Short communication

Quantitative detection of doping substances by a localised surface plasmon sensor

Mark P. Kreuzer [a], Romain Quidant [b,*],
Gonçal Badenes [b], M.-Pilar Marco [a,*]

[a] *Department of Biological Organic Chemistry IIQAB-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain*
[b] *ICFO, Institut de Ciències Fotòniques, Jordi Girona 29, Nexus II, 08034 Barcelona, Spain*

Abstract

Within this communication, consistent evidence of a quantitative biosensing principle for steroidal residue analysis is presented. Our approach uses a simple method for the quantitative determination of an anabolic agent called stanozolol (Sz). Sz (Mw 328) is widely used in sports, horse racing and as a growth promoter in animals for human consumption. Through the use of localised surface plasmons (LSPs), sustained by three-dimensional noble metal nano-structures, we have developed a highly specific, label-less immunosensor for the detection of this small organic molecule to low levels (nM range). A main practical advantage over conventional flat extended film surface plasmon resonance (SPR) systems is the simplicity of the optical configuration, since there is no need for cumbersome total internal reflection illumination, thus making integration easier. In addition, the active area of the LSP-based sensor is smaller, decreasing the minimum detectable number of molecules involved in the binding event. Assay times are short and the set-up is comprised of relatively cheap instrumentation. Detection levels found here are comparable with SPR, even at this early stage of development and with further modifications, we envisage sensing down to pM (10^{-12}) levels.

Keywords: Optical sensing; Localised surface plasmons; Immunosensor; Stanozolol

1. Introduction

Among their multiple potential applications (Barnes et al., 2003), noble metal nano-structures sustaining localised surface plasmons (LSPs) have recently shown to be well suited for sensing purposes (Okamoto et al., 2000; Raschke et al., 2003; McFarland and Van Duyne, 2003). This method of sensing is advantageous when compared to conventional flat extended film surface plasmon resonance (SPR) systems (Xu and Käll, 2002; Xinglong et al., 2003; D'Orazio, 2003) in the simplicity of the system and in the minimum detectable number of molecules involved in the binding event. Whilst LSP sensors have shown current prominence, with recent demon-

strations of single particle sensing (Raschke et al., 2003; McFarland and Van Duyne, 2003) their real-life application has not been greatly exploited. In fact, of the reported systems in the literature, emphasis has been on model systems like biotin/streptavidin and with large proteins (IgGs and albumins) (Raschke et al., 2003; Lyon et al., 1999; Frederix et al., 2003; Haes and Van Duyne, 2002). Label-less detection of binding events occurring with these systems take advantage of their exceptionally high affinities (5 or 6 orders of magnitude higher for biotin/streptavidin over other biomolecules) and of the strong modification in the shallow dielectric environment of the particle, due to their large size. In this communication, the analytical potential of LSPs for the analysis of small organic molecules (500× smaller than model molecules) is shown by using immunoreagents specifically developed against an anabolic agent such as stanozolol

* Corresponding author. Tel.: +34 93 400 6100; fax: +34 93 204 5904.
E-mail address: mpkqob@iiqab.csic.es (R. Quidant, M.-P. Marco).

(Sz, Mw 328), widely used in sports, horse racing and as a growth promoter in animals for human consumption (Teale et al., 1991).

The use of androgenic steroids with anabolic effect to improve athletic performance has been banned by the International Olympic Committee (IOC) and other international sports associations (IAAF) since 1974. The IOC mainly uses for doping inspections chromatographic methods reaching the minimum performance required levels (MRPL) of Sz that is $2 \mu g L^{-1}$ (Merode, 1998; Schaenzer and Donike, 1993; Ferchaud et al., 1997). However, these sophisticated methods need expensive instrumentation, specialized personnel and complex and tedious sample preparation procedures that decrease analytical efficiency (De Brabander et al., 1998; Huenerbein et al., 2003). In this context, the results reported here suggest that LSP biosensing principle could offer alternative advantages for doping control. Immunosensors incorporating labels have largely been unsuccessful, due to loss in label activity or stability, and thus methods such as piezo-electricity, SPR and impedance spectroscopy offer alternative sensing methods (Mascini et al., 1998; Minunni et al., 2005). The characteristic physico-chemical changes associated to the recognition event have allowed the exclusion of these labels (enzymes, fluorescent markers, tags), thereby making the overall system more simplistic, robust and durable.

