Scientific Papers of the University of Pardubice,
Series A; Faculty of Chemical Technology
24 (2018) 109–118.

University
of Pardubice
Faculty
of Chemical Technology

Different approaches for preparation of AuNPs-based conjugates as a signal-generating unit for electrochemical biosensors

Pavla Murasová*, Aneta Kovářová, Jana Srbová, and Zuzana Bílková

Department of Biological and Biochemical Sciences,
The University of Pardubice, CZ–532 10 Pardubice, Czech Republic

Received: June 4, 2018; Accepted: August 15, 2018

With the advent of micro- and nanotechnology, major advances and breakthroughs have taken place in many areas, such as biotechnology, medicine, pharmacy or material industry. Gold nanoparticles (AuNPs) with their unique optical and physical properties as well as biocompatibility have already proved themselves to be powerful tool in biological applications. In this work, AuNPs were selected as a potential electrochemically active label. With the aim to use them as an electrochemical active tag, two types of AuNPs were tested and suitable protocols for their functionalization were optimized. Non-functionalized AuNPs were modified with thiolated DNA oligonucleotides using Au-S bond. Such conjugates could be potentionally used as a DNA probe. Functionalized AuNPs containing carboxyl groups were also modified, but with specific antibodies. In this case, the goal was to optimize suitable protocol for effective modification performed with carbodiimide crosslinker enabling direct and covalent immobilization. Finally, quality of AuNPs-IgG was defined by experiments via detection of bacterial cells in model samples.

Keywords: Gold nanoparticles; Bioconjugation; Label; Immunosensor

Introduction

Gold nanoparticles (AuNPs) are defined as a stable colloidal solution of clusters of individual atoms of gold in the size range from 1 to 100 nm [1]. Because of their very small dimensions, they differ in physical and chemical properties

* Corresponding author, ✉ Pavla.Murasova@upce.cz

compared to other small species or gold in common elementary form; in fact, AuNPs behave like a giant molecule [2]. In addition, this form of gold is characterized by a high absorption coefficient which ensures sufficiently high sensitivity for optical detection [3]. Thanks to yet another unique feature, which is surface oscillation (used for signaling, imaging and sensor technology), AuNPs are advantageously used in the field of biomedicine and biotechnology. They are routinely used in microscopic imaging methods or in therapy as drug delivery systems. Their use is also important in diagnostics to detect not only biomolecules as nucleic acids or proteins but also whole bacteria cells [4].

For modification of AuNPs by oligonucleotides, different types of chemical bonds can be used. One of the most common linkages between thiolated oligonucleotides and gold molecule is the Au-S binding. Due to the high affinity of the thiol group toward gold atoms, a very stable conjugate is then formed [5]. AuNPs are negatively charged, as well as the entire DNA molecule, resulting in the repulsive forces. This can be avoided by the process of gradually salting the solution with sodium chloride during the reaction [6–7]. The binding buffer and pH are also decisive, needing to be optimized.

Another type of binding between AuNPs and biomolecules could be via additional functional groups. These can be immobilized onto the surface of AuNPs by functionalizing heterobifunctional polyethylenglycol (PEG) with a thiol group at one end of the chain. On the second end of the chain, one can attach e.g. biotin, lectin, fluorescent label or different functional groups, such as amino group ($-NH_2$) or carboxyl group (-COOH) subsequently applicable, for example, to bind the protein [8].

The main goal of this work was to optimize the binding strategy for modification of AuNPs with thiolated oligonucleotides or specific antibodies. Two different types of AuNPs, two different bonds and two different types of biomolecules were selected for preparation of conjugates potentially usable as a signal generating molecule for electrochemical biosensors.

In one case, we used particles of colloidal gold without any functional group on their surface to prepare the DNA-oligonucleotides conjugates as a potential probe for detection of food borne pathogens by using a piezoelectric biosensor. Four different protocols for binding between thiolated DNA oligonucleotides and AuNPs basically rising from previously published papers had been applied [9–11]. In the second part of this work, AuNPs functionalized with PEG introducing carboxyl groups on their surface were modified by specific anti-*Listeria* antibodies. This second type of conjugate was finally tested as a part of electrochemical immunosensor for detection of whole bacterial cells in some model samples.

