Infusion of dye molecules into *Red clover necrotic mosaic virus*

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Supporting information

Materials and Methods

RCNMV propagation and purification

Growth and purification of RCNMV were as described [1]. In brief, RCNMV RNA transcripts were inoculated on *Nicotiana clevelandi* plants and maintained under standard greenhouse conditions for 7 to 10 days. Virus were collected from infected leaves and purified. The concentration of virus is determined by absorbance measurement with an extinction coefficient of 6.46.

Infusion of dye molecules into RCNMV

1.25mg/ml of RCNMV was dialyzed in a Slide-A-Lyzer 10K MWCO dialysis cassette (Pierce Biotechnology, Rockford, IL) against 200mM EDTA at pH8 for 5-6hr. Dialyzed RCNMV was collected and incubated with 2000:1 ratio of dye-to-virus at 4^{o}C for 12-16hr. After the incubation period, the sample was dialyzed against 200mM Ca^{2+} at pH6 for 24hr. The sample, which contained RCNMV-infused dye ($RCNMV_{dye}$), was then collected. Excess dyes were removed by Microcon filter unit with 30kDa cutoff (Millipore, Billerica, MA) by centrifugation at 16,000g for 30min. This filtration was repeated until the fluorescence signal of the $RCNMV_{dye}$ was reduced to the background level, an indication of complete removal of excess dyes from the sample. The identical protocol as described above was also performed to infuse Doxorubicin into RCNMV ($RCNMV_{Dox}$). After dialyzed against EDTA, 5000:1 ratio of Doxorubicin-to-virus was incubated for 12-16hr, followed by dialyzed against Ca^{2+}. The excess Doxorubicin was then removed using sucrose gradient centrifugation as described below.

RCNMV was also exposed to EGTA (ethylene glycol tetraacetic acid) to specifically remove Ca^{2+}, using the identical protocol as EDTA treatment, followed by incubation

with dyes and dialyzed by Ca^{2+}. EGTA is a chelating agent that binds with higher affinity to calcium than magnesium. The stability of complex formed between calcium and EGTA is approximately two-fold higher than the complex formed between magnesium and EGTA (log K_{Ca} =11.0, log K_{Mg}=5.2). [2 3].

Quantification of dyes or Doxorubicin infused per RCNMV

The infused dyes or Doxorubicin was released from the capsid by EDTA treatment at pH10. 200mM EDTA at pH10 was added into a Microcon filter unit with 10kDa cutoff (Millipore, Billerica, MA), which contained 0.5mg/ml of $RCNMV_{dye}$ or $RCNMV_{Dox}$ with a volume ratio of 1:1. After ~30min of incubation at room temperature, the sample was centrifuged for 30min at 16,000g. The dyes or Doxorubicin released from the disassembled capsid were separated and quantified by fluorescence measurement. To determine the capability of infused dyes to diffuse through pores, the $RCNMV_{dye}$ or $RCNMV_{Dox}$ was treated by 200mM EDTA at pH8 with a volume ratio of 1:1. After ~30min of incubation, the dyes or Doxorubicin that diffused through the re-opened pores was separated from the capsid by centrifugation for 30min at 16,000g in a Microcon filter unit (10kDa cutoff). Standard curves of native RCNMV, dyes and Doxorubicin were generated for quantitative measurement to determine the ratio of dyes or Doxorubicin infused per RCNMV.

Sucrose density gradient centrifugation

To expedite the process of removing the excess Doxorubicin, RCNMV-infused Doxorubicin ($RCNMV_{Dox}$) was loaded onto a 20-50% sucrose gradient centrifugation (180000g, 5^{o}C, and 55min) in a SW-55 rotor with a model L8-70 Beckman ultracentrifuge. The preformed gradients were prepared by layering, in succession, 1ml of 20, 30, 40 and 50% (w/w) sucrose into ultra-clear centrifuge tubes (Beckman). The gradients were kept for overnight at 4 ^{o}C to allow diffusion to form linear sucrose density gradients. The native RCNMV (0.5mg/ml), as a control sample, was also studied under the same experimental conditions. Fifteen 0.25ml fractions of the sucrose gradient were collected from bottom to top for absorbance measurement at 260nm. The fraction with the highest absorbance value (purified $RCNMV_{Dox}$) was collected for further analysis.

