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Supported silver clusters as nanoplasmonic transducers for protein sensing



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ABSTRACT

Transducers for optical sensing of proteins are prepared using cluster beam deposition on quartz substrates. Surface plasmon resonance phenomenon of the supported silver clusters is used for the detection. It is shown that surface immobilisation procedure providing adhesion of the silver clusters to quartz and functionalisation of cluster surfaces for antibody coupling are the key issues for cluster stability and protein detection. Focus was put on these tasks and the processes have been optimised. In particular, conditions for coupling of the antibodies to the clusters are developed providing an enhancement of the plasmon absorption band used for the detection. Atomic force microscopy study allows to suggest that immobilisation of antibodies on silver clusters has been achieved, thus giving a possibility to incubate and detect an antigen of interest. Hence, by applying the developed preparation stages and protein immobilisation scheme the sensing of protein of interest can be assured using a relatively simple optical spectroscopy method.

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1. Introduction

Development of nanosensors is a rapidly growing field of research. The increasing interest arises from the unique physical characteristics and properties on the nanoscale that are not present in bulk materials. Therefore, nanodevices are able to deliver sensitivity, which is orders of magnitude higher compared to conventional sensor technologies, and supply additional performance advantages like short response time and portability [1]. Nanosensors also allow for building integrated systems, thus providing a platform for intelligent devices having significant data storing, processing and analyzing power. Intelligent nanosensors have a great potential to become very attractive as autonomous systems or to be spread out in a large number to form networks.

Among nanosensors, biorecognition systems are of significant importance for environmental, bioprocess and food quality controls as well as for medical and pharmaceutical applications [2,3]. A biosensor is an analytical device that interfaces a biological object to be recognised with a physical or chemical transducer to generate a signal which is then registered and analysed. There are a number of various approaches in realisation of detection [4]. Localised surface plasmon resonance (LSPR) biosensors were among the first

demonstrated and since then they have gradually become a very powerful label-free tool. One of the great advantages of label-free detection is that the target molecules are not altered, i.e. they are detected in their natural forms. Nanoparticles (NPs) are typically used as transducers generating optical signals. At the same time they are similar in size to some organic molecules such as enzymes and proteins, thus, being ideal transducers used in detection. Many state-of-the-art biosensors utilising LSPR were demonstrated to provide a relatively high degree of sensitivity [5]. However, there are still a number of aspects to be considered in order to produce a reliable and selective sensor. Among them formation of a stable transducer, design of the detection scheme and surface immobilisation chemistry are challenging tasks.

NPs prepared through sol–gel processes starting with different salt containing solutions are the most widely used as transducers. A major disadvantage of this approach is the relatively low stability and short shelf life time of the particles, leading to a rapid decay of their sensing properties. There is also a poor size selection and high tendencies to agglomeration of NPs. Additionally, it is hard to control surface coverage by NPs. Alternatively, NPs deposited from cluster beams have been demonstrated to be an attractive approach [6,7]. One of the main advantages of this technique is that the clusters are first formed in a gas phase that provides both a high level of flexibility and precision in the control of cluster composition and size. Thereafter, the clusters can be deposited on the required substrate with control of surface coverage. Deposition which is carried

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out in vacuum allows avoiding contamination. Moreover, the cluster beam technique gives a possibility to control the kinetic energy of the particles thus providing conditions for pinning of clusters or nanostructuring of surfaces [8,9], in other words widening the spectrum of possible applications.

In the current paper, we present first results on the formation of transducers for protein sensing using deposition of silver clusters on modified quartz surfaces. The research is focused on the development of the surface immobilisation procedure to provide reasonable adhesion of the silver clusters to quartz, functionalisation of cluster surfaces for protein coupling and testing the applicability of the sensing scheme utilising LSPR.

