2.1. ABSTRACT

With an objective of developing nano-bioconjugates, we have synthesized monodisperse protein-gold nanoparticles (AuNPs), wherein \(\gamma\)-globulin, a blood plasma protein acted as a capping, reducing as well as a templating agent. As analyzed through various photophysical, biophysical and microscopic techniques such as transmission electron microscope (TEM), atomic force microscope (AFM), conductive AFM (C-AFM), scanning electron microscope (SEM), dynamic light scattering (DLS), optical polarization microscopy (OPM), circular dichroism (CD), and fourier transform infrared spectroscopy (FTIR), we observed that the initial photoactivation of \(\gamma\)-globulin at pH 12 for 3 h resulted in small protein fibres of
ca. 20 ± 5 nm in size. Subsequently, these fibres found to assist in the alignment of the colloidal gold nanoparticles of average diameter of ca. 2-2.5 nm on the surface of protein. At this particular irradiation time, the nano-bioconjugate thus formed, exhibited negligible surface plasmon resonance (SPR) absorption, but showed an intense photoluminescence at 680 nm. Further, irradiation of the solution for 24 h led to the formation of self-assembled long fibres of the protein of ca. 2 ± 0.5 μm in diameter and ca. 18 μm in length. Interestingly, such a morphological transformation resulted in the sequential knock out of the anchored gold nanoparticles resulting in the clustering of the nanoparticles of size ca. 5-6 nm and observation of the surface plasmon resonance band at 520 nm, with the concomitant quenching of luminescence intensity at 680 nm. The observation of light triggered self-assembly of the protein and its effect on controlling the fate of the anchored nanoparticles can be compared with the naturally occurring process such as photomorphogenesis. Furthermore, our approach offers a way to understand the role played by the self-assembly of the protein in ordering and knock out of metal nanoparticles and also in designing of nano-biohybrid materials for medicinal and optoelectronic applications.

2.2. INTRODUCTION

Integration of biotechnology and nanotechnology during the last decade led to the development of hybrid nanostructures, which can have potential applications in photonics, electronics, medicine and catalysis. These new nano-biohybrids
incorporate the properties of both biomaterials and nanoparticles (NP) and both of which can in principle undergo self-assembly to form 1D, 2D and 3D architectures.\(^2\) Among the various synthetic strategies reported, the top-down and bottom-up approaches are being actively explored for the assembling of complex nanostructures.\(^3\) These self-assembly processes are critical for the “bottom-up” construction of nanostructures, which enable the fabrication of three-dimensional structures on the nano- and micrometer length scale.\(^4\) The use of biological systems as templates for creating such nanoarchitectures is being recently explored as a feasible bottom up approach because of the advantages like simplicity, ease of shape control and time effectiveness.\(^5\) The nature is a beautiful example, wherein bacteria, viruses, DNA, and proteins are regularly involved in the creation of unique nanoassemblies to achieve and maximize efficacy of various biological processes.\(^6\)

Despite the remarkable advancements of nanoscience in recent years, relatively little is known about the effects of nanoscale objects on biological systems and their potential applications.\(^7\) The understanding of the interaction of the proteins with nanoparticles is still of great concern for their successful use in nanomedicine, nanotoxicology and nanophotonics.\(^7\) Acquiring of knowledge on the three-dimensional structure of the protein is important for understanding its functions. The proteins are nitrogen-containing macromolecules, which are one of the basic units of life systems. These macromolecules are made up of amino acids as the monomeric units and are joined by peptide linkages.\(^8\) Interactions of the
nanoparticles with the proteins, may alter the protein conformation, or perturb its normal function, and thereby induce unexpected biological reactions.

Of the various biological building blocks, the peptides/proteins and their unique property of self-assembly, have attracted a lot of attention in recent years as platforms for the bottom-up approach in the design of the nanoarchitectures. For example, Wang and co-workers have demonstrated that T1 peptide RGYFWAGDYNF, undergoes self-assembly to form nanofibers with lengths of up to several micrometers and average diameters of 10 nm. By using the similar strategy, Pochan and co-workers have synthesized sheets of gold nanoparticles. In these cases, the non-twisted laminated β-sheet peptide fibrils acted as a template, and which induced the gold nanoparticles to undergo intercalation within fibril laminates. The formation of the aggregates of nanoparticles were induced due to the electrostatic interactions between the positively charged lysine side chains of the fibrils and the charged nanoparticles. The assembly driven by electrostatic interactions between the nanoparticles and peptide scaffolds were very effective for the creation of supramolecular architectures of small negatively charged nanoparticles (<6 nm). However, their wide applications were limited by the size of the nanoparticles, since the larger nanoparticles were known to normally bind randomly to the fibril surfaces.

