



Developing Virus-like Particles as High-density Photoreporting Platforms using Noncanonical Amino Acids and Click Chemistry

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Mark T. Smith, Recipient
Bradley C. Bundy, Advisor
Brigham Young University
Department of Chemical Engineering

ABSTRACT - Light-based imaging and microscopy techniques are compelling diagnostic tools that provide straightforward data. Furthermore, single-particle targeting and tracking has the potential to provide new insight on myriad phenomenon, such as small particle diffusion, particle-membrane interaction, single-particle microfluidics, and many aspects of microbiology and physiology. However, limitations exist in current technologies' ability to achieve tracking of individual nanoparticles due to inability to sufficiently label or stain single targets with traditional probes. This work proposes a method to overcome probe density by creating individual probes based on virus-based nanoparticles densely decorated with many reporter probes. We present the steps to produce such reporters. This technology has the potential to provide insight into single particle pathways that might otherwise only be measured with bulk properties such as single-particle dynamics in microgravity.

Antonie van Leeuwenhoek ushered in a new age of science and technology when he reported seeing single-celled organisms with his high quality microscopes. The science of microbiology was born, leading to better understanding in fields of physiology, infectious disease, and medicine. Of broader impact, the technology of high quality micro-scale imaging opened doors for biologists, chemists, and material scientists alike. Technologies such as confocal microscopy, TEM, SEM, and AFM undoubtedly stem from the fundamental findings of Leeuwenhoek.

Imaging and light microscopy are excellent diagnostic tools because of their straightforward data collection. Specifically, items can be directly visualized with minimal disturbance in lieu of indirect or perturbing data collection methods, such

as IR or applying an electrical current. Furthermore, some imaging technologies allow for real-time data collection (e.g. dark-field microscopy). Finally, imaging can be made quite inexpensive, as demonstrated recently by a doctor in Tanzania, who transformed a smartphone into a functional field microscope for about \$8.¹ The combination of these characteristics makes microscopic imaging a compelling diagnostic tool.

However, there are limitations to light-based imaging techniques based on diffraction and wavelength.² One way to skirt these issues is to directly label or "stain" the object to be imaged with fluorescent probes and use specific wavelengths and filters to control what is visualized (i.e. fluorescence microscopy).² In so doing, items of interest can be more easily contrasted from the background and higher spatial resolutions can be achieved.

When the object of interest become extremely small and single-particle tracking is desired, (such as cases of a single motor protein or virus particle), it becomes difficult to achieve sufficient density of stain to resolve the object's position both spatially and temporally. We propose a novel method to overcome reporter-density limitations for single-particle tracking applications by developing virus-based particles that are modified to be stain-dense particles capable of targeting single molecules for single particle tracking. In so doing, we hope to aim at a single target (i.e. motor protein) with a "single" reporter (i.e. reporter-decorated particle) to achieve high spatial and temporal resolution.

Potential future applications of these particles is broad, including simple and economic medical diagnostics, single-particle analysis for diffusion and transport, nanoparticle microfluidics, membrane-particle interactions, and highly sensitive analysis of particle purification.

Technological Background

The development of such reporter platforms requires the simultaneous application of two major technologies: noncanonical amino acid (ncAA) incorporation and copper-catalyzed azide-alkyne cycloaddition (“click” chemistry). To facilitate these techniques in a single product, we employed cell-free protein synthesis.

