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Au-labeled antibodies to enhance the sensitivity of a refractometric immunoassay: Detection of cocaine

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ABSTRACT

An integrated platform for a very sensitive detection of cocaine based on a refractometric biosensor is demonstrated. The system uses a waveguide grating biosensor functionalized with a cocaine multivalent antigen-carrier protein conjugate. The immunoassay scheme consists of the competitive binding of cocaine-specific antibodies to the immobilized conjugates. A 1000-fold enhancement of the sensor's sensitivity is achieved when using gold conjugated monoclonal antibodies instead of free antibodies. Together with the optimization of the assay conditions, the setup is designed for a quick identification of narcotics using automated sampling. The results show that the presence of cocaine in a liquid sample could be identified down to a concentration of 0.7 nM within one minute. This value can be reduced even further when longer binding time is allowed (0.2 nM after 15 min). Application of the system to detection of narcotics at airport security control points is discussed.

1. Introduction

The main route for contraband trade of cocaine into European countries is by air and sea since the major production sites are located in South America. Therefore, law enforcement agents operate principally at the external frontier of the European Union and in particular at airport security controls. Systematic investigation of passengers and luggage at check points is very difficult due to the time required for each analysis with conventional detectors. Moreover, it is the sampling procedure – consisting in the swiping of luggage or clothes with a cotton swab – that precludes systematic screening for drugs. Therefore direct sampling of cocaine from the vapor phase would be a substantial improvement of current detection instruments. However, due to the low vapor pressure of cocaine this approach is very challenging. In this contribution, we present the development of an immunoassay for the detection of cocaine based on an evanescent wave biosensor. By using antibodies conjugated to gold nanoparticles the sensitivity is enhanced by three orders of magnitude. This improvement in sensitivity paves the way for automated sampling from the gas phase avoiding the need of manual intervention.

Available commercial solutions for the detection of narcotics at security check points are based on two main technologies: infrared spectroscopy (FTIR or Raman) and ion-mobility spectrometry (IMS). Although the analysis is usually performed within seconds, sampling relies on the collection of drug particles and is performed manually. Another approach for selective and sensitive detection of narcotics is based on immunoassays. Several lateral flow assays are commercially available and many of them proved sufficiently accurate and reliable to detect the presence of cocaine or its metabolites (e.g. benzoylecgonine) in saliva (Kuijten, 2009). Using cocaine-specific antibodies, *Biosensor Applications*® developed instruments based on piezoelectric quartz crystal microbalance (QCM) technology. Remarkable sensitivity is attained with this technology. At the moment the system is still based on the collection of particles with a cotton swab but recent developments indicate that direct air sampling is within reach (Frisk et al., 2006).

One can estimate the required limit of detection of a biosensor based on sampling in the gas phase using the following approximations: in standard conditions, the vapor pressure of cocaine is $\sim 1.9 \times 10^{-7}$ Torr (Lawrence et al., 1984). The detection is rendered even more difficult by the fact that drugs are usually kept in sealed packaging. Therefore the volume of air saturated with cocaine that can be effectively sampled is only a few cm^3. If this gaseous volume saturated with cocaine is entirely transferred into the liquid phase, the required limit of detection of the sensing system is in the sub-nM range (liquid sample volume: 50 μL). To attain such low detection limits with a robust refractometric immunosensor requires the application of sensitivity enhancement strategies.

Here we present a fast and sensitive method for the detection of cocaine in liquid samples. The system is designed for systematic monitoring of luggage at airport security controls. Therefore

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special focus was set on automation and signal enhancement in order to attain the speed and sensitivity required for the target application. In brief, the system consists of a waveguide grating sensor chip functionalized with cocaine antigen-protein conjugates. Due to the small size of the target analyte, a competitive immunoassay detection scheme was selected. The main finding of this work is that the sensitivity can be substantially improved by using antibodies conjugated to gold nanoparticles (AuNPs). Moreover, a flow injection analysis system was developed in parallel so as to reduce the analysis time to only a few minutes. Under optimized conditions, a lowest detectable concentration of 0.7 nM is reached within only 1 min.