Moreover, there were also reports wherein the modifications in the protein fibre can be catalyzed by the interaction with nanophase materials leading to the growth of the extended assemblies. Thus, the process of protein self-assembly has
been studied extensively and was widely established that such a process has been found to be sensitive to the external stimuli like pH, light, pressure and temperature.\textsuperscript{14} By having control over the process of self-assembly of the proteins, it is possible to generate systems that can be effectively utilized as template/scaffolds for the synthesis of the ordered nanostructures. Moreover, the realm of usefulness of the well defined nanoscale properties of the metal nanostructures can be significantly enhanced by harnessing interparticle properties.\textsuperscript{15} Thus, the synthesis of controllable ordered nanoparticle assemblies has been challenging in the context of their potential applications in the development of nanoscale electronic and optical devices.\textsuperscript{14-16} The conformational changes occurring in the protein upon self-assembling processes is, often, crucial for its various functions. A large number of efforts have been devoted to the fabrication of the nanoparticle assemblies,\textsuperscript{17} whose peculiar recognition mechanisms to-date remain elusive. However, there are important scientific and practical issues that need additional attention to further improve these methods and also to determine, which applications are best targeted with superstructures constructed by using these biological building blocks.

Among the various biomolecules for functionalization of the nanoparticles, we chose $\gamma$-globulin, since it belongs to a class of blood plasma proteins and is considered as antibody that helps to fight infections and diseases.\textsuperscript{18} Herein, we describe the synthesis of monodisperse gold nanoparticles employing $\gamma$-globulin as a capping, reducing as well as templating agent. We observed pH, light and concentration dependent morphologies as analyzed through photophysical,
biophysical and microscopic techniques such as atomic force microscopy (AFM), transmission electron microscopy (TEM), scanning electron microscopy (SEM) and optical electron microscopy (OPM). Interestingly, the initially UV irradiation triggered self-assembly of γ-globulin found to control the anchoring of the nanoparticles, whereas on further irradiation led to the formation of long fibres, which eventually knocked out the ordered nanoparticles from the protein matrix. Interestingly, we observed that the protein γ-globulin acts as a capping, reducing as well as templating agent for ordering of these nanoparticles. Uniquely, this approach offers a way to understand the role played by the self-assembly of the protein in sequential ordering and knock out of the metal nanoparticles and also in the design of novel nano-biohybrid materials for medicinal and optoelectronic applications.

2.3. RESULTS AND DISCUSSION

2.3.1. Synthesis of Protein Functionalized Gold Nanoparticles

Synthesis of the protein functionalized biocompatible gold nanoparticles (AuNPs) involved the reaction of the aqueous solution of hydrogen tetrachloroaurate hydrate (HAuCl₄) with γ-globulin. The formation of the gold nanoparticles, thus formed, was found to depend on various external factors such as the reaction temperature and pH, in addition to the concentration of the capping agent used. These factors significantly altered the reduction and capping capability of the protein and hence the reduction kinetics, the nucleation and crystal growth and thereby, the formation of stable monodisperse gold nanoparticles. To study the
effect of concentration on the formation of the gold nanoparticles, we have varied the protein concentration (w/v = 0.1, 0.2 and 0.5%) and carried out the reaction with HAuCl₄. The variation in the protein concentration led to the formation of the nanoparticles with different sizes. It is well-known that the optical properties of the metal nanoparticles are strongly size and shape dependent.⁴,⁵ Under these conditions, we observed the formation of size and shape dependent surface plasmon resonance (SPR). The formation of the surface plasmon resonance band was a clear indication that the γ-globulin can act as both reducing agent as well as a capping agent, thereby indicating the formation of stable gold nanoparticles. For example, at ca. 0.1% (w/v) of γ-globulin, we observed the formation of AuNPs having the surface

![Graph](image_url)

**Figure 2.1.** Changes in the surface plasmon resonance of γ-globulin-Au nanoparticles (AuNPs) synthesized at 60 °C from a solution containing a) 0.1%, b) 0.2% and c) 0.5% (w/v) of γ-globulin and HAuCl₄ (2 mM).
plasmon band at 548 nm (Figure 2.1), whereas at protein concentrations ca. 0.2% and 0.5% (w/v), we observed a blue shifted SPR bands at 540 and 528 nm, respectively. This blue shift in the absorption was attributed to the eventual narrowing of the size by varying the globulin concentration. However, the gold nanoparticles thus formed under these conditions were found to be highly polydisperse in nature.

