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# Hybrid Virus−Polymer Materials. 1. Synthesis and Properties of PEG-Decorated Cowpea Mosaic Virus

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Cowpea mosaic virus was derivatized with poly(ethylene glycol) to give well-controlled loadings of polymer on the outer surface of the coat protein assembly. The resulting conjugates displayed altered densities and immunogenicities, consistent with the known chemical and biological properties of PEG. These studies make CPMV potentially useful as a tailored vehicle for drug delivery.

## Introduction

Viruses are both important biological entities and architectural assemblies of fascinating structure and diversity, which have been harnessed by chemists only recently. Chemistry-based studies have included the organization of inorganic materials in or around virus cages[1−4] and the conjugation of a toxin to the packaged genome of a bacteriophage.[5] We have recently initiated a broad exploration of virus particles as chemical building blocks, focusing on cowpea mosaic virus (CPMV) as a prototype.[6−8] This plant virus can be made and purified in large quantities,[9,10] is structurally characterized to near atomic resolution,[11] is stable to a variety of conditions compatible with both hydrophobic and hydrophilic molecules,[6] and can be manipulated at the genetic level to introduce mutations at desired positions.[12−16] We have taken advantage of these properties to demonstrate the attachment of lysine- and cysteine-reactive small molecules to specific positions on the CPMV coat protein (capsid).[7,8] One of our goals is to bring new functions to CPMV by attaching macromolecules to the capsid, thereby generating novel species with medicinal or diagnostic applications. As a first step, we describe here the selective attachment of poly(ethylene glycol) (PEG) units to CPMV and the chemical and physical characterization of such constructs. PEG has previously been attached to the capsid surface of adenovirus in attempts to moderate the immune response, which must be controlled if viruses are to be used as delivery vectors for gene therapy.[17−21] Tobacco mosaic virus has also been derivatized with PEG.[22]

## Experimental Section

**Reagents and Procedures.** Fluorescein-PEG-NHS (MW 2000 and 5000) and methoxy-PEG-NHS (MW 2000 and 5000) were purchased from Shearwater Co. Unless otherwise noted, "buffer" refers to 0.1 M potassium phosphate, pH 7.0. Wild-type cowpea mosaic virus was prepared by the standard method[9,10] and was stored at approximately 10 mg/mL. Size exclusion columns for purification of virus from reaction mixtures were prepared and used as previously described.[7] Sucrose gradient ultracentrifugation analysis (as in Figure 3B) was performed on 30 mL gradients made of 20% (w/w) sucrose solution in 0.1 M potassium phosphate buffer pH 7.0, frozen at −20 °C, and defrosted just before use, with centrifugation at 28 000 rpm for 3 h using a Beckman SW28 rotor, giving rise to well-separated bands. Transmission electron microscopy (TEM) analyses were performed by depositing 20 $\mu$L aliquots of each sample onto 100-mesh carbon-coated copper grids for 2 min. The grids were then stained with 20 $\mu$L of 2% uranyl acetate and viewed with a Philips CM100 electron microscope. Electronic spectra were recorded using an Agilent 8453 spectrometer. Fast protein liquid chromatography (FPLC) analyses were performed with AKTA Explorer (Amersham Pharmacia Biotech) equipment, using Superose-6 size-exclusion columns and 0.05 M potassium phosphate buffer (pH 7.0) as eluent. Intact virions show retention times of approximately 25 min at an elution rate of 0.4 mL/min, whereas broken particles and individual subunit proteins elute after 50−60 min.

