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Success and Failure in Antibody Recognition by Surface-Type Sensors: Essential Prerequisites †

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Abstract: In order to determinate small molecular compounds (so called haptens) in biological media, especially when the concentrations of compounds are at trace concentrations, it is necessary to produce antibody with high affinity and high selectivity. Since the hapten is not able to stimulate the animals to produce specific antibodies directly it should be bound to protein carrier. The manner of chemical binding of the hapten to a protein determines the character of the antibody specificity to small molecules analyzed. One of the high sensitive methods in the small molecule determination at low concentration is competitive SPR-based immunoassay. To use the method effectively the definite sensor surface sensitive to specific antibody is needed to achieve the lowest value in limit of detection (LOD) in immunoassay. The most evident class of small molecules to be determined in biological media at the lowest concentration is the steroid hormones, particularly estrogens. We have developed the sensor surface by binding the target molecules (estradiol, E2) directly to the gold surface through the specific linker to provide the closest distance to the surface along with biocompatibility to get maximal response in antibody-antigen interaction. As an antibody we have used a commercial monoclonal antibody raised to the 6-position in E2 with BSA (E26*BSA_CC). The specific binding the antibody to the sensor surface has failed to observe. Then, we suggested that the main factor hindering this interaction is the wrong choice regarding the hapten–carrier conjugated with carrier protein. In order to confirm the assumption we took the serum obtained from animal immunizing by the antigen where BSA- is attached to 3- or 17-position in E2 (E23*BSA_CC or E217*BSA_CC) instead of 6-one. Only the polyclonal antibody obtained with E23*BSA_CC expectedly resulted in its successful binding to sensitive sensor surface.identification of low molecular weight analytes.

Keywords: low molecular weight analytes; 17β-Estradiol; antibody recognition

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1. Introduction

One of the most urgent challenges specific for biochemical safety of environment, food control, medical diagnostics, is the need for quick analysis of low molecular weight compounds (LMWC) that exhibit strong biological activity, such as potent estrogenic steroids and their derivatives. Small biomolecules (<2 kDa) plays crucial role in a wide range of biochemical processes in the human body. The molecules are typically found in the blood stream and their concentrations presents the main indicative factor in the biochemical functioning of the organism. This is particularly true of a major class of small molecule which belongs to the steroids. Analysis of such hormones has been used for investigating human physiology, monitoring of reproductive cycles in humans and animals, and in the

diagnosis and treatment of hormonal disorders. On the other hand, the interest in the detection of steroids has been stimulated by the fact that most of them have estrogen effects and these compounds are called as environmental endocrine-disrupting chemicals (EDCs) [1]. Although the environmental concentrations of hormones are very low, their adverse effect on reproduction of organisms is significant. The main methods applied for the detection of hormones are the combination of chromatographic and mass-spectroscopic techniques, e.g., GC–MS, LC–MS, HPLC [2]. These techniques exhibited advantages of sensitivity, specificity, and accurate determination. However, because of necessary cleanup procedures, time-consuming processes, and expensive equipment, detection methods with simplicity, specificity, low-cost and less required time has been developed.

There are several commonly used immunoassays involving radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLISA), fluoroimmunoassay (FIA), fluorescence polarization (FP), surface plasmon resonance-based immunoassay (SPR), electrochemiluminescence immunoassay (ECLIA),and lateral flow assay (LFA) [3]. SPR is considered to be the best immunoassays method due to a very sensitive transduction technique where only small changes in refractive index are required to generate a response [4]. SPR biosensor assays have the advantages that information about antibody—antigen binding can be collected in real-time as the sensorgram (plot of response vs. time) and so there is no need to wait for the response development of an entire immunoassay plate to obtain results [5].

Generally speaking in small molecule immunoassays hapten (a small molecule with low molecular-weight) is used in preparing both an antigen to get specific antibodies and a sensitive layer on the sensor surface to form recognition domain. The last is established by grafting hapten through a linker to the surface and such combination is called as the targeted compound (TC). Since hapten has low molecular-weight it does not possess any immunogenicity. Therefore, it is necessary to modify its original structure to introduce accessible functional groups which provide the possibility to bond to both protein carriers and linker. Hapten–carrier conjugate (HCC) can be regarded as the artificial antigen, possessing immunogenicity. So, the same hapten can be applied to perform immunoassays in its two key steps by using TC and HCC. It should be noted that there appear definite difficulties connected with relationship between the core molecule structure and the presence of functional groups which correlates with the affinity of antibody obtained from CC with TC formed on the sensor surface to generate the response in their interaction.

Few attempts to evaluate 17β-Estradiol (E2) level in environmental samples by enzyme immunoassay (EIA, ELISA) kits were rather inconclusive and the ambiguous; overestimated results were explained by a matrix interference associated with coextracted humic substances [6,7]. The commercially available immunological methods have been widely used for measuring of different steroids in biological fluids (plasma, urine, cerebrospinal fluid, saliva etc.) [8] as well as in tissues as brain [9]. However, only using special detection formats, as radioimmunoassay (RIA) [10], fluoroimmunoassay [11,12], luminescence enhanced immunoassay [13–15], provided the requested limit of detection (LOD) for the actual analytical tasks but those techniques utilize special equipment and require high-cost and hazardous labeling of active components. So conventional current analytical methods for their detection and quantitation require laborious extraction and pre-concentration steps prior to analysis as well explore expensive and sophisticated equipment [16,17]. The biosensor approach is reasonable alternative to solve this problem [18,19].

