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Melamine Functionalized Silver Nanoparticles as the Probe for Electrochemical Sensing of Clenbuterol

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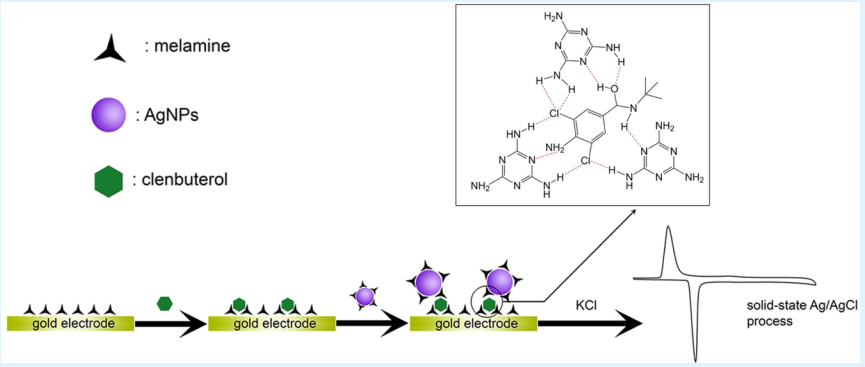

ABSTRACT: Clenbuterol, a member of β-agonist family, has now been a serious threat to human health due to its illegal usage in the livestock feeding. Herein, we describe the application of melamine functionalized silver nanoparticles (M-AgNPs) as the electrochemical probe for simple, fast, highly sensitive and selective detection of clenbuterol. Generally, AgNPs are prepared and functionalized by melamine. After interacting with melamine modified gold electrode in the presence of clenbuterol, M-AgNPs can be immobilized on the surface of the electrode via the hydrogen-bonding interactions between clenbuterol and melamine. This sandwich structure permits sensitive and selective detection of clenbuterol. Since M-AgNPs can provide a couple of well-defined sharp silver stripping peaks, which stands for a highly characteristic solid-state Ag/AgCl reaction, a rather low detection limit of 10 pM can be achieved. The detection range is from 10 pM to 100 nM, which is quite wide. This developed biosensor can potentially be used for clenbuterol detection in biological fluids in the presence of various interferences.

KEYWORDS: *silver nanoparticles, clenbuterol, biosensor, melamine, self-assembly*

INTRODUCTION

Clenbuterol, a sympathomimetic amine that belongs to β-agonist family, is commonly used as a therapeutic drug for pulmonary disease.[1] It also has the ability to increase the growth rate and produce more muscle when fed to animals.[2] However, the accumulation of β-agonists in animals for human consumption is a serious threat to human health, which may lead to acute poisoning with the symptoms of headache, cardiac palpitation, muscular disorders, nausea, fever, chills, and so on.[3] Due to these side-effects, the abuse of clenbuterol is banned in many countries for human or even animal use. However, there is still illegal usage in the livestock feeding for economic motivations. To monitor illegal applications of clenbuterol (e.g., additive in pig feed), the development of highly sensitive and selective biosensors for clenbuterol is of great importance and urgent requirement.

At present, a number of analytical methods have been used including colorimetric method,[4,5] surface-enhanced Raman scattering assay,[6] electrochemical immunoassay,[7,8] liquid chromatography sensor,[9] enzyme-linked immunosorbent assay, and surface plasmon resonance immunosensor.[10] However, some techniques lack sufficient sensitivity,[11] some may inherently rely on expensive instruments,[12,13] and some are involved with complicated and time-consuming processes such as purification, preconcentration and traditional isolation procedures. Therefore, developing novel methods for rapid, cheap, convenient, and sensitive detection of clenbuterol is still much desirable.

In recent years, nanomaterials have attracted increasing attention in material science,[14] analytical science,[15] and biomedical applications.[16] The noble metallic nanoparticles, including gold nanoparticles (AuNPs),[17] silver nanoparticles (AgNPs),[18,19] and platinum nanoparticles (PtNPs),[20] have become the most popular nanomaterials due to their unique nanoscale properties. For biosensing purposes, these nanoparticles have been functionalized via the conjugation of nucleic

