Physical Chemistry Chemical Physics

ARTICLE

Critical Role of Tyrosine-20 in Formation of Gold Nanoclusters within Lysozyme: a Molecular Dynamics Study

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Lysozyme is one of the most commonly used proteins for encapsulating gold nanoclusters, yielding Ly-AuNC complexes. While possible applications of Ly-AuNCs in environmental, biological and trace metal sensing in solution have been demonstrated, there is currently a poor understanding of the physical characteristics of the Ly-AuNC complex. In this study we have employed fully atomistic Molecular Dynamics simulations to gain an understanding of the formation of Au clusters within the protein. It was found that in order to form AuNCs in the simulations, an approach of targeted insertion of Au atoms at a critical surface residue was needed. Tyrosine is known to be crucial for the reduction of Au salts experimentally, and our simulations showed that Tyr20 is the key residue for the formation of an AuNC beneath the protein surface in the in the α-helical domain. It is hoped these observations will aid future improvements and modification of Ly-AuNCs via alterations of the alpha-helix domain or Tyr20.

Introduction

The development of novel fluorescent probes with unique emission characteristics sensitive to their surroundings is critical in the development of new spectroscopy and microscopy techniques for observing biological nanoscale processes[1–4]. Of major interest currently, due to their unique fluorescent properties, are protein encapsulated gold nanoclusters (AuNCs)[5]. Protein encapsulated AuNCs predominantly fluorescence in the red regime of the spectrum making them desirable for imaging organic tissue[6], however the emission wavelength has been shown to be tunable[7]. They also display extremely long fluorescence lifetimes of 1-2 μs[8], are non-photobleaching[9] and can be excited by a wide range of wavelengths[10], making them ideal candidates for biological imaging. However, in order for protein encapsulated AuNCs to see wide spread use as a fluorescent probe in biological imaging, it is important to understand how AuNCs are synthesized and how that may affect the encapsulating protein's native function, yielding better understanding of the physical basis for any changes in fluorescence emission observed. Elucidation of the growth mechanism and properties of protein encapsulated AuNCs has seen a rapid advancement in recent years[11–15]. Of most interest to researchers has been AuNCs encapsulated with either serum albumin or lysozyme type proteins due to their well-studied nature and critical biological function[16–21]. Previously, the location of AuNCs when encapsulated within Human Serum Albumin (HSA) was uncovered through the use of Molecular Dynamics (MD) simulations and fluorescence based spectroscopy techniques[10,22]. MD calculations of singular gold atoms interacting with a single serum albumin protein indicated that gold preferably nucleated in a number of hydrophobic sites situated near cysteine residues. We were able to narrow the field of possible AuNC sites by using Forster Resonance Energy Transfer (FRET) between the single tryptophan in the protein and the Au clusters; only one candidate satisfied the separation requirements. This information was critical for understanding changes to the fluorescence characteristics of HSA-AuNCs under different environmental conditions and during drug binding interactions[23]. Similarly, if the location of AuNCs within lysozyme proteins could be discovered, then changes to the fluorescence characteristics of Ly-AuNCs under different conditions would be better understood, making AuNC fluorescence a powerful tool in studying lysozyme-molecule interactions.

Lysozyme is a smaller, more compact protein in comparison to serum albumin; consisting of 129 amino acid residues with a mass of 14,307 Da[24]. The lysozyme protein is typically described as having two distinct domains and is rigid due to 4 disulphide bridges cross linking the two domains. Lysozyme is used by the immune system as a natural anti-bacterial defence mechanism that hydrolyses and breaks glycosidic bonds within the cell walls of bacteria. The large active cleft between the two domains of the protein bind to peptidoglycan molecules, destroying them, causing the bacteria cell to burst due to osmotic pressure[22,25].

