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Plant virus-based materials for biomedical applications: Trends and prospects

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ABSTRACT

Nanomaterials composed of plant viral components are finding their way into medical technology and health care, as they offer singular properties. Precisely shaped, tailored virus nanoparticles (VNPs) with multivalent protein surfaces are efficiently loaded with functional compounds such as contrast agents and drugs, and serve as carrier templates and targeting vehicles displaying e.g. peptides and synthetic molecules. Multiple modifications enable uses including vaccination, biosensing, tissue engineering, intravital delivery and theranostics. Novel concepts exploit self-organization capacities of viral building blocks into hierarchical 2D and 3D structures, and their conversion into biocompatible, biodegradable units. High yields of VNPs and proteins can be harvested from plants after a few days so that various products have reached or are close to commercialization. The article delineates potentials and limitations of biomedical plant VNP uses, integrating perspectives of chemistry, biomaterials sciences, molecular plant virology and process engineering.

Contents

Abbreviations: 4CL2, 4-coumarate:CoA-ligase 2; AFM, atomic force microscopy; AMI, acute myocardial infarction; BMSC, bone marrow stem cell; BMV, brome mosaic virus; BSA, bovine serum albumin; CalB, *Pseudozyma* (*Candida*) *antarctica* lipase B; CCMV, cowpea chlorotic mottle virus; CMV, cucumber mosaic virus; CP, coat protein; CPMV, cowpea mosaic virus; CRISPR, clustered regularly interspaced short palindromic repeats; CT, cholera toxin; Cy5, cyanine 5; Cy7.5, cyanine 7.5; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Dy, dysprosium; ECM, extracellular matrix; EDC, endocrine-disrupting chemicals; EGFR, epidermal growth factor receptor; ELISA, enzyme–linked immunosorbent assays; eVLP, empty VLPs; FMDV, foot-and-mouth disease virus; GBCA, gadolinium (Gd)-based contrast agents; Gd, gadolinium; GISAXS, grazing-incidence small-angle X-ray scattering; GOx, glucose oxidase; HBV, hepatitis B virus; HepG2, hepatocellular carcinoma cell line; HRP, horseradish peroxidase; ICG, indocyanine green; ICTV, International Committee on Taxonomy of Viruses; ISPMF, International Society for Plant Molecular Farming; LBL, layer-by-layer; MCF-7, human breast cancer cell line; MP, movement protein; MRI, magnetic resonance imaging; M.T, microtubule; NA, NeutrAvidin; NIR, near infrared; OEG, oligo-ethylene glycol; OVG, optical viral ghost; PAH, poly(allylamine hydrochloride); PAI, photoacoustic imaging; PC3, prostate cancer cells; PCR, polymerase chain reaction; PEG, poly(ethylene glycol); PEG-DA, poly(ethylene glycol) diacrylate; Pen, penicillinase; plant VNP, plant virus nanoparticle; POXylated, poly(2-oxazoline)-modified; PPC, pancreatic progenitor cell; PVA, potato virus A; PVX, potato virus X; PVY, potato virus Y; QCM, quartz crystal microbalance; Qd, quantum dot; RCNMV, red clover necrotic mosaic virus; RGD, Arg-Gly-Asp tripeptide motif; SA, streptavidin; SEB, staphylococcal enterotoxin B; SeMV, *Sesbania* mosaic virus; SPAB, staphylococcal protein A domain B; SPR, surface plasmon resonance; STS, stilbene synthase; TBSV, tomato bushy stunt virus; TEM, transmission electron microscopy; TMV, tobacco mosaic virus; TMV_{Cys}, cysteine-mutant of TMV; TMV-Lac, lactose-TMV; TMV_{Lys}, lysine-mutant of TMV; TMV-Man, mannose-TMV; TYMV, turnip yellow mosaic virus; VCAM, vascular cell adhesion molecule; VLP, virus-like particle; VNP, virus nanoparticle; ZYMV, zucchini yellow mosaic virus..

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1. Introduction

Virus-based building blocks and regulatory elements have become increasingly attractive for various applications, as their naturally optimized capacities and shapes may help to exceed limits of current technical fabrication. This yields novel types of precisely formed soft-matter nanocontainers and templates, self-assembling and hierarchically arranged two- and three-dimensional platforms, or bioactive compounds. Plant viral derivatives can be harvested in commercially reasonable amounts and lack potential risks for mammals by contaminating pathogens, a fundamental healthcare consideration. Biodegradable but robust multivalent plant viruses and virus-like particles (VLPs) are now regarded valuable for clinical and biomedical purposes, and are experiencing intense research and development. This article intends to provide a concise overview of the major and most advanced approaches making use of plant virus derivatives in medical contexts, and to assess the potential of newly emerging concepts. A critical literature review takes account of original reports on significant biomedical achievements, book chapters and review contributions. Owing to the interdisciplinary authors team, these consider not only medical accomplishments, but also molecular biology and plant virology, biological self-organization and materials formation on distinct scales, fundamental and applied sciences of interfacial engineering and biotechnology, (bio-) organic synthesis, and biomedical fabrication and processing.

Most important for the majority of uses described for plant virus nanoparticles (plant VNPs), they can serve as efficient soft-matter carrier vehicles for molecules with complex functionality such as enzymes and antibodies, for active pharmaceutical ingredients, but also for organic and inorganic (metallic and non-metallic) bulk compounds and pre-formed particles. This enables to design and realize bio-inspired artificial effector structures like vaccines, nanoobjects for drug delivery, energy and chemical conversion systems, signal-responsive arrays, expanded 'smart' films and three-dimensional materials e.g. for biosensing, cell differentiation and/or tissue engineering, and even actuated nanomachines. An impressive burst of review articles and books demonstrates the expansion and promises of these developments e.g. [1,2–27]. Plant viral nucleic acid elements ranging from promoters and enhancers up to ribosome-affecting sequences have convinced as excellent genetic tools in the heterologous expression of proteins or VLPs [28–31], and as scaffolds or switches controlling the semi-autonomous organization of technically not accessible small structures e.g. [32,33–36]. Certain plant viral proteins may affect intracellular processes such as microtubule organization and transport pathways [37,38], with potential future therapeutic applications. All these opportunities have been realized and investigated throughout the last three decades. Hence the old idea of exploiting plant viral assemblies for testing and improving technological advances, as demonstrated long ago e.g. in the history of electron microscopy [39], was taken up again and is now accelerated especially in the field of biomedicine.

Fast progress has been made in the preparation of plant VNP-based vaccines, accessible in just a few days through 'farming' in plant leaf tissues [2,29,40]. It seems likely that broadly applied influenza vaccines may soon be of plant VNP origin. Therefore, a separate contribution in this issue of Advanced Drug Delivery Reviews provides a detailed description of plant virus-based vaccination strategies [41], for which reason this aspect is not treated in a special section below. This article first covers fundamentals of plant virus properties and production routines. Subsequently, it spans from promising in vitro applications outside patients, namely biomedical detection, via uses at the interface between artificial and live systems (such as tissue engineering and the processing of implant coatings), to prophylaxis, diagnostics and therapeutics in vivo. Potential limitations of such approaches, and risks of plant viruses applied to animal and human patients, are discussed briefly, as well as the industrial impact of plant virus-enabled medical progress. Some examples of non-conventional strategies and additional opportunities are added, before the article also reminds of the world-wide underestimated field of phytomedical issues responsible for major ecological, economic and social problems, which might profit from novel developments based on plant VNPs as well. Fig. 1 categorizes the distinct routes of plant virus-deduced biomedical developments and denotes the corresponding section numbers in this article.

2. Properties of plant viral assemblies attractive for biomedical uses

2.1. Plant virus-based nanoparticles: natural organization and historically developed uses

During the recent few years, sequencing of 'metagenomes' from environmental samples has uncovered an unexpected prevalence of

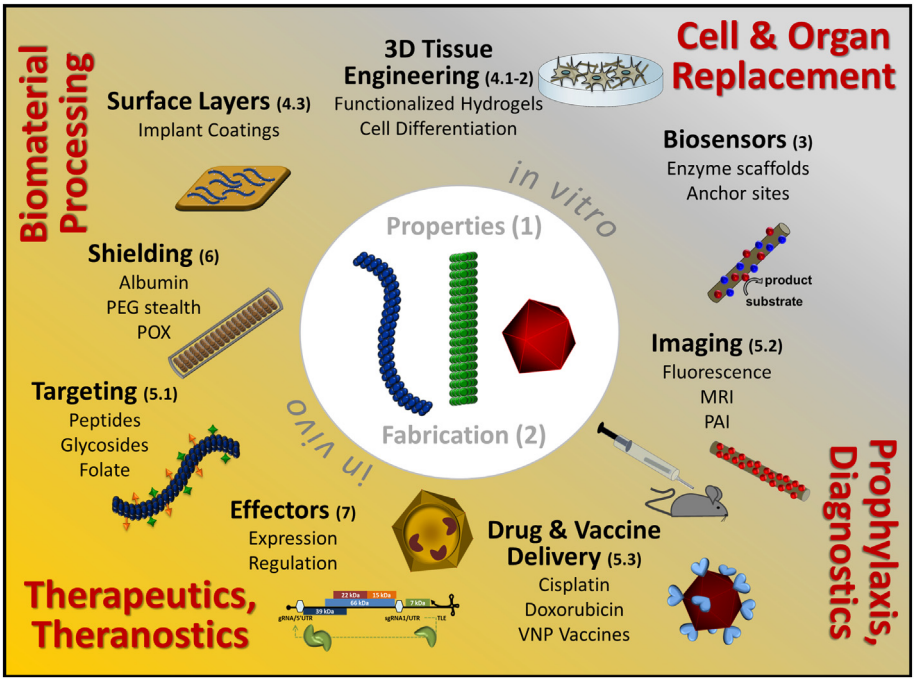

Fig. 1. Plant virus-derived materials for biomedical applications: opportunities and article sections. Differently shaped plant viruses may be engineered or processed to yield nanoparticles and building blocks adapted to specific uses, ranging from the fabrication of organoid tissues up to personalized medicine. Major strategies treated in this article are assigned to the respective section numbers (in brackets).

distinct viruses in all types of biotopes and organisms so that even the virus classification conventions of the International Committee on Taxonomy of Viruses (ICTV) were changed [42]. The term 'metaviromics' was coined ([43], and references therein) for the discipline that unravels the existence of viruses by way of high-throughput sequencing virtually everywhere. Although in many cases, the corresponding particles (*syn.* virions) of new viruses have not yet been isolated, there is little doubt that the majority of all virions exhibit the architectural principles known for viruses since decades: They are organized as regularly shaped supramolecular complexes of a low number of biomolecule types, with multiple protein subunits forming in most cases icosahedron-like capsules, or, alternatively, helically elongated core structures [44,45]. Quasi-equivalent interactions [46,47] of the capsid (or coat) protein (CP) monomers of a single or few distinct species typically result in the formation of a repetitively organized protein shell protecting the viral DNA or RNA genome, referred to as (nucleo)capsid [48]. Many viruses infecting (and exiting) animal cells via passage of their outer membranes are enveloped by a biological phospholipid bilayer originating from the virus-producing cell, in several cases equipped with protein domains or spikes protruding from the envelope membrane [45]. Bacteriophages and most plant viruses, however, are non-enveloped, i.e. the CPs are directly accessible [48], which is advantageous for several medical applications as described later. Depending on the overall virus architecture, such virus particles may span a size regime of below 20 nm diameter up to more than a μm in diameter or length, with less than a hundred up to several thousands of CP subunits. Fig. 2 depicts the types, sizes and morphologies of viruses infecting plants [49–51].

The stability of plant viral nanoparticles against variable conditions of pH, temperature and solvents is strikingly different and may depend on both protein-protein and protein-nucleic acid interactions, in combination with accessory compounds such as polyamines and divalent ions contributing to the biomolecular interactions [48]. Virus infections of plants may be completely symptomless in many cases, but can also induce a large variety of inconspicuous up to striking alterations of plant growth and development. These may result in considerable economic effects due to reduced yield or crop quality, and complete losses upon certain plant virus epidemics. Mosaic-like dark- and light-green patterns on the leaves of infected tobacco, however, also sparked intense scientific research on the causal agent from the 1880s on [53], and similarly the spectacular yellow-green mosaic-like variegation of ornamental *Abutilon* plants in the end of the 19th century (for the history of plant virology, see e.g. [27,54,55,56]). In the subsequent three decades it became clear that plant viruses are non-bacterial entities and depend on living cells [57]; and in 1935, the "tobacco mosaic virus" was isolated as a crystalline material initially considered a protein [58]. This led to a burst of in-depth investigations, which only one year later identified tobacco mosaic virus (TMV) as a liquid-crystalline combination of protein and a much lower amount of ribose-type nucleic acid, as reviewed in detail elsewhere [54]. During the 20th century, research on and with plant viruses did not only focus on understanding their 'life cycles' and interactions with host cells and tissues, but also led to pioneering discoveries at the edge between biology, physics, chemistry, and technical as well as materials sciences. Examples are the advancement of electron microscopy [39] and ultracentrifugation [59], or the template-assisted fabrication of nanomaterials starting in the 1990s (reviewed in [60]). Even a decade earlier, the first plant viral tools for heterologous protein and vaccine expression in plants were raised (see [61] and also section 3.1 below), moving plant viruses also into the focus of biomedical uses.