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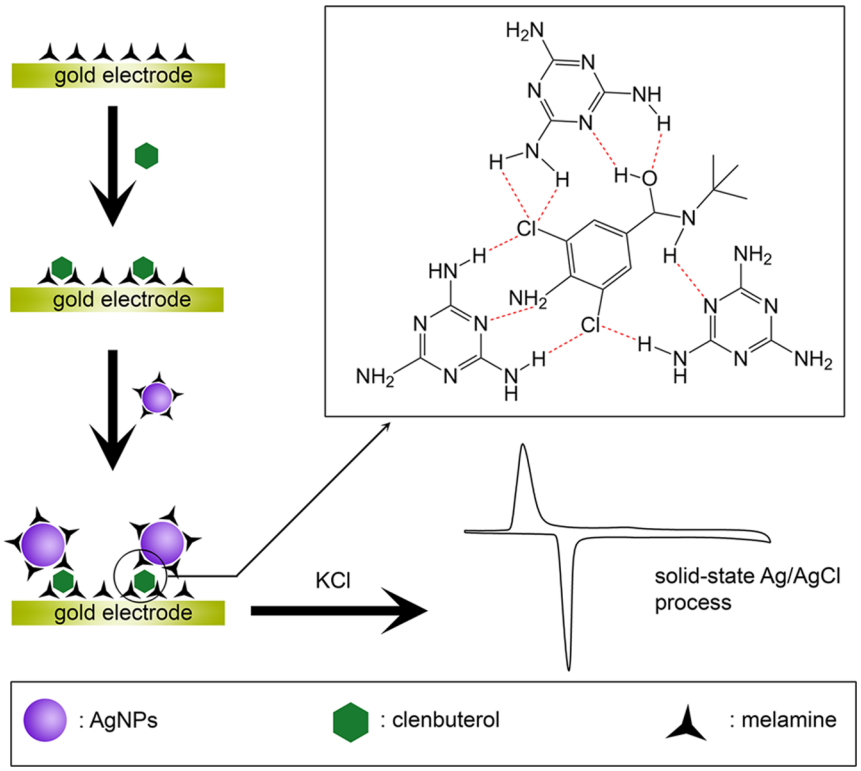
Scheme 1. Schematic Representation of the Melamine Functionalized Silver Nanoparticles-Based Electrochemical Biosensor



acids, proteins, and many other specific compounds to achieve selective recognition and signal output.[21−26] To date, AgNPs are popularly used due to the properties of high extinction coefficient, large interfacial surface, high thermal and electrical conductivity, etc. Mehrgardi and Ahangar have employed AgNPs in the amplified electrochemical detection of single base mismatches.[27] Recently, Mirkin's group has proposed a general approach to prepare nanoparticles bearing a high-density shell of nucleic acids.[28] With the progress of surface modification and novel ligand designs, there are broad prospects for further development of functionalized nanoparticles-based chemical and biological sensing.

In our previous work,[29] we have developed a PtNPs based electrochemical method for clenbuterol analysis. Clenbuterol has the ability to reduce chloroplatinic acid into PtNPs. The formed nanoparticles can then catalyze the reduction of H_2O_2 and generate electrochemical signal, which is utilized to determine clenbuterol concentration. In order to improve the sensitivity and simplify the detection process, we have herein proposed another electrochemical sensing strategy based on melamine functionalized silver nanoparticles (M-AgNPs). This method not only possesses a wide detection range from 10 pM to 100 nM and a low detection limit (10 pM) but also has antijamming properties. Therefore, this method has potential use in food safety monitoring and the agonist control.

EXPERIMENTAL SECTION

Materials and Chemicals. Silver nitrate, trisodium citrate, sodium borohydride ($NaBH_4$), melamine, and clenbuterol were purchased from Sigma-Aldrich. Other regents were of analytical grade and were used as received. The solutions used were prepared with double-distilled water, purified with a Milli-Q purification system (Branstead, U.S.A.).

Preparation of M-AgNPs. Bare AgNPs were first synthesized by the borohydride reduction of $AgNO_3$ according to previously reported method.[30] In a typical process, $AgNO_3$ and trisodium citrate solution with the concentration of 0.25 mM was prepared. 100 mL of the above solution was then mixed with 3 mL $NaBH_4$ solution (10 mM) under stirring for 30 min. AgNPs with the color of bright yellow were thus formed and left to sit in dark for 24 h. Afterward, AgNPs were purified by centrifugation at 12000g for 30 min. The size of AgNPs (5 nm) was verified through transmission electron microscopy (TEM) analysis (FEI Tecnai G20, U.S.A.). The prepared AgNPs were then incubated in 0.1 mM melamine for 24 h to achieve the conjugation.[31] Finally, excess melamine was removed by centrifuging at 12000g for 30 min.

