Denatured crude protein extracts from the sea anemone *Entacmaea quadricolor* as capping agents for silver nanoclusters

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**Abstract**

Novel nanomaterials derived from numerous biological samples including extracts from plants, animals, and even bacteria have gained prominence due to their unique properties, more environmental friendly preparation, and interesting bioactivities. Among these, protein-capped noble metal, e.g., Ag, nanoclusters (NC) have gained considerable attention for their many potential applications, and significant efforts are being directed at using a wide variety of protein sources as capping agents as well as. In this work, crude protein extracts from the sea anemone *Entacmaea quadricolor* (*E. quadricolor*) subjected to either heat or β-mercaptoethanol denaturation were prepared to investigate the effects of these processes in the formation of AgNC and on its preliminary bioactivity, measured using a hemolysis assay against human erythrocytes. Fourier-transform infrared spectrometry (FTIR) analysis showed comparable spectra for the two denatured crude protein-capped AgNC, as well with as the undenatured protein-capped AgNC, like previous reports indicating successful capping of the NC. Moreover, the spectra suggest no significant alterations if protein secondary structure upon NC formation. The crude protein extracts were also found to be very weakly hemolytic at the concentration range tested (0.5 – 1 mg protein/mL) and denaturing the protein prior to NC formation did not significantly alter its hemolytic activity as well. These results imply that *E. quadricolor* crude protein denaturation prior to use with the reduction of Ag⁺ has no deleterious effects in functioning as capping agents for the prepared AgNC.
Introduction

Over the past several decades, the field of nanotechnology has emerged as one of the crucial scientific research areas that has found invaluable use in addressing the most pressing concerns of today’s society such as energy applications, environmental bioremediation, and health and medicine, in large part brought about by the nanomaterials’ unique properties arising from their extremely small size\(^1-3\). Of particular interest among the many nanotechnology platforms are metal NC generally comprised of several to a few hundred metal atoms and capped with or conjugated to a wide range to different proteins that are thought to aid in the stabilization of the material and improving its biocompatibility. Protein-capped metal NC have been demonstrated for numerous promising applications such as the ability to fungi-synthesized iron- and cadmium-based metal nanoparticles to inhibit Tau aggregation as well as facilitate the disassembly of paired helical filaments formed as one of its consequence, showing for the first time a potential benefit of these systems against Alzheimer’s disease\(^4\). Biosensor development has also seen significant applications for protein-capped metal nanoclusters, for example in an enzyme-free electrochemical biosensor for organophosphates fabricated from a composite material of bovine serum albumin-capped Cu nanoclusters and single-walled carbon nanotubes, and in a rapid and direct determination of Hg\(^{2+}\) in environmental samples\(^5,6\).

The reported metal NC are usually prepared from noble metals, specifically Au ang Ag, although other metal centers have also been generated, such as Cu, Fe, and even Cd\(^4,7-9\). Similarly, different research has also shown the utility of various proteins as capping agents and though many reports have used bovine serum albumin due to its abundance in the blood plasma and thus high biocompatibility, other proteins and enzymes could also act as efficient scaffolds such as the case with lysozymes, human transferrin, and horseradish peroxidase\(^10-12\). In addition to these single-protein systems, numerous reports have also demonstrated the ability of crude protein extracts from fungi and bacteria, as well as from a sea anemone that we reported earlier, to serve as capping agents for the metal core where the two former platform serve as a “green” synthetic route where the organisms themselves are used to reduce the metal salts \(^7,8,13\). To further explore other probable protein templates for NC preparation, in this study, aqueous crude protein
extracts from the sea anemone *E. quadricolor* was used, as well as to explore the role of protein
denaturation, with either heat or β-mercaptoethanol addition, in the preparation of AgNC. Previously, it
was shown that denaturing the proteins both have deleterious and advantageous effects on the synthesized
NC in terms of stabilization, which is most likely dependent on the nature of the protein samples
themselves. Data from our work showed successful capping of AgNC with the crude *E. quadricolor*
extracts, and that denaturing the protein extracts had to significant effect, at least from the point of view of
NC preparation. Furthermore, preliminary hemolytic assay also showed that the AgNC prepared from both
undenatured and denatured crude proteins had similar low to no activity.

