterned, ITC-silane-covered glass substrates and kept at pH 9.0 overnight, to induce covalent coupling between the ITC and the primary amino groups at the CP_{Lys} C-termini protruding from the outer ring rims (see the schemes in Figures 5(b) and 5(c)). As controls, the ITC-coated substrates were incubated with an Atto 488–maleimide solution devoid of VLPs under the same conditions. Its dye content surpassed that of the fluorescent VLP preparation, of which non-bound Atto 488–maleimide had been removed (see Section 2). Gold-patterned glass slides without ITC silane served as the second control, in combination with the fluorescent disk preparation. For all three layouts, the spatially resolved fluorescence

signal intensities were recorded microscopically after extensive washing with water (Figure 7). To exclude signal background from the gold patterns, the glass slides were monitored from the back side – that is, opposite the immobilized components.

The Atto 488-equipped disks spread onto ITC-silane and exposed to coupling conditions at pH 9.0 yielded a homogenous fluorescence distribution over the whole transparent glass areas (Figure 7(a)). Its intensity was considerably higher than that of the ITC-silane-covered control substrates treated with an excess of plain Atto 488–maleimide (Figure 7(b)), indicating selective covalent immobilization of the dye-loaded adapter rings by way of their amino groups through thiourea bond formation. For the ITC-free substrates incubated with Atto 488-labeled disks, no fluorescence was recorded in the central regions of the bare glass surfaces. However, signals occurred along the edges of the gold profiles (Figure 7(c)). They were supposed to reflect VLPs enriched at these borders, which was confirmed in the following.

To investigate the underlying distribution, dispersal and structural integrity of the fluorescent TMV-derived nanorings, the different specimens were analyzed by AFM. The ITC silane-modified surface incubated with Atto 488-equipped disks was fully covered with randomly distributed, well-separated circular-shaped particles of 8.5 nm height (Figure 7(d), 67.6 particles/ μm^2 ; Supplementary Material Figure S2), which is close to the expected four-turn-helix length of 9.2 nm and coincides with earlier findings on some height reduction of TMV particles adsorbed on hydrophilic substrates.⁸⁵ The apparent diameter of the disks was around 28.3 nm due to tip convolution (Supplementary Material Figure S3) and thus is in agreement with the expected 18 nm according to the previous AFM measurements.^{85–88} The structural integrity of the bifunctional disks in the reaction solution at pH 9 over the extended reaction time of 16 h was also attested by TEM (Supplementary Material Figure S4). The ITC-functionalized control substrates treated with Atto 488–maleimide solution did not show any similar structures in the AFM analysis (Figure 7(e)), as the size of the dye molecules is below the resolution of the system applied. A few background particles (1.6/ μm^2) of deviating dimensions (up to a size of 32 × 114 nm) were not analyzed in more detail. Analysis of the ITC-free substrate incubated with fluorescent disks revealed negligible numbers of the corresponding particles (8.0 particles/ μm^2) in the central areas of the bare glass (Figure 7(f), rightmost area; Supplementary Material Figure S2(f)), but verified an accumulation of VLPs in the vicinity of the gold profiles. This most likely resulted from unspecific physical adsorption to the non-ITC-functionalized substrates in these areas, as it can be explained by attractive capillary forces applied to the nanorings during the drying process close to the concave edges of about 25 nm height. It might be further promoted by the strong affinity of gold to residual unreacted thiol groups⁸⁹ of CP_{Cys} in the bifunctional disks. In contrast, the post-incubation washes removed non-covalently bound disks from the well-accessible ITC-free central glass areas efficiently (Supplementary Material Figure S2(f)).

The experiments therefore proved a chemically selective immobilization of the dye-loaded TMV-based adapter disks on appropriately functionalized substrates. On ITC silane-coated glass, simple convective spreading by means of a coverslip led to even random distributions as solitary objects. Their reliable attachment was achieved by thiourea bond formation between the amino groups of the C-terminal CP_{Lys} lysines protruding from the nanorings' outer rim, and the silane's ITC functions at pH 9.0 for 16 h. The RNA-stabilized heterovalent four-turn helices equipped with Atto 488 molecules by way of their thiol-exposing CP_{Cys} cysteine residues withstood the treatment without any indication of structural changes and remained brightly fluorescent. As a whole, these tests demonstrated an easy application of the TMV-deduced nucleoprotein rings as adapter templates, enabling a predictable installation of preestablished functional clusters on technical surfaces.

