engineering proceedings

MDPI

Proceeding Paper

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

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† Presented at the 9th International Electronic Conference on Sensors and Applications, 1–15 November 2022; Available online: https://ecsa-9.sciforum.net/.

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 antibodies 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 which are analyzed. One of the highly sensitive methods in the small molecule determination at low concentration is competitive SPR-based immunoassay. To use the method effectively, a definite sensor surface sensitive to the specific antibody is needed to achieve the lowest value in the 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 achieve 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). We failed to observe the specific binding of the antibody to the sensor surface. 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 immunization by the antigen where BSA- is attached to the 3- or 17-position in E2 (E23*BSA_CC or E217*BSA_CC) instead of 6-position. Only the polyclonal antibody obtained with E23*BSA_CC resulted as expected in its successful binding to the sensitive sensor surface identification of low molecular weight analytes.

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

Citation: Kravchenko, S.; Boltovets, P.; Manoilov, E.; Poix-Shinkaruk, S.; Vellutini, L.; Snopok, B. Success and Failure in Antibody Recognition by Surface-Type Sensors: Essential Prerequisites. *Eng. Proc.* **2022**, *27*, 9. https://doi.org/10.3390/ecsa-9-13221

Academic Editor: Francisco Falcone

Published: 1 November 2022

1. Introduction

One of the most urgent challenges specific to the biochemical safety of the environment, food control, and 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) play crucial roles 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 molecules known as 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 environmental endocrine-disrupting chemicals (EDCs) [1]. Although the environmental concentrations of hormones are very low, their adverse effect on the 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 requiring less time have 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 immunoassay method due to a very sensitive transduction technique where only small changes in the refractive index are required to generate a response [4]. SPR biosensor assays have the advantages of information about antibody–antigen binding can be collected in real-time in 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 produce specific antibodies and a sensitive layer on the sensor surface to form recognition domain. The latter is established by grafting hapten through a linker to the surface and such a combination is known as the targeted compound (TC). Since haptens have low molecular weight, they do not possess any immunogenicity. Therefore, it is necessary to modify their original structure to introduce accessible functional groups which provide the possibility to bond to both protein carriers and linker. The Hapten–carrier conjugate (HCC) can be regarded as the artificial antigen, possessing immunogenicity. Therefore, 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 the relationship between the core molecule structure and the presence of functional groups which correlates with the affinity of the antibody obtained from CC with TC formed on the sensor surface to generate the response in their interaction.

The few attempts to evaluate the 17β-Estradiol (E2) level in environmental samples by enzyme immunoassay (EIA, ELISA) kits were rather inconclusive, and the ambiguous, over-estimated 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 different steroids in biological fluids (plasma, urine, cerebrospinal fluid, saliva, etc.) [8] as well as in tissues such as those in the brain [9]. However, only using special detection formats, such as radioimmunoassay (RIA) [10], fluoroimmunoassay [11,12], and luminescence-enhanced immunoassay [13–15], provided the required limit of detection (LOD) for the actual analytical tasks, but those techniques utilize special equipment and include high cost and hazardous labeling of active components. Thus, 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 a 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 special attention paid to the analysis of estrogens as important representative of environmental pollutants [7,20]. The analysis is usually carried out by gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS or MS/MS). These techniques allow precise analysis but remain expensive tools requiring a highly skilled staff. Their application also needs preliminary efficient extraction and adequate cleanup of the sample, often followed

by a derivatization step for increasing of steroid volatility or ionization efficiency for GC or LC methods, respectively [21].

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 response kinetic analysis.

Though the reagents designed for enzyme immunoassay methods are also widely used for SPR-based approaches, it is very important to understand that they are not always applicable for this task, because if classical immunoassay methods act in a 3D space, surface biosensor methods are 2D. Additionally, we will here consider such an example. We attempt to reveal some methods for the resolution of such a problem in the present 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.

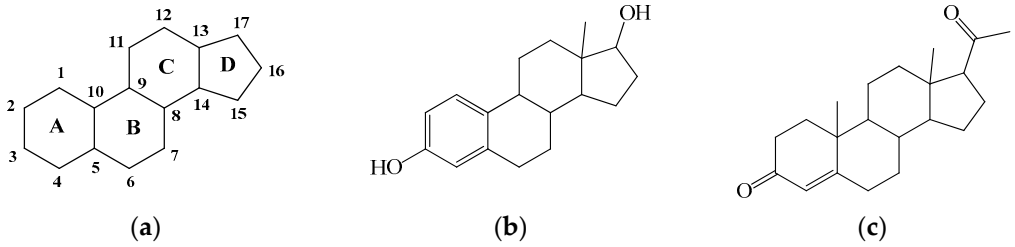
2. Methods

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 [22]. 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) [23]. 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 the Introduction, if there are any active groups in the hapten molecule, such as –COOH, –NH, –OH, they will be coupled with a 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 [24]. 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. Additionally, it is desirable that a linker group is allocated to the opposite position of the most distinct groups of the target molecule. Moreover, the modified hapten molecules must have an active group at the terminal of the linker. E2 molecule possesses steroid configuration with aromatic ring and two OH- groups in the 3 and 17 positions. These functional groups

