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Article

Dual Site-Selective Presentation of Functional Handles on Protein-Engineered Cowpea Chlorotic Mottle Virus-Like Particles

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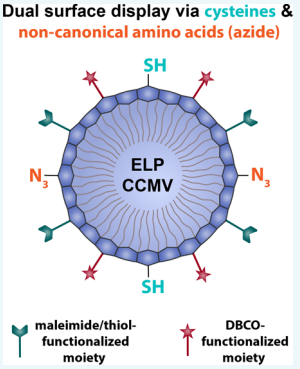
ABSTRACT: Protein cages hold much promise as carrier systems in nanomedicine, due to their well-defined size, cargo-loading capacity, and inherent biodegradability. In order to make them suitable for drug delivery, they have to be stable under physiological conditions. In addition, often surface modifications are required, for example, to improve cell targeting or reduce the particle immunogenicity by PEGylation. For this purpose, we investigated the functionalization capacity of the capsid of cowpea chlorotic mottle virus (CCMV), modified at the interior with a stabilizing elastin-like polypeptide (ELP) tag, by employing a combination of protein engineering and bio-orthogonal chemistry. We first demonstrated the accessibility of the native cysteine residue in ELP-CCMV as a site-selective surface-exposed functional handle, which was not available in the native CCMV capsid. An additional bio-orthogonal functional handle was introduced by incorporation of the noncanonical amino acid, azido-phenylalanine (AzF), using the amber suppression mechanism. Dual site-selective presentation of both a cell-penetrating TAT peptide and a fluorophore to track the particles was demonstrated successfully in HeLa cell uptake studies.



INTRODUCTION

Nanomedicine is a fast-growing field, aiming to improve healthcare in a range of applications, such as medical imaging, vaccines, regenerative medicine, and nanotherapeutics. For this purpose, a large variety of nanoparticles (e.g., liposomes, micelles, metallic, polymeric and protein-based nanoparticles) have been studied. Although the number of clinical trials is rapidly growing and some nanomedicine formulations are already approved for clinical use, there is a constant need to further control particle features in a biological context to ensure a more effective translation to the clinic.[1,2] In this regard, protein-based virus-like particles (VLPs) are of great interest because of their biocompatibility,[3] well-defined shape and size,[4,5] the possibilities of cargo encapsulation[6] and ease of modification.[7] One of the most intensely studied VLPs is derived from the cowpea chlorotic mottle virus (CCMV) due to its controllable and reversible self-assembly behavior.[8] This characteristic is of great value, since having good control over assembly and disassembly allows for controlled functionalization of either the interior or exterior. For example, the interior can be functionalized by conjugation of the molecule of interest to the capsid protein N-terminus prior to capsid assembly, whereas surface exposed functional handles at whole capsids can be used for exterior modifications.[9,10] Since the N-termini of the CCMV capsid proteins (CPs) are predominantly directed toward the inner cavity of the VLP,[11] the N-terminus has extensively been explored for cargo encapsulation. Multiple strategies have been developed,[12−16] for example via a genetic fusion of the protein of interest with the capsid protein,[12] via electrostatic interactions between cargo and the positively charged domain of native CCMV capsids,[14] or via conjugation employing a biorthogonal handle.[16] On the other hand, the number of site-selective modifications of the capsid surface is surprisingly limited and up to now mainly performed using abundantly present surface-exposed amines or carboxylic acids.[8,17,18]

To achieve site-selectivity, and hence better control over the degree and positioning of the modification, a less abundant amino acid residue at the surface of the capsid that can undergo a chemoselective reaction should be used.[19] Cysteine residues are the most commonly employed site-selective functional handles as they fulfill both criteria. However, up to now, neither of the two cysteines present in native CCMV capsids were demonstrated to be reactive toward chemical modification.[17] Efforts to introduce cysteine residues using protein engineering strategies resulted in CCMV variants with cysteines at various positions (R82C, A141C, A163C, and S102C).[17,20] Both small molecules and macromolecules were conjugated to the surface of these variants of CCMV. This strategy allowed for controlled modification of the surface of CCMV. However, the possibility to introduce multiple

