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***In vivo* Virus-based Macrofluorogenic Probes Target Azide-labeled Surface Glycans in MCF-7 Breast Cancer Cells**

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Abstract

Chemical addressability of viral particles has played a pivotal role in adapting these biogenic macromolecules for various applications ranging from medicine to inorganic catalysis. The consistent multimeric assemblies dictated by its genetic code, facile large scale production, lack of observable toxicity in humans, *Cowpea mosaic virus* possesses multiple features that are advantageous for the next generation of virus-based nanotechnology. Herein, the chemistry of the viral particles is extended with the use of Cu-free strain-promoted azide-alkyne cycloaddition reaction, or SPAAC reaction. The elimination of Cu, its co-catalyst and reducing agent simplifies the reaction scheme to a more straightforward approach, which can be directly applied to living systems. As a proof of concept, the viral particles modified with the aza-dibenzylcyclooctynes functional groups are utilized to trigger and amplify a weak fluorescent signal (azidocoumarin) in live cell cultures to visualize the non-natural sugars. Future adaptations of this platform may be developed to enhance biosensing applications.

Keywords

Bionanoparticles; Cowpea mosaic virus; strain-promoted alkyne-azide cycloaddition (SPAAC) reaction; click chemistry; fluorogenic dye; bioconjugation

INTRODUCTION

Extensive biological studies with alkynes and azides have shown that these functional groups exhibit highly narrow distributions of reactivity, with physiologically permissive reaction conditions and near zero reactivity against other biological molecules that can be

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Author Contributions

C.L.W purified the virus, conducted the ADIBO conjugations to the virus; Y.C. modified the viruses with azido-groups, DLS measurements, TEM imaging; L.C. synthesized Ac₄GalNAz and X.D. provided MCF-7 cells labeled with GalNAz; L.A.L. and Q. W. coordinated the experiments, performed live cell imaging; C.L.W., L.A.L. and Q.W. wrote the manuscript.

Supporting Information. MALDI-TOF MS of CPMV and modified CPMV particles, DLS measurements and SDS PAGE supplied as Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

readily achieved in living systems.¹⁻⁵ The incorporation of these groups by organic synthesis, chemical conjugation and/or via natural biosynthetic pathways has been employed to investigate some of the complex biological events involved in protein glycosylation, *de novo* protein synthesis and nucleic acid replication.⁶⁻¹² Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, pioneered by Sharpless and Meldal, paved a way in achieving selective modifications of alkynes and azides.^{13, 14} However, the obligate use of copper catalyst limits its use in applications wherein the cells or whole organisms must remain viable. An alternative mean of activating alkynes was proposed by Bertozzi based on the classic work by Wittig and Krebs by incorporating strained alkynes in a series of cyclooctyne reagents to undergo selective reaction with azides.^{4, 15-17} The reactivity of the cyclooctyne had been further enhanced by incorporating difluoro group or biphenyl rings that led to reagents that were several orders of magnitude more reactive than the original cyclooctyne.¹⁵⁻¹⁷ Among these reagents, commercially available dibenzylcyclooctynes that contains a nitrogen atom in the cyclooctyne ring supply a larger array of copper-free click reagents with physiologically applicable reaction kinetics.

In this study, we incorporated these reagents to expand the chemistry of protein nanoparticles, in particular virus scaffolds by using *Cowpea mosaic virus* (CPMV) as a prototype biomolecule for quantitatively labeling the molecules with azido-dibenzylcyclooctynes (ADIBO) and its corresponding azido-counterparts. The surface exposed lysine groups on native CPMV have been used to conjugate dyes, proteins, nucleic acids, polymers, and inorganic metals to demonstrate its potential use in biomedical applications (vaccines, intravital imaging, and biosensing).¹⁸⁻²⁷ Herein, we monitored the coupling efficiencies of virus-azide and virus-ADIBO with fluorescent derivatives containing the complementary groups for the desired reactions. The covalent modifications were also verified by MALDI-TOF MS of the small subunit of CPMV, and the structural fidelity of the modified particles were assessed by dynamic light scattering to measure average particle sizes, and visualized by transmission electron microscopy (TEM). As a proof of concept, the ADIBO-virus particles were used to label live cells as a macrofluorophore with the pro-fluorogenic dye, 3-azido-7-hydroxycoumarin. The relative ease of incorporating the strained alkynes on the virus particles enables the installation of a variety of functionalities in Cu(I)-catalyst free reactions for potential use in bioimaging applications, wherein signal amplification may be required.

