ANTIBODIES MODIFIED BY GOLD NANOPARTICLES OR QUANTUM DOTS FOR SPECIFIC DETECTION OF SARCOSINE ON THE MEMBRANE: USE IN RAPID DIAGNOSTICS

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Abstract

Paper tests are very cheap and fast diagnostic tools for self-testing in a wide range of health problems. Thus, the diagnostics of tumor development is of a great medical and socio-economic significance. Malignant prostate cancers are the most common malignancy in men. Procedures have been developed to diagnose this disease based on the monitoring of tumor marker molecules. Most commonly, PSA and fPSA are used. Although these tumor markers are significantly increased, the cancer at the biopsy is not always confirmed. Thus, the finding of other marker molecules is urgently needed. Recent studies have shown that changes in content of the amino acid sarcosine are associated with prostate tumours. Polyclonal chicken antibodies against sarcosine (AntiSar) were prepared. AntiSar antibodies were labelled with gold nanoparticles Au20 (size 20-30 nm, zeta potential - 38 mV) and CdTe QDs (emission 530 green QDs and 650 nm yellow QDs). Dot blot and flow lateral chromatography were used to develop a newly designed sarcosin detection procedure. The aggregation or fluorescence intensities were evaluated by newly designed software. Sarcosine was analysed in a concentration range (0-150 μM) in PBS, artificial urine and urine sample buffer. The calibration curves obtained in the concentration range (0-12 μM) were strictly linear (R^2 = 0.99) with a relative error of 10%. Using the proposed methods sarcosine concentrations as low as 250-500 nM were analysed. Moreover, the results obtained show a higher sensitivity (by 20-40%) of sarcosine detection using QDs in comparison with using gold nanoparticles.

Keywords: Sarcosine, prostate cancer, flow lateral chromatography, CdTe QDs, gold nanoparticles, nanomedicine

1. INTRODUCTION

Tumor diseases are a very common cause of death in developed countries. The nonproteinogenic amino acid sarcosine is involved in the metabolism of amino acids and methylation processes. A study on urinary sarcosin levels has been performed in patients with a diagnosed prostate cancer [1-3]. Detection of sarcosine by a variety of methods (HPLC, GC / MS) has been described [4]. In some studies, an enzymatic method using sarcosin oxidase was described for sarcosine determination [5,6]. At present, there is an increasing interest in introducing a simple urine sarcosin test. The analytical techniques described above, such as HPLC, microfluidic systems, spectrophotometry (GC / MS), are sensitive but time-consuming and require complicated sample preparation, unlike the ELISA method. This analytical method is used to quantify different antigens based on antigen-antibody interactions [7]. ELISA method is commonly used in clinical-diagnostic laboratories to determine a wide range of analytes using specific antibodies. Antibodies isolated from egg yolks (IgY) of

immunized chicken are a suitable alternative to commonly used mammalian antibodies. The main advantages of IgY are: greater amounts of IgY obtained after immunization, better reproducibility of antibody formation in a long run production, and improved responsiveness of the bird's immune system to mammalian antigens [8]. Gold nanoparticles (AuNPs) have long been used as a suitable tool for molecular biological experiments [9]. In addition, with the rapid development of nanotechnology, gold nanoparticles and quantum dots (QDs) are used as one of the main biomolecular carriers in nanomedicine and biosensor applications [10-12]. In the forefront of interest also reaches LFIA test (Lateral Flow Immuno Analysis) which principle is a combination of chromatography and immunoaffinity reactions. The aim of this work was to utilize polyclonal chicken antibodies for the detection of sarcosine on the nitrocellulose membrane.

2. MATERIAL AND METHODS

Chemicals: Chicken polyclonal antibodies were obtained from the company Hena (Czech Republic) [7,8]. The animals were immunized with an antigen containing a sarkosin-linked to 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) hydrate ($HAuCl_4 \cdot 4H_2O$), 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.

Synthesis of Gold Nanoparticles (AuNPs): AuNPs were synthesized according to the reported method with slight modification [13,14]. Thermal synthesis of AuNPs was performed using a magnetic stirrer with heating under controlled temperature, stirring for 60 minutes and preventing evaporation of liquid. Preparation of AuNPs-anti-IgG conjugate followed. One ml of AntiSar (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 blue colour development appeared after incubation in 100 µl of 1 mM TMB and 10 mM H_2O_2 (15 min, 30°C) [13]. The absorbance at 652 nm was recorded by using a reader Infinite M200 (Tecan, Switzerland). 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). To perform dot blot analysis and flow lateral chromatography, FF120HP (GE Healthcare Life Science Whatman, UK), glass fiber conjugate pad (Millipore, France) and CF 6 (GE Healthcare Life Science Whatman, UK) were used. For the defined antibody deposition, Linomat (Camag, Switzerland) was applied at a pressure of 1 bar, 1-5 µl of antibodies were applied in 10 to 50 layers according to the length of the prescribed band.