Cell-free Protein Synthesis (CFPS) is an extremely flexible system for protein production.³ The system removes the cell wall, which would otherwise behave as a barrier blocking access to the synthesis environment. The removal of this wall allows for excellent control over the synthesis environment that would be difficult for an *in vivo* system. With this access, the ability to add and carefully control external cofactors to the protein synthesis environment is made simple.⁴ Furthermore, post-production purification is also simplified compared

to *in vivo* systems, because extraneous cellular debris is removed before production. Finally, CFPS simplifies characterization of proteins because the synthesis environment can be directly sampled.³

Some 70+ different noncanonical amino acids have been successfully incorporated into appropriately mutated proteins.⁵ To achieve ncAA incorporation, the ncAA must be available in the reaction mix. The open access of the cell-free environment provides for easy control of ncAA concentration and we are able to achieve concentrations that might otherwise be unreachable *in vivo*.⁶ Beyond availability, the ncAA must be appropriately aminoacylated to tRNA and be accepted by the ribosome. There are numerous paths to achieve incorporation. Our work employs the technique of Global Replacement that is Residue Specific (GRRS) to incorporate our ncAA.⁷ Specifically, we do not add methionine to the CFPS environment, and instead replace it with one of methionine’s ncAA analogues – azidohomoalanine (AHA). In so doing, the AHA replaces locations in the protein where methionine would have been.^{7, 8} The successful incorporation of the methionine analogue results in reactive moieties in the form of azides.

The click reaction is a bioorthogonal reaction that creates a cyclic covalent bond called a triazol.⁹ Through the GRRS, we have the ability to incorporate many azides in the particles. We then select fluorescent probes that have an available terminal alkyne moiety for conjugation to and decoration of the virus-based particles.

By using these technologies in tandem, we are able to create virus-based nanoparticles that are densely decorated with fluorescent probes (Figure 1).

Particle Production and Purification

We used the coat proteins (CPs) from the bacteriophage Q β as the basis of our virus-based nanoparticle. When CPs are produced in sufficient number, they self assemble into 28 nm particles consisting of 180 CPs that are stabilized by interpeptide disulfide bonds.^{4, 10} Furthermore, previous work has demonstrated the production of similar particles in cell-free and using GRRS.⁷

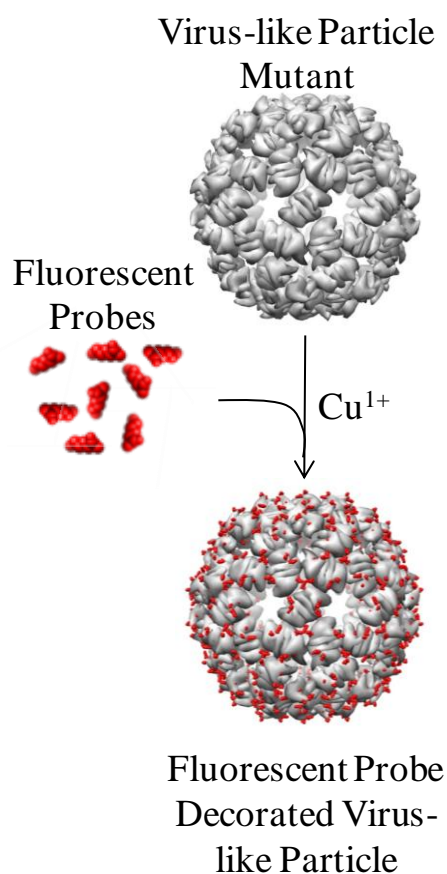


Figure 1: Scheme for Virus-like Particle Decoration. Mutant particles self-assemble from coat proteins produced using cell-free synthesis. After purification, these particles are conjugated with fluorescent probes using the copper-catalyzed click reaction.

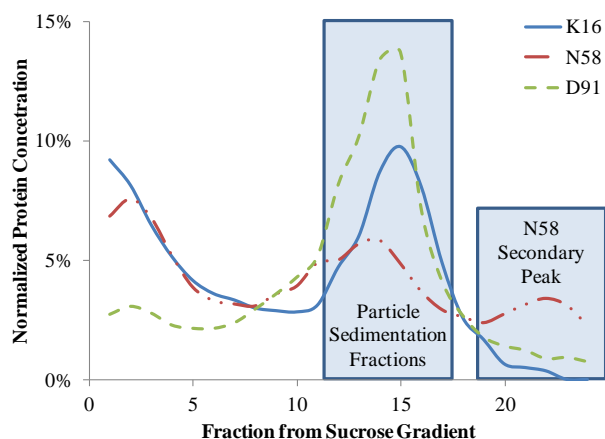


Figure 2: Velocity Sedimentation. To purify the particles from improperly or unassembled proteins, the CFPS reactions were centrifuged on a 10-40% sucrose gradient and subsequently collected in fractions. Fractions at the nominal location of properly assembled particles were collected and concentrated. The secondary peak exhibited by mutant N58 is suggestive of polymorphism in that mutant.