Plant viruses applied for diagnostic and therapeutic treatments of patients are often pretty robust so that they withstand conditions within the blood stream or even the gastrointestinal system, at least in the non-fasting stomach above pH 2 [62–66], without being pathogenic to warm-blooded animals [67]. Plant viruses are eaten and inhaled almost daily by everyone, due to their high prevalence in vegetables and plant-based products [68–70], and due to the spread of certain virus species even in fog and clouds [71], and of course cigarettes [72,73]. Apart from genome-containing virions, some viruses also produce additional empty VLPs (eVLPs) devoid of nucleic acids [31,48], whereas other virus particles rely on interactions with cognate or engineered nucleic acids for stable assembly. These possibilities are the same for both elongated helical virus types such as filamentous potato viruses (e.g. potato virus X [PVX] as type member of the

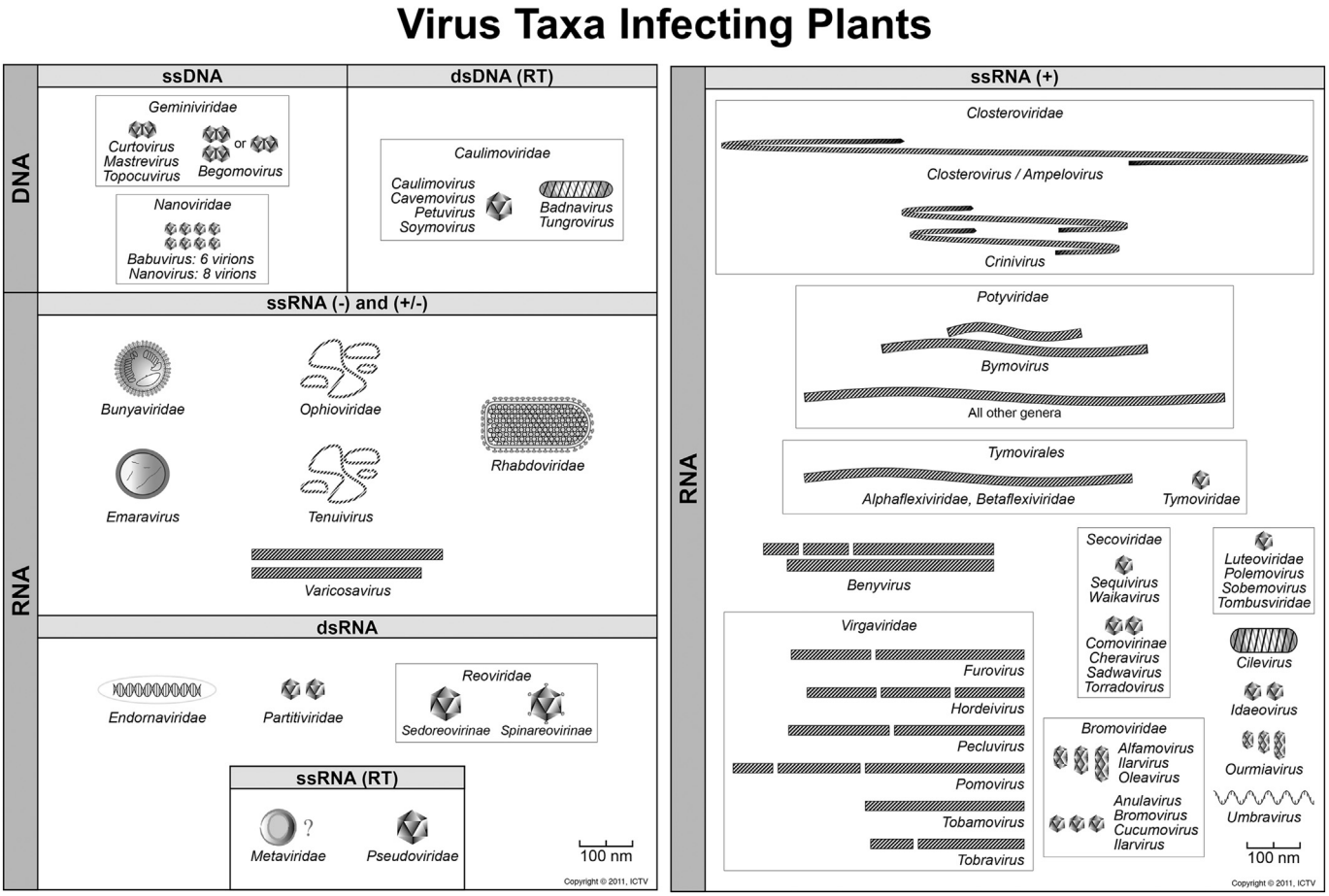

Fig. 2. Virus types infecting plants: genomes, morphologies and approximate sizes (see scale bars). Reproduced according to the Creative Commons Attribution-ShareAlike 4.0 International License from [49]; for background information, see also [50,51] and [52].

potexviruses, and potato virus Y [PVY] as that of the potyviruses), or nanotubular TMV-like tobamoviruses, and icosahedrally organized 'spherical' viruses. The latter include brome mosaic virus (BMV), cowpea mosaic virus (CPMV), cowpea chlorotic mottle virus (CCMV) or red clover necrotic mosaic virus (RCNMV), to mention but a few of the frequently applied plant viruses [8,21,74], which are in most cases named after their originally discovered host plant and characteristic symptoms [48].

2.2. Plant VNP tunability and functionalization

In summary, the features making certain plant viruses highly attractive for technical and biomedical uses are their high yields, capacities to self-assemble into well-defined, multivalent and stable non-pathogenic nanoobjects, capable of accommodating and transporting a high amount of cargo in their inner cavities, and/or densely present effector molecules on their outer surfaces. Such hybrid particles cover a size range not simply accessible by top-down pharmaceuticals preparation methods, and enable the delivery of multiple identical or blended functional molecules inside living organisms. Combinations of internally loaded and externally fashioned VNPs pave the way to multimodal functionality combining e.g. cell-type or disease-selective ligands on the outer VNP surface with the delivery of cargo compounds internalized in the viral shells (see e.g. [19] for a detailed review). A number of plant virus capsids permit an inclusion of small effector molecules on demand, either by way of pore structures that can reversibly open if triggered e.g. by pH or temperature changes, or by in vitro assembly of the CPs in the presence of molecules or nanoparticles of choice, thereby encapsidating the relevant substances (reviewed e.g. in [75], see also Dragnea and co-authors [76] in this issue). In turn, under certain cellular or externally induced conditions, transported and protected molecules may be released from the VNP containers. Not only natural virus capsids are extensively evaluated for physicochemical properties beneficial for medical delivery, but also an immensely growing number of plant VNP mutants with characteristics adapted to specific demands. In some cases, direct genetic fusion of functional proteins or peptides to the viral CPs has allowed harvesting useful VNPs directly from plant tissues inoculated with appropriate expression constructs e.g. [77,78,79]. More frequently, natural and genetically engineered chemically addressable CP variants serve as 'working horses' for the covalent coupling of linker and effector molecules, or for a non-covalent charge-mediated, but reversible equipment with functional units ([6,19] and references therein). Concomitantly, chemical, enzymatic and bioaffinity-based coupling reactions and combinations thereof [80] have been systematically optimized to functionalize viral carrier particles [81–84]. Thereby, a large variety of plant VNPs with specifically installed drugs or enzymes, biological ligands and contrast agents for in vivo imaging has been created during recent years, and is being evaluated in comparison to conventional diagnostic and therapeutic approaches worldwide [19]. This toolbox is growing year by year and now also includes plant VLPs with enzymatic, glutathione peroxidase-like activity due to the spatially ordered display of the unnatural amino acid selenocysteine [4], incorporated in an engineered bacterial expression system [85]. Since bacteriophage and mammalian VLPs equipped with non-canonical amino acids are already used for highly selective bio-orthogonal chemical conjugation reactions [86,87], this strategy may soon be expected to expand the spectrum of linkage strategies applicable to plant VNPs further. Last but not least, two more types of processing methods should be

mentioned here that are in some cases helpful, in others even essential to achieve the desired biomedical VNP functionality in vivo. The first one is an improvement of the particles' biocompatibility by shielding immunogenic CP surfaces and by enhancing clearance from the body, both efficiently achievable via stealth 'camouflage' coatings [88,89]. The second one is the adaptation of particle shapes to the intended tropism and cellular uptake behaviour after intravital administration, which may strongly depend on the nanoparticles' aspect ratio and size [90]. This can be adjusted either via choice of a respective plant virus carrier [64], via in vitro assembly of VLP shape variants [33,65,91], or by physical transformation of virus derivatives into objects of altered architecture [64,92,93]. As a whole, all VNP templates and the resulting types of hybrids introduced up to here offer many unprecedented degrees of freedom for applications as medically active nanoparticle formulations.

2.3. Layers and larger assemblies of plant VNPs: controlling the spatial organization

Plant virus particles are not only applied as colloidal objects to address certain cells or targets e.g. via the blood stream for medical purposes, they can also be deposited on biological and technical surfaces, or even converted or integrated into extended composite 3D materials [60,94]. Thereby, they may e.g. serve as tailorable adapter layers between natural and artificial components to facilitate a replacement of organs or tissues by implants, as cell differentiation-triggering agents, or as polyvalent soft-matter templates allowing a high surface-density immobilization of e.g. sensor enzymes, for analyte [1] and cell type-specific [95] detection. Such VNP uses in the preparation of functional biomaterials and implants require an interdisciplinary spectrum of methods, especially when hierarchical structures and topographies are involved in creating the functionality. The past two decades brought many new developments for the design, fabrication and characterization of such composites, to understand the behavior of the interacting components including that of the plant viral building blocks in the material formulations. A seminal atomic force microscopy (AFM) study investigated the deposition of diluted aqueous suspensions into thin TMV films on mica. The arrangements of TMV varied in dependence of the colloidal stabilizer bovine serum albumin (BSA) [96], but were also shown in other studies to reflect concentration, anisotropy and semiflexibility of the helical TMV particles [97] with their 17 CP subunits per turn ([98–102,103], and references therein), as well as their overall charge [104]. Flexible viruses such as soybean mosaic virus, a potyvirus, and PVX are widespread in nature, but so far hardly characterized in terms of structural diversity and materials properties. Some of these helically arranged filamentous VNPs were reported to share a common CP fold, with slightly less than nine subunits per helical turn [105,106]. If and how distinct helical features may influence hierarchical functionalities of virus-based materials is, however, still far from being predictable. In the past decade, efforts primarily addressed techniques to achieve structural control over 2D patterns of VNPs, for example by processing TMV precursor suspensions in confined spaces between two glass slides into large-scale stripe patterns [107].

2.4. Extended plant VNP-containing materials: from design to application

By means of electrospinning, hybrid polyvinyl alcohol-(PVA)-supported TMV-PVA nanofibers and non-woven fibrous mats were produced. It turned out that as-spun fibers did not suffer from problems such as phase separation or geometric variation. This offered best prerequisites for using the integrated TMV nanorods as templates for a spatially defined display of peptide ligands containing the tripeptide motif Arg-Gly-Asp (RGD), to interact with cell surface receptors and thereby induce cell adhesion. The RGD-fashioned TMV thus served as extracellular matrix (ECM) protein equivalents and came out to not significantly change the mechanical properties of the support polymer, but were useful to promote cell growth [108]. This concept was elaborated by use of distinct hydrogel formulations for 3D tissue engineering, as will be described later. TMV has also been subjected to a capillary flow-driven alignment at the triple contact-line of an evaporating droplet. Making use of the "coffee-ring effect", TMV particles rapidly formed 3D-assemblies with parallel orientation to the rim, as revealed by AFM and X-ray microdiffraction techniques. Grazing-incidence small-angle X-ray scattering (GISAXS) data even allowed calculating a low-resolution electron density projection along the rod axis [109]. The same group reported that virus nanofilaments could be directionally grown on a superhydrophobic surface [110]. These and further manipulation techniques guiding the self-organization of plant virus-containing superstructures [111,112] may become future clues to pre-define spatial domains with distinct ligand-exposing VNPs in meso- to microscale arrangements. These might serve as 3D templates for engineering organ-like assemblies of two or more differentiated cell types, given that sufficient control will be gained over the localization of the distinct effector VNPs and the accessibility of the ligands installed.

As mechanically adjustable and stimulable microenvironments are increasingly important for developing 'organs-on-chips' as physiological models and implantable adducts [113,114], flexuous viral templates may harbor specific advantages in such systems. Cryo-EM investigations of the filamentous bamboo mosaic virus provided evidence that viral CPs with only modest inter-subunit contacts and flexible N- and C-terminal extensions might be crucial to allow deformation, with the structural integrity of the entire virion preserved [115]. Generally, however, the comprehension of dynamic structural features of plant viruses is still limited, although common techniques such as CD spectroscopy may provide significant insights [116]. It is therefore important to increase the understanding of the relationships between molecular structure and properties of viral capsids, including virion shape, lability and capability of structural remodeling, as reviewed [117], in order to exploit the full potential of plant VNPs for biomaterials applications. Recently, a biodegradable viral nanoparticle/polymer implant was prepared via melt-processing at 95 °C, with the compression and shear forces successfully adjusted to the stability of the virus capsids [118]. This underscores the prospects of a precise knowledge on plant VNPs' responses to mechanical stress and dynamic deformation, with regard to efficient biofabrication. Well-characterized precursor formulations and predicting their properties are keys to design processes which are also suitable for up-scaling.

Spherical plant virus particles offer additional options for designing multifunctional 'smart' 3D materials: They may act as nanocontainers and thus reservoirs for effector compounds, and simultaneously display ligands on their outer surfaces. Layer-by-layer (LBL) deposition has yielded predictably combined materials with CCMV or CPMV layers, interconnected e.g. by streptavidin-biotin affinity anchors ([60], and references therein). VNP-containing hydrogels with larger gaps between the viral nanocages are highly attractive for cell culture layouts [119], whereas oligonucleotides conjugated to the particles' surfaces may order and interconnect them into large aggregates if two VNP types with complementary sequences are applied [120]. Inter-particle distances, size, packing and dimensionality of the extensive arrays can be defined via the oligonucleotide strands, and dissociation achieved by external triggers such as heating and competitor nucleic acids.

