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Viral nanoparticles as platforms for next-generation therapeutics and imaging devices

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Abstract

Nanomaterials have been developed for potential applications in biomedicine, such as tissue-specific imaging and drug delivery. There are many different platforms under development, each with advantages and disadvantages, but viral nanoparticles (VNPs) are particularly attractive because they are naturally occurring nanomaterials, and as such they are both biocompatible and biodegradable. VNPs can be designed and engineered using both genetic and chemical protocols. The use of VNPs has evolved rapidly since their introduction 20 years ago, encompassing numerous chemistries and modification strategies that allow the functionalization of VNPs with imaging reagents, targeting ligands and therapeutic molecules. This review discusses recent advances in the design of "smart" targeted VNPs for therapeutic and imaging applications.

1. Introduction: Viral nanotechnology in medicine

Advances in nanotechnology have led to the development of novel materials that can link targeting molecules with therapeutic and/or imaging reagents. Such "smart" targeted formulations promise to deliver imaging reagents and therapeutics to precise locations, producing high-contrast images and allowing treatment with higher doses of drugs while minimizing adverse effects, an important goal in the development of next-generation therapies.

Several nanomaterials are currently under investigation, including quantum dots (QDs), dendrimers, polymer vesicles, liposomes and protein-based nanostructures such as viruses.[1–4] Each of these systems has advantages and disadvantages in terms of biocompatibility, pharmacokinetics, toxicity and immunogenicity. QDs are promising as imaging tools because of their long-lasting fluorescence, broad bandwidth absorption and narrow bandwidth emission, but they are also cytotoxic.5 Dendrimers are simple and inexpensive to synthesize, but they too show *in vivo* toxicity.[6] The only platform currently approved for clinical use is liposomes, e.g. targeted liposomes containing the anti-cancer drug doxorubicin take advantage of organ avoidance and slow drug release, thus minimizing toxic side effects.[5, 7]

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Viral nanoparticles (VNPs) are virus-based nanoparticle formulations that can be used as a building block for novel materials with a variety of properties. VNPs can be bacteriophages, plant or animal viruses, and they can be infectious or non-infectious. Virus-like particles (VLPs) are a subset of VNPs expressed in heterologous systems but lacking any genomic nucleic acid, rendering them non-infectious. VNPs are dynamic, self-assembling systems that form highly symmetrical, polyvalent and monodisperse structures. They are exceptionally robust, they can be produced in large quantities in short time, and they present programmable scaffolds. VNPs offer advantages over synthetic nanomaterials, primarily because they are biocompatible and biodegradable. VNPs derived from plant viruses and bacteriophages are particularly advantageous because they are less likely to be pathogenic in humans, and therefore less likely to induce undesirable side effects.

A wide range of different VNPs is available (Figure 1), and each platform can be tailored for distinct applications. Rod-shaped VNPs, for example, can be developed as templates for mineralization and metallization reactions. Their propensity to form crystalline 1D and 2D arrays has been exploited to fabricate highly ordered hybrid materials.[8] Although VNPs are robust and stable, they are also highly dynamic structures, and many icosahedral VNPs can undergo transitions that lead to the formation of pores, thus allowing access to the interior cavity as a constrained reaction environment or storage unit. Self-assembly strategies have been developed to encapsulate materials into VNPs.[9]

In order to endow VNPs with different functions, a broad range of conjugation chemistries can be implemented.[9, 10] Ligands ranging from small chemical modifiers to peptides and proteins, and even to additional nanoparticles, can be attached by genetic engineering, chemical bioconjugation, mineralization, or encapsulation techniques (Figure 2). This article focuses on recent advances in the biomedical application of VNPs based on plant viruses and bacteriophages. Mammalian viruses (e.g. adenovirus) have also been investigated in the context of nanotechnology, but currently their main application is gene delivery rather than drug delivery or imaging.[11–13]

