Icosahedral Virus Particles as Polyvalent Carbohydrate Display Platforms

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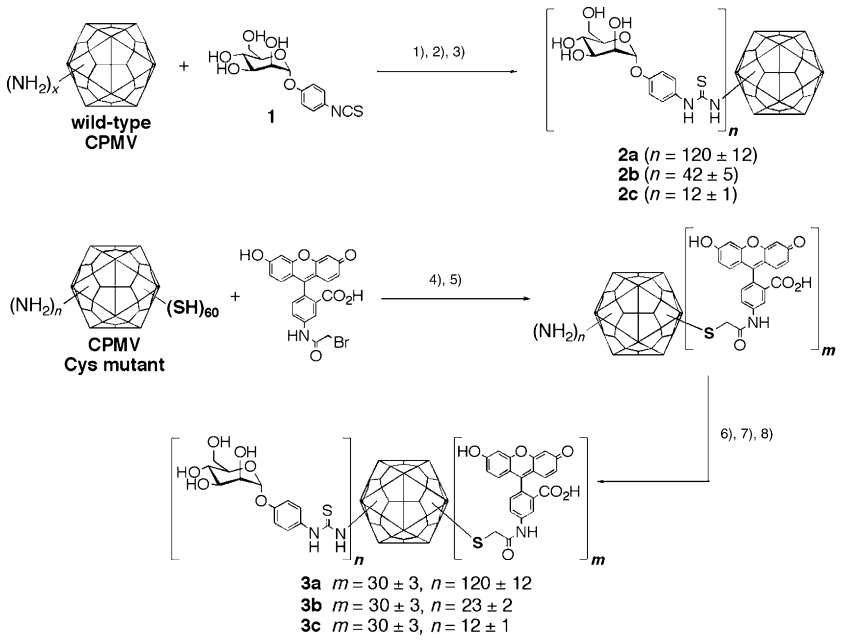
Icosahedral viruses can serve as robust and programmable scaffolds to which a wide variety of chemical and biological structures may be attached.[1] Their advantages include biocompatibility, the display of functional groups on both the inside and outside surfaces of the viral coat protein; very large size (on the chemical scale) allowing for the independent attachment of different units to noninteracting positions and their presentation to the environment on dimensions relevant to those of biological cells; and the ability to introduce amino acid residues with unique reactivity in designed locations of the capsid structure. No other type of scaffold of comparable size is available for which the structure is known to near atomic resolution. Among other applications, we seek to employ virus particles to display biochemical units of importance to cellular recognition and signaling events.[2] Carbohydrates are an obvious choice in this regard, as their interactions with receptors mediate a variety of important processes.[3]

A central theme in this area is polyvalency.[4] The display of multiple copies of carbohydrates on polymeric,[5] dendritic,[6] small-molecule,[7] and other[8] supports has been shown to provide tighter binding to cell-surface receptors and/or more effective biochemical response than is observed with monomeric carbohydrate derivatives in solution.[4] We describe here the covalent decoration of cowpea mosaic virus (CPMV) with sugar molecules, and studies of the binding behavior of the resulting multivalent particles with both carbohydrate-binding protein and cells.

We have previously established that each asymmetric unit of wild-type CPMV contains one lysine residue that exhibits diminished conjugate acid pK_a, identified as K38 of the small subunit. This residue is found on the exterior surface of the capsid and can be selectively targeted by isothiocyanate reagents,[11] such that 90% or more of isothiocyanate attachments are made at this position, up to a value of 60 per virion (the total number of K38 residues). Thus, α-D-mannopyranosyl-phenyl-isothiocyanate (**1**) was reacted with wild-type CPMV at a molar ratio 500:1 (**1** with respect to the concentration of viral protein) in 0.1 M potassium phosphate buffer (pH 7.0) containing 20% DMSO (Scheme 1). Previous experiments with fluorescein isothiocyanate (FITC) established an average covalent loading under these conditions of 50 ± 5 dye molecules per virus particle. The level of mannose loading on the product (**2b**) was measured with a modified fluorescamine assay[12] as 42 ± 5 per virion, comparable to the result with the dye reagent. In all cases, derivatized CPMV samples were purified by dialysis and size-exclusion chromatography, and isolated in ≥ 60% yield.