2. Experimental

Silver clusters were produced using the experimental setup based on magnetron sputtering which is described in detail in [10,11]. A silver target of 99.99% purity was used for the cluster production. Cluster deposition was carried out on quartz substrates with dimensions 10 mm × 10 mm at room temperature in high vacuum at a background pressure of ca. 1×10^{-8} mbar. Thus, one produces pure supported silver NPs on the quartz surface. Deposited at low kinetic (so-called thermal) energies NPs preserve almost spherical shape with a slight tendency to oblate [10]. The setup allows for size selection of clusters with a relative standard deviation of ~9–13% for particles of various diameters in the range between 5 and 23 nm [11]. However, to test the principle of detection and develop methods for surface immobilisation, the precise size of clusters is considered to be not essential for the first experiments. Therefore, silver clusters were deposited without exact size selection in this work. Mean sizes and the size distribution will be described below.

Quartz substrates were modified (functionalised) prior to the deposition. A series of earlier experiments led us to the elaboration of the methodology to improve the cluster adhesion to the substrates in relation to stability against dipping in solutions used in the following steps of transducer formation and protein deposition. Quartz substrates have been cleaned with ethanol and subsequently treated for 30 min in an ozone cleaner to remove residual organic materials and to increase the surface density of hydroxyl groups. Directly after the ozone treatment the samples were placed in a desiccator and evacuated (subjected to low vacuum) in the presence of a mixture of toluene/3-aminopropyltrimethoxysilane (APTMS) at a ratio of 3:1. The gas phase deposition was carried out for 30 min to cover the surface with approximately one monolayer of APTMS. A schematic picture of the quartz surface modification is shown in Fig. 1. After this surface functionalisation with positively charged amine groups, the quartz substrates have been used for cluster deposition as described above. The presence of the amine groups was found to be significantly improving the silver NPs adhesion to the substrate.

Substrates with as-deposited clusters were incubated with a 1 mM 11-mercaptoundecanoic acid (11-MUA) solution in ethanol

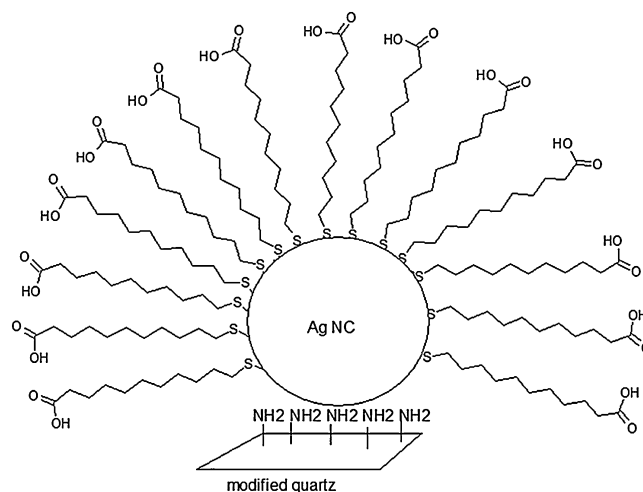


Fig. 2. Functionalisation of silver cluster surfaces using 11-MUA.

for 30 min and subsequently washed with pure ethanol to remove residual not reacted 11-MUA. The 11-MUA modified substrates have been dried under a stream of nitrogen. 11-MUA becomes selectively bound to silver NP by the sulphur-containing end and provides reaction groups for coupling of proteins (see Fig. 2). To activate these groups the samples were incubated with a freshly prepared 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/*N*-hydroxysuccinimide (EDC/NHS) mix ratio 1:1 for 20 min. Subsequently the mix has been removed from the substrates and a protein solution has been added on top of the substrates. The incubation period for protein solutions was 30 min. After the incubations the samples have been thoroughly rinsed. All proteins used for the experiments are commercially available and they have been used without further purification.

Three series of samples were prepared: the first one follows classical antibody–antigen scheme (with anti-chicken egg albumin antibody and chicken egg albumin as antigen), the second one is of inversed sequence of protein deposition (first chicken egg albumin, then the corresponding antibody) and the third one is also inversed scheme but with lysozyme as the antigen, which should not be recognised by the anti-chicken egg albumin antibody. The proteins used in this work are chosen only to test the applicability of the developing detection approach and they are not of high practical importance.

