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Crosslinking of viral nanoparticles with "clickable" fluorescent crosslinkers at the interface

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Cu (I) catalyzed alkyne-azide cycloaddition (CuAAC) reaction, a typical "click" reaction, is one of the modular synthetic approaches which has been broadly used in various organic syntheses, medicinal chemistry, materials development and bioconjugation applications. We have for the first time synthesized two dialkyne derivatized fluorescent crosslinkers which could be applied to crosslink two biomolecules using CuAAC reaction. Turnip yellow mosaic virus, a plant virus with unique structural and chemical properties, was used as a prototypical scaffold to form a 2D single layer at the interface of two immiscible liquids and crosslinked with these two linkers by the CuAAC reaction. Upon crosslinking, the fluorescence of both linkers diminished, likely due to the distortion of the polymethylene backbone, which therefore could be used to indicate the completion of the reaction.

pickering emulsion, crosslinking, nanoparticles, CuAAC reaction, click chemistry, interfacial assembly

1 Introduction

Click chemistry is a powerful and modular synthetic approach introduced by Sharpless *et al*. [1, 2]. The criteria defining a click reaction include simple reaction conditions, high product yields, little byproduct formation and simple product isolation procedures [1]. The driving force of click reaction is thermodynamic in nature which controls the modular and stereo specific nature of the reaction resulting in high product yields [3]. Of all the reactions that can be ranked under click chemistry, Cu(I) catalyzed alkyne-azide cycloaddition (CuAAC) reaction is undoubtedly the premier example of all [4, 5]. This reaction has been extensively used in materials development [6–9], polymer synthesis [10, 11], dendrimer synthesis [12–16] and drug discovery [17–19]. CuAAC reaction serves as a great tool for bioconjugation applications [20–29], mainly due to (i) facile synthesis and easy incorporation of alkyne and azide moieties into biomolecular frameworks, (ii) compatibility to other functionalities yielding highly specific products, (iii) compatibility to water which is crucial for biomolecular systems, (iv) mild reaction conditions which will help maintaining the properties of biomolecules, and (v) stability of the resulting triazole ring to the hydrolytic cleavage, oxidation and reduction [2, 3, 9].

For a biomolecular system, chemical cross-linking of two biomolecules or proteins is highly significant in identifying structure-function relationships in proteins, monitoring protein-protein interactions, and developing novel biomaterials for biomedical applications. Over the last 25 years, a variety of mono-functional and hetero-bifunctional reagents have been developed that target different side chains of proteins for coupling. Crosslinkers with reaction chemistries such as

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photoactivatable molecules [30], fluorescent linkers [31], cleavable linkers [32], antibody-antigen complexes [33], metal complexes [34], and biotin-streptavidin complexes [31, 35] have been reported for protein couplings. However, few crosslinking methods possess properties such as good reaction specificity, high product yields, limited side reactions and ability to account the crosslinking efficiency [31, 35]. In this regard, the synthesis of a crosslinker which serves as a crosslinker as well as a label indicating the reaction completion is invaluable.

Cyanine dyes are one of the most common fluorescent compounds that have been applied in a variety of applications due to their facile synthesis, relative stabilities, high polarity, high molar extinction coefficients and broad wavelength range of fluorescence emission [36–40]. In our previous publications we have demonstrated the synthesis of novel cyanine and hemicyanine dyes [39], and their application in selectively modifying viruses [41]. We hereby report the synthesis of two new dialkyne cyanine dyes bearing polymethylene chains and their applications as chemical crosslinkers to ligate two biomolecules via the CuAAC reaction. A plant virus, turnip yellow mosaic virus (TYMV), was used as the model to demonstrate the reaction potential of these dyes in crosslinking two particles. Recent studies indicate that TYMV can be employed as a great tool in drug delivery, imaging and tissue engineering applications [6, 42–46]. This symmetric bionanoparticle (BNP) could self-assemble at the liquid-liquid interface to yield stable emulsion systems and highly ordered long range arrays at the flat interface [44]. Due to the availability of facile amino acid coat protein structure these particles could be crosslinked using simple chemicals such as glutaraldehyde. In lieu of the fact, that a labeling technique which can help in determining the reaction completion will be very useful for different applications, we propose to crosslink the self assembled TYMV particles at the interface using CuAAC reaction with fluorescent cyanine dyes which lose their fluorescent nature after the reaction.

