Tian et al. Chem. Biol. Technol. Agric. (2018) 5:5
https://doi.org/10.1186/s40538-018-0117-0

Chemical and Biological Technologies in Agriculture

REVIEW

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Antibody production and application for immunoassay development of environmental hormones: a review

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Abstract

Environmental hormones, also called environmental endocrine-disrupting chemicals (EDCs), are produced by various human activities and environment pollution, and they are composed of industrial chemicals and environmental pollutants. They play a harmful role in breaking the balance of normal physiological metabolism, causing serious diseases and disrupting reproductive development in wildlife and human beings by inhibiting or imitating the action of normal gonadal hormones. Strict controls of maximum residue levels to some environmental hormones in foodstuff have been set up by many countries. Traditional detection methods, due to their high accuracy and maturity, are used extensively, such as HPLC, HPLC–MS, and GC–MS. However, these methods are time consuming and require extraordinary skills of operators, and sometimes they cannot meet the requirements of field testing. Immunoassay has the advantages of high specificity, sensitivity, simplicity, convenience, and the ability to achieve high throughput, playing an irreplaceable role in the field of rapid detection. This review focuses on the antibody production and the development of immunoassays to detect environmental hormones in food and environment. Therefore, estrogen, progesterone, and testosterone are selected as examples to introduce a series of procedures for the detection of environmental hormones including antigen synthesis, antibody production, and antibody-based detection methods.

Keywords: Environmental hormones, Antigen, Antibody, Immunoassay

Introduction

Environmental hormones, also called environmental endocrine-disrupting chemicals (EDCs), are mainly produced by various human activities and environment pollution. These hormones have similar structures to natural hormones, and they can easily interfere with the normal hormone function of human body [1]. Most of them have estrogen effects. Although the environmental concentrations of hormones are very low, their adverse effect on reproduction of organisms is significant. Moreover, they can also cause strong biological effects: reduce the biological immune ability, damage the biological nervous system, and cause a serious threat to human's health and safety. Some papers have reported that obesity, juvenile delinquency, attention, and out-of-control emotions, and other abnormal conditions are likely to have a relationship with environmental hormones [2–4].

More than 70 kinds of environmental hormones have been confirmed so far, of which only three are heavy metals including Pb, Cd, and Hg, while others are organics. They tend to be existing at the same time, exerting the influence mutually and intricately. In addition, most of the EDCs have a common structure with benzene ring, with a molecular weight in the range of 200–400 [5]. Due to their chemical stability, once they get accumulated in the bodies, it is difficult for them to decompose [6]. EDCs will influence the health of a given organism, but even its offspring. Because of high lipophilicity, EDCs can get easily accumulated in human and animal's breasts, and delivered to the fetus through the placenta, or delivered to the infants through lactation [2, 7]. Typical representatives of EDCs are natural and synthetic hormones, which are always used as regulating metabolism drugs for human or animals, such as estrogens, progestagens, and

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testosterones. Estrogens and progestagens are classes of steroid hormones that are all synthesized in the human body, initially from cholesterol [1]. Depending on the precursor and the enzyme mediating the synthesis, there are four types of natural structures of estrogen: estrone (E1), 17β-estradiol (βE2), 17α-estradiol (αE2), and estriol (E3). Similarly, progestagens have four types involving progesterone, 20α-dihydroprogesterone, mestranol (M), and the synthetic contraceptive 17α-ethinylestradiol (EE). Their chemical structures are depicted in Fig. 1. Besides these, polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAHs), dioxin, nonyl phenol (NP), and bisphenol A also can be classified into environmental hormones [8].

Since the 1990s, there has been an increasing awareness about the presence of widespread hormone residues in environment or in food and about the consequent potential human health hazards [9]. Europe, America, Japan, and other developed countries and some of the united organizations (such as OECD, WHO, and WWF) attach great importance to the study of hormone substances [10]. The hazard is identified, but the risks are not quantified. Therefore, the survey about the hormone pollution and the study of the quantitative