**Methodology**

**Materials**

The sea anemone sample *E. quadricolor* was obtained from a local store in Pasay City, Philippines. The sample was instantly carried to the laboratory in a thermal cooler filled with ice. The phosphate-buffered saline (PBS, 0.1 M phosphate, containing 2.7 mM KCl and 137 mM NaCl, pH 7.4) and bovine serum albumin (BSA) were both acquired from Sigma, while the Bradford reagent was purchased from Bio-Rad. All other reagents were standard reagents and used as received.

**Crude Extract Preparation**

The extraction of crude protein was performed using the method carried out in previous reports. The animal sample was homogenized in a 1:2 (w/v) ratio of sea anemone and distilled water using a blender. The obtained mixture was then centrifuged at 5000 x g for 15 minutes. The supernatant was then collected and passed through a 0.45µm filter to obtain the crude extract which was subsequently lyophilized. Using the freeze-dried sample, the total protein content was then determined using a Bradford assay in a BioTek ELx800 microplate reader, with BSA as protein standard. All freeze-dried samples were stored in a freezer prior to use.
Preparation of protein-capped Ag nanoclusters

The freeze-dried crude extract was divided into three parts and dissolved with enough distilled water to obtain individual solutions with a final concentration of 50 mg/mL. One part was denatured by heating the solution in 100 °C for 5 minutes (referred to as CE-H), a second part was denatured by adding β-mercaptoethanol to a final concentration of 5% (v/v) (referred to as CE-M), while the third part was undenatured (referred to as CE).

From the denatured or undenatured protein samples prepared, 5-mL aliquot portions were introduced into clean 50-mL centrifuge tubes. Then, 5 mL of freshly prepared 10 mM AgNO₃ was added to each tube and the solution was stirred for 3 minutes at room temperature using a magnetic stirrer. Afterwards, 0.5 mL of 1 M NaOH was added to each of the solutions and was continuously stirred for another 30 minutes. To reduce the Ag⁺ and prepare the nanoclusters, freshly prepared 10 mM NaBH₄ was added dropwise until a visible color change occurred (Espiritu and Rebustillo, 2017). Reduction control experiments were also carried out for each of the protein samples following the same protocol described above, except the addition of AgNO₃. The synthesized protein-capped Ag nanoclusters were then lyophilized and stored in a freezer for later use.

FTIR Characterization

Fourier transform infrared spectroscopy (FTIR) was employed for the characterization of the changes in protein secondary structure upon nanocluster formation. The KBr method was utilized using the freeze-dried undenatured, heat-denatured, and β-mercaptoethanol-denatured samples. FTIR spectra were obtained using a Thermo Scientific Nicolet 6700 from 400 cm⁻¹ and 4000 cm⁻¹.

Hemolysis Assay

The two milliliters of fresh human blood were collected and stored in a centrifuge tube with 18 mL of PBS buffer. The resulting suspension was placed in a Jouan Br4 centrifugation chamber and centrifuged twice at 2000 rpm for 5 minutes. The pellet was collected and mixed with 20 mL of the same buffer and
vortexed. Afterwards, a 2 mL aliquot from this suspension was mixed with 18 mL of PBS buffer resulting to a 1% hematocrit suspension, which was used in the hemolysis assay.

The released hemoglobin was quantified from the ruptured erythrocytes through the following method. Using a micropipette, 190 µL of the 1% hematocrit suspension was added to a 0.5 mL Eppendorf tube, followed by the addition of 10 µL of the corresponding protein or protein-capped Ag nanoclusters sample. After vigorously mixing, the suspensions were incubated in a water bath at 37 °C for 1 hour. The samples were then subjected to centrifugation at 2000 rpm for 5 minutes and 50 µL of the resulting supernatant were transferred into a 96-well microplate. Absorbance measurements from triplicate samples were then carried out at 450 nm using a BioTek ELx800 microplate reader. The released hemoglobin in each sample was quantified using the formula: \[ \% \text{ hemolysis} = 100 \times \frac{(A_{\text{protein}} - A_{\text{PBS}})}{(A_{\text{SDS}} - A_{\text{PBS}})} \]. The absorbance reading of PBS corresponded to zero, whereas that of the SDS was interpreted as 100% hemolysis.

