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Ciências

Disposable immunosensor for diagnosis of Human Cytomegalovirus infection

Antibodies immobilization

Hugo Flávio Cunha da Silva

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Orientador: Prof. Doutora Ana Cristina Dias-Cabral
Co- Orientador: Prof. Doutora María Julia Arcos Martínez

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Abstract

Human Cytomegalovirus (HCMV) is a herpes virus that establishes a lifelong latent infection which, in most of the immunocompetent individuals is normally subclinical. Severe infections occur more frequently in immunocompromised ones, or those with immature immune system, for which can be fatal. Glycoprotein B (gB) is the dominant antigen of HCMV envelope being regarded as a promising component in the establishment of new diagnostic tests.

Nowadays there are several diagnosis methods, however, these are expensive, or/and require long time to perform, or/and need skilled operators, or even leads to the possibility of false results. So, in previously work, a disposable immunosensor was developed based on electrochemical silver oxidation as response for gB concentration increasing, using screen-printing carbon electrodes. This method allows a faster and inexpensive way to detect that viral protein. Despite that, a lack on device reproducibility and sensitivity, due to the randomly antibody adsorption was found. It is known that the oriented immobilization has become critical for optimized antigen detection on solid surfaces; therefore, those results lead us to employ other immobilization techniques to improve the immunosensor performance.

In this work the immobilization of antibodies was carried out through glutaraldehyde cross-linking, by covalent immobilization using diazonium salts and finally by using the boronic acid affinity towards the carbohydrate present on the antibody molecules. The results were conclusive only for the cross-linking, which has disadvantages when compared with adsorption.

Additionally, the importance of the gB on diagnosis tests for HCMV, lead us to study its isolated electrochemical behavior. Questionable results were found demonstrating the impossibility of its use in the determination of gB in biological samples.

Keywords

Immunosensor, antibody immobilization, Human cytomegalovirus, glutaraldehyde, diazonium salts, boronic acid.

Resumo alargado

O Citomegalovírus humano (HCMV) é um herpes vírus que pode originar infeções primárias, a partir das quais a reativação poderá ser frequente. Quando os hospedeiros deste agente viral são indivíduos imunocompetentes, é geralmente associada uma latência do HCMV que eventualmente poderá resultar em sintomas subclínicos. No entanto, em indivíduos imunocomprometidos, tais como os portadores do vírus da imunodeficiência humana (HIV) e os sujeitos a terapêuticas imunossupressoras ou ainda em indivíduos cujo sistema imune é imaturo, como é o caso de fetos e recém-nascidos, a infeção adquire proporções graves, podendo resultar na morte dos mesmos.

Este vírus apresenta um invólucro rico em proteínas virais das quais a glicoproteína B (gB) se destaca, pois está presente em aproximadamente 100% dos indivíduos infetados, sendo também reconhecida por desencadear a produção de anticorpos neutralizantes que levam à eliminação das células infetadas. Assim, esta proteína pode ser vista como um componente essencial no diagnóstico da infeção por HCMV.

A definição de um diagnóstico ideal para o HCMV tem sido difícil de implementar devido às desvantagens apresentadas pelos métodos existentes, que se baseiam em informações clínicas e imunológicas. Definido como método convencional, o isolamento do vírus em culturas de fibroblastos, obtido a partir de biópsias ou de fluidos dos hospedeiros, tem associadas as desvantagens de exigir assepsia total e longos períodos de tempo para a sua execução. Para contrapor as dificuldades desta técnica, um método idêntico, o “Shell-vial”, reduz o tempo do ensaio através de um passo de centrifugação que aumenta a penetração do HCMV nos fibroblastos, que por sua vez pode ser avaliada por imunofluorescência. No entanto, o sucesso deste método continua a depender das condições assépticas usadas. Por outro lado, o PCR (“Polimerase Chain Reaction”), analisa amostras clínicas com uma rápida performance e elevada sensibilidade, conseguida na amplificação de ADN viral. Contudo, o elevado custo e a dificuldade de realização contrapõem-se ao seu uso como técnica de diagnóstico corrente. Outra técnica, ELISA (“Enzyme-Linked Immunosorbent Assay”), deteta a presença de anticorpos específicos no sangue, o que pode levar a falsos positivos devido a reações cruzadas com o fator reumatoide, anticorpos antinucleares e outros membros da família *herpesviridae*. Usado para medir a afinidade e avidéz do anticorpo para o antígeno viral, o “Western Blotting” apresenta uma baixa disponibilidade comercial conjugada também com a possibilidade de falsos positivos. Por último, testes citológicos/histológicos permitem observar inclusões virais em biópsias de tecidos do hospedeiro, contudo apresentam igualmente uma baixa sensibilidade tendo apenas 50% de sucesso na identificação de falsos negativos.

Todos os métodos têm associadas desvantagens quanto a falsos resultados, ou equipamentos e/ou procedimentos caros, que podem estar ou não relacionados a uma difícil manipulação e elevado tempo de realização. Desta forma, para contrariar estes inconvenientes, num estudo anteriormente realizado neste grupo de investigação, foi desenvolvido um imunossensor eletroquímico descartável para a deteção do HCMV, tendo por base uma imunorreacção do tipo sandwich na qual o anticorpo secundário estava marcado com nanopartículas de ouro (AbNPs). Esta marcação permitiu a posterior deposição catalítica de nanopartículas de prata (AgNPs), que geraram um sinal eletroquímico na sua redissolução anódica por voltametria de pulso diferencial. O uso de elétrodos serigrafados (SPEs) como base para o immunosensor, acrescenta vantagens tais como a miniaturização, baixo custo, versatilidade e principalmente a possibilidade de uso como “point of care”. Adicionalmente, a facilidade de produção destes dispositivos por impressão sequencial de camadas de tintas, oferece vantagens na manipulação de padrões e geometrias conforme o pretendido.

O maior desafio na construção deste tipo de biossensores, passa pela imobilização dos anticorpos na superfície dos elétrodos. Está descrito na literatura que a imobilização de anticorpos de forma orientada resulta numa melhor exposição dos locais de ligação aos antigénios, exibindo melhores capacidades de ligação e posterior deteção dos mesmos. De facto, foram detetadas algumas limitações relacionadas com este passo de construção do imunossensor, uma vez que os anticorpos anti-HCMV se encontravam adsorvidos de uma forma aleatória na superfície do elétrodo de trabalho. Desta forma, as moléculas de anticorpo apresentam uma orientação nem sempre ideal afetando a reprodutibilidade e a sensibilidade do dispositivo na resposta a concentrações de gB do HCMV.

Assim, com o objetivo de melhorar as características do immunosensor descartável, neste trabalho foram aplicadas várias técnicas para a imobilização dos anticorpos anti-HCMV, tais como a reticulação, imobilização covalente através de sais de diazónio e por último usando a afinidade do ácido borónico para os açúcares presentes nas moléculas de anticorpo. O glutaraldeído, usado como agente reticulante, permitiu a imobilização das moléculas de anticorpo anti-HCMV na superfície dos SPEs. O sistema mostrou responder às concentrações incubadas de gB, levando a respostas dependentes das mesmas. Comparativamente aos resultados obtidos para a adsorção, a reticulação demonstrou ter associadas algumas desvantagens em termos de reprodutibilidade e de sensibilidade, devido à imposição da ligação dos anticorpos pelos domínios de ligação ao antigénio. Por outro lado, em condições favoráveis, a adsorção pode resultar numa orientação favorável dos anticorpos, uma vez que as moléculas têm a liberdade para se adaptarem à superfície pela conjugação de diversos fatores, nomeadamente a sua reorientação favorável na zona de saturação. Quanto à imobilização covalente foram encontradas interferências na ativação da superfície dos elétrodos. As vias condutoras de prata presentes nos elétrodos serigrafados geram uma espécie desconhecida de prata que impossibilita o estudo da eficiência de imobilização, levando a uma incompatibilidade com o método de deteção usado no imunossensor. Por fim,

uma primeira abordagem foi realizada para a imobilização dos anti-HCMV através da afinidade do ácido borónico para os resíduos de açúcar presentes na estrutura dos anticorpos. Os resultados preliminares demonstraram ser promissores para uma futura aplicação neste imunossensor.

Finalmente, a reconhecida importância da gB no diagnóstico do HCMV remeteu-nos para o estudo do seu comportamento eletroquímico. Este estudo permitiu ainda inferir sobre a possibilidade de esta proteína viral interferir no sinal obtido durante a redissolução anódica das AgNPs no imunossensor. A análise foi primeiramente conduzida em SPE, os quais não possibilitaram a procura de um sinal associado à gB, permitindo no entanto concluir que não há interferência desta molécula no imunoensaio. Quando aplicado num sistema eletroquímico convencional, a gB gerou um sinal em meio tamponado, o que não se confirmou quando aplicado a amostras reais (urina).

Palavras-chave

Imunossensor, imobilização de anticorpos, Citomegalovírus humano, glutaraldeído, sais de diazónio, ácido borónico.

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I. Introduction

1. Human cytomegalovirus

Human cytomegalovirus (HCMV) is a highly host-specific herpes virus that provides a primary infection followed by the establishment of lifelong latent infection, from which periodic reactivation is common (Cannon and Davis, 2005; Khattab *et al.*, 2009). During reactivation it can be a potential killer. For this reason is regarded a virus of paradoxes (Revello and Gerna, 2002).

1.1. General characteristics

Cytomegalovirus (CMV) (from the Greek cyto-, 'cell', and -megalo-, 'large') is a viral genus of the Herpes virus group, which belongs to the Betaherpesviridae subfamily of *herpesviridae*. In humans, the designated Human cytomegalovirus (HCMV) or human herpes virus 5 (HHV-5) is a common virus that infects most people at some point during their lives (Khattab *et al.*, 2009).

Known as the largest human virus, it is morphologically indistinguishable from other human herpes virus (Revello and Gerna, 2002). In this specific type, the virions consist in three major structural regions (Figure 1.1.). The icosahedral viral capsid, containing the double-stranded DNA; the tegument composed of many proteins, which help in viral replication and that are highly immunogenic. And at last, enclosing the capsid and the tegument, a lipid bilayer envelope consisting of two glycoprotein complexes, which plays a vital role in infection (Sanchez *et al.*, 2000; Vadlapudi *et al.*, 2012).

It is a ubiquitous double-stranded DNA virus and its genome, which has approximately 230 kb in size, encodes 200 proteins including glycoprotein B (gB) and glycoprotein H (gH), found only in Betaherpesvirus (Boeckh and Geballe, 2011; Malm and Engman, 2007).

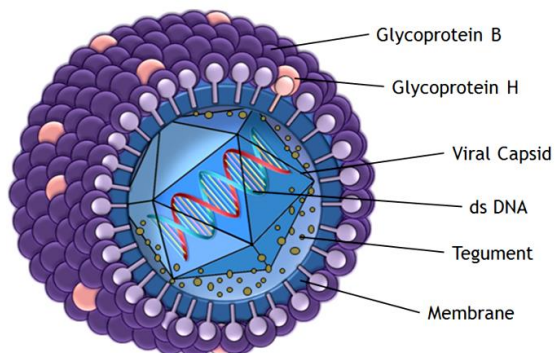


Figure 1.1. Representation of Human cytomegalovirus (HCMV).

Like others human herpesviruses, the assembly of this infectious HCMV particle is a complex and poorly understood process. It is generally accepted that virus establish interactions, to enter in host cells, by binding and fusing with the plasma membrane receptors, including integrins and possibly growth factor receptors, in a process mediated by virion glycoproteins (gB and gH) (Boeckh and Geballe, 2011; Sanchez *et al.*, 2000).

1.2. Viral infection and transmission

HCMV's infection, first detected in newborn's urine during the early 20th century, is endemic and occurs throughout the year, having no seasonal pattern and occurring early in childhood, being about 40-80% of individuals seropositive at puberty (Almeida *et al.*, 2001; Boeckh and Geballe, 2011). This opportunist virus, as previously described, can provide an initial infection, known as primary infection, followed by establishment of a lifelong latent infection, from which periodic reactivation is common (Cannon and Davis, 2005). The primary infection is defined as the detection of HCMV in an individual previously found to be HCMV seronegative, whereas reactivation or recurrent infection is defined as new detection of virus in patient, due to a reactivation of a latent virus or reinfection, by respectively endogenous or exogenous causes (Ljungman *et al.*, 2002). After a primary infection, the virus can remain in the body fluids for months to years (Cheeran *et al.*, 2009; Nassikas and Tsaples, 2013).

It is believed that humans are the only receptor for HCMV, and transmission occurs by direct or indirect contact through multiples routes, like contaminated urine, saliva, semen, cervical secretion, breast milk, tears, sexual activity and organ transplantation. The transmission between individuals can also be achieved during blood transfusions by infected lymphocytes and mononuclear cells, establishing a latent infection in mononuclear leucocytes and organs like kidneys and heart (Domingues *et al.*, 2005). Seroprevalence is due to hygienic circumstances, geographic location and socio-economic factors (Almeida *et al.*, 2001; Malm and Engman, 2007).

1.2.1. Congenital infection

HCMV infection is one of the most common viral causes of congenital infections (Malm and Engman, 2007), in which a pregnant mother transmits the virus via placenta to the fetus, being a major public health concern (Nassikas and Tsaples, 2013).

Different incidences are found for a primary or a recurrent infection. The seroconversion, defined as the detection of anti-HCMV antibodies that were previously absent, during the pregnancy is reported in 1-4% of seronegative women and the risk for viral transmission to the fetus is 30-40% (Malm and Engman, 2007; Manicklal *et al.*, 2013). On the other hand, a recurrent maternal infection is moderately protective against sequelae, but these infants are still in risk (Ijpellar, 2010). The reactivation occurs in 10-30% of seropositive women and the

risk of transmitting the virus to the fetus is about 1-3% (Malm and Engman, 2007). Furthermore, the infection time relative to the pregnancy is a crucial factor in establishing the risk to the fetus *in utero* transmission (Cheeran *et al.*, 2009). That is because, during early pregnancy, HCMV has a teratogenic potential to the fetus (Malm and Engman, 2007). Therefore, the infection during the first semester is more likely to cause HCMV disease, once organogenesis takes place in this period (Nassikas and Tsaples, 2013), being associated with more severe symptoms, sequelae and abnormalities (IJpellar, 2010).

A variety of disabilities, alone or in combination, may be caused by congenital infection in children, resulting in clinical manifestations such as mental retardation, autism, learning disabilities, cerebral palsy, epilepsy, deafness or hearing impairment, visual deficit or blindness, (Malm and Engman, 2007) microcephaly, small body size and hepatomegaly (Domingues *et al.*, 2005).

1.2.2. Non-congenital infection

Despite concerns about the congenital infection, HCMV can be a problematic issue to other individuals. Throughout the world; 40-100% of adults in different populations become infected by the fourth decade of their life (Eddleston *et al.*, 1997), principally due to sexual relations and occasionally via blood transfusions or transplanted organs, or even via saliva and urine from children (Boeckh and Geballe, 2011).

In immunocompetent individuals, a primary infection is normally subclinical, producing a mononucleosis-like syndrome, which is generally mild and self-limiting and rarely develops a fulminant infection (Eddleston *et al.*, 1997). Severe infections occur more frequently in immunocompromised individuals like transplantation recipients and those infected with HIV (Human immunodeficiency Virus), or others with immature immune system like newborns (Domingues *et al.*, 2005), being a common cause of death among them (Eddleston *et al.*, 1997). For these individuals the diagnosis is pneumonia, retinitis, colitis and encephalopathies (Domingues *et al.*, 2005).

With this worrying pathology and multiple routes of infection and transmission, HCMV is capable of affecting many types of population in endemic scale, being a global concern for prevention and treatment.

1.2.3. Prevention of virus transmission

The greatest importance has been given to the management of those infected individuals, mainly to confine the disease spreading. Particularly in congenital infection, this management is further difficult, first because mothers are unaware of HCMV disease and second because the associated symptoms are non-specific, turning HCMV infections undiagnosed.

Seroconversion can occur at mucosal surfaces, especially via infected urine and saliva (Nassikas and Tsaples, 2013). Thus, to minimize the transmission risk of the virus to the seronegative mothers, behavioral changes on hygienic practices should be adopted, like hand-washing and cleansing of environmental surfaces when interacting with young children. Predominantly the women who work in daycare or preschool centers should wear gloves in certain activities of child caring (Kenneson and Cannon, 2007). Concomitantly, educational interventions among population, should be adopted in preventing HCMV transmission (Kenneson and Cannon, 2007).

The major promising and priority in prevention of HCMV disease, is the possibility of developing a vaccine that can avoid infection. The Institute of Medicine declared that the development of a HCMV vaccine should be a primary goal for controlling the virus (Boeckh and Geballe, 2011). Although an available licensed vaccine is not imminent (Kenneson and Cannon, 2007), some researches have promising results to this problematic.

The most promising approach is a subunit vaccine, using the HCMV envelope gB, who has been recognizing as candidate target, with the goal of inducing a potent virus-neutralizing antibody response (Nyholm and Schleiss, 2010). Pass and co-workers developed a vaccine formulation that consists on envelope gB with MF59 adjuvant. Results of a phase II, placebo-controlled, randomized, double-blind trial showed 50% efficacy in preventing CMV acquisition of primary CMV infection in young mothers (Pass *et al.*, 2009).

1.3. HCMV immune response

As aforementioned, HCMV infection mostly occurs in immunocompromised patients possibly due to immunosuppressive drugs or to the presence of underlying disease; or in individuals with immature immune system, evidencing that humoral immunity may be important in the role of infection (Rasmussen *et al.*, 1982).

1.3.1. HCMV specific antibodies

As a response to HCMV infection, there is an increase of antibody production that usually sheds the virus asymptotically, rather than developing clinically significant diseases (Rasmussen *et al.*, 1982). High levels of virus neutralizing antibody (NAb) have been associated with a favorable clinical course. Indeed, titers of NAb have been reported to be lower in mothers who transmit HCMV to their fetus, than those who did not transmit. The protective immunity to HCMV due to humoral antibody response, plays an important role, particularly in maternal-fetal transmission and transplant recipients (Rasmussen and Cowan, 2003).

Two antibodies are produced in presence of HCMV, the immunoglobulin M (IgM) and the immunoglobulin G (IgG). This immunoglobulin production is associated with different phases of infection. The IgM antibody production may be related to the severity of the infection once is carried out during active infection, both primary and reactivated, observable with high titers of IgM. This immunoglobulin was also detected early in the course of HCMV infection, being associated with the initial excretion of the virus and providing a marker for its replication in patients. On the other hand, IgG antibody titers persist during and after symptomatic infection being similar to those asymptomatic positive individuals (Rasmussen *et al.*, 1982).

1.3.2. Glycoprotein B as dominant antigen

In acute HCMV infection, an antibody production is triggered against to numerous structural and non-structural viral proteins. Nevertheless, between more than 100 proteins encoded by HCMV, just B and H glycoproteins have the ability to induce neutralizing antibodies that will result in virus neutralization and elimination of the infected cell (Domingues *et al.*, 2005).

Glycoprotein B (gB) is the major antigen of HCMV envelope and, as was said above, has an important role in infection, being implicated in host entry, cell-to-cell virus transmission and fusion of the infected cells, as well as, being an important target for humoral and cellular immune responses. This protein has also been shown to be a major target for the production of neutralizing antibodies that comprise 40 to 70% of the total neutralizing activity against HCMV (Fan *et al.*, 2011). In fact, this envelope protein has been recognized as the immunodominant target for humoral immune response, because nearly 100% of the infected people who are seropositive have antibodies against gB (Domingues *et al.*, 2005).

Structurally gB has 3 recognition sites (figure 1.2.), that allows antibodies linkage: the antigenic domain 1 (AD-1), which lies between a.a. 552 and 635; the AD-2 between a.a. 50 and 77; and the AD-3 between a.a. 783 and 906. The AD-2 includes two sites, local I (residues 68-77) and local II (residues 50-54). A number of additional non-linear epitopes is also found in this glycoprotein. This 906 amino acid polypeptide can be proteolytically cleaved into two subunits gp116 and gp58, covalently linked by disulphide bonds (Domingues *et al.*, 2005; Speckner *et al.*, 2000).

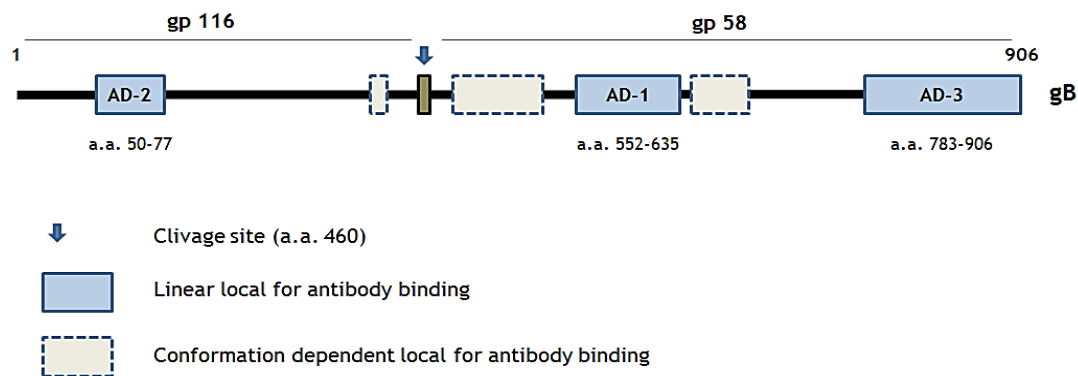


Figure 1.2. Schematic representation of gB and the antigenic domains.

The immune response is due to the antigenic domains, mainly AD-1 and site II of AD-2, that induce neutralizing antibodies production in a natural infection. AD-1 has been recognized as the immunodominant site of gB, once all seropositive individuals have antibodies against this domain, whereas only 47% of serological samples from individuals with past infection, are against local II of AD-2. The occurrence of antibody binding requires the entire AD-1 sequence and the formation of intramolecular disulfide bond between cysteine 573 and cysteine 630. This antigenic domain is highly conserved because, unlike other domains, it does not show considerable variations. This is also evidenced when the recognition ability is not affected by the glycosylation of gB, because the antibodies also recognize non-glycosylated protein form. Thus, the gB, more specifically the AD-1 fraction, plays an important role in diagnostic tests to verify the presence of neutralizing antibodies (Domingues *et al.*, 2005).

1.4. Diagnosis of HCMV infection

Diagnosis of HCMV disease has a vital importance in the management of patients, with great improved advances obtained during the past two decades. While viruses still account for substantial morbidity, mortality and associated costs, it is still difficult to establish an ideal clinical diagnosis (Boeckh and Geballe, 2011). However, there are several diagnostic methods available, which are based on clinical and immunological data (Jahan, 2010; Junqueira *et al.*, 2008). These methods must provide the ability to detect the virus or viral components on several types of samples (Ljungman *et al.*, 2002). Thus, generally the diagnosis of HCMV infection can be substantiated by virus isolation culture, shell-vial, polymerase chain reaction (PCR), Enzyme-linked immunosorbent assay (ELISA), western blotting and by cytological/histological methods. Some disadvantages of these methodologies, described below, are in conflict with the requirements for an efficient diagnosis. So, avenues for improving outcomes in HCMV diagnosis must provide rapid, efficient, simple, sensitive, sensible, specific and low cost methodology, which allows the clinician to predict which patients are infected with HCMV.

In order to avoid the over treatment with immunosuppressive drugs and to guide antiviral therapy, diagnosis of active HCMV infection is of great importance (Jahan, 2010). In fact, early diagnosis is essential in order to start preventive treatments and reduce consequent sequels (Albanna *et al.*, 2013).

