NOVEL SYNTHESIS OF 4E,10E-4-(2-PHENYLDIAZENYL)-N-((1H-INDOLE-3-YL)METHYLENE)BENZENAMINE AND ITS APPLICATION FLUORESCENT CHEMOSENSOR FOR Ag⁺ ION, CYTOTOXICITY ACTIVITY AND DFT STUDIES

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ABSTRACT

A Schiff base of 4E,10E-4-(2-phenyldiazenyl)-N-((1H-indole-3-yl)methylene)benzenamine (PQ) was synthesized and confirmed by FT-IR, ¹H & ¹³C NMR spectroscopy. Fluorescence responses on photo fluorescence spectroscopy to different metal ions have been studied. The sensor PQ exhibited highly selective for Ag⁺ ion with turn-on chemosensors confirmed by emission intensity at 364 nm while another fluorescence emission intensity at 522 nm. The stoichiometric complexes PQ+Ag⁺ behavior indicated that 1:1 ratio with association constant value of detecting as was calculated to be 2.33×10⁶ M⁻¹. The concentration of EDTA solution 0.5 equiv added with PQ+Ag⁺ solution diminished fluorescence intensity at 522 nm. It takes a long time to inhibit the cytotoxicity effect of HeLa cells treated with PQ of differing concentration results. The Gaussian output value of the energy gap value of the HOMO-LUMO was found as ΔE=2.85 eV.

Keywords: Schiff Base, Fluorescence, Ag⁺ Ion, PET Mechanism, Cytotoxicity, DFT Calculation.

INTRODUCTION

Recently, in the enlargement of the modern industry, water contamination has become too serious due to the increased industrial emissions containing various metal ions. The results of many truths are food and agriculture, and also more concern for human health and environment¹. Polluted heavy metal ions have been highly worried worldwide because of their adverse effects at low concentrations on the environment and even human health. A rise in the concentration of Ag⁺ ions in aqueous environments and wastewater caused by the release of industrial waste into drinking water². Thus, a keepsake shows many roles Ag⁺ performs in everyday life, the very important effects of silver ions on biological and environmental systems through sensing quickly and simply. Different methods, such as atomic absorption, an inductive combination of plasma-mass atomic emissions, fluorescence and UV-Vis absorption spectroscopy were widely used to calculate trace amounts of Ag⁺ ions³. The high Ag⁺ ion concentration leading to true brain injury, nerve damage, adverse health effects, and immune systems. Consequently, the study of food and water resources for Ag⁺ ions has been of great importance. It is too important to develop highly sensitive and responsive sensors to detect Ag⁺ ions between samples relating to the environment and food. The U.S. EPA (EnvironmentalProtectionAgency) has effectively monitored the polluted silver by coordinating a secondary maximum pollution level (SMCL) for 0.1 mg L⁻¹ (0.93 μM) in this product⁴,⁵. Spectrophotometric methods⁶, spectrofluorometry⁷, voltammetry⁸, phosphorescence methods⁹ are various instrumented techniques. For the determination of the silver ion, flame atomic absorption spectrometry¹⁰, chemiluminescence¹¹, ICPMS¹² inductively coupled plasma optical emission spectrometry¹³, rayleigh light dispersion¹⁴ and ion-exchange chromatography¹⁵ were created. Many fluorescent and colorimetric chemosensors have been documented in recent years when Ag⁺ was formed in the organic solvent, or mixed with an aqueous solution. Sensor recording, naked eye ion detection, without the need for spectroscopic instrumentation, allows naked eye identification of Ag⁺ ions in the aqueous medium. Ag⁺ is
revealed in the imaging, pharmaceutical, and electrical industries. Fluorescence spectroscopic can be the system's second detection that has gained considerable attention due to its high selectivity, application for in situ sensitivity monitoring, etc. Numerous chemosensors used colorimetric and even fluorometric, to adjust the positive detection of specific metal ions more than in the past few decades. Most chemosensors stated to be fluorescent for Ag+ ions are essential to the fluorescence quenching mechanism. For instance, Ag+ ions inactivated in sulfhydryl enzymes and binding specific metabolites, imidazole, carboxyl groups, and amine included. Nevertheless, excess Ag+ leading to precipitation in the skin eyes to form proteins. Limited efforts have recently been made to develop highly sensitive and highly selective fluorescent sensors to classify and dispose of the toxic and its radioactive species in drinking water. That various signaling mechanisms evolve and can be implemented to target the optical detection of different organisms. Signaling mechanisms engaged the following lists as, photo-induced electron/energy transfer (PET) and C=N isomerization. Also, this imine was provided with the opportunity to generate rich fluorescent sense pathways for C=N isomerization towards probes.

