Silver nanoprism-mediated protein estimation—an ultrasensitive platform for rapid estimation of protein concentration

Katha Shyam Sundar, Jatavath Ramesh, Praveen Chinthala, Karunakar Rao, Swagata Banerjee and Shibsekhar Roy

Dept of Biochemistry, University College of Sciences, Osmania University, Hyderabad 500007, India

* Author to whom any correspondence should be addressed.

E-mail: shibsekharroy@gmail.com

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Abstract

Estimation of protein concentration in the range of nanogram level (ng/ml) is a big challenge for conventional protein estimation methods. The highly dipole sensitive spectroscopic properties of Silver nanoprism (AgNPR) has been utilized to develop a rapid and highly sensitive method for the estimation of globular protein concentration at ng/ml (or ppb) range. We have applied a unique molecular doping approach to introduce protein in the interstitial space of the Ag fcc(111) crystal planes within AgNPR structure. The presence of the doped protein induces deformation in the crystal plane arrangement of AgNPR that results in a quantitative red shift of the dipole resonance peak (D-peak) of AgNPR under UV–vis spectroscopy. The proposed method allows detection of a protein concentration range of as low as 1–20 ng ml\(^{-1}\) that is better than the sensitivity limit of conventional protein estimation techniques. This method has been successfully applied for commonly used proteins like haemoglobin (Hb), Bovine serum albumin (BSA), Trypsin (TRYP) and Lysozyme (LYS) with a very low limit of detection (LOD) within 2–6 ng ml\(^{-1}\). The lowest LOD value was shown by Hb as 2.08 ng ml\(^{-1}\). The method has further been validated by measuring Casein concentration from milk with an accuracy of 99% and 95% recovery for the concentration of 3.1 and 31 ng ml\(^{-1}\) respectively. Transmission emission microscopy (TEM) images show that the doped protein has been found to alter the size and shape of the AgNPR as a function of the dopant concentration by creating systematic deformation. This method does not require any alteration of the reaction temperature and solely depends on the physical interaction of doped protein with its neighbouring crystal structure of the nanoplanar geometry.

1. Introduction

Estimation of protein concentration is a very basic requirement of many streams of biological and related interdisciplinary fields. To estimate protein in any system the existing methods, which are currently available worldwide are Lowry assay, Biuret assay, Bradford assay. The established gold standard assays like BCA assay and Bradford assay provide a detection range of 0.2–1.0 mg ml\(^{-1}\) and 0.2–1.5 mg ml\(^{-1}\) respectively [1, 2]. However specialised kits and alternative protocols to perform specialized assay have been developed to measure protein concentration range of 1–20 μg ml\(^{-1}\) [3]. All the existing methods include heating and cooling of the reagents during reaction, which makes the process much lengthier. In addition, these methods suffer from several disadvantages like incompatibility with Cu reducing agents and DTT for BCA and Lowry method, surfactant incompatibility and protein-protein variation for Bradford method [4–7]. As the sensitivity of these methods are dependent on both the number of aromatic amino acids and peptide linkages, deficiency or abundance of a selected set of amino acids may result in compromising the accuracy of the measurement.

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With the advent of nanotechnology in the last three decades, noble metal nanoparticles, primarily gold nanoparticles have emerged as leading candidates for dye independent optical or spectroscopic probes [8–10]. Several reports have emerged describing the quantifiable concentration of proteins interacting with the nano surface. The usual technology involves non-specific or covalent attachment of protein on the noble metal nanosurface resulting in the change of the dipole environment surrounding the surface. Hence, the refractive index of the ‘protein-thickened’ nanosurface increases resulting in higher order scattering of light. So, the surface plasmon wavelength undergoes red shift, whose extent is measured as a function of protein concentration [11]. However, the active dynamic range of SPR peak, more specifically dipole resonance peak from Au or Ag nanoparticles are very small; usually 10–20 nm which limits the resolution of the method [12].

