Site-Selective Nucleation and Size Control of Gold Nanoparticle Photothermal Antennae on the Pore Structures of a Virus

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ABSTRACT: In this Article, we show that the surface of the bacteriophage Qβ is equipped with natural ligands for the synthesis of small gold nanoparticles (AuNPs). By exploiting disulfides in the protein secondary structure and the geometry formed from the capsid quaternary structure, we find that we can produce regularly arrayed patterns of ~6 nm AuNPs across the surface of the virus-like particle. Experimental and computational analyses provide insight into the formation and stability of this composite. We further show that the entrapped genetic material can hold upward of 500 molecules of the anticancer drug Doxorubicin without leaking and without interfering with the synthesis of the AuNPs. This direct nucleation of nanoparticles on the capsid allows for exceptional conduction of photothermal energy upon nanosecond laser irradiation. As a proof of principle, we demonstrate that this energy is capable of rapidly releasing the drug from the capsid without heating the bulk solution, allowing for highly targeted cell killing in vitro.

INTRODUCTION

The explosive growth in the synthesis of inorganic materials, specifically plasmonic gold nanoparticles (AuNPs) with controlled nanostructures, has led to several divergent applications in the biomedical and materials fields. Among the possible strategies to create homogeneously sized AuNPs, biomolecular templates such as proteins, nucleic acids, biological fibers, and lipids have emerged, because of the ordered and well-defined structures that these macromolecules form. In addition to these structural advantages, the utilization of biotemplates can impart additional characteristics to these nanomaterials, including solution stability, three-dimensional architectures, molecular and cellular recognition, and tunability for optimized cell uptake. These emergent characteristics permit advanced applications such as concomitant drug delivery and in vivo imaging.

Virus-like particles (VLPs), which are the noninfectious, proteinaceous nanoparticle analogues of viruses, are proven templates for the synthesis of metal nanoparticles, because they are innately monodisperse in size and can be genetically modified or chemically functionalized to attach stabilizing ligands or residues onto their surface with atomistic precision. While there have been several instructive reports on coating, absorbing or growing large AuNPs onto or within viral capsids using surface-exposed residues, reports on restricting the diameter of the nanoparticles by constraining their growth to a homogeneous sub-7 nm size have not been forthcoming. This is an important issue because nanoparticle size is critically linked to clearance from blood and tissues, and cell-specific uptake and distribution has been linked to nanoparticle diameter. To address this problem, we seek to utilize a multivalent strategy to decorate a proteinaceous biocompatible VLP with multiple small plasmonic AuNPs. From here, these small clustered AuNPs could serve as X-ray or positron emission tomography (PET) contrast agents. We are particularly interested in their use as photothermal antennae as a way to convert pulsed optical radiation to a potent yet extremely localized external stimulus to activate drug release. Indeed, the growth of AuNPs directly onto the protein surface should increase the efficiency of thermal conduction following nanosecond pulsed laser-induced heating. This improved transfer of thermal energy into the protein by direct templated-growth should promote more efficient thermally induced protein denaturation, compared to traditional bioconjugation methods used to attach gold onto protein...
surfaces via spacers or synthetic ligands. In addition, reports have shown that smaller AuNPs possess greater photothermal conversion efficiencies, because of their intrinsically higher absorption/extinction ratios. In principle, templated growth of small AuNPs directly onto a protein surface would mean the amount of gold necessary to induce any changes in protein structure could be reduced, decreasing the likelihood of detectable bioaccumulation even further. Finally, the use of nanosecond pulsed irradiation in this case will confine heating to only a few nanometers of the AuNP, permitting very high thermal-spatial resolution with negligible bulk heating of the solution. In this paper, we report the synthesis and characterization of such a system using VLP Qβ as outlined in Scheme 1. The Qβ capsid itself is an icosahedral virus with 180 identical coat proteins and contains 32 disulfide-lined pores. We reasoned that these proteins, linked like a daisy chain by either five or six disulfides to form a fixed pore, would act as a natural ligand and template for the formation of spherical gold nanoparticles upon in situ reduction of gold salts. We then show that this new composite material works as a proof-of-principle photolytically activated drug delivery vehicle. We can load the capsid with the anticancer drug Doxorubicin (Dox) noncovalently using random RNA located within the capsid as a supramolecular host, grow AuNPs over the loaded VLP and, finally, initiate release of the RNA-bound Dox from the center of the virus using nanosecond laser irradiation to disrupt the proteinaceous shell and release the drug molecules from the interior. Finally, we demonstrate highly selective drug release and cell killing of macrophage and cancer cells in vitro exclusively within the laser path while cells outside the path—even though they are in the same culture—show no drug release or death.