Therefore, a Schiff base of 4E,10E-4-(2-phenyldiazenyl)-N-((1H-indole-3-yl)methylene) benzenamine (PQ) Scheme-1, have been investigated by FT-IR, 1H & 13C NMR and ESI-mass spectrometry. Fluorescence chemosensors turn on process prope that highly selectively for detecting Ag+ ion which confirmed different applications. However, the cytotoxicity activity how much inhibition HeLa cells for ligand, and also theoretical calculated by different methods HOMO & LUMO, MEP, and Mulliken value were performed.

**EXPERIMENTAL**

**Material and measurement**

Most of the chemicals were obtained from Sigma-Aldrich, albeit without further purification in the laboratory. Selectivity analyzes of a variety of metal ions, such as Cd²⁺, Cu²⁺, Co²⁺, Sn²⁺, Ti³⁺, Sr²⁺, Zn²⁺, Pb²⁺, Cd²⁺, Hg²⁺, Ca²⁺, Mn²⁺, Al³⁺, Ni²⁺, Fe³⁺, Cu¹⁺, and Ag⁺. Nevertheless, supplementary studies for instance measurement of FT-IR based on 1H & 13C-NMR measurements were carried out on a Bruker (400 MHz for 1H and 100 MHz for 13C) and solvent used in DMSO. Of the NMR spectrum, and chemical changes, respectively, were reported. UV-vis-spectra was collected at room temperature using a liquid compound mixing solution of distilled ethanol and water with a UV-2450-Shimadzu spectrometer. The emission spectrum of FT-IR based on H&13C-NMR measurements were carried out on a Bruker (400 MHz for 1H and 100 MHz for 13C) and solvent used in DMSO. Of the NMR spectrum, and chemical changes, respectively, were reported.

**Synthesis 4E, 10E-4-(2-phenyldiazenyl)-N-((1H-indole-3-yl)methylene)benzenamine (PQ)**

In a round bottom flask, a compound (4)-4-(phenyldiazenyl)aniline (0.34 g, 2 mmol) and 20 mL of ethanol was applied to indole-3-carbaldehyde (0.29 g, 2 mmol) and continuously refluxed for 12 hours after the reaction. The solution washed with H₂O and filtered out. The recrystallized substance is from ethanol. Yield: 0.692g, 70%. M.p=219°C. FT-IR (KBr, cm⁻¹): 3390 (νN-H), 2925, 2975 (νAr-C-H), 2817 (νAli.C-H), 1610 (νC=C), 1517 (νC=N), 1432 (βC-H), 1002 (βN-H), 882-739 (ΓC-H).

**UV-Vis Titration**

Probe PQ (1×10⁻⁵M) stock solutions were dissolved in ethanol. The stock solutions were diluted with a 10 mM HEPES buffer (pH=7.0) to making the ultimate concentration (10μM) for UV-Vis experiments. The final buffer conditions for the experiment are set to CH₂OH/H₂O (1:4, v/v) and pH=7.0. In addition, a number of metal salt stock solutions (0.1 M) including Co²⁺, Sn²⁺, Ti³⁺, Sr²⁺, Zn²⁺, Pb²⁺, Cd²⁺, Hg²⁺, Ca²⁺, Mn²⁺, Al³⁺, Ni²⁺, Fe³⁺, Cu¹⁺, and Ag⁺ (50μM) were dissolved in H₂O and diluted to concentrations. Only a few seconds of stirring them UV-vis-spectra was taken at ambient temperature.

