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Exploring the Interaction Dynamics of Azo Ester Fluorophores and Insulin: A Spectroscopic and Molecular Docking Approach

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ABSTRACT:

Azo-Ester Fluorophore (AEF), a phenol blocked isocyanate compound, has antifungal, antimicrobial, properties. Diabetes is a killing disease, especially for people who cannot maintain a healthy lifestyle. People with diabetes need additional information about a supplement that can prevent and treat diabetes. The present study aims to investigate the interaction of insulin with AEF using fluorescence spectroscopy, UV–Vis spectroscopy, and molecular docking methods. As an outcome of this study, we expect to understand the contribution of AEF in insulin resistance. The fluorescence spectroscopy results showed that the peak intensity of insulin emission decreased with AEF. The interaction of insulin with AEF involved a quenching effects. The interaction occurred through hydrogen bonding, van der Waal, and was hydrophobic. The results of UV–Vis spectroscopy showed that the addition of AEF caused a peak in a red shift, and the absorbance was hyperchromic. To further investigate the nature of the interaction between insulin and the AEF, various hydrogen-bonding solutes such as urea, guanidine hydrochloride, and acetamide were introduced, and their photophysical properties were studied. The molecular docking study showed that AEF interacts via hydrogen bonding with aspargine, tyrosine, glutamic acid and arginine. Hydrophobic interactions occurred in glutamic acid and tyrosine. The interaction of electrons occurred through the π orbitals of AEF with tyrosine A and B.

1. Introduction

Insulin is a polypeptide hormone that modulates glucose levels in the blood. Deficiency in insulin's secretion causes diabetes [1, 2,] and therefore it is also used as a medical compound that is widely prescribed for the treatment of diabetes. Insulin is stored in the pancreas in complex with Zn ions as a hexamer, which upon released into the bloodstream readily dissociates into the monomer [3-7]. This is an extremely essential step as only a bioactive monomer can bind and activate receptor to regulate blood glucose levels. In vitro study indicates that in solution at low concentration and physiological pH, insulin exists as a monomer or dimer [7]. Numerous biophysical studies of insulin under variable conditions helped to understand its structure and function [2, 5, 6, 8-15]. The active monomeric form consists of two polypeptide chains, chain-A 21 residues, and chain-B 30 residues (Fig. 1). These chains are held together by two inter-chain disulphide bonds (A7-B7 and A20-B19) and one intra-chain disulfide bond in chain-A (A6-A11). Chain-B plays an important role in the association of insulin monomer into dimer and hexamer [6]. The hexamer (Zn-insulin), which is the most stable form of insulin [2, 10] is well characterized around neutral pH values [6, 8]. The hexamer is capable of transitioning among distinct conformational states, which have been given the nomenclature T6, T3R3, and R6 16 depending on the conformations of the monomer subunits. The binding of anions and phenols to allosteric sites of insulin is needed to bring the conformational change from the T- to the R-state.

Electrons in the insulin residue serve absorption, excitation, emission, and fluorescence. Fluoresce molecules have absorption at 200–700 nm [16] The electrons transition occurring from π-π* [17]. The transition π-π* of the carbonyl group absorption in the protein was found at 210 nm [18]. Proteins have a chromophore in the form of peptide bonds that appears at 220 nm [19]. Insulin has an optimum absorption at 275 nm. In a previous study, wavelengths of 250–300 nm have been used to determine insulin absorption in different

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solvents, and insulin could be detected at a wavelength of 275 nm [20]. Wavelengths 220–340 nm have been used, measuring the absorption of insulin interactions with its aptamer [21] While wavelength at 260–400 nm is used by Correia et al. [22]. Insulin has two peaks at 275 nm and 282 nm. The presence of the two peaks in insulin is probably due to the absorption of an amine group having a lone electron pair (275 nm) and an aromatic ring group (282 nm). Lone pair electrons own by NH bonds was a component of protamine. Protamine is composed of arginine, which is rich in NH bonds. Protamine is a protein that can protect insulin from being degraded by enzymes. The uptake of proteins such as protamine was detected at a wavelength of 275 nm [19]. The aromatic ring is owned by tyrosine, tryptophan, and phenylalanine also have a signals. It was seen at a wavelength of 280 nm [23] Amino acid aromatic chromophore has an absorption range of 230–300 nm [19]

When binding occurs, the electrons in the insulin residue release energy (fluorescence). At the same time, the electrons in the ligand absorb energy, and the spectra overlap. The benzene group absorption of ligand overlaps with the aromatic ring absorption of insulin. It has been reported that molecular overlap can occur against small molecules with nitrogenous base groups in the DNA chain [24]. The fluorescence emission spectra overlap with the absorbance spectra of the acceptor molecule. Possibility of energy transfer when the donor–acceptor distance is less than 8 nm or 80 Å [25].