To understand the changes observed in the surface plasmon resonance properties of γ-globulin-AuNPs at 60 °C, we have analyzed the samples through dynamic light scattering (DLS) and microscopic techniques such as TEM, AFM and SEM. At lower concentrations of γ-globulin (ca. 0.1-0.2% w/v) and pH 7, we observed predominantly a mixture of aggregated structures (Figure 2.2A) such as spherical, plate-like and irregular trigonal, hexagonal and polygonal shapes as confirmed through TEM (Figure 2.2C) and DLS analysis (Figure 2.3). The γ-globulin capped nanoparticles formed under these conditions showed an average diameter of

![Figure 2.2](image.png)

**Figure 2.2.** TEM images of the γ-globulin Au nanoparticles (AuNPs) synthesized at various protein concentrations (w/v) and HAuCl₄ (2 mM) by heating at 60 °C A) 0.1%, B) 0.2%, and C) 0.5%.
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ca. 72 nm. Moreover, the size and stability of AuNPs showed negligible changes with the further increase in concentration of the protein after ca. 0.5% (w/v) of γ-globulin. The results of these initial characterization studies have led us to select ca. 0.5% (w/v) of γ-globulin for further studies to improve their monodispersity by adopting different methods like photoirradiation and changing the pH of the reaction mixture.

Figure 2.3. Dynamic light scattering data of 0.5% γ-globulin Au nanoparticles synthesized at 60 °C.

2.3.2. Effect of pH and Irradiation on Bioconjugate Formation

To synthesize monodisperse nanoparticles, we have employed irradiation technique and varied the irradiation time (ca. 1-24 h) as well as pH of the solution (ca. 7-12). When an aqueous solution of HAuCl₄ was irradiated for 1 h with a light
source of 350 nm in presence of ca. 0.5% of γ-globulin at pH 7, we observed the formation of the surface plasmon resonance (SPR) band centred at around 526 nm, corresponding to the nanoparticles. However, after 24 h of irradiation under these conditions, there was an increase in the intensity, but we could not observe any significant shift in the position of SPR band at 526 nm (Figure 2.4). This spectral observation indicated that the nanoparticles thus formed even after 24 h irradiation were stable and homogeneous. Of the various conditions investigated, interestingly, we observed the formation of homogeneous size distribution of ca. 27 nm of AuNPs (Figure 2.5) only under the irradiation conditions.

**Figure 2.4.** Surface plasmon resonance of 0.5% (w/v) γ-globulin-Au nanoparticles (AuNPs) synthesized by irradiation with 350 nm in presence of HAuCl₄ (2 mM) after a) 1 h irradiation b) 24 h irradiation.
These protein concentration dependent studies indicate that γ-globulin having amino and thiol groups\textsuperscript{12} not only found to reduce the Au(III) ions, but also stabilize the nanoparticles. Moreover, the formation of the narrower size distribution of the nanoparticles under these conditions with the increase in the concentration of the protein supports the fact that γ-globulin acts both as a controller of nucleation as well as stabilizer as reported in the case of chitosan based gold nanoparticles.\textsuperscript{19} Furthermore, our results indicated that though γ-globulin was able to reduce Au(III) ions as well as stabilize the nanoparticles, but the homogeneous size distribution of AuNPs was observed only upon irradiation, which demonstrate the importance of light excitation in the formation of the nanoparticles as reported in the literature.\textsuperscript{20,21} In contrast, when the pH of the solution was increased to 12 and irradiated for ca. 1-3 h, we observed negligible formation of the

\begin{figure}
\centering
\begin{subfigure}{0.4\textwidth}
\includegraphics[width=\textwidth]{figureA.png}
\caption{A) TEM images of 0.5% γ-globulin capped gold nanoparticle by irradiation at pH 7 with a light source of 350 nm}
\end{subfigure}
\begin{subfigure}{0.4\textwidth}
\includegraphics[width=\textwidth]{figureB.png}
\caption{B) DLS data of 0.5% γ-Globulin capped gold nanoparticles}
\end{subfigure}
\caption{Figure 2.5. A) TEM images of 0.5% γ-globulin capped gold nanoparticle by irradiation at pH 7 with a light source of 350 nm B) DLS data of 0.5% γ-Globulin capped gold nanoparticles.}
\end{figure}
surface plasmon band. However, when the irradiation time was increased further for 12 h, we observed the evolution of the surface plasmon resonance band centered at around 520 nm and which became prominent after 24 h irradiation (Figure 2.6). In the emission spectrum, on the other hand, we initially observed intense red emission having maximum at *ca.* 680 nm along with a less prominent band at 450 nm after 3 h of irradiation (Figure 2.7). With increase in the irradiation time, we could observe quenching in the fluorescence intensity at *ca.* 680 nm. Inset of Figure 2.7 shows visual observation of fluorescence changes after irradiation of 24 h. In addition, we observed a visual color change of the solution from pale yellow to deep brown with the increase in irradiation time from *ca.* 3 to 24 h (Figure 2.8).

