



# Synthesis and Characterization of Bio-Active GFP-P4VP Core–Shell Nanoparticles

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**Abstract:** Bioactive core–shell nanoparticles (CSNPs) offer the unique ability for protein/enzyme functionality in non-native environments. For many decades, researchers have sought to develop synthetic materials which mimic the efficiency and catalytic power of bioactive macromolecules such as enzymes and proteins. This research studies a self-assembly method in which functionalized, polymer-core/protein-shell nanoparticles are prepared in mild conditions. Transmission electron microscopy (TEM) and dynamic light scattering (DLS) techniques were utilized to analyze the size and distribution of the CSNPs. The methods outlined in this research demonstrate a mild, green chemistry synthesis route for CSNPs which are highly tunable and allow for enzyme/protein functionality in non-native conditions.

Keywords: immobilization; assembly; Core-Shell Nanoparticle; protein; polymer

# 1. Introduction

Biomolecules such as proteins and enzymes possess many unique features, the speed and efficiency of which are difficult to match by synthetic materials. Proteins and enzymes are used in a wide variety of applications, including use as a biocatalyst in organic synthesis, as well as in the textile, starch, and detergent industries [1,2]. Unfortunately, many biomolecules lose their stability and functionality outside of their native environment, which limits the scope of their applications. A variety of approaches has been developed to improve their stability while maintaining their functionality [3–5]. One widely used method is to design a core-shell nanoparticle (CSNP) with proteins as its shell [6,7]. Recently, CSNP synthesis methods have been studied through electrostatic and self-assembly [8–10], direct adsorption [11–13], and covalent bonding methods, among others [14,15]. Many studies have focused on the use of proteins in forming CSNPs through direct covalent coupling methods [14–18]. The main drawback with these methods is the fact that covalent bonds are used to adhere the protein/enzyme to the nanoparticle surface. This can often result in protein instability and a loss of biological activity [11,19–24]. The loss of enzyme and protein activity has been noted in electrostatic assembly, direct conjugation, and physical adsorption methods [25–27]. Recent research has outlined an entropically driven assembly process utilizing hydrophobicity effects, similar to Pickering emulsions [23,28–30]. These interactions, along with hydrogen bonding between the polymer–protein, have been shown to result in the formation of stabilized polymer-protein CSNPs without the loss of bioactivity [23,31].



In this paper, we utilize a co-assembly method to fabricate polymer-green fluorescent protein (GFP) based-CSNPs that can stabilize the GFP structure in non-native conditions without the loss of functionality. The GFP is a 238 amino acid sequence, 26.9 kDa protein, which was discovered in the jellyfish Aequorea Victoria [32,33]. Known for its vibrant fluorescent properties, GFP has applications as a biosensor, physiological indicator, and gene expression tool [34–38]. Poly(4-vinylpyrine) (P4VP) will be the polymer core, because the nitrogen atom in the pyridine group serves as an efficient hydrogen-bonding acceptor [39,40]. P4VP is generally only soluble in organic solvents (e.g., tetrahydrofuran (THF), dimethylformamide (DMF), ethanol), however the assembly occurs in a solution which is roughly a 3:1 volume ratio of aqueous to organic solvent. The formation of the protein-based corona stabilizes the polymer throughout the assembly process and during dialysis in an aqueous solution [23,41,42]. The initial co-assembly process Figure 1) is driven by the highly chemically active surface area of the nanoparticles, which is stabilized by the biomolecules, as well as the hydrogen bonding interactions via the nitrogen atom present in P4VP [12,22,43,44].



**Figure 1.** Schematic illustration of the formation of the polymer–protein core–shell nanoparticles (CSNPs).

# 2. Results and Discussion

## 2.1. Synthesis and Purification

During the assembly process, visible changes could be observed within the solution. As P4VP was added, the clear green solution began to become opaque. After thirty minutes, the solution containing the CSNPs underwent dialysis in a buffer solution. After 48 h of dialysis, the assembly process was completed, and the particles are formed. Past research has used dialysis as a method of removing the organic solvent from the solution [23,30,42]. In this experiment, we employed a 300 kDa molecular weight cut-off dialysis device to allow for any unbonded 26.9 kDa GFP to diffuse out of the reaction mixture, as well as for the removal of the organic solvent. Removal of any excess, unbonded GFP ensures that the only remaining protein in the solution is assembled with the polymer, which is crucial for accurate protein activity testing. Figure 2, a dialysis control test, shows the fluorescence spectra of a GFP solution initially after 24 h of dialysis, and again after 48 h of dialysis. The data show that no significant amount of GFP remained in the solution after as little as 24 and 48 h of dialysis.