2 Experimental

2.1 General methods

All chemicals and solvents were obtained from commercial suppliers and used as received unless otherwise noted. ^{1}H NMR spectra were recorded on Bruker 400 NMR spectrometer and UV-vis absorption spectra were measured on Agilent 8453 spectrometer. Emission spectra were measured on Varian Cary Eclipse fluorescence spectrophotometer. ESI HRMS spectra for compounds were recorded using Micromass Q-TOF. TYMV was purified from infected Chinese cabbage leaves following the procedure as described [43]. Chemical modification of TYMV with the desired compounds and its characterization was done using the established protocols as described [42]. TEM analyses of samples were performed using Hitachi H-8000 TEM electron microscope. TEM samples of the emulsion particles were prepared by depositing 20 μL of emulsion samples on carbon coated copper grids and staining it with 2% uranyl acetate solution for 4 min. The synthesis of the dyes studied in this paper is outlined in Scheme 1. *N*-(propargyl)-2-methylbenzothiazolium bromide **1** was synthesized according to a known protocol [41]. Protein gel and Western blotting were performed on a 15% SDS-PAGE gel following the standard protocols [43]. The primary TYMV polyclonal antibody was provided by Prof. Theo Dreher (Oregon State University).

2.2 Synthesis of dyes

*Synthesis of dye **2***

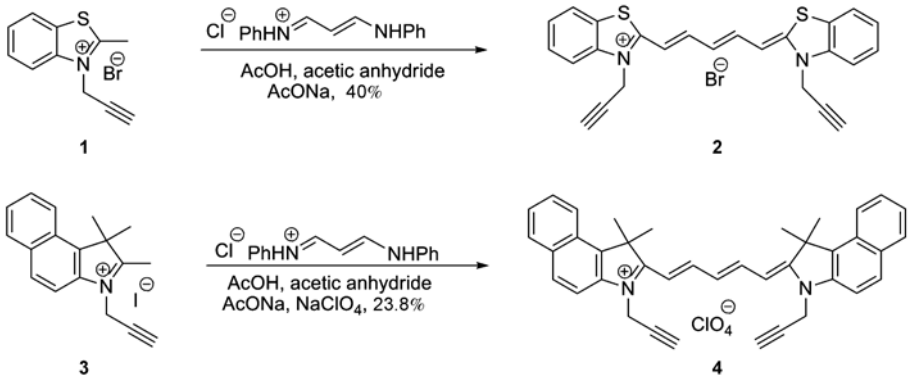
Compound **1** (0.5 g, 1.86 mmol) and malonaldehydedianil hydrochloride (0.24 g, 0.93 mmol) were dissolved in a mixture of acetic acid (2 mL) and acetic anhydride (15 mL). After the addition of sodium acetate anhydrous (0.3 g, 0.56 mmol), the reaction mixture was stirred under 90–100 °C for 1 h. After stirring, the flask was left in a refrigerator for precipitation overnight. The precipitate was filtered and washed with ethanol and water. Then the solid was recrystallized from ethanol three times to yield the product; 0.3 g (40%); mp 240–242 °C; ^{1}H NMR (400 MHz, DMSO): δ (ppm) 8.03 (d, J = 7.6 Hz, 2H), 7.84 (t, J = 12.8 Hz, 4H), 7.76 (d, J = 8.4 Hz, 2H), 7.59 (t, J = 8.4 Hz, 2H), 7.42 (t, J = 8.0 Hz, 2H), 6.67 (d, J = 13.2 Hz, 2H), 6.51 (t, J = 12.4 Hz, 2H), 5.33 (s, 4H), 3.61 (s, 2H). ^{13}C NMR (100 MHz, DMSO): δ (ppm) 164.2, 151.7, 141.0, 128.6, 125.8, 125.6, 123.7, 122.3, 113.8, 101.7, 77.4, 77.1, 36.2. ESI HRMS exact calcd mass for $(C_{25}H_{19}N_2S_2 + H)^+$ is *m/z*: 411.1000, and found *m/z*: 411.0990.

*Preparation of 7-ethynyl-1,1,2,3-tetramethyl-1H-benz[e]indolium iodide **3***

1,1,2-trimethyl-1*H*-benz[e]indolenin (4.2 g, 16 mmol) was dissolved in 20 mL acetonitrile. Iodomethane (9.03 g, 64 mmol) was added and the solution was stirred at 50 °C for 8 h. The mixture was left in a refrigerator overnight. The precipitates were filtered and washed with acetonitrile (3 × 3 mL). Thorough drying yielded **3** as a green solid; 3.2 g (54%); mp > 250 °C.