Figure 1. Qβ VLP is depicted as a ribbon model. The VLP has a diameter of 28 nm and contains a total of 32 pores. Depicted are one of the 20 large pores, each of which contains six disulfide bonds, and one of the 12 smaller pores, each of which contains five disulfide bonds.

**Scheme 1.** Qβ Possesses 32 Pores, Each Either 1.5 or 3.0 nm in Diameter, Which Are Large Enough To Permit the Diffusion of Doxorubicin into and out of the VLP; after Loading, the Pores Then Are Capped by ~6 nm AuNPs Grown in Situ

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**RESULTS AND DISCUSSION**

VLP, structurally depicted in Figure 1, is expressed as a noninfectious nanoparticle that self-assemblys around random genomic material within *E. coli*. Protein crystallographic analysis reveals 20 pores at the 3-fold axis and 12 smaller pores at the 5-fold axis of symmetry. Key to our strategy is that these pores are lined with solvent-exposed disulfides and we hoped to benefit from the affinity of disulfides toward gold species. To establish an optimized protocol for site-specific growth of AuNPs, parameters including the concentrations of Qβ and tetrachloroauric acid, reaction temperature, and incubation time were individually varied until we had minimized the formation of unstable naked gold particles. After significant optimization, the required conditions for AuNP formation turned out to be quite straightforward—specific absorption of gold by disulfide bonds was achieved by incubating Qβ and tetrachloroauric acid in water followed by addition of the reducing agent sodium borohydride. Because the water was unbuffered, the final solution was lowered to a pH of 2 by the gold acid; as an alternative, 0.1 M potassium phosphate buffer (pH 7) was used. Once all the reagents were added to the vial, the solution was allowed to stand undisturbed for at least 2 h, during which time its color quickly changed from light red to dark red (Figure S1 in the Supporting Information). Based on ultraviolet–visible light (UV-vis) kinetic analysis following the formation of the surface plasmon, the reaction is 50% complete after 5 min and is >99% complete after 20 min (Figure S2 in the Supporting Information). After the reaction, the small amount of precipitated and unbound AuNPs were removed from the crude mixture by passing the reaction solution through a size-exclusion column. We noted that the nanoparticles in the precipitate increases as we increased the concentration of the acidic gold starting material, which we attribute to a decrease in pH and concomitant protein aggregate formation, which can no longer function as a template.

The selective growth of AuNPs on the pore structures was verified by transmission electron microscopy (TEM) (Figures S3 and S4 in the Supporting Information), which indicated that nearly every VLP contained some population of AuNPs, although it appeared that there was a fairly wide distribution of the number of AuNPs per Qβ. The nanoparticle population dispersity prompted an investigation into the average number of nanoparticles per capsid, which was determined to be ~6 ± 3 per Qβ (Figure S5 in the Supporting Information), as determined by inductively coupled plasma–mass spectroscopy (ICP-MS). Dynamic light scattering (DLS) found an increase in capsule size from 23 nm to 64.83 ± 0.219 nm, which is a significant increase in the size of the capsule (Figure S19 in the Supporting Information). Negative stain was used to show the position of Qβ relative to the AuNPs. A crystallographic model of Qβ was used to map the AuNPs over the surface. The micrographs shown in Figure 2 exhibit excellent correlation to the mapped pore patterns on the right. This clear association
173 visible light (UV-vis) spectra shows an absorption centered at 526 nm, which corresponds to the surface plasmon (Figure 2B). To elucidate the role of the virus in the nucleation and growth of the AuNPs, we conducted several control experiments. When the same procedure was performed in the absence of any Qβ, a dark precipitate formed in solution. This precipitate was collected and was found to consist of spherical AuNPs, as determined by X-ray diffraction and confirmed by transmission electron microscopy (TEM), with a diameter of 48 ± 10 nm (as shown in Figure S6).