## 2.2. Size and Surface Characterization

TEM was employed to characterize the formed assembly structures. A bright field TEM micrograph in Figure 3a shows the P4VP-GFP as smooth, uniformly spherical particles. Although TEM provides useful information about the overall structure of the CSNPs, the surface coverage of the GFP in the CSNPs is another key aspect that needs to be investigated. Past research has reported polymer–protein structures in which the polymer serves as a "chaperone-like" shell wrapped around the protein [45].

In order to image the proteins on the nanoparticle surface, N-hydroxysuccinimide (NHS)-activated 15 nm gold nanoparticles were reacted with the GFP, forming covalently bonded conjugates. The high density of the gold nanoparticles provides a much higher contrast in TEM imaging [46,47]. Since they are covalently bonded to the GFP, it will allow the indirect visualization of the GFP in the CSNPs. The gold–GFP particles were then assembled with P4VP before being analyzed via TEM. Figure 3b shows a TEM image in which the gold nanoparticles are uniformly distributed on the surface of the polymer core, indicating that the GFP has good surface coverage on the P4VP sphere. To test whether the GFP is completely on the surface of the P4VP sphere or partially inside the sphere, TEM electron tomography (ET) was used to image the CSNPs. TEM ET utilizes a series of tilted TEM images that can be reconstructed to reveal a three-dimensional object [48,49]. A series of ET images of a P4VP + GFP–gold conjugate particle at different tilted angles are displayed in Figure 3c–h. It is worth noting that the dark circular region present in Figure 3c–h is the result of free P4VP + GFP–gold conjugate particle at the CSNP–TEM grid interface during TEM sample preparation.



**Figure 2.** Fluorescence spectra of the dialysis control test. The spectra show no significant amount of green fluorescent protein (GFP) remaining after 24 h.

Another key aspect of these polymer–protein CSNPs is the ability to control their relative size distribution. By adjusting the polymer–protein reaction ratio, varying unimodal size distributions can be synthesized. Figure 4 shows the TEM images of reactions G1, G2, and G3. As the polymer to protein ratio was increased, the data show that the average particle size increased as well. For each reaction, the concentration of reagents was set constant. The varying polymer–GFP ratios of 0.25, 0.60, and 1.0 were calculated by changing the total volume of the P4VP solution added to the mixture. As the P4VP–GFP ratios increased from 0.25 to 1.0 (Figure 4a–c), the particle size obtained from TEM became larger. Without the presence of the GFP, the P4VP precipitates out from the solution (data not shown). Although traditional TEM is a valuable tool for the characterization of nanoparticles, sample preparation requires samples to be dried out, which is not ideal for the characterization of colloidal systems.



**Figure 3.** (a) Transmission electron microscopy (TEM) image of Poly(4-vinylpyrine) (P4VP) + GFP CSNP. (b) TEM image of P4VP + GFP–gold conjugated particles. The high contrast of the gold nanoparticles shows the distribution of GFP on the surface of the particle. (**c**–**h**) TEM tomography images as the P4VP + GFP–gold conjugated particle is rotated. All scale bars are 200 nm.

To analyze the samples in the solution state, dynamic light scattering (DLS) was used to statistically determine the average particle size and size distribution of each of the CSNP samples, as seen in Figure 4d. Analysis of the DLS size distribution data showed distinct unimodal peaks for each sample. The data also showed the direct effect that the polymer–protein ratio had on the overall particle size, i.e., the average particle size increased as the P4VP concentration increased, similar to what was observed from the TEM results. Reactions G1, G2, and G3 generated average particles sizes of roughly 500, 750, and 900 nm, respectively. Once the size distribution of the CSNPs was determined, it was important to determine if the GFP on the surface of the P4VP was present/active. The fluorescence profile of the GFP and the various CSNPs can be seen in Figure 5. The data are consistent with known values, corresponding to an emission spectrum that contains  $\lambda_{max}$  around 509 nm followed by a shoulder peak at 545 nm [50]. The smaller peak around 425 nm present in the spectra of the CSNPs is characteristic of the P4VP fluorescence profile, as seen in Figure 5. The presence of fluorescence suggests the GFP still remains in its native structure, since unfolding leads to the loss of fluorescence [51].



**Figure 4.** (**a**–**c**) TEM images of CSNPs from reactions (**a**) G1, (**b**) G2, and (**c**) G3. (**d**) Size distribution of CSNPs determined via DLS. Scale bars for low magnification images for a, b, and c are 1  $\mu$ m. Scale bars for red box images for a, b, and c are 0.5  $\mu$ m.



Figure 5. Fluorescence spectra of GFP, P4VP, and CSNPs with various sizes.