4. Conclusion

Hetero-multivalent RNA-scaffolded nanorings assembled from a blend of two selectively addressable, engineered TMV CP variants on a 204⁺ nt RNA exhibit a unique combination of features in great demand for the construction of biohybrid arrangements with complex functionality: (a) dimensions of 18 nm × 9.2 nm (*d* × length) with a central 4 nm hole; (b) a four-turn helical organization with about 68 CP subunits enclosing and protecting the RNA that, in turn, shifts the equilibrium of the self-organizing complexes to near-homogeneous suspensions of the ring-like particles and (c) confers stability up to pH 9.0; and (d) the formation of colloidal dispersions devoid of larger aggregates between neutral and mildly alkaline pH. These characteristics enable a wide range of conjugation chemistry in order to install molecular ensembles of predefined composition and surface density on the different outer and upper/lower ends and even inner surfaces of the disks, since they can be prepared not only by use of the CP types applied here, but also from a large variety of further TMV CP mutants available in several research groups worldwide. As more than two protein variants can be combined in a single multifunctional VLP, this may provide polyvalence for several independent chemical modifications. These do not necessarily need to be introduced into the assembled disk complex consecutively, but may be performed prior to the CPs' RNA-controlled self-organization, as the authors were able to show recently,⁶² thus expanding the combinatorial possibilities further. The repetitive distances between adjacent CPs' N-termini and less ordered C-termini exposed on the outer helix surface are in the 3 nm range. Hence, they are most interesting for applications relying on ordered co-operating or interacting moieties such as enzymes or metal nanoparticles, combining into catalytic cascades or plasmonic nanostructures, respectively. Such layouts are being tested with currently accessible plant virus derivatives in different laboratories (e.g. those of Koch *et al.*,¹⁷ Szuchmacher Blum *et al.*,¹⁹ Lebedev *et al.*,⁵⁰ Zahr and Szuchmacher Blum⁵¹) and may profit from the availability of the novel short but robust TMV disk adapters. Furthermore, fluorescent dyes may be clustered with predefined spacing, thus avoiding mutual quenching, as demonstrated for a spherical plant virus before.⁹⁰

Figure 7. Immobilization of bifunctional adapter disks equipped with fluorescent molecules on ITC-functionalized glass substrates adjacent to gold profiles and corresponding control specimens. (a–c) Fluorescence intensities and (d–f) corresponding AFM analyses of (a, d) Atto 488-labeled disks on ITC-covered specimen; (b, e) Atto 488–maleimide in the absence of any TMV derivative on ITC-covered specimen; and (c, f) Atto 488-labeled disks on an ITC-free specimen. Insets in the graphs in (a)–(c) show spatially resolved images of the fluorescence signals recorded from the back of the substrates. AFM images in (d)–(f) represent left halves of specimens shown in (a)–(c). For details, refer to the text. The scan size of the AFM images is $5\ \mu\text{m} \times 5\ \mu\text{m}$

Ring-shaped flat ordering tools enabling an installation of several tens of selected molecules are particularly useful if prearranged functions are to be grafted into technical environments at spatially defined sites collectively. The short VLPs designed and tested in this study came out to undergo the desired selective dual interactions with distinct synthetic components – that is, Atto 488–maleimide and ITC-terminated silane – efficiently. Hence, they seem the most promising for advanced follow-up approaches, and compatible with upscaling, as a major benefit based on the natural viral assembly system is the highly reproducible bottom-up structure formation *in vitro*. Well-defined rings (or short barrels in the case of the second, elongated OA-containing RNA scaffold) with the length determined by the tailored RNA may be fabricated in one-pot reactions from appropriate mixtures of TMV-based building blocks. Beyond this, the assemblies maintain their structure identity and uniformity over a pH range allowing for convenient standard conjugation reactions with best efficiencies at mild basic pH. However, pH conditions below 7.2 or above 9 have to be viewed with caution, since in an acidic milieu, elongated template aggregates are formed, and under more basic conditions, disassembly occurs. Thus, it still remains important to select a suitable environment for practicing with these plant virus-derived building blocks, but the RNA core of the authors' novel structures extends their use into most of the pH regime practically relevant for biochemical modification and reaction conditions. Their good dispersal suggests uses in dilutable surface coatings whereby the effector density integrated by way of the adapter disks can be adjusted.