*Synthesis of dye **4***

Compound **3** (1.47 g, 3.92 mmol) and malonaldehyde-dianil hydrochloride (0.51 g, 1.86 mmol) were dissolved in a mixture of acetic acid (2 mL) and acetic anhydride (15 mL). After the addition of sodium acetate anhydrous (1.0 g, 12.2 mmol), the reaction mixture was stirred at 90–100 °C for 1 h. Then the reaction mixture was left in a refrigerator overnight for precipitation. The precipitates were filtered and washed with ethanol and water. The obtained solid was recrystallized in ethanol containing sodium perchlorate



Scheme 1 Synthesis of **2** and **4.**

(0.41 g, 2.88 mmol). Upon cooling, the deposit was collected to yield a solid; 0.63 g (24%); mp > 250 °C; ^{1}H NMR (400 MHz, DMSO): δ (ppm) 8.62 (t, J = 13.2 Hz, 2H), 8.31 (d, J = 8.0 Hz, 2H), 8.16 (d, J = 8.4 Hz, 2H), 8.11 (s, 2H), 7.85 (d, J = 8.8 Hz, 2H), 7.75 (s, 2H), 7.57 (s, 2H), 6.72 (t, J = 12.4 Hz, 1H), 6.57 (d, J = 13.6 Hz, 2H), 5.28 (s, 4H), 3.61 (s, 2H), 2.02 (s, 6H). ^{13}C NMR (100 MHz, DMSO): δ (ppm) 174.2, 154.6, 139.4, 133.4, 131.9, 130.8, 130.4, 128.3, 128.1, 125.4, 122.7, 112.0, 104.3, 77.5, 76.7, 51.3, 34.3, 27.2. ESI HRMS exact calcd mass for $(C_{39}H_{35}N_2 + H)^+$ has *m/z*: 531.2800 and found *m/z*: 531.2806.

2.3 Modification of TYMV and emulsion preparation

The lysine residues present on the coat protein of TYMV were modified with azide NHS-ester **5** as described [42]. The reaction was carried out in 0.01 M potassium phosphate buffer (pH 7.8), containing 10% (*v*/*v*) THF with TYMV (1 mg/mL) and a 100-fold excess of **5** at 4 °C overnight. The TYMV-azide was then pelleted out from the reaction mixture by centrifuging for 2 h at 42000 rpm (Beckman 50.2 Ti rotor). The final concentration of TYMV-N_3 was determined by UV-vis analysis of the product at 260 and 280 nm. The modification of virus particles was characterized using MALDI-MS.

Formation of emulsions and sequential crosslinking reaction

Previously, we have demonstrated that spherical virus particles such as cow pea mosaic virus and TYMV could yield stable Pickering emulsions in perfluorodecalin/aqueous phase system, which could be crosslinked together to form stable layers using glutaraldehyde [44, 46]. In this study, we employed the same system to obtain TYMV stabilized perfluorodecalin emulsions in aqueous phase and demonstrated the crosslinking of TYMV particles arranged at the interface using CuAAC reaction. The aqueous phase was prepared by dissolving 150 mM NaCl in nanopure water. To the aqueous solution, TYMV-azide was added such that the final concentration of the virus-azide was 0.1 mg/mL. A 10-fold excess of the cyanine dyes per virus subunit was added to the aqueous phase prior to the addition of perfluorodecalin. After the addition of dyes, bathocuproinedisulphonic acid (BCA), the ligand for CuAAC reaction, was added to the aqueous phase to reach a final concentration of 2 mM. A 10% (*v*/*v*) amount of perfluorodecalin was added and the mixture was shaken vigorously for 15 min to form very small emulsion droplets. The reaction vials were covered with aluminum foil in order to avoid photo bleaching of the dyes. These emulsion solutions were kept at 4 °C for 2 h before (*tris*(2-carboxyethyl)phosphine) (TCEP) (final [*c*] = 5 mM) and CuSO4 (final [*c*] = 1 mM) were added. The reaction vials were kept overnight at 4 °C and the emulsions were thoroughly washed using nanopure water before analysis.

