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Aggregation of Recombinant Human Growth Hormone in the Presence of HAuCl4

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ABSTRACT

The salt of HAuCl4 is commonly used in nanoparticle synthesis. Gold nanoclusters have strong fluorescence and biocompatibility and are widely used in molecular biology. Numerous proteins were reported as templates for nanocluster synthesis. More studies need to determine the properties of the appropriate proteins to this end. It is predictable in the nanoclusters' harsh conditions (alkaline pHs and high temperatures) the protein fold-state changes and forms aggregates. This study prepared the Au-NCs synthesis conditions to propose rhGH as a protein template, which had not been reported earlier. It was compared with the protein in the same condition without HAuCl4. The techniques used were UV-visible absorbance, fluorescence spectrophotometry, FTIR, and DLS. Further investigation of the samples was done in the presence of different ions. Even though the measurements did not support the formation of nanoparticles, significant differences were observed in the aggregates of rhGH formed in the samples. The data showed that in contrast to the aggregates formed in the absence of HAuCl4, aggregates formed in the presence of HAuCl4 have a uniform population, various UV/vis spectra, and different fluorescence behavior in response to the addition of ZnSO4. These findings display a small path in the big world of forming regular aggregates.

Keywords: Recombinant human growth hormone, Gold nanoclusters, Protein template, Aggregation, Bivalent ions.

INTRODUCTION

Au Nanoclusters (NCs) are tiny metal nanoparticles (<2 nm) that possess capping agents within their structure [1]. The capping agents are stabilizers for the metal cluster cores, with a wide range of options available, from thiolate ligands to protein templates [2]. Among NCs with different capping agents, Au-NCs coated with proteins are particularly popular, especially in the last decades. Although the precise mechanism of the synthesis remains

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unclear, it is hypothesized that the bond between gold and the thiol group of protein cysteine residues (-S-Au(I)-S-Au(I)-S-) and non-covalent interactions of gold nanoclusters with other amino acid residues of proteins contribute to the stability of the nanocluster structure. Additionally, protein amino groups at pH near 7, as well as lysine, tyrosine, and tryptophan residues at alkaline pH, facilitate the reduction of Au3+ [3, 4]. Methionine usually oxidized to methionine sulfoxide in biochemical processes, can have a role in gold nanocluster synthesis as the biocompatible reducing and capping agent [5]. Histidine residues can also be used as reducing and capping agents, containing an imidazole group, which can coordinate with metal ions, including gold, facilitating the nucleation process necessary for cluster formation [6]. It has a pKa close to physiological pH, making it sensitive to changes in pH. This property can influence the binding and release of gold ions during the synthesis and stabilization processes. The presence of histidine can also introduce functional groups on the surface of gold nanoclusters, like sites to ion attachment, enhancing their reactivity and potential applications in biomedicine and catalysis. Tyr with its phenolic group, produced good reductivity of gold ions under alkaline conditions so that its reduction potential is pH-dependent. Other amino acids like the hydrophobic phenylalanine and alanine can act as protecting agents by being located on the surface of AuNCs that throw away water molecules and decrease the fluorescence quenching [7].

Studies have demonstrated that altering the pH, the reaction temperature, and the molar ratio of protein to gold yields nanoclusters with varying sizes and optical properties [8]. Numerous papers reported various proteins from animal to plant proteins as NCs templates such as BSA, ovalbumin, lysozyme, Trypsin, pepsin, HSA, phenylalanine dehydrogenase, insulin, hemoglobin, transferrin, HRP, papain, soybean proteins and so on [9, 10]. The research on these gold nanoclusters is experiencing significant growth [11-15]. More studies using different proteins can be useful in understanding the properties of the appropriate proteins to synthesize protein-coated Au-NCs, the effect of HAuCl4 salt on them, and finally to classify them from this point of view.