To this end, the RNA-stabilized disks presented in this work might be developed further into novel species of nanoordering components for being immobilized onto other inorganic and organic (e.g. polymeric) substrates, including natural materials. The biodegradability of the nucleoprotein nanoparticles might thus bring ahead the fabrication of biologically functional hybrid structures decomposing after use. Further application perspectives may lie in the central 4 nm wide hole, as it is possible to modify genetically its inner protein lining^{55,91} to serve, for example, as reaction space in analogy to initial studies with peptide doughnuts,⁹² or in combination with top and/or bottom capping units 'plugging' and occluding an internal confined volume as shown for Hcp1 nanorings and nanotubes.⁹³ Additionally, peptides installed on the outer surfaces of the TMV-derived disks may confer selective affinities to certain material classes, or induce mineral or metal deposition from liquid precursors, thereby embedding the bioadapters into a 'bionic glue', as suggested recently.⁹⁴ In summary, the versatile plant virus-based nanorings developed and characterized in this work combine important auspicious features enabling highly diverse applications – namely, as multifunctional building blocks amenable to controlled integration into hybrid materials.

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REFERENCES

1. Nam YS, Magyar AP, Lee D *et al.* (2010) Biologically templated photocatalytic nanostructures for sustained light-driven water oxidation. *Nature Nanotechnology* **5**(5): 340–344.
2. Chiang CY, Epstein J, Brown A *et al.* (2012) Biological templates for antireflective current collectors for photoelectrochemical cell applications. *Nano Letters* **12**(11): 6005–6011.
3. Dorval Courchesne NM, Klug MT, Huang KJ *et al.* (2015) Constructing multifunctional virus-templated nanoporous composites for thin film solar cells: contributions of morphology and optics to photocurrent generation. *Journal of Physical Chemistry C* **119**(25): 13987–14000.
4. Yang C, Choi CH, Lee CS and Yi H (2013) A facile synthesis-fabrication strategy for integration of catalytically active viral-palladium nanostructures into polymeric hydrogel microparticles *via* replica molding. *ACS Nano* **7**(6): 5032–5044.
5. Bauer S, Alles M, Arpa-Sancet MP *et al.* (2016) Resistance of amphiphilic polysaccharides against marine fouling organisms. *Biomacromolecules* **17**(3): 897–904.
6. Weinert U, Pollmann K, Barkleit A *et al.* (2015) Synthesis of S-layer conjugates and evaluation of their modifiability as a tool for the functionalization and patterning of technical surfaces. *Molecules* **20**(6): 9847–9861.
7. Raff J, Matys S, Suhr M *et al.* (2016) S-layer-based nanocomposites for industrial applications. In *Protein-based Engineered Nanostructures* (Cortajarena AL and Grove TZ (eds)). Springer, Cham, Switzerland, pp. 245–279.
8. Badelt-Lichtblau H, Kainz B, Völlenkle C *et al.* (2009) Genetic engineering of the S-layer protein SbpA of *Lysinibacillus sphaericus* CCM 2177 for the generation of functionalized nanoarrays. *Bioconjugate Chemistry* **20**(5): 895–903.
9. Heddle JG, Fujiwara I, Yamadaki H *et al.* (2007) Using the ring-shaped protein TRAP to capture and confine gold nanodots on a surface. *Small* **3**(11): 1950–1956.
10. Schreiber A, Huber MC, Colfen H and Schiller SM (2015) Molecular protein adaptor with genetically encoded interaction sites guiding the hierarchical assembly of plasmonically active nanoparticle architectures. *Nature Communications* **6**: 6705.
11. Pham TA, Schreiber A, Sturm EV, Schiller S and Cölfen H (2016) Hemolysin coregulated protein 1 as a molecular gluing unit for the assembly of nanoparticle hybrid structures. *Beilstein Journal of Nanotechnology* **7**(1): 351–363.
12. Smith JC, Lee KB, Wang Q *et al.* (2003) Nanopatterning the chemospecific immobilization of cowpea mosaic virus capsid. *Nano Letters* **3**(7): 883–886.
13. Fan XZ, Naves L, Siwak NP *et al.* (2015) Integration of genetically modified virus-like-particles with an optical resonator for selective bio-detection. *Nanotechnology* **26**(20): 205501.
14. Baecker M, Koch C, Eiben S *et al.* (2017) Tobacco mosaic virus as enzyme nanocarrier for electrochemical biosensors. *Sensors and Actuators B: Chemical* **238**: 716–722.
15. Schreiter M, Gabl R, Lerchner J *et al.* (2006) Functionalized pyroelectric sensors for gas detection. *Sensors and Actuators B: Chemical* **119**(1): 255–261.
16. Zang F, Gerasopoulos K, Fan XZ *et al.* (2014) An electrochemical sensor for selective TNT sensing based on tobacco mosaic virus-like particle binding agents. *Chemical Communications* **50**(85): 12977–12980.

