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Chapter 25
Optical Immunosensor Based on Photoluminescent TiO_2 Nanostructures for Determination of Bovine Leucosis Proteins. Model of Interaction Mechanism

A. Tereshchenko, V. Smyntyna, U. Bubniene, and A. Ramanavicius

Abstract The main aspects of the interaction mechanism between nanostructured TiO_2 layer and BLV proteins *gp*51 have been evaluated during the formation of photoluminescence-based immunosensor. *Bovine leucosis* protein *gp*51 was adsorbed on the surface of a nanostructured TiO_2 thin film, formed on glass substrates. A photoluminescence (PL) peak shift from 517 to 499 nm was observed after modification of TiO_2 surface by adsorbed *gp*51 (i.e. formation of the biosensitive layer *gp*51/TiO_2). An incubation of *gp*51/TiO_2 in a solution containing anti-*gp*51 antibodies resulted in the formation a of a new structure (anti-*gp*51/*gp*51/TiO_2) and the backward PL peak shift from 499 nm to 516 nm. The PL shifts are attributed to the variations in the self-trapped exciton energy level, which were induced by the changes of electrostatic interaction between positively charged atoms and groups, provided by the adsorbed *gp*51 protein and negatively charged surface of TiO_2. The charge–charge-based interaction in the double charged layers *gp*51/TiO_2 can also be interpreted as a model based on 'imaginary capacitor', formed as a result of the electrostatic interaction between oppositely charged protein *gp*51 layer and the TiO_2 surface.

25.1 Introduction

Bovine leucosis is a lethal cancerous disease caused by *Bovine leukemia* virus (BLV) that belongs to one of the most monitored family of retro-viruses in the world. There is a significant risk that BLV can infect other mammalians like it was in the case

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of Human immunodeficiency virus (HIV). Due to lethal epidemical nature, the outbreaks of *Bovine leucosis* pose a great threat to the environment and eco-systems. As the food quality control is an inseparable part of human safety and wellbeing thus an advanced determination of *Bovine leucosis* can decrease the risks of human diseases caused by this and other dangerous viruses. For this reason, timely diagnosis of virus-induced diseases is an important direction in environmental monitoring. For such monitoring very efficient bio-analytical systems are required but old bio-analytical methods are cumbersome and slow. Therefore, the development of advanced bioanalytical systems based on photoluminescence immunosensors seems to be the most promising direction [1, 2]. However, despite of many reports on photoluminescence-based immunosensors, the mechanism of interaction of biomolecules with semiconducting materials, which are used as analytical signal transducers in the most promising PL-based immunosensors, is still very poorly evaluated [3].

Nanostructured Titanium dioxide (TiO_2) is known as a material of intense photoluminescence at room temperature [4, 5]. The application of TiO_2 photoluminescence properties in optical biosensors and immunosensors have been reported in the range of works [1–3]. TiO_2 is widely studied material as a wide-band gap semiconductor with a great combination of physical and chemical properties [6–8]. A good biocompatibility of TiO_2 nanostructures, their applicability at physiological pHs in the range of 5.5–7.0, non-toxicity and excellent chemical stability have resulted in the extensive application of TiO_2 in various biosensors [1, 2, 9]. Optical biosensors are increasingly studied class of biosensors because optical detection methods have a number of advantages. Optical methods allows to evaluate some inter-molecular interactions contactless, i.e., without contamination and/or deterioration of the aliquot, which contains biological compounds [2, 3] and it allows to avoid application of special tags or markers and enables to determinate the analyte concentration directly without any chemical/physical labels [1–3]. Among the large variety of biosensors, a special attention is paid to the development of immunosensors, based on the specific interaction between antibody and antigen that can be applied for the determination of wide variety of analytes in complex biological samples [1, 2, 9].

