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Ciências

Disposable immunosensor for diagnosis of Human Cytomegalovirus congenital infection

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Abstract

Human cytomegalovirus is a herpes virus which can cause pneumonia, retinitis, colitis and encephalopathies in immunosuppress individuals, as transplanted ones, persons infected by HIV, and individuals with immature immune system, like fetuses and newborns. In the last ones microcephaly, small body size, hepatomegaly, blindness, deafness and mental retardation can also be observed. This infection is the most frequent cause of embryogenic and fetal pathology induced by a virus.

In the actuality, the diagnosis of HCMV is based on clinical and immunologic data. There are several methods for HCMV detection: the virus isolation in fibroblasts culture, the shell-vial method, the PCR technique, the ELISA test and the western blotting technique. However all of them require a long period of time to perform or are costly which is problematic for diagnosis. Therefore, an immunosensor was developed for human cytomegalovirus glycoprotein B detection based on electrochemical stripping analysis of silver nanoparticles.

In this sandwich type immunosensor, the silver deposition solution is added to the electrode surface where the gold nanoparticles attached to antibodies, will catalyze the reduction reaction of silver ions, leading to the formation of silver nanoparticles. The higher concentration of analytes means that more amounts of gold nanoparticles are capture on the sensor surface, producing more silver nanoparticles. The silver nanoparticles are dissolved and measured by anodic stripping voltammetry. This method allows a faster and, we expect, a more sensitive way to detect HCMV.

Key words

Immunosensor, Screen-printed Electrodes; Human Cytomegalovirus; Glycoprotein B

Resumo

O Citomegalovírus humano é um vírus que pode causar pneumonia, renite, colite e encefalopatias em indivíduos imunossuprimidos, como sujeitos transplantados, infectadas com o vírus VIH e com sistema imune imaturo, como fetos e recém-nascidos. Nestes últimos, pode-se observar microcefalia, pequeno tamanho corporal, hepatomegalia, cegueira, surdez e atraso mental. Esta infecção é a causa mais frequente de patologias embriogénicas e fetais induzidas por um vírus.

Na infeção aguda por HCMV são gerados anticorpos específicos para um grande número de proteínas estruturais e não estruturais. Embora o vírus codifique mais de 100 proteínas apenas as glicoproteínas B e H induzem anticorpos capazes de neutralizar o vírus e eliminar células infetadas (anticorpos neutralizantes). A glicoproteína B (gB) é o antígeno dominante existente na cápsula de HCMV e aproximadamente 100% dos indivíduos infetados com HCMV desenvolvem anticorpos contra esta proteína.

Na glicoproteína B foram identificados três sítios de ligação ao anticorpo: domínio antigénico 1 (AD-1), 2 (AD-2) e 3 (AD-3). O domínio AD-2 compreende dois locais, local I (resíduos 68-77) e local II (resíduos 50-54). Dos três domínios, apenas o domínio AD-1 e o local II do domínio AD-2 são capazes de induzir anticorpos neutralizantes do vírus durante a infeção natural. O domínio AD-1 representa o local imunodominante da gB. Na verdade, cerca de 100% dos indivíduos infetados que são seropositivos para gB têm anticorpos contra o domínio AD-1 enquanto o domínio AD-2 é apenas reconhecido por 47%.

AD-1 é um domínio estrutural muito complexo, que tem entre 552-635 resíduos de gB. A ligação de anticorpos requer a presença da sequência completa de AD-1 e a formação de uma ligação disulfureto intramolecular entre a cisteína 573 e cisteína 610. Supõe-se que a ligação dos anticorpos a AD-1 não é afectada por glicosilação da gB, uma vez que os anticorpos também reconhecem a proteína não glicosilada. Além disto, enquanto outros domínios da molécula mostram uma variação significativa entre os isolados, o domínio AD-1 parece ser das regiões da gB mais altamente conservada.

Assim, o domínio de AD-1 pode ser visto como uma fração promissora a ser usada em testes de diagnóstico para verificar a presença de anticorpos neutralizantes e pode ser utilizado para estabelecer uma relação entre a presença destes anticorpos e a ocorrência de sintomas.

Atualmente, o diagnóstico de citomegalovírus humano (HCMV) é baseado em informações clínicas e imunológicas. Existem vários métodos para a deteção de HCMV. O isolamento do vírus em cultura de fibroblastos é o método convencional. Neste, é feito o isolamento do vírus a partir de um tecido de biopsia ou de um fluido corporal, tal como a urina. Após isolamento, o HCMV é replicado “in vitro”, incubado com fibroblastos a 36 °C durante uma a três semanas e posteriormente a incubação é analisada com o objetivo de identificar inclusões de HCMV em fibroblastos. Este método, que requer assepsia total, não é

utilizado pois requer um longo período de tempo para sua execução, dificultando assim o diagnóstico.

O método “Shell-vial” é muito similar ao anterior, mas o tempo de revelação (feito por imunofluorescência indireta) diminui para 24, 48 ou 72 horas, devido à utilização de anticorpos monoclonais contra diferentes antígenos de HCMV e à utilização de centrifugação que facilita o processo da penetração do vírus em fibroblastos.

Outro método alternativo para análise de amostras clínicas é o PCR (Polimerase Chain Reaction). É uma técnica rápida (~ 6h) que apresenta uma elevada sensibilidade, baseada na amplificação seletiva de sequências específicas de ácidos nucleicos, permitindo a detecção de ADN viral. A sensibilidade e especificidade deste método é semelhante ao método de isolamento viral, mas o PCR apresenta algumas vantagens, tais como a velocidade de obtenção do resultado e a possibilidade de uso de amostras congeladas. Esta técnica é bastante utilizada apesar do seu custo elevado e dificuldade de realização. Pode ser utilizada tanto qualitativamente (diagnóstico por PCR) como quantitativamente através da medição da carga viral, que é proporcional ao nível de ADN de HCMV.

Outra técnica é a de ELISA (Enzyme-Linked Immunosorbent Assay), esta apresenta uma sensibilidade de 100% e uma especificidade de 86% na detecção de anticorpos no sangue. No entanto, existe a possibilidade de resultados falso-positivos, causados por reações cruzadas com algum vírus da família Herpesviridae, fator reumatóide e anticorpos antinucleares.

Finalmente, outro método que pode ser usado para a detecção é o “Western Blotting”, que permite a medida da afinidade do anticorpo para o antígeno. No entanto, este método também apresenta uma disponibilidade comercial questionável, porque igualmente alguns resultados falso-positivos podem ser observados.

Como descrito, todas estas técnicas de ensaio envolvem, por vezes, ou equipamentos caros e/ou procedimentos igualmente caros, demorados, complicados e conducentes a falso-positivos.

As células eletroquímicas são de grande interesse na análise de substâncias devido à sua robustez, fabrico fácil e económico.

Uma das técnicas mais utilizadas no fabrico dos eléctrodos que se utilizam nas células eletroquímicas é a serigrafia. Esta técnica permite construir sensores químicos com uma alta reprodutibilidade e uma infraestrutura mínima. A serigrafia é um método de impressão direta, também denominado de impressão por penetração. A deposição de tintas é realizada por camadas sobre um substrato. A qualidade dos sensores químicos assim fabricados depende, em grande medida, dos materiais utilizados.

Mediante a tecnologia de eléctrodos serigrafados, é possível a miniaturização dos sensores, que oferecem a vantagem de terem baixo custo, serem versáteis, poderem ser fabricados com configurações de eléctrodo distintas e com diferentes tintas. Devido às suas características, esta tecnologia ajusta-se bem à produção em massa de eléctrodos descartáveis.

Para aumentar a seletividade dos elétrodos, estes são modificados por diferentes métodos, por exemplo imobilizando uma substância na sua superfície.

O método de imobilização é muito importante pois influencia o tempo de vida do sensor e a sua sensibilidade. Existem diferentes tipos de imobilização, designadamente: adsorção, aprisionamento, reticulação e ligação covalente.

O dispositivo desenvolvido neste trabalho, através da sua simplicidade, baixo custo e tempo de análise relativamente curto, tem como objectivo superar todas as desvantagens referidas anteriormente para as técnicas de diagnóstico normalmente utilizadas, pois combina as vantagens dos dispositivos electródicos com o uso de anticorpos específicos para a detecção de glicoproteína B.

Neste trabalho, desenvolveu-se um immunosensor do tipo sandwich. Neste é adicionada uma solução de deposição de prata à superfície onde, os anticorpos marcados com nanopartículas de ouro irão catalisar a reacção de redução dos iões de prata, levando a formação de nanopartículas de prata. Uma maior concentração de analito significa que uma maior quantidade de nanopartículas de ouro serão capturadas na superfície do eléctrodo que, por sua vez, irão levar à produção de mais nanopartículas de prata. As nanopartículas de prata são depois dissolvidas e medidas por voltametria de redissolução anódica. Este método permite uma forma mais rápida, económica e sensível para a detecção de glicoproteína B.

Palavras-Chave

Imunosensor, Electrodo Serigrafado, Citomegalovirus humano, Glicoproteína B

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Chapter 1 - Human Cytomegalovirus

Human Cytomegalovirus (HCMV) is a herpes virus that establishes a lifelong latent infection following primary infection that can periodically reactivate with shedding of infectious virus [1].

1.1. General Characteristics

Human Cytomegalovirus (HCMV) is the most usual name for human herpesvirus 5, it is a member of *Herpesviridae* family and *Bethaherpesviridae* subfamily [2, 3]. HCMV is the largest virus of the family, with 200 nm in diameter, 240 kb in size and a molecular weight of 155 kDa, and is morphologically indistinguishable from other human herpes viruses, with an icosahedral capsid, a tegument, and an envelope [2,4,5]. This virus is a double-stranded DNA virus and its genome has a high size. It also has an envelope constituted by glycoproteins that perform an important role in the initial process of binding, fusion and penetration in the host cell [3].

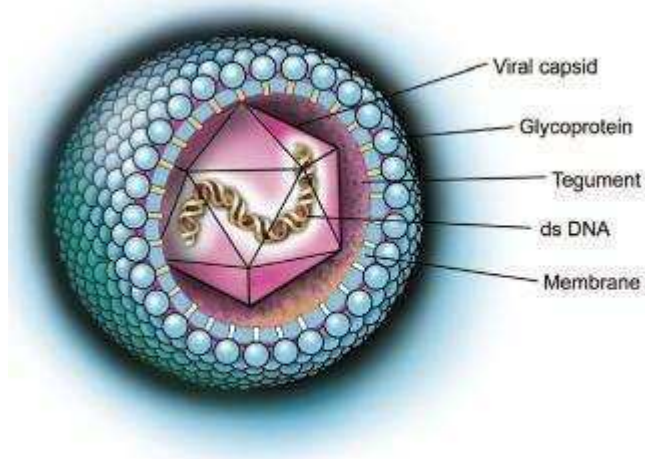


Figure 1.1. Representation of Human Cytomegalovirus (HCMV). Adapted by <http://www.asovida.org/?p=372>

Virus replication results in the formation of intranuclear and intracytoplasmic inclusion bodies, in which the nucleocapsids are formed primarily followed by several dense bodies. Nucleocapsids acquire the envelope from the nuclear membrane or cytoplasmic vacuoles [2]. However, as for other herpesviruses, the assembly of the infectious human cytomegalovirus particle is a complex and poorly understood process [4].

Like all herpesviruses, HCMV undergoes latency and reactivation in the host cell, so once a person is infected, HCMV is not eliminated from the body but persists to cause a low

grade chronic infection or remains in latent stage allowing further transmission of the virus to new hosts. [2,5].

1.2. Infection and Transmission

Human being is the only known receptor for this virus and the transmission is facilitated by mucous contact. The transmission routes are saliva, vaginal and cervical secretion, breastmilk, blood, urine, semen and tears [3].

HCMV can also be transmitted by infected lymphocytes and mononuclear cells, establishing a latent infection in mononuclear leucocytes and organs like kidneys and heart. HCMV infects all kind of cells using common receptors to the majority of cells [3].

Like all human herpes viruses, HCMV is an opportunist virus that establishes a lifelong latent infection and causes asymptomatic or little severe infections when it is reactivated [1,3]. Reactivation of HCMV to a state of active replication with potential to induce disease and transmission to a new host can occur in a situation of cellular immunity suppression, induced by disease [3].

1.3. Worldwide HCMV seroprevalence

HCMV infection was relatively common among women of reproductive age, with seroprevalence ranging from 45 to 100% (Fig. 1.2.). The fetus is most likely to suffer permanent damage if infected as the result of primary maternal infection. In children and adults, both primary HCMV infections and reactivations are typically asymptomatic; as a result, many people are unaware that they have been infected. Worldwide, HCMV seroprevalence tended to be highest in South America, Africa and Asia. However, HCMV seroprevalence was also elevated in parts of Europe (e.g. Italy and Sweden) and the Middle East (e.g. Turkey and Israel). Seroprevalence was lowest in Western Europe and in the United States. Within the United States (Fig. 1.3.), CMV seroprevalence showed substantial variation as well, differing by as much as 30 percentage points between states. HCMV seroprevalence generally increased with age [1].

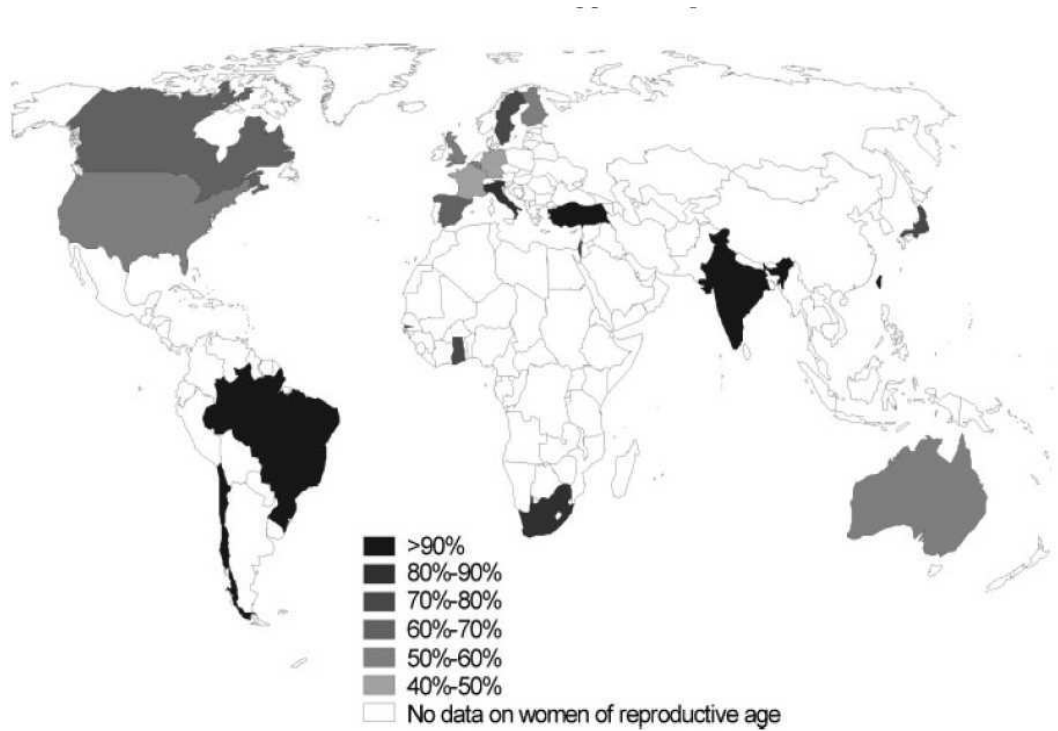


Figure 1.2. Worldwide HCMV seroprevalence among women of reproductive age. Reproductive age was generally defined as between 12 and 49 years of age. Adapted by [1]

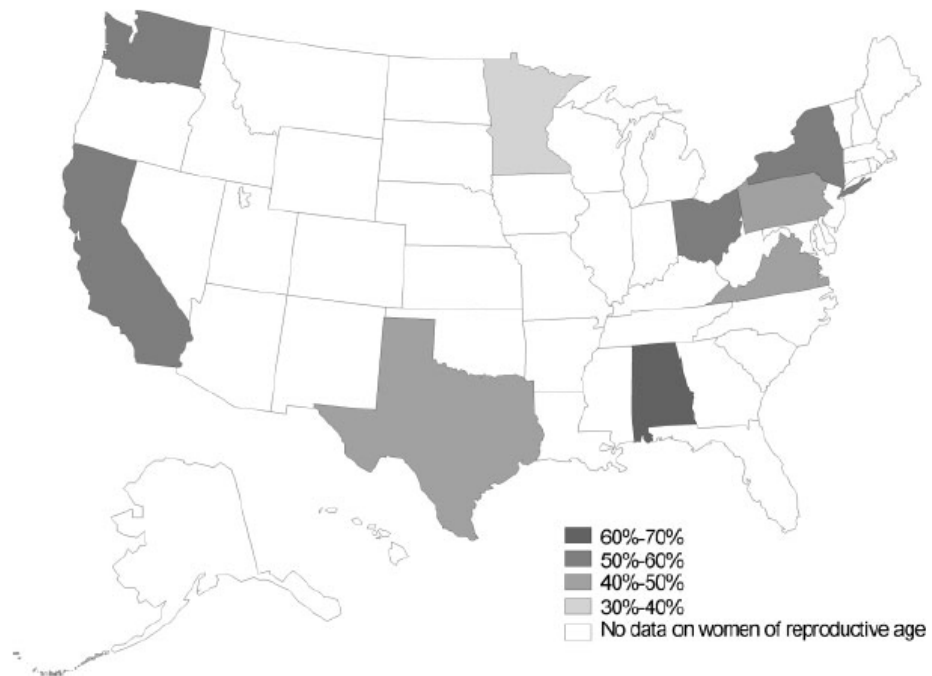


Figure 1.3. HCMV seroprevalence among women of reproductive age in United States. Reproductive age was generally defined as between 12 and 49 years of age. Adapted by [1]

1.4. HCMV pathology

Although the infection by HCMV in the most of the immunocompetent individuals does not represent a serious problem, in immunosuppressed individuals, as transplanted individuals, individuals infected by HIV or individuals with immature immune system, as fetuses and newborns, the reinfection can be severe and even fatal. This means that HCMV is a virus of paradoxes, it can be a potential killer or a lifelong silent companion [2,3,6]. In immunosuppressed individuals may be diagnosed pneumonia, retinitis, colitis and encephalopathies, while in newborns can manifest microcephaly, small body size, hepatomegaly, blindness, deafness, mental retardation, among other pathologies. [3,7]

The infection by HCMV is the most frequent cause of embryonic and fetal pathology induced by virus in the whole world, although the majority of the infected children does not manifest any symptom at birth [3]. HCMV infection is mostly controlled in immunocompromised patients by available antiviral drugs, yet it continues to maintain its role as the most dangerous infectious agent for the unborn infant [7].

In spite of primary and recurrent infections by HCMV being accepted, the primary maternal infection is a major cause of congenital birth defects [7].

In newborns, cytomegalovirus may be acquired in utero via the placenta or by exposure in the maternal genital tract during labor. In this case, the most perinatal infection by HCMV are asymptomatic, although occur hepatitis and atypical lymphocytosis and to 20% of infected child develop pneumonia [7, 8]. Severe disease acquired perinatally occurs in the most of the cases in child underweight [9].

1.5. Primary *versus* Recurrent infection - Implications for the fetus and infant

A primary infection is defined as an initial infection. Individuals who have never been infected are at risk for primary infection.

Recurrent infection is defined as a past infection and includes latent infections. More neonates with congenital HCMV are symptomatic at birth in primary maternal infection than in recurrent infection. Primary maternal infection is also associated with an increased risk of more severe sequel. However, while recurrent maternal infection is somewhat protective against sequel, these infants are still at risk. In addition the presence of symptoms at birth is associated with much higher risk of sequel. Even though most infected infants are asymptomatic at birth, some of them eventually develop serious sequel, including deafness and mental retardation [8].

1.6. Management of patients with CMV

Because the infection by HCMV is the most frequent cause of embryonic and fetal pathology, the management of pregnant women with CMV will be highlighted.