EXPERIMENTAL PROCEDURES

General

Reagents **1**, **2** and **6** were kindly provided by Dr. Andrei Poloukhine from Click Chemistry Tools. Wild-type CPMV was prepared by the method described by Siler *et al.*²⁸ The virus was stored in buffer at a concentration of about 10 mg/mL. Unless otherwise indicated, “buffer” refers to 0.1 M potassium phosphate (pH 7.0). SDS-PAGE was prepared in house as 12% acrylamide separating gel (30:1 acrylamide to bis-acrylamide) with 5% acrylamide stacking gel. Size exclusion columns for the purification of virus-containing reaction mixtures were prepared by preswelling 23 g of Bio-Gel P-100 (BioRad) in 400 mL of buffer and loading the gel into Bio-Spin disposable chromatography columns (BioRad). The

columns were allowed to drain upon standing and were then further dried by centrifugation (3 min at 800 g). For 80 μ L of virus solution (1 mg/mL), approximately 1 mL of prepared gel was used. Ultracentrifugation was performed at the indicated rpm values with a Beckman Optima L-90K Ultracentrifuge equipped with either SW41 or 50.2 Ti rotors.

CPMV conjugate to NHS-azide or NHS-ADIBO

Conjugation of NHS-ester modified reagents (NHS-ADIBO **1**, NHS-EG₄-azide **2**, and NHS-EG₄-ADIBO **9**) to CPMV was carried out following previously reported methods.¹⁸ Briefly, 2.0 mg/mL CPMV was loaded with 10 to 200 fold excess of NHS-modified reagents relative to the asymmetric unit in 5-20% DMSO/buffer (vol/vol) to ensure amidation of surface exposed lysines. The reactions were performed at 4 °C for 24 h. ADIBO or azide tethered CPMV was purified by P-100 (Bio-rad) size exclusion column (small scale, for characterization studies) or by pelleting through sucrose cushion (45K rpm, 40.2 Ti rotor) followed by resuspension in buffer (large scale, for additional conjugations and cell studies).¹⁸

SPAAC reaction of CPMV-azide and CPMV ADIBO

Azide- or ADIBO-CPMV was used to react with its complementary ADIBO or azide conjugates. Briefly, 2.0 mg/mL modified CPMV was loaded with varying concentrations of its ADIBO or azide counterpart conjugate in 5-20% DMSO in buffer. The conjugation was carried under room temperature for allocated time. Purified CPMV conjugates are analyzed by TEM, DLS, MALDI-TOF MS, UV-vis, fluorometry, and reducing SDS-PAGE. For kinetics measurement, WT-CPMV and **3** at 0.2 mg/mL in buffer were reacted with 25 equivalent of fluorogenic dye **5** with respect to virus asymmetric unit for four hours at room temperature. The fluorescence at 465 nm with emission cutoff at 455 nm was measured at five minute intervals on a 96-well plate (Ex/Em 390/465 nm, Molecular Devices, SpectraMax M2e).

Mass spectrometry of viral particles

For MALDI-TOF MS analysis, 24 μ L of virus (> 1 mg/mL) was denatured by incubating with 6 μ L guanidinium chloride (6.0 M) for 15 minutes at room temperature. Millipore ZipTip C₄ tips were prepared by rinsing with pure acetonitrile (ACN), then ddH₂O (MilliQ, 18.2 M Ω) with 0.1% TFA (trifluoroacetic acid) then adsorbing the denatured protein to the ZiptipTM (Millipore). The residual salt was removed by rinsing three times with 5% MeOH/water and then the samples were eluted with saturated solution of sinapinic acid in 70% ACN/water with 0.1% TFA to the MALDI plate. The sample was air-dried then analyzed on a Bruker Ultraflex II mass spectrometer (Bremen, Germany).