Recombinant human growth hormone (rhGH) is a therapeutic protein hormone. It has several properties that make it an appropriate candidate for synthesizing gold nanoclusters; its three-dimensional protein structure serves as a natural scaffold for Au-NC formation and stabilizes the nanoparticles during the synthesis process. RhGH exhibits four alpha-helix structures with a relatively buried Trp residue. It is a globular 25 kDa protein comprising 191 amino acid residues with a 6 His-tag on its N-terminal [16]. RhGH contains four cysteines, one tryptophan, eight tyrosine, nine lysine, and eleven arginine residues, (PDB DOI:10.2210/pdb1HGU/pdb) that as mentioned above may play a role in Au-NCs synthesis and make the protein as an option to this end. Human growth hormone features potential binding sites for metal ions such as Cu2+, Zn2+, Co2+, and Ca2+, among others. These cations can induce structural changes in hGH (human growth hormone), leading to hormone dimerization or oligomerization [17-20] which can be an opportunity to reduce the four chloroauric acids.

In this study, we posited the recombinant hGH in the gold nanoclusters synthesizing conditions, for the first time. The study followed by comparing the protein in the presence and absence of HAuCl4. Fluorescence spectroscopy revealed the emission of products in the visible wavelength at 408 nm. DLS (dynamic light scattering) analysis indicated the size of the products and the TEM imaging showed their spherical morphology. Also, the FTIR (Fourier Transform Infra-Red) spectroscopy provided insight into some properties in the secondary structure of the samples. The investigation of

the sample's behavior in the face of different ions of Cu2+, Co2+, Ni2+, Zn2+, and Mg2+ was also done. Despite the growth hormone properties being suitable for nanocluster synthesis, the data cannot completely support this proposal. However, the data indicated that the rhGH in the presence of gold salt can form aggregated structures whose size, population, and probably folded state differ from the aggregates produced in the absence of the gold salt. We aim to contribute to future studies in the field of nanoclusters useful template proteins.

MATERIALS AND METHODS

Materials

HAuCl4, Sodium hydroxide (NaOH), ions, and other chemicals utilized in this research were of analytical grade and procured from Sigma Aldrich and Merck Companies. It should be noted that the curves were drawn with the average of the data using Prism 8.4.

Expression and purification of rhGH

The pET28a plasmid containing the His6-tagged rhGH gene was utilized for gene expression in the BL21 strain of Escherichia coli in an LB medium supplemented with kanamycin, as previously described [21, 22]. Induction of gene expression was achieved using IPTG (isopropyl-beta-D-thiogalactopyranosides). Purification of rhGH was conducted using Ni-NTA agarose affinity chromatography. Following dialysis in PBS buffer for 24 hours, purification was confirmed via SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide gel Electrophoresis), and the protein concentration was estimated using the Bradford assay.

Preparation of protein solutions in Au-NCs synthesis conditions

All glassware used in the process was thoroughly washed with aqua regia (HNO3/HCl, 1:3) for 10 minutes, followed by rinsing with distilled water. Subsequently, washing was performed for an additional 10 minutes with 96% alcohol, and dishes were rinsed again with distilled water. The synthesis conditions of gold nanoclusters based on protein followed the method reported by Xie et al. in 2009 [23]. Briefly, the purified rhGH solution was poured into a container at 37°C, and while stirring with a magnetic stirrer, gold salt was slowly added drop by drop to the protein solution. The pH was adjusted to approximately 11.5 using a 1 M NaOH stock solution. The reaction was allowed to proceed for 24 hours, and the resulting product was stored in the refrigerator for subsequent analysis. The next sample of the experiment was the glassware containing all reagents without gold salt. Also, we prepared a control as intact rhGH that did not pass the high pH and temperature.

