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FULL PAPER

Metallic nanoparticles enabled sensing of drug-of-abuse, an attempt to forensic application

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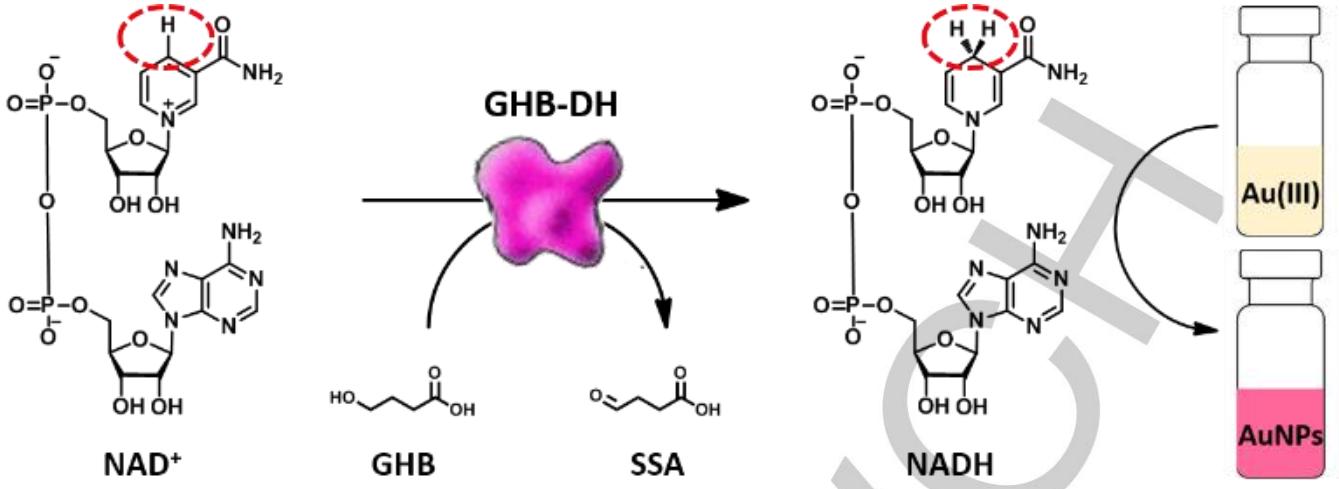
Abstract: Gamma-hydroxybutyric acid (GHB) functions as depressant on the central nerve systems and serves as pharmaceutical agent in the treatment of narcolepsy and alcohol withdraw. In recent year, GHB has been misused as recreational drug due to its ability to induce euphoric feelings. Moreover, it has gained increasing attentions as a popular drug of abuse which is frequently related to drug-facilitated sexual assaults. By now, detection methods based on chromatography exhibit extraordinary sensitivity for GHB sensing. However, such techniques require complicated sample treatment prior to analysis. Optical sensors provide an alternative approach for rapid and simple analysis of GHB samples. Unfortunately, currently reported probes are mostly based on hydrogen bonding to recognize GHB, which may raise concern such as lack of specificity. In this work, we reported a bioinspired strategy for selective sensing of GHB. Such method is based on specific enzyme recognition to allow highly selective detection of GHB with minimum interference even in complex sample matrix (e.g. simulated urine). In addition, the result can be obtained by either quantitative spectroscopy analysis as well as colormetric change observed by naked-eye demonstrating the potential application in drug screening and forensic analysis.

Introduction

Gamma-hydroxybutyric acid (GHB) is a short chain acid, which can function as depressant on the central nerve systems. GHB was used as anesthetic in 1960s[1, 2]. In addition, it has been utilized as pharmaceutical agent in the treatment of narcolepsy and alcohol withdraw[3, 4]. However, its use as therapeutic agent has been reduced mainly for the side effects such as amnesia and seizure[5]. In recent year, apart from the application as therapeutic agents, GHB has earned popularity as recreational drug due to its ability to induce euphoric feelings. It has been demonstrated that, the misuse of GHB may result in neurotoxic damage especially to maturing youths. In addition to the use as party drug, GHB has gained increasing attentions as a popular drug of abuse which is frequently related to drug-facilitated sexual assaults[6]. Considering the fact that GHB is colorless and odorless, it is barely noticeable when spiked in drinks[7]. This makes it desirable to sexual offenders. In addition, its easy availability, low cost and rapid clearance from body make it one of the most commonly used drugs of abuse in drug-facilitated sexual assaults (DFSA)[8].

