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Tobacco Mosaic Virus as a Versatile Platform for Molecular Assembly and Device Fabrication

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Viruses are unique biological agents that infect living host cells through molecular delivery of a genomic cargo. Over the past two decades advancements in genetic engineering and bioconjugation technologies have allowed the unprecedented use of these "unfriendly" biological molecules, as nanoscopic platforms for the advancement of an array of nanotechnology applications. This mini-review focuses on providing a brief summary of key demonstrations leveraging the versatile characteristics of Tobacco mosaic virus (TMV) for molecular assembly and bio-device integration. A comprehensive discussion of genetic and chemical modification strategies along with potential limiting factors that impact the assembly of these macromolecules is presented to provide useful insights for adapting TMV as a potentially universal platform toward developing advanced nanomaterials. Additional discussions on biofabrication techniques developed in parallel to enable immobilization, alignment, and patterning of TMV-based functional particles on solid surfaces will highlight technological innovations that can be widely adapted for creating nanoscopic device components using these engineered biomacromolecules. Further exploitation in the design of molecular specificity and assembly mechanisms and the development of highly controllable and scalable TMV-device integration strategies will expand the library of nanoscale engineering tools that can be used for the further development of virus-based nanotechnology platforms.

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1. Introduction

Biology offers unique solutions to current needs in nanoscale design and application. One class of biological nanostructures particularly suitable for nanomaterial engineering is viruses. Virus particles are nanoscale assemblies of proteins organized around a nucleic acid genome. They take advantage of the structural and functional capabilities of proteins for host recognition, invasion and infection, extracellular stability, and containment of genomic cargo. This unique combination of properties has made viruses useful for the development of a wide range of applications including targeted drug delivery,[1] biosensing,[2] and energy storage.[3]

Tobacco mosaic virus (TMV) is a particularly attractive substrate for the creation of nanoscale materials. Physical knowledge of TMV, including precise 3-D structural information, virion assembly mechanism, and biochemical properties are understood in unique detail.[4] The rod-shaped TMV has a high-aspect-ratio geometry (300 nm length by 18 nm diameter) and large protein copy number (≈2130) per particle that makes it an ideal substrate for the integration of tunable high-surface-area materials or densely arranged motif displays into functional devices. These properties have driven research into the use of TMV for the development of diverse applications, including anti-reflective photochemical electrodes, super-hydrophobic surfaces, improved energy storage, and transducer surfaces for sensing applications.[5,6]

Here we review the state of research into the use of TMV as a potentially universal functional nanomaterial. We will focus on three broad areas of investigation: 1) Surface modifications for display of useful elements; 2) alterations to TMV morphology; and 3) methods integrating TMV onto device surfaces for microsystems applications.

2. Methods for Engineering Surface Modifications of TMV

TMV can act as a nanoscale scaffold for the anchoring, arrangement, and display of biochemical motifs. For some applications, the native functional groups displayed on the

surface of the wild-type virus are sufficient. However, the customizability of TMV allows for genetic and post-translational modifications that permit the incorporation of various molecules and functional groups (**Table 1**).

2.1. Genetic Modifications

One strategy to functionalize virus particles genetically alters the coat protein (CP) to introduce peptide sequences to the virus surface. The well-established structure of assembled TMV CP established by X-ray diffraction[7] and cryoEM[8,9] indicates that the outer surface of the virion includes the CP N-terminus, C-terminus, and a loop containing residues 63–66.[10] Insertions of sequences at each of these three sites have produced infectious and stable virions. In general, short peptide sequences are most successful as they minimize interference with CP folding and virion assembly.[11]

The ability to express short peptide sequences as contiguous parts of the TMV CP enables the immediate use of purified virus as a highly multivalent nanoscale peptide display scaffold without further modifications. Functional peptides that have been displayed from the TMV CP include immunoreactive peptides for antibody detection in the picomolar range.[12] The display of binding peptides that target small molecules, identified from phage display libraries,[13] enabled the use of TMV as a multivalent biorecognition element for the detection of analytes such as 2,4,6-trinitrotoluene (TNT) via square wave voltammetry.[14] Additionally, display of the integrin receptor target amino acid sequence RGD allowed TMV to mimic extracellular matrix proteins with unique consistency and patterning for the culture of stem cells.[15] However, genetic insertions may reduce purifiable virus yield, and virus rods may unpredictably fail to form entirely.[16] For these reasons, many groups opt instead for post-translational bioconjugation of functional groups, including peptides, to assembled virus.

