

PACS numbers: 68.18.–g; 42.62.Be
DOI: 10.1070/QE2003v033n09ABEH002498

Immunosensor systems with the Langmuir-film-based fluorescence detection

G.K. Chudinova, I.A. Nagovitsyn, R.E. Karpov, V.V. Savranskii

***Abstract*. A method is developed for detecting protein antigens for fluorescent immunoassay using a model system based on the technique for preparation of Langmuir films. Fluorescein isothiocyanate and donor – acceptor energy-transfer pairs of markers (the Yb complex of tetraphenyl porphyrin – benzoyl trifluoroacetoneisothiocyanate and derivatives of tetra(carboxyphenyl) porphyrin – cyanine dye containing a five-membered polyene chain), which were not studied earlier, were used as markers for detecting the binding of an antigen on the surface of Langmuir films of antibodies. Fluorescence was detected in the near-IR region (for the first pair) and in the visible spectral range (for the second pair). To reduce the nonspecific sorption of a protein (antigen), a method was proposed for the preparation of a nonpolar surface by applying an even number of layers of stearic acid as a substrate for the Langmuir – Blodgett film. A high sensitivity of model systems to a protein antigen in solution was achieved ($\sim 10^{-11}$ M), the assay time being 6 – 8 min. The model system with the first donor – acceptor pair was tested in analysis of the blood plasma. The fluorescence of the Dy^{3+}, Tm^{3+}, and Yb^{3+} complexes of tetraphenyl porphyrin sensitised by diketonate complexes of lanthanides was studied for the first time and the enhancement of the IR fluorescence of these complexes in a Langmuir film was demonstrated.**

Keywords: *fluorescence resonance energy transfer, fluorescent immunoassay, immunosensors, biosensors, Langmuir – Blodgett films, lanthanides, cyanine dyes, serum albumin.*

1. Introduction

During the past decade, the development of efficient devices for medical and ecological monitoring has attracted great attention. It is very important to create simple chemical and biological sensors for the assay of dangerous infections, narcotic substances and poisons [1 – 9]. Fluorescent immunoassay is one of the promising methods, which is now being actively developed. In this connection, the search for more efficient markers and the development of new techniques for detecting fluorescence are urgent. Many papers (see, for example, Refs [1, 2, 10, 11]) were devoted to the search for such markers and the improvement of the methods for immobilisation of antibodies and enhancement of the marker fluorescence.

The efficiency of immunosensors available at present is severely limited by the intense background fluorescence of proteins and other biological objects. This fluorescence, which introduces considerable errors into measurements, is mainly caused by the nonspecific binding of biological molecules with the polar surface of the substrate. The efficiency of a sensor element is characterised by the ratio of the useful signal intensity (the fluorescence intensity of a marker) to the background fluorescence intensity.

The aim of our study was to develop a model system of a highly sensitive sensor based on simple and low-cost techniques for film preparation and fluorescent assay. We discussed the methods we used for the enhancement of the marker fluorescence intensity and the reduction of background fluorescence.

Highly sensitive systems were prepared using the Langmuir film technique, which allows one to prepare highly ordered monolayers of antibodies on the surface of an aqueous subphase and transfer them to solid substrates. We proposed the method for immobilisation of antibodies (immunoglobulins IgG) by transferring a protein – lipid monolayer, which is virtually insoluble in water, to the substrate. This method makes it possible to avoid the covalent binding of IgG with the substrate, which substantially reduces the cost of the preparation of the model system.

It is known that the fluorescence intensity increases in ordered systems [12]. The intensity of the intrinsic fluorescence of a marker can strongly increase in a Langmuir film representing a two-dimensional highly cooperative ordered system, i.e., the sensitivity of a sensor will be improved. The marker fluorescence can be further enhanced due to fluorescence resonance energy transfer from a donor marker to an acceptor marker. We used in our study for new donor – acceptor marker pairs: benzoyl trifluoroacetoneisothiocyanate (bta) (donor) – Yb complex of tetraphenyl porphyrin (YbTPP) (acceptor) [13] and derivatives of tetra(carboxyphenyl) porphyrin (MATCPP) (donor) – cyanine dye (Cy5) (acceptor) [14]. When YbTPP was used as a marker, fluorescence was detected in the near IR region, where proteins do not emit fluorescence.