Optimization of the reaction conditions

The conditions of nanocluster synthesis using rhGH consisted of the optimal concentration of HAuCl4, temperature, time, and pH optimized. The concentration of rhGH was fixed at 0.8 mg/ml, while various concentrations of Au salt (0, 0.05, 0.1, and 0.3 mM) were examined. Additionally, different temperatures (14, 27, 37, 47, and 57°C) and reaction times (1 hour to 48 hours) were investigated. The optimization process also included synthesis at different pH values (7, 8, 10, and 12). The next samples included rhGH exposed to varying temperatures and pH without the addition of Au salt (heated rhGH), as well as intact rhGH solution without exposure to temperature, pH, or salt. Fluorescence spectra of each sample were analyzed using the Perkin Elmer LS 55 Fluorescence spectrometer.

Investigation of the products

Various techniques were employed to characterize the products of the samples. UV absorbance spectra were recorded using a

Perkin Elmer Lambda 25 UV/vis spectrometer and fluorescence emission was examined with excitation at 320 nm and emission at 408 nm using a Bio Tek Cytation 3 multimode microplate reader CYT3V. FTIR spectrometry was performed in the range of 400 to 4000 cm-1 using KBr tablets containing dried samples. Dynamic Light Scattering (DLS) (Zetasizer NANO-ZS (Malvern instruments Ltd., U.K.)) was utilized to measure the hydrodynamic diameter of the reaction products, and Transmission Electron Microscopy (TEM) images were captured using a Philips EM208 microscope after negative staining with 2% uranyl acetate.

Fluorescence emission of samples in the presence of different ions

To further investigate the samples, their fluorescence emission in response to different ions was studied. Samples included intact rhGH, Agg. rhGH, Agg. rhGH- Au, and buffer as an additional negative control. Reagents included Zn2+, Co2+, Ni2+, Cu2+, and Mg2+ ions in sulfate form, as well as Ca2+, K+, and Na+ ions in chloride form. Samples were mixed with reagents at a final concentration of 0.5 M and distributed in a 96-well plate. Fluorescence emissions at 408 nm upon excitation at 320 nm were recorded using a Bio Tek Cytation 3 multimode microplate reader CYT3V. Subsequently, the sensitivity range of Agg. rhGH-Au was determined by testing different concentrations of quenching ions (0, 0.0001, 0.001, 0.01, and 0.1 M) and zinc ions (0, 0.25, 0.5, and 1 M).

RESULTS AND DISCUSSION

Characterization of aggregated rhGH in the presence and absence of HAuCl4

This work presents a detailed study of the effect of HAuCl4 salt on the recombinant human growth hormone in the nanocluster synthesis conditions. After purifying the protein, it was mixed with HAuCl4 at an alkaline pH and high temperature. UV/vis absorbance and fluorescence spectrophotometry were conducted to investigate the samples' structural changes and optical properties. The UV/vis absorption spectrum in Figure 1a shows peaks for rhGH at around 280 nm (related to Trp) and 235 nm (related to peptide bonds). About Agg rhGH, the Trp peak decreases, and the peptide bonds' absorbance increases, indicating a change in protein structure [24]. Figure 1a indicates that the Trp peak of the Agg. rhGH–Au disappears, possibly due to aggregation of the protein or Trp converting to Indole-3-acetic acid in the presence of HAuCl4 [25]. Additionally, the disappearance of the Trp peak coincides with the appearance of a redshifted Tyr peak, suggesting -OH group ionization. Typically, the Tyr peak is observed around 270 nm [26].