Iodixanol (Optiprep) density gradient centrifugation

The Optiprep gradient is formed using an aqueous solution of iodixanol, which is nonionic, water soluble and has relatively low osmotic content. Unlike sucrose and CsCl gradients, the Optiprep density gradient has been a more favorable medium for the separation of virion due to the low viscosity. As a consequence the centrifugation process does not cause serious damage to the virions [4,5]. The RCNMV-infused Doxorubicin ($RCNMV_{Dox}$) was layered on a 20-60% iodixanol gradient (Optiprep, Axis Shield) into polyallomer bell-top quick seal tubes (Beckman) and centrifuged in a SW-55 rotor with a model L8-70 Beckman ultracentrifuge (27900*g,* 16hours, 5°C). After centrifugation, the gradients were fractionated into 0.25ml fractions. 50ul of each collected fraction was mixed with equal volume of Bradford reagent (Pierce Biotechnology, Rockford, IL) and the absorbance was measured at 595nm using a microplate reader (Bio-Tek Synergy HT). The fraction with the highest absorbance value (purified $RCNMV_{Dox}$) was collected. The refractive indexes of samples were measured using a refractometer to determine the densities, based on the formula $\rho=3.4394\eta- 3.5970$, where ρ is the density (g/ml) and η is the refractive index.

Fluorometry

Fluorescence measurements were performed by Perkin Elmer LS-50B Spectrofluorometer. Rhodamine 590 Chloride was measured with an excitation wavelength at 500nm and emission wavelength at 560nm. Luminarosine was excited at 425nm and fluorescence emission was observed at 530nm. Fluorescein was measured with an excitation wavelength at 470nm and the emission wavelength at 530nm. In three cases, excitation and emission slit widths were set to 2.5nm and 5.0nm, respectively.

Transmission Electron Microscopy (TEM)

TEM images were acquired using a (Philips) CM12 microscope operating at 100kV accelerating voltage located at the University of North Carolina, School of Dentistry, Chapel Hill, NC. Images were created with a Gatan 780 DualView camera system. All

TEM samples, unless otherwise noted, were negatively stained with 2% uranyl acetate to enhance image contrast.

Figures

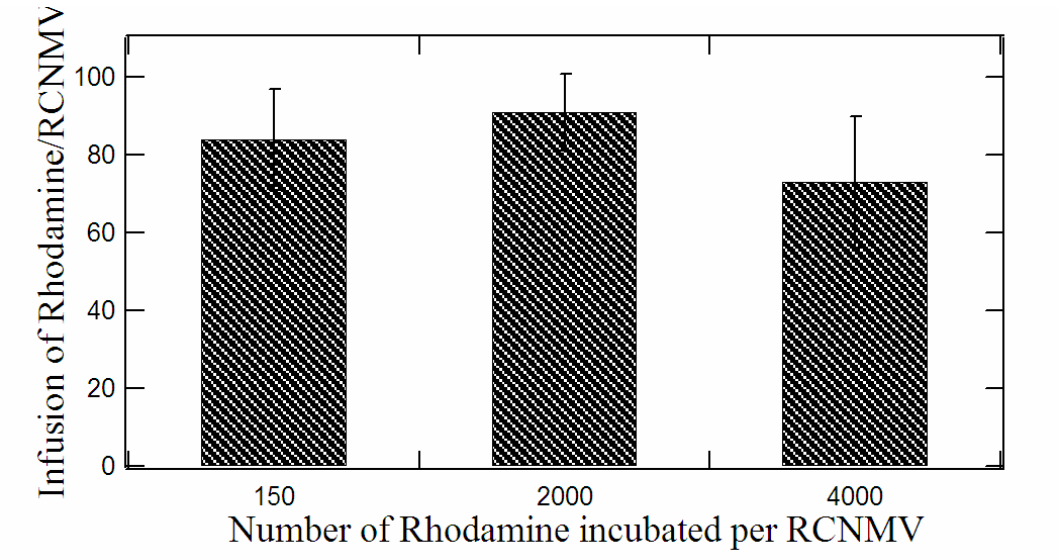

Figure S1. Number of Rhodamine infused per RCNMV under various RCNMV/Rhodamine ratios during incubation period. 3 different ratios of Rhodamine-to-RCNMV were incubated to determine the maximum loading capacity of Rhodamine per virus. Data indicated a maximum of ~90 Rhodamine can be infused per RCNMV.