A spectroscopic study was carried out to explore the interaction between phloretin and insulin. UV/Vis spectroscopy, fluorescence spectroscopy, and circular dichroism spectropolarimeter were used in the study. The addition of Phloretin has revealed that the proportion of α-helix in the insulin stabilizes its structure. Phloretin's stabilization and enhancement of the α-helix structural configuration in insulin indicate that phloretin can improve insulin resistance [26]. Yanti et al investigated the interaction of insulin with resveratrol using fluorescence spectroscopy, UV–Vis spectroscopy, CD spectropolarimeter, and molecular docking methods. The fluorescence spectroscopy results showed that the peak intensity of insulin emission decreased with resveratrol. The interaction of insulin with resveratrol involved a combination of static and dynamic quenching effects. The molecular docking study showed that resveratrol interacts via hydrogen bonding with glycine and asparagine. van der Waal interactions occurred in asparagine, phenylalanine, and cysteine. The interaction of electrons occurred through the π orbitals of resveratrol with tyrosine A and B. [27]

Interaction occurs between proteins as donors and ligands as acceptors. Donors can produce fluorescent light. The fluorescence emission spectra overlap with the absorbance spectra of acceptor molecules so that the binding sites of Try with small molecules can be predicted through fluorescence observations. The Try can be the centre of the binding regions or close to the binding regions if it experiences a quenching effect. This quenching effect can be a reference for studying the configuration of protein molecules in the solution. Changes in the microenvironment around fluorescent clusters may be relevant evidence for binding proteins to small molecules [28] Fluorescence microscopy can provide information about the excitation spectrum, emission, fluorescence intensity, quantum yield, fluorescence lifetime, etc. This value can reflect the behaviour of the molecule at various angles. Many researchers have observed molecular configurations and analysed these parameters [29, 32]

Circular dichroism (CD) spectropolarimeter is a rapid spectroscopic technique in determining protein molecules' secondary structure and fold characteristics. This technique is widely used to study protein stability and whether configuration changes can form folds or mutations. The combination of fluorescent and CD data shows that there is induction of polypeptide bonds, changes in protein conformation, and exposure in hydrophobic regions [33]. Substitution of water molecules by ligands inside and outside the enzyme molecule can cause stability problems and reduce the strength of important covalent bonds so that the enzyme becomes inactive. Enzyme function will be lost if its orientation and proximity to the substrate molecule are lost. This can be detected through the reduced amino acid binding site. [23]

Molecular docking can predict ligand-binding sites on proteins to the atomic level. Non-covalent interactions between proteins and drugs are divided into four types: hydrogen bond interactions, van der Walls interactions, hydrophobic interactions, and electrostatic interactions [34]. The distance of 2.264 nm causes Van der Waals interactions and hydrogen bond interactions on Trp (donor) and prednisolone (acceptor) [34]. Van der Waals interactions and hydrogen bonding also occur in pepsin, and naphthol yellow decease the affinity of pepsin to the substrate [23]. Studies on the interaction of insulin with various small molecules have been carried out, such as vanillin [35], vitamin E, and vitamin D3 [36], paclitaxel

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[37], DPPC (dipalmi-toylphosphatidylcholine) or POCP (1-palmitoyl-2-ole-oylphosphatidylcholine) [38].

The present study aims (i) to explore the interaction between insulin and AEF by spectroscopy, (ii) to observe the change of insulin absorbance by UV/visible spectrometer after adding AEF, (iii) to study the interaction between insulin and small molecules affecting the fluorescence properties, and (iv) to study the interaction between insulin and AEF by the molecular docking method and explore whether the addition of various hydrogen bonding solutes to the insulin- AEF complex can stabilize the secondary structure of insulin by UV/visible and fluorescence spectra. The study's outcome has potential application in medical research and the treatment of diabetic patients.

4. 3.1. Materials

4. 3.1.1. Synthesis of the dyes

The AEF was synthesized according to the available procedure. [39] Insulin was purchased from Sigma Aldrich chemical limited. Urea (SRL), acetamide (SRL) and Guanidine hydrochloride (SRL). The chemicals received were used as such without any further purification methods.

4. 3.1.2 Preparation of stock solutions of dyes and proteins

AEF stock solutions were prepared by dissolving the weighted amount of the dyes at 2 mM concentration in DMSO. Stock solutions of ovalbumin was prepared by dissolving their weighted amounts in 0.05 M Tris–HCl buffer (pH 7.9) in a concentration equal to 0.2 mg ml−1. Protein concentrations in stock solutions were equal to 4.5 μM for Insulin

4. 3.1.3 Preparation of working solutions of dyes and proteins

Working solutions of free dyes were prepared by dilution of the AEF stock solution in 0.05 M Tris–HCl buffer (pH 7.9), 0.05 M phosphate buffer (pH 6.0), 0.05 M Tris–HCl (pH 9.0), in water. Working solutions of the dyes in the presence of proteins were prepared by the addition of the aliquot of the dye stock solution to the protein stock solution. The concentrations of dye working solutions amounted to 5 μM except for absorption spectra measurements where it was equal to 10 μM. All working solutions were prepared immediately before the experiments

4.3.1.4 Spectral measurements

Spectroscopic measurements were performed in a standard quartz cuvette (10 × 10 mm). Fluorescence excitation and emission spectra were registered using the fluorescent spectrophotometer Cary Eclipse (Varian, Australia). Absorption spectra were registered using the spectrophotometers Genesys 20 (ThermoScientific) and Shimadzu UV-3600. All the spectral-luminescent characteristics of dyes were studied at room temperature.

