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Biophysical Insights into β-Lactamase-Gold Nanoparticle Conjugates: Pioneering Diagnostic Solutions for Antimicrobial Resistance

Adwaita S R Nair[1,2,3], Kunal Dhankhar[1,4], Arup Samanta[2,3], Saugata Hazra[*1,2]
[1] Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee
Roorkee, Uttarakhand 247667, India
adwaita_n@nt.iitr.ac.in
[2] Centre for Nanotechnology, Indian Institute of Technology Roorkee
Roorkee, Uttarakhand 247667, India
kunal@pe.iitr.ac.in
[3] Department of Physics, Indian Institute of Technology Roorkee
Roorkee, Uttarakhand 247667, India
arup.samantha@ph.iitr.ac.in
[4] Department of Polymer and Processing Engineering, Indian Institute of Technology Roorkee
Saharanpur, Uttarakhand 247667, India
saugata.iitk@gmail.com
*Corresponding Author: Dr. Saugata Hazra, Associate Professor, Department of Bioscience and Bioengineering, Indian Institute of Technology-Roorkee, Roorkee, Uttarakhand, India; Telephone no.: +918171064462; Email: saugata.iitk@gmail.com; saugata.hazra@bt.iitr.ac.in. ORCID ID: 0000-0002-3074-1534

Abstract **–** With the increasing mortality attributed to antibiotic resistance, the development of a rapid and early detection biosensor for β-lactamase enzyme identification has become imperative. The exceptional optical and electromagnetic characteristics of gold nanoparticles have rendered them as a prime candidate for biosensing applications. This research seeks to establish a foundation for the nanosensor development by investigating intricate interaction between gold nanoparticles and the β-lactamase enzyme, focusing on functional and conformational dynamics following conjugation. UV-visible spectroscopy has been employed to examine the stability of bioconjugates and influence of pH on their conformational state by observing changes in the localized surface resonance plasmon band (LSRP). The minor red shift of the LSPR peak following β-lactamase conjugation confirms protein conjugation and indicates the absence of gold nanoparticle aggregation due to the protein. The fluorescence quenching of tryptophan residues in β-lactamase, in the presence of gold nanoparticles, is utilized to ascertain the binding of protein onto the surface. Circular dichroism spectroscopy further provided insight into the structural integrity of bioconjugate. Intact α-helix and β-sheet peak in CD spectra confirmed that the interaction of gold nanoparticle surface did not cause unfolding or denaturation of protein. This research provides essential insights into the interaction between β-lactamase and gold nanoparticles, facilitating the advancement of sophisticated nanosensors that may be utilized in early pathogen detection and the monitoring of antimicrobial resistance.

Keywords**:** Gold nanoparticle, β-lactamase, Antibiotic resistance, Bioconjugate, FTIR

1. Introduction

Worldwide deaths due to the inability to treat diseases with current known antibiotics has brought inspection of antibiotic resistance to the limelight of research. According to a survey, deaths due to antimicrobial resistance have crossed 1.2 million as of 2019, thus bypassing the death counts due to HIV or malaria[1] Multiple studies direct towards inefficiency of current treatments for more than half of the clinical patients. Rapid dissemination of antibiotic-resistant bacteria by horizontal gene transfer specifically carbapenemase resistant ESKAPE pathogen has raised concerns among all International and National health agencies, policymakers, and researchers. Over the years bacteria have adapted multiple methods to combat antibiotics such as pump efflux, reduction of drug permeation across the bacterial membrane, alteration of target sites and β-lactamase expression. β-lactam drugs being a major arsenal against antimicrobial resistance plays a major role in the development of antibiotic resistance in bacteria. [2], [3] Hydrolysis of β-lactam drug is one of the most concerning mechanisms adapted by bacteria to overcome the effect of antibiotics. β-lactamase genes are plasmid-encoded, thus rapidly

