Effective Detection of Phenylalanine Using Pyridine Based Sensor

Vijayakumar Sathya1 · Venkatesan Srinivasadesikan2 · Shyi-Long Lee3 · Vediappen Padmini1

Received: 5 November 2021 / Accepted: 24 March 2022 / Published online: 2 May 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract
Pyridine based organic molecule as probe has been synthesized for the detection of phenylalanine (PA) biomarker. The synthesized probe is characterized by 1H and 13C NMR and mass spectroscopic studies. The photophysical properties for the probe has recorded by colorimetric and fluorimetric techniques. The quenching has been observed between the probe and PA through ICT (Intermolecular Charge Transfer Mechanism). Under optimized conditions, the probe detects PA selectively in the presence of other biologically important biomolecules. The practical application for PA has been successfully applied in human blood serum and urine.

Keywords Sensor · Phenylalanine · Pyridine derivatives · Fluorescence · Phenylketonuria · Intermolecular Charge Transfer Mechanism (ICT)

Introduction
Amino acid plays a significant role in protein synthesis, pharmaceutical industry, food industry and health product industry [1]. Natural amino acids are classified into three types as acidic, basic and neutral amino acids [2]. Phenylalanine (PA) is one of the essential, neutral aromatic amino acid. It exists in zwitterionic form. PA is being gathered from diet and endogenous proteolysis [3]. PA is found in mother’s milk and numerous vegetables (carrot, onion, palm, potato, radish and beets etc.), fruits (acai palm, apple, banana, jack fruit, kiwifruit, mango etc.), meat (chicken, beef, fish and goat), milk, lentils, peanuts, cheese and sesame seeds [4, 5].

Phenylalanine is precursor of tyrosine, dopamine, melanin, adrenaline, nor-adrenaline, phenylpyruvate and thyroxine. Conversion of phenylalanine into tyrosine by phenylalanine hydroxylase enzymes in liver. This PAH enzymes is major part involved in conversion of phenylalanine into tyrosine, further tyrosine convert to DOPA by tyrosine hydroxylase. DOPA (Dihydroxy Phenylalanine) converted to dopamine via DOPA decarboxylase then dopamine convert adenrenaline, nor adrenaline using dopamine β-hydroxylase and phenylethanolamine N-Methyltransferase [6–12]. PA plays a vital role for the production of several neurotransmitters such as dopamine, melanin, epinephrine and norepinephrine. Phenyl hydroxylase enzyme (PAH) is present in liver which used as a catalyst for the conversion of PA into tyrosine. Absence or inactivation of PAH leads to the inhibit of conversion of PA into tyrosine. The tyrosine level always depends on PA in human body. The normal and abnormal level of PA in human biofluids are 30 ± 60 µmol/L and > 30 ± 60 µmol/L, respectively. Abnormal level of PA causes Phenylketonuria disease (PKU) [13]. From this discussion one can understand that the essential of PA and the determination of the PA level in human biofluids. Literally available and predictable sensor platforms for the PA such as electrochemical sensors, modified carbon electrode and electrode surface techniques [2, 23, 26], cyclodextrin based electrode methods [5, 19, 24, 30], polymer based sensors [14, 15], liquid crystal sensing [16], photo luminescent sensor [17], bioluminescent sensor [18]. However, very limited work has been reported in the literature for the detection of PA. Particularly, detect the PA at lower concentration is the challenging.

Fluorimetric method is one of the potent analytical aid to detect various toxic biomolecules and metal ions.
Fluorescent organic compound has very high sensitivity, operational simplicity less time consuming, cost effective and wide range detection to the biomarker. In general, disease causing biomolecules and other microorganism containing primary amines, secondary amines and amides functional groups can interact with -OH group of blood glucose unit in our body through intermolecular hydrogen bonding [19].

