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Dicoumarol assisted synthesis of water dispersible gold nanoparticles for colorimetric sensing of cysteine and lysozyme in biofluids

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Abstract

The important roles of cysteine (Cys) and lysozyme (Lys) in biological systems have attracted great interest in their detection in various biofluids. Here, we report the use of dicoumarol (DIC) as a novel reducing and stabilizing agent for the synthesis of water-dispersible DIC-decorated gold nanoparticles (Au NPs) for the sensitive and selective colorimetric determination of Cys and Lys in biofluids. The characteristic surface plasmon resonance (SPR) peak at 525 nm and solution color of DIC-Au NPs undergo dramatic changes on exposure to Cys and Lys, yielding new absorption peaks appearing at 666 and 570 nm for Cys and Lys and concomitant color change from cherry red to blue for Cys and to purple for Lys, respectively. The high degree of DIC-Au NPs aggregation induced by Cys and Lys was observed at 0.4 M of NaCl. Based on this finding, we propose a method to detect Cys and Lys by naked eye and UV–visible spectrometry. The proposed sensor quantitatively measured Cys and Lys concentrations within the dynamic range of 10 – 90 μM (R^2= 0.9942) and 100 - 800 nM (R^2= 0.9961) with limits of detection of 2.2×10^{-6} and 8.7×10^{-9} M for Cys and Lys, respectively. This probe was successfully applied to detect Cys and Lys in human urine and plasma and Lys in egg white, demonstrating its great value of practical application in biological systems.

Keywords: DIC-Au NPs, Cys, Lys, UV-visible spectrometry, TEM, FT-IR, Biofluids and Egg white.

1. Introduction

Cysteine is a semi-essential α-amino acid, and it can be biosynthesized in humans. It plays an important role in the biological systems and involves many biochemical pathways.[1] It serves as a reagent in many vital catalytic reactions and plays a pivotal role in inducible, endogenous detoxication mechanisms in organs.[2,3] Lysozyme is known as a muramidase or *N*-acetylmuramide glycanhydrolase, and contains antimicrobial proteins. It is a polypeptide of 129 amino acid residues cross-linked by four disulfide bridges, with molecular weight 14.3 kDa (isoelectric point - 10 – 11).[4] It (bacteriolytic and mucolytic enzyme) is generally found in egg white and in many body fluids. It is the animals host defense and breaks down the bacterial cell wall through the muramidase activity. As a result, it showed strong antibacterial activity against Gram-positive bacteria and found tremendous practical applications in food, pharmaceutical industries and medicine.[5] Thus, the detection of both (Cys and Lys) biomolecules plays important roles in theory and practical applications in proteomics. Several analytical techniques including high performance liquid chromatography (HPLC),[6,7] electrochemistry,[8] mass spectrometry,[9-11] and capillary electrophoresis [12] have been used for the detection of Cys and Lys in various biofluids. Apart from these, the detection of Lys is often accomplished by immunoassay,[13] and enzyme-linked immunosorbent assay. [14,15] Although these methods exhibit well ability to detect Lys with good selectivity, unfortunately, most of these methods are lacking in sensitivity and required longer analysis time and tedious sample pretreatments. Further, these techniques are expensive, require significant instrumentation, and are nonportable. Therefore, it is essential to develop a simple, fast

and cost-effective method to detect Cys and Lys in biofluids with good sensitivity and selectivity.

In recent years, great progress has been made in the development of nanoparticles-based portable analytical approaches to open up new opportunities in inorganic, organic and biomolecules assays.[16-20] Due to their fascinating physico-chemical properties, metallic nanoparticles have aroused as ideal probes for selective and sensitive reorganization of wide variety molecules in environmental and biological samples.[21,22] Among the various metal NPs, Au NPs have been extensively used as colorimetric probes for selective and sensitive sensing of various molecular species in complex samples. Since Au NPs possess a high molar extinction coefficient, high stability, and strong distance-dependent optical properties, which makes them as potential candidates for colorimetric assays of wide variety molecules *via* target analytes-induced Au NPs aggregation, yielding a red shift in the SPR absorption band and a color change from red to blue.[23] The color changes induced by the association of NPs can be successfully used for the quantification of trace target analytes by UV-visible spectroscopy, which facilitate to detect the trace analytes from complex samples with high sensitivity and selectivity.[24] The molecular assembly on Au NPs plays key role to act them as persistent potential colorimetric sensors for facile, selective and sensitive detection of biomolecules in biocomplex samples with minimized sample preparations at minimal volume of samples. For example, a colorimetric method was described for detection of Cys using cetyl trimethyl ammonium bromide (CTAB)-capped Au NPs as a sensor.[25] Wang and co-workers described the use of carboxymethyl cellulose (CMC)-functionalized Au NPs as a colorimetric probe for simple and sensitive detection of Cys

in biological samples.[26] Yu's and Mao's groups developed a sensitive and selective colorimetric method for the detection of cysteine in the rat brain using aspartic acid functionalized Au NPs as a sensor.[27]