2. Materials and methods

2.1. General section

BK7 glass was purchased from Marienfield (Lauda-Koenigshofen, Germany). Gold colloid came from BBInternational (Cardiff, UK). The Ellman reagent (5,5-dithio-bis-[2-nitrobenzoic acid]) was from Pierce Biotech (Il, USA). Mercaptopropyltrimethoxysilane (MPTMS), anti-IgG-HRP (anti-rabbit IgG coupled to horseradish peroxidase), 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma–Aldrich (Madrid, Spain). Stanozolol from Sequoia Research Products (Oxford, UK). All other reagents were of the highest grade available and when required, solvents were distilled prior to use. Bovine serum albumin derivative of Sz (Sz-BSA) and Sz antiserum were a kind gift from Mr. J. Pablo Salvador. Phosphate buffered saline (PBS) used on the immunochemical methods was 10 mM with 0.001% Tween-20 at pH 7.4. Substrate buffer was 0.04 M citrate buffer, pH 5.5 containing 0.6% TMB and 1% H_2O_2. Absorbance measurements on the visible region were performed using a Spectramax microplate reader (Molecular Devices).

2.2. Optical set-up

The extinction spectra of the gold colloids were measured by conventional dark-field spectroscopy. The sample illumination was performed from a halogen lamp (100 W) using an immersion-oil dark-field condenser with high numerical aperture (NA = 1.4 − 1.2). The light scattered by the particles was collected by a long working distance objective lens (×50, NA = 0.5) and sent toward both a microspectrometer and a video camera. An adjustable diaphragm located at the microscope output was used to reduce the detection area to approximately 100 um × 100 um. Considering the bandwidth of the particles extinction spectra, the set-up allows for the determination of the central LSP wavelength with a resolution of approximately 0.5 nm.

2.3. Chemical modification of glass

Corning glass was sonicated for 1 min in ethanol (EtOH) before drying under nitrogen (N_2). Glass slide samples were then activated for 1 h at room temperature (RT) using 2 N sodium hydroxide, 50% (v/v) EtOH according to Tätte et al. (2003). After washing excessively with water and drying under N_2, mercaptopropyltrimethoxysilane in dry EtOH was applied to one side of the glass for 2 min under inert atmosphere (Argon). After washing excessively with dry EtOH and subsequent drying under N_2, gold colloid was added for 20 min at RT. Samples were then washed in MilliQ water and dried under a N_2 stream. These samples were used immediately as described in the biological section.

2.4. Quantitation of sulfhydryl groups bound to glass

Briefly, a calibration curve was prepared ($n = 8$) of cysteine hydrochloride monohydrate between 0 and 200 μM using a solution of the Ellman reagent in reaction buffer (0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA; $4 g L^{-1}$). Once the glass sample was prepared as per Section 2.3, it was immersed in the reaction buffer and the absorbance measured at 412 nm. The concentration of sulfhydryl groups was quantitated using the calibration curve.

2.5. Biological modification of gold colloid

Gold samples were first functionalized with Sz-BSA (10 μg/mL in PBS) for 30 min at RT. After washing with MilliQ water, the scattering spectrum was measured and this constituted the reference spectrum.

2.6. Stanozolol measurements by LPR

Different concentrations of Sz were mixed with the Sz antiserum appropriately diluted and incubated for 30 min in 20 μL drops. After washing, scattering curves were recorded for each Sz concentration and compared with the reference spectrum.

2.7. Colorimetric assessment

A HRP labelled anti-rabbit IgG (1:6000 in PBS) was used to assess antibody binding to the Sz-BSA derivatized gold

particles, followed by the addition of the TMB substrate solution. The colour evolved was measured at 450 nm.

