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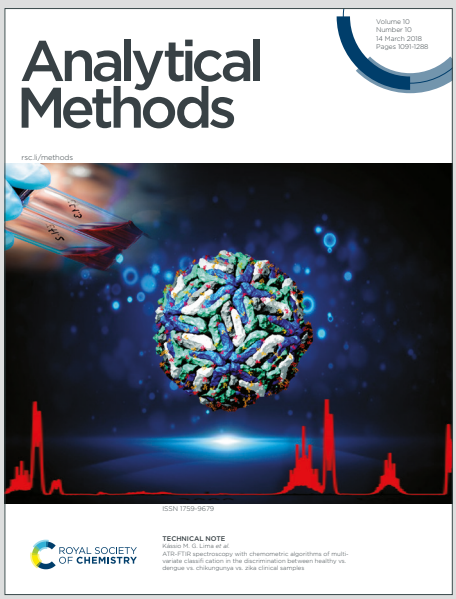

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A Multimodal Approach Integrating Spectroscopy, Deep Learning guided Molecular Docking, and Molecular Dynamics simulation for predictive assessment of Pioglitazone to albumin binding for Formulation development

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Abstract

Binding affinity is a critical parameter that can influence the state of the drug in vivo and help to define the formulation strategy. The current study implements a multimodal approach to analyse the binding affinity between Human serum albumin (HSA) and Pioglitazone. Ultraviolet (UV) absorbance and Fluorescence spectrometry analysis were performed on different combinations of human serum albumin and Pioglitazone complexes, and the absorbance and fluorescence intensities were mapped to calculate the binding constant. DynamicBind, a distinct deep-learning artificial intelligence tool, was implemented to perform in-silico docking studies using a non-conventional approach. Further, molecular dynamics simulation was also performed to generate Root Mean Square Deviation, Radius of gyration, and Root Mean Square Fluctuation values, followed by Principal Component Analysis, Probability Distribution Function, and Free Energy Landscape analysis. The simulation output was analysed to interpret the binding affinity and associated conformation of the protein-active pharmaceutical ingredient (API) complex. The binding constant calculated through UV analysis was 1.1×10^{4} M^{-1}. Fluorescence spectroscopic analysis derived a value of 1.7×10^{5} M^{-}

[1]. At the same time, DynamicBind predicted the cLDDT score for the top predicted model to be 0.634, and a binding affinity value of greater than 5, indicating a relatively moderate binding between Pioglitazone and HSA. The results from molecular dynamics simulations further complemented our earlier observations, indicating non-covalent binding interactions and a stable protein-API complex, which is desirable for developing a formulation using HSA as a carrier polymer. This orthogonal approach also provided critical information on the fate of the API and possible considerations that needed to be made during the design of the formulation process, highlighting the need for similar approaches that could provide multifaceted advantages and help in optimising R&D costs and timelines.

***Keywords*:** Orthogonal approach, Spectroscopy, Binding affinity, Deep-learning AI model, Molecular Dynamics, Molecular Docking.

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1. Introduction

In the Generic Drug User Fee Act (GDUFA) Fiscal Year 2025 report, the U.S. Food and Drug Administration (US FDA) highlighted the development of orthogonal methods as a key investment area. In previous guidance documents for the characterization of drugs and biologics, the agency has emphasized the need for orthogonal methods as part of additional techniques.[1] This focus is also reflected in ICH guidelines, which have shaped the global understanding of orthogonal and complementary techniques for drug and biologic characterization.[2]

Orthogonal techniques have been critical to elucidate the structural and functional characteristics of drugs and formulations, especially in cases where a single analytical technique is unable to provide a complete dataset. With the advancements in drug formulation and the development of nanomaterials, the emphasis on advanced techniques to characterize individual critical quality attributes (CQAs) has become an area of research interest and is now a crucial part of regulatory submissions. Structure elucidation, drug-polymer binding, and the nature of bonds have also been evaluated in numerous articles and reports.[3-8]

In the study, we aimed to assess the binding of Pioglitazone drug to human serum albumin (HSA) using two different analytical techniques and two simulation tools. The objective was to verify whether we can use HSA to formulate a polymeric nanoparticle of Pioglitazone. HSA nanoparticles have been widely used to increase the solubility of poorly soluble drugs and enhance their pharmacokinetic behaviour in vivo.[9,10,11] It has also been used as a targeted drug delivery system owing to its propensity to be internalized in tumour sites.

The analytical techniques used for the study were UV spectroscopy and Fluorescence spectroscopy. Both techniques are spectroscopic in nature but use two different measuring principles. Through our analysis, we tried to determine whether the two analytical techniques can provide comprehensive information on the binding affinity of the drug to HSA, thereby aiding our understanding of the possible *in vivo* behaviour of the formulation.[12,13]

Alongside the two spectroscopic techniques, we performed molecular docking with a deep learning artificial intelligence (AI) docking tool, DynamicBind,[14] and Molecular dynamics simulation using Desmond in Schrodinger Release 2021-1, to understand the binding efficiency between HSA and Pioglitazone. Previously reported studies considered protein to be of a static nature, which is not the case.[12,13,15,16] In our study, both the simulation tools considered the protein to be in a dynamic state, and the simulation runs took into account the natural motion

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of the protein and further equilibrated the system to derive the stable conformation, potentially aligning with actual observations.[17,18]

2. Materials & Methods

2.1. Materials

Vials containing Human serum albumin (20% solution, Grifols) and Pioglitazone hydrochloride (gift sample from Dr. Reddy's Laboratories Ltd.) were used to prepare the diluted albumin-bound Pioglitazone nanoparticle. Methanol (Rankem) and Acetone (Merck) were used as process solvents to dissolve the poorly soluble Pioglitazone HCl. A Jasco V-750 double-beam spectrophotometer was used for UV analysis, and a Horiba Fluorolog 3-21 was employed for fluorescence spectroscopy.

2.2. Methods

2.2.1. Sample preparation

A 12 µM stock solution of HSA was prepared for both spectroscopic techniques. A Pioglitazone HCl stock solution was also prepared by dissolving the drug in a 4:1 mixture of Methanol and Acetone to attain a 5 mg/mL concentration. Next, 2 mL centrifuge tubes were filled with the HSA and Pioglitazone stock solutions in different combinations. The Pioglitazone concentrations in the centrifuge tubes ranged between 4.45 and 26.7 µg/mL. The mixtures were vortexed for 1 min and analysed immediately.

