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# A novel aldehyde dextran sulfonate matrix for affinity biosensors

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**Abstract**

Aldehyde dextran sulfonate (ADS), a modified oligosaccharide polymer, was used to prepare a new matrix structure for affinity biosensors. The principal difference between the ADS matrix and similar structures developed previously results from presence of two active functional groups in the matrix, namely, aldehyde and sulfonate. These groups perform two different functions in the matrix. The aldehyde group is responsible for covalent bonding in the biomaterials, and the negatively charged sulfonate group provides electrostatic attraction of the positively charged biomolecules. By varying the ratio between the aldehyde and sulfonate groups in the matrix, one can control contributions from the two binding modes (covalent and electrostatic). A number of oligosaccharides, such as simple dextran, aldehyde dextran (AD), aldehyde dextran sulfonate (ADS) and aldehyde ethylcellulose (AEC), were used for preparation of matrix structures. The properties of the obtained matrices were analysed and compared. Surface plasmon resonance (SPR) was used as the main technique to characterize the matrix structures. 

*Keywords:* Aldehyde dextran sulfonate; Matrix; Surface plasmon resonance; Biosensor

## 1. Introduction

The method of surface plasmon resonance (SPR) is based on polariton–plasmon surface resonance in thin metal films and in semiconductors. It allows recording

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interaction kinetics for a wide range of biological objects and requires no additional labelling [1]. An analysis of the SPR angle vs. time curves (sensograms) allows investigation of the effect of physico-chemical factors on interaction between biological objects. Structural characteristics of these objects and quantitative estimation of kinetics of interaction between them can be studied as well.

Proteins are the most typical objects for employing the SPR technique. Immobilization of one of the partners of the biospecific interaction on a metal (usually gold) film exposed to a solution containing a modifying agent is a necessary condition for application of the SPR technique. Usually, protein immobilization by adsorption on gold surfaces leads to uncontrolled changes in the spatial structure of the protein and, as a result, to the loss of its biological activity [2,3]. Thus, the gold surface should be isolated from the direct contact with proteins to maintain their biological activity during the study. It is known that the SH-groups interact with gold under soft conditions [4,5] stronger than other functional groups. This is the reason why SH-containing compounds are usually used for preparation of a linker layer on the gold surface. For these purposes, aliphatic thiols with sufficiently long ($n > 10$) unbranched chains are most often used. Such compounds (or their derivatives with different functional groups at the end of the methylene chain) spontaneously form stable ordered monolayer on the gold surface. In these monolayers, the sulphur atoms are bound to the gold support and close-packed aliphatic chains make equal angles with the surface. The monolayer is stabilized by van der Waals interaction between chains [6].

A ligand could be added to the previously immobilized elastic polymer chains in order to improve sensitivity and optimise use of a thin (ca. 300 nm) layer where an evanescent wave propagates. This approach allows immobilization of protein molecules in such amount that is equivalent to several close-packed layers. The protein layer ability to interact with macromolecular biospecific partners is also retained during the immobilization. Similar method employing a polymer matrix has been developed by the leading manufacturer of SPR spectrometers ("Biacore", Sweden) and found wide application [7]. This method seems to be rather complicated in the everyday practice of SPR technique use. It involves formation of an omega-mercapto-hexadecanol monolayer on the gold surface, its epichlorohydrin activation, covalent bonding of dextran to "the activated surface" and transformation of immobilized dextran into carboxymethyl (CM)-dextran. The carboxylic groups of this layer can be used for protein immobilization. The SPR spectrometer manufacturers offer sensor chips of several types with the CM-dextran layers organized on the surface. It is known that CM-dextran matrix is not optimal for proteins with low p$I$, since immobilization of proteins carrying electric charge of the same sign as that of the matrix charge requires substantial additional modification of the matrix layer [8].

Polymethacroylyl-chloride polymer derivatives have been also suggested for protein immobilization on the gold surface [9]. However, this method allows immobilization of the protein monolayer only and does not provide increase of the amount of immobilized biomolecules provided usually by polymer matrices. The synthesis of these polymers and their bonding to the gold surface also seems to be rather complicated. Therefore, one can see need to develop a simple and universal method for modification of gold surfaces with immobilized proteins and other biological objects.

We applied 16-mercapto-hexadecane acid (16-MHDA) for monolayer modification of gold surfaces. Monolayers of omega-mercapto-carboxylic acids have been widely applied for chemical modification of the gold surfaces to develop chemical and biological sensors, for instance, a sensitive element of SPR spectrometer [7,10] or an electrode of the variable-capacitance immunosensor [11]. The carboxylic groups of the 16-MHDA monolayer are oriented towards solution and could be used for protein immobilization. However, we have modified carboxylic groups of the 16-MHDA monolayer with hydrazine to obtain a mixed monolayer of 16-MHDA and its hydrazide derivative that can react with aldehyde groups. The hydrazide groups have $pK < 4$. This fact gives them a significant advantage over the primary amino groups. The hydrazide groups are not protonated at $pH > 4$ and retain their nucleophilic properties allowing the reaction with the aldehyde group. The primary amino groups are protonated under neutral pH and, accordingly, they have no nucleophilic properties required for the reaction with the aldehyde group [12]. The hydrazide derivatives of the matrix were employed for immobilization of periodate-oxidized glycoproteins [8].

To form a polymer matrix, we have chosen polysaccharide aldehyde derivatives obtained by periodate oxidation. Such derivatives were used for synthesis of water-soluble conjugates with trypsin [13], as well as with catalase and also with superoxide-dismutase in the microemulsions of the surface-active substances in heptane [14]. However, as we know, the aldehyde dextrans have not been used for protein immobilization on sensor surfaces. Polysaccharide aldehyde derivatives can be easily immobilized on a surface carrying hydrazide groups and then ligands with amino groups can complete the immobilized polysaccharide derivatives through covalent bonding to the polysaccharide aldehyde groups.

