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An azo based colorimetric probe for the detection of cysteine and lysine amino acids and its real application in human blood plasma†

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The present work describes the rational design and synthesis of a simple azo based colorimetric chemodosimeter to selectively detect Cys and Lys in the environment of competitive different class of amino acids in HEPES buffer under physiological conditions. The probes **3a** and **3b** containing aldehyde functionality upon interaction with Cys and Lys afforded stable thiazolidine and aldimine derivatives and displayed a sensitive ratiometric response in the absorption spectra due to a change in the intramolecular charge transfer (ICT) process. The whole recognition process for amino acids gives rise to a rapid significant colorimetric response with readily detectable naked-eye sensitive color changes in the real biological sample. The mechanism of interaction between the probes and amino acids has been confirmed by the optical behavior, FT-IR, NMR and ESI-MS data analysis.

Introduction

Recently, considerable attention has been made toward the development of suitable sensing events for low molecular weight containing small amino acids. Intracellular biothiols like thiols, cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) are relevant to a variety of vital cellular processes in organisms including the maintenance of biological redox homeostasis and cellular growth.[2] The biothiol levels in plasma are associated with certain disorders.[1] For instance, a deficiency of Cys is responsible for slow growth rates, skin lesions, liver damage, hair depigmentation, lethargy, muscular fat loss, edema and weakness.[3] Similarly, among proteic α-amino acids, lysine (Lys) is an important component of the human and animal diet which is closely related to the Krebs–Henseleit cycle and polyamine synthesis.[4] The high concentration of Lys in plasma and urine indicate some congenital metabolic disorders like, cystinuria or hyperlysinemia.[5] Therefore, the recognition of amino acids like Cys and Lys is valuable in the accurate predetermination and diagnosis of various disorders. Several conventional sophisticated techniques, like high performance liquid chromatography (HPLC),[6] capillary electrophoresis (CE),[7] absorption,[8] emission,[9] FT-IR[10] and mass spectrometry[11] have been employed to detect amino acids but have shown limitation in the sense of operational convenience, skilled personnel and analysis cost.[12] However, the optical detection based on specifically designed small synthetic organic molecular scaffolds-chemosensors are becoming more attractive and reliable due to their simplicity and sensitivity.[13]

In this direction a variety of sensors and/or fluorogenic probes have been developed to recognize various classes of amino acids.[14] Yoon *et al.* reviewed the progress of optical sensors for various classes of amino acids[17] however, naked-eye sensitive colorimetric probes, especially for Cys and Lys are limited in numbers.[15–20] Therefore, the selective detection of biologically important amino acids is still demanding and it is necessary to look for some simple methods involving suitable chromo and fluorogenic probes. Recently, Strongin's group described a chemical reaction based chemodosimeter approach utilizing an aldehyde functionality containing xanthene dye to detect Cys and Hcy, by taking advantage of the unique nucleophilicity of the thiol function to form thiazolidine and thiazinane derivatives.[21] With these viewpoints we herein describe the synthesis and potential application of simple azo based colorimetric probes to recognize Cys and Lys selectively in the environment of competitive amino acids in partial aqueous medium and human blood plasma, through a chemical reaction based chemodosimeter approach.

Result and discussion

The synthesis and UV-vis absorption studies

The probes, **3a**/**3b** were synthesized by a simple azo coupling reaction between the diazonium salt of aniline derivatives (*p*-nitro and *p*-methoxy) and salicylaldehyde to afford 2-hydroxy-5-(4-nitrophenyl)-azo-benzaldehyde, **3a** and 2-hydroxy-5-(4-methoxyphenyl)-azo-benzaldehyde, **3b** in good yields (Scheme 1). The potential probes differ from each other in having electron

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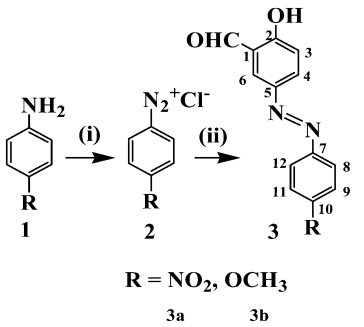


Scheme 1 (i) $NaNO_2$/HCl/0 °C/room temperature (ii) Salicylaldehyde/sodium carbonate/room temperature.

withdrawing ($-NO_2$) and donating ($-OCH_3$) functions and are expected to behave differently upon interaction with different amino acids. The UV-vis absorption spectra of the probes were acquired in HEPES buffer (DMF/H_2O; 9 : 1 v/v; pH 7.4). The absorption spectrum of **3a** shows low energy ($n\rightarrow\pi^*$) charge transfer (CT) bands at 542 nm ($\varepsilon = 8.54 \times 10^4$ mol^{-1} cm^{-1}) and ~410 nm. Similarly, probe **3b** exhibited a low energy ($n\rightarrow\pi^*$) CT band at 450 nm ($\varepsilon = 4.75 \times 10^4$ mol^{-1} cm^{-1}) along with broad shoulder at ~410–420 nm (Fig. 1). The high red-shift in the low energy CT band of **3a** clearly indicated that the existence of **3a** is predominantly in the hydrazone form while in the case of **3b** the hydrazone form dominates but the percentage of the azo form is relatively more than that in **3a**. This variation in the extent of the tautomeric forms is probably due to electronic effect of the $-NO_2$ and $-OCH_3$ groups present on **3a** and **3b**, respectively.[22]

To gain further insights about the equilibrium between the tautomeric hydrazone-azo forms acid–alkali spectrophotometric titration studies has been performed in HEPES buffer (DMF/H_2O; 9 : 1 v/v) at different pHs. Under acidic conditions (pH 1 to 4) the low energy bands with respect to the hydrazone-azo forms of both probes **3a** and **3b** diminished almost completely and a new high energy band appeared at 375 and 355 nm, respectively. Upon changing the pH from 5 to 12 the absorptivity of the high energy bands decreased with the hypsochromic shift and low energy bands corresponding to the hydrazone-azo form dominate. The typical change in the absorption pattern of **3** under acidic and alkaline conditions clearly suggested the possibility of a dynamic equilibrium between the hydrazone and azo forms as proposed in Scheme 2.