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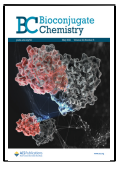

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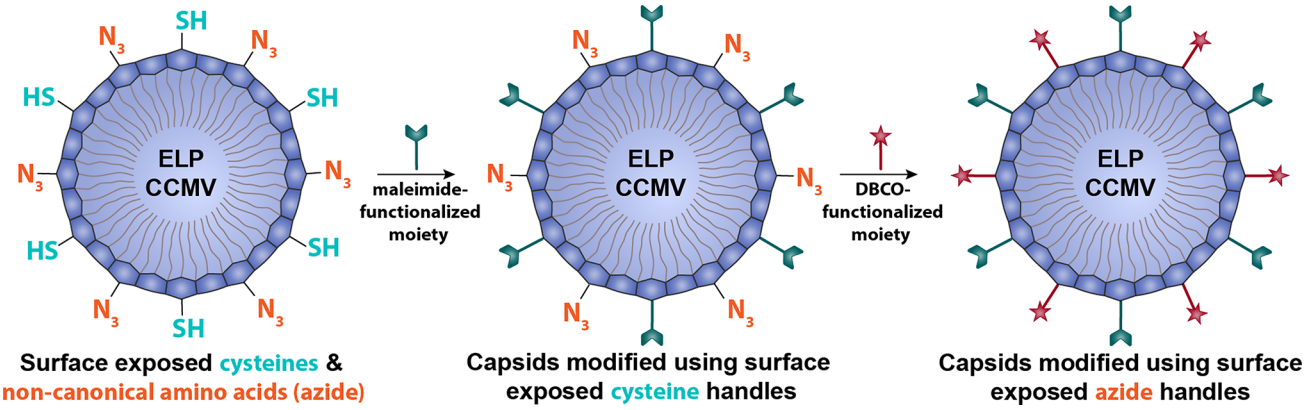

Figure 1. Schematic presentation of the exploration of dual site-selective handles on the surface of ELP-CCMV. Native cysteine residues were surface exposed in ELP-CCMV. Via protein engineering a noncanonical amino acid (azido-phenylalanine (K65azF)) was incorporated in ELP-CCMV. Protein engineered K65azF ELP-CCMV capsids therefore displayed both cysteine residues (cyan) and azide functional groups (red) (1 per capsid protein). The functional handles could be independently used for the display of moieties of interest.

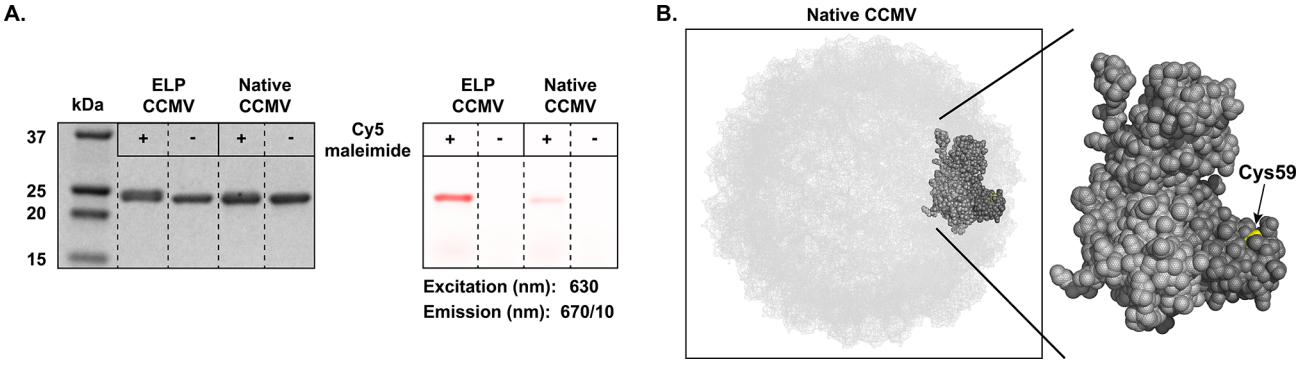

Figure 2. (A) SDS-PAGE analysis of a maleimide conjugation reaction of sulfo-Cyanine5-maleimide (Cy5) to either ELP-CCMV or native CCMV (heparin stabilized). Coomassie blue staining (left). False color images obtained by illumination (Cy5 excitation at 630 nm) and emission through a 670/10 nm (Cy5) emission filter (right). (B) Analysis of the orientation of cysteines in a native CCMV capsid ($T = 3$). Space filling model of one trimeric subunit (dark gray) to illustrate the native cysteine at position 59 (yellow) in CCMV capsids (light gray mesh; PDB: 1CWP).[26]

