



ARL-TR-8371 • MAY 2018

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Site-Specific Nanocluster Synthesis in Energy-Coupled Biomolecular Hosts

by Sasha Teymorian, Abby West, Michael Lee, Nick Bedford,
and Mark Griep

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE (DD-MM-YYYY) May 2018		2. REPORT TYPE Director's Research Initiative		3. DATES COVERED (From - To) August 2016 – November 2017	
4. TITLE AND SUBTITLE Site-Specific Nanocluster Synthesis in Energy-Coupled Biomolecular Hosts				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sasha Teymorian, Abby West, Michael Lee, Nick Bedford, and Mark Griep				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Research Laboratory Weapons and Materials Research Directorate (ATTN: RDRL-WMM-C) Aberdeen Proving Ground, MD 21005				8. PERFORMING ORGANIZATION REPORT NUMBER ARL-TR-8371	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Although several proteins have been used to synthesize fluorescent gold metal nanoclusters (AuNCs), the fundamental mechanisms of AuNC formation and stabilization in protein conjugates is still a topic of investigation. Understanding key binding residues and protein structure(s) will enable, for the first time, the ability to engineer site-specific nanocluster growth within biomolecular hosts. This report will describe our efforts to elucidate the synthesis and stabilization mechanisms of AuNCs in various protein hosts. Several mutant forms of trypsin were prepared using site-directed mutagenesis, and the mutant proteins purified and their ability to synthesize and stabilize AuNCs will be detailed. In addition, gold-coordinating ligands identified through proteolysis and peptide electrospray ionization-mass spectrometry that may yield insight into the design of novel protein nanoclusters will be explored. Finally, development of 3-D atomic models to predict gold-protein interactions based on experimentally obtained X-ray data will be discussed.					
15. SUBJECT TERMS DRI, metal nanocluster materials, protein engineering, mass spectrometry, fullRMC modeling					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 16	19a. NAME OF RESPONSIBLE PERSON Sasha A Teymorian
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (Include area code) (410) 306-4787

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1. Introduction

This research aims to discover the fundamental mechanisms and amino acid residues/protein structures controlling the synthesis and stabilization of metal nanocluster (NC) materials. An understanding of the key binding residues and required protein structure(s) will enable, for the first time, the ability to engineer and control NC growth sites within a biomolecular host. Methods to engineer energy-tuned NCs at desired locations within a biomolecule enables new bio-nano energy coupling paradigms, where both electromagnetic and magnetic fields may be coupled to control and enhance the native function of the biomolecular host, depending on the cluster composition.

2. Project Overview

Our project goal was two-fold: 1) to understand the formation and stabilization of gold NCs (AuNCs) within various protein hosts, and 2) to investigate AuNC formation within optically-active biomolecules as a means to couple bioactivity and achieve non-native photophysical properties.

Year 3 Objective: Localization and engineering of NC stabilization sites within trypsin and associated mutants.

3. Cloning, Expression, and Purification of Native and Mutant Enzymes

Amino acid (aa) residues involved in the formation and stabilization of a 25-atom gold nanocluster (AuNC) in native trypsin (human isoform 2, 247 aa) were predicted computationally to be located near the enzyme active site. A total of 7 residues were selected for alanine scanning mutagenesis—a technique in which selected aa residues are mutated to alanine, a small and chemically inert aa that acts as a placeholder in the protein sequence. A total of 4 variants were generated, yielding both point and mass mutants of native trypsin (Fig. 1a). DNA primers were designed and genetic changes were introduced using standard procedures. Purified fractions containing native and mutant proteins revealed a dominant protein band near M_r at approximately 26 kDa, corresponding to the calculated molar mass of trypsin. Presence of the protein in the wash fractions suggests the C-terminal (His)₆ tag is partially masked within the folded protein and unavailable for binding (Fig. 1b). Low concentrations of urea (2 M) were included in the binding buffer in an effort to expose the (His)₆ tag without denaturing the enzyme; however, no improvement in binding affinity was observed. Experimental reproducibility was a

challenge because of the low expression yields of native and mutant proteins, possibly due to the autocatalytic activity of trypsin and damage by trypsin to the host cell. Fast protein liquid chromatography (FPLC) was pursued as an additional form of purification in an effort to yield higher specificity in the protein purification; however, expression yields remained low and the mutant proteins exhibited poor separation.

