



Gold nanoparticles-based catalysis for detection of S-nitrosothiols in blood serum

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ABSTRACT

Accumulating evidence suggests that S-nitrosothiols (RSNOs) play key roles in human health and disease. To clarify their physiological functions and roles in diseases, it is necessary to promote some new techniques for quantifying RSNOs in blood and other biological fluids. Here, a new method using gold nanoparticle catalysts has been introduced for quantitative evaluation of RSNOs in blood serum. The assay involves degrading RSNOs using gold nanoparticles and detecting nitric oxide (NO) released with NO-selective electrodes. The approach displays very high sensitivity for RSNOs with a low detection limit in the picomolar concentration range ($5.08 \times 10^{-11} \text{ mol L}^{-1}$, $S/N = 3$) and is free from interference of some endogenous substances such as NO_2^- and NO_3^- co-existing in blood serum. A linear function of concentration in the range of $(5.0\text{--}1000.0) \times 10^{-9} \text{ mol L}^{-1}$ has been observed with a correlation coefficient of 0.9976. The level of RSNOs in blood serum was successfully determined using the described method above. In addition, a dose-dependent effect of gold nanoparticles on the sensitivity for RSNOs detection is revealed, and thereby the approach is potentially useful to evaluate RSNOs levels in various biological fluids via varying gold nanoparticles concentration.

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1. Introduction

S-nitrosothiols (RSNOs) are generated by the nitric oxide (NO^*)-dependent S-nitrosation of thiol-containing proteins and peptides, capable of initiating many biological functions including platelet deactivation [1,2], immunosuppression, neurotransmission, and host defense [3]. The RSNOs in biological fluid act as potential transport and delivery system for NO in the human circulation. However, a general consensus on reference values for physiological RSNOs concentration in biological fluids is still missing. Consequently, physiological functions of RSNOs in biological fluids have not yet been clarified. Therefore accurate detection and quantification of RSNOs are vital to understand their physiological functions in health and disease.

In the past decade, RSNOs levels have been measured by spectrophotometry [4–8], chemiluminescence [9–15], fluorimetry [16–18], high-performance liquid chromatography [19], capillary zone electrophoresis [20], mass spectrometry [21,22], electron paramagnetic resonance (EPR) spectroscopy [23] and electrochemical methods [24]. Each of these techniques has certain benefits but suffer from low sensitivity, poor specificity or expensive experimental apparatus when it comes to monitoring RSNOs con-

centration in biological fluids. For measurements of RSNOs in biological fluids, the ideal technique should selectively detect them at sub-nanomolar level with the shortest possible response time in addition to other desired properties such as miniaturization, stability, easy calibration, and ease of use.

Among these methods, electrochemical techniques are promising because of their inherent sensitivity, speed, simplicity, and low cost. Moreover, electrochemical sensors are especially suitable for the development of analytical approaches for RSNOs detection in real time. In electrochemical assay, the concentration of RSNOs is usually determined after decomposition of RSNOs by catalyst and subsequent determination of the NO released with NO-selective electrodes [25]. Thus, the catalyst selected is crucial to quantify RSNOs level accurately. Various catalysts for RSNOs decomposition have been developed for determination of physiological RSNOs level. The most common procedure for cleavage of the S–NO bond is previously achieved using copper ion as decomposition agent. However, it has been reported that Cu^{2+} catalyzes RSNO release NO with low efficiency [26], which may lead to low sensitivity in some biological systems. In comparison, Cu^+ is highly efficient in converting RSNO to NO, so it is often used as decomposition agent in the assay. However, Cu^+ is easy to be oxidized into Cu^{2+} in the presence of oxygen, thus the detection system should be deoxygenated, which makes the pre-analytical procedure complex. In addition, it is somewhat difficult to provide an accurate determination of RSNOs level due to an unknown percentage of NO

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contributed by copper(II)-ion catalyst. Recently, Meyerhoff et al. developed a series of electrochemical/amperometric RSNO sensors based on various catalysts (e.g., Cu (I/II)-complex, glutathione peroxidase (GPx), organoselenium (RSe) and organotellurium (RTe) compounds) [27–29]. Although it has been demonstrated that these sensors enable direct detection of RSNOs in complex physiological media, they lack sufficient sensitivity and detection limit to measure RSNOs at sub-nanomolar level. Hereby, efficient and specific catalyst for RSNOs decomposition is still of great importance to determine nanomolar quantities of RSNOs in biological fluids.