In this work, we have synthesized pyridine based fluorescent organic compound which selectively detects PA in the presence of other interfering biomolecules such as, leucine, asparagine, urea, creatinine, ascorbic acid, cysteine, tryptophan, alanine, glutamine and albumin. Literally, the fluorescent organic probe has been exhibit the low detection limit of $4.5 \times 10^{-7}$ mol/L towards PA.

Materials and Methods

Diaminopyridine, aldehyde, glacial acetic acid, absolute ethanol, leucine, asparagine, urea, creatinine, ascorbic acid, cysteine, tryptophan, alanine, glutamine and albumin are purchased from Sigma Aldrich and Alfa Aesar. $^1$H and $^{13}$C NMR studies have been done with CDCl$_3$ as solvent containing tetramethylsilane (TMS) as internal standard in Bruker-300 MHz spectrometer at 25 °C and Mass spectral has been recorder by MS-LCMS mass spectrometer respectively. Absorption and Emission spectral measurements are recorded using SHIMADZU single beam UV – vis spectrophotometer and Cary Eclipse spectrophotometer with 450 W xenon lamp, respectively [20–23].

Experimental Section

The 4,4’-((1E,1’E)-(pyridine-2,6-diylbis(azanylylidene))bis(methanylylidene))diphenol (probe) was synthesized by a previously reported method [24]. 2,6-diamino pyridine (1.0 mmol) was stirred in hot absolute ethanol and 4-hydroxybenzaldehyde (1.0 mmol) was added. The reaction mixture was allowed to reflux for 1 h. Upon cooling, the orange colour precipitate was filtered, washed with cold ethanol and dried (Scheme 1) [25]. The synthesized probe is characterized by $^1$H and $^{13}$C NMR and mass spectroscopic studies (S1-S5).

Results and Discussion

Photo physical properties of probe

The probe has phenolic -OH which binds with the acid group in the phenylalanine by hydrogen bond through ICT (Intermolecular Charge Transfer Mechanism) mechanism selectively. Initially the probe shows bright yellow color and with the addition of phenylalanine complete quenching has been observed (Scheme 1).

The absorption spectrum of probe shows peaks at 411 nm and 438 nm in $1 \times 10^{-6}$ M ACN/PBS (v/v, 1:9) at neutral pH which is mainly characteristics of $n \rightarrow \pi^*$ transition [26, 27] and the emission spectrum exhibits peak at 548 nm, (Fig. 1a, b).

This comparative experiments have been performed to discriminate the phenylalanine by the probe in the presence of other interference competitors (Fig. 2). The selective detection of phenylalanine at 500 µM concentration has been clearly revealed. Upon the addition of different concentrations of phenylalanine (20 µM-500 µM) to the probe, decrease in the absorbance has been observed (Fig. 3). Thus, Fig. 4 shows the linearly decreasing absorbance observed from the addition of gradual increasing the concentrations of phenylalanine (20 µM-500 µM) to the probe ($1 \times 10^{-6}$ M) [28].

The probe and analyte exhibit complete fluorescence quenching (TURN- OFF response) selectively compared with other competitors (Fig. 5). Further addition of phenylalanine (20 µM -500 µM) to probe, gradually decreases the fluorescent intensity with hypsochromic shift (Fig. 6). The corresponding bar diagram shows selectivity towards phenylalanine, in which no spectral change has been observed with other competitors. (Fig. 7) It clearly reveals the high selectivity to phenylalanine by optical experimental method [29].

The competitive experiments for the probe and analyte with interfering other biomolecules such as leucine, asparagine, urea, creatinine, ascorbic acid, cysteine, tryptophan, alanine, glutamine and albumin are carried out. These experiments indicate that phenylalanine is binding with probe. Other biomolecules do not interfere during the phenylalanine binding with probe, as represented in the bar diagram [30] (Fig. S12).

The job’s plot plotted between mole fraction (same concentration of probe and analyte) vs fluorescence intensity. Binding stoichiometric ratio of probe with phenylalanine

![Scheme 1](https://example.com/scheme1.png)
Fig. 1 (a). UV–Visible and (b). Emission spectrum of probe (1 μM) $[1 \times 10^{-6}$ M, at pH 7.2, PBS], $\lambda_{ex}=438$ nm and $\lambda_{em}=548$ nm.

