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Polysaccharide based organic frameworks with embedded nanoparticles: advanced SPR study on the antiviral activity of gold composites derived from glucuronoxylomannan †

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Abstract: The nanosized composites based on the natural polysaccharides and nanoparticles of noble metals are promising candidates for efficient antiviral drugs. However, the complexity of such objects, their diversity and novelty necessitate the development of new analytical methods for investigation such supramolecular architectures. In this work, the recently developed for SPR based instrumentation the concept of variative refraction (DViFA, density variations in fixed architectures) was used to elucidate the mechanism of the antiviral action of a polysaccharide with gold nanoparticles grown in it. The SPR data were confirmed by direct biological tests: the effect of the native polysaccharide glucuronoxylomannan (GXM) obtained from the fungus *Ganoderma adspersum* and gold nanocomposites thereon on the infection of *Datura stramonium* with tobacco mosaic virus (TMV) was investigated. Both drugs suppress the development of viral infections. However, if for high concentrations the characteristic activity of the composite is somewhat lower than for GXM, then with an increase in dilution, the effectiveness of the composite increases significantly, up to a twofold excess. It has been reasonably suggested that the mechanism of antiviral action is associated with the formation of clusters of viruses that are no longer capable of infecting cells.

Keywords: nanocomposites; variative refraction; surface plasmon resonance; antiviral activity

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1. Introduction

Nanosized composites combined organic compounds and inorganic nanoparticles extend our capabilities to form supramolecular architectures of advanced functionality. Complex macromolecules of biological origin, which can act as a matrix for the synthesis of such objects, are of particular interest. In this work, the polysaccharide glucuronoxylomannan with known immunomodulatory activitiy was chosen as such a matrix [1].

Early we considered the potential of polysaccharide glucuronoxylomannan as antiphytoviral agent. It was demonstrated that GXM isolated from *Tremella mesenterica* culture can suppress TMV infection in *Nicotiana tabacum* and *Datura stramonium* plants [2]. It was shown that GXM affects both the virus before infection and the processes that are proceeding immediately in the cell. In particular, polysaccharide can suppress virus reproduction and induce plant resistance to pathogen. It was suggested that GXM sterically block the virions, thereby suppressing its ability to infect [3]. In this regard, it is reasonable to evaluate the antiviral effectiveness of GXM nanocomposites with gold, well-known inhibitory agents, the effect of which is often associated with the activity of this metal ions

in an aqueous medium. [4] It should be emphasized that the "ionic model" of the antiviral action of nanoparticles finds fewer and fewer supporters among researchers: the authors of many publications increasingly prefer various physical processes in the field of contact of nanoparticles with biological objects when describing one or another effect of nanoobjects on biological systems.

Indeed, the nanosized composites turned out to be a fundamentally new object with unusual properties - in particular, their toxicity, which does not correlate with the properties of the material in the atomic-molecular state or in a solid. This is well illustrated by the example of the effect of nano-objects on viruses and bacteria, for which a large amount of experimental material has been collected. It is reasonable to conclude that antiviral effects of nanoparticles are due to some physical phenomenon, rather than a chemical interaction of one type or another. Nanoscale analytes induce the change of the toxicity paradigm: physical effects come to the forefront, not the features of the chemical structure. This means that we need take into account not only the chemical composition of the object, but also its geometric characteristics, such as size and shape [5].

The aim of this work was to study GXM nanocomposites with gold nanoparticles in order to elucidate their potential as antiviral drugs using the example of the TMV virions.

Since macromolecules of polysaccharides are complex branched structures, it is not possible to uniformly introduce pre-synthesized metal nanoparticles into these structures. However, taking into account the fact that polysaccharides contain a sufficiently large set of functional groups with the characteristics necessary for carrying out the corresponding redox reactions, in this work the synthesis of metal nanoparticles was carried out directly inside the polysaccharide matrix.

This polysaccharide can act both as a reducing agent and as a stabilizer due to the presence of the corresponding functional groups in the molecule. GXM consists of a linear backbone of (1→3)-linked α-D-mannose with mainly xylose and glucuronic acid in the side chains [6]. Molecule of glucuronic acid contains carboxylic acid group which gives acidic properties to GXM (acid polysaccharide). In order to clarify the peculiarities of the interaction of composites with viral particles at the molecular level, an analysis of their interaction with the tobacco mosaic virus was carried out using the SPR method. Antiviral activity of the complex was also tested *in vivo* at the *Datura stramonium L.*