Pregnant women who are susceptible to CMV infection should be advised of the importance of careful hand washing and cleansing of environmental surfaces when interacting with young children. Also, if blood transfusions are required, pregnant women should always receive CMV-negative blood, and this same type of blood should be used for any intrauterine transfusion. In addition, because of the possibility of CMV transmission through sexual intercourse, pregnant women should be urged to adopt safe sexual practices [10].

However, considering the ease of CMV virus conveying, the best way to prevent its transmission, clearly, undergoes by the development of an effective vaccine. In this light, the work of Pass et al. [11] is extremely encouraging. These authors conducted a Phase II placebo-controlled, randomized, double blind trial (Level I evidence) of a new CMV vaccine. This vaccine was prepared by recombinant technology and contained envelope glycoprotein B along with the MF 59 adjuvant.

But if the diagnosis of congenital CMV infection is confirmed, one option is pregnancy termination; another is mother treatment with antiviral agents such as ganciclovir, foscarnet, and cidofovir. However, these drugs are of moderate effectiveness in treating CMV infection in the adult, particularly the immune compromised patient. The most promising therapy for congenital CMV infection appears to be hyperimmune globulin. Nigro et al. [12] described the use of hyperimmune globulin for treatment of a mother who had a twin pregnancy, discordant for congenital CMV infection (Level III evidence). Treatment was administered at the 22 weeks of gestation. The patient received hyperimmune globulin intravenously for three days and a separate dose was injected intra-amniotically into the sac of the affected twin. The authors noted that treatment resulted in decreased placental edema, improvement in fetal growth, and an increase in maternal cell-mediated immunity. At nine months of age, both infants were negative for CMV.

1.7. HCMV specific antibodies

Humoral immunity is important to detect an infection by Human Cytomegalovirus. So tests that measure a specific type of antibody help to tell the difference between a current and a past infection. Elevated titer of Immunoglobulin M (IgM) antibodies appear during the active infection and were highest during viremia while Immunoglobulin G (IgG) antibodies last all symptomatic infection and persists during asymptomatic positive individuals and may even last a lifetime [13].

As was said above, in the acute infection by HCMV specific antibodies are generated against to a large number of structural and non-structural proteins. Although the virus

codifies more than 100 proteins just B and H glycoproteins induce antibodies capable to neutralize virus and to eliminate infected cell (neutralizing antibodies). B glycoprotein (gB) is the dominant antigen existing in the capsule of HCMV and approximately 100% of infected individuals with HCMV develop antibodies against this protein [3].

In B glycoprotein were identified three binding sites to the antibody: antigenic domain 1 (AD-1), 2 (AD-2) and 3 (AD-3) (Fig. 1.4.). AD-2 comprises two sites, local I (residues 68-77) and local II (residues 50-54). Of the three domains, just domain AD-1 and local II of AD-2 are able to induce virus-neutralizing antibodies during the natural infection. The gB protein also contains a number of additional non-linear or assembled epitopes. AD-1 represents the immunodominant site of gB. In fact, approximately 100% of infected individuals who are seropositive for gB have antibodies against AD-1 while AD-2 is recognized by 47%.

AD-1 is a very complex structural domain; it has between 552 to 635 residues of gB. The binding of antibodies require the presence of the complete sequence of AD-1 and formation of intramolecular disulfide bond between cysteine 573 and cysteine 610 [9, 3]. It is supposed that the binding of the antibodies to AD-1 is not affected by the glycosylation of gB because the antibodies also recognize the non-glycosylated protein. While other domains of the molecule show considerable variation between the isolates, AD-1 appears to be one of the regions of gB most highly conserved

Thus, the AD-1 domain can be viewed as a promising fraction to be used in diagnostic tests to verify the presence of neutralizing antibodies and can be used to establish a relationship between the presence of neutralizing antibodies and the occurrence of symptoms, in the case of vertical transmission [3].

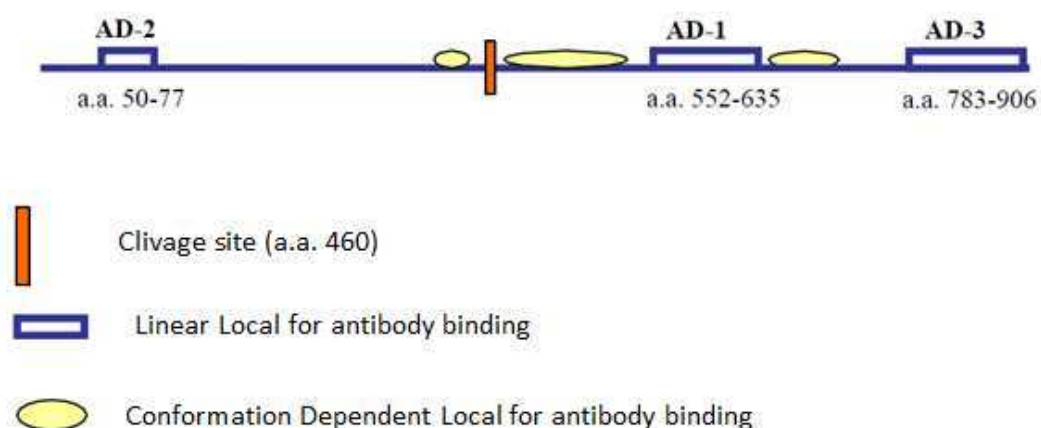


Figure 1.4. Schematic representation of antigenic domains of glycoprotein B. Adapted by [3].

1.8. Diagnosis of HCMV Infection

1.8.1 Laboratory Diagnosis

In the actuality, the diagnosis of HCMV is based on clinical and immunologic data. There are several methods for HCMV detection: the virus isolation in fibroblasts culture, the *shell-vial*, the *PCR technique*, the *ELISA test* and the *western blotting technique*.

The virus isolation in fibroblasts culture is the conventional method. It is made by virus isolation from a biopsy tissue or a body fluid, as urine. This method requires total asepsis. After the isolation, HCMV is replicate *in vitro*, incubated with fibroblasts at 36°C for one to three weeks and posteriorly analyzed with the main to identify HCMV inclusions in fibroblasts. This method is not available because it requires a long period of time to be carried complicating the diagnosis.

The *shell-vial* method is very similar to the one presented above, but the revelation time (done by indirect immunofluorescence) decreases to 24, 48 or 72h, by the use of monoclonal antibodies against different HCMV antigens and the use of a centrifuge that facilitates the process of penetration of the virus in fibroblasts.

PCR (*Polymerase Chain Reaction*) is a fast technique (~6h) and has a high sensitivity based on the selective amplification of specific nucleic acids sequences. Allow the detection of viral DNA and is an alternative method for urine our clinical samples. The sensitivity and specificity of this method is similar to the viral isolation method, but in comparison has some advantages, such as speed of the result and the possible use of freeze samples. This technique is widely used despite the high cost and difficulty to perform. It can be use both qualitatively (diagnosis by PCR) and quantitatively by the measured of viral load, that is proportional to HCMV DNA level.

The ELISA (Enzyme-Linked Immunosorbent Assay) presents a sensibility of 100% and a specificity of 86%, by detecting antibodies in the blood. However, there is the possibility of false positive results, caused by cross-reactions with some virus of *Herpesviridae* family, rheumatoid factors and antinuclear antibodies.

Finally, Western Blotting allows the measured of affinity of the antibody for the antigen. So it can be used for detection of maternal infection, but presents a questionable commercial availability because, also, some false positives results can be observed by this method [14].

Chapter 2 - Biosensors

In recent years, biosensors have been increasingly used for continuous monitoring of biological and synthetic processes and to aid our understanding of these processes. Typical applications include environmental monitoring and control, and chemical measurements in the agriculture, food and drug industries. Biosensors can also meet the need for continuous, real-time in vivo monitoring, leading to replace of intermittent analytical techniques used in industrial and clinical chemistry [15]. Thus, they become practical and useful, if not essential, tools in medicine, food quality control, environmental monitoring and research. Theoretically, they can be tailored according to individual analytical demands of almost target molecule that interacts specifically with a biological system [16].

2.1. What are Biosensors?

Biosensors are chemical sensors that are constituted by a recognition system, which uses a biochemical mechanism and a receptor transducer device, which is capable to provide selective quantitative or semi-quantitative analytical information using a biological recognition element (Fig. 2.1.) [17].

The biological recognition system, which is the biological material (e.g. tissue, microorganisms, enzymes, antibodies, nucleic acids, etc) or biological derived material (e.g. recombinant antibodies, engineered proteins, etc) is immobilized in proximity to a transducer that translates information from the biochemical domain into a chemical or physical output signal with a defined sensitivity. In the case of the simplest applications, the electrochemical reactions occur directly on the electrode surface [15, 17]. The main purpose of the recognition system is to provide a sensor with a high degree of selectivity for the analyte to be measured. [17, 18].

The transducer serves to transfer the signal from the output domain of the recognition system to, in most of the cases, the electrical domain (non-electrochemical techniques including photochemistry, piezoelectric detection and quite recently magnetic permeability have also been used to detect biological signals). Because of the general significance of the word, a transducer provides bi-directional signal transfer (non-electrical to electrical and vice versa). The transducer is also called detector, sensor or electrode [17].

Finally, very complex biochemical process that occurs in the boundary region close to the sensor interface can be described by computer-assisted mathematical modeling [15]. This type of modeling is particularly valuable in the mass production of biosensors.

The biosensor present the advantage to allow real-time analysis, which is particularly important for the rapid measurement of body analytes [19].

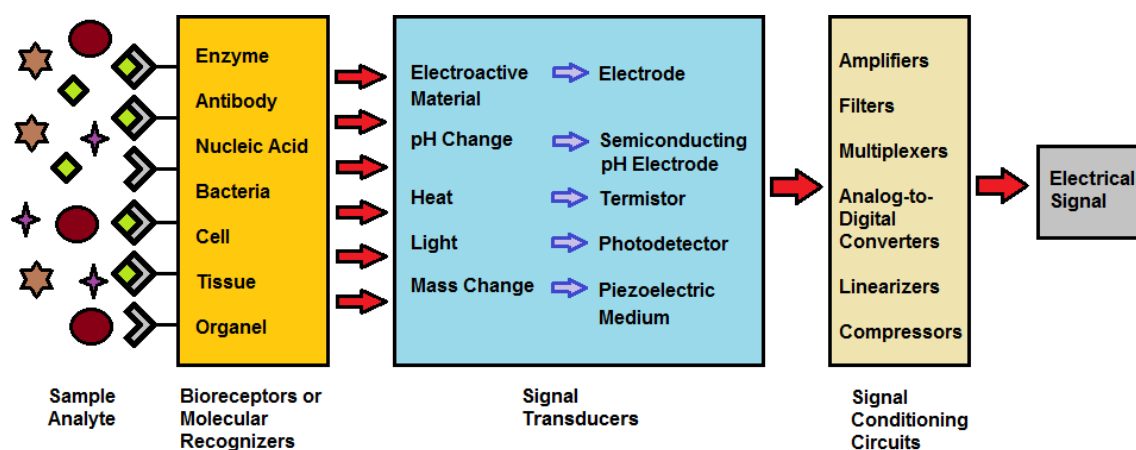


Figure 2.1. Schematic representation of a biosensor. Adapted by www.intechopen.com

2.2. Biosensors Classification

Biosensors may be classified according to the biological specificity conferring mechanism [17] or according to the transducing system used [Patel, 2002; Mello and Kubota, 2002 [20, 21].

2.2.1. Biological specificity conferring mechanism

Two modes of interaction may be identified involving either a biocatalytic or a bioaffinity recognition element.

2.2.1.1. Biocatalytic recognition element

The biocatalytic-based biosensors are the best known and studied and have been most frequently applied to biological matrices [19]. In this case, the biosensor is based on a reaction catalyzed by macromolecules, which are present in their original biological environment. These macromolecules were previously isolated or manufactured.

There are three types of biocatalyst that are commonly used:

Enzyme (mono- or multi-enzyme): These are the most common and well developed recognition system [17]. The specificity of enzymes with regard to recognition of substrates enables sensors incorporating enzymes with a limited range of substrates to achieve a greater selectivity for target species. Enzyme electrodes result from combining any type of electrochemical sensor with a layer of enzyme as thin as 10-200µm in close proximity to the active surface of the transducer. The analyte is most often a substrate or a product of the

enzyme reaction. The difficulties in achieving direct electron transfer between enzymes and electrodes has encouraged the use of small-molecule, electroactive mediators to enhance the rate at which the transfer of electrons occurs. The role of the mediator is to shuttle electrons efficiently between electrode and enzyme. Some examples of electrons transfer mediators are ferrocenes [19].

Whole Cells (microorganisms such bacteria, fungi or eukaryotic cells), cell organelles or particles (mitochondria, cell walls): In this device cells are fixed at the end of an electrode, between the sensing membrane of the probe and the retaining membrane. Dialysis membranes or microporous filter membranes typically are used for this purpose. The general principle of operation and response characteristics of cell based sensors are comparable to those exhibited by enzyme electrodes prepared with similar electrochemical detectors. These devices presents some advantages over enzyme electrodes, such reduced cost, higher biocatalytic activity, and improved stability and furthermore, they also offer the possibility of regeneration by storage in growth-medium to replenish the active cells, increasing the lifetime of the electrode. They, however, have the distinct disadvantage of poor selectivity, an inherent characteristic of microbial sensors attributable to the multireceptor behavior of intact cells. In addition, they require lengthy recovery times, often 3-4 hours, between measurements, mainly because of the close packing of the cells on the electrode, which leads to diffusion constraints. This would not be of concern for disposable devices.

These biosensors provide a simple and rapid means of measuring analytes that previously were undetectable by electrochemical methods. They may prove to be particularly valuable in the clinical analysis of biological fluids and for monitoring fermentation processes [19].

Tissue (plant or animal tissue slice): Whole tissue materials from plants or animals provide many advantages for the construction of biosensors. The enzymes remains in their natural environments which gives them high stability, high activity and low cost compared with isolated enzymes. Immobilization of the material however can be a problem and the use of tissues of sufficient thickness to maintain mechanical stability can result in slow responses, because of the long diffusion path between the analyte solution and the detector surface [19].

2.2.1.2. Bioaffinity recognition element

The biosensor operation is based on interaction of the analyte with macromolecules or organized molecular assemblies that have been isolated from their original biological environment or engineered. Thus there is no consumption of the analyte by the immobilized biocomplexing agent. These responses are monitored by the integrate detector. In some cases, this biocomplexing reaction is itself monitored using a complementary biocatalytic reaction.

Antibody-antigen interaction: The most developed examples of biosensors using biocomplexing receptors are based on immunochemical reactions receptors, i.e. binding of an antigen (Ag) to a specific antibody (Ab). Formation of Ab-Ag complexes has to be detected under conditions where non-specific interactions are minimized. Each Ag determination requires the production of a particular Ab, its isolation and, usually, its purification. In order to increase the sensitivity of immunosensors, enzymes labels are frequently coupled to Ab or Ag, thus requiring additional chemical synthesis steps. In the case of the enzyme-labelled Ab, the enzyme is there only to quantify the amount of complex produced. As the binding or affinity constant is usually very large, such systems are irreversible (single-use biosensor), or Ab may be regenerated by dissociation of complexes by chaotropic agents, such glycine-HCl buffer at pH 2.5.

These types of biosensors offer the advantage of a highly sensitive and selective approach to the detection and quantification of trace substances that makes use of the interaction between an Ab and Ag. This immunological interaction can be very specific under favorable conditions. Electrochemical immunoassay also presents an excellent detection limits that can be achieved on small sample volumes.

There are many ways to implement immunoassays based on voltammetric detection, depending on electrochemical technique, label type, and assay format. They also can be divided into heterogeneous assays, in which antibody bound antigen is separated from free antigen during the procedure, and homogeneous assays, in which there is no such separation step [19].

Nucleic Acids Biosensors: Electrochemical detection of DNA hybridization usually involves monitoring of a current response, resulting from the Watson-Crick base-pair recognition event, under controlled potential conditions. In these biosensors, the coated electrode with oligonucleotides is commonly immersed into a solution with a sequence of DNA that it will be tested. When the target DNA contains a sequence which matches to the immobilized oligonucleotide probe DNA, a hybrid duplex DNA is formed at the electrode surface. Such hybridization event is commonly detected via the increased current signal of an electroactive indicator (that preferentially binds to the DNA duplex), in connection to the use of enzyme labels or redox labels [22].

2.2.2. Used transducing systems

As mentioned before transduction can be optical, piezoelectric or electrochemical [17]. For the optical and piezoelectric systems, we can classify them as high sensitivity, but presenting the disadvantages of its complexity and equipment high cost. The oldest transducers are the electrochemical ones [23, 24] and definitely the most used to develop biosensors [23, 24, 25]. They provide an attractive means to analyze the content of a

biological sample due to the direct conversion of a biological event to an electronic signal. These devices feature high sensitivity and low detection limits, are easy to handle, have a quick response time and are not expensive [23, 26, 27]. Electrochemical detection techniques use predominantly enzymes, due to their specific binding capabilities and biocatalytic activity. However, antibodies, nucleic acids, cells and micro-organisms are also used.

Typically in bio-electrochemistry, the reaction under investigation would either generate a measurable current (amperometric), a measurable potential or charge accumulation (potentiometric) or a measurable alteration in the conductive properties (conductometric) of a medium between electrodes [10].

1) An amperometric biosensor measures the current produced when an electroactive specie is oxidized or reduced at a biomolecule-coated electrode to which an analyte interacts specifically.

2) Potentiometric devices measure changes in pH and ion concentration when an analyte in a sample interacts with a biomolecule immobilized on an electrode. The potential difference between the electrode bearing the biomolecule and a reference electrode is a function of the concentration of analyte in the sample.

3) Conductimetric and capacitive biosensors measure the alteration of the electrical conductivity in a solution at constant voltage, caused by biochemical reactions that specifically generate or consume ions. As these transducers are usually non-specific and have a poor signal/noise ratio, they have been little used.

2.3. Biosensors Preparation

Methods for the preparation of electrochemical electrodes are well established. Some of these techniques are used to prepare the conductive supporting substrate, while others are employed to achieve an efficient electrical communication between the chemical reaction site and the electrode surface, high levels of integration, sensor miniaturization, measurement stability, selectivity, accuracy and precision. In addition, the technique used to immobilize the biological recognition components of the sensor can affect biosensor performance significantly.

Traditional electrode systems for measurements of the concentrations of ions in liquids and dissolved gas partial pressures contain only a working electrode (W.E.) and an electrically stable reference electrode (R.E.), such as Ag/AgCl, though a counter electrode (C.E) is sometimes included (Fig. 2.2.) [16].

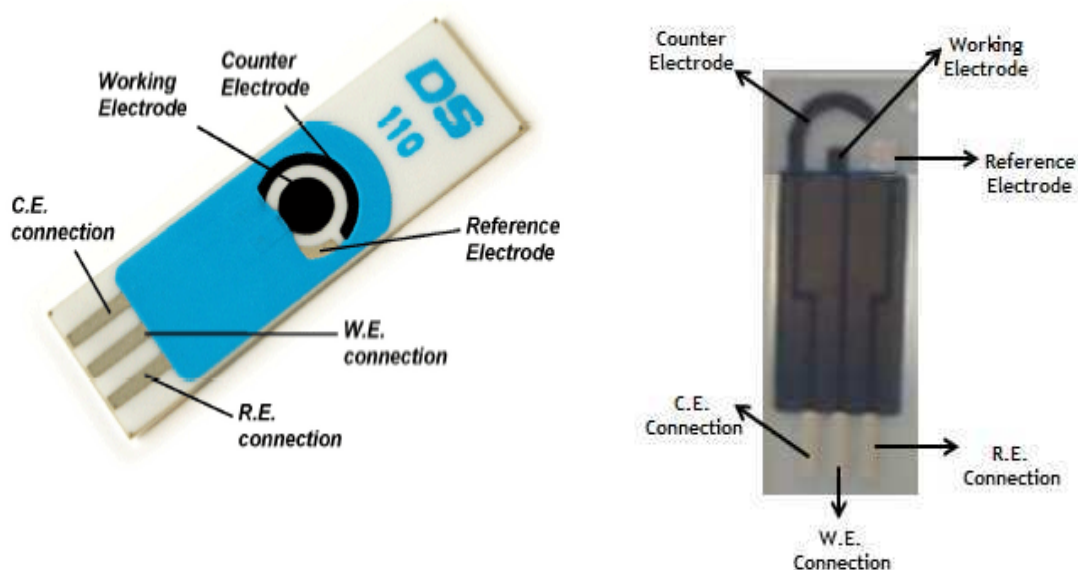


Figure. 2.2. Electrochemical electrodes and its constitution. Adapted by http://www.dropsens.com/en/screen_printed_electrodes_pag.html

2.3.1 Materials used in biosensors

The selection of materials and fabrication techniques is crucial for adequate sensor function and the performance of a biosensor.

