In vitro-reassembled plant virus-like particles for loading of polyacids

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The coat protein (CP) of certain plant viruses may reassemble into empty virus-like particles (VLPs) and these protein cages may serve as potential drug delivery platforms. In this paper, the production of novel VLPs from the Hibiscus chlorotic ringspot virus (HCRSV) is reported and the capacity to load foreign materials was characterized. VLPs were readily produced by destabilizing the HCRSV in 8 M urea or Tris buffer pH 8, in the absence of calcium ions, followed by removal of viral RNA by ultrahigh-speed centrifugation and the reassembly of the CP in sodium acetate buffer pH 5. The loading of foreign materials into the VLPs was dependent on electrostatic interactions. Anionic polyacids, such as polystyrenesulfonic acid and polyacrylic acid, were successfully loaded but neutrally charged dextran molecules were not. The molecular-mass threshold for the polyacid cargo was about 13 kDa, due to the poor retention of smaller molecules, which readily diffused through the holes between the S domains present on the surface of the VLPs. These holes precluded the entry of large molecules, but allowed smaller molecules to enter or exit. The polyacid-loaded VLPs had comparable size, morphology and surface-charge density to the native HCRSV, and the amount of polyacids loaded was comparable to the weight of the native genomic materials. The conditions applied to disassembly–reassembly of the virions did not change the structural conformation of the CP. HCRSV-derived VLPs may provide a promising nano-sized protein cage for delivery of anionic drug molecules.

INTRODUCTION

The quaternary structure of some plant viruses may undergo conformational changes to swollen states when metal ions are removed or the pH of the medium is raised (Liu et al., 2003; Oda et al., 1990). One of the most widely studied viruses is Cowpea chlorotic mottle virus (CCMV), which is stable at pH 5-0 but will undergo a 10 % radial expansion when the pH is raised to 7-0 in the absence of divalent cations (Perez et al., 2000; Tama & Brooks, 2002). This expansion, which is believed to give rise to an intermediate viral conformation during the disassembly and infection processes, caused the appearance of 60 cavities on the surface of the viral capsid. Several other plant viruses, including Tomato bushy stunt virus (Harrison & Robinson, 1982) and Turnip crinkle virus (Sorger et al., 1986), share similar properties. CCMV particles will dissociate and release the viral RNA if the pH is raised above 7-5, thus providing a simple mechanism for separating viral RNA from its coat protein (CP) (Fox et al., 1998). Moreover, the disassembly of CP appears to be reversible under specific in vitro environments. For example, CCMV can be reassembled in vitro from its RNA and CP at pH 7 (Zhao et al., 1995). In the absence of the viral RNA, the viral CP will form empty virus-like particles (VLPs) at pH 5 (Bancroft et al., 1968). Both the RNA-containing virus and the empty VLPs assembled in vitro are structurally identical to the capsid of the native virus (Fox et al., 1998). This indicates that the CPs of certain plant viruses are macromolecules that are able to self-assemble into particles of specific structures. The self-assembled empty VLPs have the potential to be used as inner constrained environments for the preparation of nano-structured materials. In particular, they may be promising as potential drug carriers, if they are shown capable of encapsulating foreign molecules (Douglas & Young, 1999). The empty VLPs derived from CCMV are capable of encapsulating foreign molecules, including the paratungstate ion (H2W12O422−), decavanadate ions (V10O6282−) and polyethoxylsulfonic acid, an anionic organic polymer, in vitro (Douglas & Young, 1998). CCMV-derived VLPs loaded with these compounds were observed to have the
same dimensions as the native CCMV. The monodispersity of these particles, along with their nano-sized dimensions, are attractive features for drug delivery applications.

However, there is to date no *in vitro* assembly study of HCRSV VLPs reported. There is also no systematic study on compound loading properties of plant viral protein cages. For the purposes of nano-material synthesis or developing a drug delivery system, it is necessary and meaningful to study what and how much foreign material can be loaded in the VLPs. In this study, we hypothesized that VLPs could be derived from the *Hibiscus chlorotic ringspot virus* (HCRSV) for the encapsulation of foreign materials. HCRSV belongs to the family *Tombusviridae* of plant viruses (Huang et al., 2000; Liang et al., 2002). It is a member of the genus *Carnovirus*, which also includes *Carnation mottle virus*, *Turnip crinkle virus* and *Cowpea mottle virus* (Büchen-Osmond, 2004). These viruses possess an ssRNA genome of about 40 000 nt and 180 CP subunits of approximately 38 kDa (Ke et al., 2004). In their hosts, the CPs assemble into capsids of 30 nm diameter that encapsulate the viral RNA (Ke et al., 2004; Lee et al., 2003). If these viruses are capable of disassembly followed by reassembly *in vitro* to form VLPs, they can be used as potential nano-scale protein cages for drug delivery (Morganova et al., 1994).