2. Methods

Preparation of GXM. The principle of the method of obtaining preparations of β-glucan from the mycelium of the fungus *G.adspersum* was the same as in obtaining β (1 → 3) -β (1 → 6) -bound glucan ("ganoderan") from other species of *Ganoderma sp.* Namely: polysaccharide preparations were obtained from lyophilized mycelium of the fungus by sequential aqueous, alkaline and acid extraction [7]. First, to remove low molecular weight compounds to the dry mycelium of the fungus *G.adspersum*, thoroughly ground in a porcelain mortar, add 85% ethanol solution (1: 5, v / v) and boil for 3 hours. The alcohol extraction procedure was repeated three times. Each time the precipitate was separated by centrifugation (10000 g, 15-20 min) and used in further work. To obtain the aqueous fraction of β-glucan, water (1: 5) was added to the mycelium purified from low molecular weight impurities and boiled for 3 hours. The procedure was repeated 5 times. The extracts were collected by centrifugation (10,000 g, 15-20 min) and combined. The resulting extract was dialyzed against running and distilled water and then evaporated to a minimum volume (1: 5) on a rotary evaporator 1/5 by volume of a mixture of isoamyl alcohol and chloroform (1:10) was then added to the concentrate, the mixture was shaken vigorously for 10 min

and then centrifuged (10000 g, 20 min) to separate the aqueous and organic phases. Deproteinization of the extract was repeated once more, the organic extracts were removed, the aqueous ones were combined and dried in freeze-drying. [8]

Synthesis of AuNPs. First, GXM water solution was prepared by dissolution of 3 mg GXM in 2.9 ml H_20, and then aqueous solution of $HAuCl_4$ (0.1 ml, 30 mM) was added to it at violent stirring. The mixture was stirred during 1 minute at room temperature, heated to 100°C with boiling during 10 minutes. The AuNPs formation was confirmed by UV-Vis Spectroscopy and TEM.

Instrumentation and Measurements. The morphological, optical, and spectroscopic properties of AuNPs were examined using the following measurements. UV-vis spectra were acquired with Umico UV-Vis spectrophotometer (data not shown). TEM was performed at 100 kV using a JEOL-1011 (JEM, Japan) instrument (data not shown). Scanning spectrometer "BioHelper" (ISP NASU, Kiev, Ukraine) was used for SPR measurements with standard chips (50 nm Au / 1.5 nm Cr / glass (n = 1.61)) and the protein A immobilization protocol described in detail in [9]. SPR measurements were carried out in a static mode without a sample flow in an open cell configuration (400 μL).

3. Results and discussion

Molecular level analysis: advanced SPR study

Conventional SPR studies typically use "2D" interfacial architectures whose thickness is significantly smaller than the penetration depth of an exponentially decaying evanescent wave in a dielectric medium [10, 11]. The use of SPR techniques for the investigation of "bigger" objects (e.g. intact virus particles so called virions, cells etc.) has some limitations since their characteristic size exceeds tens of nanometers, which is required to ensure adequacy of the quasi-linear approximation in SPR sensing.

In the conventional approach, the SPR response depends on the effective thickness of the analyte layer that is bound to the receptors on the surface, - the density of both layers (receptor and analyte) is uniform, - i.e. the variation of the SPR signal is due to the changes of the thickness. However, an SPR shift depends also on the change of the refractive index within the layer. Therefore, variations of the layer density can also affect the response value due to the variations of the refractive index inside the layer ("variative" refraction). One of the possible mechanisms is changing the packing of the objects of different size and shape within interfacial architectures on the surface (e.g. a virus, an antibody, a small molecule etc.). If the thickness of the surface layer is fixed due to the constant form of the biggest interacting components (e.g. virion), the SPR shift is a single-valued function of the molecular assembly compactness. Exactly such unconventional approach DViFA (density variations in fixed architectures) has been proposed by us early [12-14] for quantitative detection of native virus particles t (V) using SPR techniques.

Typical protocol includes the incubation of a virus-bearing material (V) with definite concentration of a specific antibody (Ab) following by the injection of statistically formed V-Ab complexes into SPR instrument with sensitive surface covered by protein A. A shift of the minimum of SPR signal depended on the compactness of the V-Ab monolayer on the surface. To realize such an approach, we took advantage of (1) specificity of protein A from *Staphylococcus aureus* to the Fc-fragment of immunoglobulins as well as development of (2) statistically stable distribution of complexes with different numbers Abs per V specific for different Ab/V ratio.