Electrodes and supporting substrates:

Electroanalytical techniques are well considered in the Analytical Chemistry field. However, they have found several restrictions and practical difficulties for some applications. One of the most common problems of these techniques has been their lack of reproducibility, associated to the complexity of obtaining identical electrodes for all the measurements. Hanging drop mercury electrodes have presented fewer problems in this respect but their toxicity has led to the development of different alternatives. In this way, the possibilities of the electrochemical techniques can be improved by means of the replacement of the classical electrodes and cell systems by disposable screen-printed devices.

Recently, the change of the conventional solid electrodes by screen-printed electrodes (SPEs) has increased the possibilities of electroanalytical techniques in the biosensor field. Various advantages including simple fabrication, low cost, small size, disposability, portability and easily mass-produced reinforce the use of screen-printed

biosensors. Moreover, SPEs avoid some common problems related to traditional solid electrodes such as the needed cleaning processes. Thus, SPEs are extensively used in the fabrication of disposable biosensors [28].

SPEs add many attractive advantages to the electroanalytical techniques, including the elimination of the surface regeneration needed in solid electrodes. Moreover, SPEs can be designed according to the analytical problem characteristics by choosing the adequate fabrication materials.

Selective and disposable biosensors can be easily obtained by biomolecules immobilization on SPEs surfaces.

Procedures based on these disposable devices have been shown as practical systems for the fast, accessible and low cost analysis of many target species, including microorganisms. In the same way, the immobilization of microorganisms on the electrode has given rise to the development of selective and sensitive disposable biosensors for the analysis of different substances [29, 30].

Usually carbon-based materials such as graphite, carbon black and carbon fiber are also used to construct the conductive phase in the SPEs. These materials have a high chemical inertness and provide a wide range of anode working potentials with low electrical resistivity. They also have a very pure crystal structure that provides low residual currents and high signal-noise ratio.

Nanomaterial, such noble metal nanoparticles (NMNPs), inorganic nanotubes/nanowires and semiconductor quantum dots, are also highly used as they exhibit unique electronic, optical, thermal and catalytic properties. Especially NMNPs possess advantages over other nanomaterials, including stability, extraordinary conductivity, biocompatibility, large surface-to-volume ratio, magnetic and optical properties.

The dimensional similarities of NMNPs with biological molecules and large surface areas provide opportunities for stable immobilization of biomolecules with their bioactivity maintained. Additionally, the stability and biocompatibility of NMNPs make them easy to conjugate with multiple species of biomolecules, chemical groups and polymer materials. Moreover, their conductivity facilitates the electron transfer between the redox center of biomolecules and electrodes surface.

Due to the significant role of NMNPs in the biosensor fabrication, how to prepare NMNPs with appropriate size, shape, assembly and surface modification becomes primary element which determines the performance of a biosensor. In general, the synthesis of NMNPs involves the chemical reduction of noble metal salt in aqueous or organic phase. However, the high surface energy of NMNPs makes them extremely unstable and easy to undergo aggregation without protection or passivation of their surfaces. As a result NMNPs are typically synthesized in the presence of a stabilizer/surface protector which binds onto particle surface to improve their stability and solubility as well as provide charge and chemical groups [27].

2.3.2. SPCPEs fabrication

Screen-printing technology is based on the sequential layer deposition of different inks on a ceramic or plastic substrate using the appropriate screen. A typical screen is made from a finely mesh of different materials including stainless steel, polyester or nylon mounted under tension on a metal frame. The finished screen has open-mesh areas through which the desired pattern can be printed (Fig. 2) [31].

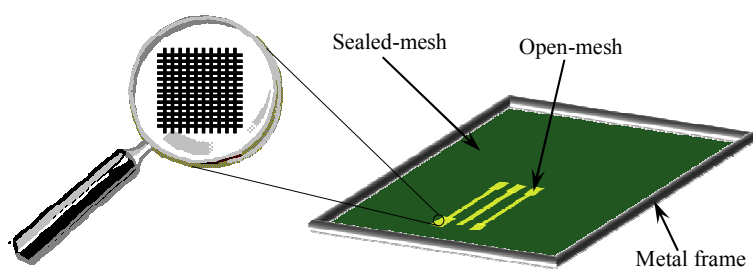


Figure 2.3. Scheme of a screen.

The screen-printing ink is then poured onto the top surface of the stencil. Then, a squeegee slowly moves from the rear to the front part of the screen, forcing the ink through the open areas. The required pattern is thus deposited onto the substrate surface, as it can be seen in Fig. 3 [31].

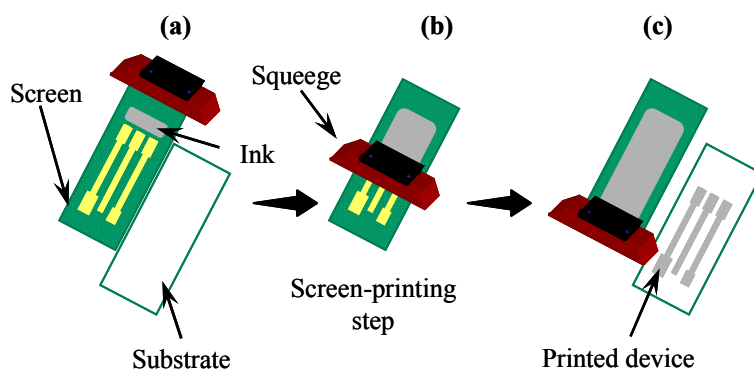


Figure 2.4. Screen-printed device fabrication process scheme: (a) Ink poured; (b) Squeegee traversing; (c) Ink deposited on the substrate surface

The next phase of the process is to dry the printed ink. Screen-printing inks usually contain various organic solvents, which are added with the aim of producing the accurate viscosity for screen printing. These solvents can be removed by drying the printed ink in an oven at an adequate temperature. After drying, the substrate retains a rigid pattern that is relatively immune to smudging. The combination of different screens and inks give rise to the

definition of the different electrodes (working, reference and auxiliary) in the same configuration unit.

Finally, the disposable electrochemical biosensor is generated by the subsequent modification of the working electrode with the biosensing material. This modification implies several steps in order to assure the robustness and durability of the developed biosensor.

Disposable biosensors generated by modification of SPEs have been successfully applied in the microbiology field. The next section involves the report of different disposable biosensors employed in such field, including the description of the different biocomponents and immobilization procedures used in their development.

Biological elements:

Biologic elements include enzymes such glucose oxidase and lactate oxidase, , cofactors based on nicotinamide adenine dinucleotide (NADH^+ and NADP^+), antibodies, antigens and nuclei acids. Also mediators, such ferrocene and its derivatives, are frequently included in order to decrease the measurement potentials and avoid interferences [15].

2.3.3. Immobilization of biological materials

Biological materials, i.e. enzymes, antibodies, cells or tissues with high biological activity can be immobilized in a thin layer at the transducer surface by using different procedures. However, the biomolecule immobilization is the critical step of the development of any biosensor. Biomolecule provides the core of the biosensor and gives it its identity. Moreover, the immobilized biomolecule needs to keep its original functionality as far as possible in order for the biosensor to work. Another common reason for biosensor failure or underperformance is the chemical inactivation of the active/recognition sites during immobilization stages. There is no universal immobilization method suitable for every application [18].

Adsorption: Is the simplest technique; the physical adsorption is based on van der Waals attraction between biomolecule and solid support surface; in this immobilization mild conditions are used, which are less disruptive to biomolecule. However, biomolecule linkages are highly dependent on pH, solvent and temperature and the adsorption occurs randomly, which does not guarantee the proper immobilization.

Entrapment: The physical adsorption occurs in matrices such gels or polymers. The biological material is usually mixed and well homogenized with the supporting material and then this solution is placed in contact with the electrode. This method carries large diffusional barriers and loss of biomolecule activity.

Crosslinking: The procedure is simple and the chemical binding of biomolecules are strong. This method is based on formation of three-dimensional (3D) links between the

biological material and bi or multifunctional reagents. The resulting modified biological material is insoluble in water and can be adsorbed on a solid surface. Crosslinking is a widely used technique in stabilizing physically adsorbed biomolecules that are covalently bound onto a support. However requires a large amount of biomolecules [16]

Covalent: A stable technique, ideal for mass production and commercialization. This method require the activation of active groups (-COOH, -NH₂, -OH) on the surface of electrode. However the procedure is complicated and time-consuming. Exists the possibility of activity losses. [16, 32]

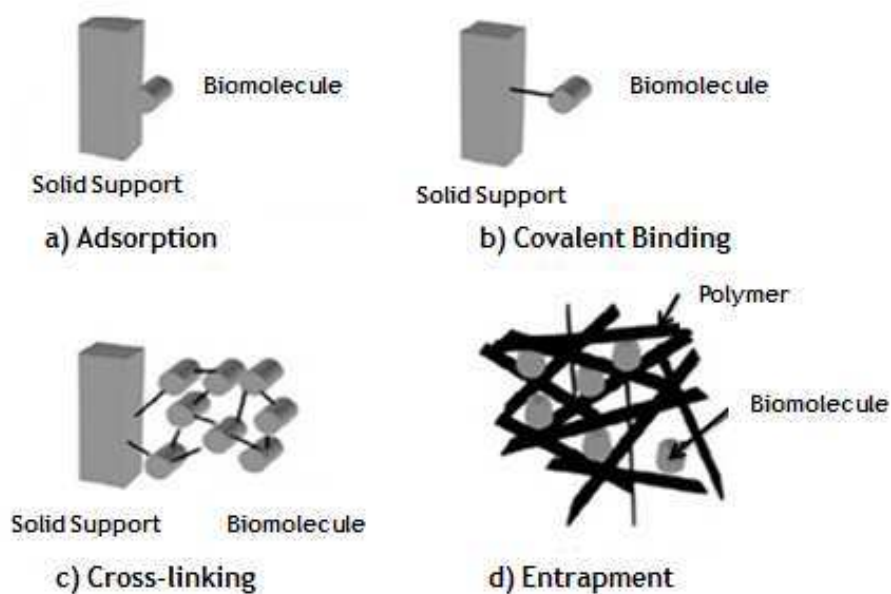


Figure 2.5. Different immobilization methods. Adapted by <http://english.tebyan.net>

Chapter 3 - Immunosensors

Immunosensors are miniaturized measuring devices, which selectively detect their targets by means of antibodies and provide concentration-dependent signals. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex. Since sensitivity is directly related to the affinity of the ligand binding the quality of immunosensors primarily depends upon the selectivity and affinity of the Abs used in the receptor unit [33].

3.1. Components of immunosensors

Just like others biosensor, immunosensor has a tripartite composition, the biological component, the signal transducer and the signal conditioning. Here, the biological component, the Ab, conveys selectivity and sensitivity to the sensor. The Ab binds the analyte with high affinity and is therefore able to detect the analyte in the presence of other substances. The transducer converts the particular biochemical or biophysical event into an electrical signal, in this case converts the binding of the analyte to Ab into an electric signal. Finally this signal is amplified and digitalized by an electronic part [33].

3.2. Immunosensors principles

Immunosensors are based on the ability of antibodies to form complexes with the corresponding antigens. This property of highly specific molecular recognition of antigens by antibodies leads to high selectivity of assays based on immune principles. The extreme affinity of antigen-antibody interactions results in great sensitivity of immunoassay methods (Fig. 3.1.) [34].

Immunosensing can be done either directly or indirectly, meaning that the detection mechanism operates either directly via Ab/Ag interaction, or through a label, such as enzymes or fluorescent molecules, that are used in order to detect if a binding event has occurred.

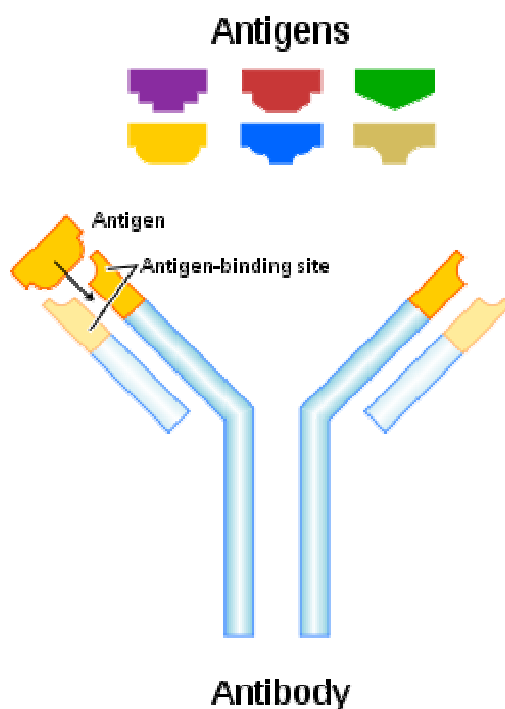


Figure 3.1. Formation of antibody/antigen complex. Adapted by www.lupusresearch.org

3.3. Electrochemical Immunosensors

Electrochemical immunosensors can be based on potentiometric, amperometric or impedimetric transduction principals. Inherent benefits of electrochemical sensors include selectivity, ease of use, low detection limits and scope for miniaturization [33].

3.3.1. Potentiometric Immunosensors

The potentiometric biosensors measure changes in pH and ion activity when an analyte in a sample interacts with biomolecule immobilized on an electrode. The potential difference between the electrode bearing the biomolecule and a reference electrode is a function of the concentration of analyte in the sample.

Challenges to the use of these devices are lack of sensitivity to distinguish between concentrations within the same order of magnitude and often a high occurrence of non-specific binding effects in comparison to their amperometric counterparts, due to their logarithmic signal response characteristics [32, 35].

3.3.2. Amperometric immunosensors

Amperometric immunosensors designs measure changes in current in response to an electrochemical reaction at a set voltage. Are generally favored over potentiometric designs in research and sensor development. They consume a small percentage of analytes during measurement, which means that either the analyte must be redox active or use of an active label will be necessary. Consumption of the analyte creates concentration gradients.

Usually amperometric sensors require a redox reagent to allow electron flow [35].

3.3.3. Impedimetric Immunosensors

The use of impedance/capacitance to detect Ab/Ag complex formation was first reported when upon formation of this complex on the surface of the electrode, the increase in dielectric layer thickness caused changes in capacitance proportional to the size and the concentration of antibodies. Binding of the antigen led to a drop in capacitance, giving immunosensors with detection limits of 1mg l^{-1} .

Impedance changes between electrode surfaces and a surrounding solution upon a binding event can be transduced into an electrical signal using a frequency response analyzer [35].

3.3.4. Voltametric Immunosensors

Voltammetry belongs to a category of electro-analytical methods, through which information about an analyte is obtained by varying a potential and then measuring the resulting current. It is, therefore, an amperometric technique. The voltage is measured between the reference electrode and the working electrode, while the current is measured between the working electrode and the counter electrode. The obtained measurements are plotted as current vs. voltage, also known as a voltammogram [36].

3.4. Antibodies immobilization

In the immobilization process, the critical problem is the antibody denaturation or conformational changes during or after immobilization, that will lead to reduced immune-sensor sensitivity. These types of antibody inactivation are frequently caused by non-specific adsorption of antibodies on solid supports. Antibody modification, especially on antigen binding sites, is also one of several factors diminishing the antigen binding sites. Random chemical tagging of antibody is often associated with this antigen binding site modification.

The oriented immobilization of antibodies has become critical for optimized antigen detection on solid surfaces (Fig. 3.2). Multiple studies have demonstrated that properly oriented antibodies, with their antigen binding sites well exposed to the solution containing the analyte, exhibit higher antigen binding capacities compared to randomly oriented antibodies. Uniformly oriented immobilization also provides high consistency for the construction of immunosensors.

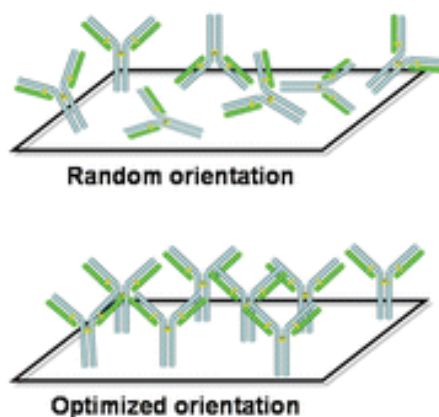


Figure 3.2. Random vs Optimal Antibodies Immobilization. Adapted by [37].

Antibody-antigen interaction on a solid substrate is also affected by nature of the spacer between the bound antibody and the solid surface. When antibodies are directly conjugated to the surface, antigen detection is often interfered with by steric hindrances and the limited mobility of bound antibodies. Antibodies immobilized *via* a long and flexible linker(such PEG) captures target antigens nearly two times more efficiently than directly-linked antibodies. Generally, following antibody immobilization, the remaining non-specific active sites must be blocked by proteins such as bovine serum albumin (BSA).

Various methods have been described for antibody immobilization [37] some of them are presented below.

3.4.1. Adsorption

Antibody adsorption on solid surface is by far the easiest method of antibody immobilization. It occurs through hydrophilic, hydrophobic or both types of interactions between antibodies and target solid substrates. This method uses solid supports including plastic surfaces (polystyrene), membranes (nitrocellulose) and various metallic surface and carbon. However adsorbed antibodies are randomly oriented and can lose their antigen binding abilities by denaturation. Moreover, as surfaces are mostly designed to capture proteins, background signals are generally high. Despite these drawbacks of antibody adsorption, the methods have been used in many applications such as ELISA, antibody arrays,

and immuno-sensors due to their simple procedure (no modifications of antibodies are required) and high antibody-binding capacity [37].

3.4.2. Covalent Immobilization and modification

Covalent cross-linking of antibodies on chemically-activated solid surfaces is the most common method for antibody immobilization. Amino groups on the antibody surface can be readily coupled with several reactive moieties, such as aldehyde, epoxy, and *N*-hydroxysuccinimide (NHS) on various solid surfaces. The surface activation process, on different substrates including glass and gold, is well established. Although chemical immobilization is a highly stable process, covalently-linked antibodies through amino groups are randomly oriented. The antigen binding capacities of randomly-coupled antibodies were 2-3-fold lower compared to the capacities of well-oriented antibodies [37].

3.4.3. Antibody-binding proteins

Immunoassays employing antibody-binding proteins for antibody immobilization regularly exhibit higher sensing abilities compared to those using conventional methods such as random covalent immobilization. One of the major concerns associated with the method is the immobilization process for antibody-binding protein coating on the surface prior to antibody immobilization [37].

3.5. Application of Nanoparticles in Immunosensors

The use of nanoparticles for biosensing is showing an increased interest between the different applications in several areas, such as clinical analysis, environmental monitoring as well as safety and security.

As the sensitivity of the immunosensor is mainly determined by what kind of label is used, different types of materials have been investigated as labels [38]. Although large signal amplification can be achieved by enzyme labeling, preparation procedure is not a straightforward route, and once prepared the activity of the conjugate must be periodically controlled owing to the inherent poor stability of enzymes. A recently new type of labels based in the use of metallic nanoparticles (NP) has been described in literature [38]. Colloidal gold nanoparticles (AuNPs) have been widely used as labels for analytical signal amplification [39]. These nanoparticles have advantages over other nanomaterials such as rapid and easy synthesis, narrow size distribution and desirable biocompatibility. Furthermore, AuNPs can be easily conjugated with biomolecules and retain the biochemical activity of the tagged

biomolecules, leading AuNPs to be excellent transducers for several biorecognition applications [39].