We proposed the method for preparing the hydrophobic

G.K. Chudinova, I.A. Nagovitsyn, V.V. Savranskii Natural Science Center, A.M. Prokhorov General Physics Institute, Russian Academy of Sciences, ul. Vavilova 38, 119991 Moscow, Russia;
R.E. Karpov Photochemistry Center, Russian Academy of Sciences, ul. Novatorov 7, 117421 Moscow, Russia

Received 23 January 2003
Kvantovaya Elektronika **33** (9) 765 – 770 (2003)
Translated by M.N. Sapozhnikov

working surface by depositing an even number of stearic acid monolayers as a substrate and showed that this can substantially reduce nonspecific sorption.

By using the methods described above, it is possible to create systems having a high sensitivity to an antigen in solution ($10^{-11} - 10^{-16}$ M) and a short assay time (6–8 min).

The results obtained in the paper are also of interest from the point of view of fundamental science. They concern the mechanism of energy transfer in condensed media. Note in this connection that the discrepancy between the Förster theory and processes of nonradiative energy transfer in condensed media has been pointed out in many papers [15, 16].

The direction of studies described below was supported by academician Aleksandr Mikhailovich Prokhorov. In this paper, we present a review of investigations performed under his direct supervision.

2. Materials and methods

As markers fluorescing in the IR spectral range, we used the lanthanide complexes ($Ln = Dy^{3+}$, Tm^{3+}, Yb^{3+}) of 5,10,15,20-tetraphenyl porphyrin (DyTPP, TmTPP, YbTPP) synthesised by V.D. Rumyantseva (M.V. Lomonosov Moscow State Academy of Fine Chemical Technology) using the method described in paper [17]. Markers 5-{4-carbamoyl[2-aminoethyl(4-aminobenzyl)]phenyl}-10,1-5,20-tris(4-carboxyphenyl) porphyrin (MATCPP), benzoyl trifluoroacetoneisothiocyanate (bta), and iodide hydroxysuccinimide ester 1-(5′-carboxypentyl)-1'ethyl-3,3,3′,3′-tetramethylindocarbocyanine (Cy5) with active groups for covalent binding with protein were placed at our disposal by A.V. Chudinov (V.A. Engel'gardt Institute of Molecular Biology, Russian Academy of Sciences). The conjugates of IgG with MATCPP and of bovine serum albumin (BSA) with Cy5 were prepared by methods described in papers [18–20]. We used fluorescein isothiocyanate (FITC), stearic acid (SA), and lipid (L-α-phosphatidylcholine dipalmitoyl) (Sigma, USA), rabbit antibodies (IgG) specific to human serum albumin (HSA) (N.I. Mechnikov Research Institute of Vaccines and Serums, Russian Academy of Medical Sciences), BSA, and mouse immunoglobulins specific to BSA (Institute of Virology, Russian Academy of Medical Sciences).

Langmuir–Blodgett films were prepared on a Joyce Loebl setup (Great Britain). Subphase was prepared using triply distilled water. The monolayers of the DyTPP-SA mixture were prepared on the subphase containing 0.03 g of $NaHCO_3$, HCl giving pH = 8.0, and 0.05 g of NaN_3 per 5 litre of a triply distilled water. In all other cases, the 0.05-M KCl solution was used (8.65 g of KCl and 0.05 g of NaN_3 per 5 litre of triply distilled water).

A monolayer of antibodies was deposited in a mixture with L-α-phosphatidylcholine dipalmitoyl. The stability of a monolayer of protein–lipid complexes in water is substantially greater than that of a monolayer containing only protein. The specific feature of the method we used is the modification of a substrate by depositing the Langmuir–Blodgett films of fatty acids. This results in the smoothing of microdefects in the substrate and modification of the substrate surface (producing of polar and nonpolar surfaces). The formation of a nonpolar surface substantially reduces the nonspecific binding of antigens.

The preparation of immunosensitive systems and the method for measuring fluorescence signals are described in Refs [13, 21]. Quartz plates (35 mm × 10 mm × 1 mm), on which 10 or 11 layers of stearic acid were preliminary deposited by the Langmuir–Blodgett technique at a surface pressure of 30 mN m^{-1}, were used as substrates. After the deposition of a protein–lipid monolayer or an LnTPP–SA mixture monolayer, we obtained the nonpolar (hydrophilic) or polar (hydrophobic) surface. The protein–lipid monolayers and LnTPP–SA monolayers were transferred on substrates by the Langmuir–Schefer method (contact method) at a surface pressure of 15–17.5 and 22.5 mN m^{-1}, respectively. The solutions were prepared in chloroform for the ratio LnTPP : SA = 1 : 1 (initial concentrations were 10^{-3} M).