Scheme 1. *1) 20% DMSO in buffer, 4 °C, 48 h; 2) dialysis; 3) size-exclusion chromatography. Reaction a, yielding product* ***2a****: pH 9.0, 500 equiv.* ***1****; reaction b, yielding product* ***2b****: pH 7.0, 500 equiv.* ***1****; reaction c, yielding product* ***2c****: pH 7.0, 100 equiv.* ***1****. 4) 20% DMSO in buffer, pH 7.0, 4 °C, 48 h; 5) Size-exclusion chromatography; 6) 20% DMSO, pH 9.0, 4 °C, 48 h; product a: 500 equiv.* ***1****; product b: 200 equiv.* ***1****; product c: 100 equiv.* ***1****.*

In order to obtain a more heavily mannose-labeled particle, the above reaction was performed in sodium bicarbonate buffer (pH 9.0) containing 20% DMSO (Scheme 1). Product **2a** was found by fluorescamine assay to bear 120 ± 12 mannose units per virion; again this is consistent with previous FITC observations. CPMV derivatives **3a**–**c**, bearing both carbohydrate and dye molecules, were similarly constructed by using a virus mutant with one cysteine residue per asymmetric unit on the exterior surface, in addition to the normal reactive lysines (Scheme 1).[9e, 11b] Fluorescein bromoacetamide was first used to address the highly accessible cysteine side chains, and the purified intermediate particles were then decorated with mannose as above. Virions bearing fewer than 60 mannose units (**2b**, **2c**, **3b**, **3c**) therefore have those carbohydrates displayed at the K38 positions. Since there are 60 component proteins per particle, samples **2a** and **3a** have approximately half of their 120 carbohydrates attached to lysine side chains

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other than K38. This is demonstrated by SDS-PAGE analysis (Figure 1), which shows covalent modification of both protein subunits. The intact nature of all mannosylated virions was verified by fast protein liquid chromatography (FPLC) and transmission electron microscopy (TEM).[13]

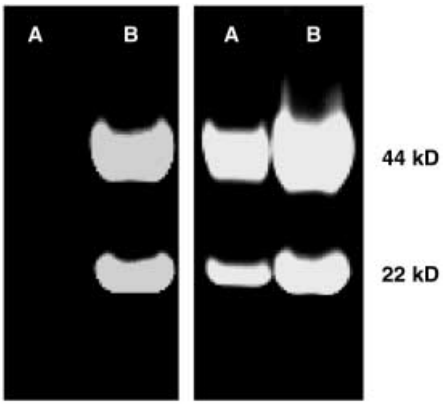

Figure 1. *SDS-PAGE analysis of mannosylated virus* ***2a****. Lanes: A) wild-type CPMV. B)* ***2a****. On the left, the gel is visualized with Pro-Q Emerald-488 stain, specific for carbohydrates; on the right, the gel is visualized with SYPRO Ruby stain showing protein-containing bands (both stains obtained from Molecular Probes, Inc.). These results reveal mannose attachment to both small and large CPMV subunits.*

The plant lectin concanavalin-A (con-A), exists as a tetramer in PBS buffer and binds up to four α-D-mannopyranoside or α-D-glucopyranoside units in the presence of Ca^{2+} and Mn^{2+} ions.[14] In analogy to the formation of aggregates between con-A and dendritic glycosides,[6b–d] the addition of a con-A solution to **2a** in the presence of 0.1 M Ca^{2+} and Mn^{2+} caused the rapid formation of a gel. This agglutination behavior was marked by increased absorbance intensity throughout the UV-visible spectrum, due to light scattering from the aggregated particles, as shown in Figure 2. The addition of a large excess of galactose, which is not bound by the lectin, had no effect on the mixture, whereas excess α-D-mannopyranoside restored the original spectrum, presumably by competing for the virus-displayed mannose units and thereby breaking up the gel (Figure 2). The rate of the aggregation phenomenon was conveniently monitored at 490 nm, at which point no absorbance of either **2** or con-A is observed (Figure 2, inset). Gel formation occurs in minutes at a virus concentration of 0.7 mg mL^{-1}, and considerably faster (to within a few seconds) at higher concentrations.