2. The toxicity, biodistribution and pharmacokinetics of VNPs

When developing novel materials for applications in biomedicine it is essential to understand their *in vivo* properties, particularly any potential toxic effects. Toxicity has certainly been a challenge when dealing with human pathogens such as adenovirus, even when using replication-deficient strains.[14–16] VNPs derived from bacteriophages and plant viruses are considered to be much safer because humans are not natural hosts for the parent viruses, although there have been few studies describing the characterization of such VNP platforms *in vivo*. Animal studies have been carried out with the plant-derived VNPs *Cowpea mosaic virus* (CPMV) and *Cowpea chlorotic mottle virus* (CCMV), as well as with the phages Qβ and M13. Toxicity studies were undertaken using CPMV and CCMV, these showed no clinical symptoms.[17, 18] Both CPMV and CCMV were detected in a wide variety of tissues throughout the body, but there was no toxicity despite this broad biodistribution.[18, 19] CPMV, Qβ and M13 particles accumulated primarily in the liver and spleen,[17, 20, 21] whereas CCMV particles were mostly found in the thyroid gland (but also in the liver, spleen, bladder and salivary glands).[18] The accumulation of VNPs in the liver and spleen is expected because these organs are part of the reticuloendothelial system (RES) and their function is to remove antigens, including proteinaceous nanoparticle structures, from circulation.[22]

As well as toxicity and biodistribution, it is also important to determine the pharmacokinetic characteristics of VNPs, since there needs to be an appropriate balance between tissue penetration/accumulation and systemic clearance, and the optimal balance is likely to differ for therapeutic VNPs and those designed as imaging tools. Longer circulation times allow drugs

and reagents to accumulate in target tissues, but the risk of toxicity and increased background noise in imaging applications is higher.23 Pharmacokinetic properties are dependent on the composition of the VNP, i.e. its surface charge24 and surface modifications such as PEGylation (see Section 3). It has been shown that positively charged nanomaterials have longer circulatory half lives, and this is also true for VNPs. CPMV and CCMV particles, which have a negative surface charge, also have short circulation times (half life <15 min17, 18), whereas Qβ particles, which have a positive surface charge, have a half life >3 h.21

Chemical modification and genetic engineering can be used to alter the surface charge of a VNP, and therefore change its *in vivo* properties. Surface lysine (Lys) residues are protonated under physiological conditions but chemical modification of the ε amine can reduce the overall positive charge, which is then reflected in the pharmacokinetics. For example, acetylation of the surface Lys residues on Qβ and M13 reduced their plasma half-lives.[20, 21] Many modification chemistries involve the covalent modification of Lys side chains, and thus may alter the surface charge properties. It is difficult to predict the *in vivo* properties of a particular formulation precisely, and each must therefore be evaluated on a case by case basis prior to clinical testing.

3. PEGylation to reduce biospecific interactions and immunogenicity

PEGylation, the attachment of polyethylene glycol (PEG), is a common strategy in biomedicine to reduce or eliminate biospecific interactions. PEG is a non-charged, hydrophilic polymer that is non-toxic and approved by the FDA. As well as reducing biospecific interactions and hence immunogenicity, it also increases the solubility and stability of molecules to which it is attached, thus increasing the plasma circulation time. PEGylated versions of VNPs have been generated based on *Potato virus X* (PVX), *Tobacco mosaic virus* (TMV) and bacteriophage MS2.[25–27] PEGylation has also been studied extensively using various CPMV formulations. 28–31 PEGylating CPMV reduces its interactions with cells *in vitro* and *in vivo*28–30 and effectively prevents the particles from inducing a primary immune response.[31] Shielding efficiency is dependent on the PEG chain length, but only minimal surface coverage (<1%) is needed to block CPMV-cell interactions.[29] Similar data have been obtained for PEGylated PVX formulations.27 This implies that limited PEGylation is sufficient for shielding while still leaving a large surface area and many attachment sites available for further modification with targeting, imaging and therapeutic ligands.