3 Results and discussion

Methylation of 1,1,2-trimethyl-1*H*-benz[e]indolenin with methyl iodide yielded indolinium salt **3** and its purity was examined using ^{1}H NMR. Treating the thiazolium salt **1** and indolinium salt **3** with malonaldehydedianil hydrochloride in a solution of glacial acetic acid and acetic anhydride produced the dialkyne-derivatized cyanine linkers **2** and **4**, respectively. The methods developed for the synthesis of the two cyanine dyes yielded gram quantities of both dyes. Like most of the cyanine dyes, dyes **2** and **4** were soluble in water/DMSO co-solvent systems and showed intense blue and green colors with maximum absorption at 663 nm and 729 nm, respectively. Both linkers display strong fluorescence emission at room temperature (Figure 1).

Based on our previous studies we have established that spherical plant viruses could self-assemble at the oil-water interface forming a monolayer of particles that could be crosslinked using crosslinkers as glutaraldehyde. The crosslinking of BNPs arranged at the interface plays a crucial role in enhancing the mechanic properties of the virus assemblies [44, 46]. Therefore, this offers an ideal platform to test the reactivity of linkers **2** and **4**. As shown in Scheme 2, TYMV particles were modified with azide **5** according to

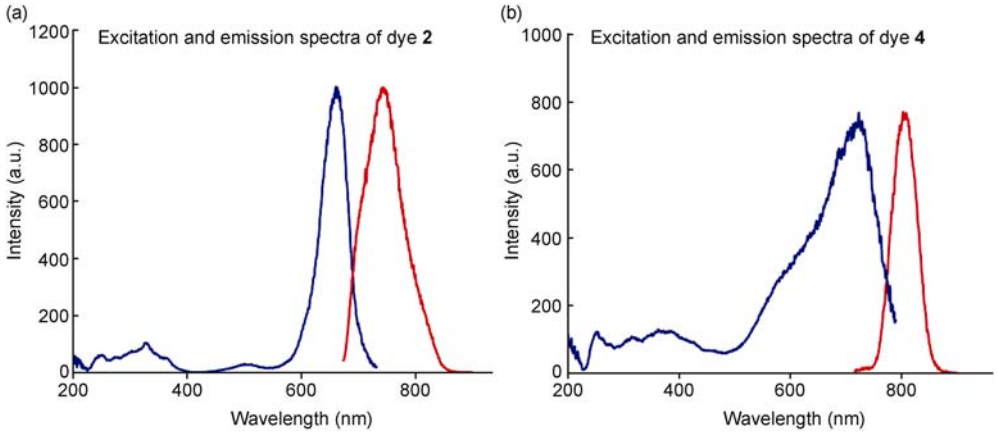
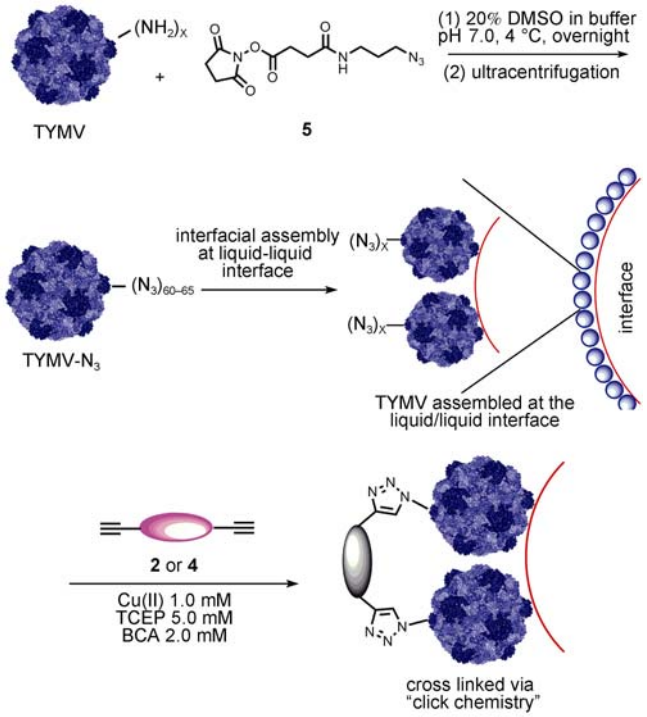


Figure 1 UV-vis and fluorescence spectra of dyes **2** (a) and **4** (b) recorded in pure DMSO. Blue curves represent absorption spectra and red curves emission spectra, respectively.