The samples were characterised after each of the above-mentioned steps in preparation of the transducer system using atomic force microscopy (AFM) and optical transmission spectroscopy. For AFM studies, an *Ntegra-Aura* (NT-MDT) system was utilised. The measurements were performed in tapping mode using commercial Si cantilevers with curvature radius of tip better than 10 nm and a spring constant of approximately 26 N/m. Optical transmission spectra were obtained by a Perkin Elmer High Performance Lambda 1050 spectrometer in the interval of wavelengths

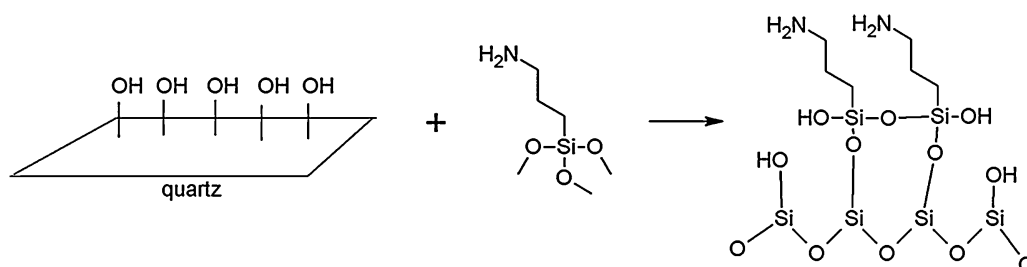


Fig. 1. Gas phase deposition of APTMS on quartz (surface functionalisation).

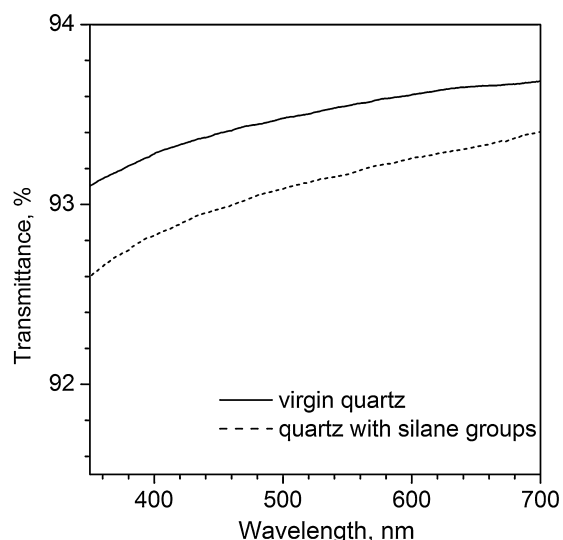


Fig. 3. Optical transmittance spectra of virgin quartz and one with surface functionalised by APTMS (silane groups).

$\lambda = 300\text{--}750\text{ nm}$ with data interval of 1 nm and acquisition time of 0.32 s for every measurement. The spectrometer was used in a standard configuration providing beam spot size of about 2 mm in diameter at the sample location.

3. Results and discussion

Silver clusters deposited on a bare quartz substrate were found to have very low adhesion to the surface leading to removal of most of them when immersing into solutions. The clusters deposited on APTMS-functionalised substrates show significantly improved adhesion. The most probable mechanism is through the formation of polarisation interaction between the amine groups and NPs. This additional APTMS layer on the surface decreases the transmittance of the substrates uniformly and only by about 0.5% as can be seen in Fig. 3, i.e. the presence of APTMS does not affect the optical sensitivity.