**General Procedure for Modification of CPMV with Poly(ethylene glycol).** Wild-type CPMV (1−2 mg/mL) was incubated with poly(ethylene glycol) NHS ester in 0.1 M $NaHCO_3$ buffer (pH 8.4) at 4 °C for 48 h. Various PEG-to-virus ratios were used to assay the reactivity of CPMV, as shown in Figure 2. In all cases the virus products were purified by passage through a P-100 size exclusion column (centrifugation at 800*g* for 3−5 min), followed by pelleting through a sucrose gradient. The latter procedure employed 30 mL gradients (made of 20% (w/w) sucrose solution in 0.1 M potassium phosphate buffer pH 7.0, frozen at −20 °C, and defrosted just before use), centrifugation at 28 000 rpm (Beckman SW 28 rotor), followed by 42 000 rpm

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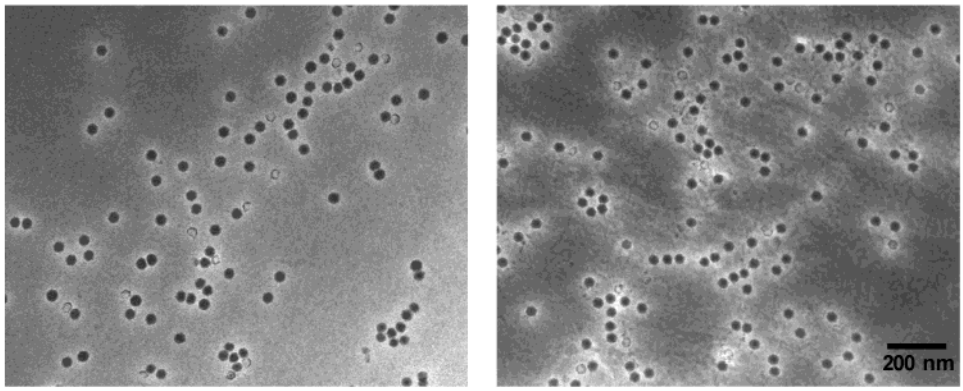
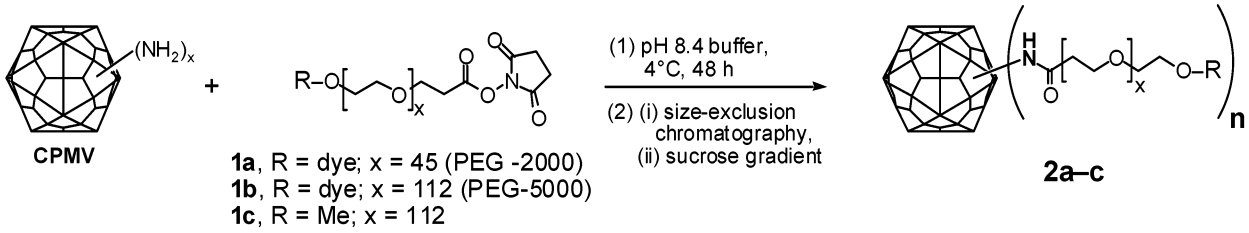



**Figure 1.** TEM images of CPMV (left) and CPMV−PEG construct **2a** (right), both at 0.2 mg/mL.

**Scheme 1**




**1a**, R = dye; x = 45 (PEG -2000)
**1b**, R = dye; x = 112 (PEG-5000)
**1c**, R = Me; x = 112

(Beckman 50.2Ti rotor), and resuspension in buffer. Mass recoveries of derivatized viruses were typically 60−80%; all such samples were composed of essentially intact particles as determined by analytical size-exclusion FPLC, TEM, and the observation of the characteristic $A_{260}/A_{280}$ (protein/RNA) absorbance ratio of 1.60 ± 0.1. Virus concentrations were measured by absorbance at 260 nm; the virus at 0.1 mg/mL gives a standard absorbance of 0.8. The average molecular weight of the CPMV virion is $5.6 \times 10^6$. The number of PEG chains attached per virion was determined from the ratio of the intensities of dye (496 nm) to protein (260 nm) absorbance peaks in the electronic spectrum, using calibrated values of molar absorptivity for the chromophores.[6] The reported PEG loadings on the virons are the average of three independent reactions, which showed a variation of less than 7%.

**Animals and Immunizations.** C57BL/6J female mice were immunized on days 0 and 56 with 10 μg of wild-type CPMV, CPMV−PEG **2c** ($n = 29 \pm 3$), or CPMV−PEG-Fluorescein **2b** ($n = 29 \pm 3$). The control group received PBS buffer. Each group was composed of five animals. Antigen was administered subcutaneously at days 0 and 56, and the animals were bled on days 0, 8, 14, 21, 28, 56, 64, 70, 77, and 84.