Negative charge of carboxymethyl-dextran is an advantage when immobilization is performed at pH lower than the isoelectric point of the given protein. This advantage originates from the electrostatic attraction of protein molecules to the matrix that promotes protein “pre-concentration” in the region adjacent to the matrix. However, this matrix is not efficient for covalent coupling of proteins with low $pI$ values since a decrease in the carboxylic groups content means that the amount of active groups through which immobilization occurs drops. In the matrix formed by periodate-oxidized polysaccharide, immobilization proceeds through uncharged aldehyde groups. However, when negative charge of the matrix is desirable, it can be obtained using periodate-oxidized dextran-sulfonate instead of periodate-oxidized dextran.

## 2. Materials and methods

### *2.1. Reagents*

Polysaccharides were obtained from “Sigma” (USA), trypsin from “Reanal’ (Hungary), bovine serum albumin (BSA) from “Dia-M” (Russia), glutaric aldehyde and hydrazine from “Fluka” (Switzerland), ethanolamine from “Ferak” (Germany). The following substances were used in the work: polyclonal rabbit antibodies against bovine serum albumin and against human fibrinogen, glucose oxidase from *Aspergillus niger*

and 1-ethyl-3-(3-dimethyl-aminoproline)-carbodiimide (EDC) from "Sigma", 16-MHDA from "Analytical μ-systems" (Germany). All compounds used in the study were of the "analytical grade" quality and were used without additional purification. Twice-distilled water was used for preparation of solutions in this study.

### *2.2. SPR: equipment and interpretation of data*

The SPR spectrometer "BIOSUPLAR-2" and Au-coated slides from "Analytical μ-systems" were used in this work. Standard refractometer IRF-22 was employed to determine the refractive indices of solutions. All experiments were carried out at room temperature and at constant velocity of solution flow through the cell of SPR spectrometer that was 2.5 ml/h (but for specially indicated cases). The change in the SPR angle position (the SPR response) was considered to be directly proportional to the change of the protein mass surface concentration [15].

The SPR technique is usually applied for monitoring of biospecific interactions at the modified interfaces. So it is more important to follow the binding activity of the modified interface than the primary modification of the solid support. Polyclonal rabbit IgG was used as a probe binding specifically to the protein primarily immobilized at the sensing interface. To analyse immobilization efficiency, we used the ratio $\Delta\theta_{ab}/\Delta\theta_{p}$, where $\Delta\theta_{ab}$ is the SPR response to binding of specific antibodies and $\Delta\theta_{p}$ is the response to protein–antigen immobilization. This value is proportional to the part of the immobilized antigen molecules that is able to interact with the dissolved antibodies. The stoichiometric ratio of the interacting proteins was determined from the direct proportionality of the SPR angle shift to the mass protein concentration on the surface. The difference between the SPR angles before and after immobilization was divided by molecular mass of the protein to calibrate the SPR angle response in the protein molar concentration [3].

### *2.3. Treatment of slides*

Sensor slides, made of TF glass, with an evaporated monolayer of polycrystalline gold were employed for studies using the SPR technique. The slides were exposed for 15 s to the as-prepared "piranha" solution (the mixture of concentrated sulphuric acid and 30% aqueous solution of hydrogen peroxide in the ratio of 3/1 v/v) and then washed with water using intense stirring. The slides after modification were washed with ethanol and dried in air. To form the 16-MHDA monolayer, the slides were immersed in the 0.0025 M solution of 16-MHDA in 96.5% ethanol for 15 h, then washed with ethanol and dried in air. All operations with the modified slides were performed in situ immediately in the SPR spectrometer cell.

### *2.4. Oxidation of polysaccharides*

The solution of the oxidizing agent $NaJO_4$ ($10^{-5}$ M/g of polysaccharide) was prepared at least 40 min prior to the usage and added to the polysaccharide water

solution (25 ml/g). Then the solution was thoroughly mixed up at room temperature and incubated in the dark for 5 h at 4 °C. A dry lyophilic material was isolated by dialysis from the mixture after 24 h.

### 2.5. Immobilization of polysaccharides

Immobilization of periodate-oxidized polysaccharides on the 16-MHDA layer was performed by the procedure described for glycoproteins immobilization on the carboxymethyl-dextran matrix [14]. The procedure was modified (see Fig. 1) and performed directly in the cell of the SPR spectrometer. This enabled us to observe and register the real-time kinetics of the immobilization process for all polysaccharides used.

After obtaining a stable SPR response for several minutes when washing cell with water, the as-prepared 0.2 M solution of EDC in 1 M solution of hydrazine-hydrochloride with neutral pH was introduced into the cell for 40 min. Then the cell was washed with distilled water until a stable response of SPR was obtained. Thus, at least a part of 16-MHDA molecules was transformed into the corresponding hydrazide. Then PBS was introduced to determine the background level. And after that, the 10 mg/ml solution of oxidized polysaccharide in the corresponding buffer (0.01 M sodium-acetate, pH 4.9 or 0.01 M sodium carbonate, pH 9.0) was introduced into the cell. Approximately 4 h later, the cell was washed with the immobilization buffer, then with 0.1 M solution of HCl (pH 2.0) until a stable response of SPR for 20–30 min was obtained, and again washed with the immobilization buffer. The amount of immobilized polysaccharide was determined by the difference between the input and output values of the SPR angle in the presence of immobilization buffer in the cell.

### 2.6. Immobilization of proteins

Polysaccharides immobilized (as described above) at the sensor surface and glutaric aldehyde (GA) (as a low-molecular mass analogue of "aldehyde-polysaccharides") were used to compare protein immobilization in polysaccharide matrices and in the absence of the matrices.