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

An accepted author manuscript of the following research article: Russell, B. A., Kubiak-Ossowska, K., Chen, Y., & Mulheran, P. A. (2019). Critical role of tyrosine-20 in formation of gold nanoclusters within lysozyme: a molecular dynamics study. Physical Chemistry Chemical Physics. https://doi. org/10.1039/C8CP06374E

Hen Egg White Lysozyme (HEWL) is the most commonly used protein for AuNC encapsulation due to its low cost and abundance. In terms of critical constituents for the synthesis of AuNCs, the HEWL protein contains 8 cysteine residues which contain the necessary sulphur atoms to stabilise the AuNCs[26]. It also contains 3 tyrosine residues, Tyr 20, 23 and 53, (one of which is fully exposed on the protein surface, one which is partially exposed and another which is buried under the protein surface) which act as a reducing agent of the gold salt used during synthesis[27]. Since tyrosine is critical in the reduction of gold salt, and subsequently the formation of AuNCs, the residues that play the reduction role most likely have to be accessible on the protein surface for the gold salt.

MD simulations are critical tools allowing for the interaction between atoms and molecules to be elucidated. In this study, we have taken two approaches to creating gold nanoclusters within HEWL in simulation. Firstly, multiple Au(0) atoms (neutral gold atoms) were randomly introduced to the simulation of a HEWL protein in solution, and the formation of clusters monitored. Then, single Au(0) atoms were introduced near the two exposed tyrosine residues since Au(0) are most likely present at these positions after the reduction of gold salt during experimental synthesis, and their subsequent interactions with the protein were studied. Indeed, we find that the latter approach yields a convincing model for the AuNC formation beneath the lysozyme surface.

It is hoped that the understanding of the AuNC growth pathway within HEWL will allow for further intelligent manipulation of Ly-AuNCs to utilize the AuNC fluorescence, and that changes of AuNC fluorescence in lysozyme proteins under various conditions can be better understood due to the appreciation of the AuNC location.

Methods

Theoretical Modelling

NAMD[28] software was used in conjunction with CHARMM27[29] to carry out all simulations. The model for the HEWL protein (1IEE) was downloaded from the Protein Data Bank and used in all simulations[30]. The conditions found during previous experimental synthesis[19,24] were emulated by solvating the protein within a water box with the dimensions: 85 Å x 77 Å x 82 Å. The system was then neutralized by adding NaCl (ionic strength of 0.05 M), resulting in a total system size of 93,000 atoms.

Two different approaches were used to simulate the growth of AuNCs within the lysozyme protein model. Firstly, 25 atoms were added to the system at random distances larger than 20 Å from the protein surface. The system was then allowed to run for 50 ns without the addition of any further Au atoms. Secondly, individual atoms were selectively inserted at targeted positions, close to Tyr residues 20 and 53, which are near the protein surface. The initial distance between the introduced gold atoms and the protein surface was no larger than 10 Å. A larger initial distance could lead to undesired initial interactions with other residues due to weak van der Waals (vdW) interactions between the atoms of interest (the strength of the vdW interaction falls rapidly to zero with increasing distance). The simulations was then left to run for 100 ns before another single Au atom was inserted at the same position relative to the protein surface.

For all electrostatic interactions the Particle Mesh Ewald (PME) method was used[31]. Gold potential parameters developed by Heinz et. al.[32] were used throughout (Lennard-Jones parameters ε = -5.29; $1/2_{min}$ = 1.4755). It is worth to emphasize that MD simulations with Au(0) provide information regarding only van der Waals interactions; because we used a standard MD approach no bonds are created or broken during the simulation.

All modelling and calculations including radial distribution functions was carried out using NAMD 2.8 software on the ARCHIE-WeSt supercomputer. VMD[33] was used to track the trajectories of all Au atoms and subsequent nucleation within the lysozyme protein using the visualisation nodes of ARCHIE-WeSt.