17. Koch C, Eber FJ, Azucena C et al. (2016) Novel roles for well-known players: from tobacco mosaic virus pests to enzymatically active assemblies. *Beilstein Journal of Nanotechnology* 7: 613–629.
18. Koch C, Wabbel K, Eber FJ et al. (2015) Modified TMV particles as beneficial scaffolds to present sensor enzymes. *Frontiers in Plant Science* 6: 1137.
19. Szuchmacher Blum A, Soto CM, Sapsford KE et al. (2011) Molecular electronics based nanosensors on a viral scaffold. *Biosensors and Bioelectronics* 26(6): 2852–2857.
20. Cuenca S, Mansilla C, Aguado M et al. (2016) Nanonets derived from turnip mosaic virus as scaffolds for increased enzymatic activity of immobilized *Candida antarctica* lipase B. *Frontiers in Plant Science* 7: 464.
21. Fan XZ, Pomerantseva E, Gnerlich M et al. (2013) Tobacco mosaic virus: a biological building block for micro/nano/biosystems. *Journal of Vacuum Science & Technology A* 31(5): 050815.
22. Payne GF, Kim E, Cheng Y et al. (2013) Accessing biology's toolbox for the mesoscale biofabrication of soft matter. *Soft Matter* 9(26): 6019–6032.
23. Mao C, Liu A and Cao B (2009) Virus-based chemical and biological sensing. *Angewandte Chemie International Edition* 48(37): 6790–6810.
24. Bittner AM, Alonso JM, Gorzny ML and Wege C (2013) Nanoscale science and technology with plant viruses and bacteriophages. *Sub-cellular Biochemistry* 68: 667–702.
25. Bai Y, Luo Q and Liu J (2016) Protein self-assembly via supramolecular strategies. *Chemical Society Reviews* 45(10): 2756–2767.
26. Uhde-Holzem K, McBurney M, Tiu BDB et al. (2016) Production of immunoabsorbent nanoparticles by displaying single-domain protein A on potato virus X. *Macromolecular Bioscience* 16(2): 231–241.
27. Yang G, Wu L, Chen G and Jiang M (2016) Precise protein assembly of array structures. *Chemical Communications* 52(70): 10595–10605.
28. Storm AJ, Chen JH, Ling XS, Zandbergen HW and Dekker C (2003) Fabrication of solid-state nanopores with single-nanometre precision. *Nature Materials* 2(8): 537–540.
29. Altunbas A, Sharma N, Lamm MS et al. (2010) Peptide-silica hybrid networks: biomimetic control of network mechanical behavior. *ACS Nano* 4(1): 181–188.
30. Royston E, Lee SY, Culver JN and Harris MT (2006) Characterization of silica-coated tobacco mosaic virus. *Journal of Colloid and Interface Science* 298(2): 706–712.
31. Royston E, Ghosh A, Kofinas P, Harris MT and Culver JN (2008) Self-assembly of virus-structured high surface area nanomaterials and their application as battery electrodes. *Langmuir* 24(3): 906–912.
32. Patwardhan SV, Emami FS, Berry RJ et al. (2012) Chemistry of aqueous silica nanoparticle surfaces and the mechanism of selective peptide adsorption. *Journal of the American Chemical Society* 134(14): 6244–6256.
33. Azucena C, Eber FJ, Trouillet V et al. (2012) New approaches for bottom-up assembly of tobacco mosaic virus-derived nucleoprotein tubes on defined patterns on silica- and polymer-based substrates. *Langmuir* 28(42): 14867–14877.
34. Müller A, Eber FJ, Azucena C et al. (2011) Inducible site-selective bottom-up assembly of virus-derived nanotube arrays on RNA-equipped wafers. *ACS Nano* 5(6): 4512–4520.
35. Alonso JM, Gorzny ML and Bittner AM (2013) The physics of tobacco mosaic virus and virus-based devices in biotechnology. *Trends in Biotechnology* 31(9): 530–538.
36. Culver JN, Brown AD, Zang F et al. (2015) Plant virus directed fabrication of nanoscale materials and devices. *Virology* 479–480: 200–212.
37. Butler PJ (1999) Self-assembly of tobacco mosaic virus: the role of an intermediate aggregate in generating both specificity and speed. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* 354(1383): 537–550.
38. Rego JM, Lee JH, Lee DH and Yi H (2013) Biologically inspired strategy for programmed assembly of viral building blocks with controlled dimensions. *Biotechnology Journal* 8(2): 237–246.