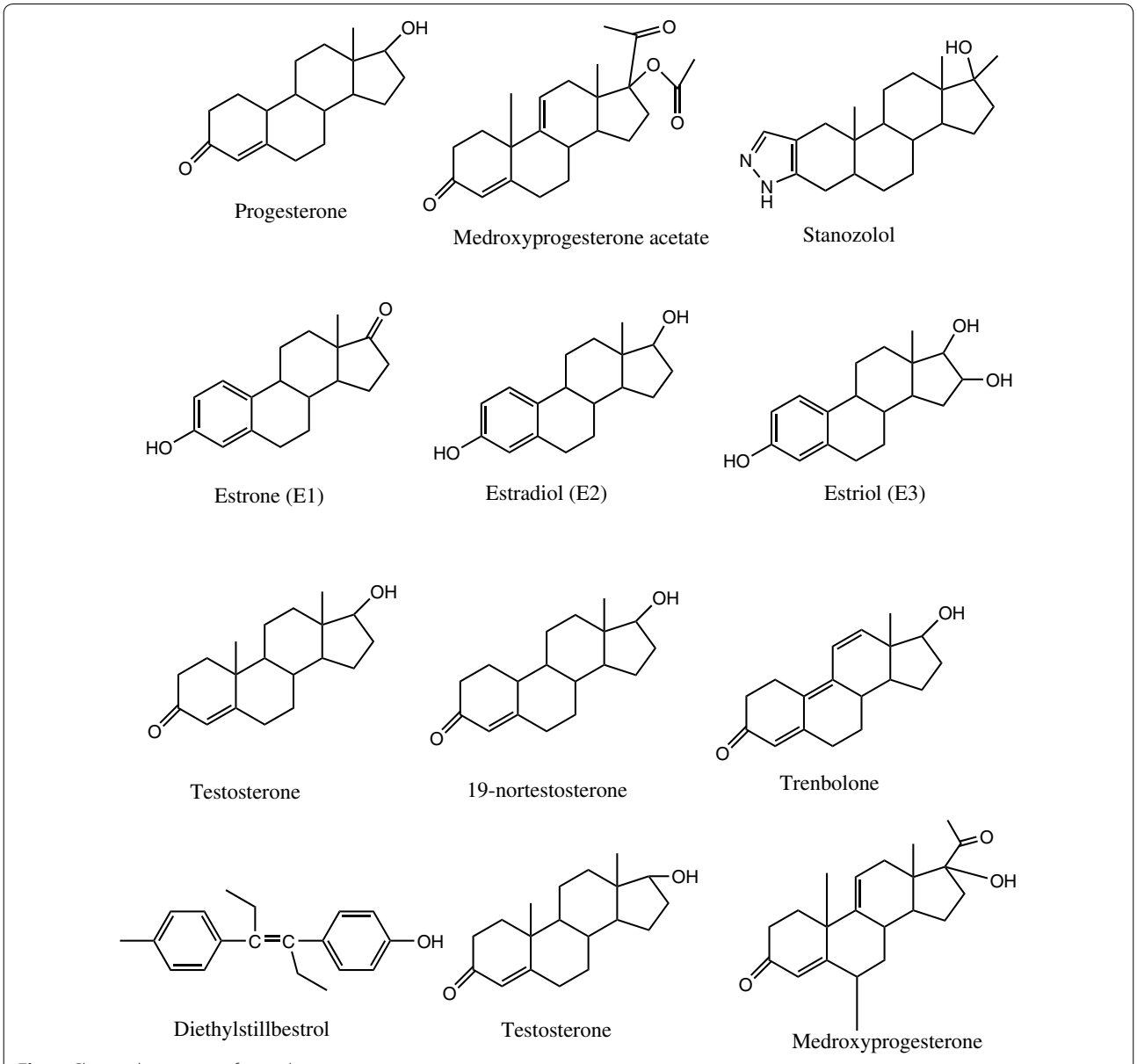


Fig. 1 Chemical structures of some hormones

measurements have become a hot issue of the environmental science in the world. In the recent decades, chromatographic methods applied for the detection of hormone using GC–MS [11], LC–MS [12], HPLC [13], as well as SPE [14] have been reported. These techniques exhibited advantages of sensitivity, specificity, and accurate determination. However, because of necessary cleanup procedures, time-consuming processes, and expensive equipment [15], methods with simplicity, specificity, low-cost, less analytical time, and high-throughput characters should be developed.

Immunoassays are based on the highly specific molecular recognition of antibodies and antigens, which meet the above requirements and facilitate the sensitive detection of biomolecules [16]. 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) [1, 17–20]. Currently, among various techniques, immunoassays are considered one of the most popular protocols for bioanalysis due to the advantage of their ability of measuring trace low-molecular-weight molecules [21]. The basic process is roughly divided into three steps: preparing for the complete antigens, immunizing animals to get antibodies, and establishing immunoassays [22]. Antibody is a key substance in establishing immunological methods, but most of the hormones are low-molecular-weight compounds without immunogenicity. Therefore, the first step is to modify the structure of origin antigens to introduce accessible functional groups, which lead to bonding with protein carriers. These generated macromolecules can be used to immune animals to get high specificity antibodies. Then through the specificity of the reaction between antigen and antibody, the results can be easily detected by prescribed signals, and thereby the goal of monitoring trace hormones can be easily accomplished [23].

In this study, current immunoassays applicable for the determination of trace environmental hormones (estrogens, progestagens, testosterone, etc.) with the emphasis on the preparation of antigen and production of antibody are mainly discussed.

