Versatile post-functionalization of the external shell of Cowpea Chlorotic Mottle Virus by using click chemistry

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We present the modification of the outer protein shell of Cowpea Chlorotic Mottle Virus (CCMV) with linear and strained alkyne groups. These functionalized protein capsids constitute valuable platforms for post-functionalization *via* click chemistry. After modification, the integrity of the capsid and the reversible disassembly behavior are preserved.

Introduction

Self-assembled, viral protein cages have shown to be of great interest as biological building blocks for material sciences.[1] With three distinct interfaces[2] that can be addressed either chemically or genetically, these protein nanocages can be adapted to produce multifunctional biological nanoparticles for applications in medicine, catalysis and nanotechnology.[3]

Due to their genetic encoding, icosahedral viral protein cages are well-defined, monodisperse in size and highly symmetrical. Hence their exterior surface shows a uniform distribution of functional handles,[4] available for chemical modification. Among others, viruses have been decorated with metal nanoparticles, fluorescent dyes, polymers, drugs, carbohydrates, peptides, biotin, DNA and luminescent quantum dots.[5] However, as a consequence of the overabundance of chemical functionalities displayed by the protein shell, selective bioconjugation is limited. Hence, in order to control the degree of functionalization, it is desirable to have a non-natural functional group present, which can be addressed orthogonally.

The copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC), often referred to as "click reaction", has proven to be a valuable bioorthogonal functionalization strategy.[6] The CuAAC reaction is known for its efficiency, compatibility with numerous functional groups and high tolerance towards different solvents (including water) as well as variations in pH and temperature. To overcome toxicity issues in *in vivo* applications, Bertozzi and co-workers have developed several strained cyclooctynes to perform copper-free click chemistry, through the Strain-Promoted Azide-Alkyne Cycloaddition reaction (SPAAC).[7] Click chemistry, either copper-mediated or copper-free, has been applied widely in, *e.g.*, drug target synthesis,[8] surface functionalization[9] and enzyme and protein modification[10] including both spherical and rod-shaped protein shells of viruses like *cowpea mosaic virus* (CPMV)[11] and *tobacco mosaic virus* (TMV).[12] These last examples emphasize the potential of click chemistry as a covalent post-functionalization method for viral protein cages. CPMV has been studied extensively due to its remarkable tolerance towards pH and temperature changes. However, compared to rod-like TMV, which can be assembled around a template *in vitro*, CPMV only has the ability to enclose specific small molecules by infusion and retain them by covalent binding or non-covalent interaction with its RNA.[13] To be able to encapsulate larger templates like polymers and metal nanoparticles, we turned to the *cowpea chlorotic mottle virus* (CCMV) as the nanocarrier of interest.

CCMV is an icosahedral plant virus consisting of 180 identical coat proteins of approximately 20 kDa, which form a capsid with triangulation number T = 3 around the single-stranded viral RNA (Figure 1A). The capsid has inner and outer diameters of approximately 18 and 28 nm, respectively. A particularly interesting feature of CCMV, compared to other spherical viruses such as CPMV, lies in its defined and reversible assembly behavior.[14] Depending on the pH and ionic strength, CCMV has the ability to disassemble into 90 coat protein dimers and reassemble into the empty, non-infectious virus-like-particle (VLP). The possibility for in vitro encapsulation of, in principle, any cargo, makes CCMV attractive as a nanocontainer in the development of smart materials.[15]