For GA modification, the monolayer of 16-MHDA with hydrazide synthesized in situ was incubated for 25 min in 7% GA solution in 0.01 M sodium-phosphate buffer, pH 7.3. Then protein immobilization was performed via both aldehyde derivatives of polysaccharides and GA. The cell was washed with water and corresponding immobilization buffer. Then 7.2 μM protein solution in this buffer was run through the cell for 90 min. After washing the cell with the immobilization buffer, the protein molecules that were not bound covalently were washed off with 0.01 M solution of HCl (pH 2.0) and again the immobilization buffer was introduced. The amount of the immobilized protein was determined by a difference between the SPR angles that corresponded to the buffer before and after immobilization.

To estimate possibility of using, if necessary (low p$I$ of a biomolecule), different functional groups of aldehyde dextran sulfonate (ADS) matrix, immobilization of glucose oxidase (GOD) (p$I$ = 4.1) was carried out in the presence and absence of sulfonate groups (in ADS and aldehyde dextran (AD) matrices) as described above.

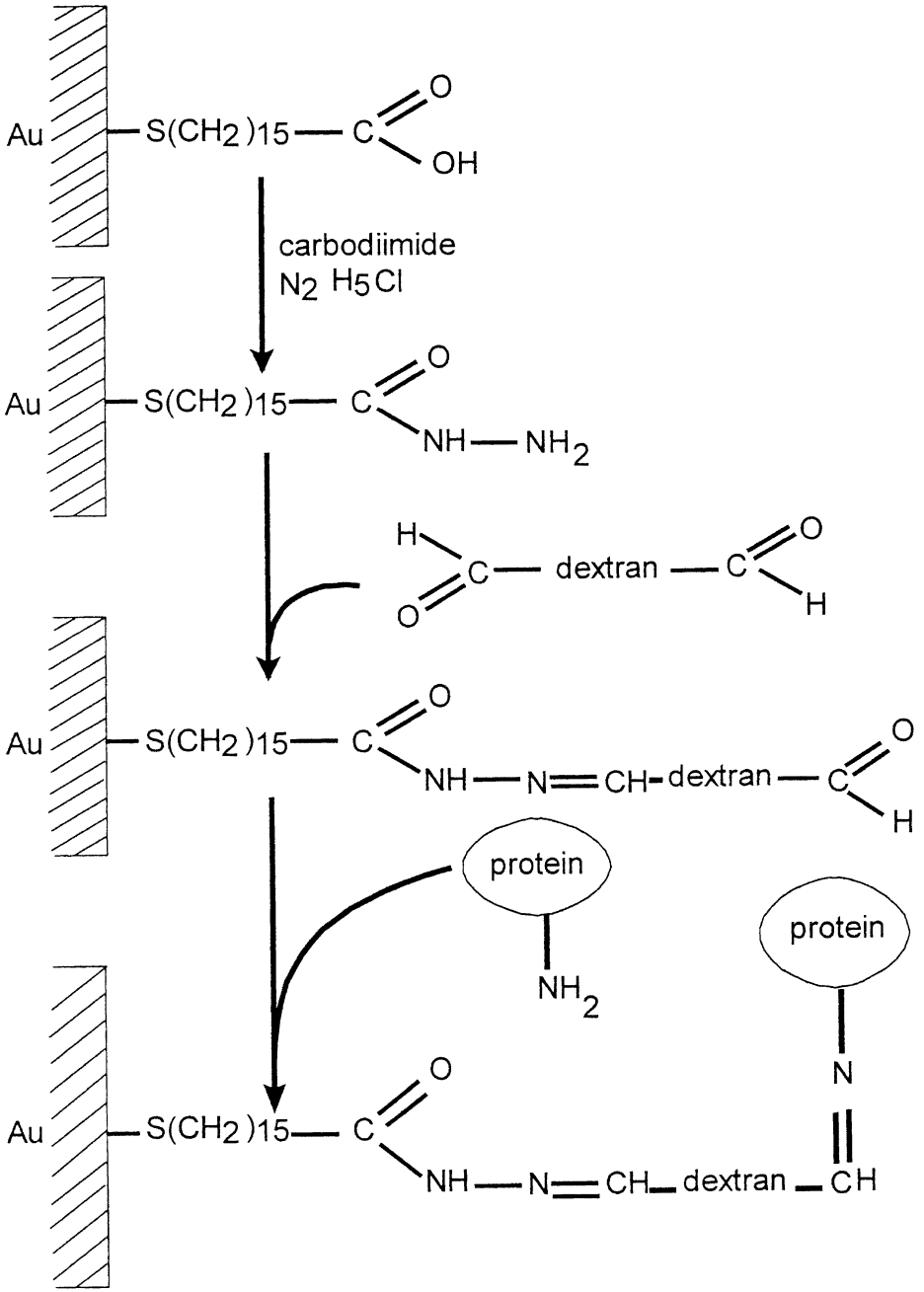


Fig. 1. Diagram of protein immobilization using a periodate-oxidized polysaccharide matrix.

### 2.7. Binding of antibodies

The polyclonal rabbit IgG against BSA (Ig$\alpha$BSA) was employed to confirm the ability of BSA molecules immobilized in the ADS matrix to participate in specific protein − protein interactions. The basic line of the SPR response was obtained by application of 0.01 M sodium-phosphate buffer (pH 7.3) containing 0.15 M NaCl and 0.02% sodium azide. Then, to determine the level of non-specific binding, 0.2 mg/ml solution of polyclonal rabbit antibodies against human fibrinogen (Ig$\alpha$Fg) in the mentioned buffer was introduced into the cell; 15 min later, it was washed for 10 min with the same buffer. Then the analogous solution of antibodies against BSA was introduced and 45 min later, the cell was washed with the above buffer. The unbound antibodies were washed off with 0.01 M solution of HCl, pH 2.0.