**Detection of CPMV-Specific Antibodies.** Anti-CPMV antibodies were detected by enzyme-linked immunosorbent assay (ELISA) by a previously described method.[26] Briefly, 100 μL/well of wild-type CPMV (10 μg/mL) in 100 mM $NaHCO_3$ (pH 8.5) was used to coat flat-bottomed 96-well plates (Immulon 2, Fisher). The plates were incubated overnight at room temperature and blocked with 3% milk in Tris-buffered saline (TBS; 0.02 M Tris, 0.14 M NaCl, pH 7.0) for 2 h at room temperature. Serum dilutions ($10^3$, $10^4$, $10^5$, and $10^6$) were made in TBS containing 0.05% Tween 20/1% milk; 100 μL of each serum dilution was used per well and the plates were incubated for 1 h at room temperature. After three washes in TBS 0.05% Tween 20, biotinylated sheep-anti-mouse Ig (Amersham) was added and incubated for 1 h at room temperature. After three washes, streptavidin-alkaline phosphatase conjugate (Amersham) was added, and the plates were incubated for 1 h at room temperature, followed by washing three times. The substrate *p*-nitrophenyl phosphate (Sigma) was added (100 μL/well), and the plates were incubated for 20 min at 37 °C. The reaction was stopped using 50 μL of 2 N NaOH and quantified using an automated ELISA reader at 405 nm.

## Results and Discussion

Commercially available poly(ethylene glycol) samples, capped at one end with an *N*-hydroxysuccinimide ester and at the other with either a methoxy group or fluorescein (compounds **1** in Scheme 1), were mixed with CPMV (pH 8.4 buffer) for 48 h at various molar ratios to accomplish attachment to lysine residues on the exterior of the virus capsid. In all cases the virus products were purified by filtration through size-exclusion resin spin columns to remove the excess PEG reagent, followed by sucrose gradient sedimentation, ultracentrifugation pelleting, and resuspension in 0.1 M potassium phosphate buffer (pH 7.0). The resulting samples were shown to be composed of intact virus particles by their sedimentation properties in sucrose gradients (see below), by the observation of characteristic $A_{260}/A_{280}$ (protein: RNA) absorbance ratios, by the observation of dominant peaks at characteristic retention times on size-exclusion FPLC, and by transmission electron microscopy analysis (Figure 1). The number of PEG chains attached per virion was determined from the ratio of the intensities of dye (496 nm) to protein (260 nm) absorbance peaks in the electronic spectrum, using calibrated values of molar absorptivity for the respective chromophores.[6]

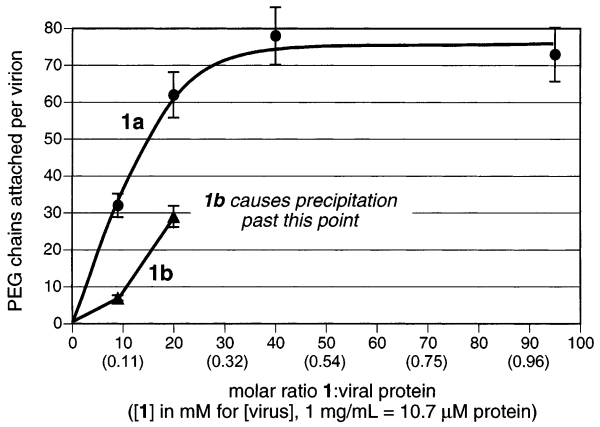



**Figure 2.** Attachment stoichiometries of PEG reagents **1a** and **1b**.

A plot of the loading of PEG on the virus vs the reagent stoichiometry is shown in Figure 2, demonstrating that significant levels of polymer attachment may be achieved. Under the most forcing conditions, more than one PEG-2000 chain per virus asymmetric protein unit (60 per particle) was covalently attached. In contrast, no more than 29 ± 3 PEG-5000 chains may be covalently attached to CPMV, presumably because of steric hindrance around the virus capsid and/or an intrinsically slower rate of bimolecular reaction with the larger PEG reagent. The use of higher concentrations of **1b**, required to get loadings higher than 30 per virion, causes precipitation of the virus, consistent with the fact that 6000−8000 MW PEG is used to precipitate CPMV out of solution in one of the steps of the virus purification protocol.[23] Lysine-38 of the small subunit has been shown to be the most reactive amine site at pH 7.0, while at pH 8.4 other exposed lysines become competitive; at most 4 lysine residues per asymmetric unit (240 per particle) may be addressed with small molecule NHS esters.[7]