**Figure 2.6.** Irradiation time-dependent changes in the absorption spectra of the solution containing HAuCl₄ and 0.5% (w/v) of γ-globulin. Irradiation time, a) 3, b) 6, c) 12 and d) 24 h.
Figure 2.7. Irradiation time-dependent changes in fluorescence of HAuCl₄ solution containing 0.5% (w/v) of γ-globulin. Irradiation time, a) 3, b) 6, c) 12 and d) 24 h. Inset shows visual observation of fluorescence changes after irradiation time, a) 3 and d) 24 h. (λₑₓ 350nm).

Figure 2.8. Visual observation of irradiation time-dependent changes in the color of HAuCl₄ solution containing 0.5% (w/v) of γ-globulin after irradiation time, a) 3 and b) 24 h.
Further, we have carried out the control experiments using γ-globulin alone under similar conditions. When γ-globulin alone was irradiated in the absence of HAuCl₄ under similar experimental conditions, it showed negligible absorption in the surface plasmon resonance region but exhibited only blue emission having maximum ca. 450 nm (Figure 2.9) as reported in the case of serum albumins.²⁵ Figure 2.10 shows the optical polarization microscopy (OPM) images monitoring the changes in the emission of γ-globulin-AuNPs at different stages of γ-globulin and HAuCl₄ (2 mM), upon irradiation using 360 nm excitation. We observed an intense red emission after the irradiation period of ca. 3-6 h of a solution containing 0.5% (w/v), which was supported by the emission observed at 680 nm (Figure 2.7). On

**Figure 2.9.** Fluorescence spectra of the γ-globulin protein fibres after the irradiation for 24 h using 350 nm light source in the absence of AuNPs. Inset shows the visual fluorescence of the protein alone.
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Further irradiation for a period of ca. 12 h, we observed the weakening of the red emission as shown in Figure 2.10B. When the solution was irradiated additionally for a period of ca. 24 h, interestingly, we observed the emission change from red to blue (Figure 2.10C). The results of the optical polarization microscopy were in accordance with the observations made in the steady-state and time-dependent fluorescence measurements for γ-globulin-AuNPs during the progress of irradiation.

![Figure 2.10](image)

**Figure 2.10.** Time-dependent optical polarisation microscopic (OPM) images showing the emission changes during irradiation of a solution containing 0.5% (w/v) of γ-globulin plus HAuCl₄ (2 mM) at pH 12. Irradiation time, A) 6 B) 12 and C) 24 h. Excitation wavelength, 360 nm. Scale bars: (A), (B), and (C) 2 μm.

### 2.3.3. Morphological Analysis of Self-Assembled Bioconjugates

To have a better understanding of the irradiation time-dependent changes in the photophysical properties, we analyzed the time-dependent morphological changes of the solution containing ca. 0.5% (w/v) of γ-globulin and HAuCl₄ at pH 12 through TEM analysis. After the initial period of irradiation for ca. 3 h, we observed the ordering of AuNPs having an average diameter of ca. 2 ± 0.5 nm into the one
dimensional array within the protein fibre size of \( ca. 20 \pm 5 \) nm (Figure 2.11). With the further increase in irradiation time, we observed the growth of the protein fibres into formation of longer fibres. At \( ca. 24 \) h of irradiation, we observed the fibres having dimensions of micrometers of \( ca. 18 \pm 2 \) \( \mu \)m in length and \( ca. 2 \pm 0.5 \) \( \mu \)m in diameter (Figure 2.12). Simultaneously, destruction of the ordered nanoparticles was observed, which sequentially knocked out of the fibre and clustered with an average size of \( ca. 5 \pm 1 \) nm (Figure 2.12B).

**Figure 2.11.** Transmission electron microscopic (TEM) images of A) 0.5\% (w/v) of \( \gamma \)-globulin capped gold nanoparticles formed on the self-assembled protein fibres by irradiation at 350 nm for \( ca. 3 \) h. Magnified images (B, C and D) of the area marked by the black box in panel A, B and C respectively.
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The negligible formation of the surface plasmon band during the initial irradiation times of less than 12 h could be attributed to the formation of smaller nanoparticles of size ca. 2-2.5 nm as evidenced through TEM analysis. The evolution of the surface plasmon resonance band centred at around 520 nm with the increase in the irradiation time further supports the fact that initially formed nanoparticles transform into bigger particles of size around 5 nm, since the observation of surface plasmon resonance band is a signature of the metal nanoparticle.\textsuperscript{22} The initial observation of an intense red emission having maximum at ca. 680 nm along with a less prominent band at 450 nm confirms the fact that the size of the arrayed gold nanoparticles was very small and of the order of ca. 2-2.5 nm. However, when the irradiation period was increased to 24 h, the emission at 680 nm was found to be

Figure 2.12. A) Transmission electron microscopic (TEM) images showing the formation of long protein fibres and sequential knockout of gold nanoparticles after 24 h irradiation of a solution containing 0.5\% (w/v) of \( \gamma \)-globulin plus HAuCl\(_4\) (2 mM) at pH 12 with a light source of 350 nm. B) Magnified image of the marked oval area of the panel A.
significantly quenched, albeit with negligible changes in the emission intensity at 450 nm.