## 3. Results and discussion

### 3.1. The geometry of immobilized polysaccharide layers

The previous measurements using a refractometer have shown the refractive index $N$ to be 1.335 for the polysaccharide solutions (concentration of 10 mg/ml) and 1.334 for

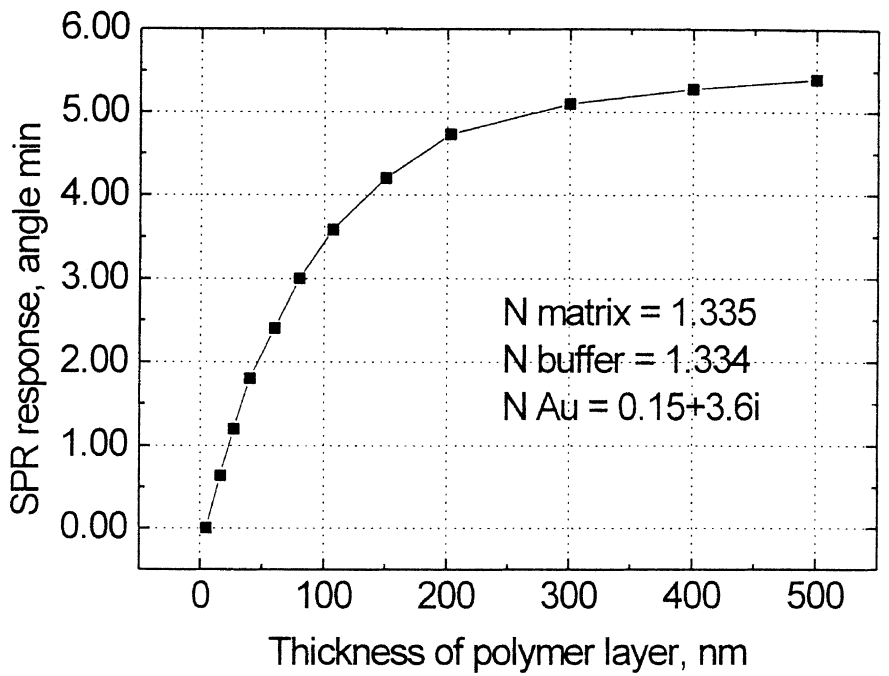


Fig. 2. The calculated dependence of the SPR response on the thickness of the polysaccharide matrix on Au surface (buffer solution $N = 1.334$).

the phosphate buffer (0.14 M NaCl, pH 7.4). We assumed that the refractive index value for the polysaccharide layer is close to that for the polysaccharide solution or higher. It is possible to estimate the thickness of immobilized polysaccharides using the theoretically calculated dependence of the matrix thickness on the plasmon resonance angle shift (Fig. 2). Fig. 3 presents typical sensograms registered after the chip surface was contacted with the solution of the attached molecules. Usually, the registered curve steadily goes up and then levels off, thus indicating at completion of the molecular layer formation on the chip surface. The quantitative data obtained after layer formation for different kinds of polysaccharides are presented in Table 1. The first row corresponds to the ADS layer formation after contact with the corresponding solution. We estimated the layer thickness of 80 − 140 nm (Fig. 4). Similar thickness was obtained for the AD layer, while aldehyde ethylcellulose (AEC) and unmodified dextran layer thicknesses were 20 − 50 nm, depending on pH buffer solution during the immobilization. It should be noted that at pH 4.9, a bigger amount of the oxidized polysaccharides can be immobilized than at pH 9.0, in spite of the fact that alkaline pH promotes the reaction between aldehyde and amino groups. This could result from the partial desorption of 16-MHDA from the gold surface that may occur at alkaline pH [11]. As expected, the amount of the unmodified dextran immobilized at the surface appears insignificant due to the absence of aldehyde groups in its structure. The amount of immobilized AEC that

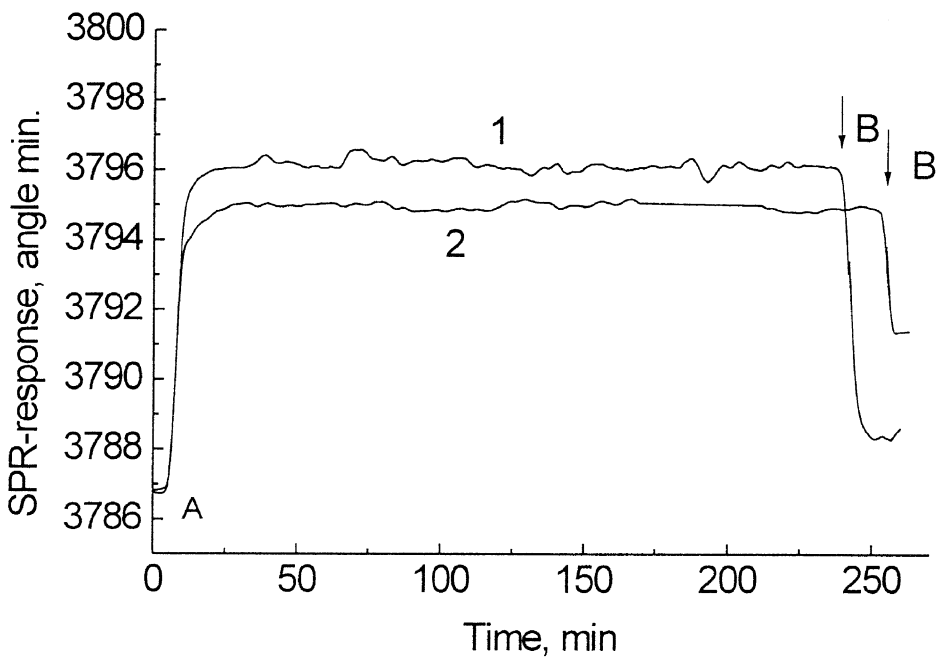


Fig. 3. Sensograms obtained upon oxidised polysaccharides immobilization. The baseline corresponds to the level of 0.1 M Na-carbonate buffer, pH 4.9. A—injection of 10 mg/ml solution of the oxidised polysaccharide in the same buffer; B—replacement of the polysaccharide solution with the buffer; 1—AEC; 2—ADS.