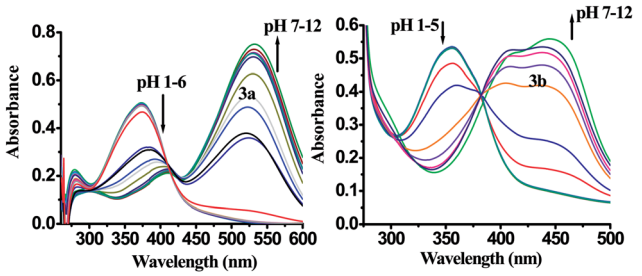


Fig. 1 The changes in the pH-absorption spectra of compounds **3a** and **3b** (10 μm) in HEPES buffer.

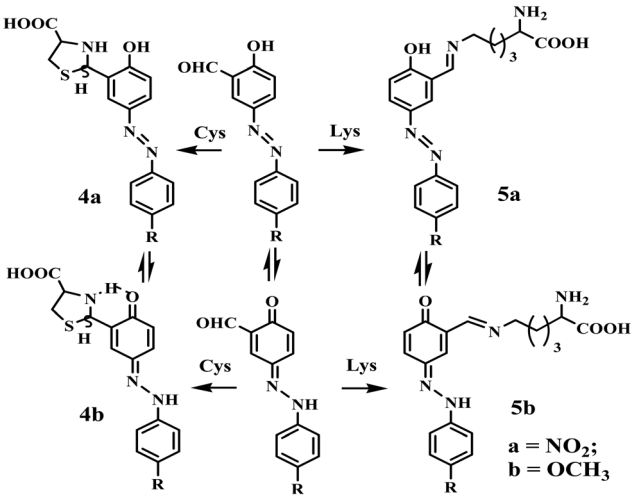
Notably, the absorption bands that appeared under acidic condition also suggested a geometrical structural change in probe **3** from *cis* to *trans*.[22] Moreover, to understand the possibility for the *cis–trans* isomerism probe **3a** and **3b** were photo-irradiated for 100–150 min at an interval of 5–10 minutes in DMF (Fig. S7†). The respective change in the absorption spectra suggests the existence of probes in geometrical *cis–trans* forms which is better in the case of probe **3a**. The variation in the extent of the actual isomeric forms is attributed to the presence of the electron withdrawing ($-NO_2$) and donating ($-OCH_3$) groups, respectively.[22]

Interaction studies with amino acids

The affinities of probes **3a** and **3b** toward different amino acids have been examined through absorption spectroscopy in HEPES buffer (DMF/H_2O 9 : 1 v/v, pH 7.4). The electronic transition spectra of **3** (10 μM) upon interaction with different amino acids (50 equiv.) showed significant modulation in the presence of Cys and Lys. In the presence of Cys the CT band of both **3a** and **3b** centered at 542 and 450 nm, respectively, diminished along with the hypsochromic shift of ~166 and 94 nm and new bands appeared at ~376 nm and ~356 nm, respectively. Similarly, in the presence of Lys the intensity of the CT band of **3a** decreased with the hypsochromic shift of ~55 nm and a new band appeared at ~487 nm along with a shoulder, at ~416 nm while with **3b** the low energy bands diminished almost completely and a new broad band appeared at ~381 nm with a blue shift of ~69 nm (Fig. 2 and S7†). Moreover, the naked-eye sensitive violet color of probe **3a** changed to an off-white and yellowish orange color with Cys and Lys respectively, while a brown-yellow color of **3b** became colorless with Cys and Lys respectively (Fig. 2, images).

Selectivity of probes toward Cys and Lys

In order to understand the selective colorimetric affinities of probes toward Cys and Lys amino acids interference studies



Scheme 2 A plausible mechanism of interactions between probes **3a** and **3b** with Cys and Lys amino acids.

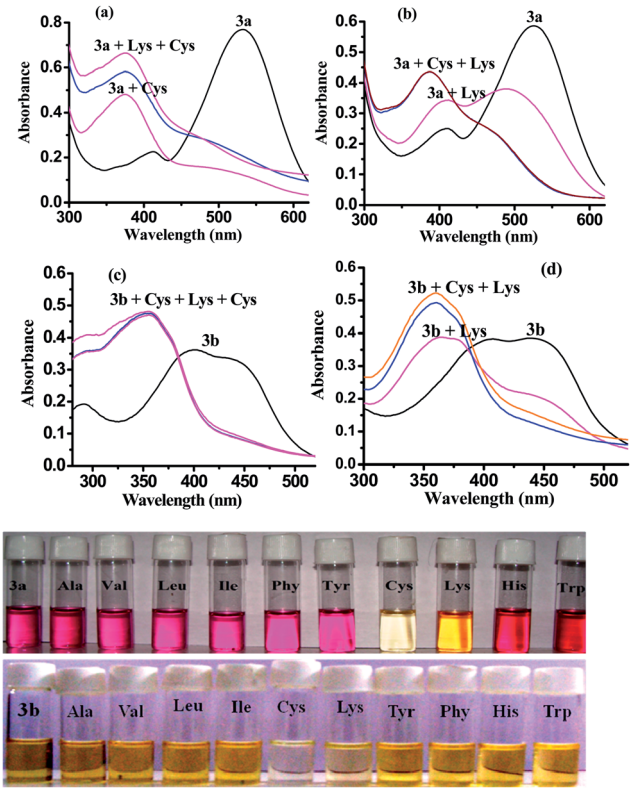


Fig. 2 The change in the absorption spectra of **3a** and **3b** (10 μm) with Cys and Lys (50 equiv.) in HEPES buffer (DMF/H_2O 9 : 1 v/v, pH 7.4). Images: the change in the color of **3a** and **3b** with different amino acids.

