β-Lactoglobulin-gold nanoparticles interface and its interaction with some anticancer drugs – an approach for targeted drug delivery

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ABSTRACT

The protein-nanoparticle interface plays a crucial role in drug binding and stability, in turn enhancing efficacy in targeted drug delivery. In the present study, whey protein β-lactoglobulin (BLG) is conjugated with gold nanoparticles (AuNP) and its interaction with curcumin (CUR) and gemcitabine (GEM) has been explored. Further, AuNP-BLG conjugate interactions with anticancer drugs were characterized using dynamic light scattering (DLS), zeta potential, UV-visible, Raman spectroscopy, fluorescence, circular dichroism along with molecular dynamics simulation. The cytotoxicity studies were performed using breast cancer cell lines (MCF-7). ~8 μM of BLG resides on AuNP (~29 nm) surface revealed by DLS. Raman scattering of AuNP-BLG conjugate showed orientation of the central calyx of BLG towards solvent. BLG fluorescence confirmed the interaction between AuNP-BLG conjugate with drugs and indicated strong binding and affinity (for CUR $K_D = 3.71 \times 10^8$ M$^{-1}$, $n = 1.83$, and for GEM $K_D = 3.78 \times 10^8$ M$^{-1}$, $n = 0.94$), enhanced in the presence of AuNP. CD and Raman analysis exhibited selective hydrophilic and hydrophobic conformations induced by drug binding. Computational studies on BLG-drug complexes revealed that the residues Pro38, Leu39 and Met107 are largely associated with CUR binding, while GEM interaction is via hydrophilic contacts which significantly matches with spectroscopic investigation. $IC_{50}$ values were calculated for all components of this loading system on MCF-7. The possible mechanisms of interaction between AuNP-BLG with anticancer drugs has been explored at the molecular level. We believe that these conjugates could be considered in the targeted drug delivery studies for cancer research.

1. Introduction

Currently, nanotechnology-based drug delivery systems are rapidly developing. Materials at the nanoscale are being effectively used for either diagnostic or therapeutic applications. Nanotechnology extends several benefits in treating human diseases, delivering medicine towards the target. Nanomaterials are synthesized/designed at the atomic level, and they are usually in the range of 1-100 nm. Their small size confers free movement in the human body fluid as compared to the larger materials (Goenaga-Infante & Larsen, 2014; Zhou et al., 2011). Nanomaterials exhibit specific properties i.e. surface to volume ratio, enhanced permeation and retention effect, electric, magnetic, and biological properties which make them effective for use as a vehicle for precise drug delivery systems to the targeted tissue/cells. Most important property of nanoparticles is that they stay for a longer time in the circulatory system which enables the release of the drugs as a specified dose (Tekie et al., 2020). It is reported that nanostructures penetrate in cell/tissue, facilitating effective internalization of drugs in cells. Further, the choice of nanoparticles depends on biocompatibility and physicochemical features of the drugs (Chandran et al., 2017). The conjugation of biomaterial with nanoparticles is a strategic approach and is growing very rapidly in recent times. The design of nanoparticle-based drug delivery systems is primarily characterized biophysically and biochemically. For example, Albumin, a major and important carrier protein in human serum that transport micronutrients fatty acids, vitamins, drugs etc. (Mariam et al., 2016). Its adsorption on silver and gold nanoparticle has been well characterized using biophysical and biochemical methods (Mariam et al., 2011; Mariam et al., 2016; Mariam et al., 2017; M. Waghmare et al., 2018).

β-Lactoglobulin (BLG, 18.2 kDa), is a globular and abundant whey protein in bovine milk. The secondary structure of β-Lactoglobulin has a lipocalin fold involving an 8-stranded β-barrel with a 3-turn helix. It possesses a specific active site known as ‘central calyx’ unlike ‘Sudlow’s site’ in albumin. BLG has been reported as a safe and biodegradable milk biomolecule, capable of binding and transporting majorly bioactive compounds such as fatty acid, retinol, flavonoids, phospholipids, and vitamins etc. The strong affinity of fatty acids such...
as oleic acid and linoleic acid with BLG exhibited an increase in thermostability and anti-tumour activity (Fang et al., 2015). The studies also showed that BLG interacts with average affinity to folic acid (hydrophilic), low affinity to resveratrol (amphiphilic) and high affinity to α-tocopherol (hydrophobic) (Liang & Subirade, 2012). To date many efforts have been taken to develop BLG as an efficient targeted drug delivery system. The physicochemical properties such as the stability to low pH, resistance to gastric proteases, and belonging to lipocalin family, provide it potential for targeted transport of drugs for colon cancer (Izadi et al., 2016). Kayani et al. demonstrated that β-lactoglobulin nanoparticles conjugated with folic acid and loaded with doxorubicin could have high therapeutic potential for breast cancer and triple negative breast cancer, facilitated drug release and exhibited targeted doxorubicin transport capabilities (Kayani et al., 2018).

The combination of anti-cancer drugs has shown enhancement of efficacy compared to the conventional therapy (Mokhtari et al., 2017). Curcumin is a major colouring agent present in spice turmeric, a multifunctional phenolic compound including its use in food. Curcumin structure comprises a β-diketone moiety, two ferulic acid molecules associated with methylene bridge at the carbon atom of carboxyl group. This bioactive polyphenolic component is extensively studied for its potential in advancement of efficient chemotherapeutics, based on its natural occurrence and effective anti-cancer activity (Zorofchian Moghadamtousi et al., 2014). Gemcitabine is a deoxycytidine analogue with 2 Fluorine substitutions - DiFluorodeoxycytidine (dFdC), possess an antineoplastic antimitabolite characteristic i.e. it blocks synthesis of DNA in cells, through the process of masked termination. Gemcitabine is today the most employed chemotherapeutic drug for treating cancers of a wide range of origins – ovarian, breast, non-small lung, biliary and predominantly pancreatic cancers, comply with no more than a 20% efficacy in penetrating cancer tissue, which lead to 5% survival rate in pancreatic cancer (de Sousa Cavalcante & Monteiro, 2014). For instance, it was reported that a potent antiproliferative activity of curcumin can also potentiate the antitumor effect of gemcitabine (Epelbaum et al., 2010). In addition, curcumin has also been tested with many such combinations with other chemotherapeutic agents, for instance, doxorubicin, irinotecan, 5-fluorouracil, cisplatin, and paclitaxel (Anitha et al., 2014; Tan & Norhaizan, 2019). Recently, the combination of superparamagnetic iron oxide nanoparticles conjugated to curcumin and gemcitabine showed enhanced gemcitabine therapeutic response in pancreatic cancer via inhibition of Sonic Hedgehog signalling (SHH signalling stimulates multiple drug resistance), and enhanced chemotherapeutic delivery in a mouse model of pancreatic cancer (Khan et al., 2019; Olive et al., 2009).

In view of the above, it is necessary to deeply explore the molecular interaction of combination of drugs with protein and nanoparticles-protein conjugates. In the present investigation, we reported the molecular interaction of curcumin and gemcitabine with BLG and AuNP-BLG conjugate using various spectroscopic approaches and molecular dynamics simulation. The cytotoxicity assay of AuNPs, BLG, CUR, and GEM was also studied. Our molecular understanding reports that AuNP-BLG-CUR and AuNP-BLG-GEM complexes could serve as a suitable nano-formulation for in vivo targeted drug delivery studies.

2. Material and methods

2.1. Material

Raw buffalo milk was procured from a private farming. Gemcitabine hydrochloride, Curcumin, and Gold (III) chloride hydrate were purchased from Sigma Aldrich. Sodium citrate, Na2HPO4, NaH2PO4, H2O, Tris-HCl, Acrylamide, bis-acrylamide, and ammonium persulfate (Sisco research laboratories). Ethanol (HiMedia), microfilters (0.22 and 0.45 μm, Merck), protein prestained marker (Puregene Genetix), Sodium dodecyl sulfate (SDS), NaCl, NaOH, HCl, Glycerol, Tetramethyl ethylenediamine (TEMED), β-mercaptoethanol and glacial acetic acid (Loba Chemie), and Coomassie Brilliant Blue R250 (SD Fine-chem). Double distilled water was used throughout the experiments.

Cell culture: The breast cancer cell line MCF7 was maintained in Roswell Park Memorial Institute (RPMI) medium (HiMedia) supplemented with 10% foetal bovine serum and 1% penicillin streptomycin antibiotic (Invitrogen). Cell cultures were incubated at 37°C, 5% CO2.