Native and mutant proteins were assayed for enzyme activity using N_{α} -benzoyl-L-arginine ethyl ester hydrochloride (BAEE) substrate. Native trypsin was found to retain its active conformation following expression and purification, whereas the mutant forms lost all enzymatic activity (Fig. 1c). Circular dichroism (CD) of the proteins following expression and purification revealed that, while the overall folded structure is maintained for the native and mutant proteins, the secondary structure is abolished in the proteins following AuNC synthesis reactions, as noted by the adoption of a random coil conformation (Fig. 1d). Native trypsin was observed to yield red-emitting AuNCs; however, AuNC formation in the mutant forms of the protein were unsuccessful (Fig. 1c, inset). These data suggest that, while having minimal effect on the secondary structure of the protein, each mutated residue is necessary in the nucleation and growth of AuNCs in trypsin. Interestingly, AuNC nucleation and stabilization was found to not impact the secondary folded structure of all proteins equally. Bovine serum albumin (BSA) and pepsin were found to retain their native folds, whereas insulin did not.

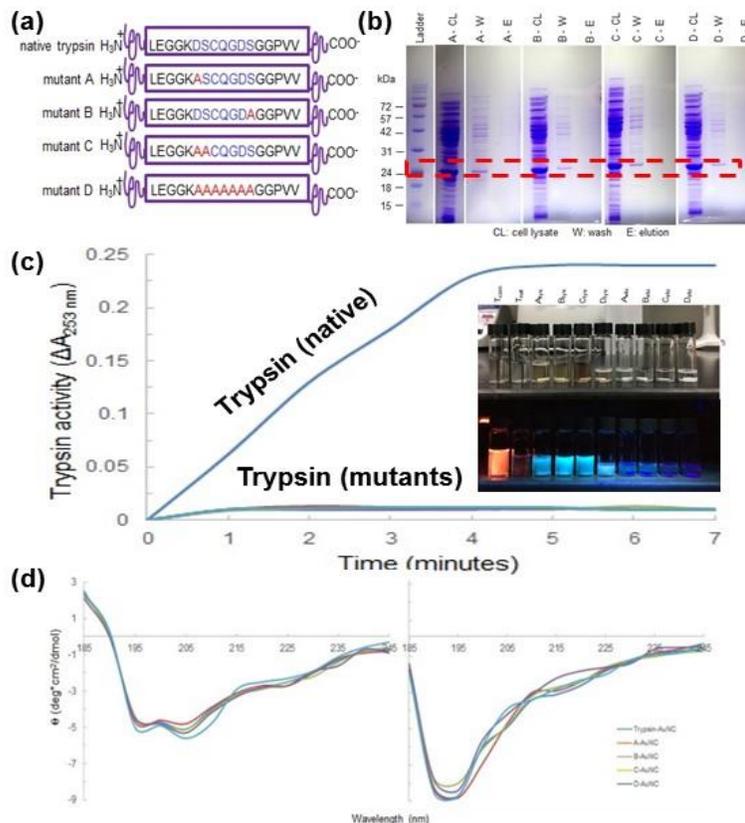


Fig. 1 a) Native and mutant protein sequences generated to investigate AuNC stabilization and growth. b) Separation of purification fractions by SDS-PAGE for mutant trypsin proteins, A–D as labeled. c) Activity assay of native and mutant trypsin, measured as the $\Delta A_{253\text{nm}}$ due to release of N_α -benzoyl-L-arginine over time. d) CD spectra of native and mutant proteins indicate the loss of secondary structure as a result of AuNC synthesis reaction.

4. Bioanalytical Mass Spectrometric Approaches to Characterize Protein-Stabilized AuNCs

The concentration of gold (^{197}Au) in the PNCs (BSA-AuNCs, trypsin-AuNCs, and insulin-AuNCs) was directly measured by inductively coupled plasma mass spectrometry (Agilent 7700X ICP-MS). The ^{197}Au concentration in the AuNC samples were determined via a calibration curve generated with gold atomic absorption standard (Fluka Analytical) and the protein concentrations were determined via the Bradford protein assay (Thermo Coomassie Plus). By combining gold quantification via ICP-MS and protein quantification via the Bradford protein assay, we determined the stoichiometry of the AuNCs provided (Fig. 2a). The gold-to-protein stoichiometry measured in this manner was lower

than that determined using fluorescence. The liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS) analysis revealed that only a fraction of the proteins are associated with gold, suggesting that the stoichiometry is under-determined due to the presence of additional proteins that are not part of the gold-stabilized nanocluster (Fig. 2b). This issue was circumvented by isolating PNCs via LC to achieve stoichiometries that match those determined via fluorescence. Ongoing studies are combining proteolysis and peptide electrospray ionization-mass spectrometry (ESI-MS) to isolate and identify the ligands that coordinate gold present in the AuNCs. These final studies are expected to provide key molecular-level details and inform on the design of novel PNCs.