Modified particle analysis

The size distribution and hydrodynamic diameter of wild type and modified CPMV were measured by Zetasizer Nano ZS (Malvern Instrument) using diluted samples. TEM analysis was carried out by drying 5 μ L of sample at a concentration at 20-40 μ g/mL onto 100 mesh carbon-coated copper grids. The grids were then stained with 10 μ L of 2% uranyl acetate and viewed with a Hitachi H-8000 TEM electron microscope at 200 kV. UV-vis and

fluorescence emission spectra of diluted samples measured were recorded on Thermo Fisher NanoDrop and FP-6200 spectrofluorometer, respectively. Stoichiometries were determined by absorbance measurements for CPMV-TMR (tetramethylrhodamine) conjugates. Since the dye contains a tetraethylene glycol linker between the ADIBO-functionality, we assumed the dye maintained its original molar absorptivity upon conjugation. For reducing SDS-PAGE, the modified virus particles were concentrated to 10 mg/mL with Amicon centrifugal filters (Millipore, 30K MWCO) by centrifuging at 14,000 rpm for 10 minutes at 4°C. Approximately 100 µg of unmodified controls and dye modified virus samples were loaded onto a 12% reducing polyacrylamide gel at 200 V for 1 h. Precision Plus Protein Standard (Bio-rad) was loaded as molecular weight standard. The gel was imaged with UVP gel imager with the proper fluorescence filters, followed by staining with Coomassie Blue to visualize all protein samples. For each lane, the fluorescence and Coomassie stained intensities were measured with ImageJ. The relative fluorescence intensities were determined by normalizing the fluorescence intensities against the intensities from Coomassie blue stain.

Cell culture and non-natural sugar labeling

Human breast cancer cell line, MCF-7, was cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum at 37°C, 5% CO₂ in humidified chamber to 70-80% confluence. The cells were treated with trypsin and passaged at 1:10 ratio. For cell labeling studies, the cells were cultured on No. 2 coverslips (VWR Micro) with an azide analog of GalNAc (Ac₄GalNAz, 50 µM) in the culture medium for three days.

Fluorescent Cell labeling assays

The cells were washed with 1x DPBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature, then blocked with 2% BSA in 1x D-PBS for 30 minutes. The blocking solution was removed and washed with 1x D-PBS, followed by incubation with 0.1 µM of **10** (assuming average MW of CPMV = 5.6 MDa) in 1x D-PBS for one hour. The virus solution was removed and a fluorogenic dye solution (50 µM of **5**, 5% DMSO in 1x D-PBS) was added for 30 minutes at room temperature. The sample was then rinsed once with 1x D-PBS, and then mounted on glass slides for epifluorescence and DSU confocal microscopy mode (Olympus IX-81). All images were taken and processed with Slidebook v5 (Olympus) with same parameters (20x lens, gain = 1, exposure time = 500 ms).

For live imaging, the cells were seeded on 35-mm glass bottom dish (Nunc) with Ac₄GalNAz (50 µM) and cultured for three days at 37°C, 5% CO₂ in humidified chamber. The cells were rinsed thrice with 1x D-PBS prior to adding a solution of **9** at a concentration of 0.1 µM in pre-warmed phenol-red free, serum-free DMEM. The dye (10 µM of **5**) was added 15 minutes after the virus solution. The preparative steps were all conducted at room temperature (~22°C). The cells were imaged without removal of the fluorogenic dyes after 15 minutes on Olympus IX-81 in DSU confocal mode with 60x oil lens (NA = 1.47).

For 96-well microplate (Greiner) cultures, MCF-7 cells were seeded at 1,000 cells per well in 100 µl complete media (DMEM/10% FBS). The cells were cultured in the same manner as for live cell imaging protocol. The last step included removal of the labeling solution and

addition of serum-free DMEM (phenol red free) prior to reading with a plate reader (Ex/Em 390/468 nm) with emission cutoff at 455 nm (Molecular Devices, SpectraMax M2e). For statistical analysis, each experiment was performed with $n = 4$; and significant differences were based on $p < 0.05$ with two-tailed Student t-test.

