Decoupling and Elucidation of Surface-driven Processes during Inorganic Mineralization on Virus Templates

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Abstract

There is a lack of fundamental information about the molecular processes governing biomineralization of inorganic materials to produce nanostructures on biological templates. This information is essential for the directed synthesis of high quality nanomaterials via biotemplating. We characterized palladium (Pd) mineralization via the individual adsorption, reduction, and nanocrystal growth processes, which simultaneously occur during the hydrothermal synthesis on Tobacco mosaic virus (TMV). The adsorption of precursor and reduction of palladium were decoupled through UV-Vis Spectroscopy and *in situ* X-ray absorption spectroscopy studies. The role of additional cysteine (Cys) residues, ionic strength, and coating density on the fundamental parameters describing these processes were quantitatively evaluated. Primary nanocrystal growth and structural orientation of Pd nanoparticles was characterized using *in situ* small angle X-ray scattering. The adsorption, reduction of Pd species, and nanocrystal sizes were significantly changed on addition of Cys residues to the amino terminus of the TMV coat protein. Reduction of Pd on an already coated

virion was dependent on the Pd surface area, and was hindered by the presence of residual salt. Furthermore, trends in Pd adsorption intensity and capacity suggested that chloride ions affected the adsorption equilibrium. Application of this fundamental approach with further optimization of parameters dictating biomineralization would facilitate directed synthesis and scale up of bioinorganic systems.

Keywords: Tobacco Mosaic Virus, cysteine, biomineralization, adsorption, reduction, nanocrystal growth, X-ray Absorption Spectroscopy, Small Angle X-ray Scattering

Graphical Abstract. Biomineralization during the hydrothermal synthesis of Pd on TMVs

Introduction

Biotemplating has become promising as a means for bottom-up fabrication of nanomaterials via directed growth and hierarchical organization. An increasing range of nanoscale biological objects such as DNA,¹⁻⁵ RNA,⁶ microtubules⁷⁻⁹, viruses,¹⁰⁻¹⁸ and assembled protein aggregates,^{9,19,20} have been employed in the controlled synthesis of inorganic and organic materials under mild and inexpensive conditions. High quality materials resulting from some of these efforts have been applied in varied systems such as chemical and biological sensors,²¹⁻²³ battery electrodes,^{12,24-26} nanocircuits,²⁷ memory devices,¹⁶ and photovoltaic devices.²⁷ Particularly, in biotemplating involving inorganic substances, rod-like plant viruses have been used for epitaxial growth of tethered isolated nanoparticles,^{28,29} solution based nanoparticles³⁰ and dense surface coatings.³¹ These templates are especially attractive due to their availability, inherent stability and malleability through genetic engineering,^{18,32} click chemistry,¹⁰ and phage display.^{33,34} The Tobacco mosaic virus (TMV), the virus template employed in this paper, has been genetically engineered in a number of ways to display additional amino acids such as cysteine,^{5,33} histidine,¹¹ lysine,³⁶ and glutamine³⁷ on their coat protein via site directed mutagenesis. Each TMV virion consists of 2,130 copies of a single coat protein (17.5 kDa), which assemble around RNA into a stiff rod-like particle with a length of 300 nm, outer diameter of 18 nm and inner hollow of 4 nm³⁵. The displayed multi-functionalized interfaces on the exterior and interior of the hollow structure serve as viable sites to direct the synthesis of nanoparticles. Previously TMV1Cys (TMV with one extra cysteine) and TMV2Cys (TMV with two extra cysteines) were investigated for increasing the biomineralization of metals such as Au³⁶, Ag^{30,32}, Pd,^{29,32,36-39} and Pt.²⁸

These studies were performed via conventional electroless deposition involving an external reducing agent. More recently, we have employed the hydrothermal synthesis to form nanowires that are uniform, dense, and monodisperse, as well as tunable along their diameters.^{17,43} The quality of nanowires produced with this synthetic method shows the advantages of controlling biomineralization via the molecular interactions on the surface of the biotemplate rather than with an external reducer. Other studies on Ag³¹ and Au³² have also demonstrated the possibility of virus-surface autoreduction of precursor. Nevertheless, controlled synthesis of nanomaterials is still elusive. Yang *et al.* noticed that under different conditions, nanoparticle coating could be unpredictable. Particles could be completely absent from the surface, mildly decorate the surface, or uniformly coat the virus surface⁴², and significant metallization of biotemplates may proceed at low precursor concentration^{17,39} instead of at higher concentration⁴¹ as would naturally be expected in chemical reactions. Also, virus surface-mediated mineralization, either in the presence or absence of an external reducer, has resulted in the production of large nanocrystals in the supernatant solution rather than on the virus surface.^{39,42}

Most synthesis has been performed without consideration of the fundamental processes involved in mineralization. Furthermore, previous attempts to characterize adsorption were performed without knowledge of the existence of surface-mediated reduction. An improper consideration of both chemical reduction and mass transfer driven processes during the experimental procedure (e.g. no stirring) led to inconsistent results.³⁶ Therefore, it is important to fundamentally characterize the governing molecular processes controlling biomineralization. We have previously shown that at high Pd concentration, reduction is autocatalytically initiated by the virus surface and then proceeds via two first order regimes at the growing Pd interface.⁴¹ Yet

the dynamic processes resulting in the different degrees of coating density, uniformity, and primary nanoparticle sizes still remain unclear. In this paper, we further elucidate biomineralization by reframing it within commonly known fundamental molecular processes. After characterizing and confirming these processes, we studied the effects of different system parameters, such as surface identity, ionic strength, and available metal surface for growth and temperature, which may act as synthetic levers for biomineralization. With this approach, directed synthesis will be achievable and scale up of processes will be simpler.