Electrostatically guided, thoroughly adjusted self-assembly of plant viral shells in the presence of different 'spacer' molecules or particles can lead to macroscopic, hierarchically arranged and highly ordered superlattices. Such co-crystals of spherical VNPs have been generated e.g. with gold nanoparticles [121], supercharged ionic polypeptides together with functional proteins [122], or photosensitive dendron molecules, the latter allowing optically induced decomposition of the crystalline arrays. Because all these nanoparticle lattices are porous, with inter-subunit channels of different width, they may be of special value for diffusion-controlled reactions in vitro, and even for intracellular tasks after uptake into the cytoplasm of target cells. They resemble

natural virus polyhedra well-known e.g. for baculovirus storage, ensuring a long-term stabilization of delicate biomolecular complexes ([122], and references therein). Artificial plant virus-based materials designed for specific biomedical applications, e.g. for tissue engineering or in vitro biosensing of relevant targets are exemplified in later sections of this review article.

3. Plant virus-like particle fabrication and processing: preparation routes and their limitations

3.1. Harvesting plant viruses and tailored VNPs: What does a virus tolerate?

Already in the 1980s, pioneering molecular biology studies demonstrated an immense potential of plant viruses to be endowed with 'designer functions', as framed in a foresightful and inspiring review article by T. Michael A. Wilson in 1989 [61]. Among numerous further approaches, initial plant virus applications comprised not only the production of bacterial enzymes in plants [123,124], but even the fabrication of self-assembling neutralizing polio vaccines based on an engineered TMV fusion CP expressed in *E. coli* [125].

This illustrates that strategies exploiting plant viral capacities for biomedical advancements have been followed for at least 35 years so far, with convincing results obtained from the outset. However, on the other hand, a significant number of concepts proposed for novel plant virus uses eventually failed, as common in the course of scientific progress. Such failures often reflect specific limitations of the plant viral modulation capability, interfering with the accumulation of genetically tailored VNPs in plant tissues or in alternative production hosts. Upon 'virus farming' in plants, even minor changes of the genetic information may inhibit the viral 'life cycle' at distinct stages. This is aggravated by the fact that most viral protein and nucleic acid elements are multifunctional and interact with partner molecules in *cis* and in *trans*, i.e. with other virus as well as host factors, often in a dynamic and cell stage-dependent manner. Thus alterations of the structural and non-structural virus proteins and of the encapsulated genome may prevent both assembly and disassembly of the virus particles inside cells, may abolish interactions with e.g. plant replicases, transcription factors and transport systems in plant tissues [48], or may trigger plant defense reactions ranging from tissue necrosis up to sRNA-mediated silencing. Hence, the frequently desired CP extensions by genetically fused protein domains or peptides, or amino acid substitutions affecting the proteins' overall pI or interactions with adjacent CP subunits are prone to abolishing an efficient virion production *in planta*. This may also confine successfully multiplied VNPs to the inoculated areas, as several viruses depend on intact particles for systemic long-distance transport inside the plant vessels [48]. Finally, many engineered virus variants also tend to undergo backmutation to more favorable sequences, leading to loss of the intended features.

3.2. Clues to customize plant virus derivatives

During the last decades, however, the understanding of several aspects of molecular plant virology has deepened, and a substantial amount of experience and persevering, creative work has overcome many obstacles. Therefore, viable and reliable concepts for producing tailored plant VNPs for biomedical purposes were established. Different particle types containing genetically encoded CP fusion proteins have become accessible [as summarized in 13,19] by way of genetic constructs exploiting e.g. the 'ribosome skip' mechanism exerted by the 2A sequence of foot-and-mouth disease virus (FMDV; [126], and references therein). This strategy leads to an expression of CP blends containing both wildtype and engineered variants, thus providing the steric prerequisites for an incorporation of CPs with voluminous extensions into otherwise naturally arranged VNPs. Heterologous expression systems of plant viral protein building blocks, which may subsequently assemble into virus-like structures under appropriate conditions, play a further important role, given that no plant-specific post-translational modifications are required for in vitro self-organization. In the case of TMV, for example, RNA-free CP helices may efficiently form by use of certain bacterially produced CP mutants with increased inter-subunit interactions [127–129], whereas an RNA-guided nanotube growth demands for N-terminally acetylated CP from plants or other eukaryotic systems [129,130], at least as additive blended with *E. coli*-made TMV CP [131]. To exclude such risks, plant-based protein and also VNP production systems have become the primary choice for plant virus-enabled, economically reasonable biomedical advancements, and have even proved to effectively generate numerous animal virus-derived and hybrid VLPs e.g. for vaccination purposes [132–135]. For a more detailed description, see original work.

A major cornerstone of eco-friendly and cost-efficient production routines in plants is the *Agrobacterium*-mediated transient expression technology (described concisely e.g. in [137-138]), which makes use of the natural T-DNA transfer mechanism of this bacterial genus (see Fig. 3). By help of 'disarmed' laboratory bacterial strains, and sophisticated replicating or non-replicating target expression constructs combining selected plant viral elements with modular cloning sites (exemplarily explained in [139,140]), a rapid and safe high-yield production of medically active protein compounds and plant VNPs is now possible. *Agrobacterium* expression inocula are typically applied into all leaves of up to hundreds of plants simultaneously by vacuum infiltration, and lead to high accumulation of the products of interest within a few days only. This makes green 'farming' of VNP vaccines and also other virus-based compounds increasingly attractive for commercialization so that specialized companies have been founded worldwide, and corresponding products have already reached phase III clinical trials [2,40,141–145]. As a side-effect, the resulting preparations are in many cases also energetically favorable. The International Society for Plant Molecular Farming is dedicated to combine forces in all continents [146], in order to advance the respective technologies on an economically competitive time scale. Importantly, for medical and food applications of the plant-borne components, "humanized" special plant lines with adapted glycosylation patterns are available [147], to minimize potential side effects of tailor-made and personalized pharmaceuticals from plants. In summary, the age of "synthetic plant virology" [74] has now come to a stage in which both proof-of-concept and market-oriented hybrid formulations are being tested for their performance, in comparison to materials employed so far. Routine fabrication and processing protocols have been established, of which many have been combined in a comprehensive methods book recently [10], providing detailed instruction on how to engineer and handle VNPs and their building blocks for generating functional materials and devices.

The following sections describe selected biomedical applications of plant VNP derivatives. They are arranged from the out- to the inside of patients, starting with virus-assisted biosensing and concluding with intravital tasks accomplished by help of plant viruses with promising outcome.

4. Applications outside patients: diagnostics by help of plant virus-assisted sensors

4.1. Biosensor improvement by multivalent plant viral nanotemplates: bioaffinity layouts

The unique properties of virus particles suggest their integration into novel diagnostic devices to serve as polyvalent nanocarriers for both receptor and signal-transducing components. A comprehensive report on similar uses of non-viral protein cages such as lumazine synthase, ferritin, encapsulin or synthetic protein nanocages is given in [148]. Here, we will highlight a few highly promising, stunning or unusual approaches employing plant virus assemblies in biomedical sensors. One of the major players in the field is the spherical CPMV in the genus *Comoviridae* [52]. CPMV particles have a diameter of 30 nm and 60

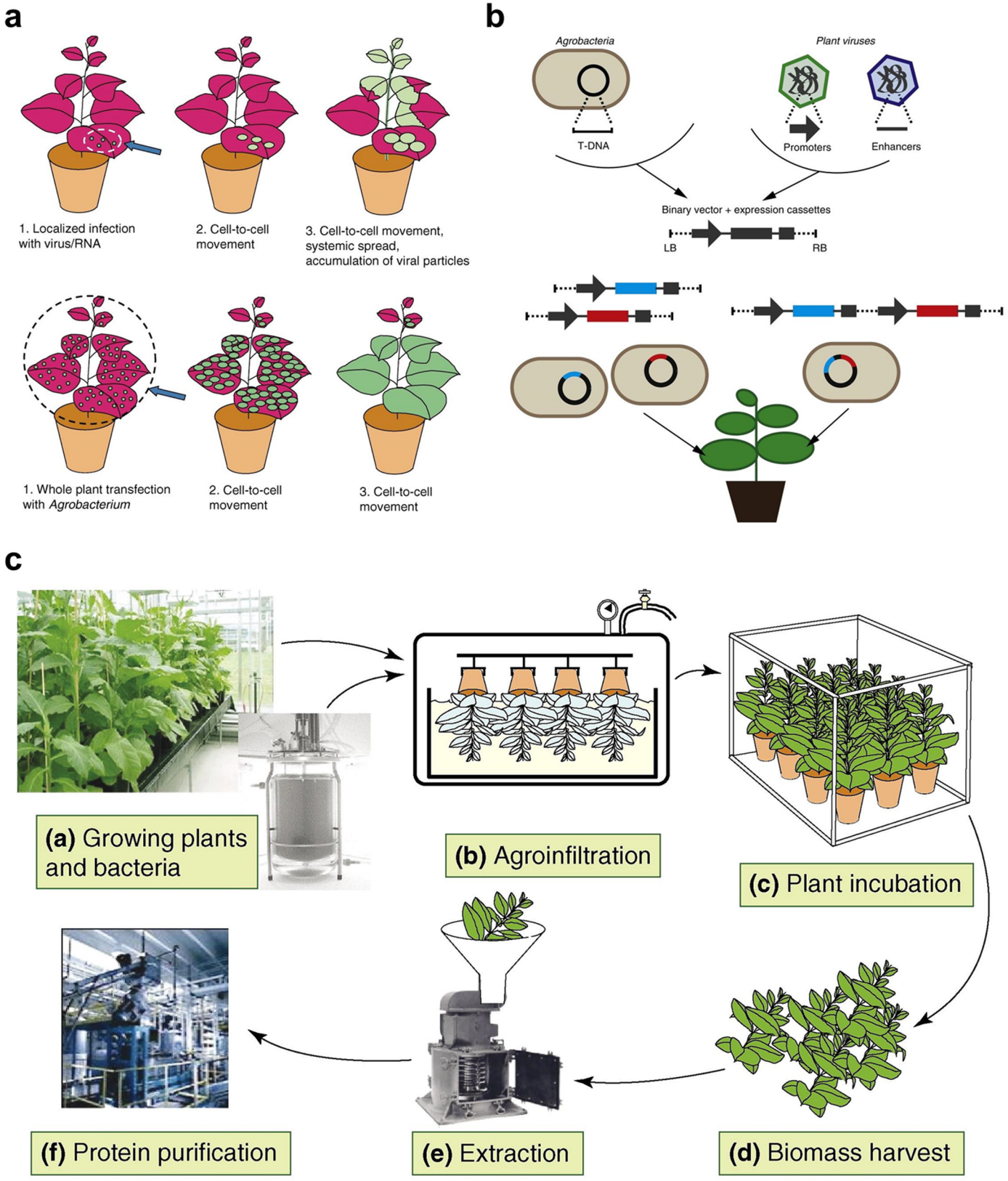

Fig. 3. *Agrobacterium*-mediated transient expression technology – farming in plants by magnifection. a) Schematic drawing of localized and whole plant transfection with expression constructs by help of agrobacteria. Adapted with permission from reference [136]. Copyright© 2007 by Elsevier. b) Cartoon of transient expression systems used in plant-based synthetic biology. Reproduced according to the Creative Commons Attribution 3.0 International Public License from [137]. c) Schematic overview of the recombinant protein production and purification in plants using "magnifection". Adapted with permission from [136]. Copyright© 2007 by Elsevier.

copies each of a minor and a major CP. Genetically engineered, stable CPMV mutants exposing 60, 120 or multiples thereof cysteine residues on their outer capsid surface enable an efficient (multi-) functionalization of the particles and thus the creation of CPMV-based biosensors (reviewed e.g. in [18]). Convincing sensor layouts were obtained e.g. for the detection of bacterial toxins. The gram-positive human pathogen *Staphylococcus aureus* is responsible for a wide range of serious diseases and can secrete a great number of potent toxins, for instance staphylococcal enterotoxin B (SEB) [149]. SEB is an extremely potent bacterial superantigen and causes serious food poisoning; even low amounts can lead to multiorgan dysfunction and death [150]. In most cases, detection of this toxin is performed by immunodiffusion or enzyme–linked immunosorbent assays (ELISAs) [149]. The integration of multi-functionalized CPMV particles in sandwich immunoassays improved their detection limit for SEB [151,152]. Fluorescent reporter molecules installed on the capsid surfaces produced significantly stronger signals compared to equal input amounts of conventional fluorescently labeled tracer antibodies [152]. A highly elaborated immunoassay based on CPMV combined with gliding microtubules (MTs) for the detection of SEB was realized two years later [151]. MTs carrying SEB-specific capture antibodies glided on kinesin-functionalized glass surfaces (Fig. 4 a). Binding of SEB down to a detection limit of 0.5 ng/mL was visualized via fluorescently labeled CPMV-anti-SEB tracers. The kinesin-MT nanomachinery used in this approach might be a first step towards novel lab-on-a-chip devices for the detection of targets in complex solutions.