To refine the DViFA approach, the investigation of TMV interaction with serum containing specific antibodies was performed, both with and without the presence of GXM-Au

composite in the sample during the incubation phase. We investigated how the nanocomposite affects the interaction of the virus with specific antibodies. During the measurements, the concentration of TMV particles was constant (at constant concentration of 100 μg/ml), and the concentration of antibodies was changed by serial dilutions of serum in the range from 1:25 to 1:1600. The results obtained were compared with the data for a virus at the same concentration preincubated with GXM-Au complex (Figure 1):

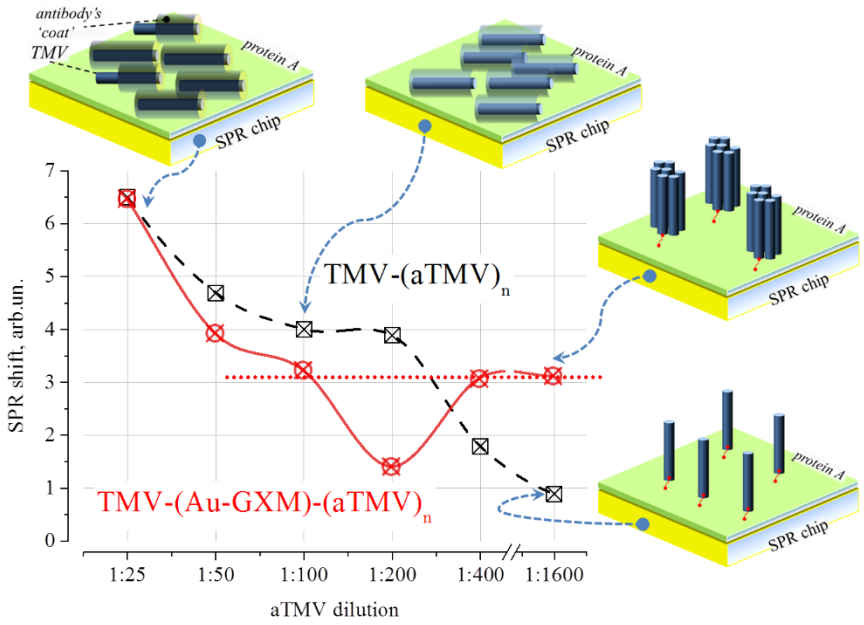

Figure 1. Comparasion of the immobilization of TMV-aTMV complexes in the presence and in the absence of Au-GXM nanocomposite. The figures around the graph show schematic representations of the surface orientation of viral particles "on side" (at low dilutions) and "at end" (at large dilutions of antibodies). Individual virus particles or aggregates are attached to the surface by "bridging" antibodies, the Fc fragment of which is bound by protein A immobilized on the surface.

At dilution levels between 1:25 and 1:200, the antibodies fill the free surface of the V-Ab cylinders. Such complexes experience a mutual orienting effect on the surface modified by protein A and their configuration is typical for the classical “car parking” problem. The general structure of such an ensemble of viral particles on the surface remains practically unchanged up to a dilution of antibodies of about 1: 200. The decrease in the SPR response in this range is due to a decrease in the number of antibodies associated with the virus, i.e. density of surface architecture. Some stabilization of the response in the range from 1: 100 to 1: 200 can be associated with two competing processes, - on the one hand, a decrease in the number of surface-bound antibodies decreases the density, and on the other hand, it increases through some convergence of individual viral particles. In the presence of a GXM-Au composite, all the considered effects are less pronounced and are observed at low dilutions, since the GXM composite blocks some of the surface binding sites of Ab on the virion surface. At a dilution level of 1: 200 and 1: 100 for a virus without and in the presence of a nanocomposite, respectively, the amount of antibodies becomes insufficient to keep the virus “on side” on the surface and some of the viral particles become “at end”, fixing pointwise on the surface with a chain of virus-antibody-protein A. In the absence of a GXM-Au composite, this process continues with further dilution (1:400-1:1600), leading to a decrease in the mass (number of attached virus particles) on the surface.

A radically different situation is observed in the case of the presence of a GXM gold nanocomposite: a decrease in the amount of antibodies at dilutions greater than 1: 200 leads to

an increase and subsequent stabilization of the response for dilutions up to 1: 1600. In this case, the stabilization of the response occurs approximately at the same level, which corresponded to the densest structure of viral particles in the position "on the side". Since the diameter of the virus particle coated with antibodies is about 40 nm, and the length of the virus particle is 300 nm, then in the orientation "on side" and "at end" the surface ensemble of viral particles practically completely overlaps the region of the highest concentration of the evanescent wave (less then 100 nm due to its exponential decay with distance from the surface). All this suggests that the presence of a polysaccharide nanocomposite stimulates the aggregation of virus particles into large clusters that are attached to the surface by only a few antibody molecules. Summarizing the obtained results, it can be argued that the nanocomposite stimulates the aggregation of viral particles into clusters, preventing their "independent" functioning.