2.2.2. UV Spectrophotometry analysis

Each sample was analysed using a Double-beam UV Spectrophotometer (Jasco V-750 Spectrophotometer). The blank contained the combination solution of Methanol and Acetone. In the sample cuvette, the different API-HSA complexes were transferred and then scanned for absorbance spectra between 200 and 400 nm with data points collected every 1 nm using application software Spectra Manager™. The absorbance and λ_{max} were noted for each complex and HSA alone. The data were next plotted as a double reciprocal graph with absorbance values at 275 nm, the λ_{max} for HSA alone being used for calculation. Based on the equation for the graph plotted, the binding constant was deduced by dividing the intercept value by the slope value.[15,16]

2.2.3. Fluorospectrometric analysis

Steady-state fluorescence spectroscopy was performed using a Horiba Fluorolog spectrophotometer. An excitation wavelength of 275 nm was applied for both HSA and API–HSA complexes. Using the Horiba Fluorolog spectrophotometer, the emission spectra were analysed for HSA alone and all the combinations of API-HSA complexes. Like UV spectroscopy data, the Emission spectra were plotted as a double reciprocal graph with emission values at 361 nm, the emission max wavelength for HSA alone. Based on the equation for the graph plotted, the binding constant was deduced by dividing the intercept value by the slope value.

2.2.4. Molecular Docking study: DynamicBind simulation

Molecular docking is an important approach to understanding protein-ligand interactions.[19,20] DynamicBind performs dynamic docking by allowing significant protein conformational changes to predict protein-ligand complexes. In this study, the Neurosnap platform was used to perform the AI-based simulation to bind Pioglitazone to HSA.[21] The protein structure of HSA (**Figure 1A**) was retrieved from the Protein Data Bank (PDB), and the ligand structure (**Figure 1B**) was obtained from PubChem. (Upon submission of both input parameters in DynamicBind (accessed in the Neurosnap platform on February 18, 2025), docking simulation were performed yielding the top 10 predicted protein-ligand models. The predicted structures were also complemented by their contact-LDDT (cLDDT) scores and binding affinity value, which were generated as part of the algorithm.[14]

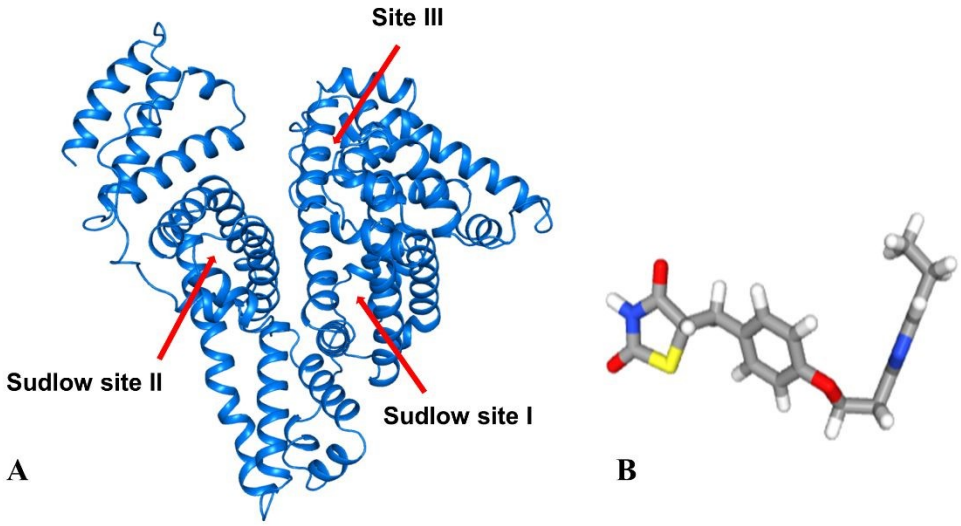

Figure 1. Structures of (A) human serum albumin (PDB ID 1AO6) and (B) Pioglitazone (PubChem CID 4829).

2.2.5. Molecular Dynamics (MD) Simulation study

Molecular Dynamics simulation study was performed using the Desmond (D. E. Shaw Research, New York, Academic License, link: https://www.deshawresearch.com/) in Schrodinger Release 2021-1 to understand the dynamic interactive behaviour of the protein-ligand system.[22] As per standard protocol, the protein-ligand system built up by Desmond software was solvated with the TIP3P water model within a predefined orthorhombic box of 10 Å size. This was followed by neutralization of the charges with an adequate quantity of counter ions. Further energy de-escalation was performed by removing steric clashes and other unfavourable interactions. As a final step, the energy-minimized system was loaded for a simulation run for 200 ns at 300 K, with a recording interval of 200 ps as per the NPT ensemble method. The output of the simulation runs was next interpreted in terms of radius of gyration (RG), root-mean square deviation (RMSD), and root-mean square fluctuation (RMSF).[23] Additionally, the free binding energy for the apo protein and protein-ligand complex was next calculated using the molecular mechanics with the generalized born and surface area solvation (MMGBSA) method.[24,25]

3. Results & Discussion

For macromolecules like HSA, fluorescence behaviour can help early researchers understand the molecular interaction between molecules of interest and HSA. Parameters that can be ascertained through fluorescence studies include binding affinity, binding mechanism, and the ligand binding site on the protein. Spectroscopic studies have played a significant role in annotating this behaviour. It has also been used to interpret the conformational changes the protein undergoes for binding to the protein.[12,15,16,23-29]

HSA is a 585 amino acid monomeric protein consisting of 17 disulfide bridges, with its secondary structure chiefly composed of α-helices. HSA (**Figure 1A**) consists of 9 loops, spanning three homologous domains named Domain I (residues 1-195), II (residues 196-383), and III (residues 384-585), with each domain consisting of two long and one short loop. Each domain is further divided into two subdomains, with the first two loops denoted as subdomain A, with the remaining being denoted as subdomain B. HSA contains 35 cysteine residues, of which all, except the C34 residue in Domain I, are involved in a disulfide bond, further stabilizing the protein structure. Although these homologous domains appear to have similar structures, each domain exhibits distinct binding characteristics.[23,30-33]