Considering the potential physiological damage to human body and social impacts associated with GHB drug abuse, scientific researchers were greatly motivated to develop effective methods for sensing and detection of GHB. By right, methods based on Gas Chromatography-Mass Spectroscopy (GC/MS) or Liquid Chromatography-Mass Spectroscopy (LC/MS) have been well-established[9-11]. Although these procedures can be used for sensitive GHB detection, such methods require considerable sample treatment prior to analysis, which is laborious and may raise concerns of unwanted loss of significant information of the original sample[12]. To this end, alternative approaches (e.g. optical sensors) have been developed to facilitate sensitive detection of GHB with minimum sample manipulation[13, 14]. Although worked in principle, most of these probes recognize GHB based on hydrogen bonding which may hamper the detection specificity due to the interference from complex sample matrix. In addition, by far, most of these probes have not been reported to distinguish GHB from its lactone precursor γ-butyrolactone (GBL) which is the point of interest in forensic applications[15]. Therefore, the development of alternative method for selective GHB analysis with minimal sample preparation would be highly desirable. In human body, GHB can be selectively oxidized through reactions catalyzed by specific enzymes (e.g. GHB dehydrogenase) and yield the oxidized form succinate semialdehyde and side reduced nicotinamide adenine dinucleotide (NADH)[16].

Inspired by this naturally occurring process, we introduce a simple yet effective approach based on highly specific enzymatic reaction for selective sensing of GHB (Scheme 1). Typically, GHB molecules in either biosamples or spiked drinks can be recognized through specific enzyme-substrate interaction with GHB degydrogenase (GHB-DH) and undergo oxidation reaction generating NADH. As an ubiquitous reducing agent, the resulting NADH molecule can promote the reduction of gold(III) complex and form gold nanoparticles (AuNPs)[17-19]. Owing to the fascinating optical properties of metallic probes which are dependent on the morphological and physiological states[20-30] (e.g. size, shape, aggregation state etc.) the distinct transformation from molecular metal complex into nanoscaled particles which is assisted by NADH reduction can be easily observed through the generation of Surface Plasmon Resonance (SPR) peak of AuNPs[31-43]. In addition, the obvious colormetric changes[44-47]



Scheme 1. Scheme illustration of a bioinspired strategy for selective sensing of illicit date rape drug GHB.

accompanied by the formation of AuNPs allows simple GHB detection through naked-eye observation. [48, 49] In addition, such selective analysis of drug-of abuse can be achieved even under complex biological sample matrix, which exhibit the great potential in future forensic application and drug screening.

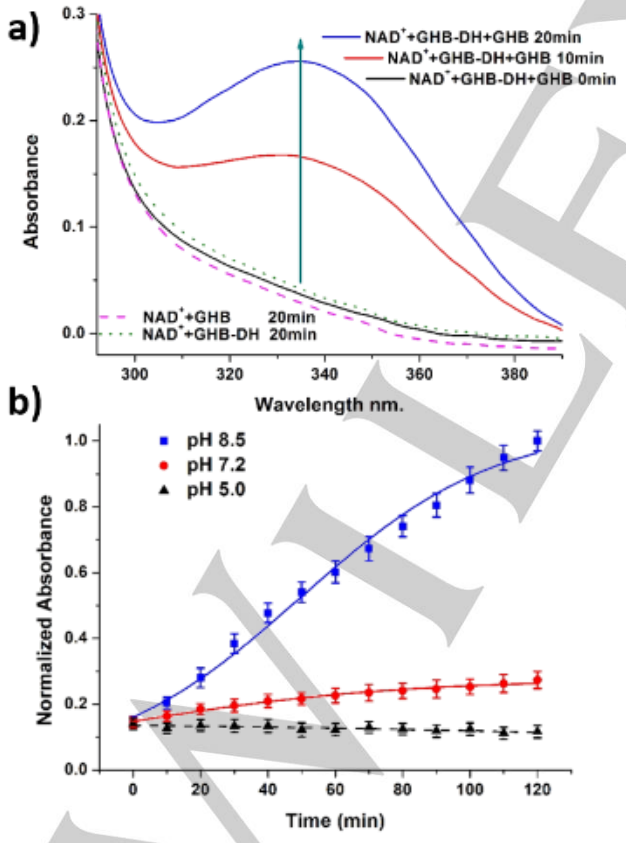


Figure 1. a) Absorbance spectra of enzyme (0.24 mg/mL) catalyzed production of NADH upon reduction of GHB (5 mM GHB in 50 mM Tris-HCl buffer pH 8.5, containing 1mM NAD^+); b) Time-dependent NADH produced by enzyme reaction under different pH conditions.