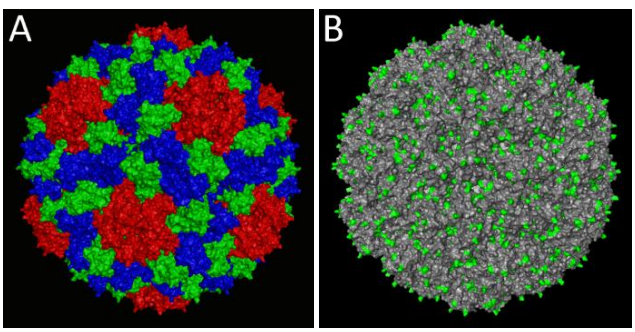
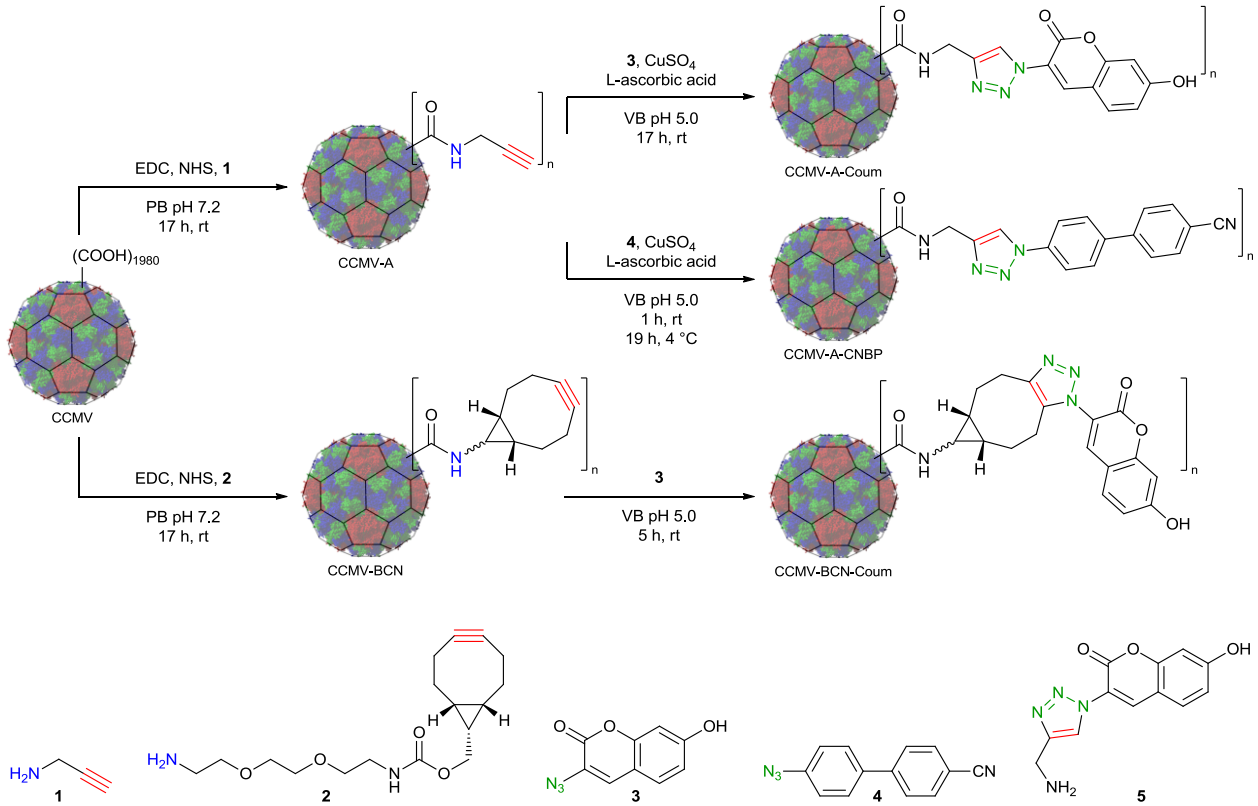


Figure 1. A) Space-filling representation of CCMV demonstrating the symmetrical protein assembly; B) CCMV where the exposed carboxyl residues are denoted in green.

CCMV has been functionalized with a variety of fluorophores, but, to the best of our knowledge, no examples have been reported for the modification of CCMV with alkyne or azide groups.