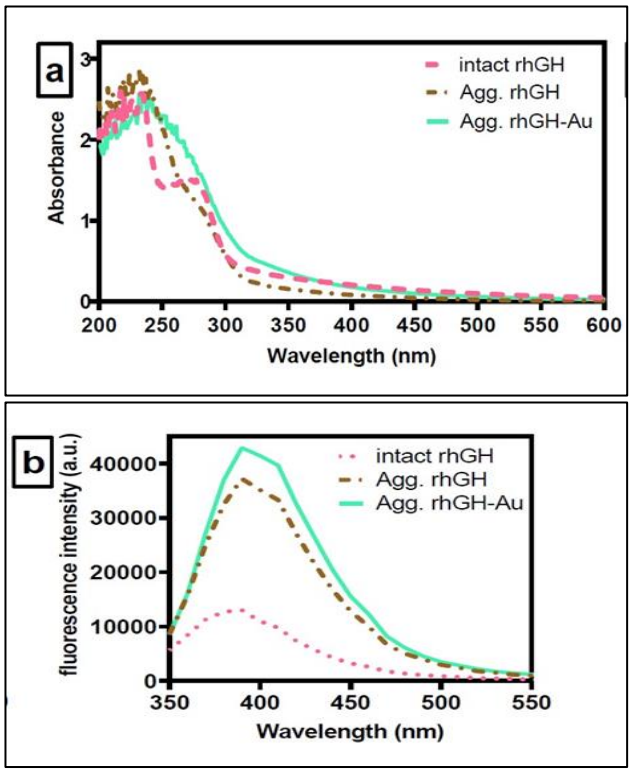

Figure 1. a) UV-visible absorption spectra of intact rhGH, aggregated rhGH in the presence of HAuCl4 (Agg. rhGH-Au), and aggregated rhGH in the absence of HAuCl4 (Agg. rhGH) **b)** Fluorescence spectra of the samples with excitation at 320 nm.

The lower absorption intensity of Agg. rhGH-Au compared to the Agg. rhGH can indicate the tryptophan residues' less exposed positions in the aggregated sample in the presence of gold salt.

To determine the fluorescence emission of the samples, excitation was varied across different wavelengths from 280 to 400 nm. The data shows that when the samples are excited at 280 nm, the intrinsic fluorescence of Agg. rhGH and Agg. rhGH- Au decreases compared to intact rhGH (data not shown). This could be due to aggregation, where peptide bonds suppress the indole fluorescence (Trp) as the amide groups come closer together, leading to increased quenching [27]. Another reason could be the protein becoming protonated in an alkaline environment. Additionally, exposing the fluorophore to a more polar environment can also reduce fluorescence. Based on the data, both aggregated rhGH samples exhibit a peak strength of around 408 nm (em.λmax) when excited at 320 nm, while intact rhGH shows an emission strength of around 350 nm (em.λmax) when excited at 280 nm. According to Figure 1b, excitation at 320 nm was chosen, revealing the strongest emission of aggregated samples between 350-450 nm. The fluorescence at higher wavelengths is attributed to Trp located in a hydrophilic environment [28]. This suggests that in intact rhGH, Trp is in a relatively hydrophobic space but in a more hydrophilic position in aggregated samples. On the other hand, as we know the minimum lambda max of gold nanocluster emission reported is 450 nm [12] which is somewhat far from the peak, we observed about the Agg. rhGH- Au.

The optimal conditions to produce the samples with the highest fluorescence emission are depicted in Figure 2. Figure 2a illustrates that a concentration of 0.1 M HAuCl4 exhibited the highest fluorescence emission intensity and was thus selected. Subsequently, the optimal temperature and time at the chosen Au salt concentration were investigated. Temperatures ranging from 14 ℃ to 57 ℃ were tested and monitored via fluorescence emission spectrophotometry over 1, 5, 24, and 48-hour intervals. The data, depicted in Figure 2c, revealed that the highest emission was achieved at 57 ℃ after 48 hours and approximately 47 ℃ after 24 hours. To ensure milder conditions, the latter point (47 ℃ after 24 hours) was selected. Finally, an experiment was conducted at the selected salt concentration, temperature, and time across different pH values of 7, 8, 10, and 12. The results indicated the highest fluorescence intensity at pH 12 (Figure. 2b).