2.2. Chemical Bioconjugation

Surface-exposed functional groups provide selective sites for chemical bioconjugation, broadening allowable modifications while avoiding assembly disruptions that sometimes result from genetic modifications. Naturally occurring bioconjugation sites include a recessed but outer-surface-exposed tyrosine (T139) and two inner-channel-accessible glutamic acids (E97 and E106). Bioconjugation to the tyrosine's phenol can occur by azo-coupling using diazonium salts, while the inner-channel glutamic acids are activated by 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and coupled to amine-linked targets via an amide bond.[17] The azo-coupling of an alkyne group to the exterior tyrosine allows the use of Cu(I)-catalyzed azide-alkyne cycloaddition reactions (CuAAC), a type of "click" chemistry,[18] simplifying further modifications. The addition of β-cyclodextrin by CuAAC, allows the noncovalent attachment of adamantyl-coupled molecules.[19]

Independent targeting of TMV's tyrosines and glutamates allows heterologous functionalization of their distinct surfaces. Given TMV's potential drug delivery and imaging applications, bioconjugated molecules include fluorescent dyes,[19,20] MRI contrast agents,[21] poly(ethylene glycol) (PEG) to tune circulatory clearance rates,[17,19] carbohydrates,[22] folic acid,[19] and peptides (cRGD)[23] to assist endocytosis, and pharmaceutical agents including doxorubicin[19,23] and cisplatin.[22] Additionally, the negative charges of the inner channel glutamates (E97 and E106) are sufficient for non-covalent loading and retention of ≈800 molecules/virion of a triply cationic porphyrin-based

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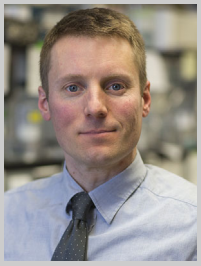

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Table 1. Overview of TMV surface functionalization strategies.

Location on TMV	TMV alteration	Purpose	Functional group	Activity	Specific uses
Inner channel	None	Site for conjugation of functional molecules	Glutamic acids (Glu97, Glu106)	Electrostatic interactions	cationic photosensitizer,[24] phenanthriplatin[25]
				EDC-activated amide attachment,	Fluorescent dye (Cy5[20]), cisplatin,[22] MRI contrast agents[21]
Outer surface			Tyrosine (Tyr139)	Azo-coupling of diazonium salts	Fluorescent dyes (Cy5,[20] rhodamine B[19]), folic acid,[19] doxorubicin,[19] PEG,[17,19] MRI contrast agents,[21] carbohydrates[22]
	Genetic alteration		N-terminal lysine	NHS-attachment	PEO_4, biotin, protein (GFP), peptide (canine papillomavirus fragment)[10]
			C-terminus proximal lysine (Thr158Lys)		Fluorescent dye (Cy5[20]), PEG,[30] peptides[30]
			N-terminus proximal cysteine (S3C or S1_Y2insC)	Maleimide attachment	Fluorescent dyes (Texas Red and Atto 488),[27] PEG,[27] glucose oxidase[28]
		Inherent functionality		Thiol-metal interactions	TMV surface attachment, metal deposition[56]
			Peptides	Target binding	TNT-binding[14]
				Antigen display	FLAG-tag[12]
				Receptor binding	RGD[15,67]

photosensitizer[24] or ≈2000 molecules/virion (≈1 molecule/CP) of the doubly cationic chemotherapeutic candidate phenanthriplatin with subsequent release in acidic environments.[25]