It contains two major drug-binding sites: Sudlow site I (subdomain IIA), which preferentially binds bulky heterocyclic compounds, and Sudlow site II (subdomain IIIA), which favors

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aromatic compounds. In addition, a third major non-Sudlow binding site has been identified in subdomain IB, capable of accommodating a wide range of endogenous and exogenous ligands. Albumin also possesses up to seven binding sites for long-chain fatty acids, each with distinct binding affinities.[33]

In the present study, subdomain IIA (Sudlow site I) is of particular interest because pioglitazone contains a 2,4-thiazolidinedione ring and a pyridine moiety, structural features that favour binding at this site. HSA exhibits intrinsic fluorescence arising from three amino acid residues (tryptophan, tyrosine, and phenylalanine). Among these, tryptophan is the dominant fluorophore, and Sudlow site I is enriched with the key residue W214. The fluorescence contribution of phenylalanine is minimal, while tyrosine fluorescence is often diminished due to ionization and interactions with neighbouring amino and carboxyl groups, including proximity to tryptophan. Consequently, fluorescence quenching experiments primarily probe interactions involving W214 in subdomain IIA, enabling precise characterization of ligand binding at Sudlow site I.[12,34] Additionally, binding of small molecule drugs may not fill the hydrophobic pocket of the Sudlow site I, thereby predisposing the site to be filled with wate molecules, and further Hydrogen bond interaction with the polar residues at the site.[33,35]

3.1. UV Spectrophotometry analysis

UV spectroscopic analysis is a simple and effective process to qualitatively and quantitatively measure the binding nature between protein and ligand. UV analysis of HSA alone revealed a λ_{max} at 275 nm. Further analysis of the HSA-drug complexes revealed a blueshift of the UV spectrum (**Figure 2**). This indicated an interaction between the amino acids in the hydrophobic pocket and those of Pioglitazone. The hypsochromic spectrum shift is predominantly associated with the structural changes that the protein undergoes to accommodate the ligand to its preferred binding site. This phenomenon has been widely reported in multiple literatures and can be considered a cornerstone for structural analysis of protein-ligand complexes.[15,16,30] Besides the shift in the UV spectrum, a proportional increase in absorption intensity was also observed. An increase in intensity with an increase in drug concentration could also be attributed to the presence of Pioglitazone. Pioglitazone has a λ_{max} of 265 nm.[35,36] In general, the presence of ligand accentuates the blueshift, but in this specific case, it also contributes to the absorption intensity for the drug-protein complexes. The increase in intensity due to higher concentrations of the drug can indicate that the drug itself did not undergo any change and was able to hold on to its functional groups. It may also point to the fact that the binding of

pioglitazone to the amino acid residues is non-covalent and the occurrence of a π-π transition with the Tryptophan residues at the binding site.[19]

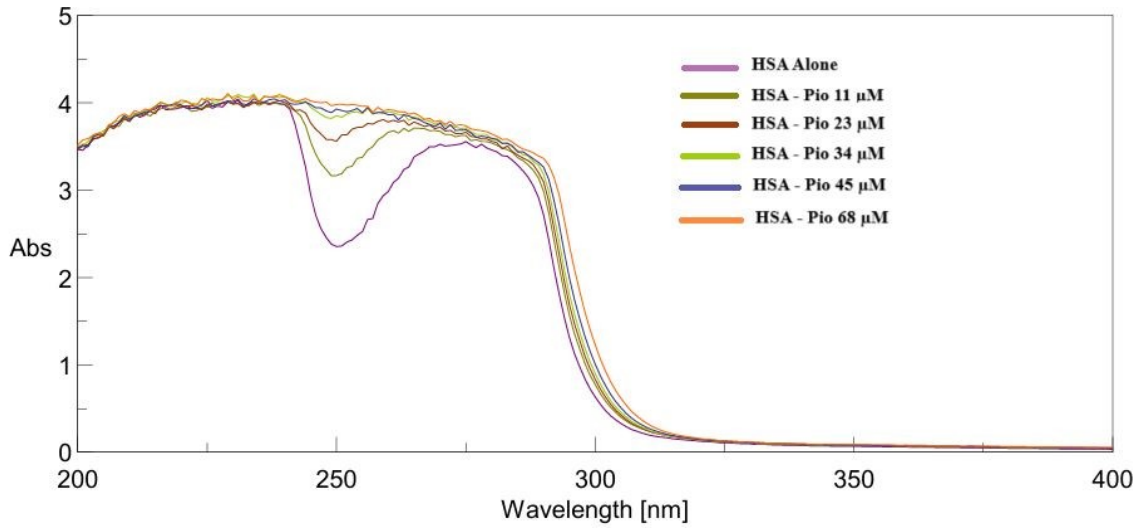

Figure 2. The overlay of the UV spectra shows the hypsochromic shift of the absorbance for the different samples, with the λ_{max} shifting to shorter wavelengths with increasing concentration and higher absorbance.

3.2. Fluorospectrometric analysis

The fluorometric analysis involves quantifying photoluminescence triggered by the absorption of UV wavelengths. As previously established, HSA exhibits known photoluminescence properties, primarily due to the presence of Tryptophan residues. A spectroscopic analysis of HSA alone revealed that an excitation wavelength of 275 nm at room temperature generated an emission spectrum with a λ_{max} at 361 nm. Like UV spectroscopy, fluorometric analysis generated emission spectra for individual drug-HSA complexes and a similar hypsochromic shift (**Figure 3)**. On the other hand, the emission intensity showed a behaviour opposite to that of UV spectroscopy. Pioglitazone does not possess any fluorophore and hence did not contribute to the fluorescence intensity. The decline in intensity can be attributed to the utilization of tryptophan residues and adjacent amino acid residues for binding to pioglitazone. The inverse relationship proved the preferred binding site to be Sudlow site I for Pioglitazone.[37-40]

