THE STUDY OF INTERMOLECULAR INTERACTIONS USING OPTICAL BIOSENSORS OPERATING ON THE EFFECT OF SURFACE PLASMON RESONANCE

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Present knowledge of the technology of optical biosensors based on the effect of surface plasmon resonance (SPR) is presented. SPR enables to register almost all molecular interactions in real time without any labels or associated processes. There are considered the main technical characteristics of SPR biosensors and the principles of analysis of kinetic, equilibrium and thermodynamic parameters of intermolecular interactions.

There are presented a number of examples of Biacore biosensors (GE Healthcare, USA) application in post-genomic researches carried out in the Institute of Biomedical Chemistry (Moscow, Russia): SPR technology has been used in the analysis of intermolecular interactions, like protein-protein, protein-low-molecular-weight compounds, protein-DNA; in the analysis of protein oligomerization and the search of dimerization inhibitors; in the search of drug prototype compounds interacting with a target protein; and in biosensor analysis of disease biomarkers.

The technology of optical biosensors operating on the effect of surface plasmon resonance is getting one of the approaches in researching various intermolecular interactions. It is of universal character and enables to record the interactions between almost any molecular objects in a wide range of concentrations. SPR-biosensors have very high accuracy, capability of reproducing and sensitivity; due to these characteristics their application is growing both in fundamental and applied researches.

Key words: optical biosensor; surface plasmon resonance; intermolecular interactions.

Optical biosensor technology based on the effect of surface plasmon resonance (SPR) enables to record directly practically any intermolecular interactions in real time without using any labels or adjoint processes [1–2].

SPR effect as physical phenomenon was discovered in the 60s of XX c. and is referred to the sphere of modern quantum nanooptics [3–4]. It is observed at the boundary of phase immiscibility in total internal reflection of incident beam (Fig. 1, *a*). Photons do not cross refracting surface, though their electric field overrides the boundary of phase immiscibility by a quarter of light wavelength. If refracting surface is covered by a thin gold layer, under certain conditions (resonance) photons can interact with free electrons in metal and transform into plasmons, and light does not reflect in this case (Fig. 1, *b*).

Surface Plasmon is quantized collective oscillation of electrons near the material surface, oscillation consisting in origination of charge density fluctuation. Electric (evanescent) field of plasmons falls beyond a gold layer, and interacts with the material of two phases (Fig. 1, *c*). Mobility of plasmons, and therefore, the degree of resonance angle depend on these interactions. If one phase is glass prism (lower phase in Fig. 1), and another is liquid (upper phase in Fig. 1), the degree of resonance angle will be proportional to refraction coefficient in a thin layer of liquid medium (~300 nm). Monitoring the changes of resonance angle,

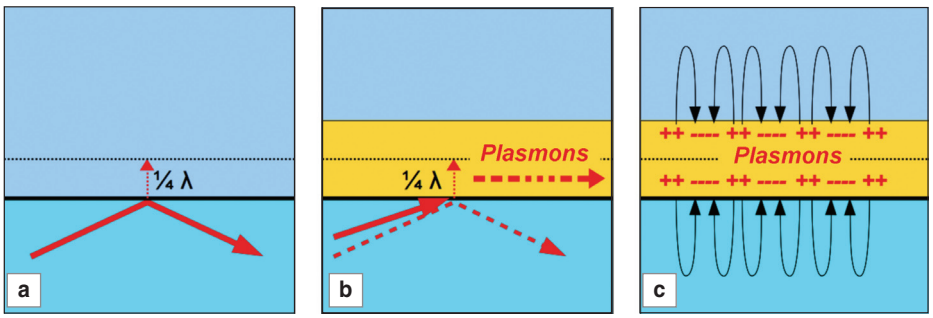

Fig. 1. Effect of surface plasmon resonance (SPR): *a* — effect of total internal reflection of light at the boundary of phase immiscibility; *b* — origination of plasmons in resonance between light quanta and free electrons in metal; *c* — electrostatic (evanescent) field of plasmons

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one can record mass transfer of any substances between free medium and this layer.

Operating principle of optical biosensor based on SPR effect

Operating principle of SPR-biosensor [5–7] is rather simple (Fig. 2). The first partner of intermolecular interaction is mounted on gold surface of optical chip that is the boundary of phase immiscibility, and biosensor records mass transfer of the second partner between the medium and measurement zone (either side). In biosensor technology chemical terminology is used: any object fastened to a carrier is called ligand, and free-floating object interacting with it is called analyte. Any molecular objects — from low-molecular weight substances to biopolymers, and even larger objects as viruses, bacteria, and cells — can be used as ligands and analytes.

The curve of biosensor signal record depending on time is called sensogram. Fig. 2, *c* shows the main stages of classic sensogram: I — record of basic signal of a biosensor before measurement; II — kinetics of forming ligand-analyte complexes, when analyte solution is injected through a measuring channel (*1*); III — kinetics of decomplexation after completing analyte injection and transfer to working buffer (*2*); IV — rapid decomplexation, and chip surface cleaning in regenerating solution injection (*3*); V — the record of basic signal of biosensor after measurement cycle after the termination of regenerating solution injection and transfer to working buffer (*2*).

A series of sensograms (Fig. 3, *a*) obtained at different analyte concentrations were mathematically analyzed, and there were calculated equilibration characteristics of intermolecular interactions – dissociation constant of complex Kd and affinity A, as well as kinetic parameters: rate constants of complexes formation k_{on} and decomplexation k_{off} (Fig. 3, *b*).

It should be noted that judging by sensogram appearance it is extremely difficult to assess the integrity of a forming complex, as it depends on the ratio of rate constants of complex formation and decomplexation rather than their absolute value. This is readily demonstrated by rate map given in Fig. 3, *c*. Points on one diagonal correspond to reactions with equal Kd value, while absolute rates of complexes formation and decomplexation and forms of sensograms are very much different due to the characteristics of interacting molecules.

From series of sensograms obtained at different temperatures, thermodynamic characteristics can be calculated: Gibbs free energy change (ΔG), the change of enthalpy (ΔH) and entropy (ΔS) (Fig. 3, *d*).

Principles of ligand immobilization on surface of optical chips

The method of ligand immobilization on optical chip surface is little different from similar ones used for lgand immobilization on other carriers (e.g., chromatographic sorbent, hard surfaces, nano- and microparticles) [7–8].

Fig. 4, *a* shows the principle of standard covalent immobilization of ligand. So called carbodiimide reaction and standard optical chips covered by carboxylated dextran layer are used for this technique. At first carboxyl groups of dextran are activated by means of carbodiimide+hydro xysuccinamide (EDC+NHS) mixture forming short-lived ether groups, which further react with ligand amino groups forming amide bond. In case of protein-ligand, lysine amino groups and amino n-groups located on protein globule surface, and N-group can be reactive groups.

In certain cases there used modifications of this reaction with ligand immobilization for thiol and aldehyde groups.

