ABSTRACT: The acid base protonation equilibria of N-acetylcysteine (Nac) and its equilibrium constants in water solutions were determined by the Hyperquad 2008 software assessment from the pH potentiometry data, which provides a diversity of statistics presentations. The effect of a number of organic solvents on the acid base protonation processes was also examined. The solution equilibria of N-acetylcysteine (Nac) were studied at \( T = 298.15 \) K in water (\( \omega_1 \)) + organic liquid mixtures \([100 \omega_2 = 0, 20, 40, 60, \) and \(80\%]\) with an ionic strength of \( I = 0.16 \text{ mol dm}^{-3} \text{ NaNO}_3\). Also, the organic solvent’s influence was studied based on the Kamlet–Taft linear solvation energy relationship. The experimental results were compared with theoretical ones obtained via the Gaussian 09 calculation computer program. The protonation equilibria of Nac were found to be important in the progress of separation systems in aqueous and non-aqueous ionic solutions. Nac showed a likely good metal dibasic chelating bioligand as the DFT calculations proved two binding sites. Spectrophotometry evaluation was also done for N-acetylcysteine bioligands at various pH values in water solutions then its absorbance ratio was measured.

INTRODUCTION

N-Acetylcysteine (Nac, Figure 1) is the acetylated type of L-cysteine, which is listed as one of vital medicines for any essential health system by the World Health Organization with an intake limit between 600 and 1800 mg per day.\(^1\) Nac is widely used as an antidote in the treatment of paracetamol overdose or acetaminophen toxicity, which causes liver failure.\(^2\) Nac is beneficially an anti-inflammatory,\(^3\) antioxidant,\(^4\) antibacterial,\(^5\) anticancer,\(^6\) neuroprotective,\(^7\) and mucolytic agent and a source of sulfur in the body.\(^8\)

Many efforts have been done to clarify the beneficial effect of Nac in medical therapy. Nac blocks human immunodeficiency virus (HIV) expression in chronic and acute infection models as well as its replication in normal peripheral blood mononuclear cells. They also claimed that Nac may help in maintaining intracellular thiol levels and replenish depleted GSH.\(^9\) Kellof et al. reported that Nac can be considered as one of the promising chemoprotective agents for bladder and colon cancer.\(^10\) De Rosa et al. claimed that HIV treatment using Nac may also increase both protection against oxidative stress and detoxification of acetaminophen and improve and increase immune system function.\(^11\) Whillier et al. confirmed that Nac can sustain glutathione (GSH) synthesis by reducing plasma cystine to cysteine that then enters human erythrocyte.\(^12\) Meyer stated that consuming Nac daily in safe amounts may prevent degenerative disease, reduce the attacks of chronic pulmonary disease, and improve insulin sensitivity.\(^13\) Many studies summarizing the role of Nac in therapy of distal intestinal obstruction syndrome,\(^14\) psychodermatological disorders,\(^15\) grooming disorders,\(^16\) Helicobacter pylori eradication,\(^17\) neurodegenerative diseases,\(^18\) addictions,\(^19\) schizophrenia,\(^20\) idiopathic pulmonary fibrosis,\(^21\) and cardiovascular disorders\(^22\) have been published in the last eight years.

A lot of solution and solid studies on bioactive binary and mixed ligands (N-acetylcysteine) complexes with divalent manganese, copper, zinc, nickel, and cobalt metal ions have been done recently.\(^23\) Solution NMR structural studies were also achieved for the binary complexes formed between

Figure 1. (a) Chemical structure of N-acetylcysteine bioligand and (b) its 3D model drawn by ChemDraw 0.3.
mercury(II), cadmium(II), and chromium(VI) metal ions and N-acetylcysteine. Thus, Nac also appears to be a potential metal chelating ligand in which Nac is able to decrease the level of lead (Pb\(^{2+}\)) in red blood cells, brains, livers, and kidneys of CD-1 mice by forming a chelate complex. Also, Güzelöglu et al. studied the equilibrium constant of Nac toward Cr\(^{3+}\), Co\(^{2+}\), Ni\(^{2+}\), and Fe\(^{3+}\) in 0.12 mol·dm\(^{-3}\) NaClO\(_4\) water solutions at 310.15 K. The potential cytotoxic activity of Ag-Nac against HeLa cells and its bioactivity against Staphylococcus aureus, Pseudomonas aerugi-nosa, and Escherichia coli microorganisms were evaluated.