Another powerful enterotoxin is secreted by *Vibrio cholerae* genotypes hosting a prophage that carries the genes encoding cholera toxin (CT) [153,154]. Due to the high pathogenicity and global spread of such *V. cholera*, and the dangerous diarrhea evoked by the toxin, a sensitive and specific monitoring of environmental and human samples is crucial for preventing cholera outbreaks. Genotyping of bacteria in the samples can be carried out by hybridizing biotinylated, PCR-(polymerase chain reaction)-amplified *V. cholera* DNA fragments to suitable DNA probes on microarrays, followed by fluorescence-based detection. Biotinylated target DNA accumulated on a CT-indicative probe spot is then typically labeled by a biotin-specific conjugate of a fluorescent dye and an avidin-like protein (e.g. NeutrAvidin, NA), as shown in Fig. 4 b. If, instead of a one-by-one conjugate, CPMV particles co-equipped with up to more than 40 fluorescent dye (cyanine 5, Cy5) and additional biotin-binding NA molecules were used for target DNA detection, the assay sensitivity was strongly increased, whereas duration and costs decreased. Due to their dual functionalization, the virus particles acted simultaneously as recognition element (by biotin-NA interaction) and as signal-generating unit (by fluorescent Cy5) [155]; see Fig. 4 b. A further variation in constructing sensors with double-modified CPMV employed it in combination with analyte-capturing moieties and a conductive 3D surface network of metal grains (gold nanoparticles), deposited on sensor chip arrays with multiple electrode pairs with a common drain [156] (Fig. 4 c): Here, the multivalent nanoshell mediated both target recognition and electronic signal transduction, because binding of the corresponding analyte resulted in reliable changes in the network conductance. Related setups taking advantage of signal amplification by molecule ensembles on multivalent VNP surfaces can be transferred to other plant virus types and analytes. Recently, for example, genetically engineered TMV-based nanorod VLPs with thousands of receptor peptides genetically fused to the CP subunits were incorporated in a capillary microfluidic sensor system for antibodies [157]. In the label-free impedance detection setup, the VLPs served as dense receptor layer on the sensor surface, allowing detection of target antibodies in the pmol range within several minutes (Fig. 4 e). Similarly employed TMV-VLPs in an optical microdisk resonator [158] created an ideal platform for the real-time detection of antibody binding events by inducing changes in the refractive index. Such sensor designs enable direct sensing under circumstances where labeling is too imprecise, costly or simply not possible.

4.2. Enhanced biosensing by way of immobilized enzymes

The sensors mentioned so far all accomplish analyte recognition by high-affinity binding to biomolecules ranging from immunoglobulins to reverse-complementary nucleic acids, the latter then undergoing a further hapten-biomolecule interaction. Another important detection route exploits the substrate specificity of enzymes, which typically do not only assemble with their targets, but also convert them into one or more products. These may be either directly detected e.g. by label-free electrochemical setups, or subjected to follow-up reactions that might involve further enzymes in cascade reactions to generate signals suited for efficient read-out. Among other systems, colorigenic substrates may evidence the analyte's presence by simple color formation (colorimetric analysis), or halochromic substrates may indicate pH changes. During recent years, plant VNPs came out to be excellent carrier templates for enzymes [82,83,159–165], stabilizing different enzyme species over repeated uses up to a year or even more [163,166] in comparison to conventional immobilization matrices. They also exerted further positive effects on sensor layouts with high relevance for biomedical applications, with some examples described in the following.

Biotin-linker-fashioned TMV nanotubes used as adapters for enzyme immobilization in microtiter plates have enabled an exceptionally efficient coupling of enzyme conjugates to utmost surface-densities, leading to 45-fold higher catalytic activities in comparison to TMV-free control samples with the same input of enzymes [163]. Thanks to the strong and specific biotin-streptavidin ([SA]) interaction it was possible to install the cooperating two-enzyme system of [SA]-conjugated glucose oxidase (GOx) and horseradish peroxidase (HRP) for the colorimetric detection of glucose [163], or [SA]-penicillinase (Pen) for antibiotic detection [164] (see also Fig. 4 d) on TMV nanoscaffolds in a straightforward manner. This ensured optimal steric accessibility of the active catalytic sites, and had astonishingly stabilizing effects on the enzymes. The protein surface of the plant viral nanotubes seems an advantageous physicochemical environment that can considerably increase shelf-life and reusability of enzymes for months or more, as recently found for penicillin detection by capacitive field-effect EIS sensors with TMV nanocarriers, which retained almost full sensitivity through one year [166]. The performance of amperometric, electrochemical biosensors for glucose detection was enhanced by TMV nanoadapters in several aspects: here, the plant VNPs conveyed highest sensitivities, extended linear detection ranges (with lower detection limits) and fastest response times [167]. These findings collectively indicate that an integration of VNPs with electronic transducers is a highly promising concept for the fabrication of durable, versatile biosensors interesting e.g. for on-site monitoring of health-relevant analytes with well-calibratable handheld devices.

In addition to serving as adapters in enzymatic detection setups, plant viruses can be used in layouts designed for enzymatic product conversion in medical applications. One decade ago, *Pseudozyma (Candida) antarctica* lipase B (CalB) could be genetically fused to CP subunits of PVX [160]. CalB is an efficient enantiospecific catalyst able to convert pyrrolidines, which are important precursors in the synthesis of selective neuronal nitric oxide synthase inhibitors [168]. Although the activity of the virus-anchored enzymes was decreased in this case, it exemplifies possible uses of plant VNP carrier templates for enzymatic processing of therapeutic drugs, or for delivering biomedically intervening enzymatic reactions to potential effector sites in vivo (see section 5 below). Another seminal work applied flexuous plant viral nanocarriers for an enzymatic production of 'functional food' components. To this end, zucchini yellow mosaic virus (ZYMV) was firstly coated with ZYMV CP-specific antibodies, which then served as interlayer mediating selective binding of enzymes fused to an antibody-binding peptide (Z33, derived from SPAB, i.e. the B-domain of *S. aureus* protein A). First, Z33 was genetically fused to 4-coumarate:CoA-ligase 2 (4CL2) [82], and later, the system was expanded to a combination of 4CL2

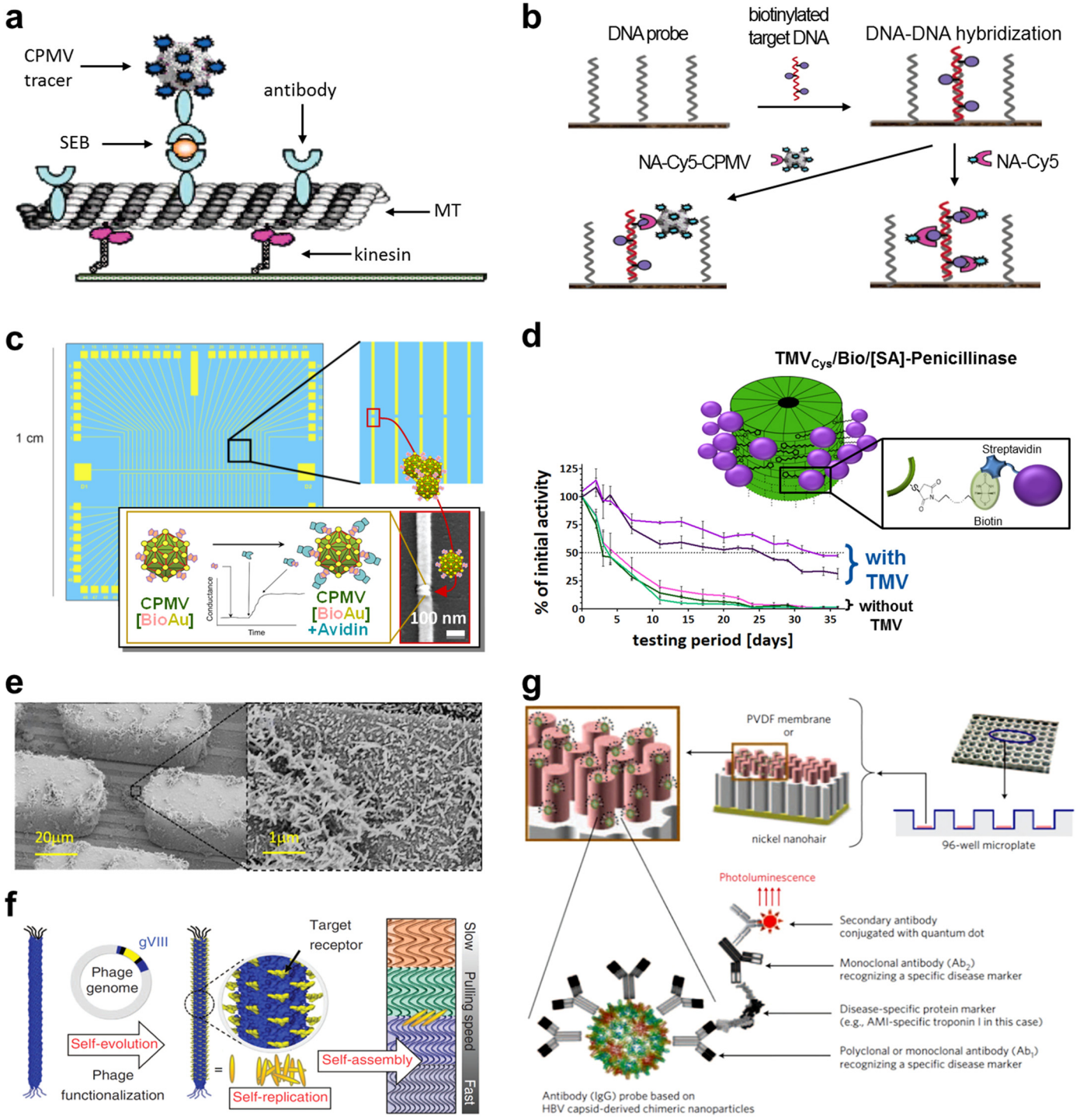

Fig. 4. Virus-assisted sensor layouts. a) Mobile sandwich immunoassay on gliding microtubules for the detection of SEB. Adapted with permission from reference [151]. Copyright© 2008 by the American Chemical Society. b) CPMV-based microarray for genotyping of *Vibrio cholerae*. Adapted with permission from reference [155]. Copyright© 2006 by the American Chemical Society. c) CPMV employed as dual-functionalized nanoparticles in the gaps between multiple electrode pairs on an electron sensor chip, leading to conductance changes upon recognizing avidin (blue) and related proteins by the exposed biotin moieties (rose) due to the interspersed gold beads (yellow). Adapted with permission from reference [156]. Copyright© 2011 with permission from Elsevier. d) Antibiotic detection with streptavidin-conjugated penicillinase ([SA]-Pen) on functionalized TMV adapters. Adapted with permission from reference [164]. e) Microfluidic sensor surface with a TMV-VLP nanoreceptor layer. Adapted with permission from reference [157]. Copyright© 2017 by the American Chemical Society. f) Self-assembly of engineered M13 phages into colored arrays composed of quasi-ordered bundled structures. Adapted with permission from reference [172]. Copyright© 2014 by Springer Nature. g) Scheme of a 3D diagnostic assay based on virus nanoparticles and nickel nanohairs for the detection of the acute myocardial infarction marker troponin. Adapted with permission from reference [179]. Copyright© 2009 by Springer Nature. For a more detailed description, see also original work.

and stilbene synthase (STS) on the backbone of potato virus A (PVA) [165]. These enzymes catalyze consecutive steps in the resveratrol synthetic pathway. Resveratrol, an abundant component e.g. in red wine, is being regarded a 'nutraceutical', despite some controversial reports on its potential therapeutic benefits. Its anti-oxidative, cardio- and neuroprotective, anti-inflammatory and certain anticancer effects are subject of clinical trials with several promising, but also conflicting data obtained [169]. Hence additional studies will have to delimit potential

indications where patients might profit from resveratrol. Therefore a cost-saving way for the biotechnological resveratrol production could, on the one hand, contribute to more conclusive clinical trials based on the pure compound, and - in the case of convincing data - also allow its fermenter production for future healthy diet. In this regard, filamentous plant VNP carrier materials such as interlinked 'nanonets' [162] might offer new prospects for a convenient biotransformation of precursors into resveratrol by way of efficiently immobilized enzymes.

4.3. Best-practice approaches with non-plant viruses

Some very promising and fascinating approaches have not been realized with plant viruses so far, but with bacteriophages or animal viruses. The largest variety of nanomaterials and biosensors has been realized in combination with filamentous phages such as M13 and fd, as reviewed thoroughly [17,170,171]. The frequent use of these phages is mainly due to their easy cultivation and handling, stability, flexibility, size (880 nm in length, 6 nm in diameter), and the possibility to display different peptides or short protein fragments on pre-selected, distinct regions of the capsid, as defined by genetic fusion with the respective CP species (CPs: pIII, pVI, pVIII, pVII or pIX; see e.g. [170] for an overview). Since the phages rely on their host cells for assembly and do not self-organize in vitro, appropriate bacteria-infectious cloning vectors are employed for the in vivo production of single- or oligofunctionalized phage particles in *E. coli*. If fashioned that way with analyte-binding peptides and, optionally, signal-generating molecules (or attachment sites), these phages can serve as antibody substitutes in a multitude of distinct detection setups. Combinations of phages and analytical devices such as quartz crystal microbalance (QCM) or surface plasmon resonance (SPR) sensors were successfully used to selectively detect human pathogens, for instance *Salmonella typhimurium* or *Listeria monocytogenes* (comprehensively reviewed in [17]).

One might assume that without a secondary modification, target-binding viruses may serve as sensing elements in certain label-free readout systems, but cannot be used as complete sensors themselves. However, they can - as recently proven: M13 phages were genetically tailored to display a tryptophan (W) – histidine (H) – tryptophan (W) sequence in their pVIII CP. A simple pulling technique led to self-assembly of these M13 variants into a structural color matrix on a flat support, due to their liquid crystalline-like behavior [172]. Depending on the pulling speed, the phages arrange into various bundle-like structures with differences in bundle thicknesses and distances [173], and thereby distinct light scattering events per area (Fig. 3 f). Binding of target chemicals leads to a change in the material's refractive index, causing easily detectable color changes of the respective chip areas [174,175]. Colorimetric sensors of this type were developed into portable point-of-care testing devices in form of a handheld smartphone camera and an adapted software (*iColour Analyser*) [172]. This allowed detection of e.g. antibiotics and endocrine-disrupting chemicals (EDCs). Both are major environmental health threats, the first well-known for provoking resistance of pathogenic bacteria to medical treatments, the latter due to their structural relation with natural hormones, which may exert deleterious effects on various organisms [174,175]. Until now, predominantly phages have been used for these special kinds of colorimetric setup, but the design should be transferable to helical plant viruses. These might be advantageous especially for multiplex analyses if various types of receptor elements have to be combined on individual particles.