4.3.1.5 Ligand and protein preparation

The 3D structures of synthesized compounds were constructed by maestro builder panel. The ligand preparation wizard was used to add hydrogen atoms and regulate the rational bond angles, geometry, and ring conformations. The most favourable ionization state of compounds was produced with aid of force field OPLS-2005 (optimized potentials for liquid simulations) and geometry minimization was carried out until it reaches a RMSD cutoff of 0.01Å [1]. The subsequent output structure of compounds was suitable for docking with protein Ova.

The crystal structure of the Insulin (PDB- 1OVA) was downloaded from the protein data bank (http://www.rcsb.org). The receptor was fully optimized in the protein preparation wizard, which involved adding polar hydrogen atoms, assigning bond orders, determining protonation states, and subsequently removing water molecules beyond a 5Å distance. Thereafter, by using force field (OPLS-2005) the energy minimization of 0.03Å RMSD was performed to reduce the steric hindrance caused by the addition of hydrogen atoms. Now the minimized Insulin receptor was suitable for docking by supplied x, y, and z coordinates 45.14, -7.99, and -8.72 Å respectively with grid box diameter (xyz) of 30×30×30 Å. The chemical structures from Chem-Draw were transformed into energy-optimized PDB (Protein Data Bank) format using Chem 3D 17.0. This conversion allows the representation of the compounds in a suitable format for further computational analysis and modelling. Pymol software was used for the visualization of the docked molecules.

UV absorption measurements (AEF variation)

Figure. 1a shows the absorption spectrum of free insulin, the absorption intensity of the insulin absorption band observed at 250 nm increased by the addition of AEF. The aqueous solution of 10-5 M of AEF shows a characteristic absorption band with maximum at 290 and 450 nm, corresponds to π-π* and n-π*, transitions respectively

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(Figure. 2a). Ligands have higher transition energy than protein which is read as a higher molar absorption coefficient (ε) value of AEF, each AEF and insulin have the ε value 22,000 and 1900 M^{-1} cm^{-1} at 250 nm and 230 nm respectively. An interaction between insulin and AEF can be proposed based on spectrum and the ε value. The largest ε value was obtained at the mole ratio of 1:1 (10^{-5} M AEF: 10^{-5} M Insulin), which was 78,000 M^{-1} cm^{-1}. The incremental additions of AEF (10^{-8}, 10^{-7} and 10^{-6} M) to the solution of $1x10^{-5}$ M Insulin result in notable changes in the absorption feature of the dye as shown in Figure. 3a. It is evident from that with stepwise addition of AEF to $1x10^{-5}$ M Insulin solution, there is both increase in absorbance of the dye molecule and Insulin along with a subtle red shift (˜2 nm) in the peak position (Table 1). This result indicates that the reason of the change in absorption spectra is formation of a ground state complex between AEF and insulin.

The hyperchromic effect in the absorption spectra, with increasing AEF concentration, may be accounted to the increased refractive index of the surrounding environment as the protein molecules are moved from the low refractive index environment to the comparatively high refractive index pockets of AEF, causing an increasing in the oscillator strength of the absorption process [40] The modulations, observed in the absorption features, with the increasing AEF concentration, certainly provide us the indication about the interaction of AEF with Insulin in aqueous solution, though the extents of these changes are not large enough to be applied for any reliable quantitative analysis.

Steady-State fluorescence measurements

Steady-state emission is a sensitive technique to obtain both qualitative and quantitative information on the host-guest binding. AEF display emission band in the red side of the visible spectral region (Figure 2b) Insulin excitation have been carried out at a wavelength range of 200–500 nm, and the optimum was at 252 nm (Figure 1b). It is well documented in literature that the quantum yield of molecule decreases with increase in the polarity of the medium [41, 42] The large structural flexibility of dye molecule and also their ability to show

