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Chemical modification of a viral cage for multivalent presentation

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Here we present generalized methods for chemically modifying the surface of a viral protein cage; this exploits the chemistry of native and engineered surface exposed functional groups for multivalent presentation of ligands.

Viral protein cages provide versatile platforms for modification[1] and multivalent presentation of ligands.[2–4] The icosahedral viral protein cage of the Cowpea chlorotic mottle virus (CCMV) assembles from 180 identical protein subunits.[5] Each subunit presents multiple surface exposed functional groups that can potentially be chemically modified. Here we present initial studies illustrating our derivitization of the viral cage using both endogenous functional groups and engineered site-specific functional groups. The cage-like architecture of viral particles defines a spatial division between the inside and outside. The inside of the CCMV protein cage has previously been shown to act as a host for the size constrained synthesis and encapsulation of both inorganic and organic materials.[1,6,7] The outside surface of CCMV presents the potential for multiple, highly symmetrically arranged, native or engineered reactive groups that can be chemically modified for site specific attachment of a variety of ligands.

We have chemically modified the exterior surface of CCMV with both fluorescent molecules and small peptides. We have quantified the degree of surface modification obtained *via* coupling of fluorophores to surface exposed amine (lysine), carboxylic acid (glutamate and aspartate), and engineered thiol (cysteine) residues. A 24 amino acid peptide was linked to the exterior surface of CCMV through exposed lysine and cysteine residues.

Based on a space filling model from the CCMV crystal structure,[8] we predict that up to 11 carboxylate groups (E63, E77, E81, E111, E148, E166, D128, D132, D153, D168, D183) on each subunit may be exposed on the exterior surface (Fig. 1). Similarly, there are up to six surface exposed amine groups (K54, K84, K87, K65, K106, K131) per subunit. In addition, we have engineered two surface exposed thiol groups (C82, C141) on the exterior of the virus. This provides an estimate of up to 1980, 1080 and 360 potential sites for each of these functional groups, respectively, on the assembled protein cage.

Chemical modification of surface exposed carboxylate groups were activated by reaction with 1-[3-(dimethylamino-)propyl]-3-ethylcarbodiimide hydrochloride (DEC) and *N*-hydroxysuccinimide (NHS) to generate the succinimidyl ester.[9] This was subsequently reacted with an amine containing fluorophore (5-((5-aminopentyl)thioureidyl)eosin (eosin cadaverine)). CCMV (100 μg, 5.2 nmol) was incubated with 550 mol excess to subunit of NHS (331.4 μg, 2.86 μmol) and DEC (546.8 μg, 2.86 μmol) at room temperature in 250 μL volume (100 mM sodium acetate, pH 5.5). After 1 h the reaction volume was increased to 1000 μL using HEPES (100 mM, pH 7.0), fluorophore was added and the reaction was allowed to progress for 2 h at room temperature. A range fluorophore concentrations was used (100–1000 mol excess of fluorophores per viral subunit, 520–5200 nmol) resulting in different degrees of modification (Table 1). Five hundred and sixty of the potential 1980 reactive sites were labelled at 1000 mol excess.

Surface exposed amine groups were derivitized using the succinimidyl ester of (5- (and-6)-carboxyfluorescein, (5(6)-FAM, SE). Typical reactions (1000 μL) were performed in HEPES (100 mM, pH 7.0) with 5.2 nmol of CCMV for 2 h at room temperature. A range of excess fluorophore was used (100–1000 mol excess of fluorophores per viral subunit, 520–5200 nmol). A maximum of 540 fluorophores could be attached to the cage by this method (Table 1).

Neither of the two native cysteines (59 and 108) in the wild type virus is reactive towards chemical modification (unpublished data). An engineered form of CCMV with surface exposed cysteine residues (R82C and A141C) was used as a template for thiol-based modifications. The cysteine thiol