This drawback regarding small spectral or optical dynamic range of measurement has been addressed through this communication by introducing noble metal nanostructures with planar geometry, focussing on Nanopirism. The advantages of nanoplanar structures are their easy seed mediated synthetic route and tunable optical properties [13–18]. Due to the ease of synthesis and high degree of sensitivity, we have selected the silver Nanopirism (AgNPR) system for the proposed work. These AgNPRs are synthesized by a planar alignment of smaller silver nanoparticles of c.a. 5 nm, referred as silver nanoseed (AgNS) (see scheme1) [19]. The asymmetry in the structure of AgNPR results in three significant spectral peaks in the visible range; namely, in plane quadruple resonance peak (Q-peak), out of plane dipole resonance peak (O-peak) and in-plane dipole resonance peak (D-peak). The D-peak of nanoplanar geometry is ultra-sensitive to its immediate dipole environment. The wavelength of D-peak usually varies from 450 to 1000 nm depending on its neighbouring dipole environment, making the active spectroscopic dynamic range more than 500 nm. This makes the AgNPR at least twenty times more sensitive than its spherical counterpart [20]. Another important attribute of these planar geometries is the dependence of the D-peak characteristics on the key geometric parameters of the AgNPR like length, thickness and internal angles. Any alteration of the dipole nature of the environment and/or the key geometric parameters of AgNPR caused by the introduction of protein can be probed instantaneously. Both the effects, i.e. effect of altered dipole environment and change in geometric parameters take place when a protein molecule is doped within the nanoseed (AgNS) interior. When about hundreds of these AgNS particles align to create AgNPR geometry, the combined effect of these two factors renders a very high sensitivity of the D-peak compared to O and Q-peaks.

However, attachment of protein on the surface of nanoplanar structure has to be uniform to yield a homogenous plasmonic response. But, at a very low concentration range like 1–100 ng ml$^{-1}$, the uniform layering of protein does not occur, which compromises with the accuracy of the assay. However, high sensitivity in the protein concentration range of ng/ml was reported by Grove et al who demonstrated the unique application of two-dimensional gel electrophoresis followed by silver staining in determining the protein concentration at that low concentration range [21]. But the process of 2D-Gel electrophoresis being too lengthy and expensive, its application as a protein estimation platform is very limited. In addition, reports are there where ng/ml level protein concentration has been detected using Ag nanoparticle system, but using an additional external fluorophores [22].

To counter the challenge, we have bypassed the conventional mode of interaction between protein surface and the nanosurface by adopting a molecular doping approach, where protein is introduced during the synthesis of nanoseed (AgNS). For a more homogenous structural response, we have selected some popularly used globular proteins namely lysozyme (LYS), bovine serum albumin (BSA), Haemoglobin (Hb), and Trypsin (TRY) for the study. The presence of a globular protein during the formation of AgNS, results in steric interaction between the protein surface and the fcc(111) crystal planes as described in scheme2. This scheme suggests that the presence of a protein disturbs the regular alignment of fcc(111) crystal units. This structural disturbance leads to deviation from the regular planar orientation of fcc(111) resulting in the loss of surface smoothness and

![Scheme 1. Synthesis strategy of AgNPR by seeding method.](image-url)
concomitant rise of ‘rough patches’. These ‘rough patches’ contribute altered optical properties of AgNPR in more than one ways.

i. Roughness enhancement: firstly, the nanoseed (AgNS), which are formed with doped protein, form a more asymmetric spheroid compared to a near perfect sphere for an undoped AgNS. Hence, scattering interaction with light becomes much different from that of AgNS. Here, one crucial difference from conventional nanosurface–biomolecule interaction must be mentioned to comment on the versatility of the novel optical properties of AgNPR. For a conventional nanosurface–biomolecule interaction, the biomolecule attaches to the surface of the nanoform by covalent or electrostatic interaction. Hence, the increase in biomolecule concentration increases the density and thickness of biomolecular layer outside the nanosurface that in turn increases the refractive index of the interface resulting in the red shift of the dipole sensitive SPR peak of a noble metal nanoform. Hence, optical response towards this interaction involves

Scheme 2. Deformation of the assembly of fcc(111) plane by a standard globular protein.
only surface attached analyte; the shape of the nanoform stays unchanged. Here lies the significant advantage of our method over the conventional ones for measuring protein concentration as it involves the quantification of shape change of the nanoform as a result of biomolecular doping.

ii. Alteration of dimension: with the introduction of a single rough patch, there is an increase of the size of its sphere of influence (SIN) for the corresponding AgNS species. With increasing protein concentration, the number of ‘rough patches’ increases, creating higher order asymmetric spheroids as described in scheme 3. Higher order asymmetry results in large increase of SIN that increases the distance between two neighbouring AgNS particles resulting in significant increase in the length of the AgNPR. With the increase of the length of the AgNPR, there should be a concomitant red shift of the D-peak. This red shift of the D-peak will be used to quantify the concentration of the doped protein.