Furthermore, the detection of Lys using NPs-based methods has gained tremendous interest due to their unique size and interparticle-distance dependent optical properties. For example, Lin *et al*. developed a dual-readout sensor (colorimetric and fluorometric) for the detection of Lys based on the interactions of Au NPs with triazolylcoumarin and Lys.[28] Lee's group developed a convenient colorimetric detection system for various proteins including Lys using aptamer−Au NPs conjugates.[29] Tseng's team functionalized Au NPs with human serum albumin (HSA) and used as a probe for the colorimetric sensing of Lys in egg white.[30] Similarly, Xiang and co-workers described the importance of electrostatically adsorbed aptamers on Au NPs for colorimetric detection of Lys with good selectivity.[31] Further, Cys-Ala-Leu-Asn-Asn (CALNN)-capped Au NPs-based colorimetric method was established for the detection of Lys in biological samples.[32] Besides, carbon nanomaterials-based methods have been developed for the detection of Lys in biological samples.[33,34] Although the reported methods have shown well ability to detect micro and nanomolar concentrations of Cys and Lys in complex samples by various analytical and nanomaterials-based colorimetric and fluorescence techniques, unfortunately they require expensive instruments, and tedious sample preparations. On the other hand, they still require multiple steps for the functionalization of Au NPs, and they have shown well ability to detect only single analyte either Cys or Lys. Consequently, the development of rapid, simple, selective, and sensitive methods for the determination of Cys and Lys in biofluids has become

particularly significant and proven to be a challenging task. Therefore, it is necessary to design a novel molecular assembly on Au NPs that can act as a reducing and capping agent for developing advanced Au NPs-based smart sensor.

In this work, we describe dicoumarol directed synthesis of water dispersible Au NPs for colorimetric sensing of Cys and Lys in real samples. This method does not need any capping agent and multiple steps, and it possesses good selectivity and sensitivity. The DIC-Au NPs have proven to be a colorimetric nanosensor for the determination of Cys and Lys using the interparticle plasmon coupling on analyte-induced aggregation of Au NPs, resulting a change in their absorption spectra and color. The target analytes (Cys and Lys) are selectively bound on the surface of DIC-Au NPs in 0.4 M of NaCl, which results a high degree of DIC-Au NPs aggregation induced by target analytes. The target analytes-induced DIC-Au NPs aggregations were characterized by UV-visible spectroscopy, dynamic light scattering (DLS), and transmission electron microscopy (TEM), respectively. Based on the sensitive assembly of DIC-Au NPs associated with solution color change and absorption spectra, this sensing system enables optical detection of Cys and Lys in biofluids with high selectivity and sensitivity.

2. Experimental section

2.1. Chemicals and materials

Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4.3H_2O$), phenyl alanine, histidine, 4-hydroxyproline, proline, tryptophan, tyrosine, alanine, isoleucine, aspargine, aspartic acid, methonine, lysine, serine, leucine, cysteine, lysozyme, and dicoumarol, were procured form Sigma –Aldrich, USA. NaCl, NaOH, Na_2HPO_4, KCl, KH_2PO_4, and

HCl were obtained from Finar Chemicals Ltd., India. Milli-Q-purified water was used throughout the practical work. All the glass wares rinsed with Mill-Q-Purified water.

2.2 Synthesis of DIC-Au NPs

The DIC-Au NPs were synthesized using DIC as a reducing and capping agent. Briefly, DIC (0.026 g) was dissolved in 2.5 mL of NaOH and rapidly added into a boiling solution of 0.5 mM $HAuCl_4$ (25 mL), under stirring condition and the resulting solution was kept continuously stirring for another 20 min. The color of solution was changed from pale yellow to cherry red, confirming that the formation of DIC-Au NPs. The obtained DIC-Au NPs solution was cooled to room temperature and then filtrated through a Millipore syringe (0.45 μm) to remove the precipitate, and the obtained DIC-Au NPs solution was stored in a refrigerator at 4 °C for further use. The size of DIC-Au NPs was about 13.84 nm as confirmed by DLS and TEM techniques. The DIC-Au NPs showed the absorbance at 525 nm and their analytical applications for colorimetric assay of biomolecules were performed.