The changes in the photoluminescence spectra (shift of photoluminescence maximum and the variation of photoluminescence signal intensity) were exploited as analytical signals for the determination of target analyte [2, 3, 9, 10]. However, the interaction mechanism of proteins with TiO_2 and the origin of the changes in the photoluminescence spectra were not discussed. Although the mechanism of the interaction between semiconductor TiO_2 and proteins is the key in solving many of problems, which are still arising during the development of TiO_2-based immunosensors, such as an improvement of sensitivity and selectivity.

This work is aiming to highlight the origin of the changes in the photoluminescence spectra of TiO_2 resulted after the protein adsorption on its surface during the formation of biosensitive layer, and after the interaction of biosensitive layer with the analyte. The proposed interaction mechanism provides the general understanding of the interaction between TiO_2 and proteins, what is a key in the development of new

PL-immunosensors and solving of many issues related to an improvement of performance of PL-based immunosensors, first of all, related to the sensors' sensitivity and selectivity.

25.2 Experiment Details

Nanostructured TiO_2 layers containing TiO_2 nanoparticles were formed by deposition of colloidal suspension of TiO_2 nanoparticles (Sigma Altrich, 99.7%, particle size of 32 nm) dissolved in ethanol. The concentration TiO_2 nanoparticles was about 0.01 mg/ml. TiO_2 layers were dryed at room temperature and annealed at 350 °C. Structural and surface characterization of the obtained samples showed that TiO_2 layers kept the anatase structure and TiO_2 nanoparticles formed a high surface area porous structure suitable for the formation of biosensitive layer. More detailed information on the deposition and characterization procedures applied for characterization of nanostructured TiO_2 layers is reported in earlier researches [3, 10].

Optical characterization of nanostructured TiO_2 layers was performed by photoluminescence measurements using 355 nm solid state laser as the excitation source. Optical setup is described previous our researches [3, 9]. The spectra were recorded in the range of wavelength from 360 to 800 nm.

The immobilization of biological molecules was carried out by incubation in a solution containing Bovine leukemia virus proteins—*gp*51, similar to the immobilization procedure described in earlier works [3, 9, 10]. In brief: a solution of PBS containing *gp*51 antigens at a high concentration was directly immobilized on the TiO_2 surface. Then the sample was placed into a Petri cup for the incubation in a medium saturated with water vapor at 25 °C. After 10 min of incubation, the surface of the sample was washed with PBS solution in order to remove non-immobilized antigens on the TiO_2 surface. As a result, an adsorption-sensitive layer TiO_2/*gp*51 was formed, selective to the one type of bio-molecules—anti-*gp*51 antibodies against leukemia proteins *gp*51. To prevent a nonspecific interaction (i.e., binding of anti-*gp*51 antibodies directly to unmodified TiO_2 surface), the surface of TiO_2 was further treated with a solution of bovine serum albumin (BSA), which filled possible adsorption sites that remained free after the modification of TiO_2 surface with *gp*51. Thus it is expected that the selectivity of the structure of TiO_2/*gp*51 was improved by this procedure based on treatment with BSA, which is frequently applied during the development of immunosensors devoted for the determination of proteins.

25.3 Results and Discussion

Analysis of the interaction between TiO_2 and *gp*51 proteins was based on the evaluation of photoluminescence of TiO_2 nanoparticles. The protein *gp*51 is specifically binding with antibodies against *gp*51 (anti-*gp*51) that were interpreted as analyte

in this research. The photoluminescence properties of the TiO_2 nanoparticles have been previously investigated by authors in the research papers [3, 9, 10] as well as the influence of *gp*51 protein adsorption on the optical properties of TiO_2 and the development of photoluminescence based immunosensor for the determination of *gp*51 antibodies [3, 9, 10].