Our doping strategy highlights the physical entrapment of the protein within the interstitial spaces of Ag fcc (111) crystal planes as described in scheme 3. The entrapment or spatial doping of globular protein exerts quantifiable space defect within the nanostructure. The globular shape of the protein has a significant role in maintaining the linear response even in the ppb scale.

2. Materials and methods

2.1. Materials
Analytical grade Silver nitrate, trisodium citrate, sodium borohydride, L-ascorbic acid were obtained from HIMEDIA laboratory, Mumbai, India. Poly(sodium 4-styrenesulfonate) (PSSS), Hb (human), BSA, LYS, TRY were purchased from Sigma-Aldrich. Casein was purified from commercially available milk with 3.1%(w/v) casein concentration using precipitation method [20].

2.2. Methods
2.2.1. Instrumentation
X-Ray diffraction patterns were recorded using a Shimadzu XRD-7000 X-ray diffractometer. Data collection was performed using Cu-Kα (λ = 0.154060 nm) radiation, over an angular range=10°–80° with an operating voltage 40 kV and operating current 30 mA. Scan speed was set at 2° min⁻¹. Samples for transmission electron microscopic (TEM) analysis were prepared by drop casting on carbon-coated copper TEM grids, allowing the grid to stand for 2 min, following which the extra solution was removed using a blotting paper. The nanoparticles thus obtained were subjected to TEM analysis on a JEOL model 1200EX instrument operated at an accelerating voltage of 120 kV. UV–vis absorption spectra were recorded on a double beam spectrophotometer.
UV3000 + obtained from Labindia analytical instruments Private limited. All the spectroscopic measurements were carried out in quartz cuvettes (10 mm × 10 mm) in the wavelength region 200–900 nm. The scan rate was kept at medium speed (600 nm min⁻¹). Baseline correction was performed prior to each measurement. Zeta potential (ZP) of the AgNS samples were measured by Electrophoretic Light Scattering (ELS) techniques using Anton-Paar Litesizer™ 500 (Anton Paar GmbH, Austria) instrument, equipped with a 40 MW single frequency Laser diode of 658 nm wavelength. The operating procedure was programmed using the Kalliope software, such that a maximum of 100 runs can be tested, each being averaged for 10 s. For measuring the zeta potential, 400 μl of the sample was inserted into the capillary cell to measure the electrophoretic mobility. This value is measured using Henry’s equation with Smoluchowski approximation (for polar solvent).

2.2.2. Synthesis of protein conjugated nanoprisms

The synthesis is a two stage process including the seeding step and elongation step, as we have described earlier [20]. We have used the protein to be quantified in the seeding stage. The whole method is described below in brief.

a. Synthesis of nanoseed (AgNS): To an aqueous solution of trisodium citrate (5 ml, 2.5 mM), 1 to 100 μl of protein of interest (Hb, LYS, BSA, TRY) from a stock solution of 1 mg ml⁻¹ was added to get a resultant protein concentration in 1–100 μg ml⁻¹ after being doped within AgNS. This was followed by the addition of 100 μl of aqueous poly(sodium styrene sulphonate) with molecular weight of 1000 kDa (500 μg ml⁻¹) and 300 μl of 10 mM freshly prepared aqueous NaBH₄. To this mixture, 5 ml of 0.5 mM aqueous AgNO₃ was added at a rate of 2 ml min⁻¹ with continuous stirring. The protein modified AgNS, which will be referred as Hb@AgNS, BSA@AgNS, TRY@AgNS and LYS@AgNS respectively, were kept at 4 °C for 15 min and then they were used for the next step. During AgNS synthesis, the molar ratio between protein and Ag⁺ present in the protein doped AgNPR having the highest protein concentration of the linear dynamic range has been calculated to be 1:270 considering haemoglobin as the representative protein. At such a small stoichiometric ratio, it was very unlikely to have any undoped or free protein molecule in the reaction system. We further investigated (section 3.5) in detail to find the estimate of the protein inclusion within AgNS structure.