2.2. Preparation of BLG and AuNP-BLG, and its conjugation with CUR and GEM

All the experiments were carried out in 0.1 M PBS of pH 7.4. The procedure for gold nanoparticles synthesis and its concentration calculation is given in SM. AuNP (0.66 nM) was mixed with different concentrations of BLG (2.0 – 20.0 μM) and incubated for 30 min at RT under mild agitation on the shaker, similar approach has been used for other conjugates. For CUR, the ethanol concentration was maintained at less than 2% and GEM was prepared in PBS. Both CUR and GEM concentrations varied from 2, 4, 6, 8, 10, 12, 16, 20, 25, 30 and 35 μM. The complexes were incubated for 30 min to form a stable complex, and used for further experiments.

2.3. Spectroscopic studies

2.3.1. Mass spectrometry measurement

Protein mass measurements were carried out on MALDI-TOF-MS (Bruker Ultraflextreme, Germany) in linear mode. The matrix used was Sinapinic acid prepared in saturated solution of 1:1 ratio of acetonitrile and 0.1%TFA v/v. 1.0 μL of each BLG and matrix (1:1) were mixed, and 1.0 μL of the mixture was spotted on the MALDI plate, then air dried and loaded into the instrument. Data acquisition was done using the FlexControl software and analysis using FlexAnalysis software.

2.3.2. Hydrodynamic size and zeta potential measurements

The average hydrodynamic radius (R.H) and zeta potential measurements of AuNPs and AuNP-BLG conjugate were...
carried out on DLS system (Zetasizer Nano ZS90, Malvern, UK) at 25 °C, provided with red (633 nm) laser and avalanche photodiode detector (quantum efficiency > 50% at 633 nm). Attenuator position (4.65 mm) will set automatically. Glass cuvette was used for size measurement and folded capillary cell for measurement of zeta potential. To obtain correct nanoconjugate size, DLS measurements were performed for fixed AuNP (0.66 nM) with different BLG concentrations (2 – 200 μM). It should be taken care that only test tubes with glass caps should be used to avoid entry of air particles. The Hückel approximation \( f(\kappa) = 1 \) was used to determine the zeta potential.

### 2.3.3. Uv-visible measurement and job plot

The exact concentration of isolated protein in 0.1 M PBS was determined spectrophotometrically using molecular absorption coefficient \( \varepsilon_{280nm} = 17335 \, M^{-1} \, cm^{-1} \). The characterization of AuNPs, AuNP-BLG, and BLG with CUR and GEM has been performed at RT using nanophotometer (Implen, Germany). The spectrum was recorded in the range 200-700 nm in quartz cuvette. The methodology for Job plot is provided in SM.

### 2.3.4 Raman micro-spectroscopy

Raman spectra were acquired using confocal Raman spectroscopy (WITec 300R alpha setup, Germany) with a 532 nm (green) diode laser excitation source at 50X objective. Spectra for all samples (except with NP) were recorded at 25-30 mW laser power over 6 accumulations of 20 s exposure. Due to the photothermal effect, samples with NPs exhibited burning/quick heating with laser, hence acquisition was done at low laser power and accumulation-exposure was adjusted for each sample to obtain good quality spectra. About 10 spectra \((n = 10)\) were recorded from different areas of the samples to examine intra-sample variability. The spectra were baseline corrected for the spectra were interpolated in 450–1750 cm\(^{-1}\) region, smoothed (Savitzky–Golay method, Window size = 3) and baseline corrected for background background signals by fitting and subtracting polynomial order = 5 using project four 4.1 software (WITec) and LabSpec software (HORIBA), followed by area normalization for a clear band’s presentation. The working concentration of BLG was 20 μM and AuNP was 1.65 nM. Additional information is provided in SM.

### 2.3.5. Fluorescence measurements

The fluorescence experiment of BLG and AuNP-BLG with CUR and GEM (2-35.0 μM) was performed using Varian Cary Eclipse fluorescence spectrophotometer (USA). The samples were excited at 280 nm and the emission recorded between 300-500 nm. Temperature dependent experiments were conducted using Single cell Peltier accessory.

The synchronous scans were performed in the range 250-400 nm with initial excitation wavelength 250 nm, emission slit width was kept 10 nm, wavelength shift (∆λ) for tryptophan (60 nm) and tyrosine (15 nm). PMT voltage was 600 V for all fluorescence experiments. BLG was used at a fixed 8 μM and both drugs varied from 2-30 μM. Excitation and emission slit widths were 5 nm in both the cases.

### 2.3.6. Circular dichroism measurement

The CD spectra were recorded using 1.0 mm path length in the Far UV range 200-260 nm, with a scan rate of 50 nm/min, bandwidth 2 nm, response time of 1 s and data interval of 0.5 nm. BLG-CUR (1:2), BLG-GEM (1:1), and AuNP-BLG (0.66 nM – 8.0 μM) were used software provided by JASCO CD J-815 spectrophotometer (Jasco, Japan) was used for spectral recording and processing. Spectral analysis for % secondary structure was performed using online software BestSel (http://bestsel.elte.hu/results.php).

### 2.4. In vitro cell culture and cytotoxicity assay

MCF-7 cells were seeded in 96-well plates at 7 × 10^3 cells/ well density and incubated at 37 °C, 5% CO\(_2\) for 24 h. In view of previous literature, the chosen concentrations curcumin for MTT assay were 10, 20, 30, 40 and 50 μM, maintained in 2% ethanol. Gemcitabine concentrations ranged from 1, 10, 100, 1000 and 10000 nM. Due to the lack of literature on BLG toxicity studies, a wide range of protein concentrations – 1, 10, 100, 1000 and 10000 nM were prepared in 0.1 M PBS. 0.5, 1, 2 and 4 nM of AuNPs were used for the assay. MCF7 cells were treated with each sample respectively and incubated for 24- and 48-hours’ time intervals. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) solution was added at a final concentration of 0.5 mg/mL and incubated for 4 h at 37 °C, 5% CO\(_2\). The resultant Purple for-mazan crystals were dissolved using 0.01 N HCl in isopropanol and absorbance was measured at 540 nm (BMG Labtech). MTT readings were recorded in triplicates and data is shown as mean ± SEM. The IC\(_{50}\) values were calculated by non-linear regression curve fit function in GraphPad Prism 8 using the formula:

\[
Y = \frac{100}{1 + 10^{(\log IC_{50} - X) \times Hill \, slope}}
\]

Where X is the log of concentration and Y is the normalized response.

### 2.5. Molecular dynamics simulation

Molecular Dynamic Simulation which was performed using Desmond 2019.2 software package. The BLG, BLG-CUR and BLG-GEM were solvated with TIP3P water model in an orthorhombic box and the system was then neutralized by replacing water molecules with sodium and chloride counter ions. The systems were typed with Optimized Potentials for Liquid Simulations (OPLS) all-atom force field for energy minimization and stability of systems. These prepared systems were equilibrated with the default Desmond protocol that comprises a series of restrained minimizations. Once the systems are equilibrated, the production dynamics was run for 100 ns and coordinates were saved in every 2 ps of time intervals. After the end of MD simulation of BLG, BLG-CUR and
BLG-GEM systems, 100 ns of trajectories for each system were obtained. These trajectories were analysed using the ‘simulation interaction diagram’ tools from the Desmond package to plot RMSD, RMSF, intermolecular interaction and ligand-protein contacts plots.

3. Results and discussion

3.1. Purification, characterization, and quantification

β-lactoglobulin isolation and purification procedure was performed as reported in our previous work (M. N. Waghmare et al., 2020). Further it was characterized by size exclusion chromatography and confirmed by SDS-PAGE summarized in supporting information (Figure S1). Mass spectrometry (MALDI-TOF) was used to analyse the molecular size and purity of the protein fraction. The analysis of protein fraction 2 showed an exact m/z = 18,213 kDa, which strongly signifies purity and presence of the BLG protein in solution (Figure 1).