The process of immobilization of *gp*51 and formation of TiO_2/*gp*51 structure was similar to that reported in earlier our works [3, 9, 10]. The immobilization of *gp*51 antigens leads to an increase in the intensity of the photoluminescence signal of TiO_2 nanostructures and a UV-shift in the position of the maximum of the photoluminescence spectra (Fig. 25.1). The application of BSA, which were used to block the free adsorption centers on the TiO_2 surface, also leads to a slight increase in the photoluminescence intensity, but the spectrum shift in this case was not observed.

Interaction between TiO_2/*gp*51 and anti-*gp*51 led to the inverse changes in the photoluminescence spectrum (Fig. 25.1), i.e. a decrease in the integral intensity of the photoluminescence and the IR-shift of spectra. Therefore, the response of the immunosensor TiO_2/*gp*51 to anti-*gp*51 can be estimated by two parameters: (i) the photoluminescence intensity and (ii) the position of the PL-maximum. The sensitivity of TiO_2/*gp*51 based immunosensor towards anti-*gp*51 was in the range of 2–8 mkg/ml [9, 10].

TiO_2 (anatase) is known as a semiconductor of n-type conductivity, usually with an 'upward' band bending of the energy levels when closing the surface of TiO_2 (Fig. 25.2) [11], which indicates the accumulation of a negative charge (bound at surface levels) on its surface. The adsorption of the most of molecules is known to

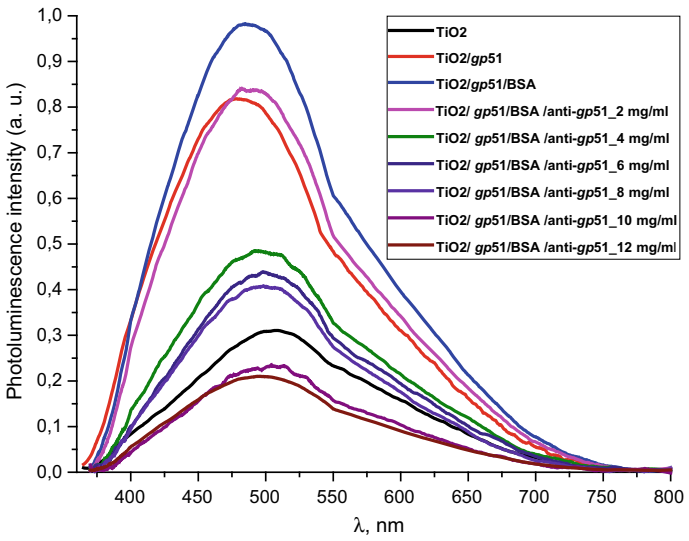

Fig. 25.1 Photoluminescence spectra of TiO_2 nanoparticles before and after the immobilization of *gp*51 antigens on the TiO_2 surface, subsequent BSA deposition and after the interaction of TiO_2/*gp*51 based immunosensor after with analyte (anti-*gp*51 antibodies) of different concentrations

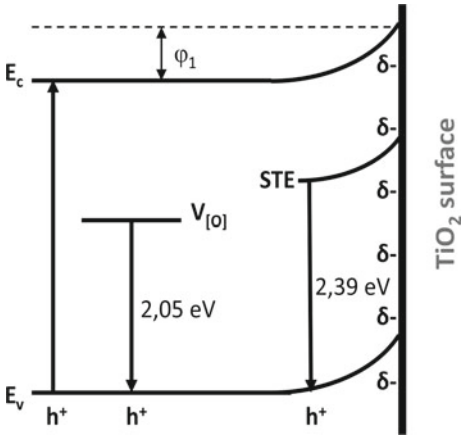

Fig. 25.2 Energetic levels of TiO_2: E_c, E_v—conductive and valence bands respectively, STE—self-trapped exiton level, $V_{[o]}$ —oxygen vacancies level

introduce an additional charge on the solid state surface and it can change the existing surface energy levels or form the additional ones that are involved in the exchange of charges with the volume of a solid material [12].