have been performed (Fig. 3). First, Cys (50 equiv.) was added to the solution of **3a** and **3b** (10 μm) then Lys was added (50 equiv.). The intensity of the CT band of **3a** centered at 542 decreased and a new band appeared at 376 nm. Similarly, in case of **3b** the broad band centered at ~450–415 nm disappeared with the formation of a new band at 356 nm (Fig. 2a and c). In contrast, when Lys was added first to a solution of **3a**/**3b**, the absorption spectra of **3a** showed a significant hypsochromic shift, ~126 nm in which the intensity of the band centered at 542 nm decreased and a new band appeared at 416 nm, whereas in the case of **3b** the intensity of the broad band diminished and a new band appeared at 356 nm. Furthermore, when Cys (50 equiv.) was added to the same solution of **3-Lys** the absorbance of the diminished ICT band of **3a**/**3b** decreased almost completely and new bands appeared at 376 and 356 nm, respectively (Fig. 2b and d).

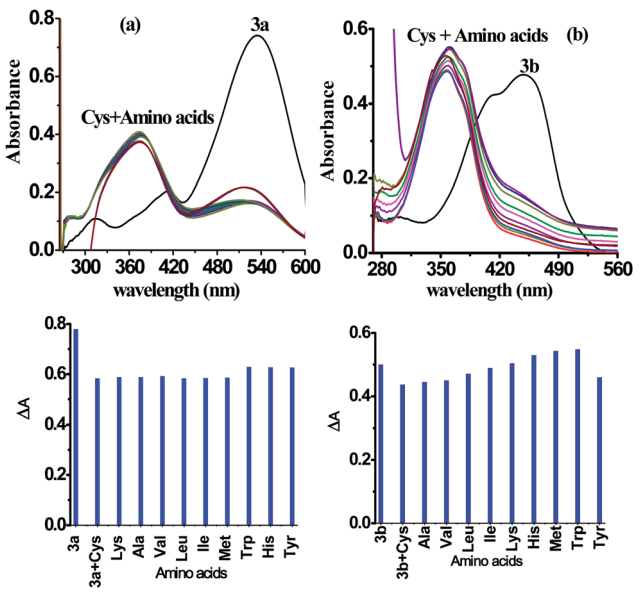


Fig. 3 The interference studies of **3a** and **3b** (10 μm) with different amino acids (50 equiv.) in HEPES buffer (DMF/H_2O 9 : 1 v/v, pH 7.4).

More precisely, when Lys and other amino acids were added to a solution of **3a + Cys** the new band appeared at 376 nm remained unchanged (Fig. 3a). Similarly, on the addition of tested amino acids to a solution of **3b + Cys** an insignificant change was observed at the newly developed band centered at 356 nm (Fig. 3b). This suggests the potential applicability of **3a** and **3b** as sensitive colorimetric and reaction based chemodosimeter probes to detect both Cys and Lys amino acids through the formation of thiazolidine and aldimine derivatives, respectively. Furthermore, other tested amino acids such as cysteine (Cys), lysine (Lys), valine (Val), alanine (Ala), leucine (Leu), isoleucine (Ile), tyrosine (Tyr), methionine (Met), tryptophan (Trp) and histidine (His) failed to exhibit any significant change in the absorption spectra of the probes, and the interference studies also suggested the relatively high selectivity of **3** for the Cys amino acid. It is important to mention that the observed typical changes in the absorption spectra of **3a** and **3b** are attributed to variations in the CT process as well as due to a typical structural change in which the stable hydrazone form changed to the azo form dominantly[22] as a result of the formation of new entities in the medium.

Additionally, we tried to see the behavior of both the probes with thiol containing compounds like mercaptoethanol (ME), glutathione (GSH) and cysteine (Cys) separately. It is interesting to observe that both **3a** and **3b** show affinities for GSH that are similar to Cys but fail to show any significant change in the absorption spectra through ME (Fig. S9†). Also, when the interference studies were performed by the addition of GSH and ME to a solution of **3** + **Cys** no significant change in the absorption spectra was observed. This assured the selectivity of **3** for Cys through a reaction based chemodosimeter approach (Fig. S10†).

The absorption titration experiments have been performed to understand extent of the binding affinities of probes **3a** and **3b** with Cys and Lys. Upon a gradual addition of Cys (0–20 equiv.) to a solution of **3a** a ratiometric spectral pattern was observed in which low energy bands centered at 542 and ~415 nm disappeared and a new band appeared at 376 simultaneously, along with formation of a sharp isosbestic point at 408 nm. An almost similar spectral pattern was observed in the case of **3b** in which the addition of Cys (0–20 equiv.) led to a decrease in the intensity of the broad band at 450–415 nm and a new band appeared at 356 nm along with the formation of an isosbestic point at 385 nm (Fig. 4). The readily detectable naked-

eye sensitive color of the solutions changed from violet to off-white and light yellow to almost colorless for receptors **3a** and **3b**, respectively. The Job's plot analysis suggested a 1 : 1 stoichiometry for an interaction between Cys and the probes **3a** and **3b** (Fig. S11†) for which association constants were estimated through nonlinear fittings of the UV absorption titration data and were found to be, $K_{ass} = 2.0 \times 10^3$ and 1.03×10^2 M^{-1}, respectively (Fig. 4) which are relatively good and comparable to others obtained from reported procedures.[17]