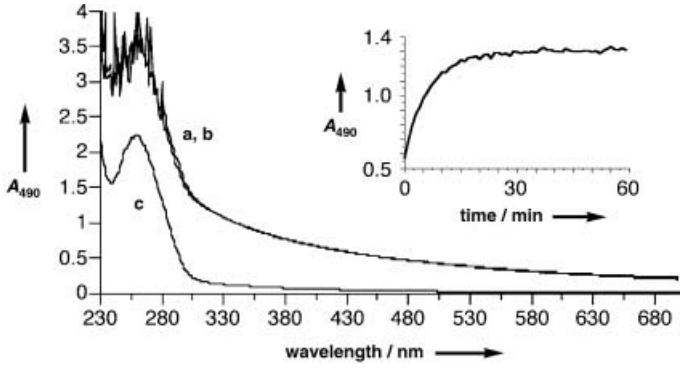

Figure 2. *UV/vis absorbance spectra. a) Mixture of* ***2a*** *(0.42 mg mL^{-1}) and con-A (0.77 mg mL^{-1}; approximately 100:1 molar ratio of con-A tetramer to virus particles) in PBS buffer with 0.1 mM of Ca^{2+} and Mn^{2+}. b) Addition of galactose (final concentration 33 mg mL^{-1}) to a. c) Addition of mannose (final concentration 33 mg mL^{-1}) to a. Inset: time course of agglutination for a mixture of* ***2a*** *(0.67 mg mL^{-1}) and con-A (0.32 mg mL^{-1}; approximately 26:1 molar ratio of con-A tetramer to virus particles) in PBS buffer with 0.1 mM Ca^{2+} and Mn^{2+}.*

Aggregation of labeled virus **2a** with con-A was also examined by TEM as shown in Figure 3. Mannose-decorated particles (Figure 3a) assembled into small aggregates within 30 minutes after the addition of con-A (Figure 3b), and then into a networked gel after 12 h (Figure 3c). The gel was broken up into discrete particles upon the addition of a large excess of glucose (Figure 3d).

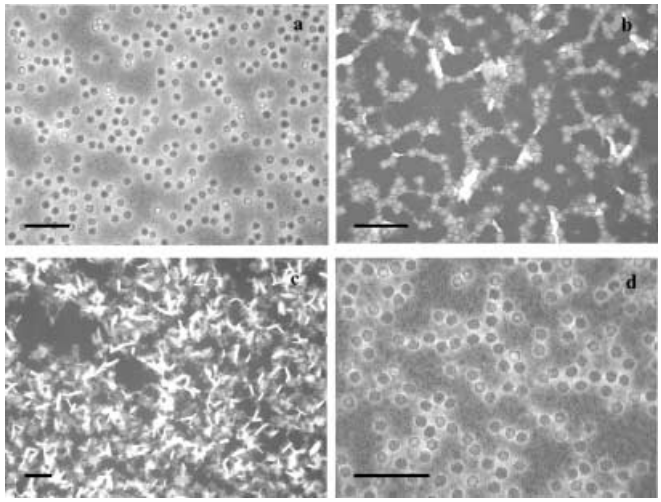

Figure 3. *Negative-stained TEM images (scale bars = 200 nm). a) Virus* ***2a*** *at a concentration of 0.2 mg mL^{-1}. b) Agglutination between* ***2a*** *(0.2 mg mL^{-1}) and con-A (93 μg mL^{-1}; approximately 116:1 molar ratio of con-A tetramer to virus particles) in PBS buffer (0.1 mM Ca^{2+} and Mn^{2+}) after 30 min at room temperature; c) Solution b after incubation at 4 °C overnight; d) Addition of glucose (70 mg mL^{-1}) to sample c.*

The sequence of aggregation and dissociation steps was also detected with fluorescence resonance energy transfer (FRET), by employing commercially available tetramethylrhodmaine-labeled con-A (**4**, with an average of 2.5 rhodamine units per con-A monomer).[13] In mixtures of **3a** and **4**, the emission intensity of fluorescence was quenched in proportion to the amount of **4** employed (Figure 4). No quenching was observed when con-A in the absence of rhodamine or CPMV-fluorescein without mannose was employed. Addition of soluble glucose or mannose (but not galactose) restored the fluorescence intensity to its former value. These observations suggest that the binding of con-A to mannosylated virus units brings some of the fluorescein and rhodamine molecules into sufficiently close proximity (55 Å being the Förster radius for this pair) to engage in FRET. The use of FRET to detect the formation of clusters of receptors for a polymer-displayed carbohydrate has been