2. Materials and methods

2.1. Reagents

All chemicals, unless specified, were obtained from Sigma–Aldrich (Switzerland). Monoclonal anti-cocaine antibodies (mAb) were provided by Securetec both unconjugated and conjugated to gold nanoparticles (Au-mAb) of 40 nm diameter. Concentrated stock solutions were kept at 4 °C. Prior to use, the antibodies were dissolved in phosphate buffered saline pH 7.4 solution (PBS). Cocaine multivalent antigen-carrier protein conjugates (APC) were synthesized by Securetec. The photo-crosslinkable polymer (OptoDex®) used to functionalize the chips was supplied by Arrayon Biotechnology (Switzerland).

2.2. Waveguide grating biosensor

Immunoassays for the detection of cocaine were performed with the so-called wavelength-interrogated optical sensing platform (WIOS) developed at CSEM. Details of the instrumentation have been given elsewhere (Cottier et al., 2003). Briefly, the detection is based on the changes of effective refractive index of a waveguide grating upon adsorption of biomolecules (Fig. 1). The waveguide chips consist of a thin Ta_2O_5 film deposited on a glass substrate (produced by Optics Balzers AG, Liechtenstein). Each chip features three columns containing each 8 input grating that can be measured individually. The surface of each functionalized input grating is 0.8 mm^2 and the flow cell (see below) is designed to circulate the solutions onto all 8 channels of a single column simultaneously. A light beam, emitted by a laser diode centered around 763 nm, is collimated and incident on the chip. The light is coupled into the waveguide through a grating region. The guided light probes the presence of bound biomolecules in the evanescent field and is then outcoupled by another grating before being eventually detected. The resonance wavelength is found by tuning the emission wavelength of the laser light source. The sensitivity of the instrument was shown to be below 10^{-6} RIU for bulk refractometry and the limit of detection for the adsorption of small molecules corresponded to a surface coverage of 0.3 pg/mm^2 (Cottier et al., 2003). Compared to standard surface plasmon resonance (SPR) instruments (Homola, 2008), the system has a similar sensitivity with the advantage to be able to measure eight channels simultaneously. This feature makes it possible to use several analytes in the same sample or, in this study, to use other input gratings as reference for the correction of matrix and temperature variations.

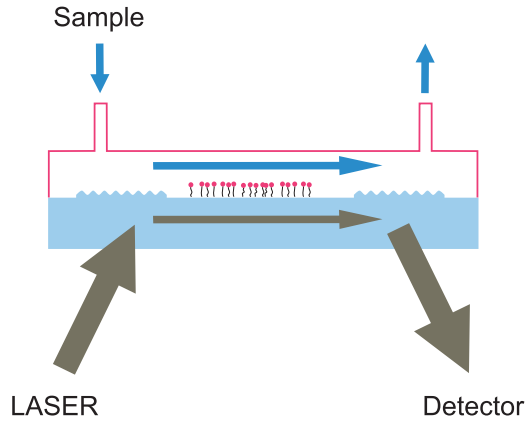

Fig. 1. Schematic drawing of the waveguide grating biosensor chip.

2.3. Chip functionalization

Multivalent cocaine APCs were immobilized on the waveguide input gratings using the OptoDex® technology developed by Arrayon Biotechnology. OptoDex® is a dextran-based photo-crosslinkable polymer that covalently binds biomolecules to surfaces upon UV exposure (Caelen et al., 2002; Sprenger et al., 2005). The reagents are spotted onto any of the eight input gratings using a NanoPlotter™ dispensing system (GeSim, Germany). In addition to the excellent stability of the bound molecules the polymer layer provides passivation, preventing the non-specific adsorption of biomolecules. In each measurement column, four input gratings were functionalized with an optimized amount of cocaine APCs and four others were used as reference channels.

