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## Detection of plant viruses using a surface plasmon resonance via complexing with specific antibodies

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### Abstract

The use of instrumental systems based on the surface plasmon resonance (SPR) for rapid diagnosis of intact plant viruses (in particular, tobacco mosaic virus (TMV)) is considered. A new approach using detection of viral antigen and antibody (IgG) complexes formed during the preincubation step (instead of their consecutive application in classical approach) is discussed. A comparison between signal level registered from the mixture of virus and specific serum and that from the sample without virus (samples deposited onto the sensor surface treated with thiocyanate and protein A *Staphylococcus aureus*) allows unambiguous detection of viral particles in the material studied. The performance capabilities of the method are discussed and illustrated by quantitative detection of virus in the actual samples (cells homogenate) at high concentration.

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### 1. Introduction

One of the most urgent problems in modern virology is rapid, low-cost and adequate detection of intact viruses and their fragments in the ambient media, clinical samples (for human and animal viruses), foodstuff and affected parts of plants (for plant viruses). Formed viral particles are present at the inactive stage of the virus life cycle. However, the availability of intact virions in the material studied indicates the beginning of the infection process, and quantitative determination makes it possible to characterize the intensity of this process. It should also be noted that the same clinical features

may be caused by different viruses. It is, therefore, necessary to develop highly specific and rapid techniques for laboratory diagnosis of viral diseases that use a direct detection of viruses.

Many techniques exist for the detection of virus specific proteins and nucleic acids, but only few can detect intact viral particles. The traditional technique for detection of intact viruses and viral specific antigens is an enzyme-linked immunosorbent assay (ELISA). This technique, however, has certain constraints. The most important stem from the thermodynamic character of the above process, i.e., no information exists on the kinetic parameters of reaction. Moreover, ELISA cannot discriminate directly the source of antigen; however, it would be of interest to determine what is the immediate cause of response. Is it the specific protein, the virus fragment or the virion itself?

In recent years, nondestructive techniques (such as biosensors) for the detection of biospecific interactions (in particu-

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lar, with intact viral particles) find ever-growing use. For instance, a possibility of specific detection of bacterial viruses with quartz microweighing was demonstrated (Dultsev et al., 2001). The same approach can be used to detect intact human (Cooper et al., 2001) and plant (Eun et al., 2002) viruses. For direct detection of viral particles the bioelectric recognition technique (based on the interaction between intact virus and intact cell) is often applied (Kintzios et al., 2001). Atomic force microscopy is an efficient technique to investigate not only intact viruses, but also the stages of their development in the cell (Kuznetsov et al., 2002; Malkin et al., 2003). Various optical techniques, e.g., interferometry (Schneider et al., 1997), also can be used to detect viral particles in samples. This is due to the fact that viral particles have higher refractive indices than those of biological media.

At the same time, the techniques using optoelectronic transducers are easy and rapid. These factors make it possible to develop handy and economical instruments for rapid diagnostics of the viral infections in the clinical setting, as well as to determine the virus content in plants. One of the most efficient techniques, which are used widely for the rapid detection of biospecific interactions is that of surface plasmon resonance (SPR). It is based on generation of surface polaritons on the state of the medium surrounding surface of a thin metal (Au, Ag, and others) film deposited onto surface of an insulator (Ulber et al., 2003; Homola, 2003). The principal advantages of such systems over the traditional techniques for study of immunochemical interactions, stems from a possibility to investigate directly, in the real-time mode, intermolecular interactions, as well as kinetic and thermodynamic parameters of the binding process. This permits the determination of the mechanism for such process and, correspondingly, optimize the developed analytical procedure; besides, there is no need to use labeled reagents (Keusgen, 2002).

The surface plasmon resonance technique is used widely for studying virus specific macromolecules. Most studies in this field deal with individual components rather than entire viral particles (Wittekindt et al., 2000; Tanaka et al., 2000). The viral peptides, recombinant and refined viral proteins can serve as a convenient model to investigate antibody affinity. However, the structure, conformation and microsurroundings of subunits, both separate and incorporated into viral particles, may differ essentially. Therefore, investigation of virus–IgG interaction under the conditions similar to those in vivo is of great importance, beyond all doubt (Schoefield and Dimmock, 1996). Indeed, each virus is a complex mixture of antigens whose diversity is determined by the number of virus specific proteins. Even a single protein usually contains a number of antigen determinants. They depend on the spatial structure of molecule and may be located in different sectors of the polypeptide chain. That is why model viruses should be used with small number of antigen determinants when developing novel approaches of detection of intact viruses. From these considerations the tobacco mosaic virus (TMV) seems to be the most convenient model to refine such approaches,

because it is of small size and contains a single envelope protein.