Preparation of CdTe quantum dots: 10 ml of a solution of Cd $(CH_3COO)_2 \cdot 2H_2O$ (0.266 g / 50 ml) + 76 ml of H_2O + 1 ml of mercaptosuccinic acid (MSA) solution (3g / 50ml) + 1.8 ml of NH_3 +1.5 ml of Na_2TeO_3 / 50ml) + 40mg $NaBH_4$ was stirred on a magnetic stirrer (VMS-C4, VWR International Ltd.,Germany) for at least 2 hours until bubbling stopped. Subsequently, the volume was adjusted to 100 ml. Two ml of the prepared solution was pipetted into the glass vials (Sigma Aldrich, USA), with a white cap (Anton Paar, Austria) and a teflon cap (Anton Paar, Austria). The thus prepared vial was placed in a microwave which was set to a power of 300 W and the heating took place for 2 minutes with green QDs and 4 minutes for yellow QDs.

3. RESULTS

Sarcosin is a very small molecule. To ensure immunological response, sarcosin hapten was coupled to KLH and applied to animals (hen). Polyclonal antibodies against sarcosine were prepared from collected egg yolks. To detect antibody binding to the antigen, we decided to label the antibodies with the Au20 gold nanoparticles (Au20NPs) or CdTe quantum dots (QDs). In the proposed experiment, several different types of polyclonal chicken antibodies to sarcosine (AntiSar13, AntiSar14, AntiSar15, AntiSar16, AntiSar17) were prepared. The following experiment was designed to test individual antibodies. The primary antibody to sarcosine (ng per spot) was bound to the nitrocellulose membrane in the usual manner. Subsequently, the membrane was blocked by BSA (1%). A solution of sarcosine (100 μM) was then added and after a 30 minute of incubation, the membrane was thoroughly washed and secondary Au20NPs-labeled antibody was added. Secondary antibodies are intensively bound to the sarcosine site, and the aggregation of gold nanoparticles produces a typical pink and purple colour (in the case of very strong aggregation).

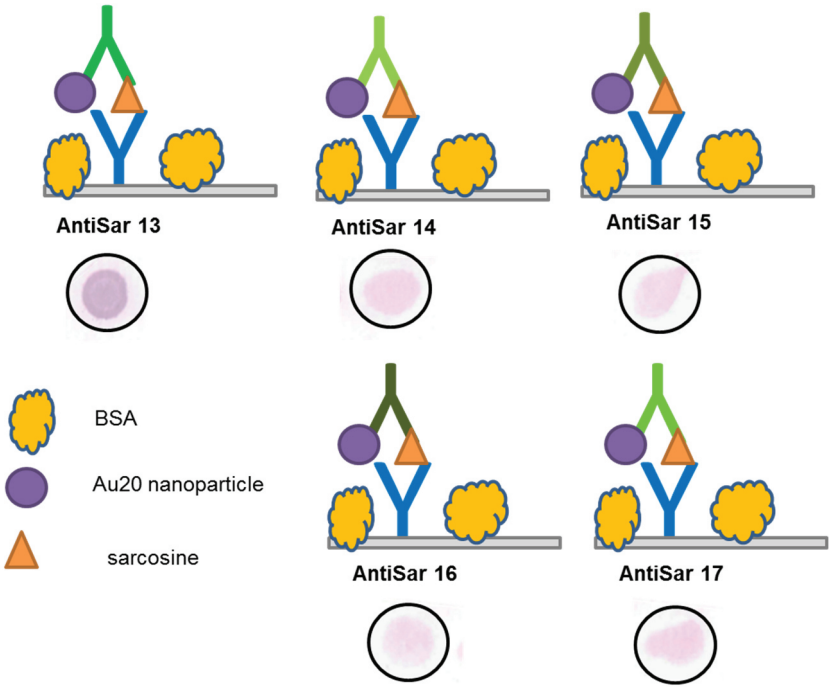

Figure 1 Interaction study performed on the membrane. The primary AntiSar antibody was bound to the membrane in the usual manner. The membrane was then blocked with BSA. Subsequently, sarcosine (100 μM) was added and washed for 30 min. A secondary antibody labelled with Au20NPs was then applied