Plates with LnTPP-SA monolayers were kept in enhancing solutions by measuring fluorescence spectra in the range between 900 and 1100 nm at intervals of 2, 4, 6, and 8 min. The enhancing solutions contained Ln acetyl acetonates, thenoyl trifluoracetone (TTA) at the concentration two orders of magnitude greater than the concentration of Ln, and polyvinyl alcohol. The initial solution contained TTA at the concentration 10^{-2} M and polyvinyl alcohol at the mass concentration 0.02 %. Less concentrated solutions were prepared by successive dilution and were used after 24 hours.

We used in our calculations the relative integrated fluorescence intensity S/S_0, where S and S_0 are the areas under the fluorescence spectrum at the instant t and before keeping the plates in enhancing solutions, respectively. The curves were plotted in the S/S_0-donor concentration coordinates and in the Stern–Volmer coordinates (quenching of S_0/S-donor concentration) [22]. The rate of a change in the integrated fluorescence intensity was calculated from the first-order kinetic equation for the first eight minutes of the reaction. The curves were plotted using the Microcal Origin v.5.0 computer program. The dependences presented in all the figures, except Fig. 2, were processed by the spline interpolation. The dependences in Fig. 2 were processed by the 'b-spline' method (due to a great scatter of the results).

The fluorescence spectra were recorded with a Shimadzu RF-5000 and a Hitachi 850 spectrofluorimeters. The absorption spectra were recorded with a Shimadzu UV-VIS 3100 and a Hitachi 330 spectrophotometers.

3. Results and discussion

The preparation of Langmuir monolayers of water-soluble proteins involves certain difficulties. For this reason, we studied the behaviour of different proteins on the water subphase surface. We found that it is possible to prepare stable, ordered monolayers by using the subphase of the appropriate ionic composition. In this case, due to salting out, the protein is not dissolved in the subphase [23].

The sensitivity of the system was tested using FITC as a marker for rabbit IgG specific to HSA. By performing the antigen–antibody reaction on the surface of a Langmuir film, we obtained a nearly linear dependence of the fluorescence intensity on the concentration of unmarked HSA in solution in the interval from 10^{-6} to 10^{-12} M (Fig. 1a), Fig. 1b shows the influence of the nonspecific agent, BSA. The linear calibration dependence is convenient because it allows one to interpret unambiguously the response of a sensor. In the case under study, fluorescence

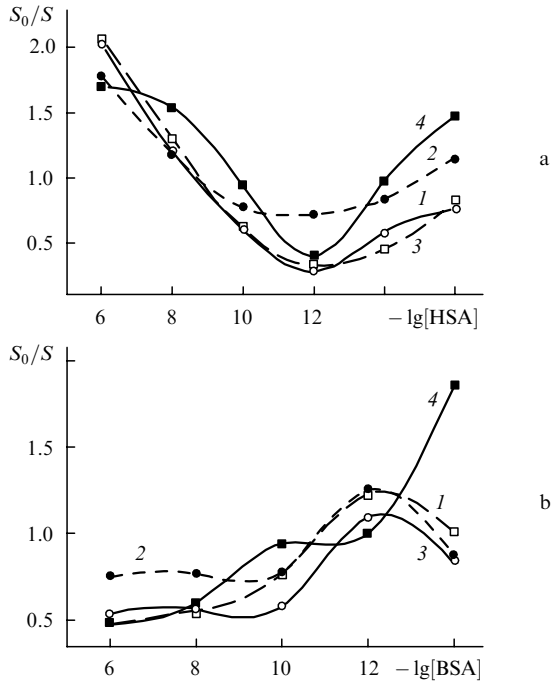

Figure 1. Variation in the fluorescence intensity in the Stern–Volmer coordinates during the antigen–antibody reaction on the IgG(FITC) monolayer surface upon the interaction with specific (HSA) (a) and nonspecific (BSA) (b) antigens for 2 (*1*), 4 (*2*), 6 (*3*), and 8 min (*4*).

was detected in the visible range. Films containing marked IgG (FITC) emit two fluorescence bands at 500 and 575 nm instead of the 525-nm band emitted by IgG (FITC) in solution [21].