2.4. Flow injection analysis system (FIA)

Automation of the system consisted in (a) the assembly of commercial fluid handling components, (b) the design and fabrication of a flow cell and (c) the generation of a common software interface to control all devices (see Fig. 2). The fluidic system was designed to handle up to 6 samples. It can also be easily interfaced with a sampling system performing the capture of cocaine vapor into a liquid sample. The samples are pumped individually with a syringe pump (Tecan Cavro® XCalibur) through a 6-port distribution valve (Tecan Cavro® SV). A 50 μL injection loop is filled with the sample in an injection valve (Vici Model 22). Thanks to the precise volume handling of syringe pumps with small syringe cylinders (50–250 μL), the dead volume is minimized. The minimal sample size for a 50 μL injection loop is around 85 μL. This value can be further reduced by using smaller loops. The carrier fluid is PBS. It is circulated with a second syringe pump (Tecan Cavro® XCalibur). In order to avoid formation of bubbles by degassing of the liquids due to the pressure drop in the small tubings (Tygon®, 0.25 mm inner diameter) and the flow cells, the carrier buffer is pumped through the system by the syringe pump placed upstream. The optical flow cell is made of a polymer housing which hosts the waveguide chip fitting it precisely on the optical setup. One flow channels addresses a single column of the chip containing eight functionalized input gratings. The flow channels are shaped in a polydimethylsiloxane (PDMS) stamp (Sylgard 184, Corning) used at the same time as a gasket ensuring watertightness at the interface with the chip. The PDMS stamp was structured by replication of a mold made of a cross-linked photoresist (SU-8) on a silicon wafer obtained by standard photolithography techniques. In order to facilitate wetting of the whole channel, an amphiphilic copolymer (Pluronic™ F127, Sigma) was added to the two-part silicone elastomer prior to cross-linking (Wu and Hjort, 2009). The channels have a height of 100 μm and a volume of 2.8 μL. Optionally, when used with gold-labeled antibodies, the concentration of Au-mAb in solution was measured in a second optical flow cell. The Z-shaped flow cell (FIA-Z-SMA, Ocean Optics, FL) is placed in series after the main sensing flow cell. Light absorption in the cylindrical channel (20 mm pathlength) is measured at two different wavelengths (530 nm and 860 nm). Light emitted by two separate LEDs is alternatively coupled into a

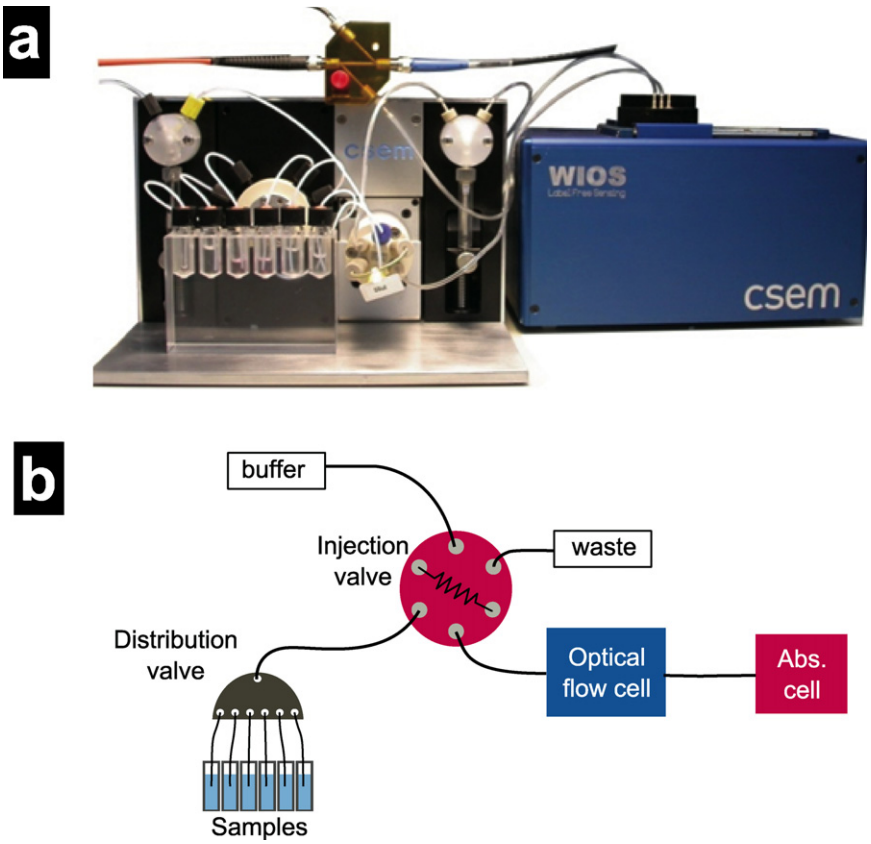

Fig. 2. Description of the fully assembled biosensing platform including fluidic components, the WIOS biosensor and an optical absorption Z-shaped flow cell. (a) Photo of the setup. (b) Schematic description of the components.