We have identified the adsorption of precursor, reduction of precursor and growth of nanoparticles as the pertinent processes at interplay as the precursor interacts with the surface amino acids. To fully characterize these, we studied the mineralization of Pd onto TMVs using UV-Vis spectroscopy along with X-ray absorption spectroscopy (XAS). Statistically relevant information on the nanoparticle size and placement during synthesis was provided by *in situ* small angle x-ray scattering (SAXS) and confirmed by transmission electron microscopy (TEM). Our results indicated the ability to decouple and quantitatively characterize the adsorption and reduction of Pd. Reduction was affected by the addition of exposed Cys to the TMV coat protein surface, the surface area of Pd coating, reaction temperature and chlorine content. The adsorption of precursor was influenced by both the introduction of exposed Cys on the TMV CP and the presence of chlorine anions in solution. Lastly, the addition of Cys to the surface of TMV also had a noticeable influence on the growth and placement of primary Pd nanoparticles during mineralization.

Experimental

Reagents and Chemicals

Sodium tetrachloropalladate (II) (99.98%, Sigma Aldrich, St Louis, MO) was used as the Pd precursor. Acrodisc Syringe Filters (Pall life sciences, 13 mm), purchased from Careforde Safety and Scientific (Chicago, IL), were used for filtration of nanoparticles. Poly(methyl methacrylate) (PMMA) plastic cuvettes (VWR Scientific Prod Midwest, Radnor, PA) were used for UV-Vis spectra acquisition. Millipore water was used for all experiments. Wild type unmodified TMV (WtTMV) and TMV mutants (TMV1Cys and TMV2Cys) were used in this study. Purification of these TMV variants was performed according to published protocols.³³ Compared to the wtTMV CP, the TMV1Cys CP contains an insertion of a Cys between Ser₁ and Tyr₂ at the amino end of its CP sequence; TMV2Cys contains two sequential Cys at the same position.^{5,32}

Ex situ UV-Vis Characterization

Experiments were performed in a constantly stirred 3.5 mL reactor vessel under controlled temperature conditions. Elevated temperature experiments were conducted below 70°C to ensure virus stability. In a typical synthesis, 1.5 mL of the Na₂PdCl₄ solution (between 0.2 mM and 3 mM) was incubated with a 2 mL virus solution (0.035 mg/mL) after they had been individually heated to the desired synthesis temperature in eppendorf tubes. During the course of the reaction, 0.4 mL aliquots of the stirred solution were collected at different time intervals. These were then filtered to remove the solid phase consisting of Pd and virus particles. The supernatant was immediately diluted three fold in water at 4°C and placed on ice to quench the reaction. Finally, the absorbance of aliquots at 256 nm and 427 nm was measured at 25°C by a

UV-Vis spectrophotometer (Varian Cary 100). The uptake of Pd was evaluated by accounting for the absorbance in the control (no virus) experiment. With this method, experiments were performed over shorter times (5 minutes) and longer times (typically between 40 minutes and 180 minutes).

In situ X-ray Absorption Spectroscopy Characterization

X-ray Absorption Spectroscopy (XAS) experiments were conducted on the TMV-Pd system at the Advanced Photon Source (Argonne National Laboratory, Lemont, IL) 10 ID-B beamline. A 3 mM Pd precursor solution was placed in the path of X-rays. The concentration was chosen to allow for a significant signal to noise ratio for data analysis. TMV:Pd ratio was maintained constant across experiments. However, since this increase in salt and TMV particle (virion) concentration will result in a greater tendency for shear driven aggregation, the solution was carefully stirred. XAS measurements of the vessel and reference materials used were taken in transmission mode near the Pd K-edge (24.4 keV). Five four-minute scans of Pd precursor solutions were averaged for the unreduced reference spectra Pd⁽²⁺⁾. Pd foil was used as the reference for Pd⁽⁰⁾. Approximately 20 spectra were taken during the course of each chemical reaction. Each spectrum was then analyzed individually but identically. The Pd K-edge energy was determined as the maximum of the first derivative in the XANES region (about 100 eV from edge) after normalization of the energy spectra to the edge energy. The EXAFS (100 – 900 eV from edge) and the XANES spectra were analyzed by standard procedures using WINXAS 3.1 fitting software.⁴⁵ For the XANES analysis, each spectrum was fit as a linear combination of the two reference spectra. EXAFS parameters were obtained by performing a least square fit of a fourier transformed k^2 -weighted normalized absorption data in R-space.