1.4.1. Virus isolation culture

Considered as the gold standard method, it is based on the virus isolation predominantly from urine or biopsy tissues. Urine was found to be the ideal specimen for detection of HCMV for containing moderate to large amounts of infectious virus particles (Albanna *et al.*, 2013). HCMV grow in human diploids fibroblast cell cultures at 36°C and HCMV inclusions in cells are identified. This technique leads to a limitation on timing, once it is required 1-2 weeks, or 6 weeks when little amount of virus is present for a visible cytopathology. Moreover, this method is associated with difficulties on technique procedures, once required aseptic techniques (Jahan, 2010; Junqueira *et al.*, 2008).

1.4.2. Shell-vial

To deal with the excessive time consumption of cells culture, this similar technique was developed to reduce the time for detection of cytopathology. Using indirect immunofluorescence monoclonal antibodies, which enable the detection of a nuclear HCMV antigen in fibroblast, the revelation time is decreased to 24, 48 or 72 hours. Unlike conventional culture, these cells suffer an additional centrifugation step, which enhance the absorption of the virus, increasing four fold the infectivity of the vial inoculums. This method has been reported to exceed the sensitivity of the conventional culture (Jahan, 2010; Junqueira *et al.*, 2008).

1.4.3. Polymerase chain reaction (PCR)

Known as, highly sensitive and rapid technique (~6 hours), the PCR is based on selective amplification of specific nucleic acid sequences. This method measures the HCMV DNAemia, which is correlated with risk and severity of HCMV disease in patients. It is versatile and can be used either qualitatively in diagnostic PCR, or quantitatively, to measure the viral load, which is proportional to the level of HCMV DNA. With sensitivity and sensibility similar to the virus isolation cultures, it brings some advantages in obtaining rapid results and enabling the possible use of freeze samples. PCR disadvantages are the highly cost, difficulty to perform and its great sensitivity, once it can detect low levels of HCMV that are not always predictive of disease. Despite its disadvantages the PCR is widely used (Jahan, 2010; Junqueira *et al.*, 2008).

1.4.4. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA technique, detects antibodies in blood with 100% of sensibility and 86% of specificity. This method is available from several manufacturers, is accurate in determining serologic status and it is easier to perform, with results in a few hours. However, there is the possibility of false positive results, caused by cross-reactions with some virus of *herpesviridae* family, rheumatoid factors and antinuclear antibodies. Also, because IgG and IgM antibodies pass through placenta, that technique is not able to be used in congenital HCMV infection (Jahan, 2010; Junqueira *et al.*, 2008).

1.4.5. Western Blotting

Western blotting allows to measure affinity and avidity of the antibody for the viral antigen. In that way it is used in detection of a primary infection. The major disadvantage of this method is the lower commercial availability and the false positive results (Junqueira *et al.*, 2008).

1.4.6. Cytology/Histology

Cytological techniques may be applied in specific diagnose of HCMV involvement in an organ, attempting to find characteristically intranuclear inclusions. Inclusion-bearing cells may be found in saliva, milk, cervical and tracheal secretions and in preparations from biopsy tissues. The microscopic hallmark of HCMV infection is the large, central, basophilic intranuclear inclusion, which is referred as “owl’s eye”; concomitantly, clusters of small intracytoplasmic inclusions are also observed. This technique has lower sensitivity relative to virus isolation, and has false negative results with only 50% successful ones. The histological examination of a small piece of tissue obtained by biopsy is prone to sampling errors (Jahan, 2010).

2. Biosensors

The modern concept of biosensors represents a rapidly expanding and active field of instruments to determine the concentration of substances and other parameters of biological interest (Turner *et al.*, 1987). The major markets for biosensors generally are the clinical and health care fields, with increasing interest in analytical devices with greater sensitivity, specificity, reproducibility, and reliability (Frew and Hill, 1987). This emerging technology results from the apparently alien marriage of two contrasting disciplines, which combines the specificity and the sensitivity of biological systems with the computing power of microprocessor (Turner *et al.*, 1987).

2.1. Biosensors principles

Defined as an analytical device that integrates a biological recognition element with a physical transducer to generate a measurable signal, proportional to the concentration of analytes (Su *et al.*, 2011), biosensors, in recent years, takes a very important role on medicine, biology and biotechnology (Koyun *et al.*, 2012).

Since the first approach for glucose detection by immobilized glucose oxidase, developed by Clark and Lyon in 1962 (Clark Jr. and Lyons, 1962), biosensors have been intensively studied and extensively used in various applications. The applications range include environmental monitoring and control, chemical measurements in the agriculture, food analysis, drug industries, bioprocess and clinical diagnosis (Mehrvar and Abdi, 2004; Zhang *et al.*, 2000).

Biosensors have recognized advantages such as, the improved performance, the elimination of slow preparation and expensive reagents, providing a low cost, portable, simple-to-operate and real-time analysis, over the conventional analytical instruments. On the other hand, some limitations are known such as, electrochemically active interferences in the sample, weak long-term stability and troublesome electron-transfer pathways (Mehrvar and Abdi, 2004).

These analytical devices contains two basic components connected in series, the biological recognition system which lead to an biochemical mechanism; and a physicochemical transducer, which is capable to provide selective quantitative or semi-quantitative analytical information (Figure 2.1.) (Thévenot *et al.*, 2001). Both components play an important role in the construction of a sensitive and specific device for the analyte analysis.

The biological recognition system, also known as bioreceptor, consists an immobilized sensitive biological element (e.g. enzyme, DNA, probe, antibody) recognizing the analyte (e.g. enzyme substrate, complementary DNA, Antigen). Its immobilization in proximity of the transducer allows the translation of information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity.

Biosensors can also be self-contained, that is, all parts are packaged together in the same unit, with the biorecognition element contacted directly with the transducing element. The main purpose of the recognition system is to provide a sensor with a high degree of selectivity for the analyte to be measured. Enzymes are the major biological elements employed in biosensors, nevertheless other biomolecules are widely used (Koyun *et al.*, 2012; Thévenot *et al.*, 2001).

The transducer part of the sensor serves to transfer the output signal resulting from the interaction of the analyte with the bioreceptor, to the electrical domain by converting the biochemical signal into an electronic one. The intensity of generated signal is directly or inversely proportional to the analyte concentration. With transducer as preferred term, this component is also called as detector, electrode or sensor; in any case, should provide bi-directional signal transfer (non-electrical to electrical and vice versa) (Thévenot *et al.*, 2001).

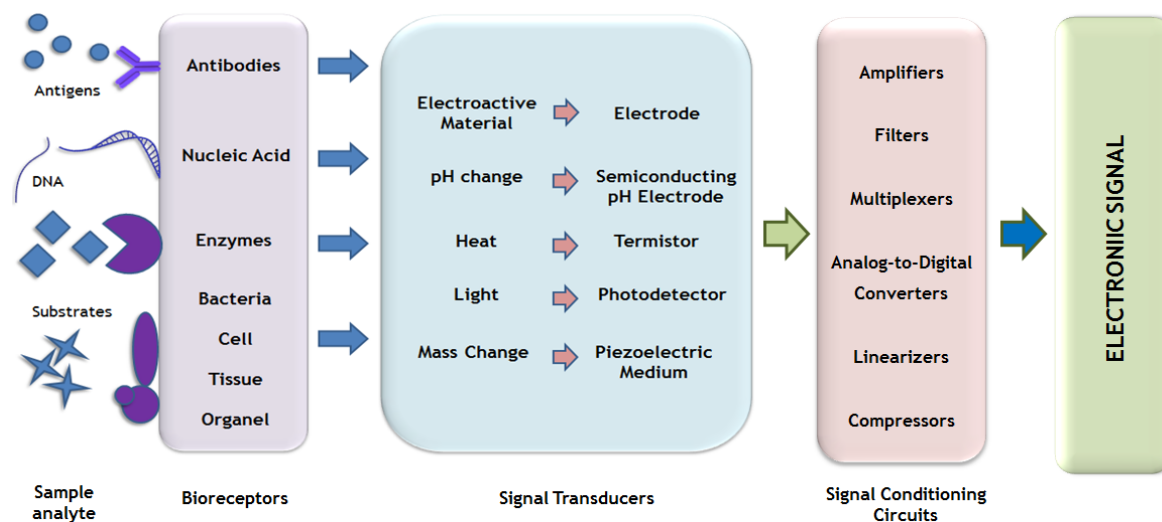


Figure 2.1. Schematic representation of biosensor device.

A third component based on computer-assisted mathematical modeling, describe the very complex biochemical processes that occur in the boundary region close to the sensor interface (Zhang *et al.*, 2000). Finally, the resulting processed signal has to be presented through an interface to the human operator (Grieshaber *et al.*, 2008).

2.2. Biosensors classification

The existing high range of biorecognition molecules and transducers, leads to high possibility of combinations to construct biosensors. These devices may be classified according to the biological specificity conferring mechanism, based on a catalyzed chemical reaction, or on an equilibrium reaction with, macromolecules that have been isolated, engineered or present in their original biological environment. Biosensors may be further classified according to the

mode of physicochemical signal transduction, and to the analytes or reactions they monitor (Mehrvar and Abdi, 2004; Thévenot *et al.*, 2001).

2.2.1. Transduction process and measurement techniques

As aforementioned, transducer domain will respond to an analyte concentration, converting the biochemical signal into an electronic one. Nowadays, biosensors are miniaturized and self-contained, with all parts packaged together in the same unit, with the biological recognition element in direct spatial contact with the transducing element. Although electrochemical transducers are widely used in biosensors development, the devices can also be based on piezoelectric, calorimetric or optical detection (Koyun *et al.*, 2012; Thévenot *et al.*, 2001).

2.2.1.1. Non-electrochemical transducers

Optical biosensors are particularly attractive for the application in direct detection systems. The light resulting of a biological and/or chemical reaction can be measured with high sensitivity. However, this sensitivity may result in a lack on turbid media analysis. Also a disadvantage is the relatively long assay time, the longest of all the biosensors, and the lack of sensitivity.

Calorimetric biosensors are based on the heat evolved from biochemical reactions of the analyte with a suitable biological active substance. In terms of performance, those biosensors have also some lack once the majority of heat evolved is lost to the surrounding media without being detected, leading to lowers sensitivities.

Finally, the piezoelectric transducers operate through the coating of the surface of the biosensor that selectively binds biologically active substances. This surface is placed in a solution containing analytes that bind to the binding substance, increasing the mass of the crystal while resonance frequency of oscillation decreases proportionally. This methodology has also some disadvantages like, lack on specificity, selectivity and sensitivity (Mehrvar and Abdi, 2004).

2.2.1.2. Electrochemical transducers

Electrochemical biosensors emerge as the most commonly used biosensors in monitoring and diagnosis tests in different areas, including clinical analysis (Mehrvar and Abdi, 2004). For extracting information from biological systems to measure electrical proprieties (Grieshaber *et al.*, 2008), those transducers are able to converts biochemical information to a processed and quantifiable electric signal. They provide selective quantitative or semi-quantitative analytical information using a biological recognition element (Thévenot *et al.*, 1999), offering

advantages such as, low cost, simple design, small dimensions (Koyun *et al.*, 2012), compatibility with instrumental sensitivity and the ability to operate in turbid media. Those transducers are also more amenable to miniaturization having the biological environment directly connected to the electronic device with a rapid response (Mehrvar and Abdi, 2004).

An electrochemical sensing device usually requires in its composition, a reference electrode, a counter or auxiliary electrode and a working electrode, shown later in figure 2.2. for a screen-printed electrode as example. The reference electrode is a kind of standard electrode and is kept at distance from the reaction site in order to maintain a known and stable potential, being commonly made from Silver/Silver chloride (Ag/AgCl) electrically stable. This functions as a redox electrode for the reaction between Ag metal and its salt, AgCl.

The working electrode where the reaction occurs in the electrochemical system, is also known as sensing or redox electrode, and serves as the transduction element in biochemical reaction. Based on Platinum (Pt), Gold (Au) or Carbon (C) (Thévenot *et al.*, 2001), they can be referred as either cathodic or anodic, depending if the reaction is a reduction or an oxidation, respectively. The working electrode is where the immobilization procedures for bioreceptor molecules are conducted.

The third component, a counter electrode, establishes a connection to the electrolytic solution, so that a current can be applied to the working electrode. Thus, the counter electrode functions are cathodic, whenever the working electrode is operates as anode, or vice versa. Both, working and counter electrodes should be conductive and chemically stable, therefore, platinum, gold, carbon and silicon compounds are commonly used, depending on the analyte (Grieshaber *et al.*, 2008; Koyun *et al.*, 2012).

2.2.1.3. Measurement techniques of electrochemical signals

Depending on electrochemical property measured by the detector system, (Bio)-electrochemical sensors can be divided into amperometric, conductometric or potentiometric, according to the detection method of biochemical changes in solution.

When the reactions under investigation generate a measurable current, the amperometry is the technique in operation, which is correlated to the reduction or oxidation of an electroactive product on the surface of a working electrode. It is usually performed by maintaining a constant potential between the working electrode and the reference electrode, generating a signal correlated with the proportional concentration of target compounds.

Conductometric or capacitive techniques are able to measure conductive properties of a medium, measuring chemical changes in the conductance between electrodes, at constant voltage. These devices need to perform an additional measurement, in an identical device

without the bioreceptor, due to parallel conductance of the sample solution, which hinders the biochemical signal analysis.

Potentiometry investigates a potential or a charge accumulation between the working and a reference electrode, measuring changes in pH and ion concentration, in function of the analyte interaction with immobilized bioreceptor. Thus, the response is a logarithmic function of analyte in sample.

Amperometric biosensors have advantages of being more highly sensitive, rapid, inexpensive, and disposable in comparison to the conductometric and potentiometric biosensors (Grieshaber *et al.*, 2008; Mehrvar and Abdi, 2004; Thévenot *et al.*, 2001).

2.2.2. Biological specificity conferring mechanism

The bioreceptor, usually biomolecules such as, enzymes, DNA probes and antibodies, or even living cells, can interact with the analyte through two types of mechanisms, a biocatalytic one or a bioaffinity mechanism.

2.2.2.1. Biocatalytic recognition element

This category is represented for enzymes, whole cells and tissue as immobilized biocatalyst, which are incorporated into the sensor, allowing the continuous monitoring of substrate(s) consumption. Those macromolecules are present in their original biological environment or have been isolated or manufactured previously.

Enzymatic biosensors, which can be mono- or multi-enzyme, are the most common and well developed recognition system, which are not consumed and can be reused. Their use as biological recognition elements is very popular owing to their commercial availability and the facility of isolation and purification from diverse sources. Other advantages, such as rapid response, selectivity, sensitivity, precision, the possibility of regeneration and the simplicity involved in constructing devices, are also recognized. The operating principle, based on the recognized specificity of enzymes to the limited range of substrates (Frew and Hill, 1987), enables the monitoring of the catalyzed reaction. The union of the substrate to the active center of the enzyme, allows the formation of the products, which when released, allows the initiation of a new reaction cycle. Thus, these are used to detect the presence of any of the substrates that participate in the reaction by detecting the disappearance of it, or in the appearance of a known product (Cock and Aponte, 2009).

Enzymes as thin as 10 - 200 μm , are incorporated in sensors for its combination in close proximity to the active surface of the transducer, leading to a great selectivity for target species. However, some difficulties in direct electron transfer between enzymes and electrodes are found as limitation. This encourages the use of small-molecule and

electroactive mediators, because they shuttle efficiently electrons between these two elements, enhancing the rate at which the electrons are transferred (Frew and Hill, 1987).

In the common applications, the detection limits are satisfactory or excessive, but the stability of enzymes and their capacity to maintain enzymatic activity over a long period of time continues to be problematic. Generally, it is resolved by enzyme immobilization allowing the increase of affinity to the substrate, reducing inhibition, increasing the optimal pH range and reducing possible microbial contamination. However, bacteria, fungus, protozoa and higher organisms become preferred as recognition molecules, when the purification of such enzymes is too difficult and costly, or when the stability is not sufficient to achieve the desirable results (Cock & Aponte, 2009).

Whole cells (microorganisms, cell organelles or particles) can be immobilized in membranes or trapped in matrix in a simpler and more economical way than enzymatic immobilization. With value in clinical analysis of biological fluids and for monitoring fermentation processes, these devices can provide a simple and rapid means of measuring analytes that previously were undetectable by electrochemical methods (Frew and Hill, 1987). These systems have the advantage of being directly used as biorecognition element, instead to be purified, having a reducing cost, improved stability and even offering the possibility of regeneration with the increase of electrode lifetime. As well, they are capable to metabolize different organic compounds, due to multi-cellular enzymatic systems that possess, generating distinct products. Furthermore, cells offer the facility to be modified genetically to improve their activity or to produce specific enzymes that do not normally appear. Nevertheless, limitations like the reduced diffusion of substrates and products result in a slower response comparing to the purified enzyme biosensor, leading to lengthy recovery times between measurements, referring their use as disposable devices. In addition, is also observed a lost on specificity owing to reactions catalyzed by others enzymes present in the cell (Cock and Aponte, 2009; Frew and Hill, 1987).

Tissue biosensors (plant or animal tissue slice), are based on determined tissues that according to their physiological function in the organism, produce specific enzymes or enzymatic systems. Some examples of used tissues are roots, leaves, fruits or seeds from plants, or animal tissues as beef liver or cortex of porcine kidney, in sliced or homogenized form (Cock and Aponte, 2009; Wijesuriya and Rechnitz, 1993). These structures have recognizable high concentrations of biocatalytic activity, due to their functions in the live being.

Low cost, simple construction, high catalytic activity, good shelf and electrode lifetime are offered by these types of sensors. Like whole cells biosensors, slow responses due to diffusional limitations; and a poor selectivity are also found. The presence of multiple enzymes, lead to the need of changing external conditions like, the use of different

substrates, enzyme inhibitors or activators and stabilizing agents (Cock and Aponte, 2009; Rechnitz and Ho, 1990).

2.2.2.2. Bioaffinity recognition element

Antibodies and nucleic acids are the biorecognition elements integrated in this category. Based on the interaction of the analytes with macromolecules or organized molecular assemblies, the biosensor reach the equilibrium and there is no further net consumption of the analyte by the immobilized biocomplexing agent, which have been isolated from their biological environment or engineered. In some cases this biocomplexing reaction is itself monitored using a complementary biocatalytic reaction. The equilibrium reaction signals are then monitored by the integrated detector, being often unable to monitor continuously the analyte concentration (Thévenot *et al.*, 2001, 1999).

Antibodies and Antigens have been used for the specific detection of their complementary partners through immunochemical reactions, where an antigen (Ag) is bound to a specific antibody (Ab). The biosensors concerned with monitoring solely Ab-Ag interaction, are also termed immunosensors, and the detection needs to be performed under conditions where non-specific interactions are minimized. Similarly to conventional immunoassays, these devices are based on the principles of solid-phase immunoassay, with either antibody or antigen immobilized at the sensor surface. These, 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 (Frew and Hill, 1987). Each determination requires the production of a particular Ab, its isolations and, usually, its purification. However, the Ab-Ag complexes formed have a particularly high binding or affinity constant, which make regeneration of the surface difficult, turn such systems irreversible (single-use biosensors). Notwithstanding, the Ab surface can be regenerated by dissociation of complexes, placing within environments with chaotropic agents, such as glycine-HCl buffer at pH 2.5.

The basic feature of immunoassay that provides the desired specificity under favorable conditions, is the complementary reaction (both on chemical binding and spatial orientation of reactive groups) between antigen and antibody. Thus, the sensitivity and specificity of an immunosensor are determined by the affinity and specificity of the binding agent. In some cases, the use of labels has been incorporated into immunosensor design to enhance sensitivity. Thus, the choice of label is another important feature, once such labelling requires an additional chemical synthesis step; and can be performed using radioisotopes, fluorescent, luminescent, light-scattering, enzyme or redox labels. In the case of labelled Ab, these devices will operate at equilibrium to quantify indirectly the amount of complexes produced (Morgan *et al.*, 1996; Thévenot *et al.*, 2001). Thus, an voltammetric based detection of immunoreactions depends on label type, assay format and electrochemical

technique, which can achieve excellent detection limits on small sample volume (Frew and Hill, 1987).

Based on the hybridization concept, **nucleic acids** (e.g. DNA and RNA) also have been used, once a known DNA sequence (probe) and an unknown counterpart (analyte) have a complementary degree between both chains. This biomolecular recognizing event occurs directly on the surface of the physical-transducer due to an immobilized single-chain of DNA specific to the target DNA analyte chain. The hybridization is stronger and more specific with the increase of complementarity degree between the two DNA chains, reaching a maximum in the case of full 100% recognition. That hybridization results in measurable changes in electrical parameters, allowing the electrochemical analysis. Like immunosensors, in this DNA-sensors design can be used various types of labels to improve the sensitivity (He *et al.*, 2011; Wang *et al.*, 1997).

2.2.3. Analytes or reactions monitored

Biosensors classification according to the target analytes or reactions that they monitor should be differentiated between, the direct monitoring of analytes and the indirect monitoring of inhibitors or activators.

Direct monitoring of the analyte: Known as the main application of biosensors, are based on the direct measurement, continuously or sequentially, of a physical phenomenon that occurs during a biochemical reaction on the transducer surface. Those devices measure the production or consumption of a given compound, being a useful tool for enzyme or living cell activities monitoring.

Indirect monitoring of inhibitor or activator of the biochemical receptor: These devices are irreversible, and their use is limited to applications which not require exact measurement of the analyte concentration. They are referred to as single-use biosensors and were developed for detecting substances, such as inhibitor or activator of the bioreceptor, which interferes with biocatalytic properties. One example of those devices applications are the monitoring of organic pesticides and inorganic substances (Mehrvar and Abdi, 2004; Thévenot *et al.*, 1999).

2.3. Screen-Printed Electrodes (SPE)

During the past decades, due to their advantages, the importance of electrochemical techniques has increased, being the main class of used of biosensors (Mehrvar and Abdi, 2004). However, in the analytical chemistry field these techniques have several restrictions and practical difficulties such as the cleaning and the surface regeneration processes needed in solid electrodes. On the other hand, conventional based stripping measurements had relied on mercury-based electrodes which have known problems with respect to their toxicity.

Additionally, there is a growing need of rapid performance and *in situ* analyses. This challenges resulted in an emerging electroanalytical field of small sized, disposable, sensitive, selective and low-cost devices, the Screen-Printed Electrodes (SPE) produced by thick-film technology (figure 2.2.) (Metters *et al.*, 2011; Nyholm and Schleiss, 2010; Renedo *et al.*, 2007; Tudorache and Bala, 2007).

These microelectronics adapted devices, offer important benefits facing the traditional methods mainly the possibility of sample direct analyses in its environment without alteration of the natural conditions (Tudorache and Bala, 2007), reinforcing their use to a more effective real life applications on industrial, clinical and environmental fields. SPE allows short assays with lowest energy consumption, making possible a small fluid manipulation, with less reagent at lower cost, increasing the sample throughput, which is also increased by the additional possibility of employing many parallel on-chip systems (Nyholm and Schleiss, 2010; Sin *et al.*, 2014).