Materials and methods

Reagents and Chemicals

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), the sodium salt of N-hydroxysulfosuccinimide (sulfo-NHS), 2-(N-morpholino)ethanesulfonic acid (MES), N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), acrylamide, *N,N´*-methylen-*bis*-acrylamide, *N,N,N´,N´*-tetramethylendiamine (TEMED), ammonium persulfate, glutaraldehyde, silver nitrate were purchased from Sigma-Aldrich. Other chemicals were of analytical grade and obtained from Penta (Chrudim, Czech Republic). All buffers were prepared from ultrapure water using a TKA purification system (model "Smart2Pure"; Thermo Scientific TKA, Niederelbert, Germany).

Gold nanoparticles, magnetic particles, antibodies, and oligonucleotides

A suspension of gold nanoparticles (with 10 nm in diameter) stabilized in citrate buffer was purchased from Sigma-Aldrich. PEG-AMINO 3500 functionalized gold nanoparticles (20 nm) were from Orion High Technologies (Madrid, Spain). A pure proteome Protein "A" magnetic beads were from Merck. Polyclonal antibodies (polyAb): rabbit anti-*Listeria* ssp. antibodies (concentration: 4–5 mg mL^{-1}) were provided by Virostat (Portland, ME, USA). 5´-Thiol (C6)-CAA ATC TTA TTT TCT TTT CAC CTT CTC TCT-Cy3-3´ DNA oligonucleotides were from Friz Biochem (Neurid, Germany).

Probe oligonucleotide-gold nanoparticles conjugation

For conjugation of AuNPs with oligonucleotides four different protocols were used; for details, see Table 1. Thiolated DNA oligonucleotides were treated with TCEP for 45 min. to cleave the disulfide bounds. Thereafter DNA oligo-nucleotides were desalted using an NAP-5 column and mixed with the AuNPs in ratio 46:1 (AuNPs:oligo). After incubation, the samples were spin for 30 min. under 13 000 rpm, the supernatant with unbound DNA oligonucleotides was removed, conjugates washed one time and resuspended in the appropriate buffer. For quantification of the bound DNA oligonucleotides, a fluorescence spectrophotometric method was used. Due to this, fluorescently labeled oligonucleotides were used for these optimizing steps. All the measurements were performed using an Infinite M200 well-plated reader (Tecan, Salzburg, Austria). The concentration of bound oligonucleotides was calculated from the respective calibration curve.

Table 1 *Overview of binding protocols / procedures*

	Gold nanoparticles		Oligonucleotides	Binding
	Size [nm]	Concentration of AuNP in reaction [nmol]	Concentration of oligonucleotides in reaction [nmol]	Protocol
1	10	230	5	Incubation for 4 h under 37 °C; in **water**; salt stabilization 0.1M NaCl, overnight 38 °C
2				**10 mM citrate-HCl buffer pH 3**; lab. temp. incubation 10 min.; salt stabilization 0.3 M NaCl; lab. temp. incubation 20 min
3				**5 mM HEPES buffer pH 9**; 16 h incubation at lab. temp.; than salt stabilization 0.1 M NaCl.
4				**10mM PBS buffer pH 7.4** , 16 h incubation at lab. temp.; add **0.01% SDS**, salt stabilization 1M NaCl, 30 min incubation at lab. temp.

