ALP-functionalized Mesoporous Silica Nanoparticles for Antibody Labeling in Immunosensors

Aneta Kovářová, Michaela Čadková, Veronika Dvořáková, Zuzana Bílková, and Lucie Korecká
Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Studentská 573, 532 10 Pardubice, Czech Republic,
E-mail: aneta.kovarova@upce.cz

Abstract
Currently, the new ways how to improve the sensitivity of commonly used ELISA methods for detection of biomarkers are being found. The possibility how to achieve lower detection limits is the use of secondary antibodies labeled with nanoparticles modified by enzymes instead of antibodies labelled only by enzymes. Here we present ALP-modified silica mesoporous nanoparticles utilized for labelling of anti-ApoE IgG antibodies for quantification of apolipoprotein E (ApoE) by sandwich-type magneto-imunosorbent enzyme assay combined with square wave voltammetry. In this arrangement we achieved linear calibration curve in the concentration range of 75 – 500 ng/ml.

Key words: Alkaline phosphatase, Silica nanoparticles, Antibody labeling, Electrochemical immunosensors, SWV.

Introduction

Electrochemical immunosensors combine the specificity of the immunochemical reaction with the advantages of an electrochemical detection, which features high sensitivity, low cost and ease of the automation [1]. The specificity of the molecular recognition of antigens by antibodies to form a immunocomplex is the principle of the analytical immunoassay [2].

The development of materials with suitable properties for enzyme immobilization is emerging area of the research. Currently, mesoporous silica nanoparticles (MSN) are used for their potential biomedical applications; for example, in drug delivery, therapeutic applications and also in the area of immunosensors for the analysis of biologically active proteins [3, 4]. Silica material is attractive due to thermal stability, chemical inertness, harmlessness to human health and commercial availability [5]. MSN is also characterized by large surface area, controllable synthesis and porous structure. Moreover, surface properties and composition can be adjusted by changing the organic component during the preparation of MSN [6]. Nanoparticles may also be used as carriers for enzyme immobilization for following use as antibody labels combined with electrochemical detection [7] and this was the aim of our work. The sensitivity of the detection is increased due to the ability of nanoparticles to bind a greater amount of the enzyme while enzyme activity is maintained [6,8].

Alkaline phosphatase is the one of the most common enzymes used in immunoassay (ELISA) as antibody label due to its broad substrate specificity. In practice, broad spectrum of substrates easily convertible to electroactive products are used for alkaline phosphatase; for example, *p*-aminophenyl phosphate, 1-naphtyl phosphate, 4-nitrophenyl phosphate etc. [9].

The aim of this work was preparation of the antibodies labelled by ALP-modified nanoparticles, which are used for quantitative electrochemical detection of an antigen by using the immunosensor based on the ELISA principle. The immunosensor consists of primary anti-ApoE antibodies (IgG) immobilized on magnetic particles, protein ApoE as an

antigen and prepared secondary anti-ApoE antibodies (IgG) labeled with ALP-modified mesoporous silica nanoparticles (MSN) (Fig. 1).

