AFRL-AFOSR-VA-TR-2022-0670

Structural studies to elucidate the mechanisms of biobased nanoparticle synthesis

Nannenga, Brent
ARIZONA STATE UNIVERSITY
660 S MILL AVE STE 312
TEMPE, AZ, 85281
USA

09/26/2022
Final Technical Report

DISTRIBUTION A: Distribution approved for public release.

Air Force Research Laboratory
Air Force Office of Scientific Research
Arlington, Virginia 22203
Air Force Materiel Command

REPORT DOCUMENTATION PAGE

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED	
20220926	Final	START DATE 20171101	END DATE 20211031

4. TITLE AND SUBTITLE
Structural studies to elucidate the mechanisms of biobased nanoparticle synthesis

5a. CONTRACT NUMBER	5b. GRANT NUMBER	5c. PROGRAM ELEMENT NUMBER
	FA9550-18-1-0012	61102F
5d. PROJECT NUMBER	**5e. TASK NUMBER**	**5f. WORK UNIT NUMBER**

6. AUTHOR(S)
Brent Nannenga

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
ARIZONA STATE UNIVERSITY
660 S MILL AVE STE 312
TEMPE, AZ 85281
USA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)	11. SPONSOR/MONITOR'S REPORT NUMBER(S)
Air Force Office of Scientific Research 875 N. Randolph St. Room 3112 Arlington, VA 22203	AFRL/AFOSR RTB2	AFRL-AFOSR-VA-TR-2022-0670

12. DISTRIBUTION/AVAILABILITY STATEMENT
A Distribution Unlimited: PB Public Release

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Protein and peptide directed synthesis of inorganic nanomaterials offers several advantages over traditional synthesis methods. These advantages include material production under more environmentally friendly conditions, extremely fine control over nanoparticle shape and size, and the ability to functionalize the nanoparticle surface through the use of protein fusions. Two of the next major advances in the field of peptide and protein driven nanomaterial synthesis will be the ability to rationally design biomolecules for novel, highly controlled
nanostructure synthesis, and the ability to engineer this capability into organisms that can then be used as nanomaterial factories. One of the biggest roadblocks to achieving these goals is the lack of understanding of how these biological molecules interface with the inorganic
nanomaterials during the early stages of nanomaterial formation. We propose that the fundamental molecular mechanisms for how these peptides and proteins influence nucleation and growth can be determined through high-resolution structural studies (protein
crystallography and cryo-electron microscopy). Furthermore, increasing our understanding of which sequences can be translated into in vivo nanomaterial synthesis systems will be accomplished by developing new combinatorial screening strategies to isolate sequences that
function in the context of a living cell.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES
a. REPORT	b. ABSTRACT	c. THIS PAGE		
U	U	U	UU	6

19a. NAME OF RESPONSIBLE PERSON	19b. PHONE NUMBER *(Include area code)*
BENNETT IBEY	000-0000

Structural studies to elucidate the mechanisms of biobased nanoparticle synthesis

Brent L. Nannenga

In the final year this project, which was part of a no cost extension, we focused on wrapping up remain experiments that were nearly completed the year before. The results from this project pave the way for the use of high-resolution structural techniques in the analysis and application of bio-enabled nanoparticle synthesis. Our new findings have been published in journals and we have given several presentations and conferences and seminars. It has also supported two graduate students in PI Nannenga's lab, one PhD student and one Master's student, for the duration of their graduate studies. Both students have recently graduated and support from this project has carried them into the next phase of their scientific careers in industry. In this final report we will discuss this work as well as the past work that was completed in previous years.