Scheme 2 Crosslinking of TYMV at the liquid-liquid interface via CuAAC reaction. BCA, bathocuproinedisulphonic acid; TCEP, tris(2-carboxyethyl)phosphine.

the protocol described previously [42]. The integrity of the modified viral particles TYMV-N_3 was confirmed by TEM and FPLC analysis. Following the reported Pickering emulsion approach, TYMV-N_3 particles were assembled at the interface of water and perfluorodecalin [46]. In order to enhance the interfacial tension between water and perfluorodecalin, 150 mM NaCl solution was used as the aqueous layer. After the addition of perfluorodecalin the mixture was shaken vigorously to form emulsion droplets decorated with TYMV-N_3 particles (Figure 2(a)). The emulsions were equilibrated for two hours before the CuAAC reaction was initiated and carried out overnight. $CuSO_4$ was used as the copper source which was reduced *in situ* to Cu(I) by TCEP, a bio-benign reducing reagent [25]. TCEP is a competent reducing agent for Cu(II); it is more hydrophilic, resistant to oxidation in air and reacts very slowly with azides [3]. Copper chelator BCA was added as a ligand to stabilize the *in situ* generated Cu(I) which catalyzed the CuAAC reaction. The successful crosslinking of TYMV-N_3 particles at the interface was first observed upon comparing the stability of emulsions to washing with water. In the absence of crosslinking the droplets collapsed into separate oil and water phases. After the CuAAC reaction, the emulsion droplets covered with crosslinked TYMV were very stable and were washed with water to remove excess dyes and other reaction components without disrupting the droplets.

As shown in Figure 2(b), after removing the excess dyes, the emulsions retained the original color of dyes. The droplets were clearly observed using optical microscope (Figures 2(c) and (d)). However, the fluorescence of dyes was completely quenched after the reaction. This loss of fluorescence may be attributed to the distortion of polymethylene chains of cyanine dyes **2** and **4** caused by the steric hindrance upon conjugation to bulky TYMV particles. The integrity of TYMV particles after CuAAC reactions was confirmed by transmission electron microscopy (TEM) analysis. TEM images show the presence of closely packed TYMV particles emulsions (Figure 3(a) and (b)). A very good surface coverage of the emulsion droplets with viral particles was observed.

We further confirmed the crosslinking of TYMV particles by SDS-PAGE and Western blot analyses. The coat protein of TYMV was recognized with anti-TYMV polyclonal antibodies. As shown in Figure S1 (Supporting Information in electronic version), a single band at 20 kDa was observed for TYMV-N_3 which corresponds to the protein subunit of TYMV. Upon crosslinking TYMV particles with **2** or **4** by CuAAC reaction, higher molecular band corresponding to the protein dimer structure at ~40 kDa