The phenomenon of surface plasmon resonance shows some limitations when applied to virology. In particular, adequate interpretation of the results is becoming difficult for measurements done over the intact viruses with length ( $L$ ) of several tens of nm. Indeed, the resolving power of instrument at quantitative (within the linear range, i.e., when  $L \ll \lambda$ ) detection of biomolecules is determined by the penetration depth ( $\lambda \approx 700$  nm) of wavelength  $\lambda$ . So the size of the receptor–analyte complex must not be over 100 nm (Liedberg et al., 1993) for linear relation between angle shift and total mass of materials on the surface (Snopok et al., 1998). This technique (in its classical version) was successfully used for the detection of specific antibodies using immobilized intact virus (Abad et al., 2002), for the analysis of reversible (low-affine Langmuir like process) interactions of viruses with specifically modified surface (Barton et al., 2001) and for the analysis of immunoglobulins–virus interactions (Hardy and Dimmock, 2003). Moreover, the surface plasmon resonance technique is quite applicable for investigation of plant viruses whose sizes are small (Sanual and Van Regenmortel, 1995; Van Regenmortel, 1999). At the same time to date, surface plasmon resonance based approaches were not used for quantitative detection of intact viruses. In accordance with this, the objective of this study was to develop the surface plasmon resonance approach for the quantitative determination of viruses, using TMV as a model system.

## 2. General approach

The traditional approach for use of the surface plasmon resonance technique is to have receptor immobilized at the sensor surface, while an analyte remains in solution. In this case, the SPR angle shift depends on the effective thickness of analyte layer specifically bound to the immobilized receptor layer; the densities of both layers being constant. This means that SPR angle shift change is due to variation of the parameters of molecular ensemble for interacting molecules in the vertical plane (Snopok et al., 2003). The shift of the angle depends not only on the layer thickness but on the refractive index variation within the layer (Snopok et al., 1998). Therefore, the molecular layer compactness will also affect the response due to refractive index variation. If, at the same time, the surface structure thickness can be fixed (due to specificity of the interaction process and constant form of the interacting components), then the shift of the angle will be a one-valued function of the biomolecular ensemble compactness.

In this study, a new approach was considered that uses the layers density variation as information parameter. For this (i) the protein A specificity towards Fc fragment of immunoglobulin and (ii) the formation of previously obtained specific IgG–virus complex were used. Depending on the relative concentration of viral particles and specific antibodies,

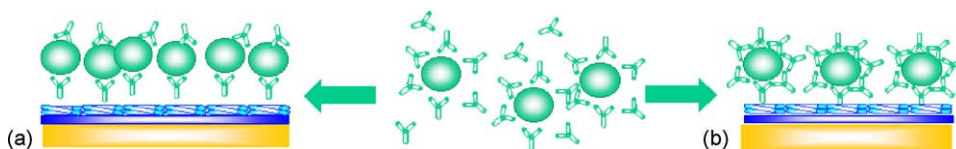


Fig. 1. Virus detection at “antiviral IgG–virus” complex immobilization: general approach: (a) high density and (b) low density.

the virion– $n$ IgG complexes will have different numbers ( $n$ ) of antibodies per virion. This determines compactness variations within the complexes, as well as in their packing at the surface. The average thickness of the whole complex will remain approximately the same, due to statistical character of interaction between the antiviral immunoglobulins and a system of corresponding epitopes at the virus surface (about 800 for TMV). In this case the shift of the angle minimum will now depend on the layer parameters in the horizontal plane (Fig. 1).