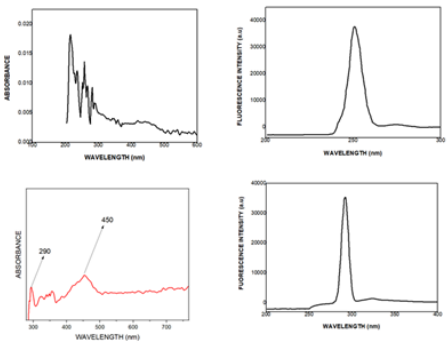

Fig 1a UV-Vis spectrum of free insulin measured at a concentration of 10^{-5} M
Figure 1b Emission spectrum of Insulin measured at a concentration of 10^{-5} M in water (Excitation wavelength = 250 nm)
Figure 2a. UV-Vis spectrum of AEF measured at concentration of 10^{-5} in H_2O
Figure 2b Emission spectrum of AEF measured at a concentration of 10^{-5} M in water

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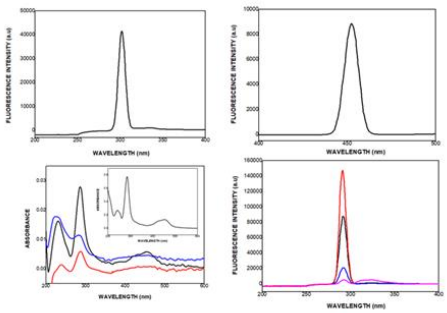

Figure 2c Emission spectrum of AEF measured at a concentration of 10^{-5} M in water (λ_{exc} = 450 nm)
Figure 3a: UV-Vis spectra of various concentration of AEF (10^{-6} M -10^{-8} M) with Insulin 10^{-5} M in Water (Inset: UV-Vis spectrum of 10^{-5}M :10^{-5}M mixture of dye and insulin in water)
Figure 3b: Emission spectra of various concentration of AEF (10^{-5} M -10^{-8} M) with Insulin 10^{-5} M in Water (λ_{exc} = 250 nm)
Figure 3c: Emission spectra of various concentration of AEF (10^{-5} M -10^{-8} M) with Insulin 10^{-5} M in Water (λ_{exc} = 290 nm)

weakly emissive state, which gets amplified in a polar medium, provide additional pathways for the excited-state of these molecules to dissipate their excitation energy in non-radiative manner, causing the AEF molecule to be weakly fluorescent. AEF contains hydrophobic units like azo, ester, N, N-dialkanol and phenyl carbamate units in it, and hence may exist in the aggregated from in neat water medium. Further, AEF molecule is also reported to undergo emissive aggregate formation in aqueous solution even at a concentration as low as 1 μM, which significantly

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interfere strongly in photophysical measurements. [39] Gradual addition of AEF (10^{-8}-10^{-6}M) to the protein solution (fixed concentration of 10^{-5} M) results into a significant increase in fluorescence intensity along with a gradual red shift in peak maxima as shown in Figure. 3 b and listed in Table 1. The fluorescence spectra of insulin in the presence of varying concentrations of dye, recorded at excitation wavelengths of 290 nm and 450 nm, exhibit trends similar to those observed at 250 nm excitation (Figure 3c, 3d). Specifically, a red shift in emission maxima and an increase in fluorescence intensity are observed with increasing dye concentration. However, it is important to note that the overall emission intensities at 290 nm and 450 nm excitation are lower compared to those obtained at 250 nm excitation. (Figure 3c and 3d).

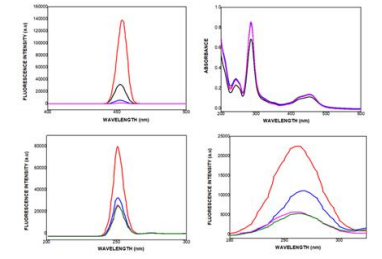

Figure 3d: Emission spectra of various concentration of AEF (10^{-5} M -10^{-8} M) with Insulin 10^{-5} M in Water (λ_{exc} = 450 nm).
Figure 4a: UV-Vis spectra of various concentration of Guanidine hydrochloride (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water
Figure 4b: Emission spectra of various concentration of Guanidine hydrochloride (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M : 10^{-5} M) in Water (λ_{exc} = 250 nm)
Figure 4c :Emission spectra of various concentration of Guanidine hydrochloride (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M : 10^{-5} M) in Water (λ_{exc} = 290 nm)

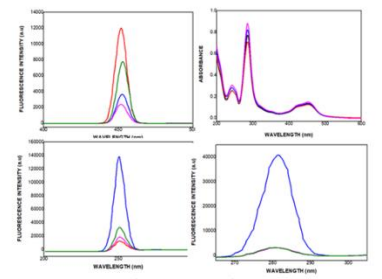

Figure 4d Emission spectra of various concentration of Guanidine hydrochloride (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M : 10^{-5} M) in Water (λ_{exc} = 450 nm).
Figure 5a: UV-Vis spectra of various concentration of urea (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M : 10^{-5} M) in Water.
Figure 5b Emission spectra of various concentration of urea (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water (λ_{exc} = 250 nm)
Figure 5c Emission spectra of various concentration of urea (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water (λ_{exc} = 290 nm)