Several bioanalytical approaches were applied for the determination of the steroid level in environmental samples (wastewater, sludge, soil etc.) with the special attention to the analysis of estrogens as important representative of environmental pollutants [20,21]. The analysis is usually carried out by gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS or MS/MS). These techniques allow precise analysis but remain still expensive tools and require a highly skilled staff. Their application

also needs preliminary efficient extraction and adequate cleanup of the sample, often followed by derivatization step for increasing of steroid volatility or ionization efficiency for GC or LC methods, respectively [22].

A rapid and convenient alternative to conventional analytical methods for monitoring of steroids and steroid-specific reactions can be chemical or biochemical sensor based on different physical phenomena, namely surface plasmon resonance (SPR). The main advantage of SPR based techniques is the low-cost analysis in real time, which is especially significant for monitoring human hormonal responses kinetic analysis.

Though the reagents designed for enzyme immunoassay methods are widely used for SPR based approaches also, it is very important to understand that they are not always applicable for this task, because if classical immunoassay methods act in 3D space, surface biosensor nethods are 2D. And here we will consider such an example. We attempted to reveal some ways for the resolution such a problem in presented article. The target E2 molecules were grafted to the gold surface through the linkers containing aliphatic chains with ethylene glycol moiety to form the combination of lipophilic and hydrophilic domains in the surface sensitive layer.

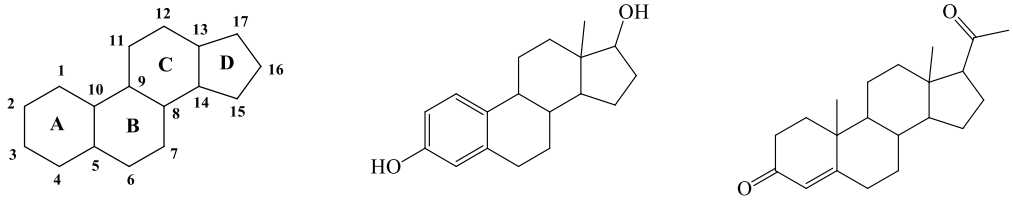
2. Methods

17-beta Estradiol (ESTR-1) a mouse monoclonal antibody raised against 17-beta Estradiol conjugated to BSA was purchased from Santa Cruz Biotechnology, Inc.

The immunospecific detection of E2 was performed with a scanning SPR spectrometer "BioHelper-01" designed in the V.Ye. Lashkaryov Institute of Semiconductor Physics, NAS of Ukraine [23]. The 20 × 20 × 1 mm glass plates, covered with a 50 nm gold layer on a 2 nm chromium adhesive layer, were fixed on a sustaining glass prism with immersion liquid (polyphenyl ether) whose refractive index is close to that of glass (1.61) [24]. To determine the position of the minimum in the SPR curve, the angular dependence was approximated by a third-order polynomial function.

3. Results and Discussion

A steroid is a biologically active organic compound with four rings arranged in a specific molecular configuration which is typically composed of seventeen carbon atoms, bonded in four "fused" rings: three six-member cyclohexane rings (rings A, B and C) and one five-member cyclopentane ring (the D ring) (Figure 1a). Steroids vary by the functional groups attached to this four-ring core and by the oxidation state of the rings, e.g., sex steroid hormones Estradiol, E2 (Figure 1b) and Progesteron, P4 (Figure 1c).



(**a**) (**b**) (**c**)

Figure 1. Steroid molecules. (**a**) steroid configuration. (**b**) Estradiol, E2. (**c**) Progesteron, P4.

As was mentioned in Introduction if there are any active groups in the hapten molecule, such as –COOH, –NH, –OH, they will be coupled with carrier and linker directly. If not, the hapten should be redesigned. The design principles of hapten molecules with respect to low-molecular compounds were proposed in [25]. Antibodies are thought to recognize part of the hapten molecule with specific characteristic. Therefore, the new structure should possess similar physicochemical properties as much as possible. And it is desirable that a linker group is allocated to the opposite position of the most distinct groups of the target molecule. Besides, the modified hapten molecules must have an active

group at the terminal of the linkerE2 is characterized by steroid configuration with A aromatic ring and the presence of two OH- groups in 3 and 17 positions. These functional groups are likely to be the most suitable for their attachment to substrate, i.e., carrier and linker, from chemical point of view. At the same time some physicochemical properties are changed in particular at the expense of proton substitution. It should be emphasized that redesign of E2 changing cyclohexane, cyclopentane or aromatic rings is associated with great consumption in chemicals and time. Therefore, we attempted to attach E2 to linker employing 3 position and carrier employing 3 or 17 position. A sensitive layer on SPR sensor surface contains so called recognition domain (RD) where antibody-antigen (Ab-Ag) interaction occurs. This RD can be in 3D (Ab-Ag binding is both in the volume and on the surface of matrix) or 2D (the binding is only on the surface of one) configurations. Typical procedures using various surface configurations were proposed for E2 detection in biological media used 3D layer based on hydrogel (with a carboxymethylated (CM) dextran coating (Ref. [26] Figure 2a (c = 2,3,4; n = 4), ref. [8,9] Figure 2a (c = 3; n = 1), ref. [27] Figure 2b) or poly(N-isoproprylacrylamide) (PPAA) coating (Ref. [28] Figure 2b) and 2D based on SAM (Ref. [29], Figure 2b).