Table 1

| *N* | Immobilized molecule | Type of Au surface coating | Type of buffer solution/pH | SPR minimum shift after layer immobilization/ layer thickness | SPR minimum shift after exposition to HCl (pH = 2) |
|---|---|---|---|---|---|
| 1 | ADS | thiol-Au | Na acetate/4.9 | 4.4′/140 nm | |
| | AEC | thiol-Au | Na acetate/4.9 | 1.2′/30 nm | |
| 2 | BSA | ADS | Na acetate/4.9 | 73′ | 67′ |
| | BSA | AD | Na acetate/4.9 | 51′ | 21′ |
| | BSA | GA | Na acetate/4.9 | 23′ | 13′ |
| | BSA | AEC | Na acetate/4.9 | 18′ | 7′ |
| 3 | BSA | ADS | Na acetate/3.8 | 40′ | |
| | BSA | ADS | Na acetate/4.9 | 65′ | |
| | BSA | ADS | Na phosphate/7.2 | 50′ | |
| | BSA | ADS | Na carbonate/9.0 | 3′ | |
| 4 | IgGaFg | BSA/ADS | Na-phosphate/7.3 | 1–4′ | |
| | IgGaFg | BSA/GA | Na-phosphate/7.3 | 1–4′ | |
| 5 | IgGaBSA | BSA/ADS | Na-phosphate/7.3 (1st) | 72′ | 18′ |
| | IgGaBSA | BSA/ADS | Na-phosphate/7.3 (2nd) | 54′ | 18′ |
| | IgGaBSA | BSA/ADS | Na-phosphate/7.3 (2 weeks) | 30′ | 5′ |
| | IgGaBSA | BSA/GA | Na-phosphate/7.3 | 37′ | 8′ |
| 6 | Trypsin | ADS | Na-phosphate/8.0 | 28′ (0.25′/kDa) | 0′ |
| | BSA | ADS | Na-phosphate/4.9 | 70′ (1′/kDa) | 62′ |
| | GOD | ADS | Na-acetate/3.9 | 48′ (0.25′/kDa) | 48′ |

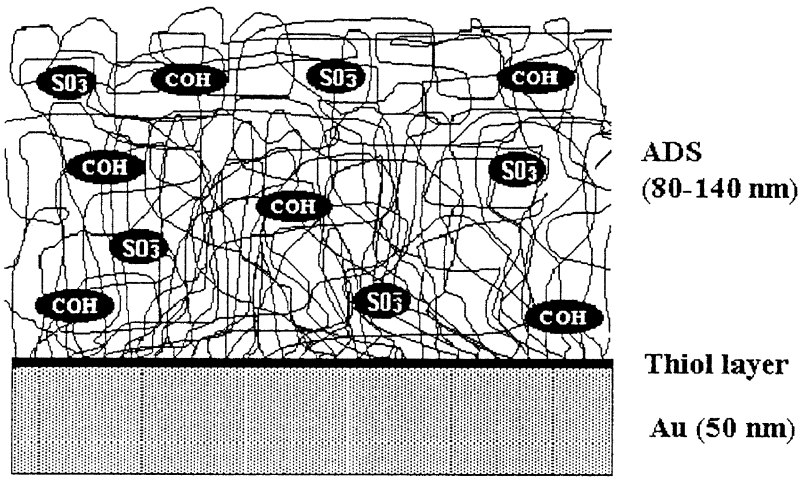


Fig. 4. Schematic presentation of the modified SPR sensor surface with an ADS layer.

depends on the conditions of oxidation appears to be unexpectedly low. The similar thickness of ca. 30 nm was also observed upon immobilization of dextran.

### *3.2. Effect of the presence and type of matrix on the amount of immobilized protein*

Presence of a polymer three-dimensional matrix provides significant increase of the amount of immobilized BSA as compared to the case of immobilization on thin linker layers (Fig. 5; Table 1, row 2). ADS allows immobilization of the highest amount of BSA. It is clear from Fig. 5 that the number of BSA molecules in the area of influence of evanescent wave increases more rapidly (Fig. 5, curve 4) than for other matrices. This may result from more pronounced effect of protein "pre-concentration" in the volume adjacent to the Au surface due to electrostatic interaction of positively charged (at the used pH value) BSA molecules with negatively charged sulfonate groups of polysaccharide. (In the absence of charged polysaccharide, this effect cannot be excluded completely owing to the presence of carboxylic groups of 16-MHDA on the gold surface.) Due to electrostatic attraction, the real concentration of BSA molecules near the chip surface is higher than in the solution, enhancing the reaction between the protein amino groups and aldehyde groups. This result, comparable to an insignificant amount of the immobilized AEC, enables one to draw a conclusion about the obvious advantages of ADS matrix. That is why we performed further experiments mainly with ADS and AD.

