Pharm Res (2010) 27:2509–2513
DOI 10.1007/s11095-010-0251-2

COMMENTARY

Application of Plant Viruses as Nano Drug Delivery Systems

Yupeng Ren • Sek Man Wong • Lee Yong Lim

Received: 2 June 2010 / Accepted: 16 August 2010 / Published online: 2 September 2010

ABSTRACT Nano-sized drug delivery systems based on virus-derived platforms have promising delivery and targeting efficiencies. To date, much of our understanding of these systems is obtained from studies of animal viruses. Application of plant viruses for drug delivery is in the nascent stage, but it is becoming apparent that plant viral particles can be engineered to possess novel properties to meet the unique requirements of targeted drug delivery. Chemical functionalization of a plant viral particle surface can impart stealth properties to prolong *in vivo* circulation half-life and/or targeting capability to direct drug delivery to diseased tissues. The amino acid sequence of the viral coat protein can be genetically manipulated to yield protein cages of specific chemistry and morphology, while the conformation of the protein cage can be directed, via the external environment, to disassemble, then reassemble *in vitro* to exchange native viral genomic material with exogenous cargo. The purpose of this commentary is to evaluate current literature to assess the potential of nano-scale plant-virus-based drug delivery systems for the targeted delivery of chemotherapeutic agents.

Y. Ren
Shanghai Institute of Materia Medica, Chinese Academy of Sciences
Shanghai, China

S. M. Wong
Department of Biological Sciences, National University of Singapore
Singapore, Singapore

S. M. Wong
Temasek Life Sciences Laboratory
Singapore, Singapore

L. Y. Lim (✉)
Pharmacy Program
School of Biomedical, Biomolecular and Chemical Sciences
University of Western Australia (M315)
35 Stirling Highway
Crawley, WA 6009, Australia
e-mail: limly@cyllene.uwa.edu.au

KEY WORDS coat protein · drug delivery · plant virus · protein cage

ABBREVIATIONS

CP	coat protein
CCMV	*Cowpea chlorotic mottle virus*
CPMV	*Cowpea mosaic virus*
CMV	*Cucumber Mosaic Virus*
Dox	doxorubicin
HCRSV	*Hibiscus chlorotic ringspot virus*
mw	molecular weight
MTT	3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
PEG	polyethylene glycol
PSA	polystyrenesulfonic acid
PC	protein cage
RCNMV	*Red clover necrotic mosaic virus*
TEG	tetraethylene glycol
TMV	*Tobacco mosaic virus*

INTRODUCTION

Plant viruses have potential as drug delivery systems. As monodisperse particles with predictable surface morphologies, they are attractive vectors for gene and drug delivery. Although the development of plant viruses for drug delivery is a recent phenomenon, it has been shown that the conformational manipulation of the protein cages (PC) can lead to the exchange of the viral genomic material with exogenous cargo. Chemical functionalization of the plant viral particle can also impart stealth properties and drug targeting capability.

Plant viruses present predominantly as rods or spheres. The *Tobacco mosaic virus* (TMV) is an example of a rod-

shaped virus, having a straight rod protein shell about 300 nm long. Most plant viruses, like animal viruses, appear as icosahedrons. The *Cowpea mosaic virus* (CPMV) is an example of an icosahedral plant virus. It has a PC of diameter ~30 nm comprising of 180 units of coat proteins (CP). The PC maintains its integrity even under harsh conditions, e.g. 1 h exposure at pH over a broad range of 3.5 to 9, or when subject to chemical modifications, such as conjugation with polyethylene glycol (PEG) (1). However, a high pH (>7.0), together with low ionic strength (I=0.1), may cause 2-nm openings along the 3-fold axes of symmetry (2). The CP assembly into the stable icosahedral PC has been shown to occur *in vitro*, providing opportunities for the loading of exogenous cargo and the development of plant viruses into nanoscale platforms for drug delivery.