3. Results and discussion

3.1. Quantitation of sulfhydryl groups bound to glass

Using the standard Ellman test, MPTMS was found to cover the surface of the glass at a concentration of 1.846×10^{-11} moles/mm^2 from the calibration curve ($y = 0.0109x - 0.0151$; $R^2 = 0.9992$). MPTMS is prone to forming disulphide bonds (–S–S–) over time, and therefore it is necessary to control carefully the conditions by removing residual oxygen and light from the modification step. Otherwise, this reagent becomes less reactive for subsequent binding of the gold colloid. When careful control of these conditions was kept, a 20% increase in sulfhydryl density was recorded/mm^2.

3.2. Density of gold colloid

This label-less LSP sensor consists of isolated gold nanoparticles ($\varnothing = 100$ nm) randomly arranged and chemically immobilized on top of a glass substrate through the use of the silylating agent, MPTMS. The absence of an adhesion absorbing metallic layer (Ti or Cr) prevents attenuation and broadening the LSP resonance of each particle (Frederix et al., 2003). The chemical conditions chosen allow for a consistent control of the density of gold dots. The conditions have been optimized to avoid clustering of the gold particles (Fig. 1a), thereby limiting the spectral broadening of the ensemble's resonance (Fig. 1c). Indeed, the bandwidth of the resonance determines the resolution on the reading of the central peak wavelength, and thus the sensitivity of the sensor.

3.3. Optimisation of localised plasmon sensing

To achieve the sensing format, immobilized gold nanoparticles were coated with a Sz-BSA conjugate. Binding of the anti-Sz antibody was observed by a shift of the resonance wavelength, measured after just 30 min incubation and 15 s

recording the signal. One-dimensional dose–response experiments for both, the coating antigen, Sz-BSA and the binding antiserum, were used to select optimum concentrations for both immunoreagents. For Sz-BSA, a concentration reaching saturation of the gold colloids was used whilst binding of antiserum to this concentration of coating antigen, occurred at antiserum dilutions between 1/100 and 1/10. A maximum resonance change ($\Delta R_{\max}$) of 13 nm was noted under these conditions. No significant increase was observed if higher antiserum concentration was used. This result was also confirmed by colorimetric means (data not shown).

3.4. Specificity of the sensor

Non-specific binding has been a problem for systems of a similar nature (Haes et al., 2004). As a means to investigate the specificity of the sensor, a control was prepared by coating the gold nanoparticles with an atrazine-BSA derivative (Fig. 1b, part 2). Atrazine (Mw 216) is a pesticide, differing chemically and structurally from Sz. Therefore, the Sz antibody would see a similar surface topography as for the Sz-BSA coated gold nanoparticles case, but molecular recognition of the atrazine or the BSA should not occur. As it is shown in Fig. 1c, when this sensor was made in contact with the Sz antibody, a shift of only 0.6 nm was observed in the resonance peak (ca. 4% in repetitive experiments).This value is on the limit of the resolution of the sensor system. This result indicates that the sensor response is solely due to the specific binding of the antibody to Sz-BSA.

3.5. Localised plasmon sensing of stanozolol

Results from the one-dimensional experiments were used to determine Sz under a competitive configuration. As can be seen from Fig. 2, a clear Sz dose-dependent decrease in the resonance shift was observed. The dynamic response of the unoptimized biosensor ranged between 10 and 1000 nM Sz. Below concentrations of 10 nM, the system approaches the zero control (no analyte) and above 1000 nM, there is no distinction between the background control (no antibody).
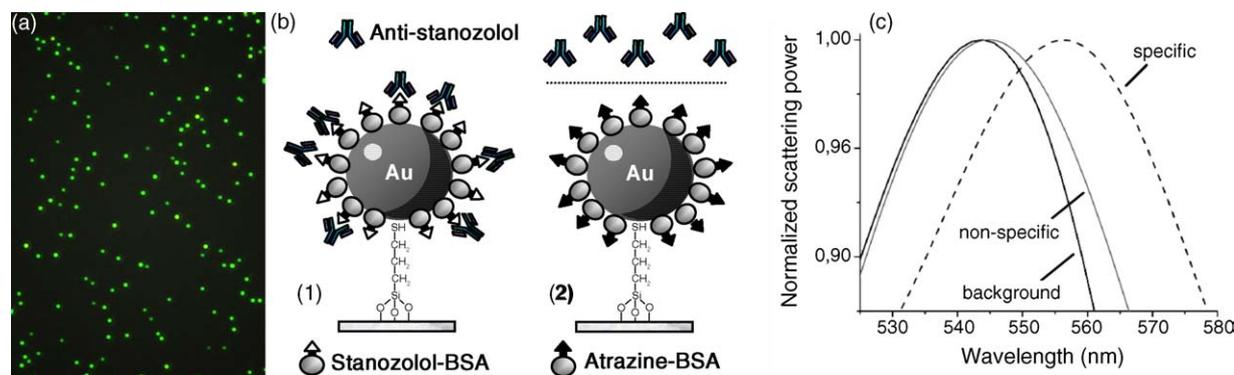