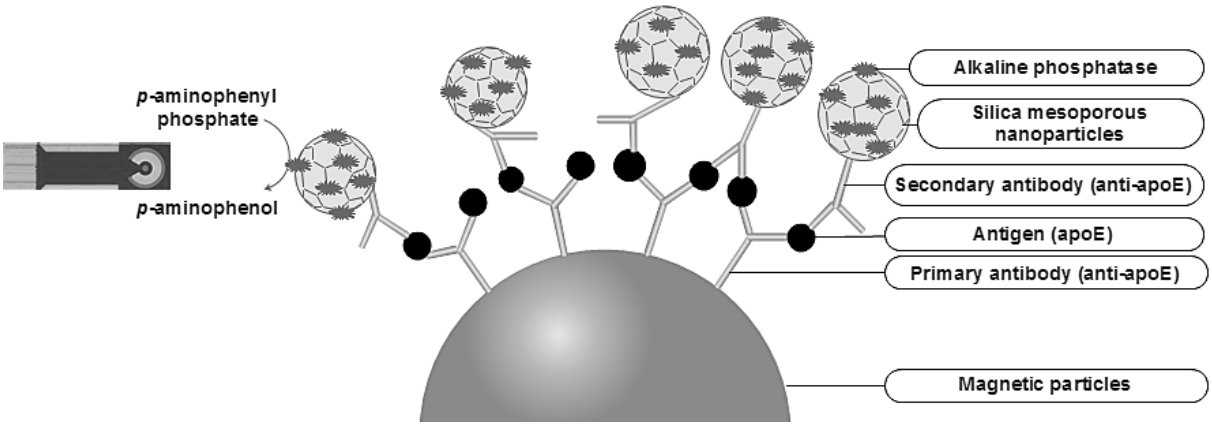

Fig. 1. Scheme of the final immunomagnetic electrochemical biosensor for the detection of the antigen apolipoprotein E.

Experimental

Alkaline phosphatase (Sigma Aldrich, St. Louis, MO, USA), mesoporous silica nanoparticles with amino functional groups, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, N-hydroxysulfosuccinimide sodium salt were obtained from Sigma Aldrich (St. Louis, MO, USA). Human recombinant ApoE3 was supplied by BioVision (USA). Rabbit polyclonal affinity purified anti-ApoE antibodies were produced by Moravian-Biotechnology (Czech Republic). Lightning-LinkTM ALP conjugation kit was from Innova Biosciences (UK). *p*-aminophenyl phosphate (PAPP) was supplied by DropSens (Spain). All other chemicals were of analytical grade purity and were from Sigma-Aldrich (USA) or Penta (Czech Republic).

All electrochemical measurements were performed with portable electrochemical interface PalmSens (PalmSens, The Netherlands) controlled by software PSTrace 4.7 and connected to the three electrodes screen printed sensors with carbon working, a platinum auxiliary and a silver pseudoreference electrode (DropSens, Spain).

Labeling of anti-ApoE antibodies by alkaline phosphatase and electrochemical detection by square wave voltammetry

Firstly ALP was immobilized onto mesoporous silica nanoparticles with amino functional groups via standard one-step carbodiimide method (EDC, S-NHS). The activity of the ALP after immobilization onto the nanoparticles was verified spectrophotometrically by using the substrate solution of 15,2mM *p*-nitrophenyl phosphate at 405 nm. Subsequently, the thus prepared enzyme-modified nanoparticles were conjugated with polyclonal rabbit anti-ApoE IgG (Moravian Biotechnology, Brno, Czech Republic) by use of oriented immobilization technique after periodate oxidation of antibodies. Functionality of conjugate labeled by ALP-modified nanoparticles (anti-ApoE$^{NPs\text{-}ALP}$ IgG) was then verified using magnetic microparticles (SiMAG-carboxyl, Chemicell, Germany) modified by ApoE antigen. Formed immunocomplex in 0.1 M carbonate buffer pH 9.4 containing 0.1 % BSA and 0.05 % Tween-20 was detected after addition of the substrate 3mM *p*-aminophenyl phosphate (in 0.1 M Tris-HCl buffer pH 8.9). Electroactive product *p*-aminophenol was measured electrochemically by square wave voltammetry by using screen-printed three-electrode sensor with carbon working electrode. Enzyme activity was monitored and evaluated by the recording the peak height at the tenth minute and the potential ±0.035 V. Parameters of voltammetric technique were as

following: potential range from -0.3 V to 0.3 V, step potential 0.005 V, amplitude 0.02805 V, and frequency 20 Hz. Purification of electrodes by using cyclic voltammetry (potential range from -0.4 V to 0.4 V, step potential 0.005 V, scan rate 0.1 V/s, number of scans 10) was performed before sample measurement.