### *3.3. Effect of pH on BSA immobilization*

The effect of pH on the protein immobilization results from the pH sensitivity to several processes occurring during immobilization. In particular, electrostatic interaction of BSA with the sulfonate groups of ADS that can cause protein "pre-concentration" in the matrix is a pH-dependent process. The pH decrease is required to enhance this

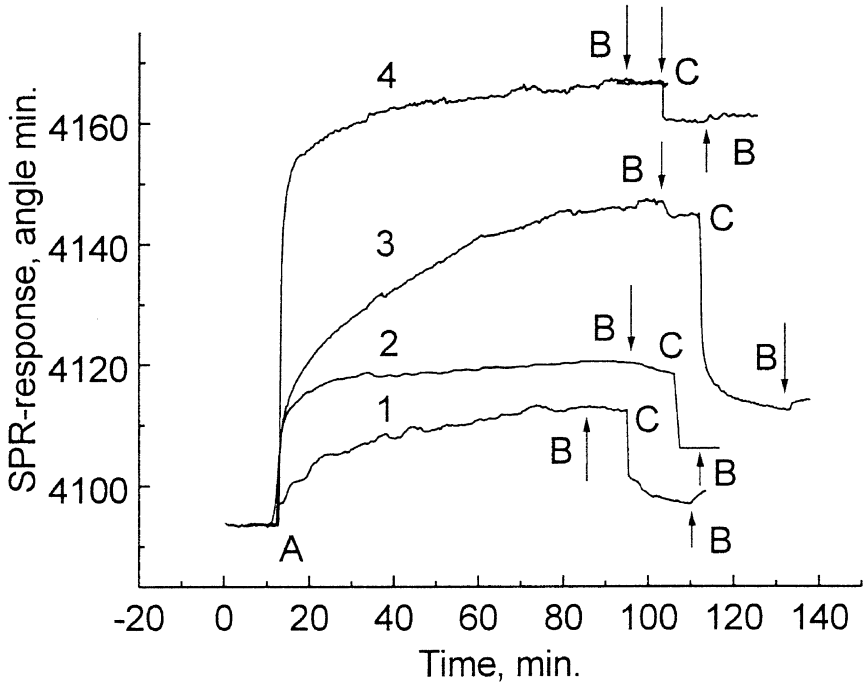


Fig. 5. Sensograms obtained upon BSA immobilization. Each baseline corresponds to the level of the buffer used later during the immobilization process. A—injection of 0.5 mg/ml BSA solution; B—injection of the buffer used during immobilization; C—washing with 0.01 M HCl, pH 2.0. Molecules attached to the surface before BSA immobilisation: 1—AEC; 2—GA; 3—AD; 4—ADS. Immobilization buffer: −0.01 M Na-acetate, pH 5.0.

process, since the total positive charge of the BSA molecule increases with lowering of pH. At the same time, the protein amino group should be deprotonated for the reaction with the aldehyde group that requires the alkaline pH. So it was necessary to determine experimentally which of the above processes is of importance for immobilization and what pH value is optimal for the BSA immobilization on the obtained matrix consisting of ADS.

We found that the optimal pH = 4.9 for the BSA immobilization is lower than p$I$ = 5.1 of protein (Fig. 6; Table 1, row 3). The BSA immobilization almost does not occur, probably due to complete protonation of the protein amino groups under these conditions. The efficiency of the BSA immobilization is not very high at pH > p$I$, as a result of electrostatic repulsion between the negatively charged BSA and sulfonate groups of the matrix. Besides, at pH far from the isoelectric point, the electrostatic repulsion between protein molecules themselves may prevent immobilization.

### *3.4. Binding of antibodies*

Non-specific antibody adsorption on the matrix obtained with immobilized protein is insignificant as is shown by curves corresponding to binding of antibodies specific to

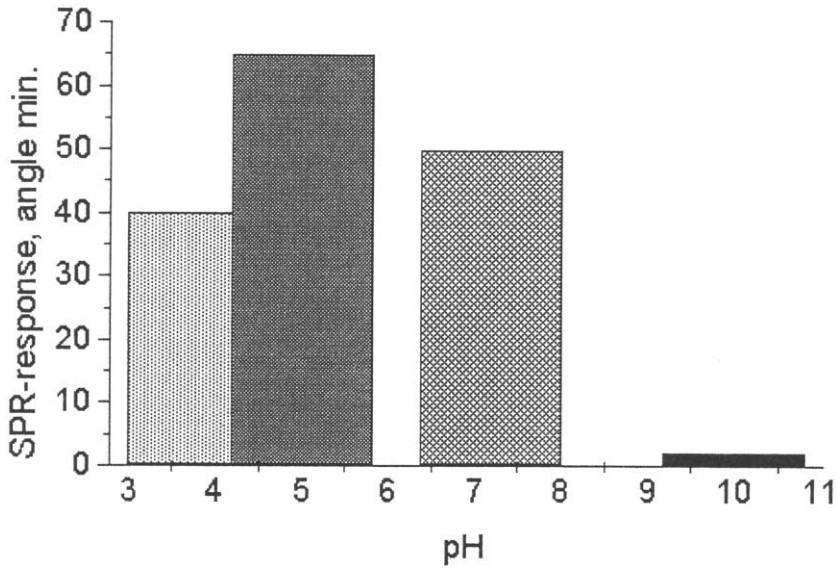


Fig. 6. pH-dependence of the BSA immobilization. Concentration of all buffers is 0.01 M, pH 3.8 and pH 5.0 —Na-acetate buffer; pH 7.2—phosphate buffer; pH 9.0—Na-carbonate buffer; 0.5 mg/ml solutions of BSA were incubated in the SPR-cell for 60 min and then replaced with the corresponding buffers.