39. Wu Z, Mueller A, Degenhard S et al. (2010) Enhancing the magnetoviscosity of ferrofluids by the addition of biological nanotubes. *ACS Nano* 4(8): 4531–4538.
40. Eber FJ, Eiben S, Jeske H and Wege C (2015) RNA-controlled assembly of tobacco mosaic virus-derived complex structures: from nanoboomerangs to tetrapods. *Nanoscale* 7(1): 344–355.
41. Zimmer D (1977) The nucleotide sequence at the origin for assembly on tobacco mosaic virus RNA. *Cell* 11(3): 463–482.
42. Durham AC, Finch JT and Klug A (1971) States of aggregation of tobacco mosaic virus protein. *Nature: New Biology* 229(2): 37–42.
43. Carpenter JM (1970) The stacked-disk structure of tobacco mosaic virus protein. *Virology* 41(4): 603–614.
44. Durham AC and Klug A (1971) Polymerization of tobacco mosaic virus protein and its control. *Nature: New Biology* 229: 42–46.
45. Butler PJG, Bloomer AC and Finch JT (1992) Direct visualization of the structure of the 20 S aggregate of coat protein of tobacco mosaic virus – the disk is the major structure at pH 7.0 and the proto-helix at lower pH. *Journal of Molecular Biology* 224(2): 381–394.
46. Bhyravbhata B, Watowich SJ and Caspar DLD (1998) Refined atomic model of the four-layer aggregate of the tobacco mosaic virus coat protein at 2.4-angstrom resolution. *Biophysical Journal* 74(1): 604–615.
47. Raghavendra K, Kelly JA, Khairallah L and Schuster TM (1988) Structure and function of disk aggregates of the coat protein of tobacco mosaic-virus. *Biochemistry* 27(20): 7583–7588.
48. Vogel D (1982) Neutral salt effects on the polymorphism of tobacco mosaic virus protein – a contribution to the understanding of its mechanism of aggregation and virus reassembly. *Biochimica et Biophysica Acta* 706(1): 65–79.
49. Stauffer H, Srinivasan S and Lauffer MA (1970) Calorimetric studies on polymerization-depolymerization of tobacco mosaic virus protein. *Biochemistry* 9(2): 193–200.
50. Lebedev N, Griva I, Dressick WJ et al. (2016) A virus-based nanoplasmonic structure as a surface-enhanced Raman biosensor. *Biosensors and Bioelectronics* 77: 306–314.
51. Zahr OK and Szuchmacher Blum A (2012) Solution phase gold nanorings on a viral protein template. *Nano Letters* 12(2): 629–633.
52. Miller RA, Presley AD and Francis MB (2007) Self-assembling light-harvesting systems from synthetically modified tobacco mosaic virus coat proteins. *Journal of the American Chemical Society* 129(11): 3104–3109.
53. Hou C, Luo Q, Liu J et al. (2012) Construction of GPx active centers on natural protein nanodisk/nanotube: a new way to develop artificial nanoenzyme. *ACS Nano* 6(10): 8692–8701.
54. Finbloom JA, Han K, Aanei IL et al. (2016) Stable disk assemblies of a tobacco mosaic virus mutant as nanoscale scaffolds for applications in drug delivery. *Bioconjugate Chemistry* 27(10): 2480–2485.
55. Dedeo MT, Duderstadt KE, Berger JM and Francis MB (2010) Nanoscale protein assemblies from a circular permutant of the tobacco mosaic virus. *Nano Letters* 10(1): 181–186.
56. Zhang J, Zhou K and Wang Q (2016) Tailoring the self-assembly behaviors of recombinant tobacco mosaic virus by rationally introducing covalent bonding at the protein–protein interface. *Small* 12(36): 4955–4959.
57. Mueller A, Kadri A, Jeske H and Wege C (2010) In vitro assembly of Tobacco mosaic virus coat protein variants derived from fission yeast expression clones or plants. *Journal of Virological Methods* 166(1–2): 77–85.
58. Kadri A, Maiss E, Amsharov N et al. (2011) Engineered tobacco mosaic virus mutants with distinct physical characteristics in planta and enhanced metallization properties. *Virus Research* 157(1): 35–46.
59. Bruckman MA, Soto CM, McDowell H et al. (2011) Role of hexahistidine in directed nanoassemblies of tobacco mosaic virus coat protein. *ACS Nano* 5(3): 1606–1616.