escalating their presence in all the corners of the world. Their ability to cleave all classes of β-lactam drugs including carbapenem, which is usually the last resort, and the absence of clinically accepted inhibitors makes the understanding its study need of the hour. The concerning rate of β-lactamase spreading across the world directly points at the urgent need for deep understanding and remedy for the same. Thus, thorough literature search and mechanistic study of emergence, activity, spreading and remedies for more β-lactamase should be foremost and noteworthy. Colloidal gold nanoparticles (AuNPs) have attracted considerable interest in recent years due to their remarkable characteristics, including size and shape-dependent optoelectronic properties, an extensive surface-to-volume ratio, minimal toxicity, and good biocompatibility. Researchers globally have advanced in regulating its dimensions, morphology, and functionalization, alongside various synthesis techniques. Distinct characteristics, including surface plasmon resonance, size-dependent absorption color, and fluorescence quenching capability, significantly enhance their extensive utility in diagnostics, treatments, and imaging.[4] The simplicity of functionalization and biocompatibility render gold nanoparticles exceptionally advantageous for interaction with biomolecules, including enzymes. Research on bioconjugate nanoparticles has garnered significant attention due to their potential uses in luminescence tagging, imaging, medical diagnostics, multiplexing, and biosensors. The attachment of protein to the nanoparticle surface may considerably influence its shape and functionality. As studied by Park,[5] comprehending the conformational alterations and unfolding mechanisms of proteins is crucial for advancing the biomedical uses of nanoparticles. β-lactamase protein, being a major concern has been in the limelight of research, yet there has been limited study upon its interaction with nanoparticle. This limits the usage of nanoparticle for sensing and therapeutics of antibacterial resistance associated β-lactamase protein. In this study, we aim to ascertain if β-lactamase protein may adhere to the surface of gold nanoparticles. We investigate the conformational alterations experienced by the protein upon binding and whether this interaction may induce any denaturation of the protein.

2. Material and methods

The SME gene tagged with six histidine at the N-terminal, was introduced into the pET28A vector and transformed into E. coli BL21 (DE3) cells for protein purification. Following cell lysis and subsequent supernatant collection, the kanamycin-resistant gene was isolated utilizing Ni-NTA agarose gel via affinity chromatography. In the final purification stage, 2 ml of the protein mixture was subjected to size exclusion chromatography using a Sephadex G-200 gel filtration column pre-equilibrated with the same buffer. All collected fractions were analysed using a 10% SDS gel to verify the presence of pure SME-E166A protein in the fractions (fig 2.e). The concentration of the fractions containing pure protein was quantified via UV-visible spectroscopy at 595 nm with the Bradford assay. The determined protein content was 15 mg/ml. Ultimately, pure proteins were cryogenically frozen with liquid nitrogen and preserved at -80 ºC for subsequent experimental applications.

Gold nanoparticles were synthesized by the thermal reduction of chloroauric acid by using sodium citrate, a method initially introduced by Turkevich and further modified by Frens[6], [7] Synthesis of nanoparticle from auric acid was confirmed by the change in color to ruby red from yellow. For roughly 1 hour, histidine-tagged SME-E166A was incubated with gold nanoparticles in varying concentration ratios to facilitate conjugation based on histidine affinity towards metal surface. The surface morphology and size synthesized gold nanoparticles were assessed using a field emission scanning electron microscope (FESEM, Apreo S LoVac, Thermo Fischer Scientific) and transmission electron microscopy (TEM TALOS F200-X). Malvern zetasizer was used to evaluate the surface charge changes on gold nanoparticle with protein interaction. Tryptophan fluorescence was studied for interaction using Horibo fluorescence spectroscopy. Secondary structure analysis using circular dichroism (CD) spectroscopy were carried out in a Jasco J-1500 spectrometer with a Peltier-effect temperature controller. Quartz cells with a 0.1 cm path length were used.