The autofluorescence of proteins in the visible region is one of the main factors reducing the sensitivity of fluorescent immunoassay. The background fluorescence of proteins is virtually absent in the red (600–850 nm) and near-IR (920–1000 nm) regions. It was assumed that IR fluorescence markers can enhance the sensitivity and selectivity of immunoassay. We used YbTPP as such a marker. The ytterbium porphyrin complexes exhibit intense fluorescence at 975 nm. However, the fluorescence intensity in water is low, the quantum yield of IR fluorescence of YbTPP being only $\sim 10^{-4}$ [12, 24].

The fluorescence intensity can be increased by using newly synthesised complexons – efficient fluorescence sensitisers, by introducing the Ln complex to a highly ordered system, and using energy transfer to sensitise the fluorescence of a marker (in the case of lanthanide complexes, the enhancement of fluorescence of one of the lanthanides is called co-fluorescence).

It is known that the fluorescence band of Yb porphyrin in liposomes in water is narrower and its intensity is greater than that for Yb porphyrin in water by a factor of 1.5 [12]. It was shown in papers [25–30] that the fluorescence of Ln complexes, mainly Eu^{3+} complexes, can be enhanced in Langmuir films. However, in these papers, only fluorescence in the visible region was studied. It was found that Ln complexes with weak ionic fluorescence, in particular, Y^{3+} complexes, enhance fluorescence [26]. The energy is transferred from the ligand of the enhancing ion to the ligand of the Eu^{3+} ion and from the triplet level of the Eu^{3+} ligand to the Eu^{3+} ion.

We studied the intrinsic fluorescence of LnTPP complexes and their co-fluorescence induced by Ln thenoyltrifluoroacetonates [$Ln(TTA)_3$] in Langmuir films and found that the introduction of porphyrin complexes with lanthanides into Langmuir–Blodgett layers resulted in the increase in their fluorescence intensity by a factor of 3–3.5 compared to that in solution. The sensitisation of fluorescence of LnTPP by thenoyltrifluoroacetonate complexes of other lanthanides led to the enhancement of the fluorescence intensity of Ln approximately by an order of magnitude compared to that in solution and by a factor of 1.4–8 compared to the intensity of intrinsic fluorescence of LnTPP in the film in the absence of $Ln(TTA)_3$ [31–33].

We found that the IR fluorescence of LnTPP films is mainly determined by the fluorescence of the TPP ligand. The intensity and shape of the fluorescence spectra of Ln porphyrins depend on the temperature and structure of the porphyrin ligand [12, 34]. The shape of the fluorescence spectra of LnTPP that we observed in the Langmuir films is similar to that for Yb tetraxy(p-hydroxyphenyl) porphyrin in solutions at room temperature [34].

The fluorescence spectra of YbTPP, DyTPP, TmTPP, and TPP in films in the region from 900 to 1100 nm consist of one broad band at 978, 952, 960, and 970 nm, respectively. We can assume that lanthanide ions play in our case the same role as impurity centres in crystals, in which the relaxation of electronic excitation takes place. This is confirmed by the fact that the maximum fluorescence enhancement is achieved when the concentration of the enhancing complex is approximately 2–4 times grater than the concentration of LnTPP in the film (the latter was 10^{-10} M). In addition, we found that the enhancement of the IR fluorescence of TmTPP and DyTPP depended on the ratio of the ionic radii of Ln in LnTPP and $Ln(TTA)_3$ (Figs 2 and 3). The maximum fluorescence enhancement by factors of ~ 2 and 8 was achieved in the case of $Y(TTA)_3$ and $Tm(TTA)_3$ for TmTPP and DyTPP, respectively. The fluorescence of TPP was enhanced by a factor of 1.4–2.8 by lanthanide complexes.

The weakest enhancement of IR fluorescence, not exceeding 1.2–1.4, was observed for YbTPP. Unlike the cases considered above, the maximum enhancement was achieved in the range of concentrations comparable with the

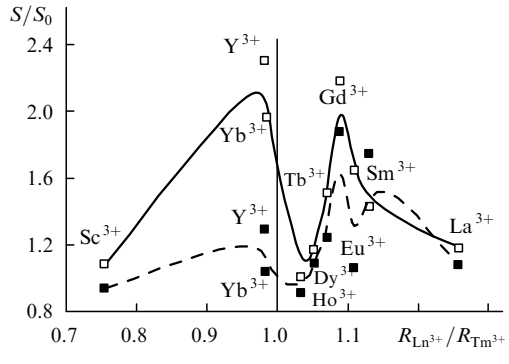

Figure 2. Variation in the relative integrated fluorescence intensity of TmTPP caused by the action of $Ln(TTA)_3$ as a function of the ratio $R_{Ln^{3+}}/R_{Tm^{3+}}$ of the ionic radii of Ln^{3+} and Tm^{3+} for polar (solid curve) and nonpolar (dashed curve) surfaces for the $Ln(TTA)_3$ concentration equal to 10^{-5} M. Fluorescence was excited by light at 330 nm (where TTA absorbs).