Conjugation of gold nanoparticles with antibodies

Anti-*Listeria* antibodies were immobilized on the surface of AuNPs by one- or two-step carbodiimide method in different ratio of AuNPs and antibodies. In the case of one-step method, all the reagents were mixed at the same time (300 µg of EDC, 50 µg of sulfo-NHS, 30 µg of AuNPs and 15 resp. 30 µg of antiodies depending on the ratio chosen). In the case of two-steps method, particles were firstly preactivated with EDC and sulfo-NHS for 10 minutes, washed, and antibodies were added to pre-activated particles at the aforementioned ratios. Particles with antibodies were incubated overnight moderately rotated at 4 °C. After incubation, the particles were washed and the complex antibody-AuNPs purified on Pure proteome protein "A" magnetic particles to remove the unbound (naked) AuNPs. The binding efficiencies were determined indirectly using a standard of tris/glycin SDS-PAGE, followed with silver staining and densitometric detection. The amount of immobilized antibodies was calculated from the difference in the densities of antibodies in the initial solution and the total antibodies in the supernatant after completing the immobilization and washing steps. Prepared conjugates were checked by electrochemical detection via conditions specified in the next paragraph.

Electrochemical detection of *Listeria* ssp. bacteria cells

Magnetic immunosorbent with specific anti-*Listeria* antibodies (0.1 mg, prepared according to previously published [12]) was mixed with 50 µL of PBS buffer containing 0.05% Tween 20 (PBS-T) spiked with 500 *Listeria* CFU and with 75 µL of the electrochemically active probe (75 µL containing 7.5 µg of AuNPs and 7.5 resp. 3.75 µg of Ab).

In the case of negative control, 50 μL of PBS-T buffer was used instead of bacteria suspension. Incubation for 1 hour at room temperature took place under rotation regime. After that, the resulting magnetic particle-bacteria-conjugate complexes were magnetically separated and washed five times with 1 mL of PBS-T buffer. All electrochemical measurements were performed with a portable PalmSens interface (PalmSens, GA Houten, The Netherlands) controlled by software PSTrace 4.8 (the same manufacturer) and connected with a screen-printed three-electrode system with carbon as the working and auxiliary electrodes and a silver pseudo-reference (DropSens, Spain) completing the cell.

The *Listeria* cells were detected after 5 min. incubation in the presence of 100 μL 0.1 M HCl. The Au(III) ions were monitored by square-wave cathodic stripping voltammetry (SWCSV) coupled with the SPC-3-EL-cell. Parameters of the SWV ramp were as follows: deposition potential, 1.2 V for 180 s; condition potential, 0.15 V for 120 s; potential range, 1.2 V – 0.0 V vs. ref.; step potential, –0.003 V; pulse amplitude, 0.02805 V; and SWV-frequency, 25 Hz.

Results and discussion

In this work, we have tested potential of AuNPs as an electrochemically active label especially for their unique optical and physical properties. Our goal was to prepare conjugates using different types of particles and different types of analyte bound onto their surface.

AuNPs are usually prepared by citrate reduction method and stabilized via the negative charges by citrate ions [13–14]. Under standard conditions, the DNA molecules are highly negatively charged and being repelled by AuNPs. To screen the negative charge of AuNPs, a solution with high concentration of NaCl should be added although this addition of highly concentrated salt may lead to the irreversible aggregation of particles. This problem can be solved by the process called "salt aging", when such a salt is gradually added together with the molecules of DNA. While DNA is still at an excess against the salt, the aggregation of particles is then prevented. In this work, four various protocols working with different buffers and different processes using the salt aging method were performed and compared; see Table 1. The proposed procedures were taken from the literature and modified. For optimizing steps, the DNA molecules labelled with fluorescent tag Cy3 were used for easier determination of capture efficiency, when the intensity of fluorescence correlated with the concentration of bound DNA oligonucleotides. The capture efficiency expressed by numbers of DNA chains bound on the surface of AuNPs was calculated from calibration curve. Because there exists a mathematical relationship for predicting suitable saturation of the nanoparticles surface with oligonucleotides, we could compare our results with theoretical assumption. Namely, one should bound around 68 ± 10 chains per one unit of AuNP with diameter 10 nm.