The proteins consist of amino acids that might contain positively and/or negatively charged radicals that are determining the charge of the different protein domains [13]. A large quantity of negatively charged groups such as aldehyde (–CHO), hydroxyl (–OH), carboxyl (–COOH) and positively charged primary amine ($–NH_2$) and some other groups, which are involved into the structure of amino acids, are responsible for the partial (δ+ and δ−) charges of particular protein domains (Fig. 25.3) [14]. Therefore the proteins are characterized by electrostatic properties, and sometimes even significant electrostatic 'asymmetry of protein molecule' because the atoms

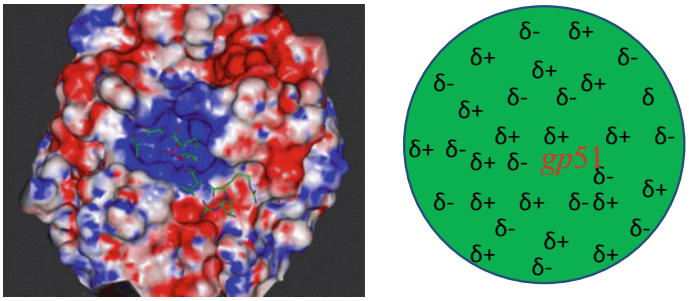

Fig. 25.3 **a** Charge distribution in calciumneurin protein: positive charge—blue color, negative charge—red color, neutral charge—white color [15]; **b** schematic image of charge distribution in *gp*51 antigen protein: δ+ and δ− are partial positive and negative charges respectively

and functional groups forming the protein molecules are charged differently both in their sign and in absolute charge value. Naturally, the charges at least partly are compensating each other, but since the ternary structure of proteins is relatively rigid and the charged groups have only limited degree of freedom to move within the protein globule, therefore in some parts of the protein some uncompensated charge on the surface and inside of the protein still remains [14]. The distribution of charged groups on the surface of the protein depends on the sequence of amino acids, which is pre-determined by the genome that was developed during billions of years lasting evolution and selected genes promoting the synthesis of proteins whose structure the most efficiently matches their function.

It should be taken into account that even if the structure of the most proteins is at some extent 'rigid' there is some degree of flexibility because both secondary and tertiary structures of the protein are supported by a large number of hydrogen bonds but many of them are not very strong [13, 16]. The electrostatic bonds, which are based on Coulomb forces, between the opposite charges, van der Waals forces and disulfide bonds also play an important role in the formation of both secondary and tertiary structures of protein.

25.3.1 Mechanism of Interaction Between TiO_2 and Proteins

A *gp*51 protein molecule has a molecular mass of 51 KDa. The characteristic geometric size of the *gp*51 molecules adsorbed on the TiO_2 surface is about 6 nm in diameter [17]. The authors, which have published a research on the formation of *gp*51 virus based capsid of BLV, have constructed an image of *gp*51 virus structure from the X-ray crystallography data and they have reported that this protein is extra-flexible, which provides very high functionality and the ability to associate and/or dissociate of BLV capsid from the membrane of BLV infected cell [18]. Therefore, it is expected that on the surface of TiO_2 *gp*51 forms well-ordered monolayer. The formation of such layer was confirmed in other our researches by spectroscopic ellipsometry [10, 19, 20].

*Gp*51 protein is not a redox-protein therefore the charge transfer between *gp*51 and TiO_2 is not possible [18, 14]. However the *gp*51 protein like many others, contains a number of partially charged groups and domains, represented as partial charges "δ−" and "δ+" in Fig. 25.3b, which mostly are lower in value than the total electron charge (1.6×10^{-19} coulombs) per charged atom or group. The presence of these partial charges suggests that the electrostatic influence on the surface charge of TiO_2 from the side of partially uncompensated charges in those parts of the *gp*51 protein that located on the surface of TiO_2 is responsible for the adsorption of this protein on the TiO_2 surface. The Coulomb interaction takes place between charged groups in the *gp*51 protein and the negatively charged surface of the TiO_2 because such electrostatic interactions are very strong at a distances ranging from several Angstroms to few nanometers. Therefore, among the others interactions such as hydrogen bonds, disulfide bonds, Van der Waals interaction, etc., which also have