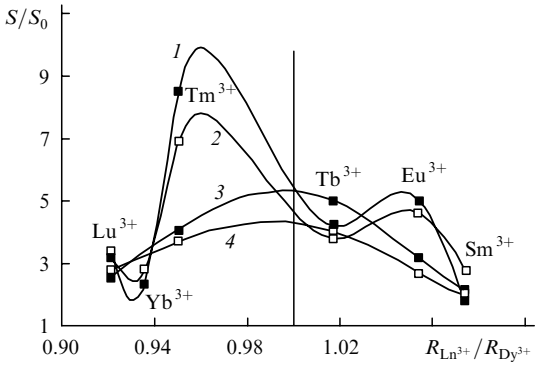

Figure 3. Variation in the relative integrated fluorescence intensity of DyTPP I the region 900 – 1100 nm caused by the action of $Ln(TTA)_3$ as a function of the ratio $R_{Ln^{3+}}/R_{Dy^{3+}}$ of the ionic radii of Ln^{3+} and Dy^{3+} for nonpolar (*1*, *3*) and polar (*2*, *4*) surfaces for the $Ln(TTA)_3$ concentration in the enhancing solution equal to 10^{-5} M (*1*, *2*) and 10^{-7} M (*3*, *4*). Fluorescence was excited by light at 330 nm (where TTA absorbs).

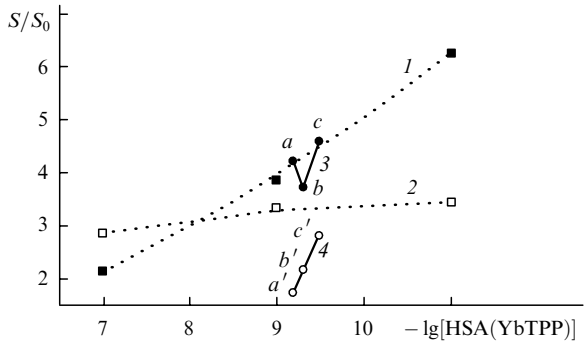

Figure 4. Variation in the integrated fluorescence intensity of HSA(YbTPP) in the Langmuir – Blodgett layer (dashed curves) and the influence of the nonspecific BSA antigen (solid straight lines; sorption of proteins was performed from the HSA(YbTPP) : BSA = 1 : 2 mixture (*a*, *a*′), 1 : 1 (*b*, *b*′), and (2 : 1) (*c*, *c*′) for the initial concentrations of the mixture components equal to 10^{-9} M) for nonpolar (*1*, *3*) and polar (*2*, *4*) surfaces. Fluorescence was excited by light at 330 nm.

concentration of the porphyrin complex in the film or lower. This can be explained by a strong fluorescence of the Yb^{3+} ion, which efficiently quenches the excited state of TPP due to intramolecular energy transfer, resulting in a weak increase in the integrated fluorescence intensity.

Therefore, we have demonstrated the principal possibility of a significant enhancement of the IR fluorescence of porphyrin complexes with rare-earth ions using energy transfer from β-diketonate energy donors. Because it is difficult to mark proteins involved in immunoreactions by rare-earth TTA complexes, we decided to use metal-free bta and YbTPP as markers for antigens and antibodies, respectively.

The results of the study of a sensitive immunosensor system based on energy transfer from bta to YbTPP are presented in paper [13]. As in Ref. [21], polyclonal antibodies, rabbit IgG specific to the antigen (HSA) were used. However, the system proposed in this paper can be used not only for determining serum albumin but also for protein and non-protein antigens by immobilising in a Langmuir film the antibodies specific to these antigens.

The criteria of the efficiency of a sensor system are its sensitivity to different concentrations of specific antigens and a very weak response to the binding of nonspecific antigens. Fig. 4 shows the calibration curves of the sensor system: the dependences of the enhancement of the integrated fluorescence intensity of HSA(YbTPP) sorbed on the film surface on its initial concentration in solution (dashed curves). The solid straight lines represent the relative integrated intensities S/S_0 that were obtained in experiments with the addition of the nonspecific antigen BSA. One can see that the system with a nonpolar working surface [curve (*1*)] has a higher sensitivity than the system with a polar surface [curve (*2*)], i.e., in the former case, the dependence of the ratio S/S_0 on the antigen concentration is stronger. The selectivity to a specific antigen is also higher for curve (*1*) because the values of S/S_0 in experiments with the addition of BSA and without it are virtually coincident. The close values of S/S_0 in curve (*2*), obtained for different concentrations of HSA(YbTPP), can be explained by a high degree of nonspecific binding – by sorption of polar albumin molecules on polar lipid 'heads'.