Biological experiments in vivo

To verify the antiviral activity of the nanosized composite the classical biological test has been performed. Aqueous solutions of Au-GXM (in the concentration range 1-500 μg/ml) were added to a suspension of TMV (10 μg/ml) and the mixture was incubated for 30 minutes at room temperature. Then, the left halves *Datura stramonium L.* were inoculated with mixture, whereas the right halves were infected with the virus at the same concentration without composite.

The degree of viral infection suppression (in percentage terms) was calculated from the number of necrotic local lesions on the test and control leaves using the following expression [8]

$$I = ((C - P)/C) \cdot 100\%, \quad (1)$$

where I is the degree of viral inhibition in percent; C - local lesions number on the control half; P – number of local lesions on the test half. The results of the calculation of necrosis were subjected to statistical processing by parametric criteria, calculating their average number (M) and the ratio of these data in the experiment and control, as well as the average error of the ratio (m). On the graph, the data of statistical evaluation of the results were expressed as M ± m (Figure 2).

The in-vivo experiment showed that both native GXM and GXM-Au composite inhibit the development of viral infection: this is manifested in a much smaller number of necrosis on the experimental halves of the leaves of the studied plants (see Insert in Figure.2).

In order to compare the effectiveness of potent antiviral drugs, a concentration dependence of the percentage of necrosis on the experimental leaf halves to the number of necrosis on the control leaf halves on the drug concentration was constructed (i.e., the lower the value, the more effectively the virus is suppressed). For the preparation of native GXM, a clear linear (exponential in linear coordinates) dependence of the degree of viral infection suppression on the concentration was demonstrated. The difference in the concentration range of 0.5 mg/ml - 0.001 mg/ml is c.a. 50%. It was shown that at high concentrations (0.5 mg/ml) the activity of the GXM is higher than the activity of composite. However, the effectiveness of the GXM-Au composite is significantly less dependent on the concentration of the drug. This leads to the fact that the antiviral activity of the composite is much higher at low concentrations. In particular, for a concentration of 0.001 mg / ml, the degree of viral infection suppression more than twice better for Au composite in respect to native glucan.

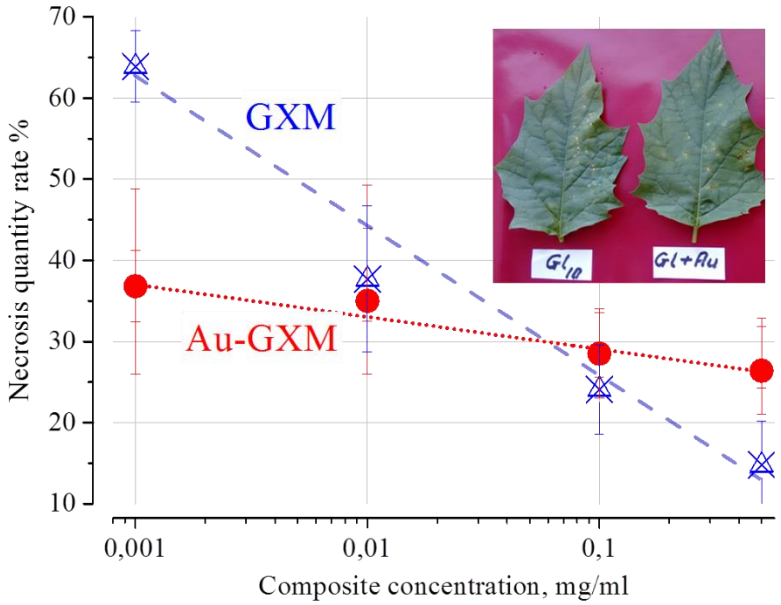

Figure 2. Percentage of necrosis per leaf with native glucan (GXM) and Au-glucan composite (Au-GXM). Insert: *Datura stramonium L* leaves infected with TMV in a mixture with native glucan GXM (labeled "Gl_{10}") and GXM-Au nanocomposite (labeled "Gl+Au").

The results of molecular analysis and *in vivo* studies suggest that polysaccharide matrices with embedded gold nanoparticles have a stronger antiviral effect at low concentrations in comparison with natural polysaccharides. The mechanism of this action is due to the fact that the metal composite induces the aggregation of viral particles into clusters incapable of subsequent infection of plant cells.

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