Fig. 1. (a) Dark-field image of Au particle distribution. (b) Schematic of Au nanoparticles with (1) specific binding of Sz-BSA and antiserum and (2) non-specific control using atrazine-BSA. (c) Normalised resonance curves obtained for both conditions described in caption b.
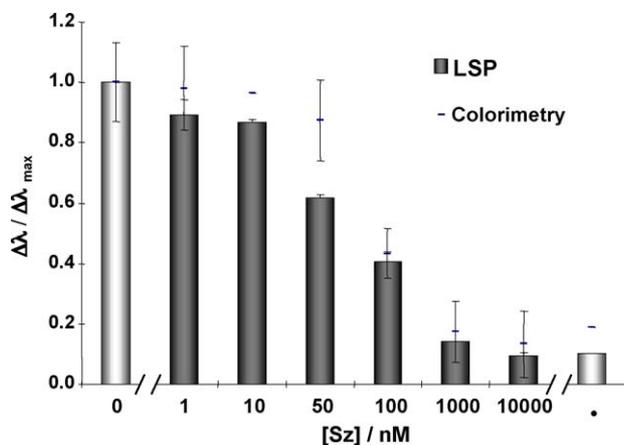
Fig. 2. LSP sensor response (bar chart) and the associated colorimetric response (scatter plot) to different concentrations of Sz in a competitive mode ($n = 2$). $\Delta\lambda / \Delta\lambda_{max}$ is the ratio of the signal to the maximum signal obtained without Sz present.

The IC_{50} value (the concentration that inhibits the curve by 50% from the zero, or when no analyte is present) has been determined at 70 nM with a conservative limit of detection of 20 nM ($6 \mu g L^{-1}$). This detection level, found here, is comparable with conventional SPR (Gillis et al., 2002; Cui et al., 2003) and with further modifications, we envisage sensing down to pM (10^{-12}) levels.

As a way to confirm the LSP result, we used a method based on assessing antibody binding to the Sz-BSA-gold particles through the use of a secondary labelled antibody. The signal recorded is, in this case, only dependent on the biological binding event, and not of the LPS resonance. The results showed (Fig. 2, scatter plot) that competition takes place in about the same Sz concentration interval as in the LSP measurements. As can be seen in Fig. 2, the LSP method gave reproducible measurements, based on an averaged relative standard deviation for all the points of the curve (RSDave: 3.3%; $n = 2$), whilst the colorimetric method was only used as an indicative approach. This uncertainty in the colorimetric approach was attributed to the fact that the absolute colour change was very low (0.15 AU) and variations stem from transferring of colour solution from the colloid samples to the wells of a microtiter plate to measure the subsequent colorimetric signal.

4. Conclusions

In our opinion, there is great potential for further development of an efficient analytical procedure using this biosensing principle. The simplicity of the approach and the speed of the response points also to the possibility of developing single-use disposable devices. Once the surface has been prepared, measurements can be made after 30 min incubation time or even less. In the present format, the features of this immunosensing principle compare favourably with literature reported values and necessary detection levels for anabolic

steroid systems (Merode, 1998; Schaenzer and Donike, 1993; Ferchaud et al., 1997). Improvement upon these parameters is envisaged through further optimization of the system, including particle shaping (Kottmann et al., 2000) and coupling (Enoch et al., 2004), protein type, immobilization strategies and concentration considerations. These investigations are in progress.

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