Label-free real time optical detection of binding of living cells and biopolymers

O V Morozova\textsuperscript{1,2}, E I Isaeva\textsuperscript{2}, and D V Klinov\textsuperscript{1}

\textsuperscript{1} Federal Research Clinical Center of Physical-Chemical Medicine of the Federal Medical and Biological Agency of Russia, 1a Malaya Pirogovskaya Street, 119435, Moscow, Russian Federation
\textsuperscript{2} Ivanovsky Institute of Virology of the National Research Center of Epidemiology and Microbiology of N.F. Gamaleya of the Russian Ministry of Health, 16 Gamaleya Street, 123098, Moscow, Russian Federation

E-mail: omorozova2010@gmail.com

Abstract. Biosensor based on long range surface waves on one-dimensional photonic crystal (PC) surface in microfluid channel was used to detect binding of cells, viruses, nanoparticles and proteins. Covalent attachment of biopolymers to polyaminated PC with glutaraldehyde cross-linking in microfluid channel results in functionally active proteins, living eukaryotic, bacterial cells and viruses. The evident advantages of the optical biosensor include native conformations of biopolymers, detection of reversible or permanent specific binding with ligands, broad detection scale from eukaryotic cells to low-molecular-weight ligands and observations in real time with possible calculations of affinity and kinetic constants. However, relatively low sensitivity limit near 0.1 \( \mu \)g/ml of proteins is significantly less those of ELISA and xMAP immunofluorescent analysis (approximately 1 pg/ml). Integral adlayer thickness does not reveal molecular events on each cell with possible variations. The label-free real-time optical detection allowed us to estimate cytotoxicity of currently used and novel drugs and to explore the antiviral properties of new compounds.

1. Introduction
Optical sensors are currently used in biomolecular interaction analysis, providing kinetic data in real time without labelling. The advantages of the label-free real time measurements are the absence of time-and-money consuming pre-labelling and the elimination of steric interference from labels. The disadvantages of all label-free techniques including the surface plasmon resonance (SPR) devices are deficient sensitivity to a specific signal and undesirable susceptibilities to non-specific signals, e.g., to the volume effect of refractive index (RI) variations. These variations arise from temperature fluctuations and drifts, and they are the limiting factor for many optical biosensors. To overcome these disadvantages, the biosensor on the base of registration of PC surface waves angle and the critical angle was used [1-2]. Angular interrogation of the optical surface wave resonance was used to detect changes in the thickness of an adsorbed layer, while an additional simultaneous detection of the critical angle of total internal reflection provides independent data of the liquid refractive index. The changes in RI of the medium and the adlayer thickness can be derived from the changes in these angles by linear method based on Taylor expansion using a home-made acquisition software [1-2].
Protein microarrays and microfluid devices are widely used for proteomic and clinical assays due to their high throughput fashion and simplicity. Adsorption of biopolymers is essential stage for lab-on-a-chip type devices. Immobilized proteins should retain their activity, remain stable and not desorb during subsequent steps.

Our research was aimed at development of optical detection of binding of living cells and proteins with specific ligands in real time.

2. Materials and methods

2.1. Proteins
Polyallylamine (PAA) 65 kDa, glutaraldehyde and bovine serum albumin (BSA) were purchased from “Sigma–Aldrich”. Ultrapure Milli Q water was used to prepare aqueous solutions. Hepatitis B virus (HBV) surface antigen HBsAg was purchased from Jena Bioscience GmbH, Germany. Monoclonal antibodies against HBsAg were kindly provided by Dr. L.E. Matveev (“BioSan”, Novosibirsk, Russia). Monoclonal antibodies against HIV-1 p24 antigen were kindly provided by Dr. V. N. Morozov (Institute of Theoretical Experimental Biophysics of the Russian Academy of Sciences, Moscow, Russia).

2.2. Tissue cultures
Madin-Darby Canine Kidney (MDCK) epithelial cells, mouse subcutaneous connective tissue L929, green monkey kidney Vero cells and porcine embryo kidney PS cells were obtained from the Russian State Tissue Culture Collection (National Research Center of Epidemiology and Microbiology, Moscow, Russia) and grown in Eagle minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (HyClone, “Thermo Scientific”, USA) in the presence of 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in the presence of 5% CO₂ for 24 hours. Then cellular monolayers were treated with trypsin and resuspended in PBS before analysis in microfluid channel of the biosensor.

2.3. Viruses
Influenza A virus of subtypes H1N1 (strain A/swine Iowa 15/30) and H3N2 (strain A/Aichi/1/68 isolated in China in 1968) were from the Russian State Collection of Viruses (National Research Center of Epidemiology and Microbiology, Moscow, Russia).