3. Result and discussion

3.1 Physicochemical Characterization of Synthesized Gold Nanoparticles

Fig. 1 illustrates the physicochemical characterization of the synthesized gold nanoparticles, employing various techniques to assess their properties. The colloidal gold nanoparticles exhibited a reddish color and displayed a distinct absorption peak at 520 nm. (Fig. 1a and Fig. 1b) This intense absorption peak could be attributed to the electronic oscillation

i.e. surface plasmon resonance. Fig. 1c and Fig. 1d presents the SEM and TEM images, respectively of gold nanoparticles synthesized via the Turkevich method. The SEM micrograph reveals a uniform dispersion of gold nanoparticles with minimal agglomeration. Additionally, the size of the icosahedral-shaped gold nanoparticles was estimated to be approximately 13 nm (Fig. 1e). Fig. 1f depicts the particle size distribution curve obtained through dynamic light scattering (DLS), indicating moderate polydispersity with a PDI of 0.272 and a hydrodynamic size of 38.3 nm. The surface charge measurement, as assessed by DLS, was -32.6 mV, reflecting their stability in colloidal state.

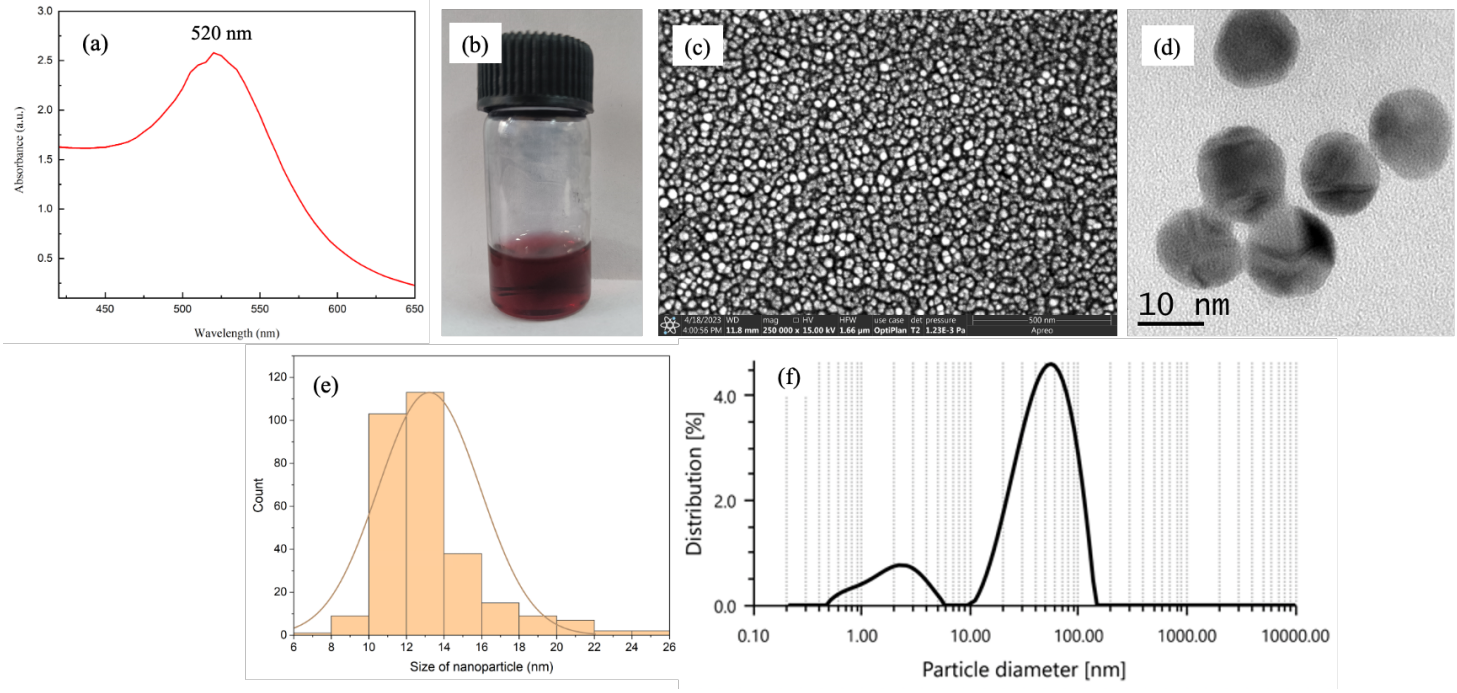

Fig. 1: Gold nanoparticle characterization (a) Surfacr plasmon resonance peak using UV spectroscopy (b) Colloidal solution of gold nanoaparticle (c) SEM micrograph (d) TEM image (e) SPR peak (f) Zeta size analysis

3.2 Characterization of SME β-Lactamase