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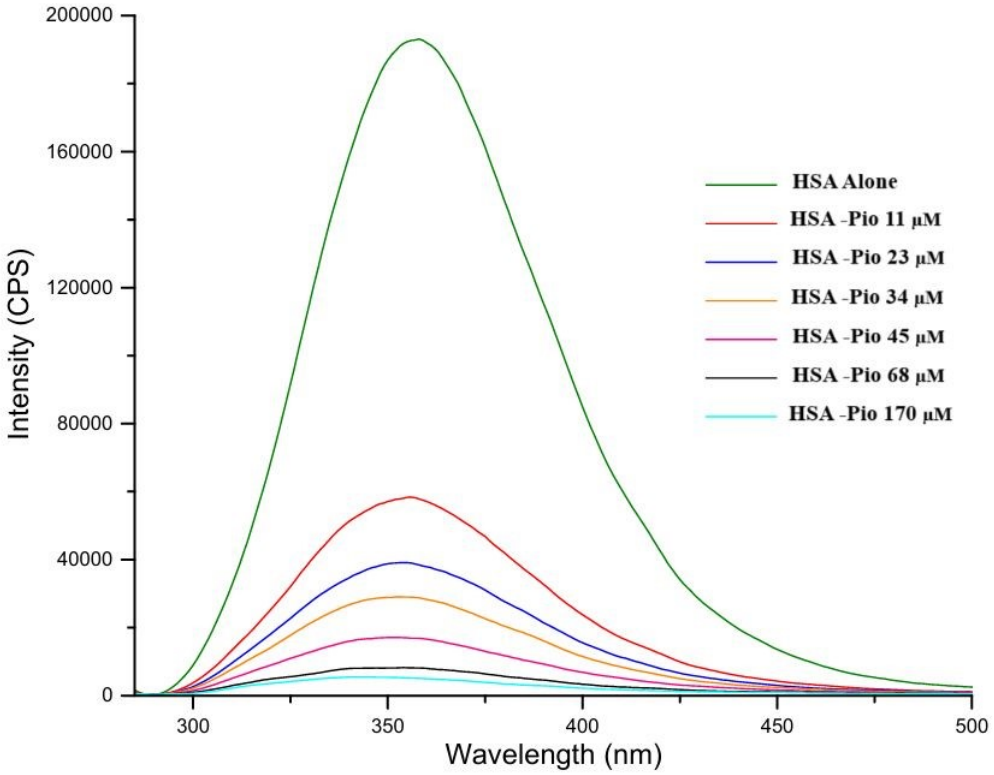

Figure 3. The overlay of the Emission spectra shows the hypsochromic shift of the fluorescence for the different samples, with the emission max shifting to shorter wavelengths with increasing concentration and lower fluorescence.

3.3. Double-reciprocal graphs to determine binding constant

A double reciprocal graph with respect to Pioglitazone molar concentration was plotted to determine the binding constant. For UV spectroscopy (**Figure 4**), the mode values of $1/(A_0\text{-}A)$ were considered for plotting the graph and plotted with regard to the inverse of the molar concentration of Pioglitazone in the samples. For Fluorescence spectroscopy (**Figure 5**), the $1/(E_0\text{-}E)$ values were plotted vs the reciprocal of Pioglitazone molar concentration of samples. In respective cases, A_0 and E_0 indicated the absorbance for HSA alone at 275 nm and the emission of HSA alone at emission wavelength 361 nm. The spectroscopic titration by individual methods was performed for individual concentrations at the aforementioned wavelengths for respective methods. For Fluorescence spectroscopic titration, an emission/ fluorescence correction (E_{cor}) was done, considering the inner filter effect study, as per Equation 1, where absorbance values of Pioglitazone at excitation (A_{ex}) and emission (A_{em}) wavelength were considered for the correction E_{obs}.[41]

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$$Ecor = Eobs * 10^{\frac{Aex}{2}+\frac{Aem}{2}}$$ …. Equation 1

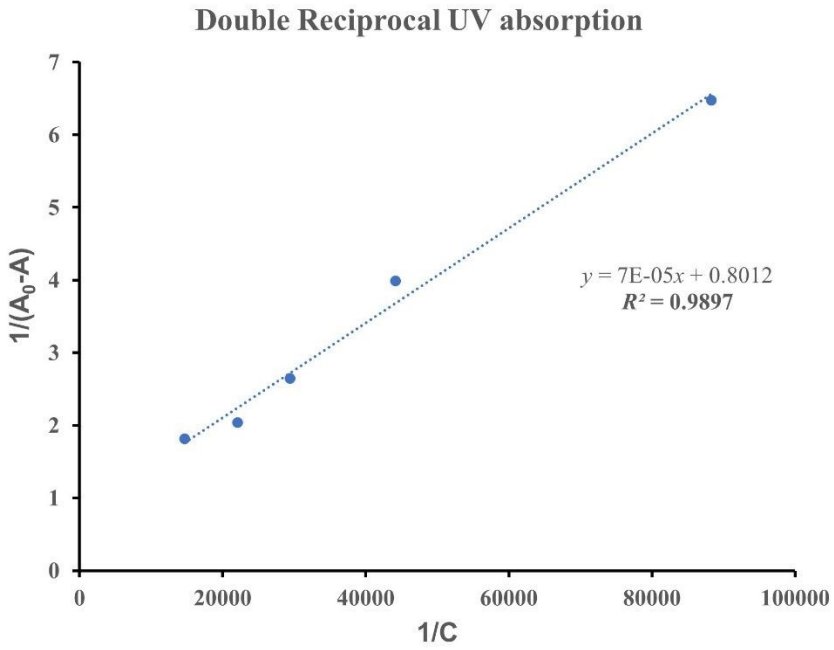

Figure 4. The double reciprocal graph of UV absorbance with different HSA-Pioglitazone complexes.