That the viral protein is indeed covalently modified in these reactions was confirmed in several ways (Figure 3). Denaturing gel electrophoresis of **2b** bearing 29 PEG-5000-fluorescein units per capsid shows three dye-labeled bands, along with the two underivatized capsid proteins at lower molecular weight. The labeled bands are assigned to CPMV subunit proteins with either one or two PEG chains attached. The positions of these bands on the gel are displaced to a greater degree than would be expected on the basis of molecular weight alone. We presume this to be due to the highly different chemical nature of poly(ethylene glycol) as compared to protein, leading to very different electrophoretic behavior. Sucrose gradient ultracentrifugation supports this analysis. For example, **2a** having 29 ± 3 PEG-5000 chains

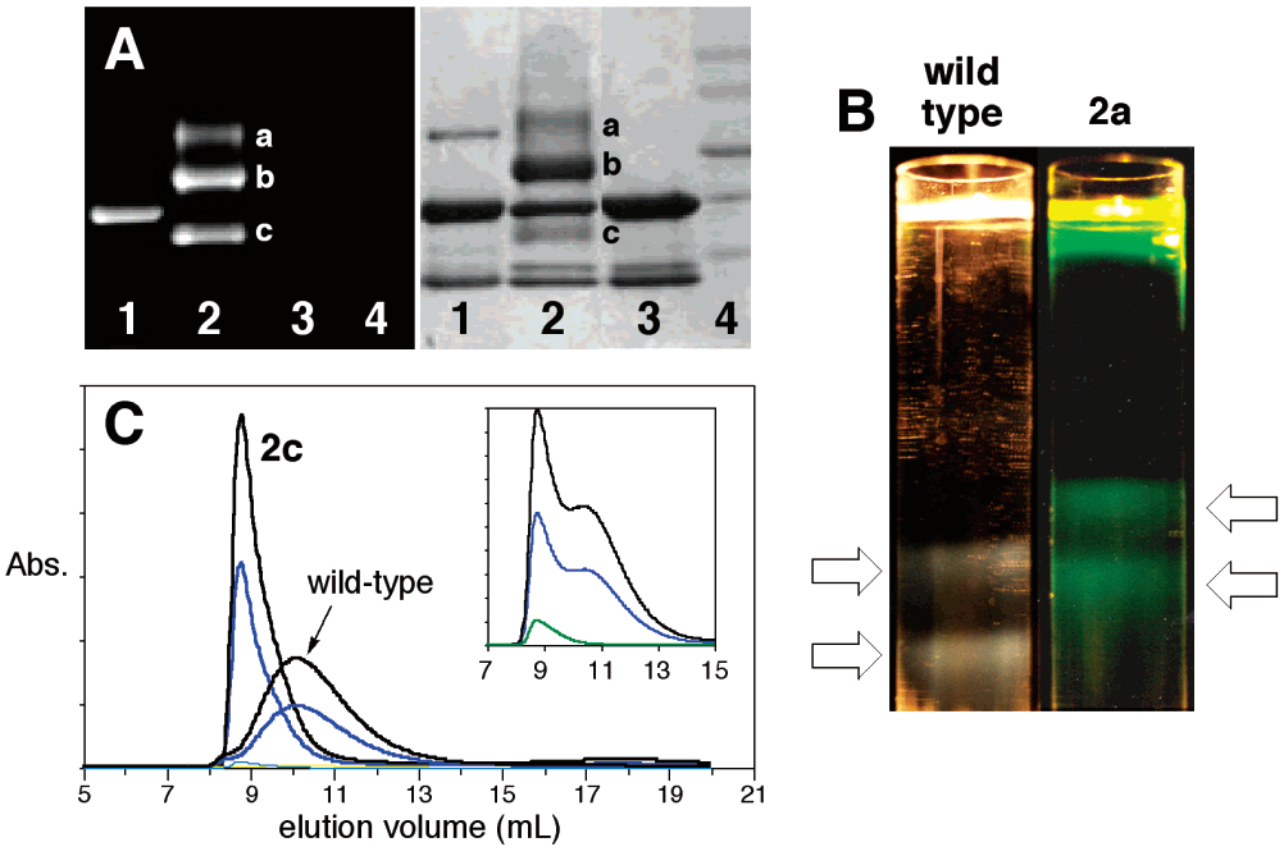



**Figure 3.** Analysis of PEG-derivatized viruses. (A) SDS−PAGE analysis of CPMV−*N*-PEG−fluorescein reactions, visualized under ultraviolet light before (dark background) and after (light background) Coumassie blue staining: (lane 1) CPMV-*N*-fluorescein, with specific dye labeling on the CPMV large subunit; (lane 2) **2b**, with 29 ± 3 PEG−fluorescein units per capsid; (lane 3) underivatized wild-type CPMV; (lane 4) protein size ladder. The marked bands in lane 2 are assigned as follows: (a) large subunit + two PEG-5000 chains; (b) large subunit + one PEG-5000 chain; (c) small subunit + one PEG-5000 chain. Note the presence of both derivatized and underivatized bands in the Coumassie stain of lane 2, consistent with the value of $n = 29$ for loading of PEG on the capsid. (B) Sucrose gradient ultracentrifugation analysis of wild-type CPMV and **2a** ($n = 29 \pm 3$). Note the green color of the fluorescein-containing PEG. The two observed bands are characteristic of the two RNA-packaged forms of CPMV.[7] (C) Overlayed size-exclusion FPLC chromatograms (Superose-6, 0.05 M potassium phosphate buffer, pH 7.0) of wild-type CPMV and **2c** ($n = 29 \pm 3$), showing the shortened retention time of the latter. (inset) FPLC of a mixture of wild-type CPMV and **2b** ($n = 29 \pm 3$). Traces recording absorbance at 260 nm (black), 280 nm (blue), and 495 nm (green) are shown.