A typical AFM image for the clusters as-deposited on the functionalised quartz is shown in Fig. 4a. Since the clusters were deposited without size-selection, mean sizes slightly vary from sample to sample. These sizes (diameters) were estimated from the height of the particles assuming near-spherical shape and found to

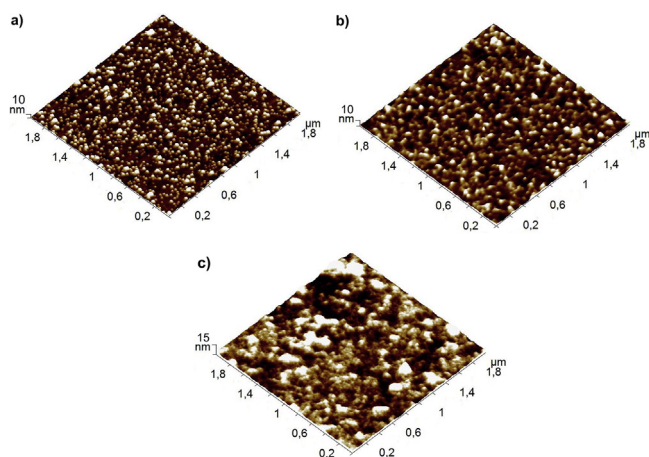


Fig. 4. AFM images of substrates (a) with as-deposited clusters, (b) after antibody–antigen incubation (classical scheme) on clusters and (c) after antigen–antibody incubation (inversed scheme) on clusters.

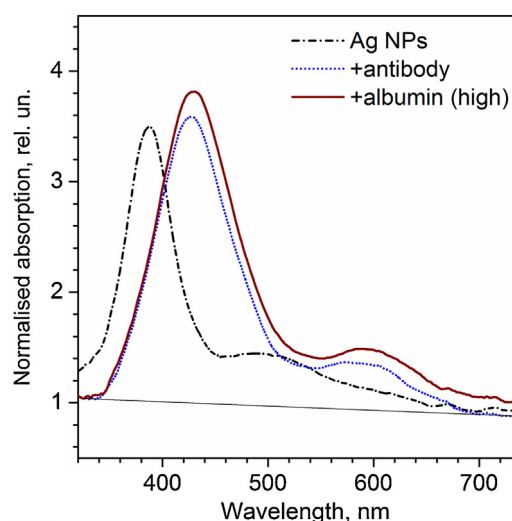


Fig. 5. Normalised optical absorption spectra of quartz substrates with as-deposited clusters followed by antibody–antigen incubation scheme (anti-chicken egg albumin antibody followed by chicken egg albumin). Straight base line is shown for one of the spectra.

be between 8 and 12 nm. In the experiments, we use substrates with very similar cluster coverages which are below one monolayer as can be seen by example in Fig. 4a. After the functionalisation of silver NPs with 11-MUA and deposition of antibodies or antigens we see only a small decrease in the NPs' coverage, demonstrating good resistance of the supported clusters when treated wet chemically.

Optical spectra of the samples with as-deposited clusters clearly demonstrate the presence of a LSPR absorption band at $\lambda \approx 390\text{--}400\text{ nm}$ in Figs. 5–7 (dash-dotted curves). These absorption spectra are obtained from transmission measurements for particular samples. The spectrum corresponding to the functionalised quartz (covered by APTMS) is subtracted from every spectrum to eliminate contribution of the substrate and emphasise the plasmonic features. One can see that the position of LSPR maximum on the wavelength scale varies slightly from sample to sample. This difference is related to a small variation in mean particle sizes between the series of samples. For quantitative comparison of LSPR

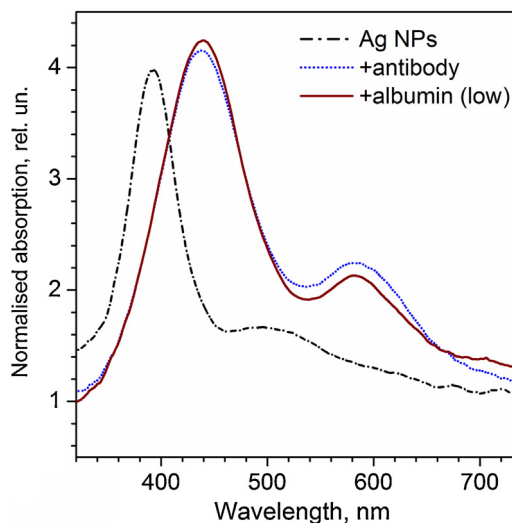


Fig. 6. Normalised optical absorption spectra of quartz substrates with as-deposited clusters followed by antibody–antigen incubation scheme (anti-chicken egg albumin antibody followed by chicken egg albumin). Albumin concentration is reduced for six times compared to the case presented in Fig. 5.