After this excursus to phage-enabled biosensors, we also like to report on an intriguing detection system based on hepatitis B virus (HBV)-derived chimeric nanoparticles. It exemplifies a cutting-edge combination of hierarchically refined surface-enhancement by both inorganic and biotemplate nanostructures, and a conventional indirect sandwich immunoassay approach. This resulted in an exceptional detection sensitivity with immense application potential: One of the most common causes of death worldwide is acute myocardial infarction (AMI) [176–178]. Early diagnosis and immediate treatment are essential to improve the survival rate of patients with AMI. The reliable and sensitive detection of cardiac biomarkers as indicator for an acute heart attack is a vital goal for clinical diagnoses. Currently, the most specific markers are troponin I and troponin T [178]. To enhance the detection sensitivity for troponin I, Park et al. developed a 3D assay system based on a nickel 'nanohair' structure, which increased the device's inorganic surface area on a first level, and attached dual-modified HBV particles [179] for a second level of surface enhancement by the polyvalent soft-matter bionanostructure. The genetically engineered viruses were designed to expose the domain B of staphylococcal protein A (SPAB) and a hexahistidine sequence so that these chimeric viruses obtained a dual affinity for antibodies (IgG; here anti-troponin antibodies) and nickel. The virus-presented IgGs came out to have maximum accessibility to troponin, which was efficiently bound and detected in a triple sandwich assay by a second antibody and a signal-generating final antibody species (see Fig. 4 g). The combination of the hair structure and virus particles boosted the sensitivity of the system to the attomolar level, which is about six to seven orders of magnitude lower than that of current ELISA assays [179].

4.4. Plant virus-based calibration standards for diagnostics and more

Plant virus derivatives may not only directly participate in biosensor layouts, they have also proven to be versatile calibration particles in different medically relevant assays. First, the precisely shaped VNPs are efficient size rulers for nanometrology, applied in electron microscopy calibration since decades, but these days also in laser-assisted, localization-based widefield superresolution microscopy [180,181] with its enormous burst of research and diagnostic applications (see e.g. [182] for a recent review), and also for scanning near-field optical microscopy [183] that can grant high-resolution access to individual labels on single virus structures. Second, the multitude of plant virus-hybrid vaccines and other heterologous epitope-displaying plant VLPs [11,12] are safe and reliable reactivity control preparations for biomedical immunodetection, be it ELISA layouts or single-use test strips. Third, the encapsidation potential of several plant virus coat proteins for heterologous nucleic acids (e.g. [184], and references therein) is highly attractive for producing animal virus mimics as positive internal controls and quantification standards for RNA- or DNA-based detection [185–187]. Fragments of target nucleic acids derived from pathogenic viruses are assembled into hybrid particles with harmless plant viral proteins, and diluted into test samples as in-tube controls for the efficient nucleic acid release from possibly contained virus cages, and detection e.g. by (RT-)PCR or isothermal amplification methods.

The above examples in this section demonstrate that during the past few decades, plant viruses and certain animal and bacteriophage counterparts have acquired new reputation as advantageous nanostructured materials with countless medically relevant potential applications in vitro. In the future, even more attention may shift to the development of highly sensitive real-time biosensors with viral adapter templates. Their profits lie in lower detection limits, increased durability and reusability, and enhanced reliability, in conjunction with reasonable costs, an eco-friendly fabrication, and a purely 'vegetarian', i.e. health-risk free origin. This aspect gains superior importance in the next sections of this review, which deal with plant VNP preparations getting into direct contact with live cells and organisms after tissue engineering, implant preparation or intravital delivery.

5. Applications at the human-artificial interface: plant virus-enabled tissue engineering in hydrogel matrices

5.1. Extracellular matrix (ECM) properties and plant VNP mimics

Both soft and hard tissues in the human body consist of an ECM into which cells are embedded. Especially in soft tissues like skin, organs or

blood vessels, the ECM is composed of various organic polymers like proteins (e.g. collagen, elastin), glycosaminoglycans (e.g. hyaluronan, chondroitin sulfate) and proteoglycans, which are partly cross-linked and swollen in water [188,189]. Therefore, the soft tissue ECM can generally be considered as a hydrogel, which has led to numerous attempts to mimic the ECM with synthetic, bio-based or hybrid hydrogels, in fundamental research or for tissue engineering and replacement [190,191]. In this context, the interaction of the cells with their surrounding matrix is particularly interesting. Cells react to numerous stimuli such as stiffness [192] and chemical composition of their environment [193], or biochemical cues integrated into the matrix [194–196]. Hence, material properties in tissue engineering have to be tailored according to the desired tissue type and the cells present therein. Plant viruses are interesting in this context since they possess defined shapes and sizes with a multitude of addressable groups, are non-pathogenic for humans, stable in aqueous suspension, and can be modified with chemical functional groups and biologically active moieties simultaneously. Therefore, their use as agents for tailoring hydrogel properties for tissue 3D construction is straightforward as selective anchoring of ECM components, excreted by distinct cell types, by plant VNPs has been demonstrated [197]. This enables addressing peptide ligands, polysaccharides and enzymes, which are among the key players in the ECM, in order to tune the resulting tissue-like materials from soft to hard types [198,199]. In a longer run, plant VNP-enabled modification of hydrogels with suitable interaction sites may become a clue to gain control over the spatial 3D arrangement of distinct cell types and their subsequent differentiation into complex organ-like entities.

5.2. Specific approaches of plant VNP-aided tissue engineering in hydrogels

Initially, biological effects of plant viruses were analyzed in much detail by way of VNP interlayers between 2D substrates and cells, as reported in numerous publications [16,200–202]. Seminal work along that line started in the late 1990s, when ~30–40 amino acids (aa) long N-terminal fragments of a fibronectin-binding protein were expressed on the surface of the icosahedral CPMV, or the filamentous PVX [197]. Fibronectin is one of the major ECM components and, due to its multidomain structure, involved in all kinds of regulatory processes including cell and tissue development and wound healing. The following decade brought several novel applications of modified plant VNPs, demonstrating their huge potential e.g. in guiding stem cell differentiation in space and time on turnip yellow mosaic virus (TYMV)-coated substrates, as revealed by osteogenic markers [203]. In another approach, endothelial cell interactions were programmed by poly(ethylene glycol)-modified ('PEGylated') CPMV [204]. As a result, the auspicious properties of both natural and modified plant viruses prompted a handful of research groups to investigate their effects in tissue engineering, where plant VNPs were expected to simultaneously act as cell anchoring and differentiation-modulating scaffolds.

Apart from rare works on cucumber mosaic virus (CMV) [205] and PVX [206], research on plant viruses in hydrogels for 3D tissue engineering has so far mainly focused on TMV nanorods. To disperse these particles inside the gel matrix, molecularly imprinted hydrogels were prepared by cross-linking poly(allylamine) in the presence of TMV around ten years ago [207]. Subsequently, a cysteine-functionalized mutant of TMV (TMV_{Cys}) was integrated at a concentration of 0.6 mg mL^{-1} into hydrogel microparticles formed from poly(ethylene glycol) diacrylate (PEG-DA) by microfluidics [208]. After using a similar technique [209], it could be shown by fluorescence labelling that TMV_{Cys} was distributed evenly throughout the hydrogel volume. Furthermore, it was also demonstrated that Pd nanoparticles on the surface of TMV_{Cys} inside the gels maintained their catalytic activity for reduction of dichromate salts. In this approach, both a physical entrapment of TMV_{Cys} in the hydrogels as well as a thiol-Michael reaction of TMV_{Cys} with PEG-DA before cross-linking possibly accounted for the final integration of the particles into the material, although no data on the resulting linkage have been presented. In a similar approach, covalent binding of TMV_{Cys} into PEG-DA hydrogels by thiol-Michael addition resulted in an increased stiffness of the hydrogels compared to preparations which contained physically entrapped wt-TMV [210]. Here, rather high TMV concentrations up to 2% (w/v) were applied. Another strategy for binding TMV to hydrogels was envisioned in a study that combined chitosan with PEG-DA hydrogels, and used the chitosan amino groups for conjugation reactions [211]. First, a strained alkyne was coupled to the amino groups and subsequently a single-stranded capture DNA attached, by means of copper-free Huisgen cycloaddition. Finally, a linker DNA was bound to both the capture DNA and the TMV RNA partially released from the viral nanotube, to immobilize the TMVs at one end via their protruding RNA on the hydrogel surface. In this approach, the small mesh sizes in the hydrogel prevented TMV from diffusing into the gel matrix. The concept was further utilized for protein conjugation enhanced by coupling to the TMV particles compared to planar substrates [212]. Although the envisioned applications of these studies were not in tissue engineering, the results show that TMV remained intact inside the hydrogels and that its functionality was accessible for small molecular compounds, as also further evidenced recently [213]. Another strategy for TMV incorporation into hydrogels made use of β-cyclodextrin-decorated TMV in combination with azobenzene-functionalized hyaluronan, but only very weak gels were obtained [214].

A series of studies revealed that grafting of cell-binding motifs to plant viruses was beneficial for cell adhesion and differentiation [215,216]. Hence, one step further towards tissue engineering was undertaken by employing porous, bio-based alginate hydrogels containing either TMV or RGD-modified TMV [217]. TMV was encapsulated in analogy to reference [208] into the hydrogel at a concentration of 0.1% simply by mixing the precursor solution with a TMV suspension. The authors could show that the majority of TMV particles were not washed out of the hydrogels and remained intact inside, by dissolving the gels and subjecting the resulting solution to transmission electron microscopy (TEM). Also a change in mechanical properties of the TMV-containing hydrogels was observed compared to TMV-free gels in unconfined compression tests, showing a smaller linear deformation regime of the TMV-supplemented hydrogels. Furthermore, the study showed that the integration of RGD-modified TMV increased the attachment of bone marrow stem cells (BMSCs) and accelerated the early differentiation stages of the BMSCs slightly. In a further work, the same team investigated the toxicity and immunogenicity of the TMV alginate hydrogels in vivo with a mouse model for four weeks [218]. Generally, the hydrogels proved to be biocompatible without provoking the immunogenic response of TMVs not immobilized in hydrogels. Similar results were obtained with rats having a cranial defect [219]. The RGD-modified TMV-containing hydrogels additionally improved bone remodeling and maturation. In the same research group, another study made use of the reactivity of the thiols present on TMV_{Cys} for its immobilization in hydrogels based on methacrylated hyaluronan [220]. For this purpose, 0.1% (w/w) of TMV_{Cys} were immobilized in the hydrogels by the thiol-Michael reaction, which proceeds rapidly in aqueous media at slightly basic pH values. No toxic effects of these hydrogels applied for BMSC cultivation were observed over 21 days. Additionally, chondrogenic differentiation of BMSCs was more pronounced for TMV_{Cys}-containing compared to hyaluronan hydrogels, evidenced by a larger collagen type II content, a higher BMP-2 expression and a greater increase in stiffness during the cell culture times. The regulation of osteogenic differentiation of rat bone marrow stromal cells was also studied on 2D substrates coated with TMV nanorods [221]. It was proposed that the early interaction of the bone marrow cells with TMV triggered signaling pathways, which led to the expression of osteocalcin and subsequent mineralization. More recently, it was discovered that conjugation of TMV with RGD also improved bone differentiation of mesenchymal stem cells [222]. In addition, the spacing (2–4 nm) of the RGD ligands on the TMV surface turned out to be important for promoting proliferation and differentiation, presumably due to a polyvalent ligand

clustering effect. Related effects were found for the growth of neural crest-derived cell lines [223]. In order to obtain β-cells for transplantation as pancreatic islet-like multicellular clusters to treat diabetes, polystyrene supports were patterned with patches of TMV-RGD particles via piezoelectric inkjet printing. Pancreatic progenitor cells (PPCs) were then seeded onto these substrates and developed into uniformly sized cell clusters [224]. The number of studies in the field is constantly rising and covers in the meantime a broad range of topics such as a rapid production of ex vivo implant materials based on multivalent plant viral bone regeneration templates [215], the design of substrates for differential adhesion strengths and morphologies [225], or specific bone tissue regulatory pathways [226–228].

The data presented so far suggest that it is worthwhile to look further into using plant viruses for tissue engineering constructs. Interestingly, already rather small concentrations of TMV in hydrogels, such as 0.1%, do have a measurable biological effect in cell culture. However, a more comprehensive understanding of the plant virus-evoked effects will be necessary to bring the materials into clinical applications. Due to the fact that research in this field started only a few years ago, this is hardly surprising. Because plant viruses can act on different properties at the same time, such as the mechanical properties and the chemical/biological composition of the materials, possible benefits and drawbacks utilizing plant viruses in tissue engineering can only be identified by a larger quantity of systematic studies.

5.3. At the interface: VNP coatings for customizing implants

Apart from their use in fabricating tissue-like progenitors for organ replacement, plant viral building blocks became just recently quite fashionable for fine-tuning biointerfaces also between artificial implants and the adherent or adjacent cells. Such customization of surfaces bears a huge potential to increase biocompatibility and tunability of biomedical materials. Generally, due to the complexity of cell-cell- and cell-surface-interactions, it is almost impossible to design universally applicable interfaces. Therefore, efficiently tailorable, multi-functional and even personalized coatings are attracting increasing attention, to enable specific molecular communication that depends on various parameters simultaneously. For long-term interaction between implants and surrounding tissues, either an intimate conjunction or 'amalgamation' of the partner components may be intended, or an integration of the foreign material with as low as possible influence on the cells. Tuning such interactions may be possible by help of plant VNP coatings, as indicated e.g. by experiments with the icosahedral turnip yellow mosaic virus (TYMV). It was employed either with its natural surface, or modified via Cu-catalyzed click reaction with RGD peptides or oligo-ethylene glycol (OEG), respectively [229]. Cell binding studies with mouse fibroblasts revealed that OEG-modified viral surfaces retarded cell adhesion, whereas RGD-modified ones improved cell proliferation.