multimode fiber and the transmitted intensity is measured with a Si photodiode. Due to the strong absorption of the gold nanoparticles around 530 nm, attenuation of the green light is proportional to the Au-mAb concentration whereas the LED emitting in the near infrared is used as a reference. The entire system, consisting of the fluidic components, the WIOS sensor and the light absorption system is controlled by a single computer interface written in LabVIEW (National Instruments) programming language. The system can be operated in two modes: sequential analysis of up to six samples or semi-continuous sampling for monitoring applications. During a typical experiment, the flow speed is adjusted automatically in order to shorten the time for a complete analysis. For example the rinsing steps are carried out at a high flow rate whereas the adsorption step is performed at a much slower rate (10 μL/min).

3. Results and discussion

3.1. Immunoassay for the detection of cocaine

An immunoassay was designed for the detection of cocaine in water samples. The waveguide chips were functionalized with multivalent cocaine antigen-protein conjugates (APC) consisting of drug molecules covalently grafted on immunoglobulin G carrier proteins. APCs were attached covalently to the surface of the waveguide chip using the OptoDex® dry-and-bond technology. This approach is based on the cross-linking of dextran-based polymer that can covalently bind virtually any organic compound by the formation of carbene radicals under UV illumination. The functionalized chips are kept at 4 °C in dried state until use. In these conditions, the shelf life of the functionalized chips exceeds one year. In addition to the ability to attach biomolecules to the chip, the OptoDex® polymer provides also excellent passivation properties. Indeed, negligible adsorption of free or conjugated antibodies was observed. As depicted in Fig. 3, the detection was carried out according to a competitive binding immunoassay scheme. The sample which may contain cocaine is mixed with a fixed amount of monoclonal anti-cocaine antibodies (mAb) and injected into the flow cell. The antibodies can either bind to free cocaine in solution or to the cocaine analogues present on the surface-bound carrier proteins. When the concentration of free analyte increases, fewer antibodies can bind to the surface. Thus, the instrument response decreases when cocaine is present in the sample.

As shown in Fig. 4 a calibration curve is obtained by injecting known concentrations of cocaine mixed with monoclonal antibodies (mAb). The concentration of mAb (10 μg/mL in PBS) as well as the surface concentration of the immobilized APCs (printed from a 100 μg/mL solution) were optimized prior to the measurement of the calibration curve. The result is a standard sigmoidal shaped curve that shows a half maximal effective concentration (EC_{50}) of 3.7 ppm (12.2 μM) and a lowest detectable concentration (EC_{90}) of 0.15 ppm (0.49 μM). Such values are typical of refractometric competitive immunoassays (Suárez et al., 2009) but slightly higher than similar experiments performed on state-of-the-art SPR instruments (Homola, 2008). As discussed earlier, the target sensitivity for the envisaged application is below in the sub-nM range. The competitive immunoassay described in this section does not match this requirement. As a consequence, the protocol was further improved by using Au-labeled antibodies in the competitive assay.

3.2. Enhancement of sensitivity with Au nanoparticles

The use of gold nanoparticles (AuNPs) to enhance the sensitivity of surface plasmon resonance (SPR) has been reported previously