The protonation and solvation properties in solutions of N-acetylcysteine are imperative to clarify the linking between its chemical aptitude and biological action in which the protonation equilibria of Nac considered in this study were evaluated in water solutions having diverse organic media. Therefore, it is valuable to elucidate analytically bioligands in organic solvents having a dissimilar number of hydroxyl functional groups to clarify its chemical, molecular, and biological mechanistic activities.

It was known that the protonation constant (pK\(_a\)) is a key central property in pharmaceutical research studies. pK\(_a\) specifies the dissociation of hydrogen atoms from the functional group of any bioligand at definite pH values. These protonation constants are used to assess the absorption, delivery, metabolism, and elimination of bioligands in many biological and environment systems. Lower pK\(_a\) values indicate that the bioligand is readily dissociated in acidic pH media, whereas high pK\(_a\) values require a basic pH to dissociate the bioligand. The protonation of hydrogen atoms of bioligand functional groups causes a negative charge of the bioligand compound, which gives higher activity of the bioligand compound to attract metal ions and bind them stronger. By means of its polarity and activity properties, water is predictable to be lower in the active site cavity of bioligands such as proteins than its appearance in bulk water. The acid base protonation processes of the N-acetylcysteine bioligand in this study were observed in water containing organic liquid mixtures since the thermodynamic properties found would be valuable to biomedicine workers. Consequently, it is useful to study systematically different bioligands such as the N-acetylcysteine bioligand in a diversity of organic solvents having various numbers of hydroxyl groups leading to understanding the mechanism of how the organic liquid solvents can affect the stability of such bioligands and its pharmacological and biological dynamic processes taking place in living cells. Data of thermodynamic and chemical properties of N-acetylcysteine are worthy of importance for interpreting the mechanism of such bidentate ligand protonation and figuring out the effect of the nature of various organic solvents and the hydrophobicity for the functional groups of N-acetylcysteine on its energetic processes.

In the present work, the acid–base equilibria for N-acetylcysteine (Nac) in water solutions and combinations of water plus organic solvents such as methanol, dimethylsulf oxide (DMSO), dioxane, dimethylformamide (DMF), and acetone were studied pH-potentiometrically. The acid base dissociation constants (pK\(_a\) values) of Nac and the interpreted thermodynamic functions for the consecutive overall dissociation processes of the Nac bioligand were determined. pH potentiometric titrations were performed in a water ionic strength of 0.16 mol·dm\(^{-3}\) NaNO\(_3\) and 10, 20, 40, and 60% 100 \(\nu_2\) organic solvent compositions, and the resulting equilibrium dissociation constants were determined. Quantum DFT calculations of the effects of each organic solvent used in manipulating the dissociation processes of the Nac ligand were determined and discussed to evaluate different factors that regulate these processes. Also, a UV–visible spectrophotometric estimation of the Nac bioligand was evaluated in different pH media.

### RESULTS AND DISCUSSION

It was known that acidic ligand protonation equilibria in water solvent proceeds through the stage of ionization, producing ion pairs, followed by the acid dissociation step that gives free ions. In a smaller amount of polar solvents, the equilibria are usually partially ionized then more complex processes can happen. The stronger the acidity of the ligand, the lower is its pK\(_a\) values and the higher the anionic species concentration and solvated protons in the aqueous solutions compared to those of the acidic ligand molecules. Nac retains two functional organic thiol (–S) and carboxyl (–COO) groups, which are able to dissociate in aqueous as well in non-aqueous solutions at definite pH values. The dissociation of Nac can be stated as follows in two equations (eqs 1 and 2):

\[
\begin{align*}
\text{H}_2\text{Nac} &\rightleftharpoons \text{HNac}^- + \text{H}^+ & K_{a1} &= [\text{H}_2\text{Nac}] \\
\text{HNac}^- &\rightleftharpoons \text{Nac}^{2-} + \text{H}^+ & K_{a2} &= [\text{HNac}^-]
\end{align*}
\]