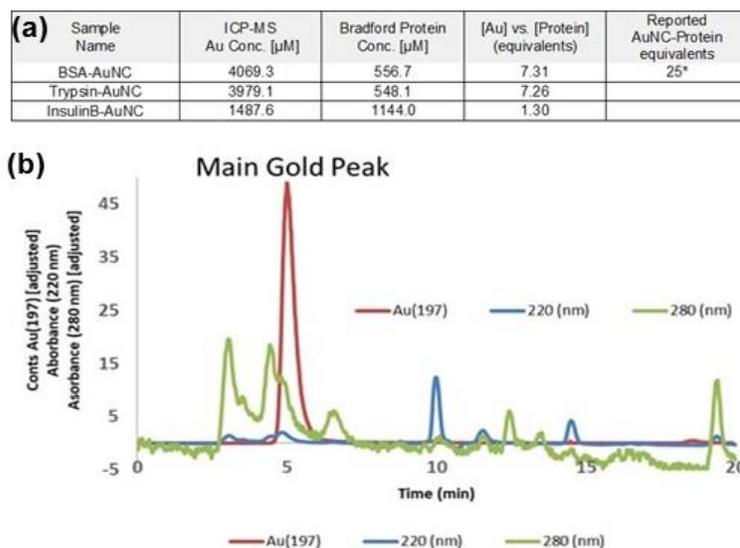


Fig. 2 a) Protein:Gold stoichiometry and b) LC-ICP-MS of trypsin PNCs

5. X-Ray Studies of Reduced Molecular Weight Protein Host and Crystallized PNC Models

Green fluorescent protein (GFP), BSA, and trypsin proved challenging to model computationally because of their large size (approximately 27 kDa, 66 kDa, and 26 kDa, respectively). A smaller PNC model made via human insulin (approximately 5 kDa), which yielded a red fluorescence (Fig. 3a, inset), was pursued in an effort to harmonize experimental pursuits with computational approaches. X-ray absorption studies (XAS) show that insulin-NCs exhibit structure that is drastically different than bulk gold, directly suggesting the formation of small clusters, consisting of S-Au and Au-Au bonds (Fig. 3a). Small angle X-ray scattering (SAXS) further shows larger-scale changes to protein morphology upon the

inclusion of AuNCs, which implies a strong interaction between the AuNCs and protein (Fig. 3b). In addition to modeling complications associated with PNC size, the lack of periodicity in the material hinders uniform X-ray data. A new method developed to create amino-acid conjugated PNC structures was shown successful in creating energy-tuned BSA, trypsin, pepsin, and lysozyme PNCs (Fig. 3c). Preliminary X-ray studies show that the form factor scattering is substantially diminished as compared to non-crystallized PNCs, potentially suggesting the materials are becoming more structured. In concert with this work, collaborators at the National University of Singapore demonstrated the ability to stabilize PNCs within a tailorable metal organic framework (MOF) structure. Further X-ray studies will determine if these synthetic PNC crystal structures yield a high degree of organized PNC-repeating structure, which would allow for potential crystallographic studies.

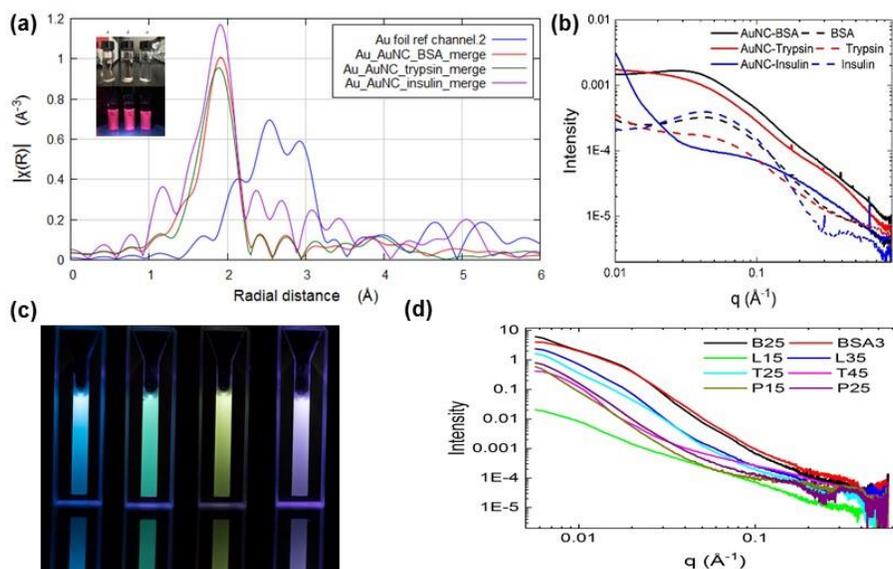


Fig. 3 X-ray studies of PNC constructs including a) PDF and b) EXAFS measurements; c) image of crystallized PNC emission and d) associated SAXS spectra

6. Computational Results

In this task, our goal was to develop 3-D atomic models of proteins that produce the observed X-ray spectra. The fullRMC package (<http://bachiraoun.github.io/fullrmc/>) was modified to accept protein input. Specifically, the topology and parameter files from the CHARMM (Harvard) molecular modeling programs were parsed into fullRMC potential terms. Finally, fullRMC's author graciously added support for the dihedral term necessary to model the protein backbone.