We obtained similar results in the human blood plasma (Fig. 5). Because it is necessary to determine antigens without markers in real physiological liquids, we used in experiments the IgG-lipid films with a preliminary prepared antibody [IgG(bta)] – antigen [HSA(YbTPP)] complex. For this purpose, a plate was kept for 6 min in the HSA(YbTPP) solution with the concentration 10^{-9} M, which resulted in the enhancement of IR fluorescence. Then, the plate was immersed into the blood plasma containing non-marked HSA at the specified concentration. The non-marked HSA is competitively binding with the antibody, and the concentration of HSA in the blood plasma can be estimated from a change in the fluorescence intensity. The change in the fluorescence intensity for a polar surface [curve (*2*)] is much weaker than that for a nonpolar surface [curve (*1*)]. This suggests that nonspecific binding is minimal on the nonpolar surface. A weaker change in the fluorescence intensity at lower concentrations of HSA being determined can be reasonably explained by the fact that a greater part of marked HSA(YbTPP) remains bound with the IgG monolayer.

At present, one of the main problems is the search for new efficient markers for biological assay methods. New compounds are being studied which can be used as markers

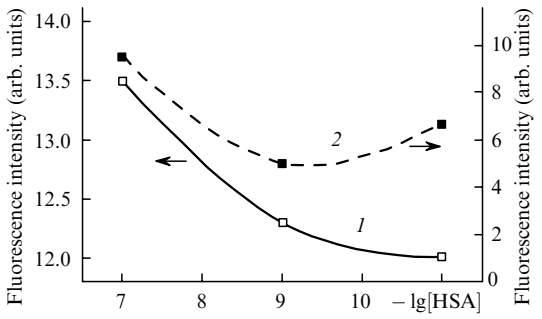

Figure 5. Response of the sensitive system in the blood plasma as a function of the concentration of non-marked specific HSA antigen for nonpolar (*1*) and polar (*2*) surfaces. Fluorescence was excited by light at 330 nm.

for DNA and proteins [10, 35], the use of donor–acceptor energy-transfer pairs being quite efficient for marking [2, 36–38].

We have made an attempt [14] to enhance the fluorescence assay signal by using a new donor–acceptor pair of porphyrin-cyanine dye markers. The markers were Cy5 and MATCPP. In this case, the antigen (BSA) was marked by the cyanine dye, while the antibody (mouse IgG) was marked by porphyrin. We observed energy transfer in films from MATCPP (donor) to Cy5 (acceptor) during the formation of the antigen-antibody complex. We found that the efficiency of energy transfer in Langmuir ordered layers was 1.5–2 times higher than that in solutions.

Fig. 6 shows the calibration curves of the sensitivity of a system with a pair of MATCPP–Cy5 markers. The response of the system (the rate constant of a change in the integrated fluorescence intensity during measurements) on the polar surface changes according to a parabolic law, with a maximum at the antigen concentration equal to 10^{-9} M, while its variation on the nonpolar surface is described by the S-like curve and its value is almost an order of magnitude lower. The difference between the rate constants of a change in the fluorescence intensity can be explained by a great amount of BSA(Cy5) on the surface due to nonspecific binding with the polar groups of lipid.

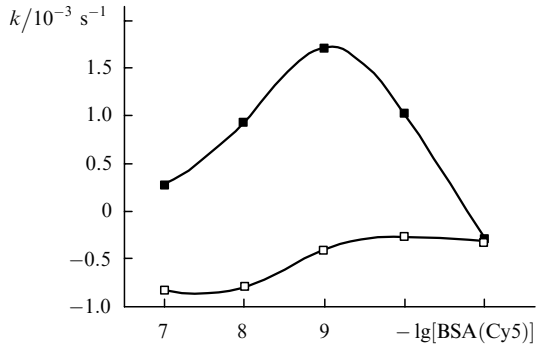

Figure 6. Dependences of the rate constant k of variation in the integrated fluorescence intensity on the concentration of the marked BSA(Cy5) antigen in solution for polar (■) and nonpolar (□) surfaces. Fluorescence was excited by light at 410 nm into the Soret band of MATCPP.

