PHOTON-UPCONVERSION NANOPARTICLES FOR SINGLE-MOLECULE IMMUNOSENSING OF CANCER BIOMARKERS AND BACTERIA

[1]Zdeněk FARKA, [2]Matthias J. MICKERT, [3]Antonín HLAVÁČEK, [1]Veronika POLÁCHOVÁ, [4]Uliana KOSTIV, [1]Matěj PASTUCHA, [4]Daniel HORÁK, [2]Hans H. GORRIS, [1]Petr SKLÁDAL

[1]CEITEC MU, Masaryk University, Brno, Czech Republic, EU,
farka@mail.muni.cz
[2]Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, Germany, EU
[3]Institute of Analytical Chemistry of the Czech Academy of Sciences, Brno, Czech Republic, EU
[4]Institute of Macromolecular Chemistry of the Czech Academy of Sciences, Prague, Czech Republic, EU

https://doi.org/10.37904/nanocon.2019.8445

Abstract

The recent progress in the field of immunoassays has been driven by introduction of various kinds of nanomaterials. In particular, photon-upconversion nanoparticles (UCNPs) proved to be excellent immunoassay labels due to their ability to emit light of shorter wavelengths under near-infrared excitation (anti-Stokes emission), which prevents autofluorescence, minimizes light scattering, and thus reduces the optical background interference. These unique photoluminescent properties allow counting of individual biomolecules labeled with UCNPs by conventional wide-field epiluminescence microscopy and enable the development of single-molecule (digital) immunoassays. We have introduced a novel label based on UCNPs conjugated with streptavidin *via* poly(ethylene glycol) and applied it in a digital upconversion-linked immunosorbent assay (ULISA) for the detection of a cancer biomarker prostate specific antigen (PSA). The digital readout based on counting of individual immunocomplexes improved the sensitivity 16× compared to conventional analog readout and allowed to reach a limit of detection (LOD) of 23 fg·mL^{-1} (800 aM). Human serum samples were successfully analyzed achieving an excellent correlation with electrochemiluminescence reference method. The conjugates of UCNPs with streptavidin are also suitable for the detection of pathogenic bacterium *Melissococcus plutonius*, the causative agent of honeybee disease European foulbrood. The ULISA assay provided an LOD of 340 CFU·mL^{-1} and successfully analyzed real samples of bees, larvae and bottom hive debris. Due to the high reliability and relatively simple detection scheme, the digital ULISA can pave the way for a new generation of digital immunoassays with a strong potential for commercialization.

Keywords: Single-molecule detection, photon-upconversion nanoparticle, bioconjugation, digital upconversion-linked immunosorbent assay

1. INTRODUCTION

Due to their high specificity, immunochemical assays are widely useful for detection of various analytes within complex matrices. The most commonly used immunoanalytical method - enzyme-linked immunosorbent assay (ELISA) - relies on signal generation by enzymes, typically horseradish peroxidase [1]. The enzymes provide highly sensitive detection, because single enzyme molecule can transform numerous molecules of substrate. However, enzymes also suffer several disadvantages, most notably low stability [2]. The recent progress in nanotechnology has provided various nanomaterials, which can be used as labels in immunoassays [3]. The detection schemes can be based on: (i) transformation of substrate, similarly to the use of enzymes, or (ii) on direct detection of the label. For the detection based on substrate transformation, various kinds of catalytic nanoparticles (nanozymes) can be employed, typically mimicking the peroxidase activity [4]. The direct detection of the label often exploits fluorescent nanoparticles, including quantum dots [5] and carbon dots [6].

The convenient readout of fluorescence signal is, however, accompanied by a serious disadvantage, the presence of background signal, which is especially important in case of complex biological matrices.

The background signals can be avoided by the use of materials exhibiting anti-Stokes emission, such as photon-upconversion nanoparticles (UCNPs). The typical UCNPs consist of a hexagonal $NaYF_4$ host crystal doped with well-defined amounts of lanthanides, in particular Yb^{3+} and Er^{3+} [7]. UCNPs strongly reduce autofluorescence and light scattering because they can be excited by near-infrared (NIR) light and emit light of shorter wavelengths [8]. Further advantages of UCNPs include a high photostability and multiple emission bands that can be tuned individually for the multiplexed assay formats [9]. The preparation of bioconjugates of UCNPs, especially with antibodies or streptavidin, allowed the development of upconversion-linked immunosorbent assays (ULISAs). ULISA assays were previously reported for detection of different analytes, including α-fetoprotein [10], cardiac troponin I [11], and sulfaquinoxaline [12]. Recently, we have developed a microscopic approach for detection of single UCNPs and applied it for the detection of cancer biomarker prostate specific antigen (PSA) [13]. The measurement was based on readout of individual sandwich immunocomplexes, consisting of anti-PSA antibody immobilized on the surface of microtiter plate, captured analyte PSA, and conjugate of anti-PSA antibody with UCNP.