Nanoparticles often have superior catalytic properties because of their nanometer size, which gives them increased surface-to-volume ratios and chemical potentials. Gold nanoparticles, as a class of nanomaterials, behave as unique catalyst in some reaction when they are arranged on appropriate supports [30]. Most recently, our research group has demonstrated that gold nanoparticles can induce NO releasing by catalyzing RSNOs decomposition [31]. Moreover, the catalysis of gold nanoparticles for decomposing RSNOs has a concentration-dependent effect. Therefore, a method for quantitative RSNOs detection can be established using gold nanoparticles catalyst. The sensitivity may be enhanced by changing gold nanoparticles concentration.

In the present study, we develop a novel electrochemical technique for determining RSNOs level based on gold nanoparticles. In the assay, RSNOs are homolytically cleaved by gold nanoparticles and subsequently the NO released is measured with NO-selective electrodes. This approach provides a sensitive and specific assay for quantification of RSNOs due to the unique property of gold nanoparticles. Further, it has been used to measure the RSNOs level within blood serum. Our observations also imply that it is a potentially useful method to determine RSNOs concentration simply and rapidly in other biological fluids.

2. Experimental

2.1. Chemicals

S-Nitroso-N-acetyl-D,L-penicillamine (SNAP, purity > 98%,) was received from World Precision Instruments (WPI; Sarasota, USA). Reduced glutathione (GSH) was purchased from Sigma-Aldrich (St. Louis, USA). Hydrogen tetrachloroaurate (III) trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$), sodium nitrate, sodium nitrite and all the other chemicals were of analytical grade and were from Beijing Chemical Reagent (Beijing, China). All H_2O was 18 M Ω , distilled through a Barnstead Nanopure water purification system.

2.2. Synthesis of gold nanoparticles

Gold nanoparticles were prepared by the citrate reduction of HAuCl_4 according to the reference [32]. The typical reaction is as follows: a 100 mL aqueous solution of HAuCl_4 (1 mM) was brought to a reflux with vigorous stirring, and then 10 mL of a 38.8 mM trisodium citrate solution was added quickly. After the solution color changed to deep red, the solution was refluxed for an additional 15 min, then allowed to cool to room temperature, and subsequently filtered through 0.45 μm membrane (Millipore). The concentration is about 0.91 mM assuming that Au^{3+} was completely reduced to gold nanoparticles. In order to obtain more concentrated gold nanoparticles solution, it can be condensed under reduced pressure to remove some water.

2.3. Characterizations of gold nanoparticles solution

Transmission electron microscopy (TEM) performed with a JEM-1011 transmission electron microscope was used to determine the

size and monodispersity of the gold nanoparticles solution. A typical sample was prepared by dropping 10 μL of dilute aqueous solution of gold nanoparticles onto a copper grid coated with a carbon film, followed by wicking the solution away. The grid was subsequently dried under vacuum and imaged. The UV–vis absorption spectra were recorded on a Hitachi U-3020 spectrophotometer. Specimens were diluted with nanopure water.

2.4. Electrochemical measurements

Electrochemical measurements were performed with the Apollo 4000 instrument (WPI Europe) connected to NO selective carbon fibre electrodes (ISO NOPF, 200 μm , WPI Europe). The electrode tip was inserted into a stirring gold nanoparticles solution (10 mL), allowing the background current to stabilize. SNAP or sample was then added in solution to generate NO. The rapid decline in the NO signals generated after the addition of each concentration of SNAP precluded the use of a cumulative protocol. Consequently, the maximum change in current (ΔpA) produced by each new addition was recorded and used to assess the generation of nitric oxide. All the experiments are carried out at room temperature.

2.5. Standard stock SNAP solution

The pH of 500 mL of nanopure water was adjusted to 9.0 and 5 mg EDTA was next dissolved in the solution. Then, 11.3 mg SNAP was weighed and added to the solution. The solution was stored in the dark and refrigerated at 4 °C, as this solution is sensitive to light and slowly decomposes at room temperature. Fresh solutions were prepared for every experiment.