Herein, we describe the functionalization of the CCMV capsid, with either linear or strained alkyne groups to create versatile platforms for post-functionalization. These platforms can be targeted either by copper-mediated or by copper-free click chemistry, which allows for the preparation of multi-functionalized virus particles bearing fluorescent dyes or mesogen moieties that promote liquid crystallinity, under mild conditions. Importantly, these virus particles retained their structural integrity and self-assembling properties after functionalization, and consequently hold potential as building blocks for the design of hybrid materials.



Scheme 1. Functionalization of CCMV with linear or strained alkyne groups and post-functionalization by click chemistry with the complementary azides. CCMV: Cowpea Chlorotic Mottle Virus; EDC: *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; NHS: *N*-hydroxysuccinimide; PB: phosphate buffer; VB: virus buffer; the wavy bond in CCMV-BCN represents the linker in **2**.

Results and Discussion

The purification of CCMV was performed according to a literature procedure.[16] The native virus, *i.e.* the protein shell encapsulating the ss-RNA, was stored in virus buffer (VB) at pH 5.0 and 4 °C.

To introduce linear alkyne groups on the external shell of CCMV, the exposed carboxyl residues (predicted to be 11 per coat protein;[4] Figure 1B) were allowed to react with seven equivalents of propargyl amine (**1**) *via* an EDC coupling in phosphate buffer (PB) at pH 7.2 (Scheme 1). The solution was left overnight and was dialyzed back to VB afterwards. The integrity and monodispersity of alkyne-modified CCMV (CCMV-A) were preserved, as confirmed by fast protein liquid chromatography (FPLC; V_{CCMV} = 10 mL, Figure S5A) and transmission electron microscopy (TEM, Figure S6A).

The same reaction was performed with amine-terminated bicyclononyne (BCN) **2** on CCMV to produce particles with a strained alkyne moiety for copper-free click chemistry. Seven equivalents of **2** were added to CCMV in PB. BCN modified CCMV (CCMV-BCN) was purified from unreacted moieties by dialysis against VB and directly used for post-functionalization.

CCMV-A was reacted with 3-azido-7-hydroxycoumarin (**3**) in a copper-mediated click reaction to verify the presence of propargyl amine on CCMV. Azidocoumarin **3** is a profluorophore, *i.e.* it shows only minor fluorescence due to a quenching effect of the azide functional group.[17] Upon formation of the 1,2,3-triazole ring this quenching effect is suppressed and the intensity of the fluorescence increases, indicating a successful conjugation. Formation of the 1,2,3-triazole product is also supported by the appearance of an extra absorption band around λ = 404 nm.

CCMV-A (8.12 mg, 1.77 nmol) in VB was incubated overnight with copper sulfate, L-ascorbic acid as reducing agent and 49 equivalents of azidocoumarin **3** per coat protein (CP). Since **3** is only slightly soluble in aqueous solution, it was predissolved in DMSO before adding it to the virus solution (DMSO being < 1% of the total buffer volume). The coumarin-functionalized virus (CCMV-A-Coum) was purified by preparative FPLC, by monitoring the UV absorbance of the effluent simultaneously at λ = 260 and 280 nm for CCMV and at λ = 340 nm for coumarin. An elution peak at V = 9.4 mL was observed for both CCMV and coumarin (Figure S5B). This co-elution of the coumarin moiety verifies the success of the click reaction. The integrity and monodispersity of the virus after functionalization were confirmed by TEM (Figure 2B).

CCMV-A was also functionalized with 4-azido-4'-cyano-biphenyl moieties (**4**), whose mesogenic structure is commonly encountered in nematic liquid crystals, such as E7,[18] which shows

liquid crystallinity at room temperature. We anticipated that fixing **4** on CCMV would improve the solubility and stability of the virus in organic solvents and liquid crystals, which act as templating matrices for nano-objects and might thus promote self-organization of the virus and its cargo.[19] The synthesis of **4** was performed according to a literature procedure.[20] To CCMV-A (2.82 mg, 0.61 nmol) in VB was added copper sulfate, L-ascorbic acid and 47 equivalents per CP of **4** and the solution was shaken overnight. Again the azide moiety was predissolved in DMSO (< 1% of the total reaction volume). The integrity and monodispersity of the cyanobiphenyl-functionalized virus (CCMV-A-CNBP) were confirmed by FPLC (Figure S5C) and TEM (Figure 2C).