Methods of noncovalent ligand immobilization are various, though actually all of them are based on one principle: specific affinity reagent is fastened on optical chip surface, and ligand is non-covalently immobilized on this reagent (Fig. 4, *b*). Specific mono- or polyclonal antibodies, streptavidin (immobilization of biotinylated ligands, Kd complex ~10^{-14} M) can act as affinity connection. In some cases there used more compound connections, e.g. covalently immobilized protein A affinily connects specific antibody, on which is affinily mobilized protein-ligand. The method of immobilization of protein-ligand containing so

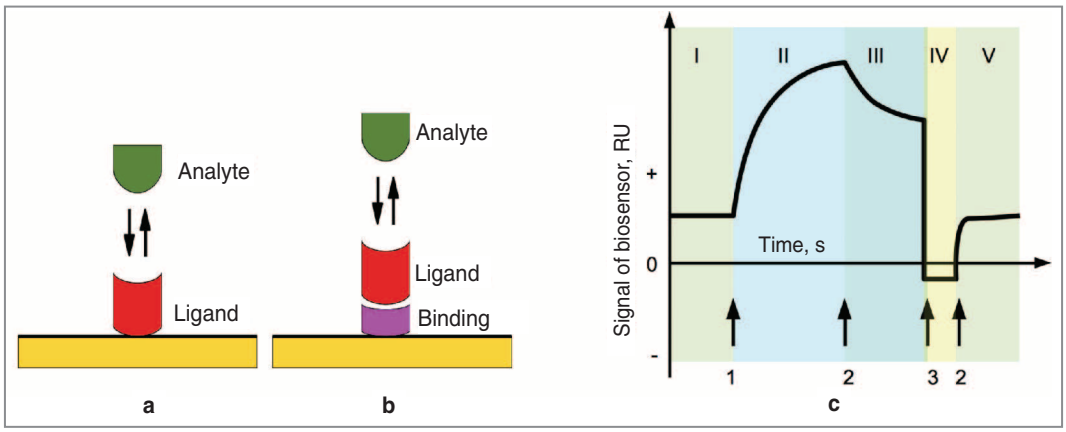

Fig. 2. Operating principle of optical biosensor: record of analyte mass transfer in interaction with ligand immobilized directly on optical chip surface (*a*) or through additional binding (*б*); *c* — structure of a typical sensogram

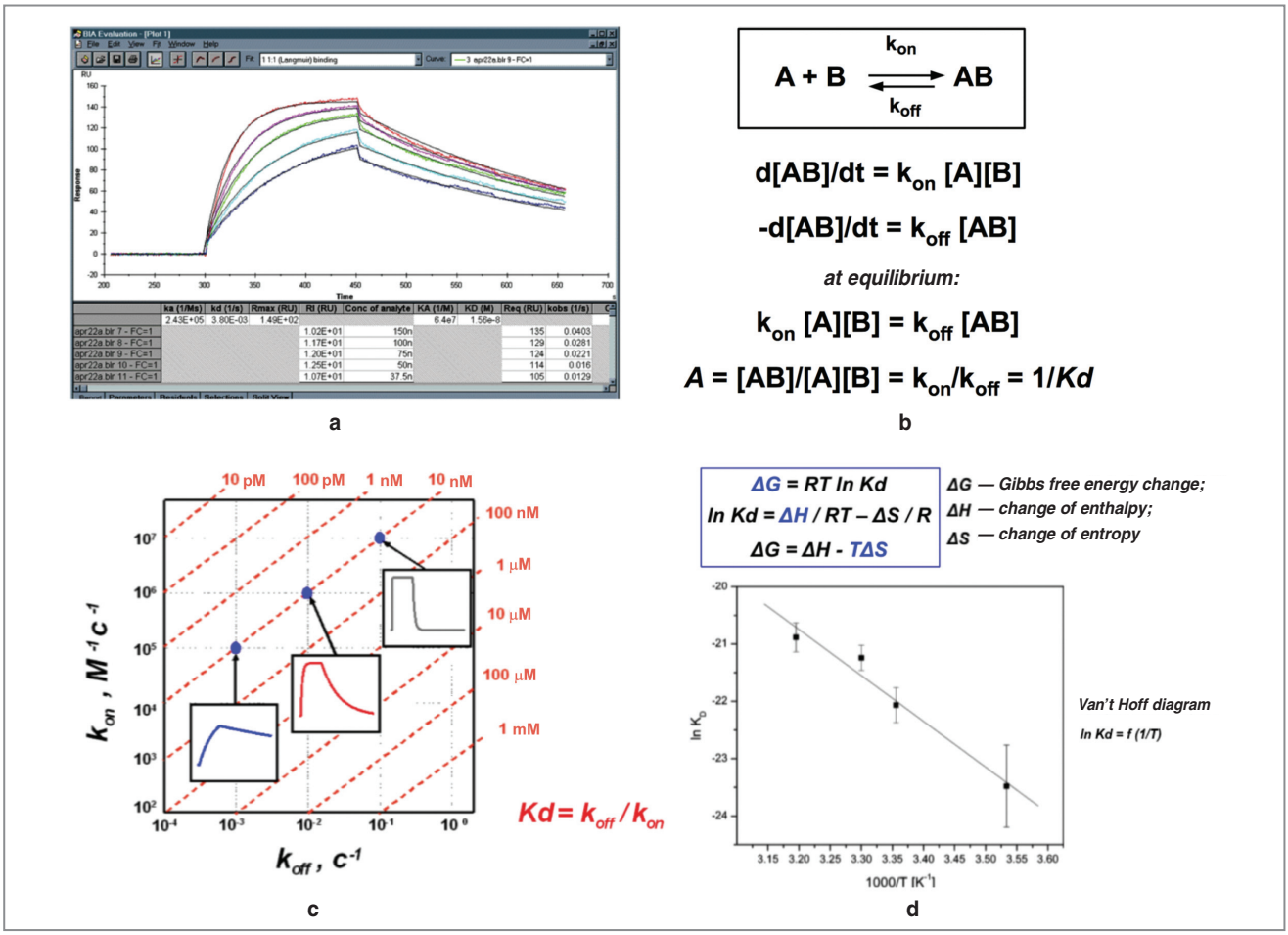

Fig. 3. Calculation principle of intermolecular interaction parameters: *a* — a series of sensograms in injections at different concentrations of analyte; *b* — calculation of interaction parameters based on chemical equation of complex formation of pseudo-first order: *c* — rate map of complex formation and decomplexation; *d* — calculation of thermodynamic parameters; A — affinity; [A], [B] — concentrations of A and B substances