4. Hybrid VNP complexes for biomedical imaging

A broad range of design principles have been established to formulate hybrid VNP systems for imaging applications. VNPs can be modified with organic fluorophores for optical imaging (section 4.1), gadolinium (Gd) complexes for magnetic resonance imaging (MRI; section 4.2), and QDs or metallic nanoparticles for detection using various spectroscopic methods (see below).[9, 32] Hybrid VNPs with metal or QD cores are plasmonic composite materials with potentially useful biosensing applications, e.g. single virus spectroscopy has been demonstrated *in planta* using *Brome mosaic virus* (BMV) containing gold cores.[33] Hybrid aggregates of CPMV and QDs have also been assembled successfully.34

Hybrid hydrogel networks consisting of chimeric M13 particles displaying cell-binding peptides and gold nanoparticles have been investigated for cell-sensing applications. The bacteriophages were able to bind to cells and undergo receptor-mediated internalization, even when incorporated into the hybrid network.[35] When developing hybrid systems that combine biocompatible VNPs with synthetic materials, it is important to determine the pharmacological properties of the hybrid materials and evaluate potential toxic side effects, as these may differ from those of the individual components. For example, recent studies have revealed the

significant cytotoxicity of QD nanocrystals.[5] The goal should be to develop biocompatible and biodegradable nanomaterials for safe use *in vivo*.

4.1 Fluorescent-labeled VNPs for intravital vascular imaging

It has recently been demonstrated that CPMV particles carrying fluorescent labels can be used for intravital vascular imaging in mouse and chicken embryos (Figure 3).[30] The particles were administered intravenously, and imaging was performed over a time frame of 72 h. CPMV was internalized by endothelial cells, thus lining the vasculature and providing high-resolution images. This specificity and resolution cannot be achieved with nanospheres, a state-of-the-art platform for optical imaging (Figure 3).[30] Fluorescent VNPs could be combined with targeting ligands to develop a powerful new tool for non-invasive disease-specific imaging. Further optimization of the system, e.g. through the use of near-infrared (NIR) probes, may improve its sensitivity. In support of this expectation, CPMV has been used successfully for NIR fluorescence tomography.[36]

4.2 VNPs as contrast agents for MRI

MRI is a powerful, non-invasive *in vivo* imaging tool based on the alignment of protons in a strong magnetic field. Contrast agents are typically used to increase the brightness of the image, and hence the sensitivity of the technique. Contrast agents such as gadolinium increase the relaxation time of protons in water, lengthening the period during which nuclear magnetization (the alignment of protons) returns to equilibrium distribution.[37] Longer relaxation times can be achieved by coupling contrast agents to macromolecular carriers such as dendrimers and liposomes.38, 39

More recently, VNPs such as CPMV, CCMV, MS2 and Qβ have been developed as contrast agent platforms.[40] VNPs are rigid structures with large rotational correlation times, which increases relaxivity. Also, because they are polyvalent they can carry several hundred chelated Gd-complexes, which can be covalently attached to the exterior or interior surfaces. Gd^{3+} ions can be complexed with encapsidated RNA molecules, or bound at intrinsic metal binding sites at coat protein interfaces.[41–44] Each of these paramagnetic VNP formulations shows extraordinarily high relaxivity, indicating that VNPs are excellent candidates for MRI contrast agents, even though *in vivo* evaluation has yet to be reported.

5. Targeted VNPs

Targeted therapy requires that drugs interact specifically with disease tissue, while avoiding healthy cells. The targeted delivery of imaging and therapeutic molecules will make diagnosis more accurate and will reduce off-target effects associated with drugs. The development of phage-display technologies has led to the identification of tumor-specific markers and their ligands, as well as vascular homing peptides.[45–48] These ligands specifically recognize receptors overexpressed on unhealthy cells. The discovery of these ligands has revolutionized the field and opened the door for the development of specifically targeted reagents.

5.1. CPMV – a VNP with natural affinity for mammalian endothelial cells

Intravital imaging studies with CPMV revealed that the VNPs are specifically internalized by endothelial cells *in vivo*.[30] This interaction is biospecific and mediated by the mammalian protein vimentin, a type III intermediate filament protein predominantly expressed in the cytosol of mesenchymal cells.49 Cytosolic vimentin plays a key role in intracellular dynamics and architecture,50, 51 but it is also found on the surface of activated macrophages,[52] endothelial cells (high-level expression in tumor tissue),[53] and the endothelial venules of lymph nodes.54 The overexpression of vimentin in tumor endothelium correlates with the uptake of CPMV in tumor endothelial cells as shown in studies using the chick choreoallantoic membrane

tumor model (Figure 4).[30] The use of CPMV as a natural endothelial probe in imaging vascular disease may provide novel insights into the expression pattern of surface vimentin.