2.3. Colorimetric sensing of Cys and Lys

Colorimetric assays for Cys and Lys were performed using DIC-Au NPs as a probe by the following procedures. ***For Cys sensing***: 100 μL of various target analytes (phenyl alanine, histidine, 4-hydroxyproline, proline, tryptophan, tyrosine, alanine, isoleucine, aspargine, aspartic acid, methonine, lysine, serine, leucine, Cys and Lys, 1 mM) were added separately into DIC-Au NPs at Tris-HCl buffer pH 7.0 in the presence of NaCl (0.4 M) and the resulting mixtures were vortexed for 1 min at room temperature. The color of DIC-Au NPs was changed from red to blue, indicating that the aggregation

of DIC-Au NPs induced by Cys. ***For Lys sensing***: Typically, the solutions of various target analytes (phenyl alanine, histidine, 4-hydroxyproline, proline, tryptophan, tyrosine, alanine, isoleucine, aspargine, aspartic acid, methonine, lysine, serine, leucine, Cys and Lys, 100 μL, 50 μM) were mixed with DIC-Au NPs solutions (1.0 mL) in the presence of NaCl (0.4 M) and then vortexed for one min at room temperature. The sensing and selectivity of Cys and Lys were monitored using UV-visible spectroscopy by changes in the SPR peak of DIC-Au NPs. Supporting Information of Scheme 1 shows the preparation of Au NPs using DIC as a reducing and capping agent for colorimetric detection of Cys and Lys.

2.4. Detection of Cys and Lys in real samples (urine, plasma and egg white)

The urine and plasma samples were collected from three healthy volunteers. The collected samples were diluted (100-fold) by water and then vortexed for 5 min at room temperature. The resulting samples were spiked with different concentrations of target analytes Cys (40, 60 and 80 μM) and Lys (0.2, 0.5 and 0.8 μM) and then added to 1.0 mL of DIC-Au NPs solutions at Tris-HCl pH 7 for Cys and at PBS pH 7 for Lys in the presence of NaCl (0.4 M). The UV-visible absorption spectra of the solutions were recorded and then analyzed by the above described procedure.

The quantification of Lys in chicken egg was carried out by the well described procedure in the literature.[30] Briefly, chicken egg was obtained from the local supermarket, the egg white and egg yolk were separated. The collected egg white was diluted with 20 mM of phosphate buffer (pH 8.0) in a 1:20 ratio before use. The above sample was added to a solution (1 mL) of DIC-Au NPs at PBS pH 7 and then vortexed

for 1 min. Finally, the concentration of Lys in egg white was determined by UV-visible spectrometry.

2.5 Instrumentation

The UV–visible spectra were measured by using a May Pro 2000 spectrophotometer (Ocean Optics, USA). Fourier transform infrared (FT-IR) spectra were recorded on a Perkin Elmer (FT-IR spectrum BX, Germany). The TEM images were taken on a JEOL 3010. DLS measurements were performed by using Zetasizer Nano ZS90 (Malvern, UK).

3. Results and discussion

3.1. Optimization of $HAuCl_4$ and DIC concentrations and temperature

UV–visible spectroscopy is very simple and useful technique to investigate the effect of reagents and temperate on the characteristic SPR peak and size of Au NPs. First, we studied the effect of $HAuCl_4$ concentration (0.1 mM – 1.0 mM) on the formation of color and absorption spectra of Au NPs using DIC (38.8 mM) as a reducing and capping agent. As shown in Figure 1, no obvious color and SPR peak change was observed using 0.1 mM of $HAuCl_4$, which indicates that Au NPs does not form. The color of the solution was slightly changed from colorless to light pink color, yielding to generate a characteristic SPR peak at 525 nm, which confirms the formation of DIC-Au NPs. The intense cherry red color and maximum SPR peak (at 525 nm) intensity were observed using 0.5 mM of $HAuCl_4$. It can be observed that the color of DIC-Au NPs is dark red in color and the typical SPR peaks (at 532 and 537 nm) were observed with slight red-shifts using 0.75 and 1.0 mM of $HAuCl_4$, indicating that the optical properties of DIC-Au NPs

were slightly changed due to the colloidal instability at high ionic strength. Therefore, we selected 0.5 mM of $HAuCl_4$ as the best concentration for the preparation of DIC-Au NPs with an average size of 13.84 nm.

Generally, metallic nanoparticles are formed by chemical reduction method using organic molecules as reducing agents. The aggregation and flocculation of NPs can be prevented by using organic molecules as protective agents or stabilizers. In order to establish DIC as a novel reducing and capping agent for Au NPs, we studied the effect of DIC concentration ranging from 18.8 to 48.8 mM for reduction of Au^{3+} ions using 0.5 mM of $HAuCl_4$ (Supporting Information of Figure S1). The color and the absorption spectra of Au NPs vary with respect to the DIC concentrations. It is noticed that the intense cherry red color and the maximum SPR peak intensity at 525 nm are observed using 38.8 mM of DIC, confirming that the formed Au NPs are well dispersed in water with spherical shape (<540 nm for the spherical Au NPs). Meanwhile, we observed that the characteristic SPR peak intensity was gradually increased with increasing DIC concentration from 18.8 to 38.8 mM, which can be well agreed with the color of solutions. However, the SPR peak of DIC-Au NPs shows a red-shift with broadening of spectrum using 48.8 mM of DIC, which is due to the self-molecular assembly of DIC onto the surfaces of Au NPs. Furthermore, negligible color and SPR peak of Au NPs are observed using 28.8 mM of DIC, indicating that the Au^{3+} ions are not completely reduced by DIC. Thus, 38.8 mM of DIC was selected as an optimal concentration for the preparation of Au NPs with good optical characteristics.