To confirm that the disulﬁdes themselves were responsible, we prepared a solution of Qβ and ﬁrst reduced the disulﬁdes to the free thiols and then alkylated them with 2-iodoacetamide. We obtained the same results as when no Qβ was present in the solution—huge particles in a black precipitate—conﬁrming the role of the disulﬁdes in the controlled nucleation (Figure S1). In our initial strategy, we assumed that reduction of the disulﬁdes was occurring with sodium borohydride, but we were able to rule this out because we did not detect any such reduction using gel electrophoresis (Figure S8 in the Supporting Information) nor was there any appreciable difference in the Ellman’s assay before and after the addition of borohydride (Figure S10 in the Supporting Information). It is important to point out that, during the synthesis, even spectroscopically pure virus particles are not fully oxidized—that is to say, it appears from nonreducing sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) that monomeric proteins exist when there should be none (Figure S9 in the Supporting Information).

Curiously, reducing the disulﬁdes to free sulfurs also fails to yield AuNP formation on the surface of the virus. Nevertheless, based on these experiments, it is quite evident that nucleation and size control is occurring as a result of the disulﬁde and pore geometry, as we get uncontrolled particle growth in their absence.

AuNP@Qβ are stable at room temperature for more than a month when left undisturbed on the bench. TEM micrographs (Figure S11 in the Supporting Information) of a month-old sample that had been left under ambient laboratory conditions show that the AuNPs are still associated with Qβ, and neither free AuNPs nor aggregation was visible. This stability is surprising, considering that (1) the nanoparticles are considerably larger than the pores and (2) there are only 10 or 12 S atoms bound to the nanoparticle. Based on the TEM data, which show varying levels of contrast on the edges of the nanoparticles, we anticipated that there were interactions between the virus and the nanoparticles beyond simply Au–S bonding. Therefore, we conducted computational analysis to determine the likely changes in the local environment around the nanoparticle. As seen in Figure 3, we modeled a 6.4 nm nanoparticle bound to the 12 S atoms of the hexameric pore structure. Molecular dynamics (MD) simulations provide a reasonable picture of the actual nanoparticle–protein interaction. From these simulations, several intriguing results could be inferred (see the expanded discussion of the theory in the Supporting Information and Figures S12–S15; interestingly, there was no signiﬁcant change in the size of the hexameric pore). However, the pore S atoms shifted radially inward, which created a deeper cavity for the nanoparticle to sit in and, as shown in Figure 3, allowed more of the loop structures present near the pore to cover the surface of the nanoparticle. Roughly 23% of the nanoparticle’s surface area was protected by surrounding proteins, which does not signiﬁcantly change the secondary structure of the VLP, as shown by circular dichroism.
inside the VLP, the genetic material inside Qβ upward of 4300 molecules of Dox. Our tests show that the red clover necrotic mosaic virus capsid tightly holds precedent, as researchers have shown that the RNA inside therapies. Binding of Dox to VLP RNA is not without intercalator Doxorubicin (Dox), which is a genetic material could be used as a supramolecular host within the fully assembled VLP. We hypothesized that this consequently, random bacterial RNA becomes entrapped recombinant Qβ particles, we followed a procedure outlined in Scheme 1. To do this, we exploited the fact that random E. coli microenvironment without damaging nearby healthy cells. 266 To this, we exploited the fact that random E. coli nucleotides serve as templates in the self-assembly of recombinant Qβ coat proteins to form the intact capsid. 271 Consequently, random bacterial RNA becomes entrapped within the fully assembled VLP. We hypothesized that this genetic material could be used as a supramolecular host capable of noncovalently trapping the strong nucleotide intercalator Doxorubicin (Dox), which is a fluorescent chemotherapeutic that is used in many different cancer therapies. Binding of Dox to VLP RNA is not without precedent, as researchers have shown that the RNA inside the red clover necrotic mosaic virus capsid tightly holds upward of 4300 molecules of Dox. Our tests show that the genetic material inside Qβ does the same: when Dox is bound inside the VLP, the fluorescence is modestly quenched in water, for instance—it fluoresces. The association of Dox to the nucleic acids inside the Qβ is sufficiently high that, after loading, we observe no leakage, even after 24 h (Figure S17 in the Supporting Information). Thus, we can monitor the release by following any fluorescence enhancement after laser irradiation.