In the specific case of biomaterials and implants, additional effects occur on various length scales which, for example, guide the mechanosensitivity of cells and macroscopic soft and hard tissue on different levels of hierarchy (see also above section 2.4: Extended plant VNP-containing materials: from design to application). For interfaces between bones and artificial limbs, e. g. of hip joint endoprostheses, the surface relief in combination with its coating may gain special importance, which is reflected also by studies with plant VNP adapters. In ECM mimicry assays, pluripotent stem cells were tested for their potential in bone regeneration on flat and topographically structured substrates coated with either natural, or phosphate-functionalized TMV [200,221] (Fig. 5 a). Already the TMV coating by itself promoted osteogenic cell differentiation on the nanofeatures, with a phosphate-exposing TMV layer inducing a significant upregulation of osteo-specific genes. Other cell adhesion and spreading assays indicated an effect of polyvalent RGD clustering on the spreading and adhesion of BMSCs, if RGD motifs were displayed on the CP surface of TYMV capsids and processed into poly(allylamine hydrochloride) (PAH)-containing composite films [216]. Recently, an electrostatic layer-by-layer construction of fibrous TMV biofilms was developed [230]. These multilayers were assembled from wildtype TMV displaying an anionic surface charge and TMV_{Lys} displaying a cationic surface charge (Fig. 5 b). This approach provided free-standing biomembranes, which could be successfully used for the adhesion of NIH-3 T3 fibroblast cells.

6. Biomedical applications inside living organisms

6.1. Targeting diseased organs and tissues by functionalized plant VLPs (shape-assisted and ligand-targeting approaches)

In the research area of nanobiotechnology and nanomedicine, the targeting of diseased organs and tissues by functionalized virions, empty and nucleic-acid containing VLPs has gained increasing attention due to the straightforward modification and manipulation of the viral CPs and genomes. Different plant VNPs can act as drug carriers with adaptable functionality and the possibility to address specific target sites by means of exposed ligands. This is due to (i) their multivalence allowing multifunctionalization, (ii) distinct distances of the CPs of individual virus species, (iii) the particles' stability, (iv) availability in different morphologies, sizes and charges, (v) their variable loading capacities for molecules inside the capsids, and (vi) their harmlessness in view of human health. In the last few years several review articles have been published on this subject, providing a fundamental overview of possible modifications and applications of VLPs as addressable drug delivery systems [19–21,74,231–239]. The primarily investigated plant viruses are listed in Table 1, summarizing the adaptions of the VLPs to target specific cell lines, tissues or organs and even pathogens. First comparative investigations on VNP targeting in mice showed that some viruses possess intrinsic preferences to enter special organs or tissues [240,241]. After intravenous (i. v.) injection, tropism and fate of the virus particles were primarily correlated with the viruses' shapes and aspect ratios (i.e. spherical or elongated with different length-to-width ratio), influencing tumor homing in mice tumor xenografts [240], and the dispersal into different mice organs such as liver, kidney or spleen [65,241]. For instance, the filamentous PVX accumulated to highest amounts in tumor xenografts. Further, it was tested whether modifications of the capsids with target-specific molecules, e.g. peptides, could render their tropism more selective. Several studies were carried out to evaluate the effects of the RGD motif, which targets the integrins of the ECM, which are often overexpressed on the surface of mammalian carcinoma cells (including the HeLa cell line) [242,243]. Indeed, the display of RGD peptides on VNP surfaces increased their association with cancer cells. Conversely, the display of an RGD1 motif (of seven amino acids) on VLP films also promoted the attachment of different cell lines (BHK or CHO cells) [215]. However, as integrins are exposed on almost all animal cells, the clear and reliable distinction of cancerous from sound cell lines was limited [244]. Hence, superior targeting peptides with higher specificity were explored to more precisely direct the viral vehicles to their targets. An improved targeting to human skin epidermoid carcinoma, colorectal adenocarcinoma and breast cancer cells (A-431, HT-29, MDA-MB-231) was achieved with filamentous PVX exposing a peptide with high affinity to the tyrosine kinase epidermal growth factor receptor (EGFR), as analyzed by flow cytometry [245]. In arteriosclerosis treatment, the peptides CREKA and GPRPP with high affinity to fibrin seem to be good candidates for the delivery of thrombolytic drugs inside blood vessels [246]. TMV particles labeled with CREKA localized at thrombosis sites in carotid arteries after tail vein injection into mice. Complementing the aforementioned strategy of blood clot targeting, the effect of the thrombolytic agent streptokinase coupled to TMV particles (TMV-PEG_8-STK) was tested in a perfusion chamber assay [247]. This approach revealed reduced thrombus formation in the presence of TMV-PEG_8-STK. Hence, in future thrombosis treatment, VLPs addressed with peptides such as CREKA

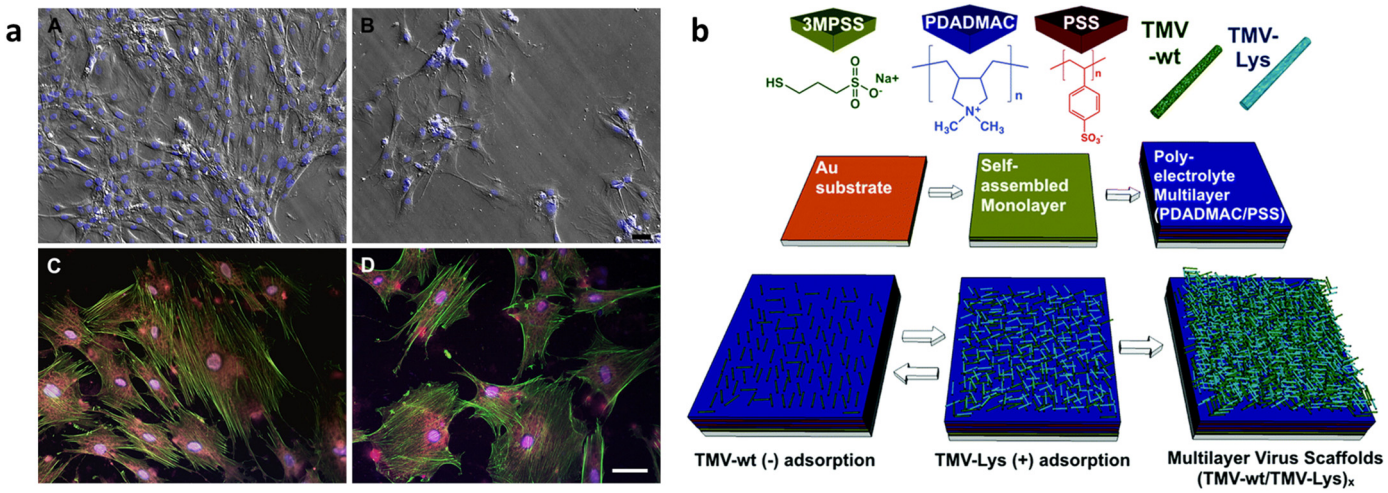

Fig. 5. TMV-functionalized supports: cell cultivation on single layers and preparation of polyelectrolyte TMV multilayers. (a) Bone marrow stem cell adhesion on supports coated (A, C) with native TMV or (B, D) phosphate-exposing TMV. (A/B): BMSCs adhering and spreading after 24 h under serum free conditions, bright-field images. (C/D): Fluorescence-based detection of the ECM protein fibronectin. Scale bar: 50 μm (all images). Blue: nucleus, green: actin, pink: fibronectin (pink). Reprinted with permission from [200]; Copyright (2010) Elsevier. b) Scheme of the sequential fabrication of multilayered TMV-containing scaffolds by way of layer by-layer (LbL) deposition (TMV-wt: wildtype TMV; TMV-Lys: lysine-exposing TMV). Reprinted with permission from [230]; Copyright (2017) The Royal Society of Chemistry.

and enzymatically activated via streptokinases are a conceivable possibility for a controlled administration of novel medical products and might once enable curative therapy via the blood stream (see also section 6.3 below). Apart from peptides, also other small molecules are applicable to equip VLPs for target recognition, such as sugars and folic acid. Glyco-decorated TMV particles displaying lactose or mannose showed differential target cell affinities: lactose-TMV (TMV-Lac) exhibited a higher affinity to asialoglycoprotein receptor-overexpressing cells (hepatocellular carcinoma cell line HepG2), while mannose-TMV (TMV-Man) showed a higher affinity to galectin-rich cells (human breast cancer cell line MCF-7) [248]. In vitro investigations of the cell viability after uptake of TMV-Lac or TMV-Man loaded with cisplatin (CDDP) confirmed the carbohydrate-specific induction of apoptosis in the different cell lines. Discrimination between two cancer cell lines, either positive (MCF7) or negative for the folate receptor (HepG2), and healthy cells (HEK) was proven as well for CCMV particles chemically modified with folic acid recognized by the folate receptor, which is overexpressed in MCF7 cells [231]. Besides tracing diseased cells for detection and, in the case of tumors, destruction, VLPs were also tested for their capability to inactivate viral pathogens such as HIV [249] or measles virus [250]. The presentation of the human viruses' epitopes or receptors could help to inhibit the spreading of the pathogens after infection, employing the plant VNPs as immunostimulants. For future usage in clinical studies, highly specific target qualities are inevitably necessary to achieve proper VNP-mediated delivery of drug cargos to their destinations inside the human bodies. Progress in cancer research may help to identify appropriate marker combinations in the treatment of various tumor types. Diseases with more characteristic marker patterns, however, may be expected to be accessible to VNP-mediated treatment first.

6.2. Plant virus-enabled imaging strategies for various diseases

Studies of plant VNPs in combination with imaging reagents fall in two categories: the first is for observing the fate of the VNP in combination with different cell lines or in vivo, the second intends diagnosis of diseases. In many cases these goals are both investigated within the same approach. Here we focus on in vivo diagnosis and/or therapy as long-term objective. Fluorescence-enabled tracing of functionalized plant VNPs is among the most frequently applied detection techniques. In addition to classical organic dyes (e.g. [240,245,259,267]) and fluorescent proteins [280], three new groups of fluorescence labels have evolved within recent years. Quantum dots (Qds) are nanoparticles well-known for their brightness, resistance to photobleaching, and their emission tuning ability by size. However, due to the fact that they are often composed primarily of heavy metals such as cadmium, toxicity is a major, widely discussed problem [281,282]. Encapsulation of Qds within a shell made up of viral proteins might be a solution to this problem as already investigated in 2006 using BMV capsids [253], and again in 2017 using CCMV CPs for encapsulation [257]. Although both studies achieved an efficient self-assembly of the CPs around the Qds and stable fluorescence of the resulting core-shell particles, in vivo-imaging studies are still pending so that the usability of the nanosystems has not yet been confirmed. Two-photon fluorescence imaging is a second technique applied with VLPs only recently. This method uses infrared (IR) light to excite appropriate dyes with a pair of photons, to emit fluorescence in the visible range. As biological materials are transparent to IR light, this results in low background and a high penetration and detection depth also in intact organisms. By coupling the dye BF3 (Fig. 6 c) to TMV nanorods and application via the tail vein, the mouse brain vasculature could be imaged (Fig. 6 d). However, after subsequent addition of freely dispersed sulforhodamine B, differences in the penetration efficiency of free sulforhodamine B and TMV-coupled BF3 were observed that hinted at a blockage of the smallest vessels by the VNPs (Fig. 6 e) [278]. Apart from two-photon imaging approaches, the good penetration of IR in biological matter can also be harnessed by directly using near infrared (NIR) fluorescent dyes, the third type of recently introduced fluorescent tracers. Free and BMV-encapsulated indocyanine green (ICG), a United States Food and Drug Administration (FDA)-approved NIR dye, were e.g. used in comparison with free and BMV-encapsulated BrCy-106 to visualize SKOV-3 tumors in mice [251]. Fig. 6 g shows that while free and encapsulated BrCy-106 resulted in a higher fluorescence intensity than the ICG formulations, the signal in the tumor was highest with encapsulated BrCy-106 (BrCy106-NHS-doped optical viral ghosts, OVGs). An additional advantage of these dyes is the potential for their use in photoacoustic imaging (PAI). In this case, part of the applied energy is adsorbed by the dye molecules and converted into heat, resulting in an acoustic wave that can be monitored by an ultrasound detector. For example, BMV-encapsulated ICG (Fig. 6 f) could be detected through 14 mm thick chicken tissue ex vivo [254]. In a dual mode imaging approach, a different NIR dye, cyanine 7.5 (Cy7.5), was used in combination with magnetic

Table 1
Examples of plant VNP-based approaches for in vivo medical applications.