Our approach makes it possible to avoid the restrictions of the surface plasmon resonance technique due to the size of particles. Indeed, when dealing with TMV, it provides formation of a uniform biomolecular layer, with TMV virions parallel to the interface. This is because of specific interaction between antibodies and virion, as well as between antibodies and immobilized protein A. Obviously such an approach requires of some relations between the concentrations of virus specific antibodies and virions:  $[\text{TMV}] < [\text{IgG}] < [\text{TMV}] \times \text{number of epitopes}$ . If  $[\text{IgG}] < [\text{TMV}]$ , then the angle shift will be proportional to the concentration of  $[\text{IgG}]$ , because TMV itself does not adsorb at a surface functionalized with protein A. If  $[\text{IgG}] > [\text{TMV}] \times \text{number of epitopes}$ , then further increase of  $[\text{TMV}]$  will not affect the shift of the angle, due to formation of a compact structure.

Two procedures were attempted aimed on quantitative determination of viruses in plant homogenates. These were (i) determination with an “open” system, when the experiment is made in an open chamber without liquid circulation (such situation is similar to the classical analysis using standard biochemical plates) and (ii) determination with a “closed” flow system (in accordance with the IUPAC recommendations for standard analytical techniques).

### 3. Experimental

For this study, the refined preparation of typical TMV virus strain (TMV-U) was carried out. Typical TMV-U is approximately 18 nm in diameter, has one envelope protein containing 158 aminoacid residues with the molecular weight 17.5 kDa. This protein serves as antigen for antibodies. Although the quantity of the protein subunits in the whole virus particle is 2130, its antigene valency is about 800 because each antigen-binding site occupies the surface of about three protein subunits.

Also for this study, we used the homogenate of green alga *Bracteacoccus minor* (Chodat) Petrova cells infected with

TMV at the zoospore stage. The homogenate was obtained by pasting up cell suspension with the carbonate buffer (pH 9.6). Then homogenate was centrifuged at 4000 rpm to remove intact cells and large organelles. The obtained supernatant was free of any precipitates. Preincubation of the virus-containing preparation with antibodies was performed at room temperature for 10 min.

The initial virus concentration  $C$  (in mg/ml) was determined by spectrophotometry using the following expression:

$$C = A_{260} \frac{N}{K_e}$$

Here  $A_{260}$  is the extinction at wavelength of 260 nm;  $N$ , the dilution of the virus preparation and  $K_e$  is the extinction coefficient (2.7 for TMV).

Investigations with the surface plasmon resonance were undertaken using a “PLASMION” (“BioSuplar”) spectrometer developed at the V. Lashkaryov Institute of Semiconductor Physics of the National Academy of Sciences of Ukraine (Rengevych et al., 1999). A GaAs laser served as source of excitation ( $\lambda = 670$  nm). The glass plates (refractive index  $n = 1.61$ ), with as-deposited (through an intermediate Cr adhesion layer 1–1.5 nm thick) gold layer (50 nm), were pretreated with the “piranha” mixture ( $\text{H}_2\text{O}_2$ – $\text{H}_2\text{SO}_4$  in the ratio 1:3) to remove organic contaminations. After this plates were fixed at a supporting prism ( $n = 1.61$ ); optical contact was realized using immersion oil (polyphenyl ether,  $n = 1.61$ ). Water solution of potassium thiocyanate, KNCS (concentration of  $10^{-2}$  M) was prepared immediately before the experiment. The glycine (pH 2.2) and carbonate (pH 9.6) buffers were prepared using the standard procedure. The protein A *Staphylococcus aureus* (Sigma) was used in a water solution (concentration of 50 mg/ml).

### 4. Results and discussion

In accordance with our procedure for immobilization of antiviral immunoglobulins (Boltovets et al., 2002), the gold surface of the converter was treated consecutively with the solutions of thiocyanate and protein A *S. aureus*. Fig. 2a presents a typical time dependence of angle shift for an open system at immobilization of the specific IgG–virus complex at a functionalized surface. Such shift is due to both specific and nonspecific adsorption of the sample component and therefore the total response depends not only from the virus concentration.