Results and Discussion

Enzymatic oxidation of GHB

To achieve selective GHB detection, we started investigating enzymatic treatment of GHB with GHB-DH. Typically, to a solution containing 1mM NAD^+ and 0.24mg/mL GHB-DH in 50mM Tris-HCl buffer (pH 8.5), GHB substrate was added to trigger the reaction. In order to achieve optimum activity of GHB-DH, the reaction mixture was kept at 37℃ based on the manufacturer's protocol. The formation of NADH through the oxidation GHB can be monitored by the absorbance peak of NADH at 334nm. As shown in Figure 1a, in the absence of GHB-DH, minimum change in absorbance spectra can be observed from the reaction mixture of GHB and NAD^+. Similarly, no obvious absorbance peak

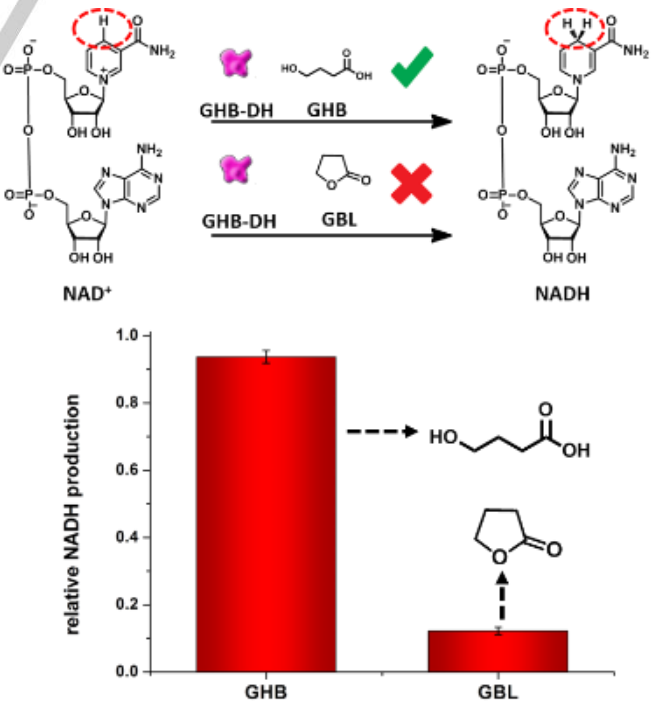


Figure 2. Selectivity of NADH formation catalyzed by GHB-DH. (5 mM GHB or GBL in 50 mM Tris-HCl buffer pH 8.5, containing 1mM NAD^+).