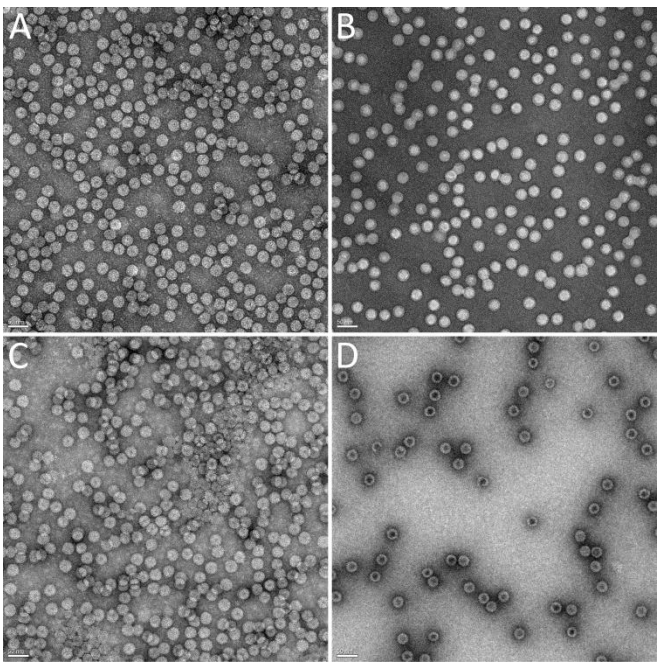


Figure 2. TEM micrographs of uranyl acetate stained capsids (scale bar = 50 nm). A) CCMV; B) Coumarin-functionalized CCMV (CCMV-A-Coum); C) Cyanobiphenyl-functionalized CCMV (CCMV-A-CNBP); D) Empty alkyne-functionalized CCMV (VLP-A).

The modification of CCMV with strained alkyne **2** was also evaluated by post-functionalization *via* a SPAAC reaction with dye **3**. CCMV-BCN (1.9 nmol) was incubated with 50 equivalents per CP of **3**. Purification by FPLC yielded an elution peak at 10.0 mL, with co-elution of the coumarin again proving its binding to the CCMV capsid (Figure S5D). TEM analysis of the coumarin-functionalized capsid (CCMV-BCN-Coum) showed intact and monodisperse capsids (Figure S6B). All capsid derivatives mentioned were also analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), displaying a single band at 20 kDa for the coat protein monomer and some protein multimers (Figure S7).

Both CCMV-A-Coum and CCMV-BCN-Coum were analyzed by UV/Vis spectroscopy, in order to evaluate the average amount of functionalization by click chemistry.

Native CCMV has its maximum absorbance at λ = 260 nm, partially due to the contribution of its RNA. The virus concentration was calculated from the absorption value of the modified capsids at this wavelength by using the extinction coefficient of native CCMV; $\varepsilon = 5.85\ L \cdot g^{-1} \cdot cm^{-1}$.[21]

A range of different coumarin-triazole analogues is described in literature, with considerably varying extinction coefficients for different analogues in different solvents. To accurately determine the degree of functionalization of CCMV, we synthesized the coumarin-triazole model by reacting **1** and **3** in a CuAAC reaction to form product **5** (Scheme 1).[22] UV/Vis analysis of **5** (10^{-4} M) showed a maximum absorbance at λ = 342 nm, yielding an extinction coefficient ε = 4600 $M^{-1} \cdot cm^{-1}$. The spectrum also demonstrates a minor contribution of the coumarin moiety to the absorption of the virus at λ = 260 nm (Figure S3), which should be taken into account upon calculating the degree of functionalization. To exclude a concentration-dependent absorbance of the coumarin moiety, UV/Vis spectra of **5** were measured over a range of concentrations. The absorbance at λ = 342 nm shows a linear dependence to the concentration (Figure S4).