To create the Dox-loaded, gold-speckled, viral nanoparticles, we followed a procedure outlined in Scheme 1. Initially, the Qβ is loaded by incubating the VLP in a 1 mg/mL solution of Dox for 10 min at room temperature. This mixture is then filtered through a cross-linked dextran size-exclusion column to remove unbound Dox to create Qβ(Dox) (Figure S18 in the Supporting Information). The filtered solution of Qβ(Dox) was then subjected to the same procedure to grow AuNPs over the surface. This solution was again passed through a size-exclusion column to remove unattached nanoparticles and excess salts to yield pure AuNP@Qβ(Dox). By TEM, we saw no discernible difference in gold loading over the pores nor any changes in nanoparticle sizes. We were pleased to see that we could load upward of 500 molecules of Dox—as determined by UV-vis spectroscopy—without interfering with the AuNP formation on the shell.

A 70 μL solution at a concentration of 0.04 mg/mL AuNP@Qβ(Dox) was then irradiated with a single 6-ns laser pulse at an energy density of 500 mJ/cm² and centered at a wavelength of 532 nm. The solution temperature was monitored using a
thermo-couple and no bulk solution temperature change was observed. Based on fluorescence analysis of the irradiated sample, a 100% enhancement in fluorescence was seen, indicating that Dox was released from the capsid. Under control conditions, with no laser irradiation, no fluorescence change was observed because the Dox is tightly bound to the RNA (Figures 4A–C). In a second control, which uses convective heat, we found we could replicate these results by boiling AuNP@Qβ(Dox) in water for 5 min, which completely denatured and destroyed the viral capsule. At room temperature, however, the AuNP@Qβ(Dox) was stable for several hours and showed little variation in fluorescence. We conducted CD and DLS spectroscopic studies (Figure 4D and Figure S19 in the Supporting Information) to determine if the photothermal energy was transferred to the protein. Specifically, we looked at samples of Qβ VLP with and without AuNPs to determine if any obvious change in the spectra of these samples could be ascertained. When AuNP@Qβ was irradiated, a decrease and slight shift in molar ellipticity could be seen, indicating changes in secondary structure and in nanoparticle absorbance (Figure 4D), suggesting some precipitation of aggregates. Agarose band shift assays (Figure S21 in the Supporting Information) showed clear Dox release by UV imaging and a loss or change in capsid structure, following irradiation. In a control experiment using laser irradiation centered at 1064 nm, no change was evident by DLS or agarose band shift (Figures S19 and S21). We therefore attribute this heat to localized absorption of the laser light to generate thermal energy via localized surface plasmon resonance (LSPR), which, in turn, denatures the capsid proteins and provides sufficient thermal energy to release the Dox. This localized heat denaturation and drug release causes a slight shift in the nanoparticle absorbance (Figure S20 in the Supporting Information), denaturation of the protein capsid, and release of the drug, as shown by bandshift assays (Figure S21) in agarose gel electrophoresis.