From the microscopic data, it was clear that when the 0.5% γ-globulin containing gold salt solution at pH 12 irradiated for 3 h resulted in fibrillar network of the protein with arrayed gold nanoparticles. The time-dependent microscopic investigations showed that the increase in the irradiation resulted in hierarchical assembly of the fibrillar network of the protein with arrayed gold nanoparticles leading to the formation of micrometer long fibre, wherein pH of the solution was found to play a crucial role in the protein aggregation. These observations clearly indicate that the emission at 680 nm was mainly due to the fact that in the initial state, the particles bound to the fibres were very small (<5 nm) having negligible surface resonance plasmon band but possess prominent red emission.\textsuperscript{23,24} While during the fibrillar growth of the protein, the particles get clustered with evolution of the surface resonance plasmon band and prominent quenching of emission intensity at 680 nm. These changes in the photophysical properties observed were coherent with the time dependent changes observed in the alignment of the ordered nanoparticles and self-assembly of the protein. Moreover, when we have done the morphological analysis of the γ-globulin alone in the absence of HAuCl\textsubscript{4} under similar experimental conditions, we could observe the formation of the protein nano fibres. Furthermore, with the increase in irradiation time, we observed the transformation of the initially formed short self-assembled γ-globulin fibres to the long fibres as evidenced through TEM analysis (Figure 2.13).
Figure 2.13. TEM images of the protein fibres in the absence of AuNPs, after A) 6 h and B) 24 h irradiation with 350 nm light source.

To further evidence the morphological transformations, we have employed other microscopic techniques and analyzed the samples under different conditions. Figure 2.14 shows the AFM images of 0.5% (w/v) γ-globulin-AuNPs irradiated for ca. 3-24 h at pH 12. As in the case of TEM analysis, the AFM analysis of samples after an irradiation period of 3 h, we observed the formation of small protein fibres of ca. 20 ± 5 nm. Further irradiation of these small protein fibres resulted in their self-assembly and finally, we observed well developed micrometer long protein fibres of ca. 18 ± 2 μm. Similar results were obtained from SEM analysis, wherein we initially observed the small fibres, which bundled to form micrometer length long protein fibres after longer periods of irradiation (Figure 2.15). Unfortunately, the initially formed regular small fibril like structures of the γ-globulin with ordered array of gold nanoparticles couldn’t be clearly visualized in the AFM and SEM images since the average size of these nanoparticles was between 2-2.5 nm.
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Figure 2.14. Atomic force microscopic (AFM) images showing the different stages of the growth of the protein fibres after irradiation of a solution containing 0.5% (w/v) of γ-globulin plus HAuCl₄ (2 mM) at pH 12 with a light source of 350 nm. Irradiation time, A) 3 B) 12 and C) 24 h.

Figure 2.15. Scanning electron microscopy (SEM) images showing the different stages of the growth of the protein fibres after irradiation of a solution containing 0.5% (w/v) of γ-globulin plus HAuCl₄ (2 mM) at pH 12 with a light source of 350 nm. Irradiation time, A) 3 B) 12 and C) 24 h.

2.3.4. Conductive AFM (C-AFM) Study of the Bioconjugates

To understand conducting properties, the electrical transport characteristics of these fibres with and without the alignment of nanoparticles were measured using conductive probe atomic force microscopy (C-AFM). Since the gold
nanoparticles were encapsulated within the protein matrix, the interparticle distance was found to be close enough to transfer electrons through the protein matrix. The $I-V$ responses at different positions of the image was measured and the representative $I-V$ curves are shown in Figure 2.16. From this figure, it was clear that

![Graph](image)

**Figure 2.16.** $I-V$ curve obtained after irradiation for a period of 3 h of 0.5% (w/v) of $\gamma$-globulin plus HAuCl$_4$ (2 mM) at pH 12 with a light source of 350 nm obtained from the aqueous solution dropcasted on HOPG substrate.

during the initial period of irradiation, where the nanoparticles were aligned, the fibres exhibited conductivity in the range of semiconductors. This result clearly demonstrates the fact that the AuNPs aligned within the protein fibrils were able to mediate the transfer of electrons liberated by the enhanced third-order nonlinear susceptibility of AuNPs near their SPR frequency. After irradiation for longer hours (24 h), these fibres behaved almost like an insulator (Figure 2.17). This conductivity measurements gave further evidence for the ordering of nanoparticles on the
protein fibre in the initial state of photoactivation since light can be transported through the aligned nanoparticles of substantially smaller sizes.\textsuperscript{6f,24c-d} The insulator behaviour of the $I$-$V$ responses after 24 h irradiation also supports the fact that the nanoparticles thus were clustered out of the long fibres after longer periods of irradiation as characterized through microscopic techniques.