The dissociation is initiated by releasing H atoms from [H\(_2\)Nac] species at its –COO group with a pK\(_{a1}\) of 3.14 to form negative charged ligand [HNac\(^-\)]\(^-\) followed by dissociation in –S groups to form free ligand [Nac\(^-\)]\(^-\) with a pK\(_{a2}\) of 9.43 as shown in Table 3. From these results, protonation equilibrium constants can be said to be accurate by the electronic property of the substituent functional groups. By comparing both first and second protonation constants (pK\(_{a1}\) and pK\(_{a2}\)) values of Nac bioligands with those of the previous research results, we found that it is in good agreement. The maximum standard deviations in all values (Table 1) were calculated to be approximately 0.19 units recognized to the

| functional group | present work | ref \(^{57}\) | ref \(^{58}\) |
|------------------|--------------|--------------|--------------|
| –COOH            | 3.14 ± 0.05  | 3.08         | 3.03         |
| –SH              | 9.43 ± 0.07  | 9.62         | 9.51         |

\(^{57}\) Potentiometric, \(I = 0.16\) mol·dm\(^{-3}\) NaNO\(_3\), \(T = 298.15\) K.

\(^{58}\) Potentiometric, \(I = 0.12\) mol·dm\(^{-3}\) NaClO\(_4\), \(T = 37\) °C.

All pK\(_a\) values were estimated using the Hyperquad 2008 computer program of the pH potentiometry technique measurements at approximately 298.15 K in I = 0.16 mol·dm\(^{-3}\) NaNO\(_3\) water solutions. The experimental pressure was maintained at 101.3 ± 10.0 kPa. The standard uncertainty value is \(u(T) = 0.15\) K. The reported uncertainties' values are shared expanded uncertainties at an approximately 0.95 level of confidence (k = 2). The uncertainties' values are defined as the maximum deviation of one experimental measurement from the average of three independent experimental measurements. Values of uncertainties were predictable by propagating the experimental errors in the parameters of the appropriate equation indicating that they were independent.
differences in the variety of experimental and methods of calculation conditions. The protonation process is illustrated in Figure 2 in which acetylated cysteine (Nac) has two binding groups, –COO and –S groups. The dissociable functional groups of Nac show a very important role in forming metal complexes. Numerous studies stated that Nac binds metal ions through these two functional organic groups, while the nitrogen organic group is not contributing in the coordination to metal ions in which it is neutralized by the supplement of an acetyl group. In acid–base equilibria for any bioligand in various organic media, the charge pattern was considered as the proportional solvation aspect for each solvent to cationic and anionic species may vary.

The influence of different organic solvents on the two pKₐ values of Nac can be deduced by means of the solvate chromic effect of the Kamlet–Taft hydrogen bond acidity, basicity (α, β), and dipolarity polarizability (π*) properties for each organic solvent used. These solvate chromic limitations may be used to measure and explain numerous interacting solvent properties on the protonation equilibria of the Nac bioligand. Our experimental results are illustrated in Table 2. To examine the role of solvation interaction modes upon solubility of the Nac bioligand in aqueous and non-aqueous solutions, a multiple-linear regression estimation has been sited based on the linear solvation energy equation. The Kamlet–Taft linear solvation energy relationship (KAT-LSER) model has been established to designate the Gibbs free energy change (ΔG°) of solvent-dependent reactions at 298.15 K. As predictable, the positive values of α, β, and π* coefficients show that the solubility of the Nac bioligand increases with the increment of dipolarity and polarizability of the organic solvent and by the increment of each hydrogen bond formation capability.

The ionizing power of any organic solvent used to convert the covalent bond of the studied acidic ligand into an ionic bond depends on its dielectric constant, its autoprotolysis constant, and the organic solvent’s properties (Table 3). The experimental minor variations in the pKₐ values of Nac in the methanol organic solvent can be mostly deduced to have been caused by the next two issues. The principal issue is the moderately high conjugate base stabilization of donor hydrogen bonds in water solutions comparative to that in the presence of a methanol solvent because of the superior affinity of water particles to release a proton in a solvent-to-solute hydrogen bond. The other issue is the larger stabilization of the proton in methanolic solutions over ionic organic solvent interactions. The detected reliability in pKₐ values of Nac in the existence of both DMF and DMSO organic solvents can be principally elucidated as resulting from the greater basicity of non-aqueous solutions such as DMF or DMSO water mixtures than water alone.

Any errors arising from the determination of pKₐ values are mirrored in standard Gibbs free energy changes (ΔG°) values (Table 4). Accordingly, an assessment of errors is essential to display how reliable are these experimental and calculated results. The main variations between the unlike procedures for the research plane of water and non-aqueous solution equilibria are due to the activity coefficients.