An immediate limitation of the Q β CPs was the lack of methionines in the native protein. The native protein only has a single methionine at the N-terminus of the peptide. The N-terminus is partially exposed on the external surface of the particle. However, this initial residue is often cleaved off at the end of protein synthesis an estimated 20-80%, of the time.⁷ To overcome this barrier, we chose three locations to genetically mutate to the methionine codon. After carefully reviewing the known crystal structure for the bacteriophage, we altered lysine 16 (K16), asparagine 58 (N58), and aspartate 91 (D91) to produce three different mutants. The K16 mutant provided another external surface exposed location. The N58 and D91 mutants provided internally exposed locations. The internalization of fluorescent probes in virus-based particles has been demonstrated previously using a similar bacteriophage and probes with thiol reactive capabilities.¹¹ Furthermore, the crystal structure of the Q β bacteriophage reveals large (9-13 nm) pores in the particle. These pores should be sufficiently large enough to allow entry of the components essential for click chemistry, namely: 1) Cu¹⁺ ions, 2) fluorescent probes approximately 2 nm in the longest conformation, and 3) the click reaction accelerating ligand that is also approximately 2 nm in its longest conformation.

To produce the particles, 0.5 mL CFPS reactions were performed at 30 °C for 8 hrs similarly to

previously reported work. Radioactive leucine (C14) was added to the reaction to aid in characterization of particles and yields. Directly following protein production, the reaction solution was dialyzed against NET buffer (150 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl, pH 7.8).

Velocity sedimentation on a sucrose gradient (10-40% wt/vol) in NET buffer proved an effective purification technique for the particles.⁴ This also provided insight into the efficiency of proper particle formation.¹⁰ Fully assembled particles sediment to the fractions 11 to 17 (Figure 2). The stronger peaks at these fractions represent higher assembly efficiencies. D91 and K16 mutant particles were efficient at proper assembly, while N58 particles nearly failed to form any properly sedimenting particles. In fact, it appears that the mutation of N58 caused the particles to form a completely different tertiary structure. Such behavior has been previously reported in other viruses and virus-based particles, and is therefore not wholly unexpected.¹² However, the low assembly efficiency largely precluded N58 mutants from being viable candidates for further modification. Therefore, we focused on K16 and D91 mutants in further work.

Particle Decoration and Characterization

We decorated the particles using a tetramethyl rhodamine (TAMRA) derivative with an alkyne moiety. Under anaerobic conditions, the virus-based particles and alkyne-TAMRA derivatives were mixed with Cu¹⁺ and a click reaction accelerating ligand.¹³ These reactions were incubated at room temperature overnight and were subsequently removed from the anaerobic environs.

Native Gel electrophoresis: Previous works have demonstrated that the maintained assembly of viruses and virus-based particles can be assessed using nondenaturing, native gel electrophoresis.^{4, 10, 14} As the particles form from the many CPs, nucleic acids are encapsulated inside of them. By staining the gels with ethidium bromide (EtBr), the nucleic acids can be visualized under UV lighting. The proteins can then be stained using a coomassie or similar stain. The location and shape of the protein stain compared to the nucleic acid stain provides insight into what particles remain assembled and what particles have lost stability. Figure 3 are the images of the nucleic acid and protein stained gel.

