Abstract

The aim of this work was the development a biological layer of recognition for universal binding application in immunoassays, using the system of self-recognition biotin-streptavidin and a spectroscopic technique the Surface Plasmon Resonance. This development was carried out at spinit® platform using microfluidic devices of the type lab-on-a-disk property of Biosurfit S.A.

The conditions of preparation and stabilization of the surfaces biotin-streptavidin recognition were optimized in terms of the following parameters: buffer solution, pH, and concentration of streptavidin in solution. It was investigated a strategy for amplification of the SPR signal using biotinylated gold nanoparticles of different sizes as model system. It was defined the optimal size for the devices concerned.

A demonstration assay was carried out to validate the model system developed. This assay consisted of an inhibition test with duration of 15 minutes (5 minutes for sample preparation and 10 minutes of the test SPR) with a sample volume of 1 μL. The analyte concentration range covered extends over two orders of magnitude and presented a logistical behavior.

Keywords: Biological Recognition Layer, Immunoassays, Surface Plasmon Resonance, Lab-on-a-disk Technology, Biotinylated Gold Nanoparticles

1. Introduction

Conventional clinical laboratory testing is rapidly trying to adapt to the increasing demand by doctors for faster and more comprehensive clinical diagnostic results that will allow for quicker and more effective patient treatment. A standard laboratory test will typically take several hours or days before it delivers results to the doctor. The need to lower healthcare costs, to provide access to more accurate clinical decisions and faster patient treatment are all drivers for a new concept in the clinical diagnostics sector.

Thus, Biosurfit S.A has developed a technology platform that allows testing with the patient, named Point-of-Care-Testing (POCT). These tests combine speed, ease
of use, low cost and reliable sensitivity and specificity of the results. The tool developed, the *spinit®*, includes a reading device and a disposable disk array disposable, where by use of a single drop of blood quantitative results can be provided over a period of 15 minutes, allowing the doctor to make then a clinical diagnosis to the patient.

The disposable disk have several detection zones that include a Biological Recognition Layer (BRL), developed using immobilized protein on a gold surface. When the sample passes through this BRL, binds to the immobilized proteins. This will cause a measurable refractive index change in the Surface Plasmon Resonance (SPR) signal. When used in conjunction with the cartridge's microfluidic control structures and the *spinit®* reader SPR sensor, this layer enables us to measure specific blood biomarkers in real-time without any additional labeling reagents. The technology behind the *spinit®* detection relies on SPR, a well established spectrometry technique, that enables the detection of molecular changes on the cartridge's biological recognition layer. SPR biosensors are based on a simple and direct optical technique that can be used to probe refractive index changes that occur in the very close vicinity of a thin metal film surface.

With this project we propose developing and establishing the enabling processes for the implementation of universal chemistry binding surfaces on *Biosurfit S.A* proprietary disposable disk cartridges. The developments will be demonstrated for assays employing gold nanoparticles in a model system. The main goals are: 1) Develop and establish the processes and protocols for the functionalisation of gold coated surfaces using universal receptors; 2) Investigate and establish gold nanoparticle sizes fitting the requirements for optical detection protocols; 3) Demonstrate the technology using a model system for an inhibitor assay.

2. Literature Review

An ongoing revolution in the area of medical diagnostics is the development of biosensors that have the potential for use in POCT devices. These would provide rapid and reliable quantitative results "anytime, anywhere", for example, clinics and hospital emergency departments. In order for POCT to become a reality, current biological testing formats must be reduced to the size of hand held devices that require only small amounts of sample and reagents, while maintaining a multi-analyte, high through put, specific and sensitive assay.

Recent advances in biosensor development, using nanoparticles and nanostructures as integral components, have enabled the development of analyte detection methods in the nanoscale. These have obvious advantages for use in POCT settings compared with traditional measurement techniques. (Healy *et al.*, 2007)

A biosensor is an analytical device that brings together an immobilized biological sensing material and a transducer, to produce a quantifiable signal that is proportional to the concentration of the analyte.
Biosensors comprise three distinct components: (i) a biorecognition element; (ii) a transducing element that converts the interactions of biomolecules into a quantifiable signal; and (iii) a readout system (Figure 1).

**Figure 1** – Schematic of a simple biosensor. (Malhotra et al., 2002)

Biosensors that use label-free transduction methods do not require a reporter molecule to signal the presence of target analyte on the sensor surface, thereby enabling the use of unmodified samples, with the possibility of “real-time” measurement.