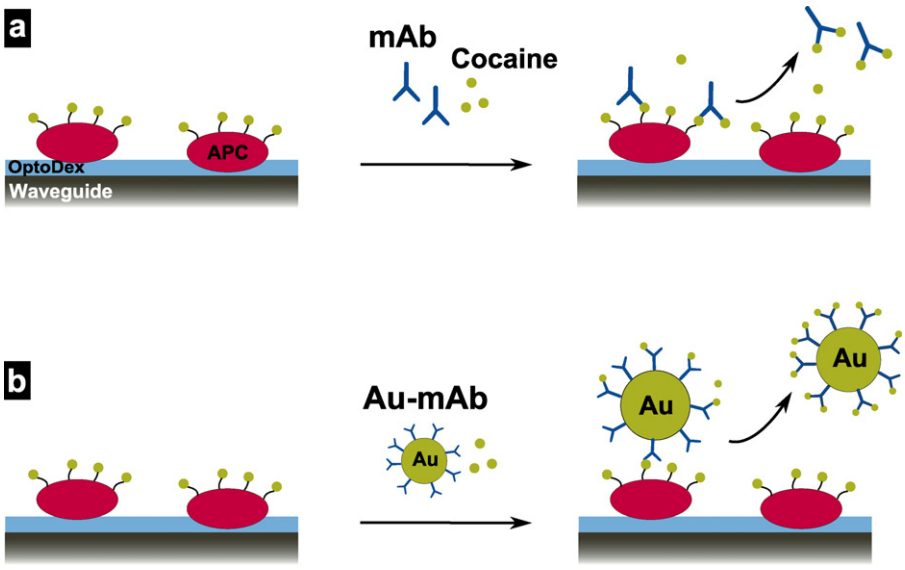

Fig. 3. Scheme of the competitive immunoassay for the detection of cocaine using (a) free monoclonal antibodies (mAb) and (b) Au-conjugated antibodies (Au-mAb). The multivalent antigen-protein conjugates (APCs) are bound to the waveguide using a photo-crosslinkable polysaccharide (OptoDex®).

(He et al., 2000; Hutter et al., 2001; Hutter and Fendler, 2004; Mitchell et al., 2005; Stewart et al., 2008). In the case of SPR this effect has been attributed mainly to electromagnetic coupling between the AuNPs and the bulk Au film. However, it was also recognized that changes in dielectric constant and surface mass had an influence (He et al., 2000). For refractometric sensors based on waveguides, no electromagnetic coupling is present and amplification would result only from variations of the local refractive index. This strategy to enhance the sensitivity of waveguide-based sensors was reported already in the 1990s by Kubitschko et al. who observed a significant improvement of the sensitivity by using nanoscale latex beads (Kubitschko et al., 1997). Here we observe that by using AuNPs on Ta_2O_5 waveguides a similar sensitivity enhancement can be achieved without the need of specific electromagnetic coupling.

Gold nanoparticles were functionalized with the same monoclonal antibodies used in the experiments described in the previous section. The resulting AuNPs are covered with a monolayer of antibodies thereby increasing significantly the particles' diameter from 40 nm to approximately 70 nm. Precise estimation of the number of antibodies attached to each AuNP is difficult. As a consequence the final antibody concentration used in the competitive assay remains unknown. To find the best assay conditions, the concentration of Au-mAb was minimized while still maintaining a suitable signal. Surface concentration of immobilized cocaine APCs was kept the same as in experiments with mAb without gold nanoparticles. The results obtained under optimized conditions show that the working range is substantially shifted towards lower concentrations of cocaine (Fig. 4). Taking advantage of the favorable signal-to-noise ratio, the presence of cocaine can be identified shortly after injection of the sample. However, for enhanced accuracy, a binding time delay of 15 min before reading the response is appropriate (Fig. 5). The lowest detectable concentration (EC_{90}) and half maximal effective concentration (EC_{50}) estimated from Fig. 4 are given in Table 1. Compared to the assay performed with antibodies without gold label, a 1000-fold enhancement is obtained with gold-labeled antibodies. As expected the sensitivity is lower if the response is read after only one minute of incubation. But due to the increased sensitivity brought by the utilization of Au conjugates a qualitative detection of the presence of cocaine in liquid samples can be obtained within one minute. The sensitivity (0.2–0.7 nM) for the competitive immunoassay using AuNPs conjugates is now well within the target range.

The enhancement of sensitivity by using AuNPs conjugates is here clearly demonstrated. In the case of SPR measurements, the improvement was mainly attributed to the presence of electromagnetic coupling between the localized plasmon resonance in the particle and the surface plasmon propagating in the film. No such

Table 1
Half maximal effective concentration (EC_{50}) and minimal detectable concentration (EC_{90}) of competitive immunoassay for the detection of cocaine using free (mAb) or gold-conjugated monoclonal antibodies (Au-mAb). The time indicated correspond to the delay observed between the injection of the sample and the reading of the response magnitude.