Natural interactions between CPMV and mammalian cells have also been demonstrated using an animal model of the human demyelinating disease multiple sclerosis, by targeting the VNPs to sites of inflammation in the central nervous system (CNS).[55] The VNPs localized mainly to the endothelium of the blood–brain barrier. In inflammatory lesions containing macrophages, microglia, and immunoglobulins (indicative of barrier failure), CPMV was also detected in the brain parenchyma. This provides opportunities for the targeted treatment of inflammatory disease of the CNS.

5.2. Designing receptor-targeted VNP formulations

Certain disease tissues express different receptors to the corresponding healthy tissues and such receptors can be targeted specifically using appropriate ligands to deliver therapeutic or imaging reagents. For example, the receptor for the iron storage protein transferrin (Tf) is overexpressed on several tumor cells. Delivering VNPs to tumor cells via the Tf receptor has been studied using MS2 and CPMV chemically engineered to display Tf on their surfaces.[56, 57] Similarly, the receptor for the vitamin folic acid (FA) is also overexpressed on tumor cells, and cell-specific delivery of VNPs based on CPMV and *Hibiscus chlorotic ringspot virus* (HCRSV) has been achieved by covalent modification of the particle surfaces to display FA, resulting in specific binding and internalization by tumor cells.[28, 58] The FA-targeting strategy has already been developed for targeted drug delivery (see section 6).[58]

6. Targeted therapeutic VNP formulations

VNPs have been designed for the targeted delivery of drugs and also for targeted photodynamic therapy (PDT), in which a photosoensitizer is excited by specific wavelengths of light to generate reactive oxygen species, killing the target cells. Derivatives of C_{60} ("Buckyball") are excellent photosensitizer candidates in PDT, but a major drawback is the aqueous insolubility of fullerene material. However, the solubility of C_{60} can be significantly enhanced through conjugation and multivalent display using CPMV or Qβ VNPs, which act as a hydrophilic carrier and facilitate delivery to target cells. Biochemical and biophysical data have shown that approximately 40 C_{60} molecules can be displayed per VNP, and *in vitro* studies have confirmed the efficient delivery of the hybrid material into cells (Figure 5).[59]

In a different approach, CCMV particles have been dual-functionalized and used to kill bacteria by PDT.[60] The CCMV particles were covalently modified to carry ruthenium complexes as the photosensitizers, and then targeted to the pathogenic, biofilm-forming bacteria *Staphylococcus aureus* through conjugation with specific antibodies. When the VNP-targeted bacteria were exposed to light emitting diodes (at a wavelength of 470 nm) they were killed by the resulting burst of reactive oxygen species. Bacteriophages M13 and fd have also been developed as antimicrobial agents using antibodies against *S. aureus*, in this case by targeted delivery of the antibiotic chloramphenicol.[61]

Small drug molecules can be covalently attached to VNPs, or encapsulated within them, and used for targeted therapies. The chemotherapeutic molecules hygromycin and doxorubicin have each been covalently attached to M13 and targeted successfully to cancer cells *in vitro* resulting in targeted cytotoxicity.[62] Doxorubicin has also been encapsulated in VNPs based on *Red clover necrotic mottle virus* (RCNMV) and HCRSV,[58, 63] the latter successfully targeted to cancer cells *in vitro* and internalized efficiently by conjugation to FA.[58] Similarly, dual-functionalized MS2 particles have been developed in which toxins or cytotoxic drugs such as ricin A or 5-fluoruoridine have been encapsulated by covalently linking them to the RNA stem loop operator. When VNPs were infused with the RNA operator, it diffused into the particles

and bound to all 90 copies of the coat protein dimer, allowing 90 molecules of the cargo to be encapsulated.[56] Specific targeting was achieved using antibodies or Tf, and cell delivery and cytotoxity were demonstrated *in vitro*.[56]

7. Conclusions and outlook

Recent advances in nanotechnology have led to the development of VNPs for potential applications in targeted imaging and therapy. Most of the studies conducted so far have focused on the *in vitro* behavior of functionalized VNPs, either in biochemical assays or using cultured cells, and there is only limited data on the performance of specifically engineered VNPs *in vivo*. Many of the studies reported thus far have demonstrated proof of concept, to underline the strong potential of VNPs as novel candidate materials for medical devices. The next hurdle will be to gain a better understanding of the fate and potential long-term side effects of VNPs *in vivo*. Targeting VNPs to specific receptors has been achieved in tissue culture but replicating these results *in vivo* will require greater insight into the way VNPs are processed by the body. The studies carried out thus far suggest that VNPs are indeed promising candidates for the development of next-generation targeted imaging reagents and drugs. The virus-chemistry interface remains an exciting place to be!