Similarly, when Lys (0–20 equiv.) was added sequentially to a solution of **3a** the band centered at 542 nm decreased with a blue shift of ~55 nm and new broad band appeared at ~405–487 nm. Additionally a new band of enhanced molar absorptivity appeared at ~322 nm along with the formation of a sharp isosbestic point at 490 nm (Fig. 5). Similarly, the addition of Lys to a solution of **3b** displayed a sharp variation in the absorption spectra in which the band at 450 nm decreased gradually with a blue shift of ~69 nm to appear at 381 nm and a new high energy band of a relatively high molar absorptivity appeared at 326 nm along with the formation of an isosbestic point at 399 nm. The color of the solutions was changed from violet to yellowish orange for **3a** and from yellow to colorless for **3b**. The Job's plot analysis consistently revealed a 1 : 1 stoichiometry for an interaction of **3a** and **3b** with Lys (Fig. S12†) for which the estimated binding constants were found to be $K_{ass} = 5.0 \times 10^2$ and $K_{ass} = 2.0 \times 10^3$ M^{-1}, respectively (Fig. 5). Thus, the formation of sharp isosbestic points and the variation in the spectral behavior clearly supported the existence of more than one species in the medium and the typical structural changes in which the

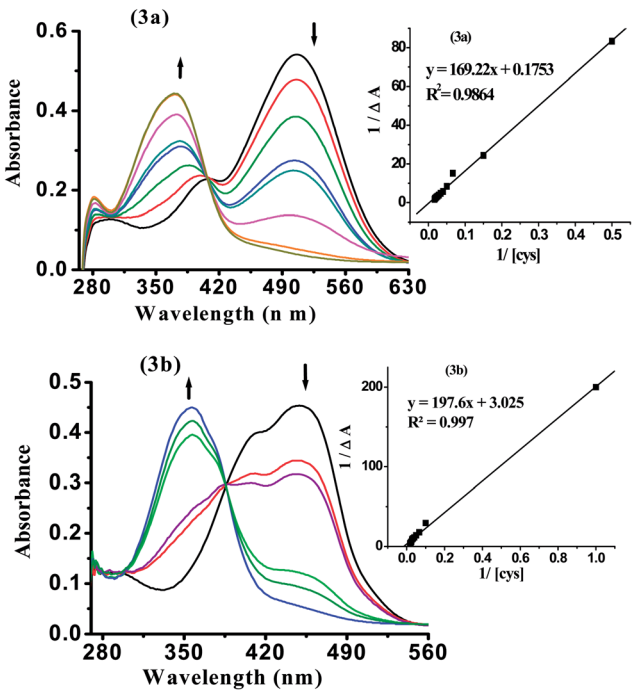


Fig. 4 The absorption titration spectra and plots of the binding constants of **3a** and **3b** (10 μm) upon the addition of Cys (0–20 equiv.) in HEPES buffer (DMF/H_2O; 9 : 1 v/v, pH 7.4).

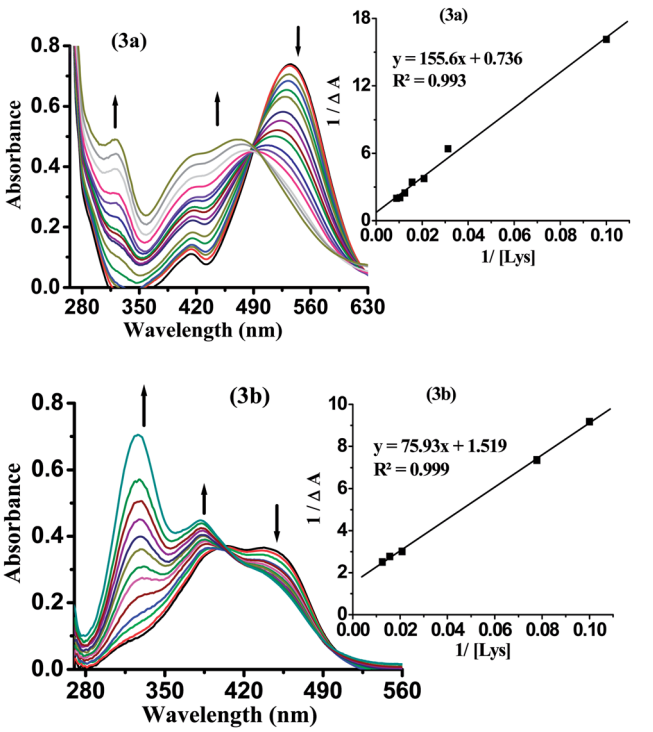


Fig. 5 The absorption titration spectra and plots of the binding constants upon the addition of Lys (0–20 equiv.) to solutions of **3a** and **3b** (10 μm) in HEPES buffer (DMF/H_2O; 9 : 1 v/v, pH 7.4).

percentage of the hydrazone form decreases and the azo form increases as well as the new entities generated in the medium which preferred to adopt the geometrically *trans* form.

Kinetic studies

The reaction kinetics have been studied to understand the response time of the potential reaction based chemodosimeters **3a** and **3b** to detect Cys and Lys (Fig. 6). After the addition of Cys and Lys (25 equiv.) to the solutions of **3a** and **3b** separately, the absorption spectra were acquired at regular time intervals of 10 min. The time *vs.* absorption kinetic plot for **3a** and **3b** displayed significant changes at the respective bands within ~60–100 min in which **3a** took relatively less time (~60–70 min) to complete the chemical reaction with Cys and Lys. Similarly, **3b** took almost the same time to react with Cys but more (~100 min) with Lys to generate new derivatives in the medium.

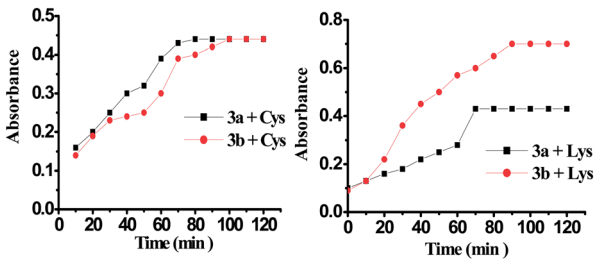


Fig. 6 The reaction kinetic plots for **3** with Cys and Lys.

This variation in the response time is reasonable and attributed to the presence of the $-NO_2$ and $-OCH_3$ substituents on **3a** and **3b**, respectively.