60. Eiben S, Stitz N, Eber F et al. (2014) Tailoring the surface properties of tobacco mosaic virions by the integration of bacterially expressed mutant coat protein. *Virus Research* **180**: 92–96.
61. Geiger FC, Eber FJ, Eiben S et al. (2013) TMV nanorods with programmed longitudinal domains of differently addressable coat proteins. *Nanoscale* **5**(9): 3808–3816.
62. Schneider AN, Eber FJ, Wenz NL et al. (2016) Dynamic DNA-controlled ‘stop-and-go’ assembly of well-defined protein domains on RNA-scaffolded TMV-like nanotubes. *Nanoscale* **8**(47): 19853–19866.
63. Goelet P, Lomonosoff GP, Butler PJ et al. (1982) Nucleotide sequence of tobacco mosaic virus RNA. *Proceedings of the National Academy of Sciences of the United States of America* **79**(19): 5818–5822.
64. Butler JG (1984) The current picture of the structure and assembly of tobacco mosaic virus. *Journal of General Virology* **65**(2): 253–279.
65. Zimmermann D (1983) An extended secondary structure model for the TMV assembly origin, and its correlation with protection studies and an assembly defective mutant. *EMBO Journal* **2**(11): 1901–1907.
66. Green MR and Sambrook J (2012) *Molecular Cloning: a Laboratory Manual*, 4th edn. Cold Spring Harbor Laboratory Press, New York, NY, USA.
67. Gooding GV and Hebert TT (1967) A simple technique for purification of tobacco mosaic virus in large quantities. *Phytopathology* **57**(11): 1285.
68. Fraenkel-Conrat H (1957) Degradation of tobacco mosaic virus with acetic acid. *Virology* **4**(1): 1–4.
69. Raghavendra K, Adams ML and Schuster TM (1985) Tobacco mosaic virus protein aggregates in solution: structural comparison of 20S aggregates with those near conditions for disk crystallization. *Biochemistry* **24**(13): 3298–3304.
70. Butler PJ (1972) Structures and roles of the polymorphic forms of tobacco mosaic virus protein: VI. Assembly of the nucleoprotein rods of tobacco mosaic virus from the protein disks and RNA. *Journal of Molecular Biology* **72**(1): 25–35.
71. Turner DR, McGuigan CJ and Butler PJ (1989) Assembly of hybrid RNAs with tobacco mosaic virus coat protein: evidence for incorporation of disks in 5'-elongation along the major RNA tail. *Journal of Molecular Biology* **209**(3): 407–422.
72. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31**(13): 3406–3415.
73. Turner DR, Joyce LE and Butler PJ (1988) The tobacco mosaic virus assembly origin RNA: functional characteristics defined by directed mutagenesis. *Journal of Molecular Biology* **203**(3): 531–547.
74. Turner DR and Butler PJ (1986) Essential features of the assembly origin of tobacco mosaic virus RNA as studied by directed mutagenesis. *Nucleic Acids Research* **14**(23): 9229–9242.
75. Talianky ME, Kaplan IB, Yarekulg LV et al. (1982) A study of TMV ts mutant Ni2519 2: temperature-sensitive behavior of Ni2519 RNA upon reassembly. *Virology* **118**(2): 309–316.
76. Kaplan IB, Kozlov YV, Pshennikova ES, Talianky ME and Atabekov JG (1982) A study of TMV Ts mutant Ni2519 III. location of the reconstitution initiation sites on Ni2519 RNA. *Virology* **118**(2): 317–323.
77. Schön A and Mundry KW (1984) Coordinated two-disk nucleation, growth and properties, of virus-like particles assembled from tobacco-mosaic-virus capsid protein with poly(A) or oligo(A) of different length. *European Journal of Biochemistry/FEBS* **140**(1): 119–127.