3.2. Physicochemical characterization

3.2.1. DLS, zeta and UV-visible analysis

The gold nanoparticles were synthesized by a chemical reduction method (see SM). UV-visible spectrum of AuNP showed absorption wavelength at around 521 nm (inset of Figure 2A), suggesting formation of spherical shaped gold nanoparticles (Gadogbe et al., 2013; Turkevich et al., 1951). The average hydrodynamic diameter ($D_H$) and surface charge distribution: $D_H = \sim 29.0 \pm 2$ nm, $\zeta = \sim 25.5$ mV, BLG $\zeta = \sim 7.0$ mV, all measured using DLS at pH 7.4 as shown in Figure 2A, S2A-B (see Table 1). The nanoparticle functionalization was achieved using β-lactoglobulin, and adsorption kinetics was characterized by both size and zeta potential. To study the adsorption of BLG on AuNP, AuNP (0.66 nM) were mixed and incubated with varying amounts of BLG (0.0-20 μM), and the change in $D_H$ of AuNP at increasing concentration of BLG monitored by DLS (Figure 2B). As a function of BLG concentration, there was a continuous increase in $D_H$ of AuNPs, further increase in protein concentration showed the saturation point at $D_H = \sim 41.0$ nm with $\sim 8.0$ μM. Similar experiments were performed and results reconfirmed using zeta potential measurements (Figure 2C). The negative potential of AuNP decreases and reaches the saturation point, showing stable potential at increased protein concentrations. The average increase in AuNP-BLG complex size was observed from 29.0 to 41.0 nm and zeta potential was $\sim 24.5$ to $\sim 7.89$ mV. Based on the above results, we estimated that $\sim 8-10$ μM of BLG concentration is required to completely

Figure 1. The characterization of BLG by mass spectrometry.

Figure 2. (A) Average $D_H$ of AuNP by DLS and inset shows UV-visible spectrum of AuNP; Adsorption of BLG to AuNP surface characterized by (B) DLS and (C) zeta potential, (D) the absorbance changes of BLG-CUR and ALA-CUR complexes at 417 nm; and the absorbance of BLG and ALA was respectively subtracted from (E) BLG-GEM and (F) ALA-GEM complex, a linear fitting showed $R^2 = 0.99773$. 
cover NP surface. Similar absorption kinetics were shown for albumin and α-lactalbumin in the previous work (M. Waghmare et al., 2018; M. N. Waghmare et al., 2020).

3.2.2. Analysis of BLG and AuNP-BLG with CUR/GEM

It has been predicted that BLG possesses multiple ligand binding sites for polar and nonpolar ligands (G Kontopidis et al., 2004). Therefore, to investigate the degree of specificity and affinity of CUR to the ligand binding sites of BLG. We used α-lactalbumin (ALA) protein that does not possess a specific binding site/pocket for CUR interaction, but it has non-specific hydrophilic and hydrophobic patches that act as a sites of binding and interaction (Delavari et al., 2018).

Therefore, by employing ALA we can show that BLG has a specific binding site for CUR whereas ALA does not possess a specific binding site. The spectra of various concentrations of CUR (2-30 μM) studied at a fixed concentration of both ALA and BLG proteins is shown in Figure S2A-B. BLG-CUR complex showed an increase in protein absorbance and blue shift from 280 nm to 267 nm, indicating biomolecular interaction. It is clear from Figure 2D that there is a difference in CUR absorbance (λ = 417 nm) in the selected range of concentration for both the proteins (see scan Figure S2A-B). To interpret this difference, we plotted a bar graph of absorbance vs concentration, and found that as the CUR concentration increases, the absorbance of ALA-CUR and BLG-CUR complexes also increases (Figure 2D). Comparatively, the absorbance of BLG-CUR complex was significantly reduced than ALA-CUR, emphasizing that the reduction in BLG-CUR absorbance could be due to the interaction between CUR with BLG, occurring at a specific binding site. Therefore, we state that CUR interacting through one of its di-ketone moieties, which is penetrated/inserted into the BLG hydrophobic calyx, while the other side of the CUR molecule is exposed to the solvent environment. CUR intensity and position of maxima depends on the amino acids surrounding the binding pocket, suggesting that CUR possibly diffuses and interacts the calyx resulting in suppression of its absorption efficiency. This deepens our understanding that BLG shows strong affinity with CUR. In spectroscopic language, it can also be assumed that the part of CUR is not available to interact with the incident light. These results are further strongly supported by a MD simulation.

There was no research published on the strength of hydrophilic interaction of BLG with GEM. The protein-ligand interaction that occurs through hydrophobic forces changes the protein conformation more significantly than the hydrophilic forces (Fujiiwara et al., 2006). The absorption spectra of BLG at increasing concentration of GEM (2-30 μM) were recorded (Figure S3C). Analysis of BLG-GEM binding showed an increase in absorbance at 280 nm, and blue shift from 280 to 271 nm (Figure S3C). The reason behind this blue shift is that the absorption maxima (shoulder) of GEM appears at 267 nm i.e. close to the protein absorption maxima. For better understanding of absorbance changes, and to investigate the molecular interaction between BLG and GEM, the absorbance of BLG was subtracted from BLG-GEM complex. The subtracted complex was plotted against GEM controls (Figure 2E) (the absorbance at 267 nm was considered). Figure 2E shows no significant change in absorbance of GEM with BLG complex, indicating weak interaction. Certainly, it can thus also be stated that the conformational changes were less sensitive to UV-visible. To confirm the interaction of GEM with BLG, we used ALA protein to study interaction with GEM. Similar subtraction experiments were performed for ALA (Figure 2F), surprisingly, the plot with ALA showed well-defined discrepancy between ALA-GEM and GEM control lines (Black and red line), i.e. there was a substantial concentration-dependent increase in absorbance of ALA-GEM at 267 nm. Thus, we substantiate that BLG possesses stronger binding to hydrophobic drugs than hydrophilic drugs, possibly due to the presence of central calyx. Further, we predict that the function of BLG in milk could be a reason to bind/carry hydrophobic and some hydrophilic compounds.

Further, we have studied the interaction of AuNP-BLG with both the drugs (Figure 3A and 3C). The spectral changes in AuNP-BLG-CUR complex showed blue shift from 278 nm to 266 nm with increase in protein absorbance and blue shift of 9 nm to 418 nm (Figure 3B). For GEM, AuNP-BLG complexes showed changes in absorbance and blue shift of 8 nm to 270 nm (Figure 3C). Besides, there was no effect on NP wavelength (~520 nm). It is evident from the graphs that interaction of CUR and GEM with AuNP-BLG complex points towards ground state complex formation. Similarly, interaction studies of AgNP-BSA complex with CUR showed a red-shift of 10 nm to 434 nm, suggesting significantly decreased auto-degradation of CUR as a result of strong affinity and binding with BSA (Jaiswal & Dongre, 2020).

In polar solvents, curcumin exists in two forms enol (424 nm) and keto (360 nm) that are nearly the same proportion. In BLG-CUR complex, we did not find any clear peak around 260 nm, an indication of CUR degradation. It was reported that the degradation of CUR was 6.7 times reduced in presence of BLG (Sneharani et al., 2010). Mass spectrometry has been employed to understand the auto-degradation of CUR. It has been recognised that CUR undergoes auto-oxidation in aqueous media, and shows no peak from innate curcumin (m/z = 369 Da) (M. N. Waghmare et al., 2020). Considering that curcumin has been consumed with milk from ancient times, CUR in presence of BLG showed a clear peak at m/z = 369 Da, revealing noteworthy interaction with the protein (Figure 3D). We then checked for BLG mass where we assumed there would be an increase in its molecular weight, but no such result was obtained, possibly because the energy used in MALDI-TOF does not keep the complex intact.
3.2.3. Job plot

Job’s plot is used to examine the protein:drug stoichiometry using UV-visible spectroscopy. The titration of BLG with CUR was conducted, and the graphical data showed the maximum mole fraction \(X_A\) at around 0.76, which implies the presence of 1:3 stoichiometry between BLG and CUR, indicating maximum three binding sites for CUR (Figure 3E). The noticeable difference in the molecular association of BLG with CUR could be explained by their nature of binding site. Naturally, the hydropathy index of BLG equal to −0.010, suggests that the native BLG structure is more nonpolar than that of albumin (-0.429). Further, in BLG-GEM complex, the affinity between BLG and GEM exhibited maxima \(X_A = 0.48\) that enables the complex formation with maximum 1:1

Figure 3. The interaction of AuNP-BLG conjugate with both the drugs; (A) AuNP-BLG-CUR, (B) the peak shift of AuNP-BLG complex with CUR, (C) AuNP-BLG-GEM, (D) Mass spectrometry characterization of BLG-CUR complex; Job plot for (E) BLG-CUR and (F) BLG-GEM.
stoichiometry (Figure 3F). This reveals that GEM possesses maximum one binding site for BLG, which could be due to the presence of hydrophilic patches as determined by the shape and distribution of basic and polar residues on the BLG surface. Finally, Job plot successfully demonstrated three binding sites for CUR and a single interaction site for GEM.