As in Ref. [21], the model system with porphyrin-cyanine dye markers is sensitive to the antigen in the region of its lower concentrations as well. The response curve in the region of BSA(Cy5) concentrations between 10^{-12} and 10^{-14} M is a straight line, the response being greater in the region of lower concentrations [14]. In this case, BSA(Cy5) molecules can be similar to impurity centres in which the relaxation of electronic excitation occurs in an ordered Langmuir film.

4. Conclusions

We have described the study devoted to the fabrication of highly sensitive Langmuir-film systems for fluorescent immunoassay. We have proposed the method for modification of the substrate surface by applying Langmuir–Blodgett films of stearic acid to obtain surfaces of different polarities. Also, the method was proposed to immobilise antibodies in the form of protein–lipid complexes in a monolayer, which allows one to avoid the covalent binding of antibodies. We measured the sensitivity of such a model system to the non-marked antigen (HSA) by using FITC as a marker for the antibody. The sensitivity of the system to the antigen in solution was not worse than 10^{-12} M.

We have studied for the first time the sensitisation of the LnTPP fluorescence in Langmuir films by $Ln(TTA)_3$ and have shown that the IR fluorescence of LnTPP can be enhanced by a factor of 1.4–8. This enhancement depends on the ratio of the ionic radii of lanthanides in the porphyrin complex and in the complex with TTA.

We used a pair of energy-transfer YbTPP–bta markers to mark the antigen and antibody, respectively. Fluorescence was detected in the near-IR range (the fluorescence maximum was located at 975 nm). The sensitivity of the system was 10^{-11} M. The visible fluorescence was detected using a pair of energy-transfer MATCPP–Cy5 markers to mark the antigen and antibody, respectively. The sensitivity was 10^{-11} M.

The pairs of energy-transfer markers were used for the first time.

The sensitivity of the sensor systems proposed in the paper exceeds that of well-known methods, for example, ELISA, which can determine the protein concentration of the order of 10^{-9} M [39]. The assay time in our case is 6–8 min, whereas the ELISA assay time is up to 2 h.

The results reported in the paper demonstrate the outlook for practical applications of these studies. The methods proposed in the paper reduce the assay time, enhance the sensitivity and selectivity of fluorescent immunoassay, and substantially reduce nonspecific binding.

Acknowledgements. The work was supported by Grant No. 00-15-96559 of the Leading Scientific Schools of the Russian Federation. The authors thank V.D. Rumyantseva (M.V. Lomonosov Moscow State Academy of Fine Chemical Technology), A.V. Chudinov (V.A. Engel'gardt Institute of Molecular Biology, Russian Academy of Sciences), and G.G. Komissarov and A.V. Lobanov (N.N. Semenov Institute of Chemical Physics, Russian Academy of Sciences) for their help in this study.

References

1. Sheikh S.H., Abela B.A., Mulchandani A. *Anal. Biochem.*, **283**, 33 (2000).
2. Grant S.A., Xu J., Bergeron E.J., Mroz J. *Biosensors & Bioelectronics*, **16**, 231 (2001).
3. Kaiser T., Gudat P., Stock W., Pappert G., Grol M., Neumeier D., Luppa P.B. *Anal. Biochem.*, **282**, 173 (2000).
4. Vo-Dinh T., Sepaniak M.J., Griffin G.D., Alarie J.P. *Immunomethods*, **3**, 85 (1993).
5. Simonian A.L., Grimsley J.K., Flounders A.W., Schoeniger J.S., Cheng T.-C., De Frank J.J., Wild J.R. *Anal. Chim. Acta*, **442**, 15 (2001).
6. Madou M., Joseph J. *Immunomethods*, **3**, 134 (1993).
7. Mullett W.M., Lai E.P.C., Yeung J.M. *Methods*, **22**, 77 (2000).
8. Volkov S.K. *Usp. Khim.*, **63** (1), 93 (1994).
9. Eremin S.A., Samsonova Zh.V., Egorov A.M. *Usp. Khim.*, **63** (1), 638 (1994).
10. Savitskii A.P. *Usp. Biol. Khim.*, **31**, 209 (1990).
11. Garcinuño R.M., Fernandez P., Perez-Conde C., Gutierres A.M., Camara C. *Talanta*, **52**, 825 (2000).
12. Gaiduk M.I., Grigor'yants V.V., Mironov A.F., Rumyantseva V.D., Roitman L.D. *Zh. Prikl. Spektr.*, **46** (1), 163 (1986).