2.4. Antivirals.
Water-soluble complex Li⁺[Ag⁺₂Cys₂(OH)₂(NH₃)₂] (short name AC-1) was previously described [3].

2.5. Biosensor-mediated optical detection
Label-free optical detection based on propagation of long-range surface waves along the interface of one-dimensional photonic crystal (PC) in a microfluidic channel was described earlier [1]. Before functionalization, PC was sonicated in isopropanol and water and then treated in the plasma cleaner Zepto B (Diener electronic, Germany) for 10 min at the maximal power (200 W) and air pressure of 0.4 mbar. All further procedures were performed in the flow cell of the Biosensor EVA 2.0 (http://www.pcbiosensors.com) with monitoring of the adsorbed layer thickness and refractive indexes in real time. The flow rate in all our experiments was 1.5 μl/sec. The inner diameter of tubes for the peristaltic pump was 0.51 mm. The baseline (the thickness and refraction index) was registered in running deionized water at the beginning of the measurements. After baseline stabilization, PC was exposed to solutions of PAA 65 kDa until stable layer were formed. After short rinsing to remove a trace amount of unbound NH₂ containing polymers, 0.1 % fresh glutaraldehyde was added to the PC-aminated surface. Then proteins, antibodies from blood sera, virions, bacterial or eukaryotic cells were injected and both adlayer thickness and refractive index (RI) changes were registered in two spatially distant channels from different areas of PC shown as green and red curves in all figures.
Measurements using the biosensor was previously described [2]. In brief, the method is based on simultaneous registration of excitation angle of two optical modes propagating along interface between PC and liquid. One of the surface mode is sensitive to volume refractive index (RI) fluctuations whereas other mode is designed to detect dielectric changes of attached biopolymer onto PC surface. Both angles of modes excitation are measured in reflected light by the CMOS matrix and analyzed using software supplied with the biosensor. The distance of optical modes excitation angle positions on the matrix correlates with the adlayer thickness of adsorbed biopolymer.

3. Results and discussion

To observe protein immobilization a new approach of optical detection in real time on the surface of photonic crystal (PC) prepared by deposition of alternate layers of SiO$_2$(L, L’) and Ta$_2$O$_5$(H) on glass was developed. Functionalization of multilayer PC with SiO$_2$ surface layer with PAA 65 kDa in microfluid channel in water solutions at room temperature for several minutes resulted in stable interface of 1-2 nm height (figure 1). For stable chemical binding of biopolymers with PC cross-linking with glutaraldehyde was required (figure 1). Attached proteins and virions retained their native conformations and ability to bind with polyclonal and monoclonal antibodies (figure 1, 2).

![Figure 1](image.png)

**Figure 1.** Real time optical detection of binding of the hepatitis B virus surface antigen S (HBsAg) with specific monoclonal antibodies. (A) adlayer thickness; (B) RI. Time in sec.
Despite specific binding of antigen with antibodies the sensitivity of the biosensor is limited. Reasonable adlayer growth was observed for protein concentrations more than 1 µg/ml. The biosensor allowed us to register antibody titers less than 1:1,000. For comparison, the sensitivity limit of widely used ELISA and xMAP with fluorescent magnetic beads is 1 pg/ml.

Broad range of molecules from low-molecular-weight (0.1 nm) to biopolymers (1-5 nm), nanoparticles (10-500 nm), viruses (100-500 nm), bacterial (1-3 µm) and eukaryotic cells (10-30 µm) can be detected by the biosensor after their adhesion onto PAA-treated PC (figures 2, 3).

![Graph](image)

**Figure 2.** Binding of the purified influenza virus H3N2 with specific polyclonal antibodies.

Binding with ligands or drugs can be permanent or reversible (figure 3). Permanent binding of AC-1 with the influenza virus (figure 3(A)), a weak binding with mouse sera and the absence of any detectable interaction with immobilized host cells suggested its potential antiviral properties and a limited toxicity. However, interaction of the influenza virus with currently used drug ribavirin was shown to be reversible (figure 3(B)) and the virus inhibition was not complete. Comparison of tissue culture toxicity concentration and biosensor detection of complexes of cellular proteins with a chemical compound suggested a high-throughput screening way to estimate toxicity of new drugs.
Figure 3. Permanent binding of novel compound - 160 mM AC-1 (A) and reversible binding of known antiviral drug - 40 µg/ml ribavirin (B) with the influenza virus H1N1.

4. Conclusion
Optical detection of binding of viable cells of different origin and biopolymers can be observed in real time by using the PC surface wave biosensor. Specific permanent or reversible interaction can be registered. However, the sensitivity limit significantly restricts the broad implementation of the biosensor for biomedical research with trace amounts of biologically active compounds.

Acknowledgements
The research was supported by the Russian science foundation (grant № 17-75-30064).

References
[1] Konopsky V N, and Alieva E V 2009 Optical Biosensors Based on Photonic Crystal Surface Waves Methods Molecular Biology Clifton 503 49–64
[2] Konopsky V N 2010 Plasmon-Polariton Waves in Nanofilms on One-Dimensional Photonic Crystal Surfaces New Journal of Physics 12(9) 093006

[3] Tretyakov V V et al 2007 Water-soluble antiviral agent based on silver-cistine compound and method of preparing thereof, WO 2007/061337 A1
(http://patentscope.wipo.int/search/en/WO2007061337)