In situ Small Angle X-ray Scattering Characterization

SAXS measurements were performed at the Advanced Photon Source (Argonne, National Laboratory, Lemont, IL) 12 ID-B beamline. As the synthesis was performed, a two-ended capillary was connected to a flow pump on one end and placed in the reaction vessel on the other end. During nanoparticle synthesis, an automated system allowed the flow of solution through the capillary while 10 SAXS scans were taken. The ten scans were averaged for high quality spectra. The TMV solution background was subtracted in order to study the Pd nanoparticles alone. Typical exposure time for experiments was kept below 0.2 s to minimize sample degradation or X-ray induced chemistry. The scattering X-rays were detected using a Pilatus 2M detector with a sample to detector distance of about 2 m. The 2D scattering patterns collected at small angles ($< 2^\circ$) were azimuthally averaged to obtain 1D scattering spectra of intensity (I) vs scattering vector (q), which is defined in Equation [1] below. The primary nanoparticles were assumed to be spherical, and either dilute or spatially correlated. In dilute systems, curves were fit using a unified method developed by Beaucage.⁴² The method assumes that for every i^{th} level of particle size, particle scattering comprises of both knee-like decays (guinier regimes) and power law decays. The data is fit to a global scattering function that is aligned to the local scattering laws (guinier law and generalized porod law) thereby accounting for overlap between these regimes. By fitting the data this way, scattering features possibly obscured by the overlap of structural levels can be distinctly identified. The 1st term in Equation [2] represents the guinier regimes while the 2nd term represents the power law regimes. Table 1 shows the definition of parameters used in the SAXS fit.

$$q = \frac{4\pi \sin \theta}{\lambda} \quad [1]$$

where θ is the scattering angle and λ is the wavelength of the incident beam of the x-rays

$$I(q) = \sum_{i=1}^n \left(G_i \exp \left[-\frac{q^2 R_{g,i}^2}{3} \right] + B_i \exp \left[-\frac{q^2 R_{g,i}^2}{3} \right] q_i^*(q)^{P_i} \right) \quad [2]$$

$$\text{where, } q_i^* = \frac{q}{\left\{ \text{erf} \left(\frac{q R_{g,i}}{\sqrt{6}} \right) \right\}^3} \text{ and } i = 2 \text{ in the system studied}$$

Under conditions with high particle concentration, good fits to data were only obtained after the scattering relationship for the lower particle level was modulated by incorporating a structure factor $S(q)$ in order to account for interparticle correlation.⁴⁷ As seen in Equation [3], the average interparticle distance (ζ) and average packing parameter (k) between particles are the two additional physical parameters that characterize the structure of the system as a result of spatial correlation of nanoparticles. The entire scattering curve for correlated systems is modeled by equation [4].

$$S(q) = \left(1 + k \frac{3 * (\sin(q\zeta) - q\zeta \cos(q\zeta))}{(q\zeta)^3} \right)^{-1} \quad [3]$$

$$\begin{aligned} I(q) = S(q) & \left(G_1 \exp \left[-\frac{q^2 R_{g,1}^2}{3} \right] + B_1 \exp \left[-\frac{q^2 R_{g,1}^2}{3} \right] q_1^*(q)^{P_1} \right) \\ & + \left(G_2 \exp \left[-\frac{q^2 R_{g,2}^2}{3} \right] + B_2 \exp \left[-\frac{q^2 R_{g,2}^2}{3} \right] q_2^*(q)^{P_2} \right) \end{aligned} \quad [4]$$

Table 1. Fitting parameters in unified fitting model for Small Angle X-ray Scattering analysis of TMV-Pd systems

^a Fitting Parameter	Symbol
Guinier prefactor	G_i
Power law prefactor	B_i
Radius of gyration of i^{th} level	$R_{g,i}$
Power law exponent	P_i
Structure factor	$S(q)$
Average interparticle distance	k
Average packing parameter	ζ

^a Analysis was performed using Irena software⁶³

Experiments were performed with 0.75 mM Pd with the same TMV:Pd ratio used in UV-Vis reduction experiments. In these experiments, second and third pulses of Pd precursor (equivalent to the same amount of Pd in the reactor initially) were added to resume a new cycle of particle coating. In order to ensure consistency in mass content and aggregate structure from the previous growth cycle, these extra coatings were performed without a washing procedure between coats.

TEM Imaging

The solutions containing nanorods were centrifuged at 14,000 rpm and redispersed in 2 mL of water. This was defined as a washing cycle. The particles were washed repeatedly to remove precursor solution. In preparation for imaging, a 4-5 μL droplet of the TMV-Pd suspension was deposited onto carbon formvar copper grids. TEM images were taken using a 200 kV Tecnai T20 microscope. Unwashed samples were also examined to observe the particles in solution along with the nanorods. A summary of the experimental setup is shown in Figure 1.

Figure 1. Schematic of the setup used to characterize the mineralization process. It is to be noted that UV-Vis, XAS and SAXS experiments were not performed at the same time.