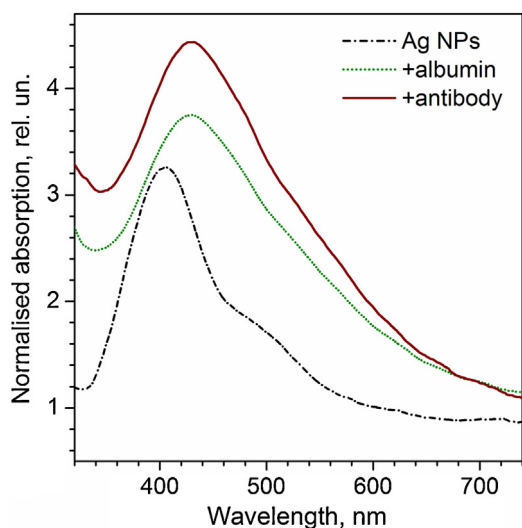


Fig. 7. Normalised optical absorption spectra of quartz substrates with as-deposited clusters followed by inversed antigen-antibody incubation scheme (chicken egg albumin followed by anti-chicken egg albumin antibody).

Table 1

Wavelength of LSPR maximum λ_m and band intensity for spectra in Fig. 5. Relative error for intensity is calculated using standard deviations for optical measurements and found to be ± 0.05 .

Sample	λ_m (nm)	Intensity (rel. un.)
As-deposited Ag clusters	390	2.32
After antibody deposition	426	2.53
After albumin deposition	430	2.77

Table 2

Wavelength of LSPR maximum λ_m and band intensity for spectra in Fig. 6. Relative error for intensity is calculated using standard deviations for optical measurements and found to be ± 0.05 .

Sample	λ_m (nm)	Intensity (rel. un.)
As-deposited Ag clusters	393	2.61
After antibody deposition	435	3.04
After albumin deposition	439	3.16

Table 3

Wavelength of LSPR maximum λ_m and band intensity for spectra in Fig. 7. Relative error for intensity is calculated using standard deviations for optical measurement and found to be ± 0.05 .

Sample	λ_m (nm)	Intensity (rel. un.)
As-deposited Ag clusters	398	2.15
After albumin deposition	421	1.60
After antibody deposition	423	1.71

intensities, a base line is introduced for every spectrum. It is defined as a straight line tangential to spectral minima in the “blue” and “red” regions. An example is shown in Fig. 5 for one of the spectra. Intensity of plasmon band is measured from this line to the maximum and the data are presented in Tables 1–3 for Figs. 5–7, respectively. One can also observe a very weak band at around 500 nm which is tentatively attributed to cluster-cluster interaction. However, it is not essential for the current study and, therefore, it is not discussed further.

After the deposition of proteins the optical spectra change significantly. The difference between the cases presented in Figs. 5 and 6 and that in Fig. 7 is in sequence of protein deposition. In the first two cases, the classical antibody-antigen scheme is used. In the third one, the inversed scheme is applied. The attachment of the antibodies to Ag NPs causes broadening of the LSPR band,

shift to longer wavelength and increase of the absorption that can be clearly seen in Tables 1 and 2 as well as in Figs. 5 and 6, respectively. The subsequent deposited of chicken egg albumin leads to further small red shift of the plasmon band (see Tables 1 and 2). The change of the band intensity is found to be dependent on the albumin concentration. The albumin/antibody ratio is 2:1 (high) for the case presented in Fig. 5 and 1:3 (low) for the case shown in Fig. 6. One can clearly see a larger increase in the band intensity for higher albumin concentration, thus, being indicative of albumin detection.