2.6. Preparation of blood serum

The mouse blood was first collected in a sterilized disposable syringe and then transferred to a sterilized conical. After 10–15 min (blood clotting) at 4 °C in the dark, the blood underwent centrifugation at $8000 \times g$ and 4 °C for 5 min. The supernatant was collected and stored at 4 °C in the dark. All the samples were freshly analyzed within 2 h.

2.7. Electrochemical determination of S-nitrosothiols

In this method, RSNOs were decomposed in the presence of gold nanoparticles and the NO released was measured using the NO-selective electrode. A calibration curve was constructed by adding different volumes of the standard SNAP to the gold nanoparticles solution. The final concentrations of SNAP were 0, 5, 20, 41, 51, 102, 203, 407, 509, 1017 nM. These standards were analyzed using electrochemical method described above, in order to generate a calibration curve. Changes of the current were plotted against SNAP concentrations added to the gold nanoparticles solution.

In order to demonstrate that the assay was specific for NO released from RSNOs, a variety of controls were performed. The biological sample in the reaction mixture was substituted with an aqueous solution of: (1) GSH, (2) nitrite, (3) nitrate and (4) SNAP. The precision of the assay was analyzed through current responses with successive additions of SNAP (10 and 20 nM final concentration, respectively) to gold nanoparticles solution.

3. Results and discussion

3.1. Characterizations of gold nanoparticles by TEM and UV–vis

The size of gold nanoparticles can be determined by TEM. Fig. 1A shows a typical TEM image of the samples. The gold nanoparticles

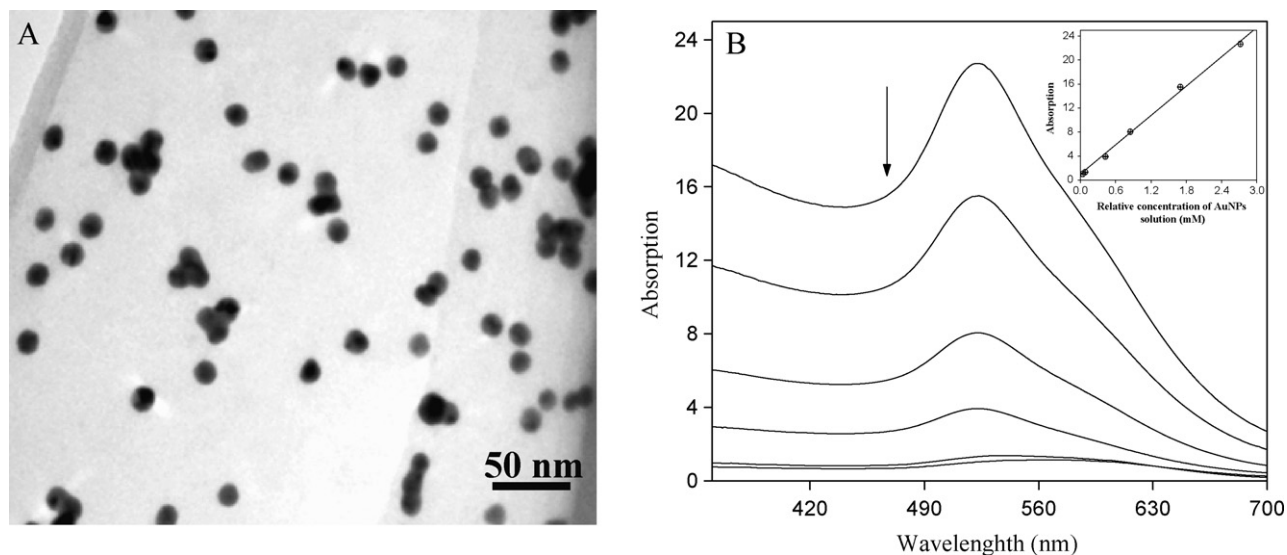


Fig. 1. (A) TEM micrograph of gold nanoparticles; (B) UV-vis spectrum of gold nanoparticles solution. The inset is correlation between intensity of plasmon peak (523 nm) and concentrations of gold nanoparticles solution.

are good monodisperse in solution. The image reveals that the prepared particles have, in general, a spherical shape with average size of 13 ± 2 nm.