In order to move toward studies in cellular systems, we first had to determine the toxicity of our particles via MTT assay, using each of the Qβ constructs without the use of laser activation (Figure 5A). The assay found no significant cell viability difference when Qβ(Dox), AuNP@Qβ, and AuNP@Qβ(Dox) were incubated with RAW 264.7 cells. On the other hand, the cells clearly responded to free Dox. A key advantage of this extreme confinement of thermal energy is that it should enable a highly targeted release of therapeutics exclusively within the path of a focused beam of light. In other words, we should be able to pinpoint the cells we wish to kill in a single culture without affecting the surrounding cells, because of convective heat loss. To demonstrate the efficacy of our approach in vitro, cell studies were performed using RAW 264.7 macrophage and A549 lung cancer cells. For the macrophage cells, 3-cm glass bottom plates were seeded with ∼1×10⁶ cells 1–2 days prior to the experiment producing cells that reached ∼80% confluency. The cells were then incubated with 240 μL of 0.2 mg/mL AuNP@Qβ(Dox)—equivalent to ∼7.8 μM Dox, as well as appropriate controls for 4 h. The cells were washed three times with PBS, stained with 200 nm Hoechst 34442 and washed again with PBS. The plates were covered with a cardboard mask pierced with an 18-gauge needle to confine the laser path to a diameter of 1.27 mm to demonstrate the spatial selectivity of release. The cells were then subject to a single 6-ns pulse of 500 mJ/cm² and immediately imaged by live-cell fluorescence microscopy.

Immediately following laser irradiation, the release of Dox in the targeted “kill-zone” to the right of the white line in Figures 5B–E was obvious in the red channel of our fluorescence microscope. The morphology of the cells, as shown in Figure 5E and at higher magnification (inset), did not immediately change following irradiation, indicating that the initial laser
itself did not affect the cells (also shown in Figure S22 in the Supporting Information). However, fluorescence imaging revealed extensive Dox release in the kill zone with the correlation between cells showing release and those within the targeted area being very high; outside of this area, no red fluorescence could be discerned. The highly targeted nature of laser irradiation makes bulk cell viability assays such as MTT ineffective; therefore, we monitored cell viability via live-cell fluorescence imaging for 12 h. Cells outside the kill zone remained intact and adherent, and their morphology was generally unchanged. Within the kill zone, the resulting cells largely detached from the plate after 12 h (Figure S23 in the Supporting Information), indicating that they were killed. When the experiment was repeated using 1064 nm laser light, which is incapable of exciting the LSPR, the results of live cell imaging clearly show no DOX release and no toxicity over that same 12 h time period (Figure S24 in the Supporting Information), again demonstrating that the release is attributed to highly localized optical excitation of the proteinaceous gold complex within the living cell. We repeated these experiments on A549 cells and obtained similar results. In the case of A549 cells, we confirmed cell death exclusively in the laser path using NucRed Dead 647 after allowing the cells to incubate for 4 h (Figure S25 in the Supporting Information). Taken together, these results show that we can release a supramolecularly bound chemotherapeutic from random RNA using photo-thermally triggered degradation of a protein complex for highly localized cell killing.

CONCLUSION

Using the strong affinity between disulfides and gold species, we have shown that AuNPs can be synthesized site-selectively in a controlled manner on the pore structures of Qβ through a simple incubation and reducing process. TEM micrographs show a well-ordered topology of AuNP@Qβ in accordance with the pattern of pores on Qβ. The growth of the nanoparticles is clearly dependent upon the existence of disulfides as reduction to free sulfurs or acylation of these sulfurs fails to yield the composite material. The resulting AuNP@Qβ is stable, when left exposed on the benchtop at room temperature for more than one month, even though the AuNPs themselves are prone to self-aggregation. Computational modeling indicates this stability arises from surface passivation by local protein physisorption. We found that the growth of AuNPs on the VLP is unaffected by loading the interior with the anticancer drug Doxorubicin. We successfully demonstrated that this new AuNP@Qβ(Dox) composite can release the Dox upon nanosecond-pulsed irradiation without heating the bulk solution and thus offers a pathway to drug delivery with very high spatial resolution. This proof-of-concept shows great promise in using laser irradiation to trigger the release of materials confined within proteinaceous capsids and adds another tool in the arsenal of photothermally activated nanotherapeutics.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b10446. Detailed experimental and synthetic procedures as well as the characterization of the materials presented in this work (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

VLP, virus-like particle; Dox, Doxorubicin; AuNP, gold nanoparticle; CD, circular dichroism; MD, molecular dynamics; TEM, transmission electron microscopy; PXRD, powder X-ray diffraction; EPR, enhanced permeability and retention

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