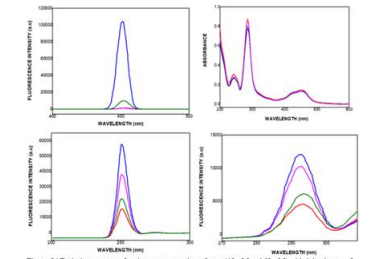

Figure 5d Emission spectra of various concentration of urea (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water (λ_{exc} = 450 nm.
Figure 6a: UV-Vis spectra of various concentration of acetamide (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water
Figure 6b Emission spectra of various concentration of acetamide (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M : 10^{-5} M) in Water (λ_{exc} = 250 nm)
Figure 6c Emission spectra of various concentration of acetamide (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M : 10^{-5} M) in Water (λ_{exc} = 290 nm)

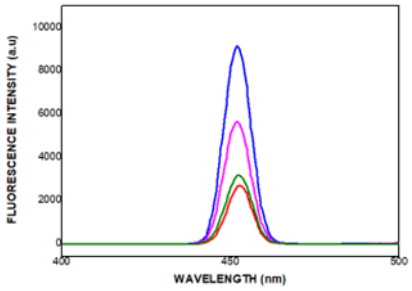

Figure 6d Emission spectra of various concentration of acetamide (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water (λ_{exc} = 450 nm)

It is important to mention here that a 10^{-5} M:10^{-5} M mixture of insulin and AEF complex results in a 15.5-fold, 26-fold, and 27.8-fold enhancement of fluorescence intensity for the peaks centred at 250 nm, 290 nm, and 450 nm, respectively, when compared to a 10^{-5} M:10^{-8} M mixture of insulin and AEF. Such a large increase in the emission intensity indicates that AEF can be a potential probe for insulin. Understandably, imposed structural rigidity experienced by the entrapped AEF in the Insulin microenvironment significantly reduces the non-radiative decay rate through its retarded molecular motions (rotation and vibration), which, in turn, results in an increase in fluorescence intensity of AEF in the presence of Insulin. Further, the reduced micro-polarity, at the binding site of Insulin, also reduces the propensity of formation of the weakly emissive state which, in turn, causes an enhancement in emission intensity of AEF in the presence of Insulin. As the AEF dye contains large hydrophobic units such ester, N,N'-dialkanol, phenyl ring and azo etc., At the10^{-5} M concentration of AEF hydrophobic environment around the AEF is increased and also due to availability of extended hydrophobic surface on protein, AEF can easily bind with protein leading to increase in its emission intensity. At the lower concentrations of the dye (10^{-7} and 10^{-8} M), hydrophobic environment is also decreased, so both the micro environment of protein and dye does not differ much, and marginal change in the emission intensity was observed (~3 times). Whereas at the 10^{-5}M concentration of AEF, protein molecules undergo structural arrangement with the substantial exposure of their hydrophobic parts and form an intermediate structure enable the most favourable interaction with dye. [43] This may lead to the formation of small water soluble insulin oligomers. [44] The observed shift in the emission maxima (~ 2 nm, from 260 nm to 262 nm for protein and ~ 4 nm from 290 nm to 294 nm, ~ 4 nm, 450 nm to 454 nm for AEF) of AEF and Insulin, can be ascribed to the increase in AEF-water hydrogen bonding interaction and enhanced

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micro-polarity of the dye surrounding, as the dye molecule moves from bulk polar water medium to less polar Insulin pocket. The noteworthy changes observed in emission features of AEF, in presence of Insulin, clearly indicate a strong interaction of AEF with Insulin protein.

Effect of guanidine hydrochloride on AEF-Insulin complexation

To get an idea about whether AEF binds selectively to the native form of Insulin or it can also bind to the denatured form of Insulin as well, the emission characteristics of AEF- Insulin complex was monitored with increasing concentration of a denaturing agent like guanidine hydrochloride (GunHCl). The GunHCl is one of the most efficient denaturizing agents, which has been frequently employed to unfold Insulin structure. At high concentration, GunHCl ruptures the secondary protein structure making them randomly coiled. As a result, most of the hydrophobic pockets present in the protein structure (in this case Insulin) get exposed to the bulk solution [45] thereby reducing both hydrophobic and electrostatic interactions for the guest molecules. In the present study, it was observed that the gradual addition of 40 µM to 160 µM GndHCl to aqueous solution containing AEF-Insulin complex (10^{-5} M:10^{-5} M), resulted in a dramatic reduction of the absorption and emission intensity of the dye, as clearly indicated from the plot in figure 4a and 4b, 4c and 4d. It is also important to note that the addition of 40 µM GunHCl to the 10^{-5} M:10^{-5} M insulin–AEF complex results in a 5-fold, 2.3-fold, and 6-fold quenching of fluorescence intensity for the peaks centred at 250 nm, 290 nm, and 450 nm, respectively (Table 2).