Although the velocity sedimentation suggests similar morphology, density, and size of the K16 and D91 mutants, the native gel points to significantly different surface charges for each particle type. The K16 mutants travel approximately 1.7x as far as the D91 mutants (compare lane 1 to 5, Figure 3). The difference in surface charge density is understood to be created by the ncAA AHA. The terminal azide group ($R-N=N^+=N^-$) of this ncAA exhibits a negative charge. The incorporation of 180 AHA on the external surface of K16 mutants causes the changes exhibited on the native gel electrophoresis.

After the click reaction has occurred, both particles experience a mild gel shift on the native gel (lanes 1 vs. 2 and 4 vs. 5). This is indicative that their respective surface charges becoming less negatively charged after the click reaction. During the click reaction, the negatively charged azides react with the alkyne moiety of the probes, resulting in an uncharged triazol bond. Furthermore, the probe itself lacks obvious negative charges. The replacement of the surface exposed terminal azides by neutral probes causes the gel shift. This also suggests that, although the D91 mutants are internally exposed (i.e. the charge shouldn't affect electrophoresis), there are sufficient N-terminal methionines being

modified to cause an overall change in the behavior of the particle during electrophoresis.

To check for gross stability of the modified particles compared to their unmodified counterparts, the particles were subjected to elevated temperatures (85 °C) for 10 minutes. The results are seen in Figure 3, lanes 3 vs. 4 and 7 vs. 8. The lack of distinct protein bands and the smearing of the nucleic acid bands imply that both the unmodified and modified particles are equally susceptible to disassembly at such temperatures.

SDS-PAGE: To examine the ratio of fluorescent probes to protein, denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed and the resulting fluorescence of the gel was imaged using UV-light (Figure 4). We used densitometry to approximate the amount of fluorescent probes per particle. The K16 mutants provided the highest ratio of probes to particle at an estimated 233 probes per particle. However, D91 also had near 197 probes per particle.

Although a high probe to particle ratio is desirable, the final product must also remain soluble and experience low aggregation. We therefore probed the solubility of the modified particles by subjecting