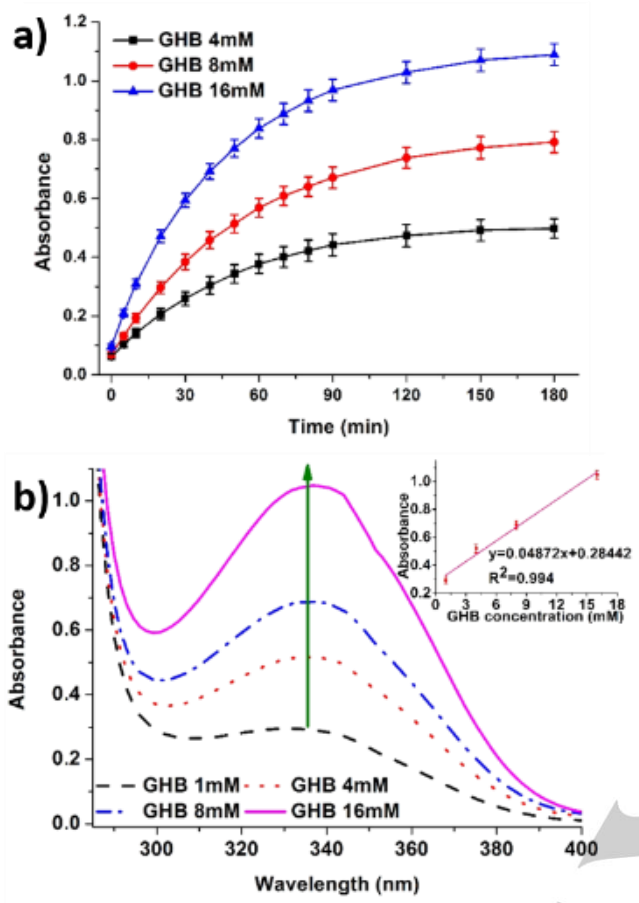


Figure 3. a) Kinetics of NADH produced through enzymatic oxidation of GHB of various concentration (50 mM Tris-HCl buffer pH 8.5, containing 1mM NAD^+); b) Absorbance spectra of NADH produced through GHB (1-16 mM) triggered enzymatic reaction under optimized conditions (75min, 50 mM Tris-HCl buffer pH 8.5) inset: linear correlation of NADH absorbance (334nm) versus GHB concentration.

appeared in the mixture of NAD^+ and GHB-DH, even with prolonged incubation, suggesting the minimal production of NADH. In comparison, obvious increase in the absorbance at 334nm was observed upon enzymatic treatment GHB with NAD^+. The absorbance of NADH increase as incubation prolonged, exhibiting time-dependent spectra change, demonstrating the enzyme promoted generation of NADH. In addition, to further facilitate the enzymatic oxidation of GHB, the enzyme activity was evaluated under different buffer conditions. In this case, GHB-DH, NAD^+ and GHB was added to various buffer solutions (acetate buffer, 100mM pH 5.0, PBS buffer 1X, pH 7.2, and Tris-HCl buffer, 50mM, pH 8.5). Kinetic studies (Figure 1b) revealed that, under acidic conditions, no obvious absorbance of NADH was observed suggesting the minimum activity of GHB-DH under such condition. At neutral condition, the presence of NADH signal can be observed at a moderate rate. However, under slightly alkaline environment, NADH can be produced rapidly in comparison to that under acidic or neutral environment. This suggests slightly alkaline condition can facilitate the GHB reduction as well as promoting NADH production. Under such enzyme favorable condition, the specificity of enzyme reaction was further investigated. While NADH was efficiently produced upon enzymatic reduction of GHB, no significant amount of NADH was detected through enzyme catalyzed reaction in the presence of GBL (Figure 2), which suggest the generated NADH is largely based on the specific interaction between GHB and GHB-DH. Such specific enzyme-substrate recognition allows us to minimize interference from GHB-derivative GBL, which is of special interest in some forensic analysis.

Optimization of GHB-DH enzyme reaction

To facilitate the future application in GHB sensing, the parameters such as reaction time, substrate concentration were optimized. Kinetic studies were performed to get the optimum reaction time of GHB with NAD^+ under the treatment of GHB dehydrogenase. As shown in Figure 3a, upon the addition of GHB, the absorbance of NADH increased gradually suggesting the generation of NADH is dependent on GHB concentration. Rapid accumulation of NADH was observed within 80mins (Figure 3a) upon addition of GHB. As reaction time prolonged, the rate of NADH production gradually decrease and finally reached a plateau. To facilitate sufficient amount of NADH for subsequent detection step as well