With the increasing sensitivity of the readout methods, the minimization of non-specific binding is getting ever higher importance. In this work, we have focused on the preparation of conjugates of UCNPs with streptavidin, based on PEG and coating by bovine serum albumin (BSA), with the focus on high sensitivity and the lowest possible level of non-specific binding. The conjugates were employed for the detection of PSA and bee pathogen *Melissococcus plutonius*.

2. MATERIAL AND METHODS

2.1. Preparation of UCNP-PEG-streptavidin labels

The $NaYF_4$:Yb^{3+},Er^{3+} and $NaYF_4$:Yb^{3+},Tm^{3+}-based UCNPs were synthesized by high-temperature co-precipitation [14]. To prepare alkyne-PEG-neridronate, sodium neridronate was mixed with alkyne-PEG-NHS, the product was dialyzed (MWCO 3.5 kDa) against water and freeze-dried. The surface of the UCNPs was modified by mixing an aqueous UCNP dispersion with alkyne-PEG-neridronate. After stirring for 24 h at room temperature, dialysis (MWCO 14 kDa) was performed. The streptavidin-azide (7 Bioscience) was coupled to the surface of UCNPs *via* copper-catalyzed click chemistry. The alkyne-PEG-UCNPs were mixed with streptavidin-azide and sodium L-ascorbate and purged with argon to remove oxygen. Afterwards, $CuSO_4$ was added to generate the Cu(I) catalyst *in situ*. The prepared conjugates were sonicated, dialyzed (MWCO 100 kDa) and stored at 4 °C [15].

2.2. Digital ULISA for PSA detection

A high-binding polystyrene 96-well microtiter plate (μClear, Greiner Bio-One) was coated with polyclonal horse anti-mouse antibody (3 μg·mL^{-1}; Vector Laboratories) in coating buffer (50 mM carbonate/bicarbonate, 0.05 % NaN_3, pH 9.6) at 4 °C overnight. All following incubation steps were performed for 1 h at room temperature. After each step, the plate was washed 4 times with washing buffer (50 mM phosphate, 150 mM NaCl, 0.05 % NaN_3, pH 7.4). After blocking with 1 % BSA in washing buffer, monoclonal mouse anti-PSA antibody (0.3 μg·mL^{-1}; Ab403, Abcam) in assay buffer (50 mM Tris, 150 mM NaCl, 0.05 % NaN_3, 0.5 % bovine gamma globulin, 0.2 % BSA, 0.01 % Tween 20, 0.2 % poly(vinyl alcohol), 1 % glucose, 5 mM EDTA, and 1 mM KF) was bound. Afterwards, the plate was incubated either with serial dilutions of PSA standard (Abcam, ab78528) or with clinical human serum samples (local hospital). Finally, biotinylated anti-PSA antibody (0.25 μg·mL^{-1}; BAF1344, R&D Systems) was bound, followed by the UCNP-PEG-streptavidin conjugate (3.5 μg·mL^{-1}).

For the conventional analog readout, dried microtiter plate was scanned using custom-built upconversion microtiter plate reader (Chameleon, Hidex). Before performing the digital readout, glycerol was added to the dry wells to minimize local heating effects during NIR excitation. A modified epifluorescence microscope (Eclipse Ti-E, Nikon) [13] was used to record 9 wide-field images per well. The total number of UCNPs was counted using software NIS elements (Nikon). For both readout modes, the average and standard deviation were calculated from three wells and analyzed using a four-parameter logistic function [15].

2.3. Preparation of UCNP-BSA-streptavidin labels

The $NaYF_4$:Yb^{3+},Er^{3+} UCNPs were first modified by carboxylated silica layer using water-in-oil microemulsion method [16]. Afterwards, streptavidin was conjugated to the UCNPs *via* BSA layer using copper-free click chemistry. First, fluorescent click-reactive BSA-alkyne was prepared. (1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl *N*-succinimidyl carbonate (BCN-NHS) was mixed with 5-carboxyrhodamine *N*-succinimidyl ester (Rh-NHS), the mixture was added to BSA solution and incubated for 4 h. The carboxyrhodamine-labelled BSA-alkyne conjugate was purified by dialysis (MWCO 12.4 kDa).