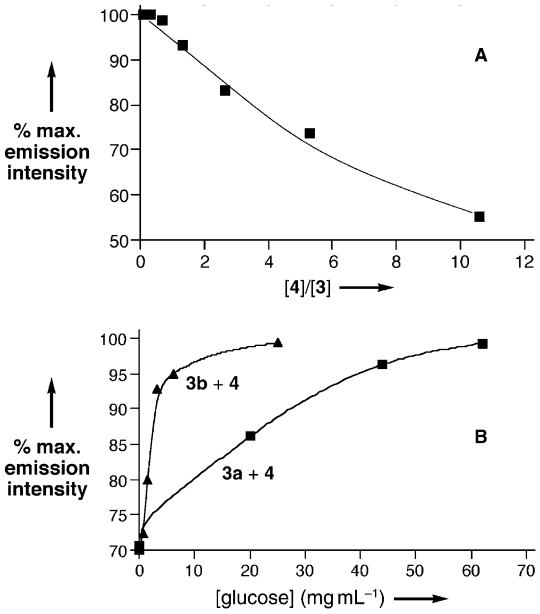

*Figure 4. A) Quenching of emission at 518 nm (excitation 495 nm) in mixtures of **3a** (0.27 mg mL^{-1}) and **4** adjusted to give the indicated ratios of dye units. B) Variation in emission intensity upon addition of glucose to the indicated mixtures of **3** (0.27 mg mL^{-1}) and **4** (0.16 mg mL^{-1}). [**3**] in plot A is the concentration of protein asymmetric unit, which is 60 times the concentration of virus particles.*

described.[15] A sensor for soluble glucose[16] of tunable sensitivity can thereby be constructed (Figure 4). Thus, while high concentrations (10 – 50 mg mL^{-1}) of glucose are required to break up the gel formed by **3a** and **4**, a more sensitive aggregate (responding to 1 – 4 mg mL^{-1} glucose) is produced with **3b**. The latter particle displays fewer mannose units and therefore forms a less robust network with con-A. Virus **3c**, containing fewer mannose groups still, does not form a gel with con-A at similar concentrations to the other particles.

The relative strength of con-A networks that use virions decorated with different numbers of mannose units was further illustrated in two ways. First, retention times on a con-A – Sepharose column, eluted with increasing concentrations of mannose, were distinctly different (Figure 5): wild-type eluted in the void volume with no mannose (20 min); virus **2c** at 43 min (ca. 0.4 *M* mannose); and **2a** at 55 min (ca. 0.7 *M* mannose).[13] Second, a quantitative estimate of the polyvalency exhibited by the virus particle was obtained with a standard hemagglutination assay by using rabbit erythrocytes.[13] Mannose units presented on virus scaffolds **2a** and **2c** were 690 and 890 times more efficient, respectively, than monomeric mannose at inhibiting erythrocyte agglutination with con-A.[13] Comparable values have been reported for mannosylated 5th- and 6th-generation PAMAM dendrimers (values of ca. 500 and 700 for structures bearing 95 and 172 sugars, respectively)[6a] and high-molecular-weight side-chain glycopolymers (values of ca. 2000).[5d]

These trends can be rationalized by considering the size of con-A tetramers, which have saccharide binding sites spaced approximately 65 Å apart.[17] Steric crowding prevents the binding of more than a few con-A units to mannose on small