different surface modifications in a site-selective manner has not yet been studied for CCMV. This is of importance for biomedical applications, as often multiple functionalities are required, for example active targeting of diseased cells using ligands combined with stealth characteristics introduced using poly(ethylene glycol) (PEG).[21]

Besides surface functionalization capacity, CCMV stability at physiological pH is also a limitation of the native capsid.[22] We have recently developed a CCMV variant which showed improved stability under physiological conditions. This was achieved by genetically fusing an elastin-like polypeptide motif (ELPs), consisting of nine repeats of Val-Pro-Gly-Xaa-Gly (VPGXG), to the N-terminus of the CCMV capsid proteins (ELP-CCMV).[23−25] The thermosensitive ELP stabilized capsid protein assembles by increasing the salt concentration or temperature to induce the formation of stable ($T = 1$) capsids, even in the absence of cargo. In our efforts to modify the ELP-CCMV capsid surface, we observed that, in contrast to the native CCMV, one of the cysteine residues was accessible for chemical modification. Herein, we describe how we have utilized this remarkable feature to develop a CCMV capsid with dual site-selective surface presentation of functional entities by additional incorporation of a noncanonical amino acid using the amber suppression method (Figure 1). For ease of comparison with native CCMV studies, the amino acid positions of both the cysteine and noncanonical amino acid in our ELP-CCMV capsid proteins are based on the wildtype CCMV sequence.

RESULTS AND DISCUSSION

Surface Exposed Cysteine Residues in ELP-CCMV Capsids. Our aim was to develop CCMV capsids that can be used in biomedical applications, for which well-controlled site-selective and multivalent display of functional moieties at the surface is necessary. Exploring the abundantly present, surface-exposed amines (~360 per $T = 1$ capsid) and carboxylic acids (~660 per $T = 1$ capsid) does not allow for the required high level of control over the degree of functionalization.[17] As the cysteine accessibility was not yet assessed for our newly developed ELP-CCMV capsids, we investigated the classical thiol-maleimide coupling first. In this study, our hydrophobic (VY1-VY8) ELP-CCMV, in which the guest residue X was a tyrosine instead of a valine in the first and eighth repeat of the ELP sequence, was used unless stated otherwise.[24,25] We compared the conjugation efficiency of both ELP-CCMV particles and native CCMV particles (heparin stabilized) when incubated with a maleimide-functionalized sulfo-cyanine5 (Cy5; Figure S3). Remarkably, SDS-PAGE analysis demonstrated successful conjugation of Cy5 to ELP-CCMV, while the native CCMV capsid only showed minimal modification (Figure 2A). Image analysis demonstrated a 10-fold increase in conjugation of Cy5 to ELP-CCMV capsids compared to

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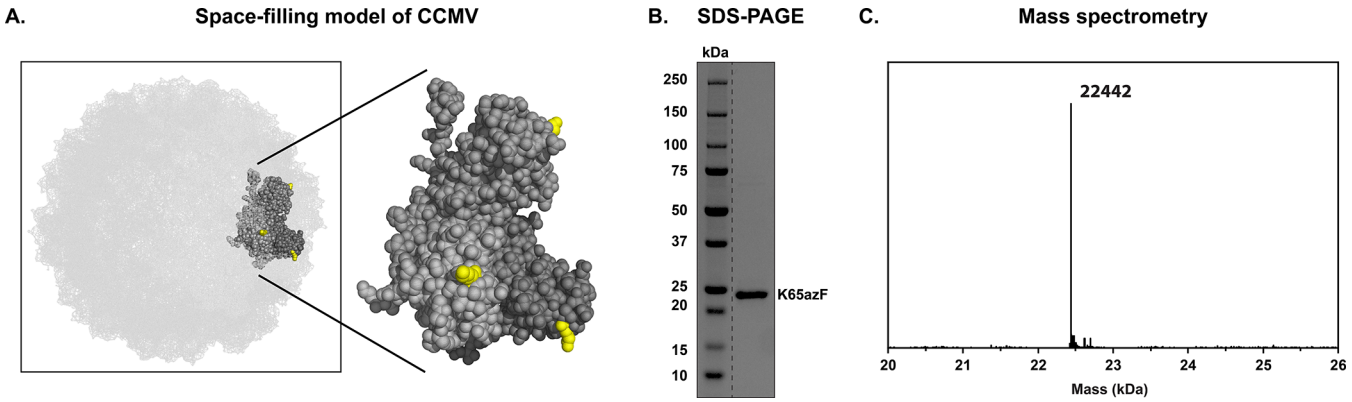