The objectives of this study were: (i) to develop methods to remove the native viral RNAs from the HCRSV capsids; (ii) to reassemble the purified CP of the virus into empty VLPs *in vitro*; and (iii) to assess the capacity of the VLPs to accommodate foreign materials of different molecular mass (Mm). Two polyacids, polystyrenesulfonic acid (PSA) and polyacrylic acid (PAA), were used as test anionic compounds, which provided different acid groups for the evaluation of anionic cargos.

**METHODS**

**Propagation and purification of HCRSV from kenaf plants.** HCRSV was originally obtained from infected kenaf (*Hibiscus rosasinensis* L.) plants exhibiting chlorotic leaf spots. The virus was propagated in kenaf plants and purified by the method described previously (Lee et al., 2003).

**Removal of viral RNA and the production of empty VLPs from native HCRSV.** Two methods were evaluated for the removal of viral RNA and the production of empty VLPs from HCRSV. With the urea denaturation method, 1 mg HCRSV dispersed in 0-1 ml resuspension buffer (50 mM sodium acetate, 50 mM NaCl, 20 mM CaCl₂, 5 mM EDTA, pH 5-4) was denatured by incubation with 1 ml urea solution (8 M urea, 1 M NaCl, 0-01 M sodium phosphate buffer, pH 7-2) at ambient temperature for 10 min (Michels et al., 1999). The dissociated viral RNA was pelleted by centrifugation at 25 000 g for 30 min at 4 °C. The supernatant was dialysed against buffer A (50 mM sodium acetate, 50 mM NaCl, 2 mM EDTA, 20 mM CaCl₂, pH 5-0) for 12 h at 4 °C to enable refolding and assembly of the CP into VLPs. For the dialysis method, 0-1 ml HCRSV (10 mg ml⁻¹) in resuspension buffer was dialysed overnight against buffer B (50 mM Tris/HCl, 5 mM EDTA, 2 mM DTT, 0-2 mM PMSF, pH 8-0) at 4 °C. It was then incubated with 10 % of its volume of 5-5 M CaCl₂ at 4 °C for 30 min, after which the solution was centrifuged at 25 000 g. 4 °C for 30 min to pellet the viral RNA. The supernatant was dialysed against buffer C (50 mM Tris/HCl, 5 mM EDTA, 0-2 mM DTT, 0-1 M NaCl, pH 8-0) for 4 h at 4 °C, followed by dialysis against buffer A for 15 h at 4 °C to allow the CP to reassemble into VLPs. The efficiency of RNA removal from HCRSV was assessed by determining the A₂₆₀/A₂₈₀ ratio of the respective supernatants, while the concentration of CP was calculated as CᵥLP (mg ml⁻¹) = A₂₈₀/VLP × 36 971 e⁻¹. The absorbance coefficient (ε) of CP was calculated as 36 200 ml mmol⁻¹ based on its amino acid sequence (Mach et al., 1992).

**Loading of foreign materials.** The capacity of VLPs to load foreign materials was studied using CP prepared by the dialysis method. Fluorescein isothiocyanate-dextran (FITC-dextran or FD) and two polyacids PSA and PAA (Fig. 1) were selected for loading tests. The CP was separately incubated with FD [Mm of 4, 10, 75 and 150 kDa, in 2:1 (w/w) ratio], PSA [Mm of 1-4, 4-3, 13, 75, 200 and 990 kDa, in 3:1 (w/w) ratio] and PAA [Mm of 450 kDa, in 3:1 (w/w) ratio] in buffer C for 8 h at 4 °C, then dialysed against buffer A for 15 h at 4 °C. The samples were put on 10-40 % sucrose gradients and centrifuged for 3 h at 100 000 g, at 4 °C using an SW41 rotor. Control samples, which consisted of 1 mg ml⁻¹ of FD, PSA, HCRSV particles, HCRSV CP, empty VLPs or mixtures of empty VLPs and polyacids in buffer A, were similarly subjected to sucrose-gradient separation. Fractions of the sucrose gradient were collected and analysed. FD samples were assayed by measuring the fluorescence of each fraction at λₑx 485 nm and λₑm 535 nm (TECAN SpectraFluor). The absorbance of HCRSV and PSA samples was determined at 260 and 262 nm, respectively, while those of CP, empty VLPs and PAA were determined at 280 nm (Beckman DU 640B spectrophotometer). Fractions that tested positive for the respective cargoes were collected and diluted with up to four times their volumes with buffer A. The solutions were put in an Amicon Ultra-15 filter device (Mm cut-off 10 000; Millipore) and centrifuged under 3000 g to concentrate to 0-5 ml for further analysis.