The BSA-alkyne was conjugated to the UCNPs to prepare a click-reactive product. The carboxyl groups of UCNPs were activated by mixture of EDC and NHS. The dispersion was sonicated for 10 min, followed by a short centrifugation and redispersion in solution containing BSA-alkyne. After 90 min of mixing, the UCNP-BSA-alkyne conjugate was purified by centrifugation (1700 *g*, 5 times).

Fluorescent click-reactive streptavidin-azide was prepared by coupling 5-azidopentanoic acid (APA) and 5(6)-carboxyfluorescein (CF) with the streptavidin. Free carboxyl groups of APA and CF were activated using EDC/sulfo-NHS and incubated for 1 h. Then, the APA and CF solutions were mixed with streptavidin and incubated for 4 h. The click-reactive streptavidin-azide was purified by dialysis (MWCO 12.4 kDa).

In the final copper-free click conjugation, UCNP-BSA-alkyne was centrifuged (1700 *g*) and redispersed with streptavidin-azide. The solution was then dialyzed against 100 mM sodium MES, pH 4.5, which allowed electrostatic attraction and enabled an efficient click-conjugation. The UCNP-BSA-streptavidin conjugates were centrifuged (1700 *g*), redispersed in 50 mM Tris, 50 mM H_3BO_3, pH 8.6, and stored at 4 °C [17].

2.4. ULISA for *M. plutonius* detection

To allow specific detection of *M. plutonius*, rabbit polyclonal antibody was prepared [18]. The rabbits were immunized by *M. plutonius* cell wall fraction, followed by a booster antigen injection after 35 days. After 10 days, rabbit serum was collected and immunoglobulin G fraction was purified on a protein G column. To prepare the detection conjugate, antibody was modified by biotin. Biotinamidohexanoic acid *N*-hydroxysuccinimide ester (NHS-LC-biotin) was added in 15 times molar excess to the anti-*M. plutonius* antibody. The Ab-biotin conjugate was purified using Amicon Ultra 0.5 mL centrifugal filters 100K (MWCO 100 kDa) and stored at 4 °C.

For the ULISA assay, a high-binding microtiter plate (Microlon, Greiner Bio-One) was coated with anti-*M. plutonius* antibody in PBS at 4 °C overnight. All following steps were carried out at room temperature, after each incubation, the plate was washed 4 times with washing buffer. The plate was blocked using 5 % powdered milk in PBS, followed by incubation either with standard dilutions of bacteria in assay buffer or spiked in bee, larvae or bottom hive debris extracts diluted in assay buffer. Afterwards, the microtiter plate was incubated with biotin-conjugated anti-*M. plutonius* antibody and UCNP-BSA-streptavidin conjugate. After drying, the microtiter plate was scanned using up conversion microplate reader equipped with a continuous 980 nm laser (Laserland) and CCD spectroscope QE65 Pro (Ocean Optics) [17].

3. RESULTS AND DISCUSSION

3.1. Digital detection of PSA using UCNP-PEG-streptavidin label

The PEG-based conjugates were employed in the sandwich ULISA for the detection of PSA (**Figure 1**). After optimization of the assay parameters, the two readout modes were compared. The digital mode provided LOD of 23 fg·mL^{-1} (800 aM), which is approximately 20 times lower than the LOD of the analog mode (410 fg·mL^{-1}). Compared to our previous work on PSA detection using UCNP-SiO_2-antibody conjugates (LOD 1.2 pg·mL^{-1}) [13], improvement of sensitivity of 50 times was achieved. This can be attributed to two factors: (1) The use of biotinylated antibody, which acts as a flexible linker facilitating the binding of the nanoparticle label. Furthermore, the high affinity of biotin-streptavidin interaction allows decreasing of UCNP label concentration, which has a positive effect on reduction of the non-specific binding. (2) The level of non-specific binding was further reduced by the PEG coating on the surface of the UCNP [15].

The optimized assay was used for the determination of PSA levels in clinical human serum samples. Random serum samples were collected in a hospital and analysed by an electrochemiluminescence immunoassay (Roche Elecsys) as a standard method. The digital ULISA provided recovery rates between 74 and 102 %. The achieved results, especially the low LOD value, demonstrate the potential of digital ULISA for monitoring of patients after radical prostatectomy, where monitoring of low PSA concentrations is required [19].