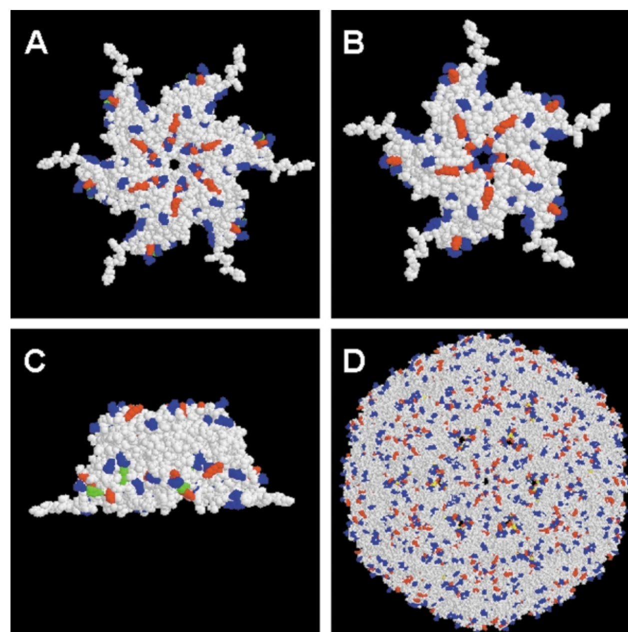

Fig. 1 Space filling model of the exterior surface of CCMV (pdb file 1CWP). Lysines are indicated in red, carboxylates in blue and the engineered cysteines in green. Panels A and B are views at the six- and five-fold axes, C a side view of a five-fold axis and D of the entire cage.

Table 1 Extent of labeling of CCMV using fluorophores that react with different functional groups

Fluorophore	Mol excess	Dye molecules (av.)
FAM (lysines)	100	180/cage
	300	380/cage
	1000	540/cage
F5M (sulfhydryls)	100	30/cage
	500	60/cage
	1000	100/cage
Cadaverine (carboxylic acids)	100	500/cage
	500	520/cage
	1000	560/cage
SAMSA (bifunctional linker to lysines)	250	45/cage
	500	95/cage
	1000	150/cage

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groups on CCMV (10.4 nmol) were reacted with a maleimide modified fluorophore (fluorescein-5-maleimide) in 1000 μL reaction volume (100 mM HEPES, pH 6.5) at room temperature for 2 h. Fluorophore was present at 100–1000 mol excess per subunit (520–5200 nmol). As shown in Table 1, a maximum of 100 thiol groups could be labelled, or approximately one third of the total number available.

An additional approach for labeling viral cages utilizes linkers having bifunctional reactive groups. Surface lysine residues were initially reacted with the bifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) in 100 mM HEPES (pH 7.0) for 2 h and subsequently purified from unreacted linker by Microcon ultrafiltration (100 kD MWCO). This modified CCMV was reacted with the deprotected fluorescent dye 5-((2-(and-3)-*S*-(acetylmercapto)succinoyl amino) fluorescein (SAMSA fluorescein) in HEPES (100 mM, pH 6.5) for 2 h at room temperature. In these reactions, using a range of fluorescein mol ratios (520–5200 nmol), we obtained derivatization of up to 150 lysine residues per protein cage (Table 1).

Using the surface exposed cysteine residues we have attached a 24 amino acid peptide having antitumorgenesis properties to the exterior of the CCMV mutant (A163C). This was achieved by an oxidative coupling of cysteine thiols on CCMV (1.8 μg, 93 pmol) to the free thiol of the peptide (1 μg, 385 pmol) in the presence of 1 mM $CuSO_4$ in 10 μL (50mM sodium acetate, pH 4.8). Based on altered mobility of the protein subunits on SDS PAGE (non-reducing) an estimate of up to 25% (45) of the subunits could be derivatized with the peptide. The biological activity of these materials is under investigation.

In all labeling experiments we determined that the virus particles were still intact and monodisperse after labeling by dynamic light scattering (DLS), transmission electron microscopy (TEM) and velocity sedimentation on sucrose gradients. DLS demonstrated an average particle diameter of 27 nm before and 37 nm after labeling (Fig. 2A and B). Analysis of the TEM images confirmed that intact particles were present before and after labeling (Fig. 2C and D) and that they underwent a similar size increase (25 nm diam. before and 32 nm after labeling). Sucrose gradients of modified CCMV were fractionated and the fluorescence of each fraction measured (Fig. 3A). The fluorescence peak of fraction 7 corresponded to the sedimentation velocity of the wild type CCMV (88S) as well as the UV absorbance (280/260) peak due to the protein and nucleic acid in the viral cage (Fig. 3B). It is apparent that, while the particles remain intact during the labeling procedure, the diameter of the particles also increases. Addition of the fluorophore alone cannot account for this size increase, which might result in part from a swelling[5] of the viral particle upon derivatization.