Fig. 2 UV–Vis spectra of probe (1 μM) upon addition of PA and different biomolecules as competitors (500 μM) in ACN/PBS (v/v, 1:9), $\lambda_{ex}=438$ nm.

Fig. 3 UV–Vis absorption spectra of probe (1 μM) upon addition of PA (20–500 μM) in ACN/PBS (v/v, 1:9), $\lambda_{ex}=438$ nm.

Fig. 4 The linear relationship of probe (1 μM) toward PA (500 μM), $\lambda_{ex}=438$ nm.

Fig. 5 Fluorescence spectra of probe (1 μM) upon addition of PA and different biomolecules as competitors (500 μM) in ACN/PBS (v/v, 1:9), $\lambda_{em}=548$ nm.
has been arrived by fractional addition method. From the jobs plot, the binding ratio of probe: phenylalanine is found to be 1:2 \[31\] (Fig. 9).

The pH dependance has been studied using Phosphate buffer solution (PBS). The different solutions were prepared at various pH level (pH 2–12). At basic medium (pH 8–12), deprotonation of the probe leads to less interaction with phenylalanine. At the neutral pH, high fluorescent intensity is observed while adding phenylalanine with complete quenching. Hence, all other experiments have been performed at neutral pH condition (Fig. S6) \[32\]. Calculation of binding constant using the Benesi-Hildebrand plot (B-H plot) as shown in Fig. S14. Slope from the plot (B) = 0.01228. The binding constant towards phenylalanine is 8.1 \times 10^7 \text{ M}^{-1} \[32\].

The fluorescence titration experiment was performed by the gradual increasing addition of PA to the probe solution (Fig. 6). From the Stern–Volmer analysis, the linear plot of \( F_0/F \) produced a linear relationship \( R^2 = 0.9469 \), which confirms that the probe has high sensitivity towards PA (Fig. S16) \[32–34\].

---

**Fig. 6** Fluorescence spectra of probe (1 \( \mu \text{M} \)) upon addition of PA (20–500 \( \mu \text{M} \)) in ACN/PBS (v/v, 1:9), \( \lambda_{\text{em}} = 548 \text{ nm} \)

**Fig. 7** Fluorescence intensity of probe (1 \( \mu \text{M} \)) upon addition of PA (500 \( \mu \text{M} \)) with probe in the presence of other competitors [1- probe alone, 2- probe + PA, 3-leucine, 4-asparagine, 5- urea, 6-creatinine, 7-ascorbic acid, 8-cysteine, 9-tryptophan, 10-alanine, 11- glutamine, 12- albumin] (500 \( \mu \text{M} \)) in ACN/PBS (v/v, 1:9), \( \lambda_{\text{em}} = 548 \text{ nm} \)

**Fig. 8** Fluorescence spectra of probe (1 \( \mu \text{M} \)) upon addition of PA in human blood serum in ACN/PBS (v/v, 1:9), pH = 7.2, \( \lambda_{\text{em}} = 548 \text{ nm} \)

**Fig. 9** Fluorescence spectra of probe (1 \( \mu \text{M} \)) upon addition of PA in human urine sample in ACN/PBS (v/v, 1:9), pH = 7.2, \( \lambda_{\text{em}} = 548 \text{ nm} \)
Colorimetric and Fluorimetric Detection of Phenylalanine

Initially the probe is dissolved in acetonitrile (ACN) solution. Then, the addition of phenylalanine to the probe leads to the colour changes from yellow to dark brown observed by naked eye. Under the UV lamp, the colour changes observed from bright yellow fluorescence. But, there is no colour change observed for the other competitors with probe (Fig. S8a, S8b). From the colorimetric and fluorimetric techniques, it is confirmed that there is a selective interaction between the probe and phenylalanine at nanomolar concentration at neutral pH [35].