SME, a typical β-lactamase derived from Serratia marcescens, was selected due to its classical carbapenemase activity, showcasing a range of catalytic capabilities. SME gene cloned in pET-28a(+) vector using NcoI and XhoI restriction enzyme were visualized using 1% agarose gel. (Fig. 2a and Fig. 2c). SME protein was purified by two-step purification process, Ni_NTA agarose followed by size exclusion chromatography.[8] The purified proteins were analsyed using gel electrophoresis, which revealed protein bands at approximately 28 kDa. (Fig. 2d and Fig. 2e) The size exclusion chromatogram displayed a highly intense singular peak corresponding to SME protein, which was collected for subsequent characterization. (Fig. 2f) The protein's secondary structure was assessed by circular dichroism spectroscopy. The CD spectrum indicates that the SME beta-lactamase possesses a distinct secondary structure, predominantly characterized by a combination of alpha-helices and beta-sheets. (Fig. 2g) The pronounced negative ellipticity near 210 nm indicates substantial alpha-helical content, whereas the positive region at shorter wavelengths implies contributions from other structures, likely beta-sheets. Furthermore, the melting temperature (T_m) of SME was determined using CD spectroscopy to evaluate its thermal stability.[9] Fig. 2h demonstrates that the T_m of SME is roughly 55°C, indicating the temperature at which fifty percent of the protein undergoes unfolding.