Preparation of Melamine Modified Gold Electrode. Prior to sensor fabrication, the gold disk electrode with the diameter of 2 mm was treated by piranha solution (98% H_2SO_4/30% H_2O_2 = 3:1) for 5 min (*Caution: Piranha solution reacts violently with organic solvents and should be handled with great care!*). Afterward, it was polished carefully on P5000 sand paper and then alumina slurry (1, 0.3, and 0.05 μm), respectively. Next, it was cleaned by ultrasonicating in ethanol and double-distilled water, each for 5 min. Then, the electrode was immersed in 50% HNO_3 for 30 min, followed by electrochemically cleaning with 0.5 M H_2SO_4 to remove any remaining impurities. After being dried with nitrogen, it was incubated in 1 mM melamine for 6 h at room temperature to achieve the immobilization.

Determination of Clenbuterol Concentration. Clenbuterol solutions with different concentrations were prepared (pH 7.0). Then, melamine modified electrodes were immersed in the above solutions separately for 30 min, followed by being rinsed with double-distilled water. Afterward, the electrodes were incubated in M-AgNPs for another 30 min. The nanoparticles were introduced onto the electrode surface and the clenbuterol-induced-sandwich structure was formed via the hydrogen-bonding between melamine and clenbuterol.

Electrochemical Measurements. Cyclic voltammetry (CV), linear sweep voltammetry (LSV) experiments were carried out on a CHI 660D electrochemical workstation (CH instruments, Shanghai, China). A three electrode electrochemical system was applied, which contains the platinum auxiliary electrode, the Ag/AgCl reference electrode and the working electrode (the modified gold electrode). CV and LSV were all performed at room temperature, and the scan rate

was 100 mV/s in 0.1 M KCl solution, which was employed as the electrolyte.

RESULTS AND DISCUSSION

Sensing Principle. The principle of this method has been shown in Scheme 1. Generally, AgNPs are synthesized and functionalized by melamine (M-AgNPs). The gold electrode is also modified with melamine through multiple strong-binding sites including amino groups of melamine.[32] The amount of melamine is excessive so as to fill the reaction-active sites on the electrode; thus, there are no spare sites for the direct immobilization of M-AgNPs on the gold surface. Moreover, due to the repulsion between amino groups of M-AgNPs and melamine, M-AgNPs cannot approach the electrode surface. Only via the hydrogen-bonding interactions between clenbuterol and melamine,[33,34] can M-AgNPs be introduced on the electrode surface, which are then employed as the electroactive labels and can be detected by a highly characteristic solid-state Ag/AgCl reaction.[35] On the contrary, in the absence of clenbuterol, no such signals can be obtained. Compared with other methods for clenbuterol determination, the proposed method does not need any expensive instruments or complicated and time-consuming processes. It is of much simplicity and has excellent analytical performance.

Cyclic Voltammetry Measurement. To verify this proposal, cyclic voltammetry measurements have been carried out to characterize the electrochemistry of the binding M-AgNPs, which is mediated by clenbuterol. As shown in Figure 1, no current peak can be observed in the cyclic voltammograms of melamine modified gold electrode. After direct incubation of M-AgNPs, there is still no peak emerges. The couple of well-defined sharp silver stripping peaks can be obtained at the presence of clenbuterol, which is due to the successful formation of hydrogen-bonding based sandwich structure.

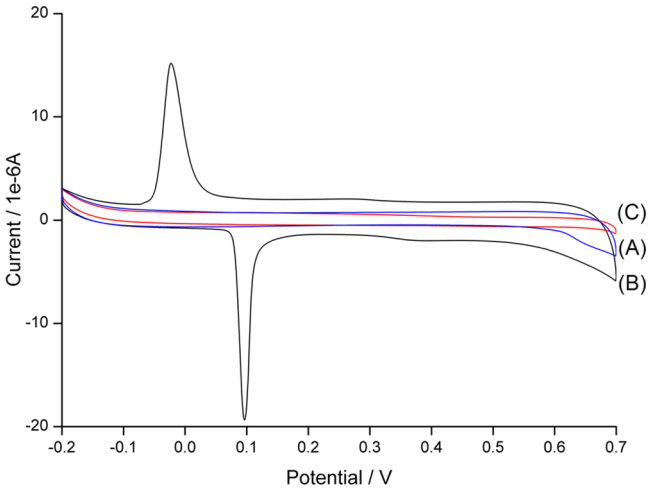


Figure 1. Cyclic voltammetry of the electrochemical biosensor for the detection of (A) 0 and (B) 1 nM clenbuterol. (C) Cyclic voltammetry of melamine modified gold electrode. The electrolyte is 0.1 M KCl; the scan rate is 100 mV/s.