Objective 1

To study structural features important for biologically controlled nanoparticle formation, we identified a system of maltose binding protein (MBP) and gold nanoparticles (AuNPs) that could be used a model for these studies. MBP, encoded by the *malE* gene, is a protein found in the periplasm of *E. coli* whose role is in the binding and transport of maltose, and has been shown to be capable of controlling the growth of silver nanoparticles (1). We found that using conditions similar to previously reported peptide driven AuNP synthesis (2), MBP could also influence the formation of AuNPs (Fig. 1). In the presence of MBP, stable red AuNPs are formed in a solution at ~4.5 pH with HEPES acting as a mild reductant (3). In the absence of MBP, the gold ion precursor is reduced, however no AuNPs and only a small amount of bulk gold is formed after a few hours of incubation. We hypothesized that in the presence of MBP the gold ions are sequestered through the formation of a higher order macromolecular aggregates which inhibit spontaneous nanoparticle formation, lead to a slower growth of AuNPs followed by capping and stabilization. The interaction of MBP with the gold ions in solution was independently corroborated through two complementary techniques: native PAGE and dynamic light scattering (DLS) (Fig. 3). When analyzed by native PAGE (Fig 3A), MBP migrates as expected with clear and distinct bands. Upon incubation with the gold salt, a shift in the MBP band migration is observed indicating significant interaction of the protein with the gold ions. A similar result is obtained by DLS (Fig 3B), which depicts a hydrodynamic diameter of ~7nm for MBP, which shifts to a larger and more polydisperse complex with an average diameter of ~50nm upon incubation with gold salt solution. In order to rule out that the shifts seen were due solely to protein aggregation as a result of the low pH of the $HAuCl_4$ solution, MBP was incubated in a sodium acetate buffer, pH 4.5 without any $HAuCl_4$. The native

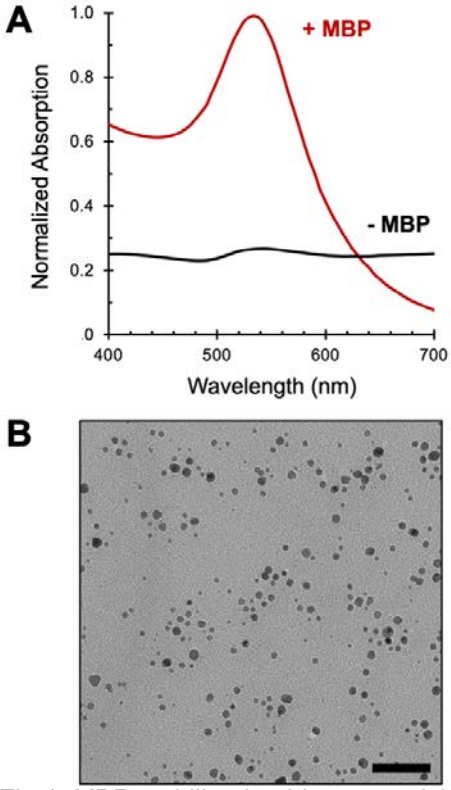

Fig 1. MBP stabilized gold nanoparticle formation. (A) The absorbance of the samples where the gold reduction occurred in the presence of MBP showed characteristic spectra associated with AuNPs, whereas control samples with no protein did not form stable AuNPs. (B) TEM analysis of MBP-AuNPs confirmed the presence of gold nanoparticles with an average diameter of 4.91 ± 1.54 nm (n = 116). Scale bar in B represents 50 nm.

PAGE shows identical migration for both MBP and MBP in low pH, and the DLS data at this pH does not show large aggregates as seen when $HAuCl_4$ is present (Fig 2). This suggests that it is the gold in solution which is causing the MBP to form larger complexes. To investigate whether these larger aggregates are the result of gold-mediated MBP complex formation or simply due to irreversible protein aggregation, MBP which had been incubated with HAuCl4 was analyzed by size exclusion chromatography (SEC). When the MBP + $HAuCl_4$ sample is ran over the SEC column containing the purification buffer without $HAuCl_4$, the MBP elutes at the same point as the MBP control sample. This shows that upon the removal of gold ions, the large MBP complexes seen by DLS are able to dissociate and return to its original monomeric state. Taken together, these results indicate that the MBP is able to sequester the gold in solution and form larger MBP-gold complexes, which are reversible (4).