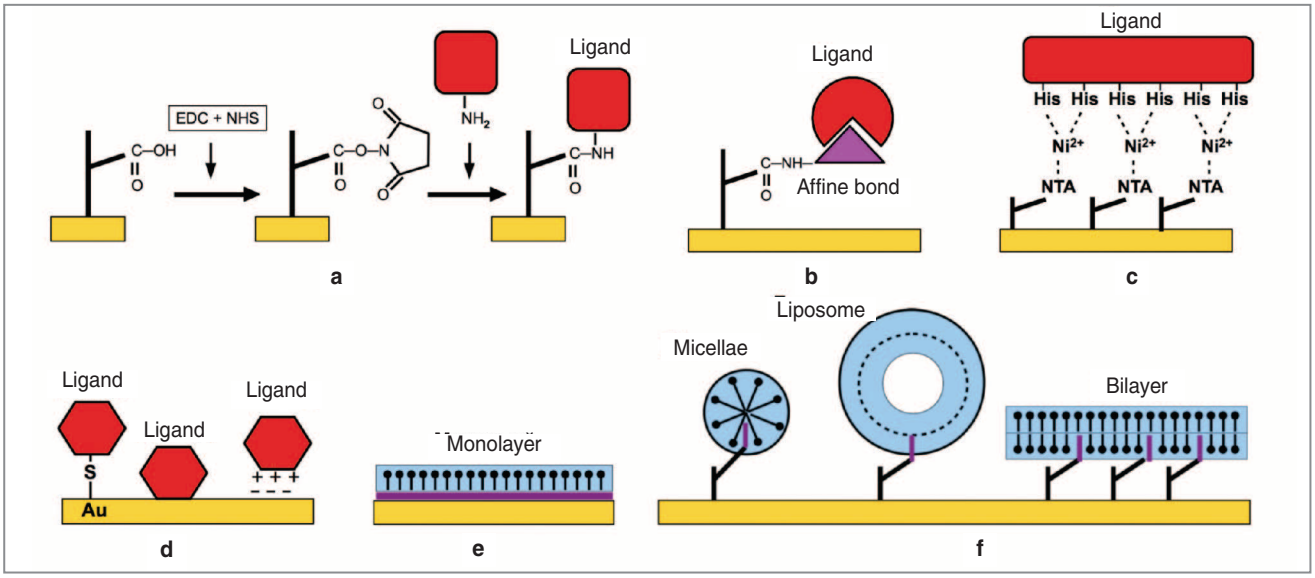

Fig. 4. Principles of ligand immobilization on optical chip surfaces: *a* — covalent immobilization of a ligand with amidation between carboxyl group of modified dextran and ligand amino group; *b* — non-covalent immobilization of ligands through affine bond; *c* — chelate immobilization of 6×His-labeled protein-ligand; *d* — covalent and non-covalent immobilization of ligands on gold surface; *e* — formation of lipid monolayers on the surface of chip with hydrophobic coating; *f* — formation of lipid structures modeling the structure of biological membranes (micellae, liposomes, and plane bilayer)

called histang (label in the form of 6 histidines sequence, 6×His) is rather popular. These proteins are immobilized on optical chip, the surface of which is modified by ion-exchange compound NTA (nitrilotriacetate) due to formation of three chelate complexes consisting of two histidines residual, one nickel ion (Ni^{2+}) and NTA molecule NTA (Fig. 4, *c*).

The method of immobilization of ligands on unmodified gold surface [8] (Fig. 4, *d*) can be regarded as a separate group due to three different processes: 1) formation of covalent link between gold and thio-groups of ligand; 2) firm adsorption of ligand on gold surface; 3) electrostatic interactions.

The methods of immobilization of lipid monolayers (Fig. 4, *e*), lipid micellae, liposomes, and plane bilayer (Fig. 4, *f*) on optical chip with hydrophobic acyl "anchors" also represent a separate group [7].

SPR-biosensors

Manufacturers of serial scientific equipment offer at the market over ten SPR-biosensors with original constructive decisions and various functional characteristics. The best known biosensors are offered by the following companies: GE Healthcare (USA), Reichert Technologies (USA), SensiQ (USA), Bio-Rad (USA), Horiba (Japan), BioNavis (Finland), Analytik Jena (Germany), MissionCouver Technologies (China), IBIS Technologies (Holland), GWC Technologies (USA), and some others. However, the analysis of scientific literature shows that the most biosensor researches are carried out using optical biosensors Biacore (GE Healthcare), and the reasons for this are the following: 1) Biacore SPR-biosensors equipped by microfluidic continuous-flow system were the first serial devices of this type, and in the past decade have been mostly accepted in different scientific and production spheres; 2) Biacore biosensors have the best characteristics in some essential parameters including: high sensitivity (in the order of 10^{-11} M of analyte); minimal noise level (less 0.01 RU, where RU is resonance unit); high stability of basic signal (drift less than 1 RU/h); record of low-molecular weight analyte practically with no restrictions on minimum molecular weight (under 50 Da); cost-effective biomaterial consumption (100 ng of protein is enough for immobilization, volume of flow cuvettes is from 20 to 60 nl); the presence of commutated microfluidic system with continuous flow nanocuvettes that determines flexibility of measurement protocols.

Practical application of SPR-technology

SPR-technologies are successfully used in various biomedical investigations:

1) in real-time analysis of interactions of various molecular and supramolecular objects (from low-molecular weight compounds and biopolymers to micellae, liposomes, viruses, bacteria, cells, and intracellular particles);

2) in analysis of affinity, specificity, and thermodynamics of molecular complexes formation;

3) in quantitative analysis of a wide range of bioactive compounds, organic substances, and biomarkers of diseases;

4) in the analysis of different antibody characteristics, and in quality control of antibody preparations;

5) in screening and testing of drug prototypes of interaction with target protein;

6) in the analysis of enzyme activity and inhibition;

7) in molecular fishing — direct affinity binding of target molecules from complex biological mixtures on optical chip surface with their further elution for identification by methods of tandem mass-spectrometry LC-MS/MS.

The variety of SPR-biosensor application can be demonstrated by investigations carried out at different times in the Institute of biomedical chemistry of RAMS [9–21].

1. *Interaction of trypsin inhibitor from Radianthus macrodactylus with different proteases* [1]. We studied the interaction of InhVJ inhibitor isolated from *Radianthus macrodactylus* with serine (trypsin, α-chymotrypsin, plasmin, thrombin, kallikrein), cysteine (papain) and aspartic (pepsin) proteinases on optical biosensor Biacore 3000. The research was carried out on standard optical chips of CM5 type. Fig. 5, *a* shows typical sensogram of InhVJ immobilization. InhVJ was found to interact with trypsin and α-chymotrypsin only (Fig. 5, *b*).

To determine kinetic and thermodynamic parameters of complexing reaction, we analyzed a series of sensograms of interaction of trypsin and chymotrypsin with InhVJ (Fig. 5, *c, d*).

Kd value for a pair InhVJ-trypsin was $7 \cdot 10^{-8}$ M, and for a pair InhVJ-α-chymotrypsin — $1 \cdot 10^{-7}$ M.

2. Search and analysis of interaction of dimerization inhibitors with HIV-1 protease monomer [10–12]. There was developed the approach to screening of potential inhibitors of HIV-1 protease dimerization functioning only in the form of homodimers. In this approach we used original biosensor test-system based on differential analysis of interaction of the compound under test with monomers and dimers of HIV protease (Fig. 6, *a, b*). There were analyzed the most prospective substances-candidates predicted by methods of virtual screening of chemical databases (Fig. 6, *c, d*). Finally, there was found one new inhibitor of HIV protease dimerization with lowest molecular weight. Further we performed biosensor analysis of thermodynamic parameters of interaction of peptide and non-peptide dimerization inhibitors with HIV protease monomers (Fig. 6, *e*). Entropic factor was found to play the leading role in complexes formation.