**Statistical Analysis**

One-way ANOVA and Tukey multiple comparison test were used to analyze significant differences \((p < 0.05)\) among the samples in their hemolytic potential using Origin Pro 8.

**Results and Discussion**

In this work, protein-capped silver nanoclusters were prepared from the crude protein extracts of the sea anemone *E. quadricolor*, and the effects on NC formation of protein denaturing via heat treatment or disulfide bond reduction with β-mercaptoethanol were explored. Furthermore, the hemolytic activities of the undenatured and denatured crude protein extracts and their corresponding derived AgNC were also investigated.

Figure 1 shows representative images of the lyophilized crude protein extracts and the protein-capped Ag nanoclusters prepared under different conditions. The lyophilized undenatured, heat-denatured, and β-mercaptoethanol-treated crude protein extracts were yellowish in color, however, in the presence of
the Ag⁺ and the reducing agent NaBH₄, the color changed to deep brown for all the protein samples (Fig. 1, A-C, top and middle images). This change in color, which has been ascribed to synthesized silver nanoparticles exhibiting surface plasmon resonance⁸,¹³, is a very common qualitative indicator of a successful reduction of Ag⁺ ions to metallic silver (Ag⁰) and nanocluster formation, as has been reported in previous studies⁶,⁷,¹⁷–¹⁹. The requirement of the Ag⁺ to Ag⁰ transition for the characteristic color change was also supported by the observation that under reducing conditions but without the addition of AgNO₃, the lyophilized samples (Fig. 1, A-C, bottom images) were comparable to the crude extracts. From a qualitative point of view, it appears that denaturing the crude extracts, either with heat or disulfide bond breaking, prior to Ag⁺ reduction does not significantly affect nanocluster formation.

**Figure 1.** Images of lyophilized undenatured crude protein extract (A, top), and the heat-denatured (B, top,) and β-mercaptoethanol-treated (C, top) crude protein extracts of *E. quadricolor*. The protein-capped AgNC
are shown in the middle set of images derived from the undenatured crude protein (A), and the heat-denatured (B), and the β-mercaptoethanol-treated (C) crude protein extracts. Samples in the presence of NaBH₄ but without Ag⁺ were also prepared for the three corresponding crude proteins and shown in the respective bottom images. CE, undenatured crude protein extract; CE-H, heat-denatured crude protein extract; CE-M, β-mercaptoethanol-treated crude protein extract; AgNC, silver nanocluster.

To have an overview of how proteins are adsorbed into the silver nanocluster core, as well as to get insights into which functional groups are involved in the capping process and stabilization of the synthesized nanoclusters, FTIR measurements were carried out. Figure 3 shows the overlay of the relevant FTIR spectra for the undenatured, heat-denatured, and β-mercaptoethanol-treated crude extracts and protein-capped silver nanoclusters, and a quick inspection shows the very close similarity of all spectra and the presence of the major signals. The broad intense signal with that peak at around ~3400 cm⁻¹ is attributable to the N–H and O–H stretching vibrations from the amine and alcohol functionalities in the crude extract components, respectively. The signal appearing at ~2900 cm⁻¹ is due to the symmetric and asymmetric stretching vibrations from sp³-hybridized C–H groups. The very intense and sharp band observed at ~ 1640 cm⁻¹ is generally attributed to the amide I vibration which is due mainly to the C=O stretching. This signal has been used previously to understand the secondary structure present in protein samples, but in the context of protein-capped nanoclusters, a shift in this signal has been interpreted as successful capping on the nanoclusters. In the prepared silver nanoclusters from the undenatured and denatured crude protein extracts, the amide I band has appeared between 1646 – 1652 cm⁻¹ slightly shifting from 1642 – 1644 cm⁻¹. Finally, the small peak at around ~ 1540 cm⁻¹ may be assigned to the amide II band due to a combination of out of phase N–H in-plane bending and C–N stretching vibration. These FTIR data provide basis of the successful protein capping and stabilization of the silver nanoclusters as has been reported previously, which could possibly be mediated by free amine and cysteine residues or electrostatic interaction with protein carboxylate groups. Moreover, the FTIR data obtained also suggest that no significant changes in protein secondary structure occurred upon nanocluster formation and protein
capping, similar to previous reports\textsuperscript{19,22}. It is worth mentioning that it was earlier suggested that for protein silver nanoclusters, significant changes in protein secondary structure was observed once the nanocluster to protein molar ratio reaches at least $10^{23}$; however, given that the study used crude protein extracts, making an accurate determination of this mole ratio is also challenging. Denaturing the proteins seem to also have no effect on the synthesis of AgNC, in fact in a previous study, a denatured bovine serum albumin that frees up 34 cysteine residues was found to more effectively stabilize silver nanoclusters than its native counterpart\textsuperscript{15}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Overlaid FTIR spectra of the corresponding \textit{E. quadricolor} crude protein extract, protein-capped AgNC, and crude protein extract without Ag\textsuperscript{+} for the corresponding undenatured (A), heat-denatured (B) and \textbeta-mercaptoethanol-treated (C) crude protein extracts. CE, undenatured crude protein extract; CE-H,}
\end{figure}
heat-denatured crude protein extract; CE-M, β-mercaptoethanol-treated crude protein extract; AgNC, silver nanocluster.