are likely to be the most suitable for their attachment to the substrate, i.e., carrier and linker, from a 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 in the redesign of E2, changing cyclohexane, cyclopentane or aromatic rings is associated with great consumption of both chemicals and time. Therefore, we attempted to attach E2 to a linker employing the 3 position and a carrier employing the 3 or 17 position. A sensitive layer on the SPR sensor surface contains known as the recognition domain (RD) is 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 a 3D layer based on hydrogel (with a carboxymethylated (CM) dextran coating (ref. [25] Figure 2a (c = 2, 3, 4; n = 4), ref. [8,9] Figure 2a (c = 3; n = 1), ref. [26] Figure 2b) or poly(N-isoproprylacrylamide) (PPAA) coating (ref. [27] Figure 2b) and 2D based on SAM (ref. [28], Figure 2b).

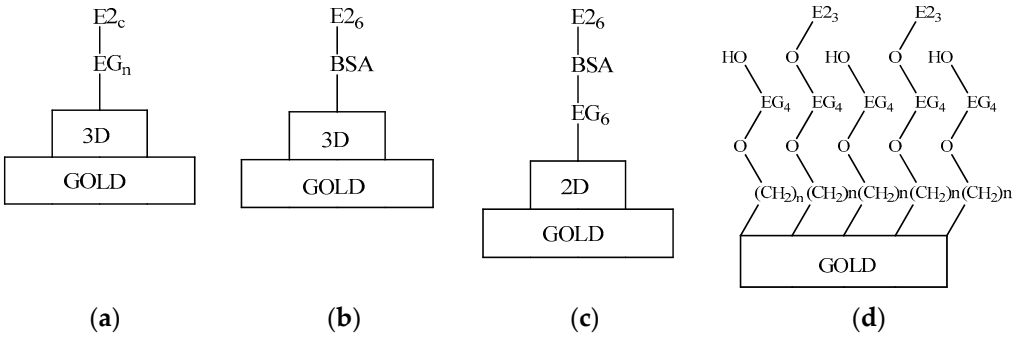


Figure 2. A sensitive layer configuration. EG_n—ethylene glycole moiety with n its units, BSA—bovine serum albumin, $E2_C$—estradiol conjugated to the surface in the c position of the steroid molecule.

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 [25], the binding of the monoclonal antibody to the surfaces was very little with the 3-E2-conjugated surface and quite strong with the 2-E2- and 4-E2-conjugated surfaces. On the other hand, as shown in [26,27], the 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 the appearance of the 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 occurred approximately 300 nm out from the sensor surface where the plasmon produces an electronic field. Since the SPR chip contains the gold sensing surface covered with a carboxymethylated (CM) dextran coating in a thickness of 100 nm, it possibly reduces the actual SPR working range. Presumably, if such a dextran layer is replaced by another shorter attachment layer, e.g., a SAM on the gold surface, further improvement in the SPR response is likely 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 the 3-position (Figure 2d).

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

The obtained results happened to be different to those described in [29,30], possibly due to the difference in hindrance between 2D and 3D sensor matrix configuration. In fact, 2D configuration with 3-E2-TC cannot provide the binding due to the lipophilic

domain. In spite of the fact that the use of polyclonal antibody failed to achieve an SPR response [25], we attempted to do so. For that reason, the desired antibodies were obtained from the serum of an immunized animal by appropriate 3-E2*BSA_CC and 17-E2*BSA_CC antigens by using a well-known procedure [31,32]. As found in SPR immunoassays, only the polyclonal antibody obtained with 3-E2*BSA_CC resulted as expected in the successful binding to sensitive sensor surface, but that was not the case in the antibody obtained with 17-E2*BSA_CC.

4. Conclusions

The 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 achieve maximal response in the antibody–antigen interaction. At that, the use a commercial monoclonal antibody raised to the 6-position in E2 with BSA resulted in no detection of the binding to the sensor surface. Then, the serum from an immunized animal was used. The immunoassays exhibited only the polyclonal antibody raised to the 3-position where E2 successful bound to the sensor surface. Thus, 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.

Author Contributions: Conceptualization, S.K. and B.S.; methodology, S.K.; software, E.M.; validation, P.B., S.K.; formal analysis, B.S.; investigation, S.P.-S.; resources, L.V.; data curation, E.M.; writing—original draft preparation, P.B.; writing—review and editing, S.K.; visualization, E.M.; supervision, B.S.; project administration, S.P.-S.; funding acquisition, L.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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