significant role during the adsorption of proteins, the electrostatic interaction plays one of the most important role during the adsorption of proteins to electrically charged surfaces, such as TiO_2. In addition, the local electric fields of charged domains of adsorbed proteins are affecting the photoluminescence centers of TiO_2 and it causes the shift in the photoluminescence spectra of TiO_2 nanoparticles. Therefore, the photoluminescence maximum caused by STE shifts from 517 to 499 nm (i.e., to 18 nm), which corresponds to ~0.086 eV that is less than 0.1 eV, and it is one of the proofs of electrostatic interaction based physical adsorption of *gp*51 [12].

The splitting of the photoluminescence spectra into Gaussian curves at each stage of the experiment shows that after the adsorption of *gp*51 protein molecules on the TiO_2 surface the energy value of excitation levels, which are responsible for the luminescence and are associated with oxygen vacancies $I_{V[O]}$, almost does not change remaining at a value of 605 ± 2 nm. At the same time, the photoluminescence maximum caused by recombination of self-trapped excitons (STE) [14, 21] shifts to short wavelengths, changing its position from 517 ($STE_1 = 2.39$ eV) nm to 499 ($STE_2 = 2.48$ eV) nm. Since the involvement of the STE level in the process of radiative recombination is regulated by the surface, this indicates that STE level is located either on the surface plane or not very deeply within the surface layer of TiO_2. The displacement of the light emitting recombination peak indicates that the energy level of STE is complex and has its 'basic' and 'excited' states [18]. The appearance of luminescence in the region of 499 nm indicates a radiative transition from the excited STE level. This indicates that the charge at the TiO_2/*gp*51 boundary controls the energy level of the STE and shows that the electronic demarcation level practically coincides with the position of the STE level, i.e. is approximately 2.39 eV above the valence band. Therefore, the appearance of proteins on the TiO_2 surface leads to a shift in the energy levels, including the light emitting centers, relatively to the electron demarcation level E_{dn}.

The blue-shift of the photoluminescence maximum by 18 nm as a result of adsorption of the *gp*51 protein, which corresponds to $\Delta E_{STE} = STE_2 - STE_1 = 0.086$ eV, also indicates that the initial value of the potential barrier on the TiO_2 surface has decreased by a value of 0.086 eV. Variation of the potential barrier means that the value of negative charge localized on the TiO_2 surface has also changed, due to the charge-charge-based interaction with adsorbed protein *gp*51. Positively charged atoms and groups, which are provided by the *gp*51 protein, partially compensates the surface charge of TiO_2 and reduces the energy of electrons localized at the surface levels, which are the most responsible for the generation of photoluminescence signal. Taking into account the fact that the total negative charge predominates on the TiO_2 surface, the positively charged parts of the *gp*51 protein electrostatically interact with the negatively charged TiO_2 surface. As a result, a partial decrease of the surface charge reduces the electric field in the TiO_2 surface region. Further interaction of TiO_2/*gp*51 structure with anti-*gp*51, which is also a protein, leads to the inverse changes in the photoluminescence spectra, i.e., to UV-shift the spectrum (Fig. 25.1) and decrease the photoluminescence intensity to the value that corresponds to the pure TiO_2. The latter effect is based on the formation an immune complex between immobilized antigens *gp*51 and anti-*gp*51 antibodies, which were present in aliquot.