Limit of detection (LOD)

Furthermore, the limit of detection (LOD) for **3a** and **3b** with Cys and Lys was estimated in HEPES buffer (DMF/H_2O; 9 : 1 v/v) as reported previously from our laboratory.[22] A stock solution of **3a** and **3b** was prepared in HEPES buffer and diluted serially from 10 to 1 μM. The absorbance spectra of different concentration solutions were acquired. The absorption *vs.* concentrations plot outfitted a linear calibration curve and the standard deviations (σ) for **3a** and **3b** were found as 1.60 and 1.24, respectively (Fig. 7a and 8a). From the slope of the absorption curves, the calibration sensitivities (m) for Cys were obtained as 26.2 and 19.6, whereas for Lys they were obtained as 106 and 87 (Fig. 7b and c and 8b and c). The LOD for Cys with probes **3a** and **3b** was estimated using eqn (1) and was found to be 0.18 μM (22.16 ppb) and 0.19 μM (22.9 ppb), respectively. Similarly, the LOD for Lys with the probes **3a** and **3b** was found to be 0.045 μM (6.61 ppb) and 0.042 μM (6.24 ppb), respectively.

Nature of interaction

FT-IR spectra. The FT-IR spectra of **3a** and **3b** showed stretching vibration bands at 1608, 3420 and 1664 and at 1606, 3437 and 1648 cm^{-1} corresponding to azo (–N=N), hydroxyl, (–OH) and aldehyde (–CHO) functions, respectively (Fig. S13 and S14†). Upon the interaction of **3a** and **3b** with Cys, the aldehyde bands at 1664 and 1648 cm^{-1}disappeared and new bands appeared at 2926 and 2931, and 1745 and 1743 cm^{-1} could be attributed to the stretching vibrations of the methine (–CH) unit of the new thiazolidine ring formed and the C=O function of the Cys unit, respectively. Moreover, the FT-IR spectrum of **3a** and **3b** upon interaction with Lys showed a strong vibration band at 1592 and 1597 cm^{-1} attributable to the –C=N function of the newly formed aldimine function with the disappearance of the aldehyde C=O function stretching vibration at 1664 and 1648 cm^{-1} (Fig. S15 and S16†). Thus, the FT-IR spectra suggested that the interaction of **3a** and **3b** with Cys and Lys occurs through the formation of thiazolidine and aldimine derivatives.

^{1}H NMR titration studies. An ^{1}H NMR titration experiment has been performed to gain an insight about the mechanism of interaction between **3** and both amino acids in DMSO-d_6 ($c = 1.13 \times 10^{-2}$). The ^{1}H NMR spectra of **3a** (Fig. 9 and S17–S19†) showed resonances as singlet peaks at δ 11.77, 10.37 and 8.23 ppm and doublet peaks at δ 7.23 (J = 8.7 Hz), 8.05 (J = 8.7 Hz), 8.16 (J = 8.7 Hz) and 8.42 (J = 8.7 Hz) ppm which are attributable to the –OH, –CHO, H6, H3, H4, H8 and H9 protons, respectively. Upon the addition of 10 and 30 equiv. of Cys to a solution of **3a** the H6, H4, H8, H9 resonances shifted upfield and new resonances appeared at δ 6.18 (s), 6.05 (d) and 8.59 ppm which could be assigned to the methine (–CH) and –NH protons of the thiazolidine derivative **4a** formed in the medium. Similarly, the –CHO resonance disappeared and the –OH signal broadened with an upfield shift to appear at δ 8.47 ppm.

Similarly, the ^{1}H NMR spectrum of **3b** due to presence of the $-OCH_3$ substituent displayed resonances as singlet peaks, due to the –OH, –CHO and H6 protons, at δ 11.39, 10.35 and 8.12 ppm, whereas doublets assignable to the H3, H4, H8 and H9 protons and a singlet due to $-OCH_3$ appeared at δ 7.87 (J = 8.4

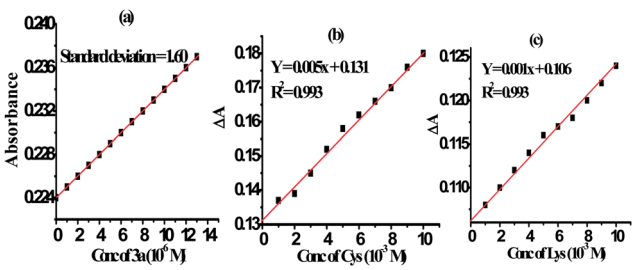


Fig. 7 (a) The calibration curve for **3a**, and the calibration sensitivities for (b) Cys (10^{-3} M) and (c) Lys (10^{-3} M) in HEPES buffer.

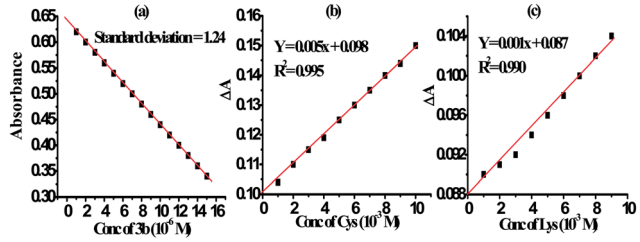


Fig. 8 (a) The calibration curve for **3b**, and the calibration sensitivities for (b) Cys (10^{-3} M) and (c) Lys (10^{-3} M) in HEPES buffer.