3. *Molecular recognition in oligomerization of bacterial L-asparaginases* [13, 14]. Substrate specificity and activity of bacterial L-asparaginase are directly connected with oligomerization process, since the enzyme is active only in the form of tetramer, and active sites are located in subunits contact zone. For the purpose of studying oligomerization process, enzyme L-asparaginase was immobilized on the surface of optical chip CM5 (Fig. 7, *a*), and using the technique of surface plasmon resonance we recorded degradation processes of tetramer complexes of the enzyme to monomeric condition and their further recovery (Fig. 7, *b*). We studied specificity of molecular recognition in oligimerization of some bacterial L-asparaginases and found nearly absolute specificity of molecular recognition: stable tetramer complexes are formed only from subunits

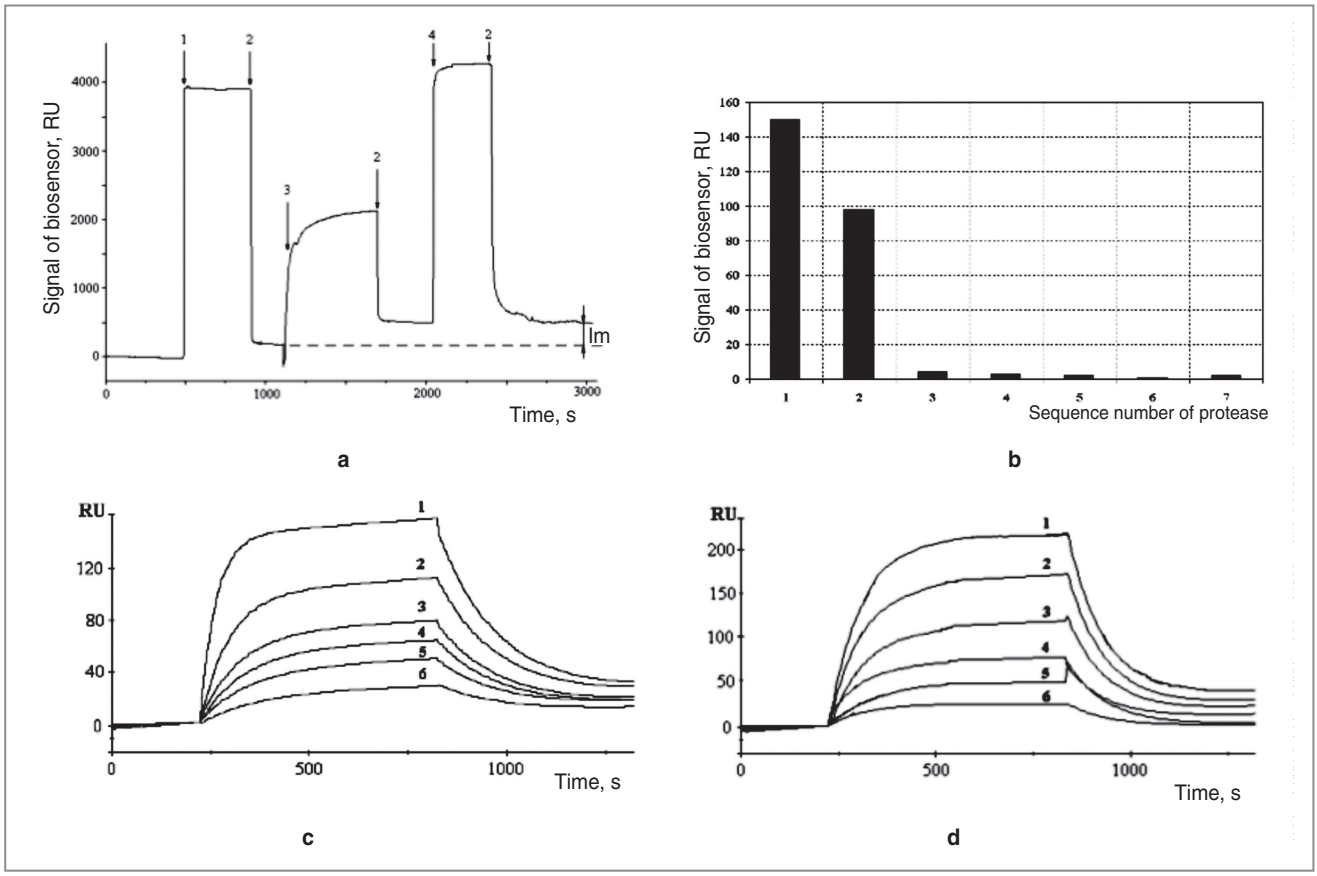

Fig. 5. Interaction of InhVJ inhibitor with various proteinases: *a* — a typical sensogram of covalent immobilization of InhVJ on optical chip CM5 (*1* — EDC-NHS mixture; *2* — HBS-buffer; *3* — InhVJ solution; *4* — ethanolamine solution; Im — immobilized protein amount); *b* — interaction of proteolytic enzymes (200 nM) with immobilized inhibitor InhVJ (*1* — trypsin; *2* — α-chymotrypsin; *3* — kallikrein; *4* — plasmin; *5* — thrombin, *6* — papain; *7* — pepsin); *c*, *d* — sensograms of interaction of trypsin and chymotrypsin with immobilized InhVJim) within the range of concentrations (1-6) from 10 to 200 nM

of the same type (Fig. 7, *c*). Through the example of two mutant variants of L-asparaginase of *Helicobacter pylori,* specificity of molecular recognition was found to decrease appreciably in the presence of even one point mutation in contact zone of subunits of highly homologous closely related enzymes (Fig. 7, *c*).

4. *Interaction analysis of the second molecule of flavin mononucleotide (FMN) with cytochrome P450 reductase (CPR)* [15]. As proof that the binding of the second FMN molecule with CPR is not crystallization artifact, there was performed SPR-analysis of the interaction. There was obtained a series of sensograms (Fig. 8, *a*) of interaction of different FMN concentrations with CPR immobilized on optical chip. The interaction was found to have rather high affinity (Kd ~10^{-7} M) and evident selective character: on examination of a series of FMN derivatives with CPR, only FMN and flavin adenine dinucleotide (FAD) interacted (Fig. 8, *b*).

5. *Interaction of DNA-aptamer constructions with thrombin* [16, 17]. On DNA-aptamer model, where aptamers selectively interact with two exosites of thrombin, there was shown that their integration in heterodimer aptamer constructions by means of poly-(dT)-linker, its length being from 35 to 55 nucleotides (Fig. 9, *a*), results in affinity increase approximately thirty times as much (Fig. 9, *b*). Finally, Kd value reached 10^{-10} M.

The analysis of thermodynamic parameters of the interaction of aptamers with thrombin showed complex formation to be characterized by negative values of both ΔH, and TΔS (Fig. 9, *c*). In case of heterodimer construction A1(35)A2 the contribution of entropy component (-TΔS) to the change of free energy ΔG in complex formation increases, while the contribution of enthalpy component (ΔH) changes slightly.