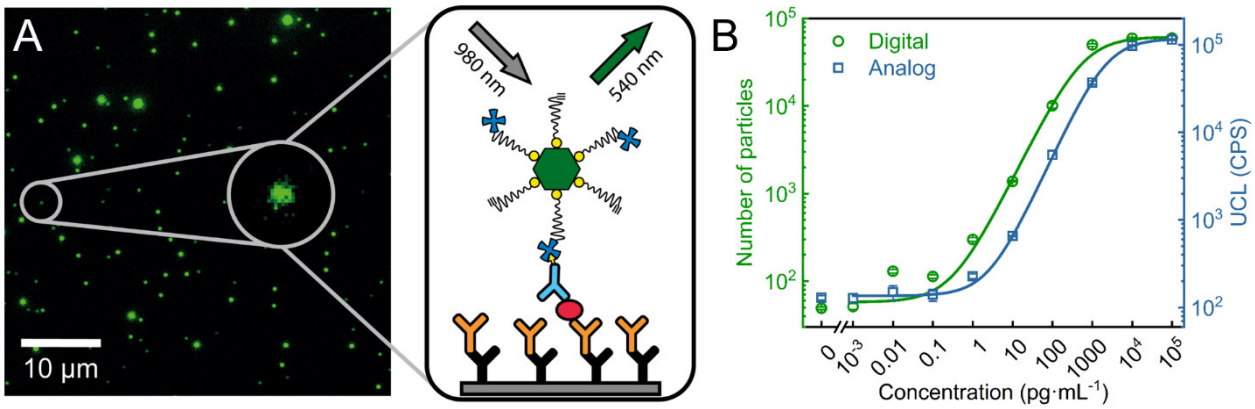

Figure 1 (A) Up conversion microscopy image of microtiter plate after specific binding of 100 pg·mL^{-1} of PSA with schematic representation of single immunocomplex. (B) Calibration curve for digital and analog ULISA assays for PSA detection [15].

3.2. Detection of *M. plutonius* using UCNP-BSA-streptavidin label

In order to detect *M. plutonius*, the causative agent of European foulbrood, rabbit polyclonal antibody was prepared. The antibodies were tested by ELISA assay (LOD 1.4×10^5 CFU·mL^{-1}), which later served also as a standard method for comparison of ULISA performance. The silica-coated UCNPs (**Figure 2A**) were modified by BSA layer with click-reactive BCN, which served for conjugation of azide-modified streptavidin. The successful bioconjugation was confirmed by dynamic light scattering and gel electrophoresis. Afterward, the UCNP-BSA-streptavidin was used as a label in the sandwich assay for *M. plutonius*. LOD of 340 CFU·mL^{-1} with a wide working range up to 10^9 CFU·mL^{-1} was achieved, which is 400 times better than the LOD of the conventional ELISA [17].

The practical impact of the assay was demonstrated on the analysis of real samples of spiked bees, larvae and bottom hive debris. The 10 times diluted real samples provided LODs in the range between 540 CFU·mL^{-1} and 8.5×10^3 CFU mL^{-1} (**Figure 2B**). Since the amount of *M. plutonius* in infected apiaries with clinical

symptoms is typically around 10^5 CFU·mL^{-1} [20], the ULISA assay is a suitable method for early-stage diagnosis of European foulbrood infections.