3.5.1. Antibodies, modification with nanoparticles

The structure of IgG, the most used antibody in immunosensing assays, has been determined by x-ray crystallography and shows a Y-shape consisting of three equal-size portions, loosely connected by a flexible tether. This antibodies are constituted by two heavy and two light polypeptide chains linked (Fig.3.3.).

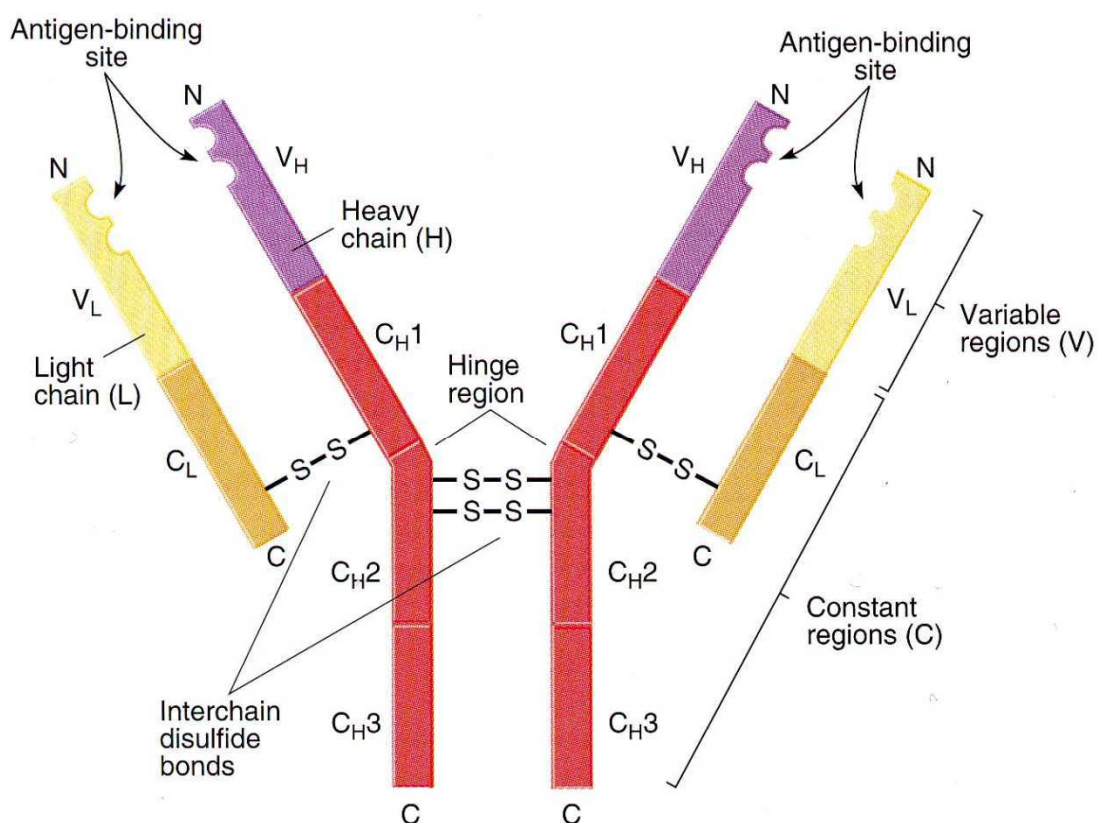


Figure 3.3. Antibody Structure

The C regions determine the isotype of the antibody whereas the variable region of one heavy and one light chain constitute an antigen binding site (ABS).

As was said above immunoassays are based on the interaction between the antibody and the antigen, in particular, between the ABS and epitope. In order to functionalize the Ig, the connection of labels, like the nanoparticles, can occur through three main groups: -NH₂, -COOH and -SH. However is preferable to connect to the carboxy-term of the antibody because the C-term region is far from the ABS and should allow the molecule interaction with the antigen. The AuNP modification of antibody can be observed by TEM [40].

3.5.2. Applications of nanoparticles to indirect electrical detections

The immunogold labeling technique is commonly used because of the high electron density of gold nanoparticles (AuNPs). However, this trace label generally requires a chemical [41] or electrochemical oxidation [42] pretreatment prior to detection of electrochemical signal.

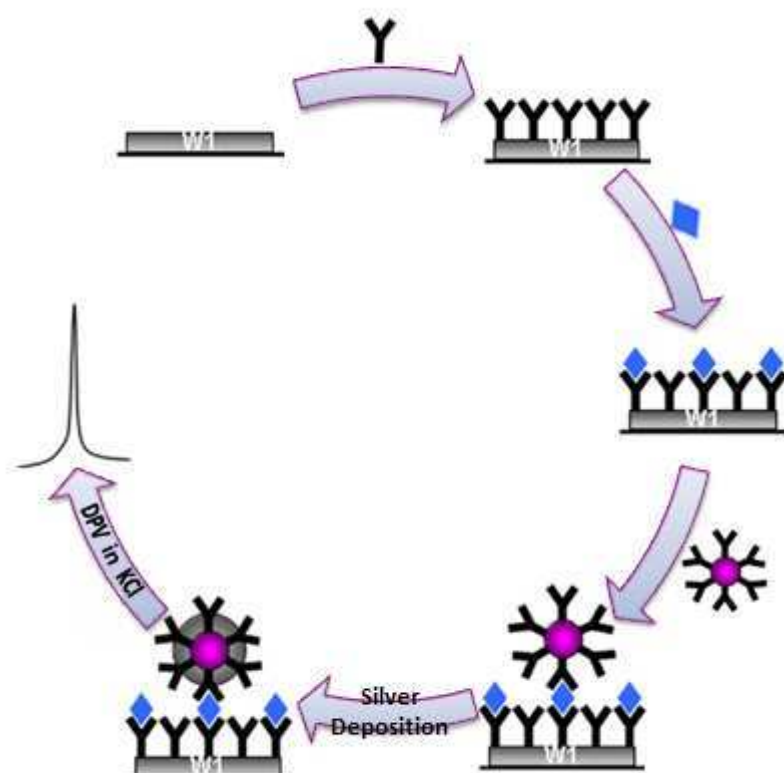


Figure 3.4. Schematic representation of preparation of immunosensor array and detection strategy by differential pulse voltammetric analysis of Ag NPs catalytically deposited on the immunosensor surface by gold nanolabels.

It is known that AuNPs exhibited a dark brown spot under an electron microscope resulting in a distinct image for labeling and that this image response can be further enhanced through immunogold silver staining; in this technique silver particles are precipitated on the surface of the AuNPs [40]. Thereby, the well-known catalytic properties of AuNPs on silver reduction developed a very sensitive methodology based on selective electro-reduction of silver ions on the surface of AuNPs. The same principle can be used for the electrochemical detection, Silver nanoparticles (AgNPs) are precipitate on the surface of

the AuNPs, and after that, the silver can be dissolved and measured by anodic stripping voltammetry [43].

3.6 Biosensors reported in the literature for CMV detection

Few reports can be found in literature associated with CMV detection. Two of them are related with the detection of amplified human cytomegalovirus DNA sequences [41, 44], by the use of miniaturized sensing devices. Azek et al. [44], proposed a low cost DNA sensor based on disposable screen-printed carbon electrodes. Target DNA was adsorbed and hybridized with a biotinylated DNA probe and the extent of hybrids formed determined with streptavidin conjugated to horseradish peroxidase. The intensity of differential pulse voltammetric peak currents resulting from the reduction of the enzyme-generated product was related to the number of target amplified DNA molecules present in the sample. Although large signal amplification can be achieved, labeling of an oligonucleotide by an enzyme is not a straightforward route, and once prepared, the activity of the conjugate must be periodically controlled owing to the inherent poor stability of enzymes. To overcome this limitation, Authier et al. [41] explored an electrochemical amplification strategy based on colloidal gold nanoparticle labels for the sensitive quantification of an amplified 406-base pair human cytomegalovirus DNA sequence. The assay relied on the hybridization of the single-stranded target CMV DNA with an oligonucleotide-modified Au nanoparticle probe, followed by the release of the gold metal atoms anchored on the hybrids by oxidative metal dissolution, and the indirect determination of the solubilized Au III ions by anodic stripping voltammetry at a sandwich-type screen-printed microband electrode. Nevertheless, both described methods [41, 44] are used in conjunction with the PCR technique for characterization and quantification of amplified products, being useless as rapid tests.

A piezoelectric affinity sensor has been also described in the literature by Susmel et al. [45] to detect gB epitope of the human cytomegalovirus. This work was a preliminary study that use antibodies immobilized on a gold electrode. Although the technique does not rely on amplified DNA, is required the use of prohibitively expensive instrumentation for its implementation.

More recently, in France, a consortium has been established in order to set-up an original device toward the screening of CMV [46]. The device consists of a disposable cartridge containing the biological sample and the reactive liquids required for immunofluorescence detection on a functionalized surface. A biological sample is applied onto a gold surface coated with CMV specific antibodies. If present in the biological sample, CMV is trapped onto the surface by means of the antibodies. After the injection of a fluorescent probe (Cy5 labelled antibodies) a fluorescent signal is detected. Nevertheless, this method was applied to a biological fluid combining fetal urine with amniotic fluid, where viral loads are high. The main drawback of this device is the low sensitivity that compromises its applicability to samples with low viral loads.

Chapter 4 - Goal of study

Primary infection, reactivation and reinfection during pregnancy can all lead to in utero transmission to the developing fetus. Congenital CMV infections are a major cause of permanent hearing loss and neurological impairment. CMV infection was relatively common among women of reproductive age, with seroprevalence ranging from 45 to 100% [1].

Another risk group is immunosuppressed individuals, as transplanted ones, persons infected by HIV or individuals with immature immune system (fetuses and newborns). As mentioned before, in immunosuppressed individuals may be diagnosed pneumonia, retinitis, colitis and encephalopathies [3].

For the diagnosis of HCMV, highlighted in first chapter, there are several methods, however, these are expensive, or/and require long time to perform, or/and need skilled operators, so it's important to develop a fast, simple and cheap method to detect HCMV.

Therefore, in this work it is proposed the development of an immunosensor for HCMV diagnosis. Combining the excellent stability of nanogold label with the convenient stripping analysis of Ag NPs and a disposable immunosensor, an electrochemical sandwich type immunoassay method will be developed for ultrasensitive detection of HCMV by the catalytic deposition of AgNPs by the captured AuNPs. The advantages of this method are that is a cheap and fast method and presents a high specificity, due to Ab/Ag interaction.

Chapter 5 - Background

5.1. Production, recovery and purification of a fusion protein containing the antigenic domain 1

For the development of reliable and inexpensive serodiagnostic tests, the AD-1 domain of HCMV glycoprotein G, which is known to bind neutralizing antibodies, was expressed in *Escherichia coli*, because it remains one of the most attractive, and has been widely used in basic research studies as a host strain for the overproduction of recombinant proteins [6]. Then, the general strategy for protein recovery includes three consecutive steps: isolation and washing of inclusion bodies, solubilization of aggregated protein, and refolding of solubilized protein [47]. Finally the isolation of fusion protein was performed by gel filtration chromatography [48].

5.1.1. Production in *Escherichia coli*

The fusion protein containing the antigenic domain (AD-1) is produced by the fermentation of a recombinant strain of *Escherichia coli* (*E.coli*) containing the plasmid Mbg58, which expresses the AD-1 (aa484-650) of human cytomegalovirus glycoprotein B as a fusion protein together with aa 1-375 aa of β -galactosidase [6].

The production of AD-1 in *E.coli* was possible because the antibody binding domain isn't affected by glycosylation. *E.coli* is widely used for production of heterologous proteins because it presents some advantageous characteristics, such as genetic metabolism and other biochemical processes well known such, the existence of several well-characterized cloning vectors, the efficiency in the production of recombinant proteins, and the fact that the processes developed based on *E. coli* are attractive in the economic level [3].

An important characteristic of promoters (*lac* and derivatives) used in recombinant protein production in *E.coli* is their inducibility. Induction by IPTG is widely used for basic research; however, its use in large-scale production is undesirable because of its high cost and toxicity. So Sousa *et al*, (2006) showed that lactose could be used as an inducer in fermentation process for production of this protein and that expression levels could exceed those achieved with IPTG. The use of lactose for protein expression in *E.coli* is extremely useful for the inexpensive large-scale production of heterologous proteins in *E.coli* [6].

5.1.2. Recovery

The expression of heterologous proteins in *E.coli* often leads to their production in form of inclusion bodies, insoluble and inactive aggregates [3]. The general strategy for protein recovery includes isolation and washing of inclusion bodies, solubilization of aggregated protein and refolding of solubilized protein. The first and very important step in recovering intracellular proteins is cell disruption. Several devices for cell disruption by chemical, enzymatic or physical methods are available. The design and operation of these methods is case-dependent and thus are difficult to generalize [47].

The lysis process used involves combining two methods, enzymatic (lysozyme) and mechanical (sonication) which improves the efficiency of cell disruption. Inclusion bodies can be isolated by centrifugation or by filtration from the cells extracts, and solubilized by strong denaturants such as urea, which promote the disruption of intermolecular interactions. Washing of inclusion bodies with Triton X-100 / EDTA promotes their isolation by elimination of the membrane proteins and other contaminants present in extract [3]. The solubilization process of inclusion bodies was based in the utilization of two consecutive extractions with urea 8M, a powerful denaturant agent that leads to breaking of molecular interactions. While the first step of solubilization eliminates the majority of lysozyme contamination, the second solubilization allow an efficient fusion protein recovery with high yield.

5.1.3. Purification

To eliminate the traces of lysozyme presents in the sample extract was made a last step of formulation. The differences molecular weight between fusion protein and lysozyme allow separation through gel filtration in a column of Superose pep grade [48].

5.2. Construction of Immunosensor

In this work we construct a disposable immunosensor that combines the excellent stability of nanogold label with the convenient stripping analysis of silver nanoparticles. The principle of this procedure consists in the fact of the attached gold nanoparticles act as nuclei and catalysts to induce the reduction of silver ion from the silver deposition solution. The higher concentration of analytes means that more amounts of gold nanoparticles was capture on the sensor surface, producing more silver nanoparticles. After this, the anodic stripping analysis of the quantitatively deposited silver nanoparticles was used for HCMV detection [49].

Silver deposition on gold nanoparticves is commonly used in histochemical microscopy to visualize the distribution of an antigen over a cell surface [50].

Chapter 6 - Material, apparatus and methods

6.1. Reagents

Recombinant *E. coli* W3110 harbouring plasmid pMbg58 was a kind gift from Dr. M. Mach (Universität Erlangen-Nürnberg, Germany).

Cytomegalovirus Glycoprotein B and Anti-Cytomegalovirus glycoprotein B antibody were purchased from Abcam, Cambridge, United Kingdom.

Bovine Serum (BSA), lysozyme, Chloroauric acid, tris(hydroxymethyl)aminomethane, nitric acid, potassium carbonate, trissodium citrate, potassium chloride, twee-20 and Silver Enhancer solutions were obtained from Sigma-Aldrich, Steinheim, Germany.

In the fabrication of screen-printed electrodes several inks were used, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) supplied by Achenson Colloiden (Scheemda, Netherlands). Commercial SPEs were obtained from DropSens (Llanera (Asturias), Spain)

6.2. Apparatus and software

Cell disruption has been achieved by sonication, centrifugation

Isolation of fusion protein was performed, on a 2.6 x 95 cm and 1.6 x 23 cm columns packed with Superose 12 - prep grade gel, (Amersham Biosciences, Uppsala, Sweden) with ÄKTApurifier 10, using Unicorn Software control (GE Healthcare Life Sciences, Uppsala, Sweden).

SPEs were produced on DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke (Figure 6.1).



Figure 6.1 - DEK 248 printing machine

Electrochemical measurements were performed Autolab PGSTAT128N electrochemical system (Fig. 6.2) and μ Autolab type II with General Purpose Electrochemical System (GPES) software version 4.9 (Eco Chemie B.V., Utrecht, Netherlands).



Figure 6.2 - Autolab PGSTAT128N electrochemical system



Figure 6.3. μ Autolab type II

6.3. Methods

6.3.1. Production, recovery and purification of a fusion protein containing the antigenic domain 1

Bacterial Strains and Plasmid

For the development of reliable and inexpensive serodiagnostic tests, the AD-1 of HCMV glycoprotein gp58, which are known to bind neutralizing antibodies, was expressed in prokaryotic systems.

Among many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains one of the most attractive, and has been widely used in basic research studies as a host strain for the overproduction of proteins from cloned genes. The lac promoter-derived expression systems are generally used for the production of heterologous proteins in *E. coli*, and one of the most commonly used strategies is its induction with the nonmetabolizable analog of lactose, .

Recombinant *E. coli* W3110 harboring pMbg58 was used for the production of a fusion protein, which is the antigenic domain 1 (AD-1) of glycoprotein B of human cytomegalovirus, fused to the truncated β -galactosidase.

Production

Cells, from a bank, frozen at -80°C , are plated on plates containing Luria-Bernati agar 35g/L and ampicillin 100 $\mu\text{g}/\text{mL}$. The growth of the strain on solid medium will take place overnight at 37°C . Then Luria-Bernati 20g/mL, supplemented with 100 $\mu\text{g}/\text{mL}$ of ampicillin was used as pre-culture and culture media. All bacterial growths were performed in flasks of 4-fold greater volume than the culture volume and agitated at 250 rev/min to provide adequate aeration. To reduce variations in expression levels, a preculture was made. The inoculation of pre-culture is made removing all well-defined cell colonies and cultivated at 37°C on a rotary shaker until OD_{600} was approximately 2.6. Culture will start with optic density (OD) of 0.2. Thereby, it should be removed a specific volume of pre-inoculum that is calculated from the follow equation:

$$\begin{aligned} & \text{OD}_{\text{pre-inoculum}} \times V_{\text{to removed from the pre-inoculum}} = \\ & = (V_{\text{to remove from the pre-inoculum}} + V_{\text{fermentation}}) \times \text{OD} \end{aligned}$$

To monitoring the growth of the recombinant *E. coli* W3110 strain it is necessary taking samples at an interval of 1 hour.

In all culture experiments, cells were induced when the optical density at 600 nm (OD_{600}) reached 0.8-1, by adding lactose (0.75 mM). Cells were cultivated for another 4h and then were harvested by centrifugation at 5445 rpm for 20 min at 4°C [6].

Recovery

Disrupt method: The cells harvested at the end of fermentation were subjected to resuspension after addition of 5 mL of lysis buffer (50 mM Tris/HCl, pH 8.0; 25% (w/v) sucrose; 1mM EDTA), then 12.5 mg lysozyme previously dissolved in 1.25 ml of lysis buffer was added to the extract. The extract was placed on ice for 30 min, proceeding to occasional stirring. After the incubation, the tubes were placed in an ice bath and subjected to 4 pulses of sonication. Each pulse lasts 30 seconds. After this, 6.250 mL of MgCl₂ 10 mM and MnCl₂ 1 mM were added and allowed to incubate at 37°C during 1 hour (until viscosity appears). After the incubation 62.5 µL of DNase were added and, once again, allowed to incubate at room temperature for 1 hour. After this, 12.5 mL of detergent buffer (0.2M NaCl; 1%(w/v) deoxycholate; 1%(v/v) Niaproof; 20mM Tris/HCl, pH7.5; 2mM EDTA) were added, and the lysate centrifuged for 15 min at 5000g for recovering the fusion protein aggregated into inclusion bodies. At this stage a sample of the supernatant (1 mL) should be taken for analysis by SDS-PAGE. The remaining supernatant was discarded.

Inclusion body isolation: The pellet was suspended in 2.5 mL washing solution (0.5% Triton X-100 plus, 1mM EDTA) and centrifuged for 10 min at 5000g. Several more pellet suspensions have been done, followed by centrifugation at 5000g for 10 min. The first one was performed in 2.5 mL of washing solution, the second in 2.5 mL of citric acid, and the last one in 2.5 mL of washing solution. The supernatant was discarded.

Inclusion Body Solubilization: The pellet was suspended in 12.5 ml of urea 8 M. The extract was placed on ice with stirring, where it remained overnight, after which was centrifuged at 18000g for 10 min at 4 ° C (after centrifugation an aliquot of the supernatant was reserved for analysis and the rest discarded). The pellet was subjected to a second suspension in 12.5 mL of urea 8 M in the same conditions described above. The agitation was maintained for approximately 10h, after what centrifugation was again carried out at 18000g for 10 min at 4 ° C. The pellet was discarded and all of the recovered supernatant conserved [48].