The potential use of noble metal nanoclusters, e.g., from silver, has been explored extensively over the years specifically for various biomedical applications such as cellular imaging tools, antimicrobial agents, and novel biosensors and drug delivery platforms arising from their good biocompatibility. To explore the physiological properties of the prepared protein capped AgNC in this work, their hemolytic activity against human erythrocytes were investigated, as summarized in Figure 3. Sea anemones are known to produce cytolytic and hemolytic toxins such as the actinoporins, but the undenatured crude extracts prepared in this study did not show any considerable hemolytic action at the concentration tested, with the highest activity at around 10% hemolysis (Fig. 3A). A comparable hemolytic activity was also observed with the protein-capped AgNC, as well as with the heat-denatured and β-mercaptoethanol-treated crude protein extracts and their corresponding AgNC (Fig. 3, B and C), suggesting that denaturing the crude protein extracts also had little to no effect at least from the hemolysis perspective. There have been rather few reports on toxins from E. quadricolor, but most of the reported cytotoxicity and bioactivity were obtained for venom directly extracted by milking the anemone, as opposed to the aqueous extraction for crude extract preparation employed in this work, which may have severely diluted the hemolytic components, if any. Despite being non-hemolytic at the conditions tested, it would still be very interesting to further characterize the bioactivity of the prepared protein-capped silver nanoclusters, for example, as possible antimicrobial agents similar to NC capped with H. crispa crude protein extracts that we reported earlier or its cytotoxicity against different cancer cell lines.
Figure 3. Hemolytic activities against human erythrocytes of the corresponding *E. quadricolor* crude protein extract, protein-capped AgNC, and crude protein extract without Ag⁺ for the corresponding undenatured (A), heat-denatured (B) and β-mercaptoethanol-treated (C) crude protein extracts. Error bars indicate standard deviation of three independent trials. CE, undenatured crude protein extract; CE-H, heat-denatured crude protein extract; CE-M, β-mercaptoethanol-treated crude protein extract; AgNC, silver nanocluster.

Conclusions

In this work, the effect of denaturation of the crude protein extracts from the sea anemone *E. quadricolor* on the preparation and hemolytic activity of AgNC were investigated. The crude extracts were either heat-denatured or treated with β-mercaptoethanol prior to reduction of Ag⁺ and was compared with
the undenatured protein extracts. Results from this work showed that denaturing the protein extracts did not have a significant effect on the capping of AgNC as all protein scaffolds showed similar successful capping based on FTIR data. Furthermore, denaturation also did not affect the hemolytic activity of the prepared NC and were comparable to the undenatured crude protein extracts. Further investigations will be needed to characterize the prepared materials, for example, its size, morphology, and stability using a combination of TEM, DLS, and zeta-potential measurements. Moreover, other bioactivity assays are also strongly recommended.

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References