Results and Discussion

According to Tait *et al.*,⁴⁸ the dominant species in the speciation equilibrium of Pd under these conditions (25°C, pH 5) is $\text{PdCl}_3(\text{H}_2\text{O})^-$. The absorbance of this species was monitored at 256 nm and 427 nm. Time-resolved characterization experiments were conducted in order to determine the change in $[\text{PdCl}_3(\text{H}_2\text{O})^-]$ as a result of its interaction with the surfaces of WtTMV, TMV1Cys and TMV2Cys. As expected, the interaction was marked by a drop in $[\text{PdCl}_3(\text{H}_2\text{O})^-]$, as it was mineralized onto the surface of the viruses. As shown in Figure 2(a) - (c), there was a swift drop (~50%) in $[\text{PdCl}_3(\text{H}_2\text{O})^-]$ within the first five minutes. This is followed by a much

slower decrease in $[\text{PdCl}_3(\text{H}_2\text{O})^-]$, which can be modeled as a first order kinetic process. At approximately 60°C, the rate constants were $2.2 \pm 0.10 \text{ hr}^{-1}\text{mg}^{-1} \text{ virus}$ for the WtTMV, $3.1 \pm 0.20 \text{ hr}^{-1}\text{mg}^{-1} \text{ virus}$ for TMV1Cys, and $3.1 \pm 0.03 \text{ hr}^{-1}\text{mg}^{-1} \text{ virus}$ for TMV2Cys. Under the same conditions, an activation energy of $45.2 \pm 2.3 \text{ kJ}$ was obtained for the hydrothermal synthesis on the surface of the TMV1Cys as shown in Figure 2(f).

Figure 2. Conversion of Pd Species on the surface of TMV biotemplates measured by UV-Vis and XAS characterization. (a) - (c) First order kinetic model fit to $[\text{PdCl}_3(\text{H}_2\text{O})]$ uptake on WtTMV, TMV1Cys, and TMV2Cys respectively. (d) and (e) Pd reduction kinetics from solution interaction with WtTMV and TMV1Cys obtained through XAS at 3mM Na_2PdCl_4 and 0.15 mg/mL virus. Experiments (a) - (e) show experiments at $57 \pm 2^\circ\text{C}$. (f) shows an Arrhenius fit for first order constants obtained from the reduction of Pd on TMV1Cys. The obtained kinetic parameters are shown in Table 2.

Table 2. 1st order rate constants describing mineralization on TMV biotemplates

Virus	Characterization Technique	1st order rate constant at 55°C (hr ⁻¹ mg ⁻¹)
WtTMV	UV-Vis	2.2 ± 0.1
TMV1Cys	UV-Vis	3.1 ± 0.2
TMV2Cys	UV-Vis	3.1 ± 0.03
WtTMV	XANES	2.2 ± 0.4
TMV1Cys	XANES	3.2 ± 0.05

Since, UV-Vis characterization only provides information on the change in a particular Pd species ($\text{PdCl}_3(\text{H}_2\text{O})^-$), it was necessary to determine if the reduction mechanism characterizes all $\text{Pd}^{(2+)}$. This was essential to investigate the possible occurrence of other chemical processes such as adventitious hydrolysis or ion-induced precipitation. In addition, another technique was needed to explain the molecular mechanism behind the swift drop in supernatant concentration at the beginning of the synthesis. Therefore, comparative XAS experiments were performed under similar conditions. Time-resolved XANES spectra were used to obtain the ratio of oxidation states of palladium i.e. $\text{Pd}^{(2+)} : \text{Pd}^{(0)}$ at different times during synthesis as was previously described by Adigun *et al.*⁴⁴ Results from these are shown in Figure 2(d), Figure 2(e) and Table 2. The kinetic constants obtained via XAS and UV-Vis were in agreement. Therefore, the uptake on the longer time scale, (about an hour) identified in the UV-Vis experiments, represents the virus surface-mediated reduction of $\text{Pd}^{(2+)}$. In comparison, mineralization is complete within 33 seconds when an effective external reducer (sodium hypophosphite) identified by Manocchi *et al* is used.⁴¹ The longer time scale of the hydrothermal synthesis allows kinetic control over the mineralization (i.e. nanoparticle size, metal density) that is absent when an external reducer is employed in synthesis. In addition, a slower reaction localized on the particle surface circumvents the polydispersity caused by local concentration

variation during an ultrafast reduction. Further uptake studies on the mineralization behavior of already coated virus were done to characterize the reduction of palladium as the coating density increases. These were achieved by resetting the supernatant concentration of $\text{PdCl}_3(\text{H}_2\text{O})^-$ to 0.75 mM at 57°C. Comparative experiments were performed either in the presence of residual salt from previous coatings (no washing) or after particle washing.