3.3. Protein conformations revealed by Raman scattering

Raman spectroscopy has been used to understand molecular vibrations originating from protein-ligand interaction to reveal intricate details of some specific molecular groups involved in protein conjugation.

3.3.1. Analysis of BLG and BLG-AuNP

Majorly, β-sheet structured BLG can be identified by characteristic vibrations of amide band I at 1668 cm\(^{-1}\) \(\nu(C = O)\) and strong intensity amide band III at 1249 cm\(^{-1}\) (coupling of \(\delta NH\) to \(\delta C\alpha-H \& \nu CN\)), which is also the region of random coil structure as shown in Figure 4A. The presence of the vibration band at 947 cm\(^{-1}\) \(\nu(N-C\alpha-C)\) indicates \(\alpha\)-helix existence. Adsorption of BLG onto AuNP surface yields substantial fluctuations in following vibration bands; a) The amide bands position shifted from 1668 to 1672 cm\(^{-1}\) and 1249 to 1240 cm\(^{-1}\) (sensitive to conformations), b) noticeable amide bands broadening, c) Drastic weakening of the \(\alpha\)-helix band at 947 cm\(^{-1}\) red shifted by 13 cm\(^{-1}\), indicates strong conformation changes in the polypeptide backbone, similar to the loss of helix content induced by adsorption of albumin onto NP (Matei et al., 2019; Silveira et al., 2019), and d) pronounced decrease in intensity of \(\delta CH_2\) and \(\delta CH_3\) vibrations observed near 1453 cm\(^{-1}\), originating from aliphatic residues (Figure 4D). A report on BLG-AuNP complex showed significant intensity reduction and redshift in amide band I from 1665 to 1673 cm\(^{-1}\), this shift could be due to an increase in protein disorder (Winuprasith et al., 2014), in agreement with our CD results. In protein-metal interaction, Raman cross-section area of the biomolecule increases possibly due to charge transfer, leading to broadening and shifting of the electronic states in the adsorbed molecule (Siddhanta & Narayana, 2012). AuNPs do not show Raman scattering in the protein spectrum range, indicating that the modification in protein vibration bands arises purely due to NP binding. AuNP also induced greater change at 1453 cm\(^{-1}\) caused by hydrophobic interaction, which agrees with our fluorescence and UV studies. In addition, there was an amide band II shoulder scattering observed at 1515 cm\(^{-1}\), appearance of this amide band implies surface enhanced Raman spectrum (Podstawka et al., 2004).

In AuNP-BLG conjugate, the bands at 1566, 1617, 1001, 571 and 499 cm\(^{-1}\) show large relative enhancement, while the bands at 1668, 1454, 1239, 957, 898, and 781 cm\(^{-1}\) showed large relative decrease, indicating that AuNP interacts with BLG backbone. Tyr doublet ratio in the complex (I\(_{850}\)/I\(_{830}\) < 1.0) denotes that Tyr acts as a strong hydrogen bond donor as compared to BLG control spectra, also showing that most of the Tyr residues are buried. Tyr ring vibrations at 836 cm\(^{-1}\) disappeared, which led us to believe that Tyr position could be close to the AuNP surface. In SERS of adsorption of pancreas \(\alpha\)-chymotrypsin on AgNP surface, Tyr doublet (I\(_{844}\)/I\(_{803}\)) suggested that Tyr hydroxyl group strongly interacts with the hydrophilic environment (Siamwiza et al., 1975).

The exposed Trp, Tyr and Phe residues show shoulder ring vibration at 1618 cm\(^{-1}\), which then becomes enhanced in the presence of AuNPs suggesting conformational changes. Likewise, Trp indole ring vibration showed significant enhancement at 1559 cm\(^{-1}\), red shifted to 1566 cm\(^{-1}\) caused by interaction of nitrogen atoms of Trp with gold surface, and similar result was previously noticed at the interface of silver surface (Grabbe & Buck, 1989). Trp stretching vibration at 888 cm\(^{-1}\) showed dramatic weakening and shift to higher frequency (898 cm\(^{-1}\)), these large changes demonstrating that AuNP-BLG interaction is taking place near exposed Trp residue. Reasonably, the surface exposed Trp61
and Tyr42 residues might be involved in adsorption of BLG onto NPs surface or in proximity which influences its vibrations (Figure S6).

Further, BLG spectrum shows weak vibrations of $\nu$S-S bands ($\sim$512, 528, 540 cm$^{-1}$) along with a strong stretching vibration at 595 cm$^{-1}$ and shoulder at 570 cm$^{-1}$ ($\nu$S-S), which is significantly enhanced, broadened and blue shifted in presence of AuNP. The appearance and enhancement of the $\nu$S-S and $\nu$C=S bands may be associated with close distance or perpendicular alignment to AuNP surface. According to researchers, it is believed that the S-S bridge binds to the silver surface through one of the sulphur atoms, resulting in enhancement of vibrations (Stewart & Fredericks, 1999). Selective enhancement, broadening, weakening, and disappearance of the vibration bands in AuNP-BLG complex help us conclude that the orientation of BLG involving central calyx and Trp61 is in the vicinity of the AuNP surface, reiterating the outcome explained by fluorescence study.

3.3.2. Analysis of BLG-CUR and AuNP-BLG-CUR

So far, there have been few researches published on Raman spectral analysis of protein-curcumin complex. Due to overlapping of Raman spectra, we selected a few bands that showed significant changes in BLG-CUR complex. The absence of $\nu$(C=O) in the region 1650-1800 cm$^{-1}$ suggests the enol form of curcumin is more stable than the ketone form in most nonpolar solvents (Mohan et al., 2012). The characteristic CUR Raman bands found are 1604 cm$^{-1}$ assigned to telescopic vibration of $\pi=\pi$ on the benzene ring and 1638 cm$^{-1}$ corresponding to $\pi=\pi$ and C=O vibrations on enol structure (Figure 4B).

The spectra of BLG-CUR (1:2) shows an overall peak shift and decrease in scattering intensity of BLG spectrum, except the 500-600 cm$^{-1}$ region, which showed major enhancement of cystine bridge vibration and blue shift to 492 cm$^{-1}$, implying direct involvement and strong binding. Hydrophobic interaction being an actual force in BLG-CUR conjugation, induces a drastic intensity drop in 1452 cm$^{-1}$ vibration. A report on Raman spectra of CUR with increasing pH, shows bands at 1581, 1486, 1387, 1260, and 1162 cm$^{-1}$, indicating clear degradation of CUR (Canamares et al., 2006). In our case, BLG-CUR does not show any Raman bands reflecting CUR degradation.

The state of Tyr residues is important to investigate the conformation of protein in a protein-ligand interaction. Aromatic residues situated near the calyx may become influenced during binding events. Tyr42 residue possesses accessible surface area and is positioned near the central calyx. BLG-CUR complexation is associated with a large number of conformational changes, evident in the selective enhancement of the band and blue shift to 818 cm$^{-1}$. The phenolic oxygen on Tyr has a partial negative charge, expected to substantially increase on conformation change, leading to increase in ring breathing vibration and enhancement of the lower component of the doublet. In addition, it may also lead to a shift of this component to lower frequency (Siamwiza et al., 1975), which can be seen in our BLG-CUR complex. BLG conformations drives the hydrogen bonding between the side chain of Gln40 and -OH of Tyr42 (Vijayalakshmi et al., 2008), which may cause an increase in partial charge on strong donor hydroxyl oxygen, also confirming our findings ($I_{850}/I_{830} = 3:10$). In BLG-CUR complex, there is noteworthy change observed in the microenvironment of Tyr, it can thus be suggested that the intensity change and band shifting is because of Tyr42 residue. Further, Tyr doublet reveals that Tyr residues are buried, in agreement with synchronous fluorescence results. Trp doublet ($I_{1349}/I_{1329} < 1.0$), indicated no change in its hydrophilic microenvironment. Thus, our close analysis suggests that CUR induced major changes in the entire BLG conformation, indicating strong association with CUR.