13. Nagovitsyn I.A., Chudinova G.K. *Dokl. Ross. Akad. Nauk*, **282** (2), 267 (2002).
14. Chudinova G.K., Nagovitsyn I.A., Chudinov A.V., Savranskii V.V., Prokhorov A.M. *Dokl. Ross. Akad. Nauk*, **282**, 404 (2002).
15. Vekshin N.L. *Fotonika biologicheskikh struktur* (Photonics of Biological Structures) (Pushchino: Department of Scientific and Technical Information, NtsBI, Akad. Nauk SSSR, 1988).
16. Estep T.N., Thompson T.E. *Biophys. J.*, **26**, 195 (1979).
17. Wong C.-P., Venteicher R.F., Horrocks W. *J. Am. Chem. Soc.*, **96** (22), 7149 (1974).
18. Egorov A.M., Osipov A.P., Dzantiev B.B., Gavrilova E.M. *Teoriya i praktika immunofermentnogo analiza* (Theory and Practice of Immunoenzyme Analysis) (Moscow: Vysshaya Shkola, 1991).
19. Mujumdar R.B., Ernst L.A., Mujumdar S.R., Waggoner A.S. *Cytometry*, **10**, 11 (1989).
20. Schobel U., Egelhaaf H.-J., Fröhlich D., et. al. *J. Fluorescence*, **10**, 147 (2000).
21. Chudinova G., Chudinov A., Savransky V., Prokhorov A. *Thin Solid Films*, **307**, 294 (1997).
22. Lakowicz J.R. *Principles of Fluorescence Spectroscopy* (New York: Plenum Press, 1983; Moscow: Mir, 1986).
23. Chudinova G.K., Pokrovskaya O.N., Savitsky A.P. *Russ. Chem. Bull.*, **44**, 1958 (1995).
24. Tsvirko M.P., Stelmakh G.F., Pyatosin V.E., Solovyov K.N., Kachura T.F. *Chem. Phys. Lett.*, **73** (1), 80 (1980).
25. Xu Y.Y., Hemmila I., Mukkala V.-M., Holttinen S., Lovgren T. *Analyst*, **116**, 1155 (1991).
26. Zhong C., Feng Y., Yang K., Zhu G. *Chemistry Lett.*, **25** (8), 775 (1996).
27. Zhong G.-L., Yang K.-Z. *Langmuir*, **14**, 5502 (1998).
28. Yang Y., Su Q., Zhao G. *J. Molecular Structure*, **525**, 47 (2000).
29. Zhang R.-J., Yang K.-Z. *Thin Solid Films*, **371**, 235 (2000).
30. Zhang R.-J., Yang K.-Z., Yu A.-C., Zhao X.-S. *Thin Solid Films*, **363**, 275 (2000).
31. Chudinova G., Rumiantseva V., Chudinov A. *Proc. European Conf. on Thin Organized Films* (Potsdam, Germany, 1998).
32. Chudinova G., Rumiantseva V., Nagovitsyn I. *Book of Abstracts VIII Intern. Conf. on Spectroscopy and Chemistry of Porphyrins and their Analogs* (Minsk, Repuplic of Belarus, 1998) p. 81.
33. Nagovitsyn I.A., Chudinova G.K., Rumyantseva V.D., Lobanov A.V. *Abstracts of Papers, VI Mezhdunarodnaya konferentsiya "Naukoemkie khimicheskie tekhnologii (VI International Conference on Scientific Chemical Technologies)* (Moscow: Russia, 1999) pp 208 – 209.
34. Meng J.X., Li K.F., Yuan J., Zhang L.L., Wong W.K., Cheah K.W. *Chem. Phys. Lett.*, **332**, 313 (2000).
35. Glazer A., Benson S. *Biotechnol. Advances*, **13**, 575 (1995).
36. Mitsui T., Nakano H., Yamana K. *Tetragedron Lett.*, **41**, 2605 (2000).
37. Youn H.J., Terpetschnig E., Szmacinski H., Lakowicz J.R. *Anal. Biochem.*, **232**, 24 (1995).
38. Tolosa L., Malak H., Raob G., Lakowicz J.R. *Sensors and Actuators B*, **45**, 93 (1997).
39. Ngo T.T., Lenhoff G. (Eds) *Immunofermentnyi analiz* (Immunoenzyme Analysis) (Moscow: Mir, 1988).