Figure 3. Characterization of purified K65azF ELP-CCMV capsid proteins. (A) Space filling model of one trimeric subunit (dark gray) to illustrate the position of K65 (yellow) in CCMV capsids (light gray mesh; PDB: 1CWP); (B) Coomassie stained SDS-PAGE of K65azF ELP-CCMV capsid proteins (22.4 kDa); (C) LC-MS Q-TOF mass spectrum of K65azF ELP-CCMV capsid. Expected mass 22441.6 Da.

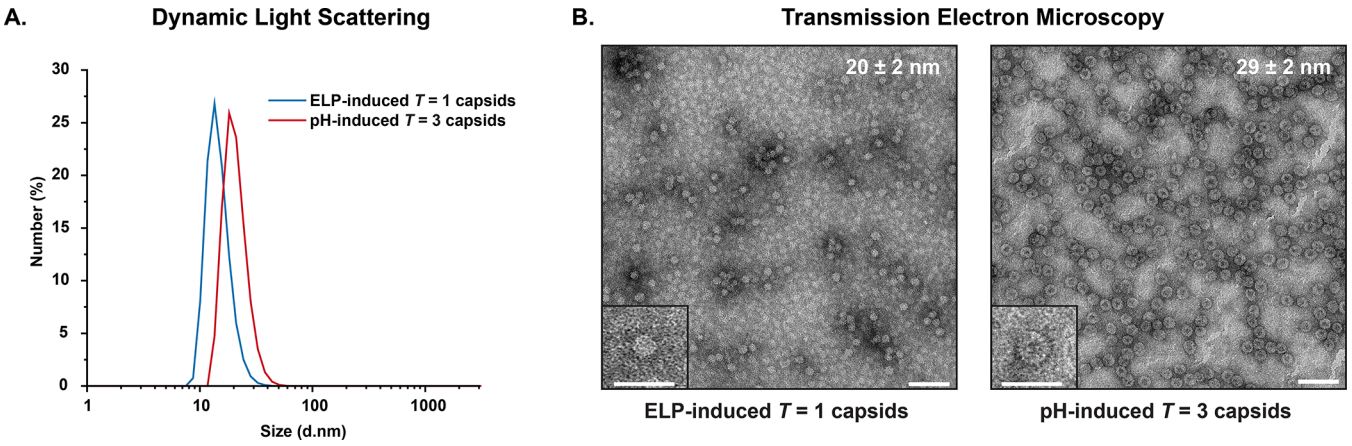

Figure 4. Characterization of purified K65azF ELP-CCMV capsids. (A) Dynamic light scattering number plot of ELP-mediated (T = 1) and pH-induced (T = 3) capsids; (B) transmission electron microscopy images of ELP-mediated capsids (20 ± 2 nm; N = 50) and pH-induced capsids (29 ± 2 nm; N = 50), scale bar 100 nm, zoomed-scale bar 50 nm.