**Fluorescence Titration**

Probe PQ (1×10⁻⁵M) stock solutions have been dissolved in ethanol. Solutions were diluted with 20mM HEPES buffer (pH=7.0) to render the final concentration (10μM) for the spectroscopic experiments and...
In order to analyze the selectivity of the absorptive activity of PQ (10 μM), various ions in metal, such as Cu²⁺, Fe³⁺, Co²⁺, Ni²⁺, Ca²⁺, Sn²⁺, Sr²⁺, Ti⁴⁺, Mn²⁺, Pb²⁺, Zn²⁺, Cd²⁺, Hg²⁺, Al³⁺, and Ag⁺ (50μM). The measurements were taken within 1 minute of response time. Many of the tests were conducted at ambient temperature. To ensure the research results were reproducible and reliable, all experiments were performed fewer than three times.

Job’s Plot Studies
The PQ stock solutions (0.0036 mg, 0.002 mmol) were dissolved in ethanol and diluted to a CH₃CH₂OH/H₂O, 20mM HEPES buffer (1:4, v/v, pH=7.0) solution to achieve a finishing concentration of (10 μM). Sensor PQ solution: (a) 5.0, (b) 4.5, (c) 4.0, (d) 3.5, (e) 3.0, (f) 2.5, (g) 2.0, (h) 1.5, (i) 1.0, and (j) 0.5 mL was taken and passed on to quartz cells. The metal solution of the Ag⁺ solution (50μM) was diluted to the CH₃OH/H₂O HEPES buffer (1:4, v/v) solution: (a) 0.5, (b) 1.0, (c) 1.5, (d) 2.0, (e) 2.5, (f) 3.0, (g) 3.5, (h) 4.0, (i) 4.5 and (j) 5.0 mL of Ag⁺ It was taken at room temperature for a three seconds, and UV-Vis spectra. They have carried out the same experimental procedures for Ag⁺.

Cell Culture and Cytotoxicity Assays
HeLa cells were grown 10 % FES supplement in the DMEM (DulbeccoModifiedEagleMedium). All cells were treated with an antibiotic antimycotic solution (100 penicillin mL units, 0.1 mg streptomycin mL⁻¹ and 0.25 mg amphotericin B mL⁻¹); and growing at 37°C (5 % CO₂, 95 % moisture content) under normal culture cell. In cultivated media the cells were seeded into 96-well plates with a density of 4x10⁴ cells per well, then added compounds of 1) 0, 2) 1.95, 3) 3.9, 4) 7.8, 5) 15.6, 6) 31.2, 7) 62.5, 8) 125, 9) 250 and 10) 500 μM (final concentration). The cells were then incubated at 37°C in a CO₂, 5% atmosphere, and 95% in the air for 24 hours; ELISA measured the cells' absorbance.

Computational Details
All calculations used in the Gaussian 09w program are performed. The PQ answer geometry optimization compound was performed using the preliminary geometry developed from the standard geometric parameters of B3LYP, which adopted this definition in both 6-31 G (d, p). Moreover, the 6-31 G (d, p) B3LYP base set processes are also used to measure Mulliken charge, polarizability, dipole moment and even hyperpolarizability. All the parameters were disrupted to settle down within all the equations given for an optimized geometry, which corresponds to the exact value of the minimal energy required by molecule organization. Frontier molecular orbital energies are important as the difference between energy values measured binding gap energy, including HOMO and LUMO. On the other hand, the chemical reactivity of the given molecule and even the MEP surface is worked on over the optimized geometry, and the title molecule's Mulliken atomic charge is modified using Gaussian 09w package software.