Development of immunosensor composed of anti-ApoE IgG – ApoE - anti-ApoE$^{NPs-ALP}$ IgG
Final immunosensor was formed by the primary rabbit anti-ApoE antibody IgG (Moravian Biotechnology, Brno, Czech Republic), which were immobilized on magnetic particles with carboxyl groups by two-step carbodiimide method. Antigen protein ApoE (in phosphate buffer pH 7.3 containing 0.1% BSA and 0.05 % Tween-20) was captured to immunocomplex and subsequently visualized by addition of anti-ApoE$^{NPs-ALP}$ IgG (in 0.1 M carbonate buffer pH 9.4 containing 0.1 % BSA and 0.05 % Tween-20). Current responses were measured by the same electrochemical system and under the same conditions of electrochemical detection, which are mentioned above.

Results and discussion
As already was stated, the ALP-modified nanoparticles, which were used for a labeling the secondary anti-ApoE IgG antibodies should amplified the electrochemical signal. The only commercial mesoporous silica nanoparticles modified by amino groups ALP were selected for immobilization, because nanoparticles with carboxy groups were considered as unusable for immobilization of the ALP. To modify nanoparticles, the different amounts of enzyme 50 U, 100 U, 200 U and 400 U were tested. The 200 U ALP was chosen as optimal, because there was sufficient enzyme activity, and the rest of free functional groups were available for subsequent conjugation with antibodies. For the verification of the functionality of the conjugate (anti-ApoE$^{NPs-ALP}$ IgG) was done with 1µg of the antigen ApoE covalently bound to the magnetic particles. The optimal dilution of the conjugate with a sufficient current response was set and the lowest possible consumption of the conjugate (Fig. 2.) was taken into account. The 10x diluted conjugate was selected as sufficient.

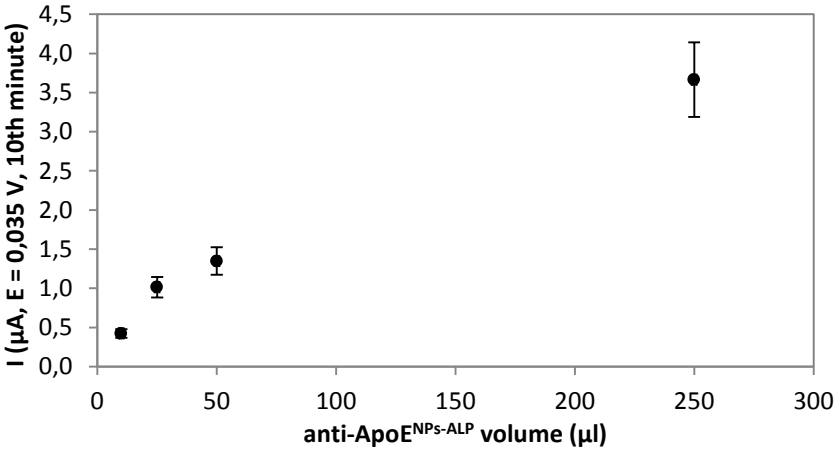

Fig. 2. Optimization of the dilution of the prepared conjugate anti-ApoE$^{NPS-ALP}$.

Last part was focused on the compiling the final immunosensor (anti-ApoE - ApoE - anti-ApoE$^{NPs-ALP}$). Firstly, the 1 µg of the primary anti-ApoE antibody bound to magnetic particles was incubated with 75 ng, 250 ng, and 500 ng antigen ApoE to form immunocomplex which was afterwards visualized by using the prepared conjugate anti-ApoE$^{NPs-ALP}$. Acquired calibration curve was compared with the calibration curve of immunocomplex labeled by the secondary anti-ApoE IgG antibody (1000x diluted) modified by the ALP by using the Lighting-Link® kit via already published procedure [10] (Fig. 3.). When the two types of

antibody labeling were compared, it was shown that the use of ALP-modified nanoparticles, unfortunately, have not led to increasing sensitivity of the assay. On the other hand the linear range of the conjugate compiled of the ALP-modified nanoparticles was determined in the range between 75 to 500 ng of the antigen ApoE, while the linearity with conjugate created by utilization the commercial kit was only in the range of 1-50 ng of the antigen ApoE.