Results and Discussion

1.1 Random insertion

Our initial attempts to simulate the growth of lysozyme encapsulated gold nanoclusters was based on previous successful attempts at simulating the growth of Bovine Serum Albumin (BSA) encapsulated gold nanoclusters[10]. Initial uptake was found to be much slower in comparison with the BSA experiment, taking 20 ns for all interacting gold atoms to be adsorbed. All initial interactions were seen to take place at arginine (Arg) and lysine (Lys) surface residues with small, randomly distributed clusters of 1 – 6 atoms in size forming as shown in Figure 1. The simulations were carried out 5 times with randomly assigned initial velocities. The lack of any interaction with the three tyrosine residues of the lysozyme protein (with one exception of a single Au which interacted with Tyr20, resulting in the atom stabilizing within the protein) suggests that the random approach to inserting Au atoms into the simulation poorly mirrors the experimental synthesis of AuNCs. It is well understood that tyrosine acts as an initial reducing agent for gold salts in solution with the protein, facilitating the start of the synthesis chemically[27].

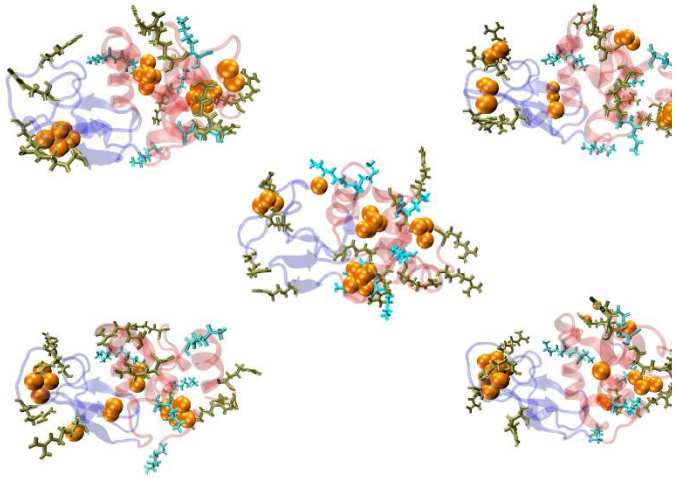
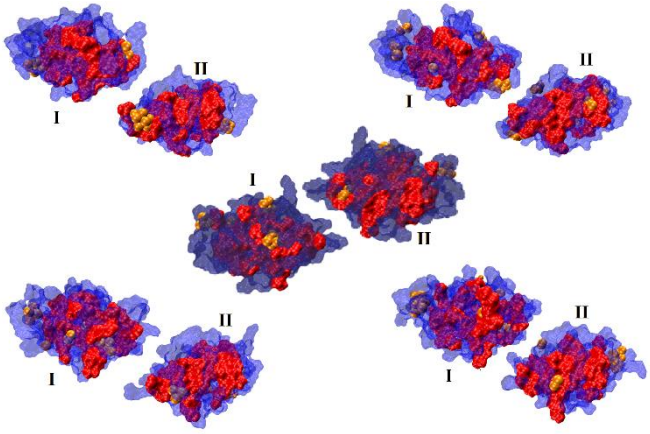


Figure 1: Final positions of Au atoms indicated in orange after 50 ns simulation after randomly inserting around lysozyme protein. Brown residues indicate Arginine positions; light blue residues indicate Lysine positions. Each image represents a unique simulation.

On the other hand it is quite reasonable that Au atoms were trapped by Arg and Lys, because they are (i) numerous and well exposed to solution at the protein surface, and (ii) known as readily able to interact with a gold surface and responsible for protein / peptide adsorption on gold[32,34–36] due to their long amphiphilic side chains. Although Tyr is also known as residue that interacts well with gold[32,34], it is not so well represented on the protein surface, and therefore all the available gold atoms, due to strong interactions with Arg and Lys, could be unavailable for Tyr interactions. Over the remaining 30 ns of the simulation the gold clusters were seen to settle on the protein surface in hydrophobic pockets consisting of leucine (Leu), methione (Met), alanine (Ala), phenylalanine (Phe) and isoleucine (Ile) residues randomly distributed around the protein surface structure as shown in Figure 2. This result contradicts with the experimental results which confirm that gold nanoclusters stabilise themselves via sulphur-gold bonds forming between the cysteine side chain containing a sulphur atom and the Au cluster[37]. The lack of cysteine within proximity with the final nucleation sites of the Au atoms again suggests that randomly inserting Au atoms into simulation is not an appropriate approach to accurately mimic the growth of lysozyme encapsulated AuNCs.