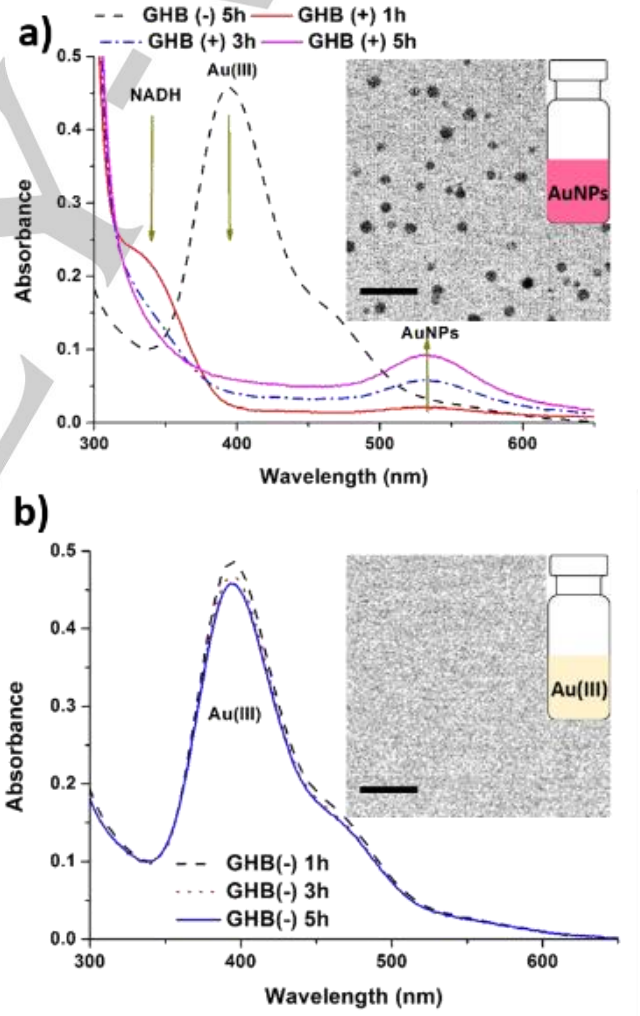


Figure 4. Absorbance spectra of growth solution containing CTAB (37mM), HAuCl4 (0.09mM) and Au seeds (1.4 x 10^{-7}mM) a) with NADH generated from enzyme catalyzed reaction (GHB 1.26 mg/mL 12.6 mM, NAD^+ 1mM, GHB-DH 0.24 mg/mL), b) without NADH generated from enzyme catalyzed reaction (NAD^+ 1mM, GHB-DH 0.24 mg/mL). Inset TEM micrograph representing the gold nanocrystal formation triggered in the presence of GHB, scale bar 50nm.

as to ensure time efficiency, 75mins reaction time was chosen as the optimized reaction period of enzymatic oxidation of GHB. Under this optimized condition, the absorbance of NADH produced in the presence of GHB exhibited a linear increase with respect to the concentration of GHB within 1 to 16mM (Figure 3b).

GHB-mediated formation of AuNPs

Moreover, to explore the possibility of selective detection of GHB through the formation of AuNPs, we exploited the catalytic growth of AuNPs by NADH directly generated from enzymatic recognition of GHB. Generally, reaction mixture containing NADH was introduced into a solution containing CTAB (37mM), $HAuCl_4$ (0.09mM) and Au seeds (1.4 x 10^{-7}mM, 10nm). The reaction was allow to proceed at 30℃. As shown in Figure 4a, without NADH, the solution exhibited a distinct absorbance peak at 392nm which is attributed to the presence of $AuCl_4^-$ species. However, upon treatment of NADH containing solution, the absorbance peak at 392nm gradually disappeared. In the meantime, the absorbance of NADH at 334nm decreased (Figure 4a) simultaneously suggesting the rapid reduction of $HAuCl_4$ (Figure S2) which is similar to previously reported studies[17]. Furthermore, as the concentration of NADH further increase, a distinct absorbance peak at ~539nm gradually appeared (Figure 4a). Upon prolonged incubation, the intensity of absorbance peak at 539nm gradually increased. Meanwhile, the colorless reaction solution gradually developed pinkish color (Figure S3), suggesting the growth of AuNPs (Figure S2), which matches well with reported studies[17]. Moreover, the growth of AuNPs in the presence of GHB induced NADH was further confirmed by TEM analysis (Figure S3a and Figure S3b). The presence of spherical particles revealed the growth of AuNPs in the presence of NADH produced from enzyme catalyzed GHB reduction. Similarly, increased hydrodynamic diameter of Au seeds also suggest the growth of AuNPs (Figure S3c). In comparison, no obvious formation of AuNPs was observed as revealed by absorbance spectra and TEM, when there is a lack of GHB (Figure 4b) or enzyme activity (Figure S4) during NADH production.