For ease of discussion, empty VLPs are denoted as VLPs, while VLPs loaded with FD, PSA and PAA are denoted as FD-VLPs, PSA-VLPs and PAA-VLPs, respectively. In addition, the prefix ‘x’ may be used for PSA-VLPs samples to denote the Mm of the PSA cargo in kDa.

**Analysis of polyacid-loaded VLPs.** Both PSA (13 kDa and greater) and PAA (450 kDa) were successfully loaded into the HCRSV-derived VLPs and subjected to further characterization. Size and zeta potential of the native HCRSV, VLPs, PSA-VLPs and PAA-VLPs were determined in a particle sizer (3000HSA zetazeter; Malvern Instruments), and their morphology was observed under a transmission electron microscope (TEM) (CM10; Philips Electronic Instruments) after staining with 1 % phosphotungstic acid. Samples resuspended in 30 % sucrose solution were loaded onto 1 % agarose...
gel and analysed at 5 V cm\(^{-1}\) for 50 min. The gel was visualized Coomassie brilliant blue (Newman \textit{et al.}, 2003). Conformational structure of the HCRSV-derived CP in various samples was analysed by circular dichroism (CD) spectroscopy (Jasco J-815 spectropolarimeter).

For the PSA-VLPs samples, the loading efficiency was calculated as the ratio of loaded PSA over the CP. The concentration of CP in the samples was quantified by bis-cinchoninic acid assay (Smith \textit{et al.}, 1985) with a calibration curve produced from purified CP. The concentration of PSA was quantified by measuring the ultraviolet absorption at 262 nm after compensating for the contribution of the CP. Loading efficiency and the number of PSA molecules loaded in each VLP were calculated by the following equations:

\[
\text{Loading efficiency (\%)} = \frac{C_{PSA}}{C_{CP}} \times 100 \%
\]

\[
n = \frac{C_{PSA} \times 36971 \times 180}{(\text{Mm}_{PSA} \times C_{CP})}
\]

**RESULTS AND DISCUSSION**

**Purification of HCRSV, removal of viral RNA and reassembly of VLPs**

The yield of purified HCRSV per 100 g infected leaves ranged from 1 to 10 mg, and the \(A_{260}/A_{280}\) ratio was 1.53 ± 0.01 (\(n=3\)). Incubation of HCRSV with 8 M urea denatured its CP sufficiently to allow the viral RNA to be removed, as indicated by the lower \(A_{260}/A_{280}\) ratio (0.7 ± 0.1, \(n=3\)) of the supernatant. Upon removal of urea by dialysis against buffer A, the CP refolded into its native \(\beta\)-sheet structure, as evidenced by the CD spectra (see Supplementary Fig. S1 in JGV Online). The CP subunits reassembled into structures resembling VLPs \textit{in vitro} (Fig. 2b). However, precipitation was noted during the dialysis process, which may have been caused by the misfolding of some protein, and the resultant VLPs did not have a well defined structure (Fig. 2b), when compared with the native HCRSV under the TEM.

Dialysis of native HCRSV against buffer B followed by incubation with 0.5 M CaCl\(_2\) and centrifugation also resulted in a supernatant with a lower \(A_{260}/A_{280}\) value of 0.6 ± 0.1 (\(n=3\)). Buffer B was designed to be devoid of calcium ions, which have been reported to be necessary for the conformational stability of several plant viruses (Fox \textit{et al.}, 1998). Incubation of HCRSV in buffer B appeared to destabilize the viral structure, causing the CP to be disassembled. The RNA was then isolated by precipitation with 0.5 M CaCl\(_2\) and the CP was reassembled into VLPs by dialysis against buffer A (Fig. 2c). As indicated by the \(A_{260}/A_{280}\) value of 0.6, the resultant samples contained purified CP with very little, if any, residual RNA or intact virions (Michels \textit{et al.}, 1999). In both cases, the reassembled VLPs appeared as spherical particles that have comparable diameter and morphology to the native HCRSV, when viewed under the TEM. Therefore, the dialysis method was chosen to prepare subsequent batches of VLPs for the loading of foreign materials.