**RESULTS AND DISCUSSION**

The compound synthesized 4E,10E-4-(phenylidiazonyl)-N-(1H-indole-3-yl)methylene)benzenamine (PQ) (Scheme-1) was characterized by FT-IR, ¹H & ¹³C-NMR, and ESI-mass spectrometry. Additionally, experiments were conducted using the spectrofluorimetric method to measure the visual tests under UV lighting. Conversely, common ions of metal such as Co⁴⁺, Sn²⁺, Ti⁴⁺, Sr²⁺, Zn²⁺, Pb²⁺, Cd²⁺, Hg²⁺, Ca²⁺, Mn²⁺, Al³⁺, Ni²⁺, Fe³⁺, Cu²⁺, and Ag⁺ ions are limited.

**Absorption Studies**
In order to analyze the selectivity of the absorptive activity of PQ (10 μM), various ions in metal, such as Cu²⁺, Fe³⁺, Co²⁺, Ni²⁺, Ca²⁺, Sn²⁺, Sr²⁺, Ti⁴⁺, Mn²⁺, Pb²⁺, Zn²⁺, Cd²⁺, Hg²⁺, Al³⁺ and Ag⁺ ions (50μM) were...
added to the HEPES buffer solution (CH$_3$CH$_2$OH/H$_2$O, 1:4; v/v, pH 7.0). The PQ UV-Vis spectrum showed two bands of absorption at a steadily increased 258 nm, and at the same time decreased slowly by 308 nm due to the electronic transformation of π-π* and π-π*, respectively. It is possible to trace the absorption band to the transition from 258 nm to 308 nm seen in π-π* and π-π* (Fig.-1). Also, binding properties of 0.5 equiv concentration of Ag$^{+}$ ion with PQ (10 μM) caused by the absorption of two absorptions at 243 nm, while 308 nm decreased were shown in Fig.-2, which shows PQ sensing Ag$^{+}$ confirmed.

Fluorescence Emission Studies

The selectivity of PQ (20 μM) for different metal ions such Cu$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Ca$^{2+}$, Sn$^{2+}$, Sr$^{2+}$, Ti$^{2+}$, Mn$^{2+}$, Pb$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Al$^{3+}$ and Ag$^{+}$ (50μM) 20mM HEPES buffer solution (CH$_3$CH$_2$OH/H$_2$O, 1:4; v/v, pH 7.0) were investigated by the fluorescence emission spectroscopy. Fluorescence chemosensors for detection of highly-selective Ag$^{+}$ ion with 'turn-on' sensing action, remarkable fluorescence emission intensity at 364 nm appeared while simultaneous another emission intensity slowly increased at 522 nm appeared Fig.-3. To investigate chemosensor PQ binding properties, many metal ions can be sensed and projected to Ag$^{+}$. This likes water molecule interaction removed to form an imine group under conditions, the excitation wavelength of 364 nm appeared while the fluorescence experiments were performed with the concomitant addition of Ag$^{+}$ (0 to 5.0 equiv.) to 20 μM PQ solution probe. The two large peaks appeared at around 364 nm to 522 nm and developed complete, the fluorescence spectrum was observed in Fig.-4.

The weakly fluorescent nature of PQ can be demonstrated in terms of intermolecular π–π interactions, as well as quenching of the PET phenomenon due to intramolecular hydrogen bonding between the imine
nitrogen atoms and Ag⁺, linked probe that NH group shifted from 3390 to 3110 cm⁻¹ decreased and while C=N group shifted 1610 to 1584 cm⁻¹. Scheme-2, PQ-Ag⁺ coordination complex results in two different effects: hampering the intermolecular π–π interactions between PQ molecules, which may increase emission intensity; coordination of PQ through its nitrogen donors, it likes to decrease the chance of PET and may be responsible for the hyperchromic shift, and intensity magnification of along with the fluorescence intensity improvement.