For example, in water–organic solution mixtures, a related electrolyte is supplemented to maintain the solution’s ionic strength changing from 0.1 to 3.0 mol·dm⁻³ NaNO₃ ionic strength. This is acceptable in some water–organic solution mixtures yet not in some organic solvents with lower dielectric constants somewhere the solubility of such electrolytes is limited. Negative calculated values of standard Gibbs free energy changes (ΔG°) values are shown in Table 4 for acid–base equilibria of the Nac bioligand viewing spontaneous and exothermic manners indicating that lower temperature is good for the acid–base equilibrium processes.

Figure 3a shows ultraviolet visible spectroscopic data of the Nac bioligand at different pH values (2.5 to 11), while Figure

Table 2. Experimental Acid Dissociation Constant (pKₐ) of N-Acetylcysteine

| 100 w₂ (%) | methanol | DMF | DMSO | acetone | dioxane |
|---|---|---|---|---|---|
| −COOH (pKₐ₁) | | | | | |
| 10 | 3.01 ± 0.05 | 3.02 ± 0.03 | 3.11 ± 0.005 | 3.02 ± 0.07 | 3.21 ± 0.02 |
| 20 | 2.82 ± 0.06 | 2.83 ± 0.08 | 2.96 ± 0.02 | 2.85 ± 0.04 | 2.91 ± 0.04 |
| 40 | 2.63 ± 0.05 | 2.71 ± 0.04 | 2.76 ± 0.04 | 2.62 ± 0.05 | 2.77 ± 0.07 |
| 60 | 2.53 ± 0.02 | 2.66 ± 0.05 | 2.78 ± 0.07 | 2.59 ± 0.05 | 2.64 ± 0.04 |
| −SH (pKₐ₂) | | | | | |
| 10 | 9.35 ± 0.05 | 9.01 ± 0.03 | 9.01 ± 0.005 | 9.01 ± 0.07 | 9.01 ± 0.02 |
| 20 | 8.89 ± 0.04 | 8.81 ± 0.08 | 8.93 ± 0.02 | 8.94 ± 0.04 | 8.98 ± 0.04 |
| 40 | 8.56 ± 0.03 | 8.72 ± 0.04 | 8.86 ± 0.04 | 8.75 ± 0.05 | 8.97 ± 0.07 |
| 60 | 8.21 ± 0.01 | 7.59 ± 0.05 | 7.78 ± 0.07 | 7.84 ± 0.05 | 7.98 ± 0.04 |

“All pKₐ values were estimated using the Hyperquad 2008 computer program of the pH potentiometry technique measurements at approximately 298.15 K in I = 0.16 mol·dm⁻³ NaNO₃ water solutions. The experimental pressure was maintained at 101.3 ± 10.0 kPa. Standard uncertainty value is u(T) = 0.15 K. The reported uncertainties’ values are shared expanded uncertainties at an approximately 0.95 level of confidence (k = 2). The uncertainties’ values are defined as the maximum deviation of one experimental measurement from the average of three independent experimental measurements. Values of uncertainties were predictable by propagating the experimental errors in the parameters of the appropriate equation indicating that they were independent.
3b,c shows the usual pH dependence of the absorption (or ratio of absorption at two different wavelengths), which fully corresponds to the expected picture for the object under study: two separated steps of protonation, one in the acidic and the other in the alkaline regions. The raw UV−visible spectral data showed two maximum absorbance wavelengths (\(\lambda_{\text{max}1} = 209\) nm and \(\lambda_{\text{max}2} = 240\) nm), and the absorbance values increased by the increment of pH values from acidic to neutral to alkaline. At acidic pH (Figure 3), the inflection of Nac was observed at pH > 3.0 where precisely the pK\(_a1\) value is 3.18. At basic pH, the inflection happens around pH 9.5 and the determined pK\(_a2\) value from the program is 9.48.

### CONCLUSIONS

Nac is a dibasic bioligand, and the −COO functional group is the first to dissociate followed by the −S group. The protonation equilibria data of Nac is important in the progress of quantification and separation systems in aqueous and non-aqueous solutions. To distinguish which form of a drug fragment exists wherein the ratio in water or organic solvent media is undoubtedly of great use in selecting a precise separation method throughout quantification processes. Lastly, Nac seems to be a likely good metal chelating biological ligand as the DFT calculations proved two binding sites.