Purification

Isolation of fusion protein was performed by gel filtration chromatography on a 2.6 x 95 cm and 1.6 x 23 cm columns packed with Superose 12-prep grade gel (Amersham Biosciences, Uppsala, Sweden). The columns were equilibrated with saline phosphate buffer (PBS 10 mM, pH 7.4, 0.14 M NaCl), at 0.8 ml/min. Protein samples, resulting from inclusion bodies solubilization, were then injected (10 ml and 1 mL respectively) and species were eluted at the same flow rate of buffer. The absorbance was monitored at 280 nm. Fractions were pooled according to the chromatograms obtained and the proteins recovered were analyzed individually by standard SDS-polyacrylamide gel electrophoresis (PAGE) [48].

6.3.2. Biosensor Construction

The operating principle of the designed biosensor is through a sandwich-type immunoreaction, antibody-functionalized AuNPs were captured onto the immunosensor surface to induce the silver deposition from a silver enhancer solution. The deposited Ag NPs could be directly measured by anodic stripping analysis. So, gold nanoparticles were prepared and antibodies labeled with these nanoparticles. After that, were tried two different methods for antibodies immobilization, but the way how this was done will be presented in the discussion.

Preparation of Gold Nanoparticles (AuNP)

Briefly 100 mL of 0.01% HAuCl₄ solution was boiled with vigorous stirring, and 2.5 mL 1% trisodium citrate solution was quickly added to the boiling solution. When the solution turned deep red, indicating the formation of Au Nps, the solution was left stirring and cooling down [51].

Preparation of Au NP-labeled antibodies

100 μ L of anti-gB of HCMV antibody was added to 900 μ L of colloidal Au NPs adjusted to pH 9.0 with 0.1 M K₂CO₃ and gently mixed for 60 min at room temperature. After centrifugation at 4800 rpm for 30 min, the supernatant containing the excessive antibody was discarded and the soft sediment was washed with 50 mM pH 7.2 Tris-HNO₃. After another centrifugation and discarding the supernatant, the resulting Au NP-labeled antibodies were suspended in 1.0 mL of 50 mM pH 7.2 Tris-HNO₃ containing 1.0% BSA and stored at 4 °C [49].

Chapter 7 - Results and Discussion

This chapter describes primarily the obtained results for the process of production and purification of glycoprotein B of Human cytomegalovirus (Section 7.1). Secondly it is described in detail all the process of immunosensor fabrication, which includes the method used to determine the glycoprotein B (gB), as well as the optimization of the concentration of the silver enhancer solutions and the silver deposition time in the immunosensor surface (Section 7.2.).

7.1. Production of antigenic domain of HCMV's gB

After antigenic domain 1 (AD-1) of human cytomegalovirus glycoprotein B expression in *E. coli*, protein recovery has been done by inclusion bodies isolation, washing and solubilization. Aliquots reserved during these steps were analyzed by SDS-PAGE (Fig. 7.2.1). Comparing figure 7.2.1. with figure 7.2.2. we can observe that this method allow the production of fusion protein containing the antigenic domain 1, because it is possible to check its presence by SDS-PAGE gel electrophoresis. However, it is clearly visible that the concentration obtained is substantially smaller than the concentration presented by S. Ferreira *et al.* (Fig. 7.1.2.) [47]. This can be concluded based on the fact that Ferreira results were obtained from 1mL of cells sample and diluted 8 fold. Several attempts were done in order to get better results, but all to no avail; thereby we suppose that probably the cell line used was not in perfect conditions, since it has been stored for a long period of time.

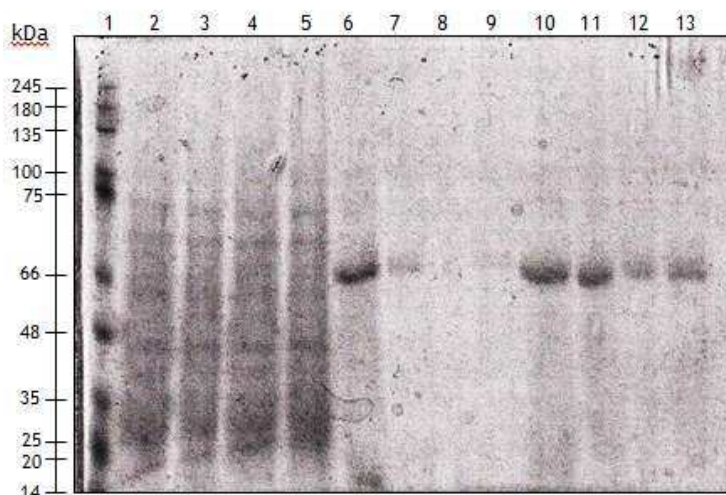


Figure. 7.1.1. SDS-PAGE gel electrophoresis of protein recovered. Lane 1 represents the molecular weight marker; lane 2 to 5 represents the resulting supernatant from disruption method; lane 6 to 9 represents the protein inclusion body solubilization with 8 M urea (first extraction); lane 10 to 13 represents the resulting protein from 8 M urea (second extraction).

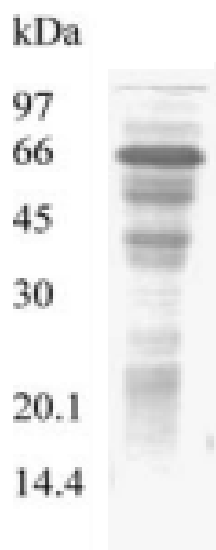


Figure 7.1.2. . Electrophoresis gel SDS-PAGE of protein recovered presented with the optimized process. The samples were obtained from 1 mL of cells and dilutes 1:8 [45].

Although, it was suspected that the cell line was not producing, to confirmation the fusion protein isolation was tried. For this, using columns of different dimensions (2.6 x 95 cm and 1.6 x 23 cm), a gel filtration chromatography was performed. Both columns were packed with Superose 12-prep grade gel. Purification was tried first with the bigger column (figure 7.1.3.), as we want to purify large amounts of fusion protein. However, analysis of previously concentrated collected fractions, by SDS-PAGE gives us no outcomes, which may be indicative of excessive dilution. Thereby, we try to use the other column, the smaller one. A better separation was achieved (Fig. 7.1.4.). Two different peaks were observed. To confirm if one of the peaks match up to our protein, an injection of two molecular weight markers was performed. The used markers were albumin, that has a molecular weight similar to the fusion protein (67 kDa), and lysozyme with a molecular weight of 14 kDa. The obtained chromatogram (Fig. 7.1.5.) shows that albumin is detected by the UV cell after, approximately 20 mL, which confirms that the first peak that appear in the chromatographic profile of protein extract obtained with the smaller Superose-prep grade column (Fig. 7.1.5.) represents the fusion protein.

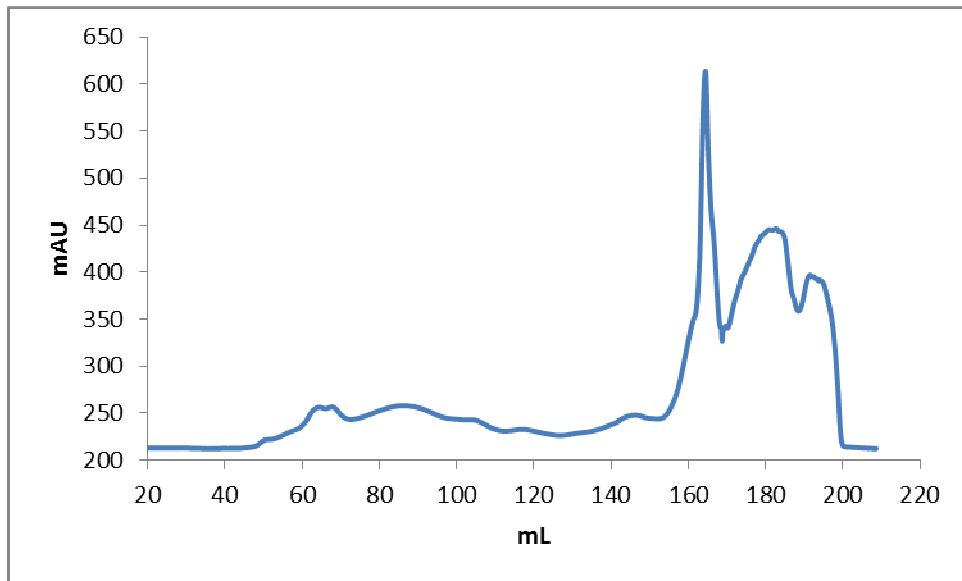


Figure 7.1.3. Chromatographic profile of protein extract obtained with the 2.6x95 cm column packed with Superose-prep grade gel.

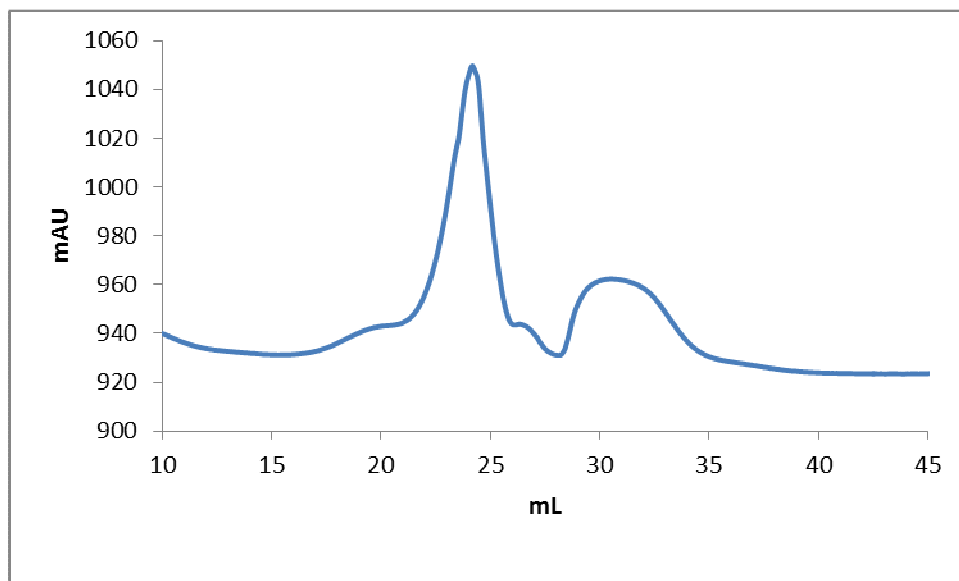


Figure 7.1.4. Chromatographic profile of protein extract obtained with the 1.6x23 cm column packed with Superose-prep grade gel.

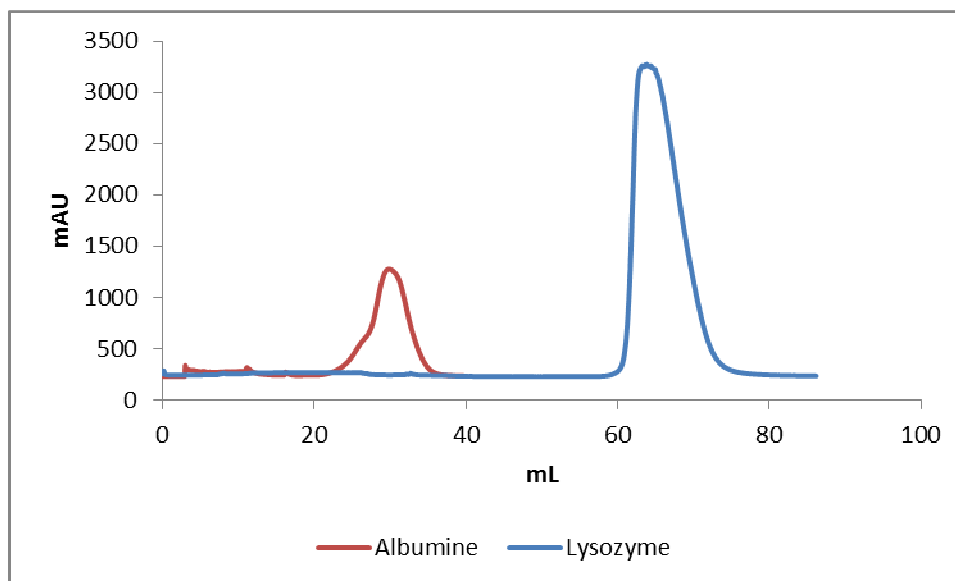


Figure 7.1.5. Chromatographic profile of albumin and lysozyme.

After purification, obtained fractions were collected, concentrated, and finally analyzed by SDS-PAGE gel electrophoresis, which again failed to give results, no band was observed, meaning that the cell line produced a very small concentration of fusion protein.

7.2. Immunosensor Construction

7.2.1. Assay to test the antibody

Firstly, to confirm that antibody was in good shape and detects the glycoprotein a dott-blotting was performed, however it does not give any signal, since low concentrations of antibody and glycoprotein B were used. Thereby, a more sensitive method was tried, isothermal titration calorimetry (ITC), to verify if the antibody recognizes the glycoprotein B. ITC is useful, in this regard, since it directly measures the heat change as interaction proceeds and this is proportional to the level of interaction. ITC is a well-established, powerful, versatile and high-sensitive technique, that is widely used for measuring the thermodynamics of equilibrium association interactions [32].

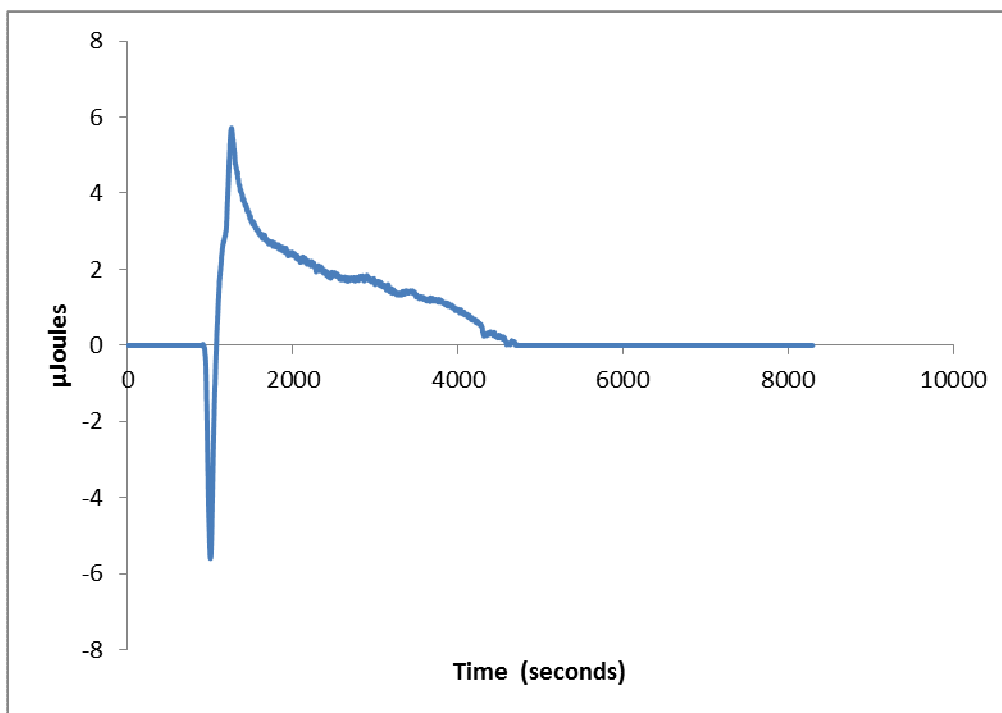


Figure 7.2.1. Profile obtained in the reaction between antibody-antigen.

The interaction between the antibody and the antigen is a very exothermic reaction, where exists a liberation of energy. As it can be observed in figure 7.2.1., an enthalpically driven process leads the interaction meaning that the antibody recognize the glycoprotein B.

7.2.2. Immobilization Procedures

In this work we tried two different screen printed carbon electrodes. The first ones are commercial electrodes, (DropSens S.L.), and the other ones are made in the University of Burgos.

For immobilization of the biological material we tried two different procedures, covalent immobilization and immobilization by adsorption.

In covalent immobilization is necessary to prepare the electrode surface for the immobilization of antibodies. This preparation consists in two steps, activation of the electrode surface with nitro groups (NO_2) and reduction of the NO_2 groups to amines groups (NH_2). Figure 7.2.1 represents schematically this procedure:

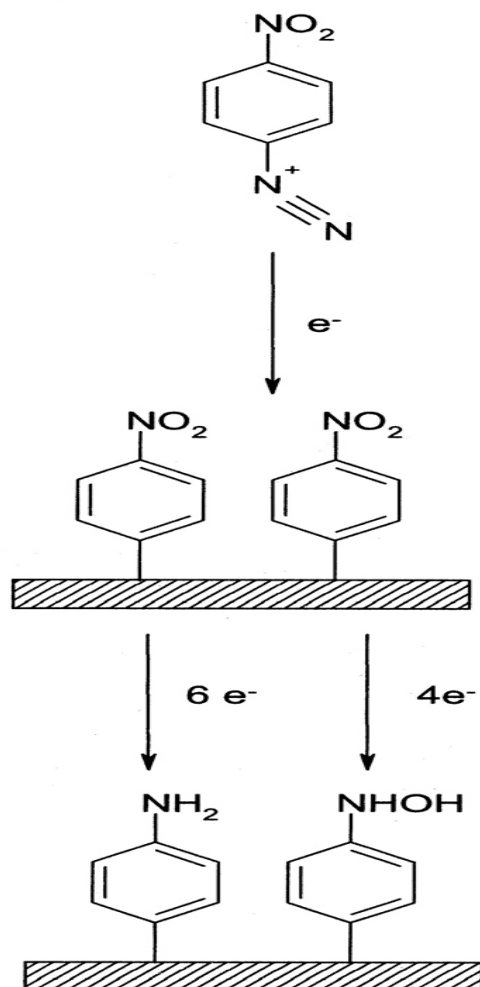


Figure. 7.2.1. Schematic representation of electrochemical process involved in covalent immobilization.

For the activation of the electrode surface, a solution of diazonium salt in acetonitrile (0.73 mg /mL) was prepared. 100 μL of this solution were placed in the working electrode surface and then 2 cycles of cyclic voltammetry were performed from 0.8V to 0.4 V at 200 mV/s. After the cyclic voltammetry scans, the shape of the observed peaks should fit the profile showed in Fig. 7.2.2.

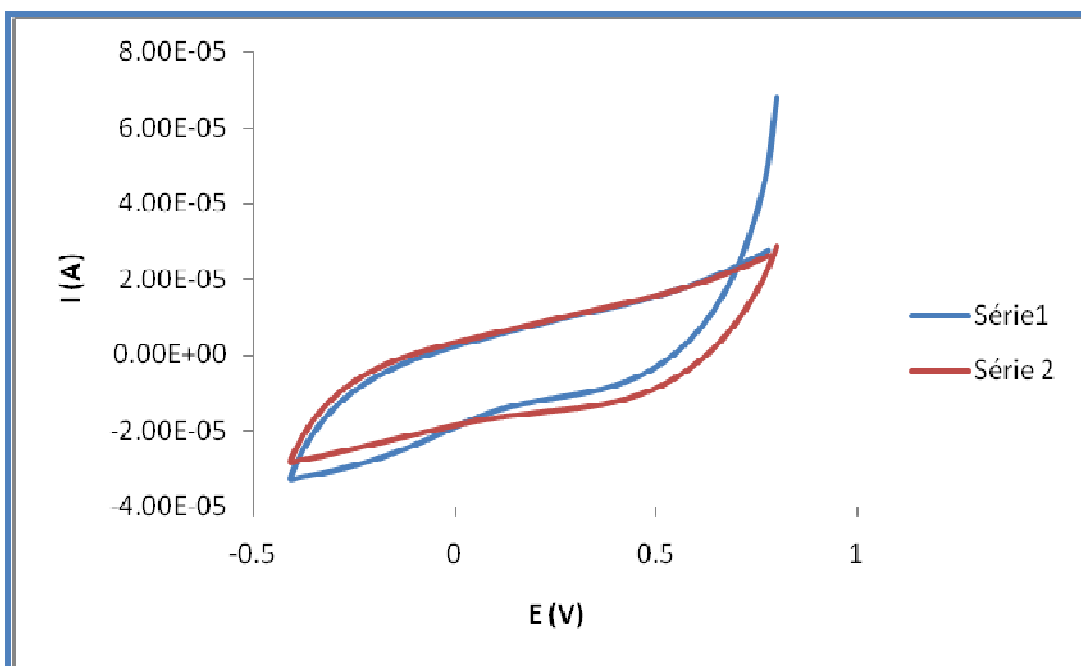


Figure. 7.2.2. Voltammograms obtained in the process of activation of the electrode with nitro groups, of a solution of diazonium salt 0.73 mg /mL, in acetonitrile.