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Abbreviations List

(BMV)	*Brome mosaic virus*
(CNS)	central nervous system
(CCMV)	*Cowpea chlorotic mottle virus*
(CPMV)	*Cowpea mosaic virus*
(HCRSV)	*Hibiscus chlorotic ringspot virus*
(MRI)	magnetic resonance imaging
(NIR)	near-infrared
(PDT)	photodynamic therapy
(PEG)	polyethylene glycol
(PVX)	*Potato virus X*
(QDs)	quantum dots
(RCNMV)	*Red clover necrotic mottle virus*
(RES)	reticuloendothelial system
(TMV)	*Tobacco mosaic virus*
(Tf)	transferrin
(VNPs)	viral nanoparticles
(VLPs)	virus-like particles

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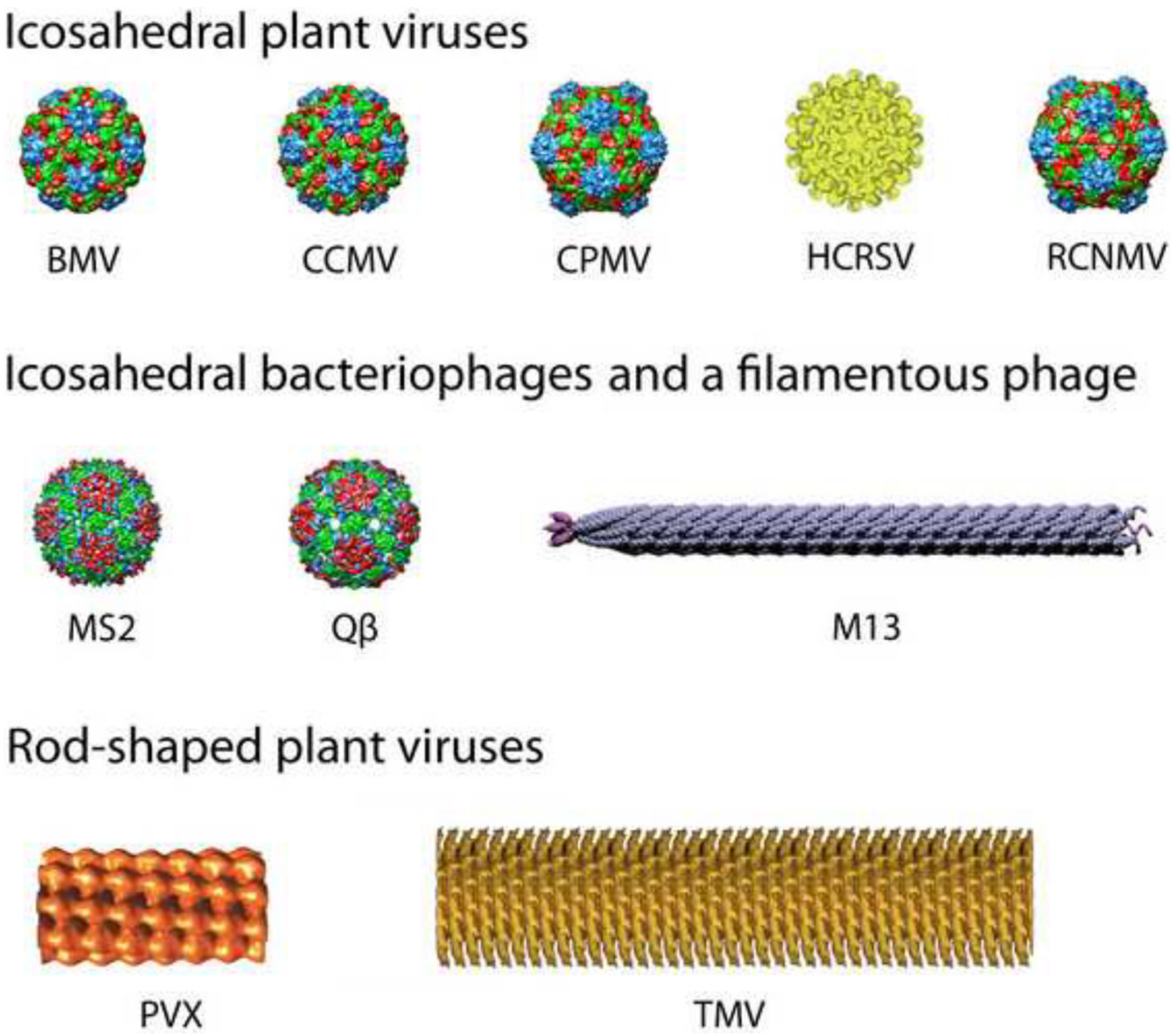