### EXPERIMENTAL SECTION

Materials, Chemicals, and Solutions. All chemical compounds, materials, and different organic solvents used in this research were of high quality and analytical grade and used as purchased by the commercial suppliers without any further purification as shown in Table 5. The main chemical material used in these experiments was the N-acytylecysteine (Nac, \(\text{C}_5\text{H}_9\text{NO}_3\text{S}\), 99% purity) organic ligand from Sigma-Aldrich (St. Louis, MO). The organic solvents: methanol, dimethylsulfoxide (DMSO), dioxane, dimethylformamide (DMF), and acetone were provided by Sigma-Aldrich, U.K. Sodium hydroxide supplied by Yakuri Pure Chemical, Kyoto, Japan, was standardized using potassium hydrogen phthalate provided by Sigma-Aldrich St. Louis, MO. Nitric acid (HNO\(_3\)) solution (Panreac, Spain; purity of 65%), used to give acid conditions in the sample solution, was prepared and standardized against sodium carbonate (Na\(_2\)CO\(_3\)) before use in determining the stability constant. Hydrochloric acid was purchased from Fischer Scientific, Bridgewater, NJ. Ionic strengths of all

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Table 3. Acidity/Basicity-Related Properties of the Studied Solvent Media

| solvent     | dielectric constant | basicity constant | hydrogen-bond-accepting properties | empirical solvato-chronic constant | hydrogen-bond-donating properties | auto-protolysis constant |
|-------------|---------------------|-------------------|------------------------------------|----------------------------------|----------------------------------|------------------------|
| H\(_2\)O\(^c\) | 81                  | 156               | strong                             | 1.17                             | strong                           | 14.0                   |
| DMSO\(^a\)  | 47                  | 362               | strong                             | 0                                | weak                             | 33                     |
| DMF\(^b\)   | 37                  | 291               | strong                             | 0                                | none                             | 29.4                   |
| MeOH\(^p\)  | 32                  | 235               | strong                             | 0.98                             | rather strong                    | 15.11                  |
| acetone\(^c\)| 21                  | 224               | moderate                           | 0.08                             | none                             | 32.5                   |
| dioxane\(^c\)| 2.2                 | 287               | moderate                           | 0                                | none                             | very high              |

\(^a\)Data taken from refs 41, 46, and 47. \(^b\)Data taken from refs 41−42, 43, 46, and 47. \(^c\)Data taken from refs 41, 44, 46, 47

Table 4. DFT Theoretical Gibbs Free Energy Change \(\Delta G^\circ\) (J·mol\(^{-1}\)) for the Dissociation Processes of N-Acetylcyesteine in Various Non-aqueous Solutions

| 100 \(w_2\) (%) | methanol | DMF | DMSO | acetone | dioxane | \(\Delta G^\circ\) (\(\Delta G^\circ\) J·mol\(^{-1}\)) |
|----------------|----------|-----|------|---------|---------|----------------------------------|
| −COOH \(\Delta G^\circ\) | 10       | 17180.15 | 17237.23 | 17750.92 | 17237.23 | 18321.69 |
|               | 20       | 16095.69 | 16152.77 | 16894.77 | 16266.92 | 16609.38 |
|               | 40       | 15011.23 | 15467.85 | 15753.23 | 14954.15 | 15810.31 |
|               | 60       | 14440.46 | 15182.46 | 15867.38 | 14782.92 | 15068.31 |
| −SH \(\Delta G^\circ\) | 10       | 53366.92 | 51426.30 | 51426.30 | 51426.30 | 51426.30 |
|               | 20       | 50741.38 | 50284.77 | 50969.69 | 51255.07 | 51255.07 |
|               | 40       | 48857.84 | 49771.07 | 50570.15 | 49942.30 | 51198.00 |
|               | 60       | 46860.15 | 43321.38 | 44405.84 | 44748.30 | 45547.38 |

\(^a\)The calculations were performed via (DFT)-B3LYP methods and 6-31+G(d) as a basis set.

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Figure 3. (a) UV−visible spectrum of the Nac bioligand and its absorbance ratios at two maximum wavelengths of (b) \(\lambda_{\text{max}1} = 209\) nm and (c) \(\lambda_{\text{max}2} = 240\) nm.
prepared solutions were sustained using sodium nitrate supplied by Showa, Tokyo, Japan. All running solutions were prepared daily and freshly before their use with ultrapure water solutions with a resistance of approximately 18.3 MΩ·cm⁻¹.