Hapten and antigen

Since most of hormones are small molecular compounds which are called haptens (molecular weight less than 1000), they will not be able to stimulate the animals to produce specific antibodies directly. However, the hapten can be modified to introduce an accessible functional group as well as a connecting arm and then combined with macromolecular carrier. This hapten–carrier conjugate can be regarded as the artificial antigen, possessing immunogenicity.

Hapten: design and synthesis

If there are any active groups in the hapten molecule, such as –COOH, –NH, –OH, they will be coupled with carrier directly. If not, the hapten should be redesigned. Goodrow et al. [24] proposed the design principles of hapten molecules and expressed the common apprehension in producing antibodies with respect to low-molecular compounds. Antibodies are thought to recognize part of the hapten molecule with specific characteristic [25]. 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 [26]. Besides, the modified hapten molecules must have an active group at the terminal of the linker. A glamorous and potential concept was proposed [27] that an immunogenic complex conjugated with more than one hapten molecule against the same carrier protein as an immunogen. It would produce polyclonal antisera that can recognize more than one compound at a time. Watanabe et al. [28] designed the hapten molecule of sex steroid hormones in which the linker group is attached to the D-ring; it would be the best approach to produce desirable antibodies that can distinguish both compounds (17β-estradiol and testosterone).

As shown in Table 1, most reaction regions of the environmental hormone molecules are located at phenolic hydroxyl group and ketone group. Using appropriate reagent and method to bring about a phenol hydroxyl acylation or a keto oximation reaction remains one of the most popular routes for generating carboxyl or amino groups of hapten derivatives. However, Estrone Sulfate (E_1S) can be directly coupled with proteins due to its structure [29].

In addition, the chemical structures of haptens have a great impact on the antibodies' production and their quality. Zhang et al. [30] drew a conclusion that hydrophobic interactions play an important role in the antigen and antibody binding based on quantitative structure–activity relationship (QSAR) techniques, and used comparative molecular field analysis (CoMFA) to predict the cross-reactivity (CR) of the PAHs in ELISA, suggesting that haptens' steric (40.4%) and electrostatic fields (59.6%) make contributions to CR, respectively. Spier et al. [31] also noted that the hydrophobic haptens would limit accessibility of the antibody, because hydrophobicity can cause conformational changes in the protein carrier and result in internalization of the hapten within hydrophobic pockets. Hence, they improved the concentration of DMF to 25% to increase the conjugation efficiency

Table 1 The synthesis of some hormonehapten

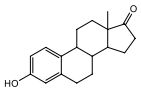
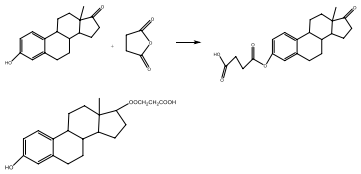
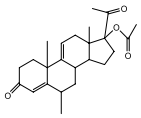
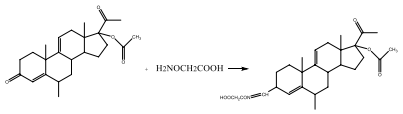
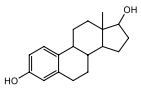
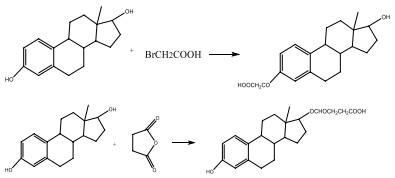
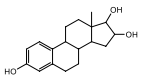
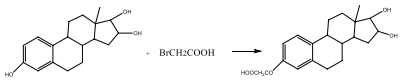
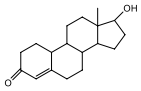
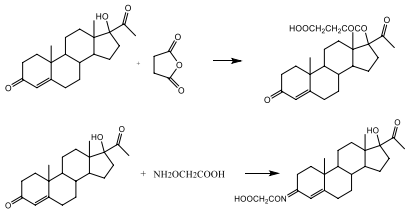
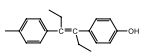
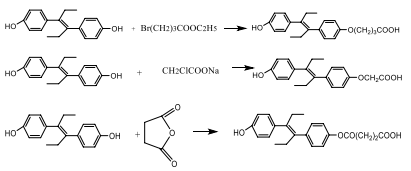
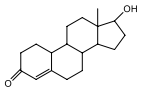
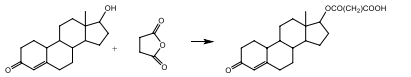
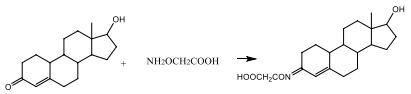
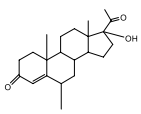
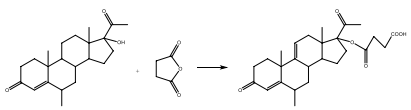
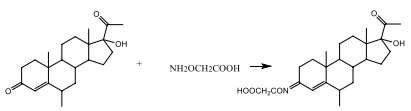
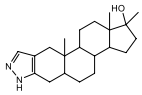
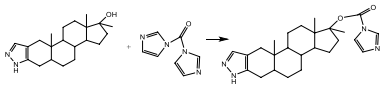
Compound	Structure	Synthetic route	References
Estrone			[73]
Medroxyprogesterone acetate			[99]
Estradiol			[38]
Estriol			[100]
			[101]
Progesterone			[102]
Diethylstilbestrol			[103]
			[104]
19-Nortestosterone			[87]
			[105]
Medroxyprogesterone			[106]
			[107]
			[108]