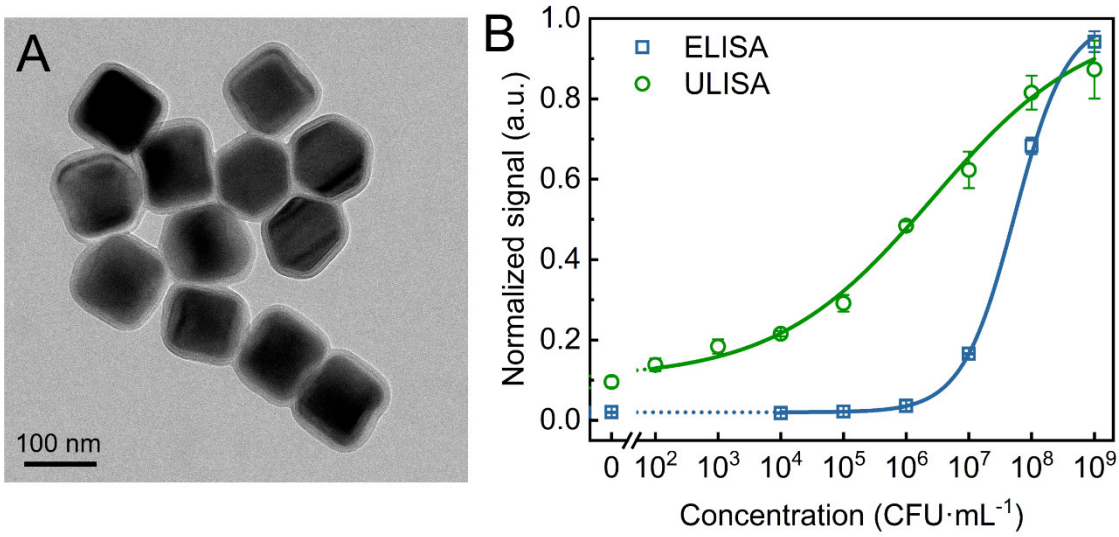

Figure 2 (A) TEM image of silica-coated UCNPs. (B) Calibration curves of sandwich ELISA- and ULISA-based detection of *M. plutonius* spiked in 10% bee extract [17].

4. CONCLUSIONS

The streptavidin-conjugated UCNPs can serve as highly sensitive labels in immunoassays based on biotinylated antibodies. We have developed a digital ULISA for the detection of PSA, which provided sub-femtomolar LOD of 23 fg·mL^{-1} (800 aM). The assay was successfully employed to analysis of clinical samples and provided excellent correlation with standard electrochemiluminescence method. Furthermore, we have developed ULISA assay for detection of *M. plutonius*, the causative agent of European foulbrood, with LOD of 340 CFU·mL^{-1}, and demonstrated its practical applicability on the analysis of real samples of spiked bees, larvae and bottom hive debris.

ACKNOWLEDGEMENTS

This research has been financially supported by the Technology Agency of the Czech Republic (project TJ01000386) and Ministry of Education, Youth and Sports of the Czech Republic under the projects CEITEC 2020 (LQ1601) and INTER-ACTION LTAB19011. CIISB research infrastructure project LM2015043, funded by MEYS CR, is acknowledged for financial support of the measurements at CF Cryo-electron Microscopy and Tomography and CF Nanobiotechnology.

REFERENCES

[1] LEQUIN, R.M. Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clin. Chem.* 2005, vol. 51, no. 12, pp. 2415-2418.

[2] WEI, H. and WANG, E.K. Nanomaterials with enzyme-like characteristics (nanozymes): next-generation artificial enzymes. *Chem. Soc. Rev.* 2013, vol. 42, no. 14, pp. 6060-6093.

[3] FARKA, Z., JUŘÍK, T., KOVÁŘ, D., TRNKOVÁ, L. and SKLÁDAL, P. Nanoparticle-Based Immunochemical Biosensors and Assays: Recent Advances and Challenges. *Chem. Rev.* 2017, vol. 117, no. 15, pp. 9973-10042.

[4] YANG, N., HUANG, Y.X., DING, G.S. and FAN, A.P. In Situ Generation of Prussian Blue with Potassium Ferrocyanide to Improve the Sensitivity of Chemiluminescence Immunoassay Using Magnetic Nanoparticles as Label. *Anal. Chem.* 2019, vol. 91, no. 7, pp. 4906-4912.

[5] ANNIO, G., JENNINGS, T.L., TAGIT, O. and HILDEBRANDT, N. Sensitivity Enhancement of Forster Resonance Energy Transfer Immunoassays by Multiple Antibody Conjugation on Quantum Dots. *Bioconjugate Chem.* 2018, vol. 29, no. 6, pp. 2082-2089.

[6] WU, Y.Y., WEI, P., PENGPUMKIAT, S., SCHUMACHER, E.A. and REMCHO, V.T. Development of a Carbon Dot (C-Dot)-Linked Immunosorbent Assay for the Detection of Human alpha-Fetoprotein. *Anal. Chem.* 2015, vol. 87, no. 16, pp. 8510-8516.

[7] HAASE, M. and SCHAFER, H. Upconverting Nanoparticles. *Angew. Chem. Int. Ed.* 2011, vol. 50, no. 26, pp. 5808-5829.