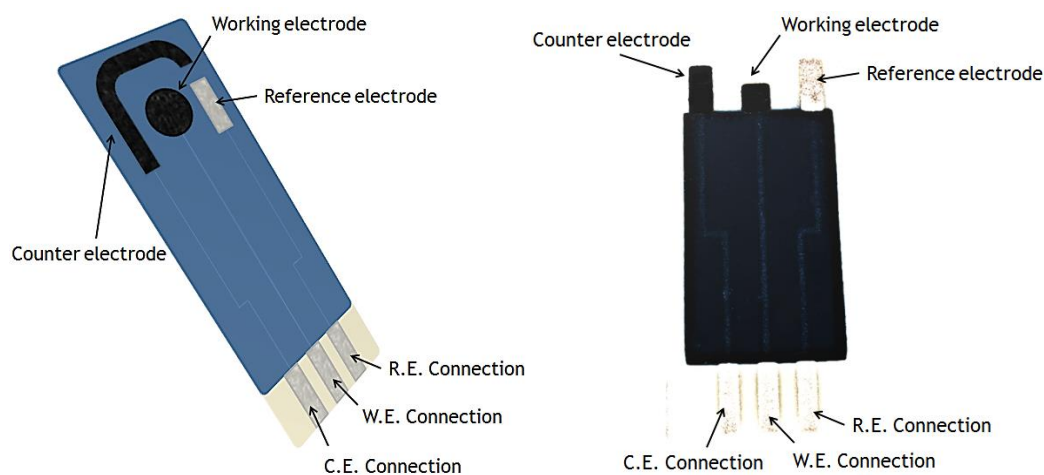


Figure 2.2. Electrochemical biosensor as a screen printed device and its constitution. Representation of working, counter and reference electrodes. At left, is represented an SPCE device produced at Burgos University.

2.3.1. SPEs fabrication

The automated and easy fabrication of these devices results in a large range of possibilities for design. The thick-film technology consists on the sequential deposition of layers of special inks or pastes onto an insulating support or substrate, being widely used on large-scale fabrication (Renedo *et al.*, 2007; Tudorache and Bala, 2007).

The deposition of different inks is carried out on a substrate using the appropriate screen, which is made from a finely mesh of different materials including stainless steel, polyester or nylon mounted under tension on a metal frame. The screen has open-mesh areas through which the desired pattern can be printed. The printing of SPE, shown in figure 2.3., is

conducted by a squeegee which slowly moves from the back to the front part of the screen, forcing the ink or paste through the open areas, depositing the required pattern onto the substrate surface. To obtain the desired electrode, between the printing patterns, is carried out a step of ink drying at an adequate temperature to allow the evaporation of the ink solvents. The requirement of this step is justified by the screen-printing inks which usually contain various organic solvents added with the aim of producing the accurate viscosity for screen-printing process. Thus, the substrate retains a rigid pattern, in the range of 20 to 100 μm of thickness (Metters *et al.*, 2011) that is relatively resistant 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. The available range of different screens, originates different types of SPEs configurations, used according to the analytical problem characteristics by choosing the adequate fabrication materials.

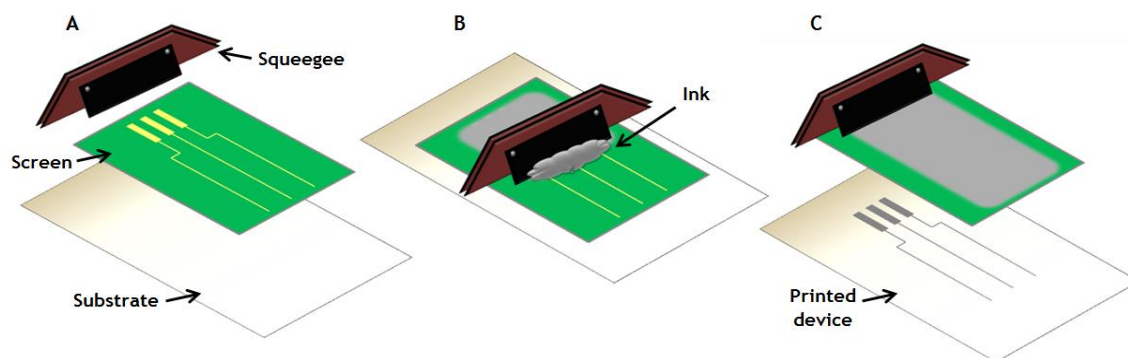


Figure 2.3. Screen printed device fabrication process scheme. A - Components for SPE fabrication, Squeegee, porous screen and substrate; B - Screen-printing step by squeegee traversing, C - Ink deposited on the substrate surface.

To be converted into a biosensor device, the SPE are further modified with the biosensing material. This modification, held at the working electrode, implies several steps in order to assure the robustness and durability of the developed biosensor (Metters *et al.*, 2011; Renedo *et al.*, 2007).

2.3.2. Materials for SPE construction

The performance of sensors fabricates by thick-film technology depends critically on the materials used and the manufacturing process. In addition, an electrode substrate of suitable condition, size, geometry, and immobilization technique has usually been taken into consideration to design appropriate electrochemical biosensors (Mehrvar and Abdi, 2004). Thus, the configuration of the SPE comprises material defining the basic structure of the electrode, the non-biological materials; and the biorecognition element with their related compounds and additives and/or cross-linkers necessary for developing the electrochemical signal (Tudorache and Bala, 2007).

2.3.2.1. Non-biological materials

Depending on the measurement technique, different materials can be used for the preparation of surfaces for biosensing applications. Currently, a wide diversity of materials is used, however, they need to fulfill special requirements, such as electrical conductivity for the electrochemical techniques (Grieshaber *et al.*, 2008). This category encompasses the materials for substrates, inks or pastes and the immobilization matrixes, additives or cross-linkers used for the bioreceptor element (Tudorache and Bala, 2007).

The first consideration is the composition of the substrate, the base on which the functional sections of the sensor are printed. The most common and well known materials used for substrate composition are alumina, ceramics, Poly(vinyl chloride) (PVC), cardboard coated with acrylic paint, and polycarbonate. The substrate must provide good electrical insulation, be chemically inert (Tudorache and Bala, 2007) and an inexpensive support (Renedo *et al.*, 2007).

On the other hand, the inks or pastes which represent the conducting part of the electrode, has an important role on the SPE performance. Commercial carbon and metal ink formulations are commonly used for printing the working electrodes, whereas silver-based inks are used for obtaining the reference electrode (Renedo *et al.*, 2007; Wang *et al.*, 1998). In fact, a broad range of pastes with different properties are available commercially, however, they are commonly adjust to make them suitable for particular purposes. These pastes usually contain binding agents, solvents and additives for dispersion, printing and adhesion tasks, that provide functional characteristics. To increase the conductivity properties, powdered metals, such as gold, platinum, silver, or palladium, or even non-metallic conductors like graphite, are added (Tudorache and Bala, 2007). Thus, the composition of the various inks used determines the selectivity and the sensitivity required for each analyses, resulting in versatile SPEs (Mettters *et al.*, 2011; Renedo *et al.*, 2007). The carbon inks, composed by graphite particles are widely used, owing to their electrochemical advantages and improvements in response, detection limit and cost. Moreover, they have an easily handling and easy preparation. Differences in ink composition (e.g., Type, size or loading of graphite particles), and in the printing curing conditions, may strongly affect the electron transfer reactivity and overall analytical performance of the resulting carbon sensors (Tudorache and Bala, 2007; Zhang *et al.*, 2000).

Attributes such as good adhesion, similar coefficients of expansion, to prevent stress-relates damage during sudden heating and cooling, the ability to retain their characteristics through the fabrication process, the ready availability, and low cost must be found at all materials mentioned above (Tudorache and Bala, 2007).

Apart from the biomolecule, some other materials can be used to functionalize the working electrode surface, as example the gold, silver, nickel, bismuth and mercury coating-films,

which increase the function of SPEs. Among the materials used, the metallic nanoparticles (Nps) are highlighted due to their important properties and multiple applications. Au, Pt and Ag have an important role on nanoparticle field for biosensing application and can be obtained by chemical or electrochemical synthesis or by UV light (Renedo *et al.*, 2007). Due to their high surface-to-volume ratio, conductivity, catalytic and magnetic properties the NPs are attractive means of signal amplification for the improvement of sensitivity detection rates and versatility of biosensing devices (Nyholm, 2005).

Furthermore, the possibility of modifying the SPE by means of depositing various substances on the surface of the electrodes, as example the immobilization matrixes, additives (e.g. cellulose acetate) and cross-linkers (e.g. glutaraldehyde) are also often involved in structures to improve the sensitivity, selectivity, stability, and reproducibility of the biosensor (Renedo *et al.*, 2007; Tudorache and Bala, 2007).

2.3.2.2. Biological material

The active part of the sensors, the layer of the biorecognition element, must be connected to different conductive pads for transducing and conversing signals. These elements, as mentioned may be enzymes, antibodies, DNA, RNA, cells, mediators and cofactors existing different forms for its deposition onto SPEs (Tudorache and Bala, 2007). Special attention will be given to antibodies in the next chapter.

3. Immunosensors

Bioanalytical assays such immunoassays (IAs), are transcendent to a variety of fields, including the biosensor field, in which offers applications for biological and medical research, diagnostic medicine, genetics, forensics, drug and pesticide testing (Frew and Hill, 1987; Renedo *et al.*, 2007). Firstly introduced by Yalow and Berson (Yalow and Berson, 1959), the concept of monitoring the specific antigen (Ag) and antibody (Ab) complexation, was used on an IA for plasma insulin in human subjects (Holford *et al.*, 2012).

3.1. Immunosensors principles and components

As already mentioned, biosensors can integrate different types of biomolecules as sensing elements. In the case of immunosensors, antibodies or antibody fragments with high specificity to detect the presence of its specific antigen, by forming a stable complex (Figure 3.1) are employed. The high affinity of the Ab is an important advantage of immunosensors, resulting in very low detection limits. However, this type of biosensor presents a general problem which is the difficulty to reversibly regenerate the sensing surface without rendering the device unusable by removing or denaturing the biological recognition element. Thus, due to the high affinities of Ab to Ag (Kd's generally lie between 10^5 - 10^{11} M), efforts are to the construction of disposable or single-use devices (Holford *et al.*, 2012; Tudorache and Bala, 2007).

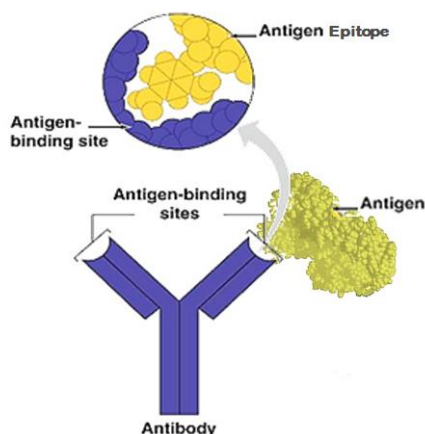


Figure 3.1. Schematic representation of complexation reaction between antibody and its specific antigen. Adapted from <http://drccercone.iculearn.com/bio2/wpcontent/uploads/Lectures/immune>

Immunosensors are small and portable instruments, designed for use by untrained personnel, for a rapid and sensitive assay. In accordance with the definition of a biosensor, the immunosensors also incorporate a transducer in which the biological component is immobilized allowing the occurrence of a biochemical or biophysical event onto the surface.

The transducer posteriorly converts its signal into an electric one, which is after amplified and digitalized by an electronic part (Hock, 1997; Holford *et al.*, 2012).

The Ab molecules convey selectivity and sensitivity to the sensor, improving its ability of analyzing specific targets in the presence of other substances (Hock, 1997). In fact, immunosensors are able to detect a specific analyte in matrices such as serum, plasma, urine or cerebrospinal fluid without sample treatment. These assays can be run either as direct sensors, which are able to detect the physical changes during the immune complex formation; or as indirect sensors, which use various types of labels for signal generation, allowing more sensitive and versatile detection modes (Holford *et al.*, 2012; Luppa *et al.*, 2001).

3.2. Electrochemical immunosensors

Immunoassays with electrochemical detection combine the specificity of bioaffinity assays with the sensitivity and low detection limit afforded by modern electrochemical techniques. As an alternative to the existing immunochemical tests, they bring benefits such as, enhanced sensitivity, reduced instrumentation and costs, selectivity, ease of use and low limits of detection with fast results (Holford *et al.*, 2012; Renedo *et al.*, 2007). These methods also provide the possibility of automation and integration in compact analytical devices with great potential in point of care diagnosis (Wan *et al.*, 2013). The classification of such devices can be divided into potentiometric, impedimetric, amperometric and voltammetric immunosensors.

3.2.1. Potentiometric immunosensors

Potentiometric transducers electrodes detect potential changes between the reference electrode and the electrode bearing the bioreceptor, due to a particular ion activity in function of the analyte interaction with the immobilized bioreceptor. An advantage of these sensors is the simplicity of the operation; however, problems of sensitivity to detect concentration gradients and nonspecific effects of binding or signaling are found, due to influences from other ions present in the sample. Especially, the signal-to-noise ratio causes analytical problems, which are difficult to circumvent.

3.2.2. Impedimetric immunosensors

In this type of immunosensors, the formation of Ab/Ag complexes on the electrode surface increase the dielectric layer thickness, causing capacitance changes proportional to analytes size and concentration. The binding of the antigen leads to a drop in capacitance, giving immunosensor with detection limits of 1mg/L. The high ionic strength of biological matrices

makes it difficult to record the relatively small net conductivity changes caused by the signaling reaction.

3.2.3. Amperometric immunosensors

Amperometric immunosensors are designed to measure a current flow generated by an electrochemical reaction at a set voltage. They consume a small percentage of analytes during the measurement, resulting in linear analyte concentration gradient, which also results in the need of electrochemically active labels (directly or as products of an enzymatic reaction). The main disadvantage for amperometric immunosensors of having an indirect sensing system is compensated for an excellent sensitivity due to the analyte consumption (Holford *et al.*, 2012; Lippa *et al.*, 2001).

3.2.4. Voltammetric immunosensors

Within the amperometric techniques, the voltammetry is the most versatile technique in electrochemical analysis. The information about an analyte is obtained by varying the potential and then measuring the resulting current. The position of the peak is related to the specific chemical and the peak current density is proportional to the concentration of the corresponding species. The voltage is measured between the reference electrode and the working electrode, while the current is measured between the working and the counter electrodes. Advantages of this technique are the low noise and the ability to detect multiple compounds since they have different peak potentials, providing higher sensitivity to the biosensor (Grieshaber *et al.*, 2008).

3.3. Antibodies immobilization

The use of antibodies as bioselective molecules has become a powerful tool in biosensor technology, due to the diversity of the immune response, which can produce Ab against almost any molecule (Nisnevitch and Firer, 2001). Consequently, Abs have been used as bioaffinity receptor elements in immunosensor construction, improving its ability to detect only their complementary partners, the antigens. In fact, immunosensors seem to be a promising attractive technique, offering high specificity, non-destructive approach to the sample, simple operation and uncomplicated sample preparation. Notwithstanding, the immobilization of those biomolecules onto the sensor surface remains a crucial challenge for sensor optimization, since sensitivity, stability and durability of the device depends on this (Makaraviciute and Ramanaviciene, 2013; Trilling *et al.*, 2013).

The recognition layer immobilization is an essential prerequisite for fabrication and development of electrochemical immunosensors, aiming to promote specific binding and

suppress non-specific binding (Wan *et al.*, 2013). It is known that the choice of the immobilization method greatly affects Ab-Ag interactions on the assay surface, resulting in specific or random orientation of the Abs. It is also known that the aim of the immunoassays will determine which immobilization procedure is more effective, since some assays require maximum sensitivity while others demand more rigorous consistency or lower cost. So, a variety of ideal methods for Ab immobilization are originated with each assay type. However, the similarity on Ab structures leads to many common aspects that strongly affect the Ag sensing ability of the immunosensor (Jung *et al.*, 2008).

Currently, several approaches for Abs immobilization are presented in literature, though a universal methodology is not available (Trilling *et al.*, 2013). Therefore, the structure and the functional groups of the Ab molecules must be highlighted to achieve and define the most appropriate technique for their immobilization onto the sensor surfaces.

The Abs are included in the protein field, representing a small class of glycoproteins with a well-defined structure. They consist of two identical light chains and two identical heavy chains, held together by disulfide bonds to form the characteristic Y-shape (Figure 3.2).

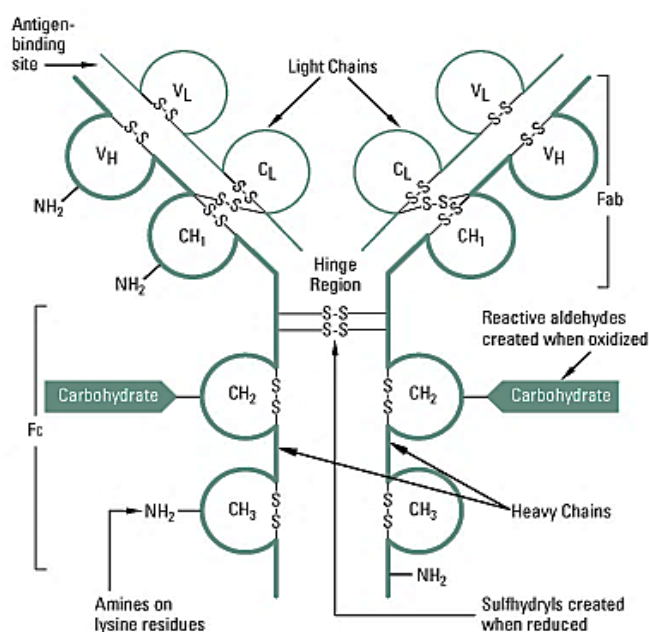


Figure 3.2. Antibody structure. Adapted from <http://www.piercenet.com/method/antibody-labeling-immobilization-sites>.

The chains are divided into constant (C) and variable (V) regions. Half of the heavy chain, the Fc region, incorporates the C-terminal which determines distinct properties. This region has a relative homogeneity that allows the definition of immobilization protocols with general applicability. As a glycoprotein, Abs have a single N-glycosylation site in the secondary heavy chain constant region (C_{H2}), showing a carbohydrate moiety. On the other hand, the variable domains present at the N-terminus bearing hypervariable regions, are known as

complementarity-determining regions (CDRs). Those CDRs are responsible for the specific Ab-Ag interaction, allowing the recognizing of virtually specific structures of the Ag molecules. The diversity in this area allows the endless supply of Abs with different specificity and binding strength (affinity) (Nisnevitch and Firer, 2001; Trilling *et al.*, 2013; Yoo *et al.*, 2002).

Chemically, the Ab molecules exhibit several functional groups which can be used to promote an oriented or random orientation in the attachment to the sensor surface. Generally shown in figure 3.3., those functional groups are the amine groups at lysine residues and at N-terminus; the sugar chain on C_H2, which can also be oxidized to originate a reactive aldehyde; the C-terminus which is also the natural affinity locus for intermediate proteins such protein A and G; the thiol groups formed from the reduction of the disulphide bonds and finally the nucleotide binding site.

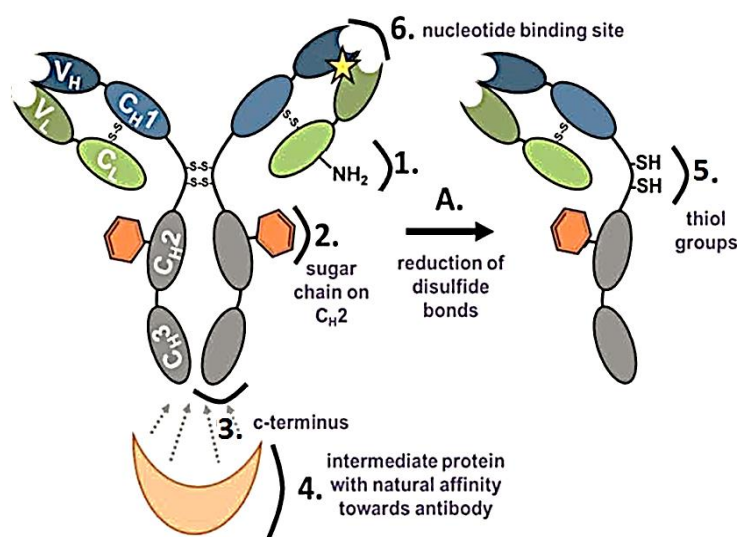


Figure 3.3. Schematic representation of the IgG functional groups used for random and oriented immobilization onto surfaces. 1. Amine group at lysine residues and at N-terminus; 2. Sugar chain on C_H2; 3. C-terminus; 4. Natural affinity locus for intermediate protein; 5. Thiol groups formed from the reduction of the disulphide bonds (A.) and 6. Nucleotide binding site. Adapted from (Trilling *et al.*, 2013).

The functional groups presented in Ab molecules have been part of numerous strategies, which can usually be made compatible with the surfaces of various materials, by functionalizing such surfaces with specific groups. A variety of reports on different assay platforms demonstrate how to control the antibody immobilization, mainly directing the orientation, stability, and density of bound antibodies.

The requirements for ideal Ab immobilization include proper and uniform orientation, lowest Ab modification, and mild incubation conditions (Jung *et al.*, 2008; Trilling *et al.*, 2013). Thus, the key problem during the immobilization is how fully maintain the protein

conformation (Wan *et al.*, 2013), without changing their binding activity and specificity, since denaturation or conformational changes can occur, reducing the immunosensor sensitivity.

Ideally, the Abs should display free CDRs after immobilization (figure 3.4. A) to improve the analyte binding and enhance the biosensor sensitivity, however the orientation is also dependent of the dimensions (Jung *et al.*, 2008) and the self-organizing capacity of the molecules, which may be steered by specific reactive groups on the surface or on the Ab, or on both. In addition, several factors, such as steric hindrance caused by neighboring Abs at high surface concentration, the distance between coupled Ab and the support surface and the modified conformation also affect the binding capacity (Lu *et al.*, 1996).

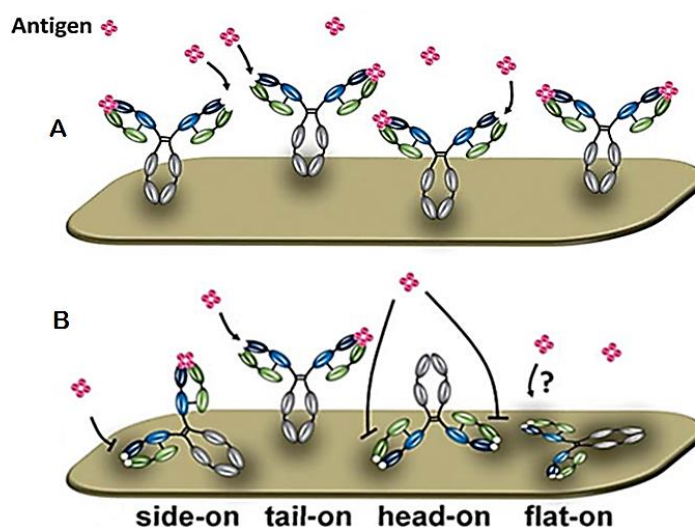


Figure 3.4. Illustration of Ab configurations when immobilized in the sensor surface. A - Oriented immobilization of the antibodies with free antigen binding sites. B - Randomly immobilized antibodies configurations; side-on, tail-on, head-on and flat-on. Adapted from (Trilling *et al.*, 2013).

3.3.1. Random immobilization techniques

Random orientation is generally characterized by weak attachment of the biomolecules, resulting from the proteins interaction with the electrode surfaces via non-covalent interactions, mainly electrostatic forces, ionic bonds, hydrophobic interactions and van der Waals forces (Trilling *et al.*, 2013; Wan *et al.*, 2013). Usually the result of such interactions is a combination of different orientations (figure 3.4. B): side-on (one Fc and one Fab attached to the surface), tail-on (Fc attached to the surface), head-on (both Fabs attached to the surface) or flat-on (all three fragments attached to the surface)(Trilling *et al.*, 2013; Wan *et al.*, 2013).