Determination of Clenbuterol by Linear Sweep Voltammetry. We have then carried out linear sweep voltammetry (LSV)[36,37] to quantitatively determine clenbuterol concentration using the anodic peak, which stands for the oxidation of M-AgNPs. Optimized incubation time of melamine and M-AgNPs have also been identified by LSV technque (Figure 2). Data shown in Figure 3 reveals that

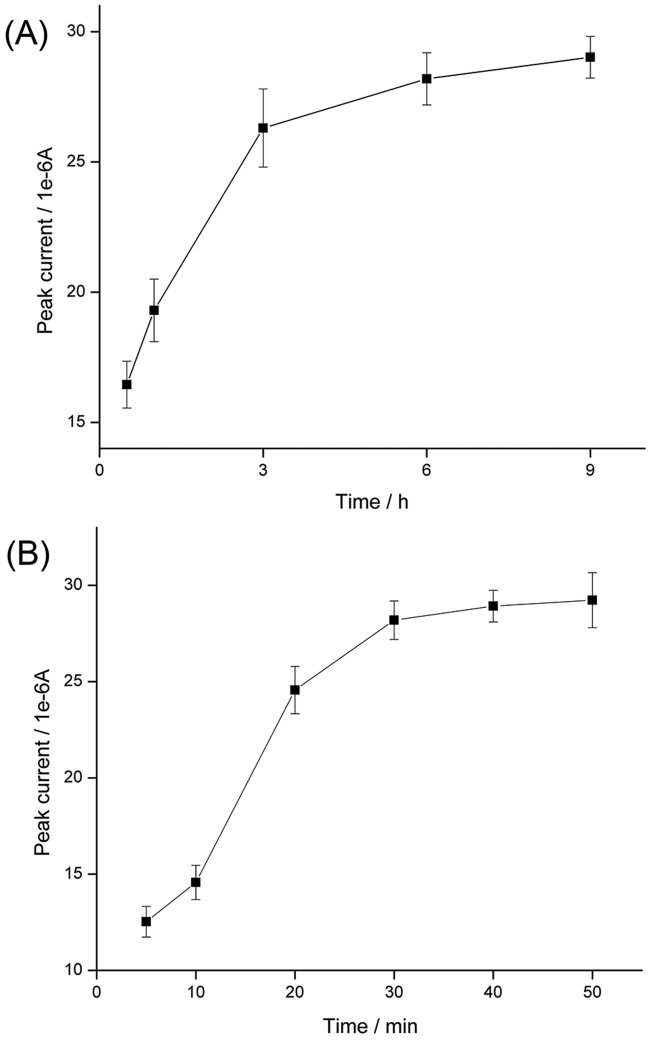


Figure 2. Relationship between peak current of linear sweep voltammetry and the incubation durations of the electrode in (A) melamine solution, (B) M-AgNPs solution at the presence of 100 nM clenbuterol. Error bars represent standard deviations of measurements (n = 3).

increase of clenbuterol level enlarges the electrochemical signal. Also, the inset in Figure 3 indicates that there is a good linear relationship between the peak current (0.1 V) and the logarithm of clenbuterol concentration. The fitting equation is $y = 73.51 + 6.67\,x$ (n = 3, R^2 = 0.99), where y is peak current and x is the logarithm of clenbuterol concentration. The detection limit is 10 pM (signal-to-noise ratio of 3). A comparison of analytical performance between the proposed method and some previously published methods for clenbuterol assay is summarized in Table 1. The detection range of this method covers 5 orders of magnitude, which is quite wide compared with the listed methods. Moreover, to the best of our knowledge, the detection limit of this work is among of the lowest ones that have been reported so far.

Interference Study. The selectivity of this clenbuterol biosensor is then checked by introducing various interferents, including urea, glucose, NaCl, trypsin, and glycine at a relatively high concentration (1 μM). The current values (0.1 V) of the

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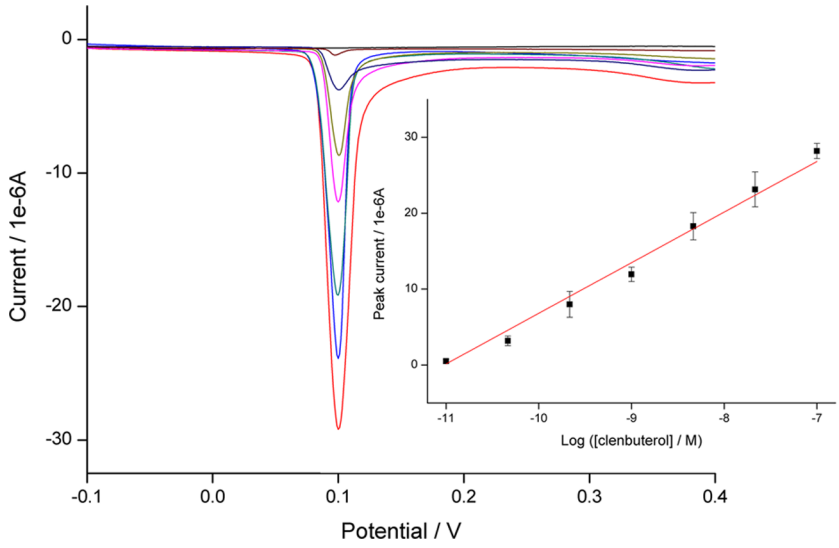