Earlier results suggested that the chemical reaction rate drastically depends on temperature which plays an important role in the reduction of Au^{3+} ion to Au^0 using

various organic reagents.[35] In order to establish DIC-Au NPs as a good nano-biosensor, we studied the color and the absorption spectra of Au NPs using 38.8 mM of DIC as a reducing reagent at different reaction temperature from 0 to 100^{o}C (Supporting Information of Figure S2). It was found that the formation rate of DIC-Au NPs with good color and SPR peak intensity was very good at 100^{o}C as expected than lower temperatures. The color of DIC-Au NPs obtained at 100°C was drastically differed from that of those produced at 0^{o} – 75^{o}C reaction temperatures. It can be observed that the color and the SPR peak at 525 nm of DIC-Au NPs do not show obvious differences, and no obvious difference is found in the full width at half maximum of the peak profiles (Supporting Information of Figure S2). Therefore, we selected 100^{o}C as an optimum reaction temperature for synthesis of Au NPs using DIC as a reducing and capping agent. The effect of optimal experimental parameters (reagent, precursor concentrations and reaction temperature) on the acceptable DIC-Au NPs for colorimetric assays is listed in Table 1.

3.2. Characterization

The synthesis of Au NPs using DIC as a novel reagent was illustrated by UV-visible, FT-IR, DLS and TEM data. The UV-visible spectroscopy is one of the simplest technique to characterize Au NPs and to calculate the sizes of Au NPs based on their SPR peak. The UV–visible spectrum of DIC-Au NPs shows the SPR peak at 525 nm (Figure 1). The stability of DIC-Au NPs is usually monitored by their absorption characteristics, no change in the absorption characteristics of one month aged DIC-Au NPs was

observed. Based on the UV-visible spectra of DIC-Au NPs, we also calculated the average size of DIC-Au NPs by using the following formula.[36]

$$d = exp\ (B_1 \frac{A_{spr}}{A_{450}} - B_2)$$

Where d (nm) is the size of a given Au NP sample, A_{spr} and A_{450} are absorbance at the SPR peak and 450 nm respectively, B_1 = 3.55 and B_2 = 3.11. The UV-visible data were confirmed the formation of DIC-Au NPs with maximum SPR peak intensity at 525 nm, indicating that the DIC-Au NPs are well dispersed with an average size of 13.84 nm those are confirmed by DLS and TEM.

The DIC contains two hydroxyl groups at fourth positions, which proves to be the main functional groups for the reduction of Au^{3+} ions (Supporting Information Scheme S1). During the oxidation-reduction process between DIC and Au^{3+} ion, the two hydroxy groups of DIC loses electrons to form carbonyl group (keto). The carbonyl keto groups of DIC molecules would adsorb on the surface of Au NPs, which allows to produce well dispersed DIC-Au NPs. To confirm this, we studied the FI-IR spectra of DIC and DIC-Au NPs (Supporting Information of Figure S3). The FT-IR spectrum of pure DIC exhibited characteristic absorption bands at 2900 – 3500 cm^{-1} corresponded to hydroxyl groups and to stretching vibrations of aromatic C-H and aliphatic C-H groups. The peaks at 1600 and 1557 cm^{-1} correspond to stretching and vibrations of aromatic C=C groups. The C=O group stretching and vibrations are observed at 1630 and 1652 cm^{-1}, indicating that DIC contains lactone keto group. It is noticed that the entire spectrum was drastically changed by redox reaction between Au^{3+} ion and DIC. As a result, the characteristic peak of -OH at ~3400 cm^{-1} was completely disappeared due to the oxidation of hydroxy groups, which also confirms the formation of Au NPs (Au^{3+} ion to Au^0). Furthermore, the

absorption bands at 1641 and 1491 cm^{-1} correspond to in-plane vibrations of aromatic –C=C– in DIC and the peaks at 1400 – 1700 cm^{-1} represent to the deformations of C=O and -C–H groups onto the surfaces of Au NPs, indicating that interaction of keto group (oxidized –OH group) with the surfaces of Au NPs. From FT-IR spectra, it is inferred that –OH groups of DIC molecules are responsible for the reduction of Au^{3+} ion and the stabilization of DIC-Au NPs.