b. Alignment of AgNS to AgNPR: A reaction mixture was prepared by mixing 5 ml distilled water, 75 μl of 10 mM aqueous ascorbic acid, and 300 μl of seed (AgNS) solution. To this mixture, 3 ml of 0.5 mM aqueous AgNO₃ solution was added at a rate of 1 ml min⁻¹ with continuous stirring. The protein doped AgNPRs will be referred as Hb@AgNPR, BSA@AgNPR, TRY@AgNPR and LYS@AgNPR from here on. To the as synthesized AgNPR, 2 ml of 0.5 mM trisodium citrate was added to stabilize the nanoform for subsequent use and storage for longer period. Hence, the final Protein concentration range becomes 1–100 ng ml⁻¹. The samples were characterized by conventional UV–vis spectroscopy and TEM. The spectroscopic data was used to construct calibration curve for each protein using linear regression method.

2.2.3. Purification of Casein from milk

The extraction of Casein was performed as per the standard protocol [23]. 50 ml of milk (Vijaya Dairy, Hyderabad, India), as purchased was centrifuged at 4000 rpm for 25 min at room temperature. After centrifugation, fat was removed from the surface by using a stainless steel spatula. After that, this skimmed milk was transferred in to a 100 ml glass beaker and an equal volume of distilled water was added and the mixture was stirred. Then the pH of the diluted milk was adjusted to 4.8 by adding 0.5 N HCl using a burette, with constant stirring. At this pH the casein was precipitated and the precipitate was allowed to settle at room temperature for 30 min. Supernatant is removed and suspension was filtered using Whatman number1 filter. The moist precipitate was washed three times with 10–15 ml of distilled water to remove the salts. After the two water-washing steps, further washing routines were performed with 10 ml of ethanol and diethyl ether each. The casein cake obtained after washing, was transferred form the funnel onto a clean petri plate. It was then uniformly spread on a petri plate and was allowed to dry at room temperature overnight. The weight of the dried casein sample is measured and percent of yield is calculated.

2.2.4. Comparison with conventional methods

We compared our method with the conventional ones like Biuret, Lowry and Bradford method. The test concentration range was chosen from 1 ng ml⁻¹ to 100 μg ml⁻¹. The detail procedure of each of the methods are described in ESI as paragraph P1.
3. Result and discussion

3.1. UV visible spectroscopy based characterization

The protein doped AgNPRs have been initially characterized by analysing their UV–vis spectra. The spectral profile of each of the doped AgNPRs doped with 100 ng ml\(^{-1}\) protein have been described in figure 1. The figure shows the spectra of BSA@AgNPR, LYS@AgNPR, HB@AgNPR and TRY@AgNPR along with the control AgNPR. The figure shows ca. 150 nm of red shift for the D-peak (\(\Delta D\)) for all the proteins. The profile also highlights that the protein-protein variation during the assay is very little. The individual spectral profiles of Hb@AgNPR, LYS@AgNPR, BSA@AgNPR and TRY@AgNPR in the concentration range of 1–20 ng ml\(^{-1}\) are observed in figures 2(a)–(d) respectively. The fundamental spectra for each protein doped AgNPR are characterized by three peaks (D-peak, Q-peak, O-peak) which are clearly observed in all the sets. The O-peak and Q-peak doesn’t undergo any significant spectral shift with respect to the protein concentration; however, the D-peak shows an appreciably high red shift with protein concentrations. The difference in the position of D peak

![UV–vis spectra of protein doped AgNPR](image)

**Figure 1.** UV–vis spectra of protein doped AgNPR is shown for the protein concentration of 100 ng ml\(^{-1}\) along with control. A red shift of ca. 150 nm is observed during the interaction.