Figure 3. Linear sweep voltammetry of the electrochemical biosensor for the analysis of 0, 0.01, 0.0464, 0.215, 1, 4.64, 21.5, 100 nM clenbuterol (from top to bottom). Inset is the calibration curve of peak current value versus the logarithm of clenbuterol concentration. Error bars represent standard deviations of measurements (n = 3).

Table 1. Comparison of Different Methods for the Detection of Clenbuterol

techniques	detection strategies	detection range (nM)	LOD (nM)	ref
colorimetry	$HAuCl_4$ reduction	2340−600000	722	4
colorimetry	gold nanoparticles	0.28−280; 280−1400	0.028	5
differential pulse voltammetry	graphene oxide−Ag composites	0.036−36	0.025	7
differential pulse voltammetry	platinum nanoparticles	100−800	43.96	29
differential pulse adsorptive stripping voltammetry	multiwalled carbon nanotube composite	19.9−47600	1.38	38
linear sweep voltammetry	melamine functionalized AgNPs	0.01−100	0.01	this work

LSV have been compared in Figure 4. The results demonstrate that none of these interferents can cause the immobilization of M-AgNPs and the corresponding sharp silver stripping peak.

Detection of Clenbuterol in Real Samples. We have also employed spiked pig urine as samples for clenbuterol analysis in order to test the applicability of this method in real samples. The pig urine was obtained from local markets, which is first diluted with distilled water (1:20). Afterward, different amount of clenbuterol is added, which is then detected by this proposed method. The concentration of clenbuterol is calculated according to the calibration curve obtained above. Since the solid-state voltammogram has a much sharp and intense peak, target peak can be easily distinguished from any interference, which would be advantageous in real sample analysis. The recoveries and the relative errors of the proposed method are listed in Table 2. The results are satisfactory and demonstrate that the proposed biosensor is effective for quantitative analysis in real samples.

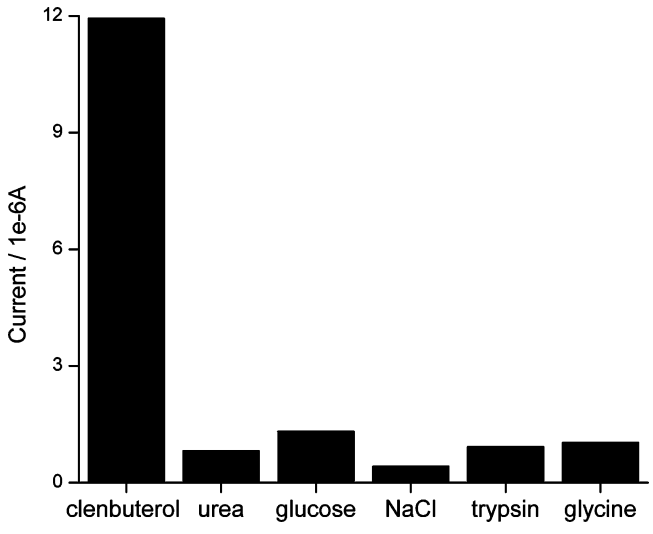

Figure 4. Selectivity of clenbuterol measurement (1 nM) over other interferents (1 μM).

Table 2. Measurements of Clenbuterol Spiked in Pig Urine Samples (n = 3)

spiked (nM)	found (nM)	recovery (%)	relative error (%)
0.1	0.112	112.0	3.9
0.5	0.486	97.2	8.6
1.0	0.978	97.8	5.3

CONCLUSIONS

In summary, we have developed a novel electrochemical method for clenbuterol detection based on M-AgNPs. This proposed biosensor not only has a wide linear range, but also has high sensitivity with fast response. It is also cost-effective and can be easily operated. Moreover, it is antijamming and can be applied directly in pig urine samples with a satisfactory recovery. The experiment results ensure the biosensor great potential application in the food safety monitoring and the agonist control.

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Notes

The authors declare no competing financial interest.

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