6. *Proteome analysis and SPR validation of isatin-binding proteins in mice and rats* [18, 19]. Isatin (indole-2,3-dione) is endogenous indole that presents in mammal brain and other tissues. Its content changes in stress and anxiety state. Isatin and its derivatives demonstrate a wide range of pharmacological activity. We performed proteome analysis of isatin-binding proteins isolated from murine tissues using affine molecular fishing. As a result, we identified over 60 individual proteins. The ability of some of them to interact with isatin was validated in biosensor experiments with 5-aminoisatin immobilized on optical chip surface (Fig. 10, *a*). The study proteins were found to interact actually with isatin with sufficiently high affinity — Kd values were within the range of 10^{-6}-10^{-7} M.

There was also performed biosensor investigation of the interaction of human recombinant cytokeratines with isatin analogues (5-aminoisatin and 5-aminocaproilisatin)

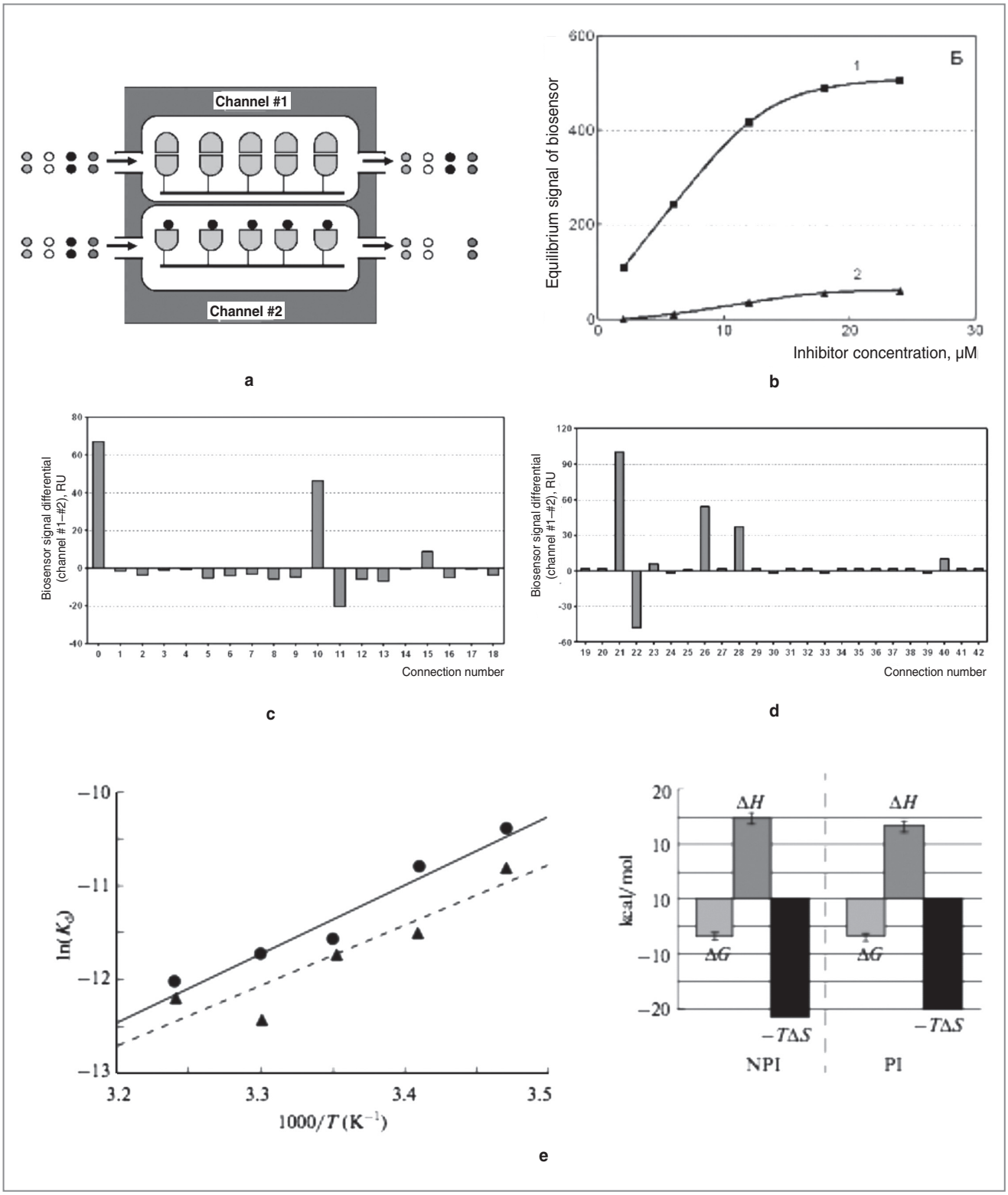

Fig. 6. Differentiated biosensor test-system of potential inhibitors of HIV protease dimerization: *a* — principle of differential biosensor test-system of analysis of interaction of potential inhibitors of dimerization with dimers and monomers of HIV protease (in channel #1 dimer protease form is immobilized and stabilized, in channel #2 — monomer protease form; binding of testing compound only with monomers in channel #2 is considered to be a positive result); *b* — validation of differential biosensor test-system using Schramm inhibitor — known peptide inhibitor of HIV protease dimerization; *c*, *d* — screening results of 42 potential inhibitors of HIV protease dimerization (0 — known peptide Schramm inhibitor — positive control); *e* — analysis of thermodynamic parameters of interaction of peptide and non-peptide inhibitors of dimerization with HIV protease monomers: *a* — Van`t Hoff diagrams for determination of thermodynamic parameters of interaction of peptide (PI, dashed line) and non-peptide (NPI, full line) dimerization inhibitors with HIV protease monomer; *b* — comparison of thermodynamic parameters of interaction of PI and NPI with HIV protease monomer