Binding of AuNP-BLG conjugate with CUR demonstrated changes in amide bands, Trp, Tyr and Cys vibrations (Figure 4E). We noticed small enhancement of the amide I (1670 cm$^{-1}$) and III (1239 cm$^{-1}$) bands, with no major frequency shift as compared to AuNP-BLG spectra. Trp, Tyr and Phe residues showed benzene ring breathing vibration at 1615 cm$^{-1}$, while Trp indole ring vibration exhibited significant blue shift from 1566 to 1556 cm$^{-1}$. Tyr doublet ($I_{1360}/I_{1340} < 1.0$), signifies that Tyr-OH remains buried. Furthermore, Trp doublet ($I_{1380}/I_{1340}$) yielded similar results to those of AuNP-BLG and BLG-CUR complexes, proving that Trp is away from the central calyx and AuNP surface. For $\nu$S-S vibration, we observed disappearance of disulphide of $\sim$530 cm$^{-1}$ and weakening of 500 and 570 cm$^{-1}$ ($\nu$S-S-S-C) band vibration, indicating significant modification of $\nu$S-S. The formation of complex between AuNP-BLG and CUR showed weak signal at 1380 cm$^{-1}$, implying minor CUR degradation as suggested (Canamares et al., 2006).

3.3.3. Analysis of BLG-GEM and AuNP-BLG-GEM

The bands at 1250, 1620, and 1670 cm$^{-1}$ were enhanced indicating binding of GEM to the amide groups of BLG, although these changes were more prevalent for CUR binding. $\delta$(N-C$_{\alpha}$C) vibration showed a change in intensity and red shift to 952 cm$^{-1}$, suggesting changes in the $\alpha$-helical conformation of BLG. A report showed that gradual loss of side helix structure leads to broadening and weakening in intensity of this band (Frushour & Koenig, 1975). The aromatic side chain of Trp at 887 cm$^{-1}$ displayed pronounced intensity drop, with no considerable change in Trp doublet. Tyr doublet ($I_{850}/I_{830} = 1.15$) showed an increased ratio compared to BLG, suggesting strong interaction of GEM with exposed hydroxyl groups. We observed that S-S bond and central calyx has a direct involvement in ligand interaction, since a greater change in the conformation of disulfide vibration was observed at 527 and 595 cm$^{-1}$, manifesting itself in band broadening and blue shift of 5-7 cm$^{-1}$. Overall, with GEM, the selective hydrophilic conformational changes in BLG points towards hydrophilic nature of GEM binding.

AuNP-BLG conjugate binding with GEM does not alter the characteristics of amide bands, unlike the BLG-GEM complex. In contrast, larger changes were observed in aromatic signals, such as enhancement of bands at 1617 cm$^{-1}$, disappearance of 1565 cm$^{-1}$ and red shift of 3-4 cm$^{-1}$ in 898
and 1341 cm$^{-1}$ as shown in Figure 5A. Tyr doublet ($I_{852}/I_{832} = 0.8$) shows that Tyr residue is buried in the protein scaffold, indicating change in the tertiary structure of BLG. For both AuNP-BLG-GEM and BLG-GEM complexes, there was a significant impact on disulfide bands leading to weakening of the vibration modes, thus demonstrating close relation of the active site of GEM binding with S-S bridge conformation. For GEM, the enhancement at 1318 cm$^{-1}$ band associated with the $\alpha$-helix region (absent in AuNP-BLG conjugate), indicates interaction with the $\alpha$-helix. BLG adsorption on AuNPs causes a decrease in the possible hydrophilic nature of GEM-protein interaction. However, GEM compensates by binding with the helical region of the protein. This means that GEM interacts or has weak binding with the $\alpha$-helix structure of free BLG. From the above data, we can extrapolate the definite ways of orientation of adsorbed BLG to the AuNP surface, revealing that some $\alpha$-helical region faces the solvent whereas $\beta$-sheets on the opposite region may be masked by AuNP. Raman bands are provided in Table 2.

3.4. Fluorescence quenching studies

The strong ultraviolet fluorescence signal originating from tryptophan and tyrosine in proteins can be employed as a sensitive tool for inspecting the strength of interaction or affinity, protein conformation upon binding with ligands. In BLG, the fluorescence contribution from buried Trp19 is superior to the contribution of Trp61 (Essemine et al., 2011).

3.4.1. Spectral changes induced by ligands

The interaction of BLG (8 $\mu$m) with increasing concentration of CUR (2-30 $\mu$m) is depicted in Figure S4A. The emission maximum ($\lambda_{max}$) of BLG was observed at 333 nm, attributable to average nonpolar environment of aromatic residues in BLG relative to emission maxima of BSA ($\lambda_{max} = 344$ nm, indicating polar environment). BLG-CUR complex exhibits significant fluorescence quenching, and emission with an average 3-4 nm red shift. This energy shift was due to hydrogen bonding and interaction with the amino group. The interaction of CUR was also coupled with a slightly increased exposure of Trp residue to aqueous phase and structural deformation of BLG. Furthermore, the red shifting is an indication of restricted motion and rigid interaction between BLG and CUR, revealing that the binding site of CUR might be away from Trp residues. In the $\beta$-lactoglobulin-serotonin complex, the position of Trp residues was in a more polar environment (Taheri-Kafrani et al., 2011). Figure S4C represents the intrinsic fluorescence quenching spectra BLG-GEM complex, showing no major shift in emission maxima, and reports weak quenching as compared to CUR.

Adsorption of BLG proteins on AuNP caused strong fluorescence quenching, i.e. the adsorbed protein underwent larger structural conformations, indicating strong binding and interaction as illustrated in Figure S4B. Generally, the interaction of protein with NPs is largely driven by hydrophobic and hydrophilic forces (Mariam et al., 2011; 2017). This large fluorescence quenching showed average redshift of 2-3 nm, resembling selective quenching of surface Trp residue i.e. Trp61, which is nearly accessible to solvent. The characteristic interaction of AuNP-BLG conjugate with GEM showed emission at $\sim 335$ nm with net redshift of $\sim 2-3$ nm relative to nanoconjugate (Figure S4D). In particular, the change in emission wavelength is associated with the collective spectral environment of Trp and Tyr residues in protein, which can be influenced by drug interaction. The blue shifted Trp residues are mostly inaccessible to quenching, and red shifted residues nearly accessible in hydrophilic solvent (Glandières et al., 2000). In our case, it can be suggested that half of the Trp residues are accessible to the drugs, the red shifted BLG-CUR complex demonstrating that Trp61 is nearly accessible to quenching while Trp19 remains inaccessible.

3.4.2. Mechanism of fluorescence quenching and interaction parameters

The fluorescence quenching was employed in detail to estimate protein conformation, quenching efficiency, binding constant and binding sites of BLG, and AuNP-BLG complex with CUR and GEM. Molecular quenching of the fluorophore takes place either by dynamic and static quenching phenomena. Also, quantitative assessment of protein-ligand interaction can be obtained using the Stern-Volmer equation (SM equation 1). Generally, the quenching data are represented by a plot of $F/F_0$ vs $[Q]$, where the slope of the line will provide Stern-Volmer (SV) quenching constant ($K_{SV}$), $k_q$ is the bimolecular quenching constant, and $[Q]$ is the quencher concentration.
Stern-Volmer plot of BLG-CUR complex showed a clear upward curvature (Figure 6A), the curvature denoting possibility of both dynamic and static quenching (\(K_{SV} = 0.42 \times 10^5 M^{-1}\)), revealing that \(\sim 23.8 \mu M\) of CUR could quench 50% fluorescence of BLG. Both static and dynamic quenching can be differentiated by the dependence on temperatures, higher temperature results in faster diffusion of molecules and therefore \(K_{SV}\) increases. BLG-CUR was tested at varied temperatures from 310-333 K (Figure 6E), and the result indicated an increase in \(K_{SV}\) confirming the presence of quenching.