Addition of GunHCl results in the unfolding of the secondary structures of Insulin and hence the hydrophobic pockets which are buried inside Insulin molecules are exposed to the bulk water. [46] Thus, the AEF molecules which were attached to the specific Insulin pockets initially are easily released to the bulk aqueous phase in the presence of GunHCl. Accordingly; there is a systematic reduction in the fluorescence intensity of the AEF- Insulin system with the increasing concentration of GunHCl as shown in table 2. The addition of 40 µM GunHCl to the 10^{-5} M:10^{-5} M insulin–AEF complex results in a 36% quenching of fluorescence intensity for the peak at 250 nm. However, a more significant reduction in emission intensity is observed for the peaks centred at 290 nm and 450 nm. This could be due to the formation of a more stable complex between GunHCl and the dye than between insulin and GunHCl. Thus, these results clearly suggest that AEF selectively binds to the native form of the Insulin as compared to its denatured form. Note that the quenching observed in the present system by addition of GunHCl might also be caused by the variation in pH of the solution due to molar level of addition of GunHCl into the solution. To check this aspect, we added 7.5 M GunHCl to the AEF-Insulin complex, which do not cause any changing of pH of the solution. We further checked if there was any specific quenching interaction of the free AEF probe with GunHCl that might contribute to the quenching observed for the AEF - Insulin complex by addition of GunHCl. However, the addition of GunHCl to the free AEF probe leads to an increase in emission intensity that can be assigned to the change in the pH of the solution. (figure not shown). From these results, it is clearly understood that the addition of GunHCl to the 10^{-5} M:10^{-5} M insulin–AEF complex not only quenches the insulin fluorescence peak centred at 250 nm but also reduces the fluorescence intensity of the AEF dye peaks centred at 290 nm and 450 nm.

Effect of Urea on AEF-Insulin complexation

The interaction of globular protein like BSA with urea derivatives which possess hydrophobic groups and hydrogen-bonding moieties in aqueous solution is primarily through hydrogen-bonding interaction and the concentration of the denaturant influences the excited state properties. Apart from hydrogen bonding, the factors that influence the extent of denaturation comprise the presence of hydrophobic moieties, solute–solvent, protein–solvent and solute–protein interactions [47] In the present study, it is observed that the gradual increase in the concentration of urea from 40 uM -160 uM to aqueous solution containing AEF-Insulin complex (10^{-5} M:10^{-5} M), resulted in a reduction of the absorption and emission intensity of the dye Figure 5a and 5b-d respectively. On the addition of 40 µM solution of urea to the 1:1 mixture of Dye: Protein 38 % of the emission intensity, centred at 250 nm, is quenched, accompanied with a significant red shift (2~ nm). Specifically, 63% and 21% quenching of fluorescence intensity were observed for the peaks centred at 290 nm and 450 nm, respectively, accompanied by a red shift. (Table 3) [48]

A report suggests that urea denatures protein by binding favourably to hydrophobic amino acid side chains. [49, 50] The occurrence of 62 % fluorescence quenching in Insulin on addition of a very low concentration of (40 µM) urea clearly reveals that urea directly attacks the tyrosine amino acid in the protein sequence of the Insulin (Figure 5b-d). The prominent red-shifted peak at such a low concentration of urea signifies its strong interaction with

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the tyrosine amino acid. According to the mechanism, urea molecules replace water in the first shell around peptides. Urea has a planar distribution of charges, which enables it to occupy positions that are inaccessible to water. [51, 52] Hence, it is predicted that urea unfolds the protein by masking the hydrophobic interactions between the aliphatic and aromatic amino acid side chains. The occurrence of rapid quenching at a low concentration of 40 µM can be explained to be due to the presence of several aromatic amino acids within the quenching radius of urea molecules. Thus, the simultaneous effective binding of urea with aromatic amino acids and the possibility of the existence of several aromatic amino acids (signified by the aggregations present in insulin) within the quenching radius of a fewer urea molecules should be the reason for the observed remarkably efficient quenching.

Effect of Acetamide on AEF-Insulin complexation

Addition of acetamide to AEF-Insulin results in fluorescence enhancement up to 160 uM. The absorption and emission spectra of AEF-Insulin complex with various concentrations of acetamide is provided in the figure 6a and 6b-d. respectively. The shift in the emission maxima on the addition of acetamide is and the variation in the fluorescence intensity is provided in the figure 6b-d. Similarly, the addition of 40 µM acetamide to the 10^{-5} M:10^{-5} M insulin–AEF complex initially causes a 93% quenching of the emission intensity at 250 nm. However, a gradual increase in acetamide concentration from 80 µM to 160 µM leads to an increase in emission intensity from 15,300 to 57,300 units—a 3.8-fold enhancement in fluorescence. A similar trend was observed for the peaks at 290 nm and 450 nm. This initial quenching at 250 nm at 40 µM acetamide is attributed to the disruption of the dye–insulin complex, with the subsequent formation of a more stable insulin–acetamide complex at higher acetamide concentrations. This is further supported by the initial quenching followed by fluorescence enhancement at 290 nm and 450 nm, which correspond to dye emission peaks. Interestingly, the shift in the emission maximum is only towards the blue region even at very low concentration of acetamide (40 uM).