Interestingly, the gold atoms were not seen to penetrate the protein surface after the initial interaction, as previously observed for BSA-AuNC simulations[10]. The longer uptake time and lack of penetration may arise due to lysozyme's more rigid, less flexible structure in comparison to the larger BSA protein. Au atoms have been shown to be highly sensitive to solvent exposure[38], thus, Ly-AuNCs would likely have very weak fluorescence in comparison to other protein encapsulated AuNCs if they were located on the lysozyme surface, which is not the case[27].

Figure 2: Hydrophobicity map (I – front of molecule, II – rear of molecule) of the surface residues. Dark blue indicates Hydrophillic residues; red indicates hydrophobic residues



(Kyte-Doolittle Hydropathy scale[29]). Au atoms, indicated in orange, can be seen residing in surface hydrophobic pockets. Each set of images represents a unique simulation.

1.2 Targeted insertion

Due to the inadequacies arising from the attempt using the previously successful random inserted Au method, a more Lysozyme contains three tyrosine residues in total in positions 20, 23 & 53, with only Tyr20 and Tyr53 on the surface of the protein structure. In order to simulate the initial interactions between protein and Au atoms more accurately the Au atoms were introduced individually within less than 10 Å of the surface tyrosine residues 20 & 53. Examples of the initial Au atom targeted insertion into the simulation are shown in Figure 3.

The initial uptake of Au by protein was extremely fast, taking place within 100 ps due to the small separation distance and therefore strong vdW interactions. The introduction of Au atoms at Tyr20 and Tyr53 yielded different outcomes. Au atoms inserted at Tyr53 resulted in the Au atoms migrating randomly around the protein surface with no formation of an Au cluster, whereas insertion at Tyr20 (in the α-helical domain) gave the nucleation of a clear Au cluster made up of 14 atoms added at this site, shown in Figure 4.

Moreover, inserting Au atoms at Tyr20 lead to penetration of the protein surface and Au cluster nucleation within a hydrophobic pocket containing two cysteine residues, whereas inserting at Tyr53 resulted in the Au atoms randomly migrating across the protein surface until they settled within hydrophobic crevices on the protein surface. The coverage of the nucleated Au cluster from targeting Tyr20 can clearly be seen in Figure 5 to be beneath the hydrophilic surface of the protein compared to Au atoms inserted at Tyr53. Therefore, the Au cluster located within the α-helical domain of lysozyme is the most likely location based on these simulations. Here it is protected from the solvent and likely to have the strong fluorescence observed experimentally.

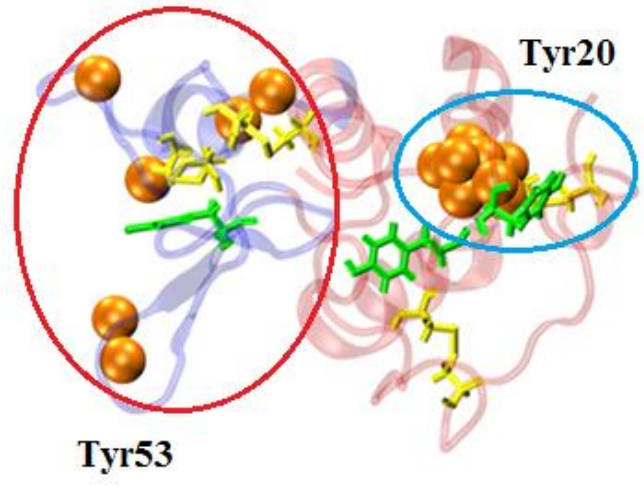


Figure 4: Final positions of Au atoms indicated in orange after targeted insertion at Tyr 20 and 53 (Atoms within red circle indicate insertions at Tyr53, atoms within blue circle indicate insertion at Tyr20). Green residues indicate Tyrosine; yellow residues indicate Cysteine.