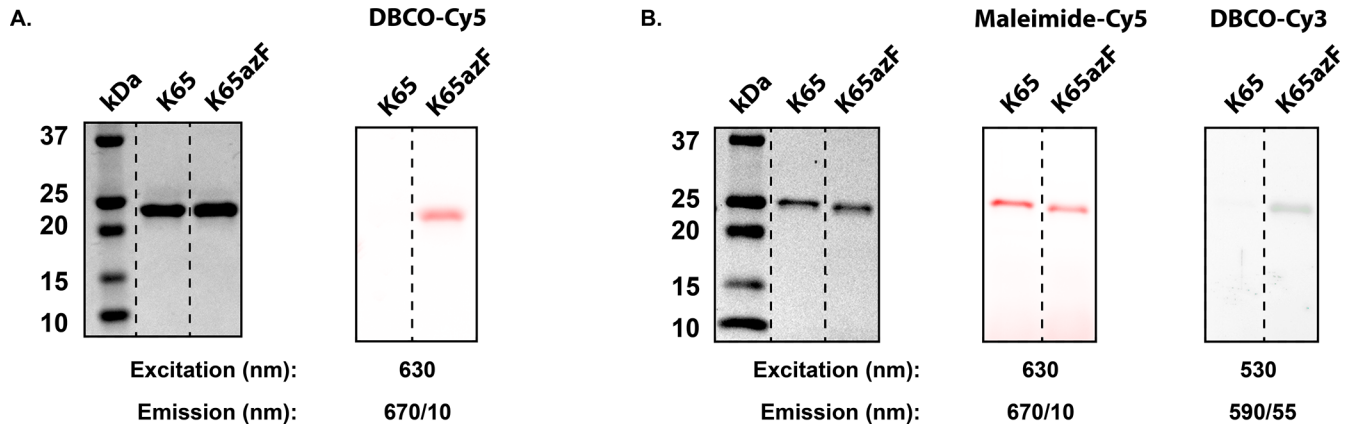

Figure 5. SDS-PAGE analysis of fluorophores labeled to surface-exposed functional handles of ELP-CCMV. (A) Coomassie stained gel of K65 and K65azF reacted with DBCO-Cy5 (left). False color images obtained by illumination (Cy5 excitation at 630 nm) with a LED light source (LED Trans illuminator BT509) and emission through a 670/10 nm (Cy5) emission filter (right). (B) Coomassie stained gel of dual labeled K65 and K65azF with Maleimide-Cy5 and DBCO-Cy3 (left). False color images obtained by illumination (Cy5 excitation at 630 nm) and emission through a 670/10 nm emission filter (middle) and by illumination (Cy3 excitation at 530 nm) and emission through a 590/55 nm emission filter (right).

native CCMV capsids. From literature it is known that the cysteines in native CCMV are not accessible.[17] The reason we observed efficient modification of our CCMV variant can be explained by a conformational change due to the introduction of the ELP-tag, exposing the normally hidden Cys 59 (Figure 2B).[26]

Display of Biomolecules via the Native Cysteine Residue. Next, we investigated the accessibility of the cysteines to more sterically demanding biomolecules, a

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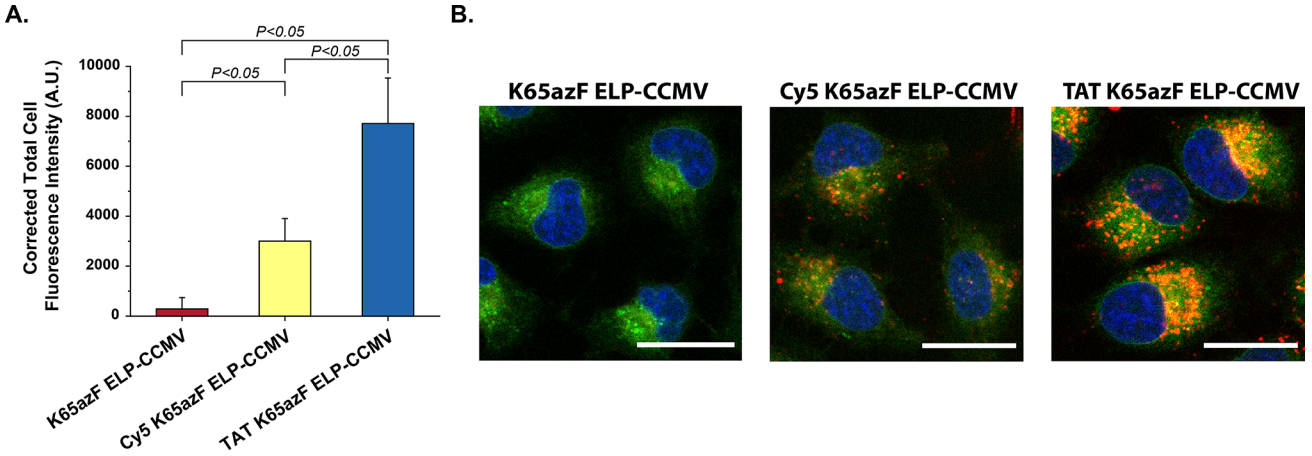