Results show that the kinetic constant decreased to zero when a new coating cycle was performed without the removal of ionic products (Figure 3(a)). The opposite observation was made for the mineralization of washed particles i.e. the kinetic constants significantly increased as coating density increases. In fact, the rate constant shown in Figure 3(b) is a linear function of the exposed surface of the palladium nanorods ($2\pi RL$) at the beginning of the coating, indicating that the metal reduction is primarily catalyzed by the already deposited palladium surface. Although the identity of the reducer is still unknown, a surface area driven growth indicates that the reductant is not limiting as new coatings are added to the nanorod surface. The observed drop in reductive activity at higher ionic strength could result from a few possibilities. The ionic strength of the solution increased as residual salt consisting of Na^+ and $\text{Cl}^-_{\text{free}}$ were released from the dissolved Na_2PdCl_4 when the Pd species was hydrolyzed and then reduced as shown in Equations (I) and (II),

An increase in ionic strength will provide more ions in the diffuse layer on the surface of the palladium coating. These Cl^- ions would hinder reduction by providing ionic mass transfer resistance, thereby inhibiting the surface accessibility for reactant ions. Alternatively, at higher ionic strength, salt-induced aggregation caused by screening of the repulsive forces between particles could result in a significant decrease in accessible surface area for reduction.

Figure 3. Coating of layers of Pd on TMV1Cys during the hydrothermal synthesis. (a) Effect of washing on Pd reduction kinetics. (b) Change in the kinetics of Pd reduction as nanorod radius increases. The radius of nanorods was obtained from the SAXS experiments by Freer *et al.*⁴⁰ Black dashed (---) lines identify a linear correlation of the rate constants to parameters.

In a system with much higher Pd concentration, salt build up will increase the ionic strength of the solution and could initiate this Cl^- driven inhibition even before all of the Pd has reduced. This observation was made in the entryway XAS study performed by Adigun *et al.*⁴⁴ in which a much fast reduction was followed by a slower reduction after an initial layer of Pd had grown on the biotemplate surface. This observation about the effect of salt concentration during

the mineralization process is also particularly important in view of accessing the future possibility of halide controlled nanocrystal growth in these systems.^{49,50}

The mechanism responsible for the swift drop in concentration at the beginning of synthesis remained unclear after UV-Vis kinetic characterization. A swift drop was not observed in the comparative XAS analysis as shown in Figure 4(a), where the XANES spectra at five minute is identical to that of unreduced Pd. Therefore, the drop in concentration of Pd species is not caused by reduction of Pd species but rather by rapid surface sorption at the protein-salt solution interface. We conclude that at less than 5 minutes, the system is adsorption or mass transfer dominated and at longer times, the system is reduction dominated as is confirmed in the reaction analysis above. With this finding, the two processes could be completely decoupled. With biosorption studies on short time scales, the thermodynamic properties and kinetics of the adsorption process could be accessed and compared for different TMV variants. Different concentrations of the Pd precursor were incubated with virus at 25°C in order to slow the chemical reduction. Figure 4(b) shows that there was an increase in uptake (q_t) of $\text{PdCl}_3(\text{H}_2\text{O})^-$ until a flattening of the curve at 150 seconds. The concentration of the flat region was assumed to be the equilibrium concentration (C_e) for $\text{PdCl}_3(\text{H}_2\text{O})^-$ species adsorption at a particular incubation concentration (C_{in}). That is, at this concentration, the adsorption process is complete while the reduction of $\text{Pd}^{(2+)}$ is negligible. At adsorption equilibrium, the $\text{PdCl}_3(\text{H}_2\text{O})^-$ uptake and equilibrium concentration were fit to either the Langmuir or Freundlich adsorption isotherms identified in Equations [5] and [6] respectively. A change from the Langmuir (WtTMV and TMV1Cys) to Freundlich (TMV2Cys) model as the best fit to describe the thermodynamics of adsorption indicates the change to a more heterogeneous surface as thiol groups are added to the TMV surface.

$$q_e = q_{max} \frac{aC_e}{1+aC_e} \quad [5]$$

$$q_e = K_f C^{-n} \quad [6]$$

By comparing the Langmuir model fits, it was observed that the adsorption capacity decreased on addition of the first Cys (WtTMV to TMV1Cys) but then increased on addition of the second Cys (TMV1Cys to TMV2Cys). This result further elucidates the role of the thiol functionality during biomineralization. From Prior studies, which have been performed in the presence of an external reducer, it has been postulated that an increased Pd nanoparticle content on the surface of Cys engineered TMV is a result of additional Cys coordinating sites.^{32,36} Following this reasoning, the Pd species will individually nucleate on the added thiol sites along with other native sites (other sorbing species or functionalities on WtTMV) and then act as an origin for nanoparticle growth. However, results in Figure 5(b) show that an increase in adsorption capacity was not observed as more thiol groups were added to the surface of the WtTMV. A more nuanced description of the mineralization process is therefore necessary. Furthermore, in experiments performed in the presence of a reducer, the increased Pd nanoparticle content in the nanowire product could be caused by the attachment of already reduced Pd⁽⁰⁾ atoms rather than an affinity for Pd⁽²⁺⁾ species. Moreover, although the chemical affinity of Pd⁽⁰⁾ for thiol groups is well documented, the affinity of PdCl₃(H₂O)⁻ is not yet fully elucidated^{51,52} while that of PdCl₄²⁻ has been shown to be negligible.⁵³