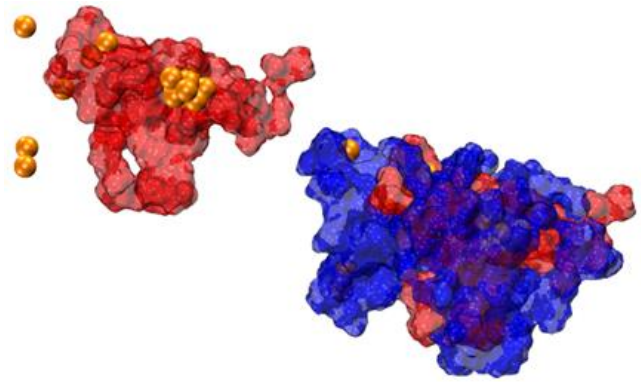


Figure 5: Red indicates protein hydrophobic structure, dark blue indicates protein hydrophilic structure, orange indicates gold atoms (See Figure 4 for insertion target of Au atoms). The left-hand image shows the inner hydrophobic structure of the protein (beneath the residues of its surface layer) while the right-hand image shows the full structure. Gold atoms inserted at Tyr20 can be clearly seen to nucleate under the protein surface, whereas gold atoms inserted at Tyr53 are scattered on the protein surface.

To quantify the degree to which atoms inserted at Tyr20 are encapsulated compared to atoms inserted at Tyr53, radial distribution functions were calculated between the Au atoms and the water molecules of the solvent, shown in Figure 6. It can be seen that the Au atoms on average are move exposed to water when introduced to Tyr 53 in comparison to Au atoms introduced to Tyr20. This indicates that the Au atoms introduced at Tyr20 are encapsulated and stabilised by the protein since it is less likely to find water molecules at smaller distances, lending further weight to the theory that Tyr20 is the critical interaction site of Lysozyme where the Au salt used experimentally during synthesis is reduced before forming AuNCs.

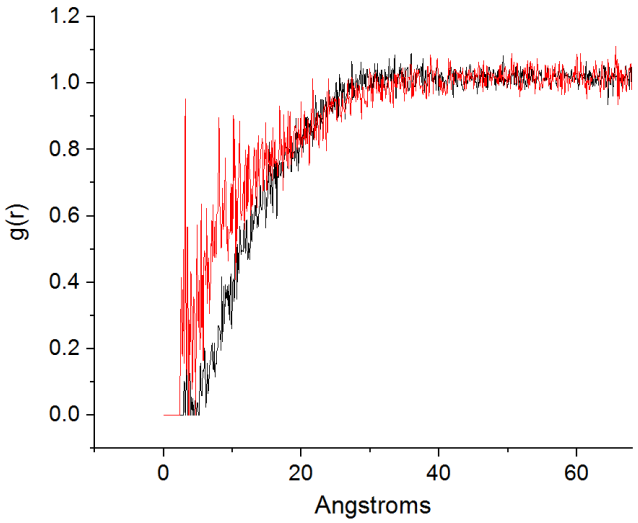


Figure 6: Radial pair distribution function plots of Au atoms to water molecules in simulation. Red indicates Au atoms introduced at Tyr53; black indicates Au atoms introduced at Tyr20.

Conclusions

Molecular Dynamics simulations of the nucleation of Au clusters within proteins have previously been shown to aid in the understanding and development of protein encapsulated gold nanoclusters[10,22,23]. In this study we present observations which elude to the major nucleation site of Ly-AuNCs to be within the α-helical domain of lysozyme, with Tyr20 playing a major role in the formation of the AuNC. Tyr20 has been shown to have a major effect on the nucleation of an AuNC when compared to direct Au atom insertion at Tyr53 or random insertion to the protein surface. We believe this information will allow for the development of new strategies for improving and using Ly-AuNCs as fluorescent probes, centred around modifying either the lysozyme α-helical domain structure or altering the synthesis of Ly-AuNCs through the Tyr20 residue.

Acknowledgements

Results were obtained and analysed using the ARCHIE-WeSt High Performance Computer (www.archie-west.ac.uk) based at the University of Strathclyde

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