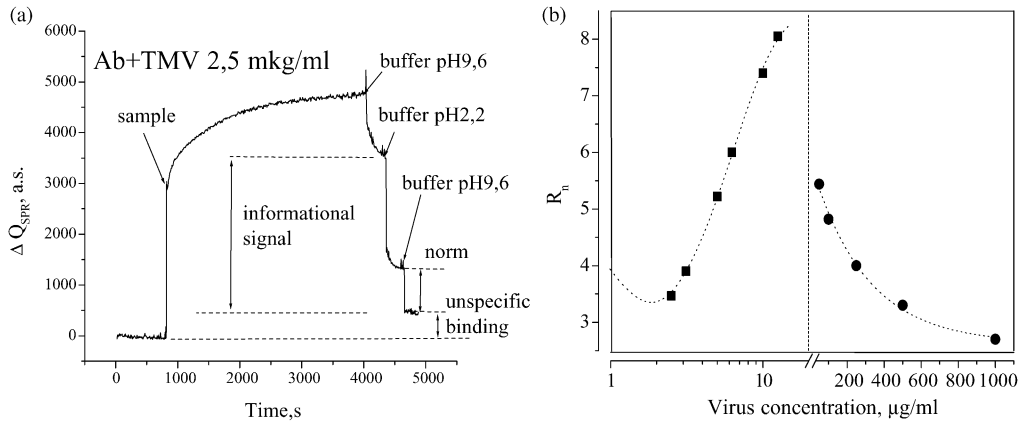


Fig. 2. Dependence of the virus concentration in sample from the angle shift in the open system. The mean linear deviation of the data is not more, than 5% ( $\pm 0.2$  in relative units). (a) Time dependence, (b) calibration curve.

Taking into account that sensitivities of different sensor elements may differ, the shift of the angle when passing from one standard buffer to another (shown in Fig. 2a as “norm”: water–carbonate buffer exchange, whose refractive indices are known and constant) was used for standardization of the measurements for different samples. Fig. 2b presents the calibration dependence (normalized in accordance with  $R_n = \text{“inform signal”}/\text{“norm”}$ ) for “open” system. The informative signal is the specific (i.e., appropriate interactions between virus-specific immunoglobulins complexes and protein A) part of the total response that remains after removal the non-specific signal by a buffer (that remains after washing by a buffer with pH 2.2 which breaks the specific bonds between the Fc-fragment of immunoglobulin and protein A).

At dilution 1:10 of the antiviral serum a monotone dependence of the angle shift from the virus concentration (concentration range from 2 to 20  $\mu\text{g/ml}$ ) was observed. At high ( $>100 \mu\text{g/ml}$ ) virus concentrations the signal level drops. This is a result of paracrystalline structures formation in the strong solution (when virus concentration exceeds 10%). Thus, the use of an open chamber enables one to detect quickly virus presence and their concentration in a samples and (using calibration curve as in Fig. 2b). It is necessary to stress that concentration of antibodies determine the position of calibration curve along the virus concentration axis owing to required relations between [IgG] and [TMV] discussed above; if the antibody concentration decrease the calibration curve (and correspondingly method detection limit at given [IgG]) shift to the lower virus concentrations. In spite of the fact that the open cell is suitable for the rapid analysis limitations inherent to the static sample preparation (i.e., diffusion and concentration gradients) can substantially affect accuracy of the analysis.

Bearing in mind, the importance of the quantitative analysis of virus-containing samples, flow system (in accordance with the IUPAC requirements) was developed. This enabled us to improve control of the experimental conditions and automate both the measurement process and sample preparation (involving different dilutions of the virus specific serum).

Four sets of qualitative experiment were attempted: (i) immobilization of specific IgG from antiviral serum at a surface treated with KNCS and protein A (negative control); (ii) immobilization of the specific IgG–refined virus complex at a surface treated with KNCS and protein A (positive control); (iii) immobilization of the specific IgG–homogenate of *B. minor* cells (infected with TMV at the zoospore stage) complex; (iv) immobilization of the specific IgG–homogenate of *B. minor* cells (infected with TMV at the zoospore stage and double-reseeded) complex.