Table 1 continued

Compound	Structure	Synthetic route	References
Stanozolol			[109]

between haptens and proteins. Zhang et al. [32] believed some specific coupling sites and bonds would affect the production of antibodies. They chose NH_2MQCA as the hapten and succeeded in obtaining highly specific monoclonal antibody with an IC_{50} of 17.7 ng/mL.

Conjugation

To synthesize an artificial complete antigen requires the participation of a carrier. Because of its specificity structure and immunogenicity, carrier can not only increase the relative molecular mass of the hapten, but also induce the body's immune response for both carrier and hapten-carrier [33]. Usually, the primary carrier proteins are bovine serum albumin (BSA), ovalbumin (OVA), keyhole hemocyanin (KLH), human serum albumin (HSA), and rabbit serum albumin (RSA). It is possible to elicit antibodies with affinity to haptens by conjugating to such protein forming an immunogen [34]. Among these, BSA is the most popular one, due to its stability of physical and chemical properties, low price, easy obtainability, and highly successful rate of conjugation.

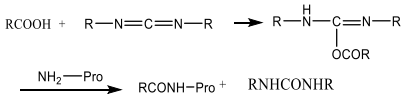
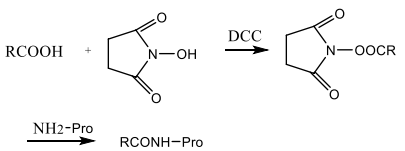
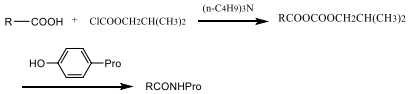
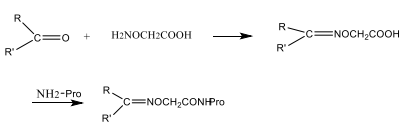
The manner of chemical binding of the hapten to a protein determines the character of the antibody specificity. Different ways of conjunction can be chosen according to different active groups: carbon imide method, mixed anhydride method, active ester method, and *O*-carboxymethyl hydroxylamine method are often used in hormone antigen synthesis. The following table lists the methods of conjugating proteins commonly used in hormones (Table 2).

Verification

Verification is a critical step to prepare a stable artificial antigen through the whole process. After preparation of antigen, the quality and conjunction ratio should be determined. Some correlation between antibody quality and the amount of haptens combining on the carrier protein has been reported. Since the approaches and theories to produce antibody are complex and dynamic, there is no clear statement regarding which incorporated ratio is the best [35]. However, Fodey et al. [36] affirmed that a high combination can lead to high antibody titer but low affinity, so reducing the extent of coupling appropriately can make it easier to produce sensitive antibody. Singh et al. [37] once obtained high-quality antibody with about 15 hapten molecules per carrier protein.

Several different methods can be used to check for the quality of artificial antigen, such as UV spectroscopy, SDS-PAGE, and mass spectrometry. Chemical linkage of haptens to proteins can bring changes in their biological

Table 2 Common methods of coupling hapten derivative with carrier protein

Compound	Active groups	Coupling methods	Reaction principle	References
Estradiol	—COOH	Carbon imide method		[110]
Estradiol	—COOH	Active ester method		[38]
Diethylstilbestrol 19-nortestosterone	—COOH	Mixed anhydride method		[87]
Medroxyprogesterone acetate	C=O	*O*-Carboxymethyl hydroxylamine method		[99]

functionality. Therefore, characteristic peaks would be changed: for example, the UV spectra of BSA, E2, and E2–BSA (Fig. 2, [38]).

As can be seen, the three curves do not coincide, and they have different peaks. The E2–BSA conjugate has a shift compared with the unconjugated E2 hapten (from 272 to 276 nm [38]). The typical UV absorption peak of protein (BSA) was found at 280 nm. These findings confirmed that the antigen was successfully linked to protein.

The conjugation ratio means molecules of protein carrier per molecule of hapten. At present, the methods of measuring the conjugation ratio are mainly composed of ultraviolet spectrophotometry (UV), electrophoresis, etc. UV spectrophotometry is a method to measure the absorbance of the complete antigen and the carrier protein under the characteristic of hapten absorption wavelength. In accordance with the following formula, ratio can be calculated out [37].

$$\text{Conjugation ratio} = \left(K_{\text{complete antigen}} - K_{\text{carrier protein}}\right) / K_{\text{hapten,}}$$

where K represents molar absorption coefficient.