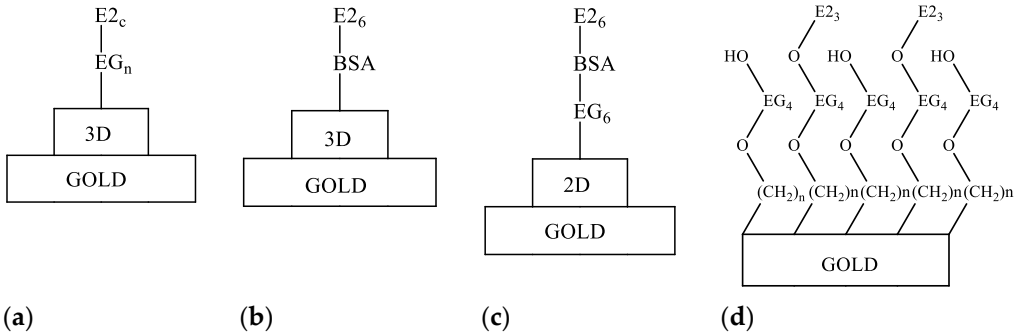


Figure 2. A sensitive layer configuration. *EGn—ethylene glycole moiety with n its units, BSA—bovine serum albumin, E2c—estradiol conjugated to the surface in c position of steroid molecule*.

Since 6-position conjugation involves attachment that does not compromise existing functional groups in E2 an antibody raised to the 6-position of E2 is employed in all procedures. This suggested that the antibody would have a relatively high specificity to both free E2 in biological media and E2 grafted to the surface. As reported in [26] the binding of monoclonal antibody to the surfaces was very little to the 3-E2 conjugated surface and quite strong to the 2-E2 and 4-E2 conjugated one. On the other hand as shown in [27,28] SPR response to the 3-E2 conjugated surface was observed and the surface structure differed from the previous one by decreasing EG moiety. This fact, namely appearance SPR response as described above has important implications in SPR immunoassays because the 3-position is widely used in enzyme and chemiluminescent label conjugations of E2.

Any SPR response is occurred approximately 300 nm out from the sensor surface where the plasmon produces an electronic field. Since SPR chip contains the gold sensing surface covered with a carboxymethylated (CM) dextran coating in thickness of 100 nm it possibly reduces the actual SPR working range. Presumably, if such dextran layer is replaced by other shorter attachment layer, e.g., a SAM on the gold surface, further improvement in SPR response is like to be achieved. Therefore, the need is to form the specific surface layer on the SPR chip providing maximum SPR response. In doing so E2 was grafted to the gold surface through the linker containing aliphatic and EG moieties employing 3 position (Figure 2d).

It was quite surprising to find that the use of commercial monoclonal antibody in SPR immunoassays resulted in no detectable binding to the surface layer formed by such manner.

Obtained results has happened to be different as to ones described in [30,31] possibly in virtue of the difference in hindrance between 2D and 3D sensor matrix configuration.

In fact, 2D configuration with E23-TC cannot provide the binding due to the lipophilic domain. In spite of the fact that the use of polyclonal antibody failed to get SPR response [26], we attempted in doing so. For that reason desired antibodies were obtained from serum of immunized animal by appropriate E23*BSA_CC and E217*BSA_CC antigens by using well-known procedure [32,33]. As found in SPR immunoassays only the polyclonal antibody obtained with E23*BSA_CC expectedly resulted in the successful binding to sensitive sensor surface, but that was not the case in the antibody obtained with E217*BSA_CC.

4. Conclusions

Polyclonal antibody is the earliest antibody to be applied in research. It is simple to prepare and easy to obtain by means of traditional immunization procedures. The drawbacks of polyclonal antibodies are their general lack of specificity and unsuitability for routine use in practice. These shortcomings limit the application of polyclonal antibodies to a certain extent, and our research has been proposed to broaden the limited extent.

We have developed the sensor surface by binding the target molecules (estradiol, E2) directly to the gold surface through the specific linker to provide the closest distance to the surface along with biocompatibility to get maximal response in antibody-antigen interaction. At that the use a commercial monoclonal antibody raised to the 6-position in E2 with BSA resulted in no detection the binding to the sensor surface. Then, the serum from immunized animal was being used. The immunoassays exhibited only the polyclonal antibody raised to the 3-position in E2 successful binding to the sensor surface. So, we have shown that there is possibility for using available functional groups without special redesign in E2 and polyclonal antibodies to perform successive SPR immunoassay

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