Animal viruses, with their inherent cytoinvasive property, have provided significant insights into viral-based gene delivery vectors. By comparison, the potential of plant viruses for biomedical applications is only beginning to gain attention among pharmaceutical scientists. An underlying reason may be the greater understanding now of differences between plant and animal viruses in their mechanisms of infection and transmission. The major mechanism of transduction for animal viruses involves the interaction of the viral CP with receptors on host cells. However, successful infection of plants by a plant virus necessitates mechanical damage to the cell wall followed by opportunistic penetration. The proliferation of plant viruses in the host is either by direct cell-to-cell contact or via manipulations of the plasmodesmata vascular system in the plant. Therefore, novel strategies need to be developed in order to optimize plant viruses for drug delivery and therapeutic applications.

APPLICATION AS TEMPLATES FOR COMPOUND CONJUGATION AND EXPRESSION

There are several ways that the plant viral PCs may be used for drug delivery. They can serve as conjugation templates to produce novel nanosized materials. Vaccines have been generated by introducing antigens, such as the peptide of the canine parvovirus, onto the CP of CPMV (3). An insertion of the Cys amino acid into the CP of CPMV by site-specific mutation provided sulfhydryl moieties that enabled surface conjugation of the viral PC with gold nanoparticles (4). Surface modification of the CPMV viral PC with PEG molecules resulted in PEG-CPMV particles, which showed a poorer immune response in mice than the native virus (1). The surface of a plant virus PC can further be modified to impart tissue-targeting capabilities. Folic acid, a cancer-cell-targeting ligand, has been successfully conjugated onto the PC of the *Hibiscus chlorotic ringspot virus* (HCRSV), and the resultant PCs were shown to be efficiently internalized by cancer cells *in vitro* (5).

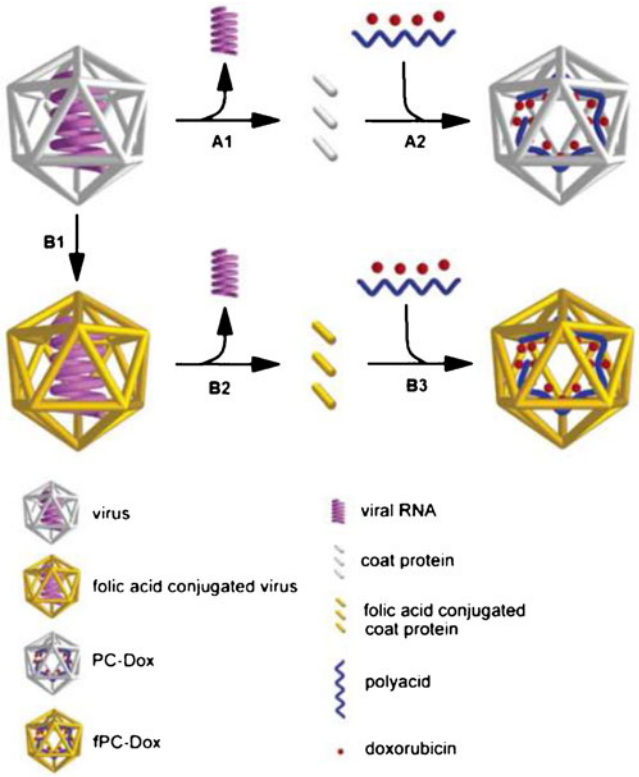
CPMV particles conjugated with the dye, Alexa Fluor 555, have been applied as probes for the *in vivo* imaging (6) of vasculature in mice. A single injection of these particles (50 μg to 1 mg) resulted in localization in the vascular endothelium for up to 30 days, enabling the visualization of vasculature to a depth of 500 μm, and the mapping of the capillary network and vessels entering and exiting a tumor mass. The preferential uptake of the CPMV by the venous endothelial cells also allowed arteries and veins to be differentiated. In addition, since the dye-conjugated CPMV were stably internalized by the endothelial cells, a stepwise administration of CPMV conjugated with different dyes, e.g. Alexa Fluor 555 and 488, would enable the monitoring of angiogenesis as a function of time, and permit the mapping of newly formed vasculature around the tumor cells.

