Asian Journal of Chemistry; Vol. 34, No. 3 (2022), 740-744

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23524

Denatured Crude Protein Extracts from the Sea Anemone *Entacmaea quadricolor* as Capping Agents for Silver Nanoclusters

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Received: 8 September 2021; *Accepted*: 25 January 2022; *Published online*: 14 February 2022; AJC-20712

Silver nanoclusters (AgNC) have gained considerable attention for their potential applications. In present work, crude protein extracts from the sea anemone *Entacmaea 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 of silver nanoclusters showed no significant alterations in protein secondary structure upon nanocluster formation for all samples. All samples, including the crude protein extracts, were found to be weakly hemolytic at the concentration range tested (0.5-1.0 mg protein/mL). 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.

Keywords: Hemolysis, Protein, Nanoclusters, Sea anemone, *Entacmaea quadricolor*, Silver nanoclusters.

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 nanocluster 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 nanoclusters 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 metal nanoclusters are usually prepared from noble metals, specifically Au and 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 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 nanocluster preparation, in this study, the aqueous crude protein extracts from the sea anemone *Entacmaea 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. It was shown that denaturing of the proteins both have deleterious and advantageous effects on the synthesized nano-cluster in terms of stabilization, which is most likely dependent on the nature of the protein samples themselves [14,15]. In this work, the effect of denaturation of crude protein extracts from the sea anemone *E. quadricolor* on the preparation and hemolytic activity of silver nanoclusters (AgNC) were investigated. Furthermore, preliminary hemolytic assay also showed that the AgNC prepared from both undenatured and denatured crude proteins had similar low to no activity.

EXPERIMENTAL

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 acquired from Sigma-Aldrich (USA), 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 [7,16]. 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 × g for 15 min. The supernatant was then collected and passed through a 0.45 μm 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 silver 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 immersing the samples in a hot water bath at 100 ºC for 5 min (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 $AgNO_3$ was added to each tube and the solution was stirred for 3 min 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 min. To reduce the Ag^+ and prepare the nanoclusters, freshly prepared 10 mM $NaBH_4$ was added dropwise until a visible color change occurred [7]. Reduction control experiments were also carried out for each of the protein samples following the same protocol described above, except the addition of $AgNO_3$. The synthesized protein-capped silver nanoclusters were then lyophilized and stored in a freezer for later use.

The 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 in the range of 4000-400 cm^{-1}.

Hemolysis assay: Fresh human blood (2 mL) 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 min. 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 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 silver nanoclusters sample. After vigorously mixing, the suspensions were incubated in a water bath at 37 ºC for 1 h. The samples were then subjected to centrifugation at 2000 rpm for 5 min 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:

$$\text{Hemolysis (\%)} = \frac{A_{protein} - A_{PBS}}{A_{SDS} - A_{PBS}} \times 100$$

A_{PBS} and A_{SDS} corresponded to the absorbance of the negative and positive control, respectively.

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 (AgNC) were prepared from the crude protein extracts of the sea anemone *E. quadricolor* and the effects on nanoclusters 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.

Fig. 1 shows representative the images of the lyophilized crude protein extracts and the protein-capped silver nanoclusters prepared under different conditions. The lyophilized undenatured, heat-denatured and β-mercaptoethanol treated crude protein extracts were yellowish in colour, however, in the presence of Ag^+ and the reducing agent $NaBH_4$, the colour changed to deep brown for all the protein samples (Fig. 1a-c, top and middle images). This change in colour, which has been ascribed to synthesized silver nanoparticles exhibiting