However, to examine the selectivity of PQ for Ag⁺, competitive experiments involving Ag⁺ have been conducted with specific concentrations of different metal ions and, as a result, three metal ions such as Ni²⁺, Co²⁺ and Ca²⁺ are interfering in a normally significant degree Fig.-4. To determine the stoichiometry ratio of the PQ-Ag⁺ complex, it was the duty of Job’s plots to build them using a continuous, specific method. As shown in Fig.-5, at a constant total concentration (5x10⁻⁵ M⁻¹) the fluorescence intensity at 522 nm versus the molar fraction of Ag⁺ is plotted. Extremely, a plot of a 1:1 PQ-Ag⁺ complex job shows the minimum fluorescence power reached at 0.5 molar fractions. Using the Benesi - Hildebrand equation, the constant (Kᵚ) relationship between the PQ and Ag⁺ was evaluated graphically (Fig.-6).

From the line's slope and intercept, it was determined that Kᵚ was 2.33×10⁶ M⁻¹. The reversibility and regeneration were checked with non-negligible variables when developing new chemosensors for practical use. The effectiveness of sensor PQ binding to Ag⁺ was tested by using the EDTA solution to react to ethylenediamine tetraactic acid. A reduction of the fluorescence strength at 522 nm can be achieved by adding EDTA (0.5 equiv.) into a PQ-Ag⁺ mixture (0.5 equiv). However, this complexation of the PQ-Ag⁺ complex was seen (Fig.-7). Therefore, such reversibility of PQ to Ag⁺ was positive for the development of Ag⁺ ion-sensing products.

The Cytotoxicity Activity
The cytotoxicity activity with ligand PQ was demonstrated against the HeLa cell lines. The HeLa cells were prepared at various concentrations at 37 °C in an atmosphere of CO₂ 5 % and 95 % air for 24 h. Different concentrations applied to cell lines at a specific temperature were prepared for the ligand PQ, which results in comparison with that ligand, there is no application ligand from Fig.-8a, in the image was
empty cells. Third, cells were greatly blocked from Fig.-8c cell death. However, many ligand-treated cells take a long time to fully cell death due to inhibition Fig.-8b. Calculation of the IC\textsubscript{50} value for different cell viability concentrations increases the degree, as shown in Fig.-9. The PQ value for IC\textsubscript{50} is shown in Table-1.

![Fig.-8: HeLa Cells induced of PQ exhibited (10 mM) treated with (a) Cells, (b) After treatment with cure cells, (c) Cells death for a long time.](image)

### Table-1: IC\textsubscript{50} Value calculated with Cell Viability

| Concentration (\(\mu\text{M}\)) | PQ  |
|-------------------------------|-----|
| 0                            | 100 |
| 0.9                          | 86  |
| 1.95                         | 74  |
| 3.9                          | 59  |
| 7.8                          | 43  |
| 15.6                         | 31  |
| 31.2                         | 25  |
| 62.5                         | 17  |
| 125                          | 12  |
| 250                          | 7   |
| 500                          | 3   |

**HOMO and LUMO Analysis**

The theoretical estimation of the HOMO-LUMO value substratum for the prediction of the energy gap by Gaussian 09 was investigated. HOMO energy assumed that the electron-donating power described by the LUMO could be capable of accepting the energy gap HOMO-LUMO, as shown in Fig.-10. The compound PQ then measured the lowest energetic gap (almost \(E_{\text{gap}}=2.85\text{eV}\)), the lower gap being the softest molecule involved. The PQ compound that has the most HOMO power is (EHOMO=-5.271eV). But this higher energy would make it possible to be the biggest donor of electrons. The compound PQ that has the lowest LUMO energy is (ELUMO=2.416eV), which signifies that it can be the greatest electron acceptor respectively. The two properties such as the principle of electrophilicity and softness are so important; the configuration of these two properties helps one to measure the absolute electronegativity(\(\chi\)) (and absolute hardness (\(\eta\)). These two parameters are related respectively to the HOMO and LUMO single-electron orbital energies. Compound PQ has the lowest electronegativity value (\(w=5.176\text{eV}\)), the far better donor of electrons. Compound PQ has the largest value of the softness (s=0.353eV), so it was the much stronger acceptor of electrons. The chemical reactivity varies with the identified structures of molecules. The chemical hardness (softness) value of the compound 1 (\(\eta=1,427\text{ eV}, S=5,176\text{eV}\)) for all molecules is less than (greater). Compound PQ has a higher electronegativity value (\(\chi=3.844\text{eV}\)) than other compounds, so it was the strongest acceptor of electrons. The rate of distance \(\omega\) for compound PQ (\(\omega=2.854\text{eV}\)) indicates which is more than the stronger electrophiles than all compounds. This modified orbital is a similar molecule of reactivity; the compound PQ may have a smaller orbital gap where it was also better polarized and associated with a high chemical reactivity with low kinetic stability and called the soft molecule. The B3LYP method followed a scheme such as Mulliken charge, dipole moment, polarisability, and also hyperpolarizability is shown in Table-2.