The plotted graph showcased a linear relationship with a correlation coefficient higher than 0.9 for both spectroscopic procedures. The binding constant derived from the graph was next calculated by dividing the intercept value by the slope of the graph. The binding constant calculated through UV analysis had a value of 1.1×10^4 M^{-1}, while Fluorescence spectroscopic analysis derived a value of 1.7×10^5 M^{-1}. Both these values are indicative of medium affinity (expected values to be between 10^3 and 10^6 M^{-1}).[12,15,16,37,39]

The binding affinity of a drug to the protein site can influence its therapeutic effectiveness in vivo. The ability of a drug to diffuse from systemic circulation and reach its intended target depends much on its binding affinity to plasma proteins like HSA. Our study aimed to evaluate the binding affinity and nature of interactions of Pioglitazone to HSA. A drug weakly bonded to HSA will have poor bioavailability as it will be poorly distributed, rapidly metabolised, and eliminated, thereby having a shorter half-life. Strongly bonded drugs, in contrast, will not be efficiently released to be available, resulting in sub-therapeutic effectiveness. Moderately bonded drugs ensure that the drug will efficiently diffuse through the biological barrier and have the required half-life to be transported through the systemic circulation to its intended activity site. At the same time, the affinity does not impede its release, thereby ensuring the availability of the drug in sufficient concentration to exert its therapeutic impact. The binding

affinity values generated through the orthogonal studies complement the understanding with regard to the values derived from spectroscopic measurements.[15,16,30,38]

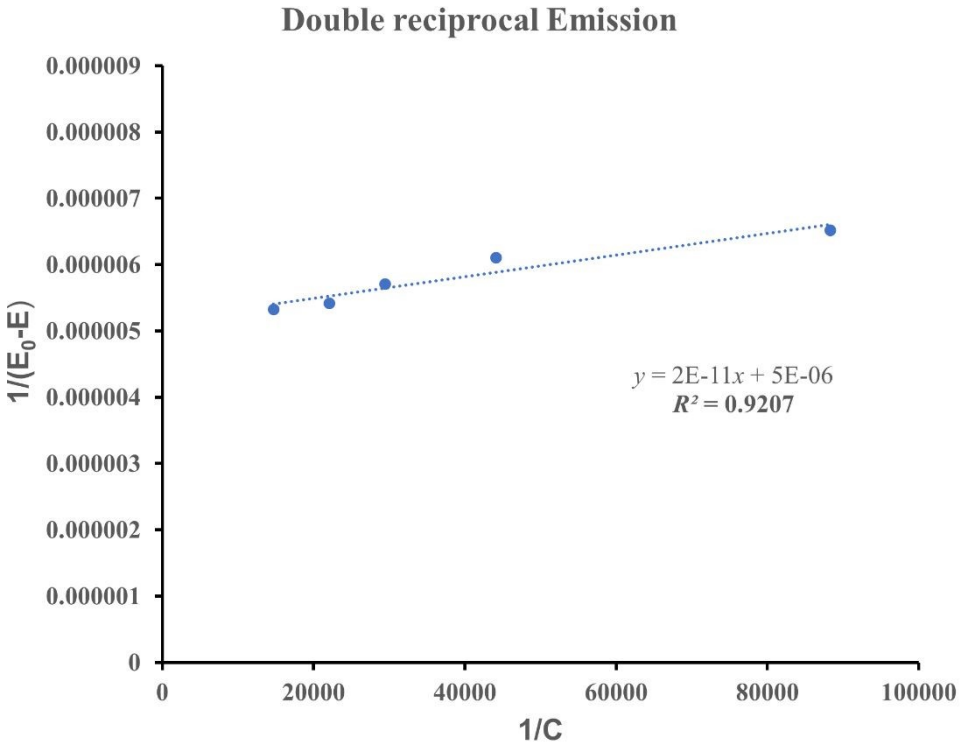

Figure 5. The double reciprocal graph of Fluorescence emission with different HSA-Pioglitazone complexes.

3.4. DynamicBind simulation

DynamicBind is a geometric deep generative model that considers proteins not to be in a state of rigid configuration while docking.[14] This ability of proteins to transition between multiple conformations is central to their functional character *in vivo*. Ligands, especially drug moieties, can only bind to specific conformations of the targeted protein molecule, thereby regulating the overall impact of the drug in the biological system. It outperforms other models in predicting possible ligand poses for known protein datasets (PDB and MDT). It does so by being able to predict protein configuration with lower Root Mean Square Deviation (RMSD) scores for regions around the binding sites, providing a protein conformation resembling its native state. The cLDDT scoring developed as a measuring parameter for DynamicBind generated models is well aligned with the auROC score, showing its effectiveness in selecting the most appropriate model from amongst its multiple predicted protein-ligand complexes. DynamicBind also calculates Binding affinity as a negative logarithm value of dissociation and

complements the cLDDT score to assess the quality of ligand interaction with the protein binding site.[14,42]

The DynamicBind predicted the cLDDT score for the top predicted model to be 0.634, with the range varying between the top model between 0.634-0.472, with the absolute value of 1 indicative of near native contact.[21] The binding affinity value greater than 5 indicates a relatively moderate binding between Pioglitazone and HSA.[43] The results are consistent with the practical observations from the spectroscopy analysis, where we predicted the affinity to be of medium intensity. Therefore, DynamicBind can serve as a usable tool for initial screening and understanding. The fact that it demands far less computational power compared to conventional docking tools, makes it lucrative for researchers working on early-stage projects.

Aside from the binding interaction deduced through spectroscopic analysis, the simulated docking study also identified other interactions and their nature. A π-π stacked interaction is evident between the pyridine ring and the tryptophan residue (W214), with the electron-rich indole ring of tryptophan contributing to such non-covalent interactions, inadvertently stabilizing the protein-ligand system (**Figure 6**).