Electrophoretic method is used to obtain the relative molecular mass of the product by comparing with Marker. The difference of the relative molecular weights of the artificial complete antigen and the carrier protein can be calculated as conjugation ratio.

$$\text{Conjugation ratio} = \left(M_{\text{complete antigen}} - M_{\text{carrier protein}}\right) / M_{\text{hapten,}}$$

where M means relative molecular mass.

In addition, the structure of artificial antigen has been changed compared to hapten. Substitution during conjugation can adversely affect the activity and specificity of antibodies produced, and it also can determine the analytical strategies of protein conjugates employed. Structural and immunogenic effects of hapten highlight the effect of new bio-conjugated substance [39]. MALDI-TOF MS and MALDI-TOF/RTOF MS proved to be highly valuable tools for the characterization of protein conjugates at a peptide level. Based on the technology, not only the modification site can be exhibited, but also the subsequence of Lys located on the protein's surface can be assessed [40].

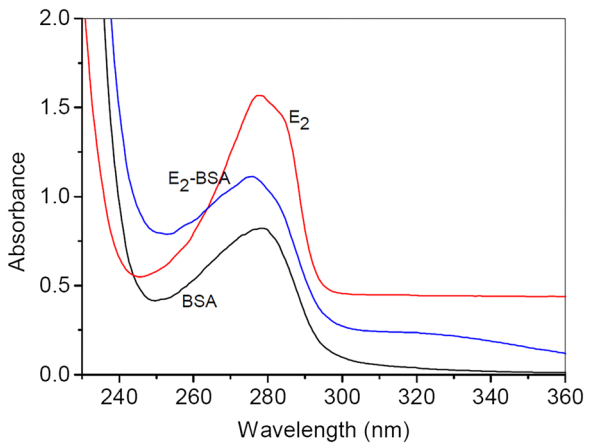


Fig. 2 UV-Vis spectra for the artificial antigens of E2–BSA, BSA, and E2 [38]

Antibody production

The key to establish an immunoassay for small molecular compounds and guarantee precise measurements is to produce antibody with high affinity and high selectivity, especially when the concentrations of compounds are at trace levels [41]. Figure 3 depicts the processes of antigen and antibody production.

Polyclonal antibody (pAb) is the earliest antibody to be applied in research [42]. It is simple to prepare and easy to obtain by means of traditional immunization procedures. The general procedure is to emulsify the purified antigen and adjuvant, and then immunize animals. After triggering an immune reaction in animals, the immune system produces corresponding antibody, which can be isolated from serum. The drawbacks of pAb are their general lack of specificity and unsuitability for routine use in practice. These shortcomings limit the application of pAb to a certain extent. Ryshchanova et al. [43] obtained specific antisera against estradiol by immunizing rabbits on schedule. For obtaining hyperimmune serum, there are specific materials to be applied in the production of various immunobiological samples. Chen et al. [44] developed three immunogens and obtained three pAbs to PCBs, with all of them exhibiting high antibody titers (1:32–64, agar diffusion test). Milan et al. [45] developed ELISAs based on pAbs for coplanar congeners of PCBs, which showed a good stability and high specificity with the working range of 0.1–20 ppb, enough for real sample analyses.

Monoclonal antibody (mAb) has higher specificity and better repeatability than polyclonal antibody. Therefore, it is more suitable for the detection of the substance of a particular structure. By virtue of benefits drawn from their specific, sensitive, rapid, simple, and high-throughput characters, mAbs can be widely used in practical testing. Despite being useful, it also has some disadvantages. The approaches for preparing antibodies are mostly empirical and guided by nonstandardized protocols and individual experience [46]. During the experiment, great care has to be exercised to dilution factors and cultivation conditions such as the use of feeder cells [47].

Moreover, screening for single hybridoma cell clones that produce high-quality antibody is mainly done by ELISA. When using this method to isolate target cells, a large number of cells would be lost. This makes the whole process extremely time consuming and labor intensive [48]. Furthermore, the characteristic of antibody is vulnerable to be impacted by the feature of antigen. For example, the extent and yield of the antigen against protein's chemical conjugation may be very important to mAb. Acevedo et al. [49] proved that combining double-chemically modified carrier proteins with hetero-functional crosslinkers allows preparing tailor-made hapten protein carrier conjugates. Wang et al. [50] prepared a generic mAb and developed a sensitive ic-ELISA for detecting phenothiazines in animal feed. The limit of detections of the method ranged from 1.1 to 15.3 μg/kg. Han et al. [51] produced an mAb with broad specificity to nitroimidazoles, dimetridazole, ipronidazole, ronidazole hydroxydimetridazole, and hydroxyipronidazole, with the IC_{50} values of 4.79, 0.47, 5.97, 23.48, and 15.03 μg/L, respectively. Peng et al. [52] used arsanilic acid as a hapten, and produced the suitable mAb for simultaneously determining organoarsenic compounds including nitarsone, carbarsone, and arsanilic acid in animal tissue and feed.