Calculation of the ratio of coumarin over CCMV from the UV/Vis spectra shows an average of approximately 450 coumarin moieties on CCMV-A-Coum. This is comparable to the degree of functionalization of the carboxylate residues found by Gillitzer *et al.*,[4] only in this case we used a much smaller excess of reagents. For CCMV-BCN-Coum (Figure 3A) the number of functionalization is increased up to more than 950 coumarin moieties per capsid. Considering a maximum of 1980 carboxyl groups exposed on the exterior of each capsid, and taking into account that some residues are more solvent-accessible than others, the values we obtain for the amount of functionalization are very satisfactory. The significantly shorter reaction time for the SPAAC reaction between CCMV-A and **3** (Scheme 1), proves that, in this case, the copper-free procedure outperforms the copper-mediated procedure. For the capsid with the highest degree of functionalization, the fluorescence spectrum was measured upon excitation at λ = 380 nm (Figure 3B). The maximum emission was found at λ = 463 nm, which is characteristic for coumarin-based moieties.

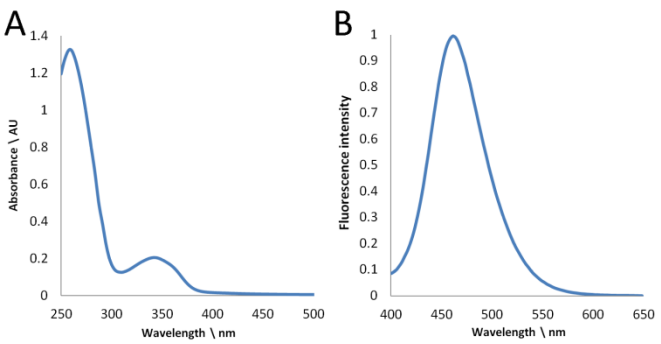


Figure 3. Characterization of CCMV-BCN-Coum by A) UV/Vis absorbance; B) fluorescence spectroscopy (normalized).

The controlled and reversible assembly behavior of CCMV constitutes one of its most salient features. By modifying the virus capsid and thereby changing the properties of the coat proteins, this mechanism can be disrupted. To verify that cargo loading of CCMV is still possible after functionalization and post-functionalization, CCMV-A was subjected to controlled disassembly, by increasing the pH and simultaneously precipitating the ss-RNA with calcium chloride. Afterwards, the coat proteins were reassembled at reduced pH and high salt concentration to form the empty alkyne-functionalized VLP (VLP-A). Upon FPLC analysis, an increased absorption of the empty capsid at λ = 280 nm (the absorption maximum of the coat protein)[23] with regard to the absorption at λ = 260 nm illustrates the successful removal of the viral RNA (Figure S5E). TEM analysis also shows fully reassembled and monodisperse T = 3 particles, which have a dark interior, due to the larger void volume that can be occupied by the staining solution (Figure 2D). The controlled and reversible disassembly, as described above, was also performed on CCMV-A-Coum, albeit with less

efficiency. Examination of the 260/280 nm absorbance ratio from the FPLC chromatogram of empty CCMV-A-Coum (VLP-Coum) (Figure S5F), shows that the decrease in efficiency is likely related to incomplete removal of the RNA. Nevertheless the reversible disassembly mechanism proves to still be operative.

Conclusions

In summary, we have developed the tools to functionalize the exterior of the CCMV capsid, by using the mild conditions offered by click chemistry. Click chemistry provides an avenue for the easy preparation of versatile biological nanocages, which also exhibit a reversible assembly mechanism for VLP formation. Further studies on the scope and potential applications of this work will involve cargo loading and incorporation of the virus in templating matrices to create novel platforms for material science.