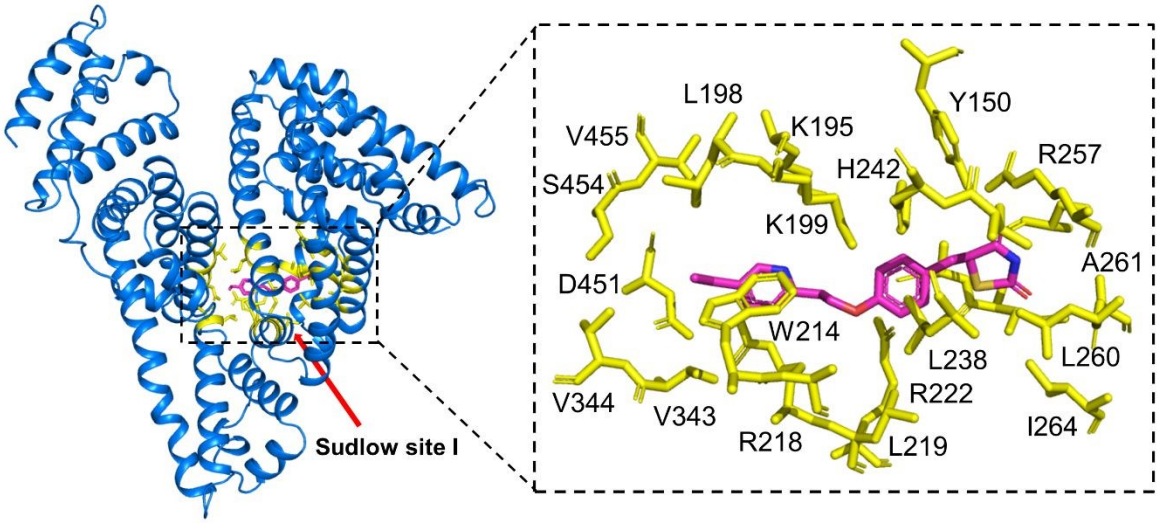

Figure 6. Possible binding mode of Pioglitazone (shown as a pink stick model) within the binding site of human serum albumin (HSA, shown as a cartoon representation). Interacting residues are highlighted in yellow.

A π-alkyl interaction between W214 and the alkyl chain adjacent to pyridine is also predicted, owing again to the indole group of W214.[44,45] The other predicted interaction involving amino acid residues other than W214 includes a π-cation interaction of the pyridine ring of

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Pioglitazone with arginine residue (R218) owing to the positively charged guanidinium group,[46] a π-alkyl interaction between the benzene ring of Pioglitazone and leucine residue (L238), and a hydrogen bond between R257 residue and the thiazolidinedione ring (**Supplementary Figure S1**). The possible nature of binding interactions is thereby non-covalent, further supporting our earlier assumptions derived from the previously executed studies.

3.5. Molecular dynamics (MD) Simulation study

The molecular dynamics simulation was run for a period of 200 ns, and the stability of the protein-API system was assessed. The assessment was done on the RG, RMSD, and RMSF values derived for the protein in both the apo and bound states. The RMSD values, as observed from **Figure 7**, indicate that the protein-ligand system is as stable as the apo-protein structure, while the RG values for both protein systems show similar compactness, corroborating the previous observation obtained through DynamicBind simulation. The residual mobility of the bounded and unbounded form of human serum albumin was further computed, and the resulting RMSF values for the residue seem to be comparable with the apo protein, showing more structural flexibility than the bounded form.[47,48]

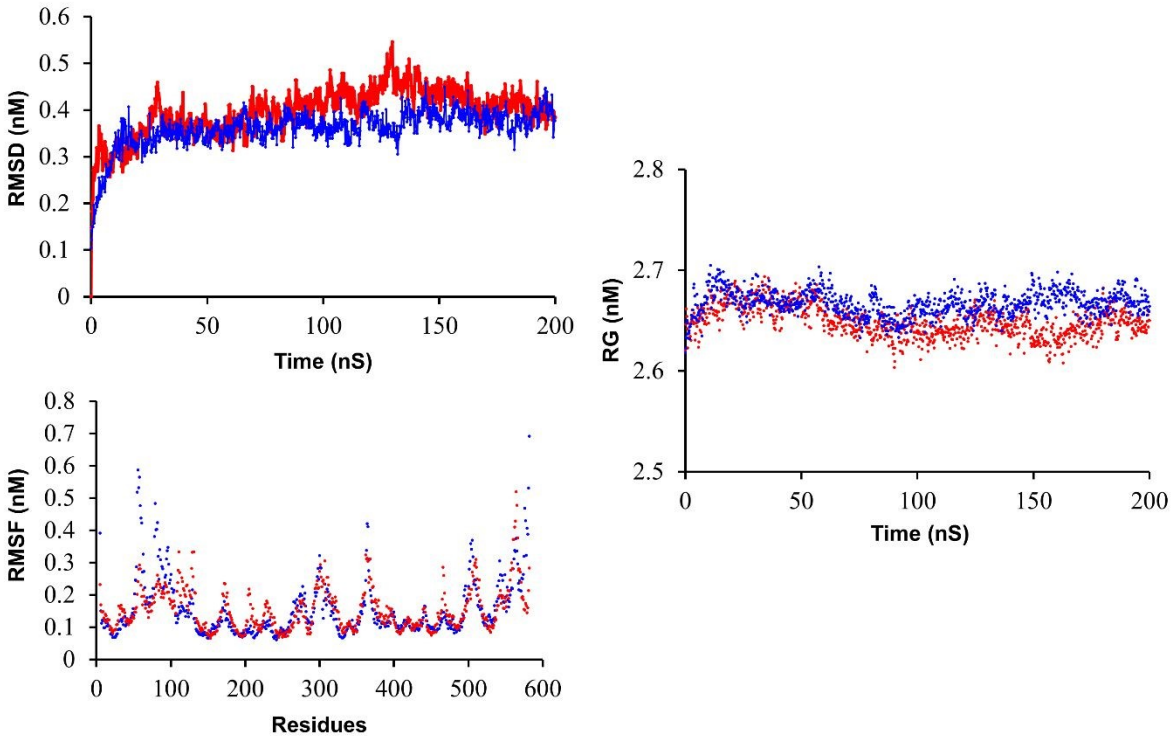

Figure 7. Comparison of RMSD, RMSF, and RG of apo-human serum albumin (blue) and its complexes with Pioglitazone (red).

Hydrogen-bond analysis of the MD trajectory revealed stable and persistent interactions between Pioglitazone and key HSA residues. R257 exhibited the highest hydrogen-bond occupancy (~77%), followed by S287 (~34%) and R222 (~16%), indicating their critical roles in ligand stabilization. Additional hydrogen-bond interactions were observed with R218 (~13%), L260 (~8%), and A261 (~1%). As shown in **Supplementary Figure S2**, the timeline analysis illustrates the dynamic evolution of Pioglitazone-HSA interactions, highlighting both the overall interaction frequency and residue-wise contact persistence throughout the simulation. Collectively, the high occupancy and temporal stability of these hydrogen bonds support the favourable binding orientation of Pioglitazone and confirm the dynamic stability of the Pioglitazone-HSA complex during the MD simulation.