We were not far from this prediction, so there was no need to change the protocol to increase the capture efficiency. Except the binding in water (63 chains of DNA per one AuNP), there were no significant differences among the individual protocols; see Fig. 1. The most effective was the procedure with 10mM PBS containing 0.01% SDS used as the detergent avoiding undesirable aggregation of AuNPs. We could estimate that, by this protocol, 154 of DNA oligonucleotide chains were bound on the surface of one nanoparticle. For future application, protocol with citrate buffer was chosen. As we have estimated, around 134 of DNA oligonucleotide chains were bound on the surface of one nanoparticle. Moreover, from the sufficient binding efficiency, this protocol was not time-consuming and the conjugates could be prepared in 1.5 hours.

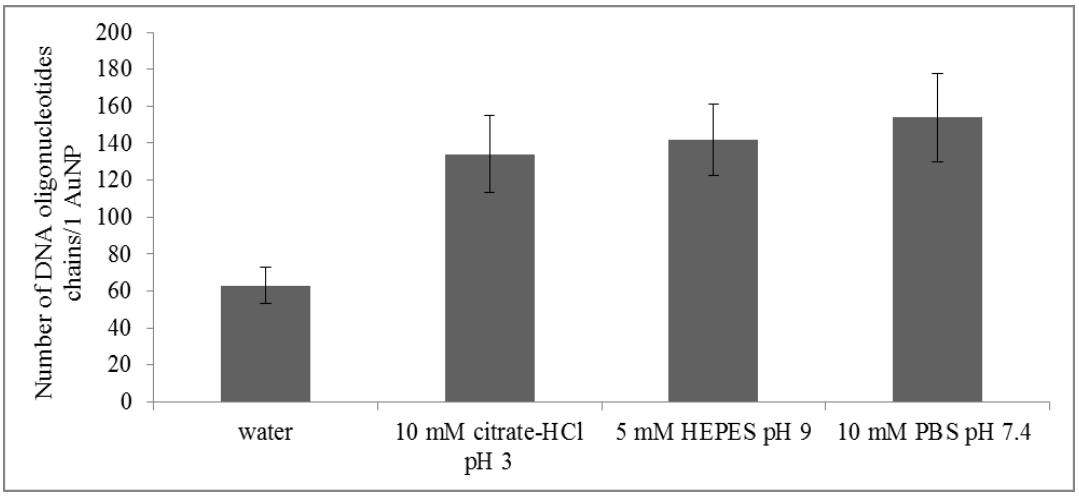

Fig. 1 *Immobilization of reduced oligonucleotides on AuNPs in four different buffers*

Second part deals with the modification of AuNPs by antibodies via crosslinker. On the surface of AuNPs containing functional groups, the specific anti-*Listeria* antibodies were bound. One- or two-step carbodiimide methods were tested. In addition, the different ratios of AuNPs and antibodies (1:1 or 2:1, resp.) were optimized. The advantage of one-step method is the absence of washing which extends analysis time. On the other hand, this washing step removes redundant reagents that could affect the subsequent procedures so we had to evaluate the quality and quantity of final product. The binding efficiencies were estimated by the SDS-PAGE via densitometry evaluation. Binding efficiencies were expressed as the percentage of molecules of antibodies immobilized onto the surface of AuNPs versus the initial amount of antibodies used for immobilization. As shown in Fig. 2, concerning the one-step method, the capture efficiency of antibodies was around 68 % (initial ratio of AuNPs:Ab was 1:1) and in the case of two time surplus of AuNPs (initial ratio 2:1) the capture efficiency was around 60 %. In the case of two-step method, the binding efficiency was substantially lower, being around 36 % resp. 31 %.