Biosensors combine high analytical specificity with the processing power of modern electronics to achieve highly sensitive detection systems. There are two different types of biosensors: biocatalytic and bioaffinity-based biosensors. The biocatalytic biosensor uses mainly enzymes as the biological compound, catalyzing a signaling biochemical reaction. The bioaffinity-based biosensor, designed to monitor the binding event itself, uses specific binding proteins, lectins, receptors, nucleic acids, membranes, whole cells, antibodies or antibody-related substances for biomolecular recognition. In the latter two cases, these biosensors are called immunosensors.

Biosensors are extensively used as diagnostic tools, predominately in point-of-care testing.

In this context, it was developed a layer of binding recognition for universal use in immunoassays from a detection system that involves a high binding affinity between biotin and streptavidin and uses the SPR spectroscopic technique.

**Streptavidin and Biotin Interaction: a model system**

Streptavidin is a tetrameric protein that has a high affinity for its ligand biotin with an association constant of about $10^{15} \text{ M}^{-1}$ (Figure 2). The formation of the streptavidin-biotin complex does not involve any covalent bonds, but it solely involves hydrogen bonds, electrostatic and hydrophobic interactions. The binding site is built by sharing regions of two subunits.

**Figure 2** - Affinity reaction between biotin and streptavidin.

One of the numerous applications of this high-affinity system comprises the streptavidin-coated surfaces of bioanalytical assays which serve as universal binders for straightforward immobilization of any biotinylated molecule. Proteins can be immobilized with a lower risk of denaturation using streptavidin-biotin like a model system. (Välimaa, 2008)

The streptavidin-biotin interaction can be used to detect a number of different molecules of interest with three different basic configurations:
a) The streptavidin is labeled with a detectable molecule, for example, a fluorescent or chemiluminescent enzyme, a metal, or another specific group. While that biotin is present in another reagent (antibody, etc.) binding to the molecule of interest with a marking system (Figure 3A). This form of detection is widely used in immunoassays.

b) Streptavidin is used without labelling, binding biotinylated ligands with also biotinylated molecule detection (Figure 3B). This arrangement has the advantage over the previous one, of the multiple binding sites in molecule of the biotin in each streptavidin. This assay is also used in immunoassays and DNA hybridization techniques.

c) The third method combines effectively with the principles of (a) and (b) to construct a system significantly more sensitive (Figure 3C). This method consists contact, under controlled conditions, streptavidin proteins without labeling proteins and a biotinylated reagent detection. The multiple biotin binding sites on streptavidin may form a matrix. (Nara et al., 2008)

Surface plasmon resonance

Many of the biosensors currently available on the marketplace use the optical properties of lasers to monitor the interactions of biomolecules. Surface Plasmon Resonance (SPR) is an optical technology that involves the interaction of light with the electrons of a metal.

SPR is sensitive to refractive index changes on a suitable metal surface and can be used to measure the adsorption of proteins in a label-free manner. The surface must be chemically modified to allow coupling of the ligand and to ensure that it exhibits very low non-specific binding of proteins.

A disposable disk with a thin gold surface for the detection system gives the surface such properties. To transport samples and reagents to the adsorption and detection spot, an integrated liquid handling system was developed. These three components - SPR, biocompatible sensor chips, and integrated fluidics - together enable researchers to quantify protein-ligand interactions. The binding and dissociation of the analyte to the immobilized ligand are followed in real-time and presented in a “sensorgram” of response versus time (Figure 4). The
response signal from the SPR detector is proportional to the mass of protein/surface area.

**Figure 4** - Response is followed with time in a sensorgram, and is proportional to the mass adsorbed proteins. (Szabo et al., 1995)

The analyte in the sample plug is adsorbed on the immobilized ligand, and from the adsorption profile the association rate constant can be determined. After the sample pulse has passed, the biospecific complex so formed will dissociate, and from the logarithmic decay the dissociation rate constant can be calculated.

Thus, the technique enables rapid, label-free monitoring of biomolecular reactions in real-time, and requires only a small amount of sample. SPR measures changes in the refractive index and thus in the resonance angle at which polarized light is reflected from a surface, which is, in turn, related to a change in layer thickness or mass upon gold nanoparticles biotinilated binding to an streptavidin-coated gold layer.