The hydrodynamic diameters of the as-synthesized DIC-Au NPs were measured by DLS. The size distribution of DIC-Au NPs is shown in Figure 2a. The DLS data of DIC-Au NPs show the synthesized DIC-Au NPs are well dispersed with an average diameter of 13.84 nm, which is well agree with TEM data. To confirm the size and morphology of DIC-Au NPs, we studied the TEM image of DIC-Au NPs (Figure 3a). On the basis of the TEM image, the size distribution of DIC-Au NPs was calculated and shown in Figure 3b. The size histogram was derived by analyzing TEM micrograph of Figure 3a for at least 20 particles and the average particle size is 14±3.5 nm, indicating that the synthesized DIC-Au NPs are well dispersed with an average size of 14 nm. However, the synthesized DIC-Au NPs have different shapes because the morphology and dimension of Au NPs depend on the concentrations of the seed particles and reagent, in addition to the reactants (Au^{3+} and DIC). The above factors are found to be interdependent and can give rise to interesting combinations for various shapes.[37] Even though the synthesized DIC-Au NPs have different shapes, no obvious changes in color and in SPR peak were observed, which indicates that the DIC-Au NPs can be used as a nanosensor for colorimetric detection of Cys and Lys.

3.3. Colorimetric sensing of Cys and Lys

The sensing ability of DIC-Au NPs with various biomolecules including phenyl alanine, histidine, 4-hydroxyproline, proline, tryptophan, tyrosine, alanine, isoleucine, aspargine, aspartic acid, methonine, lysine, serine, leucine, Cys and Lys (1.0 mM) were added, and then vortexed for 1.0 min. The resultant solutions were investigated by UV-visible spectroscopy (Figure 4). In order to develop DIC-Au NPs as a probe, we studied the effect of pH and NaCl concentration on colorimetric sensing of Cys and Lys.

3.4. Effect of pH

It is well known that the analytical applications of Au NPs are mainly dependent on the stability of Au NPs. The pH of the NPs solution plays key role to act as a nanoprobe for colorimetric sensing of target analytes. In order to establish DIC-Au NPs as a sensor for detection of Cys and Lys, we studied the effect of various buffer media including ammonium acetate, phosphate-buffered saline (PBS), Tris-HCl pHs ranging from 2 to 12.0 (Supporting Information of Figures S4 – S7). It can be observed that the color and absorption spectra of DIC-Au NPs were changed for both analytes in three buffer media at pH from 2.0 to 4.0, which is due to the surface charge neutralization of DIC-Au NPs. The color and absorption spectra of DIC-Au NPs were significantly changed by the addition of Cys at three buffer media pH from 6.0 to 12.0 (Supporting Information of Figures S4-S5), the maximum color intensity and absorption ratio at A_{666}/A_{525} were observed for Cys at pH 7 using Tris-HCl as a buffer medium (Figure 5a). Similarly, the color and the absorption ratio at A_{570}/A_{525} nm were increased with increasing pH of three buffer media from 6.0 – 12.0 (Supporting Information of Figures

S6-S7), indicating that the strong interactions between the surfaces of DIC-Au NPs and Lys. To confirm the best buffer pH, we compared the absorption spectra of DIC-Au NPs by the addition of Lys at three buffer media including Tris-HCl (pH 6), PBS (pH 7.0) and ammonium acetate (pH 10) (Figure 5b). It is noticed that the maximum absorption ratio (A_{570}/A_{525}) was observed for Lys at pH 7 using PBS. At higher pH, the absorbance ratio of Lys-induced DIC-Au NPs aggregation was decreased, which might be due to opening of the lactone ring under stronger alkaline conditions. Meanwhile, the stability of DIC-Au NPs is also studied in the presence of three buffers pHs (2.0 to 12.0) without addition of Cys and Lys, since the surface charges can be influenced by the pH, which implies that the aggregation of DIC-Au NPs without Cys and Lys at low pH 2.0 to 4.0. As shown in Supporting Information of Figures S8-S9, no obvious spectral and color change were observed at pH 6.0 to 10.0, indicating that the used buffer media did not induce the aggregation of DIC-Au NPs. Based on the above results, we selected Tris-HCl pH 7 and PBS pH 7 as the best buffer pHs for colorimetric sensing of Cys and Lys using DIC-Au NPs as a probe.

3.4. Effect of NaCl on the sensitivity

It is well known that the addition of salt will screen the charge on the surface of Au NPs, resulting the high degree of Au NPs aggregation, which allows to detect target analytes with improved sensitivity. Therefore, we studied the effect of NaCl on the color and absorption spectra of DIC-Au NPs (Supporting Information of Figure S10). It can be observed that the color and absorption spectra of DIC-Au NPs did not change up to 0.4 M of NaCl, however, there was a red-shift in the absorption spectra of DIC-Au NPs after