![UV–vis spectra of protein doped AgNPR at various concentrations](image)

**Figure 2.** UV–vis spectra of protein doped AgNPR is shown for the protein concentration of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 ng ml\(^{-1}\) in (a) Hb@AgNPR (b) LYS@AgNPR (c) BSA@AgNPR and (d) TRY@AgNPR. Each spectra also shows the colour of the corresponding solution having protein concentration of 0, 2, 5, 10, 14 and 20 ng ml\(^{-1}\).
of protein doped AgNPR compared to that of the undoped AgNPR (referred as $\Delta \text{D}$) was calculated for different concentrations of protein and the plot of $\Delta \text{D}$ versus concentration of protein is shown in figure 3. Although the concentration of protein was varied from 1 to 100 ng ml$^{-1}$, good linear fit with $R^2 > 0.95$ was observed in the range of 1–20 ng ml$^{-1}$. The linear fits of all the protein doped AgNPRs up to protein concentration 20 ng ml$^{-1}$ are observed along with fitness ($R^2$) value (The complete dataset of 1–100 ng ml$^{-1}$ can be found in the ESI as figure S1 (available online at stacks.iop.org/NANOX/3/025002/mmedia)). From the linear fit value, we have calculated the limit of detection for each of the protein using the formula of LOD = $3.3 \sigma / \text{s}$; where, $\sigma$ is the standard deviation of response and ‘s’ being the slope of corresponding calibration curve. The obtained LOD values have been tabulated in table 1 which ranges between 2–6 ng ml$^{-1}$, which were significantly lower than the detection range as reported by commercially available protein estimation methods like Biuret assay, Bradford, Lowry’s method.

3.2. Validation by estimating Casein from milk
We have validated our method by estimating Casein from commercially available toned milk and compared the conventional protein estimation techniques like Biuret, Lowry and Bradford method. The Casein concentration has been mentioned in the product description as 3.1 mg/100 ml. We have chosen to validate the method by calculating the recovery percentage for at least two given concentration of protein within and around the range of the method’s detection range. The UV–vis spectra of the estimation is described in figure 4. In figure 4(a), the whole spectra of Cas@AgNPR are described in the range of protein concentration of 1–100 ng ml$^{-1}$ We have appropriately diluted the stock to attain reaction sets having Casein concentration as 3.1 and 31 ng ml$^{-1}$. The calibration curve for Casein doped AgNPR (Cas@AgNPR) was obtained within a range of 1–100 ng ml$^{-1}$. The standard curve obtained by plotting $\Delta \text{D}$ against protein concentration as described in figure 4(b). The calibration curve was fitted to a linear regression model with the equation $y = 1.993x + 7.8853$; where ‘x’ is the protein concentration in ng ml$^{-1}$ and ‘y’ being $\Delta \text{D}$ and $R^2 = 0.98$. Cas@AgNPR were synthesised with final casein concentration of 3.1 and 31 ng ml$^{-1}$ and their UV-visible spectra were recorded. The concentrations of the doped casein were determined from the calibration curve using the $\Delta \text{D}$ value for each casein doped AgNPR.

![Figure 3. Standard curves of the protein doped AgNPRs for the protein concentration within the range of 1–20 ng ml$^{-1}$ is shown for (a) Hb@AgNPR (b) LYS@AgNPR (c) BSA@AgNPR and (d) TRY@AgNPR.](image)

| Protein | Goodness of fit ($R^2$) | Slope(S nm$^{-1}$) | Standard Error ($\sigma$) | LOD = $3.3\sigma / \text{s}$ (ng-ml$^{-1}$) |
|---------|------------------------|-------------------|--------------------------|----------------------------------|
| TRYP    | 0.94                   | 10.66             | 18.3                     | 5.66                             |
| Hb      | 0.99                   | 5.9               | 3.7                      | 2.08                             |
| BSA     | 0.97                   | 14.14             | 18.2                     | 4.24                             |
| LYSO    | 0.95                   | 6.54              | 9.9                      | 4.97                             |
The concentrations determined from the calibration curve were 3.07 and 29.65 ng ml$^{-1}$ respectively that provided a recovery percentage of 99.56% and 95.67%. When we checked concentration of Casein less than 1 ng ml$^{-1}$ and more than 100 ng ml$^{-1}$, the recovery percentage is not acceptable [data not shown], as the linear trend levels off at higher protein concentration. The excellent recovery percentage, especially within 1–20 ng ml$^{-1}$ range has strongly established the application potential of this platform as a protein estimation method in this lower concentration range.