Formation of this immune complex besides the van der Waals interaction and other interactions at a very high extent is based on the interaction between oppositely charged domains, functional groups and atoms in *gp*51 and anti-*gp*51 molecules (including the formation of number of hydrogen bounds, which can be estimated as specific kind of electrostatic interaction). It can be assumed that uncompensated charges (δ+ and δ−) of both proteins are involved in electrostatic interactions during the formation of immune complex. As a result, some of the charged groups that were originally involved in the interaction between *gp*51 and TiO_2 are at least partially compensated by the opposite charge of the anti-*gp*51 protein groups, thereby reducing the direct electrostatic effect from immobilized *gp*51 proteins to the charged surface of TiO_2 and to light emitting centers.

The effects described above cause the shift of photoluminescence maximum and decrease in the potential barrier on TiO_2/*gp*51 interface due to the charge-charge interaction between TiO_2 and *gp*51. The potential barrier at the interface between TiO_2 and *gp*51 has greater value in TiO_2/*gp*51 structure in comparison with that in TiO_2/*gp*51/anti-*gp*51 due to partial compensation (decrease in value) and/or delocalization of charges, which were initially involved into interaction between TiO_2 and *gp*51 after formation of TiO_2/*gp*51 structure.

The distribution of charges in TiO_2/*gp*51 structure can also be interpreted as a model based on an 'imaginary flat capacitor' (Fig. 25.4), formed as a result of the electrostatic interaction between oppositely charged protein *gp*51 layer and the TiO_2 surface. The capacitor is formed as a result of protein *gp*51 adsorption on TiO_2 surface, after which the charges are distributed in energetically most favorable way, partially compensating each other. Consequently, the positive 'imaginary capacitor plate' is based on the positive charges, which are predominant in the protein *gp*51 area that after adsorption appears in close proximity to TiO_2/*gp*51 interface and/or due to the negative electrostatic effect of TiO_2 are induced/attracted closer to negatively charged surface.

These charged atoms/groups/domains of *gp*51 that are localized in the close proximity to the TiO_2 surface and they electrostatically affect the TiO_2 emission centers and the energy value of the surface potential barrier. Hence, the position of the

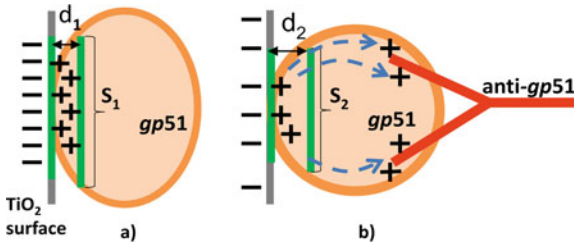

Fig. 25.4 Flat capacitor based model of the charges interaction between TiO_2 surface and *gp*51 proteins: **a** electrostatic interaction of uncompensated charges of immobilized protein *gp*51 with charges located on the surface of TiO_2; **b** model of interaction that takes into account the electrostatic interaction of charges within *gp*51 antigens and anti-*gp*51 antibodies

energy levels of the TiO_2 emission maximum depends on TiO_2 surface modification stage (TiO_2 or TiO_2/*gp*51) shifts from/backwards the initial position of the demarcation level. Figure 25.4a represents an imaginary flat capacitor consisting of a negatively charged plate on the surface of TiO_2 and an 'imaginary positively charged plate' formed in *gp*51 protein in close proximity to TiO_2/*gp*51 interphase. Hence, the interaction of TiO_2/*gp*51 with anti-*gp*51 antibodies and the formation of *gp*51/anti-*gp*51-based immune complex leads to a 'deformation' and the reduction of charge 'stored' on 'the positive imaginary capacitor plate' (Fig. 25.4b). This is mainly due to the redistribution and partial compensation of charges during the formation of the *gp*51/anti-*gp*51 immune complex, which in turn reduces the charge of 'the imaginary capacitor plate' based on *gp*51 ($q_2 < q_1$). Due to this reduced charge it can be interpreted as the reduction of the area of the same plate (S_2) and/or the increase of the distance (d_2) between the two imaginary capacitor plates based on *gp*51 and TiO_2 which leads to the decrease of capacitance according to (25.1).