Figure 4. Evolution of Pd species early in the mineralization process. (a) A comparison between XANES reference spectra of Pd solution and spectrum at five minutes. (b) Uptake of $\text{PdCl}_3(\text{H}_2\text{O})^-$ obtained by UV-Vis absorbance experiments at 256nm. Experiments were performed at six different $\text{Pd}^{(2+)}$ concentrations and three of them are shown here. (c) Pd adsorption fit to a pseudo second order model in remaining adsorption sites. Dashed lines indicate acceptable linear fits

Figure 5. Adsorption isotherms from adsorption of Pd species on TMV surfaces during the first 5 minutes. Plots of mass of Pd adsorbed on virus against solution concentration at equilibrium. (a) Langmuir fit of data for adsorption on WtTMV. (b) Langmuir fit of data for adsorption on TMV1Cys (c) Freundlich and Langmuir fit of data for adsorption on TMV2Cys. The Langmuir fit has a correlation coefficient of 0.94. Although, the Langmuir isotherm is not the best fit of data, it is used for comparison with the other TMV variants. (d) Langmuir fits of data for adsorption on TMV1Cys at different total chlorine concentrations. The best fits for the model to the data resulted in a correlation coefficient >0.99 .

We propose a different explanation for how additional Cys residues on the TMV CP surface affect mineralization. Results from Figures 2 and 5 show that both the reductive capacity and thermodynamic affinity for $\text{Pd}^{(2+)}$ are altered when one or two cysteine groups are added between the Ser₁ and Tyr₂ on the WtTMV CP surface. The reported isoelectric points of different TMV variants (3.5 for WtTMV³⁸ and 2.6 for TMV2Cys²⁹) dictate that their surfaces possess a net negative charge during mineralization, which occurs between pH 5 and 3.5. Also, the exposed amino acid sequences on the TMV variant surfaces, shown in Table S1, are either neutral or negatively charged. Therefore, an electrostatic adsorption of the negatively charged $\text{PdCl}_3(\text{H}_2\text{O})^-$ is unattainable. Conversely, adsorption on the surfaces of all three TMV variants must occur by covalently driven chemisorption (ligand exchange) of Pd species by functionalities on the TMV surface. The tendency of ionic Pd species to form complexes with hydroxyl, amines and aromatics is well known.^{54–57} In the consideration of reductive activity, amino acids on the WtTMV surface such as tyrosine, serine, threonine and tryptophan have been identified elsewhere as fast reducers.⁵⁸

Wang *et al.* in their systematic study on the mineralization of AuCl_4^- by amino acids, showed that in the adsorption (or affinity) and reduction of precursor on a specific amino acid are modulated in different ways depending on their neighboring amino acids in the biopolymer.⁵¹ This means that along with its (amino acid) individual (or isolated) affinity for absorbate molecules, the spatial context of the amino acid has a significant influence on its ability to interact with precursor molecules. The existence of this modulating behavior explains the observed change in surface-controlled adsorption and reduction. In the case of AuCl_4^- , on which tyrosine and serine show substantive potential for reduction, the presence of histidine attenuates the reduction on tyrosine while it augments that on serine.⁵¹ We deduce that the addition of Cys

between Ser₁ and Try₂ drives a comparable modulating effect, so that the reduction of Pd is increased on addition of the first Cys. A similar modulating behavior also caused the adsorption capacity of Pd species to drop on TMV1Cys (NH₂-ser-tyr-) compared to WtTMV (NH₂-ser-cys-tyr-), and then increase on addition of the second Cys (NH₂-ser-cys-cys-tyr-). In summary, chemisorption and reduction of Pd species by certain amino acids are affected by the locally driven modulations caused by adjacent amino acids on the TMV virus surface. It is therefore important to be aware of the nearby amino acids when directing biomineralization, rather than merely attempting to introduce additional amounts of functionality on the virus surface.

The kinetics of the adsorption of Pd species as the adsorption approaches equilibrium was also investigated. As shown in Figure 5(d), the process can be modeled by a pseudo second order mechanism in the amount of remaining adsorption sites ($q_e - q_t$) according to Equation [7].⁵⁹

$$\frac{dq_t}{dt} = k_{ads}(q_e - q_t)^2 \quad [7]$$

becomes

$$\frac{t}{q_t} = \frac{1}{k_{ads} q_e^2} + \frac{t}{q_e} \quad [8]$$

where k_{ads} is the 2nd order constant and q_t is the amount of PdCl₃(H₂O)⁻ adsorbed at time = t .