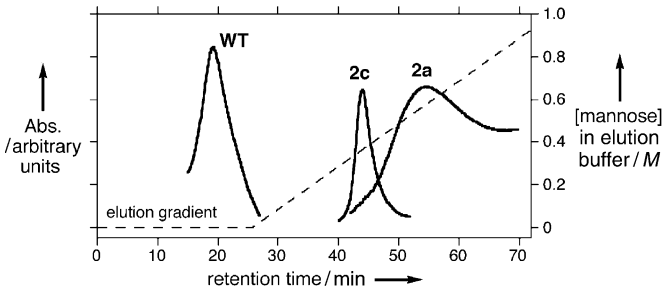

*Figure 5. Chromatographic analysis of underivatized wild-type (WT) and mannosylated (**2a** and **2c**) CPMV by using a con-A-Sepharose column. An overlay of the major peaks (comprising > 80 % of the integrated area) from three samples is shown; the remainder of the chromatograms are omitted for clarity.*

dendrimers,[6a,d] whereas the virus scaffold is large enough to allow for efficient binding. The side-chain glycopolymers are highly flexible; this is thought to alleviate steric crowding and allow for high effective polyvalencies. Wild-type CPMV provides mannose spacings well matched to the con-A dimension: K38 sites are 42 Å apart within each pentamer unit and 86 Å apart between pentamers. The aryl spacer used to connect each mannose to the lysine side chain may allow the con-A tetramer to bind tightly to two adjacent mannose fragments within pentamers and certainly between pentamers. Of course, the distances between carbohydrates on **2a** and **3a** are not certain, since lysines other than K38 are derivatized.

The larger polyvalent effect in the case of the virion with a lower density of mannose units on the surface (**2c** vs. **2a**), presumably reflects a case of diminishing returns—not all of the additional mannose units on **2a** can be bound, and so the affinity per mannose unit is not as high: on a per-virion basis, **2a** outperforms **2c** (83 000 vs. 10 700 per mole of virus vs. mole of free mannose). Virus **2a** interacts more strongly with con-A units in the chromatography discussed above, because it presents more opportunities for contact with the surface-bound lectin, and a networked structure is not involved. Further investigations of the effect of tether length, flexibility, position, and overall loading on the polyvalency of carbohydrate-decorated virus particles are underway.

We have shown here for the first time that a virus can be artificially patterned with carbohydrate units in a well-defined fashion and that the products function as polyvalent particles in interactions with a complementary lectin. Furthermore, the nature of the polyvalent interaction was shown to depend upon the density and position of functionalization. The use of a scaffold such as CPMV, with a known and relatively rigid protein structure, provides the opportunity to place such functional groups as sugar molecules at precise distances from each other and in a variety of steric environments. We anticipate that many applications of such polyvalent structures to biological and materials science targets, will benefit from these attributes.

Experimental Section

General Procedure for Modification of CPMV with Chemical Reagents: Organic reagents were introduced into a solution of

virus, such that the final solvent mixture was composed of 80% buffer and 20% DMSO. Following incubation at 4 °C for 24 – 48 h, the mixture was purified by passage through a P-100 size-exclusion column (centrifugation at 800 *g* for 3 – 5 min). This filtration was repeated with fresh columns until all the excess reagents were removed (typically 2 – 3 times). Purification of larger quantities of derivatized virus (> 1 mg) was performed by ultracentrifugation at 42 000 rpm (Beckman 50.2 Ti rotor) over a 2 cm-high sucrose cushion, followed by solvation of the resulting material in buffer. Mass recoveries of derivatized viruses were typically 60 – 80%; all such samples were composed of > 95% intact particles as determined by analytical size-exclusion FPLC. Virus concentrations were measured by absorbance at 260 nm; virus at 0.1 mg mL^{-1} gives a standard absorbance of 0.8. Fluorescein concentrations were obtained by measurement of absorbance at 495 nm, and applying an extinction coefficient of 77 000 M^{-1} cm^{-1}, determined experimentally by mixing measured quantities of dye with CPMV (1 mg mL^{-1}). Each data point is the average of values obtained from three independent parallel reactions. The average molecular weight of the CPMV virion is 5.6×10^6.

CPMV – Mannose Conjugate 2b: Wild-type CPMV (10 mg) and α-D-mannopyranosyl phenylisothiocyanate (50 μmol, approx. 500-fold molar excess per viral subunit) were gently agitated in a mixed solvent of DMSO (0.25 mL) and $NaHCO_3$ buffer (pH 9, 0.1 M, 1 mL) at 4 °C for 48 h. The reaction mixture was purified by one passage through a P-100 size-exclusion column and then by dialysis (M_W cutoff of membrane = 12 000) into potassium phosphate (0.1 M) at pH 7. This was followed by two passages through P-100 size-exclusion columns to give **2b** (6.1 mg, 61% yield).

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