A RAPID ELISA METHOD FOR THE DETECTION OF SARCOSINE USING PSEUDOPEROXIDASE ACTIVITY OF GOLD NANOPARTICLES

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Abstract

Sarcosine is an amino acid that is formed by methylation of glycine and is present in trace amounts in the body. Increased sarcosine concentrations in blood plasma and urine are associated with sarcosinemia and some other diseases such as prostate cancer. In addition, sarcosine may be administered as a drug in some psychiatric disorders. Primarily, sarcosine in biological fluids is analyzed using liquid chromatography techniques, but this technique is not suitable for routine analysis. For this purpose, sarcosine detection using the nanomedicine approach was proposed. In the presented test, Au20 gold nanoparticles (size 20-30 nm, zeta potential -38 mV, absorption maximum at 529 nm), which exhibit pseudoperoxidase activity and convert a suitable substrate such as TMB, ABTS (1 mM) to a distinctive color product, were used. In the following experiments, the Au20 nanoparticle was linked to the chicken anti-sarcosine antibody. Subsequently, the ELISA method for the determination of sarcosine was prepared. Firstly, the construct for direct detection of sarcosine bound to the antibody was prepared by both the direct and the indirect method (dependencies were strictly linear, R^2 = 0.996). The subsequent construct uses rabbit antibodies against the anti-sarcosine chicken antibody labelled with Au20 nanoparticle. The bound product is then monitored by the conversion of the TMB (655 nm) or ABTS (425 nm) substrate (the dependencies are strictly linear, R^2 = 0.998). To further increase the sensitivity of the method (for more than 90% of the original signal), an aggregation agent was added to the used Au20 nanoparticles, which further increased adherence of gold nanoparticles to the antibodies. This procedure succeeded in achieving subnanomolar detection limits for the amino acid sarcosine.

Keywords: Sarcosine, sarcosinemia, ELISA, pseudoperoxidase activity, gold nanoparticles, nanomedicine

1. INTRODUCTION

Sarcosine (N-methylglycine) is an amino acid that is formed by methylation of glycine. Physiologically, sarcosine is present in the organism in trace amounts. Elevated values of sarcosine are found in blood plasma and urine during sarcosinemia due to sarcosine dehydrogenase deficiency and glutaric aciduria type II. Sarcosinemia is a very rare metabolic disorder that does not have well-defined clinical manifestations. In this disease increase in the sarcosine level in both urine and blood plasma is observed [1]. The analysed values of sarcosine can thus be in the range of tens to hundreds of micromolar concentrations in the studied biological sample. N, N-dimethylglycine was tested for a potential antioxidant effect during fertilization. It has been found that use of N, N-dimethylglycine produces a better development of bovine embryos [2]. In addition, changes in levels of selected amino acids (in urine, blood plasma) are indicators of potential cardiovascular risk [3]. Therapeutically sarcosine is used in high concentration (2 g / day) as adjunctive therapy of mental and psychiatric disorders, especially schizophrenia [4,5]. During its use glutamate receptor is affected and sarcosine is probably glycintransporter-1 (GlyT-1) inhibitor [6].

In a number of experimental studies, amino acid profiles were observed in relation to prostate tumors [7-9]. Elevated urinary sarcosine levels have been identified as a potential marker of malignant tumors, especially prostate tumors [10,11]. The content of sarcosine in prostate cancer tissue was studied. The median of sarcosine content was by 7% higher compared to the control group of non-tumor samples. The level of sarcosine was not associated with tumor stage, tumor grade or biochemical recurrence. This study failed to demonstrate the suitability of sarcosine as a tumour marker [12]. In addition to the above-mentioned reasons for the analysis of sarcosine in a biological sample, sarcosine is available as a food supplement in a common consumer network. In this case, its increased concentrations should probably be monitored to assess the maximum appropriate doses of this amino acid. Due to the clinical significance of this metabolite, some attempts have been made to find ways how to modify these methods for use in clinical laboratories and to allow quantitative determination of sarcosine in patient biological fluids as part of a diagnostic examination. Analysis of sarcosine in clinical laboratories in normal operation is currently not possible. In order to implement it, it is necessary to introduce a suitable methodology, including the work with individual components and a rather demanding optimization of the method. In our previous work we dealt with the use of pseudo-peroxidase activity associated with gold nanoparticles [13]. The aim of this work was to propose the detection of sarcosine using polyclonal hen antibodies.