Antibodies	Time (min)	EC_{50}	EC_{90}
Au-mAb	1	6.6 nM	0.7 nM
Au-mAb	15	9.6 nM	0.2 nM
mAb	15	12.2 μM	0.5 μM

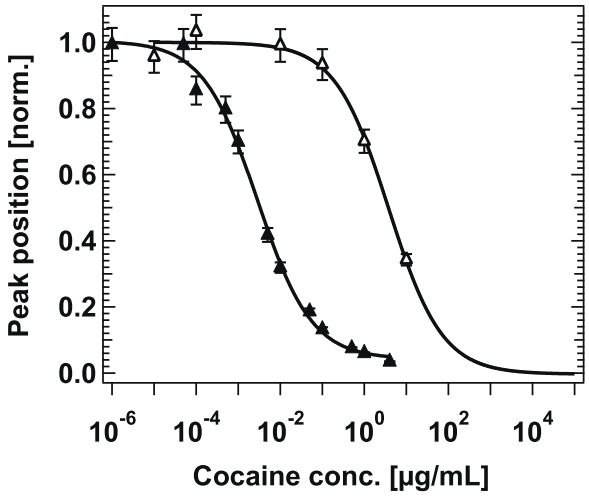

Fig. 4. Calibration curves for the competitive immunoassay performed with free monoclonal antibodies mAb (open triangles) or conjugated with gold nanoparticles Au-mAb (closed triangles). The error on the measurement is estimated at ±5% of the signal. From this one can estimate a limit of detection (3 times signal-to-noise ratio) of 0.2 ppb (0.7 nM) for Au-mAb and 0.26 ppm (0.85 μM) for mAb. These values are similar to the minimal detectable concentration calculated from EC_{90}.

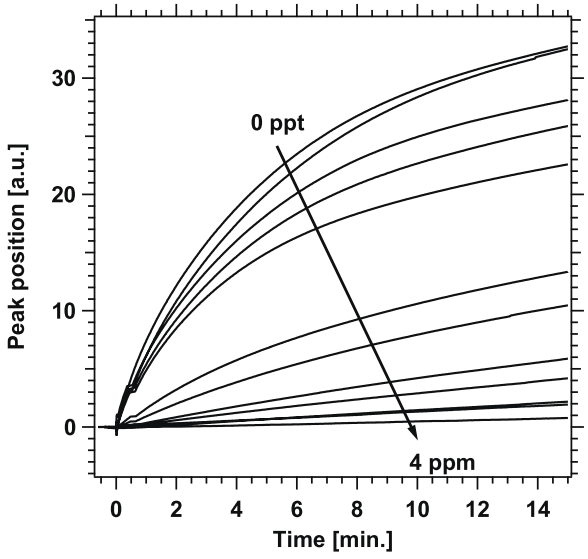

Fig. 5. WIOS sensorgrams for a competitive assay using a fixed amount of Au-mAb and various concentrations of cocaine: 0 ppt, 50 ppt, 100 ppt, 500 ppb, 1 ppb, 5 ppb, 10 ppb, 50 ppb, 100 ppb, 500 ppb, 1 ppm and 4 ppm of cocaine in PBS.

effect is expected in Ta_2O_5 waveguides. Additionally, the resonance of the localized plasmon in 40 nm AuNPs does not overlap with the wavelength of the WIOS probe laser (λ_{WIOS} = 763 nm). Therefore the effect is mainly attributed to the dielectric properties of the composite particles comprising of gold and antibodies that amplify the observed changes of effective refractive index. As reported earlier by Kubitschko et al. (1997) such effects can also be observed with polystyrene nanoparticles. However, due to the limited penetration depth of the evanescent wave (~130 nm) it is preferable to use small metallic nanoparticles (<100 nm diameter).