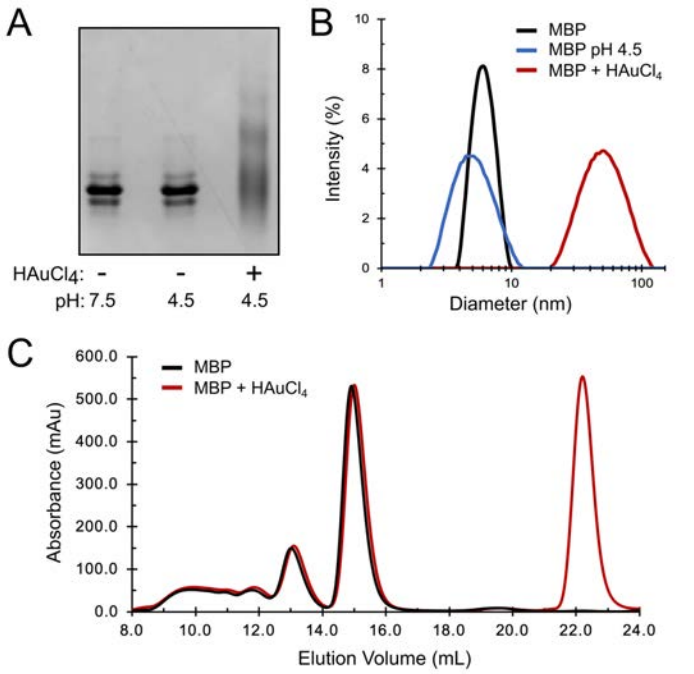

Fig 2. (A) When MBP is run on native PAGE, the majority of the protein migrates as a single band. When MBP is incubated with $HAuCl_4$, the migration pattern shifts indicating that the gold ions are interacting with MBP. (B) A similar shift in size is seen when these samples are analyzed by DLS. (C) When two samples of MBP, with and without $HAuCl_4$, were analyzed by SEC in buffer without $HAuCl_4$, the overlaid chromatograms are nearly identical indicating the larger aggregates rapidly dissociate once the gold is removed.

Structure studies of this MBP-AuNP system were carried out by using X-ray crystallography. We identified crystallization conditions that produce large, well-diffracting MBP crystals under pH 4.5-4.6 conditions, which is the same as what is used for the synthesis of AuNPs in solution. MBP crystals were soaked with $HAuCl_4$ and diffraction data were collected to approximately 2Å. The gold soaked MBP structure determined showed strong density for a gold atom near M322 of MBP. There is also a second potential gold atom near a second methionine, M205; however, the electron density for this atom is not as strong. When the function of the residues near this gold binding site were tested by mutation studies, we could find no difference in the mutants versus wild type. This indicates that while gold can bind to this site, it is not the only site responsible for the effects we see with MBP and there are other, most likely many other, sites where gold interacts with MBP.

To continue to understand the structural features of this system, the crystal structure of gold ion bound MBP was further characterized. When mutations were made of residues near the gold binding site, significant differences in MBP-facilitated gold nanoparticle synthesis could not be seen. Because we hypothesized that there are multiple important interactions of MBP with gold, we aimed to decouple this particular gold site from the rest of the MBP structure. To this end, a peptide was selected based on the residues nearest to the gold atom and connected via a serine and glycine linker. This peptide, AT1 (YPFGGSGGSGM) was tested for nanoparticle synthesis under similar conditions to those used for MBP, except using increased concentrations of peptide relative to protein. We found that using 100μM of AT1 peptide, but not an all serine/glycine control peptide, was able to produce stable gold nanoparticles (3) that were similar to those produced by