![Figure 2.17. I-V curve obtained after irradiation for a period of 24 h of 0.5% (w/v) of $\gamma$-globulin plus HAuCl$_4$ (2 mM) at pH 12 with a light source of 350 nm obtained from the aqueous solution dropcasted on HOPG substrate.](image)

2.3.5. Conformational Analysis of the Bioconjugates

With an aim to understand the protein conformational changes, we have investigated the chiroptic properties of $\gamma$-globulin-AuNPs system during different irradiation time intervals through circular dichroism (CD) spectroscopy. The CD experiments were performed at 25 °C and all the spectra were recorded between 190 and 350 nm at a rate of 50 nm/min. The CD spectrum of 0.5% (w/v) of $\gamma$-
globulin alone in the native state exhibited the characteristic CD response with a negative ellipticity at 218 nm (Figure 2.18). However, after the irradiation for 24 h, we observed a red shift of ca. 18 nm and the protein displayed a negative CD signal at 236 nm. In contrast, when a solution of 0.5% (w/v) of γ-globulin containing 2 mM of HAuCl₄ at pH 12 was irradiated for 3 h, we observed both negative and positive CD signals at around 232 and 250 nm, respectively (Figure 2.19). On further irradiation for 12 h, we observed the decrease in intensity of ca. 66% and 27%, respectively, of both the negative and positive CD signals. Furthermore, after irradiation for 24 h, the CD spectrum of the solution showed a blue shift of ca. 5 nm of the negative signal, while the positive signal showed ca. 80% decrease in the

![CD Spectral Changes](image)

**Figure 2.18.** CD spectral changes of 0.5% (w/v) of γ-globulin in aqueous medium a) before irradiation and b) after irradiation for a period of 24 h.
intensity. The observed red shifted far UV-CD signal could be assigned to the antiparallel β-sheet structure on the basis of literature reports,\textsuperscript{27,28} while the positive CD signal around 250 nm could be likely due to tertiary folding of the polypeptide chain.\textsuperscript{29}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{cd_spectral_changes.png}
\caption{CD spectral changes during the protein fibre growth after photoirradiation of 0.5\% γ-globulin capped gold nanoparticles in aqueous medium for a period of a) 3 b) 12 and c) 24 h with a light source of 350 nm.}
\end{figure}

Additional information for the observation of the protein conformational changes was obtained through fourier transform infrared (FTIR) spectroscopy. The FTIR spectrum of the solution containing 0.5\% (w/v) of γ-globulin alone exhibited characteristic protein amide I and amide II bands at 1639 cm\textsuperscript{-1} and 1549 cm\textsuperscript{-1}, respectively. In contrast, when the protein alone was irradiated for 24 h, we
observed a red shift of ca. 58 cm\(^{-1}\) in the case of the amide I peak (1697 cm\(^{-1}\)), while the amide II band showed a blue shift of ca. 3 cm\(^{-1}\) (1546 cm\(^{-1}\)).

The FTIR analysis of a solution containing 0.5% (w/v) of \(\gamma\)-globulin and HAuCl\(_4\) showed similar spectrum as that of the protein alone in the dark. However, after irradiation of this solution for 3 h, we observed the amide I band at ca. 1698 cm\(^{-1}\) with a bathochromic shift of ca. 59 cm\(^{-1}\), while the weak amide II band appeared at ca. 1545 cm\(^{-1}\) with a blue shift of ca. 4 cm\(^{-1}\). On further irradiation for 24 h, we observed both the amide bands I and II at 1648 and 1501 cm\(^{-1}\), respectively, thereby confirming the protein conformational changes in accordance with the observations made through time-dependent fluorescence, CD and microscopic techniques. These results confirmed the fact that there are secondary structure perturbations in the protein due to the array of gold nanoparticles. The observation of an intense band for amide I region at 1639 cm\(^{-1}\).