This method was compared with Biuret, Lowry and Bradford method to investigate the linear dynamic range and LOD for the methods. The result has been tabulated in table 3 where as the standard curve for each of the methods are described in ESI as figure S2. Table 3 suggests that the LOD calculated for our method is at least 3 orders less compared to the most sensitive conventional methods.

3.3. Understanding the detection mechanism using TEM and XRD
To understand the mechanism of the detection of protein, TEM images of undoped and protein doped AgNS and their corresponding AgNPR samples were tested. The TEM image of these samples are presented in figure 5 along with their approximate shape diagram. Figures 5(a)–(c) shows the TEM image of Hb@AgNS at Hb concentration of 0, 5 and 20 ng ml$^{-1}$ respectively. The size of the undoped AgNS was calculated to be 4.1 ± 1.2 nm. With increasing protein concentration, the size of the Hb@AgNS was found to increase to 5.1 ± 1.1 nm and 11.6 ± 2.5 nm for 5 and 20 ng ml$^{-1}$ Hb concentration respectively. This is indicative of a systematic increase in the size of Hb@AgNS with increasing protein concentration.

Figure 4. (a) The UV–vis spectra for Casein doped AgNPR with Casein concentration of 0, 2, 5, 10, 20, 30, 50, 70 and 100 ng ml$^{-1}$. (b) Standard curve of the CAS@AgNPR.
In figure 5(d), the undoped AgNPR has been found to be a nearly perfect equilateral triangle of side length of ca. 60 nm. In figure 5(e), the TEM image of Hb@AgNPR ([Hb] = 5 ng ml\(^{-1}\)) shows the geometry of a triangle whose tips have lost the sharpness and have become relatively ‘blunt’. In figure 5(f), the TEM image of Hb@AgNPR ([Hb] = 20 ng ml\(^{-1}\)) shows a significant shape change of the AgNPR from triangular to pentangular one which is a direct result of differential alignment of protein doped AgNS during the NPR forming step, where the individual nanoseeds (Hb@AgNS) are positioned more distant to each other compared to the undoped AgNSs (see scheme 3). The approximated shape for the Hb@AgNPR at ([Hb] = 0, 5 and 20 ng ml\(^{-1}\)) are described in figures 5(g)–(i) respectively.

To further investigate the structural effect of protein doping on AgNPR, firstly we performed XRD studies on protein doped AgNS keeping Hb as the model protein. The size difference between small AgNS particles doped with different concentration of protein was found to be very less as discussed in the previous section because of a very small size distribution range of 5–11 nm for all the samples. The regular XRD profiles of Hb@AgNS and Hb@AgNPR can be found in the supporting material (ESI figures S3 and S4), which do not show any remarkable change in the peak location or intensity in the presence of various protein concentrations either. This is also explainable by the fact that the change of protein concentration within the AgNS or AgNPR interplanar regions alter the network within the crystal plane assembly but does not alter their population density or chemical nature. Hence, an accurate assessment of the geometry of protein doped AgNS assembly can explain the differentiation of spectral properties of corresponding protein doped AgNPRs.

When Hb@AgNSs form crystalline assembly during sample dehydration, the size of the crystal can be accurately measured from the XRD profile, using the Debye–Scherrer formula as presented below [24].
Where, ‘τ’ is the mean size of the ordered (crystalline) domains of AgNS, ‘k’ is the shape factor, usually taken as 0.9, but it varies as the shape of the actual crystallite varies; ‘λ’ is the wavelength of x-ray that is 1.5406 Angstrom for CuKα1 line, ‘β’ is the full width half maximum (FWHM) in radians and ‘θ’ is the Bragg’s angle of the crystal. It is understood that individual nanoseeds are too small to generate strong and sharp diffraction peaks. So, any XRD peak observed, is the result of diffraction from a crystalline assembly of the nanoseeds rather than an individual nanoseed. Hence, to simplify the comparison study between different nanoseeds, one important assumption was made that the number of different protein doped nanoseeds required to attain the crystalline alignment are equal.