Optimization of AuNPs based GHB sensing

We have proved the concept of selective sensing illicit drug GHB through the growth of AuNPs. To better apply this strategy for the application of drug sensing in spiked drink, reaction of the sensing step (the formation of AuNPs) has been optimized. Kinetic investigations on the growth of AuNPs upon interaction with NADH were carried out. It can be observed that upon the addition of NADH, the absorbance of reaction mixture at 539 nm gradually increased (Figure S5a), suggesting the formation of AuNPs is dependent on the concentration of NADH. Within the first 2 hours, no obvious increase in the absorbance was detected. As reaction time prolonged, the absorbance at 539 nm gradually increase over 24 hours (Figure S5a). However, the slow reaction rate and limited sensitivity (relatively low absorbance) are the major concerns in future application. To address these issue, the growth of AuNPs in the presence of different concentration of catalyst (Au seeds) was evaluated. As shown in Figure S5b, higher concentration (7.0 x 10^{-7}mM, 1.4 x 10^{-6}mM) of catalyst (Au seeds) can dramatically increase the absorbance of AuNPs within a shorter period of time suggesting the great improvement in the rate of AuNPs formation which is desirable for enhanced sensitivity and detection efficiency. Therefore, the amount of catalyst (Au seeds) was optimized as 1.4 x 10^{-7}mM. Under this condition, the absorbance of AuNPs formed in the presence of GHB exhibited a linear increase with respect to the concentration of GHB within 4 to 16 mM (Figure 5a). In addition, the sensitivity was calculated to be 3.51mM which is comparable to previously reported methods[13, 14].

Visual analysis of GHB containing samples

In addition, the platform was investigated for the application of detection of GHB spiked in different types of drinks. Typically, GHB-free and GHB-spiked beverage was incubated with GHB dehydrogenase and NAD^+ for 75min. The reaction mixture was subsequently used to promote the reduction of $HAuCl_4$ and the different types of beverages including mineral water, soda as well as alcoholic drink (e.g. white wine and rice wine Figure 5b growth of AuNPs. As shown in Figure 5b, pink color of AuNPs formed in

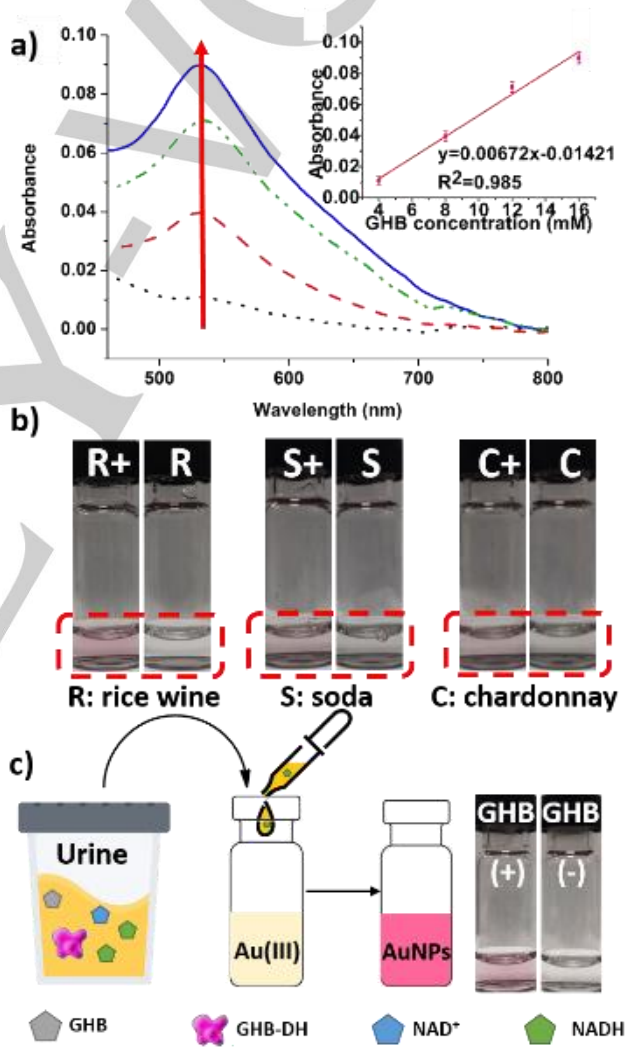


Figure 5. a) Absorbance spectra of AuNPs produced upon addition of GHB (4-16 mM) under optimized conditions (CTAB 37mM, $HAuCl_4$ 0.09mM, Au seed 1.4 x 10^{-6}mM and GHB black:4mM, red 8mM, green 12mM, blue 16mM, incubation 1h) inset: linear correlation of AuNPs absorbance (539nm) *versus* GHB concentration. b) Photograph of drinks spiked with (R+, S+, C+) or without (R, S, C) GHB (10 mM) R: rice wine, S: soda, C: chardonnay. c) Illustration of GHB analysis in simulated urine sample by simply mix and observe. (Bottom photo: simulated urine sample containing GHB exhibits obvious pinkish colormetric change compare to sample without GHB.)