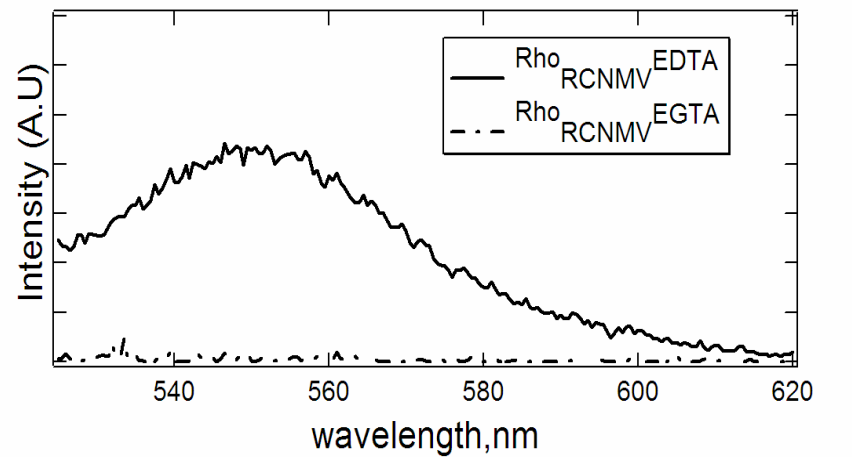

Figure S2. Emission spectrum of Rhodamine released from RCNMV pre-treated with EDTA (^{Rho}RCNMV^{EDTA}) and RCNMV pre-treated with EGTA (^{Rho}RCNMV^{EGTA}). The protocol to infuse Rhodamine into RCNMV-pretreated with EGTA, followed by the removal of excess Rhodamine and the released of infused Rhodamine was identical as described for infusion of Rhodamine into RCNMV-pretreated with EDTA. After EDTA, pH 10 treatment, Rhodamine released from the disassembled capsid was measured. The Rhodamine intensity from ^{Rho}RCNMV^{EGTA} was negligible if compared to the Rhodamine

intensity from ^{Rho}RCNMV^{EDTA}. In other word, the failure to open the pores leads to low or no Rhodamine infusion.

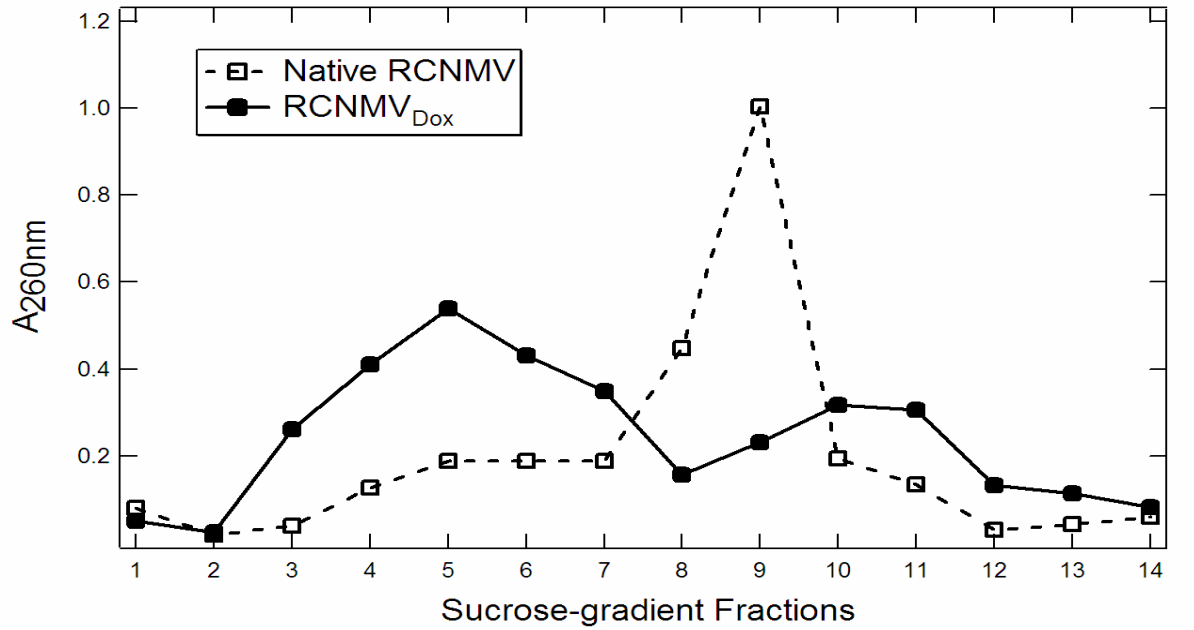

Figure S3. Absorbance of each sucrose gradient fractions obtained from native RCNMV and RCNMV$_{Dox}$. Data are presented with the densest fractions to the left. The highest absorbance for native RCNMV was observed at fraction 9, which corresponded to 30% sucrose. The infusion of Doxorubicin into the RCNMV capsid increased the density of the particle, and banded at fraction 5, equivalent to 40% of sucrose.

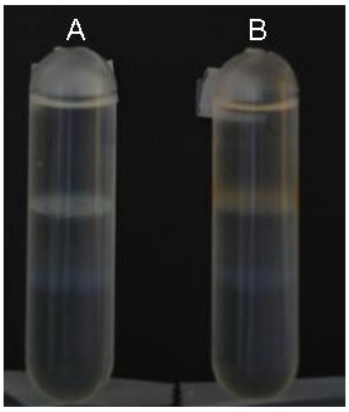

Figure S4. Iodixanol (Optiprep) gradient centrifugation. The distribution profile of (A) native RCNMV and (B) negative control sample through a 20-60% iodixanol gradient after centrifugation. The negative control sample was prepared by incubation of Doxorubicin with untreated RCNMV (not treated with EDTA or EGTA).

References

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