3.3.1.1. Adsorption

The adsorption of antibodies onto the electrode surfaces is by far the easiest method for antibody immobilization, with no required antibody modification. It occurs through unspecific hydrophilic, hydrophobic or both interactions between antibodies and the surfaces. However, adsorbed antibodies are randomly oriented and can lose their antigen binding ability. Adsorption gives low control over the orientation of Abs, with only less of 10% of bound antibodies active for antigen binding. Indeed, Zhao and co-workers (Zhao *et al.*, 2012) studied the Ab adsorption onto silica surfaces by varying the pH and salt concentration. They concluded that the salt concentration and the pH did influence the amount of adsorbed Ab and the analyte binding, but did not influence the orientation, since flat-on configuration were predominantly. Some other improvements on this type of immobilization pass through the surface modification to enable a partially oriented Ab (Jung *et al.*, 2008; Trilling *et al.*, 2013; Wan *et al.*, 2013).

3.3.1.2. Cross-linking

Like the adsorption, crosslinking procedures have been recognized as simple, with the advantages of offering a strong chemical binding of the biomolecules, preventing conformational change and the unfolding of the antibodies. Thus, enhancing the immunosensor storage times and resistance to extremes pH and temperature. The methodology is based in the formation of three-dimensional links, between the Abs and the solid surface by using multifunctional agents, such as glutaraldehyde, carboxymethyl dextran, aminobenzoic acid, carbodiimide and other reagents. Those possess functional groups, which mainly react with amine or carboxyl groups on the Ab, creating the cross-links. The nonspecific linkage may result in different Ab orientations that might prohibit formation of Ab-Ag complex. For instance, if multiple lysine groups are present on the surface of an antibody molecule, multiple attachments might occur (Holford *et al.*, 2012; Lu *et al.*, 1996; Makaraviciute and Ramanaviciene, 2013; Saerens *et al.*, 2008; Zhang *et al.*, 2000).

Several approaches of using glutaraldehyde (GA) as cross linker are available in the literature, once this agent has been the most widely used for proteins crosslinking. It can react with amine, thiol, phenol, and imidazole of the proteins. Lai *et al.* (Lai *et al.*, 2012) developed an electrochemical immunosensor for the detection of tumor markers, on which the immobilization was carried out using a firstly deposited chitosan coating and after a GA cross-linking to immobilize the antibodies molecules, achieving promising results in immunosensor performance.

3.3.2. Oriented immobilization techniques

The imposition of an orientation of the Abs molecules avoids most of the problems associated with random immobilization techniques. The main purpose is to release the CDRs to the sample phase of the immunoassay, attaching the biomolecules on the support through the Fc domain (figure 3.4. A), mainly by the C-terminus, the carbohydrate moiety or by pre-formed thiol groups on Ab fragments.

3.3.2.1. Covalent immobilization

Covalent methods require chemical activation of the surfaces to allow the exposing of chemically active groups with affinity to the functional groups on the Ab surface. Covalent binding provides a robust structure, involving the chemically active groups on the transducer in order to react with amino acids of proteins (Alonso-Lomillo *et al.*, 2009). This type of immobilization procedures can be carried out onto various types of electrodes by its modification with different functional groups. Numerous strategies for linking biomolecules to solids supports through covalent interactions are described in literature. Some examples are briefly described below.

The use of diazonium organic salt-modified electrodes is a promising approach, which has already been demonstrated for a wide range of conducting materials such as carbon, carbon nanotubes, silicon, metals and diamond. Data from Radi and co-workers (Radi *et al.*, 2009) describe the covalent immobilization of antibodies onto gold electrodes, thanks to the surface functionalization with amine groups reduced from the nitro groups originated by a diazonium mixture. This immunosensor was considered satisfactory with respect to precision, accuracy and stability to the detection of ochratoxin A.

Other works describe both, Abs and surfaces modification. For instance, Ferreira and Sales (Ferreira and Sales, 2014) presented an approach of a disposable immunosensor for detection of an oxidative stress biomarker. The propose relied on gold electrodes, is based on the surface functionalization with amine groups assigned by cysteamine, with the parallel modification of the Ab molecules, by the activation of carboxylic functions through the use of N-hydroxysuccinimide and Carbodiimide hydrochloride. The amine groups on the electrode surface bind to the carboxylic groups at Fc previously activated on the Ab molecules, providing a tail-on configuration. This strategy is efficient, since only the aspartic and glutamic acids have carboxylic acid side chains, poorly affecting the oriented immobilization process.

3.3.2.2. Bioaffinity immobilization

Other approaches to achieve oriented Ab immobilization were developed based on different bioaffinity interactions, by using antibody binding proteins, His-tag systems, DNA-directed immobilization, the sugar moieties with affinity for a range of functional groups and so on (Wan *et al.*, 2013).

Protein A and G could selectively bind with Fc region of different types of IgG with high affinity, resulting in the oriented immobilization of antibodies with significantly improved antigen binding capacity, sensitivity, and stability (Wan *et al.*, 2013). This technique is often used, with the advantage of the non-required Ab modification and additionally with the demonstrated high sensing abilities when compared to those using conventional methods such as random techniques (Jung *et al.*, 2008). However, an additional step of surface coating with the protein is required prior to Ab immobilization. This additional step can result in a random immobilization of the proteins, which can lead to a random immobilization of the Abs. Several schemes for avoiding this are described in the literature, such as pH manipulation and the engineering of those proteins, which can produce cysteine-functionalized protein G or fusing them with a gold-binding protein which makes them amenable for immobilization on gold electrodes (Trilling *et al.*, 2013).

Also widely used to obtain an oriented immobilization of Ab molecules is the sugar moiety. Taking advantage of the affinity of boronic acid towards sugars moieties of Ab molecules, Ho *et al.* (Ho *et al.*, 2010) have prepared a carbon surface presenting boronic acid groups on which anti-biotin Abs have been immobilized. They achieve an ultrasensitive electrochemical immunosensor, detecting concentrations of biotin as low as 0.19 pg.

3.4. Immunoassay signaling

As aforementioned, the immunosensor can run either as direct or as indirect assay. However, most of the electrochemical sensing techniques require the labelling of either antigen or Ab, since their binding is accompanied by only small physico-chemical changes (Hock, 1997; Renedo *et al.*, 2007).

3.4.1. Antibodies labelling

Abs and antigens are usually not electrochemically active within the desired potential range, thus, redox-active compounds can be applied as labels for indication (Warsinke *et al.*, 2000). The type of labels used in antibody functionalization, including radioisotopes, enzymes, fluorescence compounds, latex particles, nanoparticles (NPs), liposomes and metal compounds, have been investigated (Renedo *et al.*, 2007; Yin *et al.*, 2011). Those tracers provide signal generation or amplification, being recognized as a robust method on

biosensing. The most of the electrochemical immunosensors are based on the sandwich-type immunoreaction (Wang, 2012), which relies on the analyte sandwiching between the antibodies immobilized on electrode surface; and the detector Ab for signal output. Electroactive labels, must provide redox reactions used to detect the amount of captured analyte, increasing the sensitivity of the assay (Nyholm, 2005; Sin *et al.*, 2014).

The labelled type determines the sensitivity of the immunosensor. Enzymatic labels like horseradish peroxidase (HRP) and alkaline phosphatase (ALP) are conventionally used, due to the electroactivity of their enzymatic products, which allow electrochemical measurements. However, the amplification obtained with this conventional labels are not high enough for ultrasensitive detection (Wan *et al.*, 2013). So, the use of nanomaterials has been reported for this purpose with remarkable advantages.

3.4.2. Nanoparticles in signal amplification

Nanosized particles of noble metals, especially gold (AuNPs), silver (AgNPs), platinum (PtNPs), among others, aroused great interests due to their attractive stability, conductivity, biocompatibility, low cytotoxicity and size-related electronic, magnetic and optical properties. Among them, AuNPs are the most widely employed because of their unique characteristics such as excellent biocompatibility, but especially due to their remarkable ability to catalyze electrochemical reactions resulting in enhanced electrochemical signal (Wang, 2012; Xu *et al.*, 2014).

Some applications for the use of AuNPs are reported as an enhancement component with catalytic activity. With great interest on metalloimmunoassay, is the silver enhancement catalyzed by AuNPs, once those NPs have well-known catalytic properties on silver ions reduction. Thus, the use of a sandwich type immunoassay with a secondary Ab labelled with AuNPs, allows the precipitation of silver nanoparticles (AgNPs) on the surface of the AuNPs. In this manner it is possible to improve the sensitivity of immunosensors, dissolving the AgNPs and measuring its oxidation by anodic stripping voltammetry. Stripping analysis is a powerful electroanalytical technique for trace metal measurements. Its remarkable sensitivity is attributed to the preconcentration step, during which the target metals are accumulated onto the working electrode. The inherent high sensitivity of stripping metal analysis, combined with the remarkable signal amplification resulting from the catalytic precipitation of silver onto AuNPs tags, make this technique a powerful tool for electrochemical immunosensors (Chu *et al.*, 2005).

II. Groundwork and Aim

Nowadays, the diagnosis of HCMV is based on clinical and immunologic data, being available numerous methods for its detection. However, these are expensive, or/and require long time to perform, or/and need skilled operators, or even leads to the possibility of false results, being not effective for on-site use. So, as previously stated in section 1.5., new diagnosis techniques for this virus should be rapid, efficient, simple, sensitive, sensible, specific and low cost.

Combining the advantages of the biosensor field with this problematic, some authors have proposed biosensing approaches for HCMV detection. Approaches for HCMV biosensing are related with HCMV DNA sequences (Authier *et al.*, 2001; Azek *et al.*, 2000); HCMV determination by piezoelectric affinity sensor (Susmel *et al.*, 2000), and immunofluorescence (Wacogne *et al.*, 2011). However, these proposes have some gaps for the recommended criteria to be used as point of care. Both DNA detection methods are used in conjugation with PCR techniques, already implemented on HCMV diagnosis, for the characterization and quantification of amplified products, being useless as rapid diagnosis and adding costs to the assay. Disadvantages on HCMV determination by piezoelectric affinity sensors rely principally in the use of costly equipment, which causes a deficiency for the use as recurrent diagnosis technique. In the last approach, the Abs are immobilized in a disposable cartridge where the biological samples and the reactive liquids are placed. The complexation reaction between the immobilized Abs and the HCMV in the sample allow the attachment of a secondary and labelled Ab, resulting in fluorescence, which can be detected. However, that technique results in a lower sensitivity, that limits its use in low viral load samples.

To circumvent the problematic for Human cytomegalovirus diagnosis, Pires developed an inexpensive, simple and disposable immunosensor as an alternative methodology for the existents procedures (Pires *et al.*, 2013). Established in screen-printed electrodes produced at Burgos University, in Spain, this immunosensor was design to use electrochemical methodologies on detecting the presence of glycoprotein B in human fluids. This approach is based on a sandwich-type immunoassay, with the HCMV glycoprotein B sandwiched between the Anti-HCMV antibody adsorbed onto the working electrode, and the Anti-HCMV labeled with gold nanoparticles. In presence of a silver deposition solution, the gold nanoparticles catalyze the reduction reaction of silver ions, leading to the formation of silver nanoparticles. Subsequently, the silver nanoparticles are dissolved and its oxidation measured by anodic stripping voltammetry in a KCl solution (shown later in figure 3.3.5.). The basic principle is that a higher concentration of HCMV glycoprotein B means more amount of captured gold

nanoparticles on the sensor surface, producing more silver deposition with higher signals at the measurement.

This study achieved variations in the peak intensities of silver oxidation, directly correlated with variations in HCMV glycoprotein B concentration. A saturation profile was reached for 15 ng/mL of HCMV glycoprotein B. The calculated detection limit was 2.02 ng/mL of HCMV glycoprotein B and the reproducibility 16.6%, obtained for calibration curves of different assays. The reproducibility was evaluated by the residual standard deviation (RSD) and the value was considered consistent with that obtained for this type of sensor.

These promising results provide excellent guidelines for the device improvement. However, a deficiency on antibodies immobilization was found, since those were randomly adsorbed onto the working electrode, affecting the stability and the reproducibility of the immunoassay. Thus, to improve the immunosensor performance on HCMV detection is proposed the study of different antibody immobilization strategies, such as the glutaraldehyde cross-linking, the covalent immobilization with diazonium salts and the immobilization of the Ab through the affinity of the boronic acid towards the sugar moiety. The results will be evaluated by voltammetric techniques on stripping analysis of silver nanoparticles, previously deposited in catalytic gold nanoparticles which in turn are attached to the sandwich immunoassay.

Although the great and recognized specificity of the immunosensors, conferred by the antibodies molecules, it also becomes relevant to study the electrochemical behavior of the glycoprotein B, once it is the main dominant protein in HCMV infection, and to the best of our knowledge, until now it wasn't described in literature.

III. Materials, Apparatus and Methods

1. Reagents

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

Bovine Serum Albumin (BSA), hydrogen tetrachloroaurate (III) trihydrate (HAuCl_4), tris(hydroxymethyl)aminomethane, nitric acid, potassium carbonate, trissodium citrate, potassium chloride, tween-20, glutaraldehyde, 4-aminobenzoic acid, 3-aminophenylboronic acid, N-hydroxysuccinimide (NHS), 4-nitrobenzenediazonium tetrafluoroborate ($\text{N}_2\text{C}_6\text{H}_4\text{NO}_2\text{BF}_4^-$), tetrabutylammonium tetrafluoroborate (NBu_4BF_4) and silver enhancer solutions, were obtained from Sigma-Aldrich, Steinheim, Germany. Sodium dihydrogen phosphate (NaH_2PO_4), di-Sodium hydrogen phosphate (Na_2HPO_4) and Sodium carbonate (Na_2CO_3) were obtained from Merck, Darmstadt, Germany. Sodium perchlorate monohydrate (NaClO_4), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and Hydrochloric acid were purchased from Fluka, Steinheim, Germany. Sodium nitrite and Dimethyl Sulfoxide (DMSO) were obtained from Panreac, Barcelona, Spain.

Different inks were used in the fabrication of screen-printed carbon electrodes (SPCEs), namely C10903P14 (carbon ink) and D2071120D1 (dielectric ink) (Gwent Electronic Materials, Torfaen, UK), Electrodag 418(Ag ink) and Electrodag 6037SS (Ag/AgCl ink) (Acheson Colloiden, Scheemda, The Netherlands). On covalent immobilization is required a resistive insulating dielectric ink 242-SB (ESL Europe, Agmet Limited, Reading, UK), due to the use of organic solvents such as acetonitrile. A polyester substrate (0.5mm thickness) from HiFi Industrial Film, Dardilly, France was used.

All reagents used were of analytical grade. Milli-Q water (Millipore, Bedford, MA, USA) was used for preparing aqueous solutions.

2. Apparatus and software

A DEK 248 screen-printing system (DEK, Weymouth, UK) was used to fabricate the SPEs, using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke (Figure 3.2.1.).



Figure 3.2.1. DEK 248 screen-printing system (DEK, Weymouth, UK).

Electrochemical measurements were performed with Autolab PGSTAT128N electrochemical system and μ Autolab type III (Figure 3.2.2.) with General Purpose Electrochemical System (GPES) software version 4.9 (Echo Chemie, Utrecht, The Netherlands).



Figure 3.2.2. At left the μ Autolab type III and at right the Autolab PGSTAT128N electrochemical system.

The electrochemical study of the HCMV glycoprotein B was conducted using a three-electrode cell containing a Ag/AgCl reference electrode, a platinum auxiliary electrode (model-mf 2010) (bioanalytical systems, west Lafayette, USA) and a carbon doped diamond as working electrode (Windsor scientific Ltd, Slough, UK).

3. Methods

3.1. Immunosensor construction

A sandwich-type immunoreaction is the basis principle for the immunosensor operation, where the viral glycoprotein B (gB) is sandwiched between immobilized and labelled Anti-HCMV antibodies. Several strategies, present bellow, were tried for the Anti-HCMV antibodies immobilization onto the carbon working electrodes of the SPCEs. The labelling of the secondary antibodies with gold nanoparticles (AuNPs) allows, in presence of a silver enhancer solution, the catalytic reduction of silver ions onto the gold surface, which can be directly measured by anodic stripping analysis. The different strategies for antibodies immobilization may reflect improvements in the analysis, mainly in the reproducibility and in the achievement of lowest detection limits.

3.1.1. Antibodies labelling

3.1.1.1. Preparation of gold nanoparticles (AuNPs)

The gold nanoparticles were prepared from 100 mL of a 0.01% HAuCl₄ solution. This solution is boiled with vigorous stirring, and 2.5 mL of 1% trisodium citrate solution was quickly added. When the solution turned deep red, indicate the formation of colloidal AuNPs, and was left stirring and cooling down (Gao *et al.*, 2011).

3.1.1.2. Preparation of AuNPs-labelled antibodies

Firstly, 10 µg of anti-HCMV was added to 0.990 mL of colloidal AuNPs, and the pH of this solution was adjusted to 9.0 with 0.1 M K₂CO₃. The solution was gently mixed for 60 minutes at room temperature. After a centrifugation at 4800 rpm for 30 minutes, the supernatant containing the excessive antibody was discarded and the soft sediment was washed with Tris-HNO₃ (50mM, pH 7.2). After a second centrifugation and discarding of supernatant, the resulting pellet of AuNP-labelled antibodies were resuspended in 1.0 mL of Tris-HNO₃ (50 mM, pH 7.2) containing 1.0 % BSA and stored at 4 °C (Lai *et al.*, 2012).

3.1.2. Antibodies immobilization procedures

3.1.2.1. Glutaraldehyde cross-Linking

The working electrode surface was firstly activated by incubating 3 μL of 2.5 % glutaraldehyde solution in Tris- HNO_3 (50 mM, pH 7.2), during 2 hours at 4 °C. When the activation time was over, 3 μL of 1 $\mu\text{g}/\text{mL}$ of anti-HCMV antibody solution was placed on working electrode surface, followed by a new incubation at 4 °C overnight at moisture-saturated environment. Subsequently the excess of antibodies were washed using sequentially washing buffer (Tris- HNO_3 , 50 mM, pH 7.2, with 1% of Tween 20) and with Tris- HNO_3 (50 mM, pH 7.2). Finally, a 3 μL of blocking solution (BSA 5 mg/mL) was applied on the electrode surface, and incubated at 4 °C during 60 minutes in a moisture-saturated environment (Lai *et al.*, 2012) (Figure 3.3.1).

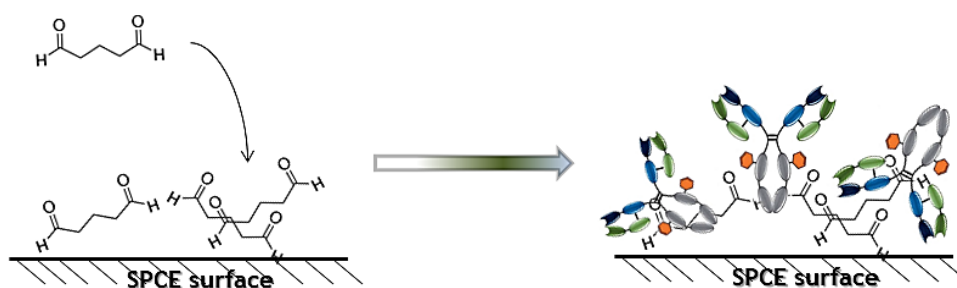


Figure 3.3.1. Schematic representation of Abs cross-linking through glutaraldehyde molecules.

3.1.2.2. Covalent immobilization using diazonium salts

Briefly, the covalent immobilization procedure pass through three main steps: the working electrode modification by forming a monolayer of nitro groups on working electrode surface, the reduction of those previously deposited nitro groups to amine groups, and finally the antibody immobilization through the use of EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) and NHS (N-Hydroxysuccinimide), which may activate the carboxylic functions on the antibody molecules (Figure 3.3.3.).

Formation of a monolayer of nitro groups

Firstly, the nitro groups (NO_2) were deposited on working electrode surface using a 3mM of diazonium salt dissolved in 0.1M of $\text{N}(\text{BU})_4\text{BF}_4$ (Tetrabutylammonium tetrafluoroborate) in acetonitrile. 50 μL of that solution were placed to cover the working area of the electrode 2 cycles of a cyclic voltammetry were preformed between 0.8 and -0.4 V, at a step potential of 0.012 V and a scan rate of 0.2 V/s.

Reduction of nitro group to amine groups

After the surface activation with nitro groups, those may be reduced to amine groups, to become amenable for the antibody immobilization. The reduction was achieved performing 2

cycles of cyclic voltammetry between 0 V and -1.7 V, at step potential of 0.012 V and a scan rate of 0.2 V/s, using 100 μ L of 0.1M KCL in 1:9 of ethanol in water (v/v).

Antibody immobilization through NHS and EDC

Finally, the antibody immobilization was achieved through the combination of the antibody molecules with 40 mM of NHS and 80 mM of EDC, both in 10 mM of phosphate buffer at pH 6.0. After an incubation at 4°C, during 90 minutes in a moisture-saturated environment the electrodes were washed with washing buffer (Tris-HNO₃, 50 mM, pH 7.2, with 1% of Tween 20) and Tris-HNO₃ (50 mM, pH 7.2); and a drop of blocking solution (BSA 5 mg/mL) was applied in working electrode, with a new incubation at 4°C during 60 minutes in a moisture-saturated environment.

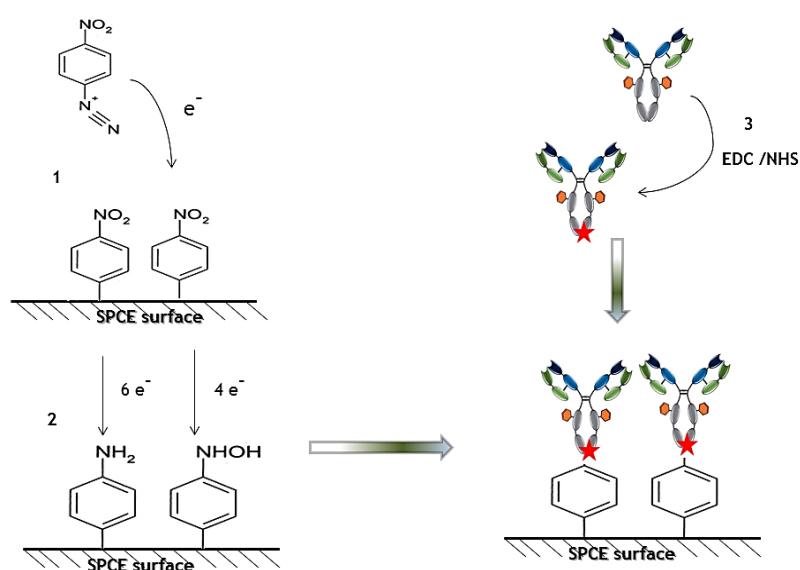


Figure 3.3.3. Schematic representation of covalent immobilization. 1- Electrochemical deposition of diazonium salt in electrode surface, exposing NO₂ groups; 2 - Electrochemical reduction of NO₂ to NH₂ groups; 3 - Activation of carboxylic function of the antibody molecules through the use of EDC and NHS.

3.1.2.3. Immobilization using the affinity of boronic acid (APBA)

The aim of this technique passes through the use of the boronic acid affinity towards the sugar moiety present in the antibody molecule. For that propose, the SPCE was previously modified to present boronic acid groups on the surface, which will interact with the carbohydrate unit on the Anti-HCMV antibody. This modification involves three steps: surface functionalization with carboxyl groups using 4-aminobenzoic acid followed of the boronic acid linkage through EDC and NHS, and finally the incubation with the anti-HCMV antibodies (Figure 3.3.4.).