**Loading of foreign materials**

VLPs loaded with PSA were screened at 262 nm, which corresponded to the \(\lambda_{\text{max}}\) of PSA (Fig. 3). When subjected to sucrose-gradient centrifugation, free PSA molecules at all Mm used were concentrated in fractions collected from the top of the gradient. For the VLPs loaded with PSA of
Based on these results, it is evident that the larger PSA molecules were more successfully loaded in the HCRSV-derived VLPs than the smaller PSA molecules, a phenomenon that may be attributed to the poor retention of the smaller PSA molecules within the VLPs. The VLPs are expected to share a similar structure to the native HCRSV in having 180 U of CP and nano-scale holes in the viral shell (Doan et al., 2003; Douglas & Young, 1998), which would allow small molecules to pass through but would effectively trap larger sized molecules within the VLPs. Thus, a cargo of small molecules, like the 1-4 kDa PSA, would diffuse readily across the holes and would be poorly retained within the VLPs upon dilution. PSA molecules of 13 kDa or greater might be too large to pass through the holes and were therefore effectively retained within the VLPs after sucrose-density-gradient centrifugation. The partial retention of the 4-3 kDa PSA in the VLPs suggests that the Mm of PSA molecules is close to the passage limit of the cavities.

One PAA sample of Mm 450 kDa was loaded into the VLPs. Compared with PSA, which contains strong sulfonic acid groups (pK_a = 1), PAA contains weaker carboxylic acid groups (pK_a = 4.5) that would be partially ionized at pH 5. The PAA-VLPs had a sucrose-gradient separation profile similar to those observed for the PSA-VLPs in that the absorbance for both the PSA-VLPs and PAA-VLPs samples corresponded to the native HCRSV fraction (Fig. 3d) after centrifugation in a sucrose gradient, suggesting that they were comparable in size and density.

VLPs successfully loaded with PAA (450 kDa) and PSA (13 to 990 kDa) were analysed further for size, surface charge and protein conformation. Regardless of the Mm of the cargo, the samples were comparable to HCRSV in morphology (Fig. 2d–h). The mean size of the particles measured by the particle size analyser, which represents the hydrodynamic volume, fell within a narrow range of 44 (HCRSV) to 53 nm (200 PSA-VLPs). Zeta potential values were -2.4 mV for the HCRSV, -2.3 to -2.9 mV for PSA-VLPs and -2.3 mV for PAA-VLPs and there was no significant difference between them (P > 0.05). Native gel electrophoresis was in agreement with this and bands corresponding to the PSA-VLPs and PAA-VLPs were located at positions comparable to that of the native HCRSV. CD spectra for all samples showed a minimum at 220 nm, indicating that the purification of CP and loading of PSA and PAA did not affect the b-sheet structure in the CP (see Supplementary Fig. S1).

The collective data indicated that the loaded PSA and PAA conferred a negative charge to the VLPs that was equivalent to that of the native RNA in HCRSV. Given the differences in Mm and acid type among PSA, PAA and native viral RNA, it appears that the total charge conferred was independent of the type and Mm of the polyacids contained within the viral structure. It was also not influenced by differences in the degree of ionization of the three polyacids at pH 5. This phenomenon may be attributed to the existence of a buffering system in the VLPs: possibly the...
basic amino acids in the CP could neutralize the anionic charges on the polyacid cargo.

In contrast to samples loaded with PSA and PAA, all samples in which the neutral FD (4–150 kDa) was mixed with the CP for reassembly into FD-VLPs showed strong fluorescence only in those fractions located at the top of the sucrose gradient, at positions corresponding to the respective control-free FD samples. Negligible fluorescence was detected in middle fractions collected from the sucrose gradient, suggesting that FD was not loaded into the VLPs. This failure to load might be attributed to FD not being encapsulated when the CP reassembled into VLPs, or to the entrapped FD being poorly retained within the VLPs after being subjected to sucrose-gradient centrifugation. The former hypothesis appears more likely because two of the FD samples (75 and 150 kDa) showed higher Mm than the successfully loaded 13 kDa PSA molecules. If they were successfully encapsulated within the VLPs, they would be effectively retained upon dilution because they were unlikely to pass through the cavities in the viral capsid.