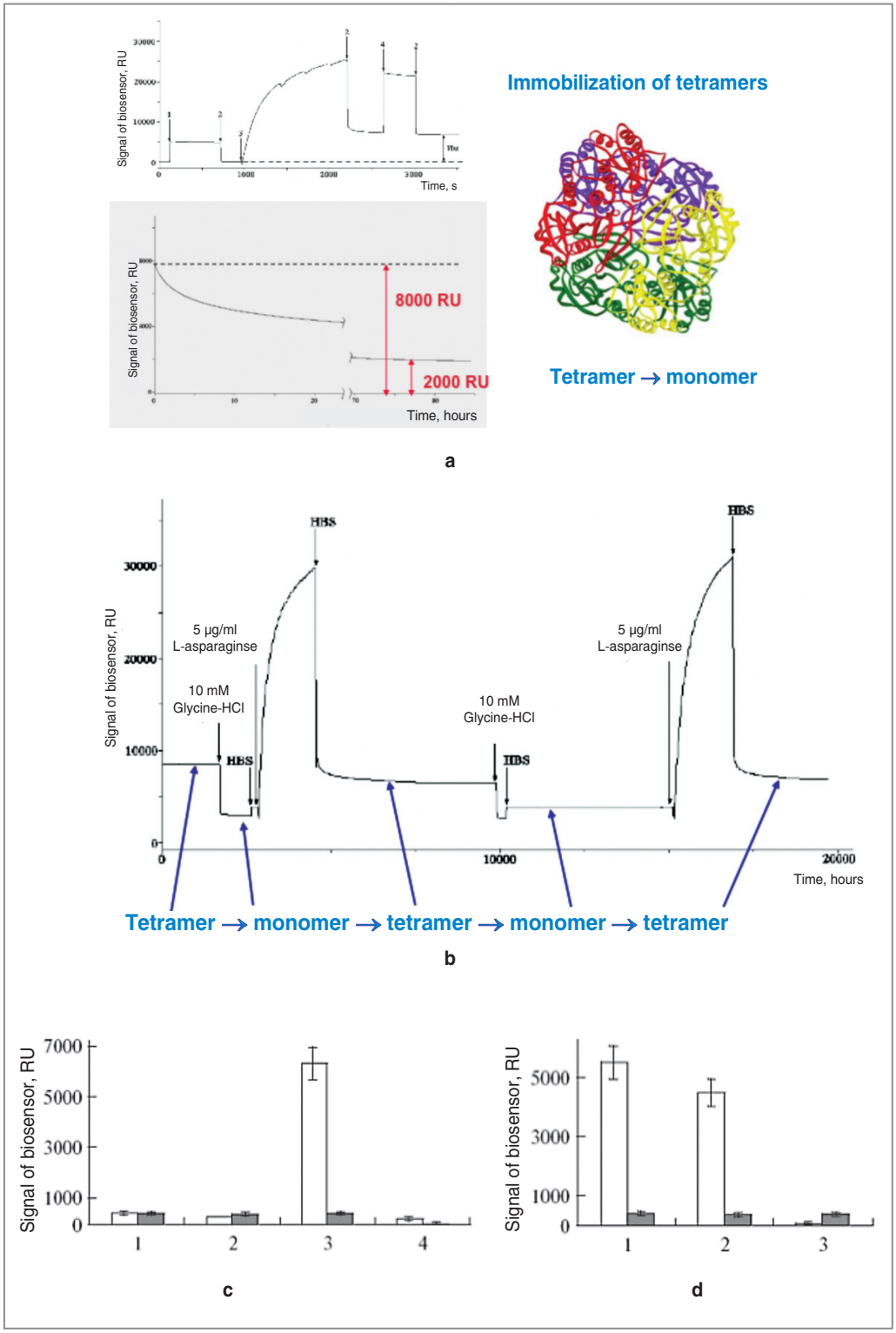

Fig. 7. Molecular recognition in oligomerization of bacterial L-asparaginases: *a* — structure of L-asparaginase tetramer, immobilization scheme of L-asparaginase of *E. carotovora* on optical chip CM5 and spontaneous decomposition of protein tetramers in long transmission of working buffer; legend of injections: *1* — EDC-NHS mixture; *2* — HBS-buffer; *3* — L-asparaginase; *4* — ethanolamine; Im — immobilized protein amount; *b* — decomposition of L-asparaginse tetramers in transmission of glycine-HCl solution (pH 2.5) and further recovery of enzyme tetramers in transmission of L-asparaginase solution; *c* — oligomerization of L-asparaginases in the channel of optical biosensor with immobilized monomers of L-asparaginase from *E. coli* (white colour) and in blank channel (control, grey colour). Legends of injections of asparaginase solutions: *1* — *H. pylori J99*; *2* — *H. pylori 26695*; *3* — *E. coli*; *4* — *Erw. chrysanthemi*; *d* — oligomerization of L-asparaginases in channel of optical biosensor with immobilized monomers of L-asparaginase from *H. pylori J99* (white colour) and in blank channel (control, grey colour). Legends of injections of asparaginase solutions: *1* — *H. pylori J99*; *2* — *H. pylori 26695*; *3* — *E. coli*

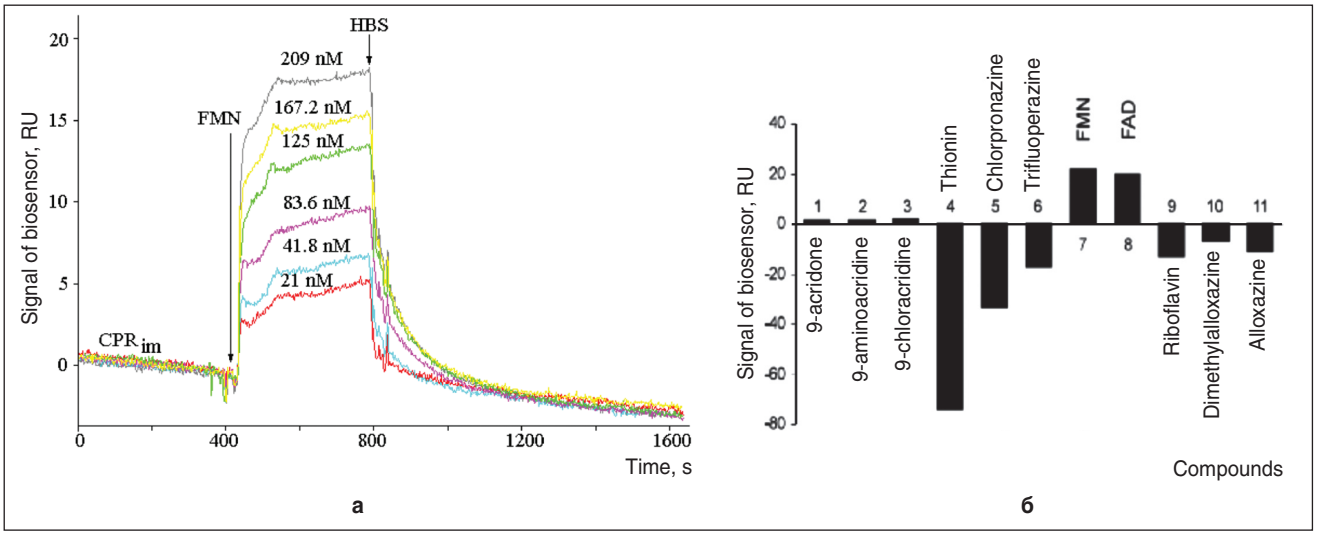

Fig. 8. Analysis of interaction of FMN with CPR: *a* — a set of sensograms of interaction at different FMN concentrations; *b* — SPR-analysis of interaction selectivity