Table 2. shows Raman shift (wavenumber cm\(^{-1}\)) of BLG and BLG-AuNP interaction with CUR and GEM. Blue shift (BS) and red shift (RS).

| Region         | Control | BLG-AuNP | BLG-CUR | BLG-NP-CUR | BLG-GEM | BLG-NP-GEM |
|----------------|---------|----------|---------|------------|---------|------------|
| S-S (~510)     | 514     | –        | 515     | –          | 513, 518| 511, 519   |
| [G-G-G]        |         |          |         |            |         |            |
| S-S (~495, ~525) [T-G-G] | 525    | 497, 529 | 528     | 497        | 529     | –          |
| S-S (~540)     | 541     | –        | –       | –          | 541     | 542        |
| [T-G-T]        |         |          |         |            |         |            |
| Tyrosine Doublet (~830/~850) | 833, 858| 832, 847 | 836, 847 | 830, 845   | 836, 843| 831, 852   |
| Cx-C          | 946     | 940      | 955     | –          | 952     | 952        |
| (~940)         |         |          |         |            |         |            |
| Phenylalanine (~1000) | 1002    | 1005     | 1001    | 1004       | 1002    | 1003       |
| Amide III      | 1249    | 1241     | 1244    | 1237       | 1250    | 1238       |
| (~1240)        | BS      | BS       | BS      | –          | BS      | BS         |
| C-H bending (~1450) | 1454    | 1455     | 1454    | 1449       | 1457    | 1454       |
| Amide I        | 1669    | 1672     | 1671    | 1670       | 1672    | 1669       |
| (~1670)        | RS      | RS       | -       | -          | RS      | -          |
| Tyrosine Doublet (~830/~850) | 833, 858| 832, 847 | 836, 847 | 830, 845   | 836, 843| 831, 852   |
| C-H bending (~1450) | 1454    | 1455     | 1454    | 1449       | 1457    | 1454       |
| Amide I        | 1669    | 1672     | 1671    | 1670       | 1672    | 1669       |
| (~1670)        | RS      | RS       | -       | -          | RS      | -          |

Figure 6. Stern-Volmer plots for (A) BLG-CUR, (B) BLG-GEM, (C) AuNP-BLG-CUR, (E) AuNP-BLG-GEM; (E) the temperature dependent study of BLG-CUR; the double logarithmic Stern-Volmer plot for (F) BLG-CUR, (G) BLG-GEM, (H) AuNP-BLG-CUR, (I) AuNP-BLG-GEM.
of static and dynamic quenching mechanism (Figure S4E). Furthermore, the curvature in $K_{SV}$ plot was also seen for BLG-retinol complex specifying accessibility of exposed Trp in BLG protein to quencher molecules (Khorsand Ahmadi et al., 2015). However, the interaction of BLG with CUR has previously shown a static type of quenching (Sneharani et al., 2010). The quenching of BLG-oxaliplatin complex studied at 25 and 37°C, showed $K_{SV}$ value $\approx 1.3 \times 10^3$ M$^{-1}$. Likewise, other investigators have shown protein-drug binding such as doxorubicin, ciprofloxacin, and kanamycin by showing straight line plots, which specified dominancy of static quenching in these complexes (Aguadé et al., 2012; Ghalandari et al., 2015; Mehraban et al., 2017). For GEM, the quenching study of BLG with GEM exhibits linear SV plot with slope $10^4$ M$^{-1}$, which confirms the quenching of BLG-CUR as higher than BLG-GEM complex (Figure 6B). At physiological temperature, the interaction of HSA/BSA with gemcitabine also showed static quenching in $10^5$ M$^{-1}$ order (Kandagal et al., 2006).

AuNP-BLG conjugate interaction with drugs has been investigated (Figure 6C-D). BLG-CUR and AuNP-BLG-CUR complexes did not show any major difference in quenching constant (Table 3), which means that Trp61 is exposed to an aqueous environment and not facing the nanoparticle surface. Compared to BLG-CUR complex, the upward curvature does not appear in case of AuNP-BLG interaction with CUR. The interaction of GEM with AuNP-BLG illustrated a linear quenching plot that confirms GEM acts as a static quencher molecule (Figure 6D). Generally, the extent of fluorescence quenching relies on the strength of molecular interaction between acceptor and donor molecules. In this study, the $K_q$ values are larger than the diffusion-controlled limit ($1 \times 10^{10}$ M$^{-1}$s$^{-1}$), suggesting an efficient static quencher. The interaction of BLG with both CUR and GEM drugs showed $K_q = 1.55 \times 10^{13}$ and $K_q = 2.82 \times 10^{13}$ M$^{-1}$, respectively (Table 3). Both drugs presented higher $K_q$, suggesting that both drugs bind and interact with BLG.

The interaction parameters such as binding constant ($K_b$) and binding sites ($n$) can be calculated using the double logarithmic Stern-Volmer plot, and the expression is provided in SM equation 2. The binding constant for BLG-CUR and BLG-GEM are in the order of $10^8$ M$^{-1}$ and $10^9$ M$^{-1}$, respectively (Figure 6F-G). The number of binding sites for CUR was found to be 1.83, indicating that BLG has more than one binding site for CUR. Previously, in the Job plot, we estimated the stoichiometry ratio for BLG-CUR as 1:3, hence, it can be precisely suggested that BLG possesses maximum 3.0 and minimum 2.0 sites for CUR. Additionally, the physico-chemical properties show that CUR possesses topological polar surface area of around 93.1 Å$^2$. It is acceptor of six and donor of two hydrogen bonds, which shows that CUR can also interact through the available hydrophilic region. In BLG-GEM complexation, the slope of the graph shows $n = 0.94$ site, exhibiting maximum 1:1 stoichiometry ratio, in a strong agreement with Job plot result (see Table 3). The interaction of HSA/BSA with gemcitabine also showed binding constant $10^8$ M$^{-1}$ and a single binding site (Kandagal et al., 2006).

It was proved that BLG on interaction with AuNP, undergoes conformational changes. Accordingly, BLG alters its responses to drugs interaction, and hence $K_b$ and $n$ values may increase or decrease. Indeed, in our case, the interaction of AuNP-BLG with CUR showed change in binding constant i.e. $10^5$ M$^{-1}$, which is slightly increased compared to BLG-CUR complex. Surprisingly, the number of binding sites for AuNP-BLG-CUR also showed little improvement, elucidating that the central calyx remains exposed to solution and the conformational changes driven by AuNP play a crucial role in increasing binding with CUR (Figure 6H). In AuNP-BLG-GEM complex, there was no change in the binding constant and even in the binding sites, thus proving that the conformation changes by NP adsorption do not alter BLG interaction with GEM (see Figure 6I and Table 3).

### 3.4.3. Fluorescence accessibility

Quenching measurements can reveal the accessibility and position of fluorophores to quenchers. In BLG, there are two Trp residues, accessible (Trp61) and another inaccessible (Trp19). Stern-Volmer plot displays upward curvature, which we observed previously for the BLG-CUR complex (Figure 6A). This kind of curvature in Stern Volmer plots states that the binding of CUR to BLG instigates conformational changes which may lead towards partial exposure of previously shielded Trp residues (i.e. Trp19). To study the accessibility of Trp, the fraction of accessible fluorescence ($f_a$) can be derived from a modified form of Stern-Volmer equation 3 provided in SM. The $f_a$ can be calculated by plotting $F_0/\Delta F$ vs $1/[Q]$, and intercept yields $f_a^{-1}$. Complete quenching is expected for accessible residue (Trp61) that displays $f_a = 1$, while, for the buried Trp, $f_a < 1$. Figure 7A shows $f_a = 0.42$ for BLG-CUR complex, indicating that about half of the total fluorescence is accessible to quenchers. Therefore, we believe that the exposed Trp61 is completely quenched, and conformational changes trigger Trp19 to be quenched leading to partial alteration in its microenvironment. It was

| Name          | Binding constant ($K_b$, M$^{-1}$) | Binding sites (n) | Binding sites (Jobs plot) | Quenching constant ($K_q$, M$^{-1}$) | Accessible fluorescence ($f_a$) | Melting temperature (°C) |
|---------------|-----------------------------------|-------------------|---------------------------|--------------------------------------|-------------------------------|--------------------------|
| β-LG          | 1.3 × 10$^3$                      | 1.54              | 3.29                      | 0.42 × 10$^4$                        |                               | 45.72                    |
| β-LG-CUR      | 3.71 × 10$^4$                     | 1.83              | −                         | 0.43 × 10$^4$                        |                               | –                        |
| AuNP-β-LG-CUR | 3.78 × 10$^4$                     | 0.94              | 0.95                      | 0.76 × 10$^4$                        |                               | –                        |
| AuNP-β-LG-GEM | 4.36 × 10$^5$                     | 0.95              | −                         | 0.99 × 10$^4$                        |                               | –                        |
demonstrated that the binding of retinol to BLG quenches the fluorescence of Trp19 residue, situated beneath the central calyx (Papiz et al., 1986). In the BLG-GEM complex, it was found $f_a = 0.13$, which gives the impression that lone exposed Trp61 is accessible to GEM (Figure 7B). This also evidenced that GEM interacts only with the Trp residues on the surface protein and thus quenching them, GEM could not penetrate the hydrophobic interior of protein. For AuNP-BLG-Drugs (Figure 7C-D), we did not observe major differences in fluorescence accessibilities, except slight increase seen as a result of CUR interaction which may be due to its strong binding (see Table 3). Trp61 is positioned at the surface and is adjacent to strand I which takes part in binding at BLG dimer interface (Croguennec et al., 2003). In fluorescence accessibility analysis, there is a strong relation between central calyx and buried Trp, in other terms, the buried Trp fluorescence quenching is purely associated with specific binding of CUR to central calyx conformation.