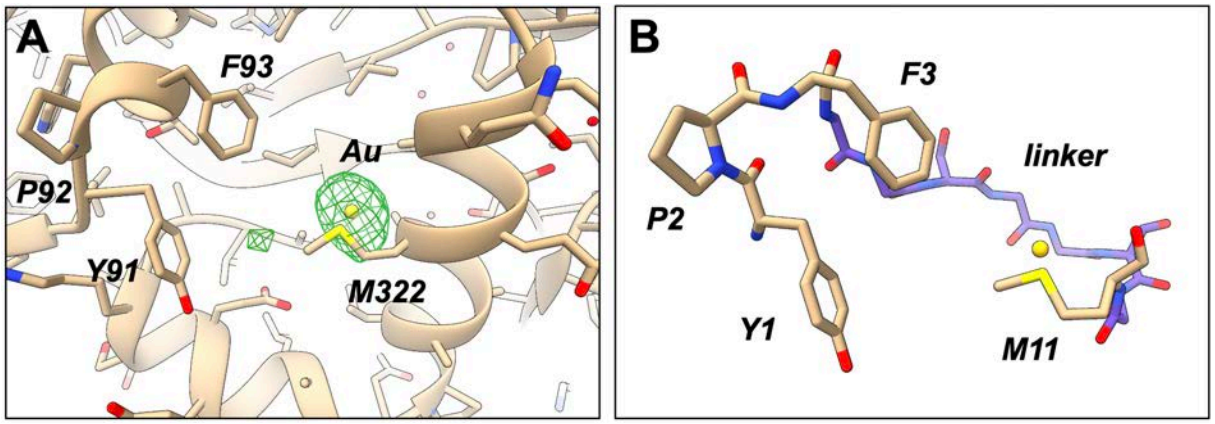

Fig 3. High-resolution structure of gold atom bound MBP and structure guided designed peptide AT1. (A) When examining the electron density maps of MBP soaked with gold, a very strong signal for gold can be seen near methionine 322 in the difference map (green and blue, contoured at 5σ and 15σ respectively. (B) A peptide, AT1, was designed using the closest residues in the structure (tan) to the bound gold, which were connected by a serine-glycine linker (purple).

the full MBP protein. Additionally, the use of the A3 peptide, a previously identified gold binding peptide (5), was incapable of producing stable nanoparticles under these conditions, indicating there is a link between MBP driven synthesis and the AT1 peptide. This study, which was recently published in *Biotechnology and Bioengineering* (3), represents the first time, to our knowledge, of a high resolution structure being used to select a novel peptide capable of aiding in the synthesis and stabilization of nanomaterials.

One unexpected outcome of the work on MBP facilitated gold nanoparticle synthesis was that we found that if we use the MBP system described above at higher pH (pH 7.5), the reaction is inhibited, and the solution remains clear because no AuNPs are formed. When this clear MBP, gold, and HEPES solution is irradiated with X-rays, we can again drive AuNP formation in the presence of protein. We found the synthesis of the AuNPs by this method to be dose dependent where the color of the AuNPs shift from blue to red as dose is increased (Fig 4), and we have used this to control the size and shape of gold nanoparticles with MBP. As an application of these system, we have developed it as a protein-nanoparticle enabled radiation sensor that is capable of the detection of therapeutic levels of X-ray radiation (6). While it was not our goal in this project to develop any sorts of sensors or applications for protein-nanoparticle systems, it was a very interesting byproduct of the more fundamental research that is the focus of this project. This study was done in collaboration with cancer researchers at a local hospital and was also published in *Biotechnology and Bioengineering* (4).

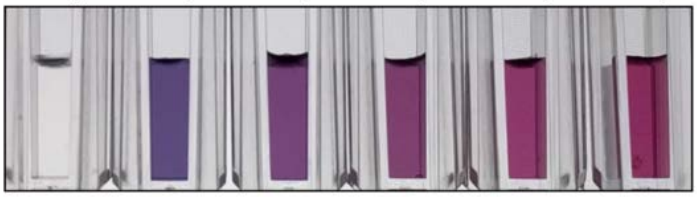

Fig 4. This system produces AuNPs that show a dose-dependent shift in maximum absorbance wavelengh. We can use this to tune the color and size of the resulting nanoparticles.

Objective 2

In order to study how nanoparticles bind to proteins after the nanoparticles have been formed, we employed single particle cryo-electron microscopy (cryo-EM) analysis. In order for cryo-EM to be successful larger proteins are required. Therefore, we made use of the protein GroEL, a 840 kDa