3.6. Probability density function (PDF) analysis

The Probability Density Function plots analysed the different conformations of the apo and ligand-bound protein. They mapped them based on the highest (in blue) and lowest (in red) occurring conformations (**Figure 8**). The differences in RMSD value between the highest and lowest conformers for both the apo protein seemed to be higher than that of the ligand-bound protein, indicating a higher flexibility for the unbound protein than that of the bound protein. In case of RG values, the differences between the highest and lowest conformers of the apo-protein seem to be narrower than those for the API bound protein, indicating structural changes and slight relaxation of the protein secondary structure.[47,49]

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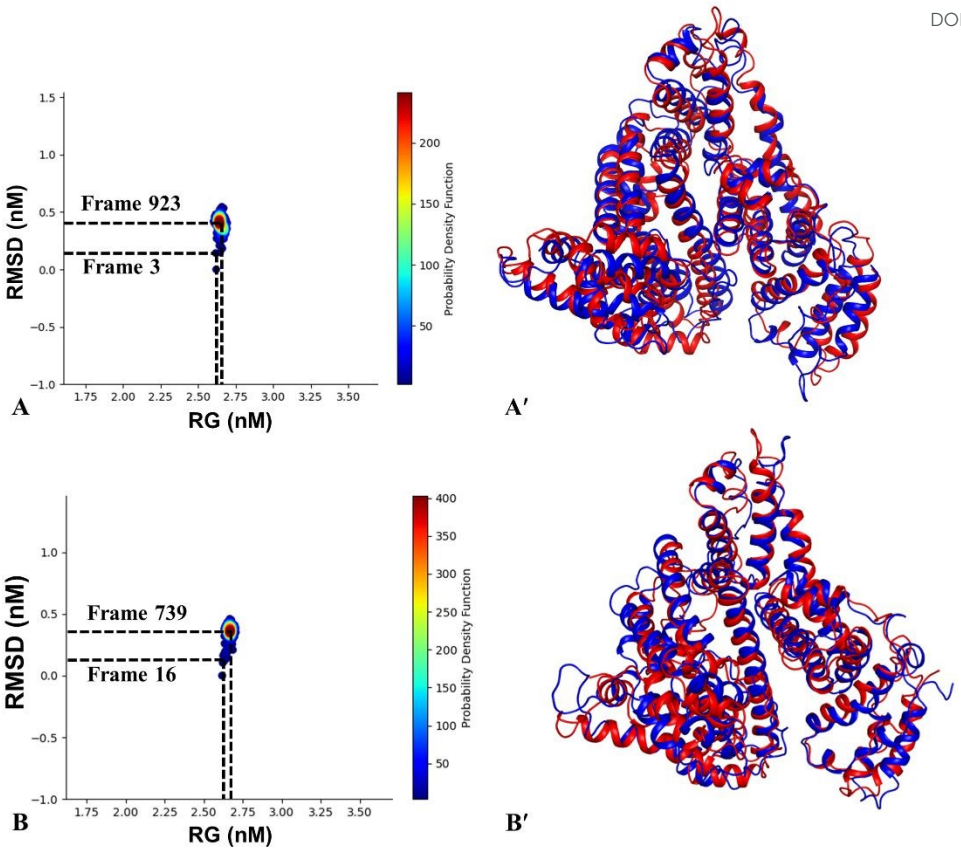

Figure 8. Probability density function (PDF) plot along with corresponding largest (blue), sparsely populated (red) conformation in case of apo-HSA (A, A´), Pioglitazone-bound HSA (B, B´).

3.7. 3D Free Energy Landscape (FEL) analysis

The 3D FEL plots (**Figure 9**) were prepared to map the free energy variation in accordance with the RMSD and RG values for different conformations of the unbound (apo) and bound protein conformations. The 3D plot for the apo protein showcased a wider cluster of low-energy peaks, which may be a result of multiple conformations being in a metastable state, owing to the absence of a ligand to restrict the apo protein to a stable conformer (**Figure 9A**). On the other hand, a narrow cluster of low-energy peaks was derived for the API-bound protein complex, indicating a more stable conformation (**Figure 9B**). The observations were comparable to those observed through PDF analysis and further demonstrate the correlation between RMSD and RG values to free energy variation, to deduce conformer stability.[47,49]

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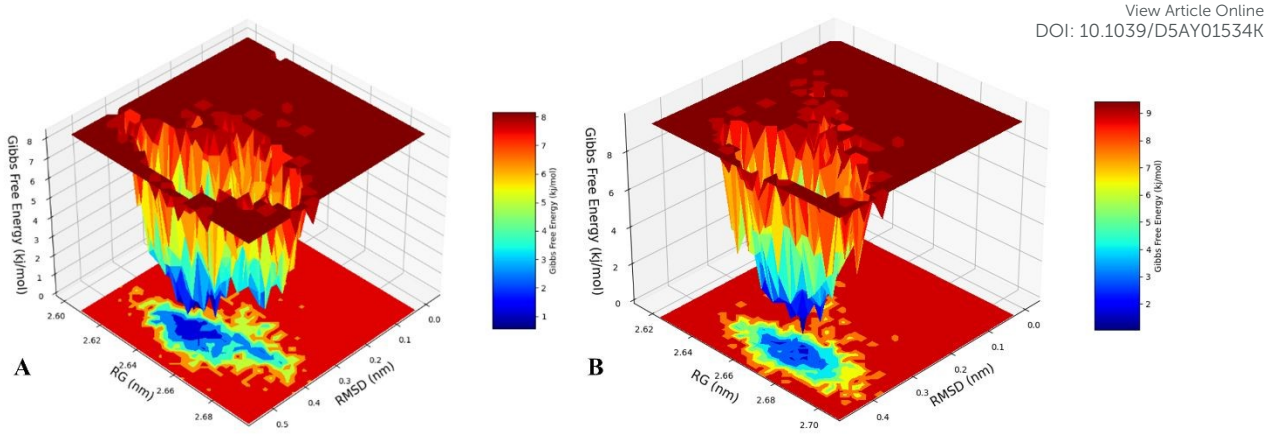

Figure 9. Free energy landscape (FEL) plot of (A) apo-HSA, and (B) Pioglitazone-bound HSA.