Figure 6. Trafficking of stabilized K65azF ELP-CCMV, Cy5 K65azF ELP-CCMV and TAT K65azF ELP-CCMV in HeLa cells. (A) Epi-fluorescence microscopy was used to track capsids in HeLa cells. Within a technical replicate, 3 × 10 cells were analyzed with analysis software in every captured image and intensities were corrected for the size of each corresponding cell. In order to generate biological triplicates, experiments were repeated three times with different batches of HeLa cells, passage number and batch of particles. The mean values of the corrected total cell fluorescence intensity of the biological replicates (N = 90 cells per condition) were plotted. The cell uptake of the TAT labeled particles (TAT K65azF ELP-CCMV) was significantly higher ($P < 0.05$) compared to the particles without TAT (Cy5 K65azF ELP-CCMV). B) Confocal laser scanning microscopy was used to study the localization of the particles (blue = nuclei, green = lysosomes, red = capsids, yellow = colocalization of lysosomes (green) and capsids (red). Scale bar = 25 μm.

negatively charged ssDNA (~6.8 kDa) and a positively charged cell-penetrating peptide, TAT (~1.5 kDa). Both molecules were successfully conjugated to the ELP-CCMV capsids via a maleimide reaction or disulfide bond formation, respectively (Figures S4 and S5). In order to verify that cysteine residue C59 was targeted, the cysteine at position 59 was substituted to a serine (C59S) in our more hydrophobic VW1-VW8 ELP-CCMV (Figure S6). Data indicated that the introduction of this mutation completely prevented labeling with the TAT peptide, demonstrating that C59 was the surface-exposed handle in the ELP-CCMV structure (Figure S7).

Introduction of a Surface Exposed Noncanonical Amino Acid. After establishing that ELP-CCMV capsids have one site-selective cysteine handle available, we moved toward introducing a second bio-orthogonal handle on the capsid surface. For this purpose, we investigated the well-established amber suppression method, which allows for the facile, site-selective introduction of a noncanonical amino acid. The amber suppression method has been used previously in MS2 and tobacco mosaic virus (TMV) capsids to add new functional handles but has not been studied for CCMV before.[27,28] To efficiently use this method to our advantage, amino acid K65, located at a surface-exposed loop (Figure 3A),[26] was predicted to be innocuous with regard to capsid stability and hence could be easily replaced. We thus decided to substitute this lysine for an azido-phenylalanine (K65azF) to provide us with one bio-orthogonal functional handle per capsid protein. Expression and purification were successful, as demonstrated with SDS-PAGE and mass spectrometry (Figure 3B,C). CCMV capsids were next assembled from these K65azF capsid proteins. Two methods were employed; using the traditional pH-induced assembly upon incubation in pH 5 buffer yielded T = 3 particles. T = 1 particles were formed upon salt-induced, ELP-mediated assembly at pH 7.5. Both assembly states were confirmed with DLS and TEM (Figure 4) and were comparable to ELP-CCMV capsids assembled from the original capsid proteins. By using the incorporated noncanonical amino acid, a fluorophore containing a DBCO group (sulfo-Cyanine5-DBCO) was attached via the copper-free, strain-promoted azide−alkyne cycloaddition (SPAAC) reaction to the azido-handle. SDS-PAGE analysis (Figure 5A) indicated successful and specific labeling of the fluorophore to the K65azF variant, whereas no fluorescence was observed for the CCMV variant without a surface exposed azido-group (K65 ELP-CCMV). Since ELP-CCMV capsids allow for controlled assembly and disassembly, Cy5-labeled capsid proteins were mixed with unlabeled capsid proteins in various ratios, followed by the induction of self-assembly.[8] In order to verify that we mixed capsid proteins instead of capsids, complete disassembly was first confirmed by DLS (Figure S8A). Subsequently, capsid assembly was confirmed with size exclusion chromatography (Figure S8B). SDS-PAGE analysis showed a high level of control over the average incorporation of Cy5-labeled K65azF ELP-CCMV capsid proteins into ELP-CCMV capsids (Figure S9). Furthermore, the thermal stability of the K65azF ELP-CCMV was comparable to the original ELP-CCMV variant and was thus not affected by the incorporation of the noncanonical amino acid (Figure S10).[25] This finding is essential for future applications when these particles with multiple surface-exposed functional handles will be used for nanomedicine purposes. We investigated the possibility of using both surface-exposed handles as a strategy for dual-labeling of the surface. Sulfo-derivatives of Cyanine5-maleimide and DBCO-Cyanine3 were used to ensure solubility in aqueous solutions. SDS-PAGE analysis demonstrated the site-selectivity of this dual- labeling strategy as only K65azF ELP-CCMV was functionalized with both dyes (Figure 5B).