Genetic engineering antibody, to a large extent, overcomes the shortcomings of traditional antibodies. With the advantages of wide screening, short production cycle, simple operation, and low production cost, the application range of the genetic engineering antibody has been greatly expanded. At present, the antibodies developed by genetic engineering to environmental hormones mainly include chimeric antibody, humanized antibody, antibody Fab, Fv antibody, single chain antibody (ScFv), and so on [53–56]. Xaver et al. [57] built a set of 16 different variable light (V_L) and heavy (V_H) chain combinations in order to investigate the interaction of V_L and V_H. One conclusion may be that V_H was more important for binding affinity, and V_L determined the sensitivity of assay. Keiko et al. [58] explained the principle that the binding affinities of V_H and V_L are usually weak in the absence of the antigen, while it would be strengthened in its presence. Therefore, they developed a novel transcriptional regulation system using separated V_H and V_L. This new method can be used in many fields such as gene therapeutics. Hideyuko et al. [59] designed the scFv antibodies that can be satisfactorily used to monitor PCBs with no obvious difference for mAb.

Application in immunoassays

Antibodies have been widely used as analytical materials in various assays and techniques in the development for food and environmental research and hazard control [60]. Among the various rapid-detection techniques, immunoassay is the most widely used one because of its adaptability of different kinds of samples and its convenience of operation. Highly sensitive detection of EDCs can be performed by the following assays.

Radioimmunoassay (RIA)

Radioimmunoassay (RIA), as the first developed immunoassay in 1959 by Yalow and Berson, has been regarded as a classical immunoassay method for several years [61]. Besides antigen and antibody, this method also requires radioactive labels (e.g., ^{125}I, ^{32}P, and ^{14}C). By measuring the radioactivity, it is possible to determine the concentration of the target antigen in the sample. It is reliable

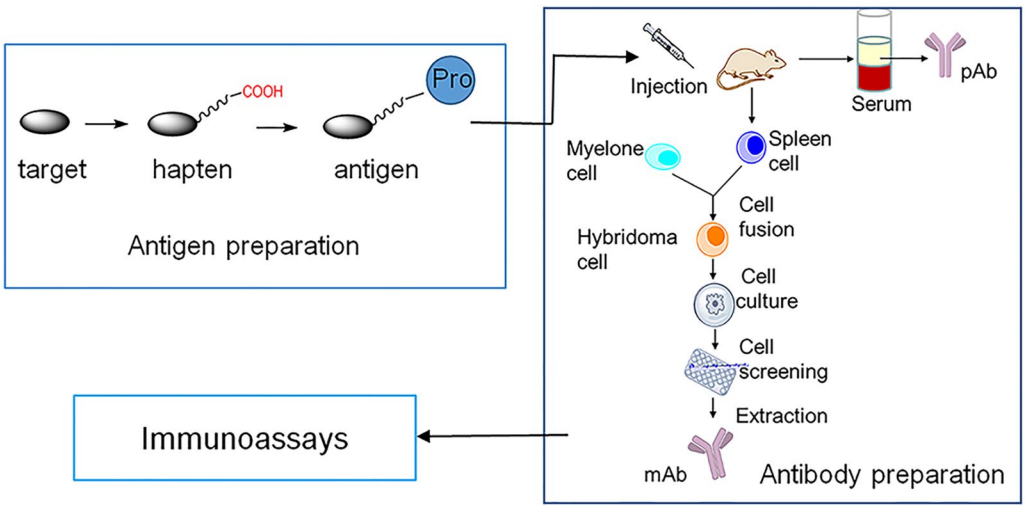


Fig. 3 Processes of antigen and antibody preparation

and sensitive, so RIA has been used to measure melatonin [62], progestagens [63], and estriol [64, 65]. Naicker et al. [66] implemented an application of radio-immunoassays to assess the fate of estrogens (estrone, estradiol, and estriol) in wastewater effluents of South Africa. Results indicate that RIA can be employed as a fast tool for detecting natural estrogens in water. However, the use of radiolabel restricts the application of practical detection, because of its harmful effects to human health.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a simple and rapid method based on antigen and antibody recognition and is highly specific for particular residues [67, 68]. There are various forms of ELISA methods. Direct ELISA is the simplest one, and other methods are indirect ELISA, sandwich ELISA, competitive ELISA, and direct competitive ELISA [69, 70].