Since FD did not contain ionizable groups and was neutral in pH during loading, the results suggest that electrostatic interaction may be necessary for the encapsulation of cargo in the VLPs during CP reassembly. This is in agreement with previous findings that the amino acid groups in the interior of a plant virus capsid play an important role in material loading (Douglas & Young, 1998, 1999). In the case of HCRSV, the positively charged Arg and Lys moieties located at the N terminus of the CP would render the inner cavity of the VLPs attractive for the binding of negatively charged compounds, as demonstrated by the successful loading of polyacids during CP reassembly (Fig. 3).

Two lines of evidence affirmed that the polyacids were encapsulated inside the protein cage of the VLPs rather than conjugated on the surface. The first evidence is that polyacids with small Mm were not retained by the VLPs. If the polyacids were loaded simply by conjugation onto the outer surface of the VLPs, the VLPs should be able to link with polyacids regardless of their Mm. The failure to load small polyacids indicated that conjugation is not the mode of loading by the VLPs. The other evidence is that the large Mm polyacids could not be loaded if they were added after the CP was reassembled into VLPs. Experiments in which reassembled VLPs were incubated with either the PSA (13–990 kDa) or PAA (450 kDa) failed to show successful loading of any of the polyacids. In this case, the failure to load could be attributed to the barrier posed by the reassembled VLPs. For successful loading of the cargo molecules, they must enter the VLPs through cavities on the viral capsid and be retained. However, the cavities were too small for large molecules to permeate into the interior of the viral capsid. Sole incubation with already reassembled VLPs will not permit loading of large Mm molecules into the VLPs. Taken together, it is demonstrated that both polyacids were loaded into the VLPs before CP reassembly and not through conjugation of the molecules to the exterior of the viral capsid.

**Quantitative assay of loaded PSA**

To determine the PSA loading efficiency with respect to Mm, PSA-VLPs samples were purified by sucrose-gradient centrifugation and the PSA and CP contents were separately quantified by UV spectroscopy and bis-cinchoninic acid assay. The results, expressed as percentage PSA relative to CP, are given in Table 1. Despite a 75-fold difference in Mm, PSA of 13–990 kDa were encapsulated with comparable efficiency into the VLPs. The PSA loading efficiency was determined to be 15–20% (Table 1). This suggests that the VLPs of HCRSV were capable of encapsulating foreign materials to an amount equivalent to the mass of their native genomic materials.

The number of PSA molecules encapsulated per VLP was approximated based on the PSA loading data and the corresponding PSA Mm (Table 1). For the largest PSA molecule, only one molecule was accommodated in each of the VLPs. This restriction in cargo loading size is not surprising, and is in fact an acknowledged limitation in virus-based gene delivery systems (Dong et al., 1996). A linear relationship \((n=1/Mm \times 10^6, r^2=0.9969)\) was observed between the number of PSA molecules loaded into each VLP \((n)\) and the Mm of the PSA. Each VLP functions as a protein cage that shows considerable capacity for loading foreign materials.

The knowledge obtained from this research is meaningful for future work in nano-material application and provides useful guidance for loading anti-cancer agents inside VLPs. HCRSV could be disassembled to release its native RNA and the resulting CP reassembled into VLPs in vitro. Reassembly of the CP could be carried out with concomitant loading of foreign polyacids of 13 kDa or greater. Compared to the formation of nano-particles by polymer condensation, the reassembly of CP into VLPs occurred by a more precise mechanism to yield particles that were mono-dispersed and had uniform morphology and consistent properties. The structural features of the VLP as a protein cage was

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<tr>
<th>Mm of PSA (kDa)</th>
<th>Loading efficiency (%)</th>
<th>No. PSA molecules/VLP</th>
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<tbody>
<tr>
<td>13</td>
<td>19.6 ± 1.7</td>
<td>101</td>
</tr>
<tr>
<td>75</td>
<td>17.1 ± 1.7</td>
<td>15</td>
</tr>
<tr>
<td>200</td>
<td>20.0 ± 1.0</td>
<td>7</td>
</tr>
<tr>
<td>990</td>
<td>15.8 ± 2.1</td>
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independent of its cargo and the VLP was comparable in size, morphology and protein conformation to the native virus with its RNA load. In addition, the CP could be modified and the surface of the protein could be linked with targeting agents. These features render HCRSV-derived VLPs a potential vehicle for drug delivery applications.

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). Y. R. is a recipient of NUS graduate scholarship.

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