Enhanced Cell-Uptake Using Surface Modified Capsids. To demonstrate the ability to label the particles with functionally relevant moieties, particles were functionalized with the cell-penetrating peptide (TAT; Figure S11), and a fluorophore using both site-selective handles to allow cell-uptake studies to be performed. To ensure stability of the empty capsids in culture media, a homobifunctional cross-

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linker BS3 (bis(sulfosuccinimidyl)suberate) was used to react with amines present in the capsid proteins (Figure S12).[25,29] First, the cell-penetrating peptide, TAT, was conjugated to K65azF ELP-CCMV capsids. The conjugation proceeded successfully even with the additional cross-linking procedure. Second, for particle tracking in HeLa cells, capsids were labeled with the fluorophore sulfo-DBCO-Cyanine5 (Figure S13).

Previously, it was shown that capsids were internalized by cells even without targeting moieties.[30] Capsids labeled with TAT showed, however, a 2.5 times enhanced uptake compared to capsids lacking the CPP (Figure 6A). The localization of particles in HeLa cells was studied by confocal laser scanning microscopy and demonstrated that most internalized capsids accumulate in the lysosomes (Figures 6B and S14). A viability assay further confirmed that capsids were not toxic to the cells (Figure S15). The observed uptake of our capsids occurs thus in living cells.

CONCLUSION

Herein, we have demonstrated a facile route to introduce two different functionalities on the surface of CCMV capsids in an orthogonal manner. Remarkably, when using CCMV capsid proteins, which were N-terminally modified with the stabilizing elastin-like polypeptide tag (ELP-CCMV), one of the cysteine residues proved to be sufficiently accessible for modification, in contrast to the native CCMV capsids. A noncanonical amino acid, azido-phenylalanine, was subsequently incorporated as a second handle in a surface-exposed loop using the amber suppression method. Both handles could be addressed effectively and independently of each other, without affecting the capsid stability. Using both handles, capsids displaying both cell-penetrating peptides, TAT, and fluorophores enhanced the uptake in HeLa cells compared to capsids without TAT. This study shows the potential of these multifunctional capsids for future biomedical applications in which often various and multi labeling strategies are necessary at the same time, e.g., targeting moieties, fluorophores, or PEGylation. The use of these site-selective handles enables a much better control over the degree of functionalization compared to traditional nonspecific modifications using amines or carboxylic acids, improving the reproducibility of the particle features, which is an important property for biomedical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.1c00108.

Experimental methods as well as figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

CCMV, cowpea chlorotic mottle virus; ELP, elastin-like polypeptide; CPP, cell-penetrating peptide; AzF, azido-phenylalanine; TAT, transactivator of transcription; VLPs, virus-like particles; CP, capsid protein; PEG, poly(ethylene glycol); SPAAC, strain-promoted azide−alkyne cycloaddition; BS3, bis(sulfosuccinimidyl)suberate; Cy5, cyanine5

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