In **Figure 1**, typical records for the individual tested antibodies are shown. As is evident from the results, all tested antibodies have a very good ability to recognize the studied antigen (sarcosine). The detected immunoreactivity of the AntiSar antibodies to sarcosine was good and it was possible to monitor the effect of various sarcosine concentrations. Different concentrations of sarcosine (0-150 μM) were prepared and intensity of Au20NPs aggregation was determined by densitometry software developed by us. The obtained dependence is shown in **Figure 2** and the maximum of Au20NPs aggregation at the applied sarcosin concentration (100-150 μM) is detectable. When we analyzed sarcosin concentrations in the range of 0-12 μM, the obtained dependence was strictly linear (R^2 = 0.995) with the regression equation y = 0.0006x - 0.0004 and the lowest determined sarcosine concentration was 500 nM. In the case that we have used for the detection CdTe QDs the following construct has been formed. The antibody to the chicken antibody (Anti IgG) was bound to the membrane and then it was bound to the AntiSar antibody labelled with CdTe QDs. Different concentrations of sarcosine were added to this QDs labelled antibody. The binding of sarcosine to the antibody caused a decrease in observed fluorescence. Therefore, different concentrations of sarcosine (0-100 μM) were

prepared and the analysis principle is shown in **Figure 2**. The obtained dependence and a detectable maximum of fluorescence intensity decrease of CdTe QDs at an applied sarcosin concentration of 0-100 μM is shown in **Figure 2**. If we analyzed shorter interval of sarcosin concentrations (0-12 μM), the obtained dependence was strictly linear (R^2 = 0.9946) with regression equation y = 0.0101x + 0.4146 and the lowest determined sarcosine concentration was 250 nM. Fluorescence detection of sarcosine in this simple arrangement was more sensitive compared to the use of gold nanoparticles (**Figure 2**).