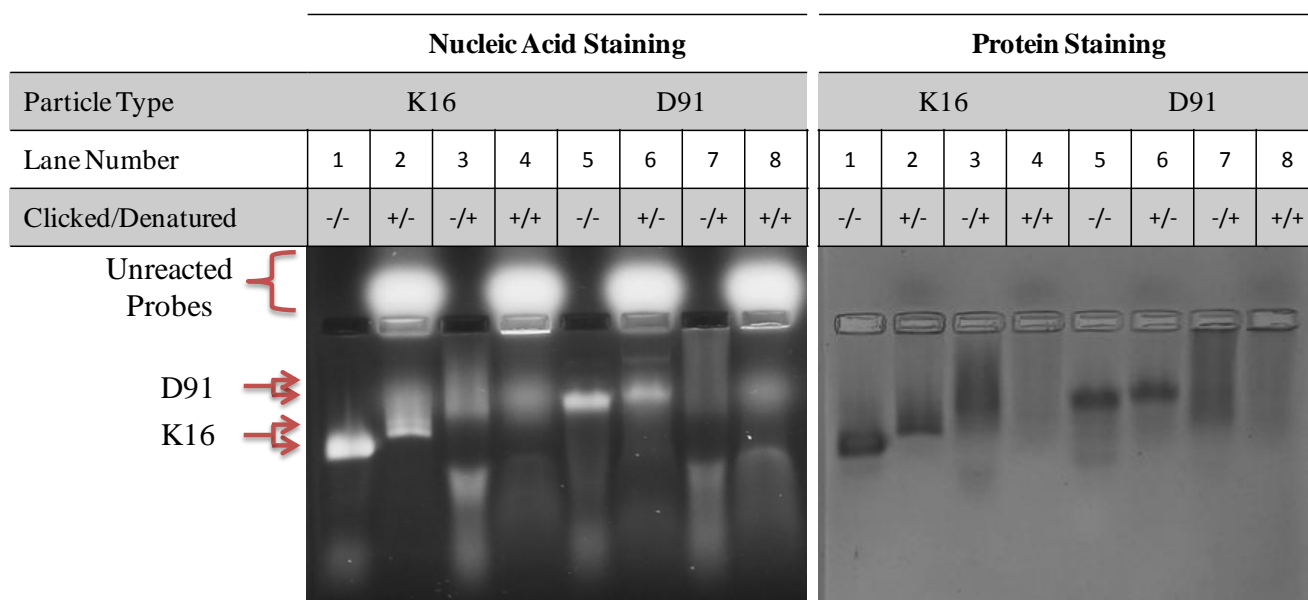


Figure 3: Native Gel Electrophoresis. Nondenaturing native gel electrophoresis provides a simple assay of assembly and gross stability. Furthermore, it offers insight into the external surface character of the particles and how that character changes with conjugation of fluorescent probes. Odd lanes are mutant particle controls, and contain no alkyne-modified fluorescence probes. Even lanes are click reaction products. Lanes 3, 4, 7, and 8 have been incubated at elevated temperature for 10 minutes prior to loading onto the gel. *Left image:* Fluorescent staining of the nucleic acids and fluorescence from conjugated and free probes. *Right image:* Staining of protein in the gel with SimplyBlue Safestain (Invitrogen). The elevated temperatures caused near complete disassembly of the particles.

portions of them to centrifugation before SDS-PAGE (lanes 2, 4, 6, Figure 4). The highly decorated K16 mutants experienced high aggregation rates and displayed an aqueous solubility of only 36% of the total particles. On the other hand, the D91 mutants displayed an 87% solubility, or nearly 2.5x higher solubility than the K16 particles. We hypothesize that the high density of probes on the external surface of K16 mutants shifts the properties of the exposed surface to become much more hydrophobic due to the hydrophobicity of the externally conjugated fluorescent probes. The D91 mutants avoid this hydrophobicity in part by internalizing the probes.

To further explore the solubility, we hypothesized that intermingling hydrophilic moieties amongst the hydrophobic probes on K16 mutants would result in higher overall solubility. We performed a new click reaction of K16 mutants with the addition of alkyne-terminal polyethylene glycol (PEG-2000). The resulting SDS-PAGE is seen in Figure 4 and shows a distinct advantage in solubility over the non-PEGylated K16 particles. Densitometry reveals that 69% of the PEGylated K16 particles remain soluble while still achieving fluorescent probe density of 164 probes per particle. This is a near 200% improvement in solubility with only a 30% loss in

probes per particle. However, the D91 mutants exhibited the highest overall solubility while maintaining a high ratio of probes per particle.

Conclusion

The use of cell-free protein synthesis combined with ncAA incorporation and click chemistry provided a solid basis for controlled decoration of virus-based nanoparticles. Through careful engineering and exploration of potential sites, we demonstrated the ability to produce and characterize different configurations of ncAA incorporation. The ability to directly select targets for decoration in lieu of nonspecific residue decoration gives the user more precise control over the final characteristics of the particles. For example, by internalizing the conjugation site of the fluorescent probe, the particle characteristics are less affected by high density decoration.

It should be possible to fine-tune other aspects of the future particles in a similar manner. For example, a mutant might be developed that contained both the K16 and D91 mutations. The particle could be first PEGylated externally and conjugated with fluorescent probes internally. During the initial step of PEGylation, the PEG will be too large to fit into the particle's pores and would only decorate the