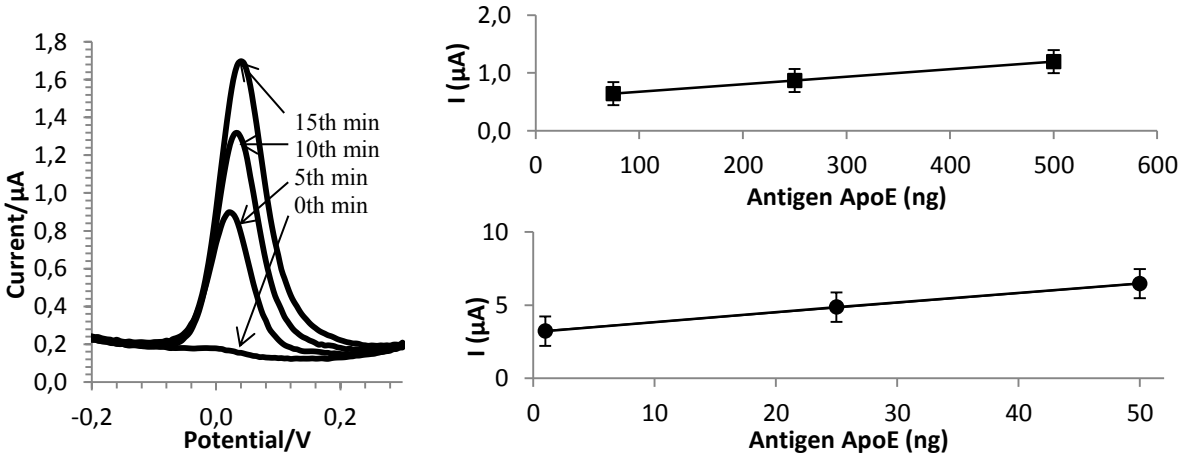

Fig.3. Square wave voltammogram and dependence of the current response (10th minute, E = ±0,035 V) on the antigen amount with the utilization of the conjugates anti-ApoE$^{NPs\text{-}ALP}$(■) and anti-ApoE^{ALP}(●). Device and conditions of the electrochemical detection are specified in the experimental part.

Conclusion

In this work conjugate comprised of ALP-modified nanoparticles for detection antigen ApoE was created. Unfortunately, it was shown, that this approach is insufficient for increasing the detection sensitivity when we compared it with other possible labeling approach. However, the sufficient sensitivity for detection of selected biomarkers and linear range much wider than in the conventional approach was achieved.

Acknowledgement

Support of the University of Pardubice, Faculty of Chemical Technology (Project No. SGFChT07/2016) and Czech Science Foundation Grant No. 15-16549S and is gratefully acknowledged.

References

1. Wan Y., Su Y., Zhu X., Liu G., Fan Ch.: Biosens. Bioelectron. *47,* 1 (2013).
2. Luppa P. B., Sokoll L. J., Chan D. W.: Clin. Chim. Acta. *314(1-2),*1 (2001).
3. Tang F., Li L., Chen D.: Adv. Mater. *24*(*12),*1504 (2012).
4. Liu G., Lin Y.: Talanta *74*(*3),* 308 (2007).
5. Nandiyanto A. B. D., Kim S.-G., Iskandar F., Okuyama K.: Micropor. Mesopor. Mat. *120,* 447 (2009).
6. M. Yang, Li H., Javadi A., Gong S.: Biomaterials *31(12),* 3281 (2010).
7. Rusling J. F., Sotzing G., Papadimitrakopoulosa F.: Bioelectrochemistry. *81(4),*189 (2009).
8. Wu Y., Chen Ch., Liu S. et al.: Anal. Chem. *4*, 1600 (2009).
9. Kokado A., Arakawa H., Maeda M.: Anal. Chim. Acta. *407(1-2),* 119 (2000).
10. Cadkova M., Dvořáková V., Metelka R, Bílková Z., Korecká L.: Monatsh. Chem. *147*, 69 (2016).