Test Strip Method

Test strip method is a confirmation method to selective detection of phenylalanine. Filter papers were soaked into the probe solution (a), probe with analyte (b) and probe with other competitors (c). The strips (a) and (c) shows yellow color and colourless, respectively. No color change has been observed with the other competitors (Figure c) under UV lamp. It can be concluded that the pyridine derivative selectively detect the phenylalanine [36, 37].

The Fluorescence Quantum Yield

The fluorescence quantum yield (Φ) has been determined for probe by the equation (I) as in supplementary material (S9). Rhodamine 6G was as a standard in ethanol, the quantum yield is 0.95. The probe concentration is $1 \times 10^{-6}$ M and the integrated area is determined from the absorption spectrum. The calculated quantum yield is 0.88 [38–40].

Phenylalanine Sensitivity in Human Biofluids

Human biofluids such as blood and urine are used for the real sample test following standard addition method. Separation of serum from the biofluids was effected by centrifuging in REMI centrifuge with 2,000 rpm in 1 h time interval. After that the blood serum was kept at 0 °C before testing.

Initially, a known concentration of probe was added into the human serum and urine samples. Then, different concentrations of phenylalanine like 50 μM, 100 μM, 200 μM, 300 μM, 400 μM and 500 μM are spiked into the blood and urine sample (Figs. 8 and 9). Which was incubated for 15-20 min. The results were then recorded in Tables S10.1 and S10.2 with respect to blood and urine sample [41, 42].

The fluorescence titration experiment was performed by the biofluids like blood and urine samples with the probe solution (Figs. 8 and 9). From the Stern–Volmer analysis, the linear plot of $F_0/F$ produced a linear relationship for blood ($R^2 = 0.9885$) and urine ($R^2 = 0.989$) samples, which confirms that the probe has high sensitivity towards PA with the biofluids (Figs. S17 and S18) [32].

For the first time, a good detection limit of phenylalanine is $4.5 \times 10^{-7}$ mol/L was obtained and compared with the previous optical, potentiometric, electrochemical and amperometric sensors as shown in Table 1.