3.3. Automation

Besides the necessity for very low detection limits, the second major requirement for the systematic investigation of cocaine in luggage at security control points is a short analysis time. In general the time needed for an optical immunoassay (e.g. using SPR) is 10–15 min. The timescale is mainly defined by mass transfer issues and not by binding kinetics (Squires et al., 2008). Therefore the careful design of the flow cell and the assay conditions (e.g. flow rate) can substantially accelerate the response of the sensing device. Here, we designed a flow cell with a small height (100 μm) but a relatively large area to limit mass transport issues. To ensure tight contact with the waveguide chip a PDMS gasket defining the channel was designed. Additionally, the PDMS was modified by including an amphiphilic copolymer in the two-part silicone solution prior to thermal cross-linking. As a consequence the hydrophilicity of the surface of the PDMS gasket is substantially improved. Therefore wetting of the channels is improved and trapping of air bubbles, which is a major issue in automated devices, is reduced.

The automated delivery of the sample was designed to minimize the sample amount, ensure bubble- and contamination-free operation and reduce the overall analysis time. Therefore the sample is loaded via a two-position injection valve and the carrier buffer (PBS) is pumped through the system using a syringe pump placed upstream. In this configuration it is possible to control very accurately the flow rates and the volume dispensed in the measurement cell. For example in an optimized protocol, rinsing steps are performed at a relatively high flow rate (typically 85 μL/min) and only the step where the sample is in contact with the functionalized waveguide is performed at a slow rate (10 μL/min) to maximize the capture of antibodies. In such conditions, a full analysis cycle including sample uptake, detection and rinsing could be performed in 7.5 min. The system is entirely controlled by a single software interface. This includes an optimized sequence of fluid delivery operations but also the full control of the biosensing device (WIOS) and the nonlinear fitting procedures to find the resonance peaks corresponding to the particular wavelength of the light coupled into the waveguide.

3.4. In-line measurement of the concentration of antibodies

In order to improve the reliability of the system, a calibration method was implemented to measure the concentration of the antibodies contained in the injected samples. In a competitive assay, variations in the amount of the antibodies added to the sample can lead to erroneous measurements (false positive or false negative). We take advantage of the strong coloration of gold nanoparticles to estimate the concentration of antibodies directly in the flow path by using light absorption spectroscopy. This approach is demonstrated in the case of the immunoassay based on gold-conjugated monoclonal antibodies (Au-mAb). Indeed, monodisperse gold nanoparticles solutions show a strong absorption band in the visible range of the electromagnetic spectra due a size-dependent localized plasmon resonance (Murray and Barnes, 2007). The spectrum of the solution of Au-mAb features a sharp absorption peak centered at 532 nm (see Supplementary data). The absorbance of the sample solution is measured in a second flow cell placed in line after the main assay cell. The Z-shaped cell is illuminated through a multimode optical fiber with two LEDs with emission bands corresponding to the nanoparticles' absorption peak (green) and in the near infrared for an estimation of the background optical absorption (see Fig. 2A). The transmitted light is collected by another optical fiber and finally detected with a common photodiode. The optoelectronic compounds are controlled with the same software interface as for the fluidics platform and the waveguide sensing device. After building a calibration curve (see Supplementary data), the concentration can be estimated with a standard error of 2%. The inline optical measurement of the concentration is therefore a valuable addition enabling some freedom on the sampling procedure to capture cocaine in the vapor phase.

4. Conclusions

The main goal of this work was to develop a sensing system able to detect cocaine in air contained, for example, in luggage. A major challenge is the low vapor pressure of cocaine which necessitates efficient sampling procedures and very low detection limits. Here, we developed a biosensing platform based on a competitive immunoassay performed on functionalized waveguide chips. It is shown that using monoclonal antibodies conjugated to gold nanoparticles, the sensitivity of the assay was improved by three orders of magnitude. This illustrates the potential of signal enhancement of AuNPs for evanescent sensors. The platform includes automated fluid handling, customized flow cells and a single software interface controlling all components. In optimized conditions, cocaine can be detected within one minute with a minimum detectable concentration of 0.7 nM. This value can be further reduced to 0.2 nM if the response is read after 15 min. The realized device demonstrates the potential of optimized biosensing systems for the detection of narcotics at very low levels compatible with the tiny vapor pressures of such compounds. Additionally, the waveguide chip design makes it possible to measure several analytes simultaneously such as other narcotics or explosives opening new applications in the field of security.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.01.033.

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