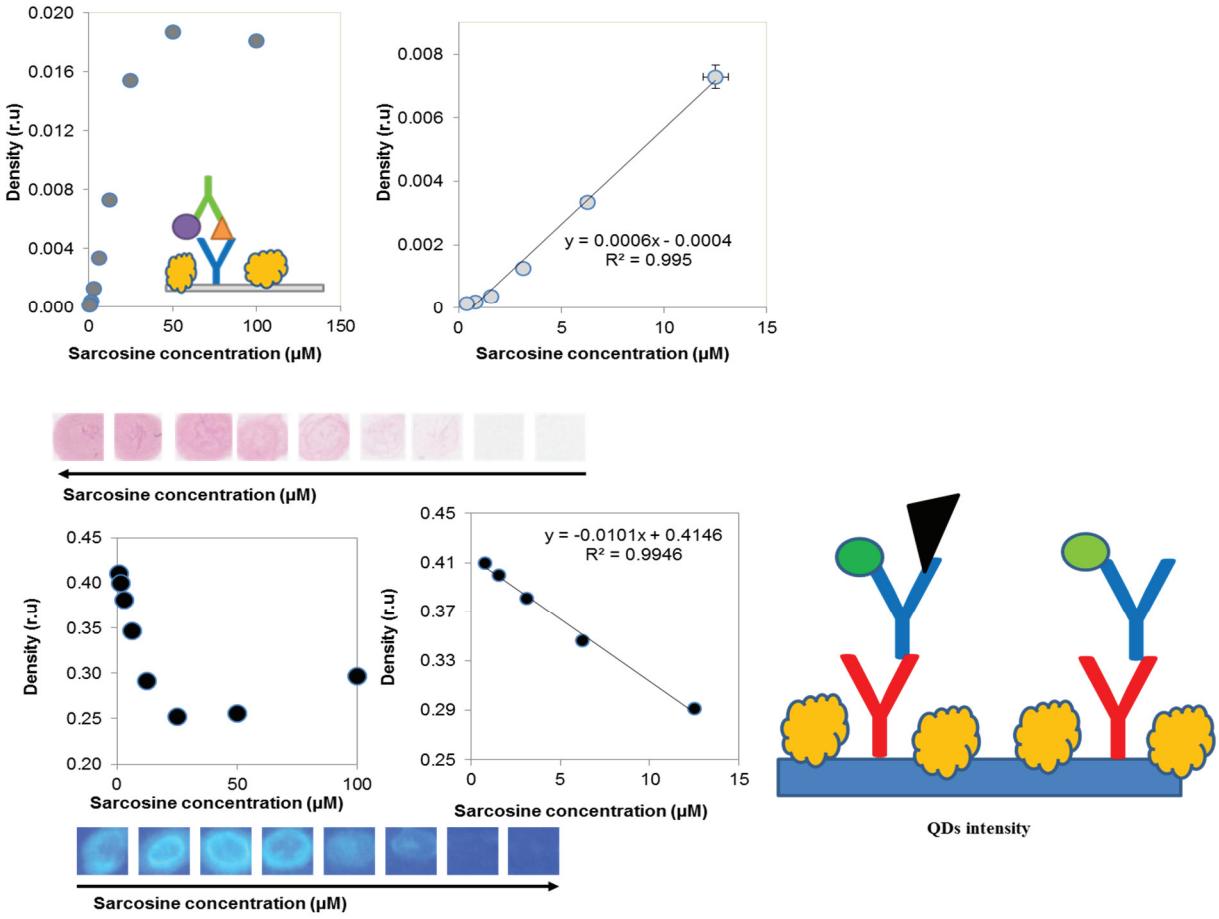

Figure 2 Detection of sarcosine on the nitrocellulose membrane was observed as the aggregation of gold nanoparticles or the fluorescence intensity of CdTe QDs in PBS buffered media. Interaction study was performed on the nitrocellulose membrane. The primary AntiSar antibody was bound to the membrane in the usual way, then the membrane was blocked with BSA (1%). Sarcosin (at various concentrations) was then added and after 30 minutes the membrane was washed. Subsequently, the secondary antibody labelled with Au20NPs was applied. In the case of the use of CdTe QDs, the primary IgG antibody was against the chicken antibody

The obtained results in a buffered environment allowed us to perform experiments in complex biological matrices. First, we performed a test in arteficial urine (150 mM sodium chloride, 55 mM potassium chloride, 30 mM sodium phosphate, 350 mM urea, 20 mM creatinine, 700 μM bovine serum albumin) and urine. Different concentrations of sarcosine (0-100 μM) were prepared and analyzed. The obtained dependence is shown in **Figure 3**, and the maximum of Au20NPs aggregation is detectable at an applied sarcosin concentration of 50-100 μM. If we analyzed sarcosin concentrations in the range of 0-12 μM, the obtained dependence was strictly linear (R^2 = 0.995) with regression equation y = 0.0006x - 0.0003 and the lowest determined sarcosin concentration was 500 nM. A similar pattern was obtained for the urine sample (R^2 = 0.995) with the regression equation y = 0.0005x - 0.0003 and the lowest determined sarcosine concentration was 500 nM (**Figure 3**).

In the following experiment, knowledge gained in dot blot analysis was used to construct a flow lateral chromatographic assay. The nitrocellulose membrane of length 10 cm was taped by polyester backing. On the prepared membrane, two sets of antibodies were applied in thin lines using Linomat device. The test line was plated at 100 µg / ml and diluted in PBS. Control line formed by the antibodies was plated 3 mm above the test line at 100 µg / ml. Antibodies were immobilized on the nitrocellulose membrane overnight at 37 °C. The second day the suction strip was attached to the membrane at the bottom. Furthermore, the membrane was cut into individual strips by means of a cutter. The strips were glued to the stand in a vertical position. An absorbent paper was also adhered to the top edge of the stripe, for faster rising of the mixture along the strip. A reaction mixture was applied to each strip. The reaction mixture was prepared by mixing sarcosine in reaction buffer (PBS, 2% BSA, 1% PEG and 0.5% Tween) and Au20NPs antibody conjugate.The mixture was allowed to rise freely along the strip. To further improve the detection properties of Au20NPs aggregation, it was advantageous to use the addition of gold acid (5 mM) and hydroxylamine (10 mM) in a developing solution containing NaCl at a concentration of 0.15 mM, KCl of 3 mM, Na_2HPO_4 8 mM, KH_2PO_4 2 mM, bovine serum albumin 0.5%, polyoxyethylene (20) -sorbitan monolaurate (Tween20) at a concentration of 1%.