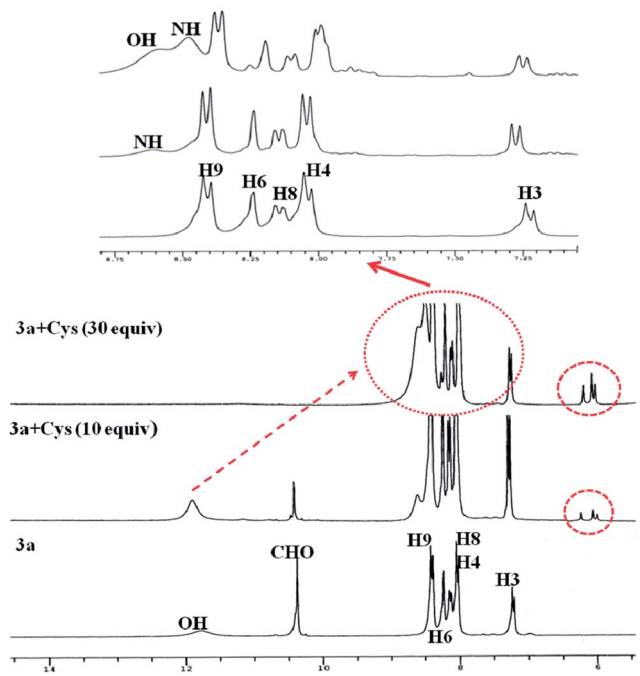


Fig. 9 The ^{1}H NMR titration spectra of **3a** upon the addition of Cys (0, 10 and 30 equiv.) in DMSO-d_6.

Hz), 8.06 (J = 9 Hz), 7.19 (J = 8.7 Hz), 7.13 (J = 8.7 Hz) and 3.86 ppm, respectively (Fig. 10). Upon the addition of Cys (10 and 30 equiv.) to a solution of **3b** the H6, H4, H8 and H9 resonances shifted upfield and new signals attributed to the methine (–CH) proton of the thiazolidine derivative **4b** appeared at δ 6.14 (s) and 6.06 (d) ppm. Interestingly, the –OH signal broadened with an upfield shift to appear at δ 8.46 ppm along with the resonance of the –NH proton and $-OCH_3$ proton which shifted downfield to appear at 4.07 ppm, respectively. The broadened resonances attributed to the –NH/–OH protons suggested the existence of the hydrazone and azo forms and probable hydrogen bonding interaction between –OH and –NH functions.

Furthermore, to ascertain the formation of the aldimine derivative **5** upon the interaction between **3** and Lys the ^{1}H NMR spectra were acquired (Fig. 11 and S20†). The ^{1}H NMR spectrum of **3a** (in $CDCl_3$) showed –OH and –CHO resonances as singlets at δ 11.38 and 9.98 ppm with the H6, H3, H4, H8 and H9 resonances at δ 8.21, 7.10 (J = 9.0 Hz), 7.96 (J = 8.4 Hz), 8.16 (J = 9 Hz), 8.33 (J = 8.4 Hz) ppm, respectively. Upon interaction with Lys the –CHO signal (at δ 9.98 ppm) disappeared and a new resonance attributed to the aldimine function (–N=CH) appeared at δ 8.97 ppm while the protons of the phenyl ring and –OH (appeared at 10.6 ppm) function shifted upfield, respectively. The upfield shift in the –OH signal is possibly due to a better H-bonding interaction with the aldimine function.[20] Thus, the ^{1}H NMR experiment clearly supported the formation of the thiazolidine and aldimine derivatives **4** and **5** upon the interaction of probe **3a** and **3b** with the Cys and Lys amino acids (Scheme 2).

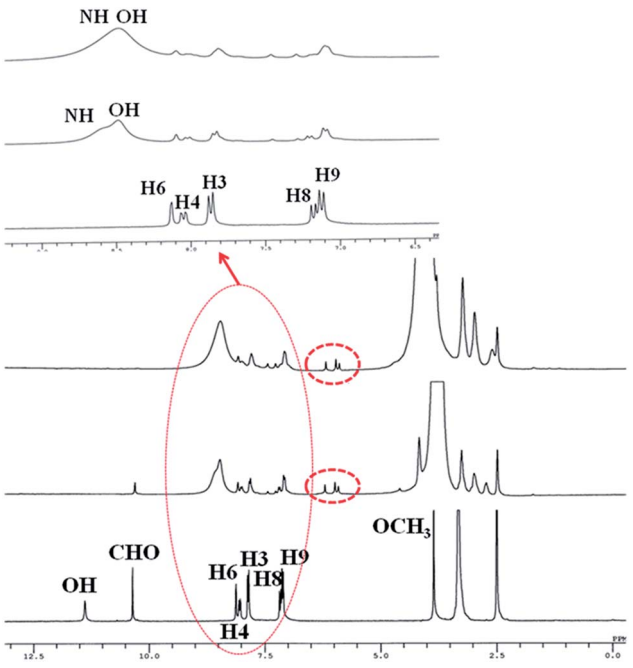


Fig. 10 ^{1}H NMR titration spectra of **3b** upon the addition of Cys (0, 10 and 30 equiv.) in DMSO-d_6.

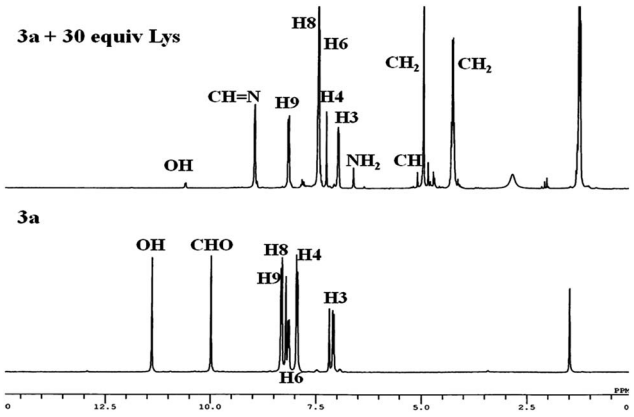


Fig. 11 ^{1}H NMR spectra of **3a** with Lys (30 equiv.) in $CDCl_3$.