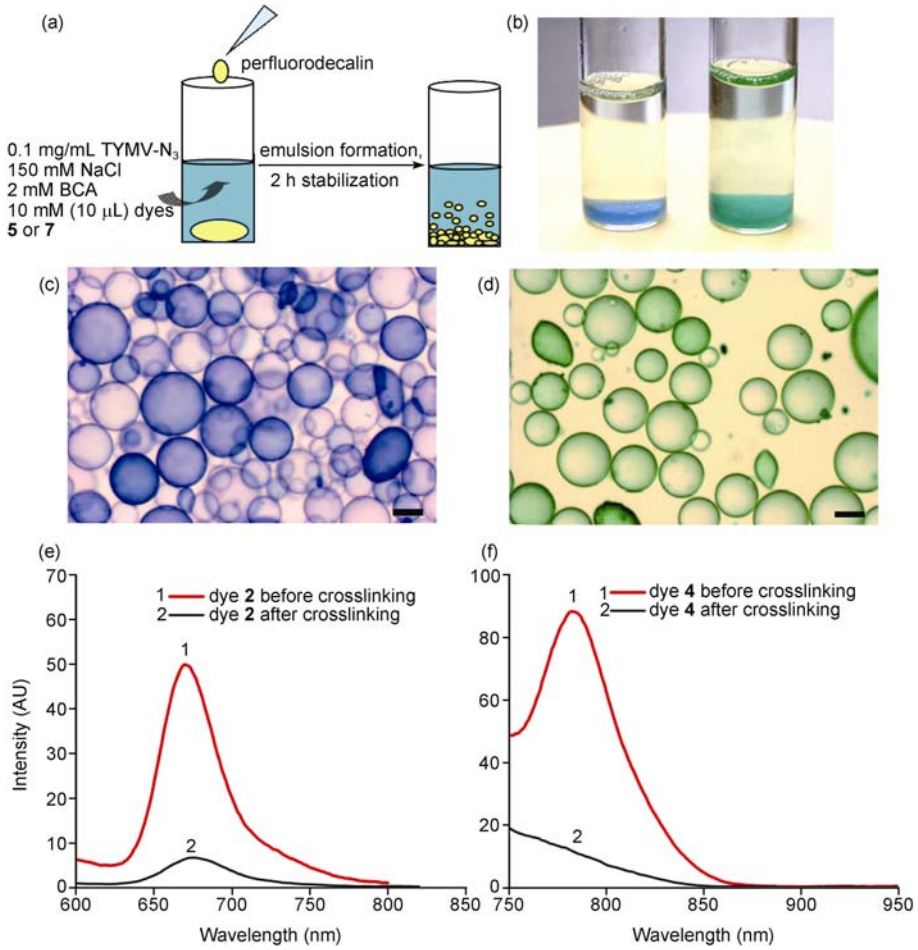


Figure 2 (a) Schematic illustration of the emulsification process. (b) Optical image of TY an MV particles covered emulsions after thorough washing (bottom layer) crosslinked with **2** (blue color) and **4** (green color). (c, d) Emulsions visualized using an optical microscope show that after crosslinking reaction the dyes retained their respective blue and green colors. Scale bars are 100 μm. (e, f) Fluorescence spectra of dyes **2** and **4** before and after crosslinking via CuAAC reaction, showing the fluorescence of the original dyes was quenched upon crosslinking.

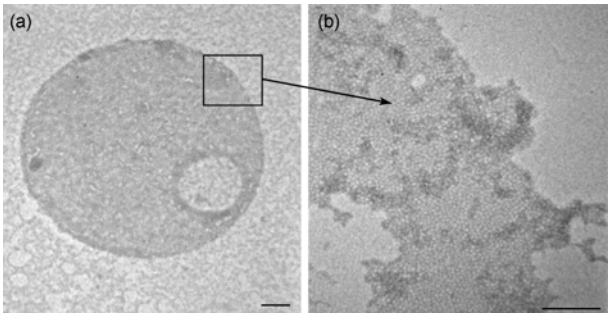


Figure 3 (a) TEM image of the emulsion droplet (the scale bar is 50 μm). (b) A magnified TEM image of the emulsion boundary showing the arrangement of closely packed TYMV particles (the scale bar is 500 nm).

was observed in addition to the 20 kDa monomer band, which was attributed to the crosslinking among TYMV particles at the interface. The faint bands at ~58 kDa might be attributed to the formation of small quantities of trimers during the crosslinking. As the control reaction, a solution of TYMV-N_3 in water (1.0 mg/mL) was treated with either **2** or **4** under the same CuAAC reaction conditions. However, after the overnight incubation, no crosslinking was observed in either case. Considering the azido groups on TYMV are fairly hindered, the close proximity of two viral particles is necessary to promote an efficient crosslinking reaction [41]. At the water-oil interface of Pickering emulsions, the local concentration of virus particles is very high and the reactive sites of TYMV are relatively fixed, which therefore result in much more efficient CuAAC reactions.

4 Conclusions

In this paper we have reported the synthesis of two fluorescent dialkyne linkers **2** and **4** which can be utilized in crosslinking two azide-carried entities by CuAAC reaction. Plant virus TYMV was employed as a prototypical biomacromolecule in our study. We found the linkers could be efficiently employed to crosslink TYMV particles that were arranged at the interface of oil and water. Due to the high

local concentration of TYMV particles at the interface and close proximity of the particles the crosslinking reaction occurred very efficiently. Both linkers retained their absorption properties after the crosslinking but lost their fluorescence completely. This is the first time that dialkyne fluorescent linkers were reported for the crosslinking applications by CuAAC reaction. Due to the biocompatibility of azido and alkyne groups, as well as the bioorthogonal feature of the CuAAC reaction, this crosslinking strategy is highly specific. In particular, less side products are produced in comparison to conventional crosslinkers as glutaraldehyde or bis-NHS esters.

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