The absorption spectra of gold nanoparticles in solution were observed using a UV-vis scanning spectrophotometer. The corresponding spectra are shown in Fig. 1B. In Fig. 1B, the spectra show a continuous rising background towards higher energies which is due to Mie scattering from the nanoparticles solution [33]. Superimposed on the background, between 500 and 550 nm, is a peak corresponding to a surface plasmon polariton generated within the gold nanoparticles [34]. The inner picture of Fig. 1B shows that the integrated intensity of this absorption increases linearly with increasing the concentration of gold nanoparticles. The position for the maxima (523 nm) remains approximately fixed for all nanoparticles mixtures. The lack of concentration dependence indicates that there is no significant aggregation of particles within the solution.

3.2. Effect of gold nanoparticles concentration on sensitivity

According to our previous work [31], gold nanoparticles catalyze RSNOs to generate NO in dose-dependent manner. Hence, it is necessary to investigate the effect of gold nanoparticles concentration on the sensitivity of detection RSNOs. In the process, 20 nM SNAP was added into 0.085, 0.43, 0.85, 1.7, 2.72, and 4.23 mM gold nanoparticles solutions respectively. The NO was released immediately as soon as SNAP was added to the gold nanoparticles solution, which is due to the catalysis of gold nanoparticles to SNAP decomposing. Especially, in 4.23 mM gold nanoparticles solution, the amount of NO release is out of the capacity of the instrument (Fig. S1). The sensitivity was evaluated according to the current responds. The results are shown in Fig. 2. It can be seen that the level of NO release increases with the concentration of gold nanoparticles enhanced. Namely, the sensitivity of this method depends on the concentration of gold nanoparticles. It suggests the RSNOs in different biological fluids can be detected with NO electrodes by changing concentrations of gold nanoparticles.

3.3. Selectivity of gold nanoparticles for S-nitrosothiols

Interference-free measurement of RSNOs in biological fluids is a great analytical challenge and requires us to deal with it critically.

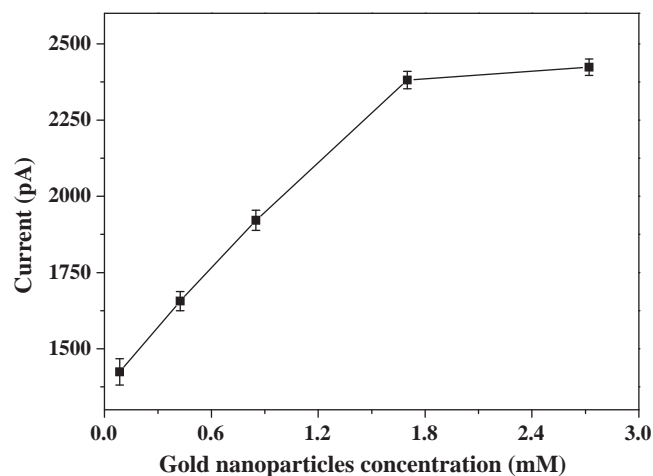


Fig. 2. Effect of gold nanoparticles concentration on the amount of NO released from SNAP.

The main problem encountered is mainly due to the low concentration of RSNOs in biological fluids. Consequently, the NO_x (e.g., nitrite and nitrate) and other compounds occurring in biological fluids and in buffers and chemicals, act as contaminants and make the detection of RSNOs artifact-prone. Therefore, it is very important for accurate quantification of RSNOs to evaluate the selectivity of proposed method.

In the assay, RSNOs in biological sample are not detected in their intact form, but are measured after conversion their S-nitrosocysteiny groups to NO. Thus, the analysis of RSNOs basically relates with the detection of NO, and all methodological problems associated with the NO analysis also apply to RSNOs. Most significantly, however, is the contribution to NO by NO unrelated to RSNOs, e.g. endogenous NO₂⁻ and NO₂⁻ from exogenous sources such as chemicals and laboratory materials, during sample treatment. Additionally, the chemical lability of RSNOs in the presence of thiols further complicates the problem. Thus, elimination of thiol reducing agents (RSHs; e.g., glutathione and cysteine) is stringent for accuracy of the method. Previously, RSHs were often removed by using N-ethylmaleimide (NEM). According to our previous research, removal of RSHs can be accomplished by formation Au-thiolate bond between thiol-group and gold nanoparticles. In