Proceeding activation, the reduction of NO_2 to NH_2 groups is pursued (Fig. 6.3.3). In this way, a solution of 0.1M of KCl in ethanol:water (1:9) was prepared. Then a drop of this solution was placed in the electrode surface and 2 cycles of cyclic voltammetry from 0.8 V to -1.7 V at 200mV/s were applied.

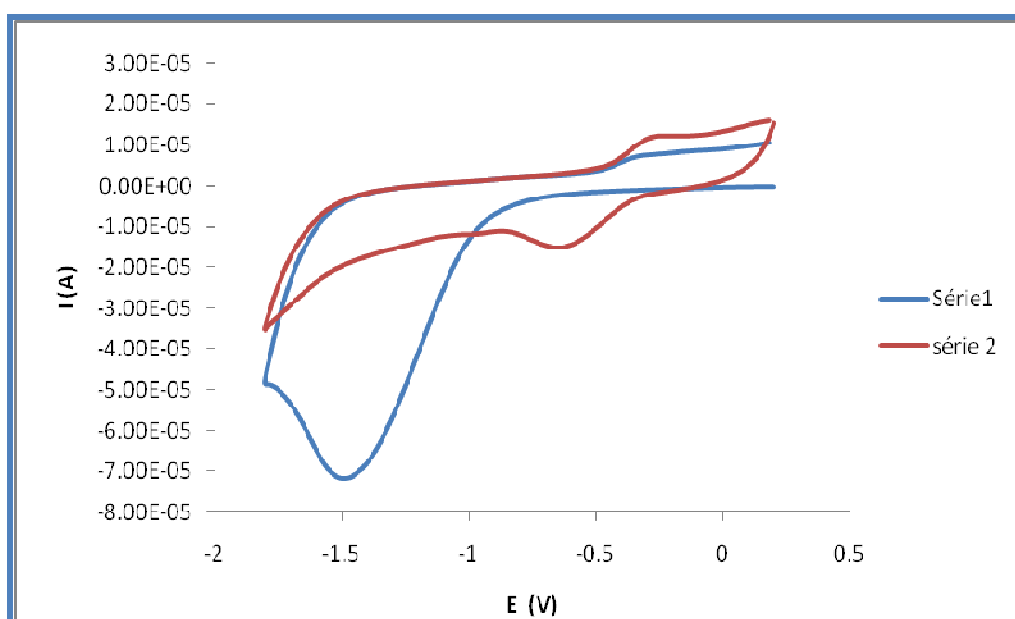


Figure. 7.2.3. Cyclic Voltammograms obtained after electrode activation with nitro groups, in 0.1 M KCl in ethanol:water (1:9)

Finally, the antibody immobilization is performed. For this 3 μL of antibody, 2 μL of hydroxysuccinimide (0.0046g/mL in NaH_2PO_4 , pH 4, 10mM) and 2 μL of carbodiimide (0, 0146 g/mL in NaH_2PO_4 , pH 4, 10mM) are added.

However, this method cannot be applied because it was verified that both used types of electrodes were not compatible with covalent immobilization. The University of Burgos electrodes cannot be used in covalent immobilization because the ink that is used in its construction is dissolved in acetonitrile. The DropSens electrodes are constituted by an ink that resists to acetonitrile, however, when we proceed to the activation of the electrode and to the reduction of groups NO_2 to NH_2 the profiles obtained are different from those expected.

Figure 7.2.4 A shows the voltammogram obtained in commercial electrodes for the activation of the electrode surface. The activation process consists in adding a solution of diazonium salt in acetonitrile (0,73 mg/mL), this solution will add NO_2 groups to the electrode surface, and to verify that, a cyclic voltammetry will be made. After the cyclic voltammetry it should be observed the profile presented in the figure 7.2.2. As we can see, the obtained voltammogram (Fig. 7.2.4. A) for the surface electrode activation corresponds to the expected behavior, being consistent with what was explained.

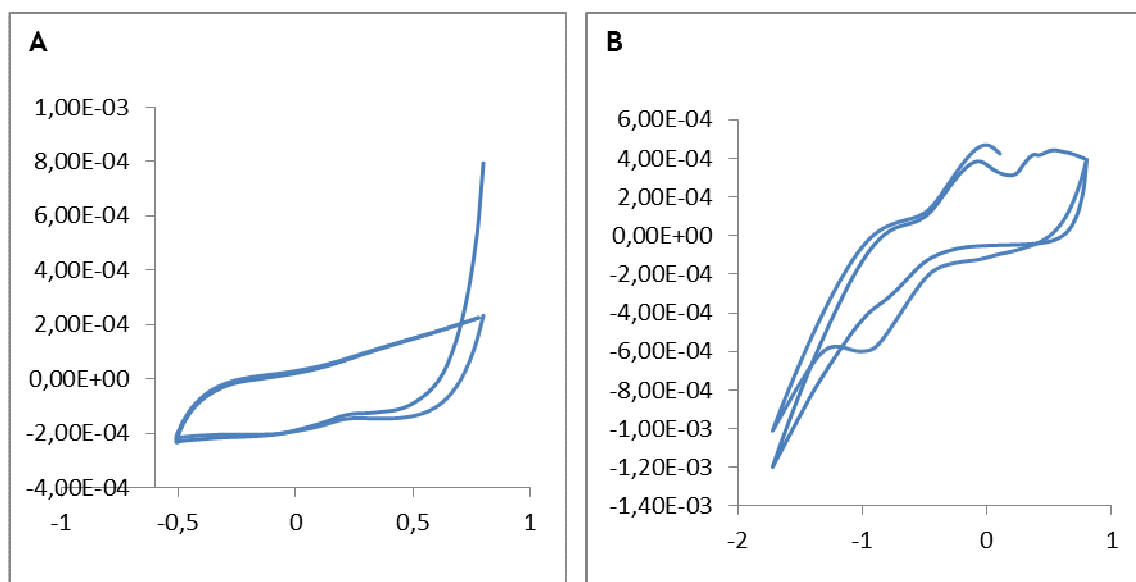


Figure 7.2.1. Comparison of modification process on electrodes surface. A) Obtained profile for activation of DropSens electrode surface with 0.73mg of diazonium salt in 1 mL of acetonitrile. B) Obtained profile for reduction of NO_2 to NH_2 groups in DropSens electrodes with 0.1 M of KCl in ethanol:water (1:9).

The reduction of NO_2 groups to NH_2 groups existing in the electrode surface can be verified by cyclic voltammetry in a solution of 0.1 M of KCl in ethanol:water (1:9). After the cyclic voltammetry it should be observed the profile presented in figure 7.2.3. In figure 7.2.4 B., the profile for reduction of NO_2 to NH_2 is different from the profile observed in figure 7.2.3. So, it could be conclude that probably the reduction of this group does not occur, and subsequent immobilization of antibody would not be possible.

So, this work just focuses immobilization by adsorption. This method has the advantage to be simpler and less laborious than covalent immobilization. And in both cases the immobilization occurs randomly.

7.3. Glycoprotein B Determination

The sandwich developed immunosensor demonstrated respond sensitively to the concentration of the glycoprotein. This particular configuration presents a silver deposition enhanced by gold nanoparticles. The silver deposition solution is added to the electrode surface and then the Au NPs attached to antibodies acts as nuclei and catalysis the reduction reaction of silver ion, leading to the formation of Ag NPs. Finally, the Ag nanoparticles suffer an oxidation process, by means of anodic stripping, and the analyte can be quantified. In summary, increasing analyte concentration will increase the amount of Au PN, so more of Ag NP will be produced.

7.3.1. Voltammetric measurements

The first thing we tried was to verify if it the developed device detect the presence of the target analyte. For those previous measures, we start using linear sweep voltammetry and the commercial electrodes (DropSens electrodes). We started whit the commercial electrodes, since it had a larger surface area, so they should give higher currents and therefore more sensitive results. In fact, we verified that in those conditions, the intensity of the signal increased with the concentration of the analyte. The figure 7.3.1 shows a clearly defined peak due to a glycoprotein B concentration of 10 ng/mL in a pH 7.2 Tris- HNO_3 buffer.

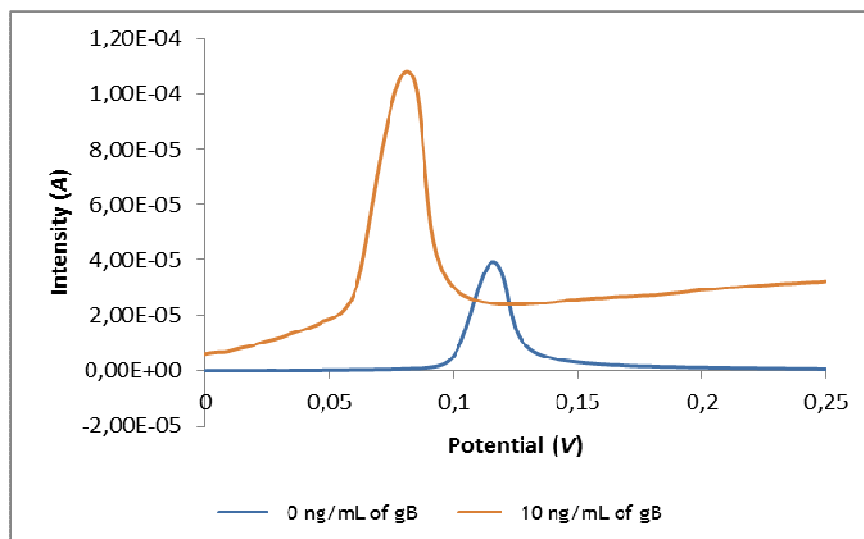


Figure. 7.3.1. Linear-sweep voltammetric curves of Ag NPs Anodic stripping with DropSens Electrodes

However, we have also verified that the background of observed electrochemical signal was very high and the peaks show a low analytical quality. On the other hand, their potentials suffer a considerable displacement. This displacement is related with the low quality, as reference, of the Ag printed reference electrode of the commercial screen-printing devices (DropSens) used in those experiments.

The displacement of the peaks and the high background currents affects the quality of the signals making impossible the realization of a correct measuring. So these electrodes procedure will lead to inaccurate determinations of glycoprotein B.

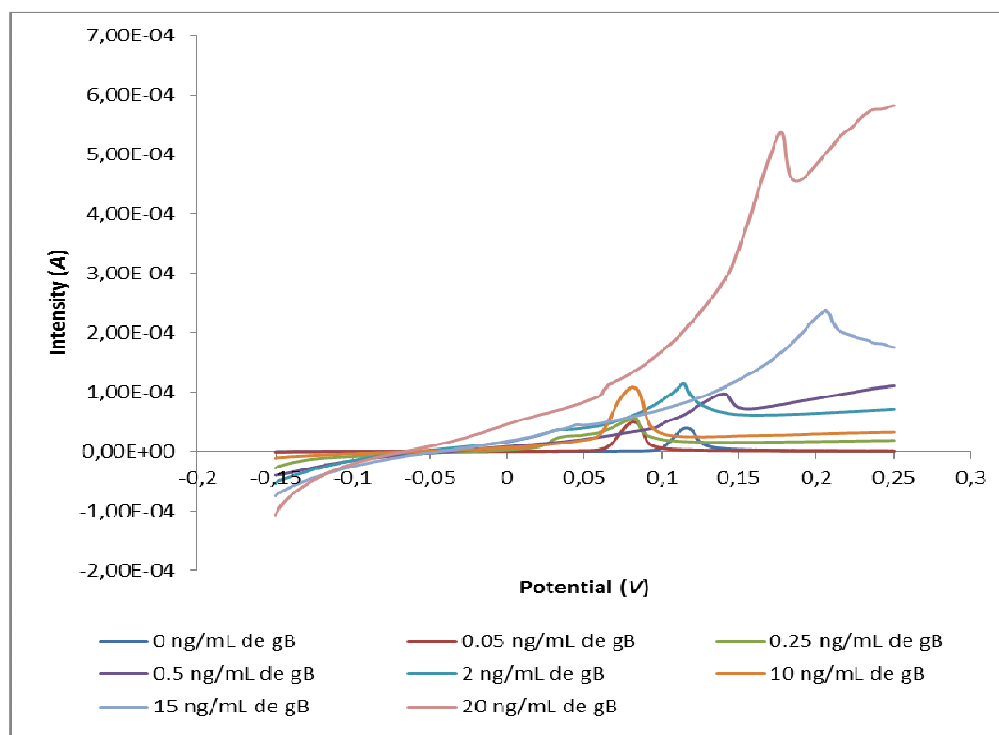


Figure 7.3.2. Background observed in electrochemical signal.

7.3.2. Differential Pulse Voltammetry measurements

To improve the sensitivity of method, we also use another voltammetric method like differential pulse voltammetry, that is a more sensitive technique, because, unlike linear sweep voltammetry, where the potential varies linearly with the time, in differential pulse voltammetry a pulse of potential is applied and the measurements are realized immediately before the pulse application and at the end of the pulse, and the difference between the two currents is registered. Therefore, the capacitive current and the residual faradaic current, that arise from trace impurities, from the electrode material, from the solvent and supporting electrolyte, dies away faster than the faradaic current [52,53]. However, in figure 7.3.3. it can be noted that the peaks are still very displaced.

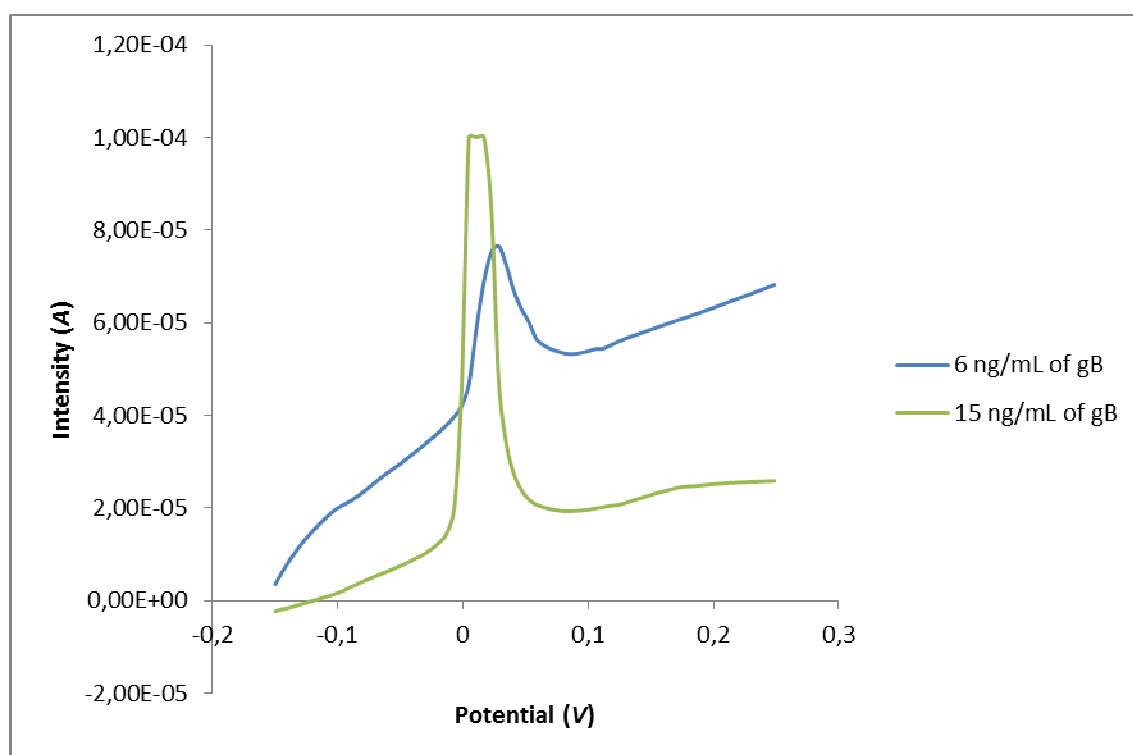


Figure. 7.3.3. Differential pulse voltammetric curves of Ag nPs Anodic Stripping with DropSens electrodes.

In order to avoid the problems associated with the displacement of the peak we tried with another screen-printed carbon electrode (screen printed made in Burgos University) and we verify, just as happened in the DropSens electrodes, that the signal increased with the analyte concentration (Fig. 7.3.4) until reach a constant value of current corresponding to saturation of the electrode surface. Moreover peaks are better defined and no displacement was observed (Fig. 7.3.5).

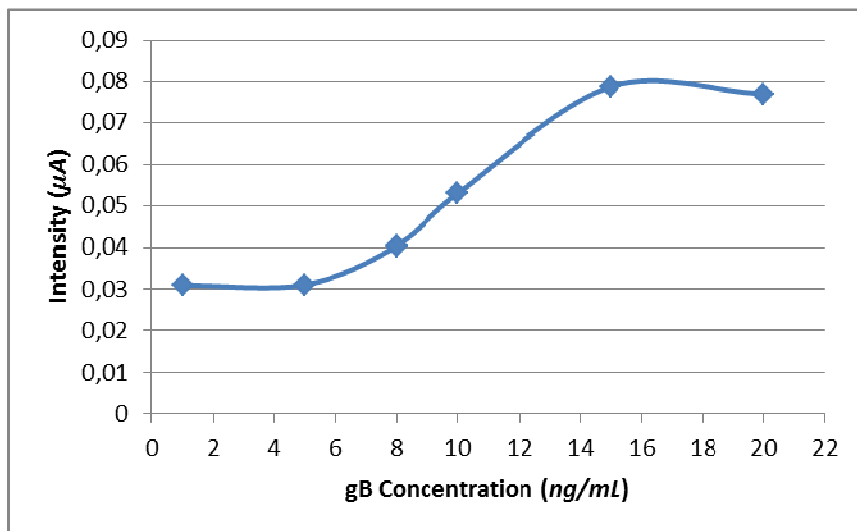


Figure 7.3.4. Assays results for differential pulse voltammetry with electrodes made in University of Burgos. In this detection was used a gB concentration of 1, 5, 8, 10 and 15 ng/mL. gB was diluted in Tris-HNO₃ buffer

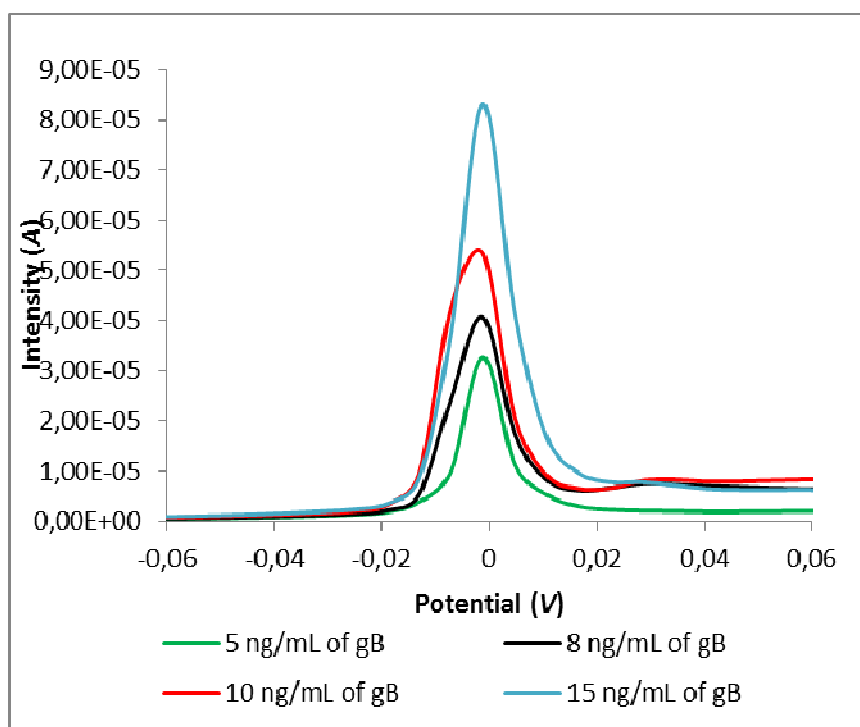


Figure 7.3.5. Differential Pulse Voltammetric Curves of Ag NPs anodic stripping with electrodes made in Burgos University.