Figure 6 shows the comparison of Ag fcc(111) peaks for Hb@AgNPR having Hb concentration of (a) 0, (b) 5 and (c) 20 ng ml\(^{-1}\). The obtained FWHM values (Black double arrows) were used to calculate the diameter of the respective crystalline nanoclusters (Hashed line circle).

**Table 2.** Crystal parameters obtained from Debye Sheerrer formula.

|            | Angle, 2θ (degree) | (hkl) | FWHM (deg) | β FWHM (rad) | 0.9∗λ/βcosθ (λ = 0.1541 nm) |
|------------|--------------------|-------|------------|-------------|-----------------------------|
| AgNPR control | 37.1               | 111   | 0.35       | 0.006109    | 24.16                       |
| AgNPR + P05  | 36.98              | 111   | 0.26       | 0.004538    | 32.53                       |
| AgNPR + P20  | 37.01              | 111   | 0.17       | 0.002967    | 49.75                       |

**Table 3.** Comparison of AgNPR based detection with conventional methods.

| Method    | Linear dynamic range for concentration (μg ml\(^{-1}\)) | LOD (μg ml\(^{-1}\)) |
|-----------|--------------------------------------------------------|----------------------|
| Biuret    | 1–20 μg ml\(^{-1}\)                                   | 5.4                  |
| Lowry     | 1–20 μg ml\(^{-1}\)                                   | 2.2                  |
| Bradford  | 1–20 μg ml\(^{-1}\)                                   | 12.3                 |
| AgNPR     | 1–20 ng ml\(^{-1}\)                                  | 0.0056               |

\[
\tau = \frac{k\lambda}{\beta \cos \theta}
\]

Figure 6 shows the comparison of Ag fcc(111) peaks for Hb@AgNS with Hb concentration of 0, 5 and 20 μg ml\(^{-1}\). The result is summarized in table 2. The 2θ maximum of the (111) peak was shifted from 37.6° for Hb concentration of 0 ng ml\(^{-1}\) to 37.1° and 37.04° for 5 ng ml\(^{-1}\) and 20 ng ml\(^{-1}\) of Hb concentrations respectively; indicating an alteration of the crystal plane growth. The deviation from the original crystal plane alignment was further investigated by comparing the FWHM of the corresponding peak. The values for ‘β’ were measured as 0.35, 0.26 and 0.17 rad for Hb concentration of 0, 5 and 20 ng ml\(^{-1}\). The crystal size obtained from these values were 24.2, 32.5 and 49.8 nm respectively for Hb concentration of 0, 5 and 20 ng ml\(^{-1}\). Hence, there is a significant increase in the size of the crystalline assembly, indicating the increase of the size as well as ‘surface roughness’ of the AgNS as a function of concentration of the doped protein. As a result, during the synthesis of the nanoprisms, protein doped AgNS will be aligned to form the corresponding AgNPR with increased side length and decreased sharpness that result in quantifiable spectroscopic properties of the D-peak of protein doped AgNPR.

**Figure 6.** Analysis of Ag fcc(111) plane from XRD data for Hb@AgNPR having Hb concentration of (a) 0, (b) 5 and (c) 20 ng ml\(^{-1}\). The obtained FWHM values (Black double arrows) were used to calculate the diameter of the respective crystalline nanoclusters (Hashed line circle).
3.4. Assessment of the probe stability