3.5. Analysis of BLG conformations

3.5.1. Fluorescence synchronous: Microenvironment of Trp and Tyr

To assess the microenvironment of Trp and Tyr and conformation of BLG, we employed fluorescence synchronous methodology. BLG-CUR complex showed fluorescence quenching in both Trp and Tyr spectra (Figure 5A-B). Synchronous data demonstrated the conformational adjustments are induced in BLG-CUR complex, but no major change in the microenvironment of the Trp and Tyr. An investigation of BLG-retinol complex showed a blue shift of 3 nm, indicating changes in the conformation and microenvironment of Trp and Tyr (Khorsand Ahmadi et al., 2015). For BLG-GEM, we observed pronounced Trp synchronous quenching and with no significant impact on Tyr fluorescence or the emission maxima (Figure SSC-D). This result confirms that the position of GEM binding was closer to exposed Trp than Tyr residue. $\beta$-lactoglobulin-capsaicin complex showed an increase in fluorescence intensity and blue shift in both Trp and Tyr spectra, demonstrating that capsaicin interacts in the vicinity of Trp and Tyr residues within a hydrophobic environment (Zhan et al., 2020). In view of the above, synchronous data showed that CUR has a significant impact on Trp and Tyr conformations, and suggests that GEM binding site is closer to Trp61.

3.5.2. Structural changes induced by AuNP, CUR, and GEM

Circular dichroism (CD) spectroscopy was used to investigate quantitative effect of CUR and GEM binding on the secondary structure of BLG and AuNP-BLG conjugate (Figure 8). In native state of BLG, CD peaks are clearly visible at 214 nm, stipulating a higher percentage of $\beta$-sheets and a lesser percent of $\alpha$-helix (Figure 8A). The effect of CUR interaction on BLG (2:1) does not show any changes in $\lambda_{max}$ but showed distributed changes in secondary structures of BLG such as increase in $\alpha$-helix, $\beta$-sheet and $\beta$-turns, and reduction in random coil structures from 45.05% to 43.60% (Table 4). Neha et al. observed similar changes in $\alpha$-helix content in albumin linked AuNP elucidated by CD study (Kumari et al., 2019). This indicates a ligand having affinity to central calyx does not necessarily need to show major conformational changes except some changes in weak molecular forces (mainly hydrophobic) due to structure adjustments. These conformations also support specific interaction of CUR with the central calyx; hence, it may be possible to predict that BLG does not
necessarily undergo major conformations. Similar outcomes were described by Zhan and co-authors for β-lactoglobulin-capsaicin complex, they stated that capsaicin carries out a concentration-dependent change in the ellipticity, but does not lead to a substantial change in β-sheet (Zhan et al., 2020).

The interaction of BLG with GEM (1:1) showed major changes in β-sheet (31.75%), β-turns (14.85%), random coils (42.70%), and small changes in α-helix region (Figure 8B and Table 4). This shows that GEM binding site is located away from the calyx region, where it interacts with β-sheet or α-helix that instructs the protein to undergo conformation to fit/accommodate the GEM molecule. Polar drugs act through ionic and electrostatic interaction or through hydrogen bonding, and here, it appears like GEM interacts with polar amino acids of β-sheet and random coil structure. A scientist and his group used molecular docking to predict the possible binding sites for hydrophobic and hydrophilic ligands such as milk sugar and lactose binding with monomeric and dimeric BLG. They noticed that lactose binds in the dimer interface and another site C at K141 residue: a probable site in the BLG dimer interface (Domínguez-Ramírez et al., 2013).

In addition, it was stated that Trp61 was positioned at protein surface and is adjacent to strand I involved in binding at dimer interface (Croguennec et al., 2003). The binding of GEM with BSA also showed significant modifications in secondary structure percentage (Kushwah et al., 2017).

For AuNP-BLG, careful investigation of percent modification induced by AuNP demonstrated about a 23% decrease in α-helical region relative to other secondary structures as shown in bar graph (Figure 8C). The covalent backbone of protein comprises thousands of individual weak bonds, many of which can freely rotate in exposed and buried secondary structures environment. It can therefore be predicted that the restricted rotation leads to large changes in helical structure, suggesting that the major alterations in α-helical region of BLG are due to NP interaction. For AuNP-BLG-CUR complex, we found a large increase in α-helix from 8% to 11.40% and slight increase in β-turns. There was reduction in β-sheets from 30.50% to 29.10% and random coil from 45.80% to 43.65%, and changes were also seen in β-sheets and β-turns. We noticed almost similar structural changes in the BLG-CUR complex. Therefore, it could be considered that the central calyx is facing the solvent environment in conjunction with the BLG-CUR results. Most of these small changes are well detailed in Raman scattering study. For AuNP-BLG-GEM, a large increase in α-helix from 8% to 10.53%, whereas small amounts of decrease in other secondary structures were seen. The helical structures increase in binding both drugs, suggesting that calyx faces the solvent. The secondary structural changes induced by CUR were considerably more than GEM, confirming that CUR binds strongly to BLG and AuNP-BLG complex than GEM.

| Content (%)         | BLG control | BLG-CUR (1:2) | BLG-GEM (1:1) | AuNP-BLG | AuNP-BLG-CUR | AuNP-BLG-GEM |
|---------------------|-------------|---------------|---------------|----------|--------------|--------------|
| α-helix (±0.65)     | 10.35       | 10.95         | 10.75         | 8.00     | 11.40        | 10.53        |
| β-sheets (±0.78)    | 28.6        | 29.1          | 31.75         | 30.5     | 29.1         | 29.83        |
| β-turn (±0.26)      | 16.00       | 16.30         | 14.85         | 15.60    | 15.90        | 15.00        |
| Random coil (±0.52) | 45.05       | 43.60         | 42.70         | 45.80    | 43.65        | 44.60        |

Figure 8. The change in % secondary structure characterized by CD spectroscopy shown in A) and B), and C) shows AuNP induced maximum % change in α-helix of BLG.
3.6. Molecular dynamics simulation

3.6.1. Root mean square deviation (RMSD)

In MD simulation, the trajectory stability for BLG, BLG-CUR, and BLG-GEM complexes was assessed and verified by the study of the RMSD of Cα atoms in protein from the initial structure. The RMSD of the free BLG and with CUR and GEM drugs is plotted against time (0 to 50 nsec) (Figure 9A-B). For free BLG, it was understood that the RMSD values reached equilibrium and oscillated around the same value after about 6 nsec (Figure 9A). Further, we noticed that there was slight structural re-organization and short-range oscillations in BLG-CUR complex. BLG backbone atoms have no major deviation during 7.0-50 nsec. The RMSD values observed for both showed 1.0 - 1.8 Å (BLG) and 4.8 - 7.2 Å (BLG-CUR) respectively, demonstrating stability of protein-ligand complex. The lower RMSD value in the presence of ligand RMSD specifies CUR stability with respect to BLG and its binding pocket.