2. MATERIAL AND METHODS

Chicken polyclonal antibodies were obtained from Hena (Prague, Czech Republic) [14,15]. The animals were immunized with an antigen containing a sarkosin-linked KLH. Rabbit anti-chicken antibody (anti-CHIC), and bovine serum albumin (BSA) were supplied by Sigma Aldrich (USA). 3,3′,5,5′-tetramethylbenzidine (TMB), ABTS and sodium citrate dihydrate, hydrogen tetrachloroaurate(III) tetrahydrate ($HAuCl_4 \cdot 4H_2O$), hydrogen peroxide (H_2O_2), hydroxylammonium chloride ($NH_2OH \cdot HCl$), sodium carbonate anhydrous (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$), sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), acetic acid (HAc),and sodium acetate (NaAc) were purchased from Merck (Germany). All reagents were of analytical grade and used without any further purification. AuNPs were synthesized according to the reported method with slight modification [16]. Thermal synthesis of AuNPs was performed using a magnetic stirrer with heating under controlled temperature, stirring for 60 minutes and preventing evaporation of liquid.

The nanoparticles were characterized using the following equipment and approaches: ZetaSizer, spectrophotometry, fluorimetry, and electrochemistry. Preparation of AuNPs-antibody conjugate followed. One ml of antibody (1 mg/ml) was added into 10 ml of AuNPs suspension with pH at 9.0 (0.1 M Na_2CO_3 and 0.1 M HCl were used to adjust pH). The mixture was incubated at room temperature for 1 h. Then, 1 ml of 10% BSA solution was injected under stirring to block the unspecific binding of proteins to antibodies on AuNPs, followed by incubation at room temperature for 0.5 h. The conjugate was then centrifuged (16,000 g) at 4°C for 15 min, and the soft sediment was washed and re-suspended in PBS solution. The colour development appeared after an incubation in 100 µl of 1 mM TMB/ABTS and 10 mM H_2O_2 (15 min, 30°C). The absorbance or spectra were recorded by using a reader Infinite M200 (TECAN, Switzerland) or spectrophotometr (UV-3100PC, VWR Germany). Electrochemistry: measurements were carried out in a volume of 1.0 ml; where the electrolyte was 0.2 M acetate buffer (pH 5). Parameters of voltammetry were as follows: initial potential −1.2 V; end potential −0.3 V; potential step 3 mV; and potential of deposition −1.0 V. Electrochemical measurements were performed using a minicomputer-connected potentiostat 910 PSTAT mini (Metrohm, Switzerland).

3. RESULTS

Pseudoperoxidase activity of the gold nanoparticles is intensively investigated and certainly brings a number of advantages compared with the use of naturally-occurring enzyme [17,18]. In our experiments we are very intensively engaged in the pseudoperoxidase activity of gold nanoparticles (**Figure 1**), where we have recently

published their physicochemical characterization [13]. Proposed procedure was used in the modification (labeling) of the antibody to maximize the amplification of the resulting colored product (TMB and ABTS).