fibrinogen (Fig. 7; Table 1, row 4). The ratio between the amount of bound specific antibodies and that of immobilized BSA ($\Delta\theta_{At}/\Delta\theta_{Im}$) is considerably higher when immobilization in the polymer matrix is used (50/13) than in the case of immobilization on a flat gold surface through GA (30/10). This may be explained by steric hindrance, preventing antibodies from binding to the BSA molecules immobilized immediately near the surface, in the case of epitope "unsuccessful" orientation to the surface. Besides, the free space near the layer of BSA molecules may be insufficient to bind relatively big antibody molecules to each BSA molecule immobilized on the surface. In the case of immobilization in the polymer matrix, BSA molecules are accessible almost from all sides and actual distances between the immobilized molecules are longer than those between the molecules on the surface. The ratio $\Delta\theta_{At}/\Delta\theta_{Im}$ ($50/13 = 3.8$) exceeds that between the molecular masses of antibodies and BSA ($160000/69000 = 2.3$). This fact indicates that some BSA molecules are bound to more than one antibody molecule. Such a conclusion is confirmed by the fact that there is sufficiently free space near immobilized BSA molecules. Thus, it is possible to consider that steric hindrances caused by immobilization do not substantially hinder the interactions of the BSA molecules immobilized on the ADS matrix with the high-molecular-mass biospecific partner that are similar to interaction in a solution.

### *3.5. Stability of immobilized protein*

The protein-modified chips should demonstrate long-term stability for practical applications. Therefore, stability of immobilized BSA was estimated from its ability to

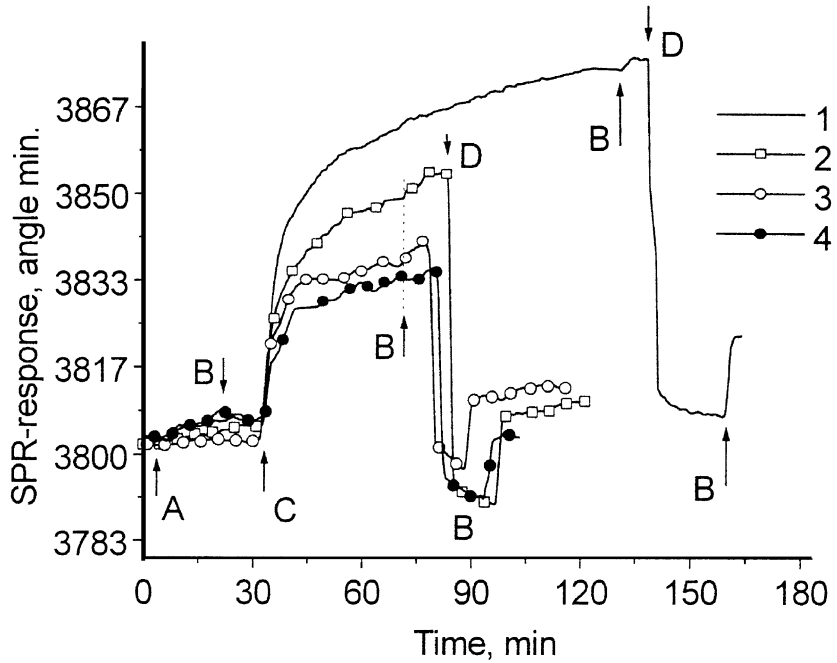


Fig. 7. Sensograms of the IgG binding by BSA. A—injection of 0.5 mg/ml solution of anti-human fibrinogen IgG in 0.01 M phosphate buffer (0.15 M NaCl and 0.01% sodium azide, pH 7.3); B—injection of the same buffer; C—injection of 0.5 mg/ml solution of anti-BSA IgG in the same buffer; D—washing with 0.01 M HCl, pH 2.0. 1, 2, and 4—BSA immobilized to the ADS previously attached to the surface; 3—BSA immobilized to GA. 1—the 1st injection after the BSA immobilization; 2—the 2nd injection after the BSA immobilization performed on the day of immobilization; 4—the same experiments performed after 2-week storage of the sensor chip at room temperature in the same buffer solution.

bind specific antibodies when storing the modified chips at room temperature in 0.01 M sodium phosphate buffer, pH 7.3, containing 0.15 M sodium chloride and 0.02% sodium azide. Binding of antibodies after storing the chip with immobilized BSA in air for 15 days decreased approximately by 30% but it remained vastly greater than non-specific binding (Fig. 7; Table 1, row 5). The chip degradation may be explained either by spontaneous denaturation of immobilized protein or by splitting of the covalent bonds between the aldehyde and amino groups.

Thus, the chips with immobilized proteins are capable to determine qualitatively the high-molecular-mass biospecific partner in a solution at least for 15 days.

### *3.6. Comparison of the activity of different immobilized proteins*

To estimate ranges of application of the ADS matrix, proteins with isoelectric points from 4.1 to 10.5 were immobilized on it. These were GOD (p*I* 4.1), BSA (p*I* 5.1) and trypsin (p*I* 10.5) (Fig. 8; Table 1, row 6). Taking into account the decisive effect of the protein charge on the immobilization reaction, each of the proteins was immobilized at