The results have shown (Fig. 3) that the total shifts of angle for virus-containing samples was slightly higher then for virus-free samples (as in the case of open cell). Only small difference of the total responses was due to efficient adsorption (both specific and nonspecific) of different mixture components at the physical converter surface. After removal of

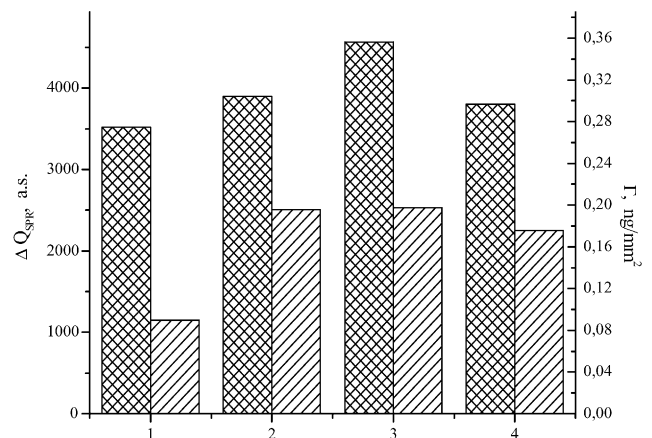


Fig. 3. A comparison between the SPR shifts to nonspecific and specific adsorption of the IgG–virus complex.  $\Delta Q_{\text{SPR}}$ , SPR angle shift; s.a., second of arc;  $\Gamma$ , degree of surface coating; 1, antiviral serum; 2, antiviral serum and TMV; 3, antiviral serum and homogenate of the *Bracteaococcus minor* cells infected with TMV at the zoospore stage; 4, antiviral serum and homogenate of the *Bracteaococcus minor* cells infected with TMV at the zoospore stage and twice reinoculated. (⊗) Nonspecifically adsorbed complex. (⊘) Specifically adsorbed complex.



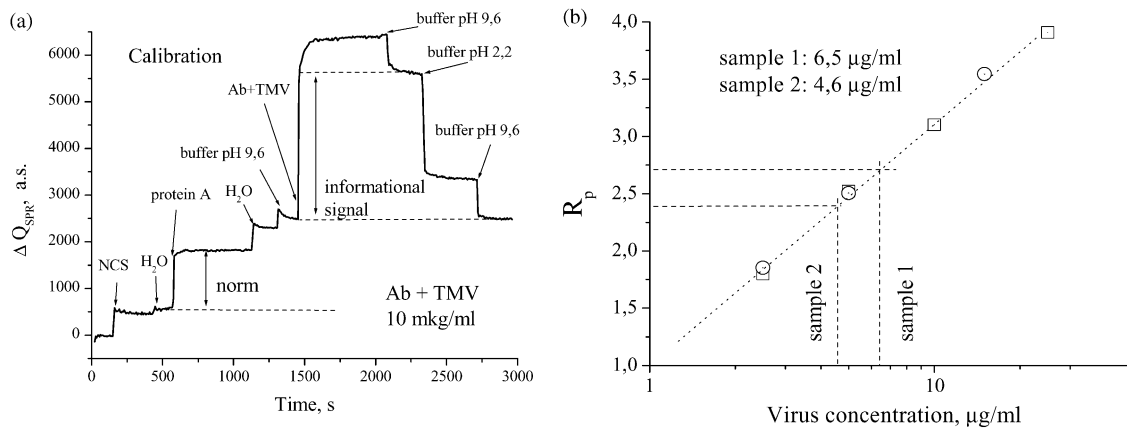


Fig. 4. The dependence of the virus concentration in sample from the SPR angle shift in flow system: sample 1, *Bracteacoccus minor* cells infected with TMV at the zoospore stage and sample 2, *Bracteacoccus minor* cells infected with TMV at the zoospore stage and twice reinoculated. The mean linear deviation of the data is not more than 3% ( $\pm 0.1$  in relative units). (a) Time dependence, (b) calibration curve.