Diazotization of 4-aminobenzoic acid and carboxyl group functionalization of the SPCE surface

4-aminobenzoic acid (2 mg) was dissolved in HCl (0.2 mL) and cooled in an ice-water bath. 0.2mM sodium nitrite (38 μ L) was added to this solution with constant stirring and then a mixture was stirred for 5 minutes. A drop of this diazonium solution (50 μ L) was placed onto the SPCE to cover the entire working area and then 10 cyclic voltammograms were recorded over the range from 0 to -1 V at 0.2 V/s. Finally, the SPCE was sonicated in d.d. water for up 1 minute, washed with MeOH, and dried under a stream of N₂.

Aminophenylboronic acid-modified SPCE

EDC and NHS (0.018 mmol each) were dissolved in DMSO (0.5 mL). To activate the electrode surface, a drop of this mixture EDC/NHS (3 μ L) was placed onto the working electrode of the carboxyl group-functionalized SPCE and left to react for 3 hours. The electrode was washed sequentially with d.d. water and MeOH and then dried briefly under a stream of N₂. A drop of 50 mM 3-aminophenylboronic acid (10 μ L) was placed on the activated surface for 3 hours. The electrode was rinsed sequentially with distilled deionized water and MeOH and then dried at a stream of N₂ to provide the aminophenylboronic acid-modified SPCE for antibody immobilization.

Site-specific antibody immobilization

An aliquot of Anti-HCMV antibody solution (0.2 mg/mL, pH 7.5, 5 μ L) was added to the APBA/SPCE working electrode, which was then left at 4 °C overnight. The Ab/ABPA/SPCE was then rinsed with 10 mM PBS to remove any unbound Ab, providing a modified SPCE for immediate use in the immunosensing of the HCMV. Finally, a drop of blocking solution (BSA 5 mg/mL) was applied in working electrode, with a new incubation at 4 °C during 60 minutes in a moisture-saturated environment.

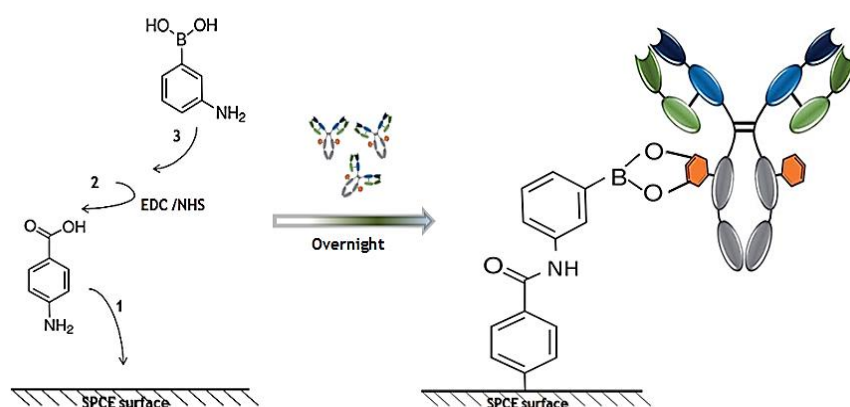


Figure 3.3.4. Schematic representation of ABPA immobilization. 1- Electrochemical deposition of 4-aminobenzoic acid and carboxyl group functionalization of the SPCE surface; 2 - Activation of the electrode surface carboxylic function through EDC and NHS; 3 - Aminophenylboronic acid surface modification, and the attachment of antibodies molecules through the sugar moiety.

3.1.3. Sandwich immunoreaction

Once immobilized the anti-HCMV antibodies, and after the blockage of the surface with BSA to avoid unspecific interactions, several HCMV gB concentrations were placed on the modified surface. After 1 hour of incubation at 4 °C in moisture-saturated environment, the electrodes were washed using sequentially washing buffer (Tris-HNO₃, 50 mM, pH 7.2, with 1% of Tween 20) and with Tris-HNO₃ (50 mM, pH 7.2); and 3 µL of the AuNP-labelled antibodies solution were placed onto the working electrode surface and incubated for 1 hour. After this, the electrodes were again washed, and a 140 fold diluted silver enhancement solution was incubated during 4 minutes in the dark, at room temperature. Finally, the electrodes were rinsed with water and a differential pulse voltammetry were performed at a windows range from -0.06 to 0.06 V, using KCl (1M) as support electrolyte (figure 3.3.5.) (Pires *et al.*, 2013).

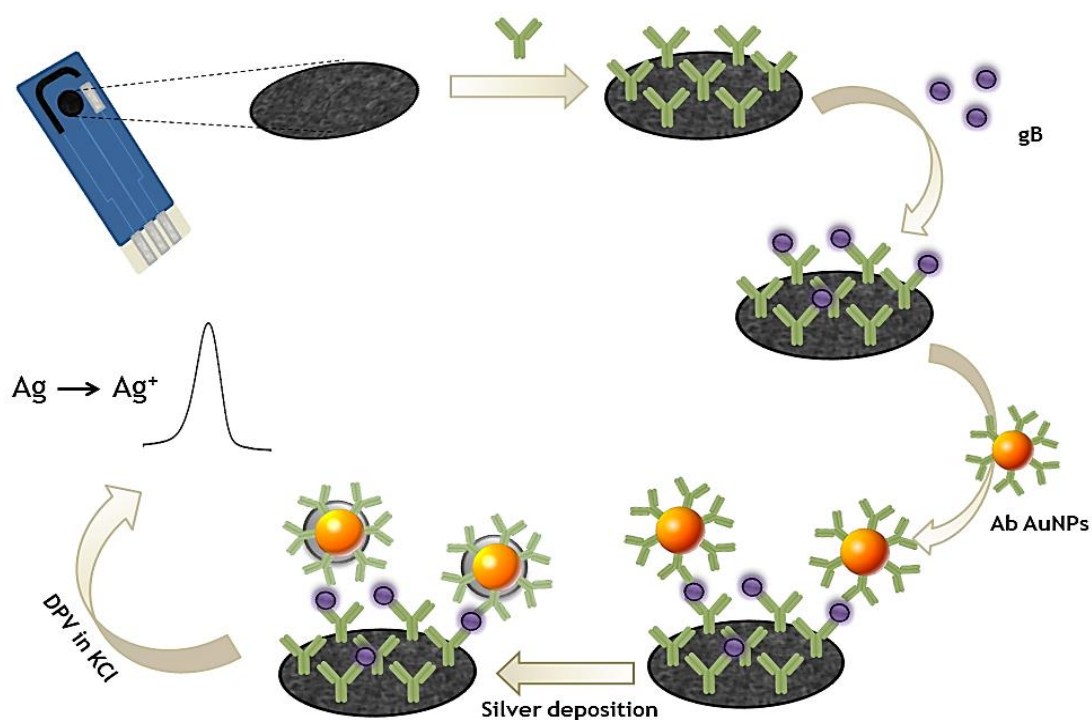


Figure 3.3.5. Schematic representation of immunosensor construction and detection strategy by differential pulse voltammetric analysis (DVP) of Ag NPs catalytically deposited on the immunosensor surface by gold nanolabels (Ab AuNPs).

IV. Results and Discussion

1. Electrochemical behavior of HCMV glycoprotein B

Glycoprotein B (gB) is the predominant protein found in the HCMV envelope, having an important role in infection (Fan *et al.*, 2011), so in this work is proposed an electrochemical study of the HCMV gB, in order to investigate its electrochemical behavior, with the prospect of generation of a characteristic electrochemical signal. Additionally, this assay will allow us to determine if that generated signal results in interferences for the previously proposed immunosensor. At last, urine samples were analyzed once that biological fluid was found to be the ideal specimen for detection of HCMV by containing moderate to large amounts of infectious virus particles (Albanna *et al.*, 2013).

1.1. Electrochemical behavior in SPCEs

Due to their recognized advantages when compared with the conventional electrodes, the first attempt for the electrochemical study of HCMV gB was conducted in SPCE, home-made at Burgos University.

In assay development, buffered mediums were subjected to a preliminary electrochemical screening to find a potential range where interferences are reduced. Three buffered mediums were tested: Tris-HNO₃ (50 mM, pH 7.2), once is medium used by Pires *et al.* in the immunoassay scheme (Pires *et al.*, 2013); carbonate buffer (0.01 M, pH 10) used by Koppang and co-workers for the study of polyamines (Koppang *et al.*, 1999), and at last an biological buffered medium, the phosphate (10 mM, pH 7.2).

The study was based on cyclic voltammetry, that allows finding both oxidation and reduction signals. However, within large windows potential of screening, it was evident that the SPCEs suffer degradation once the carbon of the working electrode reacts with the medium, being observable its degradation. On the other hand, even if the potential range were minimized, it was observable that the buffer without the presence of the analyte originates oxidation and reduction signals. Despite that, the study of the gB were conducted to see if the eventual presence of an electrochemical signal overrides the buffer signal noise. The cyclic voltammograms, presented at the figure 4.1.1. (a-c), represent the increment of gB concentrations on the different buffered solutions.

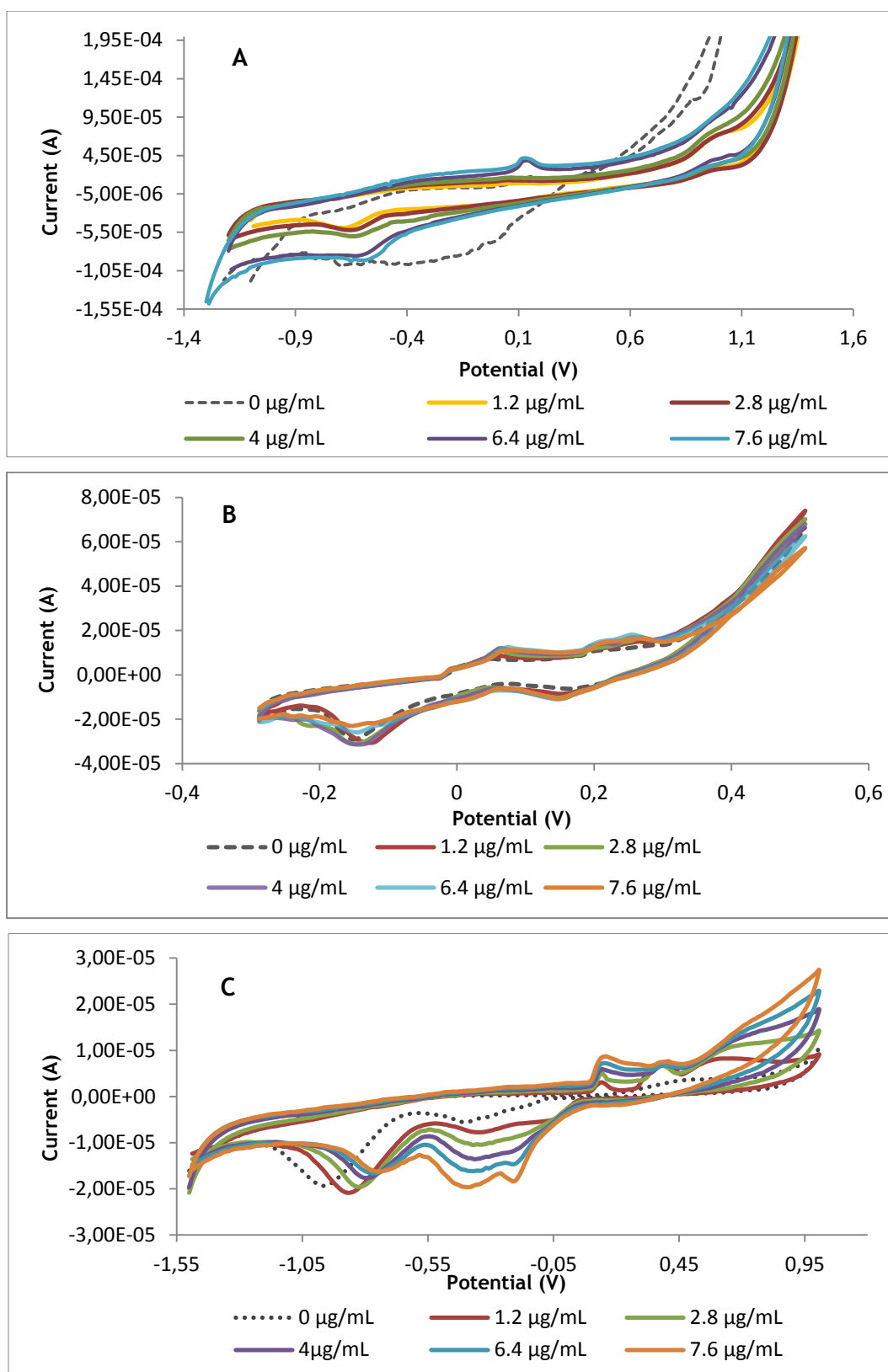


Figure 4.1.1. Cyclic Voltammograms obtained for the three buffered medium tested: A - 50 mM of Tris-HNO₃ at pH 7.2, B - 0.01 M of Carbonate buffer at pH 10, and C - 10 mM of Phosphate buffer at pH 7.2.

Evaluating the cyclic voltammograms above shown, it is visible that in Tris-HNO₃ (Figure 4.1.1. A), the signal for the blanc has a high response, which is weakly altered by the addition

of gB concentrations. When carbonate buffer was used as supporting electrolyte, the gB does not alter the behavior of the blanc, being not possible to observe the generation of an electrochemical signal. On the other hand, when the gB is added to phosphate buffer, a signal between -0.55 and -0.05 V is generated, being possible to distinguish in relation to the blanc. To confirm the origin of such signal, should not be observed any signal generation on scans performed in phosphate buffer at absence of gB. However, like is demonstrated in figure 4.1.2, that signals amplification it was also observed for the buffered medium without gB. Thus, we can conclude that may be due to the SPCE surface modification, preventing to draw conclusions about the generation of an electrochemical signal related with the gB molecules.

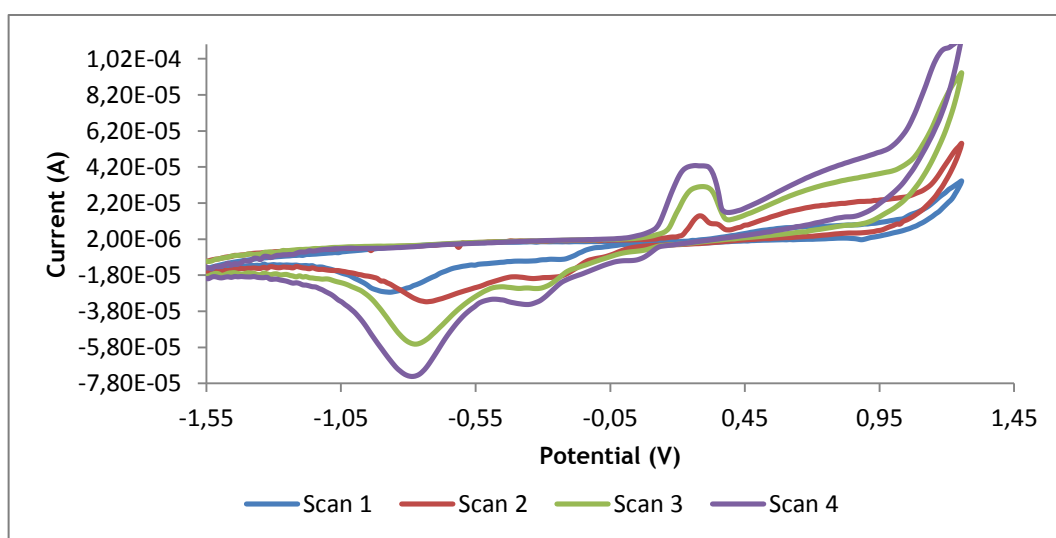


Figure 4.1.2. Cyclic Voltammograms obtained for 10 mM of Phosphate buffer at pH 7.2, in absence of gB concentrations.

That behavior can be attributed to the SPCEs, once in their constitution they contain many components including organic solvents and ink coagulants, which may interfere in the assay results (Renedo *et al.*, 2007). The observed response may be correlated with the modification of the SPCEs, and not to the gB concentration increasing. So, taking into account the results obtained, it is not possible observe the electrochemical response of the gB using the SPCEs.

1.1.1. Electrochemical interference of gB on immunoassay

As stated in chapter II, an immunosensor was developed in order to achieve a response for HCMV gB, using voltammetric techniques on detection of silver oxidation signal. The obtained response of such oxidation lies between a range potential from -0.06 to 0.06 V. Once in that assay the gB was diluted in Tris-HNO₃, it is possible to determine if that molecule interfere electrochemically.

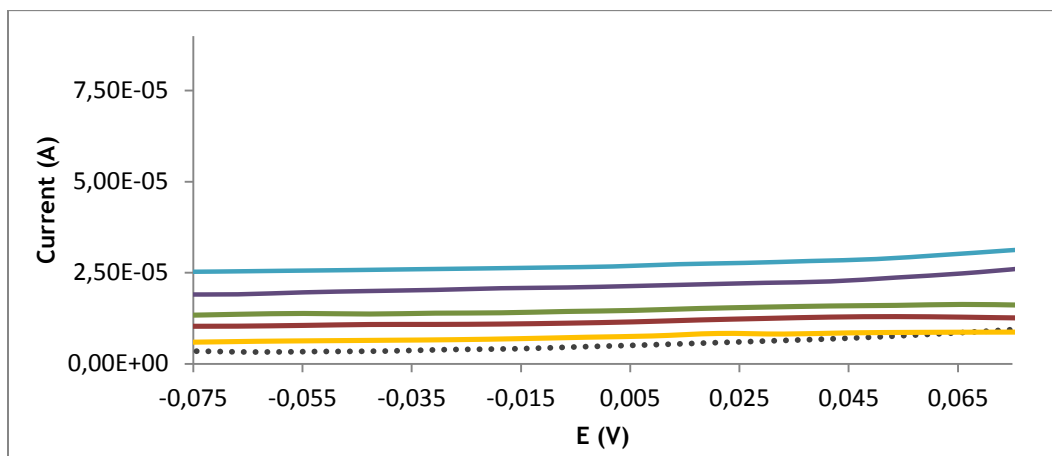


Figure 4.1.1.1. Cyclic Voltammetric response of gB obtained in 50 mM of Tris-HNO₃ at pH 7.2, within the potential range for stripping analysis of the AgNPs of the immunoassay.

Analyzing the figure 4.1.1.1., which is an amplification of the figure 4.1.1. for the potential range of silver oxidation, is evidenced that the HCMV gB addition in Tris-HNO₃ buffer leads to a clean response, with no observed oxidation signals. Thus, the gB is not able to be considered an interference on the sandwich immunoassay.

1.2. Electrochemical behavior in conventional electrodes

To avoid the problematic observed with the SPCE, conventional electrodes of Ag/AgCl as reference electrode, platinum electrode as auxiliary electrode and a carbon-doped diamond as working electrode were used.

The adsorptive voltammetric measurements consisted in accumulation of gB molecules at a fixed potential onto the electrode surface, by stirring for a given period of time followed by a rest period of 10 s, during this time the SW voltammogram from 1.0V to 1.9 V was recorded. Experimental parameters, such as the supporting electrolyte, time and potential of deposition must be optimized in order to get the best analytical signal. In the next sections optimum operating parameters were selected.

1.2.1. Buffer electrochemical screening

A carbonate buffer (0.01M, pH 10) was chosen as the medium for the measurement, taking into account the results obtained by Koppang and co-workers for polyamines oxidation (Koppang *et al.*, 1999). The screening was performed using cyclic voltammetry (CV) to found the potential range with minimal interferences. After that, a known amount of gB was added to the buffer solution and the cyclic voltammograms registered, between 0 to 2 V (figure 4.1.3).

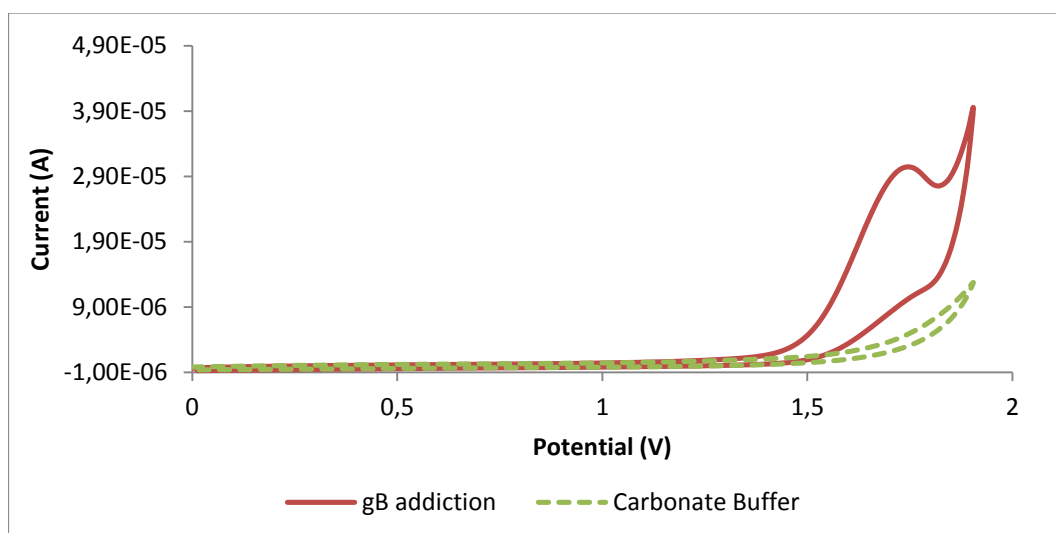


Figure 4.1.3. Cyclic voltammograms obtained in the carbonate buffer (0.01M, pH 10) screening (Dotted-green), and when the addition of a 5 $\mu\text{g}/\text{mL}$ of HCMV gB (Red).

As illustrate in the Figure 4.1.3. carbonate buffer alone does not present any oxidation or reduction signals in the analyzed potential range. However, when 5 $\mu\text{g}/\text{mL}$ of gB is added to the buffer, it is observed the appearance of an oxidation signal at an potential of approximately 1.7 V. At this potential the oxidation of the amine groups ($-\text{NH}_2$) has been described (Koppang *et al.*, 1999). Thus, we believe that this signal is related to oxidation of the amine groups on gB molecules.

1.2.2. Glycoprotein B deposition Potential

In this assay were studied the influence of deposition potential in the voltammetric peak of the HCMV gB, by varying its value between -1.5 and 1.3 V and maintaining the other parameters. Based on the results obtained for the buffer electrochemical range, the Square Wave Voltammetry (SWV) was performed between 1 and 1.9 V.

The starting conditions for deposition were defined as 30 s of deposition time in stirring mode at different deposition potentials, with 10s of equilibration time. The analyzed concentration was 5 μg of HCMV gB per milliliter of carbonate buffer (0.01 M, pH 10) (Figure 4.1.4.).

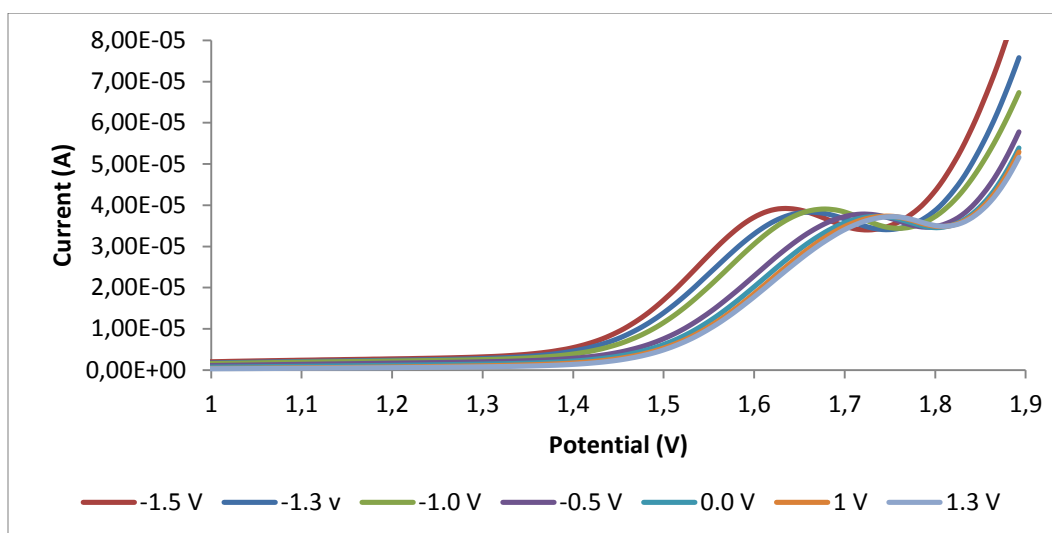


Figure 4.1.4. Square wave voltammograms obtained by varying the deposition potential for 5 $\mu\text{g/mL}$ of gB. Deposition: 30 s of deposition time and 10 s of equilibration time.