tetradecameric molecular chaperone, for our cyro-EM studies. We have produced conditions where GroEL facilitates the formation of platinum nanoparticles (PtNP), and to study how the GroEL may interact with the platinum, we prepared cryo-EM samples and imaged using our Titan Krios cryo-TEM. In our initial analysis, the raw images showed that the majority of PtNPs are associated with a GroEL molecule. Upon preliminary analysis by 2D classification and 3D reconstruction (~11Å 3D reconstruction), we could clearly identify regions of the protein that prefers to interact with the PtNPs in solution. This led us to optimize sample preparation procedures in order to collected a higher quality and larger data set of PtNP bound GroEL in order to determine if the method could produce a high-resolution structure of the protein-nanoparticle complex. We collected thousands of high-resolution images of GroEL-PtNPs, which allowed us to create a data set that contained approximately 200,000 particles, which is a substantial improvement over the approximately 6,000 particle data set used to generate the initial structure. In the first round of single particle analysis using the program cisTEM (7), we were able to reconstruct the GroEL-PtNP to around 4.5Å resolution without applying any symmetry. In this case, no symmetry was applied because the nanoparticle breaks the native D7 symmetry of GroEL. After analyzing this initial structure, it was apparent that the nanoparticle broke the 7-fold symmetry of GroEL, however the 2-fold symmetry between the heptameric rings could still be used. Therefore, we used C2 symmetry in subsequent refinements and achieved an improvement in resolution to 3.8Å (Fig 3). This higher resolution map allowed us to place the atomic model for GroEL within the density map and identify the relative orientation of the nanoparticle within the protein core, and we found that the nearest residues to the platinum nanoparticle are two arginine residues (R268) from adjacent GroEL monomeric units that extend into the central cavity of GroEL. This structure represents the first time single particle cryo-EM has been used to study a protein-nanoparticle complex at this resolution, and the paper is currently submitted for publication

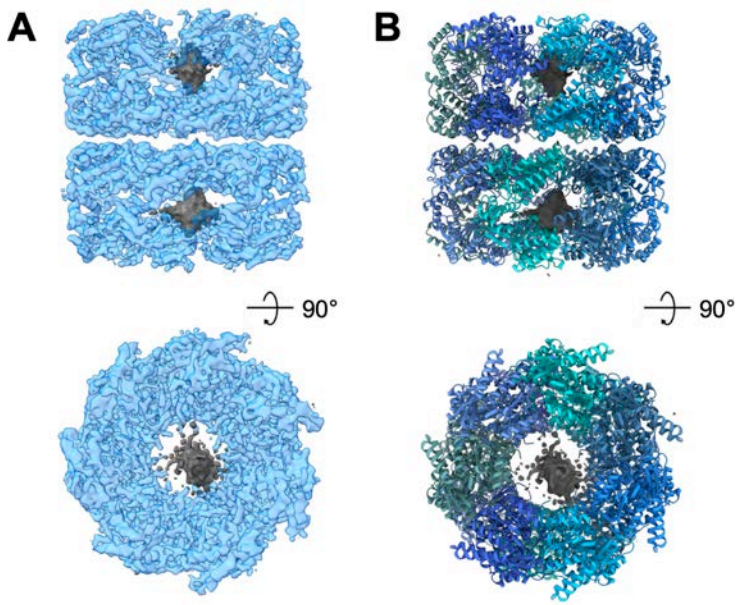

Fig 3. 3.8Å structure of a GroEL-PtNP complex by single particle cryo-EM. The potential map (A) shows density (black density) for the nanoparticle situated in the central cavity of GroEL (A), and the atomic model of GroEL fits well inside reconstructe map (B). Analysis of the nanoparticle shows that two arginine residues (R268-colored red) from adjacent monomers (colored blue and green) are the closest in position to the PtNP.

Objective 3

Much of the project focused on objective 1 and 2, which required more time and resources than was initially planned. Because of this little progress was made on screening and identifying new metallic binding peptides *in vivo*. We were able to identify $HAuCl_4$ toxicity ranges for *E. coli* grown in LB. These toxicity levels (50-100 μM) were significantly higher than previously reported values (8), and attempts to rescue toxicity using known gold mineralizing proteins and peptides (MBP fused to A3 peptide) were unsuccessful. Future work with this system using minimal media are

under way in an attempt to limit potential complicating effects of the components of the rich growth media.