**Potentiometry and Spectrophotometry Measurements.** pH potentiometric titrations (Table 6) were done using autotitrator Metrohm 888 Titrando equipment attached to an 805 Dosimat, ion-selective “Ecotrode Plus” electrodes, and an 802 rod stirrer with an 804 Ti stand.

Table 6. pH-Potentiometric Titrations and UV–Visible Spectrophotometric Experimental Parameters*  

| experimental systems | solution compositions | methods | T (K) | nₑ₅₀ | nₑₐ | data acquisition and methods of calculation | titration and spectrophotometric systems | E° \(\text{NaC} \) | NaC: \(\text{N-acetylcysteine} \) ligand |
|----------------------|-----------------------|---------|-------|-------|-------|---------------------------------|---------------------------------|----------------|---------------------------------|
| GLEE: glass electrode evaluation | [NaC] range (mol dm⁻³) 10, 20, 40, and 60% w⁻₁ solvent composition | pH potentiometric titrations in the range pH 2.5–11 | 298.15 K | 60–80 point | three titration processes | TiNet version 2.4; GLEE; Hyperquaud 2008; HySS 2009; ChemDraw 3.0, and Gaussian 09 software | autotitrator Metrohm 888 Titrando equipment, attached to an 805 Dosimat, ion-selective “Ecotrode Plus” electrodes, and an 802 rod stirrer with an 804 Ti stand | Jasco V-550 spectrophotometer |
|                      |                       |         |       |       |       |                                 |                                 | Number of titration points per each titration run. | Number of titrations per each titration process. |

*Number of titration points per each titration run. Number of titrations per each titration process. NaC: N-acetylcysteine ligand.

Using autotitrator Metrohm 888 Titrando equipment attached to an 805 Dosimat, ion-selective “Ecotrode Plus” electrodes, and an 802 rod stirrer with an 804 Ti stand. Spectrophotometry measurements were performed using a Jasco V-550 spectrophotometer (Table 6). The prepared tested solutions were placed in 10 mm path length quartz standard glass cuvettes, and the spectra were detected in the wavelength range between 200 and 500 nm. pH potentiometric titration experiments were performed such as done in our previous solution studies. A volume of 50 mL of each tested solution was acidified to approximately pH 2.5 using 3 × 10⁻² mol·L⁻¹ HCl acid. The ionic strength (I) for all tested solutions during the pH- otentiometric titration and UV–visible spectrophotometric titrations was kept constant at 1.5 × 10⁻¹ mol·L⁻¹ using NaN₃ solutions. Each tested solution was left to stand at an appropriate temperature of approximately 25 °C for 5 min before the pH titration run. A 1 × 10⁻¹ mol·L⁻¹ free CO₂ sodium hydroxide base (NaOH) titrant was prepared to alter the pH until 11.0 for each titration run. In an automatic pH potentiometric technique, 100 experimental pH potentiometric titration data points with the rate of addition (titration point/S s) monitored were recorded by TiNet 2.4 titration software.

**Glass Electrode Calibration.** The calibration process of the glass electrode was carried out using GLEE (glass electrode evaluation) software before and after each experimental run using three standard buffer solutions, pH 4.01 (0.05 mol·L⁻¹ potassium hydrogen phthalate), 6.87 (0.05 mol·L⁻¹ disodium hydrogen phosphate + 0.025 mol·L⁻¹ potassium dihydrogen phosphate), and 9.18 (0.01 mol·L⁻¹ sodium tetraborate decahydrate) prepared according to IUPAC reference recommendations. Typically, standardization and calibration of the glass electrode system in water and non-aqueous solutions was performed using Gani’s method. The electrode potential (E) was maintained after each adding acid or base to achieve the standard electrode potential of the cell (E°). The glass electrode in the background solution was titrated using a strong base in similar investigational experimental ionic strength, temperature, and solvent composition. Generally, around 12 additions are enough to confirm that E° is precisely measured that gives initial pH changes of the background solution from pH ~2, which is a value of approximately 2 units lower than the pK_w of the Nac bioligand.