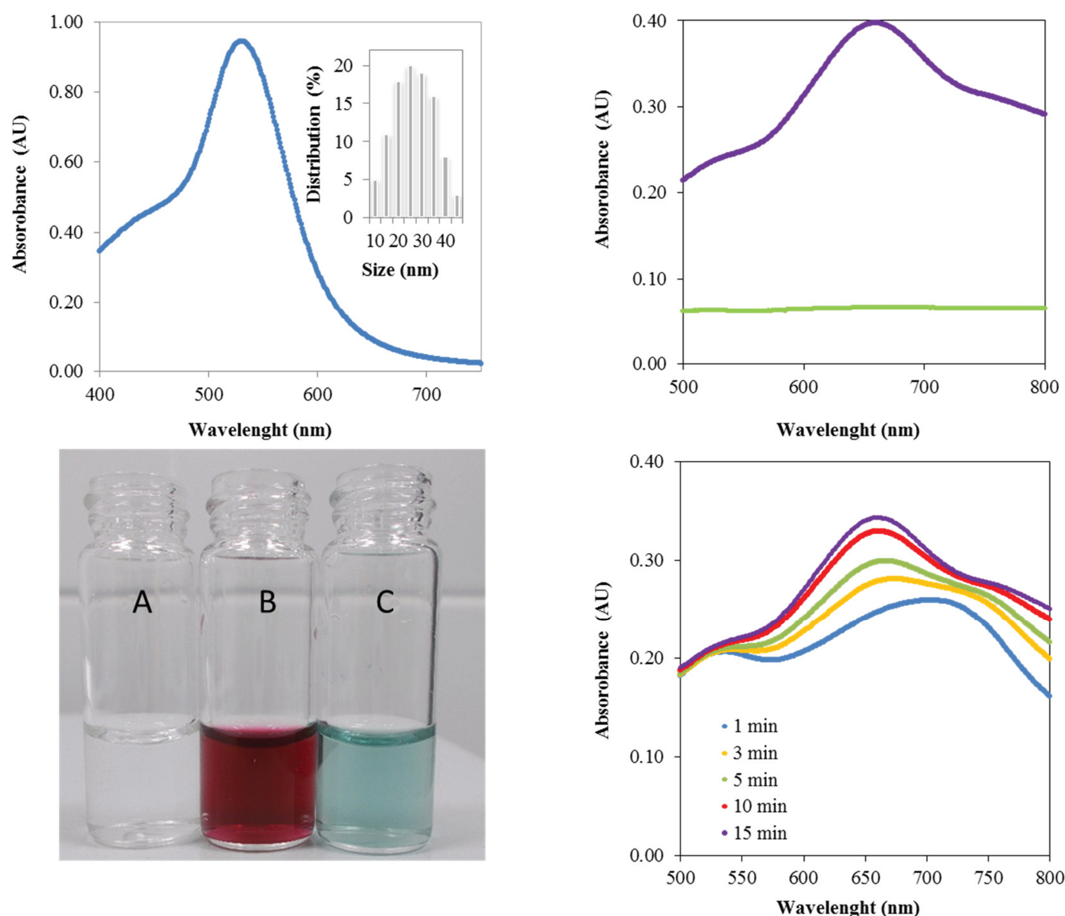

Figure 1 Basic characteristic of the prepared gold nanoparticles Au20. Spectra were obtained at absorption maximum of 530 nm, size of 20-30 nm, and zeta potential of 38 mV. Au20 nanoparticles exhibit pseudoperoxidase activity, which can be used as a marker. A- substrate itself; B- Au20 itself; C-pseudoperoxidase reaction in Au20. Peroxidase activity of Au20 nanoparticles in the presence of TMB. The reaction dependence on the time. Spectra are very well observable at 650 nm and 700 nm (0.5 M acetate buffer pH 4, 30% H_2O_2, 5 mM TMB)

Substrates TMB and ABTS exhibit a very good course of reaction depending on the type of gold nanoparticles used. Chicken antibodies against sarcosine were prepared by immunizing animals (antigen bound to KLH). Individual polyclonal AntiSar antibodies that have a good affinity for sarcosine were isolated from egg yolks. This fact led us to propose the immunodetection of sarcosine by ELISA. Two main objectives for the determination of sarcosine concentration have been suggested: (a) direct determination; (b) indirect determination. In order to verify the functionality of the proposed methodology, the following experiment was carried out: The microtiter plate was coated with a primary anti-sarcosine-specific antibody (AntiSar, in the amount of tens of nanograms per well of the microtiter plate). Antibody binding was performed in 0.05 M carbonate buffer, pH 9 at 37 °C for 1 hour. The fluid was then aspirated and the plate surface was blocked with a 1-5% solution of albumin or dried white whey or milk powder for 40 minutes at 37 °C. The blocked plate was washed with PBS-T buffer.

Sarcosine, which was bound to the surface of the gold nanoparticle, was applied to this plate and incubated for 1 hour at 37 °C. After incubation, the sample from the plate was sucked off and washed again with PBS-T

buffer. Subsequently, a protocol for monitoring the pseudoperoxidase activity of gold nanoparticles was used. Dependence on the concentration of applied sarcosine with good repeatability (error of determination up to 10%, n = 10) was obtained (**Figure 2**). The obtained calibration curve was strictly linear (R^2 = 0.9947) with the equation y = 0.0159x + 0.0115. The lowest concentrations of gold sarcosine analysed reached the value of 1 µM. The obtained experimental results showed that the proposed procedure can be used for the detection of sarcosine. Therefore, another experiment was carried out: The microtiter plate was coated with a primary antibody (rabbit Anti-IgG) at a rate of tens of nanograms per well of the microtiter plate. Binding of the primary antibody was carried out in 0.05 M carbonate buffer pH 9 at 37 °C for 1 hour. The fluid was then sucked off and the plate surface was blocked with 1-5% albumin or dried protein or dried milk for 40 minutes at 37 °C. The blocked plate was washed with PBS-T buffer, AntiSar antibody (1: 5000) was pipetted and incubated for 1 hour at 37 °C. After incubation, the sample from the plate was sucked off and washed again with PBS-T buffer. Sarcosin and gold sarcosine (50 µg / ml) were applied to the plate prepared in this way. Incubation was carried out for 1 hour at 37 °C. Subsequently, the plate was washed with distilled water.