For the proper functioning of a chemical probe, its thermal as well as other physico-chemical stability parameters become very important. The probing efficiency was also checked along with various stabilisation states. The initial characterization methods of the probe were performed at ambient conditions like temperature of 25 °C and pH between 6.5 and 7.0. To test the rigour of the detection system, we have tested the thermal stability and probing sensitivity of AgNPR at a temperature ranging from 30 to 80 °C. To ascertain maximum stability with optimum functionality, we have varied the concentration of PSSS, which is the internal stabilizer for AgNPR. We varied PSSS concentration from 1 to 20 μg ml⁻¹ with a fixed protein concentration of 10 μg ml⁻¹. The result shows excellent thermal stability at all the PSSS concentrations and is described in figure 7. Figures7 (a)–(e) demonstrate the spectra of Hb@AgNPR stabilised with 1, 2, 5, 10 and 20 μg ml⁻¹ of PSSS respectively. For figures 7 (a) and (b), there is hardly any blue shift of the D-peak over the complete temperature range. However, the position of D-peak is too much red shifted (875 and 850 nm respectively) and did not show a very high sensitivity of protein concentration figures 7 (c)–(e) describes a larger extent of a more blue-shifted D-peak and their further blue shift at higher temperature. The temperature response of the D-peaks for Hb@AgNPRs with varying PSSS concentrations are summarized in figure 7(f). It shows a gradual shortening of the zone of absolute thermal stability of Hb@AgNPR from PSSS concentration of 5 to 20 μg ml⁻¹. The almost D-peak trend line parallel to the temperature axis starts to nosedive at higher temperature values like 65 °C for 5 μg ml⁻¹ PSSS, 60 °C for 10 μg ml⁻¹ PSSS and 45 °C for 20 μg ml⁻¹ PSSS. However, the probe efficiency was found to be maximum for 5 μg ml⁻¹ PSSS concentration. Hence, the PSSS concentration of 5 μg ml⁻¹ was chosen as the detection system. Similarly, we also checked the solution stability of Hb@AgNPR at varying pH and found very good stability in the range of pH 4 to 9. The profile can be found in figure S5 in the ESI.
3.5. Mechanism and integration efficiency of protein with AgNPR

We have designed a zeta potential monitoring based experiment to identify the localization of protein after the formation of Hb@AgNS. The hypothesis was based on the expected alteration of zeta potential distribution of AgNS when coated or wrapped with a protein layer. The experimental design is demonstrated in figure 8(a). The scheme describes the two possible fates of the Hb@AgNS when Hb interacts with AgNS. In case of an ideal seeding process, the whole protein molecule, or at least a significantly larger fraction of it, will be integrated inside the AgNS. On the other hand, if seeding is inefficient, the protein wraps around the AgNS. To simulate these two situations, we varied the mode of protein addition to AgNS keeping all the other reaction conditions and stoichiometry unchanged. Firstly, we seeded AgNS with a final concentration of Hb as 100 μg ml⁻¹ to synthesize Hb@AgNS using the methodology discussed in the experimental section. In another set of synthesis,
we did not add any protein during nanoseed synthesis to prepare undoped AgNS. Then the same amount of Hb solution, as used during the seeding process, was added to the unmodified AgNS solution and stirred well to allow the protein to interact with the undoped AgNS. Finally, both these samples along with the reference undoped AgNS were measured for the zeta potential. The result is described in figure 6(b), which shows that there is a very small change of ca. 5 mV for the zeta potential peak of the seeded Hb@AgNS with respect to the undoped AgNS but there is a significantly high shift of around 40 mV for the zeta potential peak of AgNP-Hb adduct where Hb was added post AgNS synthesis. As we can consider the second case as 100% surface wrapping of AgNS by Hb, the amount of protein exposure during seeding process can be approximated to be ca. 12%. This result indicates that during seeding process almost the entire protein (ca. 90%) gets integrated within the AgNS’s interior rather than wrapping around its surface.

Additionally, the significantly less molar concentration of protein compared to that of Ag⁺ (1:270) will hardly allow it to wrap the AgNS. Even if partially covered, the exposed protein on AgNS, as found to be around 12%, will be buried under Ag⁺ layer during the elongation stage, i.e. the second stage of AgNPR synthesis. Though the system has been developed for a single protein detection, it can be extended to a two-protein detection system using weighted average slope calculation method discussed in the section P2 of the ESI.

4. Conclusion

Our method has shown tremendous potential to be used as a standard method for the estimation of the protein concentration in the range of 1–20 ng ml⁻¹ which is significantly lower than the conventional techniques like Bradford or BCA assay. As an added advantage, this method does not require any heating or cooling during any step of the procedure. The time required for the assay and estimation is only 15 min—making it a very rapid method of estimation as well. Current report focussed on the globular proteins. Future work will address the issue of structural property of a protein, considering its globular or fibrous shape, on the estimation efficiency of this method.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

ORCID iDs

Shibsekhari Roy @ https://orcid.org/0000-0002-9681-9517

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