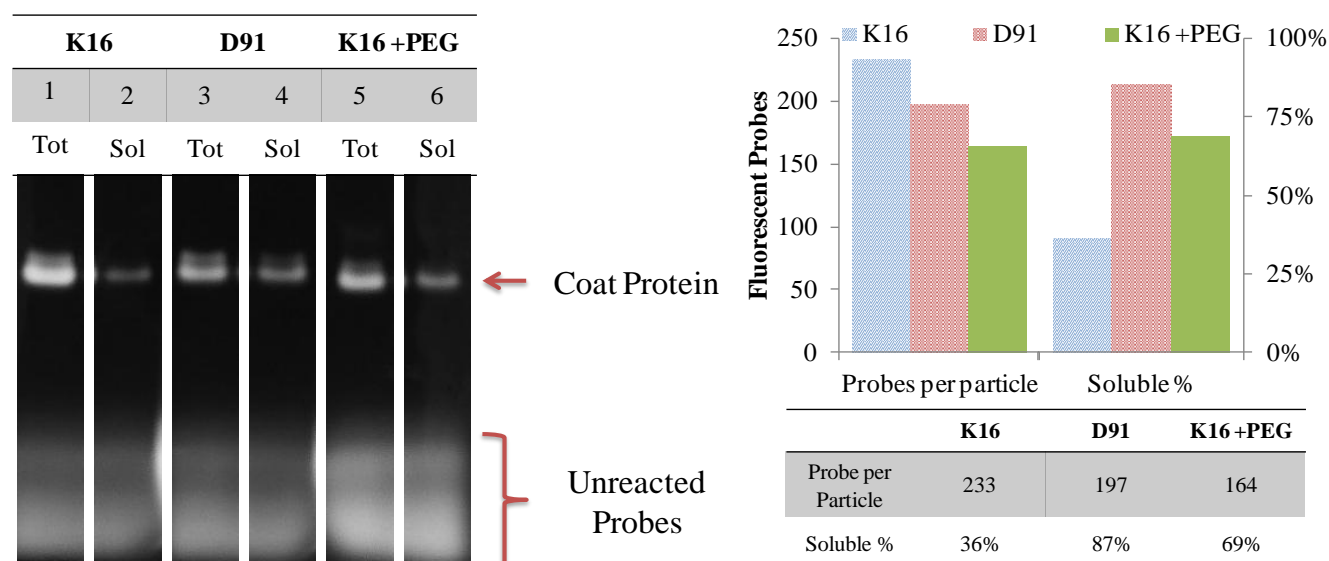


Figure 4: Sodium Dodecyl Sulfate – Polyacrylamide Electrophoresis and Analysis of Solubility. *Left:* SDS-PAGE is a denaturing gel electrophoresis and provides a simple assay of conjugation. Fluorescent probes attached to coat proteins of the virus-based particles will travel to location of the coat proteins. Densitometry was used to analyze the number of probes conjugated and the soluble fraction of particles after centrifugation. Even number lanes were the soluble fraction of the click reactions. *Right:* The graphic is a representation of the table below it. Here are reported the number of probes per peptide and the percentage of particles that remained soluble following centrifugation.



outside surface. Such a modification might be able to produce particles that are more stable and soluble while maintaining a sufficiently high probe density to remain useful for imaging and tracking applications.

Another promising example of combining internal and external modifications is that of targeting. By using a sufficiently large aptamer (e.g. oligonucleotide), the outside of the virus-based particles could be heavily decorated with 100+ aptamers for targeting or signaling. Again, internal sites could be conjugated with probes or other small molecules of interest.

The bacteriophage Q β offers a unique way to potentially target items in the future using a single, individual aptamer. Specifically, the bacteriophage Q β naturally incorporates a single, unique, and externally exposed protein called the A2 protein.¹⁵ We have demonstrated previously using CFPS technologies that we can produce virus-based particles with single and surface exposed A2 protein.⁴ This provides a potential location for a single aptamer to be attached to the particle. Such a step would greatly expand the applications of probe-decorated virus-based particles to nanometric single-target applications. Our current research is pursuing this compelling technology.

This potential for virus-based particles is large, but not restricted to merely imaging and diagnostics. The ability to carefully control the internalization and externalization of ligands brings many possible technologies within reach, such as dynamic single-particle tracking, drug delivery, therapeutic gene delivery, tumor targeting, and *in vivo* particle tracking.

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