**Table 2.1.** Data summarizing FTIR spectroscopic changes during the protein fibrillar growth.

<table>
<thead>
<tr>
<th>Protein irradiation conditions*</th>
<th>Amide I</th>
<th>Amide II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein alone at pH 12</td>
<td>1639 cm(^{-1})</td>
<td>1549 cm(^{-1})</td>
</tr>
<tr>
<td>Protein alone at pH 12 after 24 h irradiation</td>
<td>1697 cm(^{-1})</td>
<td>1546 cm(^{-1})</td>
</tr>
<tr>
<td>0.5 GLAN* after 3 h irradiation</td>
<td>1698 cm(^{-1})</td>
<td>1545 cm(^{-1})</td>
</tr>
<tr>
<td>0.5 GLAN* after 24 h irradiation</td>
<td>1648 cm(^{-1})</td>
<td>1561 cm(^{-1})</td>
</tr>
</tbody>
</table>

*0.5 GLAN = 0.5 % \(\gamma\)-globulin gold nanoparticle conjugate, \(\lambda_{ex} = 350\) nm.
for the protein alone indicate that the native globulin is in the β-sheet-rich conformation, but on irradiation and in the presence of nanoparticles, it transformed to antiparallel β-sheet conformation as evidenced through the characteristic peak at 1698 cm\(^{-1}\) and literature reports. The conformational change was further evidenced by the FTIR analysis of the solution, which was irradiated for 24 h, wherein we observed the shift of the amide I band to 1648 cm\(^{-1}\), indicating thereby the reversal of the conformation to the β-sheet. These results clearly demonstrate that the self-assembly of the protein proceed through the conformational rearrangement from β-sheet to antiparallel β-sheet and random coiled conformations, which act as template for the ordering and annihilation of nanoparticles during the irradiation conditions.

A schematic representation showing the irradiation induced assembly of the AuNPs at pH \(\text{ca. } 12\) could be clearly understood from Figure 2.20. The initial photoactivation of the γ-globulin in the presence of HAuCl\(_4\) resulted in the nucleation of the gold nanoparticles. Further photoactivation of the solution for 3 h led to the formation of the protein fibres of \(\text{ca. } 20 \pm 5\) nm, which interestingly assisted the alignment of the colloidal gold nanoparticles of average diameter of \(\text{ca. } 2-2.5\) nm. As evidenced through various microscopic and spectroscopic analysis, the further photoactivation of the solution for 24 h resulted in the growth of the initially self-assembled small protein fibres into micrometer long fibres of \(\text{ca. } 18 \pm 2\) μm. Such transformation resulted in the knock out of the aligned nanoparticles from the surface of the protein fibre and clustering of the nanoparticles outside the self-assembly.
Figure 2.20. Schematic illustration of the hierarchical assembly process in the fibril formation A) protein (γ-globulin), B) nucleation & growth of AuNPs, C) alignment of the nanoparticles over the self assembled γ-globulin protein fibre and D) knocking out of the aligned nanoparticle from the γ-globulin fibre.

assembled protein fibre. The ascertained morphological changes that occurred during the photoactivation process can be likened to the naturally occurring photomorphogenesis. The entire process of photomorphogenesis proposed, consists of three successive phenomena viz., generation, ordering, and sequential knock out of AuNPs from the γ-globulin protein fibre and which were predominantly controlled by the irradiation time dependent conformational changes of the protein.
2.4. CONCLUSION

In summary, the monodisperse biocompatible γ-globulin capped gold nanoparticles have been synthesized, wherein the protein acted as a capping, reducing as well as templating agent. Interestingly, γ-globulin when irradiated at pH 12 resulted in the self-assembly of the protein, which eventually grown into extended protein fibres of micrometer in length. We have further observed that the hierarchical assembly process plays a crucial role in aligning the nanoparticles and growth of the protein fibres causes the sequential knock out of the gold nanoparticles. Moreover, the observation of light triggered self-assembly of the protein and its effect on controlling the fate of the anchored nanoparticles can be compared with the naturally occurring process such as photomorphogenesis. Uniquely, our approach offers a way to understand the role played by the self-assembly of the protein, in particular γ-globulin in sequential ordering and knock out of the gold nanoparticles and also in the design of nano-biohybrid materials for medicinal and optoelectronic applications.

2.5. EXPERIMENTAL SECTION

2.5.1. General Techniques

The electronic absorption spectra were recorded on Shimadzu UV-3101 or -2401 PC UV/Vis/NIR scanning spectrophotometer. The fluorescence spectra were recorded on a SPEX-Fluorolog F112X spectrofluorimeter. IR spectra were recorded on a Perkin–Elmer Model 882 IR spectrometer. The pH measurements were carried
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out using ELICO Model L1-120 digital pH meter, which was calibrated using standard buffer solutions of pH 4 and 9.2. CD spectra were obtained on a JASCO-J-810 Circular Dichroism Spectropolarimeter. Irradiation experiments were carried out using Rayonet Photochemical Reactor (RPR 350 nm). All experiments were carried out at room temperature (25 ± 10 °C), unless otherwise mentioned.