APPLICATION AS CAGES FOR COMPOUND ENCAPSULATION

The PC of plant viruses can be directed to undergo reversible conformational changes, including swelling, disassembly and reassembly, outside the host cell. The diameter of *Cowpea chlorotic mottle virus* (CCMV) was observed to increase by approximately 10% when medium pH was increased from 5.0 to 7.0 in the absence of Ca^{2+} ions (2). This swelling was caused by a radial expansion and was accompanied by the appearance of 60 2-nm apertures in the PC. The swelling phenomenon was readily reversible by lowering the pH and re-supplying the medium with Ca^{2+}. Such chemical switches provide unique molecular gating mechanisms to control, contain and release the encapsulated cargo. Disassembly of the native plant viral capsids has been achieved by dialysis of the viral dispersion against a buffer of high pH of 7.5 or higher. The viral RNA can then be separated from the CP by centrifugation, and the purified CP reassembled at a lower pH into empty PCs, which are identical in shape and size to the native virus (2,7).

Plant viral PCs have been used to load materials including drugs, ions, polymers and nanoparticles. A pH-dependent gating mechanism permits the loading of charged species, e.g. tungstate ions (WO_2^{-4}). Swelling of the CCMV PC at pH 6.5 led to the opening of the surface apertures and the diffusion of water-soluble WO_2^{-4} into its interior. A lowering in pH reversed the conformational change in the virus PC, and the closure of apertures ensured the successful encapsulation of the nanosized precipitated tungstate within the PC (2).

Electrostatic interaction appears to be critical for the loading. The viral CPs have highly basic N-termini,



Schema 1 Schematic illustration of loading materials into the reassembling PC. Steps A1 and B2 are indicative of the removal of viral RNA from the plant virus and purification of coat proteins. Steps A2 and B3 involve the encapsulation of polyacid and doxorubicin during the reassembly of protein cage. Step B1 refers to the conjugation of folic acid onto the viral protein coat (5).

facilitating the encapsulation of negatively charged exogenous material. Therefore, to encapsulate gold nanoparticles, carboxylated terminated functionality was used to impart an anionic charge. The resultant electrostatic interactions between the gold particles and the viral CP initiated the PC reassembly process to yield a virus-like nanostructure with a gold core (8). Our study (7) suggested an apparent threshold mw, as indicated by the successful encapsulation of polystyrenesulfic acid (PSA) with mw no less than 13 kDa into the HCRSV PC. It was likely that the smaller PSA molecules were poorly retained in the reassembled PC due to their ability to leach out through the surface apertures.

A method named "polyacid association" (5) has been successfully employed to load doxorubicin into the HCRSV PC (Schema 1). PSA with mw of 200 kDa was utilized as the adjuvant, the long polymer chain ensuring adequate doxorubicin binding sites without compromising the net anionic charge essential for initiating CP reassembly. The reversible electrostatic interactions between doxorubicin and PSA allow for subsequent drug release, the final system showing a sustained drug release profile over 24 h at simulated physiological conditions.

A different approach was explored using the *Red clover necrotic mosaic virus* (RCNMV) (9). Doxorubicin molecules were "infused" into the RCNMV capsids without disruption of the viral structure or removal of the native RNA. This method yielded an extremely high loading efficiency of 1000 doxorubicin molecules per RCNMV virus, which was comparable to that achieved by the "polyacid association" method in the HCRSV PC. The high loading efficiency of the "infusion" method was not unexpected in view of the inherent capacity of doxorubicin to intercalate with viral RNA. Consequent to the different loading mechanisms, although the doxorubicin-loaded HCRSV and RCNMV particles appeared as perfect spherical structures indistinguishable from the respective native viruses (Fig. 1), doxorubicin release from the RCNMV for therapeutic activity could be limited and would require the disassembly of the capsid. Nevertheless, the differences between the doxorubicin-loaded RCNMV capsids and HCRSV PC suggest that plant viruses may be used to produce drug delivery systems with specific release profiles for different therapeutic purposes.