11-MUA molecules are attached to silver NPs via the sulfhydryl group. The other end of the molecule with its carboxyl group is used to form a covalent amide bond to the antibody, thus, providing chemisorption of the protein. Strong chemical bonding of the antibody to the NP changes the dipole characteristics leading to an enhancement of the SPR absorption as seen in Figs. 5 and 6. The enhancement may be caused by the charge transfer between the NP and 11-MUA, however additional studies of this phenomenon are required. Chicken egg albumin is smaller compared to the anti-chicken egg albumin antibody and its subsequent attachment to the antibody changes the NP-antibody interaction only a little, therefore, the spectral shift of the band is small. However, we are able to see an increase in the band intensity which is found to be concentration dependent, thus, demonstrating the detection of albumin by the transducers. It is worth noting that due to the relatively small spectral changes the definition of the absorption intensity may have substantial effect on the quantitative evaluation. Among alternative methods, the defining intensity from the base line, which is chosen in this work, results in most consistent numbers.

An AFM image of a sample with antibody-antigen deposited in a classical scheme can be seen in Fig. 4b. The coverage of silver NPs is slightly decreased compared to the sample with as deposited clusters (see Fig. 4a) but the height and lateral dimensions of the bumps representing NPs are increased. Taking into account the lateral sizes of the antibodies (ca. 4 nm) and supported clusters (8–12 nm), one can suggest that one or two antibodies become attached to each NP, then providing a coupling of albumin molecules. The same tendency of a single molecule coupling to an individual cluster was earlier found for a few different types of proteins immobilised by gold NPs supported on graphite [12,13]. In the case of several antibodies located on the individual NP the image would represent much larger bumps and more significant changes in the topography.

For the inversed scheme of protein incubation (the case shown in Fig. 7), the small sizes of albumin proteins (1–2 nm) cause non-selective coating of the substrate. It means that albumin is not only coupled to the clusters, but also fills the gap between them. This scenario of massive albumin deposition (with immobilisation on the clusters and physisorption on the quartz) is confirmed by AFM. As one can see in Fig. 4c, the clusters are hardly recognised in the topography. This is related to the fact that chicken egg albumin covers the entire surface. The sequentially incubated anti-chicken egg albumin antibodies are consequently situated randomly around the NPs changing the sample topography dramatically compared to that with as-deposited clusters. According to the optical spectra presented in Fig. 7 and corresponding parameters in Table 3, the intensity of the LSPR band after albumin deposition is decreased, the band broadens and experiences a red shift due to the change in dielectric environment for NPs. Sequential deposition of the antibody molecules further reduces the transmittance over the whole interval of wavelengths (entire spectrum in Fig. 7 is shifted up) but intensity of the LSPR band is affected very little. It can be still assumed that albumin molecules are attached to antibodies but since the antibodies are not directly coupled to the nanoparticles one cannot see any specific change in the plasmon band related to the presence of albumin. Thus, it can be concluded that no detection of albumin is possible for the inversed deposition scheme of proteins.

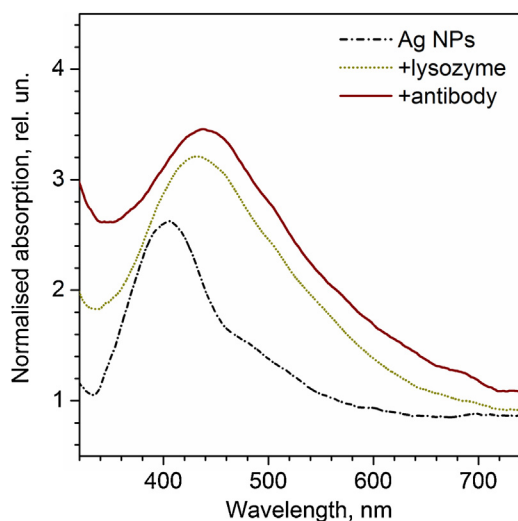


Fig. 8. Normalised optical absorption spectra of quartz substrates with as-deposited clusters followed by inverted antigen–antibody incubation scheme (lysozyme followed by anti-chicken egg albumin antibody).