Figure 9. MD simulation; RMSD analysis of backbone conformation protein (A) BLG and BLG-CUR, and (B) BLG and BLG-GEM; RMSF values for (C) BLG-CUR and (D) BLG-GEM; the number molecular contacts/forces contributed by amino acid residue interaction shown for (E) BLG-CUR and (F) BLG-GEM; the protein-ligand contacts for (G) BLG-CUR and (H) BLG-GEM.
Similarly, MD simulation was also performed to understand the stability of GEM interaction with BLG (Figure 9B). The RMSD value of protein backbone reached equilibrium state at 8 nsec, thereafter, BLG-GEM showed oscillations and short overlap between 12-30 nsec. Ligand RMSD value seems to be rapidly fluctuating between 3.0 – 48 nsec, illustrating that the GEM binding is weak as compared to CUR. The backbone conformation of BLG seems to be stable (RMSD = 1.0 – 1.8 Å) after interacting with GEM (12-48 Å). We observed a small order of overlap between 20 – 34 nsec with ligand ΔRMSD value 10 Å for BLG-GEM complex. Based on the RMSD results, we noticed that GEM oscillations were significantly larger than the protein, thus the likely conclusion could be diffusion of the ligand away from its initial binding site or weak interactions of GEM with BLG.

Conversely, the analysis of myosin conformations was studied with CUR compounds using MD simulation, strongly supporting the influence of our multi-spectroscopic study. They showed that curcumin led to a significantly higher fluctuation in the myosin tail, which suggested that the myosin tail has higher flexibility (Zhang et al., 2020).

3.6.2. Root mean square fluctuation (RMSF)
Root mean square fluctuation (RMSF) values of BLG-Drug complexes were calculated and plotted against residue numbers at the simulation trajectory (Figure 9C-D). There are various regions in BLG involved in binding with CUR, which was investigated in terms of amino acid residue numbers with RMSF value [(50, 1.2 Å), (65, 1.5 Å), (86, 1.9 Å), (110, 2.3 Å), (130, 1.0 Å), and (140, 1.2 Å)] showing higher fluctuation as compared other residues (see Figure 9C). Additionally, there are some movements of side-chains within the central calyx caused by the ligand interaction. Precisely, Phe105 and Met107 entail considerable repositioning to produce room for the ligand (Ragona et al., 2000). BLG-CUR complex showed significant decrease in fluctuation of loops near residues number 77 and 86, belonging to the EF loop (shown by green lines in Figure 9C). Previously, it has been reported that ligand binding influences EF loop conformation and the binding showed reduction in the RMSF, i.e. it induced a restriction in the flexibility of BLG structure (Ragona et al., 2003).

For GEM, the average RMSF manifests exactly double residual displacement of protein conformation in BLG-GEM than BLG-CUR complex, indicating that CUR interacts stronger with BLG and induces restriction on protein structure dynamics. α-helices and β-sheets as represented by blue and pink bars were rigid, whereas white bars represent the loop regions that fluctuate the most during simulation (Figure 9D). The loops at the closed end of the calyx (BC, DE, and FG) are short, whereas those at the open end (AB, CD, EF, and GH) are longer and more mobile. The fluctuation of protein region near residues number with RMSF peak values [(65, 1.6 Å), (78, 1.4 Å), (86, 4.1 Å), and (110, 2.3 Å)] demonstrated higher fluctuation than other residues. Likewise, the specific constraints were observed after GEM interaction near residues such as 50, 110 and 125 (indicated by green lines in Figure 9D), these residues suggest that GEM binding site is close to these loops in BLG protein. We noticed large mobility near residue 86 (EF loop) followed by residue at 110 belonging to the GH loop, indicating that EF loop is not a binding site for GEM drugs. The average RMSF reveals strong CUR binding with BLG and relatively weak interaction with GEM, which agrees with spectroscopy studies.

3.6.3. Protein-ligand interaction fraction and contacts
The protein-ligand interaction fraction can be characterized by four types of interactions such as; hydrogen bond,
hydrophobic and ionic forces, and water bridges. Here, BLG-drug interaction is represented through the ‘simulation interaction diagram’ as illustrated in Figure 9E-F. For BLG-CUR, the simulation proved that there is dominance of hydrophobic interactions (20 hydrophobic contacts), water bridges (15 contacts), and hydrogen bonding (15 contacts). Besides, it was noticed that nonpolar amino acids residues participating in most of these interactions are Pro38, Leu39, Val41, Ala86, Leu87, Phe105, Met107, and Ala118. This study agrees with the simulation studies reported previously on nonpolar ligands, such as retinol and cholesterol (George Kontopidis et al., 2002). For BLG-GEM complex, the molecular contacts were lesser as compared to CUR (Figure 9F). In the interaction fraction, water bridges were recognised to be pronounced in GEM binding, and followed by hydrogen bonding which also strongly participated, an evidence for the experimental results obtained by spectroscopy studies. On the other side, One-half of the curcumin structure binds to BLG within its calyx, dominated by three nonpolar residues participating the most as shown in Figure 9G. Pro38 interacts via hydrophobic contacts and through water-bridge with one of the diketone moieties of CUR, while Leu39 and Met107 possess only hydrophobic contacts. The rest of the curcumin molecule is exposed to the solvent. GEM shows large solvent exposure areas that participate in water bridge and hydrogen bond contacts (Figure 9H). We conclude that the spectroscopic outcomes of BLG with both CUR and GEM binding are in strong agreement with molecular dynamics simulation.

3.7. In vitro cytotoxicity

The in vitro cytotoxicity of AuNPs, BLG, CUR, and GEM was assessed by MTT assay. The percent cell viability of breast cancer cell line MCF-7 on different treatments is represented in bar graph Figure 10.

MCF-7 on treatment with AuNP for 24 and 48 h showed IC_{50} value of 1.445 nM and 0.863 nM respectively, which is much larger concentration than our spectroscopic experiments. AuNP showed dose dependent decrease in cell viability (Figure 10A-B). The IC_{50} value for CUR at 24 and 48 h was 1.521 μM and 0.8666 μM, while that of GEM was 12.84 nM and 11.15 nM, respectively (Figure 10C-D). The action of GEM on MCF7 cells seems to be slow acting in terms of cytotoxicity trend as shown in Figure 10E-F, while, Sara et al. results showed IC_{50} value of 0.4 μM for GEM, probably because a saturating concentration is delivered to pancreatic cancer Cells (Trabulo et al., 2017). It was shown that curcumin was non-toxic, and remains active after complexation (Sahu et al., 2008). BLG was also tested for cytotoxicity, at higher concentration of 10 μM BLG increases the cell viability (Figure 10G-H). This could be because there are enough BLG molecules available to interact with cells. We noticed significant cytotoxicity at 0.10 M PBS concentration, it was very high for cell line studies, thus recommending lower concentration of PBS or other compatible buffer solvent for further cell line studies. Further, in detail investigation of BLG and AuNP-BLG conjugated with CUR and GEM will be carried out on different anticancer cell lines.

4. Conclusion

The present study demonstrates the biophysical characterization of BLG and AuNP-BLG with anticancer agents (curcumin and gemcitabine) using multi-spectroscopic approach and molecular simulation dynamics with biocompatibility/toxicity study on breast cancer cells. The concentration dependent process of physical adsorption of BLG on AuNP surface has been characterized by DLS and zeta potential. It is observed that BLG possesses maximum three binding sites for curcumin and single binding site for gemcitabine, there was no significant change binding affinity even after BLG was adsorbed to AuNP surface. In addition, the affinity for curcumin has been enhanced by protein-nanoparticle binding. Interesting results have been obtained by CD; both the drugs have adequately altered the BLG conformation. Raman spectroscopy revealed that the central calyx is facing the solvent environment, and could be accessible to incoming drug molecules. Further, auto-oxidation of curcumin was significantly prevented when bound to BLG, and the binding site for gemcitabine was also available on BLG via S-S bridge. The hydrophobic and hydrophilic interaction of curcumin with central calyx was confirmed, demonstrating strong affinity with both BLG and AuNP-BLG. The MD simulation study showed curcumin binding via hydrophobic forces through some amino acid contacts such as Pro38, Leu39, Val41, Ala86, Leu87, Phe105, Met107, and Ala118, along with the involvement of hydrogen bonds and water bridges. Gemcitabine showed hydrophilic interactions with residue numbers 55, 110, 125, as well as hydrogen bonds and water bridges, suggesting binding position is close to loops in BLG protein. Thus, we believe that a comprehensive multi-spectroscopic study of AuNP-BLG conjugate for uploading of cancer drugs serving as a concrete vehicle for drug design in targeted drug delivery.

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Disclosure statement

The authors declare no conflict of interest.

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