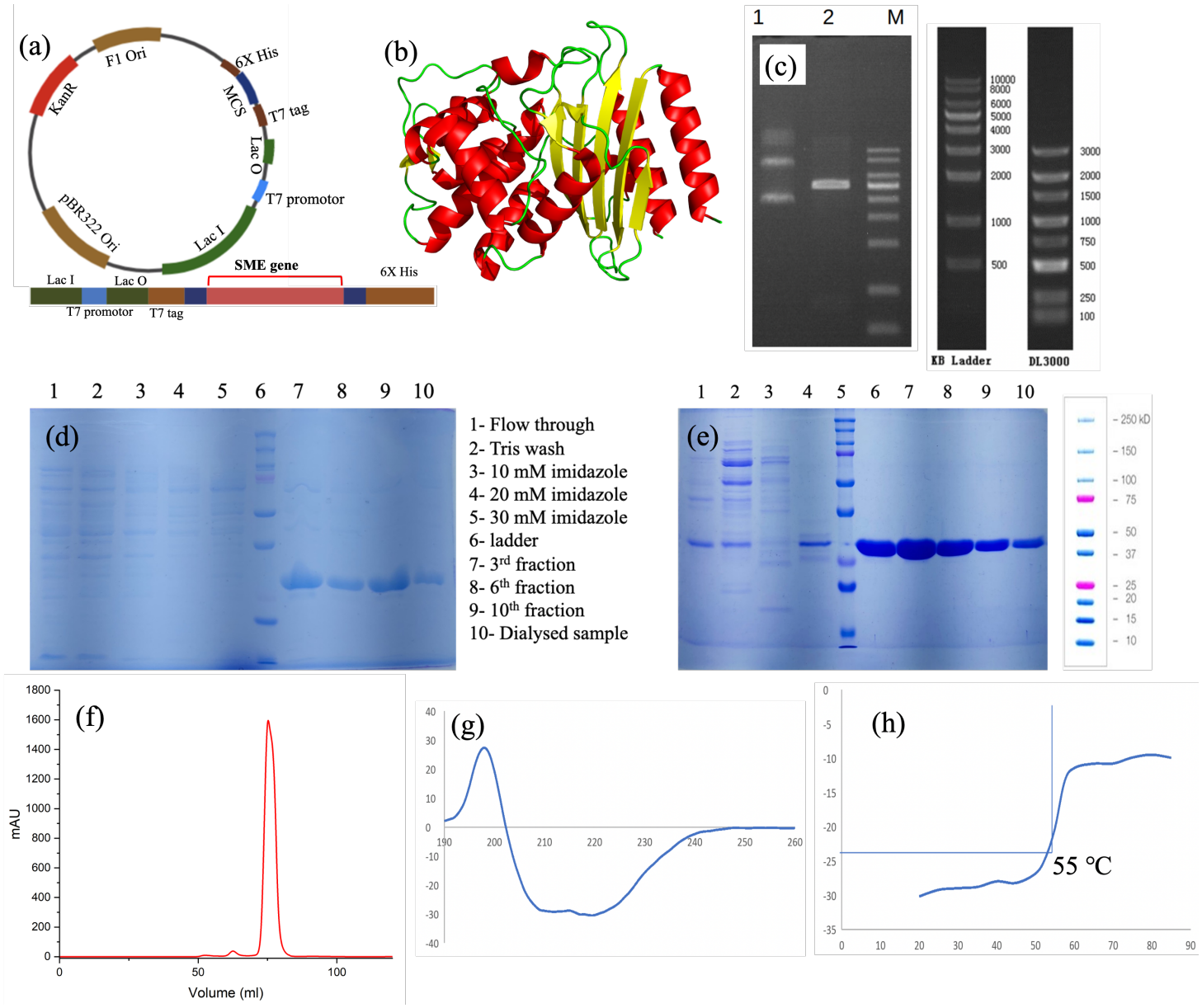

Fig. 2: SME protein biophysical and biochemical characterisation (a) Plasmid Map (b) 3D structure of SME PDB ID – 1DY6 (c) 1% agarose gel (d) Gel electrophoresis of purified protein (e) Gel electrophoresis of purified protein (f) Size exclusion chromatogram (g) Circular dichroism (h) Melting temperature study

3.3 Evaluation of Protein-Gold Nanoparticle Conjugation Under Variable Conditions

The SPR peak spectra were utilized to evaluate the *in-situ* conditions of protein-nanoparticle formation at each pH level (3, 6, 7, 8, 11) examined in this investigation. A shift in the SPR peak signifies alterations in the chemical environment surrounding gold nanoparticles, hence allowing its assessment to correspond with protein interactions. Fig. 3a illustrates that citric acid-capped gold nanoparticles have a surface plasmon resonance peak at 540 nm. The presence of protein alters both the peak's broadness and its position. The red shift, indicative of protein desorption or aggregation, suggests that an acidic media is unsuitable for β-lactamase bioconjugation. At elevated pH levels, the SPR peak exhibits a minor red shift accompanied by considerable broadening. Consequently, a pH range of 7-8 may be deemed optimal for the enhanced stability

of the β-lactamase bioconjugate. Further using the same UV-Vis spectral approach, the optimum np to protein ratio was studied. Up until 1:1000 ratio of np to protein, the SPR peak doesn't show significant shift. (Fig. 3b) But further increase shows shifting and broadening of SPR peak indicating aggregation of gold nanoparticle. This suggest that bioconjugates are not stable at higher concentration ratios.