To expand TMV's functional capability, several modifications have introduced new functional groups to the TMV CP. The insertion of cysteine residues near the N-terminus of the CP enables well-established thiol-to-maleimide bioconjugations including fluorescent dyes (Texas Red[26] and Atto 488[27]) and biotinylated PEG, which allowed the subsequent attachment of streptavidin-labeled enzymes including horseradish peroxidase[27] and glucose oxidase[28] with a 50% conjugation efficiency.[28] A lysine inserted at the N-terminus[10] or C-terminus[29] similarly allows for bioconjugation to the exterior of TMV through a reaction with an N-hydroxysuccinimide (NHS)-ester-labeled target. This enabled surface display of biotin or maleimide for the attachment of avidin-/streptavidin-linked immunogenic peptides and whole proteins such as GFP[10] or cysteine-containing peptides improving silica surface deposition.[30]

3. Impact on Assembly and Particle Morphology

An understanding of the mechanisms underlying TMV self-assembly has allowed for targeted changes altering the particle's structural properties. These include changes to the CP, to the RNA scaffold around which the CP assembles, or to solution conditions driving assembly and disassembly. The resulting alterations range from the shapes and dimensions of the particles to the longitudinal distribution of CP components.

One general method for structural TMV modification exploits the ability of disassembled TMV CP to reassemble with single-stranded RNA containing an origin of assembly sequence (OAS) motif.[31] Modified CP that fails to form rods in vivo can be incorporated into rods in vitro by the addition of unmodified CP mixture.[23,32] Furthermore, various CPs with distinct functionalities (bioconjugation sites, peptide tags, etc.) can be organized in discrete sections along the length of the particle by sequential introduction of the different CP variants to the RNA scaffold in vitro. This is due to the assembly process which progressively adds CP to the 5' end of the RNA OAS,[27] a process which can be more tightly controlled by the use of DNA oligonucleotides that can hybridize with or release from specific sequences of the exposed RNA to halt or allow continued assembly.[33] Because the TMV rod length is primarily constrained by the length of its RNA, custom length RNA containing the OAS can be used to control particle length in vitro[34] or in planta.[35] In addition, the use of branched nucleic acid scaffolds has even been used to introduce kinked or branched structures, creating "nano-boomerangs" and "multipods."[36]

Other mechanisms to control TMV morphology depend on manipulating interactions between CP subunits. Significantly, an electrostatic repulsion between carboxylate-containing residues on adjacent subunits can be controlled by solution pH and ionic concentration to promote assembly or disassembly and alter particle length or aspect ratio. TMV CP alone typically forms only short rods, disks or monomers at neutral pH and low ionic strength.[37] A 400 mM acetate solution promotes the assembly of a four layer disk consisting of two bilayer disks arranged in a "head-to-head" orientation.[38] Alternatively, lowering the pH below ≈5.5 induces CP assembly into rods,[39] allowing hydrophobic interactions between layers of CP to drive assembly. TMV retains a negative surface charge above its isoelectric point near pH 3.3, so positively charged polymer precursors accumulates on the long TMV fibers that form when the pH is near 5 and can be stabilized by the surface deposition and

polymerization of aniline,[40] pyrrole,[41] or dopamine.[42] The hierarchical arrangement of TMV rods into larger-scale bundles and lattices has also been accomplished using metal nanoparticles,[43] organometallic compounds,[44] and polysaccharides.[45] Distinct from variations on the typical rod-like form of TMV, spherical nanoparticles from TMV CP have been made by altering CP interactions through simple thermal treatments[46] and through chemical modification.[47]

A final method to alter particle assembly uses engineered changes to the CP gene. A circular permutant rearranges the large sections of the gene to move the N- and C-termini to the inner channel.[48] The replacement of destabilizing carboxylates E50 and D77, which interact axially between neighboring CP monomers, with uncharged analogs (E50Q and D77N) generates RNA-free virus-like particles of various lengths.[49] The E50Q mutation alone has been used to promote particle assembly and stability for longer particle lengths.[50] Another tactic introduces covalent bonding between subunits to stabilize the helical protein assembly – a cysteine introduced to the flexible loop at the virus's inner channel surface (T103C) positioned to interact with neighboring cysteines generated disulfide bonds that stabilized helical rods formation under conditions which limit wild-type CP assembly.[51] Additional work introducing cysteines to the outer-surface-exposed N-terminus further allowed for the organization of disks or rods into pH- and ion-dependent regular arrays or bundles.[52]

4. TMV-Device Integration Strategies

The nanoscale geometrical characteristics and molecular specificity of the engineered TMV particles has allowed them to be exploited in numerous advanced micro/nano devices.[6] It is critical to understand some of the key technologies developed for TMV-device integration that have allowed TMV's versatile functionality to be leveraged in various device applications.