78. Caspar DLD and Namba K (1990) Switching in the self-assembly of tobacco mosaic virus. *Advances in Biophysics* **26**: 157–185.
79. Niu Z, Bruckman M, Kotakadi VS et al. (2006) Study and characterization of tobacco mosaic virus head-to-tail assembly assisted by aniline polymerization. *Chemical Communications* **28**(28): 3019–3021.
80. Wyckoff RWG (1937) An ultracentrifugal study of the pH stability of tobacco mosaic virus protein. *Journal of Biological Chemistry* **122**(1): 239–U2.
81. Perham RN and Wilson TMA (1978) Characterization of intermediates formed during disassembly of tobacco mosaic-virus at alkaline pH. *Virology* **84**(2): 293–302.
82. Yi H, Nisar S, Lee SY et al. (2005) Patterned assembly of genetically modified viral nanotemplates via nucleic acid hybridization. *Nano Letters* **5**(10): 1931–1936.
83. Yi H, Rubloff GW and Culver JN (2007) TMV microarrays: hybridization-based assembly of DNA-programmed viral nanotemplates. *Langmuir* **23**(5): 2663–2667.
84. Soto CM and Ratna BR (2010) Virus hybrids as nanomaterials for biotechnology. *Current Opinion in Biotechnology* **21**(4): 426–438.
85. Knez M, Sumser MP, Bittner AM et al. (2004) Binding the tobacco mosaic virus to inorganic surfaces. *Langmuir* **20**(2): 441–447.
86. Wadu-Mesthrige K, Pati B, McClain WM and Liu GY (1996) Disaggregation of tobacco mosaic virus by bovine serum albumin. *Langmuir* **12**(14): 3511–3515.
87. Imai K, Yoshimura K, Tomitori M et al. (1993) Scanning tunneling and atomic force microscopy of T4 bacteriophage and tobacco mosaic virus. *Japanese Journal of Applied Physics Part 1 – Regular Papers Short Notes & Review Papers* **32**(6B): 2962–2964.
88. Zenhausern F, Adrian M, Tenheggelerbordier B, Ardizzoni F and Descouts P (1993) Enhanced imaging of biomolecules with electron beam deposited tips for scanning force microscopy. *Journal of Applied Physics* **73**(11): 7232–7237.
89. Grönbeck H, Curioni A and Andreoni W (2000) Thiols and disulfides on the Au(111) surface: the headgroup–gold interaction. *Journal of the American Chemical Society* **122**(16): 3839–3842.
90. Soto CM, Blum AS, Vora GJ et al. (2006) Fluorescent signal amplification of carbocyanine dyes using engineered viral nanoparticles. *Journal of the American Chemical Society* **128**(15): 5184–5189.
91. Zhou K, Li F, Dai G, Meng C and Wang Q (2013) Disulfide bond: dramatically enhanced assembly capability and structural stability of tobacco mosaic virus nanorods. *Biomacromolecules* **14**(8): 2593–2600.
92. Djalali R, Samson J and Matsui H (2004) Doughnut-shaped peptide nano-assemblies and their applications as nanoreactors. *Journal of the American Chemical Society* **126**(25): 7935–7939.
93. Ballister ER, Lai AH, Zuckermann RN, Cheng Y and Mougous JD (2008) In vitro self-assembly of tailorable nanotubes from a simple protein building block. *Proceedings of the National Academy of Sciences of the United States of America* **105**(10): 3733–3738.
94. Altıntoprak K, Seidenstücker A, Welle A et al. (2015) Peptide-equipped tobacco mosaic virus templates for selective and controllable biomaterial deposition. *Beilstein Journal of Nanotechnology* **6**(1): 1399–1412.

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