Uraipong et al. [71] employed the direct ELISA method to measure testosterone in water, and the results showed that IC_{20} (as the limit of detection) value is 0.05 ± 0.01 μg/L, and IC_{50} value is 0.33 ± 0.18 μg/L. Besides, the assay rarely showed cross-reactivity against other steroidal compounds, but was affected by environmental matrices to some degree. Moreover, Chang et al. [72] also screened testosterone by immunoassay. To their difference, they added a simple step of sample pretreatment to solve the matrices matters, and this assay allowed the detection of 0.078–68.4 ng/mL, with an IC_{50} value of 1.8 ng/mL. The limit of detection (LOD) of the assay was 0.032 ng/mL. So it is obvious that analyses require highly sensitive methods combined with efficient pretreatment techniques. Wang et al. [73] established a SPE-ELISA method to detect estrone in aquatic samples using the multiwall carbon nanotube as a new sorbent. Much attention has been paid to change the way of substrate color system, or to expand the detection signal to change the ELISA detection limit. Part of the studies had obtained ideal result by biotin-avidin system. The sensitivity of the ELISA can be improved by bounding biotin to antigen (as labeled-antigen) to amplify detection signals. Haraya et al. [74] confirmed that as the number of lysine residues increasing the assay would be more sensitive. When they used five lysine residues, the detection limit was 2.36 pg/assay.

Chemiluminescence immunoassay(CLEIA)

Chemiluminescence immunoassay which is based upon secondary antibodies format possesses higher sensitivity, selectivity, and reproducibility than ELISA. It uses the horseradish peroxidase (HRP)-luminol-H_2O_2 chemiluminescent system as the detection system [75]. CLEIA has been successfully used for detecting 17β-estradiol in wastewater; detection limit was 1.5 pg/mL, and linear range was 2.5–1600 pg/mL [76]. This method also exhibited great potential for detection of estradiol in serum [27].

Because the concentration of hormone in environment sometimes is extremely low, it is necessary to purify and concentrate the samples before measurement. This is a valid method to cope with sensitivity issue. Magnetic nanoparticles have been developed rapidly in recent years as a way of separation and enrichment. Many scholars innovatively combined the frontier technology with chemiluminescence method and established an ultrasensitive detection method. Zhao et al. [77] developed a micro-plate magnetic CLEIA (MMCLEIA) to detect 17β-estradiol in water samples. The method showed high sensitivity and specific recognition of estrogen, with the working range of 10–3000 pg/mL and the test limit of 5.4 pg/mL. It is efficient to be applied to the preliminary detection of the seawater. Xin TB et al. [78] used magnetic particles (MPs) as the separation tools and used C18 cartridges solid-phase extraction for the removal of matrix effects. They proposed a new CLEIA for estradiol in water samples with detection limit of 2.0 pg/mL, linear range of 20–1200 pg/mL, and total assay time of 45 min. Xin et al. [65] also used this method to determine E2 in human sera successfully, and the result showed a good correlation compared with the commercial RIA kit. CLEIA can be associated with a variety of technologies to form a novel detection means. Yong et al. [79] combined microchip electrophoresis and on-line magnetic separation with chemiluminescence detection, which can be used to detect simultaneously multiple analytes (i.e., testosterone, hydrocortisone, digoxin, estriol, and corticosterone).

Fluorescence immunoassay (FIA)

Combined the immunological method and fluorescence labeling technology, fluorescence immunoassay (FIA) is generated with high specificity and good practicability.

Fluorescence polarization immunoassay (FPIA) is a kind of quantitative FIA [80]. The method is suitable for detecting small and medium moleculars, such as drugs and hormones. Trapiella-Alfonso et al. [81] applied one-step FPIA for progesterone, the whole process spent only 7 min for 10 samples. Besides, Tan et al. [82] and Zhang et al. [83] used this technology to detecting the progesterone in serum and estrone in environment or food, respectively.

Time-resolved fluorescence immunoassay (TRFIA) is developed on the basis of the fluorescence analysis. It is a special kind of FIA using lanthanide marked antigen or antibody. According to the luminescence characteristics of chelate, fluorescence is measured by time-resolved

technique, which can effectively eliminate the interference of nonspecific fluorescence, then greatly improve the sensitivity of analysis. A direct competitive inhibition TRFIA was developed by Yong-ping Tang to detect unconjugated estriol in serum [84]. They employed estriol-3-carboxymethyl ether-BSA as labels and Eu^{3+} as the probe for signal detection. The limit of detection was 0.35 nmol/L.