Early efforts have established robust routes for immobilization of TMV particles onto targeted surfaces. The methods for TMV immobilization can be categorized into three major approaches (**Figure 1**A): 1) non-specific surface binding;[53,54] 2) specific surface binding via either nucleic acid hybridization or covalent binding;[55,56] and 3) use of polymer binders.[57,58] In general, the latter two approaches result in more reliable and reproducible device performance. The nucleic acid hybridization scheme has been successfully adapted by partially exposing the RNA from one end of TMV's rod-like structure through pH-controlled CP disassembly.[55] The cysteine modification enabled "thiol-Au" binding as a simple, direct, and uniform strategy for TMV immobilization.[59,60] As aforementioned, the enhanced binding affinity to metal with surface thiols also has enabled conformal coating of metal layers over the self-assembled TMV surfaces via electroless plating.[56] This allowed process integration with conventional micro/nano fabrication techniques to incorporate additional electrochemically active materials, eventually leading to a library of highly functional TMV-assembled energy storage electrodes.[6]

In parallel, techniques for patterning TMV immobilization have been developed to acquire controllability over device functionality. Learning from standard microfabrication processes, an electrode patterning technique, "lift-off," has been applied for patterning TMV-based nanostructured electrodes.[61] TMV is robust enough to withstand harsh process conditions, including maintaining its functional integrity after the final patterning step using organic solvents. Other techniques specifically developed for patterning bio/chemical molecules on solid substrates also have been applied for patterning TMV particles. For instance, micro-contact printing (μCP) technology – one of the most widely adapted biofabrication techniques – has been successfully utilized for nanoscale patterning of TMV

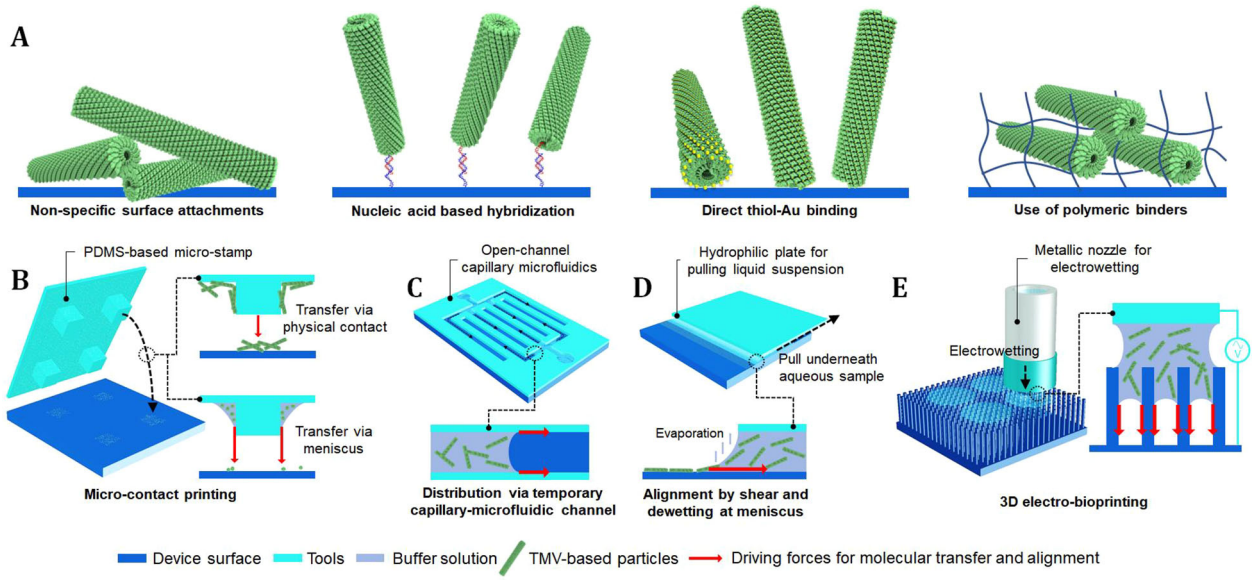

Figure 1. Schematics of TMV-device integration strategies; (A) immobilization and (B–E) patterning/alignment on device surfaces.

