Fluorescence site-encoded DNA addressable hapten microarray for anabolic androgenic steroids

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We report a new strategy for immunochemical screening of small organic molecules based on the use of a hapten microarray. Using DNA-directed immobilization strategies, we have been able to convert a DNA chip into a hapten microarray by taking advantage of all the benefits of the structural and electrostatic homogeneous properties of DNA. The hapten microarray uses hapten-oligonucleotide probes instead of proteins, avoiding the limitations of preparing stochiometrically-defined protein-oligonucleotide bioconjugates.

As proof of concept, we show here the development of a microarray for analysis of anabolic androgenic steroids. The microchip is able to detect several illegal substances with sufficient detectability to be used as a screening method, according to the regulations of the World Anti-Doping Agency for sport and the European Commision for food safety.

The results that we show corroborate the universal possibilities of the DNA chip, and, in this case, they open the way to develop hapten microarrays for the immunochemical analysis of small organic molecules.

Keywords: Anti-doping; DNA chip; DNA-directed immobilization (DDI); Fluorescence; Food safety; Hapten microarray; Immunochemical screening; Proof of concept; Small organic molecule; Steroid

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1. Introduction

Microarrays are defined as bidimensional molecular receptor arrays that allow simultaneous automation of assays to show recognition of specific biological markers. The use of this technology permits simultaneous detection of a large number of substances and is ideal for high-throughput analysis. Multiplexation, miniaturization and detectability are goals pursued by many researchers. Improvement in analytical efficiency and the consequent reduction of time and cost of analysis are some of the advantages of multiplexing. Technological advances in micro(nano)biotechnology have provided the possibility to develop multiplexing bioassays through two strategies:

a) achieving spatial multiplexing (planar microarrays); or,
b) using multiple quantitation tags (non-planar microarrays).

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Planar microarrays, in which the identity of the target analyte is encoded by its location with a secondary reporter (i.e. fluorescent dye) providing quantitative data, comprise the most widely used approach. Relevant growing areas such as proteomics, genomics and pharmacogenomics are possible thanks to the availability of protein and DNA microarrays based on this principle. More information can be found in recent reviews and books dealing with this topic [1–4]. The technology behind the well-known Affymetrix GeneChip started in the late 1980s as result of advances in semiconductor-manufacturing techniques and combinatorial chemistry [5]. Today, these arrays are considered standard tools for analyzing complex genetic information. The main users in analyzing the relationship between genes and human health are companies working in pharmaceuticals, biotechnology, agrochemicals and diagnostics, in addition to academic, government and other non-profit research institutes.

Besides genomics, protein microarrays represent a big challenge in diagnostics. For example, microarrays can be very useful for drug development, allowing the analysis of the interactions of chemicals or important pharmacological targets with proteins. Protein arrays may have a strong impact on interaction-screening assays to determine the mode of action of therapeutically interesting drugs. However, protein-microarray technology is not as straightforward as DNA technology, due to the molecular variability and complex nature of proteins (e.g., different hydrophobicities, acidic or basic characters, and functionality). Unlike nucleic acids, which are relatively homogeneous in terms of structural and electrostatic properties, proteins can be extremely diverse regarding chemical structure and biological properties. Preventing protein denaturation and maintaining structural conformations are key issues in microarray technology (see [6] for a recent review on immobilization strategies). This is the reason for DNA microarrays being much more standardized.

An alternative to circumvent some of the limitations of protein-microarray technology is to use oligonucleotide probes with well-known sequences and their subsequent hybridization with their complementary oligonucleotides previously immobilized on the surface. This strategy, known as DNA-directed immobilization (DDI), has been used for spatial assembly of mixtures of molecular components (e.g., nanoparticles, proteins and polypeptides [7–13]). It not only provides greater immobilization efficiency than conventional adsorption techniques [8], but also allows reversible immobilization of biomolecules, allowing development of reusable microarrays and biosensor chips. In combination with antibodies, DDI may also provide a useful strategy to construct antibody microarrays, expanding the number of substances that can be analyzed, considering the wide variety of selectivities provided by the antibodies and their exceptional features as natural bioreceptors [14]. However, DDI strategies to immobilize proteins would ideally require a 1:1 molar ratio for oligonucleotide:protein plus careful control of the site at which the oligonucleotide is attached. These are very difficult to achieve by the usual chemical-bioconjugation procedures. In this context, site-specific labeling strategies of recombinant proteins with DNA oligonucleotides and peptide nucleic acid (PNA) strands have been reported [6,15–19].

Small organic molecules are frequently analyzed under competitive immunochemical configurations on indirect formats by immobilizing a haptenized protein on the surface of a microplate, a microchip or a transducer. Thus, using this format, protein microarrays have been reported for the immunochemical analysis of steroids in urine samples [20,21] or antibiotics [22,23]. However, these approaches also suffer from the drawbacks and the limitations of protein arrays mentioned above. In order to circumvent these drawbacks, we report an alternative strategy for small organic molecules, by combining DDI with haptens, instead of antibodies or haptenized proteins. Hapten microarrays could be excellent screening platforms for the immunochemical analyses of small organic molecules (e.g., metabolites and drugs). Hapten-oligonucleotide probes can be prepared with well-defined stochiometry and chemical structure to avoid the problems associated with immobilization of proteins.

There are no records of reports on applying the DDI approach to the multiplexed construction of hapten microarrays. Thus, in an attempt to exploit the strengths of DNA hybridization and microarray technology, the aim of this work was to develop a universal microarray platform for small organic molecule analysis, using DDI in combination with hapten-oligonucleotide conjugates and antibodies. Control of the stochiometry and the conjugation site of these conjugates could be straightforward in comparison to those of DNA-protein constructs.

As proof of concept, we focused on the development of a microarray for determination of anabolic androgenic steroids (AASs). These substances are used illegally to improve athletic performance in sports and to increase meat production in the agro-alimentary field. AASs are completely prohibited by the World Anti-Doping Agency (WADA) [24,31] and the European Community (EC) through Directive 96/23/EC and Directive 2003/74/EC [25,26]. In order to control the use of these substances, WADA and EC have established regulations and requirements, which call for screening methods able to meet the requirements in order to ensure fair competition at athletic events and to improve the health status of the population, respectively.

2. Experimental

2.1. Reagents and immunoreagents

The immunoreagents for stanozolol (St), boldenone (B) and tetrahydrogestrinone (THG) used in this study have