DFT Studies

The experimental finding of pyridine based organic probe detect the PA at lower concentration were further explored using DFT atomic level simulation. All the calculations have been carried out using Gaussian 09 [53] suite of program. The probe and complex were optimized at B3LYP/6-31 g(d,p) [54] level of theory in the gas phase. The optimized structure of probe and complex were further confirmed with no imaginary frequencies using frequency calculation. The optimized structure of probe and complex were placed in Fig. 10 (see Fig. 10a, b). The single point energy calculation of frontier molecular orbital and electrostatic potential on the optimized structure of pyridine based organic probe and PA complex were employed at B3LYP/6-31 g(d,p) level in the gas phase. The Electro Static Potential (ESP) of probe was placed in Fig. 10c. It can be observed from Fig. 10c that the red region represents the electrophilic nature and blue region represents the electrophobic nature. The frontier molecular orbital (FMO) structure of probe and complex was placed in the Fig. 11a, b, respectively. The oscillator strength at HOMO-1 to LUMO +1 observed to be 0.519 for the organic probe and the molecular orbital gap was calculated as 4.52 eV. The computed wave length for the probe was 300.88 nm. Moreover, the oscillator strength at HOMO to LUMO +2 observed to be 0.769 for the complex and the molecular orbital gap was calculated to be 3.82 eV. The computed wave length for the complex was 369.43 nm. The observed lower band gap in the complex was confirmed the biomolecule interaction with organic probe and complex formation. In probe, at HOMO the electron cloud was observed at both the phenolic moiety and at LUMO +1 was observed at pyridine moiety. However, at HOMO and LUMO +2 levels the electron cloud in the complex was observed at the phenolic and pyridine moiety.
| Sl. No | Types of sensor platform                                                                 | Techniques                          | LOD                      | References |
|-------|-----------------------------------------------------------------------------------------|-------------------------------------|--------------------------|------------|
| 1     | Electrochemical sensor by using polyaniline modified carbon electrode for phenylalanine detection | Electrochemical Sensor              | $1.0 \times 10^{-9} \text{ mol L}^{-1}$ | [43]       |
| 2     | Electrochemical sensor by molecular imprinted techniques                                | Electrochemical sensor              | $1.0 \times 10^{-9} \text{ M}$   | [44]       |
| 3     | Fluorescence quenching by using combination of cucurbit unit with palmatine hydrochloride | Fluorescence quenching method       | $1.27 \times 10^{-8} \text{ mol/L}$ | [45]       |
| 4     | Electrochemical enantio selectivity sensor by using Graphene-ferrocene electrode        | Electrochemical sensor              | 27 nM and 52 nM           | [46]       |
| 5     | Colorimetric and fluorescent multifunctional chemosensor by Rhodamine derivative        | Colorimetric and fluorescent chemo sensor | $3.0 \times 10^{-7} \text{ M}$   | [47]       |
| 6     | Potentiometric chiral sensor based on Cross linked polymer                              | Potentiometric chiral sensor        | 1.37 µM                  | [48]       |
| 7     | Spectrofluorimetric method based on fluorescence enhancement of europium ion           | Spectrofluorimetric Method           | $5.2 \times 10^{-6} \text{ molL}^{-1}$ | [49]       |
| 8     | Electrochemical enantio selectivity biosensor based on graphene Oxide                  | Electrochemical biosensor           | 0.10 µM and 0.15 µM       | [50]       |
| 9     | Biosensor by using fluorescence protein                                                | Biosensor                           | 3.7 µM                   | [51]       |
| 10    | Electrochemical biosensor by Gold Electrode Modified with Graphene Oxide Nanosheets and Chitosan | Electrochemical biosensor           | 416 mM                   | [52]       |
| 11    | **Optical biosensor by using fluorescent organic compound for the detection of phenylalanine** | Optical biosensor                    | $4.5 \times 10^{-7} \text{ mol/L}$ | Present work |

**Fig. 10** Optimized at B3LYP/6-31 g(d,p) in gas phase (a). Probe, (b). Complex & (c). ESP at B3LYP/6-31 g(d,p)// B3LYP/6-31 g(d,p) in gas phase. Scale: $-6.901e^{-2}$ to $6.901e^{-2}$
Fig. 11 Frontier Molecular Orbital diagram obtained at B3LYP/6-31 g(d,p)//B3LYP/6-31 g(d,p) in gas phase (a). Probe, (b). Complex

Conclusion

The fluorescent pyridine based derivative, 4,4′-(1E,1′E)-(pyridine-2,6-diylbis(azanylylidene))bis(methanylylidene)) diphenol, is proved to be selective and sensitive for phenylalanine detection. The intermolecular hydrogen bonding interaction occurs between phenolic -OH in lone pair electrons with acid group in PA through ICT (Intermolecular Charge Transfer Mechanism) mechanism. This optical biosensor displays turn-off response towards PA. The binding stoichiometric ratio is 1:2 with the excellent detection limit and the binding constants towards phenylalanine is $4.5 \times 10^{-7}$ mol/L and $8.1 \times 10^{7}$ M\(^{-1}\) respectively.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10895-022-02944-z.

Acknowledgements The author VS acknowledged DST, New Delhi for the INSPIRE fellowship (IF180132). Further, we also acknowledge the BRNS, Mumbai for the UV-Visible instrument facility. Also we thanks to DST-FIST, DST-PURSE and RUSA programs for the higher solution NMR, FT-IR and fluorescence spectrophotometer facilities, respectively in School of Chemistry, Madurai Kamaraj University.

Author's Contributions Vijayakumar Sathya: Conceptualization, writing, original draft. Venkatesan Srinivasadesikan: Software resources. Shyi-Long Lee: Software resources. Vediappen Padmini:Supervision, Writing-review.