addition of 0.5 M of NaCl, which is due to salt-induced aggregation, resulting a color change from cherry red to blue. Based on these observations, we selected 0.4 M of NaCl as an optimum concentration for colorimetric sensing of both analytes with improved sensitivity. Figure 5 demonstrates the UV-visible absorption spectra of DIC-Au NPs in the presence of Cys and Lys at 0.4 M of NaCl. It can be observed that both analytes are successfully induced the aggregation of DIC-Au NPs with high degree and maximum absorption ratios at A_{666}/A_{525} and A_{570}/A_{525} are observed for Cys and Lys, indicating that the addition of NaCl plays key role to increase ionic strength of the system, which leads to a drastic decrease in inter-particle distance of DIC-Au NPs. Therefore, both analytes can effectively binds with DIC-Au NPs, reducing negative charges on the surfaces of DIC-Au NPs, and facilitating subsequent cross-linkage of DIC-Au NPs *via* hydrogen bonding between both analytes, which results a high degree of DIC-Au NPs aggregations. As a result, the analyte-mediated aggregation of DIC-Au NPs upon the addition of salt is dependent on both analytes concentrations. The ratios of the absorption at A_{666}/A_{525} nm and A_{570}/A_{525} are employed to quantify Cys and Lys. Therefore, we chose 0.4 M of NaCl as an optimum concentration for quantification of both analytes with improved sensitivity.

3.5. Sensing mechanism

A schematic representation of the mechanism for the colorimetric sensing Cys and Lys is shown in Supporting Information Scheme 1. As described in characterization of DIC-Au NPs, the DIC molecules are successfully adsorbed onto the surfaces of Au NPs to form DIC-Au NPs. It can be observed that DIC contains easily ionized keto group to

oxide group, facilitating to acts as an electrostatic probe to interact with the opposite charged molecules. At Tris-HCl pH 7, the maximum absorption ratio was observed due to the strong electrostatic interaction between negatively charged keto groups of DIC molecules and positively charged Cys. Since pK_a value of DIC is 4.40 and pI value of Cys is 5.02. Similarly, the strong electrostatic interactions can be attributed for Lys at PBS pH 7, since Lys gets positive charge below < 11.35 (pI = 11.35), yielding a strong electrostatic interaction between DIC-Au NPs and Lys. As a result, a large DIC-Au NPs nanostructured network can be formed between DIC-Au NPs and analytes *via* electrostatic and π-π interactions, facilitating to induce the color and absorption spectra of DIC-Au NPs. The characteristic SPR peak of DIC-Au NPs at 525 nm is drastically decreased with the generation of new SPR absorption peaks at 666 nm and 570 nm for Cys and Lys, leading to a change in color from cherry red to blue for Cys and to purple for Lys, respectively.

To confirm the aggregation mechanism, we studied the DLS and TEM of DIC-Au NPs aggregation-induced by target analytes (Cys and Lys). DLS of DIC-Au NPs was measured before and after the addition of Cys and Lys (Figure 2b-c). From the particle size analysis, the aggregation of DIC-Au NPs induced by Cys and Lys was confirmed. Before addition of Cys and Lys, the average hydrodynamic diameter of DIC-Au NPs is 13.84 nm (Figure 2a), however, the average hydrodynamic diameter of DIC-Au NPs is increased to 899 nm (69-fold) and to 115 nm (8-fold) after addition of Cys and Lys (Figure 2b-c). Further, the evidence of Cys- and Lys- induced DIC-Au NPs aggregations can be seen in the TEM images (Figure 3c-d). It is noticed that the size and morphology of DIC-Au NPs were greatly changed by the addition of Cys and Lys, indicating that the

aggregation of DIC-Au NPs induced by Cys and Lys, which results to increase their size from 14 nm to 899 and to 115 nm for Cys and Lys. These results indicate that the addition of Cys and Lys to DIC-Au NPs solution triggers DIC-Au NPs aggregations as is evident from the TEM images (Figure 3b-c). The aggregation propensity of DIC-Au NPs depends on the concentrations of Cys and Lys, which facilitates to develop NPs-based analytical tool for sensing of target analytes based on the aggregation of DIC-Au NPs induced by target analytes.

3.6. Sensitivity of DIC-Au NPs for Cys and Lys

In order to use DIC-Au NPs as a probe for detection of Cys and Lys, the absorption ratios at A_{666}/A_{525} and A_{570}/A_{525} were monitored as function of Cys and Lys concentrations in the presence of 0.4 M NaCl and the corresponding colorimetric responses and absorption spectra were shown in Figure 6a-b. As it can be seen, with the increase of target analytes (Cys and Lys) concentrations, the color of DIC-Au NPs solution was changed from cherry red to blue for Cys and to purple for and Lys, which could be easily observed by naked eye (Figure 6a-b). Upon the addition of increasing concentration of Cys and Lys, the absorbance at 525 nm decreased, and new absorption peak appeared progressively at a longer wavelength range. Finally, the new absorption peak was red shifted to 666 nm (Cys) and to 570 nm (Lys), resulting the color of DIC-Au NPs was changed from cherry red to blue for Cys and to purple for Lys, respectively. As shown in Supporting Information of Figures S11-S12, the calibration graphs were constructed between the absorption ratios (A_{666}/A_{525} for Cys and A_{570}/A_{525} for Lys) and concentrations in the range of 10 to 90 μM and of 100 to 800 nM for Cys and Lys

respectively. A good linear relationship between intensity ratios (A_{666}/A_{525} and A_{570}/A_{525}) and the concentration ranging from 10 to 90 μM (R^2=0.9942) and 100 to 800 nM (R^2= 0.9961) for Cys and Lys, respectively. The limit of detection was calculated by the equation LOD=K×S_0/S, where K is a numerical factor chosen according to the confidence level desired, S_0 is the standard deviation of the blank measurements (n=3, K=3), and S is the slope of the calibration curve. The detection limits were found to be 2.2×10^{-6} and 8.7×10^{-9} M Cys, and Lys, respectively. In addition, we also compared the detection limits of the present with those of the other methods reported to detect Cys and Lys (Table 2). These results indicated that the present method shows better performance and can be comparable with the reported methods.[25,27,28,30-34]