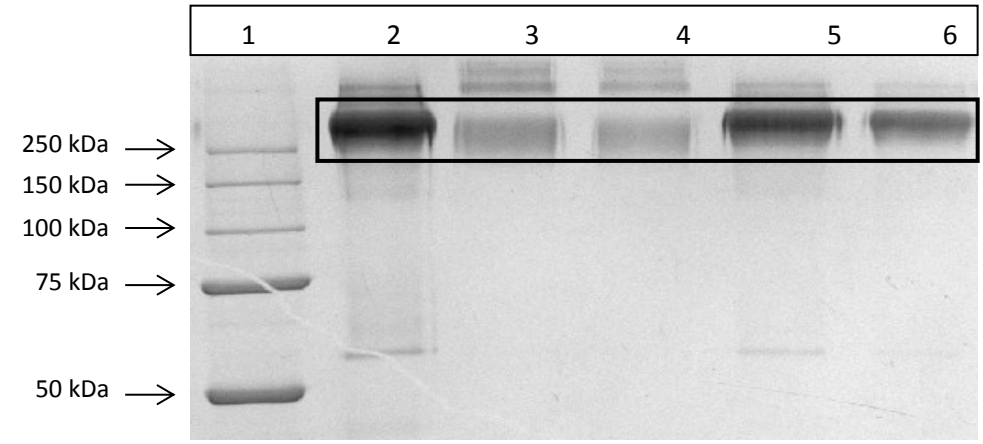

Fig. 2 *SDS-PAGE analysis followed by silver staining Lanes*

1 – molecular weight marker (10–250 kDa); 2 – initial experiment (sample);
3 – binding fraction of one step carbodiimide method – AuNPs:Ab ratio 1:1;
4 – binding fraction of one step carbodiimide method – AuNPs:Ab ratio 2:1;
5 – binding fraction of two steps carbodiimide method – AuNPs:Ab ratio 1:1;
6 – binding fraction of two steps carbodiimide method – AuNPs:Ab ratio 2:1.

Prepared conjugates (contaminated by naked AuNPs) were purified by magnetic particles containing protein "A". This substance specifically bound antibodies via their Fc fragments so that the conjugates had consisted of antibodies and AuNPs could be captured. Naked AuNPs that might affect subsequent experiments were thus separated off. To control the nonspecific sorption of AuNPs on carrier with protein "A", the aliquot of naked particles was used. In Fig. 3, one can see a voltammogram with the efficient quantitative elution of conjugated molecules from carrier with protein "A". In the case of all four conjugates, the current signals were monitored being typical for gold with appropriate peak height (–1.049, –0.695, –1.2, and –1.759 μA). These results have suggested us successful preparation and purification of anti-*Listeria* conjugates.

The functionality and usability of these conjugates were then verified by model samples with *Listeria* cells. For preliminary experiments, two conjugates were used (prepared by one- or two steps method with initial ratio of AuNPs:Ab 2:1). Conjugates were mixed together with anti-*Listeria* magnetic immunosorbent and sample spiked with *Listeria* cells. During 60 min. incubation, *Listeria* cells should be captured by magnetic immunosorbent and so caught cells were labelled with the conjugates at the same time. After incubation, these complexes were captured from the samples (due to magnetic immunosorbent), washed, and the signal generated from electrochemically active conjugate measured on SPCE. In Fig. 4, a voltammogram with positive and negative control is drawn clearly showing the differences. The signals of both negative controls were nearly 0 μA and for positive samples provided signals –0.157 μA and 0.165 μA, resp. These results indicate that the prepared conjugates give rise to an electrochemically measurable signal enabling to distinguish the samples with *Listeria* cells from negative samples.