All results obtained thus far are from experiments at a standard Pd:Cl ratio. Because our results show that the adsorption process can now be characterized using UV-Vis, the effect of Cl⁻ concentration on the process was examined. This is important to investigate because previous results performed by Lim *et al.*³⁶ showed high variability in mineralization at high ionic strength. Adsorption isotherms shown in Figure 5(d) were collected at constant [Cl_{total}⁻]. This was achieved by the addition of NaCl to the Na₂PdCl₄ solution so that,

$$[\text{Cl}^-_{\text{total}}] = [\text{Cl}^-_{\text{bound}}] + [\text{Cl}^-_{\text{free}}] \quad [9]$$

Table 3 shows the $[\text{Cl}^-_{\text{free}}]$ along each curve. Results from Figure 5(d) indicate that $[\text{Cl}^-_{\text{total}}]$ had a significant effect on the adsorption equilibrium of Pd. As $[\text{Cl}^-_{\text{total}}]$ increases, the adsorption of $\text{PdCl}_3(\text{H}_2\text{O})^-$ on the sparsely occupied virus surface (labeled as (i) in Figure 5(d)) decreases. The converse relationship is observed when the surface is fully occupied as in the region labeled (ii). As $[\text{Cl}^-_{\text{total}}]$ increases, the adsorption capacity for $\text{PdCl}_3(\text{H}_2\text{O})^-$ increases. To explain these trends, a closer examination of the solution content within the interfacial double layer is required.

According to DLVO theory,⁶⁰ the interface of the virion surface consists of tightly bound positively charged species (Na^+ and H_3O^+) in the Stern layer surrounded by a loosely associated diffuse layer, which contains some anionic species. In the sparse loading region (labelled (i)), the solution has a free chlorine ion concentration ($[\text{Cl}^-_{\text{free}}]$) but a low Pd concentration. The Cl^- anions will compete with $\text{PdCl}_3(\text{H}_2\text{O})^-$ anions in the diffuse layer. Since Cl^- has a tendency to occupy the inner anionic layers, overall electrostatic charge balance dictates that the concentration of $\text{PdCl}_3(\text{H}_2\text{O})^-$ will decrease in the diffuse layer as $[\text{Cl}^-_{\text{free}}]$ increases.⁶¹ The mass transfer driving force for adsorption of $\text{PdCl}_3(\text{H}_2\text{O})^-$ onto the virion surface will consequently decrease. Thus, adsorption is limited by electrostatically controlled mass transfer resistance. As can be seen in Table 3, the $[\text{Cl}^-_{\text{free}}]$ to $[\text{Pd}^{(2+)}]$ ratio is much less at high loading. The Cl^- anions, which are in lower concentration, will noticeably affect the complexation of $\text{Pd}^{(2+)}$ species instead of remaining as free competing anions. That is, as $[\text{Cl}^-_{\text{total}}]$ increases, speciation will shift to produce more charged Pd species ($\text{PdCl}_3(\text{H}_2\text{O})^-$ and PdCl_4^{2-}). The concentration of anionic Pd species then increases in the diffuse layer while that of complexing $\text{Cl}^-_{\text{free}}$ further decreases. This leads to greater mass transfer to the virion surface and, thus, higher Pd adsorption with

increasing $[\text{Cl}^-_{\text{total}}]$. The adsorption behavior is regulated by a tradeoff between Cl^- controlled Pd speciation and ionic competition near the virion surface.

Table 3. The incubation concentrations of $\text{Pd}^{(2+)}$ and Cl^- used for adsorption experiments at 25 °C in Figure 5(d). A single adsorption curve can be followed down the column. The first row shows the low Pd loading region labelled (i) in Figure 5(d). The bottom row represents high Pd loading (ii). The ratio of $[\text{Cl}^-_{\text{free}}]$ to Pd is very high (at least 33) in the low Pd loading region compared to the high Pd loading region (at most 10).

$[\text{Cl}^-_{\text{tot}}]$ mM	3.7	4.4	5.7	6.9
$[\text{Pd}^{2+}]$ mM	$[\text{Cl}^-_{\text{free}}]$ mM			
0.1	3.3	4.0	5.3	6.5
0.2	2.9	3.6	4.9	6.1
0.3	2.5	3.2	4.5	5.7
0.4	2.1	2.8	4.1	5.3
0.5	1.7	2.4	3.7	4.9

The growth of the virus-Pd nanoparticles into nanorods was investigated using *in situ* SAXS conducted under similar conditions to the XAS and UV-Vis studies. The 1D SAXS scattering curves for growth on the three TMV variants are shown in Figure 6(a) - (c). Significant changes in scattering could not be observed until the concentration on the virus surpassed the detectability limit of the detector. The growth of nanoparticles on the WtTMV was not distinguishable in the SAXS curve even after three coating cycles (and about 3 hours of reaction time). On the other TMV variants, nanocrystal growth was apparent after the addition of the second pulse of Pd. The unified fit identified two levels of nanoparticle size growth as is shown in Figure 6(d). Figure 7(a) shows that nanoparticles grown on TMV2Cys ($R_g = 2.4$ nm, $R = 3.1$ nm) are approximately 1 nm larger than those grown on TMV1Cys ($R_g = 1.8$ nm, $R = 2.3$ nm). The presence of these particles is confirmed in TEM images in Figure 8 where the primary nanoparticles on the surface of these TMV variants are of similar sizes to those obtained from the

lowest size level in the unified SAXS models. With these results, we assert that, along with the catalyzing of precursor reduction, the presence of the Cys groups on the multifunctional surfaces has a significant effect on the crystallization behavior of the growing particles. This observation is particularly significant since the reduction rate is similar on both TMV variants.