Thus, our study demonstrates that providing an appropriate deposition and immobilisation protocol is very essential for the application of silver NPs as transducers for optical sensing of particular proteins, i.e. for providing conditions for a sensitive detection. To further proof this concept we prepared one more series of samples in which lysozyme was used instead of albumin and incubated with the same antibody as before. In other words, we carried out the sequential deposition of two proteins which cannot be recognised by each other. The optical spectra are presented in Fig. 8, from which one can see that the deposition of lysozyme and then antibody decreases the sample transmittance over the whole interval of wavelengths and causes the red shift of the plasmon band. It also results in slight and unspecific damping of the band intensity, thus, indicating no possibility for detection of the antigen similar to the case of inversed deposition scheme shown in Fig. 7. Surface topography of the sample (not shown) is very similar to that presented in Fig. 4c.

4. Conclusion

Transducers for optical sensing of proteins are prepared using silver cluster beam deposition on quartz substrates. The supported silver NPs exhibit a specific LSPR band used in the following detection scheme. The conditions for functionalisation of both the substrate prior to the deposition and cluster surface after that are optimised providing considerable NP adhesion to quartz as well as formation of chemical bonds coupling the antibodies to NPs. This coupling enhances the intensity of the LSPR band that is used as an “optical signature” for sensing. Our AFM study of the samples allows to suggest that there is immobilisation of an antibody on individual NP. Bonding of the antibody to NP then provides a possibility to attach and detect the antigen of interest, which is chicken egg albumin in the current study. It is proven that appropriate preparation stages and immobilisation schemes are the key issues for the application of silver NPs as transducers for optical sensing of particular proteins. Thus, by applying the correct protocol the assured protein detection with high sensitivity can be reached while using a simple optical spectroscopy method. The developed detection approach may be transferred to other proteins, of which sensing is of higher practical importance compared to those used in the current experiments.

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Biographies

Peter Fojan received his Ph.D. in Biotechnology at the Graz University of Technology, Austria in 1997. He initially worked on industrial genetics of eukaryotic organisms. During his postdoc time at Aalborg University (AAU) at the Department of Biotechnology he moved into the area of protein physics and molecular modelling. In 2004, he joined the Department of Physics and Nanotechnology of AAU where he became an Associate Professor in 2009. His research interests are centred around biological and small molecules and their interactions with cells and surfaces in general, for medical, sensor applications and as antibacterial agents.

Muhammad Hanif graduated in Physics and Mathematics at Bahauddin Zakariya University in 2008. Then, he obtained M.Sc. in Condensed Matter Physics from the Norwegian University of Science and Technology in 2011. Since 2012 he is a Ph.D. student at Aalborg University under the supervision of Dr. Vladimir Popok. His current research interests are in formation of size-selected metal nanoparticles using cluster beam technique and their application for development of biosensors.

Stephan Bartling received his Diploma in Physics at the University of Rostock in 2010. He is currently a Ph.D. student at the same university. His research interests are the characterisation of catalytically active metal oxide nanoparticles under reactive conditions.

Hannes Hartmann obtained his Diploma in Physics at the University of Rostock in 2011. He is currently a Ph.D. student at the same university. His research deals with excitation processes in molecular aggregates and the coupling between nanoparticles and molecules.

Ingo Barke obtained his Ph.D. in Physics in 2004 at the University of Dortmund (now TU Dortmund University), followed by several years as postdoctoral fellow at the University of Wisconsin, Madison and at the University of Rostock. Since 2013 he is a senior researcher in Rostock. His research interests include low-dimensional systems at surfaces, structural and electronic properties of clusters and nanoparticles, and excitations in self-assembled molecular aggregates and hybrid nanostructures.

Vladimir N. Popok received his Master in Physics and Ph.D. degrees from the Belarusian State University (BSU) in Minsk in 1990 and 1995, respectively. Then he worked as researcher at BSU. Between 1999 and 2011 he was affiliated with the University of Gothenburg in Sweden and the University of Rostock in Germany having different positions. Since 2011 he is an Associate Professor at Aalborg University in Denmark. Main fields of research interest are related to formation and study of nanostructures and nanocomposite materials with focus on electronic and optical properties.