particles.[54] Compared to conventional μCP, Balci et al. have introduced a careful stamp inking and drying process prior to transferring TMV molecules from the stamp edges to the target substrate via the meniscus, achieving pattern resolution as low as single virion size (Figure 1B). In addition to the μCP-based technique, there are other biofabrication techniques, including the ones developed based on AFM technology, which can help achieve patterning and alignment of the TMV-based macromolecules at nanometer scale.[62]

Given the known stability of TMV in a range of aqueous environmental conditions, many additional works have demonstrated techniques to pattern and align TMV particles by controlling the solid (device substrate) – liquid (TMV solution) interfacial properties.[54,63] Capillary forces in microfluidic channels or glass tubes have been utilized to autonomously deliver the TMV particles to pre-defined patterns[64] or to align the anisotropic particles at the liquid meniscus formed at the solid-liquid-air interface,[65] (Figure 1C,D, respectively). The latter approach leveraging the self-alignment of TMV particles at the liquid meniscus allowed long range coatings of TMV-based nanowires, demonstrating an innovative strategy for directional arrangement of the anisotropic nanoparticles.

Additional efforts have focused on incorporating 3-D microstructured device components toward demonstrating enhanced and scalable performance. Initial works integrating TMV scaffolds with relatively low density micropillar array electrodes have successfully demonstrated scalable enhancement in device surface area for energy storage applications.[59,66] Recent work has highlighted the limited structural wetting property as a major bottleneck in biomaterial integration with high density 3-D micro/nano structures.[63] This has been overcome by applying an electrowetting technique which selectively introduces TMV solution into targeted 3-D structures by electrically modulating surface wettability (Figure 1E). The simple technique potentially allows uniform and selective immobilization of TMV on any 3-D substrate for enhancing biomaterial-integrated devices and systems.

While the techniques that allow patterning of relatively bulk amount of TMV-based materials, in spontaneous surface arrangement, have enabled demonstrating a range of functional device components, it has been difficult to incorporate the capabilities of high-precision patterning and alignment of TMV particles with nanoscale resolution for potentially enhancing the device functions. Considering the anisotropic one-dimensional structure of TMV, precise control over alignment, patterning, and assembly of TMV particles could allow unique designs in electrical, mechanical, and optical characteristics with high directionality and periodicity in device structures.

5. Conclusion

TMV has been extensively exploited in recent decades as a potentially universal molecular-assembly platform for numerous nanotechnology-driven applications. The diverse molecular engineering strategies and the core understanding behind viral self-assembly mechanisms provide essential knowledge for expanding the versatility of TMV particles with enhanced functionality and robustness. The technologies developed for integrating TMV particles with various device surfaces have enabled utilization of the unique biological functions in creating highly functional components for advanced micro/nano devices and systems.

Numerous efforts so far have focused on utilizing TMV as a 3-D platform for arranging bio/chemical molecules. The future efforts, as aforementioned, can focus on incorporating those high-precision biofabrication techniques for utilizing TMV as macromolecular 1-D base building block for creating complex or rationally designed 3-D nano/micro structures and systems. This will require capability to design TMV-based particles or subunits displaying heterogenous surface ligands with spatial binding specificity. The technological trend developing virus-like-particles, which engineers CPs for heterologous virion assembly without RNA, should provide solutions for the future revolution of TMV-based functional nanoparticles. More application-driven research leveraging the versatile biological platform should continue bringing unprecedented opportunities in bio-nanotechnology areas including bio/chemical sensing, energy storage, bio-imaging, and drug-delivery.

Abbreviations

CP, capsid/coat protein; CuAAC, Cu(I)-catalyzed azide-alkyne cycloaddition; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; OAS, origin of assembly sequence; PEG, polyethylene glycol; TMV, Tobacco mosaic virus; TNT, 2,4,6-trinitrotoluene; VLP, virus-like particle; μCP, micro-contact printing.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

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