Information on the spacing of nanoparticles on the surfaces is presented in the structure factor parameters in Figure 7(c) and (d). On both TMV1Cys and TMV2Cys, the packing parameter (ζ) indicates that there are approximately 2 particles within a distance, $2R$, from the center of a single Pd nanoparticle. The average interparticle distance is about 5.6 nm, which is physically reasonable and greater than the average intersubunit distance on adjacent coat proteins (≈ 2.9 nm). Taken together, these SAXS studies successfully characterize the nanoparticle sizes, which are otherwise difficult to discern in a system with significant packing and layering of the small nanocrystals on the biotemplate surface. When compared to Pd nanoparticles mineralized onto TMV1Cys in the presence of reducers, these nanoparticles are much smaller.⁴¹ Also, the *in situ* studies show that under the conditions studied, the nanoparticles remain approximately the same size during synthesis. The small sizes and much denser packing are attractive properties for surface area dependent applications.

At lower q , where a higher size level of particle scattering is distinguishable, radii of gyration (R_g) of 21 nm and 19 nm are observed for Pd growing for TMV1Cys and TMV2Cys respectively (Figure 7(b)). The radius of gyration for the radius for a rigid rod (with $L > 2.5R$) should correlate to $R^2 = \frac{4}{3}R_g^2$. This will result in approximately 30 nm radius using the observed radii of gyration.⁶² However, the nanorod particle radii are 7.5 nm and 10 nm on TMV1Cys and TMV2Cys respectively (Figure 8). This discrepancy is likely due to the presence the organic nanotube in the core of the particle. The scattering object, composed of a weakly

scattering organic nanotube covered with a thin layer of Pd, resembles a core-shell cylinder rather than solid rod. To fully characterize this size, scattering data is required at lower q to obtain parameters defining the form factor of the particle. USAXS analysis is currently ongoing

to achieve this.

Figure 6. 1D SAXS patterns as palladium nanoparticles grow on of TMV variants at 25°C. Black Arrows in (a) to (c) shows an increase in the invariant over time which indicates growth (a) WtTMV shows minimal growth. (b) and (c) show significant increase in Pd content on TMV1Cys and TMV2Cys respectively. (d) Typical fit of data to the Beaucage unified model. Residuals from right axis confirm a good fit. Arrows show size levels and primary fitting model at each size level.

Figure 7. Relevant size and structural information on Pd nanocrystal mineralization during the 2nd growth cycle at 25°C hydrothermal synthesis on TMV viruses. (a) and (b) show the two levels of sizes (R_g) obtained from the unified guinier/power law fitting. (c) shows the average packing on the nanoparticles and (d) shows the average interparticle distances.

Figure 8. Transmission Electron Microscope images of virions coated with Pd at 25°C during in situ SAXS studies. Particles were washed and deposited after the reaction was quickly quenched. (a) Studied TMV-1Cys-Pd particles (b) High resolution image of TMV1Cys-Pd. FT of this image (not shown) confirms Pd reduced to Pd⁽⁰⁾ (c) TMV1Cys-Pd showing both levels of particle sizes (d) TMV2Cys-Pd showing both levels of particle sizes.

Conclusions

Virus-mediated metal mineralization has been characterized to obtain fundamental parameters and insights into pertinent processes during biomineralization. Importantly, the adsorption of Pd species and reduction to Pd⁽⁰⁾ were decoupled allowing both processes to be individually probed using XAS and UV-Vis. The reduction of Pd exhibits first order kinetics and the adsorption of Pd species proceeds via pseudo-second order kinetics in unoccupied sites. In both processes, the effect of the sulfhydryl groups on the surface was elucidated. A Langmuir isotherm best describes adsorption on the WtTMV and TMV1Cys surface while the Freundlich isotherm best fits adsorption on TMV2Cys. Rather than a linear correlation between cys groups and mineralization, it was deduced that both the adsorption and reduction of Pd species by amino acids is modulated by the adjacent amino acids via complex local relationships between functionalities. Reduction of Pd species on the Pd coated surface was found to be dependent on the exposed Pd surface area while residual salt was found to hinder this reduction. The Cl⁻ content was also found to affect the adsorption process. Its effect was explained by suggesting a trade-off between its complexation with Pd and its competition with other Pd anions within the stern layer. The growth of Pd nanoparticles was also directly examined. Under the conditions studied, more nanoparticles mineralized onto TMV1Cys and TMV2Cys compared to the Wt virus, as expected from earlier reduction study projections. The growth of primary nanoparticle sizes and structure resulting from the virus-controlled synthesis were probed for the first time using SAXS. In conclusion, the studies in this paper have succeeded in reframing biomineralization within well-understood molecular processes. These processes have been quantitatively characterized thereby elucidating previously unexplained phenomena. Therefore, this study serves as a springboard for directed nanocrystal synthesis on biotemplates. Application

of this more fundamental approach to biomineralization systems will facilitate easier directed synthesis and scale up of bio-inorganic systems.

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