### 3. Materials and Methods

**Coating of gold surface**

Streptavidin was diluted in an appropriate coating solution which consisted of phosphate buffered (PB) 0.1M at pH 6.0; 7.0, 8.0 at several concentrations. The streptavidin-coated surfaces were blocked with a commercial solution and washed with a buffer solution.

**Biotinylated gold nanoparticles preparation**

Biotinylated gold nanoparticles (AuNPS) used in assays were delivered from Cytodiagnostics and diluted in PBS in appropriate concentration.

### 4. Results

**Immobilization of streptavidin on a gold surface**

The Figure 5 displays the variation of the SPR sensor signal as a function of the concentration of the streptavidin solutions used in its immobilization on gold surface. The three values of the pH solutions – 6.0; 7.0 and 8.0 – are above the isoelectric point (pl) of the streptavidin that is between 5 and 6.
Figure 5 - Variation of the SPR sensor signal obtained depending on the concentration of sAv coating conditions for the three different pHs. The experimental points were obtained from the average for n Δθ_{SPR} detection zones obtained in each sensorgram, and vertical bars indicate standard deviations.

From Figure 5 it can be seen that at pH 7 adsorption curve is similar to a Langmuir isotherm. At low concentrations of streptavidin, the SPR signal has a linear variation with a positive slope, which corresponds to a rapid adsorption of protein molecules in the substrate until a concentration range where the SPR signal reaches a maximum and the variation ceases to be significant. This level corresponds to the formation of a saturated monolayer streptavidin.

The minimum concentration at which maximum signal is obtained from the SPR sensor is 50 µg/mL.

Amplification of the sensor signal

It was analyzed the variation of the SPR sensor signal obtained from the connection between the streptavidin immobilized on the detection zones with biotinylated AuNPS. The SPR response of the sensor is proportional to the mass adsorbed to the surface. For this reason, it was investigated a range of sizes of AuNPS as substrate to evaluate the increase in size on the SPR signal amplification.

Figure 6 - Representation of the variation of the average Δθ_{SPR} according to the diameter of the biotinylated gold nanoparticles to optical density (O.D) 12.5 (blue line) and the O.D 25.0 (red line) using as a coating condition PB 0.1M pH 7 at 50 µg/ml of streptavidin.

In Figure 6 it can be seen that the curves of the SPR signal as a function of the AuNPS diameter, they both curves have a maximum for the optical density.

At the same optical density, the SPR signal increases with the mass of the adsorbed species but decreases with the concentration of those same species. From the maximum signal there is the opposite effect, that is, the SPR signal decreases with the mass of the adsorbed species but increases with the concentration thereof.
Assay using biotinylated BSA as a sample detection system as binding affinity streptavidin-biotin

An assay was conducted to demonstrate the model system developed that consisted of an inhibition test with a duration of 15 minutes (5 minutes for sample preparation and 10 minutes of the test SPR) with a sample volume of 1 µL.

5. Conclusions

The main objective of this dissertation is the development of a biological recognition layer (BRL) with universal character and for use in immunoassays and combined with the use of the surface plasmon resonance technique for detection.

For the BRL functionalization it was used the streptavidin-biotin complex. The high affinity between these two proteins ensures the formation of a complex practically inseparable, allowing its use for numerous applications, including the development of immunoassays.

The design of this BRL primarily consisted of streptavidin immobilized to the gold substrate to obtain a saturated layer. The immobilization process was optimized by the selection of buffer solution, pH and concentration of streptavidin in solution.

It was analyzed the amplification of the SPR sensor signal. Was studied the effect of varying the optical density (OD) and the size of the biotinylated gold nanoparticles (AuNPs) on the SPR signal. It was observed a non linear behavior with the size of the nanoparticles due to competitive effect between the mass increase with decreasing concentration of species in solution.

In order to verify the sensitivity of the streptavidin-biotin interaction, it was made an inhibition assay in which the biotinylated BSA is used as a sample solution to yield a signal revealing biotinylated gold nanoparticles. It is concluded that the sensor signal decreases with increasing concentration of biotinylated BSA. The describing this behavior is a logistic function with the concentration of biotinylated BSA ranging from 10 to 100 µg/mL.
Therefore, the application of SPR technique has been a powerful tool in this study, allowing the detection of the biomolecular detection system, biotin-streptavidin, in real time without the use of reagents markers, and revealing sensitivity and selectivity.

Although the measurements demonstrate specificity and reproducibility the statistical parameters of standard deviations and coefficients of variance are close to the acceptable limit. Thus, further work should be carried out in order to stabilize the BRL.

References


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