It is observed that the optimum potential for HCMV gB deposition onto the electrode is -1.5 V, because a maximum on current and a well-defined oxidation peak were obtained. It was also observed that the more positive potentials result in less defined signals, leading to the peak deformation and consequently to a minor current generation.

1.2.3. Deposition time

To search the optimum deposition time, the previously found conditions were used, being the potential range defined between 1.0 and 1.9 V, in a deposition potential of -1.5 V, with an equilibration time of 10 s. The time variation effect may result in the achievement of highest current intensity proportional to the time for deposition. However, when the experiment was carried out, it was observed that the HCMV gB have a very fast adsorption on the electrode surface, once 1 s of stirring result in the maximum signal generation. For that reason, it was assumed that the minimum deposition time required for the protein deposition is 1 s. However, and taking into account the results obtained for the previously results, 30 s of deposition time was adopted as the optimum.

1.2.4. HCMV glycoprotein B concentration determination

With the previously defined parameters is now possible to explore the electrochemical response of the system to the HCMV gB concentration increasing. Summarizing, the chosen parameters were a deposition potential of -1.5 V, with 30 s of stirring time on the deposition process, followed by an equilibration time of 10 s after this deposition; in a potential window from 1 to 1.9 V. Using these conditions, several HCMV gB concentrations were studied to construct the calibration curves (Figure 4.1.5.).

As shown in figure 4.1.5. the increase of a gB concentration leads to an increase in the measured current. Thus, it was possible to define a response for gB in the system.

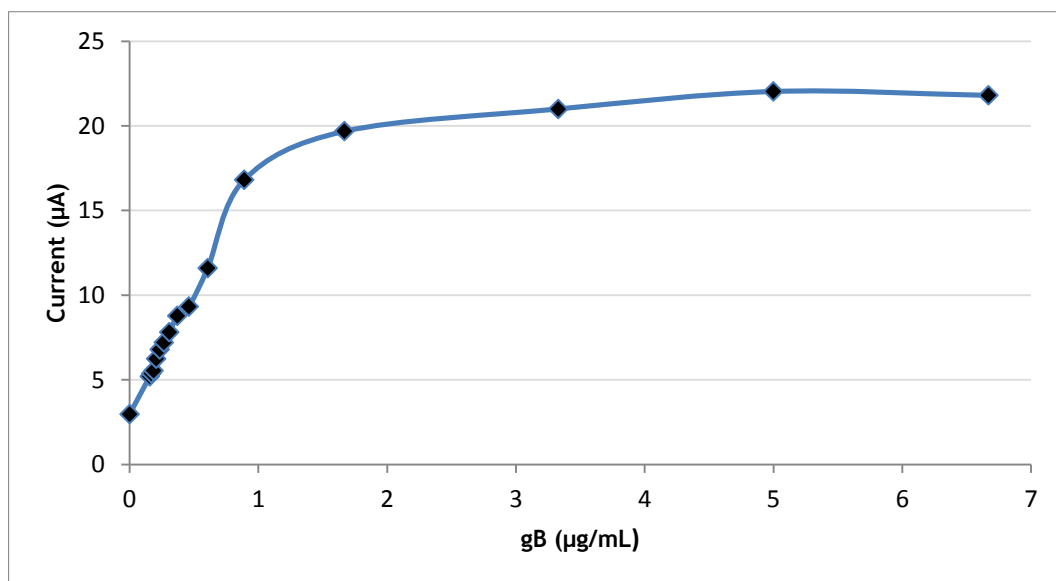


Figure 4.1.5. Square wave voltammograms response of the generated current with the increase of HCMV gB concentration in the system.

It is observable that the saturation of electrode surface occurs for values of gB above of 1.5 µg/ml, from which the intensity in current remains approximately constant. In the same manner, a less marked difference is observable between the lowest gB concentrations, suggesting that the quantification limit is around that range. A linear range of concentrations is found between these two extremes, suggesting that the calibration can be carried out in the range of concentrations from 0.15 to 1.5 µg/mL of gB.

1.2.4.1. Calibration

Under the defined optimal conditions, the system has a current response with the increase of the analyte concentration. A good linear correlation is observed between peak current and gB concentration, after anomalous points exclusion (Figure 4.1.6.). The linear response obtained is shown at equation (1), with a R^2 of 0.9933.

$$\text{Current} = 15.28 \text{ gB} + 2.95 \quad (1)$$

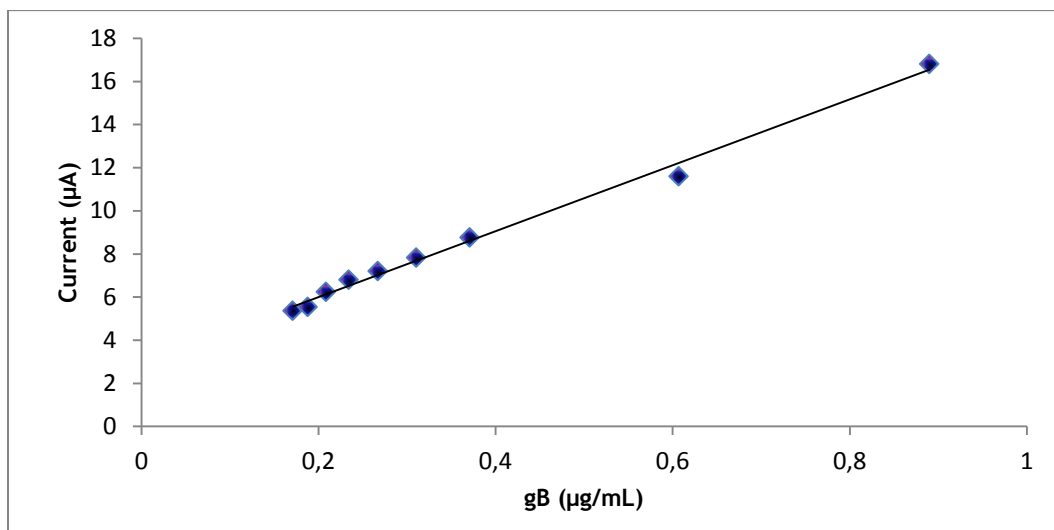


Figure 4.1.6. Calibration curve found for the linear range of HCMV gB concentrations (0.17, 0.18, 0.20, 0.23, 0.26, 0.31, 0.37, 0.60, 0.89 µg/mL).

1.2.4.2. Reproducibility

To evaluate the method reproducibility, the slopes of calibration curves, constructed with gB concentrations in the linear range previously defined, were analyzed (Figure 4.1.7., Table 4.1.).

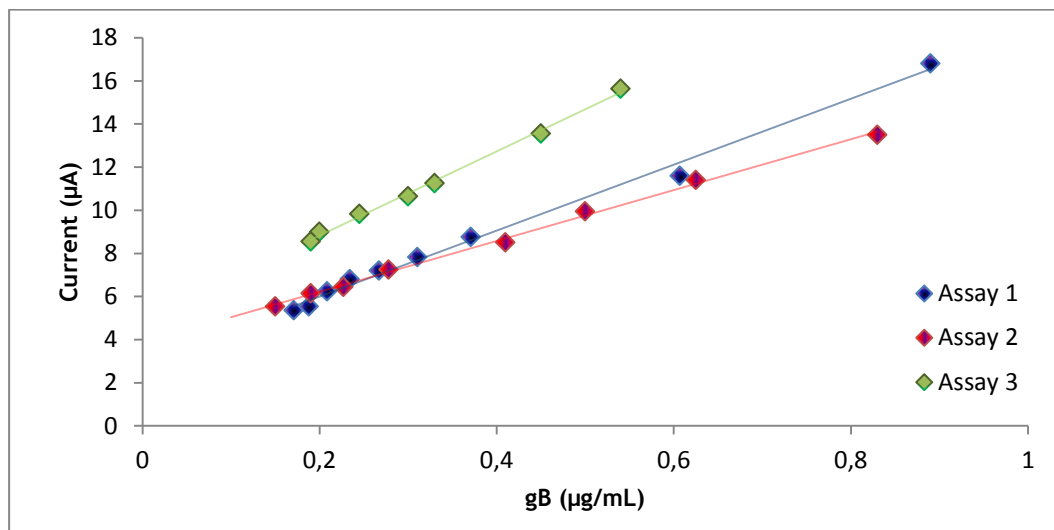


Figure 4.1.7. Calibration curve triplicates representation of the linear range of HCMV gB concentrations.

It can be observed, from table 4.1., that the different calibration curves exhibit good linear correlation coefficients (after elimination of anomalous points), allowing the establishment of slopes residual standard deviation (RSD), which is 17.07%.

Table 4.1. Results to determine relative standard deviation (RSD) of the method.

Assays	Equations	R ²	Slopes	RSD (%)
1	$Current = 15.28 \text{ gB} + 2.95$	0.9933	15.28	
2	$Current = 11.80 \text{ gB} + 3.90$	0.9973	11.80	17.07
3	$Current = 19.51 \text{ gB} + 4.92$	0.9966	19.51	

The calculated RSD value does not convey advantages in terms of reproducibility when compared with the one obtained for the disposable immunosensor developed for Pires *et al.*, which was 16.60 %.

1.2.4.3. Limit of detection (LOD)

In the analytical field, the ability to obtain the lowest detection limit (LOD) is the most critical feature, making one technique preferable among the already known and defined techniques. LOD is defined as the smallest concentration of the analyte that can be detected to a specify degree of certainty, being an important parameters for the technique evaluation.

The first stage in the LOD calculation was the determination of a linear relation between the concentration and the signal, shown in last section. The parameters of these calibration curves must be optimally evaluated. The incorrect adjustments due to the existence of anomalous points, were avoided using least median squares regression (LMS) (Massart *et al.*, 1986; Rousseeuw and Leroy, 1989). In order to check the value of LOD of the technique, the calibration curves data were assessed using the DETARCHI (Sarabia and Ortiz, 1994), a program for the calculation of detection limits with evaluation of the probability of false positive (α) and negative (β), according to ISO11843-2 (ISO11843-2, 2000). An average limit of detection of 46.00 ng/mL for HCMV gB has been obtained for $\alpha=\beta=0.05$.

This value is higher than the one obtained for the immunosensor developed for Pires *et al.*, which was about 2.02 ng/mL of gB (Pires *et al.*, 2013). In this way, the used scheme is unable to determine the trace concentrations detected by the disposable immunosensor.

1.3. Urine samples testing

As final stage of the assay, the system was tested using urine samples. One milliliter of urine was diluted in three milliliter of carbonate buffer (0.01 M, pH 10), and square wave voltammetry was performed using the considered optimal parameters, previously defined.

The Figure 4.1.8. illustrates the system electrochemical behavior using the urine sample. In absence of urine the system replies in the expected way to buffer, with no oxidation signal

observed. However, when urine is added to the system, an oxidation signal is observed at approximately 1.45 V.

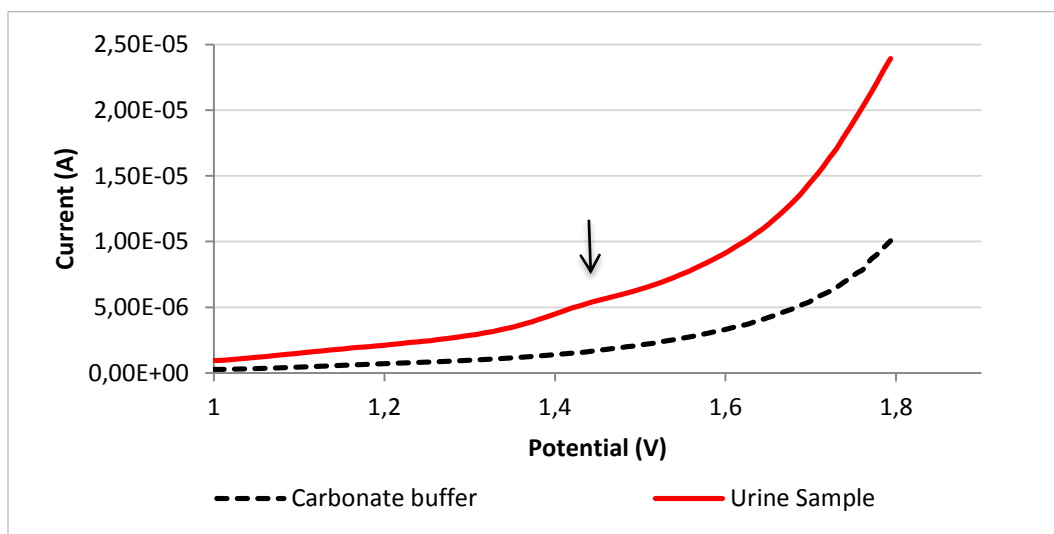


Figure 4.1.8. Electrochemical signal generated by 1:4 (V/V) of urine in carbonate buffer (0.01 M, pH10). The arrow notes the curvature observed at 1.45 V.

That oxidation signal may be a result of other species present in the biological sample, which may be considered as interference to the method. Nevertheless, to see if the system respond to the addition of gB, the urine sample was enriched with 1 and 2 $\mu\text{g}/\text{mL}$ of gB, and the electrochemical signal registered (Figure 4.1.8.). These gB concentrations may generate an good electrochemical response, once there are present at the beginning of the electrode saturation, previously shown in figure 4.1.5.

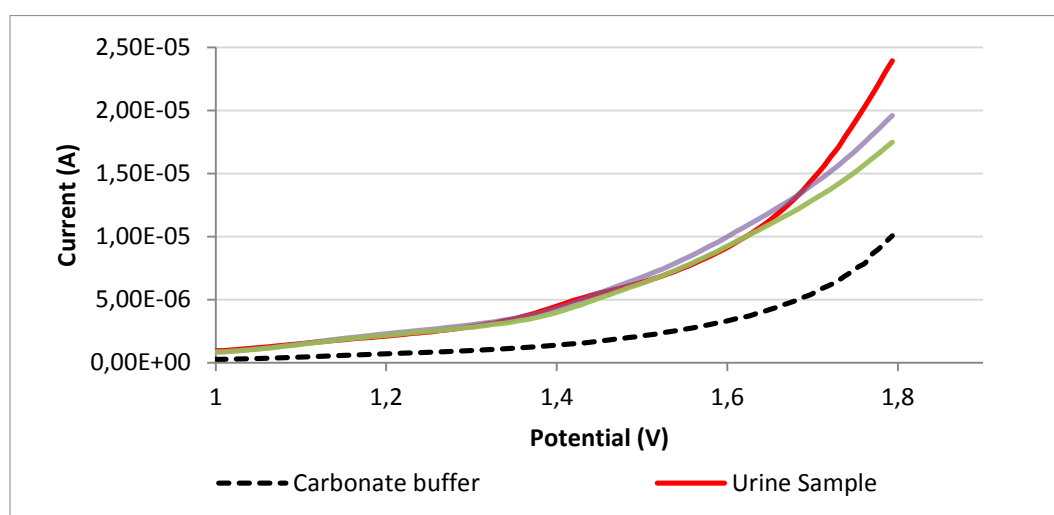


Figure 4.1.9. Electrochemical signal generation of carbonate buffer (0.01 M, pH 10), when the addiction of urine sample 4 fold diluted and in a gB enrichment with 1 and 2 $\mu\text{g}/\text{mL}$.

Analyzing the profile obtained for the addiction of HCMV gB (Figure 4.1.9.), we can deduce that enrichment does not lead to the expected oxidation signal generation. Thus, the system

will not be feasible for the HCMV diagnosis in biological samples, once urine bring several interferences which avoid the correct detection of such viral protein. Therefore, the electrochemical propose of an SPCE disposable immunosensor is preferable, bringing enhanced selectivity, specificity and lower detection limits. Additionally, avoid the need of cleansing of the conventional electrodes, simplifying the analysis without the need of an elaborated mounting scheme.

2. Anti-HCMV antibodies immobilization on SPCE

Pires *et al.* developed a disposable immunosensor based on the adsorption of antibodies on carbon surface of the SPCEs. The immunosensor assay was based on a sandwich immunoreaction, between adsorbed Anti-HCMV antibodies, in presence of HCMV gB by the subsequent attachment of a secondary Ab labelled with gold nanoparticles, which catalysis the deposition of silver nanoparticles (AgNPs) from a silver solution. This AgNPs are then electrochemically oxidized, by anodic stripping analysis, and the current intensity of such oxidation is measured by differential pulse voltammetry (Pires *et al.*, 2013).

The main concern with this preliminary assay is the random adsorption of the antibodies onto working electrode surface, which may result in technique reduced reproducibility.

It is well known that the different techniques for Ab immobilization may result in random or oriented distribution of such molecules on the SPCE surface, leading to different immunoreaction propensities and electrochemical current measurements. It is also known that a well oriented Ab molecules distribution results in improved sensitivity and sensibility of the immunosensor (Jung *et al.*, 2008; Trilling *et al.*, 2013). Thus, following the previously study of Pires *et al.* (Pires *et al.*, 2013), is suggested to try different immobilization techniques of Anti-HCMV Abs, in order to achieve improved characteristics of the device.

2.2. Anti-HCMV cross-linking through glutaraldehyde

Well established for enzymes immobilization, the cross-linking methodology uses several agents that allow the linkage of these biocatalysts with electroactive surfaces (Renedo *et al.*, 2007). Although the few reports of this methodology on immunosensor field, some promising results (Lai *et al.*, 2012) and the simplicity on the electrodes preparation, lead us to try to conduct that procedure. So, it is suggested to immobilize the Anti-HCMV Ab molecules through the use of glutaraldehyde (GA) as cross linker agent. The GA has been recognized due to its ability to react with protein groups (Migneault *et al.*, 2004). When the proteins are antibodies molecules, it was expected that the GA reacts with the N-terminus of those molecules, once in its structure GA present reactive carboxyl groups.

2.2.1. HCMV glycoprotein B concentration determination

The GA may allow the stable attachment of the Anti-HCMV Abs on the electrode surface, resulting in an increment on current intensity proportional to gB increase. To verify that behavior, several gB concentrations were incubated on the working electrode, previously modified with the Anti-HCMV cross-linked with GA. As illustrated in figure 4.2.1., the differential pulse voltammograms obtained for the silver oxidation, represent the increase on current intensity when gB concentrations are also increasing.

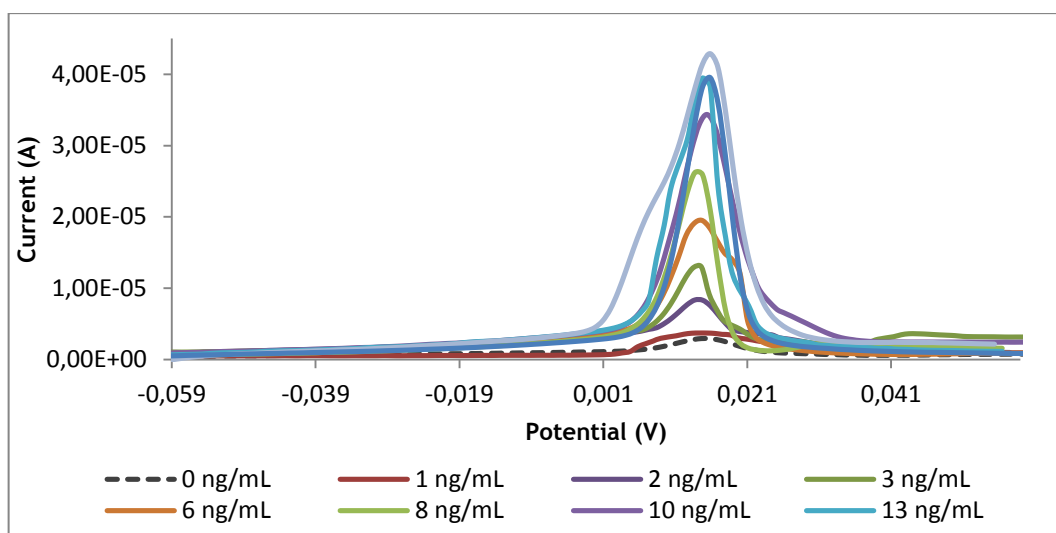


Figure 4.2.1. Differential pulse voltammetric signals of the Ag NPs anodic stripping carried out in SPCE made in Burgos University. In this detection were used gB concentrations of 0, 1, 2, 3, 6, 8, 10, 13, 20 and 30 ng/mL, diluted in Tris-HNO₃ buffer.

In figure 4.2.2. we can observe the variation of the peak current intensity with the HCMV gB concentrations, suggesting that the sandwich immunosensor is efficient on its determination. The observed behavior shows a constant current intensity for gB concentrations above approximately 12 ng/mL. So, the ability to distinguish concentrations is limited to values until 12 ng/mL of gB, once at upper values, the electrode surface saturation is observed. In the same way, for the smallest concentrations, a constant current intensity is noted between the absence and the presence of 1 ng/mL of HCMV gB, which lead us to conclude that is the limit of quantification (LOQ) for this procedure is about 1 ng/mL.

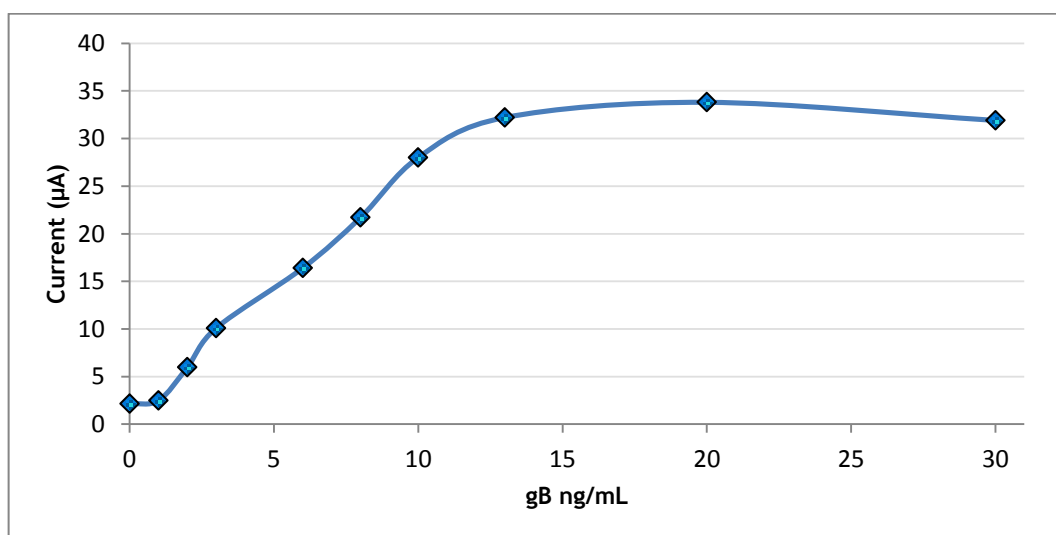


Figure 4.2.2. Current intensity behavior according to the HCMV gB concentrations increment (0, 1, 2, 3, 6, 8, 10, 13, 20 and 30 ng/mL).