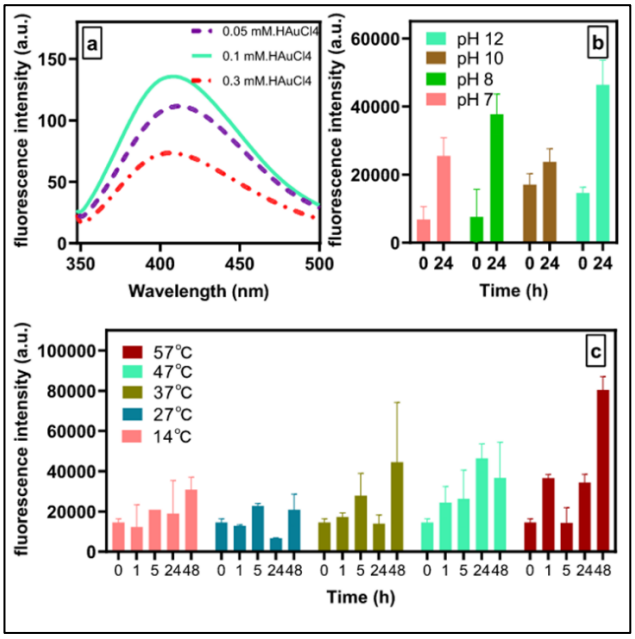

Figure 2. a) Fluorescence spectra of rhGH in the presence of different concentrations of HAuCl4, the spectra recorded by Perkin Elmer LS 55 Fluorescence spectrometer. **b)** Fluorescence spectra of Agg. rhGH-Au at different temperatures and times, **c)** Fluorescence spectra of Agg. rhGH-Au at different pH using fluorescence emission intensity. Excitation, 320nm. Emission, 408nm. The data of b and c was recorded by the Bio Tek cytation 3 multimode microplate reader CYT3V.

FTIR spectrophotometry was utilized to analyze the pattern changes between rhGH and

aggregated protein in the presence and absence of HAuCl4 to compare their secondary structures. The absorption bands in Figure 3 indicate a broad band at 3650-3250 cm-1 for N-H and O-H vibrations, a weak band at 2070 cm-1 for aromatic groups on the surface [29] that is relatively deep in the aggregated rhGH and can be a sign of aggregation, a deep band at 1639 cm-1 for C=O stretching of amid I, weak bands at around 1413 cm-1 and 1460 cm-1 for C-N and N-H vibrations of amides and amide III, a peak at 1045 cm-1 for C-N stretching, and a peak at approximately 682 cm-1 for Trp or C-S stretch [30]. Additionally, a peak at around 2360 cm-1 in aggregated rhGH may be due to CO2 interaction during drying [31]. The FTIR spectra of rhGH, aggregated rhGH in the absence of Au salt, and aggregated rhGH in the presence of Au salt show differences in peak intensity without any significant shift or disappearance, possibly indicating inter- or intramolecular bonding in rhGH-Au-NCs [32, 33].

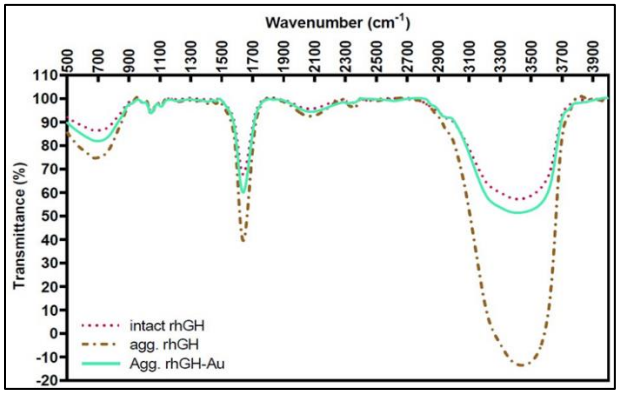

Figure 3. FTIR spectra of intact rhGH, aggregated rhGH, and aggregated rhGH in the presence of HAuCl4.

The subsequent study aimed to assess the size and morphology of the samples using DLS and TEM imaging. Figure. 4a showed that the hydrodynamic diameter of rhGH is about 6.2 nm, whereas it was approximately 90 nm related to the Agg. rhGH-Au. Although the DLS data like em.λmax does not support the formation of nanoclusters (big size; 90 nm), it confirms the mono-population of Agg. rhGH- Au. In contrast, the Agg. rhGH are larger and have heterogeneous populations; one around 164 nm and another around 615 nm (Figure. 4a). The homogeneity and smaller size of the Agg. rhGH- Au in comparison with Agg. rhGH can be a sign of forming different architecture and relatively ordered aggregates. It can also be a reason for the different optical behavior observed in the absorption spectrum. The TEM image indicated the morphology of the Agg. rhGH-Au (Figure 4b).