nonspecifically bound component by buffer, the shift of the angle in the virus containing samples studied is more than twice of that without virus. For example, for antiviral serum this shift was 1150 s.a. ( $0.090 \text{ ng/mm}^2$ ) compared to 2510 s.a. ( $0.196 \text{ ng/mm}^2$ ) in the samples with TMV. The difference between the  $\Delta Q_{\text{SPR}}$  values obtained for the preparations containing homogenates of nonseeded and seeded cells is not as significant as in the previous experiment: these values are 2530 s.a. ( $0.198 \text{ ng/mm}^2$ ) and 2250 s.a. ( $0.176 \text{ ng/mm}^2$ ), respectively. This fact indicates the formation of a compact surface layer in all the three cases. From the data presented in Fig. 3 following conclusion can be made: if there are viruses in the sample and  $[\text{Ab}] > [\text{TMV}] \times \text{number of epitopes}$ , then the angle shift is about double of that for a virus-free sample. This makes it possible to identify virus-containing samples reliable and quickly. Moreover, the result defines dynamic range of the SPR angle shift inherent the proposed approach: the angle shift with close-packed virion-IgG complexes is two times higher than in a case of single antibody. The calibration curves presented in Fig. 2b confirming this conclusion.

To provide quantitative determination of virus content in samples, calibration curve was plotted using the results of measurements for refined viruses (Fig. 4a). Taking into the account sensitivity variations for individual transducers and number of immobilized receptor centers of the protein A, a level of protein A immobilization was used as a more precise norm (which include differences between the chips discussed above). The calibration curve (normalized in accordance with  $R_p = \text{“inform signal”}/\text{“norm”}$ ) is presented in Fig. 4. Similarly, for the open cell a dependence of the angle shift from the virus concentration in a sample (concentration range from 2 up to  $20 \mu\text{g/ml}$  at dilution 1:10 of the antiviral serum) was observed. The virus concentrations in samples 1 and 2 from the homogenate of green alga *B. minor* cells infected with TMV are 6.5 and  $4.6 \mu\text{g/ml}$ , respectively. Concurrent detection of virus in the above samples made with ELISA, confirmed high concentration of virus antigen in the *B. minor*

culture infected with TMV at the zoospore stage (Boltovets et al., 2002). In the *B. minor* culture infected with TMV at the zoospore stage and double-reseeded, concentration of virus antigen (determined with ELISA) is significantly lower. The values from SPR angle shift measurements and ELISA results for the same samples correspond, which proves that surface plasmon resonance technique can be adapted for virus detection.

## 5. Conclusions

The detection and quantitation of virus content in samples by immobilizing them complex with antiviral immunoglobulins at the surface modified with NCS and protein A was demonstrated. The general sensitivity of the technique is about  $250 \text{ s.a.}/\mu\text{g}$ . With instrumental error of  $\approx 3\text{--}5 \text{ s.a.}$ , this provides signal-to-noise ratio up to 70 for the full range of angle shift. The dynamic range of virus concentrations at this value of serum dilution is about 10-fold variation of concentration: for dilution 1:10 this range is  $\approx 2\text{--}20 \mu\text{g/ml}$  which correspond changes of interfacial structure from loose to close-packed. The detection limit at a given serum concentration determined by the conditions that  $[\text{TMV}] = [\text{IgG}]$ . The absolute detection limit depends on the area of the sensitive part of sensor surface (since the surface plasmon resonance is surface-based method) and the amount of analyte in probe enough to cover the sensitive area of surface. For the cell 1.5 mm in diameter this limit is determined by the ratio of total surface area ( $\pi \times (1.5 \times 10^{-3} \text{ m})^2$ ) and area occupied by one virion ( $300 \times 18 \times 10^{-9} \times 10^{-9} \text{ m}^2$ ; TMV is a 300 nm tube with diameter above 18 nm) and equal above  $5 \times 10^9$  virus particles. The absolute detection limit decreases proportionally to the area of sensitive surface, and at the diameter of  $1.5 \mu\text{m}$  this will be around  $10^3\text{--}10^4$  virus particles (if the lower limit of the linear range of physical converter is 0.1 pg; typical limit of detection for surface plasmon resonance is  $10^{-5} \text{ pg}/\mu\text{m}^2$  (Snopok, 2002)). It is necessary to stress the re-

liability of the method for virus detection: if  $[TMV] \geq [Ab]$  and amount of complexes is enough for full coverage the surface response for the virus containing probes will be twice as high as probe which contain only specific antiserum (negative control). At lower concentrations the angle shift will be proportional to the TMV concentration.

The proposed approach extends the application of various surface plasmon resonance spectrometer for a broad range of virological samples and can be easily utilized using on many commercially available instruments (Bard and Myszka, 2001; Homola, 2003).

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