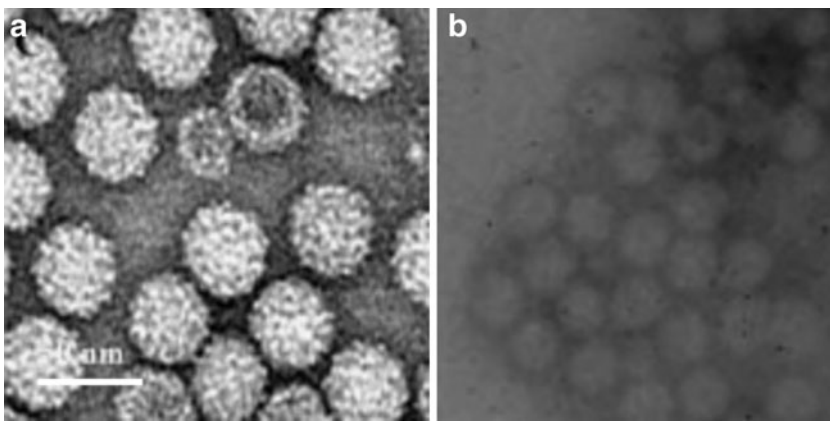


Fig. 1 Doxorubicin-loaded (**a**) HCRSV PC (5) and (**b**) RCNMV capsids (9) appeared as spherical structures indistinguishable from native viruses. Scale bar represents 50 nm.

TOXICITY, BIO-DISTRIBUTION AND UPTAKE

A number of the plant viruses have been shown to be non-cytotoxic at the doses required to effectively administer the drug load. *In vitro* MTT cytotoxicity assays indicated that the HCRSV PC was not cytotoxic against the CCL-186, OVCAR-3 and CNE-1 cells, at concentrations of up to 1 mg/ml (10). No overt toxicity was observed in mice inoculated intravenously with up to 100 mg/kg of the CPMV (11). Higher CPMV concentrations elicited immune responses, although the immunogenicity of these viral particles could be markedly reduced by surface conjugation with PEG of 2000 or 5000 Da (1). The low toxicity and favorable immunogenic profile of the PEGylated CMPV particles would make this platform very attractive for applications in drug delivery.

The native CPMV also retained its integrity after incubation with simulated gastric and intestinal fluids, indicating sufficient stability for application as a peroral delivery system (12). Furthermore, peroral absorption has been demonstrated. Upon oral gavage of CPMV in mice, its RNA was detectable in the blood, while the viral particles persisted in a variety of tissues over 3 days. Such trafficking to selective tissues, such as kidney, liver, lung, bone marrow and brain, could be advantageous for the targeted delivery of drug cargo to these tissues. Cellular uptake of CPMV by human cells has been demonstrated by Koudelka *et al.* (13), who showed the uptake of CPMV by HeLa and MFT-6 to occur rapidly via a specific endocytic process. The mechanism involved a direct binding of the virus with vimentin, which is present within the enriched plasma membrane of human cells.

For more specific targeting, chemical modification of the plant viruses may further enhance their interaction with specific receptors on animal cells. In this regard, folic acid as a cancer-cell-targeting ligand has been explored using the PC of CPMV (14) and HCRSV (5). The folic-acid-modified plant viruses showed a punctuate distribution within cancer cells, suggesting successful cellular internalization (5). Further research on these plant-virus-based drug delivery systems will be necessary to establish therapeutic efficacy and safety.