It is very clear that the concentration of the amides and the presence of hydrophobic moieties in the amide molecular frame work influence the emission spectral properties of the tyrosine moieties in Insulin. The above observation also signifies that the mode of hydrogen- bonding interaction of acetamide with Insulin is presumably different from that of Urea and GunHCl. Kumaran and co-workers reported that the carbonyl oxygen of urea derivatives is presumably not responsible for denaturation and only the N–H hydrogen of urea derivatives and the hydrophobic moieties in urea play a major role in denaturation.[53] Thus, unlike urea interaction with Insulin, where in the N–H hydrogen of urea is responsible for denaturation of protein, acetamide act as a hydrogen-bonding donor as well as an acceptor with the amino and carboxylic moieties of Insulin there by resulting in protein unfolding and denaturation. Amides denature Insulin through hydrogen-bonding interaction and the presence of one methyl and two methyl moieties in the amides is quite insignificant in the denaturation process. Further, it was studied that the efficiency of denaturation of protein decreases with the decrease in the number of hydrogen-bonding donor sites in the amides, wherein Urea and GunHCl which possesses four hydrogen-bonding donor sites is a better denaturing agent compared to acetamide which possesses only two hydrogen-bonding donor sites. The gradual increase in the concentration of acetamide from 40 uM to 160 uM to the AEF-Insulin complex results in fluorescence enhancement. (Table 4).

Energy transfer from insulin to AEF

Fluorescence energy transfer (FRET) is a process the transfer of excited state energy from a donor to an acceptor. It takes place simultaneous quenching of the donor fluorescence and electronic excitation of the acceptor in the process [54] According to Förster's non-radiative energy transfer theory, the energy transfer depends on the overlap of fluorescence spectrum of the donor (Insulin) with UV-vis absorption spectrum of the acceptor (AEF) and the distance of approach between donor and acceptor. The energy transfer efficiency E can be calculated under the condition of 1:1 situation of donor and acceptor concentrations by using the Equation (1)

$$E = 1 - [F / F0] \quad \text{(1)}$$

Here, F and F0 are fluorescence intensities of insulin with and without AEF. The obtained value of E by using equation (1) was 0.6136 at the equal concentrations of insulin and AEF, which suggests the non-radiative energy transfer from insulin to AEF.

Molecular Docking Studies

In this section, molecular docking method is employed to predict the structure of the intermolecular complex formed between AEF and insulin. The ligand, AEF was docked with insulin and the best docked pose in all possible ten poses of AEF is shown in figure. 7a. In the docked complex, the AEF has four hydrogen bonds acceptor interactions with the hydroxyl oxygen atom of

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TYR19, ASN A18, GLU A17, ARG B12 of active site of insulin. The conventional hydrogen bonding distance involving a protein with a ligand is usually in the order of 1.7–2.6 Å, beyond which either non-conventional hydrogen bonding or hydrophobic interactions exist. In the present study it is augmented that there exist the conventional hydrogen bonding between AEF with insulin. The docked AEF -insulin

complex and its docked interactions are shown in figure. 7b and Table 5, respectively. It is evident that the complex is stabilized mostly due to hydrogen bonds and van der Walls interactions. Docking score, docking interaction, binding values of AEF compound with insulin are exhibited at the end of the molecular docking. The obtained binding energy value of -4.98 kcalmol-1 show the affinity of ligand and how well bonding was done with insulin as given in Table 6. The docking results reveal that AEF compound was held in the binding site by various hydrogen bonds and van der Waals interactions with bovine insulin. These interactions are very important in enhancing binding affinity and the biological activity of the compound. These results may be help for related studies to evaluate its advanced clinical studies.

Table 1 Photophysical properties of AEF, insulin and various concentrations of AEF with 10^{-5}M of insulin.