### 3. Experimental Section

## 3.1. Chemicals and Materials

Poly(4-vinylpyridine) (P4VP; Mw 60,000) and N,N-Dimethylformamide (DMF; anhydrous, 99.8%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES; 99%) was purchased from Acros Organics (Fair Lawn, NJ, USA). Sodium chloride was purchased from Fisher Scientific (Hamptons, NH, USA). Sodium hydroxide pellets were purchased from the Ricca Chemical Company (Arlington, TX, USA). The GFP was prepared (4 mg/mL) in a 20mM tris(hydroxymethyl)aminomethane (Tris) pH 7.0, 80mM NaCl, and 2mM EDTA buffer solution. All reagents were used as received. All water used in this experiment was of ultrapure type I purity (18.2 MΩ·cm) obtained via an Elga Purelab Flex2 system. Float-A-Lyzer®G2 dialysis devices (300 kDa) used for dialysis were purchased from Spectrum Labs (Cincinnati, OH, USA). All dialysis devices were pre-treated via a 10% ethanol bath for ten minutes before being thoroughly rinsed in DI H<sub>2</sub>O, as per manufacturer's instructions. The 15 nm NHS-activated gold nanoparticles were purchased from Cytodiagnostics Inc (Burlington, Canada).

#### 3.2. Sample Preparation

The preparation methods for the GFP have been published [52]. The synthesis of the CSNPs was as follows: For sample G1, a solution containing P4VP (Mw 60 kDa) in DMF (2.0 mg/mL, 0.05 mL) was added dropwise, in 40  $\mu$ L increments, to a 3.7 mL glass vial containing the GFP (0.8 mg/mL, 0.5 mL) in a 20 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.0, 80 mM NaCl, and 2 mM EDTA buffer solution. The solution was constantly stirred during the addition of the P4VP. The vial was then sealed for thirty minutes before undergoing dialysis. Dialysis was done in a 1.0 L 10 mM HEPES and 250 mM NaCl solution. The dialysis solution was replaced by a fresh solution after 4, 6, 10, and 12 h. Once dialysis was complete, the solution containing the CSNPs was then retrieved from the dialysis tube using a pipette. All samples in this research were synthesized using the above procedure, with only variations in the amount of P4VP that was added. P4VP volumes of 0.12 and 0.20 mL were used for G2 and G3, respectively.

#### 3.3. Characterization

Dynamic light scattering (DLS) and zeta potential measurements were performed using a Nanobrook Omni particle size and zeta potential analyzer by Brookhaven Instruments. Typically,  $100 \mu$ L of concentrated sample was diluted to 2 mL and loaded into a polystyrene cuvette. The samples underwent a five-minute equilibration time before undergoing five five-minute scans. All data were collected at a 90° scattering angle.

Transmission electron microscopy (TEM) was performed using a JEOL JEM2100F field emission transmission electron microscope. The samples were prepared on 400 mesh carbon-coated copper grids by submerging the grids in 100  $\mu$ L droplets containing a 50:50 mixture of sample and ultra-pure water. The grids were allowed to soak for twenty minutes before being transferred to a 100  $\mu$ L droplet of ultra-pure water to rinse. The rinsing process was repeated an additional time before being allowed to dry.

The fluorescence profiles were measured using a NanoDrop<sup>TM</sup> 3300 fluorospectrometer (ThermoFisher Scientific). For fluorescence measurements, the samples were placed directly, in 2  $\mu$ L aliquots, onto the microvolume pedestal. No sample preparation was required.

#### 3.4. Gold–GFP Conjugation

Covalent conjugation of the gold–GFP particles was performed in accordance with the manufacturer's instructions. In short, all reagents were allowed to warm up to room temperature before the supplied protein re-suspension buffer was added to the lyophilized GFP to create a 2 mg/mL solution. In a microcentrifuge tube, the supplied reaction buffer (600  $\mu$ L) was combined with the

GFP solution (480  $\mu$ L). An amount of 900  $\mu$ L of the new mixture was then transferred to a glass vial containing the lyophilized NHS-activated gold nanoparticles. The resulting solution was mixed thoroughly using a pipette. The vial was then allowed to incubate at room temperature for two hours before the supplied quencher solution (10  $\mu$ L) was added to the vial to stop the reaction. The solution was then centrifuged at 15,000 RPM for 30 min. The supernatant containing any unbound protein was then discarded. An amount of 1 mL of a 10 mM HEPES and 250 mM NaCl buffer was added to the vial to re-suspend the conjugate. The centrifugation process was then repeated two additional times to

# 4. Conclusions

remove any unreacted free protein.

This research outlines a direct, stepwise assembly process for the synthesis of bio-active GFP-P4VP CSNPs. GFP has been shown to be present and stable on the surface of the polymer nanoparticles. The non-covalent bonding interaction provides a secure, non-hindering interaction between the GFP and the polymer. We have shown that the size range of the CSNPs can be easily controlled by adjusting the polymer–protein ratio as confirmed by the DLS and TEM images [53]. This procedure demonstrates a method for the stabilization of bio-active molecules via polymer-core CSNPs. These particles will allow for a variety of applications, from industrial to biomedical, to utilize the power and efficiency of bio-active molecules outside of their native environment.

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