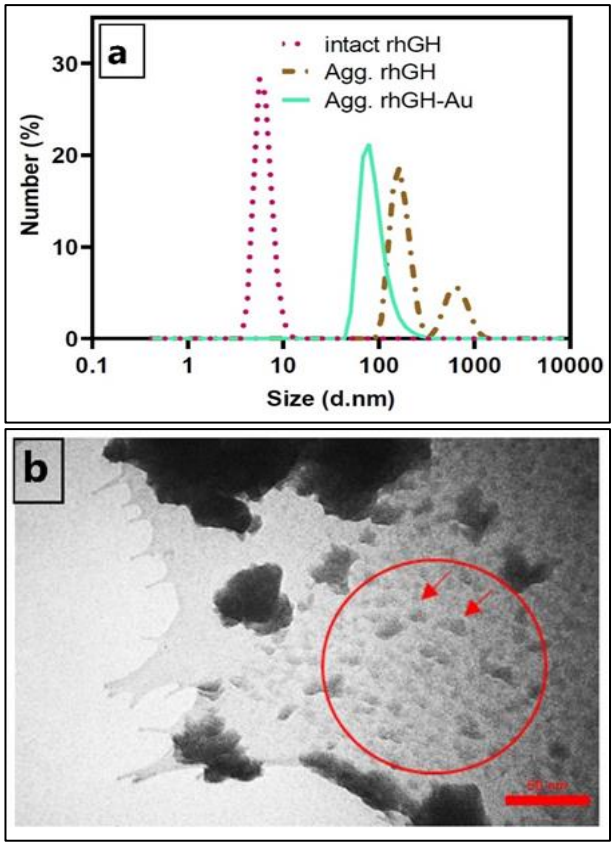

Figure 4. a) DLS spectra of rhGH, Agg. rhGH, and Agg. rhGH- Au. **b)** TEM image of Agg. rhGH- Au.

Investigation of the sample fluorescence signal in the presence of different ions

Further investigation focused on examining the effect of various ions on the fluorescence signal of the samples. Given the presence of sites for bivalent cation attachment on human growth hormone [19], the emission of the samples as an

experimental sensitivity was analyzed in the presence of 0.5 M solutions of CuSO4, NiSO4, ZnSO4, MgSO4, CoSO4, CaCl2, KCl, and NaCl. Figure 4 illustrates that the fluorescence emission of samples was quenched by copper, nickel, and cobalt ions. The magnesium ions enhanced the fluorescence of both aggregated samples and quenched the intact rhGH. However, the effect of zinc ions on the samples is different, it enhanced the fluorescence of intact rhGH and Agg. rhGH-Au, but quenched the signal of Agg. rhGH. This suggests structural differences supported by our UV/Vis spectrophotometry and DLS results. Specifically, zinc ions quench the Agg. rhGH emission, but enhance the intact rhGH emission by approximately 2.25 times and Agg. rhGH-Au emission by approximately 1.35 times.

To further investigate the sensitivity of the Agg. rhGH- Au, they were exposed to various dilutions of the target ions. Figure 6a illustrates that as the ion concentration increased, the fluorescence intensity decreased, with significant quenching observed in the presence of 0.1 M NiSO4, and 0.01 M CoSO4 and CuSO4. Conversely, Figure 6b demonstrates that the fluorescence intensity of the Agg. rhGH- Au increased even in the presence of 0.25 M zinc ion.

Key amino acid residues involved in the binding of zinc ions and hGH including H18, H21, D171, and E174, as E174 is crucial for Zn2+ binding [34]. The emission patterns of Agg. rhGH- Au and intact rhGH suggest that the presence of gold probably retains the topography of E174 to some extent, unlike in Agg. rhGH (Figure 5 and 6).