CONCLUSION

Plant virus protein cages present with specific properties that made them attractive carriers for drug delivery. Compared with non-viral nanoparticles, plant viruses are uniformly sized, robust nanostructures that are precise in chemical and conformational structures, and they could be safer than animal viruses for therapeutic applications. The plant virus PC can be used as a platform for drug delivery, with the drug loaded either by chemical conjugation or by physical encapsulation. While significant insights have been gained recently with regards to the mechanisms for cargo loading and PC reassembly *in vitro*, much about the plant-virus-based systems remains to be established before they can be accepted as viable drug delivery systems. The mechanism of release of encapsulated drug molecules and their toxicity and immunogenic profiles upon repeated and prolonged dosing would have to be evaluated. In addition, viable methods for the scaled-up production of CPs capable of reassembling into PCs *in vitro* would have to be developed. These are not insurmountable barriers, and as more is understood of plant virus platforms, it is reasonable to predict that advanced nano-sized drug delivery systems based on plant virus PCs will become available in the not-too-distant future.

ACKNOWLEDGEMENTS

This work was supported by the ARF grants R-148-000-045-112 and R-154-000-252-112 from the National University of Singapore (NUS).

REFERENCES

1. Raja KS, Wang Q, Gonzalez MJ, Manchester M, Johnson JE, Finn MG. Hybrid viruspolymer materials. 1. Synthesis and properties of PEG-decorated cowpea mosaic virus. Biomacromolecules. 2003;4(3):472–6.
2. Douglas T, Young M. Host-guest encapsulation of materials by assembled virus protein cages. Nature. 1998;393(6681):152–5.
3. Langeveld JP, Brennan FR, Martinez-Torrecuadrada JL, Jones TD, Boshuizen RS, Vela C, *et al.* Inactivated recombinant plant virus protects dogs from a lethal challenge with canine parvovirus. Vaccine. 2001;19(27):3661–70.
4. Wang Q, Lin T, Tang L, Johnson JE, Finn MG. Icosahedral virus particles as addressable nanoscale building blocks. Angew Chem. 2002;41(3):459–62.
5. Ren Y, Wong SM, Lim LY. Folic acid-conjugated protein cages of a plant virus: a novel delivery platform for doxorubicin. Bioconjug Chem. 2007;18(3):836–43.
6. Lewis JD, Destito G, Zijlstra A, Gonzalez MJ, Quigley JP, Manchester M, *et al.* Viral nanoparticles as tools for intravital vascular imaging. Nat Med. 2006;12(3):354–60.
7. Ren Y, Wong SM, Lim LY. *In vitro* -reassembled plant virus-like particles for loading of polyacids. J Gen Virol. 2006;87(Pt 9):2749–54.
8. Chen C, Daniel MC, Quinkert ZT, De M, Stein B, Bowman VD, *et al.* Nanoparticle-templated assembly of viral protein cages. Nano Lett. 2002;6(4):611–5.
9. Loo L, Guenther RH, Lommel SA, Franzen S. Infusion of dye molecules into Red clover necrotic mosaic virus. Chem Commun (Camb). 2008;7(1):88–90.
10. Ren Y. Application of HCRSV protein cage for anticancer drug delivery. A thesis of Doctor of Philosophy, National University of Singapore. 2007;chapter 2:38–67. [Ph.D thesis].

11. Singh P, Prasuhn D, Yeh RM, Destito G, Rae CS, Osborn K, *et al.* Bio-distribution, toxicity and pathology of cowpea mosaic virus nanoparticles *in vivo*. J Control Rel. 2007;120 (1–2):41–50.
12. Rae CS, Khor IW, Wang Q, Destito G, Gonzalez MJ, Singh P, *et al.* Systemic trafficking of plant virus nanoparticles in mice via the oral route. Virology. 2005;343(2):224–35.
13. Koudelka KJ, Destito G, Plummer EM, Trauger SA, Siuzdak G, Manchester M. Endothelial targeting of Cowpea mosaic virus (CPMV) via surface vimentin. PLoS Pathogens. 2009;5(5): e1000417.
14. Destito G, Yeh R, Rae CS, Finn MG, Manchester M. Folic acid-mediated targeting of Cowpea mosaic virus particles to tumor cells. Chem Biol. 2007;14(10):1152–62.