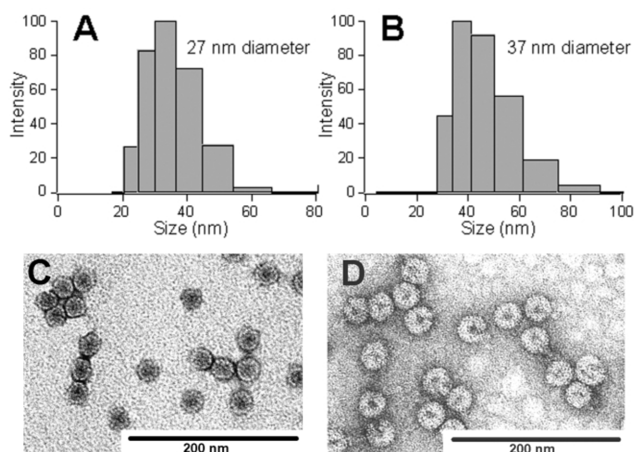

Fig. 2 Dynamic light scattering of unlabelled (A) and fluorophore labelled (B) CCMV. Transmission electron micrograph of unlabelled CCMV (C) and fluorophore labelled CCMV (D). Size bar is 200 nm.

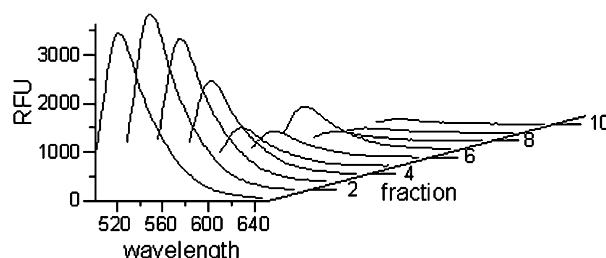

Fig. 3 Fluorescence spectra of labelled viral cages fractionated on a sucrose gradient. Peaks of fluorescence were observed at the gradient top (fractions 1–4) as well as in fraction 7. The fluorescence at the top of the gradient is due to the presence of unincorporated fluorophore. The peak in fraction 7 corresponds to presence of the viral cage protein in this fraction.

These results illustrate the range of chemistry available for modification of these versatile protein cages. The degree and spatial distribution of the modification can also be controlled. Our data indicates that the extent of labeling is lower than the upper estimate predicted from consideration of the crystal structure but provides a better representation of the reactive functional groups on the virus particle. This has been demonstrated using endogenous reactive groups, distributed over the surface of the protein cage, and using engineered reactive groups at site-specific locations. This approach is equally amenable to the attachment of both small organic molecules and large polymeric species. The future of this approach will be to use these multivalent protein cages to attach different ligands to a single common platform. Modifications to the exterior surface, coupled with the ability to act as a host for molecular entrapment, greatly enhances the utility of these viral templates for both materials and biomedical applications.

Notes and references

1 T. Douglas, E. Strable, D. Willits, A. Aitouchen, M. Libera and M. Young, *Adv. Mater.*, 2002, **14**, 415.
2 Q. Wang, E. Kaltgrad, T. Lin, J. E. Johnson and M. G. Finn, *Chem. Biol.*, 2002, **9**, 805.
3 Q. Wang, T. Lin, J. E. Johnson and M. G. Finn, *Chem. Biol.*, 2002, **9**, 813.
4 Q. Wang, T. W. Lin, L. Tang, J. E. Johnson and M. G. Finn, *Angew. Chem., Int. Ed.*, 2002, **41**, 459.
5 J. A. Speir, S. Munshi, G. J. Wang, T. S. Baker and J. E. Johnson, *Structure*, 1995, **3**, 63.
6 T. Douglas and M. Young, *Nature*, 1998, **393**, 152.
7 T. Douglas and M. Young, *Adv. Mater.*, 1999, **11**, 679.
8 V. Reddy, P. Natarajan, B. Okerberg, K. Li, K. Damodaran, R. Morton, C. Brooks III and J. E. Johnson, *J. Virol.*, 2001, **75**, 11943.
9 G. T. Hermanson, in *Bioconjugate Techniques*, ed. G. T. Hermanson, Academic Press, San Diego CA, 1996, pp. 169–186.