RESULTS AND DISCUSSION

Virus Conjugation by SPAAC

Surface modifications of native CPMV with N-hydroxysuccinimide (NHS) ester functionalized small molecules, dyes and linkers have been extensively documented.^{18-21, 24, 29} In previous studies, extending with azides or alkynes at surface exposed lysines afforded “universal” handles to allow efficient coupling through the highly selective CuAAC reaction at low concentrations.^{30,31} However, a limitation of CuAAC reaction has been the requirement of copper(I) to catalyze the reaction, which can be chelated by proteins and viral nanoparticles. Despite its essential function for physiological processes at trace levels, the presence of copper at higher concentrations can accelerate aggregation of proteins,³² and induce toxic effects in the thymus and spleen in mice even at sublethal doses.³³ To avoid such concerns, alternative conjugation strategies have been sought by using the strain promoted cyclooctyne functional group to selectively target the azide handle.^{34, 35} Several variations of these cyclooctyne moieties have been developed by other groups in recent years to address the poor solubility and slow reaction kinetics of the original cyclooctynyl group.^{36, 37} Here, we utilized the strain promoted aza-dibenzocyclooctyne (ADIBO) functional group on native CPMV followed by copper-free SPAAC reaction as a macrofluorophore for labeling non-natural sugars substituted with azido groups (Figure 1). A polyvalent cyclooctyne nanoparticle was obtained by reacting CPMV with an excess of the NHS ester reagent **1** at varying concentrations (Figure 2A). This procedure has been reported to primarily modify the two of the five surface exposed lysine side chain amines per asymmetric unit of CPMV.^{18, 20} The use of the CuAAC ligation reaction eliminates the need for protecting group manipulations and allows for reproducible attachments to be achieved with a minimum amount of material. The fluorogenic dye **5** (3-azido-7-hydroxycoumarin)³⁸ was reacted with the ADIBO-conjugated virus particles to monitor the reaction in aqueous solutions (Figure 2A). Alternatively, the azido functional group was anchored to the viral particles to demonstrate either chemical approaches were viable. Modifications with extended linkers containing tetraethylene glycol (**9**) could also be achieved to yield CPMV-EG₄-ADIBO (**10**) followed by its subsequent conjugation with dye **5** to generate the final virus conjugate (**11**) (Figure 2). The intermediate products were monitored by MALDI-TOF MS of the small subunit (Supplementary Figure 1), with the unmodified coat protein having a peak at 20,957 m/z , and several additional peaks $> 23,000$ m/z . The small subunit of CPMV has two species of small subunit molecular weight of ~ 23.6 kDa prior to proteolytic cleavage, and lower mass of ~ 21 kDa.^{39, 40} ADIBO with azidocoumarin conjugate shifted the peak from 20,957 to 21,525 m/z , which corresponds to the calculated mass difference of 577. The small subunit of azide-CPMV conjugate and the ADIBO-TMR conjugate revealed a peak at 21,231 m/z and at 22,166 m/z , respectively (Figure 2B). The relative intensity of the large subunit (~ 42 kDa) was too low to be considered for this study (*data not shown*).

Characterization of modified viral particles

The unmodified CPMV particles have a characteristic 260/280 nm absorbance ratio, and the addition of **3** had no observable difference to the native particles. ADIBO modified particles with **7** afforded two characteristic peaks at 350 nm and 400 nm attributed to the conjugated dye **5** in aqueous solution (Figure 3A), whereas the addition of the compound **5** directly to unmodified, native CPMV particles provided a single absorbance peak at 320 - 330 nm (Figure 3A *inset*).⁴¹ Native CPMV with and without dye **5** were used as two different control samples for characterization by fluorescence with excitation wavelength set at 390 nm and the emission wavelength scanned from 400 to 600 nm (Figure 3B). The peak emission at 470 nm was observed for ADIBO-CPMV particles upon addition of **5** (50 – 250 μ M), whereas the virus alone or native particles with **5** exhibited no fluorescence (Figure 3C). The incremental addition of **5** to ADIBO-CPMV reached maximal fluorescence at concentration above 100 μ M (Figure 3C). The dye-labeled particles were also analyzed by reducing SDS-PAGE alongside molecular weight standard (lane 1) unmodified CPMV (lane 2) for comparison (Supplementary Figure 2). Fluorescence gel imaging revealed both large and small subunits of ADIBO-CPMV being incrementally modified by **5** (Supplementary Figure 2, lanes 4-8) and reaching a maximal emission level at 100 μ M of **5** (Supplementary Figure 2, lane 6).