Lateral flow assay (LFA)

Lateral flow assay (LFA) combines unique advantages of ELISA, monoclonal antibody and immune marked technology. Labels used in LFA play an important role in determining sensitivity of analysis. Colloidal gold immunoassay with colloidal gold as markers use specific antigen–antibody reaction to amplify detection signals, and the result can be observed directly. Rapidity, low cost, high specificity, good sensitivity, and portability of the device are unique advantages related to LFA strips [85, 86]. Jiang et al. [87] presented a colloidal gold-based immune chromatographic assay to detect 19-nortestosterone (NT); the test limit is 1 ng/mL in PBS. NT in beef and pork can be detected at 2 and 2 μg/kg, respectively. Jiang et al. [88] established a lateral flow colloidal gold immunoassay strip to determine estradiol in milk, with screening a panel of monoclonal antibodies specifically. The test limit was 37.14 pg/mL, and the detection range was 180.42 ± 3.17 pg/mL. The recovery range was 86.7–93.5%. Deserved to be mentioned, the test only required less than 8 min. Peng et al. [89] developed a gold-based immunochromatographic assay for 3-methyl-quinoxaline-2-carboxylic acid from porcine muscle and the liver, the LOD were 10 and 50 μg/kg.

Other assays

Continuous research offers additional methodologies, which have some difference from the traditional assays, for the determination of several small weigh compounds. In recent years, more and more new methods based on antibody or combined with antibody have been excavated, such as immunosensor [1, 90–92], molecularly imprinted technique [93, 94], surface plasma resonance sensing [95], and so on. Maybe, the new combination is prone to bring high sensitivity for the assay.

Conclusion

Antibody-based assays have attracted great interest in food quality, environment safety, and clinical diagnoses because of their sensitivity, accuracy, precision, and no complex pre-extraction. A several forms of detection methods have been developed, especially for the common progesterone, 19-nortestosterone, E2, and E3. However, there are still some deficiencies and lack of potential in these techniques. The progress of development of immunoassays is still limited by the insufficient availability of antibodies with the required affinities and specificities for given applications. With the aggravation of environmental pollution and people's strong pursuit of maintaining health, the pressure coming from various aspects to the detection method has put forward the need to fulfill higher requirements. For PAHs, in spite of the different qualities of antibodies resulting in high- and low-detection levels, most of the immune methods can detect PAHs at the concentration of 200 μg/kg in food [96]. Further, for estrogens, their concentrations were measured at nanogram levels frequently. Regardless of large-scale precision instruments or rapid immunoassays, they are generally in the ng/g or ng/mL range. In real samples, matrix effect is a critical point that must be cleaned up [97]. The current trend is simplification of laboratory works, reduction in overall cost per test, improvement of detection sensitivity, and portability for field test. Thereby, efforts are still to be made.

To achieve these innovative strategies based on antigen- and antibody-specific recognition, antibodies for both common and new contaminants and pollutants, and even generic antibodies should be developed first. PAb is produced by traditional immunization procedures. A certain drawback lies in the fact that it is impossible to produce exactly the same specific antibodies, even in the same batch of animals. While mAb is still the most widely applied one, it is also limited by its low predictability. In many cases, the design of hapten is empirical and blind. The steps of cell fusion and monoclonal antibody screen are tedious. However, antibody engineering and production of recombinant antibodies are a very promising field both for research and application. The gene technologies focus on mutagenesis to that part of gene which determines the structure and affinity of antigen-binding site.

Beyond that, a novel method, simultaneous immunoassay technique, has also gained much attention, which can test two or more analytes at the same time in a single attempt. This technology achieves a major breakthrough in the detection of organophosphorus pesticides by designing systemic of haptens, screening of broad spectrum antibodies, and optimizing the experimental conditions. It also provides a reference for the simultaneous detection of hormones in food and environment. Although hormones are extremely similar in structures and they have a lot of metabolites, which create a great amount of difficulties in the preparation of antibodies, it has been reported that the proposed method for EH and TH can realize testing of E and testosterone at the same time [98]. Unfortunately, this kind of report is still rare.

In addition, most attention has to be paid on combining multiple techniques and improving signal-detecting

system. Just like colloidal gold, quantum dot or magnetic nanoparticles can be utilized to improve sensitivity by combining with ELISA, LFA, CLEIA, and so on. The use of new labels is beneficial for more dilution of the sample with less matrix interference or for samples with lower concentrations. Therefore, the research on preprocessing technology and the signal-amplification system will be a hot topic in the future.

For commercial applications, it is important to improve the reproducibility of the tests. However, it is uncertain that the antibodies used in the commercial market would have the same quality and quantity over a long period. Thus, researchers should facilitate the application of their technologies on a larger scale focusing on optimizing the experimental conditions and improving the precision.

Authors' contributions
The author WT contributed to the writing of the manuscript. The author LW provided the charts and tables, and adjusted the whole manuscript's format. The author HL, YS and ZX reviewed and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This study was supported by the Natural Science Foundation of China (U1301214).

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data and material are fully available without restriction.

Consent for publication
All the authors agree to publish.

Ethics approval and consent to participate
Not applicable.

Funding
This study was funded by the Natural Science Foundation of China (U1301214).

Publisher's Note

Received: 1 November 2017 Accepted: 13 January 2018
Published online: 05 March 2018

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