An increase on current intensity with the increase of the gB concentrations is observed for a range between 1 and 12 ng/mL, being the used to construct calibration curves.

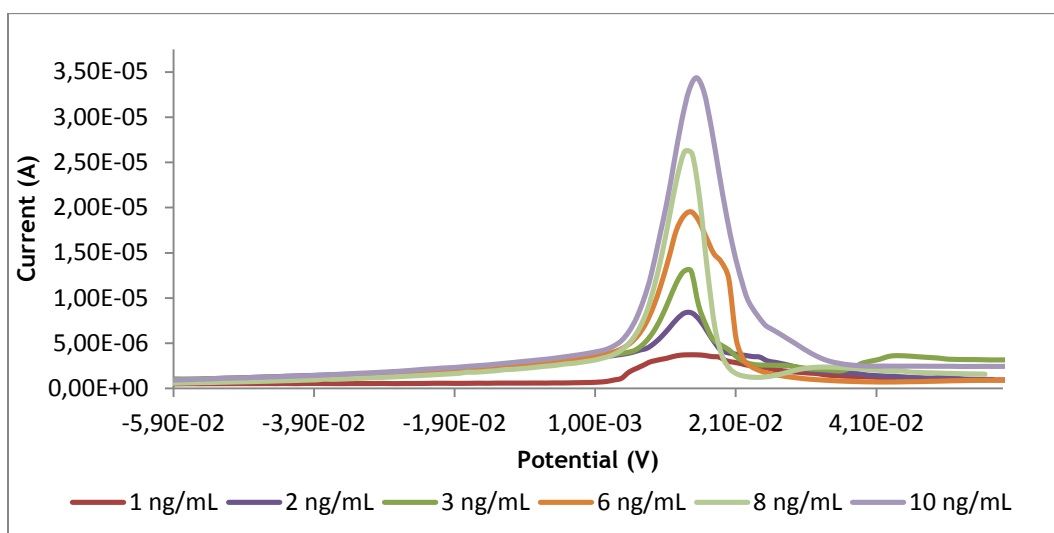


Figure 4.2.3. Differential pulse voltammetric signals of the Ag NPs anodic stripping carried out in SPCE made in Burgos University. The gB concentrations of 1, 2, 3, 6, 8 and 10 ng/mL were used to draw calibration curves.

2.2.1.1. Calibration

The linear range response, illustrated in figure 4.2.4., exhibit a good linear correlation coefficient between the peak currents and the gB concentrations. Thus, the concentrations between 1 and 12 ng/mL will be used to define new calibration curves which will allow the determination of reproducibility and the method detection limit. The linear response obtained is shown at equation (2), with a R^2 of 0.9932.

$$\text{Current} = 2.71 \text{ gB} + 0.56 \quad (2)$$

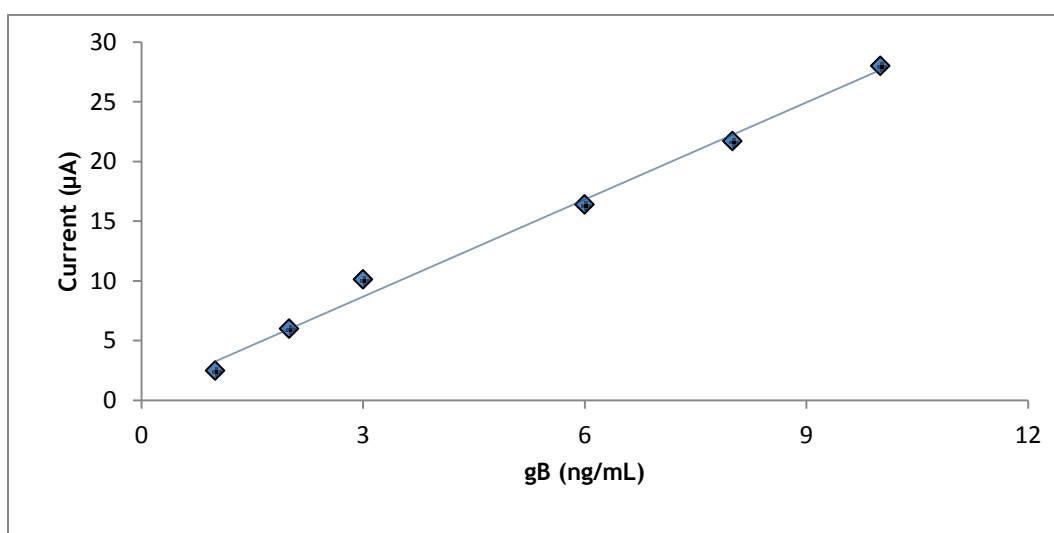


Figure 4.2.4. Calibration curve for 1, 2, 3, 6, 8 and 10 ng/mL of gB.

2.2.1.2. Reproducibility

The reproducibility of the method was evaluated by the estimation of the RSD associated with the slopes of the calibration curves. These calibration curves were constructed with different immunosensors, which were incubated with different gB concentrations. In figure 4.2.5., are represented the triplicates of the linear range for current generated vs gB concentrations, exhibiting good linear coefficients (anomalous points were excluded).

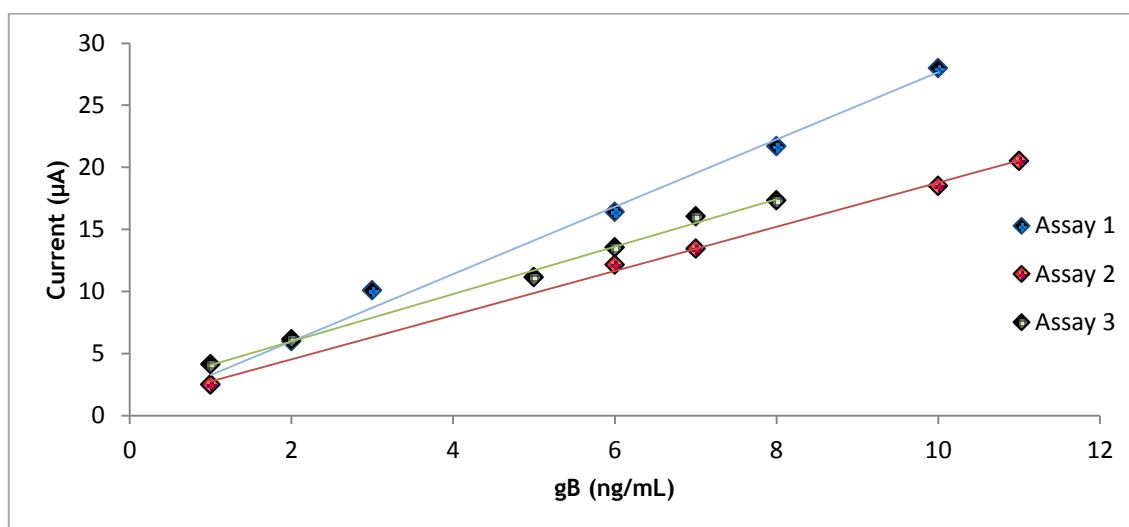


Figure 4.2.5. Calibration curves triplicates representation.

Table 4.2. Results to determine relative standard deviation (RSD) for cross-linking immobilization.

Assays	Equations	R ²	Slopes	RSD (%)
1	$Current = 2.71 gB + 0.56$	0.9932	2.71	18.04
2	$Current = 1.78 gB + 0.97$	0.9950	1.78	
3	$Current = 1.91 gB + 2.03$	0.9955	1.91	

The RSD value results from the slope analysis, gives us information about the method reproducibility and sensitivity. An RSD value of 18.04 % (table 4.2) was obtained by the GA cross-linking of anti-HCMV antibodies. This value is consistent with the ones obtained for type of sensors. We can conclude that the cross-linking reproducibility have some variation associated with the different experiments. To reduce the error from inter days experiments a calibration curve should be made before any gB detection.

Despite the observed difference, which is not substantial between the methods, the 16.60 % obtained for the adsorption represents better reproducibility when compared with the ones obtained for the Anti-HCMV cross-linking.

2.2.1.3. Method sensitivity

Sensitivity of one method is a measure of its ability to differentiate between small differences in analyte concentration. This parameter it is also dependent of the slopes of the calibration curves (Table 4.2.), a higher slope results in higher sensitivity for the gB concentration. The slopes of the calibration curves show some deviation, which affect the sensitivity of the method between different experiments. Despite that deviation, when the cross-linking immobilization is compared with the adsorption, we can concluded that the cross-linking results in lower sensitivity, once the average value of the calibration slopes, which was 2.13, is lower than the 4.27 obtained for the calibrations from adsorption (Pires *et al.*, 2013).

2.2.1.4. Limit of detection (LOD)

LOD was calculated from the differential pulse voltammetric responses in the concentration range 1.0 to 12 ng/mL. Similar to previously performed in section 1.2.4.3., after calibration curves delimitation, the anomalous points were excluded using least median squares regression (LMS) (Massart *et al.*, 1986; Rousseeuw and Leroy, 1989). The corrected data were accessed using DETARCHI program (ISO11843-2, 2000; Sarabia and Ortiz, 1994), in order to check the value of LOD for this immobilization technique. The evaluation of the probability of false positive (α) and negative (β) equal to 0.05 results in average limit of detection of 2.10 ng/mL for HCMV gB.

Evaluating this LOD result we can conclude that between this value and the 2.02 ng/mL obtained on Pires *et al.* work (Pires *et al.*, 2013) a minimal difference is observed.

The results obtained in sections 2.2.1.2., 2.2.1.3. and 2.2.1.4., may be justified by the influence associated with the different methods on the antibodies immobilization. Once both techniques results in random distribution of the antibodies molecules onto the SPCE, similar results for the GA cross-linking and adsorption were expected. Indeed, for the limit of detection and for the limit of quantification similar results were obtained. Nevertheless, evaluating the sensitivity and the reproducibility, it is observable that evaluation results in advantage for adsorption technique, exhibiting improved yields.

As previously said, GA molecule may prefer the attachment towards the NH₂ groups, which are mainly present at lysine residues and at N-terminus of the antibodies molecules. It is also known that the N-terminus is the locus for the antigen binding, thus the immobilization using cross-linking may result in CDR site blocking. The antibodies should display free CDRs after immobilization to improve the analyte binding and to enhance the biosensor sensitivity, however, considering the role of the GA in the Anti-HCMV immobilization, we can conclude that the cross-linking imposes antibody binding to SPCE surface through the NH₂ groups,

avoiding the spatial arrangement allowed by adsorption. When antibodies are adsorbed onto a surface, a degree of orientation may be achieved, once it also depends on molecular dimensions (Jung *et al.*, 2008) and self-organizing capacity of that biomolecules. In addition several factors, such as specific groups, charges on Ab and/or SPCE surface, steric hindrance caused by the neighbors Abs, distance between coupled Ab and the surface and the Ab conformational changes, also affect the orientation and consequently the binding capacity of the such molecules (Lu *et al.*, 1996). Thus, the conditions used by Pires *et al.*, provide better assay results than the ones used in cross-linking of Anti-HCMV.

2.2.2. Immunoreaction time optimization

The time for immunoreaction which is, the time used to the complexation reaction between antigen and antibody, is a primordial optimization that can be carried out in this sandwich-type immunosensor. This optimization can reduce the time needed to complete the assay.

Time optimization was carried out through the incubation of 20 ng/mL of HCMV gB, during 0, 10, 20, 30, 40, 50 and 60 minutes. The evaluation of the current generation of the differential pulse voltammograms and the minor dispersion observed for the different times, may lead us to conclude about which time is a preferable choice.

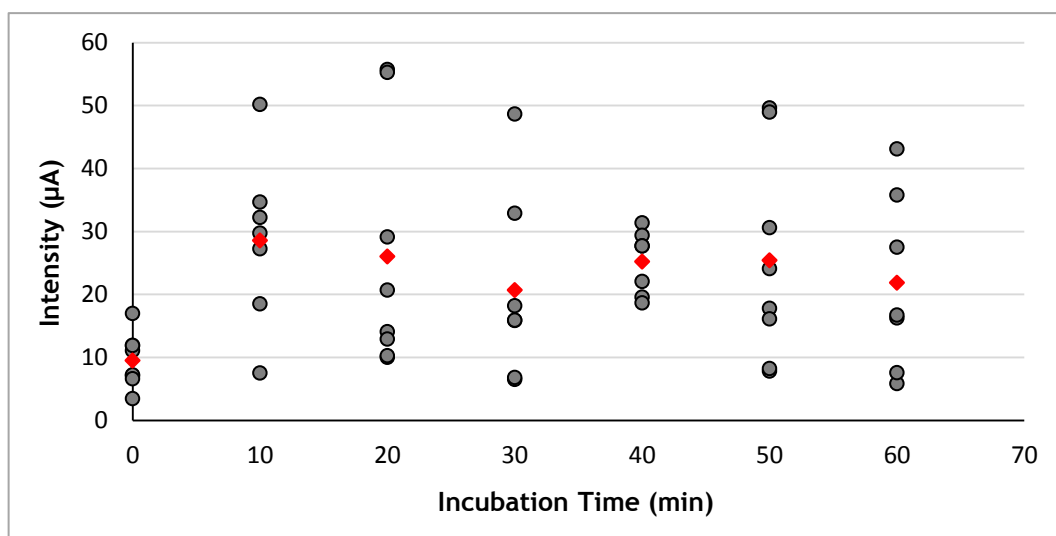


Figure 4.2.6. Total of current intensities obtained for each incubation time and the respective average value. For each time are represented several measurements (grey), and the average of that values (red).

Analyzing the results resumed in figure 4.2.6, we can observe the dispersion associated with this immobilization method. Each point represents an electrode incubated during a specific time. In a general overview, 10 minutes of incubation is enough to reach a good electrochemical signal generation, however the high dispersion associated with 10 minutes may result in lower reproducibility of the method.

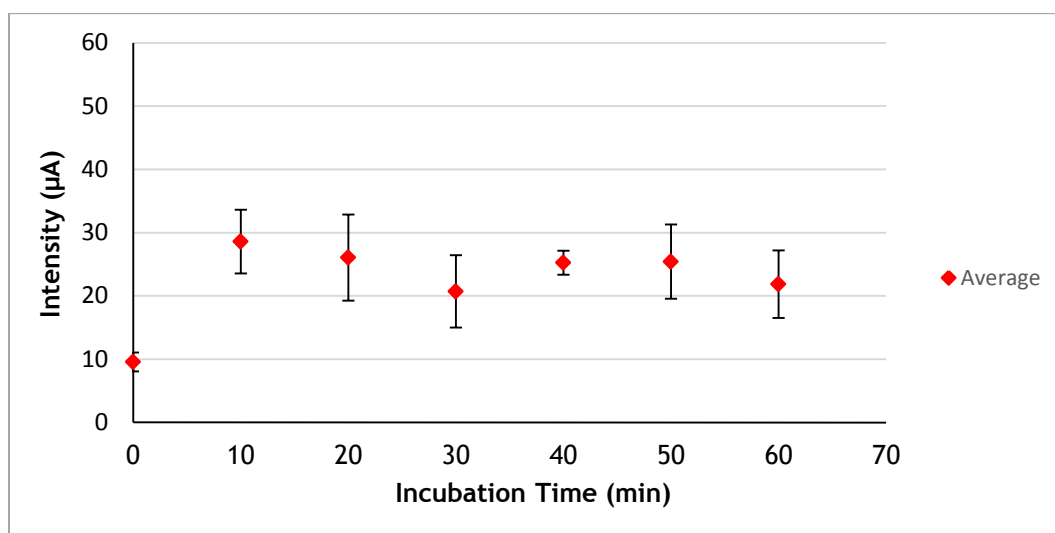


Figure 4.2.7. Averages of the total points obtained for each time incubation of HCMV gB, and the representation of the standard error bars.

In figure 4.2.7., the average of the total points for each time, represented with the associated standard error deviation lead us to choose the 40 minutes as ideal time for the immunoreaction occurrence. The 40 minutes result in smaller observed dispersion and consequently in a minor standard error, conjugated with a good signal generation in the differential pulse voltammograms.

Interestingly, the same study conducted for the adsorption leads to the same conclusions (unpublished work of Pires *et al.*). Thus, we can infer about the independence of such reaction of the immobilization procedure conducted for Anti-HCMV antibodies.

2.3. Covalent immobilization of Anti-HCMV through diazonium salts

Covalent bonding, which provides a robust structure, is based on the bond of a carbon pertaining to the surface of the SPCE and a carbon from an organic molecule. The reaction involves the activation of chemical groups on the transducer in order to react with the amino acids of the proteins (Alonso-Lomillo *et al.*, 2009).

This immobilization procedure uses organic solvents like acetonitrile, which makes it incompatible with the electrodes previously used for cross-linking immobilization, once the insulating ink is dissolved in acetonitrile. Thus, the electrodes used for this immobilization procedure are constituted by resistive insulating dielectric ink 242-SB.

2.3.1. Functionalization of SPCEs

In covalent immobilization is necessary to prepare the carbon surface of the working electrode for the anti-HCMV antibodies immobilization. That surface modification involves two phases.

Firstly, the activation of the electrode surface with nitro groups (NO_2) was achieved by electrochemical grafting of a 3 mM solution of diazonium salt, in acetonitrile, 0.1M of $\text{N}(\text{Bu})_4\text{BF}_4$. 100 μL of this solution was placed in the working area of the SPCE and a cyclic voltammetry was performed at 200 mV/s from 0.8 V to 0.4 V. NO_2 electrochemical grafting results are illustrated in the cyclic voltammograms of figure 4.3.1., results that are in agreement with the ones described in the literature (Alonso-Lomillo *et al.*, 2009).

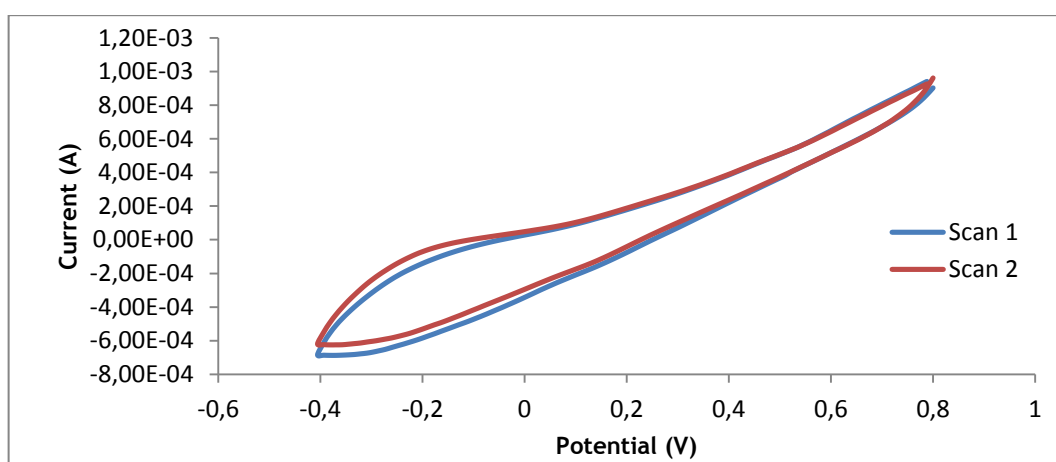


Figure 4.3.1. Cyclic Voltammograms of SPCE obtained for the process of electrode modification with diazonium monolayer, providing nitro groups (NO_2).

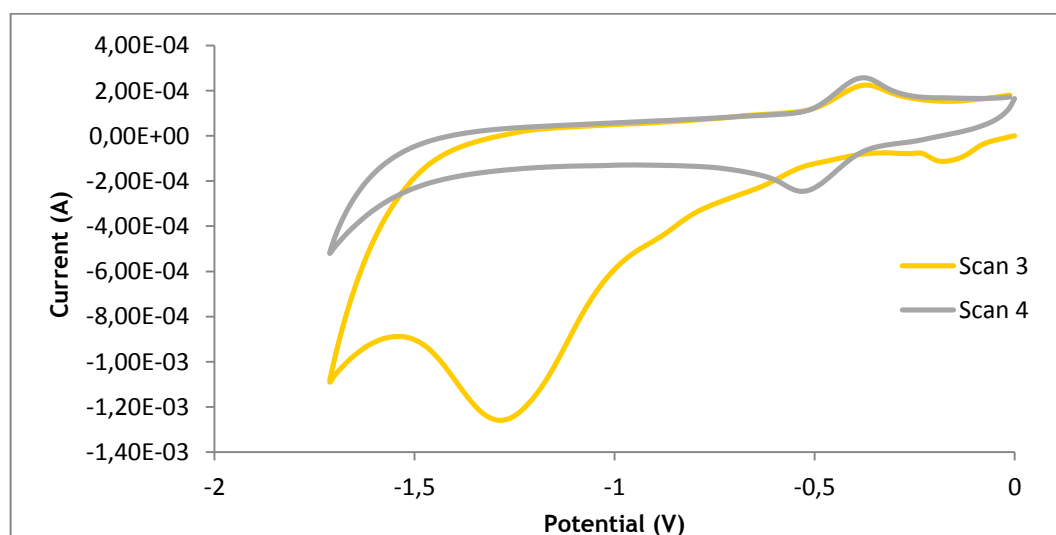


Figure 4.3.2. Cyclic voltammograms for nitro groups (NO_2) reduction to amine groups (NH_2). The yellow CV corresponds to the NO_2 reduction and grey CV shows the reversible redox couple NH_2/NHOH .

In the second step, the electrochemical reduction of the NO_2 groups to NH_2 groups was achieved by cyclic voltammetry from 0.8 V to -1.7 V at a scan rate of 200 mV/s in a 9:1 (v/v) water/ethanol solution of 0.1 M KCl.

In figure 4.3.2., it is observed from the third to the fourth scan, the disappearance of the peak at -1.375 V, which confirms the reduction of the NO_2 groups. According to the cyclic voltammograms, it can be concluded that the diazonium monolayer formation was successfully conducted, once the surface exposed reduced groups that will be able to attach carboxylic groups on the bioreceptor molecules, the anti-HCMV antibodies.

Finally 3 μL of antibody conjugated with 2 μL of N-Hydroxysuccinimide (40 mM) and 2 μL of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (80 mM), both prepared in NaH_2PO_4 (10 mM, pH 6), were deposited onto the working electrode area. The EDC and NHS were used to activate the carboxylic function of the anti-HCMV molecules. Chemically, EDC is a good leaving group with the ability to bind at the carboxylic groups present at the antibody molecules. This carbodiimide allows, after the coupling of the NHS molecule at the carboxylic groups of the anti-HCMV molecules which are then activated, the antibodies attachment to the reduced NH_2 groups, previously coated onto SPCE.

2.3.1. HCMV glycoprotein B concentration determination

Similarly to the performed for the cross-linking gB determination, several concentrations of gB were incubated onto the modified working electrode, and the sandwich immunoassay was carried out. The silver stripping analysis was conducted by differential pulse voltammetry from -0.06 to 0.06 V in 1M of KCl, and the response registered (figure 4.3.3.).