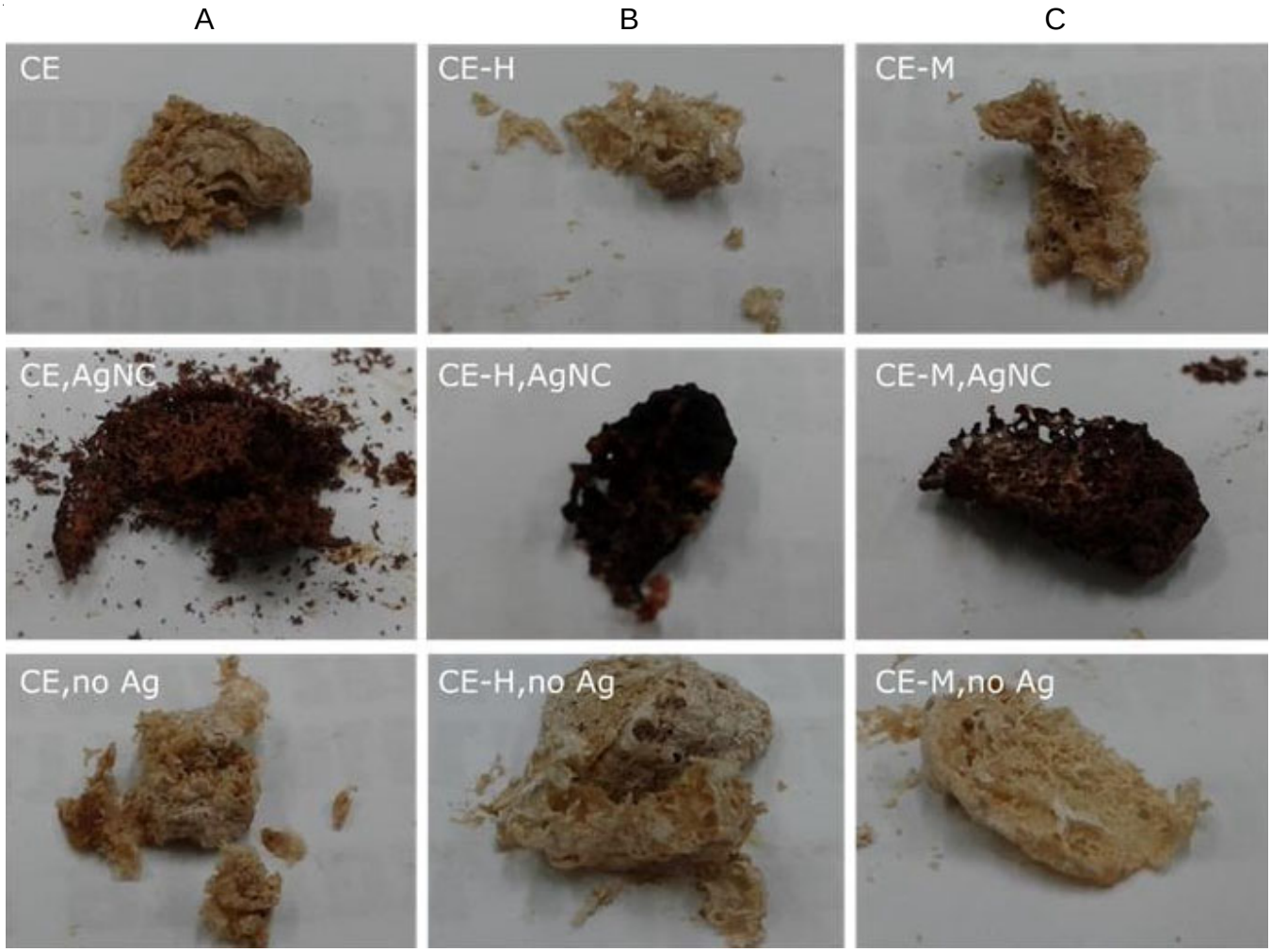

Fig. 1. Images of lyophilized undenatured crude protein extract (A, top), and the heat-denatured (B, top,) and β-mercaptoethanol-treated (C, top) crude protein extracts of *E. quadricolor*. The protein-capped AgNC are shown in the middle set of images derived from the undenatured crude protein (A), and the heat-denatured (B), and the β-mercaptoethanol-treated (C) crude protein extracts. Samples in the presence of $NaBH_4$ 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

surface plasmon resonance [8,13], is a very common qualitative indicator of a successful reduction of Ag^+ ions to metallic silver (Ag^0) and nanocluster formation, as has been reported in previous studies [6,7,17-19]. The requirement of Ag^+ to Ag^0 transition for the characteristic colour change was also supported by the observation that under reducing conditions but without the addition of $AgNO_3$, the lyophilized samples (Fig. 1a-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.

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. Fig. 2 shows the overlay of the relevant FTIR spectra for 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 sp^3-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 [20], but in the context of protein-capped nanoclusters, a shift in this signal has been interpreted as successful capping on the nanoclusters [6,7,9,17]. In the prepared silver nanoclusters from the undenatured and denatured crude protein extracts, the amide I band has appeared between 1652-1646 cm^{-1} slightly shifting to lower region of 1644-1642 cm^{-1}. Finally, the small peak at around ~ 1540 cm^{-1} may be