Virus	Target	Stealth	Imaging	Therapeutics	Toxicity
AMCV	HIV-1 2F5 neutralizing epitope [249]			Doxorubicin [249]	
BMV	Intraperitoneal tumors (SKOV-3) in mice [251]		Au-NP encapsidation [252] QD encapsidation [253] Photo acoustic imaging [254] Near-IR imaging (indocyane green, BrCy106 [251]) Iron oxide for MRI [255]		Inflammatory response in mice [254]
CCMV	Cell cultures: HEK293[a] [256], HeLa[b] [257], Raw 264.7[c] [257]		Gd^{3+} [258] Texas Red [259], ^{125}I [259], Qdot® 605 ITK™ [257]	Upload: hydrophobic molecule [260], prediction of cargo TFP [261], sortase-mediated [262] Gene therapy [263]	Biodistribution in mice, histology, immunogenicity and pharmacokinetics [259] Cell viability [257]
CPMV	Cell cultures: HeLa[b] (targeting peptide: RGD) [242], B16F10[d] [264]			Doxorubicin [264]	Immunogenicity [264] Gastrointestinal stability [62]
PVX	Cell cultures: B16F10[d] [264], HT-29[e] [245], A-431[f] [245], BT-474[g] [245], RAW264.7[c] [240,245], MDA-MB-231[g] [245,265], A2780[h] [265], HeLa[b] [265] Tumor xenograft models: avian embryo (human fibrosarcoma HT1080 or human epithelial carcinoma HEp3 tumors) [240], mouse (MDA-MB-231[g] [265], HT-29[e] [244]) Targeting peptides: GE11 (EGFR) [245], RGD [244]	PEG [66,244,265,266]	Alexa Fluor 647 [240,245], Alexa Fluor 555 [240]	Doxorubicin [265]	Cell killing ability tests [265] Biodistribution in mice [240,241,266] Clearance [241,266] Immunogenicity [241,266] Persistence in blood [241] Hemolysis, teratogenic effect on chicken embryos [67]
RCNMV	HeLa[b] cells (targeting peptide: ADH304) and CD46 (CD45 targeting peptide) [250]			Doxorubicin [250]	
SeMV	Entry into HeLa[b], B16F10[d], BT-474[g], CB 704[g] and HMECs 704[h] cells [267]		Alexa Fluor 488 [267]	Monoclonal antibodies (D6F10, anti-α-tubulin and Herclon) [267]	Biodistribution, hematology, histopathology, clearance and behavior in mice [268]
TBSV	None				Biodistribution in mice, persistence in blood and immunogenicity [241] Hemolysis and teratogenic effect on chicken embryos [67]
TMV and TMV-derived SNP	Phosphate display for Bone Marrow Stem Cells [221] BHK[i] and CHO[k] cells (targeting peptides: RGD1, RGD7, P15, DGEA, PHSRN [215] Fibrin clots (targeting peptides: CREKA, GPRPP) [246] HeLa[b], MCF-7[g], HepG2[l] cells (targeting sugars: mannose, lactose) [248] Atherosclerosis-VCAM-1 (targeting peptide: VCAM) [269] MDA-MB231[g], MCF-7[g] cells [270] B16F10[d] cells [271] Karpas 299[m] cells [272] HeLa[b] (targeting peptide: cRGD) [243]	PEG [64,88,247,269,273] PMeOX [273] Serum albumin [88]	Gd(DOTA) [269,274,275] Dys(DOTA) [276] Dual MR and Fluorescence imaging [269] Flow cytometry studies CY5-TMV [64,269,270,273,277] Zn-EpPor [271] Two-photon imaging [278]	Doxorubicin [243,270] vcMMAE [272] Cisplatin [248] Streptokinase [247] Zn-EpPor [271] Phenanthriplatin [279]	Biodistribution in mice [269,270,279] Pharmacokinetics in mice [64,88,273] Hemolysis [64] Cell viability/internalisation (RAW264.7 [273], MCF-7, MDA-MB-231 [270], Karpas 299 [272], B16F10 melanoma cells [271], A2780 [279], A2780/CP70 [279], OV81.2 [279], 8988 T [279], MIT.LNCAP [279], HeLa [243]) Liver enzyme testing [279] Histology [64,269,279] Behavioral analysis [279]

[a]human embryonic kidney; [b]human cervical cancer; [c]murine macrophage; [d]murine melanoma; [e]human colon cancer; [f]human epidermal carcinoma; [g]human breast cancer; [h]human ovarian cancer; [i]baby hamster kidney; [k]chinese hamster ovarian; [l]human liver carcinoma; [m]non-Hodgkin's lymphoma.

resonance imaging (MRI) [276]. A dysprosium (Dy)/DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (Dys) complex, i.e. a MRI contrast agent suitable for ultra-high-field MRI, and Cy7.5 were conjugated to glutamic acid residues within the inner channel of the nanotubular TMV, while the outer TMV surface was covered with mPEG and, in the case of targeting VLPs, with the collagen-mimetic peptide DGEA (Fig. 6 a). Efficient binding to cancer cells and a negligible cytotoxicity of the VLP constructs was determined on prostate cancer cells (PC3), followed by in vivo testing for piloting to tumor xenografts in mice. Both, NIR imaging and ultra-high-field MRI proved the functionality of the dual modal imaging VLP and the highest accumulation of contrast agent in the tumor after intravital application of the targeted VLP via the tail vein (Fig. 6 b). However, in addition to further expected signals in liver, spleen and kidney, quite strong signals were also observed in the lung, which might be explained by the presence of $\alpha 2\beta 2$ integrins in the respective tissues, or might reflect agglomeration of the VLPs. Despite Dys, common gadolinium (Gd)-based contrast agents (GBCAs) for MRI have also been used in several studies to produce MRI-active VLPs [93,258,274,275,283]. While GBCAs often show low proton relaxivities, coupling of the Gd chelates to VLPs at high numbers and in close vicinities to each other increases the signal output substantially, thus much less Gd has to be used for imaging, circumventing conventionally high doses, which are known to have toxic effects. In vivo dual-modal magnetic resonance and fluorescence imaging may be especially promising also for the fast and efficient diagnostics of common vascular diseases such as coronary artery

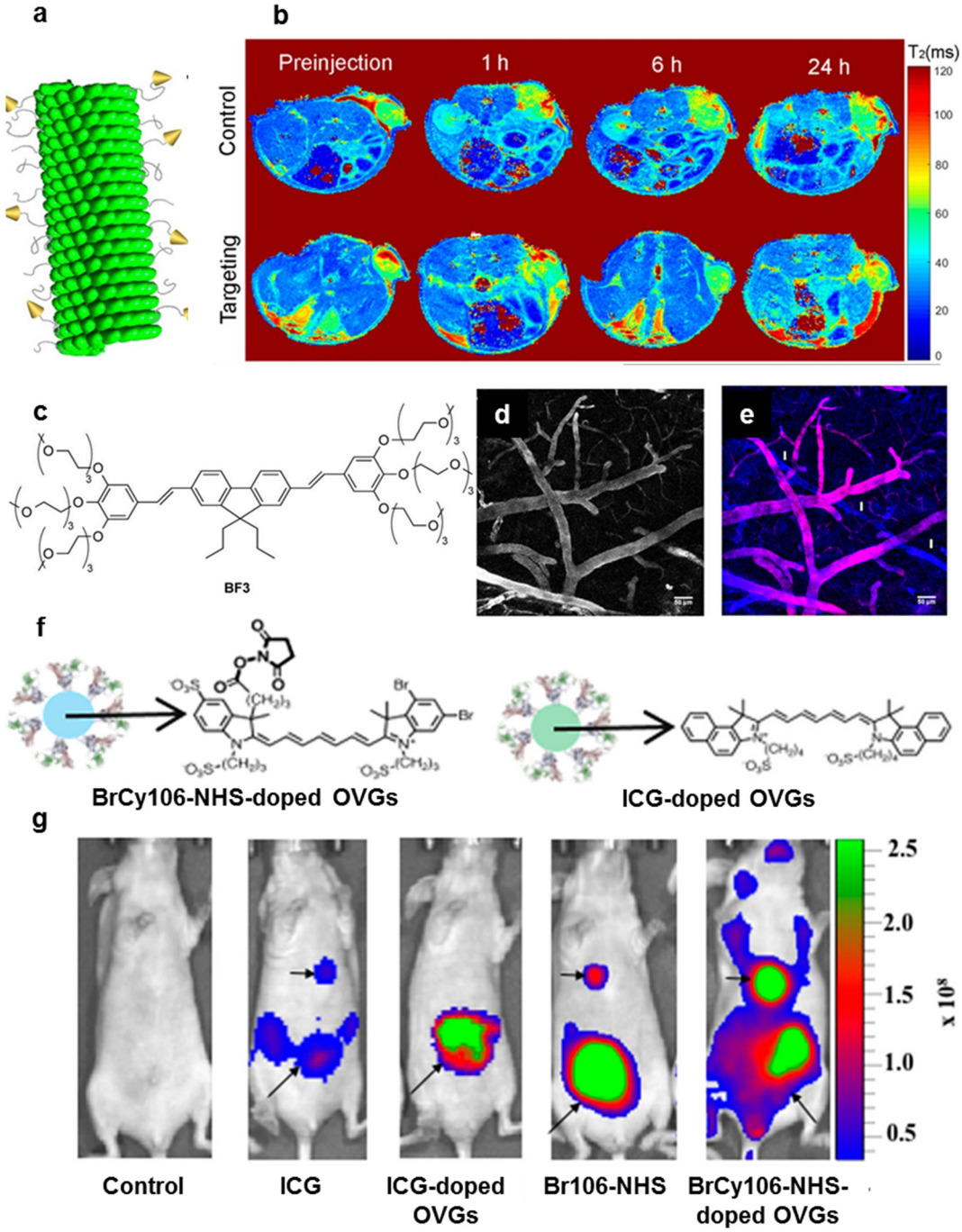

Fig. 6. Plant VNP-enabled in vivo imaging. a) Externally modified TMV with mPEG and PEG plus the targeting peptide DGEA, internal modification with Cy7.5 and Dy(DOTA) not visible. b) In vivo T_2-mapping MRI of subcutaneous PC-3 ($\alpha_2\beta_1$) prostate tumors in athymic nude mice ($n = 3$) obtained before and 1, 6, and 24 h after intravenous injection of Dy-Cy7.5-TMV-mPEG (control group), or Dy-Cy7.5-TMV-DGEA (targeting group). Reprinted with permission from [276]. Copyright (2017) American Chemical Society. c) Structure of the two-photon fluorophore BF3. d) Intravital imaging of the mouse brain vasculature 1 h after intravenous injection of TMV-BF3 particles. e) Same observation window but after an additional injection with free sulforhodamine B (red fluorescence, TMV-BF3 fluorescence shown in blue) adapted from [278]. Copyright © 2016 Niehl, Appaix, Boscá, van der Sanden, Nicoud, Bolze and Heinlein. f) Scheme for nano-encapsulation of BrCy106-NHS and indocyanine green (ICG) by the coat protein of brome mosaic virus (optical viral ghosts, OVG). g) Coronal in vivo near infrared fluorescence imaging of intraperitoneal SKOV3 tumors 2 h post injection control (RNA assembly buffer) and with various optical agents in immune-deficient mice. Adapted with permission from [251]. Copyright (2017) American Chemical Society.

disease and stroke [269]. TMV nanoparticles functionalized with NIR dyes and chelated Gd ions specifically recognized vascular cell adhesion molecule (VCAM)-1 on endothelial cells of atherosclerotic plaques [269], which could gain high importance for screening programs and preventive measures. Taken together, VLP-based MRI contrast agents seem to be very promising for imaging diseased tissues in vivo, either due to the nanoparticles' inherent tissue distribution and tumor homing properties, or through installing selective disease- or cell type-specific targeting molecules.

6.3. Intravital therapeutics and combined theranostics approaches

Since several excellent review articles of the therapeutic potential of plant VNPs have been written recently (e.g. [19,20,234,236,284-286],

and further work referenced above), this section will outline only some major routes of research and development in this field, and mention a few corresponding studies. Among the most promising VLP applications is the targeted drug delivery of cytotoxins or therapeutic enzymes, which, under ideal conditions, are inactive during their circulation in the blood stream and inert against healthy or non-target tissue. This may be realized by the encapsidation or immobilization of such molecules either inside, or on the surface of VLPs, respectively, intravenous administration, and release of the drugs only after entry of the VLP vehicles into the cells of diseased organs or tissues. In-proof-of-principle experiments for cancer therapy, the cytotoxin doxorubicin (see examples in Table 1) was investigated either immobilized on VLP surfaces [264,265], or encapsulated inside [243]. For both strategies, the desired cytotoxic effect was observed after cellular uptake by endocytosis of the functionalized VLPs in different human cancer cell lines (B16F10, A2780, MDA-MB-231). Similarly, cisplatin [248] and phenanthriplatin [279] were loaded into the inner channel of TMV particles for cell type-specific release of this payload, thereby protecting non-target cells from the encapsulated toxin. An alternative concept made use of a drug conjugate designed as a noneffective pro-drug, but with a specific cleavage site for the release inside cancer cells. This was demonstrated for the antimitotic valin-citrulline monomethyl auristatin E conjugated to TMV particles, resulting in effective killing of non-Hodgkin's lymphoma cells after protease-mediated release from the capsids via the valin-citrulline cleavage site [272]. A further perspective of applying functionalized VLPs is the combination of drug delivery and imaging at the same time, i.e. therapeutics and diagnostics also called 'theranostics'. TMV particles modified with a photosensitizer allowed simultaneous recording of B16F10 melanoma cells via fluorescence and their disruption due to the generation of reactive oxygen species upon exposure to light with a wavelength of 430 nm and an energy of 18.1 J/cm^2, resulting in apoptosis and necrosis [271]. Besides the development of VLPs for cancer therapy, they were also tested for their suitability in cardiovascular diseases such as thrombosis. In this context, TMV particles were activated with a streptokinase to degrade fibrin clots in an in vitro assay [247]. The TMV-streptokinase construct prevented the formation of new clots efficiently, providing a novel platform for enzyme-mediated thrombosis treatment (see also section 6.1 above). As several plant VLPs are able to encapsidate foreign nucleic acids, a prospective therapeutic application of plant VLPs is also gene therapy. This was tested for CCMV in mammalian BHK cells for the delivery of recombinant RNA, which was protected against degradation by the capsid proteins before its release [263]. Due to the exceptionally versatile application and modification possibilities, VNPs offer wide therapeutic treatment options, although clinical testing and approved uses in human therapies are still pending.