The inverse approach, in which the virus was modified with **2** followed by **6** then purified by SEC, was monitored by UV-Vis absorbance (Figure 4A) and fluorescence spectroscopy (Figure 4B). The results from both analytical methods indicate the final dye conjugate was successfully conjugated to the azide-CPMV and quantitative labeling of the dye to the viral capsid was achieved (Figure 4C). Dynamic light scattering measurements indicated highly uniform particle diameters for the virus particles (~31 nm) with low dispersity indices (PDI < 0.1) (Supplementary Figure 3). Small increases (1-2 nm) in the hydrodynamic diameter for **7-10** were observed, but particle size distribution remained highly uniform (PDI < 0.1). Virus conjugates **8-10** contain a tetraethylene oxide linker which would account for the slight increase in diameter and PDI values. Transmitted electron microscopy analysis indicated the modified particles were intact with some particles showing hollow cavities (Supplementary Figure 4). The sequential conjugations and the structural integrities of the viral particles were well within the previous reported studies, and both ADIBO and azides are convenient chemical handles for a variety of molecules with or without a PEG linker.

In situ reaction kinetics

The copper-free “click” reaction kinetics was evaluated *in situ* with **3** at concentration of 0.2 mg/mL in buffer and 25-fold excess of **5** with respect to the concentration of CPMV asymmetric unit. The reaction was monitored with a plate reader setting at 398 / 465 nm ($\lambda_{\text{Ex}}/\lambda_{\text{Em}}$) with an emission cutoff at 455 nm. The relative fluorescence intensity steadily increased over time and reached near maximum after 120 minutes (Figure 5). On the other hand, the addition of **5** to unmodified CPMV provided no increase in fluorescent signal over the 4 hour period (Figure 5). Based on the MALDI-TOF results, we estimate a near complete modification of the DBCO with **5** had been achieved. In a previous study, Kumzin *et. al.* reported near complete reaction on ADIBO functionalized surfaces with azide modified dyes at approximately 100 minutes,³⁷ which is consistent with our observed

results. However, our reported system did not require the removal of the precursors, since only the final conjugated product gave the fluorescent signal.³⁸

SPAAC reaction with fluorogenic probe in cells

Cancer cells often exhibit incomplete cell surface glycosylation, and this aberration can lead to shorter carbohydrate structures, such as the Tn-antigen (innermost *O*-linked GalNAc-*O*-Ser/Thr).^{42, 43} MCF-7 cells express higher levels of the Tn-antigen and are often used to probe the molecular basis of aberrant *O*-glycan synthesis.⁴³⁻⁴⁵ Supplementing the cells with an azido labeled sugar analog (azidoacetylgalactosamine, GalNAz) affords a bio-orthogonal chemical tag for selective modification with alkynes. Since ADIBO-CPMV particles possess ~60 reactive groups that undergo selective 1,3-dipolar cycloaddition reaction with azides, we demonstrate that this polyvalent carrier is an ideal candidate that can provide fluorogenic signal by linking with the azide sugar derivative and dye **5** (see Figure 1). The sequential addition of **5** and **10** in cells without GalNAz provided a slight increase in fluorescence (Figure 6A), likely due to the vimentin receptor targeting moiety on native CPMV particles.²² The presence of a relative short PEG linker (~ 500 Da) has been shown to eliminate the natural binding of CPMV in KB and HeLa cells,^{46, 47} however the shorter tetraethylene glycol linker used in this study did not sufficiently block the natural vimentin binding sites. As expected, the addition of **5** alone with GalNAz labeled cells did not provide any fluorescence (Figure 6B), and the sequential addition of reagents in GalNAz labeled cells enhanced the overall fluorescence around the cells (Figure 6C). The differences in fluorescence were further assessed by using a 96-well fluorescence plate reader in live cells. The samples lacking **10** provided the lowest fluorescence intensities, with the addition of **5** and **10** increasing fluorescence intensity, and the presence of GalNAz provided the highest intensity (Figure 6D). Similarly, the labeling study was conducted with live MCF-7 cells on glass bottom 35-mm dishes, wherein the cells were pre-labeled with the sugar analog for 24 hours. The ADIBO-EG₄ modified viral particles (**10**) were added to the live cells for 15 minutes at a concentration of 100 nM, followed by its immediate removal and addition of the fluorogenic dye at a concentration of 10 μ M (**5**). Without further washing, the cells were imaged in real time (Figure 7). Several punctuated fluorescent signals were observed on the cells. The results herein demonstrate the potential use of the virus scaffolds as polyvalent ligands in Cu-free conjugation reactions and real-time imaging of azide-labeled biomolecules.