[AEF]: [Insulin]	Intensity of absorption			Fluorescence intensity		
	λ_{abs} =250 nm	λ_{abs} =290 nm	λ_{abs} = 450 nm	λ_{exc} =250 nm	λ_{exc} =290 nm	λ_{exc} = 450 nm
10^{-5} M:0 (free AEF)	0.22	0.78	0.19	9000	35479	8895
0: 10^{-5} M (free insulin)	0.019	--	--	36520	-	-
10^{-5} M: 10^{-5} M	0.25	0.75	0.1	220598	147537	139100
10^{-6} M: 10^{-5} M	0.017	0.03	0.006	,121693	88437	32100
10^{-7} M: 10^{-5} M	0.019	0.01	0.004	42249	21367	5000
10^{-8} M: 10^{-5} M	0.002	0.005	0.001	14111	5474	1400

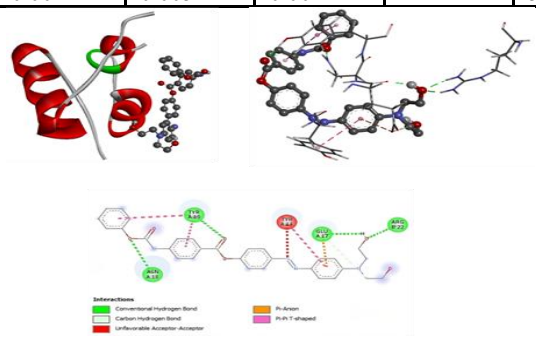

Figure 7 Docked 3D and 2D interactions of Azo-ester dye with Insulin

Table 2 Photophysical properties various concentration of Guanidine hydrochloride (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water

[AEF]: [Insulin]	Intensity of absorption			Fluorescence intensity		
	λ_{abs} =250 nm	λ_{abs} =290 nm	λ_{abs} = 450 nm	λ_{exc} =250 nm	λ_{exc} =290 nm	λ_{exc} = 450 nm
10^{-5} M:0 (free AEF)	0.22	0.78	0.19	9000	35479	8895
0: 10^{-5} M (free insulin)	0.019	--	--	36520	-	-
10^{-5} M: 10^{-5} M	0.25	0.75	0.1	220598	147537	139100
10^{-6} M: 10^{-5} M	0.017	0.03	0.006	,121693	88437	32100
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Table 3 Photophysical properties various concentration of urea (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10^{-5} M: 10^{-5} M) in Water

[GunHCl]	Intensity of absorption at			Fluorescence intensity		
	λ_{abs} =250 nm	λ_{abs} =290 nm	λ_{abs} = 450 nm	λ_{exc} =250 nm	λ_{exc} =290 nm	λ_{exc} = 450 nm
40 µM	0.3	0.9	0.19	79693	22642	12140
80 µM	0.2	0.88	0.18	33021	11238	7800
120 µM	0.25	0.8	0.1	25579	5732	3651
160 µM	0.25	0.7	0.1	24372	5328	2395

Table 4 Photophysical properties various concentration of acetamide (40 µM to 160 µM) with 1:1 mixture of AEF and Insulin (10 -5 M: 10 -5 M) in Water

Conformer	Convectional HB (cHB)	Non-Convectional HB (NcHB)	Pi-Alkyl	Pi-Lone Pair	Pi-Anion	Pi-Pi Stacked	Pi-Pi T-Shaped	Total Number of Molecular Interactions
AEF-Insulin	4	1	-	-	1	-	3	9

Table 5: Representation of overall Interaction existing between AEF and Insulin

[Urea]	Intensity of absorption at			Fluorescence intensity		
	λ_{abs} =250 nm	λ_{abs} =290 nm	λ_{abs} = 450 nm	at λ_{exc} =250 nm	λ_{exc} =290 nm	λ_{exc} = 450 nm
40 µM	0.35	0.89	0.2	13842	40951	105054
80 µM	0.3	0.83	0.1	33299	3284	10000
120 µM	0.25	0.79	0.1	18977	3224	1209
160 µM	0 .25	0.8	0.1	12891	3011	1113

Table 6: AEF-Insulin molecular interactions

[Acetamide]	Intensity of absorption at			Fluorescence intensity		
	λ_{abs} =250 nm	λ_{abs} =290 nm	λ_{abs} = 450 nm	at λ_{exc} =250 nm	λ_{exc} =290 nm	λ_{exc} = 450 nm
40 µM	0.28	0.8	0.1	15373	4584	2712
80 µM	0.25	0.85	0.1	21731	6140	3187
120 µM	0.2	0.8	0.1	33901	10278	5643
160 µM	0 .20	0.8	0.1	57337	12094	9162

CONCLUSION

The interaction of insulin with AEF which is a phenol blocked isocyanate molecule was investigated with spectral and molecular docking studies. Obtained results showed that AEF has strong ability to quench the tyrosine fluorescence of insulin with formation a ground state complex. The energy transfer calculations performed based on the Förster theory indicates the non-radiative energy transfer from insulin to AEF. The hydrogen bonding and van der Waals forces are dominant forces in stabilizing the complex. According to molecular docking calculations, the complex includes four hydrogen bonds with ASN A18, TYRA 19, GLUA 17, and ARGB27 residues of insulin. Furthermore, the obtained experimental results have been confirmed with molecular docking results which show that the compound may act as an antidiabetic agent against insulin. In addition, this study can be a guide to evaluate the interaction behaviour of an AEF and insulin with experimental and computational applications.

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