7. Potential toxicity and further risks of plant viral NPs taken up into living bodies

It is quite generally accepted that plant virus-derived VLPs are safe for the use in humans, if their inherent immunogenicity is considered and shielded in the case of intravital high-dose applications, as outlined below (and reviewed in detail e.g. in [287]). Although detectable in almost everyone as a result from their uptake through vegetable and plant products, plant-infective viruses containing their full genomes are not known to cause any animal disease, but are even discussed to have commensalic properties ([62], and references therein; [288]). Their lack of infectivity is primarily due to the different architecture of animal and plant tissues. Intercellular cytoplasmic connections (plasmodesmata) crossing the compact cell walls of plants are the main, continuous cell-to-cell movement routes of plant viruses, also allowing entry into the solute stream of sieve tubes in the vascular bundles where long-distance traveling into new leaves typically occurs. In animal tissues, the respective viruses often passage the cellular plasma membranes (lacking appropriate channels) after receptor-mediated docking, through fusion with the outer viral envelope membrane, or via endocytotic pathways in the case of non-enveloped viruses. In turn, release of newly produced viruses into next cells or organs often involves budding or exocytosis, respectively, apoptotic cell lysis or newly generated extracellular compartments [289]. The vast majority of plant viruses, however, are non-enveloped and thus lacking an outer membrane so that they cannot exploit membrane fusion-based mechanisms at all; lytic release from cells of plant tissues is not known to exist. Instead, these viruses rely on mechanic delivery into plant host cells e.g. by sucking insects, and systemic transport from cell to cell and in the plants' vascular bundles. This is aided by specific viral proteins, e.g. 'movement proteins' (MPs) dilating the plasmodesmata channels [48]. Hence such viruses cannot establish an infection, accumulate or spread in animal tissues, with the exception of a few enveloped plant viruses that indeed are propagated additionally in their insect transmission hosts (with some evidence for replication of certain plant viruses in plant-adapted fungi as well) [48,290]. In contrast, the long-term co-evolution between most animals and their food plants may account for a 'peaceful coexistence' without common pathogenic viruses. Pharmacokinetic studies show that plant viruses are cleared from the body relatively fast with half-lives in the range of several minutes, with spherical VLPs exhibiting longer circulation times than elongated ones as discussed in [241]. However, anisotropic VLPs show intrinsic tumor homing abilities [240,279], advantageous for medical approaches.

Nevertheless, uptake of large amounts of plant VNPs directly into the blood, or their presence in high concentrations in implanted hybrid hydrogel formulations is not a natural situation. In the course of such applications, one of the disadvantages of viral and other protein-based nanoparticles is their immunogenicity [241,254,259,266]. This results in faster clearance and precludes treatments involving repetitive administrations. For this reason, there has been a number of investigations on the effects of unmodified as well as PEGylated, poly(2-oxazoline)-modified ('POXylated') and also albumin-coated naïve plant viruses mainly on mice in recent years [64,88,266,273]. The persistence time of VNPs inside live organisms can be increased significantly by applying stealth molecules, hiding the virus particles from the immune system [64,266]. It was also shown that even the chain length of the attached polymer has a significant influence on the fate of a VLP by changing its interaction with blood proteins [291]. The biodistribution of different VLPs has been investigated applying different analysis techniques. On the one hand, fluorescently labeled VLPs have been used allowing observing their distribution directly in the living organism, in addition to quantitative evaluations of the fluorescence intensity of excised organs [64,240]. Alternatively, radioactively labeled VLPs using ^{125}I have been employed [259]. The distribution of differently labeled VLPs within healthy mice seemed to be usually comparable, independent of the labeling method, with the single exception that in contrast to its unlabeled counterparts, ^{125}I-labeled CCMV accumulated also in the thyroid in addition to liver and spleen. The biodistribution of unlabeled VLPs was determined by RT-PCR for *Sesbania* mosaic virus (SeMV) [268] and by DAS-ELISA for TBSV (tomato bushy stunt virus) and PVX [241]. Probably due to the higher sensitivity of the latter method, PVX proteins could be detected for up to seven days in contrast to 48–96 h with the other methods. While VLPs seem to not specifically target to the brain, an earlier study on the biodistribution of CPMV administered orally or i.v. to mice revealed the presence of low amounts of VLPs in brain tissue, making targeting to the brain possible [63]. Ex vivo analyses indicate that plant VLPs do not induce hemolysis or clotting of blood (e.g. [64,67]) and that no histopathological differences are induced in brain, liver kidney, lung or spleen [64,259,268,269,279]. Also liver enzyme analysis did not result in any significant effects of naïve VLPs [279]. However, when combined with therapeutics, imaging agents and other modifications, great care has to be taken as these medical effector materials are often cytotoxic and can result in necrosis or differences in cellular enzyme activities [279]. So far, many investigations of combined

tumor targeting and drug delivery have used only cell culture comparisons for the efficacy of a targeting process, and as reviewed in [292] for synthetic nanoparticles, different cell lines may react very differently. For interactions of VLPs with proteins/enzymes within the body, additional unforeseen reciprocal effects cannot be excluded at present and demand for further in vivo studies. The overall prospects of using plant VNPs in diagnostic and therapeutic formulations, however, seem convincing and are unlikely to affect the condition of the treated individuals: Orally or intravenously delivered plant VLPs did not exert any adverse effects on the behavior of mice as well as on their weight [268,279].

8. Outlook

As developed above, an intriguing variety of functionalized plant VNPs and 'smart' materials based thereon has become available during recent years, with many of their intended applications in biomedicine. A growing number of journals and conferences accelerate the international scientific and real-life evaluation of the different approaches. This yields increasing evidence that in many cases, tailored plant VNPs do not only keep, but surpass the initial promises, which points at bright prospects for a long-term socioeconomic impact. Consequently, companies have started to exploit the commercial potential of plant virus-assisted novel biohybrid nanostructures and production pathways [2,40,141–143] (see also section 3.2). Plant-produced vaccines, antibodies, pharmaceuticals, food additives and other 'biologicals' are frequently termed 'biorisk-free', since they cannot transmit any pathogen from mammalian cell cultures or animal hosts. This is - in addition to energy-efficient production and superior performance - another argument paving the road to the market. The International Society for Plant Molecular Farming (ISPMF) has become a well-known platform for exchanging experience and knowledge ([146], and http://societyformolecularfarming.org/). For sure, only part of the plant VNP-exploiting enterprises will turn into luscious 'growing mushrooms', whereas others will be replaced by improved concepts sooner or later. These encompass techniques for the fabrication of fully artificial, virus-resembling particles from heterologous compounds including biogenic and synthetic polymers [293–296], also named 'viromimetic assemblies' [297]. Generally, potential disadvantages or adverse effects of VLPs in specific applications still need thorough accompanying research. This includes long-term monitoring e.g. of strategies for shielding VLP surfaces to avoid immune reactions after administration of large amounts, or the robustness and thus economic viability of process chains for the mass production of non-infectious VLP types in plants.

However, plant VNPs have found their way into first approved and standardized products in applications where conventional approaches are tedious, inefficient, prone to drawbacks, or simply too expensive for being profitable, and in those fields where they offer previously unknown opportunities for clever solutions [298]. Different from several potent plant-derived therapeutic enzymes and antibodies now available as FDA-approved formulations [143,144,298,299], the field of plant VNP applications in humans is still limited to different clinical vaccination trials up to phase III, with exceptional allowance for emergency use in the case of a pandemic influenza VLP vaccine [25,143,144,298–300]. An up-to-date review is found in this themed issue [41]. For veterinary uses, the United States Department of Agriculture (USDA) is responsible and has approved further highly effective plant-produced preparations [300]. A wider spread of the different plant-made VNPs and other plant virus-enabled biopharmaceuticals on the starting blocks is soon expected, be it for intravital imaging and drug delivery [20,22,234,301], or even nucleic acid-based treatments including gene therapy [238]. However, their fabrication in sufficient amounts, according to the rules of Good Manufacturing Practice (GMP) especially for pharmaceuticals applied in humans, can only start after admission. Regulatory procedures differ between U.S., European and the various national agencies. To promote approval and enable broader uses and evaluations, sufficient acceptance by politicians and the public will be essential (for a detailed description of the state-of-the-arts, see [301]).

It is well conceivable that future research on plant viruses may bring along a plenitude of further prospects. For instance, as briefly touched in the introduction, the effector potential of plant viral proteins and nucleic acid elements in animal and human cells is still largely unexplored, but may be enormous. For comparison, only a decade ago, probably no-one would have predicted the recent, sudden burst of applications based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR-associated sequences (Cas; i.e. the CRISPR-Cas) system, originally evolved as RNA-guided defense system against foreign nucleic acids of phages and plasmids [302]. Now, countless successful applications covering genome editing of viruses, bacteria, higher plants, mouse- and human-derived organoids and animal models are published day-to-day [303–308]. The plant viral toolbox is just as likely to contain valuable elements for influencing physiology and regulatory cornerstones in pathways of animal cells. For example, many different gene products interfering with non-coding small RNA-based regulatory pathways, which play important roles in human pathogenesis [309], have evolved in plant viruses, because gene silencing is the major antiviral counter-defense of plants [310]. Due to the frequently small size of plant viral genomes, a multitude of uncommon genetic elements exists, ensuring the efficient spatial organization realization of the encoded information and its interplay with the host cell [48]. Various elements have already been shown to function in heterologous, including animal cells (see also introduction), but the vast majority remains to be tested in mammal cells, with good chances to identify potent regulators. This also might apply to plant viral proteins that determine the fate of newly multiplied viruses by manipulating the delicate interactions between cellular endomembrane and cytoskeletal systems. These and many other prospects would, however, demand for a separate large article to be worked out thoroughly.

With regard to the design of further advanced composites for tissue engineering and implants, new horizons in biotechnological biomaterials may be reached on the basis of novel synergies between the research fields on plant viruses and biomineralization. As outlined in the previous sections, plant viral materials are intensely used to develop ECM-like scaffolds for osteogenic cell differentiation and implant regeneration. A more general approach would be to tackle the complexity of ECM interfacial design from an evolutionary background of biomineralization [199], and references therein [311]. Many ECM compounds including several molecules involved in biomineralization are highly conserved even in invertebrates [198,199]. This might provide broader access to promising developments, if different molecular features of biomineralization-promoting proteins could be displayed on materials and interfaces by means of tailored plant viruses, and their properties compared.

Last but not least, the impact of plant viruses in all types of environments is considerably underrated, with recent metagenomics studies uncovering the striking prevalence of known and probably many more unknown virus species [312]. While the almost ubiquitous plant viruses can be economically and ecologically highly dangerous pathogens of crops, they are now receiving new attention as promising tools for therapeutics and prophylaxis not only in animals and humans, but also in phytomedicine. The majority of approaches in this field makes use of plant viral elements or engineered viruses interfering with the accumulation or spread of also viral pathogens, as reviewed elsewhere e.g. [313,314,315], or impairs virus transmission by insect vector biocontrol [316]. Since a few years, however, parallelizing the gain of knowledge on VNP applications in 'classical' medicine, disarmed plant viral agents have also proven efficient in the treatment of major non-viral plant diseases. Auspicious pieces of work show e.g. a convincing protection of crops against economically highly relevant root diseases caused by nematode pests,

through efficient nematicide delivery into the soil by RCNMV nanoparticles [317], or the production of antimicrobial peptide precursors directed against both plant and human bacterial pathogens by way of TMV [318,319]. Hence phyto- and human medicine have started an inspiring exchange so that future joint developments are always likely, and will be promoted further by the inspiring interdisciplinary conferences associated with this field of research.

9. Conclusions

Small, smart and sustainably produced plant VNPs might soon help to exceed current limits of several biomedical procedures. This is due to novel properties arising from combinations of the various multivalent soft-matter nanostructures with functional compounds, and from the amenability of both virions and their building blocks to supramolecular assembly, up to the formation of extensive 2D and 3D hybrid materials. The resulting non-pathogenic, biocompatible and biodegradable nanoarchitectures and matrices are efficiently loaded with effector molecules ranging from drugs and contrast agents to antibodies and enzymes. Multimodal functionality is easily achieved due to the plant VNPs' repetitive organization. The precise product layouts with effector ligands incorporated or installed at high and/or well-controlled densities often bring about both superior performance and novel capacities in comparison to current medical tools. This might advance prophylactic, diagnostic, therapeutic and combined intravital medical treatments, cell culture and tissue engineering, implant fabrication, and biodetection of health-relevant analytes. A remarkably fast increasing number of application studies indicate high reliability of plant VNP-assisted procedures, for which reason numerous companies are now farming plant virus-enabled products worldwide. Vaccines are practically relevant for veterinary purposes already, and are at the forefront of clinical testing in humans. Various further plant virus-based strategies are in the developmental pipeline and/or in the stage of animal testing. Worldwide, plant VNP applications are currently passing the bottlenecks of GMP-compliant upscaling, and approval for human healthcare and treatment.

The rational design, realization and in-depth examination of plant virus-based concepts, however, are challenging, as this depends on an exceptionally interdisciplinary network of collaborations to meet all prerequisites of durable medical developments. This article has attempted to integrate aspects and viewpoints of the different partner disciplines. From the angle of chemistry, plant VNPs are versatile carrier and adapter templates amenable to multifunctionalization, given that appropriate reaction types and partner molecules are found. From the perspective of materials sciences, plant viral derivatives may be applied at bio-inorganic interfaces e.g. of implants, and as additives in extensive materials such as tissue engineering matrices, taking advantage of interactions of the viral with heterologous compounds that also influence biophysical characteristics of the hybrids. Hierarchical arrangements furthered by viral self-assembly contribute to the large potential of material adaptation by help of plant VNPs. Process engineering has a focus on the feasibility of optimum formulations and their conversion into applicable products, from surface coatings of prostheses up to tissue supports and injectable, resorbable hydrogels. The view of molecular biologists and plant virologists intends to select best-suited virus and ligand types for the desired purpose, and to tailor them as efficiently as possible to meet all demands of the partner components. Their instrumentarium has to consider both natural constraints and bioengineering-enabled opportunities, and the relevant cornerstones of the envisaged use. This has to be defined in detail by medical scientists and practitioners, who will also delimit best-choice formulations, administration routes and treatment plans. Eventually, they will scrutinize viability, performance and significance of the plant VNP approaches, which will be decisive for the future of this young and highly attractive field of novel prospects.

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