Deliverables

The following publications and presentations were a direct result of the funding

Publications (5 total)

S. Sen, A. Thaker, L. Sirajudeen, D. Williams, and B.L. Nannenga. 2022. Protein−Nanoparticle Complex Structure Determination by Cryo-Electron Microscopy. ACS Applied Biomaterials. https://doi.org/10.1021/acsabm.2c00130

A. Thaker, L. Sirajudeen, C.R. Simmons, and B.L. Nannenga. 2021. Structure-guided identification of a peptide for bio-enabled gold nanoparticle synthesis. Biotechnology and Bioengineering. 118:4867-4873

A.M. Levine, G. Bu, S. Biswas, E.H.R. Tsai, A.B. Braunschweig and B.L. Nannenga. 2020. Crystal Structure and Orientation of Organic Semiconductor Thin Films by Microcrystal Electron Diffraction and Grazing-Incidence Wide-angle X-ray Scattering. Chemical Communications. 56:4204-4207

B.L. Nannenga. 2020. MicroED methodology and development. Structural Dynamics. 7:014304

A. Thaker, K. Pushpavanam, T. Bista, S. Sapareto, K. Rege, B.L. Nannenga. 2019. Protein-facilitated gold nanoparticle formation as indicators of ionizing radiation. Biotechnology and Bioengineering. 116:3160-3167

Presentations (7 total)

B.L. Nannenga (*invited presentation*) – Combining MicroED and GIWAXS for determining structure and orientation of organic semiconductor thin films. 25th Congress of the International Union of Crystallography. Prague, Czech Republic. 2021

B.L. Nannenga (*invited seminar*) – High-resolution molecular structure determination: Methods development and applications. Colorado School of Mines - Department of Chemical and Biological Engineering Seminar Series. Golden, CA. 2021

A. Thaker, and B.L. Nannenga (*presentation*) – High-Resolution Structural Studies of Protein Directed Nanomaterial Synthesis. AIChE Annual Meeting. Pittsburg, PA. 2018

A. Thaker, and B.L. Nannenga (*presentation*) – Development of Protein-Gold Nanoparticle Based Colorimetric Radiation Sensor. AIChE Annual Meeting. Pittsburg, PA. 2018

A. Thaker, and B.L. Nannenga (*presentation*) – Protein and Gold Nanoparticle Based Radiation Sensor. AIChE Annual Meeting. Minneapolis, MN. 2017

A. Thaker, and B.L. Nannenga (poster presentation) – Structural studies of protein based nanoparticle synthesis. AIChE Annual Meeting. Minneapolis, MN. 2017

B.L. Nannenga (*presentation*) – Structural Studies to Determine the Mechanisms of Biobased Nanoparticle Synthesis. AIChE Annual Meeting. San Francisco, CA. 2016

References

1. C. D. Grosh, D. T. Schwartz, F. Baneyx, Protein-Based Control of Silver Growth Habit Using Electrochemical Deposition. *Crystal Growth & Design* **9**, 4401-4406 (2009).
2. J. M. Slocik, M. O. Stone, R. R. Naik, Synthesis of gold nanoparticles using multifunctional peptides. *Small* **1**, 1048-1052 (2005).
3. A. Thaker, L. Sirajudeen, C. R. Simmons, B. L. Nannenga, Structure-guided identification of a peptide for bio-enabled gold nanoparticle synthesis. *Biotechnology and Bioengineering* **118**, 4867-4873 (2021).
4. A. Thaker *et al.*, Protein-facilitated gold nanoparticle formation as indicators of ionizing radiation. *Biotechnol Bioeng* 10.1002/bit.27163 (2019).
5. J. M. Slocik, J. S. Zabinski, D. M. Phillips, R. R. Naik, Colorimetric response of peptide-functionalized gold nanoparticles to metal ions. *Small* **4**, 548-551 (2008).
6. K. Pushpavanam, E. Narayanan, J. Chang, S. Sapareto, K. Rege, A Colorimetric Plasmonic Nanosensor for Dosimetry of Therapeutic Levels of Ionizing Radiation. *ACS Nano* **9**, 11540-11550 (2015).
7. T. Grant, A. Rohou, N. Grigorieff, cisTEM, User-friendly software for single-particle image processing. *eLife* **7**, e35383-e35383 (2018).
8. T. P. Shareena Dasari, Y. Zhang, H. Yu, Antibacterial Activity and Cytotoxicity of Gold (I) and (III) Ions and Gold Nanoparticles. *Biochem Pharmacol (Los Angel)* **4**, 199 (2015).