3.7. Selectivity of DIC-Au NPs towards Cys and Lys

To examine the selectivity of DIC-Au NPs as a sensor, we studied the absorption spectra of DIC-Au NPs by the addition of Cys and Lys in the presence of other common amino acids (phenyl alanine, histidine, 4-hydroxyproline, proline, trypophan, tyrosine, alanine, isoleucine, aspargine, aspartic acid, methonine, lysine, serine and leucine, 1 mM). As shown in Supporting Information of Figure S13, the intensities of absorption spectra in the presence of Cys and Lys were strikingly higher that of other aminoacids and the SPR peak red-shifts were observed just like as the addition of Cys and Lys to DIC-Au NPs, indicating that no obvious interferences were noticed with the addition of other common aminoacids for the detection of Cys and Lys. Furthermore, the selectivity of the probe was assessed by challenging it with several other biologically and environmentally relevant inorganic species (metal ions - Na^+, K^+, Br^{2+}, Ca^{2+},Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+}, 1.0 mM, and anions - Cl^-, Br^-, I^- , F^- , SO_4^{2-} ,S^{2-} , PO_4^{3-} and

$Cr_2O_7^{2-}$, 1.0 mM) and pesticides (chlorpyrifos, quinalphos, hexaconazole , tricyclazole, acephate, metsulfuron, isoproturon, and chlopropham, 1.0 mM) and measured absorption spectra of resultant solutions. As shown in Supporting Information of Figures S14-S16, the absorbance at 525 nm showed slight changes in intensity, and new SPR peak (at 666 and 570 nm) did not appear upon the addition of only above inorganic species and pesticides, however the absorbance changes caused by Cys and Lys with inorganic species and pesticides were similar to that caused by Cys and Lys alone. These results reveal that the present probe is highly selective to Cys and Lys and even the probe could detect Cys and Lys with similar efficiency in the presence of other biomolecules, inorganic species and pesticides. Hence, none of the species (biomolecules, inorganic species and pesticides) interferes in the aggregation of DIC-Au NPs induced by Cys and Lys.

3.8. Analysis of Cys and Lys in real samples

To test the practical utility of DIC-Au NPs for colorimetric sensing of Cys and Lys, we analyzed both analytes in urine and plasma and Lys in egg white. The collected samples were spiked with Cys (40, 60 and 80 μM) and Lys (0.2, 0.5 and 0.8 μM) separately, and then analyzed by the aforesaid procedure. Satisfying recoveries were obtained in the range of 95.9 – 108.3 % for Cys and of 94.3 – 104.8 % for Lys in urine and plasma. Furthermore, the recoveries of Lys from egg white were also studied (Figure 7 and Supporting Information of Figure S17). The calibration curve for Lys was obtained by detecting the sample extracts spiked with known concentration of Lys. According to this calibration curve, the recoveries in spiked egg white vary from 108.4 % to 110.6 %

and the results are depicted in Supporting Information of Table S1. Additionally, Figure 7 shows the changes in absorption spectra and color variations of DIC-Au NPs solutions in the absence and presence of different concentrations of Lys in egg white. Along with the increase of Lys concentration, the absorption spectra and color of the ensemble DIC-Au NPs solutions become closer to the original DIC-Au NPs. The color change of the ensemble DIC-Au NPs solutions can be distinguished by naked eye (Figure 7 and Supporting Information of Figure S17). The addition of Lys concentration ranging from 0.2 to 0.8 μM to egg white led to the percentage recovery of about 110.6 % with the relative standard deviations <2.6. These results suggest that the DIC-Au NPs were successfully acted as a probe to detect Cys and Lys in real samples (urine, plasma and egg) and might become a promising method for Cys and Lys assays in other biological samples.