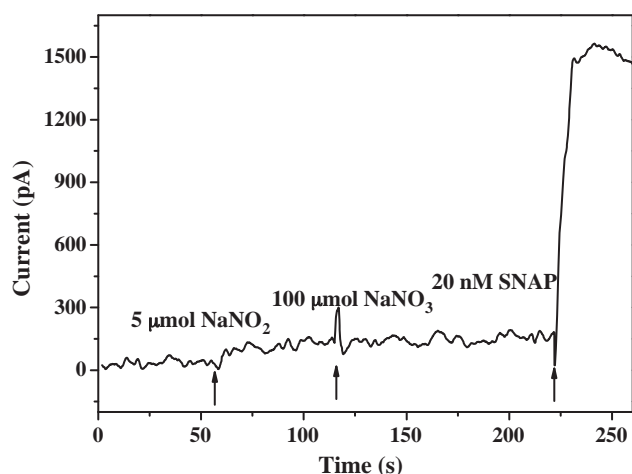


Fig. 3. Current responses when sodium nitrate, sodium nitrite and S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) were added to gold nanoparticles solution. Physiological concentrations of nitrite (5 μ M), nitrate (100 μ M) and SNAP (20 nM) were used.

order to identify it, 100 μ L 1 mM GSH was added to the 10 mL 0.91 mM gold nanoparticles solution, the signal amplitude almost kept constants (Fig. S2), which indicate RSHs are not interfering the detection in the assay. On the other hand, nitrite (5 μ M), nitrate (100 μ M) at physiological concentrations and SNAP (20 nM) were added to the gold nanoparticles solution, respectively. The corresponding responses were demonstrated in Fig. 3. There was no current change when adding 100 μ M nitrate. Although the current response was detectable when 5 μ M nitrite solution was added, the response was less than 2% compared with that of 20 nM SNAP. It indicates that both nitrite and nitrate are not prone to interfere with the detection of RSNOs in physiological condition.

On the other hand, NO-selective electrode coated with WPI NO selective membranes not only eliminates the interference of dopamine and other biomolecules, but also exhibits tremendous resistance towards electrode fouling caused by macromolecular nonspecific absorption [25]. Consequently, the method based on the gold nanoparticles decomposing RSNOs is special, it can be used to detect RSNOs in biological system and obtain accurate results.

3.4. Linear range, detection limit and precision

Because the effect of gold nanoparticles on the sensitivity is in concentration-dependent manner, the detection limit is reduced

Table 1

Comparison of detection limits for determination of RSNO.

Method/regent(s) used	Detection limit (nM)	References
Chemiluminescence	0.002–0.005	[35]
Spectrofluorimetry/DAN	20	[36]
Spectrofluorimetry/DABODIPY	1.2	[37]
Electrometry/ Cu^+	2	[38]
Electrometry/organoselenium	20	[39]
Electrometry/gold nanoparticles	0.05	This paper

with concentration of gold nanoparticles increased. Moreover, the linear range is wide at high concentration of gold nanoparticles. Therefore, this method can satisfy the requirement of detection RSNOs concentration in different biological fluids via varying gold nanoparticles concentration. In this paper, the recorded data were used to construct a calibration curve in 0.91 mM gold nanoparticles solution. The calibration curve can be simply constructed by plotting the signal output vs. the concentration of SNAP added at that time. The inset of Fig. 4A shows the responds of NO electrode (WPI) in gold nanoparticles solution with successive additions of SNAP. The microsensor clearly showed substantial increase current upon successive additions of SNAP. The standard curve was constructed using steady-state currents upon addition of SNAP (Fig. 4A), the linear range was from 5 nM to 1000 nM. The approximate limit of detection of the assay was 5.08×10^{-11} M at a signal/noise ratio (S/N) of 3. Compared with other assays, except for chemiluminescence, the detection limits of the proposed method was lower than those of other methods (Table 1), which indicate it has high sensitivity.