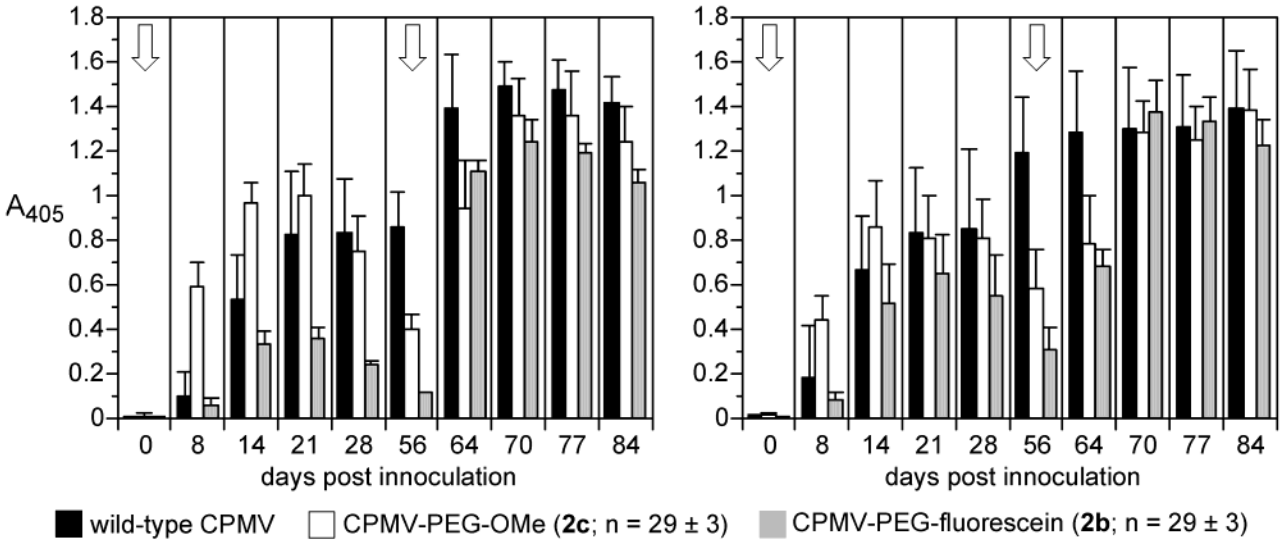



**Figure 4.** Relative concentrations of serum antibodies against CPMV in mice determined by ELISA as a function of the species used for inoculation (at days 0 and 56, indicated by arrows): (left) inoculations performed without alum as adjuvant, (right) inoculations performed with alum. Values for control experiments (inoculation with buffer) are all <0.02 absorbance units and are therefore not shown.

attached shows the two bands characteristic of CPMV, covalently labeled with green fluorescein dye. However, the positions of these bands show the PEGylated virus to be substantially less dense than the CPMV starting material, although only 145 kDa of mass was added by the PEG groups to the $5.6 \times 10^6$ Da particles. Size-exclusion FPLC is also consistent, showing shorter retention times for the CPMV−PEG conjugates, consistent with greater effective molecular size (Figure 3).

PEGylation of biomacromolecules is a common strategy to reduce their immunogenicity.[24,25] Adenovirus has been investigated intensively, with the goals of moderating the mammalian immune response or improving the bioavailability and/or pharmacokinetics so as to facilitate the use of adenovirus vectors for gene delivery.[17−21] We are interested in using tailored CPMV particles to selectively target tissues for diagnostic and therapeutic purposes, so we have performed a preliminary assessment of the change in immunogenicity occasioned by PEG coverage at the level described above. CPMV-specific antibodies in the serum of mice inoculated subcutaneously with CPMV or one of two CPMV−PEG conjugates was monitored by a standard ELISA protocol over nearly 4 months, as shown in Figure 4. PEG-decorated virus **2c** gave rise to comparable or enhanced levels of immune response compared to wild-type virus immediately after the initial challenge, but serum antibody levels decreased markedly to well below those for wt-CPMV-immunized mice by day 56. PEG-fluorescein conjugate **2b** proved to be more effective in suppressing primary antibody responses throughout the initial 56-day period while the secondary responses were similar to wild type. Inoculations in the absence of adjuvant gave clearer distinctions between the CPMV species used than those using alum adjuvant, and a boost injection of CPMV conjugates at day 56 induced strong immunogenic responses throughout.

Together these results suggest that in particular the fluorescein-PEG moiety is most effective at shielding the CPMV particles from inducing a primary antibody response. While these results are difficult to compare quantitatively with those for PEG-decorated derivatives of adenovirus (which are analyzed by other methods),[17,20] the efficiency of PEG in reducing the primary mammalian antibody response toward virus particles is roughly equivalent for the two systems. Since a fluorescein group at the terminus of a PEG chain is likely to induce much different conformational and/or dynamic properties of the polymer compared to the methoxy-terminated analogue, we speculate that PEG−fluorescein conjugation was more effective than PEG−OMe in moderating the immune response because of differences in the solution-phase presentation of the PEG chains in these two cases. Alternatively, the relative sizes of PEG or PEG−fluorescein might influence their differential ability to shield the particle from antibody recognition. In addition, it is possible that a higher number of attachments of lower molecular weight version of PEG might provide more efficient shielding of the particle surface and a further reduction in immunogenicity; these studies are in progress.

The results described here demonstrate that the exterior surface of the CPMV coat protein can be derivatized with poly(ethylene glycol) and that the resulting constructs have markedly different physical and immunogenic properties compared to the native virus. Studies involving variations in the density, location, and type of coverage of CPMV with PEG and other polymers, and the effects of such modifications on biological properties, are ongoing in our laboratories.

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