Figure 1. A snapshot of the viral nanoparticles (VNPs) currently being developed for applications in medicine

Icosahedral plant viruses: *Brome mosaic virus* (BMV), *Cowpea chlorotic mottle virus* (CCMV), *Cowpea mosaic virus* (CPMV), *Hibiscus chlorotic ringspot virus* (HCRSV), *Red clover necrotic mottle virus* (RCNMV). Icosahedral bacteriophages: MS2 and Qβ, and the filamentous phage M13. Rod-shaped plant viruses: *Potato virus X* (PVX), *Tobacco mosaic virus* (TMV). Images of the following VNPs were reproduced from the VIPER database (www.viperdb.scripps.edu): BMV, CCMV, CPMV, RCNMV, MS2, Qβ. The structure of HCRSV was reproduced from Doan DN et al. (2003) *J Struct Biol* 144(3): 253–261. M13 was reproduced from Khalil AS et al. (2007) PNAS 104(12): 4892–4897. The structure of PVX is from Kendall A et al. (2008) *J Virol* 82(19): 9546–9554. The cryo-reconstruction of TMV was provided by Bridget Carragher and Clint Potter; data were collected and processed at the National Resource for Automated Molecular Microscopy (NRAMM) at the Scripps Research Institute.

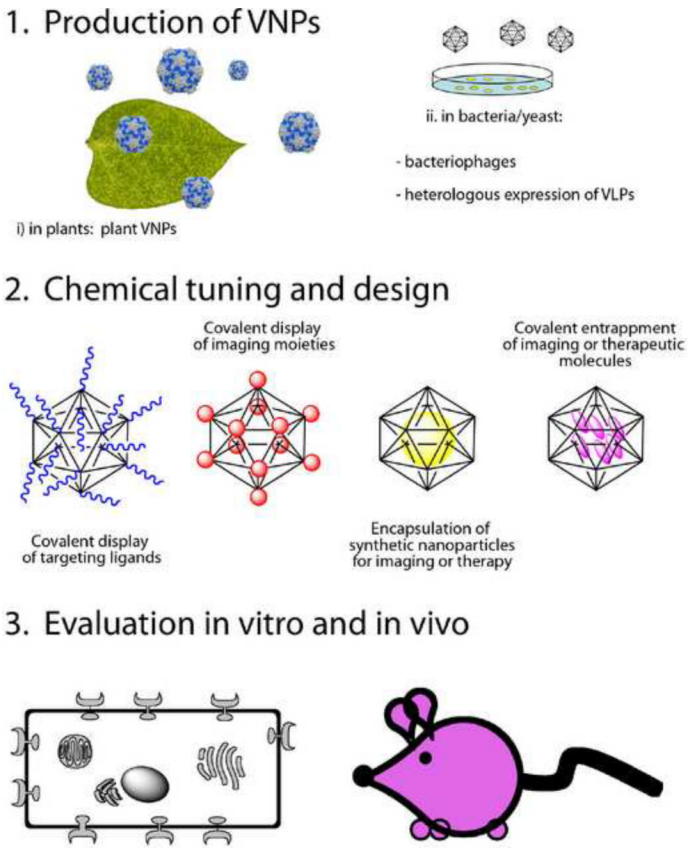

Figure 2. Viral nanotechnology – the assembly line

1. VNPs can be produced in their natural hosts: plants when using plant viruses, bacteria when using bacteriophages, mammalian cells when using mammalian viruses. Heterologous expression of VLPs in bacteria and yeast is also a common production technique. 2. Once purified, chemical tuning and design is carried out to attach and encapsulate molecules that confer different functionalities. 3. The hybrid and functionalized VNP is then evaluated *in vitro* and *in vivo*.