The detection of cysteine in human blood plasma. To our own interest we further intended to observe the utility of potential colorimetric chemodosimeters **3a** and **3b** to detect Cys in real biological samples. In blood plasma, cysteine occurs either free or linked to other amino acids like a cysteine–glycine conjugate and glutathione. In blood, the low-molecular weight aminothiols (Cys and homocysteine) are bound to proteins in a large percentage, which can be freed in blood plasma after reduction processes. Moreover, in human blood plasma free low-molecular weight aminothiols have Cys as the main component (*ca.* 83% of the total aminothiols) along with glutathione (*ca.* 3%), cysteine–glycine (*ca.* 11%) and homocysteine (*ca.* 3%).[23]

In the present preliminary assays of the determination of aminothiols in real samples we have used human blood as a source of plasma. To release the protein-bound thiols, the disulfide bonds were reduced by a vigorous mixing of blood plasma (100 μl) with an appropriate amount sodium borohydride (0.5 mM) followed by incubation for 3 min at room temperature.[24,25] This plasma solution was used for interaction and titration studies with our probes **3a** and **3b** (10 μM) and we followed the changes in the UV-visible absorption spectra. Upon the addition of human blood plasma (10 μl) to a solution of **3a** in a HEPES buffer the bands centered at 542 nm ($\varepsilon = 6.4 \times 10^4$ mol^{-1} cm^{-1}) and 413 nm ($\varepsilon = 1.8 \times 10^4$ mol^{-1} cm^{-1}) diminished and new bands appeared at 570 nm ($\varepsilon = 1.8 \times 10^4$ mol^{-1} cm^{-1}) and ~395 nm ($\varepsilon = 3.0 \times 10^4$ mol^{-1} cm^{-1}). Similarly, in the case of **3b** the bands centered at 448 ($\varepsilon = 6.0 \times 10^4$ mol^{-1} cm^{-1}) and ~410–420 nm decreased and a new band appeared at 385 nm ($\varepsilon = 4.9 \times 10^4$ mol^{-1} cm^{-1}) (Fig. 12, inset).

Furthermore, the usability of the chemodosimeter **3** for the determination of cysteine in biological samples has been shown by titration studies in HEPES buffer (Fig. 12). Upon a gradual addition of blood plasma (0–10 μl) to a solution of **3a**, the bands centered at 542 nm decreased ratiometrically and new bands appeared at 570 and 395 nm with isosbestic points at 615 and 445 nm. Similarly, upon addition of blood plasma (0–10 μl) the absorption spectra of **3b** displayed a gradual

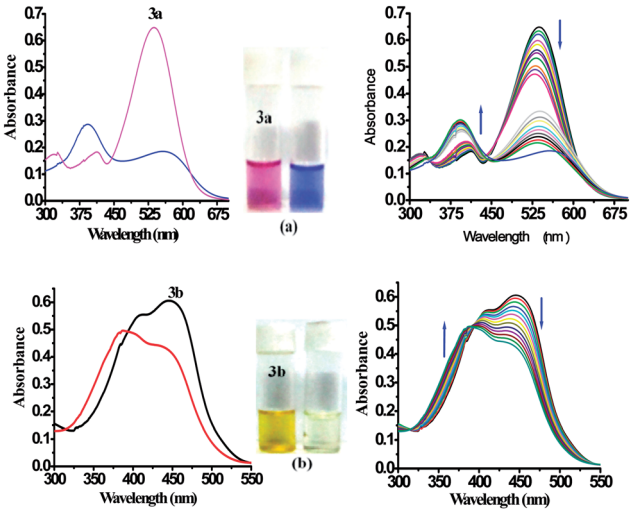


Fig. 12 The interaction and titration studies of **3a** and **3b** (10 μm) with blood plasma in HEPES buffer (pH 7.4) and the respective change in the color of the probe solutions.

decrease at 445 nm and a new band appeared at 385 nm along with the formation of an isosbestic point at 393 nm. This suggested the existence of more than one species in the medium (Fig. 12). The readily detectable naked-eye sensitive color of the solutions changed from violet to blue and yellow to almost colorless (Fig. 12, images).

Furthermore, the limit of detections (LOD) for **3a** and **3b** for Cys in human blood plasma was estimated in HEPES buffer (pH, 7.4) as mentioned above. From the slope of the absorption curves the calibration sensitivities (m) were found to be 83.5 for both probes **3a** and **3b** corresponding to Cys in human blood plasma (Fig. 13) for which, the LOD was estimated using eqn (1) and found to be 0.057 μM (6.95 ppb) and 0.045 μM (6.57 ppb), respectively. It is important to mention that in human blood plasma the changes observed in the case of **3a** are relatively more sensitive and prominent than those of **3b** however the observed pattern was more or less similar when the probes were tested with Cys in solution without blood plasma. Therefore, it is worth mentioning that the probes can also be used to detect Cys selectively in a relatively more complex native human plasma.

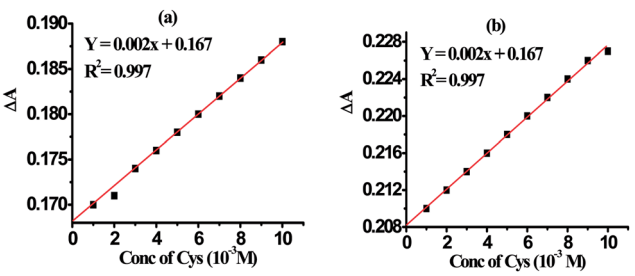


Fig. 13 The calibration sensitivity for Cys in human blood plasma (a) **3a** and (b) **3b** in HEPES buffer.

Conclusion

In summary, we have designed and synthesized simple azo based colorimetric chemodosimeters to detect selectively Cys and Lys in the environment of competitive different class of amino acids through the formation of thiazolidine and aldimine derivatives in HEPES buffer as well as under physiological conditions. The probes upon reaction with Cys and Lys showed a sensitive spectrophotometric behavior and a significant colorimetric response with readily detectable naked-eye sensitive color changes in the real biological sample. The colorimetric determination of thiols is very important and significant for the detection of aminothiols in urine and blood in biological and medical diagnosis. In a preliminary experiment we utilized our systems to detect aminothiols in human blood plasma which demonstrated the practical applicability and sensitivity of the present sensing event through a reaction based chemodosimeter approach and can be utilized further in diagnostic studies.