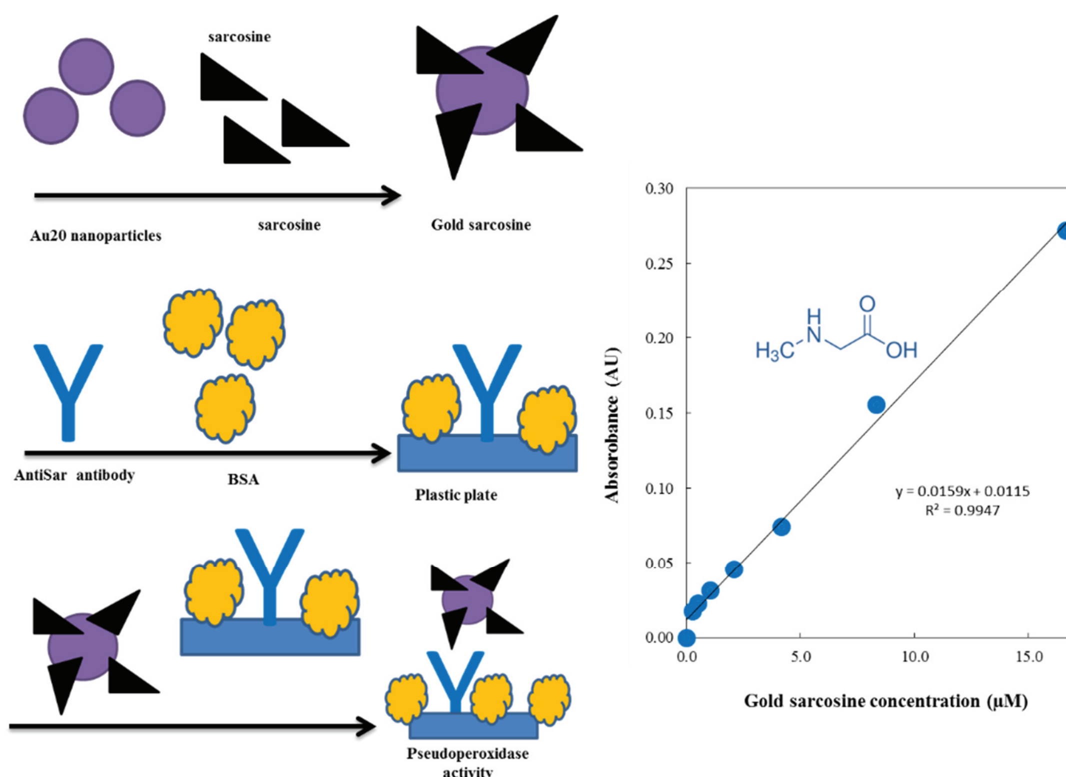

Figure 2 Detection of gold sarcosine using polyclonal chicken antibody (AntiSar). First, the Au20 gold nanoparticles were modified with amino acid sarcosine (gold sarcosine). Antibodies (AntiSar) were immobilized on the surface of the plastic plate by a conventional procedure and the free surface of the plate was blocked with BSA (1%). For the test, gold sarcosine was added at different concentrations. After binding to the AntiSar antibody, a substrate for pseudoperoxidase activity was added. The change was monitored and evaluated over time as three repetitions. The obtained calibration curve was prepared from 5 independent repetitions.

Strictly linear relationships (R^2 = 0.99) with good repeatability (determination error up to 12%, n = 10) were obtained (**Figure 3**). If a TMB was used as the substrate, a calibration curve with y = 0.01x - 0.0015, R^2 = 0.9989 was obtained and the same for the ABTS substrate (y = 0.0135x + 0.0014, R^2 = 0.9948). The lowest analysed sarcosine concentrations reached the value of 0.5 µM. The obtained experimental results show that the proposed procedure can be used for the detection of sarcosine using ELISA method. To increase

the pseudoperoxidase activity of the gold nanoparticles, 5 mM gold acid and 10 mM hydroxylamine hydrochloride in a ratio of 1: 1 was added and the reaction was allowed to react at room temperature for 20 minutes. After 20 minutes of incubation at room temperature and washing with water, chromogenic substrate (TMB or ABTS) was added. A colour reaction was carried out and the absorbance was measured in the specimens using a spectrophotometer. Obtained results of the sarcosine determination using the modified method enabled it to be determined at concentrations reaching 0.5 μM.