7.3.3. Optimization of Detection Conditions

7.3.3.1. Effect of Silver Deposition time on stripping current

To obtain better analytical performance, the effect of silver deposition time was studied in both electrodes.

In the case of DropSens electrodes it was not possible to conclude anything, because the peaks intensity randomly varied. But, it was verified that by using smaller deposition times the peaks showed less noise.

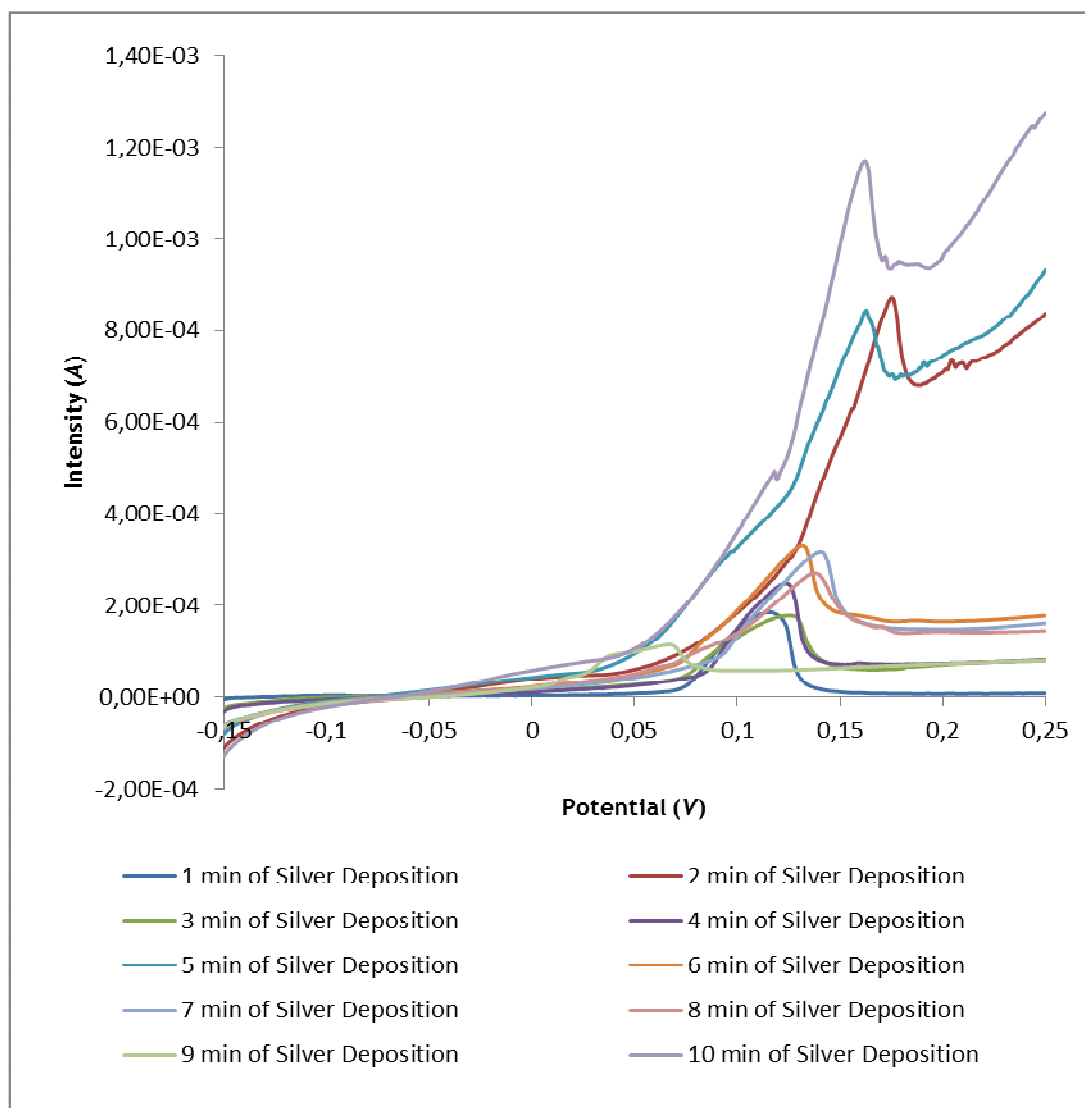


Figure 7.3.6. Effect of silver deposition time on stripping-current response of AgNPS in DropSens electrodes. Glycoprotein B 15 ng/mL.

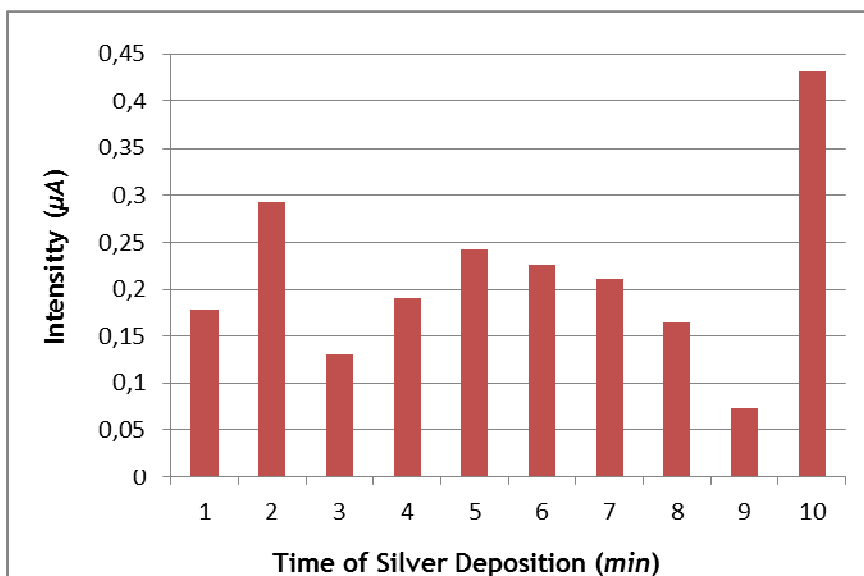


Figure 7.3.7. Effect of silver deposition time in the intensity of the peaks, using DropSenses electrodes. Glycoprotein B 15 ng/mL.

In the case of the electrodes made at the University of Burgos, it was verified that the signal increased with the increasing silver deposition time, nevertheless, after 4 min of silver deposition time the intensity of the signal tended to be constant but with more noise (figures.7.3.8 and 7.3.9). Thus 4 min was adopted as the optimal time for silver deposition.

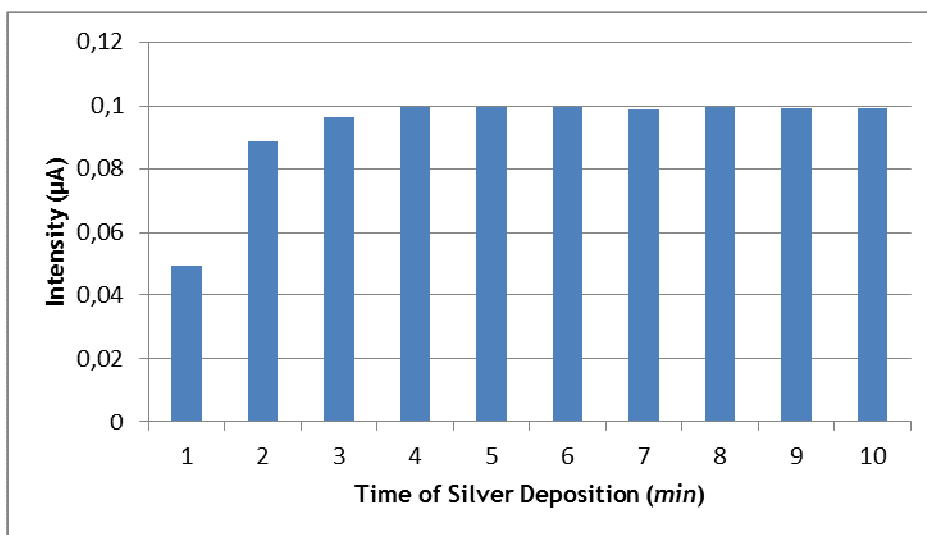


Figure 7.3.8. Effect of silver deposition time in the intensity of the peaks, using electrodes made in University of Burgos. Glycoprotein B 15 ng/mL.

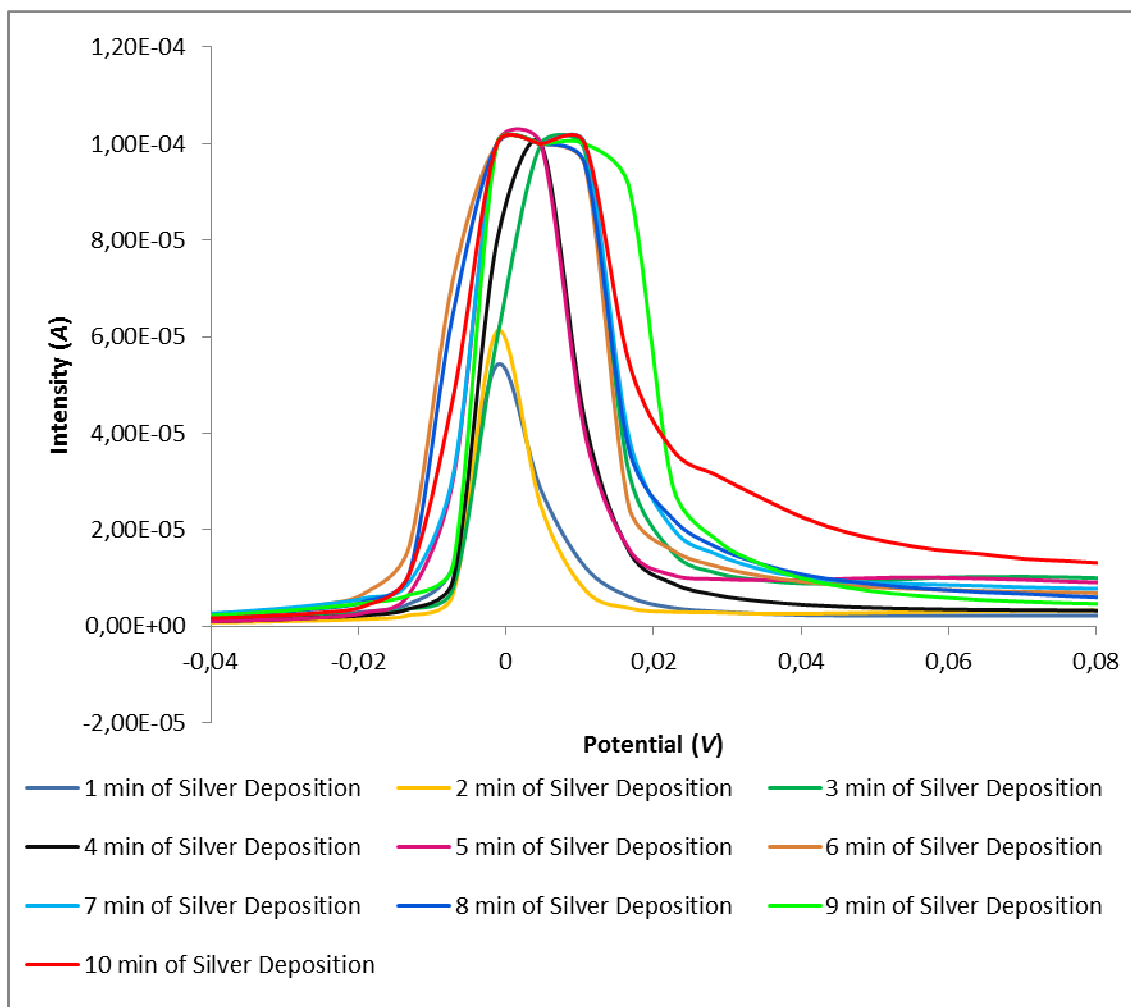
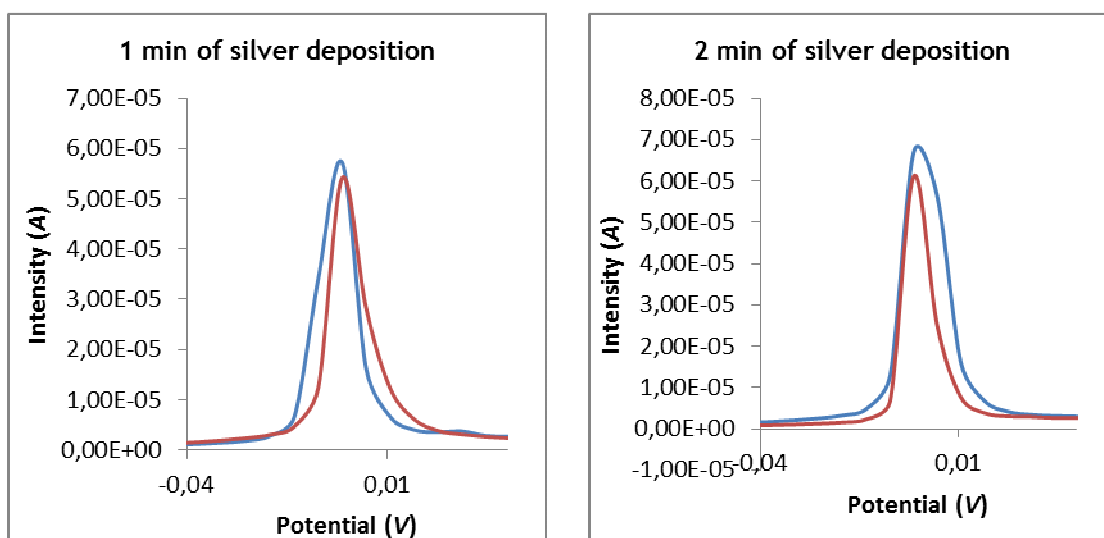
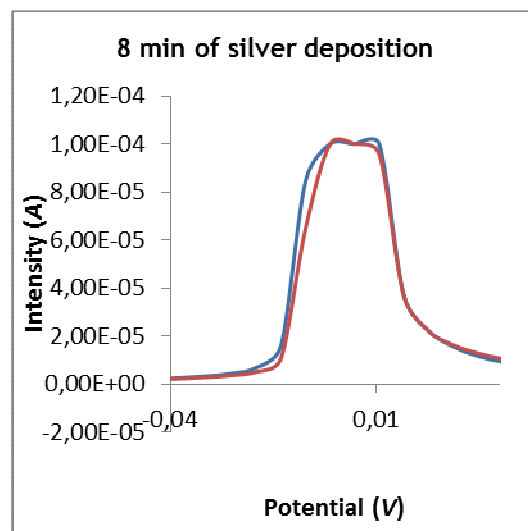
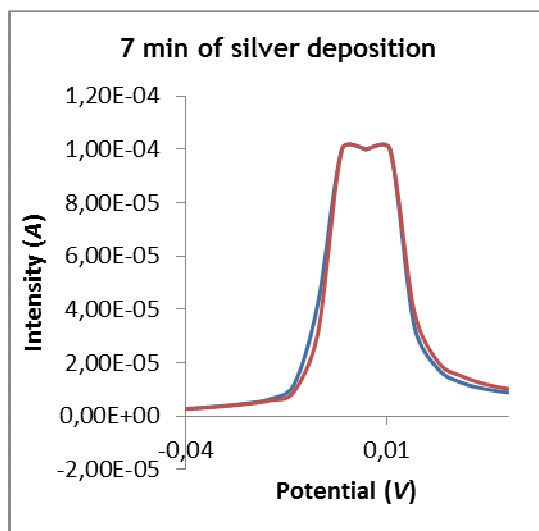
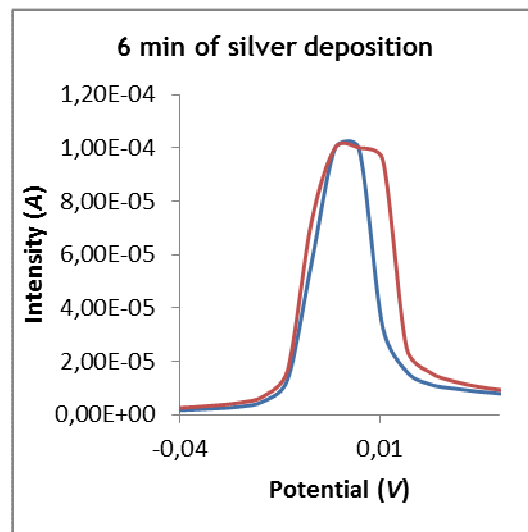
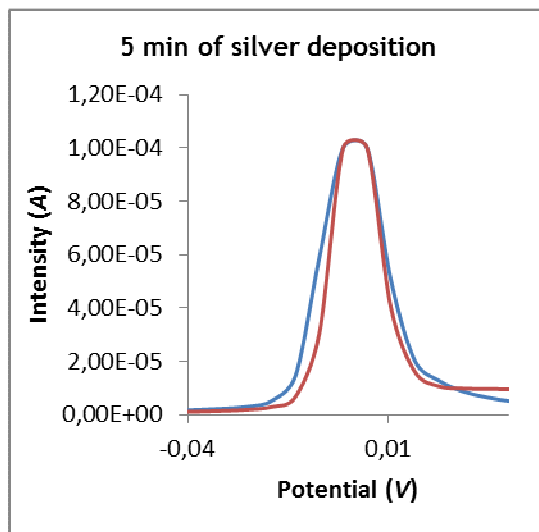
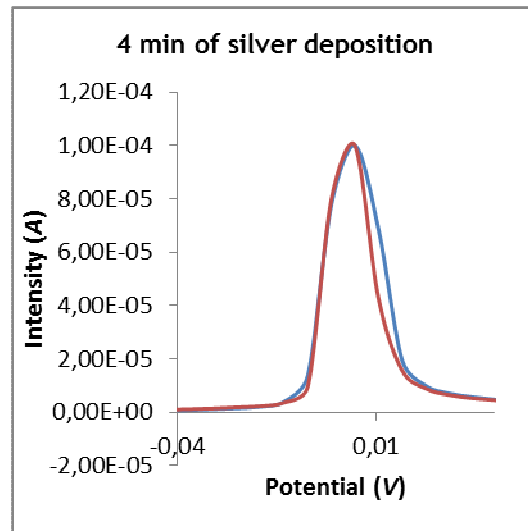
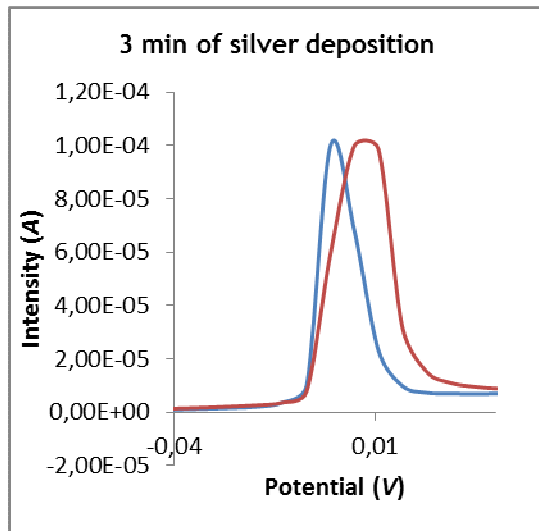


Figure 7.3.9. Effect of silver deposition time on stripping-current response of AgNPS in electrodes made in University of Burgos. Glycoprotein B 15 ng/mL





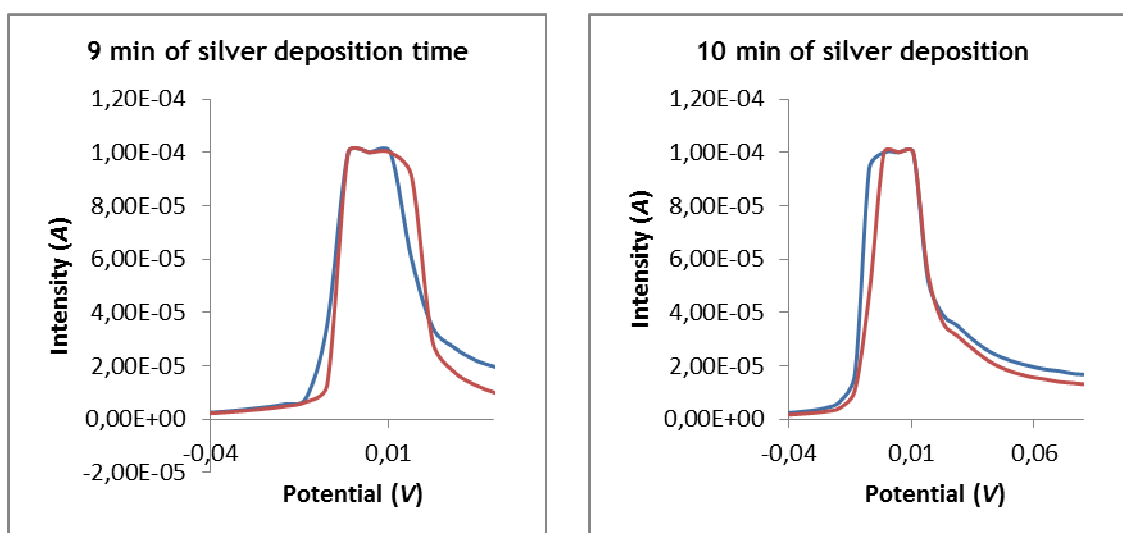


Figure 7.3.10. Different stripping-current response of Ag NPs with the time and duplicates comparison of electrochemical signal for different times of silver deposition. Glycoprotein B 15 ng/mL.