Experimental

General

Salicylaldehyde, aniline (nitro, methoxy) and sodium nitrite were purchased from Sigma Aldrich Pvt. Ltd. Ethanol and metals as their nitrate salts were purchased from Merck India Pvt. Ltd. Spectroscopic grade solvents were used in spectroscopic studies. The absorption spectra was recorded at room temperature on a Shimadzu 1700 and Perkin-Elmer Lambda 35 spectrophotometer using a quartz cuvette (path length = 1 cm). The FTIR spectra (KBr pellets) were recorded on a Varian-3100 spectrometer. The NMR spectra (chemical shifts in δ ppm) were recorded on a JEOL AL 300 FT-NMR (300 MHz) spectrometer using tetramethylsilane (TMS) as an internal standard.

Limit of detection (LOD) has been estimated using eqn (1);

$$\mathrm{LOD} = 3\sigma/m \quad (1)$$

where, σ stands for the standard deviation for the blank solution of probes and m indicates the calibration sensitivity for Cys/Lys and Cys present in human blood plasma in HEPES buffer (DMF/H_2O; 9 : 1 v/v).

The synthesis of 2-hydroxy-5-(4-nitrophenyl)-azo-benzaldehyde compound (3a)

To a well stirred cold solution of 4-nitroaniline (1.38 g, 10 mmol) in aqueous HCl (3 ml), an aqueous solution of sodium nitrite (1.38 g, 20 mmol) was added slowly for 30 min, and the temperature was maintained around 0–5 °C. After 1 h of constant stirring the reaction mixture became dark brown. Next, a solution of salicylaldehyde (1.22 ml, 10 mmol) in Na_2CO_3 (1 g in 10 ml H_2O) was added drop wise to the reaction mixture for 30 min and the reaction mixture was stirred further for 4 h. The brown yellowish color solid so obtained was filtered to afford the product in a 73% yield. ^{1}H NMR ($CDCl_3$) δ (ppm): 11.39 (d, 1H, CHO), 9.98 (s, 1H, OH), 8.33 (d, 2H, J = 8.4 Hz, benzyl), 8.21 (s, 1H, benzyl), 8.16 (d, 2H, J = 9 Hz, benzyl), 7.96

(d, 2H, $J = 8.4$ Hz, benzyl), 7.10 (d, 1H, $J = 9$ Hz, benzyl); IR (KBr) ν_{max} (cm^{-1}); 3102, 2840, 1664, 1608, 1577, 1526, 1478, 1286, 1171, 1143, 1102, 906, 855, 751, 719, 687, 578, 452. ESI-MS of **3a** at $m/z = 271.0$ (calculated) and 271.9 (observed).

The synthesis of 2-hydroxy-5-(4-methoxyphenyl)-azo-benzaldehyde (3b)

Yield 70% ^{1}H NMR ($CDCl_3$): 11.26 (s, 1H, CHO), 10.013 (s, 1H, OH), 8.145 (s, 1H, benzyl), 7.90 (d, 1H, $J = 6$ Hz, benzyl), 7.11 (d, 1H, $J = 8.7$ Hz, benzyl), 7.02 (d, 1H, $J = 7.8$ Hz, benzyl), 3.89 (s, 3H, OCH_3); IR (KBr) ν_{max} (cm^{-1}); 3422, 2837, 1648, 1600, 1580, 1500, 1481, 1325, 1278, 1246, 1149, 1105, 1034, 838, 774, 692, 583, 506, 454. ESI-MS of **3b** at $m/z = 256.0$ (calculated) and 257.8 (**3b** + H^+, observed).

The synthesis of compound 3a with lysine

To a solution of compound **3a** (138 mg, 1.0 mmol) in ethanol (5 ml) containing NaOH (80 mg, 2.0 mmol), lysine (146 mg, 1.0 mmol) was added and the reaction mixture was refluxed for 2 h (monitored on TLC). The precipitate so obtained was filtered, washed with water and dried in air. Yield = 72%. Mp > 200 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 10.6 (s, 1H, OH), 8.96 (s, 1H, aldimine), 8.17 (d, 1H, $J = 8.4$ Hz, benzyl), 7.47 (t, 2H, $J = 12.3$, benzyl), 7.0 (m, 2H, benzyl), 6.63 (s, 2H, NH_2), 5.11 (s, 1H, –CH), 4.97 (d, 2H, $J = 0.3$ Hz, CH_2), 4.73(d, 1H, $J = 8.1$ Hz, CH_2), 4.31(m, 2H, CH_2); IR (KBr) ν_{max} (cm^{-1}); 3418, 2928, 1597, 1507, 1389, 1250, 1148, 830. ESI-MS of **3a-Lys** at $m/z = 399.0$ (calculated) and 423.1 (**3a** + Lys + Na^+ + H^+, observed).

The synthesis of compound 3a with cysteine

To a solution of compound **3a** (138 mg, 1.0 mmol) in ethanol (5 ml), cysteine (121 mg, 1.0 mmol) was added and the reaction mixture was refluxed for 2–3 h. The precipitate so obtained was filtered, washed with water and ethanol and dried in air. Yield = 70%. Mp > 200 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 8.59 (s, 1H, benzyl), 8.47 (s, 1H, benzyl), 8.37 (d, 1H, $J = 8.1$, benzyl), 8.25 (s, 1H, NH), 8.19 (s, 1H, OH), 8.11 (d, 2H, $J = 8.4$ Hz), 8.0 (d, 2H, $J = 6$ Hz, benzyl), 7.25 (d, 2H, $J = 9$ Hz, benzyl), 6.18 (s, 1H, CH), 6.05 (d, 1H, $J = 14.1$ Hz, –CH), 4.17 (s, 2H, CH_2); IR (KBr) ν_{max} (cm^{-1}); 3422, 2926, 1518, 1403, 1277, 1088, 855. ESI-MS of **3a-Cys** at $m/z = 374.0$ (calculated) and 430.1 (**3a** + Cys + Na^+ + H^+, observed).

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