[8] ZHOU, B., SHI, B.Y., JIN, D.Y. and LIU, X.G. Controlling upconversion nanocrystals for emerging applications. *Nat. Nanotechnol.* 2015, vol. 10, no. 11, pp. 924-936.

[9] EHLERT, O., THOMANN, R., DARBANDI, M. and NANN, T. A four-color colloidal multiplexing nanoparticle system. *ACS Nano.* 2008, vol. 2, no. 1, pp. 120-124.

[10] LUO, Z.B., ZHANG, L.J., ZENG, R.J., SU, L.S. and TANG, D.P. Near-Infrared Light-Excited Core-Core-Shell UCNP@Au@CdS Upconversion Nanospheres for Ultrasensitive Photoelectrochemical Enzyme Immunoassay. *Anal. Chem.* 2018, vol. 90, no. 15, pp. 9568-9575.

[11] SIRKKA, N., LYYTIKAINEN, A., SAVUKOSKI, T. and SOUKKA, T. Upconverting nanophosphors as reporters in a highly sensitive heterogeneous immunoassay for cardiac troponin I. *Anal. Chim. Acta.* 2016, vol. 925, no. 82-87.

[12] HU, G.S., SHENG, W., ZHANG, Y., WANG, J.P., WU, X.N. and WANG, S. Upconversion Nanoparticles and Monodispersed Magnetic Polystyrene Microsphere Based Fluorescence Immunoassay for the Detection of Sulfaquinoxaline in Animal-Derived Foods. *J. Agric. Food. Chem.* 2016, vol. 64, no. 19, pp. 3908-3915.

[13] FARKA, Z., MICKERT, M.J., HLAVÁČEK, A., SKLÁDAL, P. and GORRIS, H.H. Single Molecule Upconversion-Linked Immunosorbent Assay with Extended Dynamic Range for the Sensitive Detection of Diagnostic Biomarkers. *Anal. Chem.* 2017, vol. 89, no. 21, pp. 11825-11830.

[14] WANG, F., HAN, Y., LIM, C.S., LU, Y.H., WANG, J., XU, J., CHEN, H.Y., ZHANG, C., HONG, M.H. and LIU, X.G. Simultaneous phase and size control of upconversion nanocrystals through lanthanide doping. *Nature.* 2010, vol. 463, no. 7284, pp. 1061-1065.

[15] MICKERT, M.J., FARKA, Z., KOSTIV, U., HLAVÁČEK, A., HORÁK, D., SKLÁDAL, P. and GORRIS, H.H. Measurement of Sub-femtomolar Concentrations of Prostate-Specific Antigen through Single-Molecule Counting with an Upconversion-Linked Immunosorbent Assay. *Anal. Chem.* 2019, vol. 91, no. 15, pp. 9435-9441.

[16] LIU, F.Y., ZHAO, Q., YOU, H.P. and WANG, Z.X. Synthesis of stable carboxy-terminated $NaYF_4$:Yb^{3+}, Er^{3+}@SiO_2 nanoparticles with ultrathin shell for biolabeling applications. *Nanoscale.* 2013, vol. 5, no. 3, pp. 1047-1053.

[17] POLÁCHOVÁ, V., PASTUCHA, M., MIKUŠOVÁ, Z., MICKERT, M.J., HLAVÁČEK, A., GORRIS, H.H., SKLÁDAL, P. and FARKA, Z. Click-conjugated photon-upconversion nanoparticles in an immunoassay for honeybee pathogen *Melissococcus plutonius*. *Nanoscale.* 2019, vol. 11, no. 17, pp. 8343-8351.

[18] FRÁNEK, M., DIBLÍKOVÁ, I., ČERNOCH, I., VASS, M. and HRUŠKA, K. Broad-specificity immunoassays for sulfonamide detection: Immunochemical strategy for generic antibodies and competitors. *Anal. Chem.* 2006, vol. 78, no. 5, pp. 1559-1567.

[19] GILJOHANN, D.A. and MIRKIN, C.A. Drivers of biodiagnostic development. *Nature.* 2009, vol. 462, no. 7272, pp. 461-464.

[20] ROETSCHI, A., BERTHOUD, H., KUHN, R. and IMDORF, A. Infection rate based on quantitative real-time PCR of *Melissococcus plutonius*, the causal agent of European foulbrood, in honeybee colonies before and after apiary sanitation. *Apidologie.* 2008, vol. 39, no. 3, pp. 362-371.