the presence of GHB can be easily observed in and Figure S6) suggesting the feasibility of such platform for the application of GHB sensing in a variety type of beverages. More importantly, the application of GHB detection in biological samples was evaluated. In this case, GHB-DH and NAD^+ was added to simulated urine sample and allow for enzyme reaction. Subsequently the reaction mixture was dropped into the growth solution (CTAB 37mM, $HAuCl^4$ 0.09mM, Au seed 1.4 x 10^{-6}mM) and stirred at 30℃. As shown in Figure 5c, obvious pinkish color appeared in the simulated urine sample spiked with GHB. In comparison, sample without GHB remained clear colorless solution. Such results suggested that our bio-inspired sensing technique based on enzyme reaction is capable of analyzing the presence of GHB in complex sample matrix (e.g. beverage and biological sample). Moreover, the facile visual analysis can be achieved through simply mix and observe which can largely reduce the laborious and sophisticated sample pretreatments.

Conclusion

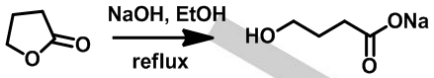
To conclude, we have developed a simple yet effective approach based on highly specific enzymatic reaction for selective sensing of GHB. Typically, the drug-of abuse can be recognized through specific enzyme-substrate interaction and generate a side product NADH. The resulting NADH can facilitate the reduction of gold(III) species and form AuNPs which can be analyzed through either absorbance spectrometer or naked-eye observation. Investigations have demonstrated the ability of such platform to detect GHB drug in a variety type of spiked drink. Moreover, such strategy can be utilized for analyzing GHB in complicated biological samples suggesting the potential of our strategy in the application of drug screening and forensic investigation.

Experimental Section

Materials: γ-butyrolactone (GBL), nicotinamide adenine dinucleotide (NAD^+) were purchased from TCI chemical (Japan). Tris(hydroxymethyl)aminomethane (Tris), Diaphorase (from Clostridium kluyveri), gold(III) chloride trihydrate ($HAuCl_4•3H_2O$), cetrimonium bromide (CTAB), gold colloid (10 nm) were acquired from Sigma-Aldrich. GHB-DH was order from prima biomed (U.S.). Simulated urine was prepared according to reported methods[50]. All theese reagents indicated above were used directly without further purification.

Instruments: NMR spectra were acquired on Bruker Avance 400 spectrometers. Mass spectra were obtained on a ThermoFinnigan LCQ Fleet MS. UV-VIS spectra were recorded on a SHIMAZU UV-1800 UV spectrophotometer. Fluorescence spectra were recorded on a Shimazu RF-5301PC spectrofluorophotometer.

Synthesis of GHB sodium salt: To a solution of GBL (23.2 mmol, 2 g) in EtOH (6 ml) a solution of NaOH (23.2 mmol, 928 mg) in water (4 ml) was added. The mixture was refluxed for 5 hours and the solvents were evaporated in vacuo. The product was white solid, 1.8 g (62%). 1H NMR (400 MHz, MeOD) δ 3.60 (t, *J* = 6.5 Hz, 2H), 2.26 (t, *J* = 7.4 Hz, 2H), 1.93–1.74 (m, 2H). M/Z=103.20 (negative mode)



Scheme 2. Synthetic scheme of GHB (sodium salt)

Enzymatic reduction of GHB: Enzymatic reduction of GHB sodium salt was monitored at 37℃ in 1mL of 1mM NAD^+ in 50mM Tris-HCl buffer (pH 8.5) containing 0.24mg/mL GHB-DH. The reaction was triggered by the addition of GHB sodium salt to 20mM .The reaction process was monitored in a cuvette of 1cm light path by the absorbance of NADH centered at 334nm.

GHB-mediated formation of AuNPs: The GHB-mediated formation of AuNPs was carried out in 2 steps. Generally, a solution of Tris-HCl buffer 50mM (pH 8.5) containing NAD^+ (1mM), GHB-DH (0.24mg/mL) and different concentration of GHB was allow to react at 37℃. Then 100uL aliquot of the resulting mixture was added to a growth solution (120uL) containing CTAB (37mM), $HAuCl_4$ (0.09mM) and AuNPs (1.4 x 10^{-7}mM, 10nm). The reaction was performed at 30℃, pH=4.0. The absorbance spectra of the solution were recorded at different time duration.

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Keywords: colorimetric detection • drug sensing • Au NPs

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