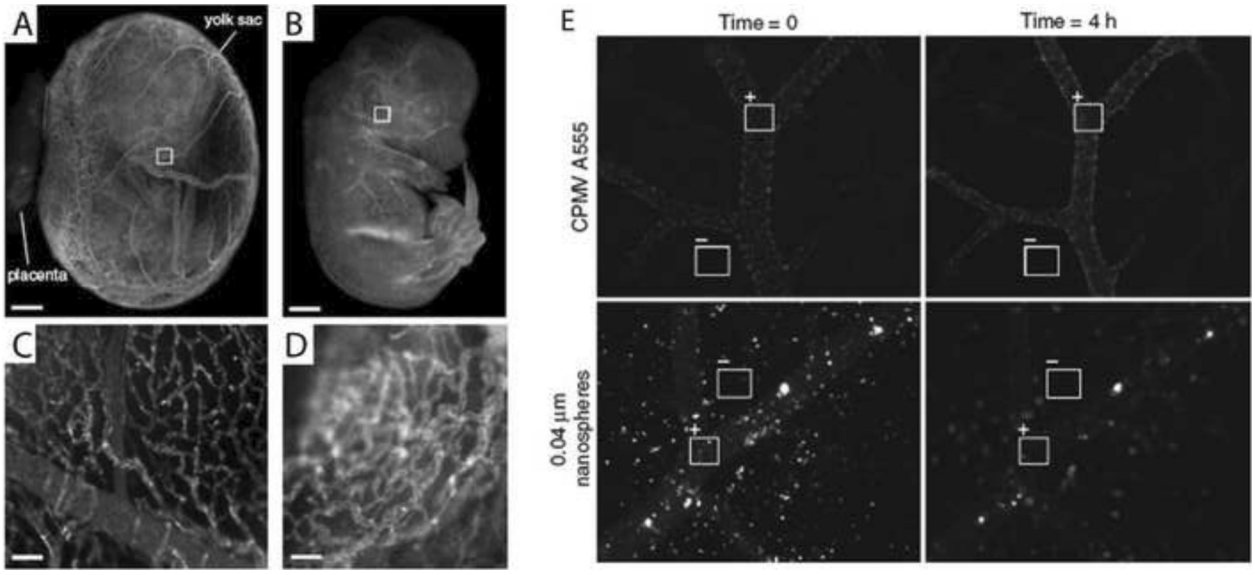

Figure 3. Intravital imaging using viral nanoparticles

Fluorescent-labeled CPMV probes (CPMV-A555) in either mouse (A–D) or chick (E) embryos. A+B. Imaging of CPMV-A555 perfused into 11.5-day-old mouse embryo with intact yolk sac (A) and removed yolk sac (B). White boxes indicate the regions magnified in (C) and (D). Comparison of intravital staining intensity over time in the chick embryo (E) using either CPMV-A555 or nanospheres as fluorescent probes. Representative images captured immediately after and 4 h after injection. Reproduced from Lewis JD et al. (2006) Viral nanoparticles as tools for intravital vascular imaging. Nat Med 12: 354–360.