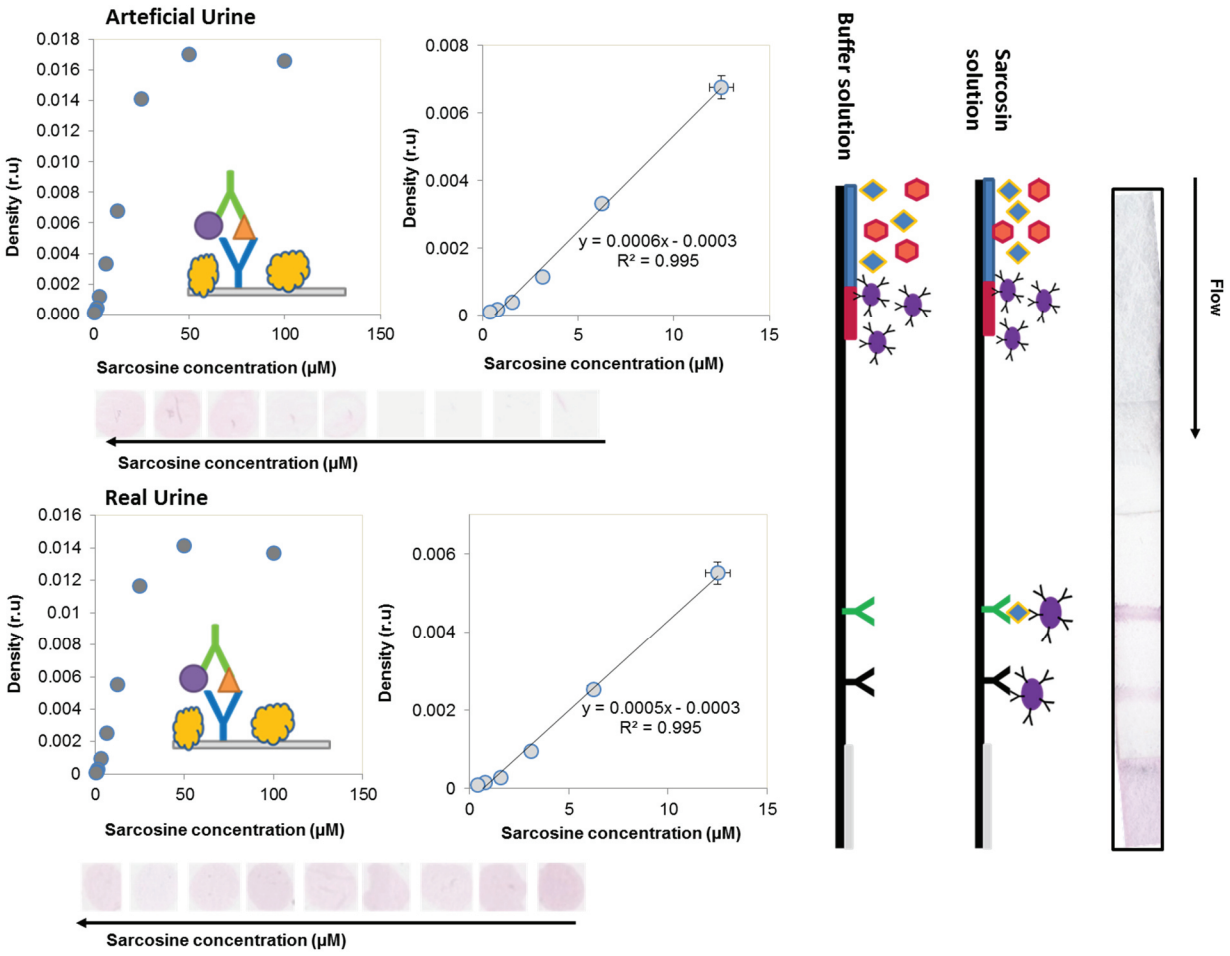

Figure 3 Detection of sarcosine on the nitrocellulose membrane was observed as an aggregation of gold nanoparticles. Interaction studies performed on the membrane in arteficial urine and real urine. The primary AntiSar antibody was bound to the membrane in the usual manner, after which the membrane was blocked by BSA. Subsequently, sarcosine (at various concentrations) was added and washed away after 30 minutes. A secondary antibody labeled with Au20 NPs was then applied. Principle of flow lateral chromatography for the detection of sarcosine in a real biological sample

A diagnostic strip was prepared from an embedded nitrocellulose membrane (Whatman, GE Healthcare Life Sciences, UK) of a length B of 6.0 cm and a width of 0.5 cm. The sample zone had a length of 2.0 cm and was composed of three layers (a glass fiber filter was placed on the adhesive plate - a first 2 cm zone, followed by a glass fiber conjugate (Au-AntiSar, QD- AntiSar) - zone 2, which was covered with a 2 cm long glass fiber filter , third zone extending beyond the first zone F1 and the second F2 by 0.3 cm The prepared layers of sample zone may be dried at laboratory or slightly elevated temperature, utilization of low pressure and utilization of lyophilization.

4. CONCLUSION

Fast paper tests will bring a significant simplification of the diagnosis of a number of serious illnesses. In addition, it will allow regular self-testing of a large group of people in a given population. In our work, simple tests were designed to identify the amount of sarcosine using polyclonal chicken antibodies labeled with gold nanoparticles or QDs. We succeeded in obtaining a very good response of the proposed system with detection limit of sarcosin 250 nM

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