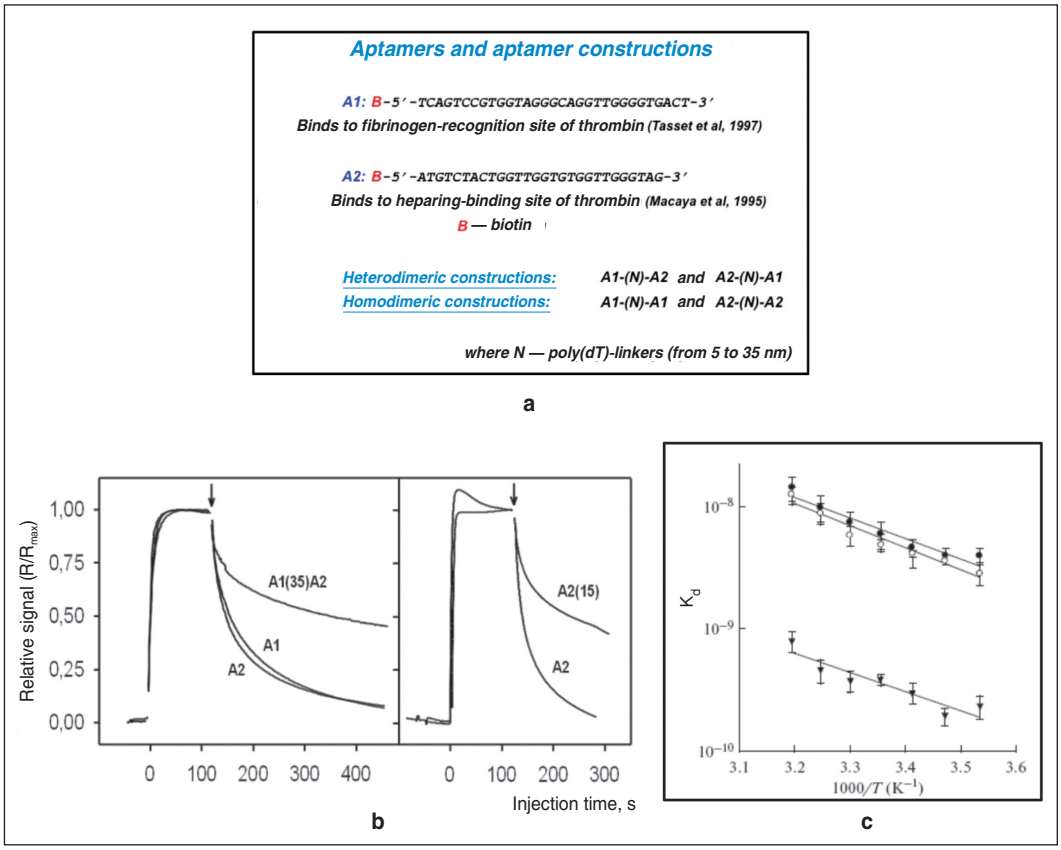

Fig. 9. Interaction of DNA-aptamer constructions with target protein — thrombin: *a* — primary structures of two aptamers to thrombin and their dimer constructions; *b* — sensograms of interaction of aptamers and dimer constructions with thrombin; *c* — Van`t Hoff diagrams for determination of thermodynamic parameters of interaction of aptamer constructions with thrombin

immobilized on optical chip CM5. The interaction in both cases was revealed to have high affinity, e.g. for a pair CK8-5-aminocaproilisatin Kd=1.7·10^{-6} M (Fig. 10, *b*).

7. *Biosensor signal enhancement using gold nanoparticles in recording telomeric repeats* [20]. On optical chip modified by streptavidin there was immobilized biotinulated oligonucleotide (sense) complimentary interacting with oligonucleotide consisting of antisense and several telomeric repeats (Fig. 11, *a*). And then suspension of gold nanoparticles modified by antisenses to telomeric repeats was injected in biosensor channel. Finally, there was achieved biosensor signal enhancement

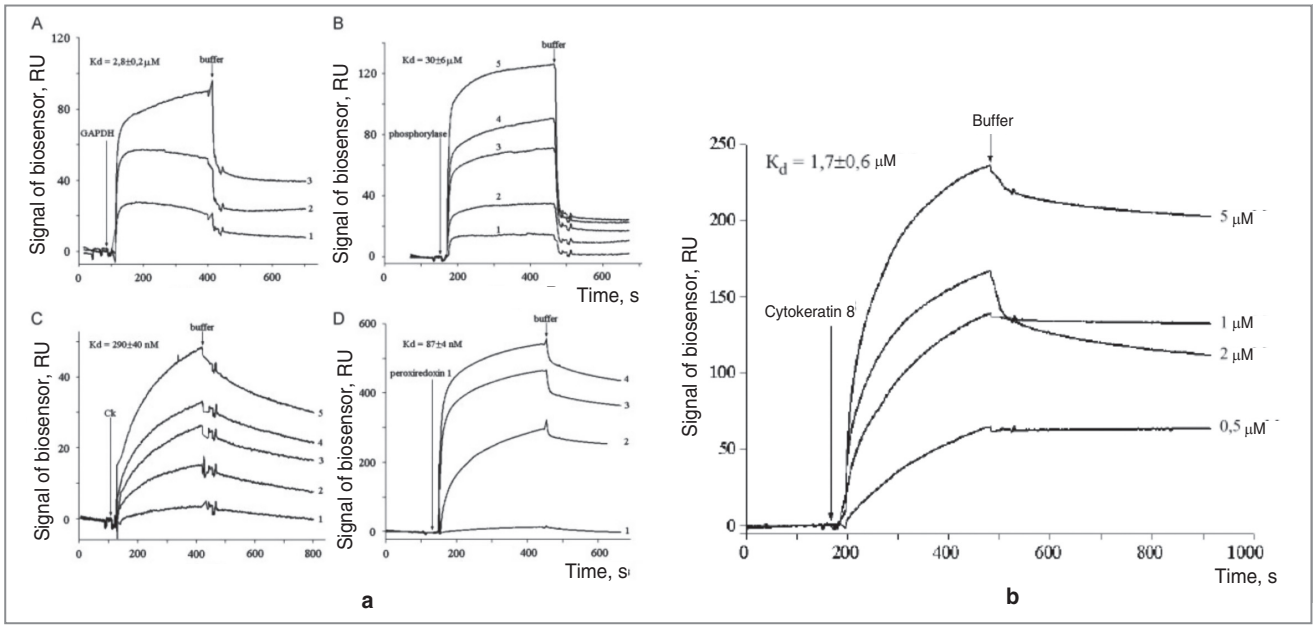

Fig. 10. SPR validation of interaction of isatin-binding proteins with isatin: *a* — interaction with immobilized 5-aminoisatin of indibidual enzymes: GAPDH (*A*), phosphorylase (*B*), creatine kinase (*C*), peroxiredoxin (*D*); *b* — sensograms of interaction of cytokeratin CK8 with 5-aminoisatin immobilized on optical chip