Funding The authors received no specific funding for this work.

Availability of Data and Material All relevant data are within the paper and its Supporting Information files.

Declarations

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflicts of Interest The authors have no conflict of interest in this research.

References

1. Wang X, Wolfbeis OS (2014) Chem Soc Rev 43:3666
2. Luo C, Jia J, Gong Y, Wang Z, Fu Q, Pan C, Appl ACS (2017) Mater. Interfaces 9(23):19955–19962
3. Sharma H, Kaur N, Singh A, Kuwara A, Singh N (2016) J Mater Chem C 4:5154–5194
4. Udhayakumaria D, Suganya S, Velmathia S, Ali DM (2014) J Mol Recognit 27:151–159
5. Udhayakumaria D, Naha S, Velmathi S (2017) Anal Methods 9:552–578
6. Petty MC (2002) Studies in Interface Science 16:317–367
7. Zhang M, Zhao X, Zhang G, Wei G, Su Z (2017) J Mater Chem B 5:1699–1711
8. Komoda T, Matsunaga T (2015) Biochem Med Profession 25–63
9. Bhagavant NV, Ha CE (2015) Essentials of medical biochemistry with clinical cases 227–268
10. Olguin HJ, Guzman DC, Garcia EH, Mejia GB (2016) Oxid Med Cell Longev 9730467
11. Slominski A, Zmijewski MA, Pawelek J (2012) Pigment Cell Melanoma Res 25(1):14–27
12. Kapalka GM (2010) Nutritional and Herbal Therapies for Children and Adolescents 141–187
13. Li N, Su X, Lu Y (2015) Analyst 140:2916–2943
14. Pacheco LG, Barroso MF, Nowis HPA, Morais S, Matos CD (2017) Curr Develop Biotechnol Bioeng Bioproc 24:4603–4626
15. Ali J, Najeed J, Ali MA, Aslam MF, Raza A (2017) Biosens Bioelectron 8:235
16. Pejic B, Marco RD, Parkinson G (2006) Analyst 131:1079–1090
17. Danborsky P, Svitel J, Katrik J (2016) Essays Biochey 60:91–100
18. Kirsch J, Siltanen C, Zhou Q, Revzin A, Simonian A (2013) Chem Soc Rev 42:8733–8768
19. Krishnan SK, Singh E, Singh P, Meyyappan M, Nalwa HS (2019) RSC Adv 9:8778–8881
20. Mehrrota P (2016) J Oral Biol Craniofac Res 6:153–159
21. Yang Z, Mao Z, Xie Z, Zhang Y, Liu S, Zhao J, Xu J, Chi Z, Aldred MP (2017) Chem Soc Rev 46:915–1016
22. Forini A, Lucenti E, Bottab C, Cariati E (2018) J Mater Chem C 6:4603–4626
23. Valeur B, Santos MNB (2011) J Chem Educ 88:731–738
24. Mohammed HA, Taha NI (2017) IJOC 7:412–419
25. Taha NI, Tapabashi NO, Subeyhi MN (2018) Int J Org Chem 8:309–318
26. Norouzi P, Ganjali MR, Ahmadalinezhad A, Adib M (2006) J Braz Chem Soc 17(7):1309–1315
27. Shylaja A, Roja SS, Priya RV, Kumar RR (2018) J Org Chem 83:14084–14090
28. Doraberi RZ, Norouzi P, Ganjali MR (2009) J Hazard Mater 171:601–605
29. Kakenajidifard A, Esna-ashari F, Hashemi P, Zabardasti A (2013) Spectrochimica Acta Part A 106:80–85
30. Chipem FAS, Mishra A, Krishnamoorthy G (2012) Phys Chem Chem Phys 14:8775–8790
31. Mahapatra AK, Sahoo P, Goswami S, Chantrapromma S, Fun HK (2009) Tetrahedron Lett 50:89–92