Experimental

Materials

All organic solvents were of analytical quality. Buffers were prepared with ultrapure water (milliQ®). For EDC couplings, phosphate buffer (PB) containing 100 mM phosphate and 1 mM EDTA at pH 7.2 was used. For click reactions and storage of native and modified CCMV, virus buffer (VB) containing 100 mM NaOAc, 1 mM EDTA and 1 mM NaN_3 at pH 5.0 was used. Samples were stored at 4 °C. Amine-terminated BCN **2** was purchased from SynAffix (Nijmegen, NL). 3-azido-7-hydroxycoumarin (**3**) was obtained from Carbosynth (Berkshire, UK). All other chemicals were purchased from Sigma Aldrich.

Functionalization of CCMV

The functionalization of CCMV with linear or strained alkyne groups was performed with eight equivalents of both EDC and NHS and seven equivalents of **1** or **2**, respectively. The reactions were incubated in PB for 17 h at room temperature and subsequently dialyzed to VB and purified by preparative FPLC.

Post-functionalization of CCMV

The post-functionalization of CCMV-A (8.12 mg, 1.77 nmol) with coumarin was performed with 1.12 mg of copper sulfate (7.0 µmol, 22 equiv. per CP), 3.6 mg of L-ascorbic acid (20.4 µmol, 64 equiv. per CP) and 3.2 mg of **3** (15.4 µmol, 49 equiv. per CP). For the conjugation of mesogen **4** to CCMV, CCMV-A (2.82 mg, 0.61 nmol) was incubated with 0.45 mg of copper sulfate (2.82 µmol, 26 equiv. per CP), 1.4 mg of L-ascorbic acid (7.9 µmol, 72 equiv. per CP) and 1.2 mg of **4** (5.2 µmol, 47 equiv. per CP). The post-functionalization of CCMV-BCN (1.9 nmol) was performed with 3.5 mg of **3** (17 µmol, 50 equiv. per CP). Modified virus samples were purified by several centrifugation and dialysis steps, followed by preparative FPLC.

The synthesis of compounds **4** and **5** is described in the Supporting Information.

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Notes and references

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Electronic Supplementary Information (ESI) available: Detailed experimental procedures, compound characterization (NMR, ESI-TOF UV/Vis), analyses of functionalized virus particles (FPLC, TEM, SDS-PAGE). See DOI: 10.1039/c000000x/

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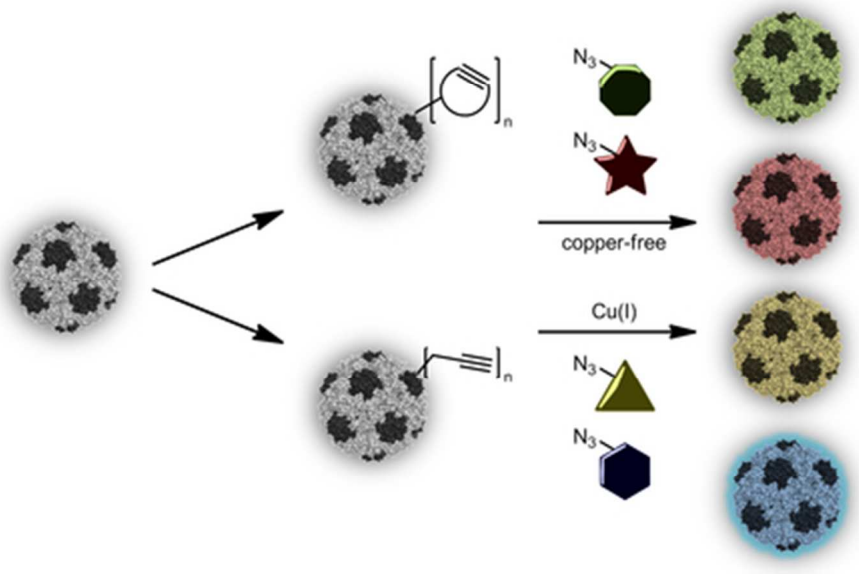

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