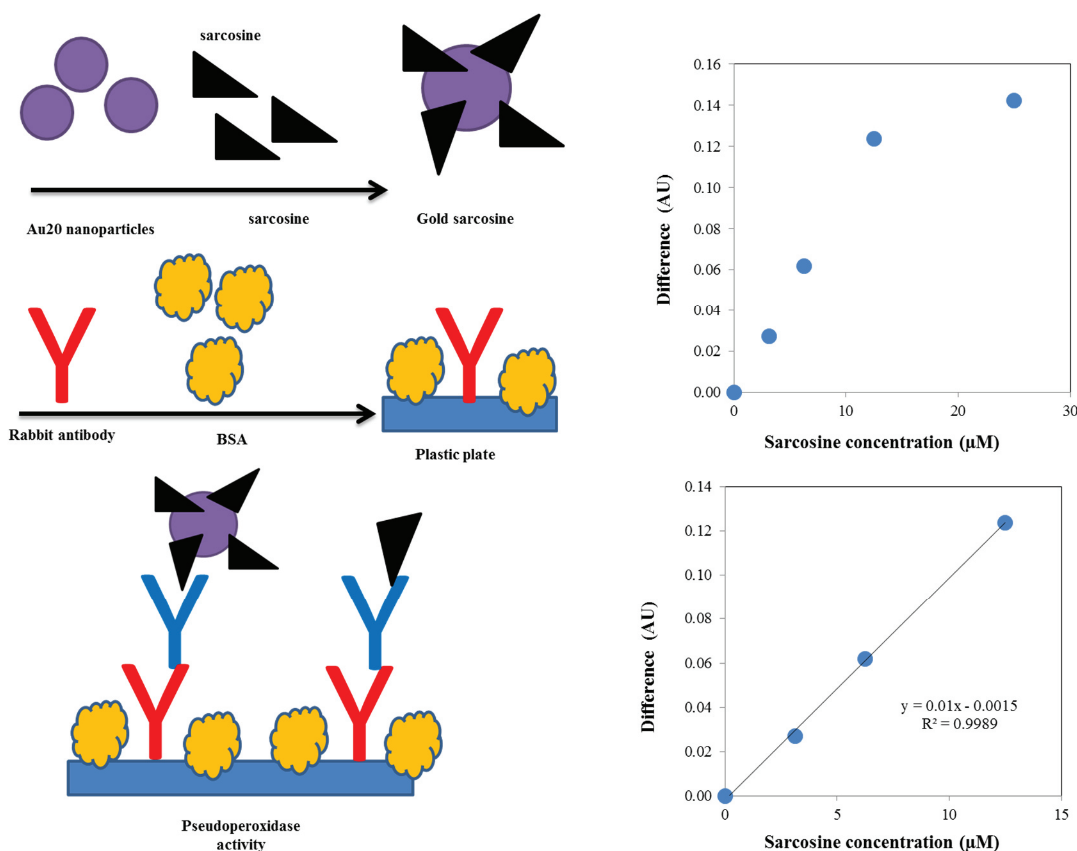

Figure 3 Detection of sarcosine using rabbit Anti-IgG antibody. Antibodies (Anti-IgG) were immobilized on the surface of the plastic plate by a conventional procedure, and the free surface of the plate was blocked by BSA (1%). Au20 gold nanoparticles were modified by the amino acid sarcosine (gold sarcosine). Subsequently, polyclonal chicken AntiSar antibody was applied. For the test, gold sarcosine at a concentration of 50 mg / ml and sarcosine at different concentrations were added. After binding to the AntiSar antibody, a substrate for pseudoperoxidase activity was added. The change in the absorption signal was monitored over time and evaluated as three replicates. The calibration curve obtained is prepared from 5 independent repetitions using the TMB substrate.

4. CONCLUSION

From a number of published data, it is clear that the role of sarcosine can be significant in both physiological and pathophysiological processes. The amount of sarcosine is bioanalytically determined mostly by liquid chromatography, which is not appropriate. Therefore, new ways for sarcosine determination are sought. In our experiments, polyclonal chicken antibodies were used for its sensitive detection. To increase the sensitivity of the assay, pseudoperoxidase activity of gold nanoparticles was utilized. It has been found that ABTS can be used as a suitable substrate in addition to TMB. Using the proposed procedure we were able to analyse sarcosine at micromolar concentrations.

ACKNOWLEDGEMENTS

This work was supported by the project for conceptual development of research organization and the realisation of the experiment was supported by project SarcoTest (PM165/2017)

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