2.5.1.1. Transmission Electron Microscopy (TEM): TEM analysis was performed on JEOL 100 kV high resolution transmission electron microscope. A drop of nanoparticle solution was placed on the top of carbon-coated Cu grid. The grids were mounted on reverse tweezers and the samples were dried by a vacuum pump under reduced pressure for 1 h at room temperature. The accelerating voltage of the transmission electron microscope was 100 kV and the beam current was 65 A. Samples were imaged using a Hamamatsu ORCA CCD camera.

2.5.1.2. Scanning Electron Microscopy (SEM): SEM studies were carried out using ZEISS EVO MA and LS series scanning electron microscope. The operating range was between 100-230V at 50-60Hz single phase with a consumption of 2.5 kVA. The aqueous solution of the nanoparticle was drop casted directly on the top of the aluminium grid and the solvent was allowed to evaporate at ambient conditions. The obtained sample was coated with copper in order to attain the easy passage flow of electrons.

2.5.1.3. Atomic Force Microscopy (AFM): The AFM images were recorded under ambient conditions using a NTEGRA (NT-MDT) and operated with the use of tapping mode regime. Micro-fabricated TiN cantilever tips (NSG10) with a
resonance frequency of 299 kHz and a spring constant of 20-80 Nm\(^{-1}\) were used. AFM section analysis was done offline. Samples for the imaging were prepared by drop casting the solution of nanoparticle on freshly cleaved mica surfaces at the required concentrations and at ambient conditions.

2.5.1.4. Conductive Atomic Force Microscopy (C-AFM): The conductive atomic force studies have been done by using tapping mode to image protein-nano particle conjugate with a resonance frequency of 299 kHz and a spring constant of 20-80 Nm\(^{-1}\). Micro-fabricated diamond coating was doped with nitrogen cantilever tips (DSP11) and employed an operator-activated external circuit to switch to contact mode for point contact electrical characterization of the selected positions. After completion of the measurement, we have deactivated the external circuit to prevent the damage of the protein fibres. Samples for imaging and electrical properties were prepared by drop casting the sample on HOPG at required concentrations at ambient conditions.

2.5.1.5. Dynamic Light Scattering Experiments (DLS): The DLS study of the nanoparticle solutions were carried out on a Nano Zeta Sizer, Malvern instruments. The samples were prepared in water at required concentrations. The light scattering experiments were performed under low polydispersity index by using glass cuvettes. The hydrodynamic diameters and polydisperse indices of the nanoparticles were determined using a Malvern Zeta Nano-ZS system.

2.5.1.6. Optical Polarized Microscopy (OPM): The optical polarized microscopy was performed using NikonHFX35A Optiphot-2 polarized-light optical
microscope, equipped with a Linkam THMS 600. For this analysis, the samples of nanoparticles were prepared in water. We drop casted the samples on a fresh glass plates and evaporate at ambient conditions with an excitation wavelength of 360 nm. The morphological transformations were followed, monitored and the images were taken using a CCD camera attached to NikonHFX35A.

2.5.2. Materials

Tetrachloroaurate hydrate (HAuCl₄), and γ-globulin were purchased from Sigma Aldrich and were used as such without further purification. Doubly distilled water was used for all the studies.

2.5.3. Synthesis of Gold Nanoparticles

2.5.3.1. Thermal Synthesis of Gold Nanoparticles in Presence of γ-Globulin: Aqueous solution of hydrogen tetrachloroaurate hydrate (2 mM) was mixed with different concentrations of γ-globulin (w/v = 0.1, 0.2 and 0.5%) and was heated at 60 °C for 4 h (GLAu). The reaction was cooled to room temperature, filtered and ultra-centrifuged to remove the unbound proteins. The nanoparticles synthesized were further characterized through AFM, SEM, TEM and DLS analyses.

2.5.3.2. Synthesis of Gold Nanoparticles in Presence of γ-Globulin by Irradiation: Aqueous solution of hydrogen tetrachloroaurate hydrate (2 mM) was mixed with different concentrations of γ-globulin (w/v = 0.1, 0.2 and 0.5%) and subjected to irradiation (3 h-24 h) in quartz tubes using a Rayonet Photochemical
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Reactor (RPR 350 nm). The resulting colloidal solutions were characterized using AFM, SEM, TEM and DLS analyses.

2.5.3.3. Synthesis of Gold nanoparticles by Irradiation and Changing the pH: In a typical experiment, 2 mM aqueous solution of hydrogen tetrachloroauration hydrate (99.999%) was mixed with 0.5% (w/v) of γ-globulin. NaOH solution (0.5 mL, 1 M) was added drop-wise to the above mixture after 2 min with vigorous stirring and the mixture was irradiated using Rayonet Photochemical Reactor by using a light source of 350 nm. The color of the solution changed from light yellow to brown. The nanoparticles on the protein fibres synthesized were further characterized through AFM, SEM, TEM and OPM analyses at different time intervals.

2.6. REFERENCES


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