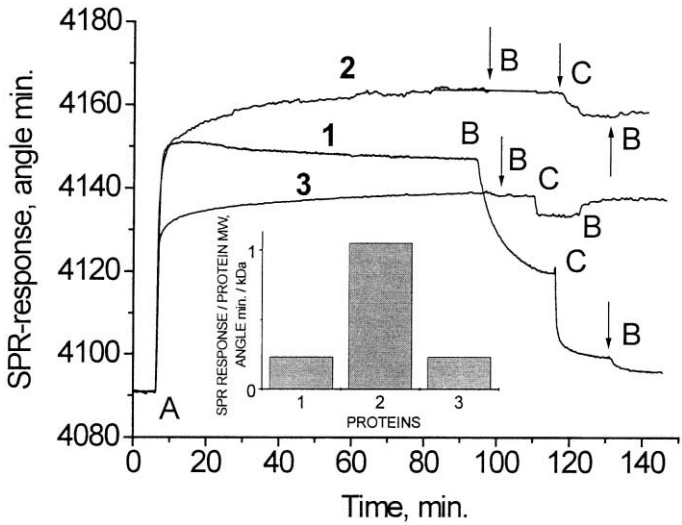


Fig. 8. Sensograms of the immobilization of proteins with different isoelectric points in the ADS matrix. 1—trypsin in Na-phosphate, pH 8.0; 2—BSA in Na-acetate, pH 5.0; 3—GOD in Na-acetate, pH 3.9. Injections: A—protein solution, 7.25 μM in corresponding buffer; B—the same buffer without protein; C—0.01 M HCl, pH 2.0. Inset: SPR responses to protein immobilization are divided by the corresponding MW (to make them proportional to the number of immobilized macromolecules). MW of trypsin, BSA and GOD are assumed to be 25, 69 and 175 kDa, respectively.

pH somewhat lower than its isoelectric point: GOD at pH 3.9, BSA at pH 4.9, trypsin at pH 8.0 (it is undesirable to use higher pH because of possible desorption of 16-MHDA). The number of immobilized protein molecules was expected to increase with pH since the basic pH enhances the reaction between the primary amino groups of proteins and aldehyde groups. The amount of immobilized molecules (Fig. 8, inset) was low when we used protein with low p$I$ (e.g., GOD) originating from the big molecular mass of the protein. However, the SPR response (Fig. 8, sensogram) registered upon the GOD immobilization is relatively high. The amount of immobilized trypsin molecules proved to be less than it was expected, probably due to autolysis that proceeded simultaneously with immobilization of this protein.

### *3.7. Differentiation of the activity of the functional groups*

The process of glucose oxidase immobilization ($pI = 4.1$) in the AD matrix at pH = 3.9 proceeds more slowly than similar process in ADS. This results from the absence of electrostatic attraction provided by the sulfonate groups of ADS (Fig. 9). Part of molecules that were not bound covalently and were washed off upon washing with HCl is more significant. Finally, the amount of immobilized protein in the AD matrix exceeds approximately by $1/3$ that of protein immobilized in ADS. Since in the case of the uncharged matrix, electrostatic interactions have no essential effect on the immobilization process, it is possible, in principle, to vary pH of the buffer solution over wide

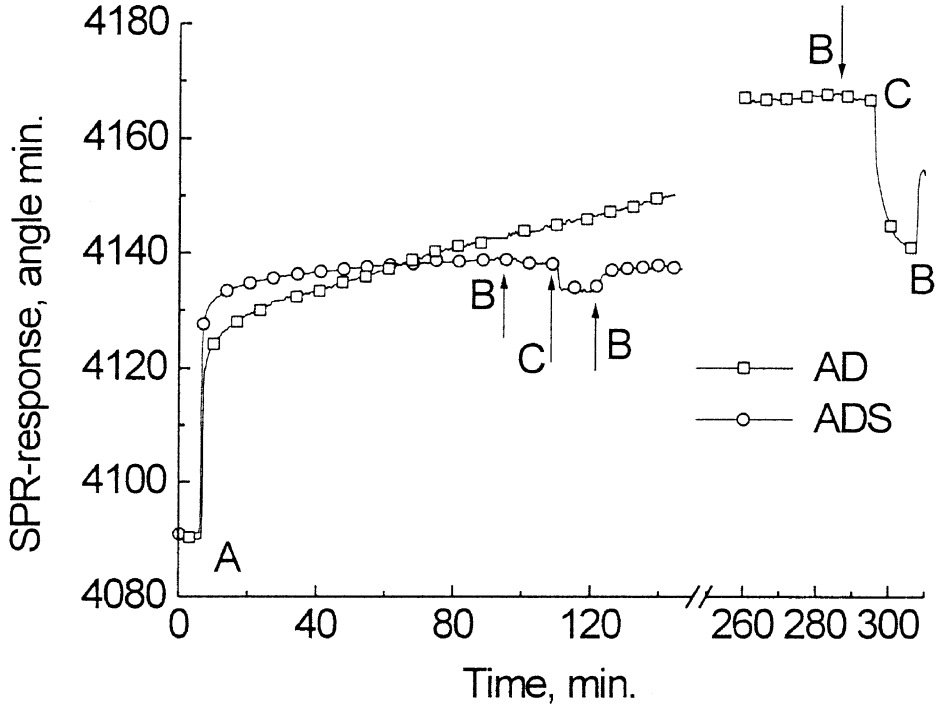


Fig. 9. Sensograms of the immobilization of GOD (p$I$ = 4.1) in the different matrices. Squares—ADS matrix; circles—AD matrix. Injections: A—protein solution, 7.25 μM in Na-acetate buffer, pH 3.9; B—the same buffer without protein; C—0.01 M HCl, pH 2.0.

ranges, selecting pH optimal for the reaction used for the immobilization (including pH which is higher than p$I$ of the chosen protein). This permits optimisation of the parameters of the immobilization process in the matrix for biomolecules with low p$I$.

## 4. Conclusion

We have shown that ADS is the most efficient material for protein immobilization. The proposed ADS matrix increases by several times the value of the SPR response after protein immobilization in its volume, as compared to the case of immobilization on the surface. The ADS matrix environment promotes biological reactions of specific interaction proceeding in the matrix. Differentiation of functions of electrostatic attraction and covalent binding between sulfonate and aldehyde groups of the ADS matrix allows to extend the dynamic range of affinity biosensors by p$I$ of the studied biological objects. It is possible to develop the general procedure for covalent binding of biomolecules in the polymer matrix without additional steps. In this case, determination of the optimal parameters of the environment where the interaction between molecules will take place becomes more important.

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