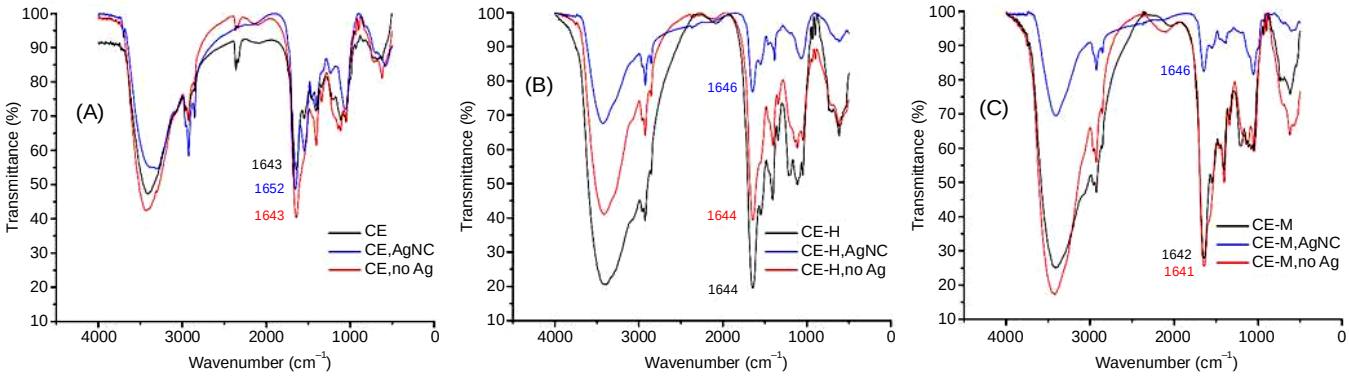

Fig. 2. Overlaid FTIR spectra of the corresponding *E. quadricolor* crude protein extract, protein-capped AgNC, and crude protein extract without Ag^+ for the corresponding undenatured (A), heat-denatured (B) and β-mercaptoethanol-treated (C) crude protein extracts. CE, undenatured crude protein extract; CE-H, heat-denatured crude protein extract; CE-M, β-mercaptoethanol-treated crude protein extract; AgNC, silver nanocluster

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 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 [13,18,21]. 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 [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 silver nanoparticle 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 AgNCs than its native counterpart [15].

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, novel biosensors and drug delivery platforms arising from their good biocompatibility [6,15,18]. 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 Fig. 3. Sea anemones are known to produce cytolytic and hemolytic toxins such as the actinoporins [24], 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-mercapto-ethanol treated crude protein extracts and their corresponding AgNC (Fig. 3b-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 bio-activity were obtained for venom directly extracted by milking the anemone [25,26], 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 nanoclusters capped

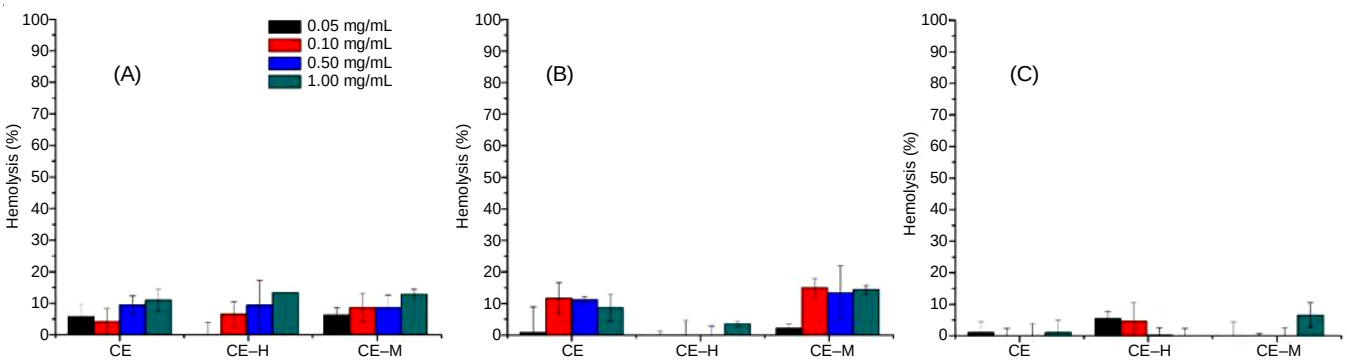

Fig. 3. Hemolytic activities against human erythrocytes of the corresponding *E. quadricolor* crude protein extract, protein-capped AgNC, and crude protein extract without Ag^+ for the corresponding undenatured (A), heat-denatured (B) and β-mercaptoethanol-treated (C) crude protein extracts. Error bars 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

with *H. crispa* crude protein extracts that reported earlier [7] or its cytotoxicity against different cancer cell lines.

Conclusion

In this work, the effect of denaturation of the crude protein extracts from the sea anemone *Entacmaea quadricolor* on the preparation and hemolytic activity of silver nanocrystals 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 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 nanocluster and were comparable to the undenatured crude protein extracts.

ACKNOWLEDGEMENTS

The authors thank the Molecular Science Unit Laboratory of De La Salle University for providing the research equipment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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