The protein complexes for GFP and BSA were first generated by the programs VMD (University of Illinois at Urbana–Champaign) and Chimera (University of California, San Francisco) and then input to fullRMC for initial X-ray spectra computation and optimization. Comparison of initial computed spectra to experimental spectra was promising (Fig. 4a). However, optimization has proven to be too cumbersome at this time. The main issue is that the protein complexes are too large to accurately optimize with fullRMC. This observation led to the experimental work on smaller systems including insulin-NC, which yielded a significantly closer model match to the X-ray data (Fig. 4b). We are currently in the process of analyzing this data and expect this to be the subject of future collaborative efforts.

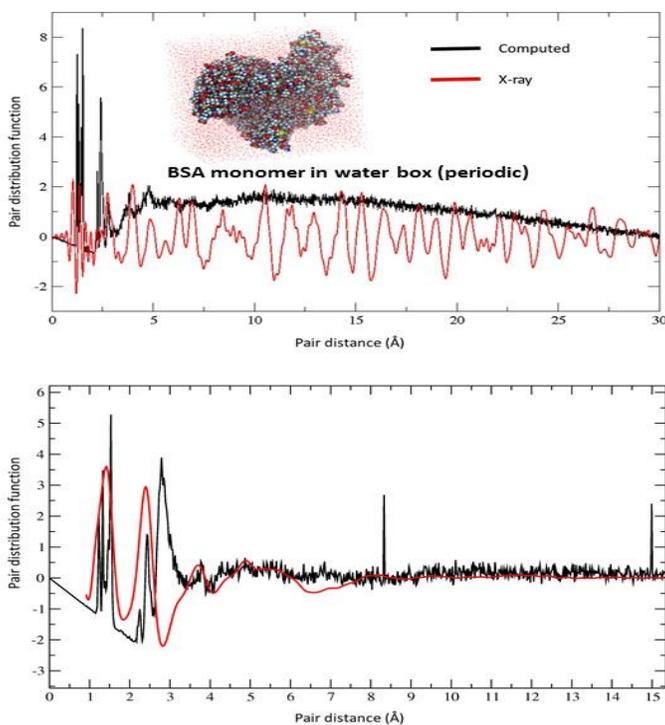


Fig. 4 fullRMC modeling compared to experimental PDF data for a) BSA-NCs and b) insulin-NCs

7. Conclusion

The formation of AuNCs within native trypsin resulted in the loss of secondary structure and enzyme activity; enzyme activity could not be regained through refolding attempts. Native trypsin was observed to yield red-emitting AuNCs; however, AuNC formation in the mutant forms of the protein was unsuccessful. Taken together, these data suggest that each mutated residue is necessary in the nucleation and growth of AuNCs in trypsin. Further insight on the design of novel

PNCs through ESI-MS and X-ray studies are poised to provide key detail on gold-coordinating ligands and molecular-level interactions. Finally, in the development of a 3-D atomic model to predict gold-protein interactions from experimentally obtained X-ray data, the transition to smaller protein systems is poised to assist in the analysis and prediction of gold nanocluster formation within biomolecular hosts.

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List of Symbols, Abbreviations, and Acronyms

3-D	3-dimensional
aa	amino acid
ARL	US Army Research Laboratory
AuNC	gold nanocluster
BAEE	N _α -benzoyl-L-arginine ethyl ester hydrochloride
BSA	bovine serum albumin
CD	circular dichroism
CHARMM	Chemistry at Harvard Macromolecular Mechanics
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization-mass spectrometry
FPLC	fast protein liquid chromatography
fullRMC	Fundamental Library Language for Reverse Monte Carlo
GFP	green fluorescent protein
ICP-MS	inductively coupled plasma mass spectrometry
LC	liquid chromatography
MOF	metal organic framework
MW	molecular weight
NC	nanocluster
PNC	metal nanocluster
SAXS	small angle X-ray scattering
VMD	Visual Molecular Dynamics
XAS	X-ray absorption studies

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