$$C = \frac{\varepsilon \varepsilon_0 S}{d} \tag{25.1}$$

This effect is observed because some of the *gp*51 protein charges move from the TiO_2/*gp*51 interface towards interacting anti-*gp*51 protein and are partially compensated by charge present in anti-*gp*51, whereby the imaginary positive *gp*51-based capacitor plate of the capacitor is reduced in imaginary surface area and/or correspondingly moving apart from the negative TiO_2 plate. This effect leads to a decrease in the capacitance of this imaginary capacitor and the electric field induced by *gp*51 becomes reduced. Therefore, after the interaction of TiO_2/*gp*51 with anti-*gp*51 antibodies and the formation of *gp*51/anti-*gp*51 complex, which is involved into TiO_2/*gp*51/anti-*gp*51 structure, the electrostatic effect of *gp*51 initially adsorbed on TiO_2 towards the TiO_2 surface significantly decreases.

25.4 Conclusions

The main aspects of the interaction mechanism between nanostructured TiO_2 layer and bovine leukemia virus proteins *gp*51, during the formation of photoluminescence based immunosensor, have been developed. Bovine leukemia virus protein *gp*51, adsorbed on the surface of nanostructured TiO_2 thin film, formed the biosensitive layer (glass/TiO_2/*gp*51) that resulted in the TiO_2 photoluminescence peak shift from 517 to 499 nm. The interaction glass/TiO_2/*gp*51 structure with specific antibodies against *gp*51 (anti-*gp*51) has shifted the photoluminescence peak backwards from 499 nm to 516 nm. These photoluminescence shifts are attributed to the variation of STE energy level, which was induced by changes of electrostatic interaction between adsorbed *gp*51 and negatively charged TiO_2 surface. The displacement of the light emitting recombination peak confirms that the energy of STE level is complex and has its ground and excited states. The blue-shift of the photoluminescence

maximum by 18 nm as a result of adsorption of the *gp*51 protein, which corresponds to $\Delta E_{STE} = I_{STE2} - I_{STE1} = 0.086$ eV, indicates that the initial value of the potential barrier on the TiO_2 surface has decreased by a value of 0.086 eV. Variation of the potential barrier means that the value of negative charge localized on the TiO_2 surface has changed due to the charge-charge-based interaction with adsorbed protein *gp*51. Positively charged atoms and groups, provided by the *gp*51 protein, partially compensate the surface charge of TiO_2 and reduce the energy of electrons localized at the surface levels, which are the most responsible for the generation of photoluminescence signal.

The charge-charge-based interaction in the double charged layers TiO_2/*gp*51 can also be interpreted as a model of 'flat capacitor', formed as a result of the electrostatic interaction between oppositely charged protein *gp*51 layer and the TiO_2 surface. The capacitor is formed as a result of *gp*51 protein adsorption on TiO_2 surface, after which the charges are distributed in energetically most favorable way, partially compensating each other. Consequently, the positive 'imaginary capacitor plate' appears in the close proximity to TiO_2/*gp*51 interface, which is based on the positive charges of protein *gp*51, predominant after its adsorption on the TiO_2 surface. The positive charges are attracted closer to negatively charged surface due to the negative electrostatic effect of TiO_2. The interaction of TiO_2/*gp*51 with anti-*gp*51 antibodies and formation of *gp*51/anti-*gp*51-based immune complex leads to a deformation and reduction of charge in 'the positive imaginary capacitor plate', caused by redistribution and partial compensation of charges during the formation of the *gp*51/anti-*gp*51 immune complex, which in turn reduces the charge of 'the imaginary capacitor plate' based on *gp*51 adsorbed on the TiO_2 surface.

The highlighted origin of the changes in the photoluminescence spectra of TiO_2 as a result of the formation of biosensitive layer and after its interaction with the analyte, bring us closer to an understanding of the interaction mechanism between TiO_2 and proteins, that is the key in the solving of many issues related to an improvement of biosensor performance.

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