Furthermore, magnesium ions enhance the emission intensity of aggregated samples but reduce the emission of intact rhGH (Figure. 5). Unlike zinc ions, magnesium does not cause growth hormones to dimerize [20]. However, it minimally alters the protein structure and aromatic residue accessibility, leading to decreased fluorescence emission [35] as can be observed about intact rhGH. In both aggregated samples, protein aggregation occurs in a manner that may limit the impact of magnesium on the Trp position.

Figure 5 indicates that the fluorescence of samples was reduced in the presence of at least 0.01M copper, 0.1M nickel, and 0.01M cobalt ions, which are paramagnetic, unlike zinc and magnesium. This suggests that the quenching can be due to a paramagnetic effect where fluorophore samples transfer the electrons to the d orbital of the ions in a non-radiative way [36]. Additionally, copper ions can quench fluorescence through internal charge transfer (ICT) [36]. Moreover, Cobalt ions were found to alter the protein structure of BSA-Au-NCs, exposing tryptophan and causing fluorescence quenching [28]. On the other hand, the binding of Cu2+, Co2+, and Ni2+ ions to the growth hormone can induce dimerization, potentially leading to aggregation-caused quenching (ACQ) [33]. However, the manner of our study was not related to amyloid formation, but other effects of ion binding to rhGH can be indirectly involved in quenching the sample's emission.

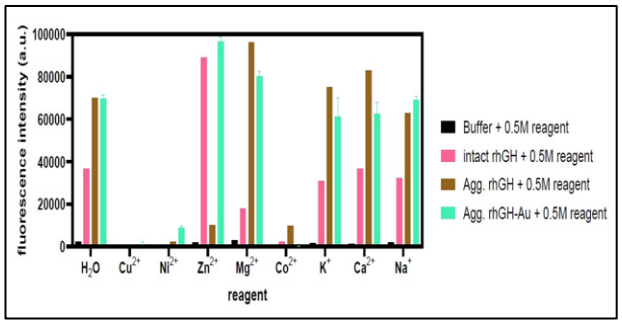

Figure 5. Fluorescence signals of samples in the presence of different ions. The ions are copper, nickel, zinc, magnesium, cobalt, potassium, calcium, and sodium solutions in a concentration of 0.5 M.

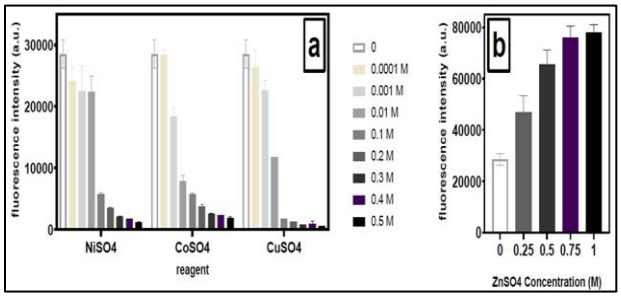

Figure 6. The fluorescence intensity of Agg. rhGH- Au in the presence of different concentrations of NiSO4, CoSO4, CuSO4 **a)**, and ZnSO4 **b)**.

CONCLUSION

In summary, the present study provides a comprehensive analysis of the rhGH in the conditions of nanocluster synthesis, the rhGH in the same condition without HAuCl4, and also intact rhGH, along with the investigation of their sensitivity to various ions. It highlights the importance of understanding the behavior of recombinant human growth hormone and its potential in the synthesis of gold nanoclusters. It seems that the tested conditions in this study cannot cause the successfully synthesized gold nanoclusters. However, the aggregates formed in the presence of HAuCl4 salt significantly differ from the aggregates formed in the absence of the salt. Moreover, the investigation of the samples in the presence of different ions revealed distinct fluorescence responses. In conclusion, the study provides valuable insights into the characteristics and behavior of rhGH in the presence of gold salt, laying the groundwork for further research in this area.

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