The precision of assay was tested by measuring NO currents at different concentrations of SNAP. Fig. 4B shows the current responds upon adding SNAP to gold nanoparticles solution. For intra-day precision, the relative standard deviation (RSD) was 3.98% ($n=5$) at 10 nM SNAP and 2.06% at 20 nM SNAP ($n=5$). The inter-day precision was shown to be within a RSD of 4.82% at 10 nM and 2.79% at 20 nM SANP. Consequently, the results have demonstrated the high reproducibility of the RSNOs measurements.

3.5. Determination of RSNOs in blood serum

It is of great significance to quantify the RSNOs that mediate various physiological and pathophysiological processes in the blood systems. The level of RSNOs in mouse blood serum was detected with the proposed method above (Fig. 5). Samples were analyzed for five times using the same recommended procedures and conditions. All samples for RSNO measurement were performed

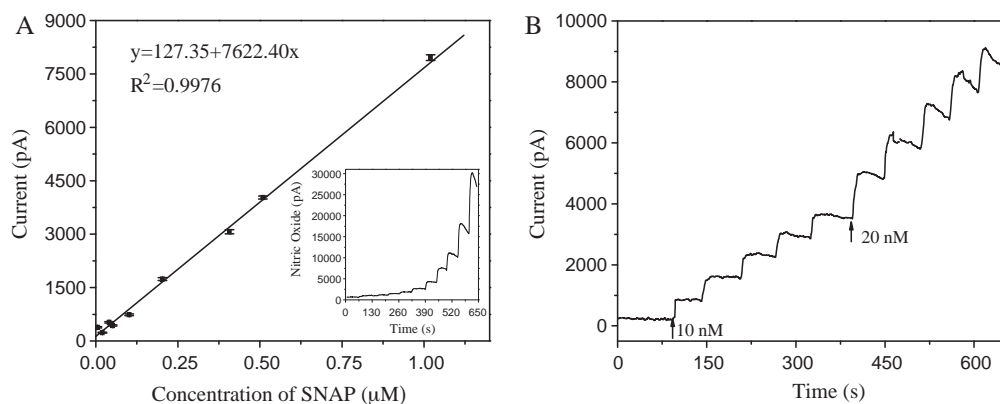


Fig. 4. (A) S-Nitroso-N-acetyl-D,L-penicillamine calibration curve for the electrochemical method used for the analysis of S-nitrosothiols in biological fluids. The inset is the amperograms of NO released due to different concentrations of SNAP addition to the gold nanoparticles solution; (B) Current responds based on gold nanoparticles catalysis with successive addition SNAP at concentration of 10 nM and 20 nM.

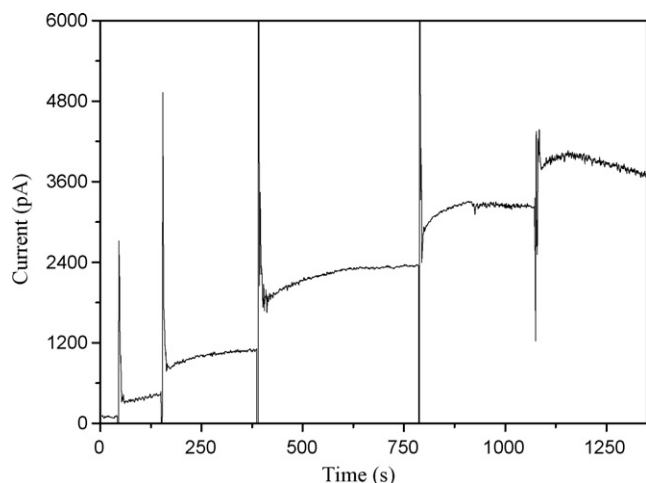


Fig. 5. Typical amperograms with successive adding blood serum to gold nanoparticles solution.

immediately without freeze-thaw. By this assay, the 510 ± 50 nM NO signal was detected in blood serum, which is consistent with the results of fluorescent assays (Supporting Information).

4. Conclusion

In summary, the electrochemical assay was developed to determine RSNOs based on the efficient catalysis of gold nanoparticles for RSNO decomposition. The approach displayed high sensitivity for RSNOs with a low detection limit to 5.08×10^{-11} M and was free from interference of some endogenous substances such as NO_2^- , NO_3^- and GSH co-existing in blood serum. The results indicate the possible applicability of the method in biological systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.talanta.2011.07.028](https://doi.org/10.1016/j.talanta.2011.07.028).

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