CONCLUSION

Viruses are the biological equivalent of dendrimers with chemically accessible functional groups, but also containing features that are genetically malleable by altering the viral genome. Several types of viruses have been demonstrated to be robust scaffolds by coupling azides and alkynes for displaying a variety of biomolecules.^{31, 48, 49} The rates of Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reactions had been reported to be comparable to that of the cysteine-maleimide reactions, which is one of the most rapid and reliable bioconjugation reactions.^{18, 30, 50} However, while CuAAC reaction has become commonplace for many bio-orthogonal reactions, its mandatory use of the transition metal and its potential detrimental effects on proteins and cells limit the utility of the method.

Alternatively, the triazole bond formation between azide and internal alkynes in the strained eight member ring can be achieved without the catalyst, but at a slower reaction rate.¹⁷ Substitutions around the unsaturated carbons in the cyclooctyne ring have been reported to improve its reaction rates significantly. For example, the biarylazacyclooctyne reported by Boons and Popik was associated with a rate-constant of $0.31 \text{ M}^{-1} \text{ s}^{-1}$.^{37, 51, 52}

Using CPMV as a convenient protein macromolecule, we perform a litany of tests involving bioconjugation reactions in viral nanoparticles. A large number of attachments to each particle are readily achieved while preserving the tertiary and quaternary structures of the virus as demonstrated by DLS and TEM analysis. In this work, we demonstrate the combination of a fluorogenic probe and a polyvalent carrier to visualize the azido-sugar substituted glycoproteins in cells. Direct, controlled reactions between ADIBO and azides on viral nanoparticles in aqueous solutions were efficient, and the observed reaction kinetic was in agreement with the reported reaction rates. The study suggests that the sequential attachment of ADIBO or azide on CPMV can be effectively used as bio-orthogonal chemical handles in the similar manner as the previous studies with CuAAC reactions.

The applications of viral nanoparticles by ADIBO-azide conjugation reactions could be used in lieu of the CuAAC reactions to display small molecules, peptides, proteins, complex sugars and polymers. The direct incorporation of an azide containing non-natural amino acid that is strategically positioned will be a powerful tool in exploring how architectural features of the virus would modulate the mammalian immune response and other cell surface receptor clusters. The future studies will exploit the intrinsic advantages of viral particles (their geometrical symmetries and genetically encoded building blocks) to probe the complex biological events. The use of Cu-free SPAAC reaction with strained alkynes has great potential in labeling of biomolecules and in vivo imaging. In a recent study, Bertozzi and colleagues reported thiacycloheptynes as promising class of reagents for Cu-free click reactions to selectively undergo reactions with azides under ambient temperatures and at reaction rates faster than any other reported cyclooctyne reagents.⁵³ The utilization of such reagents on complex macromolecules (i.e. viruses and virus-like particles) is likely to promote developments of new functional materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

CPMV	Cowpea mosaic virus
NHS-ester	N-hydroxysuccinimide ester
ADIBO	aza-dibenzylcyclooctyne
TMR	tetramethylrhodamine
MALDI-TOF	Matrix assisted laser desorption ionization – time of flight

Figure 1.

Reaction scheme with ADIBO modified CPMV for labeling azide-sugars displayed on cell surface glycoproteins. The azide-labeled glycans on the cells are subsequently labeled with the polyvalent viral scaffolds and fluorogenic azidocoumarin dye. Only upon the formation of the triazole linkage between the azide-labeled glycans, virus and the dye will yield the fluorescent signal, whereas the virus-dye alone will wash away and the unconjugated dye contributes little to the observed fluorescence.

Figure 2.