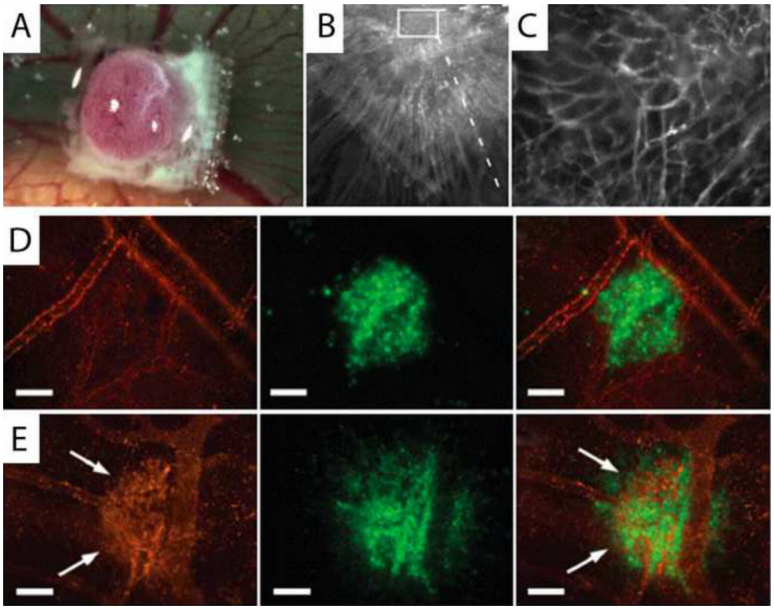

Figure 4. Fluorescent CPMV nanoparticles highlight tumor angiogenesis: intravital imaging in a CAM/HT1080 fibrosarcoma model

A) Bright-field image of HT1080 tumor on-plant on the chick chorioallantoic membrane (CAM) at 7 d. Opaque object is a nylon mesh grid used for quantification of angiogenesis. B) Fluorescence image of tumor on-plant after injection of embryo with CPMV-AF555. C) High magnification image of tumor interior shown in b; tumor microvasculature is clearly observed. D,E) Visualization of HT1080 tumor angiogenesis using CPMV-A555. D) Left, visualization of pre-existing vasculature in the CAM immediately after HT1080 tumor cell injection with CPMV-A555. Middle, GFP-expressing HT1080 tumor bolus under the surface of the CAM. Right, merge. Scale bar, 100 mm. E) Left, visualization of pre-existing CAM vasculature and neovasculature arising from tumor angiogenesis 24 h after tumor-cell injection. Middle, GFP expressing HT1080 tumor bolus. The extensive migration over 24 h indicates a high level of tumor-cell viability. Right, merge. Scale bar, 100 mm. Reproduced from Lewis JD et al. (2006) Viral nanoparticles as tools for intravital vascular imaging. Nat Med 12: 354–360.

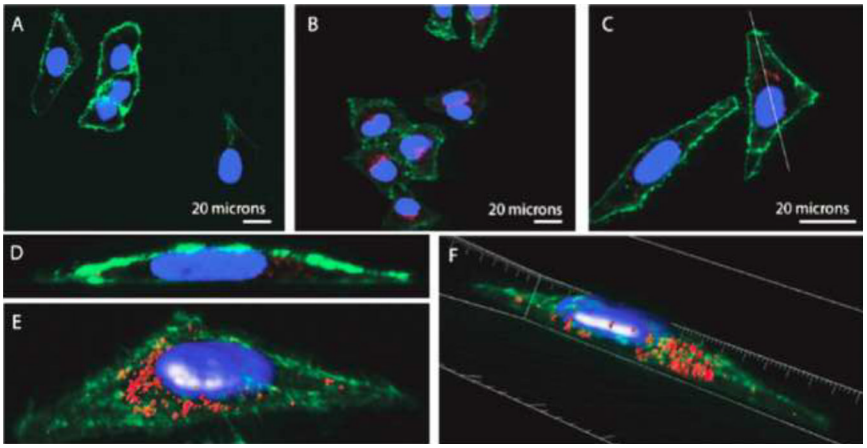

Figure 5. VNP-C_{60} conjugated in cancer cells

(A) HeLa cells only. (B–F) Cells treated with Qβ-PEG-C_{60}-A568 particles. Color key: blue, nuclei (DAPI); red, Qβ-PEG-C_{60}-A568; green, A488-labeled wheat germ agglutinin. (D) Z-section image (1.2 μm deep) recorded along the line shown in (C); step size 0.3 μm. (E, F) Same cell as shown in (D), image reconstructions using Imaris software. Reproduced from Steinmetz NF et al. (2009) Buckyballs meet viral nanoparticles: candidates for biomedicine. JACS 131: 17093–17095.