![Fig.-10: Energy Diagram of HOMO and LUMO Orbitals of PQ and its corresponding Metal Complexes calculated at the DFT Level using a B3LYP/6-31G* Basis Set.](image)

**Table-2: DFT calculated for Dipole moment, Polarisability and Hyperpolarisibility**

| Parameter | Dipole moment | Polarisability | Hyperpolarisibility |
|-----------|---------------|----------------|---------------------|
| \(\mu\)  | 5.3894        | 11.0           | 1.25                |
| \(n_\mu\)| 2.8177        | 11.0           | 1.25                |
| \(n_\nu\)| -0.3083       | 16.24          | 1.6275              |
| Total    | 0.0003        | 15.66          | 25.881              |
| \(\alpha\)| -0.340        | 15.66          | 25.881              |
| \(\beta\)| 0.26          | 28.891         | 32.17               |
| \(\alpha(\text{cm}^{-1}\times10^6)\)| 2.17           | 23.76           | 2.099               |
| \(\beta(\text{cm}^{-1}\times10^6)\)| 5.91           | 2.699           | 5.48                |
| \(\chi\) | -0.0457       | 5.68           | 5.48                |
| \(\eta\) | 5.68           | 5.48           | 5.48                |

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BENZENAMINE AND ITS APPLICATION

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Molecular Electrostatic Potential and Mulliken Charge

The MEP widely provided through the distribution of charges in the space around the molecule structure is used to understand the sites of electrophilic attacks and nucleophilic reactions for the study of the biological detection system and also the interactions between hydrogen bonding. In order to predict those much the molecular reactive sites, the PQ compound MEP diagram is determined using the B3LYP method, as shown in Fig.-11.

![Molecular Electrostatic Potential Surface of PQ](image1.png)

![Mulliken Atomic Charges for PQ](image2.png)

The surface is represented by various colors due to different values on the molecular electrostatic potential. The product of the PQ compound expressed an increase with potential order blue > green > yellow > red where blue color had indicated the lowest potential electrostatic energy and red color. Mulliken PQ charging calculations performed computational chemistry, as shown in Fig.12, lowest atomic molecular orbital is N7, C6 atoms are highest atomic molecular orbital, much of decrease atomic charge.

CONCLUSION

In summary, Schiff base of 4E,10E-4-(2-phenyldiazenyl)-N-((1H-indole-3-yl) methylene) benzenamine (PQ) fluorescent sensors for turn-on chemosensor with detection of highly-selective Ag⁺ ion. Through further metal tests, the Ag⁺ ions confirmed the fluorescence strength of 364 nm and simultaneously increased emission intensity at 522 nm. The PET mechanism linked (C=N) isomerization and nitrogen binding PQ-Ag⁺ complexes. Complex PQ-Ag⁺ job plot checked 1:1 ratio, Benassi – Hildebrand equation measured Kₛ from the slope, and line intercept, Kₛ was measured as 2.33x10⁶ M⁻¹. Reversibility tests EDTA solution 0.5 equiv increase concentration diminished the fluorescence intensity at 522 nm. The cytotoxicity effect of inhibition strong force to HeLa cells analyzed with the compound. Also measured was the B3LYP of the process using Gaussian 09, HOMO-LUMO energy difference, molecular electronic potential and Mulliken charge.

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