7.3.3.2 Effect of Silver Enhancer Concentration

The amount of deposited Ag NPs was studied in order to increase performance. It was verified that 20-fold dilution of silver enhancer is a very concentrate solution, no difference was found in the signal intensity by changing the concentration of analyte. So, it was tried different dilutions of silver enhancer solutions to found the concentration where it is verified that the gold nanoparticles have the ability to amplify the signal. In order to found this optimum concentration of silver deposition solution, we have compared the oxidation signal of silver nanoparticles onto the bare electrode surface and on the electrode modified with immobilized antibodies labeled with gold nanoparticles.

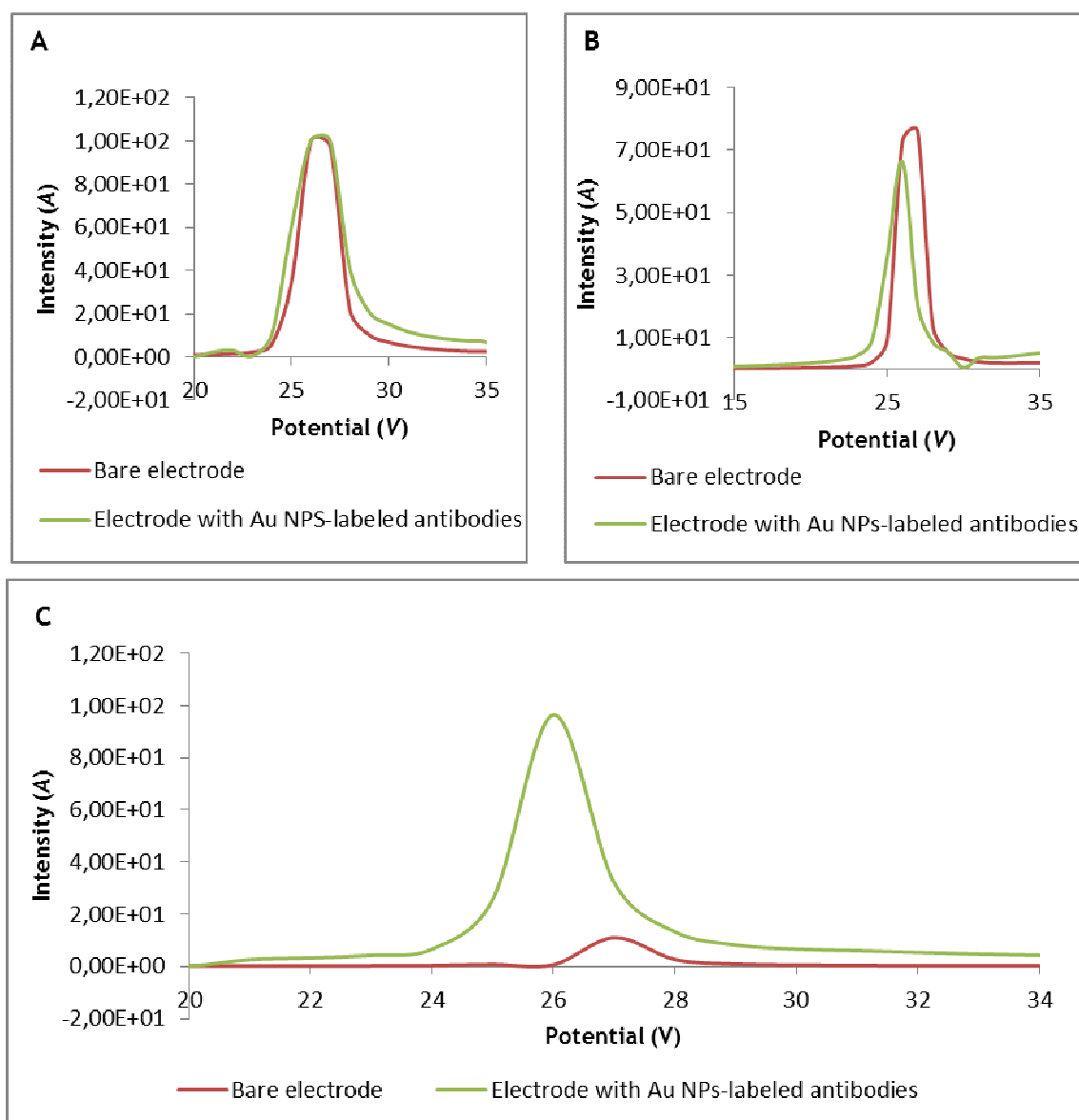


Figure 7.3.11. Variation of signal intensity with silver enhancer solutions with different dilutions. A) 20-fold dilution of silver enhancer solutions. B) 100-fold dilution of silver enhancer solutions. C) 140-fold dilutions of silver enhancer solutions.

As we can see in figure 7.3.11 we obtain a bigger peak with antibodies labeled with gold nanoparticles with a dilution of 140-fold of Silver Enhancer Solutions. So this dilution was adopted as the optimal concentration for silver enhancer.

7.3.4. GB Determination with Optimal Conditions

With a sandwich-type immunoassay format, the quantitatively deposited Ag NPs on the immunosensor could be easily detected by anodic stripping voltammetric analysis in KCl solution. In figure 7.3.12 we can observe the variation of the peak intensity with the

glycoprotein B concentration. And the behavior noted show a constant intensity in current until 5 ng/ml of glycoprotein B and after 15 ng/mL of glycoprotein B. Between these two values the current increased with the glycoprotein B concentration. The profile leads to conclude that the signal of detection of this procedure is about 1 ng/mL and that the electrode surface suffers saturation after 15 ng/mL.

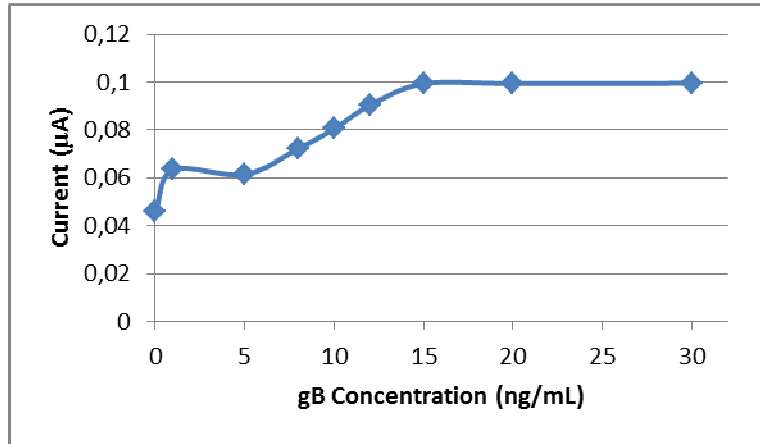


Figure. 7.3.12. Assays results for differential pulse voltammetry with electrodes made in University of Burgos. In this detection was used a gB concentration of 0, 1, 5, 8, 10, 12, 15, 20 and 30 ng/mL. gB was diluted in Tris-HNO₃ buffer.

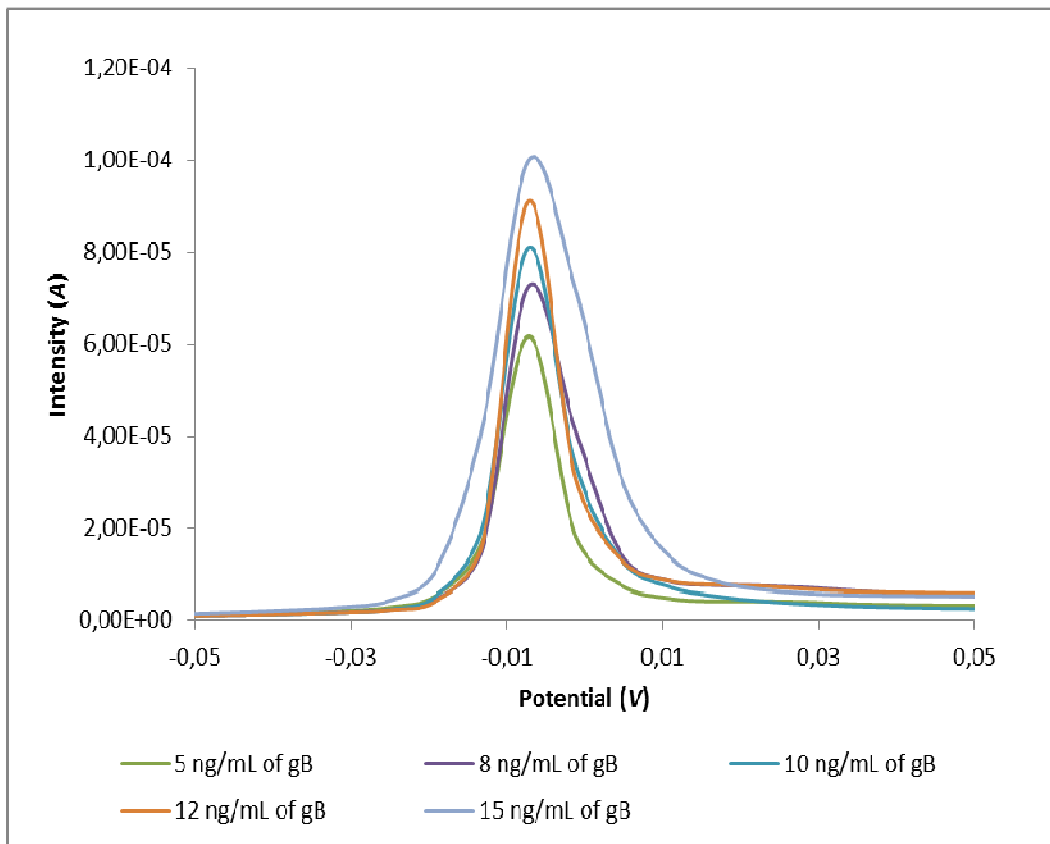


Figure. 7.3.13. Differential Pulse Voltammetric Curves of Ag NPs anodic stripping with electrodes made in Burgos University.

7.3.5. Calibration

Under the optimal conditions, the current responses from the sharp stripping peaks of the immunosensor increased linearly with increasing concentrations of analyte. Calibration plots showed good linear correlation coefficients between the peak currents and the values of gB concentrations.

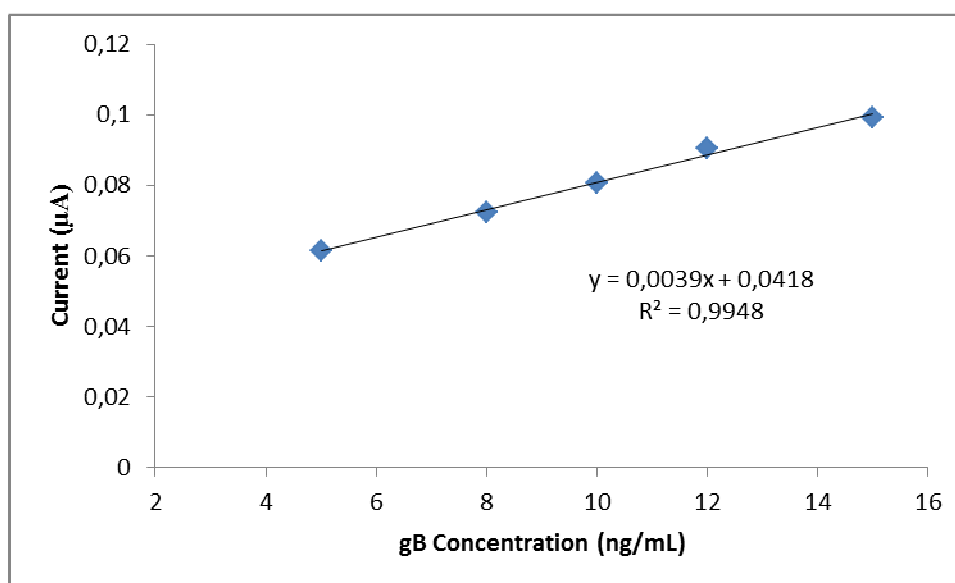


Figure 7.3.14. Calibration curve for 5, 8, 10, 12 and 15 ng/mL of gB.

7.3.6. Reproducibility

The reproducibility of the method was also checked. The slopes of the three calibration lines (having eliminated the anomalous points), constructed with different immunosensors were analyzed. The calibration curves were constructed for gB concentrations ranging from 5 ng L⁻¹ to 15 ng L⁻¹.

The residual standard deviation (RSD) associated with the slopes of these calibration curves was 16.6 %. This value is consistent with that obtained for this type of sensors.

Table 7.3.1. Results to determine relative standard deviation (RSD)

Calibration curves names	Equation	Slope	RSD (%)
A	$y = 0.0035x - 0.0028$	0.0035	16,60041356
B	$y = 0.0039x + 0.0418$	0.0039	
C	$y = 0.0054x + 0.0197$	0.0054	

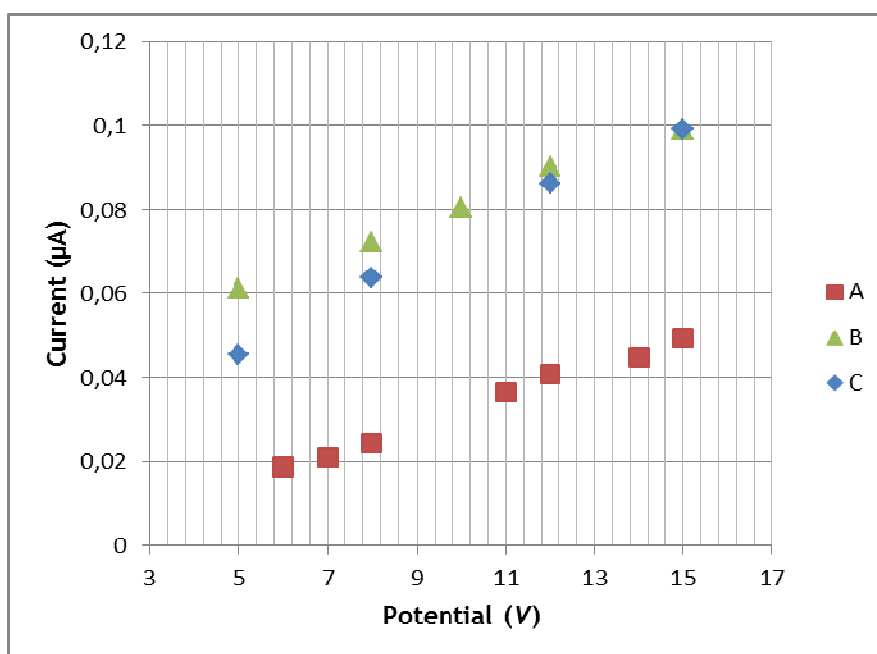


Figure 7.3.15. Calibration triplicates representation.

7.3.7. Detection Limit

A key feature of any analytical method is its detection limit or LOD; the smallest concentration of the analyte that can be detected to a specified degree of certainty.

The LOD was calculated from the DPAdSV responses on the basis of calibration curves recorded under the optimum experimental conditions, in the concentration range of 6.0 to 8.15 nM for SPCE immunosensors. The parameters of these calibrations were optimally evaluated. Least median squares regression (LMS) [54, 55] was used in order to avoid incorrect adjustments due to the existence of anomalous points that might be detrimental to sensitivity. Then, the detection limit was calculated using the DETARCHI program, which evaluates the probability of false positive (α) and negative (β) [56, 57]. The LOD averages obtained for $\alpha = \beta = 0.05$ and were 2.02 nM.

Chapter 8 - Conclusion

The different results can be summarized in the following conclusions for the respective section:

Production, recovery and purification of fusion protein

The cell line used was maintained for a long period of time in the freezer, which may lead to its loss. After trying different cells banks and after making a new one, it was observed that the cells lost the capability to produce the glycoprotein B of HCMV. This was realized after the production, recovery and purification process, low concentration of glycoprotein B was obtained by gel filtration and analysis of concentrated collected samples by SDS-PAGE does not detect any glycoprotein B.

Assay to antibody test

By isothermal titration calorimetry was verified that the interaction between the antibody and the antigen originates an exothermic peak indicating that the antibody binds to the antigen.

Immobilization Procedures

In this work were tried two different immobilization methods to develop this device: covalent immobilization and immobilization by adsorption. The method that showed better results was immobilization by adsorption. So this one was chosen for glycoprotein B detection.

Optimization of Silver deposition time

The optimal time for silver deposition is 4 minutes. This time give us well defined peaks with bigger current intensity.

Optimization of Silver Enhancer Concentration

After trying different concentrations of silver enhancer to achieved a bigger peak in presence of gold compared with the gold absence a 140 fold dilution of silver enhancer solution was choose has the best concentration.

Determination of glycoprotein B with silver enhancer

In disposable electrochemical immunosensor to detect Human Cytomegalovirus was combined the silver deposition catalyzed by gold nanoparticles with convenient stripping analysis. The silver enhancement along with the electrochemical stripping analysis of the deposited silver nanoparticles in a solution of KCl allows the detection of glycoprotein B of Human Cytomegalovirus in a wide linear range and convenient operability. However the sensitivity of the disposable immunosensor has to be improved to provide a great promise in clinical applications. Though the sensor reproducibility is considered acceptable is our goals improve it further.

Chapter 9 - Future work

In future work will be important try to achieve a lower detection limit, since Authier and co-workers managed to get a detection limit of 5 pM. In this way, to improve the sensibility of our SPCE immunosensor we can try a bigger surface area.

Another problem inherent to all immunosensors is reproducibility; thereby it is also need its improvement. For this enhancement, other immobilization techniques should be tried to guarantee the correct immobilization of the antibody.

A way to ensure the correct functioning of this immunosensor is guaranteeing the recognition of glycoprotein B by the antibodies. Thus it is necessary to optimize the incubation time of glycoprotein and antibody labeled with gold nanoparticles in order to allow the affinity interaction between these biomolecules. Furthermore, is also very important reduce at maximum the non-specific bindings, so the concentration of BSA needs to be optimized too.

Finally, this immunosensor will be tested to detect Human cytomegalovirus in samples of human fluids like blood and urine.

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