4. Conclusions

In conclusion, we have rationally designed and synthesized Au NPs by using DIC as a reducing and capping agent. The synthesized DIC-Au NPs act as a nanoprobe for selective and sensitive molecular recognitions event into an appreciable color change *via* the aggregation of DIC-Au NPs induced by Cys and Lys. The assay described in this work is easily readout with the naked eye or using UV-visible spectrometer. We carried out several studies on the effect of various buffer media pH in the range of 2 – 10 for effective Cys- and Lys- induced aggregation of DIC-Au NPs. The target analytes-induced DIC-Au NPs aggregations were confirmed by DLS and TEM. The calibration graphs exhibited good linearity between the absorption ratios and concentrations of target

analytes. This probe shows good selectivity for the detection of Cys and Lys over other aminoacids, inorganic species and pesticides. The present method was successfully applied to detect Cys and Lys in real samples (urine, plasma and egg white), which opens a new way for the detection of Cys and Lys in biological samples.

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Figure captions

Figure 1. UV-visible absorption spectra of Au NPs using DIC (--- mM) as a reducing and capping agent at different concentrations of auric chloride from 0.1 to 1.0 mM. Inset image of corresponding solutions.

Figure 2. DLS of (a) DIC-Au NPs and the aggregation of DIC-Au NPs induced by (b) Cys and (c) Lys.

Figure 3. TEM images of (a) DIC-Au NPs and (b) size distribution diagrams of DIC-Au NPs, and the aggregation of DIC-Au NPs induced by (b) Cys and (c) Lys.

Figure 4. UV-visible absorption spectra of DIC-Au NPs in the presence of various biomolecules (phenyl alanine, histidine, 4-hydroxyproline, proline, trypophan, tyrosine, alanine, isoleucine, aspargine, aspartic acid, methonine, lysine, serine, leucine, Cys and Lys, 1.0 mM). (b) Photographs of DIC-Au NPs solution containing different biomolecules.

Figure 5. (a) Comparison of absorption ratio (A_{666}/A_{525}) of DIC-Au NPs with Cys at different buffer media (Tris HCl pH 7, PBS pH 9 and ammonium acetate pH 10). (b) Comparison of absorption ratio (A_{570}/A_{525}) of DIC-Au NPs with Lys at different buffer media (Tris HCl pH 6, PBS pH 7 and ammonium acetate pH 10)

Figure 6. (a) UV-visible absorption spectra of DIC-Au NPs upon the addition of Cys concentration in the range of 10-100 μM at Tris-HCl pH 7 in the presence of 0.4 M NaCl. (b) UV-visible absorption spectra of DIC-Au NPs upon the addition of Lys concentration in the range of 50-1000 nM at PBS pH 7 in the presence of 0.4 M NaCl. The corresponding color changes of DIC-Au NPs with increasing concentrations of Cys and Lys, respectively.

Figure 7. Colorimetric detection of Lys in egg white using DIC-Au NPs as a probe. The samples were spiked (a) without and with (b) 0.2 μM, (c) 0.5 μM and (d) 0.8 μM of Lys at PBS pH 7.

Table 1. Effect of reagents concentrations and reaction temperature for the preparation of Au NPs using DIC as a reducing and capping agent.

Parameters		$A_{(spr)}$	$A_{(450)}$	Color	Size (nm)	SPR peak (nm)
Concentration of DIC (mM)	18.8	0.713	0.978	Colorless	0.38	522
	28.8	1.241	1.196	Purple	1.77	522
	38.8	1.489	1.145	Red	4.51	525
	48.8	1.749	1.645	Black	1.92	533
Concentration of $HAuCl_4$ (mM)	0.10	0.119	0.111	Colorless	2.00	524
	0.25	0.525	0.447	Light pink	2.89	524
	0.50	1.330	0.447	Red	4.71	525
	0.75	1.820	1.421	Dark red	4.20	534
	1.00	1.740	1.500	Dark red	2.74	536
Temperature (^{0} C)	0	1.241	1.103	Black	2.42	581
	25	1.021	0.909	Dark red	2.42	541
	50	1.722	1.569	Light pink	1.32	533
	75	1.954	2.046	Dark red	1.32	524
	100	1.635	1.450	Red	2.44	524
Stability (h)	2	1.381	0.944	Red	2.03	525
	4	1.350	1.002	Red	4.09	525
	6	1.720	1.429	Red	3.28	526
	8	1.700	1.469	Red	2.71	525
	24	2.161	1.958	Red	2.06	525

Table 2. Comparison of the present method for the detection of Cys and Lys with other reported methods.

Probe	Capping agent	Analyte	LOD (M)	Reference
Au NPs	Cetyltrimethylammonium bromide	Cys	23.9×10^{-9}	25
Au NPs	Aspartic acid	Cys	1×10^{-7}	27
Au NPs	Triazolylcoumarin	Lys	5×10^{-8}	28
Au NPs	Human serum albumin	Lys	5×10^{-8}	30
Au NPs	Cysteamine	Lys	3.5×10^{-6}	31
Au NPs	Cys-Ala-Leu-Asn-Asn	Lys	5.59×10^{-12}	32
graphene	---	Lys	5×10^{-10}	33
Carbon nanotube	-----	Lys	8.62×10^{-7}	34
Au NPs	DIC	Cys and Lys	2.2×10^{-6} and 8.7×10^{-9}	Present method