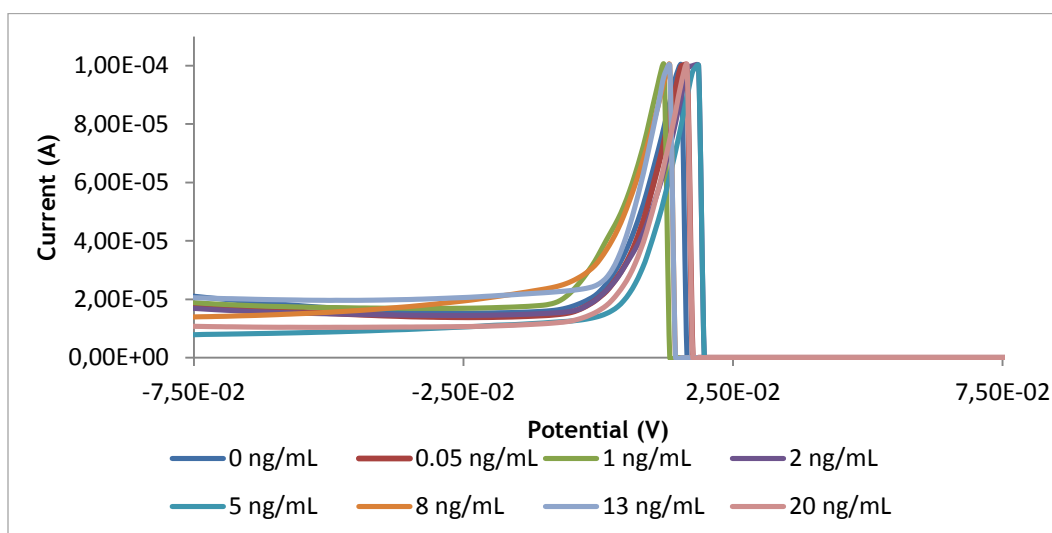


Figure 4.3.3. Differential pulse voltammetric signals of the Ag NPs anodic stripping carried out in SPCE modified by covalent immobilization. The gB concentrations of 0, 0.05, 1, 2, 5, 8, 13 and 20 ng/mL were studied.

The DP voltammograms, illustrated in figure 4.3.3., show a very large oxidation peak generated when we try to study the dependence between the current intensity and the gB concentrations. It is noted that all the concentrations tested, including the blanc, responds in the same manner, being impossible to draw conclusions about the efficiency of the immobilization procedure. In an attempt to justify this huge signal generation, a DP voltammetry was applied at each step of the immunoassay, however the oxidation peak was always present.

This observation only allows us to conclude that the sandwich component does not modify the behavior of the electrochemical signal, which may be hindering the desired signal generation. It should be noted that the potential on which this signal appear is the same for the silver oxidation. The SPE devices used have silver in the constitution of the paths/connectors and at the pseudo reference electrode of Ag/AgCl. Thus, the immobilization procedure may results in interfering species derived from the SPCE device.

2.3.1.1. Interference studies

To understand the appearance of such interference, an SPCE for covalent immobilization was subjected to DPV measurements, repeatedly performed in the same KCl (1M) solution. If a silver specie it is being formed with this repeated scans, an oxidation signal may be observed.

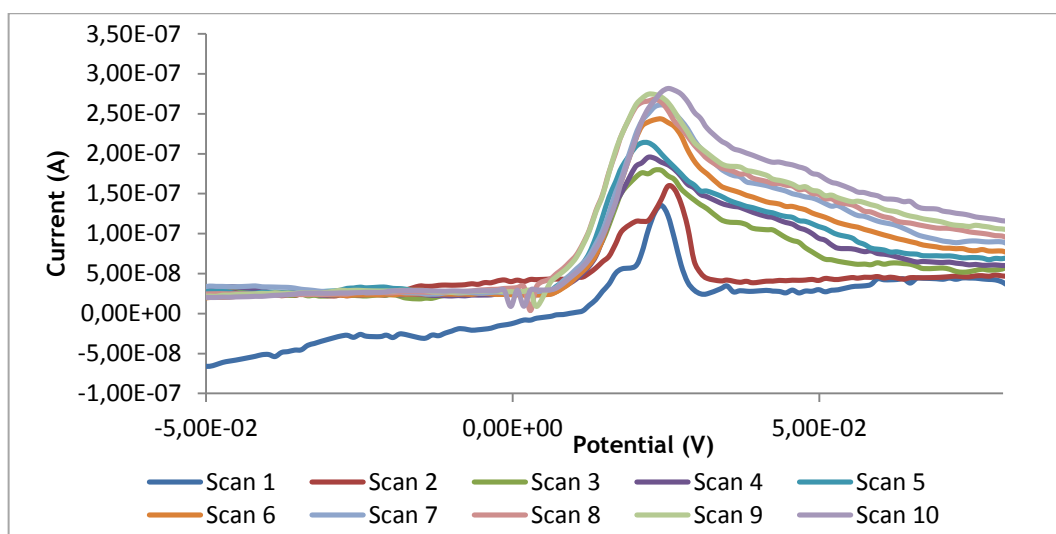


Figure 4.3.4. Differential pulse voltammetric signals of the SPCE for covalent immobilization made at Burgos University, in 1M of KCl.

In figure 4.3.4., it is observable the increase of the signal generation with the increase in the number of preformed DPV scans, in absence of any compound of the sandwich immunoassay. It is also interesting to note, that the current intensity generated does not exceed the 3.00×10^{-7} amperes, which is a very low value when compared with the previously obtained current of 1.00×10^{-4} amperes.

Notably, when the same SPCE, previously subjected to that repeated scans, is washed with d.d. water and newly exposed to an DPV in 1M of KCl, a strong oxidation peak is observed (Figure 4.3.5.).

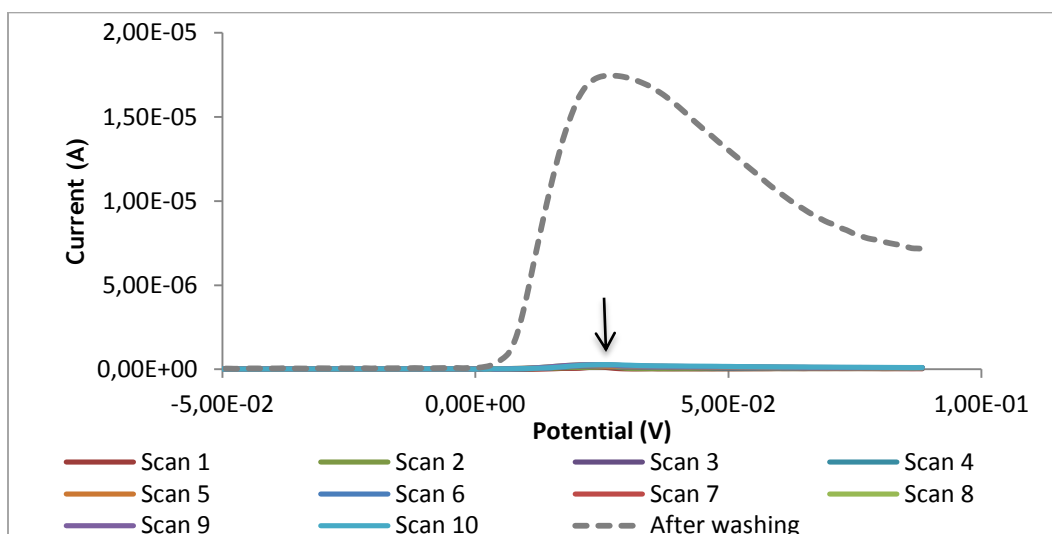


Figure 4.3.5. Differential pulse voltammetric signals of the SPCE for covalent immobilization, in 1M of KCl (represented with the arrow). Dotted line represents the signal by DPV in 1M of KCl after SPCE washing.

Despite the current intensity was not as big as previously observed for the covalent modification (figure 4.3.3.), we can suspect about the formation of an unknown specie, probably related with silver, on the SPCE device during its functionalization in presence of KCl. Thus, silver constituents of the SPCE may be reacting with the KCl, yielding that interference. As mentioned SPE devices used have silver in the constitution of the paths/connectors and at the pseudo reference electrode of Ag/AgCl. To infer if the working electrode is also involved in the interference process, a similar study was conducted using a screen-printed gold electrode (SPAuE), constituted by a working electrode made of a gold ink, silver paths/connectors and a pseudo-reference of Ag/AgCl. If an identical behavior is observed, we can confirm that the electrode silver compounds exhibits interferences when repeatedly measured in KCl solution, thus making the covalent immobilization incompatible with the detection method used in the sandwich immunoassay.

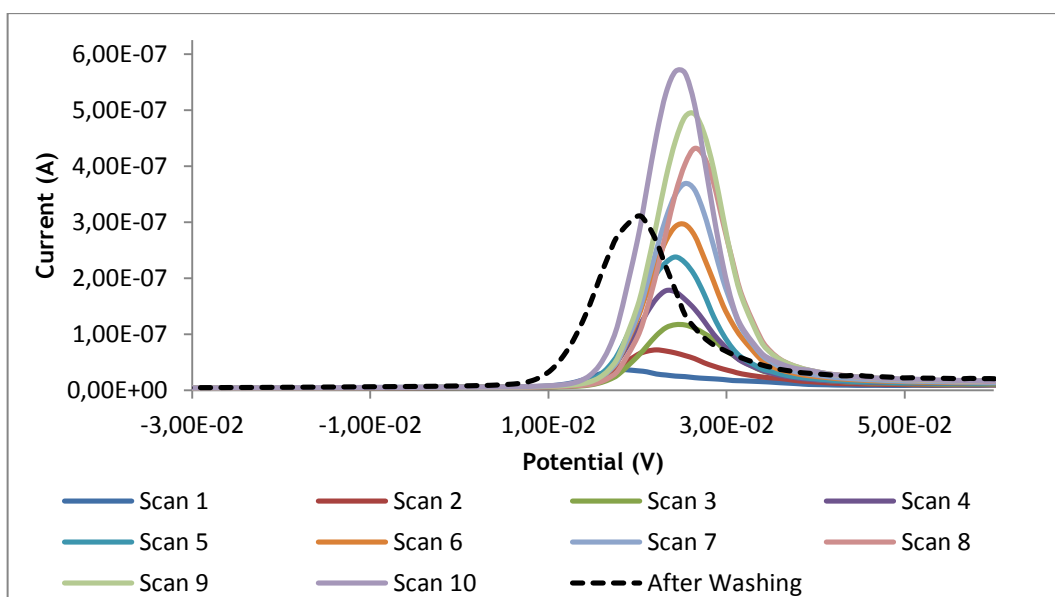


Figure 4.3.6. Differential pulse voltammetric signals of the SPAuE, in 1M of KCl. Dotted line represents the signal generation by DPV in 1M of KCl after the SPAuE washing.

Analyzing figure 4.3.6., where the DPV obtained for the SPAuE in KCL 1M are registered, we concluded that the electrode alone leads to a signal generation similar to the ones obtained for silver oxidation in the immunosensor assay. In the present electrode the signal is cleaner, presenting a lower noise ratio, when compared to the signal obtained by the SPCE electrode. This may be due to the high conductivity known for the gold materials, when compared with the carbon conductivity, allowing better kinetics on the signal generation. Similarly, after the washing step of the SPAuE, a new measurement was performed, and the signal generation recorded. As illustrated, a signal is generated with a current intensity higher than the one originated by the first scan. Thus, we can conclude that the electrode is being modified by the scans, which increases the interference. However, this signal has a minor current intensity when compared with the 10th scan, which may be due to the different adsorptive proprieties of the gold and carbon materials. Carbon is known to present a higher surface area. So, that lower adsorptive proprieties may result in less retained silver unknown specie and consequently in a lower current generation.

Finally to infer from which, silver paths or Ag/AgCl pseudo reference electrode, the interference is originated, the same study was performed in SPCEs made by carbon paths. If the interference was derived from the silver paths, in the present electrodes the oxidation signal observed on the other electrodes should not be present. The Ag/AgCl pseudo-reference is very stable (Renedo *et al.*, 2007) and is not expected to interfere in the measurements.

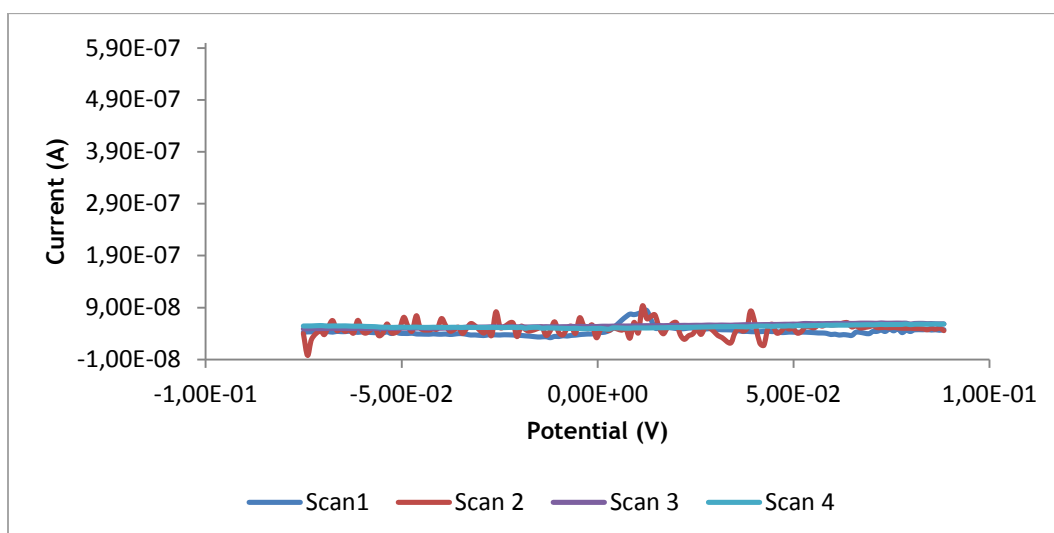


Figure 4.3.7. Differential pulse voltammetric signals of the SPCE with carbon paths, in presence of 1M of KCl.

As visible from figure 4.3.7., no signal generation is present at the potential for silver oxidation when the SPCE with carbon material as conducting paths is used. Once this SPCE also uses the same pseudo-reference of Ag/AgCl, is possible to conclude that the interference observed for the covalent modification is due to the silver paths. We also can conclude that an unknown compound is probably formed on SPCE with silver paths, during the second step of the covalent modification, once a KCl solution is used. That specie formation is enhanced with the increase of the scans performed on the same electrode, resulting in amplified interference for the stripping analysis of AgNPs of the sandwich immunoassay. Taking this into account, we can conclude that this immobilization procedure is not compatible with the used detection system.

2.4. Anti-HCMV immobilization through affinity of boronic acid

Ho *et al.* (Ho *et al.*, 2010) developed an ultrasensitive immunosensor, by taking advantage of the affinity of boronic acid towards the carbohydrate unit of the antibodies. This technique has being associated with good orientation of such molecules in SPCE. Likewise covalent immobilization, a surface modification is conducted, but in this case by linking boronic acid groups for the further attachment of the Ab molecules. This immobilization procedure was also tried for the determination of HCMV gB. The obtained response for the gB determination is presented in figure 4.3.8.

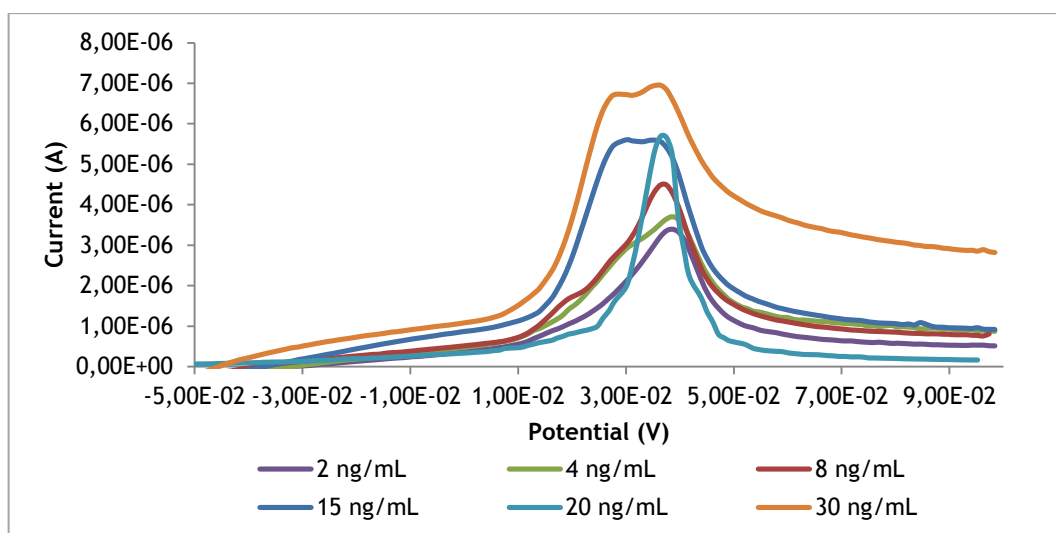


Figure 4.3.8. Anodic stripping differential pulse voltammetric signals of the Ag NPs carried out for ABPA immobilization for different gB concentrations (2, 4, 8, 15, 20 and 30 ng/mL).

Initially, it was detected a random response, but after the exclusion of the anomalous currents, a preliminary response in function of gB concentration was observed (figure 4.3.8.). It was also observed that the signals have some noise associated, which may be due to lack of optimization of experimental conditions of the immunoassay. In future work the optimization of these experimental conditions (namely Ab concentration and electrode area) may avoid this behavior. Illustrated in figure 4.3.9., a preliminary compartment is draw, showing a largest range of linear response between the current generated and the gB concentrations, when compared to immunoassays performed using in SPCEs modification adsorption or GA cross-linking.

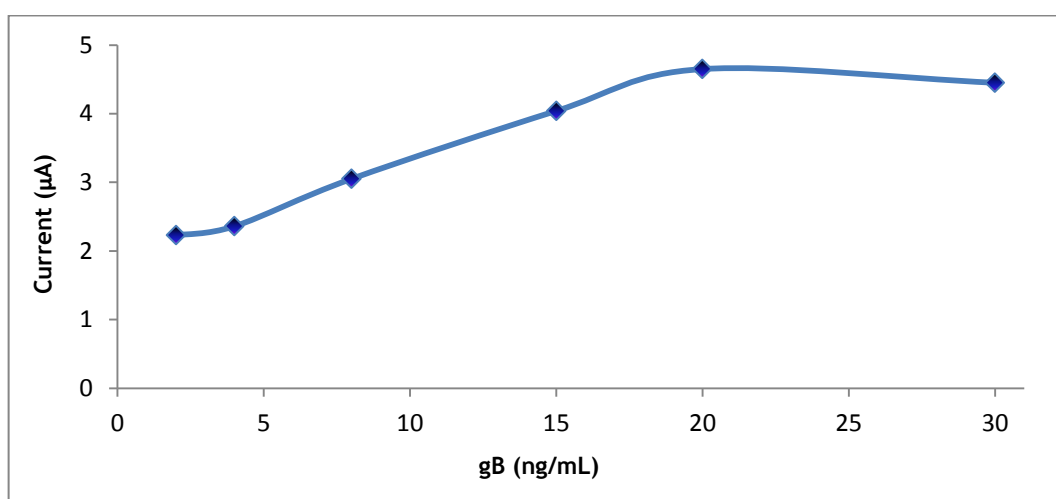


Figure 4.3.9. Current intensity behavior according to the HCMV gB concentrations increment (2, 4, 8, 15, 20 and 30 ng/mL).

So far is not possible to draw more conclusions about this technique, however the promising results and the described in the literature, makes this type of immobilization a path to be followed in the optimization of the immobilization procedure.

V. Conclusions

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

Glycoprotein B electrochemical behavior: In this work, an electrochemical screening of HCMV gB was proposed, in order to investigate the generation of a particular electrochemical signal. Also allowing to determine about its interference in the immunosensor results. The electrochemical study performed on a diamond-doped electrode shows a well-defined SW adsorptive stripping voltammetric peak at 1.7 V (vs Ag/AgCl), under the optimized conditions. The optimal conditions were achieved by applying a deposition potential of -1.5 V, with 30 s of deposition time and 10 s for equilibration time. When diluted in carbonate buffered medium, an electrochemical signal increasing with HCMV gB was detected, resulting in a RSD of 17.07% for the method reproducibility and in an average limit of detection of 46 ng/mL ($\alpha=\beta=0.05$). However, the presence of such viral protein in urine samples, does not lead to the expected response, making of the technique infeasible for use as diagnostic methodology.

In SPCE was evidenced that the HCMV gB addition in Tris-HNO₃, doesn't results in interferences on the silver oxidation potential range, where the stripping analysis is conducted for the immunosensor assay.

Anti-HCMV immobilization through glutaraldehyde cross-linking: The Anti-HCMV immobilization through GA cross-linking was tested under the same conditions used on Pires *et al.* work. A response for the silver anodic stripping was observed in terms of current generation versus HCMV gB concentrations suggesting that the sandwich immunosensor is efficient on its determination. The reproducibility was evaluated by the RSD value, which was 18.04 %, being consistent with the ones obtained for this type of sensors. This RSD value doesn't significantly differ from the one obtained when adsorption is used for Ab immobilization. However, a lower sensitivity was found, resulting in half of the observed when Ab adsorption onto the SPCE surface is used. On the other hand, the LOD obtained for cross-linking is quite similar. These results are briefly resumed in table 4.3.

Table 4.3. Review of the parameters obtained for the adsorption and cross-linking of the Anti-HCMV antibodies.

Anti-HCMV Immobilization	RSD (%)	Sensitivity (Slopes average)	LOQ (Limit of quantification)	LOD (Limit of detection)
Adsorption (Pires <i>et al.</i> , 2013)	16.60	4.27	1 ng/mL of HCMV gB	2.02 ng/mL of HCMV gB
GA Cross-linking	18.04	2.13	1 ng/mL of HCMV gB	2.10 ng/mL of HCMV gB

The optimization of immunoreaction time was conducted resulting in 40 minutes as ideal time, once smaller dispersion and good signal generation for the differential pulse voltammograms were observed.

Anti-HCMV covalent immobilization: For this procedure, when the AgNPs anodic stripping was performed, interferences were found. A strong oxidation signal was observed at the potential for the silver oxidation. After sequential experiments it was concluded that in presence of KCl solution, the silver paths of the SPCE are related with the formation of the unknown specie. That interference was associated with the surface modification step, where simultaneous cyclic voltammetric scans were performed in a solution of KCl. Thus, it is not possible to draw conclusions about the covalent immobilization performance, once the analysis is based in silver oxidation signals.

Anti-HCMV immobilization using ABPA affinity: A first attempt was performed for this immobilization procedure. Despite the few results obtained, this technique may be worked and optimized for future application on HCMV immunosensor.

VI. Future Perspectives

This work focuses on the main point for the construction of an immunosensor device, by investigating several methods of antibodies immobilization onto the working electrode. However, as we can see from the obtained results, the detection used on the sandwich immunoreaction doesn't allow the use of techniques such as covalent immobilization and others using SPAuEs. Thus, in future work, other labelling techniques should be attempted, like the enzymatic labelling. Additionally, the study of the glycoprotein B labelling must also be tried, once it can result in more inexpensive assay, by avoiding the need of a secondary labelled antibody. The proposed alternatives, may improve the immunosensor sensibility leading to lower detection limits, may also improving the device reproducibility.

The Anti-HCMV immobilization using the boronic acid should also be deeply studied, due to the promising results obtained and the ones described in the literature for this affinity immobilization.

The optimization of the parameters, namely antibody concentration, BSA concentration and the time necessary for each step should be performed after the choice of the immobilization procedure, once it can results in enhanced recognition of the gB molecules.

Additional studies should be undertaken in order to understand the process of antibodies distribution and orientation onto the electrode surface, when adsorption is used as immobilization procedure.

Finally, the immunosensor must be tested to detect HCMV in biological fluids like urine, and validated, by comparing results with the ones obtained by other diagnostic technique (e.g. PCR).

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