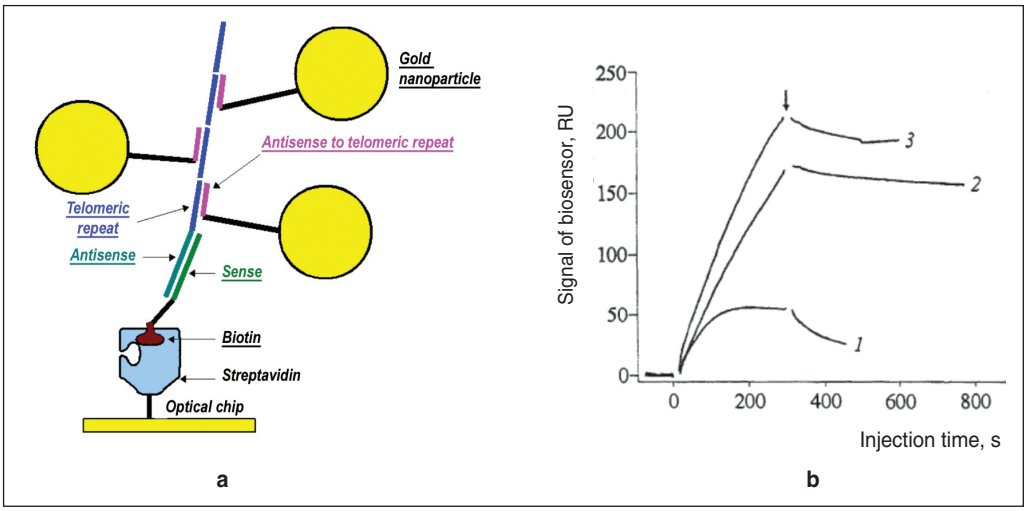

Fig. 11. Principle of biosensor signal enhancement using modified gold nanoparticles (*a*) and biosensor analysis of telomeric repeats with signal enhancement (*b*); injections: *1* — CX probe (20 nmol); *2* — conjugate of CX probe with streptavidin (10 nM); *3* — conjugate of CX probe with gold nanoparticles (0.13 nM)

approximately by 600 times compared to the record of interaction of antisense to telomeres without gold nanoparticles (Fig. 11, *б*).

8. *SPR detection of cardio-myoglobin in blood plasma* [21]. Sensitivity enhancement of biosensors for developing new diagnostic techniques is critical task, since the content of detected biomarkers in blood plasma is generally extremely low. There has been developed technology of optical biosensor signal enhancement using antibody conjugates gold nanoparticles to detect cardiomyoglobin for diagnosis, severity estimation and therapy monitoring of myocardial infarction.

The lowest concentration of a marker that has been determined in direct analysis is $4 \cdot 10^{-9}$ M, while in the analysis using antibody conjugates with gold nanoparticles — $2 \cdot 10^{-11}$ M (Fig. 12), i.e. there has been achieved biosensor signal enhancement by more than two sequences higher.

Conclusion. The technology of optical biosensors operating on the effect of surface plasmon resonance is getting one of the approaches in researching various intermolecular interactions. It is of universal character and enables to record the interactions between almost any molecular objects in a wide range of concentrations. SPR-biosensors have very high accuracy, capability of

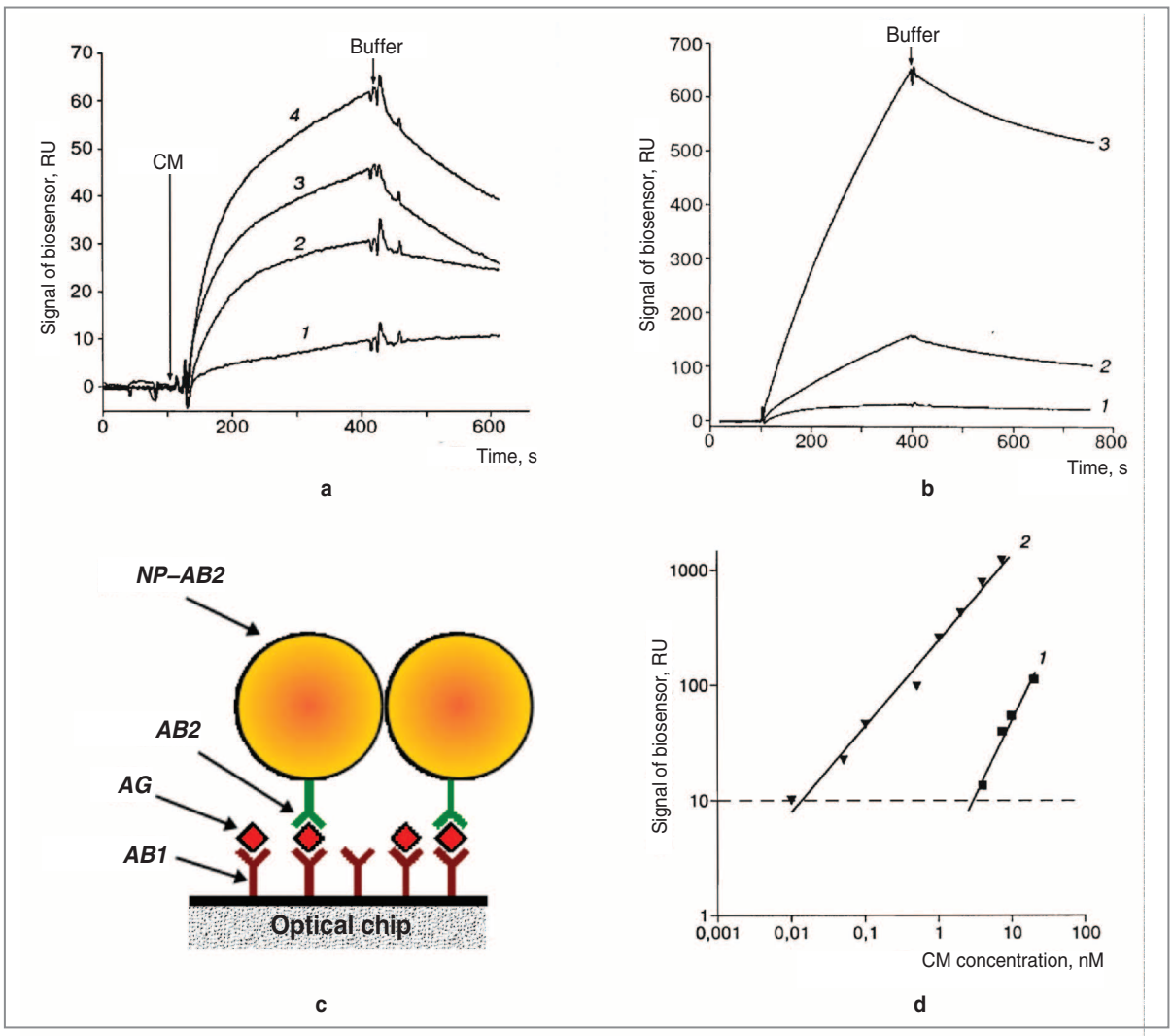

Fig. 12. The use of gold nanoparticles (NP) for optical biosensor signal enhancement in detection of cardiomyoglobin in blood plasma: *a* — interaction of cardiomyoglobin (CM) at concentrations of 1 nM (*1*); 4 nM (*2*); 7.5 nM (*3*) and 10 nM (*4*) with immobilized AB1 antibodies; *b* — interaction of samples CM (*1*); CM-AB2 (*2*); CM-AB2-NP (*3*) with immobilized AB1; *c* — principle of forming sandwich from antigen (AG), two antibodies (AB1 and AB2) and gold nanoparticles (NP) on optical chip surface; *d* — dependence of biosensor signal on CM concentration in model samples in blood plasma: *1* — direct analysis; *2* — analysis with the use of conjugates of antibodies with gold nanoparticles

reproducing and sensitivity; due to these characteristics their application is growing both in fundamental and applied researches.

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