3.8. Principal Component Analysis (PCA)

Furthermore, principal component analysis of the MD trajectory data was performed to account for any alteration in the major global atomic motion of amino acid residues in unbound (apo) and bound HSA. The coherent internal 3D motions were captured using a covariance matrix, with the resulting eigenvectors representing dominant modes of motion in the trajectory. Notable differences in trajectory clustering were observed for both the unbound (apo) and bound protein (**Figure 10**).

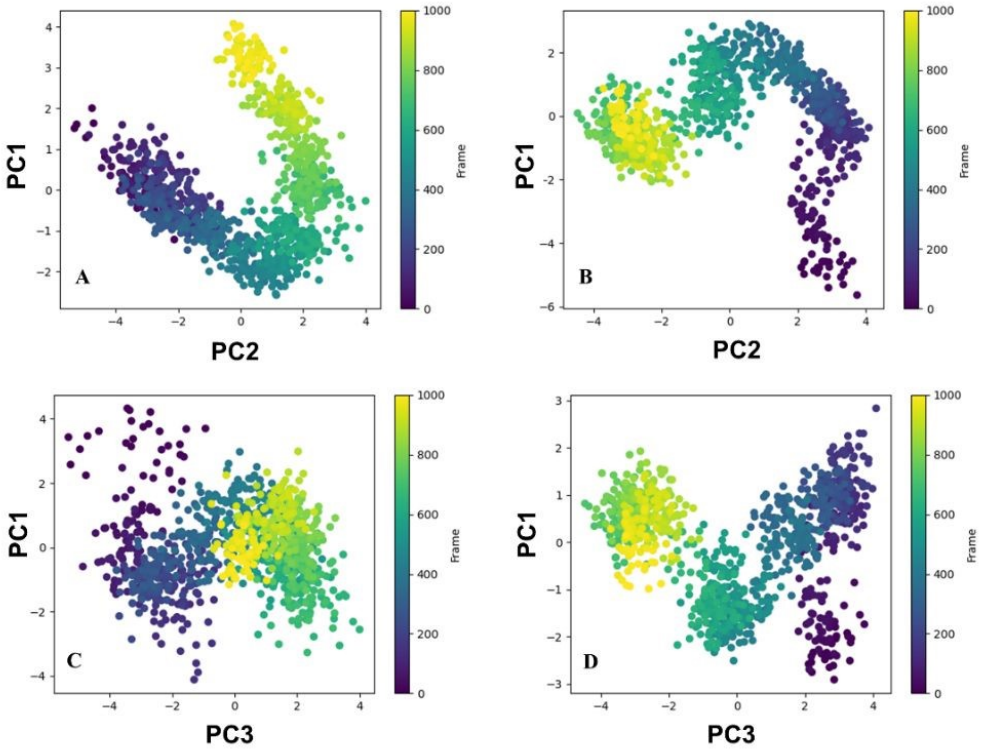

Figure 10. PCA plot for [A/C] for HSA and [B/D] for Pioglitazone-bound HSA.

In the case of apo-HSA, all intermediate frames (excluding initial frames) clustered along PC1 vs. PC2, indicating global conformational shifts. Interestingly, the terminal frames (800-1000) of the Pioglitazone-bound HSA complex scattered together, suggesting a trend toward convergence, unlike the more dispersed apo structure. Meanwhile, in the PC1 vs. PC3 projection, the terminal apo-HSA frames aligned along PC3, indicating subtle side-chain rearrangements (**Figure 10**). These shifts likely reflect the presence of multiple low-energy conformers, consistent with findings from previous FEL analyses (**Figure 9**).

4. Conclusion

In this study, we tried to assess the implementation of a few simple techniques to determine the tested parameter. The spectroscopic techniques discussed are commonly available instruments. Compared with advanced tools such as nuclear magnetic resonance, our approaches require simpler sample preparation, instrument handling, and data interpretation, making them more feasible for early-stage researchers. Notably, this study employed a deep learning-based artificial intelligence framework to perform non-conventional, dynamic molecular docking. In contrast to similar-type of previously reported studies[40,50] that typically treat HSA as rigid entities, the present work considers the HSA in a dynamic state during docking. Furthermore, the use of DynamicBind via the user-friendly Neurosnap platform eliminates the need for high-performance computing resources, thereby broadening accessibility to the wider research community. Complementary 200 ns molecular dynamics simulations provided additional insights into binding efficiency, protein-ligand complex stability, and conformational behavior, collectively strengthening the reliability and mechanistic interpretation of the results.

The understanding derived is pivotal for designing formulation strategy and predicting probable behaviour of the formulated product involving HSA as a carrier for Pioglitazone *in vivo*. The moderate binding affinity values of Pioglitazone to HSA, derived through spectroscopic techniques, further corroborated to an extent by the simulation tools, provided affirmative proof of the suitability for developing an HSA-bound formulation system. The spectroscopic analysis and docking study indicated that the binding between the drug and protein is non-covalent and exhibits the requisite affinity, which is expected to improve the drug's bioavailability. The simulated docking studies further shed light on the possible formulation pathway. The cLDDT score suggests that for the interaction to occur, a slight modification is required of the native protein conformation. These findings indicate that

incorporating an energy-input step into the process design may facilitate binding. Therefore, through this pre-formulation study, which aimed to determine the binding affinity via an orthogonal approach, we derived information that could help us design the process and predict *in vivo* behavior. It can also prove beneficial as it will help reduce R&D cost and timeline, showing the utility of orthogonal techniques beyond regulatory stipulations.

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CRediT authorship contribution statement

Saswata Banerjee: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Writing- original draft, Visualization. **Sk. Abdul Amin:** Investigation, Data Curation, Formal Analysis, Writing- original draft, Visualization. **Shovanlal Gayen**: Data Curation, Formal Analysis, Writing- original draft, Supervision. **Gautam Singhvi:** Writing- review & editing, Supervision. **Rajeev Taliyan:** Writing- review & editing, Supervision. **Sakshi Priya:** Data Curation, Writing- review & editing. **Yashika Tomar:** Data Curation, Writing- review & editing.

Conflict of Interest

There are no conflicts of interest to declare.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.