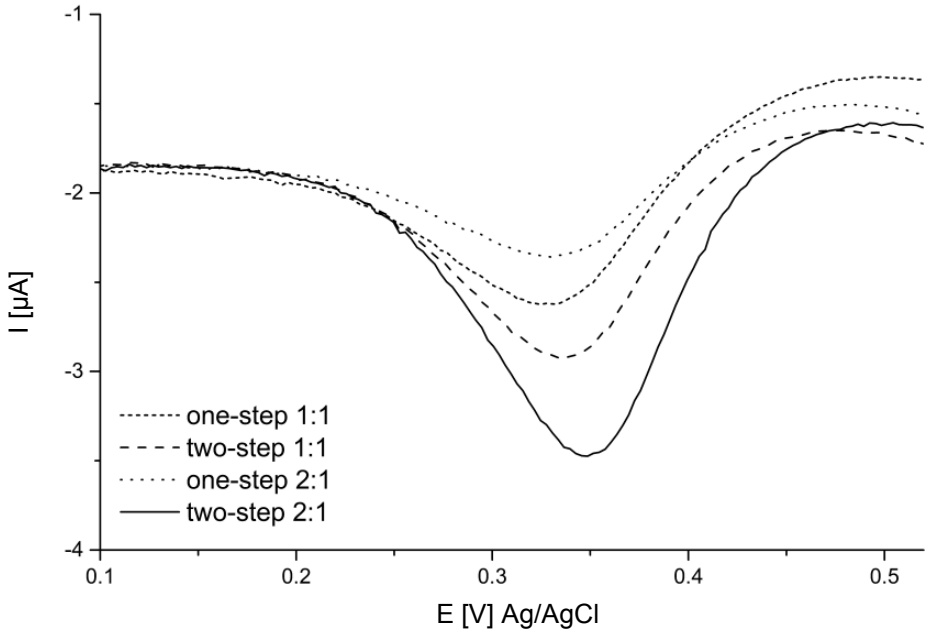

Fig. 3 *Square-wave voltammograms of conjugates prepared one- or two-step method and with different ratio of AuNPs and anti-Listeria antibodies*

Measurements were performed with SPCE under conditions for electrochemical detection specified in the experimental part ("Materials and methods").

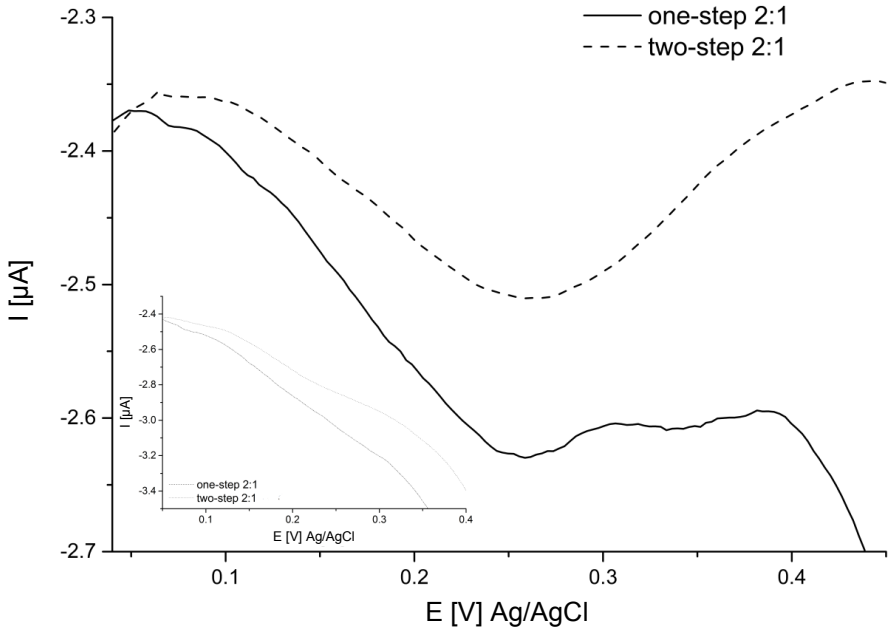

Fig. 4 *Square-wave voltammograms of Listeria-cells detection with the aid of conjugates prepared and serving as a part of biosensor*

Initial ratio of AuNPs:Ab is 2:1, both one- and two- steps method are compared. Measurements were performed with SPCE under conditions for electrochemical detection specified in Experimental. Voltammogram with negative control is inserted.

Conclusions

This work deals with the modification of AuNPs for their future use as a signal generating unit in biosensor configurations. Two types of AuNPs were chosen. Non-functionalized nanoparticles were used for conjugation with thiolated DNA oligonucleotides to create the DNA probe. This bond utilizes the affinity of surface of the colloidal AuNPs and thiol-conjugated DNA oligonucleotides. The main problem was to be repulsion between the negatively charged nanoparticles and DNA strains, which could however be eliminated using salt-aging protocol. In this work, we successfully modified AuNPs by thiolated oligonucleotides with four various protocols. Such prepared DNA probe could then be used in other applications; e.g., for detection with piezoelectric biosensor.