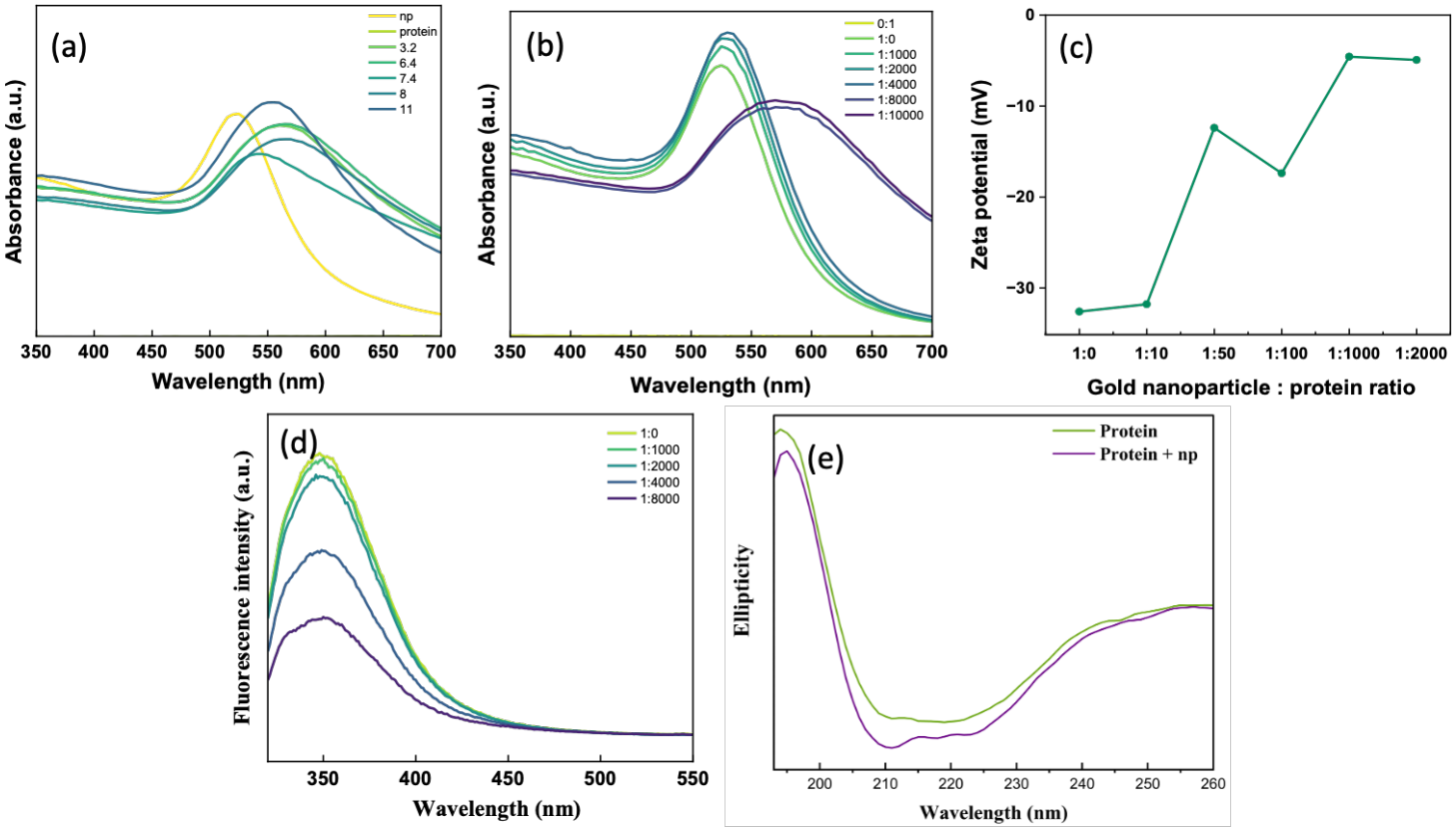

Fig. 3: Nanoparticle and protein bioconjugate characterisation (a) pH study using UV-Vis spectra (b) SPR peak analysis at varying ratio of nanoparticle and protein (c) Zeta potential analysis (d) Tryptophan fluorescence analysis (e) Circular dichroism spectra of protein and bioconjugate