In details, the GLEE program provides an approximation of base carbonate contamination, pseudo-Nernstian standard electrode potential (E°), electrode slope (s), the base concentration, and pK_w. It uses a non-linear least-squares modification to fit an improved Nernst equation (eq 3) to measure the value of the electrode potential (E)

\[ E = E° + s \log[H^+] \tag{3} \]

where [H⁺] signifies the hydrogen ion concentration.

In acidic media, [H⁺] is determined from the nitric acid concentration, \(T\text{H} \) is estimated from eq 4 in which log[H⁺] = log(T_H).
where \((a_{H}, \text{mol}\cdot\text{dm}^{-3})\) is the concentration of nitric acid, \(v_{0}\) (cm\(^3\)) is the solution volume added to the titration container, \(b_{H}(\text{mol}\cdot\text{dm}^{-3})\) is the concentration of sodium hydroxide base, \(v_{1}\) (cm\(^3\)) is the volume of the sodium nitrate electrolyte solution background, \(v\) (cm\(^3\)) is the volume of the free CO\(_2\) sodium hydroxide base titrant, \(\gamma\) is the base concentration correction factor, and \(y_{bH}\) is the calculated free CO\(_2\) sodium hydroxide base concentration.

In alkaline media, the real concentration of the sodium hydroxide base typically decreases by the occurrence of a minor CO\(_2\) contamination amount. The degree of this contamination is predicted using Gran’s plot. Originally \((E^\circ)\) is assessed from the acid region as \(s\) is provided as the ideal Nernstian slope. Then, eqs 5 and 6 are examined by linear least-squares fitting as shown below:

\[
(v_0 + v_1 + v)10^{E-E_0/s} = m^a v + c^a
\]

(5)

\[
(v_0 + v_1 + v)10^{E-E_0/s-pK_b} = m^b v + c^b
\]

(6)

Two estimated values of the volume of sodium hydroxide base consumed at the equivalence point are detected using slopes and intercepts measured from the estimation of the designed Gran’s plot fitted lines: \(v_a = -c^a/m^a\) from the acidic region and \(v_b = -c^b/m^b\) from the basic region media.

The nitric acid concentration \((T_{H})\) in the basic region media is formerly given by eq 7 as shown below:

\[
T_{H} = \frac{a_Hv_0 + \gamma b_H v}{v_0 + v_1 + v}
\]

(7)

**pH Potentiometric Data Analysis.** Most of the experiments were performed in a triplicate process with a reproducibility of an approximately ±0.02 pH unit. Using the Hyperquad 2008 program,\(^{56}\) the acid–base equilibrium constants from were determined. For this determination, a fitting principle by the alteration between the theoretically calculated and experimentally found data of the pH potentiometric titration curves was used as in the following equation (eq 8).

\[
\chi^2 = \sum \frac{(\text{calculated} - \text{experimental})^2}{(\text{experimental})^2}
\]

(8)

Hyperquad2008 software gives protonation constant detection of different various protonated and un-protonated species that formed in the aqueous or non-aqueous solutions instantaneously. Numerous models with a probable composition of composed species were suggested in the program, and the model that provided the finest statistical fit and chemical functional was selected. Principal estimation must be made for the acid–base equilibrium constants of all composed species followed by Newton–Raphson iteration refinement. To interpret the modifications in acidic, basic, dielectric constant, and ion activities properties for non-aqueous solutions relative to water solutions, pH values of each solution were improved by assembly use of the procedure designated by Douheur et al.\(^{57,58}\)

**DFT Calculations.** The Gaussian 09 program was used to estimate the computational density functional theory calculations to study the thermodynamic acid–base dissociation processes behavior of the Nac biomolecule in aqueous and in non-aqueous solutions.\(^{59–69}\) The model structures of these protonated species were improved using density functional theory (DFT) with the Becke’s three-parameter hybrid, Lee-Yang-Parr correlations, LYP (B3LYP) method, and finally the 6-31G basis set was achieved with diffuse and polarization + d for hydrogen and heavy atoms. Laterly, with geometry optimization, the frequency analysis was completed to achieve the thermodynamic properties of the formed species. The B3LYP/6-31+G(d) basis conventional set was designated subsequently showing one of the appropriate basis sets to achieve the molecular orbital of different species. The validity of different formed structure optimizations was tested using normal-mode frequency investigation. The modeling was achieved by using a number of conventions as simplification, such as the convention of the fully deprotonated Nac bioligand, not considering the use of salt to keep constant the solution’s ionic strength.

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**Notes**

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