The second type of AuNPs had had functional carboxyl groups. On such type of particles, various proteins should be bound with the help of carbodiimide crosslinker via the respective amino groups. We have tried to optimize the most effective procedure that would provide the most useful conjugate. Such conjugates are meant to be applied as the electrochemically active part of biosensor for detection of bacteria. Regarding this, we were able to prepare the conjugates of AuNPs and anti-*Listeria* antibodies. These conjugates were found electrochemically active and, during preliminary experiments, it was confirmed that they could be used as a part of electrochemical biosensors for detection of bacteria cells.

Acknowledgement

This work was supported by the European Union's LOVE-FOOD 2 MARKET project under the contract No. 687681.

References

[1] Parida U.K., Biswal S.K., Nayak P.L., Bindhani B.K.: Gold nano particles for biomedical applications. *World Journal of Nano Science and Technology* **2** (2013) 47–57.

[2] Zhang X.: Gold Nanoparticles: Recent advances in the biomedical applications. *Cell Biochemistry and Biophysics* **72** (2015) 771–775.

[3] Parida U.K., Nayak P.L.: Biomedical applications of gold nanoparticles: opportunity and challenges. *World Journal of Nano Science and Technology* **1** (2012) 10–25.

[4] Das M., Shim K.H., An S.S.A., Yi D.K.: Review on gold nanoparticles and their applications. *Toxicology and Environmental Health Sciences* **3** (2011) 193–205.

[5] Su S., Zuo X.L., Pan D., Pei H., Wang L.H., Fan C., Huang W.: Design and applications of gold nanoparticle conjugates by exploiting biomolecule-gold nanoparticle interactions. *Nanoscale* **5** (2013) 2589–2599.

[6] Hurst S.J., Lytton-Jean A.K.R., Mirkin C.A.: Maximizing DNA loading on a range of gold nanoparticle sizes. *Analytical Chemistry* **78** (2006) 8313–8318.
[7] Herne T.M., Tarlov M.J.: Characterization of DNA probes immobilized on gold surfaces. *Journal of the American Chemical Society* **119** (1997) 8916–8920.
[8] Tiwari P., Vig K., Dennis V., Singh S.: Functionalized gold nanoparticles and their biomedical applications. *Nanomaterials* **1** (2011) 31–63.
[9] Hill H.D., Millstone J.E., Banholzer M.J., Mirkin C.A.: The role radius of curvature plays in thiolated oligonucleotide loading on gold nanoparticles. *ACS Nano* **3** (2009) 418–424.
[10] Zhang X., Servos M.R., Liu J.: Instantaneous and quantitative functionalization of gold nanoparticles with thiolated DNA using a pH-assisted and surfactant-free route. *Journal of the American Chemical Society* **134** (2012) 7266–7269.
[11] Chen S., Wu V.C.H., Chuang Y., Lin C.: Using oligonucleotide-functionalized Au nanoparticles to rapidly detect foodborne pathogens on a piezoelectric biosensor. *Journal of Microbiological Methods* **73** (2008) 7–17.
[12] Srbova J., Krulisova P., Holubova L., Pereiro I., Bendali A., Hamiot A., Podzemna V., Macak J., Dupuy B., Descroix S., Viovy J.-L., Bilkova Z.: Advanced immunocapture of milk-borne Salmonella by microfluidic magnetically stabilized fluidized bed. *Electrophoresis* **39** (2018) 526–533.
[13] Turkevich J.: Colloidal gold. Part II. *Gold Bulletin* **18** (1985) 125–131.
[14] Kimling J., Maier M., Okenve B., Kotaidis V., Ballot H., Plech A.: Turkevich method for gold nanoparticle synthesis revisited. *Journal of Physical Chemistry* **110** (2006) 15700–15707.