3.4 Fluorescence Quenching Analysis

Quenching of tryptophan fluorescence in presence of gold nanoparticle was investigated to better understand the interaction and quenching mechanism of gold nanoparticle with β-lactamase protein. While the maximum emission wavelength (about 350nm) was nearly same in all cases (fig. 3c), fluorescence intensity decreased as with increasing GNP concentration which could be ascribed to gold nanoparticle quenching. The degree to which protein was conjugated with gold could be used to directly correlate to the degree at which fluorescence intensity was reduced. This confirms the interaction of nanoparticle to the protein residues.

3.5 Zeta potential analysis

The zeta potential was employed to monitor the change in surface charge after protein conjugation to the nanoparticle surface as it can provide insights into the stability of bioconjugate. At 1:0 ratio, the gold nanoparticle seems to have a surface charge of approximately -35 mV, which could be attributed to the citrate group used as capping agent. (fig. 5d) With increasing protein concentration, zeta potential increases towards positive charge. This indicates interaction of positively charged residues with the nanoparticle surface. At higher concentration ratio (of above 1:1000) there is no further change in

the surface charge as no further absorption of protein occurs. After 1:100 nanoparticle to protein ration, the zeta potential increase to more positive value >-10 mV, suggesting instability. Thus, zeta potential study corroborates with UV-Vis study, indicating instability at higher ratio.

3.6 Structural Integrity Assessment

Circular spectroscopy was used to assess whether β-lactamase was able to retain its native structure after conjugation. As discussed earlier also, spectrum in fig. 5e illustrates the inherent structure of the protein. The peak at 195–200 nm shows the existence of β-sheet or random coil structures, whereas the dip at 210–220 nm signifies α-helical content. The absence of significant shift of ellipticity peak and decrease in intensity suggest indicating the presence of intact β-lactamase protein upon nanoparticle conjugation. The spectral study indicate that the protein doesn't undergo much conformational alteration upon bioconjugation.

4. Conclusion

The increasing prevalence of antibiotic resistance resulting in fatalities has brought β-lactamase to the forefront. These β-lactam hydrolyzing proteins have been in the limelight of research, since the recognition of AMR as global threat by UNESCO. The increasing prevalence of newly found β-lactamase mutations has once more elicited worries among researchers and health officials. These new mutations pose a threat for the development of suitable countermeasures. With the advent of nanotechnology, many nanoparticles are utilized for the detection and theranostics of diseases. Among these gold nanoparticles stand out owing to its remarkable properties such as surface plasmon resonance, size-based properties and ease of synthesis. In this study, we have sought to comprehend the interaction between gold nanoparticles and the β-lactamase protein, delving into the molecular mechanism underpinning their conjugation. Gold nanoparticles, 13 nm in size were assessed and appear to interact with β-lactamase protein at a pH of 6-7, with no alteration to its secondary structure. The fluorescence spectra demonstrate the quenching efficacy of gold nanoparticles on tryptophan residues. Further surface charge study indicates strong interaction of protein with nanoparticle. The CD spectra indicate no alteration in the secondary structure of the protein upon interaction with the gold nanoparticle. This study demonstrates that β-lactamase protein can create a bioconjugate with gold nanoparticles in their intact state. While this study could serve as a foundation for the advancement of sensors utilizing gold nanoparticles, further kinetic study needs to be evaluated to understand the functional changes upon bioconjugate formation. This research offers a critical instrument in the fight against antibiotic resistance by utilizing the distinctive properties of AuNPs to develop innovative solutions for the detection and monitoring of β-lactamase activity.

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