Reaction scheme on CPMV. (A) The first approach to conjugate ADIBO (1) or ADIBO-EG₄ (9) on viral particles to yield particles (3) or (10), respectively. ADIBO modified particles (3) are then reacted with an azide functionalized coumarin dye (5) to generate the dye modified particles (7). ADIBO-EG₄ particles (10) modified with dyes yielded (11). Inverse modification strategy places the azide functional group followed by the reaction with ADIBO functionalized dyes. (B) Mass spectrometric analysis of the small subunit of CPMV (black) and modified subunits indicate the near complete sequential conjugations of (1) then 5 (blue) to the virus, and the inverse conjugations with (2) followed by 6 (red). The presence of EG₄ linker appears to slightly decrease the reaction efficiency.

Figure 3.

Characterization by UV-Vis absorbance and fluorescence spectroscopy. **(A)** UV-Vis absorbance measurements of unmodified particles (black) has a characteristic absorbance for 260/280 nm. ADIBO-CPMV has a slight shoulder at 310 nm (red) due to the presence of ADIBO, and the sequential modification with **5** reveals two absorbance peaks at 350 nm and 400 nm (blue). Only a single peak at 320 nm is observed with the addition of **5** to native CPMV (green). **(B)** Fluorescence measurements ($\lambda_{\text{ex}} = 395$ nm) indicate maximal emission peak at 463 nm for ADIBO-CPMV-coumarin conjugates. No fluorescence was detected with unmodified particles or addition of **5** to the native virus particle. **(C)** The maximal fluorescence intensities ($\lambda_{\text{max}} = 463$ nm) plotted versus concentration of **5** suggest 100 μM was sufficient to saturate the fluorescence. Each sample was performed in triplicates.

Figure 4.

Dye conjugations on azide-CPMV particles. **(A)** UV-Vis absorbance measurements of unmodified particles (black) and azide-CPMV (blue), and the subsequent modification with **6** reveals two absorbance peaks at 515 nm and 550 nm (red). No change in absorbance is observed with the addition of **6** to native CPMV (green). **(B)** Fluorescence measurements ($\lambda_{\text{ex}} = 545$ nm) indicate maximal emission peak at 570 nm for azide-CPMV-TMR conjugates. No fluorescence was detected with unmodified particles or addition of **6** to the native virus particle. **(C)** The dye per particle plotted against dye concentrations reveal similar loading capacity as previous studies. Experiments were performed in triplicate and results are represented by mean $\pm$ SD.

Figure 5.

SPAAC kinetics on BNPs. CPMV (black) and **3** (blue) at 0.2 mg/mL in buffer were reacted with 25 equivalent of fluorogenic dye **5** at room temperature for four hours. The fluorescence at 465 nm with emission cutoff at 455 nm was measured on a 96-well plate. Error bars denote $\pm$ s.d. Sample sets ($n = 4$) were statistically analyzed by using a two-tailed equal variance Student t -test.

Figure 6.

The use of CPMV as a macrofluorogenic probe in cells by SPAAC in one pot. (A) Both labeling reagents, **5** and **10**, were added to fixed cells cultured without GalNAz for one hour at room temperature. The sample is washed once then imaged. Fluorescence is still observed, likely due to the natural cell affinity of CPMV. (B) The addition of **5** to GalNAz labeled cells does not provide any fluorescence, whereas (C) the addition of **5** and **10** to GalNAz labeled cells provide the most intense signal. Scale bar is 25 microns. (D) All cell samples were rinsed once with phenol-red free media prior to analysis. All of the samples were incubated with **5** at 10 μ M. The cells with GalNAz and **5**+**10** provide the highest fluorescence intensity, but noticeable background is observed for the negative controls containing **5** and **10** only, likely due to the natural cell surface binding motif of CPMV. Sample sets ($n = 4$) were statistically analyzed by using a two-tailed equal variance Student t-test. * $p < 0.05$ and ** $p < 0.01$.

Figure 7.

Live cell labeling with GalNAz, ADIBO-CPMV and fluorogenic dye. Cells were pre-labeled with GalNAz for 24 hours followed by incubation with **10** for 15 minutes, followed by addition of **5** and imaged with inverted fluorescent microscope using a 60x oil lens at room temperature. Scale bar is 10 microns.